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In silico Analysis and Homology Modelling of Antioxidant Proteins of Spinach

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Abstract

Spinach is an important dietary vegetable often associated with beneficial health effects. In this paper, a bioinformatics and molecular modeling approach was adopted to explore properties and structure of spinach antioxidant proteins. The antioxidant proteins selected for this study are ascorbate peroxidase (APX), dehydro ascorbate reductase (DHAR), phospholipid hydroperoxide glutathione peroxidase-like protein (PHGPX) and 2-Cys peroxiredoxin BAS1 (2-CPs). Physico-chemical characterization interprets properties such as pI, EC, AI, GRAVY and instability index and provides data about these proteins and their properties. Prediction of motifs, patterns, disulfide bridges and secondary structure were performed for functional characterization. Three dimensional structures for these proteins were not available as yet at PDB. Therefore, homology models for these antioxidant proteins were developed. The modelling of the three dimensional structure of these proteins shows that models generated by Modeller were more acceptable in comparison to that by Geno3D and Swiss Model. The models were validated using protein structure checking tools PROCHECK and WHAT IF. These structures will provide a good foundation for functional analysis of experimentally derived crystal structures.

Keywords: Spinach; Computational tools; Isoelectric point; Disulphide bridge; Homology model

Introduction

Spinach (*Spinacia oleracea*) is a flowering plant in the family of Amaranthaceae. Though Spinach is most often commonly used as a food, it has medicinal value as well. Dietary supplementation with blueberries, spinach, or spirulina reduces ischemic brain damage (Wang et al., 2005). Natural antioxidant mixture (NAO), a water-soluble extract obtained from spinach leaves has been shown to have anti-inflammatory (Lomnitski et al., 2000), antiproliferative (Nyska et al., 2003) and antioxidative properties (Bergman et al., 2001) in biological systems. A detailed analysis of the antioxidant protein sequences, their probable structures and mode of action has yet to be accomplished.

Reactive oxygen species (ROS) such as superoxide radicals (O_2-), hydroxyl radical (OH-), H_2O_2, and hydroperoxides (ROOH) are generated by exogenous sources, including prooxidant allelochemicals. Stress/starvation is an important endogenous source that generates ROS (Ahmad and Pardini, 1990). Antioxidants and antioxidant enzymes interrupt the cascades of uncontrolled oxidation in some organelles (Shigeoka et al., 2002). The antioxidant defense is primarily constituted by the actions of glutathione peroxidase (GPX), superoxide dismutase, catalase and ascorbate peroxidase (Barbehenn, 2002). In this study the antioxidant proteins of spinach have been selected for which three dimensional structures were not available at the protein data bank (PDB). These proteins are ascorbate peroxidase (APX), dehydro ascorbate reductase (DHAR), phopholipid hydroperoxide glutathione peroxidase-like protein (PHGPX) and 2-Cys peroxiredoxin BAS1 (2-CPs).

APX exist as isoenzyme and plays an important role in the metabolism of H_2O_2 in higher plants. APX isoenzymes are distributed in four distinct cellular compartments: stromal APX (sAPX), thylakoid membrane-bound APX (tAPX) in chloroplast, microbody (including glyoxisome and peroxisome) membrane bound APX (mAPX), and cytosolic APX (cAPX) (Ishikawa et al., 1998). DHAR is responsible for regenerating ascorbic acid from an oxidized state. It regulates the cellular ascorbic acid redox state, which in turn affects cell responsiveness and tolerance to environmental ROS. Ascorbic acid is a major antioxidant that serves many functions in plants including involvement in the detoxification of ROS, which are produced during aerobic metabolic processes such as photosynthesis or respiration (Asada and Takahashi, 1987). Ascorbic acid also participates in the regeneration of α-tocopherol (vitamin E) from the tocopheroxyl radical (Asada, 1994).

2-Cysteine peroxiredoxins (2-CPs) constitute a ubiquitous group of peroxidases that reduce cell-toxic alkyl hydroperoxides to their corresponding alcohols (Baier and Dietz, 1999). Despite the presence of elaborate enzymatic and nonenzymatic antioxidative defense mechanisms, ROS escape from detoxification and oxidize organic compounds such as proteins, nucleic acids, terpenoids and fatty acids to the respective peroxides (Baier and Dietz, 1998). In addition, alkyl hydroperoxides are formed by enzymatic reactions in chloroplasts, e.g. lipoxygenase catalyzes peroxidation of fatty acids and other desaturated organic biomolecules, such as carotenoids (Canfield et al., 1992). Detoxification of alkyl hydroperoxides is important because they can act as long-distance mediators of oxidative damage by oxidizing other biomolecules and initiating radical chain reactions (Elstner, 1990). Another plant protein, PHGPX has been identified in several plant species, including tomato (Herbette et al., 2002). PHGPX forms reduce phospholipid and cholesterol hydroperoxides and thereby play an important role in protecting biological membranes

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against oxygen toxicity. The function of this protein in the removal of phospholipid hydroperoxides, which are generated as products of lipoxygenase catalyzed oxygenation of fatty acids has been reported (Ursini et al., 1985).

Computational tools provide researchers to understand physico-chemical and structural properties of proteins. A large number of computational tools are available from different sources for making predictions regarding the identification and structure prediction of proteins. The major drawbacks of experimental methods that have been used to characterize the proteins of various organisms are the time frame involved, high cost and the fact that these methods are not amenable to high throughput techniques. *In silico* approaches provide a viable solution to these problems. The amino acid sequence provides most of the information required for determining and characterizing the molecule's function, physical and chemical properties. Computationally based characterization of the features of the proteins found or predicted in completely sequenced proteomes is an important task in the search for knowledge of protein function. In this paper the *in silico* analysis and homology modelling studies on antioxidant proteins of spinach was reported. Three dimensional structures for these proteins were yet not available. Hence to describe its structural features and to understand molecular function, the model structures for these proteins were constructed.

Materials and Methods

Sequences of antioxidant proteins of spinach were retrieved from the SWISSPROT, a public domain protein database (Bairoch and Apweiler, 2000). Table 1 shows the protein sequences considered in this study. The antioxidant proteins sequences were retrieved in FASTA format and used for further analysis.

Physico-chemical characterization

For physico-chemical characterization, theoretical isoelectric point (pI), molecular weight, total number of positive and negative residues, extinction coefficient (Gill and Von Hippel, 1989), instability index (Guruprasad et al., 1990), aliphatic index (Ikai, 1980) and grand average hydropathy (GRAVY) (Kyte and Doolottle, 1982) were computed using the Expasy's ProtParam server (Gasteiger, 2005) (*http://us.expasy.org/tools/protparam.html*). The results were shown in Table 2.

Functional characterization

The SOSUI server performed the identification of transmembrane regions. Table 3 represents the transmembrane region identified for these antioxidant proteins. Disulphide bonds are important in determining the functional linkages. Table 4 shows prediction of "SS" bonds using the primary structure (protein sequence data) by the tool

Antioxidant Proteins	Accession No.	Length	Description
APX	Q42459	250	Ascorbate peroxidase (Cytosolic ascorbate peroxidase)
	Q7DN63	365	Stromal ascorbate peroxidase
	Q41371	309	Ascorbate peroxidase
	O46921	415	Thylakoid-bound ascorbate peroxidase
	Q7DN73	415	Thylakoid-bound ascorbate peroxidase
	Q7GDV4	365	Stromal ascorbate peroxidase
DHAR	Q9FVE4	266	Dehydro ascorbate reductase
PHGPX	O23814	171	phopholipid hydroperoxide glutathione peroxidase-like protein
2-CPs	O24364	265	2-Cys peroxiredoxin BAS1, chloroplastic

Table 1: Protein sequences considered for the study.

Antioxidant Proteins	Accession Number	Sequence Length	M.wt	pI	-R	+R	EC	II	AI	GRAVY
APX	Q42459	250	21555	5.41	37	28	21680	39.23	76.56	-0.394
	Q7DN63	365	39516.3	8.46	43	46	46995	44.89	67.26	-0.518
	Q41371	309	34471.8	6.25	40	37	16515	40.19	81.78	-0.442
	O46921	415	45015.7	8.62	47	51	51465	46.7	71.37	-0.447
	Q7DN73	415	44987.7	8.61	47	51	51465	45.57	71.37	-0.446
	Q7GDV4	365	39544.3	8.46	43	46	46995	46.18	67.26	-0.519
DHAR	Q9FVE4	266	29901.0	8.28	30	32	33982.5	39.57	78.76	-0.439
PHGPX	O23814	171	19047.6	5.92	23	22	15992.5	21.90	79.77	-0.384
2-CPs	O24364	265	28895.8	7.69	28	29	20002.5	47.14	84.98	-0.095

Table 2: Parameters computed using Expasy's ProtParam tool.

Antioxidant Proteins	Accession No.	Transmembrane region	Length	Type of protein
APX	O46921	PTNYFLNIMIVIGVLAVLSYLAG	23	Transmembrane
	Q7DN73	PTNYFLNIMIVIGVLAVLSYLAG	23	Transmembrane

Table 3: Transmembrane regions identified by SOSUI server.

Antioxidant Proteins	Accession No.	CYS_REC
APX	Q42459	-
	Q7DN63	Cys70-Cys194
	Q41371	Cys82-Cys272 Cys252-Cys304
	O46921	Cys70-Cys194
	Q7DN73	Cys70-Cys194
	Q7GDV4	Cys70-Cys194
DHAR	Q9FVE4	Cys61-Cys75
PHGPX	O23814	-
2-CPs	O24364	-

Table 4: Disulphide (SS) bond pattern of pairs predicted, by CYS_REC.

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Antioxidant Proteins	Accession No.	Motif Found	Profile	Position in the protein	Description
APX	Q42459	PEROXIDASE_2 PEROXIDASE_1	PEROXIDASE_4	33-250 33 - 44 155 - 165	Heme-binding peroxidases carry out a variety of biosynthetic and degradative functions using hydrogen peroxide as the electron acceptor. They play a key role in hydrogen peroxide removal in the chloroplasts and cytosol of higher plants.
	Q7DN63	PEROXIDASE_1	PEROXIDASE_4	98 - 349 225 - 235	
	Q41371	PEROXIDASE_2 PEROXIDASE_1	PEROXIDASE_4	15-256 34 - 45 147 - 157	
	O46921	PEROXIDASE_1	PEROXIDASE_4	98 - 349 225 - 235	
	Q7DN73	PEROXIDASE_1	PEROXIDASE_4	98 - 349 225 - 235	
	Q7GDV4	PEROXIDASE_1	PEROXIDASE_4	98 - 349 225 - 235	
DHAR	Q9FVE4	-	GST_NTER GST_CTER	65 - 143 129 - 266	Glutathione S-transferases (GSTs) are involved in detoxification of xenobiotic compounds and in the biosynthesis of important metabolites. The N-terminal thioredoxin-like domain participate in binding the glutathione moiety via its thioredoxin-like domain while the C-terminal domain contains several hydrophobic α-helices that specifically bind hydrophobic substrates.
PHGPX	O23814	GLUTATHIONE_PEROXID_1 GLUTATHIONE_PEROXID_2	GLUTATHIONE_PEROXID_3	2-171 32 – 47 69 - 76	Glutathione peroxidase is an enzyme that catalyzes the reduction of hydroxyperoxides by glutathione. Its main function is to protect against the damaging effect of endogenously formed hydroxyperoxides.
2-CPs	O24364	-	THIOREDOXIN_2	73 - 232	Thioredoxins participate in various redox reactions via the reversible oxidation of an active center disulfide bond.

Table 5: Functional characterization of proteins of spinach at Prosite.

Antioxidant Proteins	APX						DHAR	PHGPX	2-CPs
Secondary structure	Q42459	Q7DN63	Q41371	O46921	Q7DN73	Q7GDV4	Q9FVE4	O23814	O24364
Alpha helix	38.8%	29.86%	36.89%	37.83%	38.31%	30.41%	36.09%	23.98%	26.42%
310 helix	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Pi helix	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Beta bridge	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Extended strand	10.80%	12.88%	12.94%	11.81%	12.05%	11.23%	19.92%	21.64%	19.25%
Beta turn	8.40%	6.30%	5.18%	5.54%	4.34%	6.85%	6.39%	11.70%	6.04%
Bend region	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Random coil	42.00%	50.96	44.98%	44.82%	45.30%	51.51%	37.59%	42.69%	48.30%
Ambiguous states	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Other states	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%

Table 6: Calculated secondary structure elements by SOPMA.

CYS_REC (*http://sunI.softberry.com/berry.phtml?topic*). CYS_REC identifies the position of cysteins, total number of cysteins present and pattern, if present, of pairs in the protein sequence. Prosite is a database of protein families and domains (Falquet et al., 2002). Table 5 represents the output of Prosite that was recorded in terms of the length of amino residues of protein with specific profiles and patterns.

Secondary structure prediction

SOPMA (Geourjon and Deleage, 1995) was employed for calculating the secondary structural features of the antioxidant protein sequences considered for this study. The results were presented in Table 6.

Model building and evaluation

The modeling of the three dimensional structure of the protein was performed by three homology modeling programs, Geno 3D (Combet et al., 2002), Swissmodel (Arnold et al., 2006) and Modeller (Sali and Blundelll, 1993). The constructed 3D models were energy minimized in GROMACS force field using steepest descent minimization Algorithms (Van der Spoel et al., 2005). The overall stereochemical property of the protein was assessed by Ramchandran plot analysis (Ramachandran et al., 1963). The validation for structure models obtained from the three software tools was performed by using PROCHECK (Laskowski et al., 1996). The models were further checked with WHAT IF (Vriend, 1990). The results of PROCHECK and WHAT IF analysis was shown in Table 7 and Table 8 respectively. Structural analysis was performed and figures representations were generated with Swiss PDB Viewer (Guex and Manuel, 1997).

Results and Discussion

Table 1 shows antioxidant proteins of spinach considered in this

Server	Antioxidant Proteins	APX	DHAR	PHGPX	2-CPs
		Q42459	Q9FVE4	O23814	O24364
Geno3D	Residues in the most Favored Region	84.4%	79.8%	83.5%	83.0%
	Residues in additionally allowed region	13.7%	16.6%	16.5%	13.9%
	Residues in generously allowed region	0.9%	1.8%	0.0%	1.2%
	Residues in disallowed region	0.9%	1.8%	0.0%	1.8%
Swiss model	Residues in the most Favored Region	92.8%	84.0%	81.0%	90.8%
	Residues in additionally allowed region	7.2%	13.3%	17.6%	8.6%
	Residues in generously allowed region	0.0%	1.7%	1.4%	0.0%
	Residues in disallowed region	0.0%	1.1%	0.0%	0.6%
Modeller	Residues in the most Favored Region	96.2%	80.0%	95.0%	91.5%
	Residues in additionally allowed region	3.3%	18.2%	5.0%	7.9%
	Residues in generously allowed region	0.5%	1.8%	0.0%	0.0%
	Residues in disallowed region	0.0%	0.0%	0.0%	0.6%

Table 7: Ramachandran plot calculation and Comparative analysis of the models from Geno3D, Swiss-model and Modeller computed with the PROCHECK program.

Antioxidant Protein	Accession No.	RMS Z-score for bond angles
APX	Q42459	1.084
DHAR	Q9FVE4	1.123
PHGPX	O23814	1.053
2-CPs	O24364	1.208

Table 8: RMS Z-score for bond angles of modeled protein structure using WHAT IF.

study. These antioxidant protein sequences were retrieved from the SWISSPROT, a public domain protein database. These protein sequences were retrieved in FASTA format and used for further analysis. Parameters computed using Expasy's ProtParam tool was represented in Table II. The calculated isoelectric point (pI) will be useful because at pI, solubility is least and mobility in an electro focusing system is zero. Isoelectric point (pI) is the pH at which the surface of protein is covered with charge but net charge of protein is zero. At pI proteins are stable and compact. The computed pI value of APX (Q42459, Q41371) and PHGPX were less than 7 (pI<7) indicates that these antioxidant proteins were considered as acidic. The pI of APX (Q7DN63, O46921, Q7DN73, Q7GDV4), DHAR and 2-CPs are greater than 7 (pI > 7) reveals that these proteins were basic in character. The computed isoelctric point (pI) will be useful for developing buffer system for purification by isoelectric focusing method. Although Expasy's ProtParam computes the extinction coefficient for 276, 278, 279, 280 and 282 nm wavelengths, 280 nm is favored because proteins absorb light strongly there while other substances commonly in protein solutions do not. Extinction coefficient of AFPs at 280 nm is ranging from 15992.5 to 51465 M^{-1} cm^{-1} with respect to the concentration of Cys, Trp and Tyr. The high extinction coefficient of APX (Q7DN63, O46921, Q7DN73, and Q7GDV4) indicates presence of high concentration of Cys, Trp and Tyr. The computed extinction coefficients help in the quantitative study of protein–protein and protein–ligand interactions in solution. The instability index provides an estimate of the stability of protein in a test tube. There are certain dipeptides, the occurrence of which is significantly different in the unstable proteins compared with those in the stable ones. This method assigns a weight value of instability. Using these weight values it is possible to compute an instability index (II). A protein whose instability index is smaller than 40 is predicted as stable, a value above 40 predicts that the protein may be unstable (Guruprasad et al., 1990). The instability index value for the spinach antioxidant proteins were found to be ranging from 21.90 to 47.14. The result classified APX (Q42459), DHAR (Q9FVE4) and PHGPX (O23814) as stable protein (Table 2).

The aliphatic index (AI) which is defined as the relative volume of a protein occupied by aliphatic side chains (A, V, I and L) is regarded as a positive factor for the increase of thermal stability of globular proteins. Aliphatic index for the antioxidant protein sequences ranged from 67.26 – 84.98. The very high aliphatic index of all antioxidant protein sequences indicates that these antioxidant proteins may be stable for a wide temperature range. The lower thermal stability of APX (Q7DN63 and Q7GDV4) was indicative of a more flexible structure when compared to other antioxidant protein. The Grand Average hydropathy (GRAVY) value for a peptide or protein is calculated as the sum of hydropathy values of all the amino acids, divided by the number of residues in the sequence. GRAVY indices of APX are ranging from - 0.095 to -0.519. This low range of value indicates the possibility of better interaction with water.

Functional analysis of these proteins includes prediction of transmembrane region, disulfide bond and identification of important motifs. SOSUI distinguishes between membrane and soluble proteins from amino acid sequences, and predicts the transmembrane helices for the former. The Transmembrane regions and their length were tabulated in Table 3. The server SOSUI classifies APX (O46921 and Q7DN73) as membrane protein and other spinach antioxidant proteins as soluble proteins. SOSUI server has identified one transmembrane region in these proteins. The transmembrane regions are rich in hydrophobic amino acids. As disulphide bridges play an important role in determining the thermostability of these proteins. CYS_REC was used to determine the Cysteine residues and disulphide bonds. Possible pairing and pattern with probability were presented in Table 4. Result shows that except APX (Q42459), PHGPX (O23814) and 2-CPs (O24364) all proteins contain disulphide linkages.

The functions of antioxidant proteins of spinach were analyzed by submitting the amino acid sequence to Prosite server. Sequence of a particular cluster of residue types, which is variously known as a pattern, motif, signature or fingerprint. These motifs, typically around 10 to 20 amino acids in length, arise because specific residues and regions thought or proved to be important to the biological function of a group of proteins are conserved in both structure and sequence during evolution (Christian et al., 2002). Prosite analysis suggested the functionality of these proteins with profiles and patterns identified for characteristic functionality were represented in Table 5.

The secondary structure of spinach antioxidant proteins were predicted by SOPMA (Self Optimized Prediction Method with Alignment) which correctly predicts 69.5% of amino acids for a state description of the secondary structure prediction (Geourjon and Deléage, 1995). The secondary structure indicates whether a given

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amino acid lies in a helix, strand or coil. Secondary structure features as predicted using SOPMA were represented in Table 6. The results revealed that random coils dominated among secondary structure elements followed by alpha helix, extended strand and beta turns for all sequences. The secondary structure were predicted by using default parameters (Window width: 17, similarity threshold: 8 and number of states: 4).

Three dimensional structures are predicted for proteins where such data is unavailable. There is lack of experimental structures for these proteins considered. Out of six ascorbate peroxidase isoenzymes sequences, three dimensional structure was modeled for only Q42459 since it has been reported that the steady-state transcript level of cytosolic APX altered in stress condition (Bergman et al., 2001). The other three proteins for which the three dimensional structures were modeled includes DHAR, PHGPX and 2-CPs. The modeling of the three dimensional structure of the protein was performed by three homology modeling programs, Geno 3D, Swiss Model and Modeller. The constructed three dimensional models were energy minimized in GROMACS force field using steepest descent minimization Algorithms. The Φ and Ψ distribution of the Ramachandran Map generated by of non glycine, non proline residues were summarized in Table 7. A comparison of the results obtained from the Geno 3D, Swiss Model and Modeller, three different software tools in Table 7 shows that the models generated by Modeller was more acceptable in comparison to that by Geno3D and Swiss Model. The final modeled structures were visualized by Swiss PDB Viewer that was shown in Figure 1.

The stereo chemical quality of the predicted models and accuracy of the protein model was evaluated after the refinement process using Ramachandran Map calculations computed with the PROCHECK program. The assessment of the predicted models generated by modeller was shown in Figure 2. The main chain parameters plotted are Ramachandran plot quality, peptide bond planarity, Bad non-bonded interactions, main chain hydrogen bond energy, C-alpha chirality and over-all G factor. In the Ramachandran plot analysis, the residues were classified according to its regions in the quadrangle. The red regions in the graph indicate the most allowed regions whereas the yellow regions represent allowed regions. Glycine is represented by triangles and other residues are represented by squares. The result revealed that the modeled structure for APX, DHAR, PHGPX and 2CPS has 96.2%, 80.0%, 95.0% and 91.5% residue respectively in allowed region. The distribution of the main chain bond lengths and bond angles were found to be within the limits for these proteins. Such figures assigned by Ramachandran plot represent a good quality of the predicted models.

The modeled structures of spinach antioxidant proteins were also validated by other structure verification servers WHAT IF. Standard bond angles of the four models are determined using WHAT IF. The results were shown in Table VIII. The analysis revealed RMS Z-scores were almost equal to 1 suggesting high model quality. The predicted structures conformed well to the stereochemistry indicating reasonably good quality.

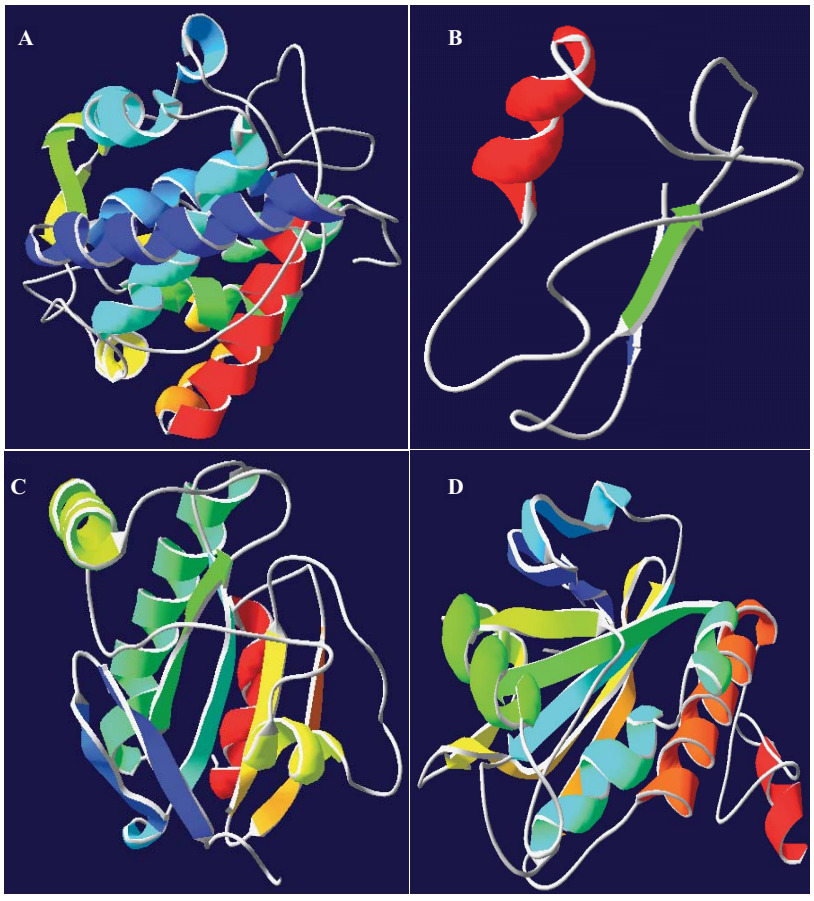

Figure 1: Modeled Structure of spinach proteins (A) Q42459 (B) Q9FVE4 (C) O23814 (D) O24364.

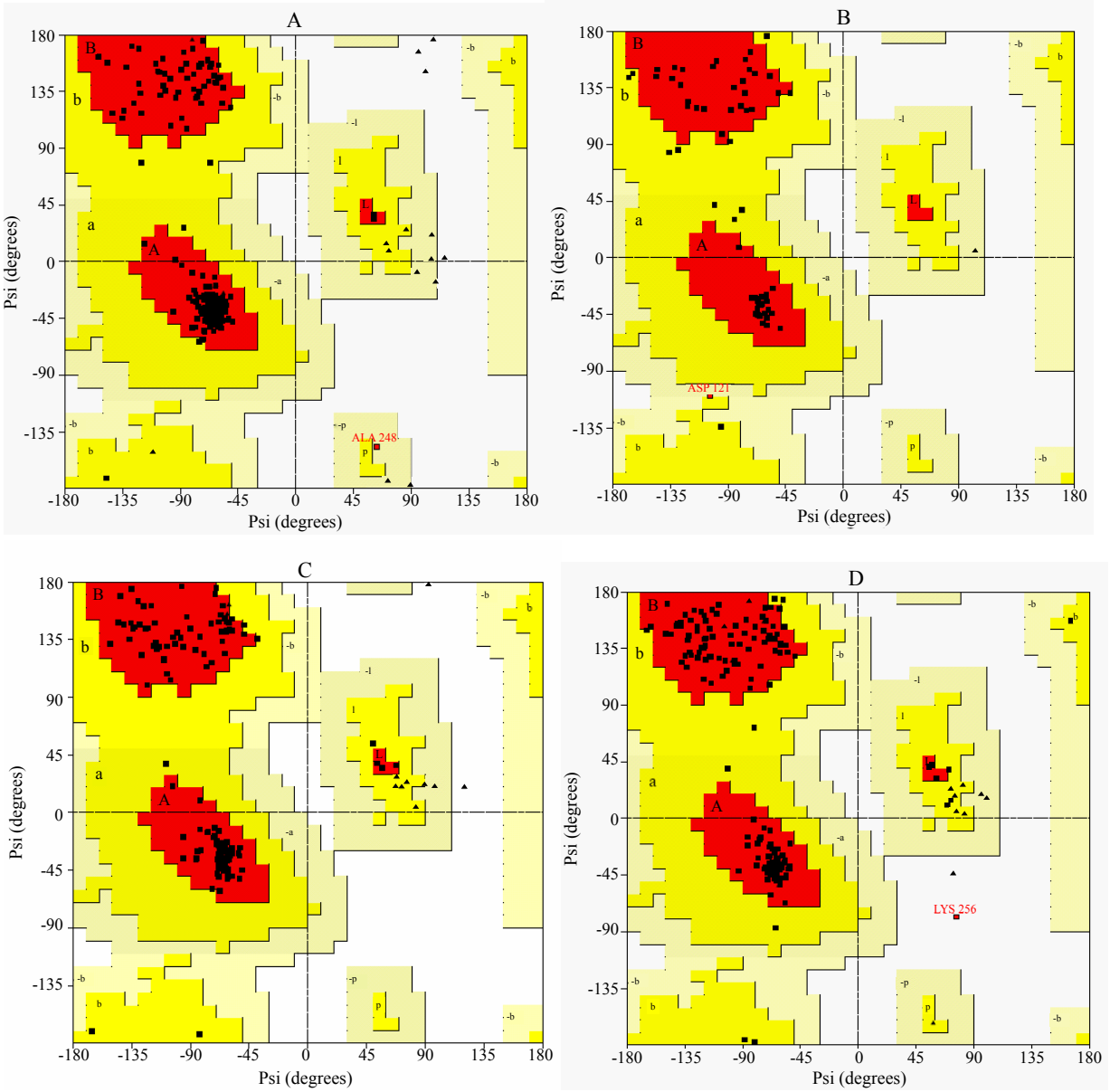

Figure 2: Ramachandran's Map of spinach proteins. (A) Q42459 (B) Q9FVE4 (C) O23814 (D) O24364.

Conclusion

In this study antioxidant protein of spinach were selected. Physico-chemical characterization were performed by computing theoretical isoelectric point (pI), molecular weight, total number of positive and negative residues, extinction coefficient, instability index, aliphatic index and grand average hydropathy (GRAVY). Functional analysis of these proteins was performed by SOSUI server. For these proteins disulphide linkages, motifs and profiles were predicted. Secondary structure analysis revealed that random coils dominated among secondary structure elements followed by alpha helix, extended strand and beta turns for all sequences. The modelling of the three dimensional structure of the proteins were performed by three automated homology programs, Geno 3D, Swiss model and Modeller. The models were validated using protein structure checking tools PROCHECK and WHAT IF. These structures will provide a good foundation for functional analysis of experimentally derived crystal structures.

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