Chapter III

Structural characterization of mutant form of ubiquitin UbEP42 and its single mutant derivatives

3 Structural characterization of mutant form of ubiquitin UbEP42 and its single mutant derivatives

3.1 INTRODUCTION

The small, compact globular structure of ubiquitin is noteworthy because of its extreme sequence conservation throughout eukaryotes as well as its stability under various physicochemical conditions. Yeast, plant and animal ubiquitin sequences differ only by change in three amino acid residues (Schlesinger et al., 1975; Schlesinger and goldsteiner 1975; Watson et al., 1978; Gavialnes et al., 1982; Wilkinson et al., 1986; Vierstra et al., 1986). Structurally, ubiquitin contains α-helix, five β-strands, nine reverse turns and two β-bulges. Structural integrity of ubiquitin is imposed by hydrophobic core of the protein formed by compact arrangement of helices and sheets (Vijay kumar et al., 1987).

Main objective of the study was to understand the significance of sequence conservation in the protein. In vitro mutagenesis is a powerful technique for generating mutants of a gene which in turn may encode proteins with altered structure and function. In this technique the gene encoding a protein is subjected to several rounds of random mutagenesis to mimic natural evolution. Our laboratory has generated library of ubiquitin mutants with the help of error prone PCR and they were screened in SUB60 strain of *Saccharomyces cerevisiae*. As mentioned previously the strain SUB60, lacks polyubiquitin coding gene. Mutant UBEP42 conferred dosage dependent lethality on SUB60 cells at permissive conditions. The protein sequence of UbEP42 unravels replacement of four amino acid residues as shown in Figure 3.1 accompanied by its secondary structure where the mutation has occurred (Prabha et al., 2010).

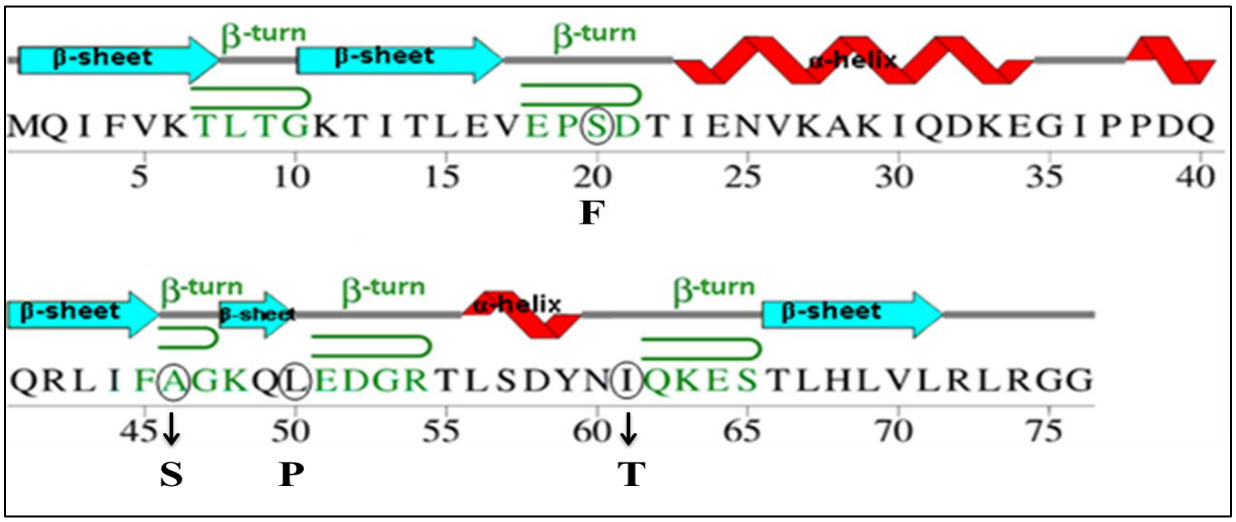

Figure 3.1: Secondary structural features of ubiquitin along with position of four mutations *viz.* S20F, A46S, L50P and I61T (Prabha et al., 2010).

Four substitutions were segregated to study its individual effects on cellular functions of the cell by site-directed mutagenesis and are named as UbS20F, UbA46S, UbL50P and UbI61T. UbEP42 and single mutants were transformed in SUB60 stain followed by evaluating its effects on cell. Our results established that UbEP42, UbL50P and UbI61T affected various functions of cells, it was hypothesized that the structure of ubiquitin protein must have changed due to replacement of amino acid residues (Doshi et al., 2014; Doshi et al., 2017). To study structural aspects we used UbF45W, also known as pseudo wild type as structurally and functionally UbF45W mimics wild type ubiquitin with no significant alteration to biology of the expressing cell (Khorasanizadeh et al., 1993). Mutations were incorporated using site-directed mutagenesis technique in ubiquitin gene and they were cloned in bacterial expression vector. Variants of the protein as well as wild type ubiquitin were purified through size exclusion chromatography. Circular Dichroism (CD) spectroscopy and Fluorescence spectroscopy were used as tool to study structural aspects.

3.2 MATERIALS AND METHODS

3.2.1 Strains and media

E.coli DH5α (F-, 80dlacZ M15, endA1, recA1, hsdR17 (rk-,mk+), supE44, thi-1, gyrA96, relA1, (lacZYA-argF) U169) strain was used for cloning purpose. For protein expression and purification *E.coli* BL21(DE3) (F^{-}*ompT*gal *dcmlonhsd*$S_B(r_B^{-}m_B^{-})$ λ(DE3

[*lacI*lac*UV5-T7 gene 1 *ind*1 *sam*7 *nin*5]) was used. Bacterial cultures were grown at 37° C at 200 rpm in Luria broth (Hi-media) under antibiotic selection of 50 µg/ml ampicillin. Bacterial transformations were done using calcium chloride method and Plasmid DNA isolation was done by standard alkaline lysis method (Sambrook et al., 2001).

3.2.2 Mutagenesis of yeast ubiquitin

Mutants of yeast ubiquitin were generated by site directed mutagenesis. Synthetic yeast ubiquitin gene was used for generating variants (Figure 3.2). pKK223-3 was used for expressing mutant ubiquitin which carries mutant form of ubiquitin gene for UbF45W, was a generous gift from Prof. Mark Searle's laboratory (School of Chemistry, Nottingham, United Kingdom) (Figure 3.3). To study the structural properties of ubiquitin using intrinsic tryptophan fluorescence, phenylalanine at 45^{th} position of wild type ubiquitin was replaced by tryptophan, as UbWt lacks suitable chromophores for conventional optical measurements to study stability and folding kinetics (Ecker et al. 1987a). It contain a single potential optical probe i.e. Tyr 59, which exhibits only small changes in fluorescence and absorbance while folding in solvent perturbation experiments (Jensen et al., 1980). Earlier studies conferred that F45W is biologically active variant of ubiquitin with marginal differences in the structure as compared to UbWt (Khorasanizadeh et al., 1993). The synthetic yeast ubiquitin gene contains several restriction sites so as to facilitate introduction of novel mutations through cloning. Mutations representing S20F, A46S, L50P and I61T were incorporated with the help of mutagenic and non-mutagenic primers listed in Table 3.1. Confirmation of mutation incorporation was done by DNA sequencing.

Figure 3.2: UbF45W, synthetic yeast ubiquitin gene containing restriction sites. Residues which are subjected to point mutations are encircled.

PRIMER	SEQUENCE OF THE PRIMER
UB FR	5' ACA GAA TTC ATG CAG ATC TTC GTC AAG 3'
UB RE	5' GCC AAG CTT CGC TCA ACC 3'
UbEP 42-20FR	5' CC ATA ACT CTT GAA GTT GAA TCT TTC GAT ACC ATC GAC 3'
UbEP 42-20RE	5' GTC GAT GGT ATC GAA AGA TTC AAC TTC AAG AGT TAT GG 3'
UbEP 42-46FR	5' GA TTG ATC TTT TCC GGT AAG CAG CTG GAG GAC GGT AG 3'
UbEP 42-46RE	5' CT ACC GTC CTC CAG CTG CTT ACC GGA AAA GAT CAA TC 3'
UbEP 42-50FR	5' G ATC TTT GCC GGC AAG CAG CCT GAG GAC GGT AG 3'
UbEP 42-50RE	5' CT ACC GTC CTC AGG CTG CTT GCC GGC AAA GAT C 3'
UbEP 42-61FR	5' CT GAT TAC AAC ACT CAG AAG GAG TCC ACC TTA CAT CTT G 3'
UbEP42-61RE	5' C AAG ATG TAA GGT GGA CTC CTT CTG AGT GTT GTA ATC AG 3'

Table 3.1: List of mutagenic and non-mutagenic primers used for generating mutant forms of ubiquitin.

3.2.3 Cloning of yeast ubiquitin variants in pKK223-3 vector

pKK223-3 carrying ubiquitin gene for UbF45W was used for cloning and expressing mutant ubiquitins. In pKK223-3 expression of protein is driven by strong *Tac* promoter present upstream to the multiple cloning site. Ampicillin cassette is present as selection marker. Variants of ubiquitin generated by site directed mutagenesis were PCR amplified with gene specific primers and were cloned between *Eco*RI and *Hind*III restriction sites of pKK223-3 vector followed by transformation in *E. coli* DH5α strain and screening. Positive clones were verified further by sequencing.

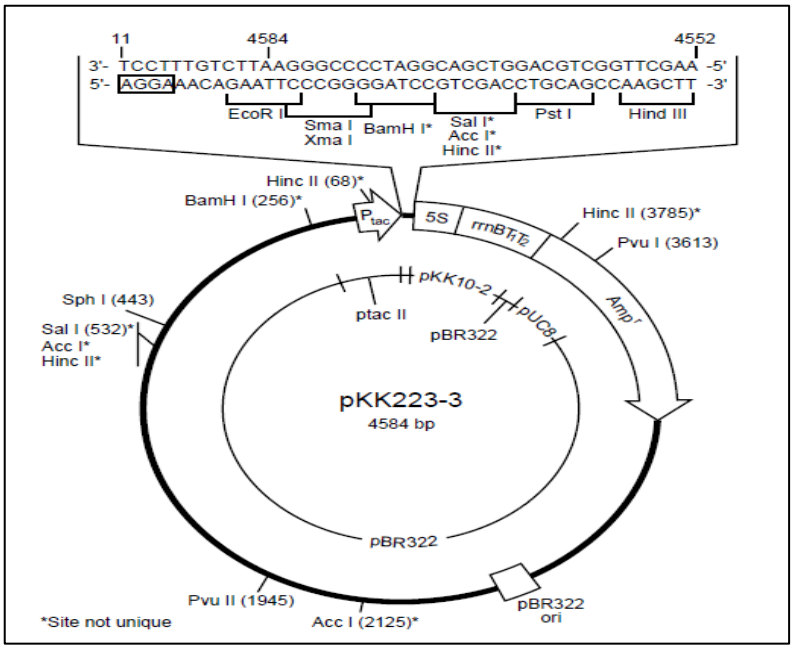

Figure 3.3: Map of pKK223-3 bacterial expression vector.

3.2.4 Expression and purification of proteins

Ubiquitin variants F45W, UbEP42, UBS20F, UbA46S, UbL50P and UbI61T were purified using the methods given by Ciechanover et al., 1980 and Ecker et al., 1987. Mutant proteins were expressed in *Escherichia coli* strain BL21 (DE3) strain. Transformants were grown in Luria−Bertani medium (Himedia) containing 50 µg/ ml ampicillin at 37° C up to log phase. Proteins were overexpressed by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) at final concentration of 1mM and cells were allowed to grow for another 5-6 hours. Cells were harvested, washed with phosphate buffered saline pH 7.4 and resuspended in lysis buffer (5% glycerol, 50mM Tris and 2mM EDTA, pH7.4). Lysis of cells was carried out by sonication (50 duty cycles for 20 sec/3-4 times in an interval of 5 sec with 20% amplitude, 2

sec on/2sec off) followed by clearing the lysate by centrifuging it at high speed to collect cell lysate containing soluble cellular proteins. Protease inhibitor cocktail and 1 mM phenylmethylsulfonyl fluoride (PMSF) were added to cell lysate. Ubiquitin is a heat stable protein. So in order to remove heat sensitive proteins, cell lysate was subjected to 85° C for few minutes in presence of 1mM dithiothreitol (DTT) followed by treating with 0.2% polyethyleneimine (PEI). Precipitates were removed by centrifugation at 12,000 rpm for 30 minutes. Salting out procedure was utilized to precipitate ubiquitin protein from the pre-cleared lysate. Finally, lysate was dialyzed against 20mM Tris-Cl buffer, pH 7.4. Dialyzed lysate was used for purifying protein by size-exclusion chromatography.

Column was filled with Sephadex G-50 beads which act as a stationary phase and it was also equilibrated by 2-3 column volumes of 20 mM Tris-Cl buffer, pH 7.4 which acted as mobile phase. Flow rate of the column was set at 1ml/ min followed by applying lysate. Fractions of 2ml volume were collected. Presence of protein was confirmed by recording absorption at 280 nm. The fractions in which protein was detected were loaded on 15% SDS-PAGE to check the purity and intactness of the protein. Ubiquitin containing fractions were confirmed by matching bands with molecular weight marker and were pooled. The protein was concentrated by salting out procedure. This was dialyzed against 20mM Tris-Cl buffer, pH 7.4 and stored in -80°C till further use.

3.2.5 Structural characterizations

Structural features of the mutant ubiquitin proteins were studied by Circular dichroism (CD) and fluorescence spectroscopy by using protocols established in our laboratory (Mishra et al., 2009; Mishra et al., 2011; Prabha and Sasidhar, 1998; Prabha and Sasidhar, 2000; Prabha and Rao, 2004; Joshi et al., 2015; Joshi and Prabha, 2016).

3.2.5.1 Circular dichroism spectroscopy

CD spectra were recorded using a Jasco J-815 spectropolarimeter. Wild type ubiquitin as well as mutant ubiquitin protein samples were prepared in 10 mM Tris buffer, pH7.4 for recording spectra. For far UV studies, spectra were recorded in a range of 195nm to 260nm using cells of 1mm path length, 2 nm resolution, 0.2nm data pitch and 50nm/sec scan speed. For far UV studies 30 µM protein concentration was used. For near UV studies, spectra were

recorded between 250nm to 350nm using cells of 1cm path length and 50 µM protein concentration was used. Five spectra were accumulated to reduce the noise in both far UV and near UV. Proper blanks were prepared for all samples and the spectra of the samples were blank corrected.

3.2.5.2 Fluorescence spectroscopy

Fluorescence spectra were recorded using Hitachi Fluorescence Spectrophotometer FL-7000. Protein was used at a final concentration of 0.1mg/ml. Protein solutions were prepared in 10mM Tris HCl, pH7.4. Intrinsic fluorescence was recorded by exciting at 280 nm and recording spectra in the range of 300-400nm. To study guanidine hydrochloride denaturation, protein samples were incubated increasing concentrations of guanidine hydrochloride (0M to 6M) and spectra were recorded. Proper blanks were prepared for all samples and the spectra of the samples were blank corrected. All the experiments were repeated thrice to negate error.

3.3 RESULTS

3.3.1 Generation of mutant forms of yeast ubiquitin by site directed mutagenesis using recombinant PCR

Mutants of ubiquitin were generated on the gene for UbF45W, using recombinant PCR employing sets of mutagenic and non-mutagenic primers (Figure 3.4). Mutant genes so generated were amplified using gene specific primers. While designing mutagenic primers with each point mutation of the residue nearby restriction site was also mutated for easy screening. For example, incorporation of UbEP42 contain non-functional *Xba*I and *Xho*I RE sites, UbS20F contain disrupted *Xba*I, UbA46S and HUbL50P carries *Xho*I disrupted RE site and UbI61T have *Sal*I site which is disrupted (Figure 3.5). Sequence analysis confirmed replacement of amino acids at desired positions.

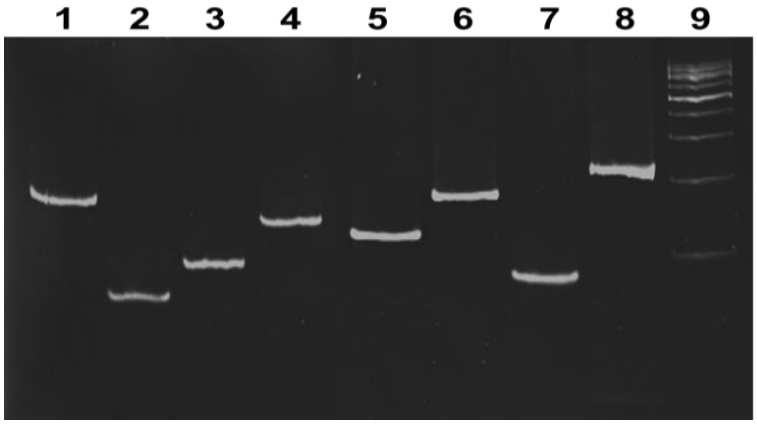

Figure 3.4: 15% DNA-PAGE showing fragments of mutagenic amplicon of ubiquitin. Fragments were PCR amplified by using mutagenic and non-mutagenic primers. Lanes 1 to 8 show fragments amplified by UBFR/20RE, 20FR/UBRE, 46FR/UBRE, UBFR/46RE, 50FR/UBRE, UBFR/50RE, 61FR/UBRE and UBFR/61RE and lane 9 shows 100bp ladder.

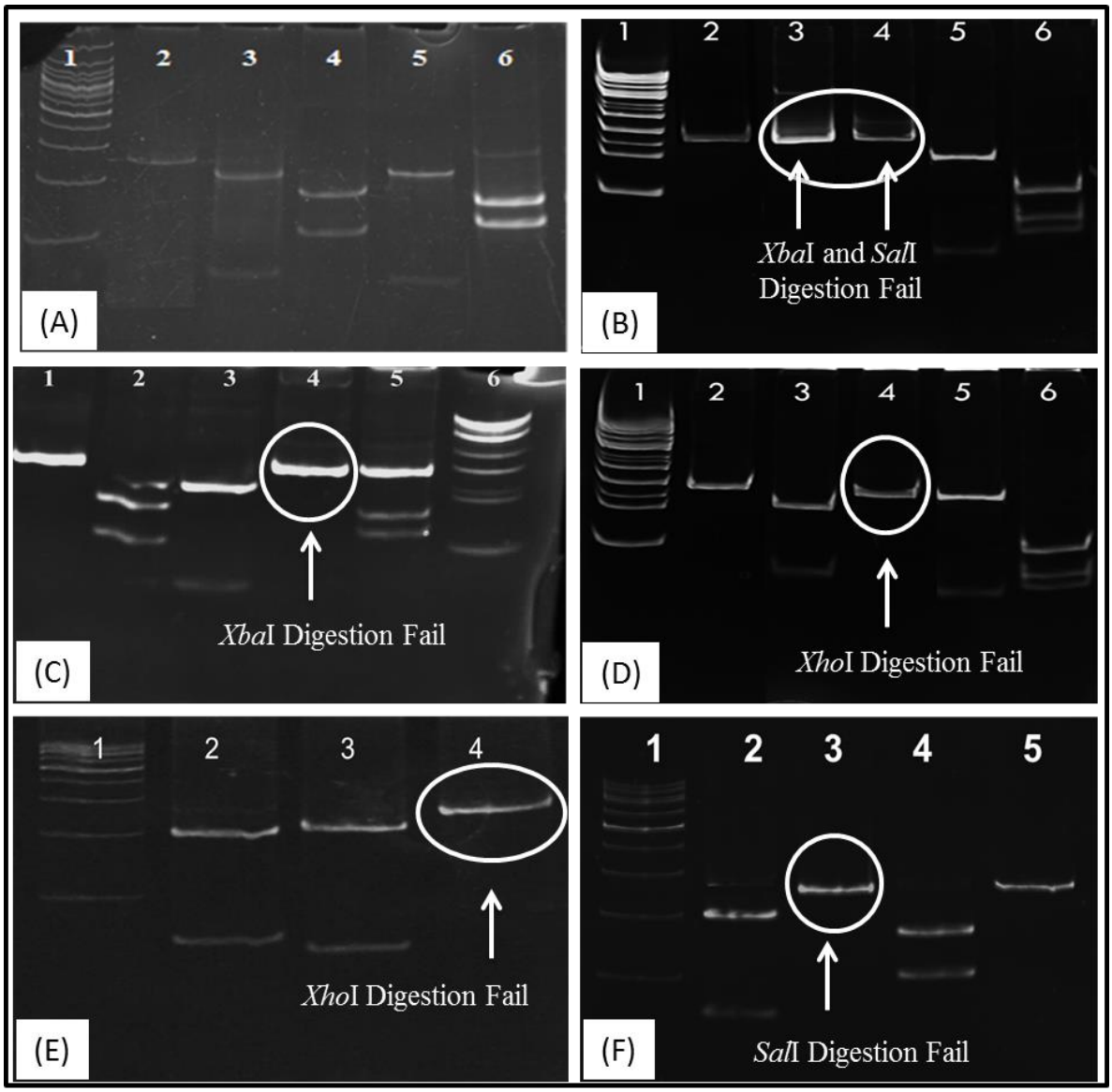

Figure 3.5: Restriction enzyme digestion pattern of yeast ubiquitin wild type as well as variant ubiquitin amplicons. (A) UbWt, which gets digested by *Xba*I, *Xho*I and *Sal*I (B) UbEP42 remains undigested upon *Xba*I, *Xho*I digestion (C) UbS20F, *Xba*I digestion failed (D & E) UbA46S and UbL50P, *Xho*I site remains undigested in both the mutants (F) UbI61T, which shows disruption of *Sal*I disruption as its not getting digested. All these sites are used for primary mutagenesis screening.

3.3.2 Cloning of yeast ubiquitin variants in bacterial expression vector

Mutants of yeast ubiquitin were cloned in pKK 223-3 bacterial expression vector where in the expression was carried out under the control of *Tac* promoter in response to IPTG.

PCR product of mutant protein was inserted between *Eco*RI and *Hind*III restriction sites. Confirmation of successful cloning was done by releasing the ubiquitin gene by digesting vector with restriction enzymes (Figure 3.6).

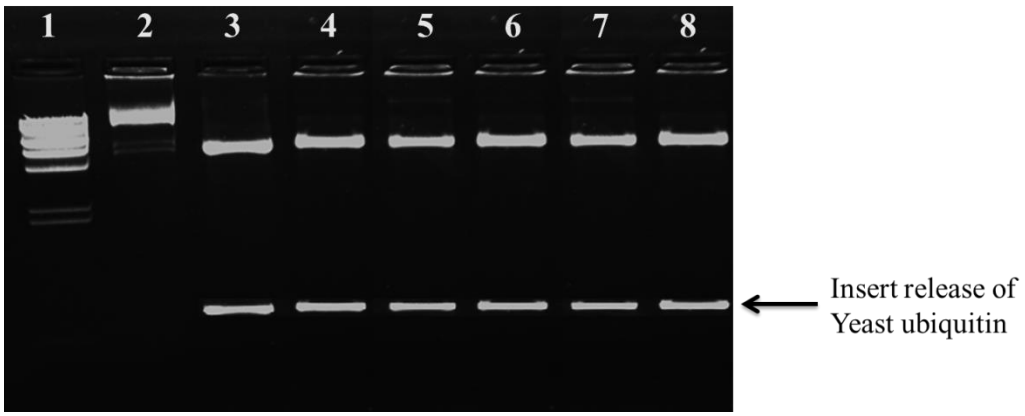

Figure 3.6: Confirmation of cloning of ubiquitin mutations by insert release. Vectors carrying mutant forms of ubiquitin gene were digested with *Eco*RI and *Hind*III, to confirm cloning through insert release. Lane 1 shows 1 Kb DNA ladder, Lane 2 shows undigested pKK 223-3 vector, Lanes 3 to 8 show insert release of UbWt, UbEP42, UbS20F, UbA46S, UbL50P and UbI61T respectively.

3.3.3 Expression and purification of yeast ubiquitin variants

Expression of mutant forms of ubiquitin, UbEP42, UbS20F, UbA46S, UbL50P, UbI61T and UbF45W were carried out by adding IPTG to *E. coli* BL21 cells containing pKK 223-3 vector harbouring mutant forms of ubiquitin gene. IPTG was added at a final concentration of 1mM to cells expressing UbF45W, UbS20F, UbA46S, UbL50P and UbI61T once the culture reached to log phase and incubate overnight whereas cells expressing UbEP42 were incubated for 5-6 hours after adding 1mM IPTG as UbEP42 was forming inclusion bodies and proteins were found in cell pellet when incubated overnight using 1mM IPTG. To avoid use of denaturant while purifying UbEP42 incubation period was varied. Overexpression of mutant ubiquitin was shown in Figure 3.7.

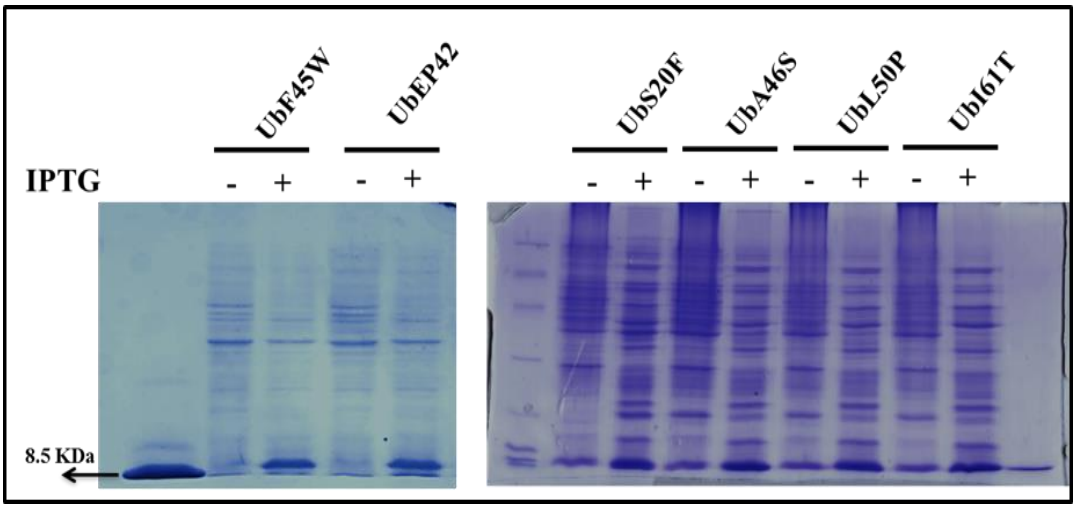

Figure 3.7: 15% SDS-PAGE showing the overexpression of ubiquitin variants. Cells were induced by 1mM IPTG and grown overnight for 5-6 hours. Lysate was checked for the presence of overexpressed ubiquitin variants.

Purification procedure includes various treatments to remove proteins other than ubiquitin. Wild type ubiquitin is heat stable protein and so this feature was used to eliminate various heat sensitive proteins. Lysate was subjected to temperature ranging from 50^0 C to 90^0 C for 10 minutes and lysates were loaded on SDS-PAGE to check stability of ubiquitin variants at increasing temperatures. The results conferred that UbF45W and UbS20F, UbA46S, UbL50P and UbI61T are heat resistant and so a strong ubiquitin band is seen on SDS-PAGE. However, UbEP42 which was found to be heat sensitive was degraded during heat treatment (Figure 3.8).

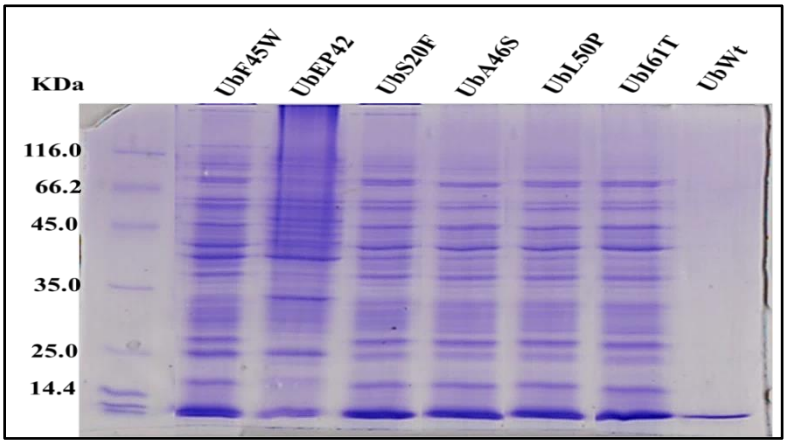

Figure 3.8: 15% SDS-PAGE showing presence of ubiquitin protein after heat treatment.

Size-exclusion chromatography was carried out to purify ubiquitin mutants as well as wild type protein and fractions corresponding to the size of ubiquitin were loaded to check ubiquitin's purity (Figure 3.9).

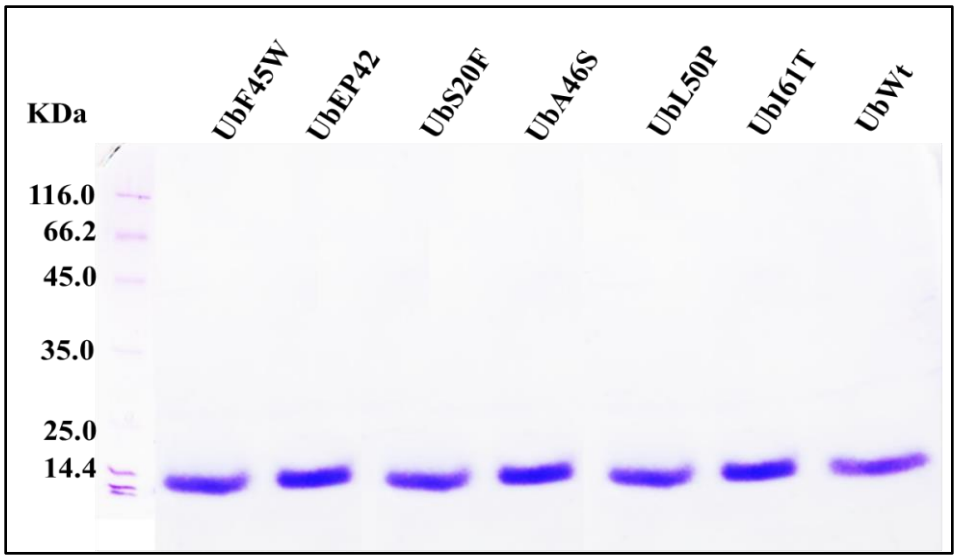

Figure 3.9: 15% SDS-PAGE showing purified ubiquitin proteins. The collected eluents were loaded on SDS-PAGE to check its purity. UbWt was also loaded as positive control.

3.3.4 Far and near UV CD spectra of variants of ubiquitin

Circular Dichroism (CD) spectroscopy was utilized to study the changes in the secondary structure of mutant ubiquitin protein. To attain this, far UV CD spectra of the mutant forms of ubiquitin viz., UbEP42, UbS20F, UbA46S, UbL50P and UbI61T were recorded. UbF45W was used as control. The spectra of UbS20F and UbA46S showed minimal changes with respect to that of UbF45W, providing an explanation for wild type like functional behaviour observed with these two mutant forms (Figure 3.10). On the other hand, UbEP42, UbL50P and UbI61T largely altered spectra when compared to that of UbF45W, displaying more of β-sheet character as their dominant feature and less of helical structure. Obtained CD spectra were analysed for the components of secondary structure using CDPro software. The results of CDPro analysis indicated reduction in helical content and enhancement of β-sheet structure in UbEP42, UbL50P and UbI61T mutants (Table 3.2).

These results point towards loss of ubiquitin folding in lethal mutations. The near UV CD spectroscopy was carried out to study differential conformational changes in tertiary structure of mutant and wild type ubiquitin protein. The aromatic amino acid side chain of protein can place in chiral environment which leads to rise in CD signals and hence can serve as characteristic fingerprints of the native structure. The results inferred that UbEP42, UbL50P and UbI61T got affected due to the mutation and caused deviation in CD signals and so in tertiary structure as compared to wild type ubiquitin protein. However, UbS20F and UbA46S remain unaffected (Figure 3.11).

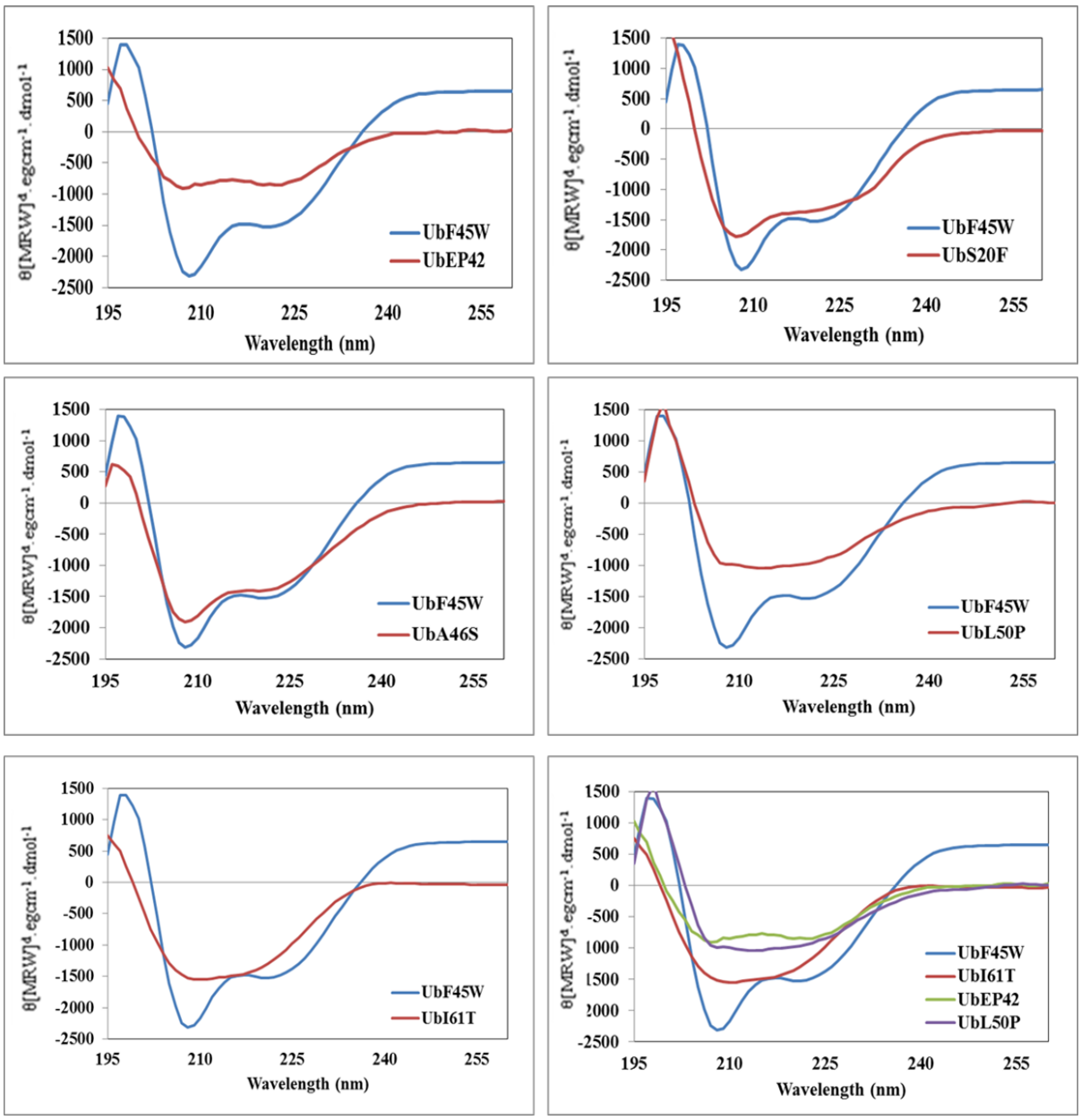

Figure 3.10: Far UV circular dichroism spectra of mutants and wild type ubiquitin

protein. Far UV CD spectra were recorded in the range of 195 nm to 250 nm in 10 mM Tris buffer, pH 8.0, to analyze the secondary structural features. Results show alteration in the secondary structure content of UbEP42, UbL50P and UbI61T as compared to wild type ubiquitin protein. UbS20F and UbA46S displayed mild changes in secondary structure with respect to UbWt.

Protein	% α-Helix	% β-Sheet	% Turns & Random coil
UbWt (X-ray)	16	32	52
UbWt	17.3	32.2	50.5
UbF45W	16.7	34.7	48.5
UbEP42	6.7	45	48.4
UbS20F	15.1	35.4	49.5
UbA46S	17.6	34	48.5
UbL50P	5.4	48	46.5
UbI61T	10.2	41	48.8

Table 3.2: Secondary structural analysis of far UV CD spectra of the wild type and mutant forms of ubiquitin using CD Pro software.

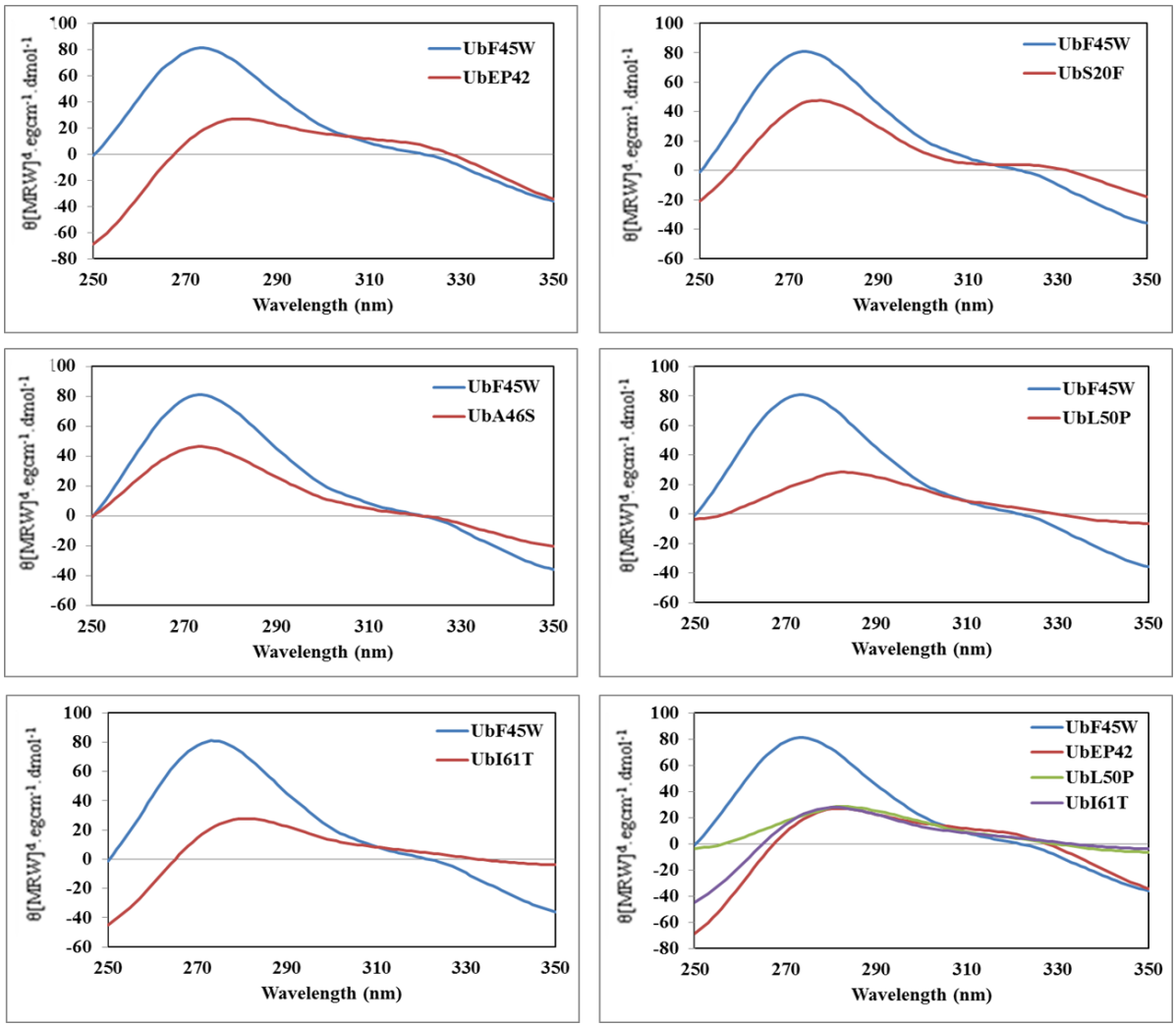

Figure 3.11: Near UV circular dichroism spectra of mutants and wild type ubiquitin protein. Near UV CD spectra were recorded in the range of 250 nm to 350 nm in 10mM Tris buffer, pH 8.0, to analyze changes in tertiary structure of the proteins. Results show changes in tertiary structures of UbEP42, UbL50P and UbI61T as compared to wild type ubiquitin protein.

From the earlier structural studies of the ubiquitin protein it is evident that the protein contains more of helical structure. Lethal mutants UbEP42, UbL50P and UbI61T were found to have more of β-sheet structure than wild type ubiquitin. Increased β-sheet content indicates possible decrease in stability of helical structure. To see if α helix can be stabilized, far-UV CD spectra of UbEP42, UbL50P and UbI61T were recorded in presence of 20% and 40% TFE (2,2,2 - trifluoroethanol). TFE is a solvent known to stabilize helical structure in peptides which can form α-helices under ideal conditions which otherwise remain in

extended conformations. Results displayed no significant changes in the far-UV spectra of UbEP42, UbL50P and UbI61T (Figure 3.12).

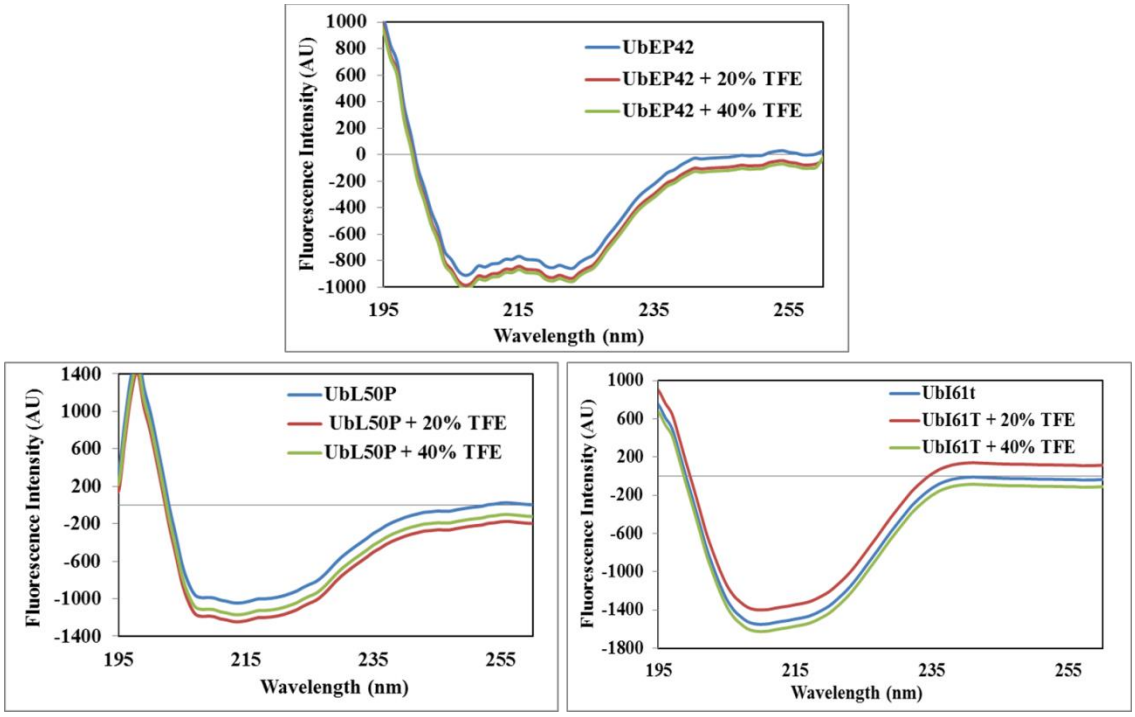

Figure 3.12: Far-UV circular dichroism spectra of UbEP42, UbL50P and UbI61T in presence of 20% and 40% TFE.

3.3.5 Fluorescence spectrum of ubiquitin mutants

UbF45W a variant of ubiquitin has been found to behave like wild type ubiquitin (Khorasanizadeh et al., 1993). In fluorescence spectroscopic studies UbF45W was used in place of UbWt. UbF45W and mutant forms of ubiquitin were excited at 280nm to record fluorescence spectra. Intensity of fluorescence emission was less with UbEP42, UbL50P and UbI61T as compared to UbF45W. This may be due to the changes in the environment of protein near intrinsic fluorophore. However, there is no significant change in fluorescence emission with UbS20F and UbA46S (Figure 3.13).

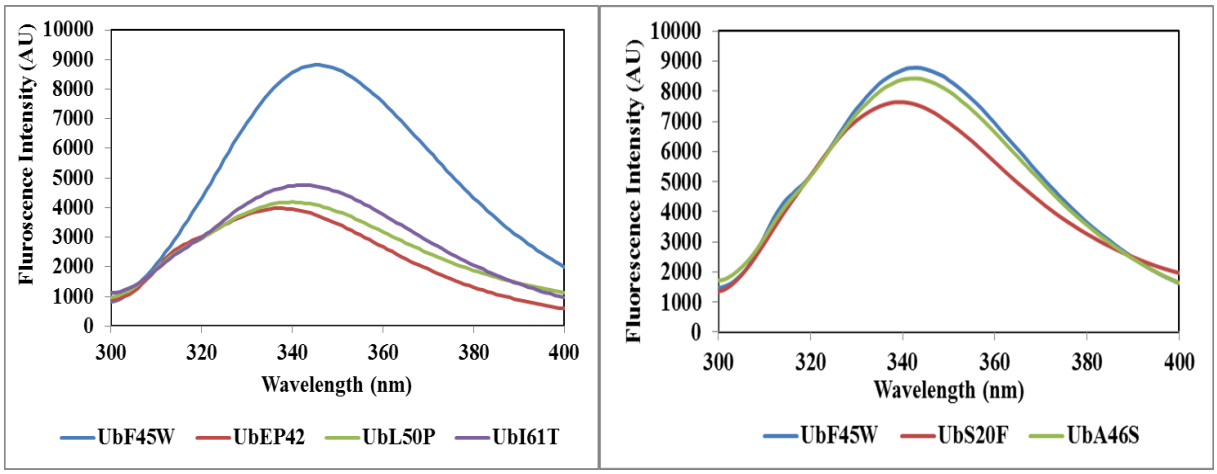

Figure 3.13: Fluorescence emission spectra of UbEP42, UbS20F, UbA46S, UbL50P and UbI61T in comparison with UbF45W recorded by exciting the protein at 280nm.

Unfolding of ubiquitin was studied by using guanidine hydrochloride (Gdn HCl), a potent chaotropic agent which unfolds protein and stabilizes unfolded state over folded state either macroscopically or through specific interactions. Increasing concentration of Gdn HCl leads to loss of secondary and tertiary structures and subsequent increase in the fluorescence intensities, along with a shift in λmax of emission to longer wave length. Protein which is more stable tends to lose its structure at higher concentrations of denaturant. Along with emission intensity, shift in the λmax of spectra is also taken into consideration while evaluating protein stability. Fluorescence emission spectra of ubiquitin mutants were recorded and shift in λ_{max} of emission of all mutants as well as wild type proteins were shown in Figure 3.14. Collectively, results show that the λ_{max} of fluorescence emission of UbEP42, UbL50P and UbI61T did not show much change, while those of UbS20F, UbA46S and UbF45W shifted to longer wavelength upon exposure to increasing concentration of Gdn HCl. It can be concluded that mutant proteins UbS20F, UbA46S have tertiary structure similar to UbF45W.

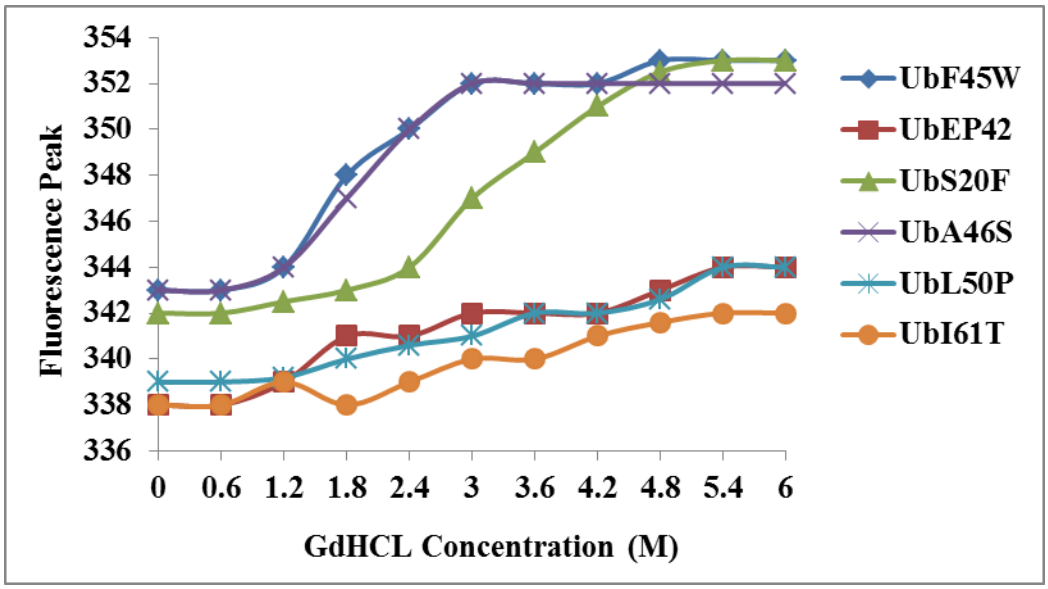

Figure 3.14: Changes in λ_{max} of intrinsic fluorescence emission of UbF45W and the ubiquitin mutants UbEP42, UbS20F, UbA46S, UbL50P and UbI61T as a function of guanidine hydrochloride concentration.

Ubiquitin type	Structural feature of the protein where substitution occurred	Kyte-Doolittle indices for the original/ substituting residues	Secondary structural preference for the original/ substituting residue
UbWt	-	-	-
UbEP42	-	-	-
S20F	3rd residue of a Type I β turn	-0.8/ 2.8	1.06/0.96
A46S	2nd residue of Type III β turn	1.8/ -0.8	0.96/1.23
L50P	β-sheet	3.8/ -1.6	1.30/0.55
I61T	Between two turns in a turn rich region	4.5/ -0.7	1.60/1.20

Table 3.3: Secondary structure alteration due to amino acid replacement. The hydropathy scales of original and substituting residues by Kyte- Doolittle, 1992, secondary structural preferences of original and substituting residues for β-turns as observed by Hutchinson and Thornton, 1994 and for β-sheets by Chou and Fasman, 1978 are also indicated (Doshi et.al., 2014).

3.4 DISCUSSION

Ubiquitin is very well studied, conserved protein of eukaryotes. Stability of a protein in various physico-chemical conditions depends on its structural integrity. Mutations occurring in essential proteins due to evolution, either reducing stability or affecting function are removed by the process of natural selection. Structural stability of ubiquitin is essential as it plays pivotal role in numerous processes in eukaryotic cells. The structure of ubiquitin is stabilized by its globular shape with a hydrophobic core and extensive hydrogen bonding. Ubiquitin gene was subjected to random mutagenesis to understand the importance of conserved residues in the maintenance of ubiquitin structure. One of the mutations generated as a result was the dosage dependent lethal UbEP42. UbEP42 harbours four mutations with replacement of amino acid residues i.e S20F, A46S, L50P and I61T. These mutations were further segregated to study their individual effects on structural and functional aspects. Site-directed mutagenesis was carried out to clone these point mutations and UbS20F, UbA46S, UbL50P and UbI61T were generated. Ubiquitin mutations namely, UbEP42, UbL50P and UbI61T generated in our laboratory displayed dosage dependent lethal phenotype in *Saccharomyces cerevisiae* and *Candida albicans*. UbS20F and UbA46S did not display any adverse effects on these organisms under laboratory conditions.

From the results of circular dichroism, we observed radical alteration in secondary structure of lethal mutants UbEP42, UbL50P and UbI61T as compared to wild type protein. These substitutions led to loss of helical structure with increase in sheets content (Doshi et al., 2017). Mutations caused change in tertiary structure as well, evident from near UV CD spectra and fluorescence spectra. However, these mutants did not show any remarkable difference in presence of TFE, meaning that the conformations displayed by UbEP42, UbL50P and UbI61T are stable.

The substitutions of Ser20 to Phe and Ala46 to Ser are present in type I and type III turns respectively (Figure 3.1) (Vijay-kumar et al., 1987), as the substituted residues show similar preference for the secondary structures. Moreover, it is reported that Ala46 forms H-bond with ε-amino group of Lys48. Ser20 and Ala46 are surface residues. Replacement of Ser which is hydrophilic to Phe which is hydrophobic and hydrophobic Ala to hydrophilic Ser leads to the difference in hydropathy scale of these mutant proteins. However, the hydrophathicity remained approximately constant as of wild type ubiquitin protein (Table 3.3). This could be the probable reason responsible for maintaning functionality in spite of replacement of amino acid residues. Mutation Leu50 to Pro has considerably changed protein structure. This replacement occurred in the last residue of β-strand. Side chain of Leu is buried in the interior of the protein. Moreover, Pro has very low propensity for forming β-strand due to its restricted geometry which produces kink in the protein backbone. Additionally, both Ala46Ser and Leu50Pro occur closer to the two functionally important residues Ile44 and Lys48 and hydropathy of mutant differed significantly and so this mutation is not favourable. Thr shares similar preference for secondary structure as of Ile61. However, Ile61 is reported to be the first residue which is protected from H-D exchange during refolding of ubiquitin (Briggs & Roder, 1992). Side chain of Ile61 remains buried in a hydrophobic pocket formed by Ala46 and Leu67 of the wild type protein and hence the replacement nonpolar residue by polar residues is unfavourable. It is also evident from the hydropathicity of I61T which is significantly different as compared to wild type ubiquitin (Prabha et al., 2010; Doshi et al., 2014).

Ubiquitin mutant namely, UbEP42, UbL50P and UbI61T were found to be destabilized due to residues replacement. The substitutions are likely to disturb the hydrophobic patch of ubiquitin formed collectively by Leu^{8}, Ile^{44} and Val^{70} as side chain of the Ala46 and Leu50 buried in the hydrophobic patch. Hydrophobic patch is crucial for the recognition of ubiquitin in proteasome mediated degradation of proteins and Ile^{44} in association with Phe^{4} promotes binding site that interacts with components of endocytic machinery (Beal et al., 1996; Shih et al., 2000; Sloper-mould et al., 2001). Moreover, Ile44 of hydrophobic patch displays protective effects against destabilization of K63 linked transactivator-promoter complexes (Archer and Kodadek, 2010).

It is reported that Leu^{8}, Ile^{44} and Val^{70} hydrophobic patch is crucial for degradation as

replacement of these residues with alanine severely inhibited degradation of Ub-substrate conjugates and hindered attachment of polyubiquitin chain to S5a subunit, a regulatory complex of the 26S protease (Beal et al., 1996). In addition, Ile^{44} is important for endocytosis (Fisher et al., 2003), MVB biogenesis (Swanson et al., 2003), kinase regulation (Wu et al., 2006), autophagy (Varadan et al., 2005) and DNA repair (Wang et al., 2005) in association with various ubiquitin binding domains. UbEP42, UbL50P and UbI61T displayed compromised K48 linked polyubiquitination, hampered protein trafficking and unbalanced protein kinase levels as presented in **Chapter 2** suggesting that replacement of conserved residues of ubiquitin may have led to alteration in hydrophobic patch.

Surprisingly, owing to four substitutions, UbEP42 shows temperature sensitive phenotype, whereas other mutant forms of ubiquitin which harbour single residue replacement i.e. UbS20F, UbA46S, UbL50P and UbI61T remain thermo-resistant as of wild type ubiquitin. The Salt bridges have been proposed to play a crucial role in promoting increased thermostability in proteins and contrifbute marginally to stability of protein at room temperature. K11 and E34 of ubiquitin are surface residues and display spatial orientation characteristic of a salt bridge (Makhatadze et al., 2003). It is evident from the earlier studies that, the single-site substitution remain structurally similar to the wild type counterpart and usually do not lead to large structural perturbations despite the dramatic functional alterations (Matthews, 1993; Keefe et al., 1994; Matthews, 1995; Matthews, 1996; Takano et al., 2000; Delbruck et al., 2001; Vaughan et al., 2002). However, UbEP42 with mutations at four places shows differences in charge and polarity of amino acids. Such drastic changes are mainly responsible for the dramatic differences observed in secondary structure content of UbEP42, UbL50P and UbI61T and compromised thermostability of UbEP42 from wild type ubiquitin.