The effect of some osmolytes on the activity and stability of mushroom tyrosinase

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The thermodynamical stability and remained activity of mushroom tyrosinase (MT) from *Agaricus bisporus* in 10 mM phosphate buffer, pH 6.8, stored at two temperatures of 4 and 40°C were investigated in the presence of three different amino acids (His, Phe and Asp) and also trehalose as osmolytes, for comparing with the results obtained in the absence of any additive. Kinetics of inactivation obeye the first order law. Inactivation rate constant (k_{inact}) value is the best parameter describing effect of osmolytes on kinetic stability of the enzyme. Trehalose and His have the smallest value of k_{inact} (0.7×10^{-4} s^{-1}) in comparison with their absence (2.5×10^{-4} s^{-1}). Moreover, to obtain effect of these four osmolytes on thermodynamical stability of the enzyme, protein denaturation by dodecyl trimethylammonium bromide (DTAB) and thermal scanning was investigated. Sigmoidal denaturation curves were analysed according to the two states model of Pace theory to find the Gibbs free energy change of denaturation process in aqueous solution at room temperature, as a very good thermodynamic criterion indicating stability of the protein. Although His, Phe and Asp induced constriction of MT tertiary structure, its secondary structure had not any change and the result was a chemical and thermal stabilization of MT. The enzyme shows a proper coincidence of thermodyanamic and structural changes with the presence of trehalose. Thus, among the four osmolytes, trehalose is an exceptional protein stabilizer.

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1. Introduction

Tyrosinase (EC 1.14.18.1) is a copper-containing mono-oxygenase responsible for the biosynthesis of melanins and other polyphenolic compounds (Lerch 1981). It catalyses both the orthohydroxylation of monophenols and the oxidation of o-diphenols to o-quinones. Tyrosinase is widely distributed in mammals, plants and micro-organisms (Robb 1984). Various strategies to increase the stability of enzyme include chemical modification (Ryan *et al* 1994) using osmolytes (Taneja and Ahmad 1994) and special organic solvents (e.g. polyethylene glycol) (Ozaki *et al* 1998). Osmolytes can be polyols, sugars, polysaccharides, neutral polymers, amino acids and their derivatives, and large dipolar molecules like trimethylamine N-oxide (Yancey *et al* 1982; Fan-Guo *et al* 2001; Murphy 2001). It is light that physico-chemical properties of proteins affected from bulk properties of the solvent environment. Osmolytes as solvent additives favorably affect protein stability and solubility.While they can increase stability of proteins while protecting them from thermal denaturation, the enzymatic activity is not reduced (Arakawa and Timasheff 1983).

Trehalose [α-D-glucopyranosyl (1-1)- α-D-glucopyranoside] is a nonreducing disaccharide in which the two glucose

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Abbreviations used: Asp, Aspartic acid; CA, caffeic acid; DTAB, dodecyl trimethylammonium bromide; His, histidine; MRW, mean amino acid residue weight; MT, mushroom tyrosinase; PBS, phosphate buffer solution; Phe, phenylalanine; T_m, protein melting point; Tre, trehalose.

units are linked in an a,a-1,1-glycosidic linkage. This sugar is present in a wide variety of organisms including bacteria, yeast, fungi, insects, invertebrates, and lower and higher plants where it may serve as a source of energy and carbon (Evansand and Dethier 1957; Wyatt and Kalf 1957; Nwaka and Holzer 1998; Elbein *et al* 2003). An important *in vivo* and *in vitro* study showed that trehalose protects cells from heat by stabilizing proteins at high temperatures. These investigations showed that enzymes are better able to retain activity during heat shock in cells that are producing trehalose (Singer and Lindquist 1998). In yeast and plants, trehalase may also serve as a signalling molecule to direct or control certain metabolic pathways or even to affect growth. In addition, trehalose can protect proteins and cellular membranes from inactivation or denaturation caused by a variety of stress conditions, including desiccation, dehydration, heat, cold, and oxidation (Crowe *et al* 1984; Elbein *et al* 2003).

Mushrooms contain large amounts of carbohydrates including polysaccharides (such as glucans and glycogen) and monosaccharides and disaccharides (such as trehalose), sugar alcohols (such as mannitol), and chitin. It has been found that *Agaricus bisporus* white and brown strains contained 4.5% and 4.6% (fresh wt.) total carbohydrates. *A. bisporus* also contain significant amounts (20–30%, dry wt.) of the sugar alcohol mannitol, and 1 to 3% of the disaccharide, trehalose (Ajlouni *et al* 1993; Mattila *et al* 2002).

Thus synthesis and intracellular accumulation of certain small organic solutes known as "organic osmolytes" is a common mechanism evolved by organisms for protecting proteins (Brown and Simpson 1972; Stewart and Lee 1974; Pollard and Wyn Jones 1979; Yancey *et al* 1982). These naturally occurring solutes include specific amino acids, certain polyols, and particular methylamine species (Yancey *et al* 1982).

There are some reports on the effect of some of these substances on the structure and function of different enzymes and proteins (Yancey *et al* 1982; Saboury *et al* 1999; Shahjee *et al* 2002) . Since tyrosinase as an important enzyme which is involved in crucial physiological process through animals, plants, fungi, insects and other organisms, and its deficiency may cause problems as well as its over activity, the study of its activation and/or stabilization is as important as its inhibition. Osmolytes can increase the stability of proteins while protecting them from thermal denaturation meanwhile the enzymatic activity is not reduced (Arakawa and Timasheff 1983). Persuing our previous studies on inhibition (Karbassi *et al* 2004; Gheibi *et al* 2005), stability (Karbassi *et al* 2003; Gheibi *et al* 2005), modification (Saboury *et al* 2004) and structure and function of mushroom tyrosinase (MT); the effect of trehalose (as a sugar), and various amino acids because of their different nature and polarity, e.g. phenylalanine (Phe) as hydrophobic amino acid, histidine (His) as positive amino acid and aspartic acid (Asp) as negative amino acid has been investigated on the stability and activity of MT.

2. Materials and methods

2.1 *Materials and buffer preparation*

Mushroom tyrosinase (MT; EC 1.14.18.1), specific activity 3400 units/mg, was purchased from Sigma (UK). Caffeic acid (CA) and trehalose were from Merck (Germany). Phenylalanine, histidine and aspartic acid were obtained from authentic samples. Dodecyl trimethylammonium bromide (DTAB) was obtained from Sigma. The buffer used in the assay was 10 mM phosphate buffer solution (PBS), pH=6.8, which its salts were obtained from Merck. All experiments were carried out in 20°C and solutions were prepared in doubly distilled water.

2.2 *Kinetic measurements*

The kinetic assays of catecholase were carried out using Cary spectrophotometer, 100 Bio model, with jacketed cell holders. Freshly prepared enzyme and substrate solutions were used in this work. All enzymatic reactions were run in phosphate buffer (10 mM) at pH=6.8 in a conventional quartz cell thermostated to maintain the temperature at 20°C. The selected conditions of solvent, buffer, pH, temperature, and enzyme concentration were applied for assaying the oxidase activity of MT according to our previous studies (Gheibi *et al* 2005). In catecholase reactions, depletion of CA were measured in 311 nm for 2 min using an enzyme concentration of 11.8 μM, 40 unit/ml. All assays were repeated at least three times. Definitions of units were defined by the vender. Accordingly, catecholase activity is equal to the 0.001 change in the optical density of ascorbic acid per minute at 265 nm in 3 ml of the reaction mixture (25°C and pH = 6.5), when catechol or L-dopa is used as the substrate.

2.3 *Long-term and thermal inactivation of MT*

Since the enzyme is labile in lab temperature (25°C), its long-term inactivation was investigated at 4°C for 360 h. Residual activity of the enzyme was investigated by UV spectrophotometry through catecholase reaction in the absence and presence of 1 mM trehalose, and 5 mM concentrations of Phe, His and Asp. Aliquots of incubated solutions containing 11.8 μM (40 unit/ml) enzyme together with above concentrations of osmolytes were removed at

various times and assayed for enzyme activity after addition of CA, as a substrate. Stability of the enzyme against heat was studied by incubating definite concentration of enzyme in the absence and presence of trehalose, Phe, His and Asp, at 40°C, for 90 min. Then the catecholase activity of each sample was assayed as usual.

2.4 *Protein stability measurements*

To obtain effect of osmolytes on the thermodynamical stability of the enzyme, chemical denaturation profiles of MT were recorded by titration of a protein solution 0.12 mg/ml with aliquots from a 50 mM stock solution of DTAB, as a cationic surfactant. These experiments were carried out in the absence and presence of 1 mM trehalose, and 5 mM concentrations of Phe, His and Asp. Conformational change of the protein was obtained at wavelength of 280 nm by the spectrophotometric technique.

2.5 *Circular dichroism spectroscopy*

The far UV region (190-260) that corresponds to peptide bond absorption was analysed by an Aviv model 215 Spectropolarimeter (Lakewood, USA) to give the content of regularly secondary structure in MT. For far UV spectra, MT was studied at concentration of 0.21 mg/ml with 1 mm path length quartz cell. Enzyme solutions were prepared in the 10 mM PBS buffer at pH 6.8. Molar ellipticity ([θ]) of the enzyme solutions in the absence and presence of 1 mM trehalose, and 5 mM concentrations of Phe, His and Asp after its incubation for at least 5 min. All spectra were collected in a triplicate from 190 to 260 nm and a back ground-corrected against osmolytes and buffer blank. The data were smoothed by applying the software, including the fast Fourier-transform noise reduction routine, which allows the enhancement of most noisy spectra without distorting their peak shapes. The results were expressed as ellipticity [deg cm^2 $dmol^{-1}$] based on a mean amino acid residue weight (MRW) of 125 for MT having the average molecular weight of 120 kDa (Strothkemp *et al* 1976). The molar ellipticity was determined as $[\theta] = (100\times(MRW)\times\theta_{obs}/cl)$, where θ_{obs} is the observed ellipticity in degrees at a given wavelength, c is the protein concentration in mg/ml and l is the length of the light path in cm. All measurements were carried out at 25°C with the help of a thermostatically controlled cell holder attached to a Neslab's RTE-110 circulating water bath with an accuracy of ± 0.1°C.

Thermal denaturation profiles of MT (0.21 mg/ml) were also obtained by circular dichroism (CD) technique following the ellipticity in 222 nm. The experiment were carried out in the absence and presence of definite concentrations of trehalose, His, Phe and Asp, as well.

2.6 *Fluorescence measurements*

Fluorescent intensity measurements were carried out on a Hitachi spectrofluorimeter, MPF-4 model, equipped with a thermostatically controlled cuvette compartment. Emission spectra were recorded from 300 to 450 nm with excitation at 280 nm in increments of 1 nm and the intrinsic fluorescence of MT, in the absence and presence of 1 mM trehalose, and 5 mM concentrations of Phe, His and Asp. MT complexes were measured by exciting the protein solution (0.17 mg/ml) in 10 mM PBS buffer at pH 6.8 and 25°C, in 1 ml semi-micro quartz cuvettes with a 1 cm excitation light path.

3. Results

3.1 *Long term and thermal inactivation*

Enzyme inactivation alongside of time and thermal condition in the absence and presence of trehlose and three amino acids, His, Phe and Asp are showed in figure 1. The kinetics of inactivation obeys the first order law:

$$\ln A/A_0 = - k_{inact}\, t. \quad (1)$$

The inactivation rate constant (k_{inact}) value is the best parameter describing the effect of osmolytes on the kinetic stability of the enzyme. A and A_0 are the enzyme activity in each time and initial time, respectively.

Magnitudes of k_{inact} in long-term and thermal condition can be obtained from insets of figure 1a, b and illustrated in table 1. The magnitudes of k_{inact} show stabilization of MT in the presence of this four osmolytes through long-term inactivation. In the thermal inactivation trehalose and His have the smallest value of k_{inact} (0.7×10^{-4} s^{-1}) in comparison with control (without any osmolyte) (2.5×10^{-4} s^{-1}).

3.2 *Chemical and thermal denaturation*

Chemical and thermal stability of MT was assessed in the absence and presence of osmolytes. Chemical and thermal denaturation profiles obtained from DTAB titration and thermal scanning, respectively, in the absence and presence of trehalose, Phe, His and Asp in definite concentrations are depicted in figures 2 and 3. Each profile is a sigmoidal curve, thus this process is described as a single denaturant-dependent step according to the two-step theory (Pace *et al* 1990). The determination of standard Gibbs free energy of denaturation ($\Delta G°$), as a criterion of conformational stability of a globular protein, is based on two state theory as follows:

$$\text{Native } (N) \Leftrightarrow \text{Denatured } (D). \quad (2)$$

By assuming two-state mechanism for protein denaturation, one can determine the process by monitoring changes in the absorbance or elipticity (Moosavi-Movahedi *et al* 1997; Saboury and Karbassi 2000), and hence calculate the denatured fraction of protein (F_d) as well as determination of the equilibrium constant (K).

$$F_d = \frac{(Y_N - Y_{obs})}{(Y_N - Y_D)}, \quad (3)$$

$$K = \frac{F_d}{(1 - F_d)} = \frac{(Y_N - Y_{obs})}{(Y_{obs} - Y_D)}. \quad (4)$$

Where Y_{obs} is the observed variable parameter (e.g. absorbance or elipticity) and Y_N and Y_D are the values of Y characteristics of a fully native and denatured conformation, respectively. The standard Gibbs free energy change (ΔG°) for protein denaturation is given by the following equation:

$$\Delta G^\circ = -RT \ln K. \quad (5)$$

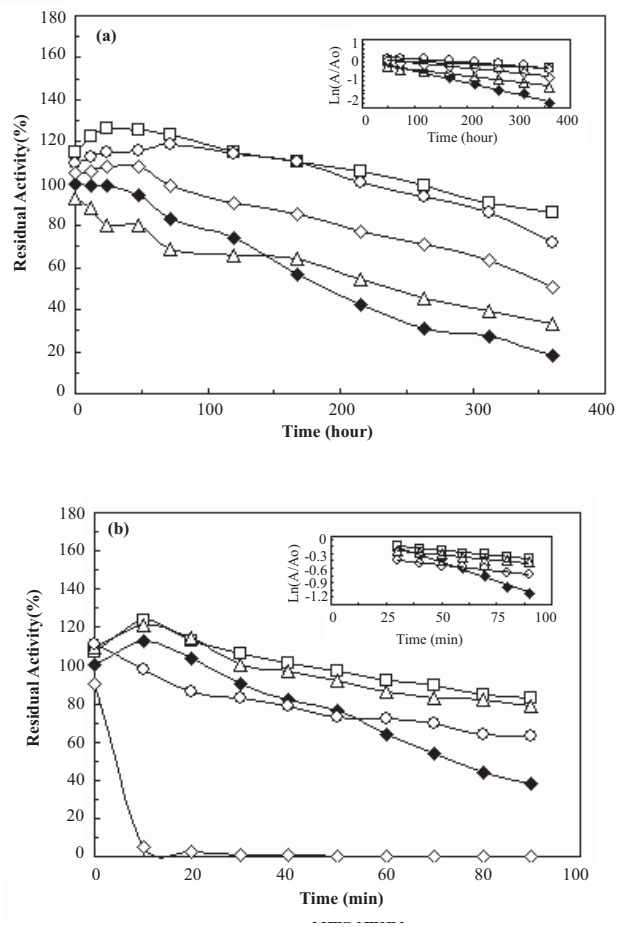

Figure 1. Inactivation of MT through catecholase reactions with 100 μM CA in 10 mM PBS and pH 6.8, in the absence (■) and presence of 5 mM, Asp (◇), Phe (o), His (Δ) and 1 mM trehalose (□) after long-term incubation in 4 °C (**a**), and thermal inactivation through incubation in 40°C (**b**). *Insets*: Rate constants of inactivation k_{inact} were obtained from a linear fit according to firs-order equation of inactivation.

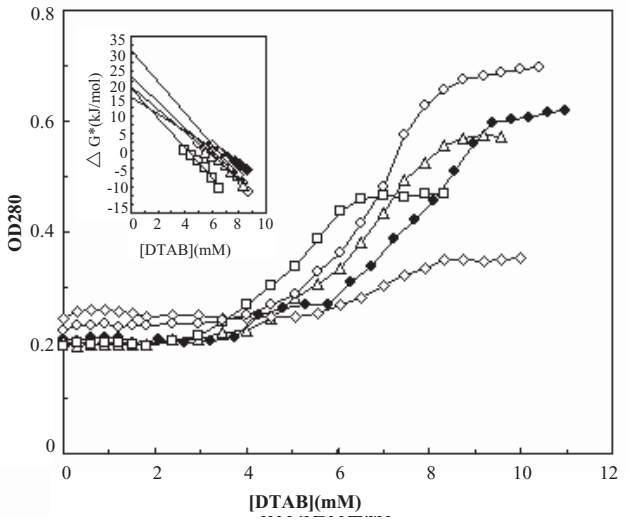

Figure 2. Chemical denaturation curve of 0.12 mg/ml MT by DTAB solution at 280 nm in 10 mM PBS , 20°C and pH 6.8, in the absence (■) and presence of 5 mM, Asp (◇), Phe (o), His (Δ) and 1 mM trehalose (□). *Inset*: Linear extrapolation method for calculation of free energy and $[DTAB]_{1/2}$, in the absence (■) and presence of 5 mM, Asp (◇), Phe (o), His (Δ) and 1 mM trehalose (□).

Table 1. Inactivation and denaturation parameters from effect of different osmolytes on the MT.

Long-term inactivation		Thermal inactivation	Chemical denaturation		Thermal denaturation	
k_{inact}×10-6/s-1		k_{inact}×10-4/s-1	[DTAB]1/2(mM)	$\Delta G^\circ_{(H2O)}$ (kJ/mol)	T_m(ºC)	$\Delta G^\circ_{(25ºC)}$ (kJ/mol)
Sole MT	1.44	2.5	7.5	17.8	44.3	8.4
Tre 1 mM	0.33	0.7	4.8	20.4	56.1	11.7
Phe 5 mM	0.42	0.76	6.7	23.7	57.3	11.3
His 5 mM	0.61	0.7	6.4	21	57.8	12.3
Asp 5 mM	0.75	-	6.6	31.4	57.7	13

Tre, trehalose; MT, mushroom tyrosinase; Phe, phenylalanine; His, histidine; Asp, aspartic acid.

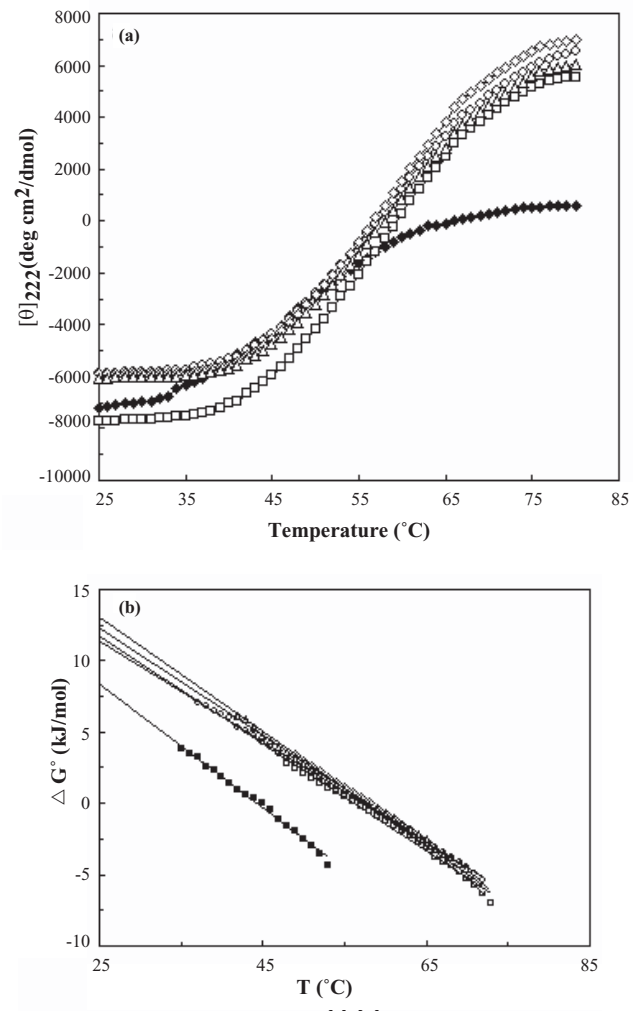

Figure 3. Thermal denaturation curves obtained from molar ellipticity [Θ] at 222 nm with 0.21 mg/ml MT in 2.5 mM PBS, 25°C and pH 6.8, in the absence (■) and presence of 5 mM, Asp (◇), Phe (o), His (Δ) and 1 mM trehalose (□) (**a**). Linear extrapolation method for calculation of free energy and T_m, in the absence (■) and presence of 5 mM, Asp (◇), Phe (o), His (Δ) and 1 mM trehalose (□) (**b**).

Where R is the universal gas constant and T is the absolute temperature. $\Delta G°$ varies linearly with denaturant concentration ([DTAB]) and temperature (T) over a limited region.

$$\Delta G° = \Delta G°_{H2O} - m\,[\text{denaturant}]. \quad (6)$$

$\Delta G°_{H2O}$ is the free energy of conformational stability in the absence of denaturant and m is a measure of the dependence of $\Delta G°$ on the denaturant concentration. Considering two state assumption, the amount of $\Delta G°_{H2O}$ (the standard Gibbs free energy of protein denaturation in the absence of denaturant) and $\Delta G°_{25}$ (the standard Gibbs free energy of protein denaturation at 25°C) can be obtained by equation 6 of Pace analysis (Pace *et al* 1990). $\Delta G°_{H2O}$ and $\Delta G°_{25}$ are the most valuable criterions of protein conformational stability in the process of chemical and thermal denaturation, respectively. These criterions are obtained from the least-square analysis illustrated in insets of figures 2 and 3. $\Delta G°_{H2O}$ and $\Delta G°_{25}$ are taken from Y-intercepts of these replots (inset of figures 2 and 3b). In chemical denaturation, $[DTAB]_{1/2}$ is the denaturant concentration that need for protein receiving to half of its two-state transition. In thermal denaturation, protein melting point (T_m) is a temperature that need for protein receiving to half of its two-state transition. Magnitudes of the $\Delta G°_{H2O}$, $\Delta G°_{25}$, $[DTAB]_{1/2}$ and T_m determined from replots, are summarized in table 1. $\Delta G°_{H2O}$ is estimated to be the highest for Asp (31.4 kJ/mol), which is a negative charged amino acid. For Phe, it is 23.7 kJ/mol may be due to the hydrophobic interactions which make the protein get a more stable structure, and for His as a polar amino acid with a positive charge it is higher than the sole enzyme (21 and 17.8 kJ/mol, respectively). In the case of trehalose, $\Delta G°_{H2O}$ is obtained 20.4 kJ/mol. The magnitudes of $[DTAB]_{1/2}$ is obtained 7.5 mM for sole enzyme, 6.5, 6.4 and 6.5 mM for Phe, His and Asp, respectively, and 4.8 mM for trehalose. Comparison of these magnitudes, reveals that the nature of enzyme medium in the presence of amino acids differs from trehlose as a sugar osmolytes.

3.3 *Secondary and tertiary structure analysis of MT*

To understand the structural differences of the enzyme in the absence and presence of the trehalose and three amino acids, CD spectra of MT in far-UV region were recorded (figure 4). Detailed structural differences can be calculated, which show no significant structural changes in the ratio of well ordered secondary structures.

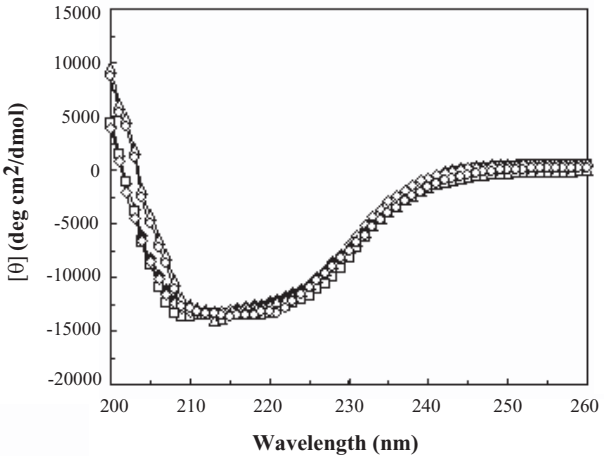

Figure 4. Far-UV CD spectra as ellipticity [Θ] of the 0.21 mg/ml MT in 2.5 mM PBS , 25°C and pH 6.8 in the absence (■) and presence of 5 mM, Asp (◇), Phe (o), His (Δ) and 1 mM trehalose (□).

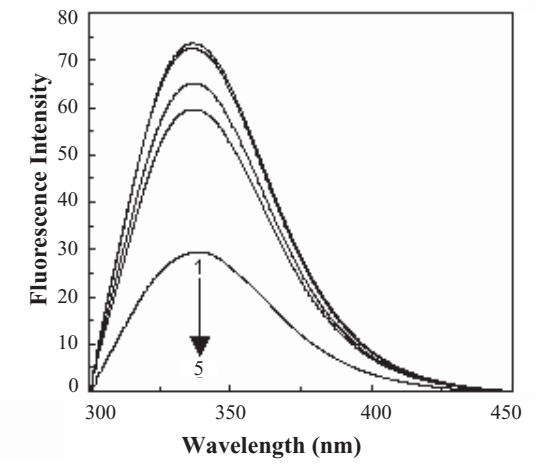

Figure 5. Intrinsic fluorescence emission spectra of the 0.17 mg/ml MT in 2.5 mM PBS , 25°C and pH 6.8 and the excitation wavelength was 280 nm: Sole enzyme (1); Phe (2); Asp (3); His (4); and Trehalose (5).

Intrinsic fluorescence curves in figure 5 show changes in tertiary structure of MT in the absence and presence of osmolytes in definite concentrations. Phe in figure 5 shows a minor reduction in maximum emission intensity of enzyme. After addition Asp to MT, the intensity decreases and shows a partial change in tertiary structure. Incubation of His with MT in figure 5 resultes to decrease in its maximum emission. In the case of trehalose there is a pronounced reduction in maximum emission of intrinsic fluorescence.

4. Discussion

Trehalose, Phe and His not only acts as stabilizer of MT, but also increase its activity. Although Asp stabilize the enzyme against time inactivation, it suppresses MT activity in thermal condition and shows inhibitory effect on its activity. Enzyme activity in the presence of Asp suppresses immediately after first 5 min of its incubation (figure 1b). Trehalose, Phe and His protect the enzyme from heat inactivation, while in the presence of Asp, the enzyme is inactivated. As shown in figure 1, relative activity of enzyme increases in the presence of defined concentrations of trehalose, Phe and His. In contrast, Asp decreases the relative activity of the enzyme. In the latter case, there are various studies about aromatic carboxylic acids on the tyrosinase activity and inhibitory effect of these compounds. This inhibition involves in important role of their carboxylic moiety as a chelator of copper in tyrosinase active site. In benzoic series, the esterification of carboxyl group greatly decreases but does not suppress the inhibitory property, i.e. the affinity of the molecule for the enzyme (Pifferi *et al* 1974). When a carboxyl group is present either directly bound to the benzene cycle (benzoic series) or to the conjugated double bonds (cinamic series or sorbic acid), it can form a complex with the copper at the active center. In this case, when such a structure is present in the same molecule together with an o-diphenolic function, the interaction of copper with the o-diphenolic part would be greatly reduced (Pifferi *et al* 1974; Janovitz-Klapp *et al* 1990), such an effect is observed with caffeic and protocatechuic acids (Janovitz-Klapp *et al* 1989, 1990; Colaco *et al* 1992). These studies persist that reduce in enzyme activity with Asp is belong to its carboxylic moiety.

The results of these compounds emphasize on the prevention of enzyme from time and thermal inactivation. Among of them trehalose as a sugar osmolyte introduce as the best choice, specially in the case of this enzyme source, *Agaricus bisporus,* known to contain a significant amounts of trehalose (Ajlouni *et al* 1993; Mattila *et al* 2002). Sugars are known to protect proteins against loss of activity (Taylor *et al* 1995; Carnici *et al* 1998), chemical (Back *et al* 1978; Sola-Pena *et al* 1995), and thermal denaturation (Lee and Timasheff 1981; Somero 1986; Xie and Timasheff 1997). Among several sugars, trehalose is known to be a superior stabilizer and a compatible osmolyte that gets accumulated in organisms under stress conditions (Somero 1986; Singer and Lindquist 1998).

Different proteins are expected to interact with cosolvent molecules in varied ways depending on their physicochemical properties, e.g. trehalose is observed to provide protection to different proteins to various extents and the efficacy of protection depends on the nature of the protein (Sola-Pena *et al* 1997; Carnici *et al* 1998). Osmolytes also affect the viscosity and surface tension of water (Schein 1990). Environmental factors such as pH, viscosity, ionic strength, temperature, prosthetic groups, solvent composition etc. can affect the protein dynamics and structure, especially the protein flexibility. Some evidences show that an increased viscogenic cosolvent enhances low frequency, large amplitude fluctuations in the polypeptide structure (Almagor *et al* 1992; Gonnelli and Strambini 1993). Thus, besides of probable structural changes of protein, its activity is affected from above osmolytes. It is light that physico-chemical properties of proteins are affected from bulk properties of the solvent environment. Bolen and coworkers (Liu and Bolen 1995; Qu *et al* 1998; Bolen and Baskakvo 2001), based on carefully conducted transfer studies of amino acids and model compounds, have shown that cumulative interactions between amino acid side chains and osmolytes (including sucrose) favour protein unfolding, whereas their overall stabilization is achieved due to unfavourable peptide-osmolyte interactions. The exact nature of interactions that govern the osmolyte-mediated stability of proteins is, therefore, not yet very clear.

Overall, the protein stability depends upon a fine balance between favourable and unfavourable interactions of the native and the denatured protein states with the cosolvent

molecules (Timashef 1998). The stabilizing effect, thus, depends on the nature of the protein as well as the cosolvent molecules and generalization of the effect may not be possible. Phe with hydrophobic characteristics, His as a positive charged amino acid and Asp as a polar amino acid with negative charge, show some differences and similarities in their effect on MT. Their presence increase the free energy of MT and its stability, without a significant change in the secondary structure. In the case of Asp and His, a significant decrease in the emission fluorescence and constriction of the tertiary structure is in agreement with thermodynamic parameters of the enzyme. Trehalose as a sugar osmolyte shows the best coincidence in both thermodynamic and structural characteristic. Trehalose induces enzyme stabilization in the chemical and thermal denaturation. These changes show a good agreement with conformational studies and enzyme constriction of tertiary structure with the presence of trehalose. Thus it should be consider as an exceptional protein stabilizer.

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