Elucidation of Antioxidant Activity of Phosvitin Extracted from Egg Yolk using Ground Meat

Samooel Jung¹, Cheorun Jo¹, Mingu Kang¹, Dong Uk Ahn^{2,3}, and Ki Chang Nam^{*}

Department of Animal Science and Technology, Suncheon National University, Suncheon 540-742, Korea

¹*Department of Animal Science and Biotechnology, Chungnam National University, Daejeon 305-764, Korea*

²*Department of Animal Science, Iowa State University, Ames, IA, 50011, USA*

³*Department of Agricultural Biotechnology, Seoul National University, Seoul 151-921, Korea*

Abstract

Phosvitin was extracted from a chicken egg yolk and the iron-binding, along with antioxidative activity of the extracted phosvitin, was determined after mixing with ground beef at the concentrations of 100 and 500 mg/kg of meat. The electrophoretic pattern of the extracted phosvitin on SDS-PAGE was found to be identical to that of the standard phosvitin. The extracted phosvitin at 1,000 µg/mL showed an ability to bind approximately 65% of the iron in a 3 mM iron solution. Lipid oxidation was inhibited in the ground beef mixed with 500 mg/kg of the extracted phosvitin, during storage at 4°C compared to that of the control ($p < 0.05$). Additionally, color stability of ground beef containing the extracted phosvitin was enhanced ($p < 0.05$). The pH, cooking loss, texture, and sensory properties of the ground beef were not affected, by adding up to 500 mg/kg of the extracted phosvitin. This result suggests that the phosvitin extracted from egg yolk could be used as an antioxidant reagent. In particular, phosvitin would be more amenable for use in meat products because it is a natural protein derived from animal products.

Key words: phosvitin, ground beef, antioxidant activity, iron binding

Introduction

Lipid oxidation is a serious problem in meat products because the oxidation products reduce the shelf life of food and lead to the deterioration of food quality factors such as flavor, color, texture, and nutritional value (Mielnik *et al.*, 2006). Lipid oxidation in meat products can be caused by several factors including handling and cooking processes, storage, and endogenous iron (Terevinto *et al.*, 2010). Meat products are more susceptible to lipid oxidation through mincing processes because grinding overexposes the muscle surface to air, microbial contamination, and metal oxidation catalysts (Devatkal *et al.*, 2010; Mitsumoto *et al.*, 2005). To prevent lipid oxidation in meat products, much effort has been spent to identify safe and effective antioxidants. Consequently, synthetic chemicals (BHT and BHA) and natural materials (such as ones derived from grains, oilseeds, honey, fruits, and veg-

etables) have been found to inhibit lipid oxidation. However, synthetic antioxidants are associated with potential for adverse health effects, and some natural antioxidants were not suitable for use in meat products because of their inherent characters (Mitsumoto *et al.*, 2005; Shahidi and Wanasundara, 1992).

Iron is an essential dietary metal ion required by animals since it is needed for heme proteins that are involved in oxygen transport and storage in the body (Carlsen *et al.*, 2005). However, iron is released from heme during the meat production processes as free iron (non-heme iron) which promotes lipid oxidation in meat products through Fenton reactions (Fe^{2+} or $\text{Fe}^{3+}/\text{H}_2\text{O}_2$) (Carlsen *et al.*, 2005; Estévez and Cava, 2004). Therefore, the elimination of free iron in meat products can be a way to inhibit lipid oxidation.

Phosvitin is a phosphoglycoprotein to be found in chicken egg yolk (Anton *et al.*, 2007). It is known to have a specific amino acid composition that is approximately 50% serine, and 90% of these serine residues are phosphorylated (Clark, 1985). This specific structure makes phosvitin a potent metal chelator. Phosvitin isolated from chicken egg yolk contains 2-3 atoms of iron per mole-

*Corresponding author: Ki Chang Nam, Department of Animal Science and Technology, Suncheon National University, Suncheon 540-742, Korea. Tel: 82-61-750-3231, Fax: 82-61-750-3230, E-mail: kichang@snu.kr

cule, which is around 95% of yolk iron, and its maximum potential iron binding ability is about 60 atoms per molecule (Albright *et al.*, 1984; Tarborsky, 1963; Webb *et al.*, 1973). Previous studies suggested that phosvitin could inhibit lipid oxidation promoted by iron in meat (Lee *et al.*, 2002; Lu and Baker, 1986). In addition, phosvitin could be more suitable for use in meat products than natural antioxidants derived from grains, oilseeds, honey, fruits, and vegetables because it is a natural protein derived from animal product. Previous studies reported that changes in the color or flavor in cooked beef, chicken, and goat meat patties resulted from adding natural antioxidants such as ones derived from tea, kinnow, pomegranate, or grapes (Devatkal *et al.*, 2010; Mitsumoto *et al.*, 2005; Selani *et al.*, 2011).

Nevertheless, phosvitin is not used as antioxidant in meat products because of the complicated procedures and cost to produce. Recently, a simple and cost-effective method was established for extracting phosvitin from chicken egg yolk using NaCl and alcohol (Ko *et al.*, 2011). The aim of this study was to evaluate antioxidant activity of the extracted phosvitin when present in ground beef.

Materials and Methods

Materials

Chicken eggs and a ground beef were obtained from a local market (Daejeon, Korea). Egg yolk phosvitin (used as a standard) was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals used in this study were of analytical grade and supplied by Sigma-Aldrich or Fulka Co. (Buchs, Switzerland).

Purification of phosvitin from egg yolks

Phosvitin was extracted from chicken eggs according to the method by Ko *et al.* (2011). Egg yolk was separated from egg white, and the egg yolk membranes and chalaza were removed by filtering through a testing sieve (1000 µm). The filtered egg yolk was centrifuged at 4,070 g for 30 min after homogenization with two volumes of distilled water. The precipitate was homogenized with four volumes of 85% ethanol, and centrifuged at 4,070 g for 30 min to remove phospholipids. The precipitate was then homogenized with five volumes of 12% NaCl solution and centrifuged at 4,070 g for 30 min to extract the phosvitin. The supernatant was collected, filtered by an ultrafiltration system to remove NaCl (Quixstand Benchtop System using a membrane column with a 10 kDa molecular weight cut-off, GE Healthcare, Waukesha, USA). At

the end of ultrafiltration, the solution without NaCl was centrifuged at 4,070 g for 30 min after the pH was adjusted to 4.0. The supernatant was lyophilized.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

For the identification of extracted phosvitin, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with a 10% polyacrylamide gel. Proteins in the gels were stained using Coomassie Brilliant Blue with 0.1 M aluminum nitrate as previously described (Hegenauer *et al.*, 1977) and destained in a 10% acetic acid solution.

Iron binding capacity of phosvitin

Iron binding capacity of the isolated phosvitin was determined by a modified colorimetric ferrozine method (Riemer *et al.*, 2004). A sample solution was made using the following procedure. A mixture of 1.66 mL distilled water, 20 µL of 1.0 to 5.0 mM FeCl₃ in distilled water, and 20 µL of 1,000 µg/mL phosvitin in distilled water was transferred to conical tubes (15 mL). The mixture was left at room temperature for 1 min. Next, 0.2 mL of 5 mM ferrozine was blended with 0.1 mL of 1% ascorbic acid in distilled water and added to the tubes. After 5 min at room temperature, the final color change was monitored using a spectrophotometer (DU530, Beckman Instruments Inc., USA) at 562 nm using distilled water as a blank. The iron binding capacity was calculated with the following equation:

$$\text{Iron binding capacity (\%)} = [(A_0 - A_1)/A_0] \times 100$$

Where A_0 indicates the absorbance of the sample solution without the phosvitin solution, and A_1 indicates the absorbance of the sample solution with the phosvitin solution.

Preparation of ground beef samples

Beef loin muscles (*Longissimus dorsi*) were purchased from 3 different markets, trimmed, and ground separately through a 3 mm-plate. The ground beef from a market was divided into three treatment groups as follows: 1) the control (ground beef with 1% distilled water (v/w) without phosvitin), 2) GBP 100, ground beef with 1% distilled water containing phosvitin; 100 mg/kg of meat, and 3) GBP 500, ground beef with 1% distilled water containing phosvitin; 500 mg/kg of meat. After adding the phosvitin solution, the ground beef was thoroughly mixed with a

food mixer for 2 min after being mixed by hand for 1 min. Aliquots of ground beef (100 g) were individually placed in petri dishes (8.5 cm×1.5 cm) on each storage day. The samples were aerobically stored at 4°C for 7 d and the 2-thiobarbituric acid reactive substances (TBARS) values and instrumental color values were determined. Tests to measure the pH, cooking loss, texture, and sensory qualities of the ground beef were conducted after 24 h of storage at 4°C.

2-Thiobarbituric acid-reactive substances (TBARS) value

A sample of ground beef (5 g) in 15 mL of distilled water was homogenized (T25b, Ika Werke GmbH & Co. KG, Germany) for 1 min. The sample homogenates (1 mL) were transferred to a test tube, and lipid oxidation was determined as the TBARS value by following the method described by Jung *et al.*, (2010). Briefly, 50 µL of butylated hydroxyanisole (7.2%) and 2 mL of TBA-TCA solution (20 mM 2-thiobarbituric acid in 15% trichloroacetic acid) were added to the test tube. The tubes were then heated (90°C) in a boiling water bath for 30 min, cooled, and centrifuged at 2,090 g for 15 min. Absorbance of the supernatants was measured at 532 nm with a spectrophotometer (DU530, Beckman Instruments Inc., USA). The TBARS value was reported as mg of malondialdehyde/kg of meat.

Color evaluation of the ground beef

Lightness (L^*), redness (a^*), and yellowness (b^*) of the ground beef was measured with a spectrophotometer (CR-300, Minolta Inc., Japan). The instrument was calibrated with a black and white tile before analysis. Measurements were taken perpendicularly to the ground beef surface at two different locations per sample in three samples from each treatment group. A numerical total color difference (ΔE) between ground beef on 0 and 7 d of storage was calculated as follows:

$$\Delta E_{0-7} = [(L_7 - L_0)^2 + (a_7 - a_0)^2 + (b_7 - b_0)^2]^{1/2}$$

Sensory evaluation

A semi-trained panel with eight members evaluated the beef patties which were cooked as the condition measuring cooking loss. The panelist observed the color, odor, tenderness, juiciness, flavor, and overall acceptability of each sample according to a 9-point hedonic scale (1 = extremely dislike, 9 = like extremely). Patties were cooked just before serving and water was distributed for rinsing

the mouth between samples.

Statistical analysis

The beef from a market was treated as a replication and this study was performed in triplicate. Analysis of variance was performed using the raw data, and the mean values and standard errors of the means (SEM) were calculated by the Statistical Analysis System (SAS, 2002). Differences among the means were determined by Duncan's multiple range test with $p < 0.05$ indicating statistical significance.

Results and Discussion

Purification of phosvitin

The concentration of phosvitin in chicken egg yolk has been reported to be up to 12 mg per 1 g of egg yolk (Juneja and Kim, 1997). This protein consists of two polypeptides, α - and β -phosvitin, with different numbers of sub-units (three or four sub-units of 35 to 40 kDa for α -phosvitin; four or five sub-units of 45 kDa for β -phosvitin) (Anton *et al.*, 2007). The electrophoretic patterns of the extracted phosvitin on SDS-PAGE are shown in Fig. 1. Bands of the extracted phosvitin were between 37.8 and 46.2 kDa in size and almost identical to those of the phosvitin standard. This result indicates that phosvitin was successfully purified by a simple extraction method using NaCl.

Fig. 1. Polyacrylamide-gel electrophoretic patterns of the phosvitin standard (1) and the phosvitin extracted from egg yolk (2).

Iron binding capacity of phosvitin

Iron binding capacity of the extracted phosvitin was measured and compared to that of the phosvitin standard (Fig. 2). Both the extracted and standard phosvitin were shown to bind iron although the binding capacity of the extracted phosvitin was lower than that of the phosvitin standard. Albright *et al.* (1984) reported that unlike the phosvitin standard the extracted phosvitin bound almost 95% of irons present in egg. This could also be one of the reasons why the iron binding capacity of the extracted phosvitin was lower than that of the phosvitin standard. Castellani *et al.* (2003) suggested that iron bound to phosvitin can be completely removed by anion exchange chromatography. However, this process is costly and therefore may not be applicable for industrial use. The results of our study show that the iron binding activity of the extracted phosvitin is still effective.

TBARS values of the ground beef

Based on the iron binding capacity of the extracted phosvitin, it can be assumed that this reagent will inhibit lipid oxidation in meat products. Ishikawa *et al.* (2004) reported that phosvitin decreases the generation of hydroxyl radical from Fenton reactions responsible for the catalysis of lipid oxidation. The TBARS values for the control ground beef increased from 0.65 to 1.49 mg of malondialdehyde kg^{-1} meat stored for 7 d at 4°C (Table 1). However, the TBARS values of the GBP 500 stored under the same conditions increased from 0.62 to 1.14. These results indicate that adding 500 mg/kg of phosvitin to ground beef significantly decreased lipid oxidation ($p < 0.05$). However, 100 mg/kg of phosvitin inhibited lipid oxidation for only 3 d of storage.

Fig 2. Iron binding ability of the phosvitin extracted from egg yolk and phosvitin standard. ^{a,b}Points with different letters in the lines for the standard and extracted phosvitin differ significantly in value ($p < 0.05$).

Table 1. 2-Thiobarbituric acid-reactive substances values (mg of malonedialdehyde/kg of meat) in ground beef containing phosvitin during storage at 4°C

Treatment	Storage (d)			SEM ¹⁾
	0	3	7	
Control	0.65 ^b	1.44 ^{ax}	1.49 ^{ax}	0.025
GBP 100 ²⁾	0.63 ^c	1.28 ^{by}	1.54 ^{ax}	0.008
GBP 500 ³⁾	0.62 ^c	1.00 ^{bz}	1.14 ^{ay}	0.016
SEM ¹⁾	0.008	0.024	0.017	

¹⁾Standard error of the means (n = 9)

²⁾Ground beef with phosvitin 100 mg/kg

³⁾Ground beef with phosvitin 500 mg/kg

^{a-c}Figures with different letters within the same row differ significantly ($p < 0.05$).

^{x-z}Figures with different letters within the same column differ significantly ($p < 0.05$).

The antioxidant activity of phosvitin in pork was previously reported by Lee *et al.* (2002). They reported that phosvitin has no effect on the inhibition of lipid oxidation in raw ground pork but is significantly more effective in cooked pork. In contrast, significant inhibition of lipid oxidation by the extracted phosvitin in raw ground beef in this study was probably due to the difference in free iron concentrations between beef and pork. Beef contains higher concentrations of free iron compared to pork (Channon and Trout, 2002). Estévez and Cava (2004) reported that the amount of free iron is increased by cooking and storage, and the highest concentrations are found in meat that is stored after being cooked. Importantly, Castellani *et al.* (2004) found that phosvitin maintained a high iron binding capacity when it was treated by thermal (90°C) or high pressure processing (600 Mpa). For these reasons, the extracted phosvitin from egg yolk could be used as a valuable antioxidative reagent in processed meat products which are subject to heat and storage before consumption.

Instrumental color evaluation

Meat color stability is very important because it is the basis for the initial impression of meat products; consumers do not typically want to purchase discolored meat (Glitsch, 2000). Discoloration more easily develops in ground meat compared to whole muscles (Rojas and Brewer, 2008). Instrumental color values of the ground beef were significantly influenced by adding the extracted phosvitin (Table 2). Lightness (L^* values) of the GBP 100 and GBP 500 were slightly higher than that of the control patties after refrigerated storage for 7 d.

Redness (a^* values) and yellowness (b^* values) were gradually decreased over time regardless of addition of

Table 2. Color changes in the ground beef containing phosvitin during storage at 4°C

Treatment	Storage (d)			SEM ¹⁾
	0	3	7	
	L*			
Control	43.0 ^{ay}	42.8 ^{by}	42.8 ^{bz}	0.02
GBP 100 ²⁾	43.9 ^{ax}	43.4 ^{cx}	43.5 ^{bx}	0.02
GBP 500 ³⁾	43.8 ^{az}	42.8 ^{cy}	43.2 ^{by}	0.02
SEM ¹⁾	0.02	0.01	0.02	
	a*			
Control	24.4 ^{ax}	15.7 ^{by}	12.0 ^{cz}	0.01
GBP 100	23.9 ^{ay}	16.6 ^{bx}	12.8 ^{cy}	0.03
GBP 500	23.9 ^{by}	15.7 ^{by}	14.1 ^{cx}	0.15
SEM ¹⁾	0.01	0.15	0.03	
	b*			
Control	23.2 ^{ax}	18.9 ^{bz}	17.9 ^{cy}	0.03
GBP 100	23.0 ^{ay}	19.7 ^{by}	17.9 ^{cy}	0.03
GBP 500	22.9 ^{ay}	20.0 ^{bx}	18.2 ^{cx}	0.03
SEM ¹⁾	0.03	0.01	0.04	
	Control	GBP 100	GBP 500	
ΔE	13.51 ^a	12.22 ^b	10.96 ^c	0.045

¹⁾Standard error of the means (n = 9)²⁾Ground beef with phosvitin 100 mg/kg³⁾Ground beef with phosvitin 500 mg/kg^{a-c}Figures with different letters within the same row differ significantly ($p < 0.05$).^{x-z}Figures with different letters within the same column differ significantly ($p < 0.05$).

phosvitin. The rates (over 0 to 7 d of storage) of decreased redness in the control, GBP 100, and GBP 500 samples were 51% (24.4 to 12.0), 46% (23.9 to 12.8), and 41% (23.9 to 14.1), respectively. Yellowness (b^* values) was relatively stable in GBP 500 samples when compared to that of the control during storage. Overall color change (ΔE) also demonstrated that the addition of phosvitin protect against discoloration of the beef patties ($p < 0.05$).

These results indicate that the color stability of ground beef can be improved by adding the extracted phosvitin and agree with previous studies reporting that the activity of antioxidants in beef patties and frankfurters improves color stability (Estévez *et al.*, 2005; Sánchez-Escalante *et al.*, 2001). Discoloration of meat products result from the conversion of oxymyoglobin to metmyoglobin by oxidation and is accelerated through lipid oxidation and/or modification of the heme molecular structure during protein oxidation (Estévez *et al.*, 2005; Rojas and Brewer, 2008). The iron binding properties of phosvitin could decrease lipid and protein oxidation caused by free iron (Extévez and Cava, 2004).

Table 3. Sensory analysis of the beef patties with phosvitin

Treatment	Sensory parameter					
	Color	Odor	Tender-ness	Juiciness	Flavor	Accept-ability
Control	5.0	5.1	2.1	2.1	4.6	4.4
GBP 100 ²⁾	4.9	5.4	2.4	2.3	4.9	4.8
GBP 500 ³⁾	5.0	5.6	2.5	2.4	5.1	4.9
SEM ¹⁾	0.17	0.31	0.32	0.33	0.39	0.38

¹⁾Standard error of the means (n = 24).²⁾Ground beef with phosvitin 100 mg/kg³⁾Ground beef with phosvitin 500 mg/kg

pH, cooking loss, texture analysis, and sensory evaluation

After 24 h of storage, the pH, cooking loss, and texture parameters of the control, GBP 100, and GBP 500 samples were analyzed (data not shown), but there were no significant differences. Results of the sensory analysis showed that the ground beef mixed with the extracted phosvitin (GBP 100 and GBP 500) did not show differences in any of the sensory parameters that were tested (Table 3). This result demonstrates that the addition of extracted phosvitin to ground beef may not have any effect on the sensory qualities of the meat. However, Jo *et al.* (2003) reported that the addition of natural antioxidants from plant sources such as green tea leaf extracts can impair the sensory quality of raw and cooked pork.

Conclusion

Phosvitin was prepared using a relatively easy and cost-effective method. The addition of the extracted phosvitin at concentrations of 100 and 500 mg/kg of meat effectively reduced oxidation and improved color stability in ground beef compared to the untreated control. Sensory properties of the ground beef were not adversely affected by addition of phosvitin. Based on the results of this study, it can be concluded that the extracted phosvitin could be used in manufacturing meat products as a highly effective antioxidant. Furthermore, the extracted phosvitin appears to be more amenable for use in meat products because it is a natural protein derived from animal sources.

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