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Role of Mannitol in Modulating the Activity, Structure and Aggregation Propensity of Superoxide Dismutase during Various Neurodegenerative disorders

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Abstract

The accumulation of small organic molecules and other low molecular weight metabolites is a widespread response that may protect diverse range of organisms against the environmental stress conditions. Accumulation of high concentrations of these organic metabolites, organisms adapt to the environmental perturbations that may lead to the structural alterations in their cellular proteins. Mannitol is one the main organic osmolyte synthesized in huge amounts as a stress-responsive factor especially in brain cells to retain cellular integrity when cells are exposed to environmental stresses. We intended to investigate the effect of mannitol on functional, structural stability and aggregation pattern of superoxide dismutase, which plays a key role against oxidative stress. We observed that mannitol increased the activity of superoxide dismutase several folds by stabilizing the secondary and tertiary structure of SOD. In addition to this, mannitol inhibited the formation of fibrils. Higher concentrations significantly decreased the formation of high molecular weight oligomers. Thus, Mannitol could be a promising scaffold for the development of therapeutic agent in a variety of neurodegenerative disorders.

Key Words: Mannitol, Oxidative stress, Superoxide Dismutase, Aggregation, Amyloid.

Introduction

Oxidative stress is linked to a number of degenerative pathways that contribute to cellular dysfunction and eventually cellular death[1]. Cells defend the oxidative stress by production and accumulation of various small organic molecules to protect the macromolecular structure under these stressful conditions[2]. Such organisms undergoing stresses accumulate higher concentrations of these molecules especially polyhydroxy compounds (up to 400 mM) in their cellular tissues during oxidative stress disorders[3]. Mannitol is one of the important metabolite that has been found to get accumulated in multiple oxidative stress disorders especially neurodegenerative diseases[4]. The prime and most vulnerable target for oxidative stress induced

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damage is the anti-oxidant system like Superoxide dismutase, Catalase and glutathione peroxidase[5].

Copper, zinc superoxide dismutase (Cu, Zn SOD) is an oxido-reductase enzyme that dismutates the deadly superoxide radical into molecular oxygen and hydrogen peroxide in a two-step process using alternate reduction and oxidation of the active-site copper[6]. The reactive agents of oxygen, such as the superoxide, escape from the respiratory chain and inflict havoc to the cell. Superoxides are the free radical molecules that promptly accept electrons, which mark them extremely reactive. They shred the electrons from cellular molecules that are essential for proper cell function, triggering cellular dysfunction and possibly resulting in the cellular apoptosis[7]. Oxygen is also a very highly reactive compound[8]. Cu, Zn superoxide dismutase is the crucial antioxidant defense in almost all the cells exposed to oxygen[9]. It is believed that It forms a very crucial constituent of the cellular response to oxidative stress by the detoxification of the superoxide radical by a specialized reaction called as dismutation[10].

Cu/Zn Superoxide dismutase is the metalloprotein having structural weight of 32,500, containing both the copper as well as zinc ions in each of the subunit. Each enzyme subunit is composed of eight antiparallel β strands that form a flattened cylinder, and the external three loops. The active site Cu(II) and Zn(II) lie 6.3 Å apart at the bottom of this long channel; the Zn is buried, while the Cu is accessible to solvent[11]. The side-chain of His61 makes a bridge between the Zn and copper and is coplanar with them[11].

Most enzymes that produce and require superoxide are in the peroxisomes, together with superoxide dismutase, catalase and peroxidases[9]. If SOD is defective or inefficient, superoxide is not degraded properly and can destroy the cells. Hydrogen peroxide is also one of the fatal agent that needs to be detoxified[12]. The cells use the enzyme catalase, a ubiquitous heme protein that catalyzes the dismutation of hydrogen peroxide into water and molecular oxygen[13]. Current studies are focused on the strategy of enhancing the activity of superoxide dismutase using mannitol as a small organic molecule that accumulate upto various millimolar concentrations in the cells during the oxidative stress stages of the cell. Mannitol functions as structural stabilizers and aggregation inhibitors for many proteins in the cell especially Parkinson's diseases, where it has been reported that mannitol inhibits the fibrillation of α-syn protein[14-20]. Thus we aimed to investigate the possible role of mannitol in attenuating the cellular oxidative stress by modulating the structure of superoxide dismutase which may inturn enhance the functional capability of SOD. The increased activity of SOD may effectively protect the cell during periods of oxidative stress.

Materials and methods

Superoxide dismutase and 4-anilino-1 naphthalene sulphate (ANS) were obtained from Sigma Chemical Co. whereas di-sodium hydrogen orthophosphate, sodium di-hydrogen orthophosphate, mannitol were purchased from Himedia laboratories. NBT, hydroxylamine

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hydrochloride, EDTA was received from Merck, Darmstadt, Germany and all other reagents used were of analytical grade.

The stock solution of SOD was prepared in 0.05 M sodium carbonate buffer solution at pH 10.2. The entire buffer and other stock solutions were prepared using double distilled deionized water. Before using the protein, it was dialyzed for 24 hours against the double distilled water at 4^{o}C. All the buffer solutions were degassed prior to use. Hydroxylamine hydrochloride and nitroblue tetrazolium solution for activity studies were freshly prepared. The stock solution of mannitol was prepared in 50 mM buffer of pH 7.4. The pH of the stock solutions was checked for any change upon addition of the mannitol.

Activity measurement of superoxide dismutase

The activity of Superoxide dismutase was estimated by Beauchamp and Fridovich method (1971) and monitored at 560 nm by using UV-Visible Spectrophotometer[21]. The reaction mixture consists of sodium carbonate buffer, NBT (nitro blue tetrazolium) and EDTA. The reaction was initiated by the addition of hydroxylamine hydrochloride and the change in absorbance was recorded at the wavelength of 560 nm at 25^{o}C for 1 hr. The activity of SOD was assessed by the amount of enzyme required to inhibit 50% of NBT.

The initial velocity (v_o) from each progress curve of the kinetic plots of enzymes at a given substrate concentration is determined from the straight portion of first 30 seconds of the kinetic curve. The reaction fits into the first order rate kinetics in which rate of reaction is dependent on the substrate concentrations.

FTIR Spectra Measurements of Secondary Structure of SOD

FTIR spectra of superoxide dismutase in absence and presence of highest concentration of mannitol were recorded on a Bruker FTIR Alpha spectrometer using a Golden Gate ATR accessory equipped with a single-reflection diamond crystal. The temperature during the measurements was kept at 25°C using the temperature controller. For each spectrum, 256 scans were collected with a resolution of 4 cm^{-1}. The spectrometer was purged with dry nitrogen to diminish water-vapor contamination of the spectra. All FTIR spectra were water subtracted and corrected using an advanced ATR correction algorithm. This method allowed us to obtain information concerning a secondary structure of the protein and its changes in the presence of mannitol by observing changes in the amide Bond of SOD with highest mannitol concentration.

Intrinsic Fluorescence Spectrometry:

The fluorescence spectrophotometry was employed to monitor the changes in the tertiary structure of superoxide dismutase in the presence and absence of different concentrations of mannitol at 25±0.1°C and pH of 7.0. The fluorescence spectral measurements were run in the emission wavelength range of 300–500 nm with an excitation wavelength of 278 nm and the

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bandwidth was set at 10 nm. The necessary blanks for each sample were also run to correct the background fluorescence.

Extrinsic fluorescence (ANS):

ANS is the dye which binds to the exposed hydrophobic residues of the proteins[22]. The dye acts as a probe to monitor the conformational changes of the protein. The assay was employed to look for any changes in the exposure of surface hydrophobic patches of superoxide dismutase in the presence of increasing mannitol concentrations (0.25, 0.50 and 0.75 M). The wavelength for ANS excitation in this study was set at 360 nm and the emission was recorded in the wavelength range of 400-600 nm using SOD concentration of 0.2 µM. All the samples were run in triplicates. All the samples were prepared in dark to avoid photo-decomposition of ANS dye. The concentration of ANS was 16-folds greater than that of the concentration of protein used.

Acrylamide quenching:

The quenching of intrinsic fluorescence of SOD by acrylamide at 280 nm was done by titrating the protein with acrylamide as quencher in presence and absence of mannitol. The data thus obtained was analyzed by Stern-Volmer model using equation 1. The stern volmer constant gives us the information about the extent of unfolding or compaction of protein using the sensitive intrinsic fluorescence as probe. The stern volmer constant was calculated (K_{sw}) was calculated as per the equation 1:

$$\frac{F_0}{F_i} = 1 + K_{sw}\,[Q] \qquad \longrightarrow \quad (1)$$

Results and Discussion

Effect of osmolytes on superoxide dismutase function

The activity of superoxide dismutase (SOD) was determined by the dismutation reaction of SOD on the reduction of nitroblue tetrazolium (NBT). The reaction was spectrophotometrically monitored at the wavelength of 560 nm. The activity of SOD in absence and presence of different concentrations of mannitol (0.25, 0.50 and 0.75) were observed by monitoring the change in intensity of blue colored product which absorbs at maximally at 560 nm. Kinetic curves obtained by plotting initial velocity versus substrate concentration were analyzed for relative activity (Fig. 1). Our study reveals that mannitol has significant effect on superoxide dismutase activity. By increasing the concentration of mannitol, the superoxide dismutase activity was progressively increased. The catalytic activity of SOD reached a maximum when mannitol concentration was increased to 0.75 M. The enhancement in the activity of superoxide dismutase by mannitol might be attributed towards the structural stabilization of superoxide dismutase by polyol osmolyte (mannitol) by causing more preferential hydration effects on the protein surface which ultimately stabilizes the protein molecules to the greater extent. To further validate the functional enhancement of SOD activity, structural studies of protein were also undertaken to evaluate the structural modifications of SOD in presence of mannitol.

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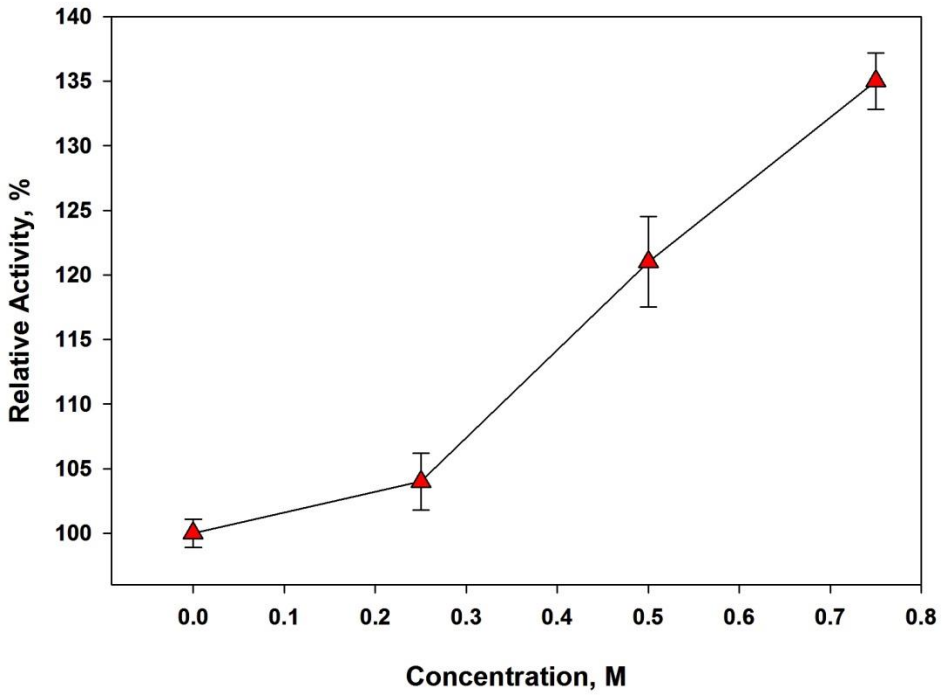

Fig. 1: Relative activity of superoxide dismutase in presence of varying concentrations of mannitol.

Fourier-transform infrared spectroscopy measurement

FTIR spectroscopy is employed to obtain information about the secondary structure, misfolding, and aggregation of proteins. The infrared absorption spectrum of superoxide dismutase and mannitol treated SOD was measured in transmission by FTIR spectroscopy in the Amide I region (1700 to 1600 cm^2) where the absorption of the C=O peptide group allows the identification of the secondary structural elements of the protein[23]. Results concerning FTIR spectroscopic analysis of mannitol−SOD interaction are presented in the Fig 2. FTIR results specify that the 0.75 M of mannitol well maintained the secondary structural elements of superoxide dismutase. The slight increase in the intensity of amide band at 1653 cm^{-1} in presence of 0.75 M mannitol represented the stabilized secondary structure of SOD as compared to control one.

The slight increase in the band intensity in presence of mannitol may be due to the increase in hydrogen bond strength between water and protein surface which provides extra stability to the secondary C=O and N=H bonds in the protein surface.

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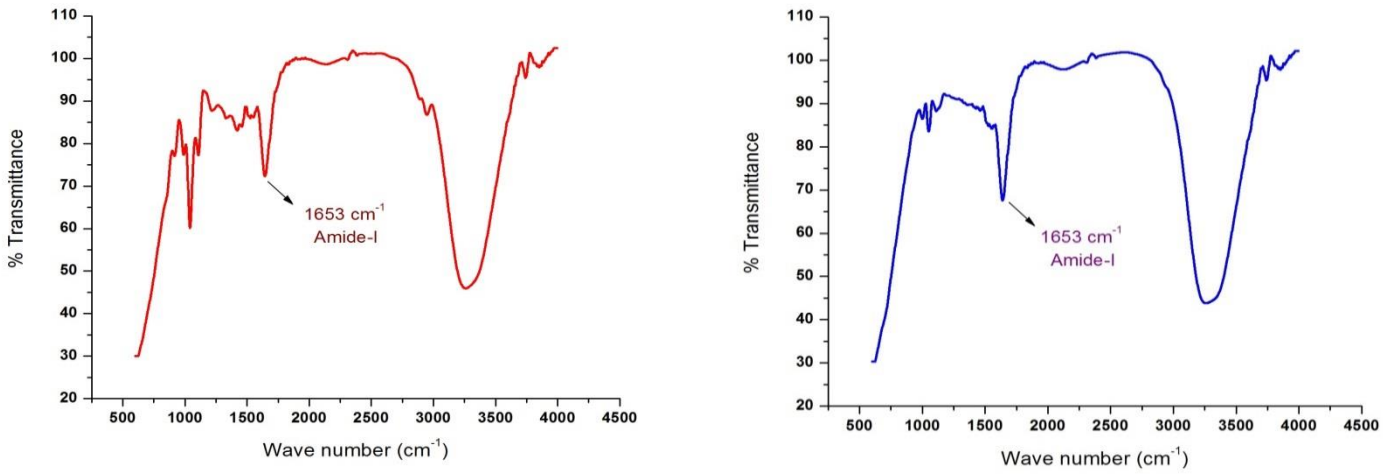

Fig. 2: FTIR Spectra of Cu/Zn Superoxide dismutase in absence and presence of mannitol. Left panel represents the IR spectra of native SOD and Right one is mannitol treated.

Intrinsic Fluorescence:

Intrinsic (Tryptophan) fluorescence can provide information about the variations in three dimensional protein structure[24]. Fluorescence spectra of SOD with various concentrations of mannitol were recorded. Fig. 3 denotes the quantum yields for SOD after interaction with increasing mannitol concentrations. The changes in the emission spectrum of tryptophan may be caused by protein conformational transitions. It was observed that the incubation of SOD with different concentrations of mannitol caused the decrease in the maximum fluorescence intensity with the blue shift of 3 nm at 0.75 M of mannitol. This serial decrease in the protein intensity with increasing mannitol concentration might be attributed towards the fact that addition of mannitol caused more compaction of protein which causes further burying of exposed tryptophan residues towards more interior of the protein and hence the fluorescence intensity of tryptophan is decreased.

Trp fluorescence can be quenched by neighboring acrylamide. The decrease in emission of intrinsic fluorescence of superoxide dismutase in presence of highest concentration of mannitol supports the possibility of less quenching, which might be due to the increased distance between Trp and quenching groups. The stern volmer quenching constant (K_{sw}), which gives information about the microenvironment of the proteins. The quenching constant (K_{sw}) value was reduced to 1.07 in presence of mannitol while it was found to be 1.71 in control, The decreased K_{sw} values in presence of mannitol indicate reduced exposure of the trp residues to the surrounding aqueous environment. This signifies that the protein molecule was compacted and further folded into the more stabilized tertiary conformation (Fig. 4).

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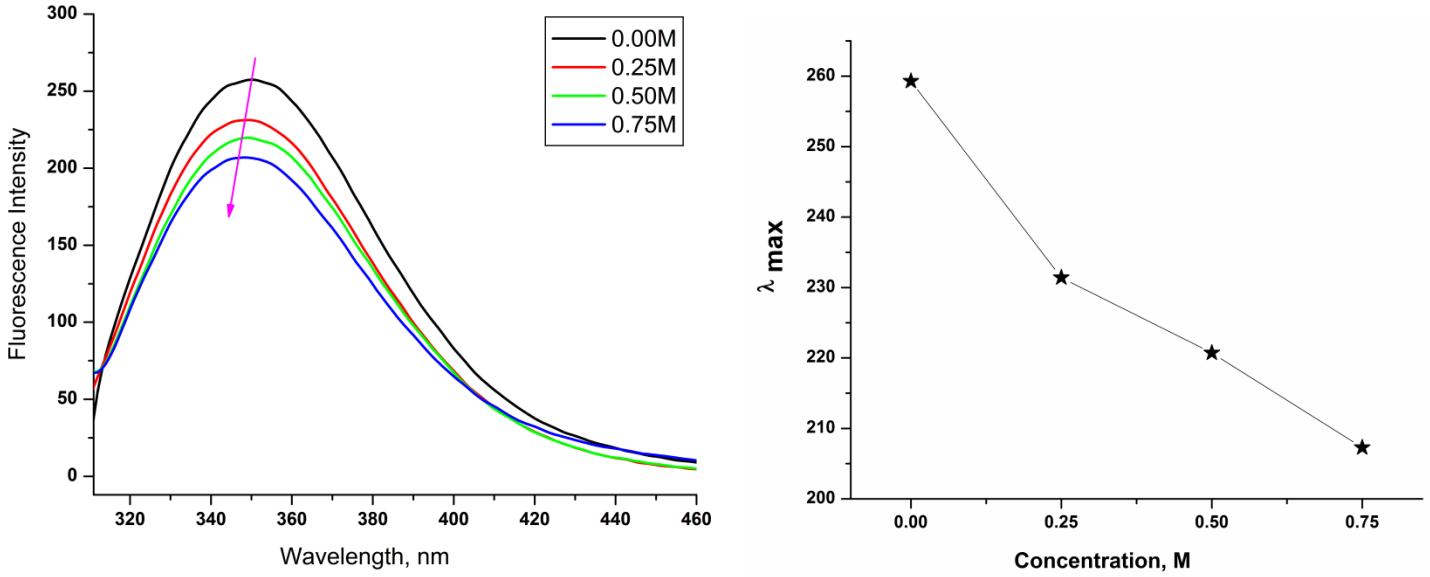

Fig. 3: Intrinsic tryptophan fluorescence of SOD in absence and presence of varying mannitol concentrations. Right panel shows the change in intensity versus concentration.

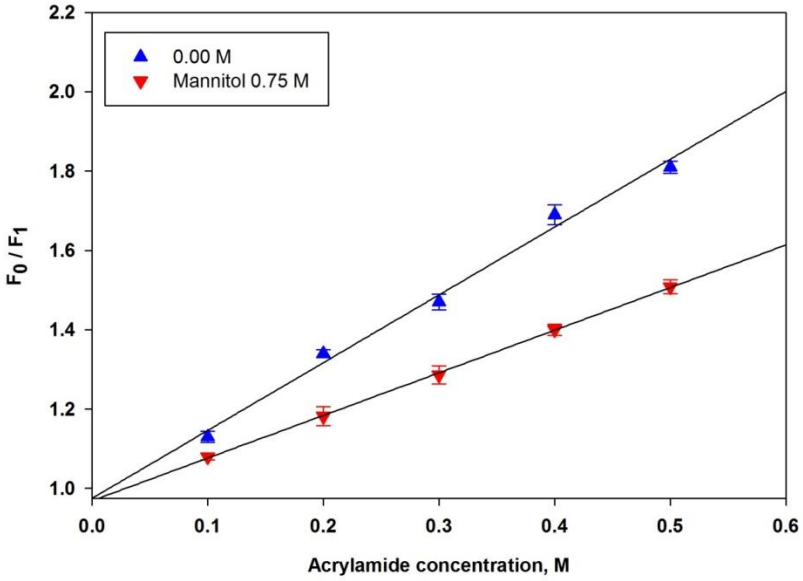

Fig. 4: Stern volmer plots for the quenching of SOD by various concentrations of acrylamide as quencher with 0.75 M mannitol concentration (Red) and control (Blue).

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Extrinsic (ANS) fluorescence:

To monitor for any change in the surface hydrophobicity of superoxide dismutase (SOD), due to alterations in the tertiary structure of SOD in presence of the different concentrations of mannitol (0, 0.25, 0.50 and 0.75 M), ANS binding experiments were carried out. With an increase in mannitol concentrations, a progressive decrease in ANS fluorescence intensity with a prominent blue shift of about 18 nm with 0.75 M mannitol concentration (reduced binding) was observed (Fig. 5). The shift of wavelength towards the lower wavelength in presence of 0.75 M concentration of mannitol indicates reduced exposure of hydrophobic residues to the solvent thus further burying of exposed tryptophan residues to the more interior thus favors compaction of protein. The result was in good conformity with that of intrinsic fluorescence measurements.

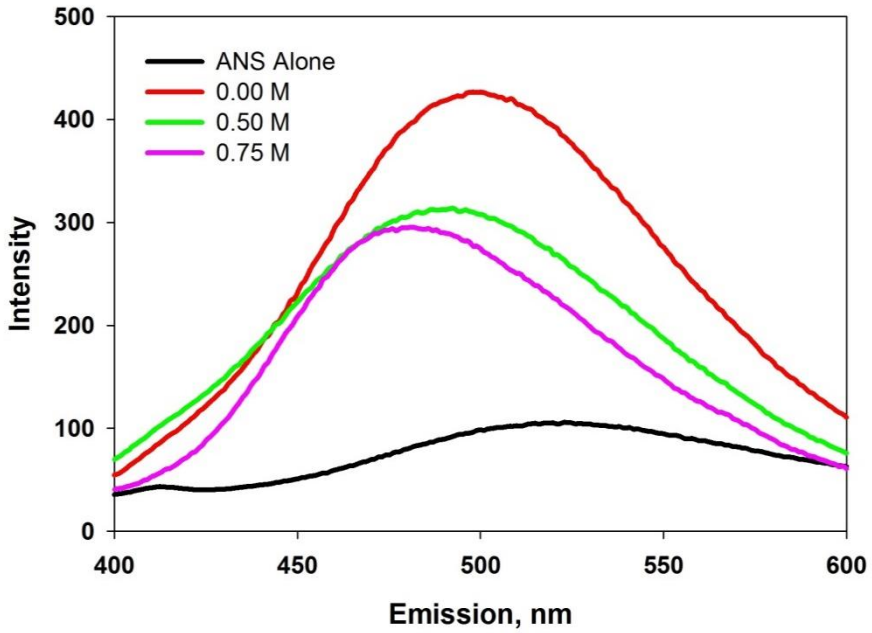

Fig. 5: ANS binding assay of SOD in absence and presence of different mannitol concentrations.

Aggregation Kinetics:

Aggregation kinetics of superoxide dismutase were studied systematically both in the absence and presence of various concentrations of mannitol. Thioflavin T (ThT) is a dye known to bind to aggregates of the protein with cross beta structure which gives rise to a large enhancement in the fluorescence intensity[25]. The profile of the aggregation kinetics using ThT as marker, the fluorescence intensity of aggregated fibrils was observed. The increase in the fibril formation correlates with an enhancement in the Thioflavin-T fluorescence. ThT fluorescence spectrum follows the sigmoidal curve characterized by the initial lag phase, which is followed by a sharp rise in fluorescence, and the final plateau phase. The initial lag phase usually is interpreted as the time in which aggregation nuclei develop. Consequently, followed by a sharp increase, which signifies fibril elongation and/or secondary nucleation, the maximum velocity is a suitable way

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for comparing the rates and the final plateau which signifies the maximum value reached where the extreme amount of β-sheet conversion has arisen (in many cases, the fluorescence after reaching the plateau, a reduction in ThT fluorescence is detected when fibrils associate laterally). The kinetic spectrum was followed for 40 h. From the ThT fluorescence kinetics, there was an increase in the lag time with a decline in the maximum plateau and slope values that signify effective inhibition in presence of increasing concentration of mannitol (Fig. 6). The concentration of 0.75 M mannitol had the maximum effect on the aggregation kinetics of the protein. When compared with the control, we have found that the rate of aggregation is higher (i.e. with less lag time) for SOD as compared to the treated ones (Fig. 6). Thus mannitol may prove as a potential candidate for the aggregation inhibitor of SOD thus may elevate the anti-oxidant potential in the cell by reducing the chances of deactivation or suppression of SOD by oxidant or free radical generated species in the living organisms.

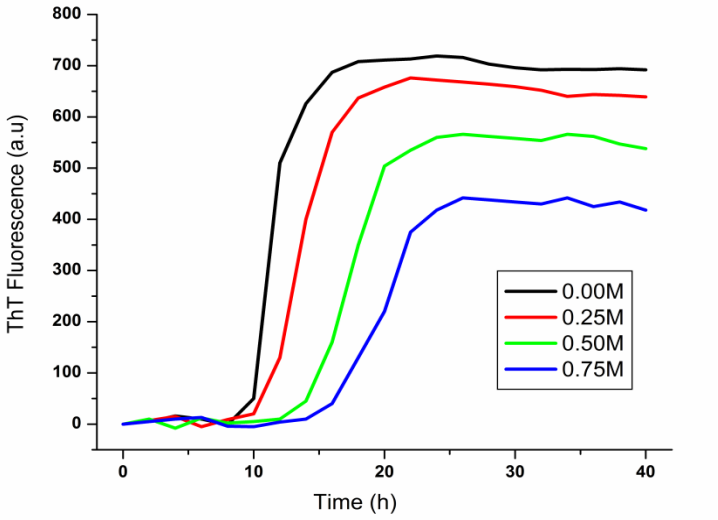

Fig. 6: Aggregation pattern of SOD with increasing concentrations of mannitol using Thioflavin-T dye as probe for 40 hrs.

Conclusion

Superoxide dismutase contains Cu^{2+} and Zn^{2+} as the cofactors. Cu has been found to be involved for the primary function of SOD (dismutation reaction), The superoxide dismutase (SOD) has been found to be an important member of the anti-oxidant system of the cell. Thus the stability and efficiency of SOD is of greater concern during different oxidative disorders. Cells during oxidative disorders have been found to upregulate the formation and accumulation of various smaller organic molecules during the oxidative stress disorders. One of the molecule that has been found to be accumulated in oxidative stress is mannitol. Our study has identified mannitol as a potent stabilizer of superoxide activity. Furthermore, mannitol also lead to the decrease in the formation of aggregation species by stabilizing the overall tertiary structure of superoxide dismutase. The mechanism of effect of mannitol on SOD stability and aggregation propensity, will eventually serve in guiding the development of possible agent which can inhibit or regulate

protein aggregation process during various oxidative stress and neurodegenerative disorders where stability of proteins is compromised. In fact, such a study would be an excellent modal study for the discovery of new stabilizing agents and aggregation inhibitors for an important protein systems in the body.

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