CHAPTER 3

CONSTRUCTION, EXPRESSION, PURIFICATION OF UbS65D AND ITS STRUCTURAL ANALYSIS

3.1. INTRODUCTION

The high degree of conservation of ubiquitin sequence suggests that the conserved residues in the protein might have direct or indirect role to play either in structure or in the function of ubiquitin and tampering with any of the residues may have a drastic effect either on the structure or on the function (Mishra et al., 2009, in press). Present study focuses on the β-bulge present in the C-terminal region of the protein, which includes Glu64, Ser65 and Gln2 (Vijay-Kumar et al., 1987). The substitution mutation E64G has been well accommodated in the protein, failing to show any drastic effect on structure. Interestingly, the mutation increased the stability of the protein (Mishra et al., 2009, in press). Results of the study are presented in Chapter 2. Ser65 which is at the second position in the parallel β-bulge is the last residue of a type II turn accompanying β-bulge (Vijay-Kumar et al., 1987). According to percentage frequency of occurrence of amino acids in β-bulge Ser65 has less preference having a value of 1.5 (Chan et al., 1993). To investigate the importance of Ser in 65th position, the site directed mutant UbS65D was constructed replacing Ser by Asp, a residue with percentage frequency of 2.4 (Figure 3.1).

Amino acid	I	F	V	L	W	M	A	G	C	Y	P	T	S	H	E	N	Q	D	K	R
Faai/bulge	0.21	0.52	0.54	0.80	0.57	0.40	0.54	0.54	0.00	1.0	0.00	1.0	1.5	0.44	2.0	0.91	3.1	2.4	2.6	2.7
Naai	2	3	7	8	1	1	4	4	0	5	0	8	8	1	9	3	9	10	15	11

Faai/bulge - % frequency of occurrence of amino acid i in a bulge.
Naai - Total number of amino acid i found in a bulge.

Figure 3.1. Percentage frequencies of amino acids in the second position of β-bulge. (Chan et al., 1993)

Since Asp is favoured structurally in comparison with Ser, it is expected to be well accommodated in the second position of the β-bulge. Though, the percentage frequency of Asp is not the highest, it makes an interesting choice, as it is found in this position in most of

the structural homologs of ubiquitin (Jentsh et al., 2000). Sequence comparison of ubiquitin with its structural homologs is shown in Figure 3.2.

Figure 3.2. Sequence comparison of ubiquitin with its homologs. (Jentsh et al., 2000)

The structure of mutated protein UbS65D was studied using CD and fluorescence spectroscopic techniques.

3.2. MATERIALS AND METHODS

3.2.1. Construction and expression of UBS65D-pKK 223-3

Mutation UbS65D was introduced using recombinogenic polymerase chain reaction into synthetic yeast ubiquitin fluorescent gene UbF45W of Plasmid pKK 223-3, described in Chapter 2. The sequences of two sets of primers used for recombinogenic PCR are given in Table 3.1. Primers B2 and B1 were designed to incorporate one more mutation that destroys a *Sal* I, to facilitate easy detection of incorporation of S65D mutation. However, *Sal* I site mutation being a silent mutation does not change any amino acid residue. Figure 3.3 presents the strategy adopted for site directed mutagenesis.

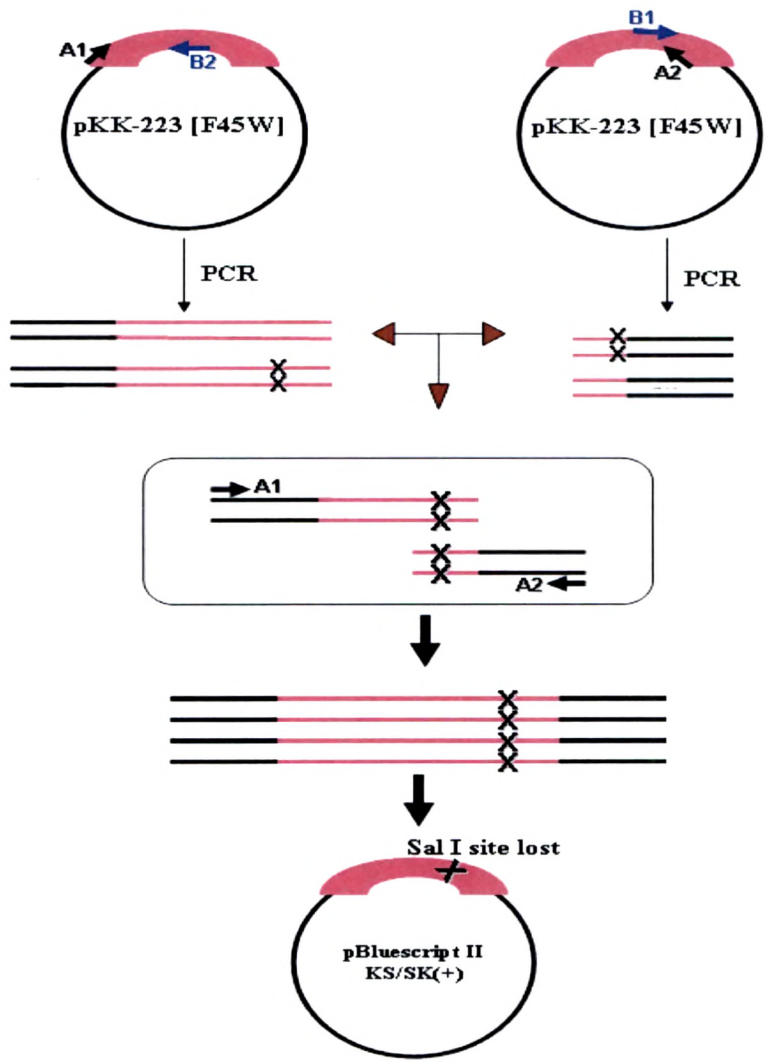
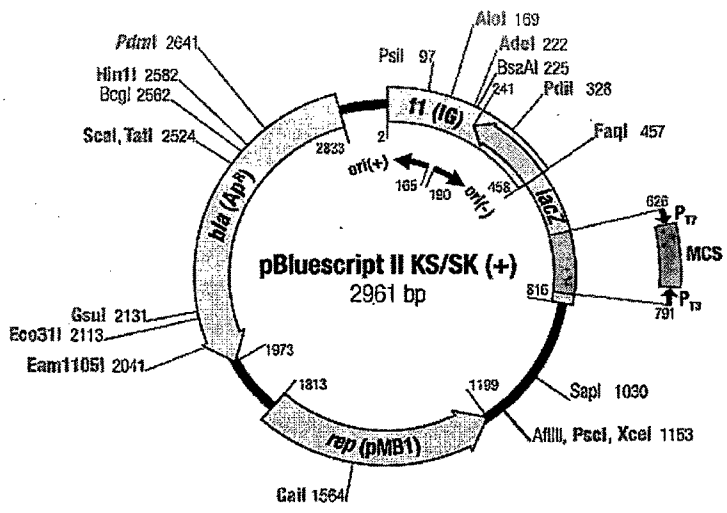


Figure 3.3. Strategy adapted for recombinogenic PCR.

Table 3.1. Sequence and direction of primers used for recombinogenic PCR

Primer and its direction	Primer sequence
Forward Primers **A1**	5′ACAGAATTCATGCAGATCTTCGTCAAG3′
Reverse Primers **A2**	5′GCC AAGCTTCGCTCAACC3′
Forward Primers **B1**	5′CAAGATGTAAGGTATCCTCCTTCTGAATG3′
Reverse Primers **B2**	5′ATTC AGAAGGAG GATACCTTACATCTTG3′

Blunt ended recombinogenic PCR product was cloned in pBluecsript II KS/SK(+) at *Eco* RV restriction site. Introduction of mutation was confirmed by loss of *Sal* I site and subsequently by DNA sequencing. The sequence data confirmed introduction of mutation. The *Hind* III- *Eco* RI fragment from pBluescript II KS/SK(+) vector (figure 3.4) was cloned back and pKK 223-3/UbS65D was obtained. Expression was confirmed on 15% acrylamide gel after incubating with 0.5mM IPTG for two hours.



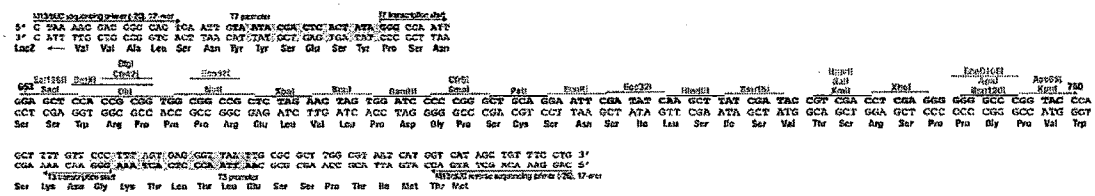


Figure 3.4. Vector back bone pBluescript II KS/SK(+) and sequence of multiple cloning site

3.2.2. Purification of UbS65D protein

Protein purification and protein estimation were done using methods given in Materials and Methods section (2.2.3.) of Chapter 2.

3.2.3. Circular dichroism and fluorescence Spectroscopy

CD spectra were recorded using a Jasco J-715 spectropolarimeter. For far uv CD spectra cells of 1mm path length were used for 200 to 250nm. Spectra were recorded at 2nm resolution. The data pitch was 0.2nm. Scan speed was 50nm/sec. Five spectra were accumulated to reduce the noise. Protein solutions were prepared in 10mM Tris HCl, pH7.4 and concentration of protein was 0.2mg/ml (23.4μM). The near uv CD spectra of the proteins were recorded between 250 and 320nm. Path length was 1cm and protein concentration was 1mg/ml (117μM). Spectra for far and near uv were also recorded at buffer of pH2.2 (Gly-HCl), pH5 (sodium acetate buffer) and pH10 (Gly-NaOH) with 10mM concentration final. Proper blanks were prepared for all samples and the spectra of the samples were blank corrected.

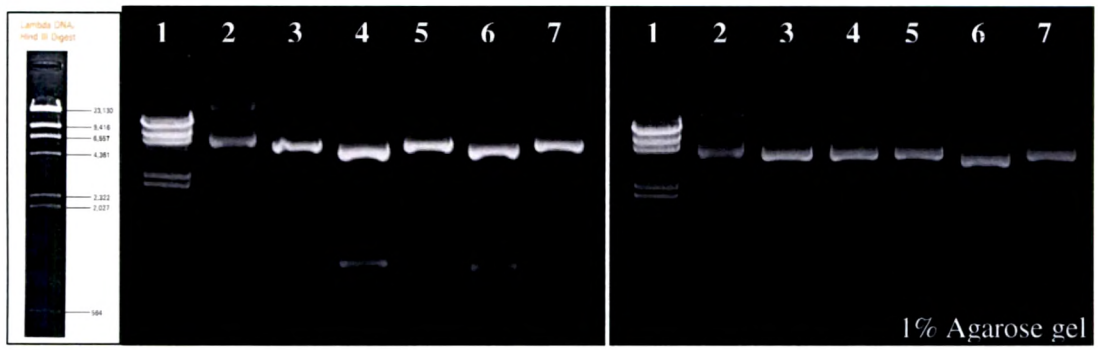
Fluorescence spectra were recorded using Hitachi F-4010 fluorescence spectrophotometer. Slit width was 5nm. Protein concentration was 0.2mg/ml (23.4μM). Samples were excited at 295nm to record the intrinsic fluorescence due to tryptophan. Emission was recorded between 320-440nm.

Bis-ANS was used as an extrinsic fluorophore at a concentration of 10μM. Bis ANS was excited at 390nm and emission was recorded between 450-600nm. Slit width was 5nm. Protein concentration was 0.2mg/ml (23.4μM). Protein solutions were prepared in Tris HCl buffer, pH 7.4. The experiment was repeated with 50μM ANS. Other parameters were not changed.

3.3. RESULTS

3.3.1. Construction, expression and purification of UbS65D from pKK 223-3

Construction of pKK 223-3-UbS65D was done by using recombinogenic PCR in vitro site directed mutagenesis protocol. The ubiquitin gene thus constructed is a double mutant at 45^{th} and 65^{th} position. S65D replacement was screened by loss of *Sal* I site (Figure 3.5), which was destroyed after the mutation is introduced (Figure 2.5). UbS65D protein was expressed by using pKK 223-3-UbS65D vector and purification was done by same method as used for UbWt and the purity was checked on 15% SDS-PAGE.



(a) **(b)**

Figure 3.5. Restriction digestion patterns of (a) pKK223-3-UbF45W and (b) pKK223-3-UbS65D. Lane 1. Contains λ *Hind* III digest as marker. Lane 2. Contains undigested plasmid of pKK223-3-UbF45W (a) and pKK223-3-UbS65D (b). Lane 3. Contains *Eco* RI digest. *Eco* RI has a unique restriction site. Lane 4. Contains *Sal* I digest. *Sal* I has two sites in the vector backbone one in the ubiquitin gene and other out side the gene (a) and releases a 728bp fragment and the site is lost after subcloning of UbS65D in (b) resulting in no fragment release. Lane 5. Contains *Xho* I digest and gives a single band of 4791bp fragment with pKK223-3-UbF45W (a) and also for (b) pKK223-3-UbS65D, which is the actual size of the vector. *Xho* I site is present near the *Sal* I site. Lane 6. Contains *Sal* I-*Xho* I double digest which releases 684bp. Lane 7. Contains *Hind* III digest which is also a unique restriction site in the vector. UbS65D gene was cloned in *Eco* RI and *Hind* III.

3.3.2. Sequence analysis of UbS65D gene in pKK223-3

Introduction of the mutation was also confirmed by sequencing of the UbS65D gene.

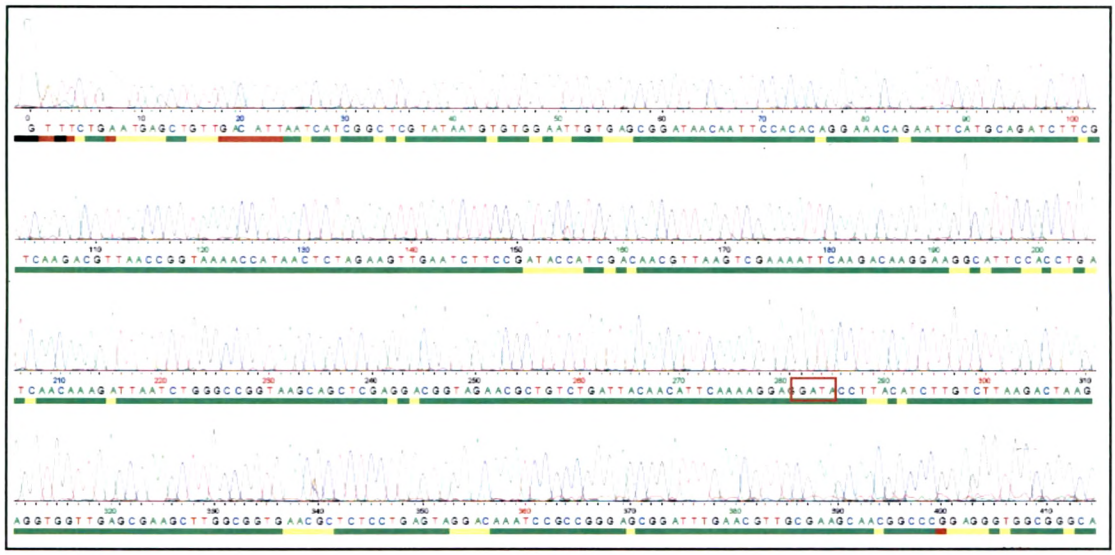
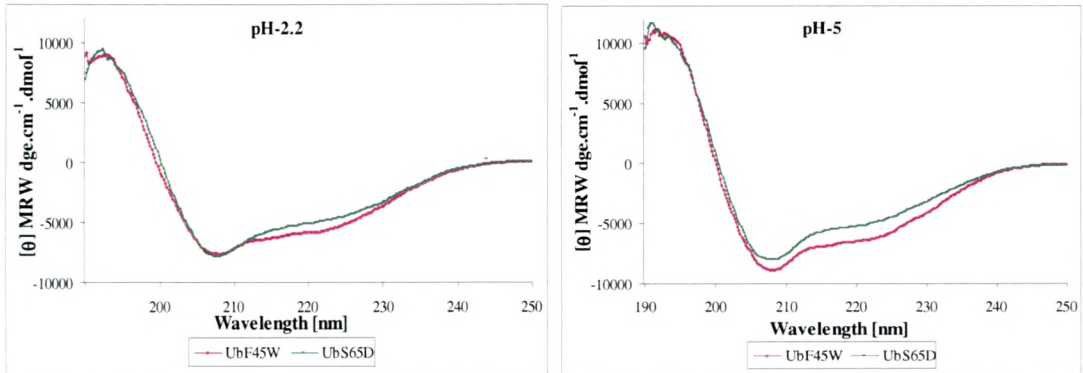


Figure 3.6. Electrophoretogram of UbS65D gene in pKK223-3 vector. The marked region confirms the UbS65D substitution.

3.3.3. Far and near UV CD spectra of ubiquitin variants UbF45W and UbS65D

Far uv CD spectra for UbS65D and UbF45W were recorded to study the changes in secondary structure due to mutations in the sequence of ubiquitin. The spectra for UbF45W were used as reference in far and near uv CD spectra. UbS65D shows a slight change in secondary structure with respect to UbF45W (Figure 3.7.).



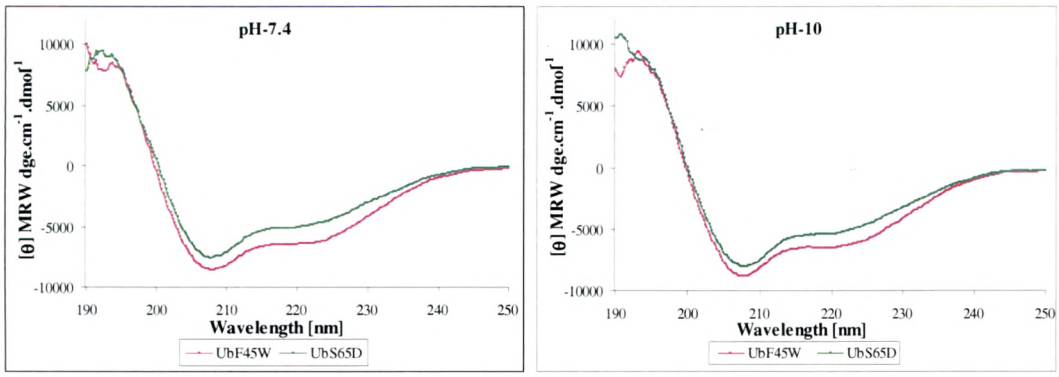


Figure 3.7. Far uv CD spectra of the two variants of ubiquitin UbF45W and UbS65D at various pH buffers 2.2, 5, 7.4 and 10.

Based on near uv CD spectra, the tertiary structure of UbS65D and UbF45W are alike (Figure 3.8.).

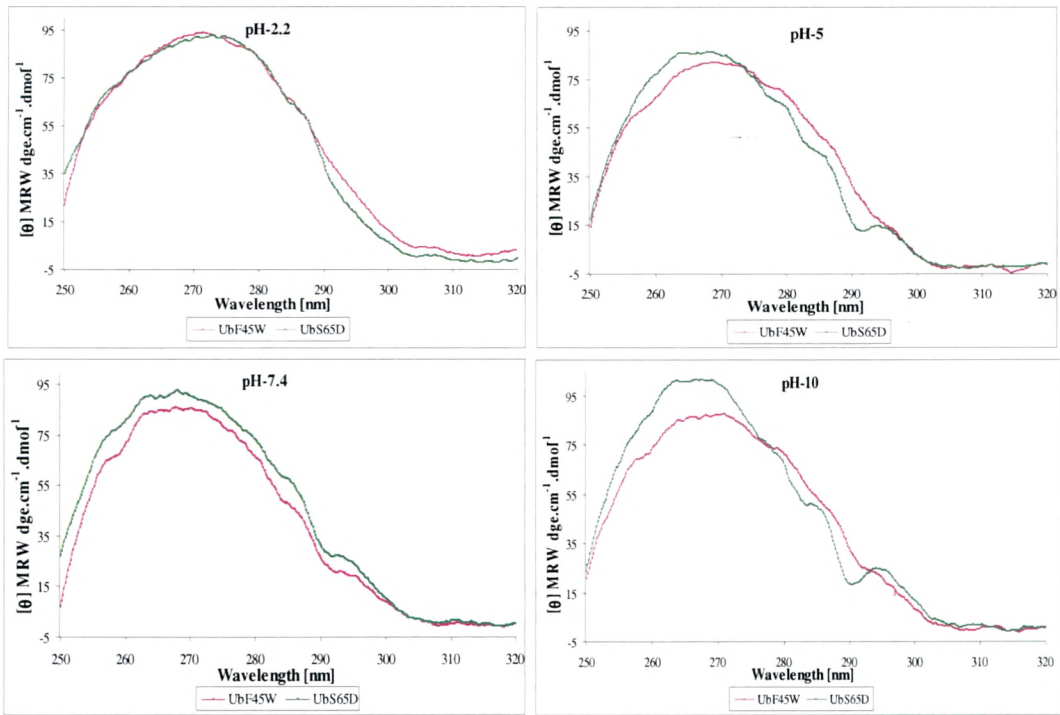
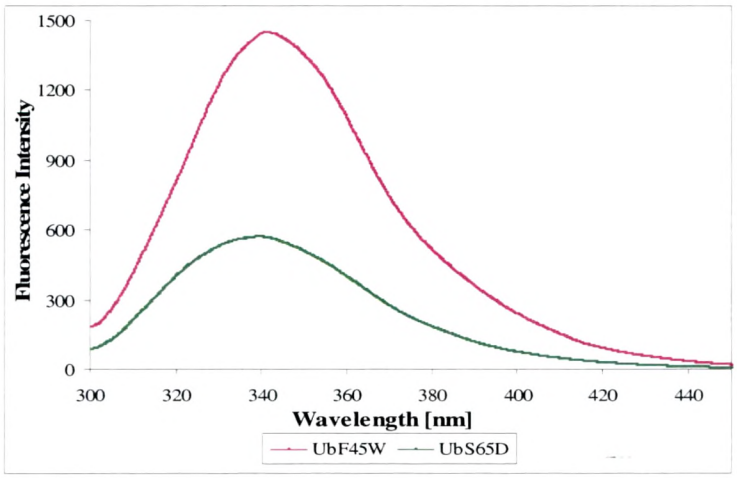


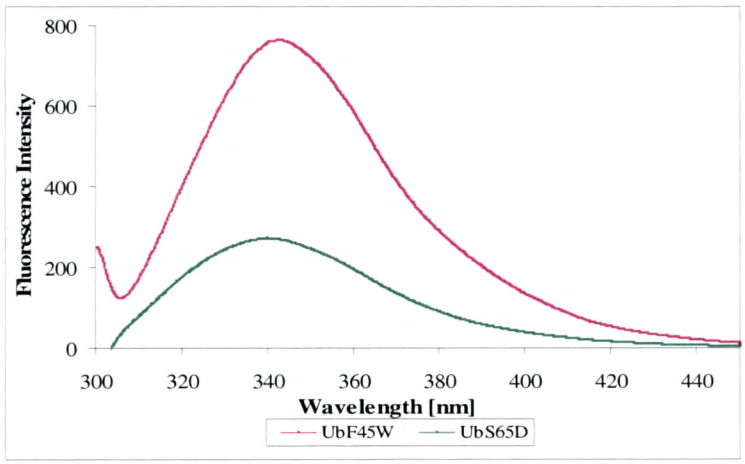
Figure 3.8. Near uv CD spectra of UbF45W and UbS65D at various pH values 2.2, 5, 7.4 and 10.

3.3.4. Fluorescence spectrum of UbF45W and UbS65D

Fluorescence spectra for UbS65D and UbF45W were recorded by exciting the protein at 280nm. UbS65D and UbF45W showed difference in the intensities of fluorescence, indicating that the environment around aromatic amino acid residue is changed by the substitution. Moreover, the λ_{max} of emission of UbS65D showed a blue shift, an indication for Trp being located in a comparatively nonpolar environment. This fact was supported by the spectra obtained by exciting Trp at 295nm (Figure 3.9.).



(a)

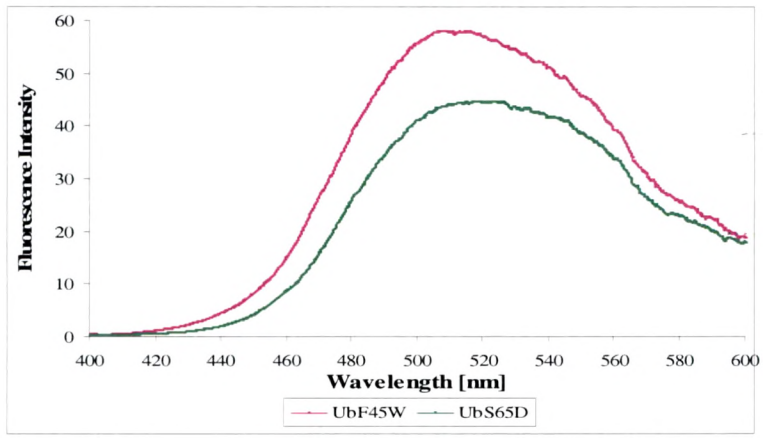


(b)

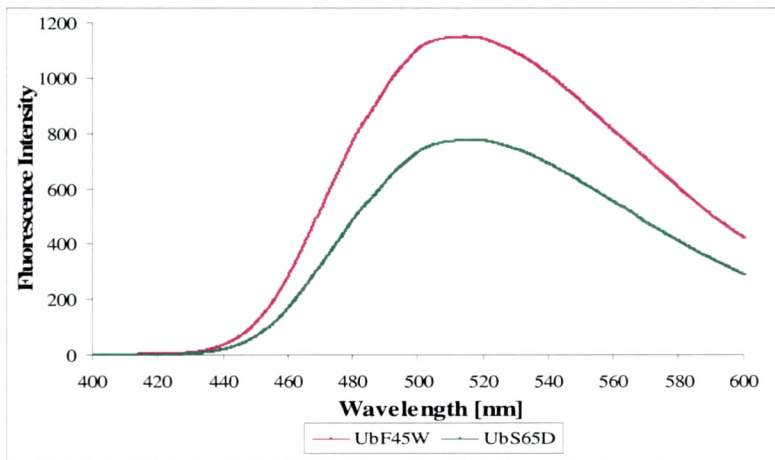
Figure 3.9. Fluorescence emission spectra of UbF45W and UbS65D recorded after exciting the proteins at 280nm (a) and 295nm (b)

3.3.5. ANS binding studies with UbS65D and UbF45W

Fluorescence spectra of ubiquitin with extrinsic fluorophore ANS and Bis-ANS showed lesser intensity with UbS65D compared to UbF45W. ANS is known to bind hydrated hydrophobic residues and emit fluorescence. Higher intensity of ANS and Bis-ANS fluorescence indicates greater exposure of hydrophobic residues to the surface. Results observed with two extrinsic fluorophores Bis-ANS and ANS indicated that the structure became more compact after substitution (Figure 3.10.).



(a)



(b)

Figure 3.10. Fluorescence emission spectra showing the fluorescence of extrinsic fluorophores ANS and Bis- ANS bound to the two variants of ubiquitin UbF45W and UbS65D shown in panels (a) and (b) respectively.

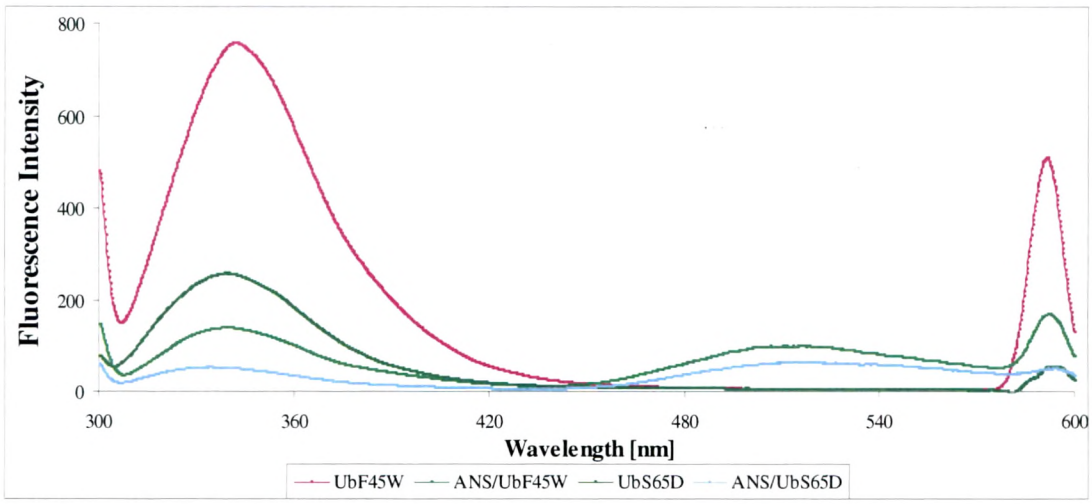


Figure 3.11. Spectra showing fluorescence resonance energy transfer between tryptophan of ubiquitin UbF45W and UbS65D in absence and presence of ANS.

Fluorescence resonance energy spectrum demonstrating energy transfer between Trp and ANS was recorded, by exciting Trp at 280nm.

3.3.6. Thermal denaturation/ renaturation profiles of UbS65D and UbF45W

Thermal denaturation experiments showed that the two proteins follow same path during unfolding (Figure 3.12). The process of thermal denaturation was found to be incomplete due to the characteristic of high thermal stability observed with this protein. The observation corroborates with the fact that the heat treatment step used for purification of wild type protein could be adopted for purification of UbS65D without any modification.

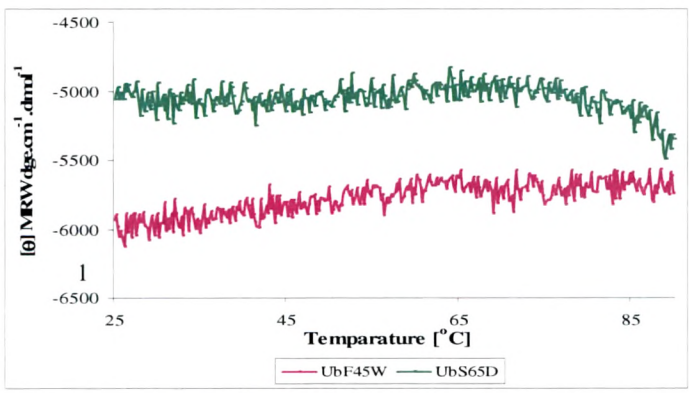
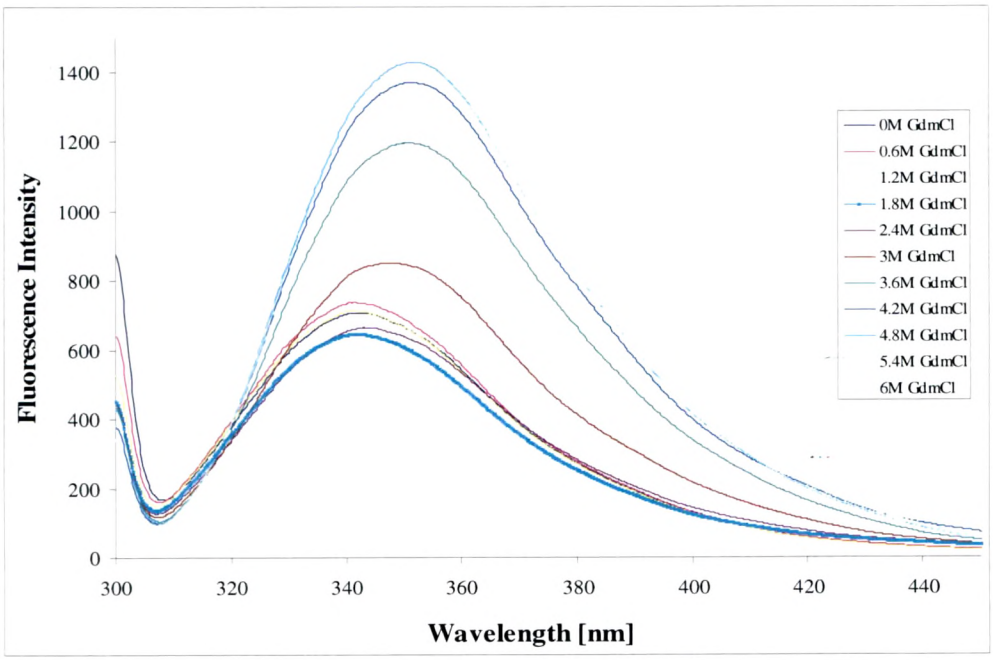


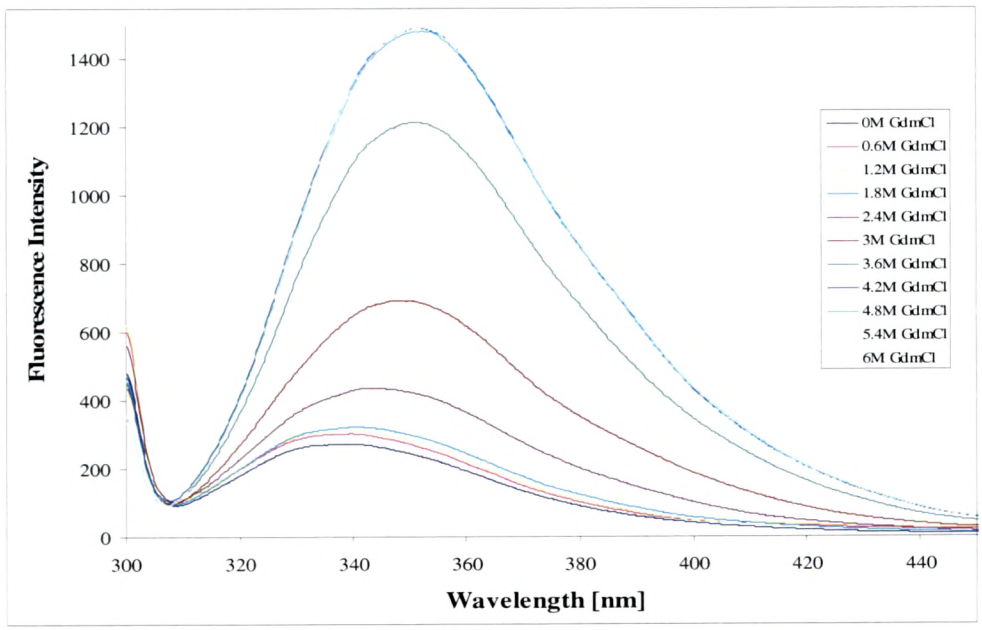
Figure 3.12. CD spectra showing thermal denaturation of UbF45W and UbS65D.

3.3.6. Guanidine hydrochloride denaturation of UbS65D and UbF45W

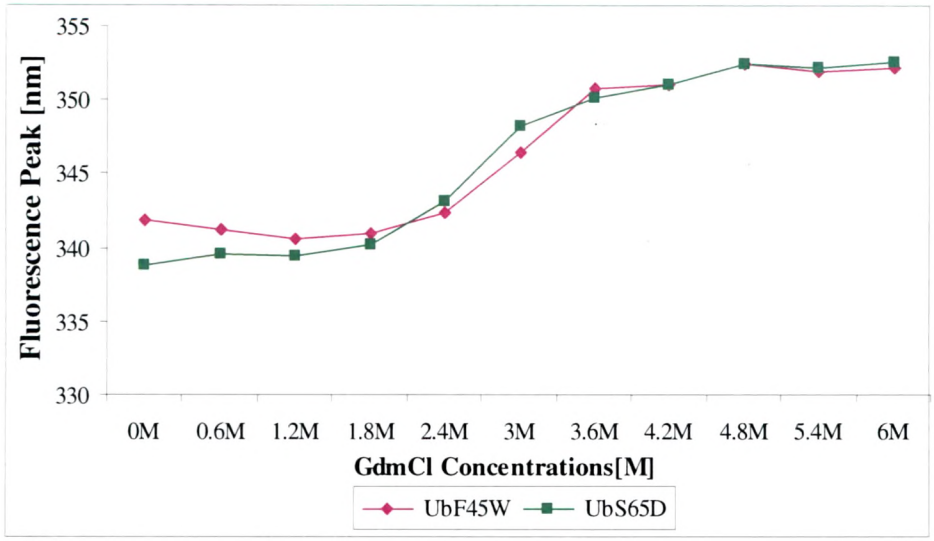
The denaturation of UbS65D and UbF45W were carried at different concentration of guanidine hydrochloride to study the stabilities of the proteins (Figure 3.13).



(a)



(b)



(c)

Figure 3.13. Intrinsic fluorescence spectra for guanidine hydrochloride denaturation of (a) UbF45W and (b) UbS65D. (c) Guanidine hydrochloride denaturation curves of UbF45W and UbS65D.

3.4. DISCUSSION

The results establish that S65D substitution has been well accommodated in ubiquitin locally. However, replacement of Ser by Asp has affected the overall conformation of the protein, by altering the surface hydrophobicity. Besides, the protein UbS65D was found to be as stable as UbF45W towards guanidine hydrochloride denaturation under equilibrium conditions (Figure 3.13.c). UbS65D could be purified by adapting the heat treatment described under experimental procedures section (section 2.2.3.) of Chapter 2, used for purification of UbWt, UbF45W and UbE64G, because of its thermal stability. Since UbE64G and UbF45W are thermostable, it appears that thermal treatment does not lead to complete denaturation (Figure 3.12). The thermal denaturation profiles appear similar. Thermal unfolding of UbS65D also did not differ from that of UbF45W.

In conclusion, replacing the conserved Ser at position 65 with Asp leads to subtle changes in structure, such as change in the exposed hydrophobic regions of the protein. This study demonstrates the point mutation produces slight structural alteration in the protein.