

New approach to stability assessment of protein solution formulations by differential scanning calorimetry

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Abstract

A central composite rotate second order design was used to evaluate chicken egg-white lysozyme (lysozyme) thermal stability at different pH, and lysozyme, sucrose and 2-hydroxypropyl- β -cyclodextrin (HP β CD) concentrations, by means of differential scanning calorimetry (DSC). Four measurements were used to characterize the thermogram: the calorimetric enthalpy (ΔH_{cal}), the temperature at maximum heat flux (T_m), the ratio of maximum heat flux over thermogram area (C_{pT_m}/area), and the ratio of calorimetric enthalpies from the second heating cycle to the first enthalpy ($R_{\Delta H_{\text{cal}}}$). These parameters were interpreted using the three step equilibrium model for protein degradation (irreversible degradation following reversible unfolding).

In addition to degradation, increased lysozyme concentration leads to a sizeable decrease in ΔH_{cal} and area ratio, showing how it causes protein aggregation; which in turn promotes protein degradation. ΔH_{cal} and T_m reach maxima at pH 5, $R_{\Delta H_{\text{cal}}}$ at pH 4.19, while C_{pT_m}/area increases linearly with pH, revealing a specific base catalysis of the irreversible degradation step. The role of sucrose concentration in lysozyme stabilization is linked to the stabilization of the unfolded moiety; it neither affects ΔH_{cal} nor C_{pT_m}/area , but increases both T_m and $R_{\Delta H_{\text{cal}}}$. No influence of HP β CD on the stability of lysozyme was observed, probably due to low concentrations employed.

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1. Introduction

It is well known that degradation processes in proteins commonly take place in two steps, the first reversible, corresponding to the unfolding process, and the second irreversible, leading to the degradation of the unfolded protein molecule. This behavior is well described by the Lumry–Eyring model (Lumry and

Eyring, 1954).



Where N denotes the native proteins, U the unfolded state and F the final state. This transition from the native to the denatured state has been investigated in many proteins using urea, guanidine hydrochloride, temperature increase, changes in pH and ionic strength as denaturing agents (Pfeil and Privalov, 1976; Gekko and Ito, 1990; Conejero-Lara and Sánchez-Ruiz, 1991; Charman et al., 1993; Martínez et al., 1995; Funahashi et al., 1996). Polyols and sugars are

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well-known protein stabilizers, but their mechanisms are still under discussion. While Lee and Timasheff (1981) found that the enthalpy of thermal unfolding shows little dependence on the concentration of sucrose, Arakawa and Timasheff (1982), Gekko and Koga (1983), Gekko and Ito (1990), and Kulmyrzaev et al. (2000) found an increase in unfolding enthalpy. Cyclodextrin has also been described as a stabilizing agent reducing protein aggregation and precipitation processes, but favoring thermal degradation (Cooper, 1992; Branchu et al., 1999).

Differential scanning calorimetry (DSC) is reported as one of the most frequently used techniques in studying the thermal stability of proteins (Sánchez-Ruíz et al., 1988; Conejero-Lara and Sánchez-Ruíz, 1991; Cooper and McAuley, 1993; Sanz et al., 1993; Martínez et al., 1995; Tello-Solís and Hernández-Arana, 1995; Conejero-Lara and Mateo, 1996; Funahashi et al., 1996; Libouga et al., 1996; Branchu et al., 1999; Kulmyrzaev et al., 2000), and as one of the most sensitive for measuring the thermodynamic parameters of thermal protein unfolding (Takano et al., 1997, 1999; Vogl et al., 1997). Its application has been a key element in the evaluation of factors that affecting protein stability and is therefore considered a powerful instrument for determining the most appropriate conditions for stabilizing liquid formulations of proteins (Remmele and Gombotz, 2000).

The temperature at the maximum point of the heat capacity curve (T_m) is commonly used as a stability indicator, fundamentally for comparative purposes (Conejero-Lara and Sánchez-Ruíz, 1991; Cooper, 1992; Sánchez-Ruíz, 1992; Azuaga et al., 1996; Remmele et al., 1998; Branchu et al., 1999; Takano et al., 1999). Several authors (Conejero-Lara and Sánchez-Ruíz, 1991, 1996; Azuaga et al., 1996; Branchu et al., 1999) also report the use of protein sample reheating to evaluate thermal reversibility of protein degradation.

The aim of this study is the evaluation of the effect of some formulation factors: lysozyme, 2-hydroxypropyl- β -cyclodextrin (HP β CD) and sucrose concentrations, as well as pH, on the thermal stability of lysozyme, using a comprehensive evaluation of DSC thermograms allowing the stabilization of the native protein to be differentiated from that of the unfolded moiety.

2. Materials and methods

2.1. Chemicals

Lysozyme was obtained from Sigma, HP β CD was kindly provided by Janssen Pharmaceutical. Disodium hydrogen phosphate dihydrate, citric acid monohydrate and sodium chloride were from Merck and sucrose from Avocado. All chemicals used were of analytical grade and distilled water was used throughout the experiments.

2.2. Calorimetric analysis

The calorimetric analyses were performed in a Mettler Toledo calorimeter, model DSC 821^e, using 120 μ l medium pressure crucibles with 70 μ l of sample; the instrument was calibrated with indium. Scanning calorimetry was performed with the Star[®] evaluation program, at a heating rate of 1 $^{\circ}\text{C min}^{-1}$, in the temperature range 30–90 $^{\circ}\text{C}$. After the end of the first heating round, the protein sample was quickly cooled (we set the cooling rate to the maximum accept, $-100^{\circ}\text{C min}^{-1}$) to 30 $^{\circ}\text{C}$, and evaluated again after 5 min stabilization time at 30 $^{\circ}\text{C}$. Base lines, obtained by filling both cells with the corresponding buffer, sucrose and HP β CD for each particular condition, were subtracted from the sample experimental trace, to give the heat capacity profile.

Thermograms were evaluated using Star[®] program. After baseline correction, areas after the first and second heating cycle ($\text{J s K}^{-1} \text{mol}^{-1}$), maximum heat flux, C_{pT_m} ($\text{J K}^{-1} \text{mol}^{-1}$), and temperature at maximum heat flux, T_m (K) were recorded for each analysis. Areas were transformed into calorimetric enthalpies dividing by 60 s K^{-1} (inverse of heating rate) and by 1000 to convert J to kJ. In order to normalize C_{pT_m} relative to the native fraction of protein at the start of the experiment, the ratio C_{pT_m}/area (s^{-1}) was used instead of C_{pT_m} .

2.3. Experimental design and sample preparation

The study was planned and developed following a central composite rotatable second order experimental design with four factors—lysozyme (X_1), HP β CD (X_2) and sucrose (X_3) concentrations, and pH (X_4) and five levels for each factor. All assays were performed

in a randomized order (Cochran and Cox, 1957). Statistical analysis was performed using the MATHEMATICA program, version 4.0. All coefficients with $P < 0.05$ were considered significant and incorporated into the model.

According to the proposed experimental design, lysozyme (molecular weight 14,300 Da) and HP β CD concentrations were fixed at 3.25, 4.25, 5.25, 6.25 and 7.25 mM for coded values -2 , -1 , 0 , 1 and 2 . Sucrose concentrations were set at 2.0, 4.0, 6.0, 8.0 and 10.0% (w/v) for the aforementioned coded values, and pH at 3.0, 4.0, 5.0, 6.0 and 7.0. Citrate buffer

was employed (Diem, 1965) and NaCl to maintain the ionic strength (μ) constant in the system, $\mu = 0.51$.

3. Results

A characteristic single endothermic transition was detected in all experiments. The calorimetric enthalpy ΔH_{cal} , T_m and relative C_{pT_m}/area for the first cycle, and the area ratio between the second and first heating rounds are listed in Table 1 for each experiment. ΔH_{cal} range from 226.0 to 406.7 kJ mol $^{-1}$, T_m from

Table 1
Design conditions and calorimetric data for ΔH_{cal} , T_m , C_{pT_m}/area and $R_{\Delta H_{\text{cal}}}$

Design number	X_1^a	X_2^b	X_3^c	X_4^d	ΔH_{cal} (kJ mol $^{-1}$)	T_m (K)	C_{pT_m}/area (10^3 s $^{-1}$)	$R_{\Delta H_{\text{cal}}}$
1	-1	-1	-1	-1	322.5	348.7	2.43	0.70
2	1	-1	-1	-1	282.2	348.0	2.87	0.59
3	-1	1	-1	-1	356.2	349.0	2.45	0.64
4	1	1	-1	-1	296.7	348.4	2.50	0.69
5	-1	-1	1	-1	406.7	349.8	1.75	0.74
6	1	-1	1	-1	283.7	349.1	2.68	0.67
7	-1	1	1	-1	347.8	349.8	2.34	0.70
8	1	1	1	-1	226.0	349.5	2.80	0.61
9	-1	-1	-1	1	334.8	348.5	2.82	0.40
10	1	-1	-1	1	274.7	348.1	3.19	0.23
11	-1	1	-1	1	296.0	348.4	3.10	0.45
12	1	1	-1	1	322.7	347.6	3.09	0.22
13	-1	-1	1	1	275.3	349.1	3.08	0.78
14	1	-1	1	1	257.7	348.6	3.19	0.40
15	-1	1	1	1	285.5	349.3	2.64	0.49
16	1	1	1	1	241.0	348.7	3.18	0.24
17	-2	0	0	0	401.5	349.7	3.04	0.68
18	2	0	0	0	292.3	348.6	2.62	0.56
19	0	-2	0	0	350.0	349.5	2.58	0.61
20	0	2	0	0	277.2	349.6	2.71	0.69
21	0	0	-2	0	276.2	348.3	2.82	0.45
22	0	0	2	0	313.3	350.6	2.67	0.77
23	0	0	0	-2	253.0	343.9	2.18	0.68
24	0	0	0	2	237.0	346.7	2.95	0.00
25	0	0	0	0	302.2	349.0	2.66	0.57
26	0	0	0	0	289.3	349.2	2.79	0.54
27	0	0	0	0	314.3	349.7	2.77	0.73
28	0	0	0	0	302.5	349.7	2.84	0.69
29	0	0	0	0	416.7	349.6	2.52	0.76
30	0	0	0	0	381.2	349.5	2.41	0.75
31	0	0	0	0	287.5	349.3	2.78	0.75

^a Coded lysozyme concentration.

^b Coded HP β CD concentration.

^c Coded sucrose concentration.

^d Coded pH.

Table 2
ANOVA of calorimetric data

Response	Model equation ^a	P ANOVA	S ₂
ΔH_{cal} (kJ mol ⁻¹)	$327.7_{(14.5)}^b - 27.31X_{1(7.81)}^b - 20.55X_{4(7.15)}^2^b$	5.25×10^{-2}	1.46×10^3
T_m (K)	$349.42_{(0.254)}^b + 0.5X_{3(0.137)}^b - 0.934X_{4(0.137)}^2^b$	7.98×10^{-4}	4.51×10^{-1}
C_{pT_m}/area (s ⁻¹)	$2.68 \times 10^{-3}_{(9.37 \times 10^{-5})}^b + 2.51 \times 10^{-4}X_{4(3.06 \times 10^{-5})}^b$	0.048	6.15×10^{-8}
$R_{\Delta H_{\text{cal}}}$	$0.692_{(0.033)}^b - 0.0624X_{1(0.0179)}^b + 0.0553X_{3(0.0179)}^b - 0.147X_{4(0.0179)}^b - 0.0891_{4(0.0164)}^2^b - 0.0529X_1X_{4(0.0219)}^b$	2.86×10^{-5}	7.71×10^{-3}

^a Coded variables: X_1 , lysozyme concentration; X_2 , HP β CD concentration; X_3 , sucrose concentration; X_4 , pH.

^b S.D.

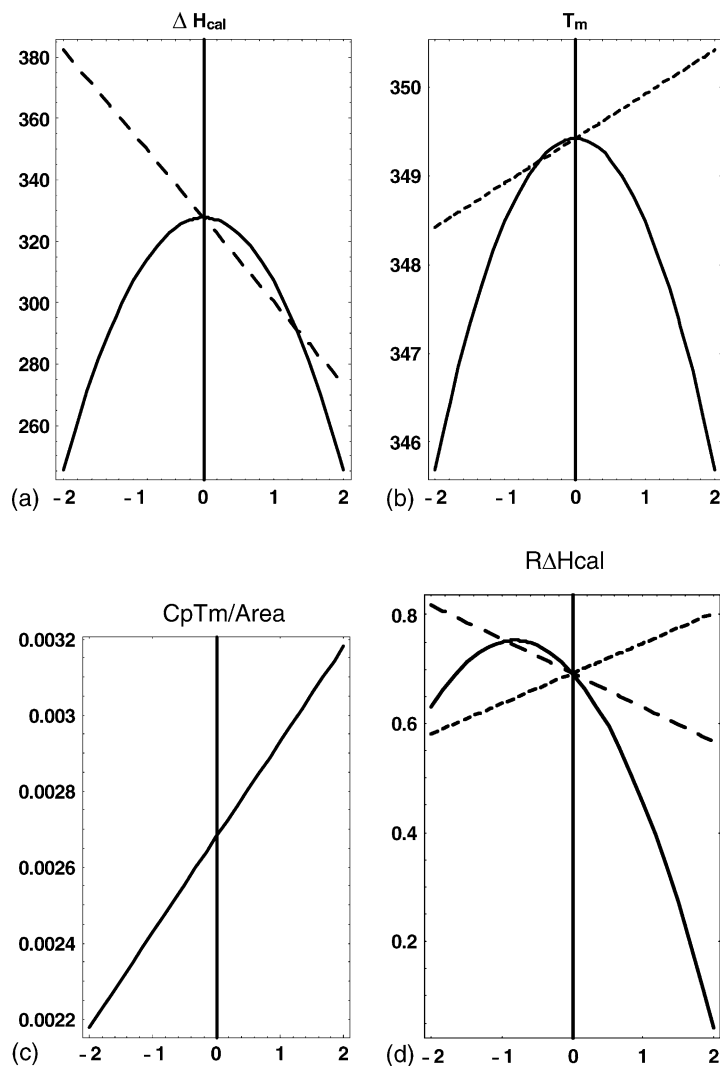


Fig. 1. Dependence of ΔH_{cal} (a), T_m (b), C_{pT_m}/area (c) and $R_{\Delta H_{\text{cal}}}$ (d) on lysozyme concentration (---), sucrose concentration (---) and pH (—). Abscissa as coded variable. Each curve corresponds to the functions in Table 2, keeping the remaining factors at zero (central point of experimental design).

347.6 to 350.6 K, C_{pT_m} area from 1.75×10^{-3} to $3.19 \times 10^{-3} \text{ s}^{-1}$, and the area ratio from 0.0 to 0.87. The analysis of linear regression and ANOVA results are given in Table 2, including those coefficients for which the null hypothesis for $\alpha = 0.05$ was rejected. These functions are plotted in Fig. 1; abscissa values correspond to the coded values for each variable included: pH (continuous line), sucrose concentration (short dotted line) and lysozyme concentration (long dotted line).

4. Discussion

The single endothermic transition observed for lysozyme in all the conditions studied is explained by the breaking of the H-bonded structure and the loss of hydrophobic interactions, in which water successfully competes with backbone and side-chain groups in the protein molecule (Branchu et al., 1999). However, the direct interpretation of the calorimetric responses, ΔH_{cal} , T_m , C_{pT_m} , and area ratio is cumbersome because they reflect rather differently the displacement of the equilibrium between the native and unfolded protein, and the modification of the conversion rate of the unfolded protein to the final degraded state.

Several authors have developed suitable theoretical models for interpreting protein degradation during DSC experiments. Most general models are based on the three state scheme depicted previously; the main difference among these is the relative rate of the unfolding process with respect to the irreversible degradation rate of the unfolded protein. The model studied by Lepock et al. (1992) included three rate constants for unfolding, refolding and degradation pathways, while that developed by Sánchez-Ruíz (1992) assumes an equilibrium between the folded and unfolded fractions of protein. However, this difference is not relevant if only a semi-quantitative interpretation of calorimetric data is intended, thus we will use the equations derived by Sánchez-Ruíz (1992) for the three step equilibrium model. According to this author, the equilibrium constant between the unfolded and natural protein, K_{eq} , can be expressed as

$$K_{\text{eq}} = \frac{x_U}{x_N} = \frac{k_1}{k_2} = \exp\left(-\frac{\Delta H}{R} \left(\frac{1}{T} - \frac{1}{T_{1/2}}\right)\right) \quad (1)$$

where x_U and x_N are the fractions of unfolded and natural protein, respectively, ΔH the unfolding enthalpy, $T_{1/2}$ the temperature for which $K_{\text{eq}} = 1$ and R is the ideal gas constant. The constant rate of degradation of the unfolded protein is provided by the Arrhenius equation

$$k_3 = \exp\left(-\frac{E_a}{R} \left(\frac{1}{T} - \frac{1}{T^*}\right)\right) \quad (2)$$

where E_a is the activation energy and T^* is the temperature for which $k_3 = 1 \text{ time}^{-1}$. The fraction of protein in the natural (x_N), unfolded (x_U) and degraded (x_F) forms is given by the equations

$$x_N = \frac{1}{1 + K_{\text{eq}}} \exp\left(-\frac{1}{v} \int_{T_0}^T \frac{k_3 K_{\text{eq}}}{1 + K_{\text{eq}}} dT\right) \quad (3)$$

$$x_U = \frac{K_{\text{eq}}}{1 + K_{\text{eq}}} \exp\left(-\frac{1}{v} \int_{T_0}^T \frac{k_3 K_{\text{eq}}}{1 + K_{\text{eq}}} dT\right) \quad (4)$$

$$x_F = 1 - \exp\left(-\frac{1}{v} \int_{T_0}^T \frac{k_3 K_{\text{eq}}}{1 + K_{\text{eq}}} dT\right) \quad (5)$$

being v the heating rate (K s^{-1}). On the other hand, the apparent excess heat capacity, C_p^{ex} , is given by

$$C_p^{\text{ex}} = -\Delta H \frac{dx_N}{dt} \quad (6)$$

Solving for dx_N/dt and substituting on the previous equation.

$$C_p^{\text{ex}} = \frac{K_{\text{eq}} \Delta H}{(1 + K_{\text{eq}})^2} \left(\frac{k_3}{v} + \frac{\Delta H}{RT^2}\right) \times \exp\left(-\frac{1}{v} \int_{T_0}^T \frac{k_3 K_{\text{eq}}}{1 + K_{\text{eq}}} dT\right) \quad (7)$$

In order to interpret the experimental data we must bear in mind the following conclusions drawn from the previously equations. First, both the three step equilibrium model and the three step non-equilibrium model assume that the DSC enthalpy corresponds exclusively to the unfolding process; therefore, ΔH would be equal to ΔH_{cal} , and the higher ΔH_{cal} is, the higher will be the fraction of folded protein at the start of the calorimetric experiment (assuming the same total protein concentration). Second, when the irreversible degradation step is not present ($k_3 = 0$), the temperature corresponding to the maximum flux heat, T_m , is that for which $x_N = x_U = 0.5$. For both the equilibrium

and non-equilibrium three state model T_m decreases non-linearly when k_3 increases, but it is also dependent on the heating rate. Third, maximum heat flux C_{pT_m} corresponds to the maximum rate of the denaturalization process; it is obviously related to the amount of protein in the native state at the start of the experiment, but also to those variables affecting the denaturalization and degradation rate. To avoid dependence on the amount of protein in native state we have used the ratio C_{pT_m}/area , whose units are the inverse of time (s^{-1}). Fourth, the area ratio between the second and the first heating cycle, $R_{\Delta H_{\text{cal}}}$, is equal to $1 - x_F$ evaluated at the end of the first heating round, and as can be concluded from Eq. (5) this is related in a rather complex form to all the parameters involved in the model.

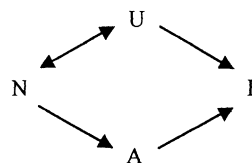
Fig. 1 depicts the influence on each response, ΔH_{cal} (a), T_m (b), C_{pT_m}/area (c) and $R_{\Delta H_{\text{cal}}}$ (d) of the three variables showing a statistically significant effect: pH (continuous line), sucrose concentration (short dotted line) and lysozyme concentration (long dotted line), setting in each case the remaining variables at zero (this corresponds to the central point of the experimental design). Abscissa values correspond to coded variables.

Analysis of our results provides in evidence that pH plays an important role in the conditions at the start of the experiment as well as in the irreversible degradation step. Fig. 1a shows the predicted response of ΔH_{cal} as a function of pH, with LYS set at 0 (see equations in Table 2). Maximum value was reached for pH = 0, equivalent to pH = 5, identifying the conditions for which the folded fraction of protein is maximal. T_m maximum is also reached for $X_4 = 0$, i.e. pH = 5.0 (see predicted response setting $X_1 = X_3 = 0$ in Fig. 1b), a result found previously (Branchu et al., 1999). However, the ratio C_{pT_m}/area increases linearly with pH; this behaviour would be due to the hydroxyl catalysis during the unfolding process. Thus, it is not surprising that the calorimetric enthalpies ratio (see Fig. 1d) shows a maximum for $X_4 = -0.81$, equivalent to pH = 4.19. Indeed, it would be expected from both ΔH_{cal} and T_m that maximum thermal stability would be reached at pH 5; however, at this pH the rate of unfolding is greater than at lower pH, as is concluded from the dependence of C_{pT_m}/area on pH. Thus, it is expected that at some pH < 5 an optimum point would be reached corresponding to a compromise between the fraction of folded protein stable at

the start of the experiment and the minimum unfolding rate.

Regression analysis showed that DSC enthalpy is not significantly affected by sucrose concentration within our experimental interval. According to the above interpretation of this parameter, the conclusion must be that sucrose does not affect the initial equilibrium between the folded and the unfolded moieties. However, the area ratio increased, evidence of the stabilizing role of sucrose. T_m also increased with sucrose concentration; Lee and Timasheff (1981) also observed that the enthalpy of the unfolding process shows little dependence on sucrose concentration; these authors explained the stabilization effect as a consequence of the high cohesive force of the solvent due to the strong interaction between water and sucrose, excluding the protein from the system and leading to the stabilization of the folded protein. Thus, the increase of T_m with sucrose concentration reflects the need for higher energy to form a cavity in the water–sucrose mixture to accommodate the protein molecule. It is worth noting that once the energy of activation is attained, the unfolding rate is independent of sucrose concentration.

The third studied variable influencing lysozyme stability is its concentration. Fig. 1 shows the influence of lysozyme concentration on the calorimetric enthalpy, and calorimetric enthalpies ratio, with the remaining variables set at zero. As before, the interpretation of the calorimetric enthalpy is straightforward: the decrease in this response is due to a reduction of the folded protein in the solution. This is not unexpected, since it is known that proteins at high concentration tend to aggregate. This tendency seems to be important because of the large reduction in calorimetric enthalpy, from 380 to 280 kJ mol^{-1} . In addition, higher lysozyme concentration reduces thermal stability, but neither T_m nor the ratio C_{pT_m}/area is affected. This fact suggests that aggregation of lysozyme is partly responsible for thermal instability by a mechanism independent of protein unfolding, i.e. following the scheme:



where A stand for the moiety of aggregated protein. In any case, the fact that the enthalpies ratio also depends on the interaction of lysozyme concentration with pH is clearly important.

These considerations lead us to the conclusion that there are several mechanisms involved in the thermal stabilization of the studied lysozyme formulations, and therefore there is no expected correlation among the four variables used in the interpretation of thermograms. In short, DSC analysis lead us to identified three types of mechanism in the stabilization of lysozyme.

First, those which displace the equilibrium between the folded and unfolded protein towards the folded state. This mechanism is identified by an increase in ΔH_{cal} . For example, setting pH equal to 5.0.

Second, those which reduce the irreversible degradation rate. This mechanism is identified by and increase in T_m and a reduction in $C_{pT_m}/area$. For example, low pH (note that the pH affects the lysozyme stability through two different mechanism).

Third, those which stabilize the folded protein but not displace the equilibrium towards the folded moiety nor reduce the irreversible degradation of the unfolded protein. This mechanism is identified because neither ΔH_{cal} nor $C_{pT_m}/area$ are affected but there is an increase in T_m . Area ratio gives a weighted measure of the effects of the different factors involved in protein stabilization.

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