



## *In vitro* stability of various enzymes by proline from H<sub>2</sub>O<sub>2</sub> mediated oxidative damage

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Plants under stress need to favour certain pathways so as to survive the stress period. Protection of specific enzymes by proline and other osmolytes could be one such mechanism to favour some pathways/processes. Therefore, the influence of osmolyte proline on conformational changes of various proteins caused by hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) was studied by intrinsic and extrinsic fluorescence emissions. H<sub>2</sub>O<sub>2</sub> caused conformational change in proteins. Results indicated that for Alcohol dehydrogenase (AD) and Glutamate dehydrogenase (GD) enzymes, H<sub>2</sub>O<sub>2</sub> induced conformational change was high and that for Glucose 6-phosphate dehydrogenase (G6PDH) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was low. Fluorescence and far-UV, CD measurements of catalase demonstrated that the H<sub>2</sub>O<sub>2</sub> stabilized the protein secondary structure at low concentrations but destabilized it at higher concentrations. Intrinsic and ANS fluorescence results showed that proline at a concentration of 1.0 M prompted a reduction in the H<sub>2</sub>O<sub>2</sub>-induced exposed hydrophobic surfaces of studied enzymes, to different degrees which suggests its differential protective effect. Furthermore, SDS-PAGE studies revealed that proline was not able to reduce or inhibit the H<sub>2</sub>O<sub>2</sub> mediated aggregation of GAPDH.

**Keywords:** ANS, CD, Conformation change, Fluorescence, Protein aggregation, Spectroscopy

Osmolytes, also called chemical chaperons, accumulate in bacteria, animals, as well as in plants under stress conditions. They include carbohydrates or polyols (trehalose, sucrose, glycerol, myoinositol, and sorbitol), methylamines [trimethylamine N-oxide (TMAO)], betaine, and glycerophosphoryl-choline), amino acids, and their derivatives [proline, serine, taurine, glycine, arginine, and gamma amino butyric acid (GABA)]<sup>1-3</sup>. They are known to be accumulated under salt, water, temperature, and pressure stresses in animals and plants<sup>1-3</sup>. They help bacteria, animals and plants to tolerate these stressful conditions. On the other hand, denaturants or chaotropic agents destabilize the proteins. Positive interaction of denaturant like urea with amino acid side chains of proteins results in protein destabilization<sup>1,4</sup>. However,

the exact mechanism of action of osmolytes is unknown. Various theories have been postulated regarding their functions as protein stabilizers, anti-oxidants, or energy storage compounds<sup>2,4,5</sup>. Exclusion of the osmolytes (*e.g.* TMAO) from the protein surface as a result of negative interaction with amino acids leads to minimization of the protein surface without changing its conformation resulting in “preferential hydration” of the protein<sup>4,5</sup>. Another theory proposes that, changes in the hydrodynamics of water molecules on protein surface result in protein stabilization. However, the most accepted theory, proposed by Bolen and co-workers<sup>2,4</sup> postulates that, more than the side chains, the protein backbone interaction with osmolytes is important. Negative interaction with the backbone is more prominent, as compared to interaction with protein side chains, and as an outcome, the protein backbone tries to hide itself from the osmolytes and acquires its folded form. Beside this, osmolytes also protect proteins from aggregation and assist in refolding from the denatured state<sup>3,7</sup>.

Numerous osmolytes have been studied *in vitro* against different enzymes to find out the mechanism

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**Abbreviations:** AD, Alcohol dehydrogenase; GD, Glutamate dehydrogenase; CA, Carbonic anhydrase; GOx, Glucose oxidase; CAT, Catalase; HRP, Horseradish peroxidase; G6PDH, Glucose 6-phosphate dehydrogenase; GAPDH, Glyceraldehyde 3- phosphate dehydrogenase; FLU, Fluorescence

of action. Proline provide good level of protection for hen egg-white lysozyme<sup>6,8</sup>. Other glycine-based osmolytes like glycine, sarcosine, and betaine have been shown to stabilize the proteins<sup>6,8</sup>. The glycerol also prevented creatine kinase from thermal inactivation and aggregation in a concentration-dependent manner<sup>9</sup>. The spectroscopic measurements suggested that the protective effect of glycerol was a result of enhanced structural stability of native creatine kinase<sup>9</sup>. Differential protective effect of various osmolytes on a particular enzyme was observed earlier<sup>10</sup>. For trypsin and chymotrypsin, TMAO was observed to be the strongest stabilizer among tested osmolytes<sup>10</sup>. Kumar and Venkatesu<sup>11</sup> reported that the stabilizing effects of polyols on chymotrypsin is highest with trehalose, followed by sucrose, glycerol, and sorbitol. Sucrose, sorbitol and trehalose also increased the re-naturation of guanidine hydrochloride-denatured trehalose-6-phosphate<sup>12</sup>.

Polyols and sugars differ in their osmoprotective effect on proteins from amino acids, and its derivatives. Under different pHs, polyols (glycerol and sorbitol) and sugars (glucose, fructose, galactose, sucrose, raffinose and stachyose, trehalose) have displayed an increase in the  $\Delta G_D^\circ$  of proteins in a pH-dependent manner<sup>13</sup> and thus, had a pH-dependent stabilizing effect<sup>14</sup>. However, amino acid osmolytes (glycine, proline, isoleucine, leucine, phenylalanine, and valine) and amino acid derivatives (taurine and  $\beta$ -alanine) neither altered  $\Delta G_D^\circ$ <sup>15</sup> nor showed significant pH-dependent stabilization effects on proteins, although they increased the  $T_m$  of proteins at physiological pH<sup>15</sup>.

Proline is an important osmolyte accumulated by plants under stress conditions<sup>16</sup>. It has been proposed that proline acts like a protein folding chaperone due to the formation of an ordered amphipathic supramolecular assembly of proline molecules<sup>6,8</sup>. It is accumulated significantly in many plants *e.g.* in maize root tips<sup>2</sup> and sugarbeet leaves<sup>16</sup>.

Proline's impact on proteins varies because of different interactions with protein side chains. Proline protects proteins from thermal unfolding, increases the refolding of chemical or thermal stress-denatured proteins, and prevents protein-aggregation<sup>12,17,18</sup>. It has been reported that proline protected PersiXyn2<sup>19</sup>, lactate dehydrogenase<sup>20</sup> under stress conditions. Proline prolonged the thermal unfolding of citrate synthase<sup>21</sup> and firefly luciferase<sup>22</sup>. This indicates its

stabilizing ability on protein structure and conformation. Renaturation of denatured proteins in the presence of proline was enhanced for lysozyme, creatine kinase<sup>9,17</sup>, citrate synthase<sup>21</sup>, and arginine kinase<sup>23</sup>. Osmolytes are known to prevent protein aggregate formation<sup>18</sup>. Chattopadhyay *et al.* (2004)<sup>21</sup> showed that proline protected citrate synthase from aggregation. High concentrations of proline protected catalase from thermal aggregation<sup>15</sup>. Proline also improves the solubility of proteins. Proline at high concentrations promoted the solubility of sparingly soluble proteins *in vitro*<sup>8</sup>. Diehl *et al.* (2021)<sup>24</sup> concluded that proline shows unfavorable interactions (exclusion from) with amide oxygens and aliphatic hydrocarbon surfaces exposed during unfolding, thus stabilizing proteins. Moreover, because of its unfavorable interactions with anionic (carboxylate and phosphate) and amide oxygens, and aliphatic hydrocarbons on the surface of cytoplasmic proteins and nucleic acids, it increases the osmolality of cells<sup>24</sup>. Therefore, it is very important to understand the role that proline plays in protection of proteins under stress.

Plants produce Hydrogen peroxide ( $H_2O_2$ ) under stress conditions.  $H_2O_2$  is a small, diffusible, and ubiquitous molecule. It is recognized as an abundant intracellular messenger due to its comparatively longer life span, localized biosynthesis, and its prompt response to a stimulus. However, at higher concentrations,  $H_2O_2$  can cause damage/injury/modifications to proteins, DNAs, membranes, lipids, and proteins containing FeS clusters and mononuclear iron centers<sup>25,26</sup>. It mainly acts on the sulfhydryl (-SH) groups of proteins by converting them into sulphenic (-SOH), sulphinic (-SO<sub>2</sub>H), sulphonic (-SO<sub>3</sub>H) acids and also form disulfide bonds (-S-S-) from adjacent sulfhydryl (-SH) groups by oxidizing methionine, cysteine, histidine, and tryptophan residues. These conversions lead to conformational changes in the secondary and tertiary structure of proteins and also, play a role in protein inactivation. The presence of cysteine residues in the active site of proteins leads to its irreversible oxidation and locking by inhibiting the enzyme activity in the presence of even very low concentrations of  $H_2O_2$ <sup>25</sup>. This mechanism might be used by  $H_2O_2$  to sense the redox status of cells and block the functioning of specific pathways under mild oxidizing conditions. Notably, dehydratases were found to be susceptible to micromolar concentrations of  $H_2O_2$ <sup>26</sup> due to the oxidation of [4Fe-4S] cluster.

Under stress conditions, it is possible that the localized concentrations of H<sub>2</sub>O<sub>2</sub> reach up to milli mole amounts per gram fresh weight in various leaf tissues<sup>27</sup>. Depending on the nature and extent of stress to plants, particularly at a specific concentration of H<sub>2</sub>O<sub>2</sub>, different proteins are expected to show differential susceptibility in terms of oxidation, aggregate formation, conformational, and activity modulation favoring certain biochemical pathways. Similarly, accumulation of proline is also expected to favor certain pathways by protecting certain enzymes damaged by H<sub>2</sub>O<sub>2</sub>. Therefore, we investigated the modulations in conformational changes of eight enzymes related to H<sub>2</sub>O<sub>2</sub> and nicotinamide adenine dinucleotide phosphate-reduced (NADPH) metabolism and photosynthesis that are simultaneously subjected to oxidative stress by H<sub>2</sub>O<sub>2</sub> and protection by amino acid, proline. Conformational changes were studied through extrinsic and intrinsic fluorescence. In addition, catalase was studied through CD spectroscopy, GD and GAPDH through thermal melting, and GAPDH aggregation using non-reducing gels.

## Materials and Methods

### Materials

Eight enzymes were purchased from Sigma-Aldrich Chemical Company, USA for this study except HRP. The sources of the enzymes were *S. cerevisiae* for AD (product #A7011-75KU) and G6PDH (product #G6378-500UN). The GD (product#19359-10MG-F) was from *Pseudomonas* sp. The other enzymes were from *Aspergillus niger* (GOx, product #49180-1G), bovine erythrocytes (CA, product # C3934-500MG), bovine liver (CAT, product # C9322-5G) and rabbit muscle (GAPDH, product # G2267-5KU). The HRP was from horseradish, CDH Fine Chemicals (Product # 185195). L-Proline (product # P5607-100G) and DTT (product # D9779-1G) were of analytical grade (Sigma-Aldrich, USA). Proteins were checked for their purity by the presence of a single band in SDS-PAGE (Fig. 1). ANS was fluorescence grade (Sigma-Aldrich, Product #10417-5G-F).

### Treatment of Proteins with H<sub>2</sub>O<sub>2</sub> and/or proline

3.0 mL solutions of different enzymes (Table 1) in Potassium-phosphate buffer (pH 7.0), were treated with various concentrations of H<sub>2</sub>O<sub>2</sub> at room temperature by adding <10 µL of freshly prepared H<sub>2</sub>O<sub>2</sub> (Fisher Scientific, catalog # M1072981000) from 30% stock solutions. 6 M proline stock solution

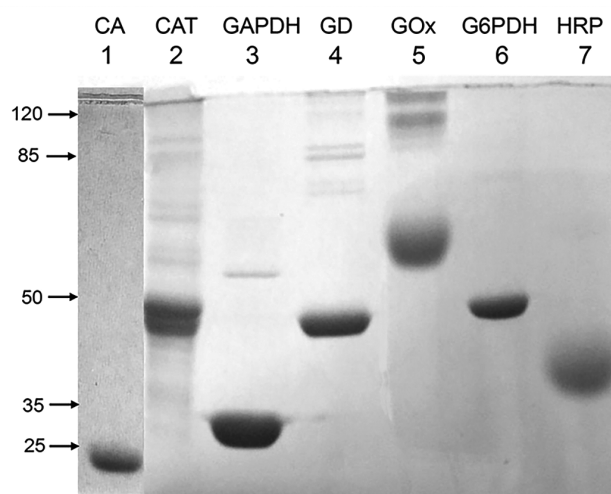


Fig. 1 — SDS-PAGE (10%) for checking the purity of proteins. Lane 1, CA, carbonic anhydrase; Lane 2, CAT, Catalase; Lane 3, GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; Lane 4, GD, Glutamate dehydrogenase; Lane 5, GOx, Glucose oxidase; Lane 6, G6PDH Glucose 6-phosphate dehydrogenase (G6PDH); Lane 7, HRP, Horseradish peroxidase

was freshly prepared and 500 µL of it was added to 2.5 mL of protein solution to get 1 M final concentration of proline. An equivalent volume (500 µL) of buffer was added to non-proline solutions. All the samples were incubated for 30 min before taking the respective spectra.

### Circular dichroism spectroscopy

Circular dichroism (CD) spectra were recorded only for catalase with a JASCO J-1500 spectropolarimeter. A quartz cell of path length 2 mm was used to hold 50 µg mL<sup>-1</sup> catalase sample in phosphate buffer saline (PBS, pH 7.0) at 25°C. Far-UV spectra were measured from 190-260 nm. Three scans were accumulated at a scan speed of 50 nm min<sup>-1</sup>.

### Intrinsic fluorescence

The intrinsic tryptophan fluorescence spectra of the 50 µg mL<sup>-1</sup> native enzymes solutions in the presence of relevant additives (H<sub>2</sub>O<sub>2</sub>, Proline and H<sub>2</sub>O<sub>2</sub> + Proline) were recorded with JASCO J-1500 spectropolarimeter (multiprobe spectropolarimeter that can be used for obtaining fluorescence as well as CD spectra) in 1 cm quartz cuvette, at 25°C between 300 and 400 nm (excitation 285 nm). The pH of 1 M proline was found to be 6.9 at 25°C in a sodium-phosphate buffer prior to the experiment. The stability of fluorescence signals was confirmed as a function of time after addition of H<sub>2</sub>O<sub>2</sub>, Proline and H<sub>2</sub>O<sub>2</sub>+Proline. For GD, 2X protein (100 µg mL<sup>-1</sup>) and

Table 1 — Fluorescence of different enzymes in the presence of H<sub>2</sub>O<sub>2</sub> and/or proline, and DTT in 50 mM phosphate buffer, pH 7.0 at 25°C

S. No.	Enzyme (concentration)	H <sub>2</sub> O <sub>2</sub> conc., mM	Proline conc., M	Fluorescence peak, nm	ΔPeak, nm	% change in Fluorescence	SD	Mol.wt, Molarity
1	Alcohol Dehydrogenase (100 µg/mL)	0	0	333				146 kD, 0.681 µM
		1	0	334	+1	-8.88	1.76	
		0	1	334	0	-33	2.88	
		1	1	334	0	-40	2.97	
		0	0	334	0	-7.06	0.87	
2	Carbolic Anhydrase (100 µg/mL)	1	0	334	0	-19.5	1.82	55.4 kD, 1.8 µM
		0	0	341				
		1	0	344	+3	-5.92	0.39	
		0	1	341	0	-32.9	3.03	
		1	1	342	+1	-35.7	2.86	
3	Glutamate Dehydrogenase (100 µg/mL)	0	0	341	0	-7.09	0.50	49.2 kD, 1.626 µM
		1	0	341	0	-1.51	0.03	
		0	1	337				
		1	1	336	-1	-9.67	1.37	
		0	1	338.5	+1.5	-25	4.78	
4	Horse Raddish peroxidase (100 µg/mL)	1	1	337	0	-27.7	4.39	40 kD, 2.5 µM
		0	0	334	-3	+1.03	0.22	
		1	0	337	0	-23.4	5.23	
		0	0	340				
		1	0	340	0	-0.55	0.2	
5	Glucose 6-phosphate dehydrogenase (100 µg/mL)	0	1	342	+3	-15.3	2.1	1.28 kD (dimer), 59.2 kD (monomer), 0.78 µM (dimer), 1.69 µM (monomer)
		1	1	345	+5	-16.5	4.3	
		0	0	341	+1	+5.46	0.46	
		1	0	341	+1	-5.05	0.48	
		0	0	NP				
6	Glyceraldehyde 3-phosphate dehydrogenase (100 µg/mL)	1	0	NP				36 kD monomer, 2.22 µM
		0	0	337				
		1	0	338	+1	+4.69	0.55	
		0	1	336	-1	-23.18	2.30	
		1	1	335.5	-1.5	-21.09	2.41	
7	Glucose oxidase (100 µg/mL)	0	0	338	+2	+10.18	1.67	80 kD monomer, 0.625 µM
		1	0	337	+1	-16.67	2.33	
		0	0	340				
		1	0	340	0	-3.51	1.11	
		0	1	340	0	-19.45	2.21	
8	Catalase (100 µg/mL)	1	1	340	0	-18.75	3.65	250 kD (tetramer), 0.4 µM
		0	0	NP				
		1	0	NP				
		0	0	335.5				
		1	0	337	+1.5	-2.76	0.15	
		0	1	336	+0.5	-27.2	2.22	
		1	1	336	+0.5	-26.4	1.09	
		0	0	335.5	0	-0.6	0.05	
		1	0	335.5	0	-2.82	0.20	
		0	0	NP				

Abbreviations: NP-Not performed, DTT-Dithiothriitol, kD-kilodalton, mM-milimolar, µM-Micromolar

H<sub>2</sub>O<sub>2</sub> concentration was used because we did not achieve a good above baseline signal at 50 µg mL<sup>-1</sup>. The excitation and emission slit widths were set to 5 and 10 nm, respectively.

#### Extrinsic fluorescence

For making 50 µg mL<sup>-1</sup> protein solution in 50 mM phosphate buffer (pH 7.0), 250 µM of 1-anilino-8-naphthalene sulfonic acid (ANS) was added to it

after dissolving in 5% ethanol and the fluorescence spectra were recorded at room temperature (JASCO J-1500 spectropolarimeter) between 450 nm and 600 nm using an excitation wavelength of 375 nm. The slit width of excitation and emission wavelengths were 5 and 10 nm, respectively.

#### Thermal denaturation of GD and GAPDH

The melting temperature,  $T_m$  was calculated by subjecting these enzymes (along with additives as described earlier) to incremental thermal denaturation @ 1°C/min, from 25 to 95°C and recording the fluorescence values.  $T_m$ , where theoretically 50% of the protein molecules are native and 50% are unfolded, were determined to assume that during a thermal transition two distinct populated states of enzymes were present, the native (N) and the denatured/unfolded state (D).  $T_m$  values are not affected by irreversibility and  $f_N + f_D = 1$ , where  $f_N$  and  $f_D$  representing the fraction of protein in the native and denatured conformations, respectively. The fluorescence signal  $F = f_N F_N + f_D F_D$ , where  $F_N$  and  $F_D$  are the values of  $F$  characteristic of the native and unfolded states, respectively, under the conditions at which  $F$  is being measured.  $K = (F_N - F) / (F - F_D)$  and  $\Delta G = -RT \ln K$ , where  $R$  is gas constant with a value of 1.98 cal M<sup>-1</sup> K<sup>-1</sup>. The values of  $F_N$  (native protein fluorescence) and  $F_D$  (denatured protein fluorescence) in the transition region were determined by linear extrapolation of pre-transition and post-transition regions. A graph is plotted between  $\Delta G$  and temperature.  $T_m$  is the temperature where  $\Delta G = 0$ <sup>28</sup>.

#### Aggregation studies on GAPDH

100  $\mu$ L of GAPDH (from rabbit muscle; 0.50 mg mL<sup>-1</sup>) in Potassium-phosphate buffer (pH 7.0), was treated with various concentrations of H<sub>2</sub>O<sub>2</sub> and H<sub>2</sub>O<sub>2</sub>+Proline at 37°C for 30 min. Immediately after that, the samples were mixed with 1/4<sup>th</sup> of the volume of SDS-sample buffer containing 250 mM Tris-HCl (pH 6.8), 2% SDS, 30% glycerol, 0.01% bromophenol blue in the presence (reduced) or the absence (non-reduced) of 100 mM DTT. Samples were separated by 10% SDS-PAGE and were stained using Coomassie Brilliant Blue.

#### *In silico* analysis of GAPDH for Cystine dimer formation

The presence of disulfide bond formation between four cysteines in GAPDH was checked with the neural network prediction algorithm using DiANNA. The PDB:1J0X was downloaded from RCSB (PMID:14646080). The fasta sequence was checked for the prediction of disulfide bonds between 150,

154, 245, and 282 using DiANNA webserver (PMID: 15980459). The distance measurement module of Chimera was used to check the inter-atomic distances between cysteines.

## Results

#### Purity of enzymes

The enzyme purity validation was performed for seven enzymes that are used in this study. Most of the enzymes were of high purity with the presence of a single band in SDS-PAGE (Fig. 1). However, a small amount of higher molecular weight bands was observed for catalase and GOx which are most likely aggregates, generated due to oxidation of proteins or it contains a little impurity.

#### Kinetic parameters of proline binding to catalase

In the present study, we used intrinsic and extrinsic fluorescence to understand the interaction mode of different enzymes with proline/H<sub>2</sub>O<sub>2</sub>. To study the kinetic parameters of proline binding with catalase, it was titrated with increasing concentrations of proline (0.02, 0.04, 0.07, 0.10, 0.15, 0.20, 0.30, 0.40, 0.60, 0.90, 1.20, and 2.40 M) by adding from 6 M stock solutions. The fluorescence decrease (Fig. 2A), due to the increase in the volume of solution with the addition of proline solution, was corrected by accounting for fluorescence decrease caused by the addition of an equivalent amount of buffer in control. Proline decreased the fluorescence intensity of emission peak (336 nm) for catalase (Fig. 2A) and other studied proteins (Table 1).

Binding properties of the ligand/fluorophore to proteins has been studied using the fluorescence quenching<sup>29-31</sup>. To know the binding constants and binding stoichiometry for the interaction of catalase with proline,  $\log(F_0 - F)/F$  was plotted against increasing “log concentrations of proline” (Fig. 2D), where  $F_0$  and  $F$  are fluorescence intensities in the absence and presence of quencher, respectively. A modified stern–Volmer equation  $\{\log[(F_0 - F)/F] = \log K_s + n \log(Q)\}$  was used for analyzing the mechanism of fluorescence quenching, where slope  $n$  corresponds to the binding stoichiometry,  $K_s$  is the binding constant, and  $Q$  is the quencher (proline) concentration. The results indicate a good linear relationship (Fig. 2D). From this plot, binding stoichiometry,  $n$  was calculated to be 0.89. Binding constant of 5.35 M<sup>-1</sup> was calculated from the intercept (Fig. 2D).

The biomolecular quenching,  $K_q$  was estimated according to the stern–Volmer equation:  $F_0/F = 1 + K_D$

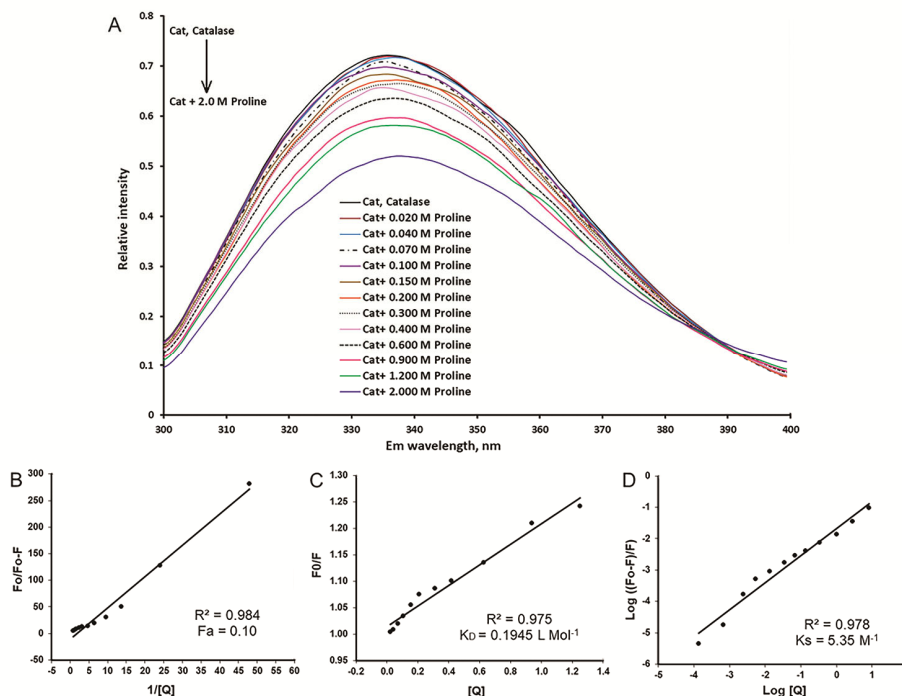


Fig. 2 — Titration and Binding of proline with catalase (A) Fluorescence emission spectra of catalase–proline interaction in 50 mM phosphate buffer (pH 7.0) at 25°C temperature. Excitation and emission were at 5 nm and 10 nm slit widths. Identical spectra were obtained during three independent experiments.  $\lambda_{ex}=285 \text{ nm}$ ,  $\lambda_{em} = 300\text{-}400 \text{ nm}$ ; (B)  $F_0/F_0-F$  vs  $1/[Q]$  to measure the fraction of accessible sites  $f_a$ , to quencher.  $R^2=0.984$ ,  $f_a=0.10$ ; (C) Stern–Volmer plots for measuring fluorescence quenching constant ( $K_q$ ) for catalase–proline binding at different proline concentrations to determine static or dynamic quenching.  $R^2=0.975$ ,  $K_D=0.1945 \text{ L Mol}^{-1}$ ; and (D) Modified Stern–Volmer plot of  $\text{log}(\Delta F/F)$  against  $\text{log}[Q]$  to determine binding constant and number of binding sites.  $R^2=0.978$ ,  $K_s=5.35 \text{ M}^{-1}$ ,  $n=0.89$

$[Q]$ , where  $F_0$  and  $F$  are the fluorescence intensities in the absence and presence of quencher,  $[Q]$  is the quencher concentration, and  $K_D$  is the Stern–Volmer quenching constant, which can be written as  $K_D = K_q \cdot \tau_0$ , where  $K_q$  is the biomolecular quenching rate constant and  $\tau_0$  is the lifetime of the fluorophore (5 ns for catalase)<sup>32</sup> in the absence of quencher.  $K_D$  value of  $0.1945 \text{ L mol}^{-1}$  was obtained from the linear regression equation and the biomolecular quenching constant ( $K_q$ ) was calculated to be  $3.89 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$  for proline (Fig. 2C). Further, by plotting  $F_0/(F_0-F)$  with  $1/[Q]$  (Fig. 2B), a low fraction of quenching sites ( $f_a$ , 0.10) accessible for quencher, proline was obtained.

#### Treatment of catalase with a low concentration of $\text{H}_2\text{O}_2$

We further analyzed the effect of  $\text{H}_2\text{O}_2$  on different enzymes with fluorescence spectroscopy. Upon treatment of catalase with a very low concentration of  $\text{H}_2\text{O}_2$ , ranging from 0–200  $\mu\text{M}$ , there was a marginal change in fluorescence emission of catalase enzyme (data not shown). At this concentration of  $\text{H}_2\text{O}_2$ , CD spectra of catalase showed a decrease in negative

ellipticity ratio of 222/208 nm at 200  $\mu\text{M}$   $\text{H}_2\text{O}_2$ , whereas, the increase in the ellipticity at 208 nm is higher than at 222 nm (Fig. 3). Since there was not much change in the fluorescence, higher  $\text{H}_2\text{O}_2$  concentrations were used for further experimentation, as described below.

#### Treatment of different proteins with higher concentrations of $\text{H}_2\text{O}_2$

Hydrogen peroxide concentration, ranging from 10–10000  $\mu\text{M}$ , caused a gradual decrease in the maximum fluorescence emission of catalase at 336 nm (data not shown). We chose 1 mM concentration of  $\text{H}_2\text{O}_2$  where the reduction in emission was prominently visible and significant, to check for differential susceptibility of eight enzymes to  $\text{H}_2\text{O}_2$ . The percentage-decrease in the fluorescence intensity of six enzymes (100  $\mu\text{g mL}^{-1}$ ) in decreasing order was  $\text{AD} > \text{GD} > \text{CA} > \text{GOx} > \text{CAT} > \text{HRP}$ . The other two enzymes, GAPDH and G6PDH showed a reverse order at low  $\text{H}_2\text{O}_2$  *i.e.* increase in fluorescence (Table 1), however, upon treatment with a higher concentration of  $\text{H}_2\text{O}_2$ , GAPDH and G6PDH also showed a decrease in fluorescence (data not shown).

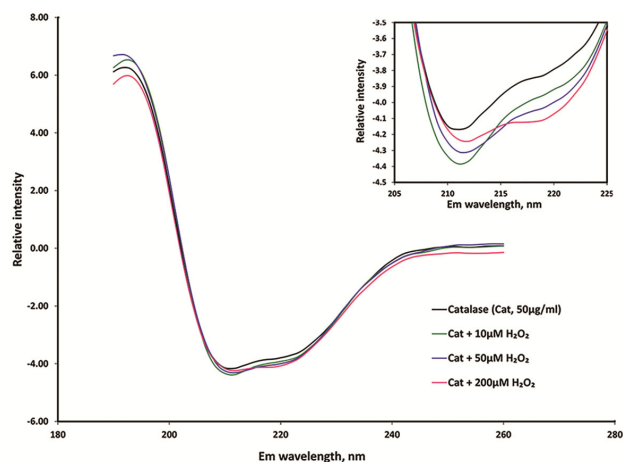


Fig. 3 — Increased secondary structure in Catalase by low H<sub>2</sub>O<sub>2</sub> concentrations. 222/208 ratio increased by ~2.3% and ~6.4% in presence of 50 and 200 mM of H<sub>2</sub>O<sub>2</sub>, respectively indicating an increase in the secondary structure ( $\alpha$ -helix) in catalase (Cat; 50 mg mL<sup>-1</sup>). CD spectra were performed with JASCO 1500 spectropolarimeter at 25°C. Blank/buffer absorption values were subtracted from obtained spectra. Incubation for protein samples was at room temperature (25°C) for 30 min

There was no significant  $\lambda_{em}$  shift upon addition of 1 mM H<sub>2</sub>O<sub>2</sub> to HRP, G6PDH, and GOx (Table 1). However, we observed  $\lambda_{em}$  red-shift (1.0-3.0 nm) in the case of AD, GAPDH, CA, and catalase (Table 1).

#### Combined treatment of proteins with proline and H<sub>2</sub>O<sub>2</sub>

Further, the addition of proline alone also caused  $\lambda_{em}$  red-shift in the case of HRP, GD, and G6PDH. However, the study from the five proteins (AD, CA, GD, GOx, and CAT), showed a differential relative intensity in fluorescence (Table 1) as compared to the fluorescence decrease by proline alone (Fig. 4). We selected four dehydrogenases based on their fluorescence changes, AD and GD displaying maximum decrease and G6PDH and GAPDH displaying maximum increase, for thermal unfolding and extrinsic fluorescence studies.

#### ANS fluorescence

In the presence of H<sub>2</sub>O<sub>2</sub>, there was a significant increase in ANS fluorescence of AD (Fig. 5), as compared to other enzymes. The ANS fluorescence has considerably increased in AD with higher blue-shift in the presence of 1 M proline as compared to GD and G6PDH. No blue-shift was observed in GAPDH. The relative ANS fluorescence intensity of AD is much lower in the presence of proline than H<sub>2</sub>O<sub>2</sub>, however, it increased to a higher extent in other enzymes.

There was a partial reversal of H<sub>2</sub>O<sub>2</sub> mediated blue-shifted-peak and an increase in the fluorescence was observed in AD by the addition of proline. In

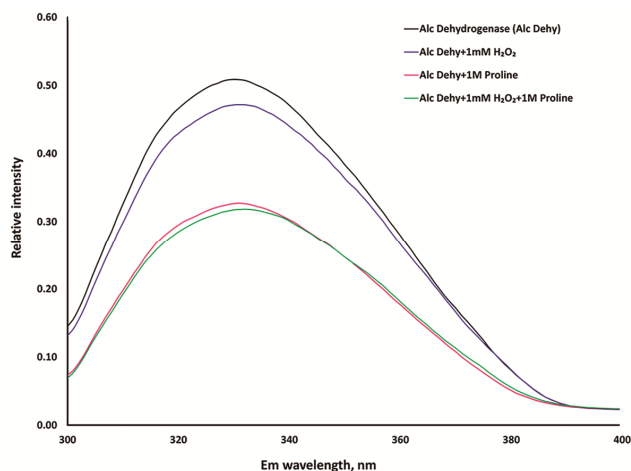


Fig. 4 — Protection by proline. Intrinsic fluorescence spectra of alcohol dehydrogenase showing that the additive decrease in the flu from H<sub>2</sub>O<sub>2</sub> and from proline individually is more as compared to combined flu (proline+H<sub>2</sub>O<sub>2</sub>). Experimental conditions were as for (Fig. 2). Incubation for protein samples was at room temperature. All measurement was carried out at 25°C. AlcDehy-Alcohol dehydrogenase

other three proteins, there was no reversal of increased fluorescence and shifted-peak by proline (Figs 5 & 6). With the addition of proline+H<sub>2</sub>O<sub>2</sub> in GAPDH and G6PDH, the ANS peak did not increase to an extent equivalent to individual additions of proline or H<sub>2</sub>O<sub>2</sub> (Fig. 6).

Further, we demonstrated the thermal unfolding of four proteins in the presence of ANS. The fluorescence of AD, G6PDH, and GAPDH (Figs 5 & 6) increased during heating up to 75, 62.5, and 65°C, respectively, and blue-shifted drastically close to their T<sub>m</sub>. However, their fluorescence decreased when the temperature was further increased up to 95°C. We observed a different fluorescence profile of GD in comparison to the other three proteins where there was no blue-shift and a continuous increase in fluorescence was detected upon heating up to 95°C.

#### Thermal denaturation of GD and GAPDH

The hydrogen peroxide treatment of GD and GAPDH decreased their T<sub>m</sub> by 1.45±0.505 and 2.18±0.896°C, respectively, (Table 2). In the presence of proline, T<sub>m</sub> increased from 61.25±0.212°C to 62.65±0.495°C for GD and from 60.1±0.424°C to 61.2±0.566°C for GAPDH. Addition of proline to enzyme + H<sub>2</sub>O<sub>2</sub> solution was accompanied by the increase in T<sub>m</sub> by 2.1±0.788°C in case of GD and 1.53±0.931°C in the case of GAPDH.

#### GAPDH aggregation

The hydrogen peroxide treatment of GAPDH is accompanied by the appearance of a smaller band

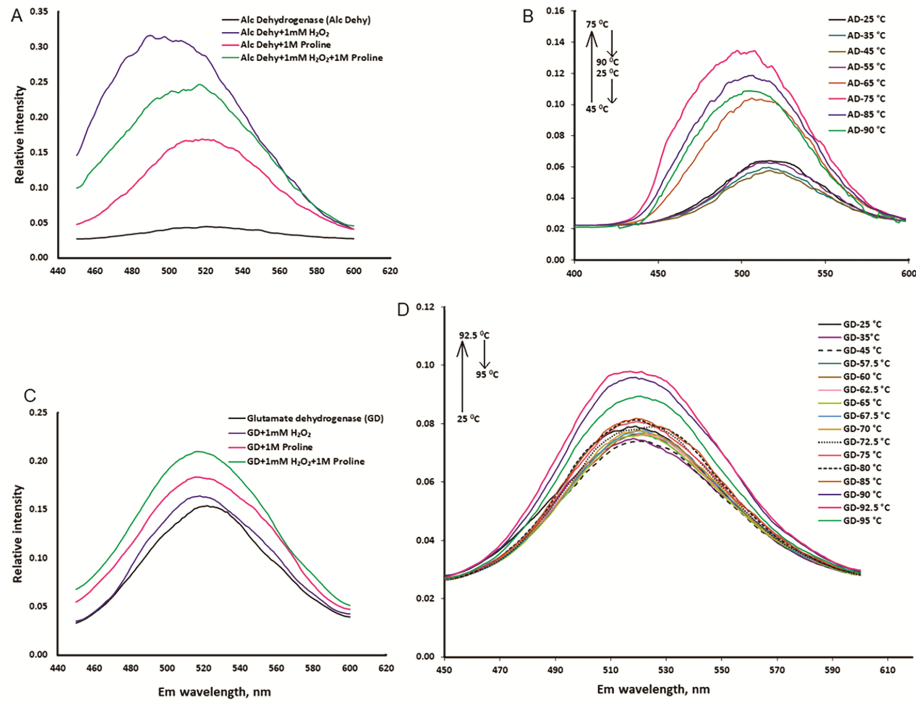


Fig. 5 — ANS-binding to enzymes AD and GD (A) ANS-binding of AD in presence of H<sub>2</sub>O<sub>2</sub> and/or proline; (B) ANS-fluorescence of AD during heating from 25°C at the rate of 1°C min<sup>-1</sup>; (C) ANS-binding of GD in presence of H<sub>2</sub>O<sub>2</sub> and/or proline; and (D) ANS-fluorescence of GD during heating from 25°C at the rate of 1°C min<sup>-1</sup>. Enzymes were incubated for 30 min in presence of H<sub>2</sub>O<sub>2</sub> and/or 1.0 M proline, 5 M equivalents of ANS was added and the solution was further incubated for 1 min before recording fluorescence emission spectra at respective temperatures

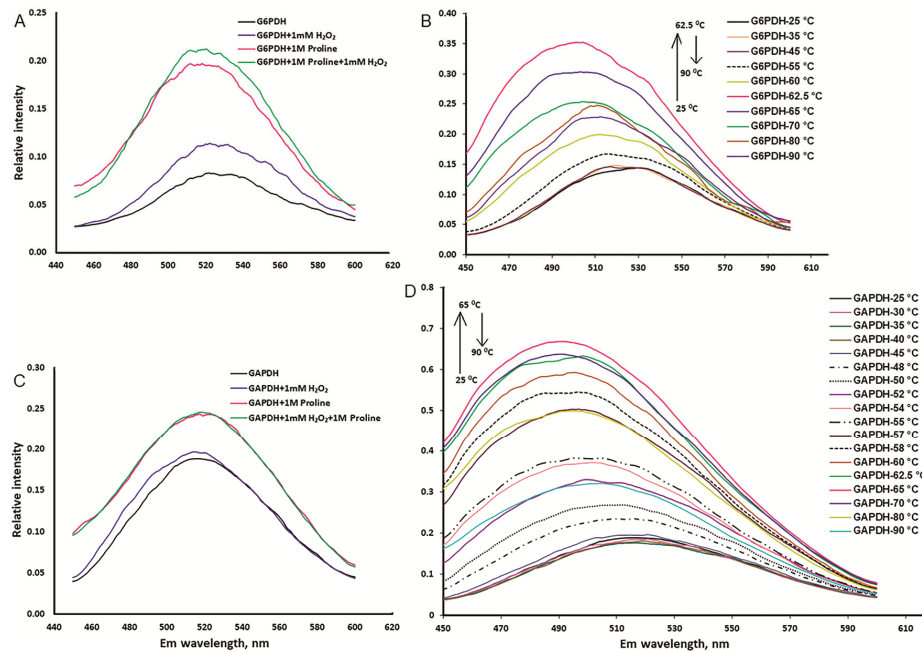


Fig. 6 — ANS-binding to enzymes (A) ANS-binding of G6PDH in presence of H<sub>2</sub>O<sub>2</sub> and/or proline; (B) ANS-fluorescence of G6PDH during heating from 25°C at the rate of 1°C min<sup>-1</sup>; (C) ANS-binding of GAPDH in presence of H<sub>2</sub>O<sub>2</sub> and/or proline; and (D) ANS-fluorescence of GAPDH during heating from 25°C at the rate of 1°C min<sup>-1</sup>. Enzymes were incubated for 30 min in presence of H<sub>2</sub>O<sub>2</sub> and/or 1.0 M proline, 5 M equivalents of ANS was added and the solution was further incubated for 1 min before recording fluorescence emission spectra at respective temperatures

Table 2 — Thermal denaturation of Glutamic Dehydrogenase (GD) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the presence of H<sub>2</sub>O<sub>2</sub> and/or 1 M Proline

Name of Enzyme	H <sub>2</sub> O <sub>2</sub> concentration.	Enzyme alone		Enzyme +H <sub>2</sub> O <sub>2</sub>		Enzyme + 1M Proline		Enzyme + H <sub>2</sub> O <sub>2</sub> + 1 M Proline	
		T <sub>m</sub>	SD	T <sub>m</sub>	SD	T <sub>m</sub>	SD	T <sub>m</sub>	SD
Glutamic Dehydrogenase (100 µg/mL)	2 mM	61.25	±0.212	59.8	±0.293	62.65	±0.495	61.9	±0.424
GAPDH (50 µg/mL)	1 mM	60.1	±0.424	57.92	±0.472	61.2	±0.466	59.45	±0.459

SD: Standard Deviation; M : Molar; mL: millilitre; H<sub>2</sub>O<sub>2</sub> : Hydrogen peroxide

(~30 kD) with significant intensity, just below the 36 kD band of GAPDH monomer (Fig. 7A). This band was also increasing in intensity with increasing H<sub>2</sub>O<sub>2</sub> *i.e.* 5 µM -5 mM (Fig. 7A). Upon treatment with 100 mM DTT, this band disappeared. Higher mass bands composed of GAPDH aggregates, with simultaneous diminution of the intensity of the band corresponding to the 36 kD GAPDH monomer, were observed with 1-20 mM H<sub>2</sub>O<sub>2</sub> (Fig. 7B) treatment, although, high molecular-weight aggregates were not visible (30 kD band was visible) at micromolar (lower) concentrations (5 and 10 µM) of H<sub>2</sub>O<sub>2</sub>. Appearances of all new bands were reversed by 100 mM DTT (Fig. 7B).

Proline when present in 1.0 M concentration could not prevent the aggregate formation of GAPDH induced by treatment with 1 mM or higher concentration of H<sub>2</sub>O<sub>2</sub> at 37°C. Additionally, proline could also not prevent degradation of GAPDH to form a disulfide (-S-S-) linked 30 kD band which was induced even at lower H<sub>2</sub>O<sub>2</sub> concentration (5-10 µM).

#### *In silico* analysis of GAPDH

The computational simulation of GAPDH protein revealed that two Cys residues are in the interior (150, 154) and two are on the surface (245, 282) (Fig. 8). The below threshold value from DiANNA server output indicated the absence of intra-monomer disulfide bonds formation. The distance between neighboring cysteines in 3D was less than 5Å which also indicated the absence of intra-molecule disulfide bonds. The overall analysis indicated that the intra disulfide bond is not present in monomer but the surface Cys may be involved in inter-monomer disulfide bond formation in the native tetramer associations as well as with the proteins in the vicinity during aggregate formation.

#### Discussion

Proline accumulates as an osmolyte at very high concentrations in plants under stress conditions without perturbing regular metabolic events<sup>1</sup>. Due to

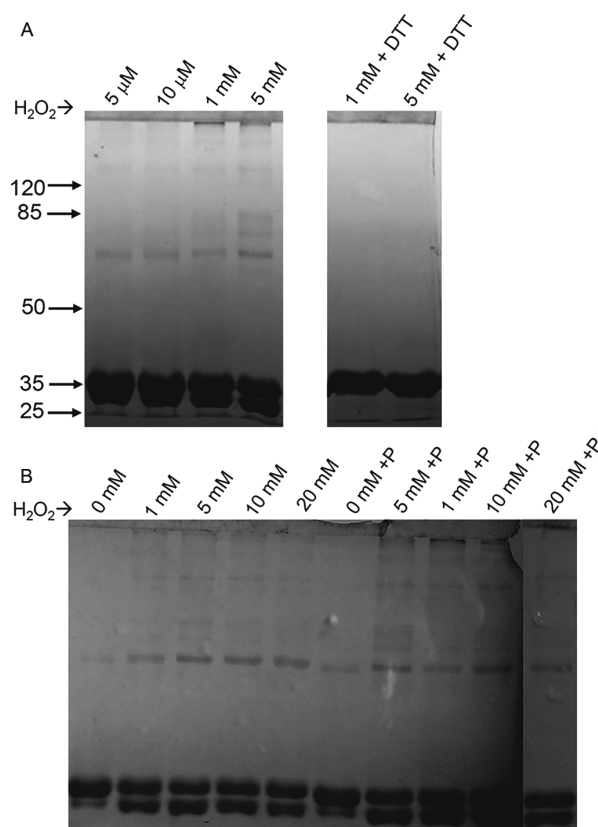


Fig. 7 — Effect of proline on aggregation of GAPDH by H<sub>2</sub>O<sub>2</sub>. (A) Appearance of high MW aggregates of GAPDH and ~30 kD fragment under non-reducing conditions with increasing concentration of H<sub>2</sub>O<sub>2</sub> from 5 µM - 5 mM. Reducing agent (100 mM DTT) caused the disappearance of all disulfide bonded species. 100 mL GAPDH (0.5 mg mL<sup>-1</sup>) was treated with given concentrations of H<sub>2</sub>O<sub>2</sub> at 37°C for 30 min. The samples were mixed with SDS sample buffer in the absence or presence of 100 mM DTT. 10 mL of each sample was subjected to 10% SDS-PAGE followed by CBB staining; and (B) Addition of 1 M proline (P) at the initiation of incubation did not inhibit H<sub>2</sub>O<sub>2</sub> (from 1 mM-20 mM)-dependent aggregation of GAPDH under non-reducing conditions

its very high solubility (up to 7 M) in water, it can tremendously influence the osmotic potential of the cell. It is reported to protect many enzymes under stress conditions<sup>11,20</sup>. To investigate the possibility

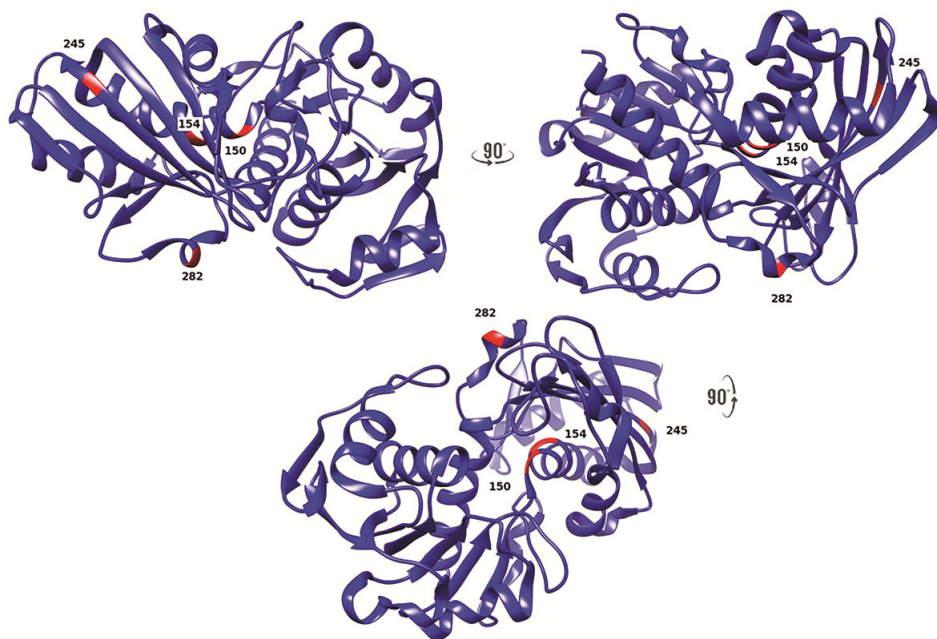


Fig. 8 — *In silico* analysis of GAPDH. Structure representation of GAPDH with rabbit muscle (PDB:1J0X; Sigma Cat No. G2267). DiANNA webserver was used for prediction of Cystine dimer formation (PMID:15980459). The protein is colored in blue and the Cys residues with corresponding residue numbers are colored in red. Two Cys are in interior (150, 154) and two are on the surface (245, 282)

that proline might be protecting enzymes in a differential way to favor specific pathways under stress conditions, we tested it on a range of enzymes damaged by  $H_2O_2$ , commonly accumulated oxidant in plants under stress conditions.

#### Kinetic parameters of proline binding to catalase

Intrinsic fluorescence spectroscopy is a type of electromagnetic spectroscopy, where fluorescence of the sample can be analyzed using UV-light to excite the electrons of a certain compound. In the present study, we have used fluorescence spectroscopy to know the binding mechanism or binding mode of different enzymes with proline. Proline decreased the fluorescence emission peak of catalase (Fig. 2A and Table 1) which has been reported earlier by Sepasi Tehrani *et al.* (2013)<sup>15</sup>. Tryptophan (Trp) gives a very common response with respect to a smaller change in protein conformation. This decrease in fluorescence may be mainly due to the interaction with the excited fluorophore within the protein. Almost all enzymes contain Tryptophan (Trp), Tyrosine (Tyr) and Phenylalanine (Phe) which might contribute to the intrinsic fluorescence but among them only Trp residue is mostly responsible for the intrinsic fluorescence. The possibility of the involvement of other two amino acids to fluorescence is much less because Phe has very little quantum yield and the

fluorescence of Tyr gets totally quenched in the presence of any ionized groups ( $NH_2$  or  $COOH$  *etc.*).

Number of binding ligands obtained from the modified Stern-Volmer equation plot was calculated to be 0.89, suggesting that proline interacts with the catalase in a one-to-one ratio. The binding constant of  $5.35 M^{-1}$  indicates a very weak binding of proline with catalase (Fig. 2D). The structure of catalase from different organisms was also investigated to postulate the binding site of proline. The oligomeric nature of the enzyme makes it difficult to make a prediction of proline binding sites on catalase. Proline is also known to alter the activity of catalase suggesting the binding interaction of proline with the enzyme<sup>4</sup>.

Bimolecular quenching constant ( $K_q$ ) was calculated to be  $3.89 \times 10^7 M^{-1}s^{-1}$  for proline (Fig. 2C) as per Stern-Volmer equation. The maximum scatter/dynamic collision quenching constant for various kinds of quenchers to biopolymer is  $2.0 \times 10^{10} M^{-1}s^{-1}$ <sup>33</sup>. The calculated quenching constant  $K_q$ , is lesser than what is allowed for a scattering process ( $2.0 \times 10^{10} M^{-1}s^{-1}$ ), therefore it suggests that the fluorescence quenching of catalase is mainly by dynamic quenching rather than static. Furthermore, a very low fraction of quenching sites ( $f_a$ , 0.10; Fig. 2B) were accessible to proline. All the above data support the hypothesis that proline is not tightly binding to

catalase, rather frivolous interaction by dynamic process happens at a single binding site in catalase.

#### Low concentration of H<sub>2</sub>O<sub>2</sub> stabilized catalase

At the low concentration of H<sub>2</sub>O<sub>2</sub> (50 and 200  $\mu$ M), CD spectra of catalase showed small but continuous decrease (after an initial increase at 10  $\mu$ M), with increasing H<sub>2</sub>O<sub>2</sub> concentration, in the negative ellipticity at 222 nm (inset of Fig. 3). The ratio between the two peaks *i.e.* 222/208 is used to determine the change in the  $\alpha$ -helix content of the protein. As can be seen from Figure 3, this ratio is clearly increasing from 0 to 200  $\mu$ M H<sub>2</sub>O<sub>2</sub> which shows that the  $\alpha$ -helix of the protein is increasing with increasing concentration of H<sub>2</sub>O<sub>2</sub>, indicating the stabilization of catalase enzyme by H<sub>2</sub>O<sub>2</sub> upto 200  $\mu$ M concentration. This increase may also be due to a higher induction of  $\alpha$ -helix form by the activation of the enzyme in the presence of substrate.

#### H<sub>2</sub>O<sub>2</sub> caused conformational changes in enzymes to different degrees

The treatment of catalase with increasing concentrations, from 10 to 10000  $\mu$ M of H<sub>2</sub>O<sub>2</sub>, leads to decreased maximum fluorescence emission at 336 nm (data not shown). Previously, bovine liver catalase has been shown to increase its activity up to 70 mM H<sub>2</sub>O<sub>2</sub><sup>34</sup>; this in conjunction with our study imply that although catalase activity increases with increasing concentrations of H<sub>2</sub>O<sub>2</sub>, still conformational changes lead to decreased fluorescence at high H<sub>2</sub>O<sub>2</sub> concentrations (1-10 mM). Based on the results of catalase, we explored susceptibility of eight different enzymes to a suitable concentration of H<sub>2</sub>O<sub>2</sub> *i.e.* 1 mM since the lower concentration was not having an easily perceptible effect and higher concentrations could be physiologically irrelevant. The percentage of decrease in the fluorescence emission peak of these enzymes in decreasing order was from AD to HRP (AD > GD > CA > GOx > CAT > HRP). The other two proteins showed an increase in fluorescence peak (G6PDH > GAPDH), however, upon treatment with a higher concentration of H<sub>2</sub>O<sub>2</sub>, GAPDH and G6PDH also showed a decrease in fluorescence (data not shown). Hydrogen peroxide has no fluorescence of its own and the decrease in fluorescence intensity of protein is possibly due to the interaction of the excited state of the fluorophore with its environment *i.e.* H<sub>2</sub>O<sub>2</sub><sup>35</sup>. Henceforth, the quenching of the fluorescence indicates either a change in the tertiary structure of proteins caused by the oxidation of amino acids in the presence of hydrogen peroxide or H<sub>2</sub>O<sub>2</sub> binding to the

proteins leads to direct quenching by H<sub>2</sub>O<sub>2</sub>. This conclusion is also supported by the T<sub>m</sub> results, where we have confirmed that H<sub>2</sub>O<sub>2</sub> treatment caused a decrease in the thermal stability of GD and GAPDH enzymes (Table 2).

Emission peaks ( $\lambda_{em}$ ) of HRP, G6PDH, and GOx did not significantly shift by the addition of 1 mM H<sub>2</sub>O<sub>2</sub> (Table 1) which suggests that the buried hydrophobic residues of these enzymes are not exposed to a polar environment (water) upon incubation with H<sub>2</sub>O<sub>2</sub> and these hydrogen peroxide molecules may be located at close proximity to the chromophores, causing the quenching of fluorescence. However,  $\lambda_{em}$  shifted towards longer wavelengths (1.0-3.0 nm) in case of AD, GAPDH, CA, and catalase (Table 1) suggesting exposure of hydrophobic buried residues to polar-solvent and a subsequent conformational change. It has been reported earlier that GAPDH *in vitro* activity on bolus treatment with H<sub>2</sub>O<sub>2</sub> was decreased in the presence of a higher concentration of H<sub>2</sub>O<sub>2</sub>, which occurs through oxidation of the active site cysteine by forming intramolecular disulfide bonds and cysteic acid<sup>36</sup>. However, continuous treatment with physiologically relevant low concentrations of H<sub>2</sub>O<sub>2</sub> caused no significant changes in three tested yeast GAPDH isoforms<sup>37</sup> which suggested a relatively higher robustness of GAPDH to H<sub>2</sub>O<sub>2</sub>. Noticeable in the current study, we observed similar GAPDH stability outcome to hydrogen peroxide.

Physiological concentrations of H<sub>2</sub>O<sub>2</sub> are low which casts doubt about its direct role in damaging proteins *in vivo*, although the presence of locally high concentrations are reported<sup>27</sup>. Many enzymes are known to be susceptible to H<sub>2</sub>O<sub>2</sub> at low concentrations as far as amino acid modifications are concerned. However, the tertiary structural changes are not observed until high concentrations of H<sub>2</sub>O<sub>2</sub>, *e.g.* the *Pseudozyma antarctica* lipase B showed changes in the tertiary structure after incubation in 2.0 M H<sub>2</sub>O<sub>2</sub> for 3 h at 40°C<sup>38</sup>. Different amino acids in proteins may be susceptible to different levels of oxidation at a particular H<sub>2</sub>O<sub>2</sub> concentration. An earlier report on active site cysteines in yeast AD have shown that cysteine-43 was 78% oxidized as compared to cysteine-153 whereas, later was oxidized only up to 27% at 1:1600 molar ratios of “yeast AD: H<sub>2</sub>O<sub>2</sub>”<sup>39</sup>. Moreover, in cells, other compounds/derivatives produced with H<sub>2</sub>O<sub>2</sub> are possibly more harmful than H<sub>2</sub>O<sub>2</sub> itself *e.g.* AD was inhibited by ferryl derivatives of H<sub>2</sub>O<sub>2</sub> formed with hemoglobin (methemoglobin) at

a concentration where  $H_2O_2$  could not inhibit the enzyme *per se*<sup>40</sup>.

#### Proline protected the enzymes from $H_2O_2$ mediated damage

Both proline and  $H_2O_2$  decreased the fluorescence emission of the most tested enzymes/proteins (Fig. 4 & Table 1). Treatment of proteins with  $H_2O_2$  and proline together is expected to exhibit an additive decrease in the fluorescence. However, five proteins in this study (AD, CA, GD, GOx, and catalase) revealed no decrease or even increase in fluorescence intensity (Table 1) as compared to the fluorescence-decrease by proline alone (Fig. 4) which suggests a protective effect of proline on proteins. Proline is protecting the proteins by reversing the fluorescence decrease by  $H_2O_2$  e.g.  $H_2O_2$  and proline decreased the fluorescence of GD by 9.67% and 25%, respectively. Conversely, if there is no protection by proline, the fluorescence is expected to additively decrease by 34.67% ( $25+9.67$ )<sup>41</sup>, however, due to the protective effect of proline, the fluorescence decreased by only 27.7%. Our Tm data also showed  $H_2O_2$  mediated damage and Proline mediated protection of GD. Protection of catalase have been demonstrated earlier by increasing concentrations of sucrose, mannitol, and betaine, in terms of increased Tm, enzyme activity, and secondary structure<sup>42</sup>. Circular dichroism studies to determine the secondary structure changes in enzymes could not be performed due to very high ellipticity shown by L-proline.

#### Extrinsic Fluorescence

Hydrophobic dye 1-Anilino-8-naphthalene sulfonic acid (ANS) is generally used to probe the exposed hydrophobic surfaces on proteins. We observed a blue-shift in fluorescence peak due to the addition of protein in a protein-free solution of ANS as expected. Among the four dehydrogenases studied for thermal unfolding, ANS fluorescence increased in case of AD, G6PDH and GAPDH (Figs 5 & 6) up to 75°C, 62.5°C and 65°C, respectively, and blue-shifted drastically around their Tm which indicates the hydrophobic residue exposure of proteins due to heating. However, their fluorescence decreased when the temperature was further increased up to 95°C which may be due to the complete disruption of protein structure at a higher temperature since non polar ANS binding regions are not present in completely denatured proteins<sup>43</sup> or due to temperature-induced quenching of fluorescence or as a result of protein aggregation. Contrastingly, there was no blue-shift in the emission peak and a continuous increase in the fluorescence of

GD up to 95°C (Fig. 5) suggesting the partial structural-stability of fluorophore even at a very high temp in GD.

In AD, there was a significant increase in ANS fluorescence upon treatment with  $H_2O_2$  (Fig. 5) however, it increased barely in case of other enzymes which implies comparatively higher exposure of hydrophobic areas in AD. These results correlate with intrinsic fluorescence data showing high susceptibility of AD to  $H_2O_2$ . In contrast to GAPDH where no blue-shift was observed, the AD showed a high blue-shift and increase in ANS fluorescence upon treatment with proline as compared to GD and G6PDH, possibly due to ANS binding to hydrophobic pockets formed by proline assemblies<sup>8</sup>. Proline and arginine, at higher concentration form an ordered supramolecular assembly with distinct solvent-exposed hydrophobic pockets that is conducive for ANS binding and leads to increase in fluorescence<sup>8,18</sup>. Emission maxima of ANS was reported earlier to be blue-shifted by 25 nm from 524 to 499 nm in the presence of proline<sup>8</sup>. We observed the same trend in this study.

Partial reversal of  $H_2O_2$  mediated blue-shifted-peak, and fluorescence increase of ANS in AD, upon addition of proline, suggests the refolding of exposed hydrophobic surfaces in AD to some extent. Since both  $H_2O_2$  and proline, individually increased the ANS fluorescence in GAPDH (Figs 5 & 6), and their concurrent presence did not result in the substantial increase of ANS fluorescence (Fig. 6), it is suggested that GAPDH is also protected from  $H_2O_2$  mediated hydrophobic surface exposure. These results are corroborated with the thermal denaturation wherein an increase of 1.45°C in Tm of GAPDH was obtained by the addition of proline.

Further,  $H_2O_2$  can also cause the damage to proteins by inducing their oligomerization/aggregation. GAPDH is an excellent example of a moonlighting molecule with various oligomerization states. Therefore, we checked the effect of  $H_2O_2$  on GAPDH aggregation with the capability of proline to protect GAPDH from this aggregation.

#### Proline is unable to prevent GAPDH-aggregation upon $H_2O_2$ treatment

GAPDH is a homotetramer composed of four identical subunits with a molecular mass of 36 kD. GAPDH aggregation has been observed in patients of alcoholic liver cirrhosis and necrosis spots *etc.*<sup>44</sup>. Therefore, protein aggregation is believed to stem primarily due to the intermolecular hydrophobic interactions among the folding molecules or by the

formation of disulfide bonds. *In silico* analysis with DiANNA web server found that two surface Cys residues (Cys-245 and Cys-282, Fig. 8) might be involved in Cys-Cys disulfide bond formation by interacting with other chains of proteins during aggregate formation. Apart from disulfide bonds, methionine-46 oxidation was proposed to be involved in GAPDH aggregation<sup>45</sup>. Coincidentally, GAPDH has an oxidation-sensitive SH-group at its active site.

Incubation of GAPDH with 1 mM H<sub>2</sub>O<sub>2</sub> at 37°C resulted in the production and increase in the intensity of a ~30 kD band below the monomeric form (36 kD) of GAPDH with increasing H<sub>2</sub>O<sub>2</sub> concentration from 5 µM to 5 mM or 1 to 20 mM (Fig. 7A & B). This lower band disappeared upon treatment with 100 mM DTT, indicating the oxidation or formation of disulfide (-S-S-) bonds in ~30 kD band formation. Similarly, incubation of GAPDH in higher H<sub>2</sub>O<sub>2</sub> concentrations (1-20 mM) resulted in high molecular weight aggregates formation (Fig. 7A), which were also reversed by 100 mM DTT (Fig. 7A) suggesting the oxidation or presence of disulfide bonds in aggregate formation. They might also be resulting from the Cys-S-OH modification by H<sub>2</sub>O<sub>2</sub> which is reversed by DTT. The diminution of 36 kD GAPDH monomer with increasing aggregates confirms that the aggregates are formed from monomers. The inability of lower concentrations of H<sub>2</sub>O<sub>2</sub> (5 and 10 µM) to form high MW aggregates suggest that a very high concentration of H<sub>2</sub>O<sub>2</sub> accumulated in the tissues can only cause GAPDH-aggregate formation. GAPDH aggregation by H<sub>2</sub>O<sub>2</sub> to form high molecular weight products have been reported earlier<sup>7</sup>. Similar to our result, Nakajima *et al.* (2007)<sup>45</sup> detected the formation of GAPDH aggregates (dimers, trimers, tetramers, and multimers) by treatment with different nitrosylating/oxidizing agents [(±)-(E)-4-ethyl-2-[(E)-hydroxyimino]-5-nitro-3-hexenamide, S-nitrosoglutathione, S-nitroso-N-acetyl-DL-penicillamine], as well as with H<sub>2</sub>O<sub>2</sub>, and re-formation of monomers in the presence of 100 mM DTT<sup>45</sup>. On the other side proline is known to prevent protein aggregate formation. In one study, proline blocked the early stages of aggregate formation when aggregate sizes were modest, however, it could not prevent once larger aggregates were formed<sup>46</sup>. Although prevention of aggregation of catalase induced by incubation at 55°C for 70 min by 250 mM of proline has been reported<sup>15</sup>, contrastingly, our results revealed that 1.0 M proline could not prevent

H<sub>2</sub>O<sub>2</sub> induced degradation and aggregate formation of GAPDH. The GAPDH protein abundance is significantly high in plant and animal cells. Separate mechanisms exist for its active site thiolate reactions with H<sub>2</sub>O<sub>2</sub>, and glycolytic activity<sup>47</sup>. Based on its very high active-site-cysteine's sensitivity to the H<sub>2</sub>O<sub>2</sub> mediated reversible oxidation and subsequent S-glutathionylation, along with its high abundance, Peralta *et al.* (2015)<sup>47</sup> proposed that GAPDH act as a sensor and scavenger of H<sub>2</sub>O<sub>2</sub> till its reactivation by NADPH-dependent reducing system, upon arrival of suitable conditions. The GAPDH and AD both are shown to increase their gene expression and protein abundance under stress conditions<sup>7,48</sup>. In addition, the inhibition of the glycolytic pathway mediated by oxidation of GAPDH in presence of H<sub>2</sub>O<sub>2</sub> has been observed in plants under different stresses. This might increase the flux of glucose through oxidative pentose phosphate pathway (OPPP) which is used to increase the cytoplasmic NADPH/NADP<sup>+</sup> ratio<sup>48</sup> that is beneficial for cells. Likewise, proline, which is also accumulated under stress conditions, will be beneficial for cells by protecting these stress-accumulating enzymes. Further investigations are needed to determine if there are any clear differences in proteins accumulating in normal and stress conditions *vis-à-vis* the protective ability of proline.

## Conclusion

Our data suggest differential responses of H<sub>2</sub>O<sub>2</sub> and proline in terms of intrinsic, extrinsic fluorescence, and T<sub>m</sub> (GAPDH and GD) of different proteins. AD and GD were found to show prominent reduction in flu peak by H<sub>2</sub>O<sub>2</sub> as compared to G6PDH and GAPDH. This indicated H<sub>2</sub>O<sub>2</sub> mediated conformational change and proline partially reversed changes in these enzymes suggesting protection. Although only fluorescence-based results cannot definitely claim damage or protection, the reversed direction of fluorescence change indicates the protection by proline. Further studies *e.g.* enzyme assays need to be carried out to confirm these results. In terms of T<sub>m</sub>, proline caused stabilization of GD and GAPDH and partially reversed the decrease in T<sub>m</sub> caused by H<sub>2</sub>O<sub>2</sub>. Different proteins exhibited different responses in the presence of proline, making them differentially protected from H<sub>2</sub>O<sub>2</sub> mediated damage. These specific effects of proline may have major repercussions as to which pathways in the cells/organisms are favored and which pathways are inhibited during stress, giving them better ability to adapt to stress conditions.

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### Conflict of interest

All authors declare no conflict of interest.

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