Conserved changes in secondary structure and aggregation properties of *in vitro* evolved proteins for thermo stability

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Abstract

Most of the screening strategies of directed evolution involved in thermo stability deals with aggregation of proteins either directly or indirectly. Here in this work I investigated what happens in aggregation property and secondary structure of the protein when it improved its thermo stability by incorporating certain amino acid changes in the protein. To study these changes I picked randomly 12 different proteins and I analyzed their 25 different thermo stable mutants. I used open access online Software to get the aggregation propensity values and values for different secondary structure elements propensities of proteins. I compared the aggregation propensity and predicted secondary structure values of thermo stable mutants with their parent Wild type proteins. The stable mutants followed three different conserved patterns to improve their thermo stability.

Introduction

There are different strategies employed to improve the thermo stability of the protein like modifications, oligomerisation, domain shuffling, protein engineering. Among these strategies protein engineering is widely used for its promising positive results for thermo stability improvement in proteins which are having industrial and therapeutic applications. Most of the screening strategies of protein engineering involved in thermo stability deals with aggregation of proteins either directly or indirectly because, most of the proteins upon thermal denaturation will undergo to an irreversible aggregation. There is an unbreakable link in between the aggregation property and thermal melting of the protein. When you change the amino acids in proteins, along with changes in aggregation propensity values there will be a definite or slight changes will happened in the secondary structure of the protein.

Here in this work I want to study is this changes are following any trend among the thermo stable mutants. So in this study I compared trends in different parameters either decreasing or increasing.

To study these changes I used Online Software to get the aggregation propensity values and predicted propensity values for different secondary structure elements of proteins. I compared the aggregation propensity and predicted secondary structure values of thermo stable mutants with their parent Wild type proteins which are less stable than mutants at given temperature. For this study, I picked randomly 12 different proteins and their 25 different thermo stable variants (5,6,7,8,9,10,11,12,14).

Methods and Material

Proteins Used in this Study

	Parent Protein	**Mutants**	**Mutations**	**Tm(in Degree Celsius)**
1	*Bacillus subtillis* Lipase			55.5
		6B	A15S, F17S, A20E, N89Y, G111D, L114P, A132D, I157M and N166Y M134E, M137P and S163P	75.6
2	Pseudomonas Stutzeri Phosphonate Dehydrogenase			39.7
		OPT14	V71I,T101A,E130K,Q132R,Q137R,T146S,I150F,Q215L,R275Q, L276Q,I313L,V315A,A319E,A325V	64.4
3	*Candida Antarctica* Lipase			
		CALB7	P218N,L219K,F220T,V221S	7 Folds High activity

				than WT at 60degree
		CALB 168	A57T,T89A,G226R,R168K	14 Folds Activity than WT at 60 degree
4	HHV8P Protein ORF49			48.05
		ORF49-1	K8N, A36T, K186R	57.8
		ORF49-2	L90I, E116Q, A155V, G157D, T239I	51.92
		ORF49-3	Q207H	53.08
		ORF49-4	V218I, C294Y	50.66
		ORF49-5	A155V, D278N, Q302E	51.3
		ORF49-6	K119E, L125V, Q207H	51.82
		ORF49-7	K187E, T193S	57.65
		ORF49-8	V218I, R273S	54.43
		ORF49-9	Q207H, K242I	53.45
5	*Serratia* sp.strain MK1 Phosholipase A1			70
		TA3	Q32P,T39A, E105V,M153I,S158I	77
		TA13	E35Q, S101N, M153K, L157W,L280Q,K290N	81
6	HHV1 capsid Triplex protein UL18			54.43*(Tagg)
		UL18-1	S212L	60.65*
		UL18-2	S212L, T247M	61.03*
7	*Thermus thermophilus* Beta-glucosidase			3.8 ± 0.4(t1/2)
		HF5	S266P A285Y, H321D, F395Y, L403I	17.9 ± 0.8
8	*Escherichia coli*			50.5

	and Edwardsiella ictaluri Fructose-bisphosphate aldolase			
		4-43D6	Multiple Mutants	61.7
9	HHV11 Tegument protein UL14			44.23
		UL14-1	V77D, V81L, A85G	54.53
10	*Streptomyces aureofaciens* Non-heme bromoperoxidase BPO-A1			80.6
		HT117	R114H,N146H	82
		HT507	G106S,V148I	84.5
11	NUD18 HUMAN 8-oxo-dGDP phosphatase			40.27
		NXR1-1	V45M, E91V	66.85
		NXR1-2	E95K, S107F	55.76
12	*Bacillus subtilis* Para Nitro Benzyl Esterase			52.5
		6sF9	I60V,L144M,L313F,H322Y,A343V,M358v,Y370F,G412E,I437T	66

Software Used in this Study

Four different software freely available online software were used in this study. Those are TANGO, AGGRESCAN, PASTA2.0 and CHOUFASMAN.

http://tango.crg.es/protected/academic/calculation.jsp

http://protein.bio.unipd.it/pasta2/

http://cho-fas.sourceforge.net/

http://bioinf.uab.es/aggrescan/

For total protein aggregation propensity values, TANGO Agg and Amylo values, AGGRESCAN a3vSA value and in PASTA2.0 number of Amyolids and for Secondary structure prediction TANGO Secondary structure propensities, PASTA Percentage of different secondary structure elements, CHOUFASMAN Percentage of different secondary structure elements were taken(1,2,3,4). Every software I used at their default settings. For analyzing the Local aggregation propensity value changes at the mutated regions I used TANGO (helix agg or Beta agg) and AGGRESCAN (a3v) (10, 12). To check local secondary structure values TANGO

Helix propensity at the mutated region or Beta sheet propensity or Turn propensity and CHOUFASMAN were used. To check aggregation prone regions in the protein an open access tool WALTZ was used (Data not shown). For a mutant out of three different values from different software which two give a similar trend like either decrease or increase when compared with wild type that trend was considered for that mutant.

When there are three different software values for a given mutant, TANGO values of aggregation propensity and secondary structure prediction propensities were taken consideration or the changes in these parameters at that particular mutated region were analyzed.

Results and discussion

Lipase

Table-1: Predicted secondary structure propensities in WT and Thermo stable Mutant (6B)

	WT	**6B**		**WT**	**6B**		**WT**	**6B**
CFSSP Helix	55.2	49.2	**PASTA Helix**	27.62	20.99	**TANGO Helix**	37.4528	33.2371
CFSSP Beta	43.6	43.1	**PASTA Beta**	26.52	28.73	**TANGO Beta**	401.536	353.099
CFSSP Turn	10.5	11.6	**PASTA Coil**	45.86	50.28	**Tango Turn**	140.245	150.232

Table-2 Predicted aggregation propensity values of WT and Thermo Stable Mutant (6B)

	WT	**6B**		**WT**	**6B**		**WT**	**6B**
Aggrescan a3vSequence Average (a3vSA)	-0.007	-0.052	**PASTA Amyloids**	20	9	**TANGO Agg**	844.27	868.45
						TANGO Amylo	256865	2081.0

The comparison of predicted secondary structure elements propensities of wild type lipase with stabilized mutant 6B showed there is a decrease in alpha helical content and beta sheet in 6B but there is an increase in overall Turn content in 6B. Comparison of wild type aggregation propensity values with 6B showed significant decrease. There is slight increase in Tango agg value of 6B but Tango Amylo value is too less for 6B than wildtype. At the every mutated position the aggregation propensity values were decreased (data was not shown).This aggregation resistance behavior of 6B was reported earlier also (5).

Phosphonate dehydrogenase

Table-3 Predicted secondary structure propensities in WT and Thermo stable Mutant (OPT14)

	WT	OPT14		WT	OPT14		WT	OPT14
CFSSP Helix	85.4	84.5	**PASTA Helix**	54.46	57.74	**Tango Helix**	934.86	962.18
CFSSP Beta	38.7	36.3	**PASTA Beta**	8.03	6.25	**Tango Beta**	494.941	463.198
CFSSP Turn	9.2	9.2	**PASTA Coil**	37.5	36.01	**Tango Turn**	128.275	129.577

Table-4 Predicted aggregation propensity values of WT and Thermo Stable Mutant (OPT14)

	WT	OPT14		WT	OPT14		WT	OPT14
PASTA Amyloids	6	4	**a3vSA**	0.005	-0.001	**Tango agg**	1728.15	1556.57
						Tango amylo	194968	170539

Opt14 (thermo stable mutant of phosphonate dehydrogenase) mutation Q215 to L showed increase in alpha helical content at 213-229 compared to wild type protein and the overall alpha helical content also improved. There is a significant decrease in overall aggregation propensity values and beta sheet values also observed in Opt 14 when compared with wild type.

Candida Antarctica Lipase

Table-5 Predicted secondary structure propensities in WT and Thermo stable Mutants (CALB7, CALB168)

	WT	CAL B7	CALB 168		WT	CAL B7	CALB 168		WT	CAL B7	CALB 168
PASTA Helix	22.7	23.4	25.87	**CFSSP Helix**	49.8	46.7	55.2	**Tango Helix**	110.959	110.956	82.2844
aPASTA Beta	13.25	10.73	11.36	**CFSSP Beta**	68.5	66.6	68.1	**Tango Beta**	683.425	671.482	687.805
PASTA Coil	64.4	65.93	62.78	**CFSSP Turn**	15.1	15.5	15.1	**Tango Turn**	182.755	186.446	178.492

Table-6 Predicted aggregation propensity values of WT and Thermo Stable Mutants (CALB7, CALB168)

	WT	CAL B7	CALB 168		WT	CAL B7	CALB 168		WT	CAL B7	CALB 168
Aggrescan a3vSA	0.032	0.01	0.031	**PASTA Amyloids**	4	1	6	**Tango Agg**	557.666	546.569	512.084
								Tango Amylo	2.24911	2.24911	5.9216

Candida Antarctica lipase thermo stable mutants CALB7 and CALB168 stabilized in two different ways.CALB7 stabilized by decreasing the aggregation Propensity and CALB168 improved it's stability by improving Alpha helix content and there is slight decrease in beta sheet also observed.

ORF49

Table-7 Predicted secondary structure propensities in WT and Thermo stable Mutants By Choufasman algorithm

	WT	ORF49-1	ORF49-2	ORF49-3	ORF49-4	ORF49-5	ORF49-6	ORF49-7	ORF49-8	ORF49-9
CFSSP Helix	74.5	73.8	75.5	74.5	74.5	74.5	74.5	74.5	74.5	74.5
CFSSP Beta	82.5	82.5	82.8	82.5	82.5	83.1	82.5	82.5	82.5	82.5
CFSSP Turn	8.6	8.6	8.6	8.6	8.6	8.6	8.6	8.6	8.6	8.3

Table-8 Predicted secondary structure propensities in WT and Thermo stable Mutants by PASTA2.0

	WT	ORF49-1	ORF49-2	ORF49-3	ORF49-4	ORF49-5	ORF49-6	ORF49-7	ORF49-8	ORF49-9
PASTA Helix	72.19	66.87	74.83	72.52	72.52	72.19	70.53	73.84	72.19	72.52
PASTA Beta	0.66	0.99	0.33	0.66	0.66	0.99	1.32	0.33	0.66	0.66
PASTA Coil	27.15	30.13	24.83	26.82	26.82	26.82	28.15	25.83	27.15	26.82

Table-9 Predicted secondary structure propensities in WT and Thermo stable Mutants by TANGO

	WT	ORF49-1	ORF49-2	ORF49-3	ORF49-4	ORF49-5	ORF49-6	ORF49-7	ORF49-8	ORF49-9
Tango Helix	138.416	129.259	161.374	137.616	141.328	142.157	121.092	137.45	138.173	139.279
Tango Beta	757.468	769.189	751.532	755.929	751.545	762.237	771.329	756.552	746.498	758.71
Tango Turn	138.647	138.905	136.868	139.422	138.296	132.949	138.564	138.79	138.687	139.006

Table-10 Predicted aggregation propensity values of WT and Thermo Stable Mutants by Tango

	WT	ORF49-1	ORF49-2	ORF49-3	ORF49-4	ORF49-5	ORF49-6	ORF49-7	ORF49-8	ORF49-9

Tango Agg	2752.09	2657.27	2778.93	2720.23	2751.31	2730.21	2721.36	2483.02	2758	3191.66
Tango Amylo	275053	161947	275054	275053	275053	275054	275054	275054	275054	275054

Table-11 Predicted aggregation propensity values of WT and Thermo Stable Mutants by Aggrescan

	WT	**ORF49-1**	**ORF49-2**	**ORF49-3**	**ORF49-4**	**ORF49-5**	**ORF49-6**	**ORF49-7**	**ORF49-8**	**ORF49-9**
Aggrescan a3vSA	0.089	0.086	0.092	0.09	0.092	0.095	0.092	0.087	0.093	0.099

Table-12 Predicted aggregation propensity values of WT and Thermo Stable Mutants by PASTA

	WT	**ORF49-1**	**ORF49-2**	**ORF49-3**	**ORF49-4**	**ORF49-5**	**ORF49-6**	**ORF49-7**	**ORF49-8**	**ORF49-9**
PASTA Amyloids	20	20	20	20	20	20	20	20	20	20

Thermo stability screening of ORF49 mutants is aggregation dependent, out of nine mutants ORF49-1, ORF49-7 showed decreased aggregation propensity values. ORF49-2 showed increase in alpha helical content. ORF49-3 (Q207H) a single mutant showed both improvement in alpha helix and decrease in aggregation propensity but ORF49-6 mutant had along with ORF49-3 mutation two extra mutations which are decreasing Tm by 2degrees of single mutant.ORF49-6 showing same aggregation propensity values like ORF49-3 but there is a significant decrease in alpha Helix content. The Decrease in Tm of ORF49-6 is due decrease in alpha helix content.ORF49-9 mutant also had this single mutation (Q207H) along with one more mutation and it doesn't showed any significant change in Tm but there is increase in aggregation propensity values. Alpha Helix content of ORF49-9 is slightly more than ORF49-3. ORF49-4 and ORF49-8 had one common mutation (V218I) but there is Tm difference of 4 degrees present in between these mutants the second mutation in ORF49-4 improving the beta sheet, in ORF49-8 the second mutation causing slight increase in local alpha helical content. Previous mutations in different proteins showed increase beta sheet content decrease the stability so the decrease in Tm of ORF49-4 is due increase in Beta sheet. ORF49-5 showed improvement in alpha helix content and showed decrease in aggregation propensity value.

Phospholipase A1

Table-13 Predicted secondary structure propensities in WT and Thermo stable Mutants (TA3,TA13)

	WT	**TA3**	**TA13**		**WT**	**TA3**	**TA13**		**WT**	**TA3**	**TA13**
CFSSP Helix	67.8	69.1	67.2	**PASTA Helix**	44.06	44.06	43.13	**Tango Helix**	302.898	324.614	294.616
CFSSP Beta	60.3	61.16	60.3	**PASTA Beta**	8.13	9.38	8.44	**Tango Beta**	601.823	626.761	591.179

CFSSP Turn	12.5	12.8	13.1	**PASTA Coil**	47.18	46.56	48.44	**Tango Turn**	123.858	116.439	128.095

Table-14 Predicted aggregation propensity values of WT and Thermo Stable Mutants (TA3, TA13)

	WT	**TA3**	**TA13**		**WT**	**TA3**	**TA13**		**WT**	**TA3**	**TA13**
Aggrescan a3vSA	-0.104	2.812	-0.122	**PASTA Amyloids**	0	2	0	**Tango Agg**	1427.32	1469.86	1428.42
								Tango Amylo	401632	352083	391301

In Phospholipase mutants TA13 improved its aggregation resistance. It was also observed that, mutated regions had less aggregation propensity values than WT protein. TA3 doesn't show any decrease in aggregation propensity values but improved its alpha helical content.

Triplex capsid protein UL18

Table-15 Predicted secondary structure propensities in WT and Thermo stable Mutants (UL18-1, UL18-2)

	WT	**UL18-1**	**UL18-2**		**WT**	**UL18-1**	**UL18-2**		**WT**	**UL18-1**	**UL18-2**
CFSSP Helix	70.1	71.7	71.7	**PASTA Helix**	35.22	35.22	35.22	**Tango Helix**	809.331	809.718	800.114
CFSSP Beta	47.2	47.2	47.2	**PASTA Beta**	16.98	16.98	16.98	**Tango Beta**	687.585	671.071	663.695
CFSSP Turn	11.3	11.3	11	**PASTA coil**	47.8	47.8	47.8	**Tango Turn**	140.643	140.816	140.8

Table-16 Predicted aggregation propensity values of WT and Thermo Stable Mutants (UL18-1, UL18-2)

	WT	**UL18-1**	**UL18-2**		**WT**	**UL18-1**	**UL18-2**		**WT**	**UL18-1**	**UL18-2**
PASTA Amyloids	20	20	20	**Aggrescan a3vSA**	0.09	0.095	0.099	**Tango Agg**	1846.89	2008.86	2008.89
								Tango Amylo	20.3624	20.3624	20.3624

Both thermo stable mutants of UL18 had common mutation (S212L) which increases the Local Alpha helix content decreases the beet sheet values and the UL18-2 had one more mutation that decreases the beet sheet value furthermore. The decrease in beta sheet value and local improvement alpha helix is cause of stabilization both mutants.

Beta-glucosidase Thermus thermophiles

Table-17 Predicted secondary structure propensities in WT and Thermo stable Mutant (HF5)

	WT	HF5		WT	HF5		WT	HF5
CFSSP Helix	63.8	62.6	**PASTA Helix**	45.24	44.78	**Tango Helix**	772.383	899.635
CFSSP Beta	57.1	57.1	**PASTA Beta**	9.74	10.21	**Tango Beta**	757.952	747.42
CFSSP Turn	12.8	12.8	**PASTA Coil**	45.01	45.01	**Tango Turn**	139.408	134.842

Table-18 Predicted aggregation propensity values of WT and Thermo Stable Mutant (HF5)

	WT	HF5		WT	HF5		WT	HF5
PASTA Amyloids	7	7	**Aggrescan a3vSA**	-0.014	-0.013	**Tango Agg**	1971.09	2053
						Tango Amylo	1.35E+06	1.35E+06

Thermo stable mutant of Beta glucosidase showed significant increase in alpha helical values at the mutated regions. Showed decrease beta sheet values. But there is no significant change in overall aggregation propensity values although two mutations of this protein showed little decrease at their positions.

Fructose bisphosphate aldolase

Table-19 Predicted secondary structure propensities in WT and Thermo stable Mutant (4-43D6)

	WT	4-43D6		WT	4-43D6		WT	4-43D6
CFSSP Helix	72.6	73.1	**PASTA Helix**	43.58	43.7	**Tango Helix**	165.262	266.39
CFSSP Bete	32.4	41.7	**PASTA Beta**	14.25	14.85	**Tango Beta**	854.948	817.164
CFSSP Turn	13.4	12.3	**PASTA Coil**	42.18	41.46	**Tango Turn**	154.457	152.639

Table-20 Predicted aggregation propensity values of WT and Thermo Stable Mutant (4-43D6)

	WT	4-43D6		WT	4-43D6		WT	4-43D6
Aggrescan a3vSA	-0.04	-0.009	**PASTA Amyloids**	2	2	**Tango Agg**	603.945	795.203
						Tango	0.670351	67.2146

						Amylo		

Aggregation propensity values for WT protein is too less. Thermo stable mutant 4-43D6 showed increase alpha helix content and also showed there is an increase aggregation propensity values. But this mutant stabilized because of improvement in alpha helix content.

Tegument UL14

Table-21 Predicted secondary structure propensities in WT and Thermo stable Mutants (UL14-1)

	WT	**UL14-1**		**WT**	**UL14-1**		**WT**	**UL14-1**
CFSSP Helix	67.6	67.6	**PASTA Helix**	52.97	53.88	**Tango Helix**	523.461	523.489
CFSSP Beta	36.1	36.1	**PASTA Beta**	1.83	0	**Tango Beta**	312.36	272.687
CFSSP Turn	15.5	16	**PASTA Coil**	45.21	46.12	**Tango Turn**	125.093	126.852

Table-22 Predicted aggregation propensity values of WT and Thermo Stable Mutant (UL14-1)

	WT	**UL14-1**		**WT**	**UL14-1**		**WT**	**UL14-1**
PASTA Amyloids	0	0	**Aggrescan a3vSA**	-0.266	-0.285	**Tango Agg**	287.803	287.803
						Tango Amylo	0.994051	0.994045

Aggregation propensity values for WT protein is too less. Aggrescan values showed there is an aggregation resistance although there is change in Tango values. Thermo stable mutant of this viral protein showed significant increase in alpha Helix content.

Non-heme bromoperoxidase

Table-23 Predicted secondary structure propensities in WT and Thermo stable Mutants (HT117, HT507)

	WT	**HT117**	**HT507**		**WT**	**HT117**	**HT507**		**WT**	**HT117**	**HT507**
CFSSP Helix	68	68	69.5	**PASTA Helix**	35.27	35.64	32	**Tango Helix**	181.586	159.313	186.417
CFSSP Beta	33.8	33.8	33.8	**PASTA Beta**	19.64	19.64	21.82	**Tango Beta**	478.027	477.883	483.261
CFSSP Turn	13.5	13.1	13.5	**PASTA Coil**	45.09	44.73	46.18	**Tango Turn**	160.112	149.923	166.113

Table-24 Predicted aggregation propensity values of WT and Thermo Stable Mutant (HT117, HT507)

	WT	**HT117**	**HT507**		**WT**	**HT117**	**HT507**		**WT**	**HT117**	**HT507**

PASTA Amyloids	4	5	4	**Aggrescan a3vSA**	-0.103	-0.102	-0.106	**Tango Agg**	740.837	761.513	741.129
								Tango Amylo	57167.2	57167.6	56933.9

The mutant HT117 mutation in the position 114 showed decrease beta sheet value but second mutation showed increase in beta sheet value and decrease in alpha helix value. But second thermo stable mutant HT507 showed significant decrease in aggregation propensity values and increase in alpha helix values. In Comparison both mutants HT507 is more thermo stable than HT117 although the mutations located same regions but different positions.

NUD18 HUMAN 8-oxo-dGDP phosphatase

Table-25 Predicted secondary structure propensities in WT and Thermo stable Mutants (NXR1-1, NXR1-2)

	WT	**NXR 1-1**	**NXR 1-2**		**WT**	**NXR 1-1**	**NXR 1-2**		**WT**	**NXR 1-1**	**NXR 1-2**
CFSSP Helix	71.2	71.2	71.2	**PASTA Helix**	42.14	43.65	41.18	**Tango Helix**	475.349	485.176	544.606
CFSSP Beta	48	49.2	48	**PASTA Beta**	13.13	12.38	14.24	**Tango Beta**	762.048	719.833	748.521
CFSSP Turn	12.7	12.4	12.7	**PASTA Coil**	44.27	43.96	44.58	**Tango Turn**	87.1818	87.1366	87.3529

Table-26 Predicted aggregation propensity values of WT and Thermo Stable Mutants (NXR1-1, NXR1-2)

	WT	**NXR 1-1**	**NXR 1-2**		**WT**	**NXR 1-1**	**NXR 1-2**		**WT**	**NXR 1-1**	**NXR 1-2**
Aggrescan a3vSA	0.006	0.013	0.014	**PASTA Amyloids**	20	20	20	**Tango Agg**	3191.53	3192.64	3225.56
								Tango Amylo	31653.7	55977.9	31652.9

The First thermo stable mutant NXR1-1showed significant increase in Alpha helix. There is decrease in beta sheet compared to second mutant NXR1-2. NXR1-2 also showed significant increase in alpha helix at their mutated regions and decrease in beta sheet value. The aggregation propensity values when compared with WT slightly increased.

p- Nitro Benzyl Esterase

Table-27 Predicted secondary structure propensities in WT and Thermo stable Mutant (6sF9)

	WT	6SF9		WT	6SF9		WT	6SF9
CFSSP Helix	76.5	76.3	**PASTA Helix**	40.7	41.2	**Tango Helix**	793.101	1015.04
CFSSP Beta	28.8	29.7	**PASTA Beta**	15.54	14.72	**Tango Beta**	1099.84	1085.02
CFSSSP Turn	14.5	14.5	**PASTA Coil**	43.76	43.56	**Tango Turn**	272.585	256.468

Table-28 Predicted aggregation propensity values of WT and Thermo Stable Mutant (6sF9)

	WT	6SF9		WT	6SF9		WT	6SF9
Aggrescan a3vSA	-0.035	-0.037	**PASTA Amyloids**	20	20	**Tango Agg**	2346.69	2277.06
						Tango Amylo	3626.39	32.9687

p-Nitro Benzyl Esterase thermo stable mutant 6sF9 showed an improvement in overall alpha helix content and decrease in overall aggregation propensity values and there is a decrease in beta sheet value also.

Conclusion

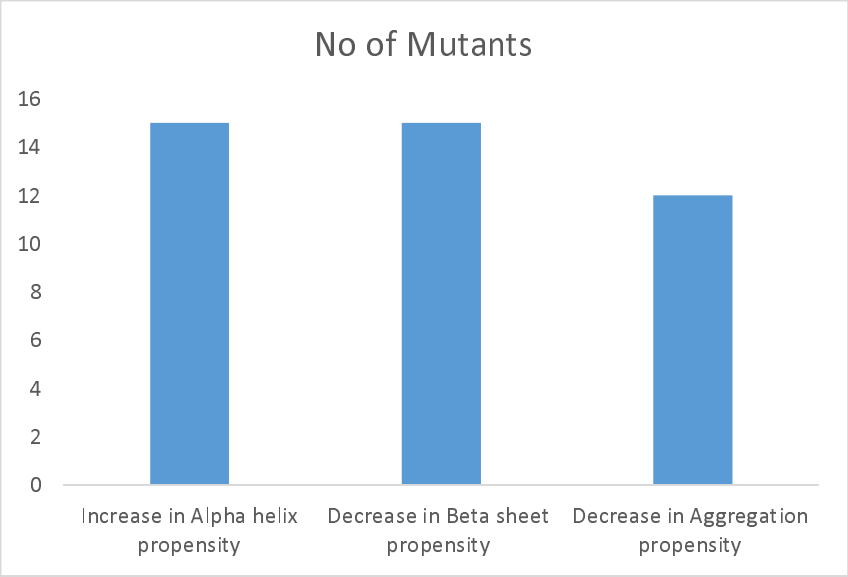
All thermo stable mutants analyzed in this study, out of 25 mutants 18 showed either there is decrease in aggregation propensity values or increase in alpha helix secondary structure prediction values. Remaining 7 mutants showed these changes at their mutated regions and almost of all of this mutants showed the decrease in overall beta sheet values and it was already reported that involvement beta sheet in thermal aggregation (13). The proteins which are thermo stabilized analyzed in this study showed are tabulated into three different groups.

Table-29 Changes in three different parameters happened in stabilized mutants

	Parent Protein	Mutants	Decrease in Aggregation propensity value	Increase in Alpha helix Value	Decrease in Beta sheet Value	Tm(in Degree Celsius)
1	Bacillus subtillis Lipase					55.5
		6B	+		+	75.6
2	Phosphonate Dehydrogenase					39.7
		OPT14	+	+	+	64.4
3	Candida Antarctica Lipase					
		CALB7	+		+	7 Folds High activity than WT at 60degree
		CALB168		+		14 Folds Activity than WT at 60 degree
4	HHV8P Protein ORF49					48.05

		ORF49-1	+			57.8
		ORF49-2		+	+	51.92
		ORF49-3	+	+		53.08
		ORF49-4	+		+	50.66
		ORF49-5		+		51.3
		ORF49-6	+	+		51.82
		ORF49-7	+		+	57.65
		ORF49-8	+		+	54.43
		ORF49-9	+	+		53.45
5	Phosholipase A1					70
		TA3		+		77
		TA13	+		+	81
6	Triplex capsid protein UL18					54.43*(Tagg)
		UL18-1		+		60.65*
		UL18-2			+	61.03*
7	Beta-glucosidase Thermus thermophilus					3.8 ± 0.4(t1/2)
		HF5		+	+	17.9 ± 0.8
8	Fructose-bisphosphate aldolase					50.5
		4-43D6	-	+		61.7
9	HHV11 Tegument protein UL14					44.23
		UL14-1		+	+	54.53
10	Non-heme bromoperoxidase BPO-A1					80.6
		HT177			+	82
		HT507		+		84.5
11	NUD18 HUMAN 8-oxo-dGDP phosphatase					40.27
		NXR1-1		+	+	66.85
		NXR1-2			+	55.76
12	Para Nitro Benzyl Esterase					52.5
		6sF9	+	+	+	66

OPT14 (thermo stable mutant of phosphite dehydrogenase) and 6sF9 (thermo stable mutant of parnitro benzyl esterase) improved their Tm in a greater extent (OPT 25degrees, 6sF9 14.5degrees than their respective parent protein) with less number mutations. These thermo stable mutants of phosphite dehydrogenase and para nitro benzyl esterase showed all three existing parameters changed and this changes shown additive effect to improve the thermo stability of these mutants in greater extent. This *in vitro* evolved proteins for thermo stability showed three types conserved changes, including their secondary structure and aggregation propensity. Sometime this changes are local at the mutated region and doesn't show global changes in protein structure, but others showed significant change at local mutated region and overall global change in the protein.



Changes in three different parameters happened in stabilized mutants

Future Directions

This study can help in rational designing of protein for improving their stability in future to minimize the errors in it.

References

1. Chou PY, Fasman GD (1974). "**Conformational parameters for amino acids in helical, beta-sheet, and random coil regions calculated from proteins**". Biochemistry 13: 211–222.

2. Fernadez-Escamilla AM, Rousseau F, Schymkowtiz J, Serrano L, **Prediction of sequence dependent mutational effects on the aggregation of peptide and protein,** Nat Biotechnology, e-pub,2004.

3. Ian Walsh, Flavio Seno, Silvio C.E. Tosatto and Antonio Trovato. **PASTA2: An improved server for protein aggregation prediction**.Nucleic Acids Research, **accepted**. (2014)

4. Ignacio Asial1, Yue Xiang Cheng1, Henrik Engman1, , Binghuang Wu1,Pa¨r Nordlund1,2 & Tobias Cornvik1, **Engineering protein thermostability using a generic activity-independent biophysical screen Inside the cell.** Nature Communications, Dec 2013.

5. Jae kwang song, Joon shick Rhee, **Simultaneous enhancement of thermostability and catalytic activity of phospholipase A1 by evolutionary Molecular engineering.** Applied Environmental Microbiology, Mar.2000, p.890-894

6. Jijun Hao and Alan Berry, **A thermostable variant of fructose bisphosphate aldolase constructed by directed evolution also shows increased stability in organic solvents.** Protein Engineering, Design & Selection vol. 17 no. 9 pp. 689–697, 2004

7. Kamal MZ, Ahmad S, Molugu TR, Vijayalakshmi A, Deshmukh MV, Sankaranarayanan R, Rao NM **In vitro evolved non-aggregating and thermostable lipase: structural and thermodynamic investigation.**J Mol Biol ,2011, 413:726–741.

8. Lori Giver, Anne Gershenson, **Directed evolution of thermo stable Esterase.** Proc. Natl. Acad. Sci, 1998, Vol95, 12809-12813.

9. Michael J, McLachlan, **Future improvement of phosphite dehydrogenase thermo stability by saturation mutagenesis.** Biotechnology bioengineering, 2008;99: 268-274.

10. Oscar Conchillo-Solé**,** Natalia S de Groot**,** Francesc X Avilés**,** Josep Vendrell**,** Xavier Daura **and** Salvador Ventura, **AGGRESCAN: a server for the prediction and evaluation of "hot spots" of aggregation in polypeptides.** BMC Bioinformatics 2007, **8**:65

11. Ryosuke Yamada, Tatsutoshi Higo, Chisa Yoshikawa, Hideyasu China, Hiroyasu Ogino, **Improvement of the stability and activity of the BPO-A1haloperoxidase from Streptomyces aureofaciens by directed evolution** Journal of Biotechnology 192, 2014, 248–254

12. Xiang-Qian Peng, **Improved Thermostability of Lipase B from Candida Antarctica by Directed Evolution and Display on Yeast Surface.** Appl Biochem Biotechnol, 2013, 169:351–358

13. Yong-Bin Yan, Qi Wang, Hua-Wei He, and Hai-Meng Zhou* **Protein Thermal Aggregation Involves Distinct Regions: Sequential Events in the Heat-Induced Unfolding and Aggregation of Hemoglobin** Biophys J. 2004 Mar; 86(3): 1682–1690.

14. Zhuo-Lin Yi, Shuai-Bing Zhang a, Xiao-Qiong Pei, Zhong-Liu Wu, **Design of mutants for enhanced thermostability of b-glycosidase BglY from Thermus thermophiles.** Bioresource Technology 129, 2013, 629–633