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Seed Storage Proteins of Foxtail Millet: Structural and Functional Analysis using Computational Approach

Gupta S*, Yadav R, Bhatnagar SK and Sengar RS

Abstract

Nine seed storage proteins of foxtail millet have been chosen mainly to study their physico-chemical properties, primary and secondary structures using computational tools and servers. Primary structure analysis revealed that most of the seed storage proteins under study are balanced in terms of amino acids composition and four of them contain disulphide linkages. Physico-chemical characterization gave a good idea about the properties like isoelectric point (pI), extinction coefficients (EC), instability index (II), aliphatic index (AI) and Grand average hydropathy (GRAVY) that are essential and vital in providing data about the proteins and their properties. Secondary structure analysis predicted that all of them contain mixed secondary structure of random coils, extended sheets, alpha helices and beta turns. Since, secondary structure prediction remains an important step in full tertiary structure prediction; findings of the present study can provide new insight in foxtail millet seed storage protein characterization and their nutritional utilization as grain.

Keywords

Foxtail millet; Seed storage proteins; *In silico* analysis; Transmembrane helices

Introduction

The seed storage proteins of cereals are of immense importance in determining the quality and end use properties of the grain [1]. Because of their abundance and economic importance, seed storage proteins were earliest of all the proteins to be characterized. There is a general distinction among plant types with reference to the type of seed storage proteins. Dicot plants, such as legumes contain globulin type, while monocots such as cereals usually contain predominantly the prolamin types [2]. Globulins and prolamins are quite different in their structures and in mechanisms by which they are synthesized and deposited in the seed [3].

Seed storage proteins of our major crops have some serious imbalances with respect to their essential amino acids. Generally the cereals proteins are deficient in lysine, tryptophan and threonine and legume storage proteins are deficient in sulphur containing amino acids, cysteine and methionine [4]. The long term objective of most of the current research work on seed storage proteins is to modify these proteins in such a way that they are more suited to the dietary needs of human being and other monogastric animals. However, the caution has to be taken that the changes must be compatible with those properties of the storage proteins that enable them to fulfill their physiological role.

Seed storage proteins of panicoideae tribe (maize, sorghum, millets, sugar cane) which mainly belongs to prolamins are much more variable in structure than 7S or 11S globulins due to they might have separate origin [3], Foxtail millet, a member of the Panicoideae, is considered as a crop of poor people. It has good nutritional profile and is comparable to staple cereals as rice and wheat in terms of protein, fiber, minerals and vitamins [5,6]. However, the potential role of foxtail millet seed as functional food has remained unrealized and unexploited. Use of proteins in food applications are largely dependent on their physico-chemical and functional properties.

Computer- assisted characterization of the features of the proteins found or predicted in complete sequence proteomes is an important task in the search for knowledge of protein function. In this context, our work aimed at providing a deeper insight into the grain proteome of foxtail millet with special focus on seed storage proteins, their types, presence of secondary structure motifs in the protein and accumulation forms etc. using an *in silico* approach.

Materials and Method

Retrieval of the seed proteins sequences of foxtail millet and protein family assignment

All the sequences of seed proteins of foxtail millet present in NCBI [7] were retrieved in FASTA format by the end of January 2015 and their redundancy was removed. Among the 51 seed proteins selected, only nine (XP_004980234.1, XP_004961615.1, XP_004979705.1, XP_004979702.1, XP_004986765.1, XP_004962290.1, XP_004960933.1, XP_004980741.1 and XP_004960634.1) were found under the category seed storage proteins. For characterization of the selected Foxtail millet seed storage proteins to the specific protein family, Pfam analysis was conducted [8]. Proteins sequences were assigned SCOP domains using the SUPERFAMILY hidden Markov models with the help of Superfamily$_{1.75}$ HMM Library and Genome Assignments Server. Further, PROSITE server was used to find out the patterns in the selected seed storage proteins.

Physio-chemical characterization of seed proteins of foxtail millet

The amino acid composition of seed storage proteins of foxtail millet and physico-chemical characterization of sequences of proteins was done using Expasy's ProtParam server [9]. Apart from computing the molecular weight (MW) it also computes isoelectric point (pI), total number of negative (-R) and positive (+R) residues, extinction coefficients (EC) [10], instability index (II) [11], aliphatic index (AI) [12], and Grand average hydropathy (GRAVY) [13].

Functional characterization of seed proteins of foxtail millet

To check the disulphide bridge and type of proteins i.e. membrane protein or soluble protein, functional characterization of seed proteins of foxtail millet was done using CYS_REC and SOSUI software

***Corresponding author:** Gupta S, Department of Molecular Biology and Genetic Engineering, College of Biotechnology, Sardar Vallabhbhai Patel University of Agriculture and Technology, Meerut-250 110, U.P, India, Tel: 91 121 288 8502; E-mail: sgupta_mbge@yahoo.com

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[14], respectively. The SOSUI server performed the identification of transmembrane regions. The predicted transmembrane helices were visualized and analyzed using helical wheel plots generated by the programme Pepwheel included in EMBOSS 2.7 suit. Target P software was used to define the secretary vs non-secretary nature of the selected proteins. Subcellular localization of a protein indicates the location at which it functions. Sub cellular localization of the seed storage proteins were predicted with the help of Prot Comp 9.0 and CELLO software.

Prediction of secondary structure of seed proteins of foxtail millet

For calculating the secondary structural features of the seed protein of foxtail millet, SOPMA (Self Optimized Prediction Method with Alignment) [15] was used using default parameters [16].

Results and Discussion

Protein family assignment to the seed storage proteins of foxtail millet

Cereal grain protein composition has long been defined on the basis of their solubility in different media. It was found that sequential extraction relying on these solubility properties enabled the whole grain proteome to be simplified and a deeper and more comprehensive description of the protein composition to be obtained. As per the results obtained from Pfam analysis, among nine of the identified seed storage proteins of foxtail millet, five (XP_004979705.1, XP_004979702.1, XP_004986765.1, XP_004962290.1 and XP_004980741.1) were falling in Zein protein family, two (XP_004961615.1 and XP_004960933.1) were in TRP alpha amyl (protease inhibitor/seed storage/LTP family) family and one each in cupin 1 (XP_004980234.1) and Glaidian (XP_004960634.1) families. Thus, most of these proteins were identified as prolamins. Three seed proteins (XP_004961615.1, XP_004960933.1 and XP_004980234.1) were identified as 2S albumin proteins. In particular, 2S albumins are grouped in prolamin superfamily. 2S albumin storage proteins are becoming of increasing interest in nutritional and clinical studies as they have been reported as major food allergens in seeds of many mono- and di-cotyledonous plants. Results for assigning SCOP domains using the SUPERFAMILY hidden Markov models with the help of Superfamily$_{1.75}$ HMM Library and Genome Assignments Server and pattern detection using PROSITE server are given in Table 1. Its only one protein (XP_004960933.1) in which a single pattern of 'N-6 Adenine specific DNA methylase signature' was noticed between amino acid sequences 172-178.

Physico-chemical characterization of seed proteins of foxtail millet

Seed storage proteins of foxtail millet were normally heterogeneous in terms of polypeptides size (no. of amino acids), charge and amino acid composition. The number of amino acids in selected seed storage proteins was ranging from 137 to 409. The amino acid composition of selected foxtail millet seed storage proteins are presented in Table 2 along with the essential amino acid composition according to FAO/WHO/UNU (2007) requirements. It can be seen that the seed protein XP_004980234, XP_004961615.1 and XP_004960634.1 are almost ideal proteins in terms of essential amino acids and meet WHO recommendations. However, in rest of the proteins amount of Lysine was a bit lower but rest of the essential amino acids were quite balanced. Thus, it was clear from the given table that the millet proteins contain all the essential amino acids in balanced proportion and are also comparable to FAO composition. The same was also reported by Mohamed et al. [17], who experimentally identified the essential amino acid pattern of foxtail millet protein and suggested their possible use as a supplementary protein source to most cereals because their protein is rich in lysine, tryptophan and threonine which are the limiting amino acids in most cereals.

The average molecular wt of seed storage proteins calculated is 25542.34Da (Table 3). The computed isoelectric point (pI) value, which was greater than 7 for all the proteins, indicates that all these proteins are basic in nature. This information is of great importance when the buffer has to be developed for their purification by isoelectric focusing method. Instability index (II) of all the seed storage proteins except XP_004980234.1 and XP_004960634.1 was found greater than 40. Due to high instability index it is concluded that these proteins, except XP_004980234.1, were unstable in nature, i.e., has a short half-life. Value of aliphatic index (AI) is an indicative of stability of a protein for a wide temperature range. Comparatively lower values of AI of proteins XP_004980234.1, XP_004961615.1 and XP_004960933.1 indicates that these proteins may be less stable for a wide temperature range than the other proteins. Negative GRAVY values of proteins XP_004980234.1 and XP_004961615.1indicates their hydrophilic proteins structure while rest of the proteins is considered to have hydrophobic proteins structure. It is noteworthy here that the protein XP_004960634.1 was showing some unusual physico-chemical properties as for this protein the values of instability Index, Aliphatic Index and GRAVY were not computed by the ProtParam software.

Functional characterization of seed proteins of foxtail millet

Besides all other physicochemical characterization, functional characterization of Seed proteins of foxtail millet was also performed including prediction of disulphide bonding pairs and transmembrane (TM) region identification, etc.

CYS_REC recognize the presence of cysteine residues and disulphide bonds in seed proteins. There was total absence of cysteine residues in seed protein XP_004962290.1. In rest of eight proteins number of cystine residues were ranging from 1(XP_004979702.1)) to 12 (XP_004960634.1). Out of the seven proteins which were showing the presence of two or more cystine residues, no possible pairs of disulfide bond(s) were noticed in four proteins. The total number of cysteins present, position of cysteins and pattern of pairs, if present, in the protein sequences are presented in Table 4.

The three proteins which revealed the presence of disulphide bond were belonging to 2S protein family and possible pairs of disulfide bonds were in a range of 2 to 5. However, unlike other cereal grains, the 2S albumin of foxtail millet were not exhibiting well conserved skeleton of cystein residues [18] and hence these were considered as different from those allergenic 2S albumin proteins.

The SOSUI server classified two proteins (XP_004980234.1 and XP_004986765.1) as soluble and rest of seven as membrane proteins, Prediction of transmembrane helices play an important role in the study of membrane proteins. Features of transmembrane helices of these membrane proteins with their corresponding sequence, sequence length, type and other parameters were identified. The results of this analysis are tabulated in Table 5. All the seven membrane proteins were showing the presence of a single primary type of transmembrane helix except two (XP_004980741.1 and XP_004960634.1) in which along with primary type a secondary type of transmembrane helix

Table 1: Assigning of protein families, domains and patterns to seed storage proteins of foxtail millet.

SN	Seed Storage Protein ID	Name of Protein (as Available on NCBI)	Pfam Analysis	Assigned SCOP domains		Patterns by Prosite
				Superfamily(Domain region)	Family	
	XP_004980234.1	PREDICTED: 13S globulin seed storage protein 1-like	Cupin 1	RmlC-like cupins (47-396)	Germin/seed storage 7S protein	-
	XP_004961615.1	PREDICTED: 19 kDa globulin-like	Tryp alpha amyl(protease inhibitor/seed storage/LTP family)	Bifunctional inhibitor/ lipid transfer protein/seed storage 2S albumin (37-102, 146-171)	Proteinase/alpha amylase inhibitor	-
	XP_004979705.1	PREDICTED: kafirin PSKR2-like	Zein	-	-	-
	XP_004979702.1	PREDICTED: zein-alpha PMS1-like	Zein	-	-	-
	XP_004986765.1	PREDICTED: zein-alpha PZ22.1/22A1-like	Zein	-	-	-
	XP_004962290.1	PREDICTED: zein-alpha ZA1/ M1-like	Zein	-	-	-
	XP_004960933.1	PREDICTED: zein-beta-like	Tryp alpha amyl (protease inhibitor/ seed storage/LTP family)	Bifunctional inhibitor/ lipid transfer protein/seed storage 2S albumin (5-162)	Proteinase/alpha amylase inhibitor	N-6 Adenine specific DNA methylase signaure (172-178)
	XP_004980741.1	PREDICTED: LOW QUALITY PROTEIN: zein-alpha 19D1-like	Zein	-	-	-
	XP_004960634.1	PREDICTED: prolamin PPROL 17D-like	Glaidin(cys-rich gliadin N-terminal)	Bifunctional inhibitor/lipid transfer protein/ seed storage 2S albumin (4-125)	Seed storage protein 2S albumin	-

Table 2: Amino acids composition of seed storage proteins of foxtail millet compared with WHO recommendations of essential amino acids.

Protein Name	Ala	Arg	Asn	Asp	Cys	Gln	Glu	Gly	His	Ale	Leu	Lys	Met	Phe	Pro	Ser	Thr	Trp	Tyr	Val
	A	R	N	D	C	Q	E	G	H	I	L	K	M	F	P	S	T	W	Y	V
XP_004980234.1	10.0	4.4	2.2	5.4	1.5	2.7	4.9	10.0	2.2	4.6	8.1	6.4	2.9	4.9	5.4	7.1	5.9	1.5	1.7	8.3
XP_004961615.1	9.6	12.4	0.6	2.3	5.1	6.8	8.5	13.6	0	0.6	8.5	1.7	4.5	2.8	5.6	2.8	2.3	1.1	5.1	6.2
XP_004979705.1	19.4	1.0	6.2	0	0.3	18.1	0.3	0.7	0.7	3.8	19.4	0.3	0.7	4.2	9.0	6.2	2.8	0.7	2.4	3.5
XP_004979702.1	15.0	2.5	5.4	0	1.2	17.1	0.4	2.1	1.7	4.2	17.1	0.4	2.5	3.8	8.3	6.2	2.9	2.1	2.1	5.0
XP_004986765.1	16.1	0	5.5	0	0.8	16.5	0	1.3	2.1	5.1	16.5	0.4	1.3	4.7	6.8	7.6	5.5	1.7	1.7	6.4
XP_004962290.1	17.6	2.0	5.4	0	0	18.1	0.5	2.0	1.0	4.9	13.7	0.5	1.0	4.9	7.8	6.9	3.4	2.5	3.4	4.4
XP_004960933.1	17.8	3.9	0.6	1.7	5.6	12.2	1.1	7.2	0.6	0	10.6	0.6	5.6	0.6	10.6	6.1	2.2	2.2	5.6	5.6
XP_004980741.1	12.4	2.5	5.4	0.4	1.2	16.5	0.8	1.7	1.2	3.3	16.9	0.4	2.5	3.3	9.1	6.6	4.1	2.1	2.5	6.6
XP_004960634.1	10.9	3.6	0.7	0	8.8	10.2	4.4	7.3	2.9	2.9	10.2	1.5	4.4	1.5	6.6	8.1	5.5	2.2	4.4	4.4
FAO/WHO/UNU									1.5	3.0	5.9	4.5	1.6*	3.8**	-	-	2.3	0.6	**	3.9

*** Met+Cys, ** Phe+Tyr**

Table 3: Physico-chemical characterization of seed proteins of foxtail millet.

SN	Protein Name	a.a.	MW	pI	-R	+R	EC	II	AI	GRAVY
	XP_004980234.1	409	43939.5	8.12	42	44	43617.5	36.49	83.72	-0.032
	XP_004961615.1	177	19731.6	8.92	19	25	24660	56.81	62.88	-0.444
	XP_004979705.1	288	30949.9	9.30	1	4	21430	69.96	120.24	0.345
	XP_004979702.1	240	26426.9	9.81	1	7	35012.5	63.99	112.38	0.258
	XP_004986765.1	236	25494.6	7.88	0	1	28022.5	58.70	118.51	0.483
	XP_004962290.1	204	22437.7	9.77	1	5	37930	59.97	103.09	0.143
	XP_004960933.1	180	19145.2	8.34	5	8	37212.5	67.02	75.06	0.101
	XP_004980741.1	242	26804.4	9.26	3	7	36732.5	62.24	110.54	0.202
	XP_004960634.1	137	14951.3	7.48	6	7	14367	-	-	-

(Where, MW: Molecular Weight; pI: Isoelectric Point; -R: Number of negative residues; +R: Number of Positive residues; EC: Extinction Coefficient at 280 nm; II: Instability Index; AI: Aliphatic Index; GRAVY: Grand Average Hydropathicity)

was also noticed. The length of helices was in range of 21-23 a.a. and are rich in hydrophobic amino acids.

The identified transmembrane regions were visualized and analysed using helical wheel plots generated by EMBOSS Pepwheel tool and are shown in Figure 1. A helical wheel is a type of plot or visual representation used to illustrate the properties of alpha helices in proteins. It is useful for highlighting amphipathicity and other properties of the residues around the helix. Figures 1(a)-1(i) revealed that the hydrophobic amino acids are concentrated on one side of the helix. Rest of the other transmembrane regions exhibited intertwined amphipatic helices arrangement.

Knowledge of the subcellular localization of a protein can significantly improve target identification. Experimentally determining the subcellular localization of a protein is a laborious and time consuming task. Taget P server identified all the seed storage proteins to be secretary in nature, except protein XP_004980234.1. Secretary nature of the proteins was further confirmed with the help of Prot Comp 9.0 software. Investigating the sub-cellular localization of these protein revealed that these proteins were extracellular localized. Sub-cellular localization was further confirmed, through CELLO analysis. Apart from having extracellular location, these proteins were

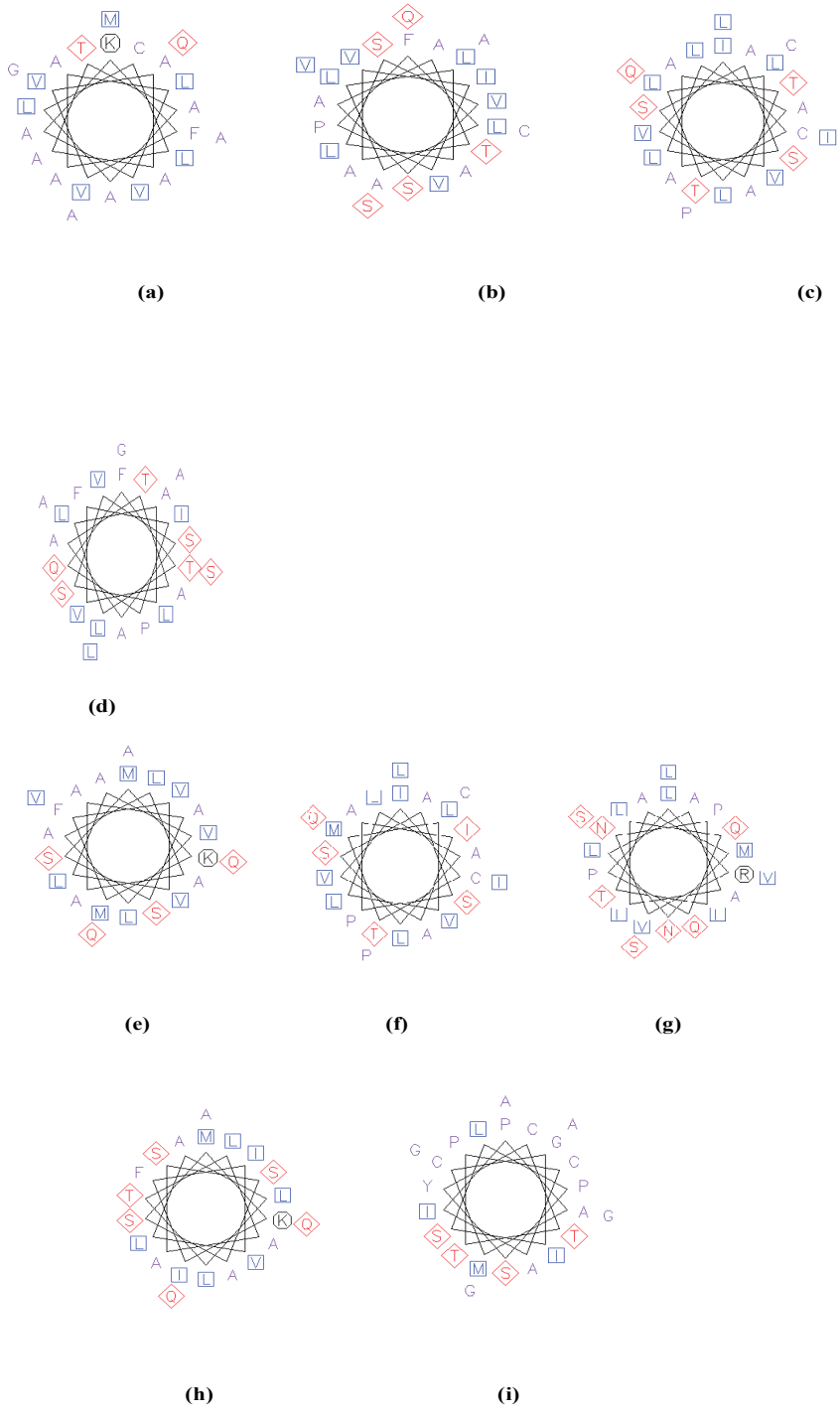

Figure 1: Helical wheel representations of predicted transmembrane helices of proteins (a) XP_004961615.1 (b) XP_004979705.1 (c) XP_004979702.1 (d) XP_004962290.1 (e) XP_004960933.1 (f) & (g) XP_004980741.1 (h) & (i) XP_004960634.1.

Table 4: Predicted disulphide bridge(s) present in seed proteins of foxtail millet.

S.N.	Protein Name	No. and Positions of Cysteine		Possible pairs of Cystine
1	XP_004980234.1	6	119, 233, 244, 288, 294, 329	-
2	XP_004961615.1	9	14, 38, 50, 79, 80, 90, 92, 161, 168	79-90, 80-92,
3	XP_004979705.1	1	27	-
4	XP_004979702.1	3	6, 27, 51	-
5	XP_004986765.1	2	50, 79	-
6	XP_004962290.1	-	-	-
7	XP_004960933.1	10	40, 49, 71, 78, 79, 91, 95, 155, 164, 168	40-168, 49-95, 71-78, 79-155, 91-164,
8	XP_004980741.1	3	6, 27, 58	-
9	XP_004960634.1	12	31, 39, 78, 82, 116, 124, 128, 58, 65, 66, 136, 137	31-65, 39-78, 58-124, 66-116, 82-128

Table 5: Predicted transmembrane regions present in seed proteins of foxtail millet.

SN	Protein	Type of Protein	Feature of Transmembrane Helix of Membrane Protein					Target P	Sub cellular Localization	
			Sequences of Transmembrane Helix	N-Ter -minal	C-Ter -minal	Type	Length		Prot Comp 9.0	CELLO
	XP_004980234.1	Soluble	-	-	-	-	-		ExC (S)	C
	XP_004961615.1	Membrane	KFVVAAATAALCLAALVAMAAGQ	3	25	Primary	23	-	ExC (S)	ExC
	XP_004979705.1	Membrane	FLALLALSVSAATAVIVPQCSVA	8	30	Primary	23	S	ExC (S)	N,ExC, PM
	XP_004979702.1	Membrane	ICTLLVLLALSASAATAVLIPQC	5	27	Primary	23	S	ExC (S)	ExC, PM, N
	XP_004986765.1	Soluble	-	-	-	-	-	S	ExC (S)	ExC, PM,
	XP_004962290.1	Membrane	FTLLALSVSAATAVFIPQGSLAA	9	31	Primary	23	S	ExC (S)	ExC, PM,
	XP_004960933.1	Membrane	MKMFVVLAVLALAAAASSAQQV	1	22	Primary	22	S	ExC (S)	ExC, N, PM,
	XP_004980741.1	Membrane	ICTMLVLLALSASPATAVLIPQC	5	27	Primary	23	S	ExC (S)	ExC, PM,
			LRVNPLTAMNLAALLQQPLVSS	220	241	Secondary	22	S		
	XP_004960634.1	Membrane	MKIFIVLALLTLAASSASAQQ	1	21	Primary	21		ExC (S)	ExC
			PAMCGISLPSYCTTPCAIAGGGA	113	135	Secondary	23	S		

Table 6: Predicted secondary structures present in seed protein of foxtail millet.

SN	Protein Name	a.a.	Alpha Helix (%)	Extended sheet (%)	Beta Turn (%)	Random Coils (%)
	XP_004980234.1	409	22.98	26.16	12.22	38.63
	XP_004961615.1	177	49.72	9.04	6.78	34.46
	XP_004979705.1	288	60.07	4.51	5.90	29.51
	XP_004979702.1	240	51.25	9.17	5.00	34.58
	XP_004986765.1	236	70.34	2.54	3.39	23.73
	XP_004962290.1	204	51.47	7.84	3.92	36.76
	XP_004960933.1	180	55.00	3.33	6.67	35.00
	XP_004980741.1	242	49.59	12.40	5.79	32.23
	XP_004960634.1	137	43.80	5.11	9.49	41.61

also shown to contain plasma membrane and nuclear localization signals, except for the protein XP_004980234.1 which was localized in chloroplast (Table 5). Determining subcellular localization is important for understanding protein function and is a critical step in genome annotation.

The secondary structures predicting programme SOPMA infers that seed storage proteins of foxtail millet have a mixed secondary structure of random coils, extended sheets, alpha helices and beta turns. But the frequency of these secondary structures varies between seed storage proteins and the results are presented in Table 6. In protein XP_004960634.1 percentage of alpha helix and random coils were almost equal. In protein XP_004980234.1 percentage of random coils was higher (38.63%) followed by extended sheet (26.16%), alpha helix (22.98%) and beta turn (12.22%). In rest of the seven proteins, the percentage of alpha helix was highest (ranging from 49.72% in protein XP_ 004961615.1 to 70.34% in protein XP_ 004986765.1) followed by random coils and extended sheets. Modern protein secondary structure prediction methods exploit the evolutionary information contained in multiple sequences.

From this study it is evident that the seed storage proteins of foxtail millet may be promising source to use as new food protein ingredient. The amino acid pattern of all the proteins were either *at par* or higher than FAO/WHO requirement. Since, seed storage proteins have been nutritionally and functionally valuable in the food industry, all the estimated nutritional parameters based on amino acids composition showed that foxtail millet seed proteins have a good nutritional quality and suggests their possible use as a supplementary protein source. Protein structure-function studies are valuable in modifying proteins for enhanced functionality. Hence, more basic applied research using actual food system is required. Further, the knowledge derived from this research can provide baseline data for crop breeders to produce superior or enhanced genetic lines of industrially important crops. The knowledge of common structural and physicochemical features of inherently allergenic protein families such as the 2S albumin could help to gain insight into the molecular basis of allergenicity, as well as to predict the potential allergenicity of novel and genetically modified foods.

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Author Affiliation

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Department of Molecular Biology and Genetic Engineering, College of Biotechnology, Sardar Vallabhbhai Patel University of Agriculture and Technology, Meerut-250 110, U.P, India