EFFECT OF HEAT STRESS OF THE CELL WALL OF THE YEAST, *SACCHAROMYCES CEREVISIAE*: PHOSPHORYLATION OF RIBOSOMAL PROTEIN S10-B BROUGHT ABOUT BY ENZYMES OF THE GLYCOLYTIC PATHWAY.

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ABBREVIATIONS

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
1,3 BPG	*1,3-bis* phosphoglycerate
CWP	Cell wall protein
DHAP	Dihydroxyacetone phosphate
ER	Endoplasmic reticulum
GDP	Guanosine 5-diphosphate
GTP	*Guanosine* 5-triphosphate
G-3-P	Glyceraldehyde-3-phosphate
GEF	Guanine nucleotide exchange
GAPDH	Glyceraldehyde phosphate dehydrogenase
kDA	kilo Dalton
LEA	Late embryogenesis abundant
MS	Mass spectrometry
MALDI-TOF	Matrix assisted laser desorption/ionization-time of flight
MAP	Mitogen activation pathway
NADH	Nicotinamide adenine dinucleotide (reduced form)
NaOH	Sodium hydroxide
PGM	Phosphoglycerate mutase
3PG	3-phosphoglycerate
rpm	rotation per minute
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TPI	Triose phosphate isomerase
YPD	Yeast peptone dextrose

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Chapter 1.

1.1 General introduction

Yeast cell wall architecture

The cell wall is a dynamic and elastic structure that provides osmotic and physical protection to the yeast cell (1). As such, it forms the immediate site of contact between the yeast cell and its environment (2), and is essential for maintaining cell integrity and shape. Cell growth and development demand that the cell wall is not rigid and unchangeable as the cell needs to adjust the wall composition and structure during growth and development stages such as morphogenesis, flocculation, cell-cell recognition and pathogenicity, and in response to changes in environmental conditions (1, 2, 3, 4). Although the chemical compositions of many fungal cell walls are known, the interactions of the various macromolecules and the assembly processes have not yet been elucidated (5). Electron microscopic analysis showed that the cell wall of *S. cerevisiae* is a two-layered structure about 70-100 nm thick depending on growth conditions (6) and comprises 15 % to 30 % of the dry weight of the cell and 25 % to 50% of the volume (5, 6). The major components of the cell wall are the β-1,3 and β-1,6 glucans, mannoproteins and chitin, these molecules being synthesized at different sites in the cell.

The inner layer of the cell wall, which is largely responsible for the mechanical strength of the cell, consists mainly of three macromolecular polymers of glucose, namely β1,3-glucan, β1,6-glucan and chitin. This layer also provides attachment sites for proteins that form the outer layer (1). β1,3-glucans are the major filamentous

component of the cell wall and are responsible for its rigidity. They consist of about 1500 glucose residues and form 50 % - 60 % of the total dry weight of the cell wall (6). *FKS1* and *GSC2/FKS2* encodes alternative catalytic subunits of β1,3-glucan synthase complex (7). *FKS1*gene is preferentially expressed under optimal conditions and is cell cycle-regulated. *GSC2/FKS2* expression is independently controlled by various inputs; thus growth at high temperatures leads to increased expression of this gene (7) The two β1,3-glucan synthase complexes found in yeast contain either Fks1p or Gsc2p/Fks2p as the catalytic subunits depending on the environmental conditions (7).

Both Fks1p and Fks2p are multiple-spanning trans-membrane proteins with the corresponding genes well conserved among fungi (7). β1,6-glucans are small (on average about 130 glucose monomeric subunits), highly branched, water-soluble polymers (1) that connect glycosylphosphatidylinositol-dependent cell wall proteins to the β1,3-glucan networks. They may also function as an acceptor site for chitin, particularly in case of cell wall stress (1). The site of β-1,6 glucan syntheses has yet to be determined although studies by Bussey et al. (9) suggested a plasma membrane location, an endoplasmic reticulum location or a combination of these. Chitin accounts for 1 % - 2 % of the cell wall content where it is located in a ring at the base of the bud and bud scar as well as in a dispersed fashion in the lateral wall (1). The structure of α-chitin, a hydrogen-bonded anti-parallel linear polymer of β-1,4-N-acetylglucosamine units, is similar to that of cellulose (5). Chitin is glycosidically linked to the non-reducing end(s) of β1,3-glucan and β1,6-glucan chains in the cell wall. This interaction aids in strengthening the skeletal layer (11).

The outer layer of the cell wall consists mainly of mannoproteins, which are involved in cell-cell recognition. The yeast wall mannoproteins are highly glycosylated polypeptides that carry N-linked glycans with additional α-linked mannose units. Phosphorylation of the mannosyl side chains gives yeast its anionic surface charge. The resulting negative charge reduces the permeability of the cell wall thereby protecting the plasma membrane from attack by foreign enzymes and membrane-perturbing compounds (5). As a result of this negative charge, the cell wall may bind positively charged proteins present in the medium that originate from lysed cells (8). The carbohydrate side chains are also responsible for the hydrophilic properties of the yeast cell and it has been suggested that they are involved in water retention during water stress.

There are two main classes of proteins (Figure 1) covalently coupled to cell wall polysaccharides (1). These are:

(i) Glycosylphosphatidylinositol (GPI)-dependent cell wall proteins (GPI-CWPs)

(ii) Protein with internal repeats (Pir) proteins (Pir-CWPs)

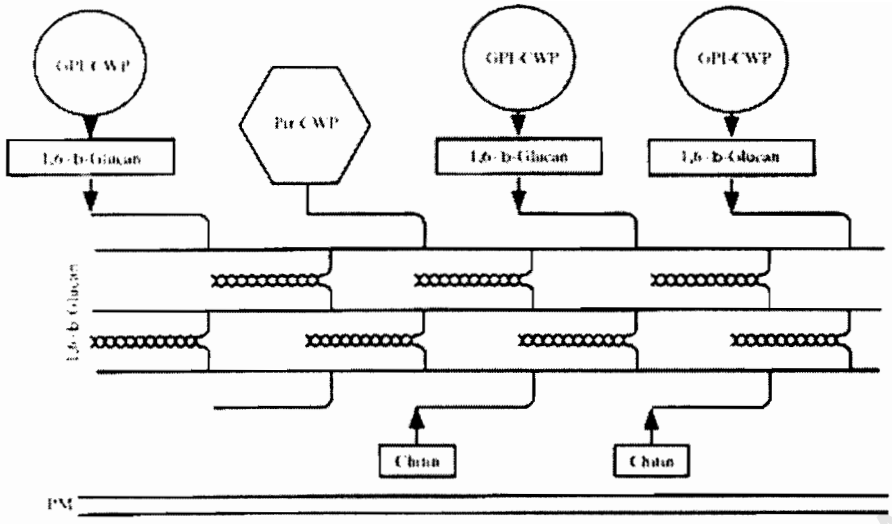


***Figure 1**. Yeast cell wall molecular architectural model. The internal skeletal layer consists of ß1,3 glucan molecules that form a three-dimensional network aligned and strengthened by hydrogen bridges. At the outside of the skeletal layer, cell wall proteins are linked to the non-reducing ends of ß1,3-glucan molecules either directly (Pir-CWPs) or indirectly through an interconnecting ß1,6-glucan moiety and the remnant of a GPI-anchor (GPI-CWPs). Some GPI-CWPs, such as Cwp1p, may be linked both ways (2).*

The yeast genome encodes different glycosylphosphatidylinositol (GPI)-dependent cell wall mannoproteins. The genes encoding GPI-CWPs correspond to serine- and threonine-rich proteins that posses a N-terminal signal peptide, often contain an internal repeat (10), and terminate in a hydrophobic sequence. The hydrophobic sequence is replaced in the endoplasmic reticulum (ER) by a GPI anchor. The most extensively studied GPI-CWP is Sag1p, which is believed to be involved in sexual agglutination (11). Cell wall proteins containing GPI attachment signals have these

GPI anchors attached in the ER before being passed through the secretory pathway to the cell membrane. The GPI anchor is then cleaved, the protein component transferred to form a glycosidic linkage with the branched β1,6 glucans through a trimmed form of their original GPI-anchor (11).

Pir proteins (Pir-CWPs) are directly linked to β1,3-glucans through an alkaline-sensitive linkage. Four such proteins have been found to exist in *S. cerevisiae* (2,12). *PIR* genes encode a family of proteins sharing the following characteristics: they have a N-terminal signal peptide, a Kex2 recognition site followed by a repeat-containing region with up to 11 repeats as well as a highly conserved C-terminal region with 4 conserved cysteine residues (12). Pir1p, Pir2p/Hsp150p, Pir3p and Pir4p/Cis3p have all been shown immunologically to be present on the external face of the cell wall. Several additional proteins, e.g. Sps100p and Ypg1p, which are involved in spore wall maturation, have been predicted to contain the N-terminal signal peptide but not the additional signal for a GPI anchor. This implies that their final destination is not the medium but rather the cell wall to which they become linked in a Pir-CWP-like fashion (12).

The yeast cell wall is remodelled in response to changing environmental conditions, during different stages of vegetative growth and during pheromone-induced morphogenesis (1,2). This remodeling in response to environmental stimuli is thought to reinforce the cell wall and minimize the damage caused by high temperatures, hydrolytic enzymes and osmotic stress (3). The yeast cell wall remodelling is mediated by yeast cell wall signalling pathway (Figure 2).

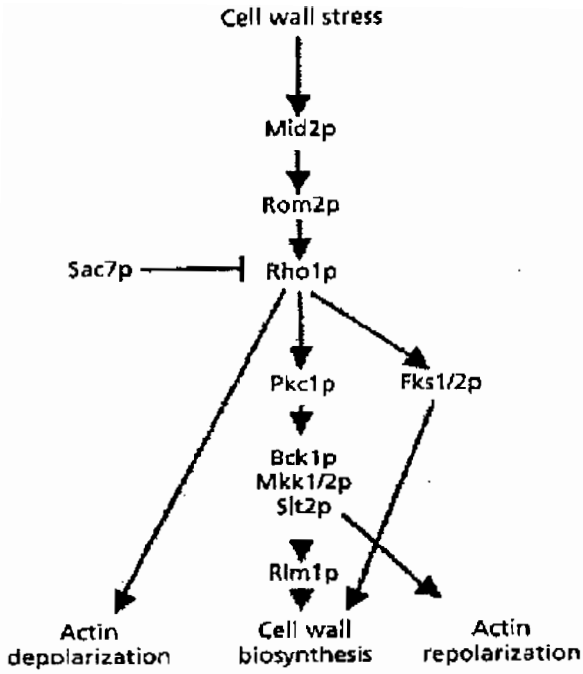


Figure 2. *A model of the yeast cell wall signaling pathway (2). Cell wall stress leads to alterations and adaptations in the actin cytoskeleton and cell wall biosynthesis, a pathway involving Rho1p and Pck1p. Pkc1p is the control kinase activated by cell wall stress. This in turn activates the ensuing MAP kinase pathway.*

It has been proposed that the proteins Wsc1p - Wsc4p or Mid2p sense stress at the cell wall. These proteins, which are highly O-glycosylated, reside in the plasma membrane of *S. cerevisiae* and are thought to act as mechanosensors (19, 20). They have extracellular domains extending into the periplasmic space and these domains have been proposed to act as rigid probes for the extracellular matrix (19). Conformational changes in the cell wall are thought to alter the conformation of Wsc1p and/or Mid2p. This signal is transmitted via the C-terminal cytoplasmic domains of these proteins to Rom2p and thence to Rho1p, which is activated by exchange of GDP for GTP. Activated Rho1p in turn activates the protein kinase Pkc1p (19); subsequent phosphorylation results in the expression of cell-wall-related genes such as *FKS1*,

GAS1 and *CHS3* as well as several genes encoding cell wall proteins such as Cwp1p, Ssr1p and several Pir proteins (18) Some proteins like the PIR family possess structural roles and their increase has been implicated in stabilizing the cell wall (32). Other proteins are involved in cell wall metabolism such as the biosynthetic and the hydrolytic glucanase enzymes (21). Up-regulation of these proteins leads to alterations in the amount of glucan and chitin in the cell wall, as well as the type of linkages between wall constituents. These are believed to change the overall rigidity of the cell wall.

One problem is to distinguish authentic cell wall proteins from proteins whose association with the cell wall is brought about by ionic interaction of proteins from lysed cells from the medium. Members of the Hsp (heat-shock protein) family and glycolytic enzymes such as glyceraldehyde-3-phosphate dehydrogenase (Tdh1p, Tdh2p and Tdh3p) have been found on the cell surface (14). It is not clear whether these proteins originate from lysed cells or, as frequently suggested, are the result of export by an unknown non-conventional secretory mechanism. (8). Heat-shock proteins and glycolytic enzymes have also been found in the medium of regenerating protoplasts. Removal of the yeast cell wall in an iso-osmotic medium leads to the formation of protoplasts, incubation of which in regenerating medium results in expression of genes involved in cell wall construction (13). During regeneration, numerous proteins are not retained in the nascent cell wall but are secreted into the medium. These proteins identified using 2-D electrophoresis and mass spectrometry, included known cell wall proteins, heat shock proteins and glycolytic enzymes suggesting that the presence of heat shock proteins and glycolytic enzymes is not the result of interaction between the negatively charged mannoproteins and positively

charged proteins present in the medium (13). Proteomic studies have shown the presence of glycolytic enzymes in the cell wall. In addition, enolase, fructose bisphosphatase and glyceraldehyde-3-phosphate dehydrogenase have been shown using immunocytochemistry to be present in the cell wall where they have been shown from studies of whole cell enzyme activity that they are in a catalytically active state (14, 15). The presence of glycolytic enzymes has also been reported in other microorganisms e.g. *Candida albicans, Streptococcus pyogenes, Schistosoma mansoni, Kluyveromyces marxianus* and *Entamoeba hystolyica* (16). Recently Motshwene et al (23) showed using immunocytochemistry that phosphoglycerate mutase is present in the cell wall of the yeast *S. cerevisiae* (23). Additionally, phosphoglycerate mutase could be extracted under alkaline conditions both from whole cells as well as from isolated cell walls suggesting that this enzyme is not only present in the cytoplasm but also in the cell wall.

Pardo et al. (13) used two-dimensional polyacrylamide gel electrophoresis and mass spectrometry to identify thirty-two different proteins secreted by regenerating protoplasts. This included known cell wall proteins, glycolytic enzymes, heat shock proteins and proteins involved in several other processes (13). All the enzymes of the lower ATP-forming part of the glycolytic pathway from triose phosphate isomerase to alcohol dehydrogenase were identified (13). Thus the yeast cell wall can theoretically convert dihydroxyacetone phosphate to ethanol using enzymes present in the cell wall. This was shown by Motshwene et al. (23) to indeed be possible. In contrast, enzymes corresponding to the ATP-using part of glycolysis were not found, although their possible existence was not dismissed. There is still very little information on whether the glycolytic enzymes present in the cell wall have some specific function in

this localization and if their activity is the same as in the cytoplasm. Recent studies have suggested a category of proteins called 'moonlighting' proteins (17). These proteins have been proposed to display multiple roles depending on their localization; in which cell type they were present, their oligomeric state, the concentration of the ligand, cofactor or product (17). Phosphoglucoisomerase is a cytosolic enzyme that catalyzes the second step in the glycolytic pathway, the inter-conversion of glucose-6-phosphate and fructose-6-phosphate. It is also secreted by mammalian cells and plays at least four other different roles: as a neuroleukin which acts as a cytokine, as a nerve growth factor, as the autochrine motility factor and as a mediator of differentiation and maturation of cells in human myeloid leukemia (17). It has been proposed that the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) displays diverse activities unrelated to glycolysis in different subcellular locations. GAPDH might act as a receptor, mediating various interactions in *Streptococci* and *Staphylococcus aureus* (14), as a flocculant in *Kluyveromyces marxians* (22), as a fibronectin receptor in the pathogenic yeast *Candida albicans* (16), and associated with resistance to schistosomiasis in humans (14). Cell surface glycolytic enzymes might therefore have functions in *S. cerevisiae* completely unrelated to their classical role in metabolism.

Modification of the cell wall must inevitably cause breakage and reformation of glycosidic linkages as well as other covalent and non-covalent interactions. It has been proposed that this might be a mechanism used to increase incorporation of proteins into the cell wall. Thus it has been shown (32) that stress up-regulates specific genes, and the protein products of these genes are incorporated into the cell wall following stress. Gozalbo et al. (26) found that cell wall-associated GAPDH

activity increased up to 10-fold in response to either starvation or temperature shift. In addition, expression of a GAPDH: invertase fusion protein resulted in similar increases in both cell wall GAPDH and cell wall invertase activities following starvation. Since this fusion protein lacked the signal sequence required for secretion, it was inferred that incorporation of GAPDH protein into the cell wall in response to stress does not require *de novo* protein synthesis, i.e. that pre-existing cytosolic enzyme was incorporated (26). Proteins lacking a secretory signal sequence are exported through a non-classical pathway in mammalian cells; such a pathway appears to be also present in *S. cerevisiae* and fungal species such as *Coprinus cinereus* (33).

Motshwene et al. (23) used NaOH to extract proteins from the cell walls of the yeast *S. cerevisiae*. This treatment, shown not to disrupt the yeast cells, extracted several proteins including the glycolytic enzyme phosphoglycerate mutase (PGM) and the stress response protein Hsp12p. When yeast was grown in the presence of 1 M mannitol, these two proteins were found to be present at an elevated concentration in the cell wall. The presence of both PGM and Hsp12p in the cell wall was confirmed by immunocytochemical analysis. In addition, not only was PGM found to be active in the yeast cell wall, but also that whole yeast cells were able to convert 3-phosphoglycerate in the medium into ethanol provided that the necessary cofactors were present. These finding have led to the suggestion that the glycolytic enzymes present in the cell wall of *S. cerevisiae* provide energy in a form of ATP for cell wall remodelling in response to stress.

1.2 Aim of this study.

The aim of this study was to determine the extent of the glycolytic pathway in *S. cerevisiae* cell walls, to investigate whether ATP was formed in the cell wall, the relationship between this ATP formed and stress, and the possible role of ATP in cell wall protein phosphorylation.

1.3 Methods

1.3.1 Yeast strains and culture conditions

The yeast strain W303 (*trp 1-1 can 1-100 leu2,3-112 his 3-11,15 ura 3-1*) of the baker's yeast *S. cerevisiae* was used in this study. Cells were initially grown on YPD plates (1 % yeast extract, 2 % bactopeptone, 2 % glucose, 2 % agar) at 30 °C for 2 days. A single colony was then subcultured into 10 ml of YPD medium (1 % yeast extract, 2 % bactopeptone, 2 % glucose), grown overnight at 30 °C with shaking. The cell density of the subculture was then determined by light scattering at 600 nm. An equal number of cells based on the 600 nm scattering were used to inoculate large (up to 800 ml) cultures, which were grown at 30 °C as before. The growth curve of this culture was determined (Figure 3).

To expose yeast cells to either heat or salt stress, 800 ml cultures cells were first grown in YPD medium at 30 °C to mid-log phase after which the culture was divided into eight 100 ml cultures. Solid NaCl was directly added to 0.5 M to four of these (salt stress) or the cultures were incubated at 37 °C (heat stress) by moving the cultures from a 30 °C room to a 37 °C room. These cultures were allowed to grow for a further 4 h after which time the cells were harvested.

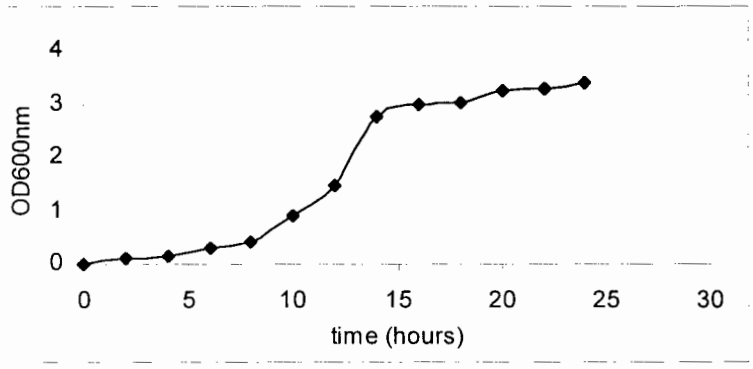


***Figure 3**. Growth curve of S. cerevisiae, strain W303 in YPD at 30 ℃. The time points for different stages of growth at 30 ℃ were extrapolated from the graph generated. Mid-log was reached at about 12 hours, early stationary at 14 hours and late stationary at about 22 hours.*

1.3.2 Determination of enzyme activities of whole yeast cells

All enzyme assays were performed at room temperature (20 ^{0}C) in a 1 ml reaction mixture. Yeast cells were grown to early-stationery phase, washed and re-suspended in wash buffer (100 mM Tris-HCl 50 mM NaCl pH 7.4) to an A_{600} of 3.0. 100 µl of this suspension was used for each enzyme assay. The reaction was allowed to proceed without the presence of substrate to determine the control rate of oxidation or reduction of NAD^+/NADH + H^+ from the change in the A_{340}. Substrate was then added and the reaction allowed to proceed for the times stated. At each time point, the cells were rapidly spun down and the A_{340} of the supernatant determined. The supernatant and precipitate fractions were then immediately re-combined by briefly vortexing the mixture and the reaction allowed to proceed. Results are reported in µmoles.min^{-1}.A_{600} $^{-1}$.

1.3.3 Phosphoglycerate mutase enzyme assay

Phosphoglycerate mutase *(PGM)* catalyzes the following reaction:

3-phosphoglycerate (3 PGA) ↔ 2-phosphoglycerate (2 PGA)

The activity of whole yeast cells was assayed as described previously from this laboratory [23]. Essentially 2 PGA was converted to phosphoenolpyruvate (PEP) using added enolase. The PEP was then converted to pyruvate using added pyruvate kinase and ADP. This was finally converted to lactate using added lactate dehydrogenase and NADH + H^+. The rate of oxidation of NADH + H^+ was measured at 340 nm to determine the PGM activity.

1.3.4 Glyceraldehyde-3-phosphate dehydrogenase enzyme assay

Glyceraldeyde-3-phosphate dehydrogenase (GAPDH) catalyzes the following reaction:

Glyceraldehyde-3-phosphate + NAD^+ + Pi ↔*1,3-bis*phosphoglycerate + NADH + H^+

The GAPDH activity of whole yeast cells was assayed as described [28, 38]. The assay mixture contained: 90 mM triethanolamine 1.43 mM $MgSO_4$ 0.6 mM EDTA 1 mM Na_2HPO_4 (or 1 mM Na_2AsO_4) 1 mM NAD^+ 1 mM ADP 10.5 mM D, L-glyceraldehyde-3 phosphate pH 7.8. The rate of reduction of NAD^+ was measured at 340 nm to determine the GAPDH activity. In some experiments Na_2AsO_4 replaced Na_2HPO_4 as this prevents further metabolism as the 1-arseno 3-phosphoglycerate is unstable and breaks down to arsenate and 3-phosphoglycerate (see text).

1.3.5 Glycerol kinase and glycerol-3-phosphate dehydrogenase enzyme assays

Glycerol kinase (GK) catalyzes reaction (1) and glycerol-3-phosphate dehydrogenase (Gly3PDH) catalyzes reaction (2):

$$\text{Glycerol} + \text{ATP} \leftrightarrow \text{Glycerol-3-phosphate} + \text{ADP} + H^+ \quad (1)$$

$$\text{Glycerol-3-phosphate} + NAD^+ \leftrightarrow \text{Dihydroxyacetone phosphate (DHAP)} + \text{NADH} + H^+ \quad (2)$$

The GK and Gly3PDH enzyme activities of whole yeast cells were assayed as a pair, as described [37, 38]. The assay mixture contained: 90 mM triethanolamine 1.43 mM $MgSO_4$ 0.6 mM EDTA 1 mM Na_2HPO_4 1 mM NAD^+ 1 mM ATP 2 mM glycerol pH 7.8. The rate of reduction of NAD^+ was measured at 340 nm to determine the GK/Gly3PH activity.

1.3.6 Triose phosphate isomerase enzyme assay

Triose phosphate isomerase (TPI) catalyzes the following reaction:

Dihydroxyacetone phosphate ↔ Glyceraldehyde-3-phosphate

The TPI activity of whole yeast cells was assayed as described [28]. The activity was determined by following the reduction of NAD^+ to NADH + H^+ brought about by conversion of the glyceraldehyde-3-phosphate formed to 1,3-bisphosphoglycerate in the absence and presence of added GAPDH. The assay mixture contained 25mM KH_2PO_4 1 mM NAD^+ 5 mM ADP 1mM DHAP and 48,5 U/ml commercial TPI

(Sigma Chemical Co.) pH 7.4. Excess TPI was added to ensure that the following reaction, catalyzed by GAPDH, was the rate limiting step in NADH + H^+ formation.

1.3.7 Yeast cell wall ATP assay

Whole yeast cell ATP was determined as described [39] using a luciferin/luciferase-catalyzed assay:

Luciferase + luciferin + ATP ↔ luciferase-luciferyl-AMP + PP_i.....1

Luciferase-luciferyl-AMP + O_2 ↔ luciferase + oxyluciferin + AMP + CO_2 + hv.....2

The 560 nm luciferin emission, which is proportional to the ATP concentration (30, 31), was determined using a Turner Designs TD 20/20 Luminometer. Since the concentration of luciferin in the reaction mixture has to be appropriate for the level of ATP present in the assay mixture, a standard curve (Figure 4) was generated using different ATP concentrations. For the reaction, 100 µl of whole yeast cells were added to the assay mixture. After 10 minutes the cells were precipitated and the ATP concentration determined in 100 µl of the supernatant. Whole yeast cells suspended in distilled water were used as a control.

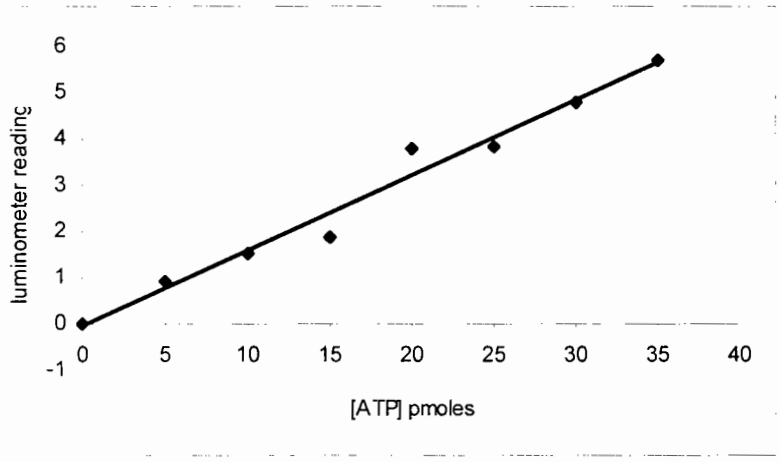


***Figure 4**. ATP standard graph generated from samples with known concentration, from 0 to 35 pmoles of ATP. Light production is in direct proportional to the ATP present or shows linearity up to concentration of 35 pmoles and deviates from linearity after this value.*

1.3.8 Extraction of cell wall proteins (CWP) and SDS-PAGE.

All operations were performed essentially as described previously [23]. Yeast cells in mid-log phase, pre-exposed to either heat stress (incubation at 37 °C) or salt stress (YPD with added 0.5 M NaCl), were harvested by centrifugation (5 min 7000 g). The pelleted cells were washed three times in wash buffer and weighed after which 1 ml/g of ice-cold 1 M NaOH was then added. The pellet was gently re-suspended, incubated on ice for 30 minutes and the cells collected by centrifugation as before. The resulting supernatant fraction was immediately subjected to SDS-PAGE (27) analysis to minimize NaOH degradation of the extracted proteins. Gels were stained with Coomasie R250 brilliant blue and destained using 25 % ethanol 7 % acetic acid in distilled H_2O. The gels were scanned and the band intensity of each band normalized

relative to the total intensity present in that lane using TNimage densitometry software.

Radiolabeled cell wall proteins were identified after electrophoresis on a SDS gel. The gel was stained and destained as described before being autoradiographed using Konica Medical X-ray film together with an intensifying screen. After incubation for 16 h at -70 °C, the film was developed, and protein bands corresponding to radiolabeled bands excised. The excised section was analyzed by MALDI TOF mass spectrophotometry after digestion with trypsin.

1.3.9 In gel tryptic digest and MALDI-TOF MS protein identification

A small section of the Coomasie stained gel was excised from the centre of the unknown protein band, with a similar sized section excised from a protein-free portion of the gel as a control. These sections were cut into small pieces and sequentially incubated in 50 % (v/v) CH_3CN for 5 minutes, in 50 % CH_3CN 50 mM NH_4HCO_3 for 30 minutes and in 50 % CH_3CN 10 mM NH_4HCO_3 for a further 30 minutes before being lyophilised. The pieces were re-hydrated in 10 mM NH_4HCO_3 buffer containing 0.1 μg trypsin (Promega) per 15 mm^3 of gel. Digestion was allowed to proceed for 16 hours at 37 °C. 1 μl of the tryptic digest was mixed with 1 μl matrix (10 mg/ml α-cyano-4-hydroxycinnamic acid in 60 % acetonitrile 0.3 % trifloroacetic acid) after which the mixture was subjected to MALDI-TOF mass spectrometry for peptide mass analysis (29). All peaks unique to the unknown sample were used to search the SWISS-PROT database using Pept-Ident free software [SWISSPROT DATABASE] allowing for 1 missed cleavage site and a mass tolerance of 2 Da.

1.3.10 Metabolic labeling of S. cerevisiae yeast cell wall proteins

Yeast cells were grown in YPD at 30 °C to mid-log phase. 50 μCi [^{32}P] orthophosphate (^{32}P-Pi) was then added to 20 ml culture, which was incubated at 37 °C. Cells were harvested by centrifugation at various times after which the cell wall proteins were extracted using 1M NaOH at 0 °C and analyzed by SDS-PAGE.

1.4 Results

Soon after enolase was found to be present in the cell walls of the yeast *S. cerevisiae* (15), Pardo et al. (13) showed that enzymes belonging to the glycolytic pathway are secreted by regenerating yeast protoplasts, suggesting their presence in mature cell walls. Glycolytic enzymes have also been shown to be present in the cell walls of yeast by mass spectrometry of cell wall proteins after separation by 2-dimensional SDS-PAGE (36), as well as by the binding of antibodies against specific glycolytic enzymes to the exterior of whole yeast cells (14, 15, 16). More recently Motshwene et al. (23) used immunocytochemistry to demonstrate that significant quantities of PGM is present in the cell walls of yeast, and showed that whole yeast cells could convert 3-phosphoglycerate in the medium to ethanol in the presence of the required cofactors.

We initially repeated this experiment by determining the PGM activity of whole yeast cells in exponential phase (Figure 5). The step from 3-phosphoglycerate to 2-phosphoglycerate catalyzed by PGM was the only step catalyzed solely by the yeast cells. All other enzymes required for the linked assay, namely enolase, pyruvate kinase and lactate dehydrogenase were added to the cuvette together with the necessary co-factors and substrates. No NADH + H^+ oxidation was found to occur when yeast cells were incubated in the assay buffer without the addition of the substrate, 3-phosphoglycerate. Addition of this substrate resulted in the oxidation of NADH + H^+ as determined by the decrease in the 340 nm absorption as a function of time. The PGM activity was determined to be 10.7 $\mu mole.min^{-1}.A_{600}^{-1}$.

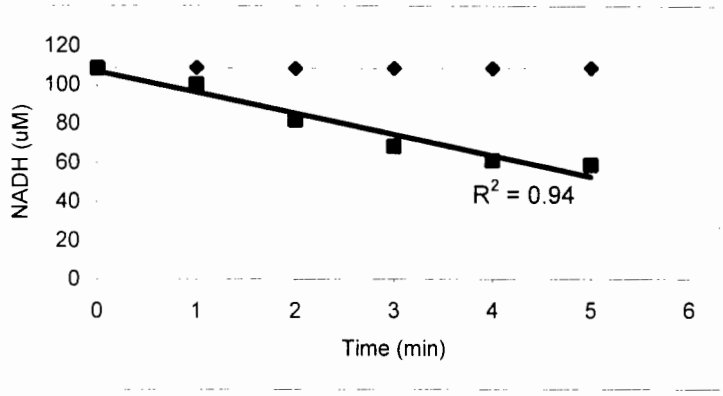


***Figure 5**. PGM activity of intact whole yeast cells. Exponentially growing cells were used to determine the PGM activity in unstressed yeast cells using a linked enzymatic assay. The activity was measured by monitoring the rate of oxidation of NADH + H^+ to NAD^+ at 340 nm (■——■). No reduction occurred in the absence of added substrate (♦- - - ♦). The PGM activity was found to be 10.7 µmole.min^{-1}.A_{600}^{-1}. The data displayed are typical data of experiments repeated three times.*

Motshwene et al. (23) suggested that all the enzymes of the glycolytic pathway that could convert 3-phosphoglycerate to pyruvate were present in the cell wall since whole yeast cells could convert 3-phosphoglycerate to ethanol in the presence of the necessary co-factors. Although some of the enzymes of the glycolytic pathway above PGM have been proposed to be present in the cell wall after their identification in a cell wall protein extract (13, 23), it is not known whether these enzymes are active in the cell wall. We therefore next assayed whole yeast cells for GAPDH activity (Figure 6), the enzyme that converts glyceraldehyde-3-phosphate to *1,3-bis*phosphoglycerate. This assay was performed by determining the rate of reduction of NAD^+ at 340 nm as a function of time. Instead of using inorganic phosphate as the substrate to be incorporated into glyceraldehyde-3-phosphate, arsenate was used in its place.

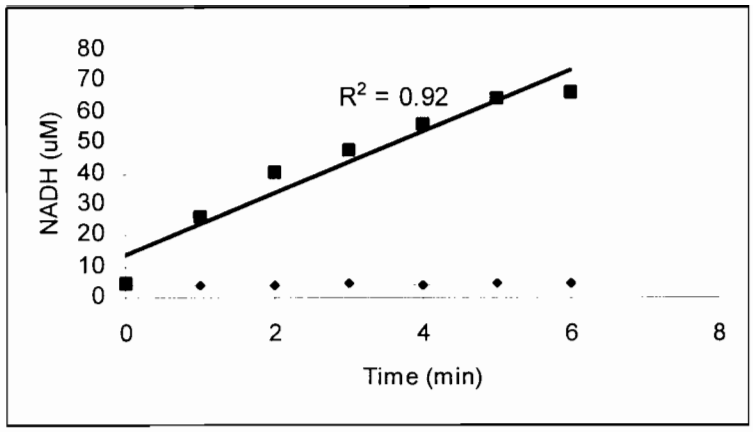


Figure 6. *GAPDH activity of intact whole yeast cells using arsenate as the substrate. Exponentially growing cells were used to determine the GAPDH activity in unstressed yeast cells by determining the rate of reduction of* NAD^+ *to NADH +* H^+ *at 340 nm* (■———■). *No reduction occurred in the absence of added substrate* (♦- - - - - ♦). *The GAPDH activity was found to be 9.9* $\mu mole.min^{-1}.A_{600}^{-1}$. *The data displayed are typical data of experiments repeated* three times.

This resulted in the synthesis of 1-arseno 3-phosphoglycerate, which is unstable and breaks down to 3-phosphoglycerate and arsenate (28, 38). In addition, the necessary co-factors required to convert pyruvate to ethanol were not added allowing the NADH + H^+ formed to accumulate. No enzymatic activity was observed when yeast cells were incubated in the assay buffer in the absence of substrate. Addition of the substrate resulted in an increased 340 nm absorption from which the activity of GAPDH was calculated to be 9.9 $\mu mole.min^{-1}.A_{600}^{-1}$.

Since *1,3-bis*phosphoglycerate (1,3 BPG) is an expensive and unstable substrate, the activity of GAPDH was determined prior to that of *1,3-bis*phosphoglycerate kinase. Subsequent to a successful demonstration that GAPDH was present in the cell wall, conversion of glyceraldehyde-3-phosphate to ethanol in the presence of phosphate rather would only be successful if the yeast cell wall contained an active *1,3-bis*phosphoglycerate kinase. Since NAD^+ is both reduced and oxidized during this process, it cannot be used to assay glyceraldehyde-3-phosphate metabolism. The change in the ATP concentration was therefore used, with the assumption that equal quantities of ATP would result from the *1,3-bis*phosphoglycerate kinase and pyruvate kinase steps. We therefore incubated yeast cells together with glyceraldehyde-3-phosphate and the necessary substrates and co-factors required to convert this compound to ethanol, and determined the ATP concentration using a luciferin/luciferase-based assay. We found that 0.5 $\mu mole.min^{-1}.A_{600}^{-1}$ of ATP was formed, a value approximately one order of magnitude less than the activities of the glycolytic enzymes measured. This might be due to a number of factors including rapid use of the ATP synthesized, inhibition of the luciferin/luciferase-based assay by whole yeast cells or poor access by the luciferin substrate to the ATP in the yeast cell wall.

Having established that whole yeast cells could convert glyceraldehyde-3-phosphate (G3P) to ethanol in the presence of the necessary co-factors and substrates, we next investigated whether dihydroxyacetone phosphate (DHAP) could be converted to G3P using whole yeast cells triose phosphate isomerase (TPI). TPI was assayed (Figure 7) as for GAPDH except that DHAP was used as the substrate in place of G3P.

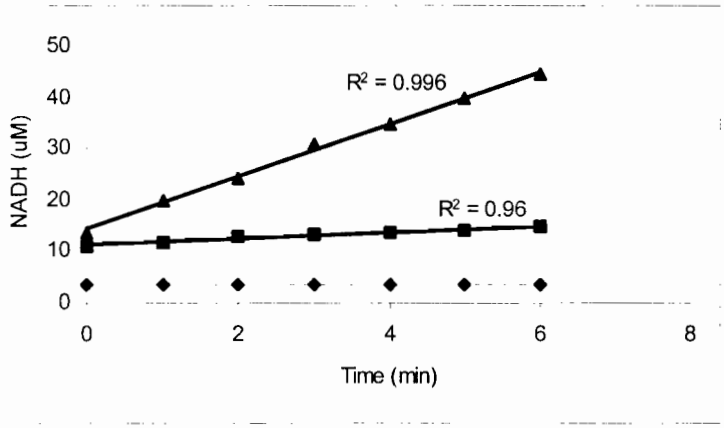


***Figure** 7. TPI enzyme activity of whole yeast cells. Exponentially growing cells were used to determine the TPI activity in unstressed yeast cells by determining the rate of reduction of NAD^+ to NADH + H^+ at 340 nm using inorganic phosphate as the substrate. No reduction occurred in the absence of added substrate (♦- - - - - ♦). The TPI activity of whole yeast cells (■———■) was found to be 0.7 $\mu mole.min^{-1}.A_{600}^{-1}$. The activity increased to 5.5 $\mu mole.min^{-1}.A_{600}^{-1}$ upon addition of exogenous GAPDH (▲———▲). The data displayed are typical data of experiments repeated three times.*

We found that very low levels of enzyme activity were observed after the addition of DHAP to whole yeast cells although this assay was performed in the presence of inorganic phosphate as the substrate rather than arsenate. The rate of NAD^+ reduction was 0.7 $\mu mole.min^{-1}.A_{600}^{-1}$, compared to the rate of GAPDH-mediated NAD^+ reduction in the presence of arsenate of 9.9 $\mu mole.min^{-1}.A_{600}^{-1}$. Although these data confirm the presence of an active GAPDH enzyme on the yeast cell wall, it suggested that either yeast cell walls display a very low inherent TPI activity or that this is the result of poor substrate, either phosphate or DHAP, access. To differentiate between these alternates, exogenous GAPDH was added and the rate of NAD^+ reduction re-assayed. The assay was performed by determining the rate of NADH + H^+ formation

for 6 min by whole yeast cells alone after which exogenous GAPDH was added and the activity determined for a further 6 min. It was now found that the activity increased from 0.7 μmole.min^{-1}.A_{600}^{-1} to 5.5 μmole.min^{-1}.A_{600}^{-1} on addition of exogenous GAPDH, suggesting that the access of G3P formed by TPI in the cell wall to GAPDH was the rate-limiting step.

Glycerol is known to serve two important functions in yeast, as a sink for excess NADH + H^+ produced during glycolysis and as an osmolyte to counter high external osmotic pressure. Glycerol can also be used as a carbon source for yeast after phosphorylation and NAD^+-mediated oxidation to DHAP. The data in Figure 7 suggested that if DHAP can be converted to glycerol-3-phosphate in yeast cell walls, this must proceed at a far slower rate than the conversion of DHAP to 1,3-bisphosphoglycerate since NADH + H^+ was formed during this assay. To investigate whether yeast cell walls could convert glycerol to DHAP, whole cells were incubated together with ATP and NAD^+ and the rate of NAD^+ reduction determined. We found that no reduction occurred suggesting that no pathway between glycerol and DHAP exists in yeast cell walls.

A major question in yeast is the role of the enzymes of the lower half of the glycolytic pathway in the yeast cell wall. Since the lower half of the glycolytic pathway is responsible for ATP synthesis, two possible roles would be the provision of ATP as an energy source, or utilizing the ATP synthesized to phosphorylate cell wall proteins as a control mechanism. ATP-derived energy might drive the incorporation of new building blocks and/or alterations in the linkages between existing cell wall components, necessary for the adaptation of the cell wall to stress. The recent

discovery of the presence of the enzymes of the lower half of the glycolytic pathway in the sarcoplasmic reticulum of cardiac muscle tissue (25) has led to the proposal that their function is to provide energy for calcium transport. It has recently been shown that the yeast cell wall GAPDH activity increased in response to starvation and temperature (26) and that cells exposed to osmotic stress possessed elevated levels of PGM in the cell wall (23). These data suggested that an increased cell wall ATP concentration should result from exposure of yeast cells to stress. Yeast cells were therefore grown to mid-log phase before being exposed to either salt (0.5 M NaCl) or to heat (37 °C) stress for 1 h, 2 h, 4 h or 6 h. At these times the yeast were centrifuged, re-suspended in the assay buffer at room temperature and the GAPDH activity (Figure 8) was determined by measuring the rate of reduction of NAD^+.

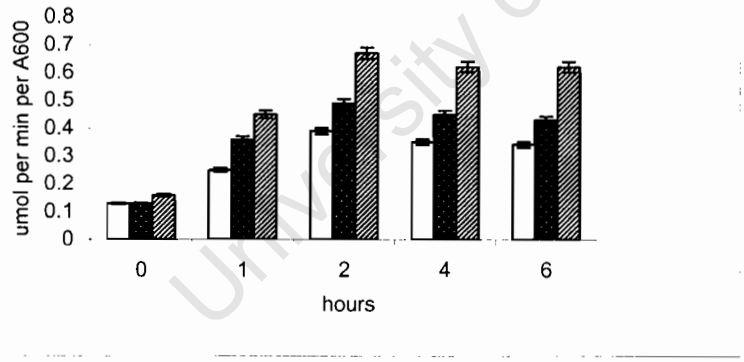


***Figure 8**. Effect of heat and salt stress on whole yeast cells GAPDH activity. Exponentially growing cells were not stressed (open bars) or exposed to 0.5 M NaCl (filled bars) or to 37 ^0C (hatched bars) for 1, 2, 4 or 6 h and the GAPDH activity determined from the rate of reduction of NAD^+. The data displayed are typical data of experiments repeated three times in duplicate.*

We found that the GAPDH activity of control cells increased 3-fold from 0.1 µmole.min-1.A_{600}^{-1} to 0.4 µmole.min^{-1}.A_{600}^{-1} over 2 h and then decreased to 0.33 µmole.min^{-1}.A_{600}^{-1} over the next 4 h. In contrast, the GAPDH activity of salt stressed cells and heat stressed cells increased 5-fold to 0.5 µmole.min^{-1}.A_{600}^{-1} and almost 7-fold to 0.68 µmole.min^{-1}.A_{600}^{-1} respectively over 2 h. The GAPDH activities of stressed cells also decreased after 2 h incubation. The GAPDH activity determined by measuring the ATP formed was approximately 15 % of that determined by measuring the rate of NADH + H^+ formation. This might be due to poor access of the ATP substrate to the luciferin substrate in the linked assay or to utilization of the ATP produced within the time frame of the experiment.

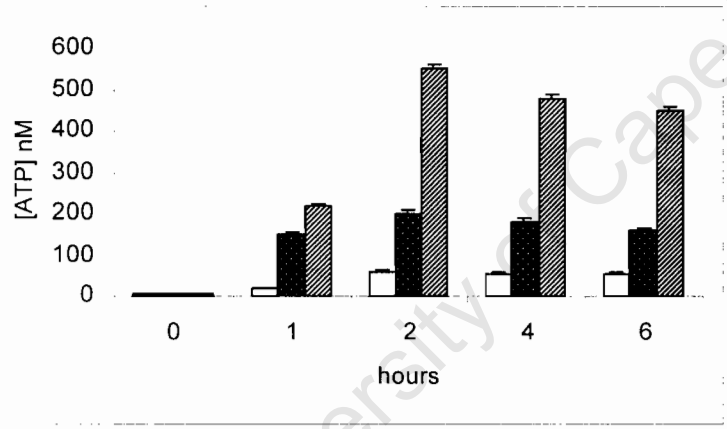


Figure 9. *Yeast cell wall ATP concentration determined using a luciferin/luciferase-based assay. ATP production was found to be increased maximally after 2 hours of stress. Heat stress (hatched bars) resulted in approximately a 3-fold greater response than salt stress (filled bars), while control cultures (open bars) at mid-log phase remained almost constant during this period. The data displayed are typical data of experiments repeated three times in duplicate.*

Demonstration that the GAPDH activity of yeast cell walls increased after exposure to stress suggested that the ATP concentration in the medium also increased as a result

of stress. Samples of the medium in which the yeast cells were incubated prior to determination of the GAPDH activity were therefore assayed to determine the ATP concentration. We found that the ATP concentration (Figure 9) increased from 5 nM to 200 nM over 2 h as a result of salt stress and from 5 nM to 550 nM after heat stress. Continued incubation resulted in a decreased ATP concentration detected at later time points.

We next wished to investigate the response to stress of individual proteins in the cell wall. Extraction with NaOH at 0 ^{0}C and subsequent SDS-PAGE has been used to identify such proteins. Motshwene et al. (23) investigated the effect of different NaOH concentrations on cell wall protein extraction and found 1M NaOH resulted in the efficient extraction of proteins without causing cell lysis, in that the cells appeared intact when viewed under an electron microscopy. Yeast cells were grown to mid-log phase in YPD medium at 30 ^{0}C before exposure to either 0.5 M NaCl or 37 ^{0}C. Cells were removed after 1, 2, 4 and 6 h, the cell wall proteins extracted with 1.0 M NaOH and electrophorised on a SDS gel. We found (Figure 10) that two proteins, one migrating with a molecular size of approximately 25 kDa (arrow 1) and one migrating with a molecular size of approximately 12 kDa (arrow 2) showed increased expression with increased exposure to both stresses. The gel shown in Figure 10 was scanned and the band intensity of each band normalized relative to the total intensity present in that lane of the gel. This showed that the approximately 25 kDa protein increased by a factor of 3 from 1 % of the total protein extracted from cell walls of mid-log phase yeast cells to 3 % after 2 h of either salt or heat stress. Similarly the approximately 12 kDa protein increased from 6 % of the extracted cell wall protein of mid-log phase yeast cells to 29 % after 2 h of either salt or heat stress.

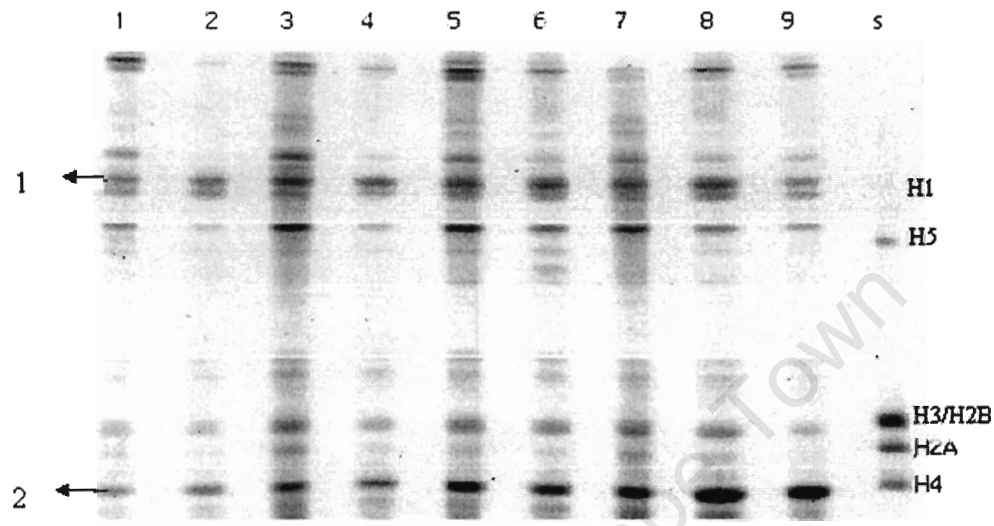


Figure 10. *SDS-PAGE of proteins present in the cell wall of yeast exposed to 0.5 M NaCl or to 37 °C heat stress. Cells were grown in YPD at 30 °C to mid-log phase (lane 1) before being exposed to salt stress (lanes 2 – 5) or heat stress (lanes 6 – 9). Proteins were extracted with 1.0 M NaOH after 1 h (lanes 2 & 6), 2 h (lanes 3 & 7), 4 h (lanes 4 & 8) and 6 h (lanes 5 & 9). The standard (lane S) is a total extract of chicken erythrocyte histones, the approximate molecular masses of which are: H1: 22.5 kDa; H5: 18.5 kDa; H2A: 14.0 kDa; H2B: 13.7 kDa; H3: 15.3 kDa; H4: 11.2 kDa.*

Motshwene et al. (23) identified PGM as a yeast cell wall protein with an apparent molecular mass of 25 kDa, the concentration of which increased due to osmotic stress. To investigate whether the 25 kDa protein (arrow 1, Figure 10) was indeed PGM, the

center of the Coomasie stained band (Figure 10, lane 3) was excised and subjected to MALDI-TOF mass spectrometric analysis after trypsin digestion (Figure 11).

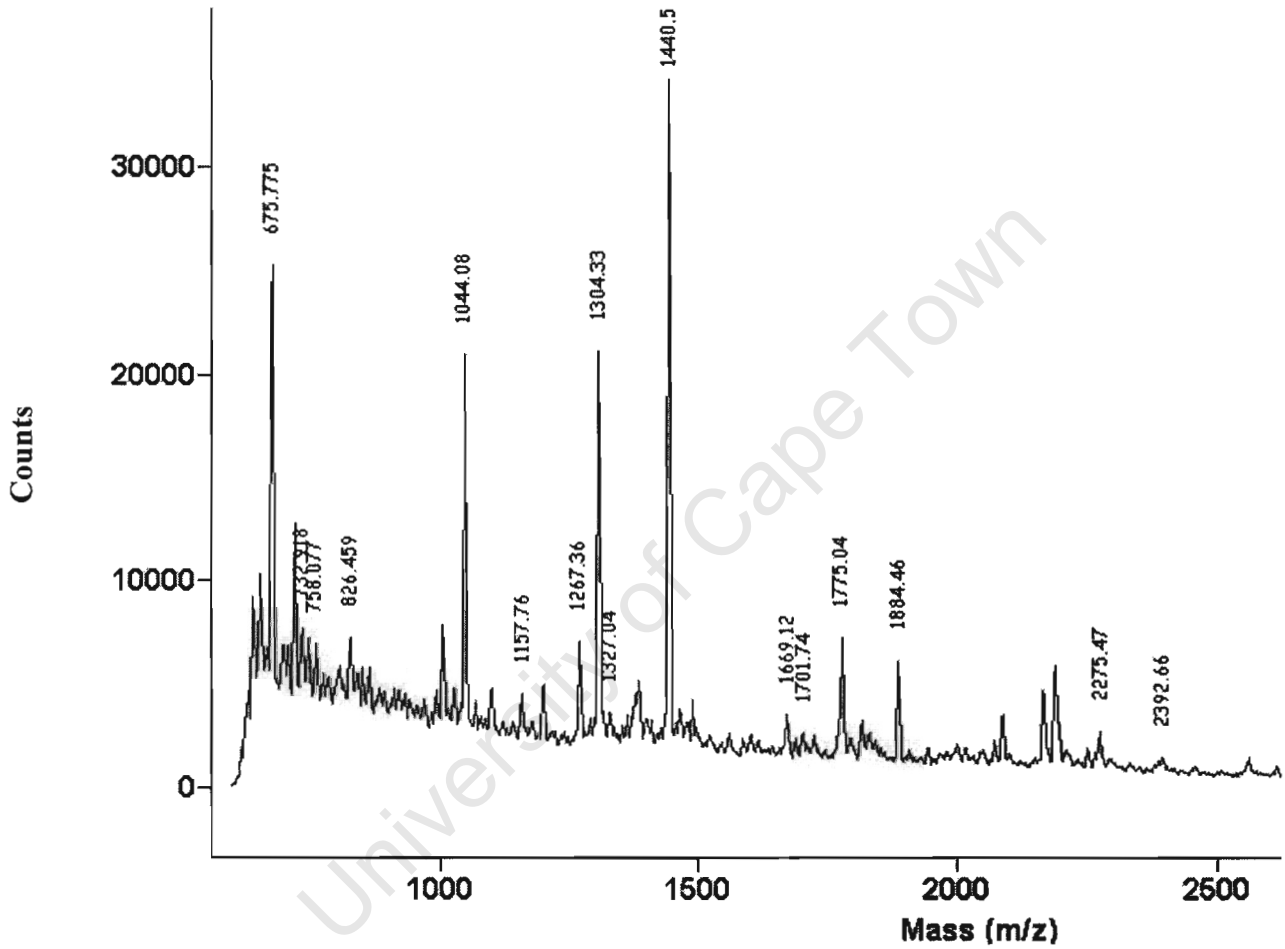


Figure 11. *MALDI-TOF analysis of peptides present in a trypsin digest of the approximately 25 kDA band PGM. Peptides with masses of 758, 826, 1157, 1267, 1327, 1440, 1669, 1701, 1884, 2275 and 2392 Da matched peptides produced by in silico digestion of the PGM sequence on the SWISS-PROT database.*

PGM was identified as the most probable protein as 11 out of 16 peptides with masses between 700 and 2700 m/z present in the mass spectrum corresponded with the masses of theoretical peptides produced by tryptic digestion of the translated PGM gene sequence in the SWISS-PROT database. These peptides had masses of m/z 758, 826, 1157, 1267, 1327, 1440, 1669, 1701, 1884, 2275, and 2392. The next possible protein was the bZIP transcription factor Hac1p, which has a molecular size of 29.6 kDa. In this case 7 out of the 16 peptides present matched the theoretical peptides produced by tryptic digestion. Apart from the lower match detected, it is unlikely that Hac1p would be observed on a Coomasie stained gel since transcription factors are generally present in very low concentrations in cells.

Motshwene et al. (41) also identified Hsp12p to be a yeast cell wall protein. This protein has a molecular mass of 12 kDa and has been shown (40) to increase in concentration after exposure of yeast to a variety of stresses including heat and salt stress. MALDI-TOF mass spectrometric analysis of the 12 kDa protein band (arrow 2, Figure 10) after the trypsin digestion showed that 9 out of the 13 peptides used matched the masses of theoretical peptides produced by tryptic digestion of the translated *HSP12* gene sequence (Figure 12) in the SWISS-PROT database.

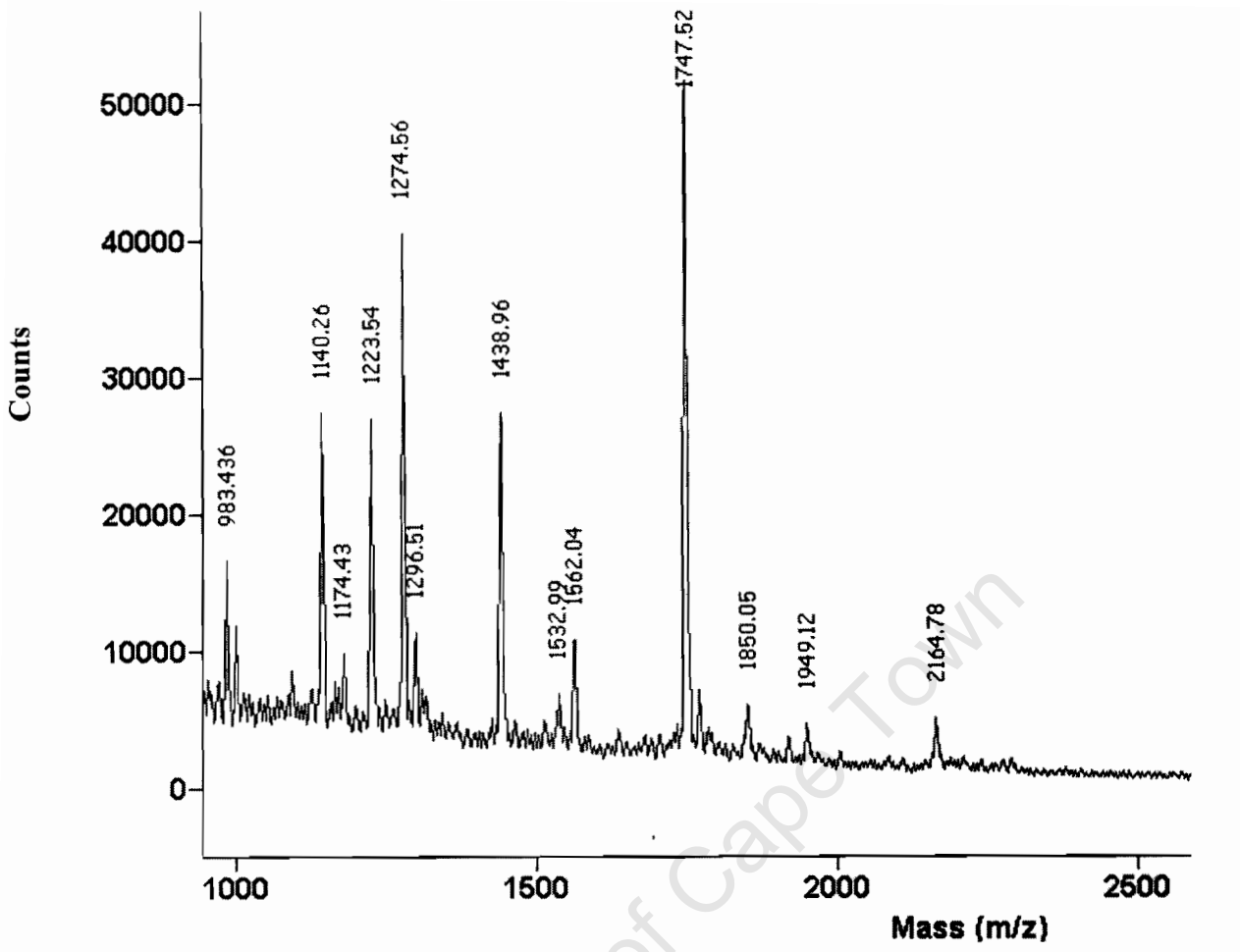
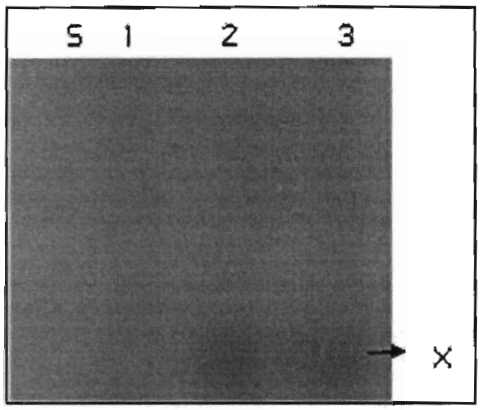


Figure 12. *MALDI-TOF spectrum of peptides present after trypsin digestion of the 12 kDa band (arrow 2, Figure 10). Peptides with masses of 665 (not shown on the mass spectrum), 1139, 1173, 1222, 1273, 1437, 1531, 1562 and 1745 Da matched peptides produced by in silico digestion of the HSP12 sequence on the SWISS-PROT database.*

The next most likely protein was the hypothetical 12 kDA protein from the APA1/DTP-PD11 intergenic region. In this case, 4 out of the 13 peptides present matched the theoretical peptides produced by tryptic digestion. The presence of unidentified peptides in a MALDI-TOF mass spectrum is not uncommon. These peptides can arise from incomplete digestion of the protein, post-synthetic modification of the protein or chemical modification of either the protein or the peptide during the isolation procedure.

It was recently demonstrated that cardiac sarcoplasmic reticulum contains a localized ATP-generating pool comprising the enzymes of the lower part of the glycolytic pathway. This was shown to provide energy exclusively for calcium transport (26) and suggested that the synthesis of ATP in the yeast cell wall might provide energy for re-modeling the cell wall after exposure to stress. The ATP formed might also be used to phosphorylate cell wall proteins and thereby activate or de-activate specific re-modeling pathways. Yeast cells were therefore grown to mid-log phase in YPD medium at 30 ^{0}C after which they were exposed heat (37 °C) stress for up to 4 h in the presence of ^{32}P-Pi. The ATP formed as a result of the bisphosphoglycerate kinase step would contain ^{32}P-γ-ATP, a suitable substrate for protein phosphorylation. After 1, 2 or 4 h incubation, cell wall proteins were extracted from the yeast cells and electrophoresed on a SDS gel. The gel was autoradiographed overnight at -70 °C (figure 13a) before being stained with Coomasie blue (Figure 13b) allowing the identification of radioactive proteins. The Coomasie-stained protein corresponding to the region of maximum exposure of the radioactive band on the autoradiograph was excised as before and subjected to MALDI-TOF mass spectrometry (Figure 14) as before. The bands corresponding to PGM and Hsp12 were also excised and identified using MALDI-TOF mass spectrometry as before (not shown).



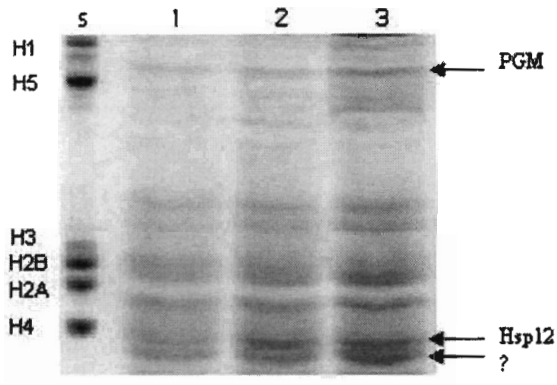


Figure 13. *A: autoradiograph of radioactive proteins extracted from the yeast cells after incubation together with 50μCi carrier-free ^{32}P-Pi for 1, 2, and 4 hours (lanes 1, 2 and 3). B: stained gel of proteins extracted from the yeast cells after incubation together with 50μCi carrier-free ^{32}P-Pi for 1, 2, and 4 hours (lanes 1, 2 and 3). Protein band marked X, showed radioactivity after 2 hours and 4 hours incubation. Standards as per Figure 10.*

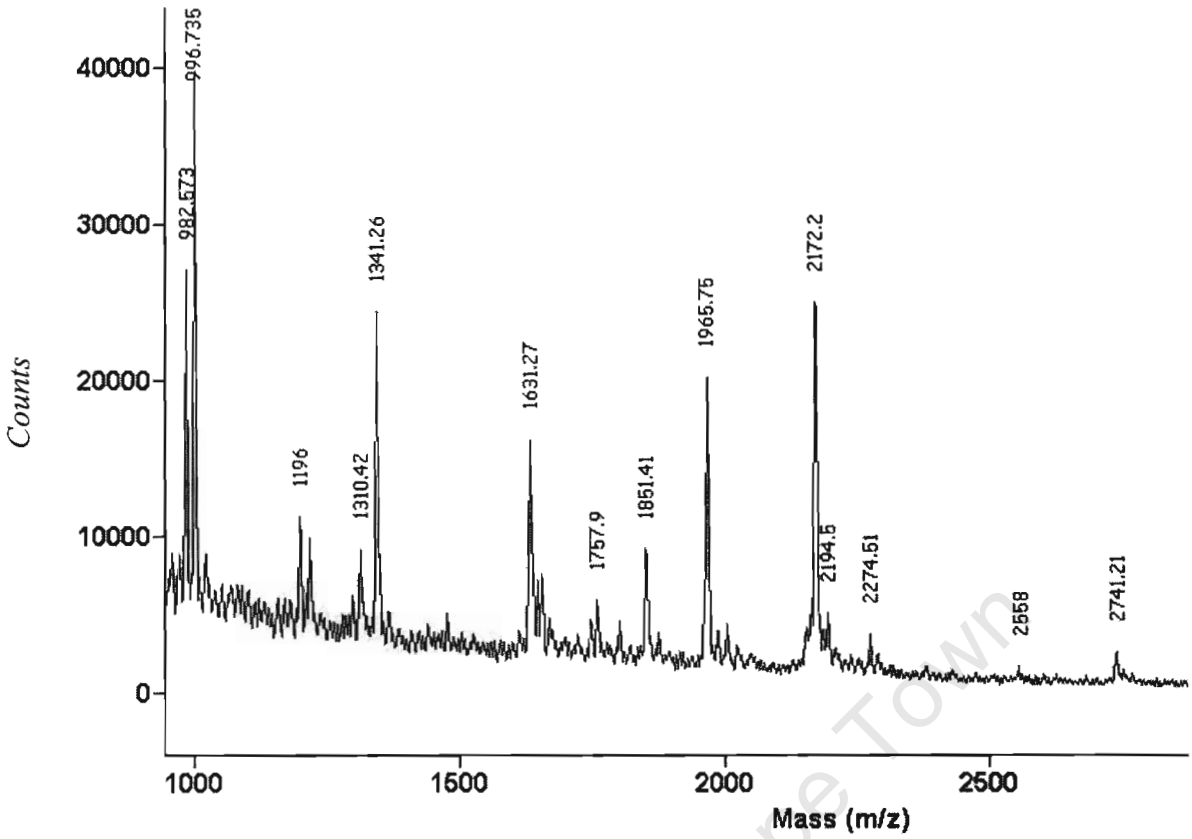


Figure 14. *MALDI-TOF spectrum of peptide present after trypsin digestion of the isolated unknown protein (band X in figure 13). Peptides with masses of 982, 1630, 1758, 2171 and 2739 Da matched peptides produced by in silico digestion of the 40S ribosomal protein S10-B sequence on the SWISS-PROT database.*

Of the 16 peptides present with masses between m/z 600 and m/z 2800, 8 corresponded with the masses of theoretical peptides produced by tryptic digestion of the translated 40S ribosomal subunit protein S10-B gene sequence in the SWISS-PROT database. These peptides had masses of m/z (647, 847, 850) not shown on the mass spectrum, 982, 1630, 1758, 2171 and 2739 Da. The next possible candidate was the 40S ribosomal subunit protein S10-A where 7 peptides with masses of m/z (847, 850) not shown on the mass spectrum, 996, 1630, 1758, 2171 and 2739 matched the

theoretical peptides produced by tryptic digestion. Proteins S-10A and S-10B have been reported to be virtually identical (97 %) showing only 3 amino acid differences in the 105 residues. The only difference in the peptide match was that the m/z 647 peptide was only present in the S10-B tryptic digest, and the m/z 982 peptide in the S10-B tryptic digest was replaced with a peptide with m/z 996 in the S10-A tryptic digest. The next most likely protein was the hypothetical 12 kDA protein from the MRPL31/APL2 intergenic region, making it an unlikely candidate. In this case, 6 out of the 16 peptides present matched the theoretical peptides produced by tryptic digestion.

1.5 Discussion and Conclusion.

Although every Biochemical textbook states that glycolysis occurs in the cytoplasm of cells, a number of authors have clearly found evidence that glycolytic enzymes are present in the yeast cell wall (13, 14, 23). The real question is as to what is their function. The majority of the glycolytic enzymes have been discovered in isolation and as a result it has been proposed that these enzymes are 'moonlighting' and that they might act as receptors during flocculation in *K. bulgaris* (22) or during invasiveness in *C. albicans* (16). The recent finding (23) that whole yeast cells could be convert 3-phosphoglycerate to ethanol has resulted in a re-assessment of their role, since this work demonstrated that not only are the enzymes of the lower part of the glycolytic pathway present in the yeast cell wall but that these enzymes are also active.

This report has investigated the extent of the glycolytic pathway present in the yeast cell wall. The initial research confirmed previous data (23) that whole yeast cells could convert 3-phosphoglycerate to ethanol in the medium providing the necessary co-factors were present. For this reaction to occur within the cytoplasm the 3-phosphoglycerate would either have had to diffuse or be transported into the cytoplasm and the resulting NAD^+ would have had to diffuse or be transported out of the cell. This is unlikely as cell membranes are relatively impermeable to charged molecules and since no 3-phosphoglycerate or NAD^+/NADH transport mechanisms have been reported in yeast.

We then demonstrated that whole yeast cells could convert dihydroxyacetone phosphate to *1,3-bis*phosphoglycerate in the presence of NAD^+ and arsenate with an enzymatic activity similar to the rate of conversion found for the catabolism of 3-phosphoglycerate to ethanol. Since the lower part of the glycolytic pathway generates NADH + H^+ as a result of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the NADH + H^+ must be oxidized to allow continued flux through the pathway. This can either be achieved by conversion of pyruvate to ethanol (via acetaldehyde) or by conversion of dihydroxyacetone phosphate to glycerol-3-phosphate. However no NAD^+-linked metabolism of glycerol was detected suggesting that the branch between dihydroxyacetone phosphate and glycerol was not present in the yeast cell wall. These enzymes have also not been detected by other workers.

Delgado et al. (26) focused on the enzyme GAPDH in *S. cerevisiae* and demonstrated that it was present on the cell wall by indirect immunofluoroscence and by flow cytometric analysis using a polyclonal antibody against GAPDH. They also showed that cell wall GAPDH activity increased in response to starvation and increased temperature and that *S. cerevisiae* responded to stress by increased incorporation of GAPDH into the cell wall. This response did not require *de novo* protein synthesis, which indicated that pre-existing cytosolic enzymes were incorporated into the cell wall. Since GAPDH lacks a secretory signal sequence, this led to a conclusion that GAPDH is exported to the cell wall through a non-classical pathway. We were unable to identify an increased GAPDH protein content in a whole yeast cell protein extract although we were able to demonstrate increased PGM and Hsp12p protein levels in the cell wall after stress. These data confirmed previous findings that PGM (23) and Hsp12p (41) are in the cell wall and the concentration of these proteins increased after stress.

We were, however, able to show an increased GAPDH activity in the cell wall after either heat or salt stress, by demonstrating an increased ATP content as a result of such stress. This ATP content reached a maximum after 2 hours of exposure, which correlated with the finding that increased PGM and Hsp12p protein levels were at a maximum 2 h after exposure of the yeast cells to stress. Heat stress was found to have a greater effect on the ATP concentration than salt stress. One possibility for this might be that salt would result in decreased cell wall flexibility (24) whereas heat would have the opposite effect by stimulating the cell integrity pathway.

Having demonstrated that ATP is produced in the yeast cell wall as a result of reactions of the lower part of the glycolytic pathway, the question is as to the role of this ATP. Two possibilities exist, namely that it could be used as an energy source for cell wall re-modelling or as the substrate for a kinase in a signal transduction pathway. To investigate this latter possibility, we used ^{32}P-Pi to synthesise ^{32}P-γ-ATP and showed that a protein of molecular mass around 10 kDa was phosphorylated after stress. This protein was identified to be either the 40S ribosomal subunit protein S10-B or the 40S ribosomal subunit protein S10-A. These proteins are remarkably similar, both having molecular masses of 12.7 kDa, making it impossible to separate them by SDS-PAGE. Moreover it is unlikely that these proteins could be separated by 2-dimensional IEF/SDS-PAGE since the proteins have very similar isoelectric points, pH 9.05 for S10-B and pH 8.73 for S10-A. S10-B is the major phosphorylated protein present in the 40S ribosomal subunit and contains the sequence RASSLK at the C-terminal end. This sequence is very similar to the phosphorylation site on the rat liver small ribosomal subunit protein S6, which has the sequence RRLS*S*LRA where the * indicates the sites of phosphorylation (42). Interestingly, both these proteins are,

like Hsp12, very hydrophilic with hydropathicity indices of -0.90 for S10-B and S10-A compared with -1.21 for Hsp12.

Other researchers have demonstrated the presence of ribosomal proteins in the yeast cell wall. The yeast ribosomal "stalk", a lateral protuberance on the 60s ribosomal subunit, contains four similar (35) acidic proteins, P1A, P1B, P2A and P2B, required for elongation factor 2 binding and GTP hydrolysis. Grankowski et al. (34) have recently showed using immunocytochemistry that these P-proteins were present both in the cytoplasm as well as in the cell wall, where they were phosphorylated.

A number of possibilities have been proposed to explain the occurrence of cytosolic proteins in the yeast cell wall:

- They may be incorporated into the cell wall as a result of increased cell wall flexibility brought about by stress. However this is unlikely since, using agarose as a model system for the β-glucan layer of the cell wall, Karreman et al. (24) demonstrated that osmotic, salt and ethanol stress results in decreased rather than increased cell wall flexibility.

- They may 'hitch-hike' to the surface by leaking into the secretory pathway during formation of transport vesicles or when transport vesicles fuse with their target organelles (8).

- They may get secreted by a nonclassical, nonsignal sequence-based secretory pathway since glycolytic enzymes lack signal for GPI as well as signal peptide (15, 16).

- They may result from aging cells or cells damaged from shearing forces and since glycolytic enzymes have high isoelectric points and thus often positively charged depending on the pH of the culture medium, may bind to the phosphodiester bridges on the cell wall (8, 14). This is also unlikely as Motshwene et al. (41) were unable to show that Hsp12p, which has a pI of 5.1, in the cell wall, was the result of cell lysis or leakage and subsequent uptake.

Our findings are therefore not unprecedented, but add to the growing group of proteins that have been discovered in the yeast cell wall. Continued work will no doubt elucidate their function in the future.

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