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 αs1-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), YQEPVLGPVRGPFPIIV (f193-206), EPVLGPVRGPFPIIV (f195-206), PVLGPVRGPFPIIV (f196-206), VLGPVRGPFPIIV (f197-206), and LGPVRGPFPIIV (f198-206) for β-CN; and APFSDFNPIGSENSEKTTMPLW (f176-199), SFDIPNIGSENSEKTTMPLW (f178-199), SDIPNIGSENSEKTTMPLW (f180-199), DIPNIGSENSEKTTMPLW (f181-199), IPNIGSENSEKTTMPLW (f182-199), PIGSENSEKTTMPLW (f185-199), IGSSENSEKTTMPLW (f186-199), and SENSEKTTMPLW (f188-199) for αs1-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; Martin-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 (Arunchalam, 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* (*L. rhamnosus*), *Streptococcus thermophilus* (*St. thermophilus*), *Lactobacillus lactis* (*L. lactis*), *Lactobacillus helveticus* (*L. 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-
tides (Genay et al., 2009; Gilbert et al., 1996; Miclo et al., 2012; Pastar et al., 2003; Sadat-Mekene 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* BI 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, antimicrobials, 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* BI 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 angiotensin 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-histidyl-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 cutoff 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 at 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 resuspended 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™ 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 cutoff 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 205-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 protolitic 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 |
|-----------------|-----------------|-----------------|
| A_{228}         | 0.715±0.027     | 0.375±0.011     |
| Activity (%)    | 0               | 62.3            |

All assays were carried out in triplicate.
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9156 in milk.

**Peptides generated from milk casein during fermentation by \textit{Bifidobacterium longum} KACC9156**

The peptides generated from casein were 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 \textit{Bifidobacterium longum} KACC9156. The results indicated that CN was the preferential substrate of \textit{B. longum} in spite of the presence of whey protein in skim milk, similar to a strain of \textit{St. thermophilus} (Chang et al., 2014). A total of 28 peptides were generated, corresponding to 11 β-CN, 16 α\textsubscript{51}-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\textsubscript{51}-CN, and 2 κ-CN) in bovine CN hydrolysates obtained from a 0.1% CN solution with the same strain of \textit{B. longum}. This difference could be explained by a difference in the accessibility of peptides for hydrolysis by \textit{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 \textit{Lactobacillus helveticus} (Sadat-Mekmene et al., 2011a), \textit{Lactobacillus delbrueckii} subsp. lactis CRL 581 (Hebert et al., 2008), \textit{Lactobacillus bulgaricus} (Tsakalidou et al., 1999), \textit{Lactobacillus lactis} subsp. cremoris (Reid et al., 1991), and \textit{S. thermophilus} (Miclo et al., 2012). On the other hand, the N terminus of β-CN was resistant to hydrolysis by \textit{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 \textit{B. longum} (Chang et al., 2013).

### 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 \textit{Bifidobacterium longum} KACC9156

| 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, MPFPKYVPEFTEQSL | 1995.9216 | 998.9681 | 1995.9652 | 998.9899 | 2  |
| f193-206, YQEPVLGPRGFPFP | 1554.7750 | 778.3948 | 1554.8195 | 778.4170 | 2  |
| f193-209, YQEPVLGPRGFPFPII | 1880.0123 | 941.0134 | 1880.0560 | 941.0353 | 2  |
| f194-209, QVEQVLGPRGFPFPIV | 1699.9307 | 850.9726 | 1699.9662 | 850.9904 | 2  |
| f195-206, EPVLGPRGFPFP | 1263.6628 | 632.8387 | 1263.6975 | 632.8561 | 2  |
| f195-209, EPVLGPRGFPFPII | 1588.9012 | 795.4579 | 1588.9341 | 795.4743 | 2  |
| f196-209, PVLGPRGFPFPIIV | 1459.8593 | 730.9369 | 1459.8915 | 730.9530 | 2  |
| f197-209, VLGPRGFPFPIIV | 1362.8054 | 642.4100 | 1362.8387 | 642.4266 | 2  |
| f198-209, LGPRGFPFPIIV | 1263.7390 | 632.8768 | 1263.7704 | 632.8925 | 2  |
| f199-209, GPVRGFPFPIIV | 1150.6611 | 576.3378 | 1150.6863 | 576.3504 | 2  |
| α\textsubscript{51}-CN | f11-21, LPQEVLENLLRF | 1302.6389 | 652.3267 | 1302.6796 | 652.3470 | 2  |
| f11-23, LPQEVLENLLRF | 1583.8263 | 792.9204 | 1583.8672 | 792.9409 | 2  |
| f12-34, APFPEVFKG | 990.4937 | 496.2541 | 990.5175 | 496.2660 | 2  |
| f176-190, APSFDPNIPGSENKTMPP | 2022.9614 | 1012.4880 | 2022.9915 | 1012.5125 | 2  |
| f176-199, APSFDPNIPGSENKTMP | 2449.0955 | 1225.5550 | 2449.1472 | 1225.5808 | 2  |
| f179-199, FSDNPIPGSENKTMP | 2362.0474 | 1182.0310 | 2362.1152 | 1182.0648 | 2  |
| f180-199, SDIPNIPGSENKTMP | 2214.9893 | 1108.5020 | 2215.0466 | 1108.5306 | 2  |
| f181-199, DIPNIPGSENKTMP | 2127.9514 | 1064.9830 | 2128.0146 | 1065.0146 | 2  |
| f182-198, IPNIPGSENKTMP | 1808.8452 | 905.4299 | 1809.8978 | 905.4562 | 2  |
| f182-199, IPNIPGSENKTMP | 2028.9335 | 1015.4740 | 2028.9827 | 1015.4986 | 2  |
| f185-199, IPNIPGSENKTMP | 1704.7677 | 853.3911 | 1704.8029 | 853.4087 | 2  |
| f186-199, IGSENKTMP | 1613.6985 | 807.8565 | 1613.7372 | 807.8759 | 2  |
| f188-199, IGSENKTMP | 1421.6108 | 711.8127 | 1421.6497 | 711.8321 | 2  |
| f199-209, GPVRGFPFPIIV | 1150.6611 | 576.3378 | 1150.6863 | 576.3504 | 2  |
| κ-CN | f151-169, EVIESPEINTQVTSTAV | 2033.9755 | 1017.9950 | 2034.0133 | 1018.0139 | 2  |

CN: casein, m/z = mass to charge ratio, where z = number of positively charged ions.
and by the proteases of *S. thermophilus* (Chang et al., 2014; Miclo et al., 2012) and *L. helveticus* (Sadat-Mekmene et al., 2011a).

For αs1-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 αs1-CN. The reason for the different cleavage patterns might be that the structure of αs1-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 αs1-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 αs1-CN.
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 \textit{B. longum} could induce a structural change due to weak bond of glycan chains on this glycomacropeptide region that would allow weak bond of glycan chains on this glycomacropeptide region that would allow \textit{B. longum} to access \( \alpha_{s_2} \)-CN for hydrolysis.

As the previously study by Chang \textit{et al.} (2013) who reported that no peptide detected from \( \alpha_{s_2} \)-CN from 0.1% to weak bond of glycan chains on this glycomacropeptide region that would allow weak bond of glycan chains on this glycomacropeptide region that would allow \textit{B. longum} to access \( \alpha_{s_2} \)-CN for hydrolysis.\n
As the previously study by Chang \textit{et al.} (2013) who reported that no peptide detected from \( \alpha_{s_2} \)-CN from 0.1%

### Table 3. Antihypertensive and potential antihypertensive peptides generated by fermentation by \textit{Bifidobacterium longum} KACC 91563 after 24 h at 37°C

| Sequence | Fragment | Source | Proteolytic agent | References |
|----------|----------|--------|-------------------|------------|
| LNVPGEIVE | \( \beta \)-CN(16-14) | milk | \textit{Lb. bulgaricus} | Gobbetti \textit{et al.}, 2000 |
| DKIHPF | \( \beta \)-CN(47-52) | milk | \textit{L. lactis} subsp. cremoris | Gobbetti \textit{et al.}, 2000 |
| LVYPEP | \( \beta \)-CN(58-63) | milk | \textit{B. bifidum} | Gonzalez-Gonzalez \textit{et al.}, 2013 |
| NIPPLQTPV | \( \beta \)-CN(73-82) | milk | \textit{Lb. bulgaricus} | Gobbetti \textit{et al.}, 2000 |
| EMPFPK | \( \beta \)-CN(f108-113) | casein | milk starter + pepsin and trypsin | Pihlanto-Leppala \textit{et al.}, 1998 |
| HLPLLP | \( \beta \)-CN(f134-140) | casein | pepsin | Del Mar Contreras \textit{et al.}, 2009 |
| SQSKVLVPQ | \( \beta \)-CN(f166-175) | sodium caseinate | \textit{Lb. animalis} | Hayes \textit{et al.}, 2007a |
| SKVLPPQ | \( \beta \)-CN(f168-175) | \( \beta \)-casein | protease of \textit{Lb.helveticus} | Yamamoto \textit{et al.}, 1994 |
| KVLPPQ | \( \beta \)-CN(f169-175) | \( \beta \)-casein | protease of \textit{Lb.helveticus} | Maeno \textit{et al.}, 1996 |
| RDMPIQAF | \( \beta \)-CN(f183-190) | \( \beta \)-casein | protease of \textit{Lb.helveticus} | Yamamoto \textit{et al.}, 1994 |
| LLYQPVLG-PVRGPFP | \( \beta \)-CN(f191-209) | \( \beta \)-casein | protease of \textit{Lb.helveticus} | Yamamoto \textit{et al.}, 1994 |
| YQEPVL | \( \beta \)-CN(f193-198) | casein | milk starter + pepsin and trypsin | Pihlanto-Leppil \textit{et al.}, 1998 |
| YQEPVLGPVR | \( \beta \)-CN(f193-202) | milk | \textit{Lb casei} ssp. \textit{rhamnosus} | Rotka \textit{et al.}, 1997 |
| YQEPVLGPVRG-PFP | \( \beta \)-CN(f193-208) | casein | trypsin | Maruyama and Suzuki, 1982 |
| YQEPVLGPVRG-PFP | \( \beta \)-CN(f193-209) | \( \beta \)-casein | protease of \textit{Lb.helveticus} | Yamamoto \textit{et al.}, 1994 |
| QEPVLGPVRG-PFP | \( \beta \)-CN(f194-209) | milk | \textit{L. lactis} + chymosin/trypsin/ chymotrypsin | Gobbetti \textit{et al.}, 2002 |
| GPVPGPD-PP | \( \beta \)-CN(f199-209) | Manchego cheese | protease in Manchego cheese | Gomez-Ruiz \textit{et al.}, 2002 |
| AVYPQR | \( \beta \)-CN(f176-182) | milk | lactic acid bacteria | Hernandez-Ledesma \textit{et al.}, 2004 |
| YQEP | \( \beta \)-CN(f191-198) | Gouda cheese | Proteases from \textit{Cynara cardunculus} | Silva \textit{et al.}, 2006 |
| GPFPV | \( \beta \)-CN(f203-209) | milk | protease of \textit{Lb.helveticus} | \textbf{Yamamoto \textit{et al.}, 1994; Hayes \textit{et al.}, 2007b} |
| RPKHIHKH | \( \alpha_{s_1} \)-CN(f1-9) | Gouda cheese | Proteases from \textit{Cynara cardunculus} | Silva \textit{et al.}, 2006 |
| FF | \( \alpha_{s_1} \)-CN(f23-24) | casein | trypsin | Maruyama and Suzuki, 1982 |
| FFVAP | \( \alpha_{s_1} \)-CN(f23-27) | casein | trypsin | Maruyama and Suzuki, 1982 |
| FFVAPPEVFGK | \( \alpha_{s_1} \)-CN(f23-34) | sodium caseinate | \textit{Lb. animalis} | Hayes \textit{et al.}, 2007a |
| YKVPQL | \( \alpha_{s_1} \)-CN(f104-109) | \( \alpha_{s_2} \)-casein | protease of \textit{Lb.helveticus} | Maeno \textit{et al.}, 1996 |
| LAYPPY | \( \alpha_{s_2} \)-CN(f142-147) | \( \alpha_{s_2} \)-casein | protease of \textit{Lb.helveticus} | Maeno \textit{et al.}, 1996 |
| DAYPSGAW | \( \alpha_{s_2} \)-CN(f157-164) | \( \alpha_{s_2} \)-casein | milk starter + pepsin and trypsin | Pihlanto-Leppala \textit{et al.}, 1998 |
| TTMPLW | \( \alpha_{s_2} \)-CN(f194-199) | \( \alpha_{s_2} \)-casein | pepsin | Maruyama and Suzuki, 1982; Pihlanto-Leppala \textit{et al.}, 1998 |
| FALQYLYK | \( \alpha_{s_2} \)-CN(f174-181) | \( \alpha_{s_2} \)-casein | trypsin | Tauzin \textit{et al.}, 2002 |
| AMKWIQPK | \( \alpha_{s_2} \)-CN(f189-197) | \( \alpha_{s_2} \)-casein | protease of \textit{Lb.helveticus} | Maeno \textit{et al.}, 1996 |
| MKPWIQPK | \( \alpha_{s_2} \)-CN(f190-197) | \( \alpha_{s_2} \)-casein | protease of \textit{Lb.helveticus} | Maeno \textit{et al.}, 1996 |
| TKVIP | \( \alpha_{s_2} \)-CN(f198-202) | \( \alpha_{s_2} \)-casein | protease of \textit{Lb.helveticus} | Maeno \textit{et al.}, 1996 |
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), YQEPVLGPVRGPFPIIV, QEPVLGPVRGPFPIIV, GPVRGPFPPIIV 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. (2000), 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, Ile and Ser called aliphatic amino acids at the N-terminal (Jao et al., 2012). The three peptides, YQEPVLGPVRGPFPIIV, QEPVLGPVRGPFPIIV, GPVRGPFPPIIV 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 αs1-CN. Peptide, RELEELNVPGEIVE (f1-14) released from β-CN identified in present study contains LNVPGEIVE reviewed by Gobbetti et al. (2000) as ACE inhibitory peptide. Another peptide, YQEPVLGPVRGPFPIIV (f193-202) from β-CN, contains also the ACE inhibitory peptide, YQEPVLGPVRGPFPIIV (Rokka et al., 1997). The others from β-CN, the fragment GPVRGPFPPIIV reviewed by Gomez-Ruiz et al. (2002) as antihypertensive peptide was included in 4 peptides, EPVLGPVRGPFPIIV, VPVLGPVRGPFPIIV, VEPVLGPVRGPFPIIV, LPGVRPGPFPIIV.

For peptides obtained from αs1-CN, the 9 peptide, APSFSDFNPQIGSENSEKTTMPLW at the extremity of C-terminal and Ala, Val, Ile and Ser called aliphatic amino acids at the N-terminal (Jao et al., 2012). The three peptides, YQEPVLGPVRGPFPIIV, QEPVLGPVRGPFPIIV, GPVRGPFPPIIV 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.

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After milk fermentation with B. longum KACC 91563 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 gastrointestinal enzymes 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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