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# Use of Caseinophosphopeptides as Natural Antioxidants in Oil-in-Water Emulsions

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Chelators are valuable ingredients used to improve the oxidative stability of food emulsions. Caseins and casein peptides have phosphoseryl residues capable of binding transition metals. Thus, the ability of enriched caseinophosphopeptides to inhibit lipid oxidation in corn oil-in-water emulsions was investigated. Enriched caseinophosphopeptides (25 $\mu$M) inhibited the formation of lipid oxidation at both pH 3.0 and 7.0 as determined by lipid hydroperoxides and hexanal. Calcium (0−100 mM) had no influence on the antioxidant activity of the enriched caseinophosphopeptides. Casein hydrolysates were more effective inhibitors of lipid oxidation than the enriched caseinophosphopeptides at equal phosphorus content. Thus, antioxidant properties might not be uniquely attributed to chelating metals by phosphoseryl residues but also by scavenging free radicals. Overall, the observed antioxidant activity of casein hydrolysates means they could be utilized to decrease oxidative rancidity in foods.

**KEYWORDS: Antioxidant; casein; caseinophosphopeptides; chelator; emulsion; lipid oxidation**

## INTRODUCTION

Transition metals are major prooxidants in many foods including emulsions, for example, beverages, sauces, soups, salad dressings, and mayonnaise (*1*). Reduction of the prooxidant activity of transition metals is a very effective method to decrease rancidity in emulsions (*2*, *3*). Currently the food industry controls metal reactivity with chelators. However, the commercial chelators currently available have several drawbacks including labeling issues (EDTA), ineffectiveness (citric acid) (*4*, *5*), and instability (polyphosphates) (*6*), and they can have an impact on the functionality of other food components; for example, polyphosphates alter the functionality of proteins (*7*). Recent work in our laboratory has shown that proteins can bind metals and inhibit oxidative reactions in oil-in-water emulsions (*8*, *9*), suggesting that proteins could be effective natural chelators in foods. This may be especially true of phosphorylated proteins and peptides originating from milk, that is, caseins and caseinophosphopeptides.

Caseins ($\alpha_{s1}$, $\alpha_{s2}$, and $\beta$) have polar domains that contain phosphorylated serine residues, and their characteristic sequences, -SerP-SerP-SerP-Glu-Glu-, are effective cation chelators that form complexes with calcium, iron, and zinc (*10*, *11*). Thus, phosphorylated caseins and/or their peptides in the aqueous phase could be a source of natural chelators to control lipid oxidation in food emulsions by binding and partitioning transition metals away from the emulsion droplet. The use of proteins as metal chelators in foods could be problematic because protein denaturation alters the metal-binding properties of proteins and can cause loss of protein solubility and formation of protein aggregates that alter food quality parameters. Phosphorylated peptides originating from milk proteins containing unique anionic clusters are known as caseinophosphopeptides. Caseinophosphopeptides could provide a more practical source of natural protein-based chelators having activity that is not influenced by denaturation. In addition, caseinophosphopeptides are known to increase mineral bioavailability (*12*−*14*), enhance immune response (*15*), stimulate bone mineralization, and act as anticariogenics (*16*−*18*). Caseinophosphopeptides' ability to both increase food stability and promote health makes them potential multifunctional food ingredients.

Our objective was to evaluate caseinophosphopeptides for their ability to inhibit lipid oxidation in oil-in-water emulsions to determine if they could be used as natural chelators in foods.

## MATERIALS AND METHODS

Sodium salt casein from bovine milk, *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) trypsin (EC 3.4.21.4) from bovine pancreas, sodium acetate anhydrous, imidazole, thimerosal, ammonium thiocyanate, $FeSO_4 \cdot 7H_2O$, $BaCl_2$, $CaCl_2 \cdot 2H_2O$, and cumene hydroperoxide were from Sigma Chemical Co. (St. Louis, MO). Polyoxyethylene lauryl ether (Brij 35) enzyme grade was from Fisher Biotech (Fair Lawn, NJ). Isooctanol, 2-propanol, methanol, and butanol were of HPLC grade from Fischer Scientific (Fair Lawn, NJ). Corn oil was purchased at a local supermarket. Double-distilled water was used throughout the experiments.

**Sample Preparation.** Casein (10%) was hydrolyzed by trypsin (1:100 enzyme/substrate) in a shaking water bath at 50 °C while the pH was maintained at 8.0 by the addition of 5 M NaOH at 15 min

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intervals. After 2 h, hydrolysates were immersed in a water bath at 80 °C for 5 min to inactivate trypsin and then transferred to a 40 °C water bath, where the pH was decreased to 4.6 and left for 2 h to flocculate undigested protein (*19*). To separate soluble peptides, casein hydrolysates were centrifuged at 10400*g* for 10 min at 4 °C. To produce low molecular weight (LMW) casein hydrolysates, casein hydrolysates were ultrafiltrated in a Molecular/Por stirred cell (Spectrum, Los Angeles, CA) with a Diaflo ultrafiltration membrane (Amicon, Beverly, MA) at 50 psig to collect both phosphorylated and nonphosphorylated peptides smaller than 10000 Da. Peptide fractions were then freeze-dried.

Enriched caseinophosphopeptides, a gift from Meiji Seika Kaisha Ltd. (Tokyo, Japan), were 86% caseinophosphopeptides (as determined by manufacturer) obtained by a calcium chloride and ethanol selective precipitation of casein hydrolysates (*20*). Considering $\alpha_{s2}$-casein amino acids (1−32) and $\beta$-casein amino acids (1−28) as the main components for the enriched caseinophosphopeptides (*20*), an average molecular weight of 3428 Da was used. For some experiments, one part of enriched caseinophosphopeptides (10% solution) was demineralized by dialysis in 100 parts of 10 mM acetate/imidazole buffer at pH 7.0 at 4 °C using a Spectra/Por 7 membrane (Spectrum, Rancho Dominguez, CA) with a molecular weight cutoff of 1000 Da. Buffer was changed three times at 4, 8, and 24 h. Dialyzed peptides were then freeze-dried.

Phosphorus and nitrogen contents were measured in all of the samples at the University of Massachusetts Microanalysis Laboratory. For phosphorus analysis, samples were acid digested as described by Ma and Rittner (*21*), and the final assay was performed using a standard inductively couple plasma method. Nitrogen content was measured by adapting the micro-Dumas method in a Perkin-Elmer 240 elemental analyzer (*21*).

**Emulsion Preparation.** Emulsions were prepared with 5% corn oil and 95% 17 mM Brij 35 in 10 mM imidazole/acetate buffer at pH 7.0 with 1 mM thimerosal to inhibit microbial growth during storage. A coarse emulsion was made with a Biohomogenizer M 133/1281-0 (Biospec, Bartlesville, OK) set at I for 1 min before homogenization with three passes at 3000 psi through an APV-100 two-stage homogenizer (APV Americas, Wilmington, MA). Particle size distribution was measured using a Beckman Coulter (Miami, FL) LS 230 particle sizer; the average droplet size ranged from 0.21 to 0.38 $\mu$m and did not change during the course of the experiments. The pH of the emulsions was adjusted (3.0 or 7.0) when necessary, and casein or the different peptide sources were added at various concentrations before 1.0 mL samples were placed in 10.0 mL vials sealed with rubber septa and aluminum seals. Emulsions were stored at 37 °C (pH 7.0) or 55 °C (pH 3.0) in the absence of light.

**Lipid Oxidation Markers.** Lipid hydroperoxides were measured according to the method of Mancuso et al. (*3*). Lipid hydroperoxides were extracted by mixing 0.3 mL of emulsion with 1.5 mL of 3:1 isooctane/2-propanol, vortexing three times for 10 s each, and centrifuging at 3400*g* for 2 min in a benchtop centrifuge. The isolated organic phase (0.2 mL) was added to 2.8 mL of 2:1 methanol/butanol followed by 30 $\mu$L of a thiocyanate/ferrous solution (freshly prepared by mixing equal volumes of 0.144 M $FeSO_4$ and 0.132 M $BaCl_2$, centrifuging it for 3 min, and then mixing equal volumes of this clear ferrous solution and 3.94 M ammonium thiocyanate). After 20 min of incubation at room temperature, absorbance at 510 nm was measured. The hydroperoxide content was determined from a standard curve generated from known concentrations of cumene hydroperoxide (0−200 $\mu$M).

To measure hexanal, samples were heated for at least 15 min at 55 °C in a Hewlett-Packard 19395A headspace sampler connected to a GC-17A Shimadzu gas chromatograph equipped with an HP methyl silicone (DB-1) fused silica capillary column of 50 m, 0.31 mm i.d., and 1.03 $\mu$m film thickness and coupled with a Class-VP chromatography laboratory automated software system (Shimadzu, Columbia, MD). Conditions for the method were described by Nuchi (*22*) with the only modification of the flame ionization detector temperature was 250 °C.

All experiments were repeated at least two times, and each treatment was measured in triplicate. Differences between treatments were statistically determined by using Student's *t* tests.

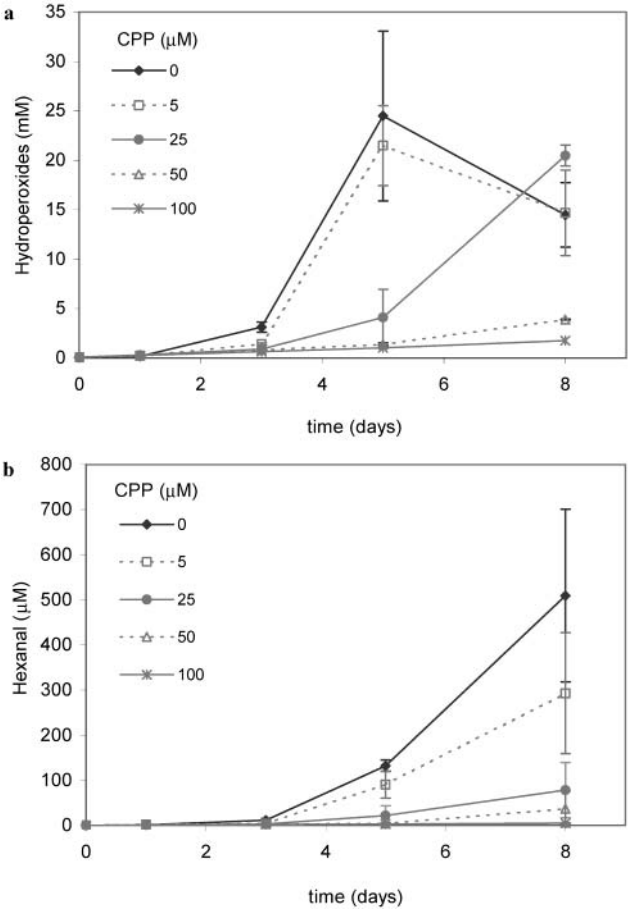


**Figure 1.** Ability of enriched caseinophosphopeptides (0–100 $\mu$M) to inhibit lipid hydroperoxide (a) and hexanal (b) formation in corn oil-in-water emulsions (pH 7.0) stored at 37 °C. Data points represent means ($n = 3$) ± standard deviation.

## RESULTS

When the enriched fraction of caseinophosphopeptides was added in the range of 0−100 $\mu$M to the corn oil-in-water emulsions at pH 7.0, a positive dose dependence was observed as higher concentrations of enriched caseinophosphopeptides produced slower rates of lipid hydroperoxide and hexanal formation (**Figure 1**). After 5 days of storage at 37 °C, lipid hydroperoxide concentrations were 12, 83, 94, and 96% lower than the control in the presence of 5, 25, 50, and 100 $\mu$M enriched caseinophosphopeptides, respectively (**Figure 1a**). Following the same trend, hexanal concentrations were 32, 84, 97, and 98% lower than the control in the samples with 5, 25, 50, and 100 $\mu$M enriched caseinophosphopeptides, respectively (**Figure 1b**).

Emulsions were also prepared at pH 3.0, and the incubation of these samples was performed at 55 °C to increase differences in oxidation markers between controls and caseinophosphopeptide treatments because the oxidation of Brij-stabilized emulsions decreases with decreasing pH (*23*). Enriched caseinophosphopeptides (25 $\mu$M) were also able to retard the formation of lipid oxidation at pH 3.0 with hydroperoxide concentrations being 10 times higher and hexanal concentrations 4.5 times higher in the control than in the caseinophosphopeptides samples after 14 days of storage (**Figure 2**).

The antioxidant activity of 25 $\mu$M enriched caseinophosphopeptides in corn oil emulsions was tested in the presence of calcium (0−100 mM) at pH 7.0 to determine if calcium would interfere with the ability of caseinophosphopeptides to chelate

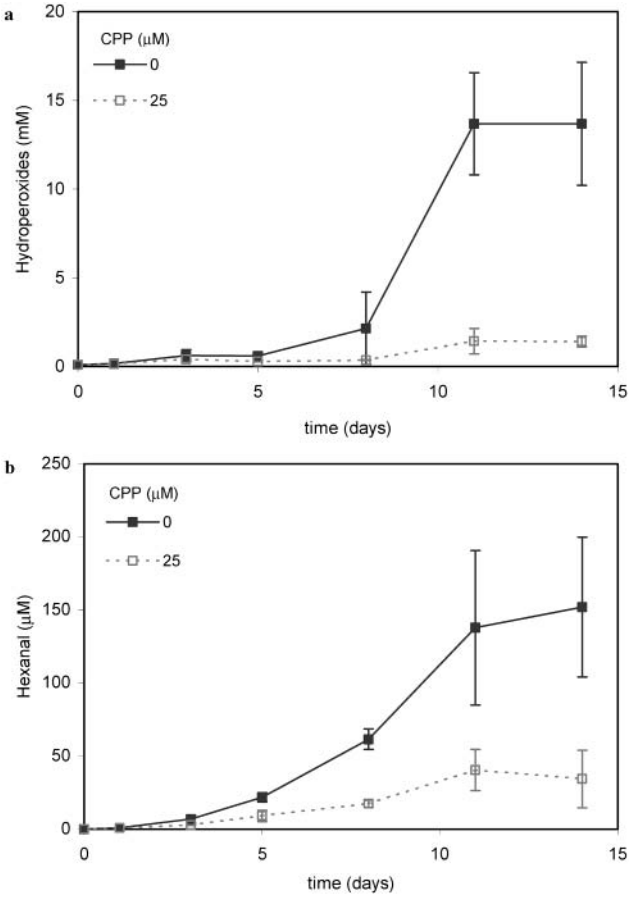


**Figure 2.** Inhibition of lipid hydroperoxide (a) and hexanal (b) formation in corn oil-in-water emulsions at pH 3.0 in the presence of 0 $\mu$M (control) or 25 $\mu$M enriched caseinophosphopeptides (CPP) (storage at 55 °C). Data points represent means ($n = 3$) ± standard deviation.

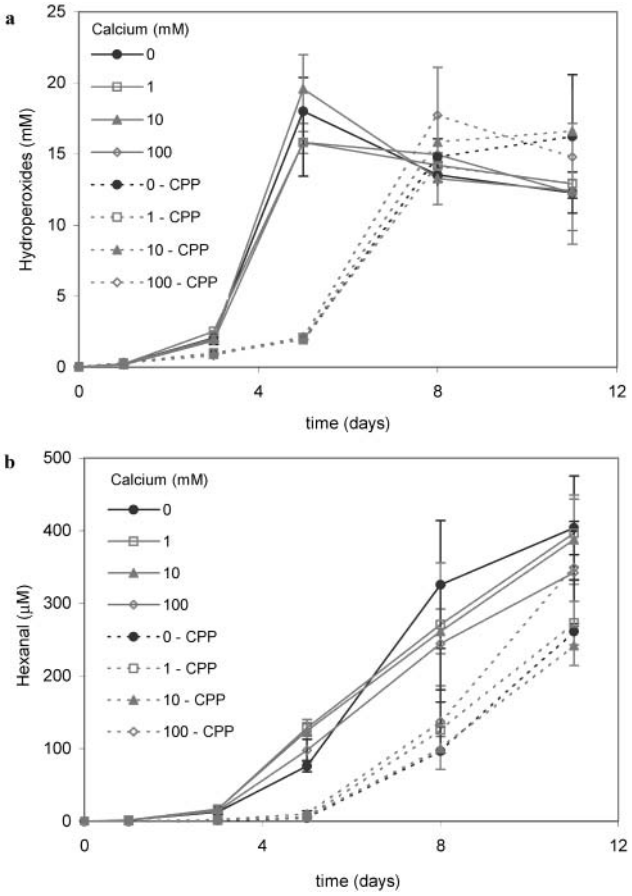


**Figure 3.** Effect of calcium addition (0–100 mM) on the formation of lipid hydroperoxides (a) and hexanal (b) at 37 °C and pH 7.0 in corn oil-in water emulsions with 25 $\mu$M enriched caseinophosphopeptides (CPP). Data points represent means ($n = 3$) ± standard deviation.

prooxidant metals. For these experiments dialysis was performed to remove any endogenous salts in the enriched caseinophosphopeptides. The addition of calcium to the emulsion systems did not cause major changes in lipid hydroperoxides or hexanal formation in the controls without caseinophosphopeptides ($p < 0.05$ compared at 5 days of storage) (**Figure 3**). None of the calcium concentrations tested affected the ability of enriched caseinophosphopeptides to inhibit lipid hydroperoxide or hexanal formation when compared to the enriched caseinophosphopeptide treatments in the absence of calcium ($p < 0.05$ compared at 5 days of storage) (**Figure 3**).

To evaluate alternative sources of metal-binding peptides and proteins from casein, the antioxidant activities of casein, casein hydrolysates, LMW casein hydrolysates, and enriched caseinophosphopeptides were compared in the corn oil-in-water emulsion system. The major component responsible for chelation of transition metals in casein and its peptides is the phosphoseryl groups (*24*, *25*). Therefore, antioxidant activity was tested on equal phosphorus concentrations of 0−310 $\mu$M (equivalent to 0−100 $\mu$M enriched caseinophosphopeptides). At 155 and 310 $\mu$M phosphorus, all casein derivatives were found to inhibit both lipid hydroperoxide and hexanal formation at pH 7.0 (data not shown). At 78 $\mu$M phosphorus and day 8, inhibition of lipid hydroperoxide formation was in the order of LMW casein hydrolysates ≅ casein hydrolysates (94% inhibition) > casein (80% inhibition) > enriched caseinophosphopeptides (14% inhibition) (**Figure 4a**). LMW casein hydrolysates inhibited lipid hydroperoxide formation (86% inhibition) more than casein hydrolysates (53% inhibition) when compared to the control at 15 $\mu$M phosphorus and 8 days of storage (**Figure 4b**). At 78 $\mu$M phosphorus and 8 days of storage, inhibition of hexanal formation was in the order of LMW casein hydrolysates ≅ casein hydrolysates ≅ casein > enriched caseinophosphopeptides (**Figure 5a**). When compared at 15 $\mu$M phosphate and 8 days of storage, inhibition was in the order of LMW casein hydrolysates > casein hydrolysates > casein ≅ enriched caseinophosphopeptides (**Figure 5b**).

Antioxidant activity of casein and its derivatives was also compared on equal nitrogen concentration of 728 $\mu$M (the nitrogen equivalent to 25 $\mu$M enriched caseinophosphopeptides) in corn oil-in water emulsions at pH 7.0 at 37 °C. For all casein derivatives the onset of lipid hydroperoxide formation was later than the control. After 8 days of storage, inhibition of lipid hydroperoxides compared to the control was in the order of LMW casein hydrolysates (90%) ≅ casein hydrolysates (83%) > caseinophosphopeptides (62%) > casein (9%) (**Figure 6a**). Differences between casein hydrolysates and LMW casein hydrolysates were not observed until 14 days of storage, with hydroperoxides being lower in the presence of LMW casein hydrolysates (**Figure 6a**). Similar trends were observed for hexanal formation with the antioxidant activity of LMW casein hydrolysates being the highest (**Figure 6b**). Inhibition of hexanal formation by casein and enriched caseinophosphopeptides were similar, although casein was not more effective than enriched caseinophosphopeptides at inhibiting hexanal formation.

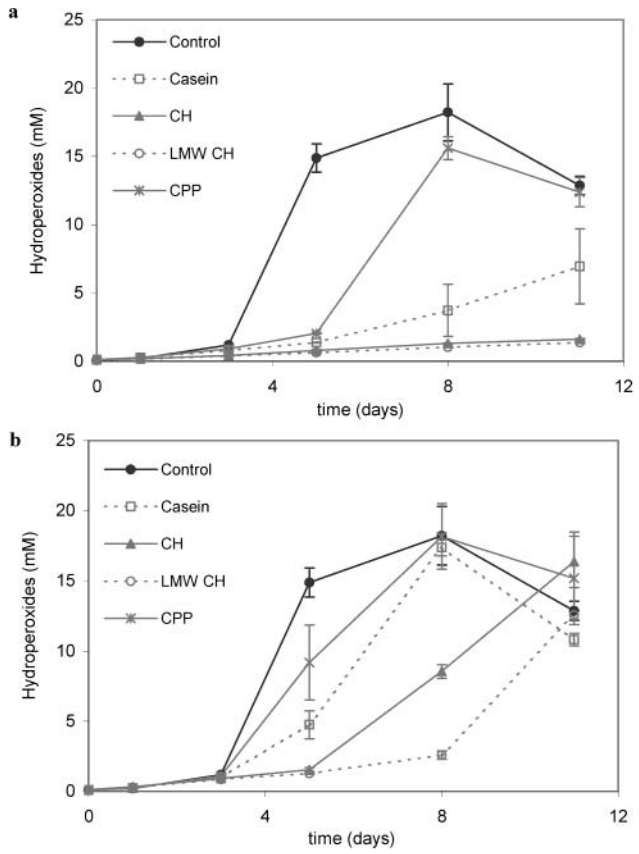


**Figure 4.** Lipid hydroperoxide formation in corn oil-in-water emulsions at pH 7.0 stored at 37 °C in the presence of casein, casein hydrolysates (CH), low molecular weight casein hydrolysates (LMW CH), or enriched caseinophosphopeptides (CPP) at a concentration of (a) 78 $\mu$M or (b) 15 $\mu$M phosphorus. Data points represent means ($n = 3$) ± standard deviation.

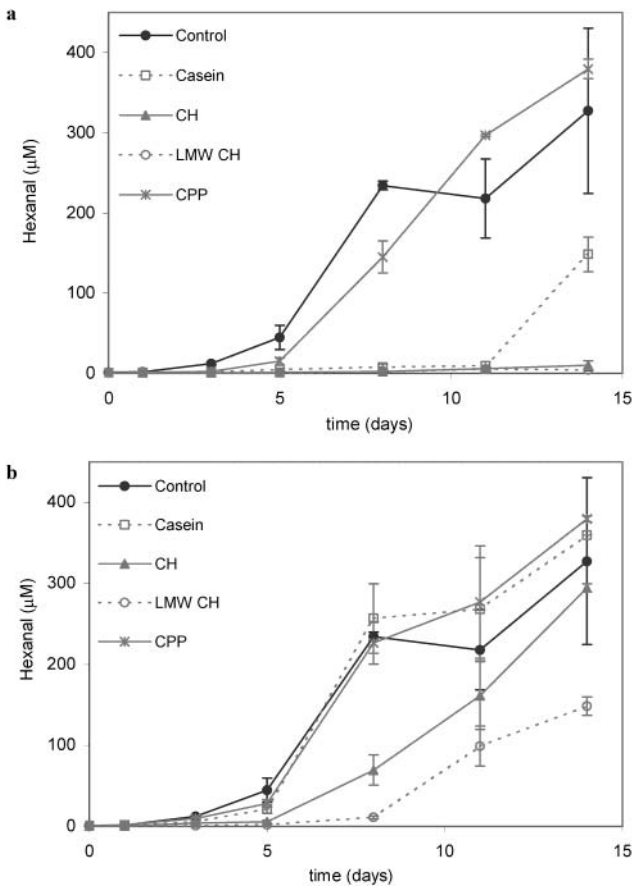


**Figure 5.** Hexanal formation in corn oil-in-water emulsions at pH 7.0 stored at 37 °C in the presence of casein, casein hydrolysates (CH), low molecular weight casein hydrolysates (LMW CH), or enriched caseinophosphopeptides (CPP) at a concentration of (a) 78 $\mu$M or (b) 15 $\mu$M phosphorus. Data points represent means ($n = 3$) ± standard deviation.

## DISCUSSION

Chelation of transition metals is an effective manner by which to inhibit lipid oxidation in food emulsions. Chelators can prevent rancidity in oil-in-water emulsions by changing the chemical reactivity of metals, impairing a metal's ability to participate in redox cycling, forming insoluble metal complexes, sterically hindering metal−lipid interaction, and/or altering the metal's physical location (*1*, *26*).

Caseins are known to bind iron, and many studies have reported the participation of phosphoseryl groups in iron binding (*27*−*29*). Caseins also accelerate the oxidization of ferrous iron by binding tightly to ferric, an activity that is lost by dephosphorylation (*24*, *30*). Thus, in oil-in-water emulsions the iron binding by phosphoseryl residues would most likely inhibit iron-promoted lipid oxidation by promoting the partitioning of iron away from the lipid droplet and/or by maintaining iron in the less reactive ferric state. The enriched caseinophosphopeptides effectively inhibited lipid oxidation in corn oil-in-water emulsions at pH 7.0 when added in the range of 0−100 $\mu$M (**Figure 1**). At more acidic pH values protonation of chelating groups can prevent metal binding and thus reduce antioxidant activity. Enriched caseinophosphopeptides (25 $\mu$M) maintained antioxidant activity at pH 3.0, suggesting that if chelation was occurring, metal-binding activity was not lost (**Figure 2**). These results agree with the findings that binding of iron to caseins is pH independent in the range of 2.5−8.0 (*10*, *28*, *29*, *31*). This is of primary importance if caseinophosphopeptides are intended to be used as food ingredients over a wide pH range. Increasing calcium concentrations did not influence the ability of enriched caseinophosphopeptides (25 $\mu$M) to retard lipid oxidation (**Figure 3**), indicating that the phosphopeptides would be effective in calcium-rich foods. These results were expected because it has been reported that $\beta$-casein has a higher affinity for iron than for calcium (*10*, *31*).

The observation of differences in antioxidant activity between the different casein derivatives at equal phosphorus concentrations suggests that chelation by phosphoseryl groups was not the only antioxidant mechanism (**Figures 4** and **5**). At equal phosphorus concentrations the less purified casein derivatives contained higher concentrations of proteinaceous material. If this nonphosphorylated proteinaceous material had antioxidant properties, casein, casein hydrolysates, and LMW casein hydrolysates would more effectively inhibit lipid oxidation compared to the enriched caseinophosphopeptides. The most effective antioxidants were casein hydrolysates and LMW casein hydrolysates, which were intermediate in phosphorus content (**Table 1**). Even though casein hydrolysates and LMW casein hydrolysates were similar in phosphorus and nitrogen contents, LMW casein hydrolysates were more effective at inhibiting lipid oxidation. These observations suggest that casein and casein derivatives are also inhibiting lipid oxidation by chelation with nonphosphorylated groups such as Glu and Asp (*32*) and/or by nonchelating mechanisms such as free radical scavenging. Dephosphorylated casein and casein hydrolysates are weak iron chelators yet are able to inhibit lipid oxidation (*33*). The antioxidant mechanism of dephosphorylated casein derivatives has been postulated to be due to free radical scavenging activity

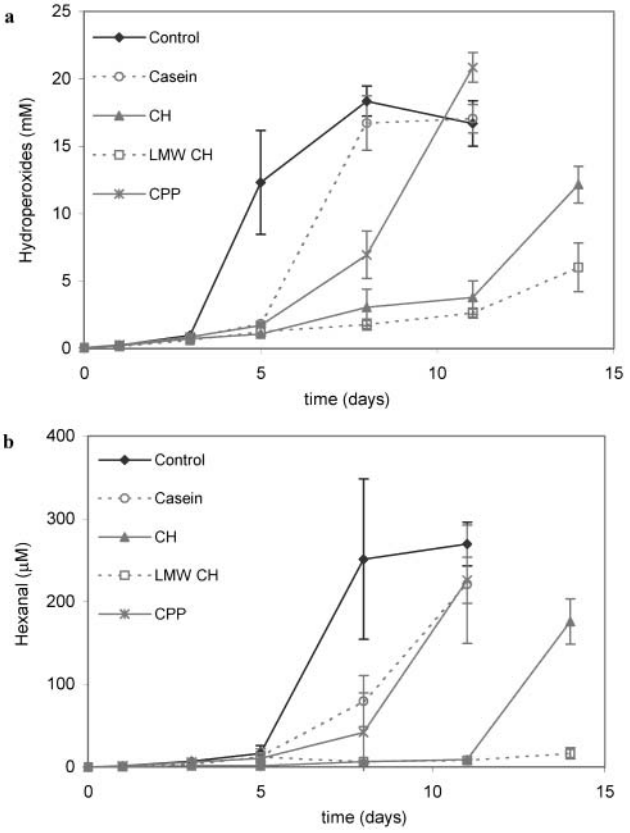


**Figure 6.** Formation of lipid hydroperoxides (a) and hexanal (b) in corn oil-in-water emulsion (pH 7.0) with casein, casein hydrolysates (CH), low molecular weight casein hydrolysates (LMW CH), or enriched caseinophosphopeptides (CPP), all at 728 $\mu$M nitrogen. Data points represent means ($n = 3$) ± standard deviation.

**Table 1.** Phosphorus and Nitrogen Contents in Casein and Casein Derivatives

| | P ($\mu$g/mg) | N (%w/w) | P/N ($\mu$g/mg) |
|---|---|---|---|
| casein | 7 | 14.3 | 52 |
| casein hydrolysates | 11 | 12.5 | 91 |
| LMW casein hydrolysates | 8 | 10.9 | 75 |
| enriched caseinophosphopeptides | 28 | 11.9 | 237 |

(*34*). It is not yet clear which mechanisms predominate in the casein derivatives used in this study.

In conclusion, enriched phosphorylated peptides from casein effectively inhibit lipid oxidation in corn oil-in water emulsions. Caseinophosphopeptides were able to inhibit lipid oxidation at pH 3.0 and 7.0, and antioxidant activity was not affected by calcium up to 100 mM. Casein and less purified sources of phosphopeptides also inhibited lipid oxidation. Overall, the results suggest that casein and casein peptides inhibit lipid oxidative reactions under the conditions expected in foods and thus could be considered as potential antioxidants. Casein hydrolysates and LMW casein hydrolysates, the less purified casein derivatives, seem to be the more economically feasible sources of phosphopeptides that could be used as "natural" ingredients to improve the shelf life of food emulsions.

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