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Research Article

EFFECT OF VARIOUS OSMOLYTES ON THE EXPRESSION AND FUNCTIONALITY OF ZEBRAFISH DIHYDROFOLATE REDUCTASE: AN *IN VIVO* STUDY

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Abstract: *In vivo* study of Zebrafish dihydrofolate reductase (zDHFR), a 23 kDa monomeric protein was studied to check the expression level in the presence of sorbitol, glycerol, proline, glycine, glucose, sucrose and betaine. Different osmolytes, also known as chemical chaperones, assist in expression enhancement of protein. In the present study, it was observed that a few osmolytes effectively enhance the expression and functionality of zDHFR and hence provide substantial stability to the protein with glucose, sucrose and betaine being the exception. A general strategy to improve the activity of recombinant proteins would be to increase the cellular concentration of viscous organic compounds, termed osmolytes, or of molecular chaperones that can prevent aggregation and convert it into native active species of commercial and pharmacological importance.

Key words: Zebrafish Dihydrofolate Reductase; osmolytes; protein expression; protein stabilizer and chemical chaperone.

Introduction

Escherichia coli is one of the most suitable and attractive host for recombinant protein production (Rosano and Ceccarelli, 2014). Due to the advantage of the fast growth in an inexpensive medium, the availability of genetic information and various cloning vectors establishes *E.coli* as most popular expression platform which provide rapid, enhanced and economical production of recombinant proteins (Auslender *et al.*, 2015). For this reason, there are many molecular tools and protocols for the high-level production of recombinant proteins, such as, vast categories of expression plasmids, availability of a large number of engineered strains and many techniques to optimize heterologous protein overexpression and production in *E. coli* which greatly enhanced the yield of the foreign eukaryotic protein (Sivashanmugam *et al.*, 2009).

The expression of recombinant proteins in *E.coli* has become an inevitable method to get an enhanced yield of functional proteins for pharmaceutical, biochemical and biophysical research. Although this approach is simple, the yield of soluble and correctly folded, biologically active proteins are often reduced by misfolding and aggregation of recombinant proteins (Chaudhuri *et al.*, 2001). There are so many factors like the rate of protein expression the inherent nature of recombinant protein or the concentration or intermediates during protein folding, which affect folding process and end up in protein aggregation known as inclusion body which are non- native in nature and can create major problems in their biochemical and structural analyses (De Marco *et al.*, 2005). Protein misfolding may lead to a large number of neurodegenerative disease states. These include

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cystic fibrosis, Alzheimer's disease, and Huntington's disease (Dobson, 2001; Anfinsen, 1973; Paul *et al.*, 2007).

Although bacteria itself has some defense mechanisms by which it can protect native proteins from aggregation or misfolding caused by osmotic stress, which include the intracellular accumulation by uptake of osmolytes (Yancey *et al.*, 2001), cells have two important and effective mechanisms to survive under stress and to reduce aggregation by using molecular chaperones and chemical chaperone also known as osmolytes (Saunders *et al.*, 2000). Osmolytes accumulated in the cells enhance protein folding and stability of the native state of proteins. It comprises of small molecule of organic compounds which are water-soluble include electrolytes and non-electrolytes and are everywhere in the living system. The mechanism of osmolyte action on proteins has been proposed by "Osmophobic theory" which suggests the solvophobic thermodynamic force which is responsible for the osmolytic action (Sharma *et al.,* 2012).

Dihydrofolate reductase (DHFR, EC 1.5.1.3) is an important enzyme which converts dihydrofolate into tetrahydrofolate using NADPH as the electron donor. A methyl group shuttle is required for the de novo synthesis of purines, thymidylic acid, and certain amino acids. DHFR has become very important nowadays as anti-cancer drug target due to its inhibition by methotrexate. In addition to its importance as a pharmacological target, DHFR has attracted the attention of protein chemists as a model for the study of enzyme structure/function relationships because of its small size and the availability of purified enzyme (Schweitzer *et al.*, 1990). *In vivo* folding pathway of any recombinant protein is an important parameter for understanding its ability to fold by itself inside the cell, which always dictates the downstream processing for the purification.

Zebrafish DHFR is an excellent model for developmental biology research. Apart from this application, its significant resemblance with human DHFR provides ample evidence which suggests the use of zebrafish DHFR as an *in vitro* assay system for folate related studies and drug discovery (T.T. Kao *et. al.*,2008). In the present study, we have tried to enhance the *in vivo* expression of zebrafish DHFR in *E. coli* cells by investigating the effect of osmolyte supplementation on the production of functional zDHFR protein (Sivashanmugam *et al.*, 2009). We observed that a combination of osmolytes and high salt concentration in the growth media enhances protein solubility. In addition, expressing recombinant protein under these conditions can improve the yield of functional protein. Subsequent studies showed that these various classes of osmolytes from carbohydrate, amino acid, methylamine and carbamide sources stabilize proteins *in vivo* (Bhojane *et al.*, 2014, Hailu *et al.*, 2013).

Materials and Methods

Materials

BL21 (DE3) Rosetta *E.coli* strains were used for expression and purification of zDHFR. The plasmid, DHFR in the pET 43.1a vector containing $(His)_6$ was obtained from Dr. Tzu-Fun, Taiwan. Isopropyl β-D-1-thiogalactopyranoside (IPTG), Dihydrofolate (DHF), and Nicotinamide Adenine Di-nucleotide Phosphate (NADPH) were purchased from Sigma Chemical Company (USA). High purity grade imidazole and sodium chloride were purchased from Merck, India. All other reagents used were of analytical grade.

Double colony selection

Competent BL21 (DE3) Rosetta *E. coli* cells were prepared according to the $CaCl_2$ method (Sambrook *et al.*, 1989). The competent cells were transformed with zDHFR plasmid in pET43.1a vector and spread on LB agar medium containing ampicillin with final concentration of 100µg/ml. Several colonies appeared after incubation for 16-20 h at 37°C from which few selected colonies were picked up and expression were checked by 12% SDS-PAGE using IPTG as expression inducer. The colony with highest protein expression was selected for another round of selection. The colonies selected by the double selection were used for preparation of glycerol stocks and stored at -80°C.

IPTG induced expression

Transformed cells bearing zDHFR gene were grown at 37°C with agitation at 250rpm and

induced with 100µM IPTG when OD_{600} reaches to 0.8- 1.0. The cells were further incubated for 6h at 25°C with agitation at 250rpm. The expression of zDHFR was confirmed by analyzing the cell extract on 12% SDS-PAGE (Laemmli, 1970).

Expression of recombinant zDHFR in presence of various osmolytes

Transformed E.coli cells bearing zDHFR gene were grown in LB Amp medium containing optimized concentrations of various osmolytes. When the culture for each osmolyte along with an expression control i.e. without any osmolyte, reached an OD_{600} of 0.8- 1.0, it was induced with 100µM IPTG and incubated at 25°C for 6 h.

Purification of recombinant zDHFR

Cells obtained from the IPTG induced culture of BL21 Rosetta E.coli cells bearing zDHFR gene were harvested by centrifugation at 6,000 rpm for 20 mins. Expression of the protein was confirmed by analysis on 12% SDS-PAGE. Harvested cells were resuspended in lysis buffer (20 mM sodium phosphate, pH 7.4; 500mM NaCl) and 0.1mg/ml DNAse I, 1 mM PMSF, 0.2 M MgCl2 and 0.1mg/ml Lysozyme were added followed by incubation for 30 mins on ice. The cells were lysed by sonication. Cell debris was removed by centrifugation at 13,000 rpm for 40 min at 4°C. The supernatant was collected and filtered through a 0.22 micron Millipore syringe filter. Purification of N-terminal Histidine tagged zDHFR was carried out by Immobilized Metal Ion Affinity Chromatography (IMAC) using Ni2+ as chelating agent as per Kao, 2008 with necessary modifications in AKTA FPLC system (GE Health care, USA). Different purifies fractions of the protein were assessed by 12% SDS PAGE. The same protocol for purification was followed for all the cultures of IPTG induced E.coli cells bearing zDHFR gene having various osmolytes in the media.

Enzymatic activity assay of zDHFR

zDHFR activity was determined by the fact that it catalyses a chemical reaction (Figure 1) where, the decrease in NADPH concentration was monitored by measuring its absorbance at 340 nm

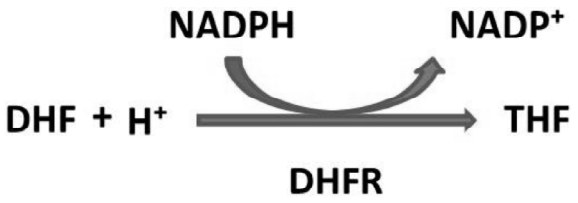


Figure 1: **Reaction catalysed by DHFR. Where, DHF and THF are dihydrofolate and tetrahydrofolate, respectively. The decrease in NADPH concentration was monitored by measuring its absorbance at 340 nm at 25°C.**

at 25°C. The composition of assay buffer was (25mM Tris-HCl, 20mM KCl, pH 7.4), 100 µM dihydrofolic acid and 140 µM NADPH. One unit was defined as the amount of enzyme required to oxidize 1 µmole of dihydrofolate per min, based on a molar extinction coefficient of 12,300 M–1 cm–1 at 340 nm (Hillcoat *et al.*, 1967).

Results and Discussion

The most of the expressed recombinant proteins cannot reach to a correct conformation and undergo proteolytic degradation or associate with each other to form insoluble aggregates of non-native proteins known as inclusion bodies. Hence, there is an ever-growing interest in developing strategies to avoid protein aggregation or to enhance protein refolding yields. Dihydrofolate reductase (DHFR; 5,6,7,8-tetrahydrofolate: NADP+oxidoreductase), a 23 kDa protein, catalyzes the NADPH-dependent reduction of dihydrofolate (H2folate) or folic acid to tetrahydrofolate (H4folate) and is considered to be a key enzyme in folate metabolism. H2folate is the product of thymidylate synthetase and must be recycled to H4folate in order to be incorporated into the tetrahydrofolate metabolic pool. After reduction of H2folate, H4folate receives one carbon unit and acts as a one-carbon donor in the biosynthesis of purines and pyrimidines and in the interconversion of amino acids. Tetrahydrofolate acts as a methyl group shuttle re-quired for the de novo synthesis of purines, thymidylic acid, and certain amino acids.

In the present study, the pET 43.1a vector containing the DHFR gene under control of T7 promoter, was used for over-expression of zebrafish DHFR in that has machinery for T7 RNA polymerase under control of Lac promoter. The chemical induction of the *Lac* promoter was

accomplished by the addition of the non-hydrolysable analogue of lactose, IPTG. While larger biomass content before induction is necessary for enhanced expression of the recombinant protein, the cells over-producing recombinant proteins should also be sufficiently active at the time of induction. Thus, pre-induction cell concentration (OD_{600}) plays a crucial role for over-expression of recombinant proteins. Expression of DHFR was obtained when induction was carried out in the mid-exponential phase. The protein was purified from the soluble fraction of the over expressed protein, using IMAC Ni^{+2}-chelating chromatography.

Over expressed recombinant proteins in bacterial cell often tend to misfold and accumulate as soluble aggregates and/or inclusion bodies. A strategy for improving the level of expression of recombinant proteins in a soluble native form is to increase the cellular concentration of osmolytes or chaperones. Osmolytes are compounds affecting osmosis and they protect organisms from stress induced by osmotic pressure.

Osmolytes are naturally occurring organic compounds, which represent diverse chemical categories including amino acids, methylamines, and polyols. Due to increased concentrations of osmolytes, organisms may undergo some conformational changes in cellular proteins. Osmolytes shift equilibrium toward natively-folded conformations by increasing the free energy of the unfolded state. Osmolytes mainly affect the protein backbone. This balance between osmolyte–backbone interactions and amino acid side chain–solvent interactions decides protein folding process.

In the present study, bacterial cells were grown in the presence of high salt, sorbitol, glucose, sucrose, proline, urea, glycerol, glycine and betaine (Table 1). The understanding of the molecular mechanisms by which osmolytes and specific molecular chaperones act in stressed and non-stressed bacterial cells are very important to design the protocols to produce optimal amounts of natively folded recombinant proteins. The presence in the cell of physiological amounts of compatible osmolytes, such as proline, glycine, betaine and sorbitol can significantly increase the stability of native thermo-labile proteins. Figure 1 shows the typical reaction catalyzed by DHFR enzyme. It has been observed that colony selection was one of the important factors for high-level protein production using high density bacterial expression methods (De Marco *et al.*, 2005). The presence of zDHFR gene was confirmed by double digestion using restriction enzymes, Xho I and Nde I and visualized by 1% agarose gel electrophoresis which is shown in Figure 2. The full-length isolated zDHFR cDNA (EU145591) was 570 bp and encodes a protein of 190 amino acids (Kao *et al.*, 2008).

Expression of zDHFR in presence of various osmolytes was confirmed by 12% SDS-PAGE, shown in figure 3. It was observed that protein shows good over expression in presence of 100µM IPTG. Amount of folded protein in a cell can be estimated based on the principle that the proteins with correctly folded structure are soluble in the cytoplasm and in aqueous buffer, however, denatured proteins are insoluble and occur as aggregates (Chaudhuri *et al.*, 2001). The concept

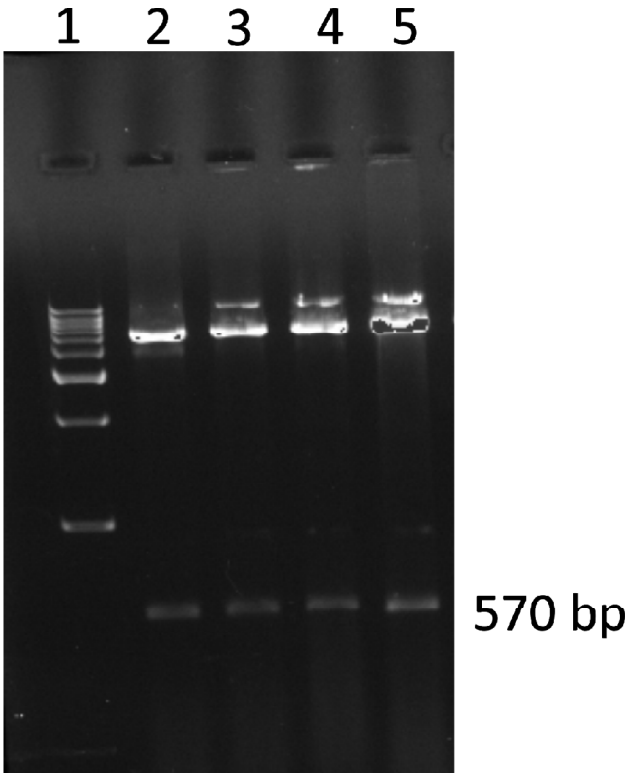
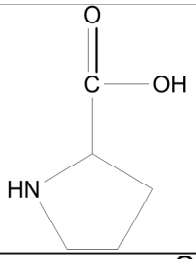
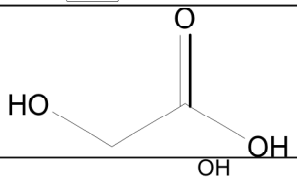
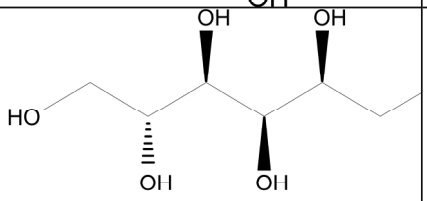
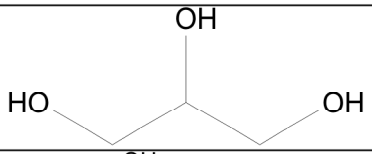
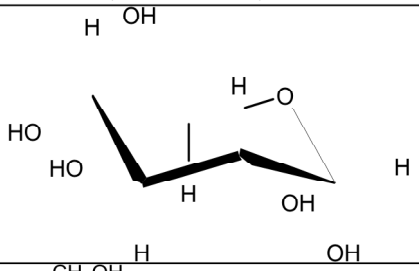
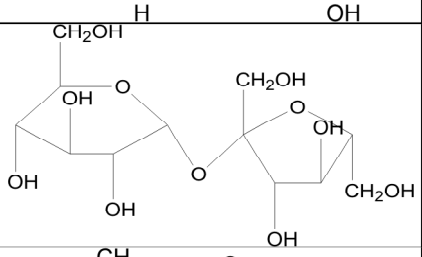
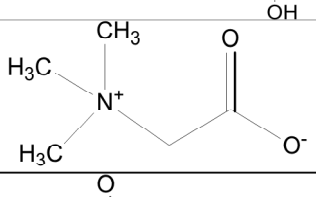
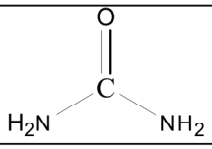


Figure 2: **The presence of zDHFR gene was confirmed by double digestion using restriction enzymes, Xho I and Nde I and visualized by 1% agarose gel electrophoresis. Lane 1, 1 kb DNA ladder; lane 2-5, closed, nicked and linear DNA (top to bottom) of zDHFR.**

Table 1
Various osmolytes and their details (adapted from Sharma *et. al.*, 2012)

S.no.	*Osmotic Class*	*Name*	*Structure*	*Molecular weight (Da)*
1.	Amino acids and their derivatives	Proline		115.13
		Glycine		75.06
2.	Carbohydrates	Sorbitol		182.17
	Half saccharide	Glycerol		92.09
	Monosaccharide	D-Glucose		180.16
	Disaccharide	Sucrose		342.30
3.	Methylammonium and methylsulfonium solutes	Glycine betain		117.14
4.	Carbamides	Urea		60.06
5.	Salts	Sodium Chloride	NaCl	58.44

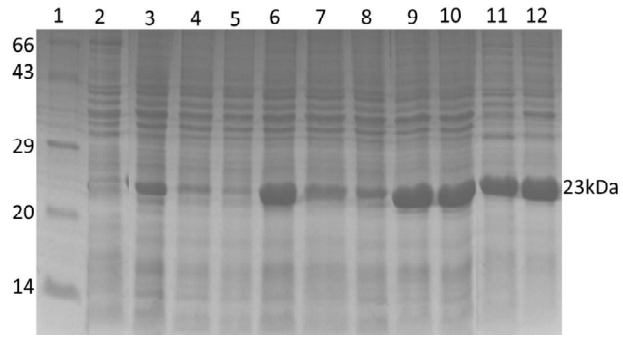


Figure 3: **Expression of zDHFR in presence of various osmolytes confirmed by 12% SDS-PAGE. Lane1, low molecular weight protein marker; lane 2, uninduced BL21 (DE3) Rosetta E.coli cells; lane 3, 100 µM IPTG induced cells; lane 4, IPTG induced cells in the presence of 0.2% glucose; lane 5, 1mM betain; lane 6, 0.5M sorbitol; lane 7, 2M glycerol; lane 8, 0.5M sucrose; lane 9, 1mM proline; lane 10, 100mM glycine; lane 11, 0.5M NaCl and lane 12, 200mM urea. IPTG induced protein sample was considered as expression control.**

of folded or native protein in soluble fraction may be utilized to confirm the enhancement of expression by osmolytes because of the fact that osmolytes stabilizes the protein so that it may attain its native or folded conformation. Normalization of the cell culture was done such that the same numbers of cells was taken for the analysis of each sample along with the control (un-induced cells). *In-vivo* protein over-expression was checked in the presence of 0.2% glucose; 1mM betain; 0.5M sorbitol; 2M glycerol; 0.5M sucrose; 1mM proline; 100mM glycine; 0.5M NaCl and 200mM urea. IPTG induced protein sample was considered as expression control. The concentrations of osmolytes were optimized prior to checking the comparative expression enhancement in zDHFR (data not shown). Protein shows enhanced expression of soluble protein in the presence of sorbitol, proline, urea and glycine but not much effect of glycerol and NaCl whereas glucose, sucrose and betain showed the inhibited expression (Chen *et al.*, 2015; Oganesyan *et al.*, 2007). The level of *in vivo* zDHFR expression in presence of various osmolytes has been presented by the bar graph (Figure 4) which shows level of in vivo zDHFR expression where an optimized concentration of different osmolytes was used along with 100 µM IPTG. zDHFR was purified by Ni-NTA affinity chromatography. Figure 5 shows FPLC chromatogram of zDHFR at 280nm using imidazole gradient from 0-500mM. An imidazole gradient of 0-500 mM in 16 column volumes was used to elute zDHFR. The protein was eluted between 150-200 mM imidazole concentrations. The fractions collected in the prominent peak region and 12% SDS-PAGE shows purification of Zebrafish DHFR by Nickel Affinity Chromatography contain very high concentrations of purified zDHFR (Figure 6). The fractions were pooled and the concentration of pure zDHFR was determined using an extinction coefficient of 24,075 $M^{-1}cm^{-1}$ at 280nm. Typically, 20-30 mg of pure zDHFR was obtained from 1L of culture. Further, purification was performed for all the protein samples obtained from different cultures having various osmolytes.

After purification, the enzymatic activity of zDHFR was monitored at 340nm determined by the method described previously by Kao *et al.* with necessary modifications, in Beckman coulter DU800 spectrophotometer (USA). Each assay mixture contained 25mM Tris-HCl, 25mM KCl, 140µM NADPH, 100µM DHF and 0.2µM Zebrafish DHFR (pH 7.4) at 25°C in a total volume of 500µl. (Hillcoat et al., 1967). All enzyme assays were done in triplicate and to minimize the degradation of substrate and cofactor, NADPH and DHF were prepared fresh, incubated in ice and consumed within 2-3 hours of experimentation. The results were compared for all osmolytes as presented in the bar graph (Figure 7).

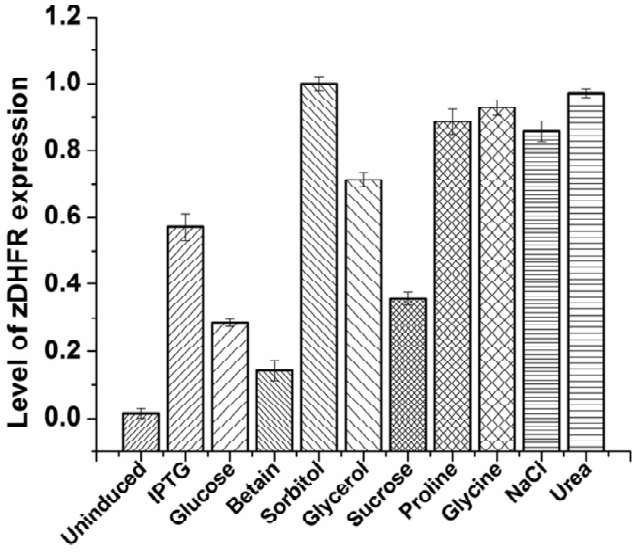


Figure 4: **Level of *in-vivo* zDHFR expression where an optimized concentration of different osmolytes was used along with 100 µM IPTG.**

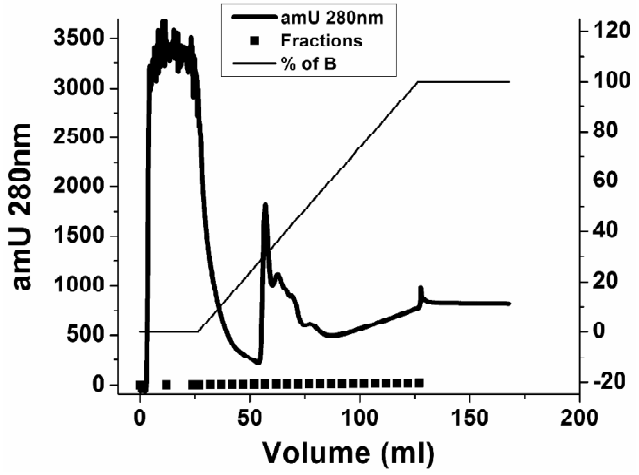


Figure 5: **FPLC chromatogram of zDHFR at 280nm using imidazole gradient from 0-500mM.**

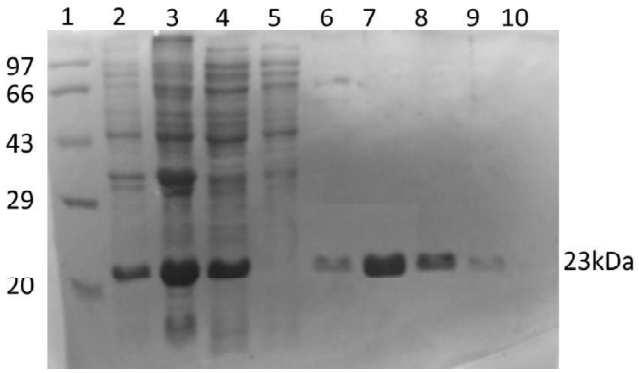


Figure 6: **12% SDS-PAGE shows purification of Zebrafish DHFR by Nickel Affinity Chromatography: Lane M, Low molecular weight protein marker; lane 1, pellet obtained after sonication of lysed induced cells; lane 2, sonicated supernatant loaded on affinity column; lane 3, flow through during load; lane 4-7, different fractions obtained after affinity chromatography.**

From the bar graph, it is very clear that the impact of the glucose, sucrose and betain on the expression of recombinant protein is less than the traditional IPTG induced expression resulting in lower enzymatic activity, whereas sorbitol shows highest expression level as well as activity with zDHFR having six hydrogen bond donor and acceptor count. For the same reason glycerol also shows higher level of activity having three H-bond acceptor and donor count. This shows the protecting nature of osmolytes like glycerol and sorbitol, which increases the free energy of the unfolded form by interacting with the peptide bond in an unfavourable manner and hence, favouring the folded conformation of zDHFR.

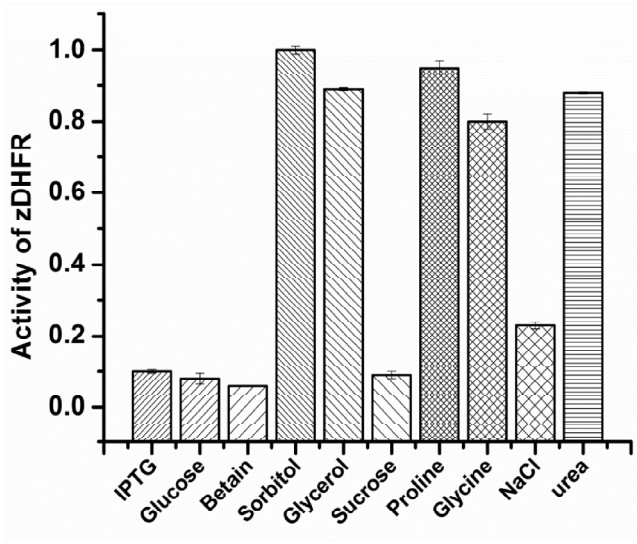


Figure 7: **Enzymatic activity of zDHFR was monitored at 340nm determined by the method described previously by TT Kao et. al. with necessary modifications, in Beckman coulter DU800 spectrophotometer (USA). Each assay mixture contained 25mM Tris-HCl, 25mM KCl, 140µM NADPH, 100µM DHF and 0.2µM Zebrafish DHFR (pH 7.4) in a total volume of 500µl. (Hillcoat et al., 1967). All enzyme assays were done in triplicate and to minimize the degradation of substrate and cofactor, NADPH and DHF were prepared fresh, incubated in ice and consumed within 2-3 hours of experimentation.**

Proline and glycine being hydrophobic amino acids, which buried inside the protein core also have H-bond acceptor count which can show almost same activity level.

It is evident from the present study that osmolytes plays a crucial role in enhancement of expression to a substantial level in case of zDHFR. Also, there are many reports suggesting the *in-vivo* role of osmolytes in correcting the folding defects of proteins for example, glycerol can correct the temperature sensitive folding defect of the human cystic fibrosis transmembrane conductance regulator mutant protein and tumor suppressor protein in cells (Sharma *et al.*, 2012)

Conclusion

Osmolytes have been used to prevent misfolding and aggregation in many proteins *in-vitro* while *in-vivo* studies using osmolytes are little difficult to perform because the higher concentrations of osmolytes used might not favour the bacterial cell growth. In the present study, we have reported that the *in-vivo* effect of supplementation of optimized concentrations of osmolytes on the

expression and functionality of zDHFR protein. We observed that a combination of osmolytes and high salt concentration in the growth media enhances protein solubility. Under these conditions the expression of recombinant protein as well as enzymatic activity of the purified protein can improve its stability substantially. Subsequent studies showed that these various classes of osmolytes from carbohydrate, amino acid, methylamine and carbamide sources stabilize proteins in vivo and assist in their enzymatic activity.

Acknowledgments

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Abbreviations

zDHFR, zebrafish Dihydrofolate reductase; IPTG, Isopropyl β-D-1-thiogalactopyranoside; DHF, Dihydrofolate; NADPH, Nicotinamide Adenine Di-nucleotide Phosphate and IMAC, immobilized metal ion affinity chromatography.

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