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Effect of Recovery Methods on the Characteristics of Antioxidative Milk Peptides

Supanida Pattorn and Parichat Hongsprabhas*

ABSTRACT

This study investigated the influences of peptide recovery methods after tryptic (E.C.3.4.21.4) hydrolysis, together with the pH before freeze-drying on the antioxidant capacity of milk peptides. Isoelectric precipitation of milk hydrolysate yielded hydrophobic milk peptides while 50% ethanol (EtOH) precipitated the hydrophilic ones. The hydrophobic peptides had significantly higher antioxidant capacity than the hydrophilic peptides ($P < 0.05$). The antioxidant capacity of hydrophobic peptides was 1.24 and 1.15 µmol Trolox equivalent (TE)/mg protein measured by the oxygen radical absorbance capacity-fluorescein ($ORAC_{FL}$) and Trolox equivalent antioxidant capacity (TEAC) assays, respectively. However, the hydrophilic peptides had $ORAC_{FL}$ antioxidant capacity of 0.62 and TEAC antioxidant capacity of 0.37 µmol TE/mg protein. Nevertheless, the hydrophobic peptides were prone to flocculation and aggregation, particularly when freeze-dried at an acidic pH of 2.0 in lactose excipient. However, freeze-drying in lactose excipient at pH 6.5 resulted in reconstituted hydrophobic peptides with higher $ORAC_{FL}$ antioxidant capacity than the freshly prepared peptides. This study suggested that the high antioxidant capacity of hydrophobic peptides could play significant roles as natural antioxidants after tryptic digestion in the gastrointestinal tract.

Keywords: antioxidant, hydrolysis, milk, peptide, trypsin

INTRODUCTION

Bioactive peptides (BPs) from milk are specific protein fragments that are released from milk proteins and have a positive impact on body functions or conditions, which may ultimately influence health. These peptides can be generated *in vitro* by the action of digestive enzymes. They can be obtained during the production of milk products (cheese, yoghurt) or under the action of endogenous enzymes of milk (plasmin or cathepsin) or of microorganisms (Kitts and Weiler, 2003; Bouhallab and Bougle, 2004; Korhonen, 2009). The biological effects of BPs include antioxidative, angiotensin-converting enzyme inhibitory, antithrombotic, antimicrobial, immunomodulatory, ion binding and opioid antagonist activities. There is considerable evidence indicating that many BPs serve multifunctional bioactivities and often have common structural features based on their amino acid sequences (Gobbetti *et al.*, 2002).

Caseins in milk are considered the most important source for the production of BPs known as caseinophophopeptides (CPPs). CPPs are released from the proteolytic digestion of casein during cheese ripening or gastro-intestinal digestion. CPPs play essential roles as carriers

Department of Food Science and Technology, Faculty of Agro-Industry, Kasetsart University, Bangkok 10900, Thailand.
* Corresponding author, e-mail: parichat.h@ku.ac.th

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for calcium and iron (Meisel and FitzGerald, 2003), as well as functions like antioxidants (Kitts, 2005). CPPs have been produced at the industrial scale by proteolytic hydrolysis of individual casein. Enzymatic hydrolysis, by means of at least one proteolytic enzyme, such as pancreatin, trypsin and α˘chymotrypsin, is carried out within a pH range of 7˘8.5 and in a temperature range of 37˘40 °C. The recovery of CPPs is by ultrafiltration, followed by aggregation induced by divalent cations to isolate the phosphopeptide constituents (Brule *et al.,* 1989; Qi *et al.*, 2003; Lapointe *et al.*, 2004). Generally, the bioactivities of BPs are governed by the purity of the protein substrate, the specificity of the proteolytic enzymes used, the process parameters (pH, temperature, ionic strength, activator) employed during hydrolysis, the degree of hydrolysis, the techniques used for enzyme inactivation (heat treatment, acidification, or membrane filtration) and the use of post-hydrolysis treatments (adsorbents for free amino acids, membrane) (Gauthier and Pouliot, 2003). To date, studies have been limited on mixed proteins containing all fractions of casein and whey proteins as the substrate for the production of BPs.

Clausen *et al.* (2009) demonstrated that caseins were quantitatively the major radical scavenging species in milk. Milk proteins and peptides can act as antioxidants via primary and secondary mechanisms (Cervato *et al*., 1999; Kitts, 2005). The former involves the process of antioxidants accepting free radicals to delay the initiation step, or interrupting the propagation step in autooxidation. The latter, however, includes the chelation of transition metals, replenishment of hydrogen to the primary antioxidants, oxygen scavenging and deactivation of reactive species (McClements and Decker, 2000).

It was hypothesized in the present study that peptides with different antioxidative characteristics could be fractionated from the tryptic hydrolysates of mixed proteins in milk by using different peptide recovery methods namely, EtOH precipitation versus isoelectric precipitation. This was based on the distinct characteristics of the casein molecules, which have anionic clusters, and hydrophilic and hydrophobic domains in their primary structure (Singh *et al*., 1996; Farrell *et al.*, 2004; Panouille *et al.*, 2005). In addition, the increase in the protein concentration during the dehydration process, which brings the proteins in close proximity, may alter the biological activities of the peptides due to aggregation during dehydration. The mechanisms responsible for irreversible protein aggregation in the solid deterioration of protein powder usually include sulfhydryl-disulfide (SH-SS) interchange, β-elimination followed by thiol-catalysed disulfide bond formation and formaldehyde-mediated pathways, amongst others (Costantino *et al.,* 1994, 1995). These interactions are favored at neutral and alkaline pH values.

Therefore, the investigation also covered the influence of the pH prior to the freeze-drying process on the alterations of physicochemical properties and the antioxidant capacity of tryptic milk peptides. A better understanding of milk peptide aggregation during the freeze-drying process may help to control the alterations in bioactivity of the peptide powder after reconstitution.

MATERIALS AND METHODS

Materials

Fresh skimmed milk (8.73% total solids, 3.01% protein and 0.10% fat; AOAC, 2000) was donated by Friesland Foods Foremost PLC. (Bangkok, Thailand). Trypsin (E.C. 3.4.21.4.; product code No.T8003) was purchased from Sigma-Aldrich, St. Louis, MO.

Preparation of milk protein curd

Six litres of fresh skimmed milk was batch pasteurized at 63 °C for 30 min and stored at 5 °C before use. The pasteurized skimmed milk

was pre-heated at 40 °C prior to the addition of 0.20 M calcium lactate to obtain the added concentration of 30 mmol Ca/kg skimmed milk. After the precipitation had been allowed to proceed at 40 °C for 15 min, the temperature was raised to 75 °C at the rate of 3 °C/min and remained at this temperature for 15 min. Whey was drained from the milk protein curd for 30 min through cheese cloth. The curd was dried in a tray dryer at 60 °C for 6 h. Milk protein powder was ground using a hammer mill, sifted through a 150 µm aperture screen and stored in a refrigerator at 5 °C before use.

Preparation of milk protein hydrolysate

Milk protein powder was dissolved in the deionized water, added with trypsin (1 mg/mL in 1 mM HCL) using a milk protein concentration of 2.5% (w/v) as substrate, and a trypsin to protein ratio of 1:80. The hydrolysis was performed at 40 °C for 30 min at pH 8.0 (adjusted by 1.0 M NaOH) unless stated otherwise. The hydrolysate was divided into two fractions. The peptides in the first fraction were recovered by EtOH precipitation using 99.9% EtOH with a ratio of hydrolysate to EtOH of 1:1 (v/v). In the second fraction, 1 M HCl was added to precipitate the peptides at the isoelectric pH of 4.6. After adding EtOH or HCl, the aggregation was allowed to proceed for 10 min. The peptide was collected by centrifugation at 6000× *g* for 10 min. The precipitated peptides were determined for protein concentration by Lowry s method (Lowry *et al.,* 1951) and the percentage of peptide recovery (% yield) was determined. The molecular weight (MW) profiles of peptides, Z-average size and antioxidant capacity were determined as described below.

Freeze-drying of milk peptides in lactose excipient

The EtOH and isoelectric precipitated peptides were mixed with lactose solution at pH 2.0 or pH 6.5 prior to freeze-drying. The ratio of peptide to lactose was 0.3:0.7 (w/w). The solutions were flash frozen in liquid nitrogen and kept frozen at -40 °C for 36 h, freezed-dried in a freeze-dryer (HETO model FD 2.5, Heto Lab Equipment, Allerϕd, Denmark) for 20 h to lower the moisture content to below 10%, ground, passed through a 150 µm sieve and stored in a freezer (-20 °C) before use. The effect of freezing before sublimating on the aggregation of milk peptides was also investigated. The frozen peptides were thawed at room temperature and evaluated for particle size distribution using the method described below.

Characterization of freshly prepared milk peptides and reconstituted peptides from different recovery methods

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The molecular weight (MW) profiles of protein and milk protein hydrolysate were determined using the Bio-Rad Mini Protein II electrophoresis apparatus (Bio-Rad Laboratories, Hercules, CA, USA) (Laemmli, 1970), using a 4% stacking gel and a 15% separating gel. The continuous buffer contained 0.125 M Tris˘HCl, pH 6.8 and 0.1% SDS for the stacking gel and 0.375 M Tris˘HCl, pH 8.8, and 0.1% SDS for the separating gel. The running buffer contained 0.124 M Tris, 0.959 M glycine and 0.1% SDS, pH 8.3. All samples were added to dissociating buffer (0.5 M Tris˘HCl, pH 6.8, glycerol and 1% (w/v) bromophenol blue). Each solution was heated at 100 °C for 4 min, cooled and centrifuged at 5,000 rpm (Labnet Spectrafuge 16 M, Labnet International, Edison, NJ) for 5 min to remove insoluble material. An aliquot of the sample solution containing 0.01 mg protein for the milk protein sample, or 0.08 mg protein for the hydrolysate sample and 4 µL MW standards were loaded into each well. Electrophoresis was run at a constant voltage of 150 V. Gel slabs were fixed and stained simultaneously using Bio-Rad

Coomassie Blue R-250 stain solution (40% methanol, 10% acetic acid, 0.1% Coomassie Blue R-250) for 30 min, and then de-stained by Bio-Rad Coomassie Blue R-250 de-staining solution for 5 h with 2˜3 changes of the de-staining solution. The MW of proteins was determined using full-range rainbow MW markers of approximately 10˜250 kDa (RPN 8000, Amersham Biosciences, Buckinghamshire, UK) and low-range MW markers of approximately 2.5˜45 kDa (RPN 755, Amersham Biosciences, Buckinghamshire, UK) as the MW standards.

Gel filtration chromatography

The chromatographic separation was performed using the Fast Protein Liquid Chromatography (FPLC) system (Pharmacia Biotech AB, Uppsala, Sweden). Milk hydrolysate (16 h hydrolysis) was centrifuged at 10,000× *g* for 10 min. The supernatant (100 µL) was loaded onto a Sephacryl S-100 HR 16/60 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) pre-equilibrated with 20 mM Tris-HCl buffer (pH 8) containing 300 mM NaCl and 0.01% NaN_3. The aliquot was eluted with the same buffer at the flow rate of 0.3 mL/min. Qualitative analysis of peptides was monitored by on-line measurement of the absorbance at 280 nm (OD_{280}). The column was calibrated with three peptide standards: aprotinin (M=6.5 kDa), ribonuclease A (13.7 kDa) and carbonic anhydrase (29 kDa) (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

Particle size distribution

The size distribution and Z-average size of milk peptides in the hydrolysate and reconstituted peptides were measured using a Zetasizer (Zen 3600, Malvern Instruments Ltd., Worcestershire, UK). Double deionized water filtered through a 0.22 µm Millipore filter was used in all experiments. Before measurement, samples were reconstituted to obtain 1 mg protein/mL.

Oxygen radical absorbance capacity-fluorescein ($ORAC_{FL}$) assay

The $ORAC_{FL}$ assay was carried out using a BMG Fluostar Optima Microplate Reader (Molecular Devices, Sunnyvale, CA), which was equipped with injectors, an incubator and wavelength adjustable fluorescence filters. The temperature of the incubator was set to 37 °C. A fluorescence filter with an excitation wavelength of 485 nm and an emission wavelength of 520 nm was used. The procedures were based on the methods described by Prior *et al.* (2003) and Davalos *et al.* (2004). The final $ORAC_{FL}$ values were calculated and reported as µmol Trolox equivalent (TE)/mg protein.

Trolox equivalent antioxidant capacity (TEAC) assay

The TEAC assay described by Re *et al.* (1999) was used to evaluate the relative capacity of antioxidant in the scavenging of ABTS radicals ($ABTS^{\cdot+}$) compared to the antioxidant potency of Trolox, and reported as µmol TE/mg protein. The $ABTS^{\cdot+}$ was generated by mixing 5 mL of 7 mM ABTS with 88 µL of 140 mM $K_2S_2O_8$ in the dark for 24 h at room temperature before use. $ABTS^{\cdot+}$ stock solution was diluted to get an absorbance of 0.700 ± 0.020 at 734 nm with phosphate buffer saline (PBS). TEAC was measured spectrophotometrically (Genesys 10 UV, Thermo Electron Corporation, Madison, WI) by analyzing the decolorisation of the $ABTS^{\cdot+}$ at 734 nm after 4 min of reaction at 30 °C using PBS as a control.

Statistical analysis

Milk proteins and peptides were precipitated and freeze-dried in 2 separate trials. Each trial was evaluated in duplicate. The data were analyzed by analysis of variance (ANOVA) with significance determined at $P < 0.05$. Significant differences among mean values were determined by Duncan s multiple range test. All statistical analyses were performed using the SPSS Software Version 12 (SPSS Inc., USA).

RESULTS AND DISCUSSION

The MW profiles of the proteins in the milk curd showed typical bands of caseins as major proteins (Figure 1). Minor proteins were also found in milk coagulum, with MW values of around 50˜75 kDa and 10˜25 kDa. These proteins were most likely whey proteins since the pasteurization step and the last step of the milk protein preparation involved heat treatment at 75 °C for 15 min. This last step could lead to the formation of a complex mixture of whey proteins and casein micelles (Vasbinder and de Kruif, 2003; Donato and Guyomarc h, 2009).

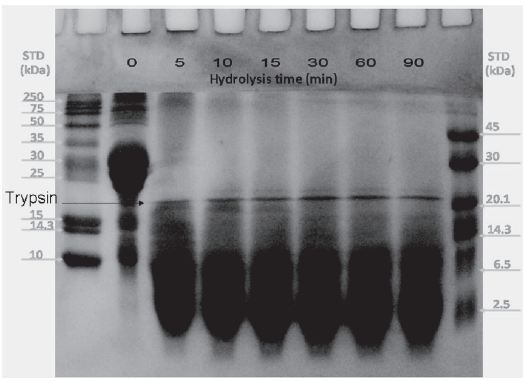


Figure 1 SDS-PAGE showing molecular weight (MW) profiles of milk proteins before and during tryptic hydrolysis.

The MWs of most of the milk peptides in the tryptic hydrolysates were below 14.3 kDa after 30 min of hydrolysis (Figure 1). These peptides were further characterized for their MWs by gel filtration (Figure 2). Fractions I and II corresponded with the peptides having a MW between 13.7 and 6.5 kDa, while fractions III, IV and V corresponded to peptides having a MW lower than 6.5 kDa. Both SDS-PAGE and gel filtration chromatography indicated that prolonging the hydrolysis time longer than 30 min did not shorten the milk proteins further. Milk protein hydrolysate prepared by tryptic hydrolysis for 30 min was then recovered for peptides using EtOH or isoelectric precipitation.

Table 1 shows that the EtOH precipitated peptides, which were fractionated due to their low solubility in 50% EtOH, had lower antioxidant capacity than the isoelectric precipitated milk peptides. The $ORAC_{FL}$ antioxidant capacity of EtOH precipitated peptides was 0.62 µmol TE/mg protein and their TEAC antioxidant capacity was 0.37 µmol TE/mg protein. Nevertheless, the hydrophilic milk peptides retained a small Z-average size of 260 nm.

Isoelectric precipitation resulted in a higher yield of peptides recovered than the EtOH precipitation. Lowering the pH from 8.0 during

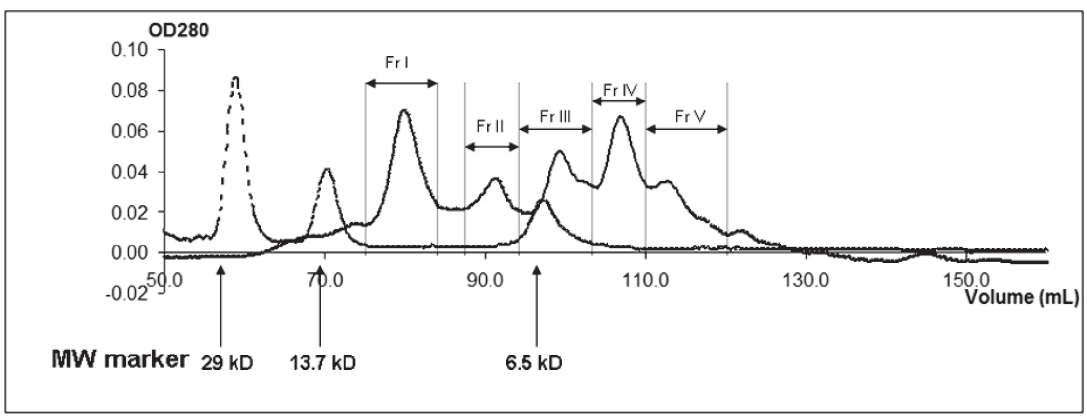


Figure 2 Gel filtration chromatogram of tryptic milk protein hydrolysate after 16 h hydrolysis by trypsin. (Fr = fraction).

tryptic hydrolysis to 4.6 by the addition of 1 M HCl led to a reduction in the surface charge of the peptides. The peptides were then precipitated due to the net zero charge in the aqueous phase. Isoelectric precipitation thus resulted in large sizes of peptide clusters, approaching almost 1,000 nm. However, these peptides had high antioxidant capacity, that is, 1.24 µmol TE/mg protein determined by $ORAC_{FL}$ assay and 1.15 µmol TE/mg protein by TEAC assay (Table 1).

The antioxidant capacities of milk proteins and peptides are most likely encrypted within the amino acid sequences of the polypeptide chains. All caseins have distinct anionic clusters, and hydrophilic and hydrophobic domains in their primary structure. For example, α_{s1}-casein has hydrophobic and polar domains. The α_{s2}-casein contains 4 domains in a single polypeptide chain namely , an N-terminal hydrophilic domain with anionic clusters, a central hydrophobic domain, another hydrophilic domain with anionic clusters, and a C-terminal positively charged hydrophobic domain. β-casein is the most hydrophobic polypeptide of the caseins. The N terminal sequence of β-casein is composed of charged amino acids and a phosphoserine cluster. This initial sequence is different from the second half of the molecule, which is rich in neutral and hydrophobic amino acid residues. κ-Caseins consist of a major carbohydrate component with varying degrees of phosphorylation and glycosylation. Heat treatment of native κ-casein resulted in aggregation caused by the SH-SS interchange with whey proteins (Singh *et al.*, 1996; Farrell *et al.*, 2004; Panouille *et al.*, 2005).

After the hydrolysis of milk proteins by trypsin, which cleaved peptide bonds at the C-terminal side of lysine and arginine, (except when either is followed by proline [Bruins *et al.*, 2009]), subsequent peptide recovery methods used in this study could fractionate peptides based on different degrees of hydrophilicity. EtOH, which is less polar than water, could precipitate the hydrophilic peptides, while isoelectric precipitation was likely to precipitate more hydrophobic peptides. The difference in physicochemical characteristics of peptides suggested that the species of amino acid residues, rather than particle size, played an important role in the antioxidant capacity of the milk peptides.

Both hydrophobic and hydrophilic peptides obtained after different recovery methods were mixed with lactose solution at pH 6.5. Lactose was chosen as the excipient to protect the peptides from aggregation during freezing and drying (Haque and Roos, 2006). Figure 3a indicates that the particle size distribution of peptides precipitated by EtOH was not affected by lactose although the peptide to lactose ratio was 0.3:0.7. The hydrophilic peptides had a similar particle size distribution with a Z-average size of 260 nm. Apparently, the presence of lactose did

Table 1 Effect of precipitation methods on the characteristics of milk peptides obtained after tryptic hydrolysis of calcium lactate milk protein for 30 min.

	Precipitation method used in peptide recovery	
	EtOH precipitation	Isoelectric precipitation
Yield (% of initial protein)	30.80[b]	41.92[a]
Z-average size (nm)	262[b]	977[a]
$ORAC_{FL}$ (µmol TE/mg protein)	0.62[b]	1.24[a]
TEAC (µmol TE/mg protein)	0.37[b]	1.15[a]

Means in the same row having different superscript letters are significantly different ($P < 0.05$).

not cause depletion flocculation of the hydrophilic peptides.

However, the presence of lactose at that ratio (peptide to lactose of 0.3:0.7) altered the size distribution of the peptides obtained from isoelectric precipitation (Figure 3b). The majority of the peptides in the absence of lactose had the size of around 825 nm. The presence of lactose resulted in a bimodal distribution of peptides, with the majorities having the sizes around 260 nm and 1,500 nm. The hydrophobic characteristics of the peptides from isoelectric precipitation could have been responsible for the sensitivity of the peptides to depletion flocculation induced by the polar molecule of lactose.

The size of the reconstituted hydrophobic peptides freeze-dried at pH 2.0 was even larger than that before freeze-drying (Table 2). This suggested that freeze-drying at the acidic pH of 2.0 further increased the particle size of the peptide clusters. Nevertheless, hydrophobic peptides freeze-dried at pH 6.5 had higher $ORAC_{FL}$ antioxidant capacity than did the freshly prepared peptides. However, the influence of drying the peptides in lactose excipient on the increase in antioxidant capacity requires further investigation.

The size of fresh hydrophobic peptides from each state of freeze-drying was significantly different (Table 2). The thawed and reconstituted peptides prepared at pH 6.5 had a bimodal distribution (Figure 4). Under the acidic condition of pH 2.0, the thawed peptides and reconstituted peptides in the lactose excipient increased in size, compared to the freshly prepared peptides (Figure 4a). Peptide aggregation into a large cluster could occur during freezing rather than during sublimation in the freeze-dryer. The frozen concentration of peptides may have induced their aggregation since they came in close proximity in the amorphous lactose matrix.

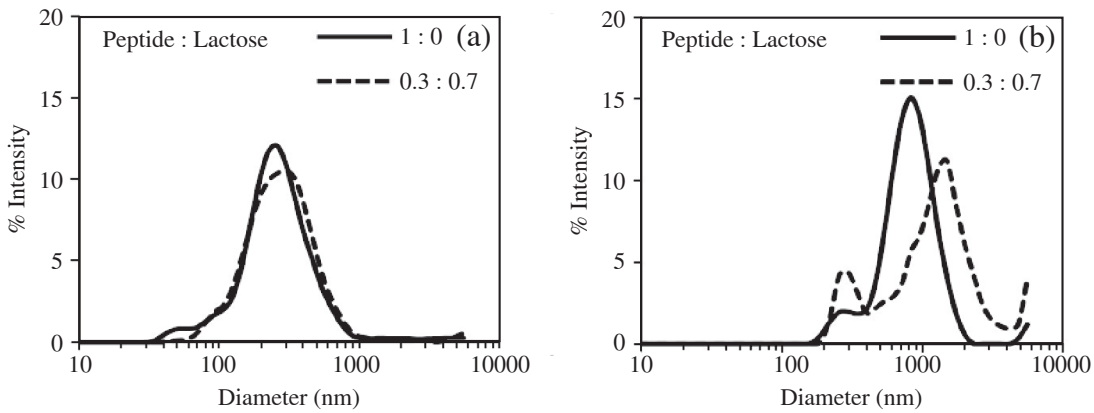


Figure 3 Effect of lactose on particle size distribution of milk peptide nanoclusters prepared by (a) EtOH precipitation and (b) isoelectric precipitation.

Table 2 Effect of pH of suspension prior to freeze-drying in lactose excipient on the characteristics of hydrophobic milk peptides recovered by isoelectric precipitation.

Characteristics	Fresh peptide	Treatment before freeze-drying	
		pH 2.0	pH 6.5
Z-average size (nm)	984[b]	2,455[a]	1,454[b]
$ORAC_{FL}$ (μmol TE/mg protein)	1.24[b]	1.08[b]	1.79[a]
TEAC (μmol TE/mg protein)	1.16[a]	0.80[a]	0.83[a]

Means in the same row having different superscript letters are significantly different ($P < 0.05$).

The degree of aggregation of hydrophobic peptides during freezing could be lowered if they were frozen at pH 6.5 (Figure 4b). The thawed hydrophobic peptides had a bimodal distribution of the size around 825 nm (a similar size to the freshly prepared peptides) and 2,300 nm. Nonetheless, sublimation during the drying step increased the size of the peptide cluster to almost 2,300 nm. Despite the alteration in the size distribution, the antioxidant capacity of reconstituted hydrophobic peptides remained high for both $ORAC_{FL}$ and TEAC antioxidant capacity (Table 2).

The particle size distribution of peptides from EtOH precipitation and their TEAC antioxidant capacity are illustrated in Figure 5. The peptides had similar size distribution and Z-average size of around 260 nm, both before and after freeze-drying and the TEAC antioxidant capacity remained unchanged after reconstitution. Apparently, the hydrophilic peptides were quite stable with regard to freezing and sublimating

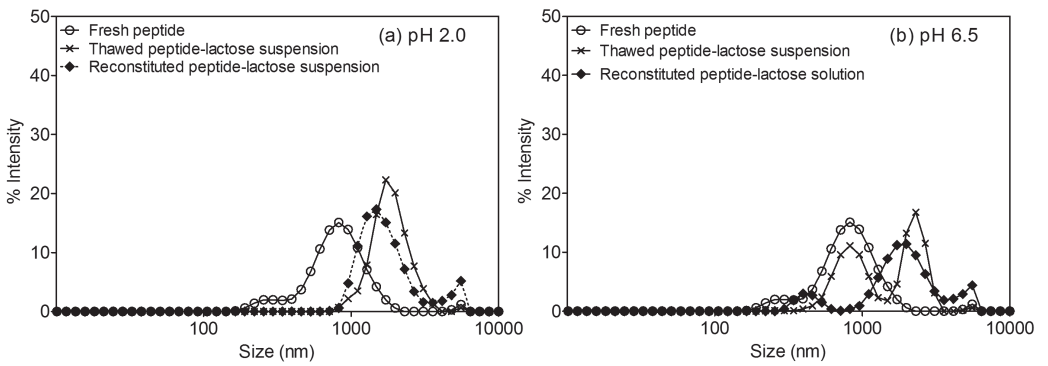
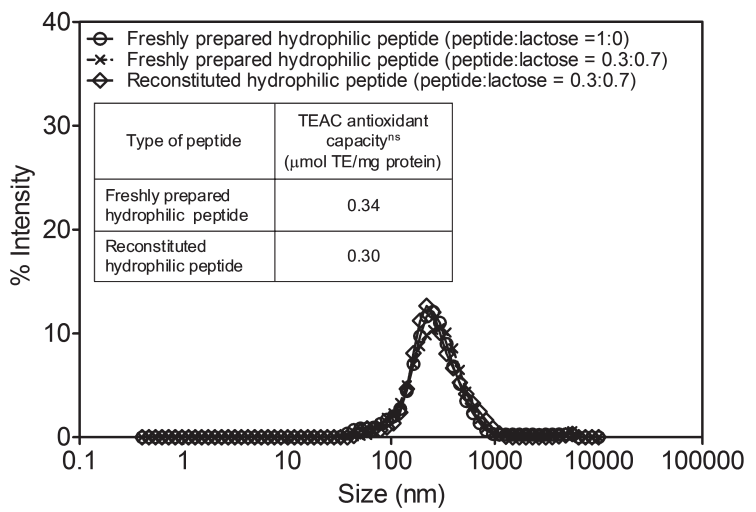


Figure 4 Effect of lactose on depletion flocculation of hydrophobic milk peptides recovered by isoelectric precipitation before freezing, thawed peptides and reconstituted peptides at (a) pH 2.0 and (b) pH 6.5.



Type of peptide	TEAC antioxidant capacity^{ns} (μmol TE/mg protein)
Freshly prepared hydrophilic peptide	0.34
Reconstituted hydrophilic peptide	0.30

Figure 5 Effect of lactose and drying on particle size distribution and TEAC antioxidant capacity of hydrophilic milk peptides recovered by EtOH precipitation and freeze-dried at pH 6.5. (ns = not significant ($P \geq 0.05$)).

during the freeze-drying process. However, it should be noted that the antioxidant capacity of the hydrophilic peptides was much lower than that of the hydrophobic peptides. It is likely that the reactive groups responsible for the antioxidative mechanisms of the hydrophilic peptides were different from those of the hydrophobic peptides.

The present study indicated that milk proteins were a rich source of natural antioxidants after tryptic hydrolysis had been performed. The antioxidant capacity of the hydrophobic milk peptides obtained from isoelectric precipitation was much higher than that of the hydrophilic milk peptides freeze-dried at both acidic and neutral pH. The $ORAC_{FL}$ antioxidant capacity of 1 g of milk protein after reconstitution, which was around 0.27˜0.57 μmol TE/mg protein depending on the methods used in milk coagulation (Pattorn and Hongsprabhas, 2008), was equivalent to that obtained from 10˜21 g of fresh strawberry, which had an $ORAC_{FL}$ antioxidant capacity of around 27 mmol TE/kg (Erkan *et al.,* 2008). Tryptic-hydrolyzed milk peptides prepared by isoelectric precipitation investigated in the present study had a much higher $ORAC_{FL}$ antioxidant capacity than that of milk proteins (1.79 μmol TE/mg protein). The high antioxidant capacity of hydrophobic peptides implicated their significance as natural antioxidants after tryptic digestion in the gastrointestinal tract.

CONCLUSION

This study indicated that peptide recovery methods influenced the characteristics of milk peptides obtained in terms of antioxidant capacity, size distribution and susceptibility to aggregation in the presence of polar molecules like lactose. EtOH precipitation yielded hydrophilic peptides which were stable to downstream processing in terms of particle size distribution but had lower antioxidant capacity than milk hydrolysate. Conversely, isoelectric precipitated peptides were prone to aggregation but could retain high antioxidant capacity close to that of hydrolysate after freeze-drying. In summary, this study showed that the production of antioxidative milk peptides from mixed proteins in milk was possible and it may not be necessary to fractionate individual casein for its use as pure substrate for tryptic hydrolysis.

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