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THE EFFECT OF GLUCOSE ON BOVINE SERUM ALBUMIN DENATURED AGGREGATION KINETICS AT HIGH CONCENTRATION
The master plots method study by DSC

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The effect of glucose (0–15 mass%) on the kinetics of bovine serum albumin (BSA) denatured aggregation at high concentration in aqueous solution has been studied by differential scanning calorimetry. The observed denatured aggregation process was irreversible and could be characterized by a denaturation temperature (T_m), apparent activation energy (E_a), the approximate order of reaction, and pre-exponential factor (A). As the glucose concentration increased from 0 to 15 mass%, T_m increased, E_a also increased from 514.59409±6.61489 to 548.48611±7.81302 kJ mol^{-1}, and A/s^{-1} increased from 1.24239E79 to 5.59975E83. The stabilization increased with an increasing concentration of glucose, which was attributed to its ability to alter protein denatured aggregation kinetics.

The kinetic analysis was carried out using a composite procedure involving the iso-conversional method and the master plots method. The iso-conversional method indicated that denatured aggregation of BSA in the presence and absence of glucose should conform to single reaction model. The master plots method suggested that the simple order reaction model best describe the process. This study shows the combination of iso-conversional method and the master plots method can be used to quantitatively model the denatured aggregation mechanism of the BSA in the presence and absence of glucose.

Keywords: *BSA, denatured aggregation, glucose, DSC, iso-conversional method, master plots method, simple order reaction model*

Introduction

Proteins often exist in their physiological environment at high concentrations or in crowded environments. 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 [1].

BSA is a relatively large globular protein (66 kDa) with well-characterized physiochemical properties [2, 3]. Such the experimental and mechanistic aspects of the heat, scanning rate, pressure, changes in pH or ionic strength as denaturing agents and additives induced denaturation and aggregation of proteins were previously reported by different kinds of investigations [4–25], very little light was shed on the kinetic aspect of these processes, which BSA is at high concentration. Previous studies have shown that sugars increase the thermal denaturation temperature of globular proteins in aqueous solutions [12, 26–30]. Sugars can alter the protein gelation mechanism in a number of ways [31–35]. Recent experimental and theoretical studies indicate that the major cause of the stabilizing effect of sugar molecules on thermal unfolding of globular proteins is the excluded volume effect [36]. Baier [37] studied binary cosolvent systems (glycerol–sucrose mixtures) on the heat-induced gelation mechanism of bovine serum albumin, but didn't give the process activation energy (E_a). Baier [38] study the influence of sugars on the thermal stability and gelation of food proteins by using a well-defined globular protein system (BSA) and by using kinetic and thermodynamic models to interpret the data. But the gained E_a, the approximate order of reaction, and pre-exponential factor (A) were suspicious. Because the BSA concentration was so high and they still used Sanchez-Ruiz, Lopez-Lacomba, [39] method. In the same time only one scanning rate and one method were employed which were more insufficient than Sanchez-Ruiz, theory. The estimations based on fitting data to single step kinetic models work poorly, and tend to be misleading [40]. More and more evidences show that kinetic methods that use single heating rate data are very limited in their applicability. Sugars are well-known protein stabilizers, but their mechanisms are still under discussion. In this study, we focus on the influence of glucose on the kinetics of BSA dena-

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tured aggregation at high concentration in aqueous solution by the integral 'model-free' method [41].

Several authors have developed suitable theoretical models for interpreting protein degradation during DSC experiments. The model studied by Lepock *et al.* [42] included three rate constants for unfolding, refolding and degradation pathways, while that developed by Sánchez-Ruíz [43] assumed an equilibrium between the folded and unfolded fractions of protein. But kinetic analysis of the denatured aggregation of protein at high concentration is very complex. Generally speaking, correlative parameters can be obtained starting from model and combining experiment result. But it is necessary to emphasize that setting up model must depend on experiment result. The kinetic integral 'model-free' method can gain kinetic parameters starting from experiment result. And the master plots method will give more insight into the mechanisms. Analysis of the activation energy dependency will provide important clues on reaction mechanism [44–47]. Also, the ability of isoconversional methods to reveal the reaction complexity is, therefore, a crucial step toward the ability to draw mechanistic conclusions from kinetic data. Theoretical master plots are reference curves depending on the kinetic models but generally independent of kinetic parameters of the process. Experimental master plots constructed on the basis of experimental data are independent from the temperature schedules. Comparing experimental master plots with theoretical ones allows us to choose the appropriate kinetic model of the process under investigation, at least, of the type of appropriate kinetic models, without doubt [48]. The knowledge of kinetic model, deduced from such a simple graphical method, is very helpful for further detailed kinetic analysis by avoiding a probable miscalculation of kinetic parameters due to wrong model being assumed.

The aim of this work was to study the effect of glucose (0–15 mass%) on and develop kinetic models for the kinetics of BSA denatured aggregation at high concentration in aqueous solution by the master plots method with differential scanning calorimetry.

Theoretical approach

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

$$G(\alpha)=\left(\frac{AE_a}{\beta R}\right)P(u) \qquad (1)$$

where α is the extent of conversion, β the linear heating rate, $f(\alpha)$ or $G(\alpha)$ the function of degree of conversion in a differential form or an integral form, and $P(u)=\int_{\infty}^{u}-(e^{-u}/u^2)du$, $u=E_a/RT$. 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}], ΔH_{tot} total peak area [J g^{-1}]. Because the exponential integral, $P(u)$, has no analytical solution. An approximation formula of high accuracy [49] is used.

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

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

$$\ln\left(\frac{\beta}{T^{1.894661}}\right)=\ln\left[\frac{AE_a}{RG(\alpha)}\right]+3.635041-$$
$$-1.894661\ln E_a-1.001450\frac{E_a}{RT} \qquad (3)$$

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 $-1.00145033E_a/R$. Then, the apparent activation energy E_a can be calculated from the slope. Inserting $\alpha=0.5$ into Eq. (1), one can get:

$$G(0.5)=\left(\frac{AE_a}{\beta R}\right)P(0.5) \qquad (4)$$

where $u_{0.5}=E_a/RT_{0.5}$, $T_{0.5}$ is the temperature when α equals to 0.5. When Eq. (1) is divided by Eq. (4), the following equation is obtained:

$$\frac{G(\alpha)}{G(0.5)}=\frac{P(u)}{P(u_{0.5})} \qquad (5)$$

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_a 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. So this 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 form the slope of the plot of $G(\alpha)$ *vs.* $E_aP(u)/\beta R$.

Experimental

Materials

BSA (A0281, ≥99% (agarose gel electrophoresis)) was purchased from Sigma, which is essentially fatty acid free, essentially globulin free and lyophilized

powder. Analytical grade glucose (SCRC63005518, AR) was used. Distilled and deionized water was used for the preparation of all solutions. The pH of the BSA solution with 150 mM NaCl used in this study was measured by a pH meter (Orion 828) to be 6.89.

Methods

Differential scanning calorimetry (DSC)

The influence of glucose on the thermal denaturation and aggregation of BSA solutions was measured using a differential scanning calorimeter (Mettler Toledo calorimeter, model DSC 822^e). The instrument was calibrated with indium. Initially, a 20 mg mL^{-1} BSA solution was prepared by dispersing powdered protein into a glucose solution (0–15 mass%) contain 150 Mm NaCl and stirring for at least 2 h. The protein solution (80 μL) was then placed in the sample cell (100 μL medium pressure crucibles) of the DSC instrument, and a glucose solution with the same glucose concentration was placed in the reference cell. Scanning calorimetry was performed with the Star® evaluation program, at different heating rates of 1.0, 1.5, 2, 2.5 K min^{-1}, in the temperature range 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 [50]. The first scan was therefore of native protein, whereas the second scan was of heat-treated protein. Measurements were carried out on two or three separate samples (replicates) and reported as the average.

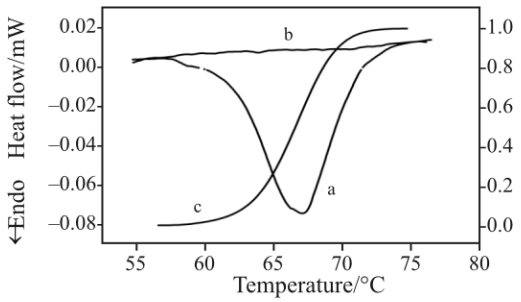

Fig. 1 DSC curve for 20 mg mL^{-1} BSA in 150 mM NaCl aqueous solution (pH=6.89) scanned at 2 K min^{-1}, after baseline subtraction and calculation of BSA denatured aggregation conversion (α) by the Star® software using the integral tangential baseline. The a represents native protein, the b represents heattreated protein and c is the conversion graph

Results and discussion

The scan of the native protein exhibited an endothermic transition between 55 and 80°C, which had a single peak at ~67°C, whereas the scan of the heat-treated protein exhibited no thermal transition

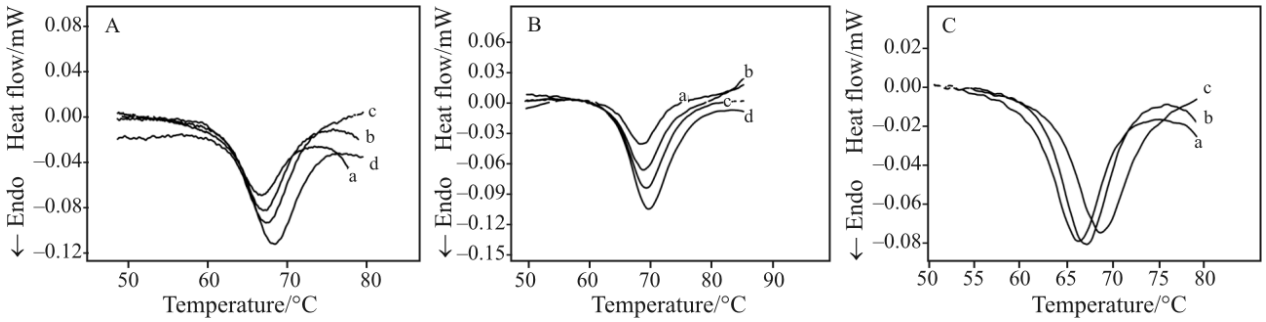

Fig. 2 A – DSC curves for the influence of glucose (5 mass%) on the thermal denatured aggregation of 20 mg mL^{-1} BSA (solution pH=6.89) at a – 1, b– 1.5, c – 2 and d – 2.5 K min^{-1} heating rates; B – 20 mg mL^{-1} BSA with 15 mass% glucose at: a – 1, b – 1.5, c – 2 and d – 2.5 K min^{-1} and C – influence of glucose concentration on the temperature dependence of the heat flow of 20 mg mL^{-1} BSA solutions; a – 0, b – 5 and c – 15% glucose, heating rate 1.5 K min^{-1}.

Table 1 20 mg mL^{-1} BSA T_m in the influence of glucose (0–15 mass%) at different scan rates

Scanning rate/K min^{-1}	1	1.5	2	2.5
Glucose content/mass%	T_m/°C			
0	65.81+(–0.14)	66.14+(–0.085)	66.72+(–0.077)	66.87+(–0.087)
5	66.55+(–0.051)	67.15+(–0.043)	67.46+(–0.003)	68.09+(–0.057)
15	68.17+(–0.135)	68.83+(–0.075)	69.08+(–0.052)	69.55+(–0.095)

(Fig. 1). The repeat curve subtracted from the first measurement curve, which serves as the baseline. These results indicated that BSA was irreversibly denatured during the heating process. The conversion (α) was calculated by the Star® software, and the conversion graph and table (inclusion the corresponding denaturation temperature (T_d)) were also displayed. A typical DSC curve and the conversion graph are also displayed in Fig. 1. Via the conversion graph C, the α–T data was immediately obtained.

DSC curves at various heating rates are shown in Fig. 2. A characteristic endothermic transition was detected in all experiments. All the thermal denaturation and aggregation transitions were found calorimetrically irreversible as reflected by the lack of transition in the second run of all the samples. The influence of glucose concentration on the temperature dependence of the heat flow of 20 mg mL^{-1} BSA solutions is also displayed in Fig. 2. Concentrated BSA formed a transparent gel when DSC endothermic peak was achieved. And from the Figs 2A and B it can also be found that the net enthalpic changes were always endothermic, and of a greater magnitude at faster heating rates. This was interpreted to infer that denatured aggregation at lower heating rates led to formation of a gel structure in which potential bondings were more completely accomplished [35]. The thermal transition temperature (T_m), defined as the temperature at which a maximum occurred in the endothermic peaks, was recorded for each analysis after baseline correction and reproducible to within 0.2°C. The T_m at different rates was listed in Table 1. The T_m is dependent on the scan rate and glucose concentration – an increase in the T_m with increasing scan rates and glucose concentration.

Due to denaturation, hydrophobic interaction can occur, and exposed thiol groups can form disulfide bonds, which result in irreversible behavior [51, 52]. For all of the glucose concentrations used (0–15 mass%) the heat-treated protein showed no thermal transition, which indicated that BSA was irreversibly denatured by the heating process in the presence and absence of glucose. The maximum in the DSC scans (T_m) shifted to higher temperatures as the glucose concentration increased from 0 to 15 mass% (Table 1). The rise in T_m was attributed to the increased thermal stability of the globular state of BSA relative to its native state.The stabilization increased with an increasing concentration of glucose which was attributed to its ability to alter protein denatured aggregation kinetics. 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. 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 [34, 53, 54].

Non-isothermal kinetics for the denatured aggregation of BSA solutions in the absence of glucose. Iso-conversional method for estimating activation energy dependence

It is well known that the iso-conversional method easily gives estimate of activation energy regardless of reaction mechanism [40, 55]. Using the α–T data obtained from DSC conversion plots, according to Eq. (3), apparent activation energy of the thermal denatured aggregation of the BSA was estimated from the isoconversional plot of $\ln(\beta/T^{1.894661})$ *vs.* $1/T$ (Eq. (3)) at several degrees of conversion within α=0.2–0.8 [49]. All plots had fairly high linear correlation coefficients greater than 0.99. As shown in Fig. 3, the value of activation energy hardly varied with the degree of conversion and the average value of activation energies is 514.59409± 6.61489 kJ mol^{-1}. Due to little dependence of the activation energy on the extent of conversion, a simple reaction mechanism may be used for reaction progressing [56, 57].

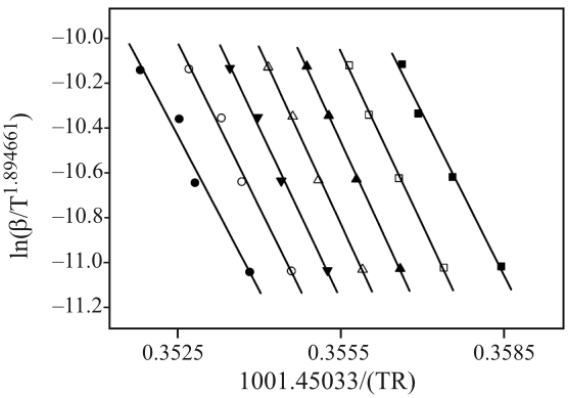

Fig. 3 Plots for determination of activation energy of 20 mg mL^{-1} BSA denatured aggregation in the absence of glucose at α: ■ – 0.2, □ – 0.3, ▲ – 0.4, Δ – 0.5, ▼ – 0.6, ○ – 0.7 and ● – 0.8. Solid lines are linear fitting corresponding to different α

Master-plots method for determining kinetic model

Using the predetermined value of E_a, along with the temperature measured as a function of heating rate, the experimental master plots for DSC data of BSA was constructed according to Eqs (2) and (5). The experimental master plots of $P(u)/P(u_{0.5})$ *vs.* α constructed from experimental data under different heating rates are

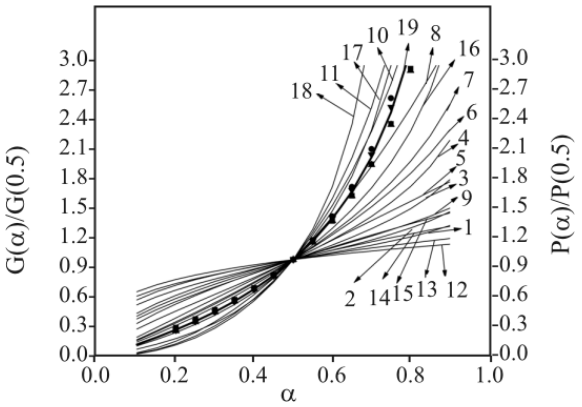

Fig. 4 Master plots of theoretical $P(u)/P(u_{0.5})$ *vs.* α for various reaction models (solid curves, as enumerated in [41], and curve 19 represents function $G(\alpha)=\frac{1}{1-1.6}-\frac{(1-\alpha)^{1-1.6}}{1-1.6}$) and experimental data for the BSA denatured aggregation in the absence of glucose at ▲ – 1, ▼ – 1.5, ■ – 2 and ● – 2.5 K min^{-1} heating rates

shown in Fig. 4. The theoretical master plots of various kinetic functions [58] are also shown in Fig. 4.

It is shown that all of these experimental master plots match with each other closely. These facts indicated that a single kinetic model could describe the observed kinetics process of BSA denatured aggregation. 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,

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

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 participate in the system.

Evaluation of pre-exponential factor and kinetic exponent

The accommodated F_n model with a non-integral exponent, which could describe the denatured aggregation process, was suggested by the comparison of the experimental master plots with theoretical ones. Also, 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

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

In Eq. (6), $P(u)$ can be calculated according to Eq. (2). Plotting

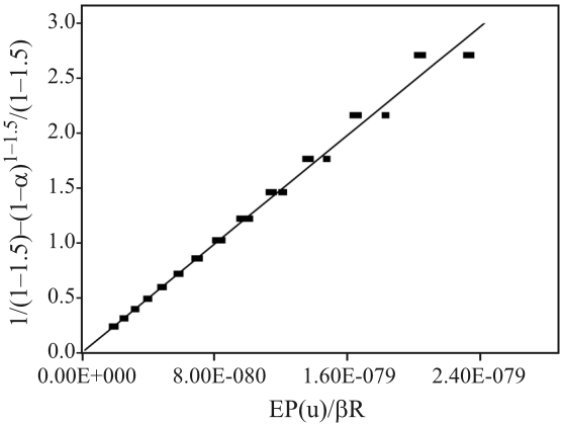

Fig. 5 Plotting $\frac{1}{1-n}-\frac{(1-\alpha)^{1-n}}{1-n}$ *vs.* $\frac{E_a}{\beta R}P(u)$ at n=1.6 for BSA denatured aggregation in the absence of glucose at various heating rates and their linear-fitting drawing (solid line)

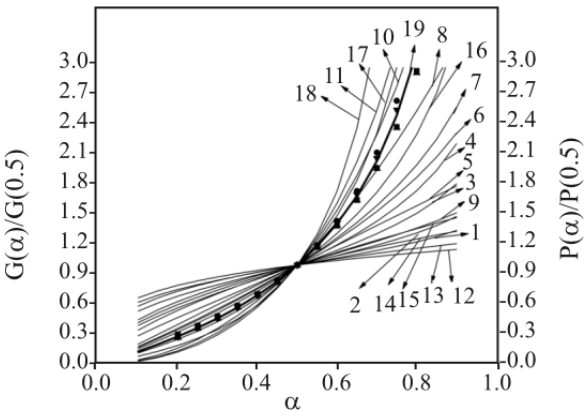

Fig. 6 Master plots of theoretical $P(u)/P(u_{0.5})$ *vs.* α for various reaction models (solid curves, as enumerated in [41], and curve 19 represents function $G(\alpha)=\frac{1}{1-1.7}-\frac{(1-\alpha)^{1-1.7}}{1-1.7}$) and experimental data for the BSA denatured aggregation in the presence of 5 mass% glucose at ▲ – 1, ▼ – 1.5, ■ – 2 and ● – 2.5 K min^{-1} heating rates

$$\frac{1}{1-n}-\frac{(1-\alpha)^{1-n}}{1-n}\ vs.\ \frac{E_a}{\beta R}P(u)$$

from n=1 to 2 with a step of 0.1, 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=1.6 led to the highest linear correlation coefficient 0.99606 with A=1.24239E79 s^{-1} from the slope of the line. The plots of

$$\frac{1}{1-n}-\frac{(1-\alpha)^{1-n}}{1-n}\ vs.\ \frac{AE_a}{\beta R}P(u)$$

at n=1.6 at various heating rates and their linear-fitting drawing through the zero point are shown in Fig. 5, respectively.

The approximate order of reaction was found to be 1.6, suggesting that a mixture of first- and second-order reactions participate in the system [59].

Kinetic parameters for BSA denatured aggregation

The DSC conversion data of BSA denatured aggregation had also been subjected to iso-conversional method for estimating activation energy dependence. Little dependence of the activation energy on the extent of conversion for the BSA denatured aggregation indicated that no change in reaction mechanism took place with reaction progressing in a high probability. Furthermore, their most possible kinetic models were determined by using the master plots method. Also, their pre-exponential factors and kinetic exponents were evaluated. All the kinetic parameters determined during the main stage of BSA denatured aggregation in the presence and absence of glucose were summarized in Table 2, and the reported E_a values corresponding to the average calculated in the α range 0.3–0.7. As shown in Table 2, the class of kinetic models, F_n, can describe the denatured aggregation process of BSA.

Kinetic triplets for the thermal denatured aggregation of BSA solutions in the presence of glucose (0–15 mass%)

The same procedures were followed for the denatured aggregation of BSA solutions in the presence of glucose (0–15 mass%). It is found that the activation energies are nearly independent of conversion and the mean activation energies are 525.86935±8.73981 and 548.48611±7.81302 kJ mol^{-1} respectively. These facts indicate that there exists a high probability for the presence of a single-step reaction for the denatured aggregation of BSA solutions with 5, 15% glucose, respectively. Their experimental master plots of $P(u)/P(u_{0.5})$ *vs.* α constructed from experimental data and theoretical master plots are shown in Figs 6 and 7, respectively. Figures 6 and 7 indicate that the class of kinetic models, F_n, best describes with the most probable kinetic models for the denatured aggregation of BSA solutions with 5, 15% glucose, also. Their logarithmic values of preexponential factors, kinetic exponents, and corresponding local heating rates are also presented in Table 2.

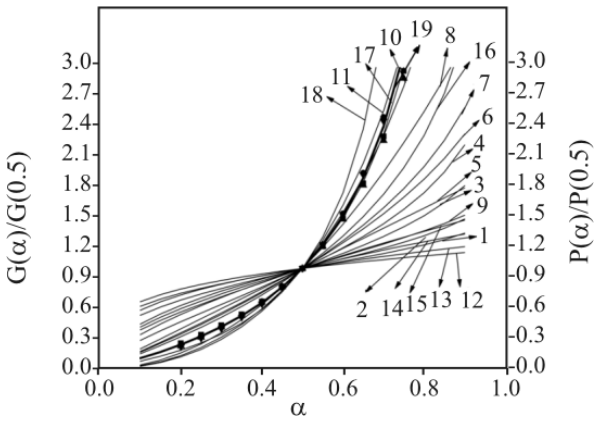

Fig. 7 Master plots of theoretical $P(u)/P(u_{0.5})$ *vs.* α for various reaction models (solid curves, as enumerated in [41], and curve 19 represents function $G(\alpha)=\frac{1}{1-2.1}-\frac{(1-\alpha)^{1-2.1}}{1-2.1}$) and experimental data for the BSA denatured aggregation in the presence of 15 mass% glucose at ▲ – 1, ▼ – 1.5, ■ – 2 and ● – 2.5 K min^{-1} heating rates

It can be concluded from Table 2 that the possible mechanisms for the denatured aggregation of BSA solutions in the presence of glucose (0–15 mass%), are simple order reaction. The measurements suggest that the possible forms of $G(\alpha)$ for the denatured aggregation of BSA solutions in the presence of glucose (0–15 mass%) are

For: in the absence of glucose,

$$\frac{1}{1-1.6}-\frac{(1-\alpha)^{1-1.6}}{1-1.6}$$

for in the presence of 5 mass% glucose, and

$$\frac{1}{1-1.7}-\frac{(1-\alpha)^{1-1.7}}{1-1.7}$$

for in the presence of 15 mass% glucose.

Table 2 Kinetic model and parameters for the BSA denatured aggregation in the presence and absence of glucose

Glucose content/mass%	E_a/kJ mol^{-1}	A/s^{-1}	n	r	$G(\alpha)$
0%	514.59409±6.61489	1.24239E79	1.6	0.99606 ó	$\frac{1}{1-1.6}-\frac{(1-\alpha)^{1-1.6}}{1-1.6}$
5%	525.86935±8.73981	4.60538E80	1.7	0.99814	$\frac{1}{1-1.7}-\frac{(1-\alpha)^{1-1.7}}{1-1.7}$
15%	548.48611±7.81302	5.59975E83	2.1	0.9966	$\frac{1}{1-2.1}-\frac{(1-\alpha)^{1-2.1}}{1-2.1}$

$$\frac{1}{1-2.1}-\frac{(1-\alpha)^{1-2.1}}{1-2.1}$$

Compared with an idealized Avrami–Erofeyev equation, a non-integral value of kinetic exponent n is more appropriate to describe the actual process. The activation energy (E_a), pre-exponential factor (A) of the thermal transition and the transition temperature (T_m) increased as the sucrose concentration in the aqueous phase increased (Tables 1 and 2).

Conclusions

This study has shown that only one characteristic single endothermic transition was detected in all experiments. All the thermal denaturation and aggregation transitions were found calorimetrically irreversible as reflected by the lack of transition in the second run of all the samples. Sucrose increases the thermal denaturation temperature of BSA and it changes kinetic triplets activation energy (E_a), the approximate order of reaction, and pre-exponential factor (A). Considering little dependence of the activation energy on the extent of conversion, it could be postulated that the denatured aggregation of BSA solutions in the presence of glucose (0–15 mass%) is carried through a single-rate process. The master plot method indicated that the most possible kinetic models for the process be described by using an accommodated Avrami–Erofeyev equation, $\frac{1}{1-n}-\frac{(1-\alpha)^{1-n}}{1-n}$. A simple order reaction model can satisfactorily describe the kinetics of BSA denatured aggregation at high concentration in the presence of glucose (0–15 mass%). The process studied in this paper does not follow rigorously first-order kinetic model or other integral order reaction models. The preexponential factors and exact kinetic exponents for the process were finally determined, respectively.

Using the integral composite procedure above, it is possible to describe the kinetic aspects of protein denatured aggregation at high concentration in the presence and absence of influence reagent. By this method, it seems to be easier, valid and less time consuming to estimate the kinetic triplets of non-isothermal protein denatured aggregation at high concentration kinetics satisfactorily.

Acknowledgements

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