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Investigating the effects of plasma pretreatment on the formation of ordered aggregates of lysozyme

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ABSTRACT

We investigated the influence of plasma pretreatment on fibril formation and aggregation properties of lysozyme by using the Congo red binding assay, transmission electron microscopy, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), far-ultraviolet circular dichroism, and 1-anilinonaphthalene-8-sulfonic acid (ANS) fluorescence spectroscopy. Our Congo red binding and transmission electron microscopy findings indicated that plasma pretreatment may suppress the formation of ordered fibrillar lysozyme aggregates. The inhibitory effect triggered by plasma pretreatment was observed to be positively correlated with the duration of plasma pretreatment. Compared to the untreated controls, our ANS fluorescence results suggested that fewer solvent-exposed hydrophobic clusters in lysozymes were formed upon pretreatment with plasma. Moreover, HEWL samples with and without plasma pretreatment showed considerably different molecular profiles. We believe the outcome from this work may not only help develop potential strategies for the attenuation of ordered protein aggregation, which is implicated in amyloid pathology, but also present a nice example of plasma-based medicine.

1. Introduction

Multimeric insoluble proteins that have lost their native forms and functions are generally regarded as protein aggregates. Due to this change in property, aggregates can interfere with the production and characterization of therapeutic proteins/peptides and are considered a nuisance in the biopharmaceutical industry [1–3]. In addition, over 30 different proteins that can form amyloidogenic aggregates *in vivo* have been identified as causes for a wide-range of debilitating degenerative diseases, such as hemodialysis amyloidosis, type-II diabetes, Parkinson's diseases, and Alzheimer's disease. These diseases are collectively known as conformational diseases [4–6]. Although these amyloidogenic proteins have dissimilar sequences, structures, and functions, they all form similar highly ordered protein aggregates or amyloid fibrils that exhibit several common morphological- and histochemical-staining properties, such as protease resistance, insolubility in most solvents, cross-β motif structure, fibrillar morphology, and birefringence upon staining with aromatic dye Congo red [4,7,8].

Recent studies have indicated that ordered aggregates, including fibrillar and protofibrillar species that are derived from amyloid-forming proteins and peptides, are correlated with cytotoxicity [9–11]. However, for certain proteins or peptides, the propensities for aggregation can be altered by nonphysiological conditions, such as ultraviolet (UV) light, ultrasound, and plasma. By affecting the conformational features, biological activities, and at times the aggregating propensities of certain proteins or peptides [12–15], these nonphysiological methods are being used to suppress and/or reverse malicious aggregation (*i.e.*, amyloid fibrillogenesis of proteins and peptides) and are now becoming subjects of increasing interest for treating protein aggregation diseases [16–20].

The fact that under appropriate *in vitro* conditions, ordered protein aggregation or amyloid fibrillogenesis can also occur

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in proteins that are irrelevant to any known amyloid diseases [1,8,21], provided the basis for the conjecture that the ability to form amyloid fibrils can be considered merely a generic property of polypeptide chains and is in fact dependent upon the polypeptide backbone rather than the specific amino acid sequence [1,4,7]. Studies that exploit this generic property using nondisease-associated proteins can help further the understanding of possible inhibition toward amyloid aggregation.

Hen egg white lysozyme, a 129-residue enzyme, is involved in catalyzing the hydrolysis of the β-linkage between N-acetylmuramic acid and N-acetylglucosamine subunits in the peptidoglycan polymers of bacterial cell walls and causing the bacteria to lyse. The native protein is a monomer with four disulfide bridges [22] and predominately adopts a helical conformation [23]. There is a wealth of information associated with hen egg white lysozyme, including its three-dimensional structure, folding–unfolding mechanism, aggregation behaviors, and stability information. It is generally regarded as one of the best characterized proteins [24–26]. The enzyme is also structurally homologous to human lysozyme (~60% sequence homology), the protein speculated to be responsible for hereditary non-neuropathic systematic amyloidosis [27,28]. In conclusion, with it being such a well-known protein, having structural similarity to human lysozyme, and exhibiting propensity to form fibrils under acidic conditions [29–32], hen egg white lysozyme has become an excellent model to study the phenomena associated with amyloid fibrillogenesis of proteins.

Results from previous investigations indicated that plasma is capable of inactivating bacteria, promoting fibroblast proliferation, and destroying the tertiary structure of DNA [33,34], suggesting that plasma plays a crucial role in various medical and therapeutic practices [33,34]. While the enzymatic activity and structure of proteins/peptides have been reported to be influenced by plasma [12,13], only limited information about how plasma affect amyloid fibril formation and/or aggregation of proteins/peptides is available [35,36]. In this work, we explore the effects of plasma pretreatment on the aggregative and fibrillogenic behaviors of lysozyme at pH 2.0 and 55 °C using several spectroscopic techniques, SDS-PAGE electrophoresis, and transmission electron microscopy. Our results demonstrated that marked differences in the structural properties and species distributions occur between lysozyme samples with and without plasma pretreatment. In addition, lysozyme was observed to display attenuating fibril-forming propensity upon plasma pretreatment. The inhibitory activity toward formation of ordered protein aggregates and/or amyloid fibrillogenesis was found to be dependent upon the duration of plasma pretreatment. We believe the outcome from this work may not only shed light on a rational design of potential strategies for the attenuation of ordered protein aggregates, which are highly associated with several protein aggregation diseases, but also demonstrate a nice example of plasma-based medicine.

2. Materials and methods

2.1. Materials

Hen egg white lysozyme (HEWL; EC 3.2.1.17) was purchased from Merck (Germany) and used without further purification. Hydrochloric acid, potassium chloride, and sodium chloride were obtained from Nacalai Tesque (Japan). Unless otherwise mentioned, all other chemicals were purchased from Sigma (USA).

2.2. Experimental setup

The schematic of the experimental setup is shown in Fig. 1 and has been described in detail previously [37–39]. In brief, a

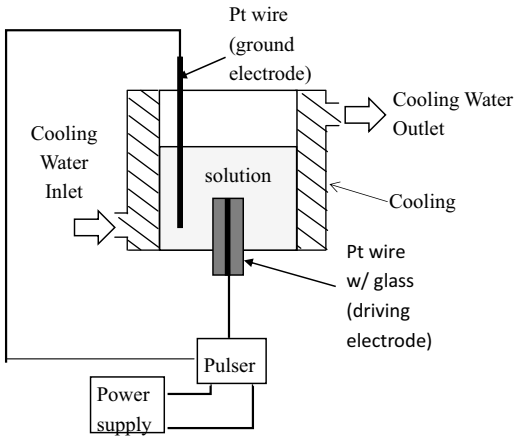

Fig. 1. Schematic of the experimental setup of plasma pretreatment.

cylindrical Pyrex™ glass cell (3 cm inner diameter, 8 cm in height) with a cooling water jacket was used. A platinum wire (0.5 mm diameter) covered with a glass tube was used as the driving electrode to precisely define the area in contact with the solution, while the grounding electrode was a platinum wire with the same diameter immersed in the solution. The pulsed power source consisted of a DC power source (GWinsek, GPR-60H15D) connected to a pulsing unit (YOU-SHANG, Model#PG06R-900-10) with tunable t_{on} (voltage on) and t_{off} (voltage off) between 10 μs and 999 ms. In this study, we used a t_{on} of 0.1 ms and t_{off} of 4.9 ms with a voltage amplitude of 480 V for the operating conditions, which allow for the generation of stable plasmas while minimizing heating of the solution and electrode damage [40]. The cooling water was set at 15 °C. With the above apparatus settings and experimental conditions, the plasma is ignited in the gas layer (bubble) at the tip of the driving electrode tip [41].

2.3. Preparation of HEWL sample solutions

Sample solutions of 0.5 mg/mL HEWL were prepared by dissolving HEWL powders in hydrochloric acid with salts (136.7 mM NaCl, 2.68 mM KCl, pH 2.0) and 0.01% (w/v) NaN_3.

2.4. Plasma pretreatment of HEWL sample solutions

We applied plasma pretreatment on the HEWL sample solutions for 0 (control), 1, 3, and 5 min, and then incubated the samples at 55 °C while stirring at 960 rpm to induce the formation of HEWL fibril.

2.5. Congo red binding assay

Congo red binding studies were performed to assess the presence of amyloid fibrils. Congo red dye was dissolved in phosphate buffer (PB; 10 mM Na_2HPO_4, 1.76 mM NaH_2PO_4, pH 7.4) with 0.01% (w/v) NaN_3 to a final concentration of 20 μM; the HEWL sample solutions were then added in a volume ratio of 1:8 (HEWL sample: 20 μM Congo red solution) and incubated at room temperature in the dark for at least 30 min. The spectra of the samples were measured from 400 to 700 nm with a 1-cm light path quartz cuvette in a Cary 50 UV-Vis spectrophotometer (Varian, USA).

2.6. 1-Anilinonaphthalene-8-sulfonic acid (ANS) fluorescence spectroscopy

ANS dye was dissolved in PB (10 mM Na_2HPO_4, 1.76 mM NaH_2PO_4, pH 7.4) with 0.01% (w/v) NaN_3 to a final concentration