# Effect of polyamines on thermal inactivation of hen egg white lysozyme

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## ABSTRACT

Lysozyme is considered as part of the innate immune system. It has stimulated considerable interest as a natural food preservative. Lysozyme has been shown to be effective in preserving a variety of foods such as fresh fruits and vegetables, meats, seafood and wine, for which many Japanese patents have been granted. The relatively high thermal stability of lysozyme also makes it attractive for use in pasteurized and heat-sterilized food products, possibly allowing reduced thermal processes, and therefore, minimized nutritional and sensory quality loss. In this study, we investigated effect of polyamines on the thermal inactivation of lysozyme by kinetics curves. Our results showed that polyamines can decrease the thermal inactivation of lysozyme; the effect of spermine on the thermal inactivation of lysozyme was more than that of the spermidine.

**Keywords:** lysozyme; polyamine; thermal inactivation.

## INTRODUCTION

Lysozyme (1,4-β-*N*-acetylmuramidase; EC 3.2.1.17) is a lytic enzyme, which degrades a constituent of bacterial cell wall. This enzyme is found in tears, saliva, milk, respiratory and cervical secretions but also in the small intestine where it is secreted by the Paneth cells. Lysozyme is considered as part of the innate immune system. It has stimulated considerable interest as a natural food preservative [1].

Enzymes are continuously exposed to several stresses during food processes, such as heating or mechanical forces involved in mixing, spray drying, pumping, etc. In the last decade there has been an increased interest in the study and development and new methods of food preservation that minimize the negative effects of heat on food quality [2]. Lysozyme has been shown to be effective in preserving a variety of foods such as fresh fruits and vegetables, meats, seafood and wine, for which many Japanese patents have been granted [3]. Lysozyme is currently used in Europe mainly as an alternative to nitrates, to combat late blowing caused by *Clostridium tyrobutyricum* during the manufacture of semi-hard cheeses such as Edam and Gouda. The relatively high thermal stability of lysozyme also makes it attractive for use in pasteurized and heat-sterilized food products, possibility allowing reduced thermal processes, and therefore, minimized nutritional and sensory quality loss [4, 5]. Many studies of thermal denaturation of lysozyme by differential scanning calorimetry (DSC) have been polished [6-8] but these results are of little value for prediction of residual enzyme activity after heat treatments.

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 [9, 10]. 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

[9-15]. Also, it was shown that polyamines in high concentration (100mM) prevent thermal aggregation of proteins [16].

The purpose of this study is to evaluate the effect of polyamines, as chemical chaperone, on thermal inactivation 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 [17]).

### *Thermal inactivation kinetics*

Thermal inactivation kinetics of lysozyme was investigated at temperatures ranging from 40°C to 95°C. Samples containing 0.2 mg/ml lysozyme in the presence or absence of 1 mM additives in 66 mM sodium-phosphate buffer (pH 6.2) were incubated at 40°C–95°C. After heat treatment for 135 minute periods, residual activities were estimated. The inactivation rate constant ($k_d$) of each heating temperature was calculated from a plot of logarithmic residual activity versus heating time.

### *Calculation of thermodynamic parameters on thermal inactivation:*

The effects of polyamines on preventing the thermal inactivation of lysozyme were analyzed as follows. The temperature dependence of the inactivation rate constant was determined using the Arrhenius equation [18]

$$k_i = k_0 exp(-E/RT) \quad (1)$$

where $E$, $k_0$, $R$, and $T$ are the activation energy, frequency factor, gas constant, and absolute temperature, respectively. $E$ was obtained from the slope of the Arrhenius plot in Fig.3. The activation free energy ($\Delta G^*$) at each heating temperature was calculated by the Eq. 2

$$\Delta G^* = -RT\ln(k_d h/k_b T) \quad (2)$$

where $R$, $T$, $k_b$ and $h$ are gas constant, absolute temperature, Boltzmann and Plank constants, respectively. The activation enthalpy change ($\Delta H^*$) at each heating temperature was calculated using:

$$\Delta H^* = E - RT \quad (3)$$

The activation entropy change ($T\Delta S^*$) was calculated using:

$$\Delta G^* = \Delta H^* - T\Delta S^* \quad (4)$$

## RESULTS

### *Thermal inactivation kinetics of lysozyme*

Figure. 1 shows the thermal inactivation kinetics of lysozyme in phosphate buffer at different temperatures ranging from 70 to 90 $^oC$ in the presence or absence of 1 mM polyamines. At 40 $^oC$ no inactivation was observed either with or without polyamine up to 135 min (data not shown). Lysozyme was very stable against heat treatments. As seen in (Fig. 1 A–C) the plots of residual activity versus heating time indicate that the thermal inactivation of lysozyme followed kinetics on linearity. The plot of residual activity versus heating time indicates that the thermal inactivation of lysozyme followed first-ordered kinetics on linearity (Fig.2).

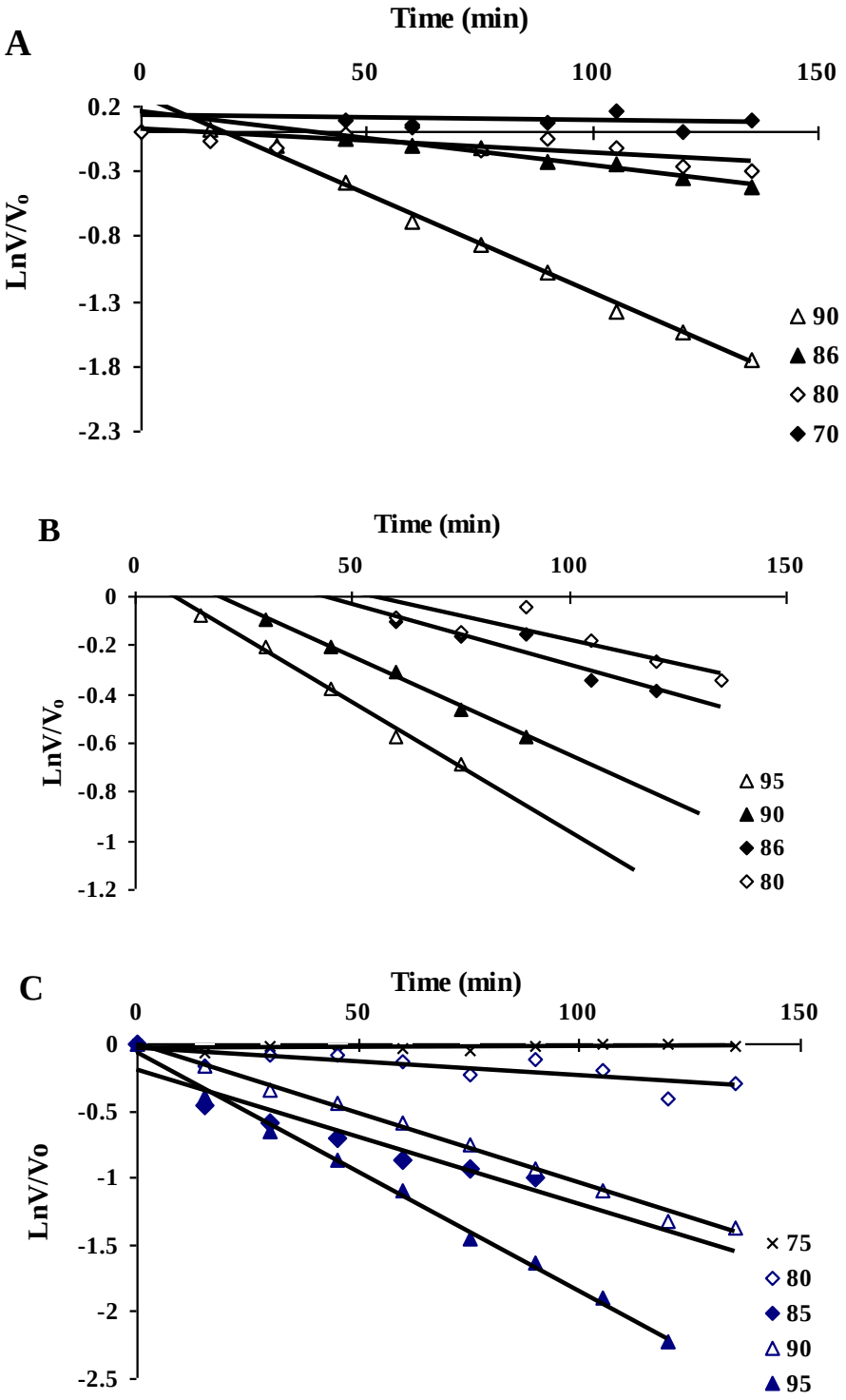


**Figure 1**. Thermal inactivation kinetics of lysozyme. Samples containing 0.1 mg.ml$^{-1}$ lysozyme with 1 mM polyamines in 66 mM sodium-phosphate buffer (pH 6.2) were heated at various temperatures for 135 min. (A) No additives, (B) spermidine, (C) spermine.

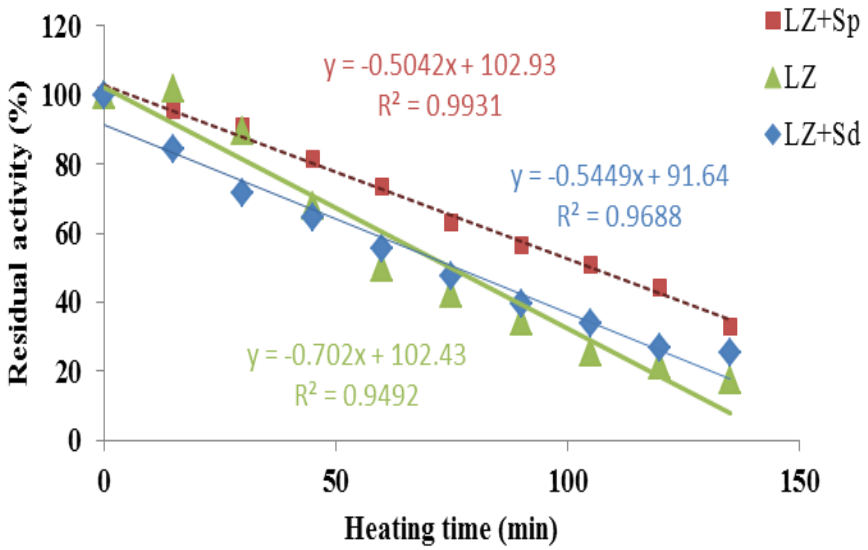


**Figure 2.** Comparison of residual activity and half-life of lysozyme in the presence of polyamines at 90 **°C.** Lysozyme alone (——), in the presence of 1 mM Sd (———), in the presence of 1mM Sp (……).

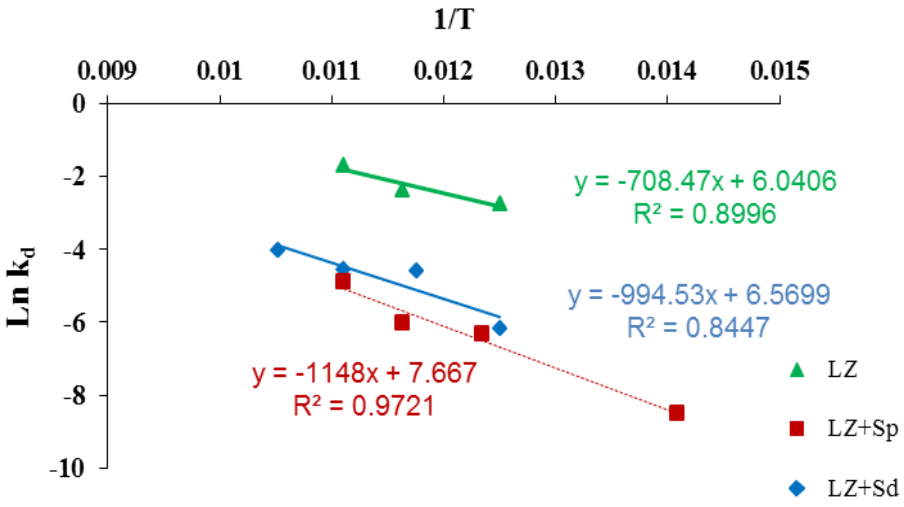


**Figure 3.** Arrhenius plots of kd calculated from the data in Figure 1

**Table 1.** Half-life, inactivation rate constant, activation energy, activation free energy, enthalpy and entropy of lysozyme in the absence or presence of polyamines at 90 °C.

| [polyamine] (1mM) | Half-life (min) | $k_d$ ($min^{-1}$) | E (J/mol) | ΔG (kJ/mol) | ΔH (J/mol) | ΔS (J/mol) |
|---|---|---|---|---|---|---|
| LZ | 74.69 | 0.0194 | 5890.22 | 376.94 | 2872.24 | -1030.5 |
| LZ+Sd | 76.42 | 0.0104 | 8268.522 | 378.78 | 5250.54 | -1029 |
| LZ+Sp | 104.98 | 0.0074 | 9544.472 | 379.79 | 6526.49 | -1028.3 |

Figure 3 shows Arrhenius plots of kd calculated from the data in Fig.1. The activation energy (*E*), activation free energy (*ΔG**), activation enthalpy change (*ΔH**), and activation entropy (*ΔS**) at 90 °C were calculated using Eqs. 1-4 (Table 1). *ΔG*, ΔH*,* and *ΔS**increased with increasing polyamine size.

## DISCUSSION

In this study, we investigated the basis of small additives (polyamine) that prevent the thermal inactivation of lysozyme. The main finding of this work is that multiple amine groups play pivotal roles in preventing the thermal inactivation of lysozyme. It is important to mention here that the concentrations of polyamines employed in this work are on the physiological range, which is on average 0.1–2 mmol in tissues (especially in nuclei), reach more than 10 mmol/l in sperm fluid [19, 20] and reach 0.26 mmol/l in plasma [21].

In order to investigate whether or not polyamines destabilizes protein structure, we analyzed the thermal inactivation of lysozyme in the presence of additives. Figure 1 showed the thermal inactivation curve of lysozyme in the presence and absent of polyamines. In the absence of additives, the inactivation rate constant ($k_d$) of lysozyme was 0.0194 $min^{-1}$ at 90 $^oC$. The $k_d$ decreased in the presence of polyamines (Table 1).

Comparison of percentage of residual activity and half-life of lysozyme in the presence or absent of polyamines at 90 $^oC$ was observed in Fig. 2. Our results showed that the half-lives of lysozyme increased in the presence of polyamines (Table 1). Half time increased with increasing polyamine size. Activation energy obtained from Fig. 3 (Arrhenius plot).

When lysozyme was heat treated, activity decreased exponentially with treatment time. We have observed that the presence of polyamines decreased the thermal inactivation rate of lysozyme. Thermal stability of lysozyme in the presence of Sp more than that of the Sd. This is in agreement with other authors' observations who have reported that polyamines (in concentration 100mM) prevent of thermal inactivation of lysozyme [16].

Kudou et al. showed that spermine (100 mM) increases the thermal stability of egg white lysozyme[16]. They concluded that spermine prevents intermolecular interactions, including disulfite exchanges and aggregation. Motonori Kudou et al. [16] shown after heat treatment at 98 $^oC$ 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. [22] showed that in lysozyme along a thermal treatment in higher temperature (89 $^oC$) sulfhydryl radicals are generated from the beginning. Probably the decrease in activity after long heating times duo to the occurrence of secondary reactions such as intermolecular aggregation or formation new disulphide bonds. . Motonori Kudou et al. [16] shown by thermal CD that at temperatures above 84 $^oC$, 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, β-elimination of disulfide bonds, and deaminations of Gln and/or Asn [23]. Polyamines interact with protein such as lysozyme [16] or α-lactalbumin [24], two homolog proteins [25]. Spermine acts directly on the enzyme in order to increase its activity.

Polyamines are relatively simple structures that are composed of multivalent amines. The pKa values of the secondary amines in putrescine, spermidine, and spermine were 8.0–8.5, whereas those of the primary amines were 10 –11.1 [26]. In biophysical aspects, polyamines can bind with nucleic acids and phospholipids, and stabilize and regulate their tertiary structures [27-29]. Although the present report did not investigate the precise mechanism of formation and inhibition of thermal inactivation, however, it can suppose the interaction of polyamine with lysozyme leading to increased electrostatic repulsion and a reduction of intermolecular interaction. Therefore, it prevents thermal inactivation and aggregation of protein. Because Sp has four amino groups is more powerful than Sd with three amine groups.

In conclusion, our results indicate that polyamines are a new class of additives which can decrease the thermal inactivation of lysozyme, even in low concentration (1mM polyamine). The effect of Sp on the thermal inactivation of lysozyme was more than that of the Sd.

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