〔食物栄養学〕
〔原著論文〕

ホスビチン及びデキストラン硫酸を用いたタンパク質の不溶化抑制メカニズムの解明

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Prevention mechanisms of heat insolubilization of proteins using phosvitin and dextran sulfate

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In industrial settings, proteins' heat insolubilization and deactivation are major challenges. Phosvitin (PV) and dextran sulfate (DS) suppress protein heat insolubilization. We explored the PV and DS action mechanism in insolubilization inhibition activity on ovalbumin, ovotransferrin, β-lactoglobulin, and thaumatin. Effects of pH on insolubilization suppression were confirmed at pH > 5. PV and DS suppressed protein insolubilization upon heating to 90°C. The molecular morphology of tested proteins varied following heat treatment. PV and DS influenced protein conformation through electrostatic interactions. Suppression of insolubilization was confirmed at low concentrations of these compounds. Insolubilization suppression capacity decreased upon NaCl addition. These results suggest insolubilization of acidic proteins can be suppressed via prevention of formation of large insoluble aggregates, whereas that of near-neutral or basic proteins can be suppressed via prevention of transition from the denatured to oligomeric state. These mechanisms may have important industrial applications in prevention of protein aggregation and insolubilization.

Keywords: Insolubilization, Phosvitin, Dextran sulfate, Electrostatic interaction, Egg white protein

キーワード：不溶化, ホスビチン, デキストラン硫酸, 静電気相互作用, 卵白タンパク質
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Introduction

Proteins have extensive application in the food industry as well as other fields ranging from industry to biological research and medicine. However, factors such as heat, pH, and ionic strength negatively influence protein properties, resulting in deactivation, denaturation, aggregation, and insolubilization. Aggregation is a key challenge that is often encountered in industrial and medical settings during protein and antibody purification. In addition, heat insolubilization and the deactivation of proteins are major challenges in industrial settings.

Various chemical compounds have been developed or applied in efforts to prevent the heat insolubilization of useful proteins [1–10]. Presently, low-molecular-weight compounds are extensively applied. The amino acid arginine is used extensively to inhibit protein insolubilization [1–5]. However,

concentrations on the order of 10 to 100 mM are required to prevent heat insolubilization. High-molecular-weight compounds, such as the milk protein casein and polyethylene glycol, reportedly have the capacity to inhibit heat insolubilization [6–8]. However, there are few studies on the inhibition of insolubilization by high-molecular-weight compounds compared with those on low-molecular-weight compounds. Previous reports have shown that phosvitin (PV), found in egg yolk, and dextran sulfate (DS), which is a polysaccharide sulfate, have the capacity to suppress heat insolubilization of proteins [7,10,11].

PV is a major phosphoprotein with a molecular weight of 42 KDa and no secondary or tertiary structure. More than 55% of proteins have serine residues; most of which are phosphorylated [12–14]. DS is a substance in which a sulfate group is bound to dextran (DX). DS is currently used as a raw material in various medicines and as an additive. A few studies have explored the suppression of heat insolubilization by PV and DS [7,10,11,15]. PV was found to suppress the heat insolubilization of egg white protein and ovotransferrin (OT) via electrostatic interactions [10]. It has been shown that the addition of DS suppresses aggregation of egg white protein via disulfide cross-linking during heat insolubilization [7,15].

Moreover, it has been reported that DS does not bind to native bovine serum albumin (BSA), but it does bind to heat-induced intermediate BSA through electrostatic interaction [11]. Since egg white protein is a complex mixture of proteins, these studies cannot differentiate which proteins PV and DS can protect from heat insolubilization. However, heat insolubilization was suppressed by PV and DS in experiments using OT and BSA. Overall, it remains unknown what proteins types that DS and PV can protect from heat insolubilization. Thus, the present study aimed to clarify the mechanism of heat insolubilization suppression by PV and DS and whether that differs depending on the type of protein. We also investigated whether aggregation is suppressed via the prevention of protein oligomerization. The novel aspects of this study were, 1. identification of protein types that PV and DS can effectively protect, 2. determination of whether the mechanism of heat insolubilization suppression differs depending on the type of protein (molecular weight and isoelectric point), and 3. determination of whether aggregation can be suppressed by preventing oligomerization. We investigated five proteins: ovalbumin (OVA), OT, β-lactoglobulin (β-Lg), thaumatin (TAU), and lysozyme (Lyz), which are found in egg white, milk, and fruits seeds. The above proteins were selected for the present study because of their varying molecular weights and isoelectric points. OVA and β-Lg are acidic proteins, OT is a neutral protein, and TAU and Lyz are basic proteins. Elucidating mechanisms of heat insolubilization suppression by PV and DS will facilitate their industrial application.

Materials and methods

Materials

OT, Lyz, β-Lg, and DS (MW: 500,000 Da) and DX (MW: 6,000 Da) were purchased from Sigma-Aldrich Japan (Tokyo, Japan). TAU was purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). OVA was crystallized with sodium sulfate from fresh egg white and recrystallized five times. Its purity was confirmed using SDS-PAGE. PV was prepared from fresh egg yolk according to the method of Mecham and Olcott [13]. Further purification was performed using ion exchange chromatography on a DEAE-Sephadex A-50 column equilibrated with 20 mM Tris-HCl buffer, pH 7.0, and eluted with a linear NaCl gradient of 0–0.6 M.10 The single peak eluted in a salt concentration of 0.4–0.5 M was collected and lyophilized after dialysis against distilled water [10]. All the other chemicals used in the current experiments were of analytical grade.

Measurement of turbidity

Protein solutions were dissolved in each of several buffers: McIlvaine buffer (0.02M sodium dihydrogen phosphate/0.01M citric acid) at pH 3.0 or 5.0, 10 mM sodium phosphate buffer at pH 7.0, or 10 mM Tris-HCl buffer at pH 9.0. Solutions were heated at 80°C for 10 min in a water bath. After heat treatment, protein solutions were cooled to room temperature. Turbidity was measured using a spectrophotometer (O.D.500).

Size-exclusion high-performance liquid chromatography

Gel filtration high-performance liquid chromatography (HPLC) was performed using a TSK Gel G3000SW column (0.75 × 30 cm, Tosoh, Tokyo). The column was equilibrated using 0.1M sodium phosphate buffer (pH 7.0) and eluted with the same buffer. A 20 µl protein solution was loaded onto the column at a flow rate of 0.5 ml/min. The chromatogram was

obtained by monitoring the effluent at 280 nm. The sample was centrifuged at 5,595 g for 10 min and then filtered.

Gel electrophoresis

SDS-PAGE was performed under non-reducing conditions on 15% poryacrylamide gels according to the standard method described by Laemmli [16]. Neutral and basic Native-PAGE were performed using 12% polyacrylamide gels according to the methods described by Davis17 and Reisfeld18, respectively. Protein bands were stained with Coomassie Brilliant Blue (CBB) R-250. Samples were centrifuged at 5,595 g for 10 min and then filtered.

Result and discussion

Effects of PV and DS on heat insolubilization of proteins

The suppressive effects of PV or DS on the insolubilization of each protein after heating were examined by measurement of the turbidity of single protein solutions or those treated with PV or DS. Table 1 shows the characteristics of each protein.

Table 1. Properties of the proteins in this study

Name	Molecular weight	Isoelectric point	Location
Ovalbumin (OVA)	45 KDa	4.5	Egg white
β-Lactogloblin (β-LG)	18.3 KDa	5.2	Milk
Ovotransferrin (OT)	77 KDa	6.2	Egg white
Lysozyme (LYZ)	14 KDa	11.1	Egg white
Thaumatin (TAU)	22 KDa	12	Fruit

Protein solutions (0.5 mg/ml) were treated with or without PV or DS (0.5 mg/ml) in various buffers with pH values ranging from 3.0 to 9.0. Mixtures were incubated at 80°C for 10 min, and absorbance was measured at 500 nm. After heating, OVA and β-Lg were insolubilized at pH 5.0 (Fig. 1 A and B), OT and TAU were insolubilized at pH 5.0–9.0 (Fig. 1C and D), and Lyz was insolubilized at pH 7.0 (Fig. 1E).

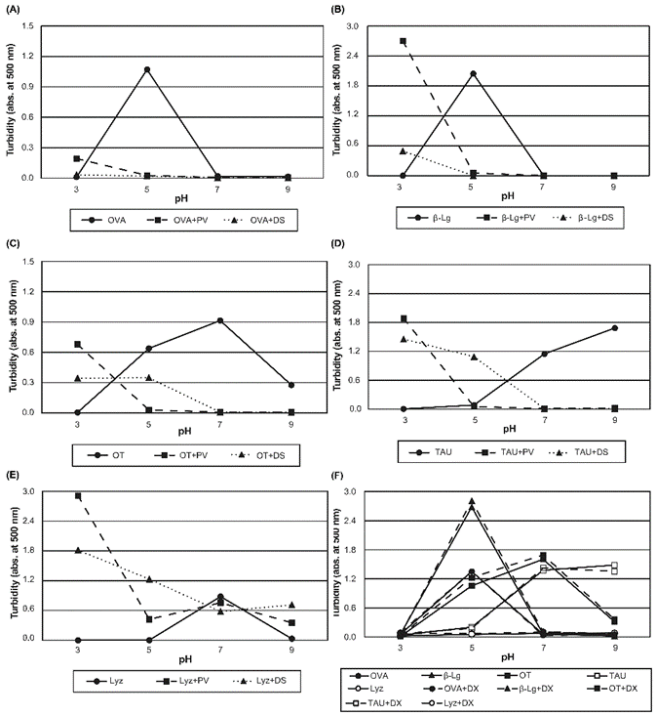

Figure 1. Effect of pH on the capacity PV or DS to inhibit heat insolubilization of proteins.

The turbidity of protein solutions (0.5 mg/ml) with or without PV, DS, and DX (0.5 mg/ml) at pH 3–9 was measured after heating at 80°C for 10 min. (A) OVA, (B) β-Lg , (C) OT, (D) TAU, (E) Lyz , and (F) proteins with or without DX.

Insolubilization of OVA and β-Lg was suppressed at pH > 5.0, and that of OT and TAU was suppressed at pH > 7.0. However, PV or DS addition enhanced insolubilization at all pH ranges following heating of Lyz.

Since the phosphate groups of PV and sulfate groups of DS are thought to be involved in the suppression of insolubilization, experiments using DX were performed. Inhibition of the insolubilization was not observed with DX (Fig. 1F). These results suggest that the sulfate group in DS was involved in insolubilization inhibition. It is conceivable that phosphate groups in PV with similar negative charges are also involved in aggregation suppression.

Following the addition of PV and DS, OVA and β-Lg were insolubilized at pH 3.0, whereas OT and TAU were insolubilized at pH 3.0–5.0. Protein charge becomes positive at pH values lower than the isoelectric point, which potentially causes insoluble aggregate because of strong electrostatic

interaction between the protein and PV or DS. Insoluble aggregates of Lyz were formed as soon as PV and DS were added at all pH values. This result is consistent with findings of a previous study [19].

PV and DS inhibit heat insolubilization of four types of protein as demonstrated in Figure 1. It has been reported that sulfated polysaccharide can stabilize proteins during heating in experiments using β-Lg [15]. DS increased the denaturation transition point of β-Lg by 5°C and contributed to heat stabilization. Therefore, we further investigated the temperature of insolubilization of the four proteins used in this study. Heating of OVA and β-Lg was performed at 65°C–90°C for 10 min at a pH of 5.0 and at a pH of 7.0 for OT and TAU. At a pH near the isoelectric point, maximum insolubilization was observed for each protein.

As shown in Figure 2, heat insolubilization occurred below 65°C in single protein solutions of OVA, OT, and TAU. Heat insolubilization of β-Lg occurred at 70°C. Conversely, OVA, OT, and TAU treated with DS or PV were not insolubilized, even following heating at 90°C for 10 min. However, treated β-Lg solutions showed some insolubilization at >85°C.

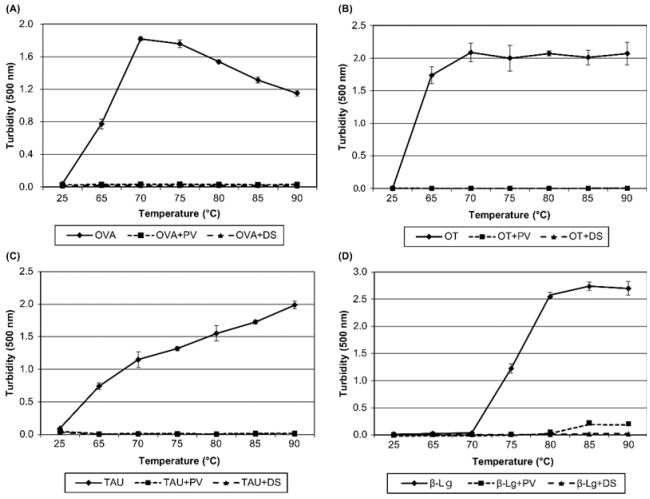

Figure 2. Effects of PV and DS on protein heat insolubilization temperature.

The turbidity of protein solutions (0.5 mg/ml) with or without PV and DS (0.5 mg/ml) was measured after heating at 65°C–90°C for 10 min. OVA (A) and β-Lg (D) experiments were performed at pH 5.0, and OT (B) and TAU (C) experiments were performed at pH 7.0.

The results suggested that PV and DS did not suppress insolubilization simply by stabilizing proteins. The tertiary structure of OT was altered during heating (data not shown). Although tertiary structures deteriorate upon heating, aggregate formation could be suppressed by PV and DS addition.

Subsequently, we investigated the minimum concentrations of PV and DS that could suppress heat insolubilization of each protein. Each protein was tested at a concentration of 1 μM. PV was tested at a molar ratio range of 0 to 1, and DS was tested at that from 0 to 0.1. The molecular weight of PV was close to that of the selected protein, whereas the molecular weight of DS was ~10 times that of the selected protein. Insolubilization inhibition by PV was observed with proteins at a protein:PV ratio of 1:0.5. (Fig. 3A).

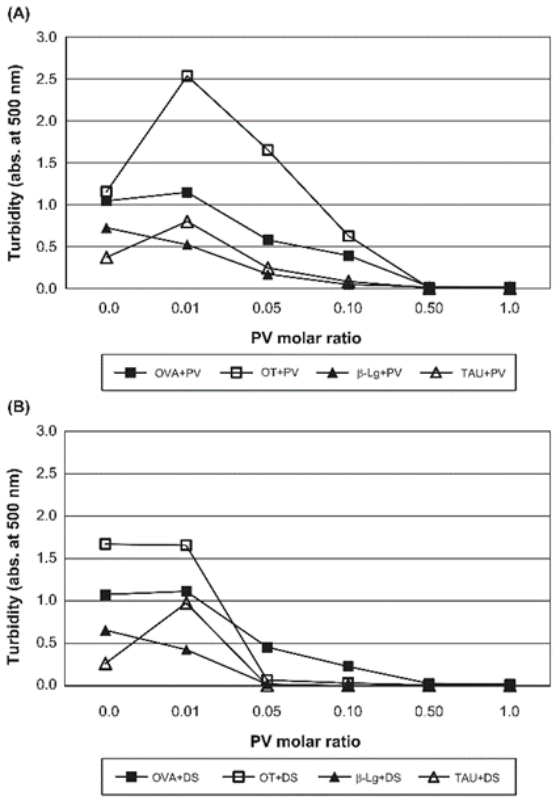

Figure 3. Effects of PV and DS concentrations on the heat insolubilization of proteins.

The turbidity of protein solutions (1 μM) with various PV (A) and DS (B) concentrations was measured after heating at 80°C for 10 min. The molar ratio ranged from 1:0 to 1:1

(protein:PV) and from 1:0 to 1:0.1 (protein: DS). OVA and β-Lg experiments were performed at pH 5.0, and OT and TAU experiments were performed at pH 7.0.

Inhibition of OVA insolubilization by DS was observed at a protein:DS ratio of 1:0.05. For the other proteins, insolubilization inhibition by DS was observed at a ratio of 1:0.005 (Fig. 3B). According to these results, insolubilization suppression was enhanced at a ratio of ≥0.5 with PV and 0.05 with DS.

Protein morphology following heating with PV and DS

To characterize the state of treated protein solutions after heating, SDS-PAGE under non-reducing conditions and neutral and basic Native-PAGE analyses were performed. Bands could not be detected in untreated single protein solutions because insoluble aggregates were formed after heating (Fig. 4A and B).

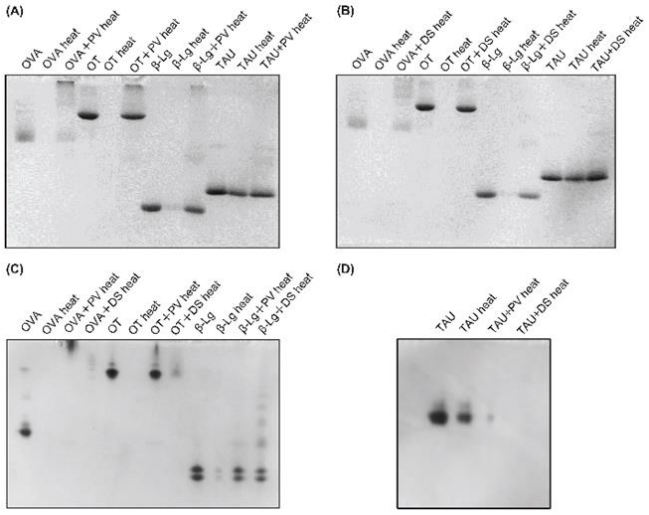

Figure 4. SDS-PAGE and Native-PAGE of proteins with PV or DS after heating.

Protein solutions (0.5 mg/ml) with or without PV or DS (0.5 mg/ml) were heated at 80°C for 10 min. OVA and β-Lg measurements were obtained at pH 5.0 and OT and TAU measurements at pH 7.0. (A) SDS-PAGE of proteins with or without PV before and after heating under non-reducing conditions. (B) SDS-PAGE of proteins with or without DS before and after heating under non-reducing conditions. (C) Neutral Native-PAGE of proteins with or without PV or DS before and after heating. (D) Basic Native-PAGE of TAU with or without PV or DS before and after heating.

In contrast, bands were detected using SDS-PAGE (Fig. 4A and B) and Native-PAGE (Fig. 4C and D) after PV or DS treatment, even following heating. PV or DS addition led to the formation of soluble aggregates of OVA following heating. However, β-Lg and OT remained monomers. A Native-PAGE band could not be detected in TAU solutions after addition of PV or DS (Fig. 4D). Acidic proteins are difficult to stain with CBB. It was speculated that the disappearance of the TAU band after PV and DS addition caused the charge to shift toward acidic because of electrostatic interactions between TAU and PV or DS.

Furthermore, molecular aggregation was confirmed by size-exclusion HPLC. The results for treated OVA and OT solutions were consistent with SDS-PAGE and Native-PAGE results (data not shown). However, DS- or PV-treated β-Lg appeared to maintain monomeric form after heating as measured by Native-PAGE but formed partially soluble aggregates as measured by HPLC (Fig. 5A, B, C and D).

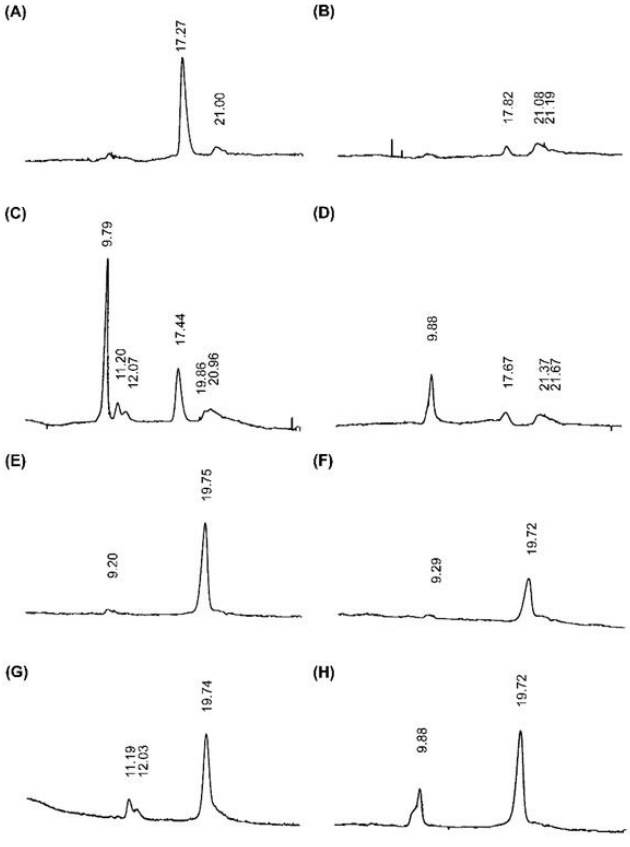

Figure 5. Gel filtration of β-Lg and TAU protein solutions without or with PV or DS after heating.
Protein solutions (0.5 mg/ml) with or without PV or DS (0.5 mg/ml) were heated at 80°C for 10 min. β-Lg and TAU experiments were performed at pH 5.0 and 7.0, respectively. β-Lg non-heated (A), β-Lg after heating (B), β-Lg + PV after heating (C), β-Lg + DS after heating (D), TAU non-heated (E), TAU after heating (F), TAU + PV after heating (G), and TAU + DS after heating (H).

In addition, TAU largely maintained a monomeric form as measured by HPLC, even after heating (Fig. 5E, F, G and H). These results show that near-neutral or basic proteins maintain monomeric forms even after heating if PV or DS is added. Thus, protein charge influences the insolubilization suppression capacity of PV and DS. Furthermore, the molecular form of each protein changes after heating depending on the charge of the protein.

Insolubilization inhibition by electrostatic interaction

It has been previously reported that the addition of NaCl impairs the ability of PV to inhibit OT aggregation [10]. PV has a large number of phosphate groups, and electrostatic interactions with proteins occur via phosphate groups. Similarly, DS has a sulfate group, and the sulfate group is thought to have electrostatic interactions with proteins [7,11]. However, it has remained unknown what types of proteins interact with PV or DS. Therefore, we investigated the extent of electrostatic interactions between PV and DS and various proteins. Mixed solutions of each protein (1 μM) and PV or DS (1 μM) and various NaCl concentrations (0–50 mM) were heated at 80°C for 10 min. As shown in Figure 6A, the capacity of PV to inhibit insolubilization decreased gradually starting at 20 mM NaCl in a concentration-dependent manner.

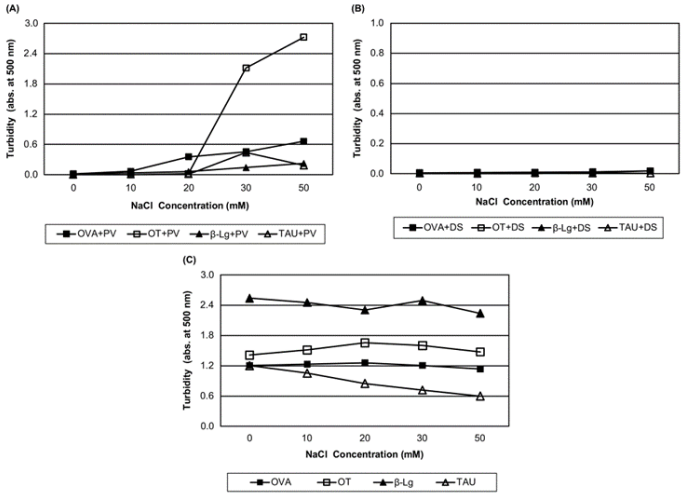

Figure 6. Effect of NaCl on the capacity of PV or DS inhibit heat insolubilization of proteins.
The turbidity of protein solutions (0.5 mg/ml) with PV (A) or with DS (B) or alone (C) (0.5 mg/ml) at 0–50 mM NaCl concentrations was measured after heating at 80°C for 10 min. OVA and β-Lg measurements were obtained at pH 5.0 and OT and TAU measurements at pH 7.0.

Conversely, as illustrated in Figure 6B, no decrease in insolubilization inhibition capacity of DS was observed, even at an NaCl concentration of 50 mM. When untreated proteins were heated with NaCl, TAU solutions had decreased turbidity, which was dependent on the NaCl concentration. However, the other proteins did not show any turbidity differences (Fig. 6C). The salting-in effect by the addition of NaCl was only observed for TAU. The results indicated that PV and DS suppress insolubilization through electrostatic interactions with proteins. Electrostatic interactions between DS and proteins are stronger than those between PV and proteins.

Considering the results presented in Figures 4, 5, and 6, two patterns of insolubilization inhibition mechanisms could be considered.

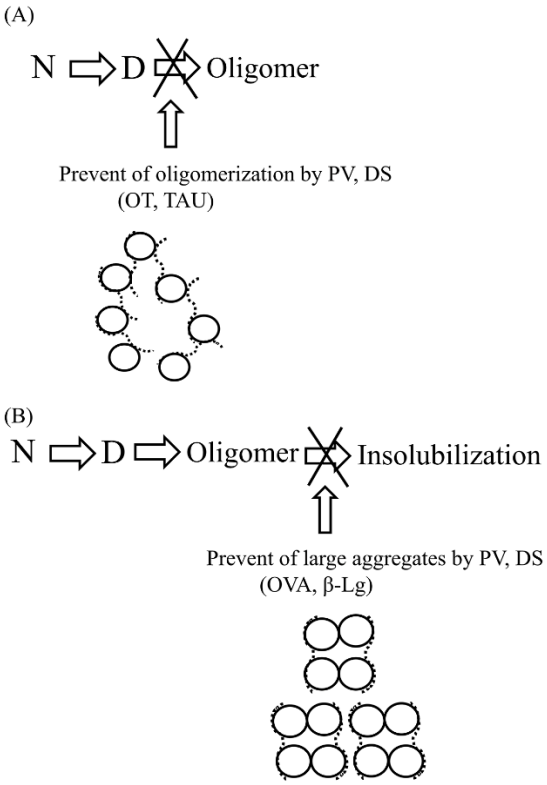

Figure 7. Working model of the mechanism of PV and DS insolubilization inhibition

(A) PV and DS suppress heat insolubilization by suppressing OT and TAU oligomerization. (B) PV and DS prevent the formation of large OVA and β-Lg aggregates, suppressing heat aggregation. N indicates "native state," D indicates "denatured state," ○ represents denatured protein, and the dotted line represents PV and DS.

First, acidic proteins such as OVA and β-Lg form oligomers following heating, whereas PV and DS suppress heat insolubilization by maintaining protein oligomer forms through electrostatic interactions. Second, neutral and basic proteins, such as OT and TAU, are denatured by heating, whereas PV and DS suppress protein insolubilization by suppressing the formation of oligomers formation through electrostatic interactions (Fig. 7). When the pH of the solution is higher than the isoelectric point, the protein is denatured by heating and the internal structure is exposed. This allows PV and DS to interact with locally positively charged regions to suppress insolubilization. It has been reported that intermediate formation by heating is important for electrostatic interaction between DS and proteins [11]. This may be because the higher-order structure of proteins inhibits binding to locally positively charged regions that are internally buried within the protein. However, when the pH of the solution is lower than the isoelectric point, the protein becomes positively charged, and PV or DS can interact with the protein to promote insolubilization.

In conclusion, we herein demonstrate that the suppressive effects of PV and DS on insolubilization depended on protein type and that the suppression mechanism depended on the isoelectric point of the protein. We found that PV and DS inhibit insolubilization by inhibiting higher polymerization of acidic proteins and by maintaining the monomeric forms of neutral–basic proteins. DS and PV can be used as chemical chaperones for neutral–basic proteins because they maintain the monomeric state even after heating. In this study, four types of proteins were used; however, further delineation of interaction between these compounds and proteins may be verified by further study with more proteins with a wider range of properties. Furthermore, in order to investigate the function of DS and PV as a chemical chaperone, it will be necessary to measure effects on enzymatic activity using various enzymes and to evaluate effects on secondary and tertiary structures.

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Reference

1) Shiraki, K.., Kudou, M., Fujiwara, S., Imanaka, T., and Takagi, M. :Biophysical effect of amino acid on the prevention of protein aggregation. *J. Biochem.*, 132, pp591–595. 2002.

2) Kudou, M., Shiraki, K., Fujiwara, S., Imanaka, T., and Takagi, M. :Prevention of thermal inactivation and aggregation of lysozyme by polyamines. *Eur. J. Biochem.*, 270, pp4547–4554. 2003.

3) Shiraki, K., Kudou, M., Nishikori, S., Kitagawa, H., Imanaka, T., and Takagi, M. :Arginine ethylester prevent thermal inactivation and aggregation of lysozyme. Eur. *J. Biochem.*, 271, pp3242–3247. 2004.

4) Matsuoka, T., Hamada, H., Matsumoto, K., and Shiraki, K., :Indispensable structure of solution additives to prevent inactivation of lysozyme for

heating and refolding. Biotechnol. Prog., 5, pp1515–1524. 2009.
5) Iwashita, K., Inoue, N., Handa, A., and Shiraki, K. :Thermal aggregation of hen egg white proteins in the presence of salts. *Protein J.*, 34, pp212–219. 2015.
6) Arakawa, T., and Timasheff, S.N. ;Mechanism of poly(ethylene glycol) interaction with proteins. *Biochemistry*, 24, pp6756–6762. 1985.
7) Matsudomi, N., Kazuichi, T., Moriyoshi, E., and Hasegawa, C. :Characteristics of heat-induced transparent gels from egg white by the addition of dextran sulfate. *J. Agric. Food Chem.*, 45, pp546–550. 1997.
8) Bhattacharyya, J., and Das, K.P. :Molecular chaperone-like properties of an unfolded protein, αs-casein. *J. Biol. Chem.*, 274, pp15505–15509. 1999.
9) Matudomi, N., Kanda, Y., Yoshika, Y., and Moriwaki, H. :Ability of αS-casein to suppress the heat aggregation of ovotransferrin. *J. Agric. Food Chem.*, 52, pp4882–4886. 2004.
10) Matsudomi, N., Ito, K., and Yoshika, Y. :Preventive effect of egg yolk phosvitin on heat-insolubilization of egg white protein and its application to heat-induced egg white gel. *Biosci. Biotechnol. Biochem.*, 70, pp836–842. 2006.
11) Chung, K.; Kim, J., Cho, B.K., Ko, B.J., Hwang, B.Y., and Kim, B.G. :How does dextran sulfate prevent heat-induced aggregation of protein? The mechanism and its limitation as aggregation inhibitor. *Biochim. Biophys. Acta.,* 1774, pp249–257. 2007.
12) Samaraweera, H., Zhang, W.G., Lee, E.J., and Ahn, D.U. :Egg yolk phosvitin and functional phosphopeptides—review. *J. Food Sci.*, 76, pp143–150. 2011.
13) Mecham, D.K., and Olcott, H.S. :Phosvitin, theprincipal phosphoprotein of egg yolk. *J. Am. Chem. Soc.*, 71, pp3670–3676. 1949
14) Abe, Y., Itoh, T., and Adachi, S. :Fractionation and characterization of hen's egg yolk phosvitin. *J. Food Sci.*, 47, pp1903–1907. 1982
15) Zhang, G., Foegeding, E.A., and Hardin, C.C. :Effect of sulfated polysaccharides on heat-induced structural changes in beta-lactoglobulin. *J. Agric. Food Chem.*, 52, pp3975–3981. 2004
16) Laemmli, U.K. :Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227, pp680–685. 1970.
17) Davis, B.J. :Disc electrophoresis. 2. Method and application to human serum proteins. *Ann. NY. Acad. Sci.*, 121, pp404–427. 1964
18) Reisfeld, R.A., Lewis, U.J., and Williams, D.E. :Disk electrophoresis of basic proteins and peptides on polyacrylamide gels. *Nature*, 195, pp281–283. 1962
19) Chiancone, E.; Bruzzesi, M.R.; and Antonini E. Studies on dextran and dextran derivatives. X. The interaction of dextran sulfate with lysozyme. *Biochemistry*, 9, pp2823–2828. 1966