Bactericidal Action of Egg Yolk Phosvitin against *Escherichia coli* under Thermal Stress

M. A. Sattar Khan,† Soichiro Nakamura,‡ Masahiro Ogawa,§ Emmanuel Akita,§ Hiroyuki Azakami,† and Akio Kato*,†

Department of Biological Chemistry, Yamaguchi University, Yamaguchi, Japan 753-8515; Department of Living Science, Shimane University, Shimane, Japan 690-8504; and Department of Food Science, University of British Columbia, Vancouver, BC, Canada V6T 1Z4

Chicken egg yolk phosvitin showed a remarkable antibacterial effect against *Escherichia coli* under thermal stress at 50 °C. *E. coli* cells (10^6/mL) completely disappeared in 1 mL of L-broth coexisting with 0.1 mg/mL phosvitin when incubated at 50 °C for 20 min, whereas a considerable amount of cells (10^5/mL) survived at the same thermal stress without phosvitin. Blocking of the chelating effect of phosvitin by the addition of Ca^{2+} ion displayed a protective effect against the bactericidal activity at 50 °C. In addition, the antibacterial activity of phosvitin was dramatically reduced by treatment with α-chymotrypsin, although the chelating effect remained. The surface properties, such as interfacial tension and emulsifying properties of phosvitin, which are an index of the affinity with the outer membrane, were greatly reduced by the α-chymotrypsin digestion. This indicates that the α-chymotrypsin-digested membrane-penetrating hydrophobic domains at the N- and C-terminal regions play an important role in antibacterial activity. These results suggest that a significant part of the bactericidal activity of phosvitin against *E. coli* resides in the synergistic effect of the high metal-chelating ability and the high surface activity under the influence of thermal stress.

Keywords: *Egg yolk phosvitin; bactericidal effect; Escherichia coli; emulsifying activity; metal chelating effect*

INTRODUCTION

Because bacterial food-poisoning frequently occurs throughout the year and causes serious results, harmless agents with a wide antimicrobial spectrum have been sought in natural compounds for food safety. Most pathogenic bacteria are classified as Gram-negative bacteria whose outer membrane with lipopolysaccharide (LPS) provides the cell with an effective permeability barrier against external noxious agents (Vaara, 1992). LPS, however, seems to be itself a target for antibacterial agents such as surfactant (Ahlstrom et al., 1998; Rana et al., 1991) and chelators (Korbashi, 1986; Vaara and Jaakkola, 1989; Temple et al., 1992). Therefore, amphiphilic proteins with chelating ability should be useful for killing Gram-negative bacteria. Egg yolk phosvitin is a good candidate for this purpose. This polyanionic phosphoglycoprotein can bind multivalent metals, such as Fe, Ca, and Mg (Taborsky, 1983), in which numerous serine residues are phosphorylated and these may be attributed to the chelating behavior (Grogan and Taborsky, 1987). In addition, this highly phosphorylated protein may have a potent affinity for lipids, as egg yolk has been often used as a natural emulsifier (Baldwin, 1986). We have reported that this phosphoprotein shows powerful emulsifying properties (Kato et al., 1987), and the activity can be enhanced by the covalent attachment of polysaccharides (Nakamura et al., 1998). The intensified affinity of phosvitin to lipid can be expected to enhance the antimicrobial potential to Gram-negative bacteria. This work demonstrates that phosvitin damages the outer membrane and kills *Escherichia coli,* a model strain of Gram-negative bacteria, under thermal stress.

* Author to whom correspondence should be addressed (e-mail kato@agr.yamaguchi-u.ac.jp; fax 0839-33-5802; telephone 0839-33-5852).

† Yamaguchi University.

‡ Ube College.

§ University of British Columbia.

MATERIALS AND METHODS

Materials. MacConkey agar, bacto-tryptone, and bacto-yeast extract were obtained from Difco (Detroit, MI). NaCl, $CaCO_3$, dialysis tube (MW 3500), and bovine α-chymotrypsin (52 units/mg) were purchased from Sigma Chemical Co. (St. Louis, MO). *E. coli* K12 used is a type strain, C600. Galactomannan (average MW 15000–25000) was obtained by dialyzing the mannose hydrolysate of guar gum supplied by Taiyo Chemicals Co. (Nagoya, Japan). All reagents used in this study were of chemical reagent grade.

Preparation of Phosvitin, Protease-Digested Phosvitin, and Phosvitin–Galactomannan Conjugate. Phosvitin was prepared from fresh egg yolk according to the method of Mecham and Olcott (1949). Phosvitin was further purified by ion exchange chromatography on a DEAE-Sephadex A-50 column (3.0 × 15 cm) equilibrated with 20 mM Tris-HCI buffer, pH 7.0, and eluted with a linear salt gradient (0–0.6 M NaCl in the equilibrated buffer). The single peak eluted in a salt concentration range of 0.4–0.5 M was collected and lyophilized after dialysis against deionized water.

Freeze-dried phosvitin (180 mg) was suspended in 12 mL of distilled water to a final concentration of 15 mg/mL, and the pH of the solution was adjusted to 8.0 with NaOH prior to the addition of α-chymotrypsin. The enzyme was added to the phosvitin at an enzyme to substrate ratio of 1:100 (w/w)

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(Goulas et al., 1996) and incubated at 37 °C for 24 h. The protease digestion was arrested by heating the digested samples at 100 °C for 3 min. The digested mixture was centrifuged (8000*g*) to precipitate a small amount of insoluble protein, and the supernatant was finally used to further experiment after dialysis against distilled water for 24 h at room temperature.

Phosvitin–galactomannan conjugate was prepared according to the method of Kato et al. (1990). Phosvitin and galactomannan were mixed in water at the weight ratio of 1:3 and then lyophilized. The powder mixture was incubated for a given time under controlled conditions (60 °C and 79% relative humidity) in a desiccator containing KBr-saturated solution in the bottom.

Binding of Calcium with Phosvitin. An equilibrium dialysis system at room temperture was used to study the binding between calcium and phosvitin. The binding of calcium to phosvitin was measured with a slight modification of the method of Tyurin (1980). Five milligrams of phosvitin (0.1%) was dissolved in 5 mL of 5 mM calcium chloride solution; the method was modified slightly by using $CaCl_2$ instead of $CaCO_3$. The pH of solution was adjusted at pH 3.6 and 7.0 with 1 N HCl and 0.1 N NaOH, respectively. The mixture solution was dialyzed against distilled deionized water for 24 h to remove the free calcium. Subsequently, the concentration of bound calcium was analyzed by the atomic absorption spectrophotometer (Shimadzu, model AA-6041-01), using a calibration curve in the range of 0–20 ppm of calcium.

Measurement of Emulsifying Properties. The emulsifying properties were determined according to the method of Pearce and Kinsella (1978). An emulsion was prepared by homogenization of 1.0 mL of corn oil and 3.0 mL of a 0.1% sample solution, using an Ultra Turrax machine (Hansen Co.) at 12000 rpm for 1 min at 20 °C. One hundred microliters of emulsion was taken from the bottom of the test tube after standing for 0, 1, 2, 3, 5, and 10 min and then diluted with 5.0 mL of 0.1% sodium dodecyl sulfate solution. The absorbance of the diluted emulsion was then determined at 500 nm. The relative emulsifying activity was represented as the absorbance at 500 nm measured immediately after emulsion formation (0 min). The emulsion stability was estimated by measuring the half-life of the decay of emulsion, estimated from the turbidity curves of an emulsion that had been standing for 10 min.

Antibacterial Assay. *E. coli* strain was used as test bacterium. This strain was subcultured and finally grown in L-broth (10 g of bactotrypton, 5 g of bactoyeast extract, 5 g of NaCl, 1 g of glucose, and water to 1 L) at 37 °C overnight. The fresh cells were diluted with L-broth and microscopically adjusted to give the concentration of 10^6 cells/mL by using a hematometer. Sample was added to the cell suspension to give a final protein concentration of 0.1 or 0.01%. Five milliliters of the suspension was incubated at 37 °C for 3 h or 50 °C for 40 min in a water bath with gentle rotation at 25 rpm to provide constant temperature and cell suspension. After a given heating time, the heated suspension was immediately put into a sterile tube immersed in an ice bath. After the suspensions were cooled to room temperature, decimal dilution was subsequently carried out in physiological saline solution adjusted to pH 7.2. A 100 μL portion was spread over MacConkey agar (Difco) plates. The number of colonies formed after incubation at 35 °C for 24 h was measured to calculate the survival ratio.

Determination of DNA Leakage. To determine the DNA leakage from the tested bacterial cells, the cell suspensions with and without 0.1 or 0.05% phosvitin were treated at 37 °C for 3 h or at 50 °C for 40 min. The absorbance of the supernatant was measured at 260 nm after centrifugation at 15000*g* for 15 min.

RESULTS

The calcium-binding capacity was estimated for the phosvitin used in the experiments. Table 1 shows the values of calcium-binding capacity of phosvitin, galactomannan–phosvitin conjugate, and α-chymotrypsin digest of phosvitin. The binding of metal ions was not observed in the native form of phosvitin. When the native and modified phosvitins were dissolved in calcium solution, the resultant calcium-binding capacities of native phosvitin, phosvitin–galactomannan conjugate, and α-chymotrypsin-digested phosvitin were suppressed at pH 3.6. On the other hand, the calcium-binding capacities of phosvitins were greatly increased at pH 7.0. This result suggests that the calcium-binding capacity of phosvitin is strengthen at physiological pH, playing a role as a chelating agent. Figure 1 shows the antibacterial effect of phosvitin on *E. coli* strain at physiological pH in the presence of 0.01 and 0.1% phosphoprotein. The log survival ratio of the ordinate gives the survival decimal fraction based on log values. No antimicrobial effect of phosvitin was observed at 37 °C for 3 h (Figure 1a). In contrast, the protein with the same concentrations showed a lethal effect under thermal stress at 50 °C (Figure 1b). When the tested *E. coli* strain was grown in the presence of phosvitin at 50 °C, the living cells were drastically decreased and had completely disappeared in the medium after 20 min, although such strong bactericidal effects were not observed in the absence of phosvitin (control medium).

The amount of DNA leaked out of the bacterial cells, when incubated with phosvitin, was monitored by the

Table 1. Determination of Calcium Binding Capacity of 0.1% Native and Modified Phosvitins

samples	bound calcium (mol/mol of phosvitin)	
	at pH 3.6	at pH 7.0
native phosvitin[a]	0	0
native phosvitin	20	148
phosvitin–galactomannan conjugate	27	151
α-chymotrypsin-digested phosvitin	17	118

[a] Value without adding $CaCl_2$. Bound calcium was determined as shown under Materials and Methods.

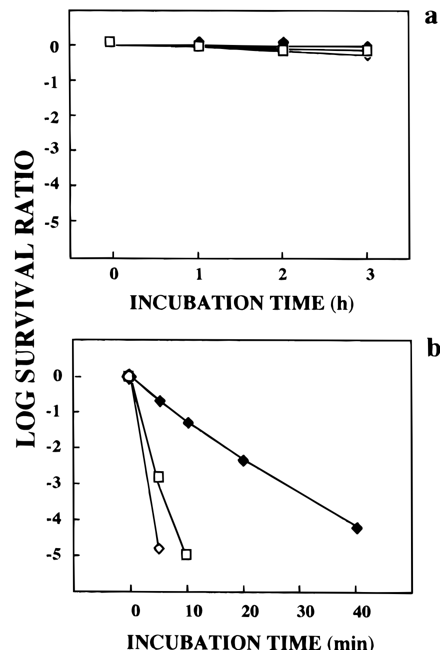

Figure 1. Bactericidal effect of phosvitin against *E. coli* at 37 (a) and 50 °C (b); control (◆); phosvitin 0.01% (□), 0.1% (◇).

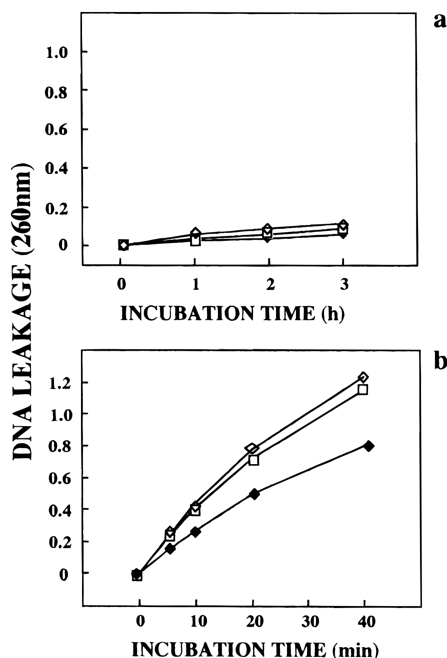

Figure 2. DNA leakage from *E. coli* at 37 (a) and 50 °C (b) in the presence of phosvitin: control (◆); phosvitin 0.05% (□), 0.1% (◇).

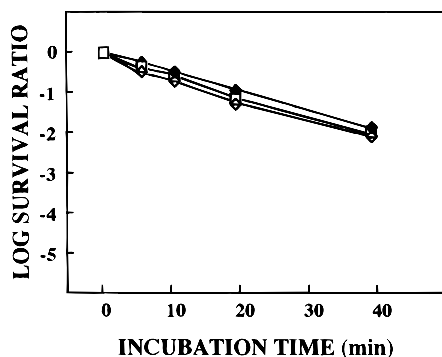

Figure 3. Bactericidal effect of calcium-bound phosvitin against *E. coli* at 50 °C: control (◆); phosvitin 0.01% (□), 0.1% (◇).

measurement of absorbance at 260 nm (Figure 2), because the 260-nm-absorbing materials are regarded as the intracellular materials leaked from damaged cells. The strain incubated at 37 °C in the presence of phosvitin showed no DNA leakage even at the long incubation period of 3 h, whereas the absorbance at 260 nm of the supernatant greatly increased within 10 min of incubation when the bacterial strain was incubated with 0.01 or 0.1% phosvitin at 50 °C (Figure 2b), indicating that a large amount of DNA was promptly leaked out of the cells. This observation suggests that phosvitin causes irreversible damage to the cellular surface of *E. coli.*

To confirm a role of phosvitin as a chelating agent, phosvitin saturated with calcium ion was subjected to the antimicrobial experiment. Figure 3 shows the antimicrobial effect of calcium-bound phosvitin on the *E. coli* strain. No bactericidal activity was detected at all at 50 °C, due to the saturation of phosphoserines with calcium ions. The effect of calcium ion on the inhibition of the antibacterial activity can be interpreted by blocking the chelating effect. Owing to inability of chelating effect, the cations from the cell wall of bacteria could not be removed under the influence of high

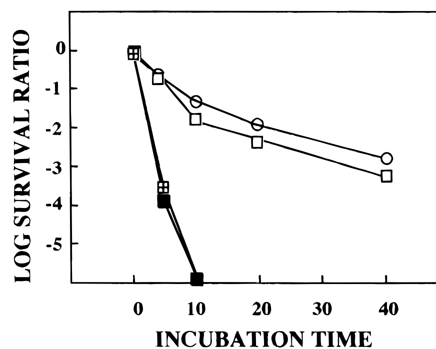

Figure 4. Antibacterial effect of 0.1% of phosvitin, protease digest, and polysaccharide conjugate against *E. coli at* 50 °C: control (○); phosvitin (⊞); phosvitin−galactomannan conjugate (■); α-chymotrypsin-digested phosvitin (□).

Table 2. Surface Properties of Native Phosvitin, Protease Digest, and Phosvitin−Galactomannan Conjugate

		emulsifying properties	
samples	interfacial tension (dyn/cm)	emulsifying activity (OD_{500})	emulsion stability (min)
oil−water	36.1	0	0
native phosvitin	30.8	1.017	>10
α-chymotrypsin-digested phosvitin	33.6	0.161	1.8
phosvitin−galactomannan conjugate	30.5	1.416	>10

temperature (50 °C). This suggests the importance of the chelating activity of phosvitin in bactericidal action. Heat treatment might weaken the compactness of cell membrane and accelerate the removal of metal ions in the outer membrane, thereby resulting in cell death.

The effect of modification of phosvitin was investigated to further elucidate the mechanism of antibacterial action. Figure 4 shows the survival cell numbers at 100 μg/mL of native phosvitin, α-chymotrypsin-digested phosvitin, and phosvitin−galactomannan conjugate at 50 °C against *E. coli.* The bacteria were killed only after 10 min of incubation in native phosvitin and phosvitin−galactomannan conjugate, whereas protease-digested phosvitin showed poor antibacterial effect. The protease treatment of phosvitin results in the truncation of N- and C-terminal domains, which act as a membrane-penetrating region (Khan et al., 1998). The main phosphoryl domain remained to have the binding capacity of the metal ions after protease digestion, as shown in Table 1. This suggests that the truncated N- and C-terminals are responsible for the lethal effect rather than chelating action. The surface properties, such as interfacial tensions and emulsifying properties of native, protease-digested, and polysaccharide conjugated phosvitins are summarized in Table 2. The surface properties of phosvitin were slightly improved by the conjugation with galactomannan, whereas they were dramatically decreased by the digestion with α-chymotrypsin. The potent antibacterial activity of phosvitin or phosvitin−galactomannan conjugate is considered to emerge from the excellent surfactant properties, which make access to the bacterial outer membrane easy, whereas the meagre bactericidal effect of protease-digested phosvitin, which lacks the hydrophobic membrane-penetrating carriers, may be responsible for the poor surfactant properties and loss of the binding capacity to the outer membrane of bacterial cells.

DISCUSSION

Phosvitin revealed a substantial antimicrobial activity against *E. coli*, owing to the synergistic effect of chelation and surfactant activities, under thermal stress at 50 °C. The antibacterial activity of phosvitin as a chelating agent was found to be induced by removal of cations, such as Mg^{2+} and Ca^{2+}, from charged polar heads of the lipid bilayer of outer membrane. It is well-known that polyanionic phosvitin can bind multivalent metals, such as Fe, Ca, and Mg (Taborsky, 1983), because numerous phosphorylated serine residues of phosvitin contribute to the chelating effect (Grogan and Taborsky, 1987). Therefore, phosvitin is extremely hydrophilic, carries a very high net negative charge, and has an unusually low proportion of hydrophobic side chains. Recent genetic data demonstrate that phosvitin consists of 124 phosphoserines, 4 phosphothreonines, and 14 dicarboxylic amino acid residues. Therefore, negative charges in phosvitin are in ionized form at neutral pH, whereas at acidic pH they are only partially ionized. Thus, the calcium-binding capacities of native phosvitin, phosvitin−galactomannan conjugate, and α-chymotrypsin-digested phosvitin were 20, 27, and 17 mol/mol of phosvitin at pH 3.6 and 148, 151, and 118 mol/mol of phosvitin at pH 7, respectively. This suggests that chelating action is strong at pH 7.0.

Calcium helps to stabilize the bacterial cell and provides heat stability, whereas magnesium functions to stabilize cell membranes, nucleic acids, and ribosomes and is also required for the activity of many enzymes. Iron plays a major role in cellular respiration, being a key component of the cytochromes and iron sulfur proteins involved in electron transport (Michael et al., 1997). Heat treatment at 50 °C might weaken the matrix of cell membrane initially to some extent by destabilizing the metal ions present in the outer membrane. The metal ions may be removed by the chelating agent, which bind the loosely embedded ions, and then the injured cells might be destroyed. It is well-known that the antibacterial activity by chemical chelating reagent EDTA was caused by the elimination of cations, such as Mg^{2+}, from the actively charged polar heads of the lipid bilayer of the outer membrane and hence the increase of the electrostatic potential between positively charged groups and the negatively charged exposed heads of the cell membrane (Ibrahim et al., 1991).

In addition to the chelating effect of phosvitin, we found that the surfactant property was important for antibacterial activity. Protease digestion of phosvitin resulted in a remarkable reduction of antibacterial activity. We have reported in a previous paper (Khan et al., 1998) that small fragments, N- and C-terminal peptide portions, were truncated from phosvitin by protease treatment and due to this reason the emulsifying properties (affinity with oil) greatly decreased. On the basis of this observation, the roles of truncated peptides must be to act as anchors to the outer membrane of bacteria. When the phosvitin accesses the Gram-negative bacterial cell envelope, the negatively charged phosvitin molecules may come in contact with or approach the positively charged metal ions present in the outer membrane. In addition to the electrostatic interaction, the truncated peptides play an important role in coming closer to the outer memebrane of cells.

In conclusion, phosvitin and phosvitin−galactomannan conjugate can effectively inhibit the growth of pathogenic Gram-negative bacteria, and this approach in food systems would provide a novel class of safe antibacterial agent.

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