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In silico Characterization of Silk Fibroin Protein using Computational Tools and Servers

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Abstract : In this paper, ten different silk fibroin proteins (SFs) retrieved from Swiss-Prot database are analyzed and characterized using *In silico* tools. Primary structure analysis shows that all the SFs are hydrophobic in nature due to the high content of non-polar residues. The presence of very few or lack of cystein in SFs shows that disulphide links are absent in the silk fibroin proteins. The presence of extensive hydrogen bonds may provide the stability to protein in absence of disulphide bonds. The aliphatic index computed by ExPasy's ProtParam infers that SFs may be stable at wide range of temperature. Secondary structure analysis shows that most of the SFs have predominant ∝-helical structure, some have β-helical structure and the rest shows mixed secondary structure. The very high coil structural content of wild silk moth, nursery web spider and earth bumble bee is due to the rich content of highly flexible glycine and hydrophobic alanine amino acids. SOSUI server predicts one transmembrane region in wild silk moth, silk moth and pine moth SFs. The predicted transmembrane regions were visualized and analyzed using helical wheel plots generated by EMBOSS pepwheel tool. The absence of disulphide bonds in SFs were confirmed by SYC_REC tool and from 3- Dimensional structure created by Rasmol tool. The cytein position identified by Rasmol tool might be correct as the evaluation parameters are within the acceptable limits for the modeled 3D structure.

Key words : Silk fibroin protein; Computational analysis; Homology modeling; Proteomic tools.

Introduction

Protein sequencehi analysis and characterization is one of the challenges in front of bioinformatics, as p rotein sequence repository increases exponentially. Computational packages and online servers are the current tools used in the protein sequence analysis and characterization. Computational tools provide researchers to understand physicochemical and structural properties of protein. A large number of computational tools are available from different sources for making predictions regarding the identification and structure prediction of proteins. The statistical parameters about protein sequence, such as sequence length, number of amino acids and the physicochemical properties of a protein such as molecular weight, atomic composition, extinction coefficient, isoelectric point, GRAVY, aliphatic index, instability index etc. can be computed by various computational tools for the prediction and characterization of protein structure. The amino acid sequence provides most of the information required for determining and characterizing the molecule's function, physical and chemical properties. Many researches reported the sequence analysis characterization of proteins using biocomputation tools (Sivakumar *et al*., 2007; King-Haw Ling *et al*., 2007; Chitta *et al*., 2005; Yuri *et al*., 2003; Rachel *et al*., 2003;

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Lesperson., 1937; Rudall, 1956). Silk is a fibrous protein secreted by labial glands in lepidoptera, hymenoptera or by accessory tubes of the genital organs of hydrophilus, by tarsal glands of embioptera and embidae, and by malpighian tubes of certain blatella, a variety of insects and arachnidan (Kaplan *et al*., 1994). The native protein of silk gland "fibrinogen" is water soluble, while passing through the spinnerets it become a tough insoluble products "fibroin" in which the molecule assume an orderly crystalline arrangement in the long axis of the fiber. The silk, which is coated with sircin, a family of glue like proteins that hold the fibroin core together. The silk fibers have been used for decades as sutures in biomedical application (Moy *et al.*, 1991) and have potential as scaffolds in tissue engineering (Min *et al*., 2004; Dal *et al*., 2003). Many report the other applications of silk fibroin in medical, agricultural fields (Tsubouchi., 2003; Sugihara *et al*., 2003; Meinel *et al*., 2005). Numerous structure and function studies have been reported from time to time from all over the world (Wang *et al*., 2007; Wang *et al*., 2008; Yang *et al*., 2007; Lovett *et al*., 2002, Gobin *et al*., 2006; Cheema *et al*., 2007).However physico-chemical characterization of silk fibroin (SF) has not been done so far. In this paper, we have report the *In silico* analysis and characterization from studies on 10 silk fibers protein, Silk fibroin (SF) of various insects and spider.

Material and Methods

Silk fibroin sequences

Silk fibroin sequences were retrieved from the manually curated public protein database SwisProt (Boeckmann *et al.*, 2003). SwisProt is scanned for the key word silk fibroin (SF). The search result yields 37 silk protein sequences including insects, ants and

Table 1 : Silk fibroin sequence retrieved from TrEMBL database

Accession number	Sequence description	Organism
Q967G5	Protein fragment	Mediterranean flour moth (*Anagasta kuehniella*)
Q8ISB3	Protein fragment	Tsar silk moth (*Antheraea mylitta*)
Q9BLL9	Protein L-chain	Wild silk moth (*Bombyx mandarina*)
A8IM39	Protein	Earth bumble bee (*Bombus terrestri*)
Q8IT50	Protein L-chain	Silk moth (*Bombyx mori*)
Q9BLL6	Protein p25	Pine moth (*Dendrolimus spectabilis*)
Q9BIU3	Protein II fragment	Nursery web spider (*Dolmedus tenebresus*)
Q9GUB4	Protein H-chain (fragment)	Wax moth *(Galleria mellonella)*
Q4F6Z3	Protein fragment	Northern candishflies (*Latrodectus Hesperus*)
A8IM76	Protein	Bull dog ant (*Myrmecia formicata*)

spiders. From this, we retrieved 8 different insect species, one spider species and one ant species by random selection and have organized a non-redundant data set (Table1). The SFs were retrieved in FASTA format and used for analysis.

Computational tools and servers

1. The amino acid composition (Table-2) of SFs sequences were computed using the tool CLC free workbench (http://w.w.w..clcbio.com/index.php?id=28). Percentages of hydrophobic and hydrophilic residues were calculated from the primary structure analysis results and tabulated in table-3. The physicochemical parameters, theoretical isoelectric point (Ip), molecular weight, total number of positive and negative residues, extinction coefficient (Gill and Hippel,1989), half-life(Bachmair *et al.*,1986; Gonda *et al.*, 1989; Tobias *et al.*, 1991; Ciechanover and Schwartz,1989), instability index (Guruprasad,1990), aliphatic index (Ikai,1980) and grand average hydropathy (GRAVY) (Kyte and Doolottle,1982) were computed using the Expasy's ProtParam(http://us.expasy.org/tools/protparam.html) prediction server and tabulated in Table-4. The tools SOPM, SOPMA (Combet *et al.*, 2000) and secondary structural content prediction (SSCP Method-1) server (Eisenhaber *et al.*, 1996) were used for the secondary structure prediction. The SOSUI (Takatsugu *et al.*, 1998) servers performed the identification of transmembrane region (table 5). The predicted transmembrane helices were visualized and analyzed using helical wheel plots (fig 2) generated by the program Pepwheel (Ramachandran and Sasikharan., 1968) included in the EMBOSS 2.7 suit. The presence of disulphide bridges ("SS" bonds) in SF IT84 and 1C1G is predicted by two methods. The first method involves the prediction of "SS" bonds using the primary structure (protein sequence data) by the tool

Table 2 : Amino acid composition (in %) of silk fibroin computed using CLC free workbench tool

Amino acids	Accession number									
	Q967G5	**Q8ISB3**	**Q9BLL9**	**A8IM39**	**Q8IT50**	**Q9BLL6**	**Q9BIU3**	**Q9GUB4**	**Q4F6Z3**	**A8IM76**
Ala	14.3	34.5	14.1	35.2	13.7	5.5	27.2	19.4	24.7	30.6
Cys	0.0	0.4	1.1	0.3	1.1	4.6	0.1	0.7	0.0	0.2
Asp	0.6	5.5	6.5	1.2	6.5	7.3	0.1	1.4	0.9	2.0
Glu	2.5	2.6	1.9	9.2	1.9	4.1	0.4	1.1	0.8	9.3
Phe	0.0	0.6	3.1	0.3	3.1	9.6	0.3	0.5	3.9	0.0
Gly	17.4	21.9	8.8	1.8	8.4	1.8	38.2	28.9	6.4	7.7
His	0.0	2.2	1.9	0.0	1.9	0.9	0.0	0.0	0.0	0.2
Ile	5.5	1.4	8.0	3.7	7.6	6.4	0.9	3.8	2.8	4.5
Lys	0.0	0.6	1.9	7.3	1.9	5.9	0.0	0.2	0.0	6.1
Leu	0.8	1.6	7.6	6.7	7.6	11.0	3.0	5.6	5.8	5.9
Met	0.0	0.8	0.8	0.6	0.8	1.4	0.1	0.0	0.0	0.9
Asn	8.7	0.6	6.9	2.1	6.9	8.2	0.9	0.5	2.9	2.9
Pro	5.0	0.8	3.4	1.8	3.8	4.6	0.7	5.2	2.3	0.9
Glu	2.5	2.6	1.9	9.2	.1.9	4.1	0.4	1.1	0.8	9.3
Arg	1.9	4.5	3.8	3.4	3.4	6.4	0.3	2.5	1.0	4.5
Ser	25.5	10.1	9.2	14.1	9.2	4.6	9.4	18.1	25.7	11.8
Thr	1.2	2.0	3.1	3.1	3.4	5.5	1.9	2.9	5.3	2.3
Val	3.7	3.0	7.3	4.6	7.6	4.6	2.7	7.4	5.5	4.8
Trp	0.6	0.8	0.8	0.6	0.8	0.9	0.0	0.5	0.0	1.8
Tyr	3.1	4.7	4.2	0.3	4.2	6.8	5.2	0.5	1.7	0.2

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. The second method involves the visualization and identification of "SS" bonds using the three dimensional structure of protein (3D coordinate data). The 3D structure of SF Q8IT50 and Q9BLL6 were generated by homology modeling using Esypred server (Lambert; 2002). The similar 3D structure for (SFs Q8IT50 and Q9BLL6 sequences) in the protein data bank (www.rscb.org) were identified by BLAST analysis (*http://w.w.wncbinlmnih.gov.80/BLAST/*). The modeled 3D structures were evaluated using the online server, Rampage (Lovell *et al.*, 2002), ProQ (Cristobal *et al.*, 2001) and CE (combinatorial Extension) (Ilya and Philip, 2001). The tool Rasmol (*http://openrasmol.org/*) is used to visualize the modeled 3D structures and to identify the cystein and presence of "SS" bonds. The three-dimensional structure of SFs Q8IT50 and Q9BLL6 modeled using the PDB template IT84 and 1C1G are shown in Figures 3 and 4,

Table 3 : Hydrophilic and hydrophobic residues content

Accession number	Percentage of Hydrophilic residues	Percentage of Hydrophobicresidues	Net hydrophobic residue content
Q967G5	44.1	55.9	High
Q8ISB3	54.6	70.1	Very high
Q9BLL9	41.4	58.1	High
A8IM39	44.4	55.6	High
Q8IT50	41.9	58	Very high
Q9BLL6	47.5	51.6	Very high
Q9BIU3	21.5	78.3	Very high
Q9GUB4	42.5	57.3	Very high
Q4F6Z3	46.9	53.1	Very high
A8IM76	28.3	71.8	Very high

Table 4 : Parameters computed using Expasy's ProtParam tool

Accession number	Sequence length	M.wt	pI	-R	+R	EC	II	AI	GRAVY
Q967G5	161	15012.9	4.59	5	3	12950	41.73	71.12	-0.138
Q8ISB3	507	45401.8	5.05	4	2	57885	20.89	54.64	-0.010
Q9BLL9	262	27638.8	5.06	2	1	27515	34.04	96.18	0.050
A8IM39	327	31980.8	7.71	3	2	12490	44.10	89.02	0.089
Q8IT50	262	27651.9	4.91	2	1	27515	34.29	96.91	0.069
Q9BLL6	219	25966.0	7.97	2	2	33975	29.90	86.39	-0.085
Q9BIU3	691	56175.4	4.41	4	2	53640	12.90	50.42	0.096
Q9GUB4	443	37197.4	7.94	1	1	14105	29.52	77.99	0.451
Q4F6Z3	1058	10551.0	4.45	1	1	26820	55.96	74.11	0.233
A8IM76	441	43377.2	5.7	5	4	45490	26.36	85.10	-0.034

M.wt; Molecular weight; pI, Isoelectric point; -R, Number of negative residues; +R, Number of positive residues; EC, Extension coefficient at 280 nm; II, Instability index; AI, Aliphatic Index; GRAVY, Grand Average Hydropathy

Table 5 : Transmembrane region identified by SOSUI server

Accession number	Transmembrane region sequence	C-terminal	Type	Length
Q9BLL9	KPIFLVLLVATSAYAAPSUTTNQ	24	Primary	23
Q8IT50	KPIFLVLLVVTSAYAAPSVTINQ	24	Primary	23
Q9BLL6	MILKLFFIFLCFGLCWAVEDPNI	23	Primary	23

respectively. The cystein position produced by CYS_REC tool and Rasmol tool in the SFs Q8IT50 and Q9BLL6 is shown in the table-6.

Results and Discussion

The result of primary analysis suggests that most of the SFs are hydrophobic in nature due to the presence of high non-polar residues content (Table 2 and 3). The presence of 4 Cys residues in SFs Q8IT50 (1.1% of Cys) (Silk moth) and 3 Cys residues in SFs A8IM39 (0.3% Cys) (Earth bumble bee) indicates the absence of disulphide bridges ("SS" bonds) in these SFs. More over primary structure analysis suggest that the SFs Q967G5 lacks aromatic amino acid, (Phe, Hist), A8IM39 lacks (Hist), Q9BIU3 lacks (Hist, Trp), QGF623 lacks (Hist, Trp) and A8IM76 lacks (Phe). The average molecular weight of SFs calculated is 41095 Da. 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 Q967G5, Q8I5B3, Q9BLL9, Q8IT50, Q9BIU3, Q4F623 and A8IM76 (pI<7) indicates that these SFs are acidic and the pI of A8IM39, Q9BLL6 and Q9GUB4 (pI > 7) reveals that these are basic in character. The computed isoelctric point (pI) will be useful for developing buffer system for purification by isoelectric focusing method. ProtParam of Expasy computes the extinction coefficient (EC) for a range of (276,278,279,280 and 282nm) wavelength, 283 nm is favored because proteins absorb strongly there while other substances commonly in protein solution, do not. Extinction coefficient of SFs at 280 nm is ranging from (12490 to 57885 M^{-1} cm^{-1}) with respect to the concentration of Q8ISB3 and Q9BIU3 indicates presence of high concentration of Tyr and Trp. Thus the high extinction coefficient is only due to the high concentration of Tyr and Trp and not due to the Cys because Cys is very low in concentration in all the 10 SFs selected. This indicates that these SFs cannot be analyzed using UV spectral methods. The computed protein concentration and extinction coefficients help in the quantitative study of protein-protein and protein-ligand interactions in solution. The biocomputed half- life of most of the SFs is greater than 25 hrs. The half-life is only 0.8 hrs for Q967G5 and Q4F623 and 2.8 hrs for SFs Q9BIU3. On the basis of instability index Expasy's ProtParam classifies the A8IM39 (Earth bumble bee) and Q4F623 (Northern Candishflies) SFs as unstable (Instability index >40) and other SFs as stable (Instability index <40). The aliphatic index (AI) which, is defined as the relative volume of a protein occupied by aliphatic side chain (A, V, I and L) is regarded as a positive factor for the increase of thermal stability of globular proteins. The lower thermal stability of Q8ISB3 and Q9BIU3 is indicative of a more flexible structure when compared to other SFs (table 4). The very high aliphatic index of all SFs infers SFs may be stable for a wide range of temperature. Grand Average Hydropathy (GRAVY) index of SFs are ranging from -0.010 to 0.451. The very low GRAVY index of SFs Q8ISB3, A8IM76 infers that these SFs could results in a better interaction with water. The secondary structure predicted with the help of programs SOPM and

Table 6 : Disulphide (SS) bond pattern of pair predicted by CYS_RE (Using primary structure) and identified Rasmol (Using 3D structure modelled)

Accession number	CYS_REC	Score	Rasmol	Bond
Q8IT50	Cys_101	-19.7	Cys_137	No SS- bond
	Cys_160	-25.7	Cys_84	No SS- bond
	Cys_190	-5.5	Cys31	Probably no SS- bond
A8IM39	Cys_101	-19.7	Cys_158	No SS- bond
	Cys_160	-25.7	Cys_142	No SS- bond
	Cys_190	-5.5	Cys190	Probably no SS- bond

Scan-window size-13

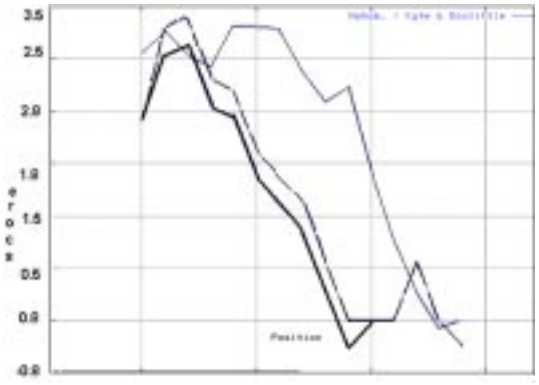

Fig. 1 : Kyte & Doolitle mean hydrophobicity profile computed for the transmembrane region of SFs A8IM78, Q8IT50 and Q9BBL9 (PRIMARY)

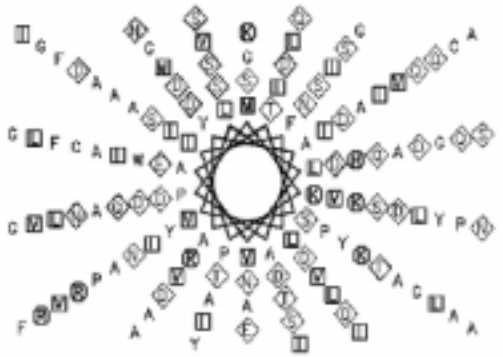

Fig. 2 : Helical wheel representation of predicted helix of Q8IT50 9 (silk moth SF)

Table 7 : PDB template (first two hits with maximum % of identity) obtained Using BLASTP search against the protein data bank

Accession number	PDB code
A8IM39	1C1G_A
Q8IT50	IT84_S

Table 8 : Validation parameters computed for the built 3D structure

Target	Template (PDB) code	Rampage percentage residues in the region of			CR MSD (A°)	ProQ	
		Favored	Allowed	Outlier		LG score	Maxsub
Q8IT50	IT84_S	76.7	18.7	4.6	0.5	2.789	0.217
A8IM39	1C1G_A	90.1	8.6	1.3	0.5	1.441	0.174

Table 9 : Criteria for a good (model) 3D structure

Rampage percentage of residues in favored region	CE RMSD (A°)	ProQ		Quality of the model
		LG score	Maxsub	
98	<2	>1.5	>0.1	Fairly good model
		>2.5	>0.5	Very good model
		>4	>0.8	Extremely good model

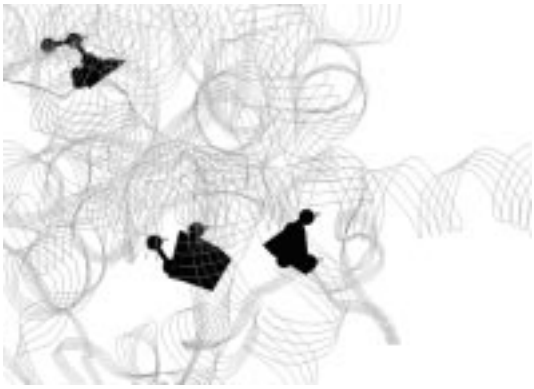

Fig. 3 : Rasmol (strands) representation of the homology modeled 3D structure of silk fibroin Q8IT50(Using PDB template IT84_S). The three cystein are shown as ball and stick models. All the cysteins are unpaired as no SS- bond is visible

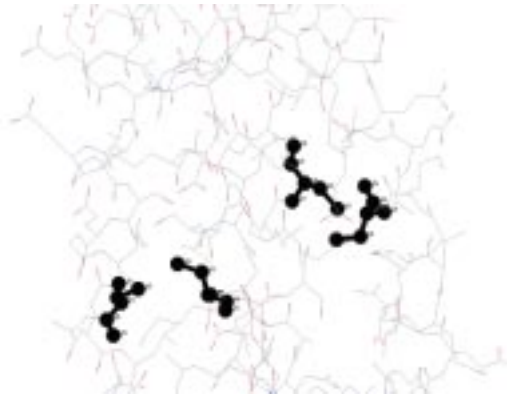

Fig. 4 : Rasmol (wire frame diagram) representation of the homology modeled 3D- structure of Silk fibroin A8IM39 (Using PDB template 1C1G_A) The cysteins are shown as ball and stick model. All the four cysteins are unpaired as no SS- bond is visible

SOPMA (Data not provided) infer that the SFs Q8ISB3 (Tsar silk moth) Q9UB4 (Wax moth) Q4F623 (Northern candish flies) and A8IM76 (Bull dog ant) have rich alanine content and mostly α-helices. SFs Q967G5 (Mediterranean flour moth), Q9UB4 (wax moth) and A8IM76 (bull dog ant) have mixed secondary structure i.e. α-helices and β-strands and coils. The very high coil structural content of Q9BIU3 (nursery web spider) 100%, Q9BLL9 (wild silk moth) 91.7% and A8IT39 (earth bumble bee) 90.4% is due to the rich content of more flexible glycine and hydrophobic alanine amino acids. The server SOSUI classifies the pine moth SFs Q9BLL6 and bull dog ant SF A8IM76 and other SFs as soluble proteins. SOSUI server has identified one transmembrane region in Q9BLL9, Q8IT50 and Q9BLL6. The transmembrane region and their length and types are tabulated in table-5. The transmembrane regions are rich in hydrophobic amino acids and it is also well documented Kyte and Doolittle (1982) meanhydrophobicity profile (Fig. 1) in which all the points are above the "0.0" line. The helix of Q9BLL9 visualized using EMBOSS Pepwheel is shown in Fig. 2. The tools CYR_REC recognize the presence of 4 cystein in SFs A8IM39 and 3 in Q8IT50 and predicted no "SS" bond pattern of pairs in both of the proteins. Though cystein is very low the protein attains the stability because of the extensive hydrogen bonding. The position of the cystein recognized by CYS_REC and Rasmol in the SFs A8IM39 and Q8IT50 are shown in

the table 6. The three dimensional structure of SFs A8IM39 and Q8IT50 were modeled using various PDB template (Table-7) selected from the hits obtained through BLASTP analysis and the modeled structures were evaluated. According to evaluative analysis, the Ramachandran plot and other parameters (Table-8) were within the standard acceptable limits for the 3Dstructure modeled using the PDB template 1C1G_A and IT84_S. Criteria for good 3D structure is identified using the three dimensional structure is given in table 9. The cystein identified using the three dimensional structure of SFs A8IM39 and Q8IT50 are shown in figure 3 and 4 respectively. The lacks of correlation between the position identified by CYS_REC and Rasmol might be due to the fact that CYS_REC identified Cystein position from primary structure. We speculated that the cystein position identified by Rasmol might be correct.

Conclusion

Ten silk proteins (fibroin) have been chosen mainly to study their physicochemical properties, primary and secondary structure by using computational tools and servers. Primary structure analysis reveals that most of the SFs under study are hydrophobic in nature and not contain any disulphide bridges. Physicochemical characterization studies give good idea about the properties such as pI, CE, AI, GRAVY and instability index that are essential and vital in providing data about the proteins and their properties. Secondary structure analysis predicts that most of these contain only α-helices, one contain irregular and remaining of these contain mixed structure. The absence of disulphide bridges is confirmed by using CYS_REC and Rasmol tools.

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