Studies on the Degradation Determinant Signals of Ornithine Decarboxylase (ODC)

THESIS
SUBMITTED TO

THE MAHARAJA SAYAJIRAO UNIVERSITY OF BARODA, VADODARA, GUJARAT

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

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October- 2012

Synopsis of the thesis on

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INTRODUCTION

The smallest well organized form of life is a cell. Cell is made up of macromolecules such as carbohydrates, proteins and lipids. Among all these, proteins gain a lot of importance because of their functional diversity, astounding structural variety and physico-chemical properties. By decoding the message hidden in DNA, protein makes a living cell functional. Structure of a protein is responsible for its function. They are involved in almost all cellular processes directly or indirectly. Proteins being important macromolecules, a highly sophisticated network exists in a cell to maintain a homeostasis between protein synthesis and degradation.

Protein Degradation:

In cell, protein degradation takes place for elimination of abnormal, nonfunctional, denatured and potentially toxic proteins. They may arise from nonsense or missense mutations, errors in gene expression or post translational damage. Protein degradation is also important in the regulation of cellular metabolism, recycling of essential amino acids and provision of nutrients during nutritional deprivation.

There are several proteases and proteolytic signals found in both prokaryotes and eukaryotes. In bacteria, degradation signals are generally found to be at N or C terminals of protein and are recognized by the target protease itself. In eukaryotes, Ubiquitin proteasome system (UPS) is implicated in the process of protein degradation (Varshavsky, 1997). All cytoplasmic degradation in prokaryotes, archaea and eukaryotes is energy dependent. For example *E. coli* has at least five ATP dependent proteases as Lon, FtsH, ClpAP, ClpXP and HslUV. These enzymes appear to have distinct substrate preferences (Gottesman *et al.*, 1998). ClpXP is the key protease responsible for degradation of SsrA-tagged substrates in most bacteria (Keiler *et al.*, 1996). Transfer messenger RNA (tm RNA) is a bacterial ribonucleic acid that adds a C terminal degradation peptide to nascent polypeptide chains on stalled ribosomes. Tsp is a periplasmic protease responsible for degradation of SsrA tagged proteins that are exported to periplasm (Gottesman *et al.*, 2010). Lysosomes and proteasomes are two major intracellular organelles to digest damaged or unwanted proteins and proteins which have completed their task. Lysosomes degrade extracellular proteins entering the cell through endocytosis, a process of

nonspecific protein degradation while specific protein degradation is mediated by intracellular proteases called as proteasomes. The proteasome is a large protein complex located in the nucleus and cytosol of eukaryotic cell. The 26S proteasome is formed by the binding of two 19S regulatory complexes on either side of 20S proteasome (Voges *et al*., 1999). As the active site of the proteasome is not in direct contact with cytoplasm, it is a self compartmentalized protease which avoids nonspecific protein degradation (Baumeister *et al.,* 1998). Substrates must enter in the proteolytic chamber for degradation. Tagging of proteins selectively by ubiquitin takes them to the 26S proteasome for specific degradation. Thus ubiquitination of a protein is a pre-degradation step.

The purpose of such complicated degradation machinery is to maintain the cellular quality control. Studies on molecular mechanism of many diseases established protein degradation to have a critical role. Either mutation or accumulation of proteins in cell may lead to defective cellular processes. Failure of degradation of such proteins by UPS system results into diseases like the Liddle syndrome, certain kinds of cervical cancers, Parkinson's, Alzheimer's, Huntington's, cystic fibrosis, and multiple myeloma. To combat such diseases where protein degradation is defective, attempts are being made to develop targeted or specific protein degradation. Manipulations at gene level such as gene knockout or antisense oligonucleotides and small interfering RNA (si RNA) were found to have various drawbacks. Probing the pathways of targeted protein degradation for the above mentioned goal has been subject of much attention in biomedical, pharmaceutical and microbial research. Efforts to develop targeted protein degradation with ubiquitin-proteasome system have not met with much success. The process of ubiquitination requires the action of 3 different enzymes, namely E1, E2 and E3. E2 and E3 are highly substrate specific to carry out ubiquitination. The 3D structure of E3 ligase complexes with their substrate protein reveals a precise geometric relationship between substrate & ligase. Further, E3s are specific to certain E2s for catalytic addition of ubiquitin onto susceptible lysine residues in target protein. These structural constraints suggest that attempts to induce ubiquitination of target proteins may prove difficult because of incompatibility between substrate & ligase (Matsuzava et al., 2005). Direct method for targeted protein degradation without ubiquitination could overcome this limitation. A small number of proteins have been identified which act as substrates of proteasome

though do not require ubiquitin for their degradation (Marian *et al..*, 2003, Coffino *et al.*, 2004). Ornithine Decarboxylase (ODC) being one of them undergoes ubiquitin independent proteasomal degradation pathway (Murakami *et al.*, 1992). ODC is an unusual proteasomal substrate as it is degraded by ubiquitin independent mechanism. Some degradation signals lie within the ODC, which along with another protein termed as Antizyme, target it for degradation (Coffino, 2001). Antizyme recognizes a stretch of 24 amino acids known as Antizyme binding element or AzBE in the α/β barrel domain of monomer and destabilizes ODC. The last 37 amino acids in the C terminus of mammalian ODC help in docking the protein on proteasome. Cys 441 in the C terminus, if mutated, stabilizes ODC (Coffino *et al.*, 2007). The yeast ODC has two intrinsic degradation determinant signals, namely the N-terminal region of 1-50 residues and Antizyme binding element (AzBE) present on the surface helices of the α/β barrel domain, from 167 to 184 residues. The stretch of last 37 amino acids of mammalian ODC, which helps in docking the protein on proteasome is not present in yeast ODC. There are evidences of N terminal being important for degradation via Antizyme (Gadre *et al.*, 2002). The N terminal region that is unique to yeast ODC acts similar to the C-terminal region of the mammalian enzyme in mediating its recognition by the proteolytic machinery. All these degradation signals also known as 'degrons' are characteristic features of ODC, thereby making ODC a favorable experimental substrate.

Ornithine Decarboxylase:

Ornithine Decarboxylase (ODC) is the first enzyme in polyamine biosynthesis pathway (Tabor *et al.*, 1984). It is approximately 450 amino acids long protein having molecular weight of 52 kDa. This protein has two domains namely N terminal α/β barrel domain and C-terminal domain. The N terminus has 9 α helices while C terminus has a β-sheet structure. It has two sheets namely S1 and S2 (Kraulis *et al.*, 1991). ODC is active in a homodimer form. Homodimer is formed by head to tail interaction of two monomers. N terminal domain of one monomer interacts with C terminal domain of another monomer and *vice versa*. Regulation of mammalian ODC is well studied (Pegg *et al.*, 2006). The crystal structure of mammalian ODC is available. However Yeast ODC protein structure has not come in focus by far. Present study tries to reveal the structural aspects of degradation signals of yeast ODC.

Structural information of any protein has been matter of utmost significance in the field of structural biology. Also it is prerequisite in drug design. It gives a visual idea of physical and chemical characteristics of protein by providing information on the structure of a protein and thus providing basis to predict function of specific protein.

Study of domain organization has been is an important area in structural studies. Domain is a part of polypeptide chain that can fold and function independently. Domain has a stable tertiary structure with its own hydrophobic core. A single protein may contain one or more domains. Many a time domains perform distinct biological functions, conferring tremendous functional and evolutionary advantage to proteins. Domains interact with other molecules in the cell and they are also involved in signal transduction pathways. Along with domain an unstructured region also has an important role in protein assembly and function (Prakash *et al.*, 2004). Unstructured stretches are involved in initiation of degradation, recognition of other molecules etc. Every structure in a protein is important with respect to its assigned function. Keeping this in mind, the idea was to focus on the structural as well as functional aspect of degradation determinant signals of yeast ODC.

The proposed peptides are:

1. α/β barrel domain of yeast ODC (α/β)
2. Entire N terminal domain (N α/β)
3. First 50 amino acid residues of yeast ODC (N50) and
4. Last 37 amino acid residues in the C terminus of mammalian ODC (37mODC).

These signals fused to target protein, along with antizyme may provide an inducible switch for targeted protein degradation. In the present study the potential of the peptides act as degrons and their ability to take up secondary and tertiary structures have been addressed.

Objectives of the present study

1 **Construction of plasmids carrying DNA sequences for proposed peptides and their transformation into *E.coli*.**
2 **Purification of all proposed peptides.**
3 **Characterization of peptides using spectroscopic techniques.**
4 **Study the potential of these degradation determinant signals as a portable tag for targeted protein degradation.**

1 Construction of plasmids carrying DNA sequences for proposed peptides and their transformation into *E.coli*:

Fragment of genes of all proposed peptides were successfully amplified from vectors pRS316 containing yeast ODC and pBSKS containing mouse ODC. Bioinformatics tools such as structure prediction softwares were used before designing the primers for peptides**.** Plasmid pET 30a was used as a bacterial expression vector, which carries histidine tag for protein purification. The cloning of DNA sequences of all proposed peptides was done successfully in pET30 vector using *Bam* HI and *Sac* I restriction sites. The clones were confirmed after sequencing. The protein expression studies were carried out in BL21 (DE3) strain of *E. coli*. These cells are designed to provide high level of protein expression and thus to ease the purification. The genes of all the proposed peptides were cloned under T7 promoter in pET30 vector which is IPTG inducible. The transformed BL21 (DE3) cells were induced with 1mM IPTG to study the protein profile. Expression of all proposed peptides was checked on SDS PAGE.

2 Purification of all proposed peptides:

All peptides were purified using affinity chromatography. Transformed BL21 (DE3) cells were grown at 37°C in Luria broth containing kanamycin (50mg/ml). At mid log phase cells were induced with 1mM IPTG and harvested after 4 hrs. of induction. Cells were lysed using lysozyme and peptides were purified by affinity chromatography using Nickel linked resin (Ni-NTA resin). Samples were dialyzed in order to remove salt.

3. **Characterization of peptides using spectroscopic techniques:**

Structural studies were carried out to characterize secondary and tertiary structure of selected peptides using Circular Dichroism and Fluorescence spectroscopy. These techniques are used for characterizing the structure and thereby inferring stability of proteins.

a) Circular dichroism studies: Circular dichroism studies revealed the secondary and tertiary structure of three peptides i.e. α/β barrel domain, entire N terminal domain and stretch of first 50 amino acid residues. CD spectra were recorded on Jasco 815 spectropolarimeter. Far UV CD spectra were recorded from 180 to 260 nm while near UV CD spectra were recorded within 250 to 320 nm range with 10mm path length. Protein samples were prepared in buffer having 10mM sodium phosphate and 10mM NaCl (pH 8). Spectra of the samples were blank corrected. The far UV-CD spectra indicate existence of well defined secondary structure in α/β and N- α/β peptides. N(50) peptide present at the N-terminal also shows evidence of structure. The near UV-CD spectra show presence of tertiary structure, which gets denatured after addition of 4M guanidine hydrochloride. At acidic pH the peptides lose secondary as well as tertiary structure.

b) Fluorescence spectroscopic studies:

Fluorescence spectra were recorded using Hitachi Fluorescence Spectrophotometer. Peptide samples were prepared in phosphate buffer (pH 8). Fluorescence studies were carried out using intrinsic and extrinsic fluorophores.

i) Fluorescence spectroscopic study using intrinsic fluorophore:

To record the intrinsic fluorescence due to tryptophan, samples were excited at 280nm and emission was recorded between 300-400nm. Urea and Guanidinium hydrochloride were used as denaturing agents. Intrinsic fluorescence spectra of α/β peptide, N α/β peptide and N50 were recorded in buffer and 5M urea. The spectra of all three peptides recorded in urea show red shift with respect to the spectrum in buffer. In guanidinium hydrochloride denaturation studies, gradual increase in guanidinium hydrochloride concentration leads to increase in fluorescence intensity and showing loss of structure of protein. These results indicate the presence of tertiary structure in all three peptides.

ii) Fluorescence spectra using extrinsic fluorophore (FRET study):

ANS was used as an extrinsic fluorophore at a concentration of 10µM. ANS is known to bind the hydrated hydrophobic residues and emit fluorescence. Fluorescence Resonance Energy Transfer (FRET) spectra of all three peptides were recorded in presence and absence of ANS. Energy transfer between Trp and ANS was recorded by exciting Trp at 280 nm and emission was recorded from 300 to 550 nm. ANS was excited at 340 nm by transfer of energy from Trp and emission was recorded between 450-550 nm. FRET studies revealed the compact tertiary structure of all three peptides.

Structural characterization of 37mODC peptide is in progress.

4 Studies on the potential of these degradation determinant signals as a portable tag for targeted protein degradation:

Cloning of the DNA fragments corresponding to the peptides into an expression vector is in progress. After cloning the peptides will be expressed as fusions of β-galactosidase. β-galactosidase activity will be assayed to assess the potential of the degrons.

Conclusion:

Our results, for the first time, establish that α/β barrel domain of yeast ODC attains structure in the absence of rest of the protein. Entire N terminal domain and α/β barrel domain can fold independently to gain the tertiary structure at physiological pH. These results are in accordance with the results of protein structure prediction softwares. The α/β barrel domain of yeast ODC harbors one degradation signal, i.e. Antizyme Binding Element (AzBE) while entire N terminal domain harbor AzBE as well as the stretch of first 50 amino acid residues. As the domain takes up structure which is mostly likely its native conformation, suggests the possibility of recognition by antizyme. This would be one of the important positive indications for evaluating the functional role of different peptides.

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Date: 29/09/2011 **Signature of the candidate**

(Dr. C. Ratna Prabha)

Guide

Off. Head **Dean**

Biochemistry Department **Faculty of science**

Swapnali Kulkarni
29/09/2011

Dept. of Biochemistry,

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To

The Registrar (Academic Section),

The M. S. University of Baroda,

Vadodara - 390 002.

Subject: Submission of synopsis of the Ph. D. work entitled – "Studies on the degradation determinant signals of Ornithine Decarboxylase."

Respected Sir,

Kindly accept the synopsis of my Ph. D work entitled – "Studies on the degradation determinant signals of Ornithine Decarboxylase." My date of registration was 30/04/2008 and registration no. is 135.

Thanking you,

Sincerely yours,

(Swapnali Kulkarni) (Dr. C. Ratna Prabha)
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Summary

Ornithine Decarboxylase (ODC) is one of the exceptional proteasomal substrate as it does not require ubiquitin for its degradation unlike most of other eukaryotic proteins. The protein sequence of ODC itself is well equipped with the degradation signals. ODC interacts with another protein termed as antizyme through one of its degradation signals and initiates the event responsible for executing degradation of ODC. Mammalian ODC has three intrinsic degradation determinant signals namely AzBE (Antizyme Binding Element), last 37 amino acid stretch and cysteine located at 441th position. In yeast also there are some reports explaining the presence of N terminal unstructured stretch and antizyme important for degradation of ODC. Yeast ODC crystal structure is yet to be resolved. Structural characterization of any protein is important as it is a basis for predicting, explaining or exploring the function of protein which is prerequisite in drug design. In an entire protein any structure i.e. domain, random coil or an unstructured stretch is important with respect to its assigned function. Our study is focused on the degradation determinant signals of ODC. The selected peptides are Nα/β peptide, α/β peptide and N50 peptide derived from yeast ODC while C37mODC peptide derived from mouse ODC. α/β peptide chosen for structural characterization is domain in nature while Nα/β peptide is combination of domain plus unstructured region at N terminus. N50 peptide and C37mODC peptide are unstructured in nature. Nα/β peptide harbors two degradation signals i.e. AzBE and N terminal unstructured tail while α/β peptide harbors one degradation signal i.e. AzBE. N50 and C37mODC peptides serve as unstructured intiation region to start with degradation process. Present study aims to focus on the structural aspects of the degradation determinant signals or degrons of yeast ODC when they are expressed independently from the rest of protein as well as functional aspects after tagging them to reporter protein.

Our results, for the first time, establish that Nα/β peptide and α/β peptide derived from yeast ODC attain 3D conformation in the absence of rest of the protein. Both domains can fold independently to gain the tertiary structure at physiological pH. These results are in accordance with the results of protein structure prediction softwares. The Nα/β barrel domain of yeast ODC harbor two degradation signals i.e. Antizyme Binding Element (AzBE) and unstructured stretch of first amino acid residues. As stated

earlier, antizyme recognizes Antizyme Binding Element (AzBE) which is situated in the N terminal α/β barrel domain of ODC, destabilizes an entire protein and directs the ODC protein towards proteasome for degradation. As Nα/β domain is in its natural conformation, the possibility of recognition by antizyme cannot be ruled out which is an important pre-degradation step for destabilization of an entire protein complex.

Our results also establish the independent folding of N50peptide. N50 peptide which is an unstructured region having 50 amino acid residues of N terminal α/β barrel domain of yeast ODC attains some tertiary structure independently in the absence of rest of the protein. This region which is unstructured in nature when present as a part of entire protein, can fold to gain the tertiary structure at physiological pH when expressed independently. These results are in accordance with the results of protein structure prediction softwares. As discussed in introduction (Under IDPs section) intrinsically disordered region has an ability to gain a structure to become functional. Rather molecular interactions or protein interaction events induce a conformation in otherwise unstructured regions. It is also noted in literature that protein or peptide can gain different conformation in different environment e.g. inside and outside the cell, physiological conditions and laboratory conditions, in buffer and in cellular crowded milieu. this could be the reason behind conformation of N50 peptide.

After successful structural characterization of Nα/β peptide and N50 peptide, it was proposed to check the portability of both peptides as a degradation tag. Nα/β peptide and N50 peptides were tagged at the N terminus of reporter protein i.e. β-galactosidase for the expression of fusion proteins Nα/β-β-galactosidase and N50-β-galactosidase respectively. Stability of reporter protein was checked by performing β-galactosidase assay. Yeast cells expressing only β-galactosidase protein has shown β-galactosidase activity. Cells expressing Nα/β-β-galactosidase fusion protein and cells expressing N50-β-galactosidase fusion protein did not show any β-galactosidase activity indicating possibility of degradation of fusion protein because of presence of N terminally tagged Nα/β and N50 peptides which are degradation signals in native yeast ODC. For the final confirmation of proteasome as the destination of proposed fusion proteins, proteasome inhibitor was added in yeast cells expressing fusion

proteins. If proposed Nα/β and N50 peptides have potential to drag the reporter protein β-galactosidase towards proteasome for degradation, then after proteasomal inhibition β-galactosidase activity was expected from cells expressing fusion proteins. No β-galactosidase activity from these cells suggesed the possibility of inclusion formation of expressed fusion protein in the cells.

Earlier it was reported that N terminal extension of yeast ODC can target the reporter protein with α helical structure and not β sheet structure. Another possibility is structural constraints esperienced by β-galactosidase protein itself cannot be ruled out.

In literature large molecular weight proteins are tagged to β-galactosidase enzyme and entire assembly is stable and functional. In our case the selected peptides are at least not allowing an enzyme to be functional, i.e. it is interfering in the folding of an enzyme or in other words destabilizing it. Actual scenario about degradation of fusion protein inside the cell is not clear but at least fusion proteins are not allowing the reporter protein to remain stable or active or functional.

CD studies revealed presence of helical structure in Nα/β peptide while CD spectrum of α/β peptide indicates sheet nature of α/β peptide. The difference between Nα/β peptide and α/β peptide is just presence of N terminal unstructured region in Nα/β peptide. This indicates significant contribution of N terminal region in folding of Nα/β peptide. α/β barrel domain is situated in between N terminal unstructured region and C terminal β sheet domain. As selected α/β peptide is not accompanied by its natural neighbouring amino acid residues, possibility of slight alteration in secondary structure was expected.

Our studies on the structural characterization of α/β peptide revealed the mechanism behind the stability of N terminally deleted yeast ODC. The α/β barrel domain of yeast ODC harbours one degradation signal i.e. Antizyme Binding Element (AzBE). Deletion of N terminal unstructured region renders stability to yeast ODC. Our results are in accordance with this finding and also explain rationale behind the stability of N terminally truncated yeast ODC. Though the domain is in tertiary conformation, it has gained β sheet conformation rather than α helix. Altered secondary structure could

affect the conformation of antizyme binding element. If AzBE is not folded in its natural conformation antizyme possibly would not recognize it. Without interaction of antizyme with AzBE ODC would not be destabilized and consequently all next steps of presenting ODC towards proteasome for degradation would be hampered. So deletion of N terminal unstructured stretch would interfere with processing by antizyme by changing the structure of α/β barrel domain. N terminally truncated yeast ODC is stable because it lacks the initiation region which starts actual degradation by providing unstructured stretch which is essential to start with proteasomal entry and subsequently degradation. We propose another possibility that instead of interference in proteasomal entry, deletion of N terminal stretch may alter the secondary structure of α/β barrel domain with altered structure may be unable to recognize antizyme and subsequently affecting destabilization and degradation of yeast ODC.

Ubiquitin is stated as vital protein for almost all cellular processes. Imbalance in its level leads to number of complications in cellular processes. Attempts to release accumulated ubiquitin conjugates, degradation of notorious proteins, and cleaving ubiquitin from inclusions to minimize the burden on cellular environment could be served by targeted protein degradation.

Though ubiquitin is recycled after presenting substrate proteins to proteasome for degradation, proteasome is final destination for degradation of ubiquitin also under certain circumstances as discussed in chapter 5. By tagging ubiquitin with C terminal tail of mouse ODC so as to make UbmODC fusion protein, we brought two degradation signals together. Our results, for the first time, have successfully shown the degradation of ubiquitin when tagged with unstructured 37mODC peptide derived from mouse Ornithine Decarboxylase (ODC). Presence of ubiquitin band and absence of C terminally tagged ubiquitin band on SDS-PAGE indicated possibility of degradation of fusion potein. Appearance of band of UbmODC fusion protein after proteasomal inhibition confirmed proteasome as a final destination of UbmODC fusion protein. Western blot study confirmed the expression of fusion protein and degradation through proteasomal way. These results show the potential of C 37mODC to act as degron for the degradation of ubiquitin.

In conclusion our study provided the mechanism behind the stability of N terminally truncated yeast ODC by revealing the altered structural aspects of α/β domain of yeast ODC. Antizyme may not recognize AzBE because of altered conformation of AzBE. This could interfere with the very initial step of ODC destabilization and consequently degradation. Our studies have also shown that degradation signals within yeast ODC can fold independently from rest of protein. Potential of C37mODC as a degron to carry out targeted ubiquitin degradation was also established.

5 Construction of pUbmODC, expression and its functional analysis in yeast

5.1 Introduction

Ubiquitin is a small protein of 76 amino acid residues present ubiquitously in eukaryotic cells hence the name is. In eukaryotic cell most of the proteins are degraded by ubiquitin proteasome system (Pickart et al., 2001). Ubiquitination of a protein is a pre-degradation step (Varshavsky, 1997). Ubiquitin is a natural tag to the protein to be degraded. Substrate protein is directed towards the proteasome for degradation with the help of ubiquitin in a series of events. An isopeptide bond is formed between COOH terminal glycine of ubiquitin and ε amino group of a lysine residue on the target protein. Ubiquitin can be attached to a free amino terminus of protein via standard peptide bond and also to threonine, serine or cysteine residues via ester or thiolester bond (Cadwell et al., 2005). A polyubiquitin chain is formed on the first ubiquitin moiety and entire assembly is dragged towards the final destination of degradation i.e. proteasome. Once ubiquitinated protein comes in contact with proteasome, recognition element or 19S subunit of 26S proteasome recognizes the ubiquitinated stuff (Lam et al., 2002) and initiates unfolding process. As protein starts unfolding 19S regulatory subunit threads the free or unstructured amino or carboxyl terminus of unfolded protein into the interior of 20S proteasome. Once an unstructured region of protein comes in contact with hollow chamber of 20S proteasome, an entire protein is taken up by the 26S proteasome for degradation. Only substrate protein is dragged and degraded by proteasome while ubiquitin is recycled. Action of certain deubiquitinating enzymes cleaves ubiquitin from substrate protein (Reyes et al., 2009; Verma et al., 2002) and ubiquitin is free for next cycles of protein recognition and degradation. So ubiquitin pool in cell is maintained sophisticatedly by recycling. Though ubiquitin is a key protein in ubiquitin mediated proteasomal degradation of proteins, entry of ubiquitin itself along with substrate inside the proteasome is restricted. A very different mechanism controls the cellular level of ubiquitin (Kimura et al., 2010). Large number of protein were identified and studied which are degraded through ubiquitin proteasome system but study on degradation and regulation of members of ubiquitin proteasome system itself is equally important

(Weissman et al., 2011). Ubiquitin proteasome system carries out specific protein degradation with the help of ubiquitin as a tag. But as far as recycling event and degradation of ubiquitin itself is concerned different notions are available in literature (Shabek et al., 2010; Weissman et al., 2011; Shabek et al., 2007; Kimura et al., 2010).

5.1.1 Fate of Ubiquitin

Ubiquitin itself is a quite stable protein. 40 to 60% of cellular ubiquitin is free (Hass et al., 1987). Ubiquitin exists in a cell as a free monomer or in the form of polyubiquitin chains (Carlson et al., 1987). Polyubiquitin chains are generally conjugated to target proteins but can also exist in free form. The levels of mono and polyubiquitin in cell are maintained by two different set of enzymes (Wilkinson et al., 2000; Nocker S, 1993; Reyes et al., 2009). First set of enzymes involves enzyme E1, E2 and E3s which are involved in recognition and ligation of ubiquitin with substrate proteins for degradation. Another set of enzymes controlling cellular ubiquitin levels are deubiquitinating enzymes (DUBs). These enzymes allow only substrate protein to enter in 26S proteasome and cleave ubiquitin from ubiquitin substrate protein complex. Cellular level of ubiquitin and its stability are dependent on the physiological condition of the cell (Shabek et al., 2010). As dwindling of ubiquitin level is seen in stress or malnutrition, it was proposed that ubiquitin is also degraded along with substrate and to overcome the ubiquitin depletion, ubiquitin synthesis is up regulated. The deubiquitinating enzymes (DUBs) can only cleave polyubiquitin chain from substrate or generate mono-ubiquitin from polyubiquitin and not degrade ubiquitin protein. Ubiquitin specific C terminal hydrolases cleave at last two glycine residues i.e. 75^{th} and 76^{th} glycine residues.

According to studies on the degradation of ubiquitin proteasome is the final destination of ubiquitin for degradation like other proteins (Kimura et al., 2010; Shabek et al., 2007). The only difference is that there are three different ways for ubiquitin to reach the proteasome unlike other proteasomal substrates. There are different ways of presenting the substrate to proteasome for degradation purpose like some proteins are tagged by ubiquitin, while other proteins need an adaptor protein for reaching proteasome (Hartman et al., 2003), some other proteins are directly

recognized by proteasome itself (Orlowski et al., 2003). Ubiquitin independent proteasomal substrate for example Ornithine Decarboxylase (ODC) is routed towards the proteasome with the help of another protein called antizyme. Different proteins have different mechanisms for degradation but generally one mechanism is executed by cell for one protein. As far as ubiquitin is concerned there are different ways to reach the proteasome. Ubiquitin can enter proteasome along with the substrate to which ubiquitin itself is linked as a tag. Ubiquitin can enter the proteasome independently as a monomer and also in the presence of C-terminal tail (Shabek et al., 2010) (Figure 5.1).

5.1.2 Pictorial representation of Ubiquitin degradation in different ways

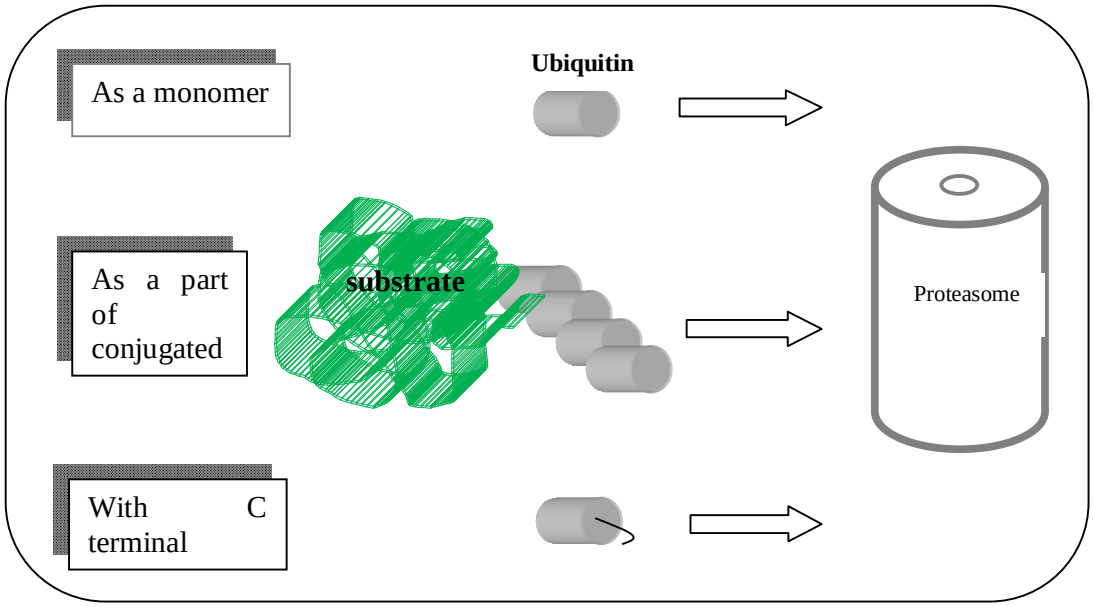


Figure 5.1 Different ways through which ubiquitin is presented to proteasome for degradation

It was hypothesized that impaired action of deubiquitinating enzymes present at the gate of proteasome results in proteasomal entry and consequently degradation of Ubiquitin along with substrate. Nitzan Shabek explained why some ubiquitin moieties follow substrate inside the proteasome (Nitzan Shabek et al, 2010). If ubiquitin moieties attached to substrates are detached earlier there is possibility of incomplete degradation of substrate. So to complete the degradation of entire substrate some ubiquitin moieties possibly are spared by cells. The reason behind proteasomal entry of only monomeric ubiquitin is unknown but believed to be a part of regulatory

mechanism. Carlson and Heroi et al proposed degradation of Ubiquitin in free monomeric form because they found that entire Ubiquitin with 76 amino acid residues and Ubiquitin without last two glycine residues i.e. having 74 amino acid residues are degraded with same rate (Carlson et al., 1987; Heroi et al., 1992). Ubiquitination of ubiquitin was also observed. Ubiquitination of ubiquitin protein itself occurs at internal lysine residues or at amino terminus. Immunobloting studies with His or Myc tagged ubiquitin reveal that lysine residue and amino terminus of ubiquitin are mandatory for ubiquitin itself to serve as a substrate. The difference between ubiquitin as a tag and ubiquitin as a substrate is requirement of lysine at 48^{th} position. Degradation followed by ubiquitination of ubiquitin was observed in cell free system as well as in cells.

As discussed in chapter 1 and 3, unstructured region is required to initiate the proteasomal entry of substrate. Ubiquitin was found to be a proteasomal substrate when extended at C terminus with few amino acids. This extension was a sequence derived from naturally present protein or any modified sequence such as His tag, HA tag and multiples or combinations of these tags. A drastic decrease in half life was observed from 8 hours of single Ubiquitin to 1.5 hours of C terminally extended Ubiquitin (Heroi et al., 1992). Table 5.1 summarizes the reports on half life and degradation of ubiquitin.

Few ubiquitinated proteins are also found in lysosomes (Laszlo et al., 1990).

5.1.3 Highlights of studies related to degradation and half life of Ubiquitin

Sr. no	System	Form of Ubiquitin studied	Result	Reference
1	Chinese hamster ovary cells	Ubiquitin histone conjugate	Ubiquitin half life- 9 hours	Wu RS et al., 1981
2	He La cells	^{125}I labeled Ubiquitin	Half life 9 hours, can be degraded as monomer	Carlson et al., 1987

3	Yeast	Wild type ubiquitin	Half life less than 2 hours	John Hanna et al., 2003
4	Cell free system	Monomeric and His, Myc tagged ubiquitin	Ubiquitin is a proteasomal substrate and need C terminal extension to enter in it.	Nitzan Shabek et al, 2007

Table 5.1 Showing highlights of various studies on half life of Ubiquitin in different systems.

5.1.4 Defective ubiquitin homeostasis and disease

Accumulation of free ubiquitin and ubiquitin conjugates is observed in various diseased conditions, few are listed in table 5.2.

Sr. no	Disease	Accumulated or non degradable ubiquitin conjugates	Reference
1	Alzheimer disease	Plaques and neurofibrillary tangles. UBB (Ub with extended 19 aa residues) accumulates and inhibits proteasome	Mori H et al., 1987 Lam et al., 2000
2	Parkinson disease	Lewy bodies	Lowe J et al., 1988
3	Pick disease	Pick bodies	Lowe J et al., 1988
4	Astrocytes	Rosenthal fibres	Lowe J et al., 1988

5	Various malignancies	Studies on immunoreactivity of ubiquitin, free and multiubiquitin chains	Ishibashi Y et al, 2004, Osada T eta l, 1997, Ishibashi Y et al, 1991
6	Alcoholic liver disease	Mallory bodies	Lowe J et al, 1988
7	Huntington disease	Accumulation of Ubb conjugates, unusual internal linkages in ubiquitin	Ciechanover et al, 2003

Table 5.2 Shows different forms of ubiquitin conjugates accumulated in various diseases.

As discussed earlier, ubiquitin synthesis is increased in stress or disease condition. In spite of increased synthesis, there is unavailability of ubiquitin for normal cellular functions. Neuronal dysfunction is initiated because of decreased ubiquitin availability as ubiquitin is trapped in accumulated inclusions.

As ubiquitin is stated as vital protein for almost all cellular processes, imbalance in its level leads to number of complications in cellular processes. Attempts to release accumulated ubiquitin conjugates, degradation of notorious proteins, and cleaving ubiquitin from inclusions to minimize the burden on cellular environment could be served by targeted protein degradation.

5.1.5 Previous studies on Ubiquitin fusions

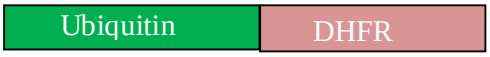
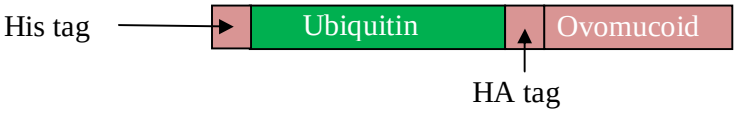
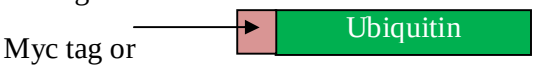
A large number of ubiquitin fusion proteins were studied to reveal the ubiquitin homeostasis and to focus on the protein degradation in normal physiological condition as well as in disease or stress condition (Table 5.3). In vivo detection of Ubiquitin conjugates is convenient because of most of the Ubiquitin fusions such as His, Myc or

GFP tagged Ubiquitin. A major finding of N end rule was explained with the help of series of Ubiquitin- β galactosidase fusion proteins having different amino acids at the junction of two proteins (Bachmair et al., 1986). A fusion protein can also be degraded by proteasome if ubiquitin is situated in the middle of fusion protein. But if ubiquitin is at the C terminus of fusion protein the entire assembly is stable. According to these findings, 26S proteasome exhibits discrimination between Ubiquitin conjugated proteins. But most studies have in common that there is a requirement of an unstructured region for initiation proteasomal degradation.

As discussed in chapter 1, the last 37 amino acid residues of mammalian Ornithine Decarboxylase (ODC) does not gain any specific conformation. This C terminal unstructured tail is important as degron for the initiation of degradation of ODC itself. It was studied that proteasome initiates the degradation of ODC by dragging first the C terminal unstructured region and then an entire protein. According to earlier reports the C terminal unstructured region of mouse ODC could successfully promote the degradation of some reporter proteins, for e.g. luciferase, GFP, DHFR etc. This unstructured region stated as 37mODC can serve as portable degradation signal because when tagged at the C terminus of reporter protein it can drag the reporter protein into proteasome. A number of studies also focus on Ubiquitin fusion proteins which are functional. With this background it would be interesting to know the fate of ubiquitin with C terminal extension of 37mODC. Whether the ubiquitin will function as wild type Ubiquitin or it will be prone to degradation due to the presence of an unstructured region.

Our study aims to check the potential of C terminal tail of mouse ODC to convert the ubiquitin tag into proteasomal substrate. UBB+1, a variant of ubiquitin with a C terminal extension of 19 amino acids is known to accumulate in Alzheimers disease. With this background, we are interested to check the fate of UbmODC fusion protein. The stretch of last 37 amino acid residues of mouse ODC was tagged at the C terminus of ubiquitin (Figure 5.2 and 5.3).

Summarizing Ubiquitin fusion proteins:

Ubiquitin fusion protein	Function	Reference
  	Used to check the stability and dual functionality of Ubiquitin fusion proteins Used to identify deubiquitinating enzyme	Quin et al., 2002 Baek et al., 1997
	Used to study N end rule	Varshavsky, 1998
	Used to study accumulation of Ubiquitin conjugates in polyglutamine disease	Maynard et al., 2009
	Used to study proteases carrying deubiquitination and degradation through proteasome	Yao et al., 2002
	Used to study split Ubiquitin as a sensor of protein interaction	Johnsson et al., 1994
	Can substitute wild type Ubiquitin in various studies.	
	**Proposed C37 mODC tag**	

(Represented size of tag is not proportional to the actual size of tag)

Table 5.3 Shows a list of ubiquitin fusion proteins made for different purposes.

5.1.6 UBIQUITIN GENE SEQUENCE

[YEAST SYNTHETIC]

```
 1  ATG  CAG  ATC  TTC  GTC  AAG  ACG  TTA  ACC  GGT
30
    Met  Gln  Ile  Phe  Val  Lys  Thr  Leu  Thr  Gly
31  AAA  ACC  ATA  ACT  CTA  GAA  GTT  GAA  TCT  TCC
60
    Lys  Thr  Ile  Thr  Leu  Glu  Val  Glu  Ser  Ser
61  GAT  ACC  ATC  GAC  AAC  GTT  AAG  TCG  AAA  ATT
90
    Asp  Thr  Ile  Asp  Asn  Val  Lys  Ser  Lys  Ile
91  CAA  GAC  AAG  GAA  GGC  ATT  CCA  CCT  GAT  CAA
120
    Gln  Asp  Lys  Glu  Gly  Ile  Pro  Pro  Asp  Gln
121 CAA  AGA  TTG  ATC  TTT  GCC  GGT  AAG  CAG  CTC
150
    Gln  Arg  Leu  Ile  Phe  Ala  Gly  Lys  Gln  Leu
151 GAG  GAC  GGT  AGA  ACG  CTG  TCT  GAT  TAC  AAC
180
    Glu  Asp  Gly  Arg  Thr  leu  Ser  Asp  Tyr  Asn
181 ATT  CAG  AAG  GAG  TCG  ACC  TTA  CAT  CTT  GTC
210
    Ile  Gln  Lys  Glu  Ser  Thr  Leu  His  Leu  Val
211 TTA  AGA  CTA  AGA  GGT  GGT  TGA
231
    Leu  Arg  Leu  Arg  Gly  Gly  End
```

Nucleotide sequence of 37 mODC:

TTC CCG CCG GAG GTG GAG GAG CAG GAT GAT GGC ACG CTG CCC ATG TCT TGT GCC CAG GAG AGC GGG ATG GAC CGT CAC CCT GCA GCC TGT GCT TCT GCT AGG ATC AAT GTG TAG

Amino acid sequence of proposed UbmODC fusion protein:

Green : amino acid sequence of His Myc tag

Black : amino acid sequence of Ubiquitin

Red : amino Acid sequence of **37mODC used as a C terminal tag** to Ubiquitin in the present study

MEIHHHHHHAGEQKLISEEDLGMQIFVKTLTGKTITLEVESSD

TIDNKSKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKEST

LHLVLRLRFPPEVEEQDDGTLPMSCAQESGMDRHPAACASAR

INVStop

Figure 5.2 Amino acid sequence of proposed UbmODC fusion protein.

Pictorial representation of proposed fusion protein:

Figure 5.3 Proposed fusion protein

5.2 Materials and Methods

5.2.1 Strains and media

E.coli DH5α strain was used for genetic manipulation as mentioned in chapter 2. Functional study has been carried out in yeast *Saccharomyces cerevisiae* BY4741 (*SPE*$^-$) as mentioned in chapter 2. Culture condition and media are already discussed in chapter 2.

5.2.2 Plasmid

Yeast expression vector pUb221 used for the present study. This plasmid carries His-Myc tagged yeast Ubiquitin under CUP 1 promoter. It has ampicillin as bacteria selection marker and Uracil as yeast section marker.

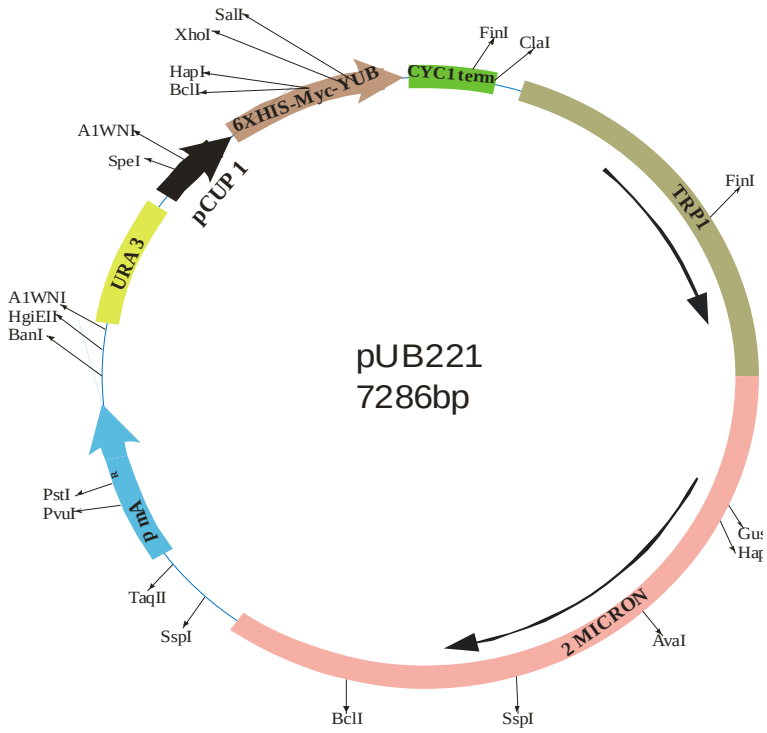


Figure 5.4 Vector map of pUb221

5.2.3 Primers used

Sr. No.	Primers used	Sequence
1	Ub mODC Forward	5'ATAGTCGACCTTACATCTTGTCTTAAGACTAAGATTC CCGCCGGAGGTGGAGGAGCAGGATGA 3'
2	Ub mODC Reverse	5'ATTGGTACCCTACACATTGATCCTAGCAGAAGC 3'

Table 5.4 Shows sequence of primers used for amplification of gene fragment coding for C37mODC peptide so as to make fusion protein named as UbmODC

5.2.4 Construction of pUbmODC

Vector pUb221 carries gene coding for yeast ubiquitin (Figure 5.4). The stop codon and last two glycine residues of ubiquitin need to be removed so as to make an in frame fusion protein and to avoid the action of few C terminal hydrolases or deubiquitinating enzymes. Fragment of gene coding for last 37 amino acids of mouse ODC was successfully amplified from pBSKS vector carrying gene for entire mouse ODC. Cloning of amplified insert was done in *Sal*I and *Kpn*I restriction sites. Few C terminal amino acid residues of Ubiquitin were deleted after *Sal*I and *Kpn*I restriction digestion. A fragment of gene coding for these amino acids was added as a flanking sequence in forward amplicon primer designed for amplifying the last 37 amino acids of mouse ODC. The 141 bp amplicon, digested by *Sal*I and *Kpn*I was ligated into pUb221 vector, which was digested with same enzymes. This ligated plasmid was transformed into DH5α strain of *E. coli*.

Confirmation of positive transformant was done by PCR using 37mODC insert specific primers. Further confirmation of positive transformant was done by PCR with different sets of primers. PCR using forward primer of ubiquitin and reverse primer of mODC, PCR using forward primer of ubiquitin designed from 50^{th} amino acid residue and reverse primer of mODC and mODC specific forward and reverse primers have shown bands of expected size confirming the insert.

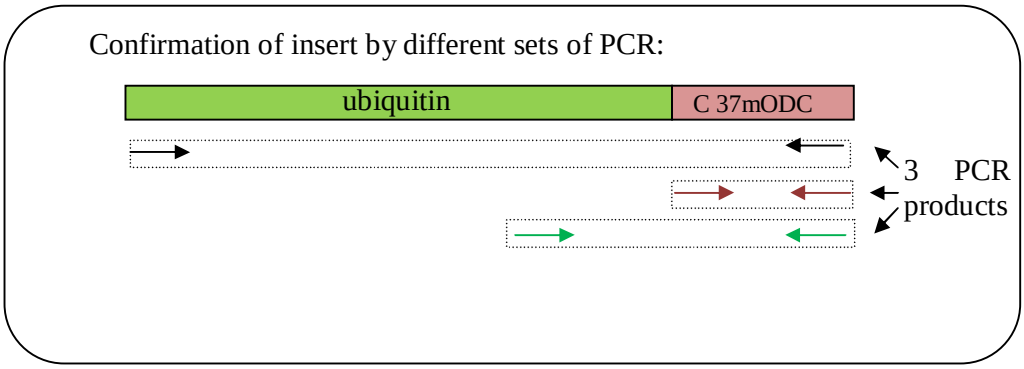


Figure 5.5 Screening strategy for construct pUbmODC by PCR using different sets of primer.

5.2.5 Yeast transformation, expression and functional analysis of fusion protein

Vector pUb221 is a shuttle vector between *E. coli* and *S. cerevisiae*. Vector pUb221 carries gene coding for ubiquitin under CUP1 promoter which expresses ubiquitin after $CuSO_4$ induction in yeast. Vector pUb221 and pUbmODC were transformed into BY4741 strain of yeast *S. cerevisiae* to check the protein profile. Both cultures i.e. yeast cells carrying vector pUb221 coding only Ubiquitin protein and yeast cells carrying vector pUbmODC coding fusion protein, were inoculated in S.D. media. At log phase cells were induced by 50µM $CuSO_4$. After 6 hours of induction cells were collected by centrifugation at 7000 rpm for 2 minutes. After three saline washes cell pellet was resuspended in 1ml of cold lysis buffer (pH 7.8). Cells were lysed by sonication and immediately loaded on 15% polyacrylamide gel to check the protein profile. The same experiment was repeated with 100µM $CuSO_4$ induction. In another set of experiment time bound induction pattern of both cultures i.e. one expressing only ubiquitin and another expressing UbmODC fusion protein was checked. After induction with 50µM $CuSO_4$ at log phase, cells were collected after every 4 hour interval till 20^{th} hour after induction and protein profile was checked on 15% polyacrylamide gel. The time bound induction pattern was also checked with 100µM $CuSO_4$ induction of yeast cells expressing UbmODC fusion protein.

5.2.5.1 Confirmation of proteasomal degradation of fusion protein

In order to confirm the degradation of fusion protein through proteasome, proteasomal inhibitor was used. MG132 is a proteasome inhibitor commonly used for yeast studies. In most of the studies on yeast cell inhibitors, erg6 mutant strain of yeast was used. In these mutant strains the final methylation reaction in ergosterol synthesis is blocked resulting in lack of ergosterol in the plasma membrane. So permeability of plasma membrane is affected and cell becomes sensitive to various inhibitors. But this strategy of making yeast cell permeable for proteasome inhibitor results in drastic side effects such as decline in tryptophan transport, reduced efficiency of genetic transformation and sexual conjugation while increased cellular levels of sodium and lithium (Gaber et al., 1989). A few studies (Pannumzio et al., 2004) used an alternate strategy of changing a nitrogen source. Generally ammonium sulphate is used as a nitrogen source in yeast minimal media. Compared to other nitrogen sources such as urea, proline and leucine, ammonium sulphate are easily taken up by the yeast cells. Proline, a poor nitrogen source, was used as an alternative to ammonium sulphate for studies on yeast cell inhibitors.

5.2.5.2 Methods to increase yeast cell permeability

A common problem with yeast studies is its tough cell membrane. As mentioned earlier use of *erg* or *pdr* mutant strains used to increase cell permeability has a few shortcomings. Instead use of simple additives such as 0.003% SDS or 150mM KCl at log phase could serve as temporary cell permealizing agents before addition of proteasome inhibitors (Pannunzio, 2004).

Yeast cells were grown in S.D. media with proline as a nitrogen source. At log phase, 0.003% SDS was added and cells were grown for another 3 hours. After 3 hours of SDS addition, cells were treated with 100μM MG132 for 3 hours. After MG132 treatment, cells were lysed by sonication and samples were loaded on 15% SDS PAGE to check the protein profile.

5.2.5.3 Western blot analysis

Yeast expression vector pUb221 used for the present study carries His-Myc tagged yeast ubiquitin under CUP 1 promoter. Western blot analysis was performed with yeast cells expressing only ubiquitin and UbmODC fusion protein. Cells were induced with 100 μM $CuSO_4$ at log phase for 3 hours in presence of 0.003% SDS. After 3 hours of induction cells were treated with 100μM MG132 for 3 hours for proteasomal inhibition. Samples for proteasomal inhibition and then for loading on PAGE were prepared as discussed above. After running discontinuous SDS-PAGE proteins were transferred on PVDF membrane at 4^0C. Anti-c-myc-horse radish peroxidase conjugated antibody (1/1000 diluted) was used for analysis (Antibody was purchased from Roche).

5.3 Results

5.3.1 Incorporation of mODC gene sequence on downstream of ubiquitin in pUb221 yeast expression vector

Using primers shown in table 5.4 gene of mODC was amplified from pBSKS. Sequence of gene fragment coding for last 10 amino acid residues of ubiquitin was included as a flanking region in forward primer designed for amplification of PCR product coding for peptide 37mODC. This modification was done for cloning so as to remove the stop codon of ubiquitin and for the expression of entire ubiquitin and C37mODC fusion protein. Insertion of mODC gene PCR in pUb221 by using *Sal*I and *Kpn*I restriction sites.

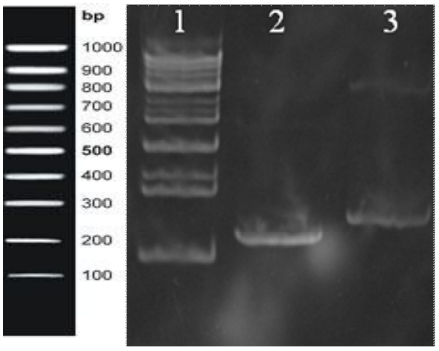
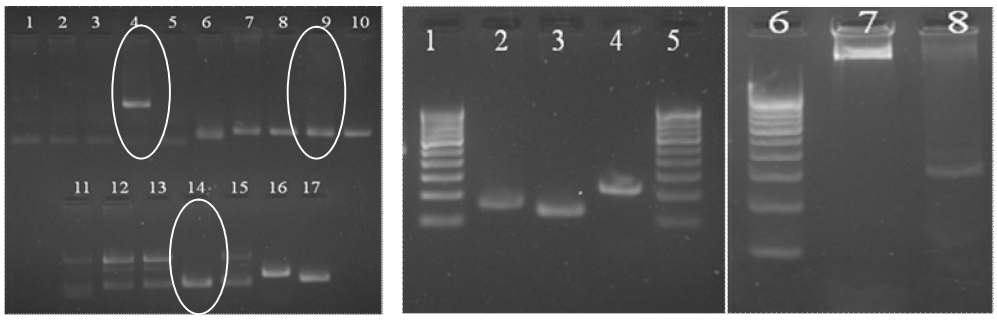


Figure 5.6 PCR product coding for peptide 37mODC

Lane 2 shows 129 bp PCR product of gene fragment coding for peptide 37mODC. Lane 3 shows 157 bp PCR product of gene fragment coding for last 10 amino acid residue of ubiquitin and C terminal tail of mODC corresponding with 100 bp marker in lane 1.

To check incorporation mODC gene in downstream of ubiquitin, various combination PCR were done by using ubiquitin and mODC primers. Further confirmation was done with restriction digestion. Finally clone conformed after sequencing.



(A) **(B)**

Figure 5.7 Screening of positive transformants of pUbmODC PCR restriction digestion

(A) Lane 1 to 5 show PCR products using forward primer of ubiquitin and reverse primer of mODC using plasmids to be screened for positive transformant as a template. Lane 6 to 10 show PCR products using forward primer of mODC with flanking sequence of ubiquitin and reverse primer of mODC. Lane 11 to 15 show PCR products using forward and reverse primer of mODC. Lane 16 shows PCR product of gene of ubiquitin. Plasmid number 4 is showing positive PCR with all 3 primer sets as shown in lane no. 4, 9 and 14 (Pictorial representation of these PCRs are as shown in figure 5). All the PCR products of plasmid no.4 were loaded with marker as shown in figure 6.

(B) Lane 2 shows 154 bp PCR product using C37mODC gene fragment specific modified forward and reverse primers. Lane 3 shows 129 bp PCR product of mODC using gene specific primers while lane 4 shows 228 bp PCR product of ubiquitin from positive transformant corresponding with 100 bp marker in lane 1 and 5. Lane 8 shows 339 bp PCR product of gene coding for entire Ub-mODC fusion protein using forward primer of ubiquitin and reverse primer of C37mODC corresponding with 100 bp marker in lane 6. Further confirmation of construct pUbmODC was done by sequencing.

5.3.2 Functional analysis of fusion protein

After confirmation, construct pUbmODC was transformed into BY4741 cells of yeast *S. cerevisiae*. Yeast cell sample expressing only ubiquitin was used as a control for functional evaluation of UbmODC fusion protein (Figure 5.8).

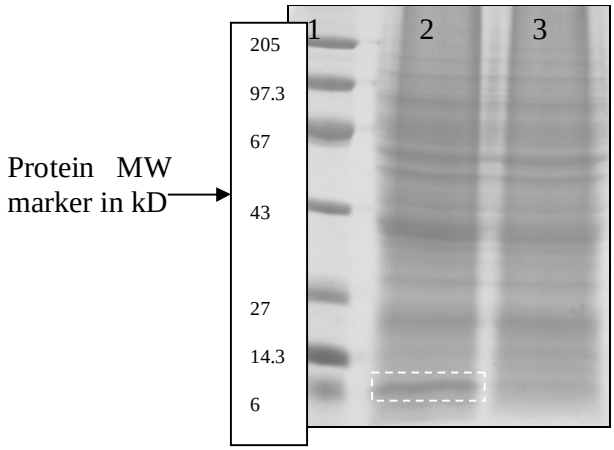


Figure 5.8 Protein profile of BY4741 cells of yeast *S. cerevisiae* transformed with plasmid pUb221 carrying yeast ubiquitin gene under CUP1 promoter

Lane 2 and 3 show induced and uninduced yeast cells respectively. Lane 2 shows 10 kD band of ubiquitin after 6 hours of induction corresponding with the protein molecular weight marker in lane 1.

After successfully transformation of pUbmODC in BY4741 cells of yeast *S. cerevisiae,* cells induced with 50µM $CuSO_4$ for 6 hours (Figure 5.9). Various method used to check the expression of mODC along with ubiquitin.

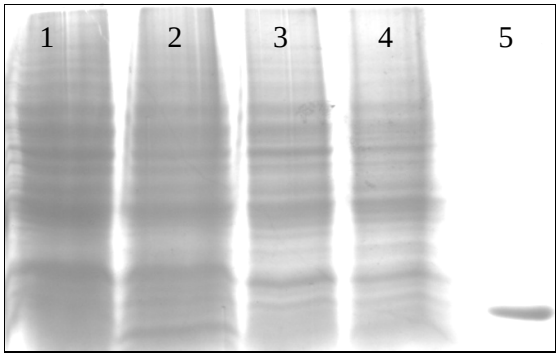


Figure 5.9 Protein profile of BY4741 cells of yeast *S. cerevisiae* transformed with plasmid pUb221 and pUbmODC and induced with 50µM $CuSO_4$ for 6 hours

Lane 1 and 2 show uninduced and induced samples of yeast cells expressing only ubiquitin respectively. Lane 2 shows induced 10 kD band of ubiquitin corresponding with 14 kD lysozyme in lane 5. Lane 3 and 4 show uninduced and induced samples of yeast cells expressing UbmODC fusion protein respectively. Lane 4 does not show any induced 15 kD band of Ubiquitin-mODC fusion protein, lagging behind the band of 10 kD ubiquitin or corresponding to the 14 kD lysozyme in lane 5.

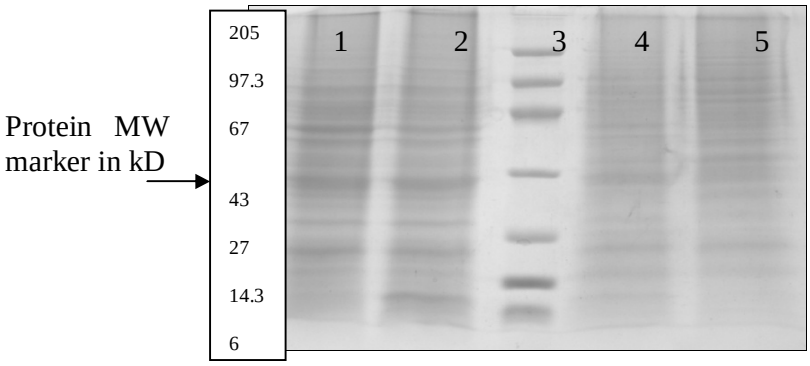


Figure 5.10 Protein profile of BY4741 cells of yeast *S. cerevisiae* expressing only Ubiquitin and UbmODC fusion protein after induction with 100µM $CuSO_4$ for 6 hours

Lane 1 and 2 show uninduced and induced samples of yeast cells expressing only ubiquitin respectively. Lane 2 shows induced 10 kD band of ubiquitin corresponding with protein molecular weight marker in lane 3. Lane 4 and 5 show uninduced and induced samples of yeast cells expressing UbmODC fusion protein respectively. Lane 5 does not show any 15 kD band of Ubiquitin-mODC fusion protein after 100µM $CuSO_4$ induction.

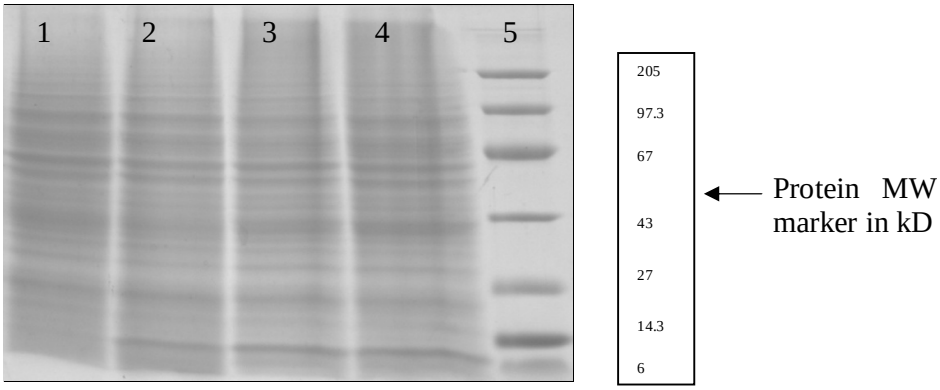


Figure 5.11 Time bound induction pattern of BY4741 cells transformed with plasmid pUb221

Lane 1 shows uninduced cells while lane 2, 3 and 4 show 4th hour, 8th hour and overnight grown i.e. 20th hour cells respectively after 50 µM $CuSO_4$ induction. After induction a gradual increase in the intensity of 10 kD band of ubiquitin is seen matching with protein molecular weight marker in lane 5.

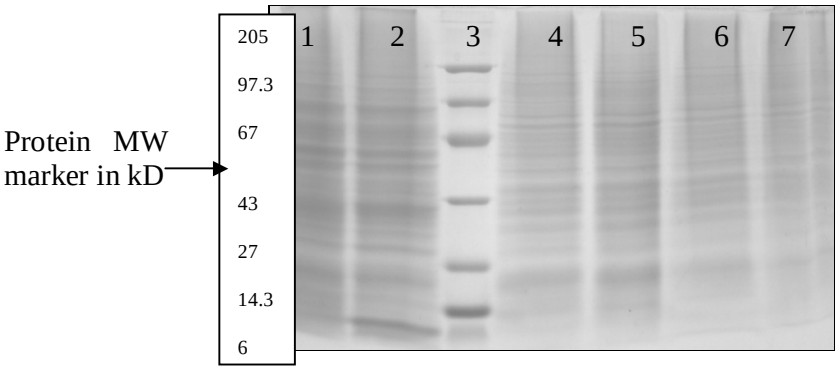


Figure 5.12 Time bound induction pattern of BY4741 cells transformed with plasmid pUbmODC after 50 µM $CuSO_4$ induction

Lane 1 and 2 show uninduced and induced yeast cells transformed with plasmid pUb221. Lane 2 shows induced 10 kD band of ubiquitin corresponding with MW Marker in lane 3. Lane 4 shows uninduced cells while lane 5, 6 and 7 show 4^{th} hour, 8^{th} hour and overnight grown i.e. 20^{th} hour cells respectively after 50 µM $CuSO_4$ induction. Any 15 kD induced band of fusion protein is not seen in any well after induction probably indicating targeted degradation of the same fusion protein by the cells.

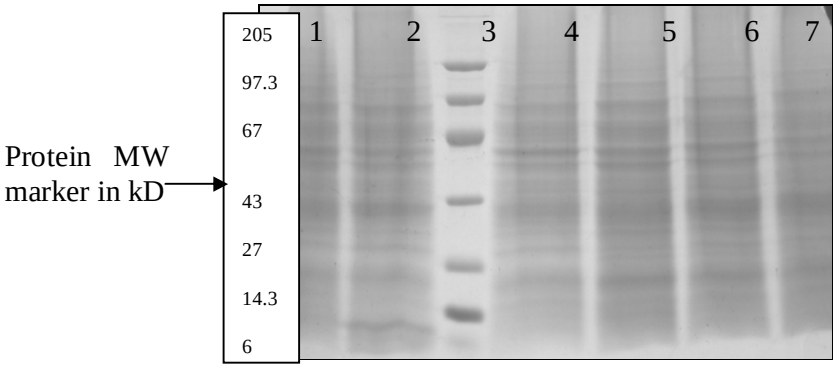


Figure 5.13 Time bound induction pattern of BY4741 cells transformed with plasmid pUbmODC after 100 µM $CuSO_4$ induction

Lane 1 and 2 show uninduced and induced yeast cells transformed with plasmid pUb221. Lane 2 shows induced 10 kD band of ubiquitin corresponding with MW Marker in lane 3. Lane 4 shows uninduced yeast cells transformed with plasmid pUbmODC while lane 5, 6 and 7 show 4^{th} hour, 8^{th} hour and overnight grown i.e. 20^{th} hour cells respectively after after 100 µM $CuSO_4$ induction. Any 15 kD induced band of fusion protein is not seen in any well after induction probably indicating targeted degradation of the same fusion protein by the cells.

After various experiment to check the expression, It was unclear that whether pUbmODC express or not. There were two possibilities, (i) protein is not expressed (ii) protein is expressed but degrade rapidly. To overcome above problem, we have inhibited degradation of protein by proteasome with proteasomal inhibitor MG132. Further we have done western blot to confirm the expression and proteasomal degradation of fusion protein.

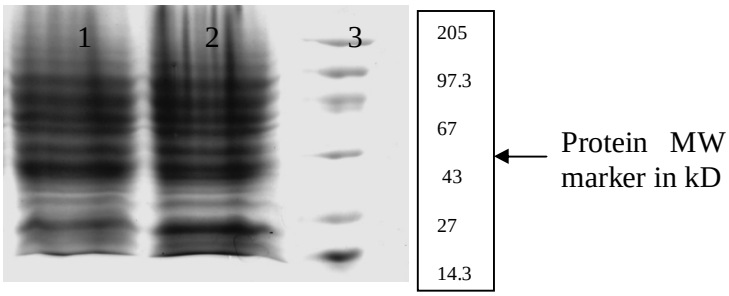


Figure 5.14 protein profile of yeast cells expressing UbmODC fusion protein treated with MG132 for 3 hours

Lane 1 and 2 show MG132 untreated and treated cells. Lane 2 shows 15 kD band of UbmODC fusion protein after proteasomal inhibition, corresponding with protein MW marker in lane 3.

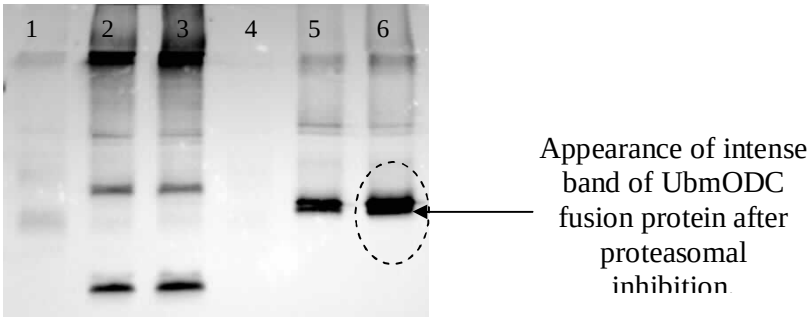


Figure 5.15 Western blot analysis of yeast cells expressing ubiquitin and UbmODC fusion protein

Lane 2, 3 and 4 show uninduced, induced without MG132 and induced with MG132 yeast cells respectively transformed with plasmid pUb221 which give expression of only ubiquitin. Lane 5, 6 and 7 show uninduced, induced without MG132 and induced with MG132 yeast cells respectively transformed with plasmid pUbmODC which give expression of UbmODC fusion protein. Western blot analysis has first time shown the band of expressed UbmODC fusion protein corresponding with protein molecular weight marker in lane 1. The intensity of band of fusion protein was increased as shown in lane 7 after proteasomal inhibition. This has confirmed that cells are expressing fusion protein and degradation of fusion protein is occurring through proteasomal pathway.

5.3.2.1 Yeast expression studies

After inducing yeast cells with 50 µM $CuSO_4$ for 6 hours, 10 kD band of ubiquitin was seen but 15 kD band of fusion protein was not seen (Figure 5.9). So yeast cells were induced with 100 µM $CuSO_4$ for 6 hours and protein profile was checked. After induction with 100 µM $CuSO_4$ also 15 kD band of fusion protein was not seen (Figure 5.10). In time bound induction pattern also an expected band of fusion protein was not seen (Figure 5.12, 5.13). As expected 15 kD band of proposed fusion protein was not seen consistently in induced cells, it was expected to be degraded by proteasome.

If the degradation of fusion protein is mediated through proteasome then fusion protein should remain stable after proteasomal inhibition. So this 15 kD band of fusion protein was expected on SDS PAGE after proteasomal inhibition. To confirm the proteasomal degradation of proposed UbmODC fusion protein, yeast cells were treated with MG132 i.e. proteasomal inhibitor and protein profile was checked. After proteasomal inhibition, expected 15kD band of fusion protein was seen (Figure 5.14). Western blot analysis also revealed the presence of expressed UbmODC fusion protein and more intense band of fusion protein after proteasomal inhibition (Figure 5.15). This confirmed that the degradation of fusion protein is occurring through proteasome only.

5.4 Discussion

Our results, for the first time, have successfully shown the degradation of ubiquitin when tagged with unstructured 37mODC peptide derived from mouse Ornithine Decarboxylase (ODC). In the present study expression of proposed UbmODC fusion protein and ubiquitin was checked in BY4741 cells of yeast *S. cerevisiae*. Cells induced for expression of fusion protein and ubiquitin were loaded on 15 % SDS PAGE and protein profile was checked. 10 kD band of ubiquitin as a control was seen after 6 hours of induction but band of fusion protein having size of 15 kD was not seen. It was assumed that absence of band may indicate the degradation of proposed fusion protein. Before concluding it was necessary to prove that degradation is occurring through proteasome, proteasomal inhibitor was added. Protein profile of yeast cells treated with MG132 has shown an expected 15 kD band of proposed

UbmODC fusion protein. This has confirmed that C37mODC is capable of targeting ubiquitin for degradation through proteasome.

In western blot analysis blots were developed after using antibody against Myc tagged ubiquitin and Myc tagged UbmODC fusion protein. Results of western blot analysis confirmed the expression of UbmODC fusion protein after proteasomal inhibition. Absence of band of fusion protein before proteasomal inhibition and appearance of the same band of fusion protein after proteasomal inhibition was confirmed by SDS-PAGE and western blot analysis respectively. Western blot analysis has also shown some amount of expressed fusion protein present inside the cell before proteasomal inhibition and increase in band intensity after proteasomal inhibition while no band was developed in uninduced samples. These results finally confirmed the degradation of UbmODC fusion protein through proteasomal pathway. In another words C37mODC peptide worked as a degradation tag to carry out degradation of natural tag for protein degradation i.e. ubiquitin.

UBB+1, a variant of Ubiquitin with a C terminal extension of 19 amino acids is known to accumulate in Alzheimers disease. But our results establish that ubiquitin extended with 37mODC is an efficient proteasomal substrate. This result is in agreement with the requirement of an unstructured initiation region for proteasomal degradation of ubiquitin. Requirement of an unstructured region is fulfilled by C 37mODC. Ubiquitin itself is not a substrate of proteasome while working as a tag for targeting substrate protein towards proteasome for degradation. Many ubiquitin fusion proteins are functional inside the cell. So our findings show another way of targeted ubiquitin degradation.

5.5 Conclusion

Accumulation of ubiquitin and ubiquitin conjugates are seen in various diseases such as neurodegenerative diseases. Various methods for carrying out targeted protein degradation had been explained in literature. C37mODC can serve as an efficient degradation vehicle to carry out targeted protein or ubiquitin degradation. Introducing adaptor protein for recognition of target proteins within cellular milieu could be an additional feature required for efficient functioning of C37mODC as a degron.

4 Construction of pRPα/β, expression and purification of α/β peptide and its structural analysis

4.1 Introduction

Structure of mammalian and yeast ODC is described in introduction. They share 40% sequence homology (Fonzi et al., 1987). Yeast ODC is composed of two domains. N terminal α/β barrel domain and C terminal β sheet domain. N terminal α/β barrel domain and C terminal β sheet structure is a characteristic of amino acid Decarboxylase enzymes (Xiaoyi Deng et al., 2010). Comparative study of mouse and yeast ODC by Fonzi et al explains conserved secondary structure in both proteins. Yeast ODC contains 466 amino acid residues while mouse ODC contains 461 amino acid residues. When 410 residues region of both ODCs was overlapped yeast ODC was predicted to contain 159 residues in α helical conformation and 63 residues in β sheet conformation. This finding also suggests the length of α helical and β sheet regions are similar in yeast and mouse ODC (Fonzi et al., 1987). As discussed in previous chapters, N terminus of yeast ODC is unstructured in nature. Significant role of N terminal unstructured stretch in the degradation of yeast ODC is proved. After N terminal extended region α/β barrel domain is present in which antizyme binding element (AzBE) is situated. This α/β barrel domain should be in a natural conformation so as to get recognized by antizyme. In order to study the structural features it was proposed to express α/β barrel domain independently from the rest of ODC protein. It would be interesting to know whether α/β barrel domain will gain the same conformation as in native protein or trimming the protein will affect the domain conformation. The selected α/β barrel domain is present in between N terminal unstructured region and C terminal β sheet domain. Other three proposed peptides in the present study are present either at N terminus (Nα/β peptide and N50 peptide) or at C terminus (C 37mODC) of ODC protein. Present chapter tries to reveal the structure of α/β barrel domain when its natural N and C terminal accompanying residues are pruned. Though domain is an independently folding and functioning unit of protein, it would be interesting to focus on α/β barrel domain of yeast ODC because it harbors important degradation signal i.e. AzBE (antizyme binding element). Degradation of ODC is due to resultant contribution of destabilization by antizyme and initiation of degradation by unstructured region in both yeast and

mammalian cells. It is already proved that deletion of N terminal unstructured region renders stability to yeast ODC. This result could be well understood by studying structure of N terminal α/β barrel domain of yeast ODC after deleting extreme N terminal unstructured stretch. Any alteration in structure could provide a clue about contribution of this region to the stability of N terminally deleted yeast ODC. With this background present study is focused to reveal the structural aspects of α/β peptide which in other words can be described as α/β barrel domain of yeast ODC with extreme N terminally deleted residues.

In mammalian ODC, α/β barrel domain is present upto 283 amino acid residues. Excluding first 50 amino acid residues of yeast ODC, range of α/β barrel domain of yeast ODC is extended upto 333rd amino acid residues. After bioinformatics study primers were designed to amplify the region starting from 39th to 346th amino acid residues so as to get a peptide of 308 amino acids length.

In the present chapter structural characterization of α/β peptide was done using Circular Dichroism (CD) and Fluorescence Spectroscopy.

Sequence of α/β barral domain (in green) of yeast ODC selected for this study.

MSSTQVGNALSSSTTTLVDLSNSTVTQKKQYYKDGETL**HNLLLELKNNQDL**
ELLPHEQAHPKIFQALKARIGRINNETCDPGEENSFFICDLGEVKRLFNN
WVKELPRIKPFYAVKCNPDTKVLSLLAELGVNFDCASKVEIDRVLSMNIS
PDRIVYANPCKVASFIRYAASKNVMKSTFDNVEELHKIKKFHPESQLLLRI
ATDDSTAQCRLSTKYGCEMENVDVLLKAIKELGLNLAGVSFHVGSGASD
FTSLYKAVRDARTVFDKAANEYGLPPLKILDVGGGFQFESFKESTAVLRL
ALEEFFPVGCGVDIIAEPGRYFVATAFTLASHVIAKRKLSENEAMIYTNDG
VYGNMNCILFDHQEPHPRTLYHNLEFHYDDFESTTAVLDSINKTRSEYPYKVS
IWGPTCDGLDCIAKEYYMKHDVIVGDWFYFPALGAYTSSAATQFNGFEQTA
DIVYIDSELD 466

Length of selected α/β peptide is 308 amino acid residues.

4.2 Materials and Methods

4.2.1 Strains, media and plasmids

4.2.1.1 Bacterial strains and media

E.coli DH5α (F', 80dlacZ M15, *endA*1, *recA*1, *hsdR*17 (r_k^-,m_k^+), *supE44*, *thi-1*, *gyrA96*, *relA1*, *(lacZYA-argF)U169*) strain was used for transforming recombinant DNA. Cultures were grown at 37°C at 200 rpm in Luria broth (Hi-media). 50µg/ml of kanamycine concentration was used for selection of plasmid. Bacterial transformation was done by $CaCl_2$ method. Plasmid isolation was done by standard alkaline lysis protocol (Sambrook 2001).

4.2.1.2 Plasmid

Vector map of plasmid pET30a used for the present study is shown in chapter 2.

The DNA sequence of the region within pET30a plasmid where the fragment of gene of α/β peptide was cloned in *Bam H*I and *Sac*I site-

CATCAT….GATATCGGATCC… fragment of gene of α/β peptide GAGCTC CGT.

His tag *BamH*I *Sac*I

4.2.1.3 Primers used for PCR

Sr No.	Name	Sequence
1	S α/β Forward	AATGGATCCCACAATCTTTTGCTTGAACTAAAG
2	S α/β Reverse	ATAGAGCTCTCACATTGCTTCATTCTCAGACA

Table 4.1 Shows sequence of primers used for the amplification of gene fragment coding for α/β peptide.

4.2.1.4 Plan of work of construction of α/β peptide sequence in pET30a bacterial expression vector

Yeast ODC contains 466 amino acid residues. The selected α/β peptide is 308 amino acids long. Primers were designed to amplify the region starting from 39th to 346th amino acid residues (Table 4.1). The plasmid pRS314 which carries entire yeast ODC gene was used as a template for PCR. (This plasmid is a kind gift from P. Coffino, University of California.)

4.2.1.4.1 Cloning strategy

Gene fragment coding for α/β barrel domain of yeast ODC was successfully amplified from pRS314 containing Yeast ODC. The 924 bp amplicon, digested by *Bam*HI and *Sac*I was ligated into pET30a vector, which was digested with same enzymes. This ligated plasmid was transformed into DH5α strain of *E. coli*. Pictorial representation of cloning strategy is as shown below in figure 4.1

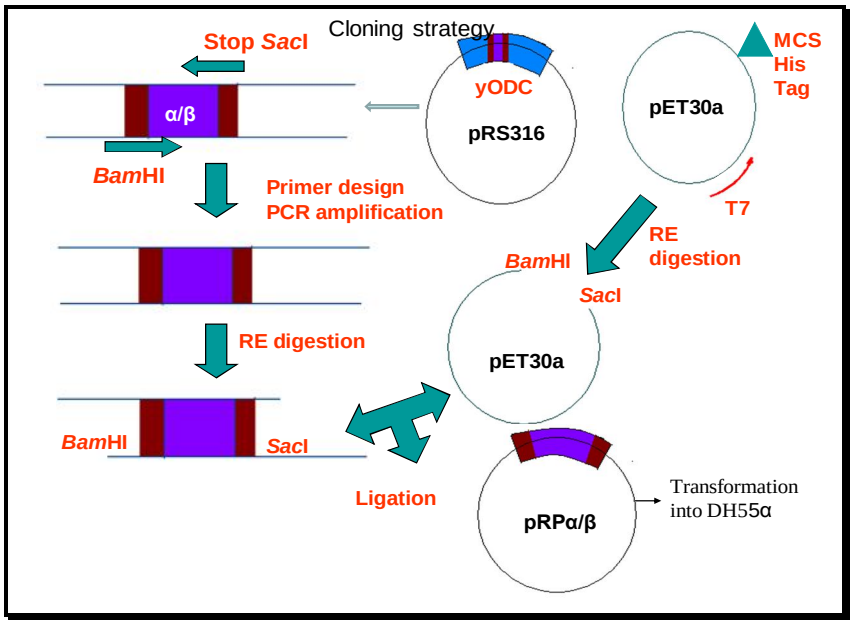


Figure 4.1 Cloning strategy for the construction of pRPα/β

4.2.1.4.2 Screening strategy

The transformants would have lost their *Eco*RI site, if the insert got ligated into the vector and hence would be resistant to *Eco*RI digestion. Further confirmation was done by checking the plasmid size, digestion with different restriction enzymes and insert release after double digestion and comparing the restriction digestion pattern with that of empty pET30a vector. Sequencing was done for final confirmation.

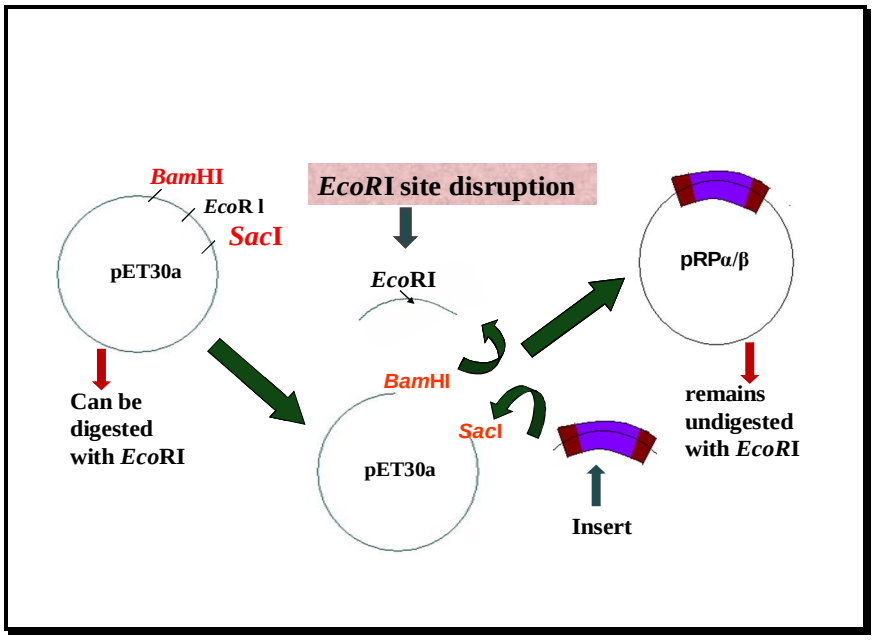


Figure 4.2 Pictorial representation of screening strategy for pRPα/β.

4.2.1.5 Expression of α/β peptide

The protein expression studies are being carried out in BL21 (DE3) strain of *E. coli.* These cells are designed for high level of protein expression and relatively easy purification.

The gene of α/β peptide was cloned under T7 promoter in pET30 vector which is IPTG inducible. The chimeric plasmid pRPα/β was transformed into *E coli* BL21(DE3) cells to study protein profile. After 1 mM IPTG induction the cells were loaded on SDS PAGE to check the expected 40kD band of α/β barrel domain of yeast

ODC. Time bound induction study was done to find out the time at which the maximum induction of desired peptide is achieved.

4.2.1.6 Purification of α/β peptide

Once induction of desired peptide is achieved, transformed *E coli* BL21(DE3) cells were inoculated for purification of target protein. Protocols for cell growth, induction, harvesting and lysis to collect peptide of interest were followed as discussed in chapter 2. Cell lysis protocol was standardized using lysis buffer without EDTA so as to use the Nickel linked resin for affinity chromatography. After cell lysis the sample was centrifuged at 5000rpm for 30 min at 4^0C. Supernatant was collected in fresh tube and pellet was resuspended in saline. Fractions of Supernatant and pellet were loaded on SDS-PAGE to check the presence of target protein. An expected band of protein was seen in pellet. To check the solubility of target protein different experiments had been carried out such as growing culture below 30^0C, minimizing the IPTG concentration, changes in time, temperature, pH, additives such as DTT. As expected band was consistently seen in pellet after centrifugation after cell lysis, urea was used to make it soluble. After collecting cell lysate the pellet was resuspended in 8M urea and incubated at 4^0C for overnight. This mixture was then centrifuged at 10000 rpm for 10 minutes at 4^0C. Fractions of both supernatant and pellet were loaded on SDS-PAGE. The expected band of α/β peptide was seen in supernatant indicating solubilization of peptide of interest. When same protocol was followed with decreasing concentration of urea as 6M, 4M and 2M, peptides remained soluble.

As solubilization of α/β peptide was achieved, transformed *E. coli* BL21(DE3) cells were inoculated for large scale expression and purification of target protein. 500 ml of Luria broth containing 50µg/ml of kanamycin was inoculated with 5 ml of overnight grown culture. Cells were induced with 0.5 mM IPTG at mid log phase. After 2 hours of induction, cells were harvested by centrifugation at 5000rpm for 10 minutes at 4^0C. Cell lysis was carried out with above mentioned protocol and peptide of interest was collected using 4M urea. This mixture is used for next step i.e. protein purification using affinity chromatography.

4.2.1.7 Affinity chromatography

Nickel charged chromatography resin was purchased from Biorad. 2ml of 50% slurry was transferred to a small polypropylene tube. The resin was washed with 5 column volumes of sterile distilled water, 3 column volumes of binding buffer and finally equilibrated with 3 column volumes of binding buffer (pH 8). For batch mode purification, the equilibrated resin was incubated with cell lysate at 4^0C for 30 minutes. It is then washed with 5 column volumes of binding buffer and washes were collected. Washing was continued until the absorbance at 280 nm of the flow through is 0.000. Bound protein was eluted using elution buffer containing 300 mM imidazole and absorbance was measured at 280nm. Using predicted molecular weight and extinction coefficient, the concentration of eluted peptide was determined. The peptide was dialyzed to remove the imidazole and the sample was concentrated. Peptide was purified for spectroscopic studies.

4.2.1.8 Structural characterization

4.2.1.8.1 Circular Dichroism

CD spectra were recorded on Jasco 815 spectropolarimeter. Far UV CD spectra were recorded from 180 nm to 250 nm with 1mm path length while near UV CD spectra were recorded between 250 to 320 nm with 1cm path length. Protein samples were prepared in phosphate buffer having 5 mM sodium phosphate and 5 mM NaCl (pH 8). Far UV CD spectra were recorded at pH 8 as well as pH 2.2. Near UV CD spectra of α/β peptide were recorded phosphate buffer (pH 8) with 0M guanidinium hydrochloride and 4M guanidinium hydrochloride. Spectra of all samples were blank corrected. Five spectra were accumulated to reduce the noise. Spectra were recorded at 0.2nm resolution and scan speed was 50nm/sec.

4.2.1.8.2 Fluorescence spectroscopic studies

Flourescence spectra were recorded using Hitachi F-4010 fluorescence spectrophotometer. Intrinsic fluorescence was studied using tryptophan residue in peptide while extrinsic fluorescence was studied by using extrinsic fluorophores such as ANS.

Fluorescence spectra were recorded in denaturants such as urea and guanidium hydrochloride (GdnCl). Guanidium hydrochloride denaturation curve was studied to check the stability of peptides.

Protein samples were prepared in phosphate buffer having 50 mM sodium phosphate and 30 mM NaCl (pH 8). To record the intrinsic fluorescence spectra, samples were excited at 295nm and emission was recorded from 300nm to 400nm. Extrinsic fluorescence was studied by using extrinsic fluorophore such as 1-anilinonaphthalene 8-sulfonate (ANS). ANS was used at a concentration of 50μM. ANS was excited at 340nm and emission was recorded between 400nm to 550nm. The same sample was also excited at 295nm and emission was recorded from 300nm to 550nm (figure 4.17).

4.2.1.8.3 Bioinformatics predictions

Some bioinformatics study of the selected peptides was carried out. Structure of all proposed peptides was predicted using prediction softwares such as APSSP, JEP-RED, SOPMA etc. This has given the primary information of peptides. Theoretical pI was calculated and hydrophobicity was checked for each peptide before designing the purification protocol.

Softwares used: SOPMA, JEPRED and APSSP.

A. **Predicted structure of α/β peptide using Swiss pdb viewer:**

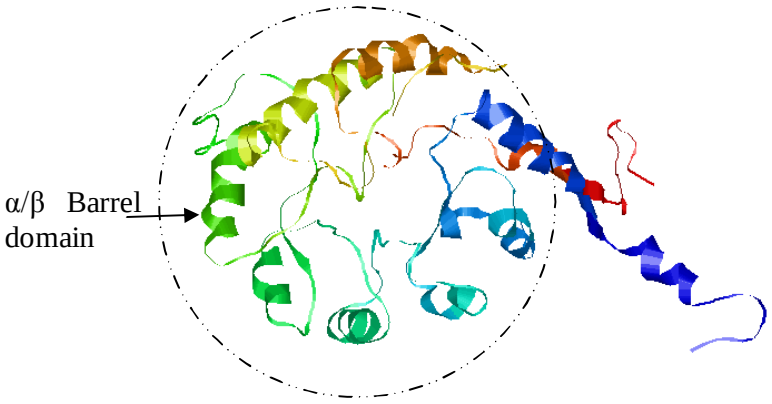


Figure 4.3 Predicted structure of α/β peptide using swiss-pdb software

B. **Structure prediction using SOPMA:**

```
10         20         30         40         50         60         70
80
         |          |          |          |          |          |
|
MHHHHHHSSGLVPRGSGMKETAAAKFERQHMDSPDLGTDDDDKAMADIGSHNLLLELKNNQDLELLPHE
Q
hhtccctttteeccccccchhhhhhhhhhttcccttcccccchhhhhhtccccchhhhhccccchhhhhhh
h
AHPKIFQALKARIGRINNETCDPGEENSFFICDLGEVKRLFNNWVKELPRIKPFYAVKCNPDTKVLSLL
A
hhhhhhhhhhhhhhhhhhhcccccccchheeehhhhhhhhhhhhhhhhhcccccceeeecccccchhhhhhh
h
ELGVNFDCASKVEIDRVLSMNISPDRIVYANPCKVASFIRYAASKNVMKSTFDNVEELHKIKKFHPESQ
L
httcccccccchhhhhhhhhttcctteeeeecccccchhhhhhhhttceeeeecchhhhhhhhhccttch
e
LLRIATDDSTAQCRLSTKYGCEMENVDVLLKAIKELGLNLAGVSFHVGSGASDFTSLYKAVRDARTVFD
K
eeeeecccccchheehccccccchhhhhhhhhhhhhhttceeeeeeeeecccccchhhhhhhhhhhhhhhh
h
AANEYGLPPLKILDVGGGFQFESFKESTAVLRLALEEFFPVGCGVDIIAEPGRYFVATAFTLASHVIAK
R
hhhtttccceeeeeetccccchhhhhhhhhhhhhhhhhcccccceeeeecttceeehhhhhhhhhhhhh
t
KLSENEAM
ccccccchh
```

Alpha helix: h, Extended strand: e, Beta turn: t, Random coil: c.

Theoretical pI: 6.17

Theoretical MW: 39822.54 D

4.3 Results

4.3.1 Construction of α/β peptide sequence of yeast ODC in pET Expression vector

In order to clone gene fragment coding for α/β peptide of yeast ODC into pET30a bacterial expression vector, PCR amplification of the gene fragment was carried out with the help of forward and reverse primers containing *Bam*HI and *Sac*I restriction sites at their 5' end respectively (Table 4.1). pRS314 plasmid was used as a template for the amplification of gene fragment coding for α/β peptide and further checked on agarose gel electrophoresis (Figure 4.4).

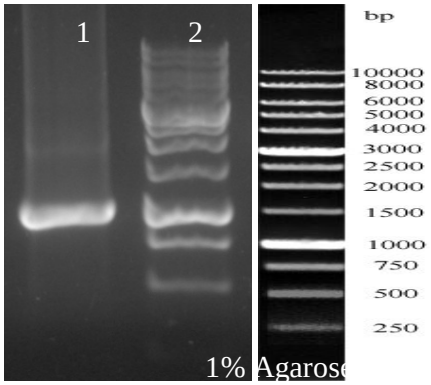


Figure 4.4 PCR product of gene coding for α/β peptide on 1% agarose.

Lane 1 Shows 942 bp amplified PCR product of α/β peptide of yeast ODC, Lane 2 shows 1 kb DNA ladder.

To clone the confirmed PCR product of α/β peptide into pET30a expression vector, both amplified PCR product and vector were double digested with *Bam*HI and *Sac*I restriction enzymes. In pET30a vector, *Eco*RI restriction site lies in between *Bam*HI and *Sac*I restriction site hence there is a loss of *Eco*RI site when the vector is double digested with above mentioned cloning enzymes. Hence loss of *Eco*RI restriction enzyme site has been used for screening purpose as recombinant plasmid remains undigested by *Eco*RI restriction enzyme.

Transformants containing positive clones for α/β peptide were screened by *Eco*RI restriction enzyme (Figure 4.5).

Clones which remained undigested with *Eco*RI were further selected for confirmation by digestion with various restriction enzymes and compared with restriction digestion pattern of empty pET30a vector as shown in figure 4.6.

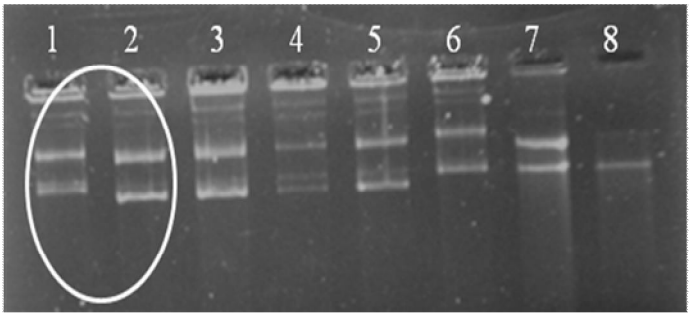


Figure 4.5 Screening of positive transformant of pRPα/β.

Lane 1, 3, 5 and 7 show undigested plasmids while lane 2, 4, 6 and 8 show *Eco*RI digests of the same plasmids. In lane 2, 4 and 6 plasmids remain undigested with *Eco*RI restriction digestion.

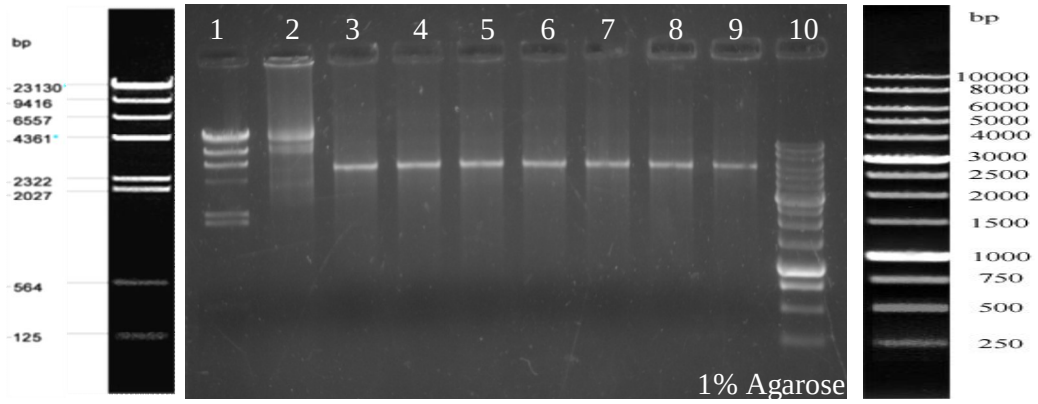


Figure 4.6 Restriction digestion pattern of empty pET30a vector

Lane 1 and 10 show λ *Hind* III marker and 1kb DNA ladder respectively. Lane 2 shows undigested 5.5kb pET30a plasmid while lane 3 to 9 show *Bam*HI, *Eco*RI, *Sac*I, *Sal*I, *Hind*III, *Not*I and *Xho*I digest of plasmid pET30a respectively.

pRPα/β clone was further confirmed by digesting it with restriction enzymes and compared with the digestion pattern of empty pET30a vector. pRPα/β remained

undigested by *Eco*RI restriction enzyme (Figure 4.7a). *Bam*HI and *Sac*I restriction enzyme have been used to release the cloned insert (Figure 4.7b).

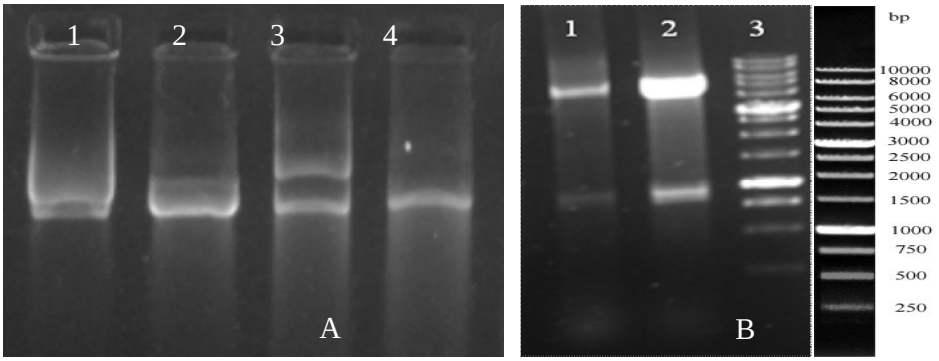


Figure 4.7: Restriction digestion pattern of pRPα/β plasmid.

In **Figure A**, Lane 1 shows undigested pRPα/β plasmid. Lane 2, 3 and 4 show *Bam*HI, *Eco*RI and *Sac*I digests of pRPα/β plasmid. Cloning for the construction of pRPα/β plasmid was done in *Bam*HI and *Sac*I restriction sites while *Eco*RI site is disrupted while cloning. Lane 2 and 4 show linearised pRPα/β plasmid while in lane 3, plasmid remained undigested after *Eco*RI digestion. **Figure B** shows further confirmation of two positive transformants of pRPα/β by checking the insert release after double digestion. Lane 1 and 2 show 946 bp insert release after double digestion of plasmid with *Bam*HI and *Sac*I enzymes corresponding with 1kb marker in lane 3.

4.3.1.1 Expression and Purification of α/β peptide

A pRPα/β plasmid construct containing the gene fragment coding for α/β peptide of yeast ODC was expressed under the control of T7 promoter in *E. coli* strain BL21 (DE3) by inducing it with 1mM IPTG at 37°C under selection pressure of 50μg/ml of kanamycin (Figure 4.8).

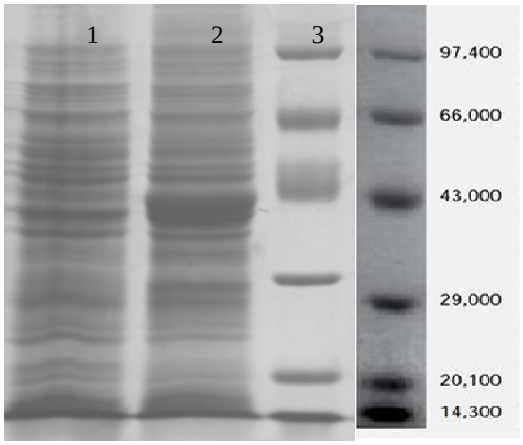


Figure 4.8 Protein profile of *E coli* BL21(DE3) cells transformed with plasmid pRPα/β

Lane 1 and 2 show uninduced and induced *E.coli* BL21(DE3) cells respectively. Lane 2 shows induced 40 kD band of α/β peptide corresponding with protein molecular weight marker in lane 3. Expression of α/β peptide was observed after inducing the cells with 1mM IPTG for 2 hours.

Expressed pRPα/β protein was further processed for the purification, it was found that the protein was not soluble in the buffer hence it remained in the pellet. To overcome the difficulty in solubilisation of protein, urea has been used to solubilise and to get the purified protein (Figure 4.9). Insolubility of the protein indicates the hydrophobic nature of the protein hence different concentration of the urea has been used to optimize the solubility of protein (Figure 4.10).

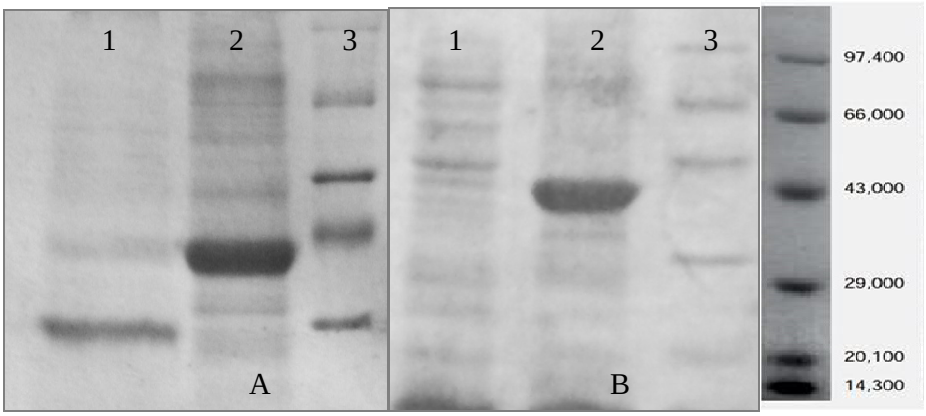


Figure 4.9 Protein profile of cell lysate having α/β peptide

Figure A, lane 1 and 2 show supernatant and pellet after cell lysis respectively. Expected 40 kD band of α/β peptide was seen in pellet corresponding with marker in

lane 3. Pellet portion was then treated with 6M urea. In **Figure B**, lane 1 and 2 show pellet and supernatant after 6M urea treatment respectively. A band of α/β peptide in supernatant indicates solubilization of α/β peptide in 6M urea.

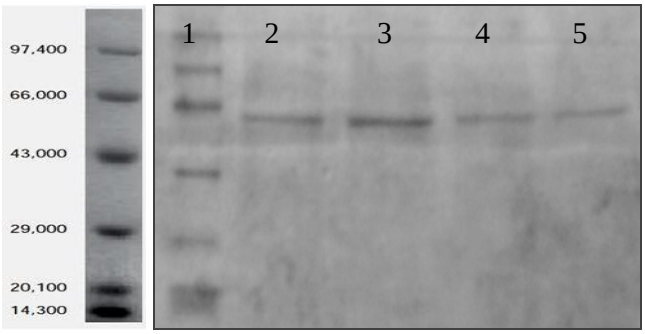


Figure 4.10 Protein profile of samples resulted from attempts to achieve solubilization of α/β peptide

Lane 2, 3, 4 and 5 show band of solubilized α/β peptide in supernatant after 6M, 4M, 2M and 1M urea treatment respectively. This indicates minimum concentration i.e. 1M of urea is sufficient to make α/β peptide soluble.

After solubilizing the protein in 1M urea, lysate was incubated with pre-treated Nickel NTA resin, unbound proteins were washed with high stringent buffer containing 20mM of imidazole. After several wash, purified protein was eluted from the resin with 300mM of imidazole and dialysed against phosphate buffer, pH-8.00 (Figure-4.11).

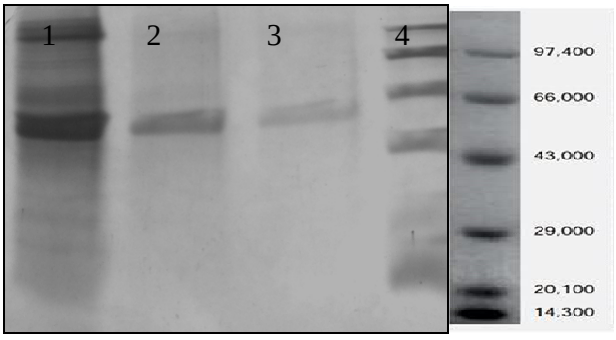


Figure 4.11 Protein profile of lysate and eluent with α/β peptide

Lane 1 shows cell lysate carrying α/β peptide used for purification using affinity chromatography. Lane 2 and 3 show 1st and 2nd eluents after elution with 300mM imidazole respectively. Both eluents show purified 40kD band of α/β peptide corresponding with protein molecular weight marker in lane 4.

4.3.1.2 Far and near UV CD spectra of α/β peptide

Far and near uv CD spectra of α/β peptide were recorded in phosphate buffer solution to study the changes in secondary and tertiary structure. Far UV CD spectra show the presence of characteristic pattern of β sheet structure with positive and negative peak (Figure- 4.12).

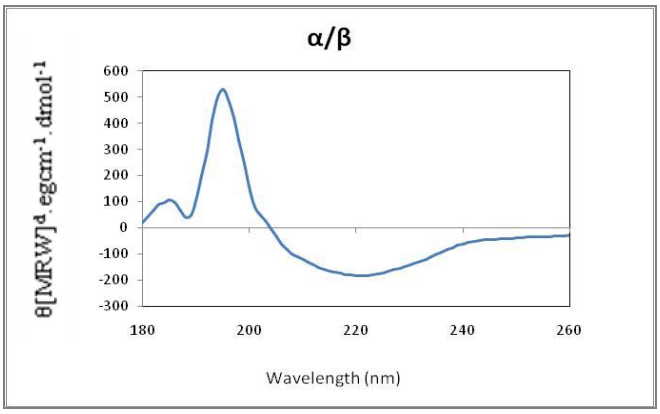


Figure 4.12 Far UV CD spectrum of α/β peptide.

To check the stability of α/β peptide in acidic environment we have recorded the spectra of α/β peptide in pH 2.2 buffer solution (Figure 4.13). Secondary structure was found to be almost same in both acidic and neutral buffer which indiates the stability of peptide in acidic condition.

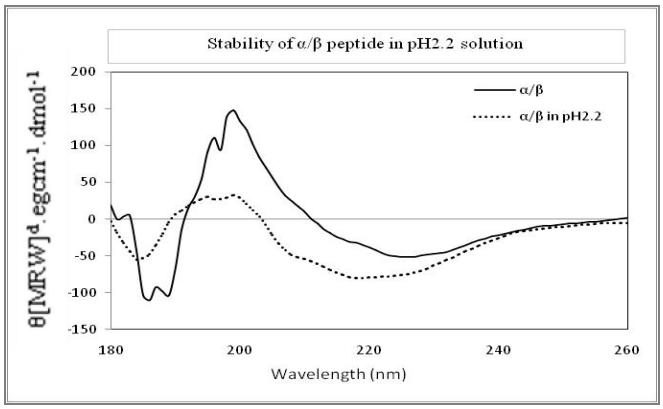


Figure 4.13 Far UV-CD spectrum of α/β peptide in pH 2.2

Near UV CD spectra of α/β peptide was recorded in phosphate buffer (pH 8) with 0M and 4M guanidinium hydrochloride. Near UV CD spectra show that the peptide has tertiary structure which is denatured in the presence of 4M guanidinium hydrochloride (Figure 4.14).

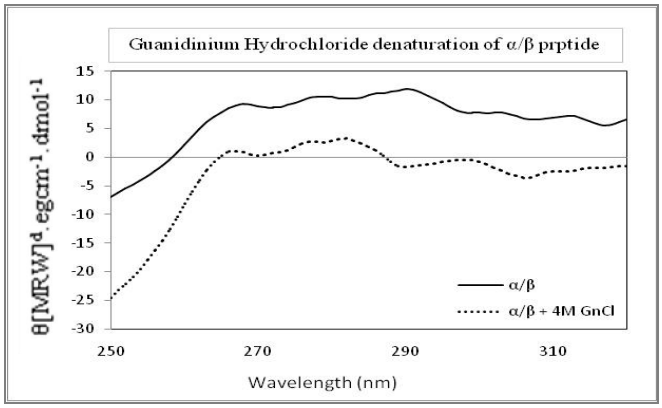


Figure 4.14 Near UV CD spectra of α/β peptide.

4.3.1.3 Fluorescence spectra of α/β peptide

Fluorescence spectra of the pRPα/β peptide were recorded by exciting the protein at 280nm. Tryptophan inside the protein can be used to probe the folding and stability of protein. Urea and guanidine hydrochloride are well known denaturant makes protein unstable. Degree of stability of the protein can be assessed by recording spectra in gradual increasing concentration of the denaturant. Denaturant exposes the buried hydrophobic regions and in turn change the intensity of protein. α/β peptide was subjected to the denaturation by using urea and guanidinium hydrochloride as a denaturant. Spectra reveal the gradual unfolding and red shift, which indicates the presence of tertiary structure as shown in figure 4.15 and 4.16.

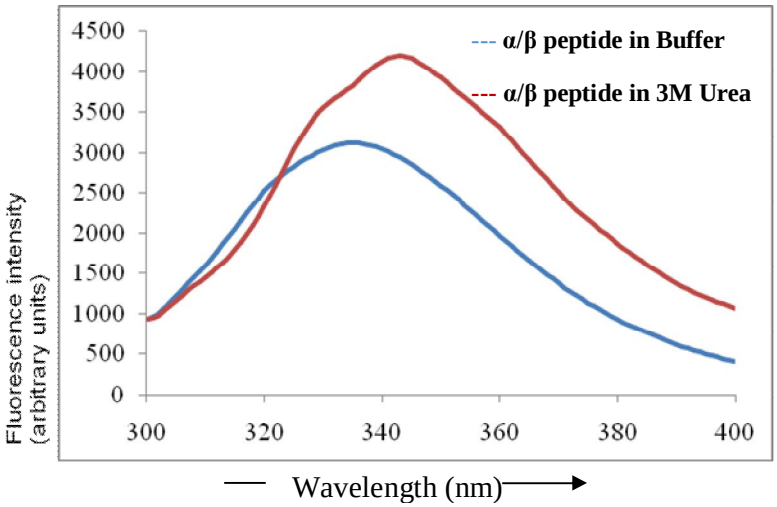


Figure 4.15 Fluorescence spectra of α/β peptide recorded in buffer and 5 M urea.

The spectrum recorded in urea (red line) shows red shift with respect to the one recorded in buffer (blue line).

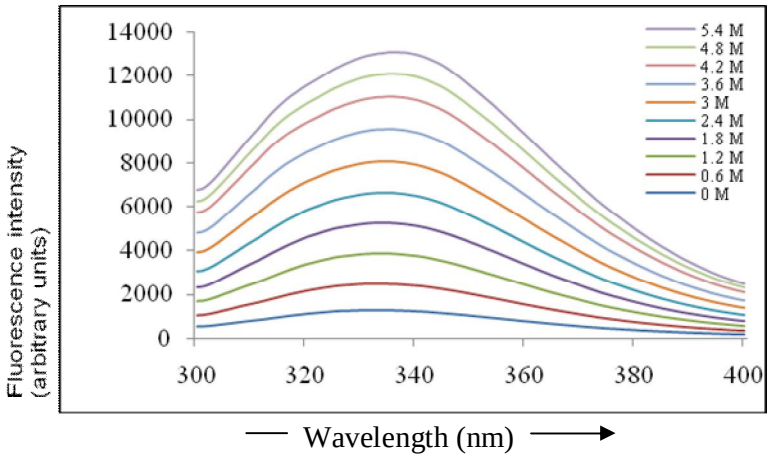


Figure 4.16 Fluorescence spectra of α/β peptide in presence of different concentrations of Guanidinium hydrochloride

Fluorescence resonance energy transfer (FRET) spectra of α/β peptide recorded in absence and presence of ANS. Decrease in emission peak in the presence of ANS between 300 to 400 nm while increase in peak between 400 to 550 nm clearly shows a successful transfer of fluorescence energy to ANS (Figure 4.17).

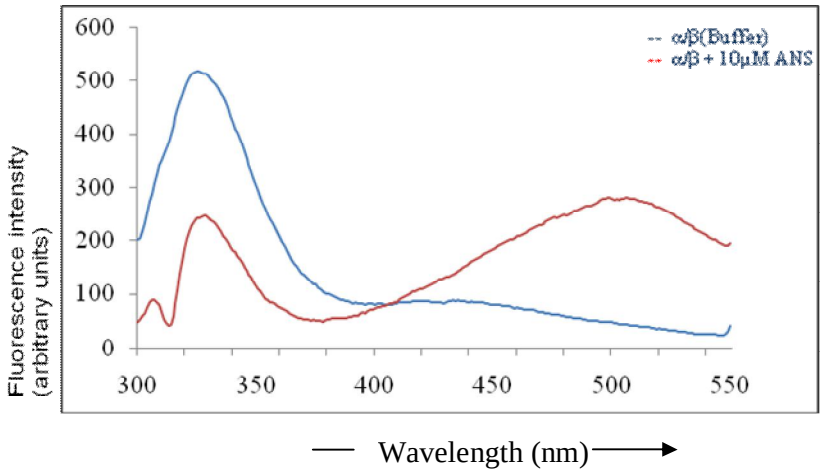


Figure 4.17 Fluorescence resonance energy transfer (FRET) spectra of α/β peptide

4.4 Discussion

Our results, for the first time, establish that α/β barrel domain of yeast ODC attains a 3D conformation in the absence of rest of the protein (figure 4.16). This domain can fold independently to gain the tertiary structure at physiological pH (figure 4.15). These results are in accordance with the results of protein structure prediction software (Figure 4.3).

As stated earlier, α/β barrel domain is situated in between N terminal unstructured region and C terminal β sheet domain. The selected α/β peptide is not accompanied by its natural neighbouring amino acid residues. Thus possibility of slight alteration in secondary structure cannot be ruled out.

CD structure revealed β sheet nature of α/β peptide when expressed independently (figure 4.12) and also stable in acidic environment (figure 4.13). CD structure of Nα/β peptide revealed α helical nature of Nα/β peptide. The difference between Nα/β peptide and α/β peptide is just a stretch of N terminal unstructured region which is not present in α/β peptide unlike Nα/β peptide. Absence of N terminal stretch changed the secondary structure of α/β region from α helix to β sheet.

The α/β barrel domain of yeast ODC harbours one degradation signal i.e. Antizyme Binding Element (AzBE). Deletion of N terminal unstructured region increases stability to yeast ODC (Gandre et al., 2002). Our results are in accordance with this finding and also explain rationale behind the stability of N terminally truncated yeast ODC. Though the domain is in tertiary conformation, it has gained β sheet conformation rather than α helix. Altered secondary structure could affect the conformation of antizyme binding element. If AzBE is not folded in its natural conformation antizyme possibly would not recognize it. Without interaction of antizyme with AzBE ODC would not be destabilized and consequently all next steps of presenting ODC towards proteasome for degradation would be hampered. So deletion of N terminal unstructured stretch would interfere with processing by antizyme by changing the structure of α/β barrel domain. N terminally truncated yeast ODC is stable because it lacks the initiation region which starts actual degradation by providing unstructured stretch which is essential to start with proteasomal entry and subsequently degradation. We propose another notion that instead of interference in proteasomal entry, deletion of N terminal stretch may alter the secondary structure of α/β barrel domain with altered structure may be unable to recognize antizyme and subsequently affecting destabilization and degradation of yeast ODC.

4.5 Conclusion

α/β barrel domain of yeast ODC attains 3D conformation in the absence of rest of protein. This domain can fold independently to gain the tertiary structure at physiological pH. α/β peptide is folding in β sheet conformation unlike Nα/β peptide. As α/β peptide is situated in between unstructured stretch and β sheet, there is change in structure of α/β peptide when expressed independently.

Deletion of N terminal stretch has altered the structure of α/β domain followed by stretch. AzBE is situated in α/β barrel domain. So change in the structure of α/β barrel domain could have changed the structure of AzBE also. Altered conformation of AzBE may not be recognized by antizyme thereby making ODC stable. Our findings are in support with the previous findings of stabilization of yeast ODC after deletion of N terminal stretch and also provide the reason behind the stabilization.

3 Structural and Functional characterization of N50 peptide

3.1 Introduction

After successful studies of structure of myoglobin and lysozyme using X-ray crystallography (Kendrew et al., 1958; Blake et al., 1965), structures of large number of proteins were elucidated. Different techniques such as NMR spectroscopy, crystallography, CD (Circular Dichroism) are available to study the 3D structure of any protein. Structure function relationship of protein is a major research area in biophysical, biomedical as well as pharmacological studies. Though the 3D structure of any protein is important, it is not an entire polypeptide chain undergoes 3D conformation but some region remains unfolded or unstructured (Gast et al., 1995; Uversky et al., 1999; Boublik et al., 1970). Earlier it was believed that polypeptide should at least gain some 3D structure to function. But it is not the case that entire polypeptide chain always gains some secondary or tertiary structure before being functional. Many a time some region of polypeptide gains some conformation while few regions are without any conformation. These unfolded regions are called as disordered regions or simply unstructured stretches. These unstructured regions are equally important as structured regions in any protein as far as function is concerned (Huber et al., 1983). Rather the conformational flexibility confers functional ease significantly rather than a rigid structure (Harauz et al., 2009). Most of the proteins have disordered region, in fact half of the eukaryotic proteins have long disordered regions especially signaling proteins. There are few examples of proteins which are fully disordered yet functionally important such as Myelin Basic Protein (MBP) (Harauz et al., 2004), prothymosine α (Gast et al., 1995) and microtubule formation promoting Tau protein (Schweers et al., 1994). Unstructured regions are needed in variety of functions such as sequence recognition, molecular ineraction (Pontius et al., 1993), protein degradation (Zhang et al., 2004). Details of disordered proteins or regions are discussed in introduction.

Structure of mammalian ODC has been resolved but crystal structure of yeast ODC is yet to be resolved. Crystal structure of mammalian ODC and some evidences of yeast ODC are discussed in introduction. Yeast ODC is composed of 2 domains namely N terminal α/β barrel domain and C terminal β sheet domain (Fonzi et al., 1987). First

50 amino acid residues of yeast ODC do not gain any conformation. So N terminal 50 amino acid stretch of yeast ODC is considered as unstructured region (Godderz et al., 2011; Palanimurugan, 2005). This unstructured region mimics with C terminal 37 amino acid residue stretch of mammalian ODC (Gandre et al., 2002). Mammalian ODC is stabilized after deletion of C terminal unstructured tail indicating degradation of mammalian ODC starts from C terminus. In the same way, when N terminal unstructured stretch is deleted, yeast ODC is stabilized indicating degradation of yeast ODC starts from N terminus (Godderz et al., 2011; Palanimurugan, 2005).

There are reports on portability of C terminal tail of mammalian ODC. Present study is focused on structural and functional aspects of N terminal unstructured stretch i.e. N50 peptide derived from yeast ODC. It would be interesting to check the conformation gained by N terminal unstructured stretch of yeast ODC when it is expressed independently from the rest of the protein. Under functional study, this unstructured stretch derived from yeast ODC was tagged to reporter protein and stability of reporter protein was checked. N50 peptide was tagged at N terminus of reporter protein as present in natural ODC protein.

Sequence of N50 peptide (in green) of yeast ODC selected for this study.

MSSTQVGNALSSSTTTLVDLSNSTVTQKKQYYKDGETLHNLLLELKNNQDLE
LLPHEQAHPKIFQALKARIGRINNETCDPGEENSFFICDLGEVKRLFNNWVKEL
PRIKPFYAVKCNPDTKVLSLLAELGVNFDCASKVEIDRVLSMNISPDRIVYANP
CKVASFIRYAASKNVMKSTFDNVEELHKIKKFHPESQLLLRIATDDSTAQCRL
STKYGCEMENVDVLLKAIKELGLNLAGVSFHVGSGASDFTSLYKAVRDART
VFDKAANEYGLPPLKILDVGGGFQFESFKESTAVLRLALEEFFPVGCGVDIIAE
PGRYFVATAFTLASHVIAKRKLSENEAMIYTNDGVYGNMNCILFDHQEPHPR
TLYHNLEFHYDDFESTTAVLDSINKTRSEYPYKVSIWGPTCDGLDCIAKEYYM
KHDVIVGDWFYFPALGAYTSSAATQFNGFEQTADIVYIDSELD 466

Length of selected N50 peptide is 51 amino acid residues.

3.2 Materials and methods

3.2.1 Structural study

As mentioned earlier in chapter 2, structural study has been carried out in bacteria.

Strains used: 1. *E. coli* DH5α (for cloning purpose)

2. *E. coli* BL21 (DE3) (for protein purification)

Plasmid: pET30a bacterial expression vector (Figure 2.1).

3.2.1.1 Primers used for PCR

Sr.No.	Name	Sequence
1	N50 Forward	TATGGATCCATGTCTAGTACTCAAGTAGGAAAT
2	N50 Reverse	ACAGTCGACTCACAAATCTTGGTTATTCTTTAGTTCAA

Table 3.1 Shows sequence of primers used for the amplification of gene fragment coding for N50 peptide.

3.2.1.2 Plan of work of construction of N50 peptide sequence in pET30a bacterial expression vector

Yeast ODC contains 466 amino acid residues. The selected N50 peptide is N terminal 50 amino acid stretch of yeast ODC. Primers were designed to amplify the region starting from 1st to 50th amino acid residues. Plasmid pRS314 which carries gene coding for entire yeast ODC was used as a template for PCR. Plasmids pRS314 and pET30a are already discussed in chapter 2. Fragment of gene coding for N50 peptide was successfully amplified from plasmid pRS314. The 165 bp amplicon, digested by *Bam*HI ans *Sal*I was ligated into pET30a vector which was digested by same enzymes. This ligated plasmid was transformed into DH5α strain of *E. coli*. Figure 3.1 shows pictorial representation of cloning strategy for the construction of pRPN50

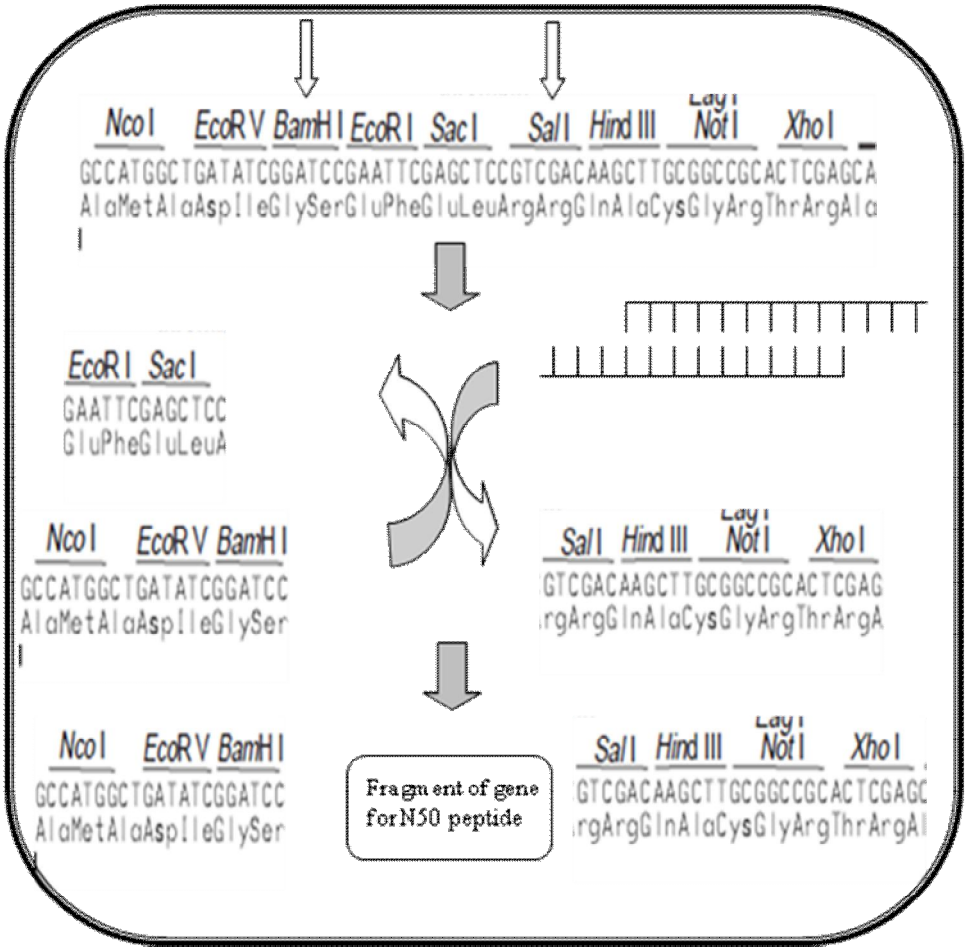


Figure 3.1 Cloning strategy for the construction of pRPN50

Screening of positive transformants was done by checking restriction digestion pattern. The transformants would have lost their *Eco*RI and *Sac*I sites, if the insert was ligated to the vector and hence would be resistant to *Eco*RI and *Sac*I digestions. Further confirmation was done by checking the plasmid size, digestion with different restriction enzymes, insert release after double digestion and comparing the restriction digestion pattern with that of empty pET30a vector. Sequencing was done for final confirmation.

3.2.1.3 Expression of N50 peptide

The protein expression studies were carried out in BL21 (DE3) strain of *E. coli*. These cells are designed for high level of protein expression and relatively easy purification.

The fragment of gene coding for N50 peptide was cloned under T7 promoter in pET30a vector which is IPTG inducible. The chimeric plasmid pRPN50 was transformed into *E. coli* BL21(DE3) cells to study protein profile. After 1 mM IPTG induction the cells were loaded on SDS PAGE to check the expected 12kD band of N50 peptide derived from yeast ODC.

3.2.1.4 Purification of N50 peptide

Protocols for cell growth, induction, harvesting and lysis to collect peptide of interest were followed as discussed in second chapter. Cell lysis protocol was standardized using lysis buffer without EDTA so as to use the Nickel linked resin for affinity chromatography. After cell lysis the sample was centrifuged at 5000rpm for 30 min at 4°C. Fractions of supernatant and pellet were loaded on SDS PAGE to check the presence of N50 peptide. The solubility of selected peptide was confirmed as the expected band was seen in supernatant. Purification of N50 peptide was done by protocol of affinity chromatography as discussed in chapter 2. The peptide was dialyzed to remove the imidazole which was used for elution protocol, the sample was concentrated and protein estimation was done.

3.2.1.5 Structural characterization

3.2.1.5.1 Circular Dichroism

CD spectra were recorded on Jasco 815 spectropolarimeter. Far UV CD spectra were recorded from 180 nm to 250 nm with 1mm path length while near UV CD spectra were recorded between 250 to 320 nm with 1cm path length. Protein samples were prepared in phosphate buffer having 5 mM sodium phosphate and 5 mM NaCl (pH 8). Far UV CD spectra were recorded at pH 8 as well as pH 2.2. Spectra of all samples were blank corrected. Five spectra were accumulated to reduce the noise. Spectra were recorded at 0.2nm resolution and Scan speed was 50nm/sec.

3.2.1.5.2 Fluorescence Spectroscopy

Flourescence spectra were recorded using Hitachi F-4010 fluorescence spectrophotometer. Slit width was 5nm. Fluorescence spectra were recorded in denaturants such as urea. Protein samples were prepared in phosphate buffer having 50 mM sodium phosphate and 30 mM NaCl (pH 8). To record the intrinsic fluorescence spectra, samples were excited at 295nm and emission was recorded from 300nm to 400nm. Extrinsic fluorescence was studied by using extrinsic fluorophore such as 1-anilinonaphthalene 8-sulfonate (ANS). ANS was used at a concentration of 50μM. ANS was excited at 340nm and emission was recorded between 400nm to 550nm. The same sample was also excited at 295nm and emission was recorded from 300nm to 550nm.

3.2.1.5.3 Bioinformatics predictions

Bioinformatics study of the selected peptides was carried out. Structure of all proposed peptides was predicted using prediction softwares such as APSSP, JEP-RED, SOPMA etc. This has given the primary information on the peptides. Theoretical pI and hydrophobicity was checked for each peptide before designing the purification protocol.

Softwares used: SOPMA, JEPRED and APSSP.

Structure prediction using SOPMA software:

```
MHHHHHHSSGLVPRGSGMKETAAAKFERQHMDSPDLGTDDDDKAMADIGSMSSTQVGNALSSSTTTLVD
L
hhtccctttteeccccccchhhhhhhhhhttcccttccccctthhhhhhhtcccchhhhhhhhhtcceeeee
h
SNSTVTQKKQYYKDGETLHNLLLELKNNQDL
ttcccccccheccttHHHHHHHHHHHHtttcc
```

Alpha helix: h, Extended strand: e, Beta turn: t, Random coil: c.

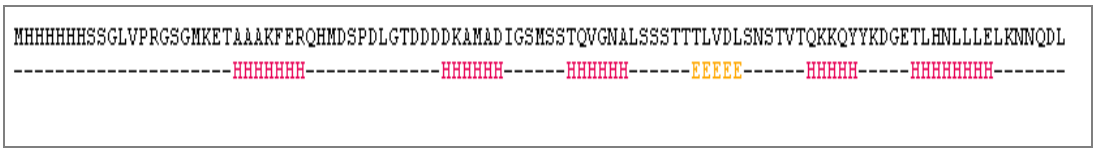


Figure 3.2 Structure prediction using Jepred software

Results of structure prediction software indicate presence of helical region in N50 peptide.

3.2.2 Functional study

Functional study has been carried out in yeast *Saccharomyces cerevisiae* BY4741 (*SPE*$^-$) as mentioned in chapter 2.

pRPSR1 Yeast expression vector (Figure 2.6) plasmid used in this study.

Culture condition and media are already discussed in chapter 2.

3.2.2.1 Primers used for PCR

Sr.No.	Name	Sequence
1	N50 Forward	TATGGTACCATGTCTAGTACTCAAGTAGGAAAT
2	N50 Reverse	ACAGGATCCTCACAAATCTTGGTTATTCTTTAGTTCAA

Table 3.2 shows sequence of primers used for the amplification of gene fragment coding for N50 peptide so as to clone in plasmid pRPSR1.

3.2.2.2 Plan of work of construction of N50 peptide sequence in pRPSR1 yeast expression vector

Fragment of gene encoding N50 peptide of yeast ODC was successfully amplified from plasmid pRS316. 165 bp amplicon, digested by *Kpn*I and *Bam*HI was ligated into pRPSR1 which was digested with the same enzymes. This ligated plasmid was transformed into DH5α strain of *E. coli*. The plasmid pRPSR1 contains one *Bgl*II site in between *Kpn*I and *Bam*HI sites which were used for cloning. If an insert got ligated into the vector, the resultant construct should remain undigested with *Bgl*II digestion. 20 colonies were screened out of which five were positive. *Bgl*II digestion failed in five positive transformants. For the further confirmation gene specific PCR was set with one of the positive tansformant as a template. One more confirmation was done by PCR using vector specific forward primer and gene or insert specific reverse primer. As both PCR products i.e. gene specific PCR of size 165 bp and another PCR of size 600bp using different primers were matching with the marker, presence of insert was confirmed. The construct pRPSRN50 was finally confirmed after sequencing.

3.2.2.3 Functional analysis in yeast

Chimeric construct pRPSRN50 was transformed into BY4741 strain of yeast *S.cerevisiae* for functional analysis of fusion protein. The gene for fusion protein is under GAL 1 promoter which is galactose inducible. The yeast cells transformed with plasmid pRPSR1 and chimeric construct pRPSRN50 were first grown in S.D. glucose media. In mid log phase cells were centrifuged at 5000rpm, media was discarded and cells were resuspended in S.D. galactose media so as to induce an expression of fusion protein. β galactosidase assay has been carried out after 6 hours of induction as well as of fully grown cultures. Yeast cells were also inoculated and grown in S.D. galactose media so as to express β galactosidase and fusion protein constitutively.

The concentration of polyamines in yeast media is 100mM. BY4741 cells were grown in 20mM, 40mM, 60mM, 80mM and 100mM of polyamines concentrations to check the viability of cells and to minimize the synthesis of cellular Antizyme. As discussed in chapter 2, though there was late log phase, cells were grown in minimum i.e. at

20mM concentration of polyamines. At log phase cells were shifted from S.D. glucose to S.D. galactose medium. In order to check an expression of fusion protein after 6 hours of induction with galactose, fraction of cells were collected and β-galactosidase assay was carried out.

Unlike fusion protein Nα/β-β-galactosidase, fusion protein N50-β-galactosidase does not have antizyme binding element i.e. AzBE region. But still β-galactosidase assay was carried out with both yeast cell cultures i.e. with cells induced for antizyme synthesis by excess polyamines and with cells without excess polyamines just to check if altered antizyme level could affect the degradation process.

3.2.2.3.1 Vacuolar inhibition to check the possibility of non-proteasomal degradation of fusion protein

As discussed in chapter 2, in yeast cells proteins have two destinations for degradation purpose. One is proteasome for the specific degradation of short lived and damaged or misfolded proteins. Another is vacuole (Armstrong, 2010). Many long lived proteins as well as a few proteins which are expressed in stress condition or starvation condition are engulfed by vacuoles. Vacuolar protein degradation is also one of the major routes of protein degradation in yeast (Li et al., 2009). To check the destination of the fusion protein of interest, vacuolar inhibitor was added in a culture at log phase. PMSF was used to inhibit vacuolar protein degradation. β-galactosidase assay was carried out after three hours of PMSF addition.

3.2.2.3.2 Proteasomal inhibition

For the final confirmation of expressed fusion protein as a substrate of proteasome and to rule out involvement of any other proteolytic machinery apart from proteasome, proteasomal inhibitor was used. MG132 is a proteasome inhibitor commonly used for yeast studies (Adle et al., 2008; Liu et al, 2007).

Methods for using proteasomal inhibitor and to increase cell permeability for proteasomal inhibitor are described in chapter 2.

Cells were grown in synthetic dextrose or SD minimal media having yeast nitrogen base with amino acids and without ammonium sulphate. Glucose and proline were used as carbon and nitrogen source respectively. At log phase cells were induced with galactose and also supplied with 0.003% SDS for three hours. After 3 hours of SDS treatment, cells were treated with 30μM MG132 and cells were taken after one hour interval till fifth hour. β-galactosidase assay was carried out with all samples.

In another set of experiment, after three hours of induction and SDS treatment, cells were treated with 100μM MG132 for three hours. After 3 hours samples were taken and β-galactosidase assay was carried out with all samples. EDTA interferes with proteasomal assembly formation. Cells were also treated with 10mM EDTA for 3 hours and β galactosidase assay was carried out.

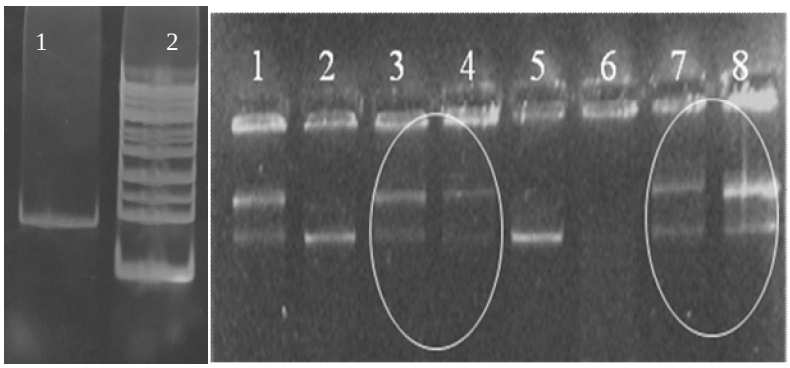
3.3 Results

3.3.1 Structural studies

3.3.1.1 Construction of N50 peptide sequence in pET30a bacterial expression vector

In order to construct N50 peptide of yeast ODC into pET30a bacterial expression vector, PCR amplification was carried out with the help of forward and reverse primers containing *Bam*HI and *Sal*I restriction sites at their 5' end respectively (Table 3.1). pRS314 plasmid was used as a template for the amplification of gene fragment coding for N50 peptide and further checked on agarose gel electrophoresis (Figure 3.3A).

Transformants containing positive clones for N50 peptide were screened by *Eco*RI restriction enzyme (Figure 3.3B).



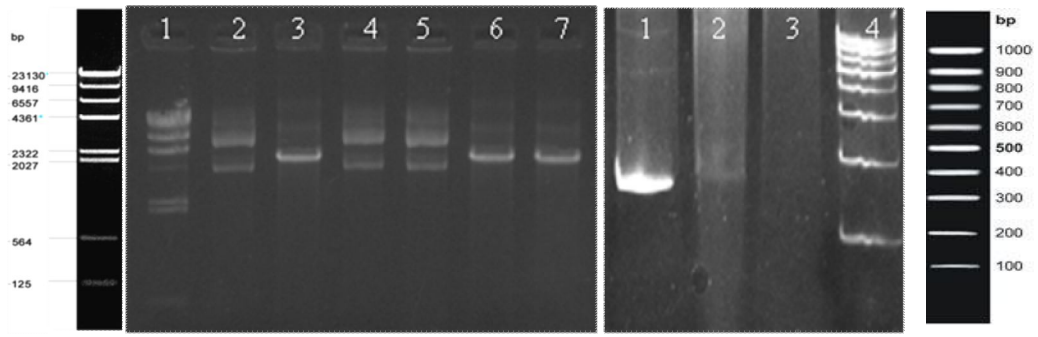
(A) **(B)**

Figure 3.3: PCR conformation and screening of pRPN50

(A) PCR amplified product of fragment of gene coding for N50 peptide on 1% agarose. Lane 1 shows 165 bp PCR product corresponding with 100 bp marker in lane 2 using primers shown in table 3.1.

(B) Screening of positive transformant of pRPN50. Lane 1, 3, 5 and 7 show undigested plasmids while lane 2, 4, 6 and 8 show *Eco*RI digests of the same plasmids. In lane 4 and 8 the plasmids remained undigested with *Eco*RI restriction digestion.

Clones which remained undigested with *Eco*RI were further selected for confirmation by digestion with various restriction enzymes as shown in figure 3.4A. pRPN50 remained undigested by *Eco*RI restriction enzyme, to insert release *Bam*HI and *Sal*I restriction enzyme have been used (Figure 3.4B).



(A) **(B)**

Figure 3.4: Restriction digestion pattern pRPN50 construct

(A) Lane 1 and 2 show λ *Hind*III marker and undigested pRPN50 vector respectively. Lane 3, 6 and 7 show *Bam*HI, *Sal*I and *Hind*III digests of vector pRPN50. Restriction sites for *Eco*RI and *Sac*I are lost while cloning for plasmid pRPN50 so plasmid should remain undigested after digestion with *Eco*RI and *Sac*I. The plasmid pRPN50 remain undigested after *Eco*RI and *Sac*I digestion as shown in lane 4 and 5 respectively.

(B) Confirmation of insert in pRPN50 construct by double digestion. Cloning of fragment of gene coding for N50 peptide was done in *Bam*HI and *Sal*I. Lane 1 shows insert release of size 159 bp after double digestion of pRPN50 construct with *Bam*HI and *Sal*I corresponding with 100 bp DNA ladder in lane 4 loaded on 15% polyacrylamide gel.

3.3.1.2 Expression and Purification of N50 peptide

A pRPN50 plasmid construct containing the sequence coding for N50 peptide of yeast ODC was expressed under the control of T7 promoter in *E. coli* strain BL21 (DE3) by inducing it with 1mM IPTG at 37°C under selection pressure of 50µg/ml of kanamycin (Figure 3.5).

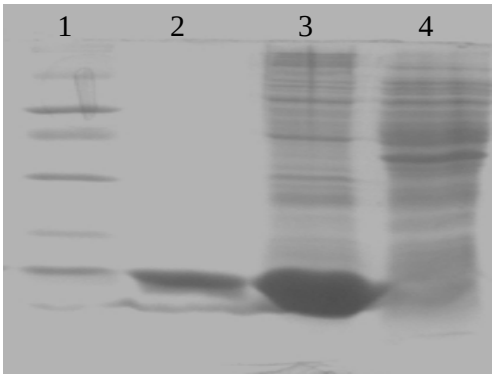
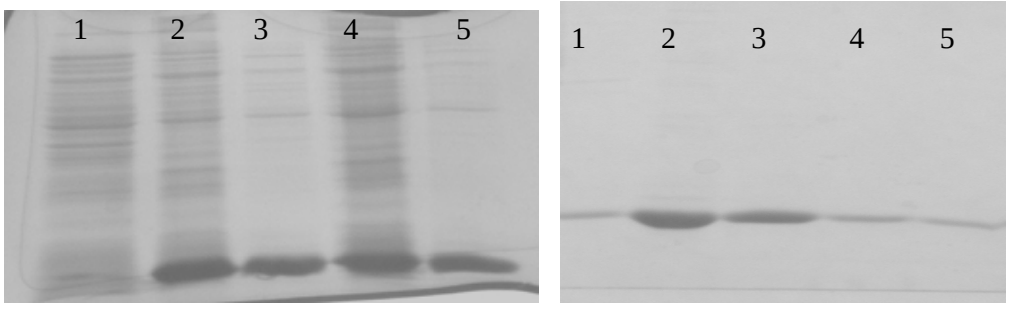


Figure 3.5 Protein profile of *E. coli* BL21(DE3) cells transformed with plasmid pRPN50.

Lane 3 and 4 show induced and uninduced BL21(DE3) cells respectively. Lane 3 shows induced 13 kD band of N50 peptide corresponding with protein molecular weight marker in lane 1. Lane 2 shows purified N50 peptide by affinity chromatography.

Expressed N50 protein was further processed for the purification. N50 showed solubility in lysis buffer (pH 8), we have directly used lyset to purified N50. Lysate was incubated with pre-treated Nickel NTA resin, unbound proteins were washed with high stringent buffer containing 20mM of imidazole. After several wash, purified protein was eluted from the resin with 300mM of imidazole and dialysed against phosphate buffer, pH-8 (Figure- 3.6).



(A) **(B)**

Figure 3.6 Protein profile of lysed *E. coli* BL21(DE3) cells with and without EDTA in lysis buffer and solubility.

(A) Lane 1 shows uninduced sample. Lane 3 and 5 show cell lysate with and without EDTA respectively while lane 2 and 4 show pellet after cell lysis with and without EDTA respectively.

(B) Lane 2 shows 11 kD band of N50 peptide in cell lysate. Lane 3 to 5 show recovered protein of interest after 2^{nd}, 3^{rd} and 4^{th} washes given to the pellet respectively. This indicates solubility of N50 peptide.

3.3.1.3 Far UV CD spectra of N50 peptide

Far and near UV CD spectra of N50 peptide were recorded in phosphate buffer solution to study the changes in secondary but it not showing tertiary structure. Far UV CD spectra show the presence of characteristic pattern of α-helical structure with two negative peak (Figure- 3.7).

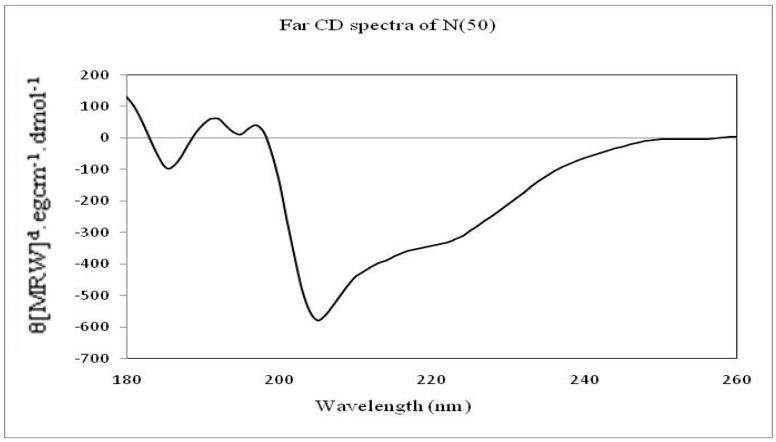


Figure 3.7: Far UV-CD spectra of N50 peptide in phosphate buffer recorded at pH 8.0.

The far UV CD spectrum shows presence of slight α helical component in its structure with two minima around 208 and 222nm.

To check the stability of N50 peptide in acidic environment we have recorded the spectra of N50 peptide in pH 2.2 buffer solution (Figure 3.8). Secondary structure was found to be almost same in both acidic and neutral buffer which indiates the stability of peptide in acidic condition.

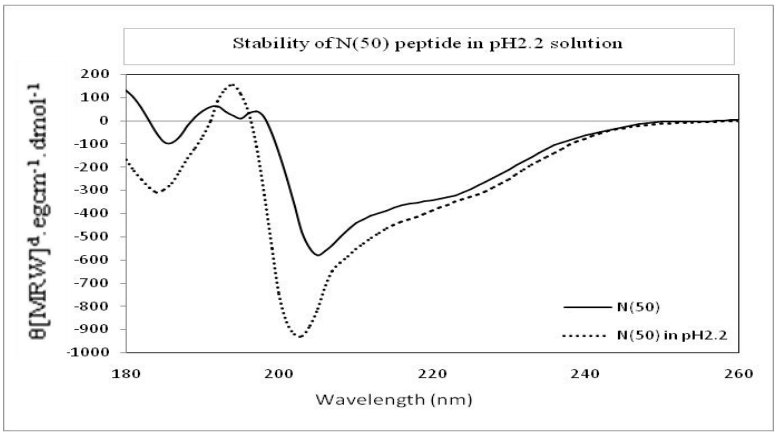


Figure 3.8: Far UV-CD spectra of N50 peptide in phosphate buffer recorded at pH 2.2.

3.3.1.4 Fluorescence spectra of N50 peptide

Fluorescence spectra of the N50 peptide were recorded by exciting the protein at 280nm. Urea and guanidinium hydrochloride are well known denaturant make protein unstable.

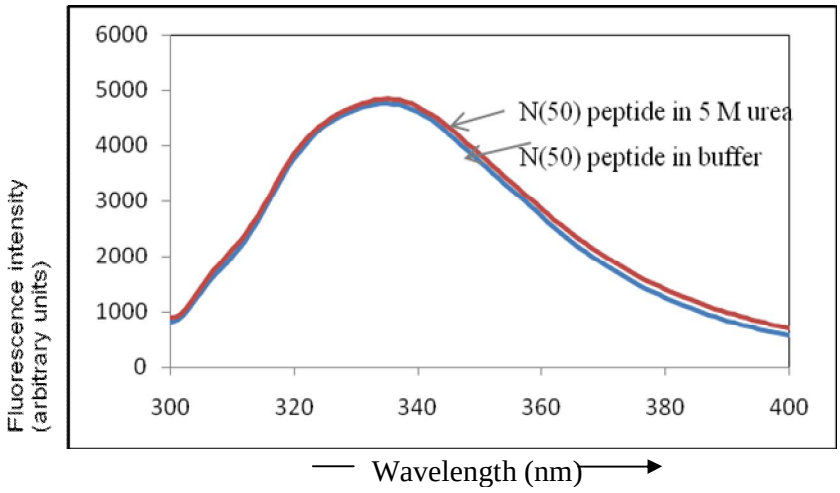


Figure 3.9 Fluorescence spectra of N50 peptide recorded in buffer and 5 M urea

The spectrum recorded in urea (red line) shows red shift with respect to the one recorded in buffer (blue line), indicating presence of tertiary structure in N50 peptide in buffer.

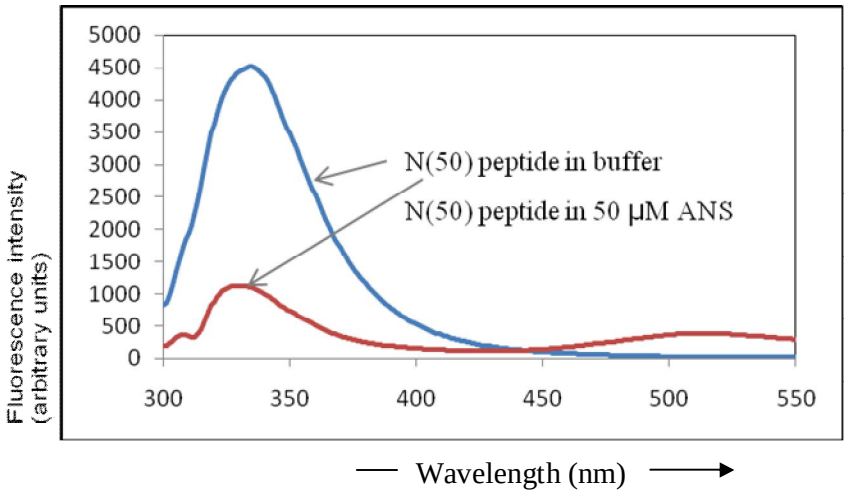


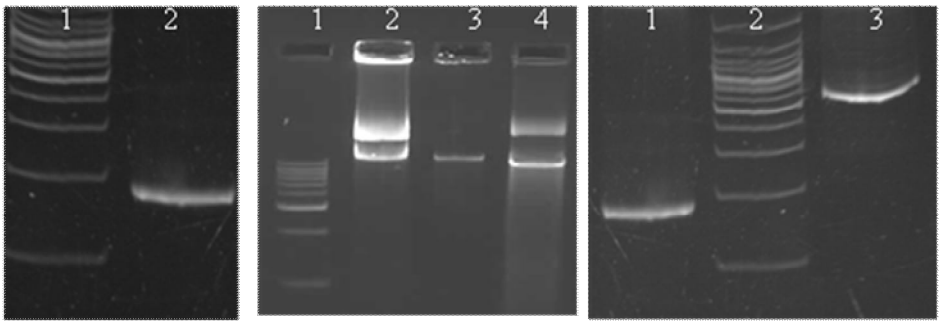
Figure 3.10 Fluorescence resonance energy spectrum of N50 peptide in the presence (red line) and absence of ANS (blue line).

Energy transfer between tryptophan and ANS is observed by Fluorescence resonance energy transfer (FRET) spectrum by exciting at 280nm. Decrease in emission peak in the presence of ANS between 300 to 400 nm while increase in peak between 400 to 550 nm clearly shows a successful transfer of fluorescence energy (Figure 3.10)

3.3.2 Functional study

3.3.2.1 Construction of the N50 gene sequence in pRPSR1

After confirming the expression and activity of test protein β-galactosidase (Figure 2.26), gene sequence of N50 peptide of yeast ODC was fused to upsteam of β-galactosidase gene sequence with the help of *Kpn*I and *Bam*I restriction enzymes (Figure 3.11).



(A) **(B)** **(C)**

Figure 3.11 PCR amplification and conformation of pRPSRN50 construct

(A) PCR product of fragment of gene coding for N50 peptide on 1% agarose. Lane 2 shows 165 bp PCR product corresponding with 100 bp marker in lane 1 using primers shown in table 3.2.

(B) Restriction digestion pattern of pRPSRN50. Lane 1 shows 1kb DNA marker. Lane 2 shows undigested 10.6 kb plasmid pRPSRN50. Lane 3 and 4 show *Kpn*I and *Bgl*II digests of plasmid pRPSRN50. Lane 3 shows linearized 10.6 kb band corresponding with marker in lane 1. Restriction site for *Bgl*II is lost while cloning for plasmid pRPSRN50. So plasmid should remain undigested after digestion with *Bgl*II. The plasmid pRPSRN50 remain undigested after *Bgl*II digestion as shown in lane 4.

(C) Confirmation of chimeric construct pRPSRN50 by PCR. Lane 1 shows 171 bp PCR product of fragment of gene coding for N50 peptide using gene specific primers and lane 3 shows 530 bp PCR product using vector specific forward primer and gene specific reverse primer corresponding with 100 bp marker in lane 2 loaded on 15% polyacrylamide gel.

3.3.2.2 Functional analysis of fusion protein

Functional analysis of fusion protein was done using β-galactosidase assay. Yeast cells transformed with control plasmid expressing only *lac Z* gene has shown β-galactosidase activity but the cells which were transformed with chimeric construct expressing fusion protein did not show any β-galactosidase activity. β-galactosidase assay of both the above mentioned cultures was carried out at log phase as well as at stationery phase. Yeast culture expressing only β-galactosidase enzyme has shown β-galactosidase activity in both log phase as well as stationary phase while yeast culture expressing fusion protein did not show any β-galactosidase activity in both log phase as well as stationary phase as shown in figure 3.12.

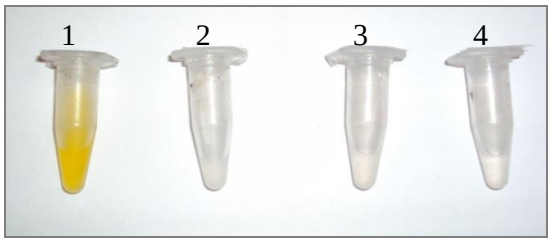


Figure 3.12: β-galactosidase assay of yeast cells transformed with pRPSR1 and pRPSRN50. Eppendorf 1 shows β-galactosidase assay of yeast cells expressing only β-galactosidase enzyme while eppendorf no 2 shows blank. Eppendorf no 3 and 4 show β-galactosidase assay of yeast cells expressing β-galactosidase with N terminal tag of N50 peptide in absence and presence of MG132 respectively. No β-galactosidase activity was observed with yeast cells expressing fusion protein in presence and absence of MG132.

These results indicate the possibility of degradation of β-galactosidase enzyme by N50 peptide which is present as N terminal in frame tag to the β-galactosidase enzyme. As discussed earlier, in yeast system there are two major destinations for the proteins to be degraded, one is vacuole and another is proteasome. To confirm the destiny of our fusion protein, vacuolar inhibitor (PMSF) was added at log phase and β-galactosidase assay was carried out after 3 hours. If destination of the fusion protein is vacuole then β-galactosidase activity was expected after addition of vacuolar protease inhibitor. Still no β-galactosidase activity was seen after addition of vacuolar protease inhibitor. This clearly rules out the possibility of vacuolar involvement in the degradation of fusion protein.

3.3.2.3 Proteasomal inhibition studies

For the final confirmation of expressed fusion protein as a substrate of proteasome, proteasomal inhibitor MG132 was used.

No β-galactosidase activity was observed with fusion protein after inhibition of proteasome while activity was seen in cells carrying only β-galactosidase protein.

3.4 Discussion

Our results, for the first time, establish that N50 peptide, an unstructured region having 50 amino acid residues of N terminal barrel domain of yeast ODC attains some tertiary structure independently in the absence of rest of the protein. This region which is unstructured in nature when present as a part of entire protein, can fold to gain the tertiary structure at physiological pH when expressed independently. These results are in accordance with the results of protein structure prediction softwares. As discussed in introduction (Under IDPs session) intrinsically disordered region has an ability to gain a structure to become functional. Rather molecular interactions or protein interaction events induce a conformation in otherwise unstructured regions. It is also noted in literature that protein or peptide can gain different conformation in different environment e.g. inside and outside the cell, physiological conditions and laboratory conditions, in buffer and in cellular crowded milieu.

After successful structural characterization of N50 peptide, it was proposed to check the portability of N50 peptide as a degradation tag. Being unstructured in nature N50 peptide of yeast ODC harbors a degradation signal within itself. A stretch of first fifty amino acid residues of yeast ODC is unstructured in nature. This unstructured region serves the purpose of requirement of an unstructured extension for initiation of actual dragging and degradation by proteasome. N50 peptide was tagged at the N terminus of reporter protein i.e. β-galactosidase and stability of reporter protein was checked by performing β-galactosidase assay. Yeast cells expressing only β-galactosidase protein has shown β-galactosidase activity while cells expressing N50-β-galactosidase fusion protein did not show any β-galactosidase activity. β-galactosidase activity was not seen in both log phase as well as stationary phase of cells expressing N50-β-galactosidase fusion protein. This indicates the possibility of degradation of fusion protein. The possibility of vacuolar involvement in the degradation of fusion protein was also ruled. Vacuolar inhibitor (PMSF) was added in cells expressing N50-β-galactosidase fusion protein. If vacuole is the destination of proposed fusion protein, then after vacuolar inhibition, β-galactosidase activity was expected in cells expressing N50-β-galactosidase fusion protein. As no β-galactosidase activity was observed, possibility of vacuolar involvement for the degradation of our proposed fusion protein is ruled out.

For the final confirmation of proteasome as the destination of proposed fusion protein, proteasome inhibitor was added in yeast cells expressing N50-β-galactosidase fusion protein. If proposed N50 peptide has potential to drag the reporter protein β-galactosidase towards proteasome for degradation, then after proteasomal inhibition β-galactosidase activity was expected from cells expressing N50-β-galactosidase fusion protein. No β-galactosidase activity from these cells has emerged out the possibility of inclusion formation of expressed fusion protein in the cells.

Another possibility of structural constraints of β-galactosidase protein itself cannot be ruled out. Earlier it was reported that N terminal extension of yeast ODC can target the reporter protein with α helical structure and not β sheet structure.

In C terminal tail of mammalian ODC Cysteine at 441^{th} position is considered as a crucial degradation signal involved in targeting ODC towards proteasome. Mutation at 441^{th} position made mammalian ODC stable. N terminal stretch of yeast ODC does not have any cysteine residue. Residue in N terminal unstructured region could prove the potential of this region to act as degradation signal.

3.5 Conclusion

N50 peptide derived from yeast ODC attains a tertiary structure in the absence of rest of protein. This domain can fold independently to gain the tertiary structure at physiological pH. Results of functional evaluation indicate the possibility of recognition of this region by proteasomal assembly which is an important initiation step for degradation of protein complex. In literature large molecular weight proteins are tagged to β-galactosidase enzyme and entire assembly is stable and functional. In the present study selected peptide is at least not allowing β galactosidase to be functional, i.e. it is interfering in the folding of an enzyme or in other words destabilizing it. Actual scenario about degradation of fusion protein inside the cell is not clear but at least fusion protein is not allowing the reporter protein to remain stable or active.

2 Structural and Functional characterization of Nα/β peptide

2.1 Introduction

Structural information of any protein has been matter of utmost significance in the field of structural biology. Structural details provide base to study function of specific protein. Structural and functional knowledge of any protein is important from basic to applied research.

Structure of mammalian Ornithine Decarboxylase (ODC) has been resolved using X-ray crystalography (Almrud et al., 2000; Kern et al., 1999) while crystal structure of yeast ODC is yet to be undertaken. A report available on yeast ODC primary structure explains that this protein has mainly two domains namely N terminal α/β domain and C terminal β-sheet domain (Fonzi et al., 1987). Dohman's Lab has revealed the unstructured nature of N terminal 50 amino acids stretch in yeast ODC (Godderz et al., 2011; Palanimurugan., 2005). The mechanism of feedback regulation of ODC another protein called antizyme (Az) is conserved between mammalian and yeast system (Hoyt, 2003; Tyagi et al., 1981). The region within ODC where antizyme binds and destabilizes the entire protein is named as antizyme binding element (AzBE) and AzBE is situated in the α/β barrel domain of ODC (Li et al., 1992; Pegg, 2006).

Study of domain and domain organization is important as domain is an independently folding and functioning unit in protein structure. A single protein can have more than one domain performing different functions. This gives protein a tremendous functional assortment (Vogel et al., 2004). According to the Structural Classification of Proteins (SCOP) database, domain is an evolutionary unit of protein (Murzin et al., 1995). In prokaryotes two-thirds of proteins contain two or more domains and in eukaryotes the number is still more. Being an important functional and evolutionary unit, structural characterization of any protein is mainly focused on domain organization, domain interaction and domain combination studies. But functional capability of any protein is contributed not only by any structured domain but unstructured stretches or regions are also equally important (Chouard et al., 2011).

Many a time conformational flexibility because of unstructured region confers functional ease to protein. Unstructured regions are needed in variety of functions such as sequence recognition, protein degradation etc (Uversky, 2010; Uversky and Dunkera, 2010). Details about unstructured regions and their functional importance are mentioned in chapter 1 and 3.

A study by Gadre et al explains the role of N terminal unstructured stretch of yeast ODC in the degradation of ODC itself (Gandre et al., 2002). This region mimics the stretch of last 37 amino acids of mammalian ODC. Deletion of first 50 amino acid residues stabilized yeast ODC against degradation confirming that the degradation of yeast ODC initiates from N terminus (Godderz et al., 2011; Palanimurugan, 2005). In case of ubiquitin dependent proteasomal degradation, apart from ubiquitin an unstructured stretch is also required to initiate the degradation process (Prakash et al., 2004). Same is the case with proteasomal degradation of yeast ODC. In antizyme mediated degradation of yeast ODC, N terminal unstructured stretch serves as docking site to proteasome (Godderz et al., 2011). So an entire N terminal domain of yeast ODC harbors two degradation signals, one is AzBE where Antizyme binds and destabilizes the protein and another is N terminal unstructured stretch which helps in dragging the protein towards proteasome. Keeping in mind an importance of domain and unstructured stretch in structural and functional point of view, entire N terminal barrel domain of yeast ODC was selected for the structural as well as functional characterization. The selected Nα/β peptide is an excellent example of mutual functioning of domain and an unstructured region as well as domain interaction with another protein (i.e. interaction of Antizyme with AzBE) for the purpose of degradation.

In the present chapter structural characterization of Nα/β peptide was done using Circular Dichroism (CD) and Fluorescence Spectroscopy. As folding in 3D structure is required for any protein to be functional, unfolding is also equally important. As discussed earlier unfolding of protein is indispensible in few cellular processes such as degradation, translocation across membrane etc (Praksh et al., 2004; Eilers, 1986). Following study tries to reveal few aspects of unfolding of compact protein structure by using denaturants such as urea and guanidium hydrochloride while fluorescence

energy transfer study was done using ANS which is an extrinsic fluorophore. Functional characterization of Nα/β peptide was done by constructing a fusion protein of Nα/β peptide with reporter protein i.e. β-galactosidase enzyme. Portability of Nα/β peptide as a degradation tag was validated by checking the stability of β-galactosidase enzyme by throgh β-galactosidase assay.

Sequence of Nα/β barrel domain (in green) of yeast ODC selected for this study.

MSSTQVGNALSSSTTTLVDLSNSTVTQKKQYYKDGETLHNLLLELKNNQ
DLELLPHEQAHPKIFQALKARIGRINNETCDPGEENSFFICDLGEVKRLFN
NWVKELPRIKPFYAVKCNPDTKVLSLLAELGVNFDCASKVEIDRVLSMNI
SPDRIVYANPCKVASFIRYAASKNVMKSTFDNVEELHKIKKFHPESQLLLR
IATDDSTAQCRLSTKYGCEMENVDVLLKAIKELGLNLAGVSFHVGSGASD
FTSLYKAVRDARTVFDKAANEYGLPPLKILDVGGGFQFESFKESTAVLRL
ALEEFFPVGCGVDIIAEPGRYFVATAFTLASHVIAKRKLSENEAMIYTNDG
VYGNMNCILFDHQEPHPRTLYHNLEFHYDDFESTTAVLDSINKTRSEYPYKVS
IWGPTCDGLDCIAKEYYMKHDVIVGDWFYFPALGAYTSSAATQFNGFEQTA
DIVYIDSELD 466

Length of selected Nα/β peptide – 346 amino acid residues.

2.2 Materials and methods

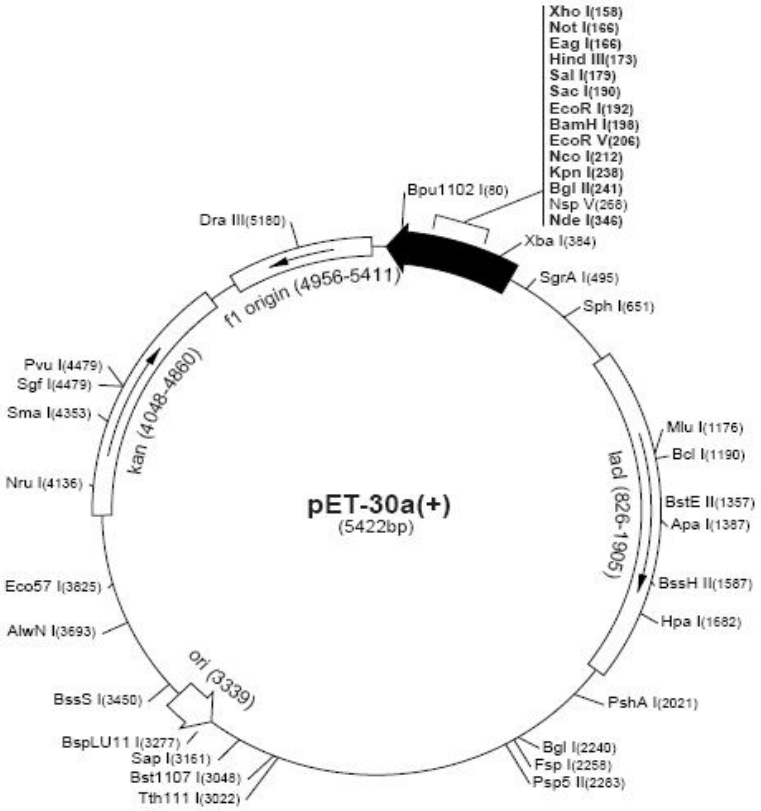
2.2.1 Structural studies

2.2.1.1 Bacterial strains and media

E.coli DH5α (F, 80dlacZ M15, *endA*1, *recA*1, *hsdR*17 (r_k^-, m_k^+), *supE44, thi-1, gyrA96, relA1, (lacZYA-argF)U169*) strain was used for transforming recombinant DNA. Cultures were grown at 37°C at 200 rpm in Luria broth (Hi-media). 50µg/ml of kanamycin was used for selection of plasmid. Bacterial transformation was done by $CaCl_2$ method. Plasmid isolation was done by standard alkaline lysis protocol (Sambrook 2001).

E. Coli BL21(DE3) pLys S F$^-$ompT hsdS_B(r_B^- m_B^-) gal dcm (DE3)pLysS(Cam^R), strain was used to express recombinant DNA. For growth and transformation same condition were used as mentioned in above paragraph.

2.2.1.2 Plasmid used in the study



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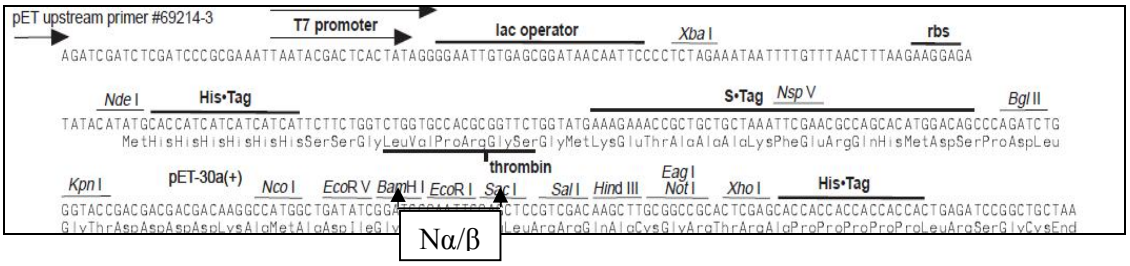


Figure 2.1 Bacterial expression vector pET30a

pET30a plasmid is bacterial expression vector used in this study. This plasmid carries histidine tag for protein purification under the control of strong T7 promoter. Expression was induced by the addition of IPTG (isopropyl-β-D-1-thiogalactoside) at a final concentration of 1mM to the medium (Figure 2.1). Confirmation of pET30a plasmid by restriction digestion pattern is as shown in figure 2.9.

The DNA sequence of the region within pET30a plasmid where the fragment of gene for entire N terminal α/β domain was cloned in *Bam*HI and *Sac*I site is as follows:

..ATCGGATCC...fragment of gene of entire N terminal α/β domain .. GAGCTCCGTCG

BamHI *Sac*I

2.2.1.3 Primers used for PCR

Sr. No.	Name	Sequence
1	S Nα/β Forward	TATGGATCCATGTCTAGTACTCAAGTAGGAAAT
2	S Nα/β Reverse	ATAGAGCTCTCACATTGCTTCATTCTCAGACA

Table 2.1 Primers used for the amplification of gene fragment coding for Nα/β peptide

2.2.1.4 Construction of Nα/β sequence of yeast ODC in pET30a expression vector

Yeast ODC contains 466 amino acid residues. The selected N terminal domain is 346 amino acids long. Primers were designed to amplify the region starting from 1st to 346th amino acid residues (Table 2.1). The plasmid pRS314 which carries entire yeast ODC gene (Figure 2.7) was used as a template for PCR (This plasmid is a kind gift from P. Coffino, University of California).

2.2.1.4.1 Cloning strategy

Entire N terminal α/β barrel domain of yeast ODC was successfully amplified from pRS314 containing gene of yeast ODC. The 1038 bp amplicon, digested by *Bam*HI and *Sac*I was ligated into pET30a vector, which was digested with same enzymes. This ligated plasmid was transformed into DH5α strain of *E. coli*.

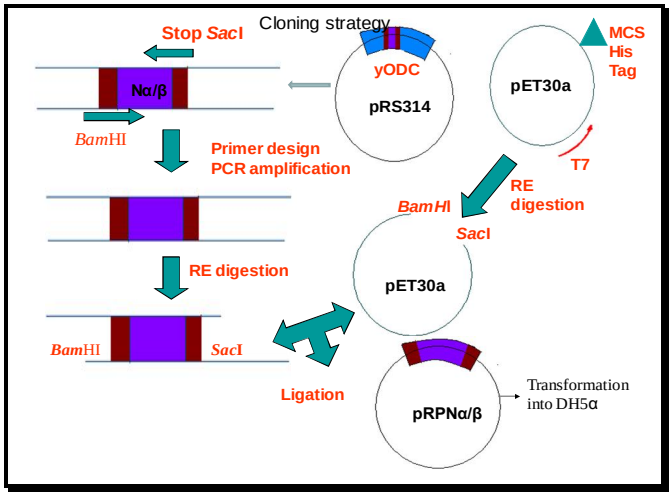


Figure 2.2 Cloning strategy for the construction of pRPNα/β

2.2.1.4.2 Screening strategy

The transformants would have lost their *Eco*RI site, if the insert was ligated the vector and hence would be resistant to *Eco*RI digestion. Further confirmation was done by checking the plasmid size and insert release after double digestion. Sequencing was done for final confirmation.

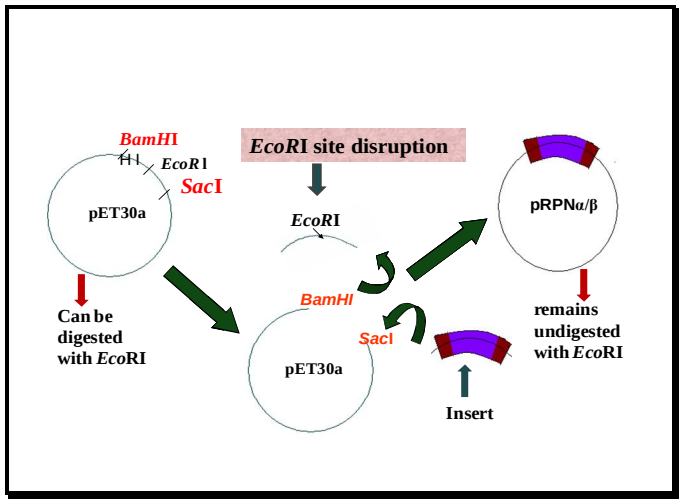


Figure 2.3 Pictorial representation of screening strategy for pRPNα/β.

2.2.1.5 Expression of Nα/β peptide

The protein expression studies were carried out in BL21 (DE3) strain of *E. coli*. These cells are designed for high level of protein expression and relatively easy purification.

To check the expression system, the empty pET30a vector was transformed into BL21(DE3) cells which were then induced with IPTG and protein profile was checked. The gene of Nα/β peptide was cloned under T7 promoter in pET30a vector which is IPTG inducible. The chimeric plasmid pRPNα/β was transformed into BL21(DE3) cells to study protein profile. After 1 mM IPTG induction the cells were loaded on SDS PAGE to check the expected 43kD band of Nα/β domain of yeast ODC.

2.2.1.6 Purification of Nα/β peptide

Once induction of desired peptide is achieved, transformed BL21(DE3) cells were inoculated for purification of target protein. 25 ml of Luria broth (LB) containing 50µg/ml of kanamycin was inoculated with 250µl of overnight grown culture. Cells were induced with 1mM IPTG at mid log phase. After 2 hours of induction, cells were harvested by centrifugation at 5000 rpm for 10 minutes at 4^0C. After three saline washes cell pellet was resuspended in 1ml of cold lysis buffer (pH 7.8). Cell lysis protocol was standardized using lysis buffer without EDTA so as to use the Nickel linked resin for affinity chromatography. Lysozyme was added to a final concentration of 1mg/ml and cell suspension was incubated on ice for 30 minutes. It is then transferred on shaker for 10 minutes after addition of DNase and RNase (final concentration 5µg/ml each). Insoluble debris was removed by centrifugation at 5000 rpm for 30 minutes at 4^0C. Supernatant was collected in fresh tube and pellet was resuspended in saline. Fractions of Supernatant and pellet were loaded on SDS-PAGE to check the presence of target protein. An expected band of protein was seen in pellet. To check the solubility of target protein different experiments had been carried out such as growing culture below 30^0C, minimizing the IPTG concentration, changes in time, temperature, pH, additives such as DTT etc. As expected band was consistently seen in pellet after centrifugation after cell lysis, urea was used to make it soluble. After collecting cell lysate the pellet was resuspended in 8M urea and incubated at 4^0C for overnight. This mixture was then centrifuged for 10000 rpm for 10 minutes at 4^0C. Fractions of both supernatant and pellet were loaded on SDS-PAGE. The expected band was seen in supernatant indicating solubilization of peptide of interest.

As solubilization of desired peptide was achieved, transformed BL21(DE3) cells were inoculated for large scale expression and purification of target protein. 500 ml of Luria broth containing 50µg/ml of kanamycin was inoculated with 5 ml of overnight grown culture. Cells were induced with 1mM IPTG at mid log phase. After 2 hours of induction, cells were harvested by centrifugation at 5000rpm for 10 minutes at 4^0C. Cell lysis was carried out with above mentioned protocol and peptide of interest was collected using 8M urea. This mixture was used for next step i.e. protein purification using affinity chromatography.

2.2.1.7 Affinity chromatography

Nickel charged chromatography resin was purchased from Biorad. 2ml of 50% slurry was transferred to a small polypropylene tube. The resin was washed with 5 column volumes of sterile distilled water, 3 column volumes of binding buffer and finally equilibrated with 3 column volumes of binding buffer (pH 8). For batch mode purification, the equilibrated resin was incubated with cell lysate at 4^0C for 30 minutes. It is then washed with 5 column volumes of binding buffer and washes were collected. Washing was continued until the absorbance at 280nm of the flow through is 0.000. Bound protein was eluted using elution buffer containing 500 mM imidazole and absorbance was measured at 280nm. Using predicted molecular weight and extinction coefficient, the concentration of eluted peptide was determined. The peptide was dialyzed to remove the imidazole, the sample was concentrated and protein estimation was done. Peptide was purified for spectroscopic studies.

2.2.1.8 Structural characterization

2.2.1.8.1 Circular Dichroism

CD spectra reveal the secondary and tertiary structure of proteins. Jasco 815 CD spectrometer was used to record CD spectra. 5 scans were accumulated to reduced the noise. Cell 1cm path length was used. Spectra were recorded at 0.2nm resolution. Scan speed was 50 nm/sec. Far UV CD spectra were recorded from 180 nm to 250 nm with 1mm path length while near UV CD spectra were recorded between 250 to 320 nm with 1cm path length. Protein samples were prepared in phosphate buffer having 5 mM sodium phosphate and 5 mM NaCl (pH 8). Far UV CD spectra were recorded at pH 8 as well as pH 2.2. Spectra of all samples were blank corrected. Near UV CD spectra of peptide was recorded in phosphate buffer (pH 8) with 0M guanidinium hydrochloride and 4M guanidinium hydrochloride.

2.2.1.8.2 Fluorescence spectroscopic studies

Flourescence spectra were recorded using Hitachi F-4010 fluorescence spectrophotometer. Intrinsic fluorescence due to the tryptophan residue in peptide was

studied while extrinsic fluorescence was studied by using extrinsic fluorophores ANS. Fluorescence spectra were recorded in denaturants urea and guanidinium hydrochloride (GdnCl).

Protein samples were prepared in phosphate buffer having 50 mM sodium phosphate and 30 mM NaCl (pH 8). To record the intrinsic fluorescence spectra due to tryptophan, samples were excited at 295nm and emission was recorded from 300nm to 400nm. 1-anilinonaphthalene 8-sulfonate (ANS) was used as an extrinsic fluorophore at a concentration of 50μM. ANS was excited at 340nm and emission was recorded between 400nm to 550nm. The same sample was also excited at 295nm and emission was recorded from 300nm to 550nm.

2.2.1.8.3 Bioinformatics predictions

Bioinformatics study of the selected peptides was carried out. Structure of all proposed peptides was predicted using prediction softwares such as APSSP, JEPRED, SOPMA. This has given the primary information on the peptides. Theoretical pI and hydrophobicity was checked for each peptide before designing the purification protocol.

Softwares used: SOPMA, JEPRED and APSSP.

2.2.1.8.3.1 Pictorial representation of predicted structure of Nα/β peptide using Swiss pdb viewer:

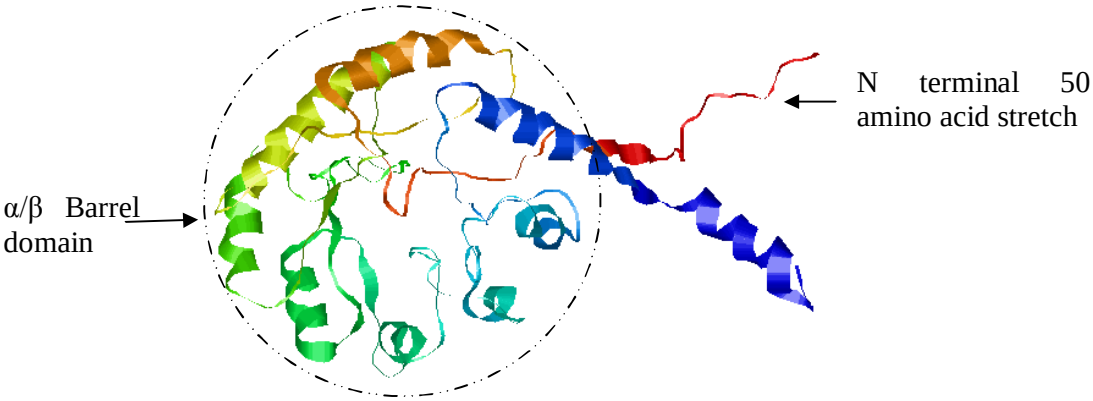


Figure 2.4 Predicted structure of Nα/β peptide using swiss-pdb software

2.2.1.8.3.2 Structure prediction using SOPMA software

```
        10         20         30         40         50         60
70
         |          |          |          |          |          |
|
MHHHHHHSSGLVPRGSGMKETAAAKFERQHMDSPDLGTDDDDKAMADIGSMSSTQVGNALSSSTTTLVD
L
hhtccctttteeccccccchhhhhhhhhhttcccttccccchhhhhhhtcccchhhhhhhhhccceeeee
h
SNSTVTQKKQYYKDGETLHNLLLELKNNQDLELLPHEQAHPKIFQALKARIGRINNETCDPGEENSFFI
C
ttccccccccccchhhhhhhhhhhhttttceeeecccccchhhhhhhhhhhhhhhhhcccccccccheee
e
DLGEVKRLFNNWVKELPRIKPFYAVKCNPDTKVLSLLAELGVNFDCASKVEIDRVLSMNISPDRIVYAN
P
hhhhhhhhhhhhhhhcccccceeeecccccchhhhhhhhhttcccccchhhhhhhhhttccccceeeec
c
CKVASFIRYAASKNVMKSTFDNVEELHKIKKFHPESQLLLRIATDDSTAQCRLSTKYGCEMENVDVLLK
A
ccccchhhhhhhttceeeeecchhhhhhhhhccttcheeeeeecccccchheeccccccchhhhhhhhh
h
IKELGLNLAGVSFHVGSGASDFTSLYKAVRDARTVFDKAANEYGLPPLKILDVGGGFQFESFKESTAVL
R
hhhttcceeeeeeeecccccchhhhhhhhhhhhhhhhhhhhhttcccceeeeeettcccccchhhhhhhh
h
LALEEFFPVGCGVDIIAEPGRYFVATAFTLASHVIAKRKLSENEAM
hhhhhhccccccceeeecttceeeehhhhhhhhhhhhhcccccch

Alpha helix: h, Extended strand: e, Beta turn: t, Random coil: c.

Sequence length :   396
SOPMA :
   Alpha helix     (Hh) :   171 is  43.18%
   Extended strand (Ee) :    59 is  14.90%
   Beta turn       (Tt) :    33 is   8.33%
   Random coil     (Cc) :   133 is  33.59%
```

2.2.2 Functional studies

As discussed earlier, degradation of ODC is a combination effect of all degradation signals within itself along with Antizyme (Az). These degradation signals are sufficient enough to carry out the degradation of ODC. In the present study we have tried to check the efficiency of the peptide to act as degradation determinant signal or degron within yeast ODC i.e. Nα/β peptide. In order to check the efficiency of Nα/β peptide to act as degron, it was proposed to express along with reporter protein by making a fusion protein. So Nα/β peptide was used as a tag for reporter protein. For the present study enzyme β-galactosidase has been selected as a reporter protein. So the proposed fusion protein has Nα/β peptide at its N terminus followed by enzyme β galactosidase. β-galactosidase assay was carried out to check the stability of reporter protein. The reason behind selecting this peptide for the functional analysis is that this peptide harbors 2 degradation signals i.e. Antizyme Binding Element (AzBE) and an unstructured stretch of first 50 amino acid residues.

2.2.2.1 Yeast strains and media

Functional study has been carried out in yeast *Saccharomyces cereviseae* BY4741 *(his3Δ1 leu2Δ0 ura3Δ0 met15Δ0, ΔODC)*. This strain lacks the ODC gene i.e. SPE1, so it cannot grow in the absence of polyamines. This strain has provided an experimental control over polyamine levels while targeting protein for degradation (Brachmann et al., 1998). *S.cerevisiae* cultures were grown in synthetic dextrose (SD) medium containing 0.67% Hi-media yeast nitrogen base (without amino acid) and 2% glucose or 4% galactose as carbon source, histidine (20 mg/lt), lucine (100 mg/lt), uracil (100 mg/lt), methionine (20mg/lt), putrescine (100mg/lt). The cultures were grown at 30ºC at 200 rpm. *Escherichia coli* DH5α strain was used for all plasmid manipulations and grown at 37ºC at 200 rpm in nutrient rich Luria broth (Hi-media). Strain was maintained under a selection pressure of 50µg/ml of ampicillin.

2.2.2.2 Plasmid

Plasmid pUb23 is yeast expression vector was used in the present study (Figure 2.5). Plasmid pUb23 carries gene coding for reporter protein i.e. enzyme β-galactosidase

under GAL promoter which is induced by galactose and which expresses functional β-galactosidase enzyme after transforming into yeast and measure activity by β-galactosidase assay.

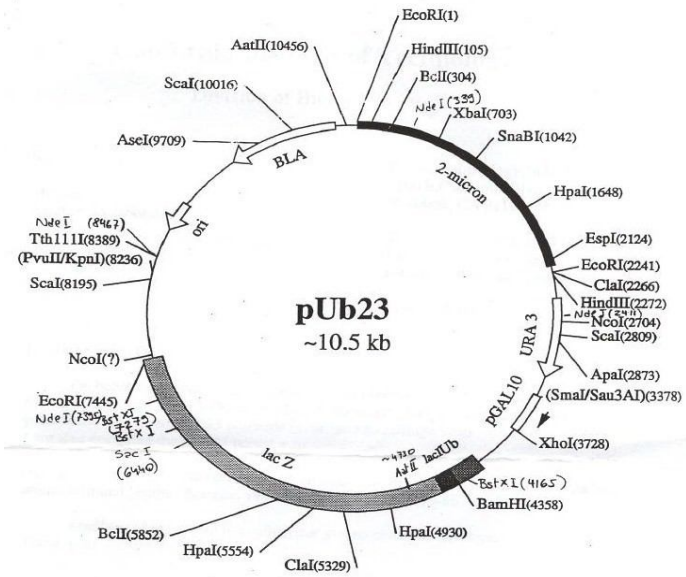


Figure 2.5 Yeast expression vector pUb23 (size 10.5 kb)

This plasmid was constructed using two other plasmids namely YEP24 and pLG669. The sequence of the fragments derived from these plasmids is known while part of the sequence is not available in the literature. Plasmid pUb23 was digested with restriction enzymes *Xho*I and *Bam*HI to release the fragment to be sequenced. This 630 bp fragment digested by *Xho*I and *Bam*HI was ligated into pBSKS plasmid, which was digested with same enzymes. This ligated plasmid was transformed into DH5α strain of *E. coli***.** The transformants would have a size of 3.6 kbp, if the insert got ligated into the vector which is of 3 kbp. 14 colonies were screened, out of which 10 were having the size of 3.6 kbp.

Digestion of the positive transformants was done using *Xho*I, which showed linearized 3.6 kbp band matching with the marker on 1% agarose gel. Double digestion of positive transformants was done using *Xho*I and *Bam*HI, which showed an insert release of 630 bp on a 1% agarose gel.

Further confirmation was done by PCR using M13 primers. Finally the clone was confirmed after sequencing. This sequence was used to design the primers to incorporate site directed mutation in the desired region of the plasmid.

2.2.2.3 Site directed mutagenesis

Plasmid pUb23 carries gene of ubiquitin which is in frame with *lac Z* thus expressing Ubiquitin-β-galactosidase fusion protein. For the present study the gene of Ubiquitin need to be removed. As this ubiquitin is a wild type one, no restriction site was available to replace the gene encoding ubiquitin. At the same time restriction sites were needed at the N as well as C terminus of *lac Z* gene so as to make the fusion proteins. Keeping this in mind the vector pUb23 (Figure 2.5) was modified by site directed mutagenesis and named as pRPSR1 (Figure 2.6).

2.2.2.3.1 Construction of pRPSR1 by Site Directed Mutagenesis

2.2.2.3.1.1 Site directed muntagenesis for N terminal fusion proteins

Original sequence

.....ACC **GGT AAA** ACC….GTT **GAA TCT** TCC….Ub seq….*Bam*HI….*Lac Z* seq….

Modified sequence

.....ACC **GGT ACC** ACC….GTT **AGA TCT** TCC….Ub seq….*Bam*HI….*Lac Z* seq….

*Kpn*I *Bgl*II

Sr No	Primer	Sequence
1	Main forward	TATCTCGAGCAGATCCGCCAGGCGTG
2	SDM Reverse	CCAATGTTATGGTGGTACCGGTCAAAGTCTTGACGAA GATCTGCATAATC
3	SDM Forward	GATTATGC**AGATCT**TCG**TCAAGA**CTTTGACC**GGTACC** ACCATAACATTGG
4	Main Reverse	ATA GGATCC GTG GAGCTC CGG ACCACC TCTTAGC

Table 2.2 Sequences of primers used for N terminal site directed mutagenesis

Mutation was incorporated in primers as shown in table 2.2. PCR product was digested with *Kpn*I and *Bgl*II to confirm the incorporation of mutations and creation of restriction sites. The 630 bp amplicon, digested by *Xho*I and *Bam*HI was ligated into pUb23 vector, which was digested with same enzymes. This ligated plasmid was transformed into DH5α strain of *E. coli***.** If the insert got ligated into the vector the transformants would have been digested with *Kpn*I and *Bgl*II. 8 colonies were screened, out of which one was positive. The positive transformant was digested with *Bgl*II .The same plasmid was digested with *Kpn*I also. This positive transformant was transformed into BY4741 strain of yeast *S.cerevisiae.* β-galactosidase assay was carried out to check whether this vector expresses an active β-galactosidase enzyme after incorporation of mutations. Transformed cells were induced with galactose at log phase and assay was carried out after 6 hours of induction. β-galactosidase activity was confirmed. Now the same plasmid was used for another site directed mutagenesis at the C terminus of *lac Z* gene.

2.2.2.3.1.2 Site directed mutagenesis for C terminal fusion proteins

Original seq:

….GCCGGTCGCTACCATTACCAGTTGGTCTGGT**GTCAAA**AATA….

Modified seq:

….GCC**GCTAGCCGGC**ATTACCAGTTGGTCTGGT**GTCGAC**AATA….

*Nhe*I *Nae*I *Sal*I

Sr. No.	Primer	Sequence
1	Main Forward	CCCATAGAGCTCCTGCACTGGATGGTGGCG
2	SDM Reverse	GTTATTATTATTGTCGACACCAGACCAACTG GTAATGCCGGCTAGCGGCGCTCAGC
3	SDM Forward	GCTGAGCGCC**GCTAGCCGGC**ATTACCAGTT GGTCTGGT**GTCGAC**AATAATAATAAC
4	Main Reverse	CCCCGTGACTGGGTCATGGCTGCGC

Table 2.3 Sequence of primers used for C terminal site directed mutagenesis

Mutation was incorporated in primers as shown in figure. PCR product was digested with *Nhe*I, *Nae*I, and *Sal*I to confirm the incorporation of mutations and creation of restriction sites. The 1800 bp amplicon, digested by *Sac*I and *Tth*III was ligated into pUb23 vector, which was digested with same enzymes. The pUb23 vector in which *Kpn*I and *Bgl* II restriction sites were created by site directed mutagenesis as mentioned above was used as a backbone here for C terminal site directed mutagenesis. This ligated plasmid was transformed into DH5α strain of ***E. coli.*** If the insert got ligated into the vector the transformants would have digested with *Nhe*I, *Nae*I and *Sal*I. 5 colonies were screened, out of which one was positive. The positive transformant was digested with *Sal*I .The same plasmid was digested with *Nhe*I and *Nae*I also. This positive

transformant was transformed into BY4741 strain of yeast *S.cerevisiae.* β-galactosidase assay was carried out to check whether this vector expresses an active β-galactosidase enzyme after incorporation of mutations. Transformed cells were induced with galactose at log phase and assay was carried out after 6 hours of induction. β-galactosidase activity was confirmed. So the construction of yeast expression vector with gene for reporter protein to express as a fusion protein was done successfully.

Cloning of fragment of gene for Nα/β peptide at the N terminus of *lac Z* was done using *Kpn*I and *Bam*HI restriction sites while *Bgl*II was used for screening. Cloning of fragments of gene of proposed peptide at the C terminus of *lac Z* will be carried out using *Nhe*I and *Sal*I restriction sites while *Nae*I will be used for screening.

pRPSR1 is yeast expression vector after creating restriction sites in upstream and downstream of *Lac Z* gene in pUb23 yeast expression vector (Figure 2.6).

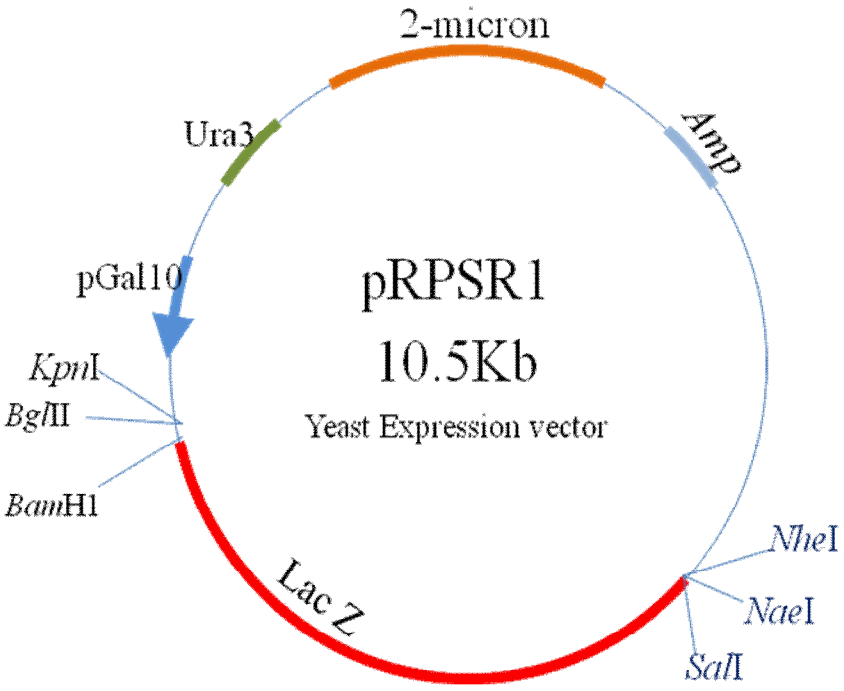


Figure 2.6 pRPSR1 yeast expression vector

2.2.2.4 Construction of pRPSRNα/β

Fragment of gene encoding an entire N terminal domain of yeast ODC i.e. Nα/β peptide was successfully amplified from plasmid pRS316. 1038 bp amplicon, digested by *Kpn*I and *Bam*HI was ligated into pRPSR1 which was digested with the same enzymes. This ligated plasmid was transformed into DH5α strain of *E. coli*. The plasmid pRPSR1 contains one *Bgl*II site in between *Kpn*I and *Bam*HI sites which were used for cloning. If an insert got ligated into the vector, the resultant construct should remain undigested with *Bgl*II digestion. 6 colonies were screened out of which one was positive. *Bgl*II digestion failed in one positive transformant. The plasmid pRPSR1 does not contain any *Sal*I site while an insert has one *Sa*lI site. So if an insert got ligated into the vector, the resultant vector should be digested with *Sal*I. The same positive transformant was digested with *Sal*I restriction enzyme confirming the presence of insert harbouring *Sal*I restriction site.

PCR was done with positive transformant using gene or insert specific primers. The 1038 bp PCR product corresponding with 1 kb marker confirmed the clone.

For further confirmation, double digestion of construct pRPSRNα/β was done using *Kpn*I and *Bam*HI, which showed an insert release of 1038 bp size on 1% agarose gel. Double digestion was also done using *Xho*I and *Bam*HI, which showed an insert release of 1468 bp size on 1% agarose gel.

2.2.3 Functional analysis in yeast

Once the chimeric construct pRPSRNα/β was ready, this vector was transformed into BY4741 strain of yeast *S.cerevisiae* for functional analysis of fusion protein. The gene for fusion protein is under GAL 1 promoter which is galactose inducible. The yeast cells transformed with plasmid pRPSR1 (as a control) and chimeric construct pRPSRNα/β were first grown in S.D. glucose media. At log phase cells were centrifuged at 5000rpm, media was discarded and cells were resuspended in S.D. galactose media so as to induce an expression of fusion protein. β-galactosidase assay has been carried out after 6 hours of induction as well as of fully grown cultures (Baker et al., 1991).

2.2.3.1 Role of an inducible switch for targeting fusion protein for degradation

The concentration of polyamines in yeast media is 100mM. BY4741 cells were grown in 20mM, 40mM, 60mM, 80mM and 100mM of polyamines concentrations to check the viability of cells and to minimize the synthesis of cellular Antizyme. Though there was late log phase, cells were grown in minimum i.e. at 20mM concentration of polyamines. At log phase cells were shifted from S.D. glucose to S.D. galactose medium. In order to check an expression of fusion protein after 6 hours of induction with galactose, fraction of cells were collected and β-galactosidase assay was carried out.

As discussed earlier an increased level of polyamines above their threshold level induces an expression of another regulatory protein named as an Antizyme. Polyamine putrescine and spermidine were added in concentrations which were above their threshold level in yeast media so as to induce an antizyme synthesis. If individual peptide and protein moieties in fusion protein went into correct conformation two possibilities can be explained. One is that after induction with galactose, cells may show positive β-galactosidase assay. Another possibility is that after addition of polyamines in medium in excess, cellular level of antizyme will be increased. This antizyme in turn may recognize an antizyme binding element (AzBE) located on the surface helices of N terminal α/β domain which is an N terminal portion of fusion protein. If AzBE is recognized by antizyme then in consecutive series of reactions, an Antizyme may direct an entire assembly of fusion protein towards proteasome for degradation. So after inducing antizyme which may be termed as an inducible switch for executing targeted degradation of fusion protein, cells are expected not to give positive β-galactosidase assay. β-galactosidase assay was carried out with both yeast cell cultures i.e. with cells induced for Antizyme synthesis by extra polyamines and with cells without extra polyamines.

2.2.3.2 Vacuolar inhibition to check the possibility of non-proteasomal degradation of fusion protein

For protein degradation yeast cells have two destinations. One is proteasome for the specific degradation of short lived and damaged or misfolded proteins, second is vacuole (Armstrong, 2010). Many long lived proteins as well as a few proteins which are expressed in stress condition or starvation condition are engulfed by vacuoles. Vacuolar protein degradation is also one of the major routes of protein degradation in yeast (Li et al., 2009). To check the destination of our fusion protein, vacuolar inhibitor was added in a culture at log phase. PMSF was used to inhibit vacuolar protein degradation. β-galactosidase assay was carried out after three hours of PMSF addition.

2.2.3.3 Proteasomal inhibition

For the final confirmation of expressed fusion protein as a substrate of proteasome and to rule out an involvement of any other proteolytic machinery apart from proteasome, proteasomal inhibitor was used. MG132 is a proteasome inhibitor commonly used for yeast studies (Adle et al., 2008; Liu et al., 2007). In most of the studies on yeast cell inhibitors, erg6 mutant strain of yeast was used (Graham et al., 1993). In these mutant strains the final methylation reaction in ergosterol synthesis is blocked resulting in lack of ergosterol in the plasma membrane. So permeability of cell membrane is affected and cell became sensitive to various inhibitors (Kaur et al., 1999). But this strategy of making yeast cell permeable for proteasome inhibitor results in drastic side effects such as decline in tryptophan transport, reduced efficiency of genetic transformation and sexual conjugation while increased cellular levels of sodium and lithium (Welihinda et al., 1994). Some studies (Liu et al., 2007) used an alternate strategy of changing a nitrogen source. Generally ammonium sulphate is used as a nitrogen source in yeast minimal media. Compared to other nitrogen sources such as urea, proline and leucine, ammonium sulphate is easily taken up by the yeast cells. Proline, a poor nitrogen source, was used as an alternative to ammonium sulphate for studies on yeast cell inhibitors (Mccusker, 1991; Liu et al., 2007).

2.2.3.4 Methods to increase yeast cell permeability

A common problem with yeast studies is its tough cell membrane. As mentioned earlier use of erg or pdr mutant strains to increase cell permeability has few shortcomings. Instead use of simple additives such as 0.003% SDS or 150mM KCl at log phase could serve as temporary cell permeabilizing agents before addition of proteasome inhibitors (Pannunzio et al., 2004; Stella et al., 2001).

Cells were grown in synthetic dextrose minimal media or S. D. media having yeast nitrogen base with amino acids and without ammonium sulphate. Glucose and proline were used as carbon and nitrogen source respectively. At log phase cells were induced with galactose and also supplied with 0.003% SDS for three hours. After 3 hours of SDS treatment, cells were treated with 30μM MG132 and cells were taken after one hour interval till fifth hour. β-galactosidase assay was carried out with all samples.

In another set of experiment, after three hours of induction and SDS treatment, cells were treated with 100μM MG132 for three hours. After 3 hours samples were taken and β-galactosidase assay was carried out with all samples

2.3 Results

2.3.1 Structural studies

2.3.1.1 Construction of Nα/β peptide sequence of yeast ODC in pET30a Expression vector

The plasmid pRS314 carries gene coding for yeast ODC was used as a template for PCR amplification of gene fragments coding for proposed peptides i.e. Nα/β peptide, α/β peptide and N50 peptide. pRS314 and pRS316 plasmids were confirmed by restriction digestion pattern with *Eco*RI and *Xho*I restriction enzymes (Figure 2.7).

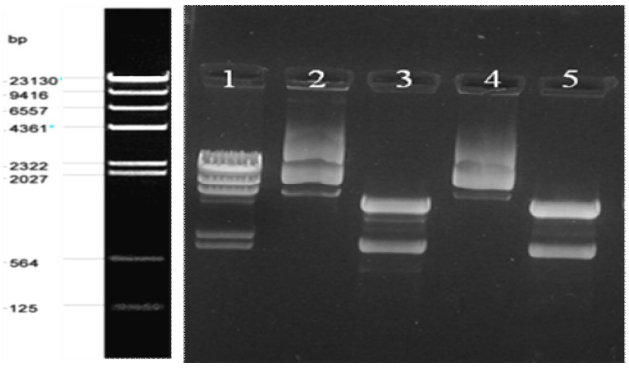


Figure 2.7 Confirmation of plasmids pRS314 and pRS316 by restriction digestion pattern

Lane 1 corresponds with λ *Hind* III marker. Lane 2 and 4 show undigested plasmids pRS314 and pRS316 respectively while lane 3 and 5 show double digestion of plasmids pRS314 and pRS316 with *Eco*RI and *Xho*I showing release of 2 kb fragment.

After confirming the pRS314 plasmid, it has been used to amplify the gene coding for Nα/β peptide and further confirmed by digesting with *Sal*I restriction enzyme (Figure 2.8). PCR product coding for Nα/β peptide was digested with *Bam*HI and *Sac*I restriction enzymess to clone in pET30a vector.

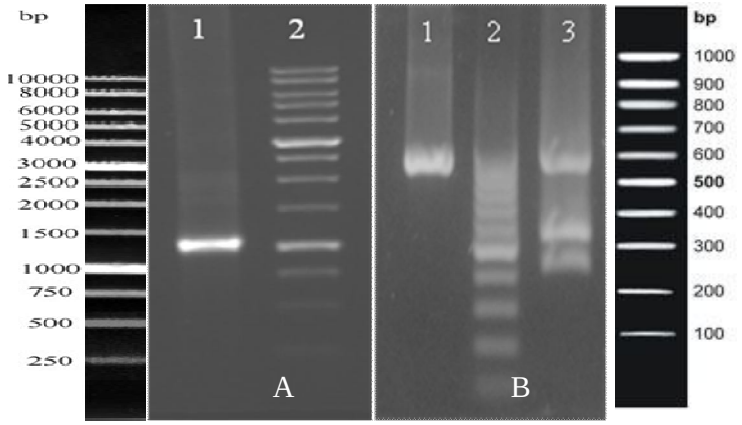


Figure 2.8 PCR amplified product of fragment of gene coding for Nα/β peptide on 1% agarose.

In Figure A, Lane 1 shows 1038 bp PCR product corresponding with 1 kb marker in lane 2. This PCR product harbors one *Sal*I restriction site. Figure B shows *Sal*I digest of 1038 PCR product. Lane 1 shows undigested PCR product, lane 2 shows 100bp DNA ladder. Lane 3 shows two fragments after digestion corresponding with 100bp marker.

In order to use pET30a expression vector for cloning purpose, it has been thoroughly checked by digesting with restriction enzyme sites which are present in multiple cloning site (Figure 2.9). All the below mentioned restriction sites are unique in pET30a vector which are used for cloning of gene fragments of selected peptides.

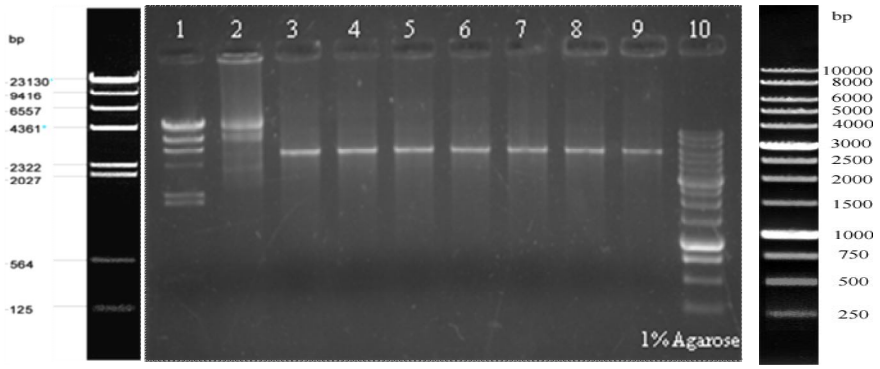


Figure 2.9 Restriction digestion pattern of pET30a vector used for cloning purpose

Lane 1 and 10 show λ *Hind* III marker and 1kb DNA ladder respectively. Lane 2 shows undigested 5.5kb pET30a plasmid, while lane 3 to 9 show *Bam*HI, *Eco*RI, *Sac*I, *Sal*I, *Hind*III, *Not*I and *Xho*I digest of plasmid pET30a respectively. All linearised 5.5kb fragments match with 1 kb marker in lane 10.

Nα/β PCR product and pET30a expression vector were double digested by *Bam*HI and *Sac*I restriction enzymes and ligated to get pRPNα/β construct. Transformants were screened by *Eco*RI restriction enzyme (Figure 2.10). After screening the construct, pRPNα/β was digested with *Bam*HI, *Eco*RI and *Sac*I to confirm the desired clone (Figure 2.11)

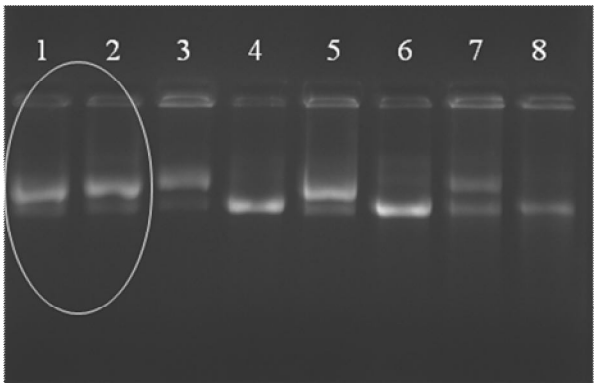


Figure 2.10 Screening of positive transformant of pRPNα/β

Lane 1, 3, 5 and 7 show undigested plasmids while lane 2, 4, 6 and 8 show *Eco*RI digests of the same plasmids. In lane 2 the plasmid remained undigested with *Eco*RI restriction digestion. Site for *E.co*RI is disrupted while cloning.

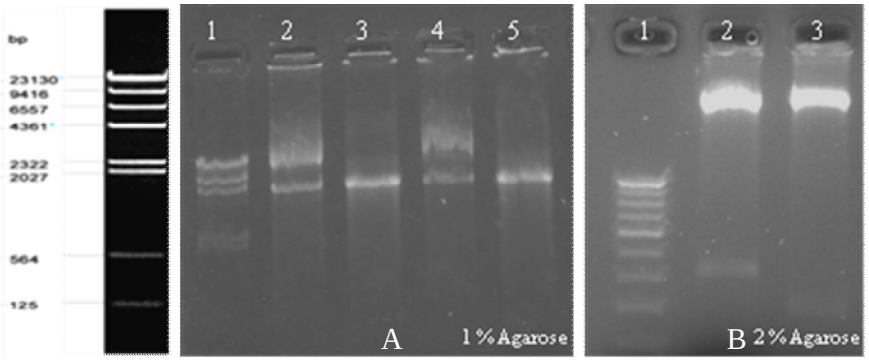


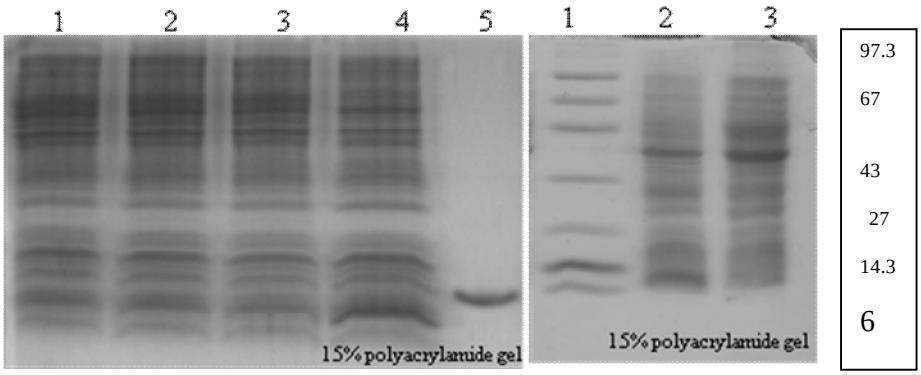
Figure 2.11 Restriction digestion pattern of pRPNα/β plasmid.

In figure A, Lane 1 and 2 show λ *Hind* III marker and undigested pRPNα/β plasmid respectively. Lane 3, 4 and 5 show *Bam*HI, *Eco*RI and *Sac*I digests of plasmid pRPNα/β. Cloning of pRPNα/β plasmid was done in *Bam*HI and *Sac*I restriction sites while *Eco*RI site is disrupted while cloning. Lane 3 and 5 show linearised 6.5 kb plasmid corresponding with marker in lane 1 while in lane 4 plasmid remained undigested after *Eco*RI digestion. In figure B, lane 2 and 3 show *Sal*I and *Hind*III digests of pRPNα/β plasmid. Restriction sites for *Sal*I and *Hind*III enzymes are unique

in pET30a plasmid while theses 2 restriction sites are also present in fragment of gene coding for Nα/β peptide. Lane 2 shows bp fragment after *Sal*I digestion while Lane 3 shows bp fragment *Hind*III digestion corresponding with 100bp marker in lane 1.

2.3.1.2 Expression and purification of Nα/β

A pET30a plasmid was expressed under the control of T7 promoter in *E. coli* strain BL21 (DE3) by inducing it with 1mM IPTG at 37°C under selection pressure of 50µg/ml of kanamycin (Figure 2.12). pET vector containing sequence coding for Nα/β peptide was expressed under the same condition as mentioned above with pET30a (Figure 2.13).



A B

Figure 2.12 Protein profile of BL21 (DE3) cells.

Figure A and B show protein profile after 0.5 mM and 1mM IPTG induction respectively. In figure A, lane 1 and 2 show uninduced and induced BL21(DE3) cells respectively. Lane 3 and 4 show uninduced and induced BL21(DE3) cells transformed with pET30a vector respectively. Lane 4 shows induced 8kD band of peptide expressed from pET30a vector corresponding with 14kD lysozyme in lane 5. In figure B, lane 2 and 3 show induced and uninduced BL21(DE3) cells transformed with pET30a vector respectively. Lane 2 shows induced 8kD band of peptide expressed from pET30 vector corresponding with protein molecular weight marker in lane 1.

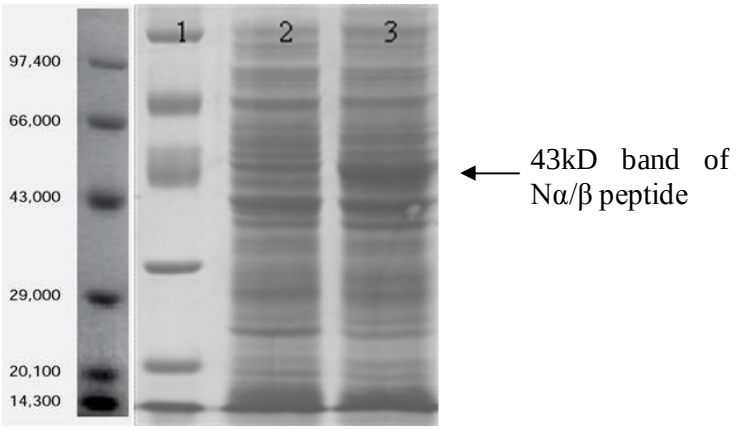


Figure 2.13 Protein profile of BL21(DE3) cells transformed with plasmid pRPNα/β

Lane 2 and 3 show uninduced and induced BL21(DE3) cells respectively. Lane 3 shows induced 43 kD band of Nα/β peptide corresponding with protein molecular weight marker in lane 1.

After confirming the expression of Nα/β, it was further processed to get purified protein. After lysis of the cells it was found that the protein was not soluble in the buffer which indicates that protein has acquired more hydrophobicity and remained in pellet even after washing steps hence urea has been used to solubilize the protein to ease the purification procedure (Figure 2.14).

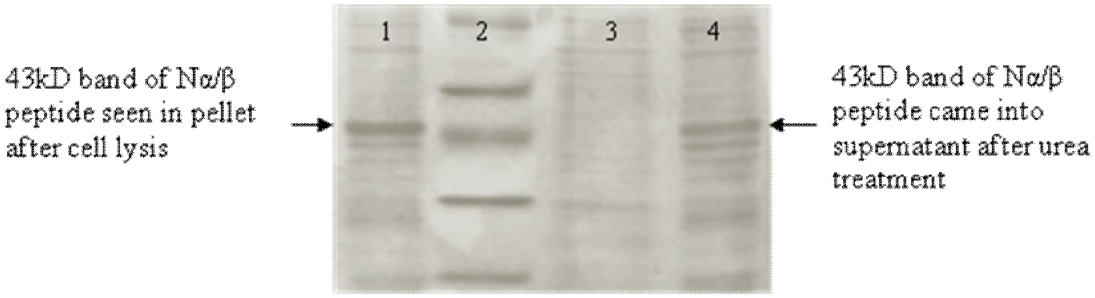


Figure 2.14 Protein profile of purification of Nα/β peptide

Lane 1 shows presence of 43kD band of Nα/β peptide in pellet after cell lysis. After treatment with 8M urea the same 43kD band has come into supernatant (Lane 4) corresponding with protein molecular weight marker in lane 2, instead of pellet (Lane 3) as shown in above figure. The same supernatant was used for purification by affinity chromatography.

After solubilizing the protein in 1M urea, lysate was incubated with pre-treated Ni-NTA resin, unbound proteins were washed with high stringent buffer containing 20mM of imidazole. After several washes, purified protein was eluted from the resin with 300mM of imidazole and dialysed against phosphate buffer, pH-8 (Figure- 2.15).

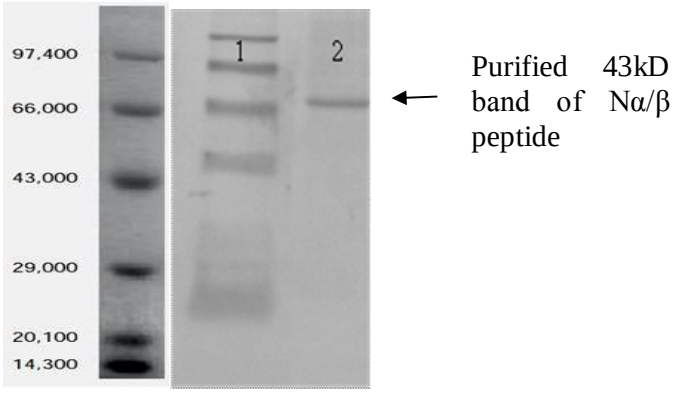


Figure 2.15 Purified Nα/β peptide

Lane 2 shows purified 43 kD band of Nα/β peptide corresponding with protein molecular weight marker in lane 1.

From SDS PAGE, purified Nα/β peptide appears to be around 43KD as expected.

2.3.1.3 Far and near UV CD spectra of Nα/β peptide

Far and near UV CD spectra of Nα/β peptide were recorded in phosphate buffer solution to study the changes in secondary and tertiary structure. Far UV CD spectrum of Nα/β peptide in phosphate buffer at pH 8.0 shows it has taken up secondary structure (Figure-2.16).

To check the stability of Nα/β peptide in acidic environment we have recorded the spectra of Nα/β peptide in pH 2.2 buffer solution (Figure 2.17). secondary structure was found to be almost same in both acidic and neutral buffer which indicates the stability of peptide in acidic condition.

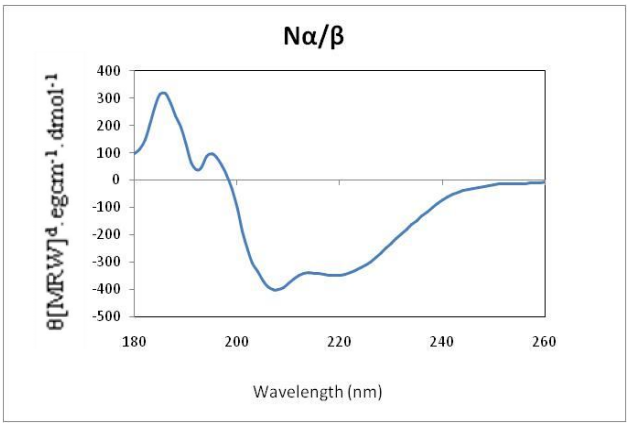


Figure 2.16 Far UV-CD spectra of Nα/β peptide in phosphate buffer recorded at pH 8.0

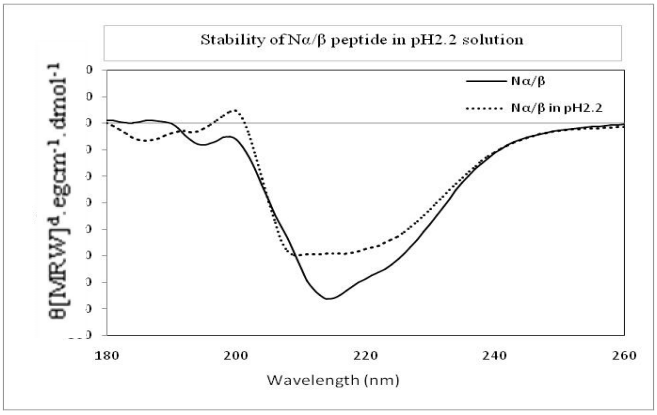


Figure 2.17 Far UV-CD spectra of Nα/β peptide in phosphate buffer recorded at pH 2.2

Near UV CD spectra of Nα/β peptide was recorded in phosphate buffer (pH 8) with 0M and 4M guanidinium hydrochloride. Near UV CD spectra show that the peptide has tertiary structure which is denatured in the presence of 4M guanidinium hydrochloride (Figure 2.18).

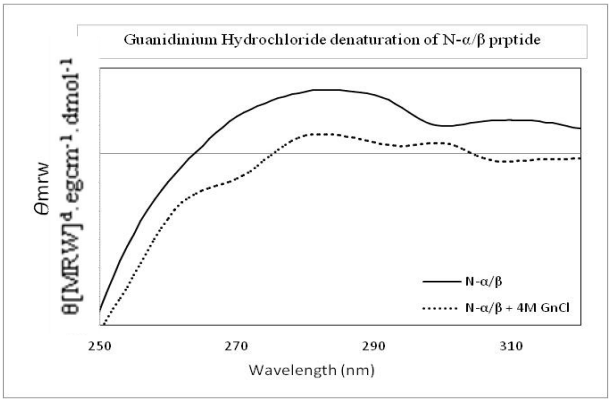


Figure 2.18 Near UV CD spectra of Nα/β peptide recorded in phosphate buffer (pH 8) with 0M guanidinium hydrochloride and 4M guanidinium hydrochloride.

2.3.1.4 Fluorescence spectra of Nα/β peptide

Fluorescence spectra of the Nα/β peptide were recorded by exciting the protein at 280nm. Tryptophan inside the protein can be used to probe the folding and stability of protein. Urea and guanidine hydrochloride are well known denaturant which makes protein unstable. Degree of stability of the protein can be assessed by recording spectra in increasing concentration of the denaturant. Denaturant exposes the buried hydrophobic regions and in turn change the intensity of protein. Nα/β peptide was subjected to the denaturation by using urea and guanidinium hydrochloride as denaturants. Spectra reveal the gradual unfolding and red shift, which indicates the presence of tertiary structure as shown in figure 2.19 and 2.20.

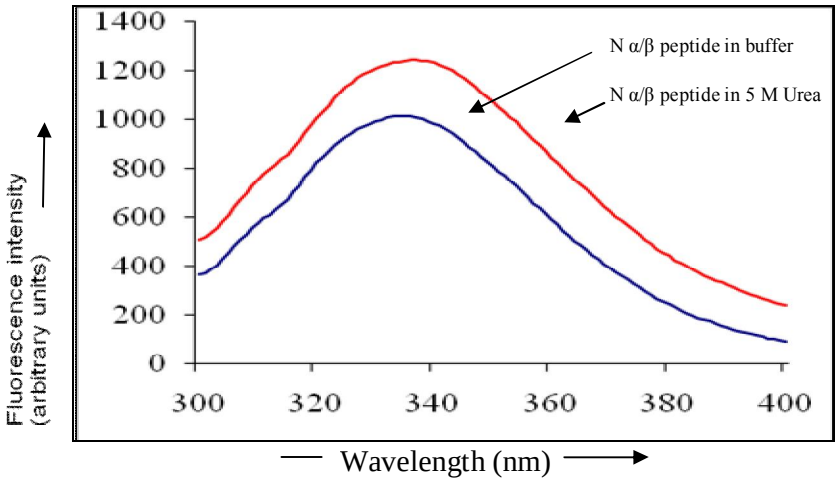


Figure 2.19 Fluorescence spectra of Nα/β peptide recorded in buffer and 5 M urea

The spectrum recorded in urea (red line) shows red shift with respect to the one recorded in buffer (blue line), indicating presence of tertiary structure in Nα/β peptide in buffer.

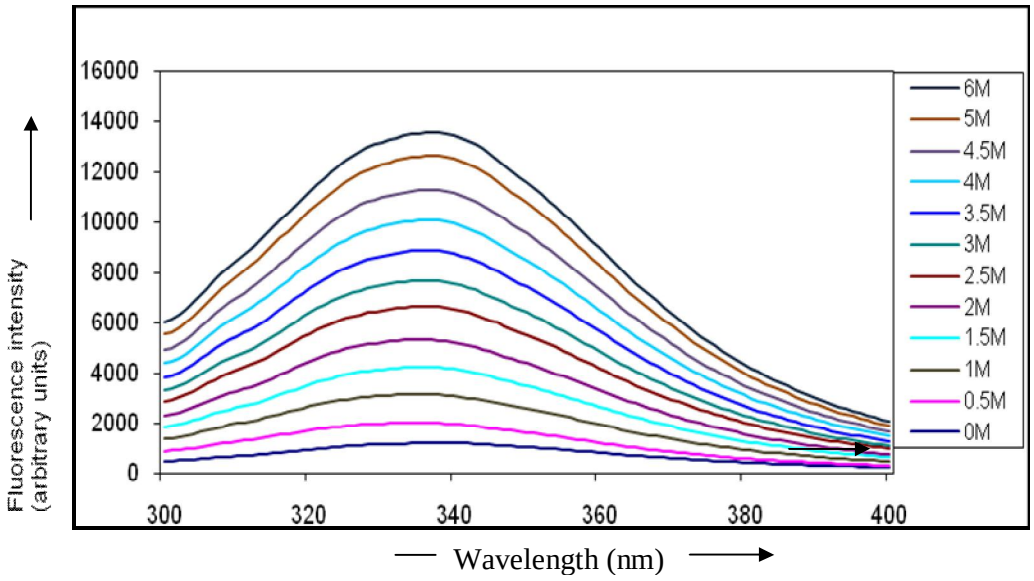


Figure 2.20 Fluorescence spectra of Nα/β peptide in presence of different concentrations of Guanidinium hydrochloride.

With the increasing GdnCl and urea concentration there is increase in emission, indicating denaturation of tertiary structure of the peptide and movement of tryptophan from hydrophobic interior to aqueous environment.

2.3.2 Functional studies

Functional studies were carried out in BY4741 strain of yeast *S. cerevisiae*. This strain is *SPE1* mutant. Gene SPE1 codes for ornithine decarboxylase. So this strain cannot synthesize polyamines. As polyamines are indispensible for cell growth and differentiation, polyamines were added in yeast media. Phenotype of the strain was checked by growing cells in presence and absence of polyamines.

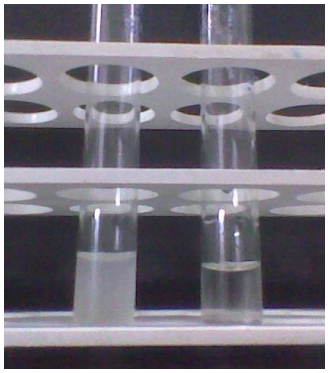


Figure 2.21 SPE1 mutant of *S. cerevisiae* with and without 100µM putresine. Tube 1 shows growth of cells in presence of Putrescine while tube 2 shows no growth in absence of Putrescine.

2.3.2.1 Construction of the Nα/β gene sequence in pUbRPSR1

Yeast expression vector pUB23 has been used as a source of test protein (β-galactosidase). Vector containing restriction sites have been confirmed by restriction digestion pattern (Figure 2.23). As the upstream sequence of pUB23 was unknown to us hence the gene sequence along with the promoter sequence was further cloned in pBSKS with the help of *Xho*I and *Bam*HI restriction enzymes, positive clone was confirmed by digestion pattern analysis (Figure 2.24)

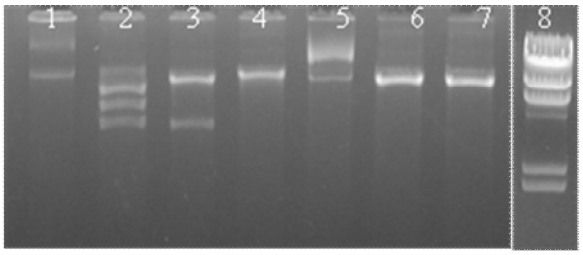
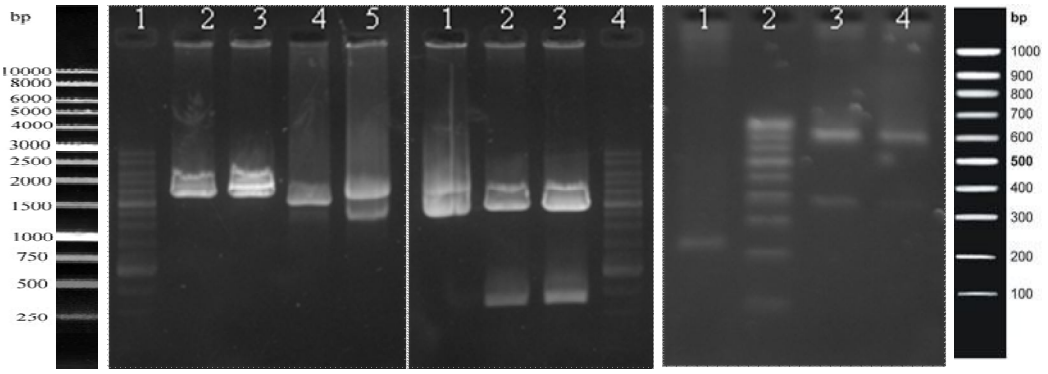


Figure 2.22 Restriction digestion pattern of plasmid pUb23

Lane 1 shows undigested plasmid. Lane 2, 3, 4, 5, 6 and 7 show *Eco*RI, *Hind*III, *Bam*HI, *Kpn*I, *Xho*I and *Sac*I digest of pUb23 respectively corresponding with λ *Hind* III marker in lane 8.



(A) **(B)** **(C)**

Figure 2.23 Subclonning of fragment from pUb23 to pBSKS

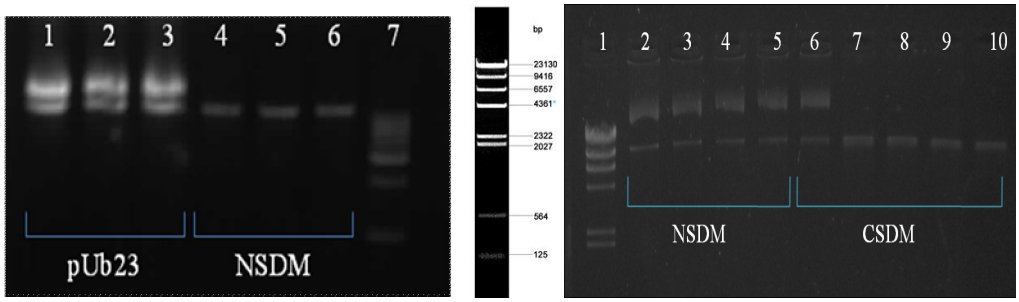
(A) The digested 3.6 kbp positive transformants and 3kbp pBSKS plasmids ran on a 1% agarose gel. Lane 2 and 3 show *Xho*I digest of 3.6 kbp plasmid corresponding with marker in lane 1. Lane 4 and 5 show *Xho*I digest and undigested pBSKS plasmid.

(B) Double digestion of positive transformants using *Xho*I and *Bam*HI showing an insert release of 630 bp on a 1% agarose gel. Lanes 1 shows undigested positive transformant. Lane 2 and 3 show 630bp insert release by *Xho* I and *Bam*HI corresponding with 1 kbp marker in lane 4.

(C) PCR products obtained using M13 primers. Lane 3 and 4 show 839 bp PCR products of positive transformants corresponding with 100 bp marker in lane 2. Lane 1 shows 209 bp PCR product of empty pBSKS plasmid.

2.3.2.2 Construction of pRPSR1 by Site Directed Mutagenesis

After knowing the sequence of upstream region of pUB23, five restriction sites have been created to facilitate the fusion of various peptides of ODC. Site directed mutagenesis technique has been used to create following sites *Kpn*I, *Bgl*II, *Nhe*I, *Nae*I and *Sal*I in pUB23 yeast expression vector (Figure 2.25).



(A) **(B)**

Figure 2.24 Restriction digestion pattern of pRPSR1 yeast expression vector before and after site directed mutagenesis.

(A) Lane 1 undigested pUb23 plasmid, Lane 2 and 3 show digestion of pUb23 with *Kpn*I, *Bgl*II respctively. Lane 4,5 and 6 show digestion of pRPSR1 with *Kpn*I, *Bgl*II and *Bam*HI respectively.

(B) Lane 1 λ *Hind*III marker. Lane 2 show undigested pUb23 plasmid, Lane 3,4 and 5 show digestion of pUb23 plasmid with *Sal*I, *Nhe*I, and *Nae*I respectively. Lane 6 show undigested pRPSR1, Lane 7, 8, 9 and 10 show digestion of pRPSR1 with *Sal*I, *Nhe*I, *Nae*I and *Sac*I respectively. After site directed mutagenesis vector pUb23 is named as pRPSR1.

2.3.2.3 β-galactosidase assay of pRPSR1

Correct frame and expression of chimeric construct having pUB23 background was checked by performing β-galactosidase activity. It has revealed that the vector is expressing and can be used further to clone proposed peptides by taking advantages of created restriction sites (Figure 2.26).

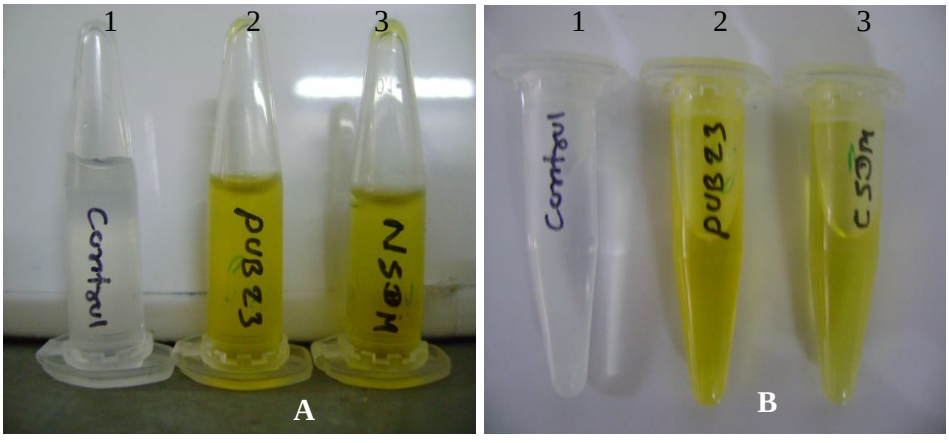


Figure 2.25 β-galactosidase assay of cells transformed with plasmid pUb23 and pRPSR1

Plasmid pUb23 was named as pRPSR1 after site directed mutagenesis. In figure A, eppendorf number 2 and 3 show β-galactosidase assay of cells transformed with plasmid pUb23 and pUb23 after N terminal SDM. In figure B, eppendorf number 2 and 3 show β-galactosidase assay of cells transformed with pBb23 and plasmid pRPSR1. In both figures A and B, eppendorf no 1 is of control. As cells are giving β-galactosidase activity after transforming it with construct pRPSR1, this construct was used for the construction of chimeric plasmid so as to express fusion protein of β-galactosidase with proposed peptides.

2.3.2.4 Construction of pRPSRNα/β

After confirming the expression and activity of test protein β-galactosidase, gene sequence of Nα/β peptide of yeast ODC was fused to upsteam of β-galactosidase gene sequence with the help of *Kpn*I and *Bam*I restriction enzymes (Figure 2.27 and 2.28).

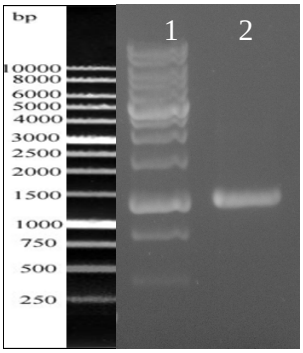
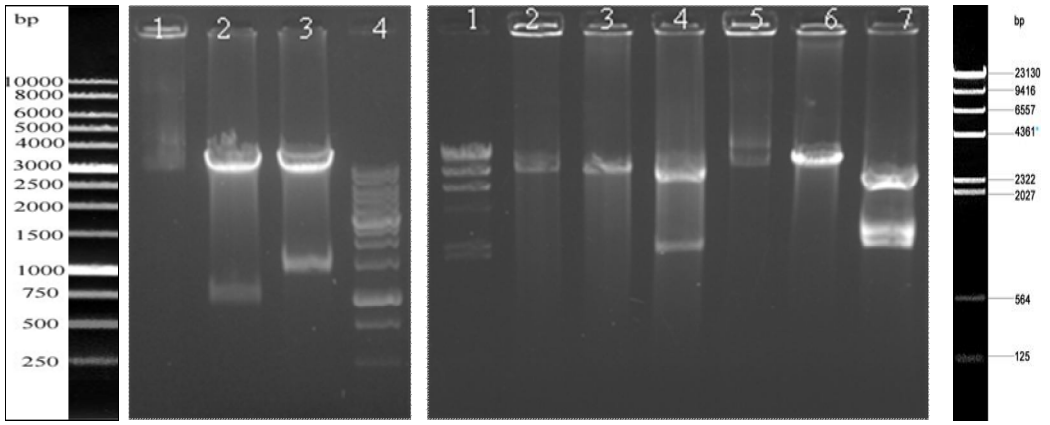


Figure 2.26 PCR amplified product of fragment of gene coding for Nα/β peptide

Lane 2 shows 1038 bp PCR product corresponding with 1 kb marker in lane 1.



(A) **(B)**

Figure 2.27 Confirmation of pRPSRNα/β construct

(A) Lane 1 shows undigested construct. Lane 2 shows insert release of 1038 bp when plasmid was digested with *Kpn*I and *Bam*HI while lane 3 shows insert release of 1468 bp when plasmid was digested with *Xho*I and *Bam*HI. After double digestions the size of released fragments are matching with 1 kb DNA marker in lane 4.

(B) Restriction digestion pattern of pRPSR1 and pRPSRNα/β. Lane 1 shows λ *Hind*III marker. Lane 2 and 5 show undigested pRPSR1 and pRPSRNα/β plasmids respectively. Lane 3 and 6 show *Bam*HI digests of plasmids pRPSR1 and pRPSRNα/β respectively while lane 4 and 7 show *Hind*III digests of plasmids pRPSR1 and pRPSRNα/β respectively. *Hind*III digest of plasmid pRPSR1 shows 2 fragments of size 8333 bp and 2167 bp while *Hind*III digest of plasmid pRPSRNα/β shows 3 fragments of size 6527 bp, 2844 bp and 2167 bp.

2.3.2.5 Functional analysis of fusion protein

Functional analysis of fusion protein was done using β-galactosidase assay. Yeast cells transformed with control plasmid expressing only *lac Z* gene has shown β-galactosidase activity but the cells which were transformed with chimeric construct expressing fusion protein did not show any β-galactosidase activity. β-galactosidase assay of both the above mentioned cultures was carried out at log phase as well as at stationery phase. Yeast culture expressing only β-galactosidase enzyme has shown β-galactosidase activity in both log phase as well as stationery phase while yeast culture expressing fusion protein did not show any β-galactosidase activity in both log phase as well as stationary phase.

These results indicate the possibility of degradation of β-galactosidase enzyme by Nα/β peptide which is present as N terminal in frame tag to the β-galactosidase enzyme. As discussed earlier, in yeast system there are two major destinations for the proteins to be degraded, one is vacuole and another is proteasome. To confirm the destiny of our fusion protein, vacuolar inhibitor (PMSF) was added at log phase and β-galactosidase assay was carried out after 3 hours. If destination of the fusion protein is vacuole then β-galactosidase activity was expected after addition of vacuolar inhibitor. Still no β-galactosidase activity was seen after addition of vacuolar inhibitor. This clearly rules out the possibility of vacuolar involvement in the degradation of fusion protein.

2.3.2.5.1 Proteasomal inhibition studies

For the final confirmation of expressed fusion protein as a substrate of proteasome, proteasomal inhibitor was used.

No β-galactosidase activity was observed with fusion protein while activity was seen in cells carrying only β-galactosidase protein.

2.4 Discussion

Our results, for the first time, establish that entire N terminal α/β barrel domain of yeast ODC attains its native conformation in the absence of rest of protein. This domain can fold independently to gain the tertiary structure at physiological pH. These results are in accordance with the results of protein structure prediction softwares. The Nα/β barrel domain of yeast ODC harbor two degradation signals i.e. Antizyme Binding Element (AzBE) and unstructured stretch of first amino acid residues. As stated earlier, antizyme recognizes Antizyme Binding Element (AzBE) which is situated in the N terminal α/β barrel domain of ODC, destabilizes an entire protein and directs the ODC protein towards proteasome for degradation. A stretch of first fifty amino acid residues of yeast ODC is unstructured in nature. This unstructured region serves the purpose of requirement of an unstructured extension for initiation of actual dragging and degradation by proteasome. The selected peptide

satisfies both criteria for being a portable degradation signal i.e. degron. As the domain has attained tertiary structure, the possibility of recognition by antizyme cannot be ruled out.

After successful structural characterization of Nα/β peptide, it was proposed to check the portability of Nα/β peptide as a degradation tag. Nα/β peptide was tagged at the N terminus of reporter protein i.e. β-galactosidase and stability of reporter protein was checked by performing β-galactosidase assay. Yeast cells expressing only β-galactosidase protein has shown β-galactosidase activity while cells expressing Nα/β-β-galactosidase fusion protein did not show any β-galactosidase activity. β-galactosidase activity was not seen in both log phase as well as stationary phase of cells expressing Nα/β-β-galactosidase fusion protein. The possibility of vacuolar involvement in the degradation of fusion protein was also ruled. Vacuolar inhibitor (PMSF) was added in cells expressing Nα/β-β-galactosidase fusion protein. If vacuole is the destination of proposed fusion protein, then after vacuolar inhibition, β-galactosidase activity was expected in cells expressing Nα/β-β-galactosidase fusion protein. As no β-galactosidase activity was observed, possibility of vacuolar involvement for the degradation of our proposed fusion protein is ruled out.

For the final confirmation of proteasome as the destination of proposed fusion protein, proteasome inhibitor was added in yeast cells expressing Nα/β-β-galactosidase fusion protein. If proposed Nα/β peptide has potential to drag the reporter protein β-galactosidase towards proteasome for degradation, then after proteasomal inhibition β-galactosidase activity was expected from cells expressing Nα/β-β-galactosidase fusion protein. No β-galactosidase activity from these cells suggests the possibility of inclusion formation of expressed fusion protein in the cells.

β-galactosidase is a large protein with tetrameric structure. Another possibility could be structural constraints experienced by β-galactosidase protein itself which cannot be ruled out.

Earlier it was reported that N terminal extension of yeast ODC can target the reporter protein with α helical structure and not β sheet structure. Playing with antizyme levels could focus more light on the existing results.

2.5 Conclusion

The Nα/β peptide of yeast ODC, which includes N terminal overhang and α/β barrel domains attains secondary and tertiary structure in the absence of rest of the protein. This domain can fold independently to gain the tertiary structure at physiological pH. This result indicates the possibility of recognition by an antizyme which is an important pre-degradation step for destabilization of intact ODC protein. In literature large molecular weight proteins are tagged to β-galactosidase enzyme and entire assembly is stable and functional. In the present case the selected peptide is at least not allowing β-gal enzyme to be functional, i.e. it is interfering in the folding of an enzyme or in other word destabilizing it. Actual scenario about degradation of fusion protein inside the cell is not clear but at least fusion protein is not allowing the reporter protein to remain stable or active.

1 Introduction

1.1 Proteins

The smallest organized form of life is a cell. Cell is made up of macromolecules such as carbohydrates, proteins and lipids. Among all these, proteins gain a lot of importance because of their functional diversity, astounding structural variety and physico-chemical properties. By enacting the message hidden in DNA, proteins make a living cell functional. Intricate structures of proteins give proteins their function. Since they are involved in almost all cellular processes directly or indirectly, a highly sophisticated network exists in a cell to maintain homeostasis between their synthesis and degradation.

1.2 Protein Degradation

In cell, protein degradation takes place for elimination of abnormal, nonfunctional, denatured and potentially toxic proteins. This may arise from nonsense or misense mutations, errors in gene expression or post translational damage. Protein degradation is also important in the regulation of cellular metabolism (Wickner et al., 1999), recycling of essential amino acids and provision of nutrients during nutritional deprivation. The process of protein degradation is executed by several proteolytic enzymes i.e. proteases found in both prokaryotes and eukaryotes. There are several proteases and proteolytic signals found in both prokaryotes and eukaryotes as discussed below. The purpose of entire degradation machinery is to maintain the cellular quality control.

1.2.1 Protein degradation in prokaryotes

E. coli has at least five ATP dependent proteases as Lon, FtsH, ClpAP, ClpXP and HslUV. These enzymes appear to have distinct substrate preferences (Gottesman et al., 1998). In prokaryotic systems, HslUV and ClpP are proteases analogous to the 20S proteasome in eukaryotes. HslUV is composed of the HslV protease and the HslU

ATPase. Hsl UV protease is ATP dependent and degrades proteins with the help of chaperone. HslV is made up of two hexameric rings stacked one above the other, with their catalytic sites facing the internal cavity. Association with hexameric rings of HslU on either side, enhances the weak peptidase activity found with HslV several fold. HslU is a chaperon of the Clp/Hsp100 family. HslU has also been shown to recognize a carboxy terminal tag sequence for a substrate called SulA, showing functional similarity with ClpX and ClpA, although the ClpP and HslV proteases are dissimilar in many respects (Sousa et al., 2000).

Carboxy terminal ssrA tagged proteins are actively degraded by ClpA or ClpX and then translocated to the ClpP proteasome for degradation (Keiler et al., 1996; Gottesman et al., 1998) like ubiquitin proteasomal system of eukaryotes. SsrA or transfer messenger RNA (tmRNA) is a bacterial ribonucleic acid that adds a C terminal degradation peptide to nascent polypeptide chains on stalled ribosomes. These peptides are degraded by RNase R (Reynald Gillet and Brice Felden, 2001). tmRNA which is a unique hybrid between a tRNA and a mRNA is exclusively present in eubacteria. Tagging by tmRNA is seen to be operative in three cases, namely, when a translating ribosome encounters a cluster of rare codons while translating an intact mRNA, when a ribosome reaches a termination codon or when there is translational 'read through' at the termination codon due a suppressor tRNA. Sometimes, a stable secondary RNA structure in an intact mRNA may also lead to tagging. tmRNA initially acts as a transfer RNA , whose 3' end is aminoacylated by an alanyl tRNA synthetase. An associated protein SmpB is required for the tmRNA to bind to a stalled ribosome at the 'A' site. The nascent polypeptide is then transferred to the tRNA portion of the alanylated tmRNA and the defective message is replaced by tmRNA ORF. Translation resumes on tmRNA internal ORF having a termination codon. The defective polypeptide I releases and might be degraded subsequently by RNase R. Tagged proteins are released by termination factors. Thus, tmRNA participates in intracellular protein quality control.

In bacteria, degradation signals are generally found to be at the N or C-terminal, which are recognized by the target protease itself. Studies have shown the presence of Tail

Specific Proteases (Tsp) in the periplasm of *E.coli* (Keiler et al., 1996). Tsp is an endoprotease that cleaves proteins at internally discrete sites, specifically degrading proteins of C-terminal tails having WVAAA residues. C terminal tails having small uncharged residues (Ala, Cys, Thr, Ser, Val) are preferred for cleavage by Tsp. Non-polar residues are also preferred in the second and third positions, but larger and more hydrophobic side chains are also cleaved. Tsp directly recognizes folded proteins by using a binding site a free α carboxyl group, at the last three positions of the substrate. Since most damaged or misfolded proteins do not have an intact C terminal sequence, the Tsp protease probably does not remove such abnormal proteins from the cell. Recent studies have shown the presence of a peptide tagging system, where proteins translated from damaged mRNA are modified by C terminal addition of a peptide with the sequence AANDENYALAA and then presented to the Tsp protease for degradation.

1.2.2 Protein degradation in eukaryotes

Eukaryotic protein degradation can be subdivided into specific and nonspecific protein degradation. Specific protein degradation is carried out by a multimeric 26S proteasome (Hershko and Ciechanover., 1998) while non specific protein degradation is carried out by lysosomes. Proteasomal substrates include short lived or regulatory proteins such as proteins involved in cell cycle, gene transcription and signaling pathways (Pickart., 2001). Proteasomes can also degrade damaged or misfolded polypeptides (Pickart et al., 2004). Lysosomal substrates include long lived proteins as well as major cytoplasmic organelles. Most of the cytoplasmic components are degraded by autophagic lysosomal pathway (Seglen et al., 1992). Present study is focused on selective protein degradation.

1.3 Proteasomal protein degradation

Proteasomes are multicatalytic proteinase complexes or macro machines of the degradation system of cell (Cuervo et al., 1995). Proteasomes are present in nucleus as well as cytoplasm of the cells (Rivette and Knecht., 1993). In literature many studies

have focused on structure of proteasome (Groll et al., 1997; Adams et al, 2003; Unno et al., 2002).

1.3.1 Structure of proteasome

Proteasome is giant macromolecular degradation machinery having size of 2.5 mega Daltons (Coux et al., 1996). 26S proteasomal complex is made up of two different subunits i.e. 20S core particle and 19S regulatory particle (Adams et al., 2003).

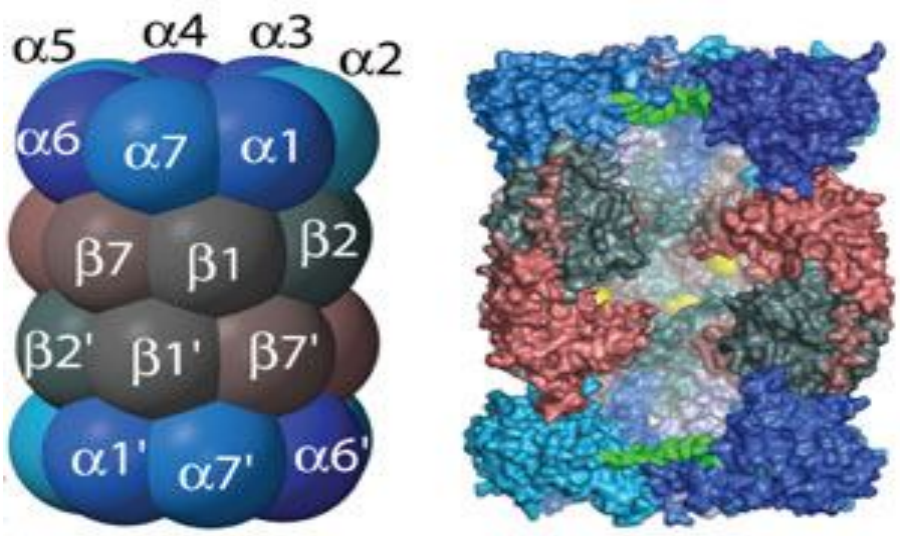


Figure 1.1 Pictorial representation of 20S proteasome (Groll et al., 1997).

1.3.1.1 Core particle

Core particle (CP) is made up of α and β subunits. The barrel shaped structure of 20S core particle is formed by four rings formed by α and β subunits. Two outer rings are formed by seven α subunits in each ring and two inner rings are formed by seven β subunits in each ring (Unno et al., 2002) (Figure 1.1). Out of seven, three β subunits of inner ring i.e. β1, β2 and β5 contain proteolytic active sites. Each active site functions specifically. β1 cleaves at the C terminus of acidic amino acid residue glutamate, β2 cleaves after tryptic residue, while β5 prefers to cleave after hydrophobic residue (Borisssenko et al., 2007). So subunits β1, β2 and β5 possess post glutamyl peptidyl hydrolase (PGH) like, trypsine like and chymotrypsin like activities respectively.

Length of 20S proteasome is 15nm and diameter is 11nm (Groll et al., 1997). Width of degradation channel is 10 to 15 Å. This narrow channel ensures entry of only unfolded polypeptide inside the 20S proteasome thereby increasing the specificity of protein degradation (Larsen et al., 1997).

1.3.1.2 Regulatory particle

Regulatory particle (RP) contains 19 subunits. The subunits are again subdivided into distal lid and proximal base particles (Glickman et al., 1998). Base is composed of 10 components, 6 of which are ATPases (Rpt), Scaffolding proteins Rpn 1, Rpn 2 and ubiquitin receptors Rpn 10 and Rpn 13. Base subcomplex is involved in substrate unfolding before proteasomal entry. Lid subunit is composed of 9 components including a well studied Rpn11 which has deubiquitinating activity (DUB). Lid subcomplex is involved in recognition of substrate and processing of ubiquitin from substrate before proteasomal entry.

1.4 Ways of presenting substrates to proteasome

There are mainly three ways by which substrate protein is presented to proteasome for degradation. Tagging by ubiquitin is the most preferred mechanism used by eukaryotic cells for selective degradation of substrate proteins (Thrower et al., 2000). This is referred as ubiquitin dependent proteasomal degradation. In another way adaptor protein acts as a bridge between substrate and protease and substrate is brought in the vicinity of proteasome (Hartmann-Petersen et al., 2003). Apart from mediator such as ubiquitin tag or adaptor protein, few protein substrates are directly recognized by proteasome for degradation (Orlowski et al., 2003). This is possible because these proteins are equipped with some specific amino acid sequences in their primary structure which can act as degradation signals. Second and third ways of degradation are referred as ubiquitin independent proteasomal degradation.

Proteasomes do not degrade protein into amino acids but into smaller peptides.

1.4.1 Ubiquitin dependent protein degradation

Ubiquitin dependent protein degradation is a complex and tightly regulated process (Wolf et al., 2004). Sequence of events such as initiation of ubiquitination, targeting towards proteasome, degradation and recycling of ubiquitin are discussed below.

1.4.1.1 Initiation of ubiquitination

Many proteins are tagged by ubiquitin for degradation after phosphorylation. Many rapidly degraded proteins contain PEST sequence which serve as phosphorylation sites important for degradation. In some cases phosphorylation at multiple sites is required for ubiquitination (e.g. yeast G1 cyclin Cln3 and Cln2) while for ubiquitination of mammalian G1 regulators cyclin E and cyclin D1 phosphorylation at single specific site is required. IκBα which is an inhibitor of NF-κB transcriptional regulator requires phosphorylation at two specific sites. Different ubiquitin ligases recognize different phosphorylation patterns (Hershko et al., 1998).

N end rule is an important finding which explains a crucial role of N terminal amino acid as a stabilizing or destabilizing residue. Methionine is considered as most stabilizing amino acid residue while proline as most destabilizing residue (Varshavsky., 1996).

Mitotic cyclins and certain cell cycle regulators possess destruction box for the purpose of degradation. Destruction box is 9 amino acid residues long sequence which is located approximately 40 to 50 amino acid residues from N terminus of protein and required for ubiquitination and degradation of protein (Hershko et al., 1998).

1.4.1.2 Ubiquitination and proteasomal degradation

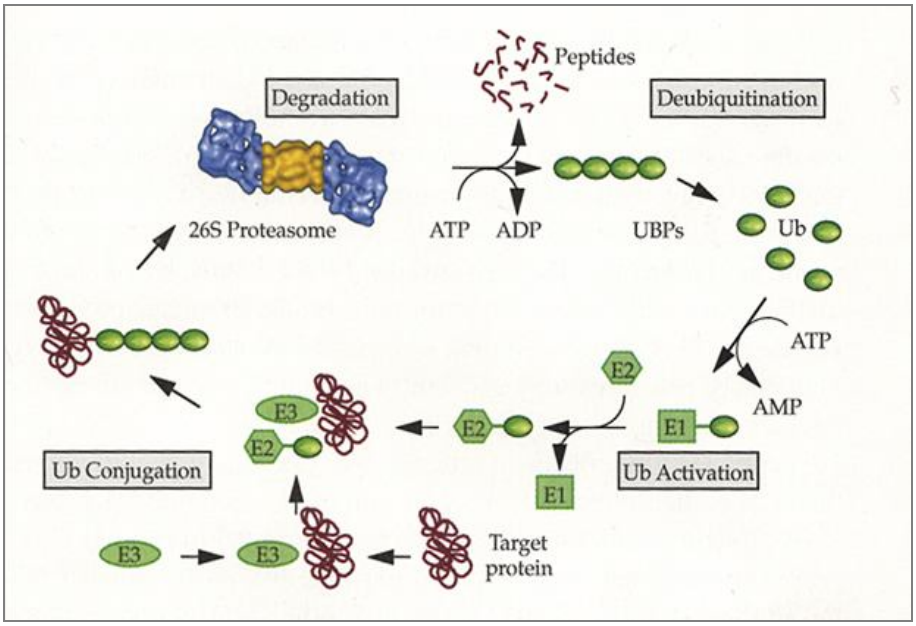


Figure 1.2 Ubiquitin mediated proteasomal degradation of protein.

The process of tagging the substrate by ubiquitin is executed by three different types of enzymes named as E1, E2 and E3 (Figure 1.2). E1 is ubiquitin activating enzyme, E2s are ubiquitin conjugating enzymes while E3s are ubiquitin protein ligases. A single E1 (Hershko and Ciechanover, 1998; Pickart, 2001) transfers Ubiquitin to approximately 20-30 distinct E2 enzymes (Scheffner et al., 1998),which in turn interact with several E3's (Wilkinson, 2000; Pickart, 2001). The specificity of the system is maintained by approximately 1000 different E3's which interact with specific substrates which may undergo a post translational modification (phosphorylation or oxidation) or an alteration in its structure (denaturation or misfolding). Carboxy terminus of 76th glycine residue of ubiquitin is attached to ε-amino group of lysine residue or amino terminus of target protein to be degraded by isopeptide or peptide bond (Varshavsky., 1997).

Further at least three ubiquitin moieties are added on lysine residue in 48th position of first ubiquitin ligated to the substrate protein. This substrate protein tagged with polyubiquitin chain is presented to the proteasome for degradation. Lid subunit of regulatory particle recognizes the polyubiquitin chain on substrate protein. Rpn 10 and

Rpn 13, subunits of regulatory complex, are involved in recognition of ubiquitin tag (Deveraux et al., 1994). Subunits Rpn 10 in yeast and S5a in mammals are localized at base lid interface of 19S particle. The interaction of these recognition subunits occurs through ubiquitin interacting motif (UIM) domain (Hofmann et al., 2001). Other subunits such as Rad 23 and Dsk 2 were also found to be involved in recognition of multiubiquitin chain and presenting the substrate to proteasome (Rao et al., 2002). Once polyubiquitin chain is recognized by proteasome, an unstructured region is required by proteasome to thread the polypeptide chain into proteolytic core (Prakash et al., 2004). The process of unfolding of substrate protein so as to enter in proteolytic core is catalyzed by regulatory particle of proteasome. At the same time trimming of polyubiquitin moiety from substrate protein by de-ubiquitinating enzymes is required for recycling of Ubiquitin. Deubiquitinating enzymes do not allow polyubiquitin chain to enter in proteolytic core (Yao et al., 2002). The process of unfolding is energy dependent (Lee et al., 2001; Rotanova et al., 2008). Stages of unfolding and translocation of protein targets are coupled to ATP hydrolysis. Once a small unstructured segment of polypeptide chain enters in core particle, remaining portion is taken up smoothly and subjected to degradation (Wolf et al., 2004). In this way, multitasking machine of the cell works for protein degradation as shown below in figure 1.3.

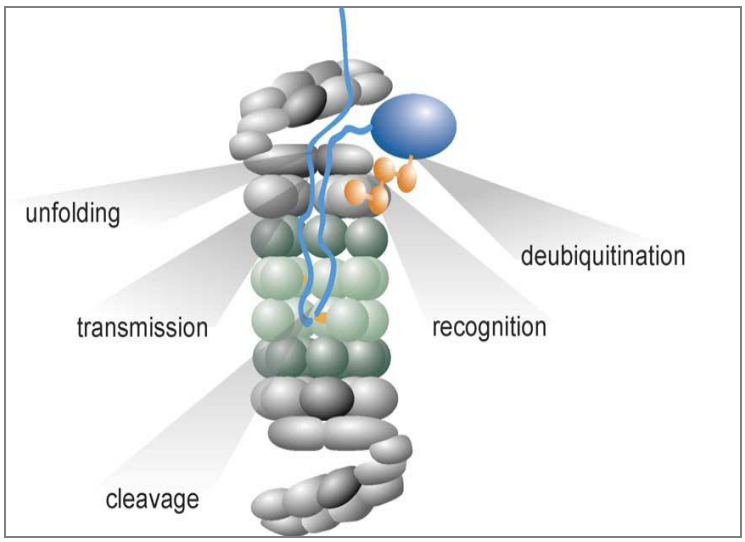


Figure 1.3 Execution of protein degradation process by proteasome in various steps.

Membrane and secretory proteins are folded in endoplasmic reticulum (ER). Nascent proteins which fail to fold in ER are degraded by ER associated degradation (ERAD) (Brodsky et al., 1999). Substrates of ERAD are translocated across the membrane of ER and degraded by cytoplasmic proteasomes (Kopito et al., 1997).

An important step in ubiquitin mediated proteasomal degradation of protein is recycling of ubiquitin moieties. Homeostasis of ubiquitin itself is described in detail in chapter 5.

1.4.1.3 Role of adaptor proteins

As discussed above Rpn 10 in yeast and S5a in mammals (subunits of regulatory complex) are involved in recognition of ubiquitin tag. Events of recognition of polyubiquitin chain, dragging of substrate protein inside the core particle and deubiquitination are carried out by proteasomal assembly. So the first event i.e. recognition of polyubiquitin chain is important to start with degradation process. This indicates Rpn 10 or S5a are indispensible for degradation. But yeast strains with deletion mutants of genes coding for these recognition subunits were viable and showed impaired protein degradation. This has given a new notion that apart from proteasomal subunits, another set of proteins exist which are involved in recognition and presenting the ubiquitinated proteins to proteasome (Rao et al., 2002). Two such extra proteasomal recognition proteins i.e. Rad23 and Dsk2 were studied. Rad23 and Dsk2 can bind ubiquitinated substrate and also to proteasome and act as bridge between proteasome and its substrate (van Laar et al., 2002). Rad 23 and Dsk 2 utilize UBA (Ubiquitin pathway Associated) domain to interact with ubiquitin chain. Ubiquitin interaction site is situated in a hydrophobic patch of UBA domain (Mueller et al., 2002). This UBA domain has weaker affinity for free monoubiquitin moiety indicating specificity of these adaptor proteins for polyubiquitinated substrates. Elsasser et al has explained an important finding that Rad23 and Dsk2 interact with Rpn1 subunit of 19S particle (Elsasser et al., 2002). Another important finding explains that Rpn10, a proteasomal subunit of yeast exists in two forms i.e. as a proteasomal subunit and also as a free form (Wilkinson et al., 2000).

1.4.1.4 Role of unstructured region for initiation of degradation

A large number of proteins involved in transferring the substrate protein to the 26S proteasome were studied in detail. Along with ubiquitin, subunits of proteasome and proteasome associated proteins another requirement to initiate the actual degradation process is presence of an unstructured region. Only recognition of ubiquitinated protein by proteasomal assembly is not sufficient but second degradation signal in the form of an unstructured region is also required (Prakash et al., 2004). Unstructured region acts as initiation site for degradation. It was proposed that unstructured region engages the unfolding machinery of proteasome. As discussed above in 'Role of adaptor proteins' Rad 23 is an adaptor protein which can bind to ubiquitinated substrates as well as proteasome. It associates with proteasome but escapes degradation (Schauber et al., 1998). This is because it lacks an effective initiation region. So proteasome can not engage the protein and unfold it. Several internal unstructured regions are present in Rad 23 but these are too short to act as initiation regions for unfolding. The short initiation regions cannot solve the purpose of docking in proteasome. Lack of effective initiation region renders it stability against degradation. A fusion protein of Rad23 along with DHFR is also stable. When a longer unstructured tail was attached to the C terminus of Rad23 the fusion protein is degraded efficiently (Fishbain et al., 2011). This could be explained by availability of sufficient length initiation region for proteasomal docking.

Efficiency of proteasomal degradation also depends on the structure of polypeptide adjacent to unstructured initiation region. If initiation region is followed by α helical region, polypeptide is degraded efficiently and if initiation region is situated after β sheet structure, degradation is impaired (Lee et al., 2001; Godderz et al., 2011). Experiments by Inobe et al revealed the details of length of unstructured initiation region required for initiation of degradation. Minimum length required for efficient degradation of reporter protein DHFR was checked by tagging 4 ubiquitin moieties and single UbL domain at N terminus of DHFR by in frame fusion. In case of Ub_4 tagged DHFR, protein with initiation region having length of 34 or more amino acid residues was degraded while protein with initiation region having length of 29 or less amino acid

residues was stabilized. In case of UbL tagged DHFR, protein with initiation region having length of 44 or more amino acid residues was degraded while protein with initiation region having length of 34 or less amino acid residues was stabilized (Innobe et al., 2011). These results indicate mutual contribution of tag and initiation region for efficient degradation of any protein.

Apart from significant contribution in the initiation of degradation process further details of unstructured region are described in detail in section 1.7 under intrinsically disordered proteins or regions.

Hsp 70 chaperon was also found to associates with proteasome via Bag1 protein for the delivery of aggregation prone substrates for degradation (Bimston, et al., 1998).
Some other proteasome associated proteins having deubiquitinating activity are also discussed including Uch37, Ubp6, Hul5 (Finley, 2009).

1.4.2 Ubiquitin independent protein degradation

There are some proteins which are degraded by proteasome but do not require ubiquitin modification (Hoyt et al., 2004; Orlowski et al., 2003,). These include ornithine decarboxylase (ODC) (Murakami et al., 1992), thymidylate synthase, calmodulin, c-Jun, TCR α subunit and troponin C (Jariel-Encontre et al., 1995; Tarcsa et al., 2000; Benaroudj et al., 2001; Sheaff et al., 2000; Asher et al., 2002).
Present study is focused on ornithine decarboxylase (ODC). ODC is well known example of ubiquitin independent proteasomal substrate. Degradation signals which lie inside ODC itself along with another protein termed as antizyme are sufficient to carry out degradation of ODC without the help of ubiquitin. Thus in case of ODC degradation, targeting role of tag is performed by antizyme. Unstructured region plays an important role in targeting protein to proteasome.

Proteins are degraded by 26S as well as 20S proteasomes in ubiquitin independent manner. Protein degradation by proteasome is also observed in eubacteria and archaebacteria (Zewick et al., 2000). Examples of proteasomal substrates include natural

unfolded proteins, some short-lived regulatory proteins, long-lived proteins, and oxygen-damaged, misfolded, mutated, or damaged proteins. Targeting of substrate towards 20S proteasome is brought by sequence within substrate itself or accessory molecule (Orlowski et al., 2003).

Janse et al proposed a different notion that once a substrate is localized to the proteasome additional signals are not required for degradation to occur (Janse et al., 2004). Proteasomal degradation of proteins without the help of ubiquitin is referred as one of the evolutionarily conserved mechanism (Hoyt et al., 2004).

1.4.2.1 An event of unfolding

During ATPase cycle the ring structures formed by AAA^+ modules undergo significant conformational changes resulted in mutual rotation of these rings changes in size of their axial opening. So AAA^+ proteins transform energy of ATP binding and hydrolysis into own mechanical movements. This develops tensions in associated protein molecules which results in unfolding of polypeptide chains (Prakash et al., 2004).

As mentioned in above paragraphs, specific as well as non specific protein degradation machinery of cell works for protein degradation.

1.5 The need for Targeted Protein Degradation

The purpose of such complicated degradation machinery is to maintain quality control in the cell. Studies on molecular mechanism of many diseases established protein degradation to have a critical role. Either mutation or accumulation of proteins in cell may lead to defective cellular processes. Failure of degradation of such proteins by UPS system results into diseases like the Liddle syndrome, certain kinds of cervical cancers, Parkinson's, Alzheimer's, Huntington's, cystic fibrosis, and multiple myeloma (Lowe et al., 1988; Ciechanover et al., 2003). To combat such diseases where protein degradation is defective, attempts are being made to develop targeted or specific protein degradation. Manipulations at gene level such as gene knockout or antisense oligonucleotides and small interfering RNA (si RNA) were found to have various drawbacks.

This prompted attempts for manipulation of protein degradation pathway of proteasome. Probing the pathways of targeted protein degradation has been subject of much attention in biomedical, pharmaceutical and microbial disciplines.

1.5.1 Drawbacks of Ubiquitin-Proteasome system

Efforts to develop targeted protein degradation with ubiquitin-proteasome system have not met with much success. The process of ubiquitination requires the action of 3 different enzymes, namely E1, E2 and E3. E3s are highly substrate specific to carry out ubiquitination. The 3D structure of E3 ligase complexes with their substrate protein reveals a precise geometric relationship between substrate & ligase. Further, E3s are specific to certain E2s for catalytic addition of ubiquitin onto susceptible lysine residues in target protein. These structural constraints suggest that attempts to induce ubiquitination of target proteins may prove difficult because of incompatibility between substrate & ligase (Matsuzawa et al., 2005).

Direct method for targeted protein degradation without ubiquitination could overcome this limitation. A small number of proteins have been identified which act as substrates of proteasome though they do not require ubiquitin for their degradation (Marian et al, 2003, Coffino et al, 2004). Ornithine Decarboxylase (ODC) being one of them undergoes ubiquitin independent proteasomal degradation pathway (Murakami et al., 1992). ODC is an unusual proteasomal substrate as it is degraded by ubiquitin independent mechanism. Some degradation signals lie within the ODC, which along with another protein termed as antizyme, target it for degradation (Coffino, 2001). Antizyme recognizes a stretch of 24 amino acids known as Antizyme binding element or AzBE in the α/β barrel domain of monomer and destabilizes ODC. The last 37 amino acids in the C terminus of mammalian ODC help in docking the protein on proteasome. Cys 441 in the C terminus, if mutated, stabilizes ODC (Coffino et al., 2007). The yeast ODC has two intrinsic degradation determinant signals, namely the N-terminal region of 1-50 residues and Antizyme Binding Element (AzBE) present on the surface helices of the α/β barrel

domain, from 164 to 187 residues. The stretch of last 37 amino acids of mammalian ODC which helps in docking the protein on proteasome is not present in yeast ODC. There are evidences of N terminal being important for degradation via Antizyme (Gandre et al., 2002). The N terminal region that is unique to yeast ODC acts similar to the C-terminal region of the mammalian enzyme in mediating its recognition by the proteolytic machinery. All these signals make ODC a favourite system for present study.

1.6 Ornithine Decarboxylase

Ornithine Decarboxylase (ODC) is the first enzyme in polyamine biosynthesis pathway (Tabor et al., 1984). The rate limiting step in polyamine biosynthesis is conversion of ornithine to putrescine which is catalyzed by ODC. Putrescine is then converted into spermidine and spermine. Spermine is most effective in supporting biological processes. ODC is approximately 450 amino acids long protein having molecular weight of 52kD and requires cofactor Pyridoxal 5'-Phosphate (PLP) for activity. Structure of mammalian (human and mouse) Ornithine Decarboxylase (ODC) is as shown below (Figure 1.4A and 1.4B)

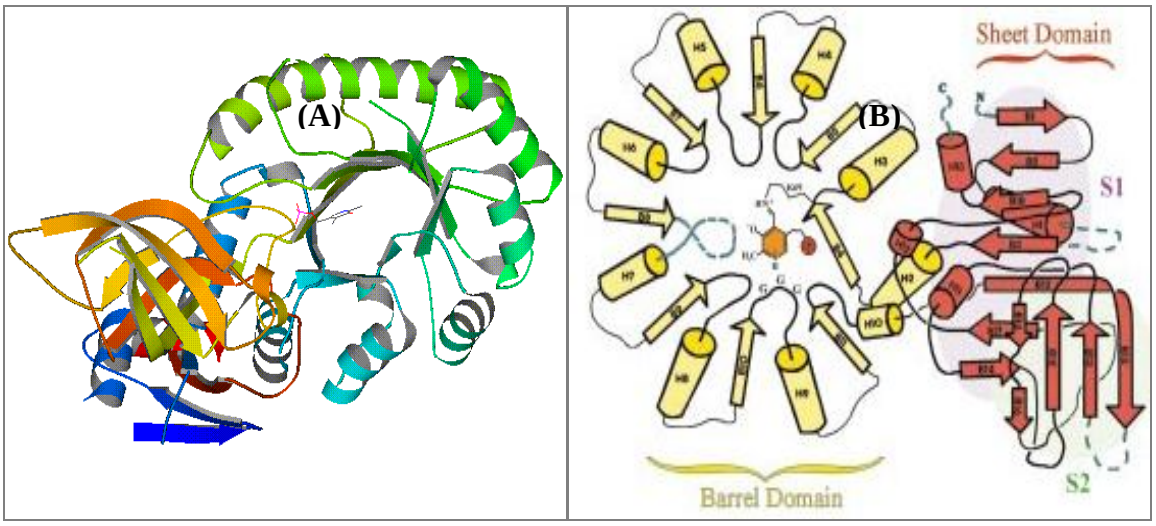


Figure 1.4 Structure of mammalian ODC

Figure A shows 3D ribbon model of human Ornithine Decarboxylase (ODC) at 2.1 Å (Almrud et al., 2000). Figure B shows features of secondary structure of mouse ODC (Kern et al., 1999).

Human ODC is active as homodimer having two fold symmetry. Homodimer is formed by head to tail interaction of two monomers. N terminal domain of one monomer interacts with C terminal domain of another monomer and vice versa. Two active sites are formed at the interface of two monomers in a dimer. Monomer of ODC consists of two domains i.e. N-terminal α/β barrel domain and C- terminal β sheet domain. The N terminus has 9 α helices and 8 β-strands. Barrel domain begins with helix H2 and ends with helix H10. β Sheet domain is composed of two sheets i.e. S1 and S2 and two helices i.e. H11 and H12. Perpendicular strands of sheet S1 and S2 forms a central hydrophobic core. Sheet S1 is composed of 6 strands which are named as B2, B18, B3, B12, B17 and B14. Sheet S2 is composed of four strands which are named as B12, B13, B15 and B16. Strand B12 connects sheet S1 to sheet S2. The loops connecting helices and strands are described as disordered loops. Protease sensitive loop is present over monomer-monomer interface towards the interior of the dimer. Lysine residue at 169^{th} position is involved in salt bridge with aspartic acid residue at 364^{th} position. Crystal structures of truncated mouse ODC and *Trypanosoma brucei* ODC were also studied (Kerl et al., 1999; Grishin et al., 1999). Primary sequence alignment and structural comparison of human ODC, truncated mouse ODC and *Trypanosoma brucei* ODC show extensive sequence and structural identity between these 3 eukaryotic ODCs. Sequence of active sites is also conserved between these 3 ODCs. 16 residues involved in active site of ODC are K69, D88, R154, K169, H197, G235-237, R274, R277, Y323, D332, Y389, C360, D361 and N398. The region from 117^{th} to 140^{th} amino acid residues is Antizyme binding element (AzBE) the region where antizyme binds and destabilizes ODC.

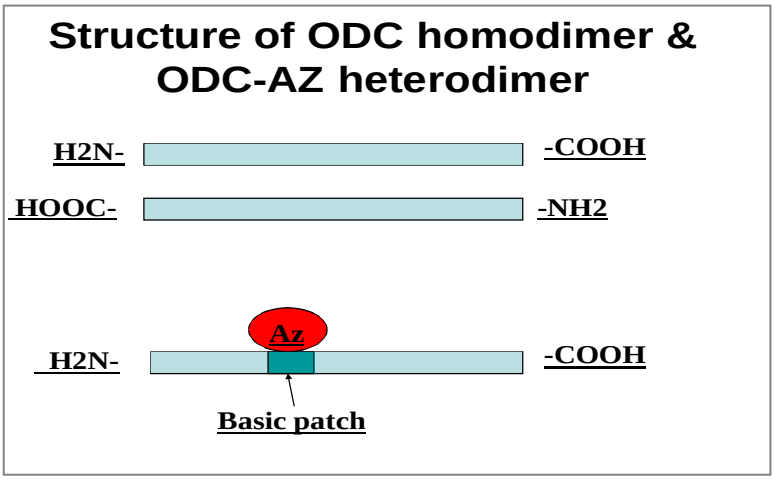


Figure 1.5 ODC homodimer and ODC-AZ heterodimer

ODC homodimer is formed by head to tail interaction of two monomers. ODC-Az heterodimer is formed by interaction of antizyme with AzBE which is a basic patch situated in N terminal α/β barrel domain of ODC.

AzBE region is a basic patch which is buried in dimeric ODC, so antizyme does not bind homodimeric ODC. Major electropositive patch of AzBE is composed of K115, K121, and R144 on 5^{th} and 6^{th} helices. Residues 428-461 are described as disordered in crystal structure of human ODC. These last 37 amino acid residues which are unstructured in nature possess basal degradation element for the degradation of ODC. This C-terminal tail of mammalian ODC is buried in structural core of monomeric as well as dimeric states of ODC. This prevents early degradation of ODC. Binding of antizyme at AzBE region induces a conformational change in β sheet domain in monomer ODC. This results in exposure of C-terminal tail which initiates the degradation of ODC. Thus binding of antizyme makes ODC susceptible to proteolysis through conformational changes. As smaller part of surface area is buried in ODC-ODC homodimer formation, ODC dimer is easily dissociable. This is important for the regulation of ODC by antizyme.

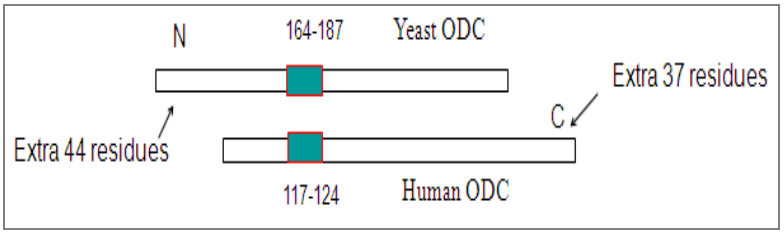


Figure 1.6 Comparison of human and yeast ODC.

As discussed earlier, mammalian ODC has C terminal unstructured region. In yeast ODC first 50 amino acid residues form unstructured stretch. In mammalian ODC AzBE region is situated at 117 to 140 amino acid residues while in yeast ODC AzBE region is present from 164 to 187 amino acid residues (Porat et al., 2008) (Figure 1.6).

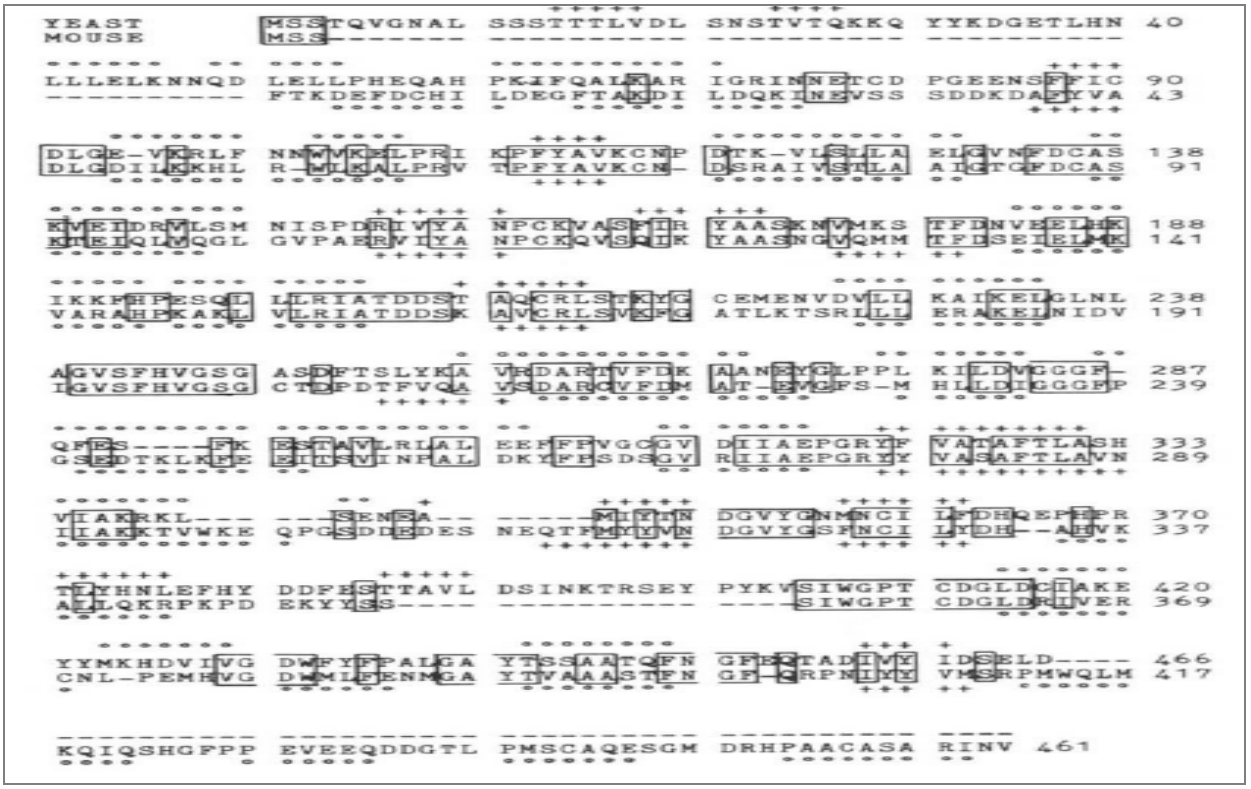


Figure 1.7 Comparison of secondary structural features of yeast and mouse ODC (Fonzi et al., 1987).

Yeast ODC amino acid sequence:

```
        10         20         30         40         50         60
MSSTQVGNAL SSSTTTLVDL SNSTVTQKKQ YYKDGETLHN LLLELKNNQD LELLPHEQAH

        70         80         90        100        110        120
PKIFQALKAR IGRINNETCD PGEENSFFIC DLGEVKRLFN NWVKELPRIK PFYAVKCNPD

       130        140        150        160        170        180
TKVLSLLAEL GVNFDCASKV EIDRVLSMNI SPDRIVYANP CKVASFIRYA ASKNVMKSTF

       190        200        210        220        230        240
DNVEELHKIK KFHPESQLLL RIATDDSTAQ CRLSTKYGCE MENVDVLLKA IKELGLNLAG

       250        260        270        280        290        300
VSFHVGSGAS DFTSLYKAVR DARTVFDKAA NEYGLPPLKI LDVGGGFQFE SFKESTAVLR

       310        320        330        340        350        360
LALEEFFPVG CGVDIIAEPG RYFVATAFTL ASHVIAKRKL SENEAMIYTN DGVYGNMNCI

       370        380        390        400        410        420
LFDHQEPHPR TLYHNLEFHY DDFESTTAVL DSINKTRSEY PYKVSIWGPT CDGLDCIAKE

       430        440        450        460
YYMKHDVIVG DWFYFPALGA YTSSAATQFN GFEQTADIVY IDSELD
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Figure 1.8 Amino acid sequence of yeast ODC.

1.6.1 Ubiquitin independent antizyme mediated proteasomal degradation of ODC

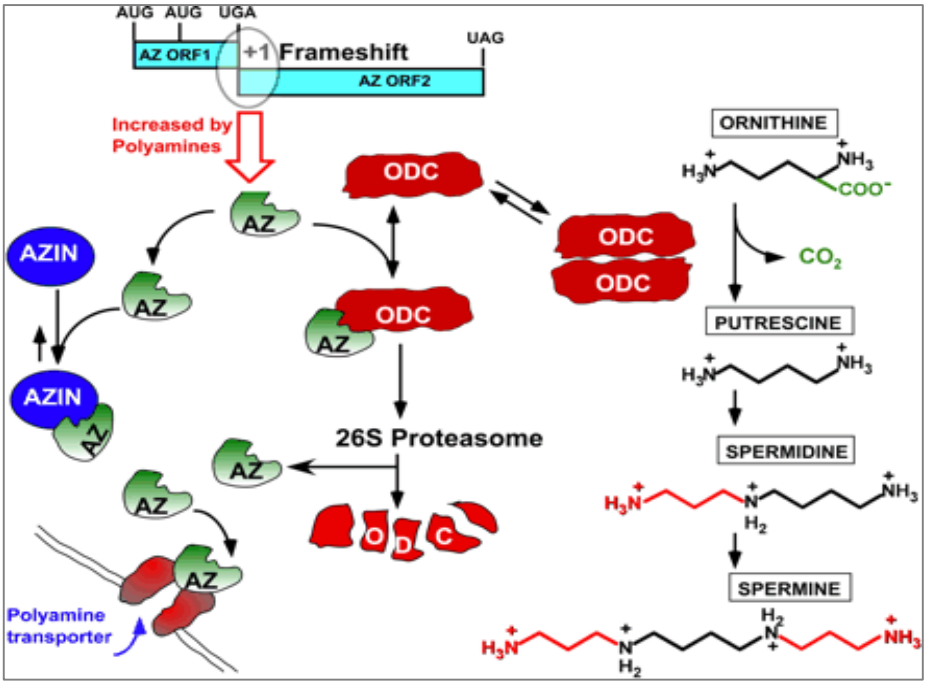

Figure 1.9 Antizyme mediated degradation of ODC (Pegg et al., 2006)

Degradation of ODC is a tightly controlled process because ODC catalyses first step of polyamine biosynthesis. Polyamines are multifunctional organic bases. They play an important role in cell growth, differentiation, apoptosis and malignant development (Pegg et al., 1986). Positively charged polyamines bind DNA and stabilize it. At physiological pH amino group of polyamine is protonated and pairs with negatively charged molecules. Spermine is most and putrescine is least effective in biological processes in which polyamines are involved because functions of polyamines depend on their electrostatic interation. As polyamines are involved in crucial mechanisms of cell, the level of polyamines is maintained strictly. First step of polyamine biosynthesis catalyzed by ODC is regulatory. ODC has extremely short half life of several minutes to one hour. Subcellular localization of ODC is cell type specific and also depends on physiological conditions such as growth, differentiation, malignant transformation and apoptosis. ODC is mainly found in cytoplasmic staining while antizyme is found in nucleus in immune staining studies (Raymond et al., 2004).

In eukaryotic cells selective degradation of proteins occur via ubiquitin modification. But there are some examples of protein including ODC, which are substrates of proteasome but do not require ubiquitin modification. Being a crucial regulator of polyamine levels a different mechanism is developed by the cell for regulation of ODC.

Degradation of ODC is well studied and characterized. In vivo and in vitro studies have proven that there is no role of ubiquitin in the degradation of ODC (Bercovich et al., 1989, Glass et al., 1987).

Level of ODC is regulated by feedback mechanism (Figure 1.9). ODC catalyzes synthesis of putrescine from ornithine. Increased level of polyamines stimulates the synthesis of antizyme by inducing translational frameshifting. Antizyme binding to ODC is reversible. Polyamine responsive element is present at 5' upstream of first open reading frame of antizyme. When present above threshold level polyamines recognize polyamine responsive element and translation of antizyme is initiated. As discussed under session 1.6.2, there are two open reading frames for antizyme synthesis. First open reading frame

has stop codon. Second reading frame does not have any start codon to start translation. Translational frameshifting should occur for the synthesis of full length active antizyme protein. Before reading the stop codon of first open reading frame, ribosome shifts forward by one base pair and continues translation. This shifting escapes the stop codon and continues with second open reading frame till the end so as to translate entire antizyme protein. In this way increased polyamine level induces synthesis of antizyme. ODC shows more affinity towards antizyme than ODC-ODC homodimer. Thus in presence of antizyme ODC-ODC homodimer is dissociated and ODC-Az heterodimer is formed. AzBE region where antizyme binds with ODC is buried in dimeric state. After dissociation of dimer, AzBE region is exposed in monomeric ODC. Antizyme binds AzBE and induces conformational change in ODC. In monomeric ODC, C terminal tail is buried inside the structural core of the protein. Interaction of antizyme with ODC induces some conformational changes in ODC because of which the buried C terminal tail flips out. This unstructured region acts as docking site for proteasomal entry. After interaction with ODC, antizyme not only destabilizes but directs ODC towards proteasome for degradation. Recognition element situated in regulatory particle of 26S proteaome recognizes antizyme bound ODC. C terminal tail is first taken up by proteasome and then an entire ODC is dragged inside the core particle for degradation. Antizyme is recycled in this process like ubiquitin. Detailed analysis of this degradation of mammalian ODC revealed three important regions inside ODC which play an important role in degradation of ODC itself. These degradation signals within ODC are AzBE (Antizyme Binding Element), last 37 amino acid residues which are unstructured in nature and cysteine at 441st position. These three degradation signals are known to be involved in efficient degradation of mammalian ODC. AzBE is the region where antizyme binds and directs ODC towards proteasome for degradation. C terminal tail of last 37 amino acid residues serves the requirement of unstructured region for initiation of actual degradation process. Role of cysteine at 441st position is not clear exactly but mutation at this position made ODC stable. Cysteine residue when situated at another positions apart from 441st position also made ODC stable (Hoyt et al., 2008).

A study by Matsuzawa et al. has shown that peptides and proteins attached to ODC undergo degradation (Matsuzawa et al., 2005). This portability of ODC as a degradation tag is contributed by degradation signals within ODC.

1.6.2 Antizyme

Antizyme is a mediator of ubiquitin independent proteasomal degradation of ODC (Coffino, 2001). C terminal half of antizyme molecule is involved in interaction with antizyme binding element i.e. AzBE situated in N terminal α/β barrel domain of ODC. But only interaction of C terminal part of antizyme with ODC is not sufficient to direct the proteasomal entry. N terminal half of antizyme is involved in directing the ODC-Az complex towards proteasome. Residues 106 to 212 are involved in interaction with ODC while residues 55 to 105 are involved in directing ODC-Az complex towards proteasome. First 70 amino acid residues are not involved in degradation process but are important for transport of Az to various locations (Pegg, 2006).

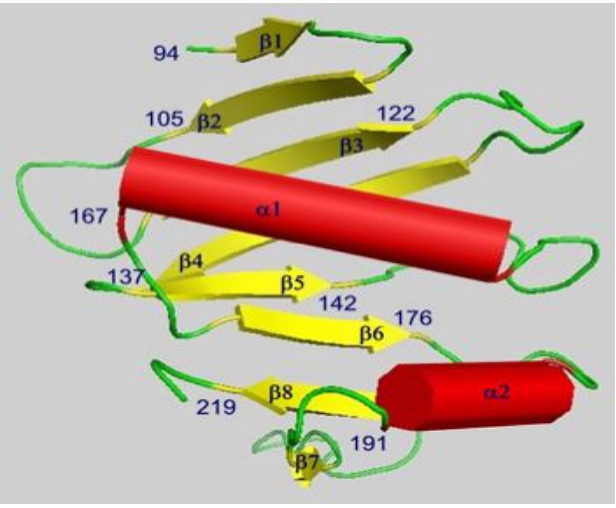

Figure 1.10 Structure of antizyme (Hoffman et al., 2005).

The expression of antizyme mRNA is interesting. There are two open reading frames for antizyme synthesis. First open reading frame has stop codon while the second reading frame does not have any start codon. Event of frame shifting is needed as second ORF

does not have any start codon. Without frame shifting only part of antizyme protein would be synthesized which is coded by first ORF. The efficiency of +1 translational frame shifting is required for synthesis of entire antizyme protein (Figure 1.11). An event of Translational frame shifting is stimulated by increased polyamine levels. Increased ODC activity leads to increased polyamine levels which in turn stimulates antizyme synthesis. Increased antizyme causes ODC degradation. Thus, ODC is regulated by feedback mechanism.

Mechanism of Translational frame shifting- Frame shifting occurs at stop codon of first open reading frame of antizyme.

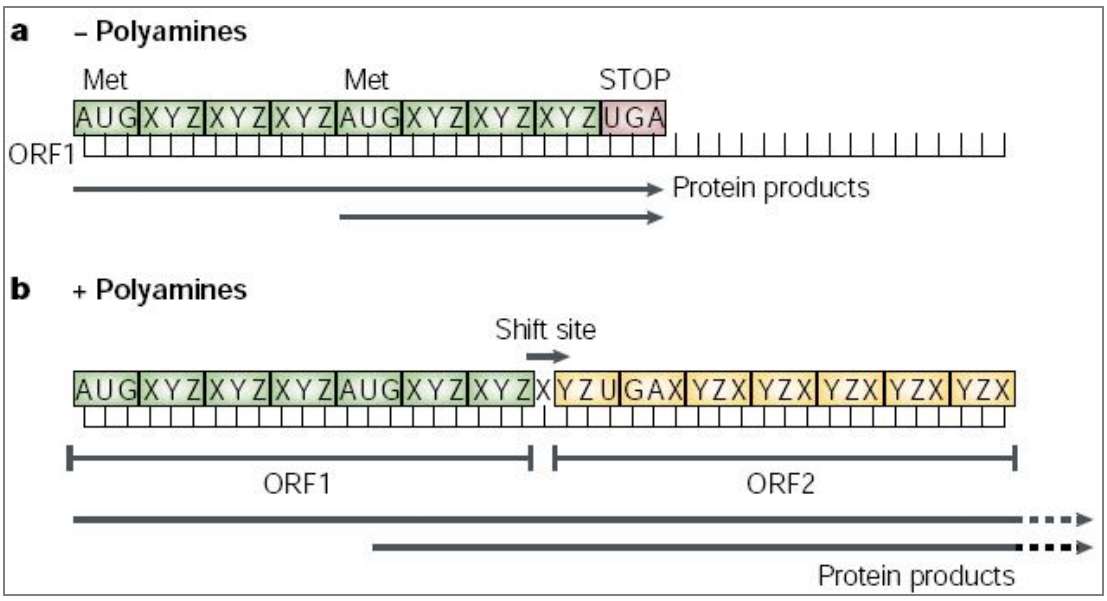

Figure 1.11 Frameshifting needed for antizyme synthesis.

In vertebrates generally the last codon is UCC. This is followed by UGA which is a stop codon. After decoding UCC which is a codon for serine, the ribosome shifts frame by +1 and continues translation. So instead of reading UCCUGA, ribosome reads as UCCGA and next codon's C. The frame shifting site in *Saccharomyces cerevisiae* antizyme mRNA is GC-GCG-UGA-C (Palanimurugan et al., 2004). Highly conserved nucleotides are involved in stimulation of frame shifting. These nucleotides are CA pair which occurs

immediately at three prime of UGA stop codon. Number of study has shown that nucleotide immediately 3' of stop codon is important because it determines the efficiency of translation termination. The CA pair following the stop codon decreases termination efficiency and stimulates +1 frame shifting. In *S. cerevisiae* decoding is carried out by rare arginine as acceptor tRNA and involves a translational pause and facilitates frame shifting (Pande et al., 1995). *S. cerevisiae* does not have a tRNA whose anticodon can form full Watson-Crick or wobble, pairing with GCG. Instead of general pairing anticodon of alanine tRNA uses unusual purine-purine opposition. This poor pairing of codon and anticodon favours frame shifting.

Till now four types of antizyme are described and named as Az1, Az2, Az3 and Az4. Az1 is potent regulator of ODC and has wide tissue distribution. Az1 has molecular weight of 26kD. Mouse Az1 is 227 amino acid residues long while human ODC has 228 amino acid residues (Tewari et al., 1994). Az2 has also equal tissue distribution but is less abundant. Az3 is found only in male germ cells in post meiotic stage of their differentiation to mature sperm. Less is known about Az4. Az1 and Az2 differ in their capacity to direct proteasomal degradation. It was studied that sixteen fold lower concentration of Az1 catalyzed significant degradation as compared to Az2.

Targeted protein degradation using antizyme has shown that proteins attached to antizyme undergo degradation by proteasome (Li et al., 1996).

The crystal structure of mammalian ODC is available. However yeast ODC protein structure has not come in focus by far. Present study tries to reveal the structural aspects as well as unstructured region of degradation signals of yeast ODC.

1.7 Intrinsically Disordered Proteins or Regions (IDPs or IDRs)

Structure of myoglobin and lysozyme were revealed by using X-ray crystallography before 50 years (Kendrew et al., 1958; Blake et al., 1965). Till then structures of large number of protein were elucidated. Different techniques such as NMR spectroscopy, crystallography, CD (Circular Dichroism) reveal the 3D structure of any protein.

Structure function relationships of proteins are a major research area in biophysical, biomedical as well as pharmacological studies. Though the 3D structure of any protein is important, often some regions remain unfolded or unstructured (Gast et al., 1995; Uversky et al., 1999; Boublik et al., 1970). Earlier it was believed that polypeptide should at least gain some 3D structure to function. But it is not the case that entire polypeptide chain always gains some secondary or tertiary structure before being functional. Many a time some region of polypeptide gains some conformation while few regions are without any conformation. These unfolded regions are called as disordered regions or simply unstructured stretches. These unstructured regions are equally important as structured regions in any protein as far as function is concerned (Huber et al., 1983). Rather the conformational flexibility confers functional ease significantly rather than a rigid structure (Harauz et al., 2009). Most of the proteins have disordered region, in fact half of the eukaryotic proteins have long disordered regions especially the signaling proteins. There are few examples of proteins which are fully disordered yet functionally important such as Myelin Basic Protein (MBP) (Harauz et al., 2004), prothymosine α (Gast K et al., 1995) and microtubule formation promoting Tau protein (Schweers et al., 1994). Unstructured regions are needed in variety of functions such as sequence recognition, molecular ineraction (Pontius et al., 1993) and protein degradation (Prakash et al., 2004; Zhang et al., 2004). IDPs are often involved in regulatory/ signaling interactions with multiple partners that require high specificity and low affinity (Uversky et al., 2005). Uversky listed some crucial roles of IDPs which include regulation of cell division, transcription and translation, signal transduction, protein phosphorylation, storage of small molecules, chaperone action, and regulation of the self-assembly of large multiprotein complexes such as the ribosome. The functions of IDPs are grouped into four broad classes: 1) molecular recognition 2) molecular assembly 3) protein modification, and 4) entropic chain activities (Uversky, 2010).

Crystallographic study revealed the unstructured nature of C terminal 37 amino acid residues of mammalian ODC (Kern et al., 1999, Almrud et al., 2000). Studies also revealed the crucial role of this unstructured region in the degradation of ODC itself. C

terminal unstructured tail of mammalian ODC acts as a portable degradation signal and directs degradation of protein to which it is tagged. Reporter proteins such as GFP, luciferase, DHFR and Ura3 were found to be destabilized when tagged C terminally with tail of mODC (Li et al, 1998; Zhang et al., 2003; Leclerc et al., 2000; Hoyt et al, 2006).

N terminal stretch of yeast ODC was also found to have a potential to act as degron when tagged to reporter protein Ura3 (Godderz et al., 2011).

After increasing evidences on significance of unstructured regions or proteins a new branch named unfoldomics has emerged which covers study on unfoldome (Dunker et al., 2008).

Unstructured regions do not fold into any specific conformation because of their amino acid sequence. Hydrophobic amino acid residues contribute for folding of polypeptide chain by pushing themselves into inner core of structure. Unstructured regions are rich in polar and charged residues and depleted of hydrophobic residues. Low hydrophobic force is responsible for less driving force for compact structure formation (Uversky, 2010). Role of unstructured regions or IDPs in neurodegeneration and protein dysfunction diseases has focused IDPs as potential drug targets.

1.8 Importance of structural characterization

Structural information of any protein has been matter of utmost significance in the field of structural biology. It is prerequisite in discipline of pharmaceutical research for establishing the mechanism of action of a drug. It gives a visual idea of physical, chemical characteristics of protein by providing information on its secondary and tertiary structure and thus providing base to predict function of a protein. Some proteins are targets for chemotherapy.

Study of domain organization has been one of the important aspect of structural study. Domain is a part of polypeptide chain that can fold and function independently. Domain has a stable tertiary structure with its own hydrophobic core. A single protein may contain one or several domains. Each domain performs one or many distinct biological

functions. This confers tremendous functional capacity to protein moieties. Domains interact with other molecules in the cell and they are also involved in signal transduction pathways. Formation of new domains and new domain combinations indicates an evolutionary mechanism of protein folding. Similarity in domain architecture of two different proteins indicates possibility of common ancestor. So study on domain helps us to understand the protein interaction, protein function and evolution too.

Sometimes unstructured region of a protein also can be a domain and has an important role in protein assembly and function (Prakash et al., 2004). These stretches are involved in initiation of degradation and recognition of other molecules. Every structure in an entire assembled protein is important with respect to its assigned function. Keeping this in mind, the idea was to focus on the structural aspects of degradation determinant signals of yeast ODC.

When protein is in a native state its structure is maintained. But during denaturation process unfolding of the molecule increases. This increases the protein solvent interface and ultimately stabilizes the structure. Large nonpolar regions and peptide bonds become exposed to the solvent and nature of interaction between protein and co-solvent changes. In the present study CD and Fluorescence spectroscopy was used for above mentioned structural characterization.

1.8.1 Structural characterization by Circular Dichroism (CD)

Circular dichroism spectroscopy is used to gain information about the secondary and tertiary structure of proteins and polypeptides in solution. The chromophores for the protein study include the peptide bond (absorption below 240 nm), aromatic amino acid side chains (absorption in the range 260 to 320 nm) and disulphide bonds (weak broad absorption bands centred around 260 nm). Different types of secondary structure (i.e. α helix, β sheet, turn) found in proteins give rise to characteristic CD spectra in the far UV region (figure 1.12).

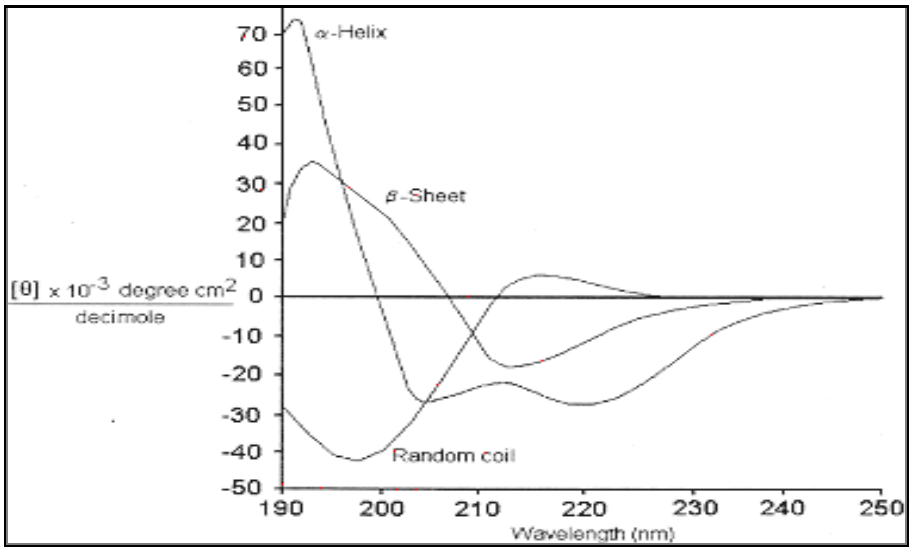

Figure 1.12 Standard CD spectra of α helix, β sheet and random coil.

1.8.2 Structural characterization by Fluorescence spectroscopy

Fluorescence has an important role in the structural determinantion of proteins. Fluorescence is the emission of radiation that occurs when a molecule in an excited electronic state returns to the ground state. For fluorescence study intrinsic (e.g. tryptophan residue) as well as extrinsic (e.g. ANS) fluorophores are used.

Protein Purification by Affinity Chromatography

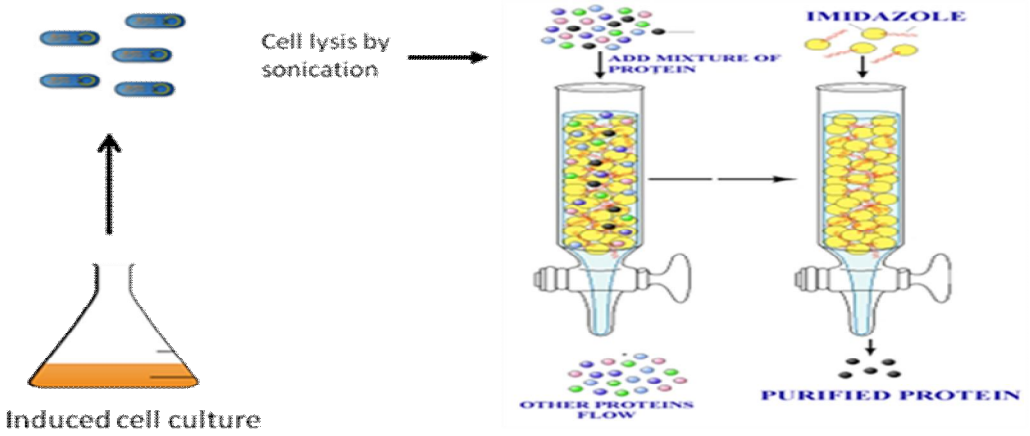

Figure 1.13 Protein purification by affinity chromatography

1.9 Importance of functional characterization

Present study tries to provide new options to carry out targeted protein degradation with the help of peptides derived from yeast and mouse ODC. As the selected peptides in the present study are degradation signals of yeast ODC, it would be interesting to check their portability. Nα/β and N50 peptides derived from yeast ODC and 37mODC derived from mouse ODC are chosen for functional characterization. Nα/β peptide is an independent domain of yeast ODC which carries 2 degradation signals namely N terminal unstructured region and AzBE region situated in α/β barrel domain. Portability of Nα/β peptide was checked by tagging it to reporter protein and then checking the stability of reporter protein. As N50 is an unstructured stretch, it would be interesting to check the potential of this peptide to act as initiator of degradation process. As discussed in chapter 5, ubiquitin is a natural tag for proteins to be degraded but regulation of ubiquitin itself is also equally important. Natural ways for degradation of ubiquitin are described in literature, but attaching a tag of another unstructured region derived from mouse ODC i.e. 37 mODC will provide a new means of targeted ubiquitin degradation (Figure 1.14), bringing together two degradation signals effectively.

The proposed peptides are:

1. Entire N terminal domain of yeast ODC (Nα/β peptide)
2. α/β barrel domain of yeast ODC (α/β peptide)
3. N terminal stretch of 50 amino acid residues (N50 peptide)
4. Ubiquitin with C-terminally tagged mODC peptide (UbmODC fusion protein)

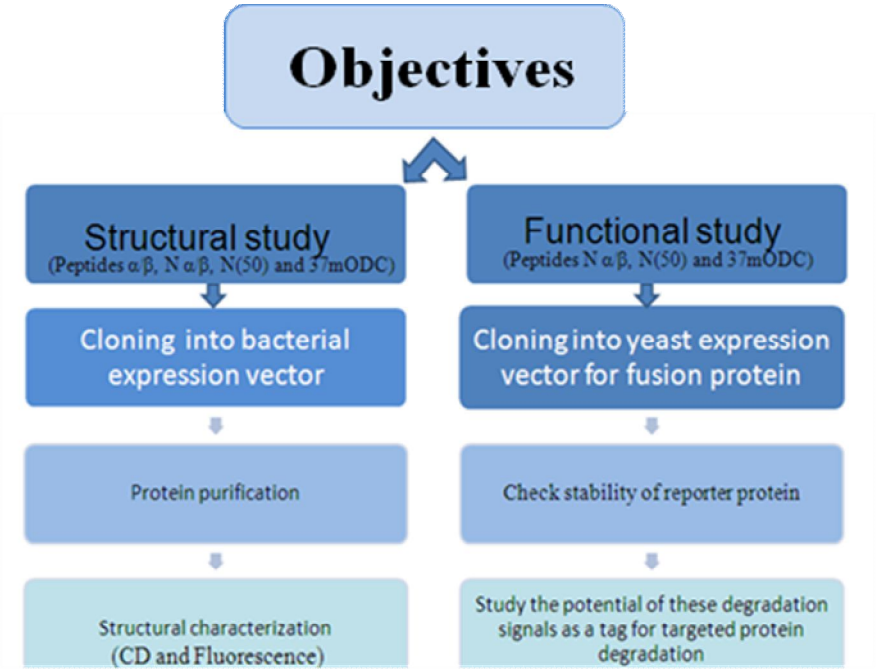

Figure 1.14 Schematic representation of proposed objectives

Proposed peptides vary in structure when present as a part of ODC. Nα/β peptide and α/β peptide are domains while N50 and 37mODC are unstructured in nature. Nα/β peptide carries two degradation signals and composed of α/β barrel domain and N terminal unstructured stretch. It would be interesting to know the structure gained by these peptides when expressed independently from the rest of the protein. It would also be important in functional evaluation of Nα/β, N 50 and 37mODC peptides.

Pictorial representation of proposed fusion proteins

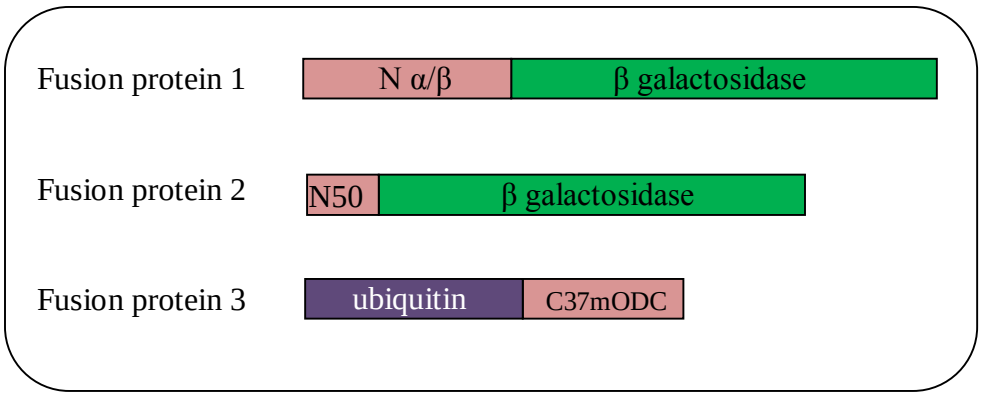

Figure 1.15 Pictorial representation of proposed fusion proteins

Chapter 1 gives general introduction about proteins, protein degradation in prokaryotes and eukaryotes, ubiquitin proteasome system, Ornithine Decarboxylase (ODC), importance of structural and functional characterization of selected peptides and rationale behind this study.

Chapter 2 is focused on structural and functional characterization of Nα/β peptide. This chapter describes in detail about the cloning of fragments for structural as well as functional study. Peptide expression and purification is also discussed herewith (Figure 1.15). Structural characterization of the Nα/β peptide was done using CD and fluorescence spectra. Under functional study, the Nα/β peptide was tagged to a reporter protein and stability of reporter protein was checked. Structural features and functional role of Nα/β peptide when it is expressed independently from rest of the protein is discussed.

Chapter 3 covers structural and functional characterization of N50 peptide. Naturally N terminal 50 amino acids stretch of yeast ODC is unstructured region. Any gain of structure by this small unstructured region was tried to resolve by using CD and fluorescence spectra when expressed independently. Under functional study, the N50 peptide was tagged to a reporter protein and stability of reporter protein was checked.

Structural features and functional role of N50 peptide when it is expressed independently from rest of the protein is discussed.

Chapter 4 covers structural characterization of α/β peptide. Standardization of purification conditions and solubility of peptide was discussed here.

Chapter 5 presents the fusion protein UbmODC. Previous reports on fate of ubiquitin and ubiquitin fusion proteins are discussed in the introduction of this chapter. During proteasomal degradation of substrate protein, ubiquitin is recycled. Potential of C37mODC peptide as a portable degradation tag to drag the substrate protein towards proteasome for degradation was already proved. In this chapter C terminal unstructured tail of mouse ODC was tagged at C terminus of ubiquitin and fate of ubiquitin was studied to understand the potential of C terminus as a degron. The construct also brings two different degrons together for increasing the rate of degradation in targeted protein degradation.

List of abbreviations

ANS	1-Anilino 8-Naphthalene sulphonic acid
Az	Antizyme
AzBE	Antizyme Binding Element
bp	Base pair
CD	Circular dichroism
DHFR	Dihydrofolate Reductase
E.coli	*Escherichia coli*
EDTA	Ethylene diamine tetra acetic acid
FRET	Fluorescence Resonance Energy Transfer
GdnCl	Guanidine hydrochloride
IDPs	Intrinsically Disordered Proteins
IDRs	Intrinsically Disordered Regions
IPTG	Isopropyl thio-ß -D-galactoside
kb	Kilo base pair
kDa	Kilodalton
LB	Luria Broth
mODC	Mouse ODC
N α/β	Entire N terminal Barrel domain of yeast ODC
N50	N terminal 50 amino acid residue stretch of yeast ODC
ODC	Ornithine decarboxylase
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase Chain Reaction
PLP	Pyridoxal Phosphate
PMSF	Phenylmethylsulfonyl fluoride
SDM	Site Directed Mutagenesis
SDS	Sodium Dodecyl Sulphate
Ub	Ubiquitin
UbmODC	Fusion protein of Ubiquitin with C terminal tail of mouse ODC
UPS	Ubiquitin Proteasome System
α/β	α/β barrel domain of yeast ODC

ABBREVIATIONS FOR AMINO ACIDS

Amino Acid	Three Letter code	Single Letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

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Studies on the Degradation Determinant Signals of Ornithine Decarboxylase (ODC)

THESIS
SUBMITTED TO

THE MAHARAJA SAYAJIRAO UNIVERSITY OF BARODA,
VADODARA, GUJARAT

DOCTOR OF PHILOSOPHY

By

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October- 2012

STATEMENT UNDER O. Ph. D 8/ (iii) OF THE M.S. UNIVERSITY OF BARODA, VADODARA.

This is to certify that the work presented in this thesis is original and was obtained from the studies undertaken by me under the supervision of **Dr. C. Ratna Prabha.**

Baroda **Swapnali Kulkarni**

Date:

This is to certify that the work presented in the form of a thesis in fulfillment of the requirement for the award of the Ph.D. degree of The M.S. University of Baroda by **Mrs. Swapnali Kulkarni** is her original work. The entire research work and the thesis have been built up under my supervision.

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ACKNOWLEDGEMENT

*First and foremost, I would like to express my deep gratitude towards my guide Dr**. C. Ratna Prabha.** Without her strong support and valuable guidance it would have been impossible for me to complete my study. She taught me from ABC to XYZ of molecular biology and protein field. Her spiritual nature and forgiveness helped me a lot to improve myself. I truly enjoyed her teaching and enjoyed working under her.*

I sincerely thank Head of the Department Prof. G. Naresh Kumar for providing me the research facilities and infrastructure in the Department. I am also thankful to him for his valuable suggestions and constant support.

I acknowledge University Grants Commission for providing UGC-RFSMS fellowship and good amount of contingency.

Thanks to Dr. Philip Coffino (University of Ccalifornia) and Dr. Tabor (University of Maryland) for providing the plasmids and strains for the research project.

Special thanks to IIT Bombay for their help in circular dichroism experiments.

I thank all the teachers of the department, Prof. Sarita Gupta, Prof. Rasheedunnisa Begum, Dr. Jayashree Pohnerkar, Dr. Pushpa Robin, Dr. S. R. Acharya, Dr. Laxmi Priya and Dr. D. Suthar for their help at various stages. I am also thankful to Prof. Tara Mehta.

My special thanks to my lab seniors Hemendra sir and Pradeep sir. They taught us not only experiments but discipline, devotion to work, planning of protocols and lot more.

Working in the lab with happy atmosphere was possible because of Mrinal, brother rather than a lab mate. I am really thankful to him for his timely help, valuable suggestions and support. It took me many days to think nice words which give me satisfaction to thank Rushikesh for his great help and support which cannot be expressed by words. Thanks to god only for offering me younger brother along with degree. Daily routine in the lab was cheerful due to presence of beautiful brainy, helping and hardworking girls Ankita, Varsha and Brinda. Thanks to Prranshu for helping all the time.

Thanks to Purna for being with me all the time whenever I needed. Her friendship helped me to improve a lot.

My special thanks to seniors Vikas sir, Keyurbhai, Maulik bhai, Niraj bhai, Shajilbhai, Gopit sir, Mukta didi, Sonal madam, Praveena madam, Hiren bhai, Prakash sir, Hina, Jyotika, Aditi and Jisha for helping me whenever there was a need.

Thanks to all research students of the Department, Nidhish, Krishma, Divya, Naresh, Mitesh, Radha, Muskan, Hemanta, Prashant, Jitendra, Chanchal, Kavita, Sumit, Ashish and Archana for making work enjoyable. Thanks to Tushar, Akhilesh, Abhay and Ujjval for keeping atleast some quantity of powa for us.

Thanks to my friends Maneesh, Purva, Darshan, Iqbal, Hiral, Anubha, Pratik who contributed something or the other.

I would also like to acknowledge M.Sc. students, Devdatta, Renuka, Pankti, Arpit, Darshan, Vijay, Dipti, Soumya, Sayantan, Shweta, Sahiti, Sejal, Grishma, Mitul and Chinmay who worked with me during the project.

Special thanks to all the office staff of the department.

I do not have words to thank my husband Dr Rohit. His support, care, love, timely guidance and most important sacrifice are the base stones of my study. My daughter Siddhi is my soul. Her smile and sacrifice always pushed me to complete my work fast. Ph.D. wouldn't be possible without help of my father in law and blessings of late mother in law. Special thanks to my brother Prasad for his enthusiastic and strong support. I am also thankful to our spiritual guru Dandavate maharaj for blessing and being with us at every moment. Last but not least are the blessings of parents. Their constant support helped me to sustain in all situations.

Swapnali Kulkarni

October 2012

Dedicated to my parents…..

Publications

Publications:

C. Ratna Prabha,* Soumya Mukhergee, Renuka Raman and Swapnali Kulkarni. The ends and means of artificially induced targeted protein degradation. Applied Microbiology and Biotechnology. (In press)

Swapnali Kulkarni, Rushikesh Joshi, **C. Ratna Prabha***. Contribution of the N terminal unstructured region to the folding of α/β barrel domain of Ornithine Decarboxylase. (Manuscript under preparation)

(*- Author for correspondance)

Oral / Poster presentations

Oral / Poster presentations:

1. Second prize in Oral Presentation in Second Annual Biotechnology conference for students held on 13th and 14th November 2010 at International Institute of Information Technology, Pune, Maharashtra. Topic: Fluorescence spectroscopic studies on the degradation determinant signals of Ornithine Decarboxylase. Swapnali Kulkarni[1], Rushikesh Joshi[2] and **C. Ratna Prabha***

2. Received Travel Award for poster presentation in Indo-US symposium & Workshop on "Modern Trends in Macromolecular Structure" held on February 21st-24th 2011 at IIT Mumbai. Topic: Structural studies on the degradation determinant signals of Ornithine Decarboxylase. Swapnali Kulkarni[1], Rushikesh Joshi[2] and **C. Ratna Prabha***

3. Oral presentation enttled "Structural Characterization of Degron Peptides From
   Ornithine Decarboxylase. Rushikesh Joshi, Swapnali Kulkarni and **C. Ratna Prabha*"** at XXVI Gujarat Science Congress in 2012.

4. Oral presentation enttled "Effect of C-terminal Degron Tail on the Stability of Ubiquitin." Swapnali Kulkarni, Rushikesh Joshi and **C. Ratna Prabha*** at Regional Science Congress on Science for Shaping the Future of India on 15th-16th September 2012.