Comparison of Antioxidant Activities of Hydrolysates of Domestic and Imported Skim Milk Powders Treated with Papain

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Abstract

Milk proteins have many potential sequences within their primary structure, each with a specific biological activity. In this study, we compared and investigated the bioactivities of hydrolysates of the domestic (A, B) and imported (C, D) skim milk powders generated using papain digestion. MALDI-TOF analysis revealed that all milk powder proteins were intact, indicating no autolysis. Electrophoretic analysis of hydrolysates showed papain treatment caused degradation of milk proteins into peptides of various size. The antioxidant activity of the hydrolysates, determined using 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and total phenolic contents (TPC) assays, increased with incubation times. In all skim milk powders, the antioxidant activities of hydrolysates were highest following 24 h papain treatment (TPC: A, 196.48 µM GE/L; B, 194.52 µM GE/L; C, 194.76 µM GE/L; D, 163.75 µM GE/L; ABTS: A, 75%; B, 72%; C, 72%; D, 57%). The number of peptide derived from skim milk powders, as determined by LC-MS/MS, was 308 for A, 283 for B, 208 for C, and 135 for D. Hydrolysate A had the highest antioxidant activity and the most potential antioxidant peptides amongst the four skim milk powder hydrolysates. A total of 4 β-lactoglobulin, 4 αs1-casein, and 56 β-casein peptide fragments were identified as potential antioxidant peptides in hydrolysate A by LC-MS/MS. These results suggest that domestic skim milk could have applications in various industries, i.e., in the development of functional foods.

Keywords: antioxidant activity, skim milk powder, hydrolysis, antioxidant peptides, papain

Introduction

Oxygen is an essential factor for human metabolism, functioning as the final electron acceptor in electron transport and creating reactive oxygen species (ROS) such as superoxide anions (O₂⁻), hydrogen peroxide (H₂O₂), hydroxyl radicals (-OH), peroxyl radicals (ROO⁻), and alkoxyl radicals (RO) (Kovatcheva et al., 2001). Free radicals are constantly generated through normal metabolism during respiration in aerobic organisms, and are necessary for the normal bactericidal activity of macrophages, used as signaling intermediates, and function in the removal of protein waste (Kovatcheva et al., 2001). Control of free radical production is necessary for proper physiologic function, as excessive amounts of reactive oxygen metabolites can result in cellular damage which, in turn, promotes chronic diseases including diabetes, atherosclerosis, DNA damage, cardiovascular disease, and cancer (Gupta et al., 2010). To neutralize free radicals, the body synthesizes antioxidant molecules that, together with the antioxidants consumed through food, form the biological antioxidant barrier. However, under certain circumstances, the defense system fails to protect the body against oxidative stress; consequently, the ability to increase antioxidant defenses is considered important in the maintenance of human health and disease prevention (Serafini et al., 2004).

Bioactive peptides derived from milk are reported to have immunomodulatory, antimicrobial, antioxidant, and antithrombotic activities (Clare et al., 2000; Kilara et al., 2003; Korhonen et al., 2003; Korhonen et al., 2007; Korhonen et al., 2009; Meisel et al., 2006, Pihlanto et al.,
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2009; Silva et al., 2005), with various milk products and fractions including milk, skim milk, whey, casein, lactoferrin having antioxidant capabilities (Cervato et al., 1999; Colbert et al., 1991; Steijns et al., 2000; Taylor et al., 1980; Tong et al., 2000). Enzymatic hydrolysis is an attractive technique to produce bioactive peptides due to the ease of controlling the reaction and the minimal formation of by-products. Adriena et al. (2010) reported that whey protein hydrolysates generated using enzymes such as alcalase, flavourzyme, protamex, and neutrase had increased antioxidant activity. These hydrolysates have a positive impact on body conditions and may influence health (Haque et al., 2009).

On June 30, 2007, U.S. and South Korean trade officials signed the proposed U.S.-South Korean Free Trade Agreement (KORUS FTA) for their respective countries after agreement with the European Union (EU). In 2013, dairy imports continued to increase because of reduced tariffs and increased tariff-rate quotas (TRQs). Skim and whole milk powders are subject to Korean import quotas that continuously expand in perpetuity (Choi et al., 2013). Korea also produces skim milk powder but human consumption of such products is still limited, while skim milk powder proteins are available in abundance. Therefore, the production of skim milk powder hydrolysates with biological activity, i.e., antioxidant activity, and improved functional properties would be of economic interest as well as processing significance. Thus the aim of this study was to show the availability of skim milk powder in dairy industry through the investigation of antioxidant activities after papain treatment and identification of the antioxidant peptides derived from it and was to show the domestic skim milk is good source to use functional food manufacture.

Material and Methods

Reagents and materials
Domestic samples (A, B) and imported samples (C, D) were obtained from the Korean market. The contents (%) of skim milk powders were 3.36-3.55 for casein, 0 for fat, 3.91-4.17 for protein, 6.01-6.24 for lactose and 10.93-11.11 for total solid. The 2,2’-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS, A1888), Folin-Ciocalteu reagent, gallic acid, dithiothreitol (DTT), and papain were purchased from Sigma-Aldrich (USA). All other chemicals used in this study were of analytical grade. Tryptic soy agar and broth were from Difco Laboratories (USA).

Autolysis: MALDI-TOF analysis
Autolysis was performed according to a previously described method (Ham et al., 2012), with minor modifications. The milk powder samples were applied as a thin film to a 96-spot steel plate (Bruker Daltonics) and visibly dried at room temperature. Subsequently, 2 µL of MALDI matrix (a saturated solution of sinapinic acid [Bruker Daltonics; USA] in 50% acetonitrile and 2.5% trifluoroacetic acid) was applied to the samples and dried. MALDI-TOF MS (Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectroscopy) was performed with a Microflex time-of-flight mass spectrometer (Bruker Daltonics) tabletop mass spectrometer using the manufacturer’s suggested settings. Ionization was achieved by irradiation with a nitrogen laser (λ = 337 nm) operating at a 3 ns pulse duration. Ions were accelerated at +19 kV with 200 nsec of pulsed ion extraction delay. Each spectrum was detected in linear positive mode and was externally calibrated using a mixture of protein standards between 1,000 and 12,000 Da. In order to increase detection sensitivity, excess matrix was removed with 10 shots at a laser power of 83% prior to acquisition of spectra with 300 shots at a fixed laser power of 70%. AutoXecute acquisition control, a software tool, was applied for automated data acquisition.

Milk powders hydrolysis
Milk powders were prepared at a concentration of 40 mg/mL in distilled water. Protein solutions (40 mg/mL) were prepared in 10 mM sodium phosphate buffer, pH 5.7, for papain and the enzyme-to-substrate ratio was 1:2000 (w/v). The reaction mixtures were incubated at 55°C. Enzymatic hydrolysis was stopped by heating for 5 min in boiling water, and an aliquot was retrieved immediately at each incubation time (1, 2, 4, 6, and 24 h). Samples were filtered through 0.45 µm filters (Whatman, UK) and stored at 20°C.

Electrophoresis
SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis), as well as gel staining/destaining was performed as previously described by Chang et al. (2013a).

OPA assay
Peptide content through an ortho-phtalaldehyde (OPA, Sigma-Aldrich) assay was measured as previously described (Chang et al., 2013b). Bacto tryptone (Difco Laboratories) at various concentrations (0.25-1.5 mg/mL) was used as a standard. To measure peptide content, each me-
formation at the National Instrumentation Center for Environmental Management (NICEM) of Seoul National University in Korea using an integrated system consisting of an auto switching nano pump, autosampler (Tempo™ nano LC system, MDS SCIEX, Canada), and a hybrid Quadrupole-TOF MS/MS spectrometer (QStar Elite, Applied Biosystems, USA) equipped with a nano-electrospray ionization source and fitted with a fused silica emitter tip (New Objective, USA). The precise method was as previously described by Chang et al. (2013a, 2013c). The injection volume was 2 \mu L into an LC-MS/MS on a Zorbax 300 SB-C18 trap column (300 \mu m i.d. \times 5 mm, 5 \mu m, 100; Agilent Technologies, USA; part number 5065-9913), at a flow rate of 5 \mu L/min, and the sample was separated on a Zorbax 300SB-C18 capillary column (75 \mu m i.d. \times 150 mm, 3.5 \mu m, 100; part number 5065-9911) at a flow rate of 300 nL/min. The gradient was carried out as follows: 2% to 35% solvent B over 30 min, then from 35% to 90% over 10 min, followed by 90% solvent B for 5 min, and 5% solvent B for 15 min. Resulting peptides were electro-sprayed and mass data were acquired automatically using Analyst QS 2.0 software (Applied Biosystems) with the 200-2000 range of m/z.

### Statistical analysis

Data were analyzed by ANOVA followed by Tukey’s multiple range test using the Statistical Analysis System Software (SAS version 9.13, SAS Institute, USA). Significant differences were set at a 5% level (p<0.05).

### Results and Discussion

#### Verification of autolysis

Prior to preparing hydrolysates of the domestic and imported skim milk powders used for this study, MALDI-TOF MS analysis was utilized to evaluate autolysis occurring during manufacture and storage. As shown in Fig. 1, domestic and imported samples showed similar patterns of individual milk proteins, with no apparent autolysis. Thus, all samples were suitable to be used to prepare hydrolysates for further study.

#### Electrophoretic pattern of skim milk powder hydrolysates

Proteolysis patterns during hydrolysis of the skim milk powders with papain treatment were monitored by SDS-PAGE (Fig. 2). Skim milk powders were prepared in distilled water. The degradation patterns of the skim milk powders during hydrolysis demonstrate that, as the incubation time increases, the concentrations of \( \alpha_s \)-casein and...
Fig. 1. MALDI-TOF analysis of skim milk powders from four different sources. A and B: domestic skim milk powders; C and D: imported skim milk powders.

Fig. 2. Electrophoresis of various skim milk powders treated with papain. Lane M: Protein standard marker; 0, 1, 2, 4, 6, 24: incubation time (h). A and B: domestic skim milk powders; C and D: imported skim milk powders.
β-casein decreased while lower molecular weight breakdown products of the caseins increased. These products, which appear in SDS-PAGE in the area between αs-casein and β-lactoglobulin, serve as substrates for proteases, leading to the formation of smaller peptides and amino acids (Ong et al., 2007). The rate of hydrolysis of αs-casein and β-casein varied between the four skim milk powders. The αs-casein and β-casein were hydrolyzed faster than other proteins in most milk powders, as shown by the disappearance of the αs-casein band during the early stages of incubation (1 h), except in hydrolysate D (2 h). Our findings are in agreement with a previous study (Chang et al., 2013) which also found that β-casein was hydrolyzed faster than other proteins during the incubation of bovine casein solution with *Bifidobacterium longum*.

Compared with other caseins, β-casein is more prone to cleavage due to its accessibility to proteases (Sadat et al., 2011). α-Lactalbumin was degraded immediately after enzymatic treatment. In contrast, k-casein and β-lactoglobulin were hydrolyzed more slowly.

**Investigation of the antioxidant activity of skim milk powder hydrolysates**

**Total phenolic contents**

Phenolic content analysis was performed to detect phenolic amino acids in the peptides and to confirm antioxidant activity. The concentration of total phenolic compounds found in the samples analyzed varied widely, with values ranged from 0 to 100 μM gallic acid equivalents (GAE). The predominant phenolics are very active as antioxidants with antiradical activity (Mansouri et al., 2005). In this study, the total phenolic content (TPC) measurement followed the Folin-Denis method (Silva et al., 2006). Table 1 shows the concentrations of total phenolic compounds measured in the four skim milk powders treated with papain. The total concentrations of phenolic compounds were significantly different in the four hydrolysates (*p*<0.05). The TPC of domestic sample A was not significantly different after 2 h of incubation (*p* >0.05). The TPCs of domestic sample B and import sample C were not significantly different after 6 h of incubation (*p* >0.05). The TPC of import sample D continuously increased with incubation time. The antioxidant activity of the hydrolysates was attributed to the presence of phenolic compounds (Al-Laith et al., 2010). The highest GE value was seen for all four hydrolysates after 24 h of incubation with papain. Chang et al. (2013c) also observed that 24 h of incubation with *B. longum* generated the highest TPC. Colbert and Decker (1991) reported an inhibition of copper-catalyzed liposome oxidation for whey protein fractions with MW < 0.5 kDa. A number of previous studies have shown that peptides derived by enzymatic treatment were thought to have higher antioxidant activity (Yang et al., 2008). A relationship was found between the degree of hydrolysis and the antioxidant activity of different hydrolysates.

**ABTS assay**

The ABTS assay used in the present study to investigate the free radical-scavenging properties of skim milk powder hydrolysates obtained using papain treatment was adapted from a previously described method (Re et al., 1999) that is widely used for studying free radical-scavenging properties with respect to antioxidant activity. The antioxidant capabilities of the samples enable the reduction of the pre-formed radical ABTS**⁺** generated by oxidation of ABTS after reaction with potassium persulfate. Therefore, if the concentration of the antioxidant in the sample is high, the reduction will be increased (Re et al., 1999), indicating that the radical scavenging effect is present. Similar to the results for TPC, the effect of radical scavenging with the hydrolyzed skim milk powders increased with reaction time (Table 2). The radical-scavenging properties of domestic sample A were not signifi-

| Table 1. Antioxidant activities using TPC assay of milk powder hydrolysates after papain treatment |
|---------------------------------|------------|------------|-----------|------------|----------|
| Skim milk powders               | 0          | 1          | 2          | 4          | 6        | 24       |
| A                               | 46.38±5.2**<sup>cA</sup> | 106.65±7.6**<sup>aA</sup> | 171.85±4.0**<sup>aA</sup> | 183.72±2.7**<sup>aA</sup> | 185.45±3.2**<sup>aA</sup> | 196.48±5.6**<sup>aA</sup> |
| B                               | 71.45±7.0**<sup>vA</sup> | 88.78±0.9**<sup>bA</sup> | 112.52±2.4**<sup>bA</sup> | 160.78±3.1**<sup>bA</sup> | 177.98±1.0**<sup>bA</sup> | 194.52±5.0**<sup>bA</sup> |
| C                               | 37.72±3.7**<sup>cA</sup> | 61.98±0.8**<sup>cA</sup> | 81.45±2.9**<sup>cA</sup> | 159.18±1.1**<sup>cA</sup> | 170.52±9.3**<sup>cA</sup> | 194.76±7.4**<sup>cA</sup> |
| D                               | 49.58±1.1**<sup>cA</sup> | 52.50±0.2**<sup>cA</sup> | 62.62±0.8**<sup>cA</sup> | 85.05±1.2**<sup>cA</sup> | 134.92±1.4**<sup>cA</sup> | 163.75±1.4**<sup>cA</sup> |

*Means±SD (n=3).

**a-dSuperscripts in rows that do not share the same letter differ (*p*<0.05).

**A-DSuperscripts in columns that do not share the same letter differ (*p*<0.05).
The antioxidant activities using ABTS assay of milk powder hydrolysates after papain treatment

| Skim milk powders | Incubation time (h) |
|-------------------|---------------------|
|                   | 0       | 1       | 2       | 4       | 6       | 24      |
| A                 | 39.38±1.1^a   | 60.95±0.2^b   | 73.66±0.3^a   | 74.81±0.2^a   | 72.85±0.1^a   | 75.33±0.8^a   |
| B                 | 34.23±0.1^b   | 50.38±0.4^b   | 57.61±0.7^ab  | 72.76±0.6^ab  | 71.85±1.2^a   | 72.09±0.2^ab  |
| C                 | 30.81±0.3^c   | 42.81±0.4^c   | 50.90±0.4^c   | 70.95±0.4^c   | 73.81±0.1^a   | 72.04±0.2^ab  |
| D                 | 28.47±0.1^d   | 29.38±0.0^d,d | 30.28±0.3^d   | 39.33±0.5^c   | 43.52±0.2^d   | 57.43±0.2^ac  |

A and B: domestic skim milk powders; C and D: imported skim milk powders.

1^) Means±SD (n=3).

a-d^Superscripts in rows that do not share the same letter differ (p<0.05).

A-d^Superscripts in columns that do not share the same letter differ (p<0.05).

Comparison of Antioxidant Activities of Skim Milk Powder Hydrolysates

The antioxidant activities of the hydrolysates were the highest following 24 h of hydrolysis (A: 75%, B: 72%, C: 72%, D: 57%). The antioxidant activity measured using the TPC and ABTS assays increased or remained the same until 24 h. This observation is consistent with that previously reported by Chang et al. (2013b) where the antioxidant activities measured by TPC and ABTS assays were detected in hydrolysates of milk casein following papain treatment. These hydrolysates were fractionated directly with a 3 kDa molecular weight cut off using an ultrafiltration membrane system. In this system, the skim milk powder hydrolysates may contain peptides which have hydrophobic amino acid residues. Hydrophobic amino acids, including aromatic amino acids (Sarmadi and Ismail, 2010), can increase the radical scavenging activity (Rajapakse et al., 2005). Ren et al. (2008) also reported that basic peptides have a greater capacity to scavenge hydroxyl radical than acidic or neutral peptides. The presence of hydrophobic amino acid residues such as Leu, Phe, and Val at C-terminal positions is consistent with the reported peptides (Table 3).

Potential peptides of antioxidant activity of skim milk powder hydrolysates

Animal protein sources of antioxidant peptides include egg whites (Chang et al., 2013a; Rao et al., 2012), hoki (Kim et al., 2007), gelatin (Kim et al., 2013), and yak milk casein (Mao et al., 2011). Antioxidant peptides are generated by enzymatic hydrolysis and milk fermentation (Haque et al., 2009). In this study, the < 3 kDa fraction of the 24 h hydrolysates was used for identification of peptides using LC-ESI-MS/MS analysis. The peptides originated from αs-casein, β-casein, α-lactalbumin, κ-casein, and β-lactoglobulin. The number of peptides derived from skim milk powders is 308 for A, 283 for B, 208 for C, and 135 for D. The different numbers of peptides generated by papain on imported and domestic milk powders could be resulted from the structure change of protein during manufacture of milk powder e.g., temperature of pasteurization and atomization. This may be caused to be difficult to access to milk protein by enzyme. It is similar to observation reported by Miclo et al. (2012) who reported that the accessibility of enzyme is different according to protection regions on milk casein structure. These results were similar to that of the OPA assay presented in Table 1. Three of the hydrolysates at 24 h, excluding hydrolysate D, showed the highest proteolytic activity releasing the highest amount of free amino groups.

The numerous reports of antioxidant peptides generated from different milk protein sources are listed in Table 3. Milk proteins are good precursors of biologically active peptides (Haque et al., 2009). As shown in Table 4, 46 peptides for hydrolysate A, 26 peptides for B, 26 peptides for C, and 24 peptides for D, generated from β-casein in this work exhibited antioxidant properties. These peptides contain the β-casein fragments VKEAMAPK (f98-105), VLPVPQ (f185-190), and AVYPQQR (f177-183) which display antioxidant activity. The fragment VKEAMAPK (f98-105) from β-casein reported in previous literature (Rival et al., 2001) was determined to have antioxidant activity through hydro-peroxide oxidation, DPPH assay, and a measurement of Fe^{2+} chelating activity. This fragment was also generated during the aging of cheddar cheese (Gupta et al., 2010) and released by hydrolysis with the cell envelop protease PrtS of Streptococcus thermophilus 4F44 in the matrix casein (Miclo et al., 2012). The fragments VLPVPQ (f185-190) and AVYPQQR (f177-183) were previously identified as antioxidant peptides by Hernandez-Ledesma et al. (2004) and Rival et al. (2001a). The peptide SKVLPVPQ, including VLPVPQ (f185-190),
was derived from two commercial fermented milk beverages with the aid of *Lactobacillus helveticus* and *Saccharomyces cerevisiae*, which exhibited antioxidant activities. Another peptide, AVPYPQR (f177-183), has antioxidant activity as demonstrated through a DPPH scavenging activity assay.

For α_{s1}-casein, 0 peptides for hydrolysates A and B, 3 peptides for C, and 9 peptides for D exhibited antioxidant properties in this study. Hydrolysis of α_{s1}-casein produced a tyrosine-containing hexapeptide (YFYPEL) with strong superoxide anion scavenging activity (Suetsuna *et al.*, 2000).

### Table 3. Antioxidant peptides identified from hydrolysates of milk protein in the literature

| Sequences | Identification of peptides from milk powder in present study | References reported |
|-----------|-------------------------------------------------------------|---------------------|
| f98-105, VKEAMAPK | GSVKVKEAMAPK GSVKVKEAMAPKH GSVKVKEAMAPKHK GSVKVKEAMAPKHKEMPFPK | Miclo *et al.*, 2012 |
| | GSVKVKEAMAPK GSVKVKEAMAPKHK GSVKVKEAMAPKHKEMPFPK | Gupta *et al.*, 2010 |
| | GSVKVKEAMAPK GSVKVKEAMAPKHKEMPFPK | Rival *et al.*, 2001 |
| | GSVKVKEAMAPK GSVKVKEAMAPKHKEMPFPK | |
Antioxidant peptides generated from αs2-casein were also detected (3 for A, 5 for B, 1 for C, and 0 for D). The fragment 144-149 (PYVRYL) has antioxidant activity and was also generated by the cell envelope protease PrtS of *Streptococcus thermophilus* (Miclo et al., 2012) and trypsin (López-Expósito et al., 2007).
For β-lactoglobulin, the number of potential antioxidant peptides detected was 4 for A, 2 for B, 2 for C, and 1 for D in this study. Hydrolysis of β-lactoglobulin with commercial proteinases produced peptides with antioxidant activity (Hernandez-Ledesma et al., 2005). Sadat et al., (2011) reported that fragment KTKIPAF (f75-82) from β-lactoglobulin has antioxidant activity. A total of 53 peptides for A, 33 for B, 32 for C, and 34 for D were identified as having potential antioxidant properties in the hydrolysates by LC-MS/MS.

**Conclusion**

To compare the antioxidant activity and antioxidant peptide release pattern, we utilized a combination of TPC and ABTS assays and LC MS/MS analysis of hydrolysates prepared from domestic and imported skim milk powders. High antioxidant activity was observed skim milk powder hydrolysate A at 24 h after papain treatment. In this fraction, 53 potential antioxidant peptides were identified. The number of released antioxidant peptides in domestic powders was higher than that of imported powders. From these results, we suggest that domestic skim milk can be utilized by various industries, i.e., in the development of functional foods. Further investigation into the antioxidant activity of peptides is an attractive line of research for the potential application of peptides in the food industry as antioxidant agents or food additives.

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**References**

1. Al-Laith, A. A. A. (2010) Antioxidant components and antioxidant/antiradical activities of desert truffle (*Tritania nivea*) from various Middle Eastern origins. *J. Food Comp. Anal.* 23, 15-22.

2. Cervato, G., Cazzola, R., and Cestaro, B. (1999) Studies on the antioxidant activity of milk caseins. *Int. J. Food Sci. Nutr.* 50, 291-296.

3. Chang, O. K., Ha, G. E., Han, K. S., Seol, K. H., Kim, H. W., Jeong, S. G., Oh, M. H., Park, B. Y., and Ham, J. S. (2013a) Novel antioxidant peptide derived from the ultrafiltrate of ovomucin hydrolysate. *J. Agri. Food Chem.* 61, 7294-7300.

4. Chang, O. K., Ha, G. E., Jeong, S. G., Seol, K. H., Oh, M. H., Kim, D. W., Jang, A., Kim, S.H., Park, B. Y., and Ham, J. S. (2013b) Antioxidant activity of porcine skin gelatin hydrolyzed by pepsin and pancreatin. *Korean J. Food Sci. An.* 33, 493-500.

5. Chang, O. K., Seol, K. H., Jeong, S. G., Oh, M. H., Park, B. Y., Perrin, C., and Ham, J. S. (2013c) Casein hydrolysis by *Bifidobacterium longum* KACC91563 and antioxidant activities of peptides derived therefrom. *J. Dairy Sci.* 96, 5544-5555.

6. Choi, S. K. (2013) Trend of agricultural import from FTA partners. *Korea Rural Economic Institute* 1, 1-44.

7. Clare, D. A. and Swaisgood, H. E. (2000) Bioactive milk peptides: A prospectus. *J. Dairy Sci.* 83, 1187-1195.

8. Colbert, L. B. and Decker, E. A. (1991) Antioxidant activity of an ultrafiltration permeate from acid whey. *J. Food Sci.* 56, 1248-1250.

9. Gupta, N., Hixson, K. K., Culley, D. E., Smith, R. D., and Panyam, J. (2010) Analyzing protease specificity and detecting in vivo proteolytic events using tandem mass spectrometry. *Proteomics* 10, 2833-2844.

10. Ham, J. S., Han, G. S., Jeong, S. G., Seol, K. H., Jang, A. R., Oh, M. H., Kim, D. H., and Park, Y. W. (2012) Determination of molecular weights of caprine milk proteins by matrix-assisted laser desorption/ionization mass spectrometry. *J. Dairy Sci.* 95, 15-19.

11. Haque, E., Chand, R., and Kapila, S. (2009) Biofunctional properties of bioactive peptides of milk origin. *Food Rev. Int.* 25, 28-43.

12. Hernandez-Ledesma, B., Amigo, L., Ramos, M., and Recio, I. (2004) Angiotensin converting enzyme inhibitory activity in commercial fermented products. Formation of peptides under simulated gastrointestinal digestion. *J. Agri. Food Chem.* 52, 1504-1510.

13. Hernandez-Ledesma, B., Dávalos, A., Bartolomé, B., and Amigo, L. (2005) Preparation antioxidant enzymatic hydrolysates from α-lactalbumin and β-lactoglobulin. Identification of active peptides by HPLC-MS/MS. *J. Agri. Food Chem.* 53, 588-593.

14. Kilara, A. and Panyam, D. (2003) Peptides from milk proteins and their properties. Critical reviews. *Food Sci. Nutr.* 43, 607-633.

15. Kim, D. W., Park, K., Ha, G., Jung, J. R., Chang, O., Ham, J. S., and Jang, A. (2013) Anti-oxidative and neuroprotective activities of pig skin gelatin hydrolysates. *Korean J. Food Sci. An.* 33, 258-267.

16. Kim, S. Y., Je, J. Y., and Kim, S. K. (2007) Purification and characterization of antioxidant peptide from hoki (*Johnius be-lergerii*) frame protein by gastrointestinal digestion. *J. Nutr. Biochem.* 18, 31-38.

17. Korhonen, H. (2009) Milk-derived bioactive peptides: From science to applications. *J. Funct. Foods* 1, 177-187.

18. Korhonen, H. and Pihlanto, A. (2003) Food-derived bioactive peptides-opportunities for designing future foods. *Cur. Pharm. Des.* 9, 1297-1308.

19. Korhonen, H. and Pihlanto, A. (2007) Bioactive peptides from food proteins. In: Hui Y. H. (ed.) Handbook of food products manufacturing. Hoboken, NJ: John Wiley and Sons Inc., 5-37.

20. Kovatcheva, E. G., Koleva, I. I., Ilieva, M., Pavlov, A., Mincheva, M., and Konushlieva, M. (2001) Antioxidant activity
of extracts from *Lavandula vera* MM cell cultures. *Food Chem.* 72, 295-300.

21. López-Expósito, I., Quirós, A., Amigo, L., and Recio, I. (2007) Casein hydrolysates as a source of antimicrobial, antioxidant and antihypertensive peptides. *Le Lait* 87, 241-249.

22. Mao, X. Y., Cheng, X., Wang, X., and Wu, S. J. (2011). Free-radical-scavenging and anti-inflammatory effect of yak milk casein before and after enzymatic hydrolysis. *Food Chem.* 126, 484-490.

23. Meisel, H., Walsh, D. J., Murray, B. A., and FitzGerald, R. J. (2006) ACE inhibitory peptides. In: Mine, Y. and Shahidi, F. (eds) *Nutraceutical proteins and peptides in health and disease.* Nutraceutical Sci. Technol. 269-315.

24. Miclo, L., Roux, E., Genay, M., Brusseaux, E., Poirson, C., Jameh, N., Perrin, C., and Dary, A. (2012) Variability of hydrolysis of β-, αs1-, and αs2-caseins by 10 strains of *Streptococcus thermophilus* and resulting bioactive peptides. *J. Agr. Food Chem.* 60, 554-565.

25. Ong, K. L., Cheung, B. M., Man, Y. B., Lau, C. P., and Lam, K. S. (2007) Prevalence, awareness, treatment, and control of hypertension among United States adults 1999-2004. *Hypertension,* 49, 69-75.

26. Pihlanto, A., Korhonen, H., and Jauregi P. (2009) Production of novel ACE inhibitory peptides from β-lactoglobulin using Protease N Amino. *Int. Dairy J.* 19, 69-76.

27. Rajapakse, N., Mendis, E., Byun, H. G., and Kim, S. K. (2005) Purification and in vitro antioxidative effects of giant squid muscle peptides on free radical-mediated oxidative systems. *J. Nutr. Biochem.* 16, 562-569.

28. Rao, S., Sun, J., Liu, Y., Zeng, H., Su, Y., and Yang, Y. (2012) ACE inhibitory peptides and antioxidant peptides derived from *in vitro* digestion hydrolysate of hen egg white lysozyme. *Food Chem.* 135, 1245-1252.

29. Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., and Rice-Evans, C. (1999) Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic. Biol. Med.* 26, 1231-1237.

30. Ren, J., Zhao, M., Shi, J., Wang, J., Jiang, Y., Cui, C., and Xue, S. J. (2008) Purification and identification of antioxidant peptides from grass carp muscle hydrolysates by consecutive chromatography and electrospray ionization-mass spectrometry. *Food Chem.* 108, 727-736.

31. Rival, S. G., Boeriu, C. G., and Wichers, H. J. (2001a) Caseins and casein hydrolysates. 2. Antioxidative properties and relevance to lipoxygenase inhibition *J. Agri. Food Chem.* 49, 295-302.

32. Rival, S. G., Fornaroli, S., Boeriu, C. G., and Wichers, H. J. (2001b) Caseins and casein hydrolysates. 1. Lipoxygenase inhibitory properties. *J. Agri. Food Chem.* 49, 287-294.

33. Sadat, L., Cakir-Kiefer, C., N’Negue, M. A., Gaillard, J. L., Girardet, G. M., and Miclo, L. (2011) Isolation and identification of antioxidative peptides from bovine α-lactalbumin. *Int. Dairy J.* 21, 214-221.

34. Sarmadi, B. H. and Ismail, A. (2010) Antioxidative peptides from food proteins: A review. *Peptides* 31, 1949-1956.

35. Serafini, M. and Del Rio, D. (2004) Understanding the association between dietary antioxidants, redox status and disease: Is the total antioxidant capacity the right tool? *Redox report,* 9, 145-152.

36. Silva, S., Gomes, L., Leitao, F., Coelho, A. V., and Boas, L. V. (2006) Phenolic compounds and antioxidant activity of *Olea europaea* L. fruits and leaves. *Food Sci. Technol. Int.* 12, 385-395.

37. Silva, S. V. and Malcata, F. X. (2005) Caseins as source of bioactive peptides. *Int. Dairy J.* 15, 1-15.

38. Steijns, J. M. and Van Hooijdonk, A. C. M. (2000) Occurrence, structure, biochemical properties and technological characteristics of lactoferrin. *British J. Nutri.* 84, 11-17.

39. Suetsuna, K., Ukeda, H., and Ochi, H. (2000) Isolation and characterization of free radical scavenging activities peptides derived from casein. *J. Nutr. Biochem.* 11, 128-131.

40. Taylor, M. J. and Richardson, T. (1980) Antioxidant oxidant of skim milk: effect of heat and resultant sulfhydryl groups. *J. Dairy Sci.* 63, 1783-1795.

41. Tong, L. M., Sasaki, S., McClements, D. J., and Decker, E. A. (2000) Mechanisms of the antioxidant activity of a high molecular weight fraction of whey. *J. Agri. Food Chem.* 48, 1473-1478.

42. Yang, S. A., Im, N. K., Ji, Y. J., Yoo, D. C., Jhee, K. H., and Lee, I. S. (2008) Radical scavenging and inhibition of platelet function by a polyphenol-rich fraction from Salvia miltiorrhiza Bunge. *Open Nat. Prod. J.* 1, 7-13.