Available online at www.sciencedirect.com
SCIENCE DIRECT
Carbohydrate Polymers 60 (2005) 193–198
Carbohydrate Polymers
www.elsevier.com/locate/carbpol

The yeast *Saccharomyces cerevisiae* stress response protein Hsp12p decreases the gel strength of agarose used as a model system for the β-glucan layer of the cell wall

Robert J. Karreman, Wolf F. Brandt, George G. Lindsey*

Department of Molecular and Cellular Biology, University of Cape Town, Private Bag, Rondebosch, 7701, South Africa

Received 8 September 2004; revised 22 December 2004; accepted 22 December 2004
Available online 3 February 2005

Abstract

Incorporation of the cell wall LEA-like stress response protein Hsp12p from the yeast *Saccharomyces cerevisiae* into the carbohydrate polymer agarose was found to reduce the gel strength of the agarose matrix. Solutes known to stimulate Hsp12p production in yeast were found to increase the gelation temperature, the turbidity and the gel strength of the agarose matrix. Increased gel strength brought about by solute incorporation was found to be reversed by co-incorporation of Hsp12p or by the chaotropic solute urea. These results suggested a mode of action for Hsp12p, assuming that agarose is a reasonable macroscopic model system for investigating the effects of cell wall proteins on the properties of the yeast cell wall.

Keywords: Rheometry; Gelation temperature; Turbidity

1. Introduction

The cell wall of the yeast *Saccharomyces cerevisiae* is composed of an outer mannoprotein layer involved in cell-cell recognition and an inner carbohydrate layer that provides much of its physical strength (Klis, 1994; Lipke & Ovalle, 1998). The glucans are the major components of this inner layer, which can be again divided into inner and outer components. The outer amorphous component is enriched in β 1,6 glucans whereas the fibrillar inner component comprises mainly β 1,3 glucans. This β 1,3 glucan layer, an insoluble triple helical arrangement of β 1,3 linked polymers of D-glucose, is thought to provide the yeast cell with its mechanical stability since removal of the mannoprotein layer and the β 1,6 glucan layer with pronase and β 1,6 glucanase respectively has been reported to have no effect on cell shape (for a review, see Klis, Mol, Hellingwerf, & Brul, 2002).

Hsp12p is a small hydrophilic Late Embryogenic Abundant (LEA)-like protein, the concentration of which increases markedly after heat shock (Varela, Praekelt, Meacock, Planta, & Mager, 1995) as well as on entry into stationary phase and when yeast are subjected to different forms of osmotic stress (Mtwisha, Brandt, McCready, & Lindsey, 1998). Immunocytochemical detection of Hsp12p has demonstrated that the protein is primarily located in the cell wall (Motshwene, Karreman, Kgari, Brandt, & Lindsey, 2004) although a smaller quantity is found surrounding the plasma membrane (Sales, Brandt, Rumbak, & Lindsey, 2000). Since Δ*hsp12* yeast displayed markedly less cell volume change upon osmotic shock, we proposed that the role of Hsp12p is to enhance the flexibility of the yeast cell wall (Motshwene et al., 2004). Indirect evidence in support of such a function is that trehalose deficient (Δ*tps*) yeast are sensitive to hydrostatic pressure (Iwahashi, Obuchi, Fujii, & Komatsu, 1997) displaying an altered morphology with cytoskeletal deformation after the application of 200 MPa pressure. This altered morphology, ascribed to resultant changes in the cell wall, was not observed when the yeast were heat shocked prior to the application of pressure (Fernandes, Farina, & Kurtenbach, 2001). Rescue of the barosensitive Δ*tps* phenotype by heat

* Corresponding author. Tel.: +27 21 650 2406; fax: +27 21 689 7573.
E-mail address: lindsey@science.uct.ac.za (G.G. Lindsey).

0144-8617/$ - see front matter © 2005 Published by Elsevier Ltd.
doi:10.1016/j.carbpol.2004.12.004

shock supports our hypothesis that Hsp12p influences cell wall flexibility.

Our hypothesis is that Hsp12p interrupts the normal hydrogen bonding pattern of the glucan polymers in a manner analogous to plasticisers in plastic polymers. In order to test our hypothesis, we would like to determine the effect of incorporation of Hsp12p on the elasticity of the β 1,3 glucan matrix. The β 1,3 glucan matrix can, however, only be solubilised after partial phosphorylation (Williams, McNamee, Jones, Pretus, Bro, & Di Luzio, 1991), which would introduce anionic groups and alter the nature of inter-chain interactions. We have therefore investigated the effect of incorporation of Hsp12p on the elasticity of the polysaccharide agarose, which has a number of similarities to β 1,3 glucan. Agarose is also a neutral copolymer but composed of alternating 1,3-linked β-D-galactopyranose and 1,4-linked 3,6-anhydro-α-L-galactopyranose residues (Arnott, Fulmer, & Scott, 1974). X-ray diffraction and optical rotation studies have elucidated the structure of agarose to be a parallel double helix, which adopts a left-handed three-fold symmetry (Arnott et al., 1974). Unlike β 1,3 glucan, agarose readily dissolves in water at 80 °C. On cooling, it will remain in a sol state until the gelation temperature, whereupon gelling begins. The mechanism for the gelation process is believed to involve the assembly of random coils in solution to more ordered double helices by hydrogen bonding and, finally, aggregation of these helices to form a porous matrix (Griess, Moreno, Easom, & Serwer, 1989). This process is accompanied by an increase in the viscosity, elasticity and light scattering properties of the solution (Arnott et al., 1974). Proteins can be readily incorporated into the agarose matrix, for example the incorporation of specific antibodies for antigen quantitation via 'rocket' electrophoresis.

In this manuscript we report that the gel strength of the agarose matrix increased upon incorporation of solutes known to induce the synthesis of Hsp12p in yeast. In contrast, Hsp12p, but not a control protein lysosyme, decreased the gel strength of the agarose. These effects were competitive and the increased gel strength of agarose incorporating lyotrophic solutes could be restored to its original value by the simultaneous incorporation of Hsp12p. The physiological significance of these findings to yeast growth is discussed.

2. Materials and methods

2.1. Agarose

Molecular Grade agarose (Whitehead Scientific, Cape Town) was used at a concentration of 1% w/v in all investigations. This grade of agarose was chosen as it possessed low electro-osmosis properties, so minimising the effect of ionic interactions between solutes and protein with the gel.

2.2. Proteins

Hsp12 was extracted from whole washed yeast cells with 0.6 M NaOH. 10 g wet packed baker's yeast (Anchor Yeast, Cape Town) was extracted with 10 ml 0.6 M NaOH for 30 min on ice before being centrifuged (10,000 × *g*, 10 min) at 4 °C. Tris was added to 10 mM to the supernatant, the pH adjusted to 7.4 with HCl and the supernatant fraction was applied to a Sephadex G 50 column incubated with 10 mM Tris–HCl 20 mM NaCl pH 7.4. The eluted protein represented Hsp12 with a purity >90% upon analysis by SDS-PAGE. Lysozyme was obtained from the Sigma Chemical Co.

2.3. Turbidity studies

Turbidity was investigated using a Shimadzu 2201 recording spectrophotometer with a temperature controlled cuvette holder. The optimum wavelength for analysis (500 nm) was chosen by determining the lowest wavelength exhibiting zero absorption for an agarose solution. A typical analysis involved placing liquid agarose at 70 °C in a plastic cuvette in the spectrophotometer with the cuvette holder maintained at 0 °C, and allowing equilibration to 50 °C to occur. The absorption and sample temperature, monitored using a Fluke Multimeter equipped with a thermocouple, was then determined over the next 300 s. Turbidity was defined as the absorption at 500 nm at 11 °C. The gelation temperature, T_g, was defined as the temperature at which the maximum rate of change of absorption occurred. The turbidity and gelation temperature were found to be linearly dependent on the agarose concentration.

2.4. Gel strength studies

Agarose was dissolved at the stated concentration in water or the solution of choice at 80 °C and the solution poured into nylon moulds size 10 mm ∅ × 10 mm height. 1-Propyl alcohol (propanol) was used instead of ethanol since the boiling point of 97.2 °C is greater than the temperature used to dissolve the agarose. Each mould was covered with a glass plate and allowed to cool to room temperature at 100% humidity to minimise syneresis. Gels were removed from the moulds immediately prior to use. Compression was applied to the agarose cylinder using an Instron Universal Tester Model 5567 using a cylindrical nylon probe with flat face geometry at a crosshead speed of 60 mm/min. This crosshead speed was used to minimise creep so that the cylinders essentially represented solid structures. The force exerted on the probe (stress) was determined as a function of probe extension (strain). Gel strength was defined as the maximum force that the sample could withstand before permanent deformation and was characterised by a sharp decline in the force exerted on the probe at a certain critical extension. Gel strength was found to be linearly dependent on the agarose concentration.

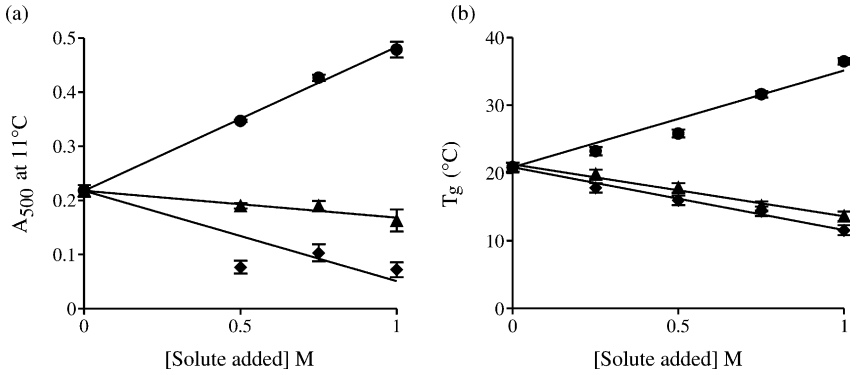

Fig. 1. The effects of included solutes on the 500 nm turbidity (a) and gelation temperature (b) of 1% agarose. The included solutes used were $(NH_4)_2SO_4$ (●—●), urea (♦—♦) and n-propanol (▲—▲). The data shown are the means ± the standard deviation of three separate experiments.

3. Results

A variety of salts have been characterised into lyotropic, neutral and chaotropic solutes on the basis of their effects on ribonuclease stability (Von Hippel & Wong, 1965). Incorporation of the lyotropic solute $(NH_4)_2SO_4$ into the agarose matrix showed a large increase in the turbidity as well as the gelation temperature. The magnitude of these increases was linearly dependent on the $(NH_4)_2SO_4$ concentration (Fig. 1). In contrast, incorporation of the chaotropic agent, urea, into the agarose matrix had the opposite effect, with a concentration dependent decrease in both the turbidity and the gelation temperature observed. Agarose gelation has been postulated to be brought about by hydrogen bonding between agarose chains. To investigate whether there was any hydrophobic contribution to this process, we incorporated propanol into the agarose matrix and again investigated the change in the turbidity on cooling. We found (Fig. 1) that propanol behaved in a similar manner to urea with regard to alteration of the gelation temperature where a concentration dependent decrease of a similar magnitude was observed. Little change in the turbidity of the agarose was observed, however, suggesting that the interaction of individual agarose chains was largely unaltered by propanol.

The changes observed in the turbidity after incorporation of various solutes suggested an alteration in the strength of the agarose matrix. The gel strength, the maximum force that the sample could withstand before permanent deformation, was therefore determined for agarose incorporating a variety of solutes at different concentrations. Solutes were found to affect gel strength in a concentration dependent manner with lyotropic solutes such as KCl increasing the gel strength and the chaotropic solute urea decreasing the gel strength (Fig. 2a). Comparison of the effects of the incorporation of the lyotropic solutes $(NH_4)_2SO_4$ and KCl showed that the presence of 1 M $(NH_4)_2SO_4$ increased the gel strength by 30% whereas the presence of 1 M KCl increased the gel strength by 50%. In contrast, the presence of 1 M urea was found to decrease the gel strength by 40% (Fig. 2b). The stress response protein Hsp12p has been shown to be expressed when yeast are exposed to hyperosmotic conditions due to the presence of a variety of different solutes including salts, ethanol and sugars in the medium. We therefore also investigated the effect of incorporation of propanol or sucrose on gel strength. We found that both sucrose and propanol increased gel strength in a concentration dependent manner (not shown) with incorporation of 1 M sucrose or 1 M propanol resulting in an identical increase of 25% in the gel strength (Fig. 2b).

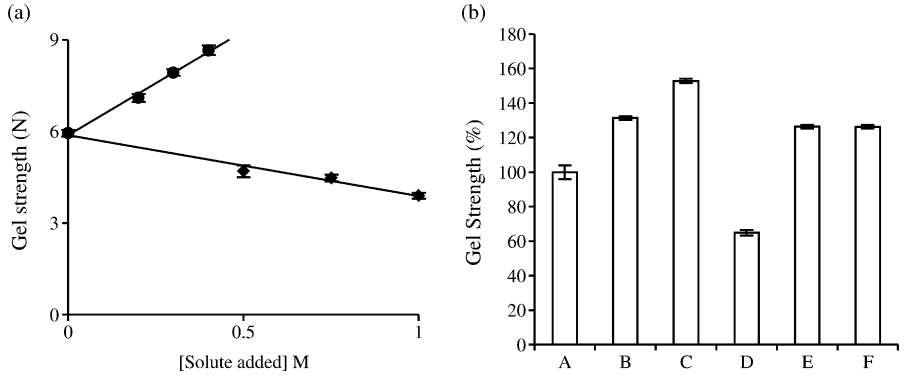

Fig. 2. (a) The effects of included solutes on the gel strength of 1% agarose. The included solutes used were $(NH_4)_2SO_4$ (●—●), urea (♦—♦). (b) Comparison of the effects of inclusion of molar concentrations of various solutes on the relative gel strengths of 1% agarose. A: no addition, B: $(NH_4)_2SO_4$, C: KCl, D: urea, E: n-propanol, F: sucrose. The results shown are the means ± the standard deviation of three separate experiments.

We next investigated whether simultaneous incorporation of a chaotropic and a lyotropic solute into the gel matrix would result in intermediate values of the turbidity, gelation temperature and gel strength dependent on the relative concentration of the chaotropic and the lyotropic solutes used. We therefore prepared agarose incorporating 0.5 M $(NH_4)_2SO_4$ together with various concentrations of urea up to 0.75 M and investigated the effect of the urea concentration on the above parameters. We found that increasing the urea concentration resulted in a concentration dependent reduction in the increased turbidity, gelation temperature and gel strength brought about by the incorporation of 0.5 M $(NH_4)_2SO_4$ (Fig. 3). The effect was most marked on the gel strength, where extrapolation of the data showed that the theoretical incorporation of approximately 0.9 M urea would have restored the gel strength to that prior to incorporation of the 0.5 M $(NH_4)_2SO_4$. The effect of the urea on the gelation temperature and the turbidity was less marked. Extrapolation of the data showed that restoration of these parameters to their original value required a higher concentration of urea, approximately 1.7 M urea in both cases.

Studies on whole yeast (Motshwene et al., 2004) showed that the presence of the 12 kDa stress response protein Hsp12p markedly affected the ability of the yeast to withstand sudden pressure changes. We ascribed this to an alteration in the elasticity of the cell wall brought about by Hsp12p interacting with the β-glucan matrix. Hsp12p was therefore incorporated into the agarose matrix at concentrations up to 4 mg/ml and the effect on the turbidity, gelation temperature and gel strength investigated. We found (Fig. 4a) that incorporation of Hsp12p had no effect on either the gelation temperature or the turbidity of the agarose matrix (not shown) but that the protein acted as a chaotrope, reducing the gel strength in a concentration-dependent manner. The gel strength was found to diminish by just over 20% when Hsp12p was present at a concentration of 4 mg/ml (0.25 mM). In contrast, incorporation of the 14.3 kDa protein lysosyme as a control at the same concentration failed to elicit any response. Since changes in the gel strength of the agarose on incorporation of Hsp12p might have been brought about by homogeneous separation of the protein and agarose on cooling, resulting in the formation of 3-dimesional agarose 'islands', the distribution of Hsp12p in the agarose matrix was determined by microscopy after staining with the protein dye Coomassie Brilliant blue. We found (not shown) a uniform distribution of stain throughout the agarose matrix suggesting that incorporation of Hsp12p into the agarose matrix had occurred during cooling. Since Hsp12p acted in a manner analogous to urea in that the agarose gel strength decreased upon its incorporation, we next investigated whether Hsp12p would restore the gel strength of agarose incorporating a lyotropic solute to that of the agarose alone. We found that this indeed occurred. Extrapolation of the data presented in Fig. 4b showed that Hsp12p at 3.4 mg/ml

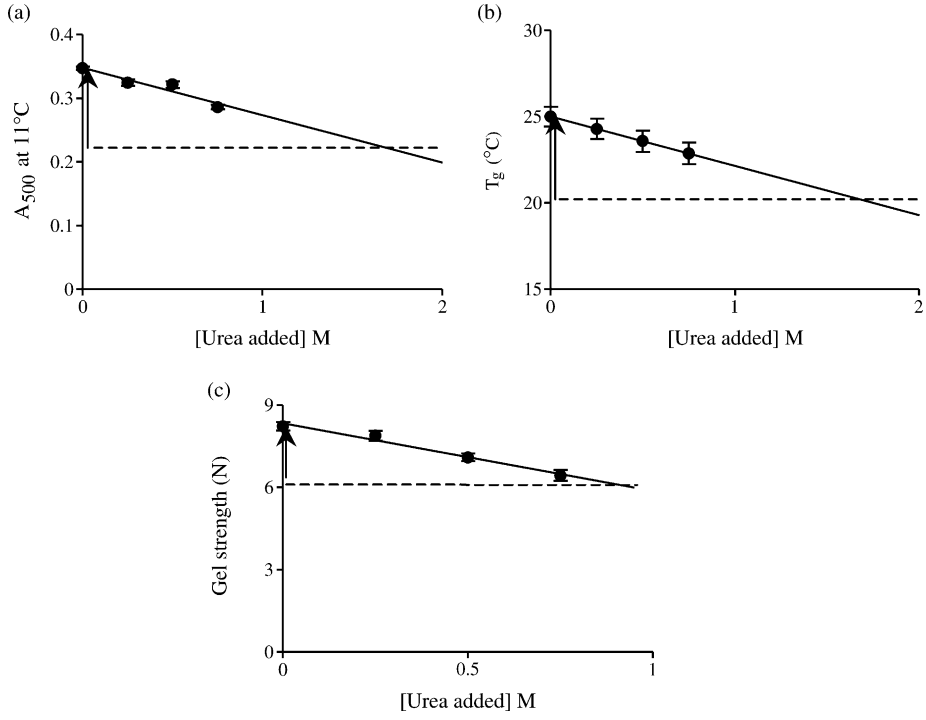

Fig. 3. Effect of the incorporation of increasing concentrations of urea on the turbidity (a), gelation temperature (b) and gel strength (c) of 1% agarose incorporating 0.5 M $(NH_4)_2SO_4$. The arrow (↑) on the ordinate shows the effect of the addition of 0.5 M $(NH_4)_2SO_4$. The results shown are the means ± the standard deviation of three separate experiments. A least squares regression line was fitted to the data and extrapolated to cross the dashed line (– – –) representing the turbidity, gelation temperature or gel strength of 1% agarose prior to the addition of 0.5 M $(NH_4)_2SO_4$.

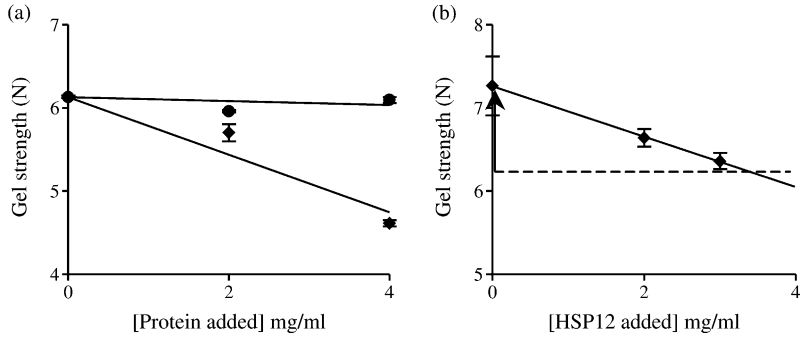

Fig. 4. (a) Effect of the incorporation of increasing concentrations of the proteins Hsp12p (♦—♦) or lysozyme (●—●) on the gel strength of 1% agarose. The results shown are the means ± the standard deviation of three separate experiments. (b) Effect of the incorporation of increasing concentrations of Hsp12p on the gel strength of 1% agarose incorporating 0.25 M $(NH_4)_2SO_4$. The arrow (↑) on the ordinate shows the effect of the addition of 0.25 M $(NH_4)_2SO_4$. The results shown are the means ± the standard deviation of three separate experiments. A least squares regression line was fitted to the data and extrapolated to cross the dashed line (– – –) representing the turbidity, gelation temperature or gel strength of 1% agarose prior to the addition of 0.25 M $(NH_4)_2SO_4$.

would have restored the gel strength of agarose incorporating 0.25 M $(NH_4)_2SO_4$ to that found for agarose alone. A similar result (not shown) was found using 0.75 M sucrose as the lyotropic solute.

4. Discussion

The results reported here show an intriguing correlation between the gel strength of the agarose matrix and factors known to stimulate Hsp12p synthesis in the yeast *S. cerevisiae*. Thus incorporation of lyotropic solutes into the agarose matrix resulted in an increased gel strength and incorporation of the chaotropic solute urea resulted in a decreased gel strength. Since gel strength measurements were all performed on agarose discs of identical dimensions, these results suggested an increased value of the Young's modulus on incorporation of lyotropic solutes and a decreased Young's modulus on incorporation of chaotropic solutes. Young's moduli were not determined as the methodology used would not produce reliable values for the Young's moduli due to a number of factors including friction between the agarose disc and the compression platforms, non-elastic compression and non-uniaxial compression. The increased gel strength observed on incorporation of lyotropic solutes could be restored to its original value by the simultaneous incorporation of Hsp12p. This is analogous to the situation in yeast, where we have demonstrated that growth in hyperosmotic media or growth in media containing ethanol resulted in an increased Hsp12p content (Mtwisha et al., 1998; Sales et al., 2000). We would therefore hypothesise that yeast would sense the increased cell wall rigidity, possibly via the cell wall integrity pathway sensors Mid2 and/or Wsc1 (Hohmann, 2002) and respond by increasing the Hsp12p content in the cell wall. This hypothesis is currently under investigation. Recently the pressure needed to break a yeast cell has been determined (Smith, Zhang, Thomas, Moxham, & Middelburg, 2000). This pressure, ~4810 kN/m^2, is equivalent to an agarose concentration of approximately 0.25%.

The gelation of agarose has been proposed to be a two-step process, with random coils in solution initially assuming double helical conformations. Upon further cooling, bundles of helices form (Arnott et al., 1974). It has been suggested that these bundles can be described as long rods, which affect the turbidity and gel filtration characteristics of the matrix (Obrink, 1968). The results reported here suggest that the size of these rods is affected by the presence of solutes during the cooling process that results in the formation of the agar matrix. This in turn alters the physical properties of the agarose matrix. Since both helix and rod formation are brought about hydrogen bonding between agarose molecules, factors that affect hydrogen bonding would be expected to alter this process. We would postulate that the presence of chaotropic solutes, which are known to disrupt hydrogen bonding, would result in a reduced helix formation and therefore smaller rods. Smaller rods would in turn reduce the turbidity due their smaller size and the gelation temperature and gel strength due to reduced hydrogen bonding. Lyotropic solutes, by increasing the bound water content of the solution, would have the opposite effect. The reduced free water content would favour the hydrogen bonding of agarose monomers to other monomers rather than to free water therefore promoting the formation of longer helical segments and therefore larger rods. Larger rods more strongly hydrogen bonded to one another would account for the increased gel strength observed. This explanation might well be an over-simplification of a complex phenomenon since the turbidity is dependent not only on the size of the particles but also on the relative refractive indices of particles and solvent.

The absence of effect of the incorporation of Hsp12p into the agarose matrix on the turbidity and the gelation temperature is perhaps not surprising. The concentration of Hsp12p used was extremely low, less than 1 mM, in contrast to the molar concentrations of solutes required to elicit a response. These data suggest that Hsp12p does not

affect either the sizes of the rods formed on cooling the agar or the overall interactions between them that occur during the cooling process. The effect of Hsp12p on the gel strength is presumably brought about by Hsp12p interchelating between the agarose rods. It has been shown that LEA proteins have little structure in solution (Lisse, Bartels, Kalbitzer, & Jaenicke, 1996; Russouw, Farrant, Brandt, Maeder, & Lindsey, 1995), a property that would enable such proteins to form hydrogen bonds to agarose rods at different positions along the primary sequence. This presumably allows the agarose rods greater movement with respect to one another without breaking hydrogen bonds, a property similar to that of plasticisers. In contrast, lysozyme failed to alter the gel strength of the agarose matrix. We would postulate that this is because lysozyme is a globular protein (Phillips, 1966) with a defined structure. This defined structure would prevent agarose rod movement without breaking hydrogen bonds, which would account for the observed lack of effect on the agarose gel strength.

Acknowledgements

G.G.L would like to acknowledge the support of South African Breweries, The National Research Foundation, THRIP and the UCT Research Fund.

References

Arnott, S., Fulmer, A., & Scott, W. E. (1974). The agarose double helix and its function in agarose gel structure. *Journal of Molecular Biology*, *90*(2), 269–284.

Fernandes, P. M. B., Farina, M., & Kurtenbach, E. (2001). Effect of hydrostatic pressure on the morphology and ultrastructure of wild-type and trehalose synthase mutant cells of *Saccharomyces cerevisiae*. *Applied Microbiology Letters*, *32*(1), 42–46.

Griess, G. A., Moreno, E. T., Easom, R. A., & Serwer, P. (1989). The sieving of spheres during agarose gel electrophoresis: quantitation and modeling. *Biopolymers*, *28*(8), 1475–1484.

Hohmann, S. (2002). Osmotic stress signaling and osmoadaptation in yeasts. *Microbiological and Molecular Biology Reviews*, *66*(2), 300–372.

Iwahashi, H., Obuchi, K., Fujii, S., & Komatsu, Y. (1997). Barotolerance is dependent on both trehalose and heat shock protein 104 but is essentially different from thermotolerance in *Saccharomyces cerevisiae*. *Letters in Applied Microbiology*, *25*(1), 43–47.

Klis, F. M. (1994). Cell wall assembly in yeast. *Yeast*, *10*(7), 851–869.

Klis, F. M., Mol, P., Hellingwerf, K., & Brul, S. (2002). Dynamics of cell wall structure in *Saccharomyces cerevisiae*. *FEMS Microbioogy Reviews*, *26*(3), 1–8.

Lipke, P. N., & Ovalle, R. (1998). Cell wall architecture in yeast: new structure and new challenges. *Journal of Bacteriology*, *180*(15), 3735–3740.

Lisse, T., Bartels, D., Kalbitzer, H. R., & Jaenicke, R. (1996). The recombinant dehydrin-like desiccation stress protein from the resurrection plant *Craterostigma plantagineum* displays no defined three-dimensional structure in its native state. *Biological Chemistry*, *377*(9), 555–651.

Motshwene, P., Karreman, R., Kgari, G., Brandt, W. F., & Lindsey, G. G. (2004). The LEA-like protein Hsp 12 is present in the cell wall and enhances the barotolerance of the yeast *Saccharomyces cerevisiae*. *Biochemical Journal*, *377*(3), 769–774.

Mtwisha, L., Brandt, W. F., McCready, S., & Lindsey, G. G. (1998). HSP 12 is a LEA-like protein in *Saccharomyces cerevisiae*. *Plant Molecular Biology*, *37*(3), 513–521.

Obrink, B. (1968). Characterization of fiber parameters in agarose gels by light scattering. *Journal of Chromatography*, *37*(2), 329–330.

Phillips, D. C. (1966). The three-dimensional structure of an enzyme molecule. *Scientific American*, *215*(5), 75–80.

Russouw, P. S., Farrant, J. M., Brandt, W. F., Maeder, D., & Lindsey, G. G. (1995). Isolation and characterisation of a heat soluble protein from pea (*Pisum sativum*) embryos. *Seed Science Research*, *5*(1), 137–144.

Sales, K., Brandt, W. F., Rumbak, E., & Lindsey, G. G. (2000). The LEA-like protein HSP 12 in *Saccharomyces cerevisiae* has a plasma membrane location and protects membranes against desiccation and ethanol-induced stress. *Biochimica et Biophysica Acta*, *1463*(2), 267–278.

Smith, A. E., Zhang, Z., Thomas, C. R., Moxham, K. E., & Middelburg, A. P. J. (2000). The mechanical properties of *Saccharomyces cerevisiae*. *Proceedings of the National Academy of Science*, *97*(18), 9871–9874.

Varela, J. C. S., Praekelt, U. M., Meacock, P. A., Planta, R. J., & Mager, W. H. (1995). The *Saccharomyces cerevisiae* HSP12 gene is activated by the high-osmolarity glycerol pathway and negatively regulated by protein kinase A. *Molecular and Cellular Biology*, *15*(11), 6232–6245.

Von Hippel, P. J., & Wong, K. Y. (1965). On the conformational stability of globular proteins. The effects of various electrolytes and non-electrolytes on the thermal ribonuclease transition. *Journal of Biological Chemistry*, *240*(10), 3913–3920.

Williams, D. L., McNamee, R. B., Jones, E. L., Pretus, H. A., Bro, I. W., & Di Luzio, N. R. (1991). A method for the solubilization of a (1-3)-β-D-glucan isolated from *Saccharomyces cerevisiae*. *Carbohydrate Research*, *219*, 203–213.