EFFECT OF SULFOXIDES ON THE THERMAL DENATURATION OF HEN LYSOZYME: A CALORIMETRIC AND RAMAN STUDY

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ABSTRACT
A multidisciplinary study of the thermal denaturation of lysozyme in the presence of three sulfoxides with different length in hydrocarbon chain (DMSO, DESO and DPSO) was carried out by means of DSC, Raman spectroscopy, and SDS-Page techniques. In particular, the $T_d$ and $\Delta H$ values obtained from the calorimetric measurements showed that lysozyme is partially unfolded by sulfoxides but most of the conformation holds native state. The sulfoxide denaturing ability increases in the order DPSO > DESO > DMSO. Moreover, only DMSO and DESO have a real effect in preventing the heat-induced inactivation of the protein and their maximum heat-protective ability is reached when the DMSO and DESO amount is $\geq 25\%$ w/w.

The sulfoxide ability to act as effective protective agents against the heat-induced inactivation was confirmed by the protein analysis. The enzymatic activity, as well as the SDS-Page analysis, suggested that DESO, having a low hydrophobic character and a great ability to stabilise the three dimensional water structure, is the most heat-protective sulfoxide. An accurate evaluation of the heat-induced conformational changes of the lysozyme structure before and after sulfoxide addition was obtained by the analysis of the Raman spectra. The addition of DMSO or DESO in low concentration resulted to sensitively decrease the heat-induced structural modifications of the protein.

KEYWORDS: Sulfoxides; Lysozyme; Thermal denaturation; Different Scanning Calorimetry; Raman spectroscopy.
1. INTRODUCTION

The thermal denaturation of many globular proteins, like lysozyme, consists in a heat-induced inactivation of the enzymatic properties. Moreover, at a fixed high temperature, the degree of the inactivation is roughly linearly related to the heating time. Indeed, under unfolding condition, the irreversible refolding or aggregation compete with the correct folding process, as is described by the classic model by Lumry-Eyring [1,2]:

\[
N \rightleftharpoons A \rightarrow B
\]  

(1)

Where N represents the native state, A a non-native state in equilibrium with the native state, and B is the thermal denatured protein. Equation (1) involves a first-order reversible folding/unfolding reaction (N \( \Delta \) A), followed up by a first or higher-order irreversible refolding or aggregation denaturation process (A \( \rightleftharpoons \) B). A similar model was recently applied to the thermal denaturation of lysozyme in the presence of glycerol [3].

The addition of many non-denaturating reagents, like arginine, spermine and spermidine, is able to increase the reversible protein refolding yield by decreasing the irreversible denaturation, thus reducing the rate of the A \( \rightleftharpoons \) B conversion and increasing the protein stability to the thermal denaturation. Also pH of medium plays an important role in the rate-depending processes involved in the overall N \( \Delta \) A \( \rightleftharpoons \) B process.

It is well known that folding, structural stability and dynamic of globular proteins are extensively controlled by the interaction of proteins with water. In particular, the addition of organic dipolar solvents, like sulfoxides that show both polar and apolar moieties, to water is able to affect the protein structure by weakening the hydrophobic interaction between apolar residues as well by perturbing the characteristic water structure around the protein molecule [4,5].

The physical properties of some sulfoxide/water systems (in particular, the dimethylsulfoxide /water and diethylsulfoxide/water systems) have been extensively studied [6-8]. These studies have
suggested that all hydrosoluble sulfoxides are able to modify the water structure by forming strong hydrogen bonds with water. Nevertheless, it should be noted that also the hydrophobic interactions play a crucial role in understanding the water-sulfoxides interactions. Indeed, dimethylsulfoxide (DMSO) destabilises the water structure over the entire concentration range, whereas diethylsulfoxide (DESO) and, in a lesser extent, dipropylsulfoxide (DPSO), stabilise the characteristic three-dimensional water structure [9].

The transfer of proteins from aqueous to organic/aqueous medium may allow to address a number of questions of relevance not only to biophysicists but also to biotechnologists. In fact, the medium replacement may enhance the thermal stability of protein (process of significant benefit in the pharmaceutical and biotechnology industries) [10] or alter the enzymatic activity [11], facilitate the study of the reciprocal processes of protein folding and unfolding, and enhance transport of topically applied pharmacological and cosmetic preparations across the epidermal barrier [12]. In this respect, both DMSO and DESO exert many interesting properties on the living systems; indeed they show cryoprotective effects on cells, tissues and organs [13, 14], have radioprotective properties [15], induce cellular fusion and increase the permeability across biomembranes [16]. In addition, topically applied DMSO is useful in the relief of certain types of chronic pains [17].

Hen egg-white lysozyme (Lyso) was chosen as model of globular proteins with enzymatic activity because its thermal behaviour and its properties have been widely studied [18,19]. Also the effect of some sulfoxides on the Lyso unfolding has just been considered but only under strong acidic conditions (pH = 3) [20].

Lyso is a relatively small enzyme (MW 14388) that attacks many bacteria by lysing or dissolving the muco-polysaccharide structure of cell wall. It is a globular protein containing 129 amino acids of which three are tyrosine, six tryptophan, and 4 pairs of cysteine residues, forming four intramolecular disulfide bonds which stabilise the protein structure [19]. It comprises two lobes consisting of four $\alpha$-helices and a triple-stranded antiparallel $\beta$-sheet, respectively, separated by a cleft containing the active state residues.
In this paper we describe the effect of sulfoxides (mainly DMSO and DESO) on the thermal inactivation of this globular protein by a multidisciplinary approach, which allows to analyse some different aspect of the protein/sulfoxide systems. In particular, the calorimetric measurements were coupled to Raman measurements and the assays of enzymatic activity, to gather a comprehensive view of the thermal inactivation effect on this globular protein.

2. MATERIALS AND METHODS

Chemicals

Hen egg-white lysozyme (Lyso) (E.C. 3.2.1.17, activity ~ 100000 units/mg) was obtained from Fluka, as BioChemika product. DMSO [(CH$_3$)$_2$S=O] was a Fluka analytical grade (purity > 99,5 % by GC) product, anhydrous on molecular sieves (H$_2$O < 0,005% w/w). DESO [(C$_2$H$_5$)$_2$S=O] and n-DPSO [(C$_3$H$_7$)$_2$S=O] were prepared and purified according to the literature [21]. Their purity was > 99.5 % and the water content, after drying on molecular sieves, was < 0,01%.

All other used chemicals were ‘analytical grade’ Merck products.

Sample preparation

The sample solutions for the DSC measurements were prepared by dissolving about 50 mg/ml of Lyso in the sulfoxides stock solutions. This concentration allowed us to obtain very clear quantifiable endotherms during upscans and Raman spectra directly from solutions. In addition, it has been reported that refolding of Lyso after denaturation is poorly affected by protein concentration up to 200 mg/ml [22]. These solutions contained sulfoxides (DXSO) in the 0.0 – 50.0 % w/w range and water (100.0 – 50.0 % w/w range). pH was adjusted to 7.0 and 5.0 by adding NaOH or HCl.

In order to evaluate both enzymatic activity and aggregation/breaking level of protein, the solutions were prepared by dissolving exactly 1.0 mg/ml of Lyso in the same sulfoxide stock solutions used
for the DSC measurements at pH 7.0. The enzymatic activity was evaluated on each samples before and after thermal treatment (15.0 min at 90 °C).

**DSC measurements**

DSC measurements were performed on Mettler-Toledo DSC 821 instrument. The samples (with a volume of about 125 μl), were preventively sealed in an aluminium pan, and then submitted to two subsequent heating-cooling cycles. The heating and cooling rates were kept at 1.0 °C/min in the 20-90 °C range. Temperature and enthalpy scales were calibrated with indium and tested in the considered thermal range by decanoic acid. Thermal cycles were repeated on at least three different samples to ensure a good reproducibility of the data; the expected experimental errors were ± 0.1 °C in the temperature and ± 5 % in ΔH values.

**Raman spectra**

Raman spectra were obtained using a Jasco NRS-2000C instrument. All the spectra were recorded in backscattering conditions with 2 cm⁻¹ spectral resolution using the 488 nm line (Innova Coherent 70) with a power of 15 mW and the total number of scans for each spectrum was 20. Measurements were made in a thermostatic cell holder at 25 °C (± 1 °C). The detector was a 160K frozen digital CCD (Spec-10: 100B, Roper Scientific Inc.).

**Activity assays**

The activity of Lyso was assayed on both not heated and heated samples by the method reported in the literature [23-25]. Reaction mixture (3 mL) was composed of Micrococcus lysodeikticus cells (2.9 mL, 0.3 mg/mL) in 0.1 M potassium phosphate pH 7.0 and Lyso (0.1 mL, 1 mg/mL). Change in absorbance at 450 nm was recorded in a Cary 1 spectrophotometer (Varian). The reaction was followed for 4–5 minutes. Specific activity was calculated as follows:
Units / mg = \Delta \frac{A_{450}}{\text{minute}} \times 1,000 \\
mg enzyme / assay

One enzyme unit is equal to a decrease in turbidity of 0.001/minute at 450 nm, at pH 7.0 and 25 °C.

The activity measurements were done at least in triplicate.

The presence of sulfoxides, after dilution of the samples in water at the level required from the method (about 100 times), was not able to affect the activity measurements.

**SDS-page analysis**

SDS-polyacrylamide (12%) slab gel (cm 7 x 5) electrophoresis of Lyso solutions (2 µg) in the absence and presence of sulfoxides, were carried out in 0.1% sodium dodecyl sulphate (SDS). After the electrophoretic run (30 mA x 2 hours), proteins were stained with Coomassie blue in 10% acetic acid and 30% methanol.

### 3. RESULTS AND DISCUSSION

#### 3.1 Calorimetric behaviour

Differential Scanning Calorimetry (DSC) is a thermal technique that provides valuable information on the overall mechanism of protein denaturation, on its reversibility as well as on its cooperativity by studying the enthalpy changes associated to thermal transitions.

Figure 1 shows the plots of the values of the denaturation peak temperatures (T_d) of Lyso as a function of the sulfoxide concentration (0.0 – 50.0 % w/w of sulfoxide content) both at pH 5.0 and 7.0, whereas the denaturation enthalpy (ΔH_d) as a function of the DXSO content at pH 5.0 is reported in Figure 2. Generally, we observed a good reproducibility of the thermal data from different samples of any mixture at the first heating cycle, showing a roughly linear decrease of T_d as the sulfoxide content increases. The slops of the T_d plots showed a strong dependence on the length of the hydrocarbon chain in the sulfoxides (DPSO > DESO > DMSO), and a less relevant
effect of pH (Fig. 1). Furthermore, the pH-dependence of the denaturation temperature became
negligible by increasing the hydrocarbon moiety. In fact, the moderate $T_d$ decrease detected in the
presence of DMSO became negligible in the DPSO-containing system.

As regards $\Delta H_d$, the values were always higher at pH 5.0 than at neutral pH, but the difference at
the two pHs was higher in presence of DMSO, lower with DESO and negligible in presence of
DPSO (Table 1).

Figure 3 shows the DSC curves relative to the thermal stability of Lyso in aqueous solutions
containing different amounts of DESO at pH 5.0 during the first heating cycle, in the 20 – 90 °C
temperature range.

The dependence of the Lyso thermal stability from alkyl chain length of DXSO is similar to what
reported on n-alkyl-alcohols [26]. This result has been explained as caused by the setting up of
more and more increasing hydrophobic interactions between the hydrophobic region of the protein
and the alkyl chain of the alcohol molecule, thus favouring the denatured state even at low
temperature. If this hypothesis is able to describe completely the effect of amphipathic molecules on
the denaturation of Lyso, we expected that no preventing effect on the thermal denaturing should be
produced. On the contrary, the $\Delta H_d$ value behaviour suggests that a more complex mechanism take
place. In fact, in all the analysed systems the $\Delta H_d$ values initially increased by increasing the
sulfoxide concentration and then started to decrease (cfr. Fig. 2). The $\Delta H_d$ reaches its maximum at a
different temperature for each sulfoxide and at different sulfoxide concentrations (30% DMSO,
20% DESO and 5% DPSO); the same trend was observed both at pH 5.0 and 7.0 (Table 1).

In a previous paper we reported that in the more diluted solutions (DMSO or DESO < 25% w/w and
DPSO < 10% w/w; molar fraction $\chi_{DMSO}$ and $\chi_{DESO}$ < 0.1 and $\chi_{DPSO}$ < 0.04), the observed
$\Delta H_d$ increase is mainly due to the changes of the protein structure arising from the water structuring
properties of the sulfoxide molecules [27]. On the other hand, by further increasing the sulfoxide
concentration, the role of the direct sulfoxide-Lyso interactions becomes more and more important,
with the consequent $\Delta H_d$ decrease.
The sulfoxide-Lyso interactions have a complex origin, involving both the polar S=O groups as well as the hydrogen bonds formed by the H atoms of the methyl or methylene groups near to the S=O group, whose importance has been pointed out in the literature [9,28,29], and also the hydrophobic apolar interactions, whose strength increases in the order DPSO > DESO > DMSO.

The hypothesis that the presence of both hydrophilic and hydrophobic moieties plays a key role in determining the overall structure of the system, could be also relevant in the pH dependence of both $T_d$ and $\Delta H_d$.

The Lumry-Eyring model of the thermal denaturation process is well confirmed from the different behaviour between the first and second heating cycle (cfr. Table 1). In fact, we observed a $\Delta H_d$ reduction in the second cycle ($\Delta H_{d2}$) and this effect appears greater if the sample is heated for a longer time. On the contrary, the $T_d$ value did not change ($T_{d2} \approx T_{d1}$), confirming thus the reversibility of the first step in the Lumry-Eyring model. In addition, the comparison between the $\Delta H_d$ behaviour of the first and the second heating curve ($\Delta H_{d1}$ and $\Delta H_{d2}$, respectively) suggests that the reversibility of the first step is increased in the presence of DMSO (from $\sim$ 75 % to $\sim$ 90 %) and even more in the presence of DESO (from $\sim$ 75 % to $\sim$ 97 %). The highest reversibility is reached when the DMSO or DESO concentration is greater than 25 % w/w. On the contrary, in the presence of DPSO, no increase of the reversibility of the refolding step is suggested from the $\Delta H_{d2}/\Delta H_{d1}$ ratio, at least within the limits of the experimental error.

Also the behaviour of $\Delta T_{1/2}$ appears to be in agreement with the previous suggested model. Indeed, as general tool, except for a little $\Delta T_{1/2}$ decrease in the presence of the smallest amounts of DMSO, a $\Delta T_{1/2}$ increase was observed after the sulfoxide addition. This $\Delta T_{1/2}$ increase was small in the presence of increasing amount of DMSO, middle in the presence of DESO and greater in the presence of DPSO (Table 1). It should be noted that the observed $\Delta T_{1/2}$ values were the same between the first and second heating cycle, at least within the limits of the experimental error.
In conclusion, our DSC results show that Lyso is only partially unfolded by sulfoxides and the unfolding ability increases in the order DPSO > DESO > DMSO; therefore, most of the Lyso conformation holds native state in water/sulfoxide mixtures. Moreover, only DMSO and DESO have a real effect in preventing the thermal denaturation of Lyso. In fact, even if sulfoxides are reported to act as denaturing agent, our $\Delta H_{d2}/\Delta H_{d1}$ ratio suggests that both DMSO and DESO are able to preserve in some extent the 3D structure of Lyso from thermal denaturation and their maximum heat-protective ability is reached when the DMSO and DESO amount is $\geq 25 \% \text{ w/w}$.

3.2 Enzymatic activity

Table 2 shows the results of the activity assay upon refolding/aggregation of Lyso in the presence of increasing sulfoxide amounts before and after heating of the samples. On the basis of the poor pH dependence of the DSC plots, at least in the considered pH range which mimics biological conditions, both the activity assays and the structural analysis of the protein were carried out only at pH 7.0. In order to avoid time- and/or concentration-depending effects, all the samples were prepared starting from the same Lyso stock solution and the time passed between the preparation of the samples and the enzymatic activity measurements was the same for all the samples (24 h at 25 °C). As a consequence of the sulfoxide addition to the protein solution, the enzyme activity was lowered to about 90% at the highest DMSO and DESO concentration. The change in the enzymatic activity was particularly evident in the presence of DPSO probably because of the higher hydrophobic property of this sulfoxide. However, in all cases the replacement of protein aqueous medium with sulfoxide/water medium was able to reduce the heating denaturing effect on the protein. In fact, in the absence of sulfoxides the thermal treatment induced a decrease of 40% in the enzymatic activity of Lyso, whereas the decrease was only of 20% in the presence of the lowest sulfoxide concentration (10 % w/w).
Among the three sulfoxides, DESO appears to increase the thermal stability of the enzyme better than the others, since the residual activity after heating was $\geq 90\%$ at all the considered concentrations.

3.3 Protein analysis

3.3.1 SDS page

The SDS-page is a powerful technique, which reveals not only if aggregation/breaking takes place, but also allows to determinate the average molecular weight of the fragment as well as the aggregates. To evaluate the aggregation/breaking level during the denaturing process, the SDS-page analysis of Lyso was carried out in the presence of DMSO or DESO, the two sulfoxides having the major capability in enhancing the thermal stability of Lyso (see calorimetric and enzymatic data). In absence of heating, the electrophoretic analysis of the Lyso solutions showed that both low and high DMSO or DESO concentrations do not affect the protein electrophoretic migration (Fig. 4A).

In absence of sulfoxides, instead, the thermal treatment ($90^\circ C$ for 15 minutes) induces a partial degradation of enzyme, confirmed by reduction of enzymatic activity and demonstrated by the appearance of several electrophoretic bands with lower molecular weight than 14 kDa (Fig. 4B).

A single electrophoretic band, corresponding to native Lyso was observed after addition of low DESO or DMSO concentrations (up to 25%), that so result able to reduce the thermal denaturating process (Fig. 4B).

On the contrary, when the thermal treatment of the Lyso solution takes place in the presence of the highest DMSO concentration (40%), SDS-PAGE revealed two electrophoretic bands corresponding at proteic aggregates with molecular weight of about 28 and 42 kDa (Fig. 4B). No changes in the Lyso electrophoretic migration occurred by adding the same DESO concentration. Since at this sulfoxide concentration the Lyso enzymatic activity is reduced of about 20% in the presence of DMSO (Table 2), this effect could be a consequence of aggregation of Lyso molecules.
Again, between the two sulfoxides DESO seems to be more able to stabilize Lyso against the thermal inactivation than DMSO, since it does not induce aggregate formation.

### 3.3.2 Raman spectroscopy

Raman spectroscopy has been proved to be a useful technique in revealing conformational changes of proteins, also in the microenvironment of the side chains. To detect the changes in the protein structure, resulting from the exposure to heat, the Raman spectra of Lyso in the absence and presence of different amounts of DMSO or DESO were recorded before and after a heating cycle (15 min at 90°C). In order to obtain the protein spectrum free of medium interferences the spectrum of water was subtracted from the overall spectrum.

As it is known, one of the potential advantages of Raman spectroscopy for the study of protein lies in the correlation between the vibrational frequencies of the peptide backbone and the various protein conformations. In particular, the amide I Raman band, which appears in the 1620-1700 cm\(^{-1}\) spectral region, may act as sensitive conformation marker [30].

From a qualitative examination of the spectra, some slight differences were evident. In particular the shape and the wavenumber maximum of the Amide I band changed by increasing the sulfoxide content in the protein solution. As an example, the Raman spectra of Lyso in DESO/water mixtures of various compositions are showed in Figure 5. In particular, the Amide I band shifted towards higher wavenumbers (from 1662 to 1667 cm\(^{-1}\)) by increasing the DESO concentration, indicating an increase in the β-sheet content of the enzyme.

Spectral modifications were also visible in the intensity of some bands due to Trp residues, in particular the doublet at 1340 and 1360 cm\(^{-1}\), marker of the hydrophobicity of the molecular environment of Trp; generally, hydrophobic interactions between the Trp indole ring and the surrounding aliphatic groups cause the 1360 cm\(^{-1}\) peak intensity decrease and the 1340 cm\(^{-1}\)
intensity increase [31]. By adding the highest DESO concentration to the protein solution, a
decrease in the intensity ratio \( \frac{I_{1360}}{I_{1340}} \) of the two components of the doublet was observed,
indicating an increase in the hydrophobic interactions between the Trp indole ring and other
aliphatic groups (Fig. 5). As regards Lyso in the DMSO/water mixtures, it was not possible to
obtain unquestionable results on Trp environment because of the overlapping of Trp bands with a
band at about 1330 cm\(^{-1}\) due to the DMSO.

As regards the 1558 cm\(^{-1}\) Raman peak, marker of the orientation in the Trp indole ring with respect
to the peptide backbone [32], its frequency did not change as a consequence of the sulfoxide
presence, but only a slight decrease in the intensity, roughly proportional to the amount of the added
sulfoxide, was observed (Fig. 5). Consequently, we draw out that no change of the Trp orientation
takes place and the intensity decrease is probably a consequence of the decrease in the dielectric
constant \( (\varepsilon_r) \) occurring after sulfoxide addition.

Some slight differences depending on the sulfoxide/aqueous medium were also observed in the S=O
stretching Raman region (900-1100 cm\(^{-1}\)), indicating some changes in aqueous environment (Fig.
6). In this region the DESO spectrum has a complex band structure that has been resolved in seven
components [29]. Among them, one of the most sensitive to the water presence is the \( \approx 1010 \) cm\(^{-1}\)
component, due to the vibration of the S=O groups directly involved in hydrogen bonds with water
molecules. Negligible differences were observed at the highest concentration of DESO in the
absence and presence of Lyso, whereas, in the system containing 25 % DESO, this band visible at
1005 cm\(^{-1}\) was shifted to 1008 cm\(^{-1}\) by adding Lyso to the sulfoxide / water solution (Fig. 6). This
behaviour suggests that the Lyso addition is able to pull out water molecules from the DESO / water
cluster structure, increasing thus the DESO concentration in the free water and behaving as a
‘structure making’ molecule. On the contrary, in presence of DMSO 40% w/w the addition of Lyso
to the solution induced a slight shift of this band toward lower wavenumbers, indicating thus a
different behaviour of DMSO and DESO towards the water structure. This is in agreement with the
literature where DMSO is reported to act as a structure breaking, whereas DESO is considered a structure making solute [8].

As far as the thermal effects on Lyso are concerned, heat treatment of Lyso solution in the absence of sulfoxides induced significant changes in the spectral features of the Amide I band, due to structural modifications undergone by the protein, in particular in the $\alpha$-helix content. In fact, this secondary structure generally gives rise to a component band at about 1650 cm$^{-1}$. On the contrary, the profile of Amide I was poorly affected by the heating treatment when the aqueous environments of the protein was modified by the sulfoxide addition.

To obtain an accurate evaluation of the heat-induced conformational changes, the percentages of the secondary structure were calculated by a method proposed by Alix and co-workers [33]. This method is based on equation (2) which permits one to express the percentages of structural contents in a protein as a linear function of some parameters of the amide I Raman band, namely in wavenumbers of the peak and the left and right widths at the half-height.

$$\% \text{ structure} = a_0 + a_1 \nu_{\text{max}} + a_2 \nu_{\text{left}} + a_3 \nu_{\text{right}}$$  \hspace{1cm} (2)

The $a_n$ constants are coefficients calculated for each class of structure. This equation has been obtained by performing a statistical multi-parametric analysis of the correlations between the structural data (obtained from X-ray crystallography) on the one hand and the spectroscopic Raman data on the other by using a large set of reference proteins. The results are reported in Table 3.

In the absence of sulfoxides, heating treatment of Lyso induced a decrease in the $\alpha$-helix content and an increase in the $\beta$-sheet percentage (both of about 10%), whereas the content of the random coil conformation was almost unaffected (Table 3). This behaviour is in agreement with the data reported in the literature indicating the heat-induced formation of $\beta$-pleated sheet structures in Lyso [34].

To better evidence the heat-induced conformational modifications in the presence of DXSO, the differences between the conformation percentages after and before the heat treatment, relative to the conformation content before heating ($|\Delta \alpha|$, $|\Delta \beta|$, and $|\Delta R|$) have been also added in Table 3.
The addition either of DMSO or DESO in low concentration (up to 25% w/w) sensitively decreased the heat-induced structural modifications of the protein (i.e. $|\Delta \alpha|$ from 9% to 3-5%), confirming the qualitative analysis of the Raman spectra and the conclusion drawn out from the calorimetric analysis (see above). At the highest concentration the two sulfoxides showed different thermal stabilizing capability: the presence of an high DESO concentration seems to reduce the thermal stability of Lyso, as shown i.e. by the heat-induced changes in the $\alpha$-helix and $\beta$-sheet percentages ($|\Delta \alpha|$ and $|\Delta \beta|$ of $\approx$ 40%, respectively), whereas DMSO was still able to restrict the heat-induced effects on the protein structure; in fact, both $|\Delta \alpha|$ and $|\Delta \beta|$ resulted to be 3% after heating. Unfortunately, the simple addition of sulfoxides to the protein solution is also capable to induce perturbation in the secondary structure of the protein and the evidenced conformational changes were ever-increasing marked by increasing the sulfoxide content in the aqueous solution. This is probably a direct consequence of the disruption of intra-molecular peptide group interactions by sulfoxides (partial unfolding).

In conclusion, also the confomational analysis confirms the ability of both DMSO and DESO to act as effective protective agent against the thermal denaturation. In particular, as just indicated above (see enzymatic assay section), DESO seems to act as more efficient protective agent since it is necessary a lesser amount of sulfoxide to restrict the heat-induced changes in the protein conformations (i.e. $|\Delta \alpha|$ and $|\Delta \beta|$ ) to their minimum values.

The heat treatment induced also some spectral changes in some bands of Tyr, a residue that frequently plays a key role in proteins through hydrogen bonding of the hydroxyl group (Fig. 7). In particular, the intensity ratio ($I_{850} / I_{830}$) of the doublet at 850-830 cm$^{-1}$, marker of the Tyr side chain environment and the state of hydrogen bonding involving the Tyr OH group [35], slightly increased both after heating of the Lyso aqueous solution and as a consequence of the sulfoxide addition to the Lyso solution before heating treatment (Fig. 8). The intensity changes in this doublet indicate that at least one of the three Tyr residues is more exposed to the solvent, in agreement with the partial unfolding taking place under the these experimental conditions.
A similar behaviour was observed after heat treatment of Lyso in aqueous/DESO mixture: the slight increase in the $I_{850} / I_{830}$ value, indicating a more exposition of Tyr residues to the solvent, can be correlated with the decrease in the $\alpha$-helix content and the increase in the $\beta$-sheet conformations, occurring under these conditions (see Table 3). On the contrary, when Lyso undergoes the heat treatment in the DMSO/aqueous medium (in particular at the highest sulfoxide concentration), the $I_{850} / I_{830}$ intensity ratio decreased, indicating that at least one Tyr residue is located in a more hydrophobic environment and mainly acts as a hydrogen-bond donor (Fig. 8). Since the trend of the overall conformational changes is similar to those revealed in aqueous/DESO mixtures and one Tyr residue is adjacent to the catalytic residue Asp-52 (Tyr-53 is hydrogen bonded with the amino group of Asp-66 [36]), the changes in the Tyr environment can be attributed to the binding of some DMSO molecules to the active site. In fact, the literature reports that the DMSO molecules are capable to bind the protein at the active cleft [37,38]. This result can be correlated with the lower residual activity found after heating in the presence of 40% DMSO than 40% DESO (Table 2) and with the different electrophoretic behaviour showed by Lyso in the 40% DMSO / water mixture (Fig. 4B). Thus, although the overall conformations of Lyso in the DMSO / water and DESO / water systems are similar, probably the two media locally induce some different significant conformational changes, may be due also to the different capability of the two sulfoxides to affect the solvatation sphere of Lyso. This conclusion can be also related with the finding of protein aggregates only in the presence of 40% DMSO.

4. CONCLUSIONS

Sulfoxides are able to stabilise Lyso structure against the thermal denaturation. The calorimetric data suggest that both the hydrophilic properties, as well as the ability to stabilise the characteristic three-dimensional water structure, are ‘cooperative’ effects against the thermal inactivation of Lyso; in particular, the $\Delta H_d$ values appear to be useful indicator of such behaviour. A weak DPSO stabilizing effect was observed only when this sulfoxide was added in small amount, preventing
thus the strong destabilizing effect due to its great hydrophobic character. On the contrary, DESO, having both a low hydrophobic character and a great ability to stabilise the characteristic three-dimensional water structure, results to be the most effective sulfoxide. As regards DMSO, it behaves as a weaker thermal inactivation-preventing agent than DESO, since it has a very limited hydrophobic character and a destabilizing ability on the water structure.

The sulfoxide ability to act as effective protective agents against the heat-induced inactivation was confirmed by the protein analysis. In particular, the electrophoretic analysis showed that low and high concentrations of both DMSO and DESO does not induce changes in the protein electrophoretic migration. However, between the two compounds, DESO carries out the better protective effect against the heat-induced inactivation, being more able to stabilise Lyso structure after heating. Conversely, high DMSO concentrations induce the formation of proteic aggregates, responsible for protein activity reduction.

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REFERENCES


LEGENDS OF THE FIGURES

Figure 1. Peak temperatures (T<sub>d</sub>) of Lyso plotted as a function of the sulphoxides concentration

● : DMSO at pH = 5.0; ○ : DMSO at pH = 7.0; ■ : DESO at pH = 5.0; □ : DESO at pH = 7.0;
*: DPSO at pH = 5.0; △: DPSO at pH = 7.0

Figure 2 Denaturation enthalpy, ΔH<sub>d</sub>, as function of the sulfoxide content at pH 5.0 (●: DMSO; ■: DESO; *: DPSO)

Figure 3 Shape of the DSC curves relative the thermal denaturation of Lyso in aqueous solutions containing a different DESO amount at pH = 5.0 (first heating cycle; DESO content: (a) = 0.0 ; (b) = 5.0 ; (c) = 10.0 ; (d) = 20.0 ; (e) = 30.0; (f) = 50.0).

Figure 4 SDS-PAGE of Lyso solutions in absence and presence of sulfoxides (A) before and (B) after thermal treatment (15 min at 90°C): 1. Dalton Markers; 2. Lyso native; 3. Lyso + DESO 10%; 4. Lyso + DESO 40%; 5. Lyso + DMSO 10%; 6. Lyso + DMSO 40%.

Figure 5 The 1700-1300 cm<sup>-1</sup> Raman region of Lyso in water (a) and water/sulfoxide mixtures: (b) DESO 10%, (c) DESO 25%, and (d) DESO 40% w/w at pH 7.0.

Figure 6 The 900-1100 cm<sup>-1</sup> Raman region of water/sulfoxide solutions containing different amount of DESO (25% w/w (a and b) and 40% w/w(c and d)) in the presence of Lyso (a and c) and in the absence of the protein (b and d).

Figure 7 The 930-750 cm<sup>-1</sup> Raman region of the Lyso aqueous solutions before (a and c) and after the thermal treatment (b and d) in the absence of DESO (a and b) and presence of DESO 25% w/w (c and d).

Figure 8 The intensity ratios of the Tyr doublet at 850-830 cm<sup>-1</sup> obtained from the Raman spectra of Lyso in water/sulfoxide mixtures (■: DMSO; ●: DESO) at pH 7.0 before (A) and after heat treatment (B).
Table 1. Table summarizing the temperature of the maximum ($T_{d1}$ and $T_{d2}$) and the enthalpy ($\Delta H_{d1}$ and $\Delta H_{d2}$) of the calorimetric peak in the first and in the second heating cycle, as well the half-width of the transition ($\Delta T_{1/2}$) in the first heating cycle at pH 5.0 and 7.0. The measurements are relative both to pure lysozyme (0 %) as well as to lysozyme in the presence of different amounts of the considered sulfoxides.

<table>
<thead>
<tr>
<th>SULFOXIDES (%)</th>
<th>pH 5.0</th>
<th></th>
<th></th>
<th>pH 7.0</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$T_{d1}$ (°C)</td>
<td>$\Delta H_{d1}$ (Kj mol$^{-1}$)</td>
<td>$\Delta T_{1/2}$ (°C)</td>
<td>$T_{d2}$ (°C)</td>
<td>$\Delta H_{d2}$ (Kj mol$^{-1}$)</td>
<td>$T_{d1}$ (°C)</td>
</tr>
<tr>
<td>0 %</td>
<td>73.6</td>
<td>550</td>
<td>7.1</td>
<td>73.3</td>
<td>415</td>
<td>73.4</td>
</tr>
<tr>
<td>5 %</td>
<td>72.4</td>
<td>605</td>
<td>6.9</td>
<td>72.2</td>
<td>473</td>
<td>71.5</td>
</tr>
<tr>
<td>10 %</td>
<td>71.5</td>
<td>637</td>
<td>6.8</td>
<td>71.5</td>
<td>534</td>
<td>69.5</td>
</tr>
<tr>
<td>DMSO 20 %</td>
<td>69.0</td>
<td>656</td>
<td>6.5</td>
<td>68.8</td>
<td>552</td>
<td>65.7</td>
</tr>
<tr>
<td>30 %</td>
<td>66.2</td>
<td>669</td>
<td>6.8</td>
<td>66.3</td>
<td>560</td>
<td>61.5</td>
</tr>
<tr>
<td>40 %</td>
<td>64.0</td>
<td>647</td>
<td>7.2</td>
<td>63.9</td>
<td>561</td>
<td>58.1</td>
</tr>
<tr>
<td>50 %</td>
<td>60.2</td>
<td>589</td>
<td>7.7</td>
<td>60.2</td>
<td>545</td>
<td>53.1</td>
</tr>
<tr>
<td>5 %</td>
<td>71.2</td>
<td>627</td>
<td>6.4</td>
<td>71.2</td>
<td>530</td>
<td>70.7</td>
</tr>
<tr>
<td>10 %</td>
<td>68.4</td>
<td>656</td>
<td>6.8</td>
<td>68.5</td>
<td>584</td>
<td>67.2</td>
</tr>
<tr>
<td>DESO 20 %</td>
<td>63.4</td>
<td>701</td>
<td>7.2</td>
<td>63.2</td>
<td>685</td>
<td>61.2</td>
</tr>
<tr>
<td>30 %</td>
<td>58.0</td>
<td>685</td>
<td>7.6</td>
<td>57.8</td>
<td>665</td>
<td>54.7</td>
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<tr>
<td>40 %</td>
<td>51.3</td>
<td>627</td>
<td>8.0</td>
<td>51.2</td>
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<td>48.1</td>
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<tr>
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<td>45.1</td>
<td>495</td>
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<td>68.0</td>
<td>428</td>
<td>68.2</td>
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<tr>
<td>10 %</td>
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<td>573</td>
<td>6.9</td>
<td>62.5</td>
<td>467</td>
<td>63.0</td>
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<tr>
<td>DPSO 20 %</td>
<td>52.0</td>
<td>498</td>
<td>7.1</td>
<td>52.0</td>
<td>348</td>
<td>52.1</td>
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<tr>
<td>30 %</td>
<td>41.7</td>
<td>405</td>
<td>7.8</td>
<td>41.5</td>
<td>310</td>
<td>41.9</td>
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<tr>
<td>40 %</td>
<td>30.9</td>
<td>299</td>
<td>9.1</td>
<td>30.8</td>
<td>232</td>
<td>30.8</td>
</tr>
<tr>
<td>50 %</td>
<td>20.5</td>
<td>95</td>
<td>9.8</td>
<td>20.7</td>
<td>72</td>
<td>20.2</td>
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</table>
Table 2. Residual Enzymatic Activity (REA) percentages of Lyso obtained before and after thermal treatment in the presence of increasing sulfoxide amounts at pH 7.0.

<table>
<thead>
<tr>
<th>LYSO + SULFOXIDES (%)</th>
<th>DMSO (% REA)</th>
<th>DESO (% REA)</th>
<th>DPSO (% REA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After heating</td>
<td>Before</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>60</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>98</td>
<td>85</td>
<td>97</td>
</tr>
<tr>
<td>25</td>
<td>98</td>
<td>84</td>
<td>95</td>
</tr>
<tr>
<td>40</td>
<td>93</td>
<td>81</td>
<td>92</td>
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</tbody>
</table>

Table 3. Percentages of the secondary structure of Lyso obtained before and after thermal treatment in the presence of increasing sulfoxide amounts at pH = 7.0. The values were obtained by the analysis of the Amide I Raman Band. $\Delta \alpha$, $\Delta \beta$, $\Delta R$ represent the
percentages (in absolute value) of the heat-induced conformational changes calculated relatively to the conformation content before heating.

<table>
<thead>
<tr>
<th>Sulfoxides</th>
<th>Lyso + α-helix (%)</th>
<th>Δα</th>
<th>Lyso + β-sheet (%)</th>
<th>Δβ</th>
<th>Random (%)</th>
<th>ΔR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After heating</td>
<td>(%)</td>
<td>Before</td>
<td>After heating</td>
<td>(%)</td>
</tr>
<tr>
<td>DMSO</td>
<td>0</td>
<td>43</td>
<td>9</td>
<td>26</td>
<td>29</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>41</td>
<td>5</td>
<td>28</td>
<td>30</td>
<td>7</td>
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<td></td>
<td>40</td>
<td>32</td>
<td>3</td>
<td>35</td>
<td>36</td>
<td>3</td>
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<tr>
<td>DESO</td>
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<td>40</td>
<td>3</td>
<td>29</td>
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<td>36</td>
<td>22</td>
<td>39</td>
<td>45</td>
<td>41</td>
</tr>
</tbody>
</table>

\[ Δα = \frac{α-\text{helix}\%\text{ before heat} - α-\text{helix}\%\text{ after heat}}{α-\text{helix}\%\text{ before heat}} \times 100 \]
Figure 1
$\Delta H$ (kJ mol$^{-1}$)

DXSO (% w/w)
Figure 3

Exothermic Heat Flow

T/°C

f
e
d
c
b
a
Figure 4

A

B

1 2 3 4 5 6
Figure 5

![Raman Spectra Image]

Wavenumber (cm⁻¹) vs. Raman Intensity

Legend:
- Amide I: 1667
- Trp, Tyr, Phe: 1620
- Tyr, Phe: 1584
- Trp: 1558
- C-H def: 1366
- C-H def: 1339

Peaks at:
- 1667 Amide I
- 1620 Trp, Tyr, Phe
- 1584 Tyr, Phe
- 1558 Trp
- 1366 C-H def
- 1339 C-H def
Figure 6
Figure 7
Figure 8

A

B

\[ \frac{I_{920}}{I_{830}} \text{ (Tyr)} \]

DXSO (% w/w)

0,0

0,3

0,6

0,9

1,2

1,5

1,8

0

10

20

30

40

0

0,3

0,6

0,9

1,2

1,5

1,8

0

10

20

30

40