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IN-SILICO STUDIES FOR COMPARATIVE ANALYSIS OF GLUTATHIONE PEROXIDAES ISOZYMES IN *HOMO SAPIENS*

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ABSTRACT

Glutathione peroxidase (GPx) is a family of protein, which is responsible for eradication of various reactive molecules like H_2O_2 and other peroxidase helps in protection from oxidative degeneration. In humans, there are 8 different isozymes of GPx families GPx1-GPx8 are isolated from various tissues. The sequence and structure based analysis of these isozymes are studied to find differences among them. Here sequence analysis is carried by MEGA6.06 software where MUSCLE an iterative alignment program was used to find multiple sequence alignment, which represents 32 conserved sites, evolutionary distances, mutation rates based on 154 sites without gap and missing residues. Structural comparison done by SPDBV based Magicfit tool to carry out carbon alpha and all atoms based structural superimposition. The results obtained by Sequence and Structure based comparison insights that there are three groups in isozymes based on evolution Group 1: GPx7, GPx8 and GPx4, Group 2: GPx1 and GPx2 , Group 3: GPx3, GPx5, and GPx6.

KEYWORDS : Conserved Sites, Superimposition, Iterative Alignment, Evolutionary Distances, Multiple Sequence Alignment

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INTRODUCTION

Glutathione peroxidase (GPx) protein is a family of multiple isozymes functionally responsible for Protection of hemoglobin in erythrocytes from oxidative breakdown in humans. These isozymes also catalyzes the reduction of H_2O_2 or organic hydroperoxides (exogenous as well as endogenous) to water or corresponding alcohols by reducing glutathione which works as an electron donor. Apart form this if the gene ontology of this class of enzymes are studied it is observed that this enzyme having wide range of activity like, UV protection activity [1], heart contraction [2], regulation of apoptotic genes like Bax and Bcl-2 [3]. In a study on Metazoan reveals that some GPxs have a selenium-dependent glutathione peroxidase activity, with selenocysteine being encoded by an opal codon TGA. In mammalian tissues, there are main eight selenium dependent GPx isozymes: (a) classical GPx (GPx1), which is found in red blood cells, liver, lung and kidney; (b) GPx (GPx2) in gastrointestinal cells; (c) GPx (GPx3) in plasma, which is present in different organs such as kidney, lung, epididymus, vas deferens, placenta, seminal vesicle, heart and muscle, (d) in phospholipid GPx (PHGPx4 or GPx4), (e) GPx5 present at epididymis in the mammalian male reproductive tract, (f) GPx6 adult olfactory epithelium, (g) GPx7 which is also broadly distributed in different tissues. GPx1, 2 and 3 act as homotetramers, whereas GPx4 is functional as a monomer [4]. Various aerobic reactions at cellular level lead to the accumulation of reactive oxygen species, which can be toxic to the cells. Biotic and abiotic stresses can trigger a dramatic increase in the generation of reactive oxygen species such as super- oxide radicals, hydroxyl radicals and hydrogen peroxide in the intracellular environment. During Evolution, aerobic organisms have developed various non-enzymatic and enzymatic activities in their systems to neutralize these compounds [5]. The enzymatic systems include a set of gene products such as superoxide dismutases, catalases, ascorbate peroxidases and glutathione peroxidases (GPx) [6]. It is observed that GPx enzyme activities in humans are reduced with age [7]. Apart from aging studies reveals that with increase in age there is a high risk of having cardiac dysfunction due to reduced activity of these enzymes [8]. Another study represents that due to reduced activity of GPx with aging the availability of hydrogen peroxide is higher in cells which results to hyperactivation of Platelets leading to thrombus formation [9]. These are very import enzymes and their malfunction or reduced activity is related to various with diseases with aging. In this study of evolution of GPx proteins in human the main objective is to find the various site that are evolutionary conserved and sites which are more prone to mutation. This will help in finding how these proteins are maintaining their conserved sites to remain functionally active as well as in the study of patterns in mutations occurred during the course of evolution.

MATERIALS AND METHODS

In this comparative study of Glutathione peroxidase protein family involves sequence data collection, structure data collection, sequence comparison, structure validation, structure prediction, structure comparison.

1. ***Sequence data collection***

All type Human glutathione peroxidase protein sequences are taken from UniProtKB database the id of the sequences are, GPx1: P07203 (203 AA), GPx2: P18283 (190 AA), GPx3: P22352 (226 AA), GPx4: P36969 (197 AA), GPx5: O75715 (221 AA), GPx6: P59796 (221 AA), GPx7: Q96SL4 (187 AA), GPx8: Q8TED1 (209 AA).

2. *Sequence comparison and Phylogenetics Tree Construction*

Sequence comparison is carried by using MEGA6.06 software, here MUSCLE[10] program is used to find multiple sequence alignment with Gap Open penalties:-2.9, Maximum Iteration: 8, Clustering method is UPGMA. Dayhoff model was used to find the rate of mutation in the entire conserved site of GPX family proteins. Jones-Taylor-Thornton (JTT) model was use find the distances between the sequences.

3. *Structure data collection*

All the structures except GPX6 are obtained from Protein Data Bank GPX1: 2F8A, GPX2: 2HE3, GPX3: 2R37, GPX4: 2GS3, GPX5: 2I3Y, GPX6, GPX7: 2P31, GPX8: 3CYN. Structure of GPX6 is not available in Protein Data Bank so its structure is modeled by homology modeling method using Schrodinger software and structure is also evaluated for its validation on various parameters.

3.1. Structure modeling and Evaluation of Glutathione peroxidase 6 (GPX6) Prime tool of Schrodinger software version 2013 is used for protein structure prediction. Here in GPX6 sequence selenocysteine is available, which causes problem in structure prediction we had modified selenocysteine to cysteine to make it acceptable to Prime Tool. GPX6 structure is also modeled by Modeller9.13[11] but on structure evaluation it is found that it Modeller 9.13 based[23], modeled structure is not well defined and failed on ERRAT2 Plot. ERRAT is a protein structure verification algorithm that is especially well-suited for evaluating the progress of crystallographic model building and refinement. The program works by analyzing the statistics of non-bonded interactions between different atom types. A single output plot is produced that gives the value of the error function vs. position of a 9-residue sliding window. By comparison with statistics from highly refined structures, the error values have been calibrated to give confidence limits.[12] Ramachandran Plot is used to find the compactness of the secondary structures.

4. ***Software used for Structure Comparison***

Structure comparison is carried by Iterative magic fit tool of SPDBV software[13, 22] available at (http://spdbv.vital-it.ch/download/binaries/SPDBV_4.10_PC.zip). This is to carry out the structure superimposition between various GPX proteins. There are two main category of atoms are taken under consideration while comparing structures one is Carbon Alpha and All atoms. Then root-mean-square deviation (RMSD) is taken for both the parameters. The RMSD is the measure of the average distance between the atoms (usually the backbone atoms) of superimposed proteins. In the study of globular protein conformations, one customarily measures the similarity in three-dimensional structure by the RMSD of the Cα atomic coordinates after optimal rigid body superposition.

$$\mathrm{RMSD} = \sqrt{\frac{1}{N}\sum_{i=1}^{N}\delta_i^2}$$

where δ is the distance between N pairs of equivalent atoms. [14]

RESULTS

The analyzed results are classified under two sections a.) Comparative Sequence analysis where only sequences are aligned and tested on various evolutionary models to find the differences between GPX proteins b.) Comparative Structure Analysis is to find the differences in the structural level by superimposing various pairs of GPX proteins.

3.1 Comparative Sequence analysis

Multiple Sequence Alignment carried out by MUSCLE tool of MEGA6.06

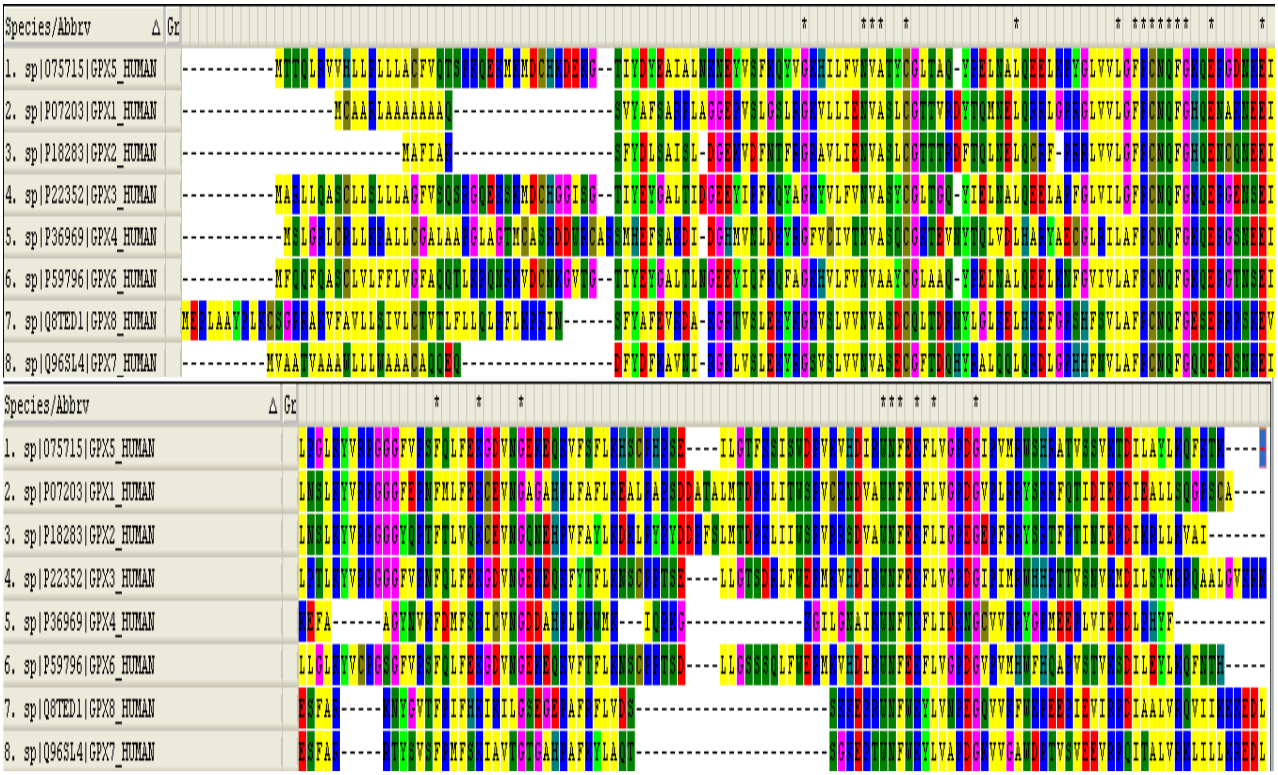

Figure 1

MUSCLE tool based multiple sequence alignment of GPx1-GPx8

Sequence comparison results shows that sequences are conserved at 70, 77, 78, 79, 82, 95, 107, 109-115, 118, 124, 129-132, 142, 147, 152, 172, 185, 187,195-197, 199, 201, 206 positions in the alignment file in various Isoforms of GPX proteins. These are the positions where all isoforms of GPX are identical, representing that these are most valuable sites for GPX functions. While there are a number of mutable sites which may undergo changes during the course of evolution.

JTT model based distances Matrix of Sequences

	1	2	3	4	5	6	7	8
1. sp|O75715|GPX5 HUMAN								
2. sp|P07203|GPX1 HUMAN	0.927							
3. sp|P18283|GPX2 HUMAN	0.997	0.431						
4. sp|P22352|GPX3 HUMAN	0.278	0.943	1.082					
5. sp|P36969|GPX4 HUMAN	1.616	1.270	1.372	1.637				
6. sp|P59796|GPX6 HUMAN	0.286	1.048	1.120	0.285	1.672			
7. sp|Q8TED1|GPX8 HUMAN	1.492	1.481	1.578	1.695	1.381	1.701		
8. sp|Q96SL4|GPX7 HUMAN	1.281	1.230	1.405	1.495	1.226	1.525	0.700	

Figure 2

Distance matrix generated by MEGA6.06 software.

JTT model of evolution reveals that which pair is having closer evolutionary distance and which pairs are distantly related. The pair in the matrix having the highest values of distances is representing greater evolutionary distance smaller the value of distance shows that pair is having least distances. The over all distance among all GPX family protein is 1.184.

Tree generated by different model of phylogenetics.

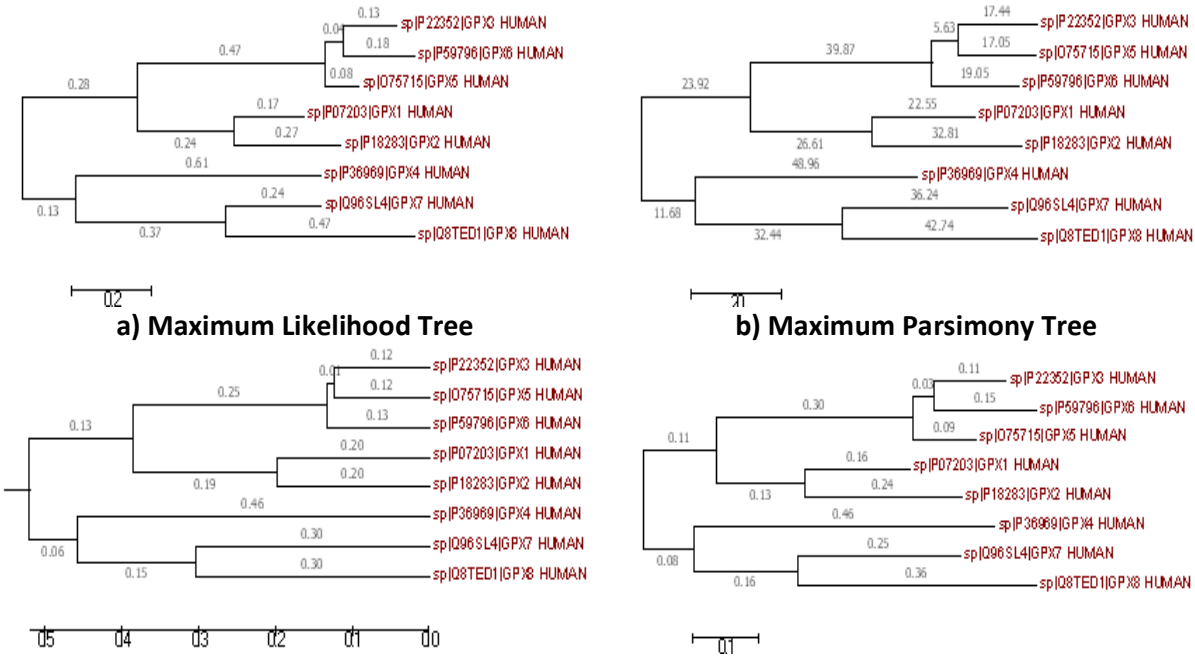

Figure3

Phylogenetic Comparision of Sequences on Various Methods

(a) Maximum Likelihood Tree[15], (b) Minimum Evolution Tree[16], (c) Maximum Parsimony Tree[17] and (d) Neighbor Joining Method Tree[18])

All the models for phylogenetic analysis are representing almost same results of evoltuion where three main groups of GPX family proteins are classified Group 1: GPX7, GPX8 and GPX4, Group 2: GPX1 and GPX 2 , Group 3: GPX3, GPX5, and GPX6. Which represent that these groups are having close diatnces.

Mutation Rates in all 154 sites (excepst Gaps and Missing data) of Gpx1-GPx8

From \To	A	R	N	D	C	Q	E	G	H	I	L	K	M	F	P	S	T	W	Y	V
A	-	0.06	0.20	0.29	0.06	0.17	0.50	1.08	0.04	0.12	0.18	0.11	0.05	0.04	0.65	1.44	1.10	0.00	0.04	0.68
R	0.12	-	0.07	0.00	0.04	0.48	0.00	0.04	0.41	0.12	0.07	1.90	0.07	0.03	0.27	0.55	0.08	0.11	0.01	0.08
N	0.44	0.07	-	2.15	0.00	0.20	0.38	0.63	0.91	0.15	0.15	1.30	0.00	0.03	0.11	1.74	0.68	0.01	0.15	0.05
D	0.53	0.00	1.85	-	0.00	0.26	2.89	0.56	0.15	0.05	0.00	0.30	0.00	0.00	0.03	0.34	0.20	0.00	0.00	0.06
C	0.16	0.05	0.00	0.00	-	0.00	0.00	0.05	0.05	0.08	0.00	0.00	0.00	0.00	0.05	0.57	0.05	0.00	0.15	0.16
Q	0.40	0.51	0.21	0.32	0.00	-	1.79	0.13	1.03	0.03	0.32	0.63	0.09	0.00	0.40	0.20	0.16	0.00	0.00	0.11
E	0.88	0.00	0.31	2.73	0.00	1.39	-	0.37	0.08	0.12	0.05	0.34	0.02	0.00	0.13	0.28	0.10	0.00	0.03	0.12
G	1.06	0.02	0.29	0.30	0.02	0.06	0.21	-	0.02	0.00	0.03	0.11	0.01	0.03	0.09	0.83	0.09	0.00	0.00	0.18
H	0.10	0.50	1.10	0.21	0.05	1.18	0.11	0.05	-	0.01	0.19	0.11	0.00	0.10	0.24	0.13	0.07	0.01	0.19	0.15
I	0.29	0.13	0.16	0.06	0.08	0.04	0.15	0.00	0.01	-	1.12	0.19	0.25	0.40	0.03	0.09	0.57	0.00	0.06	2.92
L	0.18	0.03	0.07	0.00	0.00	0.14	0.03	0.03	0.08	0.48	-	0.07	0.39	0.32	0.08	0.06	0.10	0.02	0.04	0.58
K	0.12	0.97	0.65	0.17	0.00	0.30	0.21	0.12	0.05	0.09	0.08	-	0.18	0.00	0.09	0.34	0.40	0.00	0.02	0.03
M	0.32	0.19	0.00	0.00	0.00	0.22	0.08	0.08	0.00	0.63	2.28	0.99	-	0.19	0.04	0.22	0.31	0.00	0.00	0.85
F	0.08	0.03	0.03	0.00	0.00	0.00	0.00	0.07	0.08	0.37	0.68	0.00	0.07	-	0.03	0.16	0.04	0.04	1.06	0.04
P	1.11	0.22	0.09	0.03	0.03	0.30	0.13	0.16	0.16	0.02	0.14	0.14	0.01	0.02	-	0.86	0.23	0.00	0.00	0.16
S	1.81	0.32	1.01	0.23	0.27	0.11	0.20	1.05	0.06	0.05	0.07	0.39	0.05	0.09	0.63	-	1.63	0.04	0.05	0.10
T	1.64	0.06	0.47	0.16	0.03	0.10	0.09	0.14	0.04	0.36	0.14	0.56	0.08	0.03	0.20	1.94	-	0.00	0.06	0.52
W	0.00	0.42	0.05	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.20	0.00	0.00	0.16	0.00	0.26	0.00	-	0.09	0.00
Y	0.11	0.02	0.20	0.00	0.16	0.00	0.05	0.00	0.22	0.07	0.13	0.05	0.00	1.42	0.00	0.12	0.13	0.03	-	0.09
V	0.92	0.05	0.03	0.04	0.08	0.07	0.09	0.24	0.08	1.66	0.76	0.04	0.19	0.03	0.13	0.11	0.47	0.00	0.04	-

Table 1

Mutation probabilities of amino acids from one amino acid to other in all 154 sites

Each entry is the probability of substitution (r) from one amino acid (row) to another (column). Substitution pattern and rates were estimated under the Dayhoff (1978) model 9[16]. Relative values of instantaneous r should be considered when evaluating them. For simplicity, sum of r values is made equal to 100, The amino acid frequencies are 8.71% (A), 4.09% (R), 4.04% (N), 4.69% (D), 3.35% (C), 3.83% (Q), 4.95% (E), 8.86% (G), 3.36% (H), 3.69% (I), 8.54% (L), 8.05% (K), 1.48% (M), 3.98% (F), 5.07% (P), 6.96% (S), 5.85% (T), 1.05% (W), 2.99% (Y), and 6.47% (V). For estimating ML values, a tree topology was automatically computed. The maximum Log likelihood for this computation was -2186.979. The analysis involved 8 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 154 positions in the final dataset. Evolutionary analyses were conducted in MEGA 6.06 [20].

3.2 Comparative Structure Analysis

This study is based on the fact of superimposition where the structures of similar working GPx family are studied. Here the structures are analyzed how they get are structurally alike. Here all the all structures are downloaded from PDB except GPx6 whose structure is not yet modeled. GPx6 structure was modeled, refined and evaluated on various structure parameters to make it correct according to standards. Structure comparison is carried out by superimposition of carbon alpha, to compare the backbone of various isoforms, and all atoms of the protein isoforms.

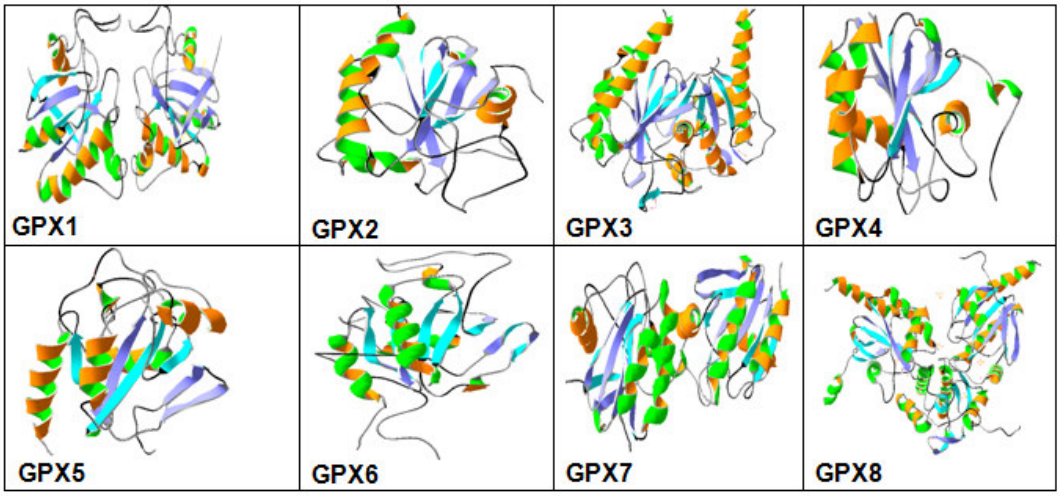

Figure 4
3 D structures GPX family Protein. Here GPX6 modeled by Prime tool of Schrodinger

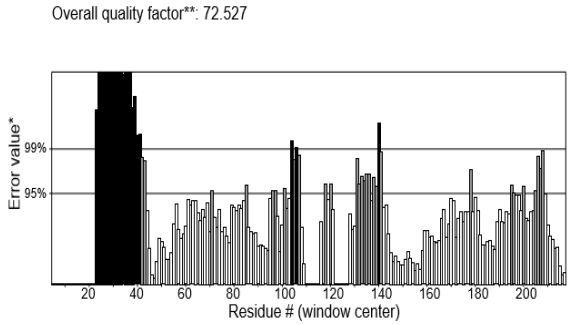

Figure 5: ERRAT Quality Factor for Modeller9.13 based GPX6 is 72.527

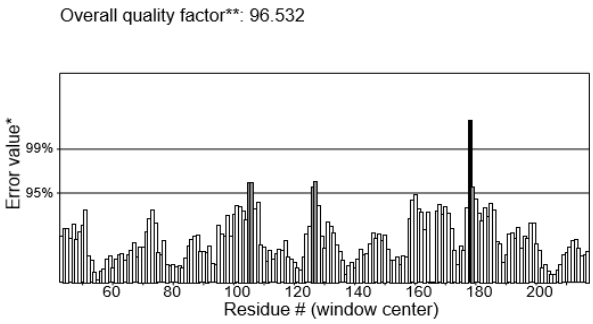

Figure 6: ERRAT Quality Factor for Prime tool of Schrodinger based Model of GPX6 is 96.532

Before going for comparison the GPx6 structure was modeled by two modeling software Modeller 9.13 (Freeware version)[23] and Schrodinger 2013 (Commercial version). In the evaluation of both modeled structure it was found that the structure generated by the Schrodinger 2013 (Commercial version) is most accurate after structural refinement and having ERRAT score of 96.532 while Modeller 9.13 based model after 64 energy minimization steps in SPDBV is have increased to a ERRAT score of 72.527 from 54.497 which is very less as compared Schrodinger 2013 based model. Model generated by Schrodinger 2013 was taken for structure comparison.

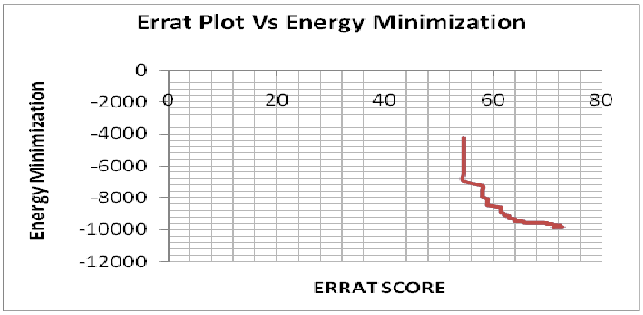

Figure 7
ERRAT plot with respect to change in energy for Modeller9.13 based structure.

To improve the Quality of Modeller 9.13 based modeled structure[24] is processed through energy minimization of tool of SPDBV. The graph represents that ERRAT score increases as the number of times energy is minimized.

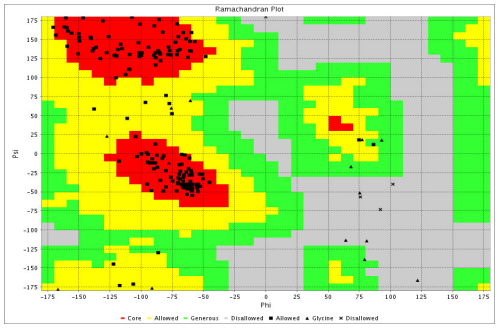

Figure 8
Ramachandran Plot of Schrodinger modeled structure.

Ramachandran Plot plotted by VADAR server[24] based in methodology of Procheck [16] software for GPX6 modeled by Schrodinger. Total 97 % of amino acids are in core and allowed region. While modeled by Modeller 9.13 is having 76% of amino acids in the core region.

	GPx-1	GPx-2	GPx-3	GPx-4	GPx-5	GPx-6	GPx-7	GPx-8
GPx-1	370	179	171	147	167	74	144	61
GPx-2	179	185	163	143	168	116	141	134
GPx-3	171	164	379	144	174	106	140	134
GPx-4	147	143	142	171	141	120	151	146
GPx-5	167	162	176	141	140	54	140	132
GPx-6	74	116	106	121	53	221	118	118
GPx-7	144	141	141	150	139	118	310	155
GPx-8	60	134	134	147	133	121	155	523

Table 2
Number of Carbon Alpha involved in structure comparison

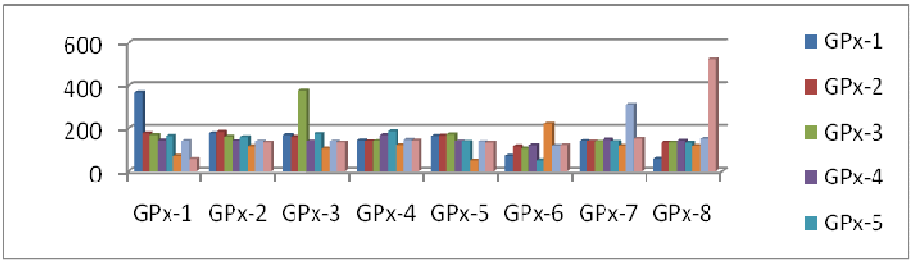

Figure 9
Graphical representation of Number of Carbon Alpha involved in structure comparison

	GPx-1	GPx-2	GPx-3	GPx-4	GPx-5	GPx-6	GPx-7	GPx-8
GPx-1	0	0.64	0.92	0.76	0.9	2	0.92	1.75
GPx-2	0.64	0	1.02	0.98	1.19	1.46	1.11	1.1
GPx-3	0.92	1.02	0	0.97	0.67	1.23	1.02	1.13
GPx-4	0.76	0.98	0.92	0	0.95	1.22	0.91	0.92
GPx-5	0.92	0.96	0	0.95	0	2.18	1.05	1.09
GPx-6	2	1.46	1.23	1.23	2.18	0	1.35	1.35
GPx-7	0.92	1.11	1.02	0.86	1.03	1.35	0	0.82
GPx-8	1.8	1.15	1.13	0.92	1.12	1.21	0.82	0

Table 3
RMSD in Å of Carbon Alpha Atoms after structural superimposing

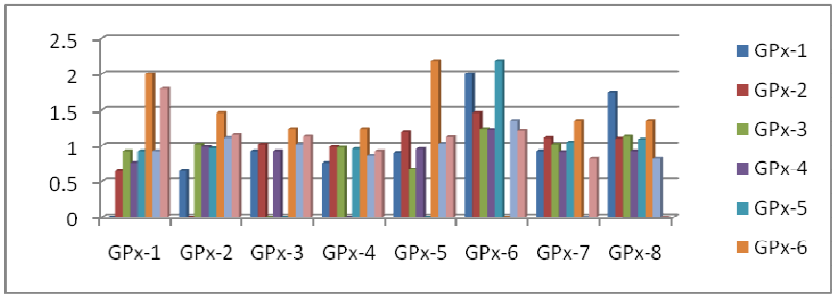

Figure10

Graphical representation of RMSD in Å of Carbon Alpha Atoms after structural superimposing

On Structural superimposition of Carbon Alpha atoms it is representing the same results as in case of sequence alignment. It is observed that all GPX are grouped in the same pattern as Group 1: GPX7, GPX8 and GPX4, Group 2: GPX1 and GPX 2 , Group 3: GPX3, GPX5, and GPX6. Where GPX6 having higher value of deviations as comapared to other pattern this may be due to the modeled structure. To verify the results we have gone through the comparison of all atoms are also implemented.

	GPX1	GPX2	GPX3	GPX4	GPX5	GPX6	GPX7	GPX8
GPX1	1480	716	684	588	668	468	576	248
GPX2	716	740	652	572	652	460	564	536
GPX3	684	656	1516	576	696	424	560	536
GPX4	588	572	568	684	564	488	604	584
GPX5	688	648	704	560	752	216	560	536
GPX6	468	460	424	488	212	884	472	472
GPX7	576	564	564	600	556	472	1240	612
GPX8	248	536	536	588	528	484	612	2092

Table 4

Number of all atoms involved in structure comparison

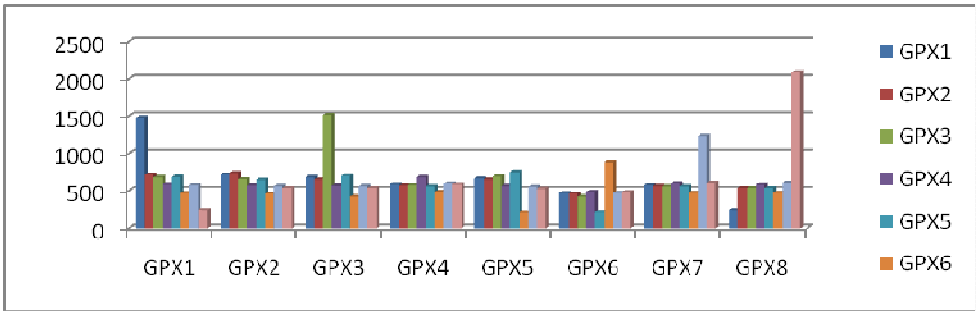

Figure 11

Graphical representation Number of all atoms involved in structure comparison

	GPX1	GPX2	GPX3	GPX4	GPX5	GPX6	GPX7	GPX8
GPX1	0.00	0.65	0.91	0.84	0.89	1.35	0.94	1.81
GPX2	0.65	0.00	1.03	1.00	1.11	1.50	1.10	1.11
GPX3	0.91	1.03	0.00	0.98	0.68	1.27	1.00	1.14
GPX4	0.84	1.00	0.92	0.00	0.97	1.30	0.91	0.91
GPX5	0.90	0.95	0.72	0.96	0.00	2.20	1.04	1.15
GPX6	1.35	1.50	1.27	1.30	2.20	0.00	1.37	1.28
GPX7	0.94	1.10	1.00	0.86	1.01	1.37	0.00	0.78
GPX8	1.82	1.14	1.14	0.95	1.15	1.24	0.78	0.00

Table5

RMSD in Å Matrix of superimposition of all Atoms

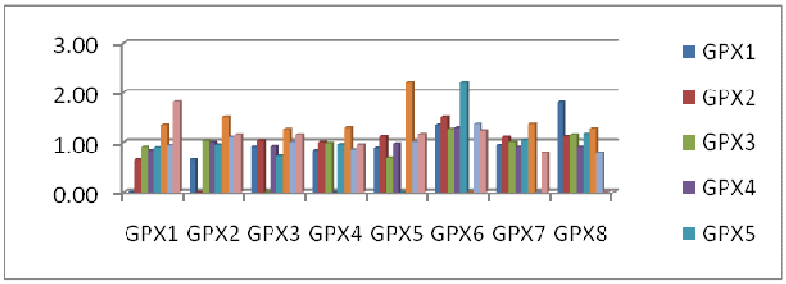

Figure 12
Graphical representation of RMSD in Å for superimposition of all Atoms

By structural superimposition of all atoms it is found that there is a deviation in RMSD values and groups the differences in results is due to involvement of side chain atoms and but even though the RMSD shows similarities with the Sequence alignment and superimposition of Carbon Alpha atoms.

DISCUSSION

The GPx family of proteins is very important in maintaining the free radicals in the body because of its peroxidase activity hence there is a great significance in comparing the sequence and structure to get the knowledge of evolutionary patterns for these proteins. On thorough analysis of the GPX family protein sequences on various Sequence analysis models by using MEGA6.06 there are total 32 positions are found conserved during the evolution hence these sites are very important for functional activity of this family of proteins. Various evolutionary models have shown that there are three major groups are available Group1: GPx7, GPx8 and GPx4, Group 2: GPx1 and GPx2 , Group 3: GPx3, GPx5, and GPx6 these goups are also tested on struture comaprison using carbon alpha atoms and found similar results. This study shows that these gropus are conserved at sequence as well at structural level. The modeled structure generated for GPx6 by Schrodinger is very much similar to other GPx proteins and also being tested on various parameters like ERRAT plot and Ramachandran Plot. This study represents that modeled structures when undergo energy minimization the ERRAT score get increased significantly but after a certain number of minimization steps its score get decreased. During comparison of carbon alpha atoms, it is observed that the backbone of all the GPx is very much similar but when we go for comprision of all the atoms in superimposition then there are lot of differnces are observed which represents even though the backbone is similar the packing of the stucture play a important role in the functioning of GPx activity. The mutation probabilites of each amino acid were calcualte in mutataion matrix. There are total 154 sites are observed which are considered for finding mutaion rate of amino acids where all gaps and missing data are eliminated.

CONCLUSION

In this study of the GPx family protein role of 32 conserved positions is represented which are very important for their function. These sites are contributing to sequence similarity and also to functional similarity. The mutation probabilities of the sequences represent which amino acid is more likely to get mutated in the GPx protein modification. This can be used in protein engineering to modulate the protein by maintaining the function of GPx i.e. for site directed mutagenesis where their activities get enhanced by the mutation. This is an important class of protein help in eradication of free radicals from Human body and preventing various diseases, thus enhance activity can help in the treatment of various free radical related problem like cancer. In further studies of GPx isozymes the docking pattern of various molecules on these proteins will be carried out to find interactions.

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