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Chemical denaturants induced folding unfolding pathway of the recombinant zebrafish dihydrofolate reductase

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Denaturation of proteins plays a crucial part in cellular activities. In this study, we have investigated the folding unfolding pathways of zebrafish dihydrofolate reductase (zDHFR) in presence of different chemical denaturants which were found to be an influential factor for the refolding yield by UV-visible spectrophotometric analysis. The activity change of zDHFR has been observed in presence of three different denaturants like Acetic Acid (AcOH), Sodium Dodecyl Sulphate (SDS), and Ethanol (C_2H_5OH). Spectrophotometric analysis reveals that protein unfolded completely at different concentrations and times by these denaturants. The spontaneous refolding experiments of chemically denatured zDHFR were also conducted to verify the spontaneous refolding yield. These investigations have helped us to decipher a picture about the denaturants contributing to achieving the refolding yield. We observed that acetic acid is a stronger denaturant among all, and the spontaneous refolding yields were higher from SDS denaturation. In the light of the above findings, higher spontaneous refolding yields were obtained from the low concentration of denaturants.

Keywords: Denaturants, Enzymatic assay, Equilibrium unfolding, Refolding yield, UV-Visible Spectroscopy

The three-dimensional shape of a protein is fundamentally important to its biological function. In living cells, nascent proteins begin to fold as soon as they are synthesized by a ribosome[1,2]. How peptide chains fold into well-defined, three-dimensional structures has long been a topic of scientific inquiry. It is quite common that the majority of over expressed recombinant proteins fail to reach a correct conformation and undergo proteolytic degradation or associate with each other to form insoluble aggregates of non-native proteins known as inclusion bodies.

Denaturation of native protein leads to changes in the protein conformation by disrupting non-covalent interactions which can be achieved by adding chemical denaturants[3-5] (Acetic Acid or SDS), high temperature, acidic pH, molecular denaturants, and combinations of these denaturing conditions on protein structures. During unfolding, there is a population shift from the native state to the denatured states through a series of different conformations[6]. Protein unfolding or misfolding is known to promote protein aggregation[7,8], which in turn is associated with several misfolding disorders, such as Alzheimer's disease, Type II Diabetes, Parkinson's diseases, etc. that are threatening human lives[9-12]. The structural transitions of proteins under non-native conditions have been actively investigated to understand protein stability and folding kinetics, as well as to identify folding intermediates[13,14].

Protein denaturation under acidic conditions such as in presence of acetic acid has gained attention because partially unfolded structures that are constructed under acidic conditions are thought to have important implications in biological systems. Sodium dodecyl sulfate (SDS), an anionic surfactant having a long hydrophobic tail and hydrophobic head[15]. SDS-induced unfolding of proteins deserves particular attention as it underlies the most common procedure for determining the molecular weight of a protein, SDS polyacrylamide gel electrophoresis. In the presence of SDS, the protein interacts with SDS to form a negatively charged SDS-protein complex. SDS could break hydrophobic interactions, hydrogen bonds, and ionic bonds interactions, while disulphide bridges are not affected by SDS. Despite being an essential part of being used in protein characterization techniques, the very mechanism by which SDS induced protein unfolding reaction has not yet been unequivocally established.

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It is generally known that the solvents containing alcohol denatures protein. In the case of Ethanol, a rapid transition in β-lactoglobulin has been observed at 20-50%[16]. At room temperature (25°C), intermolecular S-S cross-linking of BSA was detected, which, together with non-covalent interactions, contributed to the protein's aggregation. Despite multiple investigations involving DHFR, there is little information on the effects of alcohols on zebrafish dihydrofolate reductase.

Refolding of proteins to correct three-dimensional conformation is a complex process and is poorly understood. Refolding is initiated by the removal of denaturants to allow the protein to fold into its native structure[17,18]. The effect of AcOH, SDS and Ethanol as denaturants on theactivity of zDHFR and the refolding yield of this enzyme is not yet documented. Here we have performed the unfolding refolding studies in presence of three denaturants (AcOH, SDS, and Ethanol).

Dihydrofolate reductase (DHFR) converts dihydrofolate to tetrahydrofolate using NADPH as an electron donor[19]. As Tetrahydrofolate, the product of this reaction is the active form of folate in humans. Folate deficiency might be the reason for theinhibition of DHFR. Folate is needed by rapidly dividing cells to make thymine; this effect might be therapeutic. DHFR is an important pharmacological target and known model for the study of enzyme structure/ function relationships because of its small size, availability of purified enzymes, and well-developed functional assay.

Here we are reporting biochemical assay of an unexplored variant *i.e*. zebrafish DHFR (zDHFR)[20,21]. It can be used in research as an alternative to the mammalian species having adequate similarity with its human counterpart. There is no report of equilibrium unfolding of zDHFR in presence of denaturants like Acetic Acid, SDS, and Ethanol so far. Equilibrium unfolding study and investigation of the refolding process of zDHFR would provide added information on DHFR folding and comparative information of folding/unfolding behaviour on other species of DHFR proteins. Hence, our aim was to quantify the concentration of denaturant of recombinant protein and its refolding yield.

Materials & Methods

Materials

Strains of BL21 (DE3) Rosetta *Escherichia coli* cells were utilized for the over-expression and purification of zDHFR. The gene for zebrafish DHFR cloned in the plasmid pET43.1a vector were obtained from Dr. Tzu-Fun Fu, Taiwan, China. Nicotinamide Adenine Di-nucleotide Phosphate (NADPH), Isopropyl-β-D-1-thiogalactopyranoside (IPTG) were used as inducer, Dihydro folic acid (DHF), Acetic Acid (AcOH), Sodium dodecyl sulfate (SDS), Ethanol (C_2H_5OH), Lysozyme, High purity grade Imidazole, Magnesium Chloride ($MgCl_2$), Phenylmethylsufonyl fluoride (PMSF), Sodium dihydrogen Phosphate (NaH_2PO_4), Disodium Phosphate (Na_2HPO_4), Tris base, Sodium chloride (NaCl), Potassium chloride (KCl), Tris Hydrochloride (Tris HCl), Dialysis membrane along with Coomassie Blue R-250 were obtained from Himedia, India. Other reagents which were used in the experiments were analytical grade. Double distilled water or Milli-Q (Merck Millipore) water was used for all experiments.

Stock solutions

The stock solution was made for SDS (10 mM) in Sodium Phosphate Buffer (pH 7.4). Acetic acid (99.9% pure) and Ethanol (99.9% pure) was taken from a concentrated solution. Different concentrations of AcOH (10-60%), SDS (0.5-6 mM), and Ethanol (10-60%) were taken to carry out the unfolding of zDHFR.The experiments were carried out using Sodium Phosphate Buffer composed of 20 mM Tris, 25 mM KCL at 25°C pH 7.4.

Methods

zDHFR protein over expression

Over-expression of recombinant zDHFR enzyme was carried out from BL21 (DE3) Rosetta *E. coli* strain bearing recombinant plasmid vector, zDHFR-Histidine/pET43.1a encoding DHFR gene comprises of 6-Histidine codons, under Lac Z promoter. The culture of the transformed cells grown in Luria–Bertani medium comprises of ampicillin (100 μg/mL) at 250 rpm and 37°C. Bacterial cells were induced with a final concentration 1 mM IPTG once the optical density at 600 nm approaches 0.8~1.0 and then incubated at 25°C for 6 h for zDHFR protein expression. Harvesting of induced cells was done and underwent centrifugation for about 30 min at 6000 rpm. About 200 μL of culture was centrifuged and the pellet was re-suspended in SDS loading dye, boiled at 100°C for 5 min and loaded on 12% SDS-PAGE for confirming the over-expression of zDHFR.

Purification of zDHFR using affinity chromatography

The expression of histidine- tagged zDHFR was induced with 1 mM IPTG for 6 h at 25°C.Cells were harvested by centrifugation. Lysis buffer (contains

0.2 M sodium phosphate (pH 7.4), 0.2 M $MgCl_2$, 500 mM NaCl, 0.1 mg/mL Lysozyme, and 1 mM PMSF) was used for resuspension of these harvested cells further undergoes lysis of cells by sonication after incubation for 30 min. Removal of cell debris was carried out by centrifugation at 13000 rpm at 4°C for 40 min. The supernatant was retrieved and then filtered by a 0.22-micron filter. The purification process was performed by Immobilized Metal Ion Affinity Chromatography (IMAC) using Ni-Nitrilotriacetic acid (Ni^{2+}) as a chelating agent. Histidine-tagged zDHFR protein was eluted by passing Imidazole (0-500 mM) in different gradients. These different fractions containing zDHFR were monitored using SDS-PAGE. The protein was estimated by using Bradford's reagent. Aliquots with >95% purity were collected and underwent dialysis using Tris-KCl buffer (pH 7.4) where concentrated protein solution obtained by Amicon tubes (Millipore, U.S.A.), 10kDa molecular weight cut off the membrane. ProtParam tool of ExPASy was used to calculate the extinction co-efficient of purified zDHFR protein concentration which is 24,075 M^{-1} cm^{-1} (http://web.expasy.org/protparam/). Concentrated zDHFR solutions waere divided into aliquots and were stored at −80°C.

Enzyme-activity assay of zDHFR protein

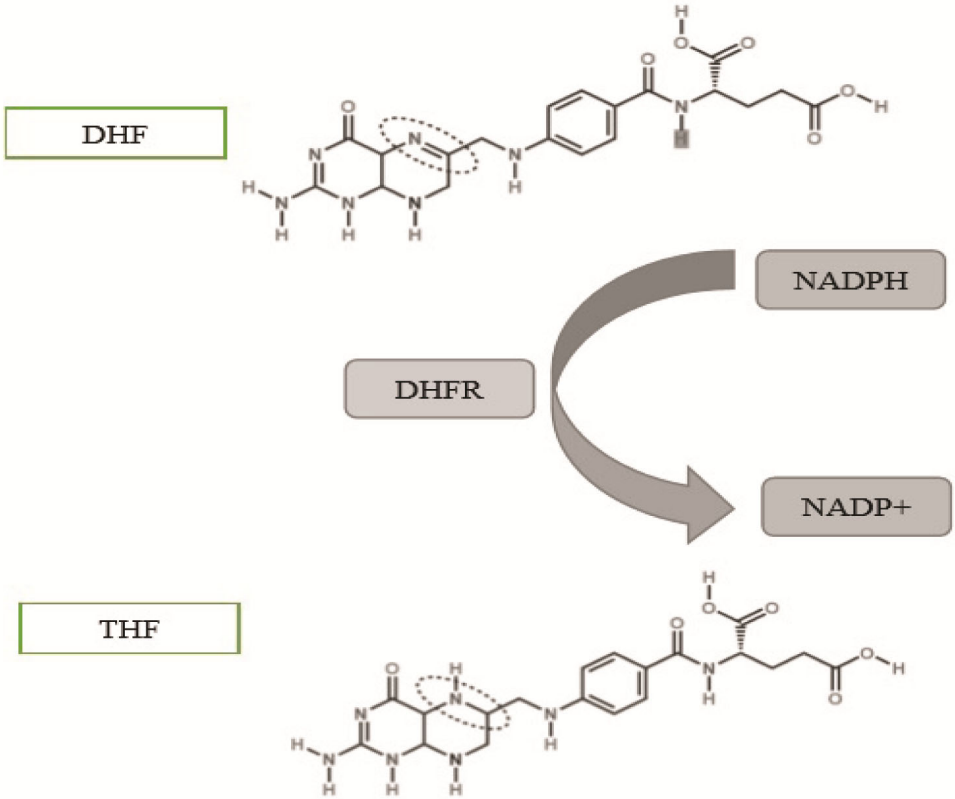
Conversion of dihydrofolate and NADPH is catalysed by zDHFR results into tetrahydrofolate and $NADP^+$ (cofactor) as represented in the following (Scheme 1).

The concentration of NADPH decreases resulting decline in the absorbance of NADPH which was monitored by calculating its absorbance at 340 nm and 25°C using a UV-visible spectrophotometer[22]. Standard assay buffer contains Tris KCl (pH 7.4), 100 µM DHF, 140 µM NADPH, and 0.2 µM DHFR.

To minimize the degradation of substrate and cofactor, NADPH and DHF were prepared fresh then incubated in ice to refrain from any degradation of component and they were consumed within 2 h of experimentation. All evaluations were done in triplicate.

Equilibrium unfolding studies by enzyme-activity assay of zDHFR with different denaturants

The equilibrium unfolding process of recombinant zDHFR protein was evaluated from the enzymatic activity with varying concentrations of AcOH (0-60%), SDS (0-6 mM), and Ethanol (0-60%) in Tris KCl buffer, pH 7.4 at 25°C. The fall in the enzymatic activity is observed using a UV-visible spectrophotometer at 340 nm. An unfolding curve



Scheme 1 — Enzymatic reaction catalysed by DHFR, where DHF and THF are dihydrofolate and tetrahydrofolate, respectively

was plotted where residual enzymatic activity was monitored against different concentrations of AcOH, SDS, and Ethanol as well as time. Amendments were made for the blank contributions.

Spontaneous refolding kinetics of zDHFR with different denaturants monitored by enzymatic activity

Here we have performed the spontaneous refolding kinetics of denatured zDHFR with different denaturants and enzymatic activity was approached by performing the assay using a UV-visible spectrophotometer. Dilution of denatured protein by AcOH, SDS and Ethanol was performed by refolding buffer (Tris KCl, pH 7.4) where the denatured protein was diluted 100-folds in the buffer. The spontaneous refolding process was done at 25°C in refolding buffer at pH 7.4 and all experiments were done in triplicates.

Results

Over-expression of recombinant zDHFR

In this study, a pET 43.1a vector bearing the zebrafish DHFR gene under the control of T7 promoter was used for the over-expression of zDHFR in BL21 (DE3) E. coli expression system. It has T7 RNA polymerase machinery which was under control of the Lac promoter. The overexpression of zDHFR was carried out by adding a non- hydrolysable analogue of lactose, IPTG which typically acts as a chemical inducer. Expression of zDHFR was observed when the induction was done in the mid-exponential phase. Over-expression of recombinant zDHFR protein was confirmed by 12% SDS-PAGE (Fig. 1).

A dense band of the over-expressed recombinant protein was observed at the position of 21.6 kDa. The figure consisting 12% SDS-PAGE confirms that the recombinant zDHFR protein was over-expressed only after the induction of cells with IPTG. Maximum expression for DHFR was measured after incubating the culture for 6 h (Fig. 1).

Purification of recombinant zDHFR

The purified recombinant protein was acquired by affinity chromatography IMAC (Fig. 2). The purified protein was obtained from a soluble fraction of over expressed protein, using IMAC Ni^{+2}-chelating chromatography. A dense band corresponding to zDHFR was found at position 21.6 kDa, confirming the presence of recombinant zDHFR in the solution. There were no other bands on the gel that suggested the solution contained purified zDHFR, and there were no other proteins in the solution (Fig. 2). For additional *in vitro* experiments, the fractions were combined, concentrated, and kept at 4°C. The concentration of protein was calculated using the Bradford protein estimation method. The protein, which was purified showed significant activity, indicating that a significant amount of protein was present in its active state.

Equilibrium unfolding studies of recombinant zDHFR monitored by enzymatic assay

Equilibrium Unfolding studies of recombinant zDHFR were monitored by enzymatic assay where the residual enzymatic activity of the protein after unfolding was plotted against the concentrations of denaturants (AcOH, SDS, and Ethanol).

AcOH induced unfolding of recombinant zDHFR

Enzymatic activity changes in recombinant zDHFR protein induced by AcOH has been studied by the enzymatic assay as shown in the (Fig. 3A & B).

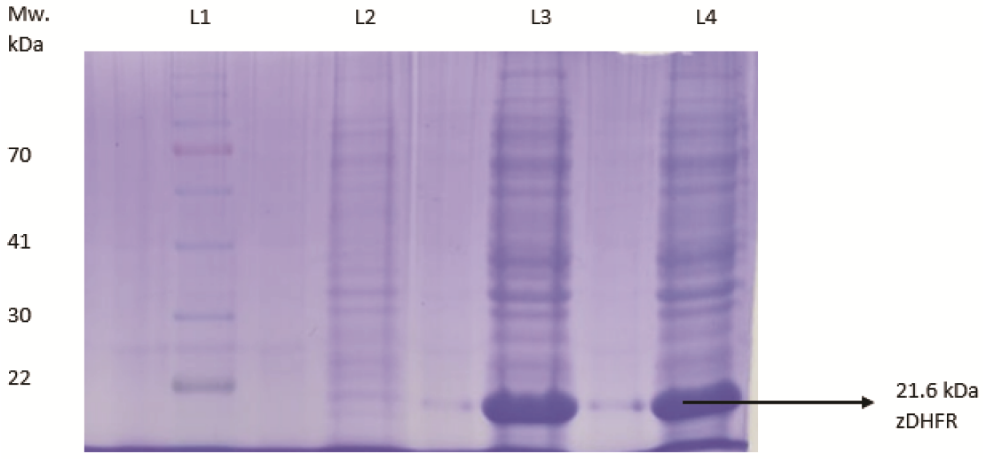


Fig. 1 — 12% SDS-PAGE shows over-expression of recombinant zDHFR. Lane 1: Low molecular weight Molecular Marker Lane 2: Uninduced Cells Lane 3, Lane 4: Induced Cells (Induced with 100 μM IPTG)

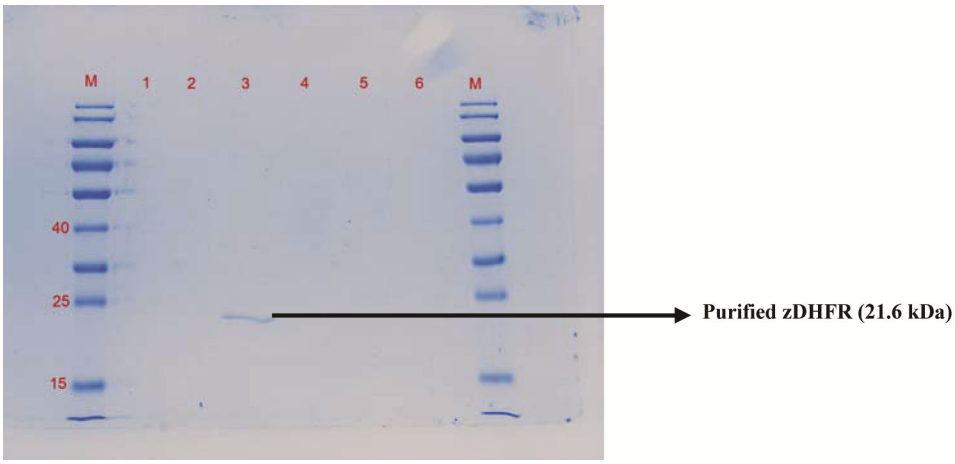


Fig. 2 — SDS-PAGE shows purified zDHFR protein obtained using immobilized metal ion affinity chromatography

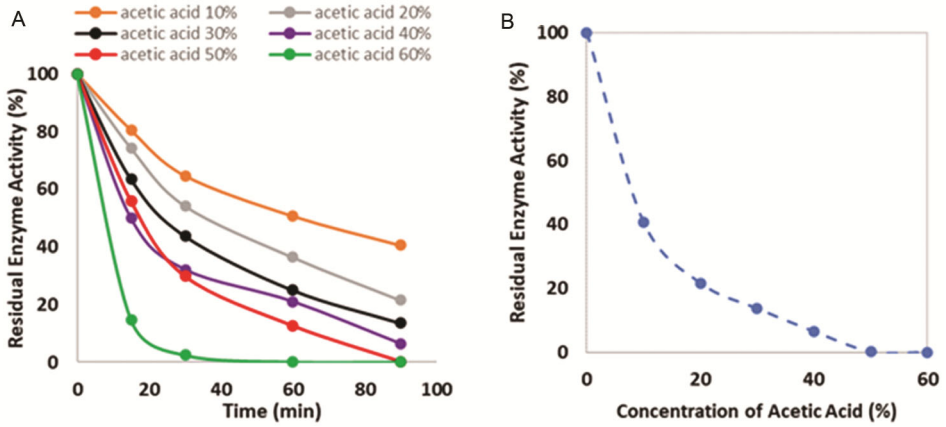


Fig. 3 — (A) Optimization of unfolding process of zDHFR protein by AcOH based on residual enzyme activity of zDHFR *vs* time (minutes); and (B) Equilibrium unfolding of AcOH induced zDHFR was monitored by UV-Visible spectrophotometer at 340 nm where residual enzyme activity (%) is plotted against concentration of acetic acid (%)

Figure 3A shows the unfolding reaction ranging from 10% to 60% AcOH concentration in Tris KCL buffer solution (pH 7.4, 25°C). At 10% AcOH concentration, the residual enzymatic activity is 40%. Further increase in AcOH concentration to 30%, the residual enzymatic activity quickly decreases from 40 to 13%, and at 50% AcOH the enzymatic activity is lost showing that AcOH has an immense effect on the enzymatic activity. Again, we observed that the addition of AcOH there is a shift in pH of the sample solution from 7.4 to 1.85 as we gradually increase the concentration of the AcOH (10%-60%).

Figure 3B depicts the recombinant zDHFR protein in 0-60% AcOH. As the concentration of acetic acid increases, the decrease in activity of the protein occurs.

SDS induced unfolding of recombinant zDHFR

The extent of inactivation of recombinant zDHFR with various SDS concentrations of 0-6 mM (Fig. 4A & B).

In Figure 4A, data shows that at low SDS concentrations, less than 0.5 mM, the residual enzymatic activity is more than 90%. Further increases of the SDS concentration to 3.5 mM, the residual enzymatic activity quickly decreases from 90% to 10%, indicating the stability has been altered significantly by SDS.

In Figure 4B, it has been shown that the enzymatic activity is decreasing continuously and at 5 mM concentration of SDS, it completely unfolds the

Ethanol -induced unfolding of recombinant zDHFR

Following a UV-visible spectrophotometer study, the impact of Ethanol as a denaturant in the range of concentrations from 0-60 % was investigated. The result is presented in (Fig. 5A & B).Ethanol have modest influence on the zDHFR at 10% concentration, then its effect increases sharply in the concentration range of 20-30%, followed by the complete denaturing action at 50%.

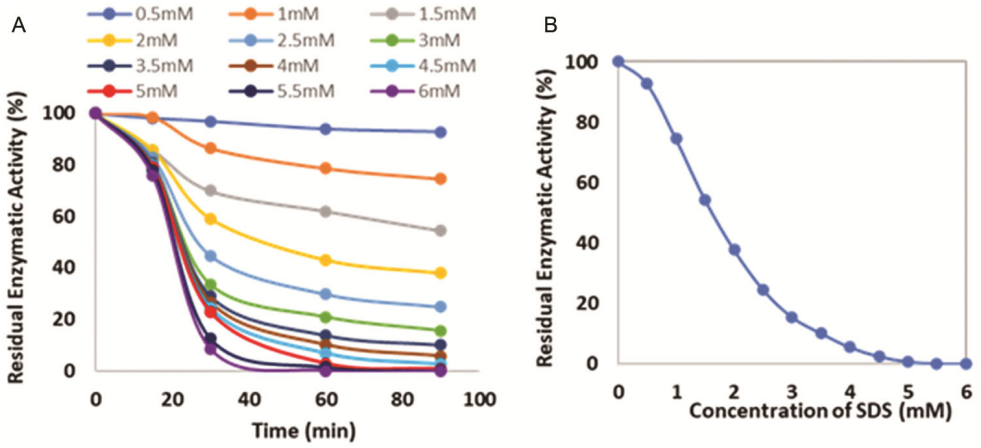


Fig. 4 — (A) Optimization of unfolding process of zDHFR protein by SDS based on residual enzyme activity of zDHFR *vs* time (minutes); and (B) Equilibrium unfolding of SDS induced zDHFR protein monitored by UV-Visible spectrophotometer at 340 nm where residual enzyme activity (%) is plotted against concentration of SDS (mM)

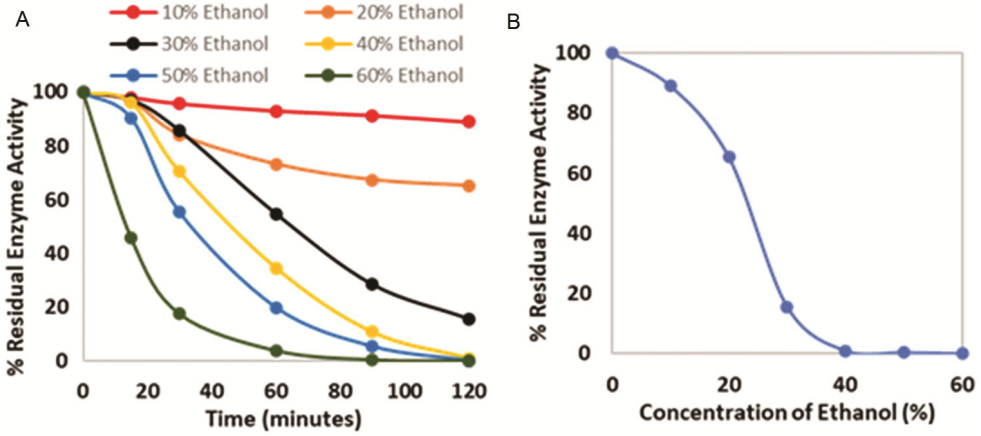


Fig. 5 — (A) Optimization of unfolding process of zDHFR protein by Ethanol based on residual enzyme activity of zDHFR *vs* time (minutes); and (B) Equilibrium unfolding study of Ethanol induced zDHFR monitored by UV-Visible spectrophotometer at 340 nm where residual enzyme activity (%) is plotted against concentration of ethanol (%)

Equilibrium refolding studies of recombinant zDHFR monitored by enzymatic assay

zDHFR protein was refolded utilizing 200 µM denatured protein, that was diluted 100 times in a refolding buffer containing 20 mM Tris and 25 mM KCl, pH 7.4 at 25°C. The refolding of protein without any denaturant was studied as control experiment. Spontaneous refolding was performed without addition of any additives and osmolytes. Changes in UV-visible spectrophotometric measurement were used to track the refolding process.

Refolding of AcOH induced denatured recombinant zDHFR

Recombinant zDHFR was initially denatured in 10%, 20%, 30%, 40% and 50% Acetic acid then after incubation the refolding buffer was added to observe the refolding yield of the protein. Figure 6 reveals that AcOH induced zDHFR protein could regain 16%, 13%, 9%, 7% and 3% enzymatic activity at 10%, 20%, 30%, 40% and 50% AcOH, respectively.

Refolding of SDS induced denatured recombinant zDHFR

Spontaneous refolding of SDS induced denatured protein was performed to recover functional protein. Results have been displayed in bar graph (Fig. 7). It shows that SDS induced zDHFR protein could regain 28%, 22%, 12%, 9% and 7% activity at 1-5 mM SDS, respectively.

Refolding of ethanol-induced denatured recombinant zDHFR

Ethanol-induced denatured zDHFR was allowed to get refold spontaneously by means of 100 folds dilution with refolding buffer at pH 7.4, 25°C. Refolding was then monitored using UV visible spectroscopy as shown in the bar graph (Fig. 8).

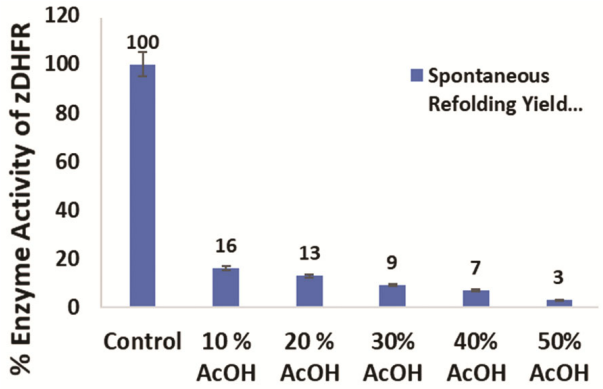


Fig. 6 — Spontaneous refolding of AcOH denatured zDHFR showed in bar graph where % enzymatic activity was plotted against different concentration of AcOH (10%, 20%, 30%, 40% and 50%) at 340 nm in UV-Visible spectrophotometer

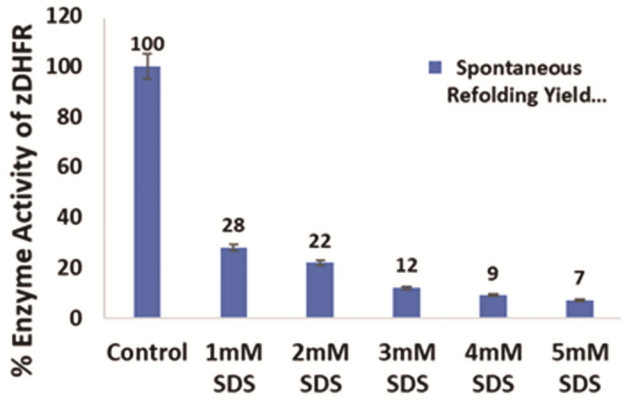


Fig. 7 — Spontaneous refolding of SDS denatured zDHFR showed in bar graph where % enzymatic activity was plotted against different concentration of SDS (1 mM, 2 mM,3 mM,4 mM and 5 mM) at 340 nm in UV-Visible spectrophotometer

The refolding process was carried out with different concentrations of denatured protein and the refolding yield of denatured protein was 13%, 11%, 7%, 4%, and 2% at 10%, 20%, 30%, 40%, and 50% Ethanol, respectively.

Discussion

Denaturation alters the protein conformation by some external stresses so that the protein will no longer be able to carry out its cellular function. The type of denaturant and concentration of denaturant regulate the protein folding processes.

In the present study, each protein solution is subjected to an activity assay to evaluate the activity of the protein, which was analysed to observe a reduction of enzymatic activity with denaturation.

The enzymatic data shows that the activity of zDHFR decreases with addition of AcOH, SDS and Ethanol where at 50% AcOH concentration the

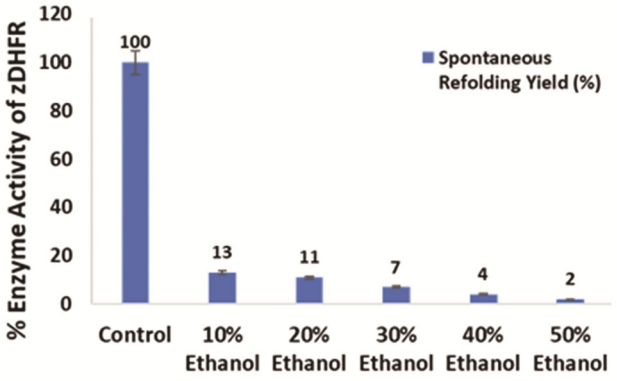


Fig. 8 — Spontaneous refolding of Ethanol denatured zDHFR showed in bar graph where % enzymatic activity was plotted against different concentration of Ethanol (10%, 20%, 30%, 40% and 50%) at 340 nm in UV-Visible spectrophotometer

enzymatic activity lost completely. In case of SDS, at 5 mM concentration, there is a sharp decrease in the residual enzymatic activity. Ethanol- induced denaturation of recombinant zDHFR protein was also done in the concentration range of 10-60% and according to the UV-visible measurements it has been observed that zDHFR starts to unfold at 10% and finally gets completely unfold beyond 50%.

As reported, when acetic acid is denaturant, protein forms 1.5 times more number of hydrogen bonds with acetic acid as compared to water[23]. Acetic acid at a final concentration of 10% lowered the pH of the reaction buffer from 7.4 to 2.65, at 20% it is 2.46, gradually when concentration at 60% pH reaches to 1.85. The enzymatic activity of zDHFR decreases sharply when the concentration of acetic acid is greater than 10% and was not detectable at all at 50% AcOH (Fig. 3B). Triyono & Agus have suggested that longer the protein reacts with acids, the more peptide bonds are hydrolyzed and caused the damage in the primary structure of the protein[24]. So this might be the case, during the unfolding of recombinant zDHFR in presence of acetic acid, which was evaluated using a UV-visible spectrophotometer at 25°C. Due to the addition of AcOH, change in pH towards acidic leads to denaturation that might disturbs the intermolecular interactions that hold protein in a folded conformation.

There are some potential factors that can affect the conformational state of recombinant zDHFR protein when ethanol induced denaturation of the protein happens like enhanced intramolecular hydrogen bonding, reduced in hydrophobicity of the protein molecule, as well as theeffect of the protein core through penetration of alcohol molecules and preferable solvation of the residues therein.

Different from AcOH and Ethanol, SDS consists of 12 carbon tails and an anionic sulfate group. SDS interacts with protein to form negatively charged SDS-protein complex, thus disrupting the non-covalent interactions of protein chains. As a result, SDS is commonly used to break hydrophobic interactions, hydrogen bonds, and electrostatic interactions in proteins[25-27].

The concentrations required for complete inactivation of recombinant zDHFR for AcOH, SDS and Ethanol are 50%, 5 mM, and 50%, respectively. The results reveal that AcOH induced unfolding of zDHFR protein is more efficient than SDS and Ethanol according to their incubation time for denaturation of protein as well as their residual enzyme activity.

It is also significant to demonstrate the spontaneous refolding process of chemically induced unfolded zDHFR at various concentrations of denaturants (AcOH, SDS & Ethanol). This can be carried out using refolding buffer (100 folds dilution) to it to decrease the concentration of denaturants in the sample solution.

After spontaneous refolding of unfolded zDHFR, induced by 50% AcOH, 5 mM SDS, and 50% Ethanol, the refolding yield are 3%, 7%, 2%, respectively. The outcome of this refolding study is a clear indication that in the case of SDS the refolding yield was more than AcOH and Ethanol although the yield is very low.

In the case of AcOH induced denatured zDHFR, it has been seen that the refolding yield is only 16% at 10% concentration of AcOH although, at this concentration of AcOH, residual enzyme activity was almost 40%. In fact, for SDS and ethanol- induced denatured zDHFR also refolding yield are low like 28 and 13%, respectively, although both of them have more than 90% residual enzymatic activity at that concentration. As a result, it appears that the extent of refolding may be governed by the protein's unfolded condition.

Our findings offer light on the activity change and loss of functionality of zDHFR protein caused by exposure to AcOH, SDS, and Ethanol. Future studies on the characterization of the denaturant -induced unfolded state of zDHFR may provide further information on this aspect and clarify the details of molecular interaction**.**

Conclusion

The Native state of proteins is characterised by highly conserved and tightly folded conformation which is biologically active. We have reported AcOH, SDS, and Ethanol- induced unfolding and spontaneous refolding after its dilution with refolding buffer using recombinant zDHFR as our model protein. According to our study, the incubation time for denaturation of zDHFR with AcOH, SDS and Ethanol are 30, 90, and 120 min, respectively, which depict that AcOH is a stronger denaturant than SDS and Ethanol.

But SDS denatured protein gives higher spontaneous refolding yield as compared to AcOH and Ethanol in their lowest concentration although the yield is very low.

The low yield acquired from the spontaneous refolding process may be due to the protein could not reach to its proper functional state instead becomes in locked in a metastable conformation.

As we know that denaturation due to ethanol can break the secondary and tertiary structure, it is difficult to regain its proper enzymatic activity spontaneously as a result showing the low refolding yield.

Unlike chemical denaturants such as guanidinium chloride or urea, which are only effective at molar concentrations due to weak binding to the protein backbone, SDS as an ionic detergent can denature proteins via strong binding to hydrophobic and charged side chains at millimolar concentrations. But when it is denaturing at room temperature, like 25°C, the residual enzymatic activity is substantially good at a lower concentrations. Hence better-refolding yield is expected.

In the case of AcOH, the residual enzymatic activity of the denatured zDHFR is less than half from its native state as it strongly denatures the protein and as a result, spontaneous refolding yield is less. Another finding has been identified from our experimental data that there is a pH shift towards acidity when AcOH concentration is increasing which may be the reason for being a strong denaturant.

With these findings, we can conclude that zDHFR probably undergoes structural shift due to which the enzymatic activity of the protein declines or during the spontaneous refolding process the protein becomes trapped in a metastable conformation as it does not refold to its functional form.

Further research is required to understand whether the unfolding is a single step process or multiple step process along with the formation of intermediates thatcan explain the proper conformational changes as well as the functionality of the protein.

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Conflict of interest

All authors declare no conflict of interest.

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