NaOH was used to extract proteins from the cell walls of the yeast \textit{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 was 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 late-embryogenic-abundant-like protein heat-shock protein 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 into ethanol, provided that the necessary cofactors were present.

Key words: alkaline extraction, hyper-osmotic stress, immunocytochemistry.

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 [1]. A number of plasma-membrane sensors, including Wsc1-4, Mid 2 and Mtl1 [1–4], 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) [3,4], which in turn activates the mitogen-activated protein kinase pathway. This pathway results in the transcription of a number of genes, including those for transcription factors such as Slt2, cell wall proteins such as the Pir proteins, enzymes possibly involved in cell wall remodelling (Fks2) [1,5], 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 characterized proteins extracted under alkaline conditions from both whole yeast cells and 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 heat-shock 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 [6], and are therefore hypothesized to be present in mature cell walls. These enzymes include those of the ATP-generating portion of the pathway as well as fructose bisphosphate aldolase and triose phosphate isomerase. Glycolytic enzymes have also been shown to be present in cell walls of yeast by MS of cell wall proteins separated by two-dimensional SDS/PAGE [6,7] or by the binding of antibodies against specific glycolytic enzymes to the exterior of whole yeast cells [8–11]. In this article 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 into ethanol in the presence of the required cofactors.

METHODS AND MATERIALS

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, South Africa) was washed with 100 mM Tris/HCl/50 mM NaCl, pH 7.4, before alkaline-soluble proteins were extracted with 4 ml of NaOH for 30 min 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 μm-diameter glass beads. The absence of intact cells was confirmed by the absence of colonies when the cell wall preparation was plated on to 1.5% (w/v) agar yeast extract/peptone/dextrose (YPD) plates and incubated at 30°C.

Isolation, trypsin digestion and MS 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/50 mM 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 × 200 mm 20% SDS gel [12]. 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) cetyl trimethyl ammonium bromide for 1 h before being electropholated at a constant current in 0.9% (v/v) acetic acid for 16 h. The extract was lyophilized, dissolved in the minimum volume of distilled water and the protein precipitated with ice-cold...
acetonitrile/20 mM HCl. The precipitate, which contained pure protein as assessed by SDS/PAGE, was washed three times with ice-cold acetonitrile, dried under vacuum and stored at −20 °C. Protein sequencing was performed as described previously [13].

Trypsin digestion and matrix-assisted laser-desorption ionization–time of flight (MALDI-TOF) MS was carried out essentially as described by Wilm et al. [14]. A piece of the SDS/PAGE gel containing the Coomassie-stained protein was cut into small pieces. Following sequential incubations in 50% (v/v) acetonitrile for 5 min, and in 50% acetonitrile/50 mM NH₄HCO₃ and 50% acetonitrile/10 mM NH₄HCO₃ for 30 min each, the gel pieces were dried under vacuum. Trypsin (Promega) digestion of the pieces re-hydrated in 10 mM NH₄HCO₃ buffer was performed for 16 h at 37 °C using 0.1 μg of trypsin/15 mm² of gel. Mass analysis was performed by mixing 1 μl of the tryptic digest with 1 μl of matrix (10 mg/ml α-cyano-4-hydroxycinnamic acid in 60% acetonitrile/0.3% trifluoroacetic acid), followed by spotting 1 μl of this mixture on to 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 [15].

**Immunoechemical methods and electron microscopy**

Pure phosphoglycerate mutase (EC 5.4.2.1), prepared by electrophoresis 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 immunize rabbits with four injections at bi-weekly intervals. Titres and specificity were determined by ELISA [16]. Immunocytochemistry was performed as described previously [17], except that the yeast cells were fixed using 0.5% glutaraldehyde in 20 mM HCl, pH 7.0, after which the extracted proteins were analysed by SDS/PAGE, at a concentration of 0.4 mg/ml in distilled water. The cutting temperature was −120 °C. Sections were retrieved on to carbon-coated, glow-discharged nickel grids and immunolabelled as described previously [18].

**Phosphoglycerate mutase assay**

Phosphoglycerate mutase activity of yeast cell extracts and electrophoresed fractions was assayed as described in [19] 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 units/ml lactate dehydrogenase (EC 1.1.1.28), 7 units/ml pyruvate kinase (EC 2.7.1.40) and 3 units/ml enolase (EC 4.2.1.11) in buffer of 100 mM Tris/HCl, pH 7.0, 100 mM KCl and 5 mM MgSO₄ was used for the assay. A 100 μl aliquot of a sample containing phosphoglycerate mutase was used to start the reaction. Phosphoglycerate mutase activity of whole yeast cells was assayed essentially as described previously [9,11]. 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 light scattering at 600 nm (D₅₀₀).

**RESULTS**

NaOH and NaOH/SDS solutions are commonly used for the extraction of proteins from purified cell walls [20–22]. We initially investigated whether dilute NaOH solutions would extract pro-

![Figure 1](image1.png)  
**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 (lane 2) or untreated (lane 3) cells

Wet packed yeast (1 g) was suspended in 1 ml of NaOH or the extracting buffer. The cells were pelleted by centrifugation after the specified treatment, and 10 μl of the supernatant was applied to the gel. The standard (S) is a total extract of chicken erythrocyte histones, the approximate molecular masses of which are: H1, 22.5 kDa; H3, 15.3 kDa; H2B, 13.7 kDa; H2A, 14.0 kDa; H4, 11.2 kDa; H5, 18.5 kDa.

![Figure 2](image2.png)  
**Figure 2**  
Electron micrograph of a whole washed yeast cell after extraction with 0.2 M NaOH

Magnification × 15000.

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 normal morphological appearance, with
Proteins subjected to SDS
therefore extracted with 1 M NaOH and the extracted pro-
ychromic medium containing increasing amounts of mannitol, were
with that after 0.2 M NaOH treatment (results not shown).

Increasing the NaOH concentration to 1 M resulted in an
increased concentration of the same proteins being present in the
cells and untreated cells were qualitatively similar (Figure 1).

The complement of soluble proteins from 0.2 M NaOH-treated
Yeast were grown in YPD medium in the absence of mannitol (lane 1) or in the presence of
0.16 M (lane 2) or 1 M (lane 3) mannitol. The arrows denote two proteins present at increased
concentrations when yeast cells were grown in the presence of 1 M mannitol. Wet packed yeast
(1 g) was suspended in 1 ml of NaOH. The cells were pelleted by centrifugation, and 10 µl of
the supernatant was applied to the gel. The standard (S) is a total extract of chicken erythrocyte
hemiloblasts, the approximate molecular masses of which are given in Figure 1 legend. Bottom
panel: scans of the gel shown in the top panel. The top, middle and bottom scans represent
0, 0.16 and 1 M mannitol respectively. The arrows denote the migration of the bands shown
by arrows in the top panel.

Figure 3  SDS/PAGE of proteins extracted from whole washed yeast cells
with 1 M NaOH

Top panel: SDS/PAGE of proteins extracted from whole washed yeast cells with 1 M NaOH. Yeast was grown in YPD medium in the absence of mannitol (lane 1) or in the presence of
0.16 M (lane 2) or 1 M (lane 3) mannitol. The arrows denote two proteins present at increased
concentrations when yeast cells were grown in the presence of 1 M mannitol. Wet packed yeast
(1 g) was suspended in 1 ml of NaOH. The cells were pelleted by centrifugation, and 10 µl of
the supernatant was applied to the gel. The standard (S) is a total extract of chicken erythrocyte
hismones, the approximate molecular masses of which are given in Figure 1 legend. Bottom
panel: scans of the gel shown in the top panel. The top, middle and bottom scans represent
0, 0.16 and 1 M mannitol respectively. The arrows denote the migration of the bands shown
by arrows in the top panel.

Phosphoglycerate mutase is present in Saccharomyces cerevisiae cell wall

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 pro-
tines subjected to SDS/PAGE (Figure 3, top panel). Two
proteins (indicated by arrows on Figure 3, top panel) appeared
increased in content in response to increased mannitol concen-
tration in the growth medium. These proteins had molecular
masses of approx. 12 and 25 kDa. The lanes representing yeast
grown without mannitol and those representing yeast grown in
the presence of 0.16 and 1 M mannitol were scanned (Figure 3, bottom panel) and the areas under the peaks representing
the 25 kDa protein calculated. These areas were found to be
in the proportions 1:1:3, confirming that the concentration of the 25 kDa protein was elevated when yeast cells were grown
in the presence of 1 M mannitol.

The 12 kDa protein was identified as HSP 12 by Western
blotting (results not shown). We have previously shown using
immunocytchemistry that HSP 12 is present in the cell in the vicinity of the plasma membrane and the cell wall [17]. 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 (results not shown). Furthermore, this
preparation failed to produce colonies when plated on to 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 localization for the unknown protein was
obtained from abrasion of 1 M NaOH-treated yeast cells. A 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 (results 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.

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. We
found 13 peptides of molecular masses 656, 701, 760, 857, 898,
1115, 1225, 1240, 1291, 1447, 1514, 2375 and 2666 Da (all
sizes ±1 Da) and used them to identify the protein as phos-
phoglycerate mutase using the SwissProt 6.12.2000 database. A
total of 11 of the 13 peptides matched the theoretical peptides
produced by trypic digestion of the translated phosphoglycerate
mutase gene sequence in the database. Peptides of the molecular
masses present were found to be 45 times more likely to have
originated from phosphoglycerate mutase than from any other
protein. The two peptides with masses of 1225 and 1447 Da were
not identified. The presence of unidentifiable peptides is not
uncommon. These can arise as a result of incomplete digestion,
post-synthetic modification or chemical modification during the
isolation procedure. To confirm that the 25 kDa protein was
indeed phosphoglycerate mutase, the protein was purified by
electroelution from a SDS/PAGE gel (Figure 4). The electro-
eluted protein, which ran as a single band on SDS/PAGE, was
subjected to N-terminal sequencing. A single N-terminal se-
quence, Pro-Lys-Leu-Val-Leu, was found. Using the Saccharo-
myces Genome Database, this sequence was found to be unique
to residues 2–6 of the phosphoglycerate mutase expressed from
the GPM1 gene. The N-terminal meth-
ionine has been reported to be cleaved from the protein during
post-translational processing [23,24]. The protein, which was
judged to be pure phosphoglycerate mutase, was used for the
preparation of polyclonal antibodies in rabbits.

The specificity and affinity of rabbit antisera to purified
phosphoglycerate mutase were tested by Western analysis of a
crude NaOH extract of whole yeast cells and by ELISA re-
spectively. Western analysis (results not shown) failed to detect

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
tickets 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
with that after 0.2 M NaOH treatment (results not shown).

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 pro-
tines subjected to SDS/PAGE (Figure 3, top panel). Two

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reactivity to contaminating proteins, with only a single band with the mobility of phosphoglycerate mutase being observed. The antisera strongly recognized the purified protein at dilutions of 1:10⁴. Thin sections of embedded yeast cells were therefore 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 in both the cytoplasm and the cell wall with an approximately equal distribution (Figure 5). 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 that, in addition, significant quantities were also present in the cell wall.

The enzymic 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 by its ability to convert 3-phosphoglycerate into 2-phosphoglycerate in the presence of 2,3-bisphosphoglycerate. The activity of this preparation was found to be 0.47 μmol/min per mg of protein. We next investigated whether the phosphoglycerate mutase present in the walls of whole yeast cells could convert 3-phosphoglycerate in the medium into 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 into the medium. It was found that the whole cell phosphoglycerate mutase was indeed active, and that the conversion proceeded at a rate of 1 ± 0.1 μmol · min⁻¹ · D₀₀⁻¹. No NADH + H⁺ oxidation occurred in the absence of added 3-phosphoglycerate, demonstrating that this oxidation occurred exogenously. Glycolytic enzymes of the lower Embden–Meyerhof pathway besides phosphoglycerate mutase have been reported to be present in the cell walls of yeast [6]. We therefore repeated this experiment in the absence of added enolase and pyruvate kinase, and found that the conversion of 3-phosphoglycerate into lactate still proceeded at a rate of 1 ± 0.01 μmol · min⁻¹ · D₀₀⁻¹. Moreover, whole cells were able to convert 3-phosphoglycerate into ethanol, as the oxidation of NADH + H⁺ still occurred when lactate dehydrogenase was omitted from the medium provided that thiamin pyrophosphate was added. The rate of reaction was, however, significantly lower, 0.4 ± 0.4 μmol · min⁻¹ · D₀₀⁻¹.

Since treatment of yeast cells with NaOH altered the exposure of phosphoglycerate mutase within the cell wall, we determined the phosphoglycerate mutase enzymic activity of NaOH-treated cells using the standard assay. Whereas this activity was 1 μmol · min⁻¹ · D₀₀⁻¹ in untreated cells, it increased to 11 ± 0.4 μmol · min⁻¹ · D₀₀⁻¹ after NaOH treatment. Omitting enolase and pyruvate kinase reduced the activity to 0.4 ± 0.05 μmol · min⁻¹ · D₀₀⁻¹.

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 in cell walls. In this report, we
have confirmed that the glycolytic enzyme phosphoglycerate mutase is present in the cell walls of \textit{S. 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 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 with those present in untreated cells, suggesting that alkaline treatment of whole cells resulted in the extraction of a small, select group of proteins. Thirdly, proteins extracted from whole yeast cells were qualitatively similar to those extracted from isolated cell walls.

Alkaline extraction has previously been used to isolate cell wall proteins from \textit{S. cerevisiae} [21,22], presumably after hydrolysis of the O-linked covalent attachment. Although it could be argued that such treatment might result in leakage of the cytoplasmic contents, we and previous authors have used immunocytochemistry to demonstrate that the proteins isolated after alkaline extraction are indeed present in the cell wall of the untreated yeast cell. 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 mass calculated from the gene sequence of 27480 Da but migrates on SDS/PAGE with an observed molecular mass of approx. 26000 Da [6,25]. The phosphoglycerate mutase released from yeast cells by alkaline treatment migrated as anticipated, migrating with the linker histones, which migrate with molecular masses in the 25000–27000 Da range. This suggests that the released phosphoglycerate mutase had no substantial post-synthetic 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 [20]. 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 in neither HSP 12 nor phosphoglycerate mutase.

We were able to demonstrate that phosphoglycerate mutase released from yeast cell walls by alkaline treatment still showed enzymic activity. Moreover, whole yeast cells were able to convert 3-phosphoglycerate into ethanol, provided that the necessary cofactors 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 to diffuse or to be transported into the cytoplasm, and the NAD\(^+\)/NADH, and since no transport mechanisms have been reported for either 3-phosphoglycerate or NAD\(^+\)/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 enzymic assays. Moreover, a number of the enzymes required to perform the conversion of 3-phosphoglycerate into 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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**REFERENCES**


