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Effect of non aqueous solvent on structural stability of α-amylase: A cost-effective prospective for protein stabilization

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ABSTRACT

The aim of the present study is to assess the effect of non-aqueous organic solvent on structural stability, molecular integrity and structure of α-amylase. The activity and thermal stability of the enzyme was measured before and after treatment with non polar solvent (i.e. hexane). The activity was found to be marginally affected and thermal stability was found to be significantly increased after treatment with hexane. The enzyme was found to be more resistant to thermal inactivation in hexane compared to in an aqueous buffer. The fluorescence measurement indicated a blue shift of 3 nm in the emission maximum (λ_{max}) probably due to a minor change in the polarity of aromatic amino acid residues after treatment with a non-aqueous solvent. Assessment of thermal denaturation profile, 1-anilino-8-naphthalene-sulfonate (ANS) binding and acrylamide quenching of the enzyme suggested an increase in the molecular integrity and overall stability of the enzyme after treatment with hexane. However, these entire molecular events were not accompanied by any major change in the secondary structure. Our findings suggest that treatment of proteins or enzymes in non-aqueous solvents could be an attractive and cost-effective strategy to improve their structural stability without compromising their biological functions.

1. Introduction

From a commercial point of view the structural stability of proteins/enzymes is considered a major concern which directly influences their applicability. To understand and maximize the stability of proteins many studies have been conducted in the recent past, which commonly involve genetic and chemical modifications. In addition, certain additives such as polyols and sugars are commonly employed to extend the shelf-life of protein based pharmaceuticals and enzymes [1–4]. However, the usage of such additives is limited only up to preservation and storage. Moreover, a protein acquires its previous state immediately after removal of the additives. Furthermore, the use of high concentration of these cosolvents may lead to undesirable physiological consequences in the enzymatic processes, which may restrict their scope to use them as universal protein stabilizers. The ability to change the amino acid sequence of a protein by amending the triplet codon at DNA level or by direct chemical modifications has become an attractive tool to improve the structural and functional properties of various proteins and enzymes [5,6]. Recently, there have been tremendous efforts to carry out enzymatic reactions in organic solvents in order to explore the enzymes in biotransformation to produce highly specific chemical molecules and optically active compounds of diverse industrial interests [7,8]. In general, the majority of enzymes and proteins are evolved and adapted to carry out their catalytic reactions in aqueous medium [9]. When such enzymes are suspended in aqueous medium they amend their molecular structure in such a way that the hydrophobic residues are located inside the core of protein molecules where as the hydrophilic residues are preferentially present on the protein surface [10,11]. Such arrangements and distribution of amino acids residue are made to avoid unfavourable interaction of polar solvents and hydrophobic residues of protein and to minimize the conformational entropy of an aqueous system. Hence, it is likely to conceive the impression that when these proteins or enzymes are placed in a non polar medium they will tend to undergo several structural adaptations, which possibly will result in inactivation, denaturation and aggregation. Nevertheless, the mounting evidences in the recent past suggest that many enzymes demonstrate improved activity and structural stability when they are placed in anhydrous non-aqueous solvents [12,13]. Therefore, it would be interesting to contemplate how the structural and functional attributes of enzymes could be altered in order to improve their performances in a non-aqueous medium.

While taking the above facts into consideration we realized the need to examine an important question; Is the stability enhancement effect of a non aqueous medium is a result of the temporary rearrangement of the functional groups of protein, merely due to change in the polarity of the medium? Or does the protein

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molecule adopt a persistent stable structure which would be continued to maintain even in the absence of non aqueous conditions? To address this issue the present study was aimed to evaluate the activity, stability and other biophysical characteristics of an enzyme (α-amylase) after treatment in non polar organic solvent (hexane). The selection of hexane as a non aqueous solvent was based on its absolute non polarity and experimental feasibility. α-Amylases (α-1,4-D-glucan glucanohydrolase, EC:3.2.1.1) have their major applications in starch saccharification and liquefaction in food, textile, paper, brewing and distillery industries [14,15]. They belong to the family 13 of glycosyl transferases which is characterized by the presence of $(\beta/\alpha)_8$ barrel and catalyze the random hydrolysis of α-1,4-glycosidic bonds in starch and result in the formation of maltose and malto-oligosaccharides [16,17]. The structure of enzyme is constituted by three domains called as domain A–C. Domain A consists of a central $(\beta/\alpha)_8$ barrel which forms the core of the enzyme molecule. Domain B is formed by a protrusion between third α-helix and third β-sheet of $(\beta/\alpha)_8$ barrel [18]. The size and structure of the domain B varies among various molecules of the α-amylase family [19]. Domain C is located at the C-terminal part of the amino acid sequence of enzyme.

2. Materials and methods

2.1. Materials

α-Amylase type II (A6380), dinitrosalisylic acid, ANS corn starch, $CaCl_2$ and potassium-sodium-tartarate were procured from Sigma Chemical Company, USA. Citric acid, *tri*-sodium citrate, NaOH, acrylamide and hexane were procured from E-Merck, Mumbai, India. Regenerated cellulose dialysis membrane was procured from Spectrum Laboratories Inc., Rancho Dominguez, CA. Enzyme solutions were prepared in 20 mM sodium citrate buffer (pH 5.9) and dialyzed extensively against the same buffer to remove the added excipients, freeze dried and stored at −20 °C for the future use. Quartz triple distilled water was used throughout the experiments.

2.2. Treatment of α-amylase in hexane

The lyophilized crystalline powder of α-amylase was subjected for dehydration in an oven at 35 °C for 24 h to remove any water. The dehydrated enzyme then suspended in pure hexane and incubated at room temperature for 24 h with mild shaking. For enzyme assay and other experiments the hexane was completely removed using vacuum centrifugation (for overnight at 5000 rpm). At the end the dried enzyme was dissolved in 20 mM sodium citrate buffer (pH 5.9) containing 2 mM $CaCl_2$, mixed by vortexing and centrifuged to remove the insoluble fractions. After estimating the protein concentration the supernatant was used for various analyses. To assess the solvent effect on the rate of thermal inactivation the enzyme was suspended in hexane and kept in air tight glass vials and incubated at 60 °C for different period of time (i.e. 2, 5, 10, 20, 30 and 40 min). Afterwards hexane was removed and dried enzyme was dissolved in the buffer, mixed thoroughly and centrifuged (10 rpm, 20 min). The supernatant was used for measuring the enzyme activity. Unless specifically mentioned all the assays were performed in 20 mM sodium citrate buffer.

2.3. Estimation of α-amylase activity

The α-amylase activity was measured according to the method of Bernfeld [20] for the estimation of reducing sugars using starch as substrate. The solution of α-amylase was prepared in the buffer for the activity measurement. The reaction mixture contains 1 ml of enzyme solution (1 μg/ml), 1 ml of 1% (w/v) starch solution. The reaction mixture was incubated at 37 °C for 5 min in a temperature controlled shaking water bath. The enzymatic reaction was terminated by addition of 2 ml of 1% alkaline dinitrosalisylic acid solution and subjected in boiling water bath for 10 min, cooled down to room temperature (25 °C) and diluted 5 times using triple distilled water. After thorough mixing the absorbance was recorded at 540 nm in Shimadzu UV-1601 UV-Visible spectrophotometer, and reducing sugar equivalent was determined using maltose as standard. One unit of enzyme was defined as the amount of enzyme required to produce 1 μmole of maltose equivalent from 1 ml of 1% starch solution in 5 min at 37 °C. Each sample had three replicates.

2.4. Determination of protein concentration

Protein concentration of α-amylase solution was determined by using its extinction coefficient ($E^{1\,cm}_{1\%}$) in aqueous medium. The absorbance of enzyme solution was recorded at 280 nm in Shimadzu UV-1601 UV-Visible spectrophotometer and concentration was calculated using $E^{1\,cm}_{1\%}$ value of α-amylase as 14.46 [21]. Alternatively protein concentration of α-amylase solution was estimated by Lowry method [22] using bovine serum albumin (BSA) as standard.

2.5. Thermal inactivation of α-amylase

The enzyme solutions were prepared in 20 mM sodium citrate buffer (pH 5.9), aliquoted and incubated at 60 °C for different period of time (i.e. 2, 5, 10, 20, 30 and 40 min). The samples were, cooled down on ice bath and residual activity was measured at 37 °C. The percentage residual α-amylase activities were calculated based on the original activity of enzyme without thermal inactivation [23,24]. Each sample had three replicates and the values represent average of three independent measurements.

2.6. Thermal denaturation of α-amylase

The thermal denaturation of native and hexane-treated α-amylase was monitored by recording the absorbance at 287 nm in the range of 30–90 °C on UV–visible spectrophotometer. The protein concentration in all the samples was maintained as 0.15 mg/ml. The apparent thermal transition temperature $(T_m)_{app}$ was obtained after normalizing the absorbance of native and denatured state of enzyme. The fraction unfolded (F_u) was determined by after normalizing the absorbance of the enzyme at different temperatures using Eq. (1). The $(T_m)_{app}$ of the enzyme was determined by plotting F_u against temperature [23,24] and interpolating the curve on temperature axis at which the F_u was found to be 0.5.

$$F_u = \frac{A_T - A_N}{A_D - A_N} \quad (1)$$

$$K_D = \frac{F_u}{1 - F_u} \quad (2)$$

where A_N and A_D are the spectral properties of native and denatured enzyme respectively. A_T is the spectral property of enzyme at temperature T (°C) and K_D is the thermal denaturation equilibrium constant.

2.7. Intrinsic fluorescence spectra

Intrinsic fluorescence spectra of α-amylase (50 μg/ml) were recorded at 25 °C in a Shimadzu fluorimeter model RF-5000, Japan, equipped with temperature-controlled water bath. The fluorescence excitation wavelength was set at 280 nm and emission spectra were recorded in the range of 300–400 nm using slit width of 5 and 10 nm for excitation and emission, respectively.

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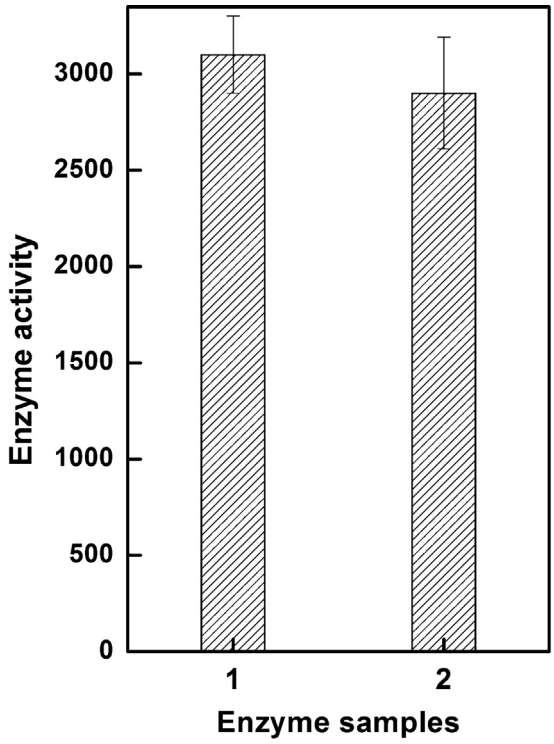

Fig. 1. Effect of hexane treatment on α-amylase activity. Samples 1 and 2 represent the enzyme activity before and after treatment of the enzyme in hexane, respectively.

3. Acrylamide quenching

Acrylamide quenching was carried out by sequential addition of acrylamide solution to enzyme solution followed by recording the intrinsic fluorescence at 340 nm. Aliquots of 10 μl of 2 M acrylamide stock solution were added to 2 ml of α-amylase (50 μg/ml) solution each time and mixed thoroughly by inverting. The fluorescence excitation wavelength was set at 280 nm and emission spectra were recorded in the range of 300–400 nm using slit width of 10 and 5 nm for excitation and emission respectively. The fluorescence quenching of α-amylase samples were analyzed by Stern–Volmer equation [25,26].

$$\frac{F_0}{F} = 1 + K_{sv}[Q] \quad (3)$$

where F_0 and F are the fluorescence intensity in the absence and presence of quencher respectively, [Q] is the molar concentration of quencher (acrylamide). K_{sv} is the Stern–Volmer constant, which was obtained from the slope of the curve of F_0/F versus quencher concentrations [Q]. The observed data were corrected for the dilution. The observed fluorescence intensities were corrected for inner filter effect for acrylamide absorbance using the equation:

$$F_{corrected} = F_{observed} \cdot 10^{-\varepsilon bc/2} \quad (4)$$

where F is the fluorescence intensity, ε is the extinction coefficient of acrylamide at 280 nm as 4.3 M^{-1} cm^{-1}, b is the path length and c is the concentration of acrylamide.

4. Results and discussion

The enzyme activity of α-amylase was measured before and after treating with hexane in aqueous buffer. As shown in Fig. 1 a marginal effect was apparent on the enzyme activity after treatment with hexane. The activity of native and the hexane-treated enzyme was found to be 3100 and 2900 units, respectively. The

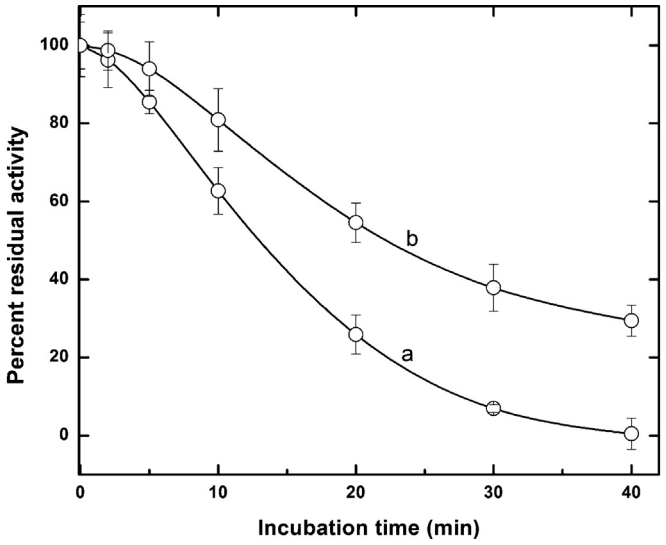

Fig. 2. Effect of hexane treatment on thermal inactivation of α-amylase. Curves a and b represent the thermal inactivation of the enzyme over a period of time before and after hexane treatment, respectively.

effect of hexane treatment on thermal inactivation of enzyme revealed a unique phenomenon. As shown in Fig. 2, the rate of thermal inactivation of the enzyme was found to be less after treatment with hexane. After 30 min of incubation at 60 °C the native α-amylase lost the complete activity where as the hexane-treated enzyme was able to retain at least ~30% of the enzyme activity (Fig. 2). This shows that the native enzyme undergoes thermal inactivation much faster compared to the hexane-treated enzyme.

To assess the direct effect of aqueous medium and non polar solvent on enzyme inactivation, the enzyme was subjected in hexane and allowed to stand at higher temperature for different period of time followed by removal of hexane and estimation of the enzyme activity in buffer. In parallel the enzyme inactivation was also carried out in buffer which served as control. As shown in Fig. 3, the enzyme losses its activity drastically in buffer when it is treated at higher temperature (60 °C) compared to the enzyme in hexane. At 60 °C after 10 min of incubation the residual activity in control was

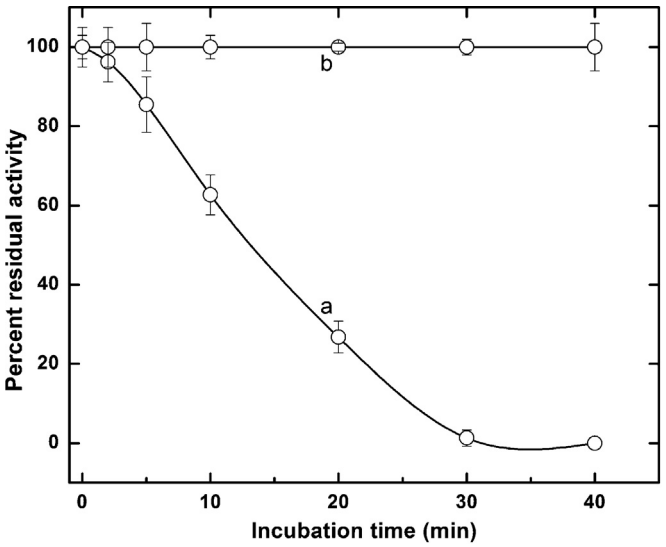

Fig. 3. Thermal inactivation of α-amylase in hexane and in an aqueous buffer. The enzyme was incubated at 60 °C in 20 mM sodium citrate buffer (a) and in hexane (b) for different period of time and enzyme activity was determined using the method described in Section 2.

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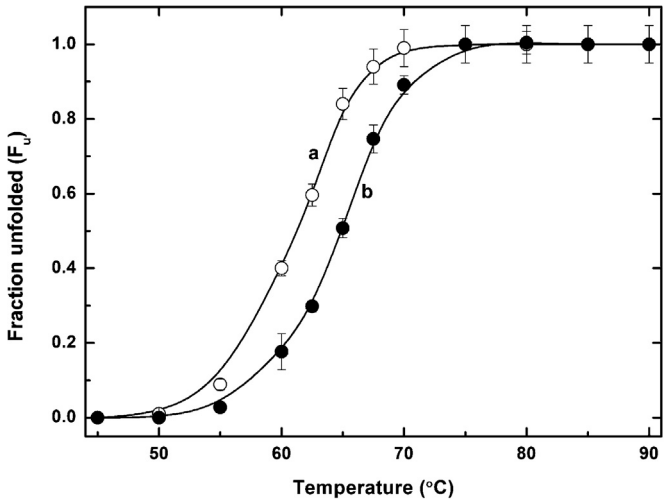

Fig. 4. Effect of hexane treatment on thermal unfolding of α-amylase. The native (without any treatment) (a) and hexane-treated enzyme (b) were dissolved in 20 mM sodium citrate buffer (pH 5.9), and absorbance was recorded at 287 nm at different temperatures ranging from 30 to 90 °C. The fraction unfolded (F_u) was determined by normalizing the absorbance of the enzyme at different temperatures using Eq. (1). The $(T_m)_{app}$ of the enzyme was determined by plotting F_u against temperature and interpolating the curve on temperature axis at which the Fu was found to be 0.5.

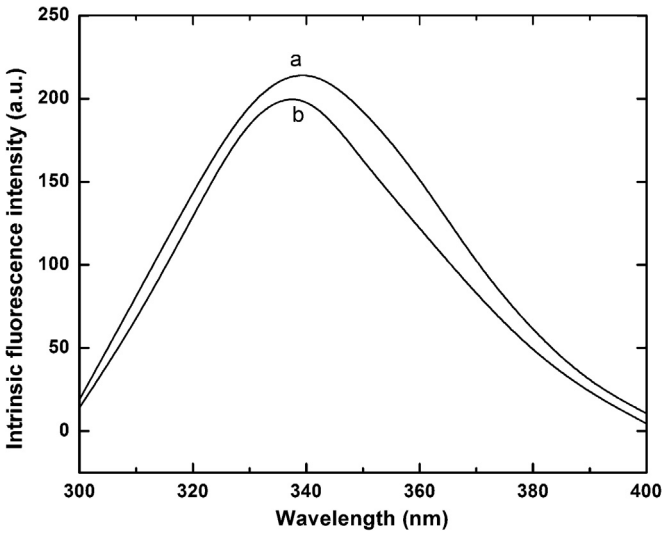

Fig. 5. Effect of hexane treatment on intrinsic fluorescence of the native (a) and hexane-treated α-amylase (b).

found to be 1500 units where as enzyme in hexane was completely protected from thermal inactivation and able to retain its complete activity. The enzyme in buffer lost its complete activity after 30 min whereas the enzyme in hexane did not show any decrease in its activity even after 60 min of incubation at the same temperature (Fig. 3). This shows that hexane has an stabilizing effect on the enzyme. This also reveals that the enzyme in non polar medium is highly resistant to thermal inactivation. The retention of enzyme activity at higher temperature after treatment with hexane indicates that hexane has structure stabilizing effect on the enzyme.

To further explore the structure stabilizing effect of hexane thermal denaturation was carried out to monitor the changes in structural stability of the enzyme after the treatment in hexane. It was performed by recording the UV-difference spectra the enzymes at 287 nm in the temperature range of 30–80 °C. The thermal denaturation temperature $(T_m)_{app}$ was considered as the temperature at which the enzyme structures remain in equilibrium between native and unfolded states. As shown in Fig. 4, the thermal denaturation profile of the hexane-treated enzyme shifted to higher temperature range. The analysis of the thermal denaturation profile provides the $(T_m)_{app}$ value as 61 °C for the untreated enzyme and 65 °C for the hexane-treated enzyme. It is evident from Fig. 4 that the thermal transition for untreated enzyme happens in the range of 55–70 °C whereas in case of hexane-treated enzyme the thermal transition range was found to be between 62 and 75 °C.

To monitor the changes in the micro environment of the aromatic amino acid residues in response to hexane treatment intrinsic fluorescence spectra of the enzyme were recorded in the range of 300–400 nm. The λ_{max} of native enzyme was found to be 340 nm where as for the hexane-treated enzyme it was recorded to be 337 nm (Fig. 5). This indicates the enhancement in the hydrophobic environment of aromatic amino acid residues in response to treatment with hexane.

The ANS fluorescence spectra of hexane-treated and native enzymes showed a marked difference in resistance against thermal inactivation. Generally, the thermal unfolding of the enzyme accompanied with exposure of nonpolar residues of protein in aqueous system which subsequently enhance the entropy of the system due to unfavourable interaction [24]. This is confirmed by an increase in the ANS fluorescence intensity. The ANS dye generally binds with the hydrophobic clusters which are exposed due to partial unfolding of protein [27]. As shown in Fig. 6, the hexane-treated and untreated enzymes do not bind significantly with ANS and therefore showed the minimum intensity. After incubation at 60 °C for 20 min the ANS intensity of the enzyme increased several fold. The hexane-treated enzyme displays lower ANS intensity compared to the native enzyme. This observation suggests that the hexane-treated enzyme is adapted to tolerate the thermal stress. To further explore the effect of hexane treatment of the enzyme acrylamide quenching was carried out to assess the conformational flexibility. The technique is based on the relative penetration and quenching of the fluorescence intensity of protein by a quencher molecules such as acrylamide. It is generally assumed that the accessibility of acrylamide in the core is proportional to the degree of fluorescence quenching and flexibility of a

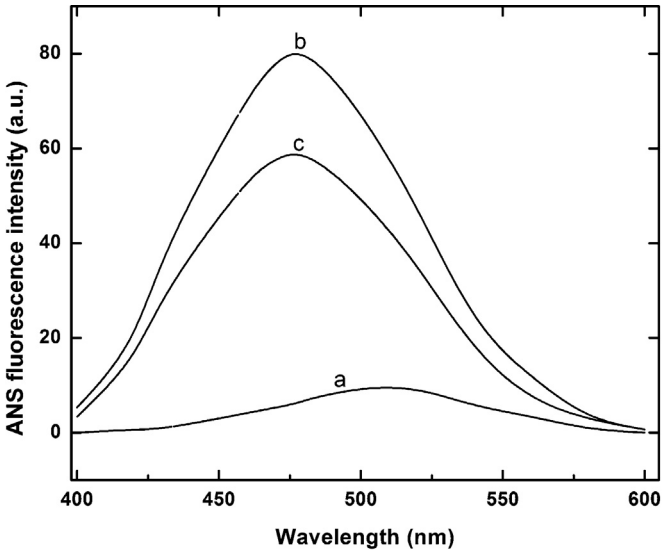

Fig. 6. The ANS fluorescence spectra of α-amylase samples after partial heat treatment. The enzyme samples were incubated at 60 °C for 5 min and then mixed with ANS solution before recording the spectra. The enzyme and ANS concentration in the samples were adjusted to 20 and 100 microM, respectively.Curve (a) represents the native enzyme without heat treatment and curves (b) and (c) represent the native and hexane-treated enzyme after thermal treatment.

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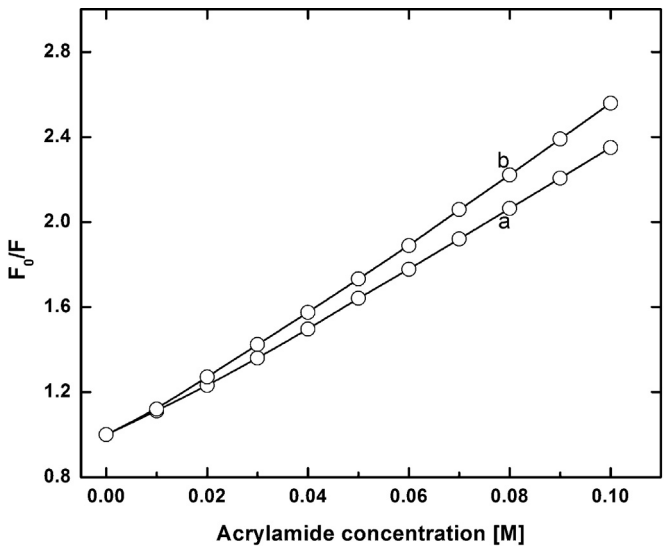

Fig. 7. Acrylamide quenching of α-amylase after (a) and before (b) treatment in hexane.

protien molecules [25]. As shown in Fig. 7, the quenching of intrinsic fluorescence is higher for the untreated enzyme compared to the hexane-treated enzyme. The values of Stern–Volmer constant (K_{sv}) of the hexane-treated and native enzyme were determined to be 13.66 ± 0.15 and $15.73 \pm 0.26\ M^{-1}$, respectively. The change in the value of K_{sv} represents the alteration in the relative compactness of the protein molecule under different conditions. Higher value of K_{sv} represents relatively lower compactness and higher quencher penetration [25]. This result shows that the hexane-treated enzyme has less conformational flexibility and higher molecular compactness compared to the enzyme without any treatment.

From the above results it is fairly evident that hexane treatment has a positive effect on overall stability of α-amylase. After hexane treatment the enzyme becomes relatively more resistant to thermal denaturation and inactivation (as evident from Figs. 2 and 4) with minor reduction (≅6.5%) in the enzyme activity (Fig. 1). However, the blue shift in λ_{max} after treatment with hexane indicates that the aromatic residues are subjected to a more nonpolar environment. Such minor changes in the microenvironment might be responsible for the positive amendment in intramolecular interactions which in turn enhance the stability. Data from quenching study further strengthen the fact that the enzyme acquires a more rigid structure after the treatment with hexane.

It is reported that 63% of protein polar side chains and 54% of charged side chain residues are hidden inside the protein molecule and therefore not inaccessible to the solvent [10]. It is also evident that the peptide bonds and buried polar groups contribute 25% more than the non polar groups in conferring protein stability [28,29]. These evidences strongly support a greater role of surface hydrogen bonding potential and electrostatic interactions in defining the protein stability. In aqueous system the hydrogen bonding potential on the protein surface is largely satisfied by interacting with water molecules (Fig. 8). When the protein is subjected to non aqueous medium the hydrogen bonding potential and electrostatic potential at the protein surface remain unsatisfied and they might possibly tend to internalize to avoid thermodynamically unfavourable interaction. In this course it is likely that the

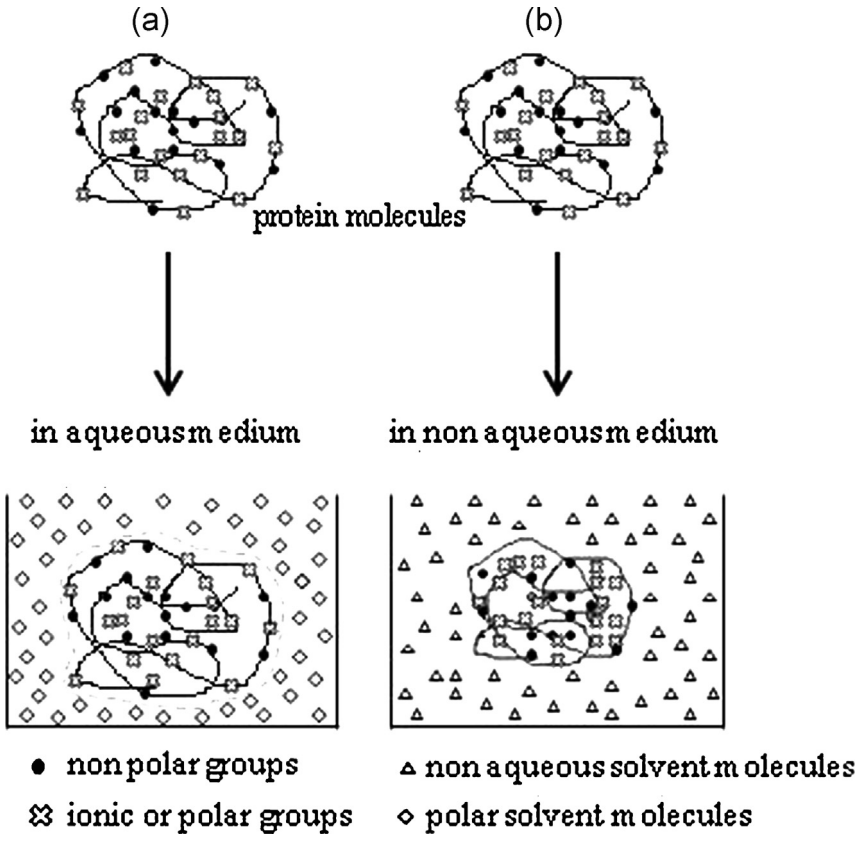

Fig. 8. Schematic representation of structural transition in the protein molecule after exposing them in aqueous and non aqueous medium. When the enzyme is dissolved in aqueous system a hydration layer of water (shown as dotted line) is formed in the immediate vicinity of protein (a) which balances the hydrogen bonding potential of the surface residues. These residues tend internalize when they are exposed to non aqueous solvents to avoid the thermodynamic unfavourable interaction and reduce the entropy of the system. This phenomenon results in the formation of stable and highly compact enzyme molecule (b).

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structure stabilizing interactions may get intensified, which subsequently may enhance the structural stability of the enzyme. While addressing the question which we asked in the beginning, we are able to observe three significant phenomena in this study, which are of fundamental importance; (1) the enzyme activity did not compromise significantly after treatment with hexane, (2) thermal inactivation was only evident when the enzyme was suspended in buffer but not in non aqueous medium and (3) the enhancement in stability was retained to some extent even after removal of non aqueous solvents and dilution of the enzyme in the aqueous buffer. These findings strongly suggest a commercially viable and an innovative approach to stabilize the protein structure without any chemical or genetic modification. This approach can further be strengthened by optimizing the stabilization processes using combinations of non aqueous solvents and treatment conditions. However, it is likely that every second type of protein may be different at the level of surface chemistry and rational distribution of polar and non polar groups, it would be necessary to optimize the stabilization process on case-by-case basis.

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