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EFFECT OF SUCROSE ON BSA DENATURED AGGREGATION AT HIGH CONCENTRATION STUDIED BY THE ISO-CONVERSIONAL METHOD AND THE MASTER PLOTS METHOD

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The kinetics of protein thermal transition is of a significant interest from the standpoint of medical treatment. The effect of sucrose (0–15 mass%) on bovine serum albumin (BSA) denatured aggregation kinetics at high concentration was studied by the iso-conversional method and the master plots method using differential scanning calorimetry. The observed aggregation was irreversible and conformed to the simple order reaction. The denaturation temperature (T_m), the kinetic triplets all increased as the sucrose concentration increased, which indicated the remarkable stabilization effect of sucrose. The study purpose is to provide new opportunities in exploring aggregation kinetics mechanisms in the presence of additive.

Keywords: *denatured aggregation, DSC, iso-conversional method, master plots method*

Introduction

Protein stability is a particularly relevant issue today in the pharmaceutical field and will continue to gain more importance as the number of therapeutic protein products in development increases. The kinetics of the thermal transition of protein is of a significant interest from the standpoint of practical applications such as laser surgery, thermal therapy, tissue engineering [1]. However, if a therapeutic protein cannot be stabilized adequately, its benefits to human health will not be realized.

One the one hand, previous studies have shown that sugars increase the thermal denaturation temperature of proteins in aqueous solutions [2–6]. The mechanism of protein stabilization with polyols has been discussed at length [7–13]. Despite these efforts, the kinetics basis for the stabilization effect is still a matter of discussion. And also most reports mainly started from the thermodynamics to study the effect of additive on protein denaturation [14–20].

On the other hand, proteins often exist in their physiological environment at high concentrations or in crowded environments. Aggregation of proteins can occur as a consequence of conformational alterations attributed to denaturation [21]. It involves the irreversible interaction of two or more denatured protein molecules [22]. The aggregation of proteins is involved in a wide variety of biomedical and biological phenomena including abnormal disorders such as neurodegenerative diseases and Alzheimer's disease [23]. Despite the increasing relevance of highly concentrated protein solutions, the unfolding and aggregation of proteins at high concentrations are incompletely understood partially because detailed studies of protein unfolding and aggregation mechanisms have traditionally been performed at low concentrations [24], such as 0.5–1.0 mg mL^{-1}. Sánchez-Ruíz method [25] based on the Lumry–Eyring model which studies the processes at very low concentration may not fit the ones at high concentration. So method that can be used directly to analyze the aggregation mechanism of protein at high concentration is thus necessary, because any change in concentration as a result of dilution may alter a protein physical state in a way that no longer represents the original physiological environment or the high concentrations necessary for certain technological and pharmaceutical applications. Alternatively, one may make use of model-free approaches represented by various isoconversional methods to give excellent results of dependencies of the activation energy on the extent of conversion of nonisothermal experiments. For complex reactions of which the mechanism is unknown, the integral 'model-free' method is very useful since no model has to be pre-selected in the evaluations of kinetic parameters. And the master plots method will give more insight into the mechanism. Analysis of the activation energy dependency will provide important clues on reaction mechanism [26–29].

Bovine serum albumin (BSA) is a well-known globular protein that has the tendency to aggregate in macromolecular assemblies [30]. However, despite

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there are a lot of papers about mechanism of aggregation [31–35], and numerous applications in kinetic analysis of thermally stimulated processes in synthetic polymers, there appears to be few reports on the application of iso-conversional method and the master plots method, to the thermal denatured aggregation of BSA at high concentration and the effect of sugar on it or any other proteins. In a previous study we have examined the influence of glucose on denatured aggregation kinetics of BSA [36]. In this study, we focus on the influence of sucrose on the kinetics of BSA denatured aggregation at high concentration by combination of the two methods using differential scanning calorimetry (DSC), in order to provide new opportunities in exploring the aggregation kinetics mechanisms in the presence of additive. This insight in turn can be used in the rational design of stable formulations of therapeutic proteins.

Theoretical approach

For a reaction under non-isothermal condition, its kinetic function can be described as the following form:

$$g(\alpha)=\frac{AE}{\beta R}P(u) \tag{1}$$

where α is the extent of conversion (to obtain α (conversion of reaction), the following equations are used: in DSC, $\alpha=\Delta H_{part}/\Delta H_{tot}$, ΔH_{part} partial area [J g^{-1}] and ΔH_{tot} total peak area [J g^{-1}]), β the heating rate, E the apparent activation energy, R the gas constant, A is preexponential factor, $g(\alpha)$ is the integral expression of kinetic model function, and

$$P(u)=\int_{\infty}^{u}-\frac{e^{-u}}{u^2}du,\ u=\frac{E}{RT}$$

Because the exponential integral, $P(u)$, has no analytical solution, an approximate formula of high accuracy, which is directly obtained from numerical integration of temperature integral without derivation from any approximating infinite series [37, 38], was used.

$$P(u)=\exp(-u)/[u(1.00198882u+1.87391198)] \tag{2}$$

Inserting Eq. (2) into Eq. (1), one can obtain:

$$\ln\frac{\beta}{T^{1.894661}}=\ln\frac{AE}{Rg(\alpha)}+3.635041-1.894661\ln E-1.001450\frac{E}{RT} \tag{3}$$

The first term at the right side of Eq. (3) is a constant corresponding to a given value of α. So for a series of experiments at different heating rates, the plot of $\ln(\beta/T^{1.89466100})$ *vs.* $1/T$ with the same conversional ratio should be a line with the slope of $-1.00145033E/R$. Then, the apparent activation energy E can be calculated from the slope. Inserting $\alpha=0.5$ into Eq. (1), one can get:

$$g(0.5)=\frac{AE}{\beta R}P(0.5) \tag{4}$$

where $u_{0.5}=E/RT_{(0.5)}$. When Eq. (1) is divided by Eq. (4), the following equation is obtained:

$$\frac{g(\alpha)}{g(0.5)}=\frac{P(u)}{P(u_{0.5})} \tag{5}$$

By plotting $g(\alpha)/g(0.5)$ *vs.* α according to different theoretical model functions, the theoretical master plots can be obtained, for different kinetic mechanisms. With E calculated from Eq. (3), the experimental master plots of $P(u)/P(u_{0.5})$ *vs.* α could be drawn from the experimental data obtained under different heating rates. Equation (5) indicates that, for arbitrary α, the experimental value of $P(u)/P(u_{0.5})$ and theoretically calculated values of $g(\alpha)/g(0.5)$ are equivalent when an appropriate kinetic model is used. The integral master plots method can be used to determine the reaction kinetic models of non-isothermal reactions. Then, the pre-exponential factor A can be estimated from the slope of the plot of $g(\alpha)$ *vs.* $EP(u)/\beta R$.

Experimental

Materials

BSA (Fraction V, purity>99.9%, Roche Chemical Company) was used without further purification. Distilled and deionized water was used for the preparation of all solutions. Analytical grade sucrose (SCRC10021418, AR) was used. The 30 mg mL^{-1} BSA solution was prepared by dispersing powdered protein into a sucrose solution (0–15 mass%) contain 150 mM NaCl and 2 M urea and stirring for at least 2 h.

Methods

Differential scanning calorimetry (DSC)

The influence of sucrose on the thermal denaturation and aggregation of BSA solutions was investigated by using a differential scanning calorimeter (Mettler Toledo calorimeter, model DSC 822^e). The instrument was calibrated with indium. The protein solution (80 μL) was placed in 100 μL medium pressure crucible, and sucrose solution of same concentration was used as reference. Scanning calorimetry was performed with the Star® evaluation program, at different heating rates of 1.0, 1.5, 2.0 and 2.5 K min^{-1}, in the temperature range of

35–95°C. After the end of the first heating round, the protein sample was quickly cooled to 35°C, and rescanned after 5 min stabilization time at 35°C. The sample is heated at a low heating rate (<2.5 K min^{-1}) to avoid thermal interference due to circulating convection currents in the sample vessel [39]. Measurements were carried out on two or three separate samples (replicates) and reported as the average. The data were collected in the presence of 2 M urea to lower the denaturation temperature (T_m) of the protein to an observable range of the instrument.

Results and discussion

A different measurement was obtained by completing two sequential scans for a single sample. The first scan of the protein exhibited an endothermic transition between 57 and 82°C, whereas the scan of the heat-treated protein exhibited no thermal transition. The protein samples extracted from the calorimetric cell showed strong aggregation. In this respect, it is noteworthy that the thermal profiles of additional scans (i.e., a third scan) of the denatured samples were essentially identical to that of the second scan, denoting that a single irreversible transition occurred over the temperature range of interest. The repeat curve subtracted from the first measurement curve, which serves as the baseline. The conversion (α) was calculated using the Star® software [40]. The conversion graph and table (including the corresponding denaturation temperature (T_d)) were also displayed.

DSC curves at various heating rates are shown in Figs 1A–D. Figure 1A shows DSC curves for the thermal denatured aggregation of 30 mg mL^{-1} BSA in the absence of sucrose at different heating rates. Figures 1B and C display DSC scans for 30 mg mL^{-1} BSA in the presence of 5 and 15 mass% sucrose at different heating rates, respectively. The influence of sucrose concentration on the heat flow of 30 mg mL^{-1} BSA solutions at scanning rate 2.5 K min^{-1} is also displayed in Fig. 1D. From Fig. 1D it distinctly shows that the maximum in the DSC scans (T_m) shifted to higher temperatures as the sucrose concentration increased from 0 to 15 mass%. This made chear that in the same condition the sucrose improve the BSA thermal denatured aggregation temperature namely heat stability. Curve e of Fig. 1D is 30 mg mL^{-1} BSA thermal denatured aggregation in the absence of sucrose and without urea. Note the T_m is much more higher in the same condition.This can show that the urea reduced BSA heat stability. For all of the sucrose concentrations used (0–15 mass%) the heat-treated protein showed no thermal transition, which indicated that BSA was irreversibly denatured aggregation by the heating process in the presence and absence of su-

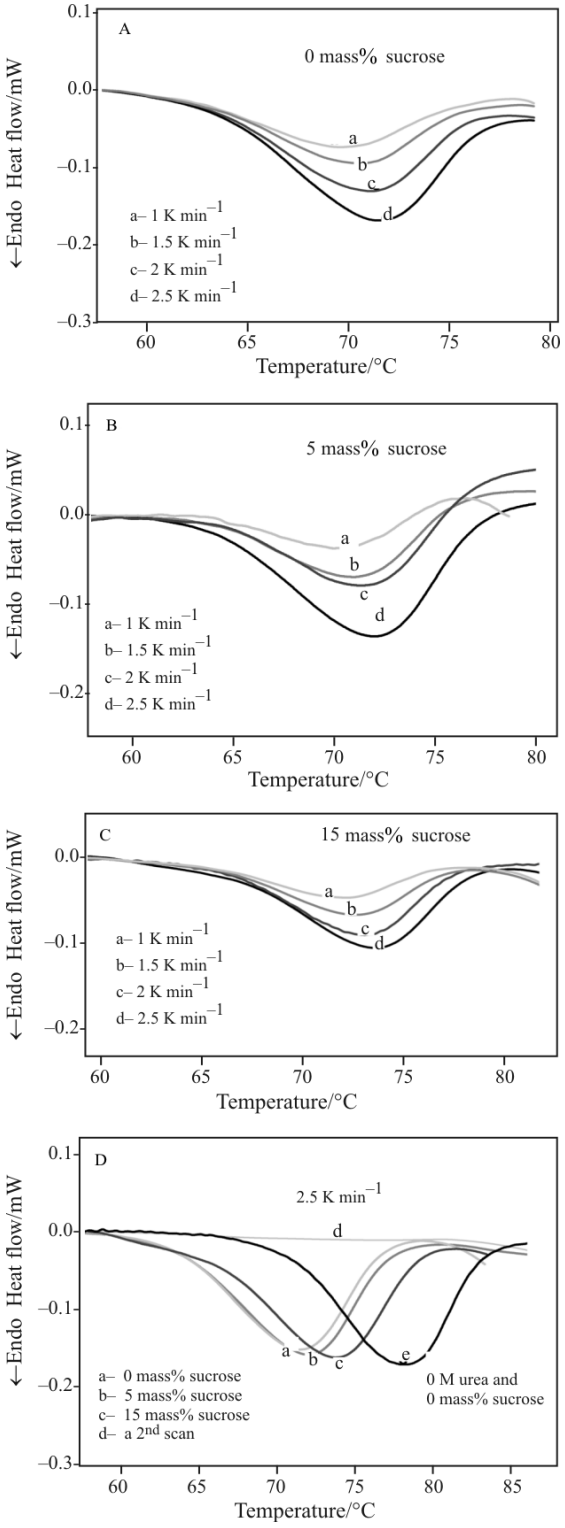

Fig. 1 DSC curves for A – 30 mg mL^{-1} BSA in the absence of sucrose thermal denatured aggregation at different heating rates; B – denotes the influence of sucrose (5 mass%) on the thermal denatured aggregation of 30 mg mL^{-1} BSA at different heating rates; C – 30 mg mL^{-1} BSA with 15 mass% sucrose at different heating rates and D – represents the influence of sucrose concentration on the temperature dependence of the heat flow in 2.5 K min^{-1} scanning rate

Table 1 T_m of 30 mg mL^{-1} BSA in present of sucrose (0–15 mass%) at different heating rates

T_m/°C	Scanning rate/K min^{-1}			
Sucrose/mass%	1	1.5	2	2.5
0%	69.40±0.09[a]	70.26±0.07	70.93±0.12	71.52±0.11
5%	70.01±0.06	70.94±0.05	71.43±0.07	71.99±0.08
15%	72.05±0.11	72.31±0.08	73.10±0.03	73.74±0.06

[a]Means±standard deviations. Standard deviations $S=Sqr(\Sigma(X-M)^2/(n-1))$, where Σ=sum of, X=individual score, M=mean of all scores, n=sample size (number of scores)

crose. BSA denaturation is irreversible probably due to the occurrence of 'side' processes such as aggregation [41]. Due to denaturation, hydrophobic interaction can occur, and exposed thiol groups can form disulfide bonds, which result in an irreversible behavior [42, 43].

The T_m was recorded for each analysis after baseline correction and reproducible to within 0.2°C. And at different rates it is listed in Table 1. It is dependent on the scan rate and sucrose concentration – an increase in the T_m with increase of scanning rates and sucrose concentration. It distinctly revealed that sucrose concentration substantially affected BSA thermal denaturation and aggregation. The highly dependent of T_m on the scan rate indicated that the thermal denaturation and aggregation process was, at least in part, under kinetic control. The T_m shifted to higher temperatures as the sucrose concentration increased from 0 to 15 mass% (Table 1). The stabilization has been explained in terms of preferential hydration or due to the strengthening of the water structure that in turn intensify the hydrophobic interactions of the protein [44]. The magnitude of the resulting thermodynamic driving force increases as the cosolvent concentration increases, and so changes in the thermal stability, conformation, or aggregation of proteins occurs when the cosolvent concentration rises [45, 46]. And thus protein denatured aggregation kinetics altered when sucrose concentration changed.

Non-isothermal kinetics for the denatured aggregation of BSA solutions in the absence of sucrose

Iso-conversional method for estimating activation energy dependence

Using the α–T data obtained from DSC conversion plots, plotting $\ln(\beta/T^{1.89466100})$ *vs.* $1/T$ by a linear regressive of least-square method, the values of E at different conversions (α) in the range of 0.2–0.8 were got and listed in Table 2. As displayed in Table 2, all these plots have linear correlation coefficients larger than 0.99. From Fig. 2, it can be vary easily observed that the value of activation energy hardly varies with the degree of conversion. And the average value of it

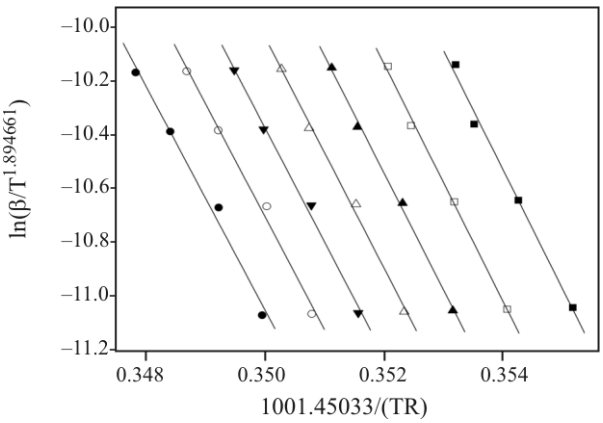

Fig. 2 Plots for determination of activation energy of 30 mg mL^{-1} BSA aggregation in the absence of sucrose at different α: ■ – 0.2, □ – 0.3, ▲ – 0.4, △ – 0.5, ▼ – 0.6, ○ – 0.7 and ● – 0.8. Solid lines are linear fitting corresponding to different α

Table 2 The apparent activation energy E and the correlation coefficients r of linear regression at different conversions α

α	E/kJ mol^{-1}	r
0.2	443.99	0.99618
0.3	442.58	0.99844
0.4	435.80	0.99863
0.5	429.76	0.99782
0.6	424.87	0.99704
0.7	421.34	0.99584
0.8	417.08	0.99424

is 430.77±10.42 kJ mol^{-1}. Due to the little dependence of the activation energy on the extent of conversion, a simple reaction mechanism could be used for reaction progressing. However, recently Vyazovkin *et al.* [1] have investigated the thermal denaturation of collagen by isoconversional method and observed a strong decrease of E with conversion that was consistent with the Lumry–Eyring model. The distinctness of study reaction systems and experiment conditions may be led to different results. In Vyazovkin research, the study system was wet collagen and the value of K fell outside the limiting cases.

While in our case, the study systems were high concentration BSA solutions; all the thermal transitions were found calorimetrically irreversible and the protein samples extracted from the calorimetric cell showed strong aggregation and the rate limiting step might always be the irreversible process itself as α in the range of 0.2–0.8, in other words, the value of *K* always fell inside the limiting case. So, a constant *E* could be observed. But it must be to point out that aggregation was probably one of the main causes of irreversibility in the thermal transitions of BSA. Therefore, the inactivation kinetics might be expected to have a higher than one reaction order [47].

The apparent activation energy is large, that is comparable to the activation energy found for other proteins, such as ovalbumin [48], glucose transporter 1 (GLUT1) [49] etc. The large value of *E* might be expected, because the highly cooperative nature of the protein implies a large Δ*H* between the folded and denatured protein and *E* is always larger than Δ*H* [48, 50].

Master-plots method for determining kinetic model

To determine the most possible mechanism, 18 basic model functions were tested as in Table 3. According to the value of *E*, the experimental master plots of $P(u)/P(u_{0.5})$ *vs.* α constructed from experimental data under different heating rates are shown in Fig. 3. The theoretical master plots of various kinetic functions [51] are also shown in Fig. 3.

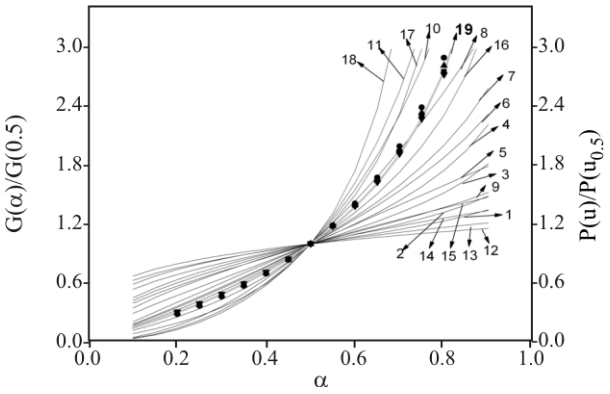

Fig. 3 Master plots of theoretical $P(u)/P(u_{0.5})$ *vs.* α for various reaction models (solid curves, as enumerated in Table 3, and curve 19 represents function $G(\alpha)=1/(1–1.36)–(1–\alpha)^{1–1.36}/(1–1.36)$ and experimental data for the BSA aggregation in the absence of sucrose at heating rates ▲ – 1, ▼ – 1.5, ■ – 2 and ● – 2.5 K min^{-1}. Models that have been used to describe the simple order reaction process are labeled as: 16 – first order reaction model; 17 – second order reaction model; 18 – third order reaction model

The superposition of experiment master plotted at different heating rates indicated that the kinetics process of aggregation of the BSA could be described by a single model function. The comparison of the experimental master plots with theoretical ones indicated that the kinetic process for the BSA denatured aggregation be most probably described by F_n model,

Table 3 The 18 model functions for the determination of the most probably model function

No.	Reaction model	Symbol	$G(\alpha)$
1	Avrami–Erofeyev, *m*=4	A_4	$[-\ln(1-\alpha)]^{1/4}$
2	Avrami–Erofeyev, *m*=3	A_3	$[-\ln(1-\alpha)]^{1/3}$
3	Avrami–Erofeyev, *m*=2	A_2	$[-\ln(1-\alpha)]^{1/2}$
4	Avrami–Erofeyev, *m*=1.5	$A_{1.5}$	$[-\ln(1-\alpha)]^{2/3}$
5	phase boundary reaction, *n*=1	R_1	α
6	phase boundary reaction, *n*=2	R_2	$1-(1-\alpha)^{1/2}$
7	phase boundary reaction, *n*=3	R_3	$1-(1-\alpha)^{1/3}$
8	one-dimensional diffusion	D_1	$1/2\alpha^2$
9	two-dimensional diffusion	D_2	$1/2[1-(1-\alpha)^{1/2}]^{1/2}$
10	three-dimensional diffusion	D_4	$1-2\alpha/3-(1-\alpha)^{2/3}$
11	Jander's type diffusion	D_3	$[1-(1-\alpha)^{1/3}]^2$
12	power law, *n*=1/4		$\alpha^{1/4}$
13	power law, *n*=1/3		$\alpha^{1/3}$
14	power law, *n*=1/2		$\alpha^{1/2}$
15	power law, *n*=3/2		$\alpha^{3/2}$
16	first order	A_1, F_1	$-\ln(1-\alpha)$
17	second order	F_2	$(1-\alpha)^{-1}-1$
18	third order	F_3	$1/2[(1-\alpha)^{-2}-1]$

$$G(\alpha)=\frac{1}{1-n}-\frac{(1-\alpha)^{1-n}}{1-n}$$

Because the experimental master plots lie between the theoretical masters plots F_1 and F_2, it is likely that the apparent mechanism of overall reaction cannot be expressed in terms of an integral order reaction model, which may be indicated mixture basic reactions participating in the system.

Evaluation of pre-exponential factor and kinetic exponent

The kinetic exponent and pre-exponential factor were determined by further calculations. The expression of F_n is introduced into Eq. (1), Eq. (6) is obtained

$$\frac{1}{1-n}-\frac{(1-\alpha)^{1-n}}{1-n}=\frac{AE}{\beta R}P(u) \qquad (6)$$

In Eq. (6), $P(u)$ can be calculated according to Eq. (2). Plotting $1/(1-n)-(1-\alpha)^{1-n}/(1-n)$ *vs.* $(E/\beta R)P(u)$ from n=1 to 2 with a step of 0.01, a series of straight lines through zero will be obtained. The most reasonable exponent n is the one with the highest linear correlation coefficient. Our calculation showed that n=1.36 led to the highest linear correlation coefficient 0.99818 with A=2.35E65 s^{-1} from the slope of the line. The plots of $1/(1-n)-(1-\alpha)^{1-n}/(1-n)$ *vs.* $(E/\beta R)P(u)$at n=1.36 at various heating rates and their linear-fitting drawing through the zero point are shown in Fig. 4, respectively. All the apparent kinetic parameters that determined during the main stage of BSA aggregation are summarized in Table 4, and the reported E value corresponded to the average of E values, which were calculated in the α range 0.2–0.8.

Kinetic triplets for the thermal denatured aggregation of BSA solutions in the presence of sucrose (5–15 mass%)

The same procedures were repeated for the denatured aggregation of BSA solutions in the presence of sucrose (5–15 mass%). It is found that the activation energies are nearly independent of conversion. The mean of them is 437.62±7.84 kJ mol^{-1} for in the presence of 5 mass% sucrose and 468.29±6.63 kJ mol^{-1} for in the presence of 15 mass% sucrose. The facts indicated that there exists a high probability for the presence of a single-step reaction for the denatured aggregation of BSA solutions with 5, 15 mass% sucrose, respectively. Further calculations showed that the class of kinetic models, F_n, best described for the denatured aggregation of BSA solutions with 5, 15 mass% sucrose, too. Their logarithmic values of preexponential factors, kinetic exponents, and corresponding local heating rates are also presented in Table 4.

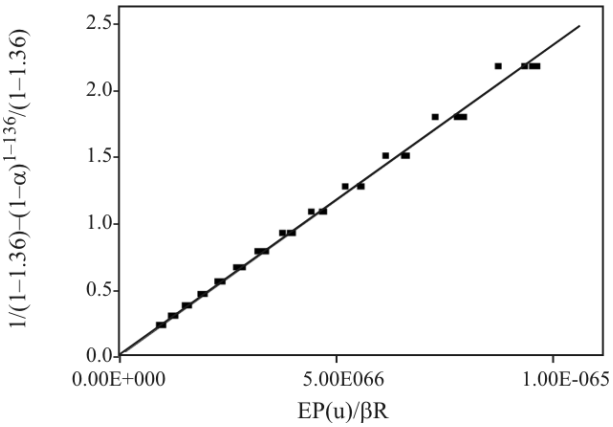

Fig. 4 Plotting $1/(1-n)-(1-\alpha)^{1-n}/(1-n)$ *vs.* $(E/\beta R)P(u)$ at n=1.36 for BSA denatured aggregation in the absence of sucrose at various heating rates and their linear-fitting drawing (solid line)

It can be concluded from Table 4 that the possible mechanisms for the denatured aggregation of BSA solutions in the presence of sucrose (0–15 mass%), are simple order reaction. Compared with an idealized Avrami–Erofeyev equation, a nonintegral value of kinetic exponent n is more appropriate to describe the actual process. The activation energy (E), pre-exponential factor (A) of the thermal transition and the denaturation temperature (T_m) increased with the increase of sucrose concentration

Table 4 Kinetic model and parameters for the BSA denatured aggregation in the presence and absence of sucrose

Sucrose/mass%	E/kJ mol^{-1}	A/s^{-1}	n	r	$G(\alpha)$
0%	430.77±10.42[a]	2.35E65	1.36	0.99818	$\frac{1}{1-1.36}-\frac{(1-\alpha)^{1-1.36}}{1-1.36}$
5%	437.62±7.84	2.50E66	1.57	0.99923	$\frac{1}{1-1.57}-\frac{(1-\alpha)^{1-1.57}}{1-1.57}$
15%	468.29±6.63	5.16E70	1.59	0.99704	$\frac{1}{1-1.59}-\frac{(1-\alpha)^{1-1.59}}{1-1.59}$

[a]Means±standard deviations. Standard deviations $S=Sqr(\Sigma(X-M)^2/(n-1)$, where Σ=sum of, X=individual score, M=mean of all scores, n=sample size (number of scores)

(Tables 1 and 4). This suggested that sucrose concentration affected BSA aggregation substantially.

Conclusions

This study has shown that all the thermal denaturation and aggregation transitions of BSA were found calorimetrically irreversible in the absence and presence of sucrose. Sucrose increased the thermal denaturation temperature of BSA and it changed kinetic triplets activation energy (E), the approximate order of reaction, and pre-exponential factor (A). A simple order reaction model could satisfactorily describe the kinetics of BSA denatured aggregation at high concentration in the presence of sucrose (0–15 mass%). The process studied in this paper did not follow rigorously first-order kinetic model or other integral order reaction models. The differential scanning calorimetric results on transition temperatures and nonisothermal kinetics analysis of the BSA aggregation in the presence of sucrose (0–15 mass%) indicated the substantial impact of sucrose on BSA denatured aggregation. The remarkable stabilization effect of sucrose was attributed to its ability to alter BSA aggregation kinetics.

This study represented the combination of iso-conversional method and the master plots method could be used to model the denatured aggregation mechanism of the protein satisfactorily.

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