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**INVESTIGATING THE ROLE OF A YEAST  
MEMBRANE PROTEIN, HSP30 IN TOLERANCE TO  
ETHANOL STRESS**

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the requirements for the degree of Master  
of Science

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# Abstract

One of the contributors to the widespread interest the yeast *Saccharomyces cerevisiae* has received is its ability to yield and tolerate high levels of ethanol. *S. cerevisiae* is able to grow and remain viable in growth media containing ethanol concentrations as high as 19.8% (w/v), a level that is toxic to many other microorganisms. Since production of ethanol is a normal event in the growth cycle of *S. cerevisiae*, this organism has evolved a number of mechanisms to cope with deleterious effects of ethanol. These include induction of heat shock proteins (HSPs). Among these, HSP30 is particularly interesting in that it is the only stress-induced protein known to be intrinsically bound to the yeast plasma membrane. Another ethanol induced HSP; HSP12 has previously been shown to have a peripheral plasma membrane localisation. It has further been shown that HSP12 protects liposomes against damage by ethanol. This study was initially aimed at investigating whether there is co-operation between HSP30 and HSP12 in this membrane protection role.

HSP30 was over-expressed in *E.coli* and *S.cerevisiae* in preparation for liposome studies and production of anti-HSP30 antibody. Both approaches yielded expression levels that were not sufficient to warrant further purification. The cause for this low expression level is discussed. Lack of expression stalled the experimental investigation of the involvement of HSP30 in membrane stabilisation. Other approaches for increasing expression level were not pursued as other work discussed here indicated that this role is unlikely. A peptide selected from HSP30 was synthesised using solid phase Fmoc-amino-acid technology and conjugated to protein carriers to produce antibodies that should cross-react with HSP30.

This study further aimed to investigate whether HSP30 provides yeast cells with a growth advantage in the absence and presence of ethanol stress. A yeast strain that constitutively expresses HSP30 protein was constructed. Assessment of growth of this strain indicated that expression of HSP30 in the absence of ethanol stress is detrimental to yeast growth. Comparison of growth of a wild type strain of yeast with a HSP30 disrupted strain (HSP30 knock-out) indicated that HSP30 provides a growth advantage to yeast in the presence of ethanol stress. The data produced are consistent with other work that indicated that HSP30 is a regulator of the plasma membrane proton ATPase of yeast.

A model of the HSP30 protein was created using current bioinformatics approaches, and revealed several remarkable features. Among these features, the putative intracellular C-terminal domain was selected for further investigation. A yeast strain expressing HSP30 protein lacking this domain was constructed. It is found that the C-terminal domain is essential for proper function and/or localisation of HSP30. Constructs for expression of site-directed mutants of HSP30 lacking putative sites of phosphorylation were made for future study.

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## Abbreviations

HSP	heat shock protein
w/v	weight per volume
ROS	reactive oxygen species
v/v	volume per volume
mg	milli gram
l	litre
°C	degrees Celsius
OD	optical density
ml	milli litre
g	gravity
s	seconds
mM	milli molar
PMSF	phenylmethanesulfonylchloride
rpm	revolutions per minute
min	minutes
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Tris	TRIS-(hydroxymethyl) aminomethane
GST	glutathione-S-transferase
IPTG	isopropyl thiopyranoside
PCR	polymerase chain reaction
µl	micro litres
dNTP	deoxynucleotide triphosphate
PGK	phosphoglycerate kinase
NaCl	sodium chloride
µl	micro litre
NaOH	sodium hydroxide
M	molar
vol.	volumes
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetra-acetic acid
Mg <sub>2</sub> Cl	magnesium chloride

μM	micro molar
pmol	pico moles
μg	micro grams
μl	micro litres
TAE	tris-actate EDTA
h	hours
SD	standard defined
ng	nano grams
Fmoc	9-fluorenylmethoxycarbonyl
DMF	dimethyl formamide
BOP	benzotriazolyl N-oxytrisdimethylaminophosphonium hexaflourophosphate
TFA	trifluoro acetic acid
MALDI-TOF	matrix assisted laser desorption ionisation - time of flight
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
PBS	phosphate buffered saline
BSA	bovine serum albumin
MBS	m-Maleimidobenzoyl-N-hydroxysuccinimide
ELISA	enzyme-linked immunosorbance assay
nm	nano meters
mA	milli amperes
pNPP	p-nitrophenylphosphate
NMR	nuclear magnetic resonance
MRNA	messenger ribonucleic acid

# Chapter one:

## Introduction

### 1.1 General introduction

The yeast *Saccharomyces cerevisiae* is well known for its high tolerance to environmental stress. Indeed, one of the contributors to the widespread interest this yeast has received is its ability to yield and tolerate high levels of ethanol in fermentation, an attribute of commercial interest. A further contributor to the widespread interest *S. cerevisiae* has received is that it is an extremely researcher-friendly eukaryotic organism. Genetic manipulation can be effected with relative ease and the organism serves well as a model for eukaryotic molecular behaviour. Many of the biochemical and molecular characteristics of *S. cerevisiae* have been found to apply in higher eukaryotes as divergent as *Arabidopsis* and human beings.

Elucidating the biochemical mechanisms of stress tolerance and adaptation in *S. cerevisiae* is therefore not only of importance to the brewing industry and other commercial beneficiaries of this organism, but also to increasing our understanding of mechanisms for stress tolerance and adaptation in eukaryotes as a whole.

### 1.2 Stress responses in yeast

Yeast cells are exposed to a number of different environmental stresses in their normal growth cycle and in industrial settings. While the number of stresses is large and the number of proteins induced by the stresses even larger, the number of transcriptional regulatory systems is low (Estruch, 2000). To date three distinct systems have been identified: the general stress response, the heat shock response and the oxidative stress response. These three basic stress responses in *S. cerevisiae* are briefly reviewed in this section.

### **1.2.1 The general stress response**

Yeast cells exposed to mild stress develop tolerance to higher doses of the same stress as well as stress resulting from other agents. This cross-protection phenomenon indicates that there exists an integrating mechanism that senses and responds to different forms of stress. This integrating mechanism is known as the general stress response. The various genes that are regulated in co-ordination through this general stress response have a common *cis* element, the stress response element (STRE), in their promoter. This element, identified earliest in the promoters of *CTT1* (Weiser *et al.*, 1991) and *DDR2* (Kobayashi and McEntee, 1990; 1993), has the core consensus AGGGG (Ruis and Schuller, 1995). The other orientation (CCCCT) is also functional.

Two *trans*-acting factors are involved in STRE-mediated gene expression (Martinez-Pastor *et al.*, 1996). These factors, Msn2p and Msn4p are zinc-finger proteins. Martinez-Pastor *et al.* (1996) showed that Msn2p and Msn4p recognise and bind the STRE element *in vitro* and *in vivo*. They further showed that these proteins are required for the induction of a *STRE-LEU2-lacZ* reporter gene in response to stresses such as heat-shock, low pH, sorbic acid and ethanol. It has further been shown that disruption of these genes (*msn2msn4* double mutant) results in higher sensitivity to different stresses, including carbon source starvation, heat shock, osmotic and oxidative stresses.

The general stress response is regulated by the cAMP-PKA signalling pathway (reviewed by Thevelien and de Winde, 1999). Many yeast genes are candidates for regulation through the general stress response pathway, based on the presence of STRE sequences in their promoter. Among them are genes involved in carbon metabolism, proteases, transporters, trehalose metabolism genes and stress protective genes HSP104 and CTT1 (Moskvina *et al.*, 1998).

### **1.2.2 The Heat Shock response**

When shifted to higher temperatures (or heat shocked) *S.cerevisiae* cells respond by the induction of synthesis of heat shock proteins (HSPs). The general stress response

(described above) is only partially responsible for this induction. The main regulatory element of the heat shock response is the heat shock element (HSE). HSEs are composed of at least three copies of the repeating sequence nGAAn (Sorger, 1991).

HSEs are binding sites for the heat shock factor (HSF also denoted Hsf1p). Hsf1p contains a helix-turn-helix DNA-binding domain (Harrison et al., 1994). The protein has two activation domains: the amino terminal activation domain, which mediates a transient response to heat shock and the carboxy terminal activation domain, which mediates both transient and sustained response to heat shock. (Sorger, 1990).

### **1.2.3 Oxidative stress response**

Various stress agents result in increased generation of reactive oxygen species (ROS) such as hydrogen peroxide and superoxide anions in aerobically grown yeast cells. Many proteins are induced in response to this oxidative stress (Gordon *et al.*, 1999) and the emerging conclusion is that while the nature of oxidative stress (i.e. the stress agent and nature of ROS produced) may differ, the response to the stress is mediated by an overlapping response pathway (Jamieson *et al.*, 1994). The main regulatory element of the oxidative stress response is the AP-1 response element, which has the core consensus sequence TGA CTCA (Harshmann *et al.*, 1988). The AP-1 response element is a binding site for a number of similar oxidative stress-responsive transcription factors, with Yap1p being the best described. Yap1p belongs to a family of b-ZIP transcription factors and its gene was originally cloned by its ability to bind an SV40-derived AP-1 site (Harshmann *et al.*, 1988).

*yap1* mutants show an increased sensitivity to H<sub>2</sub>O<sub>2</sub> and *tert*-butyl hydroperoxide. Yap1p was also further shown to be required for the induction of known ROS-scavenging proteins (Lee *et al.*, 1999).

## **1.3 Ethanol stress**

*S. cerevisiae* is able to grow and remain viable in growth media containing ethanol concentrations as high as 19.8% (w/v) (Sajibidor and Grego, 1992). However this tolerance is finite, which indicates that the presence of ethanol in culture is stressful

and soon inhibits growth and viability. This toxicity of ethanol has been attributed to the partially hydrophobic nature of the molecule, which confers capacity to weaken the water lattice structure within membranes and to destabilise hydrophobic interactions that maintain membrane integrity (Lloyd *et al.*, 1993), and protein structure (Millar *et al.*, 1982).

Preferential partitioning of ethanol to the hydrophobic interior of lipid bilayers results in disruption of membrane structure and permeability by increasing polarity in this region (Alexandre *et al.*, 1993). This in turn adversely affects many membrane-associated processes. Action of the plasma membrane H<sup>+</sup>-ATPase maintains pH homeostasis and an electrochemical potential gradient across the membrane. Increased passive proton influx due to exposure to ethanol results in dissipation of this electrochemical gradient. This in turn adversely affects vital processes such as the amino acid permease system, uptake of ammonium ions, maintenance of potassium balance and regulation of intracellular pH (Piper, 1995). Disruption of the mitochondrial membrane is also central to ethanol toxicity. The respiratory chain of the mitochondria endogenously produces reactive oxygen species (ROS). Under conditions of ethanol stress, production of ROS increases dramatically and causes severe oxidative damage to proteins, lipids and DNA (Moradas-Ferreira *et al.*, 1996).

In addition to disruption of processes due to membrane disordering, toxicity of ethanol results from denaturation of proteins; a consequence of which is the severe disruption of many crucial cellular processes. Millar *et al.* reported as early as 1982 that ethanol inhibits and denatures glycolytic enzymes *in vitro*.

Since production of ethanol is a normal event in the growth cycle of *S. cerevisiae*, this organism has evolved a number of mechanisms to cope with deleterious effects of ethanol. The ability to adapt to the stress and survive is a primary goal for the yeast. Of secondary importance is the ability to maintain viability and ability to continue growing when the insult is removed. Thus yeast cells have developed transient stress responses as well as long-term adaptation mechanisms.

Above a threshold level (4-6% v/v (Piper *et al.*, 1994), ethanol stress induces several groups of proteins: heat shock proteins (HSPs), anti-oxidant proteins as well as proteins involved in energy metabolism, protein destination and ion homeostasis. This occurs with simultaneous suppression of synthesis of many proteins made prior to the stress. Most of the down-regulated genes are involved in protein biosynthesis, cell growth, RNA metabolism and cellular biogenesis (Alexandre *et al.*, 2001). Protein levels of the essential and most abundant plasma membrane  $H^+$ -ATPase, Pma1 decline.

While HSPs were originally characterised on the basis of their strong induction by heat shock, they are also induced by a number of chemical agents including ethanol (Plesset *et al.*, 1982). Induction of some HSPs (HSP12 and HSP104) by these other stresses is mainly dependent on activation of the stress response element (STRE) by the zinc-finger proteins Msn2p and Msn4p. HSPs play diverse roles in the cell. Most widely reported is their role in ameliorating problems associated with protein misfolding and aggregation (Parsell and Lindquist, 1993). They have also been shown to influence membrane order (Sales *et al.*, 2000).

A change in protein complement is not the final damage-limitation mechanism yeast employs. Some of the proteins induced by ethanol stress lead to several secondary changes in cellular composition. Thus there is evidence of changes in lipid composition of membranes on exposure to ethanol. A relatively rapid decrease in unsaturation of lipids as well as reduced sterol content is observed (Alexandre *et al.*, 1993). The net result is to decrease the fluidity of cellular membranes.

A further response of yeast to ethanol stress that has been reported is a marked increase in levels of the disaccharide trehalose. Much data has accumulated to establish trehalose as a stress protectant molecule. Studies have shown that trehalose protects *in vitro* membrane preparations or liposomes in the presence of ethanol (Sales *et al.*, 2000). There is also evidence showing a positive correlation between trehalose levels and reversal of electrolyte leakage from ethanol-treated cells.

## 1.4 HSP30

In the previous section, the effects of ethanol stress on *S. cerevisiae* have briefly been reviewed. A glimpse of the mechanisms this yeast employs to counteract these effects has also been given. This overview has highlighted the significance of membranes; particularly the plasma membrane in ethanol stress.

HSP30P is one of the HSPs that are induced by heat-shock as well as ethanol and other chemical agents. This protein is interesting in that it is the only stress-induced protein known to be intrinsically bound to the yeast plasma membrane.

Regnacq and Boucherie reported the isolation and sequence of the yeast *HSP30* gene in 1993 indicating that it encodes a hydrophobic membrane protein of 37kDa in size. The gene shared no significant sequence similarity or identity to any other genes (from eukaryotes or prokaryotes) sequenced at that time. Other work in 1992 by Piper *et al.* showed that the plasma membrane of yeast acquired a protein of approximately this size in response to heat shock and entry to stationary phase. It was later shown that this protein, HSP30P was also induced by other stresses. Ethanol (6% (v/v)), along with exposure to weak organic acids, were shown to be particularly potent inducers of HSP30P. Most convincing were *in vivo* pulse-labelling experiments that showed that this protein was the major nascent protein intrinsically associated with the plasma membrane in stressed yeast cells (Piper *et al.*, 1994).

These data suggested a potential central role for HSP30P in the plasma membrane during stress. It was first envisaged that HSP30P might influence membrane order or might act to protect key membrane proteins. Another possibility was that it cooperates with other heat shock proteins in the translocation of polypeptides through the plasma membrane. However, conclusive data for the function of HSP30P had been elusive up to 1997 when it was reported that HSP30P appeared to regulate the plasma membrane  $H^+$ -ATPase during weak organic acid stress (Piper *et al.*, 1997). More recent data (Pearce *et al.*, 2000) has further implicated HSP30P in a pH homeostatic role through regulation of the plasma membrane  $H^+$ -ATPase.

## 1.5 Research questions

The aims and objectives of the present study are outlined below:

- The principal objective of this work is to investigate whether HSP30P has a role in influencing membrane order in stressful environments. Work conducted in our lab (Sales *et al.*, 2000) has shown that HSP12 is peripherally bound to yeast plasma membranes and protects membranes against damage by ethanol *in vitro*. This protection was optimal in liposomes prepared from positively charged phospholipids suggestive of an electrostatic interaction between the slightly negatively charged HSP12 and liposomes. *In vivo* therefore, the neutral charge of the yeast plasma membrane would not be sufficient for protection to be most effective. Since HSP30P is the single major nascent protein found at the plasma membrane on stress, we envisage that it might co-operate with HSP12 in stabilising membranes by providing the necessary means of interaction.
- As a good starting point to this work the deduced amino acid sequence of HSP30P will be analysed to reveal any interesting features which may be biochemically significant. Thus, a good working model of the HSP30P protein should be generated.
- Throughout this work ethanol stress will be used as a model stress. A convincing review highlighting the extensive similarity between ethanol stress and heat stress has been published (Piper, 1995). Much literature has in turn illustrated that heat stress has extensive similarities with other abiotic stresses.

From a practical point of view, it is relatively straightforward to effect ethanol stress in a quantitatively accurate manner. Further, ethanol production by yeast is of industrial significance, with the brewing and wine making industry being the principal beneficiaries. One of the aims of this study is therefore to investigate whether HSP30P provides yeast with a growth advantage in the presence and absence of ethanol stress.

- Other potential functions of HSP30P will also be investigated, with particular emphasis on plasma membrane ATPases.
- Any remarkable features revealed by analysis of the amino acid sequence and predicted structure of HSP30P will be investigated to determine their biochemical significance.

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## Chapter Two:

### Materials and Methods

#### 2.1 General Methods

##### 2.1.1 Yeast strains, media and growth

The *Saccharomyces cerevisiae* yeast strain FY1679-28a and the corresponding *HSP30* disrupted mutant FY1679a-28a hsp30::kanMX4 (kindly provided by Prof. Peter Piper, University College London) were used throughout this study. These strains are hereafter termed wild-type (wt) and knock-out (ko) respectively.

We routinely grew our cultures in sterile YPD medium (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose/glucose), at 30<sup>0</sup>C with shaking. Where necessary sterile standard defined (SD) medium (Sambrook *et al.*, 1989) was used. This comprised 0.67% (w/v) yeast nitrogen base without amino acids (Difco), 2% (w/v) dextrose/glucose and varying combinations of the following supplements at the stated concentrations: Adenine 20mg/l, Uracil 20mg/l, L-tryptophan 20mg/l, L-histidine 20mg/l, L-leucine 30mg/l.

Solid media were prepared as above with additional inclusion of 2% (w/v) nutrient-free agar or agarose.

##### 2.1.2 Growth assays

Pre-cultures inoculated with single cultures of the various yeast strains used in this study were grown to mid-logarithmic growth phase. These pre-cultures were used to inoculate 5ml cultures that were grown in the absence or presence of varying degrees of ethanol stress at 30<sup>0</sup>C. These cultures were routinely grown for 48 hours after which their optical density at 600nm (OD600) was recorded spectrophotometrically. OD600 of cultures grown in the absence of stress in each series was set as 100% growth and the others as a percentage of this. The media used in each case was dependent on the strain of yeast (see relevant sections in chapter 3).

### **2.1.3 Extraction of yeast Plasma membrane proteins:**

The procedure followed is essentially that of Serrano, 1988 with some modifications.

Yeast cultures were grown as prescribed in section 2.1.1. Usually a 300ml culture was used. The cells were harvested by centrifugation at 10000 g for 5 minutes at 4<sup>0</sup>C and extensively washed with water before resuspension in ice-cold homogenising buffer of the following recipe: 100mM Tris-HCl, 50mM NaCl, 0.1mM PMSF (pH7.4). Resuspension was such that the final cell suspension was at 50% (wet weight of cells in grams per final suspension volume in ml).

The cell suspension was then Ball-milled in a Braun Mill using 710-1180 micron glass beads (half the wet-weight of cells was used). Stop/start cycles of 20s/30s respectively were used to prevent heating of sample. Pressurised carbon dioxide was used for cooling.

The resulting homogenate was first centrifuged for 10 minutes at 700 g to remove debris and unbroken cells. The supernatant was further centrifuged for 20 minutes at 20,000 g. This second pellet, enriched with plasma membranes, was resuspended in 5ml 20% (v/v) glycerol with 0.1mM PMSF per 10 grams wet-weight of starting yeast. The 20,000-g pellet resuspended in 20% (v/v) glycerol was applied to a discontinuous gradient made of 8 ml 53% (w/v) sucrose and 16ml 43% (w/v) sucrose. Centrifugation was carried out for 6h at 25,000 rpm in a Beckman SW28 rotor. The purified plasma membranes banded at the 43%/53% sucrose interface. This band was carefully collected with a Pasteur pipette, diluted with four volumes of water and pelleted by centrifugation for 20min at 80,000 g.

The resulting pellet of pure plasma membranes was resuspended in 2 ml 20% (v/v) glycerol, 1% (w/v) nSDS per 10grams starting yeast. The suspension containing solubilised yeast plasma membrane proteins was loaded on SDS-PAGE gels as prescribed in section 2.1.4. The protein concentration was not measured.

#### **2.1.4 Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE)**

SDS-PAGE gels were prepared and run as originally stipulated by Laemmli (1970) and modified by Harlow and Lane (1988). 15% (w/v) and 12% (w/v) acrylamide separating gels with 5% (w/v) stacking gel were used. Gels were routinely run in Tris-Glycine buffer at a constant voltage of 150 volts for 3 to 16 hours depending on the size of gel.

### **2.2 Bacterial expression of HSP30**

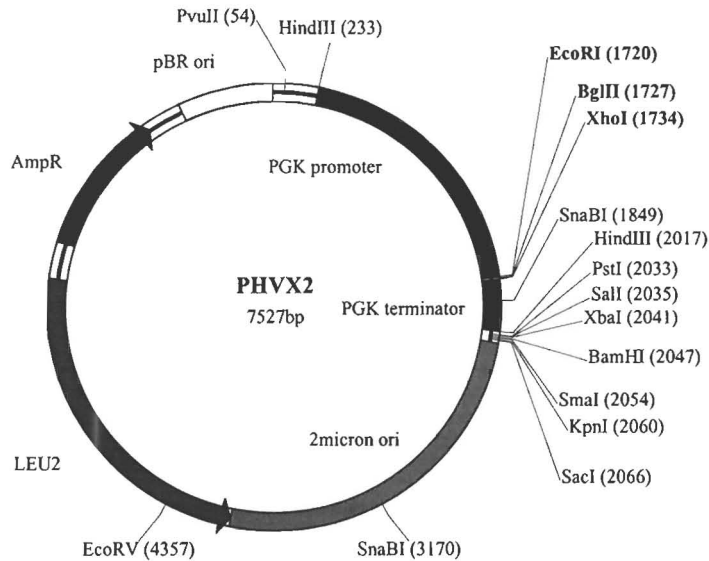
The construct used for expression of HSP30P in *E.coli* was a kind gift of Prof. Peter Piper, University College London. Briefly, the construct was prepared by Piper *et al.* as follows. pGex 2T vector was digested with EcoR1 and BamH1. The PCR-generated full-length *HSP30* gene was ligated in frame with the glutathione-S-transferase (GST) gene. The protein (HSP30P) was then expressed as a GST fusion under control of the tac promoter. The resulting construct was termed pGEX p8.

The bacterial host used was *E.coli* BL21 (DE3) and expression was induced with 0.1mM isopropyl thiogalactoside (IPTG). We routinely grew this bacterial strain in sterile LB medium (Sambrook *et al.*, 1989).

### **2.3 Construction of a yeast strain expressing HSP30 under control of the PGK promoter**

#### **2.3.1 The vector used**

The yeast shuttle vector pHVX2 was used as a vector. The map of this plasmid is shown below:



**Figure 2.1 Schematic map of pHVX2.**

This vector is derived from plasmid YEplac181, one of the classical yeast shuttle vectors of Gietz and Sugino (1988). pHVX2 was constructed by insertion of a PGK (phosphoglycerate kinase) promoter and terminator sequences (separated by three restriction sites) into the *HindIII* restriction site on position 233 of YEplac181. Presence of a LEU2 gene facilitates selection of yeast transformants using solid media lacking leucine.

pHVX2 vector was a kind gift from Prof. Prior and G. Kayingo, University of Stellenbosch.

### **2.3.2 Isolation of total yeast DNA**

A 10ml culture of *S.cerevisiae* was grown to mid logarithmic growth phase and harvested by centrifugation at 10000 g for 10 minutes at 4°C. The pellet was resuspended in 400µl homogenisation buffer: 10mM Tris-HCl, 50mM NaCl (pH 7.4).

200mg of glass beads of size 710-1180 micron were added to the suspension and the mixture vortexed at very high speed for 45 seconds to disrupt yeast cells. The homogenate was then removed from the beads and 400µl lysis solution of the following recipe was added: 200mM NaOH, 1% (w/v) SDS. The suspension was

mixed gently by inversion and left at room temperature for 10 minutes. 400µl 5M potassium acetate (pH5.4) was added; the sample was mixed by inversion and left on ice for 15 minutes. The resulting precipitate was pelleted by centrifugation in a microcentrifuge.

0.6 vol. 100% isopropanol was added to the supernatant. Precipitation of DNA was allowed to proceed for 10 minutes at room temperature. The mixture was then centrifuged for a few minutes in a microcentrifuge. The resulting DNA pellet was washed with 1ml 70% (v/v) ethanol. The mixture was spun further and the clean DNA pellet was resuspended in 100µl Tris-EDTA buffer (pH7.5).

The amount and purity of DNA was determined spectrophotometrically. This DNA preparation was used as template in the following section.

### **2.3.3 PCR amplification of the HSP30 gene**

The following primers were used to amplify the full-length *HSP30* gene from yeast:

HSP30 (F):

5' **CGCGAATTCATGAACGATACGCTATCAAGCT** 3'

**BOLD UNDERLINED** corresponds to *EcoRI* restriction site. The initiation codon is in ***BOLD ITALIC*** font.

HSP30 (R):

5' **GCGCCTCGAGCTCAAGCTGCTCTAAGCAGT** 3'

**BOLD UNDERLINED** corresponds to *XhoI* restriction site. The stop codon is in ***BOLD ITALIC*** font.

Polymerase chain reaction (PCR) amplification of the full length *HSP30* gene was carried out in a 50µl reaction volume with the following components: 200µM dNTP's, 1.5mM MgCl<sub>2</sub>, 40pmol each primer, 100ng Total Yeast DNA, 2.5 Units *Taq* DNA polymerase (Promega), 5µl reaction buffer (Promega).

The following cycling conditions were used:

1 cycle at 90<sup>0</sup>C for 2 minutes

30 cycles at:

93<sup>0</sup>C for 30 seconds

53<sup>0</sup>C for 30 seconds

72<sup>0</sup>C for 45 seconds

1 cycle at 72<sup>0</sup>C for 5 minutes

The product of the reaction was visualised on a 0.8% (w/v) agarose gel containing ethidium bromide. Bacteriophage lambda DNA digested with PstI or with Eco RI and *HindIII* was used as size marker.

#### **2.3.4 Preparation and ligation of insert and vector**

The product of the PCR reaction was gel-purified on 0.8% (w/v) agarose by gel electrophoresis. The excised band was extracted from the gel with the Qiaex II gel extraction kit (Qiagen) and eluted with 20µl water. Manufacturer's instructions were followed.

10µl of the purified PCR product was incubated at 37<sup>0</sup>C with an excess of *XhoI* and *EcoRI* for 2-3 hours. The digestion reaction was stopped by heating at 80<sup>0</sup>C for 15 minutes. 0.5µg of plasmid vector pHVH2 was incubated at 37<sup>0</sup>C with an excess of *XhoI* and *EcoRI* for 2 hours. The double digested plasmid was gel-purified on 0.8% (w/v) TAE-agarose by gel electrophoresis.

0.5 unit of T4 DNA ligase and 2µl 10X ligation buffer was added to aliquots of the double digested vector and PCR generated *HSP30*. The molar ratio of vector to insert was 1:5. Water was added to a final volume of 20µl. The tube was incubated for 16h at 16<sup>0</sup>C. 5µl of the reaction was used to transform competent *E.coli* JM109 prepared according to standard protocols (Sambrook *et al.*, 1989).

Transformants were screened for presence of insert using a standard plasmid DNA mini-prep protocol (Sambrook *et al.*, 1989) and 0.8% (w/v) agarose gel

electrophoresis stained with ethidium bromide. The resulting construct was termed pHVX30.

### **2.3.5 Transformation of *S.cerevisiae* and screening of transformants.**

A yeast culture was grown in sterile YPD to OD600 of 1.2-1.3. Cells were harvested and washed with 1/2 volume ice-cold water followed by two washes with 1/25 volume ice-cold 1M sorbitol. The cells were then resuspended in 1/200 volume cold 1M sorbitol to form a paste. 50µl of the thick suspension was then mixed with 5µl DNA (0.4mg/ml) in water and tapped into a 0.2 cm electroporation cuvette (Biorad). Electroporation was then carried out using program Sc2 on a Pharmacia Biotech electroporator.

Transformants were selected by plating on solid SD plates lacking leucine and containing 1M sorbitol. The presence of the construct was further confirmed by plasmid rescue followed by transformation of *E.coli* JM109 using standard protocols.

## **2.4 Construction of a yeast strain expressing truncated HSP30**

The method followed was essentially as outlined in section 2.3. However the following primers were used to amplify the truncated *HSP30* gene:

HSP30 (F):

5' **CGCGAATTCATGAACGATACGCTATCAAGCT** 3'

**BOLD UNDERLINED** corresponds to *EcoRI* restriction site. The initiation codon is in ***BOLD ITALIC*** font.

HSP30t (R):

5' TCATCT**CGAGCTAGCTGACTGCAATTAGCAAGTA** 3'

**BOLD UNDERLINED** corresponds to *XhoI* restriction site. The stop codon is in ***BOLD ITALIC*** font.

These primers amplified a PCR product of 829 base pairs long. A stop codon would thus be introduced after codon 270 of the full-length gene. The resulting construct was termed pHVX30t.

## 2.5 Construction of site-directed mutants of HSP30

Two different site-directed mutants of HSP30P were generated by the four-primer site-directed mutagenesis approach as described below. The procedures followed for each mutant differed only in the primer sets used.

### 2.5.1 Primer sets used

#### T181A:

Fragment1 (small fragment):

181A: 5'TCATTGATCAAGTCCGCTTACAAGTGGGGTTAT3' and  
HSP30 (R) as described in section 2.3

Fragment2 (large fragment):

181B: 5'ATAACCCCACTTGTAAGCGGACTTGATCAATGA3' and  
HSP30 (F) as described in section 2.3

#### S308A:

Fragment1 (small fragment):

308A: 5'GAAGCTGTTCCAGAGGCTCCAAGAGCATATGGA3' and  
308C: ATTGATTAGACTATTAGGCATGG

Fragment2 (large fragment):

308B: 5'TCCAGATGCTCTTGAGCCTCTGGAACAGCTTC3' and  
HSP30 (F) as described in section 2.3

### 2.5.2 PCR generation and cloning of mutant genes

Polymerase chain reaction (PCR) amplification of mutagenised gene fragments of the *HSP30* gene was carried out in a 50ul reaction volume with the following components: 200µM dNTP's, 40pmol each primer, 100ng Total Yeast DNA, 2.5 Units *Pfu* DNA polymerase (Promega), 5µl reaction buffer (Promega). For all fragments addition of 3mM MgCl<sub>2</sub> stimulated yield of product. The following cycling conditions were used:

1 cycle at 90<sup>0</sup>C for 2 min  
30 cycles at:  
    93<sup>0</sup>C for 30s  
    53<sup>0</sup>C for 30s  
    72<sup>0</sup>C for 45s  
1 cycle at 72<sup>0</sup>C for 5 min

The products of this PCR step were purified by agarose gel electrophoresis and extraction using Qiaex II gel extraction kit (Qiagen). Manufacturer's instructions were followed. The purified products were then used as template and self-primer for a second PCR step.

A 50µl reaction was prepared with the following components: 200uM dNTP's, 40pmol primer HSP30(R), 40pmol primer HSP30(F), 2µl fragment 1, 2 µl fragment2, 2.5 Units *Taq* DNA polymerase (Promega), 5µl reaction buffer (Promega). Cycling conditions were as stated above.

The product of this second PCR was run on a 0.8% (w/v) low melting point agarose gel prepared in tris-acetate buffer. The band corresponding to the full-length *HSP30* was excised and melted at 70<sup>0</sup>C for 5 minutes. This was then ligated to pGEM-Teasy vector (Promega) according to manufacturer's instructions. 3µl of the ligation reaction was used to transform competent *E.coli* JM109 prepared according to standard protocols (Sambrook *et al.*, 1989).

Transformants were screened using a standard plasmid DNA mini-prep protocol (Sambrook *et al.*, 1989) and 0.8% (w/v) agarose gel electrophoresis stained with ethidium bromide.

Each pGEM-Teasy construct was extracted from *E.coli* JM109 and purified. The coding regions were sequenced to confirm mutagenesis of the specified residues to alanine. Sequencing was carried out by the Department of Molecular and Cellular Biology specialised sequencing unit (UCT). Briefly, the Dye-primer approach using Cy5 fluorescently end-labelled primers complementary to pGEM-Teasy was

employed. An ALFexpress DNA Automated Sequencer, Amersham Pharmacia Biotech AB (Uppsala, Sweden) was used.

Mutant *HSP30* genes were then sub-cloned into the yeast shuttle vector pHVX2 (see Section 2.3.1 for plasmid map) according to standard protocols (Sambrook *et al.*, 1989). *Xho* I and *Eco*RI restriction sites were used for the sub-cloning. The resulting constructs were termed pHVX30-T181A and pHVX30-S308A.

## **2.6 Raising polyclonal antibodies against HSP30 and immunological detection of protein extracts**

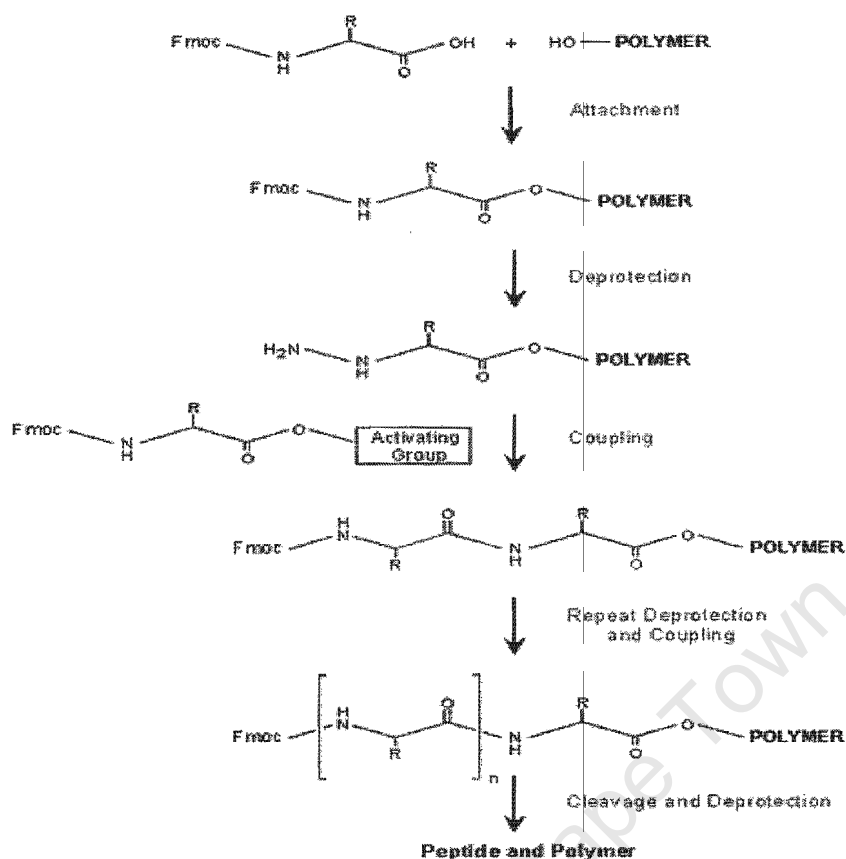
### **2.6.1 Solid phase peptide synthesis**

Solid phase peptide synthesis utilising 9-fluorenylmethoxycarbonyl (Fmoc) amino acids was carried out based on methodology proposed and reviewed by Atherton and Sheppard, 1989 and Fields and Noble, 1989. The procedure followed is essentially an adaptation of the Merrifield technique.

A peptide of the following sequence was made:



This peptide is from extracellular loop 1 of HSP30P. This sequence was selected due to its high antigenicity index (Hopp and Woods, 1981) and the prediction that it falls within exposed regions of HSP30P. A cystine residue was added to the N-terminal to maximise the variety of conjugation methods we could use.



**Figure 2. General Scheme for Fmoc Synthesis**

**Figure 2.2 Schematic representation of solid phase peptide synthesis using Fmoc amino acids.**

Figure 2.2 shows a schematic representation of the chemistry of the procedure. The peptide is synthesised from the C-terminal to the N-terminal as follows.

50  $\mu$ moles of Fmoc-glycine polymer resin was placed in a 10ml syringe fitted with a glass sinter frit (16-40 micron pores) with a gas-tight plunger. Reagents and solvents were drawn into the syringe via 0.9x120mm Teflon tubing.

The resin was washed twice with dry dimethyl formamide (DMF). The synthesis was started by deprotection of F-moc glycine by two incubations in 2ml 20%(v/v) piperidine in DMF for ten minutes with agitation. Piperidine was removed by six 5ml washes with DMF. A mixture of 80 $\mu$ moles dry benzotriazolyl *N*-oxytrisdimethylaminophosphonium hexafluorophosphate (BOP) and 80  $\mu$ moles Fmoc amino acid was dissolved in 1ml 9%(v/v) methyl-morpholine in DMF and allowed to react for 30 minutes with gentle agitation. This coupling step was repeated for

coupling of Fmoc-glutamine residues in Pep1. Excess reagent was removed by three 5ml DMF wash steps.

After completion of the synthesis and the final deprotection with piperidine, the resin-bound peptide was washed three times with 5ml dichloromethane and dried under vacuum. The peptide was detached from the resin by incubation in 5ml 9:1, trifluoroacetic acid (TFA): ethanedithiol for 20min and filtered into a clean tube through the glass sinter frit. This solution was further incubated for 15 hours at room temperature to remove side chain protecting groups. All side chain protecting groups selected were labile to this treatment. The peptide was finally precipitated in 9 volumes of diethylether and dried by lyophilisation.

The peptide was weighed and analysed by matrix assisted laser desorption ionisation - time of flight (MALDI-TOF) mass spectrometry. A 100pmol/ul solution was used.

### **2.6.2 Conjugation of peptides to protein carriers**

Small peptides alone do not generate antibodies when injected into an animal. The peptide must be conjugated to a macromolecule carrier. Each peptide was conjugated to two different protein carriers via two different coupling agents.

#### **Carbodiimide conjugation to ovalbumin**

10mg 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) was added to 50mg peptide in 1 ml water and stirred for 10 minutes at room temperature. 5mg ovalbumin in 1ml phosphate buffered saline (PBS) was then added and stirred for four hours at room temperature. Unbound peptide was removed by overnight dialysis against 20 litres of water. Conjugation was confirmed by MALDI-TOF mass spectrometry.

## Heterobifunctional coupling to Bovine Serum Albumin with MBS

50µl of 25mg/ml *m*-Maleimidobenzoyl-N-hydroxysuccinimide (MBS) in DMF was added drop-by-drop to 500 µl of a 10mg/ml solution of BSA in PBS and stirred for 30 minutes at room temperature. Excess MBS was removed using a Pharmacia G-25 column pre-equilibrated with PBS. A 2.5cm diameter, 30cm length column with medium G-25 beads was used.

Fractions containing MBS-derivatized BSA were pooled and lyophilised overnight and redissolved in 1ml PBS. Peptide (50mg) dissolved in 1ml PBS was added and allowed to stir at room temperature for three hours. Unbound peptide was removed by overnight dialysis against 20 litres of water. Conjugation was confirmed by MALDI-TOF mass spectrometry.

### **2.6.3 Injection of rabbits and analysis of antibody by ELISA**

Peptides conjugated to ovalbumin were used to inject rabbits. A booster injection was administered two weeks after first injection. The rabbits were bled prior to immunisation and at two-week intervals for three months thereafter.

Antisera from all bleeds were analysed by enzyme-linked immunosorbance assay (ELISA). Dilutions of  $10^{-1}$  to  $10^{-8}$  were prepared and analysed. Peptides conjugated to BSA were used as antigen. The procedure followed was briefly as follows.

100µl of antigen (dilute to 10µg/ml in PBS) was added to each well and incubated overnight at 4<sup>0</sup>C to coat the plate with antigen. Residual binding capacity of the plate was blocked with 2%(w/v) BSA in PBS. 100µl of each dilution of antiserum (primary anti-body) from each bleed was then added individually to the wells and allowed to incubate for 60 minutes. Wells were rinsed with TBS-0.1% (v/v) Tween and secondary antibody (goat anti-rabbit IgG conjugated to alkaline phosphatase) was added and allowed to bind for 60 minutes. 100µl pNPP substrate (1mg/ml) in 10% (v/v) diethanolamine was added and allowed to incubate for 30 minutes or until positive reactions turned yellow. The absorbance of each well was read at 405nm.

#### **2.6.4 Immunological detection of protein extracts by Western blot**

Western blots were carried out essentially as described by Harlow and Lane, 1988 using anti-HSP30-peptide antibody as primary antibody and anti-rabbit-IgG conjugated to alkaline phosphatase as secondary antibody.

Transfer of proteins was carried out at 4<sup>0</sup>C for 16 hours at 20mA constant current.

### **2.7 Bioinformatics**

#### **2.7.1 Prediction of the secondary structure of HSP30**

Attempts to model HSP30P protein structure were carried out using Swiss-Model (Guex *et al.*, 1999) at [www.expasy.ch/Swiss-Model.html](http://www.expasy.ch/Swiss-Model.html) Prediction of the secondary structure of HSP30P were carried out by input of the deduced amino-acid sequence of HSP30P (Swiss-prot accession number: P25619) into the following web-based tools:

- TM-pred (Hofmann and Stoffel, 1993) [www.ch.embnnet.org/software/TMPRED\\_form.html](http://www.ch.embnnet.org/software/TMPRED_form.html)
- TMHMM (Krogh, *et al.*, 2001), [www.cbs.dtu.dk/services/TMHMM-2.0/](http://www.cbs.dtu.dk/services/TMHMM-2.0/)
- HMMTOP 2.0 (Tusnady and Simon, 1998; 2001) [www.enzim.hu/hmmtop/](http://www.enzim.hu/hmmtop/)

#### **2.7.2 Prediction of post-translational modification of HSP30**

Potential serine/threonine phosphorylation sites were predicted using NetPhos 2.0 (Blom *et al.*, 1999) by input of the deduced amino acid sequence of HSP30P to the prediction server at [www.cbs.dtu.dk/services/NetPhos](http://www.cbs.dtu.dk/services/NetPhos).

Potential glycosylation sites were predicted using NetOglyc (Hansen *et al.*, 1998) by input of the deduced amino acid sequence of HSP30P to the prediction server at [www.cbs.dtu.dk/services/NetOGlyc](http://www.cbs.dtu.dk/services/NetOGlyc).

### **2.7.3 Other bioinformatics tools**

Antigenicity index profile of HSP30P was generated at [www.embl-heidelberg.de/JaMBW/3/1/7](http://www.embl-heidelberg.de/JaMBW/3/1/7) The service used is based on the work of Hopp and Woods, 1981.

Codon adaptation index of *HSP30* gene was calculated at [alces.med.umn.edu/webtrans.html](http://alces.med.umn.edu/webtrans.html) based on the method of Sharp and Li, 1987.

pI calculations were carried out at [www.expasy.ch/tools/pi\\_tool.html](http://www.expasy.ch/tools/pi_tool.html)

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## Chapter Three:

### Results and Discussions

#### 3.1 Predicted secondary structure and post-translational modification of HSP30

With the rapidly increasing availability of bioinformatics resources, the sequence and structure of any protein can provide a glimpse of its potential function. Regnacq and Boucherie (1993) deduced from the hydrophobicity plot of HSP30P that the protein is highly hydrophobic and that it is likely to be a multi-spanning membrane protein. Few integrally bound membrane protein structures have been determined experimentally. Their inability to easily crystallise has made X-ray diffraction structural studies of these proteins challenging (Ostermeier and Michel, 1997). Lack of resolution of structures determined by electron microscopy makes this approach unappealing. Structure determination by heteronuclear nuclear magnetic resonance (NMR) spectroscopy has required the development of novel and generally inaccessible methods due to the fact that *in vivo* membrane proteins exist in phospholipid-bound forms and NMR of proteins is traditionally carried out in aqueous solution (Siminovitch, 1998).

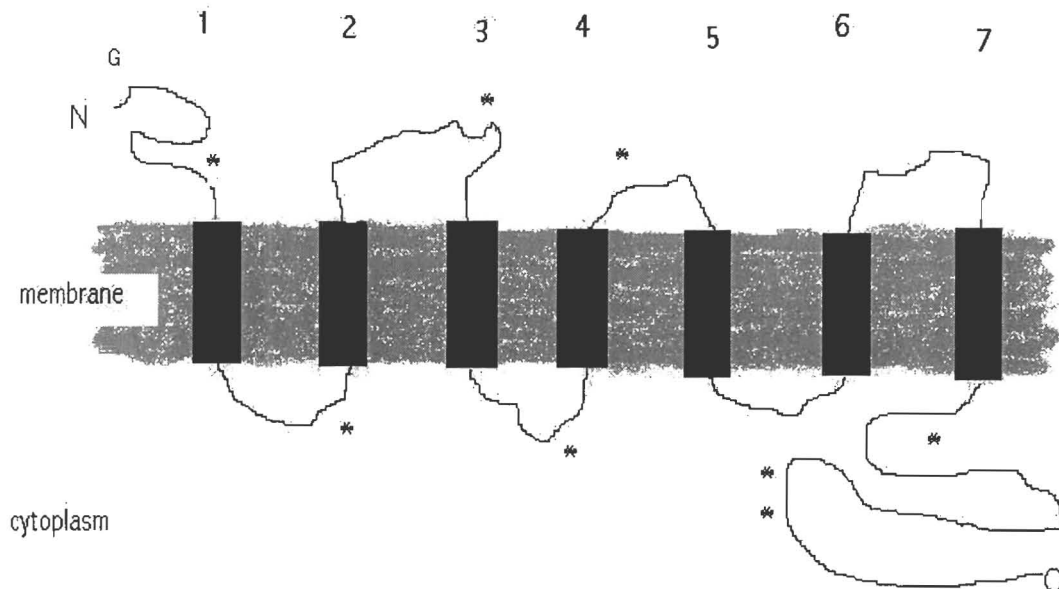
It has thus become an acceptable method to model membrane protein structures based on homology to other proteins whose structure has been resolved, or to predict their secondary structures based primarily on the knowledge that about twenty amino acid residues are sufficient to cross biological membranes in an alpha helical conformation. The high reliability of many of the widely available, published (or peer reviewed) membrane protein structure predictive tools is well established (Nilsson *et al.*, 2000).

Attempts were made to model HSP30P protein structure using Swiss-Model, a knowledge-based protein modelling tool (Guex *et al.*, 1999). These attempts were not successful due to the absence of proteins with high homology to HSP30P in protein structure databases. This result was not surprising, as homology searches at regular

intervals throughout this work did not reveal any proteins with significant homology to HSP30P. Homologous proteins whose structures have been resolved were therefore always unlikely to be identified.

Several different published programs have thus been used to predict the secondary structure of HSP30P; TMHMM (Krogh, *et al.*, 2001), TM-pred (Hofmann and Stoffel, 1993), HMMTOP 2.0 (Tusnady and Simon, 1998; 2001). All three are available through world wide web-based input of the deduced amino acid sequence. It was found that all these methods predict that HSP30P has seven transmembrane helices connected by six loops. Furthermore, the topological organisation is such that a small amino-terminal domain faces the extracellular side of the membrane while a larger carboxy-terminal, glutamic acid-rich domain faces the cytosol.

It was also important to investigate potential sites of post-translational modification as well as the nature of these modifications. One potential glycosylation site (residue 2) (NetOglyc; Hansen *et al.*, 1998) and several potential serine/threonine phosphorylation sites (NetPhos 2.0; Blom *et al.*, 1999) were found. These predicted phosphorylation sites were cross-checked with the predicted secondary structure of HSP30P. Among serine residues with the highest scores, serine 106, 150, 280, 308 and 312 fell within regions where the phosphorylated residue as well as the complete consensus recognition sequence would be exposed. Threonine 28, 64 and 181 also satisfied these criteria. Presented below (Fig.3.1) is the model of HSP30P constructed based on these *in silico*-generated data.



**Figure 3.1. Working model of the HSP30P protein based on predictive analysis. \*: potential phosphorylation site, G: potential glycosylation site (see text for amino acid locations). Horizontal black bars denote transmembrane segments numbered 1 to 7. Helix 1: amino acids 34-56, helix 2: 67-87, helix 3: 123-140, helix 4: 154-178, helix 5: 188-202, helix 6: 213-235, helix 7: 248-270.**

The predicted structure of HSP30P as presented above belongs to class 6 proteins as recognized by the structural classification of proteins (SCOP) database (Murzin *et al.*, 1995). It is more specifically reminiscent of the seven helix receptor super-family of proteins such as rhodopsin. Exhaustive bioinformatic analysis did not reveal any similarity with this family of proteins beyond secondary structural organisation.

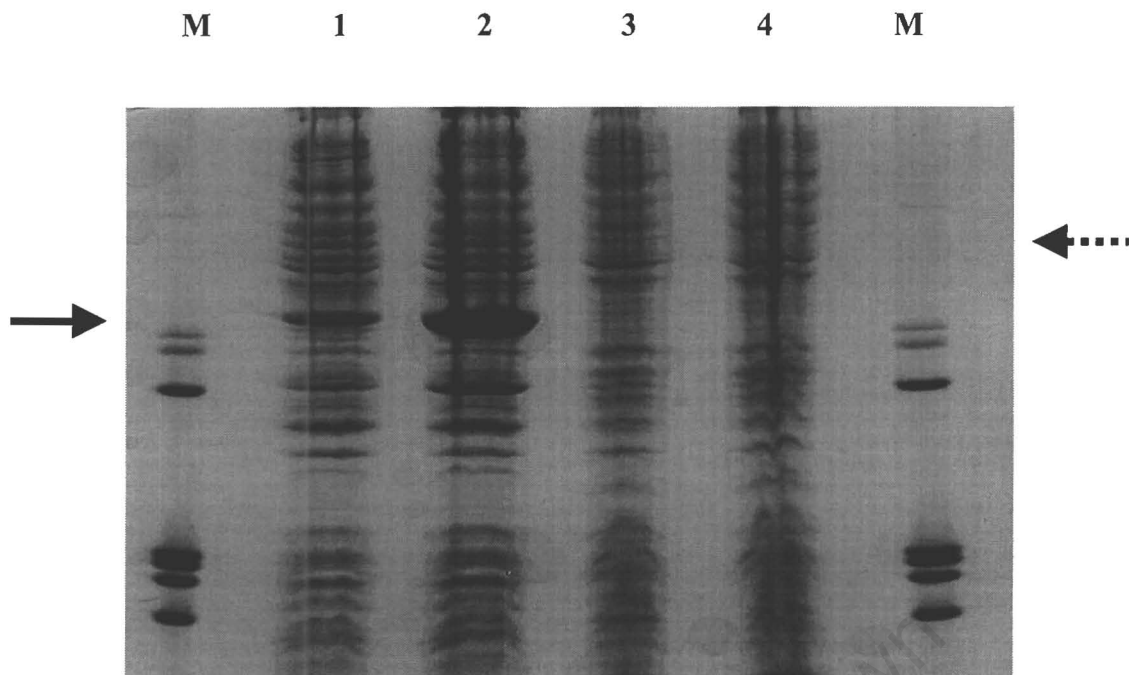
### 3.2 Expressing HSP30 for purification

One of the major initial objectives of this work was to investigate the potential role of HSP30P in influencing membrane order in stressful conditions. Previous data (Sales *et al.*, 2000) has shown that HSP12 protects membranes against damage by ethanol. This protection was optimal in liposomes prepared from positively charged phospholipids suggestive of an electrostatic interaction between the slightly negatively charged HSP12 and liposomes. *In vivo* therefore, the neutral charge of the yeast plasma membrane would not be sufficient for protection to be most effective. Since HSP30P is the single major nascent protein found at the plasma membrane on

stress, we envisaged that it might co-operate with HSP12 in stabilising membranes by providing the necessary means of interaction. Before liposomes could be prepared to investigate this, it was necessary to express large amounts of HSP30P for purification.

### **3.2.1 Heterologous expression in *E.coli***

A construct prepared for over-expression of HSP30P in *E.coli* was acquired from Prof. Peter Piper, University College London. Briefly, the construct was prepared by Piper *et al.* as follows. pGex2 vector was digested with EcoR1 and BamH1. The PCR-generated coding region of the full-length *HSP30* gene was ligated in frame with the glutathione-S-transferase (GST) gene. The construct was termed pGex-p8. We transformed *E.coli* BL21 (DE3) with this plasmid. HSP30P was then expressed as a GST fusion by inducing the *tac* promoter (a *trp* and *lac* promoter hybrid) with IPTG. In this way purification would be greatly simplified as the expressed fusion protein would be able to bind glutathione-sepharose affinity matrix. Presented below (figure 3.2) is a coomassie-blue stained SDS-PAGE gel (15% (w/v) acrylamide) showing protein extracts from induced (lane 4) and non-induced (lane 3) BL21 pGex-p8 cells. Cells were resuspended in SDS gel loading buffer and heated to extract total proteins. To test the efficacy of all our reagents, particularly the IPTG, we transformed *E.coli* BL21 cells with pGex-HSP12, to construct a bacterial strain expressing HSP12, (Sales *et al.*, 2000) as a positive control.



**Figure 3.2.** Coomassie stained 15% (w/v) acrylamide SDS PAGE gel showing total protein extracted from *E.coli* BL21 cultures. Lane 1: Non-induced pGEX-HSP12, Lane 2: Induced pGEX-HSP12, Lane 3: Non-induced pGEX-p8, Lane 4: Induced pGex-p8. M: Protein size marker (Chicken core histones) ←.....: expected size of HSP12-GST fusion (40kDa) —→: expected size of HSP30-GST fusion (65kDa).

Typical expression optimisation approaches were followed including monitoring expressed proteins at regular intervals after induction, induction at various culture OD's and induction with various IPTG concentrations. It was also thought that since HSP30P is a membrane protein, it was likely to be incorporated into membranous organelles or into inclusion bodies. Thus membrane proteins from induced cells were also analysed by SDS-PAGE

After these attempts to optimise expression as well as analysis of expressed proteins it was concluded that HSP30P is not expressed optimally in *E.coli*. Figure 3.2, lane 4 clearly shows the absence of an overexpressed protein at the expected size (dotted arrow). Unsuitability of expression host for the vector used was ruled-out as the cause for lack of expression. The BL21 strain of *E.coli* naturally lacks the *Lon* protease and is engineered to be deficient of the *OmpT* protease. We chose this strain of *E.coli* specifically to minimise degradation of our recombinant protein. Further, the pGex series of vectors harbour a *lacI<sup>q</sup>* gene that produces very high levels of the *lac* repressor. If our recombinant protein was toxic to *E.coli*, problems associated with this toxicity will have been minimised, as the protein should be produced in significant amounts only in the presence of the artificial inducer IPTG.

### 3.2.2 Homologous expression in *S.cerevisiae*

Bacterial expression of HSP30P having been unsuccessful, the endogenous level of HSP30P produced by stressed wild-type yeast cells was analysed (figure 3.4). We found that HSP30P protein levels were not sufficient to warrant further purification. *S.cerevisiae* and *E.coli* have served well as recipient organisms for cloned genes for over-expression of the encoded protein. *S.cerevisiae* has been used here as an alternative to *E.coli* to over-express our protein of interest, HSP30P.

An array of suitable vectors was available for our purposes. Briefly, there are five widely available types of yeast vectors: yeast episomal plasmids (YEp's), yeast replicating plasmids (YRp's), yeast centromere plasmids (YCp's), yeast integrative vectors (YIp's) and yeast artificial chromosomes (YACs). Our choice of vector for the present study, pHVX2 (see figure 2.1 for plasmid map) is derived from plasmid YEplac181, one of the episomal yeast shuttle vectors of Gietz and Sugino (1988). pHVX2 was constructed by insertion of the phosphoglycerate kinase (PGK) promoter and terminator sequences (separated by three restriction sites) into the *HindIII* restriction site on position 233 of YEplac181.

We constructed a strain of *S.cerevisiae* that expresses HSP30P under the control of the strong PGK promoter using this vector (Section 2.3). The full length *HSP30* gene was amplified from total yeast DNA by PCR using primers described in section 2.3.3. This was ligated to pHVX2 (a kind gift from Prof. B. Prior and G. Kayingo, University of Stellenbosch) which had been digested with EcoR1 and Xho1 to construct pHVX30. The ligation reaction was used to transform *E.coli* JM109 as outlined in section 2.3. Figure 3.3 A shows an ethidium bromide stained agarose gel loaded with plasmid DNA isolated from these *E.coli* JM109-pHVX30 cells and digested with EcoR1 and Xho1. Lanes 1,2, 4 and 6 show two bands indicating that the PCR-generated *HSP30* was successfully cloned. Addition of RNase A to the

plasmid preparation removed the co-extracted RNA seen as a large band (open arrow).

The size of the lower band (solid arrow) corresponding to the insert was confirmed as the expected 1026bp by agarose gel electrophoresis with bacteriophage lambda DNA digested with Pst1 as a size marker (figure 3.3 B).

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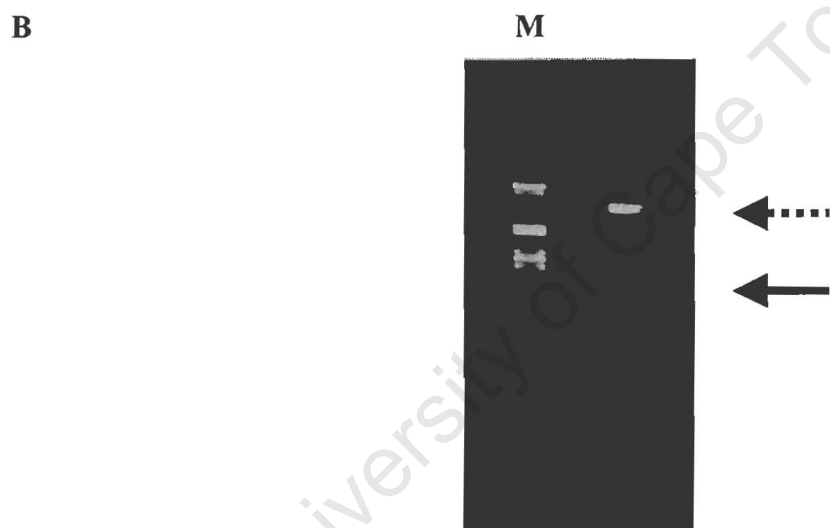
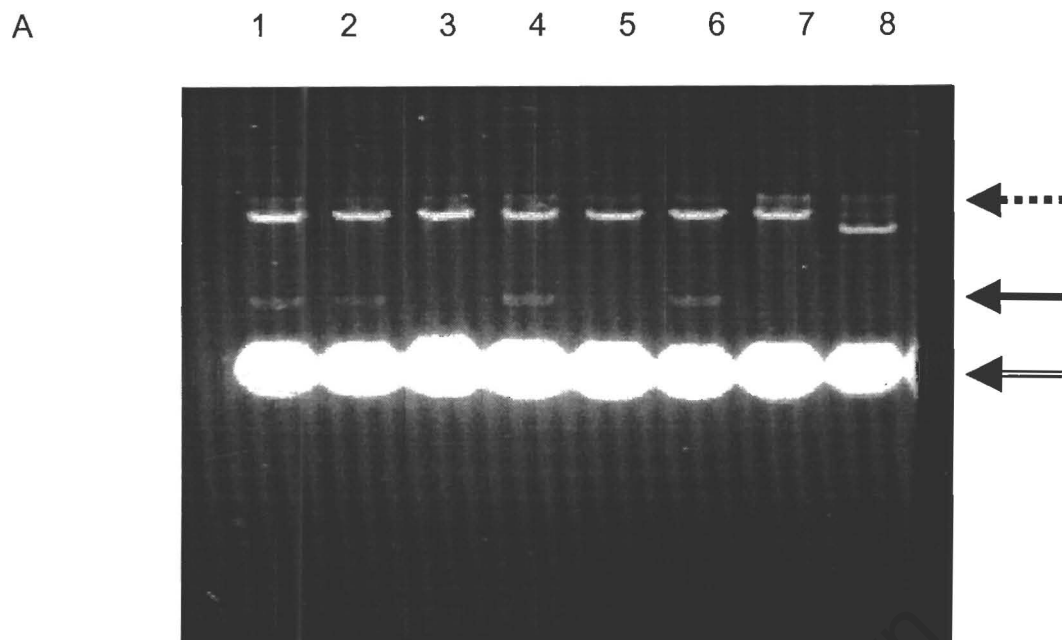


Figure 3.3. Ethidium bromide stained agarose gel showing four positive clones from double-digested plasmid DNA extracted from E.coli JM109 cells transformed with pHVX30 ligation reaction (A) and confirmation of the size of insert and vector (B).  $\leftarrow \dots$  : expected size of digested pHVX2 (7.5 kb)  $\leftarrow$  : expected size of HSP30 insert (1kb) M: Bacteriophage lambda DNA digested with PstI.  $\leftarrow \equiv$  : co-extracted RNA. Lanes 1 – 8 represent individual extracts from different transformants.

Being a membrane protein, total cellular abundance of HSP30P was expected to be considerably lower than that of soluble proteins. Gels of total protein extracts were not useful in analysing expression of HSP30P (not shown). Plasma membranes were therefore isolated from wild-type yeast transformed with construct pHVX30 and from isogenic yeast transformed with pHVX2 (Section 2.1.3) for comparison of protein levels. Yeast transformed with pHVX2 was also grown in the presence of 6%(v/v) ethanol, a stress level that has been shown to be a potent inducer of HSP30P (Piper *et al.*, 1992). In this way endogenous stress-induced HSP30P would be enriched with protein produced under control of the PGK promoter. All strains were grown in SD media lacking leucine to provide some auxotrophic selective pressure. Loss of episomal yeast vectors such as pHVX2 has been reported to be about 1% of cells per generation when grown in the absence of selective pressure. On extended growth in rich media, untransformed cells can potentially dominate.

While the molecular weight of HSP30P based on the deduced amino acid sequence is 37kDa, the protein has been reported to have an apparent mobility of 30kDa in SDS-PAGE gels. This aberrant mobility has been attributed to clustering of acidic amino acids in the region we have now denoted as the C-terminal domain (Regnacq and Boucherie, 1993).

On the gel shown in figure 3.4 a band corresponding to the expected size of 30kDa can only be seen in protein extracts from stressed cells and pHVH30-transformed cells. The relative protein levels in each extract appear to be essentially the same. It is therefore concluded that expression of HSP30P under control of the PGK promoter is not significantly higher than expression under control of its endogenous promoter (figure 3.4).

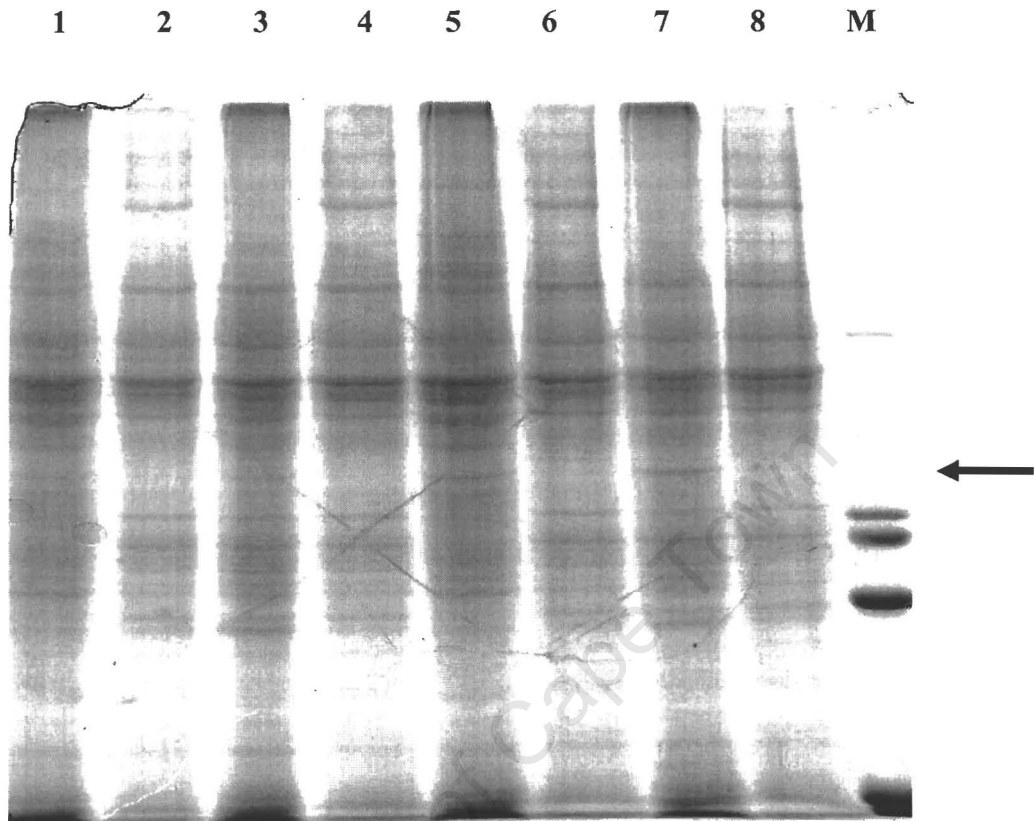



Figure 3.4. Coomassie-stained 12% (w/v) acrylamide SDS-PAGE gel showing yeast plasma membrane proteins.  : expected location of HSP30 at approximately 30 kDa. Lanes 2, 4, 6, 8 Wild type yeast cells grown in SD media. Lane 1: Wild type yeast transformed with pHVX2 grown in SD with 2% (v/v) ethanol, lane 3: wild type-pHVX2 in 3% (v/v) ethanol, lane 5: wild type-pHVX2 in 4% (v/v) ethanol and lane 7: wild type transformed with pHVX30.

### 3.2.3 Codon adaptation index of HSP30

Among the crucial factors affecting the level of expression of cloned genes are promoter strength, stability of mRNA, plasmid copy number, plasmid stability and codon choice within the gene of interest (see Old and Primrose, 1994 for review). In this study we attempted to overexpress HSP30P in a heterologous host and in its species of origin. In both cases transcription was under control of strong promoters; both plasmids used have origins of replication that are widely known to result in a high copy number and all cultures were grown under selective pressure to maximise plasmid stability. HSP30 mRNA level has been shown to increase many-fold on induction by stress and entry to the stationary phase of growth. This level is

maintained after the stress (Regnacq and Boucherie, 1993). This indicates that HSP30 mRNA is stable. Since the PCR-generated HSP30 was not sequenced to confirm efficacy of the reaction, the choice of codons within the *HSP30* gene was thus analysed to establish whether this was the cause for minimal expression. This was the remaining possible cause for lack of expression.

It is now well established that between as well as within species, the alternative codons for any one amino acid are not used randomly (Ikemura, 1985). Sharp and Li showed in 1987 that in the most studied organisms at the time, *E.coli* and *S.cerevisiae*; there is a clear positive correlation between the degree of codon bias and level of gene expression. They devised an effective and widely used measure of codon usage bias that they termed the *codon adaptation index (CAI)*. On their scale a highly expressed gene would have a CAI close to 1 while a gene that is inclined to low expression would have a CAI close to 0.

By using an algorithm based on the work of Sharp and Li it was found that the codon adaptation index of *HSP30* is 0.249 in *E.coli* and 0.230 in *S.cerevisiae*; both relatively low and indicating that HSP30P is not inclined to high expression in either organism. (For comparison we also calculated the codon adaptation index of HSP12, the protein used as a convincing positive control in section 2.3.1 (figure 3.2) and found it to be 0.394 in *E.coli* and 0.639 in *S.cerevisiae*.)

Regnacq and Boucherie, 1993; and Alexandre *et al.*, 2001 have shown that HSP30 mRNA levels are increased many-fold in response to ethanol and other abiotic stresses. Yet in our hands (Figure 3.3, lanes 1, 3 and 5), and others (Panaretou and Piper, 1994), the increase in protein levels is not appreciable. Perhaps higher levels of the protein may be detrimental to yeast cells, thus a 'bottleneck' regulatory mechanism at the translational level has evolved. This would require that tRNAs for translation of these less frequently used codons found in *HSP30* are less abundant within the tRNA pool of *S.cerevisiae*.

### 3.3 HSP30 is not likely to co-operate with HSP12 in stabilising membranes

Lack of expression of HSP30P abruptly stalled our experimental investigation of the role of HSP30P in stabilising membranes. Other approaches such as PCR based gene synthesis (see Withers-Martinez *et al.*, 1999 for example) to increase the overall codon adaptation index of *HSP30* and thus expression level were not pursued. This decision was taken with the following considerations in mind.

Our analysis of the deduced amino acid sequence of HSP30P reveals that this protein is likely to have an intracellular domain rich in glutamic acid residues (section 3.1). We anticipated that this domain would be the main site of interaction with HSP12. The theoretical pI of this domain was calculated to determine its likely charge *in vivo*. The deduced pI based on the composition of residues 271-332 is 4.5 indicating that this domain would be negatively charged at intracellular pH and is therefore unlikely to interact with the similarly charged HSP12. Indeed, a recent paper by Ito *et al.* (2001) reporting a high throughput yeast protein-protein interactome using the yeast two-hybrid system has not revealed any interaction between HSP30P and HSP12. Other work in this lab shows that HSP12, the protein envisaged to co-operate with HSP30P in its membrane stabilising role is not primarily localised at the yeast plasma membrane but in the cell wall where it appears to influence cell wall plasticity (manuscript in preparation).

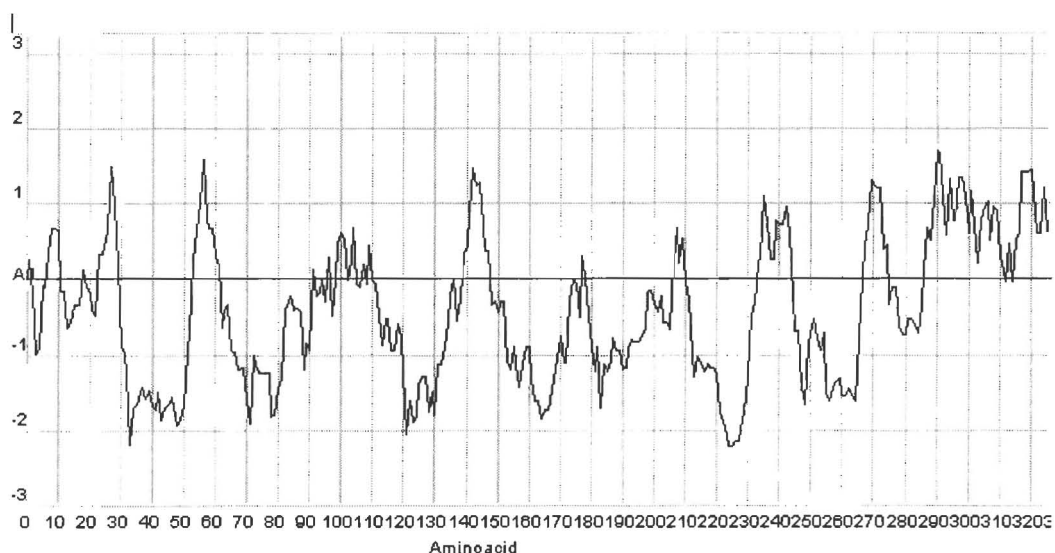
These data coupled with early work (Piper *et al.*, 1997; Braley and Piper, 1997) and the later work on the role of HSP30P in pH homeostasis through regulation of the plasma membrane ATPase (Pearce *et al.*, 2000) make the hypothetical role of stabilising the plasma membrane much less likely. In addition, as yet unknown stress inducible promoters appear to control *HSP30*. Its stress activation is largely unaffected by loss of stress transcription factors Msn2p or Msn4p (Seymour and Piper, 1999), loss of which result in prominent distortion of stress induction of HSP12. These two proteins are therefore not induced in synergy and most probably do not co-operate. The search for another candidate protein involved in membrane stabilisation in the presence of environmental stress will continue.

### 3.4 Raising polyclonal antibodies against HSP30

Immunological identification of proteins and quantification of relative expression levels is a fundamentally important tool in protein biochemistry and molecular biology. Another major objective for the unsuccessful expression of HSP30P discussed above was to ultimately produce antibodies against the purified protein. Many researchers have used synthetic peptides to raise antibodies that recognise sequences corresponding to these peptides within longer polypeptides. This approach has been used here to raise anti-peptide antibodies that should cross-react with the HSP30P protein.

To identify potential B-cell epitopes the antigenicity index plot of HSP30P (figure 3.5) was produced using an algorithm developed based on the work of Hopp and Woods, 1981. The antigenicity index plot generated is essentially the inverse of the hydropathy plot of HSP30P as presented by Regnacq and Boucherie (1993). As expected hydrophobic regions of the protein exhibit a lower antigenicity index than the mostly hydrophilic regions. This is consistent with our prediction that they would be buried within the plasma membrane, making any potential B-cell epitopes inaccessible for recognition.

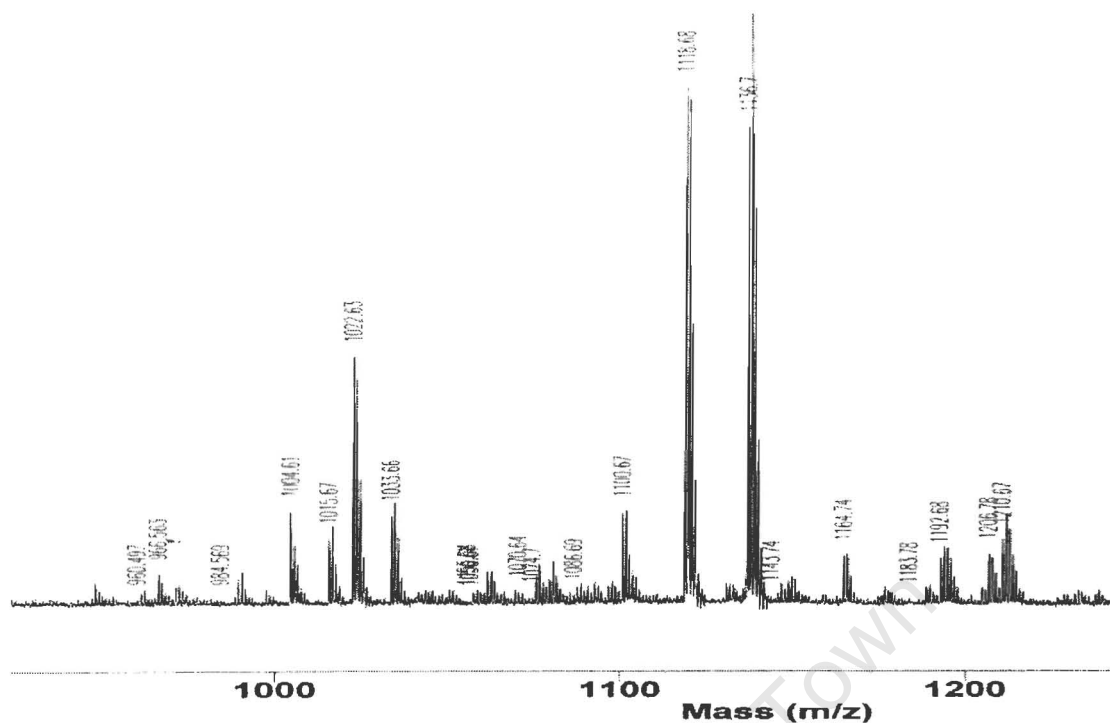
The peptide we selected for synthesis is from extracellular loop 1 of HSP30P and corresponds to amino acids 100 to 109 of HSP30P. Among the peaks shown on figure 3.5, this region had a positive but not the highest maximum. Before selecting the final peptide, we analysed antigenicity indices of 10-amino-acid sequences from regions corresponding to the peaks. We found the selected peptide to exhibit the highest antigenicity. While regions corresponding to the C-terminal domain (amino acids 271-332) exhibited equally high antigenicity, we could not synthesise peptides from this region due to our interest in investigating the role played by this domain. The potential site of glycosylation on the N-terminal domain predicted in section 3.1 further restricted our choice of peptide. If this site is indeed glycosylated *in vivo*, the epitope structure based solely on amino acid sequence in this region of the native protein may be affected.



**Figure 3.5 Antigenicity index plot of HSP30.**

The peptide was synthesised using solid-phase peptide synthesis methodology, which was pioneered by R.B. Merrifield (see Atherton and Sheppard, 1989 for a historical account of the Merrifield technique). We used a modification of this approach, which makes use of Fmoc-protected amino acid chemistry as proposed by Atherton and Sheppard and outlined in section 2.6.

After complete peptide synthesis and deprotection of the peptide side-groups, the resulting powder was analysed by MALDI-TOF mass spectrometry. The expected molecular weight of our peptide was 1118 Daltons or 1136 Daltons depending on the absence or presence of a water molecule. The spectrum for this peptide shows that the peptide powder obtained contains mainly a peptide of this size and a mixture of other peptides with larger and smaller molecular weights (figure 3.6).



**Figure 3.6 MALDI-TOF spectrum showing molecular weights of peptides in Pep1 powder.**

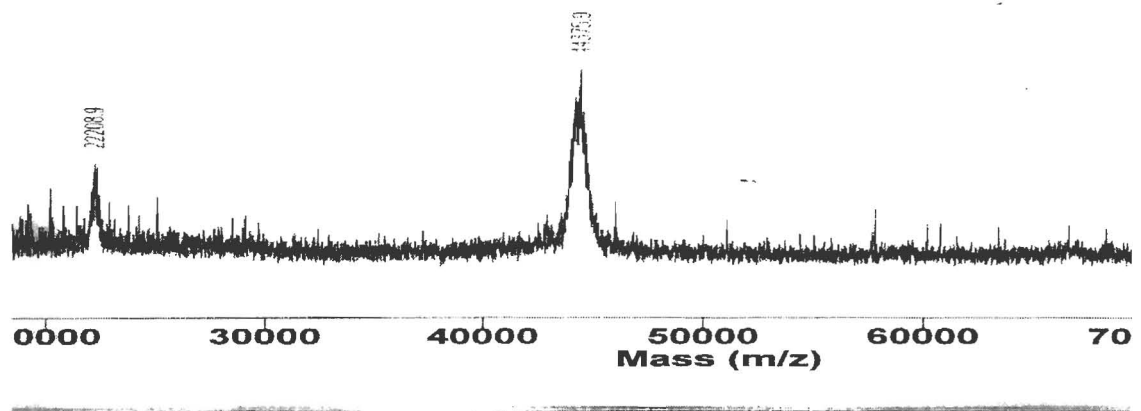
This finding was not surprising since it has been reported that, with the recommended incubation times, not all of the F-moc protecting groups are effectively removed from all amino acids at each terminal-amine deprotection step (Fields and Noble, 1989). (This is particularly the case for F-moc-glutamine residues, which must be deprotected more extensively.) Where deprotection is not complete, the subsequent amino acid cannot be coupled effectively resulting in shortened chain length. Additionally, side-chain protecting groups may also not be fully removed at the final deprotection step resulting in increased molecular weight. Atherton and Sheppard (1989) have also reported the presence of significant side reactions. For a fifteen amino acid peptide, the crude peptide powder can contain 26% of very closely related impurities.

Nonetheless, for our purpose of raising antibodies, the knowledge that the closely related peptides present in this heterogeneous mixture present uninterrupted sequences from HSP30P that are long enough to form an epitope is sufficient. As such it was not necessary to purify the peptide of the exact expected molecular weight from this mixture by HPLC.

Small peptides alone do not elicit a full immune response when injected into an animal (Hudson and Hay, 1976). They are able to bind pre-formed antibody or B-cells but will not stimulate differentiation to plasma cells and antibody production. The B-cell response to antigen requires co-operation by T-cells and the small size of synthetic peptides renders them incapable of stimulating two cells at the same time. In this study, our peptide was conjugated to chicken ovalbumin for immunization. The peptide was also conjugated to bovine serum albumin (BSA) for ELISA characterisation of the antibody. In this way we were able to differentiate between anti-ovalbumin activity and anti HSP30-peptide activity on assay. Injection with the BSA conjugate was not considered due to the common use of BSA as a blocking protein in ELISA protocols.

Conjugation was confirmed by MALDI-TOF mass spectrometry. The spectra below (figure 3.7) show that the molecular weight of the peptide-ovalbumin conjugate (Panel B) is approximately 2500Da larger than that of ovalbumin alone (Panel A) indicating that conjugation was successful. Figure 3.8 shows that the molecular weight of the peptide1-ovalbumin conjugate is on average 4000Da larger than that of BSA alone indicating that this conjugation was also successful.

A



B

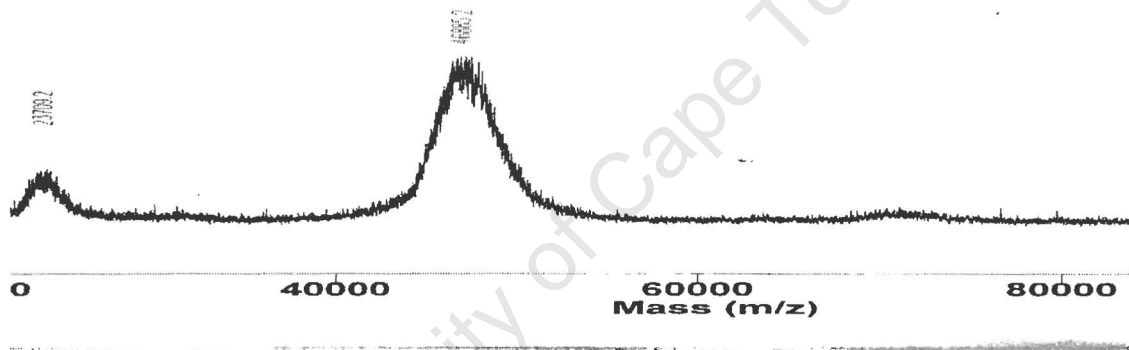
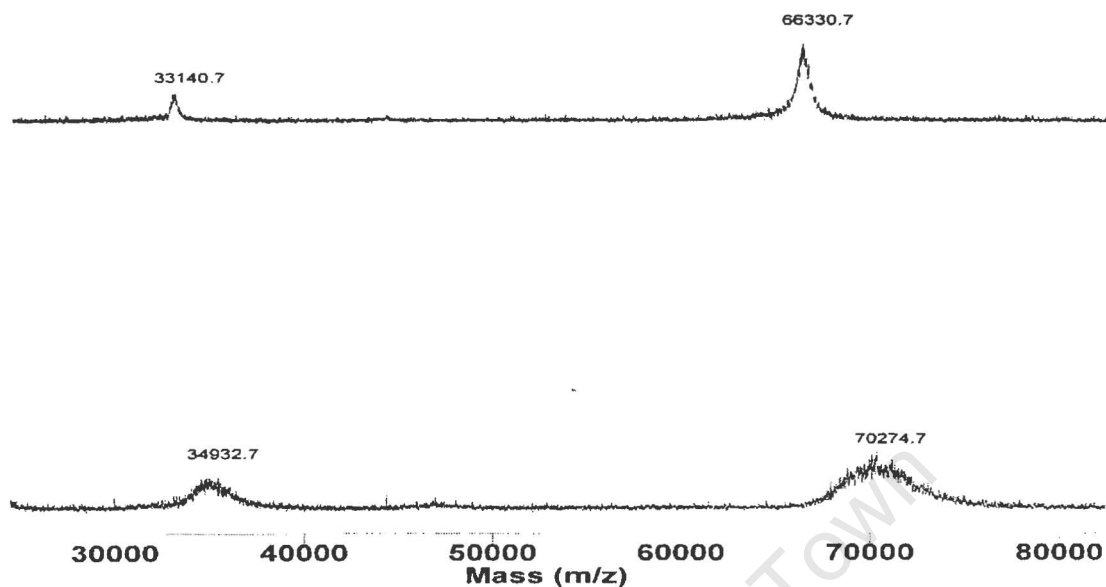


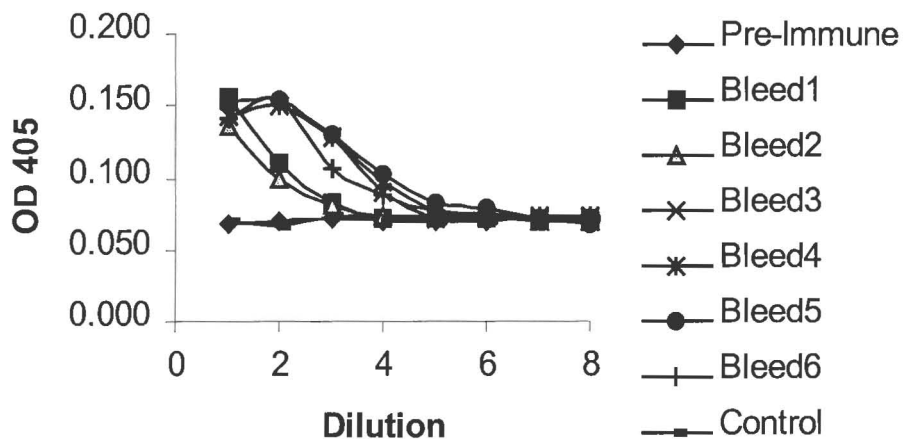
Figure 3.7 MALDI-TOF spectrum showing molecular weight of ovalbumin (Panel A) and Pep1-ovalbumin conjugate (Panel B).



**Figure 3.8 MALDI-TOF spectrum showing molecular weight of BSA (top) and Pep1-BSA conjugate (lower).**

Ovalbumin conjugates were used to immunise rabbits. Blood was collected at two-week intervals for a period of three months. After this period, antisera from each bleed were tested for anti-HSP30-peptide activity by ELISA as described in section 2.6.3. The BSA-peptide conjugate was used as antigen.

A



B

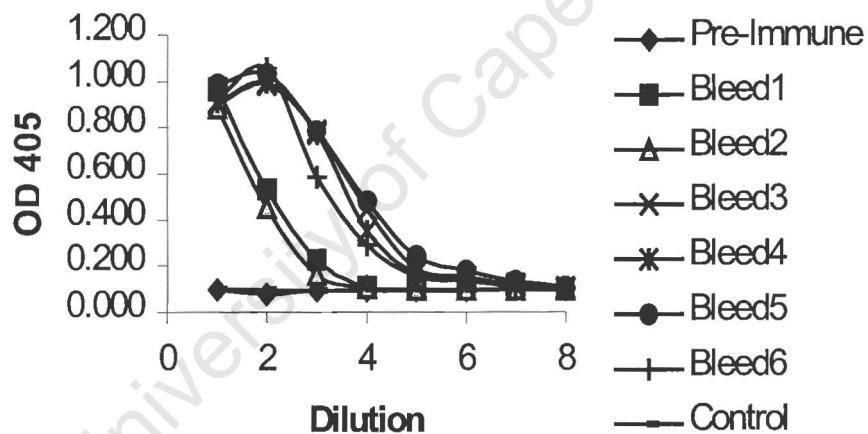


Figure 3.9 Titre determination of antisera by ELISA showing OD405 of samples after 30 minutes (A) and 16 hours (B) incubation.

It was found that the injected rabbit produced anti-HSP30-peptide antibodies. The analysis by ELISA showed that antisera from bleeds 3 and 5 show the highest anti-HSP30-peptide activity. The low OD405 reached on the standard incubation time of 30 minutes (figure 3.9 (A)) was however rather disappointing. Prolonged incubation resulted in a substantial increase in OD405 (figure 3.9 (B)). Since we have been able to show that our BSA-peptide conjugate was substantially conjugated (figure 3.8), this

suggested that there was a relatively low concentration of anti-HSP30-peptide IgG resulting in lower binding of alkaline phosphate-secondary-antibody (anti rabbit IgG) conjugate and slower conversion of pNPP to its yellow product. These results stimulated us to further investigate the 'potency' and specificity of the serum obtained. It was particularly important to establish that the anti-HSP30-peptide antibody reactivity was sufficient for chromogen based assay on nitrocellulose membranes (our Western Blot method of choice makes use of chromogenic visualisation of antibody recognition, see section 2.6.4).

10µg of antigen was blotted onto nitrocellulose paper and further processed and developed as described in section 2.6.4. Figure 3.10 shows the fully developed blot. BSA (1A), BSA-peptide conjugate (1B), ovalbumin (2A) and ovalbumin-peptide conjugate (2B) were used as antigen.



**Figure 3.10 Dot blot analysis of anti HSP30-peptide activity of serum from bleed 5. 1A: BSA, 1B: BSA-peptide conjugate, 2A: ovalbumin and 2B: ovalbumin-peptide conjugate**

It was found that the antibody did not recognise BSA alone but had good reactivity against the BSA-peptide conjugate. As expected, both ovalbumin and the ovalbumin-peptide conjugate were recognised to a greater extent.

The Hopp and Woods method for prediction of antigenic regions is widely used and has been rated as superior to other methods available (Hopp, 1993). In our case it can be seen that the antigenic peptide predicted is indeed antigenic. However other researchers have shown that software-based location of antigenic determinants has limited reliability (Van Regenmortel and Pellequer, 1994). It has particularly been shown that anti-peptide antibodies will not necessarily cross-react with epitopes in the native protein.

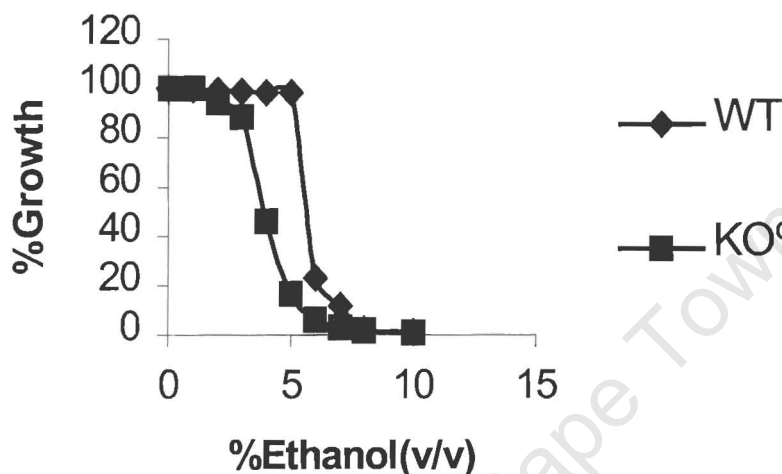
Plasma membranes were extracted from the wild type and *HSP30* knock-out strain of *S.cerevisiae* that had been grown in YPD media containing 6%(v/v) ethanol. An SDS-PAGE gel of these extracts was transferred to a nitrocellulose membrane as described in section 2.6.4. Due to its highly hydrophobic nature, transfer of HSP30P has been reported to be particularly poor (Piper *et al.*, 1997; see also HSP30 profile at the web-based yeast protein database ([www.proteome.com/ypd.htm](http://www.proteome.com/ypd.htm))). Transfer of proteins and presence of a band at the expected size for HSP30P was confirmed by staining with ponceau S. The resulting Western blot indicates that the antibody raised in this study does not cross-react with SDS-denatured HSP30P protein (not shown).

### **3.5 Manipulating Expression of HSP30 in non-stressed and ethanol stressed yeast**

#### **3.5.1 Effect of loss of HSP30 on growth in the presence of ethanol stress**

It has been shown that HSP30P is induced by ethanol stress. However, data indicating that HSP30P provides a growth advantage to ethanol treated yeast cultures has not been produced. We acquired a *HSP30* disrupted yeast strain (*HSP30* 'knock-out') (a kind gift from Prof P. Piper, University College London) and an isogenic wild type strain to investigate this. Cultures of this wild type strain and its knock-out were grown at 30°C for 48 hours in YPD media containing ethanol at concentrations ranging from 0% (v/v) (YPD medium alone) to 10% (v/v). All cultures were inoculated with pre-cultures containing approximately the same number of cells, as determined from their optical density at 600nm (OD600). OD600 reached by cells grown in YPD alone (0% ethanol) was set as 100% growth. OD600 reached by the other cultures was recorded as a percentage of this.

Wild type cells in which HSP30P is expressed normally were able to grow more successfully than the knock-out strain as the concentration of ethanol increased (figure 3.11).



**Figure 3.11 Growth of wild-type and HSP30 knock-out strains of yeast in YPD medium containing ethanol at various concentrations.**

More specifically, it was found that growth of the strain lacking HSP30P was severely compromised at ethanol concentrations as low as 5% (v/v) at which OD600 was 16% of OD600 reached by cells grown in the absence of ethanol. At this percentage of ethanol wild-type cells reach an OD600 that is as high as 98% that of cells grown in YPD alone. This percentage drops sharply to 22% in 6% ethanol. Data shown is the mean of OD600 from four independent cultures.

Extended incubation of these cultures for up to 96 hours did not result in a significant change in the profile seen in figure 3.11. This indicates that the lower OD600 reached by cultures at higher ethanol concentrations was not solely due to an extended lag phase, but a lower OD600 in the stationary phase. Effectively therefore, in the presence of ethanol the wild-type yeast cells achieved a higher final biomass than the cells lacking HSP30P.

These data conclusively showed that HSP30P provides a growth advantage to yeast cells grown in the presence of ethanol. The function of this protein is therefore of importance in the presence of ethanol stress.

As part of the introduction to this study we briefly reviewed data which indicated that HSP30P is a down-regulator of the plasma membrane  $H^+$ -ATPase. Our findings here are consistent with this view. The global effects of ethanol stress can be applied to this finding. On extended exposure to ethanol less energy in the form of ATP is produced. The electrochemical gradient across the mitochondrial membranes is dissipated. This compromises ATP synthesis by the electron transport chain with its associated ATP synthetase. Further, capacity to produce reducing power in the form of NADH and FADH is compromised due to denaturation and inactivation of enzymes associated with glycolysis and the Krebs's TCA cycle (Millar *et al.*, 1982). The plasma membrane  $H^+$ -ATPase being the single largest consumer of the ATP generated by yeast cells (Serrano *et al.*, 1991) has to be tightly regulated in these conditions. As a negative regulator of this ATPase, HSP30P can be seen as an ATP-conservation mechanism (Piper *et al.*, 1997). Perhaps the lower relative biomass yield of cells lacking HSP30P is a result of over-consumption of ATP by these cells' ATPase in the presence of ethanol stress, resulting in a lack of energy for biosynthetic purposes.

It is also possible that HSP30P further plays its ATP-conservation role by down-regulating other plasma membrane ATPases. Assessment of growth of the knock-out strain in the presence of various salts suggests that HSP30P does not regulate ATPases involved in metal ion transport across the plasma membrane. Further study will be necessary to make a firm conclusion in this regard.

### **3.5.2 Effect of constitutive expression of HSP30 in the absence of stress**

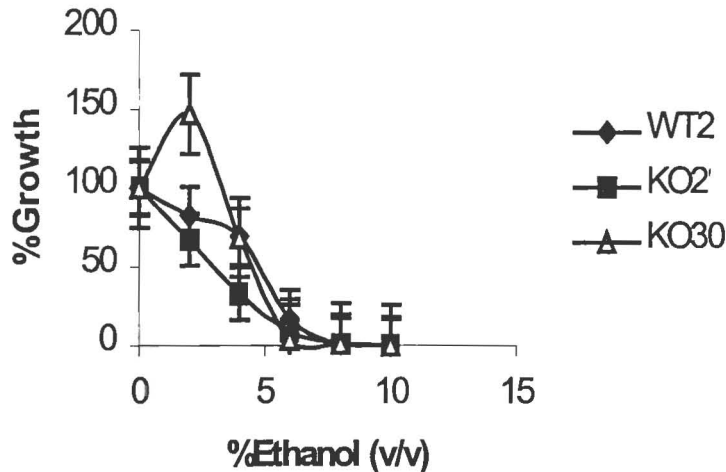
HSP30P is not normally expressed in the absence of stress. Induction in the presence of stress appears to be tightly regulated. Relative amounts of HSP30P at the plasma membrane are low and codon usage in this gene is such that expression is low. We hypothesised earlier that perhaps increased expression of this protein might be detrimental to yeast cells (section 3.2.3), probably due to the fact that this would down-regulate the plasma membrane  $H^+$ -ATPase to sub-optimal levels of activity.

Presence of HSP30P and down-regulation of the plasma membrane H<sup>+</sup>-ATPase in the absence of stress might also be unfavourable. This prospect is tested here.

*HSP30* knock-out yeast cells transformed with pHVX30 (KO30 cells) were grown in SD media lacking leucine containing 0% to 10% (v/v) ethanol. Wild-type and knock-out cells were transformed with the pHVX2 (WT2 and KO2 cells respectively) (to allow for growth in SD lacking leucine) and grown in the same conditions. In the absence of ethanol KO30 cells produce HSP30P under the control of the constitutive PGK promoter while WT2 cells do not (see figure 3.4). (The PGK promoter is induced by glucose and is termed constitutive in the presence of glucose (e.g. under standard laboratory conditions in SD or YPD media).)

All cultures were inoculated with pre-cultures containing the same number of cells, as determined from their OD600. OD600 reached by cells grown in SD alone (0% ethanol) was set as 100% growth. OD600 reached by the other cultures was recorded as a percentage of this.

The growth profile of cells that constitutively express HSP30P is dramatically different from cells expressing HSP30P under control of its endogenous promoter (figure 3.11). Data presented is the mean of OD600 from four independent cultures. It is found that KO30 cells grown in the absence of stress reach a lower OD than that reached by cells grown in the presence of 2% (v/v) ethanol.



**Figure 3.12** Growth of wild-type yeast transformed with pHVX2 (WT2), HSP30 knock-out transformed with pHVX2 (KO2) and knock-out transformed with pHVX30 (KO30) in SD medium lacking leucine containing ethanol at the stated concentrations.

More specifically, it is found that KO30 cells in 2%(v/v) ethanol reach an OD that is 146% that of cells grown in the absence of ethanol indicating that constitutive expression of HSP30P does not supply a growth advantage to non-stressed yeast. Indeed, it can be concluded that constitutive expression of HPS30 is detrimental to non-stressed yeast.

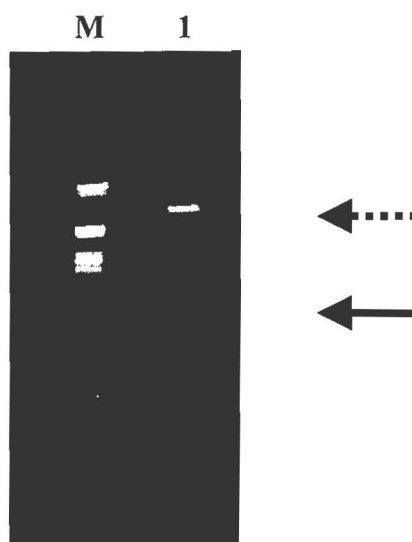
This finding is also consistent with the role of HSP30P as a negative regulator of the plasma membrane proton ATPase. Inhibition of the ATPase in the absence of stress would severely disrupt pH homeostasis and other processes that are dependent on the electrochemical gradient that this protein maintains.

### 3.6 The C-terminal intracellular domain of HSP30

Among the more interesting features highlighted by the analysis of the deduced amino acid sequence of HSP30P is its C-terminal domain. This domain has been predicted to be orientated towards the intracellular side of the plasma membrane and is highly rich in glutamic acid residues. 11 out of the 62 (18%) residues in this domain are

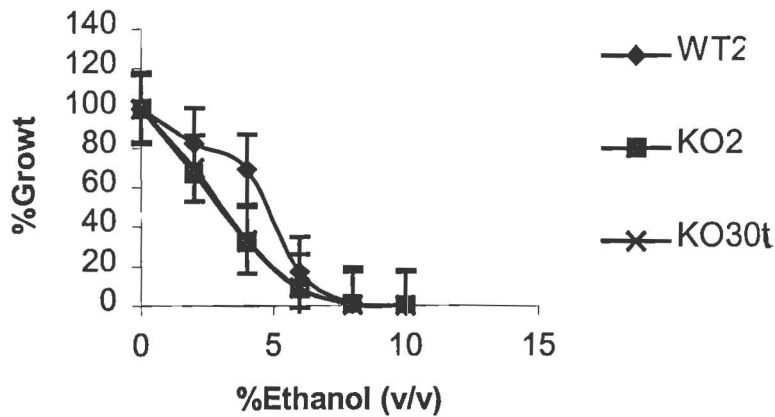
glutamic acid residues. Additionally, three of the eight predicted phosphorylation sites within HSP30P are in this region of the protein. These features suggest that this domain is likely to be important in the function of HSP30P.

A strain of *S.cerevisiae* that expresses HSP30P protein that lacks the C-terminal domain was constructed (Section 2.4). The truncated *HSP30* gene was amplified from total yeast DNA by PCR using primers described in section 2.4. In this way a stop codon was introduced after codon 270 of the full-length gene. This PCR product was digested with *EcoRI* and *XhoI*. It was then ligated to the yeast shuttle vector pHVX2 that had been digested with *EcoRI* and *XhoI* to construct pHVX30t. The ligation reaction was used to transform *E.coli* JM109 as outlined in section 2.3. Figure 3.13 shows an ethidium bromide stained agarose gel loaded with plasmid DNA isolated from these *E.coli* JM109-pHVX30t cells and digested with *EcoRI* and *XhoI*. The size of the insert was confirmed as the expected 829bp by agarose gel electrophoresis with bacteriophage lambda DNA digested with *PstI* as a size marker.



**Figure 3.13** Ethidium stained agarose gels showing cloned truncated HSP30. M: bacteriophage lambda DNA digested with PstI, 1: pHVX30t digested with XhoI and EcoRI). **←.....** : expected size of digested pHVX2 (7.5 kb) **←** : expected size of tuncated HSP30 insert (0.8kb)

pHVX30t was used to transform the *HSP30* knock-out strain of yeast (to construct KO30t cells). Cultures of this strain, the KO30 strain and the KO2 strain were grown at 30<sup>0</sup>C for 48 hours in SD lacking leucine and containing ethanol at concentrations ranging from 0% (v/v) (SD alone) to 12% (v/v). All cultures were inoculated with pre-cultures containing approximately the same number of cells, as determined from their optical density at 600nm (OD600). OD600 reached by cells grown in the absence of ethanol was set as 100% growth for each strain. OD600 reached by the other cultures was recorded as a percentage of this.



**Figure 3.14** Growth of wild-type transformed with pHVX2 (WT2), HSP30 knock-out transformed with pHVX2 (KO2), knock-out transformed with pHVX30 (KO30) and knock-out transformed with pHVX30t (KO30t) in SD medium lacking leucine containing ethanol at the stated concentrations.

It is found that truncated HSP30P had no rescue effect on yeast cells grown in the presence of increasing ethanol stress (figure 3.14). KO30t cells have a phenotype that is similar to that of *HSP30* knock-out cells. It would be premature to firmly conclude that the C-terminal domain of HSP30P is essential for the protein to function properly and provide yeast cells with a growth advantage due to its action at the plasma membrane. This observed phenotype could potentially be a result of non-incorporation of the truncated HSP30P into the plasma membrane on loss of this domain.

To confirm that the observed loss of function is a result of loss of the C-terminal domain of HSP30P and not lack of incorporation, plasma membrane proteins were extracted from the *HSP30* knock-out strain that has been transformed with pHVX30t. Analysis of this extract by SDS-PAGE was not sufficient to determine presence of the truncated protein. The full-length protein has aberrant mobility on SDS-PAGE (Regnacq and Boucherie, 1993). It was therefore difficult to predict whether this truncated protein would run at the expected 30kDa (this is incidentally the approximate size at which the full-length 37kDa protein runs).

The extract was subjected to Western blot analysis (section 2.6.4) using antibodies raised as described in section 3.3 and did not show presence of truncated HSP30P.

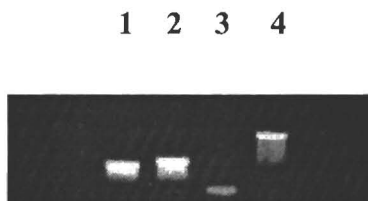
The lack of HSP30 cross-reactivity of the antibody raised in this study are discussed in section 3.3.

Truncation of proteins is a useful approach in elucidating regions that are important for proper function of a protein. In this study however, we have genetically removed a substantial portion of the protein. The putative C-terminal domain of HSP30P corresponds to no less than 16% of the total molecular weight of the full-length protein. It is thus not particularly surprising that loss of this domain results in loss of proper incorporation and/or function. We felt it was important to further pinpoint smaller regions of this domain and HSP30P as a whole that enable it to effectively regulate the plasma membrane proton ATPase. The succeeding section reports and discusses construction of site-directed mutants of one amino acid residue from the C-terminal domain as well as another residue from an extracellular loop of HSP30P.

### **3.7 Mutation of potential phosphorylation sites in HSP30**

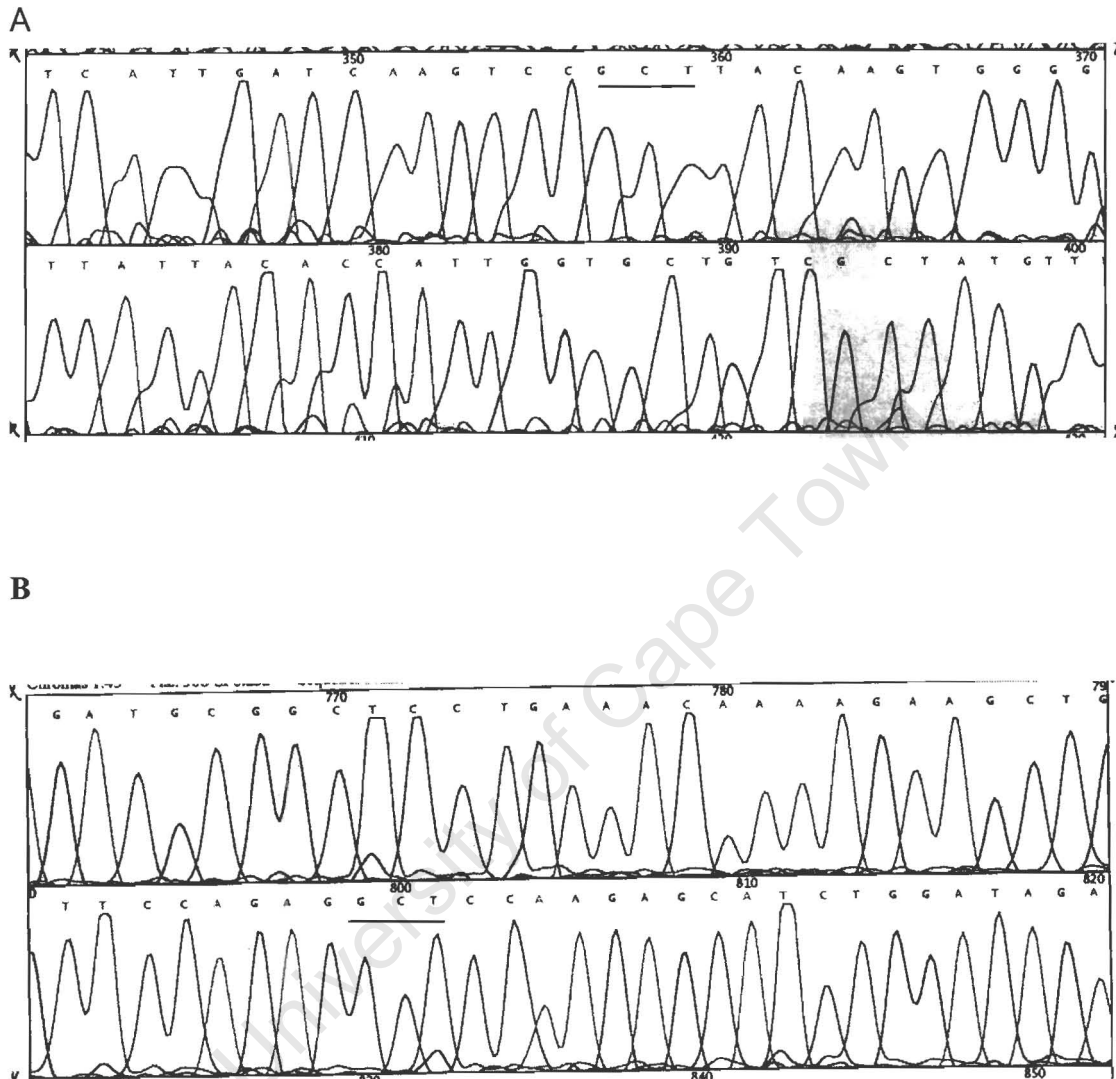
The function of several proteins *in vivo* is regulated by phosphorylation events that are catalysed by a large family of protein kinases. Catalytic domains of serine/threonine kinases share a high degree of homology and the mechanism of substrate recognition is similar among all of them. Songyang *et al.* (1994) have shown that a region of seven to twelve amino acids adjacent to the phosphate acceptor site is important for recognition. Based on this and other work a sequence and structure-based prediction tool for eukaryotic protein phosphorylation sites was developed using a neural network method (Blom *et al.*, 1999). This world wide web-based tool, NetPhos has been used here to identify serine and threonine residues that are likely to be phosphorylated in HSP30P. In section 3.1 we describe the locations of these potential phosphorylation sites. Two of these sites have been selected for mutagenesis to investigate whether they are biochemically active. Threonine 181 (from extracellular loop 2) and serine 308 (from C-terminal intracellular domain) were mutagenised to alanine residues using the four-primer method of site directed mutagenesis. Besides the various commercially available site-directed mutagenesis kits, this approach appeared to be the least tedious, most reliable and is currently more widely used than other approaches. The procedure is described in detail in Section

2.5. Mutagenised partial gene fragments were successfully amplified using *Pfu* DNA polymerase (figure 3.15). *Pfu* DNA polymerase was preferred for use in this application over *Taq* DNA polymerase due to its higher fidelity and the knowledge that this enzyme does not incorporate adenosine bases at the 3' ends of amplified DNA.



**Figure 3.15 Agarose gel showing mutagenised partial gene fragments. 1: T181A small fragment (478bp), 2: T181A large fragment (567bp), 3: S308A small fragment (313bp) 4: S308A large fragment (948bp)**

These fragments were used as template and self-primers to generate the full-length mutant *HSP30* genes, which were then cloned into pGEM T-easy (Promega) according to manufacturer's instructions. The cloned regions of the constructs prepared for each mutant were then sequenced to confirm mutagenesis. Figure 3.16 shows the sequence of the mutagenised region of each construct. Sequences corresponding to the primers used as well as the flanking regions on either side of the primer-derived sequence are shown.

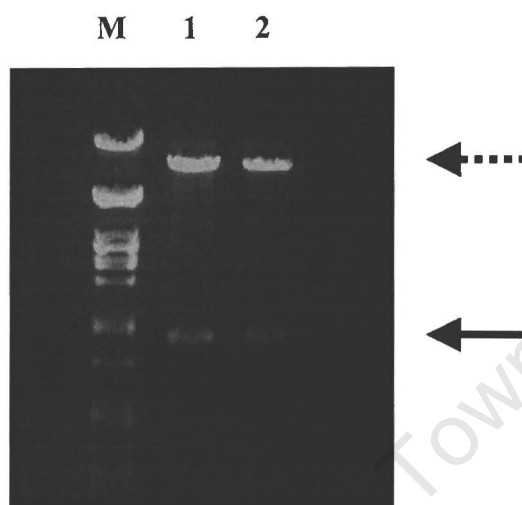


**Figure 3.16 Sequences of mutagenised regions of site-directed mutants of HSP30.**

**A: T181A, B: S308A. New alanine codons are underlined.**

It is found that both constructs incorporated a new alanine codon at the expected location within the *HSP30* gene. Comparison of these sequences with the nucleotide sequence of wild-type *HSP30* (Regnacq and Boucherie, 1993) confirms that only the mutated codons differ. Each of the mutant genes was sub-cloned into the yeast shuttle vector pHVX2. The ligation reaction in each case was used to transform *E.coli* JM109 as outlined in section 2.3. Figure 3.17 shows an ethidium bromide stained agarose gel loaded with plasmid DNA isolated from these *E.coli* JM109-pHVX30 cells and digested with *EcoRI* and *XhoI*.

Both preparations show two bands indicating that the PCR-generated *HSP30* was successfully cloned. The size of the insert was confirmed as the expected 1026bp by agarose gel electrophoresis with bacteriophage lambda DNA digested with Pst1 used as size marker. The resulting constructs were termed pHVX30-T181A and pHVX30-S308A respectively.



**Figure 3.17 Ethidium stained agarose gels showing cloned mutant HSP30. M: bacteriophage lambda DNA digested with Pst1, 1: pHVX30-T181A digested with Xho1 and EcoR1, 2: pHVX30-S308A digested with Xho1 and EcoR1. ←..... : expected size of digested pHVX2 (7.5 kb) ← : expected size of truncated HSP30 insert (1kb)**

NetPhos, the predictive tool for potential phosphorylation sites we have used here takes an “overall, general approach to kinase specificity” and includes all experimentally verified phosphorylation sites in Phosphobase at the time of creation (Blom *et al.*, 1999). *HSP30P* has a plasma membrane localisation and the compartmentalised nature of the yeast cell means that some of the phosphorylation sites predicted may not be active *in vivo*. The protein-protein interactions necessary for phosphorylation may be physically impossible.

In *S.cerevisiae*, there is a plasma membrane associated kinase, casein kinase 1 (CK1), which has been shown to regulate activity of the plasma membrane proton ATPase (Estrada *et al.*, 1996). We envisage that the stress-induced regulator of this ATPase, *HSP30P* is also a substrate for this kinase. It is quite plausible to further hypothesise that CK1 provides a means of ‘fine-tuning’ the crucial activity of the plasma membrane ATPase in the presence of ethanol and other stresses.

Future work in this lab will therefore be aimed at constructing strains of *S.cerevisiae* expressing alanine mutants of all the putative sites of phosphorylation in HSP30P. We plan to express these mutants in a yeast centromeric plasmid (an extremely stable single copy plasmid) under control of the endogenous promoter of HSP30P. In this way we will be more closely mimicking the wild type scenario in that the only major difference between these strains and wild-type yeast will be the lack of the putative site of phosphorylation. Thus normal co-ordination of gene induction and protein regulation will be intact.

University of Cape Town

## Chapter 4:

### Conclusion

The results presented in this study revealed several distinct findings.

HSP30P has been predicted here to be a multi-pass integral membrane protein that traverses the membrane via seven alpha helices. The topology is such that a small N-terminal domain faces the extracellular side of the plasma membrane while a larger C-terminal domain faces the cytosol. Analysis of the amino acid sequence of HSP30P further reveals one potential site of glycosylation and several potential sites of phosphorylation.

HSP30P has proved to be a difficult protein to manipulate. Both heterologous and homologous expression under control of strong promoters yielded amounts of protein that did not warrant further purification. Analysis of the open reading frame of *HSP30* reveals a codon adaptation index that inclines this protein to low expression in both hosts used in this study (*E.coli* and *S.cerevisiae*). We propose that this low codon adaptation index is as such because expression of larger amounts of the protein would be detrimental to yeast cells, and went some way in showing this by expressing HSP30P in the absence of stress. We show that constitutive expression is detrimental to yeast growth.

Lack of expression of HSP30P made it difficult to experimentally investigate the hypothetical role of HSP30P in stabilising membranes, the primary aim of this study. However, an analysis of the literature and other work carried out in our laboratory in parallel with this study makes this role of stabilising the yeast plasma membrane in co-operation with HSP12 unlikely.

Ethanol, the model stress used in this work has previously been shown to induce expression of HSP30P (Piper *et al.*, 1997). We have further shown in this study that this induction of HSP30P provided yeast with a growth advantage in the presence of increasing ethanol stress. Growth of a *HSP30* knock-out strain of yeast was compromised in the presence of ethanol. It has also been shown here that constitutive

expression of HSP30P is detrimental to yeast cells. These findings are consistent with the emerging consensus that HSP30P is a stress-induced regulator of the plasma membrane proton ATPase, Pma1.

Assessment of growth of a yeast mutant lacking HSP30 in the presence of various salts suggested that HSP30P is not important for regulation of ATPases involved in metal ion transport across the plasma membrane. Further study will be necessary to make a firm conclusion in this regard.

The C-terminal domain of HSP30P, which we predicted here to have an intracellular orientation, exhibits several remarkable features. These include a high glutamic acid content and three potential sites of phosphorylation. We have shown in this work that loss of this domain from HSP30P resulted in an ethanol tolerance similar to that of the *HSP30* knock-out strain of yeast indicating that the C-terminal domain is essential for the protein to function properly or become incorporated into the plasma membrane. Growth of a strain constructed to express HSP30P lacking this domain was compromised in the presence of ethanol. We further selected one out of three of the potential sites of phosphorylation in this domain; serine 308 for mutagenesis to an alanine residue. Another potential site of phosphorylation, threonine 181 from extracellular loop 2 was also mutated. We plan to construct strains of *S.cerevisiae* expressing alanine mutants of all the putative sites of phosphorylation.

The results of this work are consistent with the view that HSP30P is a stress inducible down-regulator of the plasma membrane proton ATPase, Pma1. Further work will be aimed at assessing the activity of this ATPase in all the yeast strains we constructed. Assay of ATPase activity in these strains will be more sensitive than simply assaying growth of the strains in ethanol and should enable us to make firm conclusions about the role of HSP30P, its C-terminal domain and specific residues in the presence of ethanol stress.

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