THE LOCATION AND PUTATIVE FUNCTIONAL ROLE OF A LEA-LIKE PROTEIN (HSP 12) IN YEAST.

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ABSTRACT

Cytoplasmic locations have been postulated for late embryogenesis abundant (LEA) proteins thus far purified. It has been postulated that these proteins play a role in water binding thus maintaining the shell of hydration during desiccation thereby preventing desiccation-induced cellular damage. Evidence in support of this latter hypothesis is that expression of the barley group 3 HVA I LEA-protein in rice resulted in increased tolerance to water stress. Moreover the LEA group I wheat Em protein cloned and expressed in yeast has demonstrated an osmoprotectant role in recombinant yeast grown in media of high osmolarity.

The LEA-like protein HSP 12 was identified as having a plasma membrane location in yeast. Gold particles, indicative of HSP 12, were observed to be present on the external cell wall side of the plasma membrane when yeast grown to stationary phase were subjected to immunocytochemical analysis. Growth of yeast in YPD medium containing 1.6 M mannitol resulted in an increased number of gold particles that were now observed to be present on both sides of the plasma membrane. No gold particles were observed near the plasma-membrane nor in the cytoplasm of the *hsp 12::URA 3* disruption mutant (knock-out yeast strain) of the same strain.

A model liposome system encapsulating a fluorescent dye (calcein) was constructed to investigate a putative membrane-protectant role of HSP 12 during desiccation and lyophilisation. A recombinant form (rHSP 12) of this yeast protein was cloned and expressed in *E. coli* and purified as fusion protein with the GST binding site of glutathione S-transferase for these studies. Not only was HSP 12 (either recombinant or natural) found to act in an analogous manner to trehalose and protect liposomal membrane integrity against desiccation or lyophilisation but that rHSP 12 protected these membranes at a 20 – 30 fold lower molar concentration than trehalose. The interaction between HSP 12 and the liposomal membrane was judged to be electrostatic as membrane protection was only observed with positively charged liposomes containing stearylamine and not with either neutral or negatively charged liposomes. Other proteins investigated failed to maintain more than 35 % of the structural integrity of these liposomes.

As the concentration of HSP 12 was observed to increase when yeast were grown in YPD medium containing osmolytes and since ethanol itself was an osmolyte, the ability of the wild-type yeast and mutant yeast to grow in media containing ethanol was compared. It was found that yeast not expressing the HSP 12 protein were less able to grow in media containing ethanol. HSP 12 was shown to confer increased integrity on the liposomal membrane in the presence of ethanol. This suggested that HSP 12 played a role in yeast ethanol tolerance.

ABBREVIATIONS

POPC	Palmitoyloleoyl Phosphatidylcholine
PC	Phosphatidylcholine
PS	Phosphatidylserine
DP	Dicetyl Phosphate
mRNA	messenger ribonucleic acid
kDa	kilodalton
ABA	abscisic acid
LEA	late embryogenesis abundant
EDTA	ethylenediaminetetra-acetic acid
Ca^{2+}	calcium
Å	angstrom
mm	millimeter
µm	micrometer
nm	nanometer
kilometer	kilometer
g	gram
mg	milligram
µg	microgram
ml	millilitre
µl	microlitre
mM	millimolar
rpm	revolutions per minute
A_{600}	Absorbance at 600 nm
hr(s)	hour(s)
min(s)	minute(s)
PBS	phosphate buffered saline
cm	centimeter
HCl	hydrochloric acid
BSA	bovine serum albumen
ELISA	enzyme-linked immunosorbance assay
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
E. coli	*Escherichia coli*
mA	milliamperes
TEM	transmission electron microscope
DTT	dithiothreitol
MALDI-TOF	matrix assisted laser desorption/ionisation-time of flight
TLC	thin layer chromatography
PMSF	phenylmethanesulfonylchloride
NaCl	sodium chloride
KCl	potassium chloride
$CaCl_2$	calcium chloride
GST	glutathione S-transferase
IPTG	isopropyl ß-D-thiogalactopyranoside
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulphate
NaH_2PO_4	sodium dihydrogen phosphate
°C	degrees centigrade

MW	molecular weight
g	gravity
V	voltage
NaOH	sodium hydroxide
TWEEN 20	polyoxyethylenesorbitan monolaurate
Tris-HCl	TRIS-(hydroxymethyl)aminomethane
TBS	Tris buffered saline
pNPP	paranitrophenylphosphate

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CHAPTER 1

1. GENERAL INTRODUCTION

The survival of all organisms is determined by environmental factors such as temperature and availability of water. Water deficit is the most prevalent stress experienced by plants affecting survival, growth and productivity (Covarrubias et al., 1995; Xu et al., 1996). Dehydration of living tissue results in an alteration in protein and lipid ultrastructure brought about by a change in the hydrophilic and hydrophobic interactions which usually stabilise these structures (Crowe et al., 1987; 1988; 1997). Both physical and chemical changes caused by dehydration results in irreparable changes in desiccation-sensitive tissues inevitably leading to cell death. In response to desiccation many plants have developed the ability to survive at <2% relative water content (Ingram and Bartels, 1996). Yeast (*Saccharomyces cerevisiae*) is also able to remain in the desiccated state (<5% relative water content) for long periods. The capacity of these organisms to regain vital metabolism after dehydration is termed desiccation tolerance (Crowe et al., 1992; 1997). Little is known of the mechanisms by which these organisms achieve desiccation tolerance. There is much experimental evidence indicating an upregulation of genes in plants and yeast encoding small molecules and proteins in response to desiccation stress, osmotic stress, freezing stress and heat shock (Imai et al., 1996; Kim et al., 1996; Rios et al., 1997; Steponkus et al., 1998). We are interested in the functional role played by late embryogenesis abundant or LEA proteins towards an understanding of the mechanisms of desiccation tolerance using yeast as a model organism and liposomes as a model system for investigating membrane stability.

1.1 LATE EMBRYOGENESIS-ABUNDANT PROTEINS (LEA-PROTEINS)

Desiccation-induced stress promotes protein synthesis in desiccation tolerant plants (Bewley and Oliver, 1992; Bray, 1993). This feature of desiccation tolerance was first noted in the desiccation-tolerant phase of seed embryos (Baker et al., 1988). It has been proposed that these proteins protect membranes and other cellular contents (Close et al., 1989, Dure et al., 1989) during desiccation.

Late embryogenesis abundant (LEA) proteins (Galau et al., 1986) are a subset of abscisic acid (ABA) responsive proteins. High levels of expression of LEA-proteins are observed during

embryo maturation in nearly all angiosperms. Although *lea* mRNAs and LEA-proteins are rapidly degraded upon germination, accumulation of these proteins have been reported in several plants in response to water deficit or ABA (Bewley et al., 1983; Mundy and Chua, 1988; Close et al., 1989; Cohen and Bray, 1990; Cohen et al., 1991; Russouw et al., 1995). It is proposed that transcription of these LEA-proteins increases in drought stressed plants (Baker et al., 1988; Robertson and Chandler, 1994), and that these proteins play a protective role against desiccation-induced cellular damage. LEA proteins are characteristically hydrophilic, maintaining their structure upon boiling (Blackman et al., 1991; 1995). Six groups of LEA proteins have been identified based on common amino acid sequence domains (Bray, 1993) and at least five groups have been proposed to contribute towards desiccation tolerance in the embryo. The high percentage of charged amino acids present in group I LEA-proteins are proposed to promote water binding in the form of a dipole-dipole interaction, thus maintaining the shell of hydration during desiccation. The Em protein from wheat germ, classified as a group I LEA-protein, is reported to exist almost entirely in a random coil formation promoting all its charged amino acids throughout the aqueous environment (Litts et al., 1991). Furthermore the wheat Em protein cloned and expressed in yeast has shown an osmoprotectant role when these recombinant yeast were grown in media of high osmolarity (Swire-Clark and Marcotte, 1999). Although a random coil structure has been proposed for group I LEA-proteins, circular dichroism spectroscopy of the p11 from pea seeds has indicated that the structure of these proteins is dependant on the ionic strength of the solution (Russouw et al., 1997). Group II LEA proteins or dehydrins are noted for their 15 amino acid consensus sequence EKKGIMKIKEKLPG that usually occurs near the carboxy terminus (Bray, 1993). The consensus region has a high propensity towards alpha helicity (Whitsitt et al., 1997), and it is postulated that these proteins help maintain structural integrity. Group III LEA proteins, also known as the D-7 family, are characterised by a tandem repeat of an 11-mer amino acid motif with the consensus sequence TAQAAKEKAGE (Bray, 1993; Xu et al., 1996). It is proposed that these proteins function in ion sequestration. Group IV LEA-proteins, the D-113 family, are thought to maintain the stability of membranes by forming a shell of hydration thereby maintaining membrane integrity (Bray, 1993). The Group V LEA-proteins are also called the D-29 family and are postulated to sequester ions accumulated during water loss. It is proposed that the hydroxyl side chains of these proteins mimic water molecules and thus maintain the shell of hydration around membranes and other ultrastructures during desiccation (Bray, 1993).

1.2 HEAT SHOCK PROTEINS

Heat shock proteins are classed as Group VI LEA-proteins. Exposure of nearly all organisms to an increased growth temperature causes the induction of the heat shock response (Parsell and Lindquist, 1993). These proteins are believed to assist the organism in coping with the effects of elevated temperature. Heat shock protein expression is either constitutive with induction by heat or expressed under developmental control as it is suggested that the response is important during various stages of development (Craig et al., 1993; Lee et al., 1994; Waters et al., 1996) under normal growth temperatures. It is also proposed that HSP's refold unfolded proteins thus functioning as molecular chaperones (Parsell and Linquist, 1993).

Five major classes of HSP's have been classified in eukaryotes according to their molecular masses; namely: HSP 104, HSP 90, HSP 70, HSP 60 and small HSP's (smHSP's) ~12 kDa to 30 kDa. In *Saccharomyces cerevisiae* HSP 70 and HSP 90 are essential for growth at all temperatures and levels are increased upon temperature elevation (Craig and Jacobsen, 1984; Borkovich et al., 1989). HSP 104, is expressed at low levels (Parsell et al., 1994) in cells grown at normal temperatures and only increases dramatically upon temperature elevation promoting resolubilisation and reactivation of unfolded and aggregated proteins (Sanchez and Lindquist, 1990). HSP 70 appears to prevent the premature folding of incomplete polypeptides during translation and membrane translocation (Craig and Gross, 1991; Wiech et al., 1993; James et al., 1997). HSP 60 mediates the folding of newly synthesised proteins (Craig and Gross, 1991). Other HSP's are reported to play a role in protein translocation (Bush and Meyer, 1996) as well as degradation of heat-damaged proteins or peptidyl cis-trans isomerase activity (Parsell and Lindquist, 1993).

The small heat shock proteins (smHSP's) generally range in mass from 12 kDa to 30 kDa (Helm and Vierling, 1997) and are thought to be involved in the acquisition of thermotolerance (Lee et al., 1995). These proteins have conserved carboxytermini showing less sequence similarity to the large molecular weight HSP's (Parsell and Lindquist, 1993; Kaukinen et al., 1996). The smHSP's in plants constitute about 1.0 % to 1.5 % of the total protein induced by the heat shock response (Jinn et al., 1995; Lee et al., 1995). The smHSP's have been categorised into four multigene families based on sequence similarity. Class I and

II are localised to the cytoplasm, class III to the chloroplast and class IV to the endoplasmic reticulum (Vierling, 1991; Helm et al., 1993). A fifth class has been identified in several species and found to be localised in the mitochondria (Lenne and Douce, 1994; Waters et al., 1996). A sixth class associated with membrane structures is also proposed to exist (Waters et al., 1996). There is evidence, although their function is still not clear, that the smHSP's play a role in the acquisition of thermotolerance of cells (Berger and Woodward, 1983).

1.3 ACCUMULATION OF SMALL MOLECULES IN RESPONSE TO STRESS

The accumulation of protective molecules such as the non-reducing disaccharide α-trehalose has been noted in many organisms in response to dehydration. Trehalose, which has been extensively investigated, has been found to be one of the most effective saccharides in maintaining the integrity of membranes. Trehalose mimics water in its interactions with the phospholipid bilayer. This disaccharide is proposed to replace the water of hydration during dehydration. The first glucose monomer of trehalose interacts with the phospholipid bilayer by hydrogen bonding as well as non-bonded interactions (Chandrasekhar and Gaber, 1988). There appears to be good correlation between the sequestering of such non-reducing sugars and desiccation tolerance in orthodox seeds, desiccation tolerant vegetative tissues and some animal systems (Madin and Crowe, 1975; Blackman et al., 1992; Drennan et al., 1993), suggesting that they play an important role in desiccation tolerance. Sugars are proposed to bind to the polar head groups in artificial systems and are reported to maintain liquid-crystalline structures of the bilayer during levels of low hydration (Crowe et al., 1987; 1988; 1992)

Trehalose, which is generally absent in plants, has been reported to exist in *Myrothamnus flabellifolia* (Bianchi et al., 1993) and *Selaginella lepidophylla (*Adams et al., 1990). It is proposed that another carbohydrate would have to replace trehalose in plants in order to perform this protective function and prevent lipid phase transitions (Leopold and Vertucci, 1986; Hoekstra et al., 1989; 1991; Crowe et al., 1987; 1992). An increase in trehalose concentration in yeast has also been observed upon exposure to heat (Ribeiro et al., 1997). Furthermore it is also proposed that other sugars confer the same protection to membranes that is observed using trehalose and that this effect is only observed if the sugars don't crystallise. Raffinose and stacchyose suppress sucrose from crystallising. Raffinose and

sucrose are reported to form glasses. The high viscosity of the glass would keep cellular contents in stasis, thereby preventing molecular movement (Leopold and Vertucci, 1986; Caffrey et al., 1988) and intracellular degradative processes. The regulation of many developmental processes is proposed to be brought about by the phytohormone abscisic acid (ABA). Application of ABA to normal unstressed vegetative tissues has demonstrated the ability to mimic the effects brought about by desiccation (Gomez et al., 1988; Close and Chandler, 1989; Cohen and Bray, 1990). The activation of the signal transduction pathway results in the transcription of genetic material and the activation of biosynthetic pathways to compensate for an increase in nutritional demands.

The accumulation of proline in response to stress in bacteria, algae and higher plants has also been reported (Tempest et al., 1970; Brown and Hellebust, 1978; Stewart, 1989). Accumulation of proline in drought-stressed plants is initiated by the reduction of water or an increase in salinity. Proline levels are observed to decrease after water replacement. It has been proposed that proline serves as an osmoticum during water loss to maintain cell turgor. Proline has also been reported to prevent denaturation of BSA by ammonium sulphate and ethanol (Aspinall and Paleg, 1981).

1.4 LIPOSOMES AS A MODEL SYSTEM FOR INVESTIGATING MEMBRANE STABILITY

Liposomes are artificial vesicles consisting of a bilayer of lipid encapsulating an aqueous phase. Phospholipids, the main constituent of the liposome bilayer, precipitate into monolayers in aqueous solutions. The amphiphilic nature of the lipids forming the bilayer maximises interactions with water by minimising the contact of the hydrophobic tails with the solvent. Liposomes are formed as a result of the hydration of phospholipids (Bangham, 1978). Lipid mixtures isolated from biological membranes and dispersed in water are polymorphic and spontaneously form bilayers (Bangham and Horne, 1964) or other organised structures. These 'liposomes' are identified by freeze fracture electron microscopy as spheres with bilayers consisting of onion-like concentric rings. This multilayered structure can then be converted to the unilamellar form either by sonication, Ca^{2+}-EDTA, or ether injection. In nature, the lipids constituting biological membranes are considered to form a bilayer configuration as described by the fluid mosaic model. A temperature-dependent reversible

phase transition exists in all membrane phospholipids whereby the phospholipids undergo a transformation from an ordered gel state at a temperature below the transition temperature to a more disordered liquid crystalline state at a temperature above the transition temperature (Ladbrooke and Chapman, 1969). The phase transition generally involves the disordering of the hydrocarbon chains of the lipid. It has been reported that membrane composition with regard to the presence of proteins, sterols and fatty acid saturation appears to play a role in the type of phase transition that occurs during drying (Crowe et al., 1989; Hoekstra et al., 1991; 1992). The use of liposomes as a model system for investigating biological membranes stems from the generally accepted idea that most lipids in biological systems are arranged in a similar bilayer configuration to those in the artificial liposome system (Verkleij and de Gier, 1981). One of the first uses for liposomes was as a mechanism of substance delivery *in vivo* and *in vitro* (Finkelstein and Weissmann, 1981). Liposomes have also been used as a model system for measuring the protective effect of sugars on membranes (Crowe et al., 1985, 1988) and liposome-protein interactions (Heath and Martin, 1986; Loughrey et al., 1990). It is known that membrane damage occurs during desiccation (Leopold et al., 1981), and it has been suggested that LEA-proteins protect membranes (Close et al., 1989, Dure et al., 1989). Moreover, it is suggested that the curvature of membranes in chloroplasts are altered in response to cold stress (Uemura et al., 1994; Webb et al., 1996). Cold stress has also been reported to increase the lamellar to hexagonal II phase transition of artificial membranes consisting of dioleoylphosphatidyl ethanolamine (Steponkus et al., 1998). We are interested in investigating whether various desiccation-induced proteins in yeast protect membranes against desiccation-induced damage using liposomes as a model system.

1.5 SACCHAROMYCES CEREVISIAE AS A MODEL ORGANISM

Yeast can be easily cultured in a liquid medium or on the surface of solid agar plates. Its genome has been mapped to entirety, and the stress involved in sporalation bears a close resemblance to plant seed maturation. This makes yeast an ideal model system for the investigation of the role played by proteins during desiccation. Yeast exists in both haploid and diploid states and is easily cultured in a liquid medium using glucose as the carbon source and nitrogen and phosphorous containing salts for production of the necessary trace elements. Rapid growth is obtained by supplementing the media with amino acids and other metabolites resulting in cell division occurring at approximately 90 minute intervals at 30 °C. Cell division is driven by the availability of the food source. As cell density increases, nutrient supplies decrease and the rate of cell division slows. Diploid cells are formed by the fusion of haploid cells and budding gives rise to diploid daughter cells. The rate of cell division decreases as the life cycle enters stationary phase and the food source is used up. Under these conditions, yeast reduces its water content and prepares to sporulate. These spores can be germinated into haploid cells in fresh media (Campbell and Duffus, 1988).

1.6 RESEARCH QUESTION

As living tissue dries and rehydrates, a host of ultrastructural biochemical, biophysical and physiological changes takes place. Several proteins, considered to play a role in desiccation tolerance, are induced in yeast cells by an increase in temperature, osmotic stress and desiccation. HSP 12 in yeast was characterised by Mtwisha et al., 1998 as a LEA-like protein. The aims of this thesis were as follows:

1. To find the cellular location of HSP 12 in yeast by immunocytochemistry.
2. To extract and purify HSP 12 from Baker's yeast.
3. To transform *E.coli* with an expression vector containing the HSP 12 gene cloned and expressed as a fusion protein together with glutathione-S-transferase, and isolation of the recombinantly produced rHSP 12 from competent cells.
4. To construct a model membrane system to investigate a putative functional role for HSP 12 during desiccation and lyophilisation.
5. To investigate the effect of ethanol on growth of wild-type yeast and the knock-out yeast strain.
6. To investigate the effect of ethanol on the model system in the presence and absence of HSP 12.

CHAPTER 2

MATERIALS AND METHODS

2.1 YEAST CULTURE

Sterile YPD medium (1 % yeast extract, 2 % bactopeptone, 2 % dextrose) supplemented with 100 µg/ml streptomycin and 100 µg/ml penicillin was inoculated with yeast (*Saccharomyces cerevisiae*) and the yeast grown to late stationary phase (A_{600} = 60/ml) at 30 °C under continuous agitation. Growth was monitored by measurement of the 600 nm absorbance. Cells were harvested by centrifugation at 4000 g using a Beckman JA 20 rotor for 10 min at 4 °C. The supernatant was discarded and the pellet resuspended and washed once in sterile water prior to protein extraction and immunocytochemistry.

2.2 IMMUNOLOGICAL TECHNIQUES

2.2.1 TOTAL PROTEIN EXTRACTION FROM YEAST STRAINS

Saccharomyces cerevisiae 'wild type' diploid strain 842 (*a/x, ade2-1/ade2-1, trp1-1 /trp1-1, leu2-3/leu2-112, his3-11/his3-15, ura3/ura3, can^{r}1-100/CAN*) as well as the 'knock-out' *hsp 12::URA3* disruption mutant of the same strain transformed to uracil prototrophy with linearised *HSP 12* cDNA containing a copy of the *URA3* gene inserted into the *Sty*I site (Praekelt & Meacock, 1990) and which were a kind gift from Professor Peter Meacock, University of Leicester, were streaked out and grown on agar plates at 30 °C for 24 hrs. Single pink colonies (due to the *ADE2* mutation present) were picked using sterile toothpicks and grown in YPD medium as described in section 2.1, to late stationary phase.

The cells were harvested during late stationary phase, and total protein extracted as described in section 2.5.1. HSP 12 content was determined in 4 colonies of the wild-type and compared to 4 colonies of the knock-out strain to ensure that HSP 12 was expressed, and that the knock-out had not reverted back to the wild-type.

2.2.2 PURIFICATION OF ANTIBODY WITH ANTIGEN ADSORBED TO NITRO-CELLULOSE

Polyclonal antibodies (Mtwisha et al., 1998), were purified by adsorption onto and released from HSP 12 immobilised on nitrocellulose to ensure that they were specific to HSP 12 only. This step was necessary to prevent any non-specific interaction during the immunogold labelling. Approximately 500 µg of HSP 12 made up to a final volume of 10 ml with 10 mM Tris-HCl was immobilised by adsorption onto a 6 cm x 6 cm square of nitrocellulose (Schleicher and Schuell) at 4 °C for 16 hrs under constant agitation. The nitrocellulose was thoroughly washed (three washes) with 0.05 % Tween 20 in PBS prior to blocking at room temperature for 60 minutes using a solution of 3 % bovine serum albumin (BSA) (Boehringer Mannheim) in this same buffer. After washing the nitrocellulose three times with PBS/Tween 20 to remove excess unbound BSA, the nitrocellulose was incubated at room temperature for 2 hrs together with a 1:10 dilution of the antibody in 5 % skimmed milk powder, 0.1 % Tween 20 in PBS. The nitrocellulose was washed five times with the 0.05% Tween 20 in PBS and the purified antibody eluted using 1 M Tris-HCl pH 8.5. Titres were checked by ELISA and the purified antibody was stored at -20 °C.

2.2.3 ENZYME LINKED IMMUNOSORBANCE ASSAY (ELISA)

HSP 12 was adsorbed by applying 100 µg of protein (diluted to 10 µg/ml in 1X PBS) to each well of a 96-well plate (Nunclon®) at 4 °C for 16 hrs. Unbound antigen was removed by washing three times with TBS/Tween 20. Unbound sites in the well were blocked with 3 % BSA in TBS for 1 hr at 25 °C. After thoroughly washing (three washes) with TBS/Tween 20, the primary antibody diluted in PBS in the range of 10^{-1} to 10^{-8} was incubated with the antigen at 25 °C for 60 minutes. The secondary antibody (biotinated goat anti-rabbit immunoglobulin) diluted 1:100 in PBS was added, and the plate incubated at room temperature for a further 30 minutes before rinsing with TBS/Tween 20. Antibody detection was carried out using 300 µg p-nitrophenylphosphate in 10 % diethanolamine (pNPP) pH 9.6. The reaction was stopped by addition of 50 µl of 0.1 M EDTA to each well. Absorbance was read at 405 nm in a Titertek Multiscan PLUS MKII detector.

2.2.4 WESTERN BLOT

Western blots were carried out as described by Harlow and Lane, 1988, using the affinity purified anti-HSP 12 antibody as the primary antibody and a goat anti-rabbit antibody (coupled to alkaline phosphatase) as the secondary antibody in the reaction.

SDS-PAGE was carried out on HSP 12 from Baker's yeast and recombinant HSP 12 cloned and expressed as a fusion protein together with glutathione S-transferase in *E. Coli*. The SDS-gel was transferred to an acid washed tray and thoroughly washed with the transfer buffer (0.19 M glycine, 20 % methanol, 25 mM Tris-HCl pH 8.5). The gel was underlaid with pre-soaked nitrocellulose paper (Schleicher and Schuell) supported by pre-soaked Whatman 3MM paper prior to protein transfer at 4 °C for 18 hrs at a constant current of 20 mA. After transfer, the air-dried nitrocellulose was incubated for 30 minutes at 25 °C in 20 ml of blocking buffer (50 mM NaH_2PO_4, 150 mM NaCl pH 7.4 (PBS) containing 5 % skimmed milk powder). The primary affinity-purified anti-HSP 12 antibody was added to the blocking solution. This was incubated at 4 °C for 16 hrs prior to washing in 0.05 % Tween 20 in PBS. Incubation with the secondary goat-anti-rabbit antibody (coupled to alkaline phosphatase) was carried out at 60 °C. The secondary antibody was detected with 4-nitroblue-tetrazolium chloride and 5-bromo 4-chloro 3-indoyl-phosphate in 100 mM $MgCl_2$, 100 mM NaCl, 100 mM Tris-HCl pH 8.3.

2.2.5 EMBEDDING, SECTIONING AND IMMUNOGOLD LABELLING OF YEAST

Yeast diploid strain 842 (wild-type) which produced the HSP 12 protein as well as the *HSP 12::URA3* disruption mutant (knock-out strain) were grown in YPD medium as per section 2.1. Wild-type yeast were also grown in YPD medium containing 1.6 M mannitol, an osmolyte shown to increase the concentration of HSP 12 (Mtwisha et al, 1998). These yeast were harvested by centrifugation at 4000 g in a Sorvall RC-2B centrifuge, and washed in a solution of 5 % glutaraldehyde in YPD medium for 15 minutes under constant agitation at 4 °C prior to fixing in 2.5 % glutaraldehyde in PBS at 4 °C for 16 hrs. The yeast suspension was washed three times in PBS at 4 °C and the pellet resuspended in 1 % osmium tetroxide in PBS at 25 °C for 90 minutes. After washing in PBS to remove excess osmium tetroxide, the yeast pellet was subjected to ethanolic dehydration followed by an acetone wash and final

embedding in epoxy resin (Spurr, 1969). The resin was hardened at 60 °C and 90 nm sections were cut using a Reichert Ultracut-S ultramicrotome and collected on nickel grids. The grids were sequentially floated on 0.02 M glycine in PBS, to block reactive aldehyde groups, and 1 % BSA in PBS prior to floatation on the affinity-purified antibody (diluted 1:10) at 25 °C for 16 hrs. After throughly washing in 1 % BSA in PBS these grids were floated on goat-anti-rabbit-immunoglobulin attached to 5 nm colloidal gold particles (Sigma) for 2 hrs prior to washing with PBS. The sections were fixed by floatation for 10 mins respectively on 0.1 % glutaraldehyde, 2 % uranyl acetate and 1 % lead citrate before visualisation and photography in a Zeiss EM109 transmission electron microscope (TEM) (Vardell and Polak, 1987).

2.2.6 YEAST SPHEROPLAST FORMATION

Yeast were grown in YPD medium containing 1.6 M mannitol to mid-log phase (A_{600} = 5-10/ml) or to late stationary phase (A_{600} = 60/ml) in YPD medium with no mannitol. 1 ml of culture was removed to a sterile SS34 centrifuge tube containing the osmoticum (1 M sorbitol, 25 mM Tris-HCl 10mM EDTA, 10 mM DTT pH 7.4) to a final volume of 5 mls. After centrifugation in a Beckman J2-21 centrifuge using a JA-20 rotor at 4000 g for 10 minutes, the supernatant was discarded and the pellet resuspended in 5 mls of the same osmoticum. 600 µl of the cell suspension was removed to a sterile eppendorf containing 300 µl glusulase (ß-glucuronidase/arylsulfatase) (Boehringer Mannheim) and 50 µl 0.28 M ß-mercaptoethanol. After incubation for 90 minutes at 37 °C under gentle agitation, spheroplast formation was determined microscopically using a Nikon phase-contrast microscope. Cell lysis upon addition of a droplet of water under the coverslip indicated spheroplast formation.

2.3 PREPARATION OF PHOSPHATIDYLCHOLINE FROM FRESH EGG YOLK

Phosphatidylcholine (lecithin) the main constituent of liposomes, was isolated and purified from fresh egg yolk on aluminium oxide using the procedure of Singleton et al 1965.

Fresh egg yolk weighing a total of 500 g was homogenised in 1 litre of acetone. The homogenate was stirred at 25 °C for 1 hr prior to filtration through a Whatman 4 filter. The extract was discarded and the solids thoroughly washed (three washes) with cold acetone before resuspension in a litre of 95 % ethanol. After standing at 25 °C for 1 hour, the ethanol

suspension was filtered and the ethanol removed by rotary evaporation (Buchi). After rotary evaporation, the solids were extracted twice with 300 ml of petroleum ether and the combined extracts reduced to a final volume of 200 ml by rotary evaporation 60 °C. The extract was cooled to 25 °C and poured into cold acetone to a final ratio of petroleum ether:acetone of 1:5 under rapid stirring. The precipitate which formed immediately was retained and the supernatant discarded. Excess solvent was removed by rotary evaporation. The solids were purged with nitrogen gas to prevent oxidation and stored at -20 °C.

2.3.1 PURIFICATION OF EGG YOLK LECITHIN ON ALUMINA

Aluminium oxide (75 g), pre-washed with chloroform, was applied in 80 ml of chloroform to a 4 x 14.5 cm column to a bed height of 6.0 cm. A total of 0.5 g of the crude phosphatide was dissolved in 10 ml chloroform and applied to the column. 10 ml fractions were eluted using chloroform:methanol in the ratio of 9:1. Thin layer chromatography (TLC) of the purified phosphatidylcholine was performed on Silica F_{254} plates (Merck) cut to a size of 10 cm x 20 cm and developed in a chloroform/methanol/acetic acid solution in the ratio 65:25:4. The separated lipids were detected by iodine vapour. The extracted phosphatidylcholine was found to be chromatographically pure on TLC as a single spot with an R_f value identical to a PC standard (Sigma) was detected. Molecular mass analysis of purified egg phosphatidylcholine (PC) (Figure A) showed that the composition of the phospholipid purified consisted of four species of PC ranging from 759 Da to 833 Da using trans-3-indoleacrylic acid (IAA) as the matrix (Figure A). Phosphatidylcholine is reported to have an average molecular weight of 770 Da (Batzri and Korn, 1975). Approximately 1 pmole of lipid was dissolved in 1µl of IAA (0.1 M IAA in chloroform). The differences between the various species of lipid were attributed to the length of the acyl chains and the saturation of the lipids. This suggested that the PC extracted consisted of 4 distinct species of PC. Assuming the backbone was the same for all species, various combinations of acyl chains were considered and composition of the various PC species were derived to consist of the following acyl chains $C_{16:0}$ and $C_{18:1}$ (species 1), $C_{18:0}$ and $C_{18:1}$ (species 2) , $C_{18:0}$ and $C_{20:4}$ (species 3) and $C_{20:4}$ and $C_{20:4}$ (species 4). The ratio of species 1 to 4 was 14: 4: 2: 1 respectively (Figure A).

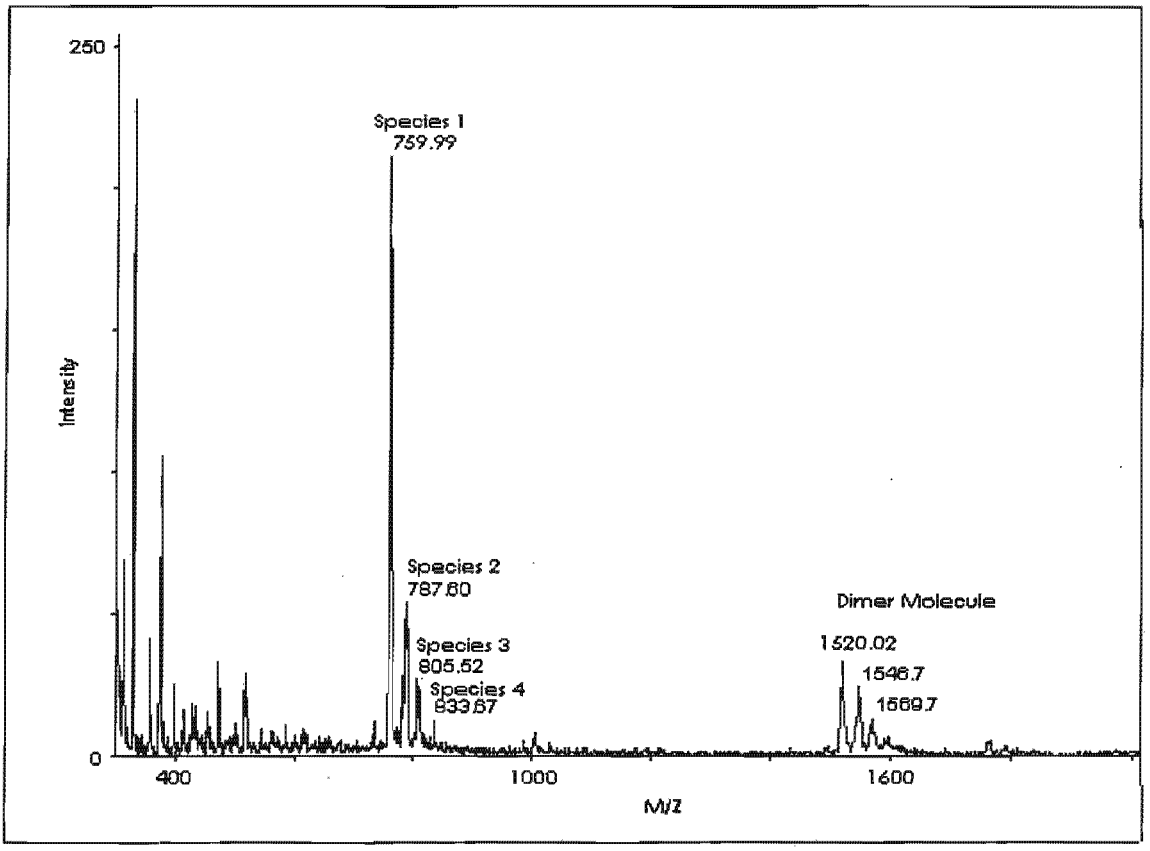


FIGURE A: Mass spectrometric analysis of egg phosphatidylcholine (PC) purified over aluminium oxide as described in section 2.3.1. PC has an average molecular mass (m/z) of 770 Da (Batzri and Korn, 1975). The matrix used was trans-3-indoleacrylic acid (IAA).

2.4 LIPOSOMES

A wide variety of lipids can be used to prepare liposomes. Generally, most liposome preparations contain phosphatidylcholine (PC) and cholesterol in a 1:1 or 1:2 ratio from egg yolk. Phosphatidylcholine has a transition temperature of –7 °C to –15 °C and the resultant liposomes are in a fluid liquid-crystal state above –7 °C (Ladbrook and Chapman, 1969). The ionic charge of the lipid used to make the liposomes will influence interactions between adjacent liposomes and especially lipid protein interactions. Charged liposomes can be prepared by incorporating charged molecules such as dicetyl phosphate or stearylamine into the lipid bilayer (Rosenberg et al, 1987). Not all lipids adopt the bilayer configuration upon hydration. Unsaturated ethanolamines, amongst others, adopt the hexagonal II configuration whereby the fatty acid tails are exposed and the phosphate head groups form a core, resulting in cylindrical structures with diameters of 50 Å -70 Å (Verkleij and de Gier, 1981). Cholesterol, a planar molecule, confers stability to membrane structures and phospholipid

bilayers as it interacts between adjacent acyl chains in the bilayer. It is proposed that cholesterol attenuates the diffusion of solutes through the bilayer (Papahadjopoulos et al., 1973 a, b). The incorporation of high concentrations of cholesterol (50 mol % and higher) is reported to abolish the gel to liquid crystalline phase transition (Todd et al., 1997).

There are two main types of liposomes; large unilamellar vesicles (LUVs) and small unilamellar vesicles (SUVs). The SUVs have diameters ranging from about 20 nm to 50 nm, whereas the LUVs have diameters of 200 nm to 500 nm, and entrap a larger volume. The disadvantages of the SUVs are that the encapsulation volume is very low, usually only 0.2 % to 1 % per mol of lipid, with an efficiency of 0.1 % to 1 % of the required material entrapped, whereas LUVs entrap between 15 % to 50 % of the aqueous phase. The preparation of SUVs requires extensive sonication, to which entrapped material may be sensitive, and they are thermodynamically unstable (Larrabee, 1979). Large unilamellar liposomes are most desirable for studies involving membrane proteins (Woodle and Papahadjopoulos, 1989). Unilamellar vesicles are liposomes with a single lipid bilayer entrapping an aqueous phase.

There are various methods for preparing liposomes, namely; cholate dialysis, reverse phase evaporation and lipid injection to name but a few. The mechanical stresses involved as well as organic solvents determines which method is the best for a given preparation.

2.4.1 LIPOSOME PREPARATION

Liposomes were prepared by reverse phase evaporation (REV) according to the method of Szoka and Papahadjopoulos, 1978. Positively and negatively charged liposomes consisted of bilayers containing 50 mol % cholesterol (Sigma) to minimise leakage and stabilise the liposomes, 45 mol % phosphatidylcholine (PC) to minimise non-specific binding and 5 mol % stearylamine or 5 mol % dicetyl phosphate to minimise aggregation of the liposomes (Rosenberg et al, 1987). The inclusion of dicetyl phosphate imparted an overall negative charge to the liposome surface, whereas stearylamine imparted a positive charge to the liposome surface. Neutral liposomes were made from PC and cholesterol only in a 1:1 ratio.

Stock solutions of 9 mM phosphatidylcholine and 214 mM cholesterol respectively were made in ratio of chloroform:methanol of 2:1. 1.8 ml PC and 150 µl cholesterol were added

and the solvent removed using a rotary evaporator (Buchi) at 25 °C under reduced pressure. The lipid film was purged with nitrogen to prevent oxidation. The lipid film was redissolved in 3 ml diethyl ether and vortexed prior to addition of 1 ml of the aqueous phase, 50 mM calcein (3,6-dihydroxy-2,3-bis[*N,N'*-di(carboxymethyl)-aminomethyl]fluoran) in phosphate buffered saline (PBS) pH7.4). The mixture was sonicated for 5 minutes until the solution cleared. The organic phase was removed under reduced pressure at 25 °C. The aqueous suspension was vortexed and sonicated for 5 minutes at 25 °C to convert the lipid bilayers from the multilamellar to unilamellar form. The suspension was immediately extruded through a 0.2 μM polycarbonate membrane. This served to decrease heterogeneity of the preparation. Any unentrapped matter was separated from the liposomes by means of Ficoll centrifugation based on the method of Fraley et al., 1980. Liposomes were mixed with an equal volume of 20 % Ficoll in a centrifuge tube and overlaid with 2.0 ml of 5 % Ficoll and 0,5 ml PBS. The preparation was centrifuged at 100 000 g for 30 mins. Liposomes banded at the 5 % Ficoll interface and were removed by aspiration in a volume of 150 μl. This method successfully removed approximately 95 % of the unentrapped material which remained in the most dense Ficoll fraction. Excess Ficoll and calcein were removed by dialysis against PBS (Osmolarity of the PBS had been determined as being 300 mosmoles). Liposomes were analysed by negative staining electron microscopy. Carbon-coated copper grids were floated on a 20 μl sample of freshly dialysed liposomes for 10 minutes. The grids were washed five times by floatation on a drop of water prior to staining with 2 % uranyl acetate followed by 1 % lead citrate. Samples were viewed and photographed in a Zeiss EM109 transmission electron microscope. The encapsulation efficiency of the liposomes was found to be 12 %. This compared quite favourably to the 14 % reported previously by Guiot and Baudhuin, 1984. Calcein was chosen over other fluors such as carboxyfluorescein because it was found that carboxyfluorescein leached out of the liposomes quite readily. Liposomes containing calcein could be prepared in advance and stored at 4 °C for a limited period.

2.4.2 THE LIPOSOME ASSAY

Liposomes consisting of approximately 10 nmoles of lipid (approximately 1.2 X 10^9 liposomes (Guiot and Baudhuin, 1984)) were diluted to 50 μl with PBS containing various concentrations of trehalose or HSP 12 before the sample was either dried using a speedy-vac at 30 °C (desiccation) or frozen in liquid nitrogen and lyophilised. The control sample

contained neither protein nor sugar. The speedy vac was used to mimic desiccation stress as the temperature which was set at 30 °C remained constant (29.5 °C ± 1.5 °C) above the transition temperature of the lipids (-7 °C to –15 °C) during desiccation. Lyophilisation after freezing samples in liquid nitrogen was used to mimic freezing and desiccation stress as the temperature was kept below the transition temperature of the lipids. The rapidly frozen liposomes were lyophilised and the dry liposomes rehydrated with water to the original volume (50 µl) and then made up to a final reaction volume of 1 ml with PBS. Calcein fluorescence was determined using an Aminco SPF 500 spectrofluorometer (excitation wavelength of 490 nm, emission wavelength of 515 nm). Membrane integrity after desiccation was calculated from the fluorescence of the sample relative to a control non-desiccated sample and the total calcein released from the sample by addition of Triton X-100 to 0.1 %. This concentration of Triton X-100 was sufficient to lyse all liposomes. Liposome experiments were performed in duplicate using different preparations of liposomes each time. Values are quoted as mean ± standard deviation.

2.5 PROTEIN EXTRACTION AND PURIFICATION

2.5.1 TOTAL PROTEIN EXTRACTION OF HSP 12 FROM BAKER'S YEAST

HSP 12 was isolated and purified as per the method of Mtwisha et al, 1998, with slight modifications. These modifications included the use of a cation exchanger to which HSP 12 was bound and eluted with a NaCl gradient. 80 g of wet packed baker's yeast *(Saccharomyces cerevisiae)* a donation from Anchor yeast, Cape Town, was ball-milled (Braun) with an equal weight of 0.45-0.50 mm diameter glass beads for 44 s in ice-cold 50 mM sodium acetate 2 mM PMSF pH 3.5. The ball-mill was cooled with CO_2 gas and operated for 11 s Bursts at 34 s intervals (a total cycle time of 45 s) to ensure the temperature remained below 5 °C to prevent proteolysis. The homogenate was transferred to an SS34 centrifuge tube and centrifuged at 15000 rpm (27000 g) using a Beckman JA 20 rotor for 10 min at 4 °C. The supernatant was transferred to a clean SS34 tube and heated at 100 °C for 10 mins. The heated fraction was then centrifuged again as before and the supernatant loaded directly onto the cation-exchange column.

2.5.2 FRACTOGEL CATION EXCHANGE CHROMATOGRAPHY

The heated extract of the baker's yeast was applied to a 10 x 100 mm column containing Fractogel EMD SO_3- 650 (s) (Merck) to a bed height of 60 mm. The matrix was pre-eluted with 50 mM sodium acetate pH 3.5. The matrix of the column carries an overall negative charge with positive counter-ions. Proteins are eluted with a gradient of 0-1 M NaCl in 50mM sodium acetate pH 3.5 at a flow rate of 2 $ml.min^{-1}$. The optical density of each fraction was analysed at 230 nm on a Beckman DU 650 spectrophotometer. Fractions were analysed by SDS-PAGE. HSP 12 was bound to the column and eluted at 0.6 M NaCl (Figure B) as a single isometric peak (arrow) between fractions 152 and 170. Separation was superior to the original method (Mtwisha et al., 1998) in which an anion exchanger was used and the protein eluted in the flow through volume. This latter method failed to remove the yeast pigment as well as some of the higher molecular weight proteins present in various batches of the commercially obtained yeast.

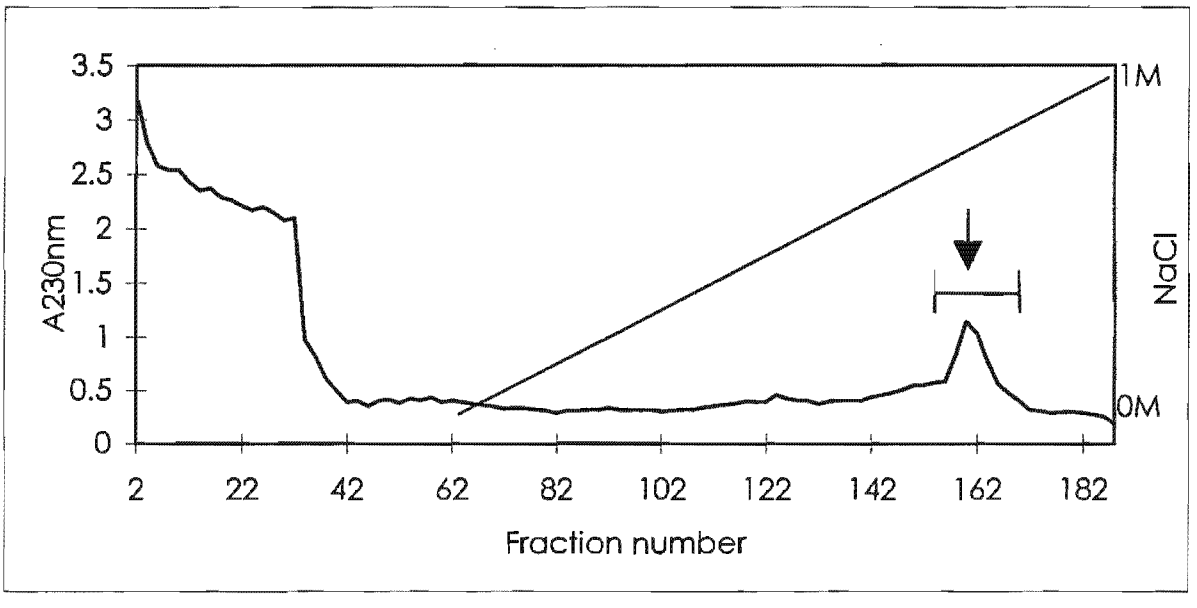


FIGURE B: A plot of Absorbane A_{230} vs Fraction number showing the elusion profile of HSP 12 from commercially obtained yeast using a 0-1 M NaCl gradient 50 mM sodium acetate pH 3.5. HSP 12 elutes as a single isometric peak (arrow) between fractions 152 and 170.

2.5.3 GEL FILTRATION CHROMATOGRAPHY

Fractions from the cation exchanger containing HSP 12 as determined by SDS-PAGE were pooled and lyophilised before applying to a 25 x 1000 mm Sephadex G-50 column equilibrated with distilled de-ionised water. Fractions were eluted with the same buffer at a flow rate of 0.5 $ml.min^{-1}$. The optical density of each fraction was analysed on a Beckman DU 650 spectrophotometer. Fractions were analysed further by SDS-PAGE before being pooled and lyophilised in preparation for HPLC.

2.5.4 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

HPLC was performed on a Millipore-Waters HPLC fitted with two model 6000A pumps, a model U6K injector, a model 441 absorbance detector equipped for monitoring absorbance at 230 nm and a model 680 gradient controller. The column used was a C_{18} Vydac column in 0.1 % heptafluorobutyric acid operated at a flow rate of 0.7 ml/min. Samples were eluted using a 0-70 % acetonitrile gradient in 0.1 % heptafluorobutyric acid.

2.5.5 SDS-POLACRYLAMIDE GEL ELECTROPHORESIS(SDS-PAGE)

SDS-PAGE was carried out according to the method of Laemmli et al., 1970, using a 20 % (w/v) separating gel and a 6.5 % (w/v) stacking gel. Gels were run at 200 V constant voltage for 3 hrs, stained with Coomassie brilliant blue, and destained using 7 % (v/v) acetic acid containing 25 % (v/v) ethanol.

2.5.6 MASS SPECTROMETRY

Biological molecular mass analysis was carried out using a model Voyager DE™-Pro (PerSeptive Biosystems) Matrix Assisted Laser Desorption/Ionisation Time Of Flight (MALDI-TOF) mass spectrometer. Approximately 1 picomole of sample was added to 1 µl of sinapinic acid (3,5-Dimethoxy-4-hydroxycinnamic acid) matrix (10 mg/ml in 50 % acetonitrile, 0.03 % trifluoroacetic acid in water) for the analysis.

2.5.7 PROTEIN CONCENTRATION DETERMINATION

Total protein concentration was quantified using the method of Lowry et al (1951), using BSA (Boehringer Mannheim) as the standard.

2.6 MOLECULAR BIOLOGY

2.6.1 CLONING OF THE HSP 12

The polymerase chain reaction (PCR) was used to amplify *HSP 12 in vitro* using *Saccharomyces cerevisiae* chromosomal DNA as the template and the sequence specific primers:

5' ATGGATCCATGTCTGACGCAGGTAGAAA 3' and

5' GTTCTTACTTTGAAAGCCTTAAGTAT 3'.

The amplified *HSP 12* was cloned into the pGEX-2T plasmid containing a thrombin site. The vector was digested with *BAM H1* and *EcoR*I restriction endonuclease and dephosphorylated by treatment with calf intestinal phosphatase to prevent the plasmid from self ligating. The PCR product was ligated into the vector . This resulted in recombinant rHSP 12 synthesised as a fusion protein together with glutathione S-transferase. Thrombin cleavage yielded pure rHSP 12. The expression vector was a kind gift from Dr Elaine Rumbak, a colleague in this laboratory.

2.6.2 TRANSFORMATION OF COMPETENT CELLS

The *Escherichia coli* strain used for the initial cloning was JM109 [*endA1 recA1 gyrA96 thi hsdR17* (r_k^-, m_k^-) *relA1 sup E44 Δ(lac-proAB)* (F' *traD36 proAB lacI*^{q}ZΔM15)] and was grown in Luria broth containing 100 μg of ampicillin/ml. Competent cells were prepared according to the method of Inoue et al., 1990. Growth was monitored by the 600 nm absorbance. Cells were grown to early log phase (A_{600} = 0.3-0.6/ml) in Luria Bertani (LB) broth before harvesting by centrifugation at 4 °C. The pellet was resuspended in 80 ml transformation buffer (10 mM Pipes, 15 mM $CaCl_2$, 250 mM KCl) at 4 °C. After incubation on ice for 10 minutes, 200 μl aliquots were transferred to sterile eppendorfs on ice, and mixed with 4 μl of the plasmid DNA. The mixture was incubated on ice for 30 minutes before

incubating for 30 seconds at 42 °C in a water bath and immediately transferring to ice. 0.8 ml of SOC medium (2 % w/v tryptone, 0.5 % w/v yeast extract, 10 mM NaCl, 2.5 mM KCl and 20 mM glucose) was added, and the cells incubated for an hour under constant agitation at 37 °C. The cells were plated out on LB agar plates supplemented with ampicillin to a final concentration of 100 µg/ml and grown at 37 °C for 16 hrs to permit selection of viable transformants. 5 mls of SOB medium (2 % w/v tryptone, 0.5 % w/v yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM of each $MgSO_4$ and $MgCl_2$) containing 100 µg/ml ampicillin was inoculated with one colony picked using a sterile toothpick and grown at 37 °C for 16 hrs until an A_{600}= 0.8/ml. In parallel 12 single transformant colonies were picked with sterile toothpicks and each inoculated in 5 mls SOB medium containing 100 µg/ml ampicillin. The cultures were grown at 37 °C under constant agitation until A_{600} = 0.3-0.6/ml. Plasmid DNA was isolated using Wizard DNA minipreps (Promega) as per the suppliers instructions. Plasmid DNA isolation was confirmed by spectrophotometric analysis.

2.6.3 TRANSFORMATION OF AN EXPRESSION HOST

The *Escherichia coli* strain BL21(DE3)pLysS [*hsdSgal (λIts857 ind1 Sam7nin5 lacUV5-T7* gene 1*) Lon⁻, F- , ompT, hsdSB, (rB-mB-), dcm, gal, (DE3), pLysS, Cmr*] was used as the expression host for the GST fusion protein as it is a protease deficient host. BL21 competent cells are reported to contain a mutation in the *ompT* protease gene. This gene has been implicated in the degradation of some recombinant proteins. BL21 cells are derivatives of *E. coli* B and naturally lack the *Lon* protease, which may affect the stability of some recombinant proteins. The transformation procedure was carried out as described in the previous section.

2.6.4 BINDING OF FUSION PROTEINS TO GST MATRIX AND THROMBIN CLEAVAGE

A culture of 5 mls of SOB medium (2 % w/v tryptone, 0.5 % w/v yeast extract, 10 mM NaCl, 2.5 mM KCl containing 100 µg/ml ampicillin, 30 µg/ml chloramphenicol, 10 mM each of $MgSO_4$ and $MgCl_2$) was inoculated with a single *E. coli* BL21 colony transformed with HSP 12 gene and incubated at 37 °C under constant agitation until the A_{600} = 0.78/ml. In parallel, a negative control culture containing only the pGEX-2T plasmid in *E. coli* BL21 was grown to

the same optical density. A larger culture of 100 ml of SOB medium containing 100 µg/ml ampicillin and 30 µg/ml chloramphenicol was inoculated with 2 ml of the starter culture and grown under constant agitation at 37 °C until A_{600} = 1.5/ml. The control culture was grown to the same optical density. 1 ml of each culture was removed prior to induction of the fusion protein for SDS-PAGE analysis. The remaining cultures were induced by addition of 100 mM isopropyl *B*-D-thiogalactopyranoside (IPTG) and grown for a further 3 hrs at 37 °C. The cells were harvested by centrifugation at 5000 g and the pellet resuspended in 5 mls of TNT buffer (40 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8.0, 150 mM NaCl, Triton X-100) containing protease inhibitors (2 µg/ml leupeptin, 2 µg/ml pepstatin, 10 mM DTT and 1 mg/ml PMSF). Lysozyme was added to a final concentration of 0.2 mg/ml and the suspension incubated on ice for 30 minutes after which 1 % Triton X-100 was added and incubated at 4 °C for 30 minutes. $MgCl_2$ and $MgSO_4$ to a final concentration of 10 mM each was added along with 100 µg/ml DNaseI prior to incubation at 37 °C for 30 minutes. The suspension was centrifuged at 5000 g for 5 mins at 4 °C and the supernatant removed to a sterile centrifuge tube containing 150 µl of 50 % glutathione-Sepharose 4B. After incubation at 4 °C for 2 hrs, the beads were pelleted by centrifugation at 2500 g for 5 mins. The supernatant was removed and kept for SDS-PAGE analysis. The beads containing the bound fusion protein were thoroughly washed (three washes) with the TNT buffer containing the same concentration of protease inhibitors pepstatin, leupeptin, DTT and PMSF as before. Each wash was kept for SDS-PAGE analysis. A Stock of 1 M thrombin was made up in phosphate buffered saline (PBS). 50 mM thrombin was added to 250 µl of glutathione-Sepharose 4B. Digestion took place for 18 hrs at 25 °C. The fusion protein was cleaved from the bead and released into the surrounding buffer. The beads were washed with PBS and the supernatant kept for protein quantification and SDS-PAGE analysis.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 IMMUNOCYTOCHEMICAL ANALYSIS OF HSP 12 IN YEAST (*SACCAROMYCES CEREVISIAE*)

HSP 12 was first identified by Praekelt and Meacock, 1990, as a putative heat shock protein on the basis of some limited sequence homology between HSP 12 and known heat shock proteins. Moreover, mRNA specific to HSP 12 was observed to greatly increase on entry into stationary phase and after changing the growth temperature from 30 °C to 37 °C. Subsequently Mtwisha et al., 1998, suggested that HSP 12 should instead be classified as a LEA-like protein. Not only was HSP 12 extremely hydrophilic and its synthesis in yeast promoted by the osmolytes NaCl and mannitol but also the concentration of HSP 12 was diminished rather than increased in yeast cultures grown at 37 °C compared with those grown at 30 °C.

No definitive role has been shown for LEA proteins although expression of the barley group 3 HVA 1 LEA protein in rice resulted in increased tolerance to water stress (Xu et al., 1996) and expression of the wheat group I LEA Em protein in yeast demonstrated an osmoprotectant role when the recombinant yeast were grown in media of high osmolarity (Swire-Clark and Marcotte, 1999). Other roles postulated for LEA-proteins include salt binding as well as the protection of membranes and other cellular contents against desiccation (Close et al., 1989, Dure et al., 1989). The initial goal of this study was to determine the cellular location of HSP 12 in yeast. Cytoplasmic localisations have been proposed for LEA group I proteins thusfar purified (Lane, 1991; Russouw et al., 1997). The strategy adopted was to probe thin sections of embedded stationary phase yeast with an affinity purified rabbit anti-HSP 12 antibody, that was shown to be specific for HSP 12 (Mtwisha et al., 1997) and to determine the location of this antibody using a colloidal gold-labelled goat anti-rabbit antibody. The yeast chosen for this study was the "wild type" diploid strain 842 (*a/x, ade2-1/ade2-1, trp1-1 /trp1-1, leu2-3/leu2-112, his3-11/his3-15, ura3/ura3, can1-100/CAN*) for which a mutant "knock-out" strain lacking HSP 12 was available.

SDS-PAGE of total soluble proteins extracted from the wild-type and knock-out strains is shown in Figure 1. Four colonies of both wild-type and knock-out strains were examined to investigate whether a selection of the wild-type colonies produced equivalent amounts of HSP 12 and to check whether any of the knock-out colonies had reverted to the wild-type. It can be seen (Figure 1) that equivalent amounts of HSP 12 (arrowed) were present in all four wild-type colonies and that no band representing HSP 12 was present in the knock-out colonies. A western blot of this gel (not shown) using rabbit anti-HSP 12 antibodies confirmed the presence of HSP 12 in the wild-type strain and showed that the knock-out strain was free of HSP 12. A protein migrating faster than HSP 12 that was not detected by this antibody was noted in the knock-out colonies. Praekelt and Meacock, 1990, reported that a truncated transcript of HSP 12 was present in the mutant knock-out strain after heat shock.

Thin sections of both the wild-type and knock-out strains grown to late stationary phase were examined by electron microscopy after probing these sections with the rabbit anti-HSP 12 and the gold-labelled goat anti-rabbit antibodies (Figures 2a and 2b). Three separate experiments done in duplicate showed that gold particles were present in all the embedded wild-type yeast cells visualised (Total of 7 different and separately embedded yeast cells) and that these gold particles were seen to be present on the exterior side of the cell membrane facing the cell wall. No gold particles were observed on the cytoplasmic side of the cell membrane. Gold particles were neither detected within the cytoplasm nor adjacent to membranes present in the cytoplasm. No gold particles could be observed in micrographs of the knock-out strain. Since growth of yeast in 0.8 M mannitol was reported to increase the amount of HSP 12 present on entry into stationary phase (Mtwisha et al, 1998), the wild-type strain was grown in YPD medium containing various mannitol concentrations between 0.8 M and 2.0 M. It was found (not shown) that the yield of HSP 12 increased upon growing yeast in YPD medium containing mannitol up to 1.6 M; growth in YPD medium was inhibited by 2.0 M mannitol. Thin sections of the wild-type strain grown to late stationary phase in the presence of 1.6 M mannitol were similarly examined by electron microscopy. It was found (Figure 2c) that the number of gold particles associated with the plasma membrane had increased and that, in addition, some gold particles were observed on the cytoplasmic side of the membrane. No gold particles could be detected in any other sub-cellular location. These results suggested an interaction between HSP 12 and the plasma membrane.

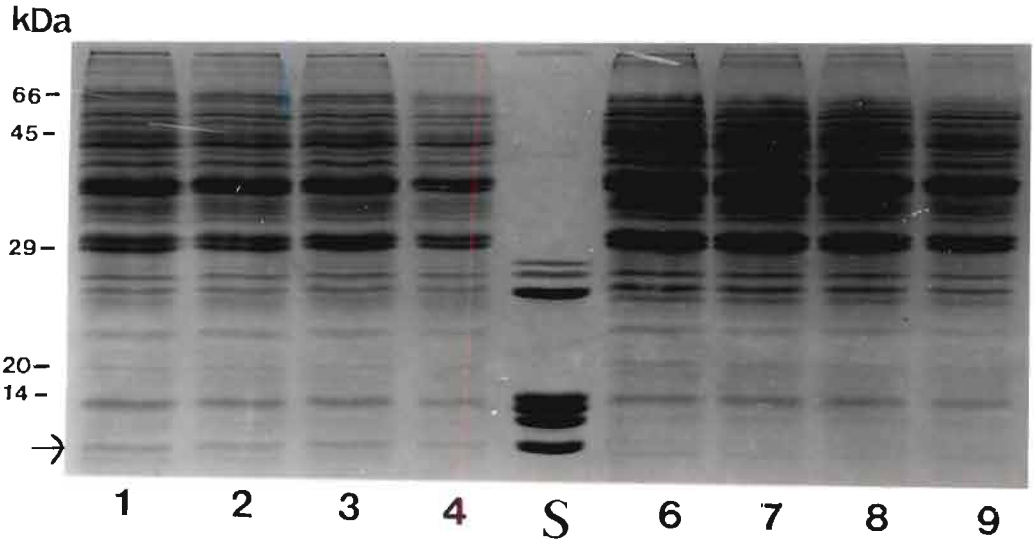
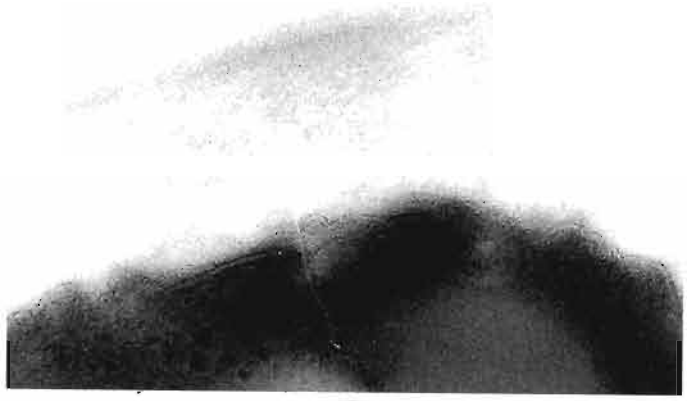
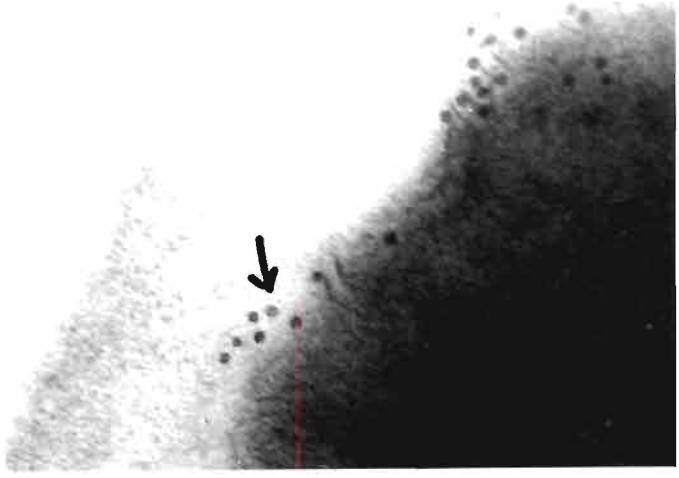


FIGURE 1: SDS-PAGE of total proteins extracted from yeast in 50 mM sodium acetate 2 mM PMSF pH 3.5. Lanes **1 to 4** represents the total protein extracted from the wild-type yeast. Lanes **6 to 9** represents the total protein extraction from the knock-out yeast strain. The molecular weight standard (**S**) is a total extract of chicken erythrocyte histones. The size of the molecular weight markers is denoted on the left hand side of the gel. The arrow shows the position of the HSP 12 protein selected for study.

A



B



C

FIGURE 2: Transmission electron micrographs demonstrating aspects of yeast ultrastructure in thin section. **A** (top left) Wild-type, x 30000. **B** (top right) Knock-out, x 30000. **C** (bottom) Wild-type grown in 1.6M mannitol, x 83000. HSP 12 is denoted by the arrow as a series of black dots positioned around plasma membrane; glutaraldehyde and OsO_4 fixation, uranyl and lead staining.

3.2 PURIFICATION AND SDS-PAGE ANALYSIS OF HSP 12 FROM BAKER'S YEAST

Initially HSP 12 was purified from commercially obtained wet packed Baker's yeast as described in section 2.5.1. SDS-PAGE of proteins extracted at each step of the purification procedure is shown in Figure 3. Pure HSP 12 eluted from the HPLC column was present as a band of approximately 11 kDa (arrow). A western blot, carried out as described in section 2.2.4, (results not shown), confirmed that this band was recognised by the HSP 12 antibody. The yield of HSP 12 in yeast using this procedure was low (± 1 mg per 200 g of commercial yeast) and varied from batch to batch. Coincident with the start of this project, production of the Baker's yeast was transferred from Cape Town to Johannesburg (1500 km away). We postulated that the transportation of the yeast might be the reason for the low yield of HSP 12 and the variance between batches compared to that found previously (Mtwisha et al., 1998).

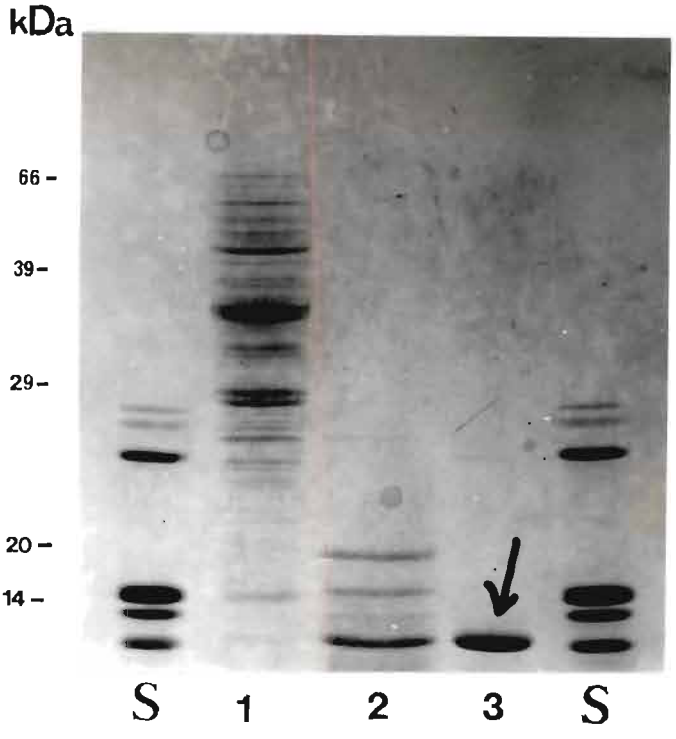


FIGURE 3: SDS-PAGE showing proteins present in each step of the purification process. The standard (**S**) is a total extract of chicken erythrocyte histones. Lane **1** represents the total soluble proteins extracted from the wet packed Baker's yeast in 50 mM sodium acetate 2 mM PMSF pH 3.5. Lane **2** represents the total eluted proteins from the cation exchange column using a 0-1 M NaCl gradient in sodium acetate pH 3.5. Lane **3** represents the purified HSP 12 after HPLC analysis.

3.3 ANALYSIS OF HSP 12 FROM BAKER'S YEAST BY MASS SPECTROMETRY

The molecular weight of the pure HSP 12 was determined by MALDI TOF mass spectrometry. The MALDI mass spectrum of HSP 12 from Baker's yeast using sinapinic acid as the matrix is shown in Figure 4. The most intense signal was that of the singly charged molecular ion with a molecular weight of 11399 Da (MH^+). The other doubly charged species $(MH_2)^{2+}$ was also present with a molecular weight of 5697.1 Da. The molecular weight calculated from the gene sequence was found to be 11715 Da. If the last three amino acids, lysine, lysine and threonine were removed by post synthetic modification and if the N-terminus were acetylated, the calculated molecular mass would then be 11398 Da. It is known that the N-terminus is blocked as HSP 12 could not be subjected to Edman degradation (Mtwisha et al., 1998).

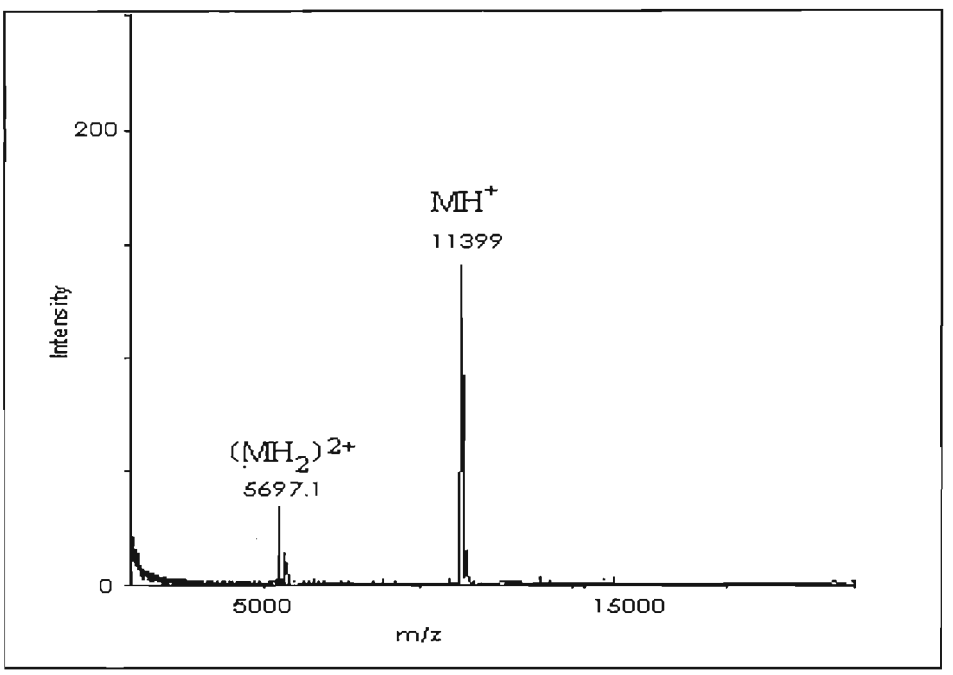


FIGURE 4: MALDI spectrum of HSP 12 in Baker's yeast with a molecular weight (m/z) of 11399 Da (MH^+) Sinapinic acid was used as the matrix.

3.4 PURIFICATION AND SDS-PAGE ANALYSIS OF HSP 12 ISOLATED AS A FUSION PROTEIN IN *E.COLI*

Because poor yields were obtained on purification of HSP 12 from Baker's yeast, it was decided to purify a recombinant form (rHSP 12) from *E. coli* which had the HSP 12 gene cloned and expressed under control of IPTG as described in section 2.6.1. Transformation and selection of viable transformants was carried out as described in section 2.6.2 and section 2.6.3.

rHSP 12 was synthesised as a fusion protein together with glutathione S-transferase. Thrombin cleavage of the fusion protein resulted in an N-terminus extension of glycine-serine to the recombinant HSP 12. *E. Coli* containing the pGEX-2T plasmid were grown at 37 °C, IPTG added and the bacteria harvested by centrifugation after 3 hrs of further growth. The bacteria were lysed and the proteins purified as per section 2.6.4. SDS-PAGE of the total proteins present in the bacterial extracts at each step in the purification process is shown in Figure 5. The *E. coli* containing the plasmid pGEX-2T containing the glutathione S-transferase vector alone were used as a control. Protein expression in the pGEX-2T is under control of the *tac* promotor which is induced by the lactose analogue IPTG. The 26 kDa glutathione S-transferase (GST) can be seen as a dark band (arrowed) 3 hrs after induction by IPTG (lane 2). No GST can be seen in the uninduced plasmid (lane 1). The total protein extracted from the uninduced *E. coli* BL21pLysS containing the inserted HSP 12 gene can be seen in lane 3. A 38 kDa fusion protein (arrowed) was present in all three cultures extracted after induction by IPTG (lanes 4 to 6). The total protein extract of three cultures were combined and the fusion protein was bound to the glutathione-Sepharose 4B beads (matrix) as described in section 2.6.4. The matrix was washed with buffer to remove any non-specifically bound proteins. Aliquots from the washes are shown in lanes 7 to 9. The fusion protein was cleaved from the matrix by thrombin. Lane 10 shows thrombin cleavage after 10 minutes of incubation indicating the presence of rHSP 12. After 18 hrs the cleavage was complete (lanes 11 and 12). Pure rHSP 12 is shown in lane 12. Lanes 13 and 14 show the glutathione-Sepharose 4B beads (matrix) after thrombin cleavage after 10 minutes and 18 hours respectively. This method proved to be fast and reliable producing a large quantity of rHSP 12. A western blot (results not shown) confirmed that this protein was HSP 12 as it was recognised by the anti-HSP 12 antibody (Mtwisha et al., 1997).

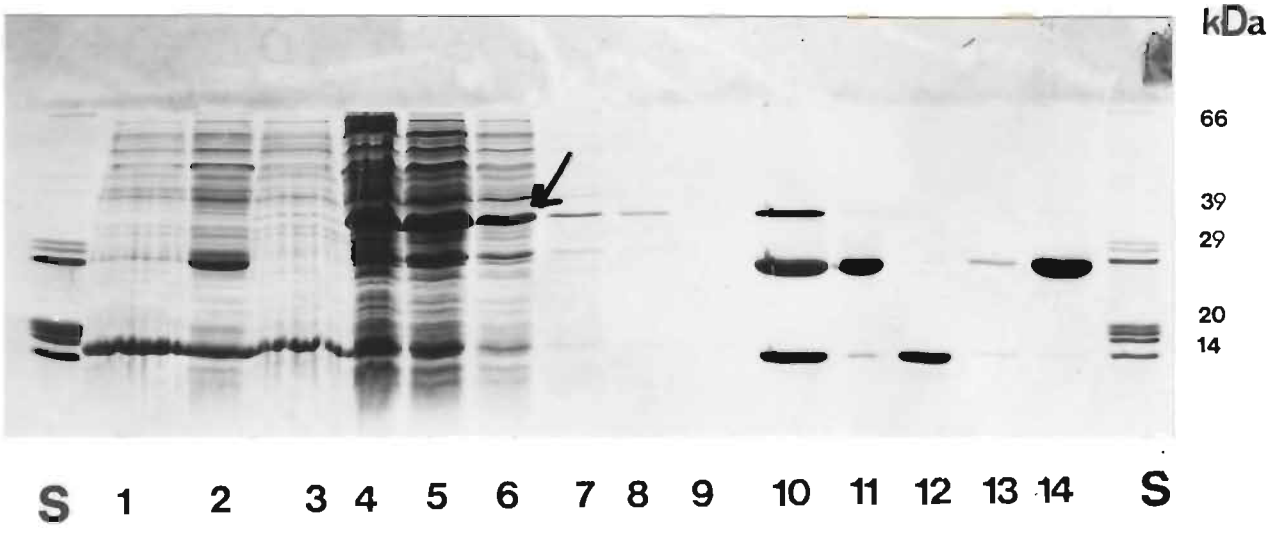


FIGURE 5: SDS-PAGE showing expression and purification of GST and rHSP 12 in *E. coli* transformed with the expression vector pGEX-2T containing rHSP 12 as a GST-fusion protein. The standard (**S**) is a total extract of chicken erythrocyte histones. Lane **1**. Uninduced pGEX-2T (control). Lane **2**. IPTG induced pGEX-2T (control). Lane **3**. Uninduced BL21pLysS containing the fusion protein. Lanes **4-6**. IPTG induced Bl21pLysS, showing the fusion protein (arrow) ~38kDa. Lanes **7-9**. Wash step showing removal of non-specifically bound proteins from the sepharose 4B. Lane **10**. Thrombin cleavage after 10min. Lanes **11, 13 & 14** (sepharose 4B pellet 10 mins and 18hrs after digestion respectively) & Lane **12** (supernatant) showing pure rHSP 12 after Thrombin cleavage (18 hrs).

3.5 ANALYSIS OF RECOMBINANT rHSP 12 BY MASS SPECTROMETRY

Mass spectrometric analysis of rHSP 12 by MALDI-TOF using sinapinic acid as the matrix (Figure 6) showed that the species with the single charge had a molecular mass of 11855 Da (MH^+). The calculated molecular mass of HSP 12 suggested by the gene sequence was 11715 Da. Glycine and serine were added to the N-terminus after thrombin cleavage of the fusion protein thus raising the molecular mass to 11823 Da. The difference between the calculated value and that determined by mass spectrometry was 32 Da. As it is not uncommon for samples to pick up water or monovalent ions upon ionisation in MALDI, this difference could be attributed to two molecules of water (equivalent to 36 Da), as this would be within the margin of error of this technique (± 0.05 % equivalent to 6 mass units). Other charged species were also present after mass analysis. The doubly charged species was present at 5925.6 Da $(MH_2)^{2+}$ as well as the species with half the charge (M_2H^+ 23723 Da). The differences between the calculated value (23710 Da) and that obtained experimentally (23723 Da) could

be attributed to the addition of a water molecule to this species (M_2H^+) as this would be within the experimental error. No water molecules appear to be associated with the species with the double charge $(MH_2)^{2+}$. The signal intensity, which is concentration dependent, of the recombinantly produced rHSP 12 on MALDI is double that of the natural form as the concentration of the recombinant form was double that of the natural rHSP 12. The high concentration of the recombinant rHSP 12 accounts for the presence of species with the half charge (M_2H^+). As analysis of the natural HSP 12 and the recombinant form was done on separate occasions using different laser intensities, it is possible that the higher laser intensity used during analysis of the recombinant form might be responsible for the addition of the water molecules.

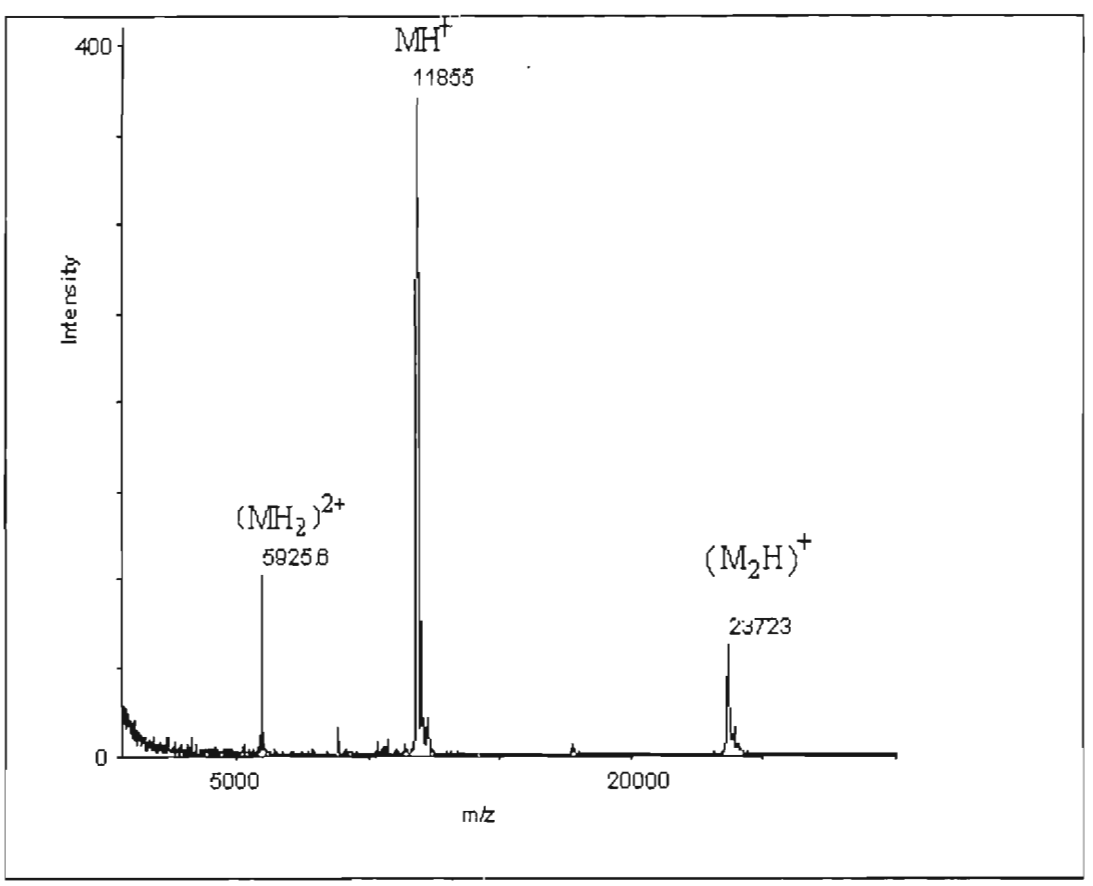


FIGURE 6: MALDI spectrum of recombinant rHSP 12 with a molecular weight (m/z) of 11855 Da (MH^+). Sinapinic acid was used as the matrix.

3.6 PREPARATION OF LIPOSOMES FOR USE AS A MODEL SYSTEM FOR INVESTIGATING MEMBRANE STABILITY.

The plasma membrane acts as the principal interface between the extracellular environment and the cytosol (Steponkus & Lynch, 1989). Since membrane systems are considered to be particularly susceptible to dehydration damage (Crowe et al., 1986; 1987; 1988; Steponkus & Webb, 1992) and since immunocytochemical analysis suggested that HSP 12 was in close proximity to the plasma membrane, we decided to investigate whether rHSP 12 protected the membrane against desiccation induced damage using an artificial liposome membrane system.

The liposome system used was constructed from phosphatidylcholine and cholesterol from egg yolk as outlined in section 2.4.1. The phospholipid molecules in liposomes in an aqueous environment are arranged in a bilayer configuration with the acyl chains in the center and the phosphate head groups on the outside. Cholesterol intercalates among the fatty acyl chains with its polar hydroxyl group interacting with the polar head groups of the membrane lipids. Liposomes were produced to incorporate the self-quenching fluorescent dye calcein (3,6-dihydroxy-2,3-bis[*N,N'*-di(carboxymethyl)-aminomethyl]fluoran) in phosphate buffered saline (PBS). The fluor calcein is autoquenching at high concentrations as the excitation and emission spectra overlap. As a result of this overlap, the transfer of electrons (fluorescence energy transfer), which is not detectable by fluorimetry, occurs between adjacent calcein molecules. Membrane damage by detergent or other means results in the release of calcein and its dispersal into solution. Whereas the distance between each calcein molecule inside the liposome is very small, that in solution is very much greater. Fluorescence energy transfer, which is inversely proportional to the sixth power of the distance between donor and acceptor molecules, markedly decreases. Thus intact liposomes are essentially non-fluorescent but once membrane damage has occurred, enhanced fluorescence is observed due to the greater distance between calcein molecules.

Large unilamellar liposomes (ca. 100 nm in diameter) are reported by Woodle and Papahadjopoulos, 1989, as being the liposomes of choice for studies involving membrane proteins. Small unilamellar vesicles (less than 50 nm in diameter) are reported to be unstable and to have a low encapsulation efficiency. Furthermore it has been reported that the content of liposomes with regard to the fatty acyl composition, cholesterol content and acidic lipid

content does not significantly alter the retention of the aqueous contents by vesicles dehydrated and rehydrated in the presence of trehalose. The susceptibility of these liposomes to leakage induced by freezing and dehydration is dependant on the size of the liposomes with the smallest liposome systems (ca. 100nm in diameter) being most stable (Harrigan et al., 1990). Reproducible large unilamellar vesicles entrapping an aqueous phase were produced using the methodology described in section 2.4.1. This method produced a homogeneous population of liposomes of 100 nm diameter when examined by transmission electron microscopy after immobilisation on copper grids and staining with uranyl acetate (Figure 7).

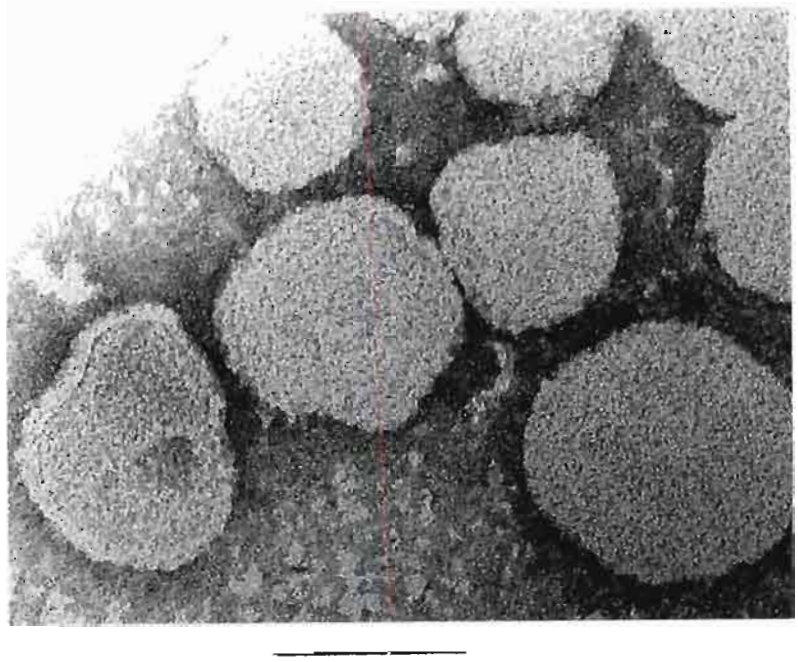


FIGURE 7: Transmission electron micrograph of a liposome preparation. Magnification x 83000. Bar represents 100 nm. Liposomes were immobilised on copper grids and sequentially stained with 2% uranyl acetate and 1% lead citrate.

3.7 STABILISATION OF MEMBRANES BY SUGARS

One of the first uses for liposomes were as vehicles for drug delivery *in vivo* and *in vitro* (Finkelstein and Weissmann, 1981). However, one of the main difficulties in using liposomes in this manner is that they are not stable for long periods (Gregoriadis, 1984). Subsequently, liposomes have been used as a model system for investigating the membrane stabilising effect of sugars (Crowe et al., 1985; 1988; 1992; Chandreskar and Gaber, 1988) as well as the effects of cold stress on membranes (Steponkus and Lynch, 1989; Steponkus and Webb, 1992; Webb et al., 1996; Steponkus et al., 1998).

Trehalose, when present on both sides of the bilayer, was seen to maintain maximum liposome integrity upon lyophilisation (Crowe et al., 1985). This suggested that the trehalose substituted for the water of hydration. It has been proposed (Crowe et al., 1988), that adjacent bilayers are held apart by the water of hydration associated with the phosphate head groups. Removal and replacement of this water caused alterations in the bilayer configuration, as demonstrated by freeze-fracture, in favour of multilamellar and oligolamellar forms. However, if trehalose were present during lyophilisation, dry liposomes were seen to exist as vesicles embedded in trehalose. These liposomes did not leak their contents into the surroundings thereby showing preservation of membrane integrity.

The initial goal of this study was to reproduce the results of Crowe et al., 1985 using liposomes made from egg phosphatidylcholine and cholesterol in a 1:1 ratio to establish the authenticity of our liposome system. This was done because these authors used a defined system of palmitoyloleoylphosphatidylcholine (POPC) and phosphatidylserine (PS) whereas the lipid composition in our system is not as defined. Analysis of the phospholipid extracted from egg yolk by mass spectrometry (Figure A) showed that 4 species of phospholipid were present. We assumed that the phospholipid backbone was the same but that the acyl chains were of different lengths and saturation levels (Section 2.3.2). From the molecular mass analysis the most likely combinations of acyl chains were $C_{16:0}$ and $C_{18:1}$ (species 1), $C_{18:0}$ and $C_{18:1}$ (species 2) , $C_{18:0}$ and $C_{20:4}$ (species 3) and $C_{20:4}$ and $C_{20:4}$ (species 4).

Liposomes were constructed to encapsulate an aqueous phase of 50 mM calcein in PBS. A fixed concentration of liposomes (approximately 10 nmoles of lipid) was added to a reaction

volume of 50 µl PBS that contained an increasing concentration of trehalose up to 30 µg trehalose per µg phospholipid. These liposomes were then subjected to desiccation in a speedy-vac at 30 °C. A preliminary study showed that the temperature of the samples in the speedy-vac remained between 28 °C and 31 °C throughout the desiccation process. Since this temperature was well above the phase transition temperature of the lipids used (-7 °C to -15 °C), it was decided that this method simulated desiccation. The dry liposomes were rehydrated with water to the original reaction volume and then diluted to 1 ml with PBS. The fluorescence of the sample was measured and the percentage structural integrity calculated as described in section 2.4.2. These liposomes made from PC and cholesterol in a 1:1 ratio maximally maintained 70 % (± 3.5 %) of their structure upon desiccation in the presence of trehalose (Figure 8a) whereas liposomes entrapping fluor only and desiccated in PBS showed total loss of membrane integrity after desiccation (Figure 8a). There was a linear relationship between the percentage structural integrity maintained and trehalose present externally up to a concentration of 12 µg trehalose per µg phospholipid. At concentrations of trehalose greater than this, no increase in the percentage structural integrity maintained was observed. Liposomes prepared with increasing concentrations of trehalose incorporated internally only and desiccated in PBS, showed total loss of all membrane integrity upon desiccation (Figure 8a). A similar effect to that observed during desiccation was seen during lyophilisation of liposomes in the presence of trehalose externally only (Figure 8b). In lyophilisation the liposomes are rapidly frozen in liquid nitrogen prior to desiccation. It has been proposed (Crowe et al., 1987) that more water of hydration is removed by lyophilisation than by desiccation. As lyophilisation involves a two step process, namely freezing and desiccation, preliminary studies were carried out to determine whether liposomes were ruptured by freezing. Liposomes rapidly frozen in liquid nitrogen and thawed to room temperature showed no signs of membrane damage as no increase in fluorescence was observed. However liposomes frozen by incubation at minus 20 °C and subsequently thawed to room temperature showed leakage of the fluor indicative of membrane damage. Lyophilisation was thus routinely carried out after freezing samples in liquid nitrogen. Lyophilisation of liposomes containing increasing amounts of trehalose externally showed that 65 % (± 3.4 %) of structural integrity was maximally maintained (Figure 8b). Once again a linear relationship was observed between structural integrity maintained and trehalose present externally up to a concentration of 12 µg trehalose per µg phospholipid. At greater concentrations there was no observable increase in membrane stability. Control liposomes entrapping fluor only, showed total loss of membrane integrity

after lyophilisation (Figure 8b). Lyophilisation of liposomes entrapping trehalose internally, showed complete loss of membrane integrity, as observed during desiccation.

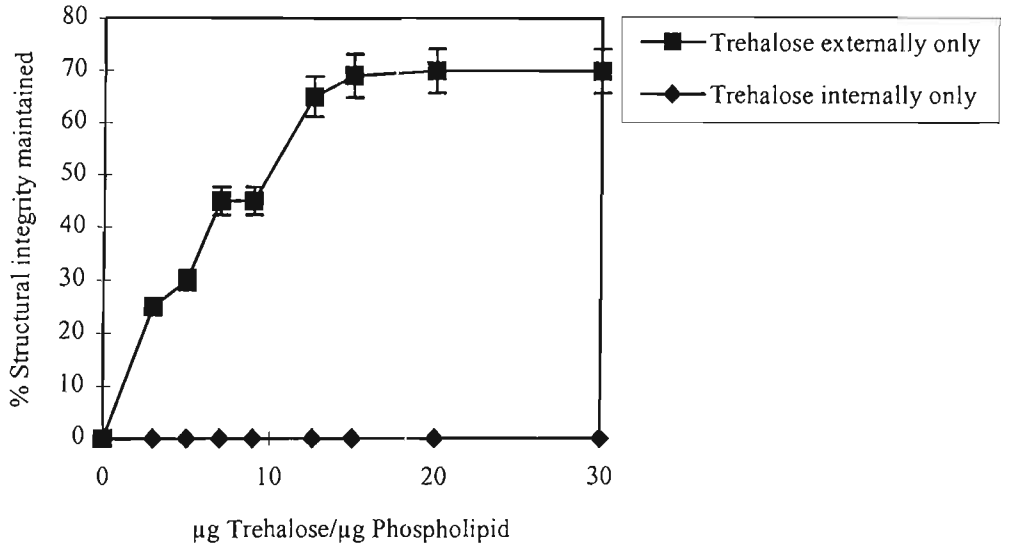


FIGURE 8a: Plot showing % structural integrity maintained (as calculated from the fluorescence in section 2.4.2) as a function of trehalose concentration (µg) per µg phospholipid. Liposomal integrity was maintained by trehalose present externally only, on liposomes encapsulating calcein during desiccation. Liposomes containing trehalose internally only showed complete leakage of fluor during desiccation.

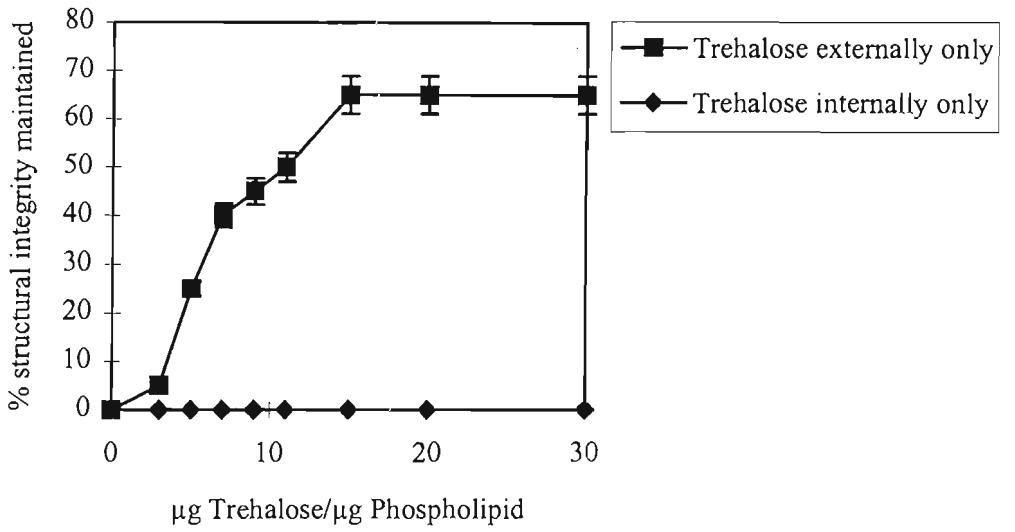


FIGURE 8b: Plot showing % structural integrity maintained (as calculated from the fluorescence in section 2.4.2) as a function of trehalose concentration (μg) per μg phospholipid. Lipsomal integrity was maintained by trehalose present externally only on liposomes encapsulating calcein during desiccation. Liposomes containing trehalose internally only showed complete leakage of fluor during lyophilisation.

Although trehalose incorporated internally had no effect on liposome membrane integrity after desiccation, we investigated whether trehalose present on both sides of the membrane would increase the amount of structural integrity maintained during desiccation compared with that observed when trehalose was present externally only (Figures 8a). Liposomes were constructed to encapsulate an aqueous phase containing 50 mM calcein and 20 μg trehalose per μg phospholipid in PBS. This amount of trehalose was chosen as it was found to maximally stabilise liposomes when present externally only during desiccation (Figure 8a) and lyophilisation (Figure 8b). Investigation of the relationship between the retention of membrane structural integrity after desiccation and the concentration of external trehalose (Figure 9a) showed that there was a linear increase in structural integrity up to 10 μg trehalose per μg phospholipid. Increasing the amount of external trehalose beyond 10 μg trehalose per μg phospholipid showed little additional effect. The maximum integrity after desiccation was found to increase to 86 % (± 4%) from 70 % (± 3.5 %). Similarly the maximum membrane integrity after lyophilisation of these liposomes increased from 65% (± 3.4 %) to 82% (± 8%) (Figures 9b) when trehalose was present both internally and externally. Although the amount of trehalose required externally to stabilise liposomes during

desiccation and lyophilisation was not altered by including trehalose internally in liposomes, the effect of trehalose on membrane stability was greater when present on both sides of the membrane during desiccation and lyophilisation. It has been proposed by Crowe et al., 1987 that dehydration of phospholipid bilayers results in fusion of adjacent vesicle bilayers and subsequent leakage of their contents. When trehalose is present internally in liposomes encapsulating the fluorescent dye calcein during desiccation or lyophilisation total loss of membrane integrity was observed upon rehydration of these liposomes (Figures 8a and 8b). It is likely that the removal of the shell of hydration surrounding these liposomes during desiccation is responsible for fusion of these liposomes and leakage of the fluor into the surrounding buffer. However with trehalose present externally on liposomes encapsulating a fluor only during desiccation or lyophilisation approximately 70 % of membrane integrity as determined from the fluorescence was maintained. This suggests that trehalose substitutes for the shell of hydration when water is removed. As 100 % membrane integrity was not maintained by the presence of trehalose externally only, this suggests that fusion is not the only mechanism that gives rise to leakage. It has been proposed by Crowe et al., 1987 that dehydrated phospholipids undergo a phase transition from liquid crystalline to gel phase. After rehydration, these lipids undergo a further phase transition from gel to liquid crystalline phase. The bilayer is assumed to be leaky during these phase transitions. We investigated whether this latter hypothesis was the reason for failure of our liposomes to maintain 100 % of the entrapped fluor during desiccation or lyophilisation using Differential Scanning Calorimetry (DSC). However as our liposomes contained 50 mole % cholesterol, the phase transition was abolished completely (Todd et al., 1997). Thus an alteration in the transition temperature or leakage as a result of the liquid-crystalline to gel transition could not explain the increase in stability of our liposomes made to incorporate trehalose internally and desiccated in the presence of trehalose externally (Figures 9a and 9b).

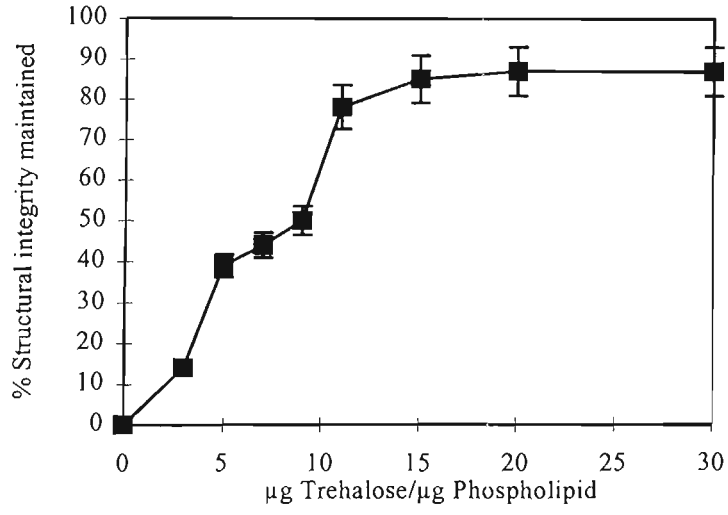


FIGURE 9a: Percentage Structural integrity of liposomal bilayers as a function of trehalose externally on liposomes encapsulating 20 µg trehalose per µg Phospholipid internally during desiccation.

Crowe et al., 1985, using a defined system of liposomes composed of palmitoyloleoyl-phosphatidyl choline (POPC) and phosphatidylserine (PS), reported that 1.8 g trehalose per g phospholipid maintained the structural integrity of lyophilised liposomes. We have shown (Figures 9a and 9b), that 12 g trehalose per g phospholipid was needed to maintain structural integrity of liposomes consisting of egg phosphatidylcholine (PC) and cholesterol during lyophilisation or desiccation. The 7 fold difference between our results and those of Crowe et al suggests that the composition of the fatty acyl chains plays an important role in membrane stabilisation. The fatty acyl chains of the liposomes used by Crowe et al., 1985 were composed predominantly of $C_{16:1}$ fatty acids. We postulate that the predominance of this single fatty acid, with one unsaturated double bond allowed uniform stacking of the acyl chains resulting in the phosphate head-groups being in close proximity. Molecular mass analysis (Figure A) suggested that egg-yolk lipids consisted of a heterogeneous array of PC with fatty acyl chains consisting of $C_{16:0}$, $C_{18:0}$, $C_{18:1}$ and $C_{20:4}$ monomers as described in section 2.3.2. Moreover, the fatty acyl chains of greater unsaturation would be more kinked and would prevent the close packing of adjacent lipid molecules. This would result in a greater distance between the phosphate head-groups and a more disordered bilayer which might explain the increased trehalose requirement needed to stabilise these bilayers.

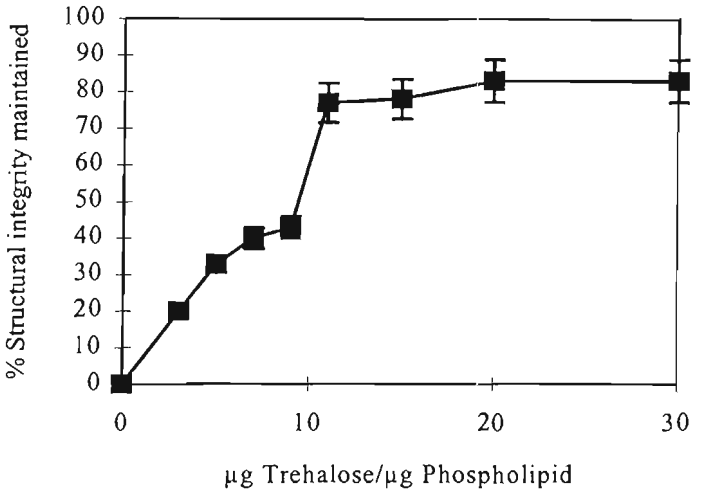


FIGURE 9b: Structural integrity of liposomal bilayers, determined from the fluorescence as outlined in section 2.4.2, by trehalose externally on liposomes encapsulating 20 µg trehalose per µg Phospholipid internally during lyophilisation.

As plants generally lack trehalose but are known to contain other non-reducing disaccharides such as raffinose, stacchyose and sucrose, we investigated whether these sugars could substitute for trehalose in the protection of membranes against desiccation (Figure 10a) and lyophilisation (Figure 10b). Liposomes incorporating calcein only were used for this study. We observed that at lower concentrations, trehalose and sucrose protected membranes more effectively compared with raffinose and stacchyose. However at high concentrations there appeared to be little difference in membrane protection between the various sugars. This effect was especially noticeable during lyophilisation. Thus the various saccharides tested appeared to be interchangeable, eliciting the same membrane protecting effect during water loss (Figure 10a and 10b) as that of trehalose.

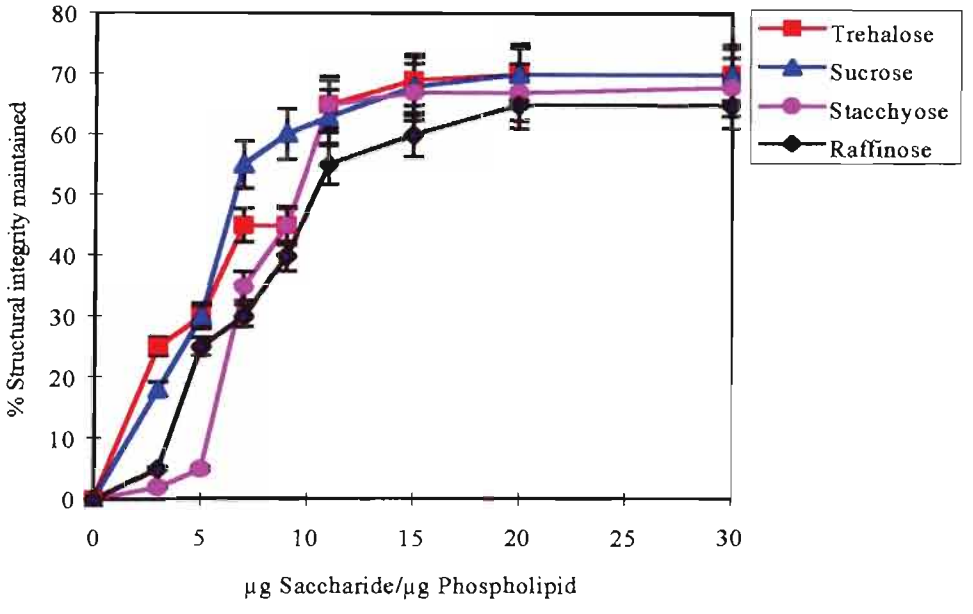


FIGURE 10a: Liposomal integrity during desiccation was maintained by the presence of various saccharides present externally only on liposomes entrapping calcein during desiccation. These disaccharides appear to be interchangeable in their ability to protect membranes.

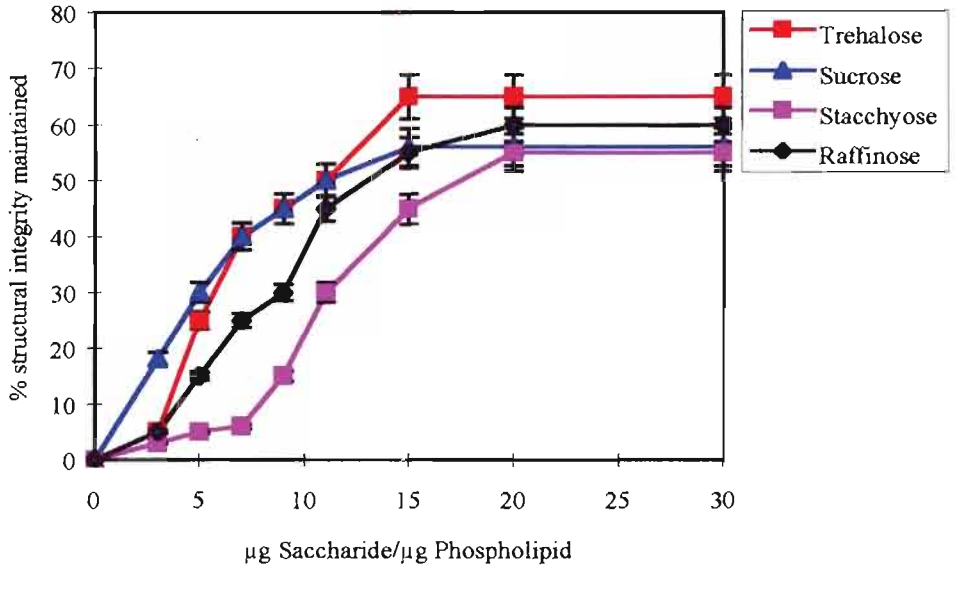


FIGURE 10b: Liposomal integrity maintained during lyophilisation as a function of disaccharide concentration present externally on liposomes entrapping calcein during lyophilisation.

3.8 STABILISATION OF MEMBRANES BY rHSP 12

The stabilisation of membranes by rHSP 12 was investigated to determine whether rHSP 12 could replace trehalose in protecting liposomes against desiccation and lyophilisation.

It was found that rHSP 12 or natural HSP 12 had little effect on membrane stability when using neutral liposomes consisting of phosphatidylcholine and cholesterol only. HSP 12 is a highly charged protein with almost half (47.8 %) of its residues consisting of the amino acids E, K, D, R, H (Table 1). We thought that the failure of rHSP 12 to stabilise neutral liposomes may have been brought about by rHSP 12 interacting with the membrane electrostatically.

TABLE 1: Amino acid content (moles %) of HSP 12 as determined from the gene sequence

RESIDUE	Mol %
Glutamic acid (E)	15.6
Lysine (K)	13.8
Aspartic acid (D)	13.8
Glycine (G)	11.9
Alanine (A)	11.9
Serine (S)	7.3
Valine (V)	6.4
Threonine (T)	1.8
Leucine (L)	2.8
Arginine (R)	2.8
Histidine (H)	1.8
Tyrosine (Y)	3.7
Phenylalanine (F)	1.8
Isoleucine (I)	0.9
Methionine (M)	1.8

Analysis of the total charge of HSP 12 as a function of pH (Figure 11) showed that the protein had an overall negative charge at pH 7.4. The composition of the liposome membrane was thus altered to include a charged group. Liposomes were prepared according to section 2.4.1, to include either 5 mol % stearylamine or 5 mol % dicetyl phosphate to produce liposomes where the membrane was either positively charged or negatively charged respectively. The effect of rHSP 12 on the desiccation sensitivity of these liposomes was investigated and no protection against desiccation was observed in liposomes containing dicetyl phosphate. Liposomes containing 5 mol % stearylamine, however, maintained 77 %

(± 3.1%) of the membrane integrity when rHSP 12 or natural HSP 12 was present externally during desiccation (Figure 12a). Membrane integrity was seen to increase linearly with increasing rHSP 12 concentrations up to 15 µg rHSP 12 per µg phospholipid. No increase in membrane integrity at higher rHSP 12 concentrations was observed.

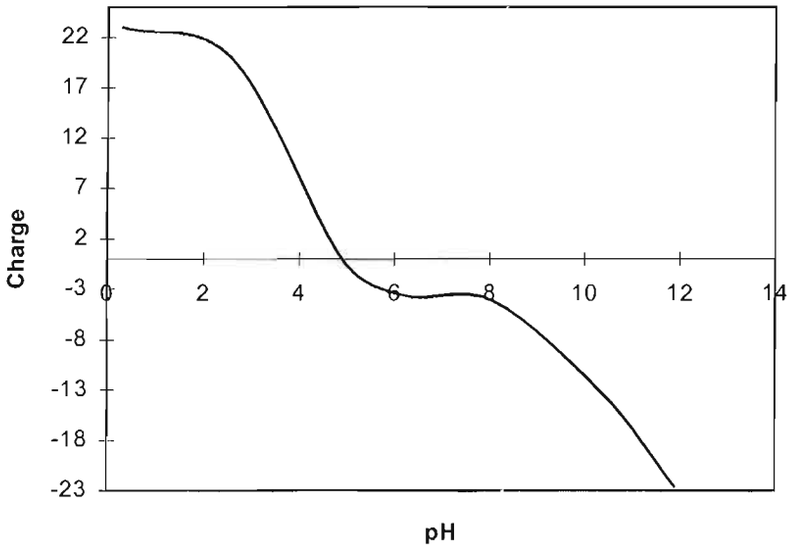


FIGURE 11: Plot of charge as a function of pH for HSP 12 based on the amino acid composition of the gene sequence.

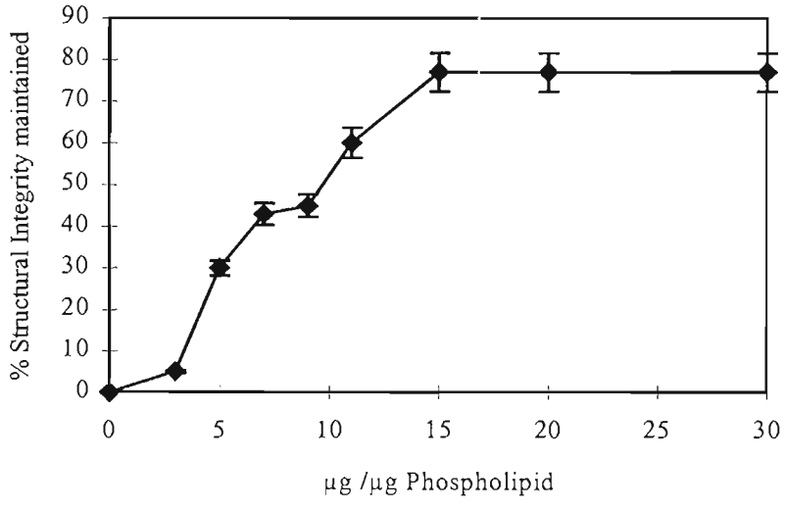


FIGURE 12a: A plot of % structural integrity maintained as a function of rHSP 12 present externally on stearylamine liposomes, encapsulating calcein in PBS during desiccation.

Since the composition and charge of the liposomes used to demonstrate rHSP 12 protection of membranes against desiccation was different to that used previously, we investigated whether trehalose also protected these liposomes upon desiccation. It was found that 65 % (± 3.8 %) of membrane integrity was maintained by trehalose at a concentration of 12 μg trehalose/μg phospholipid during desiccation. This value was similar to that determined previously (Figure 8a) despite the addition of stearylamine into the liposome membrane (Figure 12b).

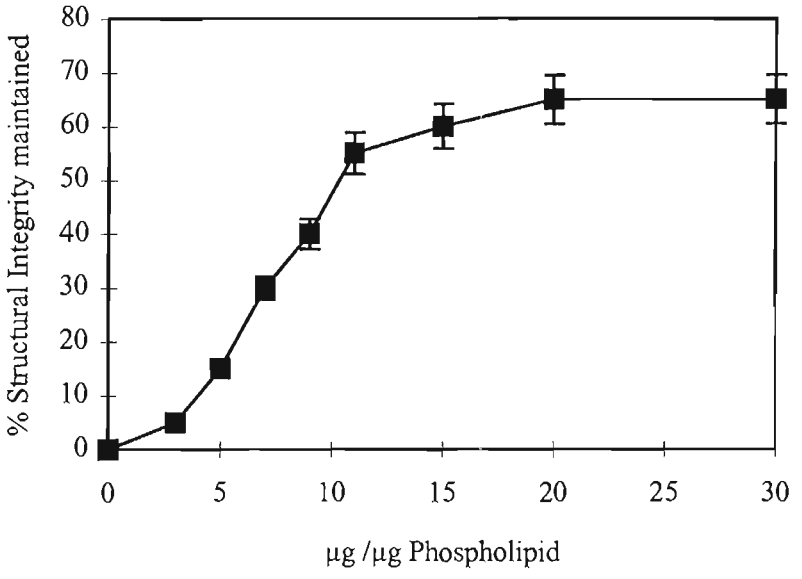


FIGURE 12b: Stabilisation of stearylamine liposomes encapsulating calcein in PBS, by trehalose externally during desiccation showing no change in trehalose requirement in these liposomes despite the addition of stearylamine to the bilayer.

We wished to investigate whether the protective effect of rHSP 12 towards liposomes during desiccation was specific to this protein or whether other proteins had a similar effect. The experiment shown in Figure 12a was repeated with myoglobin (MW 17000) or the mitochondrial membrane bound protein cytochrome C (MW 12000). These proteins were chosen because they were of similar molecular weight to HSP 12 and they were readily available. It was found that myoglobin was ineffective in maintaining structural integrity of liposomes during desiccation as only 10 % (± 1.3 %) of liposomes were protected (Figure

12c). Cytochrome C however was more effective as 35 % (± 3.4 %) of membrane stability was maintained during desiccation.

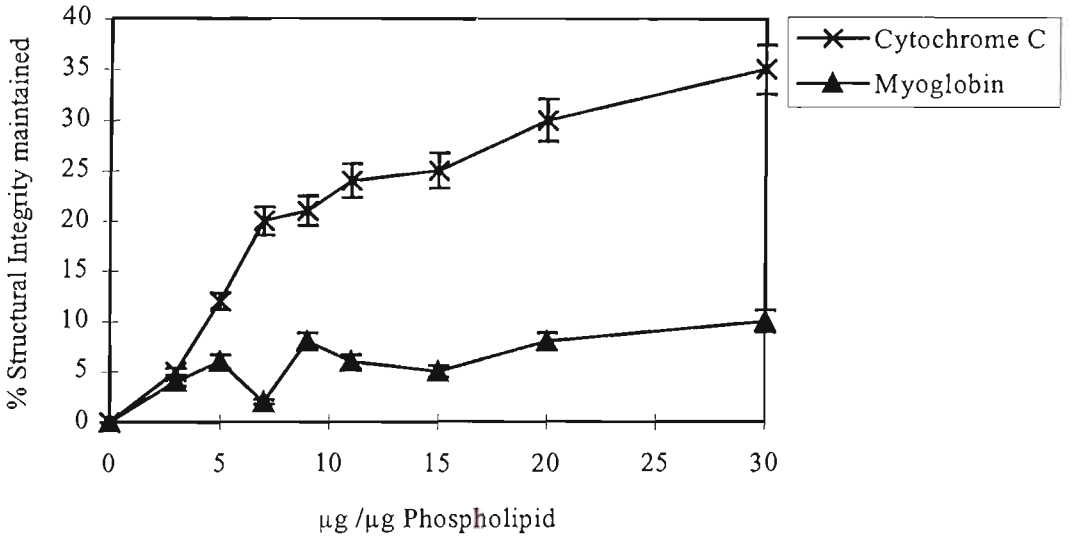


FIGURE 12c: Membrane stability of liposomes, encapsulating calcein in PBS, by myoglobin and cytochrome C during desiccation.

LEA group I proteins are reported to exist in a random coil formation promoting their charged amino acids throughout the aqueous solution (Litts et al., 1991). Myoglobin and cytochrome C are globular proteins and would thus interact differently with the membranes of these liposomes compared with HSP 12. Myoglobin, which has an isoelectric point of 7 would be weakly negative at pH 7.4. A weak interaction with the positive liposomes might therefore be proposed. This might explain the marginal protection (ca. 10 %) of liposomes by myoglobin. Cytochrome C, which has an isoelectric point of 10.6, would be positively charged at pH 7.4. However as it interacts with membranes in its natural state this may be the reason for it maintaining structural integrity of our liposomes.

Since rHSP 12 and trehalose both protected the membranes of liposomes containing 5 mol % stearylamine during desiccation, we investigated whether these molecules also protected the membranes of these liposomes during lyophilisation.

Liposomes in the presence of increasing concentrations of rHSP 12 externally were initially subjected to freezing in liquid nitrogen and subsequent thawing to room temperature. No leakage of calcein was observed during this procedure. After lyophilisation of these liposomes frozen in liquid nitrogen, a linear relationship was noted between concentration of rHSP 12 and membrane integrity (Figure 13a). It was observed that 70 % (± 4 %) of membrane integrity was maximally preserved at a concentration of 15 µg rHSP 12 per µg phospholipid after lyophilisation. The addition of 5 mol % stearylamine had no effect on the ability of trehalose to stabilise these liposomes as 60 % (± 3.4 %) of membrane integrity was maintained (Figure 13b) compared with 65 % (± 3.4 %) structural integrity maintained by trehalose when present externally on neutral liposomes (Figure 8b). Myoglobin was again ineffective in maintaining membrane stability and cytochrome C protected only 30 % (± 3 %) of the membrane structure during lyophilisation, demonstrating a similar effect during lyophilisation (Figure 13c) to that during desiccation (Figure 12c). There was no observable difference in the ability to protect membranes against desiccation between natural HSP 12 and recombinant rHSP 12. As the recombinant form was easier to produce, it was used for all liposome studies.

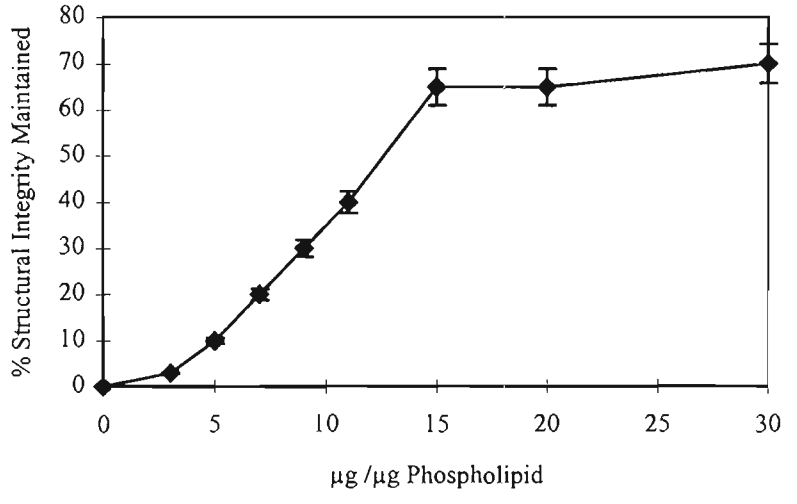


FIGURE 13a: Percentage structural integrity maintained as a function of rHSP 12 present externally on stearylamine liposomes, encapsulating calcein in PBS, during lyophilisation.

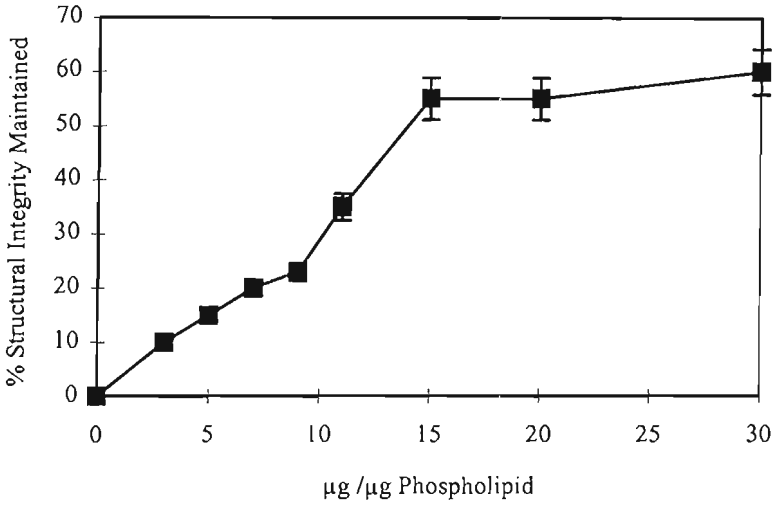


FIGURE 13b: Stabilisation of liposomal membranes containing stearylamine, encapsulating calcein in PBS, by trehalose during lyophilisation indicating that there is no change in trehalose requirement of these liposomes during lyophilisation.

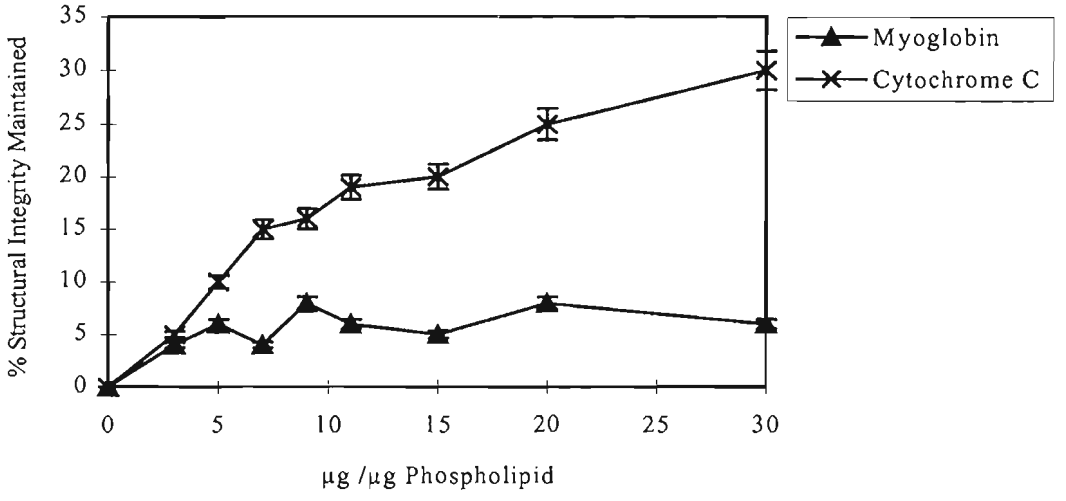


FIGURE 13c: Liposomal integrity maintained by myoglobin and cytochrome C during lyophilisation. Liposomes encapsulating calcein in PBS only.

3.8.1 INCORPORATION OF SACCHARIDES INTERNALLY IN LIPOSOMES.

The accumulation of trehalose has been reported to increase in heat stressed yeast (Ribeiro et al., 1997). Although trehalose present only internally had no observable effect on membrane stabilisation during desiccation or lyophilisation, membrane integrity was improved by approximately 20 % if trehalose were present both internally and externally. We therefore investigated whether the membrane protecting effect of rHSP 12 could be enhanced if trehalose were present internally. Liposomes were prepared as described previously to encapsulate 20 μg trehalose per μg phospholipid.

The incorporation of trehalose internally during desiccation significantly improved liposome stability in the presence of rHSP 12 externally (Figure 14) compared with liposomes that had no trehalose present internally (Figures 12a). Maximum membrane integrity was improved from 77 % (± 3.1%) (Figure 12a) to 98 % (± 1.3 %) with rHSP 12 present externally and trehalose present internally (Figure 14) during desiccation. No significant change was observed when desiccation was carried out in the presence of cytochrome C or myoglobin.

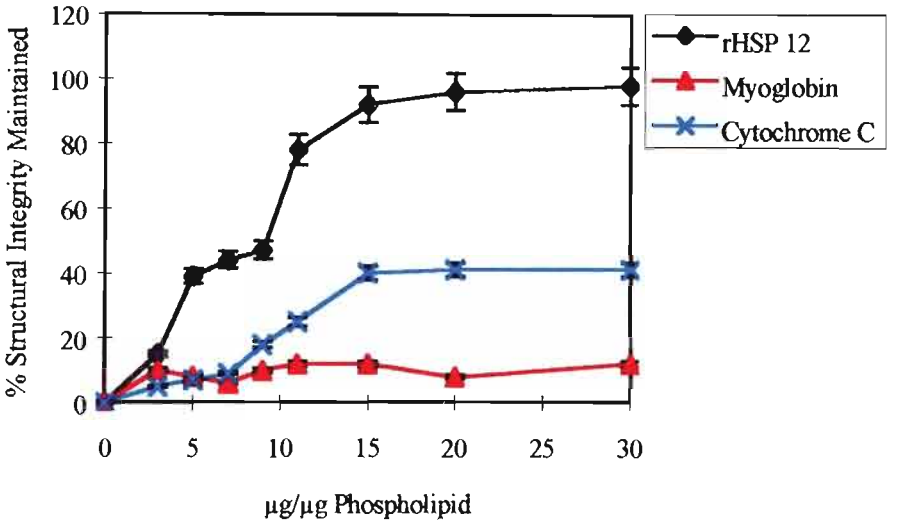


FIGURE 14: Percentage structural integrity maintained as determined from the fluorescence indicating stabilisation of liposomal membranes, encapsulating 20µg trehalose per µg phospholipid internally, by rHSP 12 during desiccation. Myoglobin and cytochrome C are protein controls of similar molecular weight to HSP 12. The membrane protective effect appears to be specific to rHSP 12.

The same liposome preparation was also subjected to lyophilisation. Membrane integrity after lyophilisation improved from 70 % (± 4 %) (Figure 13a) to 96 % (± 4%) (Figure 15) with rHSP 12 externally and trehalose present internally. Cytochrome C showed a slight increase from 30 % (± 3 %) (Figure 13c) to 40 % (± 4 %) (Figure 15) in membrane protection when present externally on liposomes incorporating trehalose internally during lyophilisation. Thus the presence of trehalose internally resulted in almost complete protection of liposomes by rHSP 12 externally during desiccation (Figure 14) and lyophilisation (Figure 15). The protective effect of myoglobin on these liposomes was negligible (less than 10 %) during lyophilisation. As complete membrane integrity of liposomes containing stearylamine was maintained with trehalose internally and rHSP 12 externally, we postulate that rHSP 12 forms a net around these liposomes during desiccation thereby preventing fusion of adjacent vesicles. It has been proposed by Crowe et al., 1987 that sugars depress the transition temperature of phospholipid vesicles. However as cholesterol abolished the phase transition of lipids constituting the bilayers of our liposome system, this explanation could not be used to understand how rHSP 12 protects these liposomes. As water is removed from surrounding

liposomes only the inside surface of the liposome bilayer is stabilised by hydrophobic interactions. This results in a conflict between the curvature of the outside surface and the inside surface of the bilayer, resulting in instability of the membrane. rHSP 12 which is highly charged from its structure interacts electrostatically with the phosphate groups and stearylamine of the bilayer thereby reducing the fusion of adjacent liposomes and the leakage of fluor. When trehalose is present on the inside with rHSP 12 externally, the bilayer is further stabilised as curvature of the membrane is prevented. This would explain the complete protection observed in these liposomes.

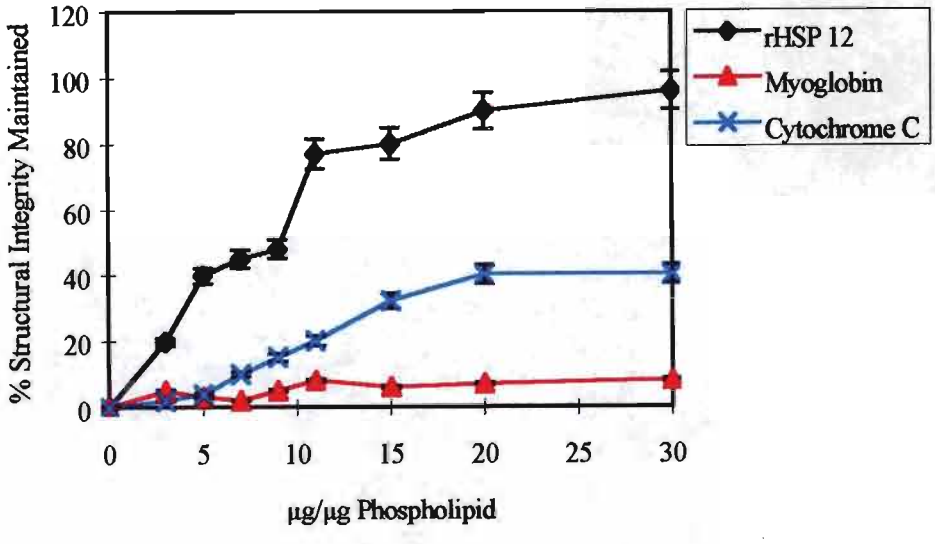


FIGURE 15: Percentage structural integrity determined by the fluorescence as outlined in section 2.4.2 indicating stabilisation of liposomal membranes, encapsulating 20µg trehalose per µg phospholipid internally, by rHSP 12 externally during lyophilisation. Myoglobin and cytochrome C are protein controls of similar molecular weight to HSP 12.

3.8.2 INCORPORATION OF rHSP 12 INTERNALLY IN LIPOSOMES.

Since rHSP 12 could replace trehalose when present externally, the internal trehalose was replaced with rHSP 12 at a concentration of 20μg rHSP12 per μg phospholipid. This concentration yielded maximum membrane protection when present externally.

The incorporation of 20 μg rHSP 12/μg phospholipid internally improved the stabilisation of the membranes in the presence of rHSP 12 and trehalose externally during desiccation (Figure 16) and lyophilisation (Figure 17) to the same extent as with trehalose present internally at 20 μg trehalose per μg phospholipid. Membrane integrity was improved from 77 % (± 3 %) (Figure 12a) to 97 % (± 1.3 %) (Figure 16) by incorporation of rHSP 12 internally during desiccation, and from 70 % (± 4 %) (Figure 13a) to 97 % (± 2.3 %) (Figure 17) by incorporation of rHSP 12 internally during lyophilisation. The incorporation of rHSP 12 internally and trehalose externally improved membrane integrity from 65 % (± 3.8 %) (Figure 12b) to 96 % (± 5 %) during desiccation (Figure 16) and from 60 ± (3.4 %) (Figure 13b) to 94 % (± 6 %) (Figure 17) during lyophilisation. This indicated that trehalose and rHSP 12 were interchangeable internally in liposomes. Cytochrome C once again maintained less than 40 % membrane integrity during desiccation and lyophilisation. Myoglobin failed to maintain structural integrity (less than 10 %) of these liposomes. Liposomes containing rHSP 12 internally only showed complete leakage of calcein into the surrounding buffer upon rehydration after lyophilisation or desiccation. rHSP 12 was thus ineffective in maintaining liposomal integrity when present internally only. This suggests that the mechanism of protection of rHSP 12 is similar to that observed with trehalose as rHSP 12 appears to be interchangeable with trehalose. Various models have been suggested for the mode of action of trehalose thusfar based on the ability of trehalose to reduce lamellar to hexagonal II phase transitions, reduce fusion of adjacent bilayers and depress the transition from liquid crystalline to gel phase (Crowe et al., 1987). We propose 2 modes of action for rHSP 12. We can speculate that when rHSP 12 was present internally only, the desiccated liposomes fused as there was nothing to substitute for the water of hydration during desiccation. This resulted in leakage of the fluor indicative of membrane damage. This fusion could also have been facilitated by the bilayer forming other organised structures or by transition from lamellar to hexagonal II conformations. We have proposed previously that rHSP 12 forms a net around the liposomes when present externally and that this net is furthermore only facilitated by

incorporation of stearylamine into the bilayer. We propose the mechanism of action of rHSP 12 to be different internally in liposomes compared with that observed with rHSP 12 externally. As complete protection of these stearylamine liposomes only occurs with rHSP 12 present on both sides of the membrane, we propose that not only does rHSP 12 externally take the place of the water of hydration during desiccation, but that rHSP 12 internally assists in preventing the curvature of the membrane thereby preventing destabilisation of the bilayer during desiccation. rHSP 12 and trehalose appear to be interchangeable in these liposomes and may thus protect these liposomes in a similar manner. However rHSP 12 is more effective than trehalose maintaining structural integrity at a 20 – 30 fold lower molar concentration than trehalose.

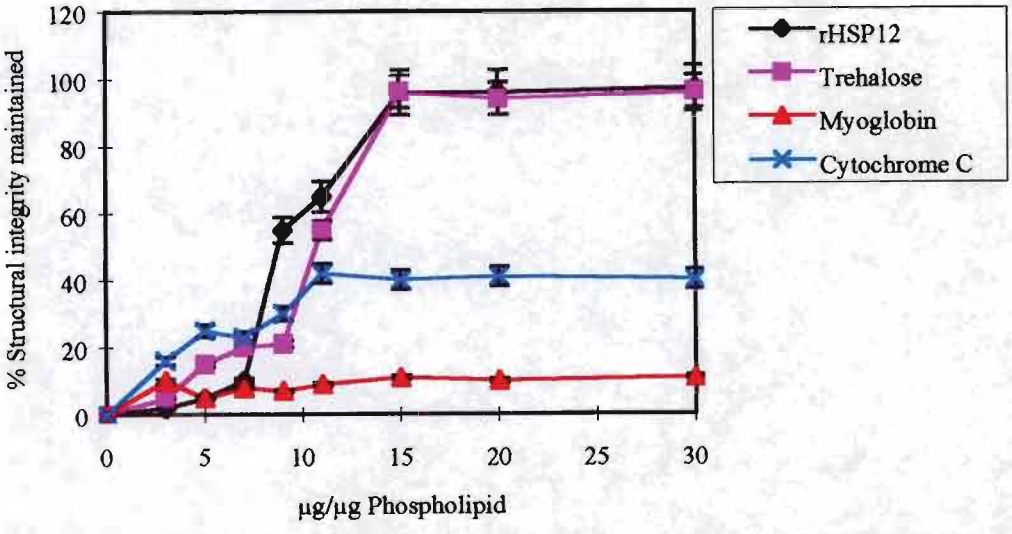


FIGURE 16: Stabilisation of stearylamine liposomes, encapsulating 20µg rHSP 12 per µg phospholipid internally, by rHSP 12, trehalose, myoglobin and cytochrome C externally during desiccation.

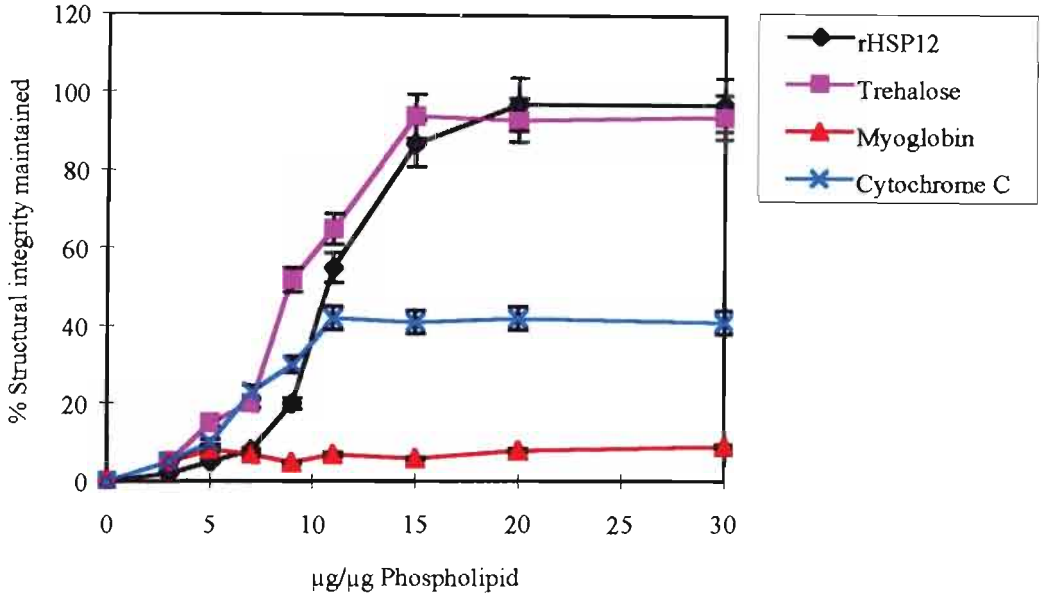


FIGURE 17: Stabilisation of liposomal membranes, encapsulating 20 µg rHSP 12 per µg phospholipid internally, by rHSP 12, trehalose, myoglobin and cytochrome C externally during lyophilisation.

3.9 PROTEIN LIPOSOME INTERACTION

As rHSP 12 protected positively charged liposomes only we postulated that an electrostatic interaction existed between rHSP 12 and the liposome membrane. In order to investigate the charge dependence of the interaction between HSP 12 and the lipid moieties, liposomes encapsulating calcein only were dialysed for 16 hrs against PBS at either pH 7.4, pH 6 or pH 3.5 before addition of rHSP 12 in the same buffer. The liposome preparation was then subjected to either desiccation or lyophilisation. The percentage structural integrity observed at pH 7.4 was set to 100 %.

The percentage structural integrity maintained was found to decrease as the pH became more acidic (Figure 18). At pH 6 the structural integrity decreased to 60 % relative to that observed at pH 7.4 and decreased even further to 30 % at pH 3.5. The amino group of the stearylamine is positively charged below pH 9 thereby repelling rHSP 12, which is positively charged below pH 5 (Figure 11). Total loss of structural integrity was not observed after desiccation or lyophilisation at pH 3.5. We speculated that some general membrane protection by proteins

proteins exists as seen previously with myoglobin and cytochrome C. We further postulated that this general effect of proteins to maintain between 10 % and 30 % of membrane integrity could be attributed to the ability of proteins to prevent adjacent bilayers from fusing or by preventing the transition from bilayer to hexagonal II formation during rehydration. This result suggested that the protection mediated via rHSP 12 and the membrane was electrostatic (Figure 18).

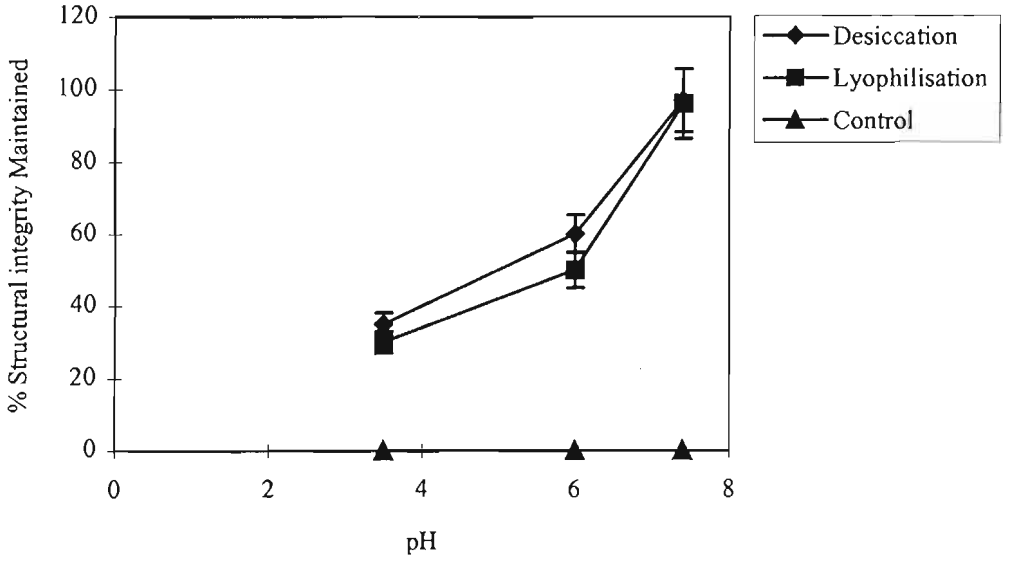


FIGURE 18: Stabilisation of membranes by rHSP 12 as a function of pH. Membrane integrity is compromised as the pH is lowered. rHSP 12 is positively charged below pH 5, and no-longer interacts with the positively charged liposome. This suggests an electrostatic interaction between rHSP 12

3.10 PROTECTION OF THE rHSP 12 AGAINST ETHANOLIC DEHYDRATION

It is proposed that the lipid composition of the plasma membrane plays a role in ethanol tolerance in yeast (Alexandre et al, 1994). These authors showed that the level of unsaturated fatty acids and phospholipid biosynthesis increases in yeast grown in cultures supplemented with ethanol. Growth in 4 % ethanol resulted in an increased membrane fluidity and a reduction in the sterol:protein ratio of the plasma membrane. Furthermore, Piper, 1995, demonstrated that ethanol exposure at sublethal doses induced a heat shock-like response in yeast. Since the concentration of yeast HSP 12 increased during conditions of osmotic stress and since ethanol, itself an osmolyte, induced the biosynthesis of proteins associated with the plasma membrane (Piper, 1995), the difference in the viability of the wild-type yeast and the knock-out strain was investigated after exposure to ethanol.

The wild-type and knock-out strains used for immunocytochemical analysis were grown in YPD media supplemented with ethanol of different concentrations up to 15 % (v/v). Cell viability was determined by spectrophotometric analysis after 24 hrs and 72 hrs using the absorbance at 600 nm. The percentage growth determined from the absorbance of the cultures containing ethanol was compared to control cultures, which contained no ethanol. Conditions were standardised, so that the same amount of cells (determined spectrophotometrically) were added to each reaction flask containing the ethanol. Although no difference in the growth of both strains was observed at 5 % (v/v) ethanol after 24 hrs (Figure 19), increasing the ethanol concentration to 10 % resulted in a diminished growth of the knock-out strain (80 %) compared with that of the wild-type strain (95 %). A further increase in the ethanol concentration resulted in zero growth of the knock out at 12 % ethanol (v/v) compared with 85 % growth of the wild type strain at this concentration. At 15 % ethanol (v/v) no growth of the wild type strain was observed (Figure 19).

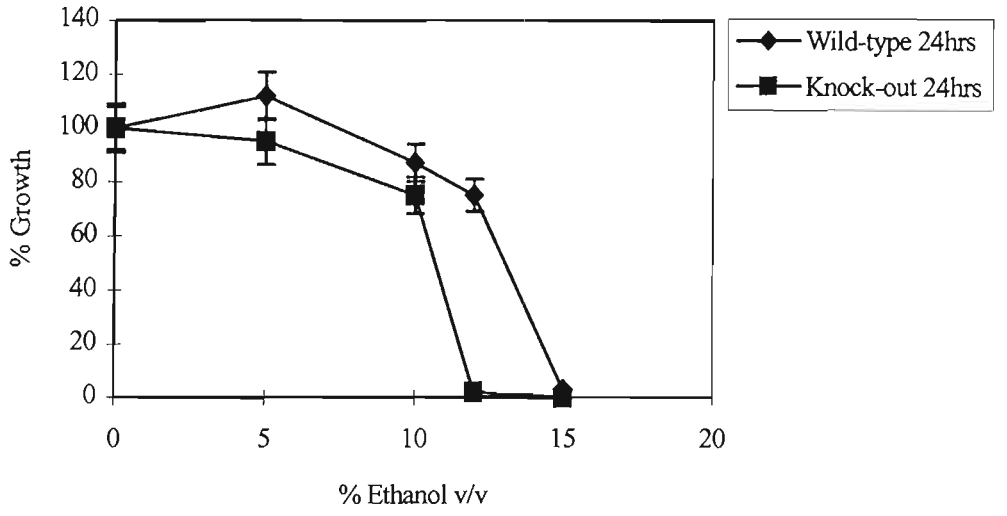


FIGURE 19: Percentage growth as a function of ethanol concentration. Yeast ethanol tolerance between wild-type and the knock-out after 24 hrs carried out at 30°C.

After growth at 30 °C for 72 hrs in the presence of increasing ethanol concentrations a linear decrease in growth was noted in both the wild-type and the knock-out strains (Figure 20). At 5 % (v/v) ethanol 80 % growth of the wild-type strain was observed compared with 60 % growth of the knock-out strain (Figure 20). At 12 % (v/v) ethanol, no growth of the knock-out strain was observed compared with 20 % growth of the wild-type strain. Total loss of growth of the wild-type strain was observed at 15 % (v/v) ethanol. Thus the wild type tolerated a greater concentration of ethanol than the knock-out strain suggesting that HSP 12 played a role in delaying the onset of membrane damage by ethanol.

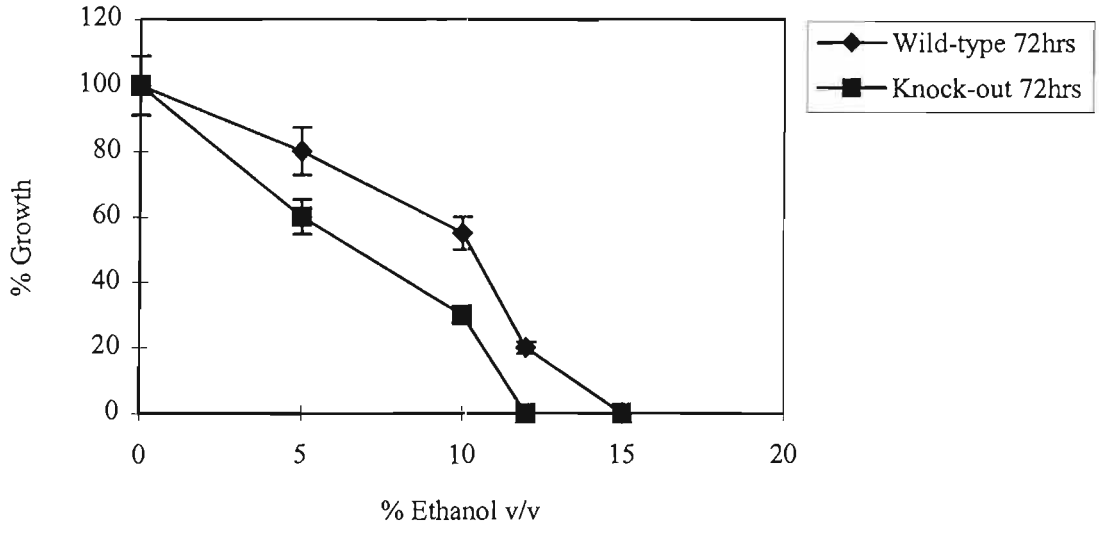


FIGURE 20: Plot of % growth as a function of ethanol concentration. Yeast ethanol tolerance between wild-type and the knock-out after 72 hrs carried out at 30°C.

The effect of rHSP 12 in protecting membranes against ethanol stress was investigated

Liposomes made from PC, cholesterol and stearylamine to encapsulate calcein were incubated with ethanol at concentrations up to 15 % (v/v). The relationship between calcein fluorescence, indicating membrane damage, and ethanol concentration was determined (Figure 21). The percentage structural integrity of liposomes containing no ethanol externally was set to 100 %. Liposomes with and without rHSP 12 present externally tolerated ethanol up to a concentration of 3 % (v/v) maintaining 100 % structural integrity. Liposomes with rHSP 12 present externally tolerated increased levels of ethanol up to a concentration of 12 % (v/v). Above 3 % ethanol (v/v), liposomes without rHSP 12 present externally showed a rapid increase in membrane permeability with increasing ethanol concentrations. Maximum fluorescence was observed in these liposomes at a concentration of 8 % ethanol (v/v) indicating that they had ruptured completely. This suggested that rHSP 12 plays a role in membrane protection against ethanolic dehydration. From these liposome studies. we could speculate that HSP 12 not only forms a net around the plasma membrane of wild-type yeast thereby preventing membrane damage by ethanol, but perhaps also maintains the liquid crystalline state of the membrane by maintaining the distances between adjacent phosphate groups, thereby reducing the van der Waals interactions between adjacent acyl chains of the

bilayer and thus reducing the liquid crystalline to gel phase transition. As there is no interface between the plasma membrane of the knock-out strain and ethanol and as ethanol itself fluidises the membrane, we propose that the water of hydration is removed from the bilayer by the ethanol resulting in an increase in the packing density of the hydrocarbon chains, leading to increased opportunities for van der Waals interactions resulting in the bilayer becoming leaky, however, the presence of the network of protein could prevent too much disorder as effected by ethanol. This would account for the reduced growth observed in the knock-out compared with the wild-type.

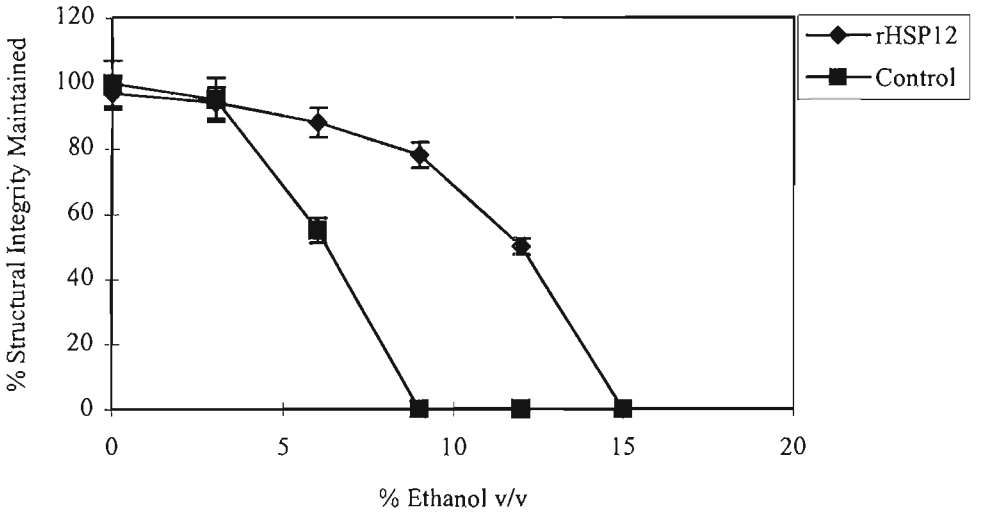


FIGURE 21: A plot of % structural integrity as a function of ethanol concentration. Maintenance of structural integrity of stearylamine liposomes containing 20 μg rHSP 12 per μg Phospholipid present externally in the presence of increasing ethanol concentrations was greater than the control suggesting that rHSP 12 played a role in membrane protection during ethanol-mediated water loss.

CHAPTER 4

CONCLUSIONS

The results presented three findings:

Firstly, immunocytochemical analysis of HSP 12 in yeast indicated a plasma membrane location for this protein. Gold particles indicative of HSP 12 were observed adjacent to the plasma membrane in wild type yeast grown to late stationary phase in YPD medium. Moreover wild-type yeast grown in YPD medium containing 1.6 M mannitol showed an increased number gold particles present. In addition to this, gold particles were observed to be present on both sides of the plasma membrane. No gold particles were observed near the plasma membrane nor in the cytoplasm of the *hsp 12::URA 3* disruption mutant (knock-out strain) of the same strain. Attempts were made to remove the yeast cell wall by enzymatic digestion using ß-glucuronidase in order to investigate the strength of the interaction between the HSP 12 and the plasma membrane. However, as the expression of the HSP 12 increased during stationary phase, so does the cross-linkage of the cell wall. Attempts to remove the cell wall by enzymatic digestion at this stage failed despite attempts to add ß-mercaptoethanol to the buffered osmoticum to break any disulphide bridges. Attempts to make spheroplasts from yeast cells grown in 1.6 M mannitol to mid-log phase, also gave a negative result apon Western blotting. Despite the formation of spheroplasts characterised under the light microscope by cell lysis upon addition of a drop of water under the cover slip, the concentration of spheroplasts was not enough to give a positive result. As it was known that the concentration of HSP 12 increased in yeast on entry into stationary phase, a time when nutrients are limited and the yeast prepares to sporulate, we attempted to produce spores in order to determine whether the increased HSP 12 present during stationary phase was to protect the spores. Unfortunately, the yield of spores was very low (less than 30 %) and we were unable to isolate a homogeneous preparation. We could thus not determine whether the HSP 12 present was due to it present in spores or yeast cells.

Secondly, we have shown that complete liposome integrity is maintained by rHSP 12 present on both sides of the membrane. Moreover the effect of rHSP 12 and trehalose internally in liposomes was observed to be interchangeable although rHSP 12 was effective at a 20 - 30

fold lower molar concentration. The protective effect that rHSP 12 showed towards liposomes during desiccation and lyophilisation was specific as neither myoglobin nor cytochrome C, of similar molecular weight to HSP 12, preserved more than 10 % to 35 % of the membrane integrity during desiccation and lyophilisation. As trehalose concentrations in yeast have been observed to increase upon heat stress (Ribeiro et al, 1997), the production of proteins in response to stress may play a role in membrane protection during desiccation. Our results also suggest that there is an electrostatic interaction between rHSP 12 and liposomes as a decrease in membrane stability was observed in positively charged liposomes as the pH decreased and rHSP 12 became more positively charged. As both the immunogold labelling and the liposome integrity studies suggest that HSP 12 is associated with the exterior surface of the plasma membrane, we suggest that there must be an intracellular transport mechanism or exocytosis to facilitate the transition of this highly charged molecule across the plasma membrane.

Thirdly, *Saccharomyces cerevisiae* has a finite ethanol tolerance during fermentation placing a limit to the industrial yield of alcohol produced by a particular batch. We have shown that wild-type yeast expressing HSP 12 grown in YPD medium containing ethanol up to a concentration of 15 % (v/v) tolerated greater ethanol concentrations than the knock-out yeast strain which did not express HSP 12. Wild-type yeast maintained 80 % growth at 12 % ethanol (v/v) compared with no growth of the knock-out after 24 hrs. The ability of the wild-type to withstand ethanolic dehydration may be brought about by the presence of HSP 12 as more leakage of calcein was observed in liposomes which had no rHSP 12 present externally.

We propose that trehalose and rHSP 12 act in a similar manner in these liposomes by substituting for the water of hydration during desiccation or lyophilisation. Despite previous reports that more water of hydration is removed by lyophilisation than by desiccation, the results presented in these experiments indicate that there is no significant difference between desiccation and lyophilisation. Furthermore as complete integrity of liposomes was not maintained by either trehalose or rHSP 12 present externally only on liposomes during desiccation or lyophilisation, but that increased integrity was observed with trehalose or rHSP 12 present on both sides of the membrane, suggests two independent mechanisms for protecting membranes. We therefore propose that trehalose and rHSP 12 not only prevents fusion of adjacent lipsomes during desiccation, but that curvature of membranes which leads to instability of the bilayer could also be prevented. As rHSP 12 delayed the onset of

membrane damage to liposomes in the presence of ethanol, and since wild-type yeast expressing HSP 12 and grown in ethanol was observed to grow at greater ethanol concentrations that the knock-out yeast strain suggests that LEA group I proteins function in dehydration protection by interacting electrostatically with its target thereby maintaining the shell of hydration during desiccation. These results suggest that the LEA group I protein HSP 12 in yeast protects membranes during desiccation.

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