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Coacervates and coaggregates: Liquid–liquid and liquid–solid phase transitions by native and unfolded protein complexes

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ABSTRACT

Coacervates are self-assemblies formed by oppositely charged macromolecules in aqueous solution. Although coacervates usually take a homogeneous spherical shape with flowability, they have the potential to adopt unexpected macroscopic structures. In this study, we investigated the influence of the interaction mode and morphology on unfolded proteins constituting coacervates and coaggregates using ovalbumin (OVA) and lysozyme (LYZ) as the model systems. The unfolded proteins were prepared via heating at 80 °C and then incubated at ambient temperature. OVA and LYZ formed complexes at pH values between their respective isoelectric points in both their native and unfolded states. The unfolded proteins were more prone to form complexes than the native proteins due to hydrophobic interactions rather than electrostatic attraction. Interestingly, native OVA and LYZ formed liquid-like coacervates with spherical shapes, whereas unfolded OVA and LYZ formed solid-like coaggregates with amorphous structures. Understanding the difference between coacervates and coaggregates will provide fundamental information regarding differences between the amorphous aggregation and liquid–liquid phase separation of proteins.

1. Introduction

Self-assemblies of macromolecules have attracted attention as new materials with potential applications as functional foods and drug delivery carriers because of their suitability for the delivery and sustained release of active substances, promoting progress in food science and pharmacology [1]. Food proteins are particularly interesting biocompatible and biodegradable matrices beyond their nutritional value [2]. They can encapsulate and protect bioactive ingredients during storage and passage through digestive organs [3]. Additionally, protein particles influence the perception of sensations within the mouth [4]. Since the assembly from food proteins was first proposed by Howell [5], numerous efforts have been devoted to understanding the formation mechanisms [6,7]. The heterogeneous interactions behind the mechanisms lead to a diversity of supramolecular structures, such as particles, fibers, ribbons, and hydrogels [8]. Coacervate is an assembly with a dense protein-rich phase in which a dilute phase coexists, and this assembly is driven by liquid–liquid phase separation throughout the electrostatic attraction between oppositely charged biomacromolecules [1,7]. Coacervates generally have a well-defined spherical shape with fluidity. However, the morphology of coacervates often becomes irregular similarly as that of aggregates depending on the composition of the coacervate and solution conditions [9,10]. Consequently, the desired functions and characteristics of the unexpected structures are impaired. Precise information about protein structures plays an important role in controlling the structure of coacervates.

The mechanism and shape of spontaneous protein assembly depend on the mode and balance of interactions between proteins, e.g., electrostatic interactions, hydrogen bonding, van der Waals forces, and hydrophobic interactions [11]. The interactions between protein molecules are also strongly influenced by the protein structure, such as native, unfolded, oligomer, and various types of aggregated states. This diversity of protein structure differs markedly from that of synthetic polymers. Changes in the three-dimensional structure of protein are probable to perturb the interactions that play indispensable roles in the association between protein molecules. One of the main obstacles to predicting such associations is the absence of knowledge regarding the protein structure, molecular interactions, and structural hierarchy of complexes.

In this report, we aim to clarify the differences in molecular mechanisms between the liquid–liquid phase separation of coacervates and liquid–solid precipitation of coaggregates. Ovalbumin (OVA) and hen egg-white lysozyme (LYZ), which are the major components of egg white, were selected as the model proteins. OVA is known as the most advanced protein material for food applications because of its abundance and versatility [12]. OVA, with a molecular weight of 45 kDa, isoelectric point of 4.5, denaturation temperature of 77.5 °C, also comprises

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54% of egg white proteins [13,14]. LYZ is a basic protein that is rare among food proteins, and it has a molecular weight of 14.3 kDa and isoelectric point of 10.7. LYZ is a stable protein with a denaturation temperature of 75.0 °C [15,16]. The investigation of coacervates and aggregates of OVA and LYZ will provide important information for managing the large diversity of supramolecular structures derived from globular proteins for the food industry as well as biophysical science.

2. Materials and methods

2.1. Materials

Hen egg white OVA (grade VI) and LYZ (six times crystallized and lyophilized) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The proteins were used without further purification. Sodium fluoride (NaF), sodium chloride (NaCl), arginine hydrochloride (ArgHCl), urea, sodium phosphate, sodium hydroxide, and hydrochloric acid were obtained from Wako Pure Chemical Inc. Ltd. (Osaka, Japan).

2.2. Preparation for unfolded state and complexation of OVA and LYZ

OVA and LYZ were dissolved at 1.5 mg/mL in pure water, and then the solution pH was adjusted to 8.0 using NaOH. Unfolded proteins were prepared as follows. Each solution was heated at 80 °C for 30 min using a water bath, immediately cooled to 4 °C, and finally incubated at room temperature. The unheated and heated OVA solutions were denoted pristine OVA (pOVA) and heated OVA (hOVA) solution, respectively. In the same manner, the unheated and heated LYZ solutions were denoted pristine LYZ (pLYZ) and heated LYZ (hLYZ) solution, respectively. Aliquots of 200 μL of the OVA and LYZ solutions were mixed with water or additive solution (NaF, NaCl, ArgHCl, and urea) adjusted to pH 8 at the equivalent volume ratio at ambient temperature. Instantly, the pH of the mixture was adjusted to 3–12 for pH-dependent experiments or 8 for additive concentration-dependent experiments by dropping 0.01–0.1 M HCl or 0.01–1 M NaOH, followed by incubation for 30 min.

2.3. Circular dichroism

Circular dichroism (CD) spectra of 3-fold diluted solutions of pOVA, hOVA, pLYZ, and hLYZ were measured using a 1-mm path-length quartz cell for far-UV in the wavelength range of 200–250 nm at room temperature using a spectropolarimeter (J-720W; Japan Spectroscopic Co. Ltd., Tokyo, Japan).

2.4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

The OVA/LYZ mixtures were centrifuged at 10,000 ×*g* for 20 min. Subsequently, 500 μL of supernatant were replaced with pure water. Centrifugation and supernatant exchange were repeated three times. Finally, 500-μL aliquots of supernatant were replaced with loading buffer solution (pH 6.8) containing 75 mM Tris-HCl, 2.4% (w/v) sodium dodecyl sulfate (SDS), 6% (w/v) sucrose, and 0.01% (w/v) bromophenol blue. The samples were incubated for 24 h at room temperature and then subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a 5–20% gradient gel (e-PAGEL; ATTO Co., Tokyo, Japan) with the entire solutions of pOVA, hOVA, pLYZ, and hLYZ as controls. The gels were then stained using Coomassie Brilliant Blue R-250.

2.5. Fluorescence assay

To measure intrinsic fluorescence spectra, the pOVA, hOVA, pLYZ, and hLYZ solutions were diluted 10-fold with pure water. To measure ANS fluorescence spectra, the solutions were diluted 10-fold with pure water containing 10 μM ANS, and then each sample was incubated for 30 min at room temperature in the dark. The fluorescence spectra were determined at 25 °C using a spectrofluorometer (FP-6500; Japan Spectroscopic) with a 1-cm path-length quartz cuvette. The emission spectra were recorded with excitation at 280 nm for intrinsic and 380 nm for ANS fluorescence. The slit width was 3 nm for intrinsic fluorescence and 5 nm for ANS fluorescence on both emission and excitation sides.

2.6. Size exclusion chromatography

pOVA, hOVA, pLYZ, hLYZ, and mixtures of these solutions were centrifuged at 10,000 ×*g* for 20 min, and then the soluble protein concentrations in the supernatant were determined via high-performance liquid chromatography (Shimadzu, Kyoto, Japan) using a system consisting of a degasser (DGU-20A_3), pump (LC-10AT), auto injector (SIL-10A_{XL}), column oven (CTO-10A), UV–vis detector (SPD-10AV), and system controller (SCL-10Avp) with a size exclusion column (3 μm, 300 mm × 7.8 mm i.d., Yarra SEC 3000; Phenomenex, Torrance, CA, USA). Isocratic HPLC was performed with a flow rate of 1.0 mL/min at 30 °C using 150 mM sodium phosphate buffer (pH 7.0). Sample aliquots of 40 μL were loaded into the column. The absorbance was monitored at 280 nm. All soluble protein concentrations were determined as the averages of three experiments.

2.7. ζ-Potential

The pH value of the pOVA, hOVA, pLYZ, and hLYZ solutions at protein concentrations of 1.5 mg/mL was adjusted to 3–12 by dropping 0.01–0.1 M HC1 or 0.01–1 M NaOH. The surface charges of proteins were measured at 25 °C using a Zetasizer Nano Z (Malvern Instruments, Worcestershire, UK). Three runs were performed for each measurement.

2.8. Turbidity measurement

The solutions of OVA/LYZ mixtures were added into a 1-cm path-length disposable PMMA cell. The turbidity at 600 nm was measured using a Jasco spectrophotometer model V-630 (Japan Spectroscopic).

2.9. Imaging of complexes via phase contrast microscopy

Aliquots of 1 μL of the OVA/LYZ mixtures were placed on a 96-well plate (Costar, Corning Inc., Lowell, MA, USA). The samples were observed using a phase contrast microscope (BZ-X710; Keyence, Osaka, Japan).

3. Results and discussion

3.1. Physicochemical properties of native and heated OVA and LYZ

Fig. 1A shows the far-UV CD spectra of OVA and LYZ before and after heating. The far-UV CD spectrum of pOVA had an α-helix–rich shape with minima at 208 and 222 nm, which is typical of the native form of OVA. On the contrary, the far-UV CD spectrum of hOVA exhibited decreased intensity compared with that of pOVA, indicating a markedly decreased amount of the α-helix. Likewise, the far-UV CD spectrum of hLYZ had decreased intensity compared with that of pLYZ. The near-UV CD spectra of OVA and LYZ also represented the different spectral shapes between before and after heating (Fig. S1 in supplementary material). These data showed that heat treatment disrupted the secondary and tertiary structures of the proteins. The structures of hOVA and hLYZ did not undergo additional changes at ambient temperature for one day.

Fig. 1B shows the SDS-PAGE data of pOVA, hOVA, pLYZ, and hLYZ under non-reducing conditions. The pOVA solution contained a monomeric form divided into two bands, and a small amount of dimer was observed on SDS-PAGE, which is similar to previous findings that OVA has multiple molecular species due to phosphorylation and