



Modeling and analysis of soybean (*Glycine max.* L) Cu/Zn, Mn and Fe superoxide dismutases

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Abstract

Superoxide dismutase (SOD, EC 1.15.1.1) is an important metal-containing antioxidant enzyme that provides the first line of defense against toxic superoxide radicals by catalyzing their dismutation to oxygen and hydrogen peroxide. SOD is classified into four metalloprotein isoforms, namely, Cu/Zn SOD, Mn SOD, Ni SOD and Fe SOD. The structural models of soybean SOD isoforms have not yet been solved. In this study, we describe structural models for soybean Cu/Zn SOD, Mn SOD and Fe SOD and provide insights into the molecular function of this metal-binding enzyme in improving tolerance to oxidative stress in plants.

Keywords: amino acid analysis, model evaluation, molecular modeling, phylogenetic analysis, superoxide dismutase (SOD).

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Introduction

Crop plants are frequently exposed to a variety of abiotic, biotic and xenobiotic stresses that cause injury, limit their growth and adversely affect their productivity. The most common result of such stress is the production of toxic reactive oxygen species (ROS). Increased levels of ROS such as superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($\bullet OH$) cause irreparable damage to cellular components such as DNA, proteins and lipids that require additional defense mechanisms (Blokina *et al.*, 2003). Plant responses to ROS toxicity involve the coordinated action of enzymatic and non-enzymatic antioxidant defense systems (Pallavi and Rama Shankar, 2005; Pallavi *et al.*, 2012). Among enzymatic defenses, superoxide dismutase (SOD, EC 1.15.1.1) is the most important enzyme because of its distinct ability to neutralize superoxide anions by dismutating them into O_2 and H_2O_2 . SOD is synthesized by all aerobic organisms and also by some air-tolerant and obligate anaerobic organisms (Fink and Scandalios, 2002).

SODs are metalloproteins that occur in four isoforms: copper zinc SOD (Cu/Zn SOD), manganese SOD (Mn SOD), nickel SOD (Ni SOD) and iron SOD (Fe SOD), all of which are highly stable because of the β -barrel structure

and low content of α -helix strands (Renu and Sabarinath, 2004). Almost all eukaryotic organisms synthesize Mn and Cu/Zn SOD whereas Fe SOD is exclusive to plants (Kwang-Hyun *et al.*, 2006). Ni SOD was recently reported in *Streptomyces griseus* and *S. coelicolor* (Garcia-Hernández *et al.*, 2002). In the *Arabidopsis thaliana* genome, three Cu/Zn SODs (cytosolic, chloroplast and peroxisomal), one Mn SOD and three Fe SODs (Fe SOD1, 2 and 3) genes have been reported, with Fe SOD2 and 3 being chloroplastic. Chloroplast Cu/Zn SOD is located in the thylakoid membranes and Fe SOD2 and 3 are located in the stroma (Myouga *et al.*, 2008).

Soybean (*Glycine max.* L) is an important legume plant that is widely cultivated for its protein and oil and is considered a miracle crop. Although the role of SOD as an antioxidant enzyme under stress conditions has been studied in different varieties of soybean (Chaitanya *et al.*, 2009; Hassan *et al.*, 2011), the genes that control the expression of these isoforms have not been identified. In this study, we undertook a molecular, structural and phylogenetic analysis of soybean SOD isoforms (Cu/Zn, Mn and Fe SOD) based on homology modeling using *A. thaliana* SODs.

Materials and Methods

Identification of EST sequences

The sequences of genes corresponding to SOD iso-enzymes in *Arabidopsis*, namely, Mn SOD (AT3G10920), Fe SOD (AT4G25100) and Cu/Zn SOD distributed in

chloroplasts (ATG12520), cytosol (AT1G08830) and peroxisomes (AT5G18100), were used as the query sequences in BLAST searches to identify the corresponding genes in soybean. These nucleotide sequences were assessed for homology at the protein level and used for phylogenetic analysis.

Phylogenetic analysis of sequences

All sequence alignments were done using ClustalW (Aiyar, 2000). Phylogenetic trees were plotted using MEGA 4.0 software with the UPGMA method (Tamura *et al.*, 2007). Soybean genes that separated along with *Arabidopsis* genes were pooled and their amino acid pattern was analyzed by constructing pretty boxes using Boxshade.

Secondary structure prediction of SOD proteins

The secondary structures of the soybean SOD isoenzymes were predicted using the PSIPRED online server based on the retrieved sequences. PSIPRED incorporates two feed-forward neural networks that analyze the data generated as an output from PSI-BLAST (position-specific iterated BLAST) (Altschul *et al.*, 1997). Validation of the procedure and performance using PSIPRED yielded an average Q3 score of 76.5%.

3D structure

The 3D structure models for soybean and *Arabidopsis* SODs were developed using 3D LigandSite. This software was used to the exact binding site of metal ions in the amino acid sequences. Dompred software was used to predict the domains and their boundaries for a given protein sequence.

Quaternary structure prediction

The quaternary structures of SOD proteins in soybean and *Arabidopsis* were predicted using the protein interfaces and surfaces tool PISA. Assemblies that could form crystals were determined by identifying the sets that represented the solutions indicated in the headings of the appropriate table (see Results). The highest values in the assemblies were considered to be the most appropriate. The MM size indicated the number of macromolecular monomeric units in that particular assembly and corresponded to an oligomeric or multimeric state. A formula was obtained to indicate the chemical composition of the assembly and denoted the number of different monomeric units. The stability of an assembly, *i.e.*, its tendency to dissociate in solution, was also determined. The solvation free energy (ΔG^{int}) was calculated as the difference in solvation energies of the isolated and assembly structures and indicated the free energy gain (kcal/M) during the formation of an assembly. The free energy of dissociation (ΔG^{diss}) represented the free energy difference between the associated and dissociated states. Assemblies with $\Delta G^{\text{diss}} > 0$ were thermodynamically more stable because positive values

were included in external energy use during the dissociation of an assembly.

Model evaluation

The dihedral angles ϕ vs. ψ of amino acid residues in the protein structures were visualized and analyzed with Ramachandran plots (Ramachandran *et al.*, 1963). The evaluation of models predicted *in silico* is essential in order to avoid errors resulting from trivial and non-trivial mistakes. To avoid ambiguities and to improve accuracy, the predicted SOD models were evaluated using the ProSA and VADAR web servers. For a specific PDB structure, ProSA calculates the overall quality score and validates a low resolution structure for approximate models using C-alpha atoms of the input structure. The output provides a z-score for the model that indicates the overall model quality; this value was determined from the plot during prediction.

Results and Discussion

ROS produced by plants are eliminated by antioxidant defense systems that enhance the tolerance of plants to environmental stress (Min-Lang *et al.*, 2012). In view of the increasing interest in the molecular modeling of the various isoforms of SOD, in this study we investigated the structure of soybean SOD isoforms and examined their phylogenetic relationships.

Phylogenetic analysis

The availability of information from various genome-sequencing projects, cDNA libraries and EST libraries offers the possibility of complementing investigations of gene function *in vivo* with parallel phylogenetic analyses of multigene families to address their evolution within and across species (Vincentz *et al.*, 2003). Within families, the protein structure and catalytic residues that determine the substrate specificity are generally conserved. Bioinformatics tools are thus useful for the functional analysis of related proteins (Henrissat *et al.*, 2001). However, many sequence-based families are polyspecific, *i.e.*, they include genes that encode proteins with different functions. This reflects gene duplication and evolutionary divergence, with the acquisition of new protein functions (Emanuele *et al.*, 2004). In the present study, the phylogenetic relationships of soybean SOD genes were evaluated with respect to *Arabidopsis* SOD genes by using the Maximum Composite Likelihood (MCL) approach implemented in MEGA (Tamura *et al.*, 2007).

Phylogenetic analysis of the soybean and *Arabidopsis* open reading frames (ORFs) provided information on the evolutionary ancestry of all the SOD groups. This analysis showed that SODs segregated into two major clusters, with cytosolic, chloroplast, peroxisomal Cu/Zn in one cluster and Mn SOD and Fe SOD in another. In this tree, soybean SOD TC332577 segregated with chloroplast Cu/Zn SOD,

tial for the survival of aerobic life and plants. Mn SODs share 65% sequence similarity with each other (Youxiong *et al.*, 2012). The degree of homology was also high among Cu/Zn SOD genes compared to that of Fe and Mn SODs. Our results indicated that the protein sequences from Cu/Zn SOD of chloroplasts, cytosol and peroxisomes had a greater number of conserved amino acid sequences than Mn and Fe SODs. The subcellular and phylogenetic distribution of SODs showed that all three SOD isoforms co-exist only in plants (Bowler *et al.*, 1994). Comparative sequence analysis of the three SOD isoforms suggests that Fe SODs and Mn SODs are more efficient than Cu/Zn SODs, and that Fe and Mn SODs most probably arose from common ancestral enzymes, whereas Cu/Zn SODs evolved separately in eukaryotes (Smith and Dolittle, 1992).

Secondary structure analysis

The secondary structure predictions for soybean and *Arabidopsis* Cu/Zn, Fe and Mn SOD proteins showed that Mn SODs had a long chain length consisting of α -helices and β -strands (Figure 3). Helices were absent in chloroplast, cytosolic and peroxisomal Cu/Zn SODs of soybean and *Arabidopsis* and their secondary structures were identical (Table 1). Soybean and *Arabidopsis* SOD proteins had a similar number of domains but their locations differed. The binding sites of the SOD proteins also differed in both plants (Table 2). The heterogen counts in the SOD genes of both plants were similar with respect to the type of heterogens present in SOD proteins (Table 3).

3D structure analysis

Proteins are complex chemical entities with a large number of variable atoms and a convoluted topology that make their description complicated (Ingale and Chikhale, 2010). The ‘indescribable nature’ of proteins also makes the quality of an experimentally determined protein structure very difficult to assess. The rapid increase in the number of genomes being sequenced and in the number of genes being deposited in databases means there is a need to identify the protein functions involved in protein interactions that form the basis of defining protein groups. In this study, three-dimensional models of soybean and *Arabidopsis* Cu/Zn, Mn and Fe SOD proteins were predicted using the software 3D LigandSite. The resulting models displayed excellent global and local stereochemical properties (Figure 4). Blue colored residues were predicted to be part of the binding. Residue conservation was calculated using the Jensen-Shannon divergence score (Capra and Singh, 2008). The ligands that formed the cluster were used to predict the metal ions shown in the space-filling format (Wass *et al.*, 2010). There was a marked distinction between the metal binding sites and normal sites without space filling that enabled us to locate the coding regions exactly. The structural symmetry of the Cu/Zn SOD groups was identical.

Quaternary structural analysis

Quaternary structure plays an important role in defining protein function by facilitating allosterism and cooperativity in the regulation of ligand binding (Matthew *et al.*,

Table 1 - Secondary structure of *Arabidopsis* and soybean SOD proteins.

Protein	Description	Amino acid number
Mn SOD <i>Arabidopsis</i>	CHAIN	1-2, 12-15, 26-48, 80-82, 107-122, 132-135, 149-154, 162-165, 172-187, 191-192, 197-206, 216-218, 231
	HELIX	3-11, 16-25, 49-79, 83-106, 123-131, 136-148, 193-196, 207-215, 219-230
	STRAND	155-161, 166-171, 188-190
Mn SOD Soybean	CHAIN	1, 19-23, 33-57, 89-91, 116-131, 141-144, 158-163, 171-174, 181-196, 200-201, 206-215, 225-227, 239-240
	HELIX	2-18, 24-32, 58-88, 92-115, 132-140, 145-157, 202-205, 216-224, 228-238
	STRAND	164-170, 175-180, 197-199
Fe SOD <i>Arabidopsis</i>	CHAIN	1-28, 52-60, 70-81, 92-102, 113-116, 131-135, 143-144, 151-162, 168-171, 174-183, 193-196, 210-212
	HELIX	29-51, 61-69, 82-91, 103-112, 117-130, 172-173, 184-192, 197-209
	STRAND	136-142, 145-150, 163-167
Fe SOD Soybean	CHAIN	1-13, 18-44, 68-76, 85-97, 108-119, 130-132, 147-151, 159-178, 184-198, 202-203, 210-217, 227-230, 246-248
	HELIX	14-17, 45-67, 77-84, 98-107, 120-129, 133-146, 204-209, 218-226, 231-245
	STRAND	152-158, 179-183, 199-201
Cu/Zn chloroplast <i>Arabidopsis</i>	CHAIN	1-3, 10-40, 52-57, 61-65, 72-76, 86-90, 99-106, 112-148, 152-155, 164-165, 168-177, 183-204, 214-216
	HELIX	4-9, 41-51, 58-60
	STRAND	66-71, 77-85, 91-98, 107-111, 149-151, 156-163, 166-167, 178-182, 205-213
Cu/Zn chloroplast Soybean	CHAIN	1-25, 34-37, 40-53, 60-64, 74-78, 87-91, 100-136, 140-143, 152-153, 156-165, 172-192, 202-204
	HELIX	Nil
	STRAND	26-33, 38-39, 54-59, 65-73, 79-86, 92-99, 137-139, 144-151, 154-155, 166-171, 193-201

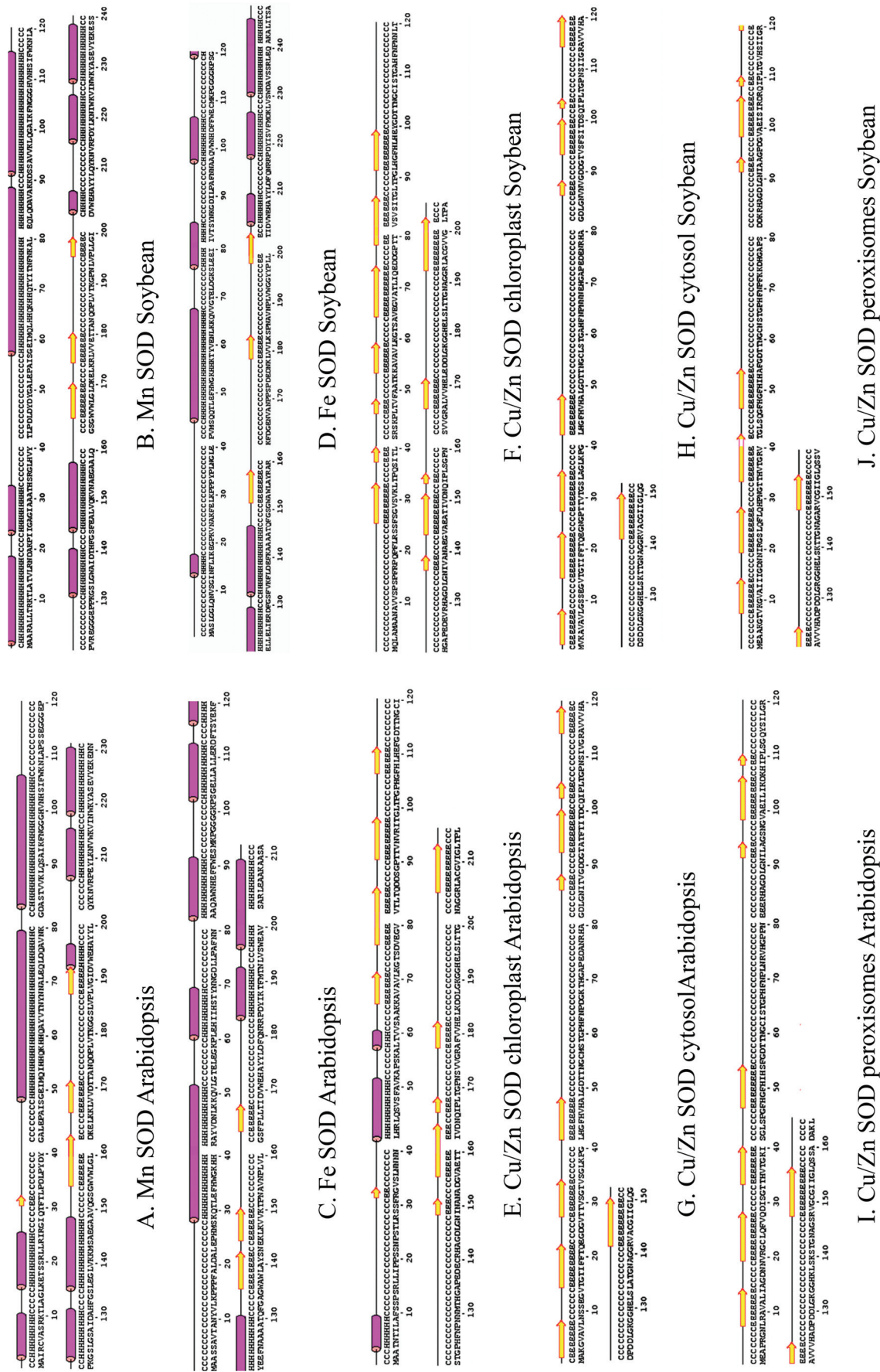


Figure 3 - Alignment of the secondary structures of soybean SOD with *Arabidopsis* SOD homologues. Identical residues are placed adjacent to facilitate comparison. The predicted secondary structure is shown for each aligned peptide; yellow arrows indicate β -sheets and pink cylinders indicate α -helices.

Table 2 - Predicted binding sites of *Arabidopsis* and soybean SOD proteins.

Protein	Plant	Residue	Amino acid	Contact	Av distance	Js divergence
Mn SOD	<i>Arabidopsis</i>	35	His	24	0	0.92
		87	His	24	0	0.92
		136	Trp	19	0.71	0.75
		169	Asp	24	0	0.92
		171	Trp	20	0.65	0.95
		173	His	24	0	0.92
	Soybean	51	His	24	0	0.92
		103	His	24	0	0.92
		152	Trp	21	0.68	0.95
		203	Asp	24	0	0.85
		205	Trp	19	0.7	0.95
		207	His	24	0	0.92
Fe SOD	<i>Arabidopsis</i>	55	His	24	0	0.92
		103	His	24	0	0.90
		155	Trp	20	0.61	0.95
		192	Asp	24	0	0.85
		194	Trp	22	0.49	0.95
		196	His	24	0	0.92
	Soybean	64	His	25	0	0.92
		112	His	25	0	0.90
		201	Asp	25	0	0.85
		205	His	25	0	0.92
Cu/Zn SOD	<i>Arabidopsis</i>	125	His	25	0	0.91
		142	His	25	0	0.92
		145	Asp	25	0	0.84
	Soybean	113	His	25	0	0.91
		121	His	25	0	0.91
		130	His	25	0	0.92
		133	Asp	25	0	0.84

Table 3 - Heterogens present in predicted binding sites.

Protein	Plant	Heterogen	Count
Mn SOD	<i>Arabidopsis</i>	Fe	22
		Fe ²	3
	Soybean	Fe	22
		Fe ²	3
Fe SOD	<i>Arabidopsis</i>	Fe	19
		Fe ²	5
	Soybean	Fe	19
		Fe ²	5
Cu/Zn SOD	<i>Arabidopsis</i>	Zn	25
	Soybean	Zn	25

2001). In this study, the quaternary structures of soybean and *Arabidopsis* SOD proteins were predicted using PISA by considering the assembly that provided the maximum

structure size with good stability as being the best (Table 4). Our results indicated that soybean and *Arabidopsis* chloroplast Cu/Zn SODs were tetramers, whereas peroxisomal and cytosolic Cu/Zn SODs were dimers; Mn SOD was a tetramer and Fe SOD was a monomer. The Biomol stability value for chloroplast Cu/Zn SOD was predicted to be 9 while that for Mn SOD was 1. All of the predicted structures were stable because of their positive ΔG^{Diss} values. There were more surface area values than buried surface values for all of the structures, indicating that these proteins had fewer folds. There was considerable similarity in the gene pattern of the soybean and *Arabidopsis* SOD enzymes, particularly with respect to protein structure. This similarity indicated a uniform evolutionary gene bank that was largely undisturbed (few mutations), as seen in our preliminary protein structural analysis.

Despite similarities in the secondary structures of cytosolic and peroxisomal Cu/Zn SOD there were many

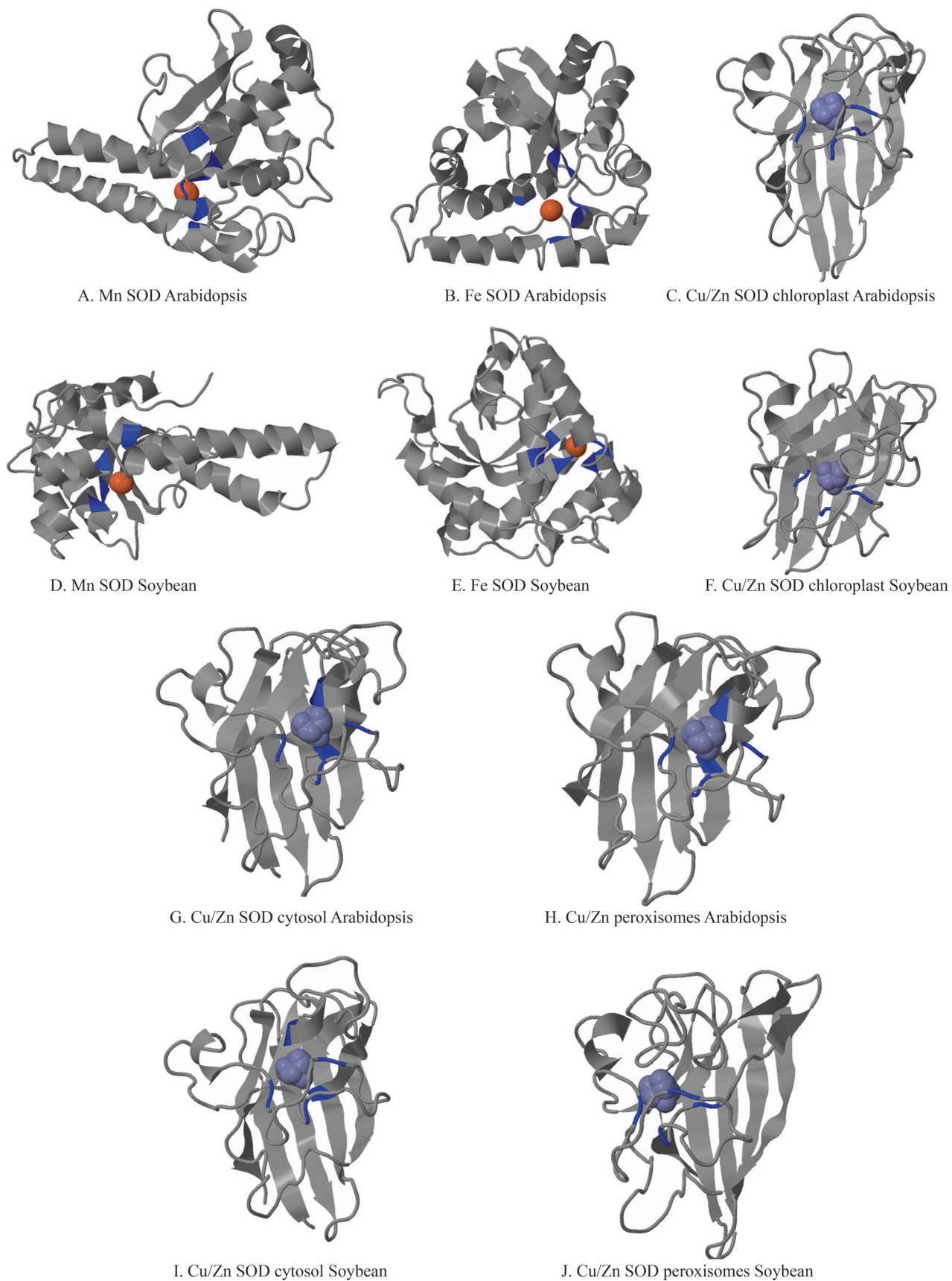


Figure 4 - Predicted secondary structure and binding sites of *Arabidopsis* and soybean SOD. Identical structures are placed below each other to facilitate comparison.

Table 4 - Quaternary structure analysis of *Arabidopsis* and soybean SOD proteins.

Protein	Size (mm)	Formula	Composition	Id	Biomol R350	Stability	Surface area (Sq A)	Buried area (Sq A)	ΔG^{int} (kcal/mol)	ΔG^{Diss} (kcal/mol)
Mn SOD Ara tetramer	4	A ₄ a ₆	A ₂ C ₂ [SO ₄] ₆	1	1	Stable	32660	8600	-115.4	17.1
	0	A	[Mn]	2	-	Stable	120	0	0	0
Mn SOD Soy tetramer	4	A ₄	ABCD	1	1	Stable	30950	8300	-40.6	12.6
	0	a	[Mn]	2	-	Stable	120	0	0	0
Fe SOD Ara monomer	2	A ₂	X ₂	1	-	Stable	18610	1740	-12.5	4.3
	0	A	[Fe]	2	-	Stable	140	0	0	0
Fe SOD Soy monomer	2	A ₂	X ₂	1	-	Stable	18610	1740	-12.5	5.3
	0	a	[Fe]	2	-	Stable	140	0	0	0
Cu/Zn Ara chloroplast tetramer	2	A ₂ a	AB[Cu]	1	-	Stable	13920	1420	-11.5	2.9
	2	A ₂ a	CD[Cu]	1	-	Stable	13690	1490	-23.6	2.5
Cu/Zn Soy chloroplast tetramer	2	A ₂	QR	1	9	Stable	13640	1340	-12.2	3.2
	2	A ₂	MN	1	7	Stable	13610	1320	-12.1	3.1
	2	A ₂	IJ	1	5	Stable	13620	1310	-12.0	2.9
	2	A ₂	KL	1	6	Stable	13680	1300	-11.9	2.9
	2	A ₂	ST	1	10	Stable	13700	1290	-11.8	2.8
	2	A ₂	WX	1	12	Stable	13570	1310	-11.7	2.7
	2	A ₂	UV	1	11	Stable	13720	1280	-11.7	2.7
	2	A ₂	OP	1	8	Stable	13640	1300	-11.7	2.6

differences between the corresponding genes in both plants. The amino acid patterns were identical in Cu/Zn SOD compared to Mn and Fe SOD. The Fe SOD structure contained more helices, as indicated by the quality index, with more omega aberrations. Cu/Zn SODs showed more homology compared to other models with no helices in their structures. Model evaluation revealed the accuracy of the predicted models and suggested possible errors that were trivial when compared to the overall quality of the proposed structure. Quaternary structural analysis showed that all of the structures are thermodynamically stable, with dissociation energies > 0 .

Model evaluation

The Z-score value, a measure of model quality that predicts the total energy of the structure (Wiederstein and Sippl, 2007), was predicted for soybean and *Arabidopsis* SODs using the PROSA server (Figure 5). The Z-score values for chloroplast, cytosolic and peroxisomal Cu/Zn SODs were -6.33, -7.05 and -6.93, respectively; the corresponding values for Mn SOD and Fe SOD were -7.59 and -7.92, respectively.

The stereochemical quality and exactness of the predicted soybean and *Arabidopsis* SOD proteins were analyzed using residue-by-residue geometry and the overall geometry of the protein structures was analyzed with Ramachandran plots (Ramachandran *et al.*, 1963). These

plots help visualize the dihedral angles ϕ vs. ψ of amino acid residues in proteins. In a polypeptide, the main chain N-C α and C α -C bonds are relatively free to rotate. These rotations are represented by the torsion angles ϕ and ψ , respectively. Figure 6 shows the quantitative evaluation of soybean and *Arabidopsis* protein structures done using Ramachandran plots provided by the VADAR web server. Soybean and *Arabidopsis* Mn SODs shared $> 90\%$ structural similarity with an equal number of residues in favored, allowed and outlier regions. There was little variation in the general and proline residue numbers in the αR , αL and β regions of both structures. The prominence of residues in the αR and β regions suggested that the structure was rigid with more right helices. Soybean chloroplast Cu/Zn SOD showed higher quality compared to the *Arabidopsis* protein, with fewer residues in the outlier region and more residues in the allowed region. The absence of residues in the αL region in both structures was an interesting feature of this protein and indicated that the structure had no left helices. Chloroplast Cu/Zn SOD of both plants had 86% of residues in the favored region (this value was lower than in previously reported SOD structures). More residues were observed in the allowed region of soybean (9%) and *Arabidopsis* (8.5%) SOD. There were more outliers in the *Arabidopsis* structure (5%) than in soybean (4%). Cytosolic Cu/Zn SODs from both plants had no residues in their

outlier regions. These soybean and *Arabidopsis* SODs also had an equal number of residues in the favored and allowed regions (95% and 4.5%, respectively). In both structures, three proline residues were distributed in the α R and β re-

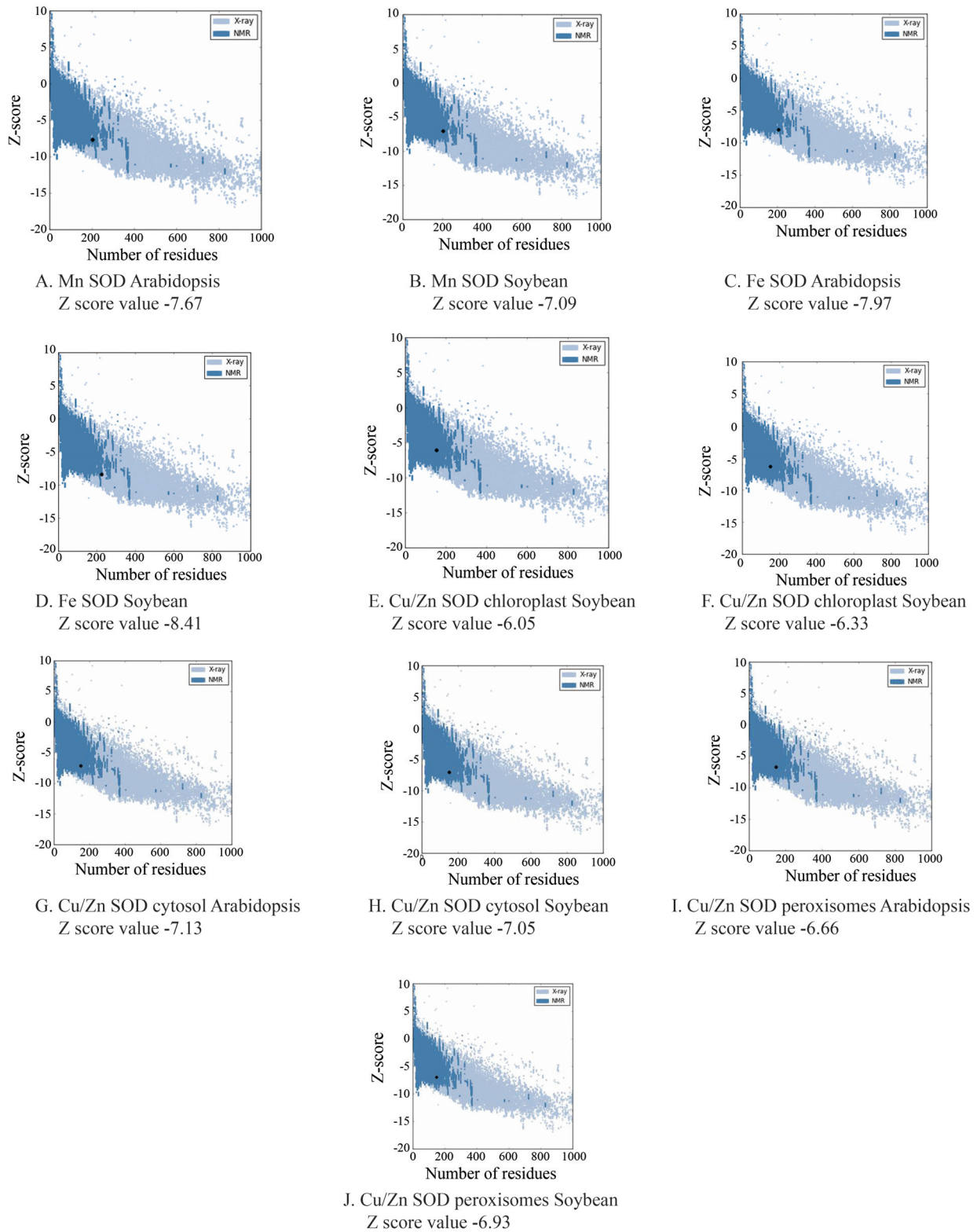


Figure 5 - ProSA-web z-score chimeric protein plot. The z-score indicates overall model quality. The ProSA-web z-scores of all protein chains in PDB were determined by X-ray crystallography (light blue) or NMR spectroscopy (dark blue) with respect to their length. The plot shows results with a z-score ≤ 10 . The z-score for SOD is highlighted as a large dot. The value is within the range of native conformations.

gions. There was a subtle difference in the sparsely populated α L region, where *Arabidopsis* had one general and one proline while in soybean both of the residues were generally in the core region. All of the Cu/Zn structures had fewer residues in the α R region compared to the β region. The Fe SOD and Mn SOD structures had good clustering of residues and a greater number of helices. The structural quality of chloroplast Cu/Zn SOD of both plants was lower than in the remaining structures. In all of the structures, the α L region was less much less populated, *i.e.*, few or no residues.

The soybean Fe SOD structure had 95% of its residues in expected regions and a negligible proportion (1.3%) in the outlier region; the corresponding values for *Arabidopsis* were 93% and 2.4%, respectively. However, *Arabidopsis* had 5% of its residues in allowed regions whereas soybean had 3.6%. Soybean peroxisomal Cu/Zn SOD had < 89% of its residues in the favored region. The number of residues in allowed and outlier regions was also high, indicating structural aberrations whereas in *Arabidopsis* no residues are observed in the outlier region and ~94% of residues were in the favorable region, indicat-

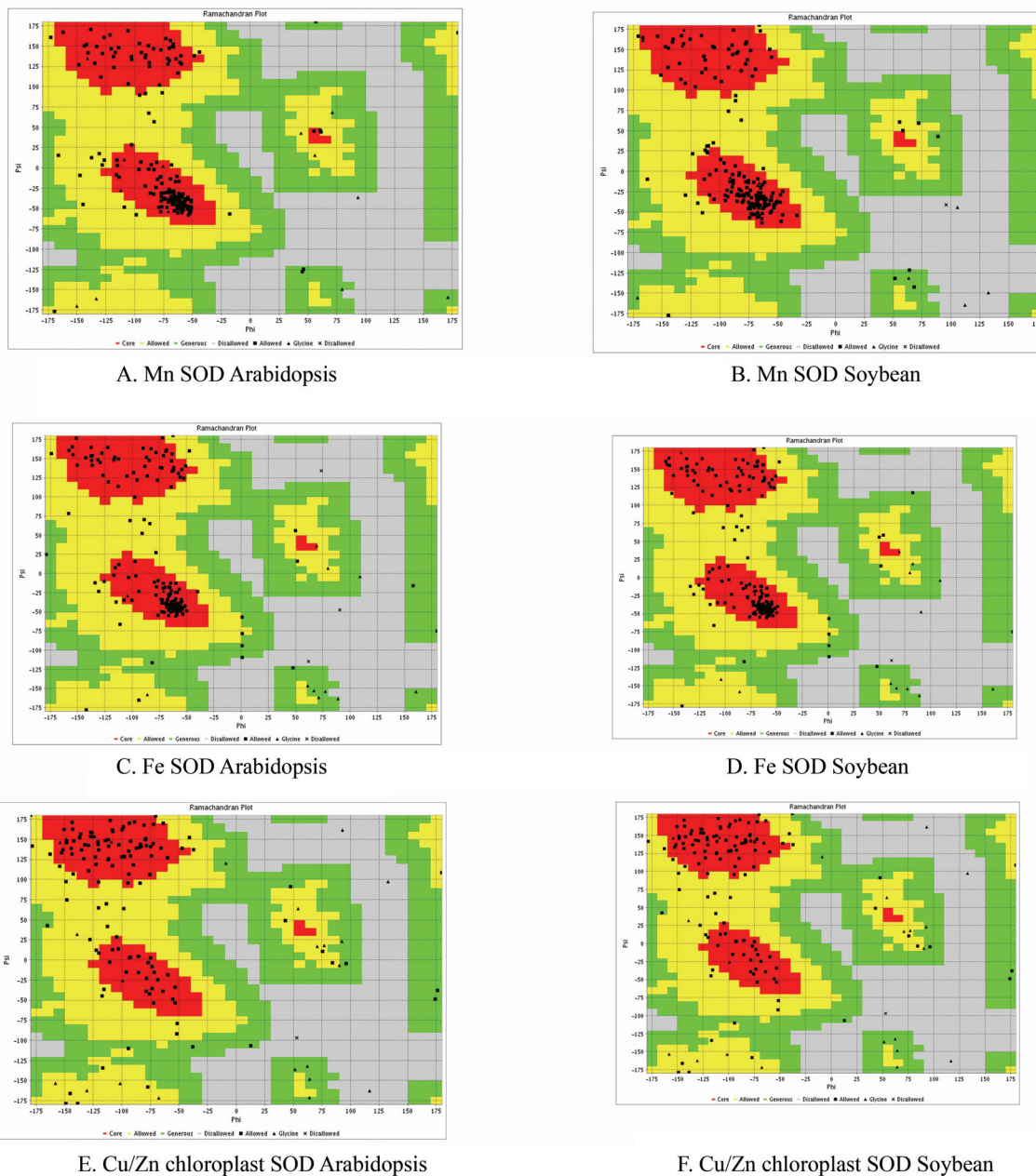


Figure 6 - Validation of SOD structures using Ramchandran plots. The Ramchandran plots revealed that > 90% of SOD amino acid residues from the modeled *Arabidopsis* structure were incorporated in the favored regions of the plot.

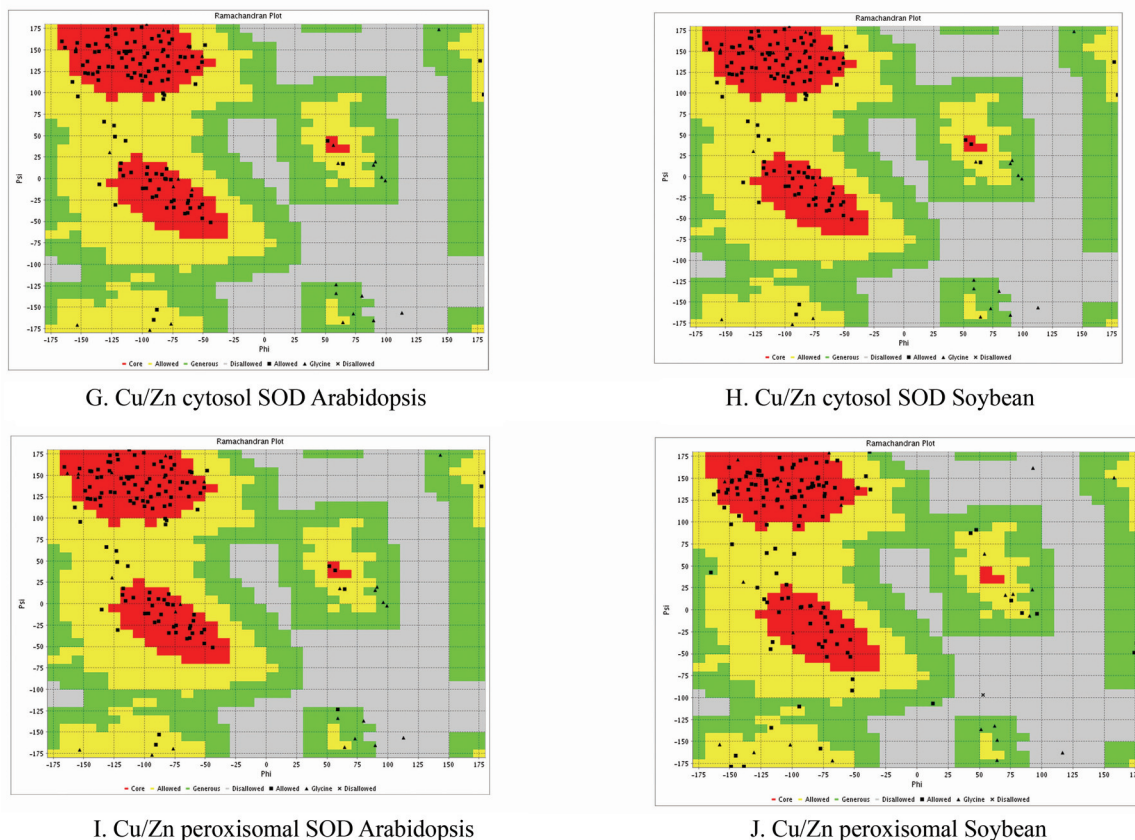


Figure 6 (cont.) - Validation of SOD structures using Ramachandran plots. The Ramachandran plots revealed that > 90% of SOD amino acid residues from the modeled *Arabidopsis* structure were incorporated in the favored regions of the plot.

ing the quality of the structure. Similar modeling and Ramachandran plot analyses to those described here have been used in the structural and functional analysis of spinach antioxidant proteins and the models were evaluated by computational tools (Sahay and Shakya, 2010).

Conclusion

Proteins are ubiquitous molecules that are involved in numerous crucial functions in organisms. Proteins accomplish their functions by positioning specific amino acids at target sites. Knowledge of the structural arrangement of amino acids is very important for understanding the molecular mechanisms by which proteins perform their functions. SOD is an important antioxidant enzyme that provides the first line of defense against ROS toxicity. The accurate and reliable molecular structural analysis of SOD isoenzymes is important for understanding their function in response to oxidative stress. In this study, structural models of soybean Cu/Zn, Mn and Fe SOD were analyzed and compared with those for *Arabidopsis*. These analyses provided insights into the molecular function of SOD isoenzymes with respect to their interactions with different cellular organelles. Further studies are in progress to understand the possible SOD gene interactions that may improve our

understanding of the role of SODs in minimizing ROS toxicity.

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Internet Resources

- Basic Local Alignment Search Tool (BLAST), <http://compbio.dfci.harvard.edu/cgi-bin/tgi/Blast/index.cgi> (January 4, 2012).
- Boxshade (version 3.21, written by K. Hofmann and M. Baron), http://www.ch.embnet.org/software/BOX_form.html (January 4, 2012).
- Boxshade: For multiple sequence analysis <http://sourceforge.net/projects/boxshade/> (January 5, 2012).
- Dompred, Server designed to predict putative protein domains <http://bioinf.cs.ucl.ac.uk/dompred> (January 5, 2012).
- 3D LigandSite, Prediction of ligand binding sites: <http://www.sbg.bio.ic.ac.uk/3dligandsite/> (January 5, 2012).
- Expasy, Translation of a nucleotide (DNA/RNA) sequence: <http://web.expasy.org/translate/> (January 7, 2012).
- Open Reading Frame Finder (ORF Finder), <http://www.ncbi.nlm.nih.gov/projects/gorf/> (January 5, 2012).
- Protein Interfaces, Surfaces and Assemblies (PISA), http://www.ebi.ac.uk/msd-srv/prot_int/cgi-bin/piserver (January 5, 2012).
- Protein Structure Prediction Server (PSIPRED), <http://bioinf.cs.ucl.ac.uk/psipred/> (January 14, 2012).
- Protein Structure Analysis (ProSA), <https://prosa.services.came.sbg.ac.at/prosa.php> (January 18, 2012).
- Volume, Area, Dihedral Angle Reporter (VADAR), <http://redpoll.pharmacy.ualberta.ca/vadar> (January 18, 2012).

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