The active subunits of the 20S Proteasome in *Saccharomyces cerevisiae* Mutational analysis of their specificities and a C-terminal extension

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Hiermit versichere ich, dass ich diese Arbeit selbst verfasst und dabei keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe.
Stuttgart, den 22.10.2007

Rui Estiveira

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Index

Abbreviations

ºC	grad Celsius
%	percentage
µg	microgram
µl	microlitre
~	approximately
β-ME	2-mercapto ethanol
5-FOA	5-Fluoroorotic acid
A	Adenine
Ala	alanine
AMC	7-amino-4-methylcoumarin
Amp	ampicillin
APS	ammonium persulfate
Arg	arginine
Asp	aspartic acid
ATP	adenosine 5'-triphosphate
bp	base pair
BrAAP	branched chain amino acids
BSA	Bovine Serum Albumin
C	Cytosine
Cbz	carbobenzyloxy
CEN	centromeric
CM	Synthetic media
C-terminus	Carboxyl-terminus of a protein
Da	Dalton
ddH_2O	double deionised water
DMSO	dimethylsulfoxide
DNA	desoxyribo Nucleic Acid
dNTP	desoxyribo Nucleotide
DTT	dithiothreitol
E. coli	*Escherichia coli*
E1	ubiquitin activating enzyme
E2	ubiquitin conjugating enzyme
E3	ubiquitin –protein- ligase
EDTA	Ethylen-diamin-N,N,N',N'-tetra acetate
Et-OH	ethanol
Fig	Figure
G	Guanine
g	gram
Gal	Galactose
Gln	glutamine
Glu	glutamic acid
Gly	glycine
h	hour
H	helix
HCl	Hydrochloric acid
Ile	Isoleucine
Kan	Kanamicin
kb	1000 base pair
kDa	1000 Dalton
l	litre
L	loop
LB	luria Broth
Leu	leucine

Lys	lysine
M	molar (mol/litre)
mA	milliampere
Met	methionine
mg	milligram
min	minute
ml	millilitre
mM	milimolar
MV	Mineral medium
MW	molecular weight
N-terminus	amino-terminus of a protein
NaCl	Sodium Cloride
NaOH	Sodium hydroxide
nm	Nanometer
Od_X	optical density at the wavelength of x nm
ORF	Open Reading Frame
p.a.	pro Analyse
pNA	para-nitroanilide
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PEG	polyethylenglycol
PGPH	peptidyl-glutamyl peptide hydrolyzing
pH	negative decimal logarithm of hydrogen positive ions concentration
Phe	Phenylalanine
rpm	rotations per minute
RT	room temperature
s	second
S. cerevisiae	Saccharomyces cerevisiae
SDS	sodium dodecil sulphate
Ser	serine
Suc	Succinyl
T	Tyrosine
TBS	tris buffer saline
TCA	Tri chloro acetic acid
TE	tris EDTA buffer
TEMED	N,N,N',N' – tetramethylendiamine
Thr	Threonine
Tris	tris(hydroxymethyl)-aminomethane
Trp	tryptophan
Tween20	Polyoxyethylensorbitan Monolaureate
Tyr	tyrosine
U	Units
UV	Ultra violet
V	Volts
Val	Valine
v/v	volume per volume (l/l)
w/v	weight per volume (g/ml)
WT	wild type
X-Gal	5-brom-4-chloro-3-indolyl-beta-D-galactopyranoside
YPD	Yeast complete media with 2% glucose
Δ	deletion

Abstract

The proteasome is a large multi-subunit complex ubiquitous in eukaryotes and archaebacteria. It contains proteolytic subunits that function simultaneously to digest protein substrates into oligopeptides.
In eukaryotic cells, it is involved in the removal of abnormal, misfolded or incorrectly assembled proteins, but additionally it has regulatory functions. For example it is responsible for the degradation of cyclins in cell-cycle control and for the destruction of transcription factors or metabolic enzymes in metabolic adaptation. Finally, the proteasome is also involved in MHC (major histocompatibility complex) class I mediated cellular immune response. These cellular functions are linked to an ubiquitin- and ATP-requiring protein degradation pathway involving the 26S proteasome whose proteolytic core is formed by the 20S proteasome.

The 20S proteasome has a cylindrical shape and is composed of four rings, each formed by seven α- or seven β-subunits and stacked in the order $\alpha\beta\beta\alpha$. In eukaryotic cells, the 20S proteasome is composed of two copies of 14 different subunits, 7 distinct α-type and 7 distinct β-type subunits. Only three of the β-type subunits are proteolytically active and have N-terminal threonine residues acting as nucleophiles. They differ in their major specificities: β5/Pre2, β2/Pup1 and β1/Pre3 are classified as having "chymotrypsin-like", "trypsin-like" and "peptidylglutamyl peptide hydrolysing" (PGPH or caspase-like) activities, respectively. This classification is based on the preferred amino acid residues found at the site of hydrolysis in peptide or protein substrates.
These three active β-type subunits have a fixed location in the proteasome, with the two β5/Pre2 copies separated from the clustered β2/Pup1 and β1/Pre3 subunits. In yeast a hierarchy of individual subunit activities for proteasomal function was established: β5/Pre2>> β2/Pup1 > β1/Pre3.

Part of this work aimed at clarifying whether this hierarchy is solely dependent on the specificities or whether topological conditions lead to the dominance of the β5/Pre2 activity over the others, which could involve inter-subunit communication mediated by interjacent inactive β-subunits. Stepwise site-directed mutagenesis of key residues forming the substrate binding pockets was used to swap specificities between the yeast β5/Pre2 and β1/Pre3 active sites. Consequences of these mutations were then analysed in regard to maturation of the modified subunits, their specificities towards peptide substrates diagnostic for chymotrypsin-like and PGPH activity and changes in their rank in the hierarchy of functional importance.

By mutating the key residue methionine 45 into an arginine, the β5/Pre2 was able to mature and showed some PGPH activity. Combinations with mutant strains, having the other active subunits inactivated, revealed that Pre2 lost its functional dominance. When other key residues were additionally replaced by those present in β1/Pre3 (A20T, V31T, I35T), instead of an further increase in PGPH activity, the β5/Pre2 subunit showed an overall decrease in activity. An unexpected exception was the *pre2-M45R-I35T* mutant with a strong increase in chymotrypsin-like activity. Maturation of the Pre2 subunit occurred normally like in wild-type in all combinations of mutations tested.
When the residue arginine 45 was mutated into a methionine in Pre3, this subunit lost any detectable peptidase activity. Attempts to stabilise the methionine 45 by introducing strategic point mutations at residue 52 were unsuccessful.
Additional alterations in the substrate binding site of β1/Pre3 (T20A, T31V, T35I) completely abolished the autolytic maturation and thus any gain of activity. Strains lacking both the Pre3 propeptide and N^{α}-acetyltransferase were used to confirm that the mutations result in activity loss, even when the autolytic removal of the propeptide was not required.

In a second project, the role of the long C-terminal extension of the yeast β2/Pup1 subunit was examined. This 37 amino acid structure embraces the β-ring neighbour subunit β3/Pup3 and reaches the next subunit β4/Pre1. It also contacts β7/Pre6 of the opposite β-ring.
Mutations of residues that could loosen contact to the surface of β3/Pup3 (Y204A, R208G and T211A) where without effect. Complete deletion of this extension or truncation of 25 residues was lethal and deletion of the last 20 amino acids caused a strong cell growth defect. When replacing the last 20 amino acids of this C-terminal extension by a FLAG tag, the growth phenotype was lost.
The lethal mutations were over-expressed in wild type strains, but the mutated β2 subunits did not incorporate into proteasomes. This indicates that removal of the distal half from the β2/Pup1 C-terminal extension impedes the integration of this subunit during early assembly stages of the 20S proteasome.

Zusammenfassung

Proteasomen sind große Komplexe aus vielen Untereinheiten und ubiquitär in Eukaryonten und Archaebakterien. Sie enthalten proteolytische Untereinheiten, die gemeinsam beim Abbau von Proteinsubstraten zu Oligopeptiden agieren.
In eukaryontischen Zellen sind sie beteiligt an der Beseitigung von abnormalen, fehlgefalteten oder inkorrekt assemblierten Proteinen, erfüllen zusätzlich aber regulatorische Funktionen. Zum Beispiel sind sie verantwortlich für den Abbau von Cyclinen bei der Zellzyklus-Kontrolle und für die Zerstörung von Transkriptionsfaktoren oder metabolischen Enzymen bei der metabolischen Anpassung. Schließlich sind Proteasomen auch in die MHC (major histocompatibility complex) Klasse I vermittelte zelluläre Immunantwort einbezogen. Diese zellulären Funktionen sind mit einem Ubiquitin- und ATP-abhängigen Proteinabbauweg verknüpft, zu dem das 26S Proteasom gehört, dessen proteolytische Grundeinheit vom 20S Proteasom gebildet wird.

Das 20S Proteasom hat eine zylindrische Form und besteht aus vier Ringen, die jeweils aus sieben α- oder sieben β- Untereinheiten gebildet und in αββα Anordnung gestapelt sind. In eukaryontischen Zellen ist das 20S Proteasom aus zwei Sätzen 14 verschiedener Untereinheiten aufgebaut, sieben unterschiedlichen α-Typ und sieben unterschiedlichen β-Typ Untereinheiten. Nur drei der β-Typ Untereinheiten sind proteolytisch aktiv und haben N-terminale Threonin-Reste, die als Nukleophile agieren. Sie unterscheiden sich in ihren wichtigsten Spezifitäten: β5/Pre2, β2/Pup1 und β1/Pre3 werden klassifiziert nach ihrer Chymotrypsin-ähnlichen, Trypsin-ähnlichen zw. Peptidylglutamyl-Peptid hydrolysierenden PGPH oder Caspase-ähnlichen) Aktivität. Diese Einteilung basiert auf den bevorzugten Aminosäure-Resten, an denen in Peptid- oder Proteinsubstraten die Hydrolyse stattfindet. Diese drei aktiven β-Typ Untereinheiten haben festgelegte Plätze im Proteasom, wobei die zwei Kopien von β5/Pre2 abgetrennt sind von den nah beieinander liegenden β2/Pup1 und β1/Pre3 Untereinheiten. In der Hefe wurde eine Hierarchie der einzelnen Untereinheiten-Aktivitäten bezüglich der proteasomalen Funktion festgestellt: β5/Pre2 >> β2/Pup1 > β1/Pre3.

Ein Teil dieser Arbeit zielt darauf zu klären, ob diese Hierarchie allein von den Spezifitäten abhängt oder ob topologische Bedingungen zu der Dominanz von β5/Pre2 über die anderen führen, was eine über dazwischen liegende inaktive β-Untereinheiten vermittelte Kommunikation unter den Untereinheiten beinhalten könnte. Schrittweise, gerichtete Mutagenese von Resten, welche die Substrat bindenden Taschen ausbilden, wurde benutzt, um die Spezifitäten der aktiven Zentren von β5/Pre2 und β1/Pre3 gegeneinander auszutauschen. Daraufhin wurden die Auswirkungen analysiert, die diese Mutationen bezüglich der Reifung der modifizierten Untereinheiten haben, ihrer

Spezifitäten gegenüber für die Chymotrypsin-ähnliche und die PGPH Aktivität diagnostischen Peptid-Substraten und Veränderungen in der Stellung innerhalb der Hierarchie der funktionellen Bedeutung.
Nach Mutation des Schlüssel-Rests Methionin 45 zu Arginin war β5/Pre2 in der Lage zu reifen und zeigte etwas PGPH Aktivität. Kombinationen mit Mutantenstämmen, die andere aktive Untereinheiten inaktiviert hatten, zeigten, dass Pre2 seine funktionelle Dominanz verloren hatte. Als zusätzlich weitere Schlüssel-Reste durch jene, die in β1/Pre3 vorliegen, ersetzt wurden (A20T, V31T, I35T), zeigte die β5/Pre2 Untereinheit statt eines weiteren Anstiegs der PGPH Aktivität einen generellen Rückgang von Aktivität. Eine unerwartete Ausnahme war die *pre2-M45R-I35T* Mutante mit einem starken Anstieg der Chymotrypsin-ähnlichen Aktivität.
Nach Mutation des Rests Arginin 45 zu Methionin in Pre3 verlor diese Untereinheit jegliche detektierbare Peptidase-Aktivität. Versuche, Methionin 45 durch Einführung strategischer Punktmutationen am Rest 52 zu stabilisieren, waren erfolglos.
Zusätzliche Veränderungen in der Substrat bindenden Region von β1/Pre3 (T20A, T31V, T35I) verhinderten die autolytische Reifung gänzlich und damit die Erlangung jeglicher Aktivität. Stämme, denen sowohl das Propeptid von Pre3 als auch die N^{α}-Acetyltransferase fehlen, dienten der Bestätigung, dass die Mutationen auch dann zum Aktivitätsverlust führen, wenn die autolytische Entfernung des Propeptids nicht erforderlich ist.

In einem zweiten Projekt wurde die Rolle der langen C-terminalen Verlängerung der β2/Pup1 Untereinheit von Hefe untersucht. Diese 37 Aminosäuren lange Struktur umschließt die im β-Ring benachbarte β3/Pup3 Untereinheit und reicht bis zur nächsten Untereinheit, β4/Pre1. Sie hat auch zu β7/Pre6 im gegenüber liegenden Ring Kontakt.
Mutationen von Resten, welche den Kontakt zur Oberfläche von β3/Pup3 lockern könnten (Y204A, R208G und T211A), hatten keine Auswirkung. Die komplette Deletion dieser Extension oder die Verkürzung um 25 Reste war letal und die Deletion der letzten 20 Aminosäuren führte zu einem starken Defekt des Zellwachstums. Dieser Phänotyp wurde aufgehoben, wenn die letzten 20 Aminosäuren dieses C-terminalen Anhängsels durch einen FLAG tag ersetzt wurden.
Die letalen Mutationen wurden in Wildtyp-Stämmen überexprimiert, jedoch wurden die mutierten β2 Untereinheiten nicht ins Proteasom inkorporiert. Dies deutet darauf hin, dass die Entfernung der distalen Hälfte der C-terminalen Verlängerung von β2/Pup1 den Einbau dieser Untereinheit während früher Assemblierungsstadien des 20S Proteasoms verhindert.

1. Introduction

1.1. Cellular proteolysis and the ubiquitin-proteasome system

During the lifetime of a cell, proteins are synthesised, maintained and also destroyed. Proteolysis is essential to eliminate misfolded and malfunctioning proteins, which are a consequence of mutations or stress conditions. Otherwise, these abnormal proteins would accumulate in the cell, endangering the cell's functionality. In addition, the activity of many regulatory proteins is also controlled by means of their degradation. A multitude of proteins involved in cell cycle control, cell type determination, and the regulation of metabolite fluxes through biosynthetic pathways are rapidly and selectively destroyed upon specific signals. [Baumeister et al., 1998; Hochstrasser, 1991]

Once the lysosome, the central organelle for non-specific proteolysis in eukaryotes, was discovered, it was thought to be the place where all cellular proteolysis occurs. Experimental evidence for non-lysosomal energy consuming pathways of intracellular proteolysis existed, but their mechanisms and constituents were not verified until the early 80's.
After the discovery of the ubiquitin-proteasome system (UPS) this issue was better understood [Ciechanover, 2005]. Ubiquitin-mediated proteolysis is highly specific and important. It involves delivery of nuclear and cytoplasmic proteins to the proteasome. Localised in the cytoplasm and the nucleus of all cells, it serves to selectively remove a huge variety of protein substrates via modification with ubiquitin and subsequent destruction by the proteasome [Knop et al., 1993; Hilt and Wolf, 1992; Seufert and Jentsch, 1991; Seufert and Jentsch, 1992].

The ubiquitin-proteasome system is indispensable for a diverse array of biologically important cellular processes, such as cell-cycle progression, signalling cascades and developmental programs. It is also involved in the protein quality control of the cell [Tanaka et al., 2004]. Its role in protein waste disposal even extends to proteins of other compartments.

Before being delivered to their place of action all proteins of the secretory pathway are folded in the endoplasmic reticulum (ER). In order to prevent misfolded or unassembled proteins from reaching their destination, a strict quality control system in the ER recognizes these proteins. Substrate proteins are extracted from the ER, polyubiquitinated (covalent attachment of ubiquitin) and delivered to the cytosolic proteasome where they are degraded. This process is named the ER associated degradation (ERAD) [Hirsch et al., 2004, Tenzer and Schild, 2005].

It was found that multiple copies of the 76 residue protein ubiquitin are covalently conjugated to a protein substrate as polyubiquitin chains marking it for selective degradation [Ciechanover et al., 1980]. The ubiquitin moieties are reusable and released immediately before the substrate degradation. Targeted proteins are recognized, and marked for degradation through the action of a cascade of enzymes; E1, E2's and E3's (fig.1-01). E1 is the ubiquitin-activating enzyme; E2's are ubiquitin conjugating enzymes and E3's ubiquitin-protein ligases. Recognition of the poliubiquitinated proteins by the 26S proteasome happens at the 19S cap.

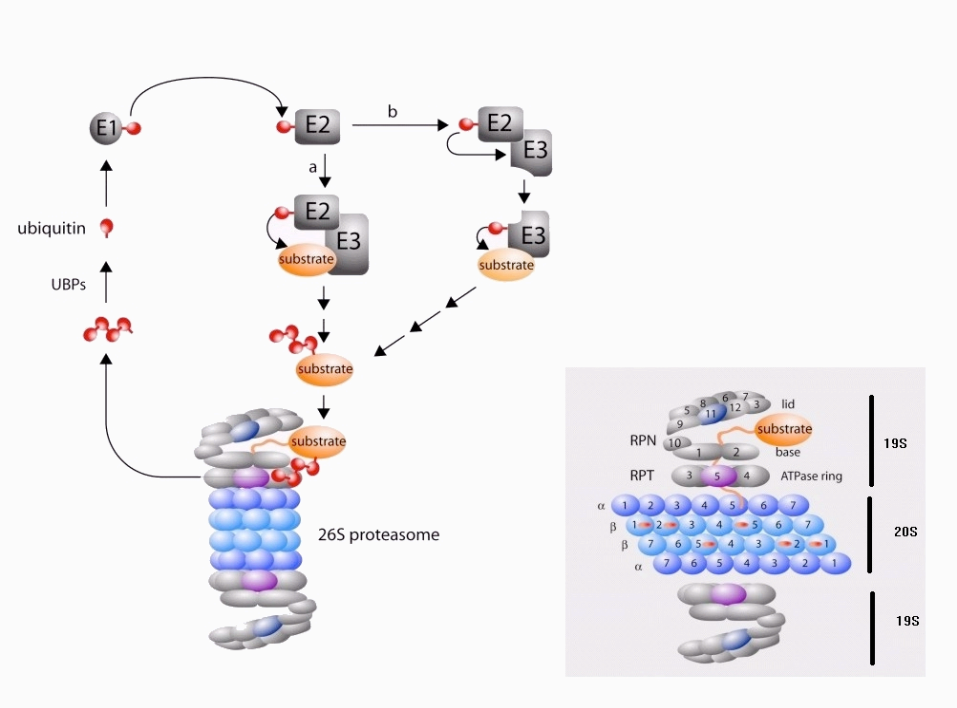


Figure 1-01 - The ubiquitin-proteasome system. Ubiquitin is conjugated to the substrate protein by the sequential action of three enzymes called E1, E2, and E3.

In a first step, ubiquitin is activated by the ubiquitin-activating enzyme (E1) consuming ATP. Then ubiquitin is transferred to an E2, the ubiquitin-conjugating enzyme. Finally ubiquitin is linked to the substrate by the means of an E3 ubiquitin-ligase.

In pathway a) a RING ubiquitin-ligase E3 works as a mediator between the substrate and the ubiquitin-conjugating enzyme. In pathway b) ubiquitin is first transferred from E2 to E3 before its conjugation to the E3-bound substrate, or the previously conjugated ubiquitin of the substrate. Multiple cycles of ubiquitin conjugation lead to a polyubiquitin chain. The polyubiquitinated substrate binds to the 19S cap receptor subunit, and the substrate is degraded into small peptides while ubiquitin is recycled via the activity of ubiquitin specific proteases (UBP's) [Hilt and Wolf, 2004].

On the right side, the 20S proteasome core is shown partially opened to reveal its constitutive subunits: 7 different α and β subunits from which β1, β2, and β5 are active and shown with a red mark; in the 19S cap an ATPase ring made of 6 RPT subunits joined to RPN1 and RPN2 forms a base subcomplex which via RPN10 is connected to a lid complex made of at least 8 RPN subunits.

In some cases specific adaptor proteins like Rad23 and Dsk2 are also involved in the delivery of substrates to the proteasome by escorting them and by binding to the 19S regulatory particle of the proteasome [Loscher et al., 2005; Elsasser and Finley, 2005]. These ubiquitin receptors have an amino-terminal region containing one ubiquitin-like (UBL) domain that is recognised by the 19S proteasome cap, while one or more ubiquitin-associated (UBA) domains bind to ubiquitin. For that reason they are called UBL-UBA proteins.
After recognition, polyubiquitin chains are disassembled, while the substrate is delivered to the 20S proteasome for degradation (fig.1-01) [Zhu et al., 2005].

1.2. Protein targeting through ubiquitination

Covalent conjugation of ubiquitin chains with a minimal length of 4 moieties to intracellular proteins is a signal for degradation by the 26S proteasome. A first ubiquitin binds with its carboxy-terminal glycine to a ε-amino group of a lysine side chain in the substrate forming an isopeptide bond and each following ubiquitin is conjugated to the lysine 48 on the previous one in analogue manner, forming a polyubiquitin chain. There exist polyubiquitin chains build via linkages to other ubiquitin lysines, in particular Lys 63. Here the marked proteins are not targeted for degradation. Such chains may be implicated on other processes, including post-replicative DNA repair or endocytosis [Thrower et al., 2000].

Conjugation is usually accomplished by the sequential action of ubiquitin-activating (E1), ubiquitin-conjugating (E2), and ubiquitin-protein ligase (E3) enzymes.
Under consumption of ATP, a thiolester bond between the carboxyl group of the C-terminal glycine 76 residue of ubiquitin and the side chain of a cysteine residue of E1 (ubiquitin activating enzyme) is formed. Then, ubiquitin is transferred to E2 (ubiquitin conjugating enzyme) by formation of another thiolester linkage. E3 (ubiquitin-protein ligase) enzymes are responsible for the final target selection and specificity. They belong to two classes. The HECT (homologous to E6-AP carboxyl terminus) domain proteins form a covalent thiolester bond with ubiquitin before transferring it to the substrate. In the second class, the RING (real interesting new gene) finger domain which co-ordinates two metal atoms, facilitates direct transfer of ubiquitin to the targeted protein without formation of the additional thiolester intermediate observed in the HECT family of E3 ligases. The 19S cap of the proteasome recognises the polyubiquitin chain and unfolds the substrate which is translocated into the proteolytic chamber of the 20S core particule for degradation [Berleth and Pickart, 1996; Scheffner et al., 1995; You and Pickart, 2001; Nandi et al., 2006].

In some cases, an E4 enzyme is required for poliubiquitination, like certain ubiquitin-chain elongation factors. Yeast Ufd2, which binds to oligoubiquitylated substrates (proteins modified by only a few ubiquitin molecules) catalyses poliubiquitin chain assembly in collaboration with E1, E2 and E3 [Hoppe, 2005].

1.3. The proteasome

Proteasomes are large multi-subunit self-compartmentalising proteases that are found in the cytosol and in the nucleus of all eukaryotic cells, existing in high amounts (up to 1% of total protein content) in these compartments.

The 26S proteasome generally acts together with the ubiquitination system, recognizing, unfolding, and digesting the protein substrates that have been marked for degradation by the attachment of polyubiquitin chains. Nonetheless, in some cases it also selectively degrades structurally abnormal proteins in an ubiquitin-independent manner. [Yano et al., 2005]

The complete 26S proteasome consists of a proteolytically active 20S core complex and a regulatory 19S cap complex which unfolds and deubiquitinates the substrates, and finally feeds them into the 20S core in an ATP-dependent manner. The 19S cap contains at least seventeen different subunits and is assembled from two main subcomplexes, a base (containing six ATPases plus two non-ATPase subunits) lying with the ATPase ring on the 20S proteasome, and a lid subcomplex sitting on the top of the base (fig.1-01) [Glickman et al., 1998b].

Inside the 20S core particle, the substrate chains are degraded into oligopeptides with a length between 3 and 20 residues [Baumeister et al., 1998; Larsen and Finley, 1997; Glickman et al., 1998].

1.3.1. Proteasome evolution

Proteasomes are found in all three domains of life: archaea, bacteria, and eukarya [Baumeister et al., 1998]. Their evolution is revealed by the different complexity existing within domains, as it can be observed when comparing the simple bacterial and complex mammalian proteasome systems.

In bacteria, HsIV is the only protease resembling the 20S proteasome. The monomers forming a dodecameric structure are distantly related to proteasomal β subunits. There is

no ubiquitin in bacteria and the substrate selection depends on intrinsic recognition signals within proteins where recognition motifs are found at the N and C termini. ATPase complexes, like HslU, associate with the HSlV protease, recognize and bind substrates, unfold and finally deliver them to the proteolytic chamber. The core particle appears to be more ancient than the ubiquitin system [Gottesman, 2003; Bochtler et al., 1999; Groll et al., 2005].

The prokaryotic prototype of a proteasome core HslV, is only a dimer of two hexameric rings stacked head to head, unlike archaebacterial and eukaryotic 20S proteasomes which have two additional α-rings resulting in four-ring structures displaying 7-fold or pseudo-7-fold symmetries (fig.1-02) [Bochtler et al., 1999].

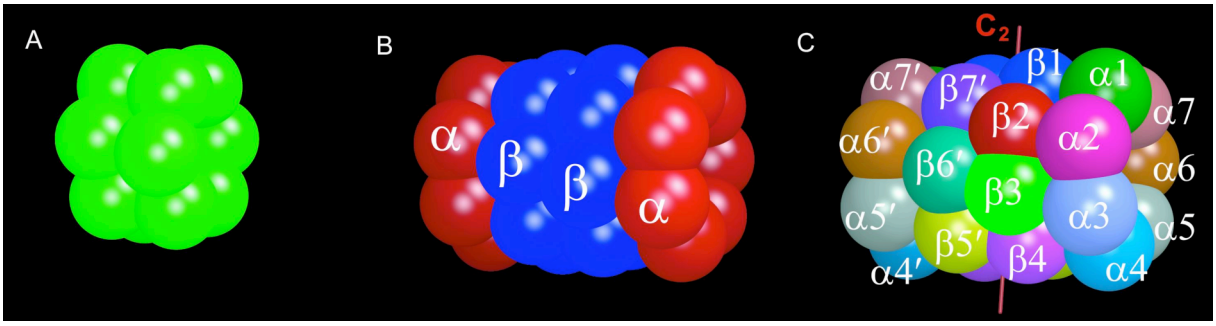


Figure 1-02 - Sphere model of a) HslV showing the dodecamer of two stacked hexameric rings in a staggered arrangement. b) sphere model of the 20S *Thermoplasma* proteasome with α and β subunits. c) eukaryotic 20S core particle, with seven different α and seven different β subunits.

In the archaeon *Thermoplasma acidophilum*, the 20S proteasome has only two kinds of subunits. Seven inactive α subunits are in each of the outer two rings and seven active β subunits in each inner ring where the N-terminal threonine provides the active-site nucleophile in each subunit. Exceptionally among eubacteria which otherwise do not possess 20S proteasomes is *Rhodococcus sp*, where two different α-type (α1, α2) and β-type (β1, β2) subunits are found [Tamura et al., 1995].

Eukaryotic 20S proteasome subunits are related in sequence to the archaeal subunits. In yeast, 14 genes (7 α-type and 7 β-type) encode the 20S proteasome subunits, and proteasomes form a uniform population in which each particle has the same 14 different subunits in unique locations (fig.1-02). This fixed topology is conserved from yeast to mammals [Heinemeyer et al., 2004].

In mammals, subcomplexes like the PA28 may bind to the 20S proteasome core, replacing the 19S cap. Together with immuno-proteasomes replacing constitutive 20S proteasomes, they serve to modulate the protein breakdown process by favouring the generation of antigenic peptide epitopes. In immuno-proteasomes, the three beta subunits containing a catalytic site are replaced by three interferon gamma inducible counterparts. The finally obtained peptides, mostly stable against further hydrolysis, are specially designed to be incorporated into class I MHC (major histocompatibility complex) molecules. These alternative forms of the proteasome emerge under conditions of an intensified immune response [Kesmir et al., 2003; Froment et al., 2005; Dahlmann, 2005; Groll et al., 2005].

The catalytic centers are harboured in the interior of the 20S particle. The central active sites are formed by N-terminal threonine residues of β-type subunits, which are liberated by precursor processing during proteasome assembly. The *Thermoplasma* proteasome has fourteen copies of a single type of active site, cleaving model peptides preferentially after hydrophobic residues (a "chymotrypsin-like" activity) whereas the eukaryotic particle has two sets of only three different active sites. Each of these distinct active β-type subunits has a different peptide cleavage preference, which, in addition to the chymotrypsin-like activity allows cleavage of peptides also after basic ("trypsin-like" activity) and acidic ("peptidylglutamyl peptide hydrolyzing" (PGPH) activity) residues [Chen and Hochstrasser, 1996; Schmidtke et al., 1996; Seemueller et al., 1996; Heinemeyer et al., 1997].

On eukaryotic, the conservation of backbone geometry and the majority of the residues making up the active site in inactive β-subunits indicate that early in evolution these subunits could have been proteolytically active. Later, they lost their activity by mutations.

The gross structures of proteasomes, such as their size and shape, have been highly conserved during evolution. They are barrel shaped structures with 3 inner chambers. The central chamber is formed by the two adjacent β-rings, while the antechambers are formed by the junction of one β and one α ring.

Both α-type and β-type subunits have the same fold, with a central sandwich of two β sheets and 5 α helices. Helixes 1 and 2 provide interaction between α and β rings and helixes 3 and 4 the contacts between the two β rings (fig.1-03).

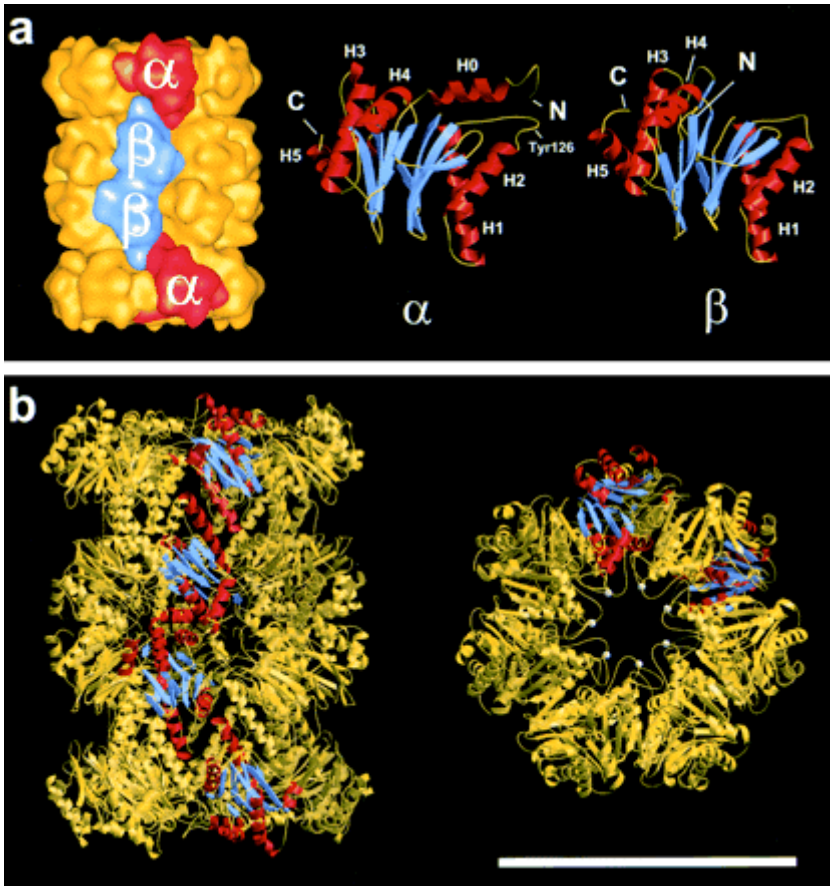


Fig 1-03 – Structure of the Thermoplasma 20S Proteasome [Baumeister et al., 1998]

(a) 12 nm resolution model derived from the atomic coordinates of the *Thermoplasma* proteasome. The α subunits form the heptameric outer rings; the β subunits, the inner rings. Individual α and β subunits are shown as ribbon drawings (right). Helixes 1-5 shown in red, β strands shown in blue.

(b) Ribbon drawing of the 20S proteasome indicating one subunit in each of the four rings by different colour coding (left). A half-proteasome (one α and one β ring) viewed down the 7-fold axis.

1.3.2. Structure and assembly of the eukaryotic 20S proteasome

Both yeast and human 20S proteasome structure has been resolved by x-ray crystallography [Groll et al., 1999; Unno et al., 2002]. The 20S proteasome in the yeast *Saccharomyces cerevisiae* is a 700 kDa cylindrically shaped particle of about 160Å in length and 120Å in diameter [Harry, 1968]. It is composed of two copies of 14 different subunits with molecular weights of 22-25 kDa, 7 distinct α-type and 7 distinct β-type subunits. These subunits exhibit a unique location, forming the 20S core which consists of two equal parts attached face to face ($\alpha_{1\text{-}7}$ $\beta_{1\text{-}7}$ $\beta_{1\text{-}7}$ $\alpha_{1\text{-}7}$) and has a structure with C2 symmetry (fig.1-04) [Hilt et al., 1993; Hilt and Wolf, 1995; Groll et al., 1997].

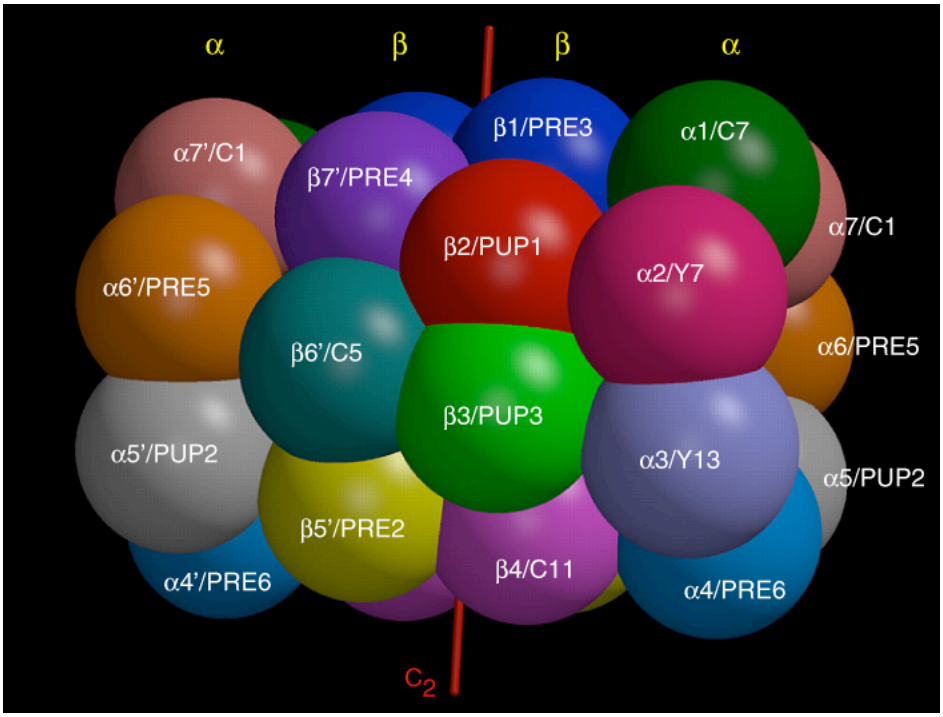


Figure 1-04 – Topology of the 28subunits of the yeast 20S proteasome drawn as spheres [Groll et al., 1997]

The mammalian 20S proteasome, similar to the yeast 20S proteasome has 7 different α and 7 different β subunits. The arrangement of the subunits is identical and the catalytic sites as well as their substrate binding regions are well conserved between the mammalian and yeast proteasomes.
Three additional catalytic active β subunits, β1i, β2i and β5i, induced by interferon-γ (IFNγ), can replace the constitutive active subunits β1, β2 and β5 respectively, leading to the formation of the "immunoproteasome".

As anticipated from their sequence similarity, the eukaryotic α- and β-type subunits have a common fold, also found in *T. acidophilum*: a four-layer α and β structure with a central β sandwich of two five-stranded sheets flanked on either side by α helices (fig.1-03a). Helixes 1 and 2 mediate the interaction of α and β rings (β-*trans*-α) by intercalating in a wedge-like fashion. Helixes 3 and 4, which are located on the opposite side, provide the dominant contacts between the two β rings (β-*trans*-β). This general architecture is the same as in the *Thermoplasma* 20S proteasome. In the yeast proteasome, there are a large number of additional specific α-*cis*, β-*cis*, β-*trans*-α, and β-*trans*-β contacts. Together with the propeptides of the β subunit precursors, these contacts ensure that the assembly proceeds in an orderly fashion, that is, that each of the 14 different subunits takes its correct place. One β-subunit in particular, β2/Pup1 has a C-terminal elongation embracing one adjacent subunit, which may be crucial to a correct placing in the proteasome assembly process.

Even with such additional specific contacts, the area between β subunits within the same β-ring is not sufficient for the complete β-ring self assembly. Additional contact provided by the α-ring is required for the complete formation of the β-ring, meaning that a dimmer of two β-rings with potentially unsealed proteolytic activity cannot be formed. Since precursors of active β-subunits are only activated after the junction of two β-rings this appears to be a fail-safe mechanism ensuring that active sites activation only occurs after proteasome assembly is complete.

The α rings play mainly a structural role, providing extra antechambers (fig.1-05) and a gating that regulates substrates access [Kwon et al., 2004; Yang et al., 1995].

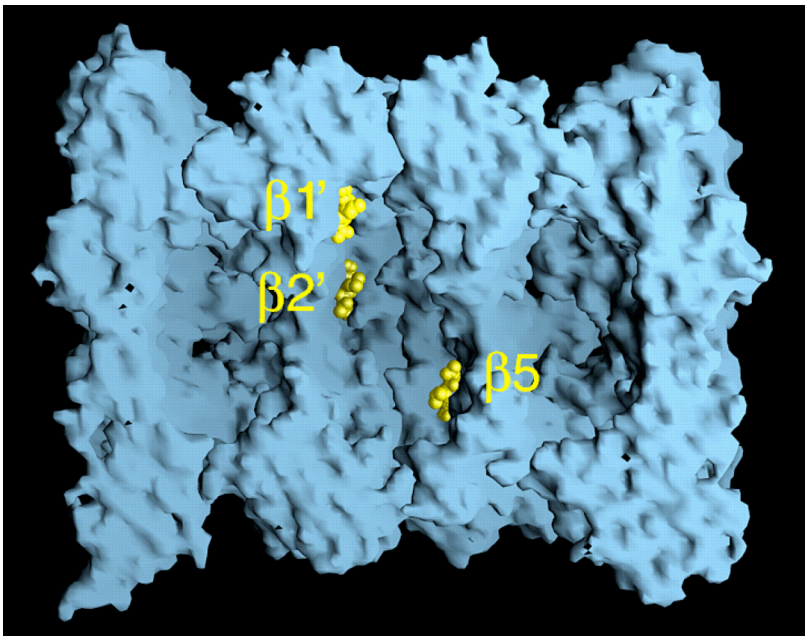


Figure 1-05 – Surface view of the 20S proteasome molecule clipped along the cylinder axis. At yellow, are shown three of six calpain inhibitor molecules bound to the active subunits, β1/PRE3, β2/PUP1 and β5/PRE2. [Groll et al., 1997]

Assembly and maturation process of the 20S complex occurs in a highly coordinate manner. In addition correct processing of β-subunit proproteins has to be guaranteed during complex maturation. The structure formation happens in different phases. First, the newly synthesized monomers have to adopt the correct tertiary structure. Subsequently the alpha-subunits probably form ring-like structures thereby providing docking sites for the different beta-subunits. The result is a double ring structure representing half-proteasomes, which contains immature proproteins. The chaperone Ump1 is then attached to the unprocessed β-subunits.

Two half-proteasomes can associate to form the assembly intermediate which still contains unprocessed beta-precursors and Ump1. Conformational changes in β-subunits occur that position their S2-S3 loops and propeptides allowing the formation of active sites and propeptide cleavage [Witt et al., 2006]. Ump1 is transiently buried inside the nascent proteasome to coordinate the processing of the β-type subunits. In its absence, not only the formation of 20S structures from two half-proteasome precursors is less efficient but also the maturation of the precursors of the three active subunits is drastically impelled. Autocatalytic processing of these precursors exposes an N-terminal threonine, allowing degradation of Ump1 and propeptides, leading to the mature, active 20S proteasome [Ramos et al., 1998; Schmidt et al., 1997; Heinemeyer et al., 2004].

The β-type subunit propeptides have both common and distinct functions in proteasome biogenesis. The Pre2 propeptide is crucial for proteasome assembly, while the Pre3 and Pup1 propeptides are dispensable for cell viability and proteasome formation. However, mutants lacking these propeptide-encoding elements are more sensitive to environmental stresses, have subtle defects in proteasome assembly and are defective for specific peptidase activities. The latter is due to a critical and common function of the propeptide, the protection of the N-terminal catalytic threonine residue against N-alpha-acetylation [Arendt and Hochstrasser, 1999]. If the N-terminal threonine is externally exposed in the cell, it becomes acetylated, and the generated subunit will not have proteolytic activity.
The main difference between α- and β- type subunits is found in a highly conserved N-terminal extension of all α-type subunits, part of which (residues 20–30) forms an α helix (H0) across the top of the central β sandwich (fig.1-03a).

The core particle is auto-inhibited by these N-terminal tails of the outer α-ring subunits. In free 20S core particles, the tails prevent substrate entry, repressing peptide hydrolysis. Deletion of this N-terminal elongation of the α3 subunit interrupts the integrity of the plug normally formed by the 7 tails, opening a channel into the proteolytically active interior chamber of the core particle, wide enough to let a peptide chain pass trough, as seen in the crystal structure of a α3 N-terminal tail deletion mutant [Groll et al., 2000].
When the regulatory particle is bound to the core particle, forming the 26S proteasome, this inhibition by the alpha-subunit tails is relieved. The opening of this channel by the 19S regulatory particle, controlling both substrate entry and product release, is specifically regulated by the Rtp2 ATPase in the base of the regulatory particle [Groll et al., 2000; Kohler et al., 2001].

1.3.3. Active site mechanism

Proteasome subunits are encoded by a family of homologous genes named the "proteasome gene family," which have evolved from a common ancestral gene [Tanaka et al., 1992]. An archaeal proteasome has a single type of each, an α- and a β- subunit, and the N-terminal threonine of the mature beta subunit is the active-site nucleophile. Yeast proteasomes have seven different β-subunits and exhibit three distinct peptidase activities, which derive from disparate active sites [Arendt and Hochstrasser, 1997].
Prosequences of varying lengths, which protect the N-terminal threonine of the active beta subunit precursors, are removed during proteasome assembly, rendering the cleft between the central β sandwich freely accessible from the central cavity. This is prototypical of the Ntn (N-terminal nucleophile) hydrolases family of enzymes to which the 20S proteasome belongs [Baumeister et al., 1998].
The Ntn hydrolase family consists of enzymes with an unusual four-layer alpha + beta fold. The amino-terminal residue (cysteine, serine or threonine) of the mature protein is the catalytic nucleophile, and its side chain is activated for nucleophilic attack by transfer of its proton to the free N terminus, although other active-site residues may also be involved. The four currently known Ntn hydrolases (glutamine PRPP amidotransferase, penicillin acylase, the 20S proteasome and aspartylglucosaminidase) are encoded as inactive precursors, and are activated by cleavage of the peptide bond preceding the catalytic residue [Seemüller et al., 1996].

The mechanism of catalysis of the maturated β-subunits in the proteasome is based on the Thr1 hydroxyl group as the nucleophile and was identified from the crystal structure of *T.acidophilum* proteasome in complex with an aldehyde inhibitor, Ac-LLnL-al [Löwe et al., 1995]. This residue is conserved in all active subunits. Thr1 deletion or mutation to Ala leads to inactivation of the enzyme [Seemüller et al., 1995] and deficiency in autolysis [Heinemeyer et al., 1997].
The nucleophilic hydroxyl group of Thr1 must be activated by a proton acceptor at the active site. The conserved Lys33, due to its electrostatic potential lowers the pK_a of the N-terminal group of Thr1, so that this group can act as the proton acceptor in proteolysis (fig.1-06a) [Groll and Huber, 2003]. The other conserved residues surrounding Thr1 (Glu/Asp17, Lys33, Ser129, Asp166 and Ser169) are also required to the catalytic mechanism in general (fig.1-07) [Heinemeyer et al., 2004].
During the autolysis reaction, as the N-terminal group is not yet available, a catalytic water molecule is found to be the proton acceptor. It is positioned to act as the general base and promote abstraction of a proton from the Thr1 hydroxyl group, initiating nucleophilic attack on the carbonyl carbon of the preceding peptide bond (fig.1-06b)

[Groll et al., 1997]. In addition to its role in intra-molecular autolysis, it also serves as a proton shuffle between Thr1Oγ and the N-terminal amino group during proteolysis.
The structures of the active sites of the 20S proteasome are therefore designed to both autocatalysis and substrate proteolysis [Groll and Huber, 2003].

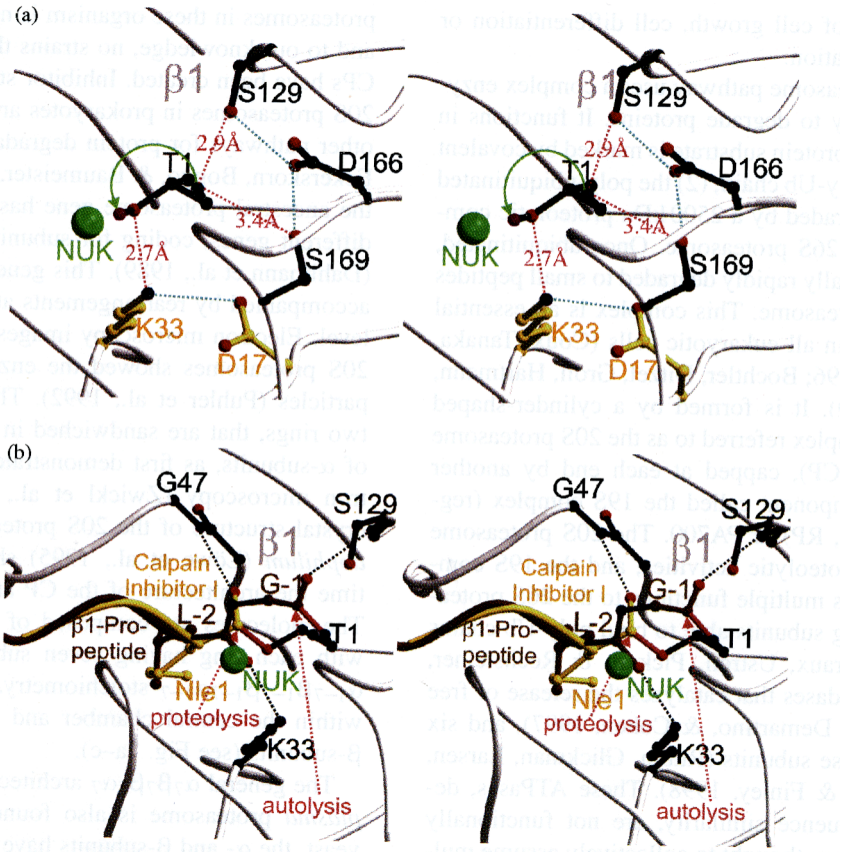


Figure 1-06 – (a) The vicinity of residue Thr1 in subunit β1. Protein backbone is drawn in white coils, while residues which contribute in particular to the active site (Thr1, Asp17, Lys33, Ser129, Asp166 and Ser169) are shown as balls-and-sticks. Lys33 is in a salt bridge to Asp17 (cyan dots) therefore presumably positively charged, lowering the pKa of Thr1Oγ electrostatical potential (purple dots). A water molecule, NUK (green sphere) is localized in the electron density close to Thr1Oγ and N, playing a role in the proton transfer (green circle).
(b) Mechanism for autolysis and substrate proteolysis at the active site of the yeast 20S proteasome subunit β1 (backbone shown as white coils and Thr1 as black balls-and-sticks). Calpain-inhibitor I and β1–propeptide are colored yellow and brown, respectively. The superposition of wild type β1 and β1T1A main chain atoms reveals an rms-deviation of 0.19Å allowing to model the Ala in the mutant with the Thr1 from the wild type CP. Proteolysis and autolysis (pink dots) are initiated by proton transfer from Thr1Oγ to water NUK (green sphere). Gly47N (black sticks) is the major constituent of the oxyanion-hole for inhibitors and substrates, lowering the energy of the tetrahedral adduct transition state. Ser129N (black sticks) is the essential part of the oxyanion-hole for the carbonyl oxygen of Gly-1, whereas Lys33Nζ (black sticks) stabilizes the carbonyl oxygen of position -2 in the propeptides. Hydrogen bonds are indicated as black dots. Addition of Thr1Oγ to Gly-1C (autolysis) or Nle1C (proteolysis) is followed by ester bond formation, which is hydrolyzed in both pathways by incorporation of water NUK into the product. [Groll and Huber 2003]

In autocatalysis, mediated by the water molecule and K33 amino group, Thr1 hydroxyl group attacks nucleophilically the Gly-1 carbonyl. This results in a ring closure, where the Gly-1 carbonyl negative charged oxygen is stabilised by the Ser129 hydroxyl. A bridging water facilitates de proton transfer from Lys33 to the amide function of the peptide bond, opening the formed ring while restoring Gly-1 carbonyl function. The N-terminal amino group of Thr1 is now free and the acetylated Gly-1 carbonyl is then deacylated by a water molecule, with protonation of K33 and S129 stabilising the negatively charged hydroxylised Gly-1 carbonyl. The ester ligation between Gly-1 and Thr1 is broken with a proton attack from K33 restoring Thr1 hydroxyl group [Schmidtke et al., 1996].

1.3.4. Active site specificities

Of the 7 different β-subunits in the yeast 20S proteasome, three have proteolytic active sites. As described in the previous chapter, each of these subunits is synthesized with an N-terminal propeptide that is auto-catalytically cleaved between the threonine at position 1 and the last glycine of the pro-sequence during particle assembly, with release of the active-site residue Thr1 [Löwe et al., 1995, Seemüller et al., 1996].
These three beta-type subunits, β5/Pre2, β2/Pup1 and β1/Pre3, have different major specificities. Their peptidase specificities, classified based upon the primary amino acid residues found at the site of hydrolysis of short artificial peptidase substrates, are respectively the "chymotrypsin-like" activity cleaving substrates after hydrophobic residues (β5/Pre2), the "trypsine-like" activity cleaving substrates after basic residues (β2/Pup1) and the "peptidyl-glutamyl peptide hydrolytic" (PGPH) or caspase-like activity cleaving after acidic residues (β1/Pre3) [Groll et al., 1997; Arendt and Hochstrasser, 1997; Heinemeyer et al., 1997].

Analysis of digestion products of protein enolase 1 confirmed these specificities as well, providing the amino acid preferences for the position P1 of the protein substrate in each active site pocket. β5/Pre2 has the strongest preference for Leu at position P1 followed by Tyr, Phe and Trp which have bulky side chains. β1/Pre3 prefers the amino acids with acidic groups, Asp followed by Glu, and β2/Pup1 prefers Arg over Lys, both with basic groups [Nussbaum et al., 1998].
Two other activities are also known to the proteasome, cleavage after branched chain amino acids (BrAAP) to β1/Pre3 and small neutral amino acids (SNAAP) to β5/Pre2, while in vivo assays have shown that cleavage after almost every amino acid was performed by the core particle [Groll et al., 2005].

The S3 pocket has specificity for polar residues in β5/Pre2 and hydrophobic in β1/Pre3 while the S4 pocket has a general preference for Pro. It was established by covalent inhibitors that P3 and P4 are important positions for substrate binding and selectivity by using different sized inhibitors with a variety of amino acid combinations at these positions [Bogyo et al., 1998; Nazif and Bogyo, 2001]. As binding pockets other then S1 have decisive influence on the cleavage preference a minimal length of 3 or 4 amino acids seem to be required for the substrates to be hydrolysed by the proteasome.

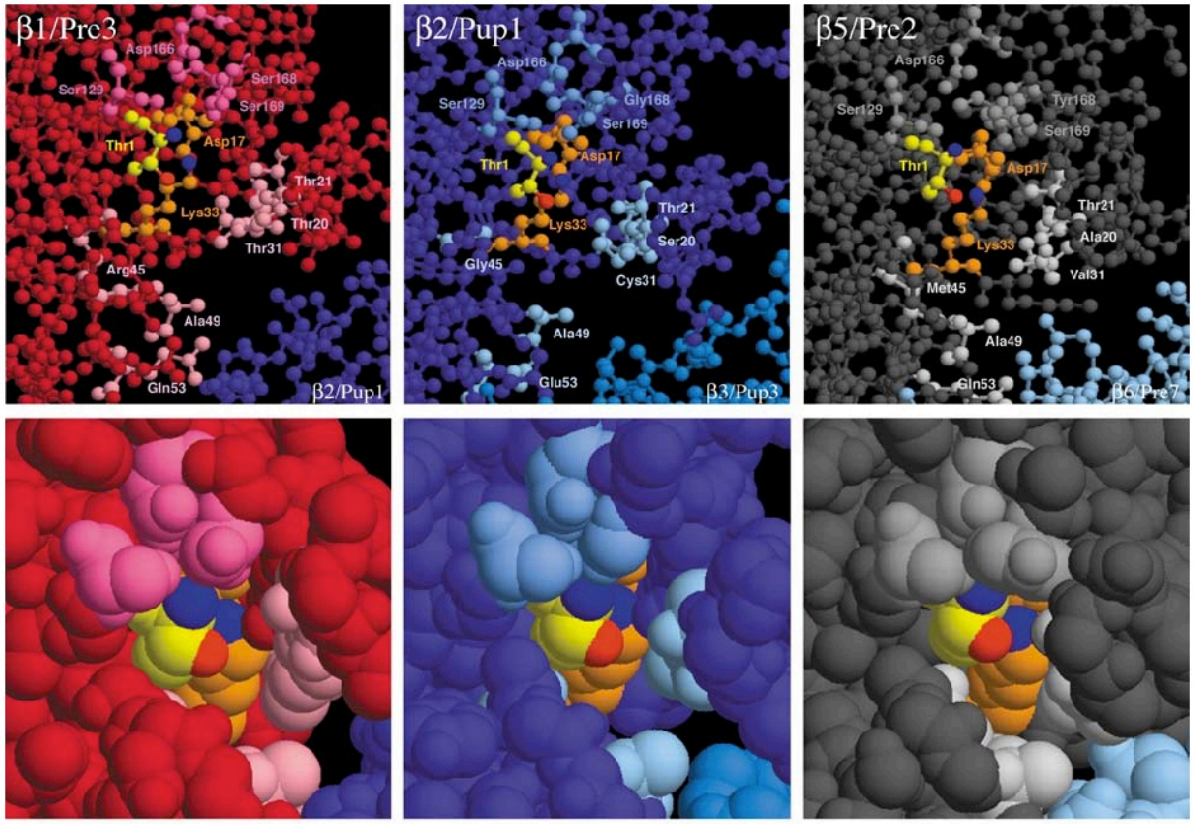


Figure 1-07 – The Structures of the three types of active sites in the *Saccharomyces cerevisiae* 20S proteasome. Upper row, ball-and-stick representations; lower row, space-filling representations of the same parts visible above. Thr1 is shown in yellow with its hydroxyl oxygen in red and the nitrogen of its free amino group in dark blue. Asp17 and Lys33 are orange except the ε-amino group of Lys33 which is in dark blue. Conserved residues Ser129, Asp166, Ser169 and variable 168 are shown with a brighter tone then the subunit colour. Variable residues at positions 20, 21, 31, 45, 49 and 53 are each coloured in a still brighter tone, forming the surface of the substrate binding pocket [Heinemeyer et al., 2004].

The distinguishing specificities of the three known active sites, based on the residues at the P1 position in artificial peptide substrates, must correlate with the characters of their S1 pockets, where the substrate is bound and cut at its C-terminal site.

In the base of the β1/Pre3 pocket, Arg45 can balance the charge of acidic P1 residues, favouring the PGPH activity of β1. The trypsin-like activity of β2/Pup1 matches with the presence of Glu53 at the bottom of its S1 pocket and an acidic side wall contributed by

the β3/Pup3 neighbour subunit. The pocket of β5/Pre2 has an apolar character (where Met45 forms the base), explaining its chymotrypsin-like peptidase activity.

In the immunoproteasome, the S1 pocket of β1i is more apolar than that of β1. Earlier studies showed that the replacement of β1 by β1i leads to a reduction of cleavages after acidic residues and to more cuts after hydrophobic residues, which is consistent with the exchange of Arg45 in β1 against Met45 in β1i. This enhances the production of MHC class I binding peptides because hydrophobic or basic carboxyl terminal residues normally serve as anchors for binding to MHC class I molecules [Toes et al., 2001; Unno et al., 2002].

1.3.5. Cooperativity, redundancy and hierarchy of active sites

In the active chamber of the 20S proteasome, the proximity between the equal and even different active subunits could lead to interactions with each other. A "bite-and-chew" model mechanism was proposed [Kisselev et al., 1999] where the conformational flexibility of active sites allowed cooperativity between pairs of active subunits across the two β-rings and between different catalytic subunits separated by inactive β-subunits.
In this model there was a mutual allosteric activation and inhibition of active centers during substrate degradation. It was based on the inhibition of the "biting" chymotrypsin-like site by PGPH substrates while the "chewing" PGPH site was activated by substrates of the chymotrypsin-like activity.
Meanwhile, other investigations challenged this model, and the binding of hydrophobic substrates (including the peptides used for proteasome activity assays) to non-catalytic sites was favoured, regulating the activity of the catalytic sites [Schmidtke et al., 2000].

The importance of active sites for cell viability was analysed with mutants having each site activity eliminated or strongly reduced. Double mutants harbouring only one active center are viable, indicating some redundancy among the catalytic centers. The exception is β1/Pre3, which alone is not enough for cell survival [Heinemeyer et al., 1997; Jäger et al., 1999].
A hierarchy of importance of individual subunit activities was established. β5/Pre2 with its chymotrypsin-like activity cleaving after hydrophobic residues has the highest importance, and β2/Pre3 with a PGPH (post acidic) activity the lowest. A strain with β5/Pre2 activity completely removed shows a strong growth defect, while mutants with β1/Pre3 inactive are not impaired in growth (β2/Pup1 importance is located in between) [Jäger et al., 1999]. This hierarchy of importance, which might be due to specificity or localization, is studied in this work.

1.4. Scope of this work

During evolution proteasomes in eukaryotic cells reduced their number of active subunits from fourteen to only six which have three distinct proteolytic specificities in defined locations (fig.1-08a). According to Jäger [Jäger et al., 1999] the different specific activities contribute differently to the cell growth. It is not clear whether this order of importance is only connected to the subunit specificities or to locations of the subunits as well. Recently another work from Kisselev [Kisselev et al., 1999] suggested a "bite-and-chew" model where the active subunits interact together in an allosteric way. In the work presented here the specificities of the active subunits β1/Pre3 and β5/Pre2 are explored in detail by mutating the substrate binding pockets with the ultimate goal of a specificity swapping.

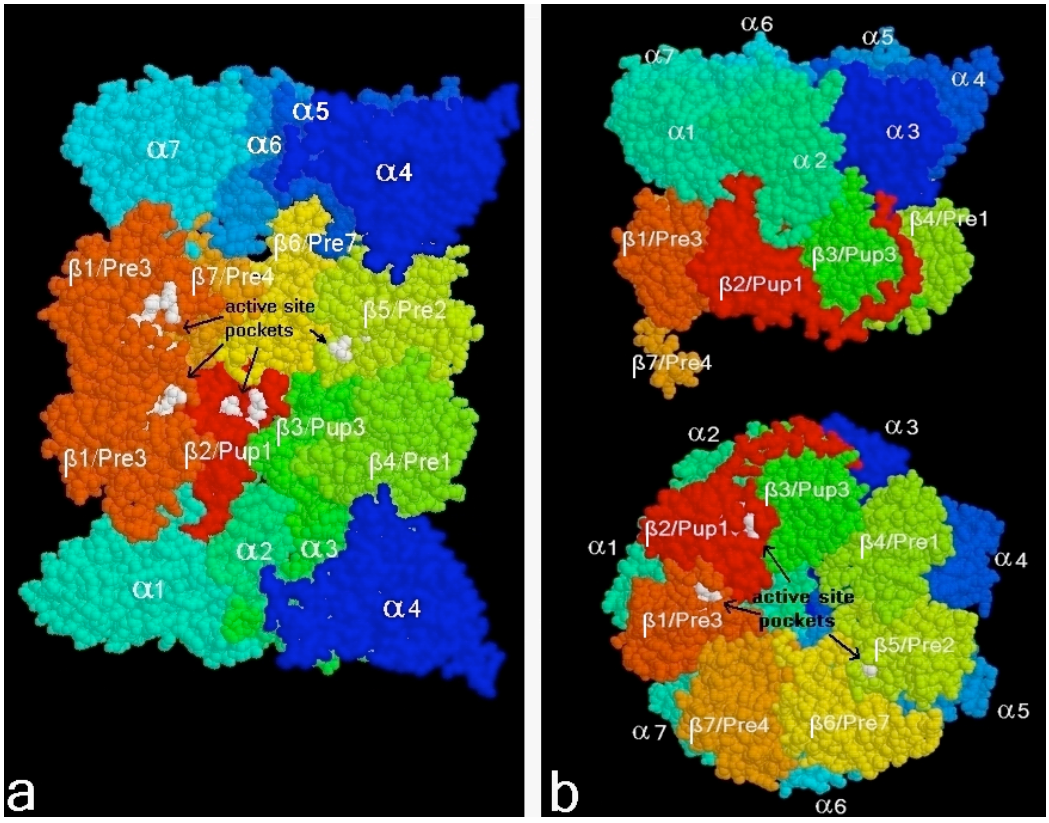


Figure 1-08 – Structural views of cut 20S proteasomes emphasizing the location of active pockets and β2/Pup1 external extension.

a) The proteasome is clipped along the cylinder axis, showing its inside. Amino acids forming the active site pockets in the active subunits are showed in white.

b) Proteasome cut along the x axis. One half showing β2/Pup1 and its C-terminal extension (top), the other viewed from the interior (bottom). Here the arrangement of the β-subunits and the locations of β2/Pup1, β1/Pre3 and β5/Pre2 active pockets are shown.

The third active β-subunit, β2/Pup1 has a unique C-terminal elongation embracing the neighbour subunit (fig.1-08b). In order to get insight into the role of such peculiar feature, this elongation is here studied as well in a mutational approach.

According to the "bite-and-chew" model [Kisselev et al., 1999] an incoming polypeptide substrate binds initially to a β5/Pre2 active pocket (with chymotrypsin-like activity), allosterically enhancing peptide binding to the other β5/Pre2 pocket and stimulating the PGPH or caspase-like cleavages. This seemed to be in accordance with the established hierarchy of importance of individual subunit activities where the subunit responsible for chymotrypsin-like activity, β5/Pre2 has the highest importance.

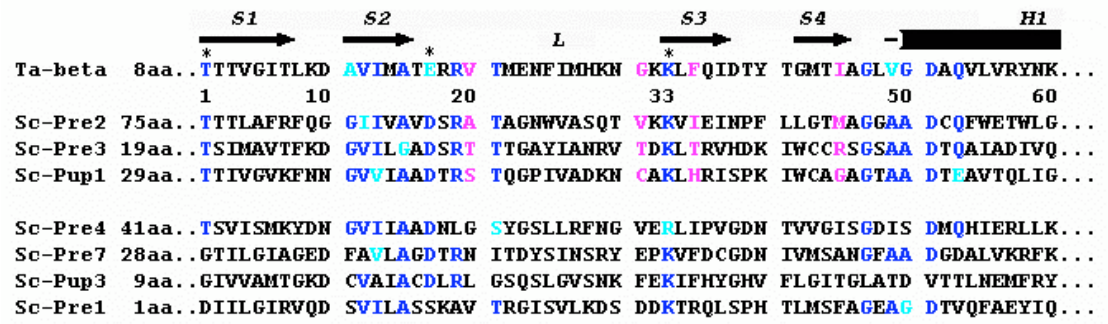


Figure 1-09 – Sequence alignment of the N terminal regions of the *T. acidophilum* proteasome β-subunit (*Ta-beta*) and the seven *S. cerevisiae* (*Sc*) β-type subunits.
The N-terminal and active pocket forming sequences of Ta-beta and corresponding fragments of the yeast proteasome active β-type subunits are aligned. The N-terminal mature parts are numbered below the Ta-beta sequence with the N-terminus of active subunits defining position 1. Omitted propeptide residues (numbers to the left of the yeast sequences) and the position of the catalytically important residues (*symbol) are indicated. Conserved and similar residues among active subunits are indicated in dark blue and in light blue respectively. Residues in pink are believed to be responsible for the subunits specificities.
The β-strands (S1, S2 S3 and S4), a loop region (L) and part of a helix (H) in the mature subunits are marked (adapted from [Heinemeyer et al., 1997 and Groll et al., 2005]).

Based on these findings, it would be very interesting to investigate the behaviour of the "bite-and-chew" mechanism and the subunit hierarchy in the situation where the specific activities were swapped between β5/Pre2 and β1/Pre3. If the chymotrypsin-like activity was associated with Pre3 and on the other hand the PGPH activity associated with Pre2, how would this affect the "bite-and-chew" model and the hierarchy?
After activity swapping, would the "first" bite still be taken by Pre2, now with a PGPH activity, and then the substrate "chewed" by Pre3, with a chymotrypsin-like activity, or would the first bite still be taken by the subunit with chymotrypsin-like specificity? Will the established hierarchy remain unchanged or will Pre3 become the most important among the active β-subunits for cell growth.

There is still a clear similarity between the sequences of the β-subunits from eukaryotic *S. cerevisiae* and the *T. acidophilum* and also among themselves, but differences prevail. Among the active subunits some of the different residues are responsible for the different substrate specificities (fig.1-09). By replacing these amino acids, it should be possible to alter the subunit specificities.
The results of this work give insight into the active site specificities by exploring how the amino acids forming the active pockets influence substrate selection. By means of point mutations specificity swapping is tried in order to understand the importance of chosen locations for each specific active subunit in the β ring of the proteasome.

Much of the sequence differences among subunits respecting the amino acids in contact with neighbour subunits should be to assure a correct assembly of the 20S proteasome. Unlike any other β subunit, β2/Pup1 has a C-terminal extension with over 30 amino acids in the shape of a tail, embracing the neighbour subunit β3/Pup3 (fig.1-08b). It also contacts with the next β subunit, β4/Pre1, and the β7/Pre6 of the opposite β ring. An important question is why such distinct feature developed in this subunit, and which might be its role in the proteasome. The C-terminal extension of the subunit β2/Pup1 is studied here by mutating residues contacting neighbour subunit and by truncating this extension in order to understand its function in the eukaryotic proteasome.

2. Materials and Methods

2.1. Materials

2.1.1. Chemicals and proteins

5-FOA (5-fluoro-orotic acid)	Toronto Research chemicals, Northyork, Canada
Acetone	Fisher Scientific, Pittsburgh, PA, USA
Acrylamide and bisacrylamide solutions	Genaxxon Biosciences, Stafflangen
Adenosine 5'triphosphate, disodium salt	Roche Diagnostics, Manheim
Agarose	Roth, Karlsruhe, Germany
Ammonium Persulfate	Merck, Darmstadt, Germany
Ammonium Sulfamate	Sigma-Aldrich, St.Louis, MO, USA
Ampicilin	Genaxxon Biosciences, Stafflangen
Bactoagar	Difco, Detroit, MI, USA
Bactotryptone	Difco, Detroit, MI, USA
β-mercaptoethanol	Sigma-Aldrich, St.Louis, MO, USA
Bovine Serum Albumin	Sigma-Aldrich, St.Louis, MO, USA
Bromophenol blue	Serva, Heidelberg, Germany
Calcium chloride	Sigma-Aldrich, St.Louis, MO, USA
Chloroform	Fisher Scientific UK
Coomassie Brilliant Blue G250	Serva, Heidelberg, Germany
Dimethilsulfoxide (DMSO)	Acros Organics, Geel, Belgium
Dithiothreitol (DTT)	Sigma-Aldrich, St.Louis, MO, USA
DNA/ladder standards	Roche Diagnostics, Mannheim, Germany
DNA polymerases	New England Biolabs, UK
EDTA	Sigma-Aldrich, St.Louis, MO, USA
EGTA	Sigma-Aldrich, St.Louis, MO, USA
Ethanol	Fisher Scientific, Pittsburgh, PA, USA
Fast Garnet GBC salt	Serva Feinbiochemica, Heidelberg
Formaldehyde	Sigma-Aldrich, St.Louis, MO, USA
Glucolase	Perkin Elmer Inc, Boston, USA
Glycerol	Riedel-de Häen, Germany
Glycine	Genaxxon Biosciences, Stafflangen
Hering Dperm DNA	Promega, Madison, USA
IPTG	Applied Biosystems, Norwalk, USA

IgG sepharose	Sigma-Aldrich, St.Louis, MO, USA
Isopropanol	J.T. Baker, VWR Scientific products, Willard, OH, USA
Lysosyme	Boerhinger Mannheim, Germany
Magnesium chloride	J.T. Baker, VWR Scientific products, Willard, OH, USA
Methanol	Fischer Scientific, UK
N-(1-Naphtyl)-Ethylendiamin	Sigma-Aldrich, St.Louis, MO, USA
Oligonucleotides	Metabion, Martinsried, Germany
Pfu DNA polymerase	Stratagene, Heidelberg, Germany
Phenylmethylsulfonyl fluoride (PMSF)	Merck, Darmstadt, Germany
Prestained (Protein) Standard marker	BioRad, München, Germany
Restriction enzymes	New England Biolabs, UK and Roche Diagnostics, Mannheim, Germany
RNAseA	Roche Diagnostics, Mannheim, Germany
Sodium Dodecyl Sulfat (SDS)	Merck, Darmstadt, Germany
Sodium chloride	Roth, Karlsruhe
Sodium Nitrite	Merck, Darmstadt
T4 DNA ligase	Roche Diagnostics, Mannheim, Germany
T4 DNA polymerase	Roche Diagnostics, Mannheim, Germany
Taq DNA polymerase	Boehringer, Manheim, Germany
Tribasic sodium citrate	Sigma-Aldrich, St.Louis, MO, USA
Trichloroacetic acid (TCA)	Sigma-Aldrich, St.Louis, MO, USA
TEMED	Merck, Darmstadt, Germany
Tris-Base	Roth, Karlsruhe
Triton X-100	Roth, Karlsruhe
TWEEN ®20	Genaxxon Biosciences, Stafflangen
Xylene Cyanole FF	Sigma-Aldrich, St.Louis, MO, USA
X-Gal	Applied Biosystems, Norwalk, USA

2.1.2. Peptide substrates

Cbz-Gly-Gly-Leu-pNAn	Bachem AG, Bubendorf, Switzerland
Cbz-Leu-Leu-Glu-βNA	Bachem AG, Bubendorf, Switzerland
Cbz-Ala-Arg-Arg-βNA	Bachem AG, Bubendorf, Switzerland
Suc-Leu-Leu-Val-Tyr-AMC	Bachem AG, Bubendorf, Switzerland

2.1.3. Miscellaneous Materials

aluminium foil	Reynolds Wrap
ExSite™ PCR-based site-directed mutagenesis kit	Stratagene, Heidelberg, Germany
Gel Extraction Kit	Qiagen, Hilden , Germany
Nitrocellulose	Pall Gelman, Roßdorf, Germany
PCR cleaning Kit	Qiagen, Hilden , Germany
Plasmid Preparation Kit	Qiagen, Hilden , Germany
PVDF membrane Hybond™-P	Amersham Pharmacia Biotech, Piscataway, NJ, USA
Quikchange II XL site-directed mutagenesis kit	Stratagene, Heidelberg, Germany
Whatman paper (filter paper)	Whatman, Hillsboro, OR, USA

2.1.4. Equipment

Blotting apparatus	Bio Rad, München, Germany
Centrifuge Rotors	Sorvall GSA Sorvall SS34 Sorvall H6000A
Centrifuges	Eppendorf centrifuge 5414 Microfuge, Biorad Biofuge Pico, Heraeus Sorvall RC-5B Refrigerated Superspeed Centrifuge DuPond Instruments Sorvall 3C-Plus Refrigerated Superspeed Centrifuge DuPond Instruments

Gel electrophoresis apparatus (poly-acrylamid)	Dual Gel Caster Mighty Small™ SE245, Hoefer
Gel electrophoresis apparatus (agarose)	Bio Rad, München, Germany
Gel electrophoresis camera	Pharmacia, Freiburg, Germany
Ice machine	Scotsman
Incubators (18ºC,25ºC,30ºC,37ºC)	VWR 1540 Sheldon Manufacturing, Inc.
Micromanipulator	Nikon, Tokyo, Japan
Microwave	Toshiba
PH meter	240 Corning
Photometer V530	Jasco, Gross Umstadt, Germany
Pipettes (2-1000μl)	Gilson
Robocycler Gradient 40 PCR grade	Stratagene, La Jolla, USA
Rotor	Roller drum, New Brunswick Scientific Co.
Scale	Meheler PJ3000
Shaker	37ºC Orbital Shaker Forma Scientific RT and 30ºC G10 Gyratory Shaker New Brunswick Scientific Co, Inc.
Spectrofluorophotometer RF-1502	Shimadzu, Tokyo, Japan
Spectrophotometer (UV/VIS)	Beckman Instruments, Inc.
Thermocycler	PTC-100™ Programmable Thermal Controller, MJ Research, Inc.
Vortexer	Vortex Mixer S/P American Scientific Products

2.1.5. Media, Buffers, Solutions

YPD

(used for growing yeast in liquid culture)

yeast extract 4.0g
bacto peptone 8.0g
D-glucose 8.0g
ddH_2O ..... to 400ml
pH 5.5, autoclaved

YP + agar

(used for growing yeast in solid media)

a)

yeast extract 12.4g
bacto peptone 6.2g
ddH_2O ..... to 300ml
pH 5.5, autoclaved

b)

agar 12.0g
ddH_2O ..... to 300ml
autoclaved

Before plating mix autoclaved YP and agar and add 2% D-glucose (autoclaved) to it.

Critical Minimum (CM) medium

yeast nitrogen base 4.15g
drop out mix 1.24g
ddH_2O ..... to 300ml
pH 5.6, autoclaved

For growing yeast on solid CM medium

Agar 12.0g
ddH_2O ..... to 300ml
autoclaved

Before plating mix 300ml of CM medium + 300ml of agar and add 30mM adenine, 20mM uracil, 100mM leucine, 60mM histidine, 100mM lysine, 40mM tryptophan, 2% D-glucose (always omit the concerned amino acid or nucleotide when selecting the yeast for that particular marker).

Sporulation Medium

a)

potassium acetate 6.0g
bacto-yeast extract 0.6g
ddH_2O ..... to 300ml
autoclaved

b)

agar 12.0g
ddH_2O ..... to 300ml
autoclaved

Before plating mix autoclaved solutions, add 2% D-glucose (autoclaved) to it.

LB (Luria Bertani) medium

(used for growing *E. coli* in liquid media)

bacto tryptone peptone 4.0g
yeast extract 2.0g
sodium chloride 2.0g
ddH_2O ..... to 400ml
pH 7.5, autoclaved

2x LB

(used for growing *E. coli* on solid media)

a)

bacto tryptone peptone 6.2g
yeast extract 3.1g
sodium chloride 3.1g
ddH2O ..... to 300ml
pH 7.5, autoclaved

b)

agar 12.0g
ddH_2O ..... to 300ml
autoclaved

Before plating mix 300ml of 2x LB and 300ml of agar

SOC

bacto tryptone 2.0g
yeast extract 0.5g
D-glucose 0.4g
NaCl 2.0mM
KCl 0.25mM
$MgCl_2$ 1.0mM
$MgSO_4$ 1.0mM
ddH_2O ..... to 100ml
PH 7.4, autoclaved

2.1.5.1. Buffers and solutions for DNA Gels

50X TAE buffer (Tris – acetate – EDTA)

Tris base 242.0g
CH_3COOH 57.1ml
0.5M EDTA pH 8.0 ..... 100ml
ddH_2O ..... to 1000ml

5X TBE buffer (Tris – boric acid – EDTA)

Tris base 54.0g
H_3BO_3 ..... 27.5g
0.5M EDTA pH 8.0 ..... 20.0ml
ddH_2O ..... to 1000ml

DNA loading buffer

glycerol 30%
bromophenol blue 0.25%
ddH_2O ..... to 100%

Gel Composition

1x TAE or TBE + 0.8-1 % Agarose (W/V) + 0.05mg/l ethidium bromide

Running Buffer

1X TAE or TBE buffer

1kb DNA Ladder

1 kb ladder 40µl
DNA loading dye 80µl
10x TE 80µl
ddH_2O 600µl

2.1.5.2. Buffers and Solutions for SDS Page

2x SDS page loading buffer

Tris-HCl pH 6.8 ..... 40mM
glycerol 20ml
SDS 2.0g
bromophenol blue 10mg
ddH_2O ..... to 100ml
One part of 1M DTT was added to 5 parts of mix before use

4x Stacking Buffer

Tris-HCl pH 6.8 ..... 0.5M
SDS 0.4g
ddH_2O ..... to 100ml

4x Separating Buffer

Tris-HCl pH 8.8 ..... 1.5M
SDS 2.0g
ddH_2O ..... to 500ml

Gel Composition

Table 1: Composition of SDS-PAGE for a final volume of 15ml

% gel	acrylamide (30%) / bisacrylamide (0.8%) [ml]	ddH_2O [ml]	4x separating buffer [ml]
10	5.0	6.25	3.75
12	6.0	5.25	3.75
15	7.5	3.75	3.75
18	9.0	2.25	3.75

Mixture was polymerised with 50µl APS (10%), 10µl TEMED

Stacking gel

30% acrylamide/0,8% bisacrylamide 0.65ml
ddH_2O 3.05ml
4x stacking buffer 1.25ml
APS (10%) 25µl
TEMED 10µl

5x Running Buffer

Tris-base 15.1g
SDS 5.0g
glycine 72.0g
ddH_2O ..... to 1l

2.1.5.3. Buffers and Solutions for Western Blotting

Western Transfer Buffer

Tris-base 30mM
Glycine 240mM
10% of methanol is added before use

10x TBS-T

Tris-HCl, pH 7.6 ..... 0.5M
NaCl 1.5M
Tween 20 ..... 1%

10x PBS-T

Na_2HPO_4 ..... 80mM
NaH_2PO_4 ..... 20mM
NaCl 100mM
Tween 20 ..... 10ml
ddH_2O..... to 1L
pH 7.5

Blocking Buffer

1x TBS-T or PBS-T + 5% (w/v) non-fat dry milk powder

Washing Buffer

1x TBS-T or PBS-T

2.1.5.4. Buffers and Solutions for Yeast Transformation

10x TE Buffer

Tris-HCl, pH 7.5 ..... 1.0mM
EDTA 10mM

10x LiAc Buffer

CH_3COOLi, pH 7.5 ..... 1M

LiTE Buffer

10x TE 1.0ml

10x LiAc 1.0ml

ddH_2O 8.0ml

PEG-LiTE

10x TE 1.0ml

10x LiAc 1.0ml

50%PEG 3350 ..... 8.0ml

ss DNA Buffer

Tris 10mM
NaCl 10mM
EDTA pH 8 ..... 1mM
ddH_2O ..... to 10ml
pH 8.0
10mg/ml of Herring Sperm DNA is dissolved in this buffer

2.1.5.5. Buffers and Solutions for Isolating Yeast Genomic DNA

Lysis Buffer

NaCl 100mM
SDS 1%
Tris-HCl pH 8.0 ..... 10mM
EDTA pH 8.0 ..... 1mM
Triton X 100 ..... 2%
ddH_2O ..... to 5.0ml

10x TE

The same as described in buffers and solutions for yeast transformation

Tris-NaCl Buffer

Tris-HCl pH7.5 ..... 10mM
NaCl 15mM
ddH_2O ..... to 5ml
10mg/ml of RNAase is dissolved in this buffer freshly

Other Solutions

phenol, chloroform, isoamylalcohol (25:24:1)

ethanol 99.9% extra pure

ammonium acetate 5M

2.1.5.6. Buffers and Solutions for isolating plasmid DNA from *E.coli*

STET buffer

glucose 8.0g
Triton X-100 ..... 5.0ml
Tris-HCl pH 8.0 ..... 25mM
EDTA pH 8.0 ..... 50mM
ddH_2O ..... to 100ml

TE buffer (0,1µg/µl RNAase A)

10x TE buffer 10ml
ddH_2O ..... to 100ml
0.1mg/ml of RNAase A is dissolved in this buffer freshly

Other Solutions

isopropanol

2.1.5.7. Stock Solutions

ampicillin 50mg/ml
APS ..... 10% (w/v)
BSA 20mg/ml
DTT 1M
ethidium bromide 10mg/ml
IPTG 1M

2.1.6. Organisms

Bacterial strains

Strain	Genotype	Source
DH5α	F^- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17(r_K^- m_K^+), λ–	Hanahan
XL1-Blue	endA1 gyrA96(nal^R) thi-1 recA1 relA1 lac glnV44 F'[::Tn10 proAB$^+$ lacI^q Δ(lacZ)M15] hsdR17(r_K^- m_K^+)	Stratagene

Yeast strains

Strain		Relevant genotype	Source
WCG4a	*MATa*	*leu2-3,112 ura3 his3-11,15 Can^s GAL2*	Heinemeyer et al., 1993
WCG4α	*MATα*	*leu2-3,112 ura3 his3-11,15 Can^s GAL2*	Heinemeyer et al., 1993
		All strains listed below are derivatives of either WCG4a or WCG4α	
YRuE001	*MAT α*	*pre2-2 pre3Δ::HIS3 [p16-PRE3*]	this work
YRuE002	*MAT a*	*pre2-2 pre3Δ::HIS3 [p16-PRE3*]	this work
YRuE003	*MAT α*	*pre3-T1A pre2Δ::HIS3 [p16-PRE2*]	this work
YRuE004	*MAT a*	*pre3-T1A pre2Δ::HIS3 [p16-PRE2]*	this work
YRuE005	*MAT α*	*pre2Δ::HIS3 [p16-PRE2]*	Heinemeyer et al., 1997
YRuE006	*MAT α*	*pre3Δ::HIS3 [p16-PRE3]*	Heinemeyer et al., 1997
YRuE007	*MAT a*	*pup1-T1A*	Heinemeyer et al., 1997
YRuE010		*pre2Δ::HIS3 [p15-pre2-M45R]*	this work
YRuE011		*pre3Δ::HIS3 [p15-pre3-R45M]*	this work
YRuE012		*pre3T1A pre2Δ::HIS3 [p15-pre2-M45R]*	this work
YRuE013		*pre2-2 pre3Δ::HIS3 [p15-pre3-R45M]*	this work
YRuE015	*MAT α*	*pre3-R45M*	this work
YRuE016	*MAT a*	*pre2-M45R*	this work
YRuE017		*pup1Δ::HIS3 [p15-pup1-std]*	this work
YRuE018		*pre2-M45R pre3-R45M*	this work
YRuE019		*pre3-R45M pup1-T1A*	this work
YRuE020	*MAT a*	*pre2-1*	(from W.Heinemeyer)
YRuE022		*pre2-2 pre3-T1A*	(from W.Heinemeyer)
YRuE026		*pre3Δ::HIS3 [p15-pre3-R45M-T52G]*	this work
YRuE027		*pre3Δ::HIS3 [p15-pre3-R45M-T52A]*	this work
YRuE028		*pre3Δ::HIS3 [p15-pre3-R45M-T52S]*	this work
YRuE029		*pre3Δ::HIS3 [p15-pre3-R45M-T52C]*	this work
YRuE030		*pre2-2 pre3Δ::HIS3 [p15-pre3-R45M-T52G]*	this work
YRuE031		*pre2-2 pre3Δ::HIS3 [p15-pre3-R45M-T52A]*	this work
YRuE032		*pre2-2 pre3Δ::HIS3 [p15-pre3-R45M-T52S]*	this work
YRuE033		*pre2-2 pre3Δ::HIS3 [p15-pre3-R45M-T52C]*	This work
YRuE034	*MAT α*	*pre3-T1A*	Heinemeyer et al., 1997
YRuE035	*MAT α*	wild type transformed with [pRS315]	(from W.Heinemeyer)
YRuE036		*pre3Δ::HIS3 [p15-PRE3-HA]*	this work
YRuE037		*pre3Δ::HIS3 [p15-pre3-R45M-HA]*	this work
YRuE042	*MAT α*	*pre3-T1A pup1-T1A*	Heinemeyer et al., 1997
YRuE043	*MAT a*	*pre3-T1A pre2-2*	this work
YRuE044	*MAT a*	*pre3-R45M pup1T1A*	this work
YRuE045		*pre3-R45M pre2-2*	this work
YRuE046		*pup1Δ::HIS3 [p15-PUP1-3HA]*	this work
YRuE047		*PUP1 [p25-pup1-mtd-HA]*	this work
YRuE048		*PUP1 [p25-pup1-ltd-HA]*	this work
YRuE049		*pup1Δ::HIS3 [p15-pup1-std-HA]*	this work
YRuE050		*pre3Δ::HIS3 [p15-pre3-T20A-R45M]*	this work
YRuE051		*pre3Δ::HIS3 [p15-pre3-T31V-R45M]*	this work
YRuE052		*pre3Δ::HIS3 [p15-pre3-T20A-T31V-R45M]*	this work
YRuE053		*pre3Δ::HIS3 [p15-pre3-T35I-R45M]*	this work
YRuE054		*pre3Δ::HIS3 [p15-pre3-T20A-T31V-T35I-R45M]*	this work
YRuE055		*pre2Δ::HIS3 [p15-pre2-A20T-M45R]*	this work
YRuE056		*pre2Δ::HIS3 [p15-pre2-V31T-M45R]*	this work
YRuE057		*pre2Δ::HIS3 [p15-pre2-A20T-V31T-M45R]*	this work
YRuE058		*pre2Δ::HIS3 [p15-pre2-I35T-M45R]*	this work
YRuE059		*pre2Δ::HIS3 [p15-pre2-A20T-V31T-I35T-M45R]*	this work
YRuE060		*pre2-2 pre3Δ::HIS3 [p15-pre3-T20A-R45M]*	this work
YRuE061		*pre2-2 pre3Δ::HIS3 [p15-pre3-T31V-R45M]*	this work
YRuE062		*pre2-2 pre3Δ::HIS3 [p15-pre3-T20A-T31V-R45M]*	this work
YRuE063		*pre2-2 pre3Δ::HIS3 [p15-pre3-T35I-R45M]*	this work
YRuE064		*pre2-2 pre3Δ::HIS3 [p15-pre3-T20A-T31V-T35I-R45M]*	this work
YRuE065		*pre3-T1A pre2Δ::HIS3 [p15-pre2-A20T-M45R]*	this work
YRuE066		*pre3-T1A pre2Δ::HIS3 [p15-pre2-V31T-M45R]*	this work
YRuE067		*pre3-T1A pre2Δ::HIS3 [p15-pre2-A20T-V31T-M45R]*	this work
YRuE068		*pre3-T1A pre2Δ::HIS3 [p15-pre2-I35T-M45R]*	this work
YRuE069		*pre3-T1A pre2Δ::HIS3 [p15-pre2-A20T-V31T-I35T-M45R]*	this work
YRuE070	*MAT a*	wild type transformed with [pRS425]	this work
YRuE071		*PUP1 [p25-PUP1-HA]*	this work

YRuE072		*PUP1 [p25-pup1-std-HA]*	this work
YRuE073		*PUP1 [p15-PUP1-HA]*	this work
YRuE074		*PUP1 [p15-pup1-std-HA]*	this work
YRuE075		*PUP1 [p15-pup1-mtd-HA]*	this work
YRuE076		*PUP1 [p15-pup1-ltd-HA]*	this work
YRuE077	*MAT* α	*pup1-T1A*	Heinemeyer et al., 1997
YRuE078	*MAT a*	*pup1-T1A pre3-T1A*	(from W.Heinemeyer)
YRuE079	*MAT a*	*pre2-2*	Heinemeyer et al., 1993
YRuE080	*MAT* α	*pre2-2*	Heinemeyer et al., 1993
YRuE081	*MAT* α	*pup1-T1A pre3Δ::HIS [p16-PRE3]*	this work
YRuE082	*MAT a*	*pup1-T1A pre2Δ::HIS [p16-PRE2]*	this work
YRuE083		*pup1-T1A pre3-T1A pre2Δ::HIS3 [p16-PRE2]*	this work
YRuE091		*PUP1 [p25-PUP1]*	this work
YRuE092		*PUP1 [p25-pup1-std]*	this work
YRuE093		*PUP1 [p25-pup1-mtd]*	this work
YRuE094		*PUP1 [p25-pup1-ltd]*	this work
YRuE095		*PUP1 [p15-PUP1]*	this work
YRuE096		*PUP1 [p15-pup1-std]*	this work
YRuE097		*PUP1 [p15-pup1-mtd]*	this work
YRuE098		*PUP1 [p15-pup1-ltd]*	this work
YRuE099		*pup1Δ::HIS3 [p15-PUP1]*	(from W.Heinemeyer)
YRuE100		*pup1-T1A pre3Δ::HIS3 [p15-pre3-T1A-HA]*	this work
YRuE101		*pup1-T1A pre3Δ::HIS3 [p15-pre3-T20A-T31V-R45M-HA]*	this work
YRuE102		*pup1-T1A pre3Δ::HIS3 [p15-pre3-T35I-R45M-HA]*	this work
YRuE103		*pup1-T1A pre3Δ::HIS3 [p15-pre3-T20A-T31V-T35I-R45M-HA]*	this work
YRuE104		*pre2-2 pre3Δ::HIS3 [p15-PRE3]*	this work
YRuE105		*pre2-2 pre3Δ::HIS3 [p15-pre3-T1A]*	this work
YRuE106		*pup1-T1A pre3Δ::HIS3 [p15-pre3-T1A]*	this work
YRuE107		*pre3-T1A pre2Δ::HIS3 [p15-PRE2]*	this work
YRuE108		*pre2Δ::HIS3 [p15-PRE2]*	this work
YRuE109		*pre3Δ::HIS3 [p15-PRE3]*	this work
YRuE110		*pup1-T1A pre3Δ::HIS3 [p15-PRE3-HA]*	this work
YRuE111		*pup1-T1A pre3Δ::HIS3 [p15-pre3-R45M-HA]*	this work
YRuE112	*MAT a*	*pre2-K33A*	Heinemeyer et al., 1997
YRuE113	*MAT a*	*pup1Δ::HIS3 [p16-PUP1]*	Heinemeyer et al., 1997
YRuE114		*pup1Δ::HIS [p15-PUP1-FLAG]*	this work
YRuE115		*pup1Δ::HIS [p15-pup1-std-FLAG]*	this work
YRuE116		*PUP1 [p25-pup1-ltd-FLAG]*	this work
YRuE117		*Nat1Δ::KAN* (YDL 040C)	EUROSCARF
YRuE118		*PUP1 [p25-PUP1-FLAG]*	this work
YRuE119		*Nat1Δ::Kan pre2-2 pre3Δ::HIS [p16-PRE3]*	this work
YRuE120		*Nat1Δ::Kan pre3Δ::HIS [p16-PRE3]*	this work
YRuE121		*Nat1Δ::Kan pre3Δ::HIS [p15-UB-pre3-R45M]*	this work
YRuE122		*Nat1Δ::Kan pre3Δ::HIS [p15-UB-pre3-T20A-T31V-R45M]*	this work
YRuE123		*Nat1Δ::Kan pre3Δ::HIS [p15-UB-pre3-T20A-T31V-T35I-R45M]*	this work
YRuE124		*Nat1Δ::Kan pre2-2 pre3Δ::HIS [p15-UB-pre3-R45M]*	this work
YRuE125		*Nat1Δ::Kan pre2-2 pre3Δ::HIS [p15-UB-pre3-T20A-T31V-R45M]*	this work
YRuE126		*Nat1Δ::Kan pre2-2 pre3Δ::HIS [p15-UB-pre3-T20A-T31V-T35I-R45M]*	this work
YRuE127		*Nat1Δ::Kan pre3Δ::HIS [p15-UB-PRE3]*	this work
YRuE128		*Nat1Δ::Kan pre3Δ::HIS [p15-UB-pre3T1A]*	this work
YRuE129		*Nat1Δ::Kan pre2-2 pre3Δ::HIS [p15-UB-PRE3]*	this work
YRuE130		*Nat1Δ::Kan pre2-2 pre3Δ::HIS [p15-UB-pre3-T1A]*	this work

2.1.7. Plasmid constructs

Plasmid constructs were mostly generated in the pRS315 vector. For over-expression of lethal mutations, the pRS425 vector was used, and for chromosomal insertion via pop in - pop out, the pRS306 vector.

Name	Vector	Insert details	Source
PRE-008	pRS315	pre3 R45M	this work
PRE-009	pRS315	pre2 M45R	this work
PRE-010	pRS315	PRE2	W. Heinemeyer
PRE-011	pRS315	PRE3	W. Heinemeyer
PRE-025	pRS306	pre2 M45R	this work
PRE-026	pRS306	pre3 R45M	this work
PRE-027	pRS315	pup1 std	this work
PRE-038	pRS315	pup1 mtd	this work
PRE-028	pRS315	pup1 ltd	this work
PRE-029	pRS315	PUP1	W. Heinemeyer
PRE-030	pRS315	pup1 T211A	this work
PRE-031	pRS315	pup1 Y204A	this work
PRE-032	pRS315	pup1 R208G	this work
PRE-034	pRS315	pre3 R45M T52G	this work
PRE-035	pRS315	pre3 R45M T52A	this work
PRE-036	pRS315	pre3 R45M T52S	this work
PRE-037	pRS315	pre3 R45M T52C	this work
PRE-044	pRS315	PRE3 (HA)	this work
PRE-045	pRS315	PUP1 (3HA)	this work
PRE-049	pRS315	pup1 std (3HA)	this work
PRE-047	pRS315	pup1 mtd (3HA)	this work
PRE-048	pRS315	pup1 ltd (3HA)	this work
PRE-050	pRS425	pup1 mtd (3HA)	this work
PRE-051	pRS425	pup1 ltd (3HA)	this work
PRE-053	pRS315	pre2 A20T M45R	this work
PRE-055	pRS315	pre2 V31T M45R	this work
PRE-056	pRS315	pre2 A20T V31T M45R	this work
PRE-059	pRS315	pre2 I35T M45R	this work
PRE-057	pRS315	pre2 A20T V31T I35T M45R	this work
PRE-052	pRS315	pre3 T20A R45M	this work
PRE-060	pRS315	pre3 T31V R45M	this work
PRE-054	pRS315	pre3 T20A T31V R45M	this work
PRE-061	pRS315	pre3 T35I R45M	this work
PRE-058	pRS315	pre3 T20A T31V T35I R45M	this work
PRE-064	pRS315	pre3 T1A	W. Heinemeyer
PRE-062	pRS425	PUP1 (HA)	this work
PRE-063	pRS425	pup1 std (HA)	this work
PRE-065	pRS425	PUP1	this work
PRE-066	pRS425	pup1 std	this work
PRE-067	pRS425	pup1 mtd	this work
PRE-068	pRS425	pup1 ltd	this work
PRE-069	pRS315	pre3 T20A T31V R45M (HA)	this work
PRE-070	pRS315	pre3 T35I R45M (HA)	this work
PRE-071	pRS315	pre3 T20A T31V T35I R45M (HA)	this work
PRE-072	pRS315	pre3 T1A (HA)	this work
PRE-073	pRS315	pre3 R45M (HA)	this work

PRE-076	pRS315	PUP1 (FLAG)	this work
PRE-077	pRS315	pup1 std (FLAG)	this work
PRE-078	pRS425	PUP1 (FLAG)	this work

2.1.8. Oligonucleotides

Name	Sequence
005-5/6F	gaaacagctatgaccatgat 5' Universal Primer for pRS315 plasmid insert region
006-5/6R	gacggccagtgaattgtaat 3' Universal Primer for pRS315 plasmid insert region
013-E2MR-for	Pho – TCt tgt acc caa taa aaa tgg gtt g 5' Primer for the point mutation of PRE2, M45 into R45
016-E2MR-rev	gct ggt ggt gcg gca ga 3' Primer for the point mutation of PRE2, M45 into R45
030-pre2A20T	Pho – gta gat tct cgt AcG act gcc ggc aat tgg g 5' Primer for the point mutation of Pre2M45R, A20 into T20
031-pre2V31T	Pho – gct tct caa act ACA aag aaa gtt att gag atc aac cca ttt tta ttg gg 5' Primer for the point mutation of Pre2M45R, V31 into T31
032-pre2I35T	Pho – aag aaa gtG aCt gag atc aac cca ttt tta ttg ggt aca aga gc 5' Primer for the point mutation of Pre2M45R, I35 into T35
014-E3RM-for	Pho – aTg tcc ggt tct gca gca gac 5' Primer for the point mutation of PRE3, R45 into M45
015-E3RM-rev	aca aca cca aat ttt gtc atg ta 3' Primer for the point mutation of PRE3, R45 into M45
026-pre3T52X-for	c ggt tct gca gca gac KST cag gcg att gcc gac 5' Primer for the point mutations of pre3-R45M, T52 into G52, A52, S52 and C52
027-pre3T52X-rev	gtc ggc aat cgc ctg ASM gtc tgc tgc aga acc g 3' Primer for the point mutations of Pre3R45M, T52 into G52, A52, S52 and C52
033-pre3T20A	Pho – gtg ctg att cac gGG cca cca ctg gtg cg 5' Primer for the point mutation of Pre3R45M, T20 into A20
034-pre3T31V	Pho – aca tag cta acc gtg tgG TCg ata aat taa cga gag tac atg ac 5' Primer for the point mutation of Pre3R45M, T31 into V31
035-pre3T35I	Pho – gat aaa tta aTT aga gta cat gac aaa att tgg tgt tgt atg tcc g 5' Primer for the point mutation of Pre3R45M, T35 into I35
028-pre3NotI-for	cct gat gaa tat gaa caa cta GGC GGC CGC taa tag acc cca aaa gaa c 5' Primer for the NotI cleavage site insertion in PRE3
029-pre3NotI-rev	g ttc ttt tgg ggt cta tta GCG GCC GCC tag ttg ttc ata ttc atc agg 3' Primer for the NotI cleavage site insertion in PRE3
040-NA1Kan-A	att cga caa ttc aaa att gag aga g 3' Primer for chromosomal Kanamycine cassete
041-NA1Kan-B	tat att tgc ttg tta gtg gac cca t 5' Primer for kanamycine insertion confirmation

042-NA1Kan-C	gac gaa agg gat tat att ttg gac t 3’ Primer for kanamycine insertion confirmation
043-NA1Kan-D	tat att ttt ctt ggg tgg tga aaa a 5’ Primer for chromosomal Kanamycine cassete
017-Pup1C-tail	tga aag taa aac ata gaa ctt 5’ Primer for the PUP1 tail deletions
018-Pup1D-tail	aag ttc tat gtt tta ctt tca aac att tgg agt caa gta at 3’ Primer for the PUP1 long tail deletion
019-Pup1E-tail	aag ttc tat gtt tta ctt tca cac agc agt tgt acc cct 3’ Primer for the PUP1 short tail deletion
020-Pup1F-tail	aag ttc tat gtt tta ctt tca cct ggg gaa ttt gta gct ttt 3’ Primer for the PUP1 middle tail deletion
021-pup1Y204A-for	gaagaaaagcagaaaagcGCTaaattcccca 5’Primer for the point mutation of pup1-Y204A
022-pup1Y204A-rev	gcttttctgcttttcttct 3’Primer for the point mutation of pup1-Y204A
023-pup1R208G-for	aaaagctacaaattccccGgTggtacaact 5’Primer for point mutation of pup1-R208G
024-pup1T211A-for	aaaagctacaaattccccaggggtacaGctgctgtgct 5’Primer for point mutation of pup1-T211A
025-pup1RGTA-rev	ggggaatttgtagcttttct 3’Primer for both point mutations pup1-R208G and pup1-T211A
036-FLAG-pup1-for	tta caa gga tga cga cga taa gtg aaa gta aaa cat aga act ta 5’ Primer for the Pup1 FLAG tagging
037-FLAG-PUP1-rev	tcg tcg tca tcc ttg taa tca gcc gtt ata tcg act tgt tc 3’ Primer for the PUP1 FLAG tagging
038-FLAG-p1std-rev	tcg tcg tca tcc ttg taa tcc aca gca gtt gta ccc ctg 3’ Primer for the pup1std FLAG tagging
039-FLAG-p1ltd-rev	tc gtc gtc atc ctt gta atc aac att tgg agt caa gta att c 3’ Primer for the pup1ltd FLAG tagging

Oligonucleotides: K – G or T; S – G or C. Capital letters are mutations. In underlined are the complementary primer sequences.

2.1.9. Antibodies

Anti-Pre2 polyclonal antibody was raised in rabbit by Eurogentech Co. [Heinemeyer W 1997]. Anti-HA and Anti-Flag monoclonal antibodies (Sigma) were raised in mouse and rabbit, respectively. Primary antibodies were detected using anti-rabbit IgG HRPO (horseradish peroxidase-coupled) secondary antibody, purchased from Sigma, Deisenhofen, Germany.

2.2. Methods

2.2.1. Molecular biology

Isolation of DNA

Plasmid preparation from *E.coli* using the Qiaprep Spin miniprep kit

Plasmid DNA with optimal purity was isolated using the Qiaprep Spin miniprep kit (Qiagen, Santa Clarita, CA, USA and PEQ lab GmbH, Germany). The plasmid isolation from bacterial cells was performed according to the manufacturer's protocol and the purity was evaluated by agarose gel electrophoresis.

Plasmid preparation from *E. coli* cultures using the boiling miniprep method

This is a fast and cheap method for isolation of plasmids from *E.coli*.

From a 3ml overnight culture (12-16h) in LB_{Amp}, 1,5ml are transferred to an Eppendorf tube and pelleted by centrifugation (1min at 13000rpm). After removing the supernatant, the cells are resuspended in 350µl of STET buffer. 350µl of the same buffer with 0.5mg/ml of lysozyme is added and mixed. The tube is heated at 100ºC during 3min30sec and a hole is made in the Eppendorf cap while heating. The solution is incubated for 10min on ice and centrifuged (10min at 13000rpm). The slimy pellet is removed with the help of a toothpick and 500µl isopropanol are added followed by centrifugation (5min, 13000rpm). The supernatant is completely removed and the DNA pellet resuspended in 50µl of TE buffer containing 0,1µg/µl RNAase A.

Total DNA isolation from *S. cerevisiae*

A 1,5ml of overnight culture or from plate, are resuspended in 200µl lysis buffer. Then 200µl of glass beads, 200µl of phenol-chloroform (1:1) and 200µl H_2O are added. The Eppendorf tubes are shacked in a vortexer for 5min at 37ºC and then centrifuged (5min at 13000rpm). The water phase is collected in a new tube, and 1ml ethanol (99.5%) is added. Precipitation at –20ºC is performed during 30min followed by centrifugation (5min at 13000rpm). The supernatant is removed, the pellet washed with 200µl ethanol (70%) and centrifuged (2min at 13000rpm). After discarding the supernatant, the DNA is resuspended in 50µl of Tris/HCl pH8.0.

DNA Agarose Gel Electrophoresis

The analytical and preparative separation of DNA molecules of different sizes was carried out on agarose gels cast in a horizontal flatbed chamber (BioRAD). Agarose was dissolved in 1x TAE or TBE buffer to a final concentration of 0.8% to 1.5% (w/v) depending upon necessity, by boiling in a microwave. Before casting, 0.5mg/L of ethidium bromide to visualize the DNA bands is added. 1x TAE or TBE buffer was used for running the gels. The DNA samples are mixed with 1μl of DNA loading buffer and run along with the molecular weight marker at 100V, until the blue dye reaches the end of the gel. The DNA bands were visualized by ethidium bromide fluorescence in UV light and photographed.

DNA extraction from gel

Usually 20-50μl of DNA is loaded in a single electrophoresis gel well. After running the gel at 100V, the desired band is excised with a sharp scalpel. Then it is purified with QIAQuick Gel Extraction Kit and resuspended in 20μl of Tris HCl pH8 buffer.

Purification of DNA

PCR products were purified by using the QIAGEN PCR purification kit, and from the agarose gels DNA was purified by using the QIAEX DNA purification kit. The DNA purification was carried after the protocol supplied by the manufacturer.

Digestion of DNA with Restriction Endonucleases

The reactions for the individual restriction enzymes were based on the information of the manufacturer's protocol. All restriction enzymes used in this study are from the Roche or New England Biolabs (NEB), the digestion reactions were performed in a total volume of 10μl, using adequate concentration of DNA, 1μl of 10x reaction buffer, 10mg/ml BSA (for certain enzymes only) and 1 to 10 U restriction enzyme were used. Reactions were carried out at 37°C to 65°C (as per manufacturer's protocol) for 1 to 3 hours. Double digestions were executed in a buffer system suitable for both enzymes or digestions were carried out subsequently.

To use digested DNA for cloning, subsequent gel electrophoresis was necessary to eliminate of other DNA fragments. DNA was then cut out of the gel and purified with the Gel Purification kit (Qiagen) according to the manufacturer manuals.

Oligonucleotides

All oligonucleotides used were supplied by Metabion.

Polymerase Chain Reaction

The polymerase chain reaction is a method to amplify defined DNA segments from either plasmid DNA or genomic DNA *in vitro*. In a first step template DNA gets denatured at 95ºC. Two short synthetic oligonucleotides, called primers determine the beginning and end of the region to be amplified. They anneal to the denatured template DNA at the calculated annealing temperature (T_m). At a third temperature the 3' primed ends of the primers are elongated, catalysed by a heat stable DNA polymerase. By repeating these steps, DNA gets amplified.

The *in vitro* amplification of specific regions was conducted using a RoboCycler Gradient 40 PCR machine (Stratagene, La Jolla) with a reaction specific program. A 50µl reaction mix was prepared in 200µl thin wall Eppendorf tubes. This reaction mix consisted of a dNTP mix, each oligonucleotide primer in a concentration of 1pmol/µl, template DNA and the DNA polymerase with the appropriate reaction buffer. The whole mix was filled up to 50µl volume with sterile ddH_2O. Volumes and concentrations for a conventional PCR reaction are listed in Table 2-01. Yeast genomic DNA and plasmid DNA were mostly used in this study as templates for the PCR reaction.

Table 2-01: Reagents and their volumes used for conventional PCR reations

Reagent	Volume [µl]	concentration/activity
DNA polymerase	1	0,1-1,0U
Reaction buffer	5	10x
dNTP's	1	25mM
Template DNA	1	0,2µg/µl
Oligonucleotide 1	1	0,1µg/µl
Oligonucleotide 2	1	0,1µg/µl
ddH_2O	add to 50µl	

The annealing temperature of the primers (T_m) was calculated with the following formula:

$T_m = 81{,}5ºC + [0{,}41x((dGTP''s + dCTP''s)/N)x100 - (675/N)]$

N is the total number of base pairs. Denaturing of template DNA was conducted for 5min in a single beginning step and then for 45s every cycle. The amplification step took place at 72ºC. One minute extension time was calculated for each 1000bp to be amplified. For a complete extension the temperature was kept at 72ºC for 5minutes in a final step. 30rounds of amplification were sufficient for a suitable yield of DNA. To look for PCR product, 3µl of the reaction mix was run on a 1% agarose gel after amplification. Purification of PCR products was carried out with the QIAquick PCR Purification Kit according to the manual instructions.

Ligation of DNA fragments

The enzyme T4 DNA ligase (Invitrogen) catalyses the formation of phosphodiester bonds between the 5'phosphate and the 3'hydroxyl group of double stranded DNA under consumption of ATP. To enhance the ligation efficiency different vector to insert ratios (1:2 or 1: 3 or 1:4) were used. A typical protocol was as follows: 2μl of vector; 6μl of insert; 2μl of 10X ligation buffer; 1μl of T4 DNA ligase (5U/μl) and ddH_2O until total volume of 20μl.

The reaction mixture was incubated for ligation at 18°C overnight.

Sequencing of plasmid DNA

To sequence certain DNA regions on a plasmid the plasmid DNA was isolated with the QIAprep Spin miniprep kit (Qiagen). Conventionally the plasmid DNA from two QIAprep reactions was eluted with 50μl of ddH_2O each and pooled in a 1,5ml reaction tube. The amount of DNA should then be between 1-2μg. Then the complete DNA got precipitated by adding 250μl of ethanol (100%) to the eluted 100μl DNA. Samples were kept at room temperature for 30min and the coagulated DNA was centrifuged (30min at 13000rpm). After carefully removing the supernatant the pellet was dried for 30-60min and samples were send for sequencing. Sequencing was conducted at MWG-Biotech, Ebersberg and primers for sequencing were sent at a concentration of 10pmol/μl.

Transformation Methods

Preparation of competent *E. coli* cells for electro-transformation

Electro-competent DH5α *E. coli* were prepared by the following way: a logarithmically growing 1l culture of *E. coli* (A_{600} 0.4-0.5) was cooled down to 4ºC on ice water, harvested at 6000rpm in a pre-chilled rotor for 10min and washed first with an equal volume and second with a half volume of ice cold 10% glycerol at 4°C. The pellet was resuspended in 50ml of ice cold 10% glycerol and, after transfer to a 50ml sterile Falcon, spun (10min at 14000rpm) in a table centrifuge. The pellet was then resuspended in 3ml of ice cold 10% glycerol; samples were stored in 160μl aliquots in sterile Eppendorf tubes at –80°C.

Transformation of electro-competent E.coli cells by electroporation

Electro-competent DH5α *E.coli* cells were taken out of the -80ºC freezer and thawed on ice. 40μl of cells were mixed with up to 1μl of ligation mixture or plasmid DNA while

keeping on ice. This mixture was transferred to a cold electroporation cuvette (0,2mm) and transformation was done using a Gene Pulser (BioRad). The settings were put to capacitance of 125µFD, resistance of 400OHM and 2,25Volt.
After the pulse, cells were immediately resuspended in 1ml of SOC medium, transferred to a 1,5ml reaction tube and incubated at 37°C for 50-60 minutes. After a quick spin, the pellet was resuspended in 50-100µl remaining SOC and plated out on an LB_{Amp} plate. Plates were then incubated overnight at 37°C.
For a so called "Blue-White" screen 100µl of X-Gal (20 mg X-Gal /ml DMF) and 40µl of a 100mM IPTG stock were distributed on a LB_{Amp} plate, which was kept open on the sterile bench for 30 min. Clones carrying a plasmid with an intact *LACZ* gene (e.g. pRS series) were able to metabolize X-Gal and turned blue. However clones carrying an insert in the multi cloning site (MCS) were not able to metabolize X-Gal and stayed white.
With this method clones carrying a DNA fragment in the MCS can be distinguished from clones without.

Transformation method for yeast *S. cerevisiae*

Yeast transformation was done by the lithium acetate method as follows: Logarithmically growing yeast cells (10ml, A_{600} 1.0) were harvested at 3600rpm and the pellet was washed first with an equal volume and afterwards with a tenth volume of autoclaved ddH_2O and once with an equal volume of TE-Li-Ac buffer. Then the pellet was suspended in 200µl of TE-Li-Ac buffer. 3µl of ssDNA and 2-5µl of plasmid DNA were added to 50µl of competent yeast cells and finally 300µl of PEG-TE-Li-Ac was added and mixed briefly. The mixture was incubated at 30°C for 30 min followed by a heat shock at 42°C for 20min. The cells were harvested at 13000rpm for 1-2sec and the pellet was resuspended in 100µl of sterile ddH_2O and spread on selective CM plates lacking the particular amino acid and incubated at 30°C for 2 days.

Site-directed mutagenesis by recombinant PCR

Two separate fragments were amplified from the plasmid pRS315 carrying a gene encoding the respective proteasomal subunit (*PRE2*, *PRE3* or *PUP1*). The first fragment was created using a forward oligonucleotide containing the desired mutation and a universal reverse oligonucleotide (5/6Rprimer). The second fragment was generated by using the universal forward oligonucleotide (5/6Fprimer) and a reverse oligonucleotide carrying the mutation. Both fragments were purified and pooled. In the mixture, two overlapping regions obtained from the oligonucleotides with the mutation sequence served as primers on one another to create the full-length PCR product in the presence of universal forward and reverse oligonucleotides (5/6Fprimer and 5/6Rprimer) (fig.2-01).

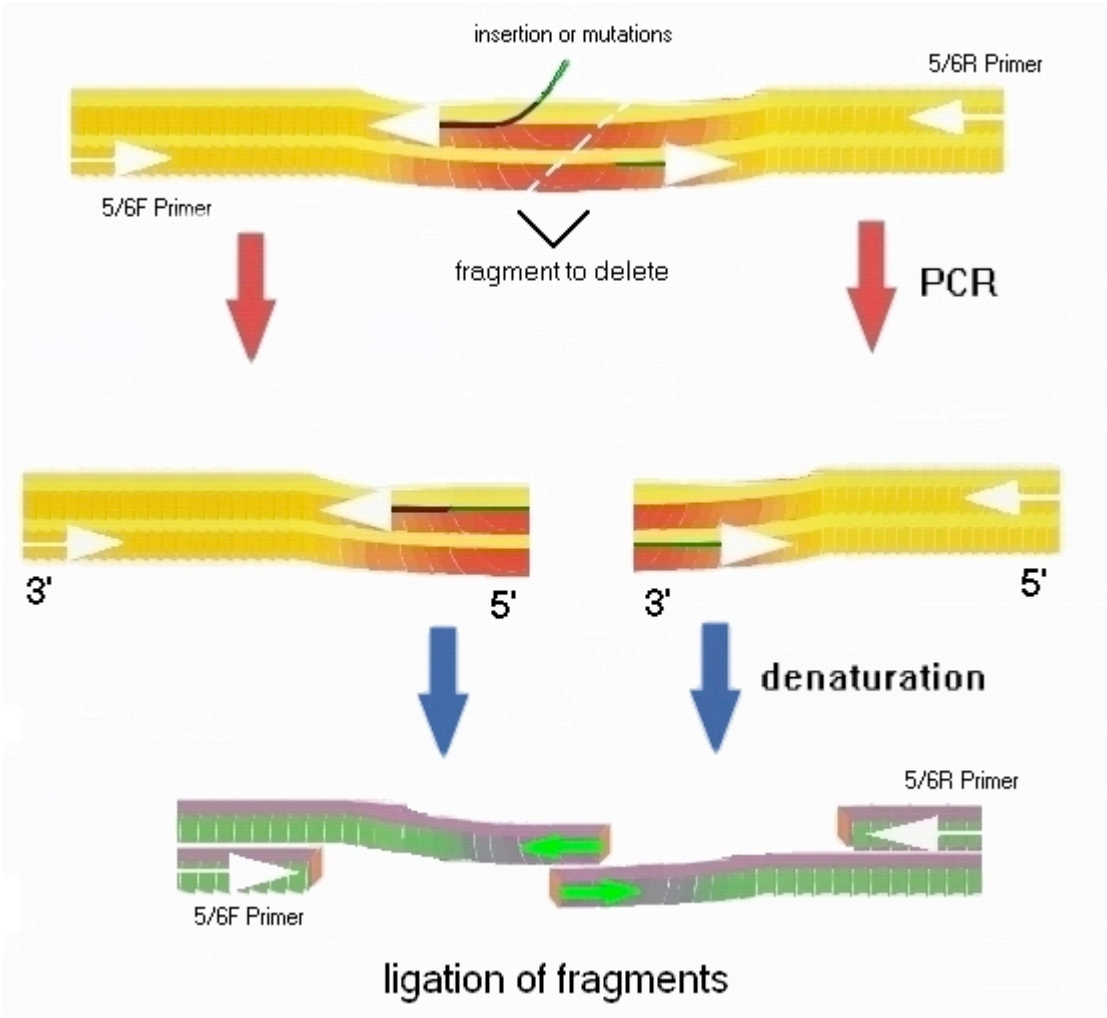


Figure 2-01 – Mutation, insertion or deletion scheme for the PCR recombination method. Two distinct PCR are done generating two different fragments with small coincident sequence at the 5'end in one and 3' of the other. Both fragments are joined with a third PCR using 005-5/6F and 006-5/6R primers.

Site directed mutagenesis by "ExSite™" mutagenesis

Site directed mutagenesis was performed as described by ExSite (ExSite™ PCR-Based Site-Directed Mutagenesis Kit). It can be used to make point mutations, large or small deletions and/or insertions (fig.2-02).

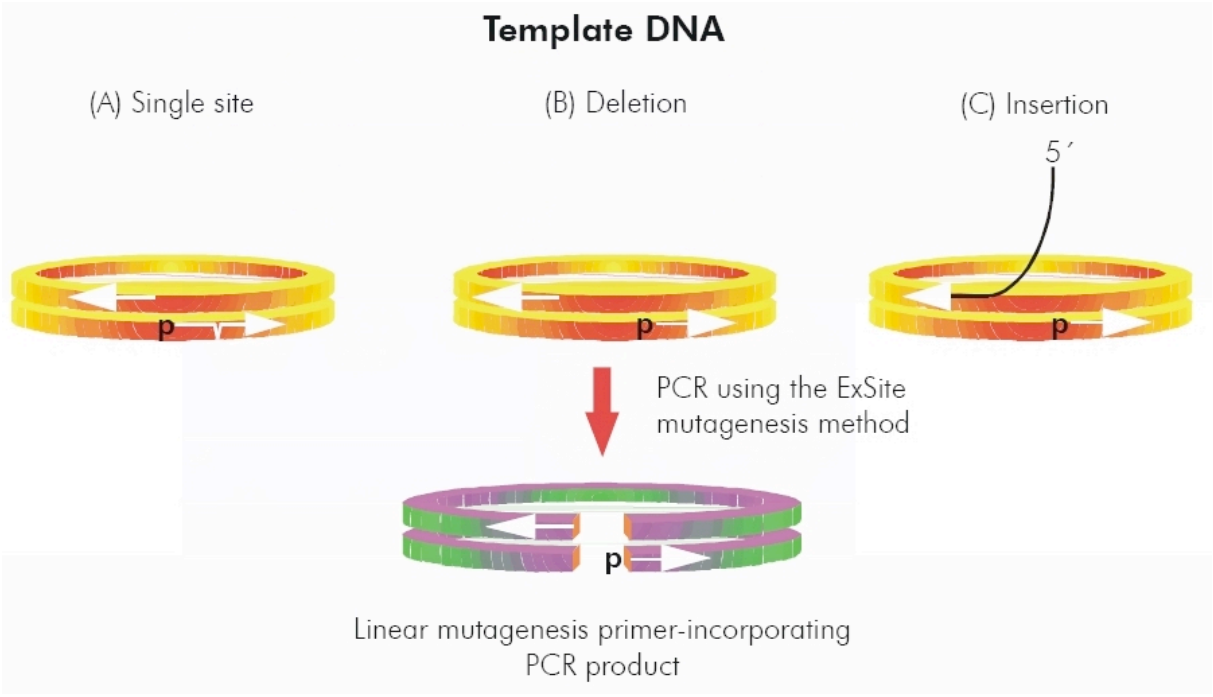


Figure 2-02 – Primer design scheme for ExSite PCR-based site-directed mutagenesis method allowing the mutagenesis of dsDNA templates resulting in (A) point mutations at a single site, (B) large or small deletions and/or (C) 5'-end oligonucleotide-directed base insertions. One of the primers must be 5' phosphorylated (p).

Therefore two oligonucleotides were designed, one or both containing the desired mutation. They should be greater then 20 bases in length. Mismatched portions are at or near 5' end of one or both primers with 15 or more bases of correct sequence on the 3' end. One or both primers must be 5' phosphorylated. 5' ends of the primers should meet but not overlap. Any nucleotides added to the 5' ends of the primers are deleted in the final product. Each primer, complementary to opposite strands of the vector are extended during PCR temperature cycling by *PfuTurbo* DNA polymerase. Following temperature cycling, the product is treated with *Dpn* I to digest the parental DNA template. Then a ligation is performed using T4 DNA ligase to circularise the product templates.

The sample was then transformed into XL-1 Blue super-competent cells and plated out on LB-Amp plates. After overnight incubation clones were picked and plasmids were tested for the mutations by restriction enzyme digestions and sequencing.

Site directed mutagenesis by "Quick-Change" mutagenesis

Site directed mutagenesis was performed as described by QuikChange (QuikChange® Site-Directed Mutagenesis Kit). It can be used to make point mutations, delete or insert single or multiple amino acids (fig.2-03).

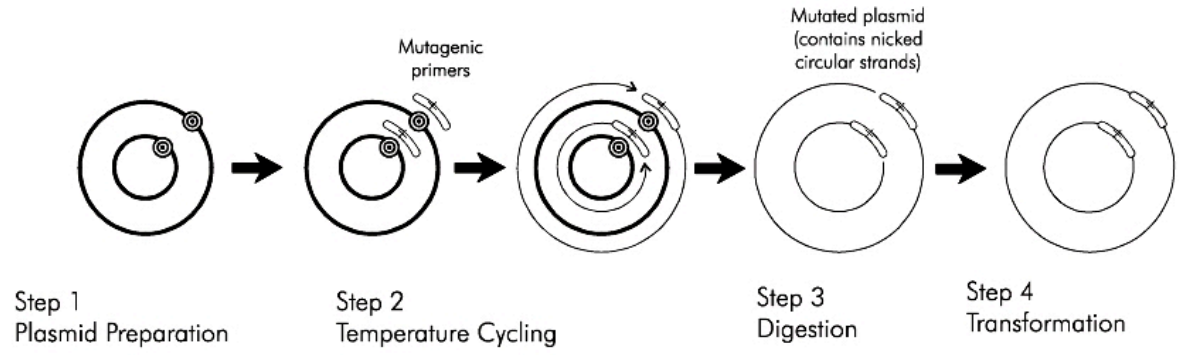


Figure 2-03 – Overview of the QuikChange site-directed mutagenesis method.

Therefore two complementary oligonucleotides were designed containing the desired mutation. They should be between 25 and 45 bases in length with a melting temperature (T_m) of ≥78ºC. Primers were also designed to carry the mutation in the middle of the primers with 10-15 bases of correct sequence on both sides. Each primer, complementary to opposite strands of the vector are extended during each step of the temperature cycling by *PfuTurbo* DNA polymerase. Following temperature cycling, the product is treated with *Dpn* I. The *Dpn* I endonuclease is specific for methylated and hemimethylated DNA and is used to digest the parental DNA template and to select for mutation-containing synthesized DNA.

The sample was then transformed into DH5α electro-competent cells and plated out on LB-Amp plates. After overnight incubation, clones were picked and plasmids were tested for the mutations by restriction enzyme digestions and sequencing.

Plasmid Shuffling method

In a yeast strain having a chromosomal deletion of the desired gene, this gene will be expressed by a plasmid having a *URA3* marker, usually a pRS316 plasmid. The new plasmid that will replace the previous should have a different marker, usually *LEU2* on a pRS315 or pRS425 plasmid. Transformation of yeast (as previously described) with the latest originates in a strain with both plasmids. This should be streaked on a 5'FOA plate, which is lethal to any cell having a plasmid with *URA3* marker. Only colonies with the pretended plasmid and without the previous one are viable.

Pop-in/pop-out replacement

A pRS306 plasmid, containing the gene with the desired mutation is linearized and then transformed into a yeast strain which then inoculated on CM medium plates lacking uracil. Surviving colonies are re-streaked on YPD plates. The plasmid should integrate into the chromosome (pop-in), and afterwards leave together with the chromosomal gene, leaving the mutated gene in the chromosome. Streaking on 5' FOA does selection

for cells that lost the plasmid (pop-out). Differentiation between mutated and non-mutated colonies is done by the identification of loss or gain of characteristics.

Crossing of yeast haploids

Haploid cells of the yeast *Saccharomyces cerevisiae* can have one of the two mating types 'a' or 'α'. When cells of opposite mating type are put together, they mate, combining both genotypes in a diploid (zygote) containing the genetic material of both haploids.

A small streak of 'a' type is mixed with 'α' type cells on an YPD plate and incubated at 30ºC for about 3-4hours. Zygotes can be observed in a microscope having a flower like shape. A very small quantity of cells is diluted in 100µl H_2O, from which 10µl are spread into an equatorial line. Using a microscope and a dissection needle, 5-6 diploids are separated at 5mm distance from each another on each side. The plate is then incubated one day at 30ºC. The surviving colonies are refreshed in YPD and incubated another day at 30ºC.

Sporulation of yeast diploids and tetrad dissection

Under starving conditions, the diploid cells suffer meiosis giving four haploid spores, called tetrads.

For sporulation, patches of the diploid cells are made on Sporulation medium and incubated during 3-4 days at 30ºC. Tetrads can be observed in a microscope. A scratch is taken and diluted in 100µl H_2O in a sterile Eppendorf tube. 1.5µl glusolase is added, mixed, and the solution incubated 5min at room temperature. 400µl H_2O is added followed by 3-4h incubation at 4ºC.

10µl are spread into an equatorial line on a YPD plate. The four spores of chosen tetrads are separated in a longitudinal line with the help of a microscope and a tetrad dissection needle. The plate is incubated overnight at 30ºC.

2.2.2. Biochemical Methods

Protein sample preparation and quantification

The yeast cells were harvested after reaching a given OD_{600} or at defined time points. The cells were lysed in lysis solution (0.25M NaOH with 1% β-mercatoethanol). The proteins were precipitated with 5.8% trichloroacetic acid, sedimented and washed with acetone. The dried pellets were resuspended in Laemmeli buffer (50mM Tris-Cl pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol and 100mM dithiothreitol or β-mercaptoethanol). Alternatively, the yeast cells were resuspended in 0.1M potassium phos-

phate buffer pH 7.0 and lysed in lysis buffer (4.5% SDS, 2.5mM EDTA and 2.5mM EGTA) with glass beads [Egner et al., 1993]. The proteins were quantified using the Bradford assay [Bradford, 1976] or according to Lowry [Lowry et al., 1951] with modifications [Ausubel et al., 1991].

Gel electrophoresis and immuno-blotting of proteins

Proteins were separated by SDS-PAGE [Laemmli, 1970) or using Tris-Tricine gel electrophoresis as described [Coligan et al., 2001] except that Tris/HCl, pH 6.8 was used for stacking gel preparation. The protein samples were electro-transferred to nitrocellulose or PVDF membrane [Towbin et al., 1979] by using a semi-dry system (Bio-Rad). The protein antigens on the membranes were probed with appropriate antibodies. The immuno-detection procedure was carried out according to the manufacturer's protocol (ECL-Kit, Amersham Biosciences).

2.2.3. Assays for proteolytic activities with fluorogenic peptide substrates

***In situ* assay for chymotrypsin-like activity of the proteasome (1)**

Overlay agar: 1% agar; 50mM Tris-HCl pH8.0

Substrate solution: 10mM Cbz-Gly-Gly-Leu-pNA (S12) in DMSO

Yeast cells are grown on YPD (or CM) agar plates at 30ºC to a minimum colony size of 3mm diameter and replica plated onto a fresh YPD agar plate covered with a sterile filter paper. The replica plate is incubated for 24h at 30ºC.

The filter is removed and transferred into a glass Petri dish. Pouring 10ml chloroform onto the filter permeabilises the cells. The Petri dish is covered with a lid and incubated for 15min. The filter is removed, dried and transferred into a plastic Petri dish. 9.7ml of overlay agar is melted and cooled to 45ºC, and 300µl of substrate solution is added and thoroughly mixed by stirring. The filter is immediately covered with an overlay of substrate agar mixture. After solidification the overlaid filter is incubated for 3h at 37ºC.

The overlaid filter is covered with 10ml of 0.1% sodium nitrite (w/v) in 1M HCl and incubated for 5min.

The sodium nitrite solution is removed and 10ml of 0.5% ammonium sulfamate (w/v) in 1M HCl is added followed by 5min incubation.

The ammonium sulfamate solution is removed and 10ml of 0.05% (w/v) 1-naphthyl-ethylendiamine in 47% (v/v) ethanol is added. Incubation proceeded until positive colonies stain pink.

***In situ* assay for chymotrypsin-like activity of the proteasome (2)**

Overlay agar: 1% agar; 25mM Tris-HCl pH8.0

Substrate solution: 2mM Suc-Leu-Leu-Val-Tyr-AMC (S51) in DMSO

Yeast cells are grown on YPD (or CM) agar plates at 30ºC to a minimum colony size of 3mm diameter and replica plated onto a fresh YPD agar plate covered with a sterile filter paper. The replica plate is incubated for 24h at 30ºC.

The filter is removed and transferred into a glass Petri dish. Pouring 10ml chloroform onto the filter permeabilises the cells. The Petri dish is covered with a lid and incubated for 15min. The filter is removed, dried and transferred into a plastic Petri dish. 9.85ml of overlay agar is melted and cooled to 45ºC, and 150μl of substrate solution is added and thoroughly mixed by stirring. The filter is immediately covered with an overlay of substrate agar mixture. After solidification the overlaid filter is incubated for 20-60min at 30ºC.

The plate is monitored under a U.V. light. Cells with proteasomal chymotrypsin-like activity will shine blue.

***In situ* assay for PGPH activity of the proteasome**

Overlay agar: 1% agar; 100mM Tris-HCl pH8.0

Substrate solution: 50mM Cbz-Leu-Leu-Glu-βNA in DMSO

Coupling reagent: Fast Garnet GBC, 50mg/ml in DMSO

Coupling buffer: 0.2M trisodium citrate pH 4.4, 4% (v/v) Tween 20

The protocol is the same as for the tests for chymotrypsin-like activity, except that the volume of overlay agar is 9.9ml and 100μl of substrate is added.

After 3hours of incubation at 37ºC, 100μl coupling reagent is mixed in 9.9ml of coupling buffer and the overlaid filter covered with the mixture until positive colonies stain red.

***In situ* assay for trypsin-like activity of the proteasome**

Permeabilization solution: 1% (v/v) toluene, 4% (v/v) ethanol, 50mM Tris-HCl pH9.3, 0.5mM EDTA, 0.5mM EGTA

Overlay agar: 1% agarose, 50mM Tris-HCl pH 9.3, 0.5mM EDTA, 0.5mM EGTA

Substrate solution: 50mM Cbz-Ala-Arg-Arg-βNA in DMSO (S54)

Coupling buffer: 0.2M trisodium citrate pH 4.4, 4% (v/v) Tween 20

Coupling reagent: Fast Garnet GBC, 50mg/ml in DMSO

Yeast cells are growth on YPD (or CM) agar plates at 30ºC to a minimum colony size of 3mm diameter and replica plated onto a fresh YPD agar plate covered with a sterile filter paper. The replica plate is incubated for 24h at 30ºC.

Cells are permeabilised by transferring the filter to a second filter soaked with 3ml permeabilisation solution and incubating for 20min. The filter with the yeast colonies is then transferred to a plastic Petri dish. 9.9ml of overlay agar is boiled and cooled down to

45ºC, and 100µl of substrate solution is added and thoroughly mixed by stirring. The filter is immediately covered with an overlay of substrate-agar mixture. After solidification the overlaid filter is incubated for 4-5h at 50ºC.
After incubation, 100µl coupling reagent is dissolved in 9.9ml of coupling buffer and the overlaid filter covered with the mixture until positive colonies stain red.

Assays for proteolytic activities of the proteasome using defined amounts of cells

Reaction buffer: 100µl 1M Tris pH8.0; 100µl substrate; 800µl H_2O
Yeast cells are grown in 3ml of YPD liquid media at 30ºC to log phase. 1OD cells (A_{600nm}) are washed in water. Chloroform is added to permeabilise the cells and incubated for 15min. 105µl H_2O are added and the aqueous solution with cells collected followed by addition of 100µl reaction buffer.
To determine the chymotrypsin-like activity 10mM Cbz-Gly-Gly-Leu-pNA (S12) or 1.5mM Suc-Leu-Leu-Val-Tyr-AMC (S51) (both in DMSO) were used as substrates. The reaction mix is then incubated at 37ºC with slow shaking for 90 (S12) or 10min (S51). The reaction is stopped by adding 1ml 99% ice cold ethanol. After centrifugation, A_{405} is measured for S12 and the fluorescence is measured at 460nm using an excitation wavelength of 380nm for S51.
The peptidylglutamyl peptide-hydrolysing (PGPH) activity is determined using 10mM Cbz-Leu-Leu-Glu-βNA (S53) as fluorogenic peptide substrate. The reaction is stopped by adding 1ml 99% ice cold ethanol. After centrifugation, the fluorescence is measured at 410nm using an excitation wavelength of 340nm.

2.2.4. Generation of plasmid constructs and yeast strains

***pre2-M45R* in plasmid pRS315**

The point mutation in residue 45 of Pre2 was made using the ExSite™ PCR Based Site Directed Mutagenesis Kit. pRS315 plasmid expressing Pre2 was PCR amplified using the following primers: 013-E2MR-for and 016-E2MR-rev.
The resulting plasmid was transformed into yeast *pre2Δ::HIS3 [pRS316-PRE2]* and through plasmid shuffling the strain *pre2Δ::HIS3[pRS315-pre2-M45R]* was created.

Integration of *pre2-M45R* into the chromosome

The chromosomal mutation was obtained by the pop-in/pop-out method. The *pre2-M45R* DNA fragment from previously made pRS315-pre2-M45R plasmid was inserted into the

pRS306 vector by an *in vitro* ligation. The plasmid was transformed into Mat a WCG4. After pop-in/pop-out, an assay for chymotrypsin-like activity was made to identify the mutants.

***pre3-R45M* in plasmid pRS315**

The point mutation in residue 45 of Pre3 was made using the ExSite™ PCR Based Site Directed Mutagenesis Kit. Plasmid pRS315 expressing Pre3 was PCR amplified using the following primers: 014-E3RM-for and 015-E3RM-rev.

The resulting plasmid was transformed into yeast strain *pre3Δ::HIS3 [p16-PRE3]* and through plasmid shuffling the strain *pre3Δ::HIS3 [p15-pre3-R45M]* was created.

Integration of *pre3-R45M* into the chromosome

The chromosomal mutation was obtained by the pop-in/pop-out method. The *pre3-R45M* DNA fragment from previously made pRS315-pre3-R45M plasmid was inserted into the pRS306 vector by an *in vitro* ligation. The plasmid was transformed into Mat α WCG4. After pop-in/pop-out, an assay for PGPH activity was made to identify the mutants.

***pre2-M45R pre3-R45M* yeast strain**

The *Mat a pre2-M45R* strain was crossed with the *Mat α pre3-R45M* strain. After tetrad dissection, the correct spore was identified through chymotrypsin-like and PGPH activity tests.

***pup1-T1A pre2-M45R pre3-R45M* yeast strain**

The *Mat a pup1-T1A* strain was crossed with the *Mat α pre2-M45R pre3-R45M* strain. After tetrad dissection, the correct spore was to be identified through chymotrypsin-like, trypsin-like and PGPH activity tests.

***pre2-2 pre3Δ::HIS3 [pRS316-PRE3]* yeast strain**

The *Mat a pre2-2 strain* was crossed with the *Mat α pre3Δ::HIS3 [pRS316-PRE3]* strain. After tetrad dissection, the correct spore was identified through chymotrypsin-like activity tests and growth in URA^- and HIS^-/URA^- selective medium.

***pre2-2 pre3Δ::HIS3 [pRS315-pre3-R45M]* yeast strain**

The plasmid pRS315-pre3-R45M was transformed in the yeast strain *pre2-2 pre3Δ::HIS3 [p16-PRE3]*. Followed by plasmid shuffling, the resulting yeast strain *pre2-2 pre3Δ::HIS3 [p15-pre3-R45M]* was made.

***pre3-T1A pre2Δ::HIS3 [pRS316-PRE2]* yeast strain**

The *Mat a pre3-T1A strain* was crossed with the *Mat* α *pre2Δ::HIS3 [pRS316-PRE2]* strain. After tetrad dissection, the correct spore was identified through PGPH activity tests and growth in URA^-, HIS^-/URA^- medium.

***pre3-T1A pre2Δ::HIS3 [pRS315-pre2-M45R]* yeast strain**

The plasmid pRS315-pre2-M45R was transformed in the yeast strain *pre3-T1A pre2Δ::HIS3 [p16-PRE2]*. Followed by plasmid shuffling, the resulting yeast strain *pre3-T1A pre2Δ::HIS3 [pRS315-pre2-M45R]* was made.

***pup1-T1A pre3-T1A pre2Δ::HIS3 [pRS315-pre2-M45R]*; and *pup1-T1A pre2Δ::HIS3 [pRS315-pre2-M45R]* yeast strains**

The *Mat a pup1-T1A* strain was crossed with the *Mat* α *pre3-T1A pre2Δ::HIS3 [pRS315-pre2-M45R]* strain. After tetrad dissection, the correct spores were to be identified through growth in HIS^-, Leu^-/HIS^- selective medium, and through trypsin-like and PGPH activity tests.

***pup1-T1A pre3Δ::HIS3 [pRS315-pre3-R45M]* yeast strain**

The *Mat a pup1-T1A* strain was crossed with the *Mat* α *pre2-2 pre3Δ::HIS3 [pRS315-pre3-R45M]* strain. After tetrad dissection, the correct spores were to be identified through growth in HIS^-, Leu^-/HIS^- selective medium, and through trypsin-like and chymotrypsin-like activity tests. Theoretically the strain *pup1-T1A pre2-2 pre3Δ::HIS3 [pRS315-pre3-R45M]* would be also generated, but was not found.

***pre3Δ::HIS3 [pRS315-pre3-R45M-T52G]*; *pre3Δ::HIS3 [pRS315-pre3-R45M-T52A]*; *pre3Δ::HIS3 [pRS315-pre3-R45M-T52S]*; and *pre3Δ::HIS3 [pRS315-pre3-R45M-T52C]* yeast strains**

The point mutations in residue 52 of β1/Pre3 were made using the Two-Stage PCR protocol from QuikChange® Site Directed Mutagenesis Kit. The pRS315-pre3-R45M plasmid was used as template. It was PCR amplified using the following primers: 026-pre3T52X-for and 027-pre3T52X-rev.

The resulting plasmids were identified with specific enzymes and sequenced. These were transformed into *pre3Δ::HIS3 [pRS316-PRE3]* yeast strain and through plasmid shuffling the strains p*re3Δ::HIS3 [pRS315-pre3-R45M-T52G]*; *pre3Δ::HIS3 [pRS315-pre3-R45M-T52A]*; *pre3Δ::HIS3 [pRS315-pre3-R45M-T52S]*; *pre3Δ::HIS3 [pRS315-pre3-R45M-T52C]* were created.

***pre2-2 pre3Δ::HIS3 [pRS315-pre3-R45M-T52G]*; pre2-2 *pre3Δ::HIS3 [pRS315-pre3-R45M-T52A]*; pre2-2 *pre3Δ::HIS3 [pRS315-pre3-R45M-T52S]*; and pre2-2 *pre3Δ::HIS3 [pRS315-pre3-R45M-T52C]* yeast strains**

The plasmids *pRS315-pre3-R45M-T52G*; *pRS315-pre3-R45M-T52A*; *pRS315-pre3-R45M-T52S*; and *pRS315-pre3-R45M-T52C* were transformed into *pre2-2 pre3Δ::HIS3 [pRS316-PRE3]* yeast strain and through plasmid shuffling the strains *pre2-2 pre3Δ::HIS3 [pRS315-pre3-R45M-T52G]*; *pre2-2 pre3Δ::HIS3 [pRS315-pre3-R45M-T52A]*; *pre2-2 pre3Δ::HIS3 [pRS315-pre3-R45M-T52S]*; and *pre2-2 pre3Δ::HIS3 [pRS315-pre3-R45M-T52C]* were created.

***pre2Δ::HIS3 [pRS315-pre2-A20T-M45R]*; *pre2Δ::HIS3 [pRS315-pre2-V31T-M45R]*; and *pre2Δ::HIS3 [pRS315-pre2-A20T-V31T-M45R]* yeast strains**

The point mutations in residue 20 and 31 of Pre2 were made using the QuikChange® Multi Site Directed Mutagenesis Kit. The plasmid pRS315-pre2-M45R was used as template. It was PCR amplified using the following primers: 030-pre2A20T and 031-pre2V31T.

The resulting plasmids were identified using specific enzymes. These were transformed into *pre2Δ::HIS3 [pRS316-PRE2]* yeast strain and through plasmid shuffling the strains *pre2Δ::HIS3 [pRS315-pre2-A20T-M45R)*; *pre2Δ::HIS3 [pRS315-pre2-V31T-M45R]*; and *pre2Δ::HIS3[pRS315-pre2-A20T-V31T-M45R]* were created.

***pre2Δ::HIS3 [pRS315-pre2-I35T-M45R]* yeast strain**

The point mutation in residue 35 of Pre2 was made using the QuikChange® Multi Site Directed Mutagenesis Kit. The template plasmid pRS315-Pre2-M45R was PCR amplified using the 032-pre2I35T primer. The PCR product was transformed in *E.coli* cells.

The resulting plasmid was identified with specific enzymes. It was transformed into yeast *pre2Δ::HIS3 [pRS316-PRE2]* strain and through plasmid shuffling the strain *pre2Δ::HIS3 [pRS315-pre2-I35T-M45R]* was created.

***pre2Δ::HIS3 [pRS315-pre2-A20T-V31T-I35T-M45R]* yeast strain**

A point mutation in residue 35 of Pre2 was made using the QuikChange® Multi Site Directed Mutagenesis Kit. The plasmid pRS315-pre2-A20T-V31T-M45R was used as template and was PCR amplified using the 032-pre2I35T primer. The PCR product was transformed in *E.coli*.

The resulting plasmid was identified with specific enzymes. It was transformed into yeast *pre2Δ::HIS3 [pRS316-PRE2]* strain and through plasmid shuffling the strain *pre2Δ::HIS3 [pRS315-pre2-A20T-V31T-I35T-M45R]* was created.

***pre3-T1A pre2Δ::HIS3 [pRS315-pre2-A20T-M45R]*; *pre3-T1A pre2Δ::HIS3 [pRS315-pre2-V31T-M45R]*; *pre3-T1A pre2Δ::HIS3 [pRS315-pre2-A20T-V31T-M45R]*; *pre3-T1A pre2Δ::HIS3 [pRS315-pre2-I35T-M45R]*; and *pre3-T1A pre2Δ::HIS3 [pRS315-pre2-A20T-V31T-I35T-M45R]* yeast strains**

The CEN plasmids containing the previously made point mutations in Pre2 residues 20, 31, 35 and 45 were transformed into *pre3-T1A pre2Δ::HIS3 [pRS316-PRE2]* yeast strain. Through plasmid shuffling the strains *pre3T1A pre2Δ::HIS3 [pRS315-pre2-A20T-M45R]*; *pre3-T1A pre2Δ::HIS3 [pRS315-pre2-V31T-M45R]*; *pre3-T1A pre2Δ::HIS3 [pRS315-pre2-A20T-V31T-M45R]*; *pre3-T1A pre2Δ::HIS3 [pRS315-pre2-I35T-M45R]*; and *pre3-T1A pre2Δ::HIS3 [pRS315-pre2-A20T-V31T-I35T-M45R]* were created.

***pre3Δ::HIS3 [pRS315-pre3-T20A-R45M]*; *pre3Δ::HIS3 [pRS315-pre3-T31V-R45M]*; and *pre3Δ::HIS3 [pRS315-pre3-T20A-T31V-R45M]* yeast strains**

The point mutations in residue 20 and 31 of Pre3 were made using the QuikChange® Multi Site Directed Mutagenesis Kit. The plasmid pRS315-pre3-R45M was used has template. It was PCR amplified using the primers 033-pre3-T20A and 034-pre3-T31V.

The resulting plasmids were identified using specific enzymes. These were transformed into yeast *pre3Δ::HIS3 [pRS316-PRE3]* strain and through plasmid shuffling the strains *pre3Δ::HIS3 [pRS315-pre2-T20A-R45M]*; *pre3Δ::HIS3 [pRS315-pre2-T31V-R45M]*; and *pre3Δ::HIS3 [pRS315-pre2-T20A-T31V-R45M]* were created.

***pre3Δ::HIS3 [pRS315-pre3-T35I-R45M]* yeast strain**

The point mutation in residue 35 of Pre3 was made using the QuikChange® Multi Site Directed Mutagenesis Kit. The plasmid pRS315-pre3-R45M was used has template. It was PCR amplified using the primer 035-pre3-T35I.

The resulting plasmid was identified with specific enzymes. It was transformed into *pre3Δ::HIS3 [pRS316-PRE3]* yeast strain and through plasmid shuffling the strain *pre3Δ::HIS3 [pRS315-pre3-T35I-R45M]* was created.

***pre3Δ::HIS3 [pRS315-pre3-T20A-T31V-T35I-R45M]* yeast strain**

The point mutation in residue 35 of Pre3 was made using the QuikChange® Multi Site Directed Mutagenesis Kit. The plasmid pRS315-pre3-T20A-T31V-R45M was used as template. It was PCR amplified using the primer 035-pre3T35I.

The resulting plasmid was identified using specific enzymes. It was transformed into *pre3Δ::HIS3 [pRS316-PRE3]* yeast strain and through plasmid shuffling the strain *pre3Δ::HIS3 [pRS315-pre3-T20A-T31V-T35I-R45M]* was created.

***pre2-2 pre3Δ::HIS3 [pRS315-pre3-T20A-R45M]*; *pre2-2 pre3Δ::HIS3 [pRS315-pre3-T31V-R45M]*;**
***pre2-2 pre3Δ::HIS3 [pRS315-pre3-T20A-T31V-R45M]*; *pre2-2 pre3Δ::HIS3 [pRS315-pre3-T35I-R45M]*; and**
***pre2-2 pre3Δ::HIS3 [pRS315-pre3-T20A-T31V-T35I-R45M]* yeast strains**

The CEN plasmids with the previously made point mutations in pre3 residues 20, 31, 35 and 45 were transformed into *pre2-2 pre3Δ::HIS3 [pRS316-PRE3]* yeast strain. Through plasmid shuffling the strains *pre2-2 pre3Δ::HIS3 [pRS315-pre3-T20A-R45M]*; *pre2-2 pre3Δ::HIS3 [pRS315-pre3-T31V-R45M]*; *pre2-2 pre3Δ::HIS3 [pRS315-pre3-T20A-T31V-R45M]*; *pre2-2 pre3Δ::HIS3 [pRS315-pre3-T35I-R45M]*; and *pre2-2 pre3Δ::HIS3 [pRS315-pre3-T20A-T31V-T35I-R45M]* were created.

***pup1-T1A pre3Δ::HIS3 [pRS315-pre3-T20A-R45M]*;**
***pup1-T1A pre3Δ::HIS3 [pRS315-pre3-T31V-R45M]*;**
***pup1-T1A pre3Δ::HIS3 [pRS315-pre3-T20A-T31V-R45M]*;**
***pup1-T1A pre3Δ::HIS3 [pRS315-pre3-T35I-R45M]*; and**
***pup1-T1A pre3Δ::HIS3 [pRS315-pre3-T20A-T31V-T35I-R45M]* yeast strains**

The CEN plasmids with the point mutations in pre3 residues 20, 31, 35 and 45 were transformed into yeast *pup1-T1A pre3Δ::HIS3 [pRS316-PRE3]* strain. Through plasmid shuffling the strains *pup1-T1A pre3Δ::HIS3 [pRS315-pre3-T20A-R45M]*; *pup1-T1A pre3Δ::HIS3 [pRS315-pre3-T31V-R45M]*; *pup1-T1A pre3Δ::HIS3 [pRS315-pre3-T20A-T31V-R45M]*; *pup1-T1A pre3Δ::HIS3 [pRS315-pre3-T35I-R45M]*; and *pup1-T1A pre3Δ::HIS3 [pRS315-pre3-T20A-T31V-T35I-R45M]* were created.

pRS315-HA-PRE3 plasmid

There was an undesired NotI cleavage site in the pRS315-PRE3 template plasmid. After cleavage with NotI, 11µl of cleaved plasmids were filled with 0,8µl 1mM GC mix (1:100) using 1µl Klenow enzyme and 0,4µ BSA 1mg/ml (1:10) in a total volume of 20µl. 0,9µl H buffer was used, and ddH_2O to fill up. The reaction took place at room temperature during 10min incubation, and stopped with 10min incubation at 75ºC.

The open plasmids were re-circularised by ligation using T4 ligase enzyme. The new plasmid was named pRS315-pre3-NotIΔ.

A new NotI cleavage site was created after the β1/Pre3 last C-terminal Leu187 codon sequence before the stop codon using the "Corrected QuickChange Site Directed Mutagenesis Kit".

The pRS315-pre3-NotIΔ plasmid was PCR amplified using the following primers: 028-pre3NotI-for and 029-pre3NotI-rev. The resulting plasmid was named pRS315-PRE3-NotI-ins.

A plasmid containing the 3xHA tag sequence was supplied by Hans Rudolf (plasmid BR394). After cleaving this plasmid with NotI, the 3xHA fragment was isolated and purified.
The plasmid pRS315-PRE3-NotI-ins (with a NotI site in a new location) was cleaved with NotI and a ligation was done to integrate the 3xHA tag sequence. The plasmid pRS315-HA-PRE3 was ready.

***pre3Δ::HIS3 [pRS315-HA-pre3-R45M]* yeast strain**

The pRS315-HA-PRE3 plasmid was partially cleaved with PflMI restriction enzyme. The fragments 5212bp and 5357bp, containing the HA sequence but not the *PRE3* location of planed mutations, were isolated and purified.
The plasmid pRS315-pre3-R45M was cleaved with BsmFI. The 2590bp fragment containing the R45M mutation was isolated and purified. Between the different fragments the sequence is complementary at both ends.
Fragments were mixed together and transformed into *pre3Δ::HIS3 [pre316-PRE3]* yeast strain. After selection for Leu^- and plasmid shuffling with 5'FOA selection, the *pre3Δ::HIS3 [pRS315-HA-pre3-R45M]* yeast strain was available.

***pup1-T1A pre3Δ::HIS3 [pRS315-HA-PRE3] and pup1-T1A pre3Δ::HIS3 [pRS315-HA-pre3-R45M]* yeast strains**

The pRS315-HA-pre3-R45M plasmid was extracted from the *pre3Δ::HIS3 [pRS315-HA-pre3-R45M]* strain.
The plasmids pRS315-HA-PRE3 and pRS315-HA-pre3-R45M were introduced into *pup1-T1A pre3Δ::HIS3 [pRS316-PRE3]* yeast strain. Using the plasmid shuffling method, the strains *pup1-T1A pre3Δ::HIS3 [pRS315-HA-PRE3] and pup1-T1A pre3Δ::HIS3 [pRS315-HA-pre3-R45M]* were made.

***pup1-T1A pre3Δ::HIS3 [pRS315-HA-pre3-T1A]*;**
***pup1-T1A pre3Δ::HIS3 [pRS315-HA-pre3-T20A-T31V-R45M]*;**
***pup1-T1A pre3Δ::HIS3 [pRS315-HA-pre3-T35I-R45M]*; and**
pup1-T1A pre3Δ::HIS3 [pRS315-HA-pre3-T20A-T31V-T35I-R45M]

The 3xHA tag was inserted into the correspondent CEN plasmids by mean of an *in vivo* ligation, using existing the pRS315-HA-PRE3 plasmid as the source for the HA sequence.
The pRS315-HA-PRE3 plasmid was partially cleaved with PflMI. Fragments near 5,3kbp, containing the HA sequence but excluding the sequence for *Pre3* planned mutations, were isolated and purified as before.

The pRS315-pre3-T1A, pRS315-pre3-T20A-T31V-R45M, pRS315-pre3-T35I-R45M and pRS315-pre3-T20A-T31V-T35I-R45M plasmids where cleaved with BsmFI and ClaI. The ~2,6kbp fragment was isolated and purified. An *in vivo* ligation between the different fragments was done as for to *pre3Δ::HIS3 [pRS315-HA-pre3-R45M]*. Fragments were mixed together and a transformation in yeast was performed in *pup1-T1A pre3Δ::HIS3 [pRS316-PRE3]* yeast strain. After plasmid shuffling, the strains *pup1-T1A pre3Δ::HIS3 [pRS315-HA-pre3-T1A]*; *pup1-T1A pre3Δ::HIS3 [pRS315-HA-pre3-T20A-T31V-R45M]*; *pup1-T1A pre3Δ::HIS3 [pRS315-HA-pre3-T35I-R45M]*; and *pup1-T1A pre3Δ::HIS3 [pRS315-HA-pre3-T20A-T31V-T35I-R45M]* were ready.

***Nat1Δ::Kan pre3Δ::HIS3 [pRS316-PRE3]* and *Nat1Δ::Kan pre2-2 pre3Δ::HIS3 [pRS316-PRE3]* yeast strains**

Using primers 040-Nat1Kan-A and 043-Nat1Kan-D, the Kanamycine gene DNA sequence is retrieved from the chromosome of the strain *Nat1αΔ::Kan* (YDL 040C) which was taken from the EUROSSCARF yeast library, consisting of about 5,000 *Saccharomyces cerevisiae* strains each deleted for a single non-essential gene. Purified PCR fragments are transformed into the yeast strains *pre3Δ::HIS3 [pRS316-PRE3]* and *pre2-2 pre3Δ::HIS3 [pRS316-PRE3]*. Fragment ends have portion of DNA corresponding to those before and after the coding region of the chromosomal Nα-Acetylase (*Nat1*) gene and a gene swapping should occur. After transformation cells are plated onto selective media, YPD with 200mg/l G418.

A PCR with primers 040-Nat1Kan-A and 041-Nat1Kan-B, as well as 042-Nat1Kan-C and 043-Nat1Kan-D was performed with the surviving colonies DNA. This confirmed the correct insertion of Kanamycine gene into the yeast chromosome, as well as the removal of the Nα-Acetylase gene. After PCR confirmation, the strains *Nat1Δ::Kan pre3Δ::HIS3 [pRS316-PRE3]* and *Nat1Δ::Kan pre2-2 pre3Δ::HIS3 [pRS316-PRE3]* were ready to be used.

Nat1Δ::Kan pre3Δ::HIS3 [pRS315-UB-PRE3]*; *Nat1Δ::Kan pre3Δ::HIS3 [pRS315-UB-pre3-T1A]*; *Nat1Δ::Kan pre2-2 pre3Δ::HIS3 [pRS315-UB-PRE3]* and *Nat1Δ::Kan pre2-2 pre3Δ::HIS3 [pRS315-UB-pre3-T1A]

Using the plasmid shuffling method, the plasmids pRS315-UB-PRE3 and pRS315-UB-pre3-T1A were each introduced into the strains *Nat1Δ::Kan pre3Δ::HIS3 [pRS316-PRE3]* and *Nat1Δ::Kan pre2-2 pre3Δ::HIS3 [pRS316-PRE3]*. This way the following yeast strains were prepared: *Nat1Δ::Kan pre3Δ::HIS3 [pRS315-UB-PRE3]*, *Nat1Δ::Kan pre3Δ::HIS3 [pRS315-UB-pre3-T1A]*, *Nat1Δ::Kan pre2-2 pre3Δ::HIS3 [pRS315-UB-PRE3]* and *Nat1Δ::Kan pre2-2 pre3Δ::HIS3 [pRS315-UB-pre3-T1A]*.

pRS315-UB-pre3-R45M; pRS315-UB-pre3-T20A-T31V-R45M and pRS315-UB-pre3-T20A-T31V-T35I-R45M plasmids

The existing plasmid pRS315-UBI/mmPRE3 (provided by Heinemeyer W. [Jager S 1999]) was completely cleaved with ClaI and then partially with BsaAI. The 2303bp fragment containing the UB sequence is isolated from agarose gel and purified.

The plasmids pRS315-pre3-R45M, pRS315-pre3-T20A-T31V-R45M, pRS315-pre3-T20A-T31V-T35I-R45M were completely cleaved with BstEII and partially cleaved with KpnI. The 5536bp fragment containing the mutations was extracted from the agarose gel and purified.

There are some base pairs in common between each fragment edges. Both fragments were mixed and transformed into wild type yeast strain, growing in selective media Leu^-. Yeast DNA was extracted from growing colonies and followed by electroporation into *E. Coli* cells.

The plasmids pRS315-UB-pre3-R45M, pRS315-UB-pre3-T20A-T31V-R45M and pRS315-UB-pre3-T20A-T31V-T35I-R45M were then made available.

Nat1Δ::Kan pre3Δ::HIS3 [UB-pre3-X-R45M]* and *Nat1Δ::Kan pre2-2 pre3Δ::HIS3 [UB-pre3-X-R45M]

The previously made plasmids pRS315-UB-pre3-X-R45M (where –X- stands for -T20A-T31V-, T20A-T31V-T35I or nothing) were transformed into the *Nat1Δ::Kan pre3Δ::HIS3 [pRS316-PRE3]* and *Nat1Δ::Kan pre2-2 pre3Δ::HIS3 [pRS316-PRE3]* yeast strains.

After plasmid shuffling the following strains were available: *Nat1Δ::Kan pre3Δ::HIS3 [p15-UB-pre3-R45M]*; *Nat1Δ::Kan pre3Δ::HIS3 [p15-UB-pre3-T20A-T31V-R45M]*; *Nat1Δ::Kan pre3Δ::HIS3 [p15-UB-pre3-T20A-T31V-T35I-R45M]*; *Nat1Δ::Kan pre2-2 pre3Δ::HIS3 [p15-UB-pre3-R45M]*; *Nat1Δ::Kan pre2-2 pre3Δ::HIS3 [p15-UB-pre3-T20A-T31V-R45M]* and *Nat1Δ::Kan pre2-2 pre3Δ::HIS3 [p15-UB-pre3-T20A-T31V-T35I-R45M]*.

***pup1Δ::HIS3 [pRS315-pup1-Y204A]* yeast strain**

The point mutation in the Pup1 residue 204 codon was made by recombinant PCR.

One fragment was done by PCR using the primers 021-pup1Y204A-for and 006-5/6R in the pRS315-PUP1 plasmid. Another fragment was made by PCR using the primers 022-pup1Y204A-rev and 005-5/6F in the same plasmid. The two different fragments have complementary ends. Both PCR products were mixed and amplified with 005-5/6F and 006-5/6R primers.

The pRS315-PUP1 plasmid was completed digested with BamHI and Bsu36I.
An *in vivo* ligation was made. The final PCR product and open plasmid vector were transformed into wild type yeast. Cells with correct circular plasmids were selected using Leu⁻ selective medium.
The yeast DNA was extracted and the correct mutation confirmed by PCR. The plasmid rescued from *E. coli* was then transformed into the *pup1Δ::HIS3 [pRS316-PUP1]* yeast strain after purification. Through the plasmid shuffling method the strain *pup1Δ::HIS3 [pRS315-pup1-Y204A]* was created.

***pup1Δ::HIS3 [pRS315-pup1-R208G]* yeast strain**

The point mutation in the Pup1 residue 208 codon was made by recombinant PCR.
One fragment was done by PCR using the primers 023-pup1R208G-for and 006-5/6R in the pRS315-PUP1 plasmid. Another fragment was made by PCR using the primers 025-pup1RGTA-rev and 005-5/6F in the same plasmid. The two different fragments have complementary ends. Both PCR products were mixed and amplified with 005-5/6F and 006-5/6R primers.
Following steps were similar to those used for *pup1Δ::HIS3 [pRS315-pup1-Y204A]* to create the *pup1Δ::HIS3 [pRS315-pup1-R208G] yeast* strain.

***pup1Δ::HIS3 [pRS315-pup1-T211A]* yeast strain**

The point mutation in residue 211 of Pup1 was made by recombinant PCR.
One fragment was done by PCR using the primers 024-pup1T211A-for and 006-5/6R in the pRS315-PUP1 plasmid. Another fragment was made by PCR using the primers 025-pup1RGTA-rev and 005-5/6F in the same plasmid. The two different fragments have complementary ends. Both PCR products were mixed and amplified with 005-5/6F and 006-5/6R primers.
Following steps were similar to those used for *pup1Δ::HIS3 [pRS315-pup1-Y204A]* and *pup1Δ::HIS3 [pRS315-pup1-R208G]* to create the *pup1Δ::HIS3 [pRS315-pup1-T211A] yeast* strain.

***pup1Δ::HIS3 [pRS315-pup1-std]* yeast strain; pRS315-pup1-mtd plasmid; and pRS315-pup1-ltd plasmid**

Sequences encoding the β2/Pup1 C-terminal extension were partially and totally deleted by recombinant PCR. From the plasmid pRS315-PUP1, two PCR fragments were first created: the first fragment with primers 005-5/6F and 018-Pup1D-tail (going from the Val196 codon for long tail deletion) or 019-Pup1E-tail (from Val213 codon for short tail deletion) or 020-Pup1F-tail (going from Arg208 codon for middle tail deletion); the second fragment with primers 006-5/6R and 017-Pup1C-tail which starts after the end of

the *PUP1* C-terminal extension sequence. The first twenty one base pairs of primers 018-Pup1D-tail, 019-Pup1E-tail and 020-Pup1F-tail are complementary with the same number of base pairs in primer 017-Pup1C-tail.

A final PCR was performed using the two fragments with complementary ends and universal primers 005-5/6F and 006-5/6R.

The pRS315- Pup1 plasmid was completely digested with BamHI and Bsu36I.

The final PCR products and the open plasmid vector were transformed into wild type yeast. The cells with re-circularized plasmids were selected using Leu^- plates.

The yeast DNA was extracted and correct mutation confirmed by PCR. The plasmids were then rescued and transformed into *pup1Δ::HIS3 [pRS316-PUP1]* yeast strain after purification.

Through the plasmid shuffling method the strains *pup1Δ::HIS3 [pRS315-pup1-std]*, *pup1Δ::HIS3 [pRS315-pup1-mtd]* and *pup1Δ::HIS3 [pRS315-pup1-ltd]* could be theoretically created (only the *pup1Δ::HIS3 [pRS315-pup1-std]* strain was viable).

pRS315-HA-PUP1; pRS315-HA-pup1-std; pRS315-HA-pup1-mtd and pRS315-HA-pup1-ltd plasmids

From the plasmid YCplac22HA-PUP1 carrying the *pup1-HA* genome (provided by Hochstrasser M. [Arendt and Hochstrasser, 1997]), the region with the *HA* sequence was removed as restriction fragment and inserted into the pRS315-PUP1 plasmid. The plasmid YCplac22HA-PUP1 was cleaved between AccI and Bsu36l sites and the 446bp fragment containing the HA sequence was then excised from agarose gel and purified. After a complete digestion with Bsu36l and partial digestion with AccI, the 7327bp fragment from pRS315-PUP1 plasmid was isolated from agarose gel. A DNA ligation was done with both isolated fragments creating the plasmid pRS315-HA-PUP1.

Using the plasmid pRS315-HA-PUP1 as a template, the ~2.1kbp fragment with HA sequence is now isolated after cleavage with BsaI. The pRS315-pup1-std, pRS315-pup1-mtd and pRS315-pup1-ltd plasmids were cleaved with BsaI and the ~5.5kbp bands were isolated and purified from agarose gel. Ligation of fragments was performed to create new plasmids pRS315-HA-pup1-std, pRS315-HA-pup1-mtd and pRS315-HA-pup1-ltd.

pRS425-HA-PUP1; pRS425-HA-pup1-std; pRS425-HA-pup1-mtd and pRS425-HA-pup1-ltd plasmids

The previously made CEN plasmids were cut using Bsl20I and SpeI restriction enzymes. The ~1750bp plasmid fragment containing both *PUP1* and *HA* sequences was isolated. These same enzymes were used in the empty pRS425 plasmid and the linearized plasmid isolated. A ligation between isolated fragments and linearized pRS425 plasmid was done to form the over-expression plasmids pRS425-HA-pup1-std, pRS425-HA-pup1-mtd, pRS425-HA-pup1-ltd, and pRS425-HA-PUP1.

***pup1Δ::HIS3 [pRS315-PUP1-FLAG]* and *pup1Δ::HIS3 [pRS315pup1-std-FLAG]* yeast strains**

The insertion of the FLAG sequence was done using site-directed mutagenesis by recombinant PCR. For the pRS315-PUP1-FLAG plasmid, a first fragment was made with primers 005-5/6F and 037-FLAG-PUP1-rev (containing the FLAG sequence), with the plasmid pRS315-PUP1 as template. The second fragment was made with primers 036-FLAG-pup1-for (also containing FLAG sequence) and 006-5/6R.

For the pup1-std-FLAG plasmid, primers 005-5/6F and 038-FLAG-p1std-rev were used with the plasmid pRS315-pup1-std generating the first fragment. The second fragment was the same made for the pRS315-PUP1-FLAG plasmid.

After the PCR step recombining both fragments, each final product was transformed into *pup1Δ::HIS3 [p16-PUP1]* yeast strain together with the open plasmid pRS315-PUP1 after this had been cleaved with BamHI and Bsu36I, isolated and purified. After plasmid shuffling, the correct cells were finally available.

***[pRS425-PUP1-FLAG]*; *[pRS425-pup1-std-FLAG]*; and *[pRS425-pup1-ltd-FLAG]* yeast strains**

For the over-expression pup1-ltd-FLAG, as for PUP1-FLAG and pup1-std-FLAG plasmids, the insertion of FLAG sequence was done with site-directed mutagenesis by recombinant PCR. Primers 005-5/6F and 039-FLAG-p1ltd-rev were used for the first fragment with the plasmid pRS315-pup1-std as template. The second fragment was the same as for *pRS315-PUP1-FLAG*. Both fragments were combined in a PCR step.

The final PCR products containing the *PUP1-FLAG* and *pup1-std-FLAG* alleles were each transformed into a wild type yeast strain together with the open plasmid pRS425-PUP1 after this had been cleaved with BamHI and Bsu36I, isolated and purified. Growth on Leu^- selective medium was required to confirm the presence of the over-expression plasmids.

3. Results

3.1. Reciprocal exchange of substrate specificities between proteasomal active subunits β5/Pre2 and β1/Pre3

The active subunits of the proteasome can cleave peptide bonds on the carboxyl side of neutral/hydrophobic, basic and acidic amino acids. The three different proteolytic activities were initially distinguishable by their sensitivity to different types of inhibitors towards the cleavage at different P1 residues in chromogenic substrates [Wilk and Orlowski, 1980; Wilk and Orlowski, 1983]. Thus, at least a chymotrypsin-like, a trypsin-like and a peptidyl-glutamyl peptide hydrolysing (PGPH) activity have been ascribed to the eukaryotic proteasome [Tanaka et al., 1988].

By genetic and X-ray crystallographic studies, the chymotrypsin-like activity was then assigned to β5/Pre2, the trypsin-like activity to β2/Pup1 and the peptidyl-glutamyl peptide hydrolysing (PGPH) activity to β1/Pre3 [Groll et al., 1997; Heinemeyer et al., 1997]. The active β-subunits carry an amino-terminal threonine residue acting as catalytic nucleophile.

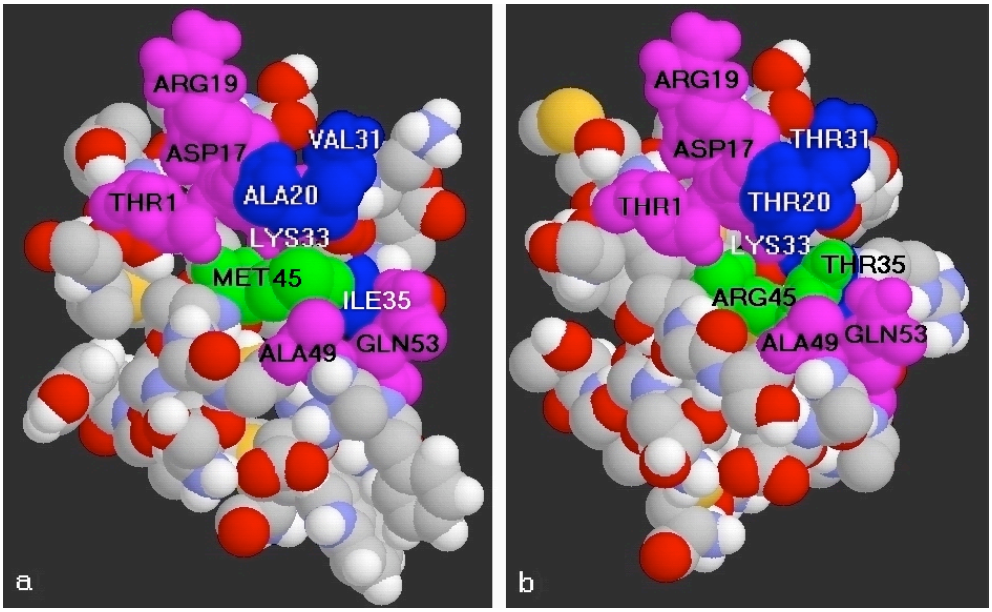


Figure 3-01 – View on the (a) β5/Pre2 and (b) β1/Pre3 active site pockets, represented in space-filling mode. Conserved residues forming the catalytic apparatus and parts of the substrate binding pocket are coloured in magenta, and those pocket forming residues that differ between both subunits, are in blue and green.

Growth phenotypes of yeast cells resulting from expression of active site mutated β-type subunits uncoupled from their propeptides and tests for viable combinations of two inactivated subunits allowed to deduce a hierarchy of the importance of the individual

proteolytic activities for proteasomal function, which is β5/Pre2>>β2/Pup1>β1/Pre3 [Jäger et al., 1999]. Although subunits β5/Pre2 and β1/Pre3 stand in the extreme positions of the hierarchy, their structures are very similar. When superimposed, their main chain C-alpha traces reveal almost identical backbone geometry [Groll et al., 1999].

Their catalytic system, mainly formed by Thr1, Lys33 and Asp17 [Groll et al., 1997], is conserved. However, several of the residues forming the substrate binding pockets differ in both subunits and thus are expected to be responsible for the different specificities (fig.3-01).

3.1.1. Mutation of methionine 45 in the S1 pocket of β5/Pre2 changes substrate specificity

The β5/Pre2 subunit is correlated with chymotrypsin-like activity, cleaving after hydrophobic residues [Groll et al., 1997].
Methionine at position 45 in the β5/Pre2 subunit is a neutral amino acid that may contribute to the selection of hydrophobic residues at position P1 in a substrate. It is replaced by an arginine in β1/Pre3, and a glycine in β2/Pup1. By changing this residue, β5/Pre2 cleavage specificity could be strongly altered.

In order to exchange the residue in position 45 of Pre2 by the one existing in Pre3 at the same position a point mutation changing the Met codon into an Arg codon was introduced into the *PRE2* gene on a plasmid, using a site-directed mutagenesis technique. The resulting *LEU2*-marked shuttle plasmid p15-pre2-M45R was introduced into haploid cells carrying the chromosomal gene deletion of *PRE2* complemented by the wild-type gene on the *URA3*-marked centromeric plasmid p16-PRE2.
The strains possessing only the modified gene variants in the *LEU2*-marked centromeric plasmid were selected on solid synthetic medium lacking leucine and containing 5-fluoro-orotic acid (5-FOA), which is toxic to *URA3*-expressing cells, thus counter-selecting against p16-PRE2 ("plasmid shuffling"). The appropriate control strains were obtained in the same manner by introducing *LEU2*-marked plasmids containing the *PRE2* wild-type gene.

Arginine at position 45 in subunit β1/Pre3 is a basic residue and responsible for the subunit's preference for acidic groups at the P1 position of substrates. The *pre2-M45R* point mutation, replacing the methionine in β5/Pre2 by the arginine existing in β1/Pre3 at the same position, should affect the specificity of β5/Pre2 by reduction or loss of

chymotrypsin-like activity and putatively gain of PGPH activity which is characteristic for the β1/Pre3 subunit.

In order to allow the detection of any PGPH activity of β5/Pre2 harbouring the M45R mutation by means of the chromogenic substrate diagnostic for Pre3 activity, the mutant strain was crossed with a strain carrying the *pre3-T1A* allele on the chromosome and the double mutant was isolated. The *pre3-T1A* mutation leads to loss of Pre3-associated PGPH activity. Therefore, any PGPH activity detectable in this double mutant has to be attributed to the mutated β5/Pre2 subunit.

Peptidase activity tests using defined amounts of permeabilized cells were performed using the chromogenic peptide substrates Cbz-GGL-pNAn and Suc-LLVT-AMC, both specific for chymotrypsin-like activity and Suc-LLE-βNA, diagnostic for the PGPH activity. Strains chromosomally carrying *pre2-1* and *pre2-2* alleles have only residual chymotrypsin-like activity and were used as negative controls. A wild type strain, with *PRE2* on the chromosome was used as positive control (fig.3-02).

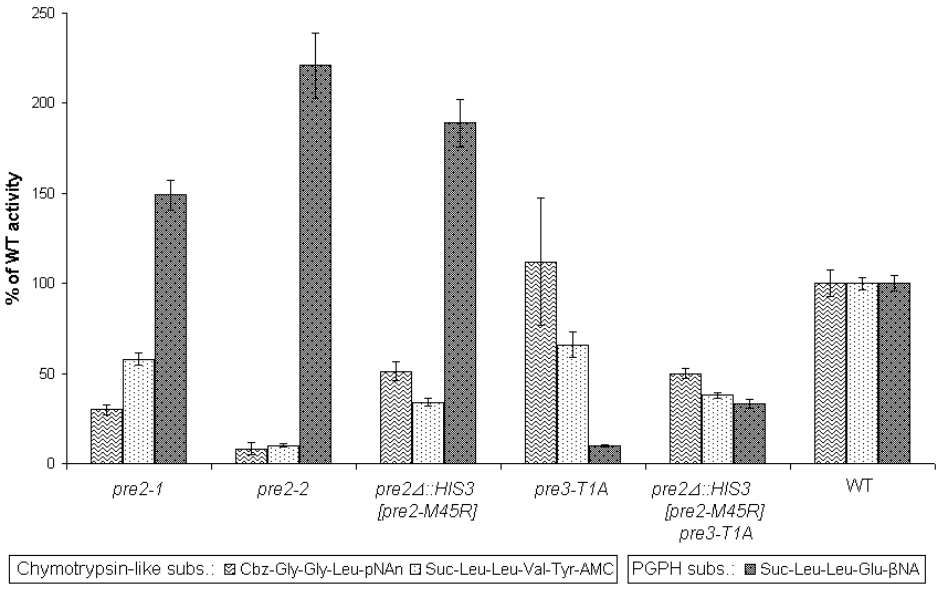


Figure 3-02 – Proteasomal peptidase activity tests with permeabilized cells of *pre2-M45R* cells and control strains. Brackets indicate expression of the allele from plasmid pRS315. Cbz-GGL-pNAn and Suc-LLVT-AMC were applied as substrates for the chymotrypsin-like activity, and Suc-LLE-βNA as substrate for the PGPH activity. The *pre2-1* and *pre2-2* cells were used as negative controls for chymotrypsin-like activity and the *pre3-T1A* cells served as negative control for PGPH activity. Error bars represent the standard error of the mean of four to eight measurements.

The β5/Pre2 subunit with the M45R point mutation shows a partial loss of chymotrypsin-like activity. This is in agreement with the supposed importance of methionine 45 for the P1 cleavage selectivity in β5/Pre2. The residual levels (40-50%) of chymotrypsin-like

activity indicate that other residues in the β5/Pre2 active pocket may be involved on its specificity as well.
The *pre2-M45R pre3-T1A* double mutant shows some PGPH activity (~35% of wild-type). This is in agreement with the importance of arginine 45 for the selection of acidic P1 residues.
The predicted correlation of Met45 and Arg45 with chymotrypsin-like and PGPH specificities, respectively, is supported by the achieved results, although no complete conversion was obtained with the single β5/Pre2-M45R mutation.

The influence of this point mutation in β5/Pre2 on the importance for proteasomal function was examined by comparing the growth of the generated mutants with *PRE2* and *pre2-T1A* strains.
A *pre2-T1A* mutant strain is unable to auto-catalytically process the β5/Pre2 propeptide, and therefore is not viable. To restore viability such a strain has to be made without propeptide at the Pre2 N-terminus. A simple deletion cannot be performed, as the propeptide fulfils essential functions for the process of proteasome assembly. Therefore the propeptide was expressed *in trans* from a separate gene and the *pre2-T1A* allele encoding the matured form of the subunit was C-terminally fused to the ubiquitin gene replacing the propeptide. Such a fusion protein with an N-terminal ubiquitin moiety is split rapidly into free ubiquitin and the mature moiety of the proteasomal subunit by ubiquitin C-terminal hydrolyses [Jäger et al., 1999].
Together with this *pre2Δ::HIS3 [UBI/mmpre2-T1A+ppPRE2]* strain, a *pre2Δ::HIS3 [UBI/mmPRE2+ppPRE2]* strain is used as WT-control (mm indicates the sequence encoding the mature moiety fused to the UBI gene, and pp is the propeptide gene expressed separately).

The *PRE2* and *pre3-T1A* strains were also used as controls for growth comparison with *pre2-M45R* and *pre3-T1A pre2-M45R* mutant strains (fig.3-03).

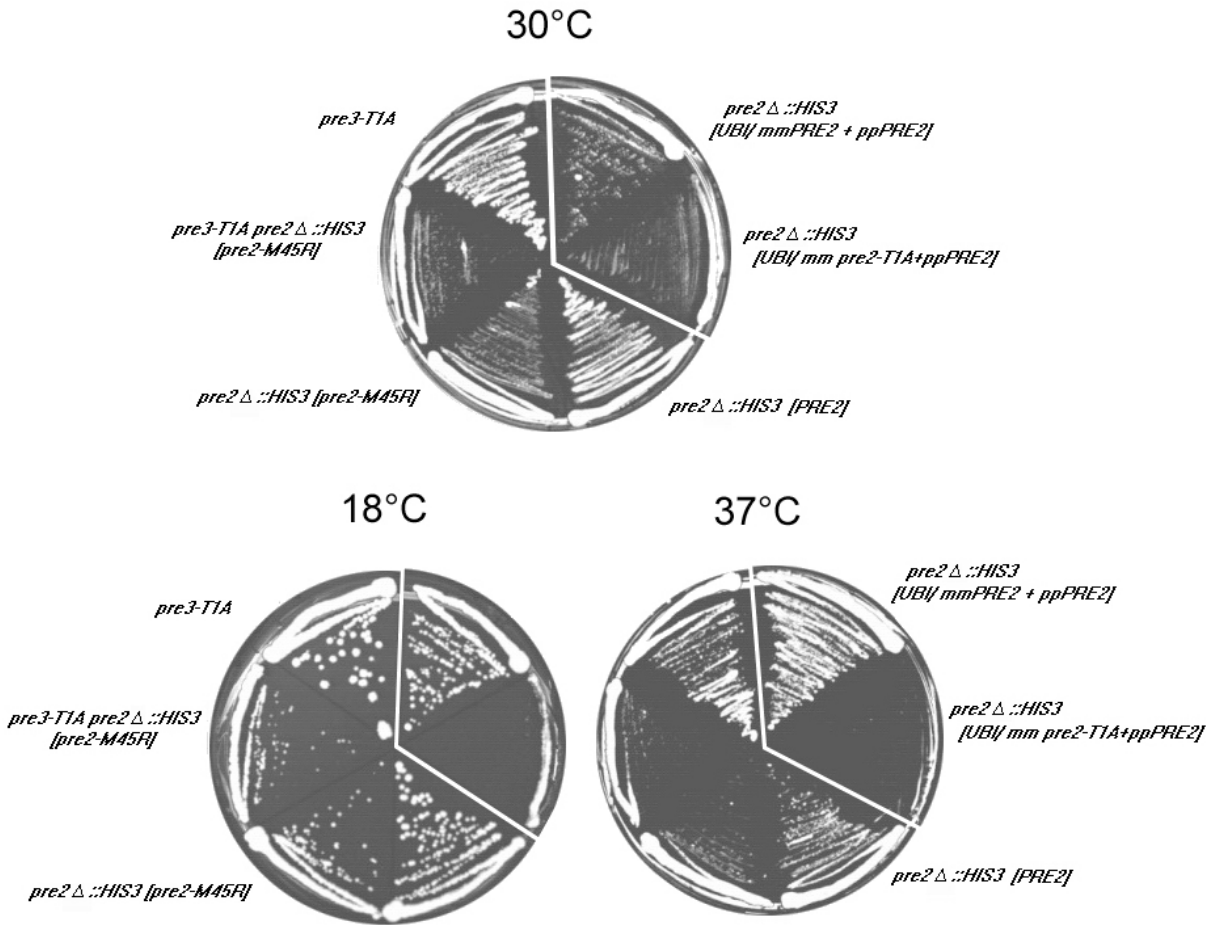


Figure 3-03 – Colony growth of yeast strains harbouring the indicated gene constructs. Brackets indicate expression from plasmid pRS315. Strains were streaked for single colonies on solid YPD medium and incubated at 30ºC and 37ºC for about two days and at 18ºC for about six days.

The *pre2-M45R* and *pre2-M45R pre3-T1A* strains form colonies with smaller size at 18ºC, when compared with *PRE2* and *pre3-T1A*. At 37ºC, growth of these mutants is almost suppressed. Only the inactive pre2 mutant expressing the *UBI/mmpre2-T1A+ppPRE2* allele has a stronger phenotype than *pre2-M45R* mutant strains.

The growth phenotype manifested by *pre2-M45R* reduces the contribution of Pre2 to a normal growth rate. This might be caused by the decrease in chymotrypsin-like activity, which is not compatible with a normal cell growth.

With 50% chymotrypsin-like activity and around 40% PGPH activity of wild type, *pre2-M45R* mutant strains have a growth rate between wild type and *pre2-T1A* strains. Interestingly, a partial loss of chymotrypsin-like activity induces a phenotype which demonstrates the importance of this specificity compared to the others in the eukaryotic proteasome.

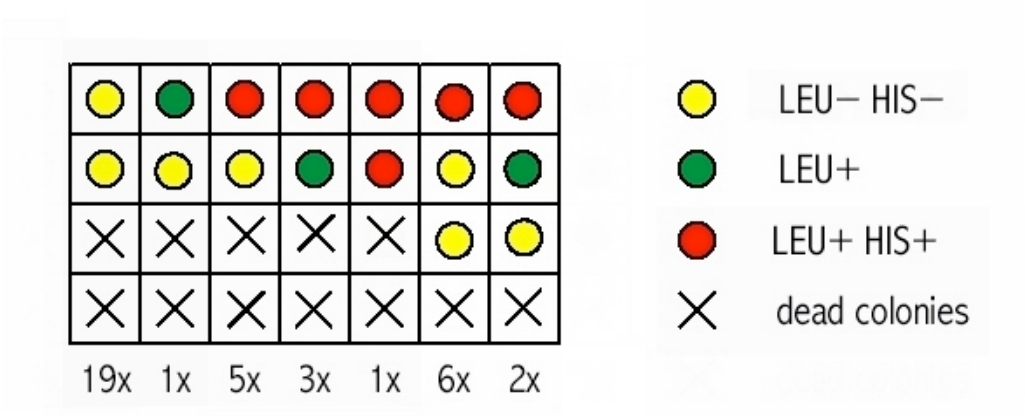
Most notably the appearance of PGPH activity in Pre2 in the *pre2-M45R* mutant strains reveals the possibility of activity shuffling or alteration of active β-subunit proteolytic specificities.

3.1.2. The *pre2-M45R* mutation is lethal in combination with *pup1-T1A*

The measurements of peptidase activities with the available peptide substrates and simple growth analysis by streaks are not sufficient for a comprehensive characterisation of the *pre2-M45R* mutant strain. It is well known that double mutant strains with an active β5/Pre2 subunit and inactivated β2/Pup1 and β1/Pre3 are viable [Jäger et al., 1999]. The β2/Pup1 activity is also sufficient for cell viability when the other two proteolytic subunits are inactivated, while strains with only β1/Pre3 active are not viable. After introduction of the M45R mutation into the β5/Pre2 subunit it is important to know whether this feature remains or whether it is altered. Therefore strains with the *pre2-M45R* mutation were combined with *pre3-T1A*, *pup1-T1A* and both by crossings.

The double mutant *pre2Δ::HIS3 [p15-pre2-M45R] pre3-T1A* was created previously for the activity assays. By crossing this double mutant strain with a strain carrying the *pup1-T1A* mutation on the chromosome, the triple mutant *pre2Δ::HIS3 [p15-pre2-M45R] pre3-T1A pup1-T1A* and the double mutant *pre2Δ::HIS3 [p15-pre2-M45R] pup1-T1A* are expected to be found among the haploid spore progeny of the diploid crossing products.

Table 3-01- Tetrad dissection results after crossing *pre2Δ::HIS3 [p15-pre2-M45R] pre3-T1A* with *pup1-T1A*. Colonies were grown first on YPD plates and then tested for growth on Leu^- and His^- selective plates.



After sporulation of the diploid, 38 tetrads were dissected. The growth of the dissected spores was tested on medium lacking leucine plus histidine (Leu^-/His^-) or lacking only

leucine. Leu$^-$ selects colonies harbouring the plasmid p15-pre2-M45R and His$^-$ selects those with the chromosomal *HIS3* marker in the *pre2Δ::HIS3* allele.

As expected, colonies autotrophic for histidine always had the *LEU2* marked plasmid (table 3-01). All the colonies growing on Leu$^-$/His$^-$, which should have the genotype *pre2Δ::HIS3 [p15-pre2-M45R]* were subjected to peptidase activity tests. These tests were made *in situ*, using the substrates specific for PGPH and trypsin-like activities.
From the 21 colonies growing without both Leu and His, seven had no PGPH activity while none gave a negative result for the trypsin-like activity. This means that seven are the *pre2Δ::HIS3 [p15-pre2-M54R] pre3-T1A* double mutant, and the remaining ones are *pre2Δ::HIS3 [p15-pre2-M54R]* single mutant strains.

According to statistical calculations, one in every two colonies should have *pre3-T1A* just like one in every two colonies should have *pup1-T1A*. Also one in every four is expected to have both the *pre3-T1A* and *pup1-T1A* mutations.
From the twenty one spores analysed, there should be around five triple mutant strains, and also around five of each one of the double mutant strains. From the number of spores dissected it can be concluded that the triple mutant strain *pre2Δ::HIS3 [p15-pre2-M54R] pre3-T1A pup1-T1A* and the double mutant strain *pre2Δ::HIS3 [p15-pre2-M54R] pup1-T1A* are not viable.

Unlike *PRE2 pre3-T1A pup1-T1A*, the *pre2::HIS3 [pre2-M45R] pre3-T1A pup1-T1A* cells are not viable. The double mutant *pre2-M45R pup1-T1A* strain, like double mutants harbouring only a Pre3 active center was also not viable. The mutation and specificity change in β5/Pre2 makes it behave like inactive Pre2.

3.1.3. Mutation of arginine 45 in the S1 pocket of β1/Pre3 leads to loss of detectable peptidase activity

The β1/Pre3 subunit is characterised by the P1 cleavage site preference in substrates, as cleaving after acidic residues [Groll et al., 1997]. The arginine at position 45 in the β1/Pre3 subunit has a basic character that may have strong influence on the selection of acidic residues.
Like the methionine 45 in β5/Pre2, the arginine 45 in the β1/Pre3 S1 pocket is predicted to be strongly related with the P1 selectivity and its mutation could alter the specificity of the subunit.
A strategy similar to the one used for β5/Pre2 was put in practice to modify the substrate selectivity of the β1/Pre3 subunit. First, a point mutation was introduced, replacing

arginine 45 by a methionine. The resulting mutant strain *pre3Δ::HIS3 [p15-pre3-R45M]* was then combined with the *pre2-2* strain, which has a defective β5/Pre2, to verify the possible development of chymotrypsin-like activity by the mutation in β1/Pre3.

The combination of *pre3-R45M* with *pre2-M45R* is not possible using expression from plasmids. With an integration of these mutations into the chromosome they can be easily combined.

From the *CEN* plasmid pRS315, the sequences of *pre2-M45R* and *pre3-R45M* were transferred into the integrative plasmid pRS306. Both *pre2-M45R* and *pre3-R45M* were individually integrated into the chromosome using the pop-in pop-out method.
These mutants were crossed to generate a double mutant, which was identified after tetrad dissection and included in the activity assays (fig.3-04).

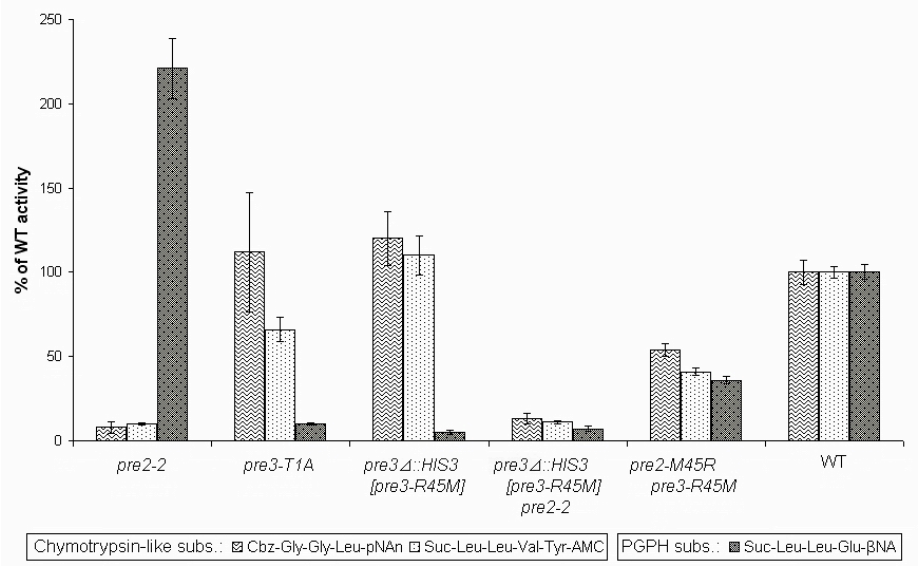


Figure 3-04 – Proteasomal peptidase activity tests with permeabilized cells of *pre3-R45M* cells and control strains. Brackets indicate expression of the allele from plasmid pRS315. Cbz-GGL-pNAn and Suc-LLVT-AMC were applied as substrates for the chymotrypsin-like activity, and Suc-LLE-βNA as substrate for the PGPH-like activity. The *pre3-T1A* cells were used as negative control for PGPH activity and the *pre2-2* cells served as negative control for chymotrypsin-like activity. Error bars represent the standard error of the mean of two to eight measurements.

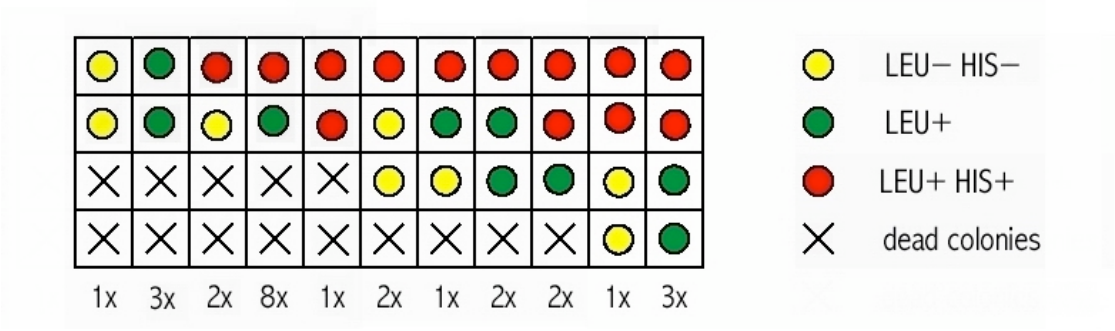
According to the activity assays, *pre3-R45M* does not show any PGPH activity, similar to the inactive mutant *pre3-T1A*. When combined with *pre2-2*, the resulting double mutant strain has only residual levels of chymotrypsin-like activity, not different from that of a *pre2-2* strain with wild type β1/Pre3.
It can be concluded that using the available specific substrates, no peptidase activity is exhibited by the mutated β1/Pre3 subunit.

3.1.4. The *pre3-R45M* mutation does not eliminate the requirement of β5/Pre2 or β2/Pup1 activity

Even with the negative results for *pre3-R45M* mutant strains obtained by activity assays, such assays do not exclude proteolytic activity of the β1/Pre3 subunit. There is always the possibility of a proteolytic activity which is not detectable by the used substrates. Therefore combinations with inactivated subunits β2/Pup1 and β5/Pre2 were tested for viability. If a novel proteolytic activity is present in the altered Pre3 subunit, there is the possibility that yeast strains with the phenotype *pre3-R45M pup1-T1A pre2-2* will be viable, unlike *pup1-T1A pre2-2* cells which have a wild type Pre3.

The double mutant strain *pre3Δ::HIS3 [p15-pre3-R45M] pre2-2* having only residual activity in β5/Pre2 was created for previous activity assays. By crossing this double mutant with a strain carrying the *pup1-T1A* mutation on the chromosome, the triple mutant *pre3Δ::HIS3 [p15-pre3-R45M] pre2-2 pup1-T1A* as well as the double mutant *pre3Δ::HIS3 [p15-pre3-R45M] pup1-T1A* are expected to be found among the haploid spore progeny of the diploid crossing products.

Table 3-02- Tetrad dissection results after crossing *pre3Δ::HIS3 [p15-pre3-R45M] pre2-2* with *pup1-T1A*. Colonies were grown first on YPD plates and then tested for growth on Leu^- and His^- selective plates.



The procedure was the same applied before with the *pre2-M45R* mutant. After crossing, the growth of the dissected spores was tested in medium lacking only leucine (Leu^-) or leucine and histidine (Leu^-/His^-). Leu^- selects colonies requiring plasmid *p15-pre3-R45M* for growth and His^- selects those with the chromosomal marker *HIS3* in the *pre3Δ::HIS3* deletion allele (table 3-02).

Similar to *pre2-M45R*, all the colonies growing on plates lacking both Leu and His, which should have the genotype *pre3Δ::HIS3 [p15-pre3-R45M],* were subjected to peptidase activity tests. These tests were made *in situ*, using the specific substrates for chymotrypsin-like and trypsin-like activities.

From twenty eight colonies, ten had no chymotrypsin-like activity indicative for the *pre2-2* mutation on the chromosome, while nine showed no trypsin-like activity and so the chromosomal *pup1-T1A* mutation.

There were no colonies without both chymotrypsin-like and trypsin-like activities excluding the viability of the triple mutant *pre3Δ::HIS3 [p15-pre3-R45M] pre2-2 pup1-T1A*.

Just like *PRE3 pre2-2 pup1-T1A*, the mutant *pre3Δ::HIS3 [p15-pre3-R45M] pre2-2 pup1-T1A* is not viable. The new mutation in Pre3 does not change its importance among the active subunits of the proteasome.

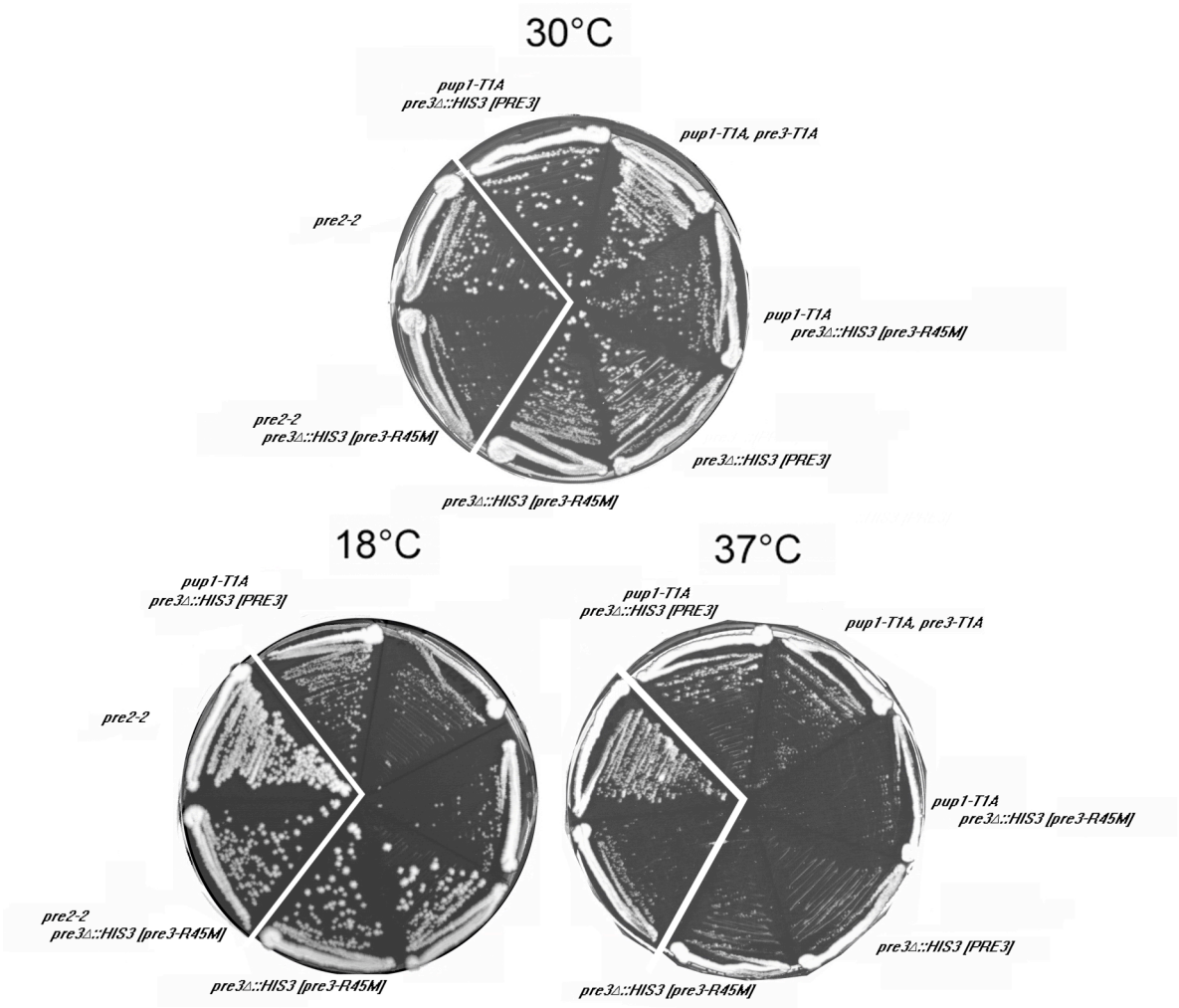


Figure 3-05. Colony growth comparison of yeast strains harbouring the indicated gene constructs. Brackets indicate expression from plasmid pRS315. Strains were streaked for single colonies on solid YPD medium and incubated at 30ºC and 37ºC for about two days and at 18ºC for about six days. All plates have the same spatial orientation.

Streaks of the *pre3-R45M* mutant strains together with the *pre2-2* and *pup1-T1A* combinations were incubated at different temperatures for growth comparison (fig.3-05).

The *pup1-T1A pre3-T1A* and *pup1-T1A PRE3* strains were used as negative and positive controls, respectively. At low temperatures, small colonies appear when *pre3-R45M* is combined with *pup1-T1A*, just like for *pup1-T1A pre3-T1A* and *pup1-T1A PRE3* cells. Slow growth of *pup1-T1A* strains at low temperatures was published [Heinemeyer et al., 1997]. The new mutation on Pre3 does not seem to cause any visible alteration of the phenotype caused by the inactive Pup1.
On the other hand, when *pre3-R45M* is combined with *pre2-2* a strong phenotype appears at 30ºC. The *pre2-2 pre3Δ::HIS3 [p15-pre3-R45M]* mutant cells develop smaller colonies then the *pre2-2* mutant strain. This is observed at high and low temperatures as well.

3.1.5. Mutations of the Thr52 aiming at the stabilisation of the β1/Pre3-R45M substrate binding pocket

The Pre3-R45M mutation gave origin to strains showing neither PGPH nor chymotrypsin-like activity of the β1/Pre3 subunit. How this single amino acid change could have such strong consequence is not clear. Modelling of the probable β1/Pre3 subunit structure after the R45M mutation leads to some hypothesis.

Some surrounding groups of position 45 are different in subunit β1/Pre3 from those in β5/Pre2. In the β1/Pre3 subunit, three hydrogen bonds stabilise the Arg45. They are situated between the Asp32 O^1, the Thr35 hydroxyl group, the Thr52 hydroxyl group and the different amino groups of the Arg45.
In β5/Pre2, a cysteine occupies the position 52 and has no interaction with the Met45. In β1/Pre3 there is a threonine at position 52 and according to the modelling of the mutant (fig.3-06) there is a possible hydrogen bond between the sulphur atom from the new methionine 45 and the hydroxyl group from threonine 52. This hydrogen bond probably changes the conformation of the methionine 45 in the proteolytic pocket. A different spatial arrangement of the important residues in the activity pocket could be responsible for the inactivity of Pre3 in *pre3-R45M* mutant strains.

To test this hypothesis, several point mutations at position 52 were planned to create a new mutant strain where the Met45 would not interact with other groups adjacent to the β1/Pre3 pocket. For replacement of threonine 52, four different amino acids were chosen – glycine, alanine, serine and cysteine.

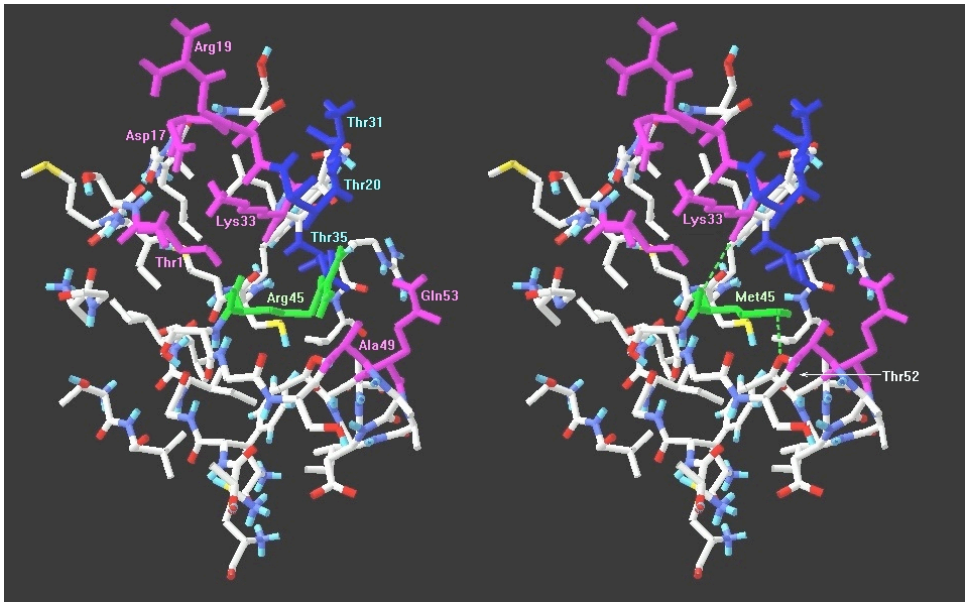
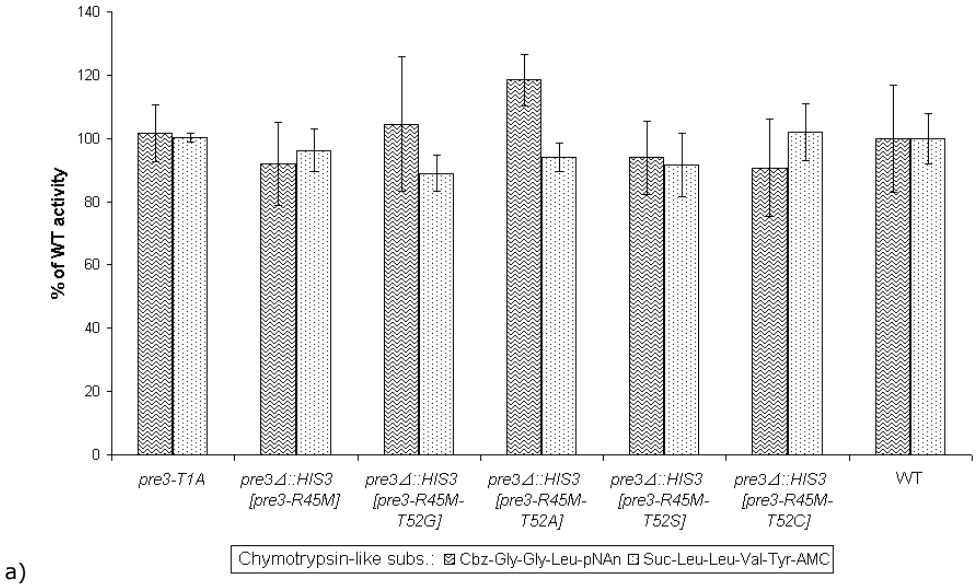


Figure 3-06 – Structure of the β1/Pre3 pocket and modelling of the Arg45Met mutated β1/Pre3, shown in stick representation. Colour representation is similar to fig.3-01, with residues forming the catalytic apparatus and parts of the substrate binding pocket in magenta and blue. The residue subject to mutation is shown in green, arginine in wild type on the left and methionine in the mutated strain on the right. Possible hydrogen bridges are shown as dashed lines.

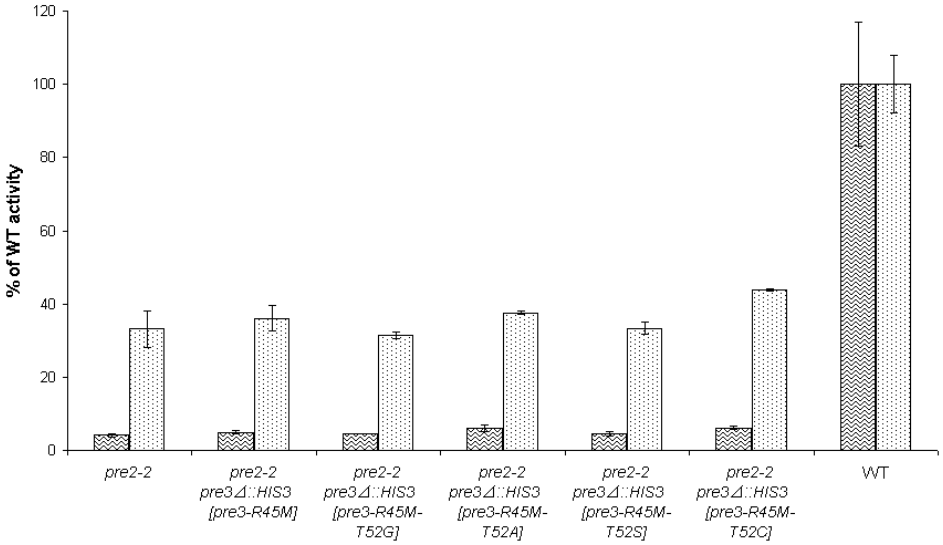
Point mutations of Thr52 were generated by means of a site directed mutagenesis kit. A primer mixture containing different DNA codons for residue 52 was used. Where sequence ACT encoded threonine, the used primers had the codons GGT (glycine), GCT (alanine), TGT (cysteine) and TCT (serine).

The different plasmids bearing the four different mutations were identified and introduced into yeast strains using the plasmid shuffling method, as previously described. The resulting mutant strains lacking the chromosomal *PRE3* and containing the *pre3-R45M-T52G*, *pre3-R45M-T52A*, *pre3-R45M-T52S*, or *pre3-R45M-T52C* alleles in plasmid pRS315 were in addition crossed with the *pre2-2* mutant strain.

Activity tests for chymotrypsin-like activities were done with the new mutant strains and their combinations with *pre2-2*, using the *pre2-2* and *pre3-T1A* strains as negative controls (fig.3-07).



a)



b)

Figure 3-07 – Proteasomal peptidase activity tests with permeabilized cells of *pre3-R45M* mutant cells and control strains. Brackets indicate expression of the allele from plasmid pRS315. Cbz-GGL-pNAn and Suc-LLVT-AMC as substrates of the chymotrypsin-like activity. a) the *pre3-T1A* strain is used as negative control, with inactivated β1/Pre3. b) the same mutants after combinations with *pre2-2*. The *pre2-2* strain is used as negative control. Error bars represent the standard error of the mean of three measurements.

According to the activity tests (fig.3-07), the additional mutations in the Pre3-R45M subunit do not lead to gain of additional proteolytic activity towards the used substrates. It can be concluded that the alteration of the amino acid at position 52 of β1/Pre3 was not sufficient to abrogate the inactivity the R45M mutation causes in this subunit. Other factors besides a possible new hydrogen bond with Thr52 may be the cause for the inactivity of β1/Pre3.

3.1.6. Other residues forming the substrate binding pocket of β5/Pre2 and β1/Pre3 – A/T20 V/T31 I/T35

Although the residue 45 in both β5/Pre2 and β1/Pre3 has a strong influence on the specificity of each active site, there are other groups which may be crucial for substrate selectivity.

Besides Thr1 there are six amino acids in contact with the substrates at their P1 positions. They are found at positions 20, 31, 35, 45, 49 and 53. The ones at position 49 and 53 are the same in Pre2 and Pre3, an alanine and glutamine, respectively.

Differing between subunits, Ala20, Val31, Ile35 in β5/Pre2 and Thr20, Thr31, Thr35 in β1/Pre3 may be also important for substrate selectivity. Those in β5/Pre2 all have apolar groups and fit well with the specificity for hydrophobic residues, while the threonines in β1/Pre3 have polar OH groups.

3.1.6.1. The importance of these amino acids for Pre2 active pocket specificity – biochemical analysis of *pre2-(A20T-V31T-I35T)-M45R* mutants

In β5/Pre2, from the amino acids found in contact with the norleucine group of calpain inhibitor, mimicking position P1 of cleavable substrates (fig.3-08), there are four which differ from the ones in β1/Pre3, including methionine 45.

New point mutations were made, replacing the existent amino acids at positions 20, 31, and 35 of β5/Pre2 by those found in β5/Pre3 at the same positions. Using the plasmid p15-pre3-M45R, the new point mutations were inserted at the mentioned positions in different combinations.

The multiple mutations were made using the "Multi Site Directed Mutagenesis Kit". After choosing five out of seven possible combinations, the following plasmids were created: pRS315-pre2-M45R-A20T; pRS315-pre2-M45R-V31T; pRS315-pre2-M45R-A20T-V31T; pRS315-pre2-M45R-I35T and pRS315-pre2-M45R-A20T-V31T-I35T. These plasmids were introduced into *pre2Δ::HIS3* yeast strains by the plasmid shuffling method.

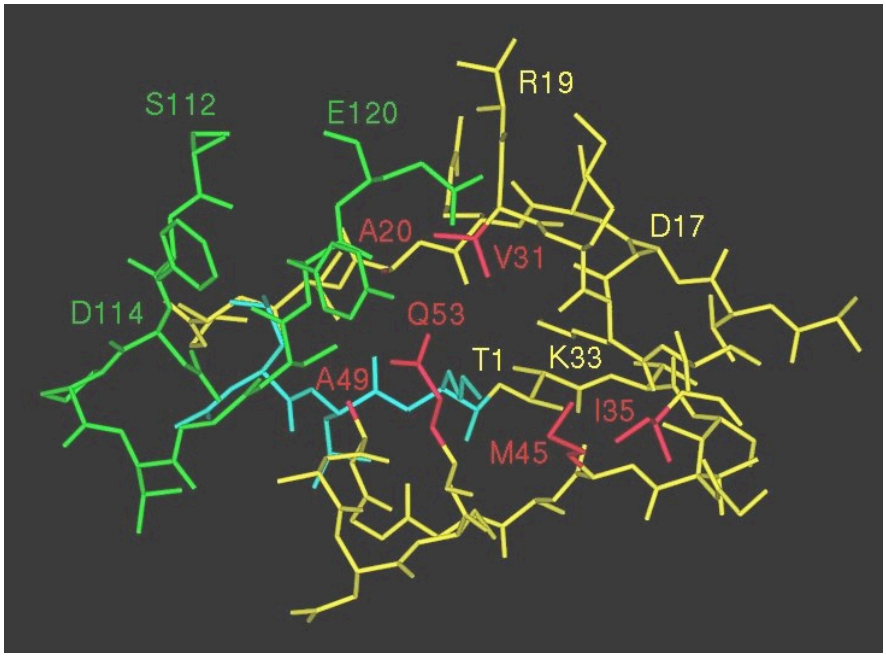


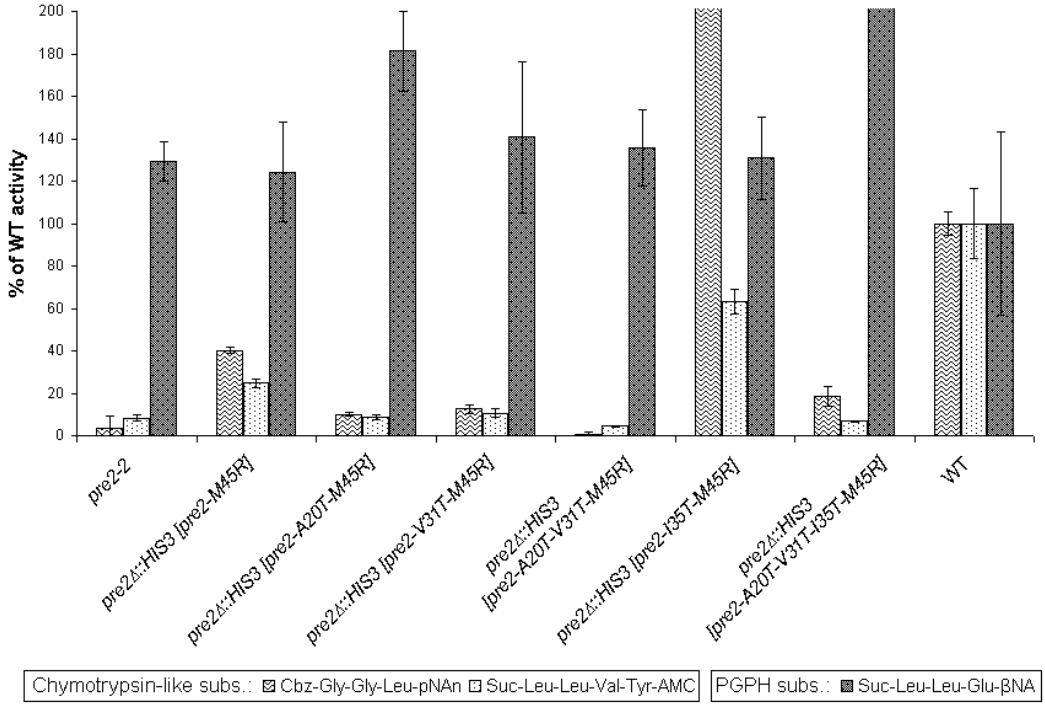
Figure 3-08 – β5/Pre2 S1 pocket with calpain inhibitor binding in stick representation. β5/Pre2 in yellow, with residues contacting P1 in red, neighbour subunit β6/C5 in green and the inhibitor in blue (taken from [Groll et al., 1997]).

After crossing all the new mutant strains with a *pre3-T1A* strain, activity tests using both substrates diagnostic for proteasomal chymotrypsin-like and PGPH specificities were performed (fig.3-09).

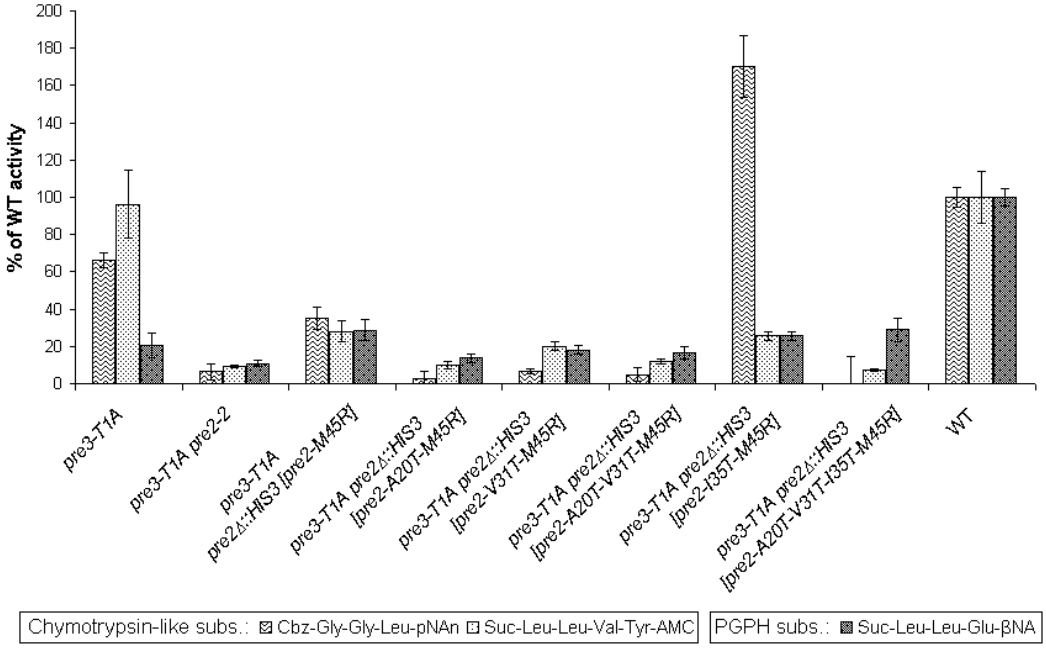
Comparing with the previously made single mutant *pre2-M45R*, the additional mutations reduce the remaining chymotrypsin-like activity to extremely low values. The only exception is *pre2-M45R-I35R*.
After combining with *pre3-T1A*, there is no significant difference in PGPH activity when comparing the new mutant strains with *pre3-T1A pre2-M45R*.

However, a very interesting result is the strong chymotrypsin-like activity revealed by the *pre2Δ::HIS3 [p15-pre2-M45R-I35T]* mutant strain. It seems to even surpass the activity values of the wild type control strain. This is more noticeable with the specific substrate Cbz-GGL-pNAn then with Suc-LLVT-AMC. When A20T and V31T mutations are added this activity is again suppressed.

In situ activity tests were then performed with the same strains. With these tests there should be a clearer visualisation and interpretation of the previous results (fig.3-10).



a)



b)

Figure 3-09 – Proteasomal peptidase activity tests with permeabilized cells of the *pre2-M45R* mutant cells and control strains. Brackets indicate expression of the allele from plasmid pRS315. Cbz-GGL-pNAn and Suc-LLVT-AMC as substrates of the chymotrypsin-like and Suc-LLE-βNA as substrate of the PGPH activity site. a) The mutant *pre2-2* is used as negative control for chymotrypsin-like activity. b) The same mutants after crossing with *pre3-T1A*. The *pre3-T1A* single and the *pre3-T1A pre2-2* double mutant are here used as negative controls. Error bars represent the standard error of the mean of three to seven measurements.

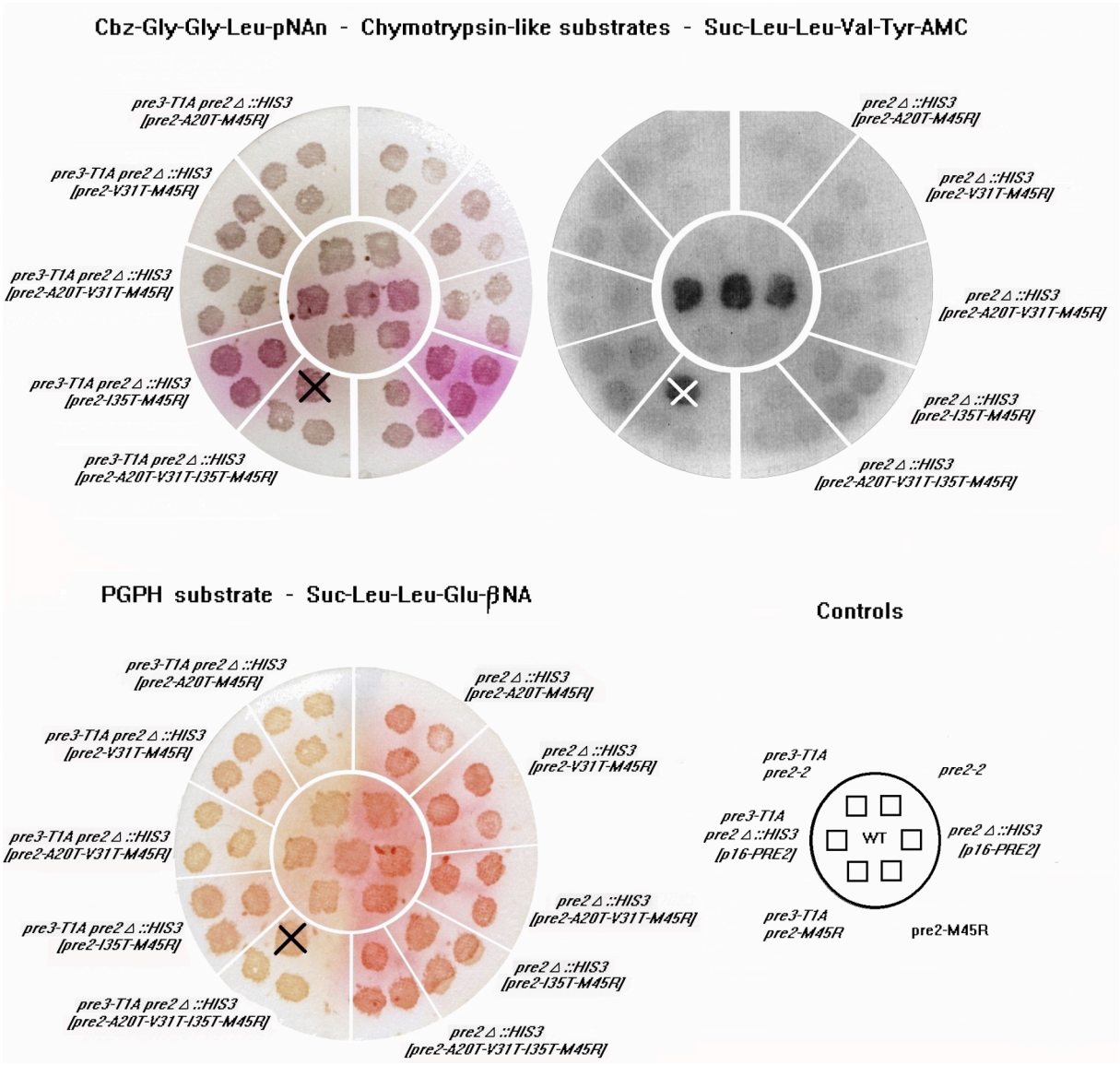


Figure 3-10 – Proteasomal *in situ* activity tests with cells from the *pre2-M45R* mutants and control strains. Brackets indicate expression of the allele from plasmid pRS315 except where indicated. Cbz-GGL-pNAn and Suc-LLVT-AMC as substrates of the chymotrypsin-like and Suc-LLE-βNA as substrate of the PGPH activity site. The one having a cross is not the correct strain and should be ignored.

Among all new multiple mutant strains, *pre2Δ::HIS3 [p15-pre2-M45R-I35T]* has a clearly stronger activity with the substrates diagnostic for chymotriptic activity. Why the other combinations do not have any activity change is unclear, and unpredictable rearrangements of the subunit pocket which make more difficult the access of substrates is the most probable reason.

The next question is whether the additional mutations affect cell growth.

Colony growth starting from different cell concentrations was monitored to compare the growth rate between the different mutants and controls (fig.3-11). Only combinations

with *pre3-T1A* were used. With a defective Pre3, the cells will be more sensitive to any phenotype caused by the mutations in β5/Pre2.

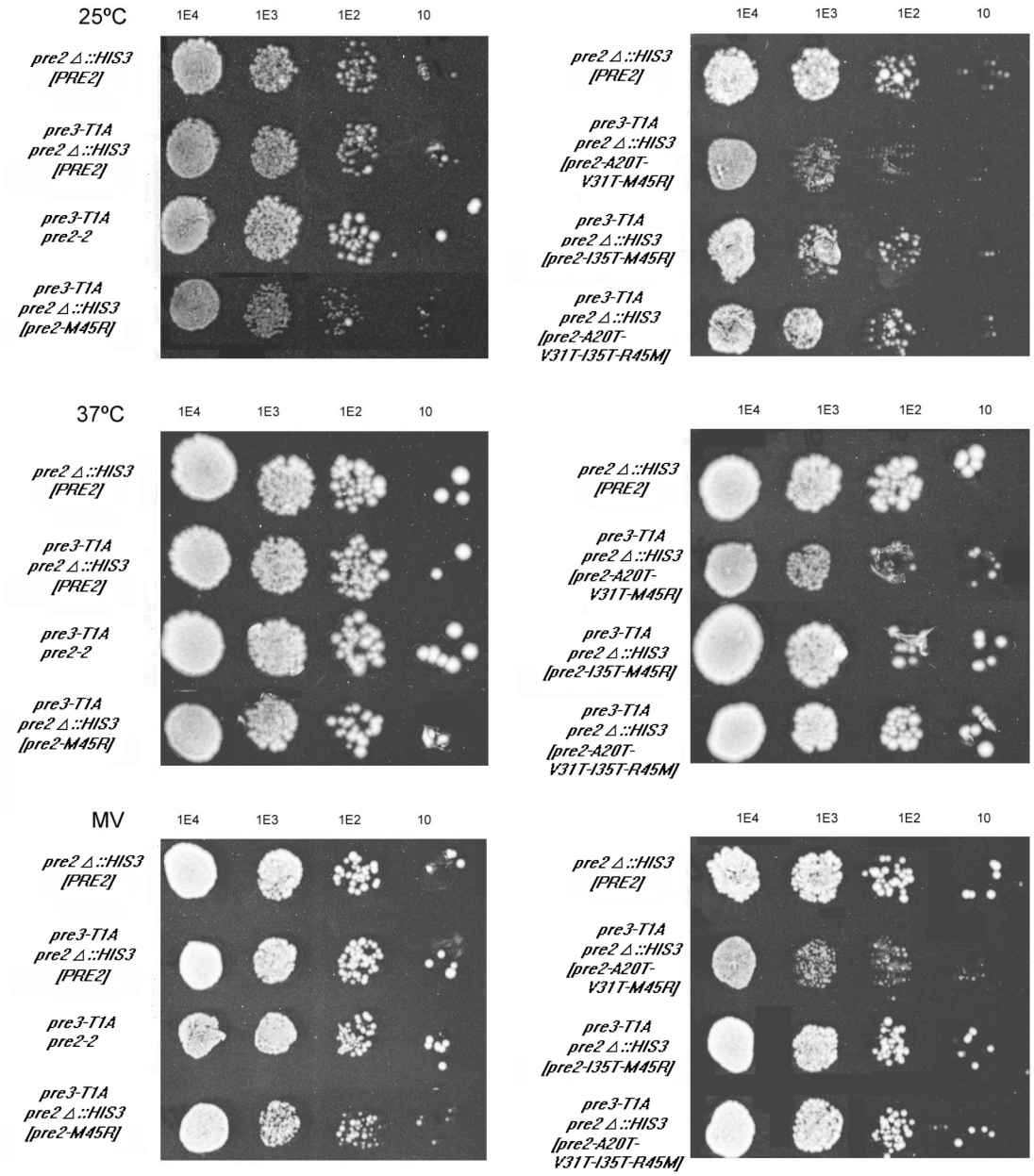


Figure 3-11 – Colony growth of yeast strains harbouring the indicated gene constructs on pRS315 plasmids (in brackets) in the respective chromosomal deletion background or integrated into the respective chromosomal locus (*pre3-T1A* or *pre2-2*) were grown in liquid YPD medium to early stationary phase, diluted to A600 = 0.5 and spotted as 2µl aliquots from tenfold serial dilutions (from left to right) onto solidified YPD or MV medium where indicated. Growth was for about two days at the indicated temperatures and at 30ºC on MV. As controls, *pre3-T1A pre2-2* and *pre2Δ::HIS3 [p16-PRE2]* were used.

Analysing the colony growth from different dilutions, it is clear that the strain *pre3-T1A pre2Δ::HIS3 [p15-pre2-M45R-A20T-V31T]* has the strongest phenotype.

What is surprising is that this phenotype is reversed by the addition of another point mutation, I35T in *pre3-T1A pre2Δ::HIS3 [p15-pre2-M45R-A20T-V31T-I35T]*.

The results with these last mutant strains, in the *in situ* proteolytic activity tests and colony growth comparison are extremely interesting. For a better visualisation and comparison, extra *in situ* activity assays were made with the most interesting mutant strains and controls to confirm the previous results (fig.3-12).

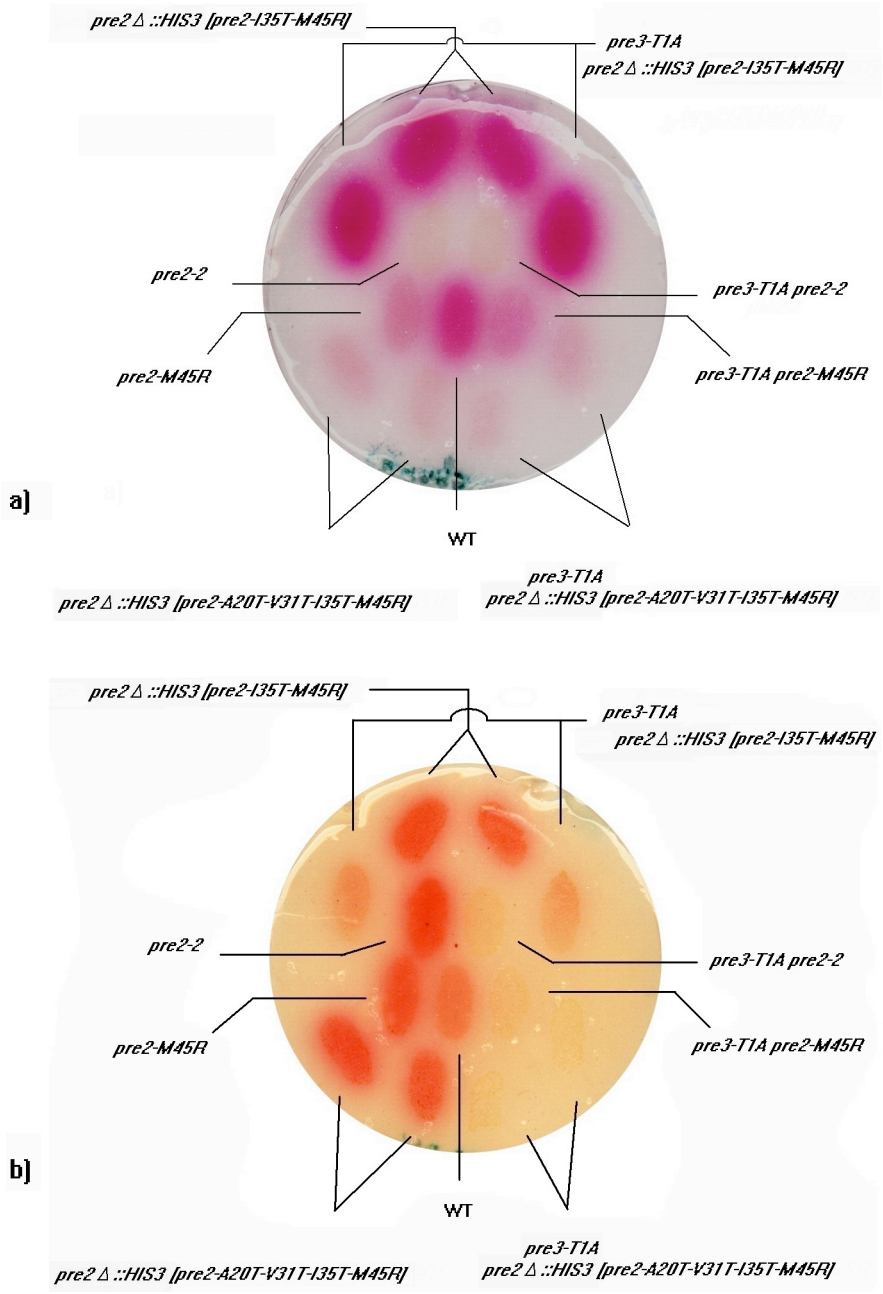


Figure 3-12 – Proteasomal *in situ* activity tests with *pre2Δ::HIS3 [p15-pre2-M45R-I35T]* and *pre2Δ::HIS3 [p15-pre2-M45R-A20T-V31T-I35T]* mutant cells, *pre3-T1A* crossings and control strains. a) chymotrypsin-like activity with Cbz-GGL-pNAn substrate. b) PGPH activity with Suc-LLE-βNA as substrate.

In the chymotrypsin-like activity test plate (fig.3-12a) it can be seen that the proteolytic activity of *pre2-M45R* mutant strains is around 50% of that of wild type (chapter 3.1.1.). With the addition of the I35T mutation, the strain *pre2Δ::HIS3 [p15-pre2-M45R-I35T]* shows an activity even stronger then the wild type control.
Although the double mutant *pre2-M45R-I35T* has enhanced chymotrypsin-like activity, even compared with wild type, when two other point mutations A20T and V31T are added, the resulting strain *pre2Δ::HIS3 [p15-pre2-M45R-A20T-V31T-I35T*] has no ability to process the substrate diagnostic for chymotriptic activity. Nevertheless there is a residual activity which can be seen by the slightly stronger pigmentation than that of negative controls *pre2-2* or *pre3-T1A pre2-2*.

When using the Suc-Leu-Leu-Glu-βNA substrate, all strains with mutated β5/Pre2 and intact β1/Pre3 have a higher PGPH activity than the wild type control. When combined with *pre3-T1A*, the resulting strain *pre3-T1A pre2Δ::HIS3 [p15-pre2-M45R-I35T]* does still have some residual activity. Similarly to the results of the test for chymotrypsin-like activity, there is no PGPH activity detectable if the additional mutations A20T and V31T are present.

The A20T and V31T point mutations in Pre2 not only cause loss of chymotrypsin-like activity, they also cause slow growth. The strain *pre2Δ::HIS3 [p15-pre2-M45R-A20T-V31T]* has a stronger growth phenotype than the *pre2-2* negative control (fig.3-11). With the addition of the I35T point mutation, growth is restored although there are no significant activity alterations (fig.3-09, fig.3-10).

3.1.6.2. The importance of these amino acids for Pre3 active pocket specificity – biochemical analysis of *pre3-(T20A-T31V-T35I)-R45M* mutants

Just like the β5/Pre2 subunit, in β1/Pre3 the amino acids which are in contact with the norleucine of the calpain inhibitor equivalent to position P1 of substrates are located at the same positions of the active pocket as of those in β5/Pre2 (fig.3-13).
As for β5/Pre2, equivalent combinations of point mutations were introduced into β1/Pre3. The amino acids at the positions 20, 31 and 35 were replaced by those existing in β5/Pre2.

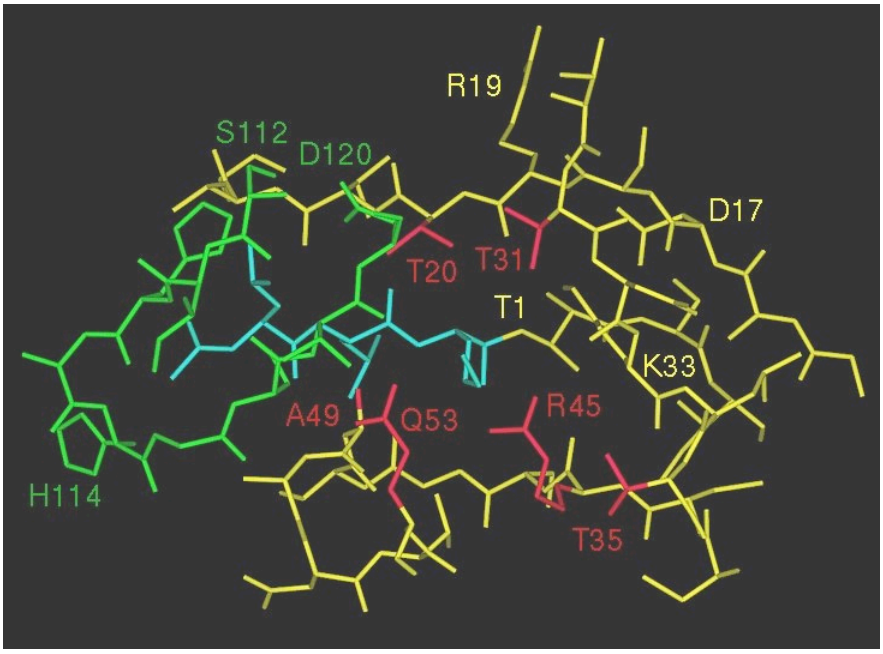
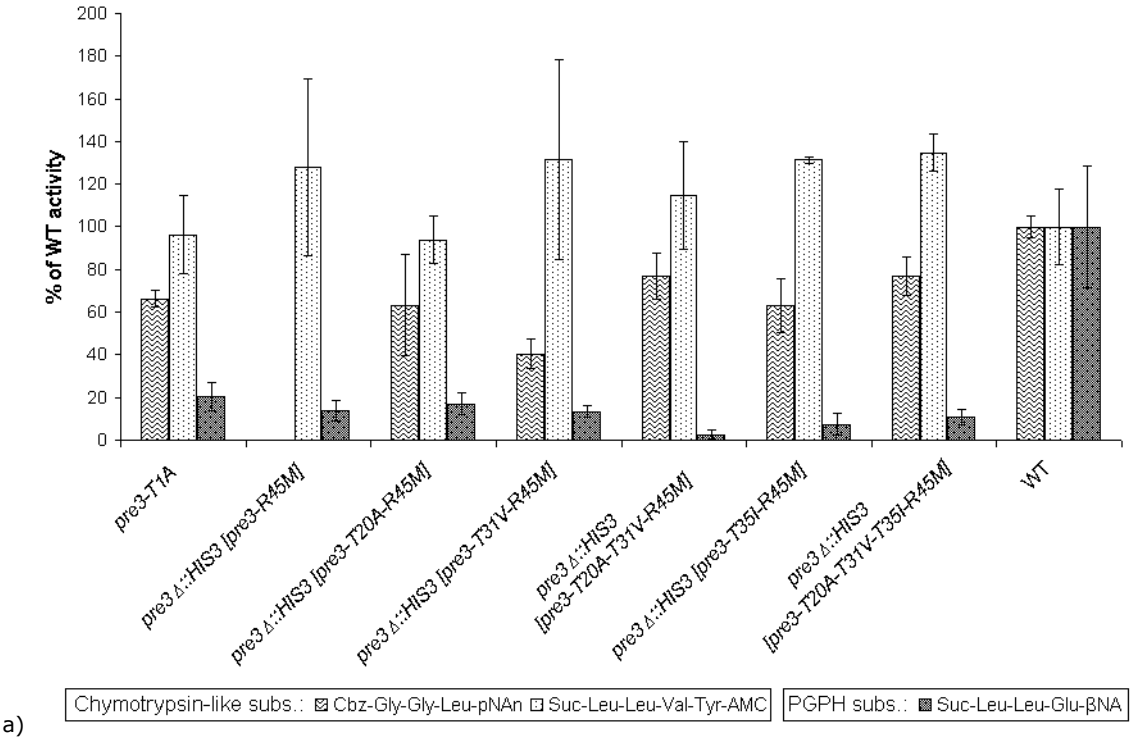


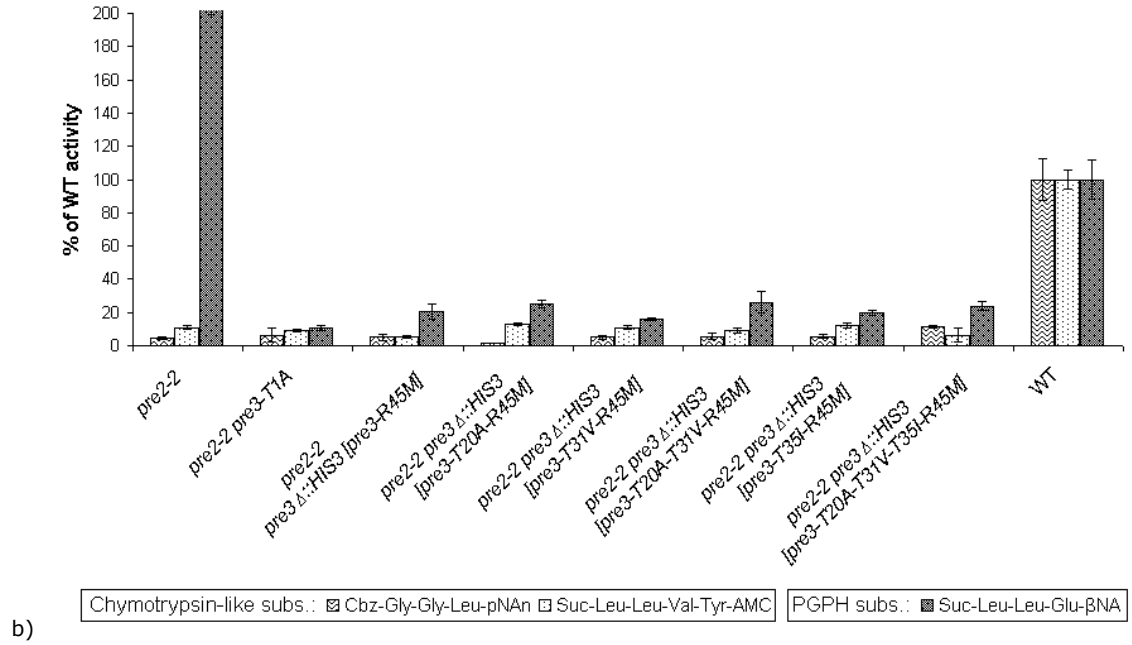
Figure 3-13. β1/Pre3 S1 pocket with calpain inhibitor binding in stick representation. B1/Pre3 in yellow, with residues contacting P1 in red, neighbour subunit β2/Pup1 in green and the inhibitor in blue [Groll et al., 1997].

Using p15-pre3-R45M as template, a variety of mutant alleles was generated using the Multi Site Directed Mutagenesis Kit: *pre3-R45M-T20A*; *pre3-R45M-T31V*; *pre3-R45M-T20A-T31V*; *pre3-R45M-T35I* and *pre3-R45M-T20A-T31V-T35I*.

After transfer of the plasmids into a *pre2Δ::HIS3* strain, the generated mutant strains were also crossed with a *pre2-2* strain. Activity tests using both substrates for chymotrypsin-like and PGPH activity were performed (fig.3-14).



a)



b)

Figure 3-14 – Proteasomal peptidase activity tests with permeabilized cells of *pre3-R45M* multiple mutant cells and control strains. Brackets indicate expression of the allele from plasmid pRS315. Cbz-GGL-pNAn and Suc-LLVT-AMC as substrates of the chymotrypsin-like and Suc-LLE-βna as substrate of the PGPH activity site. a) The *pre3-T1A* strain is used has negative control having no activity of the β1/Pre3 subunit. b) The same mutant strains after crossing with the *pre2-2* strain. The *pre2-2* strain and the double mutant strain *pre2-2 pre3-T1A* are here used as negative controls. Error bars represent the standard error of the mean of three measurements.

For the Suc-LLE-βNA substrate, the new mutants show very low values of activity, which are not different from the inactive *pre3-T1A* control strain (fig.3-14a).

Before combining with the *pre2-2* mutation, the chymotrypsin-like activity (fig.3-14a) can be associated with the intact β5/Pre2 subunit. All new *pre3* mutants with inactivated β5/Pre2 show no trace of chymotrypsin-like activity (fig.3-14b), like the *pre2-2 pre3Δ::HIS3 [p15-pre3-R45M]* strain, carrying the parental single point mutation in Pre3. Unlike with β5/Pre2 (chapter 3.1.6.1), the amino acid replacements in the β1/Pre3 S1 pocket did not lead to activity differences when compared with the single point mutant strain.

3.1.6.3. The *pre3-(T20A-T31V-T35I)-R45M* mutants do not alter the growth phenotypes of the *pup1-T1A* mutant

Although there were no changes of activity towards the used substrates induced by extra mutations in addition to R45M in β1/Pre3, a colony growth comparison was performed.
To test the possibility that the extra mutations have a non detectable activity which may suppress the growth phenotype of *pup1-T1A* mutant strains, the β1/Pre3 S1 pocket mutant strains were crossed with *pup1-T1A*.
Using tenfold dilutions, cells were grown at different temperature conditions. It was at low temperatures (25ºC) that growth phenotypes could be best observed (fig.3-15)

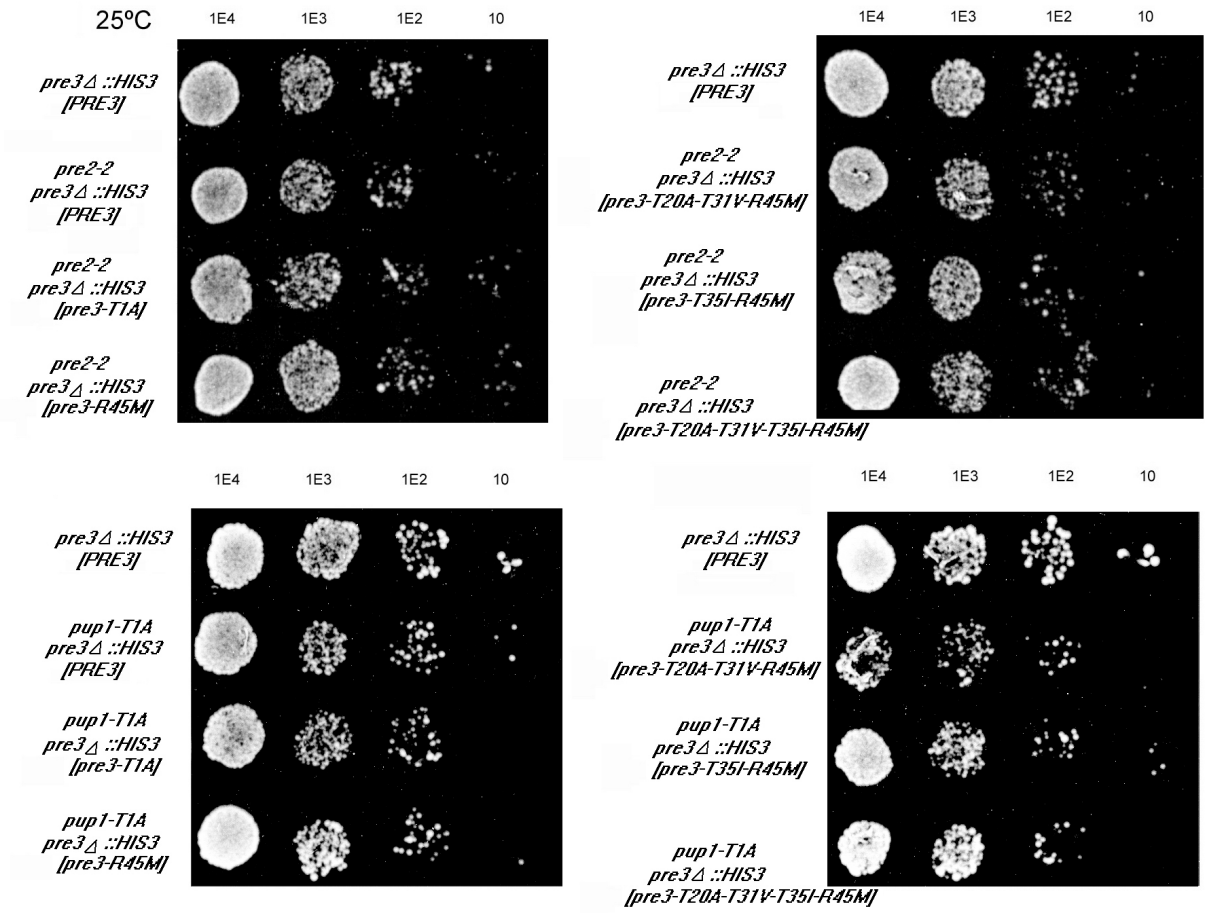


Figure 3-15 – Colony growth of yeast strains harbouring the indicated gene constructs on pRS315 plasmids in the respective chromosomal deletion background or integrated into the respective chromosomal locus (*pre2-2* or *pup1-T1A*) were grown in liquid YPD medium to early stationary phase, diluted to A600 = 0.5 and spotted as 2µl aliquots from tenfold serial dilutions (from left to right) onto solidified YPD medium. Growth was for about two days at the temperature of 25ºC. As controls strains expressing *pre3-T1A* from pRS315 were used.

The only differences in cell growth are observed between the strains with *pre2-2* or *pup1-T1A* mutations and the wild type strain. There is no visible distinction in cell growth between the different *pre3* mutant strains.

At the temperature of 37ºC (data not shown) the result was similar. After changing arginine 45 into methionine there is total loss of proteolytic activity of β1/Pre3 which is not restored by the extra imposed mutations in the S1 pocket. Different phenotypes between the strains with added mutations on Pre3 are not found either.

3.1.7. Analysis of the auto-catalytic maturation process in the different mutants

Proteasome subunits only become active after proteasome assembly by the process of propeptide self-cleavage. If by any reason a subunit is not able to auto-catalytically cleave off its propeptide it remains proteolytically inactive. The catalytic nucleophilic Thr1 hydroxyl group is not exposed and substrates won't be degraded.
The data from the activity tests made with the newly created mutants provoke the question, if the maturation of the subunits was affected by the mutations.

The processed β5/Pre2 subunit has a molecular weight of around 23,3kDa and its precursor form weights around 31.6kDa [Heinemeyer et al., 1993]. The matured β1/Pre3 subunit has a mass of about 21.5kDa while the unprocessed subunit has a mass of 23.6kDa. In absence of Pre3 self-cleavage, the proteolytically active β2/Pup1 neighbour partially cleaves the β1/Pre3 propeptide, leaving the subunit with a mass of 22,4kDa [Groll et al., 1999].

The subunit propeptides have a size between 2 and 8kDa. Although catalytically active subunits can partially cleave the unprocessed propeptides from other subunits, it can be tested by means of a Western Blot if a specific subunit was able to auto-catalytically cleave off its own propeptide.

3.1.7.1. Maturation is not affected in β5/Pre2 mutant strains

To gain an insight into putative perturbations the different S1 pocket mutations may cause to Pre2 propeptide cleavage, crude cell extracts of strains *pre2Δ::HIS3 [p15-pre2-M45R]*, *pre2Δ::HIS3 [p15-pre2-A20T-V31T-M45R]*, *pre2Δ::HIS3 [p15-pre2-I35T-M45R]* and *pre2Δ::HIS3 [p15-pre2-A20T-V31T-I35T-M45R]* were analysed by immunoblotting for the subunit β5/Pre2.

The *PRE2* wild type strain expressing a functional β5/Pre2 subunit from a plasmid shows complete Pre2 propeptide cleavage and is used as positive control. The mutant strain *pre2-K33A* does not show processing of the β5/Pre2 propeptide and therefore is used as negative control. Extracts of *pre2-2* cells and purified 20S proteasome are also used as controls.

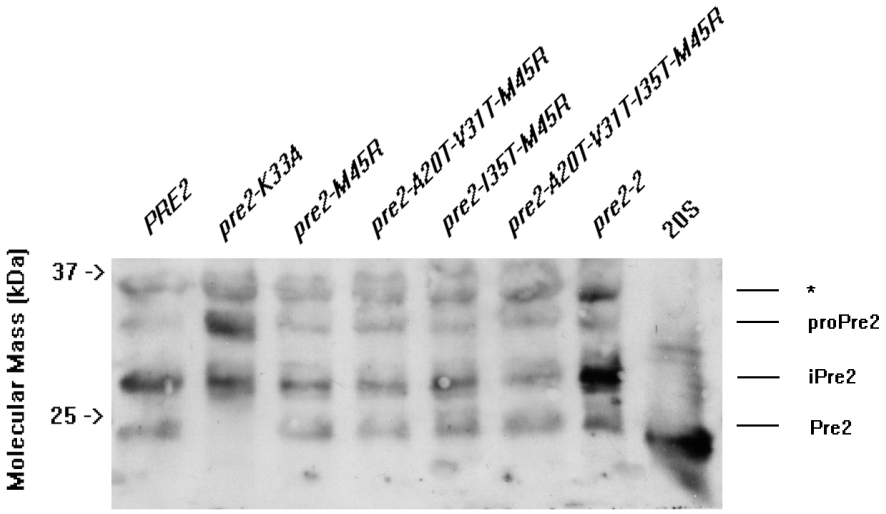


Figure 3-16 – Western Blot with β5/Pre2 S1 pocket mutant strains, and controls with antibody recognising Pre2. All *pre2* alleles are expressed from plasmid (pRS316 in *PRE2* and pRS315 in others) except controls *pre2-K33A* and *pre2-2*. 20S is purified complex. Pre2 is the respective subunit with its propeptide cleaved off; proPre2 is Pre2 with propeptide; iPre2, Pre2 with intermediately cleaved propeptide and * is for cross-reactions.

Analysis of the mutant strains extracts reveals mature forms of Pre2 in quantities comparable to those found in the control extract (fig.3-16).
As expected, the mutant strains are able to cleave off the Pre2 propeptides. There are some unprocessed subunits present in the positive control *PRE2* and in the β5/Pre2 S1 pocket mutant strains which are from free subunits or proteasome intermediate forms. The ratio of this unprocessed Pre2 form to the fully processed form was the same observed in the wild-type control, and far from the ratio revealed in the negative control *pre2-K33A*.

3.1.7.2 Maturation is strongly reduced in some of the β1/Pre3 mutant strains

Available antibodies against β1/Pre3 have many cross reactions, and do not allow a conclusive immunoblot analysis. In order to observe how the β1/Pre3 propeptides are processed in the created mutant strains, the Pre3 subunits were HA tagged. First the existing NotI cleavage site in the plasmid *p15-PRE3* was removed, and a new one was created after the codon corresponding to the last C-terminal β1/Pre3 amino acid L187 and before the stop codon. This was possible using the "Corrected QuickChange Site Directed Mutagenesis Kit". A 3xHA TAG sequence was then inserted in this newly created NotI site location.

The unprocessed Pre3 precursor has a mass of 23.6kDa. If this subunit does not auto-catalytically process is own propeptide, the neighbour subunit β2/Pup1 cleaves it partially

leaving the subunit with a mass of 22.4kDa. The mature β2/Pre3 subunit has a mass of 21.5kDa.
With only 1kDa difference between the mature Pre3 and it's partially cleaved form, the immunoblotting results would be difficult to read, and the results inconclusive. The *pup1-T1A* mutant strains have an inactive β2/Pup1 subunit and will be unable to partially cleave the β1/Pre3 propeptide. By crossing the Pre3 mutants with pup1-T1A, the partial cleavage of the β1/Pre3 propeptide is avoided.

A DNA fragment containing the HA sequence was isolated from the HA-PRE3 plasmid.
The DNA fragment containing the HA was joined with the open plasmids p15-pre3-T1A, p15-pre3-T20A-T31V-R45M, p15-pre3-T35I-R45M and p15-pre3-T20A-T31V-T35I-R45M with cohesive ends. This was done by transformation in the yeast strain *pup1-T1A pre3Δ::HIS3 [p16-PRE3]*.

The *pre3Δ::HIS3 [p15-HA-PRE3]* strain is crossed with *pup1-T1A* to be used as a positive control, whereas the strain *pre3Δ::HIS3 [p15-HA-pre3-T1A]* was combined with *pup1-T1A* to be used as negative control. Another control is the tagged *pre3Δ::HIS3 [HA-pre3-R45M]* strain, with a functional β2/Pup1.

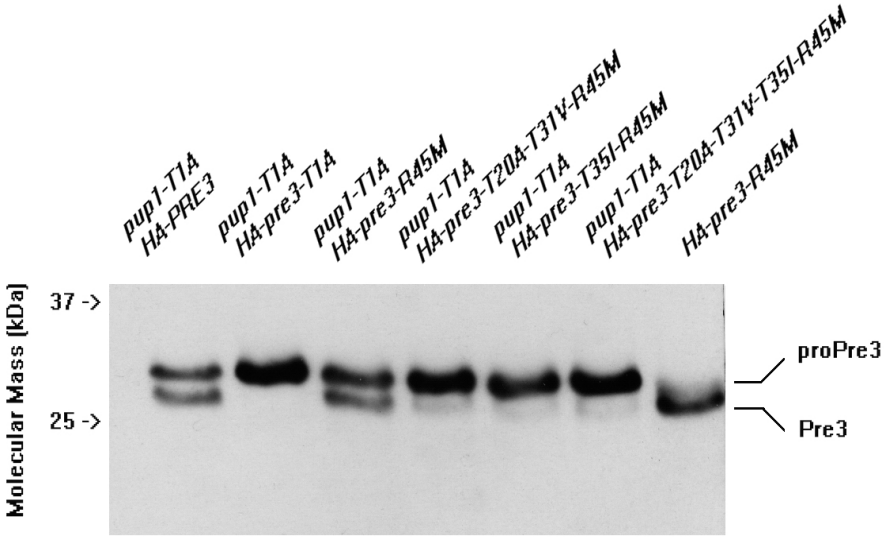


Figure 3-17 – Western Blot with HA tagged β1/Pre3 S1 pocket mutant strains after crossing with β2/Pup1, and controls. All Pre3 mutations are expressed in a pRS315 plasmid. Pre3 has complete processed propeptide; proPre3 is Pre3 with unprocessed propeptide.

Analysing the extracts, the mutant strain *pup1-T1A pre3Δ::HIS3 [p15-HA-pre3-R45M]* shows a ratio between the unprocessed and processed forms of Pre3 similar to the positive control *pup1-T1A pre3Δ::HIS3 [p15-HA-PRE3]*. When extra mutations are added to the Pre3-R45M, this ratio increases drastically. Mature β1/Pre3 subunits are much

smaller amounts and it is assumed that most of the proteasomes will possess Pre3 subunits without having their propeptides removed.
The single mutation *pre3-R45M* does not seem to affect the self-cleavage of this subunit's propeptide, only affecting the proteolytic activity. With the additional mutations T20A, T31V and T35I the auto-catalytic process is strongly compromised.

3.1.8. The β1/Pre3 mutant subunits lack detectable peptidase activity when expressed without their propeptides

The lack of activity in β1/Pre3 mutant strains is partially due to the propeptide un-processing, but without propeptides there might be some activity.
The β1/Pre3 mutant strains where additionally mutated, so that the Pre3 subunits would not have their propeptides expressed. All strains had pRS315 plasmids with *PRE3* expressed without propeptides as C-terminal fusions to ubiquitin. The ubiquitin fragment is naturally cleaved, leaving the subunit without anything before the Thr1.
The exposure of N-terminal Thr1 in the cytosol would leave to its α-acetylation and the subunit would remain inactive after proteasome assembly. The N^{α}-acetyltransferase (*Nat1*) gene was deleted, avoiding this way the acetylation of Thr1.

Plasmids with ubiquitin fusion into desired Pre3 mutations were prepared using the existent plasmid p15-UBI/mmPRE3 [Jäger et al., 1999]. From this plasmid, a DNA fragment containing the ubiquitin sequence was removed and introduced into those plasmids containing the Pre3 point mutations. Using plasmid shuffling method these plasmids were transformed into *Nat1Δ::Kan* and *Nat1Δ::Kan pre2-2* strains.

In situ activity tests were performed with the N^{α}-acetyltransferase deletion strains, for chymotrypsin-like and PGPH specificities (fig.3-18).

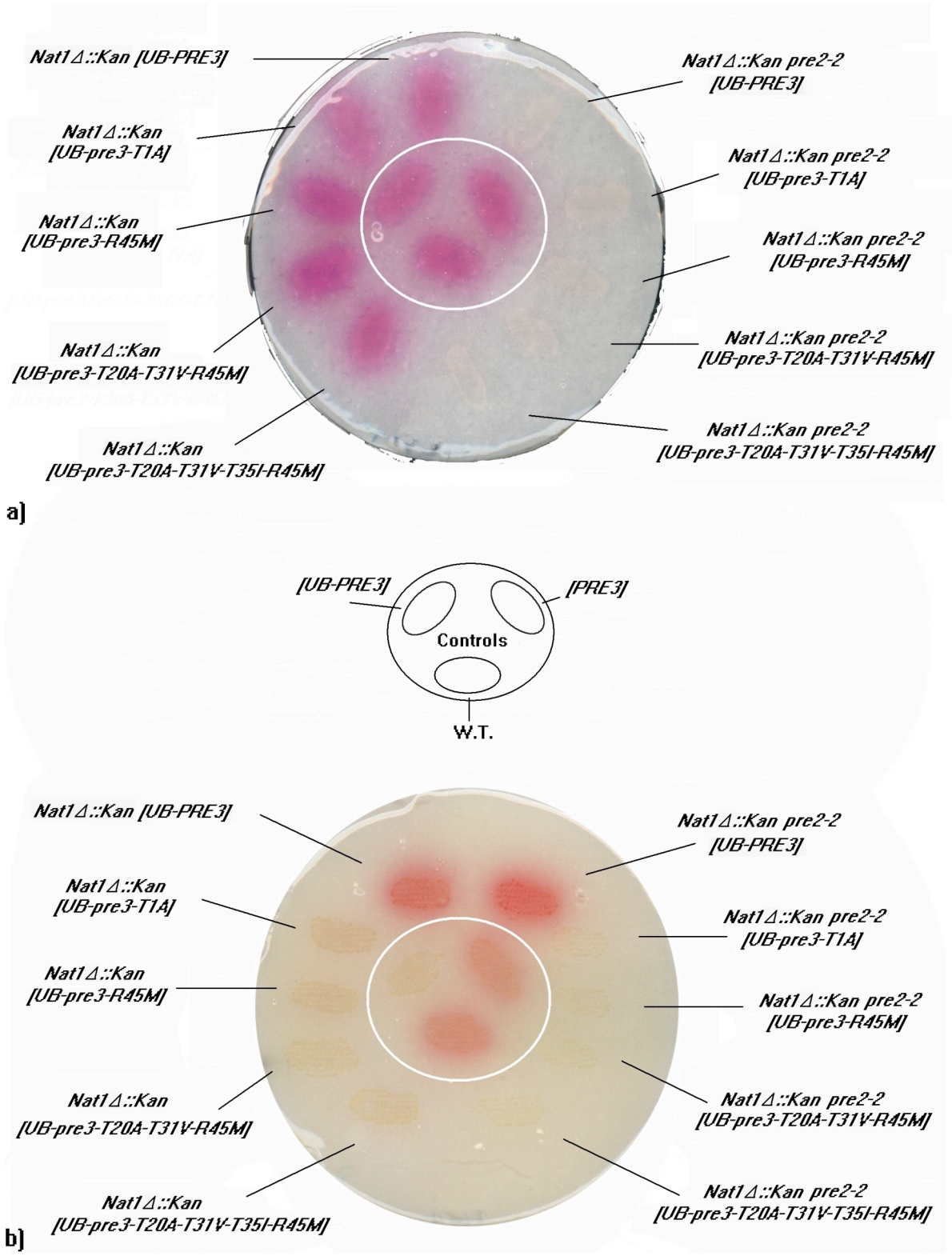


Figure 3-18 – Proteasomal *in situ* activity tests with β1/Pre3 active pocket mutant strain cells with Pre3 propeptide removed and Nα acetylase deletion, *pre2-2* crossings and control strains. Brackets indicate expression of the allele from plasmid pRS315 in the respective chromosomal deletion background.
a) chymotrypsin-like activity with Cbz-GGL-pNAn substrate. b) PGPH activity with Suc-LLE-βNA as substrate.

Observing the *pre3* mutant strains after crossing with *pre2-2* and comparing with the control *Nat1Δ::Kan pre3Δ::HIS3 [p15-UB-PRE3]*, no residual chymotrypsin-like activity is seen. The strains having a β5/Pre2 intact show full activity, just like the wild-type control. From the analysis of the chymotrypsin-like activity tests, we can conclude that after the mutations on the β1/Pre3 S1 pocket, there is no specificity swap.

The control strains *Nat1Δ::Kan pre3Δ::HIS3 [p15-UB-PRE3]* and *Nat1Δ::Kan pre2-2 pre3Δ::HIS3 [p15-UB-PRE3]* have a wild type β1/Pre3 subunit with its propeptide removed, and show a stronger PGPH activity than the wild type strain. This may be caused by free subunits. Without their propeptide or N^{α}-acetyltransferase to acetylate the N-terminal Thr1, there is the possibility that these free subunits are proteolytically active before they are assembled into full proteasomes.

By looking at the PGPH activity test, it is observed that the strains without an intact Pre2 do not have any residual activity. In combination with pre2-2, activity is diminished in all the β1/Pre3 S1 pocket mutated strains. The performed mutations in β1/Pre3 generate strains with a complete loss of PGPH activity from this subunit.

The β subunit propeptides are required for a proper assembly of the half proteasomes from which, the most important is the propeptide of the β5/Pre2 subunit. A Pre2 subunit expressed without its own propeptide causes lethality to the cells [Kimura et al., 2000]. As it is not crucial for assembly, mutant strains expressed without the Pre3 propeptide are viable but will not show proteolytic activity unless N^{α}-acetyltransferase is suppressed. Still, after the deletion of N^{α}-acetyltransferase no proteolytic activity in the mutant strains with the used substrates is found.
It could be that the limited number of used peptide substrates is not enough to say that the mutant strains have a complete loss of activity. Nevertheless it is obvious that the imposed mutations in Pre3 cause several changes on the subunit S1 pocket structure and substrate selectivity.

It can be concluded that any of the alterations done into β1/Pre3 S1 pocket somehow interfere with its ability of cleaving substrates, including to auto-cleave its own propeptide.

3.2. The role of the Pup1 C-terminal elongation

The Pup1 β subunit has, unlike the other subunits, a C-terminal extension of 30 residues. This extension is located on the outer surface of the proteasome and wraps around β3/Pup3 within the same β-ring. It could be required for some step in proteasome assembly, or even for interaction with other proteins at the outside of the 20S proteasome.

To study the relevance of this extension for the biogenesis of functional proteasomes, several point mutations and C-terminal truncations at different locations were planned.
Possible internal interactions within the proteasome were analysed by altering amino acids located between β3/Pup3 and the opposite β-ring, and putative interactions with external proteins were approached by mutating a residue pointing out from the proteasome surface. Truncations were planned by considering the shape of the Pup1 C-terminus and how it embraces the neighbour subunit. To examine how much of this elongation is required, one truncation was done by removing the entire C-terminal elongation and another by cleaving only the most distal extension located between β3/Pup3 and β4/Pre1. An intermediate truncation was also performed.
The growth phenotype of the generated mutant strains was studied at different conditions.

3.2.1. Point mutations in the β2/Pup1 elongation

To understand what could be the function of the Pup1 C-terminal elongation, a few point mutations within this external arm were planned. Instead of random mutations, amino-acids that either have extensive contact to other subunits or have a large side group oriented to the outside were chosen for directed mutagenesis.
Analysing the Pup1 arm on the proteasome's surface structure, the side chains of Tyr204 and Thr211 are oriented to the inside and contact both β3/Pup3 and β7/Pre6 of the opposite β-ring. They could thus be crucial for the joining of or contact formation between these subunits. On the other hand, Arg208 has a long basic group exposed to the outside of the proteasome, and could be involved in interactions with other proteins outside the 20S proteasome (fig.3-19).

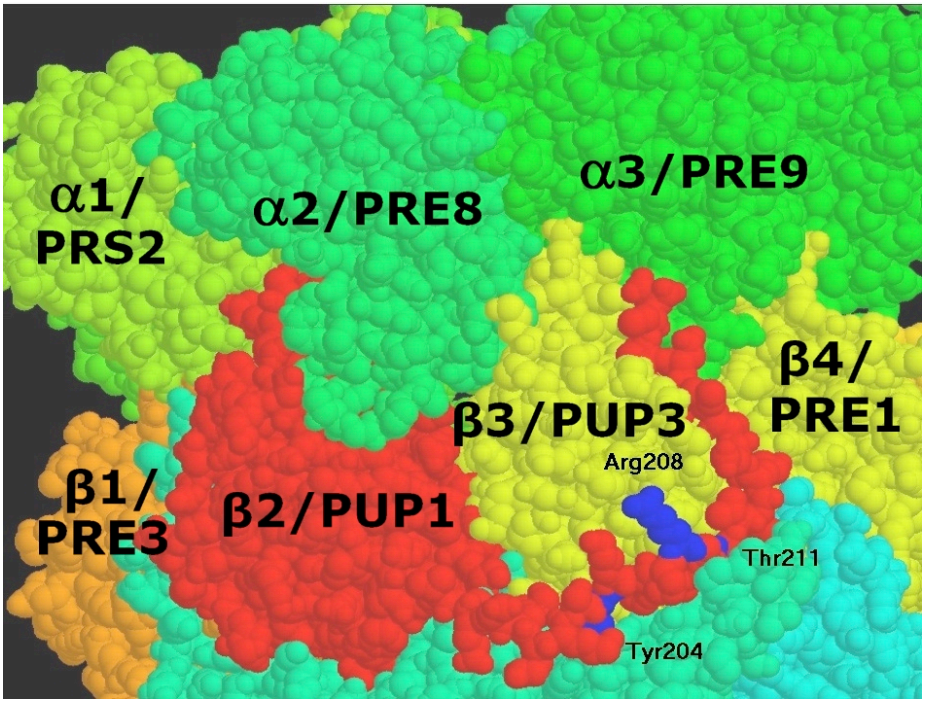


Figure 3-19 – Partial view of the 20S Proteasome in space-filling model. Different subunits are shown in different colours. Tyr204, Arg208 and Thr211 of β2/Pup1 are coloured blue (image created with RASMOL).

Using a recombinant PCR method (see methods), the independent mutations were separately introduced into the *PUP1* gene on a plasmid. The Tyr204 and Thr211 were replaced by an alanine and the Arg208 by a glycine. The resulting plasmid constructs expressing the mutations Y204A, T211A and R208G were then introduced into strains with a *PUP1* gene deletion, using the plasmid shuffling method.

If the selected amino acids have an important role, there should be a strong phenotype in the created mutant strains. Growth comparison was performed at different temperatures and on different media (fig.3-20).

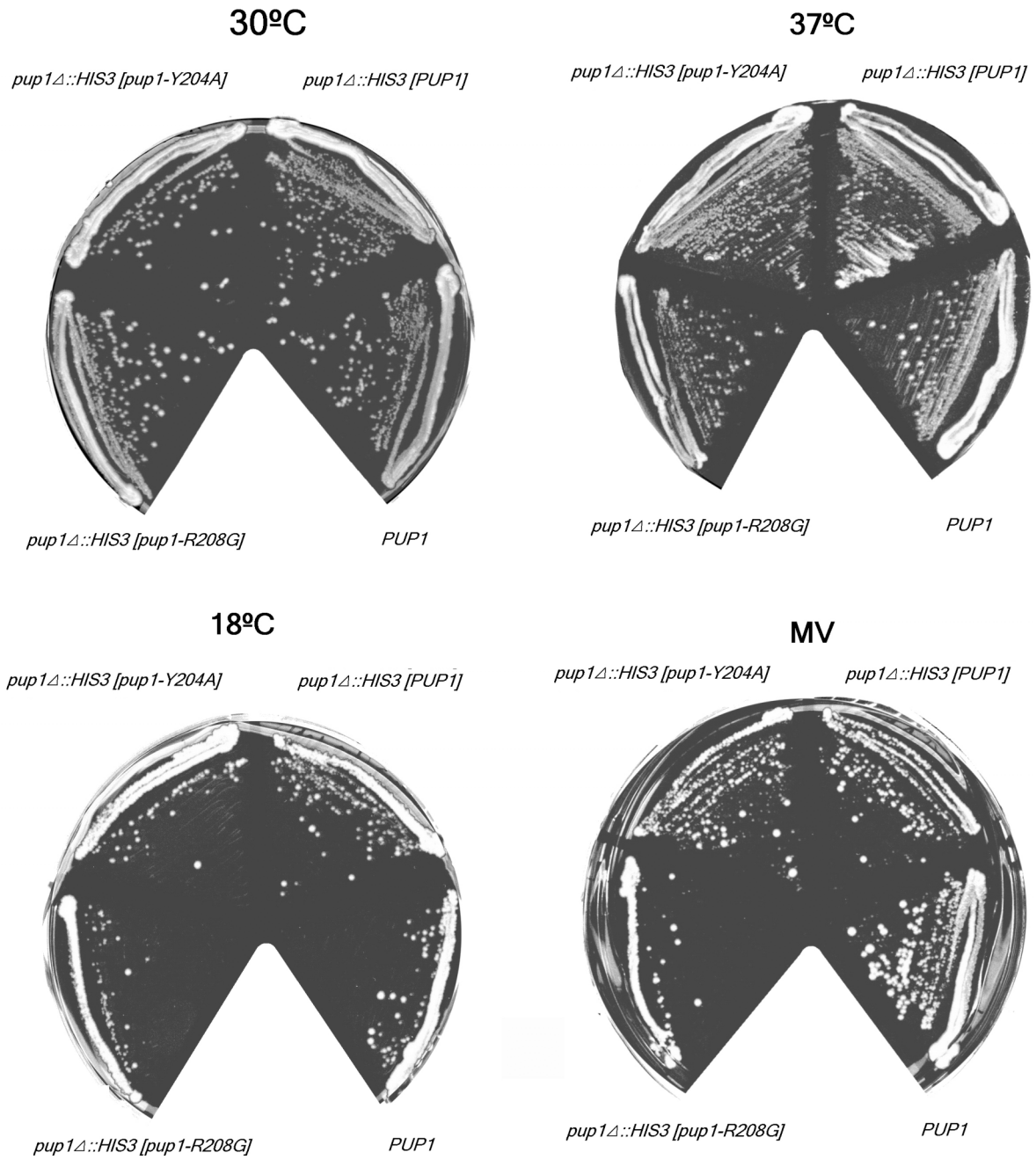


Figure 3-20 – Colony growth comparison of yeast strains harbouring the indicated gene constructs on plasmid pRS315 (indicated by brackets). Strains were streaked for single colonies on solid YPD medium and incubated at 30ºC and 37ºC for about two days and at 18ºC for about six days or MV medium at 30ºC for about two days.

Comparing the growth of the generated mutant strains with controls, there is no significant phenotype visible.

The *PUP1* chromosomal disruption causes a slight growth disturbance at 37ºC which can be observed when comparing the control *pup1Δ::HIS3 [p15-PUP1]* with the *PUP1* wild type strain. However, the strains carrying the generated point mutations Y204A, R208G or T211A (data not shown) have a growth similar to that of the *pup1Δ::HIS3 [p15-PUP1]* control strain.

According to these results it can be concluded that the chosen amino acids alone do not contribute to any important function in the proteasome or the cell.

3.2.2. Pup1 C-terminal truncations

Next, the Pup1 C-terminal elongation was completely and partially removed. Three truncations were planned, a complete or long tail deletion (pup1-ltd) by removing 37 amino acids from the β2/Pup1 C-terminus, and two partial truncations by removing 20 and 25 amino acids in a short (pup1-std) and medium (pup1-mtd) tail deletion, respectively (fig.3-21).

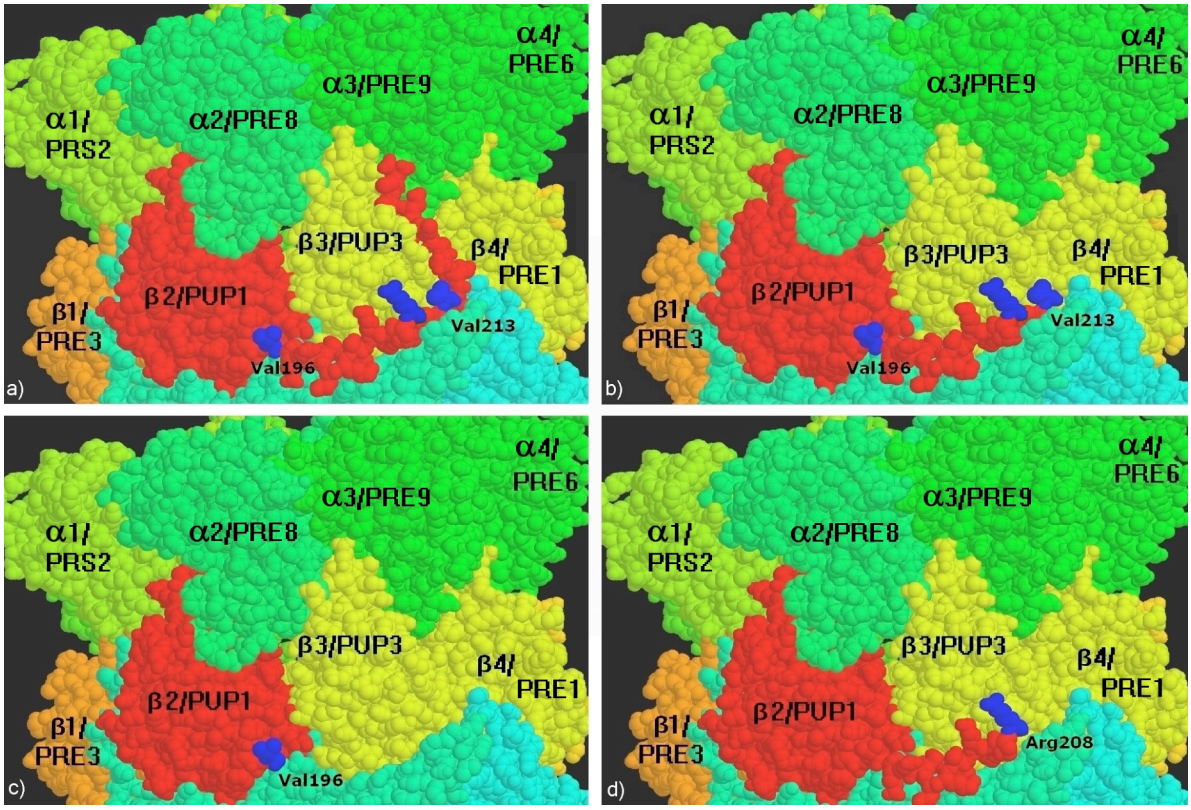


Figure 3-21 – View of the truncations on the β2/Pup1 subunit in a space-filling model. Different subunits are in different colours, amino acids after which the C-terminus was deleted are in blue. a) Intact β2/Pup1 showing the locations for truncations. b) β2/Pup1 short tail deletion (std). c) β2/Pup1 long tail deletion (ltd). d) β2/Pup1 medium tail deletion (mtd).

To perform the planned truncations, the method of recombinant PCR was applied (see methods, chapter 2.2.1.). A plasmid construct with the complete *PUP1* sequence was used as template. The resulting three different plasmids containing the sequence for each designed truncation were named p15-pup1-std, p15-pup1-mtd and p15-pup1-ltd.

With the previously described method of plasmid shuffling, plasmids containing the different *pup1* deletion constructs were brought into cells containing a chromosomal disruption at the *PUP1* gene sequence. Only the strains possessing the *pup1-std* construct produced colonies on the 5-FOA plate, whereas the other constructs failed to confer growth on 5-FOA medium.
A colony growth comparison was done with the surviving strain *pup1Δ::HIS3 [p15-pup1-std]* using *pup1Δ::HIS33 [p16-PUP1]* and *PUP1* wild type strains as controls (fig.3-22).

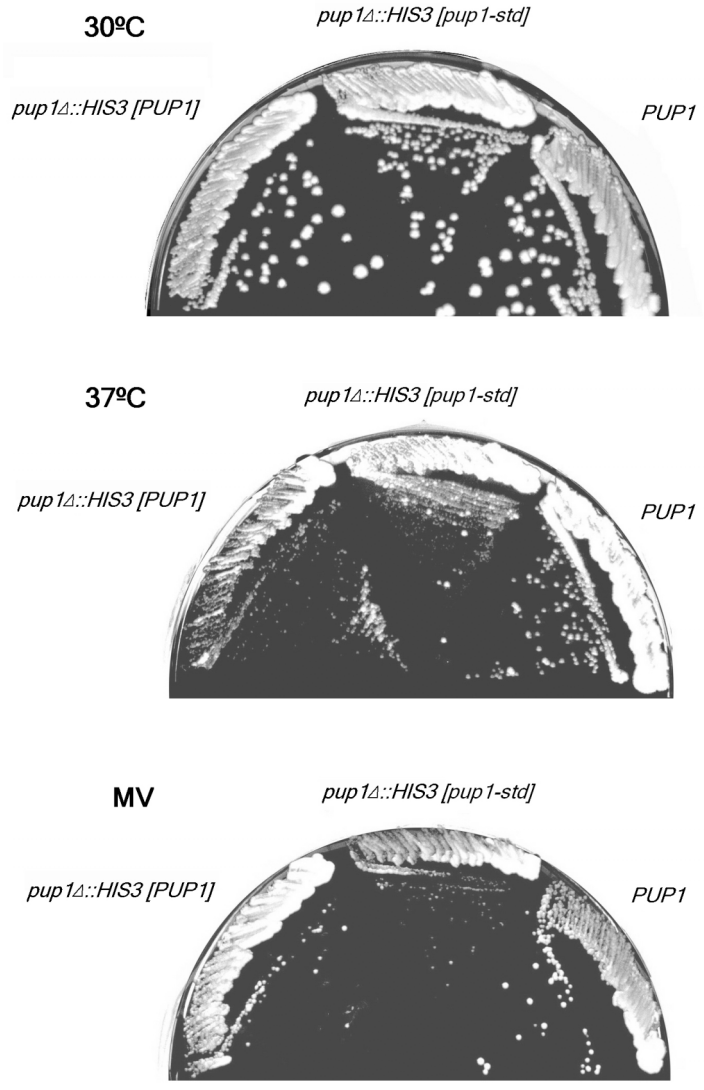


Figure 3-22 – Colony growth comparison of strains *pup1Δ::HIS3 [p15-pup1-std]* with controls *pup1Δ::HIS3 [p16-PUP1]* and WCG4 as wild type *PUP1*. Strains were streaked for single colonies on solid YPD medium and incubated at 30ºC and 37ºC or MV medium at 30ºC for about two days.

Clearly, at 30ºC a growth defect of the *pup1-std* mutant strain is seen when compared to the *PUP1* controls. At a higher temperature however (37ºC) there is slower growth of *pup1Δ::HIS3* strains expressing either *pup1-std* or *PUP1*, which is most probably provoked by the chromosomal disruption of the *PUP1* gene. On MV medium the

pup1Δ::HIS3 [p15-pup1-std] mutant strain again shows a strong growth defect when compared with both controls.

The removal of 25 or more amino acids from the elongation of β2/Pup1 is lethal and strains with 20 amino-acids removed show a strong phenotype.
The major part of the β2/Pup1 C-terminal extension is essential for the yeast cell viability, and could be required for the assembly or functionality of proteasomes.

3.2.3. Attempts to tag β2/Pup1 (internal HA, C-terminal FLAG)

The next step was to study whether the proteasome assembly is affected by the β2/Pup1 C-terminal truncations. Due to the absence of a good specific antibody against this proteasome subunit, tagging of the wild type β2/Pup1 subunit and the three truncated versions was planned. The middle and long truncations could then be over-expressed in strains containing a functional β2/Pup1. As these mutations are lethal to the cells, their over-expression in the presence of normal β2/Pup1 will permit to see if the truncated Pup1 subunits are found in mature proteasomes or in early assembled intermediates. The former result would largely exclude that the lethality is due to failure of proteasome assembly and support some unknown anomaly after assembly.

Using an existing plasmid, YCplac22HA-PUP1, containing an allele with the tag inserted into a region coding for an internal loop of Pup1 (provided by Hochstrasser, M. [Arendt and Hochstrasser, 1997]) the HA sequence was isolated as part of a restriction fragment and introduced into the plasmids expressing the Pup1 C-terminal truncations. The CEN plasmids p15-PUP1-HA, p15-pup1-std-HA, p15-pup1-mtd-HA and p15-pup1-ltd-HA were created this way.
The plasmids were introduced into yeast: p15-PUP1-HA and p15-pup1-std-HA into both *pup1Δ::HIS3* and *PUP1* strains, while p15-pup1-mtd-HA and p15-pup1-ltd-HA were introduced into *PUP1* wild type only. As these two last mutations cause lethality, they can not be used in a strain chromosomally disrupted for *PUP1*.
The fragments containing HA-tagged *PUP1* wild type and mutant sequences were then isolated from the previously made CEN plasmids and incorporated into a high copy plasmid, pRS425. The over-expression plasmids p25-pup1-std-HA, p25-pup1-mtd-HA, p25-pup1-ltd-HA, and p25-PUP1-HA were now available (see methods). The over-expression plasmids were brought into wild type strains with the *PUP1* sequence intact in the chromosome.
Strains bearing the plasmids mentioned before were grown at 30º and 37ºC (fig.3-23).

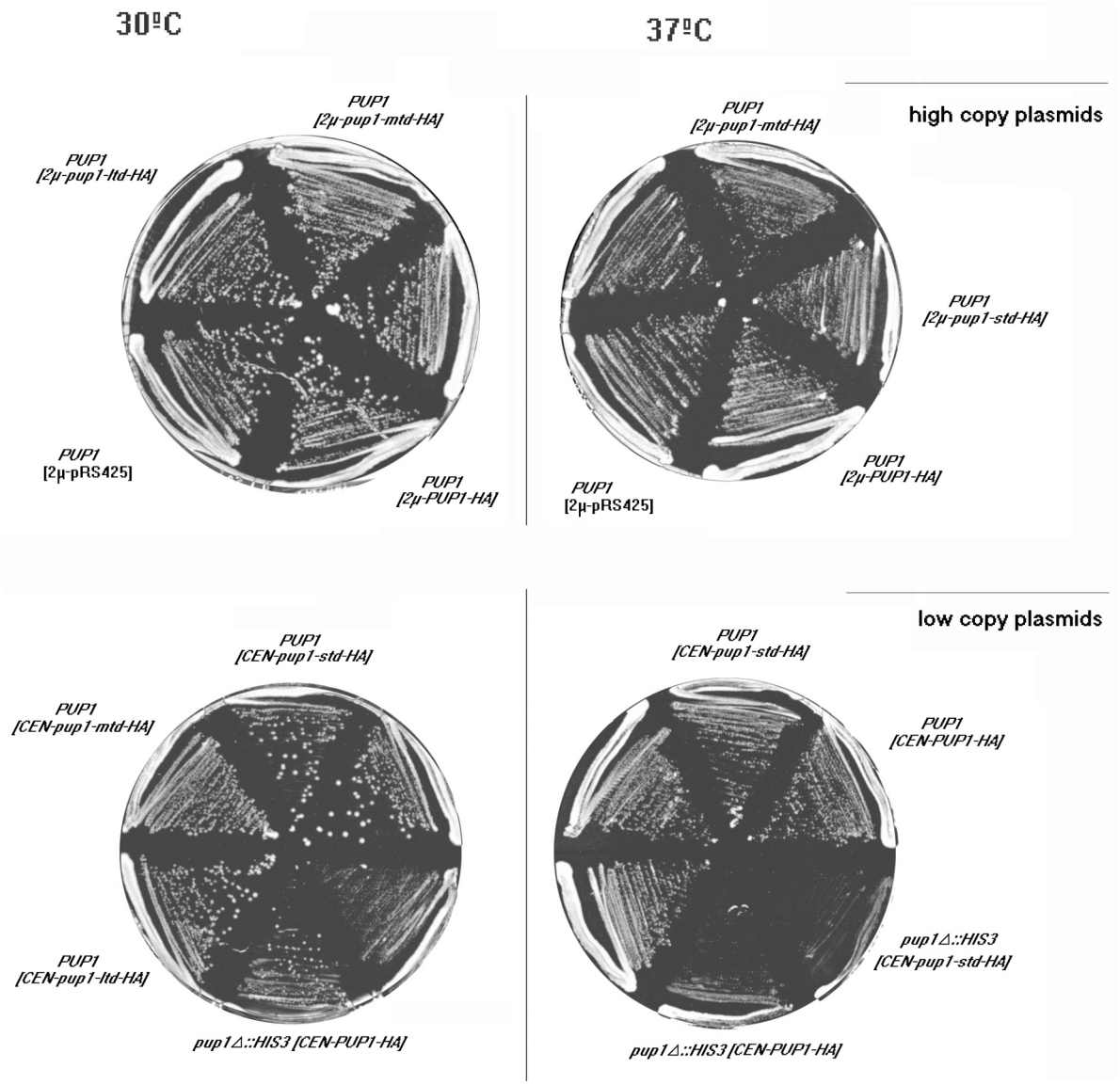


Figure 3-23 – Colony growth comparison of yeast strains harbouring the indicated gene constructs on plasmid (in brackets CEN indicates pRS315 and 2μ is pRS425). Strains were streaked for single colonies on solid YPD medium and incubated at 30ºC and 37ºC for about two days. All plates have the same strains in equal orientation. All over-expression plasmids are in strains with WT background and an empty pRS425 plasmid is used as control.

Strains having the *PUP1* sequence intact in the chromosome did not show any significant phenotype in the presence of the over-expression plasmids with the lethal *pup1* alleles encoding the middle and long tail deletions. The *pup1Δ::HIS3 [p15-pup1-std-HA]* strain has the same phenotype observed previously with the untagged *pup1Δ::HIS3 [p15-pup1-std]*, smaller colonies at both 30º and 37ºC.

The absence of any phenotype in the strains with over-expressed β2/Pup1 subunits having C-terminal truncations indicates that these subunits might either not be integrated into proteasomes at all, or that enough wild type Pup1 containing

proteasomes are assembled despite the occurrence of non-productive mutant Pup1 containing assembly intermediates.

By the means of a Western Blot it is possible to analyse to what extent the over-expressed truncated β2/Pup1 subunits can be integrated into 20S proteasomes. The β2/Pup1 subunits have a propeptide which is cleaved off upon assembly in order to become proteolytically active. According to this, it is probable that those subunits having their propeptide intact were not integrated into proteasome intermediates at all or are part of immature, non-functional proteasome species.

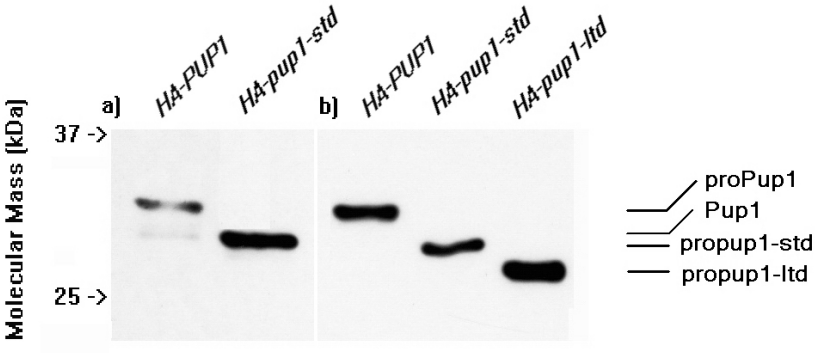


Figure 3-24 – Western Blot with HA tagged Pup1 mutant strains and controls. All *pup1* mutations are expressed from plasmids. a) *pup1Δ::HIS3 [CEN-PUP1-HA]* and *pup1Δ::HIS3 [CEN-pup1-std-HA]*. b) *[2μ-PUP1-HA]*, *[2μ-pup1-std-HA]*, *[2μ-pup1-ltd-HA]* over-expression plasmids, with chromosomal *PUP1* intact. (Pup1-HA ~28,2kD)

Surprisingly, the control strain *pup1Δ::HIS3 [CEN-HA-PUP1]* (lane 1), shows only a small portion of processed Pup1 subunits. In the strain *pup1Δ::HIS3 [CEN-HA-pup1-std]* (lane 2) no band corresponding to the processed subunit is visible and the band of the unprocessed form is much stronger then the one of the control strain (fig.3-24a). Comparison of these two strains indicates that the short C-terminal deletion might inhibit the propeptide cleavage.

When using over-expression plasmids in strains with intact chromosomal *PUP1,* the mature forms of the HA tagged Pup1 subunits are not visible in the Western Blot (fig.3-24b). There is the possibility that none of the over-expressed HA tagged subunits is assembled in proteasomes.

From the observed results it could be predicted that the β2/Pup1 C-terminal extension is required for a proper integration of this subunit into the proteasome. The band corresponding to the processed HA-PUP1 is very weak, what lead us to do some trypsine-like activity tests in order to verify the Pup1 propeptide processing. However, the activity tests performed with the *pup1Δ::HIS3 [CEN-HA-PUP1]* and *pup1Δ::HIS3 [CEN-HA-pup1-std]* strains revealed a complete absence of trypsin-like activity (data not shown). This

means that the internal HA tag is not suitable for this study as it compromises the proteolytic activity of the β2/Pup1 subunit and possibly its propeptide processing as well. Unfortunately this fact was not noticed in the studies of Arendt et al., [Arendt and Hochstrasser, 1997] using this kind of internal HA-tag. Therefore, another approach was considered. The consequent step was the tagging of the β2/Pup1 subunit with a FLAG epitope.

Unlike the HA tag, the FLAG tag was introduced after the last C-terminal amino-acid of β2/Pup1. This was done in plasmids containing a wild type *PUP1*, the short tail deletion allele *pup1-std*, and the long tail deletion allele *pup1-ltd*.
A CEN plasmid was used for the *PUP1-FLAG* and *pup1-std-FLAG* constructs. They were then introduced into strains with *PUP1* disrupted on the chromosome. As the long tail deletion was lethal, it's *FLAG* tagged sequence was introduced into a high copy plasmid, and this introduced into a wild type strain. The same was done with the *PUP1-FLAG* as a control.

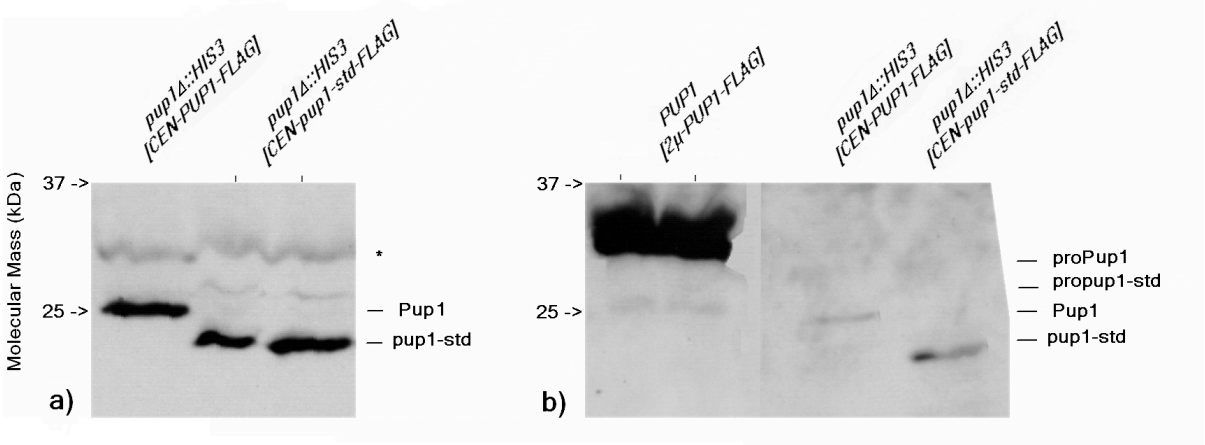


Figure 3-25 – Western Blot with FLAG tagged β2/Pup1 short tail deleted mutant strain, and controls. CEN stands for the centromeric plasmid pRS315 and 2μ for the over-expression plasmid pRS425. a) Short tail deletion FLAG tagged strain *pup1Δ::HIS3 [p15-pup1-std-FLAG]* with chromosomal disruption and *pup1Δ::HIS3 [p15-PUP1-FLAG]* as control. b) Right part, same strains as in (a), left part, *PUP1* wild type with p25-PUP1-FLAG over-expression plasmid. Labels to the right: Pup1 and pup1-std correspond to the mature subunit forms with propeptide removed; proPup1 and propup1-std are the correspondent precursor subunits; * - cross reactions.
The expected sizes for FLAG-proPUP1 and FLAG-PUP1 are 28kDa and 25kDa, respectively, while the expected sizes for FLAG-propup1-std and FLAG-pup1std are 26.8kDa and 23.8kDa, respectively.

In the case of the *pup1Δ::HIS* strains the tagged β2/Pup1 subunits are well assembled into the proteasome. It can be seen that all of the Pup1 subunits are processed and therefore must be part of mature proteasomes (fig.3-25a). It is interesting to note the difference between these and the correspondent HA tagged strains. It confirms that the used HA tag interferes with the maturation of the Pup1 subunits.

Comparing with the over-expressed PUP1-FLAG (fig.3-25b), the ratios between processed and unprocessed β2/Pup1 subunits are completely different. While most of the over-expressed subunits remain in the precursor form, only a small portion of the synthesised Pup1-FLAG subunits are integrated into proteasomes and have their propeptide processed. This could mean that the untagged subunits are preferred for integration into proteasomes, or just that the FLAG tagged subunits are expressed in such large amounts that only a few percent are used for proteasome biogenesis.

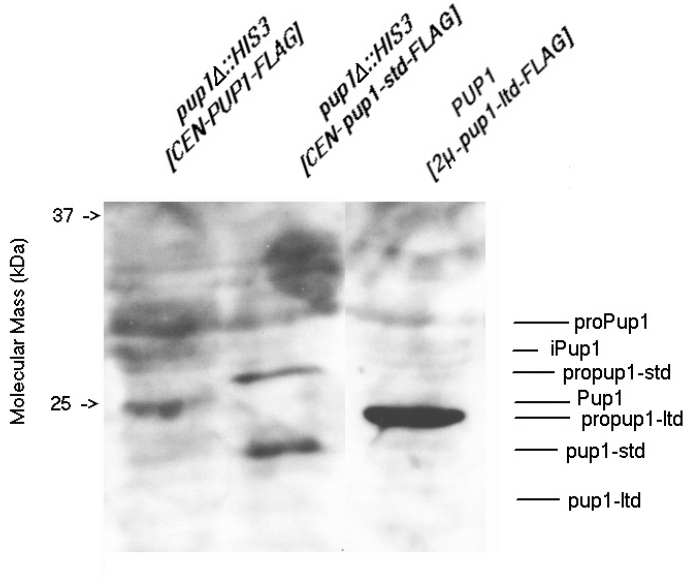


Figure 3-26 – Western Blot analysis of the strain *pup1Δ::HIS3 [p25-pup1-ltd-FLAG]* with the long tail deletion FLAG tagged Pup1 expressed from high-copy plasmid (right); *pup1Δ::HIS3 [p15-PUP1-FLAG]* (left) and *pup1Δ::HIS3 [p15-pup1-std-FLAG]* (middle) cell extracts are loaded as controls. (2μ is the over-expression plasmid pRS425 and CEN the low copy plasmid pRS315. iPup1 – intermediate forms of Pup1. No band in the expected size for pup1-ltd is visible. The expected sizes for FLAG-propup1-ltd and FLAG-pup1-ltd are 24.8kDa and 21.8kDa, respectively.

Just as with *PUP1-FLAG*, when over-expressed, C-terminally FLAG-tagged *pup1-ltd-FLAG* produces a strong Western Blot signal of its unprocessed form (fig.3-26). The mature form of Pup1-FLAG has an estimate molecular weight of 24.95kDa. By reducing the molecular weight of the last thirty seven amino acids in the Pup1 subunit, the estimate molecular weight for pup1-ltd-FLAG is 21.8kDa. Unlike for Pup1-FLAG, no band for the processed form of the long tail deleted pup1-ltd-FLAG was visible, even after longer exposure of the blot. The removal of amino-acids from the β2/Pup1 subunit C-terminal extension apparently makes it difficult for this subunit to integrate into the proteasome.
Conclusively the Pup1 C-terminus is required for the Pup1 assembly into the proteasome. This means that this elongation is recognised or recognises the neighbour subunits during assembly. Its main function should be the earlier assembly of proteasome

subunits by the proper allocation of β2/Pup1 and surrounding β-subunits on the α ring of the proteasome.

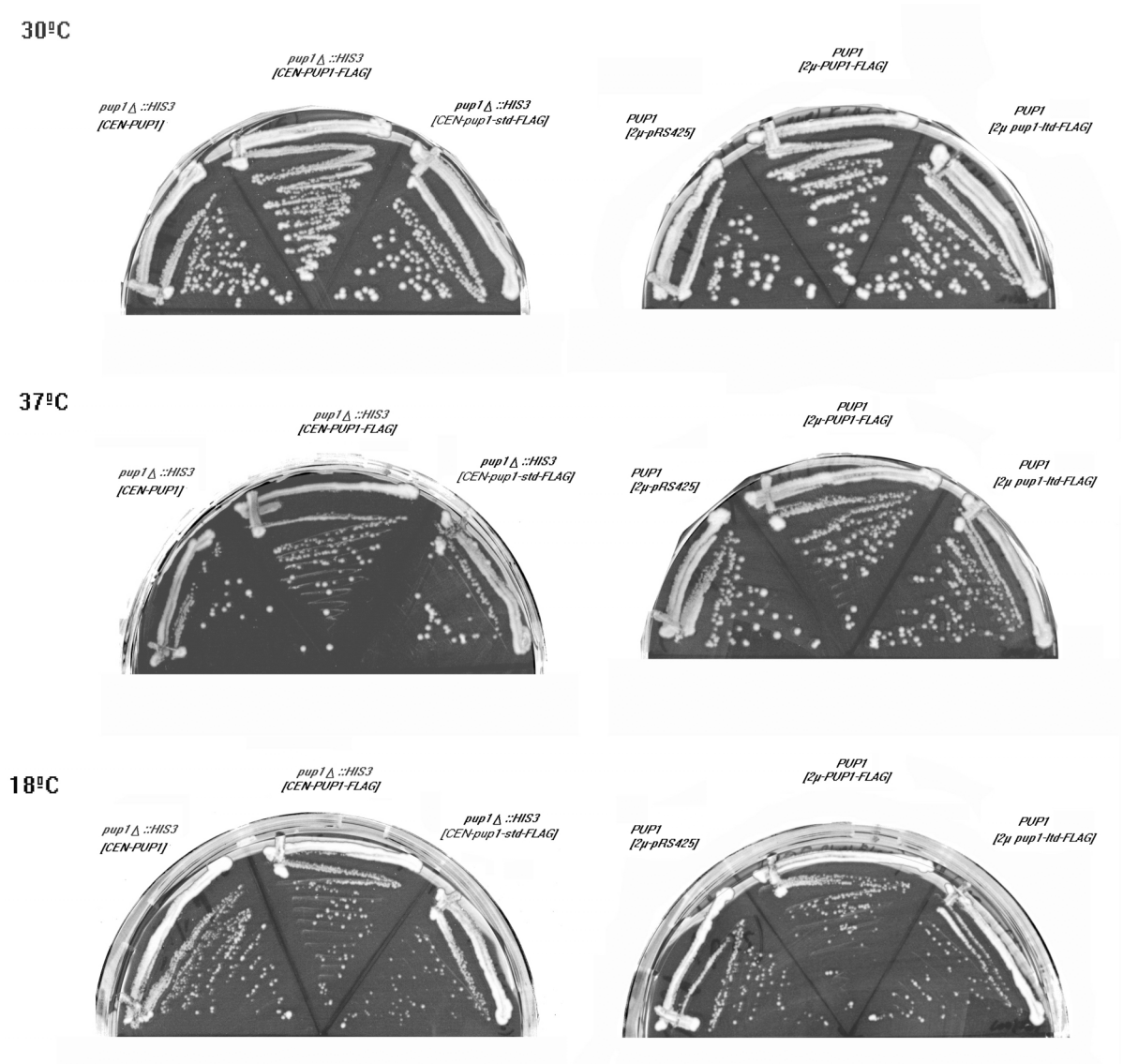


Figure 3-27 – Colony growth comparison of the FLAG tagged yeast strains and controls. Strains were streaked for single colonies on solid YPD medium and incubated at 30ºC and 37ºC for about two days and at 18ºC for about six days. All plates have the same strains in equal orientation. (2μ is the over-expression plasmid pRS425 and CEN the low copy plasmid pRS315. All over-expression plasmids are in strains with WT background.

A colony growth comparison between the existent Pup1-FLAG expressing strains was performed. Evaluating the growth of the FLAG tagged mutant strains at different temperatures no strong phenotype was found. All strains have a growth similar to their controls (fig.3-27). The long tail deletion mutation shows lethality in strains with *PUP1* disrupted on the chromosome, and some growth defect would be expected if a significant

amount of the mutant Pup1-ltd subunits would compete with wild type Pup1 for integration into proteasomes.
An interesting and surprising result is that the strain with the FLAG tagged short tail deletion Pup1 construct does not show any strong phenotype either. Before the FLAG sequence was added, this same mutant strain showed a strong growth phenotype (fig.3-23). This phenotype obviously is suppressed by the addition of the 7 amino-acid FLAG sequence. It looks like that the FLAG tag replaces the function of the removed amino acids from the Pup1 C-terminus.
The next action would be to follow the assembly of the different truncated Pup1 into proteasome complexes. This would be done with fractionation of cell extracts by gel filtration and immunoblotting of the fractions. Comparing the extracts from the different FLAG tagged mutant strains and wild type would allow conclusions about the stages of the proteasome assembly where the Pup1 C-terminal extension is required.

At this step, the project was cancelled. At first because the FLAG epitope replaces the function of the removed C-terminus until some extent what could lead to inconclusive results. And second, but not less important, a similar work was published [Ramos et al., 2004] providing answers to some of the questions that were posed here.
In this publication, a Pup1 subunit truncated for 30 amino acids with HA tag on the C-terminus was compared with Pup1-HA. This was done analysing cell extracts by gel filtration and immunoblotting. It was concluded that while the truncated Pup1 was incorporated into proteasome precursor complexes the formation of complete proteasomes containing this subunit was very inefficient.

This is in accordance with the preliminary results obtained in this work, where in the presence of wild type Pup1, none of the pup1-ltd truncated subunits are found in the processed form.

4. Discussion

4.1. The active pockets of the proteasome and their substrate selectivity

During evolution, the proteasome β-subunits developed from seven equal proteolytically active into the seven distinct subunits where only three are active but have distinct substrate selectivity: the β1/Pre3 for post-glutamyl hydrolysing (PGPH or caspase-like) activity, the β2/Pup1 for trypsin-like and the β5/Pre2 for chymotrypsin-like activity [Heinemeyer et al., 1997].
In the active β-subunits some highly conserved residues, Asp17 (Glu17 in the *Thermoplasma* β-subunit), Lys33, Ser129, Asp166 and Ser 169, surround the N-terminal nucleophile Thr1 [Heinemeyer et al., 2004] and are essential for proteolytic activity.

Some of the residues which compose the active pocket of the active β-subunits and come in contact with substrates during proteolysis differ among the three subunits. It is predicted that they are extremely important for the different substrate selectivities.
In previous crystallographic studies [Groll et al., 1997], by using a calpain inhibitor binding to yeast proteasome active subunits, the amino acids forming the S1 pocket were identified. Besides the Thr1 and Lys33, the amino acids that were in contact with the inhibitor's P1 residue are in the positions 20, 31, 35, 45, 49 and 53.
Among the three different yeast active subunits, Ala49 is conserved and Gln53 is found in Pre2 and Pre3, whereas Pup1 has Glu53. In the other positions, 20, 31, 35 and 45 each subunit shows a different residue. These residues were mutated in a few different combinations and their importance for the substrate selectivity was analysed.

By interchanging the amino acid residues at position 45 in β5/Pre2 and β1/Pre3 significant changes occurred. Using the standard specific peptide substrates it was stated that the β5/Pre2 subunit lost significant levels of its chymotrypsin-like activity, while on the other hand it gained some facility to cleave the substrate specific for PGPH activity, Suc-LLE-βNA (fig.3-02). Most significantly, when the Arg45 was replaced by a methionine in β1/Pre3, all the peptidase capacity of this β-subunit was lost (fig.3-05).
The residues Met45 and Arg45 are not directly involved in the processes of autolysis or proteolysis. Nonetheless, substitution of Met45 by arginine in β5/Pre2 breeds alterations in substrate selectivity. Why the mutation of Arg45 into Met45 in β1/Pre3 had the mentioned result can not be explained by substrate selectivity alone.

4.2. The β5/Pre2 active pocket

Similar to the archaeal active subunits, β5/Pre2 cleaves peptide substrates after hydrophobic residues and it is the most important active subunit in the eukaryotic proteasome. Removal of its propeptide is lethal and inactivation of its proteolytic function leads to significant growth defects in yeast [Jäger et al., 1999].

The active pocket of the β5/Pre2 subunit has a large size when compared with the one at β1/Pre3. By replacing Met45 with an arginine, having a larger side chain, no significant structural changes are expected. The Pre2 propeptide is properly cleaved (fig.3-18a) and the subunit is proteolytically active, but its specificity is altered (fig.3-02). There is a decrease in the chymotrypsin-like activity while some PGPH activity is noticed.
Unlike Pre2, a Pre2-M45R mutant subunit was not sufficient for viability in the presence of the *pup1-T1A* mutation and therefore had its position diminished in the established hierarchy of the active β subunits (chapter 3.1.2.).
The other S1 pocket residues involved in substrate selectivity, the residues at positions 20, 31 and 35 were additionally replaced by those present in the β1/Pre3 S1 pocket.
After replacing Ala20 and Val31 in addition to the M45R mutation, both or either of them by a threonine, the proteolytic ability to cleave the used chromogenic substrates decreases dramatically, even when compared with the single mutant strain *pre2-M45R* (fig.3-11). Both the remaining chymotrypsin-like specificity and the novel PGPH activities of Pre2-M45R are then lost. Nevertheless there are no changes in the autocatalytic processing (fig.3-18a) and it is still possible that the mutated subunit has some proteolytic activity not measurable by the used substrates.
Structural changes in the subunit's active pocket might here be responsible for the diminishment of activity. After the PGPH activity increase resulting from the M45R exchange, additional A20T and V31T mutations were expected to result in enhanced binding of substrates known to be cleaved by β1/Pre3. As it came out that instead the PGPH activity was lost, it can be concluded that structural rearrangements in the active pocket took place which are responsible for activity loss.

In addition to the M45R point mutation, the replacement of Ile35 by a threonine originated a very interesting result. When substrates specific for chymotrypsin-like activity were used, a substantial increase in activity that surpassed that of wild type strains was observed. There was no gain of PGPH activity, similar to Arg20Thr and Val31Thr replacements.
Interestingly, the chymotrypsin-like proteolytic activity was near 200% of the wild type control with one of the substrates, while it was only around 50% with the other substrate specific for chymotrypsin-like activity (fig.3-09). The substrates for chymotrypsin-like

activity used in this study are Cbz-GGL-pNAn and Suc-LLVY-AMC. The first one has much smaller residues and is preferentially cleaved by the *pre2-M45R-I35T* mutant proteasomes.
This implicates that the size of the residues of the used substrates has a strong influence on the proteolytic activity observed with the created mutant strains. With the point mutations M45R and I35T, a conformational alteration of the size of the β5/Pre2 pocket is expected to have occurred.

In the active pocket of Pre2-M45R-I35T, there is the strong possibility that the hydroxyl group of threonine at position 35 stabilises the amino group of arginine at position 45 thanks to their spatial orientation. This stabilisation might lead to a strong preference for small hydrophobic substrates.
This does not happen neither with A20T or V31T. When the additional A20T V31T mutations are combined with I35T, no chymotrypsin-like activity is observed any more, suggesting that Arg20 and Val31 in Pre2 are both required for a proper structural arrangement which gives advantage in substrate selection.
It is concluded that, during the evolutionary process, not only the active site pocket amino acids in contact with the substrates were optimised for the different achieved specificities, but those surrounding them also had to adapt. The changed amino acids belong to the β-strands S2, S3 and S4 (fig.1-03; fig.1-08). The Arg20 is found in the S2, Val31 and Ile35 in the S3 and Met45 in the S4 β-strands of the β5/Pre2 subunit.
It is likely that the interactions between the β-strands which are in direct contact with each other were compromised with the imposed mutations. One can not simply change the key amino-acids responsible for the substrate selectivity without caring for their surroundings.
With this study it was proven that different subunits can achieve different activities. Nevertheless, a complete swap of specificities can only be achieved by taking in consideration the interactions between the amino acids responsible for specificity, and those that surround and contact them. Entire active pockets swapping was tried between Pre2, Pup1 and Pre3 (data not shown) but was lethal to the cells.

A subunit is defined by both its surface, which allows its correct placement into the proteasome and is mainly composed of α-helices, plus the active pocket responsible for the activity and specificity, whose amino acids are found in β-strands. Even though the imposed mutations in the active pocket do not seem to alter the subunit surface, they are likely to affect the conformation of the active pocket altering its size and/or the orientation of the amino acids responsible for the catalytic mechanism.

4.3. The β1/Pre3 active pocket

After replacing the single amino acid arginine 45 by a methionine, the β1/Pre3 subunit lost any detectable peptidase activity (chapter 3.1.3.). Attempts to reactivate it by stabilising the residue 45 with strategic point mutations of residue 52 were done, but nevertheless the subunit remained inactive (chapter 3.1.5.).
When additional mutations of the other S1 pocket residues were performed trying to correct any structural conflict caused by the arginine 45 replacement, the β1/Pre3 subunit failed to auto-catalytically process its own propeptide (chapter 3.1.7.2.) which is a prerequisite for proteolytic activity.

The question now was whether the deficiency of activity in this mutant was due to a defect in β1/Pre3 propeptide processing alone.
Strains expressing the mature form of β1/Pre3 were used. The β1/Pre3 propeptide is dispensable for proteasome assembly *in vivo*, but its deletion inactivates the subunit since its unprotected N-terminus is subject to N^{α}-acetylation [Arendt and Hochstrasser, 1999; Jäger et al., 1999]. Inactivation of the *NAT1* gene encoding a subunit of Nat1-Ard1 N^{α}-acetyltransferase, however, was shown to restore activity of the β1/Pre3 subunit [Arendt and Hochstrasser, 1999].
Strains lacking both the β1/Pre3 propeptide and N^{α}-acetyltransferase were additionally with the point mutations in the Pre3 S1 pocket (chapter 3.1.8.). The same combinations were applied. If the unprocessed propeptide was the only reason for inactivity, these strains should have a proteolytically active Pre3. Unfortunately, although these β1/Pre3 subunits do not require auto-catalytic cleavage of their propeptide, they were still unable to cleave any of the used substrates (fig.3-19).
The structural conformation of the subunit β1/Pre3 became optimized during evolution in such way, that single changes can interfere with its proteolytic function. Any alteration seems to alter the subunit's integrity, directly affecting its activity.

4.4. The influence of the reciprocal exchange of residues 45 of β5/Pre2 and β1/Pre3 on the active site hierarchy.

There is an established hierarchy of importance among the three active subunits for the cell growth. Limited by the absence of a library composed of a variety of similar peptide substrates for the different active sites and knowing that an active site might still have proteolytic activity even when it is not able to cleave the available substrates, undetected activity can be evidenced by alterations in the established hierarchy.

With the performed mutations, aiming at altering the specificity of either β5/Pre2 or β1/Pre3, the established hierarchy of the different active subunits in the yeast proteasome could be altered as well.

It is known that a functional subunit β5/Pre2 or β2/Pup1 alone is sufficient for cell viability [Heinemeyer et al., 1997]. The mutant strains *pre2-M45R* and *pre3-R45M* were crossed with each other generating the double mutant strain *pre2-M45R pre3-R45M*. In addition, crossings with strains having defective or inactivated active subunits were performed trying to get all the possible combinations. The mutant strains *pre2-2*, *pup1-T1A*, and *pre3-T1A* were used.

The combination of *pre2-M45R* with *pre3-T1A* or *pre3-R45M* alone resulted in viable strains. Double mutant strains of *pre3-R45M* with *pre2-2* or *pup1-T1A* are as well viable.

When combinations of *pup1-T1A* with the double mutations *pre3-R45M pre2-2* or *pre3-R45M pre2-M45R* were tried, it turned out that it was not possible to create viable triple mutants. The double mutant *pre2-M45R pup1-T1A* was also not viable. Unlike wild type Pre2, the Pre2-M45R mutant subunit alone is not sufficient for cell viability. All the viable strains either have β2/Pup1 or β5/Pre2 functional (fig.4-01).

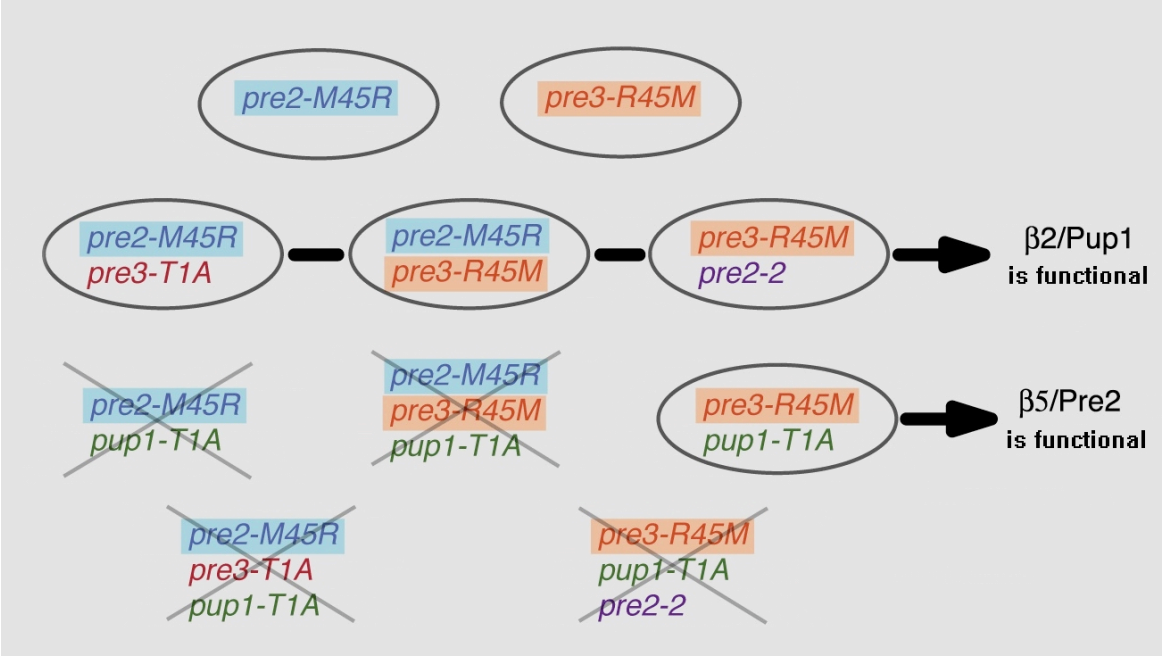


Figure 4-01 – Scheme showing the viable and no viable combinations between the pre2-M45R and the pre3-R45M mutants with the defective β5/Pre2, β1/Pre3 and β2/Pup1 strains.

Thus, the M45R mutation changed the established hierarchy of the active β subunits. On the other hand, the *pre3-R45M* mutation caused no improvement in the ranking of Pre3, and this subunit remained in the last position. If the β1/Pre3 specificity could be converted into a chymotrypsin-like activity, there would be the possibility that the combination with *pre2-2 pup1-T1A* is viable.

The Pre2-M45R subunit has 40% residual chymotrypsin-like activity and 30% novel PGPH activity when compared with the wild type Pre2 (fig.3-02). The double mutant strains with inactive Pre3 and Pup1, having only β5/Pre2 as a functional proteolytic active subunit are viable. When replacing Pre2 in these strains by Pre2-M45R it leads to lethality. Just like a *PRE2* wild type, the *pre2-M45R* mutant strain is able to auto-catalytically cleave off its own propeptide (chapter 3.1.8.) and the main difference between both is the specificity.

It might be concluded that the importance the active subunits have in the proteasome and the cell are defined by their different specificities. Thus, compared with the other active subunits, Pre2 has a bigger importance thanks to its chymotrypsin-like specificity.

Nevertheless it seems clear that the residual ~40% chymotrypsin-like activity in a *pre2-M45R* mutant was not enough for viability in combination with *pup1-T1A*. A greater ability to cleave chymotrypsin-like substrates should then be required for cell viability. It would be interesting to know what would be the minimum percentage of chymotrypsin-like activity necessary to avoid lethality.

4.5. Allosteric interactions, binding of substrates to non-catalytic sites and to unspecific active sites

When Kisselev et al., [Kisselev et al., 1999] first came with the hypothesis of allosteric regulation between active sites of the proteasome in a "bite-and-chew" model, there was much scepticism. In this model the acidic caspase substrates of Pre3 would allosterically inhibit the Pre2 chymotrypsin-like activity.
Based on this model, we tried in this work to swap activities between Pre2 and Pre3 and test if this allosteric regulation would still function if Pre2 was exhibiting PGPH and Pre3 chymotrypsin-like activity.

This model was later challenged by Schmidtke [Schmidtke et al., 2000] who postulated that control of the proteasomal chymotrypsin-like and PGPH activities is exerted through the binding of substrates to non-catalytic modifier sites. According to their findings, binding of the acidic substrates to these non-catalytic sites not only inhibits chymotrypsin-like activity, but also stimulates trypsin-like activity. This suggests that both acidic and basic substrates compete to bind into non-catalytic sites, but only binding of the acidic substrates would provoke inhibition of chymotrypsin-like activity.
When challenging the Kisselev "bite-and-chew" model, Myung [Myung et al., 2001] confirmed that specific substrates for PGPH activity inhibit the chymotrypsin-like activity

by binding to non-catalytic sites. It was observed that the specific substrates for PGPH activity do not require binding to or hydrolysis by the β1/Pre3 active site in order to produce this inhibition.

Conclusively in Kisselev's later works [Kisselev et al., 2002; Kisselev et al., 2003] it was found that the hydrophobic substrates bind to multiple non-catalytic sites activating caspase-like and trypsin-like cleavages. This is due to the inducing of the α channel opening in the 20S core particle, and does not occur in the 26S proteasome, suggesting that the binding sites for the hydrophobic substrates are located in the alpha subunit ring. Although this effect is not noticed with proteasomes containing a 19S regulator and/or the PA28 complex whose attachment to the 20S complex opens this channel in an ATP-independent manner, it might be a primitive mechanism which helps to release the cleaved products from the inside of the proteasome, avoiding their accumulation.
New high specific caspase inhibitors [Kisselev et al., 2003] enhanced trypsin-like activity 3 fold. In accordance to Schmidtke's work [Schmidtke et al., 2000] these inhibitors seem to bind also to the same non-catalytic sites where the acidic (caspase-like) and basic (trypsin-like) substrates compete for. While the acidic substrates have the effect of inhibiting chymotrypsin-like activity when binding to these non-catalytic sites, the caspase inhibitors do not affect the chymotrypsin-like sites.

The non-catalytic sites where hydrophobic substrates of the Pre2 subunit bind to in the alpha ring are unlikely to be the same where the acidic and the basic substrates of the Pre3 and Pup1 subunits, respectively, bind to in a competitive way.
Although it seems excluded that an allosteric regulation between Pre3 and Pre2 occurs, there are some interesting interactions though. Even not fulfilling one of its initial purposes, the swapping of activities between Pre2 and Pre3 subunits can still serve to substantiate these interactions. According to Myung et al. [Myung et al., 2001], acidic substrates inhibit the chymotrypsin-like activity by binding to non-catalytic sites. With their activities swapped, it would be interesting to analyse if the acidic substrates were still inhibiting the chymotrypsin-like activity expressed now by the subunit β1/Pre3 or if the β5/Pre2 was still inhibited although expressing PGPH activity. In Pre2, by the single M45R point mutation a partial specificity swap was indeed observed (chapter 3.1.1.).

New specific substrates were also developed by Kisselev and co-workers [Kisselev et al., 2003] for the caspase-like sites, Ac-Nle-Pro-Nle-Asp-AMC and Ac-Gly-Pro-Leu-Asp-AMC. These substrates also have the advantage of being cleaved exclusively at the Asp-AMC bond unlike the used substrate Suc-Leu-Leu-Glu-βNA which is also cleaved at the Leu-Glu bond [Kisselev et al., 1999]. They could be very helpful in future studies involving the specificities of the proteasome active sites.

4.6. The function of the β2/Pup1 C-terminal elongation

From all the different β-subunits, the subunit β2/Pup1 has the longest C-terminal extension with over 30 amino acids. Analysis of the crystal structure of the yeast proteasome (fig.3-19) revealed that the β2/Pup1 extension wraps around the β3/Pup3 subunit within the same ring of β-subunits. To study closely the function and roll of this specific extension, different approaches were planned.

In one approach, individual point mutations were introduced in the C-terminal extension. The amino acids Tyr204 and Thr211 were replaced by an alanine, and Arg208 was replaced by a glycine. These residues were in direct contact with the neighbour subunits or exposed to the outside of the proteasome. If these residues were important individually, the mutant strains would show a cell growth defect, but no phenotype was found for any of the three single point mutations (fig.3-20).

In another approach, different deletions of the β2/Pup1 C-terminal extension were performed (chapter 3.2.2.). With the cleavage of 20 of the C-terminal amino acids, a growth phenotype was observed at 30ºC (fig.3-22). The removal of 37 or 25 amino acids from this elongation was lethal. By over-expressing the lethal mutations in wild type strains, the mutated β2 subunits did not seem to integrate into proteasomes (fig.3-26).

To confirm this last scenario, a gel filtration and fractionation of cell extracts from both truncated and wild type FLAG tagged Pup1 was planned. It was initially planned for the strains with an internally HA tagged Pup1 which unfortunately were found not to have trypsin-like activity. This side effect was never noticed in Hochstrasser's work [Arendt and Hochstrasser, 1997]. Any alteration near the active pockets seems to be critical for the subunit activity.

By comparing the different gel filtration fractions of the cell extracts by immunoblotting it could be possible to identify at what steps of proteasome assembly the truncated Pup1 subunits are found. In addition or alternatively, a native gel analysis with crude extracts would also provide information on the relative amounts of proteasome complexes containing truncated Pup1 subunits.

Before this took place, a work of Dohmen and Ramos [Ramos et al., 2004] was published, where a similar strategy was used. Instead of over-expressing a Pup1 without the last 30 amino acids, they used a heterozygous diploid strain expressing both Pup1 wild type and a truncated Pup1, epitope-tagged with HA at its C-terminus (Pup1-Δ30C-ha).

To follow the fate of the tagged truncated Pup1 subunits, an extract from this strain was analysed by gel filtration and immunoblotting and compared with that of a wild type strain expressing Pup1-HA.

Only minute quantities of processed truncated Pup1 subunits were found to be present in fractions that typically contain 26S proteasomes. That is in accordance with our results, where no mature form of the Pup1-ltd-FLAG subunit could be found (fig.3-26).
Moreover, with the gel fractionation and native gel they verified that the truncated Pup1 accumulate mainly in the form of free subunits and in half proteasomes. These results significantly support a great importance of the Pup1 C-terminal extension for the assembly of proteasome intermediates and mature proteasomes.
When compared to wild type, the accumulation of free truncated Pup1 subunits also indicates an early requirement of the C-terminal extension for immediate association with neighbour subunits. The absence of intermediate subunit aggregates containing truncated Pup1 subunits leads to conclude that without its C-terminal extension, the Pup1 subunit has tremendous difficulties recognising its neighbour subunits. Even though the mutated subunits are incorporated into proteasomes, these are not functional.

Proteasome intermediates of 13S in mammals [Nandi et al., 1997; Schmidtke et al., 1997] and 15S in yeast [Li et al., 2007] consist of the α ring plus β2, β3, and β4. Of all the subunits β2/Pup1 buries the largest surface area against the α-ring and it has the largest *cis*-β contact primarily due to its long C-terminal extension [Li et al., 2007]. It is suggested that β2 is one of the first β subunits to assemble onto the α-ring and the Pup1 C-terminus seems to be essential for proteasome formation, being required for proper positioning of β2/Pup1 and its surrounding neighbour subunits in a very early stage of proteasome assembly.
With the results obtained in this work and in addition the work of others [Ramos et al., 2004], it can be concluded that rather then the individual amino acids composing it, it is the tail shape and length that determines the function of the β2/Pup1 C-terminal elongation for proteasome assembly.

When single amino acids were changed in β2/Pup1 subunit C-terminus no phenotype was observed and the short tail deletion mutant loses its growth phenotype with the addition of a FLAG tag, which is strange to the proteasome, to the C-terminus (fig.3-27). Although the amino acids of the Pup1 C-terminus do not seem to individually have a strong role in the function of this extension, it is possible that when a few are simultaneously mutated, a growth phenotype is then observed. On the other hand it is important to understand why the FLAG tag suppressed the phenotype seen in the case of the untagged *pup1Δ::HIS3 [p15-pup1-std]* strain. Between the *FLAG* and the missing sequence in the Pup1-std subunit, there are no similarities and therefore the size of the FLAG is what seems to be responsible for suppressing the growth phenotype of Pup1-std. To continue this work a possible strategy, besides the combination of single mutations, would be the verification of the C-terminal FLAG-tag effect by adding other "strange" sequences to the Pup1-std mutant.

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