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Arginine prevents thermal aggregation of hen egg white proteins

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ABSTRACT

The control of aggregation and solubilization of hen egg white protein (HEWP) is an important issue for industrial applications of one of the most familiar food protein sources. Here, we investigated the effects of edible amino acids on heat-induced aggregation of HEWP. The addition of 0.6 M arginine (Arg) completely suppressed the formation of insoluble aggregates of 1 mg mL^{-1} HEWP following heat treatment, even at 90 °C for 20 min. In contrast, lysine (Lys), glycine (Gly), and sodium chloride (NaCl) did little to suppress the aggregation of HEWP under the same conditions. SDS-PAGE indicated that Arg suppresses the thermal aggregation of almost all types of HEWP at 1 mg mL^{-1}. However, Arg did not suppress the thermal aggregation of HEWP at concentrations ≥ 10 mg mL^{-1} and prompted the formation of aggregates. Transmission electron micrographs revealed a high-density structure of unfolded proteins in the presence of Arg. These results indicate that Arg exerts a greater suppressive effect on a protein mixture, such as HEWP, than on a single model protein. These observations may propose Arg as a safe and reasonable additive to HEWP for the elimination of microorganisms by allowing an increase in sterilization temperature.

1. Introduction

Hen egg white protein (HEWP) is one of the most familiar protein sources in foods because of its low cost and ease of use in various food processing applications. Over the last several decades, there have been many advances in our understanding of the protein science of HEWP, such as its foaming and gelling properties (Handa, Takahashi, Kuroda, & Froning, 1998; Liu et al., 2013; Mine, 1995, 1996; Raikos, Campbell, & Euston, 2007; Van der Plancken, Van Loey, & Hendrickx, 2007). In the food industry, the control of thermal aggregation plays an important role in sterilization of HEWP. For example, it is possible to make processed foods containing HEWP safer by eliminating *Salmonella* using heat treatment at high temperatures (Mizutani, Chen, Yamasita, Hirose, & Aibara, 2006). However, unfavorable aggregates of HEWP made during the heat sterilization make problems by accumulating in the heat tube. Because HEWP has an unstable structure, unfavorable aggregates of HEWP formed easily by external stress, such as pH change,mechanical vibration, and especially heat treatment (Csaba et al., 2010; Ferreira, Hofer, & Raemy, 1997; Mine, Noutomi, & Haga, 1990). Recently, it was reported that chaotropic ions prevented the thermal aggregation of HEWP (Iwashita, Inoue, Handa, & Shiraki, 2015). These previous reports supported the rather simple application of inorganic salts in the Hofmeister series as additives to prevent the thermal aggregation of protein. However, it is also difficult to use large amounts of inorganic salts in food material because of their toxicity to human.

Arginine is a naturally occurring amino acid with a molecular weight of 174.2 Da. Arg has a guanidinium group on the side chain with the most basic isoelectric point of about pH 10.8. Arg has been applied to solution sciences and industries, e.g., for increasing solubility of poorly soluble compounds, such as drug substances of alkyl gallates (Ariki, Hirano, Arakawa, & Shiraki, 2011; Hirano, Kameda, Arakawa, & Shiraki, 2010), coumarin (Hirano, Arakawa, & Shiraki, 2008), caffeic acid (Hirano, Kameda, Shinozaki, Arakawa, & Shiraki, 2013), and an unfolded protein (Reddy, Lilie, Rudolph, & Lange, 2005); viscosity control of pharmaceutical proteins (Inoue, Takai, Arakawa, & Shiraki, 2014a, 2014b); adsorption control of proteins onto a solid surface (Hirano, Maruyama, Shiraki, Arakawa, & Kameda, 2014; Shikiya, Tomita, Arakawa, & Shiraki, 2013); improvement of protein refolding (Buchner & Rudolph, 1991; Tsumoto et al., 1998; Umetsu et al., 2003); solubilization of porcine myosin (Takai, Yoshizawa, Ejima, Arakawa, & Shiraki, 2013); and protein crystallization (Ito et al., 2011). In particular, the potential to suppress protein aggregation is one of the major applications of Arg as a solution additive (Das et al., 2007; Golovanov, Hautbergue, Wilson, & Lian, 2004; Sharma, Verma, Singh, Korpole, & Ashish, 2016; Shiraki, Kudou, Fujiwara, Imanaka, & Takagi,

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2002; Shiraki et al., 2004). The molecular mechanism underlying the activities of Arg as a solution additive is simple: (1) unique interactions between the guanidinium groups of Arg with the aromatic rings on protein, so-called cation–π interactions (Arakawa & Kita, 2014; Shiraki, Tomita, & Inoue, 2016) as well as (2) electrostatic interaction by both charged α-amino group and guanidinium group (Miyatake, Yoshizawa, Arakawa, & Shiraki, 2016).

Here, the use of Arg for prevention of heat-induced aggregation and gelation of HEWP from the viewpoint of industrial application is reported. To elucidate the molecular mechanism underlying the effects of Arg, the effects of several amino acids and salts were compared. The results of this manuscript may provide a safe sterilization method for the elimination of *Salmonella* in egg white components of food.

2. Materials and method

2.1. Materials

Guanidine hydrochloride (Gdn, PubChem CID: 10481), L-arginine hydrochloride (Arg, PubChem CID: 66250), L-lysine hydrochloride (Lys, PubChem CID: 69568), glycine (Gly, PubChem CID: 750), magnesium chloride ($MgCl_2$, PubChem CID: 5360315), disodium hydrogen phosphate (PubChem CID: 24203), and sodium dihydrogen phosphate (PubChem CID: 23672064) were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Sodium chloride (NaCl, PubChem CID: 5234) and 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES, PubChem CID: 23831) were obtained from Nacalai Tesque (Kyoto, Japan).

2.2. Preparation of hen egg white proteins

Freeze-dried HEWP was prepared as described previously (Iwashita et al., 2015). Briefly, HEWP was diluted with an equal volume of distilled water, stirred gently with a magnetic stirrer for 1 h at 4 °C, and dialyzed using a 1000 MW cut-off dialysis tube against distilled water with four changes at 4 °C to remove small molecular weight compounds and salts. The samples were then centrifuged at 10000 × *g* for 30 min to remove undesirable large aggregates for spectroscopic analysis in the following experiments. The protein content of HEWP after centrifugation was almost identical to that of the original sample. The supernatant was freeze-dried and used for further experiments.

2.3. Thermal aggregation of hen egg white proteins and determination of concentration of protein

HEWP solution was prepared by dissolving the freeze-dried HEWP in 10 mM $MgCl_2$ and 20 mM HEPES buffer with various concentrations of additives. The concentration of HEWP was adjusted to 1–100 mg mL^{-1}. Thereafter, the pH was adjusted to 7.4. Aliquots (80 μL) of each sample were placed in 200-μL microtubes. Then, the samples were heated using a temperature control system (GeneAtlasG; Astec, Fukuoka, Japan). Following heat treatment, all samples were centrifuged at 15000 × *g* for 20 min at 25 °C. The concentration of supernatant was calculated by measuring the absorbance at 280 nm using a spectrophotometer (ND-1000; NanoDrop Technologies, Inc., Wilmington, DE). The light at the wavelength of 280 nm is absorbed by tryptophan, tyrosine, and phenylalanine in the protein, and it is used to measure the concentration of protein (Pace, Vajdos, Fee, Grimsley, & Gray, 1995). The relative absorbance ($A/A_0 \times 100$) was plotted in the figures: A and A_0, each represents the absorbance at 280 nm of supernatant samples after and before heat treatment, respectively.

2.4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

The supernatants of each sample were dissolved in 62.5 mM Tris−HCl (pH 6.8) loading buffer containing 5% (v/v) 2-mercaptoethanol, 2% (w/v) sodium dodecyl sulfate (SDS), and 5% (w/v) sucrose. The proteins in the samples were completely denatured by allowing them to stand undisturbed overnight at room temperature. Next, the samples and molecular weight marker (Precision Plus Protein Dual Xtra Standards; Bio-Rad, Hercules, CA) were loaded onto a 5%–20% gradient gel (e-PAGEL; ATTO Co., Tokyo, Japan). Following electrophoresis, the gel was stained using silver nitrate (Kato, Oda, Yamanaka, Matsudomi, & Kobayashi, 1985).

2.5. Size exclusion chromatography

Soluble aggregates of HEWP were examined by high-performance liquid chromatography (HPLC) (Shimadzu, Kyoto, Japan) using a system consisting of a degasser (DGU-20A3), a pump (LC-10AT), an auto injector (SIL-10AXL), a column oven (CTO-10A), a UV–vis detector (SPD-10AV), and a system controller (SCL-10Avp) with a size exclusion column (3 μm, 300 mm × 7.8 mm i.d., Yarra SEC 3000; Phenomenex, Torrance, CA). Isocratic HPLC was conducted with a flow rate of 1.0 mL min^{-1} at 30 °C using 200 mM Arg, 50 mM HEPES buffer (pH 7.4). Samples of 100 μL were loaded into the column. The absorbance was monitored at 280 nm.

2.6. Dynamic light scattering

Dynamic light scattering analysis was performed with a Malvern Zetasizer Nano ZS (ZEN3600; Malvern Instruments, Malvern, UK), equipped with a 4 mW He–Ne laser and at a λ of 633 nm. Measurement was performed at a scattering angle of 173° from the incident beam, and the particle diameter distribution in intensity was calculated by averaging from 20 to 40 measurements. DLS experiments were performed at 25 °C with a quartz cuvette. An aliquot of 200 μL of sample was added to the cuvette.

2.7. Transmission electron microscopy

Transmission electron microscopy (TEM) images of HEWP aggregates were examined using a transmission electron microscope (H7650; Hitachi, Tokyo, Japan) with an acceleration voltage of 80 kV as follows. HEWP samples (1 mg mL^{-1}) containing 10 mM $MgCl_2$ and 20 mM HEPES (pH 7.4) were heated at 90 °C for 30 min in the presence or absence of 0.6 M additives (Arg, Lys, Gly, Gdn, and NaCl). The heated samples were diluted 100-fold in water, and 30 μL of diluted sample was negatively stained with 30 μL of 2% (w/v) silicon tungstate solution. Approximately 4 μL of the stained solution was placed on a 150-mesh copper grid covered with a carbon-coated hydrophilic film. The solution on the grid was allowed to dry for 2 days.

3. Results

3.1. Suppression of thermal aggregation by Arg

It was investigated whether Arg could suppress the thermal aggregation of HEWP as compared with the control additives. Arg has a composition of (i) basic amino acid, (ii) guanidinium group side chain, and (iii) aliphatic chain. The control additives were selected as follows (Fig. 1). Guanidine (Gdn) has a similar structure to the guanidinium group of Arg. It is possible to confirm the presence of π-cation interaction and electrostatic interaction between protein and additive by comparing Arg and Gdn. Glycine (Gly) is the smallest amino acid included in Arg main chain. It is possible to confirm the role of main chain of Arg by comparing with Gly. Lysine (Lys) has an amino acid with a positively charged side chain, which is similar structure to Arg. Thus, it is possible to confirm the role of guanidinium group of Arg by comparing with amino group. Sodium chloride (NaCl) affects to proteins electrostatically, similarly to Arg–HCl in solution. It is possible