Computational Modelling of *Pisum Sativum* L. Superoxide Dismutase and Prediction of Mutational Variations through *in silico* Methods

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Abstract: *Superoxide dismutase (SOD) is one of the major enzymes expressed in the oxidative stress pathway in plants. Its expression is also evident in other taxonomic group in oxidative reactions. Pisum sativum a common plant is being studied in the present work where SOD is characterized using computational tools. SOD sequence of P. sativum [CAA42737.1] Ala and Leu rich protein with alkaline pI value was used as query sequence and used to obtain nine similar sequences through BLASTp. Phylogenetic tree was constructed using MEGA 5.0 based on neighbour joining method. Physiochemical parameters and amino acid composition was studied and compared with query sequences and other similar sequences. Secondary structures were predicted to understand the dominant components. Homology modeling of P. sativum SOD was done using SWISS MODEL and quality was evaluated using standard methods. 27 active sites were detected in SOD predicted model which were Lys rich.*

Keywords: *BLASTp, Neighbour joining tree, Ramachandran plot, SOPMA, SWISS MODEL.*

Introduction

Oxidative stress is a major biotic stress seen in plant as well as animal cells which hampers its productivity. Higher plants employ defense strategies to cope up with various environmental stresses [21, 27]. Reactive oxygen species (ROS) are produced in both stressed and unstressed cells [1]. The univalent reduction of di-oxygen occurs in almost all aerobic cells [6, 21]. Free radicals are the chemicals which have an uncoupled electron and are capable of reacting rapidly [2]. The first product is superoxide radical anion, O_2^-. Subsequently, other toxic chemical entities such as H_2O_2 and hydroxyl radicals, OH^-, are formed. Superoxide radicals resulting in formation of H_2O_2 formation are detoxified by superoxide dismutase (SOD) [1, 21]. Within a cell, the SOD constitutes the first line of defense against ROS. Apart from SOD, plant cells equip some other major oxygen radical detoxifying enzymes such as ascorbate peroxidase (APX), glutathione reductase (GR) and catalase (CAT) [1, 21].

SODs are found in different sub-cellular locations. Based on the co-factor used by the enzyme, they are classified into three groups namely Fe-SOD, Mn-SOD and Cu-Zn-SOD. Mn-SOD occurs in mitochondria and peroxisomes. It carries a metal atom per subunit. Even though Mn- and Fe-SODs have similar primary, secondary and tertiary structure, these enzymes have diverged sufficiently that Fe (II) could not restore the function of Mn-SOD and vice versa [13]. Mn-SOD is either a hetero-dimeric or a homo-dimeric structure with one Mn (III) atom per subunit. It is not deactivated by hydrogen peroxide and is present in both prokaryotes as well as eukaryotes [1]. Once the superoxide radicals are formed, Mn-SOD dismutates it to H_2O_2 due to its enzyme activity [24].

Since these enzymes are playing a major role in oxidative stress control in plants needs to be characterized well. Apart from oxidative stress, expression of SOD is also reported in case of drought and metal toxicity also [23, 25]. There are many studies focused on the structural and functional characterization of SODs of plants. But, not many reports are available on application of computational tools for its characterization. The present work focuses on the computational characterization and structure evaluation of *P. sativum* SOD which could contribute in engineering of a plant with resistance under various stress environments especially in oxidative stress. As the stress tolerant plants are the need of the hour for increased productivity and better quality, its creation is only possible through better understanding of such stress related enzymes and the mechanisms involved in it.

Materials and methods

Sequence retrieval and BLASTp

Superoxide dismutase protein sequence of *P. sativum* [*CAA42737.1*] was retrieved from NCBI databank and BLASTp was performed to retrieve nine similar sequences of different origins. The retrieved sequences are:

- AAF50095.1 (*Drosophila melanogaster*),
- AAA29934.1 (*Schistosoma mansoni)*,
- CAA43859.1 (*Chymomyza amoena*),
- YP_001176531.1 (*Enterobacter sp. 638*),
- BAA02655.1 (*Thermus aquaticus*),
- YP_004787678.1 (*Muricauda ruestringensis*),
- YP_004272108.1 (*Planctomyces brasiliensis DSM 5305*),
- ADY29074.1 (*Cellulophaga lytica DSM 7489*),
- ADV48601.1 (*Cellulophaga algicola DSM 14237*).

Sequences obtained from BLASTp were aligned using CLUSTAL W multiple sequence alignment in BioEdit 5.0 [17]. Neighbour joining tree was constructed for the same using MEGA 5.0 software [29, 37].

Physicochemical characterization

SOD sequences were subjected to ProtParam analysis to obtain molecular weight, pI, aliphatic index, GRAVY and Instability index (II) of all sequences [15]. Percentage of amino acid occurrence was compared for these sequences. Transmembrane probability for SOD of *P. sativum* was analysed using TmPred and DAS TM segment prediction server.

Secondary structure prediction and homology modeling

Secondary structure of SODs was analyzed using SOPMA analysis [16] and the homology modeling of SOD of *P. sativum* was done on SWISS MODEL based on the template 3dc6C

[3, 22, 28]. Ramachandran plot was constructed using RAMPAGE software and quality of the model was predicted using VMD software [18]. PROSA analysis was also performed for the same. Using Swiss pdb Viewer, electron density map for the SOD was made.

Active site prediction and sub cellular localization

Active sites of SOD of *P. sativum* were predicted using automated active site prediction server of SCFBio. Amino acid composition of each cavity was studied and tabulated. As SODs are classified based on co-factors and are distributed to different sub-cellular locations. Target P analysis was performed to classify the retrieved sequences and classify them based on their possible locations.

Results and discussion

Phylogenetic analysis

Ten similar sequences were retrieved after performing BLASTp and CLUSTAL W multiple sequence alignment was done using BioEdit software. Phylogenetic tree was constructed based on *Thermus aquaticus* [*BAA02655.1*] SOD sequence as root using Neighbour joining method with boot strap of 1000 replications (Fig. 1). *P. sativum* SOD was placed as distinct branch with zero boot strap value showing least divergence from the root. SOD of *Enterobacter sp.* and *Drosophila melanogaster* exhibited highest divergence supported by boot strap value of 100.

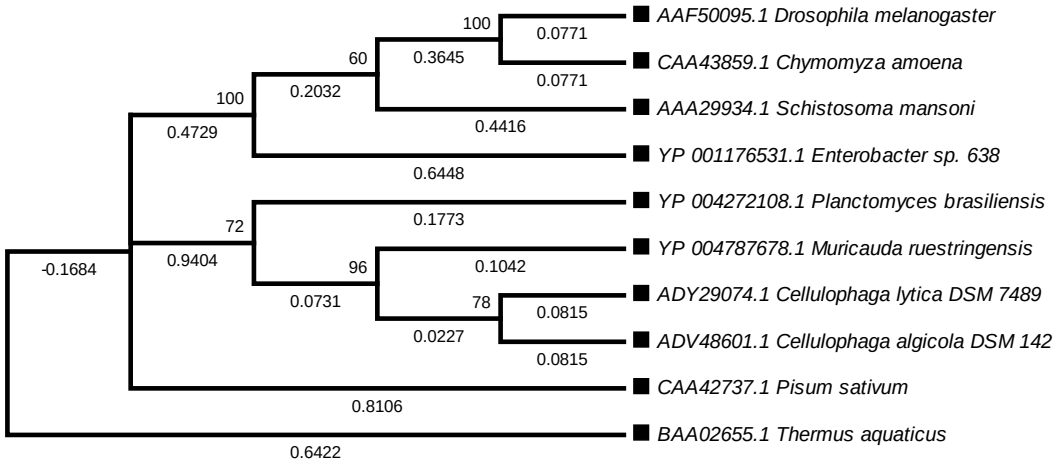


Fig. 1 Neighbour joining tree showing evolutionary relationship between various SOD sequences

Physiochemical characterization

Various physiochemical parameters like molecular weight, theoretical pI, aliphatic index, instability index, and GRAVY were predicted using ProtParam analysis in ExPASy server (Table 1). It was notable that SOD exhibited an alkaline pI which ranged from 7.72 to 9.73. *Cellulophaga lytica* SOD [ADY29074.1] showed a pI value of 6.57 which was acidic. A higher aliphatic index indicated its stability in wide range of temperatures. GRAVY value of *P. sativum* SOD was the least which ensures better interaction with water molecules. SODs of *P. sativum*, *D. melanogaster* and *Cellulophaga lytica* was classified as stable one by its Instability index (II) values. When amino acid composition of the SOD sequences were analysed, it was found that 6 SOD sequences were rich in Gly whereas for the rest of the sequences, Ala was the dominant residue (Table 2). *P. sativum* SOD had Ala and Leu dominance with a percentage of 10.8.

Sl.No	Organisms	Accession Number	No. of Amino acids	M. Wt	pI	Aliphatic Index	GRAVY	Instability Index (II)	Stability
1	*Pisum sativum*	CAA42737.1	274	29236.5	8.74	100.80	-0.014	39.56	Stable
2	*Drosophila melanogaster*	AAF50095.1	274	29049.2	8.74	98.03	-0.037	37.49	Stable
3	*Schistosoma mansoni*	AAA29934.1	274	29545.0	9.49	97.34	-0.108	50.32	Unstable
4	*Chymomyza amoena*	CAA43859.1	274	29397.6	9.10	98.39	-0.100	46.15	Unstable
5	*Enterobacter sp.*	YP_001176531.1	275	30374.5	7.72	84.04	-0.197	60.66	Unstable
6	*Thermus aquaticus*	BAA02655.1	280	30988.2	9.37	89.86	-0.236	49.16	Unstable
7	*Muricauda ruestringensis*	YP_004787678.1	255	28265.0	9.73	80.71	-0.336	56.69	Unstable
8	*Planctomyces brasiliensis*	YP_004272108.1	300	33006.9	9.36	92.23	-0.244	49.66	Unstable
9	*Cellulophaga lytica*	ADY29074.1	200	21765.0	6.57	100.30	-0.049	37.65	Stable
10	*Cellulophaga algicola*	ADV48601.1	423	46890.0	8.22	88.98	-0.131	46.04	Unstable

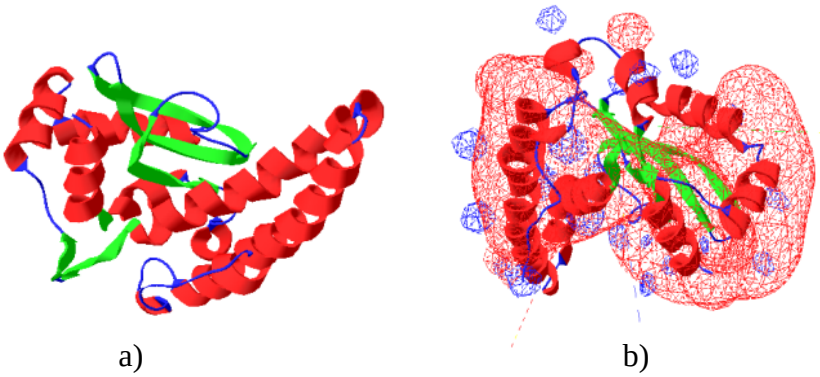
Table 1. ProtParam analysis of SOD sequences

Accession Numbers	Percentage of Occurrence (%)																				
	Superoxide Dismutase																				
	Ala	Arg	Asn	Asp	Cys	Gln	Glu	Gly	His	Ile	Leu	Lys	Met	Phe	Pro	Ser	Thr	Trp	Tyr	Val
CAA42737.1	10.8	2.5	5.4	4.6	0.4	4.2	5.4	6.7	4.2	6.2	10.8	7.5	0.8	1.7	3.8	6.2	5.4	3.3	3.8	6.2
AAF50095.1	7.2	2.0	5.2	6.5	3.3	1.3	5.2	16.3	5.2	5.9	4.6	5.9	1.3	3.9	3.3	5.9	5.9	0.0	0.7	10.5
AAA29934.1	7.1	8.2	4.9	4.9	1.6	1.1	1.6	15.2	5.4	4.9	7.6	2.2	1.6	6.0	6.0	3.8	4.3	0.5	7.6	5.4
CAA43859.1	6.5	2.0	7.2	6.5	4.6	1.3	5.9	15.7	5.2	6.5	4.6	6.5	1.3	3.3	4.6	3.9	5.2	0.0	0.7	8.5
YP_001176531.1	12.8	1.2	2.9	6.4	1.7	3.5	4.7	13.4	5.2	4.1	8.1	8.1	2.3	1.7	5.8	4.7	4.7	0.0	0.6	8.1
BAA02655.1	11.8	3.4	4.9	3.9	0.0	3.9	6.9	9.3	4.9	4.4	10.8	5.9	2.0	3.4	5.9	1.5	3.4	2.9	5.4	5.4
YP_004787678.1	9.4	2.5	6.4	5.0	1.5	1.0	7.4	11.4	4.5	5.4	6.4	5.4	2.5	5.4	5.0	6.4	3.5	2.5	4.5	4.0
YP_004272108.1	10.8	3.0	4.9	6.9	0.5	2.0	6.4	8.4	5.4	4.4	5.4	4.9	2.5	3.4	5.4	7.9	4.4	3.0	4.4	5.9
ADY29074.1	8.4	2.7	8.0	4.9	1.8	0.9	7.1	10.7	4.0	5.3	8.0	4.9	2.7	5.8	4.9	4.4	4.4	2.2	4.9	4.0
ADV48601.1	10.9	2.5	8.9	5.9	1.5	1.0	5.0	9.4	3.5	4.5	7.9	5.0	2.5	4.5	5.0	5.0	5.4	2.5	5.0	4.5

Table 2. Amino acid residue frequencies for SOD

Secondary structure of SOD sequences was analysed using SOPMA. Homology model of *P. sativum* SOD was predicted using SWISS-MODEL based on the template 3dc6C (Fig. 2a). Quality of the predicted model was analysed using PROSA software as well by Ramachandran plot analysis using RAMPAGE. RMSD value of the predicted model was calculated using VMD 9.1.1 and was found to be the best homology model.

Electron density map of the SOD model predicted was depicted in Deep Viewer (Fig. 2b). PROSITE analysis was performed for the SOD structure and found 6 distinct sites namely n-glycosylation site, protein kinase C phosphorylation site, casein kinase II phosphorylation site, N-myristoylation site, micro bodies C-terminal targeting signal and Mn- and Fe-SOD (Fig. 3). N-glycosylation site was found at helices with amino acid residues ASN111, HIS 112, SER113 and ILE114. Protein kinase C phosphorylation was also located in helices with THR95 and LYS97. Similarly N-myristoylation site (GLY107, GLY108, ASN111 and HIS112) and micro bodies C-terminal targeting signal (GLY 108, HIS109 and ILE110) also were present within helices only. Mn- and Fe-SOD had a residue in strand (ASP201) and rest in extended regions (TRP203, GLU204, HIS205, ALA206, TYR207 and TYR208). Fig. 4 depicts the manganese atom present in the SOD structure which confirms that it was Mn-SOD. SOD structure had a nuclear radius of 35.173 Å, van der Waal radius of 36.835 Å and a gyration radius of 17.262 Å from its geometric center. The mass of the structure was found to be 21080.024 g/mol.



a) b)

Fig. 2 Homology model prediction and electron density map for SOD sequence of *P. sativum* based on the template 3dc6C

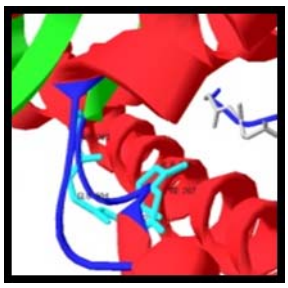


Fig. 3 PROSITE analysis of SOD model showing Mn- and Fe-SOD

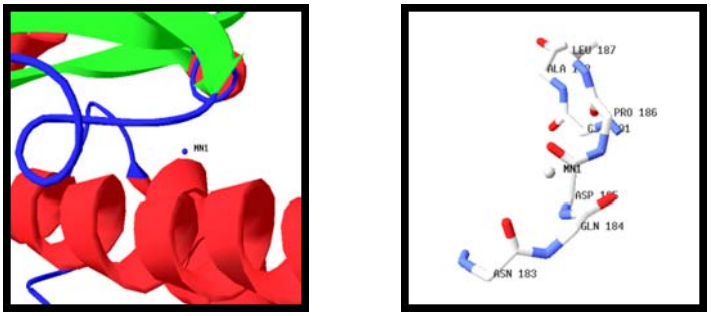


Fig. 4 Enlarged view of Manganese present in the Mn-SOD of *P. sativum*

Transmembrane probabilities

Transmembrane possibility of the *P. sativum* SOD was tested using TmPred and DAS transmembrane prediction server (Fig. 5). One strong transmembrane segment of 19 residues was predicted using TmPred from 191 to 201 with inside to outside orientation. DAS transmembrane prediction also shown a distinct high score peak in the same region which could validate the probable transmembrane nature of the same. N terminal location and total entropy of each SOD was compared (Table 3). It was found that N terminal of majority of SOD was towards outside with an exception of *AAA29934.1*. Total entropy (S) ranged from 17.0069 to 17.0174 J/mol for the SOD sequences where *P. sativum* SOD had 17.0145J/mol.

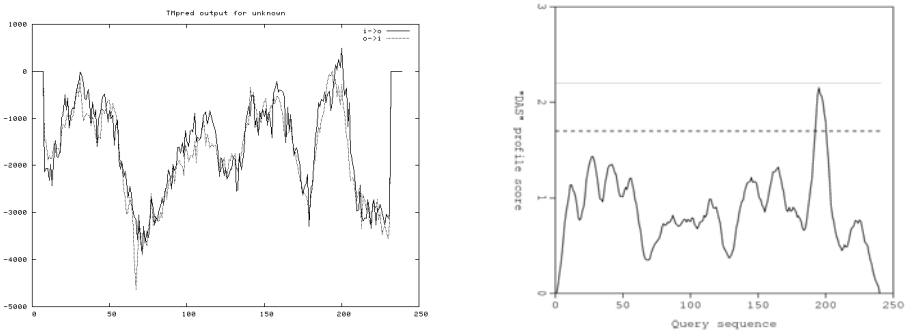


Fig. 5 Transmembrane probabilities of SOD of *P. sativum* performed using TmPred and DAS Transmembrane prediction software

Table 3. N terminal locations and entropy of SODs analyzed using HMMTOP server analysis

Accession Number	Length	N-Terminus	Total entropy	Entropy of best path
CAA42737.1	240	OUT	17.0145	17.0145
AAF50095.1	153	OUT	17.0114	17.0113
AAA29934.1	184	IN	17.0135	17.0138
CAA43859.1	153	OUT	17.0126	17.0126
YP_001176531.1	172	OUT	17.0069	17.007
BAA02655.1	204	OUT	17.0152	17.0152
YP_004787678.1	202	OUT	17.0159	17.0159
YP_004272108.1	203	OUT	17.015	17.015
ADY29074.1	225	OUT	17.0174	17.0174
ADV48601.1	202	OUT	17.0151	17.0151

Sub cellular location

Sub cellular location of SODs was predicted using Target P analysis. SOD of *P. sativum* was found to possess mitochondrial targeting peptide and located in the mitochondrion. AAA29934.1 and YP_001176531.1 sequences had a signal peptide and involved in any secretory pathway. Rest of the sequences may be found in other locations. SOD of *P. sativum* has a cleavage site of 44 residue length (Table 4).

Table 4. Target P analysis for SOD sequences showing possibilities of its cellular locations

Accession Numbers	Len	cTP	mTP	SP	other	Loc	RC	TPlen
CAA42737.1	240	0.036	0.818	0.087	0.111	M	2	44
AAF50095.1	153	0.128	0.072	0.357	0.529	_	5	-
AAA29934.1	184	0.004	0.14	0.946	0.09	S	1	22
CAA43859.1	153	0.059	0.065	0.4	0.678	_	4	-
YP_001176531.1	172	0.084	0.041	0.917	0.076	S	1	19
BAA02655.1	204	0.045	0.156	0.108	0.867	_	2	-
YP_004787678.1	202	0.079	0.122	0.169	0.818	_	2	-
YP_004272108.1	203	0.086	0.111	0.095	0.837	_	2	-
ADY29074.1	225	0.019	0.12	0.566	0.579	_	5	-
ADV48601.1	202	0.121	0.107	0.145	0.792	_	2	-

Active site on SOD

Presence on cavities on the homology model of *P. sativum* SOD was done using CASTp software. Each cavity was coloured by distinct colours (Fig. 6). Moreover, the cavities were marked in the sequence also. From the SciFBio analysis, about 27 cavities were detected in SOD structure. When the amino acid residue composition of these individual cavities was studied in detailed it was found that majority of the cavities were LYS rich with a high frequency of occurrence.

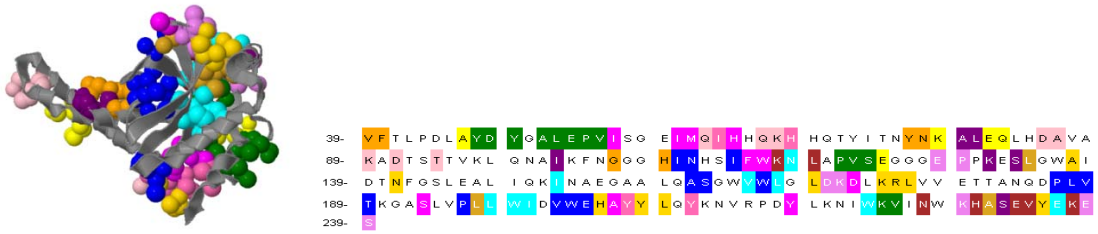


Fig. 6 Cavities in SOD homology model
(residues present in cavities are highlighted in sequence)

Mutational analysis of SOD sequences

SOD sequences were mutated using Mutate protein software with varying number of residues mutated as 10, 20, 30, 40 and 50. Changes in the protein characteristics were analysis by PEPSAT and GRAVY analysis. Amino acid frequency among the native sequence as well as mutated sequences was compared (Fig. 7). Frequency each residue were varying after mutation and residue D showed the least effect of mutation with less significant changes in its frequencies when compared to native sequence. After comparison of 7 PEPSTAT parameters, it was found that there were no significant changes in protein properties in spite of mutation of up to 50 residues through Mutate protein software (Fig. 8). Neighbour joining tree was

constructed using the native protein sequences and mutated sequences (Fig. 9). This resulted in a tree with two distinct branches. All mutated proteins and its native sequences were grouped under same branching. Divergence was exhibited among native and mutated protein which was supported by significant bootstrap values.

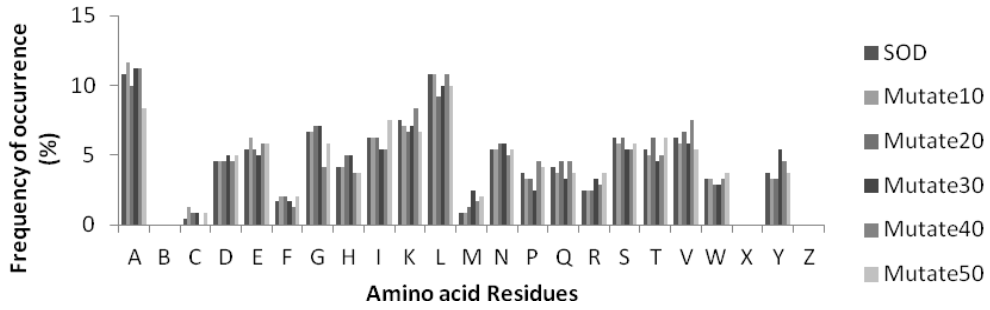


Fig. 7 Amino acid frequency comparison among SOD protein of *P. sativum* and its mutated protein sequences

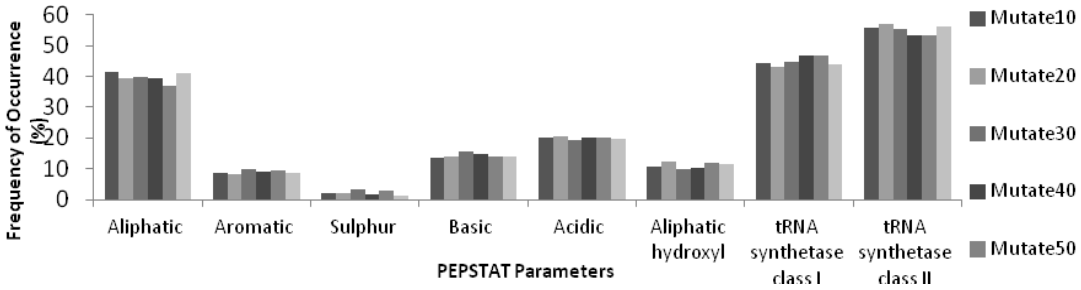


Fig. 8 PEPSTAT analysis of native and mutated amino acid sequences of *P. sativum* SOD

Discussion

Oxidative stresses are widely found in all taxonomic groups and their harmful effects are more. O_2^- is produced at any location where an electron transport chain is present, and hence O_2 activation may occur in different compartments of the cell [11], including mitochondria, chloroplasts, microsomes, glyoxysomes, peroxisomes, apoplasts, and the cytosol. It has been shown that phospholipid membranes are impermeable to charged O_2^- molecules [34]. Therefore, it is crucial that SODs are present for the removal of O_2^- in the compartments where O_2^- radicals are formed [34].

The evolutionary reason for the separation of SODs with different metal requirements is probably related to the different availability of soluble transition metal compounds in the biosphere in relation to the O_2 content of the atmosphere in different geological eras [5]. Catalysis by Mn-SODs I through the attraction of negatively charged O_2^- molecules to a site formed from positively charged amino acids present at the active site of the enzyme. The metal present in the active site the donates an electron directly to the O_2^-, reducing one O_2^- molecule, which in turn forms H_2O_2 by reacting with a proton [4, 8].

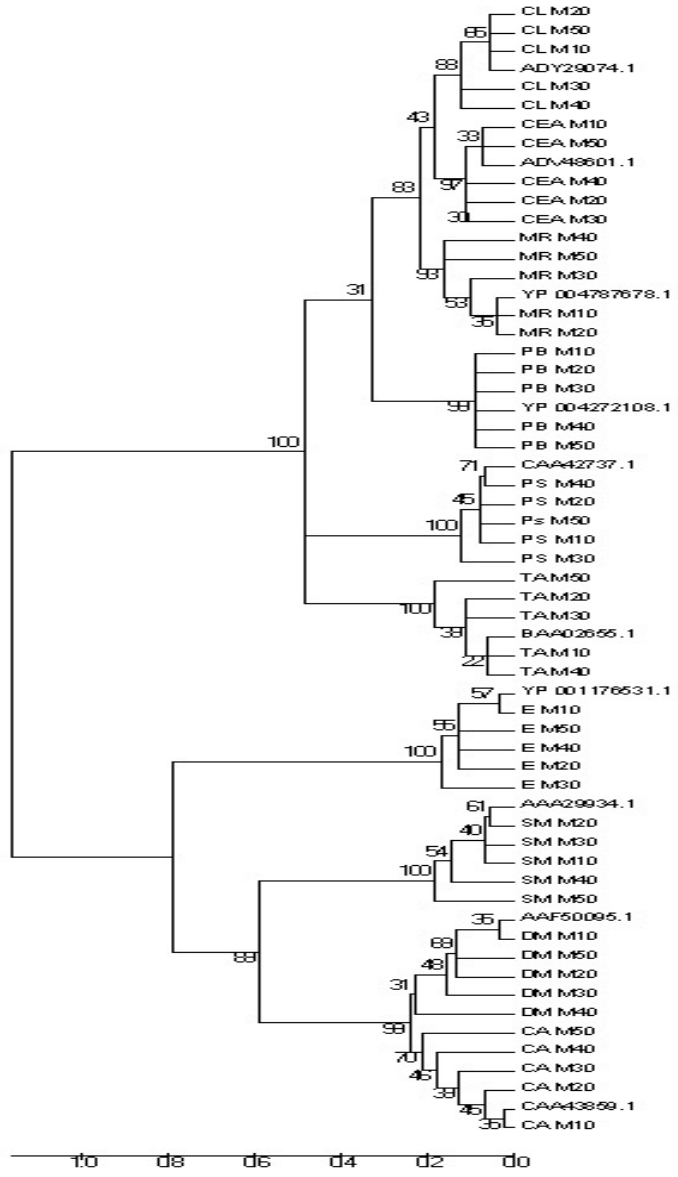


Fig. 9 Neighbour joining tree depicting the phylogenetic relation among the retrieved SOD sequences and its mutated proteins

Although Mn-SOD is known as mitochondrial enzyme of eukaryotes, a Mn containing SOD has also been located in the peroxisomes. Four genes that encode Mn-SOD were reported in maize (*Zea mays*) [41]. Deduced amino acid sequences from theses four iso-enzymes have a mitochondrial targeting sequence, indicating that all are located in the mitochondria. Multiple transcripts for Mn-SOD have been reported in human (*Homo sapiens*) tissues. These different transcripts have been found to be the product of the same gene, but result from alternative polyadenylation or alternative splicing and polyadenylation [41]. Also, it was shown that high levels of chloroplastic Mn-SOD activity protected the plant from visible injury caused by ozone, suggesting that ozone may cause oxidative stress in the chloroplast, as well as the apoplasts [38].

There are few computational tool based studies on SODs and such studies could be helpful in prediction on structural and functional aspects of SODs. Sonia et al. [32] predicted a homology model for bread wheat Mn-SOD. Moreover, it also opens up new ideology for

protein engineering in suppression or expression of these proteins through inhibitory or enhancing molecules. Vidya et al. [39] reported validations of new drug leads on SOD on *Homo sapiens* and *Plasmodium falciparum* through docking studies. There is a high scope for docking studies and anti-sense technology in the case of SOD which will contribute to the generation of more oxidative stress tolerant crop plants in the future.

Conclusion

P. sativum SOD was found to be Ala and Leu rich protein with alkaline pI value. It was classified unstable with high aliphatic index and least GRAVY value ensuring its best activity in a wide range of temperatures and better aqueous interactions respectively. 27 cavities were detected on protein surface among which majority of them were Lys rich cavities. It possesses a mitochondrial signal peptide ensuring its probable sub-cellular location. The SOD sequence when analysed after mutations, ASP showed the least frequency changes. Since SOD is an important protein involved in the oxidative stress mechanism in plants, understanding and engineering of this protein has a great scope in generating stress tolerant plants.

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