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Experimental investigation of the effects of beta-cyclodextrin on the unfolding and aggregation of human serum albumin

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ABSTRACT

Effects of β-cyclodextrin (β-CD) on the unfolding and aggregation of human serum albumin (HSA) was investigated employing isothermal titration calorimetry (ITC) at 300 K in 50mM phosphate buffer solution. β-CD inhibited aggregation and its inhibition was generally in the order of γ-CD< α-CD< β-CD. Hydrophilic β-CD reduced the thermally induced unfolding and it was suggested that β-CD destabilises native HSA or stabilises the unfolded state of HSA. The obtained heats for HSA + β-CD interactions were reported and analysed in terms of the extended solvation model, this model was used to reproduce the enthalpies of HSA interaction with β-CD in a broad range of complex concentration. The parameters δ_A^θ and δ_B^θ reflected to the net effect of β-CD on the HSA stability in the low and high cyclodextrin concentrations, respectively. The positive values for δ_A^θ indicated that β-CD stabilises the HSA structure in low concentrations. Variations of the UV-Vis and fluorescence spectra of HSA showed that β-CD in low concentrations has a strong ability to quench the fluorescence launching from HSA by reacting and forming a certain kind of new compound.

KEYWORDS

Human serum albumin;
Cyclodextrins;
Isothermal titration calorimetry;
Binding parameters.

INTRODUCTION

Human serum albumins are the most abundant proteins in plasma (50%–60% of total amount of plasma proteins) and the main transport proteins; they bind metabolites, endogenous toxins, hormones, etc. Protein stability is a particularly relevant issue in the pharmaceutical field and will continue to gain more importance as the number of therapeutic protein products increases[1,2]. Of these, protein aggregation is the most common problem in protein instability, because the aggregation process is often irreversible and the aggregates sometimes contain high levels of non-native, intermolecular β-sheet structures which have a direct impact on drug potency, immunogenicity and the unfolded protein response[3-5]. Interactions between serum albumin and ligands can provide important information about ligands storage, transportation, evacuation, etc. As a

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result, researches about this subject have attracted the attention of biologists, chemists, pharmacists and therapists[6-12].

Protein unfolding and aggregation are usually trigged by various environmental stimuli such as changes in pH, ionic strength, heating, agitation, light irradiation, adsorption on hydrophobic surface, and the addition of chemicals and organic solvents. A common method for the inhibition of protein denaturation is to add excipients/additives to the protein preparation. Cyclodextrin (CD) is one of the excipients used for the solubilisation or stabilisation of proteins. Cyclodextrin is a cyclic oligosaccharide consisting of 6-8 glucose units. The characteristic of CD molecules is the presence of a hydrophobic cavity and hydrophilic exterior. As a result, CDs can bind to hydrophobic parts of the protein surface leading to an increase the solubility and stability of proteins[13]. Recently, hydrophilic CDs, such as branched β-CDs, have been evaluated as a new class of parenteral drug carriers, because they are highly hydrophilic and have less haemolytic activity than the parent and other hydrophilic CDs[14-16].

Also, it was exhibited that lysozyme (protein/cyclodextrin) is has a more consistent protein biological activity compared to lysozyme in the absence of cyclodextrin[17-20]. On the other hand, as one of the water-soluble cyclic oligosaccharides, β-CD can form inclusion complexes with a large variety of organic and inorganic compounds, which may improve the solubility, stability and bioavailability of guest molecules and be applied particularly to the area of pharmacological science. In the drug–CD–protein ternary system, the insoluble drug was solubilised by the CD hydrophobic cavity[21]. In this work, in order to investigate the effect of β-CD on the unfolding and aggregation of HSA, the binding parameters of the interaction are measured at pH 7 by isothermal titration calorimetry (ITC). To verify the obtained results from ITC, ultraviolet and fluorescence spectra of the system were also determined and analysed.

EXPERIMENTAL

Materials

Disodiumhydrogenphosphate (Na_2HPO_4) and β-cyclodextrin (β-CD) were purchased from Sigma Chemicals Co. HSA was obtained from Sigma-Aldrich (Taiwan, China) and protein concentrations were determined from absorbance measurements at 277 nm in a 1 cm quartz cuvette. All other materials and reagents were of analytical grade, and solutions were made in 50 mM buffer phosphate using double-distilled water. The isothermal titration calorimetric experiments were carried out on a VP-ITC ultra-sensitive titration calorimeter (MicroCal, LLC, Northampton, MA). The microcalorimeter consists of a reference cell and a sample cell of 1.8 mL in volume, with both cells insulated by an adiabatic shield. All solutions were thoroughly degassed before use by stirring under vacuum.

Method

The sample cell was loaded with HSA solution (117.80 μM) and the reference cell contained the buffer solution. The solution in the cell was stirred at 307 rpm by the syringe (equipped with a micro-propeller) once filled with β-CD and later with HSA+ β-CD solution (35.242 mM) to ensure rapid mixing. Injections were started after baseline stability had been achieved. The titration of HSA with β-CD solution involved 30 consecutive injections of the ligand solution; the first injection was 10 μL and the remaining ones were 10 μL. In all cases, each injection was done in 6 s at 3-min intervals. To correct the thermal effects due to β-CD dilution, control experiments were performed in which identical aliquots were injected into the buffer solution with the exception of HSA. In the ITC experiments, the enthalpy changes associated with processes occurring at a constant temperature are measured. The measurements were performed at a constant temperature of 27.0±0.02°C and the temperature was controlled using a Poly-Science water bath. All ultraviolet-visible spectra were recorded in a UV-1100 double beam spectrophotometer (Unico) that was equipped with a temperature regulation system. All fluorescence measurements were carried out on a 100-bio recording spectrophotometer (Perkin-Elmer corporate America).

RESULTS AND DISCUSSION

We have previously shown that the heats of the macromolecules + ligands interactions that is obtained by ITC technique can be reproduced by the following equa-

tion in the aqueous solvent systems[18-29].

$$q = q_{max} x'_B - \delta_A^{\theta}(x'_A L_A + x'_B L_B) - (\delta_B^{\theta} - \delta_A^{\theta})(x'_A L_A + x'_B L_B) x'_B \quad (1)$$

where q is the heat of HSA + β-CD interaction at certain ligand concentrations and q_{max} represents the heat value upon saturation of all HSA. The parameters δ_A^{θ} and δ_B^{θ} exhibit the HSA stability in the low and high β-CD concentrations, respectively. The positive values of δ_A^{θ} and δ_A^{θ} show that HSA is substantially stabilised by β-CD at 27°C. x'_B can be expressed as follows:

$$x'_B = \frac{p x_B}{x_A + p x_B} \quad (2)$$

x'_B is a fraction of bound ligand with the protein molecule and $x'_A = 1 - x'_B$ is the fraction of unbound ligand. x_B can be defined as follows:

$$x_B = \frac{[L]}{[L]_{max}} \quad (3)$$

where $[L]$ is the concentration of ligand after every injection and $[L]_{max}$ is the maximum concentration of the ligand upon saturation of all HSA. The measured heats of HSA+ β-CD interactions, q, with 1 mM β-CD at 300 K in 50 mM phosphate buffer solution of pH=7, and the heats of dilution of β-CD with water, q_{dilut}, from ITC at many injections have been listed in TABLE 1. The heats of HSA + β-CD interactions, q, were fitted to Eq. (1) for all of the β-CD compositions. In the fitting procedure, p was changed until the best agreement between the experimental and calculated data was approached. The schematically experimental and calculated of q values have been shown in Figure 1. If the binding of the ligand at one site increases or decreases the affinity for a ligand at another site, the macromolecule exhibits positive co-operativity (p>1) or negative co-operativity (p<1), respectively. If the ligand binds at each site independently, the binding is non-cooperative (p=1). L_A and L_B are the relative unbound and bound ligand contributions to the heats of dilution in the absence of HSA. L_A and L_B can be calculated from the heats of dilution of β-CD in water (q_{dilut}) as follows:

$$L_A = q_{dilut} + x_B \left(\frac{\partial q_{dilut}}{\partial x_B} \right) \quad (4)$$

$$L_B = q_{dilut} + x_A \left(\frac{\partial q_{dilut}}{\partial x_B} \right) \quad (5)$$

δ_A^{θ} and δ_A^{θ} parameters have also been optimised to fit the data. The optimised δ_A^{θ} and δ_A^{θ} values are recovered from the coefficients of the second and third terms of Eq. (1). The small relative standard coefficient errors and the high r^2 values (0.99999) support this method. The binding parameters for HSA+ β-CD interactions recovered from Eq. (1) are listed in TABLE 2.

TABLE 1 : Heats of HSA+ β-CD interactions, q, with 1 mM β-CD at 300 K in 50 mM phosphate buffer solution of pH=7, and the heats of dilution of β-CD with water, q_{dilut}, from ITC at many injections (The precision is ± 0.1 μJ or better).

$[\beta\text{-CD}]_T$ / μM	$[HSA]_T$ / μM	q / μJ	q_{dilut} / μJ	$[\beta\text{-CD}]_T$ / μM	$[HSA]_T$ / μM	q / μJ	q_{dilut} / μJ
387.27	116.50	-908.45	31.83	2202.62	110.44	-3886.93	472.88
577.74	115.87	-1464.31	42.59	2373.81	109.87	-4050.16	525.13
766.13	115.24	-1896.26	65.81	2543.24	109.30	-4199.74	575.39
952.49	114.61	-2259.91	97.21	2710.92	108.74	-4338.21	625.28
1136.84	114.00	-2578.08	137.04	2876. 90	108.18	-4462.71	676.54
1319.22	113.39	-2855.53	185.31	3041.19	107.63	-4584.38	730.44
1499.66	112.79	-3103.22	240.36	3203.82	107.10	-4689.19	787.49
1678.19	112.19	-3322.79	299.41	3364.81	106.55	-4786.24	847.29
1854.84	111.60	-3525.77	359.32	3524.20	106.02	-4872.12	908.66
2029.64	111.01	-3713.72	417.59	3682.00	105.43	-4959.55	970.02

For a set of identical and independent binding sites, it is possible to use Eq. (6) for the calculation of K_d and g as follows:

$$\frac{\Delta q}{q_{max}} M_0 = \left(\frac{\Delta q}{q}\right) L_0 \frac{1}{g} - \frac{K_d}{g} \quad (6)$$

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where $\Delta q = q_{max} - q$. q represents the heat value at a certain ligand and biomolecule concentration, M_0 and L_0 are total concentrations of HSA and ligand, respectively, q_{max} represents the heat value upon saturation of all HSA molecules, and K_d is the dissociation equilibrium constant for the equilibrium:

$$\mathbf{M + L \Leftrightarrow ML}, \qquad \mathbf{K_d = \frac{[M][L]}{[ML]}} \qquad (7)$$

If q and q_{max} are calculated per mole of biomacromolecule, then the molar enthalpy of binding for each binding site (ΔH) will be: $\mathbf{\Delta H = \frac{q_{max}}{g}}$.

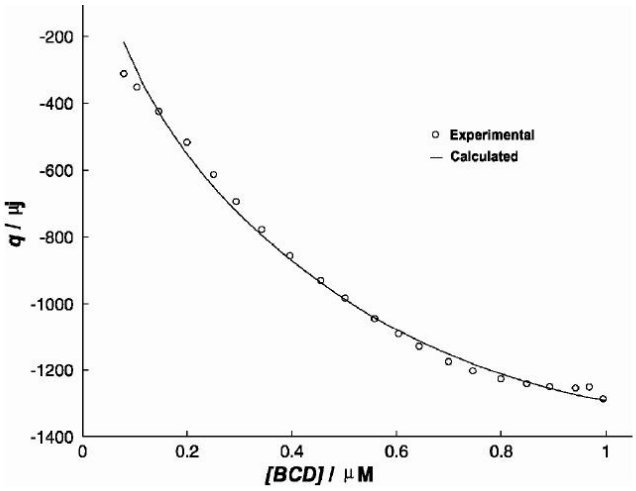

Figure 1 : Comparison between the experimental heats, *q*, (o), for HSA + β-CD interactions and calculated data (lines) via Eq. (1). *[β-CD]* are concentrations of β-CD solutions in μM at *pH*=7.

A non-linear least squares computer program has been developed to fit data into Eq. (1). The best correlation coefficient $R^2 \approx 1$ showed good support for the use of Eq. (1). K_a is the product for equilibrium:

$$\mathbf{HSA(H_2O)_g + i\,(\beta CD) \Leftrightarrow HSA(H_2O)_{g-i}\,(\beta CD)_i} \qquad (8)$$

The standard Gibbs free energy, $\Delta G°$, can be calculated from the association constant $(K_a = \frac{1}{K_d})$ as follows:

$$\mathbf{\Delta G^0 = -RT \ln K_a} \qquad (9)$$

Where K_a is the association equilibrium constant as a function of β-CD concentrations. Therefore, for the first time, we managed to calculate ΔG and ΔS values using one set of experimental data at one temperature. All thermodynamic parameters of ligand binding to HSA are summarised in TABLE 2. p=1 indicates that the binding is non-cooperative in nine binding site. The positive value of δ_A^θ shows that β-CD stabilizes the HSA structure in low concentration. The binding process for HSA+ β-CD interaction is enthalpy and entropy driven, indicating that electrostatic interaction plays an important role in the interaction of HSA with β-CD.

TABLE 2 : Binding parameters for HSA+ β-CD interaction recovered from Eq. (1) at *pH*=7.

P	1.00±0.01	ΔH / kJmol^{-1}	-4.25±0.03	δ_A^θ	3.56±0.070
g	8.90±0.03	ΔG / kJmol^{-1}	16.33±0.03	δ_B^θ	0.023±0.02
K_a / M^{-1}	697.32±12.08	ΔS / kJmol^{-1}K^{-1}	0.04±0.001		

UV–Vis absorption measurements were found to be a very convenient method to explore the structural change[30] and formation of a complex[31]. The typical absorption peaks of HSA centre at 235 nm and 279 nm (the absorption at 235 nm was not given in Figure 2 since the absorption at 235 nm was too strong to give a useful message). From Figure 2 we can see that the absorption peaks of the mixed solutions are not the simple superposition of the characteristic absorption peaks corresponding to the β-CD and HSA alone. This means that the HSA + β-CD supramolecular complex could form in aqueous buffer solution. The HSA structure is stabilized in the low concentration of the β-CD and for high concentration of β-CD is vice versa.

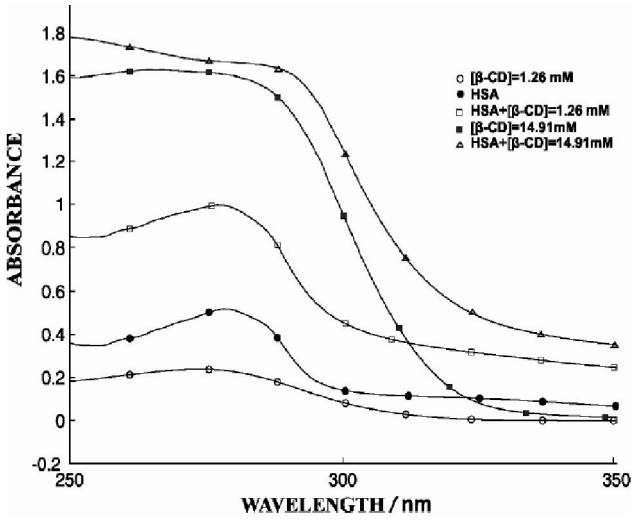

Figure 2 : Comparison between absorption spectrum of HSA in different concentrations of β-CD in phosphate buffer (50 mM), *pH*=7 and (*T*=300 K).

The fluorescence of HSA has been quenched for reacting with β-CD and forms a certain kind of new compound. HSA has three intrinsic fluorophores, including tryptophan, tyrosine, and phenylalanine residues, among which tryptophan mainly contributes to the intrinsic fluorescence of HSA[32].

In this work, the concentrations of HSA in the solutions were stabilised at 117.8 μM, and the concentrations of β-CD varied from 0 to 14.96 mM. The effect of β-CD on HSA fluorescent intensity at 300K is shown in Figure 3. It can be observed from this figure that a decreasing progression in the fluorescent intensity has been caused by the addition of β-CD; thus, it can quench the fluorescence of HSA at a low concentration.

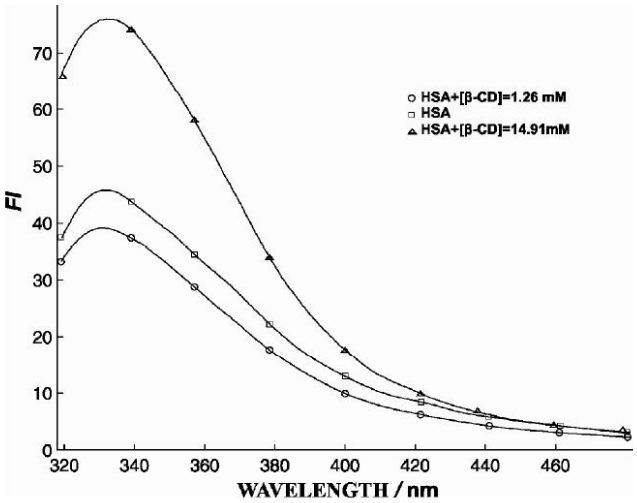

Figure 3 : The fluorescence spectra of HSA in presence of different concentrations of β-CD in phosphate buffer (50 mM), *pH*=7 and (*T*=300 K).

Earlier research demonstrated that fluorescent quenching is the decrease of fluorescent quantum yield from a fluorophore induced by a variety of molecular interactions with a quencher molecule[33]. Therefore, the quenching of HSA fluorescence indicates that the formation of a complex between the β-CD and HSA occurs. HSA contains only one tryptophan residue (214-Trp), which makes the main contribution to the fluorescent intensity of HSA in the selected wavelength range. In Figure 3, a small, but tangible red shift can be observed, which indicates that the 214-Trp residue was placed in a less hydrophobic (or more polar) environment that is more exposed to the solvent[34]. The absorption peaks of the mixed solutions in HSA+*[β-CD]*=1.26 mM are more than the simple superposition of the characteristic absorption peaks corresponding to the *[β-CD]*=1.26 mM and HSA alone, and for the mixed solutions in HSA+*[β-CD]*=14.91 mM is vice versa. This means that the HSA +β-CD supramolecular complex could form in aqueous buffer solution and β-CD stabilizes the HSA structure in the low concentration of the β-CD.

CONCLUSIONS

The inhibitory effect of β-CD may be due to a better fit of its cavity to aromatic amino acids which are involved in aggregation or in protein folding. Hydrophobic side chains of the protein interact with the cavity of β-CD leading to a hydrophilic covering layer which increases protein stability. The results showed that the higher anti-aggregation property was obtained with both the cavity size of CDs and the presence of appropriate substituent on the CD rim, indicating that they are important in the inhibition of protein aggregation. However, such interactions may affect the overall three-dimensional structure of the protein. The thermodynamic parameters for the inclusion process of β-CD with HSA gathered in TABLE 2 indicated that the inclusion complex can spontaneously form in the buffer solution through an enthalpy-driven process. The value of "H is mainly divided by two factors: (a) The polar groups of ligand molecules and those on the surface of protein molecules partly destroy their hydration layers when the ligand molecules approach the macromolecules, which cause an endothermic effect, and (b) the directly electrostatic interaction of dipole groups of the ligand molecules with peptide sections of protein molecules, which causes an exothermic effect. The experimental negative enthalpy change indicated that factor (b) is evidently stronger than factor (a) for this type of binding. The change in entropy of the binding process is thought to primarily depend on the following factors: Firstly, the direct interaction between the hydrophilic groups of the macromolecules and the ligand molecules, or the hydrophobic interaction of the drug molecules with the inner part of the binding site; Secondly, the release of combined water molecules to the buffer medium, including those from the cavities in the macromolecules, hydration layer structure on the surface of

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protein molecule and iceberg structure surrounding the hydrophobic part of the ligand molecule. Generally, the first interaction leads to a decrease in entropy, while the second leads to the opposite change in entropy. By considering the two aspects together, it could be easily understood that the latter factor becomes dominant for this type of binding. δ_A^θ and δ_A^θ values are positive and reflect to the net effect of β-CD on the HSA stability in the low and high dextrin concentrations, respectively. The positive values for δ_A^θ indicate that β-CD stabilises the HSA structure at low concentrations. The small (negative) value of δ_A^θ show that β-CD destabilises the HSA structure at high concentrations of β-CD. The obtained results indicated that there are nine binding sites with non-co-operativity for HSA+ β-CD interactions and the negative value of molar enthalpy and the positive value of molar entropy suggest that the binding process for the inhibition of HSA aggregation at the set of binding sites was both enthalpy- and entropy-driven, but that electrostatic interaction plays an important role in the binding processes. It is possible to describe the activity of HSA with the δ^θ_A and δ^θ_B values. The greater values of δ^θ_A and δ^θ_B cause the greater biological activity of HSA and vice versa. The results indicated that the use of β-CD for protein stabilisation is dependent not only on the structure and properties of β-CD but also on the concentration of β-CD; therefore, the most appropriate β-CD concentration should be used for stabilisation.

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