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ARTICLE

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 α_{s1}-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

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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 (O_2), hydrogen peroxide (H_2O_2), 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; Steijins *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 there from 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-

dium (20 μL samples and 200 μL of the OPA reagent) was mixed in a 96-well clear flat-bottomed microplate (SPL, Korea). Absorbances were measured in triplicate at 340 nm using a microplate reader (Molecular Devices, USA).

Determination of Total Phenolic Contents

Measurements of total phenolic content (TPC) were performed as previously described Chang *et al*. (2013c). The reaction mixture was prepared by mixing 60 μL of sample and 60 μL of the Folin-Ciocalteu solution (1 volume of Folin-Ciocalteu reagent with 2 volumes of distilled water). Subsequently, 60 μL of 10% sodium carbonate (Na_2CO_3) were added to the reaction mixture. The final mixture was incubated at room temperature for 1 h in the dark. The absorbance at 700 nm was measured using a spectrophotometer (Molecular Devices). Gallic acid at various concentrations (1, 2, 20, 40, 60, 80, 100, and 120 μM) was used to prepare the standard curve. Results of the TPC analysis of skim milk powder hydrolysates were expressed as micromoles of gallic acid equivalent (GE) calculated from the standard curve.

ABTS radical-scavenging assay

The 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{+\cdot}$) (Sigma-Aldrich) radical scavenging assay was used as previously described by Chang *et al*. (2013a) to determine the antioxidant activity of the milk powder hydrolysates. A solution of 7 mM ABTS and 2.45 mM potassium persulfate was prepared and incubated for 12-16 h in the dark. Subsequently, the $ABTS^{+\cdot}$ radical solution was analyzed by a spectrophotometer at 734 nm (Molecular Devices) and adjusted to an absorbance of 0.70±0.02 with distilled water. The absorbance of the reaction mixture (50 μL and 950 μL of the $ABTS^{+\cdot}$ radical solution) was measured at 734 nm after 10 min of incubation at 30°C. A standard curve was determined using gallic acid (Sigma-Aldrich). The antioxidant scavenging activities of the skim milk powders and hydrolysates were expressed as a percent: Scavenging activity (%) = $\{(A_{control} - A_{sample}) / A_{control}\} \times 100$, in which $A_{control}$ represents the ABTS absorbance of distilled water.

The activities were also expressed also as micromole GE calculated from the standard curve based on gallic acid (Chang *et al*., 2013b; Kim *et al*., 2013).

Mass spectrometry analysis

LC/MS/MS experiments were carried out as previously method by Chang *et al*. (2013c). This analysis was performed 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^{TM} 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 μL into an LC-MS/MS on a Zorbax 300 SB-C18 trap column (300 μm i.d × 5 mm, 5 μm, 100; Agilent Technologies, USA; part number 5065-9913), at a flow rate of 5 μL/min, and the sample was separated on a Zorbax 300SB-C18 capillary column (75 μm i.d × 150 mm, 3.5 μm, 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 electrosprayed 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 α_s-casein and

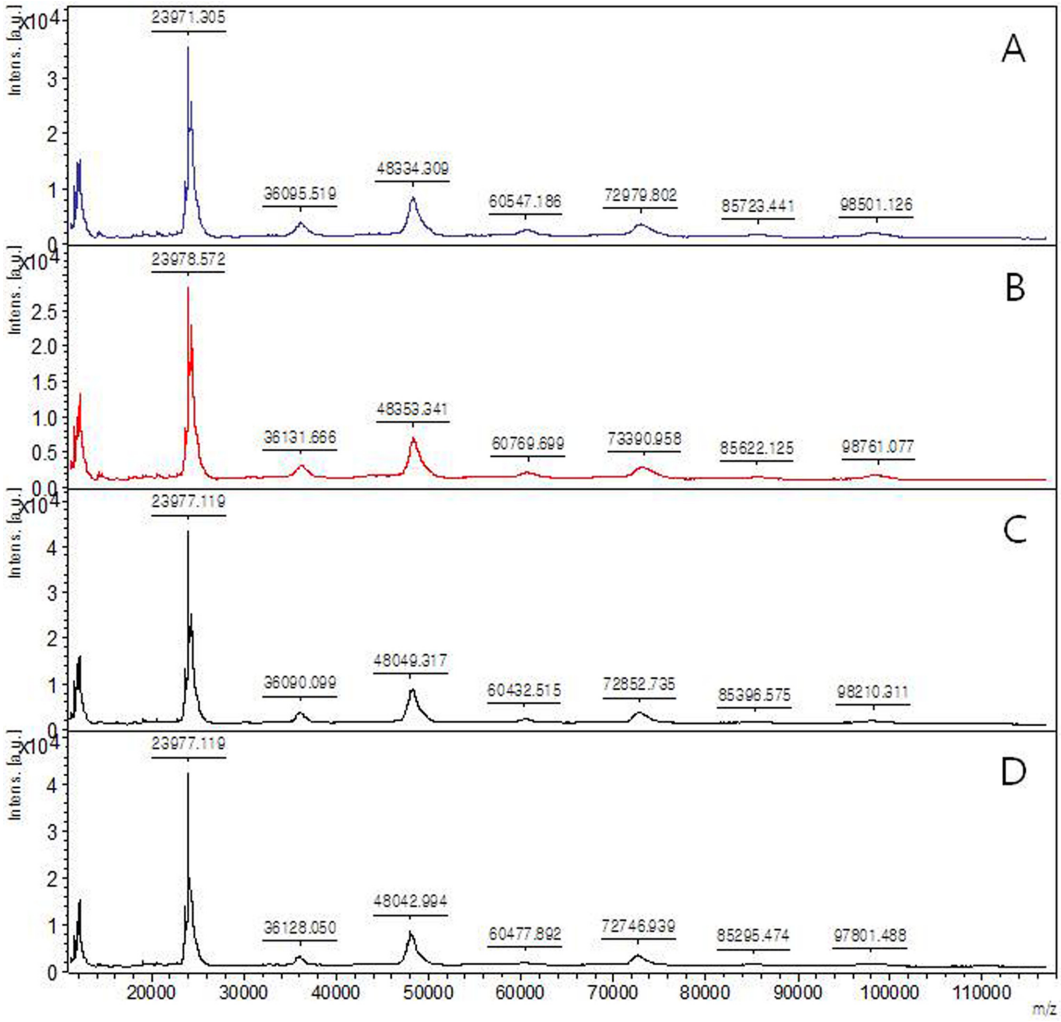

Fig. 1. MALDI-TOF analysis of skim milk powders from four different sources. A and B: domestic skim milk powders; C and D: imported skim milk powders.

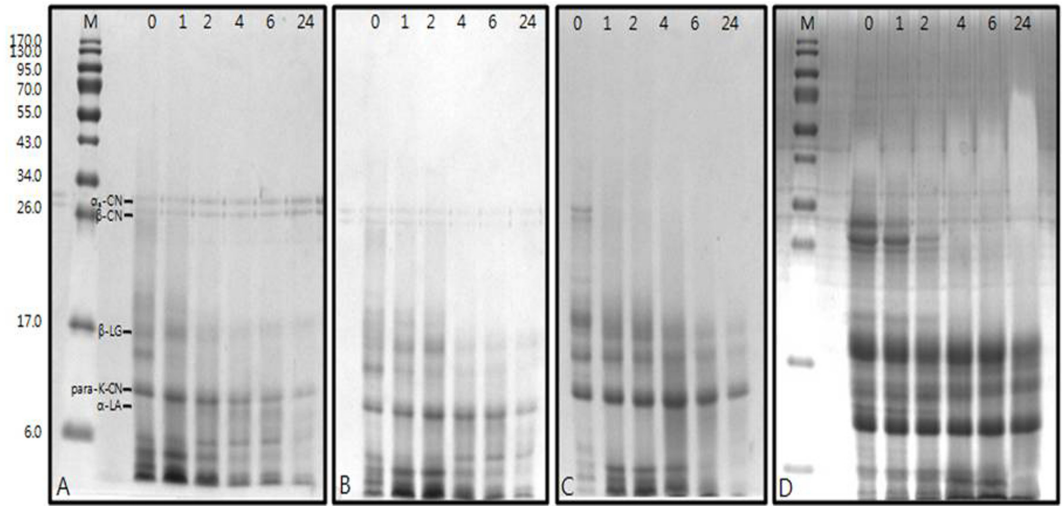

Fig. 2. Electrophoresis of various skim milk powders treated with papain. Lane M: Protein standard marker; 0, 1, 2, 4, 6, 24: incubation time (h). A and B: domestic skim milk powders; C and D: imported skim milk powders.

β-casein decreased while lower molecular weight breakdown products of the caseins increased. These products, which appear in SDS-PAGE in the area between α_s-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 α_s-casein and β-casein varied between the four skim milk powders. The α_s-casein and β-casein were hydrolyzed faster than other proteins in most milk powders, as shown by the disappearance of the α_s-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 enzyme treatment. In contrast, κ-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 $ABTS^{+\cdot}$ 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	Incubation time (h)					
	0	1	2	4	6	24
A	46.38±5.2[1)] ^{cA}	106.65±7.6 ^{bA}	171.85±4.0 ^{aA}	183.72±2.7 ^{aA}	185.45±3.2 ^{aA}	196.48±5.6 ^{aA}
B	71.45±7.0 ^{cA}	88.78±0.9 ^{cbA}	112.52±2.4 ^{bB}	160.78±3.1 ^{aB}	177.98±1.0 ^{aA}	194.52±5.0 ^{aA}
C	37.72±3.7 ^{cA}	61.98±0.8 ^{cbB}	81.45±2.9 ^{bB}	159.18±1.1 ^{aB}	170.52±9.3 ^{aA}	194.76±7.4 ^{aA}
D	49.58±1.1 ^{dA}	52.50±0.2 ^{dB}	62.62±0.8 ^{cB}	85.05±1.2 ^{bC}	134.92±1.4 ^{aB}	163.75±1.4 ^{aB}

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).

Table 2. 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[1)] ^{cA}	60.95±0.2 ^{bA}	73.66±0.3 ^{aA}	74.81±0.2 ^{aA}	72.85±0.1 ^{aA}	75.33±0.8 ^{aA}
B	34.23±0.1 ^{dB}	50.38±0.4 ^{cB}	57.61±0.7 ^{bB}	72.76±0.6 ^{aBA}	71.85±1.2 ^{aA}	72.09±0.2 ^{aB}
C	30.81±0.3 ^{eC}	42.81±0.4 ^{dC}	50.90±0.4 ^{cC}	70.95±0.4 ^{bB}	73.81±0.1 ^{aA}	72.04±0.2 ^{aB}
D	28.47±0.1 ^{dC}	29.38±0.0 ^{dcD}	30.28±0.3 ^{cD}	39.33±0.5 ^{bC}	43.52±0.2 ^{aB}	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).

cantly different after 2 h of incubation (p>0.05). The radical-scavenging properties of domestic sample B were not significantly different after 4 h of incubation and those of import sample C were not significantly different after 6 h of incubation (p>0.05). The radical scavenging activity of import sample D continuously increased with incubation time (p<0.05). 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 α_s-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 AVPYPQR (f177-183) which display antioxidant activity. The fragment VKEA MAPK (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 AVPYPQR (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
		A	B	C	D	
β-CN	f98-105, VKEAMAPK	GVSKVKEAMAPK GVSKVKEAMAPKHK GVSKVKEAMAPKH-KEMPFPK	GVSKVKEAMAPK LQPE-VMGVSKVKEAMAPK VKEAMAPKHKEMPFPK VMGVSKVKEAMAPK VSKVKEAMAPK	N.D[1]	GVSKVKEAMAPK GVSKVKEAMAPKH GVSKVKEAMAPKHK GVSKVKEAMAPKHKE GVSKVKEAMAPKH-KEMPFPK GVSKVKEAMAPKH-KEMPFPKYPVEPFT KVKEAMAPK KVKEAMAPKHK KVKEAMAPKHKEMPFPK QPEVMGVSKVKEAMAPK QVKEAMAPKHKEMPFPK SKVKEAMAPKH-KEMPFPK VKEAMAPKHKEMPFPK VSKVKEAMAPKHK VSKVKEAMAPKH-KEMPFPK	Miclo *et al*., 2012 Gupta *et al*., 2010 Rival *et al*., 2001
	f184-190, VLPVPQ	HQPHQPLPPTVMFPPQSV-LSLSQSKVLPVPQK QSKVLPVPQK HQPHQPLPPTVMFPPQSV-LSLSQSKV-LPVPQKAVPYPQR LSQSKVLPVPQK MHQPHQPLPPTVMFPPQS-VLSLSQSKVLPVPQK SLSQSKVLPVPQK SLSQSKV-LPVPQKAVPYPQ SLSQSKV-LPVPQKAVPYPQR SLSQSKV-LPVPQKAVPYPQRDMP SLSQSKV-LPVPQKAVPYPQRDMPI SLSQSKV-LPVPQKAVPYPQRD-MPIQAF SLSQSKV-LPVPQKAVPYPQRD-MPIQAFL SQSKVLPVPQKAVPYPQ SQSKVLPVPQKAVPYPQR SQSKV-LPVPQKAVPYPQRDMPIQ SQSKV-LPVPQKAVPYPQRD-MPIQA VLPVPQKAVPYPQR VLPVPQKAVPYPQRDMPI VLPVPQKAVPYPQRD-MPIQA VLPVPQKAVPYPQRD-MPIQAF VLPVPQKAVPYPQRD-MPIQAFLLYQEPVLG-PVRGPFPIIV VLSLSQSKVLPVPQK VLSLSQSKV-LPVPQKAVPYPQ VLSLSQSKV-LPVPQKAVPYPQR VLSLSQSKV-LPVPQKAVPYPQRD-MPIQA	LSQSKVLPVPQK LSQSKV-LPVPQKAVPYPQRD-MPIQAF SLSQSKV-LPVPQKAVPYPQR SLSQSKV-LPVPQKAVPYPQRD-MPIQA SQSKVLPVPQKAVPYPQ QSKVLPVPQK SKVLPVPQK VLPVPQKAVPYPQRD VLPVPQKAVPYPQRD-MPIQAF	LSLSQSKV-LPVPQKAVPYPQRD-MPIQAFLLYQEPVLGPV LSQSKVLPVPQK LSQSKV-LPVPQKAVPYPQR LSQSKV-LPVPQKAVPYPQRDMP LSQSKV-LPVPQKAVPYPQRD-MPIQAF LSQSKV-LPVPQKAVPYPQRD-MPIQAFL MHQPHQPLPPTVMFPPQS-VLSLSQSKVLPVPQK QSKVLPVPQKAVPYPQ SLSQSKVLPVPQK SLSQSKV-LPVPQKAVPYPQRD-MPIQA SQSKVLPVPQKAVPYPQ VLPVPQKAVPYPQR VLPVPQKAVPYPQRD-MPIQAF VLSLSQSKVLPVPQK VLSLSQSKV-LPVPQKAVPYPQRDMP	PQSVLSLSQSKVLPVPQK QSKV-LPVPQKAVPYPQRD-MPIQAFL SLSQSKVLPVPQK SQSKVLPVPQK SQSKV-LPVPQKAVPYPQRD-MPIQAFL	Rival *et al*., 2001 Hernandez-Ledesma *et al*., 2004

Table 3. Antioxidant peptides identified from hydrolysates of milk protein in the literature (continued)

	Sequences	Identification of peptides from milk powder in present study				References reported
		A	B	C	D	
β-CN	f177-183, AVPYPQR	AVPYPQRDMPIQ HQPHQPLPPTVMFPPQSV- LSLSQSKV- LPVPQKAVPYPQR KAVPYPQRD- MPIQAFLLYQEPVLG- PVRGPFPIIV SLSQSKV- LPVPQKAVPYPQR SLSQSKV- LPVPQKAVPYPQRDMP SLSQSKV- LPVPQKAVPYPQRDMPI SLSQSKV- LPVPQKAVPYPQRD- MPIQAF SLSQSKV- LPVPQKAVPYPQRD- MPIQAFL SQSKVLPVPQKAVPYPQR SQSKV- LPVPQKAVPYPQRDMPIQ SQSKV- LPVPQKAVPYPQRD- MPIQA VLPVPQKAVPYPQR VLPVPQKAVPYPQRDMPI VLPVPQKAVPYPQRD- MPIQA VLPVPQKAVPYPQRD- MPIQAF VLPVPQKAVPYPQRD- MPIQAFLLYQEPVLG- PVRGPFPIIV VLSLSQSKV- LPVPQKAVPYPQR VLSLSQSKV- LPVPQKAVPYPQRD- MPIQA	AVPYPQRDMPIQAFL KAVPYPQRDMP LYQEPVLGPVRGPFPI LPVPQKAVPYPQRDMPIQ LSQSKV- LPVPQKAVPYPQRD- MPIQAF SKVLPVPQKAVPYPQRD- MPIQ SLSQSKV- LPVPQKAVPYPQR SLSQSKV- LPVPQKAVPYPQRD- MPIQA TVMFPPQSVLSLSQSKV- LPVPQKAVPYPQRDMPIQ QSKV- LPVPQKAVPYPQRD- MPIQA VLPVPQKAVPYPQR VLPVPQKAVPYPQRD VLSLSQSKV- LPVPQKAVPYPQRDMP	LSLSQSKV- LPVPQKAVPYPQRD- MPIQAFLLYQEPVLGPV LSQSKV- LPVPQKAVPYPQR LSQSKV- LPVPQKAVPYPQRDMP LSQSKV- LPVPQKAVPYPQRD- MPIQAF LSQSKV- LPVPQKAVPYPQRD- MPIQAFL SLSQSKV- LPVPQKAVPYPQRD- MPIQA VLPVPQKAVPYPQR VLPVPQKAVPYPQRD- MPIQAF VLSLSQSKV- LPVPQKAVPYPQRDMP	AVPYPQRDMPIQAFLLY- EQPVLGPVR KAVPYPQRDMPIQAFL QSKV- LPVPQKAVPYPQRD- MPIQAFL SQSKV- LPVPQKAVPYPQRD- MPIQAFL	Rival *et al*. 2001
α_{s1}-CN	f144-149, YFYPEL	N.D	N.D	IHAQQKEPMIGVNQELAY- FYPELFRQ LAYFYPELFRQ	EPMIGVNQELAYFYPELFR GIHAQQKEPMIGVNQE- LAYFYPELFR IGVNQELAYFYPELFR PMIGVNQELAYFYPELFR QKEPMIGVNQELAY- FYPELFR QQKEPMIGVNQELAY- FYPELFR VNQELAYFYPELFRQF VNQELAYFYPELFRQFY YFYPELFRQFYQ	Suetsuna *et al*., 2000
α_{s2}-CN	f202-207, PYVRYL	KVIPYVRYL TKVIPYVRYL QKAMKPWIQPKT- KVIPYVRYL	PKTKVIPYVRYL PWIQPKTKVIPYVRYL PMIGVNQELAYFYPEL- FRQFY QKAMKPWIQPKT- KVIPYVRYL TVYQHQKAMKPWIQPKT- KVIPYVRYL	KPWIQPKTKVIPYVRYL	N.D	Miclo *et al*., 2012 López-Expósito *et al*., 2007
β-lg	f146-150, MHIRL	ALPMHIRLSFNPTQLEEQ- CHI HIRLSFNPTQLEEQCHI	ALPMHIRLSFNPTQLEEQ- CHI	N.D	ALPMHIRLSFNPTQ	Hernandez-Ledesma *et al*., 2005
	f75-82, KTKIPAVF	IIAEKTKIPAVF IIAEKTKIPAVFK	IIAEKTKIPAVF	IIAEKTKIPAVF IIAEKTKIPAVFK	N.D	Sadat *et al*., 2011
The number of total peptides		53	33	32	34	

[1]N.D = not detected.

Antioxidant peptides generated from α_{s2}-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 themophilus* (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 KTKIPAVF (f75-82) from β-lactoglobulin has antioxidantt 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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