International Journal of Biological Macromolecules xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Mechanism of co-aggregation in a protein mixture with small additives

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ARTICLE INFO

Article history:
Received 15 September 2017
Received in revised form
29 September 2017
Accepted 2 October 2017
Available online xxx

Keyword:
Protein aggregation
Electrostatic interaction
CARS microscopy
Additive
Arginine

ABSTRACT

Co-aggregation plays an important role in processing protein-rich food materials under heterogeneous conditions. The main cause of co-aggregation is an electrostatic attraction between oppositely charged molecules. This study investigated thermal aggregation of β-lactoglobulin (BLG) (pI = 5.1) and lysozyme (LYZ) (pI = 10.7) as a model for the heterogeneous conditions of a protein solution. BLG and LYZ were more aggregated in the mixture than in the single solutions. Co-aggregation of the BLG–LYZ mixture was not observed below 60 °C at which temperature BLG and LYZ retained their native structures. Adding sugars, salts, or amino acids to the BLG–LYZ mixture during the heat treatment revealed the co-aggregation process as follows. (i) All additives tested suppressed both the nucleation and growth of aggregates. (ii) Salts affected nucleation stage to the same degree, except arginine hydrochloride (Arg). (iii) Arg specifically suppressed both nucleation and growth of aggregates. These results indicate that co-aggregation in a protein mixture is more sensitive to the partial unfolding of proteins than that in a single protein solution, due to the presence of electrostatic attraction between different molecules. These results provide new insight into protein aggregation as well as the molecular mechanism of additives under heterogeneous conditions.

1. Introduction

Living system are composed of various types of heterogeneous proteins [1,2]. Thus, protein aggregation in food generally occurs in heterogeneous conditions. For example, egg white is one of the most abundant food materials containing 60 or more kinds of proteins [2] used in surimi products [3] and other meat products [4] to improve textual properties. Protein aggregation in heterogeneous proteins is affected by the coexistence of the kinds of proteins, leading to accelerate [5–13] or suppress [14–18] aggregation. Many of these studies only focused on understanding the molecular mechanism in a heterogeneous solution. Pioneering studies performed 20 years ago showed that electrostatic interactions have the greatest influence on protein aggregation during collaborative aggregation [5,6]. Inspired by these studies, the molecular mechanism of co-aggregation has recently been reported using ovalbumin (OVA) and lysozyme (LYZ) as a model system [13]. The co-aggregation in the report revealed that; (i) OVA and LYZ associate via stacking of multiple factors including the unfolding of OVA, electrostatic attraction, and hydrophobic interactions; followed by (ii) small aggregates of OVA–LYZ form a large network with disulfide bonds exchanged between the proteins. This is the first model of the hierarchical process of aggregation in a protein mixture, similar to a single protein solution [19,20]. However, the contribution of the multiple factors in the hierarchical process, such as nucleation and growth of aggregates, remains unclear.

Small molecular-weight additives have been used to control aggregation and stabilization of proteins [21,22]. Thus, it is thought that the intramolecular and intermolecular interactions can be analyzed based on the presence of these additives. For example, guanidine enhances denaturation of protein by interacting with the aromatic side chain and the peptide backbone [22,23], while sucrose stabilizes the tertiary structure of a protein by preferential hydration [24]. In other words, the aggregation mechanism can be visualized by the presence of Gdn and sucrose. Another example is the so-called Hofmeister series of salts [25]. An anion with a low surface charge density, typically SCN^-, tends to increase the solubility of proteins, while that with a high surface charge density, typically SO_4^{2-}, tends to decrease solubility of the proteins. In addition, arginine hydrochloride (Arg) is a well-used solvent additive; Arg is not a protein denaturant but can suppress protein–protein interactions and protein aggregation [21,26–28]. The guanidinium group of Arg interacts with aromatic or charged residues through cation–π interactions [29–31]. Taken together, these additives

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https://doi.org/10.1016/j.ijbiomac.2017.10.004
0141-8130/

enable discrimination of the interactions between molecules even in heterogeneous proteins.

In this study, we focused on understanding the mechanism of the aggregation processes in heterogeneous proteins, β-lactoglobulin (BLG) (pI = 5.1) [32] and LYZ (pI = 10.7) [33] were used as a model system for thermal aggregation of a protein mixture. LYZ has been used as a model protein of aggregation because of its clearly elucidated tertiary structure, high aromatic amino acid content, and its aggregative property in a broad range of pHs [34–36]. Lysozyme has been practically used as an antimicrobial food ingredient [37]. BLG has a similar size and denaturation temperature to that of LYZ with an opposite charge at neutral pH [33,38–40]. Accordingly, BLG and LYZ are the best combination for aggregation in a heterogeneous protein solution to investigate electrostatic interactions. This study shows the roles of unfolding of proteins, electrostatic affinity, and structural stabilization of proteins during each step of hierarchical co-aggregation. We discuss the differences in co-aggregation in a protein mixture vs. a single kind of protein. Finally, we propose that Arg is the most effective additive to prevent co-aggregation of a protein mixture.

2. Materials and methods

2.1. Materials

Hen-egg white LYZ and BLG were from Sigma-Aldrich Co. (St Louis, MO, USA). Citric acid, boric acid, sodium dihydrogenphosphate, sodium hydroxide, sodium thiocyanate (NaSCN), sodium sulfate (Na_2SO_4), arginine hydrochloride (Arg), guanidine hydrochloride (Gdn), sucrose, acetonitrile, and trifluoroacetic acid (TFA) were from Wako Pure Chemical Ibd., Ltd. (Osaka, Japan). Sodium chloride (NaCl) and silicotungstic acid 26–water were from Nacalai Tesque (Kyoto, Japan).

2.2. Heat treatment of the BLG–LYZ mixture

BLG and LYZ were dissolved in a buffer containing 10 mM sodium citrate, 10 mM sodium phosphate, and 10 mM sodium borate with 0.0–0.5 M additives. Then, the concentration of each solution was adjusted to 100 μM and pH was adjusted to pH 6.0, 7.0 or 8.0. BLG–LYZ mixtures were prepared by mixing 100 μM BLG and 100 μM LYZ solutions in specific proportions. Aliquots of 80 μL of the solutions were added to microfuge tubes. The BLG–LYZ mixed solutions were heated at 40 °C–70 °C for 30 min using a temperature control system (GeneAtlasG; Astec, Fukuoka, Japan). After the heat treatment, the samples were centrifuged at 15,000 × g for 20 min at 25 °C.

2.3. Reverse phase high performance liquid chromatography

The supernatant concentration of each protein was analyzed using a reverse phase high performance liquid chromatography (RP-HPLC) system. A 50 μL aliquot of the supernatant was added to 450 μL buffer. The diluted solution was filtered through a 0.2 μm membrane filter to remove soluble aggregates and transferred to a glass vial for the HPLC analysis. HPLC measurements were performed using a gradient high-pressure liquid chromatograph (Shimadzu HPLC Class 10A series; Tokyo, Japan) with two pumps (LC-10AT), a fixed wavelength programmable UV/VIS detector (SPD-10AV), and a Phenomenex binetex 5.0 μm 250 × 4.6 mm C18 column (Phenomenex Inc., Torrance, CA, USA). Mobile phases were A: H_2O + 0.1% TFA and B: acetonitrile + 0.1% TFA (v/v); total flow rate was always 0.5 mL min^{-1}. The elution gradient was 0–15 min, [B] = 35%–95%; 15–20 min, [B] = 95%; 20–25 min, [B] = 95%–35%; 25–35 min, and [B] = 35%. BLG and LYZ were detected by setting the detector at λ = 280 nm after separation on the column. The peak areas of BLG or LYZ were obtained as S_0 or S. S_0 and S show the peak area of BLG or LYZ in the BLG–LYZ mixtures before and after the heat treatment, respectively. The proportion of soluble BLG or LYZ protein (%) was obtained as $S/S_0 \times 100$ (%).

2.4. Circular dichroism

Far-UV (λ = 200–250 nm) circular dichroism (CD) spectra of BLG and LYZ were measured at 25 °C–70 °C using a J-720 spectropolarimeter (Japan Spectroscopic Co., Ltd., Tokyo, Japan). A 0.2 mg/ml aliquot of the BLG or LYZ solution in 10 mM sodium citrate, 10 mM sodium phosphate, and 10 mM sodium borate buffer (pH 7.0) was transferred to a quartz cell with a 1 mm path length and subjected to CD measurements at a scan rate of 100 nm/min. Near-UV (λ = 260–320 nm) CD spectra of BLG and LYZ were measured at 25 °C–70 °C. A 2.0 mg/ml aliquot of the BLG or LYZ solutions in 10 mM sodium citrate, 10 mM sodium phosphate, and 10 mM sodium borate buffer (pH 7.0) in the presence or absence of 0.5 M additives (NaSCN, NaCl, and Na_2SO_4) was transferred to a quartz cell with a 2 mm path length and subjected to CD measurements at a scan rate of 100 nm/min.

2.5. Transmission electron microscopy

Images of the aggregates were obtained using transmission electron microscopy (TEM; JEM-1400; JEOL, Tokyo, Japan) with an acceleration voltage of 80 kV; all images were obtained at 25,000 × magnification. A 1% (w/v) silicon tungstate solution was used to negatively stain 4 μL of the sample solution. Then, the 4 μL of stained solution was placed on a 150-mesh copper grid covered with a carbon-coated hydrophilic film. The solution was dried for a few hours.

2.6. Coherent anti-Stokes Raman scattering spectroscopic imaging

A home-built coherent anti-Stokes Raman scattering (CARS) microspectroscopic system was used to perform the vibrational spectroscopic analysis. The details of the experimental apparatus have been described previously [41]. Briefly, the system was a sub-nanosecond microchip Nd: YAG laser source with a 33-kHz repetition rate, 800-ps temporal duration, 1064-nm center wavelength, and 200-mW average power as a master laser source. Half of the output (100 mW) was used as the pump pulse (ω_1). On the other hand, the other half of the output (100 mW) was introduced into a photonic crystal fiber to generate a supercontinuum (SC). After the spectral filtering of the SC radiation, near infrared spectral components ranging from 1100 nm to 1700 nm were used as the broadband Stokes pulses (ω_2). Two laser pulses were tightly focused on the sample placed on a three-axis piezo stage. Typical average power at the sample position was about 10 mW for each. The CARS signal was measured using a spectrometer equipped with a charge-coupled device (CCD) camera. The imaginary part of the third-order nonlinear susceptibility (Im [$\chi^{(3)}$]), which corresponds to the spontaneous Raman signal, was numerically retrieved from the CARS spectra using the maximum entropy method.

2.7. Turbidity measurements

The process of heat-induced aggregation of proteins was measured as follows. Briefly, 100 μM BLG, 100 μM LYZ, and 40 μM BLG and 60 μM LYZ solutions in 30 mM sodium phosphate in the absence and presence of additives were prepared and adjusted to pH 7.0 with NaOH. A 2.0 mL aliquot of the solution was transferred into a 1-cm path length glass cell. The sample solutions were incubated at 70 °C, and turbidity was monitored at 400 nm using

Please cite this article in press as: S. Oki, et al., Int. J. Biol. Macromol. (2017), https://doi.org/10.1016/j.ijbiomac.2017.10.004