Chapter 2

Cloning, Purification and Structural Characterization of Four Domains of Ubiquitin Activating Enzyme E1

2.1. Introduction

Ubiquitin Activating Enzyme gene, UBA1, of *Saccharomyces cerevisiae* is a 3075 base pair long gene, which encodes a 1024 residue protein E1/Uba1 that is structurally conserved throughout evolution. The crystal structure of yeast E1 with a ubiquitin molecule bound to its adenylation site has been reported earlier (Lee and Schindelin, 2008). E1 consists of a complex arrangement of six structural domains referred to as Inactive Adenylation Domain (IAD), Active Adenylation Domain (AAD), First Catalytic Cysteine Half-domain (FCCH), Second Catalytic Cysteine Half-domain (SCCH), Four Helix Bundles (4HB) and Ubiquitin Fold Domain (UFD) **(Fig. 2.1)**.

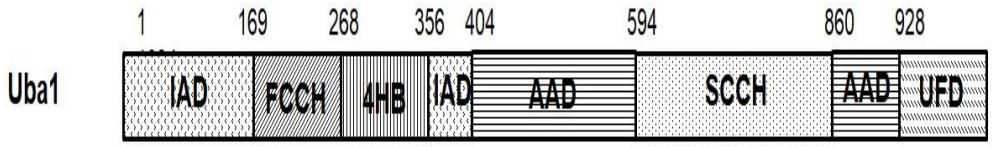


Fig. 2.1. Linear representation of yeast ubiquitin activating enzyme E1 and its domains namely, AAD (Active Adenylation Domain), FCCH (First Catalytic Cysteine Half-domain), 4HB (Four Helix Bundle), AAD (Active Adenylation domain), SCCH (Second Catalytic Cysteine Half-domain) and UFD (Ubiquitin Fold Domain).

IAD is interspersed in the N-terminal half of E1, while AAD is present on the C-terminal half of the enzyme. AAD being catalytically active serves as the site for adenylation reaction where ATP and ubiquitin are bound noncovalently (Lake et al. 2001; Walden et al., 2003). Adenylation domains of eukaryotic E1 enzyme are homologous to MoeB and ThiF proteins of bacteria (Johnson et al., 1997), as discussed earlier in Chapter 1. The domains carrying the catalytic cysteine are subdivided into two parts; FCCH and SCCH, which

are found inserted into each of the adenylation domains (Szczepanowski et al., 2005). The SCCH domain consisting of catalytic cysteine forms a thioester bond with ubiquitin. On the other hand, FCCH, which is in association with IAD, is non-functional. 4HB domain present immediately after the FCCH represents a second insertion in the IAD. The C-terminal UFD has a role in the recruitment of specific E2s (Huang et al. 2005; Lois and Lima, 2005).

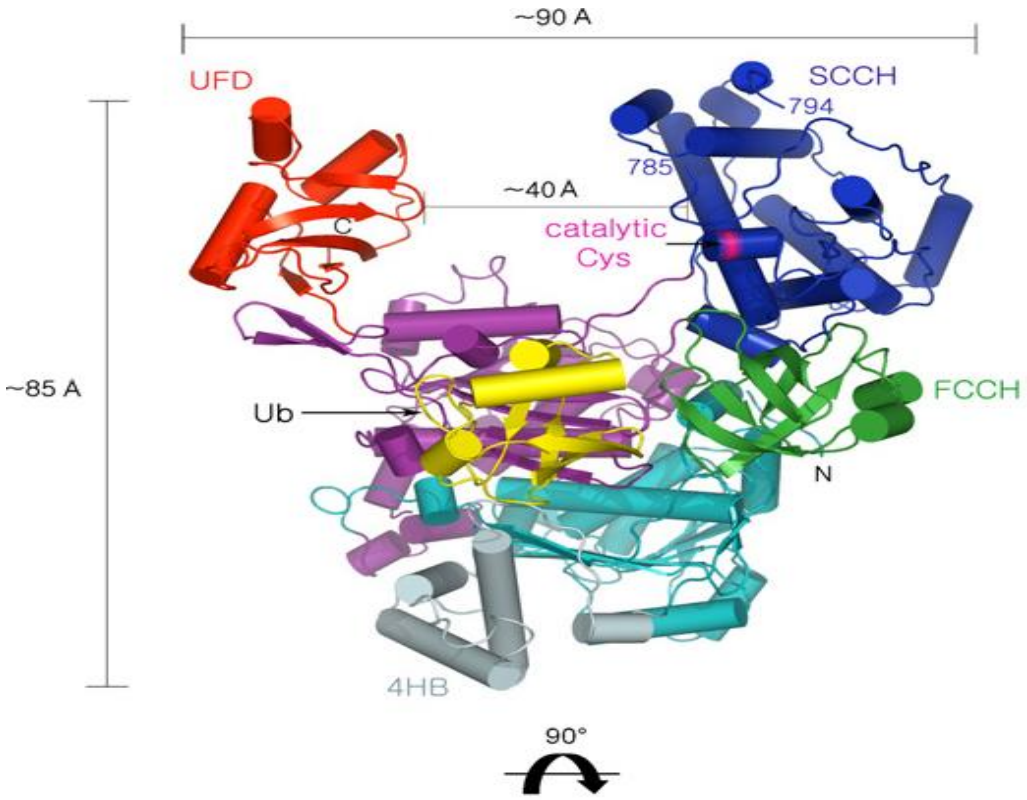


Fig. 2.2. Cartoon diagram of the E1-Ub complex (PDB ID: 3CMM). E1 contains 39% helical (46 helices; 403 residues) 16% β-sheet (47 strands; 170 residues) structure. Domain IAD is in cyan, AAD is in purple, FCCH is in green, SCCH is in blue, UFD is in red and 4HB domain is in pale cyan. Ubiquitin is displayed in yellow and the catalytic cysteine of E1 is in pink. (Lee and Schindelin, 2008)

Arrangement of the domains UFD, FCCH, and SCCH is quite crucial as they are connected to their respective adjacent domains by flexible linkers (**Fig. 2.2**). UFD, the extreme C-terminal domain is attached to AAD by an 18 residue β-hairpin loop at the end of AAD, known as the UFD linker. FCCH is attached to IAD by two long antiparallel β-strands whereas SCCH is linked to AAD by an extended 18 residue linker (Lee and Schindelin, 2008). These structural features suggest that E1 might undergo large-scale conformational changes during the ubiquitination process.

It would be interesting to study domains of E1 as independent units to understand if isolated domains can retain native structure. Hence, in this chapter, we will be focusing on four domains of ubiquitin activating enzyme E1 namely, FCCH, 4HB, SCCH and UFD. All four domains are cloned individually and all of them are subjected to overexpression and protein purification.

2.2. Plan of Work

Domains of E1, namely FCCH, 4HB, SCCH and UFD were isolated by standard PCR techniques and inserted into bacterial expression vector pET28a. All four domains were overexpressed using inducer and subjected to protein purification techniques. After which purified peptides were characterized structurally by Fluorescence spectroscopy and Circular dichroism.

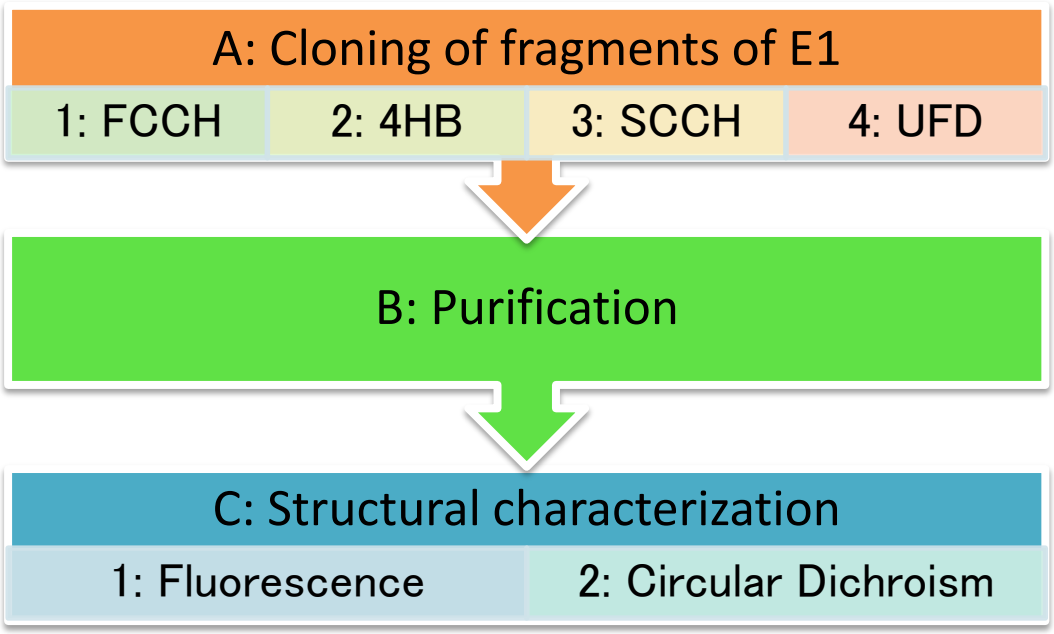


Fig. 2.3. Schematic diagram of the work plan; A) cloning of all four fragments namely; FCCH, 4HB, SCCH and UFD, B) protein purification C) structural analysis of all four peptides by Fluorescence and Circular Dichroism (CD) spectroscopic techniques.

The following are the materials and experimental procedures used to execute the objective of the study.

2.3. Materials and Methods

2.3.1. Strains and Media

Bacterial strain used for cloning purpose was *E. coli* DH5α (F^-, 80dlacZ M15, *endA*1, *recA*1, *hsdR*17 (rk^-,mk^+), *supE44*, *thi-1*, *gyrA96*, *relA1*,

(lacZYA-argF)U169). *E. coli* BL21 DE3 strain (BF^- ompT gal dcm lon hsdSB(rB^-mB^-) λ(DE3 [lacI lacUV5-T7p07 ind1 sam7 nin5]) $[malB^+]_{K-12}(\lambda^S)$ was used to express peptides for the purpose of purification. Both the strains of *E. coli* were grown on Luria broth and Luria agar (Hi-media). The selection marker for plasmid was kanamycin used at the concentration of 100μg/ml.

2.3.2. Sequence Selection

The gene sequence for yeast ubiquitin activating enzyme (*Uba1*) was taken from Swiss protein databank entry P22515. Uba1 is a 3072bp long gene which is translated into 1024 amino acid long protein. The gene for yeast ubiquitin activating enzyme (*Uba1*) inserted in vector pET28a was generously gifted by Prof. Hermann Schindelin, Rudolf-Virchow-Center, University of Würzberg, Germany (Lee and Schindelin, 2008).

2.3.3. Vector Selection

The vector used for cloning of all four domains of yeast E1 was pET28a. It is a bacterial expression vector having kanamycin resistance. It is a high copy number vector containing both N-terminal and C-terminal 6x His-tag. We used N-terminal His-tag for our studies omitting C-terminal His-tag. It has a T7lac promoter under which is induced by Isopropyl β- d-1-thiogalactopyranoside (IPTG) (**Fig 2.4**).

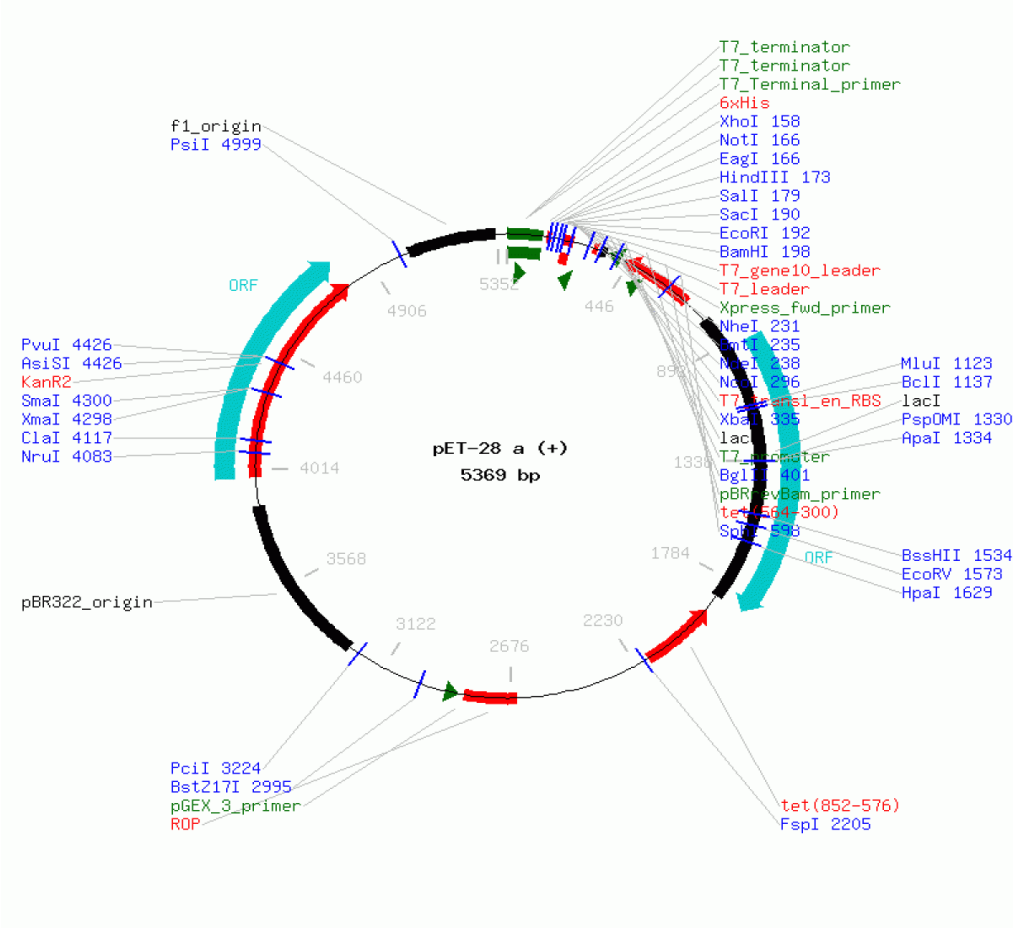


Fig. 2.4. Vector map of pET28a.

2.3.4. Cloning Strategy

DNA fragments encoding the domains FCCH, 4HB, SCCH and UFD were amplified from the gene for Uba1 using standard PCR techniques. Primers used to amplify all four domains are listed in **Table 2.1**.

Table 2.1. **Primer sequences used to amplify domains of E1**

Primer Name	Primer Sequence
FCCH FR	5’ GATAAGCTAGCGACCCAACGGGTGAAG 3’
FCCH RE	5’ GCGCACTCGAGTCATGAGATTTTACG 3’
4HB FR	5’ GCACAGGCTAGCTTGAAACAACAACTGTCC 3’
4HB RE	5’ GATAACTCGAGTCATCTTGCCTGATAGGAAAGC 3’
SCCH FR	5’ GATAAGCTAGCAAGTCTATCCCATTG 3’
SCCH RE	5’ GATACTCGAGTCAGCCAGCTATGAATTTG 3’
UFD FR	5’ GCACAGGCTAGCATGATTTGGGATAGATTTG 3’
UFD RE	5’ GTTAGCAGCCGGATCCTCGAGTCATAGATGAATGG 3’

Primers were designed to amplify residues 175-265 to make FCCH construct, residues 269-356 generated 4HB construct, residues 594-860 amplified to make SCCH construct and residues 1121-1220 amplified to generate UFD construct. To use only N-terminal His-tag additional stop codon was added at C-terminus of all the fragments. These fragments were cloned in pET28a vector between *Nhe*I and *Xho*I sites, were named as pETRPB1, pETRPB2, pETRPB3 and pETRPB4 respectively (**Fig. 2.5**).

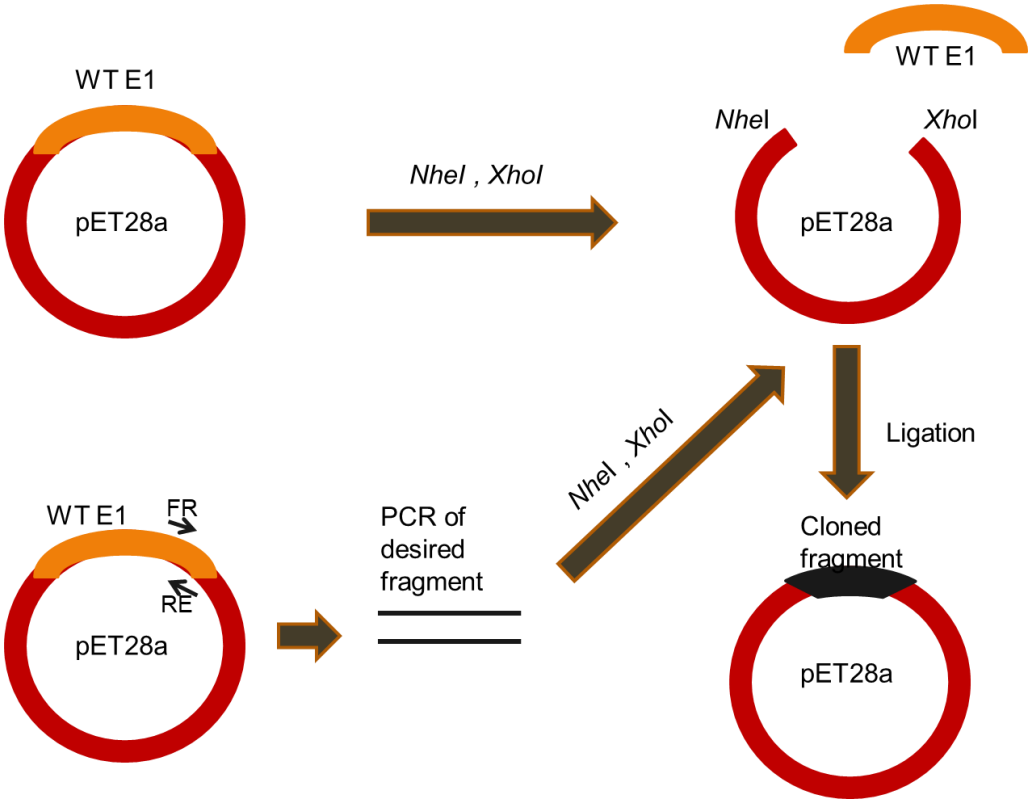


Fig. 2.5. Diagrammatic representation of the cloning strategy. PCR amplified desired fragments encoding domains were double digested with *Nhe*I and *Xho*I enzymes. Vector pET28a was also double digested with the same enzymes to form complementary sticky ends for ligation of amplicons to construct the desired clone.

After the cloning process, all four plasmids were sequenced and clones were confirmed.

2.3.5. Bacterial Transformation

After all four clones pETRPB1, pETRPB2, pETRPB3 and pETRPB4 were confirmed, they were transformed into *E. coli* strain BL21 DE3 separately using standard $CaCl_2$ bacterial transformation method for protein expression and purification purpose.

2.3.6. Expression of Protein

Cells with transformed plasmids were grown at 37 °C to an A_{600} of 0.6–1.0. After that 1mM isopropyl β-D-thiogalactopyranoside was added for overexpression and incubated overnight. Later, cells were harvested and boiled with laemmlli dye for 10 minutes. Cell lysates were loaded on 15% SDS PAGE to check the overexpression of peptides.

2.3.7. Protein Extraction

Once the overexpression of all four peptides was obtained, cells were grown in bulk culture for the purpose of protein purification. Cell lysates were prepared subjected to Ni^{2+}-NTA agarose (Qiagen) to native affinity chromatography for purification of peptide fragments corresponding to FCCH, 4HB and SCCH.

Cells transformed with one of the plasmids pETRPB1, pETRPB2, pETRPB3 and pETRPB4 individually were grown at 37°C in Luria broth (LB) containing antibiotic kanamycin (100μg/ml) to the mid-log stage. Cells were induced by 1mM IPTG overnight and harvested. Now, cells were resuspended in lysis buffer containing 50mM Tris, 2mM EDTA, 5% glycerol, pH7.8. Cell lysis was done by sonication under 40% amplitude for 4-5 minutes with an interval of 2 seconds pulse on and off. Cell lysate is now centrifuged at

12000rpm for 10 min at 4°C. A protease inhibitor cocktail was added to inhibit proteases. Following centrifugation, the supernatant containing the remaining soluble cellular proteins was collected. The collected supernatant was treated Ni-NTA chromatography and purified peptides were obtained.

The peptides were dialyzed against 20mM Tris-Cl (pH 8), 100mM NaCl buffer and concentrated by ultracentrifugation.

The peptide corresponding to UFD was purified by affinity chromatography under denaturing conditions since it was forming aggregates. All buffers used for UFD purification contained 6M Urea. Rest all the procedure followed for purification was the same native conditions. UFD was also dialyzed against 20mM Tris-Cl (pH 8), 100mM NaCl buffer and was concentrated by ultrafiltration.

2.3.8. Protein Purification by Affinity Chromatography

Vector pET28a is a bacterial expression system, consisting of affinity tag 6x-His, which was used for affinity chromatographic purification using an immobilized metal affinity chromatography (IMAC) method (PORATH et al., 1975). IMAC is based on the interaction between a transition metal ion (Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}) immobilized on a matrix. As electron donor groups on the histidine imidazole ring readily form coordination bonds with the immobilized transition metal, histidine exhibits the strongest interaction with immobilized metal ion matrices. Peptides containing sequences of consecutive histidine residues are efficiently retained on IMAC (Hochuli et al., 1987). Poly-histidine affinity tags are normally placed on either the N- or the C-terminus of target proteins depending on their characteristics. Purification using polyhistidine tags has been carried out effectively using different expression systems including bacteria (Chen and Hai, 1994; Rank et al. 2001), yeast (Kaslow and Shiloach, 1994), mammalian cells (Janknecht and Nordheim,

1992; Janknecht et al., 1991) and baculovirus-infected insect cells (Kuusinen et al., 1995; Schmidt et al., 1998). Affinity purification is the method of choice when purification based on protein's characteristics becomes difficult either due to its nature or induced structural changes because affinity purification is based on the tag attached with protein rather than characteristics of the protein itself. Tagged protein can be eluted from free imidazole as it has a high affinity towards Ni^{2+}, the concentration of imidazole lies in the range of 20-250mM imidazole (Hefti et al., 2001; Janknecht et al., 1991).

Ni-NTA resin (Qiagen) is supplied in 20% ethanol to prevent it from degradation by bacterial growth. The ethanol content in the resin was removed by washing with deionized water and subsequently washed with equilibration buffer containing 50mM NaH_2PO_4, 300mM NaCl, pH 8.0. After five-six washes resin was incubated with lysate for an hour to allow binding of His-tagged protein to the resin after incubation cell lysate was removed and resin was washed with buffer containing 50mM NaH_2PO_4, 300mM NaCl with low concentration of imidazole (20mM), pH 8.0 to remove unbound protein and to reduce nonspecific binding of host proteins with histidines. After the washing step, the target protein was eluted from the buffer containing 50mM NaH_2PO_4, 300mM NaCl, 250mM imidazole, pH 8.0. The purity of the target proteins have been checked by running them on 15% SDS-PAGE, further imidazole was removed by dialysis with buffer containing 20mM Tris-Cl (pH 8), 100mM NaCl buffer. Plasmids expressing FCCH, 4HB and SCCH were purified by the technique described above.

For purification of a plasmid expressing UFD, all the steps were followed under denaturing conditions by adding 6M urea to each buffer except dialysis buffer.

2.3.9. CD Spectroscopy

Peptide solutions were prepared in 20 mM Tris-Cl (pH 8.0) and 100 mM NaCl buffer and the concentration of peptides used in all the experiments was 23.4 μM.

Jasco J-715 CD spectrophotometer was used to record the Far UV CD spectra of all four peptides. The range for Far-UV-CD spectra was 200 to 250 nm. The path length was 1 mm. All the spectra were blank corrected. The spectra were accumulated for five times to improve the signal-to-noise ratio. Scan speed was 50 nm/ sec. The spectra were then analyzed by BeStSel software (Micsonai et al., 2018).

Separately, the secondary structure content of the peptides was predicted by PSIPRED protein secondary structure prediction tool (Buchan and Jones, 2019). PSIPRED predicts the structure based on PSI-BLAST and neural network machine learning algorithm (Jones, 1999).

2.3.10. Guanidine Hydrochloride Denaturation Studies

Guanidine hydrochloride is a strong chaotropic agent, which denatures the protein (Monera et al., 1994). At higher concentrations of guanidine hydrochloride (6M) proteins lose their structure and become randomly coiled. Fluorescence spectra were recorded using the Hitachi FL-7000 fluorescence spectrophotometer. For intrinsic fluorescence spectroscopy, the buffer used was 20 mM Tris-Cl (pH 8.0) and 100 mM NaCl buffer. The excitation wavelength was 280 nm and emission spectra were recorded in the range of 300–400 nm. Band-pass was 5 nm.

2.3.11. Fluorescence Resonance Energy Transfer Studies

An extrinsic fluorophore, 1-Anilino 8-naphthalene sulphonic acid (ANS) was used at the concentration of 50 μM. FRET assay was performed using an excitation wavelength of 300 nm. ANS was excited at 390 nm and emission spectra were recorded between 450 and 550 nm. The slit width was kept at 5 nm. Fluorescence experiments were repeated three times in independent sets.

2.4. Results

2.4.1. Construction of pETRPB1, pETRPB2, pETRPB3 and pETRPB4 plasmids

For the construction of pETRPB1, pETRPB2, pETRPB3 and pETRPB4 plasmids fragments FCCH, 4HB, SCCH and UFD respectively, were amplified from pETE1 plasmid using site-specific primers. The vector used for cloning was pET28a and restriction sites used were *Nhe*I and *Xho*I. Each of them was sequenced to confirm the insertion of fragments in vector.

2.4.1.1. Construction of pETRPB1 Plasmid

Clone pETRPB1 was constructed by inserting the FCCH fragment in the pET28a vector (**Fig. 2.6**). Samples were run on 1% agarose gel.

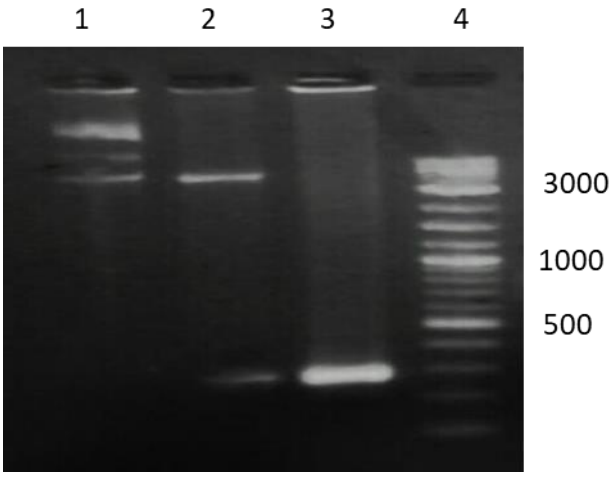


Fig. 2.6. Gel picture showing pETRPB1 clone in pET28a backbone. Lane 1 is pETRPB1 plasmid; Lane 2 is double digest of pETRPB1 plasmid by *Nhe*I and *Xho*I restriction enzyme releasing the DNA fragment of 270bp from vector pET28a (5.3kbp), Lane 3 is PCR amplification of FCCH fragment (270bp) and lane 4 is 1Kbp molecular weight marker.

2.4.1.2. Construction of pETRPB2 Plasmid

Clone pETRPB2 was constructed by inserting the 4HB fragment in the pET28a vector (**Fig. 2.7**). Samples were run on 1% agarose gel.

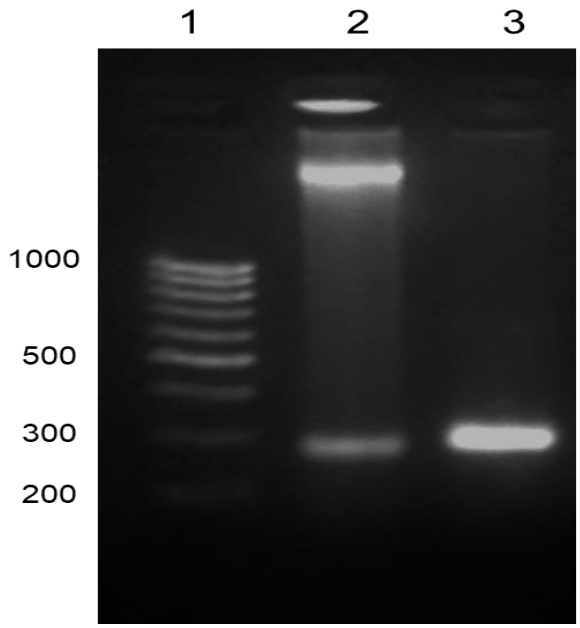


Fig. 2.7. **Gel picture showing pETRPB2 clone in pET28a backbone**. Lane 1 is 100bp ladder as molecular weight marker; Lane 2 is double digest of pETRPB2 plasmid by *Nhe*I and *Xho*I restriction enzymes releasing the DNA fragment of 237bp from vector pET28a (5.3kbp), Lane 3 is PCR amplification of 4HB fragment (237bp).

2.4.1.3. Construction of pETRPB3 Plasmid

Clone pETRPB3 was constructed by inserting the SCCH fragment in the pET28a vector (**Fig. 2.8**). Samples were run on 1% agarose gel.

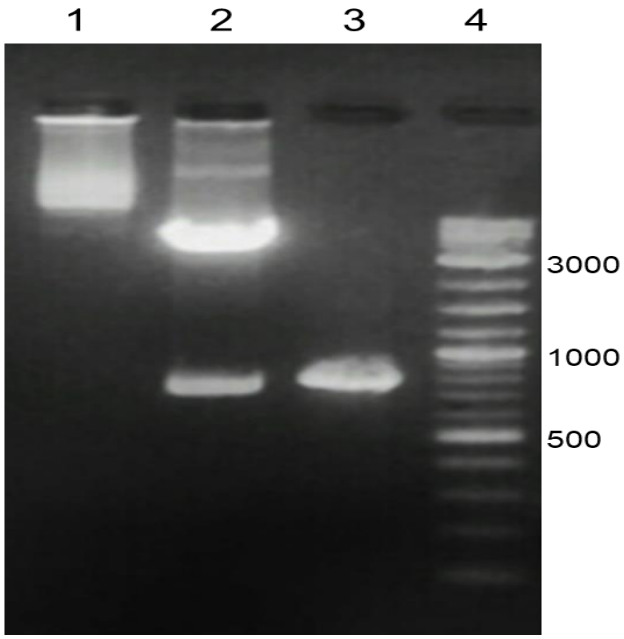


Fig. 2. 8. Gel picture showing pETRPB3 clone in pET28a backbone. Lane 1 is pETRPB3 plasmid; Lane 2 is double digest of pETRPB3 plasmid by *Nhe*I and *Xho*I restriction enzyme releasing the DNA fragment of 786bp from vector pET28a (5.3kbp), Lane 3 is PCR amplification of SCCH fragment (786bp) and lane 4 is molecular 1Kbp weight marker.

2.4.1.4. Construction of pETRPB4 Plasmid

Clone pETRPB4 was constructed by inserting the UFD fragment in the pET28a vector (**Fig. 2.9**). Samples were run on 1% agarose gel.

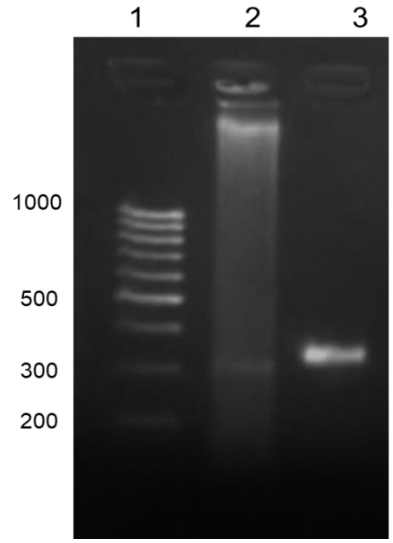


Fig. 2.9. **Gel picture showing pETRPB4 clone in pET28a backbone.** Lane 1 is 100bp molecular weight marker; Lane 2 is double digest of pETRPB4 plasmid by *Nhe*I and *Xho*I restriction enzyme releasing the DNA fragment of 300bp from vector pET28a (5.3kbp), Lane 3 is PCR amplification of UFD fragment (300bp).

2.4.2. Expression and Purification of Peptides FCCH, 4HB, SCCH and UFD from Bacterial Expression Vector pET28a

2.4.2.1. Expression and Purification of FCCH peptide in bacterial expression vector pET28a

Purified FCCH was run on 15% acrylamide gel to check the expression and purity of the product (**Fig. 2.10**).

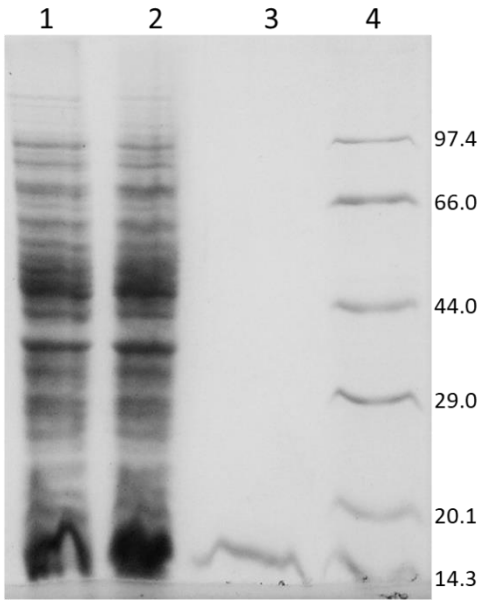


Fig. 2.10. **Gel picture showing purification of FCCH peptide.** Lane 1 is BL21 DE3 cell lysate with pETRPB1 plasmid without inducer, Lane 2 is BL21 DE3 cell lysate with pETRPB1 plasmid overexpressing FCCH peptide in the presence of 1mM IPTG, Lane 3 is FCCH peptide (~11kDa) purified by Ni-NTA affinity chromatography, Lane 4 is protein molecular weight marker 14.3 kDa-97.4 kDa range.

2.4.2.2. Expression and Purification of 4HB peptide in bacterial expression vector pET28a

Purified 4HB was run on 15% acrylamide gel to check the expression and purity of the product (**Fig. 2.11**).

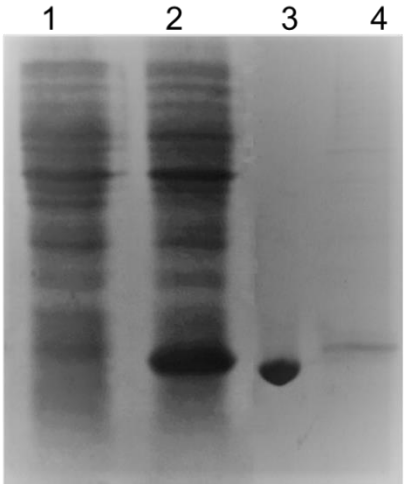


Fig. 2.11. **Gel picture showing purification of 4HB peptide.** Lane 1 is BL21 DE3 cell lysate with pETRPB2 plasmid without inducer, Lane 2 is BL21 DE3 cell lysate with pETRPB2 plasmid overexpressing 4HB peptide in presence of 1mM IPTG, Lane 3 is lysozyme protein 14.3kDa used as protein marker, Lane 4 is 4HB peptide (~11kDa) purified by Ni-NTA affinity chromatography.

2.4.2.3. Expression and Purification of SCCH peptide in bacterial expression vector pET28a

Purified SCCH was run on 15% acrylamide gel to check the expression and purity of the product (**Fig. 2.12**).

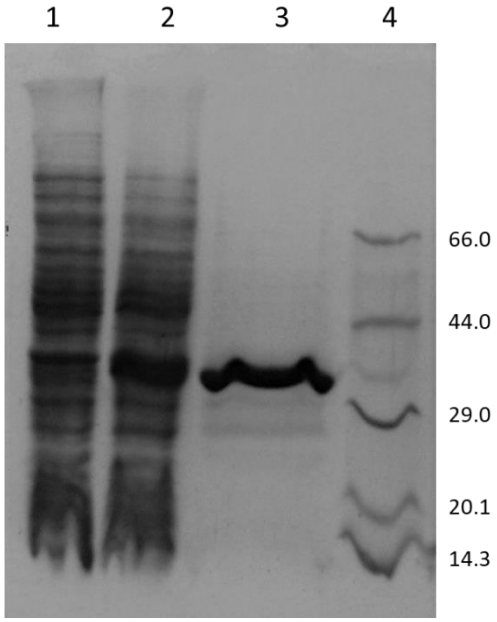


Fig. 2.12. **Gel picture showing purification of SCCH peptide.** Lane 1 is BL21 DE3 cell lysate with pETRPB3 plasmid without inducer, Lane 2 is BL21 DE3 cell lysate with pETRPB3 plasmid overexpressing SCCH peptide in presence of 1mM IPTG, Lane 3 is SCCH peptide (~30kDa) purified by Ni-NTA affinity chromatography, Lane 4 is protein molecular weight marker 14.3 kDa-97.4 kDa range.

2.4.2.4. Expression and Purification of UFD peptide in bacterial expression vector pET28a

Purified UFD was run on 15% acrylamide gel to check the expression and purity of the product (**Fig. 2.13**).

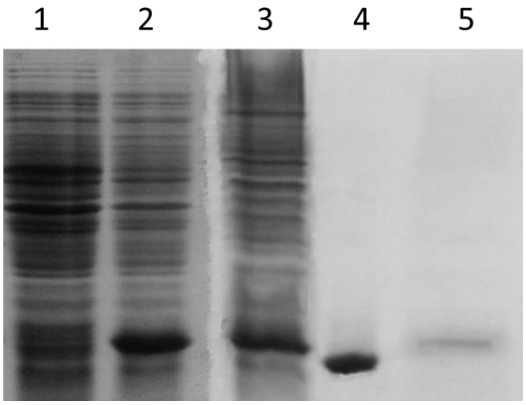


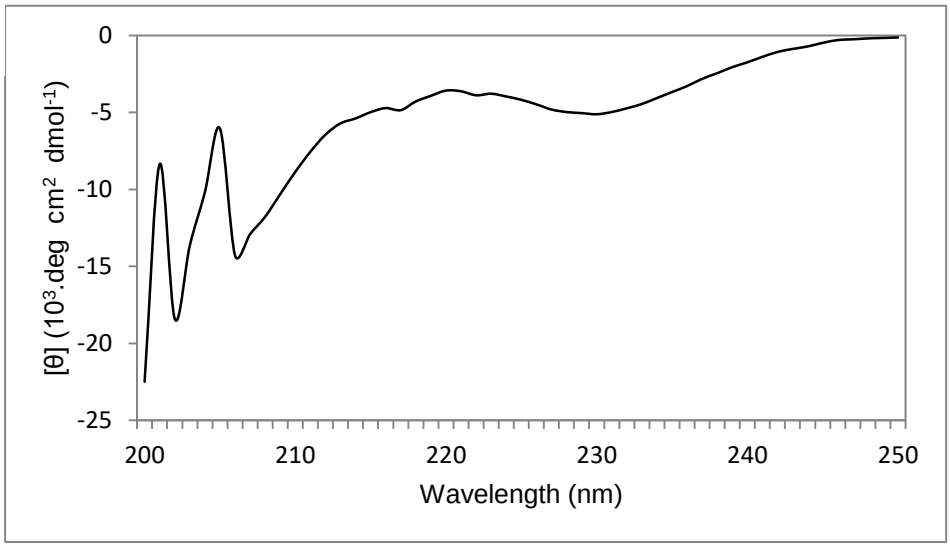
Fig. 2.13. Gel picture showing purification of UFD peptide. Lane 1 is BL21 DE3 cell lysate with pETRPB4 plasmid without inducer, Lane 2 is BL21 DE3 cell lysate with pETRPB4 plasmid overexpressing UFD peptide in presence of 1mM IPTG, Lane 3 is cell lysate, Lane 4 is lysozyme protein 14.3kDa used as protein marker, Lane 5 is UFD peptide (~11kDa) purified by Ni-NTA affinity chromatography.

2.4.3. Structural Characterization of Peptides FCCH, 4HB, SCCH and UFD by CD and Fluorescence Spectroscopy

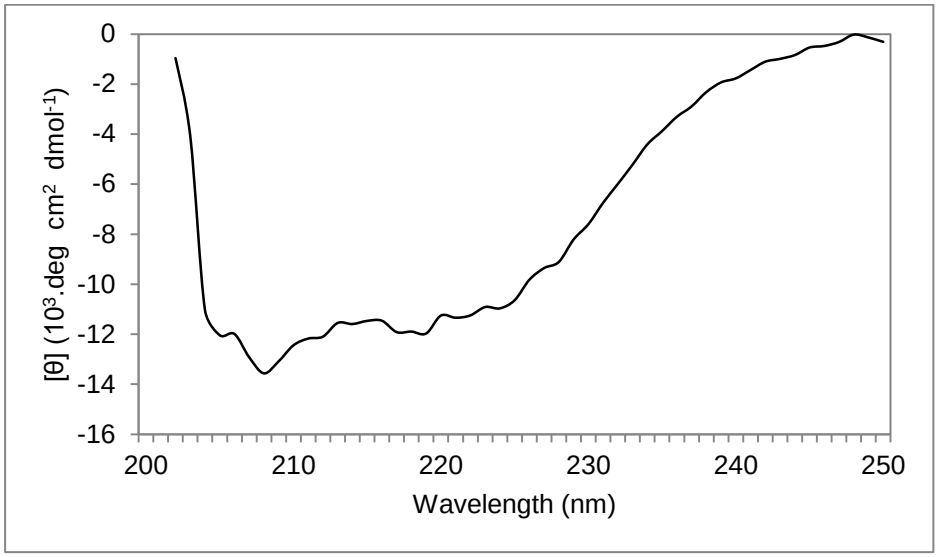
2.4.3.1. Far UV CD Spectra of FCCH, 4HB, SCCH and UFD Peptides

To assess the folding of various domains, far UV CD spectra were recorded for 4HB, FCCH, SCCH and UFD (**Fig 2.14**).

A



B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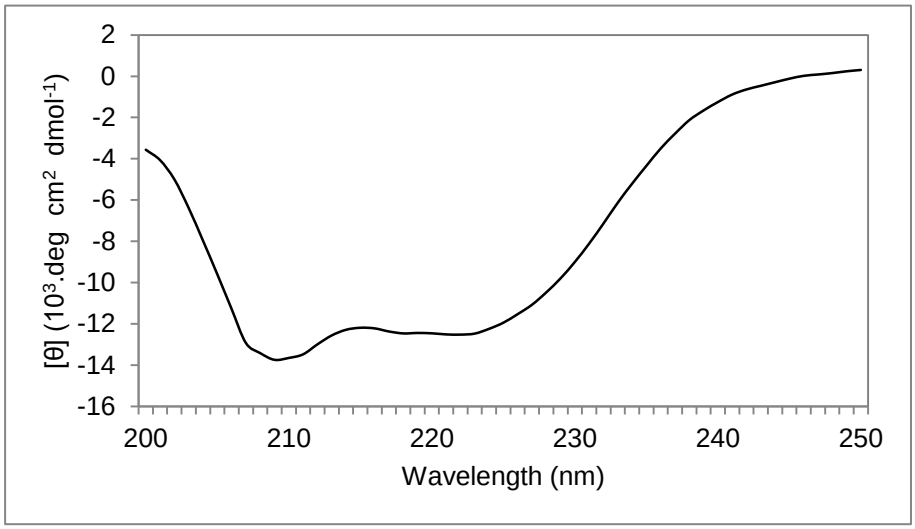
Wavelength (nm)

C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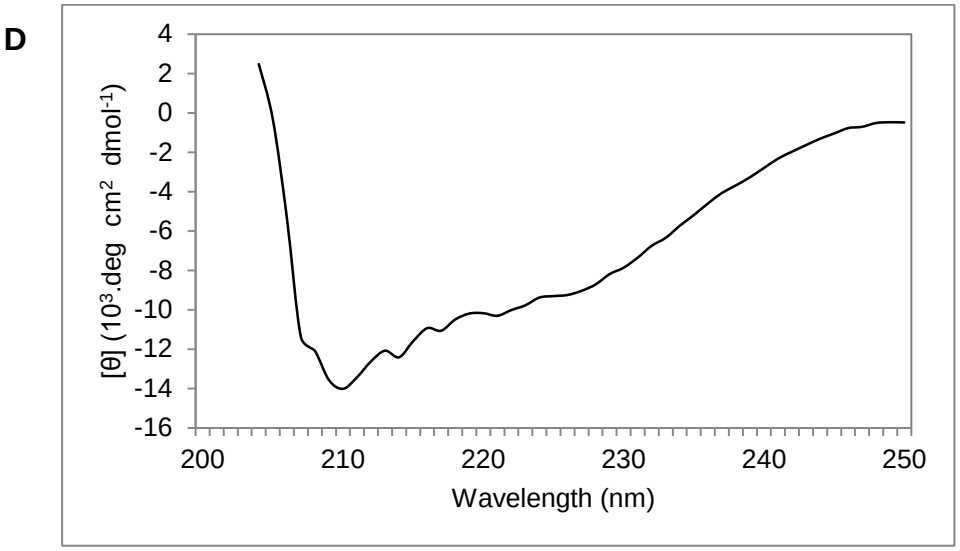


Fig. 2.14. Far-UV CD spectra of domains of ubiquitin activating enzyme E1 namely, A) FCCH, B) 4HB, C) SCCH and D) UFD peptides.

The spectra were analyzed by BeStSel software and the results were compared to values obtained by secondary structure prediction tool PSIPRED (**Table 2.2**). The far UV CD spectrum of FCCH shows mostly β-sheet structure with very little helicity, the spectra of 4HB and SCCH are predominantly helical, while that of the UFD domain has mixed α/β structure, which is in agreement with the secondary structure predictions were done using PSIPRED. Even though CD spectra of SCCH and 4HB display features corresponding to low amounts of β-sheet structure, the software fails to predict the presence of any β-sheet content. However, there are differences in results obtained with PSIPRED and CD spectra with respect to the content of helices and turns. Isolated domains were characterized in CD spectra in the absence of rest of the sequences of the protein, while PSIPRED considers the potential of the sequences under consideration based on the information from homologous sequences in whole proteins. Further, many times it is difficult to differentiate between helices and a series of turns (**Table 2.2**).

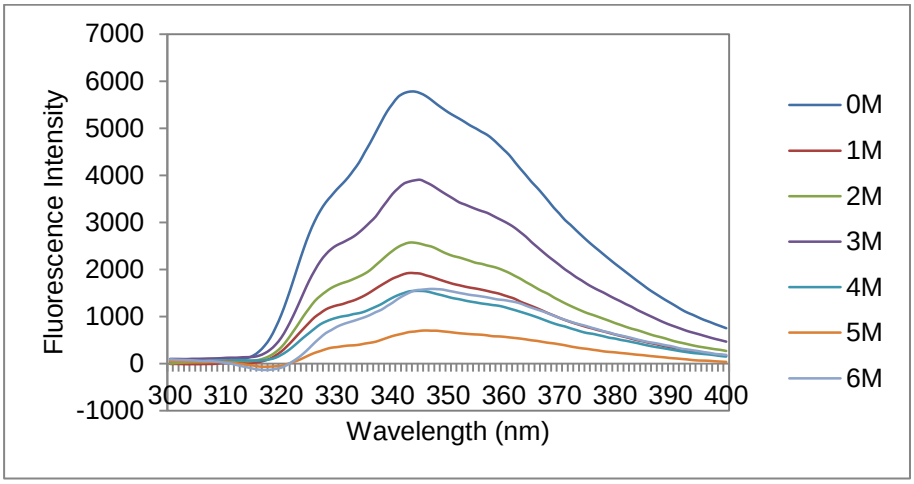
Table 2.2. Values of the secondary structure obtained from CD spectra analysis done by Bestsel and secondary structure prediction software PSIPRED shown in percentages

Peptide	**α-helix**		**β-sheet**		**Turns/coils**	
	Values obtained from PSIPRED	Values obtained from CD spectra	Values obtained from PSIPRED	Values obtained from CD spectra	Values obtained from PSIPRED	Values obtained from CD spectra
FCCH	3.3	4.9	42.85	33	53.84	62.2
4HB	60.22	52.9	0	17	39.77	30.2
SCCH	50	48.4	1.5	26	48.14	25.7
UFD	33.67	18.5	26.59	29.5	39.79	52

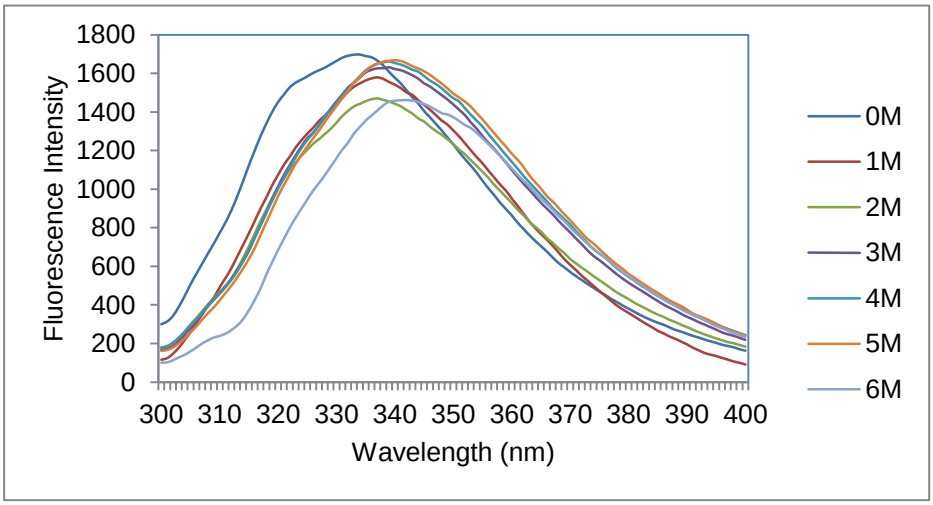
2.4.3.2. Guanidine Hydrochloride Denaturation Spectra of FCCH, 4HB, SCCH and UFD Peptides

Intrinsic fluorescence emission spectra of the domains were recorded to see if they have any structure. All four domains were individually exposed to denaturing conditions by adding guanidine hydrochloride (**Fig 2.15**).

A


7000
6000
5000
4000
3000
2000
1000
0
-1000
Fluorescence Intensity
300 310 320 330 340 350 360 370 380 390 400
Wavelength (nm)
0M
1M
2M
3M
4M
5M
6M

B


1800
1600
1400
1200
1000
800
600
400
200
0
Fluorescence Intensity
300 310 320 330 340 350 360 370 380 390 400
Wavelength (nm)
0M
1M
2M
3M
4M
5M
6M

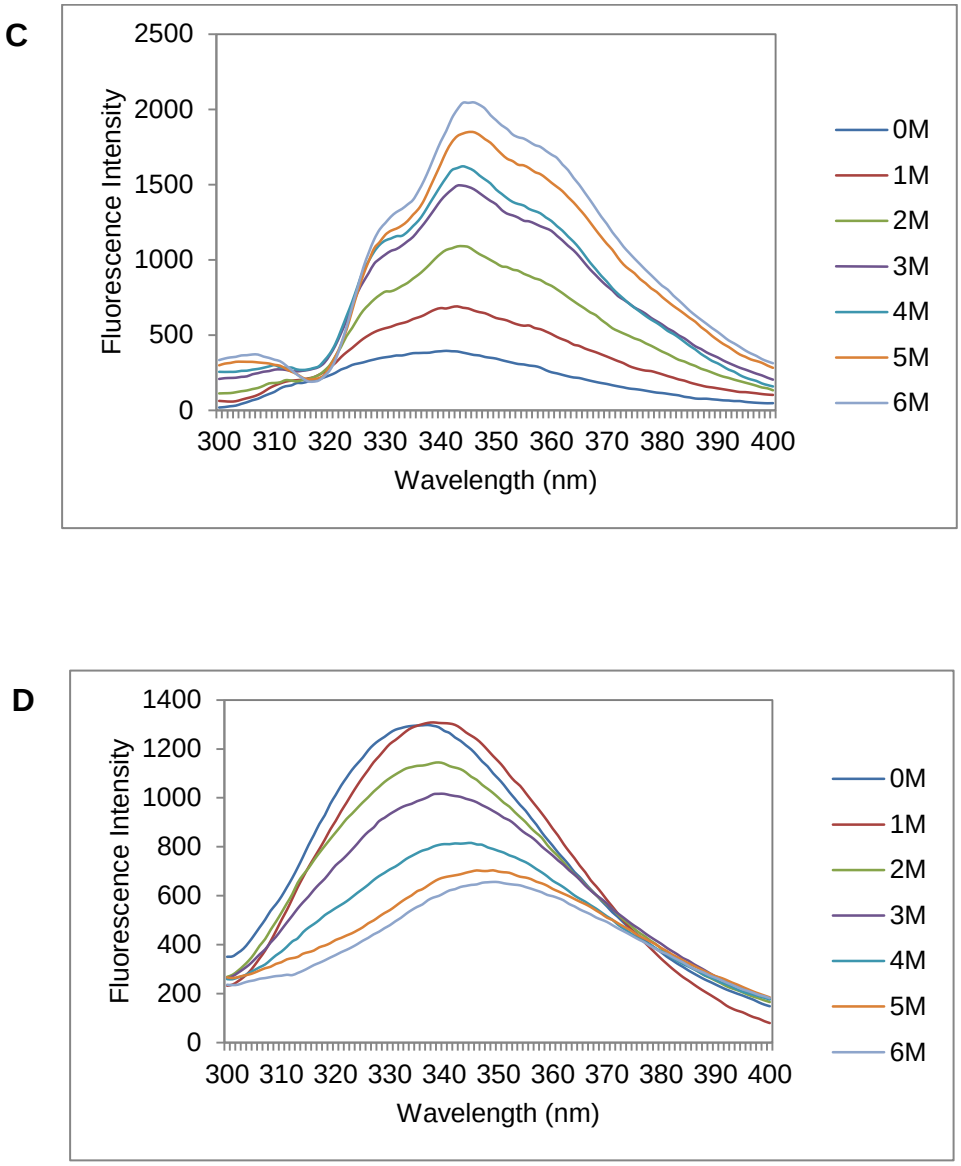


Fig. 2. 15. Guanidine Hydrochloride denaturation spectra of A) FCCH, B) 4HB, C) SCCH, D) UFD.

Change in intensity of fluorescence emission could be seen in all four FCCH, 4HB, SCCH and UFD peptides. This observation indicated peptides are changing its orientation with the change in concentration of guanidine hydrochloride.

Fluorescence spectra of the domains showed a red shift upon exposure to denaturing conditions, indicating exposure of aromatic amino acids to the polar environment as a consequence of the change in the overall structure of the domains. The results are presented in a graph showing a change in λ_{max} with an increase in guanidine hydrochloride concentration (**Fig. 2.16**).

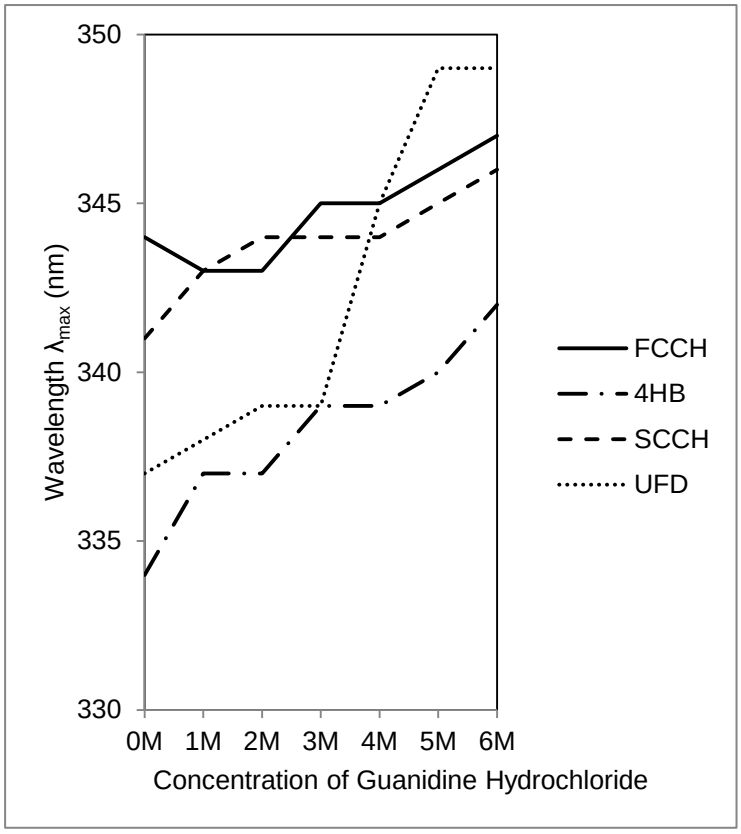
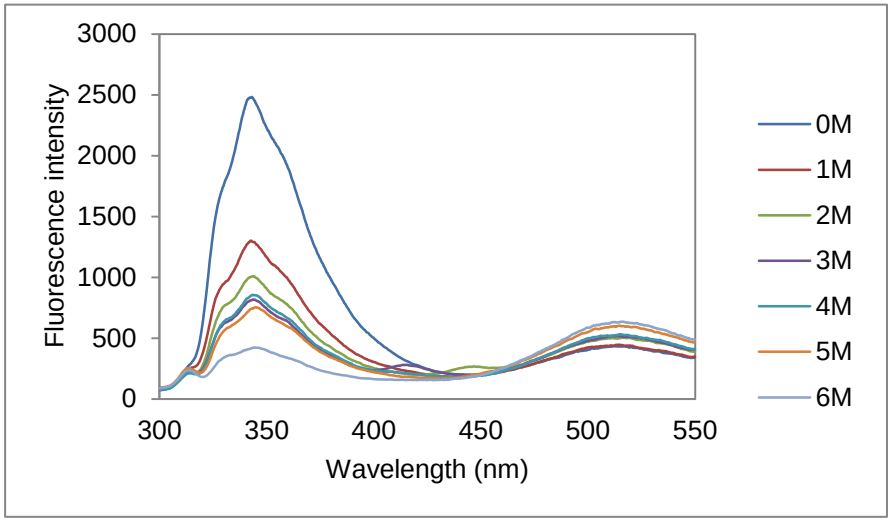


Fig. 2.16. λ_{max} values of all four peptides FCCH, 4HB, SCCH and UFD plotted against guanidine hydrochloride concentrations.

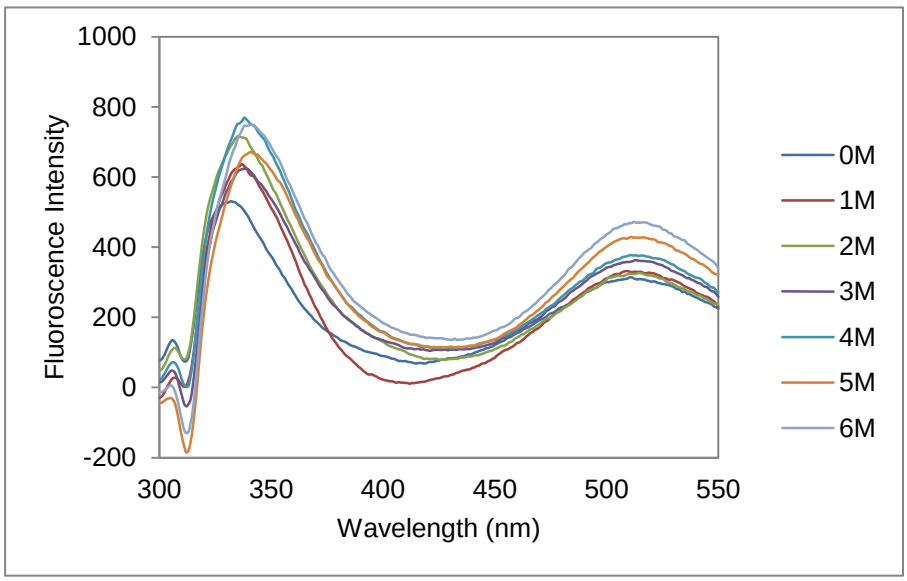
Further, an extrinsic fluorophore 1-anilinonaphthalene-8- sulfonate (ANS) was used to check surface hydrophobicity of the isolated domains. ANS binds hydrophobic residues exposed on the surface of a protein and

shows fluorescence (Hawe et al., 2008). Here, ANS was added to the domains, in the presence of various concentrations of guanidine hydrochloride to examine changes in the domain structure (**Fig. 2.17**).

A



B



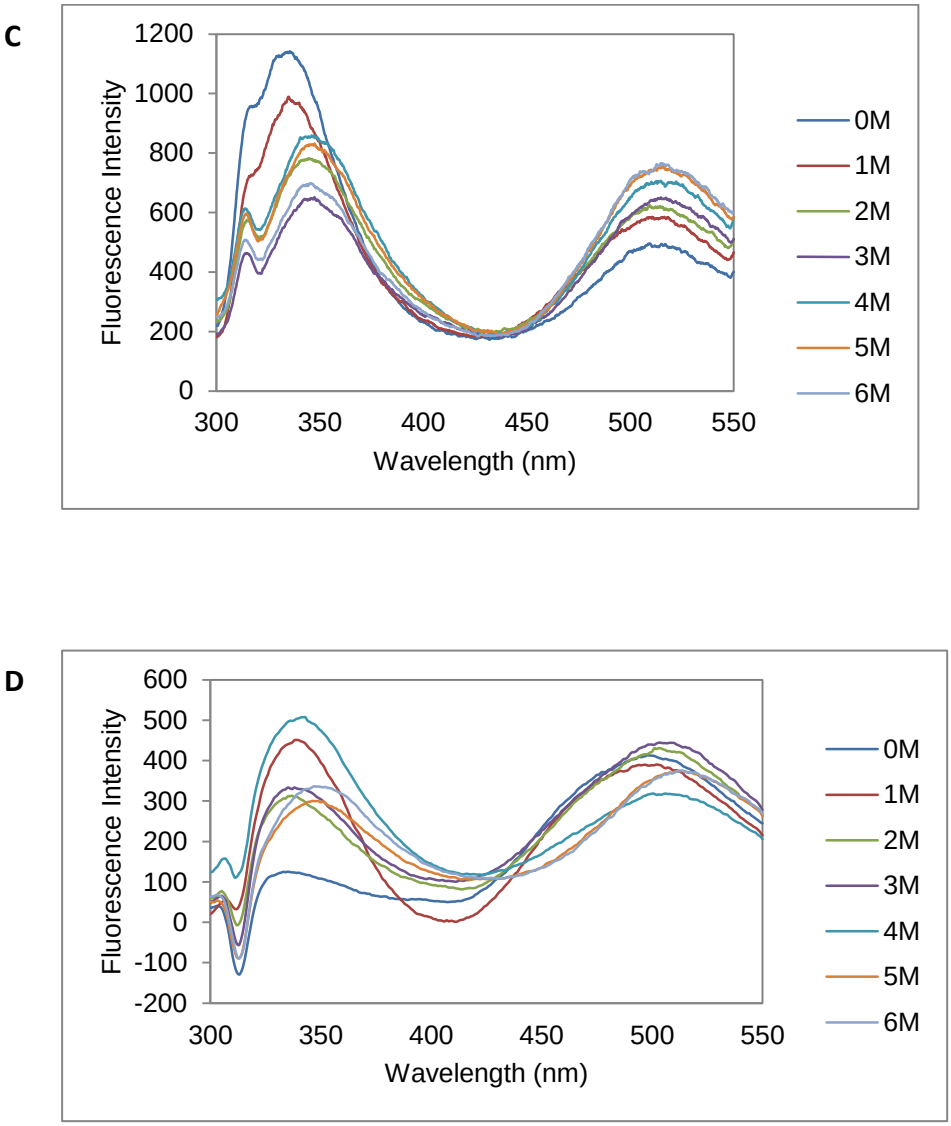


Fig. 2.17. Fluorescence emission spectra of extrinsic fluorophore ANS bound to the domains of E1 namely; A) FCCH, B) 4HB, C) SCCH, and D) UFD domains in the presence of different concentrations of the denaturant guanidine hydrochloride.

Peptides 4HB, FCCH and SCCH showed an increase in fluorescence intensity with increasing concentrations of guanidine hydrochloride, indicating that hydrophobic residues were initially buried in the core and became

increasingly accessible for binding to ANS with denaturation. With UFD, results indicated the presence of hydrophobic residues on the surface even in the absence of guanidine hydrochloride.

2.5. Discussion

Protein purification involved the basic procedure of affinity chromatography for all four peptides. However, UFD cannot be purified by the method used for purification of rest three peptides. Hydrophobicity caused insolubility of protein in solution, most of the mutant protein resided in the pellet rather than in soluble form hence it was difficult to purify the protein in standard conditions In order to get purified protein, various factors were changed and used to express and purify the peptide. Different time durations from 1 hour to overnight experimented with various concentrations of IPTG varying from 0.4mM to 1M. Later on, different concentration of denaturing agents was used to create a denaturing environment to limit excessive denaturation of the peptide. Concentrations of urea were varied from 2M to 6M to obtain purify UFD and get the maximum product. Even at lower concentrations of urea, UFD was found in inclusion bodies. Literature showed UFD is present in two different orientations when crystallized. This also indicated UFD is not under a stable state even when it is bound to the rest of the protein.

CD spectra of the domains of yeast E1 showed that they have the capacity to fold independently and have the potential to adopt a native-like structure. The CD spectrum of FCCH of yeast confirmed the presence of β-sheets as a major structural feature. Interestingly, the CD spectrum of the Mouse FCCH showed the presence of around ~40% β-sheet (Szczepanowski et al., 2005), whereas the CD spectrum of yeast FCCH showed ~33% helicity.

Mouse and yeast FCCH share ~47% sequence identity, besides both show prominent β-structure with the presence of very less or no helical content.

The CD spectrum of SCCH showed that it is predominantly helical with approximately 50% helicity, which is in agreement with the structure predicted by PSIPRED. The CD spectrum of mouse SCCH showed helical structure (Szczepanowski et al., 2005), which is strikingly similar to that of yeast SCCH at 48% helicity. Both the organisms share a ~47% sequence identity in this domain. The CD spectrum of the 4HB domain displays helicity as the prominent secondary structural feature. Since there is no information available on the mouse 4HB domain in literature, it was predicted with the help of sequence homology. 4HB domains of both the organisms share ~48% sequence identity and predominant helicity as predicted by PSIPRED. UFD domain contains the α/β structure, which matched the structure predicted by PSIPRED. E1 enzyme of yeast shares around 52% overall identity with that of mouse E1.

The fluorescence denaturation spectra for FCCH, 4HB, SCCH and UFD all four domains was carried out with intrinsic as well as by adding extrinsic fluorophore. Gradual denaturation by guanidine hydrochloride of these peptides showed the change in fluorescence intensity indicating that they are able to fold on their own. The highest pick of fluorescence emission spectra moves towards higher wavelength when aromatic amino acids are exposed and excites. A prominent red shift was observed in peptides indicating the change in the overall structure of the domains as aromatic amino acids shift towards the polar environment. An extrinsic fluorophore ANS (1-Anilino 8-naphthalene sulphonic acid) was added to all four peptides. ANS is generally used to check surface hydrophobicity of protein; it emits a higher intensity of fluorescence when comes in contact with hydrated hydrophobic residues. Increasing concentration of guanidine hydrochloride resulted in higher fluorescence intensity for peptides FCCH, 4HB and SCCH. This result

displayed the presence of hydrophobic residues in the core region during the native state. For the UFD domain, the intensity was higher at a lower concentration of guanidine hydrochloride indicating the presence of hydrophobic residues on the surface in the native state.

In summary, the domains show native-like characteristics even when they are expressed as individual units.