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Antioxidant Mechanisms of Caseinophosphopeptides and Casein Hydrolysates and Their Application in Ground Beef

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Caseinophosphopeptides (CPP) and casein hydrolysates have been shown to bind prooxidant metals such as iron, but their effectiveness as metal chelators to inhibit lipid oxidation in foods has still not been fully investigated. Thus, the antioxidant activity of CPP and casein hydrolysates was studied in phosphatidylcholine liposome model systems. CPP (<1.0 mg/mL) and casein hydrolysates (0.3−1.7 mg/mL) were effective inhibitors of TBARS development when oxidation was promoted by ferric/ascorbate. High amounts of CPP (>1.0 mg/mL) were prooxidant, whereas casein hydrolysates were observed to be only antioxidative. In the presence of peroxyl radicals, casein hydrolysates were more effective scavengers than enriched CPP (3−15 mM). In cooked ground beef, TBARS formation was inhibited 75, 39, and 17% by 0.5% enriched CPP, casein hydrolysates, and low molecular weight casein hydrolysates, respectively, after 4 days of storage. The results show that CPP and casein hydrolysates are promising sources of natural antioxidants for foods.

KEYWORDS: Antioxidant; casein hydrolysates; caseinophosphopeptides; CPP; chelator; free radical scavenger; lipid oxidation; ground beef

INTRODUCTION

Oxidation of lipids in foods can generate free radicals that lead to fatty acid decomposition and development of undesirable rancid odors and flavors (*1*, *2*). Oxidative degradation of highly unsaturated fatty acids that are extremely prone to oxidation presents a challenge in designing stable processed foods. Iron and other transitions metals act as prooxidants in many of these food systems because they accelerate the cleavage of lipid hydroperoxides into the very reactive lipid alkoxyl radicals (*3*). Effective chelators such as ethylenediaminetetraacetic acid (EDTA) can control the reactivity of transition metals and thus control rancidity. Chelating agents from natural sources may be more suitable alternatives than EDTA to produce "natural" and organic foods. Peptides derived from bovine milk caseins that contain the highly phosphorylated polar domains, ·SerP·SerP·SerP·Glu·Glu·, are capable of chelating calcium, iron, copper, and zinc (*4*−*7*). The ability of these caseinophosphopeptides and casein hydrolysates to sequester transition metals such as iron may allow them to act as antioxidants in foods. Caseinophosphopeptides and casein hydrolysates not only could improve the oxidative stability of foods but also could add value to traditional foods as enhancers of mineral bioavailability because caseinophosphopeptides and casein hydrolysates have been reported to increase the absorption of calcium, iron, and zinc (*8*−*10*).

Casein and casein peptides can bind iron (*11*−*13*), thus making them potential antioxidants in food systems. Enriched caseinophosphopeptides (CPP) and casein hydrolysates were antioxidants in corn oil-in-water emulsions at pH 7.0 and 3.0 (*14*). A direct association between phosphorus content and antioxidant activity was not observed, suggesting that CPP and casein hydrolysates might inhibit lipid oxidation by free radical scavenging as well as chelation by phosphoseryl residues. Caseins and tryptic digests of caseins also showed inhibitory properties in the oxidation of linoleate by lipoxygenase, peroxyl radicals, and hemoglobin, suggesting that casein and casein hydrolysates can scavenge free radicals (*15*, *16*). Enriched CPP and casein hydrolysates also showed free radical scavenging activity as they had the ability to scavenge the ABTS radical cation (*17*).

The objective of the present study was to compare the antioxidant activity of enriched CPP and casein hydrolysates in a cell membrane model system of phosphatidylcholine liposomes and in ground beef, with the ultimate goal of understanding the potential of CPP and casein hydrolysates as food antioxidants.

MATERIALS AND METHODS

Ground beef, 93% lean, was obtained from a local retailer. Fluorescein sodium salt (3′,6′-dihydroxyspiro{isobenzenofuran-1{3*H*},-9′{9*H*}-xanthen}-3-one), soybean l-α-phosphatidylcholine type IV-S, and 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH) were obtained from Sigma-Aldrich (St. Louis, MO). All other chemicals were of analytical grade (Sigma-Aldrich). Double-distilled water was used throughout the experiments.

Casein Hydrolysate Preparation. Casein derivatives were prepared as described in our previous study (*14*). In brief, casein (10%) was

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hydrolyzed by trypsin (0.1%) at 50 °C and pH 8.0 for 2 h, and then the pH was decreased to 4.6 to make undigested protein flocculate. Centrifugation (10400*g*) for 10 min allowed the collection of the soluble casein hydrolysates. Casein hydrolysates were ultrafiltrated through a 10000 Da membrane to obtain a filtrate containing low molecular weight (LMW) casein hydrolysates. All fractions were freeze-dried. Enriched caseinophosphopeptides were a gift from Meiji Seika Kaisha Ltd. (Tokyo, Japan); α_{s2}-casein (1−32) and β-casein (1−28) were considered as the main components for this fraction (*18*). Phosphorus content was measured using a standard inductively couple plasma method, and nitrogen content was measured by adapting the micro Dumas method in a Perkin-Elmer 240 elemental analyzer (*19*).

Liposome Preparation. Phosphatidylcholine (0.3 g) was dispersed in 50.0 mL of 10 mM potassium phosphate dibasic/0.12 M KCl buffer at pH 7.0 using a Tradesman drill press (model 8050), at speed 1, equipped with a Thomas Scientific (Swedesboro, NJ) piston-type Teflon tissue homogenizer. A Fischer Scientific ultrasonic dismembrator (model 500) connected to a tapped horn with a flat tip was used to sonicate phosphatidylcholine homogenates for 30 min at 4 °C with intermittent pulses of 0.5 s and an amplitude setting of 40%. Centrifugation (125000*g*) for 30 min yielded a clear supernatant with liposomes smaller than 100 Å (*20*). Phospholipid concentration of the prepared liposomes was estimated by measuring phosphorus content with a molybdate assay (*21*). Assuming an average molecular weight of 750 for phosphatidylcholine, concentrations ranged from 3.3 to 3.7 mg of phosphatidylcholine/mL of liposomes.

Liposome Oxidation. Liposome oxidation was promoted either by a ferric−ascorbic acid redox system or by the free radical generator AAPH. Oxidation was conducted in a shaking water bath at 37 °C. The iron-promoted systems consisted of 0.2 mg/mL of phosphatidylcholine, 15 μM $FeCl_3 \cdot 6H_2O$, 100 μM ascorbic acid, enriched CPP or casein hydrolysates, and 10 mM potassium phosphate dibasic/0.12 M KCl buffer at pH 7.0. The systems initiated by AAPH peroxyl radicals consisted of 0.3 mg/mL of phosphatidylcholine, 4 mM AAPH, enriched CPP or casein hydrolysates, and enough phosphate dibasic/KCl buffer (pH 7.0). The order of addition was buffer, peptides, ferric chloride, liposomes, and ascorbic acid for the former systems and buffer, peptides, liposomes, and AAPH for the latter systems. EDTA (0.1 mM) was added to the AAPH systems to minimize metal-promoted oxidation. Thiobarbituric acid reactive substances (TBARS) were used to monitor the formation of oxidation products (*22*). All experiments were repeated at least two times, and each treatment was measured in triplicate. Percentages of inhibition and oxidative activity (antioxidant or prooxidant) were calculated as relative to the control.

Oxygen Radical Absorbance Capacity (ORAC) Assay. A 300 mM solution of AAPH in 75 mM potassium phosphate buffer at pH 7.0 was prepared for each experiment and kept on ice. Fluorescein was dissolved to a concentration of 50 nM in phosphate buffer before each set of experiments. For each run, fluorescein was held at 37 °C in a water bath for 15 min and then brought to a final concentration of 45 nM in a system with 0.1 mM EDTA, 20 mM AAPH, 0−30 μM CPP or casein hydrolysates, and phosphate buffer (pH 7.0). Analyses were performed in a Fluorolog flourometer (Jobin Yvon Inc./Horiba, Edison, NJ) with the temperature controlled at 37 °C. The wavelength of excitation was 493 nm, and emission was 515 nm (*23*). Fluorescence was recorded every minute for 40 min, and the fluorescence relative to the initial time (F/F_0) was calculated for the fluorescence decay curve. Samples and standards were always run in duplicate.

Ground Beef Oxidation. Ground beef with 0.5% of enriched CPP and equivalent nitrogen and phosphorus concentrations of casein hydrolysates and LMW casein hydrolysates was cooked to 75 °C and stored at 4 °C. Cooking was performed by immersing test tubes containing 10 g of ground beef in a water bath at 90 °C. Oxidation was assessed by the determination of TBARS using a modification of the method reported by Park (*24*). To 1.0 g of cooked ground beef were added 4.0 mL of 50 mM phosphate buffer, 0.1% EDTA, and 0.1% propyl gallate (pH 7.0), and the mixture was homogenized in a Tissumizer (20000 rpm; Tekmar, Cincinnati, OH). The homogenates were added to 1.0 mL of 30% TCA and centrifuged at 3400*g* for 5 min. The supernatant (1.0 mL) was mixed with 1.0 mL of 0.02 M thiobarbituric acid and heated in a boiling water bath for 20 min. After cooling in the refrigerator for 30 min, samples were centrifuged (3400*g*, 5 min), and absorbance was read at 532 nm. Each experiment was repeated two times, and all of the treatments were measured in triplicate. Percentage inhibition was based on the comparison to the control samples that did not contain casein-derived peptides.

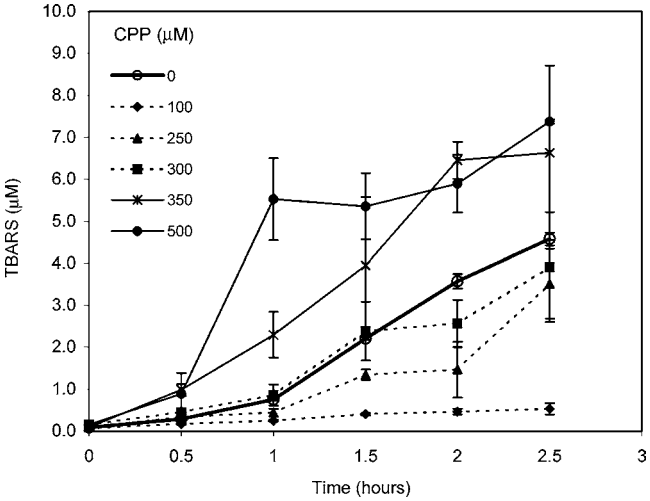

Figure 1. Effect of 0–500 μM enriched CPP on TBARS development at 37 °C in phosphatidylcholine liposomes (pH 7.0) in which oxidation was promoted by 30 μM ferric iron and 100 μM ascorbic acid.

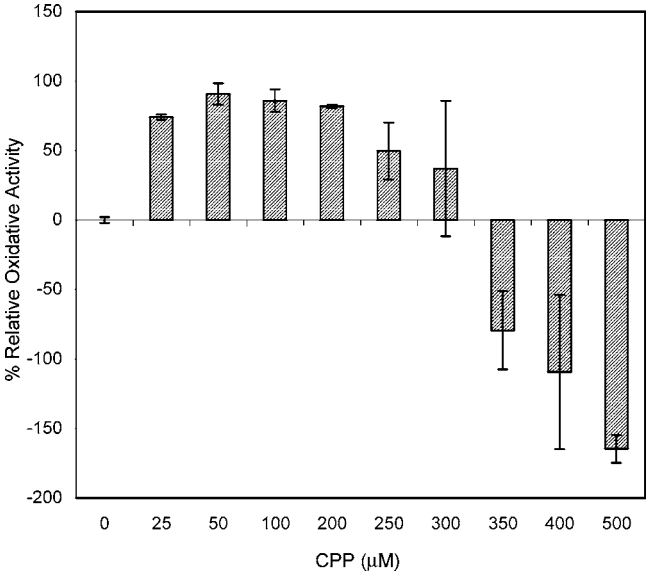

Figure 2. Percentage activity of CPP (0–500 μM) on TBARS formation in phosphatidylcholine liposomes with ferric (30 μM) and ascorbic acid (100 μM) after 90 min of incubation at 37 °C.

RESULTS

The effect of enriched CPP on phosphatidylcholine liposome oxidation induced by ferric iron/ascorbic acid was found to be dependent on the amount of CPP added to the system (**Figure 1**). After 1.5 h of incubation, 100 and 250 μM CPP caused 82 and 39% inhibition in TBARS formation, respectively. At the same incubation time, 300 μM CPP was not significantly different from the control, wheres higher CPP concentrations (350 and 500 μM) displayed prooxidative behavior with TBARS concentrations being 1.8 and 2.4 times higher than in the control (**Figure 1**). The effect of additional CPP concentrations on the oxidative activity profile of phosphatidylcholine liposomes with the ferric/ascorbic oxidizing system after 1.5 h of incubation at 37 °C is shown in **Figure 2**. CPP at 25−200 μM significantly

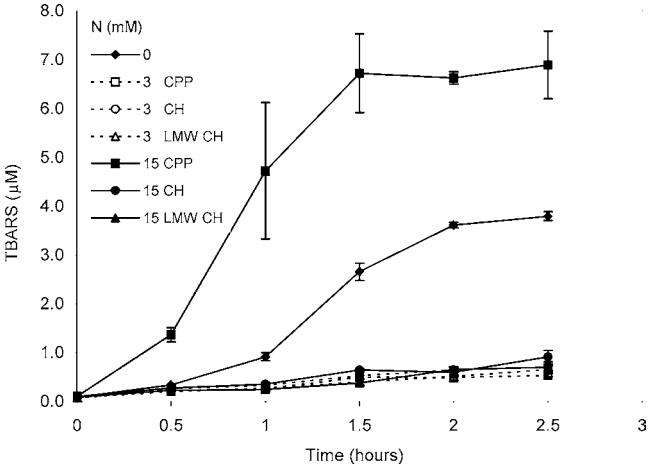

Figure 3. Comparison of antioxidant activity of enriched CPP (CPP), casein hydrolysates (CH), and low molecular weight casein hydrolysates (LMW CH) at 3 and 15 mM nitrogen contents in phosphatidylcholine liposomes with 30 μM ferric and 100 μM ascorbic acid at pH 7.0 (37 °C).

inhibited TBARS development from 74 to 91%. As CPP concentration was increased, antioxidant potential not only weakened but 350, 400, and 500 μM CPP produced 1.80, 2.10, and 2.65 times more TBARS, respectively, than the control (**Figure 2**).

The ability of casein hydrolysates and LMW casein hydrolysates at nitrogen contents of 3 mM (equivalent to 100 μM CPP) and 15 mM (equivalent to 500 μM CPP) to influence iron/ascorbate-promoted oxidation of phosphatidylcholine liposomes is shown in **Figure 3**. At 3 mM nitrogen, TBARS development was inhibited 81% by CPP, 80% by casein hydrolysates, and 85% by LMW casein hydrolysates when compared to the control at 1.5 h of incubation. Casein hydrolysates and LMW casein hydrolysates also strongly inhibited (76 and 86%, respectively) TBARS formation when the concentration was increased to 15 mM nitrogen. As previously observed, the higher concentration of CPP (15 mM nitrogen) was prooxidative, causing a 2.5-fold increase in TBARS concentration compared to the control after 1.5 h of incubation (**Figure 3**).

When oxidation was stimulated by peroxyl radicals in the phosphatidylcholine liposome model system at pH 7.0, enriched CPP in the range of 0−1000 μM inhibited lipid oxidation (**Figure 4**). After 3 h of incubation, 100, 500, and 1000 μM CPP reduced TBARS (compared to the control) by 14, 52, and 62%, respectively. Peroxyl radical-induced oxidation of phosphatidylcholine liposome was also reduced when casein hydrolysates and LMW casein hydrolysates were present at nitrogen contents of 3 mM (100 μM CPP equivalent) and 15 mM (500 μM CPP equivalent) (**Figure 5**). After 3 h of incubation, inhibition in TBARS formation by 3 mM nitrogen was 11% for CPP, 18% for casein hydrolysates, and 28% for LMW casein hydrolysates. Inhibition was greater as nitrogen concentration was increased to 15 mM, and the order of TBARS inhibition remained as CPP (46%) < casein hydrolysates (53%) < LMW casein hydrolysates (63%).

The ability of enriched CPP to scavenge peroxyl radicals was determined using AAPH-induced degradation of flourescein fluorescence, known as the ORAC assay (**Figure 6**). After 20 min at 37 °C, relative fluorescence in samples containing 2.5, 5, 10, 15, and 30 μM enriched CPP was 21, 47, 73, 80, and 89%, respectively. The blank with no CPP had only 1% of the initial fluorescence over the same incubation time. The peroxyl radical scavenging activities of casein hydrolysates and LMW casein hydrolysates at 0.15 mM nitrogen content (equivalent to 5 μM CPP) as evaluated by ORAC are shown in **Figure 7**. After 20 min, casein hydroysates and LMW casein hydrolysates retained 72 and 75%, respectively, of the initial fluorescence, whereas in the presence of CPP only 57% of the initial fluorescence remained.

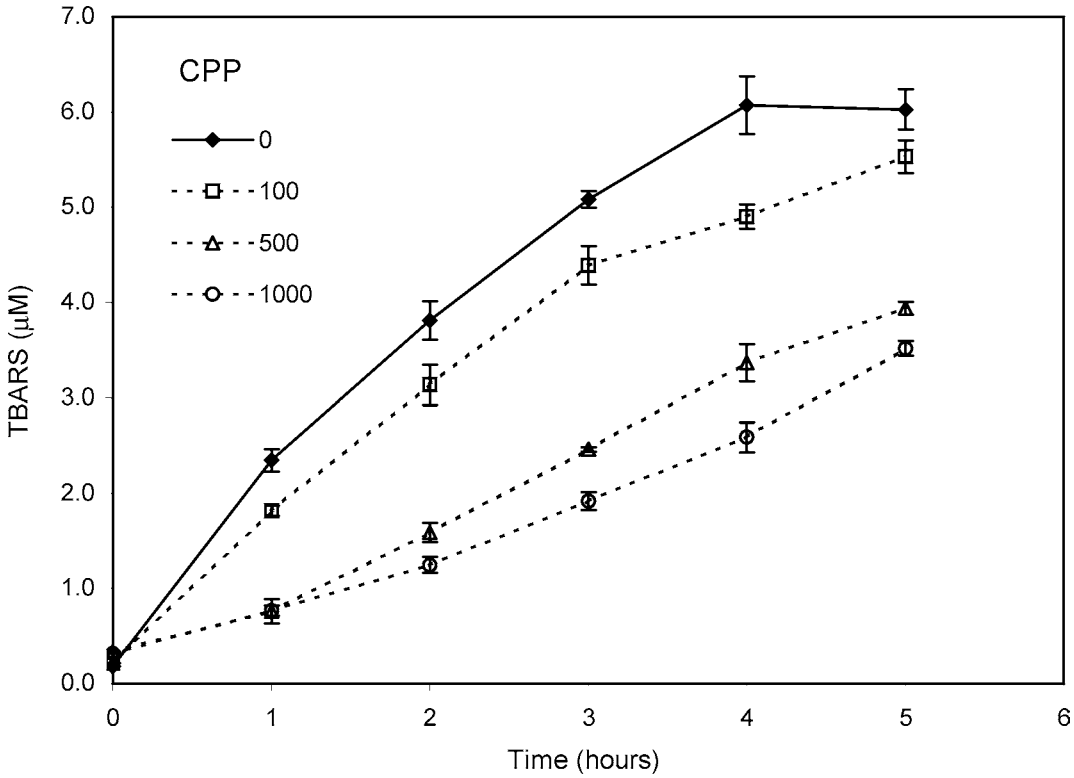

Figure 4. Ability of enriched CPP (0–1000 μM) to inhibit TBARS formation in phosphatidylcholine liposome systems (pH 7.0) in which peroxyl radicals were generated by 4 mM AAPH.

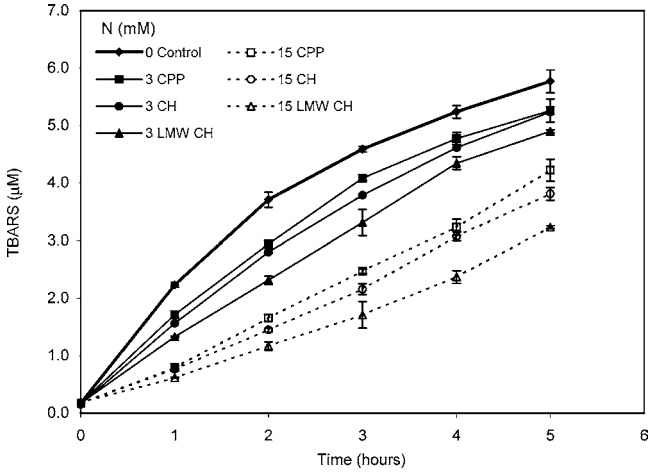

Figure 5. TBARS formation in phosphatidylcholine liposome systems incubated at 37 °C with AAPH (4 mM) and enriched CPP (CPP), casein hydrolysates (CH), or low molecular weight casein hydrolysates (LMW CH) at 3 and 15 mM nitrogen contents.

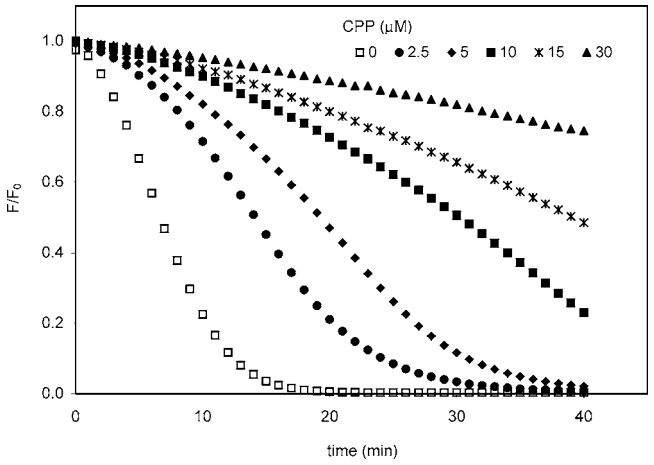

Figure 6. Changes in relative fluorescent intensity of 45 nM fluorescein (λ_{EM} 493 nm, λ_{EX} 515 nm) in the presence of 20 mM AAPH and 0–30 μM enriched CPP at 37 °C.

The antioxidant activities of enriched CPP, casein hydrolysates, and LMW casein hydrolysates were investigated in cooked ground beef stored at refrigerated temperatures. Casein hydrolysates and LMW casein hydrolysates were added at nitrogen concentrations equivalent to 0.5% CPP (60 mg of nitrogen/100 g of beef) (**Figure 8**). After 4 days of storage, TBARS development was inhibited 80, 38, and 26% by enriched CPP, casein hydrolysates, and LMW casein hydrolysates, respectively. Antioxidant activities of hydrolysates and enriched CPP in cooked ground beef were also examined at equal phosphorus levels (14 mg of P/100 g of beef, equivalent to 0.5% of enriched CPP) (**Figure 9**). Enriched CPP were still the most effective antioxidant, even though the hydrolysates, which contain lower levels of phosphopeptides, were present at a higher level (1.75% of LMW casein hydrolysates and 1.25% of casein hydrolysates). Casein hydrolysates were more effective at inhibiting TBARS formation (51% inhibition) compared to the LMW casein hydrolysates (39% of inhibition) after 4 days of storage when tested at equivalent phosphorus concentrations.

DISCUSSION

Caseins have previously been shown to be antioxidants in phosphatidylcholine liposomes, where their ability to chelate

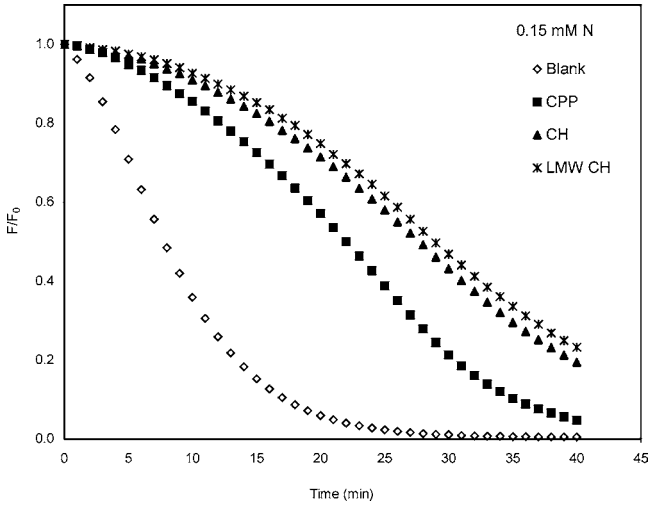

Figure 7. Influence of enriched CPP (CPP), casein hydrolysates (CH), and LMW casein hydrolysates (LMW CH) (0.15 mM N) on the fluorescence decay of flourescein (45 nM) in the presence of the peroxyl radical generator AAPH (20 mM) at 37 °C.

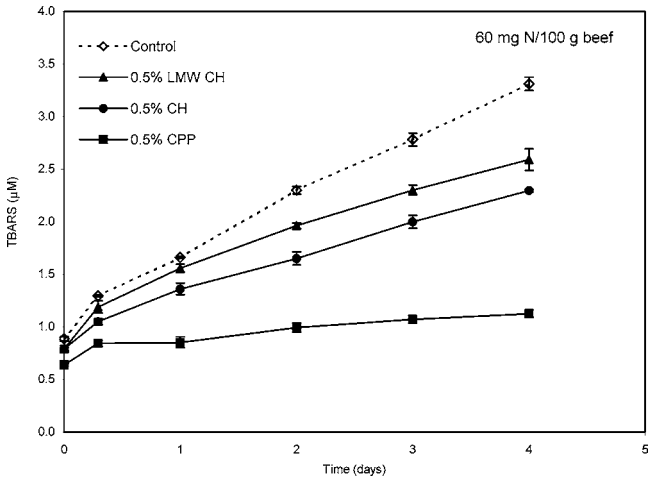

Figure 8. Effect of 0.5% of enriched CPP (CPP), casein hydrolysates (CH), and LMW casein hydrolysates (LMW CH) on the development of TBARS in cooked ground beef stored at 4 °C.

copper and scavenge free radicals by tryptophan residues inhibited the formation of conjugated diene hydroperoxides and hexanal (*25*). Caseins were also antioxidants when ferric (50 μM) and ascorbic acid (500 μM) induced the oxidation of arachidonic acid in phosphatidylcholine liposomes (*26*). In this study, the higher amount of phosphoseryl residues in the casein subunits correlated with higher antioxidant activity (i.e., $\alpha > \beta > \kappa$); however, dephosphorylation of α- and β-caseins caused only a partial loss of antioxidant activity, indicating that caseins may also act as free radical scavengers. Free radical scavenging activity was seen when caseins were able to scavenge superoxide radicals formed in the xanthine/xanthine oxidase system and hydroxyl radicals generated in the deoxyribose assay (*26*). Although casein has antioxidant properties, its addition to foods may not always be practical because casein is not soluble at pH values near 4.6 and the protein could affect food quality parameters such as texture and color. Casein hydrolysates or caseinophosphopeptides that retain the amino acid domain or sequence with antioxidant activity could be a better option to prevent rancidity in foods without affecting other food quality parameters. Enriched CPP and casein hydrolysates have previously been reported to be antioxidants in corn oil-in-water

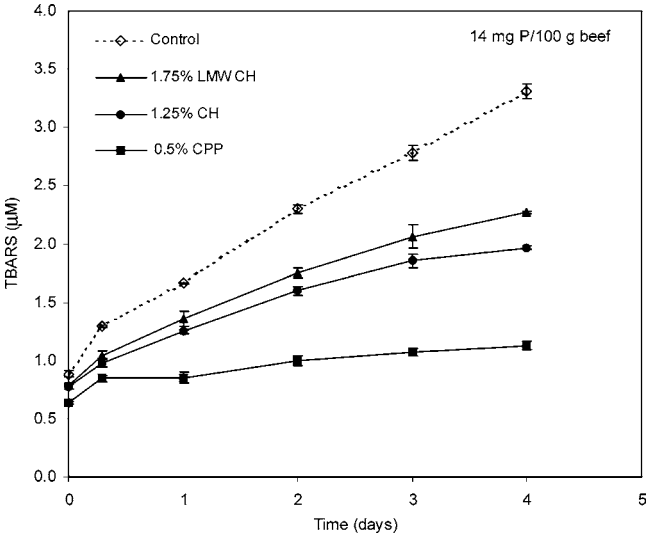

Figure 9. Comparison of the development of TBARS in cooked ground beef stored at 4 °C with 0.5% of enriched CPP (CPP), 1.75% of casein hydrolysates (CH), and 1.25% of LMW casein hydrolysates (LMW CH) (all percentages are equivalent to 14 mg of phosphorus/100 mg of beef).

emulsions with hydrolysates having greater antioxidant activity than purified CPP (*14*). Because casein hydrolysates would be more economical than highly purified CPP, hydrolysates may be more practical as food antioxidants. The present study evaluated if differences in activity between casein hydrolysates could be related to differences in the mechanisms by which they inhibit lipid oxidation.

Caseins and casein tryptic hydrolysates were proposed to act as free radical scavengers when they were observed to inhibit lipooxygenase conversion of linoleic acid in the corresponding monohydroxyperoxide (*16*). Casein and casein hydrolysates protected linoleic acid from oxidation in the presence of the peroxyl radical generator AAPH and showed free radical scavenging activity in a linoleic hydroperoxide−activated ferryl hemoglobin system (*16*). Casein hydrolysates also had the ability to scavenge the ABTS radical cation by hydrogen or electron donation (*17*). The ability of antioxidants to be effective in foods is dependent on both their chemical and physical characteristics (*27*−*29*). The limitation of the studies of Rival and Chiu is that either no lipid is present (Chiu) or that linoleic acid dispersions (Rival) might not mimic the physicochemical characteristics of food systems because free fatty acids are not the main components of food lipids. Therefore, the antioxidant activity of CPP and casein hydrolysates was tested in a phosphatidylcholine liposome system that models cellular membrane lipids.

To better understand the antioxidant activity of casein-derived peptides in the presence of prooxidant metals, the activities of casein hydrolysates and enriched CPP were tested using an iron−ascorbate redox cycling system. All CPP concentrations tested at concentrations lower than 1.0 mg/mL inhibited TBARS development, whereas higher amounts of enriched CPP caused a prooxidant effect (**Figures 1** and **2**). Prooxidant activity was observed by the CPP when the P/Fe was ≥35. Most chelators exhibit prooxidant behavior when their concentrations are lower than that of iron. Most notable is EDTA, which is prooxidative at EDTA/Fe ratios are ≤1 and antioxidative when EDTA/Fe ratios are >1 (*30*, *31*). The fact that CPP was prooxidative when its concentration was greater than that of iron is not understood. Enriched CPP, casein hyhdrolysates, and LMW casein hydrolysate activities were compared on an equal phosphorus molar basis of 1.5 mM, where the P/Fe = 50. Both casein hydrolysates and LMW casein hydrolysates displayed antioxidant properties even at this high P/Fe molar ratio (50), contrary to the prooxidant effect seen for enriched CPP (data not shown).

The ability of enriched CPP and casein hydrolysates to act as free radical scavengers was also tested in the phosphatidylcholine liposome systems. Inclusion of CPP (0−1000 μM) in the liposome systems slowed oxidation induced by peroxyl radicals generated by AAPH (**Figure 4**). Casein hydrolysates and LMW casein hydrolysates demonstrated similar antioxidant activities at the concentrations tested (3 and 15 mM N) (**Figure 5**). To further examine possible antioxidant mechanisms, the free radical scavenging properties of enriched CPP and casein hydrolysates were evaluated with the ORAC assay. Inhibition of fluorescence decay induced by AAPH was in the order of LMW casein hydrolysates > casein hydrolysates > enriched CPP (**Figure 7**), which is in agreement with the activities observed in the liposome systems containing AAPH. This order of free radical scavenging activity also agreed with the antioxidant potential seen in our previous study on corn oil-in-water emulsions (*14*). The observed superior peroxyl radical scavenging activity by casein hydrolysates and LMW casein hydrolysates over the enriched CPP might be explained by a comparison on their amino acid contents. Casein hydrolysates were reported to have 2.7, 2.2, 3.6, and 4.6 times more histidine, lysine, proline, and tyrosine, respectively, than CPP (*32*), and all of these amino acids have been previously found to act as free radical scavengers (*33*−*35*).

A feasible application of casein-derived peptides as food antioxidants is their addition to ground beef before cooking. Muscle foods are subjected to rapid oxidative reactions after being cooked, largely due to the fact that cooking causes the release of iron bound to proteins (*36*, *37*). Enriched CPP, casein hydrolysates, and LMW casein hydrolysates (0.5%) all inhibited the formation of TBARS in cooked ground beef, with the casein hydrolysates being less effective than enriched CPP (**Figure 8**). The ability of casein hydrolysates and LMW casein hydrolysates to inhibit TBARS formation in ground beef was less than that of CPP even when they were added at 1.25 and 1.75%, respectively (equivalent to 0.5% CPP on a phosphorus basis) (**Figure 9**). Casein hydrolysates and LWM casein hydrolysates have previously been shown to have better antioxidant activities than enriched CPP, but mainly as a result of the hydrolysates' superior free radical scavenging activity. As cooking ground beef increases the catalytic activity of iron, these results suggest that the stronger chelating activity of enriched CPP may make them more effective antioxidants in cooked muscle foods.

These findings suggest that CPP and casein hydrolysates are able to inhibit oxidative reactions by chelating iron and scavenging free radicals. These antioxidant mechanisms could allow CPP and casein hydrolysates to be used as food antioxidants in a variety of food systems.

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