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Research Article

COMPUTATIONAL CHARACTERIZATION OF ANTIFREEZE PROTEINS OF *TYPHULA ISHIKARIENSIS* – GRAY SNOW MOULD

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Abstract: Organisms that live in cold climates have to confront a lot of obstacles due to ice crystal formation. As the temperature dips lower than sub-zero, ice crystals gradually develop inside the cell which leads to cell rupture. Antifreeze proteins (AFPs) are produced as a specialized adaptation by these psychrophiles that permit their survival in subzero environment. Antifreeze proteins produced by fungi are thought to bind onto ice crystals quickly using a regular array of well conserved residues/motifs and alter the structure of ice in or around the host thereby hindering growth and re-crystallization of ice. In this investigation, seven different antifreeze proteins (AFPs) of *Typhula ishikariensis* retrieved from Uniprot database were analyzed and characterized *in silico* using various computational tools. Their physico-chemical properties, hydropathicity and secondary structure have been identified. Phylogenetic analysis was also performed using MEGA4. The study might be an initiation in understanding the underlying structural and functional aspects of these proteins, their common characteristics and other features for academic and industrial purposes.

Keywords: Antifreeze proteins; Physico-chemical properties; *In silico* characterization; gray snow mould; *Typhula ishikariensis*

Introduction

Computational strategies and online servers are frequently used tools in protein sequence analysis and characterization (Sivakumar, 2005). The physicochemical and structural properties of proteins are well understood with the use of computational tools. In recent times, large number of computational tools has been developed for making reliable predictions regarding the identification and structural analysis of proteins (Sivakumar *et al.*, 2007; Floudas, 2007). Information about a protein such as amino acid sequence length and physicochemical properties such as molecular weight, atomic composition, extinction coefficient, GRAVY, instability index, aliphatic index, etc. can be analyzed by computational tools for the prediction and characterization of protein structures. The amino acid sequence can provide most of the information required for insight into protein physicochemical properties and its functions (Gasteiger *et al.*, 2005; Hossain, 2012). Sequence analysis and physiochemical characterization of proteins using computation biology tools have been widely reported (Sivakumar, 2006; King-Hwa *et al.*, 2007; Yuri *et al.*, 2003; Garry *et al.*, 2004; Bell *et al.*, 2003). The present investigation applied computational tools for a special class of proteins – the anti-freeze proteins (AFPs).

AFPs or ice structuring proteins (ISPs) refer to a class of polypeptides produced by certain plants, vertebrates, bacteria and fungi that permit their survival in subzero environments (Sharma *et al.*, 2014). Antifreeze proteins bind to small ice

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crystals to inhibit growth and recrystallization of ice that would otherwise be fatal (Jack *et al.*, 2004). There is also increasing evidence that AFPs interact with mammalian cell membranes to protect them from cold injury (Inglis *et al.*, 2006). The association of AFPs with cold acclimatization has also been suggested elsewhere (Snider *et al.*, 2000). Many researchers have purified and analyzed antifreeze proteins from a number of macromolecular organisms like plants, fishes and insects and also from fungi (Hew *et al.*, 1992). To date researchers have identified seven different AFPs from the fungi *Typhula ishikariensis* (Hoshino *et al.*, 2003b).

Fungal AFPs were discovered in snow moulds which have pathogenic activities against dormant plants under snow cover (Hoshino *et al.*, 2003a; Hoshino *et al.*, 2003b; Hoshino, 2005; Schneider *et al.* 1986). Fungal antifreeze proteins have been purified and partially characterized only in the species of *Psychrophillic basidiomycete* and *Typhula ishikariensis*. These fungi have the ability to attack plants. It is a plant pathogen that can be used to destroy turf grass when it is covered for a long interval of time with snow (Evans *et al.*, 2008). Also, it can damage crops of winter wheat at low temperature under persistent snow cover. The most important winter diseases of perennial grasses and winter cereals in the United States are *Typhula* snow moulds caused by *T. phacorrhiza*, *T. incarnata* and *T. ishikariensis*. The carbohydrate reserves are depleted during the winter dormancy, and the plant becomes less resistant to disease (Boeckmann, 2000). Hence investigation of antifreeze proteins from snow moulds could have agricultural and biotechnological applications in that these proteins could be targeted in pathogenic snow moulds so that they cannot survive in cold and thus harm winter crops. However, for this long-term aim to be fulfilled certain preliminary characterization of these proteins are necessary.

Materials and Methods

Retrieval of antifreeze protein sequences

Antifreeze protein sequences were retrieved from the manually curated public protein database Uniprot (Gill, 1989). The search result yielded 15 antifreeze protein sequences of different fungi. From this, we have selected 7 different antifreeze proteins from the fungal species *Typhula ishikariensi*, the subject of current investigation, and have organized a non-redundant data set (Table 1). The AFPs sequences were retrieved in the well-known FASTA format and used for further analysis.

Table 1
Antifreeze protein sequences of Typhula ishikariensis retrieved from public protein database Uniprot

S.No.	*Accession no.*	*Sequence description*	*Sequence length*
1	Q76CE6	Antifreeze protein K3-B1	243 AA
2	Q76CE4	Antifreeze protein K3-B3	243 AA
3	Q76CE3	Antifreeze protein K3-F	243 AA
4	Q76CE5	Antifreeze protein K3-B2	243 AA
5	Q76CE8	Antifreeze protein K1-A	243 AA
6	Q76CE2	Antifreeze protein K3-G	243 AA
7	Q76CE7	Antifreeze protein K1-C	243 AA

Computational tools and servers

The amino acid compositions (Table 2) of AFP sequences of *Typhula ishikariensi* were computed using the Expasy's ProtParam tool (http://us.expasy.org/tools/protparam.html) (Gasteiger *et al.*, 2005). The physico-chemical parameters, theoretical isoelectric point (pI), molecular weight, total number of positive and negative residues, extinction coefficient (Gill, 1989), half-life (Gonda *et al.*, 1989; Tobias *et al.*, 1991; Ikai, 1980), aliphatic index (Ikai, 1980), instability index (Guruprasad *et al.*, 1990), and grand average hydropathicity (GRAVY) (Kyte *et al.*, 1982) were computed using the Expasy's ProtParam prediction server and tabulated in Table 3. The secondary structural features were predicted using Self Optimized Prediction Method from alignment (SOPMA) (Geourjon *et al.*, 1994, Geourjon *et al.*, 1995) and GOR IV (Garnier *et al.*, 1996). It is used to describe secondary features such as sequence length, alpha helix, beta turn, random coil etc (Table 4). Phylogenetic tree was constructed based on neighbor-joining method (Saitou *et al.*, 1987) using Molecular Evolutionary Genetics Analysis (MEGA) software version 4 (Tamura *et al.*, 2007).

Table 2
Amino acid composition (in %) of antifreeze protein sequences of Typhula ishikariensis.

Amino acid residues	*K3-B1*		*K1-A*		*K3-B3*		*K3-F*		*K3-B2*		*K1-C*		*K3-G*	
	NO.	*%*	*NO.*	*%*	*NO.*	*%*	*NO.*	*%*	*NO.*	*%*	*NO.*	*%*	*NO.*	*%*
Ala (A)	31	12.8	38	15.6	32	13.2	31	12.8	32	13.2	36	14.8	33	13.6
Arg (R)	2	0.8	2	0.8	2	0.8	2	0.8	2	0.8	2	0.8	2	0.8
Asn (N)	5	2.1	5	2.1	5	2.1	5	2.1	5	2.1	4	1.6	5	2.1
Asp (D)	7	2.9	4	1.6	7	2.9	7	2.9	7	2.9	6	2.5	6	2.5
Cys (C)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Gln (Q)	8	3.3	8	3.3	8	3.3	8	3.3	8	3.3	9	3.7	7	2.9
Glu (E)	4	1.6	3	1.2	4	1.6	4	1.6	4	1.6	3	1.2	4	1.6
Gly (G)	31	12.8	29	11.9	31	12.8	31	12.8	31	12.8	31	12.8	32	13.2
His (H)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ile (I)	19	7.8	18	7.4	18	7.4	18	7.4	18	7.4	18	7.4	18	7.4
Leu (L)	21	8.6	22	9.1	21	8.6	21	8.6	21	8.6	22	9.1	22	9.1
Lys (K)	7	2.9	10	4.1	7	2.9	7	2.9	7	2.9	7	2.9	8	3.3
Met (M)	2	0.8	9.1	1.2	2	0.8	2	0.8	2	0.8	3	1.2	2	0.8
Phe (F)	8	3.3	9	3.7	8	3.3	8	3.3	8	3.3	8	3.3	8	3.3
Pro (P)	8	3.3	10	4.1	8	3.3	8	3.3	8	3.3	8	3.3	8	3.3
Ser (S)	28	11.5	22	9.1	28	11.5	27	11.1	27	11.1	25	10.3	27	11.1
Thr (T)	34	14	32	13.2	33	13.6	35	14.4	34	14	37	15.2	33	13.6
Trp (W)	3	1.2	3	1.2	3	1.2	3	1.2	3	1.2	9	1.2	3	1.2
Tyr (Y)	5	2.1	6	2.5	5	2.1	5	2.1	5	2.1	6	2.5	5	2.1
Val (V)	20	8.2	19	7.8	21	8.6	21	8.6	21	8.6	15	6.2	20	8.2

Table 3
Physico-chemical parameters of antifreeze protein sequences of Typhula ishikariensis computed using Expasy's ProtParam tool

Accession no.	*Molecular wt.*	*Aliphatic index*	*Theoretical pl*	*Instability index*	*Extinction Coefficient*	*Total no. of positive residues*	*Total no. of negative residues*	*GRAVY*
Q76CE6	24093.3	100.82	4.89	22.92	23950	9	11	0.537
Q76CE4	24049.3	100.82	4.89	22.92	23950	9	11	0.546
Q76CE3	24093.3	100.41	4.89	21.11	23950	9	11	0.536
Q76CE5	24063.3	100.82	4.89	22.92	23950	9	11	0.546
Q76CE8	24198.9	102.51	9.52	26.91	25440	12	7	0.607
Q76CE2	23989.3	101.65	6.51	20.1	23950	10	10	0.566
Q76CE7	24059.3	96.91	6.12	20.35	25440	9	9	0.517

Table 4
Secondary structural features of antifreeze protein sequences of Typhula ishikariensis using SOPMA

Accession no.	*alpha helix*		*extended strand*		*beta turn*		*random coil*	
	number	*percentage*	*number*	*percentage*	*number*	*percentage*	*number*	*percentage*
Q76CE6	45	18.52	68	27.98	20	8.23	110	45.27
Q76CE4	46	18.93	71	29.22	17	7	109	44.86
Q76CE3	49	20.16	69	28.40	16	6.58	109	44.86
Q76CE5	46	18.93	77	31.69	17	7	103	42.39
Q76CE8	51	20.99	75	30.86	22	9.05	95	39.09
Q76CE2	40	16.46	71	29.22	17	7	115	47.33
Q76CE7	49	20.16	73	30.04	26	10.7	26	39.09

Table 5
Secondary structural features of antifreeze protein sequences of Typhula ishikariensis using GORIV

Accession no.	*alpha helix*		*extended strand*		*beta turn*		*random coil*	
	number	*percentage*	*number*	*percentage*	*number*	*percentage*	*number*	*percentage*
Q76CE6	34	13.99	74	30.45	0	0.00	135	55.56
Q76CE4	32	13.17	74	30.45	0	0.00	137	56.38
Q76CE3	31	12.76	74	30.45	0	0.00	138	56.79
Q76CE5	32	13.17	74	30.45	0	0.00	137	56.38
Q76CE8	69	28.40	50	20.58	0	0.00	124	51.03
Q76CE2	40	16.46	74	30.45	0	0.00	129	53.09
Q76CE7	55	22.63	68	27.98	0	0.00	120	49.38

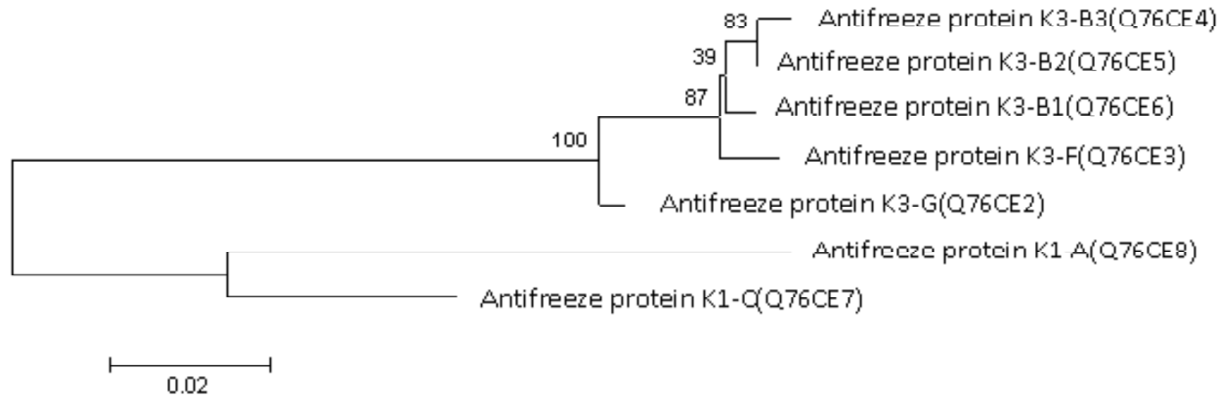

Figure 1: **Phylogenetic tree using antifreeze protein sequences of *Typhula ishikariensis* through Neighbor-Joining method using MEGA 4**

Results and Discussion

The amino acid sequences of seven antifreeze proteins of *Typhula ishikariensi* were retrieved from public protein database Uniprot having the accession numbers Q76CE6, Q76CE4, Q76CE3, Q76CE5, Q76CE8, Q76CE2, Q76CE7 (Table 1).

Physico-chemical characterization

Their amino acid composition (Table 2, Figure 2) and physico-chemical properties (Table 3) have been computed using the Expasy's ProtParam. Amino acid composition and physico-chemical properties determine the fundamental properties of the protein. The results of primary structure analyses suggest that all the antifreeze proteins of *Typhula ishikariensi* have the same amino acid composition (Table 2). Their conserved amino acid composition supports conserved and redundant functions. The large number of similar proteins provide back up copies in keeping with their significant function of protection against cold stress. All of the AFPs are hydrophobic in nature due to the presence of high content of non-polar residues (Val, Ala, Leu etc.) (Figure 2). The absence of cysteine residues indicates the absence of disulphide bridges (SS bonds) in these AFPs. Moreover, the primary structure analysis suggests that the AFPs have high percentage of aliphatic residues like Ala (31-38%), Gly (39-32%), Leu (21-22%) and Val (15-20%) and lower percentage of aromatic residues like Tyr (5-6%), Phe (8-9%) and Trp (3%) (Table 2). The hydrophobic nature of the AFPs might help them associate with membranes as well to prevent cold injury.

The computed isoelectric point for different AFPs of *Typhula ishikariensis* ranges from 4.89 to 9.52 (Table 3). It suggested that six of the antifreeze proteins are acidic in nature (Q76CE6, Q76CE4, Q76CE3, Q76CE5,Q76CE8 and Q76CE2) because their calculated pI value was less then 7 and one was basic in nature (Q76CE8) having pI value above 9 (Figure 3). Low pI and more

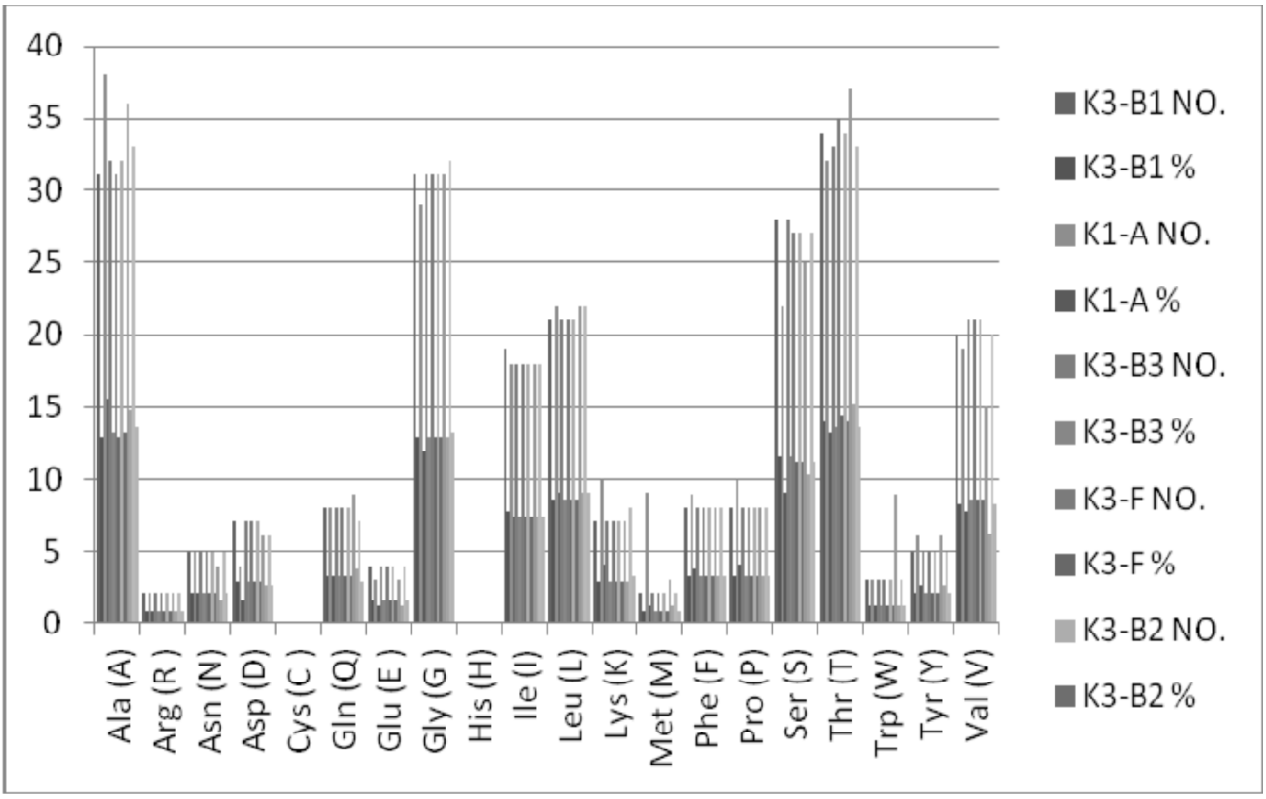

Figure 2: Amino acid composition (in %) of antifreeze protein sequences of Typhula ishikariensis.

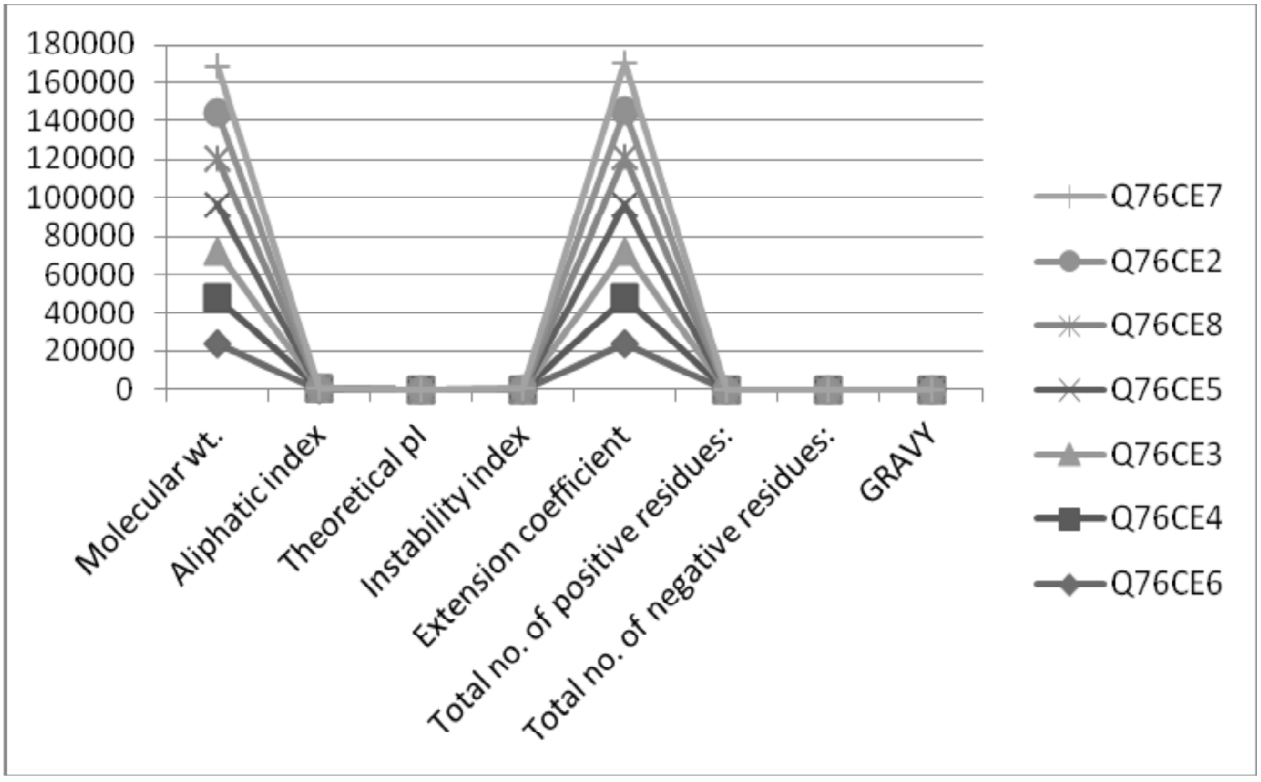

Figure 3: **Physico-chemical parameters of antifreeze protein sequences of Typhula ishikariensis computed using Expasy's ProtParam tool.**

positive charges might help them repel each other and thus prevent freezing.

The computed value of instability index lies between 21.11 and 26.91 indicating moderately stable proteins. It relies upon the occurrence of certain dipeptides along the length of the protein. The extinction coefficients of the proteins were also similar reflecting similar content of intrinsic chromophores. Aliphatic index of all the antifreeze proteins were in the range of 96.91 to 102.51. The aliphatic index refers to the relative volume of a protein that is occupied by aliphatic side chains. Higher aliphatic index of proteins indicated their structural stability (Gasteiger *et al.*, 2005). An increase in the aliphatic index increases the thermo stability of enzyme.

The grand average of 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 (Gasteiger *et al.*, 2005). Calculated GRAVY values of the proteins were found between 0.517 and 0.607 (Figure 3). A positive GRAVY value for proteins designates it to be hydrophobic in nature, as also observed above.

Secondary structural characterization

The secondary structural features of the antifreeze proteins were predicted by two online tools SOPMA and GOR IV showing that all the proteins had significant percentage of random coil and extended sheets followed by moderate content of alpha helices and beta turns (Table 4 and Table 5). The content of random coils and alpha helices predicted by the two online tools matched significantly while there was less agreement between the two tools in terms of the content of extended strands and beta turns. The results from both the tools thus revealed that in all the proteins random coils dominated among secondary structure elements (Figure 4). Such unstructured regions may be necessary for better binding to ice crystals preventing further crystallization or for binding other proteins essential for intracellular signalling (Muthukumaran *et al.*, 2011). Higher content of random coil along with absence of disulfide bridges may help the AFPs by not forming compact structures and giving more motion to them to prevent from freezing. More extended structure may also help sliding motion and their dynamics, all necessary for function.

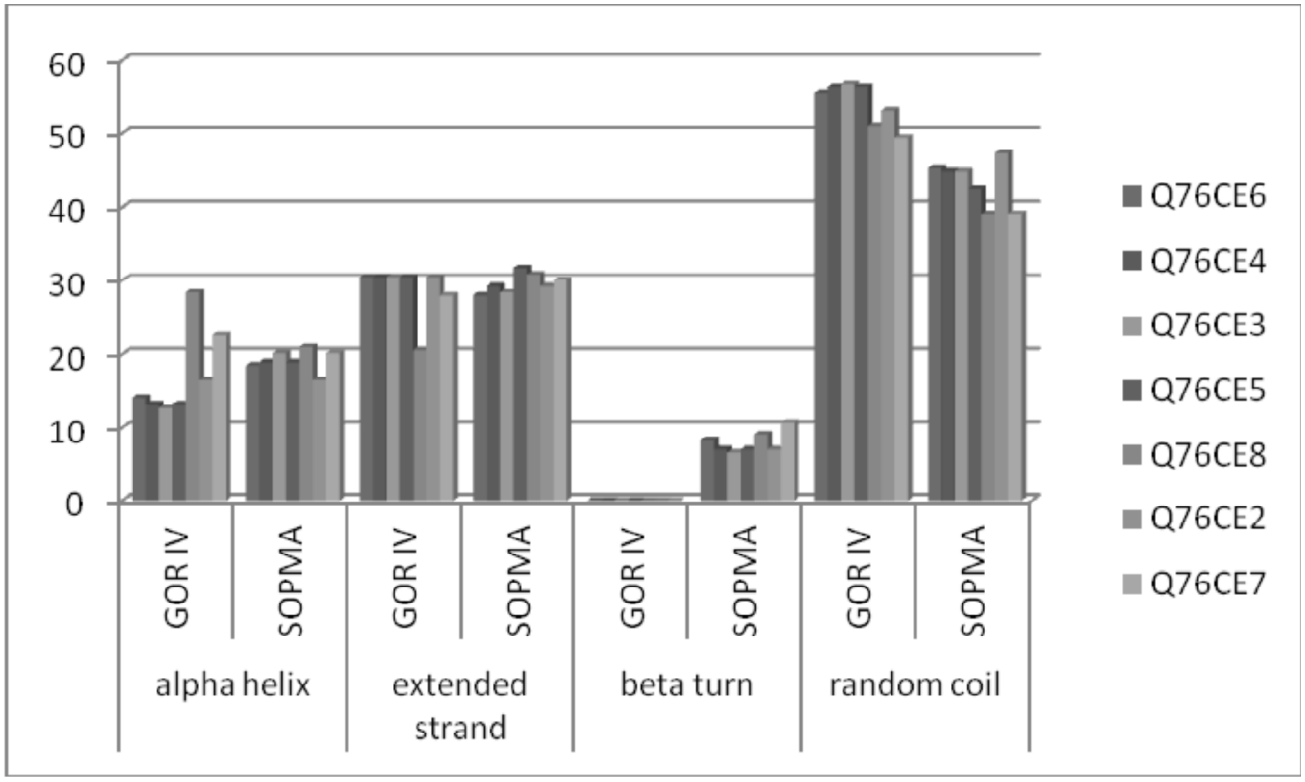

Figure 4: **Secondary structural features of antifreeze protein sequences of Typhula ishikariensis using GORIV and SOPMA**

Phylogenetic analysis

A phylogenetic tree was constructed using the protein sequences of *Typhula ishikariensi* (Figure 1) through Neighbor-Joining method using MEGA 4**, t**o clarify the phylogenetic relationship among all the proteins and infer their evolutionary history. *The tree represented the evolution of antifreeze proteins in fungi. The tree divided the sequences into two major clusters in which one cluster was again divided into two sub-clusters.* The tree has been evaluated by bootstrapping. *The analysis revealed that all the antifreeze proteins of Typhula ishikariensi* had originated from a common ancestor and during the course of evolution diverged further into sub groups. Antifreeze protein K1-A(Q76CE8) and K1-C(Q76CE7) belongs to a one sub group while antifreeze protein K3-B3(Q76CE4), K3-B2(Q76CE5), K3-B1(Q76CE6) and K3-F(Q76CE3) belongs to another sub group and were very close to each other. Antifreeze protein K3-G(Q76CE2) also belonged to the second sub group but on a different branch.

Conclusion

In the present study, an attempt has been made to perform analysis of sequence and structural features of seven antifreeze proteins of *Typhula ishikariensi* which provides insight into the ice-recrystallization inhibition process. The analyses *included their physico-chemical characterization, secondary structure prediction and phylogenetic analysis.* The dataset for AFPs was retrieved from UniProt, a public protein database. Computed pI value revealed that out of 7 AFPs, 6 antifreeze proteins were acidic in nature except one, which was basic. Their charge properties probably help the proteins to repel different segments of their polypeptide thus preventing freezing. This might also help the proteins in maintaining high content of unstructured secondary structure, which in turn helps to bind ice crystals to prevent freezing. A positive GRAVY value of the proteins and high content of apolar residues indicates their hydrophobic nature, which might be useful for their membrane association as well to prevent cold injury. The structural characteristics of the AFPs indicate their suitability as anti-freeze proteins and their high copy number, with redundant sequences and properties, maybe essential as back up copies for a significant function of survival under cold stress.

Based on these results it can also be concluded that all the antifreeze proteins may comprise of same structural and functional properties and they all could be disrupted using the same strategy in snow mould so that this fungus cannot grow in winter and so cannot destroy essential crops. However, one of them has a basic pI and can be uniquely targeted for disruption as well. We hope that *the current research will trigger further detailed understanding of* the structural and functional aspects of these proteins, which in turn will be of importance for commercial applications.

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