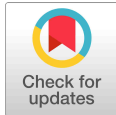




ARTICLE



Biological Potential of Novel Specific Casein-Derived Peptides

Da Young Kim¹, Jung Sik Yoo¹, Yoon Ah Cho¹, Ho Sik Yoon²
and Cheol-Hyun Kim^{1*}

¹Dept. of Animal Resources Science, Dankook University, Cheonan, Korea

²Wellrunbnf Co., Cheonan, Korea

Received: January 18, 2021

Revised: March 31, 2021

Accepted: March 31, 2021

*Corresponding author :

Cheol-Hyun Kim

Dept. Animal Resources Science,
Dankook University, Cheonan, Korea

Tel : +82-41-550-3656

Fax : +82-41-551-0924

E-mail : hichkim@dankook.ac.kr

Copyright © 2021 Korean Society of
Dairy Science and Biotechnology.

This is an Open Access article distributed
under the terms of the Creative Commons
Attribution Non-Commercial License

(<http://creativecommons.org/licenses/by-nc/3.0>)
which permits unrestricted non-commercial
use, distribution, and reproduction in any
medium, provided the original work is
properly cited.

ORCID

Da Young Kim

<https://orcid.org/0000-0003-0927-1512>

Jung Sik Yoo

<https://orcid.org/0000-0002-3758-3604>

Yoon Ah Cho

<https://orcid.org/0000-0001-9360-3959>

Ho Sik Yoon

<https://orcid.org/0000-0001-9650-9351>

Cheol-Hyun Kim

<https://orcid.org/0000-0002-1905-9948>

Abstract

This study aimed to evaluate the biological potential of functional food, i.e., specific peptides obtained from the hydrolysis of milk protein, by assessing their antioxidant and antibacterial properties. For the preparation of casein hydrolysates, commercial enzymes were added to 10% casein solution in a 1:200 (w/v) ratio, and samples were collected each hour. Based on the assessment of the degree of hydrolysis (DH) of casein hydrolysates, it was observed that the concentration of all enzymatic hydrolysates increased rapidly from 30 to 40 minutes. However, no change was observed in their concentrations after 150 minutes. Protamex[®] and Neutrase[®] exhibited the highest DH when compared to other enzymes. Furthermore, SDS-PAGE was performed for analyzing the proteolytic pattern of each enzyme, except for Flavourzyme[®], and peptides in the size range of 20–25 kDa were identified. Subsequently, peptides produced by two enzymes were isolated using a preparative liquid chromatography system. Overall, NF3, NF4, PF5, and PF6 showed higher antioxidant potential than other peptide fractions. Moreover, NF7 and PF3 exhibited the highest antibacterial activity. In this study, we evaluated the biological potential of novel casein-derived peptides that may find application in the food and healthcare industry.

Keywords

milk protein, casein hydrolysates, antioxidant activity, antibacterial activity

Introduction

Milk is made of milk fat, protein, carbohydrates, water-soluble vitamins, minerals, and water. Among them, whey protein and casein present as protein components. Casein is major component of milk protein and it is widely studied because it has high availability and nutritional importance. It is about 3% in milk and makes up about 80% of all protein in milk and it consists of α , β , γ , κ -casein, molecular weight is about 75,000–375,000 kDa [1].

Casein is a source of exogenous and endogenous amino acids and a precursor of biologically active peptides characterized by immunogenic, antioxidant, antibacterial, antiviral, antihypertensive, antitumor and opioid properties [2].

Also, the enzymatic hydrolysis of casein may act in the body as regulatory components with a hormone activity which may modulate specific physiological functions [3].

In vitro and *in vivo* enzymatic proteolysis of casein proteins produce bioactive peptides [4], which have specific biological functions throughout their ability to affect cellular function [5].

Among them, antioxidant activities of milk protein hydrolysates and milk-derived peptides obtained by fermentation have recently investigated [6]. The antioxidant

activities of the protein hydrolysates seem to be inherent to the characteristic amino acid sequences of peptides derived, depending on the specificity of protein enzyme. Results of their studies suggested that the hydrolysates from milk proteins could be used as natural antioxidant materials of functional foods and in preventing oxidation reaction in food processing. In addition, casein trypsin digests showed inhibitory properties in the oxidation of linoleate by lipoxygenase, peroxy radicals and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical [7]. Casein and casein peptides can bind iron [8], thus making them potential antioxidants in food systems. Concentrated casein phosphopeptide (CPP) and casein hydrolysates have antioxidant activities in corn oil-in-water emulsions at pH 7.0 and 3.0 [9]. In addition, in relation to the antibacterial activity of the casein hydrolysates, the antibacterial activity has been reported more than 8 times higher than the original protein [10]. A structural group of casein antibacterial peptides discovered and the association between its structure and antibacterial activity found [11].

In this regard, the aims of this study are development of specific peptides that have physiological properties of hydrolyzed casein and have similar effects to CPP, a material that is commercially available.

Materials and Methods

1. Preparation of casein hydrolysates

Commercial casein (95% protein) was obtained from Meggle food Ingredients (Wasserburg, Germany).

10% casein solution prepared by mixing 10% casein and 90% 0.1 M Potassium phosphate buffer (pH 7.4). After a sterilization for 10 minutes at 90°C of casein solution and it adjusted to pH 7.0, the optimum active pH of the enzyme, using a 1 N NaOH (Sigma, USA).

Commercial enzymes used as Alcalase® 2.4 L FG (Protease from *Bacillus licheniformis*, 5 U/g), Neutrase® 0.8 L (Protease from *Bacillus amyloliquefaciens*, 0.8 U/g), Protamex® (Protease from *Bacillus* sp., 1.5 U/g) and Flavourzyme® (Protease from *Aspergillus oryzae*, 500 U/g). All commercial enzymes purchased from Novozymes™ (Denmark).

The enzymes added at a ratio of 1:200 (w/v) based on the substrate, and then shaken at 120 rpm in a shaking water bath at 50°C. Samples taken at 30 minute intervals to inactivate the enzymes at 90°C for 10 minutes and proceeded to 240 minutes. After a cooling at 20°C, the supernatant collected by centrifugation for 20 minutes under conditions of 4,000 g and 4°C. The supernatant filtered using PVDF 0.22 Syringe membrane filter (Futecs, Korea). Samples stored at -20°C and used for the experiment.

2. Degree of hydrolysis (DH) of casein hydrolysates

The hydrolysis degree of casein hydrolysates measured using the Lowry method [12]. Lowry solution A and B prepared for the degree of hydrolysis (DH). To prepare solution A, 1% cupric sulfate (Sigma) and 2% sodium potassium tartrate (Sigma) were mixed at a 1:1 ratio (v/v), and 1.0 mL of the mixed solution and 50 mL of 2% Na₂CO₃ (DaeJung

Chemical & Metals, Korea) mixed. Solution B used to prepare a 1 N Folin-Ciocalteu reagent (Sigma).

First, 1.0 mL of casein hydrolysates and the same amount of 20% TCA (Duksan Chemical, Korea) mixed and it maintained for 1 hour to precipitate 20% TCA soluble protein. After that, it centrifuged at 18,000 ×g for 20 minutes. The supernatant and redissolved residue removed in 5.0 mL of 0.1 N NaOH (Sigma).

Thereafter, 1.0 mL of casein hydrolysates and 5.0 mL of solution A mixed and reacted at room temperature for 10 minutes. Then, 0.5 mL of solution B added and the contents immediately mixed using a vortex mixer. After reacting for 30 minutes, absorbance measured at 600 nm. Equivalent amounts of protein calculated from standard curve prepared using bovine serum albumin (Sigma). Absorbance of bovine serum albumin measured in the range from 20 to 200 µg/mL.

The amount of TCA soluble protein measured and calculated by the following equation.

$$\text{Degree of hydrolysis (\%)} = \frac{\text{Soluble protein from 10\% TCA}}{\text{Total protein}} \times 100$$

3. Protein profiling of casein hydrolysates (SDS-PAGE)

SDS-PAGE performed to obtain a profile of casein hydrolysates. The molecular weight of the casein hydrolysates measured using Bio-rad devices including gel electrophoresis Mini-protein tetra electrophoresis system [13].

12% Acrylamide gel (Bio-Rad Lab, USA) prepared to analyze a casein hydrolysates protein profile. Protein bands stained with Coomassie brilliant blue R-250 (Bio-Rad Lab). The molar mass standard used Precision plus protein dual xtra standards (Bio-Rad Lab).

4. Purification of casein hydrolysates

In order to obtain peptide fractions from casein hydrolysates, gel filtration using a preparative chromatography system performed (Waters Corp, USA).

Peptides that identified in SDS-PAGE separated throughout a preparative scale for further study. The condition of preparative liquid chromatography system is shown in Table 1. The preparative pump (Waters Corp) and Preparative liquid chromatography W600 (Waters Corp) were used with a Dual λ Absorbance detector W2487 (Waters Corp) and W717 Autosampler (Waters Corp).

The casein hydrolysates dissolved in 5 mM sodium phosphate buffer with 0.15 M NaCl (Sigma), pH 7.0. It filtered using a PVDF 0.45 µm sterile syringe membrane filter (Futechs). Next, the casein hydrolysates loaded 3.0 mL on the Hiprep 16/60 Sephacryl S-100 HR column (GE Healthcare Life Sciences, USA) and eluted at flow rate of 1.0 mL/min. A detector was set at 280 nm.

All fractions that obtained during gel filtration using Hiprep 16/60 Sephacryl S-100 HR column throughout a preparative chromatography system collected and fractions stored at -20°C.

Table 1. Condition of preparative liquid chromatography system

Instrument	Condition
Column	Hiprep 16/60 Sephacryl S-100 HR column (GE Healthcare Life Sciences, USA)
Mobile phase	5 mM sodium phosphate buffer (0.15 M NaCl, pH 7.0)
Detector (detection)	Waters dual λ absorbance detector W2487 (280 nm)
Flow rate	1 mL / min
Injection volume	3 mL

5. Antioxidant activity

1) ABTS assay

For ABTS radical scavenging activity, ABTS powder (Sigma) mixed to ethanol and 7 mM ABTS was prepared. Thereafter, ABTS mixed with 2.45 mM potassium persulfate (Sigma) in a 1:1 (v/v) ratio to prepare an ABTS solution. This solution stored in dark room for 12-16 hours. The ABTS stock solution diluted in ethanol to an absorbance of 0.70 ± 0.02 at 734 nm.

After mixing 190 μ L of the diluted ABTS solution to 10 μ L of the fraction sample, and the absorbance taken 3 min later.

The results expressed in units of Trolox (Sigma) equivalent antioxidant activity in units of mM/L.

2) DPPH assay

The 0.2 mM DPPH solution prepared by mixing 2,2-diphenyl-1-picrylhydrazyl, 2,4,6-tripyridyl-s-triazine (Sigma) and ethanol (Samchun, Korea).

Thereafter, DPPH solution and fraction sample mixed in a 1:1 ratio (v/v) and reacted in a dark room for 30 minutes. The absorbance measured at 570 nm, and results expressed in units of ascorbic acid (Sigma) equivalent capacity in units of mM. All protein concentration of fraction samples is 200 μ g/mL.

3) FRAP assay

The FRAP solution for use in the experiment was prepared. The 300 mM acetate buffer prepared by mixing Sodium acetate trihydrate (Sigma) and acetic acid (Samchun). The 10 mM TPTZ prepared by dissolving 2,4,6-tri(2-pyridyl)-1,3,5-triazine (Sigma) in 40 mM HCl (Samchun), and the 20 mM Iron(III) chloride hexahydrate solution prepared by dissolving Iron(III) chloride hexahydrate 97% A.C.S reagent (Sigma) in ethanol (Samchun). The above three solutions were mixed in a ratio of 10:1:1 (v/v).

Also, 1.5 mL of the FRAP solution pre-warmed to 37°C mixed with 50 μ L of the casein hydrolysate fraction samples and standards. The mixture vortexed and reacted in a dark room at room temperature. The absorbance measured at 593 nm.

The results expressed in units of Ascorbic acid (Sigma) equivalent capacity in units of mM. All protein concentration of casein hydrolysate fraction samples were 200 μ g/mL.

4) Hydroxyl radical assay

The 0.75 mM 1,10-phenanthroline (Sigma), phosphate buffer (pH 7.0), 2.5 mM FeSO₄ (Sigma) and casein hydrolysate fraction samples mixed in a ratio of 1:1:1:1 (v/v). The 20 mM Hydrogen peroxide (Junsei Chemical, Japan), the reaction reagent finally mixed in the same ratio (v/v). The reaction carried out in a water bath at 37°C for 90 minutes. The absorbance measured at 593 nm.

The results expressed in units of Ascorbic acid (Sigma) equivalent capacity in units of mM. All protein concentration of casein hydrolysate fraction samples were 200 µg/mL.

6. Antimicrobial activity

Antibacterial activity evaluated using a paper disc method [14]. Pathogenic bacteria *Escherichia coli* KCTC1682, *Salmonella enterica* KCTC2054, *Bacillus cereus* KCTC362A and *Staphylococcus aureus* KCTC3881 obtained from the Korean Collection for Type Cultures (KCTC) and it dispensed on Mueller hinton agar (Difco™, USA). One hundred microliter of the pathogenic bacteria culture (10³-10⁴ CFU/mL) uniformly distributed by pouring 30 mL of pre-sterilized Mueller hinton agar and allowed for solidification.

A paper disc method (Advantec, Japan), impregnated with the compound, placed on the surface of the Mueller hinton agar. The 100 µL of casein hydrolysate fractions dispensed into each paper disc and incubated in the incubator for 37°C. After incubation for 24 hours, the diameter (mm) of the inhibition clear zone around the well measured and compared to each other.

Results

1. Degree of hydrolysis (DH) of casein hydrolysates

The degree of casein hydrolysis defined as the percentage of the total number of peptide bonds in a protein that cleaved during hydrolysis [15]. The DHs of the four enzymes for casein tended to increase in a similar pattern. There was no significant difference from 150 minutes after increasing from 120 minutes to 150 minutes. Among them, the casein hydrolyzed by Protamex® measured with highest DHs, and the DHs of the casein hydrolyzed by Neutrase® and Flavourzyme® were similar (Fig. 1).

2. Protein profiling of casein hydrolysates (SDS-PAGE)

SDS-PAGE performed to confirm the hydrolysis characteristics of the casein. Protein bands with a molecular weight range of 2 to 250 kDa identified (Fig. 2). α_{s2}-casein, α_{s1}-casein, β-casein and κ-casein, etc., the main components of casein, found from bands between 25 and 37 kDa. The numbers of band in the region with lower molecular weight increase gradually with enzymatic hydrolyzation. It showed the peptides with higher molecular weight broke up during enzymatic hydrolysis.

Except for Flavourzyme®, in the case of Alcalase®, Neutrase® and Protamex® hydrolysates, α_{s2}-casein, α_{s1}-casein, β-casein and κ-casein hydrolyzed, and it confirmed that bands formed between 10 and 20 kDa. In the case of Alcalase® hydrolysates, fewer bands observed compared to other hydrolysates.

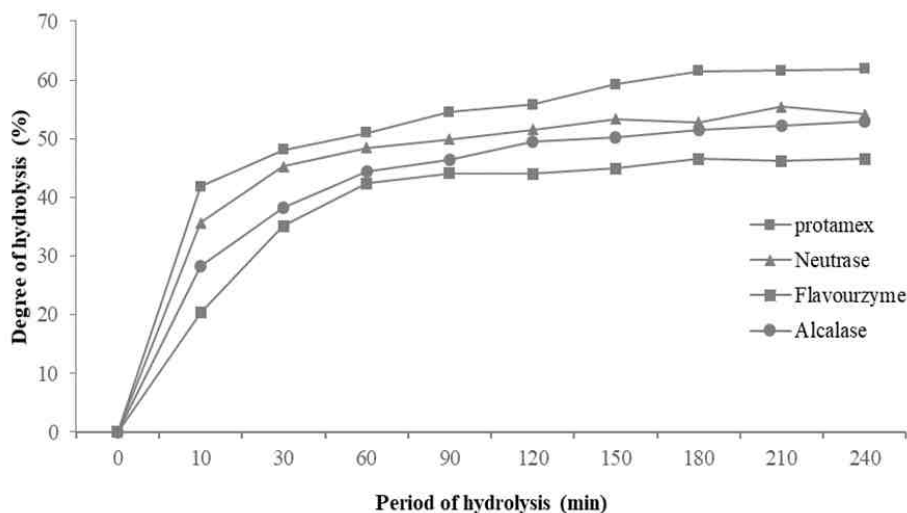


Fig. 1. Degree of hydrolysis (DH) of casein hydrolysates. Reaction were carried out in a water bath (50°C, 120 rpm). All enzymes were applied at concentration 1:200 (w/v) to the casein solution. Alcalase[®]; Neutrase[®]; Flavourzyme[®]; Protamex[®] (Novozymes[™], Denmark).

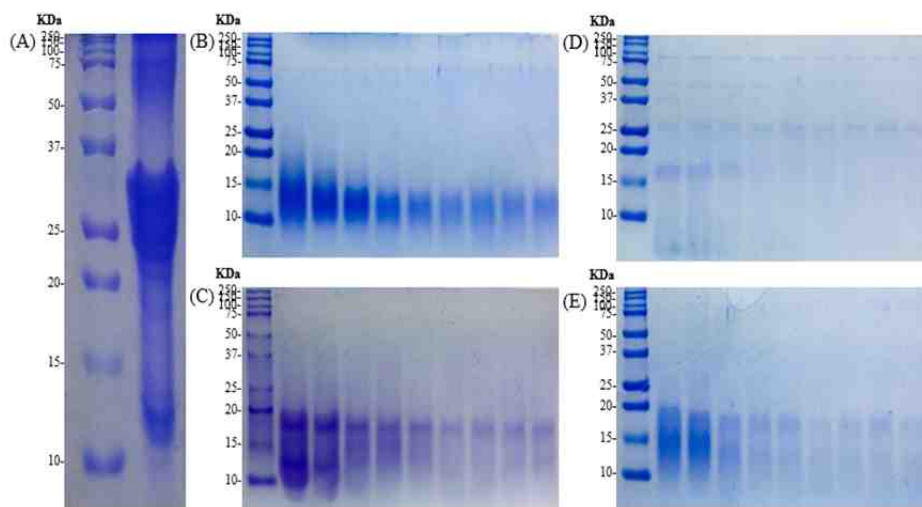


Fig. 2. SDS-PAGE patterns of casein protein hydrolyzed with commercial enzymes for each times. (A) 10% casein protein solution after pasteurization, (B) Alcalase[®], (C) Neutrase[®], (D) Flavourzyme[®], (E) Protamex[®] (Novozymes[™], Denmark). lane 1: Precision plus protein dual xtra standards (Bio-Rad Lab); lane 2, 3, 4, 5, 6, 7, 8, 9, 10: hydrolysates obtained after 10, 30, 60, 90, 120, 150, 180, 210, and 240 min of hydrolysis. All enzymes were applied at concentration 1:200 (w/v, protein) to the casein protein solution.

3. Purification of casein hydrolysates

Peptides identified in SDS-PAGE and separated throughout the preparative scale for further study. Neutrase[®] and Protamex[®] selected with base on results of SDS-PAGE and DHs. Peptide fractions of casein hydrolysates isolated using Hiprep 16/60 Sephacryl S-100 HR column throughout a preparative liquid chromatography system. Each fraction

separated by molecular weight. Total of ten peptide fractions obtained (Fig. 3 and 4). Each fraction compared with original casein hydrolysates via SDS-PAGE. As a result, it proved which the fraction by molecular weight (Data not shown).

4. Antioxidants activity

1) ABTS assay

The ABTS has a property of becoming transparent when it encounters a substance having antioxidant activity.

According to results (Fig. 5), F3, F4, F5, F6, and F7 in the hydrolysate fractions using Neutrase[®] showed similar level of activity as CPP. Except for F1, F2, F8, F9, and F10, other fractions showed higher ABTS radical scavenging activities than non-hydrolyzed casein (NHC), of which F3 had highest activity.

In the Protamex[®] hydrolysate fractions, F5 and F6 showed similar scavenging activity

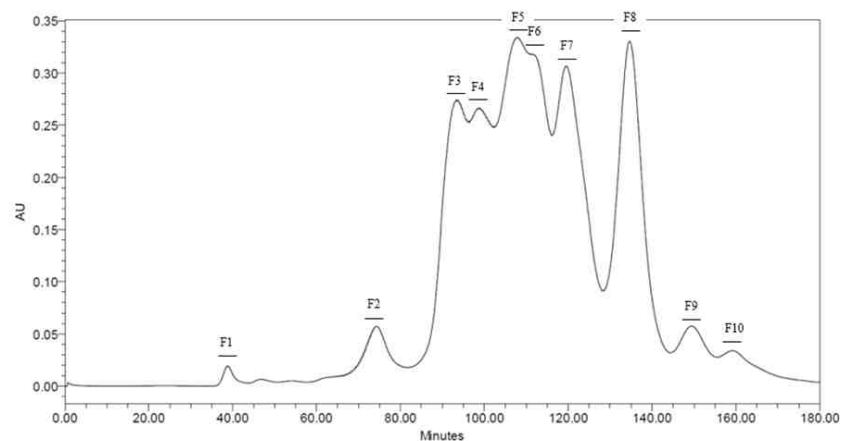


Fig. 3. Separations of casein hydrolysates using Neutrase[®] in preparative LC. F#, fraction number in prep LC system.

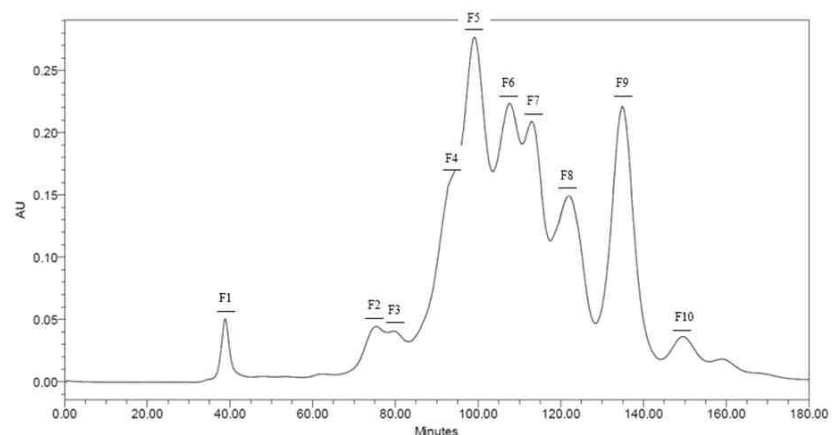


Fig. 4. Separations of casein hydrolysates using Protamex[®] in preparative LC system. F#, fraction number in prep LC system.

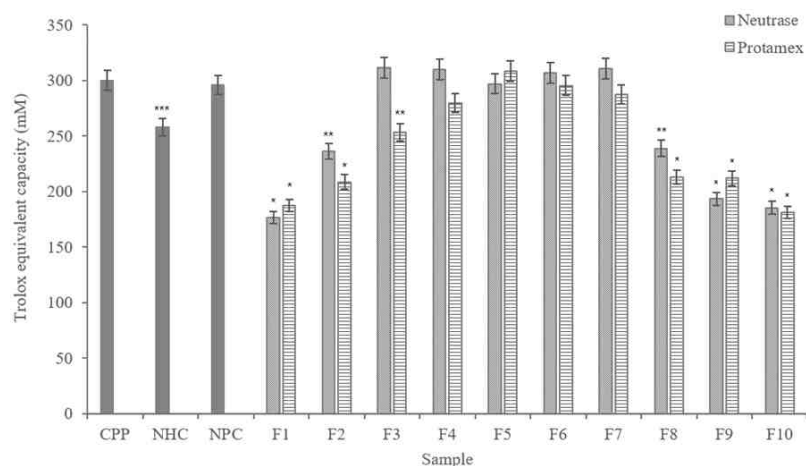


Fig. 5. ABTS radical scavenging activities of casein hydrolysate fractions. All samples protein concentration were 200 $\mu\text{g/mL}$. All values were mean \pm SD of triplicates. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; CPP vs Sample. F#, fraction number in Prep LC system; CPP, casein phosphopeptide; NHC, non-hydrolyzed casein; NPC, non-purified casein. ABTS, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid).

with the CPP. The F5 showed the highest scavenging activity than all fractions including the Neutrase[®] hydrolysate fractions. It is also higher than CPP, NHC and non-purified casein (NPC).

2) DPPH assay

The DPPH free radical is a stable free radical, which has been widely accepted as a tool for estimating the free-radical scavenging activities of antioxidants.

In this study, antioxidant activities evaluated by compared to value DPPH scavenging activity. Based on the standard curve of Ascorbic acid (Vitamin C), DPPH scavenging activity values statistically analyzed.

According to results (Fig. 6), F4 and F7 in the Neutrase[®] hydrolysate fractions showed DPPH radical scavenging ability similar with the CPP. Among them, F4, F5, and F6 showed higher scavenging ability than NHC and NPC.

The F5 in the Protamex[®] hydrolysate fractions showed excellent DPPH radical scavenging activity, similar with the CPP. F4, F6, and F7 in the fractions not significantly difference with the CPP. All fractions showed higher DPPH radical scavenging activity than NHC.

3) FRAP assay

FRAP assay is direct way to measure antioxidant activities. It is based on activities of antioxidant to reduce Fe^{3+} to Fe^{2+} in the presence of TPTZ forming an intense blue Fe^{2+} -TPTZ complex with an absorption at 593 nm. The reducing potentials of antioxidants are associated with their electron donating abilities to break the free radical chain reaction [16].

According to results (Fig. 7), FRAP measurements showed that Neutrase[®] hydrolysate

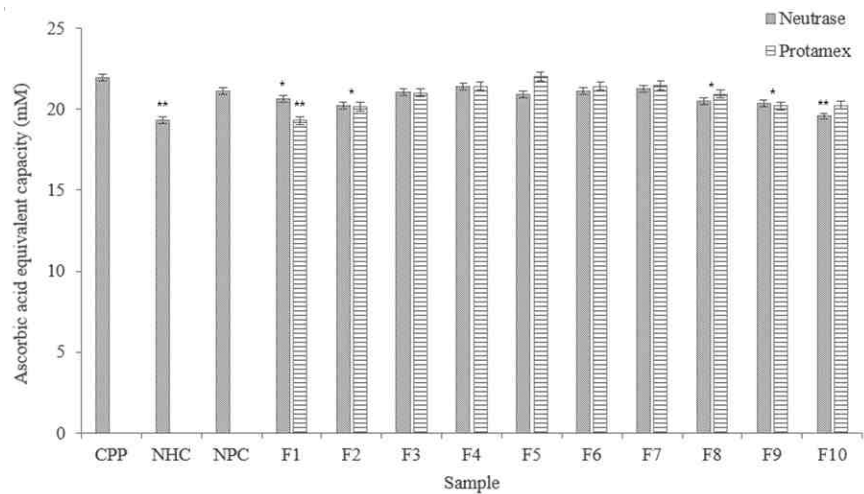


Fig. 6. DPPH scavenging activities of casein hydrolysate fractions. All samples protein concentration were 200 $\mu\text{g/mL}$. All values were mean \pm SD of triplicates. * $p < 0.05$, ** $p < 0.01$; CPP vs Sample. F#, fraction number in Prep LC system; CPP, casein phosphopeptide; NHC, non-hydrolyzed casein; NPC, non-purified casein.

fractions showed similar data in iron reducing capacity. F3 and F5 showed the most similar iron reduction capacity with the CPP compared to other fractions. F2, F9, and F10 showed lower activity than NHC.

Among the Protamex[®] hydrolysate fractions, F5 showed significantly higher iron reducing capacity, and it is higher than the CPP. In addition, all fractions have higher activities than NHC, and F7 showed a similar level with the CPP.

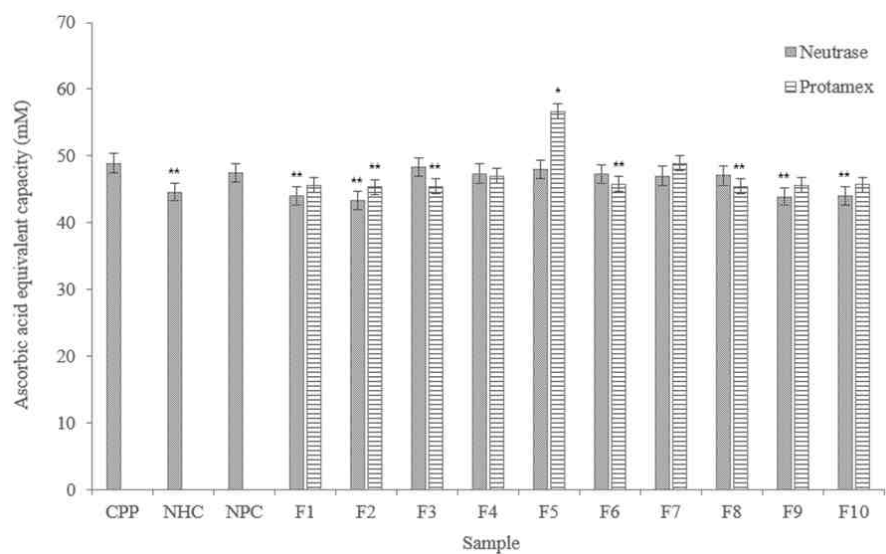


Fig. 7. FRAP activities of casein hydrolysate fractions. All samples protein concentration were 200 $\mu\text{g/mL}$. All values were mean \pm SD of triplicates. * $p < 0.05$, ** $p < 0.01$; CPP vs Sample. F#, fraction number in Prep LC system; CPP, casein phosphopeptide; NHC, non-hydrolyzed casein; NPC, non-purified casein; FRAP, ferric reducing antioxidant power.

4) Hydroxyl radical scavenging assay

Hydroxyl radical is the most reactive oxygen species produced by the reduction of three electrons from oxygen.

According to results (Fig. 8), Neutrase[®] hydrolysate fractions showed high radical scavenging activities except for F8, and it showed similar activities with NHC and NPC. Among them, F7 showed the highest scavenging activity, and F3 and F4 showed similar scavenging activity with the CPP.

In the Protamex[®] hydrolysate fractions, F5 showed similar scavenging activity with the CPP. F3, F4, and F6 showed higher radical scavenging ability than other fractions. In addition, all fractions showed higher scavenging capacity more than NHC.

5) Antibacterial activity

The agar diffusion assay (or inhibition zone assay) is a common method used to test the antibacterial activity of commonly used food, peptides and hydrolysates [17]. The ability of antibacterial activities for Neutrase[®] hydrolysate fractions measured in terms of zone of inhibition (cm) and values depicted in Table 2.

According to results (Fig. 9), In the Neutrase[®] hydrolysate fractions, the highest inhibitory fraction for all pathogens was F7. The F7 showed higher activity more than both positive control (PC) and CPP. The antibacterial activities of other fractions are similar, but it significantly low in the case of F4. All fractions have highest inhibitory for *Escherichia coli*, and it is higher than PC and CPP.

The abilities of Protamex[®] hydrolysate fractions measured in terms of zone of inhibition (cm) and values depicted in Table 3. In the case of the Protamex[®] hydrolysate

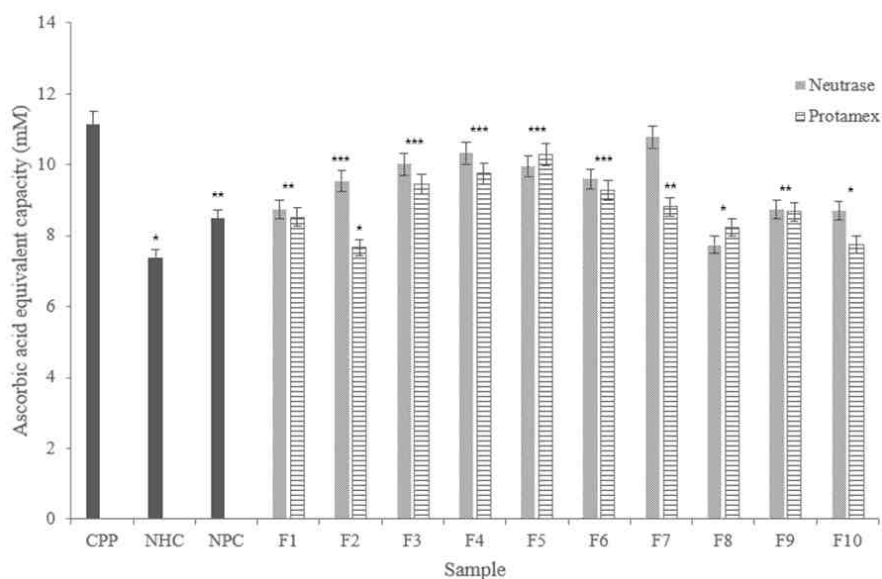


Fig. 8. Hydroxyl radical scavenging activities of casein hydrolysate fractions. All samples protein concentration were 200 $\mu\text{g/mL}$. All values were mean \pm SD of triplicates. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; CPP vs Sample. F#, fraction number in Prep LC system; CPP, casein phosphopeptide; NHC, non-hydrolyzed casein; NPC, non-purified casein.

Table 2. Antibacterial activity of casein hydrolysate fractions with Neutrase®

Sample	Degree of inhibition of peptide ¹⁾			
	<i>B. cereus</i>	<i>E. coli</i>	<i>S. aureus</i>	<i>S. enterica</i>
PC			+	
CPP	+	++	++	+
F3	++	+++	+	++
F4	+	++	+	++
F5	++	++	++	++
F6	++	++	++	++
F7	++	+++	++	++

All samples protein concentration were 200 µg/mL.

All values were mean±SD of triplicates.

¹⁾ The diameters of the clear zone of inhibition were as follows: +, 1.0≤diameter≤1.5; ++, 1.5≤diameter≤2.0; +++, diameter>2.0.

PC, positive control (0.25 N HCl); CPP, casein phosphopeptide.

Table 3. Antibacterial activity of casein hydrolysate fractions with Protamex®

Sample	Degree of inhibition of fractions ¹⁾			
	<i>B. cereus</i>	<i>E. coli</i>	<i>S. aureus</i>	<i>S. enterica</i>
PC			+	
CPP	+	++	++	+
F3	++	+++	+++	++
F4	+++	++	++	++
F5	++	+++	++	+
F6	++	++	+	++
F7	++	++	++	++

All samples protein concentration were 200 µg/mL.

All values were mean±SD of triplicates.

¹⁾ The diameters of the clear zone of inhibition were as follows: +, 1.0≤diameter≤1.5; ++, 1.5≤diameter≤2.0; +++, diameter>2.0.

PC, positive control (0.25 N HCl); CPP, casein phosphopeptide.

fractions, the inhibitory activity of F3 was the best for all pathogens. All fractions showed good inhibitory activities for *E. coli*, and the inhibitory activity for *Salmonella enterica* was almost similar except for F5.

Discussion

In this study, proteolytic enzymes used to focus on the function of specific peptides derived from the hydrolysis of casein.

As a result, the DHs of the four enzymes tended to increase in a similar pattern. There was no significant difference after increasing from 120 minutes to 150 minutes. DH of Protamex® found to increase most appropriately. All enzymatic hydrolysates maintained from 30 to 40 minutes and gradually increased and maintained after 50–60 minutes. In this study, casein contained 95% of protein, and the retention time of hydrolysis was 10 minutes.

The SDS-PAGE performed to confirm the hydrolysis characteristics according to the enzyme. α_{s2} -casein, α_{s1} -casein, β -casein and κ -casein, etc., the main components of

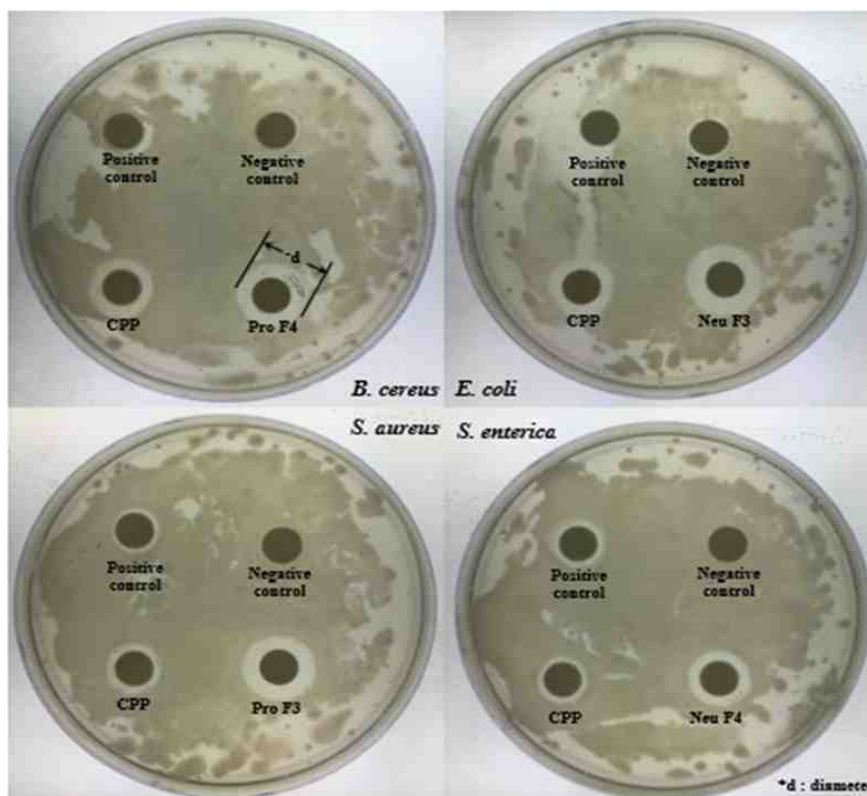


Fig. 9. Antibacterial activity of casein hydrolysate fractions. d :diameter of clear zone; diameter of disk: 1 cm. Pro F#, casein hydrolysate fractions with Protamex®; Neu F#, casein hydrolysate fractions with Neutrase®; positive control, 0.25 N HCl; negative control, distilled water; NPC, non-purified casein.

casein, found to bands between 25 and 37 kDa. In casein, hydrolyzed with trypsin, casein phosphopeptide (CPP) material obtained. α _{s2}-casein, α _{s1}-casein, β -casein and κ -casein hydrolyzed to identify bands up to 20 kDa. This result is similar with SDS-PAGE results of the enzyme hydrolysates except for Flavourzyme®. This is the basis for confirming the potential to obtain a peptide material of similar molecular weight size with the CPP.

Peptides identified in SDS-PAGE and it separated on preparative scale for further study. In this study, fractions separated by molecular weight throughout a Hiprep 16/60 Sephacryl S-100 HR column. In another study, FPLC used either size exclusion or ion exchange chromatography [18]. Chromatographic methods for peptide separation are very diverse, but the purpose of this study is obtainments of peptides similar with CPP, so fractions separated using a column by molecular weight. The fractions confirmed to be consistent with the hydrolysate molecular weight confirmed by SDS-PAGE (Data not shown).

After that, the antioxidant activities of each enzymatic hydrolysates fraction verified. According to results of ABTS, NF3, NF4, NF5, NF6, and NF7 showed similar level of activities with CPP. Also, PF5 and PF6 showed similar scavenging activity with the CPP. Comparing to results of Neutrase® hydrolysate fractions and Protamex® hydrolysates

fraction, the hydrolysis degree test results generally excellent in Protamex[®] hydrolysates fraction. As a result of DPPH, activities of NF4 and NF7 are similar with CPP, and activities of PF4 and PF6 are similar with CPP. Similarly in the FRAP and Hydroxyl radical scavenging assay results, it confirmed that certain fractions have similar activities with the CPP. According to above results of antioxidant activities, it can be seen that the antioxidant activity and iron reducing ability of Neutrased[®] hydrolysates fraction is significantly higher than Protamex[®] hydrolysates fraction. In the previous experiments, Protamex[®] found to have higher casein hydrolysis than Neutrased[®]. However, antioxidant activity confirmed that Neutrased[®] measured higher regardless of this. For example, in previous studies, egg protein that hydrolyzed with trypsin and alcalase showed the highest DHs, but the highest antioxidant activities of samples that hydrolyzed with alcalase [19]. It showed that there is no correlation between hydrolysis and antioxidant activity. This is not only because of differences in substrates but also because of the different cleavage sites for each enzyme. Since the type of amino acids located at the N- and C-terminal of the cleavage site is different, it is reported that the antioxidant activity is different.

As a result of the antibacterial activity test, several fractions showed high antibacterial activity against four pathogens. Indeed, previous studies have shown that *α*s2-casein and *α*s1-casein have antibacterial activity from pathogens such as *E. coli* and *S. aureus* [20]. In addition, it has reported that Lactoferrin obtained from Bovine lactoferrin present in milk had 8 times more antibacterial activity than the original protein. Based on this, the antibacterial activity of the peptide fraction derived from casein could be demonstrated. Therefore, these protein fractions must be obtained and produced active peptides from them to use as dietary supplements and milk based nutraceuticals. This study identified the potential of new biologically active peptides derived from milk proteins that affect the food and healthcare industry.

Conflict of Interest

The authors declare no potential conflict of interest.

Acknowledgments

This research was supported by the Ministry of Trade, Industry & Energy (MOTIE), Korea Institute for Advancement of Technology (KIAT) through the Encouragement Program for The Industries of Economic Cooperation Region (P0008626).

References

1. Choi J, Horne DS, Lucey JA. Determination of molecular weight of a purified fraction of colloidal calcium phosphate derived from the casein micelles of bovine milk. *J Dairy Sci.* 2011;94:3250-3261.
2. Pomastowski P, Walczak J, Gawin M, Bocian S, Piekoszewski W, Buszewski B. HPLC

- separation of casein components on a diol-bonded silica column with MALDI TOF/TOF MS identification. *Anal Methods*. 2014;6:5236-5244.
3. Meisel H, FitzGerald RJ. Biofunctional peptides from milk proteins: mineral binding and cytomodulatory effects. *Curr Pharm Des*. 2003;9:1289-1295.
 4. Jelen P, Lutz S. Functional milk and dairy products. In: Mazza G, editor. *Functional foods: biochemical and processing aspects*. Lancaster: Technomic Publishing; 1998. p. 439-487.
 5. Meisel H. Biochemical properties of bioactive peptides derived from milk proteins: potential nutraceuticals for food and pharmaceutical applications. *Livest Prod Sci*. 1997; 50:125-138.
 6. Pihlanto A. Antioxidative peptides derived from milk proteins. *Int Dairy J*. 2006;16:1306-1314.
 7. Rival SG, Boeriu CG, Wichers HJ. Caseins and casein hydrolysates. 2. Antioxidative properties and relevance to lipoxygenase inhibition. *J Agric Food Chem*. 2001;49:295-302.
 8. Díaz M, Decker EA. Antioxidant mechanisms of caseinophosphopeptides and casein hydrolysates and their application in ground beef. *J Agric Food Chem*. 2004;52:8208-8213.
 9. Díaz M, Dunn CM, McClements DJ, Decker EA. Use of caseinophosphopeptides as natural antioxidants in oil-in-water emulsions. *J Agric Food Chem*. 2003;51:2365-2370.
 10. Arnao MB, Cano A, Acosta M. The hydrophilic and lipophilic contribution to total antioxidant activity. *Food Chem*. 2001;73:239-244.
 11. Powers JPS, Hancock REW. The relationship between peptide structure and antibacterial activity. *Peptides*. 2003;24:1681-1691.
 12. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem*. 1951;193:265-275.
 13. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 1970;227:680-685.
 14. Karunanidhi A, Thomas R, van Belkum A, Neela V. In vitro antibacterial and anti-biofilm activities of chlorogenic acid against clinical isolates of *Stenotrophomonas maltophilia* including the trimethoprim/sulfamethoxazole resistant strain. *BioMed Res Int*. 2012;2013:392058.
 15. Adler-Nissen J. *Enzymic hydrolysis of food proteins*. London: Elsevier Applied Science Publishers; 1986. p.110-169.
 16. Gingras AC, Raught B, Sonenberg N. Regulation of translation initiation by FRAP/mTOR. *Genes Dev*. 2001;15:807-826.
 17. Devendra LP, Pandey A. Hydrotropic pretreatment on rice straw for bioethanol production. *ELSEVIER*. 2016;98:2-8.
 18. Fruebis J, Tsao TS, Javorschi S, Ebbets-Reed D, Erickson MRS, Yen FT, Bihain BE, Lodish HF. Proteolytic cleavage product of 30-kDa adipocyte complement-related protein increases fatty acid oxidation in muscle and causes weight loss in mice. *Proc*

- Natl Acad Sci USA. 2001;98:2005-2010.
19. Chalamaiah M, Jyothirmayi T, Diwan PV, Kumar BD. Antioxidant activity and functional properties of enzymatic protein hydrolysates from common carp (*Cyprinus carpio*) roe (egg). *J Food Sci Technol*. 2015;52:5817-5825.
 20. McCann KB, Shiell BJ, Michalski WP, Lee A, Wan J, Roginski H, et al. Isolation and characterisation of a novel antibacterial peptide from bovine α_{S1} -casein. *J Int Dairy*. 2006;16:316-323.