Isolation and characterisation of a LEA-like protein from yeast (*Saccharomyces cerevisiae*)

by

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ABSTRACT

LEA proteins are plant proteins that are characteristically hydrophilic and soluble at elevated temperature. The consistent correlation between desiccation tolerance in orthodox seed tissue and an accumulation of LEA proteins suggests that these proteins play an important role in protecting cells from desiccation induced damage. Yeast (*Saccharomyces cerevisiae*) has been known to desiccate as part of its normal growth cycle and to remain viable after long periods in the desiccated state. As a result of these properties this project was designed to investigate the presence of LEA-like proteins in yeast.

A protein was isolated from baker's yeast that fulfils the requirements for being a LEA protein. This protein, with a molecular mass of 11 kDa, was found to be the most prevalent heat soluble protein in the yeast extract. Antibodies raised against LEA group I proteins recognised this 11 kDa yeast protein in the total extract but failed to recognise the protein after heat treatment at 80 °C for 10 min. Amino acid analysis showed that the 11 kDa protein was highly hydrophilic - a characteristic of LEA proteins. The protein was partially sequenced (10 cycles) after CNBr digestion and the sequence obtained was compared with the sequence of known proteins in the Stanford databank. Only one protein, HSP 12, was identified to be 100 % homologous to the obtained sequence without the introduction of gaps.

Despite a previous report that HSP 12 is a heat shock protein, HSP 12 was present in a reduced concentration in yeast grown at 37 °C compared with yeast grown at 30 °C. HSP 12 was found to increase in concentration after entry into stationary phase - a time when nutrients are limiting and the yeast is preparing to reduce its water content and sporulate. This might be considered equivalent to plant seed maturation - the stage when LEA proteins are synthesised.

Moreover, growth conditions that have been reported to stimulate LEA protein biosynthesis in plants also stimulated HSP 12 synthesis in yeast. Purified HSP 12 was shown to inhibit thermal denaturation of yeast alcohol dehydrogenase (ADH) at elevated temperatures. This is a functional property of the pea seed p11 LEA group I protein. From the above results, it was therefore concluded that HSP 12 should be identified as a LEA-like protein rather than as a heat shock protein.

ABBREVIATIONS

mRNA	messenger ribonucleicacid
kDa	kilodalton
hr(s)	hour(s)
min(s)	minute(s)
g	gram(s)
mm	millimetre
ml	millilitre
mM	millimolar
s	second(s)
rpm	revolutions per minute
OD	optical density
nm	nanomole
g	gravity
µl	microlitre
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
HCl	hydrochloric acid
V	voltage
M	molar
µg	microgram(s)
NaOH	sodium hydroxide
mA	milli-amps
mg	milligram(s)

CONTENTS

Acknowledgements i
Abstract ii
Abbreviations iv

CHAPTER 1

GENERAL INTRODUCTION

1.1 Desiccation-Tolerance 1
1.1.1 Late Embryogenesis Abundant Proteins 1
1.2 Heat Shock Proteins 4
1.3 The *Saccharomyces cerevisiae* life cycle 7
1.4 Research Question 7

CHAPTER 2

MATERIALS and METHODS

2.1 Protein Purification 9
2.1.1 Total and Heat Soluble Protein Extraction 9
2.1.2 Rapid Total Yeast Extraction 9
2.2 Determination of Protein Concentration 9
2.3 TCA Precipitation 10
2.4 Gel Electrophoresis 10
2.4.1 SDS-PAGE 10
2.4.2 Two-dimensional SDS-PAGE 10
2.4.3 Tricine-SDS-PAGE 11
2.5 Western Blotting 11
2.5.1 Electrophoretic Transfer to Nitrocellulose 11
2.5.2 Colorimetric Visualisation of the Transferred Protein 11
2.5.3 Chemiluminescence Visualisation of the Transferred Protein 12
2.6 Purification of Protein via Column Chromatography 12
2.6.1 DEAE 52 Chromatography Column 12
2.6.2 Gel Filtration Chromatography 13
2.6.3 High Performance Liquid Chromatography 13
2.7 Protein Characterisation 13
2.7.1 Amino Acid Analysis 13
2.7.2 Cyanogen Bromide (CNBr) Digestion 14
2.7.3 Amino Acid Sequencing 14
2.7.4 Database and Hydropathy Studies 14
2.8 Immunological Techniques 14
2.9 Growth Conditions 15

2.10 Alcohol Dehydrogenase Assay 15

RESULTS and DISCUSSIONS

CHAPTER 3

3.1 Protein Purification and Immunological Studies 16
3.2 Purification using Chromatography Columns 23
3.3 Amino Acid Analysis 27
3.4 Digestion Studies and Sequence Analysis 30
3.5 Hydropathy Plot 31

CHAPTER 4

4.1 Investigation of HSP 12 protein accumulation during the yeast growth cycle at 30 °C and 37 °C 34
4.2 The effect of osmolytes on growth of yeast and production of HSP 12 37

CHAPTER 5

5.1 The role of HSP 12 43

CHAPTER 6: CONCLUSION 47

REFERENCES 48

CHAPTER 1

GENERAL INTRODUCTION

1.1 Desiccation-Tolerance

Water deficit is one of the most common environmental stress factors experienced by plants. It interferes with normal development and plant growth and has a major effect on plant productivity (Covarrubias et al., 1995; Xu et al., 1996). In response to this condition, plants undergo numerous metabolic, developmental and physiological changes including changes in gene expression as means of adapting to this stressful condition (Cohen et al., 1991; Robertson and Chandler, 1994; Imai et al., 1996). Some of these changes also occur during the normal embryogenesis program when seeds desiccate. Both environmental and developmental processes have been observed to be regulated by the phytohormone abscisic acid (ABA).

Application of ABA to unstressed vegetative tissues has been demonstrated to mimic many effects of desiccation on plants including changes in gene expression (Gómez et al., 1988; Close et al., 1989; Cohen and Bray, 1990). For example, total protein synthesis is reduced during water-deficit or ABA treatment, while the synthesis of several novel mRNAs and proteins is increased (Bewley et al., 1983). The stress-induced accumulation of ABA was observed to coincide with the onset of desiccation tolerance in plants. These observations have led to the suggestion that ABA may act as an *in vivo* effector of some metabolic events during the response of the plant to water deficit in order to promote tissue adaptation to water stress.

1.1.1 Late Embryogenesis Abundant (LEA) Proteins

A subset of these ABA-responsive proteins, termed LEA (Late Embryogenesis Abundant) proteins (Galau et al., 1986), were first identified during the late stages of seed development when seeds are maturing and reduce cellular water content (Dure et al., 1989; Blackman, 1991; Butler and Cuming, 1993; Wood and Goldsbrough, 1997).

Accumulation of these proteins in response to water deficit or ABA treatment has been observed in several plants including maize (Bewley et al., 1983; Pla et al., 1989), pea (Lalonde and Bewley, 1986; Russouw et al., 1995), barley (Jacobsen et al., 1986; Close et al., 1989), tomato (Bray, 1988; Cohen and Bray, 1990) and rice (Mundy and Chua, 1988). There is strong circumstantial evidence that these proteins are involved in the adaptation of the plant to water deficit. The fact that their transcripts and translation products accumulate in the vegetative tissues of a number of drought-stressed plants and the fact that desiccation treatment induces their 早熟的 precocious expression in seeds have led to the proposition that LEA proteins may play a protective role in maintaining specific cellular structures against desiccation-induced damage (Baker et al., 1988; Robertson and Chandler, 1994).

LEA proteins are characteristically hydrophilic (Baker et al., 1988; Close et al., 1989; Reid and Walker-Simmons, 1993) and remain soluble even after boiling (Blackman et al., 1991, 1995) suggesting that they have a high affinity for water (Close et al., 1989). As a result of these characteristics, LEA proteins are thought to help maintain a minimal water content during desiccation, thereby stabilising cytoplasmic structures (Lane, 1991; Bray, 1993). LEA proteins represent a group of diverse proteins often encoded by multigene families (Espelund et al., 1992; Hollung et al., 1994). They have been classified into at least five groups based on common amino acid sequence domains (Bray, 1993). Although the exact physiological function of each group is not yet known, different roles have been proposed for each, based on the predicted structures (Xu et al., 1996; Bray, 1993).

The group I LEA proteins have been predicted to have a water-binding capacity (Litts et al., 1991; Bray, 1993; Martilla et al., 1996) as a result of the high percentage of charged amino acids. These amino acids are thought to form dipole-dipole interactions with the polar water molecules, thus helping maintain a minimal water content during desiccation. One member of this group, the Em protein from wheat, is reported to exist in solution largely as a random coil (Litts et al., 1991).

This means that all the charged amino acids, which are distributed throughout the primary structure, are exposed to the surrounding aqueous environment - a property that would confer a high affinity for water molecules upon the protein (Litts et al., 1991).

The dehydrins or group II LEA proteins were originally discovered in corn (Close et al., 1989) and later in cotton (LEA D-11) and rice (Rab 21). They are characterised by a consensus 15 amino acid sequence EKKGIMKIKEKLPG that always occurs at or near the carboxy terminus and is repeated upstream of the terminus one or more times (Bray, 1993; Labhilili et al., 1995). The lysine-rich core of this domain KIKEKLPG has been found in spinach, cotton, barley, maize, rice, wheat, tomato, Arabidopsis and *Cratorostigma plantagineum* (Neven et al., 1993) and may be repeated more than once within the complete polypetide (Galau and Close, 1992). The consensus tracts were predicted to form α-helices (Whitsitt et al., 1997) which led to the postulate that dehydrins preserve the structural integrity of cells during desiccation (Bray, 1993; Martilla et al., 1996) by sequestering excessive ions. These ions might otherwise result in the formation of radicals leading to membrane impermeability due to crosslinking. Close and Lammers (1993) used polyclonal antibodies raised against the consensus carboxy terminus of plant dehydrins to demonstrate the presence of this group of proteins in the mature desiccation tolerant seeds of various species.

The D-7 family or group III LEA proteins were identified in cotton (LEA D7), carrot (DL-3), barley (pHVa1), rape (pLEA 76) and carrot (DL 8). These proteins are characterised by a tandem repeat of an 11-mer amino acid motif with the consensus sequence TAQAAKEKAGE repeated 5 to 13 times. The repetition of the 11-mer in the sequence, the lack of proline and glycine and the alternation of polar and non-polar residues, were postulated to enable the formation of an amphiphilic α-helix, (Dure III L et al., 1989; Bray, 1993; Xu et al., 1996) with the outside charged face proposed to be involved in sequestering ions whose concentration is increased during water loss (Bray, 1993).

Group IV LEA proteins (the D-113 family) are thought to replace water molecules during desiccation (Dure, 1993b) in order to preserve membrane structures. The hydroxyl side chains of these proteins mimic water molecules, so forming a hydration shell around the membranes to keep the cell structure intact upon water loss.

The D-29 family or group V LEA proteins are thought to sequestrate ions accumulated during water deficit. This group of proteins also has an 11-mer repeat in which each amino acid in the motif has similar chemical properties to those in the group III consensus sequence. Unlike the group III proteins however, they lack residue specificity at each position (Bray, 1993).

1.2 Heat Shock Proteins (HSPs)

Water stress is not only caused by desiccation but by any process that reduces the water activity within the cell. Osmotic and thermal stresses are examples of conditions whereby plants are exposed to increased water stress. Exposure to increased growth temperature cause cells of virtually all organisms studied to date to synthesise a set of proteins referred to as heat shock proteins (HSP) as an adaptive response (Nover, 1991; Vierling, 1991; Parsell and Lindquist, 1993). These proteins are believed to play an important role in helping cells to cope with the effects of high temperature. Some HSPs are either constitutively expressed and only modestly induced by heat or expressed under developmental control (Craig et al., 1993; Lee et al., 1994; Parsell and Lindquist, 1994; Waters et al., 1996). This suggests that they are important during growth and metabolism at normal growth temperatures and during various stages of development. HSP expression was observed to coincide with the acquired cellular tolerance to heat shock. This led to the proposition that HSPs protect cells against heat-induced damage and that their accumulation leads to increased thermotolerance (Kaukinen et al., 1996). Although the detailed mechanism by which these proteins protect cells is not known, most HSPs function as molecular chaperones (Parsell and Linquist, 1993) by monitoring proper protein refolding and preventing aggregation of unfolded proteins.

The major HSPs synthesised in eukaryotes, including plants, are classified into five classes according to their molecular masses: HSP 104, HSP 90, HSP 70, HSP 60, and small (sm) HSPs ~12 to 30 kDa. In yeast, *Saccharomyces cerevisiae*, two proteins HSP 70 and HSP 90 are essential at normal temperatures but are required in higher concentrations for growth at elevated temperatures (Craig and Jacobsen, 1984; Borkovich et al., 1989). In contrast, HSP 104 is expressed at very low levels at normal temperatures and is very strongly induced by heat (Parsell et al., 1994). Though HSP 104 is not required for growth at normal temperature (Sanchez and Lindquist, 1990), it plays a vital role in helping cells survive exposure to extreme temperatures by promoting resolubilisation and reactivation of unfolded and aggregated proteins.

HSPs 70 and 60 from *Escherichia coli* (also termed DnaK and GroEL) have been characterised in great detail and have been recognised as major players in cellular protein folding reaction. HSP 70s appear to prevent premature folding of incomplete polypeptides during translation and membrane translocation (Craig and Gross, 1991; Wiech et al., 1993; James et al., 1997) while HSP 60s mediate the folding of newly synthesised proteins to the native state (Craig and Gross, 1991). GroEL captures the non-native substrate protein by binding to exposed hydrophobic surfaces thus preventing aggregation of the substrate protein. The folding and concomitant release of the substrate from the hydrophobic surface of the chaperone is triggered by interaction between ATP and GroEL which is regulated by a co-chaperone, GroES - a homologue of HSP 10 (Craig and Gross, 1991; Rozema and Gellman,1996).

Other HSPs are reported to have different but related functions. HSP 90, for example, is also involved in monitoring proper refolding of denatured proteins. Yet other HSPs are reported to be involved in protein translocation (Bush and Meyer, 1996), degradation of heat damaged proteins or peptidyl cis-trans isomerase activity (Parsell and Lindquist, 1993).

In addition to these high molecular weight (HMW) HSPs, there is another group of heat shock proteins of smaller molecular weight, smHSPs, that are thought to be involved in the acquisition of thermotolerance (Lee et al., 1995). These smHSPs are characterised by a conserved carboxy terminal region (Jinn et al., 1995) and molecular masses ranging from 12 to 30 kDa (Helm et al., 1997). In contrast to the highly conserved HMW heat-shock proteins, smHSPs show less similarity in sequence (Parsell and Lindquist, 1993; Kaukinen et al., 1996). Unlike the other HSPs including mammalian smHSPs, plant smHSPs constitute the most abundant group of this type of proteins, approximately 1.0 - 1.5 % of total proteins synthesised in response to heat shock (Lee et al., 1995; Jinn et al., 1995). Following stress, these proteins remain quite stable with a half-life of 30 - 50 hrs (DeRocher et al., 1991; Waters et al., 1996) suggesting that they function during both heat stress and recovery.

Based on sequence comparisons and localisation, smHSPs are categorised into at least four multigene families, two of which are localised in the cytoplasm (class I and II), one in the chloroplast (class III) and one in the endoplasmic recticulum (class IV) (Vierling, 1991; Helm et al., 1993). A fifth class of smHSPs, localised in the mitochondria, has been identified in several species (Lenne and Douce, 1994; Waters et al., 1996) and a potential sixth class has been associated with a membrane compartment (Waters et al., 1996). Members of these families and their identified homologues, such as α-crystallin, have a tendency to form multimeric complexes ranging in size from 200-800 kDa, possibly via a GVLTV- conserved motif present in the C-terminal region (Vierling, 1991; Kaukinen et al., 1996).

Though little is known of the cellular function of smHSPs, it would seem that they play a vital role in thermotolerance of cells (Berger and Woodward, 1983). SmHSPs and their homologues may function as molecular chaperones in a manner similar to that of HMW HSPs (Horwitz, 1992; Merck et al., 1993; Parsell and Lindquist, 1993).

The expression pattern of smHSPs, in which smHSPs are undetectable in vegetative tissues under optimal growing conditions but rapidly accumulate with increased temperature (Helm et al., 1997), suggests that their function is restricted to specialised cellular conditions shared by developmental and stressed states.

1.3 The *Saccharomyces cerevisiae* life cycle

Yeast was chosen for studying the molecular mechanism of desiccation tolerance since it can be grown easily and its genome has been extensively characterized. Yeast cells exist stably in either haploid or diploid states and grow in liquid medium or on the surface of solid agar plates. They grow well on minimal medium containing glucose as a carbon source and salts that supply nitrogen, phosphorus and trace metals. However, yeast cells grow more rapidly in the presence of protein and yeast cell hydrolysates that provide amino acids, nucleotide precursors, vitamins and other metabolites that the cells would normally have to synthesize *de novo.*

During log-phase growth in such media, yeast cells divide approximately every 90 min at 30 °C. When food is available, haploid cells fuse to form diploid cells and the mother cells bud diploid daughter cells. As the cell density increases, nutrient supplies drop and the rate of cell division slows as the cells enter stationary phase. When nutrients are depleted as a result of an increased number of cells, yeasts prepare to reduce their water content and sporulate, a process equivalent to meiosis. The spores, when placed in a suitable nutrient media, germinate to form haploids and the cycle is repeated.

1.4 Research Question

Since yeast undergoes desiccation during its normal life cycle, we wished to investigate the presence of LEA-like proteins in this organism. As yeast is known to remain viable after long periods in a desiccated state, we thought that LEA proteins might play an important role in the preservation of cell structure. Using described methods for isolating and purifying LEA proteins, we examined yeast for the presence of these proteins.

This thesis reports the results of these studies whereby the yeast HSP 12 protein was identified as a LEA-like protein rather than a heat shock protein.

CHAPTER 2

MATERIALS AND METHODS

2.1 Protein Purification

2.1.1 Total and Heat Soluble Protein Extraction

Heat soluble protein was isolated from baker's yeast (*Saccharomyces cerevisiae*), a kind donation from Anchor Yeast, Cape Town. Yeast total proteins were extracted as described by Blackman et al. (1991) with a few minor adjustments. 10 g packed wet yeast was ball-milled (Braun) with 0.45-0.50 mm diameter glass beads in 20 ml ice cold extraction buffer, 50 mM Tris-HCl pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, containing 1 mM phenylmethanesulfonylfluoride (PMSF) (from a 100 mM stock solution dissolved in dimethyl sulfoxide) to inhibit proteolytic activities of proteases excreted by yeast. The ball-mill was cooled with CO_2 liquid and operated for 11 s bursts at 34 s intervals (total cycle time of 45 s) to ensure the temperature remained below 4 °C. The crude homogenate was centrifuged at 15 000 rpm (27 000 *g*) using a Beckman JA 20 rotor for 10 min at 4 °C. An aliquot of the supernatant was kept as a control sample while the rest of the sample was incubated at 80 °C for 10 min. Heat coagulated protein was removed by centrifugation as above.

2.1.2 Rapid Total Yeast Protein Extraction

2 OD units (determined spectroscopically at 600 nm) of cell culture were pelleted and washed with 10 mM NaN_3, 50 mM Tris-HCl pH 6.8 containing 2 % SDS, 10 % glycerol, 1.5 % 2-mercaptoethanol and 10 mM PMSF. 0.1 g of 0.2 mm diameter glass beads were added to the suspension which was then vortexed vigorously for 2 min before centrifugation at 12 000 *g* for 5 min. 10 μl of the supernatant fraction was applied to the SDS-PAGE gel.

2.2 Determination of Protein Concentration

The protein concentration of the yeast total extract was quantified using the method of Lowry et al. (1951). Bovine serum albumin (Boehringer Mannheim) was used as the standard.

2.3 TCA Precipitation

Trichloroacetic acid (TCA) was added to a final concentration of 13 % (one third volume of 50 % TCA solution per volume of protein sample). After incubation at 4 °C for 1 hr, the solution was centrifuged at 12 000 *g* for 10 min at 4 °C and the supernatant discarded. The precipitate was washed once with ice cold acetone containing 0.02 M HCl, centrifuged as above and once with ice cold 100 % acetone after which the pellet was lyophilised. The pellet was resuspended in 10 µl water and an equal volume of sample application buffer was added before loading on to the gel.

2.4 Gel Electrophoresis

2.4.1 SDS-PAGE

SDS-PAGE was carried out at room temperature using a 20 % separating gel containing a 6.5 % stacking gel as described by Laemmli (1970). Gels were run at a constant voltage of 150 V for 4 hrs and stained with Coomassie brilliant blue. Destaining was by diffusion in 7 % acetic acid, 25 % ethanol.

2.4.2 Two-dimensional SDS-PAGE

Two-dimensional gel electrophoresis was performed essentially according to the method of O'Farrell (1975). Heat soluble proteins were separated in the first dimension by isoelectric focusing on cylindrical gels (0.6 x 150 mm) containing 4 % acrylamide, 9 M urea, 2 % nonidet NP-40, 4 % ampholines pH 3-10. Gels were pre-focused at 200 V for 15 min, at 300 V for 30 min and finally at 400 V for 30 min. 30 µl sample containing approximately 100 µg of protein was loaded at the basic end of this first dimension gel which was electrophoresed at room temperature for 16 hrs at 400 V, after which the potential difference was increased to 800 V for 1 hr. Anode and cathode buffers were 0.01 M phosphoric acid and 0.02 M NaOH respectively. After focusing at 800 V for 1 hour, gels were extruded with a syringe and loaded directly onto the second dimension slab gel prepared as described above (section 1.4.1). The first dimension gel was then overlaid with equilibration buffer (10 %glycerol, 5 % SDS, 0.01 % bromophenol blue, 50 M Tris-HCl pH 6,8) and the gel electrophoresed as described in section 2.4.1.

2.4.3 Tricine-SDS-PAGE

Cyanogen bromide digestion products were analysed by Tricine-SDS-PAGE, as described by Schägger and von Jagow (1987). Small peptides were separated on a system consisting of a 10 % T, 3 % C spacer gel and a 16.5 % T, 3 % C separating gel polymerised together and overlaid with a 4 %, T 3 % C stacking gel. (T denotes the total percentage concentration of both acrylamide and bisacrylamide, C denotes the percentage concentration of the crosslinker relative to the total concentration T). Anode and cathode buffers were 0.2 M Tris-HCl pH 8.9 and 0.1 M tricine, 0.1 % SDS, 0.1 M Tris pH 8.25, respectively. Electrophoresis was carried out at room temperature at 90 V for 16 hrs after which the gel was fixed in 50 % methanol, 10 % acetic acid for 30 min prior to staining with Coomassie blue.

2.5 Western Blotting

2.5.1 Electrophoretic Transfer to Nitrocellulose

The gel, after SDS-PAGE, was washed with transfer buffer (0.19 M glycine, 20 % methanol, 25 mM Tris-HCl pH 8.5) and laid on pre-soaked nitrocellulose paper supported on pre-soaked Whatman 3MM paper. This was placed on a thick wad of damp nappy-liners on the anode carbon block. The gel was overlaid with pre-soaked Whatman 3MM paper and another wad of nappy-liners followed by the cathode carbon block. Care was taken to avoid bubbles being trapped in the Whatman 3MM paper "sandwich". Transfer was carried out for 16 hrs at 4 °C at a constant current of 20 mA.

2.5.2 Colorimetric Visualisation of the Transferred Protein

The air-dried nitrocellulose membrane was incubated for 30 min at room temperature in 10 ml blocking buffer 50 mM NaH_2PO_4, 150 mM NaCl pH 7.4 (PBS) containing 5 % skimmed milk powder. Primary antibody (see results for different antibodies used) was added to the blocking solution and incubation was carried out for a further 1 hr at 37 °C or overnight at 4 °C. The blot was washed three times with PBS 0.05 % Tween 20 (PBS/Tween) and once with PBS prior to incubation with the secondary antibody. Incubation with the secondary goat-anti-rabbit antibody (coupled to alkaline phosphatase), a 1:5000 dilution in PBS, was at room temperature for 60 min.

The blot was finally washed three times with PBS/Tween and once with washing buffer, 150 mM NaCl, 10 mM Tris-HCl pH 7.4. The secondary antibody was detected by incubation in the dark 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.

2.5.3 Chemiluminescence Visualisation of the Transferred Protein

The blot was incubated in a 1:100 dilution of the anti-HSP 12 primary antibody in 0.5 % blocking buffer. This was carried out essentially as described in the Boehringer Mannheim Kit. Unbound antibody was removed by washing twice for 10 min each with TBST buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5 [TBS] + 0.1% Tween 20) and twice for 10 min each with 0.5 % blocking buffer. For efficient washing, large volumes (100 ml) of buffers were used. The membrane was then incubated for 1 hr at room temperature with 8 µl horseradish peroxidase (POD) labelled secondary antibody in 0.5 % blocking buffer after which the blot was washed four times for 15 min each with TBST buffer. Excess washing buffer was drained from the blot which was then placed in a transparent plastic bag. A pre-mixed detection buffer was added and spread over the entire membrane surface. The bag was immediately sealed after eliminating air bubbles. The blot was then inserted with the protein side up into a x-ray cassette containing a pre-flashed x-ray film and exposed for 15 s. The film was developed according to the manufacturer's recommendations.

2.6 Purification of Protein via Column Chromatography

All chromatographic procedures, except HPLC, were carried out at 4 °C.

2.6.1 DEAE 52 Chromatography Column

After incubation of the total yeast extract (section 1.1.1) at 80 °C and subsequent centrifugation, the supernatant containing the heat stable proteins was applied directly to a 27 x 145 mm Whatman DEAE 52 ion exchange column previously equilibrated with 50 mM NaCl, 10 mM Tris-HCl pH 8.0. Proteins were eluted with the same buffer at a flow rate of 2 $ml.min^{-1}$. The optical density of each fraction was read on a Beckman DU 650 spectrophotometer at 230 nm. Aliquots of these fractions were analysed by SDS-PAGE.

2.6.2 Gel Filtration Chromatography

Fractions from the DEAE 52 column containing the protein of interest were pooled and lyophilised. The lyophilate was dissolved in 20 mM HCl and applied to a 2.5 x 1000 mm Sephadex G-50 column equilibrated in this same buffer with a flow rate of 0.5 $ml.min^{-1}$. 1 ml fractions were collected. The optical density of each fraction was read on a Beckman DU 650 spectrophotometer at 230 nm. Fractions were analysed by SDS-PAGE and those containing the protein of interest were pooled and lyophilised.

2.6.3 High Performance Liquid Chromatography (HPLC)

Final purification was carried out by HPLC using a C_{18} Vydac reverse-phase column in 0.1 % heptafluorobutyric acid (HFBA). 6 M urea with 0.1 % 2-mercaptoethanol was added to the lyophilised sample from the Sephadex G-50 column to unfold the protein prior to application onto the column. 0 - 70 % gradient of acetonitrile in 0.1 % heptafluorobutyric acid was used as the eluent. The HPLC was programmed as follows: 100 % buffer A from 0 to 5 min, 100 % buffer B from 5 to 65 min, 100 % buffer A from 65 to 86 min. Buffer A contained 0.1 % HFBA and buffer B contained 70 % acetonitrile in 0.1 % HFBA. All fractions corresponding to peaks detected by a fixed wavelength detector at 229 nm were collected, lyophilised and analysed on SDS-PAGE.

2.7 Protein Characterisation

2.7.1 Amino Acid Analysis

Amino acid analysis was performed to determine the amino acid composition of the isolated protein. A sample (8 μl) containing approximately 2 nmol of the purified protein was hydrolysed in constant boiling HCl (2 ml) containing 5 % phenol under N_2 gas at 110 °C for 24 hrs. The hydrolysate was lyophilised and dissolved in NLE 100 buffer (66.6 mM trisodium citrate containing the internal standard, norleucine). The sample was analysed on a Waters HPLC and the amino acids were detected by postcolumn OPA fluorescence (orthophthaldialdehyde) derivatisation.

2.7.2 Cyanogen Bromide (CNBr) Digestion

The purified protein was digested with cyanogen bromide to cleave the protein after methionine residues in the polypeptide. 100 mg of pure protein was dissolved in 70 % formic acid at room temperature. A 1000-fold molar excess of CNBr dissolved in 70 % formic acid over methionine residues was added to the protein. Digestion was carried out under N_2 gas in the dark for 16 hrs at room temperature. Excess formic acid was evaporated under a stream of N_2 gas. The residue was diluted with 10 volumes of water and lyophilised. The digestion products were then analysed on a Tricine-SDS gel.

2.7.3 Amino Acid Sequencing

A gas-liquid solid phase sequencer constructed by Hewick et al. (1981) with slight modifications by Brandt et al. (1984) was used. 1 nmol of pure protein was applied on a glass filter. The reagents, solvents and degradation cycle used were as described (Hewick et al., 1981). The converted phenythiohydatoin (PTH) amino acids were identified by an isocratic on-line HPLC system on 250 x 3 mm 3 μm Lichrosphere C_{18} (Bishoff) column as described by Lottspeich (1985). Table A (next pg) shows the yield of identified amino acids

2.7.4 Database and Hydropathy Studies

Database searching to determine the origin of the isolated protein, determination of the HSP 12 amino acid sequence and of comparative amino acid compositions were carried out using the GCG computer program (Genetics Computer Group, Madison, WI). Protein hydropathy was determined by the method of Kyle and Doolittle (1982) using a window of 4 residues.

2.8 Immunological Techniques

The LEA group 1 antibody used was raised against the pea axis p11 protein (Russouw et al., 1995). Antibodies against the group II and the group III LEA proteins were the kind gifts of Drs T. Close and M.K. Walker-Symmons respectively. The antibody against the protein isolated in this study, subsequently referred to as HSP 12, was prepared by injecting 1 mg pure HSP 12 protein in Freund's complete adjuvant into rabbits. Following the initial inoculation, the rabbits were given three booster inoculations at weekly intervals followed by two further inoculations after an interval of three weeks.

No. of Cycles	**PTH amino acids**	**pmoles**
1	Gly	435
2	Ala	417
3	Ala	503
4	Lys	149
5	Ser	42
6	Lys	161
7	Leu	166
8	Asn	113
9	Asp	183
10	Ala	252

Table A: Results of automatic gas-phase Edman degradation of the unseparated CNBr-digested HSP 12 protein

Titres and specificity were determined by ELISA. 1 µg of antigen was added to each well and left overnight at 4 °C. Unbound antigen was removed by washing three times with PBS/Tween, while the well was blocked with 1 % bovine serum albumin (BSA) in PBS at room temperature for 30 min. Bound antigen was washed three times with PBS/Tween prior to incubation with antibody diluted in PBS in the range of 10^{-1} to 10^{-8}. After incubation at room temperature for 60 min, a 1:100 dilution in PBS of goat anti-rabbit antibody coupled to alkaline-phosphatase was added and incubated for 30 min. Following five washes with PBS/Tween, antibody detection was carried out using 300 µg p-nitrophenylphosphate in 10 % diethanolamine pH 9.6 in a Titertek Multiskan PLUS MKII detector.

2.9 Growth Conditions

Yeast cells were grown in YPD medium (1 % peptone, 2 % glucose, 0.5 % yeast extract) at 30 °C under shaking. Heat shock was imposed by growing yeast at 37 °C. Osmotic shock was administered by adding 0.2 M - 0.8 M NaCl or mannitol to the growth medium. Growth was monitored by measuring the absorbance at 600 nm; samples were diluted for 600 nm absorbance determination to have an absorbance of < 0.2. Equal quantities of cells harvested at log phase, early stationary and late stationary phases were centrifuged and the pellet washed twice in distilled water prior to protein extraction (see section 1.1.1). Samples were then analysed by scanning the HSP 12 band present after SDS-PAGE using a Hoefer GS 300 scanning densitometer. Each experiment was repeated a mininum of five times and the average values determined. The seemingly different growth curves shown (results section) in different experiments was due to differences in the initial number of cells at the start of each experiment.

2.10 Alcohol Dehydrogenase Assay

Yeast alcohol dehydrogenase (ADH) purchased from Sigma was dissolved (0.1 mg/ml) in 10 mM phosphate buffer pH 7.5. 2 µl of this solution with or without HSP 12 was incubated as stated (see results) and, after cooling to room temperature, was added to the assay mixture. The assay mix contained 0.01 mM NAD^+, 0.01 M ethanol and 1 mM pyrophosphate buffer pH 8.0. The enzymatic activity was determinated by the spectrophotometric increase in NADH + H^+ at room temperature at 340 nm using a Shimadzu UV-2201 spectrophotometer. ADH activity was determined from the initial velocity of each sample.

RESULTS

AND

DISCUSSION

CHAPTER 3

3.1 Protein purification and immunological studies

LEA proteins are characteristically soluble at high temperatures. This property was therefore utilised in the initial purification process. Heat treatment of the crude extract resulted in precipitation of heat coagulable proteins as well as inactivation of some heat-labile proteases. In order to investigate for the presence of LEA-like proteins in yeast, we extracted total protein as described in section 2.1.1 and heated the extract at 80 °C for 10 min.

To release soluble proteins from yeast, cells were suspended in the extraction buffer and disrupted in a ball-mill. The homogenate had to be kept at temperatures below 4 °C to prevent proteolysis. The ball-mill was therefore cooled with CO_2 liquid and operated such that the temperature remained below 4 °C. Since heat was produced during ball-milling, the optimal time for cell disruption was determined. Obtaining optimal time required a compromise between the time taken in the ball-mill for maximum cellular disruption and speed to prevent exposure of the intracellular contents to the proteolytic enzymes. Optimal ball milling time was determined experimentally by repeatedly examining 100 μl samples of the homogenate and noting the shortest time taken to achieve total cellular lysis (determined microscopically) and maximum release of protein (measured by reading the optical density at 230 nm of the supernatant after centrifuging the homogenate at 12 000 *g* for 3 min). The results (figure 3.1) showed that maximal release of protein was achieved after 45 s of ball milling - a time that corresponded with that required for almost total cellular disruption. When proteins were extracted from these samples and analysed by SDS-PAGE they electrophoresed as a smear (data not shown). This was due to protein degradation by proteolytic enzymes. Yeasts, like many unicellular organisms, derive their nourishment from the surrounding medium by releasing hydrolytic enzymes into the medium and absorbing the products of digestion of extracellular macromolecular substrates. This problem was solved by addition of 2 mM PMSF to the extraction buffer prior to protein extraction.

Addition of the protease inhibitor increased yields of extracted proteins considerably and the protein components appeared as distinct bands on SDS-PAGE whereas previously they had electrophoresed as a smear. Figure 3.1b shows SDS-PAGE of proteins extracted at the indicated times. These results confirmed that there was maximum extraction efficiency after 45 s of ball-milling. On the basis of these results, yeast was routinely ball-milled for 45 s; protein extracted using 50 mM NaCl buffer under these conditions is shown in figure 3.2, lane 1. Proteins ranging in size from approximately 74 kDa to less than 10 kDa were extracted. If the extraction was carried out in 0.5 M NaCl buffer as described for LEA protein extraction from plants (Blackman et al., 1991), the proteins extracted were qualitatively the same (data not shown). Extraction was therefore routinely carried out in 50 mM NaCl buffer to preclude subsequent dialysis prior to ion exchange chromatography.

All LEA proteins purified so far have been found to be soluble at 80 °C (Baker et al., 1988; Close et al., 1989). This has been attributed to their high content of hydrophilic amino acids. When the total soluble extract was incubated at 80 °C for 10 min, the majority of extracted proteins were precipitated and only a few proteins were still soluble. A protein of approximately 11 kDa predominated in the supernatant fraction after heat treatment (figure 3.2, lane 2).

To determine whether antibodies against groups I, II and III LEA proteins recognised proteins from yeast, the total protein extract and the heat soluble protein fraction were subjected to SDS-PAGE and then transferred to a nitrocellulose membrane. The blot was then probed with antibodies raised against LEA proteins of groups I, II and III. Figure 3.3 shows that these antibodies all recognised proteins in the yeast total extract. The anti LEA group I antibody recognised two proteins of approximately 11 kDa and 55 kDa. The anti LEA group II and III antibodies each recognised a single protein of approximately 43 kDa and 53 kDa respectively. These results therefore suggested that the yeast 11 kDa protein was related to LEA group I protein. Previous work in this laboratory (Russouw et al.,1995) has focused on the p 11 LEA group I protein from pea seed.

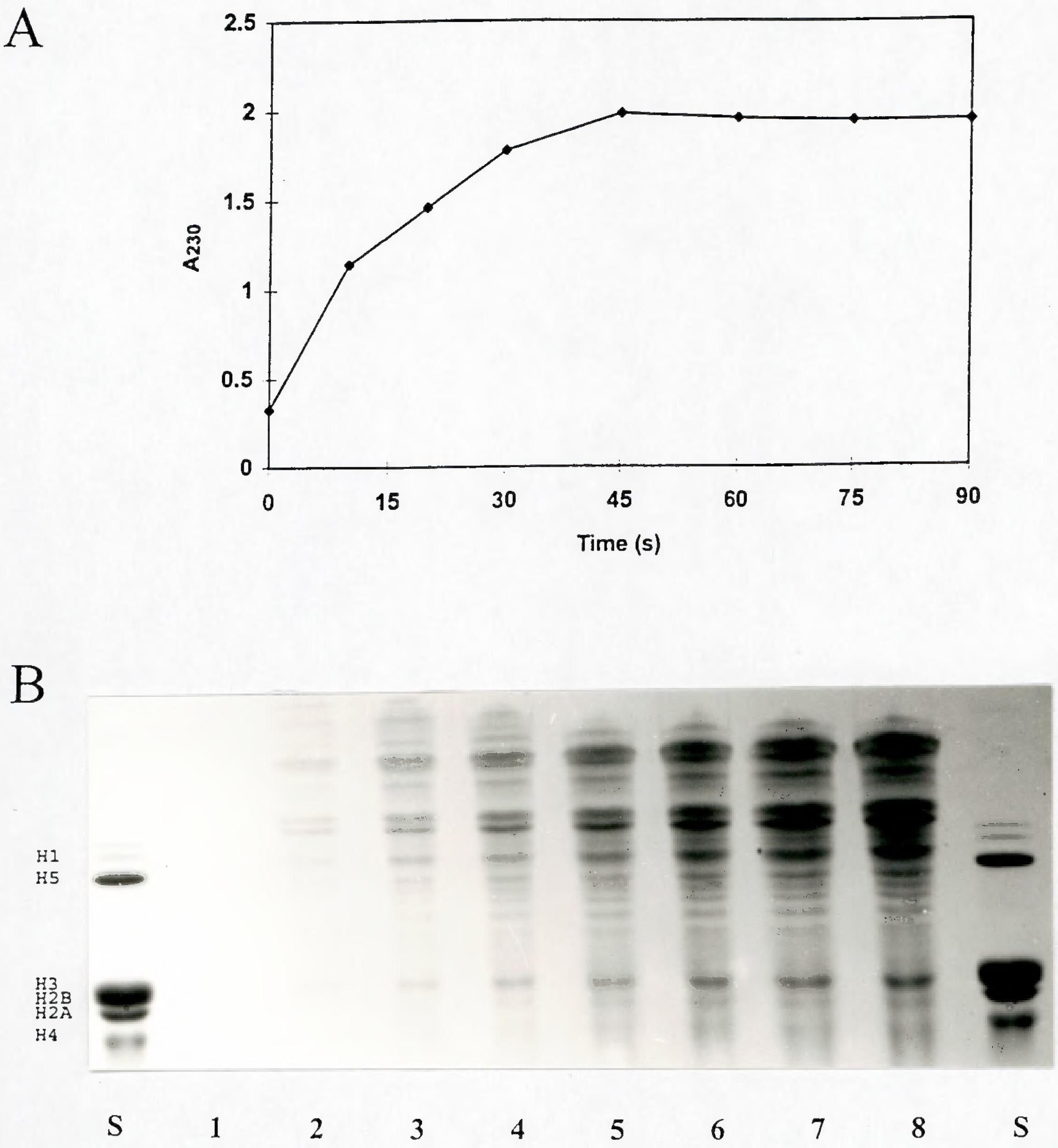

Figure 3.1 A: Time course of extraction of 230 nm absorbing material. Aliquots were removed at the time intervals indicated. B: SDS-PAGE of samples of the extract as in (A) above. Lanes 1 - 8 correspond to extraction time (s) 10, 20, 30, 45, 60, 75 and 90. The standard (S) is a total extract of chicken erythrocyte histones.

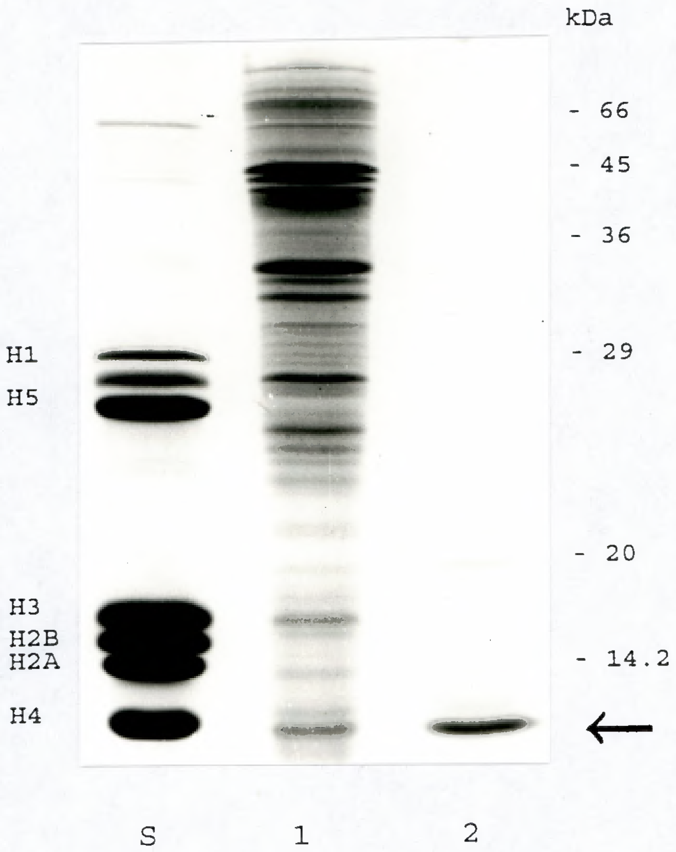

Figure 3.2. SDS-PAGE of total proteins extracted from yeast (lane 1) and those still soluble after incubation at 80 °C for 10 min (lane 2). The molecular weight markers of known size (kDa) are denoted on the right hand side of the gel. The arrow shows the electrophoretic migration of the protein selected for further study. 10 μl of sample containing 5 μg of yeast total protein extract in sample application buffer was applied to the gel; after precipitation of the heat insoluble proteins, 25 μl of the supernatant fraction was applied. Standard (S) as for figure 3.1

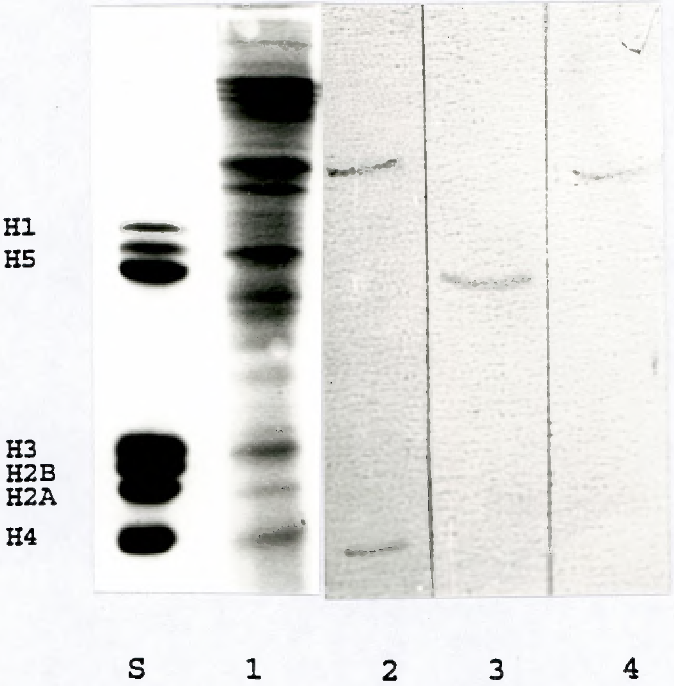

Figure 3.3. Western blot of total proteins extracted from yeast (c.f. fig. 1, lane1). Lanes S and 1: the nitrocellulose membrane was stained with amido black; lanes 2 to 4: the yeast total protein extract was probed with antibodies raised against LEA group I, LEA group II and LEA group III proteins respectively. Standard (S) as for figure 3.1.

Immunochemical identification of LEA proteins in yeast extracts was not confined to baker's yeast. Other strains of *Saccharomyces*, for example those used for production of malt whisky or wine (not shown), also tested positive for the presence of LEA group I proteins, the proteins having the same molecular mass as those recognised by the anti LEA group I antibody in *Saccharomyces cerevisiae*.

The 11 kDa protein in the heat supernatant fraction was not recognised by the anti LEA group I antibody (not shown). This may have been due to a heat-induced conformational change in the yeast 11 kDa protein, with loss of reactivity of the conformational epitopes. LEA proteins are believed to play a protective role in plants under stressful conditions and such a change in structure of the 11 kDa protein might serve the purpose of protecting cell membranes or organelles against heat-induced damage. Under non-stress conditions, the protein might exist in a dormant form in which the epitopes are preserved. Alternatively, the 11 kDa band in the total protein extract may have comprised more than one protein, one of which would have been recognised by the anti LEA group I antibody but was precipitated at 80 °C. To determine whether this was indeed so, the heat precipitated fraction was subjected to SDS-PAGE. It was found (not shown) that the 11 kDa protein was not present in the heat precipitate. Immunological analysis by western blotting using the anti LEA group I antibody was also negative. The heat soluble fraction was then analysed by 2-dimensional (2-D) SDS-PAGE (as described in section 2.4.2) to find out whether the single band in the 1-D gel contained one or more proteins. Figure 3.4 showed that the 11 kDa band in the 1-D gel indeed consisted of a single protein. This protein was also noted to have an isoelectric point close to five. These results indicated that yeast contained a 11 kDa protein that was immunologically related to group I LEA proteins. This protein was heat soluble (a characteristic property of LEA proteins), but lost immunoreactivity on heating possibly due to heat-induced conformational changes. This protein was targeted for further study.

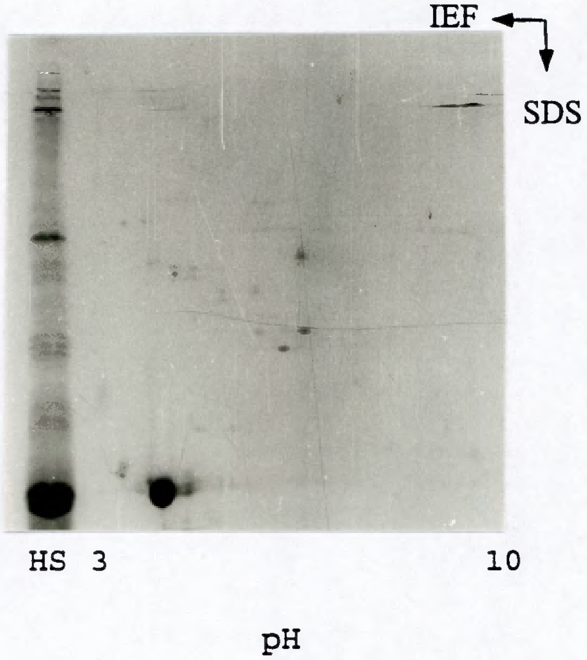

Figure 3.4. Two-dimensional electrophoretogram (isoelectric focusing from pH 3 to pH 10 in the first dimension; SDS-PAGE in the second) of heat soluble proteins. 30 µl of the heat soluble protein fraction was applied. The sample was also electrophoresed only in the second dimension on the gel (HS lane). The arrow shows the electrophoretic migration of the protein of interest.

3.2 Purification using Chromatography Columns

Various chromatographic techniques were used both to purify the protein of interest and to define some of its characteristics.

The first column used was a Whatman DEAE 52 anion exchange column. The matrix of this column, which has a pKa of 10, carries positively charged groups at pH 8.5. The protein of interest eluted in 50 mM NaCl, 10 mM Tris-HCl pH 8.5 buffer under the major peak in the elution profile shown in figure 3.5a. The yeast 11 kDa protein did not bind strongly to the matrix, since it eluted early, indicating that it was weakly negatively charged at pH 8.5. SDS-PAGE (figure 3.5b) analysis of the fractions across the peak showed that the 11 kDa protein co-eluted with a protein of approximately 20 kDa. Proteins with molecular weights between 11 and 20 kDa as well as higher molecular weight were also present in trace amounts.

After DEAE 52 chromatography, gel filtration or size-exclusion chromatography, was used to further purify the 11 kDa protein. Sephadex G-50 with a fractionation range between 1500 and 30 000 daltons for globular proteins was chosen since the molecular weight of the protein falls within this fractionation range. The elution profile is shown in figure 3.6a. SDS-PAGE (figure 3.6b) analysis of the fractions showed that the shaded part of the major peak contained the protein of interest whilst the 20 kDa contaminating protein was eluted at the shoulder on the leading edge of this peak.

Final purification, to remove any contaminating 20 kDa protein as well as any proteins present in low concentrations and any contaminants that were not detected with Coomassie blue staining was achieved with High Performance Liquid Chromatography (HPLC). The elution profile from HPLC (figure 3.7a) showed four peaks. Peaks 1 and 2 were identified to be dirt peaks. SDS-PAGE analysis of the fractions corresponding to these peaks (figure 3.7b) showed that peak 4 contained pure 11 kDa protein. The shoulder on the trailing edge of this peak, however, contained small amounts of the 20 kDa protein. Pure 11 kDa protein was obtained by appropriate pooling of fractions across this peak. Peaks 1, 2 and 3 contained material that was not detectable on SDS-PAGE after staining with Coomassie Blue. The collected material was lyophilised to remove the volatile buffer and stored at -70 °C.

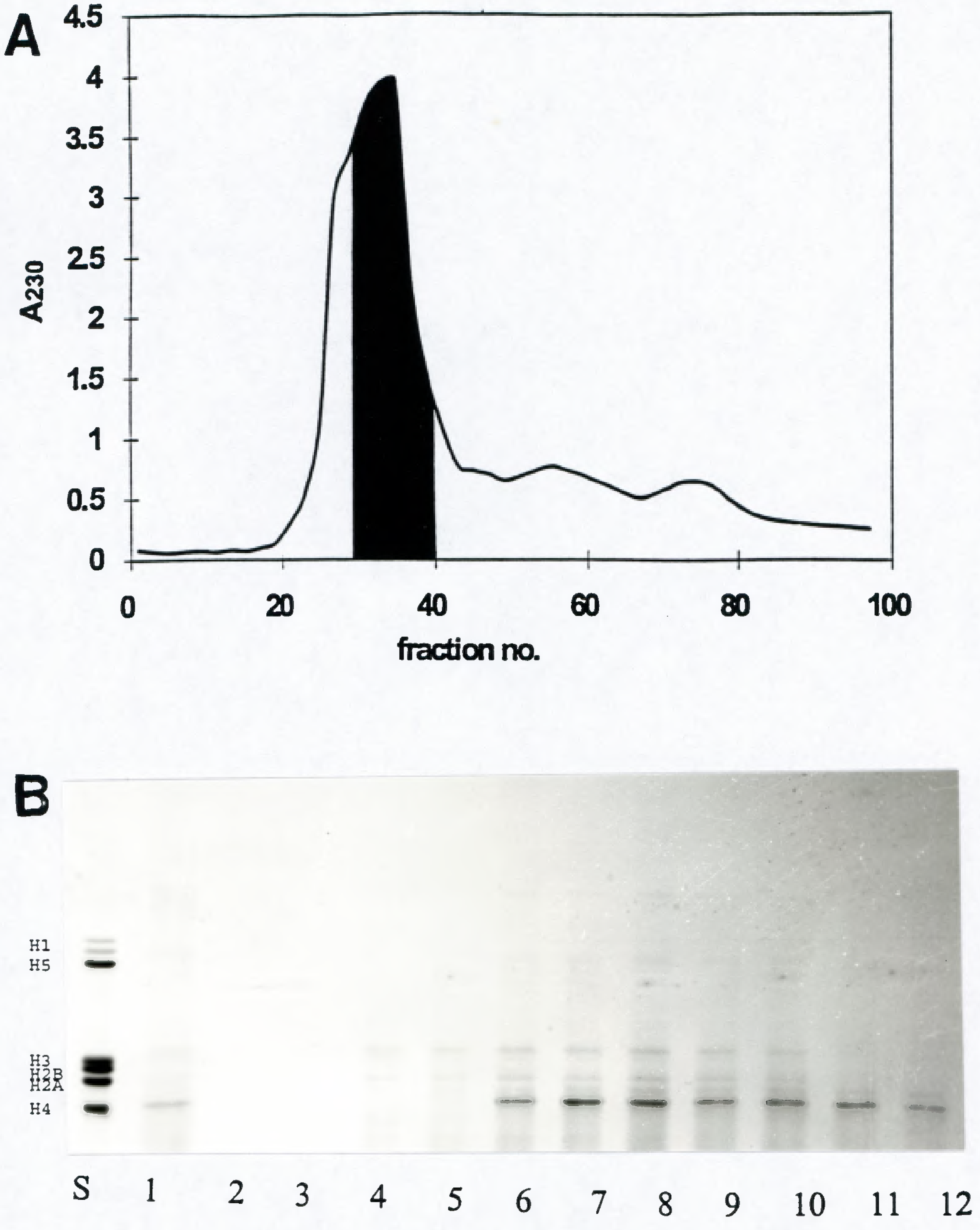

Figure 3.5 A: Chromatogram of soluble proteins eluted from the DEAE-52 column in 50 mM NaCl, 10 mM Tris-HCl pH 8.5. B: SDS-PAGE analysis of selected fractions. Lane 1: heat treated fraction before loading on the column, lanes 2-12 correspond to every second fraction from fraction 20-40. Fractions 28-40 were pooled and lyophilised. Standard (S) as for figure 3.1.

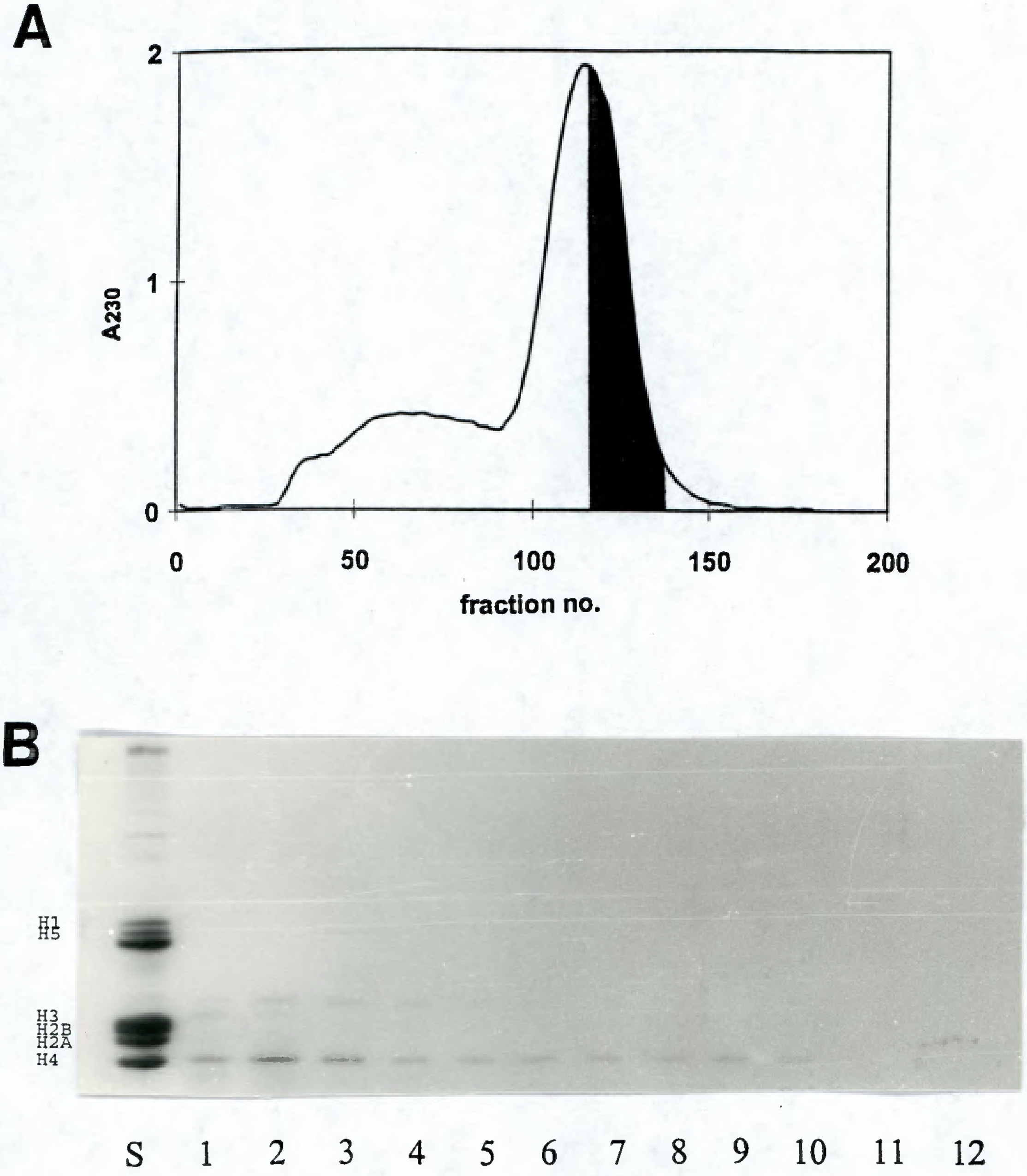

Figure 3.6 A: Elution profile of Sephadex G-50 chromatography of pooled fractions from figure 3.5. The proteins were eluted with 20 mM HCl. B: SDS-PAGE of fractions under the major peak. The samples were precipitated using 13 % TCA before SDS-PAGE analysis. Lane 1-12 correspond to every fourth fraction from fraction 108-152. Fractions under the shaded part of the peak representing fractions 120-144 were pooled. Standard (S) as for figure 3.1.

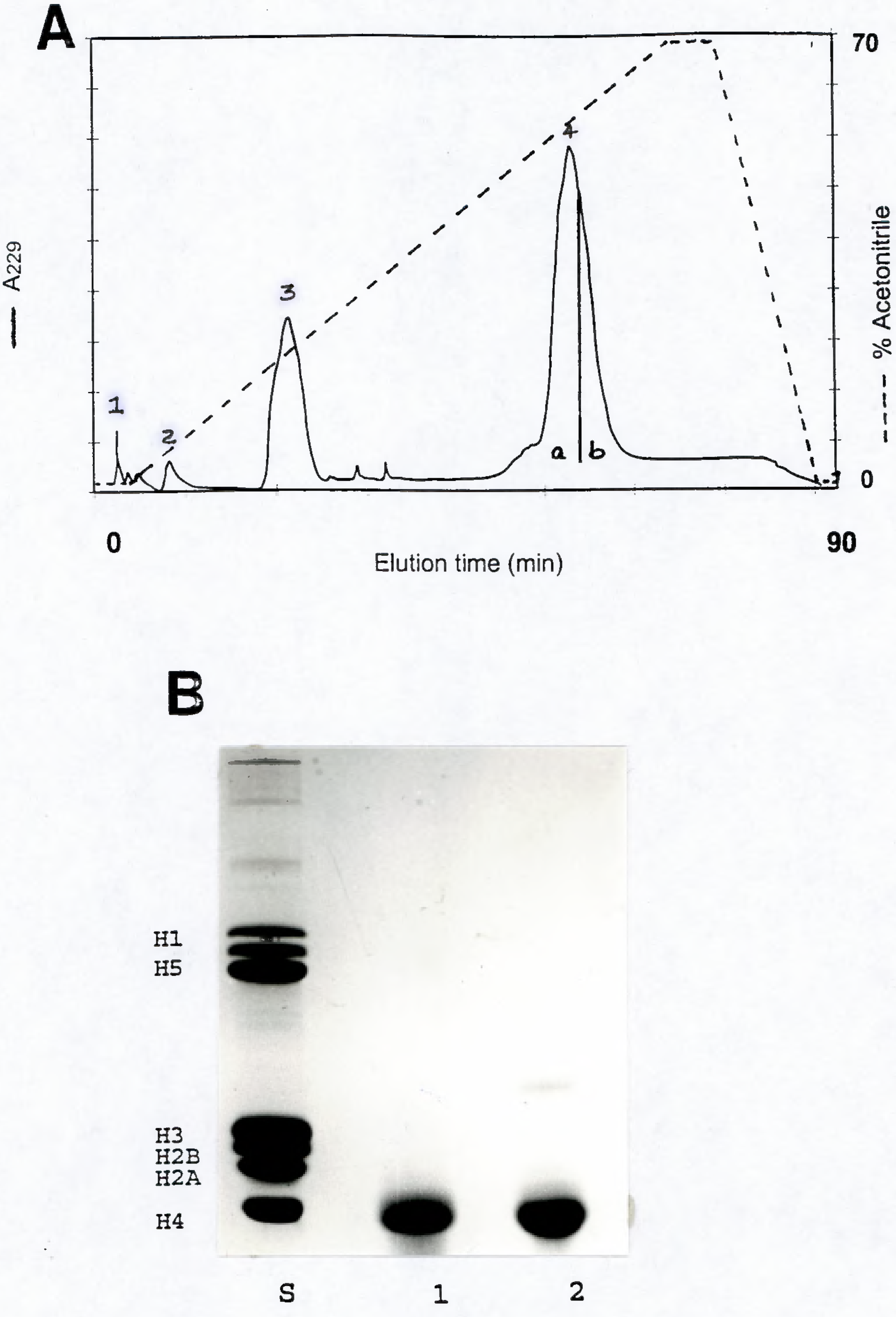

Figure 3.7 A: HPLC purification of HSP 12. B: SDS-PAGE of pure protein (lane 1) eluted in peak 4a, lane 2: contaminated HSP 12 eluted in peak 4b. Standard (S) as for figure 3.1.

3.3 Amino Acid Analysis

2 nmoles of the protein of interest were acid hydrolysed (section 2.7.1) and the hydrolysate subjected to amino acid analysis.

Table 1 shows the amino acid content (mole %) of the purified protein. The isolated protein was very rich in hydrophilic amino acids (typical of LEA proteins) particularly glutamate, aspartate, lysine, alanine and glycine. These five most abundant amino acids represented 66 % of the total composition with hydrophilic amino acids representing 59 % of the total residues present.

Compared to the pea seed p11 protein (Russouw et al., 1995), the amino acid content of the yeast 11 kDa protein showed slight differences. For example, glutamic acid was 17.2 % for the yeast 11 kDa protein and 25.0 % for the p11 protein, lysine was 12.7 % and 8.4 % for the yeast protein and the p11 protein respectively and glycine was 12.0 % for the yeast 11 kDa protein and 17.3 % for the p11 protein. The overall compositions of the two proteins were remarkably similar if their compositions were compared after adding the moles % of similar amino acids, (figure 3.8). Thus alanine + glycine together accounted for 24 % of the residues in both proteins, serine + threonine constituted 11.7 % in the yeast protein and 9.9 % in the pea seed protein. The acidic amino acids, aspartate + glutamate (including asparagine and glutamine which are deaminated during acid hydrolysis) were 29.5 % and 35.9 % for the yeast protein and the p11 protein respectively; similarly the basic amino acids arginine + lysine + histidine were 19.9 % and 15.1 % and the hydrophobic amino acids isoleucine + leucine + methionine + valine + phenylalanine + tyrosine were 15.9 % and 13.3 %. These results show a remarkable similarity between the amino acid compositions of the 11 kDa yeast protein and the p11 LEA group I protein.

Table 1. Amino acid content (mole %) of the protein purified by HPLC chromatography (A) and determined from the gene sequence (B).

Residue	Moles %	
	A	B
glutamic acid (E)	17.2	15.6
lysine (K)	12.7	13.8
aspartic acid (D)	12.3	13.8
glycine (G)	12.0	11.9
alanine (A)	11.8	11.9
serine (S)	6.9	7.3
valine (V)	6.5	6.4
threonine (T)	3.8	1.8
leucine (L)	3.6	2.8
arginine (R)	3.4	2.8
histidine (H)	2.8	1.8
tyrosine (Y)	2.7	3.7
phenylalanine (F)	1.9	1.8
isoleucine (I)	1.6	0.9
methionine (M)	0.8	1.8

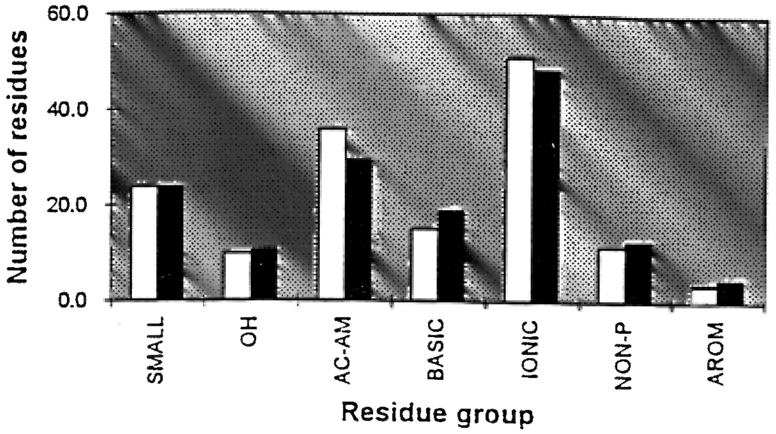

Figure 3.8. Comparative amino acid content (moles %) of the purified yeast protein (solid bars) with the pea seed LEA group I protein (clear bars) after grouping together similar amino acids. Grouped together were: small amino acids: A, G; hydroxyl containing (OH): S, T; acidic + amide (Ac-Am): D, E, N, Q; basic: H, K, R; ionic: D, E, H, K, R; non polar (Non-p): I, L, M, V; aromatic (Arom): F, W, Y.

3.4 Digestion Studies and Sequence Analysis

When the purified protein was subjected to gas phase sequencing (section 2.7.3), no sequence was obtained indicating that the protein, in common with the pea seed p11 protein and most plant proteins, was blocked at the N-terminus. The protein was therefore digested with three different agents: trypsin, which cleaves polypeptides on the carboxyl side of lysine and arginine residues; chymotrypsin, which cleaves on the carboxyl side of aromatic residues; cyanogen bromide which cleaves on the carboxyl side of methionine residues. Trypsin and chymotrypsin yielded many small fragments whereas cyanogen bromide gave two fragments (figure 3.9) and was therefore selected for use. One fragment migrated slightly faster than the parent product while the other one migrated very much faster than the parent protein. It was postulated that one fragment contained the blocked N-terminus and the other fragment contained the C-terminus together with a new N-terminus.

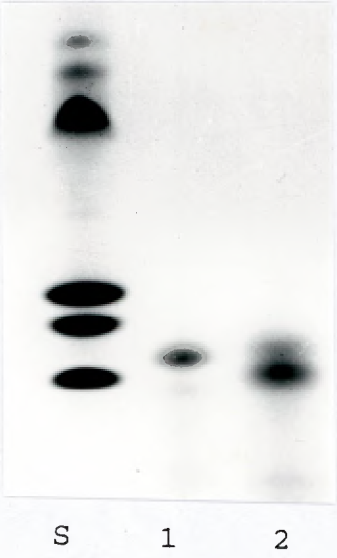

Figure 3.9. Tricine-SDS-PAGE analysis of cyanogen bromide (CNBr) digested HSP 12 protein. Lane 1: 5 μg of pure HSP 12; lane 2: 15 μg HSP 12 digested with CNBr. Standard (S) as for figure 3.1.

The entire mixture was therefore subjected to gas phase sequencing (10 cycles). A unique sequence: GAAKSKLNDA was obtained (figure 3.10). This sequence, with an N-terminal methionine, was compared with that of known proteins in the Stanford databank. Only one protein, HSP 12, was identified to be 100 % homologous with this entire sequence without any introduction of gaps. The HSP 12 amino acid composition determined from the gene sequence showed little difference with that obtained for the purified protein (table 1), confirming that the purified protein was indeed HSP 12. Though HSP 12 was previously identified as a heat shock protein as a result of increased HSP 12 mRNA in yeast cultures shifted from 30 °C to 37 °C (Praekelt and Meacock, 1990) results obtained in this report indicate that this protein is also related to LEA-like proteins. HSP 12 also showed a distinct resemblance with wheat group I LEA Em protein (figure 10b) in that charged and hydrophilic amino acids are distributed throughout the primary structure of both proteins and there are no clusters of greater than two consecutive hydrophobic amino acids occurring.

3.5 Hydropathy plots

The amino acid sequence obtained for yeast HSP 12 was used to generate a hydropathy plot (figure 3.11) according to the method of Kyte and Doolittle (1982) using a 4-amino acid window. This plot, like those of the majority of LEA proteins, has a profile with a pronounced hydrophilicity (negative values) throughout the primary structure. The average hydrophilicity value was found to be - 0.79. Nine peaks displayed positive hydrophathy though there was paucity of hydrophobic residues. There appeared to be a regular spacing pattern between these hydrophobic regions which may attribute to the function of this protein. The region in HSP 12 with the most sustained hydrophobicity is due to the presence of two valine residues and a phenylalanine residue at positions 52 to 56. Other regions that displayed positive hydrophobic values contain adjacent or nearly adjacent hydrophobic amino acids, for example leucine-alanine at positions 74 and 75. As a result of the few hydrophobic residues, that would require internalisation to minimise their interaction with solvent, HSP 12 could be postulated to exist in a random coil. This proposed conformation is presumably the reason why HSP 12 is soluble at 80 °C.

A

```
1     MSDAGRKGFGEKASEALKPDSQKSYAEQGKEYITDKADKVAGKVQPEDNK

51    GVFQGVHDSAEKGKDNAEGQGESLADQARDYMGAAKSKLNDAVEYVSGR

100   VHGEEDPTKK
```

B

```
               □-  ++   -+ □-  + -□□+□  -□ +-  □-+ -+   + □ --□+
HSP 12  1     MSDAGRKGFGEKASEALKPDSQKSYAEQGKEYITDKADKVAGKVQPEDNK
Em      1     MASGQQERSQLDRKAREGETVVPGGTGGKSLEAQENLAEGRSRGGQTRRE
                □ □□-+□□ -++ +- -□     □  +□ - □-□  - +□+  □□++-

                 □  + □ -+ +-□ - □ -□  -□ +-     +□+ □-  -  □ +
HSP 12  51    GVFQGVHDSAEKGKDNAEGQGESLADQARDYMGAAKSKLNDAVEYVSGR
Em      51    QMGEEGYSQMGRKGGLSTNDESGGDRAAREGIDIDESKFKTKS
              □  --  □□  ++   □□□--□  -+  +-  - --□+ + +□

               + --- □++
HSP 12  100   VHGEEDPTKK
```

Figure 3.10 A: The amino acid sequence of the HSP 12 protein. The underlined sequence represents the sequence of the CNBr-digest fragment that was sequenced. B: Comparison of hydrophilic amino acid distributions along the yeast HSP 12 and wheat Em protein sequences. Acidic (D, E) and basic (H, K, R) residues are denoted by - and + symbols above and below the sequences; uncharged polar residues (N, Q, S, T) are similarly denoted by □ symbols.

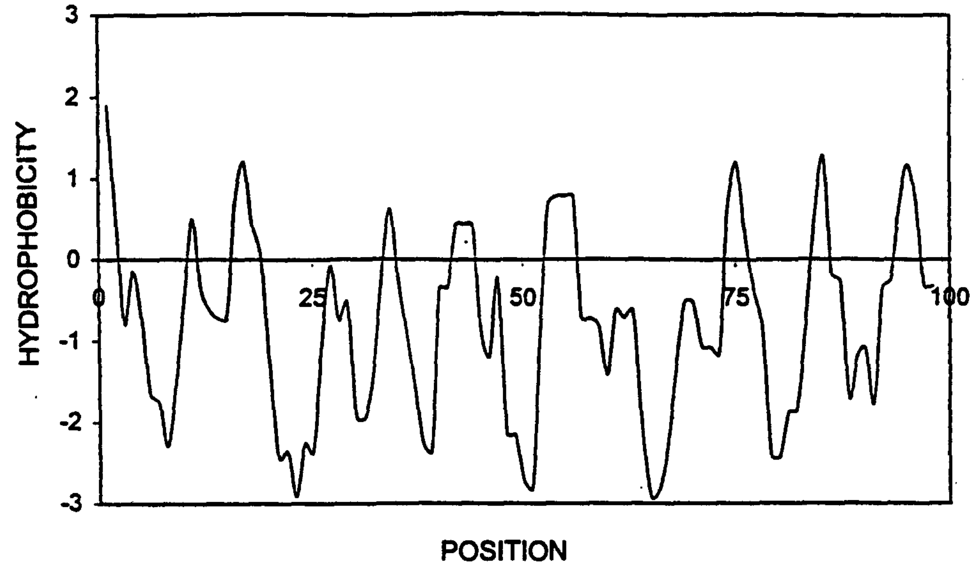

Figure 3.11. Hydropathy plot of HSP 12. Hydropathy plot is according to Kyte and Doolittle (1982) using a window of 4 residues. Negative values indicate hydrophilicity.

CHAPTER 4

HSP 12 was identified as a heat shock protein as mRNA specific for this protein increased in concentration after the growth temperature of the yeast was raised from 30 °C to 37 °C (Praekelt and Meacock, 1990). However the results obtained so far in this study suggested that HSP 12 has a number of LEA-like properties. This chapter presents and discusses results obtained in attempting to resolve this controversy.

4.1 Investigation of HSP 12 protein accumulation during the yeast growth cycle at 30 °C and 37 °C

HSP 12 content was determined in yeast grown at 30 °C and compared with the concentration of this protein in yeast grown at 37 °C. The growth cycle of yeast grown in YPD medium at 30 °C and 37 °C is shown in figure 4.1a. Growth at 37 °C was found to be slightly slower than that at 30 °C (the doubling time increased from 110 mins to 150 mins). To investigate HSP 12 protein accumulation during the growth cycle of yeast at 30 °C and 37 °C, yeast proteins were extracted at various stages of the growth cycle and analysed by SDS-PAGE. Equal loading of soluble proteins on the gel was ensured by extracting proteins from the same number of cells (indicated by the optical density of the cell culture at 600 nm) taken at different stages of the yeast growth cycle. The electrophoretogram of total soluble proteins in yeast grown at 30 °C and at 37 °C is shown in figure 4.1b. HSP 12 was found to be present in samples taken during the log phase and increased in concentration during the early stationary phase in yeast grown at 30 °C. However continued growth during the stationary phase did not result in any significant change in the HSP 12 concentration. The stationary phase of the growth cycle is a time when nutrients reach their limiting concentrations and the yeast prepares to reduce its water content and sporulate. This stage could be considered equivalent to plant seed maturation - a time when plant seeds desiccate and LEA proteins are synthesised. These results therefore showed a correlation in the timing of HSP 12 accumulation with that of LEA proteins accumulating in seeds suggesting that HSP 12 might play role during desiccation as hypothesised for LEA proteins.

Throughout the growth cycle, the HSP 12 concentration in yeast grown at 37 °C was much lower than in yeast grown at 30 °C indicating that production of HSP 12 was not induced by elevated temperatures. To confirm that the Coomassie stained band attributed to HSP 12 was indeed so, the relative amount of HSP 12 present in the various preparations was determined immunologically as shown in figure 4.1b (bottom panel). The blot also showed that yeasts grown at the elevated temperature of 37 °C had markedly lower concentrations of HSP 12 than those grown at 30 °C. The fact that its concentration remained low throughout the growth cycle at 37 °C meant that either HSP 12 biosynthesis was reduced at the higher temperature or that it was degraded more rapidly. Although it has been reported that yeast grown at 37 °C exhibit increased proteolytic activity (Gross, 1986), the protein content of the soluble fraction of yeast grown at 30 °C showed no differences at equivalent stages in growth cycle from that of yeast grown at 37 °C, indicating that the reduced HSP 12 concentration observed at 37 °C was probably not due to degradation. Moreover there was no smearing of bands as would occur if proteolytic was markedly increased.

Sequence studies showed very little homology between HSP 12 and known heat shock proteins. A computer analysis with the Swiss Prot data bank using the Fasta program of GCG showed the best homology to be with a white colony (Whl1) protein from *Candida albicans* strain WO-1 where 47.5 % homology was found over a 59 amino acid region. This protein was found to be maximally produced on entry into stationary phase and is involved in the conversion of colonies from opaque to white colonies. The second best homology was found to be with the LEA D-29 from *Gossipium hirsutum* with 33 % homology over a 97 amino acid region. In order to determine whether the homology with the Whl1 protein was significant, the HSP 12 sequence was shuffled ten times and compared to that of the Whl1 protein.The average homology was found to be 30.7 % with a standard deviation of 5.9 %. The homology between the Whl1 protein and the HSP 12 protein exceeds that of the scrambled similarity by three standard deviations and would thus appear to be significant. It is unlikely that the 33 % homology between the HSP 12 protein and LEA D-29 is significant. The evidence for HSP 12 protein being a heat shock protein is, therefore, not convincing despite the known limited sequence conservation of small heat shock proteins.

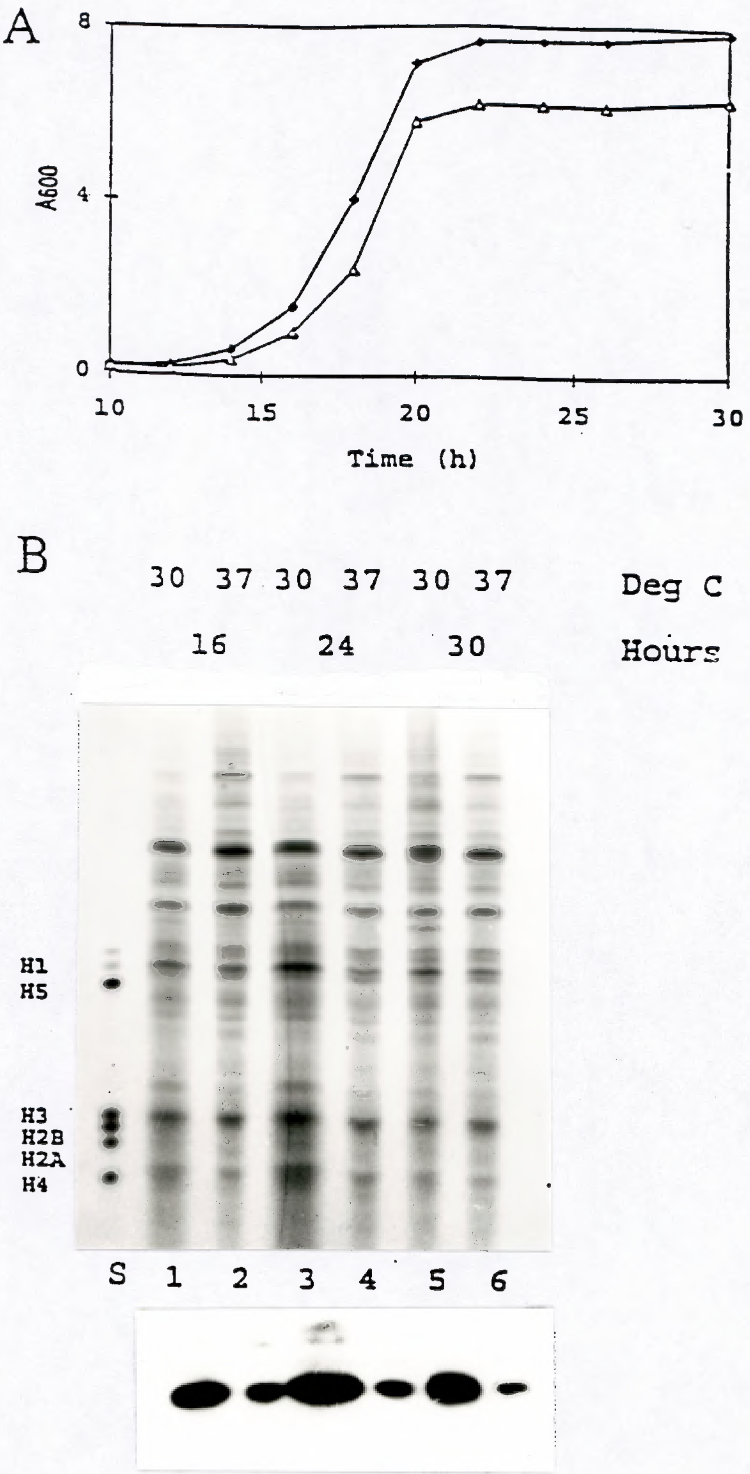

Figure 4.1. A: Growth of yeast in YPD medium at 30 °C (♦–♦) and at 37 °C (Δ–Δ). Growth was monitored by determination of the absorbance of the culture at 600 nm. B (top panel): SDS-PAGE of proteins from yeast grown at 30 °C (lanes 1,3 and 5) and at 37 °C (lanes 2,4, and 6). Cells were harvested after 16 h (lanes 1 and 2), 24 h (lanes 3 and 4) and 30 h (lanes 5 and 6). Standard (S) as for figure 3.1. Bottom panel: western blot using an anti-HSP 12 antibody of the HSP 12 portion of the gel.

4.2 The effect of osmolytes on growth of yeast and production of HSP 12

LEA proteins accumulate as seeds mature and desiccate. During this process, the osmotic strength of millieu increases.

Since the results obtained so far suggested that HSP 12 protein was related to LEA group I proteins rather than to heat shock proteins, growth conditions known to stimulate an increase in the biosynthesis of LEA proteins in plants were used with yeast to see whether they would also increase the HSP 12 content of yeast. The relationship between the production of HSP 12 and the presence of osmolytes in the yeast growth medium was investigated. A non-ionic osmolyte, mannitol, and an ionic salt, sodium chloride, were used in this study.

Mannitol was added to the yeast growth medium to final concentrations of 0.2 M, 0.4 M and 0.8 M. Compared with the control (yeast grown in YPD medium without the addition of mannitol), yeast grown in the presence of mannitol showed a slight reduction in growth rate (figure 4.2a). This reduced growth rate was shown to be inversely related to the concentration of mannitol present. Aliquots of the cell culture taken after 24 hrs and 30 hrs, representing the early stationary phase and late stationary phase respectively, were extracted. These extracts were electrophoresed on SDS-PAGE and the gel scanned to quantitate the amount of HSP 12 present. Figure 4.2b showed that the HSP 12 content increased during the stationary phase and that its biosynthesis was enhanced by increasing the concentration of mannitol present.

When sodium chloride was added to the standard growth medium in the same concentrations used for mannitol, a marked reduction in growth rate was observed (figure 4.3a). In the presence of 0.8 M NaCl, growth was completely inhibited suggesting that the yeast might have lost its viability. Varela and associates (1992) have reported that a laboratory strain of *S. cerevisiae*: YT6-2-1 L rDNA: pMIRY2 (cir^{o}, **a**, his4-519, can1) to also lose its viability upon reduction of cellular water as a result of increased salinity. The extent of this response was noted to vary with different strains.

Our results (compare fig. 4.2 and fig. 4.3) showed that yeast grown in the presence of sodium chloride has substantially greater HSP 12 protein content than yeast grown in the presence of mannitol. This was ascribed to greater sequestration of water molecules by the Na^+ and Cl^- ions which resulted in an increased osmotic shock due to dehydration although a salt toxicity effect cannot be ruled out. Mannitol only interacts with water through hydrogen bonding with a bond energy of 20 kJ as opposed to ionic bonding which has a bond energy of 250 kJ. Thus it, too, causes cellular dehydration, though not to the same extent as does sodium chloride. The results of HSP 12 measurement accord with these effects (compare fig 4.2 and 4.3).

It is of interest to note that the reduction in growth paralleled the accumulation of HSP 12 (figure 4.3b). Though the function of HSP 12 is not yet known, these results indicate that it might play a role in the protection of the yeast cells against osmotic shock-induced damage. The protective function of the HSP 12 protein was supported by the observation that cells that have been grown in medium containing 0.8 M NaCl regained their viability and showed a normal growth pattern when re-incubated in fresh standard medium (figure 4.3c).

To observe the effect of these two osmolytes on the growth rate of yeast, the growth curves of yeast grown in the presence of 0.8 M mannitol and 0.2 M NaCl (since the growth rate with higher concentration of NaCl was too low) were compared with those in control medium, (figure 4.4a). The growth rate of yeast at 30 °C in medium containing 0.8 M mannitol was slightly slower than that grown in control medium with an increase in doubling time from 110 min to 160 min. Addition of 0.2 M NaCl to the standard medium resulted in markedly reduced growth rate, with the doubling time prolonged to 290 min.

HSP 12 production under these conditions was determined by scanning the HSP 12 band present after SDS-PAGE of samples taken after 16, 24 and 30 hours, representing log phase, early stationary phase and late stationary phase respectively. It was found that HSP 12 synthesis was slightly repressed during the log phase in media containing osmolytes but was enhanced during the stationary phase, as shown in figure 4.4b. The degree of enhancement was approximately 2-fold for growth in 0.2 M NaCl and approximately 3-fold in 0.8 M mannitol.

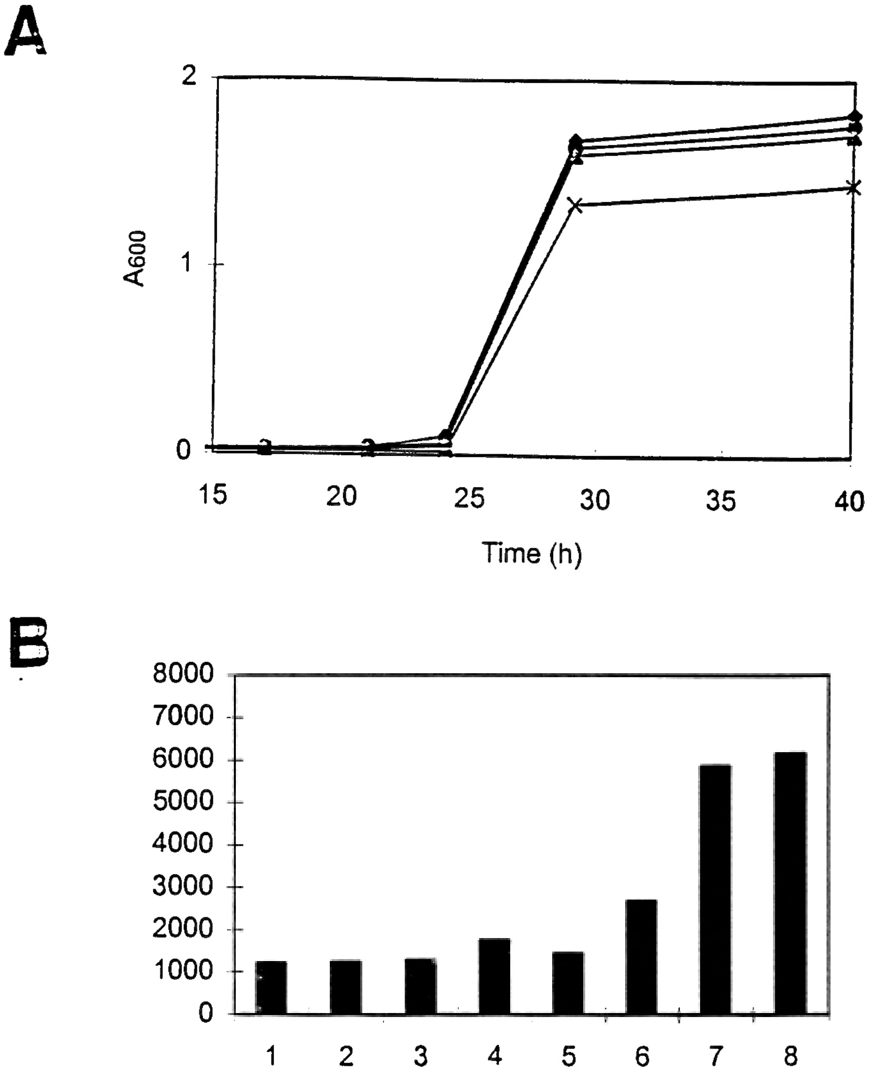

Figure 4.2. A: Growth of yeast in YPD medium at 30 °C containing 0.2 M (●–●), 0.4 M (▲–▲) or 0.8 M (× – ×) mannitol compared with the control (♦–♦). B: HSP 12 content of yeast grown at 30 °C in control medium (lanes 1 and 5), in the presence of 0.2 M mannitol (lanes 2 and 6), 0.4 M mannitol (lanes 3 and 7) or 0.8 M mannitol (lanes 4 and 8). HSP 12 content (measured in arbitrary units from the densitometer) was determined by scanning the Coomassie stained gels of the heat soluble proteins present. Yeast cells were harvested after 24 h (lanes 1 to 4) and 30 h (lanes 5 to 8).

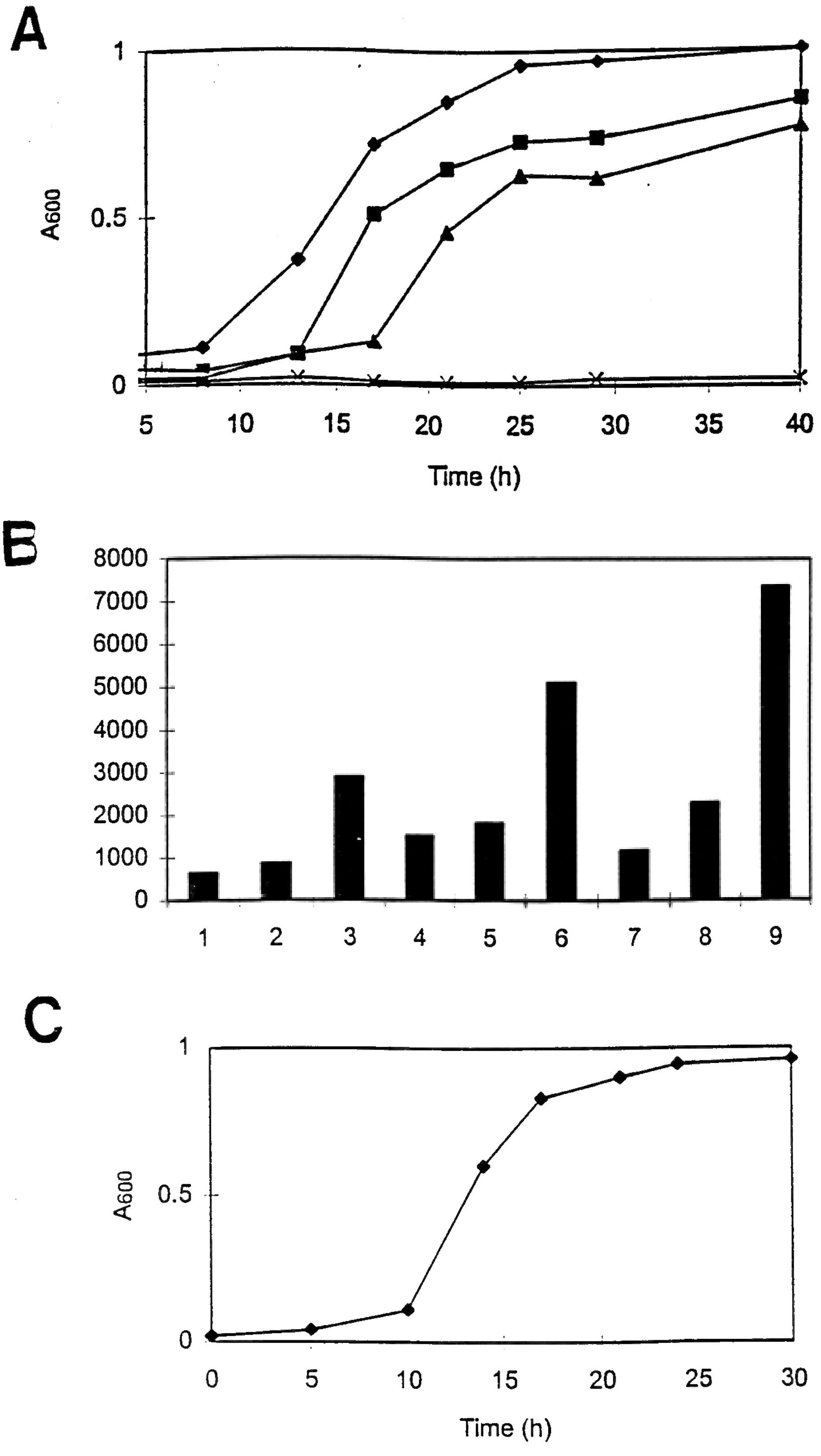

Figure 4.3. A: Growth of yeast in YPD medium at 30 °C containing 0.2 M (■ – ■), 0.4 M (▲–▲) or 0.8 M (× – ×) sodium chloride compared with the control, with no sodium chloride added (♦–♦). B: HSP 12 content of yeast grown at 30 °C in control medium (lanes 1, 4 and 7), in the presence of 0.2 M NaCl (lanes 2, 5 and 8) or 0.4 M NaCl (lanes 3, 6 and 9). HSP 12 content (measured in arbitrary units from the densitometer) was determined by scanning the Coomassie stained gels of heat soluble proteins. Yeast cells were harvested after 16 h (lanes 1, 4 and 7), 24 h (lanes 2, 5 and 8) and 30 h (lanes 3, 6 and 9). C: Growth of yeast harvested from growth medium containing 0.8 M sodium chloride and re-grown in the control medium after centrifugation and resuspension.

To confirm the identify of the band ascribed to HSP 12 in SDS-PAGE, samples representing yeast grown to late stationary phase at 30 °C in standard medium and in media containing 0.2 M NaCl and 0.8 M mannitol, were electrophoresed and probed with the anti-HSP 12 antibody. The results (figure 4.4c) show that the band was correctly ascribed to HSP 12 and confirmed the accumulation of HSP 12 caused by the two osmolytes.

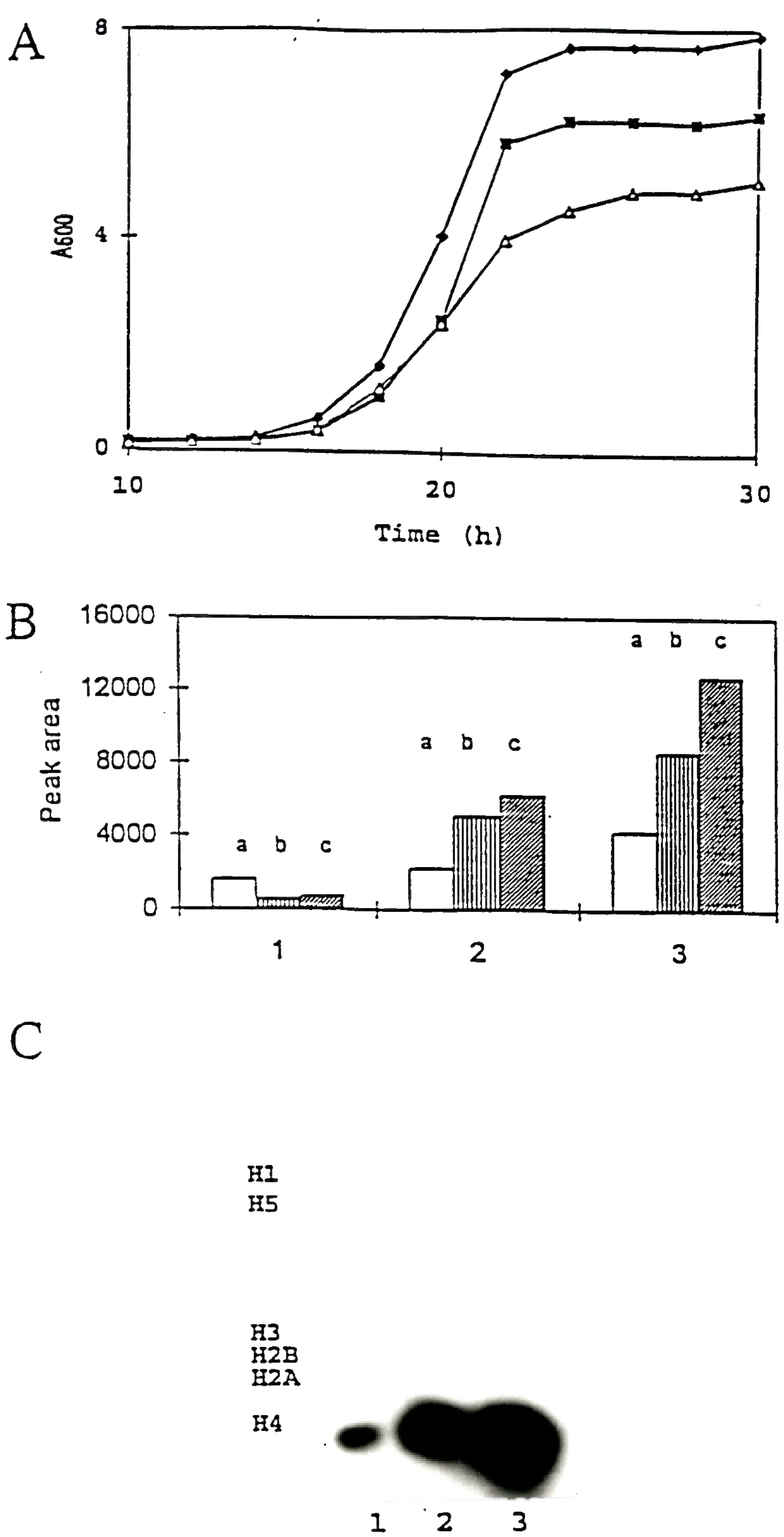

Figure 4.4. A: Growth of yeast in YPD medium at 30 °C containing 0.8 M mannitol (■ – ■) or 0.2 M NaCl (Δ–Δ) compared with that without the addition of osmolytes (♦–♦) as the control. B: HSP 12 content of yeast grown at 30 °C in YPD medium alone (a) or containing 0.2 M NaCl (b) or 0.8 M mannitol (c). Yeast cells were harvested after 16 h (1), 24 h (2) and 30 h (3). C: Western blot using an anti HSP 12 antibody of heat soluble proteins extracted from yeast grown at 30 °C in YPD medium alone (lane 1) or containing 0.2 M NaCl (lane 2) or 0.8 M mannitol (lane 3). The positions of the histone standards are indicated on the left hand side.

CHAPTER 5

5.1 The role of HSP 12

Though LEA proteins have been speculated to protect intracellular organelles and membranes against desiccation-induced damage, no work has been carried out to prove this hypothesis.

Work carried out in our laboratory by Russouw and associates was the first demonstration of a biological function of a LEA protein. The p11 LEA group I protein isolated from pea (*Pisum sativum*) seed axes was incubated with alcohol dehydrogenase (ADH) and myokinase at elevated temperatures of 50 °C and 60 °C. These enzymes were both protected against thermal denaturation in the presence of p11 protein with complete protection observed at p11 : target protein ratios approximately equivalent to the inverse of their mass ratios. The requirements for this biological function was that the protein be present in substantial amounts and the p11 protein adheres to this pre-requisite. As a result of this property, the p11 protein was postulated to carry out its biological function by forming a hydrophilic net around the target protein thereby substituting for water. Though this work is not yet published, a manuscript has been submitted to the Plant Science journal.

Since results obtained in this study classify HSP 12 as a LEA-like protein, similar functional assay was carried out with this protein. These are preliminary studies on the functional studies of LEA proteins that I will continue with as part of my doctoral studies. Because of time constraints, not enough controls were done.

To study the role of HSP 12 as a thermo-protectant, the protein was incubated with yeast alcohol dehydrogenase (ADH) to see if it would inhibit thermal denaturation of the enzyme. Figure 5.1 showed that incubation of ADH alone at 45 °C resulted in 78 % loss of enzymatic activity after 60 min.

Incubation of ADH under the same conditions in the presence of 30 mols/mol or 60 mols/mol HSP 12 resulted in the loss of only 65 % and 35 % enzymatic activity, respectively. This showed that HSP 12 protected ADH against thermal denaturation in a concentration-dependent manner. HSP 12 did not have any effect on specific activity of ADH (not shown).

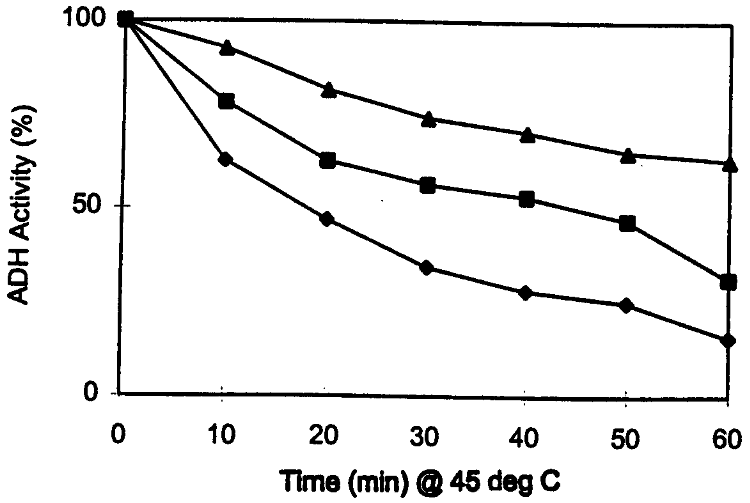

Figure 5.1. Percentage enzymatic activity of ADH versus time when incubated on its own at 45 °C (◆–◆) or together with 30 mols/mol HSP 12 (■–■) or 60 mols/mol HSP 12 (▲–▲).

Increase in the incubation temperature resulted in an increased rate of loss of ADH activity (figures 5.2a and b). Incubation of ADH at 50 °C after 60 min resulted in approximately 81 % loss of enzymatic activity (figure 5.2a) and at 55 °C with a complete loss of activity observed after 40 min (figure 5.2b). Incubation together with 60 mols/mol HSP 12 resulted in a decreased rate of loss of enzymatic activity both at 50 °C and 55 °C.

When ADH was incubated for 40 min at 55 °C in the presence of increasing concentrations of HSP 12 (figure 5.2c), increased protection against thermal denaturation of ADH was observed as a function of molar HSP 12 : ADH ratio. A 13.3 : 1 HSP 12 : ADH ratio corresponds to equivalent masses of the two proteins assuming molecular sizes of 11 kDa and 146.8 kDa respectively. 50 % ADH activity retention was observed to occur at a ratio of approximately 68 : 1. Unlike, the p11 protein, HSP 12 did not confer complete retention of enzymatic activity, but approximately 88 % ADH activity was retained. The fact that large quantities of HSP 12 are required to protect ADH might confirm the postulate that HSP 12 functions by forming a shell around the target protein.

Since small hydrophilic proteins were thought to be involved in protecting cells against desiccation-induced damage, for example thermal denaturation of target proteins, hydrophilic poly-amino acids poly-serine and poly-asparagine with molecular weight of 11.75 kDa and 10.4 kDa respectively were used in place of the HSP 12 protein. Incubation of ADH with these low molecular weight poly-amino acids at a 20 : 1 molar ratios showed no retention of enzymatic activity.

Russouw and associates (1998) repeated the experiment with different proteins using the 10 : 1 ratio of the p11 protein. No protection against thermal denaturation of ADH was observed with myoglobin (17 kDa), carbonic anhydrate (29 kDa) or ovalbumin (45 kDa). In fact, the presence of these proteins was observed to promote denaturation of ADH.

These results indicated that HSP 12 can protect ADH against thermal denaturation. This protection was afforded by interactions between the LEA proteins and ADH that increased the temperature of unfolding of the latter (Russouw et. al., 1998).

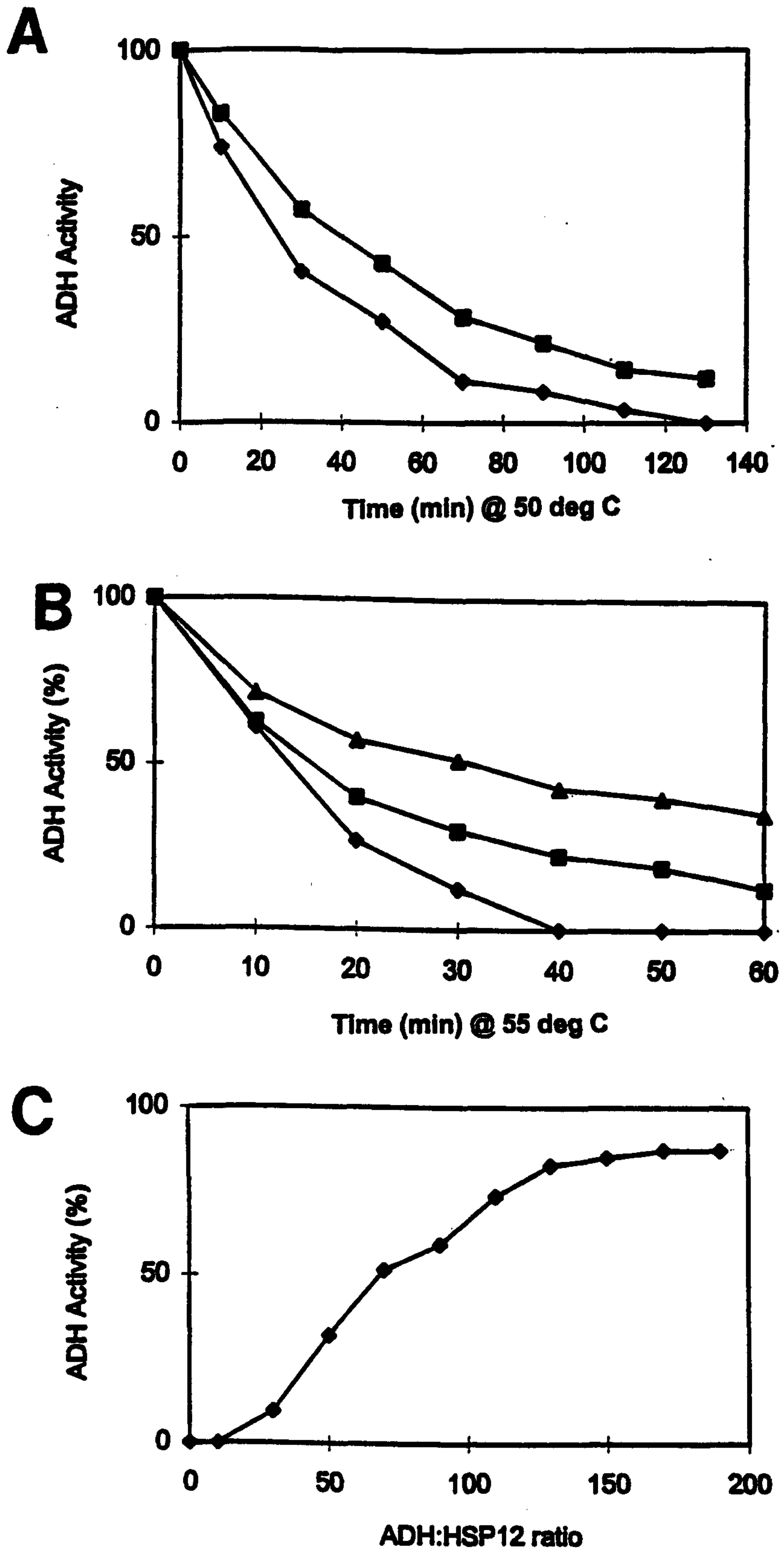

Figure 5.2 A: Percentage enzymatic activity of ADH versus time when incubated on its own at 50 °C (◆–◆) or together with 60 mols/mol HSP 12 (■–■); B: ADH incubation on its own at 55 °C (◆–◆) or together with 60 mols/mol (■–■) or 120 mols/mol (▲–▲) HSP 12; C: Percentage enzymatic activity of ADH measured upon incubation at 55 °C together with various ratios (mols/mol) of HSP 12. The incubation was for 40 mins.

CHAPTER 6
CONCLUSION

Praekelt and Meacock (1990) identified HSP 12 as a heat shock protein as the concentration of HSP 12 mRNA increased in response to a temperature shift of the growth conditions from 30 °C to 37 °C. The results reported here suggest that HSP 12 should be classified as a LEA-like protein rather than a heat shock protein. Two lines of evidence support this hyphothesis.

Fistly, HSP 12 synthesis was not induced in yeast grown at elevated temperatures of 37 °C as would be expected for heat shock proteins. Moreover, HSP 12 had several properties in common with LEA proteins. The concentration of HSP 12 was increased on entry to stationary phase of the growth cycle - a time when nutrients become limiting and the yeast is preparing to reduce its water content and sporulate. This phase is considered equivalent to plant seed maturation, the time when LEA proteins are synthesised. HSP 12 synthesis was also induced by conditions known to trigger the synthesis of LEA proteins. Growing yeast in the presence of the two osmolytes, mannitol and sodium chloride showed a markedly increase in the concentration of HSP 12 compared to the control. Like LEA proteins, HSP 12 was found to be very hydrophilic - a property that accounts for the solubility of this protein at temperatures a high as 80 °C or even 100 °C (a characteristic property of LEA proteins).

Secondly, though HSP 12 was not more homologous to known LEA proteins, this protein showed great similarity in the amino acid composition with the group I LEA proteins. Comparison of the amino acid composition of HSP 12 and the pea group I LEA p11 protein after grouping together similar amino acids showed that the overall compositions were remarkably similar.

A possible biological function of HSP 12 was identified. Functional studies showed that this protein inhibits thermal denaturation of an enzyme, a functional property also identified for the pea group I LEA p11 protein. These studies are to be continued.

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