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ARTICLE

Identification of Antihypertensive Peptides Derived from Low Molecular Weight Casein Hydrolysates Generated during Fermentation by *Bifidobacterium longum* KACC 91563

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Abstract

Angiotensin-converting enzyme (ACE) inhibitory activity was evaluated for the low-molecular-weight fraction (<3 kDa) obtained from milk fermentation by *Bifidobacterium longum* KACC91563. The ACE inhibitory activity in this fraction was 62.3%. The peptides generated from the <3 kDa fraction were identified by liquid chromatography-electrospray ionization quantitative time-of-flight mass spectrometry analysis. Of the 28 peptides identified, 11 and 16 were identified as β-casein (CN) and α_{s1}-CN, respectively. One peptide was identified as κ-CN. Three peptides, YQEPVLGPVRGPFPIIV, QEPVLGPVRGPFPIIV, and GPVRGPFPIIV, from β-CN corresponded to known antihypertensive peptides. We also found 15 peptides that were identified as potential antihypertensive peptides because they included a known antihypertensive peptide fragment. These peptides were as follows: RELEELNVPGEIVE (f1-14), YQEPVLGPVRGPFP (f193-206), EPVLGPVRGPFPIIV (f195-206), PVLGPVRGPFPIIV (f196-206), VLGPVRGPFPIIV (f197-206), and LGPVRGPFPIIV (f198-206) for β-CN; and APSFSDIPNPIGSENSEKTTMPLW (f176-199), SFSDIPNPIGSENSEKT- TMPLW (f178-199), FSDIPNPIGSENSEKTTMPLW (f179-199), SDIPNPIGSENSEKTTMPLW (f180-199), DIPNPIGSENSEKTTMPLW (f181-199), IPNPIGSENSEKTTMPLW (f182-199), PIGSENSEKTTMPLW (f185-199), IGSENSEKTTMPLW (f186-199), and SENSEKTTMPLW (f188-199) for α_{s1}-CN. From these results, *B. longum* could be used as a starter culture in combination with other lactic acid bacteria in the dairy industry, and/or these peptides could be used in functional food manufacturing as additives for the development of a product with beneficial effects for human health.

Keywords: *B. longum*, antihypertensive peptide, angiotensin converting enzyme

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Introduction

Probiotic bifidobacteria such as *Bifidobacterium longum* (*B. longum*), *Bifidobacterium breve*, *Bifidobacterium animalis*, and *Bifidobacterium bifidum* are commonly used as starter cultures in the dairy industry e.g., fermented milk, cheese and infant formulas (Chang *et al*., 2013; Davidson *et al*., 2000; Martín-Diana *et al*., 2003; McBrearty *et al*., 2001; Saavedra *et al*., 2004) because of their beneficial effects for human health. The various beneficial effects include the pathogenic species inhibition, diminution of colon cancer risk, immune response for protection effect on their function, regulation of gut microflora (Arunachalam, 1999; Chang *et al*., 2013; Collins and Gibson, 1999; Leahy *et al*., 2005).

As lactic acid bacteria (LAB) including *Lactococcus lactis* (*L. lactis*), *Lactobacillus rhamnosus* (*Lb. rhamnosus*), *Streptococcus thermophilus* (*St. thermophilus*), *Lactobacillus lactis* (*Lb. lactis*), *Lactobacillus helveticus* (*Lb. helveticus*), and *Lactobacillus* subsp. *bulgaricus* (*Lb. bulgaricus*), bifidobacteria strains for their growth also use milk protein as a nitrogen source, and their proteolytic system can produce peptides in milk (Chang *et al.*, 2013; Janer *et al*., 2005). The proteolytic system of LAB as *St. thermophilus*, *Lb. rhamnosus*, *L. lactis*, *Lb. bulgaricus*, *Lb. helveticus* is composed of 3 steps which contain the cell envelope protease (CEP), a transporter of oligopeptides and small peptides, and the various intracellular pep-

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tidases (Genay *et al*., 2009; Gilbert *et al*., 1996; Miclo *et al*., 2012; Pastar *et al*., 2003; Sadat-Mekmene *et al*., 2011b; Siezen, 1999).

The proteolytic system of bifidobacteria is not well known. Some studies have shown that peptidases of the genus *Bifidobacterium* can hydrolyze milk protein directly, including those of *Bifidobacterium animalis* subsp. *lactis* (Janer *et al*., 2005), *Bifidobacterium longum* Bl 536 (Donkor *et al*., 2007), and *B. longum* KACC 91563 (Chang *et al*., 2013) generating the biological peptides. Hydrolysis of milk protein by microorganisms during fermentation can generate various peptides, including biologically active peptides, e.g., angiotensin I-converting enzyme (ACE) inhibitory peptides, antioxidant peptides, opiates, antimutagens, immunomodulatory peptides, antimicrobial peptides, or peptides with mineral binding activity. The bioactive peptides produced by the activity of proteases or peptidases of microorganisms are well documented in the literature (Korhonen, 2009). The most studied commercial fermented milk products are "Calpis" and "Evolus", including two ACE-inhibitory tripeptides, VPP and IPP generated from β-casein by fermentation with *Lb. helveticus* including *Saccharomyces cerevisiae* for Calpis and *Lb. helveticus* for Evolus have been commercialized (Korhonen, 2009; Seppo *et al*., 2002; Takano, 2002).

In the case of bifidobacteria, the antioxidative peptides produced by *B. longum* were reported by Chang *et al*. (2013). Another study showed that the bioactive peptides of *B. longum* Bl 536 presented in *vitro* ACE-inhibitory activity, indicating that these are potential hypotensive peptides (Donkor *et al*., 2007); however, the peptides were not identified. Inhibition of ACE activity is regarded as an important component for the treatment of patients with hypertension because ACE leads to an increase in blood pressure by conversion of angiotensin I to angiogestin II or by bradykinin hydrolysis (Gobbetti *et al*., 2000; Hayes *et al*., 2007a; Miguel *et al*., 2009; Petrillo and Ondetti, 1982). Recently, some novel ACE-inhibitory peptides, including LVYPFP, were identified in *Bifidobacterium bifidum* (Gonzalez-Gonzalez *et al*., 2013). However, no hypotensive peptides have been identified in *B. longum* to date. Thus, we sought to investigate the antihypertensive activity and identify the peptides released from casein (CN) during fermentation of milk by *B. longum* KACC 91563.

Material and Methods

Materials

Chemical reagents, including hippuryl-histydil-leucine (HHL), captopril, ACE (2 mU; EC 3.4.15.1, 5.1 U mg^{-1}), and lung acetone powder from rabbit were purchased from Sigma Aldrich (USA), and all other chemicals used were of analytical grade.

Preparation and growth of *Bifidobacterium longum* KACC91563

B. longum KACC91563 was isolated from infant feces in Korea. Identification of this strain was performed according to previously described methods (Chang *et al*., 2013; Ham *et al*., 2011). After isolation, *B. longum* was grown according to the conditions described by Ruiz *et al*. (2009). *B. longum* KACC91563 was grown in de Man, Rogosa, and Sharpe (MRS) broth (BD Biosciences, USA) containing cysteine (0.05% final concentration), and the cells (bacteria) were harvested by centrifugation (Beckman Coulter, USA) at 3,200 *g* for 30 min at 4°C. The cells were incubated and stored in reconstituted skim milk (10% w/v) at 80°C.

Milk fermentation

To obtain the fermentate fraction, fermentation was performed in skimmed milk for 24 h by inoculation of 1% *B. longum* after preculture at 37°C. The fermentate was retrieved by centrifugation at 3,200 *g* for 15 min at 4°C when the fermentation was complete. The obtained fractions were separated using an ultrafiltration membrane system (Millipore, USA) with a molecular weight cut-off of 3 kDa.

Measurement of ACE inhibitory activity

ACE inhibitory activity was measured using a spectrophotometric assay as previously described (Cushman and Cheung, 1971) with slight modifications. First, crude ACE was prepared and extracted from 1 g of rabbit lung acetone powder (L0756, Sigma Aldrich) by gentle mixing of 40 mL of 0.1 M sodium borate buffer (pH 8.3) containing 0.3 M NaCl over 24 h. The supernatant containing ACE was obtained by centrifugation a 3,200 *g* for 40 min at 4°C, and was used for ACE inhibitory activity. A 50 μL sample was added to 100 μL of 0.1 M sodium borate buffer (pH 8.3) containing 0.3 M NaCl, and 50 μL of crude ACE was obtained. The reaction mixture was pre-incubated at 37°C for 5 min. Fifty microliters of 12.5 mM HHL (H-1635, Sigma Aldrich) solubilized in 0.1 M sodium borate buffer (pH 8.3) containing 0.3 M NaCl was added to the reaction mixture and left to stand for 30 min at 37°C after vortexing. To stop the enzyme reaction, 250 μL of 1 N HCl was added. Next, 1.5 mL of ethyl acetate was added

to this reaction mixture and vortexed for 15 s. One milliliter of supernatant was retrieved by centrifugation at 3,000 g for 5 min and evaporated using Concentrator Plus (Eppendorf, Germany) at 60°C for 30 min. The product was resupended in 1 mL of distilled water and its absorbance was measured at 228 nm using a spectrophotometer (Molecular Devices, USA). All assays were carried out in triplicate and the values represent the average and standard errors. Captopril (C-4042, Sigma Aldrich) was used as a positive control. The ACE inhibitory activity was calculated as follows: ACE inhibition (%) = [(C − B) − (S − B)] × 100/(C − B), where S is the absorbance of the ACE, ACE-inhibitory sample, and HHL; B is the absorbance of ACE and sodium borate buffer (pH 8.3) without HHL; and C is the absorbance of ACE, sodium borate buffer (pH 8.3), and HHL.

Identification of peptides by mass spectrometry

Liquid chromatography-electrospray ionization-quantitative time-of-flight tandem mass spectrometry experiments (LC-ESI-TOF-MS/MS) were performed at the National Instrumentation Center for Environmental Management (NICEM) of Seoul National University in Korea, according to the method described by Chang *et al*. (2013).

MS analysis experiments were carried out using an integrated system consisting of an auto-switching nano pump, autosampler (Tempo^{TM} nano LC system; MDS SCIEX, Canada), and a hybrid quadrupole-time-of-flight (TOF) mass spectrometer (QStar Elite; Applied Biosystems, USA) fitted with a fused silica emitter tip (New Objective, USA). For ionization, the nano-electrospray ionization (ESI) was applied. Two μL fractions were injected into the LC-nano ESI-MS/MS system.

Samples were first 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. The solvent A and B consisted in [water/acetonitrile (98:2, v/v), 0.1% formic acid] and [Water/acetonitrile (2:98, v/v), 0.1% formic acid]. Separation was carried out on 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 through a coated silica tip (FS360-20-10-N20-C12, PicoTip emitter, New Objective) was performed at an ion spray voltage of 2,000 eV. Peptides were analyzed automatically using Analyst QS 2.0 software (Applied Biosystems, USA). The range of m/z values was 200-2000.

Results and Discussion

Determination of *in vitro* ACE inhibitory activity in low molecular weight fermentate

Two fractions were prepared to evaluate the ACE inhibitory activity. One was the fraction obtained after *B. longum* KACC9156 fermentation in skimmed milk for 24 h. Fermentates were fractionated at a molecular weight cut-off of 3 kDa using a centrifugal ultrafiltration membrane system. This cutoff value was chosen because Gonzalez-Gonzalez *et al*. (2013) demonstrated that the <3 kDa fraction of *B. bifidum* MF 20/5-fermented milk showed higher ACE inhibitory activity than that of the >3-kDa fraction. Similarly, Miguel *et al*. (2009) showed that the 50% inhibitory concentration of ACE was higher (5.5 μg/mL) in the <3 kDa fraction than in the > 3 kDa fractions of the bovine CN hydrolysate and whole hydrolysate without molecular weight fractionation. Confirming these results, in the present study, the ACE inhibitory activity determined from the fermentate obtained from milk fermentation with *B. longum* was 62.3% (Table 1), which was higher than that of the CN hydrolysate (28.3% ACE inhibitory activity) obtained from a 0.1% CN solution in 0.05 M sodium phosphate buffer (pH 7.0) at the same incubation time (data not shown). This ACE inhibitory activity was similar to that reported previously (63.7%) in milk fermented with *B. longum* B1 536 (Donkor *et al*. 2007). During fermentation, the casein could be degraded by the protolytic system of *B. longum* which was detected on peptidase activity of their cell surface (Chang *et al*., 2013). However, the cell envelop protease of *B. longum* which is the first step of casein hydrolysis was not found. From this reason, the ACE inhibitory activity in this work could be resulted from the potential cell wall peptidase.

Using LC-ESI-MS/MS, the further study was proceeded to identify the peptides and to search the antihypertensive peptides generated by fermentation with *B. longum* KACC

Table 1. ACE-inhibitory activities in the low-molecular-weight fraction (<3 kDa) of the fermentate obtained after incubation of milk by *Bifidobacterium longum* KACC 91563

	Control	Fermentate
A_{228}	0.715±0.027	0.375±0.011
Activity (%)	0	62.3

All assays were carried out in triplicate.

Table 2. Casein-derived peptides identified by liquid chromatography electrospray ionization time-of-flight tandem mass spectrometry in the <3 kDa fraction of the 24 h fermentates obtained from fermentation of milk by *Bifidobacterium longum* KACC91563

Names	Peptide sequence	Prec MW	Prec m/z	Theor MW	Theor m/z	z
β-CN	f1-25, RELEELNVPGEIVE	1623.8091	812.9118	1623.8468	812.9307	2
	f109-125, MPFPKYPVEPFTESQSL	1995.9216	998.9681	1995.9652	998.9899	2
	f193-206, YQEPVLGPVRGPFP	1554.7750	778.3948	1554.8195	778.4170	2
	f193-209, YQEPVLGPVRGPFPIIV	1880.0123	941.0134	1880.0560	941.0353	2
	f194-209, QEPVLGPVRGPFPIIV	1699.9307	850.9726	1699.9662	850.9904	2
	f195-206, EPVLGPVRGPFP	1263.6628	632.8387	1263.6975	632.8561	2
	f195-209, EPVLGPVRGPFPIIV	1588.9012	795.4579	1588.9341	795.4743	2
	f196-209, PVLGPVRGPFPIIV	1459.8593	730.9369	1459.8915	730.9530	2
	f197-209, VLGPVRGPFPIIV	1362.8054	682.4100	1362.8387	682.4266	2
	f198-209, LGPVRGPFPIIV	1263.7390	632.8768	1263.7704	632.8925	2
	f199-209, GPVRGPFPIIV	1150.6611	576.3378	1150.6863	576.3504	2
α_{s1}-CN	f11-21, LPQEVLNENLL	1302.6389	652.3267	1302.6796	652.3470	2
	f11-23, LPQEVLNENLLRF	1583.8263	792.9204	1583.8672	792.9409	2
	f26-34, APFPEVFGK	990.4937	496.2541	990.5175	496.2660	2
	f176-190, APSFSDIPNPIGSEN	1565.6588	783.8367	1565.6974	783.8560	2
	f176-197, APSFSDIPNPIGSENSEKTTMP	2318.0234	1160.0190	2318.0737	1160.0441	2
	f176-199, APSFSDIPNPIGSENSEKTTMPLW	2633.1953	878.7391	2633.2319	878.7513	3
	f177-197, PSFSDIPNPIGSENSGKTTMP	2202.9614	1102.4880	2203.0103	1102.5125	2
	f178-199, SFSDIPNPIGSENSEKTTMPLW	2449.0955	1225.5550	2449.1472	1225.5808	2
	f179-199, FSDIPNPIGSENSEKTTMPLW	2362.0474	1182.0310	2362.1152	1182.0648	2
	f180-199, SDIPNPIGSENSEKTTMPLW	2214.9893	1108.5020	2215.0466	1108.5306	2
	f181-199, DIPNPIGSENSEKTTMPLW	2127.9514	1064.9830	2128.0146	1065.0146	2
	f182-198, IPNPIGSENSEKTTMPL	1808.8452	905.4299	1808.8978	905.4562	2
	f182-199, IPNPIGSENSEKTTMPLW	2028.9335	1015.4740	2028.9827	1015.4986	2
	f185-199, PIGSENSEKTTMPLW	1704.7677	853.3911	1704.8029	853.4087	2
	f186-199, IGSENSEKTTMPLW	1613.6985	807.8565	1613.7372	807.8759	2
	f188-199, SENSEKTTMPLW	1421.6108	711.8127	1421.6497	711.8321	2
κ-CN	f151-169, EVIESPPEINTVQVTSTAV	2033.9755	1017.9950	2034.0133	1018.0139	2

CN: casein, *m/z* = mass to charge ratio, where *z* = number of positively charged ions.

9156 in milk.

Peptides generated from milk casein during fermentation by *Bifidobacterium longum* KACC9156

The peptides generated from fermentates were identified by LC-ESI-TOF-MS/MS. As shown in Table 2, the peptides in the fermentate were identified to have been generated from CN. The results also indicated that CN was the preferential substrate of *B. longum* in spite of the presence of whey protein in skim milk, similar to a strain of *St. thermophilus* (Chang *et al*., 2014). A total of 28 peptides were generated, corresponding to 11 β-CN, 16 α_{s1}-CN, and 1 κ-CN peptide. This observation was slightly different from that reported by Chang *et al*. (2013), who identified 33 peptides (19 β-CN, 12 α_{s1}-CN, and 2 κ-CN) in bovine CN hydrolysates obtained from a 0.1% CN solution with the same strain of *B. longum*. This difference could be explained by a difference in the accessibility of peptides for hydrolysis by *B. longum* or in a structural difference of milk protein in the different matrices used, i.e., milk versus buffer.

Nonetheless, the pattern of CN hydrolysis determined in the present study was similar to that reported by Chang *et al*. (2013). In the case of β-CN peptides, the C terminus was more hydrolyzed than the N-terminal (Fig. 1). A large number of peptides were also found generated in this region from *Lactobacillus helveticus* (Sadat-Mekmene *et al*., 2011a), *Lactobacillus delbrueckii* subsp. *lactis* CRL 581 (Hebert *et al*., 2008), *Lactobacillus bulgaricus* (Tsakalidou *et al*., 1999), *Lactobacillus lactis* subsp. *cremoris* (Reid *et al*., 1991), and *S. thermophilus* (Miclo *et al*., 2012). On the other hand, the N terminus of β-CN was resistant to hydrolysis by *B. longum*, as previously reported by Chang *et al*. (2013). The C terminus of β-CN, which was determined to be a hydrophobic region in this study, is more accessible for hydrolysis by *B. longum* (Chang *et al*., 2013)

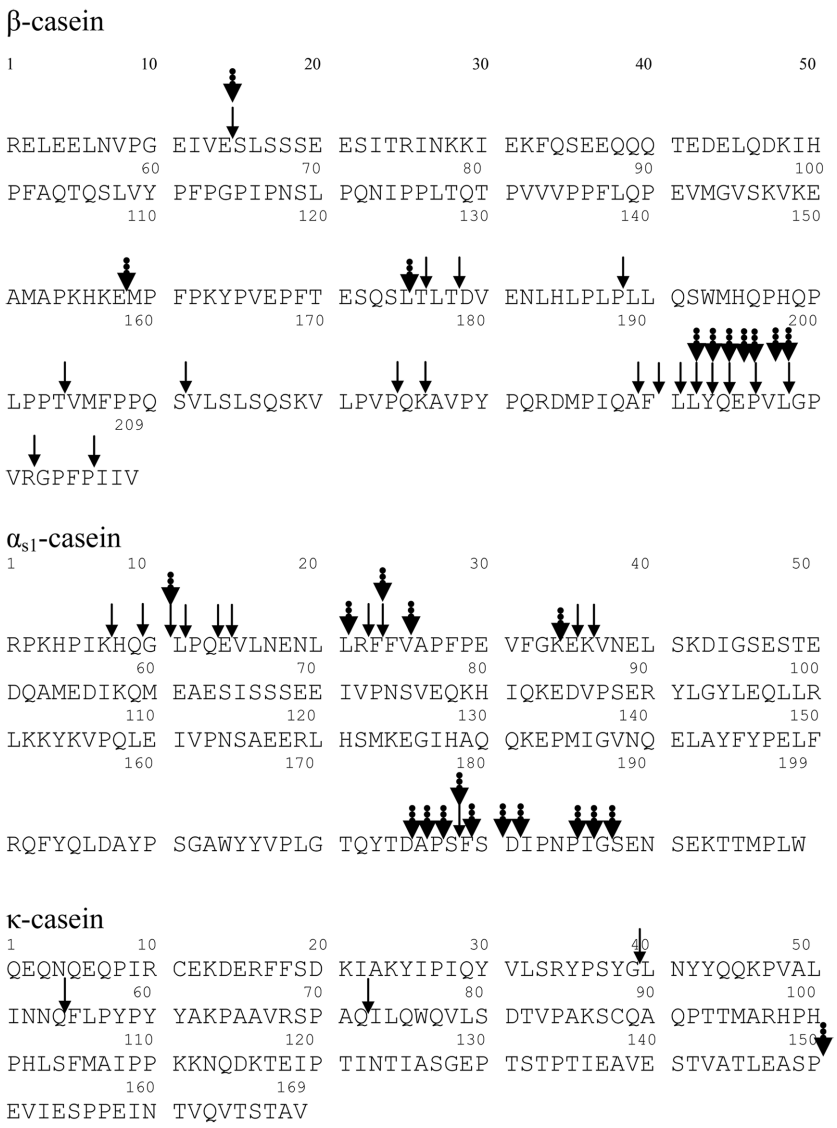

Fig. 1. Cleavage sites of peptide bonds on bovine β-, α_{s1}-, and κ-casein hydrolyzed after fermentation with *Bifidobacterium longum* KACC9156. The dot arrows cleaved peptide bonds in this study. The line arrow cleaved peptide bonds reported by Chang *et al*. (2013).

and by the proteases of *S. thermophilus* (Chang *et al*., 2014; Miclo *et al*., 2012) and *L. helveticus* (Sadat-Mekmene *et al*., 2011a).

For α_{s1}-CN, the hydrolysis pattern was also consistent with the results of Chang *et al*. (2013), who reported that *B. longum* hydrolyzed the N-terminal region to a greater extent than the C-terminal region. In spite of the similar hydrolysis patterns, the observed cleavage pattern (Fig. 1) was slightly different to the result obtained by Chang *et al*. (2013) for *B. longum*, in which we observed relatively more cleavage sites on α_{s1}-CN. The reason for the different cleavage patterns might be that the structure of α_{s1}-CN was changed during fermentation in milk to make it more accessible for hydrolysis by *B. longum.* A similar result was reported by Chang *et al*. (2014) and Sadat-Mekmene *et al*. (2011b), who also found that the proteases PrtS and PrtH of *S. thermophilus* and *L. helveticus*, respectively, were more accessible at the N terminus than the C terminus.

In this study, regions found to be resistant to hydrolysis were also identified in the β-CN and α_{s1}-CN sequences. This may be due to the presence of phosphoserine residues in these regions, which leads to high resistance to hydrolysis (Chang *et al*., 2014; Kaspari *et al*., 1996). In present work, five and eight phosphoserine residues were identified in the regions that were not hydrolyzed on β-CN and

α_{s1}-CN, respectively.

In the case of κ-CN, the cleavage site (Fig. 1) only showed the generation of one peptide, whereas Chang *et al*. (2013) found two peptides in this region. Furthermore, Zahraa (2010) reported that the glycomacropeptide (f106-169) region, composed of glycan chains, was difficult to hydrolyze because of the presence of hydrophilic amino acids and a negative charge, leading to increased electrostatic repulsion. However, in the present study, only one peptide, f150-151, of this casein was obtained. Fermentation with *B. longum* could induce a structural change due to weak bond of glycan chains on this glycomacropeptide region that would allow *B. longum* to access κ-CN for hydrolysis.

As the previously study by Chang *et al*. (2013) who reported that no peptide detected from α_{s2}-CN from 0.1%

Table 3. Antihypertensive and potential antihypertensive peptides generated by fermentation by *Bifidobacterium longum* KACC 91563 after 24 h at 37°C

Sequence	Fragment	Source	Proteolytic agent	References
A. Bioactive peptides clearly identified in the literature				
LNVPGEIVE	β-CN(f6-14)	milk	*Lb. bulgaricus*	Gobbetti *et al*., 2000
DKIHPF	β -CN(f47-52)	milk	*L. lactis* subsp. *cremoris*	Gobbetti *et al*., 2000
LVYPFP	β -CN(f58-63)	milk	*B. bifidum*	Gonzalez-Gonzalez *et al*., 2013
NIPPLTQTPV	β -CN(f73-82)	milk	*Lb. bulgaricus*	Gobbetti *et al*., 2000
EMPFPK	β-CN(f108-113)	casein	milk starter +pepsin and trypsin	Pihlanto-Leppala *et al*., 1998
HLPLPLL	β-CN(f134-140)	casein	pepsin	Del Mar Contreras *et al*., 2009
SQSKVLPVPQ	β-CN(f166-175)	sodium caseinate	*Lb. animalis*	Hayes *et al*., 2007a
SKVLPVPQ	β-CN(f168-175)	β-casein	protease of *Lb.helveticus*	Yamamoto *et al*., 1994
KVLPVPQ	β -CN(f169-175)	β-casein	protease of *Lb.helveticus*	Maeno *et al*., 1996
RDMPIQAF	β -CN(f183-190)	β-casein	protease of *Lb.helveticus*	Yamamoto *et al*., 1994
LLYQEPVLG-PVRGPFPIIV	β -CN(f191-209)	β-casein	protease of *Lb.helveticus*	Yamamoto *et al*., 1994
YQEPVL	β-CN(f193-198)	casein	milk starter +pepsin and trypsin	Pihlanto-Leppälä *et al*., 1998
YQEPVLGPVR	β-CN(f193-202)	milk	*Lb casei* ssp. *rhamnosus*	Rokka *et al*., 1997
YQEPVLGPVRG-PFPI	β-CN(f193-208)	casein	trypsin	Maruyama and Suzuki, 1982
YQEPVLGPVRG-PFPIIV[a,]	β-CN(f193-209)	β-casein	protease of *Lb.helveticus*	Yamamoto *et al*., 1994
QEPVLGPVRG-PFPIIV[a,]	β-CN(f194-209)	milk	*L. lactis* + chymosin/trypsin/ chymotrypsin	Gobbetti *et al*., 2002
GPVRGPFPIIV[a,]	β-CN(f199-209)	Manchego cheese	protease in Manchego	Gomez-Ruiz *et al*., 2002
AVPYPQR	β-CN(f176-182)	milk	lactic acid bacterias	Hernandez-Ledesma *et al*., 2004
YQEP	β-CN(f191-198)	Gouda cheese	Proteases from *Cynara cardunculus*	Silva *et al*., 2006
GPFPIIV	β-CN(f203-209)	milk	protease of *Lb.helveticus*	Yamamoto *et al*., 1994; Hayes *et al*., 2007b
RPKHPIKHQ	α_{s1}-CN(f1-9)	Gouda cheese	Proteases from *Cynara cardunculus*	Silva *et al*., 2006
FF	α_{s1}-CN(f23-24)	casein	trypsin	Maruyama and Suzuki, 1982
FFVAP	α_{s1}-CN(f23-27)	casein	trypsin	Maruyama and Suzuki, 1982
FFVAPFPEVFGK	α_{s1}-CN(f23-34)	sodium caseinate	*Lb. animalis*	Hayes *et al*., 2007a
YKVPQL	α_{s1}-CN(f104-109)	α_{s1}-casein	protease of *Lb.helveticus*	Maeno *et al*., 1996
LAYFYP	α_{s1}-CN(f142-147)	casein	milk starter +pepsin and trypsin	Pihlanto-Leppala *et al*., 1998
DAYPSGAW	α_{s1}-CN(f157-164)	casein	milk starter +pepsin and trypsin	Pihlanto-Leppala *et al*., 1998
TTMPLW	α_{s1}-CN(f194-199)	casein	trypsin	Maruyama and Suzuki, 1982; Pihlanto-Leppälä *et al*., 1998
FALPQYLK	α_{s2}-CN(f174-181)	α_{s2}-casein	trypsin	Tauzin *et al*., 2002
AMKPWIQPK	α_{s2}-CN(f189-197)	α_{s2}-casein	protease of *Lb.helveticus*	Maeno *et al*., 1996
MKPWIQPK	α_{s2}-CN(f190-197)	α_{s2}-casein	protease of *Lb.helveticus*	Maeno *et al*., 1996
TKVIP	α_{s2}-CN(f198-202)	α_{s2}-casein	protease of *Lb.helveticus*	Maeno *et al*., 1996

Table 3. Antihypertensive and potential antihypertensive peptides generated by fermentation with *Bifidobacterium longum* KACC91563 after 24 h at 37°C (Continued)

Sequence	Fragment	Source	Proteolytic agent	References
B. Potential ACE inhibitory peptides				
Sequence	Fragment	Present study[a]		References
LNVPGEIVE	β-CN(f6-14)	f1-14, RELEE**LNVPGEIVE**		Gobbetti *et al*., 2000
YQEPVLGPVR	β-CN(f193-202)	f193-206, **YQEPVLGPVR**GPFP		Rokka *et al*., 1997
GPVRGPFPIIV	β-CN(f199-209)	f195-209, EPVL**GPVRGPFPIIV**		Gomez-Ruiz *et al*., 2002
	β-CN(f199-209)	f196-209, PVL**GPVRGPFPIIV**		
	β-CN(f199-209)	f197-209, VL**GPVRGPFPIIV**		
	β-CN(f199-209)	f198-209, L**GPVRGPFPIIV**		
TTMPLW	α_{s1}-CN(f194-199)	f176-199, APSFSDIPNPIGSENSEK**TTMPLW**		Maruyama and Suzuki, 1982; Pihlanto-Leppälä *et al*., 1998
	α_{s1}-CN(f194-199)	f178-199, SFSDIPNPIGSENSEK**TTMPLW**		
	α_{s1}-CN(f194-199)	f179-199, FSDIPNPIGSENSEK**TTMPLW**		
	α_{s1}-CN(f194-199)	f180-199, SDIPNPIGSENSEK**TTMPLW**		
	α_{s1}-CN(f194-199)	f181-199, DIPNPIGSENSEK**TTMPLW**		
	α_{s1}-CN(f194-199)	f182-199, IPNPIGSENSEK**TTMPLW**		
	α_{s1}-CN(f194-199)	f185-199, PIGSENSEK**TTMPLW**		
	α_{s1}-CN(f194-199)	f186-199, IGSENSEK**TTMPLW**		
	α_{s1}-CN(f194-199)	f188-199, SENSEK**TTMPLW**		

[a]Peptides obtained from the fermentates in milk with *B. longum* in the present study.

CN solution in 0.05 M sodium phosphate buffer (pH 7.0), none of the peptides identified were found in <3 kDa fraction from fermentate. This observation could be explained by the results of Tauzin *et al*. (2002), who found a protected region due to the formation of a tetrameric complex from CN that was more resistant to hydrolysis. Miclo *et al*. (2012) also suggested that accessibility of this region depends on its protein structure change.

Milk proteins play role precursors to release many peptides relating biological activity (Korhonen, 2009), i.e., ACE inhibitory peptide as this work.

Thirty two peptides relating ACE inhibitory obtained from casein have been reported in literature (Table 3). Out of 28 peptides generated from bovine casein during fermentation in present study, only 3 peptides from β-CN identified through MS/MS analysis (ESI-Q-TOF), YQEP-VLGPVRG-PFPIIV, QEPVLGPVRGPFPIIV, GPVRGPFPIIV corresponded to ACE inhibitory bioactive peptides (Table 3A). These peptides were consistent with the previously studies reviewed by Yamamoto *et al*. (1994), Gobbetti *et al*. (2002) and Gomez-Ruiz *et al*. (2002), respectively. However no ACE inhibitory peptide released from other casein reported in previously study was present. These 3 peptides identified in this study were known the antihypertensive peptide in the literature. Thus, ACE inhibitory activity shown in Table 1 might result from these peptides.

Actually, peptides which have the high ACE inhibitory activity contain several amino acids (Trp, Phe, Tyr, or Pro) at the extremity of C-terminal and Ala, Val, IIe and Ser called aliphatic amino acids at the N-terminal (Jao *et al*., 2012). The three peptides, YQEPVLGPVRGPFPIIV, QE-PVLGPVRGPFPIIV, GPVRGPFPIIV identified in present study contain these amino acids except for 4 amino acids, Gln, Glu, Leu and Gly. Thus, from these results, the peptides identified were reasonable to show the antihypertensive activity.

Other peptides as potential antihypertensive peptides which were included the fragment having ACE inhibitory activity were also identified in this study. These peptides were listed in Table 3B displaying 6 for β-CN and 9 for α_{s1}-CN. Peptide, RELEELNVPGEIVE (f1-14) released from β-CN identified in present study contains LNVPGEI-VE reviewed by Gobbetti *et al.* (2000) as ACE inhibitory peptide. Another peptide, YQEPVLGPVRGPFP (f193-206) from β-CN, contains also the ACE inhibitory peptide, YQ-EPVLGPVR (Rokka *et al*., 1997). The others from β-CN, the fragment GPVRGPFPIIV reviewed by Gomez-Ruiz *et al*. (2002) as antihypertensive peptide was included in 4 peptides, EPVLGPVRGPFPIIV, PVLGPVRGPFPIIV, V-LGPVRGPFPIIV, LGPVRGPFPIIV.

For peptides obtained from α_{s1}-CN, the 9 peptide, APS-FSDIPNPIGSENSEKTTMPLW (f176-199), SFSDIPNPI-GSENSEKTTMPLW (f178-199), FSDIPNPIGSENSEKT-TMPLW (f179-199), SDIPNPIGSENSEKTTMPLW (f180-199), DIPNPIGSENSEKTTMPLW (f181-199), IPNPIG-SENSEKTTMPLW (f182-199), PIGSENS EKTTMPLW

(f185-199), IGSENSEKTTMPLW (f186-199), SENSEKTTMPLW (f188-199) was released (Table 3B). These peptides contain the peptide TTMPLW (f154-199) which have reviewed that this peptide has a biological activity to inhibit ACE (Maruyama and Suzuki 1982; Pihlanto-Leppälä *et al*., 1998). The antihypertensive peptide, SDIPNPIGSENSEKTTMPLW (f180-199) occurring naturally in milk, was also reviewed by Islam *et al.* (2014).

From our results, these 15 peptides obtained from β-CN and α_{s1}-CN during fermentation with *B. longum* may be presented the antihypertensive activity (Table 3B) during digestion *in vivo*. Chang *et al*. (2014) have reported that some peptides containing bioactive peptide fragment could be released during digestion. To verify whether these peptides show the ACE inhibitory activity as novel antihypertensive peptide after synthesis of these peptides, whether these peptides during digestion with gastro-intestinal enzyme i.e., pepsin, trypsin, chymotrypsin, pancreatin etc. will be short and generated as antihypertensive peptide reviewed in literature and also whether peptides generated during digestion will be novel antihypertensive peptide, the further study will need.

Conclusions

Antihypertensive activity was demonstrated in the low-molecular-weight fraction (<3 kDa) obtained from the fermentate after milk fermentation with *B. longum*. This fraction was used to identify the CN-derived ACE inhibitory peptides. Using mass spectrometry analysis, three peptides showing antihypertensive activity and 15 peptides with potential antihypertensive activity were identified from CN. Thus, our results suggest that, given its capacity to generate antihypertensive peptides, *B. longum* KACC 91563 could be used as a starter culture with other lactic acid bacteria in the dairy industry and/or these peptides could be used in functional food manufacturing as antihypertensive agents, owing to their beneficial effects for human health.

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References

1. Arunachalam, K. D. (1999) Role of bifidobacteria in nutrition, medicine and technology. *Nutr. Res.* **19,** 1559-1597.
2. Chang, O. K., Roux, E., Awussi, A. A., Miclo, L., Jardin, J., Jameh, N., Dary, A., Humbert, G., and Perrin, C. (2014) Use of a free form of the *Streptococcus thermophilus* cell envelope protease PrtS as a tool to produce bioactive peptides. *Int. Dairy J.* **38,** 104-115.
3. Chang, O. K., Seol, K. H., Jeong, S. G., Oh, M. H., Park, B. Y., Perrin, C., and Ham, J. S. (2013) Casein hydrolysis by *Bifidobacterium longum* KACC91563 and antioxidant activities of peptides derived therefrom. *J. Dairy Sci.* **96,** 5544-5555.
4. Collins, M. D. and Gibson, G. R. (1999) Probiotics, prebiotics, and synbiotics: approaches for modulating the microbial ecology of the gut. *Am. J. Clin. Nutr.* **69,** 1052S-1057S.
5. Cushman, D. W. and Cheung, H. S. (1971) Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. *Biochem. Pharmacol.* **20,** 1637-1648.
6. Davidson, R. H., Duncan, S. E., Hackney, C. R., Eigel, W. N., and Boling, J. W. (2000) Probiotic culture survival and implications in fermented frozen yogurt characteristics. *J. Dairy Sci.* **83,** 666-673.
7. Del Mar Contreras, M., Carron, R., Montero, M., and Recio, I. (2009) Novel casein-derived peptide with anti-hypertensive activity. *Int. Dairy J.* **19,** 566-573.
8. Donkor, O. N., Henriksson, A., Singh, T. K., Vasiljevic, T., and Shah, N. P. (2007) ACE-inhibitory activity of probiotic yoghurt. *Int. Dairy J.* **17,** 1321-1331.
9. Genay, M., Sadat, L., Gagnaire, V., and Lortal, S. (2009) *prtH2*, not *prtH*, is the ubiquitous cell wall proteinase gene in *Lactobacillus helveticus*. *Appl. Environ. Microbiol.* **75,** 3238-3249.
10. Gilbert, C., Atlan, D., Blanc, B., Portalier, R., Germond, G. J., Lapierre, L., and Mollet, B. (1996) A new cell surface proteinase: Sequencing and analysis of the *prtB* gene from *Lactobacillus delbrueckii* subsp. *bulgaricus*. *J. Bacteriol.* **178,** 3059-3065.
11. Gobbetti, M., Ferranti, P., Smacchi, E., Goffredi, F., and Addeo, F. (2000) Production of angiotensin-I-converting-enzyme-inhibitory peptides in fermented milks started by *Lactobacillus delbrueckii* subsp.*bulgaricus* SS1 and *Lactococcus-lactis* subsp. *cremoris* FT4. *Appl. Environ. Microbiol.* **66,** 3898-3904.
12. Gobbetti, M., Stepaniak, L., De Angelis, M., Corsetti, A., and Di Cagno, R. (2002) Latent bioactive peptides in milk proteins: proteolytic activation and significance in dairy processing. *Crit. Rev. Food Sci. Nutr.* **42,** 223-239.
13. Gomez-Ruiz, J. A., Ramos, M., and Recio, I. (2002) Angiotensin-converting enzyme-inhibitory peptides in Manchego cheeses manufactured with different starter cultures. *Int. Dairy J.* **12,** 697-706.
14. Gonzalez-Gonzalez, C., Gibson, R., and Jauregi, P. (2013) Novel probiotic-fermented milk with angiotensin I-converting enzyme inhibitory peptides produced by *Bifidobacterium bifidum* MF 20/5. *Int. J. Food Microbiol.* **167,** 131-137.
15. Ham, J. S., Lee, T., Byun, M. J., Lee, K. T., Kim, M. K., Han, G. S., Jeong, S. G., Oh, M. H., Kim, D. H., and Kim, H. (2011) Complete genome sequence of *Bifidobacterium longum* subsp.

longum KACC 91563. *J. Bacteriol.* **193,** 5044.
16. Hayes, M., Stanton, C., Slattery, H., O'Sullivan, O., Hill, C., Fitzgerald, G. F., and Ross, R. P. (2007a) Casein fermentate of *Lactobacillus animalis* DPC6134 contains a range of novel propeptide angiotensin-converting enzyme inhibitors. *Appl. Environ. Microbiol.* **73,** 4658-4667.
17. Hayes, M., Ross, R. P., Fitzgerald, G. F., and Stanton, C. (2007b) Putting microbes to work: Dairy fermentation, cell factories and bioactive peptides. Part II: Bioactive peptide functions. *Biotechnol. J.* **2,** 435-449.
18. Hebert, E. M., Mamone, G., Picariello, G., Raya, R. R., De Giori, G. S., Ferranti, P., and Addeo, F. (2008) Characterization of the pattern of as1- and b-casein breakdown and release of a bioactive peptide by a cell envelope proteinase from *Lactobacillus delbrueckii* subsp. *lactis* CRL 581. *Appl. Environ. Microbiol.* **74,** 3682-3689.
19. Hernandez-Ledesma, B., Amigo, L., Ramos, M., and Recio, I. (2004) Application of high-performance liquid chromatography-tandem mass spectrometry to the identification of biologically active peptides produced by milk fermentation and simulated gastrointestinal digestion. *J. Chromatogr. A.* **1049,** 107-114.
20. Islam, M. A., Alam, M. K., Islam, M. N., Khan, M. A. S., Ekeberg, D., Rukke, E. O., and Vegarud, G. E. (2014) Principal milk components in buffalo, holstein cross, indigenous cattle and red chittagong cattle from bangladesh. *Asian-Aust. J. Anim. Sci.* **27,** 886-897.
21. Janer, C., Arigoni, F., Lee, B. H., Pelaez, C., and Requena, T. (2005) Enzymatic ability of *Bifidobacterium animalis* ssp. *lactis* to hydrolyze milk proteins: Identification and characterization of endopeptidase O. *Appl. Environ. Microbiol.* **71,** 8460-8465.
22. Jao, C. L., Huang, S. L., and Hsu, K. C. (2012) Angiotensin I-converting enzyme inhibitory peptides: Inhibition mode, bioavailability, and antihypertensive effects. *Biomedicine* **2,** 130-136.
23. Kaspari, A., Diefenthal, T., Grosche, G., Schierhorn, A., and Demuth, H. U. (1996) Substrates containing phosphorylated residues adjacent to proline decrease the cleavage by proline-specific peptidases. *Biochim. Biophys. Acta* **1293,** 147-153.
24. Korhonen, H. (2009) Milk-derived bioactive peptides: From science to applications. *J. Funct. Foods* **1,** 177-187.
25. Leahy, S. C., Higgins, D. G., Fitzgerald, G. F., and van Sinderen, D. (2005) Getting better with bifidobacteria. *J. Appl. Microbiol.* **98,** 1303-1315.
26. Maeno, M., Yamamoto, N., and Takano, T. (1996) Identification of an antihypertensive peptide from casein hydrolysate produced by a proteinase from *Lactobacillus helveticus* CP 790. *J. Dairy Sci.* **79,** 1316-1321.
27. Martín-Diana, A. B., Janer, C., Peláez, C., and Requena, T. (2003) Development of a fermented goat's milk containing probiotic bacteria. *Int. Dairy J.* **13,** 827-833.
28. Maruyama, S. and Suzuki, H. (1982) A peptide inhibitor of angiotensin I-converting enzyme in the tryptic hydrolysate of casein. *Agric. Biol. Chem.* **46,** 1393-1394.
29. McBrearty, S., Ross, R. P., Fitzgerald, G. F., Collins, J. K., Wallace, J. M., and Stanton, C. (2001) Influence of two commercially available bifidobacteria cultures on cheddar cheese quality. *Int. Dairy J.* **11,** 599-610.
30. Miclo, L., Roux, E., Genay, M., Brusseaux, E., Poirson, C., Jameh, N., Perrin, C., and Dary, A. (2012) Variability of hydrolysis of β-, αs1-, and αs2-caseins by 10 strains of *Streptococcus thermophilus* and resulting bioactive peptides. *J. Agric. Food Chem.* **60,** 554-565.
31. Miguel, M., Contreras, M. M., Recio, I., and Aleixandre, A. (2009) ACE-inhibitory and antihypertensive properties of a bovine casein hydrolysate. *Food Chem.* **112,** 211-214.
32. Pastar, I., Tonic, I., Golic, N., Kojic, M., van Kranenburg, R., Kleerebezem, M. and Topisirovic, L., and Jovanovic, G. (2003) Identification and genetic characterization of a novel proteinase, PrtR, from the human isolate *Lactobacillus rhamnosus* BGT10. *Appl. Environ. Microbiol.* **69,** 5802-5811.
33. Petrillo, E. W. and Ondetti, M. A. (1982) Angiotensin-converting enzyme inhibitors: Medicinal chemistry and biological actions. *Med. Res. Rev.* **2,** 1-41.
34. Pihlanto-Leppälä, A., Rokka, T., and Korhonen, H. (1998) Angiotensin I converting enzyme inhibitory peptides derived from bovine milk proteins. *Int. Dairy J.* **8,** 325-331.
35. Reid, J. R., Ng, K. H., Moore, C. H., Coolbearm, T., and Pritchard, G. G. (1991) Comparison of bovine β-casein hydrolysis by PI and PIII type proteinases from *Lactobacillus lactis* subsp. c*remoris*. *Appl. Microbiol. Biotechnol.* **36,** 344-351.
36. Rokka, T., Syväoja, E. L., Tuominen, J., and Korhonen, H. (1997) Release of bioactive peptides by enzymatic proteolysis of *Lactobacillus* GG fermented UHT milk. *Milchwissenschaft* **52,** 675-678.
37. Ruiz, L., Sánchez, B., de los Reyes-Gavilán, C. G., Gueimonde, M., and Margolles, A. (2009) Coculture of *Bifidobacterium longum* and *Bifidobacterium breve* alters their protein expression profiles and enzymatic activities. *Int. J. Food Microbiol.* **133,** 148-153.
38. Saavedra, J. M., Abi-Hanna, A., Moore, N., and Yolken, R. H. (2004) Long-term consumption of infant formulas containing live probiotic bacteria: tolerance and safety. *Am. J. Clin. Nutr.* **79**, 261-267.
39. Sadat-Mekmene, L., Genay, M., Atlan, D., Lortal, S., and Gagnaire, V. (2011a) Original features of cell-envelope proteinases of *Lactobacillus helveticus*: A review. *Int. J. Food Microbiol.* **146,** 1-13.
40. Sadat-Mekmene, L., Jardin, J., Corre, C., Mollé, D., Richoux, R., Delage, M. M., Lortal, S., and Gagnaire, V. (2011b) Simultaneous presence of PrtH and PrtH2 proteinases in strains improves breakdown of the pure α_{s1}-casein. *Appl. Environ. Microbiol.* **77,** 179-186.
41. Seppo, L., Kerojoki, O., Suomalainen, T., and Korpela, R. (2002) The effect of a *Lactobacillus helveticus* LBK-16 H fermented milk on hypertension - a pilot study on humans. *Milchwissenschaft* **57,** 124-127.
42. Silva, S. V., Pihlanto, A., and Malcata, F. X. (2006) Bioactive peptides in ovine and caprine cheeselike systems prepared with proteases from *Cynara cardunculus*. *J. Dairy Sci.* **89,** 3336-3344.

43. Siezen, R. J. (1999) Multi-domain, cell-envelope proteinases of lactic acid bacteria. *Antonie van Leeuwoenhoek* **76,** 139-155.
44. Takano, T. (2002) Anti-hypertensive activity of fermented dairy products containing biogenic peptides. *Antonie van Leeuwenhoek* **82,** 333-340.
45. Tauzin, J., Miclo, L., and Gaillard, J. L. (2002) Angiotensin-I-converting enzyme inhibitory peptides from tryptic hydrolysate of bovine α_{s2}-casein. *FEBS Lett.* **531,** 369-374.
46. Tsakalidou, E., Anastasiou, R., Vandenberghe, I., Vanbeeumen, J., and Kalantzopoulos, G. (1999) Cell-wall-bound proteinase of *Lactobacillus delbrueckii* subsp. *lactis* ACA-DC 178: characterization and specificity for β-casein. *Appl. Environ. Microbiol.* **65,** 2035-2040.
47. Yamamoto, N., Akino, A., and Takano, T. (1994) Antihypertensive effect of peptides derived from casein by an extracellular proteinase from *Lactobacillus helveticus* CP790. *J. Dairy Sci.* **77,** 917-922.
48. Zahraa, N. (2010) Le peptide κ-CN(f106-109) du lait: propriétés nutritionnelles, biologiques et techno-fonctionnelles. Mémoire de M2, UHP Nancy 1.