



Identification and Characterization of a Novel Antioxidant Peptide from Bovine Skim Milk Fermented by *Lactococcus lactis* SL6

Sang Hoon Kim¹, Ji Yoon Lee², Marilen P. Balolong^{1,5}, Jin-Eung Kim³, Hyun-Dong Paik⁴, and Dae-Kyung Kang^{1*}

¹Department of Animal Resource Science, Dankook University, Cheonan 31116, Korea

²National Instrumentation Center for Environmental Management, Seoul National University, Seoul 08826, Korea

³R&D Center, Cell Biotech Co., Ltd., Gimpo 10003, Korea

⁴Department of Food Science and Biotechnology of Animal Resources, Konkuk University, Seoul 05029, Korea

⁵Department of Biology, College of Arts and Sciences, University of Philippines, Manila 1000, Philippines

Abstract

A novel peptide having free radical scavenging activity was separated, using an on-line high-performance liquid chromatography (HPLC) - ABTS screening method, from bovine skim milk fermented by *Lactococcus lactis* SL6 (KCTC 11865BP). It was further purified using reverse phase-HPLC (RP-HPLC) and sequenced by RP-HPLC-tandem mass spectrometry. The amino acid sequence of the identified peptide was determined to be Phe-Ser-Asp-Ile-Pro-Asn-Pro-Ile-Gly-Ser-Glu-Asn-Ser-Glu-Lys-Thr-Thr-Met-Pro-Leu-Trp (2,362 Da), which is corresponding to the C-terminal fragment of bovine α_{s1} -casein (f179-199). The hydroxyl radicals scavenging activity (IC_{50} 28.25±0.96 μ M) of the peptide chemically synthesized based on the MS/MS data showed a slightly lower than that of the natural antioxidant Trolox (IC_{50} 15.37±0.52 μ M). Furthermore, derivatives of the antioxidant peptide were synthesized. The antioxidative activity of the derivatives whose all three proline residues replaced by alanine significantly decreased, whereas replacement of two proline residues in N-terminal region did not affect its antioxidative activity, indicating that 3rd proline in C-terminal region is critical for the antioxidative activity of the peptide identified in this study. In addition, N-terminal region of the antioxidant peptide did not show its activity, whereas C-terminal region maintained antioxidative activity, suggesting that C-terminal region of the peptide is important for antioxidative activity.

Keywords antioxidant peptide, *Lactococcus lactis* SL6, bovine milk, fermentation

Introduction

Milk contains a wide range of biologically active compounds such as immunoglobulins, antimicrobial peptides, oligosaccharides, lipids and other minor components (Korhonen and Pihlanto-Leppälä, 2004a; Park, 2009), conferring important physiological and biochemical functions that have crucial impacts on host metabolism and health (Gobbetti *et al.*, 2007; Park 2009). In addition, milk fermentation by lactic acid bacteria releases a large number of peptides with various biological functions, such as antioxidant (Chang *et al.*, 2013), angiotensin-converting enzyme inhibitory (Ha *et al.*, 2015), immunomodulatory (Gauthier *et al.*, 2006), opioid acti-

Received March 17, 2017

Revised May 17, 2017

Accepted May 27, 2017

*Corresponding author

Dae-Kyung Kang

Department of Animal Resource Science, Dankook University, Cheonan 31116, Korea

Tel: +82-41-550-3655

E-mail: dkkang@dankook.ac.kr

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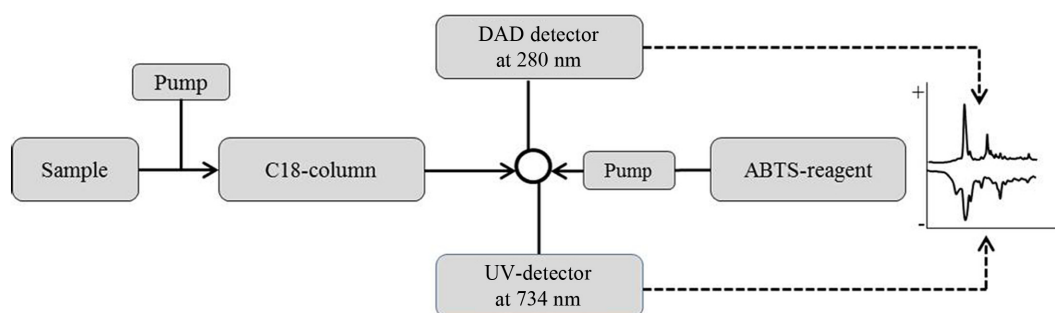


Fig. 1 Scheme for the screening of antioxidant peptides using on-line HPLC-ABTS system. Positive absorbance at 280 nm indicates peptide and negative absorbance at 734 nm indicates antioxidative activity.

vities (Park, 2009).

Among them, antioxidant peptides have free radical-scavenging activity and nonenzymatic lipid oxidation (Rival *et al.*, 2001; Suetsuna *et al.*, 2002). The side effect of oxidation in the body and in foodstuffs is well known. Excessive free radicals and other reactive oxygen species can deteriorate food quality, for example unacceptable taste, rancid flavor and shortening of shelf life (Korhonen and Pihlanto-Leppälä *et al.*, 2007b). In addition, oxidative damage plays a significant role in the number of age-specific diseases, such as atherosclerosis, diabetes, rheumatoid arthritis, Alzheimer's disease and cancer (Halliwell, 2001; Hoelzl *et al.*, 2005; Korhonen and Pihlanto-Leppälä *et al.*, 2007b). Therefore antioxidant peptides can then be utilized as an excellent source of natural ingredients to develop functional foods that may enhance physiological and metabolic functions or condition of the body to benefit the host (Clare *et al.*, 2003; FitzGerald and Meisel, 2003; Kitts and Weiler, 2003; Li *et al.*, 2004).

Different studies have demonstrated antioxidative activity of dairy products (De Gobba, C. *et al.*, 2014; Gupta *et al.*, 2009; Sabeena Farvin *et al.*, 2010). Several peptides from milk hydrolysates or fermented milk have been reported to have antioxidative activity. Rival *et al.* (2001) identified a peptide with antioxidative property from the tryptic β -casein digest. Suetsuna *et al.* (2002) and Lopez-Exposito *et al.* (2006) also reported antioxidative peptides from the peptic digests of α_{s1} - and κ -casein, respectively. Recently, Chang *et al.* (2013) identified two antioxidative peptides from the bovine β -casein hydrolysate fermented by *Bifidobacterium longum*. However, to date, only a few antioxidant peptides from fermented milk products have been identified and characterized.

The aim of this study was to identify and characterize novel antioxidant peptide from fermented milk which has

not been identified yet. Moreover, the peptide was synthesized according to the obtained amino acid sequences to confirm its antioxidative activity.

Materials and Methods

Screening of antioxidative activity with on-line HPLC-ABTS system

An on-line screening method for antioxidative activity was developed based on the method of Koleva *et al.*, (2000), as given in Fig. 1. Samples were subjected to reverse phase-HPLC (Ultimate 3000 RSLC HPLC, Dionex, Germany) to allow separation in the column (Zorbax, 3.5 μ m, 300SB-C8, 2.1*50 mm, Agilent). Each peak was detected by a diode array detector (DAD) at 280 nm. Then 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical solution was pumped into the separated fractions on-line at a flow rate of 0.5 mL/min. A peak showing a negative absorbance at 734 nm after reacting with the ABTS reagent was indicative of antioxidant activity. Antioxidative activity selection was done using an on-line ABTS reaction assay by pumping an ABTS radical solution into the separated fractions on-line with a flow rate of 0.5 mL/min. The peak showing negative absorbance at 734 nm after reaction with the ABTS reagent indicates antioxidative activity. Among the strains tested, the strain *Lactococcus lactis* SL6 was the one showing the antioxidative activity using the ABTS method and was thus used for the next experiments.

Preparation of fermented milk

Lactic acid bacteria (LAB) isolated from various traditional fermented foods were used for screening of the strain which has antioxidative activity. The LAB isolates were incubated in skimmed milk (10%, w/v) and incuba-

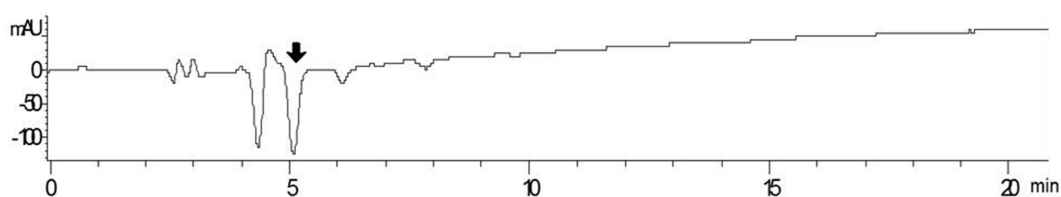


Fig. 2 RP-HPLC peptide profile of the skimmilk fermented by *Lactococcus lactis* SL6 at 734 nm. Arrow showing negative absorbance in on-line HPLC-ABTS assay indicates antioxidative activity and the corresponding fraction was collected for further analysis.

tion at 37°C for 48 h. Then the fermented milk was centrifuged at 8,000 g for 15 min at 4°C, and the supernatant was treated with ammonium sulfate (60%) to remove the precipitate. Finally the resulting supernatant was dialyzed to remove salts using Spectra/Por dialysis membrane (Spectrum labs, USA) with a molecular weight cut-off of 1 kDa and used for on-line screening of antioxidant peptides.

Identification of antioxidant peptide

Selection and identification of peptides were performed using an online platform (Fig. 2) following the methods previously described by Chang *et al.* (2013). The samples were analyzed in a Triple TOF 5600 Q-TOF LC/MS/MS system (ABSciex, CA) using a Ultimate 3000 RSLC HPLC system (Thermo Fisher Scientific Inc., USA), including a degasser, an auto-sampler, diode array detector and a binary pump. The LC separation was performed on a column with a mobile phase A (0.1% formic acid in water) and a mobile phase B (0.1% formic acid in acetonitrile). The flow rate and injection volume were 0.25 mL/min and 1-5 μ L, respectively. Mass spectra were acquired under positive electrospray ionization (ESI) with an ion spray voltage of 4500 V. Two full-scan mass spectra were acquired over an m/z range of 50-2000 on the MS mode. The data were collected using Analyst TF 1.7 software and analyzed using PeakView 2.2 and ProteinPilot 4.5 software.

Measurement of antioxidative activity

The antioxidant activity of the sample was measured as free radical scavenging activity of ABTS and 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical cation according to the previously described method by Chang *et al.* (2013) and Volta *et al.* (2014) with minor modifications.

The ABTS radical was generated by preparing ABTS and potassium persulfate at final concentrations of 7 and 2.45 mM, respectively, and incubated for 12 h in the dark

at room temperature. The ABTS radical was adjusted to an absorbance of 0.70 ± 0.02 at 734 nm by diluting with distilled water using a spectrophotometer. The sample (10 μ L) was mixed with 990 μ L of the ABTS radical solution. Changes in absorbance were monitored at 734 nm after 30 min at room temperature.

For DPPH radical scavenging assay, DPPH solution (0.1 mM) in ethanol was prepared. This solution (990 μ L) was added to synthetic peptide resolved solution (10 μ L). The mixture was mixed vigorously and incubated at room temperature for 30 min. Then, absorbance was measured at 517 nm by using UV-spectrophotometer (Shimadzu, Japan).

All assays were carried out in triplicates and recorded as means. The percentage ABTS and DPPH antioxidant activity were calculated following the equation below where A_{control} represents the initial ABTS/DPPH absorbance:

$$\begin{aligned} &\text{ABTS/DPPH Scavenging activity (\%)} \\ &= [1 - (A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100 \end{aligned}$$

The half maximal inhibitory concentration (IC_{50}) value based from the calculated standard curve and expressed as μ M of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (Sigma, USA). A standard curve was prepared using various concentrations of Trolox, a water-soluble analogue of vitamin E.

Verification of antioxidative activity of the peptides

The antioxidative peptide identified by RP-HPLC-tandem mass spectrometry and its derivatives were chemically synthesized by the solid-phase procedure by Bead-Tech, Inc. (Korea). The purity was verified as >95% by using analytical HPLC coupled with MALDI-TOF MS. The peptides were dissolved in high-performance liquid chromatography (HPLC)-grade water at a concentration of 1 mg/mL for the ABTS radical scavenging test.

Results and Discussion

Antioxidative activity of the fermented skim milk

On-line screening system of antioxidative activity using RP-HPLC was performed, as described in Fig. 1. First, samples were injected into RP-HPLC and separated by C₁₈ column, and each peak was detected by DAD detector (280 nm). Then, ABTS reagent was pumped into the separated fractions on-line using isocratic pump to mix each other well. Finally, the fraction showing negative absorbance at 734 nm after reaction with ABTS reagent was regarded indicating antioxidative activity and was collected for further experiments.

Among tested strains, *Lactococcus lactis* SL6 (KCTC 11865BP) showed strongest antioxidative activity. *L. lactis* SL6, provided by Cell Biotech, Co. (Korea), was originally isolated from kimchi. Fig. 2 shows the UV-visible chromatogram of the supernatants of skim milk fermented by *L. lactis* SL6. The retention time (Rt) 5 min showed high negative absorbance at 734 nm was its antioxidative activity was confirmed by ABTS⁺ radical cation assay (data not shown). These results indicated that the fermentation by SL6 plays an important role in the release of antioxidant peptides from milk proteins.

Identification of peptides by HPLC-MS/MS

The HPLC fraction showing high antioxidative activity was subjected to MS/MS spectrometry using a quadrupole ion trap capable of multiple stages of mass analysis from a single precursor ion. Fig. 3A shows the UV chromatogram obtained at Rt near 5 min from Fig. 2, and a dominating peak at 5.596 min was analyzed further. Fig. 3B shows the MS spectrum of the sample from Fig. 3A and one fragment which was the triply charged ion (*m/z* 788.38, 3+), indicating with an arrow, among intense fragments only matched to bovine milk peptide. Following sequence interpretation and database searching using PeakView 2.2. and ProteinPilot 4.5 softwares, the MS/MS spectrum was matched to C-terminal fragment of α_{s1} -casein (f179-199). The amino acid sequence of the identified peptide with triply charged ion (*m/z* 788.38, 3+) corresponded to the loss of three protons from the precursor ion. The corresponding amino acid sequence was identified as FSDIPNPIGSENSEKTTMPLW (2,362 Da), as shown in Fig. 4. This peptide characterized in this study has never been identified as antioxidant peptide yet, to the best of our knowledge.

To confirm whether the identified peptide has antioxi-

Table 1. Radical scavenging activity of the peptide identified in this study

| Synthetic peptide sequence | Radical scavenging activity ¹⁾ | |
|----------------------------|---|--------------------|
| | ABTS ²⁾ | DPPH ³⁾ |
| FSDIPNPIGSENSEKTTMPLW | 70.83±1.15% | 89.73±1.19% |

¹⁾Each observation is mean of triplicates of each batch. Results are expressed as mean of scores ± standard error of mean.

²⁾ABTS radical scavenging was tested at 5 μ M

³⁾DPPH radical scavenging was tested at 50 μ M.

Table 2. Comparison of radical scavenging activity of the peptide with that of Trolox, expressed by IC₅₀

| Synthetic peptide sequence | IC ₅₀ ²⁾ ± SEM ³⁾ |
|----------------------------|--|
| Trolox ¹⁾ | 15.37±0.52 μ M |
| FSDIPNPIGSENSEKTTMPLW | 28.25±0.96 μ M |

¹⁾Trolox: a water-soluble analogue of vitamin E.

²⁾The concentration of needed to inhibit 50% of the radical scavenging.

³⁾Each observation is mean of triplicates of each batch. Results are expressed as mean of scores ± standard error of mean.

dative activity, it was chemically synthesized by solid-phase procedure, and further evaluated for their antioxidative activity using both ABTS and DPPH scavenging activity assay.

As shown in Table 1, the synthesized peptide showed radical scavenging activities with ABTS scavenging of 70.83±1.15% and DPPH scavenging of 89.73±1.19%. The synthesized peptide quenched ABTS radicals more effectively. The hydroxyl radicals scavenging effect of the peptide (IC₅₀: 28.25±0.96 μ M) showed a slightly lower radical scavenging activity than that of the natural antioxidant Trolox (IC₅₀: 15.37±0.52 μ M) as positive control (Table 2).

Antioxidant activity of the peptide derivatives

Antioxidative activity of peptides is closely related to their amino acid composition, structure, and sequences (Sarmadi and Ismail, 2010). In addition, many antioxidant peptides contains at least one proline residue and higher antioxidant capacity of the peptide was related to their high proline content (Hernández-Ledesma *et al.*, 2005). Accordingly, the antioxidant peptide identified in this study contained three proline residues (Fig. 4).

In order to evaluate the importance of proline residues in antioxidative activity of the peptide, proline residues in the template peptide were replaced by alanine and checked their antioxidative activity by ABTS radical scavenging assay. As shown in Fig. 5, significant decrease in the antioxidative activity was observed in M-AAA variant in which all proline residues were replaced by alanine. In contrast, the antioxidant activity of the M-AA variant in which

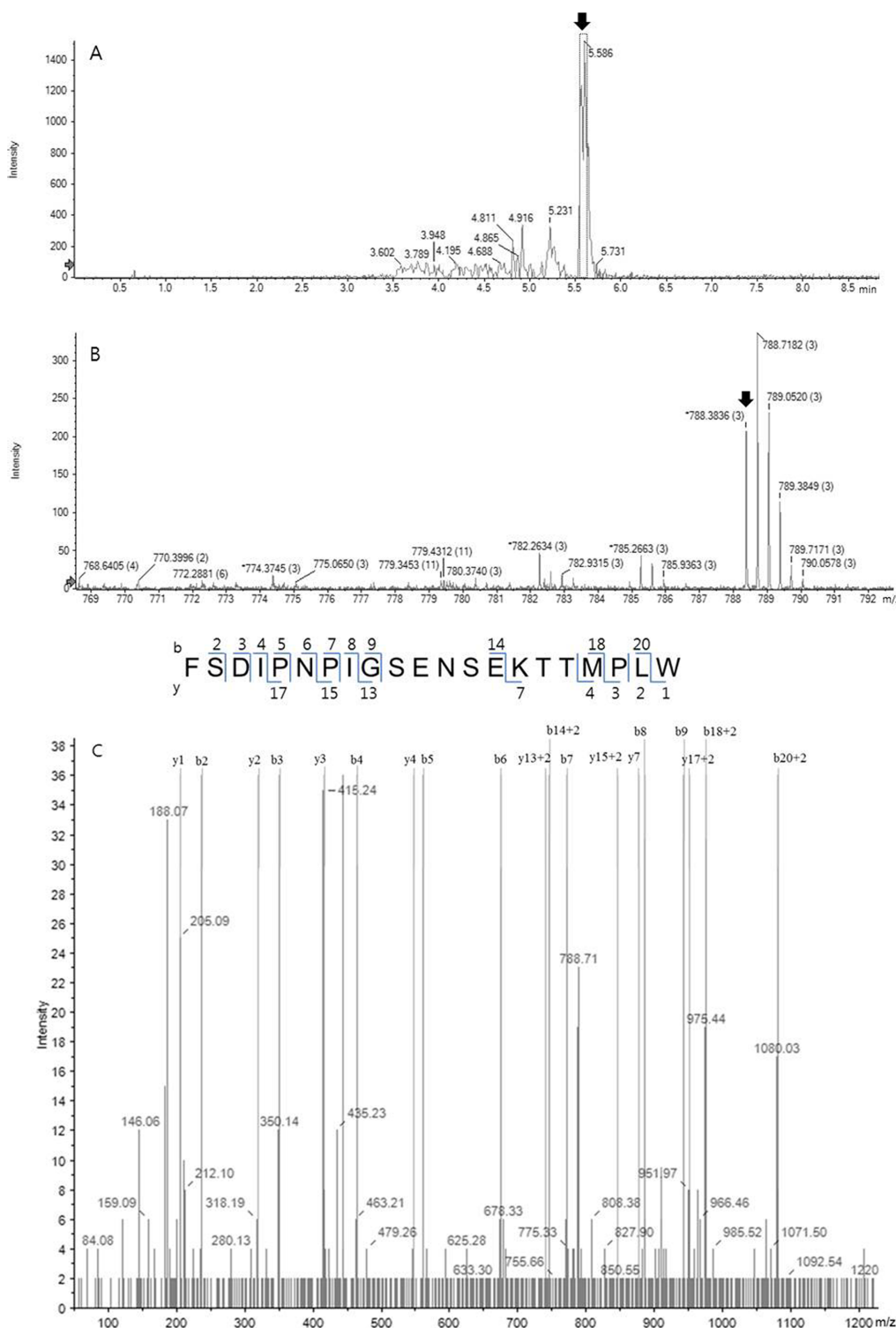


Fig. 3 (A) UV chromatogram of the fraction showing antioxidative activity (B) Mass spectrum of selected chromatographic peak in (5.568 min) in (A); (C) MS/MS spectra of triply charged ion with m/z 788.38. The sequence of this peptide is displayed with the fragment ions observed in the spectrum.

only two proline residues in the N-terminal region were replaced by alanine remained the same. These findings

suggest that 3rd proline in C-terminal region, in particular, is critical for antioxidative activity of the peptide identi-

α_{s1} -casein

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                20                               40
RPKHPIKHQG LPQEVLENENL LRFVAVPFPE VFGKEKVNEL
                60                               80
SKDIGSESTE DQAMEDIKQM EAESISSEE IVPNSVEQKH
                100                              120
IQKEDVPSER YLGYLEQLLR LKKYKVPQLE IVPNSAEERL
                140                              160
HSMKEGIHAQ QKEPMIGVNQ ELAYFYPELF RQFYQLDAYP
                180                              199
SGAWYYVPLG TQYTDAPSFSE SEKTTMPLW

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Fig. 4 Amino acid sequences of bovine α_{s1} -casein. Antioxidant peptide identified in this study was underlined. In addition, the peptide previously identified as angiotensin I-converting enzyme (ACE)-inhibitory peptide (Hernández-Ledesma et al., 2005) was boxed.

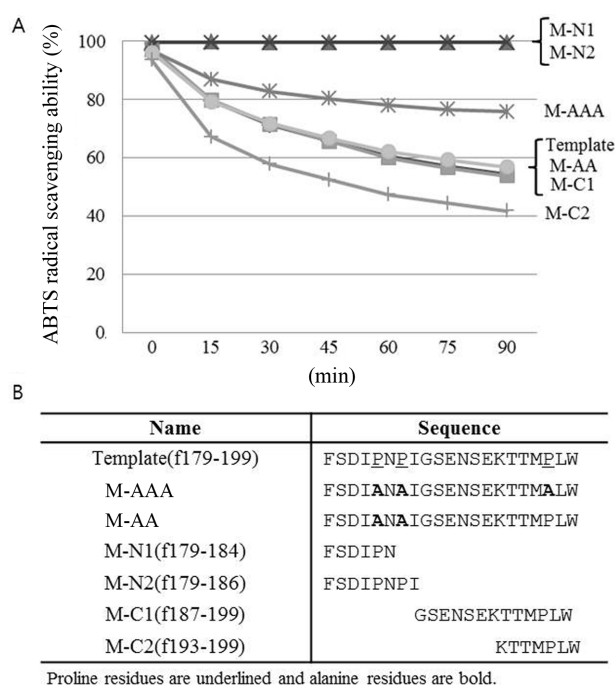


Fig. 5 (A) Changes in the radical scavenging activity of the peptide derivatives according to incubation time, **(B)** Amino acid sequences of peptide derivatives. Proline residues are underlined and alanine residues are marked in bold.

fied in this study. The distinctive cyclic structure of proline's side chain gives proline an exceptional conformational rigidity of the protein (Williamson, 1994). Therefore 3rd proline might affect the secondary structure of the peptide, then changing the antioxidative activity of the peptide. This should be elucidated in the future.

Additional manipulation of the template peptide was performed, as shown also in Fig. 5. The peptide derivatives consisting of 6 or 8 amino acids from N-terminus (M-N1 and M-N2, respectively) did not show antioxi-

dative activity, whereas peptide derivatives from C-terminus (M-C1 and M-C2) maintained antioxidative activity. Interestingly, M-C2 derivative rather showed higher antioxidative activity than template peptide. This is maybe because the native structure of the peptide was altered and bioactive functional groups particularly within hydrophobic regions become exposed (Power *et al.*, 2013). Further investigation is required in the future.

M-C2 peptide (f193-199) previously had been identified as an inhibitory peptide of angiotensin I-converting enzyme (ACE) (Hernandez-Ledesma *et al.*, 2005). ACE-inhibitory and antioxidative activity could be related each other. Future studies will focus on the multi-functional activities of peptide antioxidants and investigate structure-function relationships.

Conclusion

Novel antioxidant peptide from fermented milk by *L. lactis* SL6 was identified, and it was identified as C-terminal fragment of bovine α_{s1} -casein. Mutation experiments of the antioxidant peptide indicated proline residue at C-terminal region is important for its activity. Our findings suggest that fermentation of skim milk by *L. lactis* SL6 could have important applications in the food and pharmaceutical industries. Further studies to explore the capability of this peptide as a functional food ingredient will be of great interest in the future.

Acknowledgements

This work was supported by Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries through High Value-added Food Technology development Program, funded by ministry of agriculture, Food and Rural Affairs (314073-03-3-HD030).

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