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DMSO 对鸡蛋白溶菌酶溶液变性的影响

方盈盈[1] 胡新根[1,*] 于 丽[2] 李文兵[1] 朱玉青[1] 余 生[1]
([1] 温州大学化学与材料科学学院, 浙江 温州 325027; [2] 山东大学胶体与界面化学教育部重点实验室, 济南 250100)

摘要: 利用差示扫描量热(DSC)和温度调制差示扫描量热(MDSC)研究了鸡蛋白溶菌酶在纯水及二甲基亚砜(DMSO)/水混合溶剂中的热变性过程, 探讨了酶的浓度、扫描速率和共溶剂的含量对热变性行为的影响规律. 在纯水溶液中, 溶菌酶的变性焓(ΔH_m)随酶浓度的增大而增大. 而在 DMSO/水混合溶剂中, 变性温度(T_m)随 DMSO 体积分数的增大向低温方向移动, 变性峰变低变宽; 当 DMSO 体积分数达到 70%后, 热变性曲线变成了一条光滑的直线. 另外, 在纯水溶液中溶菌酶的 MDSC 图除了出现 DSC 中可观察到的主吸热峰(I)外, 在峰(I)的前面还出现一个小而对称的吸热峰(II), 并且当体系中有 DMSO 存在时也未能观察到此峰. 当溶菌酶浓度增大时, T_m(II)移向低温, ΔH_m(II)减小, T_m(I)与 T_m(II)之间的距离变长. 吸热峰(II)的出现被认为是由于水溶液中溶菌酶二聚体的可逆离解造成的.

关键词: DSC; MDSC; DMSO; 鸡蛋白溶菌酶; 热变性
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Effect of DMSO on Denaturation of Hen Egg-white Lysozyme in Aqueous Solution

FANG Ying-Ying[1] HU Xin-Gen[1,*] YU Li[2] LI Wen-Bing[1] ZHU Yu-Qing[1] YU Sheng[1]
([1]*College of Chemistry and Materials Engineering, Wenzhou University, Wenzhou 325027, Zhejiang Province, P. R. China*; [2]*Key Laboratory for Colloid and Interface Chemistry of the Ministry of Education, Shandong University, Jinan 250100, P. R. China*)

Abstract: A study on thermal denaturation of hen egg-white lysozyme (HEWL) was carried out by differential scanning calorimetry (DSC) and modulated temperature differential scanning calorimetry (MDSC). It was found that DSC profiles of HEWL were dependent on heating rates and concentrations of enzyme as well as cosolvent. The enthalpy of denaturation (ΔH_m) increased with increasing enzyme concentration in aqueous solution. In aqueous solution containing dimethylsolfoxide (DMSO), denaturation temperature (T_m) shifted toward lower temperature, and the transition peak became shallower and broader with increasing volume proportion of DMSO. When the content of DMSO was more than 70%, only a declining smooth line could be observed instead of a single endothermic transition curve. Interestingly, from MDSC curves of HEWL in aqueous solution without DMSO, a small and symmetrical peak in front of the main transition (I) was observed marginally, which was regarded as a pre-transition (II). Specially, this small and marginal peak could not be found in aqueous solution containing DMSO. In addition, with increasing amount of protein, T_m(II) shifted toward lower temperature, and ΔH_m(II) decreased along with the temperature gap between two transitions lengthened. The results were considered to be due to the reversible dissociation of HEWL dimers in aqueous solution.

Key Words: DSC; MDSC; DMSO; Hen egg-white lysozyme; Thermal denaturation

The stability of proteins is strongly dependent on the state of water affected by coexisting additive as a solute. Some additives stabilize proteins while others destabilize them. Solute additives such as urea, guanidinium hydrochloride, and some salts

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*Corresponding author. Email: hxgwzu@126.com; Tel: +86577-88373111; Fax: +86577-88373113.
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such as NaSCN and $MgCl_2$, destabilize proteins by binding which weakens the hydrophobic interactions between nonpolar residues of proteins[1–8]. The solute additives such as sugars, lower homologues of amino acids, and methylamine stabilize proteins by preferential hydration which strengthens the hydrophobic interactions between the nonpolar residues of proteins[9–14]. However, "solvent engineering" has been proposed as a potential approach to modulate protein structures and properties[15]. Short-chain alcohols such as methanol, ethanol, and trifluoroethanol (TFE) have been used as denaturants, in particular, TFE[16–19]. Such solvents not only stabilize nativelike secondary structures[16,20] but also transform other structural elements to nonnative structures[21–24].

Solvent environment plays an essential role in folding and unfolding of proteins. Dimethylsulfoxide (DMSO) is known to be a strong structure-perturbing reagent for proteins and peptides[25,26]. The denaturant property of DMSO could be attributed to the strong H-bond accepting ability of the sulfoxide group, whereas two methyl groups presumably interact with the hydrophobic residues of proteins[27,28]. DMSO could be a choice as denaturant, since many proteins are completely soluble in pure DMSO[25,29].

Hen egg-white lysozyme (HEWL), an enzyme with a molecular weight of 14,400 Da and a pI (isoelectric point) of 10.7, has been studied as a model protein for a long time because of its suitable properties for folding study. It is one of the most thoroughly studied proteins containing 129 residues. Its tertiary structure has been solved by X-ray diffraction and NMR. HEWL contains two domains named α and β domains. The α domain has four α helices and one 3_{10} helix. The β domain has one three-stranded antiparallel sheet, one 3_{10} helix, and one loop region. There are four disulfide bonds. HEWL is a small multi-domain protein with high content of secondary structure.

A highly unfolded state of HEWL had been reported in 100% DMSO[30,31]. A low-resolution infrared (IR) and optical rotatory dispersion (ORD) studies had indicated a structured species of HEWL in DMSO/H_2O mixtures[32]. A detailed structural characterization of a highly ordered intermediate state of HEWL in 50% DMSO/H_2O has been carried out by circular dichroism (CD), fluorescence, NMR, and H/D exchange techniques[33]. By using both wide-angle neutron and small-angle X-ray scattering (SAXS) techniques, the denaturation processes of HEWL in DMSO/H_2O mixtures have been studied in comparison with bovine α-lactalbumin[34], from which DMSO-induced denaturation was suggested to relate to the collapse of hydration shell surrounding the protein surface.

Differential scanning calorimetry (DSC), which is the most direct experimental technique to resolve the energetics of conformational transitions of biological macromolecule[35,36], has extensively been applied to the lysozyme system which is well known to exhibit reversibility upon unfolding[37–39]. Modulated temperature differential scanning calorimetry (MDSC)[40] is an advanced extension of conventional DSC, in which a sinusoidal wave modulation is applied to the standard linear temperature program. A discrete Fourier Transform algorit is then applied to the resultant data to deconvolve the sample response to the underlying (linear) and modulated temperature programs. The response to the underlying temperature program is similar to that obtained by a conventional DSC. MDSC is thus, a software development rather than a change in the basic DSC equipment. The use of the modulated temperature program improves the quality and quantity of information that may be obtained by conventional DSC. In the case of conventional DSC, the heat flow signal is a combination of "kinetic" and "heat capacity" responses. During a linear heating program, in the absence of any physical or chemical reaction, the heat flow signal is governed by the heat capacity of the sample. When temperature attains a certain value such that a kinetically controlled event occurs, the resultant heat flow signal is a combination of the heat capacity of the sample and that associated with the kinetically controlled event. MDSC is capable of separating these two processes, while conventional DSC heat flow signal represents the sum of the two types of processes. The basis of the separation of the two types of heat flow signals is their difference in response to the underlying and modulated temperature programs. MDSC thus, represents a significant advancement over conventional DSC[40,41].

In this paper, we tried to evaluate the effect of DMSO on the thermal stability of HEWL by using both DSC and MDSC techniques in comparison.

1 Materials and methods

Lyophilized HEWL (ultrapure grade, Amresco 0663) was purchased from Amresco and used without further purification. Bis-Tris and dimethylsulfoxide (DMSO), purity > 99.9%, were obtained from Sigma and Amresco, respectively. All other materials and reagents were of analytical grade. Millipore ultrapure water was used throughout the experiment. Bis-Tris solution of concentration 0.05 mol·L^{-1} with 0.1 mol·L^{-1} NaCl, pH=6.45, was used as a buffer solution. HEWL stock solution of 100 g·L^{-1} was prepared by dissolving enzyme in the buffer solution and deposited at 4 ℃. The other studied concentrations of HEWL were obtained by adding appropriate volumes of DMSO and buffer solution mixtures to certain amount of the stock solutions.

All DSC and MDSC scans were performed on a TA Q1000 differential scanning calorimeter equipped with a mechanical cooling accessory and TA Universal Analysis 2000 software. The instrument was calibrated for temperature and enthalpy using pure indium (m.p.=156.6 ℃) before experiment. Ultrapure nitrogen was used as a carrier gas at the flux of 50 mL·min^{-1} to prevent condensation of water vapor on the measuring surface. Sample solution (25 μL) was placed in aluminum pan and sealed hermetically. Vacant sealed aluminium pan of the same mass was used as a reference. DSC scanning was carried out from 20 to 100 ℃ at a programmed heating rate of 3 ℃·min^{-1}. MDSC scanning was performed at an underlying heating rate of 3 ℃·min^{-1} and a modulation amplitude of ±1 ℃ every 60 s. For each enzyme solution at least three experiments were performed.

Transition enthalpy (ΔH_m, estimated as the area of the peak), denaturation temperature (T_m, determined by the baseline extrapolation method using the peak sigmoidal horizontal option), half-hill width ($\Delta T_{1/2}$), and symmetry parameter (A_s, defined as the ratio of the slopes of the increasing and decreasing part of the peak) were computed from each thermal transition curve.

2 Results and discussion

2.1 Overall characteristics of thermal transition curves

In Fig.1, curves depict typical apparent heat flux profiles (at a heating rate of 3 ℃·min^{-1}) for the thermal denaturation of HEWL in two different solutions at the concentration of 40 g·L^{-1} lysozyme. In the case of curve (1), the denaturation temperature (T_m) is 72.68 ℃, the transition enthalpy (ΔH_m) is 1.309 J·g^{-1}, and the half-hill width ($\Delta T_{1/2}$) is 6.61 ℃. The transition is considered to be cooperative because of the shape of DSC profile and the remarkable symmetry parameter (A_s) which is close to 1.

In the case of curve (2), which exhibits the DSC profile of HEWL in aqueous solution with 30%(φ) of DMSO as solvent additive, the denaturation temperature (T_m), the transition enthalpy (ΔH_m), the half-hill width ($\Delta T_{1/2}$), and the symmetry parameter (A_s) were found to be 68.33 ℃, 1.483 J·g^{-1}, 6.17 ℃, and 0.86, respectively. The transition peak shifts to lower temperature and the transition enthalpy becomes larger compared with curve (1). The smaller value of A_s gives the evidence of less cooperative transition in the presence of DMSO.

The single endothermic transition observed for HEWL in all conditions studied is explained from the point of view of 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[41]. However, the dipolar DMSO molecule with a sulfoxide group and two methyl groups is more favorable to the hydrogen bonding and hydrophobic interactions. It could strip away water molecules from the surface of a protein and then enter the hydrophobic center to disturb the interaction of inner molecules. Two curves in Fig.1 are resembling, but the lower value of T_m in curve (2) just indicates less stability of lysozyme in the presence of DMSO.

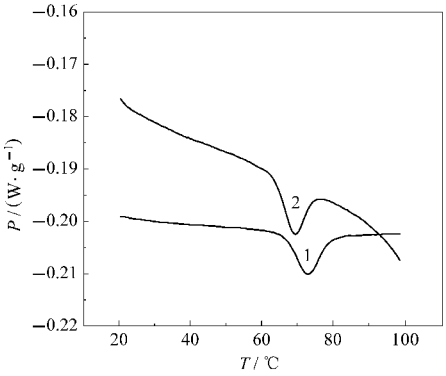

Fig.1 Typic DSC curves of lysozyme in two different solutions at a programmed heating rate of 3 ℃·min^{-1}

concentration of HEWL: 40.0 g·L^{-1}; (1) in aqueous solution; (2) in DMSO (30%, φ) aqueous solution

2.2 Concentration dependence

It is important to check whether the calorimetric profiles of proteins are concentration-dependent, because the effect of protein or additive concentration on the position of T_m gives some information about the changes in the molecularity occurring during thermal denaturation process[42].

From Fig.2 and Table 1, it can be seen that T_m decreases slightly with increasing enzyme concentration (c) while ΔH_m increases fleetly. Such concentration dependence can be ascribed to the equilibrium between the aggregation of polypeptide chains and the dissociation of aggregated protein to monomers. At lower concentration of HEWL, each protein molecule is surrounded in the cage of water molecules, and enzyme molecules are almost separated from each other. The smaller ΔH_m is clearly observed for little aggregation. On the contrary, at higher concentrations, the steric space of enzyme molecule and the density of water are obviously diminished. It is the good chance for enzyme molecules to aggregate together. So, larger heat-absorption peaks can be seen at the higher enzyme concentrations.

Many studies of thermal denaturation of proteins using DSC have clarified the thermodynamic basis of stability of the conformational states of proteins, and that at equilibrium unfolding transitions of single-domain proteins are usually two-state where only the fully folded and unfolded states are populated.

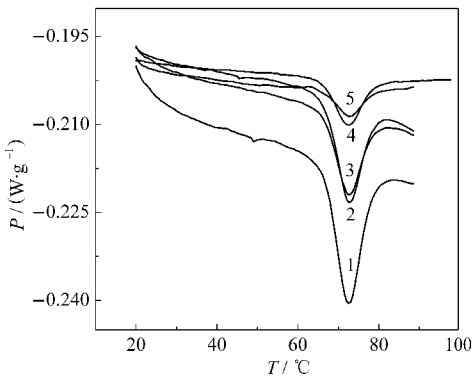

Fig.2 Typic DSC curves of HEWL in aqueous solution of various enzyme concentrations at a programmed heating rate of 3 ℃·min^{-1}

concentration (g·L^{-1}) of enzyme: (1) 100.0, (2) 80.0, (3) 60.0, (4) 40.0, (5) 20.0

Table 1 The thermodynamic parameters of thermal denaturation of HEWL in aqueous solutions of various enzyme concentrations by DSC at a programmed heating rate of 3 ℃·min^{-1}

c/(g·L^{-1})	T_c/℃	T_m/℃	ΔH_m/(J·g^{-1})	$\Delta H_{1/2}$/℃	A_s
20.0	65.97	72.73	0.824	7.47	1.30
40.0	66.71	72.68	1.309	7.40	1.00
60.0	66.69	72.62	1.965	6.79	0.93
80.0	67.05	72.60	2.358	6.61	0.86
100.0	66.70	72.54	3.508	6.81	0.98

T_c: the onset temperature, T_m: the denaturation temperature

Under equilibrium conditions the folding-unfolding transition of HEWL is shown to be a highly cooperative two-state process[43–46]. H/D exchange experiments using 2-dimensional NMR show that HEWL consists of two structural domains which differ significantly in the folding pathway. These structural domains are expected to be stabilized with different kinetics as distinct folding-domains involving parallel alternative pathways. SAXS measurements[47] on thermal denaturation of HEWL of different enzyme concentrations at pH=5 under a constant heating rate which is comparably used for DSC measurements, elucidated that thermal structural transition of HEWL depends on conformational hierarchy and concentration, namely upon heating structural fluctuation occurs at first in the polypeptide chain arrangement and next in the intramolecular domain correlation, and finally induces the significant collapse of the tertiary structure with such as the change of the radius of gyration and the surface roughness of enzyme molecule. Such a consecutive intramolecular structural fluctuation can not be described by a first-order transition but by a higher-order transition.

A lattice Monte Carlo simulation study of protein folding[48] characterized the folding ability of the polypeptide chain in terms of two intrinsic characteristic temperatures corresponding to transitions of a collapsed structure and a native conformation. This would relate to the evidences of thermal structural transition of HEWL characterized in terms of the tertiary and intramolecular structures. With increasing lysozyme concentration the above intramolecular fluctuation is suppressed to lead to the abrupt collapse of the tertiary structure at a narrow denaturation temperature in such a way of two-state transition, suggesting the increase of the protein concentration would stabilize the tertiary and intramolecular structural fluctuations under the presence of strong repulsive intramolecular interaction. In other word, at high concentration the presence of the proteins can work as macro ions to stabilize effectively the native conformation, which might be the case for native cells since the concentration of proteins in cells is around 0.15–0.18 $g\cdot L^{-1}$[49]. Recently, the oligomerization of lysozyme in aqueous solution was investigated by Monte Carlo simulation as a function of protein concentration, pH, and electrolyte screening[50]. It was observed that increasing protein concentration, or decreasing the electrostatic repulsion between protein molecules by either reducing the protein charge or increasing the ionic strength, promotes the formation of cluster.

DSC curves for HEWL in solutions containing various volume proportions of DMSO are shown in Fig.3. It is apparent that the more DMSO, the lower T_m and ΔH_m. The single peak on thermal transition curve becomes smaller and boarder, and disappears macroscopically, that is to say only a declining smooth line can be observed when the volume fraction of DMSO>70%. The related thermodynamic parameters are listed in Table 2.

In general terms, various effects on protein transition have been studied, hydrogen-bonding, hydrophobic intraction, and static interaction are usually taken into account to explain the

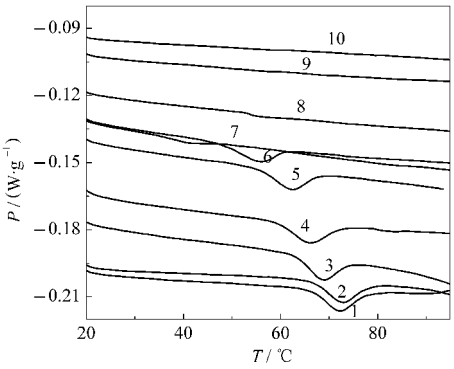

Fig.3 Typic DSC curves of HEWL in aqueous solution with different volume proportions of DMSO at a programmed heating rate of 3 ℃·min^{-1}

concentration of enzyme: 40.0 $g\cdot L^{-1}$; φ(DMSO) (%): (1) 5.0; (2) 10.0; (3) 30.0; (4) 40.0; (5) 50.0; (6) 60.0; (7) 70.0; (8) 80.0; (9) 90.0; (10) 100.0

Table 2 The thermodynamic parameters of thermal denaturation of HEWL in aqueous solution with different volume proportions of DMSO by DSC at a programmed heating rate of 3 ℃·min^{-1}

φ(DMSO)(%)	T_c/℃	T_m/℃	ΔH_m/(J·g^{-1})	$\Delta T_{1/2}$/℃	A_s
5.0	66.46	72.64	1.450	6.63	0.81
10.0	66.12	71.99	1.526	6.73	0.84
30.0	62.38	68.33	1.483	6.17	0.86
40.0	59.65	65.76	1.485	6.61	0.99
50.0	54.64	61.84	1.485	7.11	0.86
60.0	46.35	55.01	1.296	8.12	0.84

changes of calorimetric property observed in protein when a foreign substance is present. Zheng and Ornstein[51] have studied the effect of DMSO on enzyme (subtilisin) structure and dynamics using molecular dynamic and quantum mechanic methods. The simulation results indicate that DMSO is capable of stripping water molecules and metal ions away from the protein surface, and that the total number of intra-protein hydrogen bonds is increased in DMSO compared to that in water. It is well known that DMSO-water interaction is stronger compared to water-water interaction[52]. The calculated interaction energy at the 6-31G level for the DMSO-water complex [53] is also found to be stronger than that for the hydrogen bonding interactions present in protein-water system, namely (1) between a backbone carbonyl oxygen and amide hydrogen, (2) between a carbonyl oxygen and water, (3) between an amide hydrogen and water. So it is not surprising that some water molecules leave the surface of protein into DMSO solution. In addition, DMSO-amide hydrogen interaction at the 6-31G(D) level is about the same strength as the DMSO-water hydrogen bond, but is stronger at the MP2/6-31G (D) level[49]. DMSO-amide hydrogen interactions also seem to be stronger than hydrogen-bonding interactions between a backbone carbonyl and an amide hydrogen[53]. Furthermore, due to the presence of a polar hydrophilic S═O group and two hydrophobic alkyl groups in DMSO molecule, it is easy to approach the hydrophobic core, and disturb the globular structure of protein.

So the additive DMSO could strip away water molecules from the protein surface and then enter the intra-protein to disturb the stability and unfold the globular protein, which is demonstrated by experimental parameters T_m and ΔH_m, the values of which decrease with increasing volume fraction of DMSO before 70% (Fig.3). However, with the increasing of DMSO proportion, when it reaches 70%, nearly all water molecules are to be pulled out, more DMSO molecules access and enter into the hydrophobic core to make the globular protein be completely unfolded rapidly. Therefore, a degressive line was observed instead of single peak on the DSC curve when the volume fraction of DMSO＞70%.

2.3 Heating rate dependence

HEWL denaturation is also dependent on heating rate (β). In Fig.4 and Table 3, the denaturation temperature T_m and the onset temperature T_e shift toward higher temperatures and the peaks of heat capacity become smoother with increasing v.

The shift of T_m due to the heating rate change from 1.5 ℃·min^{-1} to 10 ℃·min^{-1} is about 2 ℃. This lag phenomenon is related to the possibility that the rate of thermal denaturation can not catch up with the heat flow compensation when the heating rate increases.

2.4 MDSC analysis

Fig.5 shows MDSC curves at a constant heating rate of 3 ℃·min^{-1} for HEWL in aqueous solution with various DMSO concentrations (curves 1, 2) and in aqueous solution without DMSO

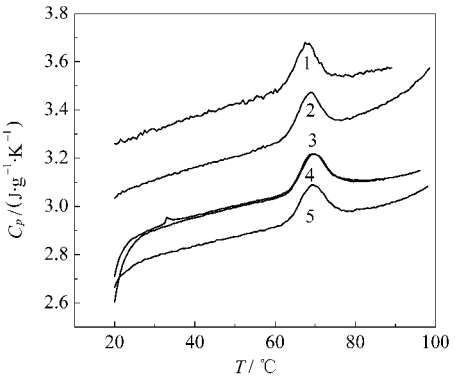

Fig.4 Heat capacity curves of aqueous HEWL solutions at different heating rates

concentration of enzyme: 40.0 g·L^{-1}; heating rate (℃·min^{-1}): (1) 1.5, (2) 3.0, (3) 5.0, (4) 7.5, (5) 10.0

Table 3 The thermodynamic parameters of thermal denaturation of HEWL at a constant enzyme concentration of 40.0 g·L^{-1} by DSC at different heating rates

β/(℃·min^{-1})	T_e/℃	T_m/℃	ΔH_m/(J·g^{-1})	$\Delta T_{1/2}$/℃	A_s
1.5	61.76	67.49	1.74	6.23	0.61
3.0	62.23	68.33	1.62	6.13	0.93
5.0	63.46	69.33	1.44	7.05	0.86
7.5	63.59	69.28	1.41	7.54	1.13
10.0	63.01	69.33	1.66	7.75	1.36

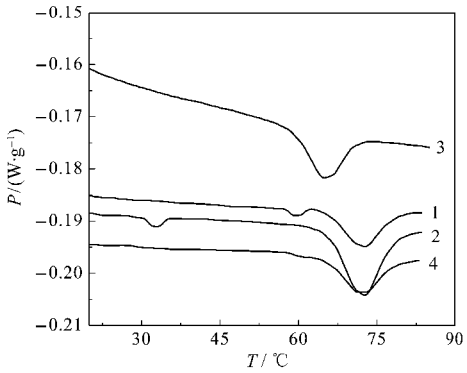

Fig.5 MDSC curves of HEWL in different aqueous solutions at an underlying heating rate of 3 ℃·min^{-1} and a modulation amplitude of ±1 ℃ every 60 s

(1) and (2): without DMSO, concentration of enzyme: (1) 40.0 g·L^{-1}, (2) 80.0 g·L^{-1}; (3) and (4): concentration of enzyme: 40.0 g·L^{-1}, φ(DMSO): (3) 20%, (4) 30%

(curves 3, 4). The values of T_m are 71.47 ℃ and 64.77 ℃ when the contents of DMSO are 20% and 30% (φ), respectively (Table 4). The shapes of curves are almost identical to the DSC curves discussed before.

Curves (3, 4) in Fig.5 are the MDSC curves for HEWL in aqueous solution without DMSO. In the studied temperature range, besides a strong and sharp main-transition (I) peak exhibiting near 72.6 ℃ which is corresponding to the one determined by traditional DSC, another smaller and symmetrical peak in front of the main transition (I) was observed, which is regarded as a pre-transition (II) event and has not been reported as yet for lysozyme. The small marginal endothermic pre-transition (II) is also of dependence on enzyme concentration. With increasing amount of enzyme, T_m(II) shifts toward lower temperatures, and ΔH_m(II) decreases along with the temperature gap between two transitions lengthened (Table 5).

Table 4 The thermodynamic parameters of thermal denaturation of HEWL in various aqueous DMSO solutions with a constant enzyme concentration of 40.0 g·L^{-1} by MDSC at an underlying heating rate of 3 ℃·min^{-1} and a modulation amplitude of ±1 ℃ every 60 s

φ(DMSO)(%)	T_e/℃	T_m/℃	ΔH_m/(J·g^{-1})	$\Delta T_{1/2}$/℃	A_s
20	65.29	71.47	1.186	7.86	0.72
30	58.73	64.77	1.557	7.39	0.93

Table 5 The thermodynamic parameters of thermal denaturation of HEWL in aqueous solutions without DMSO and with different concentrations of enzyme by MDSC at an underlying heating rate of 3 ℃·min^{-1} and a modulation amplitude of ±1 ℃ every 60 s

c/(g·L^{-1})		T_e/℃	T_m/℃	ΔH_m/(J·g^{-1})	$\Delta T_{1/2}$/℃	A_s
40.0	Trans. (I)	65.54	72.66	1.126	7.57	1.21
	Trans. (II)	57.05	59.42	0.075	2.92	0.71
80.0	Trans. (I)	65.70	72.62	2.100	7.54	0.83
	Trans. (II)	30.42	32.80	0.110	3.07	0.94

The small pre-transition might be due to the reversible dissociation of lysozyme dimers in aqueous solution. It has been proved that in aqueous solution lysozyme always exists in monomolecular or dimolecular form[54–57]. The pre-transition is a slow process, its small effect is averagely distributed into large temperature range, and therefore no obvious trace can be found on a traditional DSC curve. However, MDSC is a developed method that enables the separation of overlapping thermal transitions and the separation of reversible and non-reversible heat flow signals. All heat flow associated with processes capable of following the temperature modulation is captured in the reversing heat flow signal, while any remaining heat flow attributable to processes not capable of following the temperature modulation is captured in the non-reversing heat flow signal[58]. It resembles to prolong the lingering time right around a thermal event. Therefore the energy of weak dissociation could be detected easily by MDSC.

Specially, the pre-transition peak can not be found in aqueous solution containing DMSO. The reason is considered to be that the stronger DMSO-protein interaction leads to the collapse of dimeric structure of lysozyme in advance, and only the tertiary structure and the intramolecular structural fluctuations exist, which are responsible for the main-transition (II) of lysozyme.

3 Conclusions

The present results show that DMSO strongly affects the thermal behavior of HEWL in aqueous solution. With the proportion of DMSO increasing, T_m shifts toward lower temperatures and the peak becomes shallower and broader. When the volume fraction of DMSO > 70%, only a declining smooth line could be observed instead of the single endothermic transition. These are due to the DMSO-water interaction which is stronger than water-water interaction, and the DMSO-amide interaction which is more than the same strength as the DMSO-water hydrogen bond, so when DMSO molecules are added, water molecules are pulled out, and more and more DMSO molecules access and enter into the hydrophobic core to make the globular protein be completely unfolded rapidly.

The extension of our study was involved in the using of MDSC. Ascribed to the reversible dissociation of lysozyme dimers, another small and symmetrical peak in front of the main transition in MDSC determination of lysozyme in aqueous solution was observed, which is specially invisible in aqueous DMSO solution. The newly found pre-transition event is considered to be due to the reversible dissociation of lysozyme dimmers in aqueous solution.

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