Casein hydrolysis by *Bifidobacterium longum* KACC91563 and antioxidant activities of peptides derived therefrom

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ABSTRACT

Milk protein is a well-known precursor protein for the generation of bioactive peptides using lactic acid bacteria. This study investigated the antioxidant activity of bovine casein hydrolysate after fermentation with *Bifidobacterium longum* KACC91563 using the 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay and total phenolic content (TPC). The antioxidant activities of the 24-h and 48-h hydrolysates were higher than that of the 4-h hydrolysate (2,045.5 and 1,629.3 μ M gallic acid equivalents, respectively, vs. 40.3 μ M) in the ABTS assay. In contrast, TPC values showed activities of 43.2 and 52.4 μ M gallic acid equivalents for the 4-h and 24-h hydrolysates, respectively. Three fractions (≥ 10 kDa, ≥ 3 but < 10 kDa, and < 3 kDa) were separated from the 24-h hydrolysate by ultrafiltration. Among these fractions, the < 3 kDa fraction exhibited the highest antioxidant activity (936.7 μ M) compared with the other fractions (42.1 and 34.2 μ M for > 10 kDa and 3–10 kDa fractions, respectively). Through liquid chromatography-electrospray ionization-tandem mass spectrometry analysis, 2 peptides, VLSLSQSKVLPVPQK and VLSLSQSKVLPVPQKAVPYPQRDMPIQA, containing the fragment VLPVPQ that has antioxidant properties, were identified in the < 3 kDa fraction after 24 h of hydrolysis. The present study demonstrates the possibility of antioxidant peptide production from bovine casein using *Bifidobacterium longum*.

Key words: *Bifidobacterium longum*, antioxidant, bioactive peptide, casein hydrolysis

INTRODUCTION

Interest in probiotic bifidobacteria, which are gram-positive anaerobic bacteria found in human and animal intestinal tracts, has been increasing given their beneficial health effects including inhibition of pathogenic

species, reduction of colon cancer risk, protective effects on immune function, regulation of gut microflora resistance to microbial infections and serum cholesterol reduction (Leahy et al., 2005; Yu et al., 2002; Arunachalam, 1999; Collins and Gibson, 1999). In addition, probiotic bacteria play important roles in the prevention of allergic disease in early infancy (Kalliomaki et al., 2001). Given their beneficial effects, strains such as *Bifidobacterium longum*, *Bifidobacterium breve*, *Bifidobacterium animalis*, and *Bifidobacterium bifidum* are used as probiotic bacteria in the manufacture of milk products; for example, fermented milks, cheese, infant formulas, or food supplements (Davidson et al., 2000; McBrearty et al., 2001; Martín-Diana et al., 2003; Saavedra et al., 2004).

Bifidobacterium, a genus that belongs to the group of lactic acid bacteria (LAB; Yin et al., 2010; Cha et al., 2012), grows well in skim milk supplemented with milk protein hydrolysates producing lactic acid and exopolysaccharide (Prasanna et al., 2012), similar to other LAB, including *Lactococcus lactis*, *Lactobacillus rhamnosus*, *Streptococcus thermophilus*, *Lactobacillus lactis*, *Lactobacillus helveticus*, and *Lactobacillus bulgaricus*. They are auxotrophic for nitrogen sources (e.g., leucine and peptides; Mattarelli and Biavati, 1999). To obtain these nitrogen sources containing leucine, they use their proteolytic system as do other LAB. In fact, *Bifidobacterium animalis* ssp. *lactis* can hydrolyze milk protein to peptides using its proteolytic system (Janer et al., 2005). Some of these peptides have been reported to be bioactive. In general, bioactive peptides are generated from casein in different ways, such as (1) in vitro proteolysis and subsequent addition of the generated peptides to food, (2) in vivo gastrointestinal enzymatic digestion by microbial enzymes in the gastrointestinal tract of organisms, or (3) by lactic acid bacteria during fermentation (Korhonen, 2009). It is well known that milk proteins such as casein and whey proteins are precursor proteins that generate bioactive peptides released by the proteolytic system of LAB. Milk-based bioactive peptides released by LAB are well documented in the literature (Hayes et al., 2007; Sadat-Mekmene et al., 2011; Chang et al., 2012; Miclo

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et al., 2012). For example, the VPP and IPP peptides from β -casein are best known for their angiotensin-converting enzyme (ACE) inhibition activity, and the fermented milks Calpis (Calpis Co. Ltd., Tokyo, Japan) and Evolus (Valio Ltd., Helsinki, Finland) containing the VPP and IPP peptides are commercially available (Korhonen, 2009). In addition, many peptides generated from casein by microbial fermentation are ACE inhibitory peptides, in contrast to other bioactive peptides (e.g., antioxidant peptides, opiates, antimutagens, immunomodulatory peptides, antimicrobial peptides, or peptides with mineral binding activity).

Among the various bioactive peptides, those with antioxidant properties are also important because increased levels of reactive oxygen species such as free radicals cause cancer, DNA damage, diabetes, cardiovascular diseases, allergies, and aging (Wolff et al., 1991; Beckman and Ames, 1998; Agerholm-Larsen et al., 2000). However, the defense systems of organisms are not sufficient to prevent oxidative damage (Kullisaar et al., 2003). Therefore, other antioxidant substances originating from plants or food, particularly milk protein from dairy products, may be provided to organisms as a supplement to prevent such damage.

However, production of bioactive peptides by *Bifidobacterium* is rarely documented in the literature, except for research on the hypocholesterolemic effect of casein hydrolysate after incubation with *Bifidobacterium animalis* ssp. *lactis* strain Bb12 and of yogurt containing *Bifidobacterium pseudocatenulatum* G4 (or *Bifidobacterium longum* BB536), reported by Alhaj et al. (2010) and Al-Sheraji et al. (2012), respectively. On the contrary, we can find no reports to date of other bioactive peptides including antioxidant peptides generated by *Bifidobacterium* strains. For this reason, the objective of this work was to investigate potential antioxidant peptides released from bovine casein after hydrolysis by *Bifidobacterium longum* KACC91563.

MATERIALS AND METHODS

Materials

Chemical reagents including 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), gallic acid, Folin-Ciocalteu reagent, and the chromogenic substrate lysine-*p*-nitroanilic acid (Lys-pNA) were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals used were of analytical grade.

Isolation and Retrieval of *Bifidobacterium longum* KACC91563

Matrix and standard preparation and identification of the isolate from infant feces were performed using

a previously described method (Ham et al., 2011). After isolation of this strain, *Bifidobacterium longum* KACC91563 was grown in de Man, Rogosa, and Sharpe (MRS) broth (BD Biosciences, San Jose, CA) containing cysteine (0.05%, final concentration), and the cells (bacteria) were harvested by centrifugation (Beckman Coulter, Brea, CA) at $3,200 \times g$ for 30 min at 4°C. The cells were stored in reconstituted skim milk (10% wt/vol) at -80°C .

Growth Conditions

Before the experiment, *B. longum* was grown overnight in skim milk at 37°C , according to the method previously described (Ruiz et al., 2009). *Bifidobacterium longum* (initial inoculum, 1%) was grown at 37°C in 50 mL of MRS broth supplemented with cysteine (final concentration, 1%).

Casein Hydrolysis

Bifidobacterium longum (initial inoculum, 1%) was grown at 37°C in 50 mL of MRS broth supplemented with cysteine (final concentration, 1%) to the exponential phase (pH 5.1). Then, the cells were harvested by centrifugation (Beckman Coulter) at $3,200 \times g$ for 15 min at 20°C and washed twice with 0.05 M sodium phosphate buffer, pH 7.0, using the same centrifugation conditions. The cells were suspended in 5 mL of the same buffer and mixed with 20 mL of 0.1% (wt/vol) bovine casein (Sigma-Aldrich) solution as a substrate. The cells were then incubated at 37°C for 0, 4, 24, and 48 h, and the supernatant fractions were immediately collected after centrifugation at $13,200 \times g$ for 10 min at 4°C and filtered (0.45- μm filter). The hydrolysates obtained were stored at -20°C before their use in the following experiments.

Peptidase Activity

To verify cell surface peptidase and cell lysis activity of *B. longum*, a peptidase activity assay was performed using the chromogenic substrate Lys-pNA (Miclo et al., 2012). The substrate was prepared at a concentration of 2 mM in 0.05 M sodium phosphate buffer, pH 7.0. *Bifidobacterium longum* grown to the exponential phase (pH 5.1) was harvested by centrifugation at $3,200 \times g$ for 5 min at 20°C . The cell pellet was washed 2 times with 0.05 M sodium phosphate buffer, pH 7.0, and then resuspended in the same buffer. One hundred microliters of cell suspension was then mixed with 1 mL of Lys-pNA solution and incubated for 1 h at 37°C . The reaction mixture was centrifuged at $13,200 \times g$ at 4°C for 10 min to pellet the cells, and the absorbance

of the supernatant was determined at 410 nm using a SpectraMax spectrophotometer (Molecular Devices, Sunnyvale, CA).

Electrophoresis

Sodium dodecyl sulfate-PAGE was performed as previously described (Egito et al., 2002; Chang et al., 2012) using a 5% stacking gel and 15% separating gel. Twenty microliters (about 10 μ g of hydrolysate) was loaded into each well along with broad-range standard molecular mass markers (6, 17, 26, 34, 43, 55, 70, 95, 130, and 170 kDa). After migration, the gel was stained in 0.1% Coomassie Blue R-250 solution containing 2% (wt/vol) TCA and 50% (vol/vol) ethanol. The gel was destained using a mixture of 50% methanol, 10% acetic acid, and 40% water.

Reverse-Phase HPLC

To confirm the hydrolysis of bovine casein, the hydrolysates were separated on a Zorbax 300SB-C18 analytical column (3.0 $\times$ 150 mm, diameter 3.5 μ m, Agilent, Santa Clara, CA) connected to an HPLC system (Jasco, Oklahoma City, OK). Fifty microliters of sample were injected into the column and eluted at 30°C using a linear gradient (5 to 50%) over 90 min. The flow rate was 0.25 mL/min, and peptides were detected by UV absorption at 215 nm.

Antioxidant Activity: Radical Scavenging Assay

Free radical scavenging activity of the casein hydrolysate was determined using ABTS radical cation (ABTS^{•+}) according to previously described method with minor modifications (Re et al., 1999; Sadat et al., 2011). To generate the ABTS^{•+} radical, ABTS and potassium persulfate were prepared at final concentrations of 7 and 2.45 mM, respectively, and incubated for 16 h in the dark at 30°C. Using a spectrophotometer (Molecular Devices), the ABTS^{•+} radical was adjusted to an absorbance of 0.70 ± 0.02 at 735 nm by diluting with distilled water. Fifty microliters of casein hydrolysate was mixed with 950 μ L of the ABTS^{•+} radical solution. The decrease of the absorbance was monitored at 735 nm after 30 min at 30°C. All assays were carried out in triplicate, and the values represent the means. A standard curve was prepared using various concentrations (1, 2, 20, 40, 60, 80, 100, and 120 μ M) of gallic acid. For standard curve calculation, the percentage antioxidant activity was calculated as follows: Scavenging activity (%) = $[(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}] \times 100$, where A_{control} represents the initial ABTS absorbance. The radical scavenging activity of the casein hydroly-

sates was calculated from the equation of the standard curve based on gallic acid and expressed as micromoles of gallic acid equivalent (GE).

Antioxidant Activity: Determination of Total Phenolic Contents

Total phenolic content (TPC) measurement was followed by the previously described method (Singleton and Rossi, 1965; Yu et al., 2006; Yang et al., 2008) with minor modifications. For this purpose, a volume of 60 μ L of sample was mixed with 60 μ L of the Folin-Ciocalteu solution, which was prepared by mixing 1 volume of Folin-Ciocalteu reagent with 2 volumes of distilled water. Finally, 60 μ L of 10% sodium carbonate (Na₂CO₃) solution was added in this mixture. After incubation of the mixture at room temperature for 1 h in the dark, absorbance at 700 nm was measured using a spectrophotometer (Molecular Devices). All assays were carried out in triplicate and the values represent the means. A standard curve was prepared using various concentrations (1, 2, 20, 40, 60, 80, 100, and 120 μ M) of gallic acid. The TPC of the casein hydrolysates was calculated from the equation of the standard curve based on gallic acid and expressed as micromoles of GE.

Fractionation of Hydrolysates

Fractions with molecular weight cutoffs of 10 and 3 kDa were separated using an ultrafiltration membrane system (Millipore, Billerica, MA).

Mass Spectrometry Analysis

Liquid chromatography tandem MS (LC-MS/MS) experiments were carried out using an integrated system consisting of autoswitching nano pump, autosampler (Tempo nano LC system, MDS Sciex, Toronto, Ontario, Canada) and a hybrid quadrupole-time-of-flight (TOF) tandem mass spectrometer (QStar Elite, Applied Biosystems, Foster City, CA) equipped with a nano-electrospray ionization (ESI) source and fitted with a fused-silica emitter tip (New Objective, Woburn, MA). This experiment was performed at National Instrumentation Center for Environmental Management (NICEM) of Seoul National University in Korea. Fractions (2 μ L) were injected into LC-nano ESI-MS/MS system. Solvent A consisted of water/acetonitrile (98:2, vol/vol) with 0.1% formic acid for the high aqueous mobile phase. Samples were first trapped on a Zorbax 300SB-C18 trap column (300- μ m i.d. $\times$ 5 mm, 5- μ m particle size, 100 Å pore size, part number 5065-9913; Agilent Technologies, Santa Clara, CA) and washed for

6 min with 98% solvent A [water/ACN (98:2, vol/vol), 0.1% formic acid] and 2% solvent B [water/ACN (2:98, vol/vol), 0.1% formic acid] at a flow rate of 5 μ L/min, and then separated on a Zorbax 300SB-C18 capillary column (75- μ m i.d. $\times$ 150 mm, 3.5- μ m, 100 Å pore size, part number 5065-9911; Agilent Technologies) at a flow rate of 300 nL/min. The LC gradient was run at 2 to 35% solvent B over 30 min, and then from 35 to 90% over 10 min, followed by 90% solvent B for 5 min, and finally 5% solvent B for 15 min. Resulting peptides were electrosprayed through a coated silica tip (FS360-20-10-N20-C12, PicoTip emitter, New Objective) at an ion spray voltage of 2,000 eV. Mass data were acquired automatically using Analyst QS 2.0 software (Applied Biosystems). The range of m/z was 200 to 2,000.

RESULTS AND DISCUSSION

Production of Peptides from Casein Hydrolysis of *Bifidobacterium longum*

Bifidobacterium longum was grown to exponential phase in MRS broth containing cysteine. The cells were retrieved and incubated with bovine casein in 0.05 *M* sodium phosphate buffer, pH 7.0. Casein hydrolysates were obtained after 0, 4, 24, and 48 h of hydrolysis by centrifugation and filtration (0.45 μ m). Bovine casein was solubilized in 0.05 *M* sodium phosphate buffer at pH 7. This pH is similar to that of milk (6.6–6.8), indicating the similar environment (Miclo et al., 2012).

The casein hydrolysates were analyzed by SDS-PAGE to verify the hydrolysis patterns after the above-mentioned incubation periods. Figure 1 shows the hydrolysis of bovine casein by *B. longum* after 24 and 48 h of incubation, in particular, of β -casein compared with α_{S1} -casein and α_{S2} -casein. Similar observations have been reported in several studies on LAB in the literature, including *Lc. lactis*, *Lb. rhamnosus*, *Strep. thermophilus*, *Lb. lactis*, *Lb. helveticus*, and *Lb. bulgaricus* (Kok et al., 1988; Shahbal et al., 1993; Gilbert et al., 1996; Fernandez-Esplá et al., 2000; Pastar et al., 2003; Espeche Turbay et al., 2009; Sadat-Mekmene et al., 2011). Thus, in the case of *B. longum*, as with other LAB, β -casein is preferentially degraded compared with α_{S1} -casein and α_{S2} -casein. The different accessibilities may be explained by structural differences between individual caseins. Compared with other caseins, β -casein is more unstructured and more accessible to cleavage, and is therefore hydrolyzed to a greater extent (Holt and Sawyer, 1993; Kumosinski et al., 1993; Sadat-Mekmene et al., 2011).

This observation of casein degradation was confirmed by HPLC profiles. As shown in Figure 2, the number of peaks increased with incubation time, especially after

Figure 1. Electrophoretic patterns of bovine casein hydrolyzed by *Bifidobacterium longum* KACC91563. Twenty microliters (about 10 μ g of hydrolysate) was loaded in each well. M denotes protein molecular weight markers. Cells were grown in de Man, Rogosa, and Sharpe broth until the pH reached 5.1 and then were collected after centrifugation. Caseins were solubilized at 0.1% (wt/vol) in 0.05 *M* Na phosphate buffer, pH 7.0. One volume of cell suspension of *B. longum* was incubated with 4 volumes of caseins at 37°C and the hydrolysate used as a sample was recovered at each incubation time: (a) 0 h, (b) 4 h, (c) 24 h, and (d) 48 h.

hydrolysis for 24 and 48 h, in contrast to the minimal casein degradation seen at elution times under about 40 min at 0 h of incubation. This indicates that the casein is well degraded by *B. longum* in the present study, although no protease activity was detected for *B. longum* using the chromogenic substrate (Suc-Ala-Ala-Pro-Phe-pNa) and zymography (data not shown). This has also been proven by Janer et al. (2005), who reported the absence of protease activity of *B. animalis* ssp. *lactis* in whole cells, the cell wall-bound fraction, and cell extracts. In some strains such as *B. longum* DJO10A (accession number YP_001955201.1) or *B. longum* ssp. *infantis* ATCC 15697 (accession number YP_002322571.1), a subtilisin-like serine protease is present, but there are no reports of the genetic and biochemical characterization and identification for this protease, similar to the cell envelope protease (CEP) of LAB. In fact, some LAB use their CEP to hydrolyze milk protein for growth (Savijoki et al., 2006).

On the other hand, whole cells in the *B. animalis* strain can degrade casein after 24 h of incubation; this could be a result of cell lysis, causing degradation of casein by enzymes in the intracellular fraction, such as peptidase O (pep O) with 67.4% identity in *B. longum* (Janer et al., 2005). However, we observed no peptidase activity using Lys-pNA in cell suspensions without casein after 0, 4, 24, and 48 h of incubation in the present study, which indicates the absence of cell lysis. Therefore, the degradation of casein by *B. long-*

um was not due to intracellular peptidases released by cell lysis. However, peptidase activity using Lys-pNA was detected in the whole cell fraction [absorbance at 410 nm (A_{410}) = 0.921, which corresponds to about 38.4 nmol/min per mL of pNA released, calculated by Lambert-Beer law's equation]. This means that casein could be hydrolyzed by potential peptidases linked to the cell wall or another proteolytic system. Further study is needed to identify and characterize this peptidase.

Figure 2. Reverse phase-HPLC analysis of bovine casein (0.1%, wt/vol) hydrolysis by fermentation of *Bifidobacterium longum* KACC9156. Fifty microliters of sample was injected onto the column and eluted at 30°C by using a linear gradient (5 to 50%) of solvent B (0.1% trifluoroacetic acid in acetonitrile) within 90 min. The flow rate was 0.25 mL/min, and peptides were detected by UV absorption at 215 nm.

Figure 3. Relationship between concentration and ABTS [2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] free radical scavenging capacity (A) and total phenolic contents (B) of gallic acid as a standard. Data are expressed as the average value of triplicates. The equation for this curve is $y = 0.8106x + 4.377$, with an R^2 value of 0.99 for ABTS free radical scavenging capacity and $y = 0.0182x - 0.0039$; $R^2 = 0.99$ for total phenolic contents.

Investigation of Antioxidant Activity in Hydrolyzed Fractions Using ABTS Radical Scavenging Capacity

To investigate the free radical scavenging properties of casein hydrolysates by *B. longum* according to incubation times, the ABTS method, which is widely used to investigate free radical scavenging properties for antioxidant activity, was adapted from a previously

described method (Re et al., 1999; Sadat et al., 2011). In the present study, none of the casein hydrolysate fractions were detected with the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, another free radical scavenging activity assay (data not shown). This could be due to the nature of compounds in the sample. Arnao (2000) reported that the ABTS radical is soluble in aqueous and organic media, whereas the DPPH radical dissolves only in organic media. Therefore, in this study, the hydrolysate fractions were analyzed using the ABTS radical scavenging method.

Various concentrations of gallic acid, a strong antioxidant molecule, were used to generate the standard curve. The reaction mixture (gallic acid and ABTS cation radical) was incubated at 30°C for 30 min. The linear standard curve with a range from 0 to 120 μM gallic acid is presented in Figure 3A. From the equation of the standard curve, the IC_{50} value (the concentration that inhibits the activity by 50%) was 56 μM gallic acid.

The free radical scavenging properties of casein hydrolysates following different incubation periods are presented in Table 1. Antioxidant properties improved with increasing duration of hydrolysis with *B. longum*. The 24- and 48-h hydrolysates exhibited higher antioxidant capacities than did the other hydrolysates. Their GE values deduced from the standard curve equation of gallic acid were 2,045.5 and 1,629.3 μM, respectively (Table 1). A blank sample (casein solution after 24-h incubation without *B. longum* cells) was measured as a control for casein autolysis and showed a GE value similar to that seen at 0 h. Thus, it appears that casein autolysis does not result in antioxidant activity at 4, 24, or 48 h. The different GE values may be due to more extensive hydrolysis at 48 h than at 24 h. Consequently, the smaller peptides generated after 48 h of hydrolysis may lack antioxidant activity.

The GE values at 0 and 4 h of hydrolysis were 120.1 and 140.3 μM, respectively (Table 1), which corresponded to 5.8 and 6.8% (0 and 4 h vs. 24 h) and 7.3 and 8.6% (0 and 4 h vs. 48 h) of the later values, respectively. Thus, the 24-h and 48-h hydrolysates may contain antioxidant peptides (or antioxidant substances) from bovine casein.

Investigation of TPC in Hydrolyzed Fractions

Phenolic content analysis was performed to detect phenolic amino acids in the peptides and to confirm antioxidant activity. Gallic acid was used to generate the standard curve. The linear standard curve with a range from 0 to 100 μM gallic acid is presented in Figure 3B. The GE values of TPC in the <3 kDa fraction correspond to 6.5, 43.2, 52.4, and 20.2 μM for 0, 4, 24, and 48 h, respectively (Table 1), showing low GE val-

Table 1. Antioxidant activities in gallic acid equivalents (GE, μM) of casein hydrolysates after incubation with *Bifidobacterium longum* KACC91563

Item ¹	Incubation time (h)			
	0	4	24	48
ABTS free radical scavenging capacity	120.1	140.3	2,045.5	1,629.3
Total phenolic content (TPC)	6.5	43.2	52.4	20.2

¹ABTS = 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid); gallic acid equivalents were calculated using the standard curve equation after triplicate measurement of absorbance (at 735 nm for ABTS and at 700 nm for TPC).

ues compared with those seen with ABTS scavenging activity. Unlike the results of ABTS radical scavenging activity, the hydrolysate after 4 h of incubation had a high GE value (43.5 μM) compared with that at 48 h (20.2 μM). Regardless of the antioxidant activity measurement method, the highest GE value was seen for the hydrolysate after 24 h incubation. This fraction had a relatively low content of peptides with phenol groups (e.g., tyrosine) compared with other antioxidant peptides detected by ABTS scavenging activity.

Antioxidant Properties in Fractions Separated According to Molecular Weight by Ultrafiltration

For further analysis, the 24-h hydrolysate was fractionated according to molecular weight using ultrafiltration. Using cut-off values of 10 and 3 kDa, the 24-h hydrolysate was separated into 3 fractions (>10 kDa, 3–10 kDa, and <3 kDa). The <3 kDa fraction exhibited the highest free radical scavenging capacity after 10-fold dilution (80.3%), corresponding to 936.7 μM GE (Table 2). The capacities of the >10 kDa and 3–10 kDa fractions were 38.5 and 32.1%, respectively, which corresponded to 42.1 and 34.2 μM GE, respectively. Thus, potential antioxidant peptides derived from bovine casein hydrolysis with *B. longum* may be present in the <3 kDa fraction, given that most milk protein-derived bioactive peptides with antioxidant properties reported in the literature are small (<10 AA). Thus, this fraction was used for the identification of peptides using LC-ESI-MS/MS analysis.

Identification of Peptides Generated from Bovine Casein in the <3 kDa Fraction

The peptides generated from bovine casein were further identified by LC-ESI-quantitative time-of-flight MS analysis. In the <3 kDa fraction, the peptides were generated to a greater extent from β -casein than from other caseins (α -casein and κ -casein), consistent with results of the SDS-PAGE and reversed phase-HPLC profiles. Nineteen peptides were generated from β -casein, but only 12 were generated from α_{S1} -casein and 2 from κ -casein (Table 3).

β -Casein, compared with other caseins, could be a preferred substrate for *B. longum* as for LAB (Juillard et al., 1995; Sadat-Mekmene et al., 2011; Chang et al., 2012; Miclo et al., 2012). To hydrolyze casein, LAB use their CEP, which is linked to peptidoglycan at the cell wall by the action of sortase (Chang et al., 2012). To date, 6 types of CEP in LAB have been reported, including PrtP for *Lc. lactis*, PrtR for *Lb. rhamnosus*, PrtS for *Strep. thermophilus*, PrtL for *Lb. lactis*, PrtH for *Lb. helveticus*, and PrtB for *Lb. bulgaricus*; these facilitate bacterial growth themselves and produce peptides (Savijoki et al., 2006; Espeche Turbay et al., 2009). As mentioned above, in the case of *B. longum*, no CEP activity was detected in the cell wall using zymography and using the chromogenic substrate Suc-Ala-Ala-Pro-Phe-pNA (Sigma). Similar to *B. longum* in the present study, the *Strep. thermophilus* CNRZ1066 strain without the gene encoding CEP did not hydrolyze casein. This strain also had no intracellular peptidase activity

Table 2. Antioxidant activities of casein hydrolysate fractions obtained after 24 h of hydrolysis by *Bifidobacterium longum* KACC91563¹

Activity	Fraction		
	≥ 10 kDa	≥ 3 but <10 kDa	<3 kDa ²
Absorbance at 735 nm	0.478 $\pm$ 0.009	0.523 $\pm$ 0.020	0.185 $\pm$ 0.011
Scavenging activity (%)	38.5	32.1	80.3
GE ³ (μM)	42.1	34.2	936.7

¹All assays were carried out in triplicate.

²Sample was diluted 10-fold.

³GE = gallic acid equivalents calculated using the standard curve equation.

Table 3. Casein-derived peptides identified by liquid chromatography-electrospray ionization-tandem mass spectrometry in the <3 kDa fraction of the 24-h hydrolysate obtained by fermentation of *Bifidobacterium longum* KACC91563¹

Casein	Peptide sequence	Precursor		Theoretical		z
		MW	m/z	MW	m/z	
β-CN	f1–14, RELEELNVPGEIVE	1,623.8181	812.9163	1,623.8468	812.9307	2
	f127–138, LTDVENLHLPLP	1,359.7151	680.8648	1,359.7399	680.8772	2
	f129–138, DVENLHLPLP	1,145.5861	573.8003	1,145.6080	573.8113	2
	f139–154, LLQSWMHQPHQLPPT	1,924.9486	642.6568	1,924.9618	642.6612	3
	f162–176, VLSLSQSKVLPVPQK	1,621.9622	541.6613	1,621.9767	541.6662	3
	f162–174, VLSLSQSKVLPVP	1,365.7982	683.9064	1,365.8231	683.9188	2
	f162–189, VLSLSQSKVLPVPQKAVPYPQRDMPIQA	3,104.6990	777.1820	3,105.6897	777.4297	4
	f177–190, AVPYPQRDMPIQAF	1,631.7853	816.8999	1,631.8130	816.9138	2
	f190–202, FLLYQEPVLGPVR	1,529.8336	765.9241	1,529.8606	765.9376	2
	f191–209, LLYQEPVLGPVRGPFPIIV	2,106.2126	703.0782	2,106.2241	703.0820	3
	f191–206, LLYQEPVLGPVRGPFPP	1,780.9554	891.4850	1,780.9875	891.5011	2
	f191–202, LLYQEPVLGPVR	1,382.7690	692.3918	1,382.7922	692.4034	2
	f192–209, LYQEPVLGPVRGPFPIIV	1,993.1140	997.5643	1,993.1400	997.5773	2
	f193–206, YQEPVLGPVRGPFPP	1,554.7985	778.4065	1,554.8195	778.4170	2
	f193–209, YQEPVLGPVRGPFPIIV	1,880.0200	941.0173	1,880.0560	941.0353	2
	f194–209, QEPVLGPVRGPFPIIV	1,716.9686	859.4916	1,716.9927	859.5036	2
	f195–209, EPVLGPVRGPFPIIV	1,588.9083	795.4614	1,588.9341	795.4743	2
	f197–209, VLGPRGPFPIIV	1,362.8131	682.4138	1,362.8387	682.4266	2
	f199–209, GPVRGPFPIIV	1,150.6675	576.3410	1,150.6863	576.3504	2
α _{S1} -CN	f8–23, HQGLPQEVNENLLRF	1,905.9950	636.3389	1,906.0061	636.3427	3
	f8–22, HQGLPQEVNENLLR	1,758.9219	587.3146	1,758.9376	587.3198	3
	f10–23, GLPQEVNENLLRF	1,640.8618	821.4382	1,640.8886	821.4516	2
	f10–22, GLPQEVNENLLR	1,493.8003	747.9074	1,493.8202	747.9174	2
	f11–23, LPQEVNENLLRF	1,583.8389	792.9267	1,583.8672	792.9409	2
	f11–22, LPQEVNENLLR	1,436.7737	719.3941	1,436.7987	719.4066	2
	f12–22, PQEVNENLLR	1,323.6910	662.8528	1,323.7147	662.8646	2
	f14–23, EVLNENLLRF	1,245.6467	623.8306	1,245.6718	623.8431	2
	f15–23, VLNENLLRF	1,116.6080	559.3113	1,116.6292	559.3218	2
	f24–36, FVAPFPEVFGKEK	1,493.7756	498.9325	1,493.7919	498.9379	3
	f24–35, FVAPFPEVFGKE	1,365.6743	683.8444	1,365.6969	683.8557	2
	f179–199, FSDIPNPIGSENSEKTTMPLW	2,378.0972	793.7063	2,378.1101	793.7106	3
κ-CN	f40–54, LNYYQKQPVALINNQ	1,804.9138	903.4642	1,804.9471	903.4809	2
	f55–72, FLPYPPYAKPAAVRSPAQ	2,038.0420	680.3546	2,038.0676	680.3632	3

¹MW = molecular weight; m/z = mass to charge ratio, where z = number of positively charged ions.

upon cell lysis and no other cell wall-bound peptidase activity (Miclo et al., 2012). In contrast, in the present work, peptidase activity was detected in the whole-cell fraction without cell lysis during incubation, which was confirmed using Lys-pNA. Thus, in *B. longum*, casein could be hydrolyzed by this potential CEP or another proteolytic system.

The hydrophilic region at the N-terminal region on β-casein was resistant to hydrolysis by *B. longum* (Figure 4). This observation was also made in previous studies showing that the hydrophobic region (especially the C-terminal region) was more accessible to the CEP of *Strep. thermophilus* (Miclo et al., 2012) and *Lb. helveticus* (Sadat-Mekmene et al., 2011).

In the case of α_{S1}-casein, 12 peptides were generated by the fermentation of *B. longum*. The N-terminal region was more accessible to hydrolysis by this strain (Figure 4). The α_{S1}-casein structure is composed of 4 parts (Kumosinski et al., 1993): (1) hydrophilic region (f1–12), (2) hydrophobic region (f13–40), (3) hydrophilic region (to residue 100), (4) hydrophobic region (f100–199). In

the present study, α_{S1}-casein was degraded to a greater extent in the hydrophobic region (f8–35) than in the hydrophilic region, and only 1 peptide was generated in the C-terminal hydrophobic region (f179–199), indicating high resistance in the region of residues 40–175 and residues 181–199. This was similar to the results of Miclo et al. (2012), who reported that the region of residues 1–40 is most susceptible to hydrolysis, whereas the region 41–91 is very resistant to hydrolysis. This observation on the accessibility of each region is not consistent with that of β-casein in the present study.

Only 2 peptides were generated from the N-terminal region of κ-casein in the present work. No peptide was generated from the glycomacropeptide (f106–169) region at the C-terminus. Glycan chains may protect against hydrolysis in this region conferring hydrophilic properties and negative charge (increase in electrostatic repulsions; Zahraa, 2010).

No peptides were identified from α_{S2}-casein in this fraction. α_{S2}-Casein could be more resistant to hydrolysis by *B. longum* due to the formation of a tetrameric

Figure 4. Cleavage region of peptide bonds on bovine β-, α_{s1}-, and κ-caseins hydrolyzed after fermentation with *Bifidobacterium longum* KACC9156. The arrows indicate cleaved peptide bonds.

form of α_{s2}-casein in phosphate buffer (pH 8.1), providing protection in some regions (Tauzin et al., 2003). Investigation of the accessibility of regions containing protein structure seems to be an appropriate method to explain susceptibility to hydrolysis (Miclo et al., 2012).

Potential Antioxidant Peptide in the <3 kDa Fraction

In previous studies, antioxidant peptides have been investigated following the hydrolysis of individual caseins (Table 4). Milk proteins and fermented milk are well-known, food-based sources of natural antioxidants in the form of antioxidant peptides (Hayes et al., 2007). Díaz (2005) reported that casein hydrolysates are promising sources of natural antioxidants because they have potential free radical scavenging ability.

As shown in Table 4, 2 peptides, VLSLSQSKV-LPVPQK and VLSLSQSKVLPVPQKAVPYPQRD-MPIQA, among the peptides generated from β-casein identified through the analysis in this work exhibited antioxidant properties. These peptides contain the fragment β-casein (f185–190), VLPVPQ, which has antioxidant activity. This is consistent with studies by Hayes et al. (2007) and Hernandez-Ledesma et al. (2004), who reported that the peptide β-casein (f181–190), SQSKV-LPVPQ, may contain a potential antioxidant property, given that this peptide contains the fragment β-casein (f185–190), VLPVPQ, which was previously identified from Spanish commercial fermented milk with *Lactobacillus helveticus* and *Saccharomyces cerevisiae*. Rival et al. (2001b) reported that the peptide VLSLSQSKV-LPVPQKAVPYPQRDMPIQA, derived from milk protein after trypsin treatment and containing the pep-

Table 4. Antioxidant peptides identified from bovine milk caseins in the literature

Casein	Sequence	Identification	Reference
β -CN	f98–105, VKEAMAPK	ND ¹	Rival et al., 2001b; Gupta et al., 2010; Miclo et al., 2012
	f184–190, VLPVPQ	VLSLSQSKVLPVPQK VLSLSQSKVLPVPQKA VPYPQRDMPIQA	Hernández-Ledesma et al., 2004
	f177–183, AVPYPQR	VLSLSQSKVLPVPQKA VPYPQRDMPIQA	Rival et al., 2001a
α_{S1} -CN	f144–149, YFYPEL	ND	Suetsuna et al., 2000; Miclo et al., 2012
α_{S2} -CN	f202–207, PYVRYL	ND	Lopez-Expósito et al., 2007; Miclo et al., 2012

¹ND = not detected.

tide β -casein (f177–183), AVPYPQR, has antioxidant activity on the basis of its DPPH scavenging activity. Thus, antioxidant peptide(s) could be generated from β -casein by *B. longum*.

In addition, the peptide β -casein (f98–105), VKEAMAPK, has been reported to have antioxidant properties. Recently, PrtS of *Strep. thermophilus* 4F44 has been shown to generate this fragment (Miclo et al., 2012). In Cheddar cheese inoculated with *Lactobacillus casei* ssp. *casei* 300, this fragment was generated during aging (Gupta et al., 2010). In a synthetic peptide β -casein (f98–105), the antioxidant properties were determined through enzymatically and chemically induced oxidation of the linoleic acid oxidation system, hemoglobin-catalyzed oxidation of linoleic acid, hydroperoxide oxidation, DPPH assay, and measurement of Fe²⁺ chelating activity (Rival et al., 2001a).

For α_{S1} -casein, the peptide α_{S1} -casein (f144–149), YFYPEL, showed strong superoxide anion radical scavenging activity. This peptide was isolated from pepsic hydrolysate and exhibited superoxide anion scavenging activity and DPPH radical and hydroxyl radical scavenging activities (Suetsuna et al., 2000). The fragment PYVRYL from ovine α_{S2} -casein has antioxidant properties and corresponds to the α_{S2} -casein (f202–207) fragment (PYVRYL) from bovine α_{S2} -casein generated after hydrolysis by the cell envelope protease PrtS of *Strep. thermophilus* PB385 (Miclo et al., 2012) and trypsin (Lopez-Expósito et al., 2007).

However, no antioxidant peptides were detected from α_{S1} - and κ -casein in the present results. This could be explained by differences in protein degradation with or without proteases during incubation between LAB and *Bifidobacterium*.

CONCLUSIONS

In the present study, antioxidant activity in casein-derived peptides obtained from fermentation with *B. longum* was detected using ABTS free radical scaveng-

ing activity and total phenolic peptide measurements. Two peptides having antioxidant activity were identified through MS analysis. Based on these results, further study of the <3 kDa fraction of each casein and fermented milk is needed to investigate and identify novel peptides with antioxidant properties and to screen bioactive peptides generated by *B. longum* that have other functions, such as antihypertensive, antimutagenic, and immunomodulant peptides.

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