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Antioxidant capacity of caseinophosphopeptides prepared from sodium caseinate using Alcalase

Gyo-Nam Kim, Hae-Dong Jang *, Chong-Ik Kim

Department of Food and Nutrition, Hannam University, 133 Ojungdong Daedukgu, Daejeon 306–791, Republic of Korea

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Abstract

The antioxidant capacity of caseinophosphopeptide prepared at different pH from casein hydrolysate with Alcalase was evaluated using oxygen radical absorbance capacity (ORAC), reduction capacity and metal chelating activity. The yield of CPP production was dependent on the pH of supernatant from pH 4.6 clarified Alcalase hydrolysate of sodium caseinate. CPP of pH 3 had higher content of cysteine, aspartic acid, glycine, histidine, proline, tyrosine, and leucine, and less content of glutamic acid, serine, and isoleucine than those of other pH. The phosphate contents of all CPP were relatively similar, 5.0–5.4% (w/w) except at pH 3. The ORAC against peroxyl radical ($ORAC_{ROO\cdot}$) of CPP decreased with increasing pH (3–8) in concentrations tested and was positively correlated with its reduction capacity. The ORAC against hydroxyl radical ($ORAC_{OH\cdot}$) of CPP at 50 μg/ml increased with increasing pH to 7 and then decreased, and its metal chelating activity on ferrous ion increased linearly with increasing pH from 3 to 5, then slowly increased until pH 8, indicating that the $ORAC_{OH\cdot}$ activity of CPP might be attributed to the scavenging activity on hydroxyl radical itself as well as the chelating activity of transition metals.

Keywords: Antioxidant capacity; Caseinophosphopeptide; Oxygen radical absorbance capacity

1. Introduction

The biological and physiological activities of milk proteins are partially attributed to several peptides encoded in the native protein molecules. Among the biologically active peptide molecules, caseinophosphopeptides (CPP), phosphorylated peptides, are known to exert an effect on calcium bioavailability but also on other minerals because of the highly anionic character of CPP which makes them resistant to further hydrolysis by proteases and allows them to form soluble complexes with calcium (FitzGerald, 1998; Kitts, 2005). CPP are bioactive phosphopeptides formed in vivo from digestion of casein by gastrointestinal proteases, and produced in vitro from the proteolytic hydrolysis of casein by commercial proteases, followed by precipitation with Ca and ethanol. Some biological functions such as promotion of Ca uptake (Bennet et al., 2000; Hansen, Sandstrom, & Lonnerdal, 1996), Ca retention (Sato, Masanori, Gunshin, Noguchi, & Naito, 1991), bone calcification (Tsuchita, Goto, Shimizu, Yonehara, & Kuwata, 1996), hypotensive effect (Kitts, Yuan, Nagasawa, & Moriyama, 1992) and anticariogenicity of CPP (Reynolds, 1997) could be attributed to their chelating activity for transition metal ions.

The ability of CPP to scavenge free peroxyl radicals as well as to chelate transition metals such as calcium, iron, copper, and zinc has been reported (Díaz, Dunn, McClements, & Decker, 2003; Díaz & Decker, 2004; Rival, Boeriu, & Wichers, 2001; Sakanaka, Tachibana, Ishihara, & Juneja, 2005). It has been suggested that CPP could be used as antioxidants to prevent oxidative damage to muscle foods (Díaz et al., 2003; Sakanaka et al., 2005). The chelation of transition metals by CPP might originate from phosphoseryl and glutamyl residues contained in α-, β-, and κ-casein (Meisel, 1997). The scavenging capacity of CPP against free peroxyl radicals has been demonstrated

* Corresponding author. Tel.: +82 42 629 7393; fax: +82 42 636 0268.
E-mail address: haedong@hannam.ac.kr (H.-D. Jang).

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to be positively correlated with the amounts of histidine, lysine, proline, and tyrosine that might contribute to the antioxidant activities of some proteins and peptides (Dean, Wolff, & McElligott, 1989; Decker, Ivanov, Zhu, & Frei, 2001; Ellegard, Gammelgard-Larsen, Sorensen, & Fedosov, 1999).

These unique properties of CPP have led to interest in isolation and industrial production of these peptides. In this study, we isolated CPP using Ca/ethanol precipitation from casein digested by Alcalase that has been known as an effective commercial protease for casein hydrolysis and investigated *in vitro* for its antioxidant capacity using ORAC, reduction capacity and metal chelating activity to better understand the antioxidant mechanisms by which CPP scavenges peroxyl or hydroxyl radicals.

2. Materials and methods

2.1. Materials

All reagent grade chemicals were purchased from Sigma Co. (St. Louis, MO). Sodium caseinate was purchased from New Zealand Milk Products (Wellington, NZ). Alcalase (0.6 l, 0.6 AU/g) was obtained from Novo Nordisk Korea (Seoul, Korea).

2.2. Preparation of CPP

2.2.1. Hydrolysis of sodium caseinate

Sodium caseinate (60 g) was reconstituted at 50 °C in 1 l deionized water to give a starting protein concentration of 6% (w/v). The solution was adjusted to pH 8.3, the optimum pH of Alcalase, using 0.5 N NaOH. Enzyme was mixed with distilled water and added with stirring to the reaction vessel containing sodium caseinate. The reaction was allowed to stand at 50 °C for 8 h and the amount of released free amino groups was measured by *O*-phthaldialdehyde (OPA) assay (Church, Swaisgood, Porter, & Catignani, 1983) to monitor the extent of hydrolysis.

2.2.2. CPP preparation

CPP was isolated from the hydrolyzed sodium caseinate by aggregating with $CaCl_2$ at different pH followed by precipitation of aggregates using ethanol according to the procedure of Adamson and Reynold (1995). Sodium caseinate was completely hydrolyzed by Alcalase in 3 h. Therefore, after the sodium caseinate solution (10%) was hydrolyzed with Alcalase at 50 °C for 3 h, it was adjusted to pH 4.6 with 1 N HCl. A partially hydrolyzed caseinate was removed by centrifugal separation at 12,000*g* for 30 min from reaction mixture as a precipitate. The pH of supernatant from the pH 4.6 precipitation was brought to different value from 3 to 8 using 2 N HCl or 2 N NaOH. 10% $CaCl_2$ (w/v) was mixed with the supernatant to give the Ca^{2+} concentration of 100 mM and allowed to aggregate for 1 h at room temperature. Ninty-five percent (v/v) ethanol was then added to 50% (v/v) final concentration and the resulting precipitate was collected by centrifugation at 12,000*g* for 30 min. The obtained precipitate was dialyzed against deionized water by Amicon concentrator (Beverly, MA) overnight, freeze-dried and stored at −20 °C for the further analysis.

2.3. Chareterization of lipophilized CPP

2.3.1. Analysis of amino acid composition

Amino acid composition analysis was carried out at the Korea Basic Science Institute (Daejeon, Korea) using the following procedure. Samples were added to pyrolyzed borosilicate vial and dried under vacuum. Each vial was placed in a vacuum hydrolysis vessel with 200 μl of constant boiling 6 N HCl. For the analysis of tryptophan, 20 μl of 4 mM methanesulfonic acid was used instead of 6 N HCl. The vessel was alternately purged with nitrogen gas, evacuated for three cycles using a Waters Pico-Tag work station and then heated at 110 °C for 24 h. After vapor hydrolysis samples were dried using the work station, dissolved in 10 μl of methanol:H_2O:triethylamine (TEA; 2:1:1, v/v/v), redried, and then derivatized with 20 μl of methanol:H_2O:TEA:phenylisothiocyanate (7:1:1:1, v/v/v/v) at 20 °C for 15 min. After drying in the work station the PTC-amino acids were dissolved in 5 mM sodium phosphate, pH 7.4-acetonitrile (95:5, v/v), centrifuged at 10,000*g* for 10 min, and applied to a Waters Pico-Tag (C18) column (Waters Co., Milford, MA, 3.9 × 300 mm) maintained at 38 °C. The PTC-amino acids were eluted with binary gradient using 50 mM sodium acetate, 3.6 mM TEA, pH 6.4, and 2.5 μM ethylenediaminetetraacetic acid (EDTA) as solvent A and acetonitrile:H_2O (60:40, v/v) containing 2.5 μM EDTA as solvent B with the initial flow rate of 1.0 ml min^{-1}. For the analysis of cysteine 10 μl of formic acid:H_2O_2 (19:1, v/v) were added and maintained for 30 min before drying under vacuum.

2.3.2. Phosphorus content

Phosphorus was determined according to the AOAC (1998) method 33.7.29.

2.4. Antioxidant capacity of lipophilized CPP

2.4.1. Oxygen radical absorbance capacity (ORAC) assay

The ORAC assay was carried out using a Tecan GENios multi-functional plate reader (Salzburg, Austria) with fluorescent filters (excitation wavelength: 485 nm, emission filter: 535 nm). In the final assay mixture, fluorescein (40 nM) was used as a target of free radical attack with either 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH, 20 mM) as a peroxyl radical generator in peroxyl radical-scavenging capacity ($ORAC_{ROO\cdot}$) assay or with H_2O_2–$CuSO_4$ (H_2O_2, 0.75%; $CuSO_4$, 5 μM) as a hydroxyl radical generator in hydroxyl radical-scavenging capacity ($ORAC_{OH\cdot}$) assay. Trolox (1 μM) was used as a control standard and prepared fresh on a daily basis. The analyzer was programmed to record the fluorescence of fluorecein

every 2 min after AAPH, or H_2O_2–$CuSO_4$ was added. All fluorescence measurements were expressed relative to the initial reading. Final results were calculated based on the difference in the area under the fluorescence decay curve between the blank and each sample. All data were expressed as micromoles of Trolox equivalents (TE). One ORAC unit is equivalent to the net protection area provided by 1 μM of Trolox.

2.4.2. Reduction capacity

The reduction capacity of CPP to reduce Cu^{2+} to Cu^{+} was determined according to the method of Aruoma et al. (1998). Forty microliters of different concentrations of CPP in distilled water were mixed with 160 μl of a mixture containing 0.5 mM $CuCl_2$ and 0.75 mM neocuproine, Cu^{+} specific chelator, in 10 mM phosphate buffer, pH 7.4. The absorbance was measured with a Tecan micro-plate reader (Salzburg, Austria) at 454 nm for 60 min. Increased absorbance of the reaction mixture indicates increased reducing power.

2.4.3. Metal chelating activity

The metal chelating activity of CPP was determined by the method of Decker and Welch (1990). Two hundreds microliters of different concentration of CPP were mixed with 20 μl of $FeCl_2$ solution (1.2 mM in H_2O). The reaction was initiated by the addition of 20 μl of 2.4 mM ferrozine, and the mixture was shaken vigorously and allowed to stand at room temperature for 10 min. The absorbance of the mixture (the ferrous ion–ferrozine complex) was measured with a micro-plate reader at 562 nm. The metal chelating activity of the test sample on ferric ions was calculated using the formula below:

$$\text{Metal chelating activity } (\%) = [(A_0 - A_1)/A_0] \times 100,$$

where A_0 was the absorbance of the control, and A_1 was the absorbance of the sample.

2.5. Statistical analysis

All data are presented as means ± SD. Statistical analyses were carried out using statistical package SPSS (Statistical Package for Social Science, SPSS Inc., Chicago, IL, USA) program and significance of each group was verified with the analysis of One-way ANOVA followed by the Duncan's test of $p < 0.05$.

3. Results and discussion

3.1. Characteristics of CPP

Sodium caseinate was completely hydrolyzed in 3 h by Alcalase (Fig. 1). Therefore, CPP was prepared from sodium caseinate hydrolyzed with Alcalase for 3 h. According to Fig. 2, the yield of CPP increased in proportion to the increase of pH from 3 to 7 and it was almost similar at pH 7 and 8. Above pH 5, the yield of CPP production

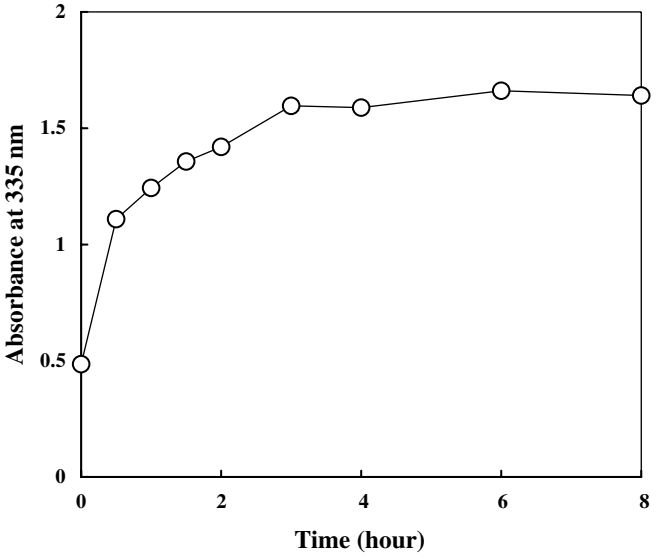

Fig. 1. Hydrolysis pattern of sodium caseinate using Alcalase.

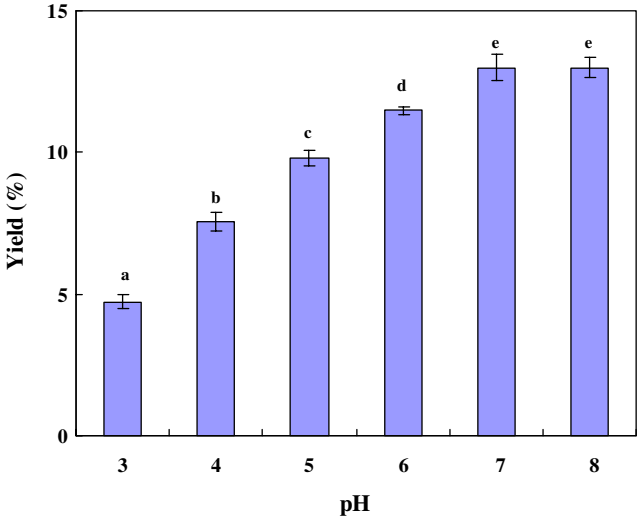

Fig. 2. Yield of caseinophosphopeptides precipitated at different pH. Columns with different letters are significantly different ($p < 0.05$).

compared well with result of other worker under identical conditions (Adamson & Reynold, 1996). This demonstrates that the selective precipitation procedure with Ca/ethanol is dependent on the pH of supernatant from pH 4.6 clarified Alcalase hydrolysate of sodium caseinate.

Table 1 shows the amino acid composition of CPP that was precipitated with Ca/ethanol at different pH. CPP of pH 3 had higher content of cysteine, aspartic acid, glycine, histidine, proline, tyrosine and leucine, and less content of glutamic acid, serine and isoleucine than those of other pH. Specially, the amount of cysteine was 2.7 (mol%), which was nine times that of other CPP. The cysteine existence in CPP of pH 3 appears to be distinctive because there has been no report about the substantial content of cysteine of CPP prepared from casein hydrolysate with trypsin or Alcalase. Considering only κ-casein among all caseins has

Table 1
Amino acid composition of caseinophosphopeptides[a]

Amino acid	pH					
	3	4	5	6	7	8
Cys	2.7	0.3	0.3	0.3	0.3	0.3
Asp	7.7	6.7	7.2	7.0	6.6	6.6
Glu	20.8	24.7	27.3	27.4	28.3	28.7
Ser	15.4	18.7	18.4	17.2	17.6	17.6
Gly	6.2	3.8	3.8	3.6	3.7	3.8
His	2.4	1.4	1.2	1.1	1.0	0.7
Arg	2.2	2.4	2.4	2.5	2.5	2.4
Thr	4.6	4.8	5.5	5.5	5.4	5.2
Ala	4.5	4.1	3.6	3.7	4.0	3.9
Pro	7.1	6.2	5.6	5.7	5.7	5.9
Tyr	1.0	0.5	0.4	0.4	0.4	0.3
Val	7.5	7.4	6.7	6.6	6.8	7.0
Met	1.8	1.6	1.5	1.4	1.5	1.3
Cys2	0.0	0.0	0.0	0.0	0.0	0.0
Ile	7.2	10.0	9.1	9.2	8.5	9.0
Leu	5.6	4.3	4.0	4.2	4.2	3.9
Phe	1.2	0.9	1.0	1.3	1.3	1.2
Trp	0.0	0.0	0.0	0.1	0.0	0.0
Lys	2.0	2.2	2.0	2.9	2.3	2.1

[a] Mol (%).

cysteine residue, CPP having cysteine residue should be derived from κ-casein digests with Alcalase. CPP from κ-casein might be precipitated at pH 3 because it has less amount of amino acid residue such as glutamic acid and phosphoserine which can be ionized and contribute to its solubility than CPP from other caseins such as α_{S1}-casein and β-casein.

The content of phosphorus of CPP is shown in Fig. 3. CPP of pH 3 had the lowest amount (3.9% w/w) of phosphorus among all CPP; other CPP had relatively similar level of phosphate content, 5.0–5.4% (w/w). The level of organic phosphorus of CPP prepared using Alcalase was higher than that of CPP from tryptic hydrolysate of casein except pH 3 (Adamson & Reynold, 1995; Díaz et al., 2003), demonstrating that Alcalase might be more effective in producing low molecular weight CPP with the high level of phosphorus content from casein than trypsin due to the broad specificity of Alcalase (Adamson & Reynold, 1996).

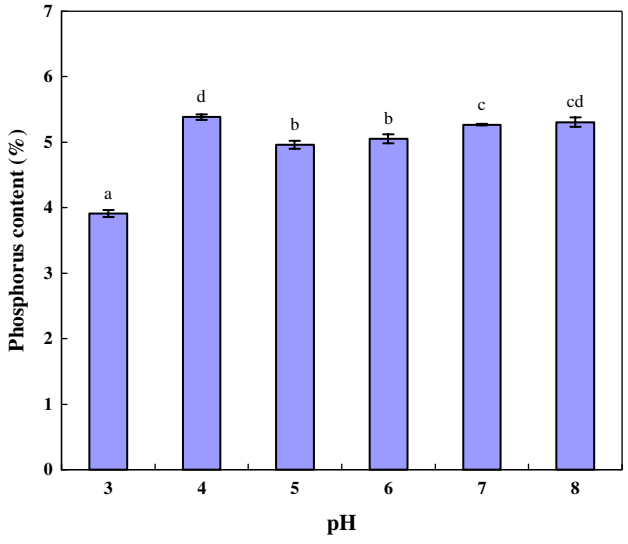

Fig. 3. Phophorus content of caseinophosphopeptides precipitated at different pH. Columns with different letters are significantly different ($p < 0.05$).

3.2. Peroxyl radical-scavenging capacity ($ORAC_{ROO\cdot}$)

The antioxidant capacity of CPP has been observed using different assay systems. CPP were investigated for their peroxyl radical-scavenging capacity using ORAC assay system, where AAPH was used as a generator of peroxyl radicals. Fig. 4 shows that the scavenging activity of CPP on peroxyl radicals generated from AAPH was dose-dependent (10–50 μg/ml). The $ORAC_{ROO\cdot}$ activity decreased with increasing pH (3–8) in concentrations tested. According to Fig. 4, at 50 μg/ml the $ORAC_{ROO\cdot}$ activity of pH 3 CPP exhibited 10.3 (Trolox equivalents, μM), which was around twice that of other pH. This data shows that the pH adjustment before precipitation has pronounced effects on the $ORAC_{ROO\cdot}$ activity of CPP prepared with Ca/ethanol from casein digest with Alcalase.

The ability of CPP to stimulate the reduction of copper ion was investigated if $ORAC_{ROO\cdot}$ activity of CPP could result from its reduction capacity donating electrons or hydrogens to peroxyl radicals. The reduction capacity of

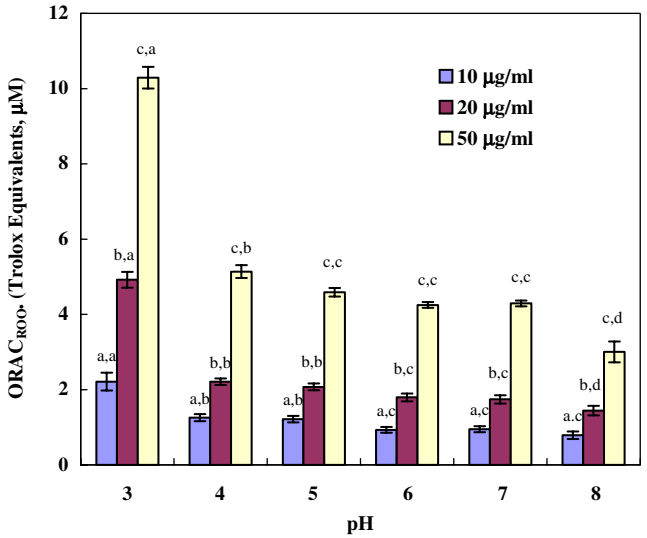

Fig. 4. $ORAC_{ROO\cdot}$ activity (Trolox equivalent, μM) of caseinophosphopeptides. The ORAC value is calculated by dividing the area under the sample curve by the area under the Trolox curve, with both areas being corrected by subtracting the area under the blank curve. One ORAC unit is assigned as the net area of protection provided by Trolox at a final concentration of 1 μM. The area under the curve fro the sample is compared to the area under the curve for Trolox, and the antioxidative value is expressed in micromoles of Trolox equivalent per liter. The results represent the mean ± SD of values obtained from three measurements. Different corresponding letters indicate significant differences at $p < 0.05$ by Duncan's test. First letter is within same pH and second one is among different pH.

CPP appears to decrease with increasing pH, which is very similar to result of the peroxyl radical-scavenging capacity assay (Fig. 5). These results suggest that the peroxyl radical-scavenging capacity of CPP could be attributed to the fact that its reducing ability reduces peroxyl radicals by donating electrons or hydrogens to make them into relatively stable compounds.

The scavenging capacity of some proteins such as casein, whey protein, soybean protein, fish protein, and their hydrolysates on peroxyl radicals has been known to be dependent on their amino acid contents and sequence (Amarowicz & Shahidi, 1997; Cervato, Cazzola, & Cestaro, 1999; Chen, Muramoto, & Yamauchi, 1995; Chen, Muramoto, Yamauchi, Fujimoto, & Nokihara, 1998; Díaz et al., 2003; Díaz & Decker, 2004; Dunlap & Yamamoto, 1995; Kim et al., 2001; Sakanaka et al., 2005). The superior $ORAC_{ROO\cdot}$ activity by casein hydrolysates and low molecular weight casein hydrolysates over the enriched CPP was explained by a comparison of their amino acid contents and profiles (Díaz & Decker, 2004). Casein hydrolysates were found to have more histidine, lysine, proline, and tyrosine than CPP, which might be contributed to the observed $ORAC_{ROO\cdot}$ activity of casein hydrolysates (Dean et al., 1989; Decker et al., 2001; Ellegard et al., 1999; Kikugawa, Kato, & Hayasaka, 1991). Cysteine carrying sulfhydryl group was confirmed to be involved in scavenging free peroxyl radical by the experiment in which *N*-ethylmaleimide (NEM) was used to bind sulfhydryl groups of the high molecular weight (HMW) fraction of whey to form a stable thiol adduct that could be unavailable for further redox reactions (Tong, Sasaki, McClements, & Decker, 2000). In same study, NEM did not completely inhibit the peroxyl radical scavenging of HMW fraction whey so that tyrosine residue was suggested as an active peroxyl radical scavenger. Therefore, the superior scavenging capacity of CPP of pH 3 on peroxyl radical to that of other pH might be attributed to its higher contents of cysteine, glycine, histidine, and tyrosine residues because among them cysteine, histidine, and tyrosine residues can effectively scavenge free peroxyl radicals. Among these amino acids, cysteine coming from κ-casein seems to play an important role in the $ORAC_{ROO\cdot}$ activity of CPP prepared from casein hydrolysate by Alcalase. Cysteine that has been known to be one of thiol antioxidants might scavenge peroxyl radicals by donating electrons and/or hydrogens through interconversions between sulfhydryl and disulfide groups (Atmaca, 2004).

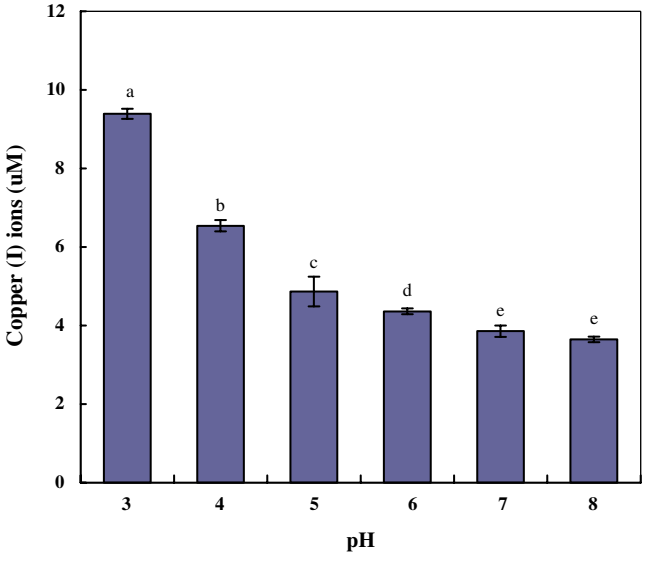

Fig. 5. Reduction capacity of caseinophosphopepetides. Columns with different letters are significantly different ($p < 0.05$).

3.3. Hydroxyl radical-scavenging capacity ($ORAC_{OH\cdot}$)

ORAC assay system has been used successfully to determine the reaction capacity with hydroxyl radical, one of most harmful and reactive oxygen species in biological systems. Hydroxyl radicals were generated by the Fenton reaction of Cu^{2+} with H_2O_2. Fig. 6 shows the dose dependency of $ORAC_{OH\cdot}$ activity of CPP in the range of 10–50 μg/ml with maximal activity occurring at 50 μg/ml. The $ORAC_{OH\cdot}$ activity of CPP at 50 μg/ml increased with increasing pH to 7 and then decreased (Fig. 6). This data demonstrates that the pH adjustment before precipitation of CPP by Ca/ethanol has the striking effects on the hydro-

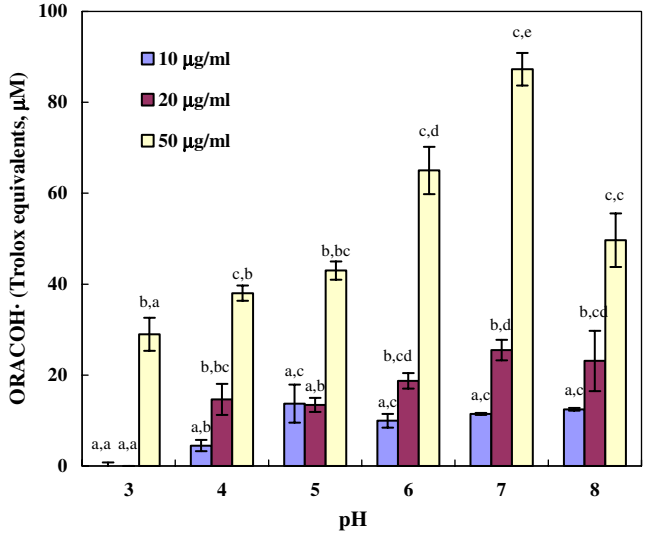

Fig. 6. $ORAC_{OH\cdot}$ activity (Trolox equivalent, μM) of caseinophosphopeptides. The ORAC value is calculated by dividing the area under the sample curve by the area under the Trolox curve, with both areas being corrected by subtracting the area under the blank curve. One ORAC unit is assigned as the net area of protection provided by Trolox at a final concentration of 1 μM. The area under the curve fro the sample is compared to the area under the curve for Trolox, and the antioxidative value is expressed in micromoles of Trolox equivalent per liter. The results represent the mean ± SD of values obtained from three measurements. Different corresponding letters indicate significant differences at $p < 0.05$ by Duncan's test. First letter is within same pH and second one is among different pH.

xyl radical-scavenging activity of CPP by ORAC assay system.

The hydroxyl radical-scavenging capacity in ORAC assay system have been known to be dependent on two mechanisms, the metal chelating activity and the scavenging activity on hydroxyl radical itself by sample. CPP can block cupric ions from the interaction with hydrogen peroxide in $ORAC_{OH\cdot}$ assay system by chelating them to inhibit the generation of hydroxyl radicals and then contribute to its $ORAC_{OH\cdot}$ activity. The metal chelating activity of CPP was determined by measuring the inhibition percentage of ferrous ion–ferrozine complex formation. Fig. 7 shows that at 0.3 mg/ml the metal chelating activity of CPP on ferrous ion linearly increases with increasing pH from 3 to 5 and then slowly increased until pH 8. However, the $ORAC_{OH\cdot}$ activity of CPP showed a little different pattern from its metal chelating activity with pH of precipitation. Therefore, the $ORAC_{OH\cdot}$ activity of CPP can not be explained by only its metal chelating activity. It seems to result from the scavenging activity on hydroxyl radical itself as well as the chelating activity on transition metal ions. This similar observation has been reported for CPP protection of deoxyribose degradation from hydroxyl radical generated at concentration of 1 mg/ml (Kitts, 2005).

The distinctive amino acid compositions of antioxidative peptides have been reported to have an important role for their chelating activity on transition metal ions such as copper and iron. Phosphoserine, glutamic acid, histidine, and cysteine have been suggested to contribute to the metal chelating activity (Baumy & Brule, 1988; Bennet et al., 2000; Tong et al., 2000). In casein and casein derived-peptides as CPP, the phosphate group and glutamic acid could confer the metal chelating activity on them through the formation of intermolecular iron bridging (Baumy & Brule, 1988; Baumy, Guenot, Sinbandhit, & Brule, 1989; Gaucheron, Famelart, & LeGraet, 1996; Gaucheron, LeGraet, Boyaval, & Piot, 1997). However, the direct correlation between phosphorus content and metal chelating activity of CPP from casein hydrolysates with Alcalase was not observed in this study, which was consistent with previous results (Díaz et al., 2003). The low metal chelating activity of CPP at pH 3 appeared to be closely related with the less glutamic acid as well as phosphorus content. This result indicates that non-phosphorylated amino acid residue such as glutamic acid as well as phosphoserine might be involved in the chelating activity of CPP on transition metal ions.

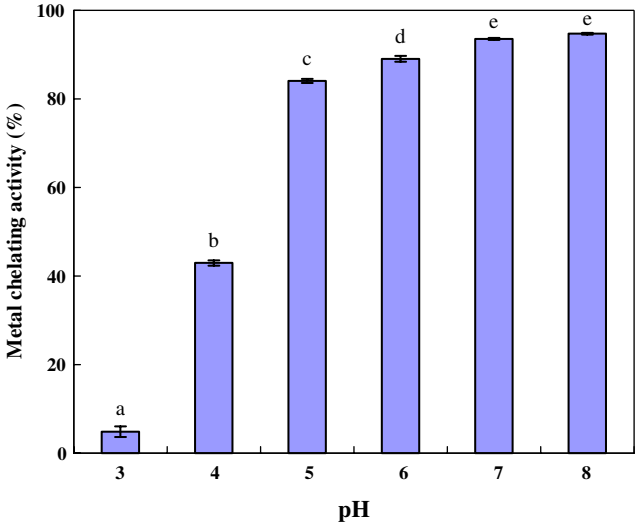

Fig. 7. Metal chelating activity of caseinophosphopepetides. Columns with different letters are significantly different ($p < 0.05$).

4. Conclusions

Alcalase hydrolyzed sodium caseinate in 3 h to release CPP and the yield of CPP increased in proportion to the increase of pH from 3 to 7. CPP of pH 3 had higher content of cysteine, aspartic acid, glycine, histidine, proline, tyrosine, and leucine, and less content of glutamic acid, serine, and isoleucine than those of other pH. The phosphorus contents of all CPP except pH 3 were relatively similar level, 5.0–5.4% (w/w), which was higher than that of CPP from tryptic hydrolysate of casein, indicating that Alcalase might be more effective in producing low molecular weight CPP with the high level of phosphorus content from casein than trypsin due to the broad specificity of Alcalase. The antioxidant capacity of CPP in terms of $ORAC_{ROO\cdot}$ activity decreased with increasing pH from 3 to 8 in concentrations tested and was positively correlated with its reduction capacity. The antioxidant capacity of CPP in terms of $ORAC_{OH\cdot}$ activity at 50 μg/ml increased with increasing pH to 7 and then decreased, and its metal chelating activity on ferrous ion increased linearly with increasing pH from 3 to 5, then slowly increased until pH 8, indicating that the $ORAC_{OH\cdot}$ activity of CPP might be attributed to the scavenging activity on hydroxyl radical itself as well as the chelating activity on transition metal ions.

Acknowledgement

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