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**YEAST CELL WALL PROTEOMICS: A TALE OF TWO PROTEINS**

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“Victories often occur after you see no way to succeed but refuse to give up”. (Bits & Pieces magazine ).

**ABBREVIATIONS**

ABC	ATP-binding cassette
ADP	Adenosine diphosphate
CTAB	Cetyltrimethyl ammonium bromide
CH <sub>3</sub> CN	Acetonitrile
Da	Dalton
EC	Enzyme commission
EDTA	Ethylenediaminetetra-acetic acid
ELISA	Enzyme-linked immunosorbance assay
FDA	Fluorescein diacetate
HSP	Heat shock protein
KDa	Kilodalton
LEA	Late embryogenesis abundant
M	Molar
MALDI-TOF	Matrix assisted laser desorption/ionisation-time of flight
NADH	Nicotinamide adenine dinucleotide (reduced form)
NaOH	Sodium hydroxide
PDR	Pleiotropic drug resistance
PGM	Phosphoglycerate mutase
SDS-PAGE	Sodium dodecyl sulphate-polyacrlamide gel electrophoresis
TEM	Transmission electron microscope
TFA	Trifluoroacetic acid
Tris-HCl	TRIS-(hydroxymethyl)-amonomethane

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## CHAPTER 1.

### 1.1 GENERAL INTRODUCTION.

#### 1.11 *Yeast cell wall architecture.*

The yeast cell wall is the immediate site of contact between the yeast cell and its environment. The cell wall is dynamic and changes structure and composition in response to a wide range of environmental conditions [8, 15]. It is not only essential for maintaining cell integrity, shape and protection but also plays a role in morphogenesis, flocculation, cell-cell recognition and pathogenicity [12, 15].

The cell wall is composed primarily of mannoproteins (designated CWP in Figure 1), glucans and chitin (Figure 1). Mannoproteins are found both in the periplasmic space and as part of the outer surface of the cell wall where they interact with the environment as well as with other mannoproteins via disulphide linkages. The glucans and chitin form the inner surface of the wall.

Mannoproteins determine cell wall porosity, act as structural proteins, and can be enzymes involved in cell wall biogenesis [11, 12]. These proteins have mannose polymers (designated sugar chains) covalently linked to them [2] either as N-glycans or O-glycans. The former consist of 8-15 mannose residues linked to nitrogen atom of the amide group of asparagine residues whereas the latter consist of between 1 and 5 mannose residues linked to the oxygen atom of the hydroxyl groups of either threonine or serine residues. Three different methods of attachment of the mannoproteins to the cell wall have been described. These are:

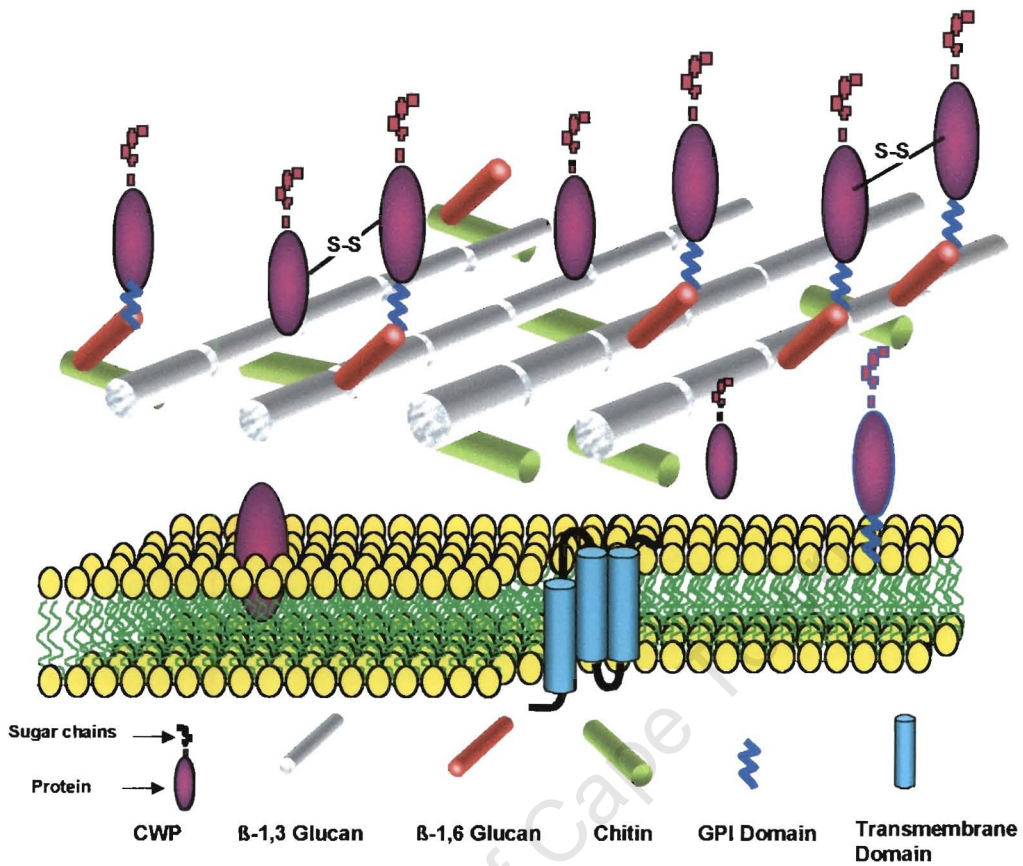
1. The attachment of C-terminus of the protein to glycosyl phosphatidylinositol (GPI) anchors. These anchors are attached to  $\beta$  1,6-glucans, which in turn are attached to chitin via  $\beta$  1,3-glucans.

2. Direct attachment to  $\beta$  1,3-glucans, for example proteins with internal repeats (Pir) [11].

3. Direct attachment to Chitin.

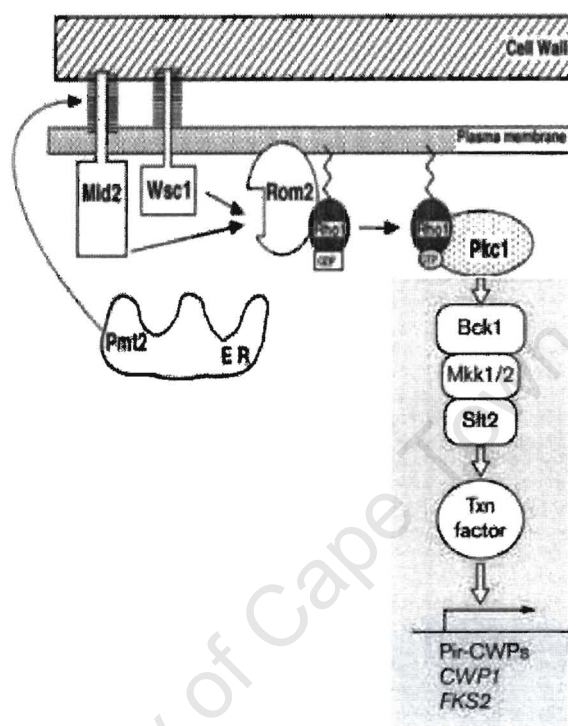
Glucans are glucose polymers that are formed either through  $\beta$ 1,3 or  $\beta$ 1,6 linkages and are responsible for cell wall strength.  $\beta$ 1,6 glucans are small molecules approximately 140 residues in length that link the components of the inner and the outer walls [9]. In contrast,  $\beta$ 1,3 glucans are the main structural components of the cell wall. They consist of about 1500 glucose residues [7] and form complexes with chitin [9]. Although the majority of the glucose residues are  $\beta$ 1,3 linked, some degree of branching via 1,6 linkages has been reported [7].

Chitin it is a linear polysaccharide that consists of  $\beta$ 1,4-linked N-acetylglucosamine repeating units. These are glycosidically linked to non-reducing ends of  $\beta$ 1,3 and  $\beta$ 1,6 glucans. It plays a structural role and has considerable mechanical strength.



**Figure 1.** A schematic representation of the yeast *Saccharomyces cerevisiae* cell wall. Major components of the cell wall are shown. (Courtesy of Professor Maria Molina [11]).

### 1.12 Molecular responses of the cell wall to changes in the environment.



**Figure 2.** A model of the yeast cell wall integrity pathway [13, 15].

The yeast cell wall is remodelled in response to environmental changes. This modelling is monitored and regulated by the cell integrity-signalling pathway [13]. A model of this pathway is shown in figure 2.

Stress is sensed by transmembrane proteins such as Wsc1-4 or Mid2 [4, 10, 13, 14]. These proteins have extracellular domains extending into the periplasmic space [13]. These domains have been proposed to act as rigid probes for the extracellular matrix [13]. Stress, sensed by these extracellular domains, activates the exchange factor Rom2, which in turn activates Rho1 by exchanging bound GDP for GTP [13]. Rho1

activates the glucan synthases Fks1 and Fks2 as well as protein kinase C (Pkc1). Protein kinase C activates the MAP kinase pathway including the MAP kinases Bck1, Mkk1/2 and Slk2.

Activation of the cell integrity-signalling pathway leads to an increase in  $\beta$ 1,3 glucan synthesis. This process is required for cell wall remodelling, due to  $\beta$ 1,3 glucans being structural components of the cell wall. The MAP kinases, which are also activated by stress, lead to the activation of genes that code for cell wall proteins, proteins with internal repeats (Pir) and cell wall biosynthetic enzymes [15].

#### 1.13 Glycolytic enzymes in the cell wall.

Recently, proteomic studies of yeast cell wall proteins have demonstrated the presence of glycolytic enzymes in addition to the already known cell wall proteins. In *S. cerevisiae*, the glycolytic enzymes enolase [5] and glyceraldehyde-3-phosphate dehydrogenase [3] have been located in the cell wall using immunocytochemical techniques. The presence of glycolytic enzymes has also been reported in the pathogenic yeast *C. albicans* [1, 6]. Pardo et al have confirmed the presence of glycolytic enzymes in the cell wall by regenerating protoplasts and showing that these enzymes are secreted into the medium. All the enzymes of the lower ATP-forming part of the glycolytic pathway from the triose phosphate isomerase mediated conversion of dihydroxyacetone phosphate to glyceraldehyde-3-phosphate as well as those responsible for the conversion of pyruvate to ethanol have been identified [12]. Thus yeast cell walls can theoretically convert dihydroxyacetone phosphate to ethanol using enzymes present in the cell wall. In contrast, enzymes corresponding to the

ATP-using part of glycolysis have not been found, although their possible existence was not dismissed.

## **1.2 AIM.**

This thesis investigates cell wall proteins, the presence of which increased in concentration as a result of stress. Two such proteins were found, phosphoglycerate mutase and Hsp 12. Studies on these proteins are reported in chapters 2 (phosphoglycerate mutase) and chapter 3 (Hsp 12).

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## CHAPTER 2.

### **SIGNIFICANT QUANTITIES OF THE GLYCOLYTIC ENZYME PHOSPHOGLYCERATE MUTASE ARE PRESENT IN THE CELL WALL OF YEAST, *SACCHAROMYCES CEREVISIAE*.**

#### **2.1 ABSTRACT.**

NaOH was used to extract proteins from the cell walls of the yeast *Saccharomyces cerevisiae*. This treatment was shown not to disrupt yeast cells as NaOH extracted cells displayed a normal morphology upon electron microscopy. Moreover, extracted and untreated cells had qualitatively similar protein contents upon disruption. When yeast were grown in the presence of 1 M mannitol, two proteins were found to be present at an elevated concentration in the cell wall. These were found to be the LEA-like protein Hsp 12 and the glycolytic enzyme phosphoglycerate mutase. The presence of phosphoglycerate mutase in the cell wall was confirmed by immunocytochemical analysis. Not only was the phosphoglycerate mutase in the yeast cell wall found to be active, but whole yeast cells were also able to convert 3-phosphoglycerate in the medium to ethanol providing that the necessary cofactors were present.

**KEYWORDS:** Phosphoglycerate mutase, yeast, cell wall, alkaline extraction.

## 2.2 INTRODUCTION.

The cell wall is the immediate site of contact between the yeast cell and its environment. Recently considerable work has been reported on the effects of cell wall perturbation caused by environmental changes [5]. A number of plasma membrane sensors, including Wsc1-4, Mid 2 and Mtl1 [5, 11, 19, 20], have been shown to activate the GTP-binding Rho1 factor via the Tor2 phosphatidylinositol-4-kinase and the Rom2 exchange factor. Rho1 activates both the glucan synthases Fks 1 and Fks 2 as well as protein kinase C (Pkc1) [19, 20], which in turn activates the MAP kinase pathway. This pathway results in the transcription of a number of genes including genes for transcription factors such as Slt2, cell wall proteins such as the Pir proteins, enzymes possibly involved in cell wall remodelling (Fks2) [5, 22] as well as several genes of unknown function.

The elucidation of such pathways has been performed by the use of deletion mutants and the analysis of mRNA transcript levels, the concentrations of which change in response to stress. Although this methodology allows the integration of a number of gene products into a pathway, no information is acquired on the relative abundance of the proteins produced by these transcripts as well as on the role of these proteins. We have adopted a different approach to investigate cell wall proteins whose syntheses are up-regulated under conditions of stress. We characterised proteins extracted under alkaline conditions from both whole yeast cells and from isolated cell walls and found that two proteins were present at an increased concentration in the cell walls of yeast grown in the presence of hyperosmolar concentrations of mannitol. One of these was the stress response protein Hsp 12, the transcript of which is known to be elevated in

response to stress. The other protein was the glycolytic enzyme phosphoglycerate mutase.

Some enzymes of the glycolytic pathway have been found to be excreted by regenerating protoplasts of yeast [18], and therefore hypothesised to be present in mature cell walls. These enzymes include those of the ATP-generating portion of the pathway as well fructose bisphosphate aldolase and triose phosphate isomerase. Glycolytic enzymes have also been shown to be present in cell walls of yeast by mass spectrometry of cell wall proteins separated by 2-dimensional SDS-PAGE [13, 18] or by the binding of antibodies against specific glycolytic enzymes to the exterior of whole yeast cells [1, 4, 6, 7]. In this manuscript we provide immunocytochemical evidence demonstrating that significant quantities of phosphoglycerate mutase are present in the cell walls of yeast and show that whole yeast cells can convert 3-phosphoglycerate in the medium to ethanol in the presence of the required cofactors.

## 2.3 METHODS.

### *2.31 Extraction of phosphoglycerate mutase from cells and cell walls.*

All operations were performed at 4 °C unless specified. A 4 g sample of wet packed yeast (Anchor Yeast, Cape Town) was washed with 100 mM Tris-HCl 50mM NaCl pH 7.4 before alkaline soluble proteins were extracted with 4 ml NaOH for 30min on ice. Cell walls were prepared by extensive abrasion at 4 °C in the Ball-mill (Braun) using an equal weight of 0.75 – 1.125 micron diameter glass beads. The absence of intact cells was confirmed by the absence of colonies when the cell wall preparation was plated on 1.5 % (w/v) agar YPD plates and incubated at 30 °C.

### *2.32 Isolation, trypsin digestion and mass spectrometry of phosphoglycerate mutase.*

Yeast cells (4 g) were treated with 1 M NaOH on ice for 30 min after which they were washed extensively with 100 mM Tris-HCl 50mM NaCl pH 7.4 before being Ball-milled for 20 s in 8 ml of the same buffer. The supernatant fraction was electrophoresed over an entire 200 mm X 200 mm 20 % SDS gel [10]. The desired area of the Coomassie stained gel was manually excised and cut into small pieces. The pieces were washed twice with distilled water and subsequently soaked in 0.5 % (w/v) CTAB for 1 h before being electroeluted at constant current in 0.9 % (v/v) acetic acid for 16 h. The extract was lyophilised, dissolved in the minimum volume of distilled water and the protein precipitated with ice-cold acetone-20 mM HCl. The precipitate, which contained pure protein as assessed by SDS-PAGE, was washed three times with ice-cold acetone, dried under vacuum and stored at -20 °C. Protein

sequencing was done in gas-liquid solid phase sequencer. 1 nmol of pure protein was applied onto a glass filter. The reagents, solvents and degradation cycle were as previously described [3]. The converted amino acids were identified by an isocratic on-line HPLC system on 250 x 3 mm 3  $\mu$ m Lichosphere C<sub>18</sub> column.

Trypsin digestion and MALDI TOF mass spectrometry was carried out essentially as described by Wilm et al., 1996. A piece of the SDS-PAGE gel containing the Coomassie stained protein was cut into small pieces. Following sequential incubations in 50 % (v/v) CH<sub>3</sub>CN for 5 minutes, in 50 % CH<sub>3</sub>CN 50 mM NH<sub>4</sub>HCO<sub>3</sub> and in 50% CH<sub>3</sub>CN 10 mM NH<sub>4</sub>HCO<sub>3</sub> for 30 minutes each, the gel pieces were dried under vacuum. Trypsin (Promega) digestion of the pieces re-hydrated in 10 mM NH<sub>4</sub>HCO<sub>3</sub> buffer was performed for 16 h at 37 °C using of 0.1 $\mu$ g trypsin per 15 mm<sup>3</sup> of gel. Mass analysis was performed by mixing 1  $\mu$ l of the tryptic digest with 1  $\mu$ l matrix (10 mg/mL  $\alpha$ -cyano-4-hydroxycinnamic acid in 60% acetonitrile/ 0.3% TFA) followed by spotting 1  $\mu$ l of this mixture onto the MALDI plate and air drying. The tryptic auto-digestion peak at 2162 Da was used as an internal standard. The identified masses were subjected to a database (SwissProt.6.12.2000) search using the MS-fit software version 3.1.1 [2].

### *2.33 Immunochemical methods and electron microscopy.*

Pure phosphoglycerate mutase, prepared by electroelution after SDS-PAGE, at a concentration of 0.4 mg/ml in distilled water, was mixed with an equal volume of Freund's complete adjuvant and used to immunise rabbits with four injections at bi-weekly intervals. Titres and specificity were determined by Elisa [8].

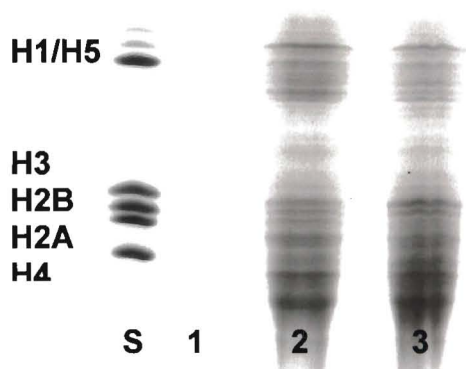
Immunocytochemistry was performed as described previously [21] except that the yeast cells were fixed using 0.5 % glutaraldehyde 4 % paraformaldehyde in  $K_2HPO_4$  pH 7.0 for 16 hours at 4°C, embedded in low melting point agarose, and infiltrated with 2.3 M sucrose as a cryoprotectant. Thereafter, they were mounted on small stubs and rapidly plunge-frozen in liquid nitrogen (-196 °C). Ultrathin sections were cut using a Reichert Ultra Cut S cryomicrotome. The cutting temperature was -120 °C. Sections were retrieved onto carbon coated, glow discharged nickel grids and immunolabelled as described by Tokuyasu, 1986.

#### *2.34 Phosphoglycerate mutase assay.*

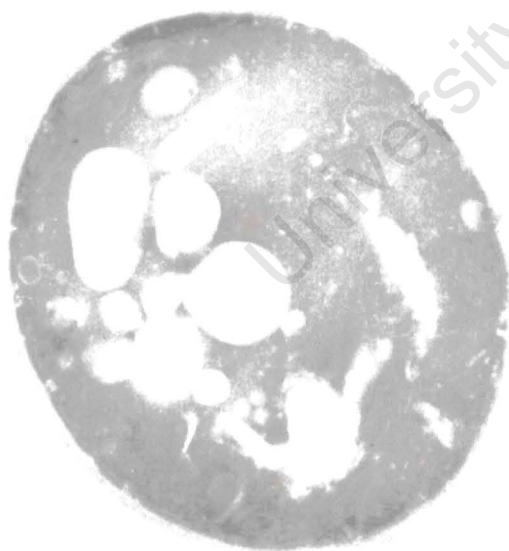
Phosphoglycerate mutase activity of yeast cell extracts and electroeluted fractions was assayed as described [23] with minor modifications. A 0.9 ml volume of a mixture containing 2 mM 3-phosphoglycerate, 0.15 mM NADH +  $H^+$ , 1 mM ADP, 0.5 mM 2,3-bisphosphoglycerate, 0.9 mM EDTA, 2.8 U/ml lactate dehydrogenase (E.C. 1.1.1.28), 7 U/ml pyruvate kinase (E.C. 2.7.1.40) and 3 U/ml enolase (E.C. 4.2.1.11) in 100 mM Tris-HCl pH 7.0 100 mM KCl 5 mM  $MgSO_4$  buffer was used for the assay. A 100  $\mu$ l aliquot of a sample containing phosphoglycerate mutase (E.C. 5.4.2.1) was used to start the reaction. Phosphoglycerate mutase activity of whole yeast cells was assayed essentially as described previously [4, 7]. A suspension of whole cells replaced the soluble sample in the above assay; the 340 nm absorption was determined after removal of the cells by brief centrifugation. Yeast cells were quantitated by the light scattering at 600 nm ( $A_{600}$ ).

## 2.4 RESULTS

NaOH and NaOH/SDS solutions are commonly used for the extraction of proteins from purified cell walls [14,15, 16]. We initially investigated whether dilute NaOH solutions would extract proteins from the cell walls of whole yeast cells without disrupting the cell and releasing the cytoplasmic contents. Whole washed yeast cells were therefore extracted with 0.2 M NaOH for 30 min at 0 °C after which the extracted proteins were analysed by SDS-PAGE (Figure 1). We found that 0.2 M NaOH extracted only a discrete number of proteins from the cell, and that when the cells were examined by electron microscopy after such an extraction, the cells had a normal morphologic appearance with internal organellar structures and an intact cell wall (Figure 2). To confirm that this treatment did not cause disruption of the yeast cell, cells were first extracted with 0.2 M NaOH and then disrupted in the ball-mill. Soluble intracellular proteins from these cells were compared with untreated cells by SDS-PAGE. The complement of soluble proteins from 0.2 M NaOH-treated cells and untreated cells were qualitatively similar (Figure 1). Increasing the NaOH concentration to 1 M resulted in an increased concentration of the same proteins being present in the extract. Again there was no evidence that cell lysis had occurred, as the cells appeared intact by electron microscopy, and SDS-PAGE of the extracted proteins was essentially identical to that after 0.2 M NaOH treatment (not shown).

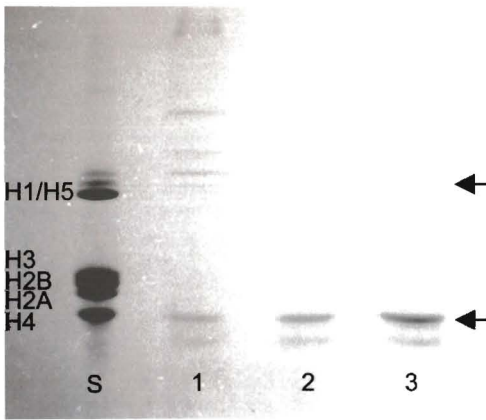


**Figure 1.** SDS-PAGE of proteins extracted from whole washed yeast cells with 0.2 M NaOH (lane 1) or by Ball-milling 0.2 M NaOH-treated cells (lane 2) or untreated cells (lane 3). The standard (S) is a total extract of chicken erythrocyte histones, the molecular weights (kDa) of which are: H1, 22.5; H3, 15.3; H2B, 13.7; H2A, 14.0 and H4, 11.2.

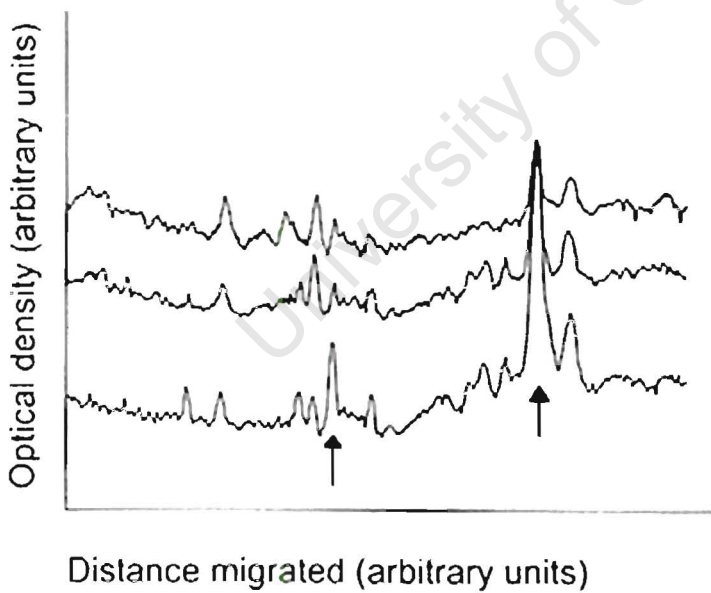


**Figure 2.** Electron micrograph (15 000 X magnification) of a whole washed yeast cell after extraction with 0.2 M NaOH.

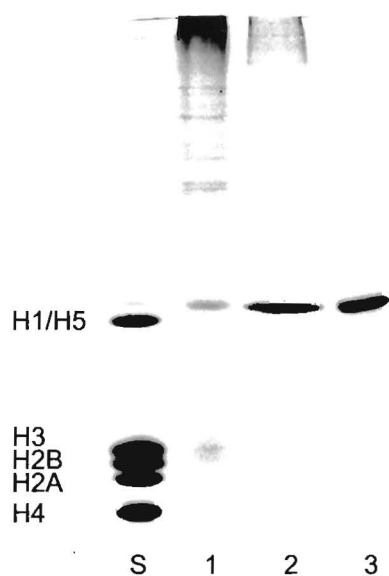
Whole washed yeast cells, grown to early stationary phase in YPD medium containing increasing amounts of mannitol, were therefore extracted with 1 M NaOH and the extracted proteins subjected to SDS-PAGE (Figure 3a). Two proteins, arrowed, were found to increase in content in response to increased mannitol concentration in the growth medium (Figure 3b). These proteins had molecular sizes of approximately 12 kDa and 25 kDa; the one protein with a molecular mass of 12 kDa was identified as Hsp 12 by Western blotting (not shown). We have previously shown using immunocytochemistry that Hsp 12 is present in the cell in the vicinity of the plasma membrane and the cell wall [21]. To demonstrate that the two proteins identified were indeed present in cell walls, a cell wall preparation was made from whole yeast cells by exhaustive (20 min) disruption in a Ball-mill. No whole cells were visible when this preparation was examined by electron microscopy (not shown). Furthermore, this preparation failed to produce colonies when plated onto YPD plates. When this preparation was extracted with 1 M NaOH, bands corresponding to Hsp 12 and the unknown protein were clearly visible on the SDS gel (Figure 4). Further evidence supporting a cell wall localisation for the unknown protein was obtained from abrasion of 1 M NaOH-treated yeast cells. 20 s abrasion in the Ball-mill of 1 M NaOH-treated yeast cells resulted in no breakage of the cells, as confirmed by transmission electron microscopy (not shown), but resulted in the appearance of Hsp 12 and the unknown protein in the medium (Figure 4). Our interpretation is that these proteins are located on the surface of the cell and were removed by the abrasion process.



**Figure 3a.** SDS-PAGE of proteins extracted from whole washed yeast cells with 1 M NaOH. Yeast were grown in YPD medium in the absence of mannitol (lane 1) in the presence of 0.16 M (lane 2) or 1 M mannitol (lane 3). The arrows denote two proteins present at increased concentrations when yeast were grown in the presence of 1 M mannitol. The standard (S) is a total extract of chicken erythrocyte histones, the molecular weights (kDa) of which are: H1, 22.5; H3, 15.3; H2B, 13.7; H2A, 14.0 and H4, 11.2.



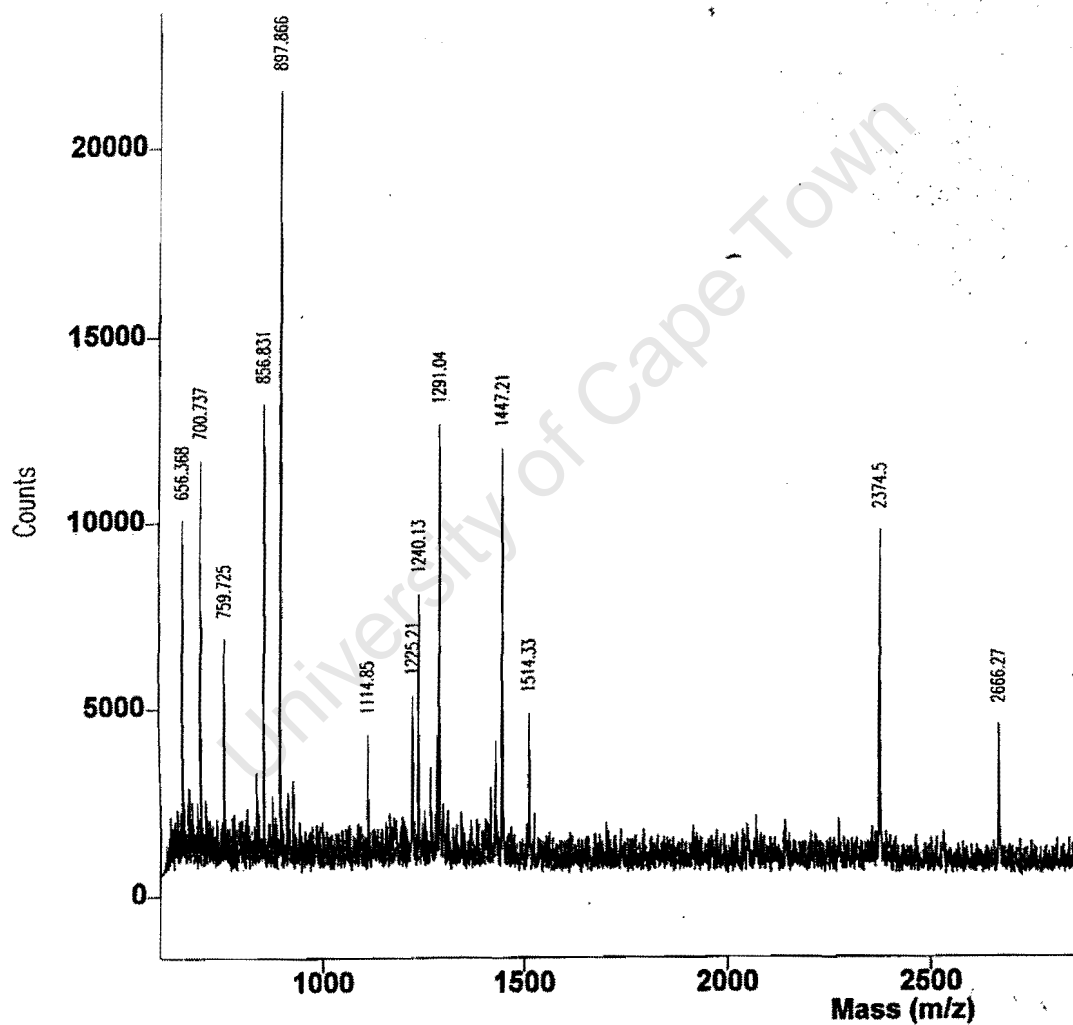
**Figure 3b.** Scan of the gel shown in Figure 3a. The top, middle and bottom scans represent 0, 0.16 and 1 M mannitol respectively. The arrows denote the migration of the bands arrowed in Figure 3a.



**Figure 4.** SDS-PAGE of proteins extracted from whole washed yeast cells with 1 M NaOH (lane 1), proteins present in the medium after Ball-milling these cells for 20 s (lane 2) and the unknown protein purified by electroelution (lane 3). The standard (S) is a total extract of chicken erythrocyte histones, the molecular weights (kDa) of which are: H1, 22.5; H3, 15.3; H2B, 13.7; H2A, 14.0 and H4, 11.2.

A stained gel slice including the unknown protein from a gel corresponding to lane 2 of Figure 4 was subjected to MALDI-TOF mass spectrometric analysis after trypsin digestion. Peptides of molecular sizes between 656 and 2666 Da were found (Figure 5) and used to identify the protein to be phosphoglycerate mutase using the SwissProt 6.12.2000 database. Peptides of this molecular size were found to be 45 times more likely to have originated from phosphoglycerate mutase than from any other protein. To confirm this identification, the protein was purified by electroelution from a SDS-PAGE gel (Figure 4) and subjected to N-terminal sequencing. The N-terminal sequence, Pro-Lys-Leu-Val-Leu, was found, using the *Saccharomyces* Genome

Database, to be unique to residues 2 to 6 of the phosphoglycerate mutase expressed from the *Saccharomyces cerevisiae gpm1* gene. The N-terminal methionine has been reported to be cleaved from the protein during post-translational processing [9, 12]. The purified phosphoglycerate mutase was used for the preparation of polyclonal antibodies in rabbits.



**Figure 5.** MALDI-TOF analysis of peptides present in a trypsin digest of the isolated unknown protein. The peaks of molecular size 1226 and 1447 daltons did not match the sizes of calculated peptides derived from phosphoglycerate mutase.

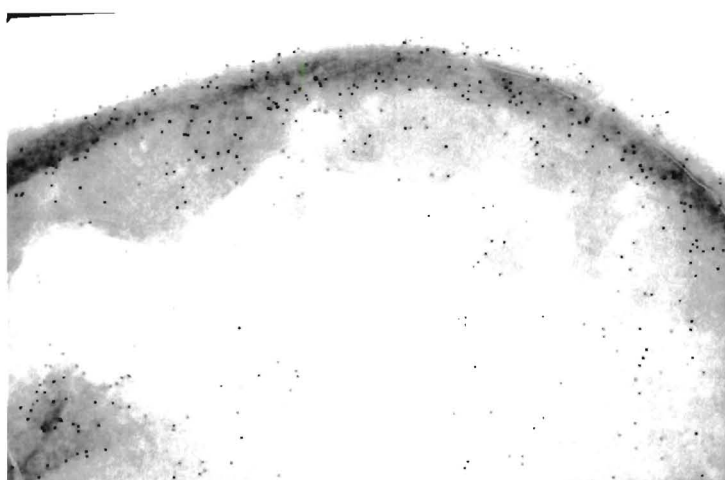
The affinity of rabbit antisera to purified phosphoglycerate mutase were tested by ELISA. Both antisera strongly recognised the purified protein at dilutions of  $1:10^4$ . Thin sections of embedded yeast cells were probed with the antiserum specific for phosphoglycerate mutase. The location of this antibody was determined by electron microscopy using a colloidal gold-labelled goat anti-rabbit antibody. Examination of the sections showed that gold particles were present both in the cytoplasm and in the cell wall with an approximately equal distribution (Figure 6). Control sections, where the location of the phosphoglycerate mutase was probed with the pre-immune serum, showed far fewer gold particles to be present, mainly in the cytoplasm. These results clearly demonstrated that phosphoglycerate mutase was not only present in the cytoplasm, but in addition, significant quantities were also present in the cell wall.

The enzymatic activity of a partially purified phosphoglycerate mutase preparation, prepared by ball-milling 1 M NaOH treated yeast cells for 20 s (Figure 4), was determined for its ability to convert 3-phosphoglycerate to 2-phosphoglycerate in the presence of 2,3-bisphosphoglycerate. The activity of this preparation was found to be 0.47 mmol/min/mg protein. We next investigated whether the phosphoglycerate mutase present in the walls of whole yeast cells could convert 3-phosphoglycerate in the medium to 2-phosphoglycerate in the medium in the presence of 2,3-bisphosphoglycerate. The enzymes and substrates required to convert 2-phosphoglycerate to lactate using the standard assay were also added to the medium. It was found that the whole cell phosphoglycerate mutase was indeed active, and the conversion proceeded at a rate of  $1 \mu\text{mole} \cdot \text{min}^{-1} \cdot A_{600}^{-1}$  (Table 1). No NADH + H<sup>+</sup> oxidation occurred in the absence of added 3-phosphoglycerate, demonstrating that this oxidation occurred exogenously. Other glycolytic enzymes of the lower Embden-

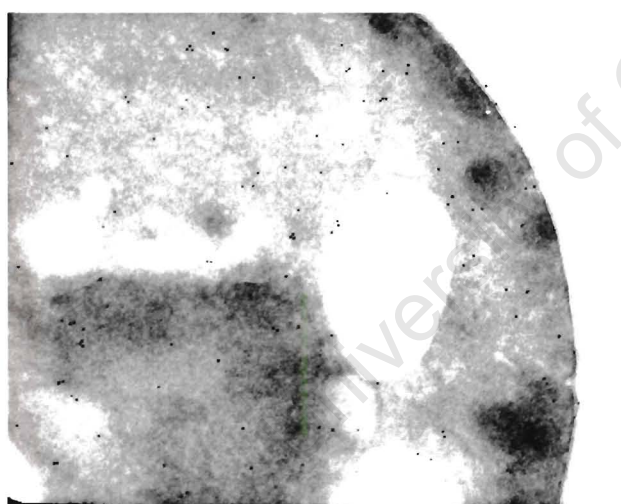
Meyerhof pathway besides phosphoglycerate mutase have been reported to be present in the cell walls of yeast [18]. We therefore repeated this experiment in the absence of added enolase and pyruvate kinase, and found that the conversion of 3-phosphoglycerate to lactate still proceeded at a rate of  $1 \mu\text{mole} \cdot \text{min}^{-1} \cdot A_{600}^{-1}$ . Moreover, whole cells were able to convert 3-phosphoglycerate to ethanol as the oxidation of  $\text{NADH} + \text{H}^+$  still occurred when lactate dehydrogenase was omitted from the medium providing that TPP was added. The rate of reaction was, however, significantly lower,  $0.4 \mu\text{moles} \cdot \text{min}^{-1} \cdot A_{600}^{-1}$ .

Since treatment of yeast cells with NaOH altered the exposure of phosphoglycerate mutase within the cell wall, we determined the phosphoglycerate mutase enzymatic activity of NaOH-treated cells using the standard assay. Whereas this activity was  $1 \mu\text{mole} \cdot \text{min}^{-1} \cdot A_{600}^{-1}$  in untreated cells, it increased to  $11 \mu\text{moles} \cdot \text{min}^{-1} \cdot A_{600}^{-1}$  after NaOH treatment. Omitting the enolase and the pyruvate kinase reduced the activity to  $0.4 \mu\text{moles} \cdot \text{min}^{-1} \cdot A_{600}^{-1}$ .

A. Anti – phosphoglycerate mutase serum.



B. Pre – immune serum.



**Figure 6.** Electron micrographs of immunogold labelled cryosections of yeast cells probed with rabbit-anti yeast phosphoglycerate mutase (A) and pre-immune serum (B). Magnifications used were X 50 000 (A) and X 37 000 (B). The gold particles used were 10 nm diameter.

**TABLE 1.****Oxidation of NADH + H<sup>+</sup> in the medium by whole yeast cells.**

Yeast cells (treatments 1, 2 and 3) or NaOH-treated yeast cells (treatments 4 and 5) in Tris-HCl buffer were incubated together with all enzymes and cofactors required to assay for phosphoglycerate mutase (1 and 4), with the enzymes enolase and pyruvate kinase omitted (2 and 5) and with lactate dehydrogenase also omitted but with TPP added (3). The substrate, 3-phosphoglycerate (3-PG) was added to start the reaction in each case. The statistics are from three independent results.

<b><u>Treatment</u></b>	<b><u><math>\mu\text{mols NAD}^+ \text{ min}^{-1} \cdot \text{A}_{600}^{-1}</math></u></b>
No 3-PG	0
1	1.0 ± 0.1
2	1.0 ± 0.01
3	0.4 ± 0.04
4	11.0 ± 0.04
5	0.4 ± 0.05

## 2.5 DISCUSSION.

Although current dogma states that glycolysis occurs in the cytoplasm of cells, several groups have reported the presence of a number of glycolytic enzymes to be present in cell walls. In this report, we have confirmed that the glycolytic enzyme phosphoglycerate mutase is present in the cell walls of the yeast *Saccharomyces cerevisiae*. Immunocytochemical studies reported here confirmed the data gathered from the alkaline extraction of whole cells and isolated cell walls and suggested that phosphoglycerate mutase is not only present in the cytoplasm, but that significant quantities are also present in the cell walls. Three pieces of evidence support our belief that alkaline extraction of whole yeast cells primarily extracted cell wall proteins. Firstly, electron microscopy of yeast cells after extraction with 0.2 M NaOH showed few morphological changes, with the cells appearing intact. Secondly, the proteins remaining after such an extraction were seemingly identical to those present in untreated cells, suggesting that alkaline treatment of whole cells resulted in the extraction of a small, select group of proteins. Thirdly, these proteins were similar to those extracted from isolated cell walls. Alkaline extraction of whole yeast cells profoundly altered the properties of the cell wall. A markedly increased abrasion resistance was found with a rough correlation observed between the resistance and the strength of the NaOH used. Our interpretation of these results is that alkaline treatment altered the conformation of the cell wall thereby releasing cell wall proteins, possibly after the hydrolysis of glycolytic linkages. Interestingly, alkaline treatment followed by abrasion in the Ball-mill resulted in the release of a few proteins into the medium, the most prevalent of these being phosphoglycerate mutase.

Phosphoglycerate mutase has a molecular weight calculated from the gene sequence of 27 480 daltons but migrates on SDS-PAGE with an observed molecular weight of around 26 000 daltons [17, 18]. The phosphoglycerate mutase released from yeast cells by alkaline treatment migrated as anticipated, migrating with the linker histones, which migrate with molecular weights in the 25 000 to 27 000 dalton range. This suggests that the released phosphoglycerate mutase had no substantial postsynthetic modification present. Two possible mechanisms might account for the interaction between the phosphoglycerate mutase and the cell wall constituents. One possibility is that the enzyme might be anchored in the cell wall by an alkaline-labile carbohydrate linkage, which was cleaved by  $\beta$ -elimination in the presence of the NaOH [14]. Since phosphoglycerate mutase has an isoelectric point of 8.95, an alternative possibility is that the enzyme, which would be positively charged at physiological pH, interacts ionically with negatively charged cell wall constituents. These interactions would not occur at alkaline pH. Interestingly, sequences reported to target proteins to the cell wall were found neither in Hsp 12 nor in phosphoglycerate mutase.

We were able to demonstrate that phosphoglycerate mutase released from yeast cell walls by alkaline treatment still showed enzymatic activity. Moreover, whole yeast cells were able to convert 3-phosphoglycerate to ethanol providing the necessary co-factors were added to the medium. We have provided evidence that this conversion occurred in the cell walls rather than in the cytoplasm. For cytoplasmic conversion to occur, the 3-phosphoglycerate would have either have to diffused or have been transported into the cytoplasm and the  $\text{NAD}^+$  produced by alcohol dehydrogenase or other enzymes would either have to diffused or have been transported out of the cell. Since cell membranes are relatively impermeable to charged molecules as well as

$\text{NAD}^+/\text{NADH}$  and since no transport mechanisms have been reported for either 3-phosphoglycerate or  $\text{NAD}^+/\text{NADH}$  in yeast, we consider it unlikely that this conversion occurred within the cell. It is unlikely that the phosphoglycerate mutase and the other enzymes required were present in the medium due to the disruption of a small percentage of the yeast cell population as cells were washed exhaustively prior to enzymatic assays. Moreover, a number of the enzymes required to perform the conversion of 3-phosphoglycerate to ethanol have been reported to be present in cell walls. Furthermore, yeast cells that had been treated with alkali were able to perform this conversion at an enhanced rate despite the increased abrasion resistance of such cells. Presumably alkaline treatment altered the conformation of the cell wall constituents allowing enhanced substrate accessibility.

The role of phosphoglycerate mutase in the yeast cell wall is intriguing. It is possible that the ATP-forming part of the glycolytic pathway is present in the wall to produce ATP as an energy source to allow cell wall re-modelling due to growth or in response to environmental changes. The incorporation of new building blocks into the cell wall during growth would require energy to drive the process. An alternative possibility is that one of the metabolites of the lower half of the glycolytic pathway is used for signalling. Experiments to investigate these hypotheses are in progress.

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## CHAPTER 3.

### **THE LEA-LIKE PROTEIN HSP 12 IS ALSO PRESENT IN THE CELL WALL OF YEAST, *SACCHAROMYCES CEREVISIAE*. WHERE IT ENHANCES WALL FLEXIBILITY.**

#### **3.1 ABSTRACT.**

The yeast, *Saccharomyces cerevisiae*, LEA-like stress response protein Hsp 12 was found by immunocytochemistry to be located both in the cytoplasm and in the cell wall, from where it could be extracted with dilute NaOH solutions. Wild type yeast expressing Hsp 12 were significantly more resistant to pressure changes than yeast with the Hsp 12 knocked out but showed slightly lower abrasion resistance. Comparison of the activities of the Pdr 5 and Pdr 12 ABC transporters in wild type and knockout yeast showed that the presence of Hsp 12 inhibited both transporters but had no effect on diffusion of the fluorescent Pdr 5 substrate rhodamine 6G into the cell.

**KEYWORDS:** Hsp 12, yeast, cell wall, elasticity, ABC transporters.

### 3.2 INTRODUCTION.

Barotolerance, the ability of organisms to remain viable after exposure to hydrostatic pressure, has been reported to be induced by heat shock treatment in yeast (*Saccharomyces cerevisiae*) [12]. It has recently been shown that yeast with the trehalose-6-phosphate phosphatase (*tps*) gene deleted are sensitive to hydrostatic pressure [8], displaying an altered morphology due to cytoskeletal deformation after the application of 200 MPa pressure. This altered morphology, which was ascribed to resultant changes in the cell wall, was not observed when the yeast were heat shocked, a treatment known to cause Hsp 12 synthesis [23, 29], prior to the application of pressure. Rescue of the barosensitive  $\Delta tps$  phenotype by heat shock therefore suggests that a heatshock protein such as Hsp 12 might fulfil some function in the cell wall.

Hsp 12 is a small hydrophilic LEA-like protein, the concentration of which increases markedly after heat shock [23, 29] as well as on entry into stationary phase and when yeast are subjected to different forms of osmotic stress [20]. We have previously shown using immunocytochemistry that Hsp 12 is located in the vicinity of the plasma membrane [25]. Using liposomes incorporating the fluorescent dye calcein as a model membrane system, we demonstrated that Hsp 12 acted in a manner analogous to trehalose in that Hsp 12 afforded protection of membrane integrity against desiccation [25].

Investigations of gene transcription induced as a result of various stress protocols [3] indicated that the transcription of the *hsp 12* gene was up-regulated, as expected, by

osmotic and salt stress as well as during the diauxic shift. However, transient up-regulation of transcription was also observed when yeast were exposed to acidic or alkaline pH, conditions that are known [22] and were found [3] to up-regulate transcription of the *pdr 12* gene, the product of which has been reported to be an ABC transporter in the plasma membrane [27].

Pdr 5 and Pdr 12 are members of the ATP-binding cassette (ABC) transporters found in the yeast plasma membrane [27]. Pdr 5 transports ionophoric peptides, steroids, anticancer drugs and has been implicated in multidrug resistance [16]. The anionic form of fluorescein has been shown to be a substrate for Pdr 12 [11] transporter, the biological function of which has still to be elucidated.

The immunocytochemical experiments reported previously were performed using thin sections embedded in epoxy resin. The results showed that most of the gold particles were observed to be located on the external side of the plasma membrane, with a few particles present on the cytoplasmic side. In addition, a few particles were also seen in the area of the cell wall. Since cryosectioning has been reported to have a number of significant advantages over embedding into resin [19], notably reduced antigen damage, increased antigen exposure and preservation of ultrastructure [5], we investigated whether this methodology might allow us to detect Hsp 12 in locations other than surrounding the plasma membrane.

This manuscript reports that Hsp 12 was detected not only in the cytoplasm but also in substantial quantities in the area of the cell wall, where its presence was found to

decrease pressure dependent cell disruption. Moreover the presence of Hsp 12 inhibited the activity of the Pdr 5 and Pdr 12 ABC transporters.

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### 3.3 METHODS.

#### *3.31 Yeast strains and growth conditions.*

Yeast strains, growth media and growth conditions were as described previously [25]. NaOH extraction of whole yeast cells was performed at 0 °C as described previously [Chapter 2].

#### *3.32 Fluorescein diacetate and Rhodamine 6G efflux assays.*

Fluorescein diacetate (FDA) assay was carried out as described by [9]. FDA (7 mg/ml in acetone) was added to a final concentration of 10  $\mu$ M to yeast cells ( $A_{600} = 3.2$ ) suspended in 10 ml of 10 mM Tris-HCl pH 7.0. After 40 minutes incubation at 20 °C to load the cells, the cells were centrifuged to remove the FDA and washed with and re-suspended in the same buffer. Fluorescein leakage was monitored by determining the fluorescence of the buffer as a function of time ( $\lambda_{EX} = 490$  nm,  $\lambda_{EM} = 525$  nm).

Rhodamine 6G loading and efflux was determined ( $\lambda_{EX} = 529$  nm,  $\lambda_{EM} = 553$  nm) as described by Kaur et al. [14], except that the buffer used was 50 mM Tris-HCl pH 7.0.

### *3.33 French Press & Ball Mill.*

The effect of pressure changes on yeast was determined using a pressure cell (Aminco) of 25.4 mm diameter. Yeast cells ( $A_{600} = 5.2$ ) in 30 ml of 100 mM Tris-HCl, 50 mM NaCl pH 7.4 buffer were placed in a pressure cell at room temperature. The cell was placed under a mechanical press and the pressure increased to the stated pressure. 1 ml of the yeast suspension was released into an Eppendorf tube at atmospheric pressure whilst maintaining the pressure on the cell. The released yeast were centrifuged for 5 min at 10 000 X g and the 280 nm absorption of the supernatant, representing yeast cellular contents, determined. The effect of abrasion on yeast was determined using a Ball-mill (Braun). Yeast cells ( $A_{600} = 3.9$ ) in 20 ml of 100 mM Tris-HCl, 50mM NaCl pH 7.4 buffer were placed together with 6g of glass beads diameter 0.75 – 1.125 microns in the vessel at 4 °C. The vessel was then shaken whilst maintaining the temperature of the vessel at 4 °C with CO<sub>2</sub>. The degree of yeast damage was determined from the 280 nm absorption of the supernatant after centrifugation.

### *3.34 Immunochemical methods and electron microscopy.*

The anti-Hsp 12 antiserum used for Hsp 12 detection was the same antiserum used previously [25]. Antibodies to Hsp 12 were separated from contaminating activities by binding the antiserum to Hsp 12, itself bound to nitrocellulose, and release at low pH [10]. Immunocytochemistry was performed as described previously [25] except that the yeast cells were fixed using 0.5 % glutaraldehyde 4 % paraformaldehyde in K<sub>2</sub>HPO<sub>4</sub> pH 7.0 for 16 hours at 4°C, embedded in low melting point agarose, and

infiltrated with 2.3 M sucrose as a cryoprotectant. Thereafter they were mounted on small stubs and rapidly plunge-frozen in liquid nitrogen (-196 °C). Ultrathin sections were cut using a Reichert Ultra Cut S cryomicrotome. The cutting temperature was – 120 °C. Sections were retrieved onto carbon coated, glow discharged nickel grids and immunolabelled as described by Tokuyasu, 1986 [28].

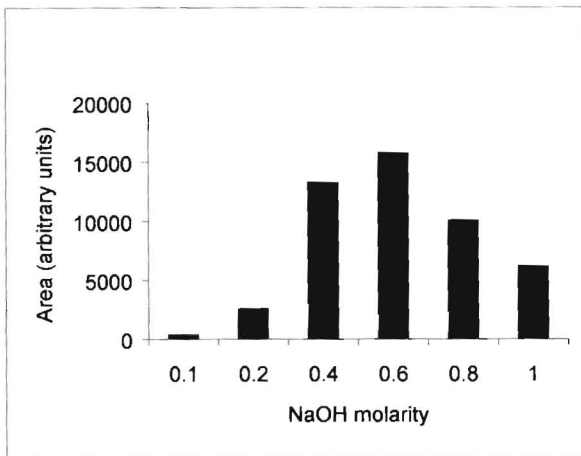
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### 3.4 RESULTS.

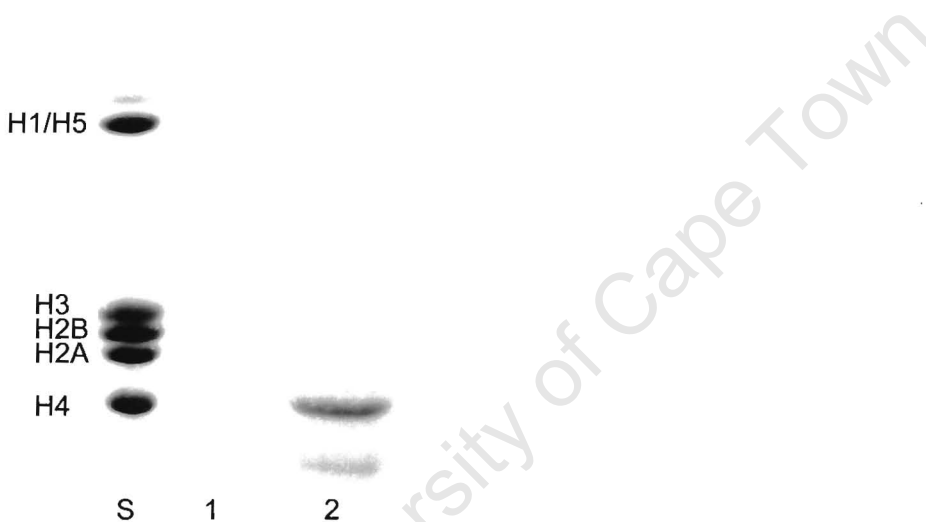
#### *3.41 Hsp 12 is present in the cell wall.*

We have shown that extraction of whole yeast cells with 0.2 M NaOH resulted in the extraction of a small, select group of proteins leaving the yeast cell intact [Chapter 2]. This group of proteins included phosphoglycerate mutase, which was shown using immunocytochemistry to be located in the cell wall, and Hsp 12. To investigate the optimum extraction of Hsp 12 from whole yeast cells, cells were incubated for 30 min at 0 °C with different strength NaOH solutions between 0.1 M and 1 M. We found that Hsp 12 was present in the extract irrespective of the strength of NaOH used between 0.1 M and 1 M, with optimum extraction observed with 0.6 M NaOH (Figure1). Since extraction of yeast cells with 0.2 M NaOH had no visible effect on the cell when treated cells were examined by electron microscopy [Chapter 2], we concluded that Hsp 12 was present in the cell wall. To confirm that Hsp 12 was indeed present in yeast cell walls, thin sections, prepared by cryosectioning yeast cells, were probed with an antiserum specific for Hsp 12. The location of this antibody was determined by electron microscopy using a colloidal gold-labelled goat anti-rabbit antibody. Examination of the sections (Figure 2) showed that gold particles were present not only close to the plasma membrane as reported previously [25], but also both in the cytoplasm and in the cell wall. The majority of the particles were found to be in the area of the cell wall. Control sections of the yeast failed to show labelling (not shown, but see Sales et al.).

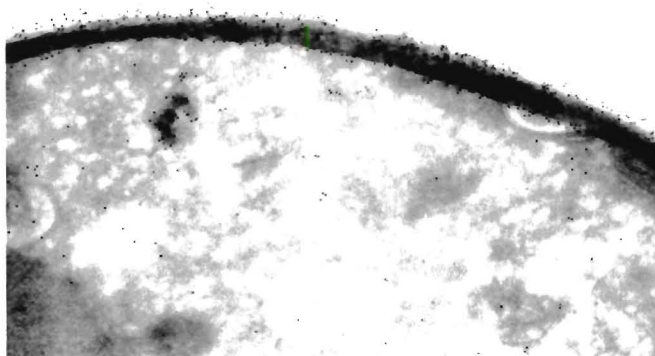
A.



B.



**Figure 1. A.** Relationship between the molarity of the NaOH used to extract whole yeast cells and the quantity of Hsp 12 extracted. Whole yeast cells were extracted with between 0.1 and 1 M NaOH and the extracts electrophoresed on SDS-PAGE (c.f. Figure 1B). Hsp 12 was quantitated by densitometry after Coomassie Brilliant Blue staining. **B.** SDS-PAGE of a 0.8 M NaOH extract of whole yeast cells (lane 2). A total heat soluble fraction of yeast cells is also shown (lane 1). The standard (S) is a total extract of chicken erythrocyte histones, the molecular weights (kDa) of which are: H1, 22.5; H3, 15.3; H2B, 13.7; H2A, 14.0 and H4, 11.2.

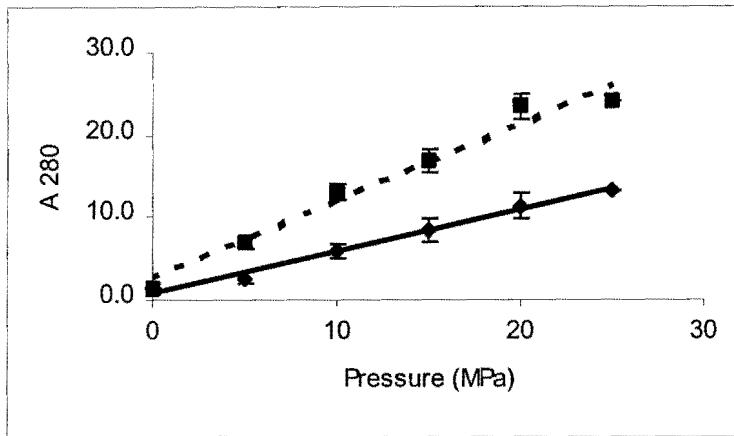


**Figure 2.** Electron micrograph of an immunogold labelled yeast cell probed with a rabbit anti-Hsp 12 antiserum. The magnification used was X 30 000. The gold particles used were 10 nm diameter. No gold particles were observed on the Hsp 12 knockout yeast strain.

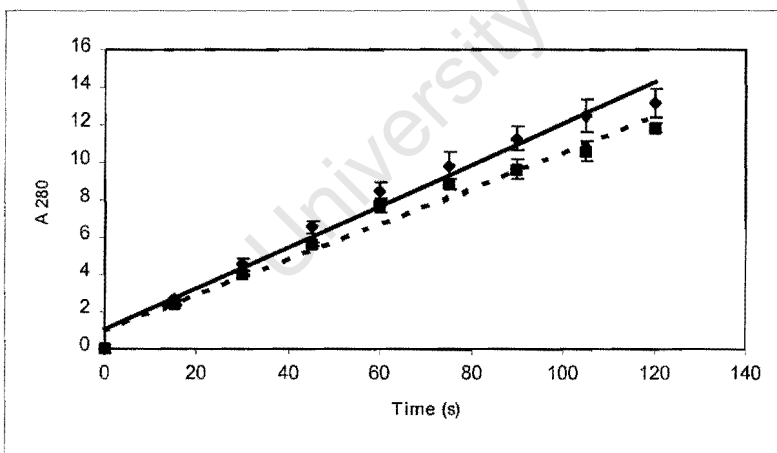
#### *3.42 Hsp 12 alters the abrasion-resistance and flexibility of the cell wall.*

A *Δtps* strain of yeast was recently shown to display an altered morphology after the application of pressure suggesting an alteration in the mechanical properties of the cell wall [8]. This strain was no longer barosensitive after heat shock, suggesting that Hsp 12 might alter the flexibility of the cell wall. We therefore compared the disruption of wild type yeast and yeast with the *hsp 12* gene “knocked out” [23] as a function of pressure. Both yeast strains were subjected to up to 25 MPa pressure in a French press pressure cell, and then returned rapidly to atmospheric pressure. Cell disruption was assayed by determination of the 280 nm absorption of the supernatant fraction after centrifugation. Approximately double the amount of disruption occurred with “knockout” yeast compared to wild type yeast irrespective of the pressure that the preparations were subjected to (Figure 3). Since we would equate the ability to

withstand rapid changes in pressure with the flexibility of the cell wall, it would appear that Hsp 12 might act as a plasticiser in the cell wall.



**Figure 3.** Wild type (◆, solid line) and knockout (■, dashed line) yeast cell disruption determined by the 280 nm absorption of the supernatant fraction after centrifugation as a function of the applied hydrostatic pressure. Hydrostatic pressure was applied using an industrial press. Statistics are from four independent results.



**Figure 4.** Wild type (◆, solid line) and knockout (■, dashed line) yeast cell disruption determined by the 280 nm absorption of the supernatant fraction after centrifugation as a function of the abrasion time. Cells were abraded in the Ball-mill at 4 °C by the action of 0.75 – 1.125 micron glass beads. Statistics are from three independent results.

We next compared the abrasion resistance of wild type and “knockout” yeast. Yeast cells were ball-milled for increasing time at 4 °C and disruption was again assessed by determining the release of soluble 280 nm chromophoric material. We found that the presence of Hsp 12 slightly decreased the abrasion resistance of yeast cells (Figure 4). Approximately 10 % less abrasion was found to occur if Hsp 12 was not present when yeast cells were Ball-milled for 120 s.

### *3.43 Hsp 12 inhibits the activities of the Pdr 5 and 12 ABC transporters.*

Microarray studies have indicated that the *hsp 12* and *pdr 12* genes are both up-regulated when yeast are subjected to certain stresses, e.g acidic or alkaline pH media [3]. To investigate the relationship between Hsp 12 and the activity of the Pdr 12 ABC transporter, we compared the efflux of fluorescein from wild type and knock out yeast cells. Fluorescein diacetate (FDA) is a nonfluorescent probe, commonly used for assessing cell viability [1, 4, 6 ], that is uncharged and can enter the cell by diffusion from the medium. Once inside the cell, it is cleaved by non-specific esterases to the fluorescent anionic molecule fluorescein [2], which is then actively pumped out of the cell by means of the Pdr 12 ABC transporter [11].

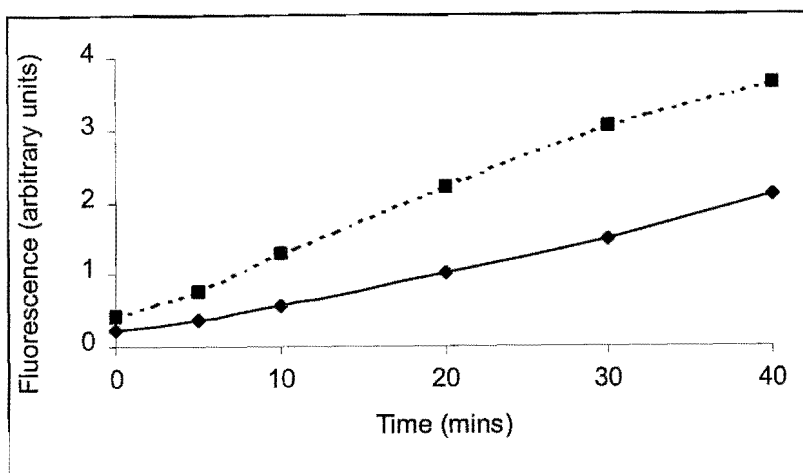
Wild type and knockout yeast cells were therefore incubated with FDA for 40 min at 20 °C during which the fluorescence of the medium was determined. We found that the fluorescence of the medium increased at approximately double the rate in the case of knockout cells (Figure 5a). After 40 min incubation with FDA, the cells were washed, re-suspended in fresh buffer and the fluorescence of the medium determined as a function of time. We found that the accumulation of fluorescein in the medium

was significantly slower in the case of knockout cells (Figure 5b). When wild type and knockout cells were examined by fluorescence microscopy after 40 min incubation with FDA and washing but before subsequent incubation in the absence of FDA, we found that whereas the wild type cells were readily visible, knockout cells showed little fluorescence (not shown). We next investigated whether the presence of Hsp 12 in the wild type cells effected the activity of the esterase responsible for cleavage of FDA to fluorescein. Total extracts of wild type and knockout cells were incubated together with FDA and the esterase activity determined by measurement of the fluorescence as a function of time. We found that no difference in the esterase activity between the two cell types could be determined (not shown). Our interpretation of these results is that the presence of Hsp 12 inhibited the activity of the Pdr 12 ABC transporter resulting in an increased intracellular accumulation and a decreased medium concentration of fluorescein.

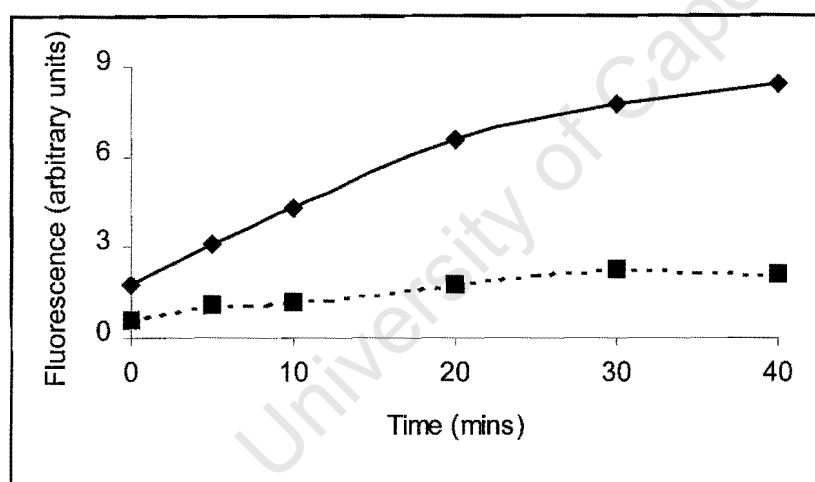
To confirm that Hsp 12 had no effect on the diffusion of small hydrophobic molecules such as FDA into the yeast cell, we compared the uptake of rhodamine 6G by wild type and knockout cells. This molecule, like FDA, is uncharged and enters the cell by diffusion, but unlike FDA strongly absorbs light in the visible region of the spectrum. Once inside the cell, it is pumped out of the cell by means of the Pdr 5 ABC transporter. Wild type and knockout cells were incubated for 2 h at 20 °C with rhodamine 6G in the presence of deoxyglucose to inhibit the activity of the Pdr 5 ABC transporter. After washing the cells, wild type and knockout cells were found to be equally stained with rhodamine 6G (not shown) demonstrating that Hsp 12 did not effect the rate of diffusion of rhodamine 6G into the cell. The cells were now incubated in buffer containing glucose to allow the Pdr 5 transporter to be operative,

and the rate of efflux of rhodamine 6G into the medium monitored by determination of the fluorescence at 529 nm. We found (Figure 6) that rhodamine 6G efflux was significantly enhanced in knockout cells suggesting that the presence of Hsp 12 inhibited both the Pdr 5 and Pdr 12 ABC transporters.

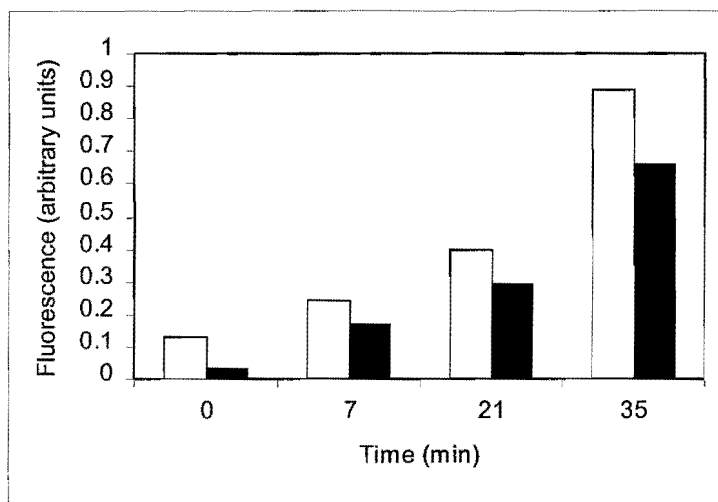
A.



B.



**Figure 5.** Fluorescein fluorescence (525 nm) in the medium of wild type (♦, solid line) and knockout (■, dashed line) yeast cells as a function of the time after the addition of fluorescein diacetate (FDA) to the medium (A). After 40 min incubation, the cells were washed, resuspended in fresh medium and the efflux of fluorescein monitored (B). The results shown are representative of separate experiments repeated four times.



**Figure 6.** Rhodamine fluorescence (553 nm) in the medium of wild type (solid bars) and knockout (open bars) yeast cells as a function of the time after the addition of glucose to the medium of cells pre-incubated with rhodamine 6G and deoxyglucose for 2 h at 20 °C. The results shown are representative of separate experiments repeated three times.

### 3.5 DISCUSSION.

In this report we have demonstrated that considerable quantities of the stress response protein, Hsp 12, are present in the cell walls of yeast, *S. cerevisiae*. Immunocytochemistry after cryosectioning rather than after embedding in resin [25] showed considerably more Hsp 12 to be present particularly in the cell wall. This was presumably because antigenic determinants present on Hsp 12 are effected by the latter process, as reported by other authors [5, 19]. Our results suggest that the role of Hsp 12 is to act as a plasticiser in the cell wall. Evidence in favour of Hsp 12 playing a crucial role in the flexibility of the cell wall is that the application of hydrostatic pressure to trehalose deficient yeast mutants resulted in cytoskeletal deformation. If these mutants were heat shocked prior to the application of hydrostatic pressure, however, no effects were observed [8]. A brief heat shock is known to result in transcription of the *hsp 12* gene [23, 29].

It is clearly important for the yeast cell to be able to respond rapidly to changes in its environment. The cell wall largely comprises polymers of uncharged sugar molecules with the  $\beta$ 1,3 glucans primarily responsible for the mechanical strength of the cell wall [13, 15]. These polysaccharides interact with one another via H bonding and, in the case of charged polysaccharides, ionic interactions. These interactions would result in stable inflexible structures akin to that of long-chain starch molecules since the  $\beta$ 1,3 glucans have been reported to exist as helical structures [18]. Yeast cells, when subjected to stress, have been shown to express a variety of cell wall proteins including the  $\beta$ -glucanases Fks 1 and 2 [7, 26]. Whilst these enzymes would allow the insertion of residues into or deletion of residues from the cell wall, a requirement for

growth and bud development, they would not allow the yeast to adapt to environmental changes that caused alterations in wall elasticity. Our hypothesis is that Hsp 12, which is a highly hydrophilic protein, interrupts the H bonding and ionic interactions between adjacent chains. We would postulate that hyperosmolar environmental conditions would result in increased hydrogen bonding between adjacent polysaccharide polymers in the cell wall as the water activity of the environment would be reduced. Hydrogen bonding between adjacent polysaccharide polymers via Hsp 12 would allow adjacent chains to move relative to one another in a manner analogous to the function of plasticisers in plastic polymers. This hypothesis would explain the synthesis of Hsp 12 in response to environmental stress. Although we have investigated the relationship between Hsp 12 and the adaptation of the yeast cell wall to environmental stress, Hsp 12 might not be the only protein involved in this process. A number of other proteins expressed after heat shock have been reported to be present in the cell wall [21, 24]. The observed decreased abrasion resistance of yeast cells expressing Hsp 12 compared with the knockout strain is similar to the decreased abrasion resistance found with plastic polymers where an inverse relationship between hardness/flexibility and abrasion resistance has been reported [17].

The observed relationship between Hsp 12 and the Pdr 5 & 12 ABC transporters is intriguing. Both transporters, which are membrane proteins [27], were inhibited by the presence of Hsp 12. These transporters have been shown to be a multi-drug transporter [16] and a transporter of weak organic acids [11, 22] respectively, although their precise roles in yeast cell physiology are unknown [27]. Since Hsp 12 levels are elevated when yeast are subjected to osmotic stress and in stationary phase,

it is possible that the role of Hsp 12 in inhibiting these transporters is to prevent non-essential ATP usage. We have previously suggested that Hsp 12 interacts with the membrane phospholipids via an ionic interaction [25]. It is possible that the presence of Hsp 12 in close proximity to the cell membrane would impede the binding of the substrates to these transporters.

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## CHAPTER 4.

### 4.1 GENERAL CONCLUSION.

Two proteins namely phosphoglycerate mutase (the product of the *gpm1* gene) and Hsp 12 were found to be up-regulated when yeast cells were grown in the presence of 1 M mannitol, a condition known to cause osmotic stress. These proteins were found not to be present only in the cytoplasm, but also on the yeast cell wall.

Hsp 12 was previously thought to occur only in the cytoplasm. The results reported here showed the presence of Hsp 12 on the cell wall using three different techniques, of which two were novel. Firstly, we developed a methodology to extract cell wall proteins using 0.2 M NaOH from intact yeast cells, which left the yeast cells both intact and viable. This result was unexpected. Subsequently, the concentration of NaOH used to extract cell wall proteins was increased to up to 1 M. 1 M NaOH did not only result in the extraction of more Hsp 12 but had a profound effect on the cell wall. The cell wall became more rigid and thus harder to break. 1 M NaOH treated cells were impossible to break in a French press. They were therefore subjected to mechanical abrasion using glass beads in the presence of a buffer. Limited abrasion resulted in intact cells together with significant quantities of a protein in the media. This protein was found to be phosphoglycerate mutase. Secondly, Hsp 12 wildtype and knockout yeast strains, were subjected to a pressure test, to measure their mechanical stability. This was accomplished using a French press. The optical densities of the resulting supernatants were measured in a spectrophotometer. We found that the Hsp 12 wild type strain was more mechanically stable than the

knockout strain, thus indirectly showing its presence in the cell wall. It has been well established that the yeast cell wall is responsible for the mechanical strength of the cell. Thirdly, we showed the presence of Hsp 12 on the cell wall by using immunocytochemical techniques. Gold particles, indicative of Hsp 12, were clearly visible on the cell wall from the transmission electron micrographs.

In order to establish the functional relationship of Hsp 12 with other proteins, we investigated its effects on the Pdr 5 and Pdr 12 transporters. We found that the Hsp 12 inhibited the activity of both these plasma membrane located proteins. There was a significant difference between the wild type and the knockout in terms of their interaction with these transporters. Of great interest was the inhibition of the Pdr 5 ABC transporter, which has been shown to confer multidrug resistance in a variety of systems. This finding is of possible medical significance, since multidrug resistance is a deterrent to prolonged chemotherapy.

Our results also showed the first reported presence of the glycolytic enzyme phosphoglycerate mutase on the yeast cell wall. Not only was this enzyme found on the cell wall, but it was also shown to be enzymatically active. In addition, we demonstrated that the enzymes of the lower glycolytic pathway were also active on the yeast cell wall, thereby implying that yeast cells can theoretically produce ethanol from 3 carbon sugars using enzymes present in the cell walls. Lastly, we developed a quick and efficient method of extracting active phosphoglycerate mutase from yeast cell walls.