

TABLE OF CONTENT

ABSTRACT	1
CHAPTER 1 INTRODUCTION	5
1.1. POST-TRANSLATIONAL MODIFICATIONS AND UBIQUITINATION	6
1.2. PROTEIN DEGRADATION	7
1.3. UBIQUITIN PROTEASOME SYSTEM (UPS)	8
1.3.1. A BRIEF HISTORY	8
1.3.2. UBIQUITIN	8
1.3.2.1. Ubiquitin Linkages - Diverse Cellular Signals	10
1.3.2.2. Ubiquitin Chains	13
1.3.3. UBIQUITINATION/UBIQUITIN PROTEASOME PATHWAY	15
1.3.4. AFTERMATH OF UBIQUITINATION	17
1.3.4.1. Regulation of Protein Turnover	17
1.3.4.2. Regulation of Gene Transcription	18
1.3.4.3. Cellular Quality Control	18
1.3.5. UBIQUITIN ACTIVATING ENZYME (UBA1)	19
1.3.6. DISTANT RELATIVES OF E1 AND UBIQUITIN	20
1.3.7. ALLELES OF UBA1	23
1.3.8. UBIQUITIN CONJUGATING ENZYME	25
1.3.9. UBIQUITIN LIGASE	28
1.3.9.1. HECT Domain E3s:	30
1.3.9.2. RING Finger E3s	31
1.3.10. 26S PROTEASOME	33
1.3.11. DE-UBIQUITINATING ENZYMES	35
1.3.12. SIGNIFICANCE OF THE UBIQUITIN PROTEASOME SYSTEM	36
1.3.13. DIVERSE FUNCTIONS OF THE UBIQUITIN PROTEASOME SYSTEM	37

1.3.13.1.	Development	37
1.3.13.2.	Apoptosis	39
1.3.13.3.	Immunity	40
1.4.	HYPOTHESIS AND OBJECTIVES OF THE PRESENT RESEARCH THESIS	41

CHAPTER 2	<u>CLONING, PURIFICATION AND STRUCTURAL CHARACTERIZATION OF FOUR DOMAINS OF UBIQUITIN ACTIVATING ENZYME E1</u>	<u>44</u>
------------------	---	------------------

2.1.	INTRODUCTION	45
2.2.	PLAN OF WORK	47
2.3.	MATERIALS AND METHODS	48
2.3.1.	STRAINS AND MEDIA	48
2.3.2.	SEQUENCE SELECTION	49
2.3.3.	VECTOR SELECTION	49
2.3.4.	CLONING STRATEGY	50
2.3.5.	BACTERIAL TRANSFORMATION	53
2.3.6.	EXPRESSION OF PROTEIN	53
2.3.7.	PROTEIN EXTRACTION	53
2.3.8.	PROTEIN PURIFICATION BY AFFINITY CHROMATOGRAPHY	54
2.3.9.	CD SPECTROSCOPY	56
2.3.10.	GUANIDINE HYDROCHLORIDE DENATURATION STUDIES	56
2.3.11.	FLUORESCENCE RESONANCE ENERGY TRANSFER STUDIES	57
2.4.	RESULTS	57
2.4.1.	CONSTRUCTION OF PETRPB1, PETRPB2, PETRPB3 AND PETRPB4 PLASMIDS	57
2.4.1.1.	Construction of pETRPB1 Plasmid	58
2.4.1.2.	Construction of pETRPB2 Plasmid	59
2.4.1.3.	Construction of pETRPB3 Plasmid	60
2.4.1.4.	Construction of pETRPB4 Plasmid	61
2.4.2.	EXPRESSION AND PURIFICATION OF PEPTIDES FCCH, 4HB, SCCH AND UFD FROM BACTERIAL EXPRESSION VECTOR PET28A	62

2.4.2.1.	Expression and Purification of FCCH peptide in bacterial expression vector pET28a	62
2.4.2.2.	Expression and Purification of 4HB peptide in bacterial expression vector pET28a	63
2.4.2.3.	Expression and Purification of SCCH peptide in bacterial expression vector pET28a	64
2.4.2.4.	Expression and Purification of UFD peptide in bacterial expression vector pET28a	65
2.4.3.	STRUCTURAL CHARACTERIZATION OF PEPTIDES FCCH, 4HB, SCCH AND UFD BY CD AND FLUORESCENCE SPECTROSCOPY	66
2.4.3.1.	Far UV CD Spectra of FCCH, 4HB, SCCH and UFD Peptides	66
2.4.3.2.	Guanidine Hydrochloride Denaturation Spectra of FCCH, 4HB, SCCH and UFD Peptides	69
2.5.	DISCUSSION	75

CHAPTER 3 CLONING AND FUNCTIONAL CHARACTERIZATION OF FOUR DOMAINS OF UBIQUITIN ACTIVATING ENZYME E1

3.1.	INTRODUCTION	79
3.2.	PLAN OF WORK	80
3.3.	MATERIALS AND METHODS	81
3.3.1.	STRAINS AND MEDIA	81
3.3.2.	VECTOR SELECTION	81
3.3.3.	CLONING STRATEGY	82
3.3.4.	TRANSFORMATION OF RECOMBINANT PLASMIDS AFTER SUBCLONING IN YEAST CELLS	83
3.3.5.	CELL GROWTH AND VIABILITY	84
3.3.6.	HEAT SENSITIVITY TEST	84
3.3.7.	ANTIBIOTIC SENSITIVITY TEST	85
3.4.	RESULTS	85
3.4.1.	CONSTRUCTION OF YRPB1, YRPB2, YRPB3 AND YRPB4 PLASMIDS	85
3.4.1.1.	Cloning of YRPB1 Plasmid Construct	86

3.4.1.2.	Cloning of YRPB2 Plasmid Construct	87
3.4.1.3.	Cloning of YRPB3 Plasmid Construct	88
3.4.1.4.	Cloning of YRPB4 Plasmid Construct	89
3.4.2.	EFFECTS OF EXPRESSION OF DOMAINS ON CELL SURVIVAL AND GROWTH	90
3.4.3.	EFFECTS OF EXPRESSION OF THE DOMAINS 4HB, FCCH, SCCH AND UFD ON CELL GROWTH UNDER HEAT STRESS	92
3.4.4.	EFFECTS OF 4HB, FCCH, SCCH AND UFD EXPRESSION ON CELL GROWTH IN THE PRESENCE OF ANTIBIOTICS	94
3.5.	DISCUSSION	96

CHAPTER 4 STRUCTURAL AND FUNCTIONAL STUDIES ON B-BULGE MUTANTS OF UBIQUITIN **97**

4.1.	INTRODUCTION	98
4.2.	PLAN OF WORK	102
4.2.1.	ASSAY OF UBIQUITIN ACTIVATION BY E1	102
4.2.2.	TESTING THE EFFECT OF DIFFERENT PHs ON B-BULGE MUTANTS OF UBIQUITIN	103
4.3.	MATERIALS AND METHODS	103
4.3.1.	STRAINS, MEDIA AND PLASMID	103
4.3.2.	PURIFICATION OF UBIQUITIN F45W AND B-BULGE MUTANTS Q2N, E64G AND S65D	104
4.3.3.	PURIFICATION OF E1 PROTEIN	105
4.3.4.	METHOD FOR ASSAY OF UBIQUITIN ACTIVATION BY E1	105
4.3.5.	METHOD FOR WESTERN BLOTTING	106
4.3.6.	METHOD TO CHECK THE EFFECT OF DIFFERENT PH ON UBIQUITIN (F45W) AND B-BULGE MUTANTS Q2N, E64G AND S65D	107
4.4.	RESULTS	108
4.4.1.	PURIFICATION OF UBIQUITIN F45W AND B-BULGE MUTANTS Q2N, E64G AND S65D	108
4.4.2.	PURIFICATION OF UBIQUITIN ACTIVATING ENZYME E1	110
4.4.3.	ASSAY OF UBIQUITIN ACTIVATION BY E1	111

4.4.4.	EFFECT OF DIFFERENT PH ON UBIQUITIN (F45W) AND B-BULGE MUTANTS Q2N, E64G AND S65D	113
4.5.	DISCUSSION	117
<u>SUMMARY</u>		<u>119</u>
<u>BIOBLIOGRAPHY</u>		<u>126</u>
<u>LIST OF PUBLICATIONS</u>		<u>151</u>
<u>LIST OF POSTER PRESENTATIONS</u>		<u>151</u>