Thermal aggregation of hen egg white lysozyme: effect of polyamines

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ABSTRACT

Protein aggregation is a serious problem for both biotechnology and cell biology. Diseases such as prion misfolding, Alzheimer's, and other amyloidosis are phenomena for which protein aggregation in our living cells is of considerable relevance. Human lysozyme has been shown to form amyloid fibrils in individuals suffering from nonneuropathic systemic amyloidosis, all of which have point mutations in the lysozyme gene. In this study, we investigated effect of small additives on the thermal aggregation of lysozyme. The main finding of this work is that multiple amine groups, spermine and spermidine, play pivotal roles in preventing the thermal aggregation of lysozyme. Our results showed that effect of spermine is more than spermidine.

Keywords: lysozyme; polyamine; aggregation.

INTRODUCTION

Protein aggregation is an unproductive phenomenon in biotechnology and cell living. To reduce aggregation *in vitro*, various techniques have been developed, such as the use of a molecular chaperone and protein mutagenesis, as well as control of pH, temperature, protein concentration, and ionic strength. A simple, practical approach to solving the aggregation problem is the utilization of small molecular additives that as aggregation suppressors. Many types of additive, such as guanidine [1, 2], proline [3], arginine[1, 4, 5], arginine ethylester [6], amino acid derivatives [7] have been reported.

Human lysozyme has been shown to form amyloid fibrils in individuals suffering from nonneuropathic systemic amyloidosis, all of which have point mutations in the lysozyme gene [8-10]. The individuals affected are heterozygous for the mutation and carry amyloid deposits in different tissues containing the variant but not wild-type lysozyme [11]. The formation of amyloid deposits is usually slow, but is generally fatal by the fifth decade of life. The only effective treatment at present involves transplantation of damaged organs, such as kidneys, when amyloid deposition causes their malfunction [11]. The properties of two amyloidogenic lysozyme mutants (I56T and D67H) have been studied in detail and, when compared to those of the wild-type protein, the mutants were found to have reduced structural stability and altered folding kinetics [12-14]. Polyamines are highly regulated polycations that are essentially involved in cell growth and differentiation. They are organic aliphatic cations with two (putrescine), three (spermidine) or four (spermine) amino groups that are fully protonated at physiologic pH [15, 16]. Various physiological roles were suggested for polyamines. For example: regulator of transcriptional and translational stages of protein synthesis, stabilizer of membranes, modulation of neurophysiologic functions, intracellular messengers, free radical scavenger, quencher of chemically generated singlet oxygen, protection of biomacromolecules from reactive oxygen species and metal chelator [15-21]. The protective role of polyamines on core histones, ubiquitin, antithrombin III, plasminogen, Hb and some other proteins against glycation was also demonstrated [17, 22]. Also, it was shown that polyamines in high concentration (100mM) prevent thermal aggregation of proteins [23].

In the present study, we have analyzed the formation of amyloid fibrils by HEVL, as this

protein is readily available and represents an excellent experimental system through which to study the determinants of protein aggregation, being highly homologous to human lysozyme in structure, although only 40% identical in its sequence [24]. The purpose of this study is to evaluate the effect of polyamines, as chemical chaperon, on aggregation of lysozyme in aqueous buffer solutions.

MATERIAL AND METHODS

Materials

Chicken egg white lysozyme (L7651), spermidine trihydrochloride (S2501) and spermine tetrahydrochloride (S2876) were purchased from Sigma Chemical Co., New York, USA. *Micrococcus lysodeikticus* ATCC No. 4698 (M3770) as the LZ substrate was bought from Sigma Co., Koln, Germany. Other used materials were of analytical grade.

Enzymatic activity

The enzymatic activity of lysozyme in presence polyamines was estimated by the turbidimetric assay method. Lysozyme activity was measured by mixing 20-µl aliquots of lysozyme solution (0.1 mg/ml) with 0.980 ml of a *M. lysodeikticus* solution (0.2 mg/ml), as substrate, and polyamines (1mM), as additive, in pH 6.2, 66 mM sodium phosphate, equilibrated at 25 °C. The samples were mixed by repeatedly inverting the cuvette for 15 s. The decrease in the light scattering intensity of the solution was monitored by absorbance at 450 nm. The residual activity was estimated by fitting the data through linear extrapolation. One unit of activity corresponds to an absorbance decrease of 0.0026/min. The concentrations of lysozyme were measured by absorbance at 280 nm using extinction coefficients of 2.63 $cm^2.mg^{-1}$ for the native form and 2.37 $cm^2.mg^{-1}$ for the reduced/denatured form [25].

Heat-induced aggregation of lysozyme

Heat-induced aggregates of lysozyme were quantified as follows. Solutions, containing various concentrations (0.1-0.7 mg/ml) of lysozyme in 66 mM sodium-phosphate buffer (pH 6.2) and 1mM additive were prepared. After heat treatment at 98 °C, the samples were centrifuged at 15 000 g for 20 min [23]. The protein concentration of supernatants was determined by Bradford colorimetric method [26], the amount of aggregates was calculated by subtraction concentration protein after and before heat treatment at 98 °C.

Native electrophoresis analysis

Native PAGE was performed using a system of a 10-20% gradient polyacrylamide gel for the Heat-induced aggregates of lysozyme (1 mg/ml) in presence and absent various concentrations additive in 66 mM sodium-phosphate buffer. After electrophoresis, the gels were stained with Coomassie Brilliant Blue G-250.

RESULTS

Heat-induced aggregation

After heating and then centrifuge the various concentrations of protein, solvated protein concentrations of supernatant were determined by Bradford method.

The amounts of aggregations were calculated by subtraction of concentration solvated protein before and after heat treatment at 98 °C (Fig. 1).

Fig. 2, 3 show activity & specific activity of lysozyme before and after heat treatment. Because of decreasing of the specific activity of lysozyme after treatment of heat, we supposed there are molecules of protein in the solution, but they are inactive (unfolded lysozyme). Amount of unfolded lysozyme was calculated by subtraction specific activity before and after treatment of heat (Fig. 4).

Native electrophoresis

PAGE electrophoresis showed that smears appeared with different electrophoretic mobilities (dimmer, trimmer, …). In comparison with Sp (1-10 mM/ml) the amounts of smears were more in the presence of Sd (1-20 mM/ml). But in high concentrations of polyamines (Sp > 10mg/ml; Sd > 20mg/ml) no smears or aggregations were observed (Fig. 6, 7). Native electrophoresis analysis lysozyme samples shows that spermine and spermidine decrease intermediate of molecules (unfold, dimmer and trimmer, …) between aggregate form and native form. In the presence of 50 mM spermidine or spermine, no intermediate of molecule was observed at the protein concentration of (1 mg/ml), and was observed that reversible of aggregation in the presence of polyamines after a week (Fig. 6B, 7B)

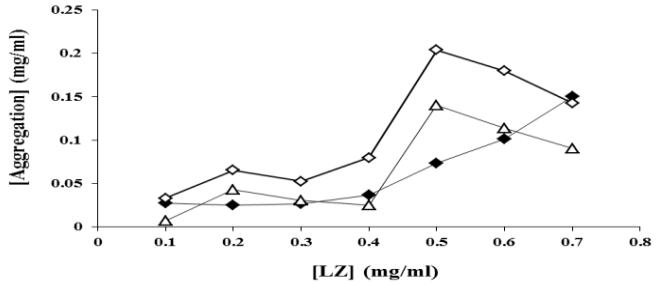

Figure 1. Thermal aggregation of lysozyme in the presence of polyamines. The samples contaning 0.1-0.7 mg/ml lysozyme in the presence 1 mM polyamines at pH 6.2 were heated at 98 ^{o}C for the 30 min. closed squares, lysozyme alone; opened squared, LZ in the presence of Sd; opened triangles, LZ in the presence of Sp.

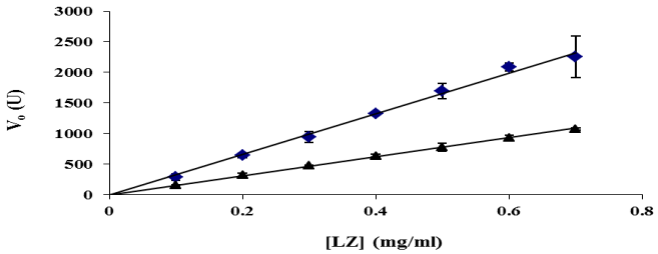

Figure 2. Activity of lysozyme before and after heating. The decrease of activity of lysozyme after treatment of heat was observed. Before thermal; closed squared, after thermal; closed triangles.

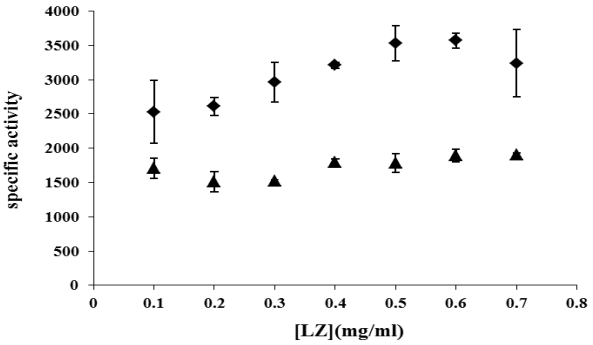

Figure 3. Specific activity of lysozyme before and after heating. The deacrease of specific activity of lysozyme after treatment of heat was observed. Before thermal; closed squared, after thermal; closed triangles.

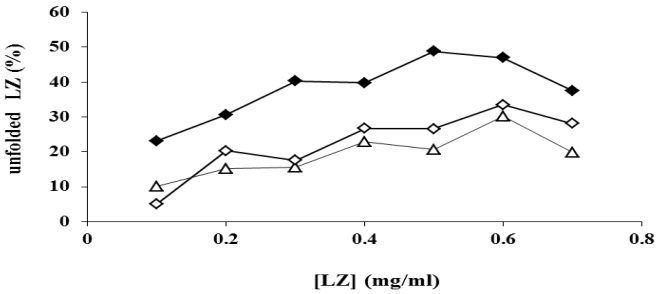

Figure 4. Unfolded lysozyme. Amount of unfolded of lysozyme was calculated by subtraction specific activity before and after treatment of heat. The same samples described in Fig. 1.

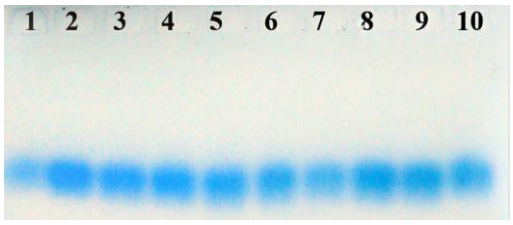

Figure 5. PAGE electrophoresis of lysozyme in the presence or absence of polyamines before treatment of heating. 1: LZ+1mM of Sp, 2: LZ+5mM of Sp, 3: LZ+10 mM of Sp, 4: LZ+20 mM of Sp, 5: LZ+100 mM of Sp, 6: LZ alone, 7: LZ+5 mM of Sd, 8: LZ+10 mM of Sd, 9: LZ+20 mM of Sd, 10: LZ+100 mM of Sd.

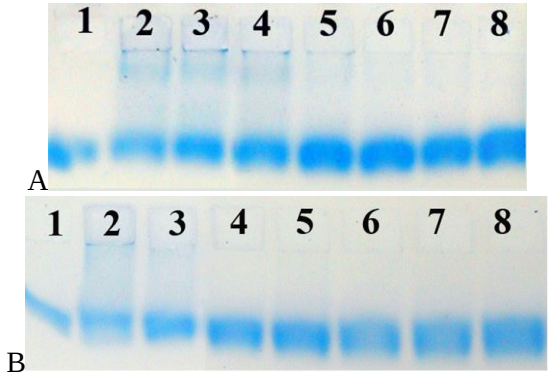

Figure 6. PAGE electrophoresis of lysozyme in the presence of spermidine after treatment of heating. (A) 1: LZ before treatment of heating, 2: LZ alone, 3: LZ+1 mM, 4: LZ +10 mM, 5: LZ+20 mM, 6: LZ+30 mM, 7: LZ+40 mM, 8: LZ+50 mM of spermidine. (B) The same samples described in (A) after a week.

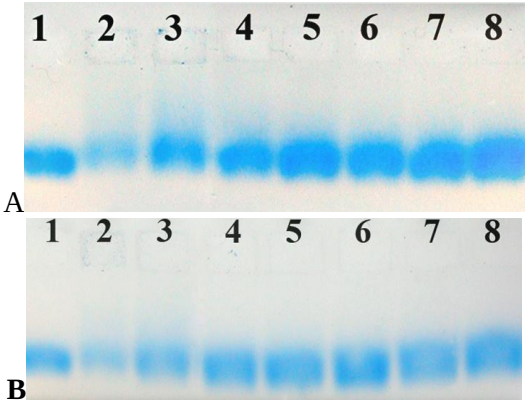

Figure 7. PAGE electrophoresis of lysozyme in the presence of spermine after treatment of heating. (A) The same samples described in Fig. 6. (B) The same samples described in (A) after a week.

DISCUSSION

Despite major efforts aimed at elucidating the genetic, cellular, and biochemical basis of amyloid diseases, relatively little is known in detail about the process that leads to the conversion of a soluble protein into a conformation that ultimately gives rise to its deposition in a living system. Amyloid diseases are of great clinical importance, and amyloid fibrils or their precursors (globular aggregates or protofibrils) have been implicated as primary causes of cell death and tissue degeneration in several such diseases [27-32]. Understanding the formation of amyloidogenic conformations of proteins and the mechanisms of aggregation may lead to novel therapeutic approaches to prevent or reverse the formation of amyloid structures in disease states. In this study, we investigated the basis of small additives that prevent the thermal aggregation of lysozyme. The main finding of this work is that multiple amine groups play pivotal roles in preventing the thermal aggregation of lysozyme.

The obtained results demonstrate that exposure of lysozyme to polyamines causes the decrease in concentration of unfolded protein which is shown in figures 4, 6A, 7A.

PAGE electrophoresis showed that smears appeared with different electrophoretic mobilities (dimmer, trimmer and so on). In comparison with Sp (1-10 mM/ml) the amount of smears were more in the presence of Sd (1-20 mM/ml). But in high concentrations of polyamines (Sp > 10mg/ml; Sd > 20mg/ml) no smears or aggregations were observed. These results illustrated in low concentration of polyamines that unfolded proteins decreased than lysozyme alone (Fig. 4). Also, our results in the case of PAGE electrophoresis showed that aggregates in the presence of polyamines are reversible (Fig. 6B, 7B).

Motonori Kudou et al.[23]shown after heat treatment at 98 ^{o}C for 30 min, no aggregates were observed in the presence of 100 mM spermidine or spermine , while 50% of the molecules were inactivated. Mañas et al. [33] showed that in lysozyme along a thermal treatment in higher temperature (89 ^{o}C) sulfhydryl radicals are

generated from the beginning. Probably the decrease in activity after long heating times due to the occurrence of secondary reactions such as intermolecular aggregation or formation new disulphide bonds. . Motonori Kudou et al. [23] shown by thermal CD that at temperatures above 84 ^{o}C, lysozyme was fully unfolded by heating. Likely in this temperature the enzyme would lose its catalytic activity by the action of heat with disulfide bond breakage and was disturbed secondary structure. Some research has reported that the heat inactivation of proteins is caused by both noncovalent and covalent modifications, including disulfide exchanges [34], β-elimination of disulfide bonds [35].

These facts propose the following mechanism, whereby the heat-induced aggregation of lysozyme is considered to follow three steps at high temperatures:

$$\mathbf{N_1} \xrightarrow{(1)} \mathbf{N_2} \xleftrightarrow{(2)} \mathbf{U} \xrightarrow{(3)} \mathbf{Agg}$$

Where, N_1 represents the native molecule, N_2 represents the like of native molecule but decreased activity (they aren't shown in electrophoresis), U represents the denatured molecules which are soluble (unfolded protein) and Agg represents the insoluble aggregates. Under Eqn (1), weakness binds (such as hydrogen and hydrophobic bonds) cleavages, Under Eqn (2) strong bond (such as disulfide) breakage. Sp and Sd prevent the intermolecular interactions shown in Eqn (2). Also, it was reported that polyamines directly bind to LZ [36]. Therefore, this interaction induces more positive charges on protein. Thus, it leading to increased electrostatic repulsion and a reduction of intermolecular interaction, in this respect Sp with four amino groups is more powerful than Sd with three amine groups. Likely polyamines interact with N_2 form of lysozyme. This finding is expected to initiate further analysis of the additive that prevents the thermal aggregation of protein.

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