Molecular Mechanism of the Excellent Emulsifying Properties of Phosvitin−Galactomannan Conjugate

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The emulsifying properties of native and N- and C-terminal-deleted phosvitin (protease digests) were compared after conjugation with galactomannan. The emulsifying properties of Maillard-type phosvitin−galactomannan conjugates were greatly improved, whereas those of the protease-digested phosvitin−galactomannan conjugates were not so dramatically improved. Phosvitin was highly glycosylated with galactomannan, whereas the protease-digested phosvitin conjugate consisting of a highly phosphorylated core peptide fragment was not. The results suggest that both N and C termini of the peptide moiety, digested by protease, were essential for the improvement of emulsifying properties of phosvitin−galactomannan conjugates. In addition, the role of N and C termini as anchors in oil droplets was supported from the comparative studies of native phosvitin, phosvitin−galactomannan conjugates, and protease-digested phosvitin−galactomannan conjugates.

Keywords: *Chymotrypsin; emulsifying properties; galactomannan; pepsin; phosvitin; trypsin*

INTRODUCTION

Egg yolks are often used as food ingredients because of their excellent emulsifying properties. Although the egg yolk proteins responsible for emulsifying properties are not well-known, phosvitin was reported to be one of the most important proteins in the emulsifying properties of egg yolk (Kato, 1987). This egg yolk phosvitin is a highly phosphorylated serine-rich protein containing about 10% phosphorus and 6.5% carbohydrate (Taborsky et al., 1967). The genomic analysis elucidated that the 217 residues of phosvitin contain a core region of 99 amino acids consisting of 80 serine residues grouped in runs interspersed by arginines, lysines, and asparagines (van het Schip et al., 1987). We proposed a mechanism of the excellent emulsifying properties on the basis of the data of proteolytic digestion of phosvitin in which the hydrophobic protein moiety of both N- and C-terminal regions played roles as anchors in oil droplets and abundant phosphorylated core peptide was essential for the emulsion stability to suppress the coalescence of the oil droplet (Khan et al., 1998). Following the loss of N- and C-terminal segments and some trimming at the two ends, the highly phosphorylated core region of the protein remained largely intact upon digestion with various proteolytic enzymes (Goulas et al., 1996). The individual phosvitin molecule is anchored to the surface of the oil droplet by a short sequence of segments at the N and C termini (containing just a few nonpolar amino acid residues), with the rest of the phosphorylated core oriented into the aqueous phase. It has been reported that phosvitin was highly glycosylated with galactomannan through the Maillard reaction, and the conjugates showed better emulsifying properties than untreated phosvitin (Nakamura et al., 1998). That finding enables us to further elucidate the molecular mechanism of high emulsifying properties of phosvitin. Both protein and polysaccharide have a role in the stabilization of oil-in-water emulsions. Proteins adsorb at the oil−water interface during emulsification to form a coherent viscoelastic layer. On the other hand, polysaccharides stabilize colloid through their thickening and gelation behavior in the aqueous phase. Therefore, protein−polysaccharide conjugates are expected to exhibit good emulsifying properties (Kato et al., 1990). This paper describes the molecular mechanisms of the excellent emulsifying properties of the phosvitin−galactomannan conjugates and discusses the importance of the N and C termini of the protein moiety as anchors.

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MATERIALS AND METHODS

Materials. Bovine α-chymotrypsin (52 units/mg), pepsin (2345 units/mg), and trypsin (12600 units/mg) were purchased from Sigma Chemical Co., St. Louis, MO. Galactomannan (average MW 15000−25000) was obtained by dialyzing the mannose hydrolysate of guar gum supplied by Taiyo Chemicals Co., Japan. Unless otherwise stated, all reagents used in this study were of reagent grade.

Preparation of Phosvitin. 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-HCl buffer, pH 7, and eluted with a linear salt gradient (0−0.6 M NaCl in the equilibrated buffer). The single peak eluted in a salt concentration of 0.4−0.5 M was collected and lyophilized after dialysis against deionized water.

Preparation of Protease-Digested Phosvitin. 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 either trypsin or chymotrypsin or to pH 2.5 with HCl prior to the addition of pepsin. Three enzymes were added to the phosvitin at the ratio of enzyme to substratre of 1:100 (w/w) (Goulas et al., 1996). Incubating temperatures were maintained at 30 °C for pepsin and trypsin and at 37 °C for α-chymotrypsin digestion. Reactions were allowed to proceed for 24 h. The protease digestions were arrested by heating the digested samples at 100 °C for 3 min. The digested mixture

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was centrifuged (8000*g*) to precipitate a small amount of undigested protein. The supernatant was dialyzed against distilled water for 24 h at room temperature.

Separation of Protease-Digested Phosvitin. A gel filtration of protease-digested phosvitin was done on a column of Sephadex G-75. The fast and slow eluting peaks were separated. The former was the extensively phosphorylated core region fragment, and the latter was the N-terminal fragment.

Preparation of Phosvitin–Galactomannan Conjugate. 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.

SDS–Polyacrylamide Gel Electrophoresis (SDS–PAGE). SDS–PAGE of phosvitin–galactomannan conjugates was done using the method of Laemmli (1970) with 15% acrylamide separating gel and 5% acrylamide stacking gel containing 0.1% SDS. Samples (20 μL, 0.2%) were prepared in Tris–glycine buffer at pH 8.8 containing 1% SDS. Electrophoresis was done at a constant current of 10 mA for 4 h in electrophoretic Tris–glycine buffer containing 0.5% SDS. Bromophenol blue was used to monitor the progress of protein separation. After electrophoresis, the gel sheets were stained for protein staining with 0.05% Coomassie blue in a solution of 0.1 M aluminum nitrate/25% isopropanol/10% acetic acid/ 1.0% Triton X-100, prepared according to the method of Hegenauer et al. (1977). Destaining was carried out in 7% acetic acid solution. For the carbohydrate staining the gel sheets were stained with Fuchsin solution (periodate) and then destained in 5% acetic acid.

Measurement of Emulsifying Properties. The emulsifying properties of sample solutions were determined according to the method of Pearce and Kinsella (1978). To prepare emulsions, 1.0 mL of corn oil and 3.0 mL of protein solution (0.2%) in 0.1 M phosphate buffer, pH 7.0, were shaken together and homogenized in an Ultra Turrax homogenizer (Hansen & Co., Germany) at 12000 rpm for 1 min at 20 °C. A 50 μL sample of emulsion was taken from the bottom of the container at different time intervals (0, 1, 2, 3, 5, and 10 min) and diluted with 5 mL of 0.1% SDS solution. The turbidity of the diluted emulsion was then determined at absorbance of 500 nm. The relative emulsifying activity was determined from the absorbance measured immediately after the emulsion formation. The emulsion stability was expressed as the half-time of the initial turbidity of the emulsion.

Determination of Free Amino Groups. The content of free amino groups in the phosvitin–galactomannan conjugate was determined according to the method of Haynes et al. (1967) using a trinitrobenzenesulfonate reagent for amino groups.

RESULTS AND DISCUSSION

The formation of the phosvitin–galactomannan conjugate was confirmed by the SDS–PAGE and the measurement of the amino groups in phosvitin during the Maillard reaction in dry heating for 3 and 5 days. Figure 1 shows the SDS–PAGE pattern of phosvitin and its conjugate with galactomannan. Covalent attachment during incubation for 3 and 5 days was confirmed from the electrophoretic profiles in which the conjugates exhibited a broad high molecular weight band for both protein and carbohydrate stains near the boundary between the stacking and separating gels, whereas it was not observed in the protein–polysaccharide mixture (0 day). Figure 2 shows the measurement of free amino groups in the conjugate to obtain further information on the binding mode. About half of the amino groups per molecule were masked during the progress of the conjugation. This suggests that phosvitin is linked to galactomannan through the carbonyl group of the reducing end in the polysaccharide with the ϵ-amino group in the protein. Figure 3 shows the emulsifying properties of the native phosvitin, phosvitin–galactomannan mixture (0 day), and phosvitin–galactomannan

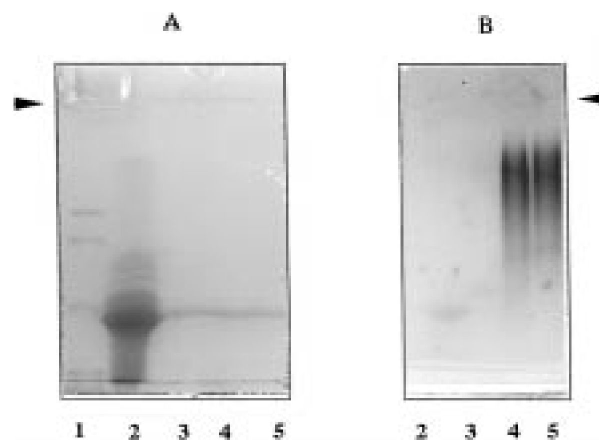

Figure 1. SDS–PAGE patterns of native phosvitin and phosvitin–galactomannan conjugates: (lane 1) molecular marker; (lane 2) native phosvitin; (lane 3) phosvitin–galactomannan mixture (0 day); (lane 4) phosvitin–galactomannan conjugates (PGC) incubated for 3 days; (lane 5) PGC for 5 days; (A) protein stain; (B) carbohydrate stain. Arrows indicate the boundary between separating and stacking gels.

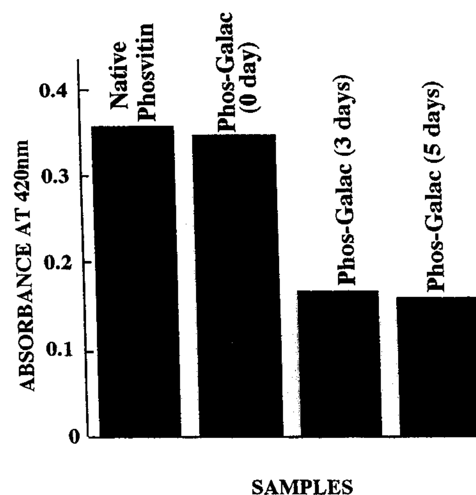

Figure 2. Quantitative analysis of free amino groups in phosvitin–galactomannan conjugates.

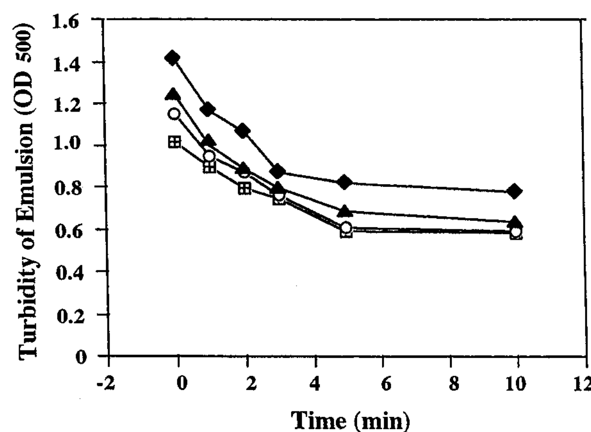

Figure 3. Emulsifying properties of native phosvitin (⊞), phosvitin–galactomannan mixture (0 day) (○), and phosvitin–galactomannan conjugates incubated for 3 days (▲) and 5 days (◆).

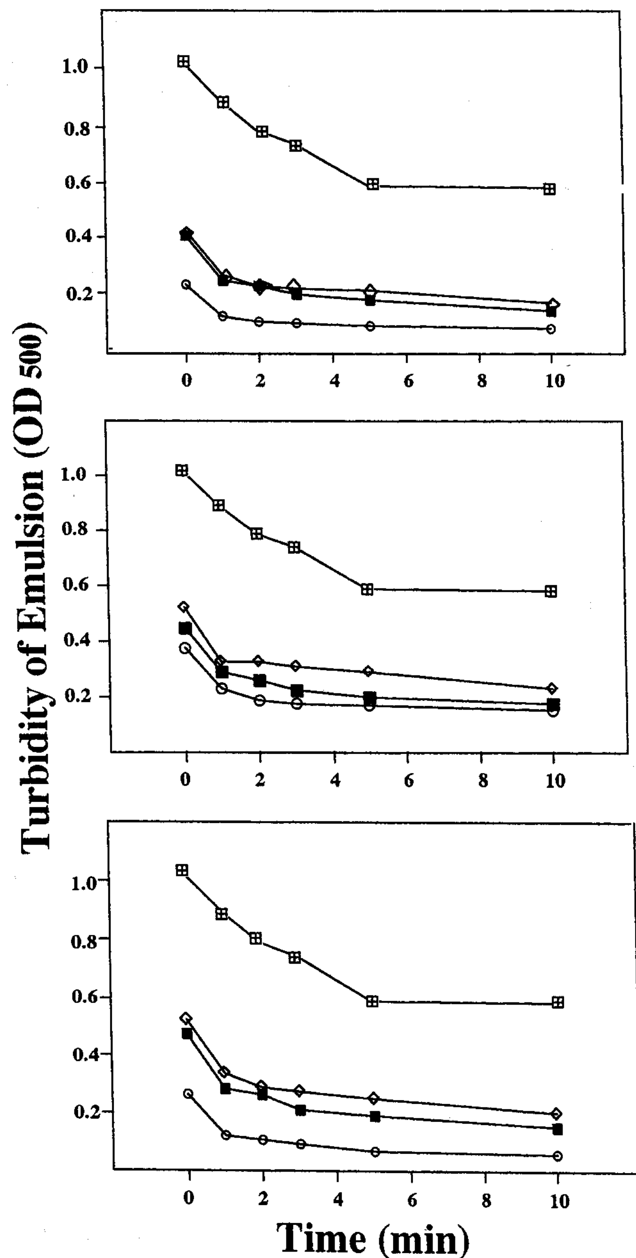

Figure 4. (Top) Emulsifying properties of native phosvitin (⊞), α-chymotrypsin-digested phosvitin–galactomannan conjugation (CDPGC) mixture (0 day) as control (○), CDPGC for 3 days (■), and CDPGC for 5 days (◇). (Middle) Pepsin-digested phosvitin–galactomannan conjugation (PDPGC) mixture (0 day) as control (○), PDPGC for 3 days (■), and PDPGC for 5 days (◇). (Bottom) Trypsin-digested phosvitin–galactomannan conjugation (TDPGC) mixture (0 day) as control (○), TDPGC for 3 days (■), and TDPGC for 5 days (◇).

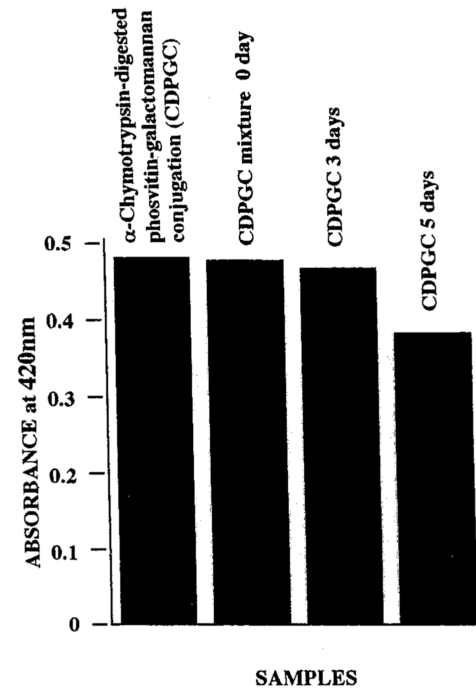

Figure 5. Quantitative analysis of free amino groups of α-chymotrypsin-digested phosvitin–galactomannan conjugates.

conjugates incubated for 3 and 5 days. The emulsifying properties of the phosvitin–galactomannan conjugates were improved significantly, compared to the native phosvitin. Both protein and polysaccharide have a role in the stabilization of oil-in-water emulsions. Proteins adsorb at the oil–water interface during emulsification to form a coherent viscoelastic layer. On the other hand, polysaccharides confer colloid stability through their thickening and gelation behavior in the aqueous phase. Therefore, protein–polysaccharide conjugates are expected to exhibit good emulsifying properties (Kato et al., 1990). As shown in Figure 3, the mixture of protein–polysaccharide showed an effect equal to that of the native phosvitin, whereas protein–polysaccharide conjugates indicated much higher emulsifying properties than the mixture. This shows a synergistic effect of the conjugation. That is, the covalent attachment of protein with polysaccharide enhances synergistically their emulsiying properties by strengthening the adsorption to the oil–water interface and by stabilizing the oil droplets surrounded by the viscoelastic layer of polysaccharide in the aqueous phase. The effect of protease digestion of the phosvitin in the phosvitin–galactomannan conjugation was investigated to get further information on the molecular mechanism of the high emulsifying properties. As shown in Figure 4 the emulsifying properties of protease-digested phosvitin–galactomannan conjugates were found to decrease drastically as compared to the untreated phosvitin. The emulsifying properties of the protease-digested phosvitin–galactomannan conjugates were not so improved as those of the untreated phosvitin. It has been recently reported by Goulas et al. (1996) that chymotryptic, peptic, and tryptic digestion of phosvitin produced three identifiable fragments: N and C termini and the extensively phosphorylated core region. We also identified and separated the extensively phosphorylated core region (50–210 fragment) and the N-terminal region (1–49 fragment) from protease digests of phosvitin in a previous study (Khan et al., 1998). The protease-digested phosvitin used in the experiment is composed of the extensively phosphorylated core region (50–210 fragment). This protease-digested phosvitin did not improve its emulsifying properties after conjugation with galactomannan. Therefore, the N- and C-terminal regions are essential for the high emulsifying properties of phosvitin or phosvitin–galactomannan conjugate. Figure 5 shows the changes of free amino groups in chymotrypsin-digested phosvitin–galactomannan conjugate. On the basis of calculations obtained from Figure 5, only a small number of lysyl residues were linked to the galactomannan, suggesting the difficulty in the progress

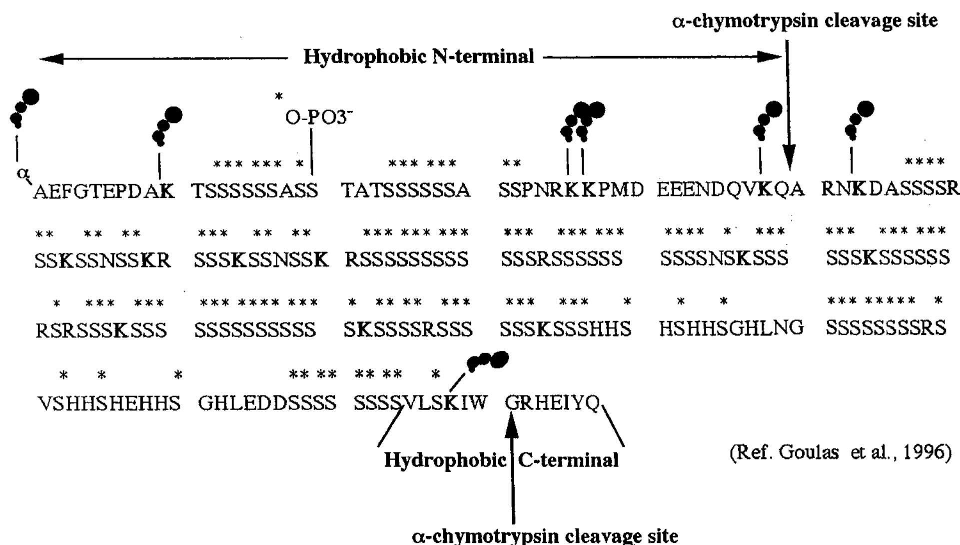

Figure 6. Amino acid sequence of phosvitin showing the α-chymotrypsin cleavage site (arrows). The active lysine residues (**K**), mostly in the N-terminal region, link to polysaccharide molecule (•●●). α denotes the additional amino group at the N terminus.

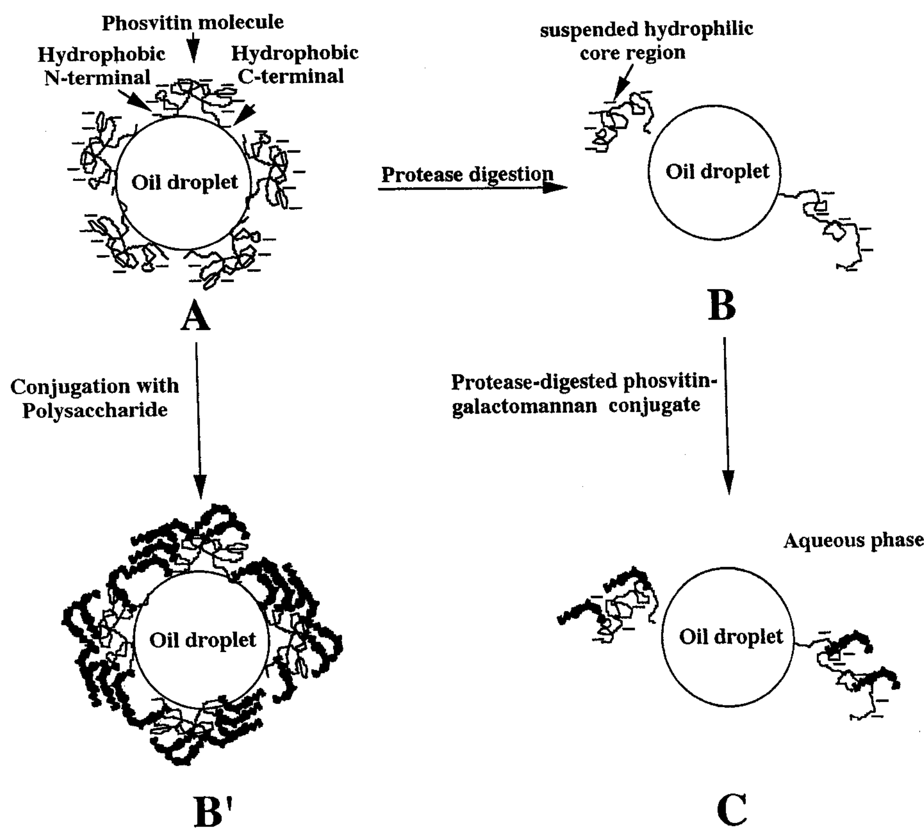

Figure 7. Schematic diagram of interaction between phosvitin and the oil droplet for the elucidation of molecular mechanisms involved in the excellent emulsifying properties of native phosvitin, phosvitin–galactomannan conjugate, and protease-digested phosvitin–galactomannan conjugate: (A) native phosvitin; (B′) phosvitin–galactomannan conjugate; (B) protease-digested phosvitin; (C) protease-digested phosvitin–galactomannan conjugate. — indicates negative charges.

of the Maillard reaction of lysyl residues in the phosphorylated core region. This indicates that most of the reactive lysine residues existed in the N terminus, and a few active residues were found in the extensively phosphorylated core region, because the lysine residues in the hydrophilic core region were arrested by a group of phosphoserines, which hinder the cross-linkage between phosvitin and galactomannan. The amino acid sequence of phosvitin and the possible binding site of galactomannan are shown in Figure 6. Four among 15 ϵ-amino groups are located in the N-terminal region, with the α-amino group at the amino terminus. The N-terminal region seems to be sensitive to the Maillard reaction between the ϵ-amino group in phosvitin and the reducing end of the carbonyl group in galactomannan. The remaining 10 ϵ-amino groups are located in the phosphorylated core regions. The amino acid sequence clearly indicates that only the C terminus, a stretch of ~13 amino acid residues, has the possibility of attaching itself to the oil–water interface. Goulas et

al. (1996) reported the emergence of different lengths of N- and C-termini of phosvitin which contain hydrophobic residues. It is probable that the N- and C-terminal hydrophobic segments adsorb to the surface of the oil droplets as anchors and the extensively phosphorylated core region covers the oil surface to inhibit the coalescence of oil droplets. Thus, the proteolytic digests without N- and C-terminal segments may result in the dramatic decline of emulsifying properties of phosvitin (Khan et al., 1998). Figure 7 shows the possible molecular mechanism of the adsorption of the native phosvitin, phosvitin–galactomannan conjugates, protease-digested phosvitin, and protease-digested phosvitin–galactomannan conjugates in the emulsion system. Phosvitin consisting of a complete protein moiety is adsorbed to the oil droplet with the help of both hydrophobic N and C termini, which are considered to be responsible for the high emulsifying properties (Figure 7A). When phosvitin is conjugated with galactomannan, the polysaccharide masks the protein moiety to enhance the coherent viscoelastic layer in the interface of oil droplets, resulting in the increase in emulsifying properties (Figure 7B′).

The protease-digested phosvitin lacks the essential hydrophobic N- and C-terminal segments responsible for the dramatic decrease in emulsifying properties (Figure 7B). Although it was conjugated with galactomannan, the emulsifying properties were not improved because of the lack of N and C termini (Figure 7C). From these results, it is confirmed that the N-terminal segment together with the C terminus, comprising about half of the hydrophobic amino acid residues, plays a role as anchor by interacting with the oil droplet, leaving the core intact fragment hanging into the aqueous phase.

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