Antioxidant Activity of a Maillard-Type Phosvitin−Galactomannan Conjugate with Emulsifying Properties and Heat Stability

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Egg yolk phosvitin was conjugated with galactomannan through a controlled Maillard reaction at 60 °C in 79% relative humidity for 1 week. Antioxidant activity of phosvitin and phosvitin−galactomannan conjugate (PGC) was assessed using a powdered model linoleic acid system. The conjugation reaction significantly ($P < 0.05$) enhanced the antioxidant activity of phosvitin. One-tenth percent PGC suppressed the relative lipid oxidation rate catalyzed with 1 mg/L Fe^{2+} to 75% and 73% in thiobarbituric acid and peroxide values, respectively, compared to those of a simple phosvitin−galactomannan mixture after 3 days at 20° C. The antioxidant effect of PGC was not affected by autoclaving (121 °C, 1.2 atm for 15 min), whereas the same treatment when applied to native phosvitin resulted in a lower affinity to inhibit iron-catalyzed lipid oxidation. The conjugation of phosvitin with galactomannan significantly ($P < 0.05$) improved both emulsifying activity and emulsion stability. The results demonstrate that the Maillard-type PGC can be used as an effective macromolecular antioxidant, with good emulsifying properties and heat stability.

Keywords: *Antioxidant activity; Maillard reaction; phosvitin−galacatomannan conjugate*

INTRODUCTION

Suppression of lipid oxidation reactions in food is critically important to the food industry in ensuring prevention of undesirable changes to food products that result in loss of nutritional value, generation of off-flavors, and production of potential toxins (Kitts, 1996). Because presently employed synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), have been suspected to be responsible for liver damage and carcinogenesis in laboratory animals (Witchi, 1986; Grice, 1988), many studies have been carried out to identify natural substrates with equivalent antioxidant potential (Frankel et al., 1996; Wijewickreme et al., 1997; Yoshimura et al., 1997).

Antioxidants can be classified into two major categories, free radical scavengers and metal chelators (Lu and Baker, 1986). Phosvitin is a phosphoglycoprotein with a molecular weight of ∼35000 (Taborsky and Mok, 1967), which contains numerous phosphoserine residues that are aligned in linear sequences of about eight residues without interruption by other amino acids (Allerton and Perlmann, 1965; Shainkin and Perlmann, 1971). Phosvitin has been identified as a protein with iron carrier activity in egg yolk, with as much as 95% of iron in yolk bound to phosvitin due to high phosphorylation properties (Powrie and Nakai, 1985). The iron−phosvitin binding strength is extremely strong, exceeding the chelating properties of citrate and nitrilotriacetic acid (Hegenauer et al., 1979). Phosvitin could therefore be used as a potent natural antioxidant on the basis of its potential to inhibit metal-catalyzed lipid oxidation.

A number of studies have demonstrated that the formation of Maillard reaction products suppresses oxidation of unsaturated fatty acids in model systems (Kirigaya et al., 1969; Kato, 1970; Lingnert and Erikson, 1980a,b; Wijewickreme and Kitts, 1997). The radical scavenging effect of ovalbumin was shown to be substantially enhanced by the conjugation with dextran or galactomannan, through a controlled Maillard reaction between the free amino groups in the protein and the reducing-end carbonyl group in the polysaccharide (Nakamura et al., 1992). In addition to the enhancement of the antioxidant capacity, a significant improvement in the emulsifying properties of proteins has been observed in the Maillard-type protein−polysaccharide conjugates (Kato et al., 1990; Kato and Kobayashi, 1991). The intensified affinity of protein−polysaaccharide conjugate to oil can be expected to enhance antioxidant potential.

In the present study, we employed a Maillard conjugate technique to produce a novel macromolecular antioxidant with emulsifying activity. This paper describes the enhanced antioxidative properties of phosvitin by the covalent conjugation with galactomannan, a mannase hydrolysate of guar gum.

MATERIALS AND METHODS

Materials. Galactomannan, a mannase hydrolysate of guar gum, was supplied from Taiyo Chemicals Co. (Japan). Nicotinamide adenine dinucleotide (NADPH), nitroblue tetrazolium (NBT), phenazine methosulfate (PMS), Celite, linoleic acid, and thiobarbituric acid were purchased from Sigma Chemical Co. All other chemicals used were of analytical grade.

Preparation of Phosvitin Conjugate with Galactomannan. Phosvitin was prepared from hen egg yolk according

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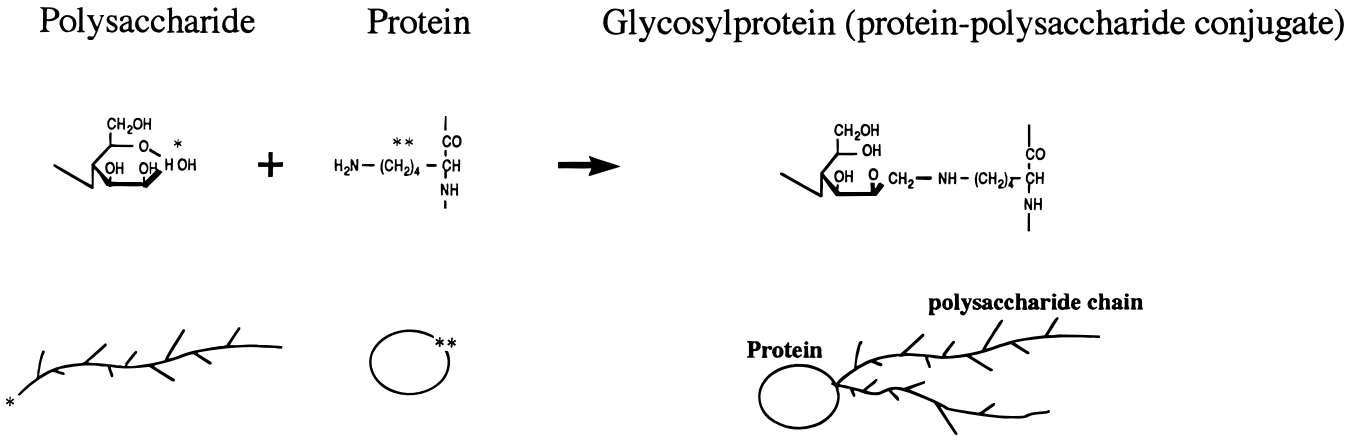

Figure 1. Scheme of protein−polysaccharide conjugate product using Maillard reaction: *, galactomannan has only one reducing end; **, free amino group (e.g., lysine) and N-terminal amino acid.

to the method of Mecham and Olcott (1949). Galactomannan was dialyzed against deionized water for 2 days at 4 °C. Phosvitin was dissolved in water and freeze-dried with galactomannan in a weight ratio of 1:3. The lyophilized mixture was incubated at 60 °C for 1 week under a relative humidity of 65%. The resulting powder sample was used as the phosvitin−galactomannan conjugate (PGC). A schematic figure demonstrating the production of the protein−polysaccharide conjugate using the Maillard reaction is shown in Figure 1.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE). SDS-PAGE was conducted according to the method of Laemmli (1970), using 10% acrylamide separating gel and 3% stacking gel containing 0.1% SDS. Protein samples (20 μL, 0.2%), were heated at 100 °C for 5 min in Tris−glycine buffer, pH 8.8, containing 1% SDS. Electrophoresis was carried out at a constant current of 15 mA for 3 h using electrophoretic buffer of Tris−glycine containing 0.1% SDS. Protein bands were stained by a CBB staining solution containing Coomassie brilliant blue G-250 (25% 2-propanol, 10% acetic acid), and carbohydrates were visualized by PAS staining with Fuchsin.

Antioxidative Assay. A powder model system was employed to monitor the antioxidant activities of phosvitin and phosvitin conjugate with galactomannan as previously described (Nakamura et al., 1992). Celite was treated with nitrohydrochloric acid for 1 week and subsequently washed with deionized water until chlorinity disappeared. The purified Celite used as a model food matrix was mixed in water with phosvitin samples and subsequently freeze-dried. To assess the heat stability of samples, phosvitin was mixed with the Celite matrix in water, autoclaved at 121 °C and 1.2 atm for 15 min, and freeze-dried. The resulting powder was mixed with linoleic acid dissolved in ethyl ether to a weight ratio of 3:1, with residual solvent being evaporated in a cabinet drier. Four grams of the powder model system was transferred to a Petri dish with a diameter of 9 cm and incubated at 20 °C in the dark without humidity control. $FeSO_4 \cdot 7H_2O$ was used as the metallic oxidation catalyst. Lipid oxidation of linoleic acid in the powdered model system was monitored by measuring peroxide value (POV) and thiobarbituric acid (TBA) values (Nakamura et al., 1992). The TBA value was determined according to the aqueous extraction procedure of Pikul et al. (1989).

Determination of Superoxide Scavenging Capacity. The scavenging capacity of superoxides was determined as described by Ponti et al. (1978). Superoxides were measured by the increasing amount of developed diformazan at 560 nm for 1 min after the addition of PMS solution to NADPH and NBT solutions under aerobic condition.

Measurement of Emulsifying Properties. The emulsifying properties of samples were determined according to the modified method of Pearce and Kinsella (1978). An emulsion was prepared by homogenization of 1.0 mL of corn oil and 3 mL of a sample solution in 0.1 M phosphate buffer, pH 7.4, using an Ultra Turrax (Hansen & Co.) at 12000 rpm for 1 min at 20 °C. A 100 μL portion of emulsion was taken from the bottom of the test tube at time 0, 1, 5, 10, 20, 40, and 80 min and immediately diluted with 5 mL of 0.1% SDS solution. The turbidity of diluted emulsion was then determined at 500 nm. The relative emulsifying activity was represented as the absorbance at 500 nm measured after emulsion formation (0 min). The emulsion stability was estimated by measuring the half-life time for emulsion decay.

Statistical Analysis. All experiments were conducted in triplicate. Data were analyzed using the Student *t* test (SAS Institute Inc., 1988) for comparing differences between treatment means.

RESULTS AND DISCUSSION

Preparation of Phosvitin−Galactomannan Conjugate. SDS−PAGE patterns confirmed that egg yolk phosvitin was successfully covalently liked with galactomannan after 1 week of incubation, under controlled reaction conditions (Figure 2). A new broad band was visualized at the boundary between stacking and separating gels for the conjugate for both protein and carbohydrate stains (Figure 2, lane 3), whereas the native protein produced a single band at a molecular mass of ~35 kDa. Because phosvitin has an expanded structure, owing to an extraordinary abundance of phosphoryl groups, every ϵ-amino group in the protein must be exposed to the molecular surface. The conjugate formation with galactomannan proceeded easily for phosvitin in this reaction system, unlike similar procedures used previously with rigid globular proteins such as ovalbumin and lysozyme (Kato et al., 1990; Nakamura et al., 1991, 1992).

Antioxidative Effects of PGC. The concentration-dependent temporal patterns of lipid oxidation by control, native phosvitin, phosvitin−galactomman mixture, and PGC in a powdered model system are shown in Figure 3. In this system, the generation of rancid odors was found to be suppressed for 1 week during storage of model lipid system by adding PGC, whereas off-odors occurred quickly in the control system without the presence of PGC (data not shown). This observation was confirmed by measuring products of lipid oxidation (Figure 3). A significant ($P < 0.05$) suppression of lipid oxidation occurred with the phosvitin−galactomannan mixture in the model system. On the other hand, the antioxidative effect of the PGC, as assessed by measurement of TBA values, was even more ($P < 0.05$) active

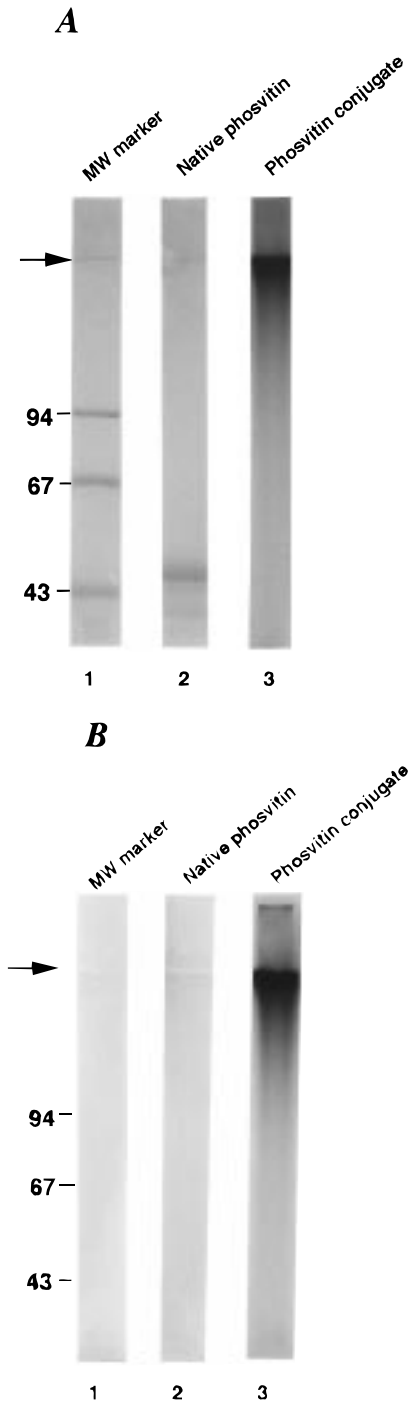

Figure 2. SDS−PAGE patterns of PGC: (A) protein stain; (B) carbohydrate stain; (lane 1) molecular weight marker; (lane 2) phosvitin−galactomannan mixture; (lane 3) PGC. Arrows indicate the position of the boundary between stacking (upper) and separating (lower) gels.

than that of the phosvitin−galactomannan mixture at both concentrations tested. A similar observation was made with POV values up to 4 days of storage. This finding suggests that the antioxidative effect of the conjugate could be attributed to the Maillard products, which were formed between phosvitin and galactomannan during conjugation formation in the controlled dry state. Individually, both phosvitin and galactomannan are basically hydrophilic compounds, and simply mixing these two substrates together would not be expected to alter this character. The fact that greater antioxidant activity was observed in the model powdered lipid

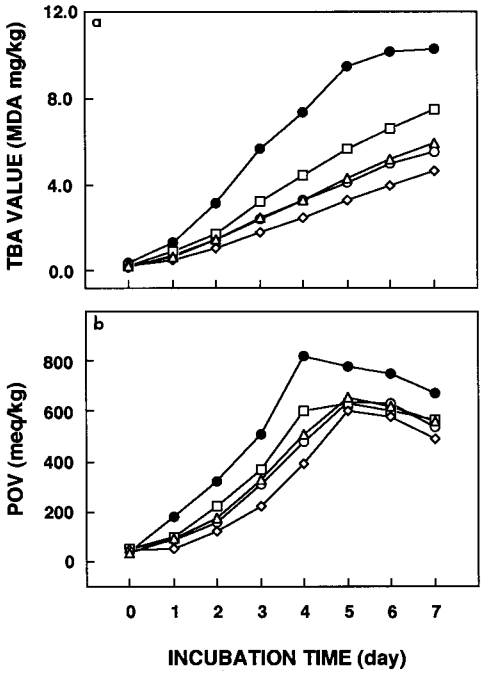

Figure 3. Antioxidative effect of PGC in a powder oil model system: (a) TBA; (b) POV; (●) control (no addition); (△) 1% phosvitin−galactomannan mixture; (□) 0.1% phosvitin−galactomannan mixture; (◇) 1% PGC; (○) 0.1% PGC.

system with the PGC derived from a Maillard reaction between phosvitin and galactomannan likely produced some hydrophobic in addition to hydrophilic character, which in turn enhanced the antioxidant activity. The suggested hydrophobic property of the PGC macromolecule would enable it to associate more closely with the oil phase. On the other hand, the hydrophilic character would provide access to polar peroxyl radicals, which, once formed on lipid oxidation, would diffuse to the polar region of the PGC macromolecule and could be removed from further propagation of the peroxidation reaction.

The inhibition effect of the PGC on metal-induced lipid oxidation was investigated using a Fe^{2+}-supplemented powdered model food system as shown in Figure 4. A typical oxidation pattern of linoleic acid, catalyzed by Fe^{2+}, is shown in the control. In the same system, the extent of lipid oxidation catalyzed by Fe^{2+} was significantly ($P \leq 0.05$) inhibited by both the phosvitin−galactomannan mixture and the PGC. The relative affinities of the phosvitin−galactomannan mixture to inhibit lipid peroxidation when catalyzed with 1 mg/L Fe^{2+} were 28% and 41%, respectively, as assessed by TBA and POV of those of control values without phosvitin after 3 days at 20° C. In contrast, PGC inhibited forced peroxidation of linoleic acid to 31% and 30% for TBA and POV, respectively. One-tenth percent PGC suppressed the relative lipid oxidation rate catalyzed with 1 mg/kg Fe^{2+} to 75% and 73% in TBA and POV, respectively, compared to the phosvitin−galactomannan mixture. These findings demonstrate that the antioxidant activity of the PGC was 1.3−1.4 times stronger than that of the phosvitin−galactomannan mixture. As represented in Figure 5, we hypothesize that the galactomannan segment of the PGC macromolecule assisted with the configuration of phosphoseryl residues of the phosvitin segment to interact with Fe^{2+} where chelation reactions occur in the microenvironment and thus contributed in part to reducing the initiation reaction of lipid peroxidation. An analogy to this finding

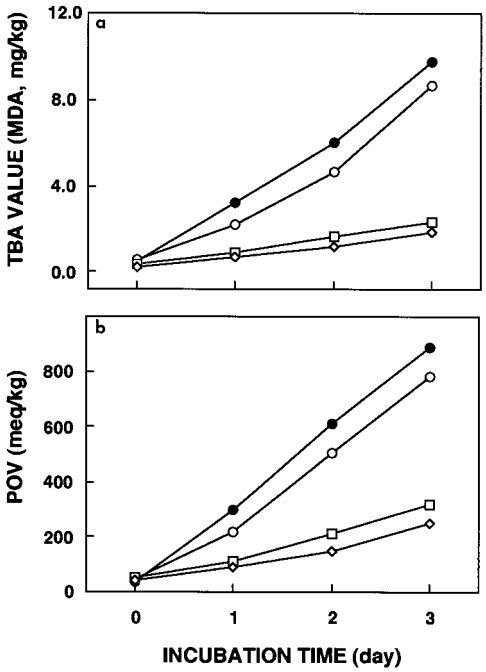

Figure 4. Antioxidative effect of PGC on Fe^{2+}-catalyzed oxidation in the powder oil model system: (a) TBA; (b) POV; (●) 10 mg/L Fe^{2+}, (○) 1 mg/L Fe^{2+}, both without phosvitin; (□) 0.1% phosvitin−galactomannan mixture with 1 mg/L Fe^{2+}; (◇) 0.1% PGC with 1 mg/L Fe^{2+}.

has been reported by Koga and Terao (1994), who demonstrated that replacing the phytyl chain in vitamin E with a phosphatidyl moiety improved the attainablity of vitamin E to polar regions where metal-catalyzed lipid peroxidation initiation reactions took place.

Effect of Heat Treatment on Antioxidant Capacity of Phosvitin Conjugate. The antioxidant activity of the phosvitin−galactomannan mixture and conjugate following a thermal process is shown in Figure 6. The efficacy of the phosvitin−galactomannan mixture to suppress lipid oxidation was reduced significantly ($P < 0.05$) following autoclaving at 121 °C and 1.2 atm for 15 min. A similar reduction in antioxidant activity for the PGC after autoclaving did not occur. Exposing the phosvitin molecule to high temperature would be expected to lead to unfolding of the protein, which on cooling would induce aggregation, due to heat-induced disruption of various noncovalent interactions. This process may be reversible in the phosvitin conjugate when covalently linked with galactomannan chains that inhibit the unfolded protein−protein interaction, whereas it may be irreversible in the phosvitin−galactomannan mixture. The reversible effect of thermal processing toward the conjugate macromolecule is one potential reason for the retained antioxidant activity of PGC, not seen in the protein−carbohydrate mixture.

Radical Scavenging Effect of Phosvitin Conjugate. Superoxides were generated using the NADPH−PMS−NBT reaction system under aerobic conditions, and its inhibitory activity of the PGC was assessed. The generation rate of superoxide from the system containing 0.1% PGC was 71.7% of the control system, whereas the rates of the phosvitin−galactomannan mixture and native phosvitin were 80.9% and 81.7%, respectively (Table 1). The inhibitory rate of generating superoxides of native phosvitin was significantly ($P < 0.01$) elevated by the conjugation with galactomannan through the controlled Maillard reaction.

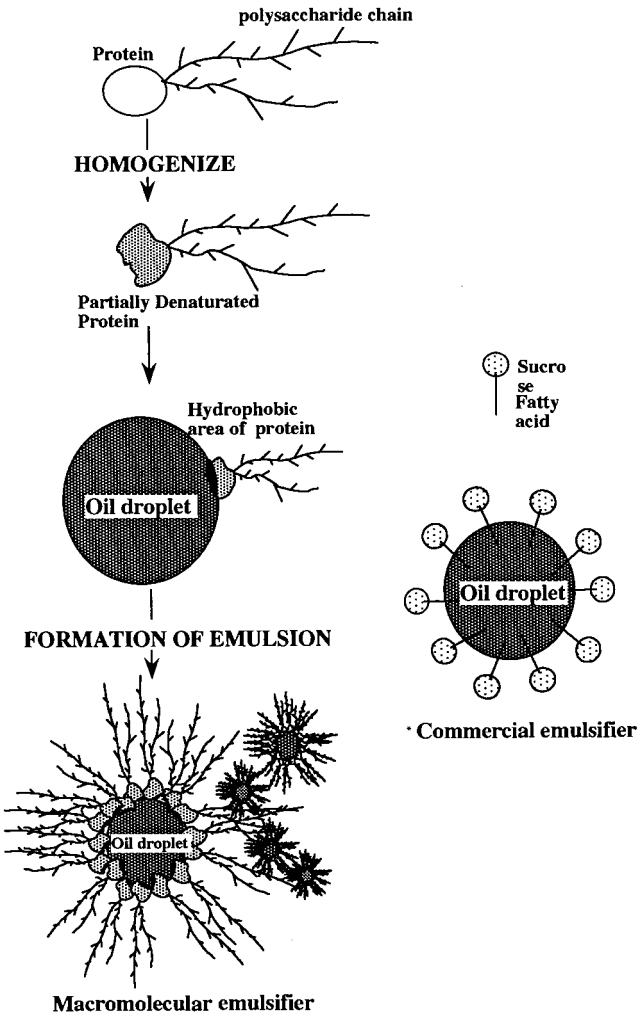

Figure 5. Diagrammatic representation of the protein−polysaccharide conjugate and interaction with oil droplet in forming a macromolecular emulsifier: *, sucrose fatty acid conjugate example in a commercial emuslifier.

Table 1. Superoxide Scavenging Effect of PGC[a]

sample[b]	superoxide generation ratio[c] (%)
control (no addition)	100.0
native phosvitin	81.7 ± 1.7
phosvitin−galactomannan mixture	80.9 ± 2.1
phosvitin−galactomannan conjugate	71.7 ± 1.5

[a] Mean ± SD, $n = 3$. [b] Sample concentration was adjusted as protein contents of 0.1%. [c] Superoxide was generated by the NADPH−PMS−NBT reaction system under aerobic conditions. NADPH, nicotinamide dinucleotide phosphate reduced form; PMS, phenazine methosulfate; NBT, nitroblue tetrazolium.

Emulsifying Properties of PGC. Emulsifying properties were measured using corn oil under neutral pH condition (0.1 M phosphate buffer, pH 7.4). As shown in Figure 7, emulsifying properties of phosvitin were further improved by conjugation with galactomannan, albeit, phosvitin in native form also has appreciable emulsifying properties. The relative emulsifying activity and emulsion stability calculated from curves in Figure 6 are summarized in Table 2 and show that the relative emulsifying activity of 0.1% PGC was ~1.4 times that of the phosvitin−galactomannan mixture at the same protein concentration. Even at an especially low concentration of 0.01%, the relative emulsifying activity PGC was much higher than that of the 0.1% phosvitin−galactomannan mixture. Moreover, the emul-

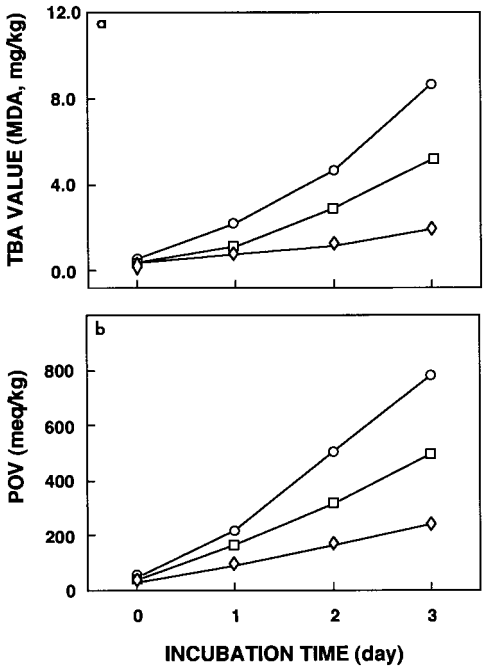

Figure 6. Effect of autoclaving on the antioxidative effect of PGC: (a) TBA; (b) POV; (○) 1 mg/L Fe^{2+} coexisting powder system; (□) autoclaved 0.1% phosvitin−galactomannan mixture with 1 mg/L Fe^{2+} coexisting powder system; (◇) autoclaved 0.1% PGC with 1 mg/L Fe^{2+} coexisting powder system.

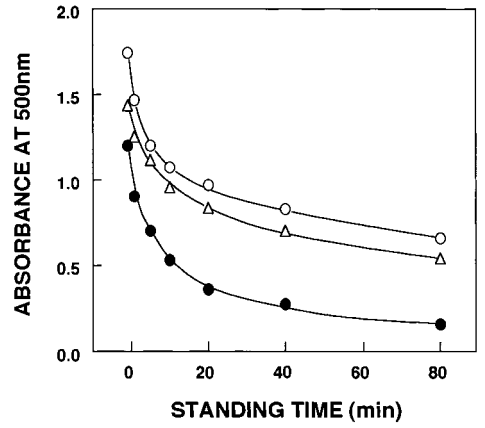

Figure 7. Emulsifying properties of PGC in 0.1 M phosphate buffer, pH 7.4: (●) 0.1% phosvitin−galactomannan mixture; (○) 0.1% PGC; (△) 0.01% PGC.

sion stability of the conjugate was also better than that of the mixture. The emulsion stability of phosvitin was improved >5 times by conjugation with galactomannan (Table 2). The fact that the superior antioxidant activity of PGC corresponded with improved emulsifying properties suggests that the functional antioxidant properties of PGC were enhanced by increased hydrophilic character associated with the carbohydrate conjugate. An important criterion in explaining the phenomena observed in this study entails the physicochemical characteristics of the test materials at the oil−air interface. On the basis of the "polar paradox" reported by Porter et al. (1989), Frankel et al. (1994) demonstrated that a partially soluble suspended ascorbic acid was more favorably oriented in an air−oil interface where surface oxidation of the oil occurs, thus explaining its unexpected antioxidant activity under this condition. In contrast, in the oil-in-water emulsion system, lipophilic antioxidants are sufficiently surface active to be oriented at the oil−water interface to better protect the

Table 2. Emulsifying Activity and Emulsion Stability of Phosvitin−Polysaccharide Conjugate[a]

sample	emulsifying activity[b] (abs 500 nm)	emulsion stability[c] (min)
0.1% phosvitin−galactomannan mixture	1.238 ± 0.039	8.7 ± 0.2
0.1% phosvitin−galactomannan conjugate	1.772 ± 0.075	36.8 ± 0.3
0.01% phosvitin−galactomannan conjugate	1.413 ± 0.033	49.3 ± 0.5

[a] Values represent means ± SD, $n = 3$. [b] Sample concentration was adjusted on the basis of protein contents. Absorbance at 500 nm measured immediately after emulsion formation. [c] Half-life time of the decay of emulsion (minutes).

oil against oxidation. By moving to the water phase, the hydrophilic antioxidants, for example, ascorbic acid, become diluted and cannot adequately protect the oil in the oil−water interface.

The powdered model system used in this study represented a bulk oil system; therefore, hydrophilic antioxidants would be expected to locate in the air−oil interface. This characteristic explains the results shown in Figures 3 and 4, respectively. Phosvitin is basically a hydrophilic antioxidant, thus locating in the air−oil interface. As a result, autoclaving the mixture should have denatured the free phosvitin, thereby reducing the antioxidant activity of the mixture. This effect was not observed in the PGC (Figure 6).

In the case of the emulsifying properties shown in Figure 7, not all of the phosvitin−galactomannan mixture can remain in the water−oil interface because galactomannan is water dispersible. Therefore, the galactomannan will diffuse away from the water−oil interface, leaving phosvitin alone in the interface. However, in the case of the PGC, it would be expected that the whole molecule would stay in the interface, thereby attracting more water molecules surrounding the oil droplets due to the hydrophilic property of the galactomannan moiety (Figure 5). Also, due to the long chain of glycosidic groups on the molecule, the surface viscosity may increase, thus further stabilizing the emulsion.

The carry-through protection afforded by antioxidants to foods that have undergone a thermal process is an especially important characteristic (Maga, 1995), thus denoting the value of the thermal stability character of food grade antioxidants. Although the metal chelating activity of egg yolk protein, phosvitin, is a favorable property for antioxidant function, its affinity to inhibit lipid peroxidation reactions is known to be decreased by high-temperature treatment such as autoclaving (Lu and Baker, 1987). In this study, we found that the inhibitory capacity of iron- catalzed lipid oxidation from PGC was not changed by autoclaving. This result is an important observation for the potential application of PGC for antioxidant use, because the heat stability character of an antioxidative agent is an important quality for employment in thermally processed foods. The safety of Maillard-type protein−polysaccharide conjugates has been investigated using both a bacterial mutagenesis test and also acute animal toxicity tests (Nakamura et al., 1992). Because the PGC tested in this study was basically prepared without chemical reagents, it can be assumed to be potentially useful as a safe antioxidant in retarding the development of rancidity in processed foods. We conclude that the improved capability of the PGC for both antioxidant and emulsifying properties was due to the unique balance

obtained between both hydrophilic and hydrophobic character using a Maillard conjugation reaction.

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