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**ASP 53, A 53 kDa CUPIN-CONTAINING PROTEIN
FROM *ACACIA ERIOLOBA* SEEDS THAT PROTECTS
PROTEINS AGAINST THERMAL DENATURATION**

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Preface

The experimental work presented in this thesis was conducted under the supervision of Associate Professors George G. Lindsey and W. Brandt and Professor Jill M. Farrant (Department of Molecular and Cellular Biology, University of Cape Town). It is hereby declared that this thesis, submitted for the degree of Doctor of Philosophy in Biochemistry at the University of Cape Town, is the result of my own investigation, except where the work of others is acknowledged.

Linda Mtwisha
November 2004

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Abstract

Acacia erioloba E.Mey, the Camel Thorn tree, grows in the semi-arid areas of Southern Africa. Since the seeds are exposed to extremely harsh environmental conditions including ground temperatures exceeding 70 °C prior to germination upon the onset of rainy seasons, the presence of unusual LEA or LEA-like proteins was investigated. ASP 53, a 53 kDa heat soluble protein was identified as the most abundant seed protein present. Amino acid analysis of ASP 53 showed that, despite being rich in hydrophilic amino acids, this glycosylated protein also contained significant quantities of hydrophobic amino acids. CD analysis demonstrated the presence of defined secondary structure of the protein. ASP 53 was rapidly degraded during germination and this coincided with the loss of desiccation tolerance in germinating seedlings. Immunocytochemistry demonstrated the presence of ASP 53 in the vacuoles and cell walls in mature seeds with the cell wall content decreasing upon germination. ASP 53 was found to inhibit all three stages of protein thermal denaturation. ASP 53 significantly decreased the rate of loss of alcohol dehydrogenase activity at 55 °C, decreased the rate of temperature-dependent loss of secondary structure of haemoglobin and completely inhibited the temperature-dependent aggregation of egg white protein. Since ASP 53 was not amenable to Edman degradation, the sequences of two tryptic peptides were obtained by PSD MALDI-TOF mass spectrometry allowing the preparation of a degenerate primer. The *ASP53* gene sequence was determined by RT-PCR and 5' RACE. Homology studies identified two cupin motifs, and only seed storage proteins exhibited close sequence similarity.

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Abbreviations

µg	Microgram(s)
µl	Microliter
µM	Micromolar
2-D	Two dimensional
A ₂₃₀	Absorbance reading at 230 nm
A ₃₅₀	Absorbance reading at 350 nm
ABA	Abscisic acid
ACN	Acetonitrile
ATP	Adenosine triphosphate
bp	Base pair(s)
cDNA	Complementary DNA
cm	Centimeter
CNBr	Cyanogen Bromide
dCTP	Deoxycytosine triphosphate
dNTP	Deoxyribonucleotide triphosphate
oligo dT	Oligonucleotide Deoxythymine
DTT	Dithiothreitol (1-threo-1,4-dimercapto-2,3 butanediol)
EDTA	Ethylenediaminetetra-acetic acid
EtBr	Ethidium Bromide
g	Gram
g	Gravity
h	Hours
IPTG	Isopropyl-β-D-thiogalactopyranoside
kDa	kilo Dalton
LB	Luria-Bertani medium
m/z	Mass per charge
min	Minutes
MOPS	Morpholinopropanesulphonic acid
n	Nucleotide(s)
NaAc	Sodium acetate
nm	Nanometer
PEG	Polyethylene glycol
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TAE	Tris-acetate-EDTA
TFA	Trifluoroacetic acid
Tween 20	Polyoxyethylenesorbitan monolaurate
X-gal	5-Bromo-4-chloro-3-indoyl-β-D-galactoside

CHAPTER 1

General Introduction

1.1 THE ROLE OF WATER IN BIOLOGICAL PROCESSES

Water is by far the most important constituent of living organisms and plays an important role in supporting life. It provides the fluid medium that allows diffusion of substrates to the active sites of enzymes, thus promoting reactions to occur. On the other hand, water inhibits deleterious reactions that would otherwise result from interaction of highly reactive molecules, by coating the reactive surfaces of these molecules. Water plays a role in facilitating metabolic reactions, as it is often either a reactant or a by-product of a number of reactions. It provides an essential milieu for the structural organization of cells. Because of its incompressible nature, water fills any space in the cell thereby providing turgor. Water also provides hydrophilic interactions that stabilize the conformation of proteins, nucleic acids and lipid bilayers. However, water can also interact hydrophobically with non-polar molecules by forming a lattice around them. Because of the tendency to form strong intermolecular hydrogen bonds between water molecules, water has an abnormally high boiling point and heat of vaporization. These characteristics help protect organisms from rapid temperature changes and also allow water to remain in the liquid state at physiological temperatures.

1.2 DESICCATION TOLERANCE

Plants being sessile are constantly exposed to adverse environmental conditions, including drought, salinity and extremes of temperatures, all of which result in cellular loss of water. Removal of water from cells has a variety of detrimental effects. The extent of damage incurred from cellular water loss depends on the severity and duration of the imposed stress, plant genotype, plant developmental stage and environmental factors imposing stress. Though most organisms cannot cope with water-deficit stress, some nevertheless can survive by tolerating intense and prolonged desiccation. This indicates that the latter group of organisms has developed mechanisms to survive water-deficit stress enabling the cells to resume operations upon rehydration. This phenomenon is known as desiccation tolerance and is widespread in the plant kingdom, though rarely occurs in the vegetative tissues of higher plants (Labhili *et al.*, 1995). Desiccation tolerance is the ability of cells to withstand stress imposed by almost complete loss of cellular water and of subsequent rehydration (Hoekstra *et al.*, 2001).

Plants, in which the vegetative tissues can withstand the deleterious effects of water-deficit stress and resume normal growth upon rehydration after long periods in a desiccated state, are known as resurrection plants (Gaff, 1977). Even though little is known about the mechanism of desiccation tolerance, resurrection plants are reported to have developed two general strategies that enable them to survive the effects of water-deficit stress. The first involves protection of cellular structures against desiccation-

induced damage, while the second involves the repair of desiccation- and rehydration-induced damage (Buitink *et al.*, 2002). In plants, the repair mechanism is more common in the lower order species (algae, ferns, bryophytes) than in higher plants (Bewley, 1979; Stewart and Bewley, 1982), with higher plants tending to protect cells during drying and relying less on repair upon dehydration. The seeds of angiosperms also utilize subcellular protection and it is believed that they employ similar mechanisms of protection as resurrection plants (Walters *et al.*, 2002). Most resurrection plants identified thus far are small herbs or shrubs, although some like *Xerophyta eglandulosa* (Gaff, 1971) and *Myrothamnus flabellifolius* (Sherwin and Farrant, 1996) can grow up to 1.5 metres.

The survival of most plants depends to a large extent on the ability of seeds they produce to withstand stressful conditions such as desiccation (Swire-Clark and Marcotte, 1999). Unlike plants, seeds undergo desiccation as part of a normal developmental programme on the parent prior to shedding into the environment. During this final developmental stage, known as late embryogenesis, seed embryos mature and undergo desiccation (Gaubier *et al.*, 1993). It has been reported that embryos in desiccation tolerant (orthodox) seeds acquire tolerance to desiccation prior to the onset of desiccation (Sun and Leopold, 1993) and lose it during germination (Berjak *et al.*, 1989; Leprince *et al.*, 1994), although the timing of loss varies among species (Senaratna and McKersie, 1983). Orthodox seeds utilize the protection strategy rather than the repair suggested for some vegetative tissues (Bewley and Oliver, 1992). Since this study is based on understanding the mechanism of tolerance

in an orthodox seed, the rest of this discussion will focus on details of the protection mechanism.

1.3 DAMAGE RESULTING FROM CELLULAR DEHYDRATION

1.3.1 General response to water loss

Removal of water from the cells results in the accumulation of solutes that in turn increase the viscosity of the cytoplasm. Changes in the hydration levels also affect the metabolic status of the cell. Slight desiccation, at a moisture level less than about -1.5 MPa, cause a switch in metabolism resulting in cessation of tissue growth (Hegarty, 1978), change in protein and nucleic acid synthesis patterns (Ingram and Bartels, 1996; Oliver *et al.*, 1998) and production of protectants (Vertucci and Farrant, 1995; Ingram and Bartels, 1996; Oliver *et al.*, 1998). Greater levels of desiccation result in metabolic imbalance caused by various reactions within metabolic pathways reacting differently to low moisture levels. At about -3 MPa, protein synthesis ceases and repair processes become inoperative (Dell'Aquila, 1992), while respiration continues to moisture levels below about -11 MPa (Farrant, 2000; Walters *et al.*, 2001). Continued respiration and light harvesting while other metabolic processes are shut down results in the accumulation of high-energy intermediates that leak out of plastids and mitochondria and form reactive oxygen species (ROS) and free radicals (Leprince *et al.*, 1993; Smirnoff, 1993). Reactions with these molecules result in cell death in desiccation sensitive organisms. The effects of free radicals and ROS on some subcellular structures are discussed below.

1.3.2 Damage to membrane structures

Membrane systems are particularly susceptible to damage induced by water-deficit stress (Crowe *et al.*, 1986; Crowe *et al.*, 1992; Oliver *et al.*, 2002). During desiccation there is an observed increase in production of ROS and free radicals. These free radicals cause extensive peroxidation and de-esterification of membrane lipids, thus decreasing the fluidity of the membrane. This leads to malfunctioning of the membranes and eventually extensive leakage of cytoplasmic components upon rehydration (Sanaratna and McKersie, 1983; Tetteroo *et al.*, 1996).

Loss of membrane structure and subsequently function is also attributed to a process known as “demixing”, a desiccation-induced compaction of molecules causing an altered lipid phase transition (Wolfe, 1987; Crowe *et al.*, 1987; Bryant and Wolfe, 1989). Under hydrated conditions, polar lipids spontaneously align to form micelles or bilayer membranes, depending on the polar head group of the lipid. Mobile acyl chains within bilayers afford considerable fluidity to the structure and enable proteins and other constituents to be inserted. Upon drying, water molecules are removed from adjacent polar head groups resulting in close packing of lipid acyl chains caused by increased Van der Waals interactions (Crowe *et al.*, 1990) resulting in greater membrane rigidity. The nature of this phase transition is determined by the molecular geometry of the membranes and their polar lipid components, the presence of other membrane constituents (proteins and sterols), temperature and the extent of water stress (Bryant and Wolfe, 1989). Two types of phase transitions have been reported: Lamellar liquid crystalline phase to gel phase, which maintains the bilayer configuration, and lamellar liquid crystalline phase to hexagonal phase in which a

non-bilayer structure is formed. The lamellar liquid crystalline phase to gel phase transition is favored by polar lipids with a rectangular-shaped head group such as phosphatidylcholine (Quinn, 1985) and by saturated long chain fatty acids and saturated free fatty acids (McKersie, 1989). This type of phase transition is completely reversible upon rehydration. On the other hand, the formation of hexagonal phases has a deleterious effect, as demixing results in loss of some of the membrane constituents as well as causing complete loss of compartmentation within the cell. These changes result in leaky membranes upon rehydration.

Although membranes might appear to be the primary site of desiccation-induced injury, the unregulated reactions brought about by the presence of free radicals also affect proteins and nucleic acids (Wolff *et al.*, 1986; Dizdaroglu, 1991).

1.3.3 Damage to proteins and nucleic acids

Removal of water from cells alters the structure of macromolecules as the hydrophilic and hydrophobic interactions that stabilize their conformation are weakened or lost (Crowe *et al.*, 1987). Proteins in desiccation sensitive tissues are irreversibly denatured during drying resulting in loss of activity upon rehydration. Enzymes such as alcohol dehydrogenase and lactate dehydrogenase are particularly labile (Prestrelski *et al.*, 1993). On the other hand, DNA is particularly stable (Wayne *et al.*, 1999), and maintains its structure in the absence of water only reversibly unfolding at high temperatures (Bonner and Klibanov, 2000). However, it becomes susceptible to desiccation damage when replicated (Deltour and Jacquard, 1974). Free radicals accumulated during drying have been observed to attack both proteins and nucleic

acids causing permanent damage. The mutagenic capacity of free radicals is due to the direct interaction of hydroxyl radicals with proteins (Wolff *et al.*, 1986) and DNA (Dizdaroglu, 1991). Hydrogen peroxide and superoxide do not directly interact with these macromolecules but instead interact with transition metals to produce the hydroxyl radicals which are responsible for the damage (Wolff *et al.*, 1986; Dizdaroglu, 1991). Hydroxyl radicals damage DNA and proteins through modification of bases/amino acid residues, DNA/protein cross-linking and fragmentation of DNA strands.

1.3 PROTECTION MECHANISMS AGAINST DESICCATION

Desiccation tolerance comprises two strategies that alleviate the effects of water stress: the first involves protection against desiccation-induced damage and is usually put in place before or during desiccation (Walters *et al.*, 2002). It involves the production, *inter alia*, of non-reducing sugars, especially sucrose and members of the raffinose family of oligosaccharides (Blackman *et al.*, 1992; Scott, 2000), stress-induced proteins such as LEA and LEA-like proteins (Bray, 1997), compatible solutes and antioxidants (Leprince *et al.*, 1994). Some of these form part of normal housekeeping metabolism while others are induced by water stress. The second strategy is based on activation of efficient repair mechanisms upon rehydration. Desiccation tolerance in seeds is based largely on the former strategy and hence only this strategy will be discussed.

1.4.1 Minimizing mechanical damage

One of the first obvious signs of water stress in cells is loss of turgor. During desiccation, cells collapse as water is removed (Vertucci and Farrant, 1995). However, it has been observed that desiccation tolerant cells of seeds exhibit minor reduction in volume during drying. This is achieved by filling vacuoles with storage proteins and by accumulating insoluble reserves such as lipids and carbohydrates in the cytoplasm (Vertucci and Farrant, 1995), thereby replacing the space previously occupied by water.

1.4.2 Minimizing metabolic stress

Desiccation tolerant organisms protect themselves against oxidative stress by reducing or shutting down their metabolic activities at low water content, thereby mitigating the consequences of unbalanced metabolism (Pammenter and Berjak, 1999). It has been proposed that coordinated down-regulation of metabolism during drying confers desiccation tolerance in seeds (Farrant *et al.*, 1997, Leprince *et al.*, 2000). During drying, the viscosity of the cytoplasm increases due to the increased concentration of solutes and this also results in a reduction in the metabolic rate.

1.4.3 Antioxidant Defense

Since ROS are natural by-products of metabolism, plants are generally well endowed with antioxidant molecules and scavenging systems (Hendry, 1993) to control the free radicals generated. Antioxidants involved include ascorbic acid, glutathione, carotenoids and enzymes such as catalase, superoxide dismutase, ascorbate peroxidase and glutathione reductase (Oliver and Bewley, 1997). Water-deficit stress,

however, is reported to increase the formation of these reactive oxygen species. Circumstantial evidence shows that there is a correlation between acquired tolerance to oxidative stress and increased antioxidant concentration in resurrection plants (Price and Hendry, 1991). Several studies have shown that tolerance to drying coincides with an increase in antioxidants quantities (Smirnoff, 1993; Farrant, 2000). In orthodox seeds scavenging systems seems to be the efficient system involved in combating the accumulated free radicals. The enzyme scavenging systems are reported to be maximally effective only during the initial stages of drying (Arrigoni *et al.*, 1992). At lower water content the enzymes lose activity and the system becomes ineffective. However, molecular antioxidants such as glutathione, ascorbate and tocopherol take over and play a dominant role in alleviating oxidative stress (Hendry *et al.*, 1992). Compounds such as quinines, polyols, flavonoids, phenolics and carotenoid pigments are also thought to be involved in the antioxidant response during drying (Oliver *et al.*, 2001).

1.4.4 Accumulation of osmolytes

During seed maturation, when seeds undergo programmed desiccation, large amounts of osmolytes are produced. These are thought to protect cells against the deleterious effects of water-deficit stress. In particular, orthodox seeds accumulate large quantities of sucrose often together with oligosaccharides such as raffinose and stachyose (Amuti and Pollard, 1977; Horbowicz and Obendorf, 1994). This results in a reduced monosaccharide content, which has been proposed to play a significant role in the acquisition of desiccation tolerance (Vertucci and Farrant, 1995). It is believed that reduced quantities of monosaccharides result in a reduction in respiratory

substrates and also impose metabolic quiescence during drying. Furthermore, monosaccharides are known to participate in Maillard-type reactions, which would cause protein structural damage (Vertucci and Farrant, 1995). The increase in concentration of sucrose and oligosaccharides coincides with the acquisition of desiccation tolerance in not only orthodox seeds (Blackman *et al.*, 1992; Koster and Leopold, 1988), but also pollen (Hoekstra *et al.*, 1989) and vegetative tissues (Schwab and Gaff, 1990) and this correlation led to the suggestion that these sugars play a role in the mechanism of desiccation tolerance. Drying experiments using model membrane systems such as liposomes demonstrated that sugars prevent membrane phase changes, which would result in leaky membranes upon rehydration. The presence of sugars during desiccation has also been shown to stabilize protein structures (Vertucci and Farrant, 1995; Oliver *et al.*, 2001). The proposed mechanism of protection of these sugars is based on the hydroxyl groups of sugars forming hydrogen bonds with the phosphate group of the polar headgroup in phospholipids and with the carboxyl group of proteins (Crowe *et al.*, 1998). As a result of these interactions, phase transitions of lipids and denaturation of proteins during drying is prevented. These data lead to the development of the water replacement hypothesis (Vertucci and Farrant, 1995) whereby sugars replace water molecules during drying, thus maintaining the structural and functional integrity of membranes, proteins and other macromolecules.

The second hypothesis is known as the glass formation or vitrification hypothesis (Leopold *et al.*, 1994; Vertucci and Farrant, 1995). As the cytoplasm dries to below $0.3 \text{ g H}_2\text{O g}^{-1}$ dry weight the accumulated sugars form an extremely viscous

amorphous metastable state in the cytoplasm referred to as a glassy state. Because of their extremely high viscosity, glasses result in the immobilization of cytoplasmic compounds, prevent fusion between membrane systems and preventing irreversible protein denaturation induced by the removal of water (Prestrelski *et al.*, 1993). Most importantly, they slow down all chemical reactions including free radical reactions (Leopold., 1994). Oligosaccharides such as raffinose and stachyose are reported to be better glass formers than disaccharides or monosaccharides (Levine and Slade, 1992; Roos, 1995). Proteins are also reported to be involved in the formation and stabilization of glasses (Kalichevsky *et al.*, 1993). They enhance the stability of glasses by increasing the glass-liquid transition temperature, T_g , (Bell and Hageman, 1996) and the average strength of hydrogen bonding of the amorphous sugar matrix (Wolkers *et al.*, 2001). Cytoplasmic glasses have been reported to also form in pollen (Buitink *et al.*, 1996) and in leaves of *Craterostigma plantagineum* (Wolkers *et al.*, 1998a). In addition to glass formation, raffinose family oligosaccharides (RFO) stabilize membranes under stress conditions through direct interaction with the phospholipid head groups (Hincha *et al.*, 2003).

1.4.5 Accumulation of stress proteins

1.4.5.1 Late embryogenesis abundant (LEA) proteins

In addition to osmolytes, antioxidants and amphiphiles, different groups of proteins have been reported to be involved in conferring desiccation tolerance to orthodox seeds. One example is the group of hydrophilic proteins known as late embryogenesis abundant (LEA) proteins. As the name suggests, they are produced in abundance during the late stages of seed development (Galau *et al.*, 1986; Baker *et al.*, 1995;

Blackman *et al.*, 1995; Oliver and Bewley, 1997), constituting up to 4 % of total cellular protein (Roberts *et al.*, 1993). They are rapidly degraded upon germination, when desiccation tolerance is lost. Though these proteins were first identified 20 years ago in cotton and wheat (Baker *et al.*, 1988), they are now known to be widespread in both prokaryotes (Garay-Arroyo *et al.*, 2000) and eukaryotes (Dure *et al.*, 1981; Galau *et al.*, 1986; Close *et al.*, 1993). They have been reported to accumulate also in the vegetative tissues exposed to water-deficit (Bray, 1993; Blackman *et al.*, 1995; Close, 1996; Castillo *et al.*, 2000). A special feature of LEA proteins is that they are thermostable, and can withstand temperatures as high as 100 °C. Based on amino acid sequences, LEA proteins have been classified into at least five groups (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 (Bray, 1993; Xu *et al.*, 1996).

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 polar water molecules, thus helping maintain the hydration shell during desiccation (Galau *et al.*, 1986; Litts *et al.*, 1991). The group II LEA proteins or dehydrins are characterized by a conserved 15 amino acid sequence, EKKGIMDKIKEKLP, (Bray, 1993; Campbell and Close, 1997) which has a high propensity to form a α -helix (Whitsitt *et al.*, 1997). This group is proposed to sequester accumulated ions during drying. The presence of amphipathic helices suggests that they might also interact with exposed hydrophobic surfaces of partially

denatured proteins to prevent protein-protein aggregation (Close, 1997; Campbell and Close, 1997). A group II LEA protein, DHN1, from maize is proposed to stabilize membranes (Koag *et al.*, 2003). Group III LEA proteins (D-7 family) are characterized by a tandem repeat of 11 amino acids that exist as an amphipathic α -helix which is involved in sequestering of excess ions, helping to reduce the effective ionic strength in dehydrating cells (Dure, 1993). Group IV LEA proteins (D-13 family), like group I LEA proteins, are believed to stabilize membranes and macromolecules by forming a hydration shell around organelles (Bray, 1993). Group V LEA proteins (D-29 family) are postulated to sequester ions.

Immunolocalization and subcellular fractionation show that these proteins are found in various places including the nuclei, mitochondria but mostly in the cytoplasm (Close, 1997; Borovskii *et al.*, 2000). Dehydrins from maize embryos (Egerton-Warburton *et al.*, 1997) and wheat (Danyluk *et al.*, 1998) were found to be associated with the cytoplasmic endomembrane and plasma membrane, respectively, suggesting that they might be involved in the protection of these membranes against desiccation. Strong circumstantial evidence shows that these proteins are involved in the adaptation of plants to water-deficit stress, since their transcripts and translation products accumulate in the vegetative tissues of a number of drought stressed plants and desiccation treatment induces their precocious expression in immature seeds (Bartels *et al.*, 1988; Baker *et al.*, 1988; Robertson and Chandler, 1994). A more general role in osmoprotection has been proposed since yeast expressing the LEA group I wheat Em protein was shown to display increased osmotolerance (Swire-

Clark and Marcotte, 1999). Similarly, overexpression of a tomato group II LEA protein (le4) in yeast alleviated the detrimental effects of ionic and freezing stress (Zhang *et al.*, 2000). Expression of barley group III LEA protein, HVA1 increased tolerance to osmotic and freeze stresses in yeast *S. cerevisiae*, (Zhang *et al.*, 2000).

1.4.5.2 Heat shock proteins

Small heat shock proteins (sHSPs) are another group of proteins that are associated with plant desiccation tolerance (Almoguera and Jordano, 1992; Wehmeyer *et al.*, 1996). They are the most prominent HSPs in plants and have monomeric molecular masses of 15 – 42 kDa and assemble into oligomers of nine to over 30 subunits (Verling, 1991). Their involvement in desiccation tolerance is supported by evidence stemming from studies on gene expression in developing seeds and in resurrection plants. In pea, Arabidopsis and sunflower seeds, the expression of sHSPs always coincides with the onset of seed desiccation and the proteins are present in large quantities in dry seeds (Coca *et al.*, 1994; DeRocher and Vierling, 1994). Further evidence comes from the observation that sHSP transcripts appear to have a coordinated expression with LEA transcripts during embryo development in response to ABA, indicating that LEA proteins, sHSPs and desiccation tolerance might have common regulatory elements (Almoguera and Jordano, 1992). In desiccation sensitive callus tissue, the expression of sHSPs and acquisition of desiccation tolerance were induced by exogenous ABA treatment (Alamillo *et al.*, 1995). Although their direct involvement in protecting plants against water stress is still unclear, most HSPs are known to function as molecular chaperones (Parcell and

Linguist, 1993) by mediating proper protein refolding upon rehydration, thus preventing protein aggregation.

1.4.5.3 Storage proteins

Another class of proteins implicated in desiccation tolerance is a group of seed storage proteins. Unlike the previously mentioned groups, involvement of this group of proteins in the acquisition of desiccation tolerance is secondary. Seed storage proteins are enzymatically inactive and known to act as amino acid reserves to feed growing seedlings (Shutov *et al.*, 2003). They are synthesized in large amounts during seed development and stored in specialized storage tissues like the cotyledons and endosperm. Membrane bound compartments such as protein bodies found within the endosperm are thought to protect the stored proteins against uncontrollable proteolysis by the proteinases that they co-exist with. However, during germination, protein degradation is triggered by newly formed proteinases targeted into the storage organelles. Degraded storage proteins then serve as the principal source of amino acids for germination and growing seedlings. Seed storage proteins are reported to exhibit some degree of polymorphism thought to arise from the presence of multigene families and in some cases, proteolytic processing and glycosylation.

Although there are various groups of seed storage proteins, only one of the major and most widely distributed groups, the storage globulins, will be discussed. Globulins are widespread in the monocotyledons, dicotyledons and fern spores (Templeman *et al.*, 1987), and are divided into two groups based on their sedimentation coefficient, the 7S vicilins and 11S legumins. Both proteins have subunits consisting of two

structurally similar domains, known as cupin domains. The term “cupin” was derived from Latin for a small barrel and is related to the β -sheet barrel-like structure characteristic of cupins. This motif is found as a single domain in fungal spherulins, fern sporulins and plant germins (Dunwell *et al.*, 2001, 2004), but two such domains are present in the globular storage proteins. The two-domain subunit in globular proteins is reported to have evolved from the duplication of a germin-like ancestral domain (Bäumlein *et al.*, 1995; Dunwell *et al.*, 2001, 2004). In plants, germins have been reported to be involved in defense against abiotic stresses (Berna and Bernier, 1999). Proteins containing cupin domains show high levels of thermal stability, a feature attributed to tight association of the protein subunits through hydrophobic interactions and hydrogen bonding. The presence of disulfide bonds in some cupins has been suggested to play a role in their stability. Since cupins may be involved in for example water-deficit stress responses, it appears that high stability might be an important facet required for their protective role.

1.4 AIM OF THE STUDY

Acacia erioloba E. Mey, the Camel Thorn tree, is an important indigenous tree that grows in the semi-arid regions of Southern Africa. It is the predominant tree in the Kalahari Desert, where the ground temperature regularly exceeds 70 °C during the summer months. Seeds of this tree mature during the autumn months and are shed in the dry winter months (June and July). The seed pods are relished by browsers and serve as an important source of nutrition for these animals. The seeds themselves are not digested, but pass through the digestive tract and are dispersed into the surrounding areas where they must withstand extremely high temperatures before germination. Interestingly the scarification which occurs in the digestive tract is a requirement for successful germination.

Since LEA proteins have been reported to play a role in the desiccation tolerance of mature seeds, the aim of this project was to investigate whether unusual LEA or LEA-like proteins are present in the seeds of *A. erioloba* and to try and elucidate their possible function. To date no work on the protein content of the seeds of this tree has been reported. This thesis reports the purification and characterization of a 53 kDa heat soluble protein that was found to protect other proteins against thermal degradation.

CHAPTER 2

Materials and Methods

2.1 PROTEIN ISOLATION

2.1.1 Plant material

Newly shed *Acacia erioloba* seed pods were collected from Auob in Southern Namibia and stored at $-20\text{ }^{\circ}\text{C}$ until the seeds were removed by crushing the pods and removing the seeds. Seeds from other *Acacia* species namely *A. haemotoxylon*, *A. tortilis*, *A. galpinii*, *A. xanthophloea*, *A. sieberiana* and *A. burkei* were the kind gift of the Community Forestry Nursery, Upington.

2.1.2 Total and Heat soluble protein extraction

Total proteins from the mature seeds of *A. erioloba* were extracted as described by Blackman *et al.* (1991). The seed coat was mechanically removed before the seeds were homogenised with an Kinematica CH-6010 Ultra-turrex homogeniser in ice cold extraction buffer, 10 mM Tris-HCl pH 7.4, 50 mM NaCl, 5 mM MgCl_2 containing 1 mM phenylmethanesulfonylfluoride (PMSF). The homogenate was centrifuged at 27 000 *g* using a Beckman JA 20 rotor for 10 min at $4\text{ }^{\circ}\text{C}$. A small aliquot of the supernatant was kept for analysis whilst the remainder of the sample was incubated at $80\text{ }^{\circ}\text{C}$ for at 30 min. Heat coagulated protein was removed by centrifugation as above. The heat treatment step was omitted for preparation of protein used for functional assays and protein secondary structure analysis.

2.2 PROTEIN PURIFICATION

2.2.1 Carboxymethyl Cellulose 52 (CM 52) Chromatography

Heat soluble protein was applied to a Whatman CM 52 cation exchange column previously equilibrated with 50 mM NaCl, 50 mM NaAc pH 5.0. Proteins were eluted with a gradient of 50 mM - 500 mM NaCl in 50 mM NaAc pH 5.0. The optical density of each fraction was read on a Beckman DU 650 spectrophotometer at 230 nm. Aliquots of specific fractions were analysed by SDS-PAGE.

2.2.2 Gel Filtration Chromatography

CM 52 column fractions containing the protein of interest were pooled and lyophilised. The lyophilate was dissolved in 20 mM HCl and applied to a Sephadex G-100 column previously equilibrated in 20 mM HCl at 4 °C. 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 ASP 53 were pooled, dialysed against distilled water and lyophilised.

Protein used for functional assays and protein secondary structure analysis was not heat-treated (see earlier) and after CM 52 chromatography was purified on a Sephadex G-100 column in 50 mM phosphate buffer pH 7.0. Proteins were eluted with this same buffer, pooled, concentrated by Amicon ultrafiltration and re-applied to this same column.

2.2.3 High Performance Liquid Chromatography (HPLC)

The lyophilised sample from the Sephadex G-100 column equilibrated in 20 mM HCl was dissolved in 6 M urea containing 0.1 % (v/v) 2-mercaptoethanol and the denatured protein applied to a C₁₈ Jupiter reverse-phase column equilibrated in 0.1 % (v/v) heptafluorobutyric acid (HFBA). Proteins were eluted using a linear 0 - 70 % (v/v) gradient of acetonitrile in 0.1 % (v/v) HFBA over 60 min after an initial 5 min wash using 0.1 % HFBA. Fractions corresponding to peaks of 230 nm absorption detected by a fixed wavelength detector were collected, lyophilised and analysed by SDS-PAGE.

2.3 GEL ELECTROPHORESIS

2.3.1 SDS-PAGE

SDS-PAGE was carried out at room temperature using a 15 % (w/v) separating gel and a 6.5 % (w/v) stacking gel (Laemmli, 1970). Protein samples were diluted in sample application buffer (2 % (w/v) SDS, 10 % (v/v) glycerol, 75 mM Tris-HCl pH 6.8, 5 % (v/v) 2-mercaptoethanol, bromophenol blue) before being applied to the gel. Electrophoresis was for 3 h at 200 V using air-cooling system to prevent overheating. Gels were stained with Coomassie brilliant blue in 50 % (v/v) methanol, 10 % (v/v) acetic acid and destained by diffusion in 25 % (v/v) ethanol, 7 % (v/v) acetic acid.

2.3.2 Two-dimensional SDS-PAGE

Isoelectric focusing (IEF) of purified protein was performed in the first dimension (O'Farrell, 1977) on cylindrical gels (0.6 x 150 mm) containing 4 % (w/v)

acrylamide, 9 M urea, 2 % (v/v) Nonidet NP-40, 4 % (v/v) ampholytes pH 3-10. The cathode reservoir buffer was 20 mM NaOH and anode reservoir buffer was 10 mM phosphoric acid. Gels were pre-focused using the following procedure: 15 min at 200 V, 30 min at 300 V and 30min at 400 V. 30 μ l sample containing approximately 20 μ g of pure protein in lysis buffer (9.5 M urea, 1 % (v/v) Nonidet NP-40, 2 % (v/v) ampholytes, 2-mercaptoethanol) was loaded on the cathode end of the 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 h. The gel was extruded from the tube using a syringe and loaded directly on the second dimension slab gel, prepared as described in section 2.3.1. The first dimension tube gel was overlaid with equilibration buffer (10 % (v/v) glycerol, 5 % (w/v) SDS, 0.01 % (w/v) bromophenol blue, 50 mM Tris-HCl pH 6.8) and the gel electrophoresed as described in section 2.3.1.

2.4 PROTEIN CHARACTERISATION

2.4.1 Determination of Molecular Weight using MALDI-MS

A total protein extract from the mature seeds of *A. erioloba* was electrophoresed on a SDS-PAGE gel before being transferred onto a polyvinylidene difluoride (PVDF) membrane. The band containing the protein of interest was excised and the protein extracted by incubating the band in extraction buffer (50 % (v/v) formic acid, 25 % (v/v) acetonitrile, 15 % (v/v) isopropanol) for 2 to 3 hrs with shaking. After extraction, 2 μ l of the solution was mixed with equal volume of sinapinic acid (10 mg/ml in 0.05 % (v/v) TFA, 25 % (v/v) acetonitrile) and analysed by MALDI-TOF mass spectrometry.

2.4.2 Amino Acid Analysis

Purified protein (8 μ l, approximately 2 nmol) was hydrolysed in constant boiling HCl containing 5 % (v/v) phenol under N_2 gas at 110 °C for 24 hrs. The hydrolysate was lyophilised and dissolved in 66.6 mM trisodium citrate containing the internal standard norleucine (NLE 100 buffer). The sample was then analysed on a Waters HPLC and the amino acids were detected by postcolumn orthophthaldialdehyde (OPA) derivatisation (Knecht and Chang, 1986).

2.4.3 In-gel protein digestion and MALDI-MS

The four protein spots detected on the Coomassie stained two-dimensional gel were excised separately and cut into smaller pieces. These were washed with 50 % (v/v) acetonitrile and dried under vacuum before being re-swollen in 10 mM ammonium hydrogen carbonate (NH_4HCO_3) containing 0.1 μ g of pure trypsin (Promega). After incubation at 37 °C for 24 hrs, an equal volume of 100 % acetonitrile was added to extract the peptides from the gel pieces. 1 μ l of the digest was mixed with 1 μ l of the internal standard and 1 μ l α -cyano-4-hydroxy-cinnamic acid. MALDI-TOF mass spectra of the peptide mixture were obtained using a PerSeptive Biosystems mass spectrometer.

2.4.4 Circular Dichroism Studies

Circular dichroism spectra over the range 184 nm to 250 nm were obtained using a Jasco J-810 spectropolarimeter using a 1 cm path length quartz cell at a protein concentration of 0.5 mg/ml in 10 mM sodium phosphate buffer pH 7.4. 1 M sorbitol

or 2 M NaCl was added to the protein solution to determine whether protein conformational changes were induced by increased osmolality. To investigate the effect of temperature on protein conformation, the spectra of samples in 100 mM K_2HPO_4 pH 7.4 were determined at temperatures between 20 °C and 80 °C. Deconvolution of the spectra was performed using the CDNN and DichroProt software.

2.4.5 Detection of glycoproteins

A SDS-PAGE gel of pure protein was soaked for 1 h in Periodic acid-Schiff (PAS) fixative (40 % (v/v) ethanol, 5 % (v/v) acetic acid), changing the solution at least once. The gel was then treated with 0.7 % (v/v) periodic acid in 5 % (v/v) acetic acid for 1 h, followed by treatment with 0.2 % (w/v) sodium metabisulfite in 5 % (v/v) acetic acid for 1 h with one solution change after 30 min. After clearing, the gel was stained with 5 % (w/v) basic fuchsin in the dark until a coloured product was apparent. Bovine serum albumin (BSA) was used as a control.

To determine the carbohydrate residues present, pure protein was applied directly to a nitrocellulose membrane and allowed to dry. The membrane was incubated in 3 % (w/v) BSA to block other protein binding sites. After two washes in PBS (172 mM NaCl, 21.5 mM KH_2PO_4 , 56.4 mM Na_2HPO_4 pH 6.8) the membrane was incubated for 30 min in 0.5 mg/ml solution of *Canavalia ensiformis* (jack bean) Concanavalin A in buffer A (10 μ M $CaCl_2$, 10 μ M $MgCl_2$, 10 μ M $MnCl_2$ in PBS). The membrane was then washed twice with PBS after which it was incubated with horseradish peroxidase (HRP) in PBS for 30 min at 20 °C. Finally the membrane was washed twice with PBS

and once in Tris buffer (50 mM Tris, 250 mM NaCl pH 7.4). Glycoproteins were detected by incubating the membrane in the visualizing reagent (3 mg/ml 4-chloro-1-naphthol in methanol, 30 % H₂O₂ in 0.1 M 50 mM Tris pH 7.4, 250 NaCl). The non-glycosylated proteins, myoglobin and bovine serum albumin (BSA) were used as controls.

2.5 IMMUNOLOGICAL TECHNIQUES

2.5.1 Raising of antisera

The antibody was prepared by injecting 1 mg of HPLC purified protein in Freund's complete adjuvant into rabbits. After the initial inoculation, the rabbit was given three booster inoculations at weekly intervals followed by two further inoculations after an interval of three weeks. Pre-immune serum was obtained before the first injection, and both the immune and the pre-immune serum were used without purification. Titres and specificity were determined by an ELISA assay.

2.5.2 Enzyme Linked Immunosorbance Assay (ELISA)

1 µg of pure protein (antigen) was applied to each well of a 96-well plate (Nunclon®) and left to bind at 4 °C for 16 hrs. Unbound antigen was removed by washing the plate three times in PBS containing 0.05 % Tween 20 (PBS/Tween), and unbound sites in the well were blocked by incubating the plate in 1 % (w/v) BSA in PBS at room temperature for 30 min. After three washes with PBS/Tween, the primary antibody, diluted between 10⁻¹ and 10⁻⁸ in PBS, was added and allowed to react at room temperature for 60 min. The plate was then washed with PBS after which the secondary antibody diluted 1:100 in PBS was added, and the plate incubated for a

further 30 min. Antibody detection was carried out using 300 μg p-nitrophenylphosphate in 10 % (v/v) diethanolamine pH 9.6 and the absorbance read at 405 nm in a Titertek Multiscan PLUS MKII detector.

2.6 WESTERN BLOTTING

2.6.1 Electrophoretic Transfer to Nitrocellulose

Western blotting was carried out as described by Harlow and Lane (1988), using antibody raised against the purified protein as the primary antibody and a goat anti-rabbit antibody coupled to alkaline phosphatase as the secondary antibody. The gel, after SDS-PAGE, was rinsed with transfer buffer (192 mM glycine, 20 % (v/v) methanol, 25 mM Tris-HCl pH 8.5) and then laid on pre-soaked nitrocellulose membrane (NitroBind, Micron Separations Inc.) supported on pre-soaked Whatman 3MM paper cut to the size of the gel. 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 in assembling this Whatman 3MM paper “sandwich” to avoid trapped air bubbles. Transfer was allowed to proceed at 4 °C for 16 hrs at a constant current of 20 mA.

2.6.2 Colorimetric Visualisation of the Transferred Protein

The nitrocellulose membrane was incubated at room temperature for 30 min in 10 ml blocking buffer, PBS (50 mM NaH_2PO_4 , 150 mM NaCl pH 7.4) containing 5 % (w/v) skimmed milk powder. Primary antibody (at a 10^{-5} dilution) was added to the

blocking solution and incubation was carried out for a further 60 min at 37 °C or for 16 hrs at 4 °C. The blot was washed three times in PBS/Tween and once in PBS prior to incubation with the secondary antibody. Incubation with the secondary antibody (Sigma) diluted 1: 30 000 in PBS, was carried out at room temperature for 60 min. The blot was finally washed three times in PBS/Tween and once with washing buffer, 150 mM NaCl, 10 mM Tris-HCl pH 7.4 before being developed. The secondary antibody was detected using 4-nitroblue-tetrazolium chloride and 5-bromo 4-chloro 3-indoyl-phosphate in 100 mM MgCl₂, 100 mM NaCl, 100 mM Tris-HCl pH 8.3.

2.7 IMMUNOCYTOCHEMISTRY

2.7.1 Tissue Processing

Sections (5 mm²) of cotyledons and axes from mature and germinated seedlings were incubated in fixative (2.5 % (v/v) glutaraldehyde containing 0.5 % (w/v) caffeine in PBS) at 4 °C for 16 hrs. The sections were washed three times in PBS before incubation in 1 % (v/v) osmium tetroxide in PBS for 60 min. After washing in PBS to remove excess osmium tetroxide, the sections were dehydrated in a graded ethanol series as follows: 10 min in 30 % (v/v) ethanol, 10 min in 50 % (v/v) ethanol, 10 min in 70 % (v/v) ethanol, 10 min in 80 % (v/v) ethanol, 10 min in 90 % (v/v) ethanol, 10 min in 95 % (v/v) ethanol, 2 x 10 min in 100 % ethanol and finally for 2 x 10 min in 100 % acetone. The samples were embedded in epoxy resin (Spurr, 1969) at a 1:1 ratio of resin:100 % acetone for 18 h, followed by 3:1 (resin:100 % acetone) for 8 h, then in 100 % resin for 18 h and finally in fresh 100 % resin for another 8 h. The samples were then hardened for 16 h at 60 °C, after which they were sectioned at a

gold interference (75 nm) with a Reichert Ultracut-S microtome and collected on nickel grids.

2.7.2 Immunogold Labelling

Sections were sequentially incubated on drops of 1 % (w/v) BSA in PBS to prevent non-specific antibody binding, and on 0.02 M glycine to block unreactive aldehyde groups, prior to incubation with the primary antibody diluted in 1 % (w/v) BSA/PBS at room temperature for 16 hrs. After washing in 1 % (w/v) BSA/PBS, the grids were floated on a drop of goat-anti-rabbit immunoglobulin, to which was attached 10 nm diameter gold particles, diluted 1:50 in 1 % (w/v) BSA/PBS. Incubation was carried out for a minimum of 60 min at room temperature, after which the grids were rinsed with 1 % (w/v) BSA/PBS. Sections were fixed by floating the grids on 1 % (v/v) glutaraldehyde for 10 min. After thoroughly rinsing the grids in water, samples were stained using 3 % (w/v) uranyl acetate and 1 % (w/v) lead citrate. Sections were examined for gold labelling using a Zeiss 200 SX (Germany) transmission electron microscope (TEM). Control sections were probed with pre-immune serum in place of the antisera.

2.8 GERMINATION

Mature seeds of *A. erioloba* were allowed to germinate to different stages on water-saturated filter paper at 30 °C in the dark. Different stages of germination were denoted by the length of the protruding radicle. The germinated seedlings were subsequently desiccated at room temperature for 12 hrs using a flash-drying apparatus (Farrant *et al.*, 1985), and thereafter rehydrated on a water-saturated filter

paper in the dark at 30 °C. Seed water content was determined gravimetrically by oven drying at 105 °C for 24 hrs. Survival of seedlings was determined after 8 days, with seedlings exhibiting re-growth of the radicle and primary leaf formation scored as viable and thus tolerant of drying imposed upon them. Total proteins were extracted from seedlings before dehydration, after dehydration and after rehydration to determine protein expression patterns.

2.9 THERMAL DENATURATION OF PROTEINS

2.9.1 Determination of Protein Concentration

Protein concentrations were determined using the method of Lowry *et al.* (1951) using BSA as the standard.

2.9.2 Light Spectroscopy

Protein aggregation induced by high temperature was monitored by measuring the light scattering of the solution in 100 mM K₂HPO₄ pH 7.4 at 350 nm using a Pye-Unicam SP1800 spectrophotometer with a custom made heating block interfaced to an IBM PC through an Oasis A/D converter. Absorbance values were recorded every 0.2 °C between 20°C and 90 °C at a heating rate of 1 °C/min. The maximum rate of thermal denaturation, the T_m, was determined from calculating the rate of change of light scattering as a function of the temperature.

2.9.3 Circular Dichroism Spectroscopy

The effect of the presence of ASP 53 on perturbations in the secondary structure of haemoglobin as a function of temperature was determined using a JASCO J-810 spectropolarimeter. Hemoglobin (0.05 mg/ml) alone or together with ASP 53 (0.6 mg/ml) in 100 mM K_2HPO_4 pH 7.4 was/were heated from 20 °C to 80 °C and the circular dichroism spectrum measured between 200 nm and 260 nm.

2.9.4 Thermal denaturation of ADH

Yeast alcohol dehydrogenase (ADH, Sigma Chemical Company) was incubated alone at 0.1 mg/ml in 100 mM K_2HPO_4 , pH 7.4 or in the presence of other proteins at 55 °C for various times. The activity was determined after cooling to room temperature. The assay mixture contained 0.01 mM NAD^+ , 0.01 M ethanol and 1 mM pyrophosphate buffer, pH 8.0. The enzymatic activity was determined 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.

2.10 PEPTIDE FINGERPRINTING BY MALDI-TOF MASS SPECTROMETRY

A gel piece of 1 mm diameter was excised from a Coomassie-stained gel, and then incubated in 75 mM NH_4HCO_3 40 % (v/v) ethanol for 30 min at room temperature. The solution was removed and discarded. This step was repeated until complete destaining had occurred after which the gel piece was washed in 100 % acetonitrile before being dried by centrifugation in a speedy-vac. 10 μ l of 5 mM dithiothreitol in

50 mM NH_4HCO_3 was added to the dried gel piece which was incubated at 60 °C for 30 min. The solution was removed and discarded after which protein alkylation was performed by adding 10 μl of 55 mM iodoacetamide in 50 mM NH_4HCO_3 . The reaction was allowed to proceed for 60 min in the dark after which it was dried as before. 10 μl of 20 % (w/v) trypsin in 50 mM NH_4HCO_3 was next added to the dried gel piece. After incubation on ice for 60 min to allow the enzyme to soak into the gel piece, the enzyme solution was discarded and 10 μl of 50 mM NH_4HCO_3 was added to the hydrated gel and digestion allowed to proceed at 37 °C for 16 hrs.

Peptides were extracted from the gel piece by 10 min sonication of the gel piece. Further extraction was performed by repeating the sonication using 10 μl of 2 % (v/v) TFA/ ACN at a ratio 1:1. 2 μl of the pooled recovered solutions was mixed with an equal volume of α -cyano-4-hydroxy-cinnamic acid and analysed by MALDI-TOF mass spectrometry. The remainder of the digest was lyophilised and stored at – 20 °C.

2.11 DERIVATIZATION OF PEPTIDES FOR PSD MASS SPECTROMETRY.

The derivatization procedure was conducted in C_{18} $\mu\text{ZipTips}^{\text{TM}}$ purchased from Millipore Corporation. To determine the efficacy of this procedure and its influence on peptide fragmentation, model peptides supplied in the CAF sequencing kit were used as controls. This two-step procedure was conducted as described in the EttanTM CAFTM MALDI Sequencing Kit manual. Model peptides and the protein digest solution were loaded onto the C_{18} $\mu\text{ZipTips}^{\text{TM}}$ after the tips had been equilibrated in 0.1 % (v/v) TFA. The peptides were guanidinated by washing the lysine modifier

solution (O-Methylisourea hydrogen sulphate in 0.25 M sodium bicarbonate, pH 10.0) five times over the sample-loaded μ ZipTips™. After the final wash, half of the lysine modifier solution was retained in the μ ZipTips™ and the reaction allowed to proceed for 16 h. The solution was removed by aspiration and the μ ZipTips™ finally washed with H₂O.

The CAF reagent (sulfonation) solution was prepared immediately before use by dissolving the CAF reagent in 60 μ l CAF buffer (0.25 M sodium bicarbonate, pH 9.4). The solution was de-gassed by vortexing and used to wash μ ZipTips™ ten times with the solution left in the tips after the last wash. The reaction was allowed to proceed for at least 3 min before the addition of 50 % (v/v) hydroxylamine solution to stop the reaction. The derivatised peptides were eluted with 80 % (v/v) ACN, 0.5 % (v/v) TFA, mixed with the matrix solution (α -Cyano-4-hydroxy cinnamic acid in 50 % (v/v) ACN, 0.1 % (v/v) TFA) before being analysed by MALDI-TOF mass spectrometry.

2.12 RNA EXTRACTION

Total RNA was extracted from mature seeds of *A. eriloba* using Trizol reagent, a commercially available guanidinium-phenol-based solution, according to the manufacturer's instructions. An aliquot of extracted total RNA was analysed on a 1 % (w/v) denaturing agarose gel.

2.12.1 Denaturing agarose gel electrophoresis

A 1 % (w/v) formaldehyde agarose gel was prepared by dissolving 0.6 g of agarose in 44 ml of milli-Q water and 6 ml of 10 x MOPS buffer (200 mM MOPS, 50 mM Na-acetate, 10 mM EDTA) at 100 °C. The solution was cooled to 55 °C before adding 8.4 ml of formaldehyde. 1 x MOPS was used as the running buffer. 1-2 µl of total RNA was mixed with 8 µl of RNA cocktail (67 % (v/v) formamide, 17 % (v/v) formaldehyde, 13 % (w/v) 10 x MOPS and 10 mg/ml EtBr) and heated for 5 min at 65 °C before the addition of loading buffer (50 % (v/v) glycerol, 0.25 % (w/v) bromophenol blue, 0.25 % (w/v) xylene cyanol FF, 1 mM EDTA, pH 8) to the sample. The gel was electrophoresed for 1.5-2 hrs at 50 V.

2.12.2 Non-denaturing agarose gel electrophoresis

A 1 % (w/v) non-denaturing agarose gel was prepared by dissolving 1 g of agarose in 100 ml TAE buffer (2 M Tris, 1 M Acetic acid, 0.5 M EDTA pH 8.0). The solution was cooled to 40 °C before adding 5 µl of 10 mg/ml EtBr. The gel was electrophoresed at 100 V for 1 h.

2.13 DNaseI TREATMENT

Contaminating genomic DNA was removed from total RNA by treatment with DNaseI. 10 µg of total RNA was incubated with 10 units of DNaseI (Promega) in 1x DNaseI buffer (40 mM Tris-HCl, pH 8.0, 10 mM MgSO₄, 1 mM CaCl₂) at 37 °C for 30 min. The reaction was stopped by incubation at 70 °C for 5 min and the DNA-free RNA analysed on a 1 % (w/v) agarose gel.

2.14 REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR)

2.14.1 First-strand cDNA synthesis

2 µg of DNaseI-treated total RNA was heat-denatured at 65 °C for 5 min in the presence of 12.5 mM anchored oligo dT primer after which the reaction was quenched on ice. The following reagents were added to the sample: 4 µl of 5 x RT buffer (Promega), 0.8 µl of 25 mM dNTP, 1.0 µl of 40 U/µl RNase inhibitor and 1 µl of 200 U/µl Moloney murine leukaemia virus Reverse transcriptase (M-MLV RT, Promega). To determine whether the reverse transcription reaction was successful, a labelled RT reaction was conducted concurrently in the presence of [α -³²P]dCTP. Both reactions were incubated at 42 °C for 60 min and thereafter at 75 °C for 10 min to inactivate the enzyme. First stand cDNA synthesis was checked by electrophoresis of the radioactive reaction on a 1 % (w/v) alkaline agarose gel and the non-radioactive sample was used for a subsequent amplification reaction.

2.14.2 Alkaline agarose gel electrophoresis

0.8 g of agarose was dissolved in 72 ml of water at 100 °C and thereafter cooled to 50 °C before 8 ml of 10 x alkaline buffer (3 ml of 5 M NaOH, 2 ml of 0.5 M EDTA, 45 ml of water) was added and the gel poured. Thin gels (3 mm) were poured because of later drying and exposure for autoradiography. An aliquot of the radioactive RT reaction was mixed with an equal volume of 2 x loading buffer (200 µl of glycerol, 46 µl of saturated bromophenol blue, 750 µl of water and 5 µl of 5M NaOH) before

being loaded onto the gel. After electrophoresis for 60 min at 70 mA, the gel was dried and exposed for autoradiography at $-70\text{ }^{\circ}\text{C}$ for 18 h.

2.14.3 Polymerase chain reaction (PCR)

5 μl of cDNA was amplified in a 50 μl PCR mixture containing 1 x PCR buffer (Super-Therm), 1.0 mM MgCl_2 , 0.2 mM dNTPs, 0.8 μM of each primer (Oligo dT and GSP 1) and 1 U Taq polymerase (Super-Therm). The sample was denatured at $94\text{ }^{\circ}\text{C}$ for 4 min before being subjected to 35 cycles of denaturation at $94\text{ }^{\circ}\text{C}$ for 1 min, primer annealing at $55\text{ }^{\circ}\text{C}$ for 1 min and extension at $72\text{ }^{\circ}\text{C}$ for 2 min, followed by a final extension at $72\text{ }^{\circ}\text{C}$ for 7 min in a GeneAmp[®] PCR System 9700, Perkin Elmer (Applied Biosystems). PCR products were analysed on a 1 % (w/v) non-denaturing agarose gel.

2.14.4 Optimisation of RT-PCR

A schematic diagram of the RT-PCR procedure is shown in Figure 2.1. Since a degenerate gene specific primer (GSP 1), the sequence of which is shown in Table 2.1, with annealing temperatures (T_m) ranging between $46.2\text{ }^{\circ}\text{C}$ and $59.5\text{ }^{\circ}\text{C}$ was used, PCR conditions had to be optimised for the actual primer used. Annealing temperatures between $42\text{ }^{\circ}\text{C}$ and $63\text{ }^{\circ}\text{C}$ were used and the products analysed for the presence of a single prominent band by electrophoresis on a 1 % (w/v) non-denaturing agarose gel. A DNA band only present in the complete reaction was excised from the gel and the DNA purified using the QIAquick[®] gel extraction kit (Qiagen) according to the manufacturer's instructions.

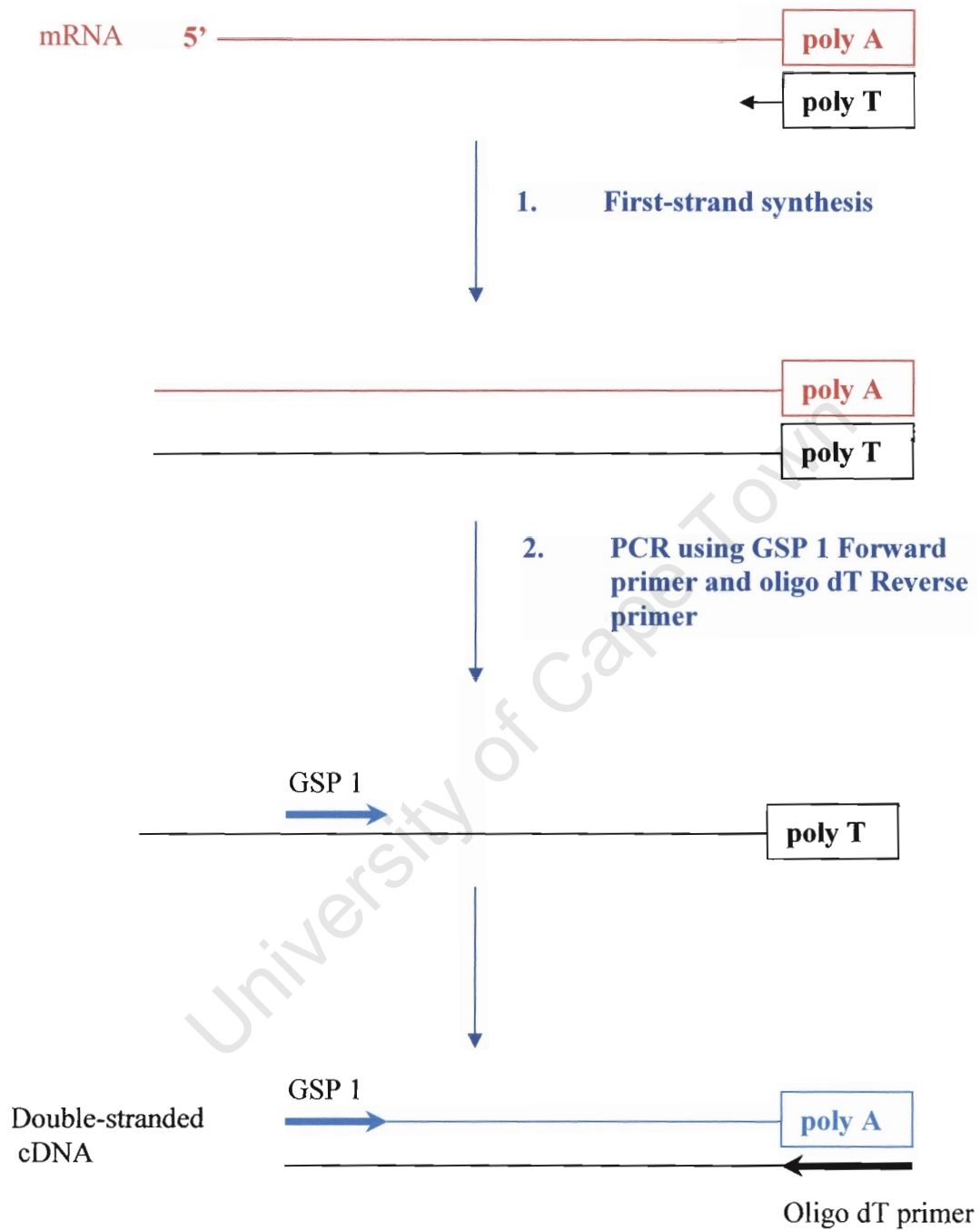


Figure 2.1: Amplification of the *ASP53* gene

Table 2.1 Primers used for RT-PCR

Primer	Sequence 5'-3'
Anchored oligo dT	GGGATCCTTTTTTTTTTTTTTTTTT(CAG)I
GSP 1	AA(CT)CA(AG)TA(CT)GA(CT)GCI(CTA)II(CA)G

I = inosine

2.15 CLONING OF TARGET cDNA

The purified PCR product was ligated into pGEM[®]-T Easy vector in a 10 µl ligation mixture containing 3 µl of the PCR product, 1 µl vector DNA, 5 µl 2x ligation buffer (60 mM Tris-HCl, pH 7.8, 20 mM MgCl₂, 20 mM DTT, 2 mM ATP, 10 % PEG) and 1 µl T₄ DNA ligase. Ligation was allowed to occur at 4 °C for 16 hrs.

2.16 TRANSFORMATION OF COMPETENT CELLS

DH5α cells were transformed with the ligation reaction by adding a 100 µl aliquot of cells to 5 µl of the ligation reaction mixture and the cells incubated on ice for 30 min. The cells were then heat shocked by incubation at 42 °C for 45 s and again placed on ice for 2 min. The transformed cells were supplemented with 800 µl LB medium (1 % (w/v) Bacto[®]-tryptone, 0.5 % (w/v) Bacto[®]-yeast extract, 0.5 % (w/v) NaCl) and incubated at 37 °C for 60 min with shaking before being plated onto LB plates containing ampicillin/IPTG/X-Gal. The plates were incubated at 37 °C for 16 hrs and white colonies, indicating the presence of an insert, were selected, grown in LB media and plasmid DNA extracted using the Qiaprep[®] Miniprep kit (Qiagen). This DNA was then sequenced (Sanger *et al.*, 1977).

2.17 5' RAPID AMPLIFICATION OF cDNA END (5' RACE)

2.17.1 First-strand cDNA synthesis

To determine the sequence of the 5' end of the RT-PCR product, 5' RACE was performed. cDNA synthesis was performed using an Expand Reverse Transcriptase kit (Roche) in a final volume of 10 μ l containing 2 μ l (1 μ g) DNaseI treated-total RNA from the mature seeds of *A. erioloba*, 1 μ M SMART IV oligonucleotide from the Creator™ SMART™ cDNA Library Construction Kit (Clontech), 1 μ M oligo dT primer, 1x first-strand buffer, 2 mM DTT, 1 mM dNTPs and 50 U Powerscript reverse transcriptase. The reaction was allowed to occur at 42 °C for 60 min and thereafter placed on ice to inactivate the enzyme. Powerscript reverse transcriptase was chosen for this reaction because it is a derivative of M-MLV reverse transcriptase and lacks RNase H activity allowing for synthesis of longer cDNA fragments. In addition this enzyme adds between 3 and 5 cytosine residues to the 3' end of the cDNA, allowing for the subsequent use of a PCR primer terminating with between 3 and 5 guanosine residues. Successful first strand cDNA synthesis was monitored by conducting a PCR using the GSP 2 and FGSP 1 primers. Both primers were designed from the DNA sequence of the prior RT-PCR product. A single band representing the expected product of 361 bp was observed when the PCR product was analysed on a 1 % (w/v) non-denaturing agarose gel. The presence of a single product demonstrated the specificity of both primers. These results not only indicated successful reverse transcription but also showed the presence of the *ASP53* gene in the newly synthesised cDNA.

2.17.2 Amplification of target cDNA

A gene specific antisense primer, GSP 2, designed from the DNA sequence of the RT-PCR product was used in conjunction with the 5' PCR primer (complementary to the SMART IV oligonucleotide that was ligated to the 3' end of the cDNA) to amplify the 5' end of the gene. The sequences of these primers are shown in Table 2.2 and a schematic diagram of this procedure is shown in Figure 2.2. PCR was performed using the Expand High Fidelity^{PLUS} PCR System (Roche); the PCR mixture contained 3 µl of cDNA, 1 µM of each primer, 1x PCR buffer, 1 mM MgCl₂, 0.2 mM dNTPs and 0.75 U Taq. PCR was performed in a GeneAmp[®] PCR System 9700, Perkin Elmer (Applied Biosystems) using 30 cycles of sample denaturation at 94 °C for 30 s, primer annealing at 61 °C for 45 s and extension at 72 °C for 2 min, followed by a final extension at 72 °C for 7 min. PCR products were analysed on a 1 % (w/v) non-denaturing agarose gel. The PCR product was excised from the gel, purified using a "High Pure" PCR product purification kit (Roche) and ligated into the pGEM[®]-T Easy vector. Colony PCR using the 5' PCR primer together with the GSP 2 primer was conducted to screen the selected white colonies. Selected positive clones were sequenced.

Table 2.2 Oligonucleotides and primers used 5' RACE

Oligonucleotide	Sequence 5'-3'
Smart IV	AAGCAGTGGTATCAACGCAGAGTGGCCATTACGGCCGGG
5' PCR primer	AAGCAGTGGTATCAACGCAGAGT
GSP 2	AGCCATGGTCGTTGGAGTAGCG
FGSP 1	TGGTGGATCCAGTGGTGATGAAG

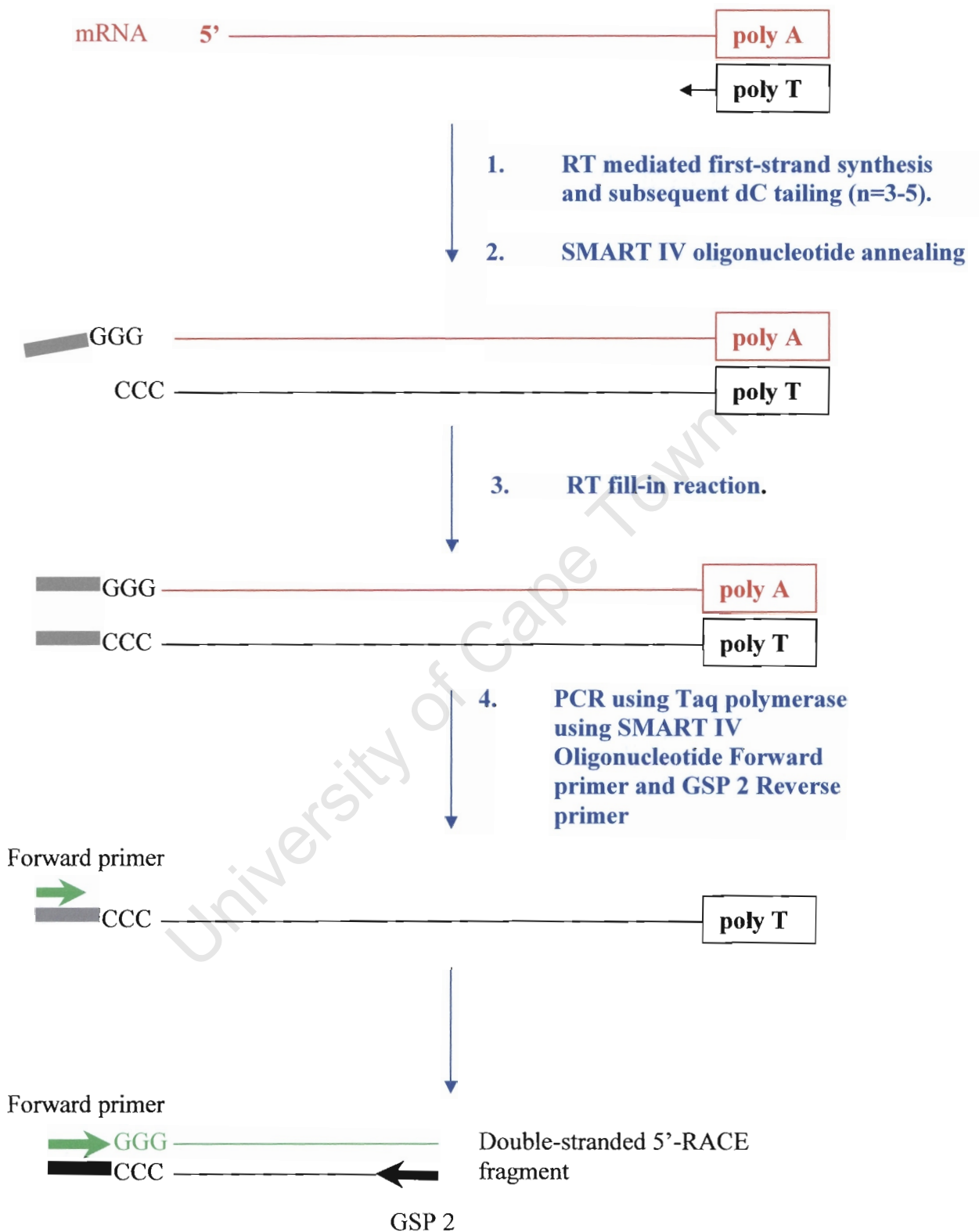


Figure 2.2: Schematic diagram showing the mechanism of the 5' RACE

CHAPTER 3

Protein purification and characterization

3.1 INTRODUCTION

The survival of many plants is dependent to a large extent on the ability of the seeds they produce to withstand environmental conditions not conducive to vegetative growth (Swire-Clark and Marcotte, 1999). Seeds of many plant species acquire desiccation tolerance towards the end of their development on the plant (Swire-Clark and Marcotte, 1999). During this stage, known as late embryogenesis, plant seeds mature and undergo desiccation (Gaubier *et al.*, 1993), an experience equivalent to plant desiccation during drought. Since tissues from both resurrection plants and orthodox seeds have been reported to experience similar subcellular stresses associated with water deficit (Walters *et al.*, 2002) both tissue types employ similar mechanisms of protection. In response to water stress, plants undergo metabolic, developmental and physiological changes including changes in gene expression as means of adapting to this stressful condition. Plants have developed two strategies to allow them to survive desiccation stress. They involve protection against damages caused by water deficit and repair of desiccation or rehydration induced damages. Resurrection plants use the protection mechanism, which involves differential expression of genes, which include lea (late embryogenesis abundant) genes (Vertucci and Farrant, 1995; Blackman *et al.*, 1992; Koster and Leopold, 1988).

LEA proteins accumulate in the seed embryos during later stages of embryogenesis when the cells desiccate (Bartels *et al.*, 1988; Dure III *et al.*, 1989; Blackman *et al.*, 1991; Butler and Cuming, 1993) and in vegetative tissues in response to the desiccation stress-response hormone, abscisic acid (Galau *et al.*, 1986; Castillo *et al.*, 2000), osmotic and low-temperature stresses (Bray, 1997; Close, 1996, 1997; Castillo *et al.*, 2000). These proteins have been well characterized, and amino acid analysis shows that they are rich in hydrophilic amino acids, a feature that enables them to remain soluble at high temperatures (Close *et al.*, 1989). They have low content of hydrophobic amino acids and display lack of tryptophan and most often cysteine residues. Even though their specific role in desiccation tolerance is still unclear, information on the secondary structure of LEA proteins have helped in giving clues to their function. Secondary structure prediction programmes show that most of these proteins exist largely in an unstructured conformation in solution with an exception of group III and group VI LEA proteins, which exhibit an α -helix conformation. However some LEA proteins show an increase in folding when water activity decreases. For example, a LEA-like p11 from pea (*Pisum sativum*) axis exhibited a salt-dependent increase in the α -helical content from 2 % in water to 15 % in 2 M NaCl (Russouw *et al.*, 1995). Similarly a LEA group III D-7 from cattail (*Typha latifolia*) pollen assumes an entirely unordered structure in solution but upon drying assumes a largely α -helical structure (Wolkers *et al.*, 2001). The predicted random coil conformation allows the protein to adjust its structure to facilitate hydrogen bonding with essentially any macromolecule or membrane surface and enables complete exposure of all charged amino acids within the structure thus promoting

efficient binding with water molecules (Eom *et al.*, 1996). This enables the protein to maintain minimal water content during desiccation. On the other hand, the amphiphilic α -helix conformation helps sequester ions accumulated during drying and interact with exposed hydrophobic surfaces of partially denatured proteins to prevent protein-protein aggregation. Correlation between the expression of LEA proteins and acquisition of desiccation tolerance suggests possible involvement of these proteins in protection of plants and seeds against desiccation-induced damages. Various ways on how these proteins protect cellular structures have been proposed and include, ion sequestration, water binding to minimise water loss, protein and membrane stabilisation or renaturation of unfolded proteins (Bray, 1993; Close, 1997). However, to accomplish these proposed roles LEA proteins are required to be present in substantial quantities. Previously, Russouw (1995) identified the most prevalent protein in the 80 °C supernatant, p11, from the pea seed axes to be a LEA group I protein. In *Saccharomyces cerevisiae*, a LEA-like protein, HSP 12, was the most abundant in the total soluble protein extract (Mtwisha *et al.*, 1998) and Wolkers (2001) purified an abundant D-7 LEA-like protein from *Typha latifolia* pollen. Although LEA proteins are highly expressed during desiccation, they have been reported to be rapidly degraded upon germination, thus serving as an amino acid source to growing seedlings (Swire-Clark and Marcotte, 1999). Even though some LEA proteins are constitutively expressed (Welin *et al.*, 1994), their expression is up regulated by water stress, and under non-stress conditions they show diverse subcellular and tissue-specific localisation patterns (Close, 1997; Nylander *et al.*, 2001). However, under stressful conditions they have been observed to be present in various places including the nuclei, mitochondria, plasma membrane and mostly in

the cytoplasm. Localization of proteins also gives insight into the functional role of the protein in the cell. Although the proposition for the function of LEA proteins lacks demonstrated supporting evidence, there is some circumstantial evidence on the effects of LEA proteins on stress tolerance. The wheat group I LEA protein, Em, confers osmoprotection when expressed in yeast (Swire-Clark and Marcotte, 1999) and LEA-like HSP 12 enhances barotolerance of yeast *S. cerevisiae* (Motshwene *et al.*, 2004). Furthermore, a barley group III LEA protein, HVA1, improved tolerance to water deficit in transgenic rice (Xu *et al.*, 1996) and wheat (Sivamani *et al.*, 2000). When expressed in *S. cerevisiae*, HVA1 increased tolerance of yeast cells to osmotic and freeze stresses (Zhang *et al.*, 2000). LEA proteins are also reported to alleviate the effects of desiccation tolerance via vitrification (Wolkers *et al.*, 1999) and, amongst other proteins, LEA group III D-7 from pollen stabilizes sugar glasses (Wolkers *et al.*, 2001). These observations clearly demonstrate the role of LEA and LEA-like proteins in ameliorating various stress effects. However, we thought it would be interesting to determine and demonstrate the specific role played by one of these protein types in combating the effects of various stresses plants are constantly exposed to.

3.2 RESULTS AND DISCUSSION

3.2.1 Protein Purification

To investigate for the presence of LEA or LEA-like proteins in the mature seeds of *A. erioloba*, the seed coats were mechanically removed and seeds homogenised in extraction buffer using an ultra-turrex homogeniser until a homogenous solution was obtained. This procedure was carried out on ice to prevent proteolysis. The total protein extract was analysed by SDS-PAGE. Figure 3.1 shows a wide variety of extracted proteins ranging in size from approximately 70 kDa to less than 10 kDa were present in this homogenate. A high molecular weight protein of approximately 50 000 Da was the most prevalent protein in the extract. Lane 2 shows that most proteins from the total protein extract, including the 50 kDa protein remained soluble after incubation at 80 °C for 30 min, suggesting that these proteins were hydrophilic in nature. The 50 kDa protein was therefore chosen for investigation in this study because it was present in substantial quantities, and its ability to remain in solution at high temperature (Baker *et al.*, 1988; Close *et al.*, 1989), properties typical of LEA proteins. SDS-PAGE of proteins extracted from hydrated and dehydrated roots and leaves of *A. erioloba* showed that the 50 kDa protein was not present (results not shown), indicating that this protein was seed specific. To identify and further characterise the protein we subsequently attempted to purify it using a variety of chromatographic techniques.

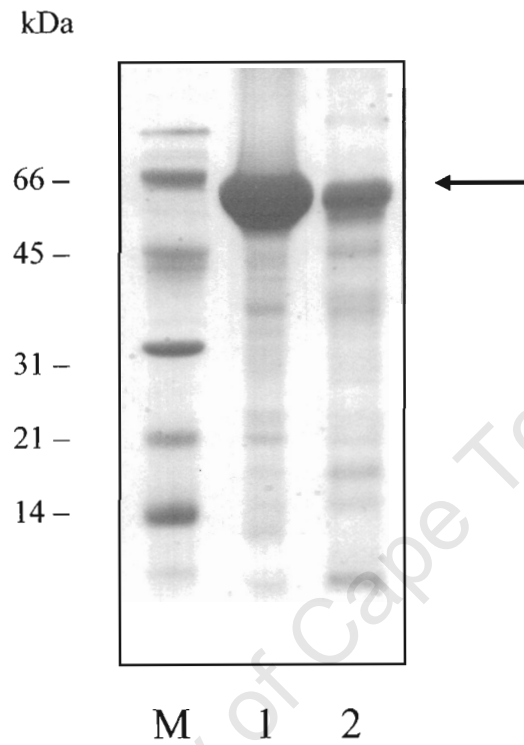


Figure 3.1 SDS-PAGE of total proteins extracted from mature seeds of *A. erioloba* (lane 1) and soluble proteins after incubation at 80 °C for 30 min (lane 2). The molecular weights (in kDa) of markers (M) of known size are denoted on the left hand side of the figure. The arrow shows the electrophoretic migration of the protein selected for further study.

3.2.2 Purification using Chromatography Columns

We initially used a Whatman CM 52 cation exchange column equilibrated in 50 mM NaCl 50 mM NaAc pH 5.0. Total soluble protein was loaded in this buffer and eluted with a gradient of 50 mM - 500 mM NaCl in 50 mM NaAc pH 5.0. Figure 3.2 shows an SDS-PAGE gel of the fractions under the major peaks. This showed that smaller proteins eluted under the first and second peaks whereas the 50 kDa protein eluted under the third major peak. These results suggested that at pH 5.0 the 50 kDa protein is positively charged. Although most proteins eluted prior to the 50 kDa protein some proteins co-eluted with it, necessitating further purification. Fractions containing the 50 kDa protein were therefore pooled and lyophilised.

To remove contaminating proteins from the sample, gel filtration chromatography using Sephadex G-100 was attempted. The lyophilised sample was dissolved in 20 mM HCl and applied onto a Sephadex G-100 column previously equilibrated in the same buffer. Figure 3.3 shows that the 50 kDa protein was eluted under the first peak of the elution profile indicating that it was not retained in the column. Although most of the high molecular weight proteins were successfully separated from the protein of interest, traces of some contaminating proteins were still visible. Fractions under the first peak containing a large amount the 50 kDa protein were pooled and lyophilised.

Final purification to remove contaminating proteins present was achieved with HPLC. The lyophilised sample from the Sephadex G-100 column was dissolved in 6 M urea with 0.1 % (v/v) 2-mercaptoethanol to unfold the protein prior to application onto the C₁₈ Jupiter reverse-phase column.

Proteins were eluted using a 0 - 70 % (v/v) gradient of acetonitrile in 0.1 % (v/v) HFBA. Figure 3.4 shows the elution profile obtained. SDS-PAGE analysis of the eluted fractions showed that a pure 50 kDa protein was eluted in peak I.

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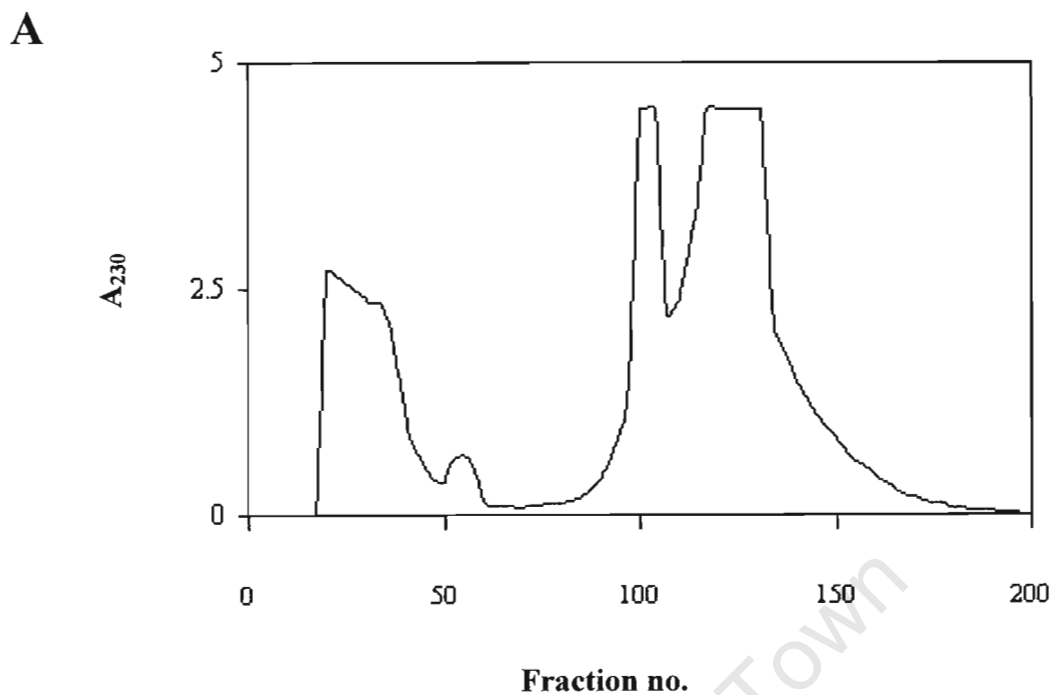


Figure 3.2 A: Chromatogram of total soluble proteins eluted from the CM 52 column in 50 mM NaCl - 500 mM NaCl in 50 mM NaAc pH 5.0. B: SDS-PAGE of selected fractions, lanes 1 – 5 corresponds to every fifth fraction from fraction 20 – 40; lanes 6 – 11 corresponds to every fifth fraction from 80 – 105; lanes 12 – 18 corresponds to every fifth fraction from fraction 110 – 140. Fractions on lanes 14 – 18 were pooled and lyophilised.

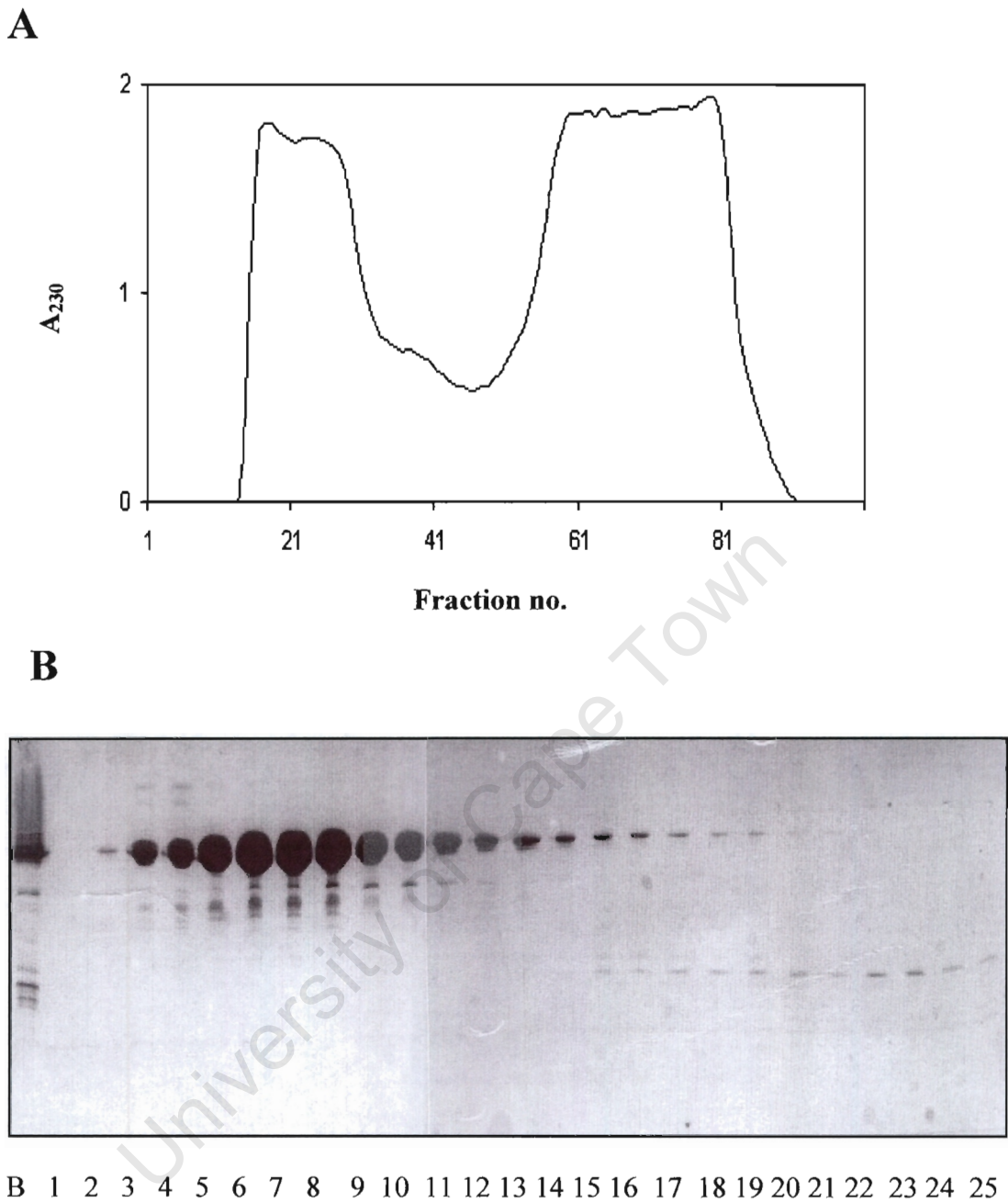


Figure 3.3 A: Elution profile of Sephadex G-100 chromatography of pooled fractions from the CM 52 column. B: SDS-PAGE of fractions under the two major peaks. Lanes 1 – 14 corresponds to every fraction from fraction 15 – 28 and lanes 15 – 25 corresponds to every second fraction from fraction 55 – 75. Fractions on lanes 3 – 11 were pooled. The sample loaded onto the column is shown in B.

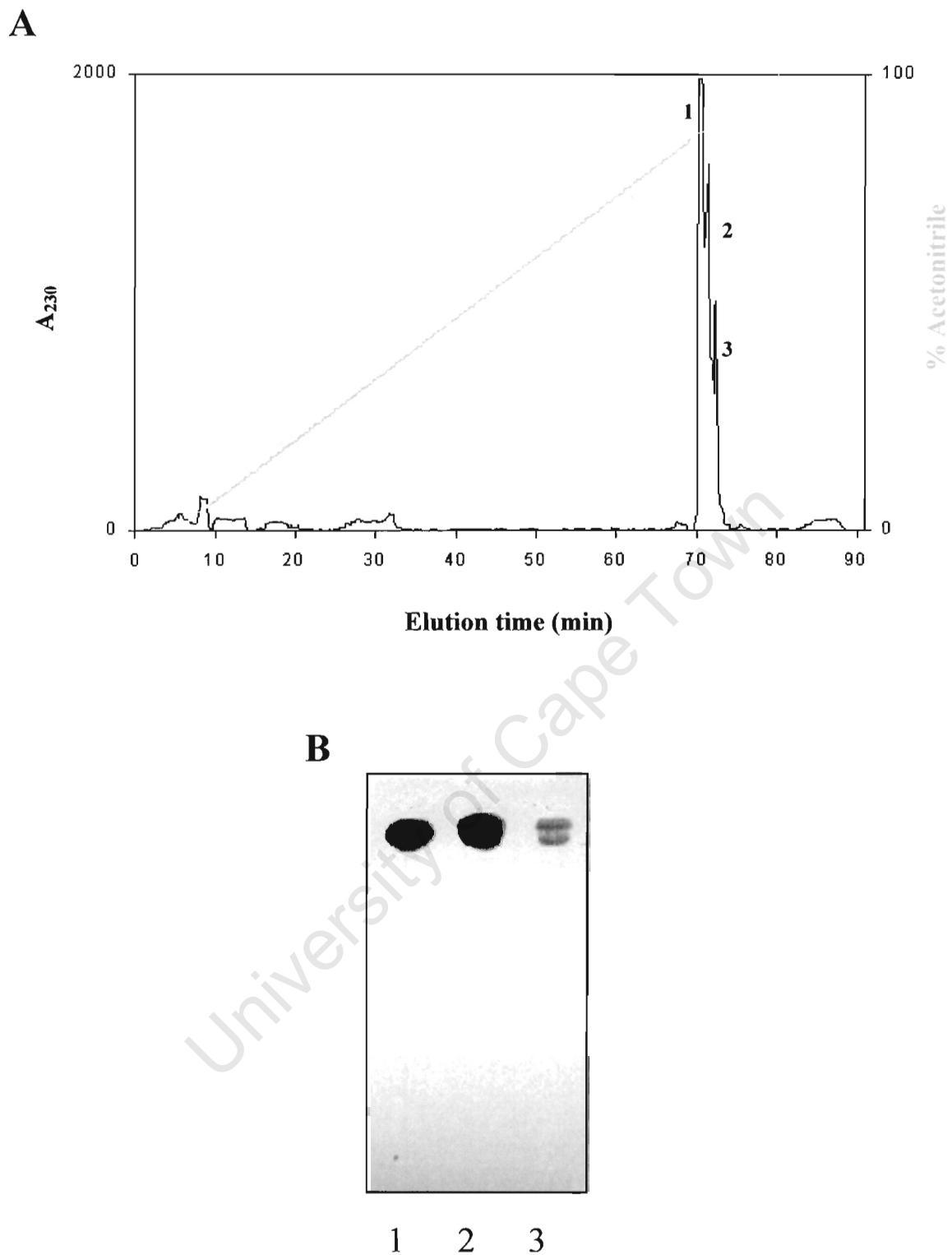


Figure 3.4 HPLC purification of the 53 kDa protein. A: Elution profile of Sephadex G-100 purified material after HPLC. B: SDS-PAGE of proteins eluted in peaks 1, 2 and 3 (lanes 1 - 3 respectively). The material eluted in peak 1 was adjudged to be pure protein and used for further characterisation.

3.2.3 Protein Characterization

3.2.3.1 Two-dimensional SDS-PAGE

To determine whether the single band observed on SDS-PAGE (Figure 3.1) represented a single protein, the HPLC-purified protein was analysed by 2-D PAGE using isoelectric focussing in the first dimension and SDS-PAGE in the second. Figure 3.5 shows that the single protein band was separated into four spots in the first dimension. These spots were of identical molecular weight but differed in their isoelectric points ranging between pH 9 and pH 10. Our interpretation of this result is that the 50 kDa protein was polymorphic, possibly as a result of amino acid substitution or post-synthetic modification, for example due to phosphorylation or glycosylation. When pure 50 kDa protein was analysed by MALDI-TOF mass spectrometry to confirm the molecular weight of the protein, the protein appeared as a relatively broad peak, suggesting that more than one individual protein species was present, with an average mass of 53 581 Da (results not shown). The protein will now be referred to as the 53 kDa protein.

To investigate the hypothesised polymorphism, the four protein spots were separately excised from the gel, digested with trypsin and analysed by mass spectrometry. Figure 3.6 shows that the sizes of the peptides present in all four digests were very similar, with the majority of the peptides common to all four digests and a few peptides only present in certain spots. We therefore concluded that this protein is present in at least four different polymorphic forms in *A. erioloba* seeds.

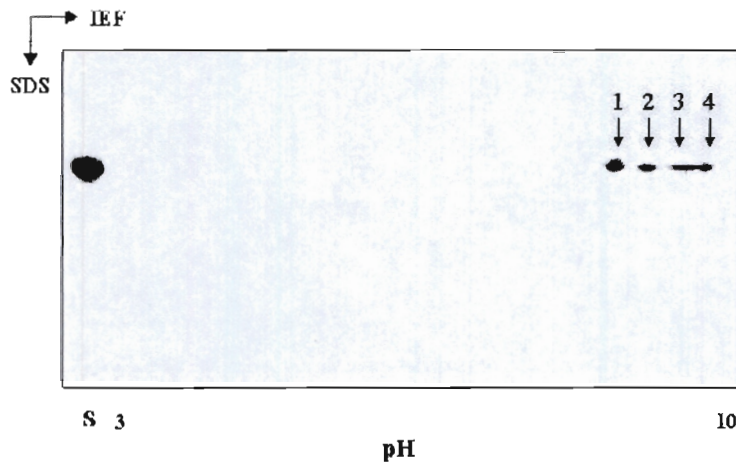


Figure 3.5 Two-dimensional IEF/SDS-PAGE electrophoretogram of HPLC purified protein. 20 μ l containing approximately 10 μ g of pure protein was electrophoresed on a first dimension isoelectric focusing gel from pH 3 to pH 10, after which second dimension SDS-PAGE was performed. The original sample was included in the second dimension (S). The numbered arrows denote the spots used for subsequent mass spectrometry (refer to Figure 3.6).

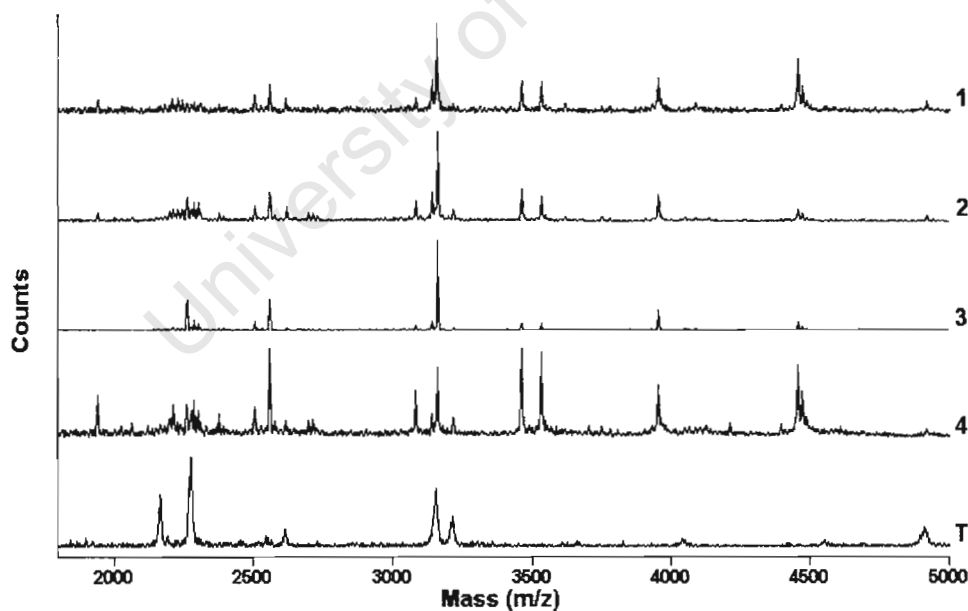


Figure 3.6 Mass analysis of peptides produced by trypsin digestion of the four protein spots (Figure 3.5). The spots on the 2-D gel were excised, treated with trypsin before being analysed by MALDI-TOF mass spectrometry. A trypsin auto-digest (T) was used as a control.

3.2.3.2 Amino acid analysis

To further characterize the protein, its amino acid composition was determined. Pure 53 kDa protein was acid hydrolysed and the hydrolysate subjected to amino acid analysis. Table 3.1 shows the amino acid content in mole % of the purified protein. The isolated protein was rich in hydrophilic amino acids, particularly glutamate, aspartate and serine. However, aspartate and glutamate are produced by acid hydrolysis of asparagine and glutamine so these are referred to as Asx and Glx respectively in Table 3.1 indicating that the aspartate and glutamate could arise from either source. Hydrophilic amino acids constituted approximately 40 % of the total amino acids present with hydrophobic amino acids constituting approximately 34 %. This result was unexpected since heat soluble proteins normally contain only relatively small quantities of hydrophobic amino acids. However, this experiment was performed only once to give a guide as to the amino acid content of the protein, the real values would be obtained from the sequence data (see Chapter 4).

In an attempt to identify the protein, we determined the amino acid sequence of the protein. Pure 53 kDa protein was subjected to gas phase sequencing. No sequence was obtained suggesting that the protein was blocked at the N-terminus. A blocked N terminus has been found in other LEA and LEA-like proteins, the group I p11 protein isolated from pea seeds and the LEA-like Hsp12p stress response protein from *Saccharomyces cerevisiae* (Mtwisha *et al.*, 1998). In order to obtain internal protein sequence, the protein was cleaved with CNBr (which cleaves after methionine residues) or trypsin (which cleaves after arginine and lysine residues).

The digest using CNBr did not produce any peptides (results not shown) indicating that CNBr did not cleave the protein.

Table 3.1 Amino acid content (mole %) of the protein after purification by HPLC.

Residue	Mole %
Glutamic acid (E)	15.0
Lysine (K)	5.2
Aspartic acid (D)	13.1
Glycine (G)	7.1
Alanine (A)	8.3
Serine (S)	8.5
Valine (V)	5.0
Threonine (T)	3.1
Leucine (L)	11.7
Arginine (R)	6.9
Histidine (H)	3.0
Tyrosine (Y)	4.0
Phenylalanine (F)	3.8
Isoleucine (I)	4.2
Methionine (M)	0.9

This might have been due to oxidised methionine residues, which are known not to be substrates for CNBr cleavage. Trypsin digestion produced a complex peptide mixture (not shown) that made it difficult to purify a single peptide for sequencing. This might have been due to incomplete trypsin digestion which would result in a more complex pattern of peptides as well as the fact that the protein mixture was polymorphic, which might add to the complexity of the digest.

To investigate whether proteins similar to the 53 kDa protein had been described previously, the amino acid composition obtained was submitted to the Swiss-Prot database. Seed storage proteins, vicilin and canavalin showed some similarity in their amino acid composition, though at low levels. The ranking scores were 61 and 36, respectively, with a score of 0 representing a perfect match, and increasing values indicating increasing difference.

We propose that the protein be named ASP 53, corresponding to an *Acacia* Seed Protein with a molecular weight of 53 kDa.

3.2.3.3 Glycoprotein test

To determine whether ASP 53 was a glycoprotein, pure ASP 53 was electrophoresed on a SDS-PAGE gel and the gel stained with Periodic Acid Schiff (PAS) reagent. This reagent interacts with aldehyde groups formed by periodate oxidation of vicinal carbohydrate hydroxyl residues. A positive reaction occurred (Figure 3.7) suggesting that some carbohydrate residues were present.

To determine which residues were present, pure ASP 53 was applied to a nitrocellulose membrane which was then probed with *Canavalia ensiformis* (jack bean) Concanavalin A (ConA). This lectin binds to glucose and mannose residues (Chatterjee and Mandal, 2003). A positive result was obtained indicating that ASP 53 was a glucose- or mannose-containing glycoprotein.

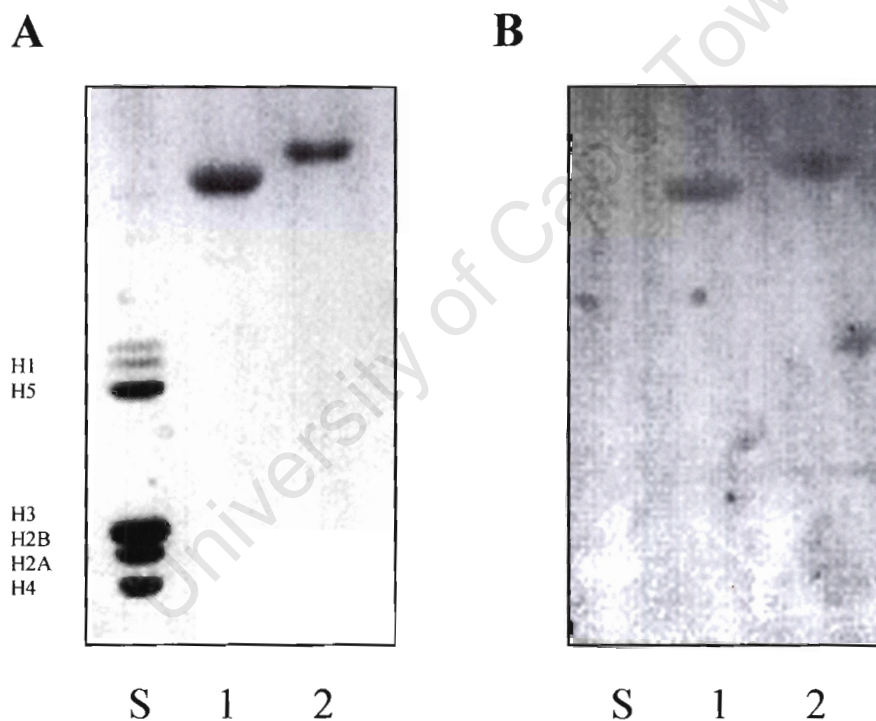


Figure 3.7 Glycoprotein test. A: Coomassie stained SDS-PAGE gel of pure ASP 53 (lane 1) and BSA (lane 2). B: The same gel stained with Periodic Acid Schiff (PAS) reagent. The standard (S) is a total extract of chicken erythrocyte histones with molecular masses (kDa) of: H1, 22.5; H3, 15.3; H2B, 13.7; H2A, 14.0 and H4, 11.2.

3.2.3.4 Circular Dichroism

To investigate the anomaly that a protein containing substantial quantities of hydrophobic amino acids still remained soluble at high temperatures, the secondary structure of native ASP 53 was determined using circular dichroism (CD) spectroscopy. ASP 53 used in this study was purified directly from the total protein extract without exposure to either elevated temperature or denaturants. CD spectroscopy was performed in 10 mM sodium phosphate buffer pH 7.4 and in the same buffer including either 2 M NaCl or 1 M sorbitol to investigate whether conformational changes were induced by increased osmolality. Deconvolution of the spectrum obtained under physiological conditions (Figure 3.8) showed that approximately 41 % of the secondary structure of ASP 53 was in the antiparallel β -sheet conformation with a further 33 % existing in a random coil conformation. Addition of either salt or sorbitol resulted in an increased ellipticity. Slight changes were observed in the secondary structure elements of the protein, with a 4 % loss of antiparallel β -sheet and 2 % increase in random coil structure. From these results we hypothesized that the solubility of ASP 53 at elevated temperatures was brought about by the hydrophobic faces of amphipathic sheets interacting with one another thereby presenting a hydrophilic face to the environment.

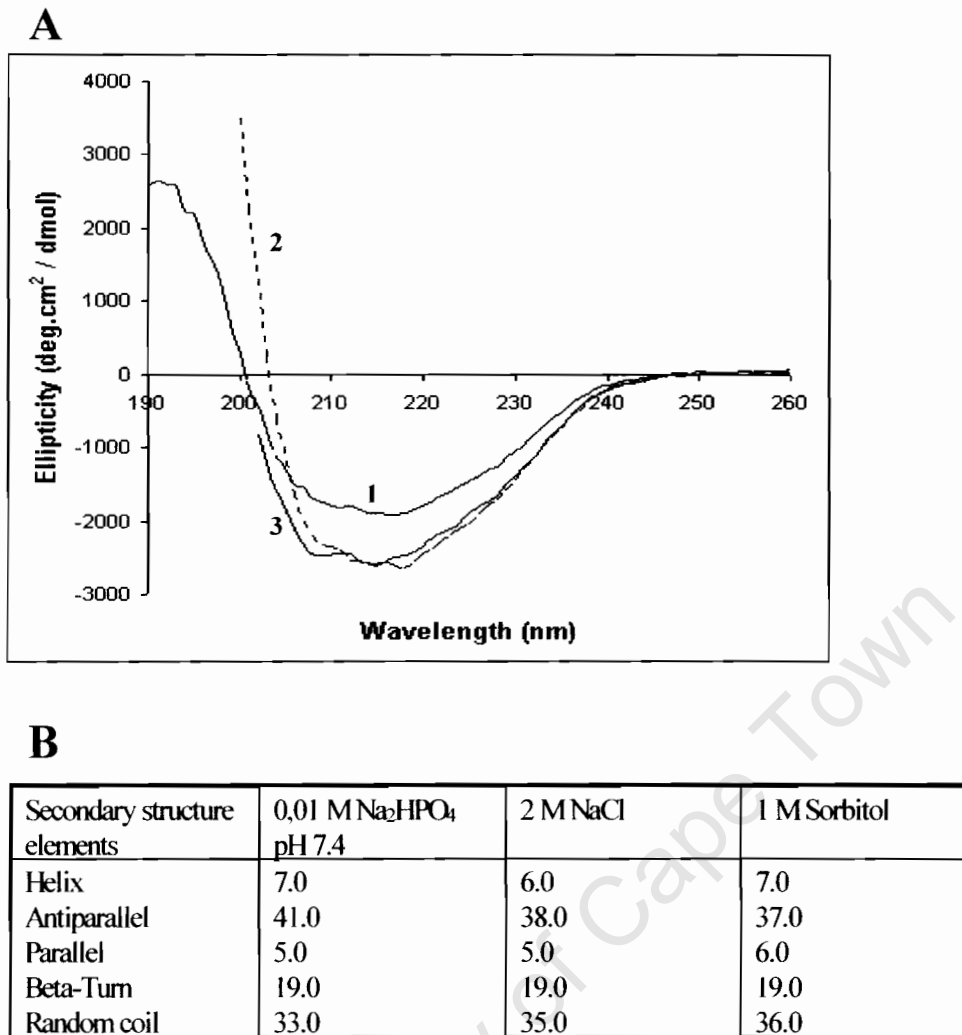


Figure 3.8 A: Circular dichroism spectra of ASP 53 in 10 mM Na₂HPO₄ pH 7.4 (1) and in this same buffer containing either 1 M sorbitol (2) or 2 M NaCl (3). B: Percentage of secondary structure elements obtained after deconvolution of each spectrum shown in (A) above.

We next determined the effects of temperature on the secondary structure of ASP 53 using CD spectroscopy. Spectroscopic measurements were performed in 100 mM K_2HPO_4 pH 7.4 at increasing temperatures from 20 °C to 80 °C, with increments of 20 °C. No conformational changes were observed when ASP 53 was exposed to elevated temperatures up to 80 °C (Figure 3.9), suggesting that the conformation of ASP 53 was very stable and temperature independent. Since CD spectroscopy is an averaging technique that reports on the structure of the entire molecule, we investigated whether tryptophan fluorescence emission spectroscopy, which reports on micro-environments of the tryptophan residues only, would yield a similar result. Accordingly the 335 nm fluorescence of the molecule was determined as a function of temperature. Since fluorescence decreases due to increased temperature, the change in ASP 53 fluorescence was compared with that of free tryptophan. Figure 3.10 shows that the fluorescence of ASP 53 decreased as a function of temperature up to 80 °C but at a similar rate to that of free tryptophan, suggesting that no structural changes had occurred over this temperature range. Above this temperature the fluorescence increased sharply suggesting that unfolding occurred.

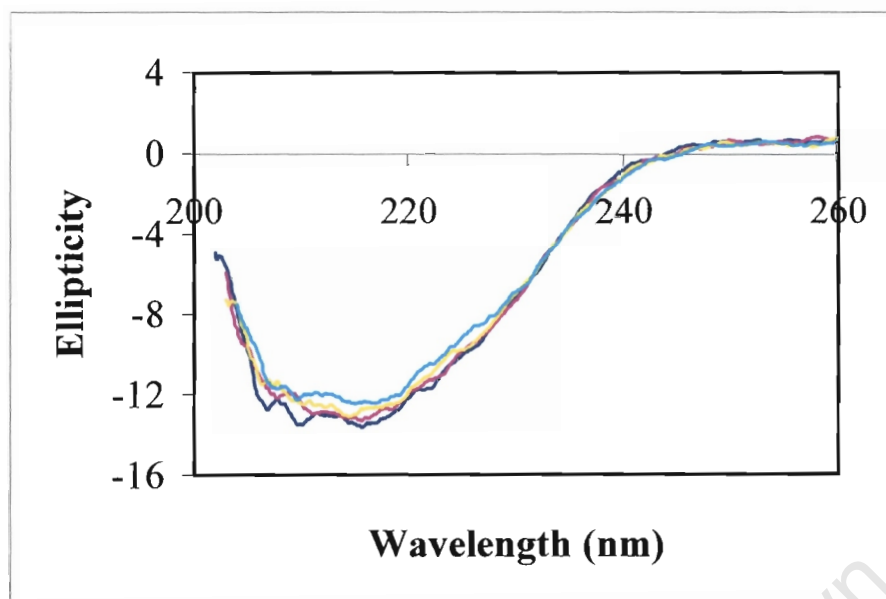


Figure 3.9 Effect of temperature on the secondary structure of ASP 53. Circular dichroism spectra of ASP 53 in 100 mM K_2HPO_4 pH 7.4 at — 20 °C, — 40 °C, — 60 °C and — 80 °C.

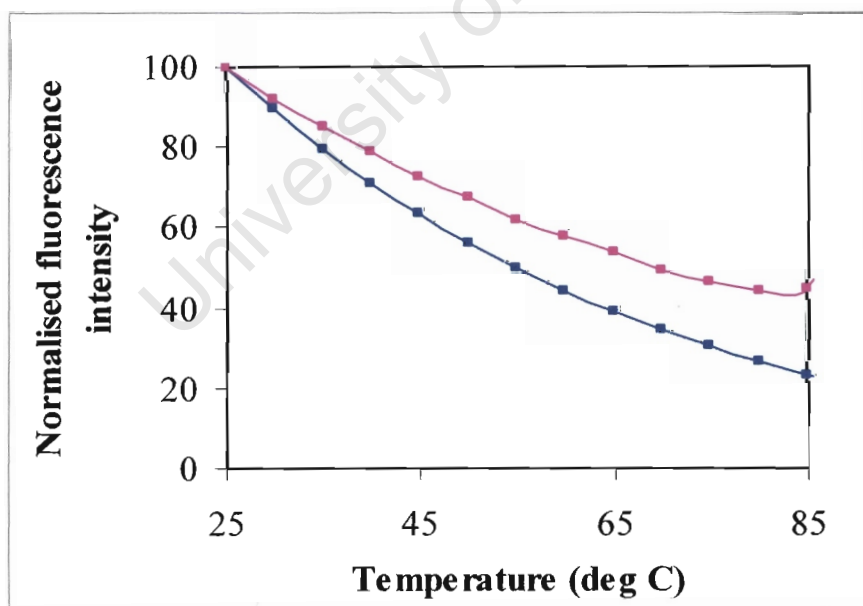


Figure 3.10 Emission fluorescence spectroscopy of ASP 53. Fluorescence of ASP 53 in 100 mM K_2HPO_4 pH 7.4 — as a function of temperature, compared with that of tryptophan in the same buffer —.

3.2.4 Western Blot

To investigate the expression profile and the localisation of ASP 53 in the cell, antibodies to this protein were produced in rabbits. An ELISA assay showed that the antibody produced could be detected using pure ASP 53 as the antigen using antiserum diluted 1:10⁸. We next investigated the specificity of the antibody produced by western blotting a gel of total *A. erioloba* seed protein with the antiserum diluted 1:10⁵. Figure 3.11 shows that ASP 53 was the only protein from the extract that cross-reacted with the antibody. No proteins were detected using the pre-immune serum as a control. The presence of proteins similar to ASP 53 in the seeds of other *Acacia* species was investigated. Total proteins were therefore extracted from the seeds of various *Acacia* species, electrophoresed on a SDS-PAGE gel, which was then western blotted using a 1:10⁵ dilution of the antibody. Proteins with a molecular size similar to ASP 53 were present in seeds of all *Acacia* species tested, except for the seeds of *Acacia burkei* (Figure 3.12.).



Figure 3.11 Western blot of total proteins extracted from *A. erioloba* seeds. A: SDS-PAGE of total proteins extract (lane 1). B: After SDS-PAGE electrophoresis, total proteins were transferred onto the nitrocellulose membrane, which was probed with the anti-ASP 53 antibody. Standard (S) as for Figure 3.7.

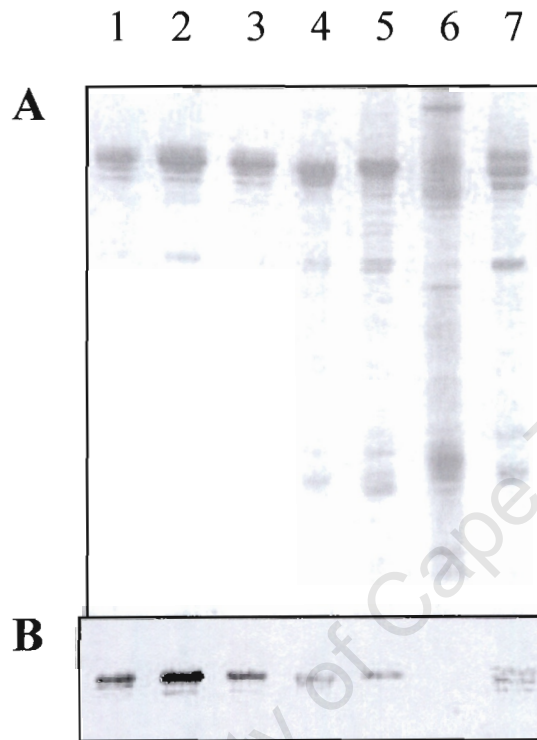


Figure 3.12 Total protein and ASP 53-like protein content in the seeds of different *Acacia* species. A: SDS-PAGE of total proteins extracted from various species of *Acacia*: lane 1. *A. haemotoxylon* 2. *A. tortilis* 3. *A. galpinii* 4. *A. xanthophloea* 5. *A. erioloba* 6. *A. burkei* 7. *A. sieberiana*. B: Western blot, using an anti-ASP 53 antibody, of the ASP 53-migrating portion of the gel. No other immunopositive protein bands were detected.

3.2.5 Determining ASP 53 content during germination

Since ASP 53 possessed the LEA protein property of heat solubility, we investigated whether this protein was present in germinating embryos. Since newly germinated embryos have been reported to retain desiccation tolerance, we also investigated whether there was a correlation between the presence of ASP 53 and the retention of desiccation tolerance. Seeds were therefore germinated to various stages as determined by the length of the radicle (15 mm, 25 mm and 50 mm). The seedlings were desiccated for 12 h at 20 °C in a stream of dry air. After rehydration in the dark at 30 °C, the viability was determined. Seedlings with radicle lengths of 15 mm, 25 mm and 50 mm had water content (on a dry mass basis) of 5.4 %, 5.0 % and 4.7 %, respectively indicating that these seedlings had been thoroughly desiccated by this procedure. The ASP 53 content as determined by SDS-PAGE was measured upon germination (Figure 3.13) and after dehydration and subsequent rehydration of the germinated seedlings. Mature seeds were used as a control. We found that the ASP 53 content, like that of most LEA proteins, decreased in both axes and cotyledons during germination, although small quantities were still present in the cotyledon of seedlings with a radicle length of 50 mm (Figure 3.13). No ASP 53 was present in the axes of these seedlings. ASP 53 degradation products, characterised by being recognised by the anti-ASP 53 antibody, accumulated as the radicle length increased. We found that seedlings with radicle lengths of 15 mm and 25 mm exhibited normal growth upon rehydration, whereas seedlings with a radicle length of 50 mm lost the ability to resume growth (not shown). This correlation between the loss of ASP 53 content in the radicle and the inability of the germinated seedling to resume growth after desiccation and subsequent

rehydration suggested a possible involvement of this protein in the acquisition of desiccation tolerance in *A. erioloba*.

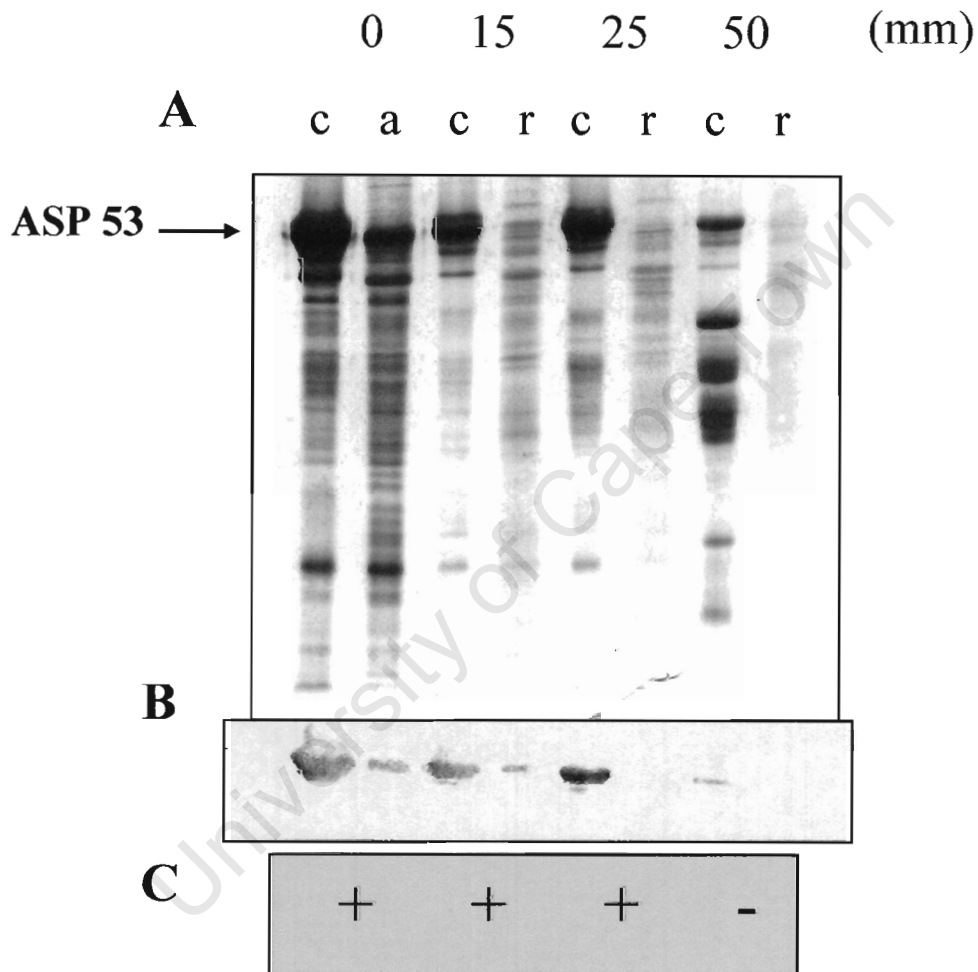


Figure 3.13 ASP 53 content of *A. erioloba* seeds during germination. A: SDS-PAGE of total proteins extracted from the cotyledons (c) and the axis (a) of mature seeds (radicle length = 0) and from the cotyledons (c) and radicles (r) of germinated seedlings. The radicle length is shown above these lanes. B: Western blot of the ASP 53-migrating portion of the gel using the anti-ASP 53 antibody. No immunopositive protein bands were detected. C: Desiccation tolerance (+) or desiccation sensitivity (-) of the seedlings used above.

3.2.6 Immunolocalisation of ASP 53

The location of ASP 53 in the axis and cotyledon of mature *A. erioloba* seeds was investigated using immunocytochemistry using the anti-ASP 53-antibody. Examination of thin sections of axes showed that gold particles, indicative of the presence of ASP 53, were only found within the cell wall (Figure 3.14.1a). Significantly more gold particles were observed when sections of cotyledons from mature seeds were examined (Figure 3.14.1b). This was not surprising as Figure 3.13 showed that considerably more ASP 53 was present in the cotyledon compared with the axis in mature seeds. The gold particles observed were present both in the cell wall and in the vacuoles in approximately equal concentrations.

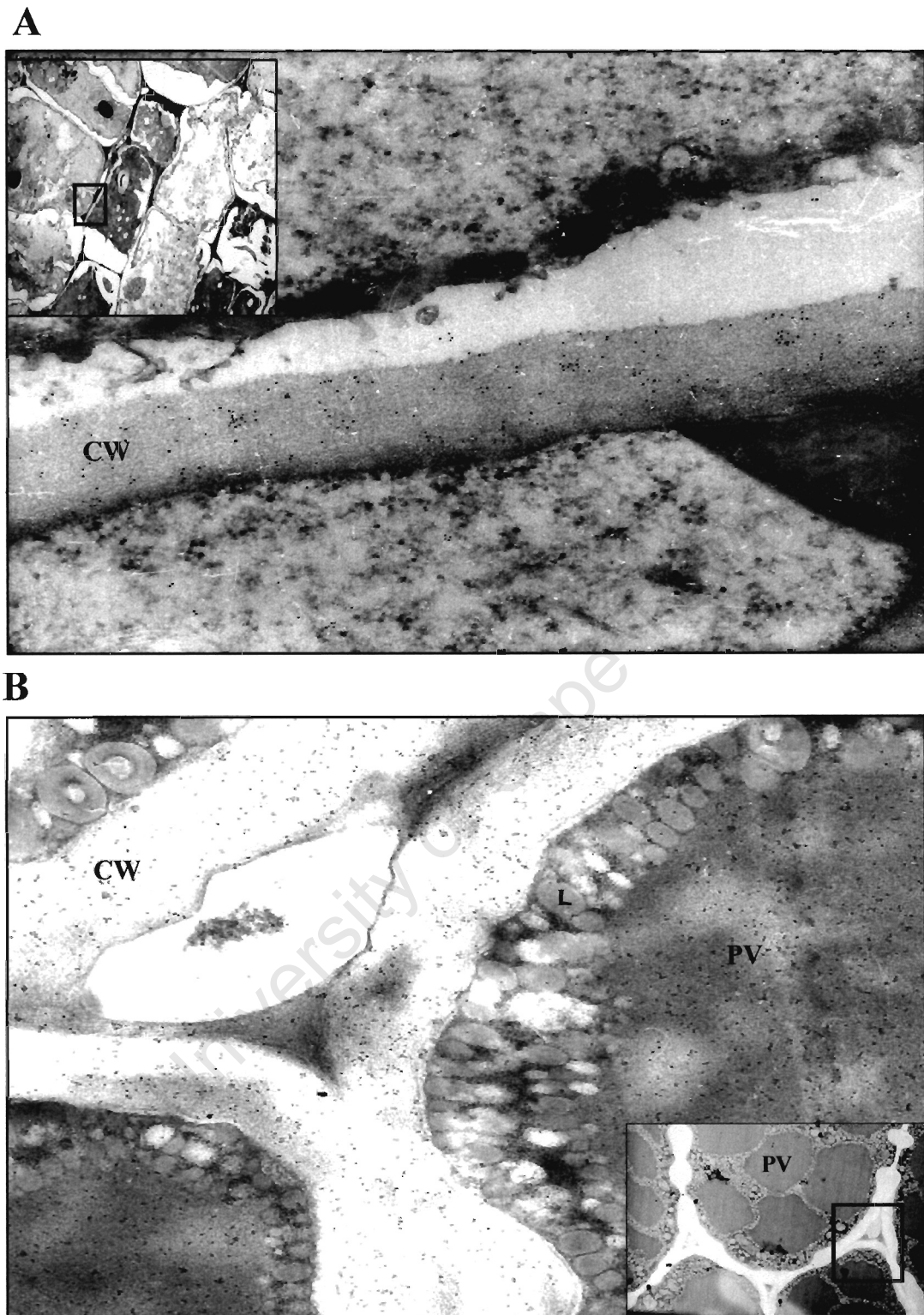


Figure 3.14.1 Immunolocalisation of ASP 53 in mature seeds of *A. erioloba* using an anti-ASP 53 antibody. We examined sections of: A: Axes and B: Cotyledons. The insets in (A) and (B) are lower magnification of axes and cotyledons, respectively from mature seeds shown to situate cells in higher magnification micrographs. CW, Cell Wall; PV, Protein vacuole; L, Lipid. Magnification: (A) x 45 000, inset x 1 000 ; (B) x 17 500, inset x 1 588.

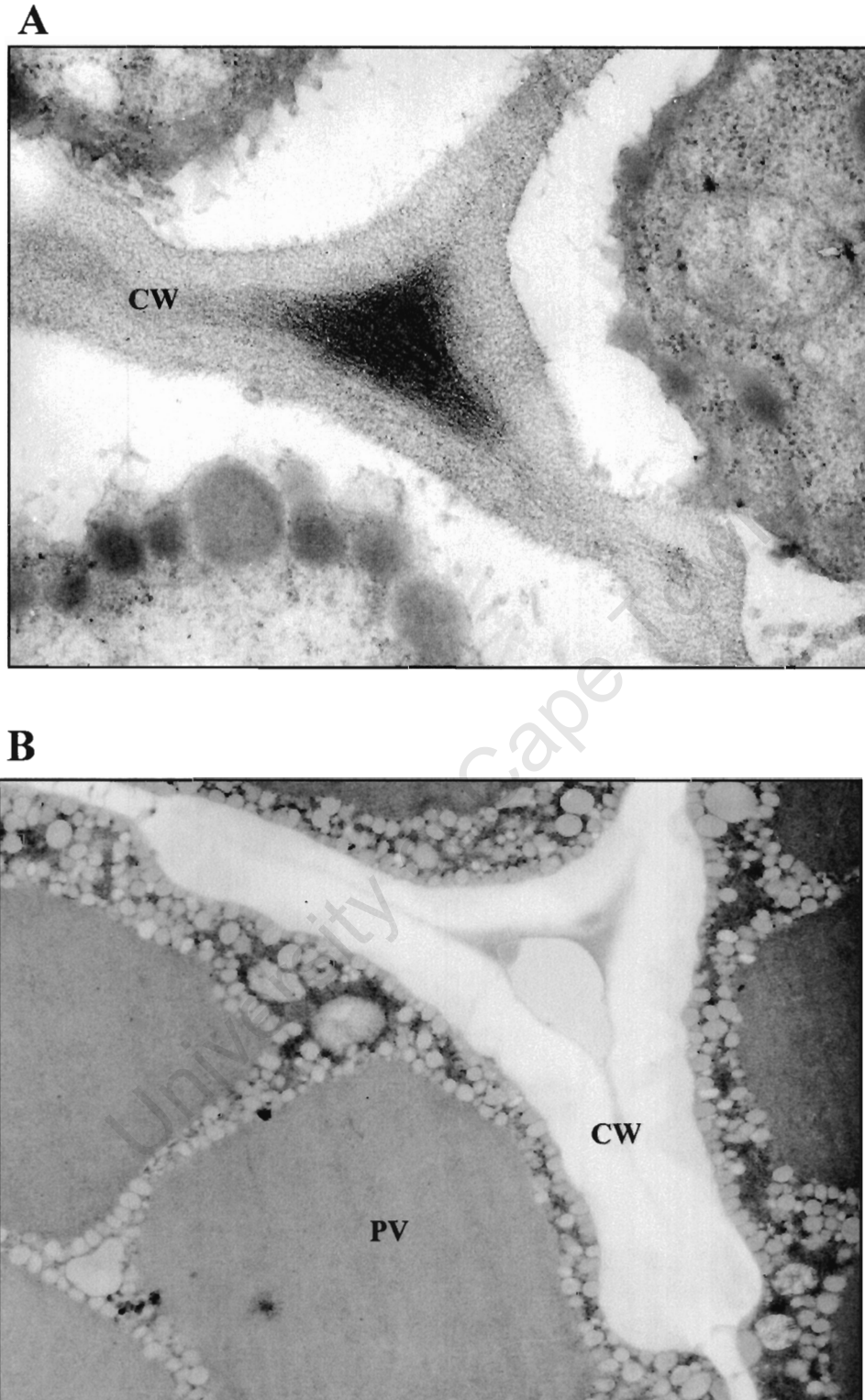


Figure 3.14.2 Sections of axes (A) and cotyledons (B) from mature seeds of *A. erioloba* probed with pre-immune serum. CW, Cell Wall; PV, Protein vacuole; L, Lipid. Magnification: (A) x 48 000; (B) x 9 420.

We next determined the location of ASP 53 in the cell during the germination process to investigate whether the loss of this protein from a particular site could be correlated with a loss of desiccation tolerance. Germinated seedlings with radicle lengths of 15 mm (desiccation tolerant) and 50 mm (desiccation sensitive) were investigated. In the case of seedlings with radicle lengths of 15 mm (Figure 3.14.3), the intensity of the gold labelling in the cell wall of the radicle decreased compared with that in mature seeds. This decrease was more pronounced in the case of seedlings with 50 mm length radicles (Figure 4.14.4). Significant ultra-structural changes were observed during germination as shown in the inset in Figure 3.14.3, including the formation of distinct starch granules in radicles of seedlings with 15 mm length radicles. These granules, with which gold particles were associated, were not present in radicles of seedlings with 50 mm length radicles. In the case of cotyledons from seedlings with radicle lengths of 15 mm (Figure 3.14.5) fewer gold particles were observed in both the cell wall and in the vacuoles, with approximately equivalent labelling observed within these two organelles. Continued germination to a radicle length of 50 mm resulted in the disappearance of gold particles in the vacuoles, although these were still present in the cell wall (Figure 3.14.6). Gold-labelled starch granules were also observed within the cotyledons of germinated seedlings with both 15 mm and 50 mm length radicles. Electron dense bodies of unknown material were present within the vacuoles of cotyledons of germinated seedlings with 50 mm length radicles (Figure 3.14.6). Gold particles were observed to interact with these bodies. No gold particles were visible when similar sections to those described above were examined using the pre-immune serum. We next determined whether the presence of gold particles in the starch-containing bodies was

brought about by antibodies to the carbohydrate portion of ASP 53 cross-reacting with the starch. Western blot analysis of a SDS-PAGE gel containing ASP 53 was performed using the anti-ASP 53 antibody in the presence of 5 % starch. Despite this, the antibody detected ASP 53 (results not shown). This was not surprising since the structures of starch and the carbohydrate moieties of glycoproteins are significantly different.

These results suggest that ASP 53 was located largely in the cell wall of the axis and the cotyledon of mature seeds. Upon germination, there was rapid decline in the number of gold particles associated with the cell wall of both the axis and cotyledon. This observed decrease in labelling correlated with the loss of ASP 53 during germination and coincided with the inability of the seedlings to resume growth after desiccation and subsequent rehydration. It would therefore appear that ASP 53 might be involved in the protection of mature seeds from damages induced by water loss via stabilisation of the cell wall. Although most reports show that LEA proteins are located in the cytosol, it has recently been shown that the yeast LEA-like protein, Hsp 12, was located in the cell wall of *S. cerevisiae* where it was postulated to modulate the elasticity of the wall (Motshwene *et al.*, 2004). Association of ASP 53 with nutritional storage organelles suggested that the protein might also act as a storage protein. Although ASP 53 possesses properties similar to LEA proteins, it also has several characteristics typical of seed storage proteins. These include the fact that it is present in substantial quantities in mature seeds, it is glycosylated, has a high aspartic acid and glutamic acid content and is rapidly degraded during germination. We therefore concluded that ASP 53 could be a seed storage protein involved in

protection of seeds against conditions of stress. Some seed storage proteins (Breiteneder and Radauer, 2004) and vegetative storage proteins (Avice *et al.*, 2003) have been reported to play an adaptive role against abiotic and biotic stresses in plants.

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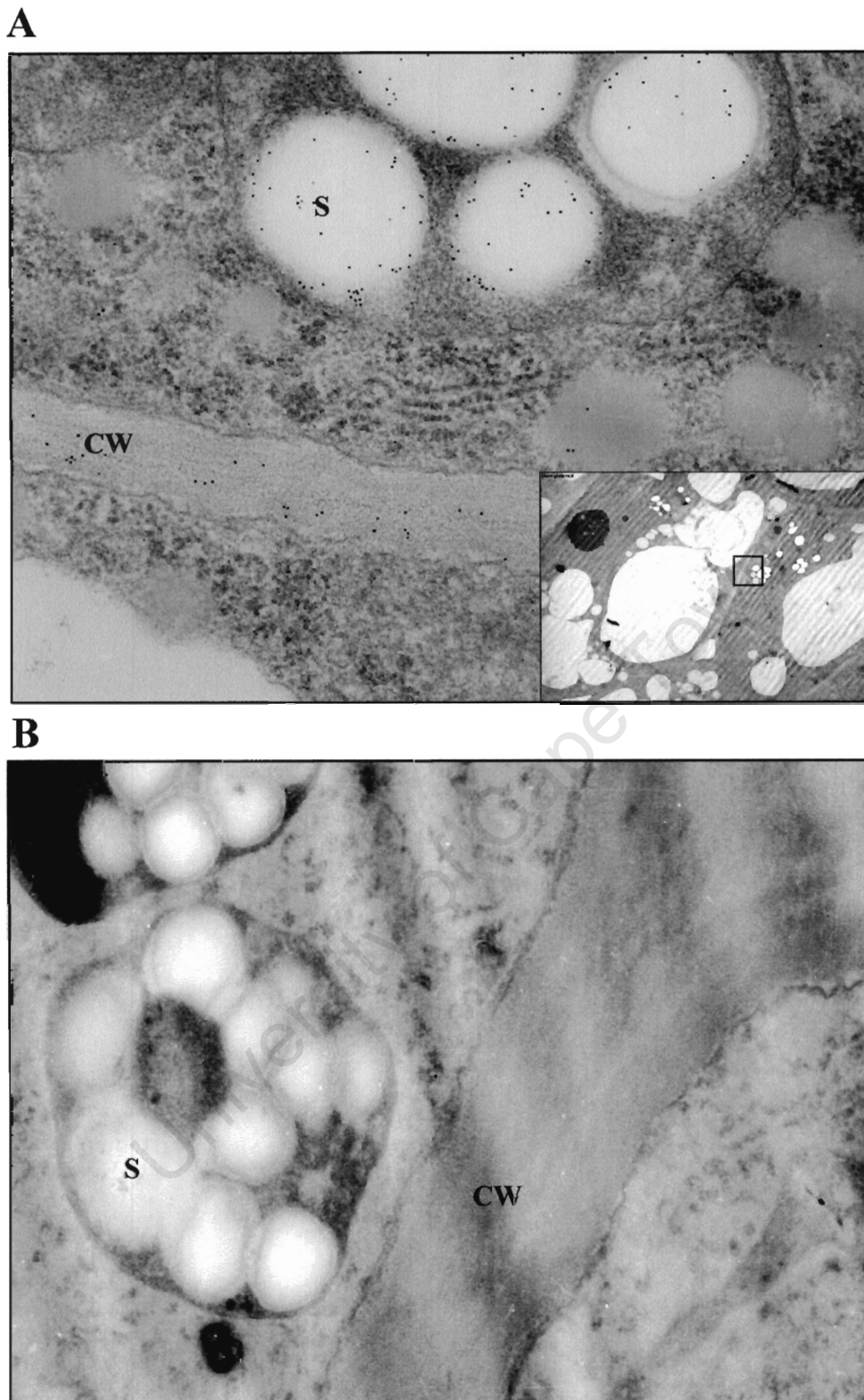


Figure 3.14.3 Immunolocalisation of ASP 53 in 15 mm radicle from germinated seedlings using an anti-ASP 53 antibody (A) or pre-immune serum (B). The inset situates Figure A. S, Starch granules; CW, Cell Wall. Magnification: (A) x 50 000, inset x 1 666; (B) x 30 000.

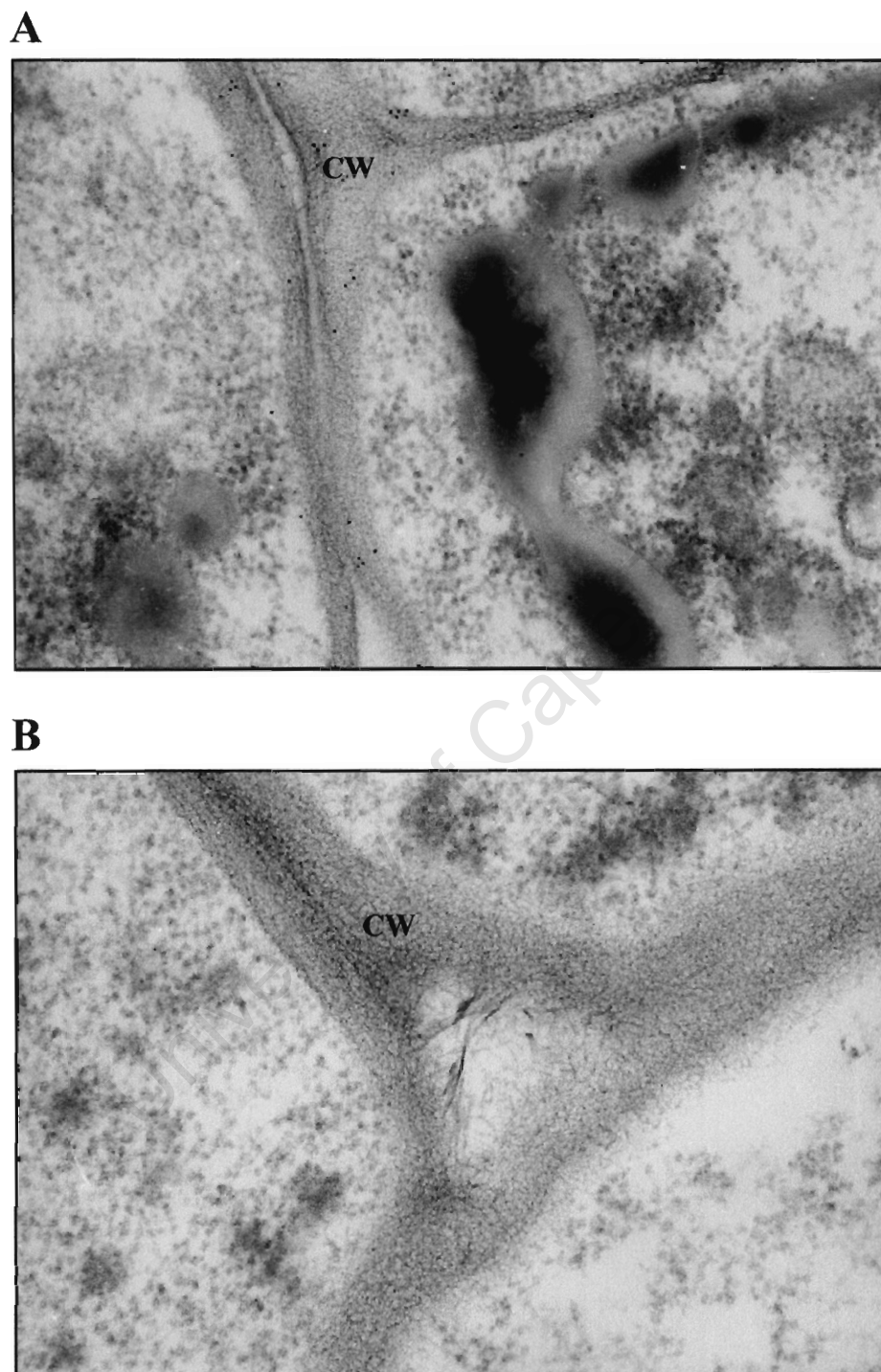


Figure 3.14.4 Immunolocalisation of ASP 53 in 50 mm radicle from germinated seedlings using an anti-ASP 53 antibody (A) or pre-immune serum (B). CW, Cell Wall. Magnification: (A) x 50 000; (B) x 65 000.

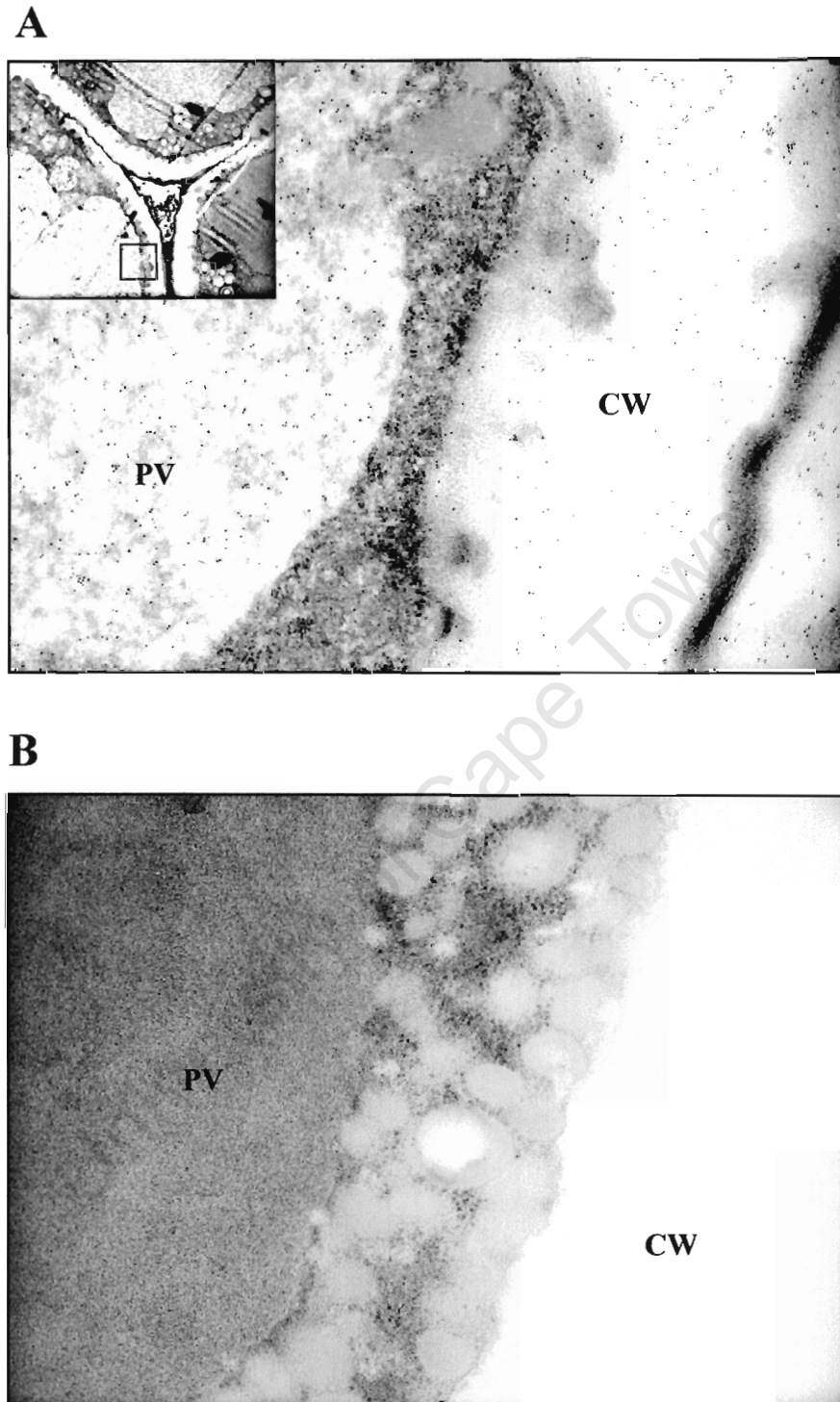


Figure 3.14.5 Immunolocalisation of ASP 53 in cotyledons from germinated seedlings with radicle length of 15 mm using an anti-ASP 53 antibody (A) or pre-immune serum (B). The inset in (a) shows the overall intracellular structure of cotyledons from germinated seedlings with radicle length of 15 mm. PV, Protein vacuole; CW, Cell Wall. Magnification: (A) x 40 5000, inset x 1 800; (B) x 36 666.

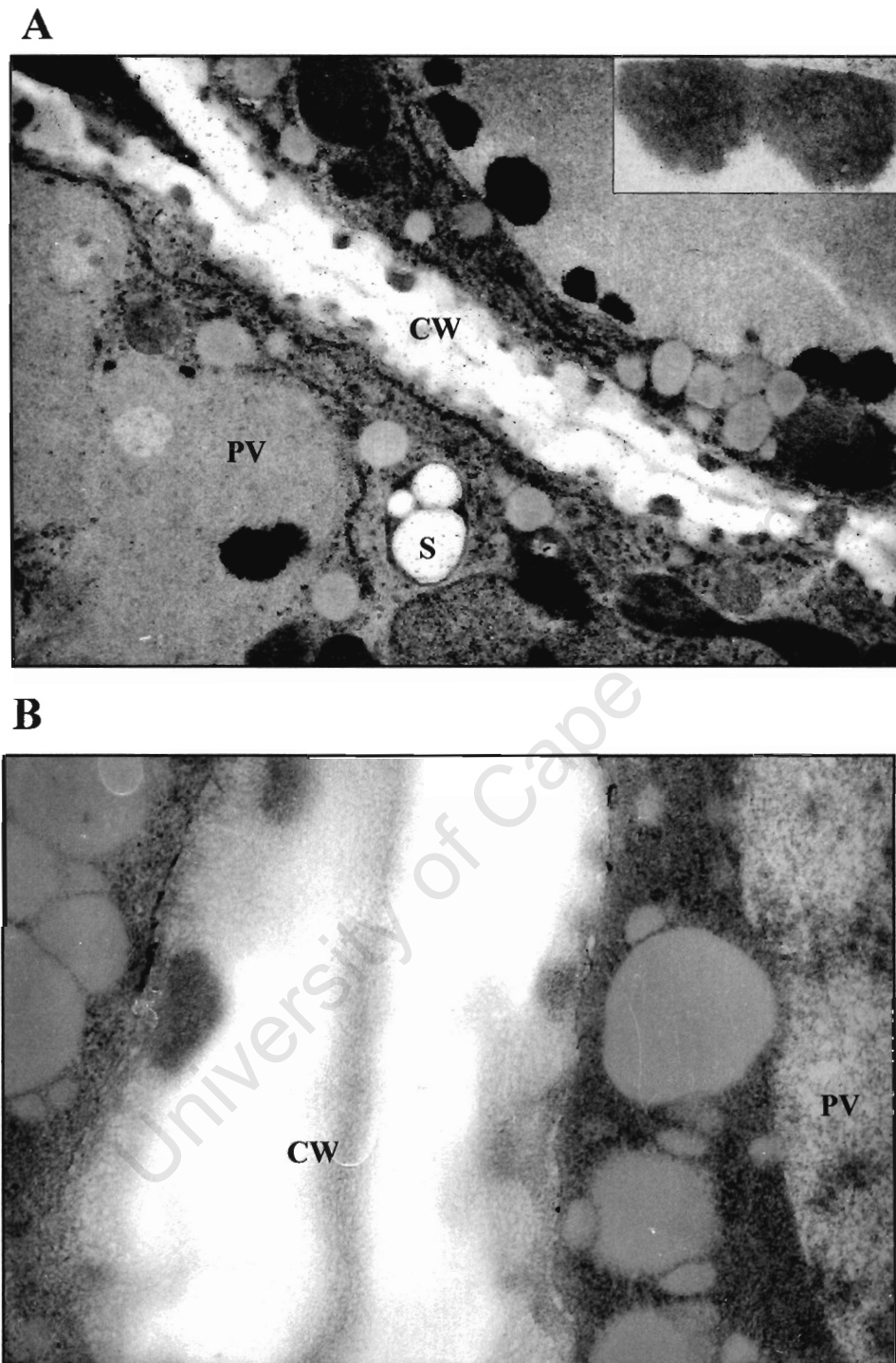


Figure 3.14.6 Immunolocalisation of ASP 53 in cotyledons from germinated seedlings with radicle length of 50 mm using an anti-ASP 53 antibody (A) or pre-immune serum (B). The inset shows the gold labelled electro dense bodies located within the vacuoles. S, Starch granules; PV, Protein vacuole; CW, Cell Wall. Magnification: (A) x 10 500, inset x 26 315; (B) x 36 000.

3.2.7 The role of ASP 53

Since we found that ASP 53 experienced no conformational changes as the temperature increased, we investigated whether the role of this protein was to protect other macromolecules against thermal stress. We chose to investigate this by determining the effect of ASP 53 on changes in target protein activity and conformation brought about by increased temperature. Thermal denaturation of proteins has been reported to occur in three phases as the temperature increases or by increased time at an elevated temperature (He *et al.*, 1997). In the first stage, enzymatic activity is lost concomitant with undetectable conformational changes surrounding the active site. In the second phase, significant conformational changes occur, which eventually result in unfolding of the protein and aggregation (third phase), brought about by hydrophobic interactions.

To investigate whether ASP 53 could prevent loss of enzymatic activity brought about by high temperature, yeast alcohol dehydrogenase (ADH) was incubated in the presence of varying concentrations of ASP 53 at 55 °C and the activity determined at various times up to 50 min. It was found (not shown) that ASP 53 had no effect on the specific activity of ADH under the assay conditions used. 55 °C was chosen as the incubation temperature as this temperature resulted in the loss of enzyme activity within 1 h. Figure 3.15 showed that whereas incubation of ADH alone at 55 °C resulted in the complete loss of enzymatic activity after 50 min, incubation in the presence of 3 mg/ml ASP 53 resulted in approximately 20 % of the enzymatic activity retained after this period. This increased to approximately 50 % when the ASP 53 concentration was increased to 6 mg/ml and to approximately 65 % in the

presence of 12 mg/ml ASP 53. To determine whether the protection conferred by ASP 53 was specific to this protein or whether other proteins had a similar effect, a protein of similar molecular weight, ovalbumin (45 kDa), was used in place of ASP 53. Incubation of ADH in the presence of 12 mg/ml ovalbumin resulted in approximately 15 % of the enzymatic activity retained after 50 min. The secondary structure of ADH was determined using CD spectroscopy before and after incubation at 55 °C that lead to a complete loss of enzymatic activity. The results (not shown) indicated that no change in secondary structure occurred due to incubation at 55 °C confirming that higher temperatures were required to induce detectable conformational changes. These were found to occur at approximately 85 °C.

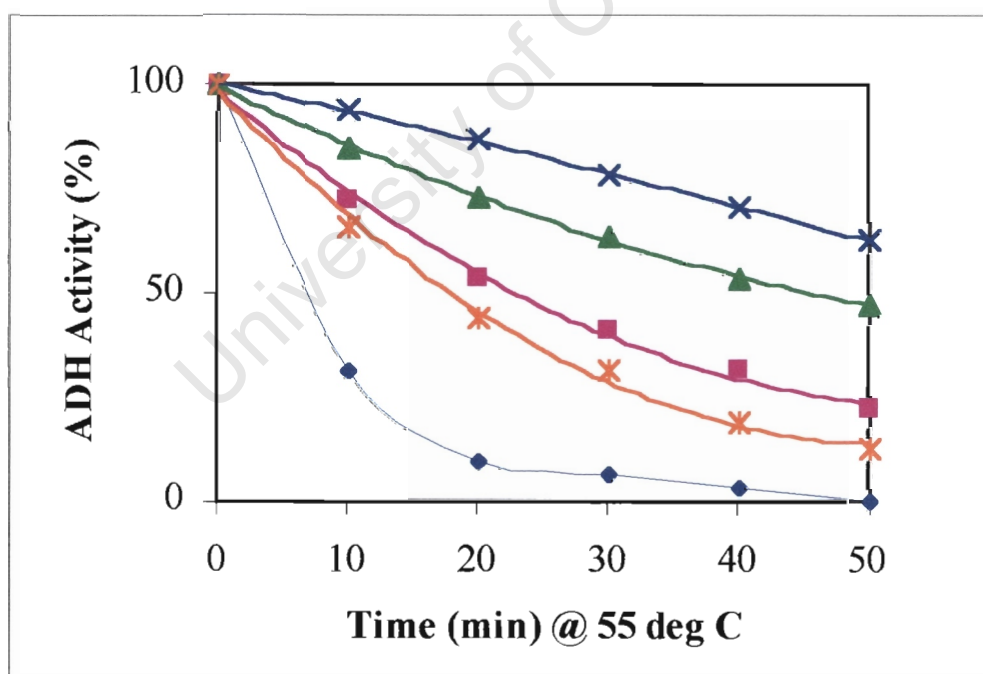


Figure 3.15 Percentage enzymatic activity of ADH as a function of time of incubation at 55 °C. ADH alone —◆—; in the presence of 3 mg/ml ASP 53 —■—; or 6 mg/ml ASP 53 —▲—; 12 mg/ml ASP 53 —×—; or 12 mg/ml ovalbumin —*—.

With ADH only exhibiting significant structural changes when incubated at 85 °C, a more labile protein, haemoglobin was chosen to investigate the effect of ASP 53 on conformational changes brought about by high temperature. This study was conducted by measuring the CD spectra of haemoglobin incubated alone or in the presence of ASP 53 at increasing temperatures, from 20 °C to 80 °C in increments of 10 °C. Figure 3.16.1 showed that no changes in the secondary structure of haemoglobin occurred when haemoglobin was incubated alone up to 50 °C (A). Small conformational changes were observed when haemoglobin was incubated at 60 °C, with significant changes apparent when the temperature was increased to 70 °C with a further conformational change observed at 80 °C. Incubation of haemoglobin in the presence of ASP 53 at these temperatures resulted in preservation of the secondary structure of haemoglobin, with a significant reduction in conformational changes observed up to 60 °C (B). At higher temperatures, 70 °C and 80 °C, conformational changes occurred but these were far lower than those observed in the absence of ASP 53. In an attempt to quantitate these changes, the effect of ASP 53 on the α -helical content (the 225 nm ellipticity) of haemoglobin was determined as a function of temperature from the data shown in Figure 3.16.2. These showed that ASP 53 protected the α -helices in haemoglobin against thermal denaturation from 40 °C but with the most pronounced relative protection observed above 60 °C.

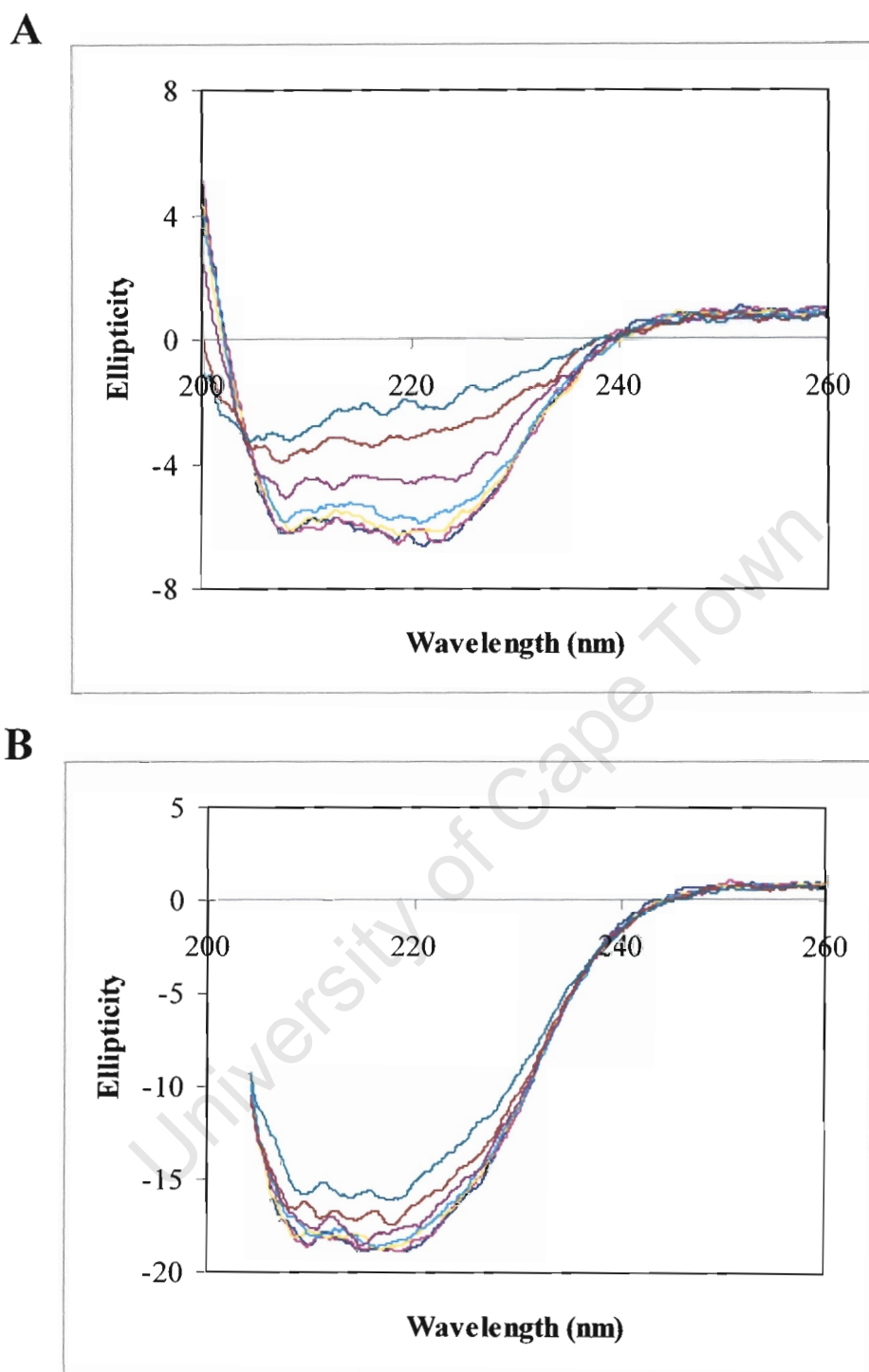


Figure 3.16.1 The effect of ASP 53 on temperature-induced conformational changes in haemoglobin. CD spectra of haemoglobin alone (A) or in the presence of 0.6 mg/ml ASP 53 (B) at: — 20 °C; — 30 °C; — 40 °C; — 50 °C; — 60 °C; — 70 °C; — 80 °C. The buffer used was 100 mM K_2HPO_4 pH 7.4.

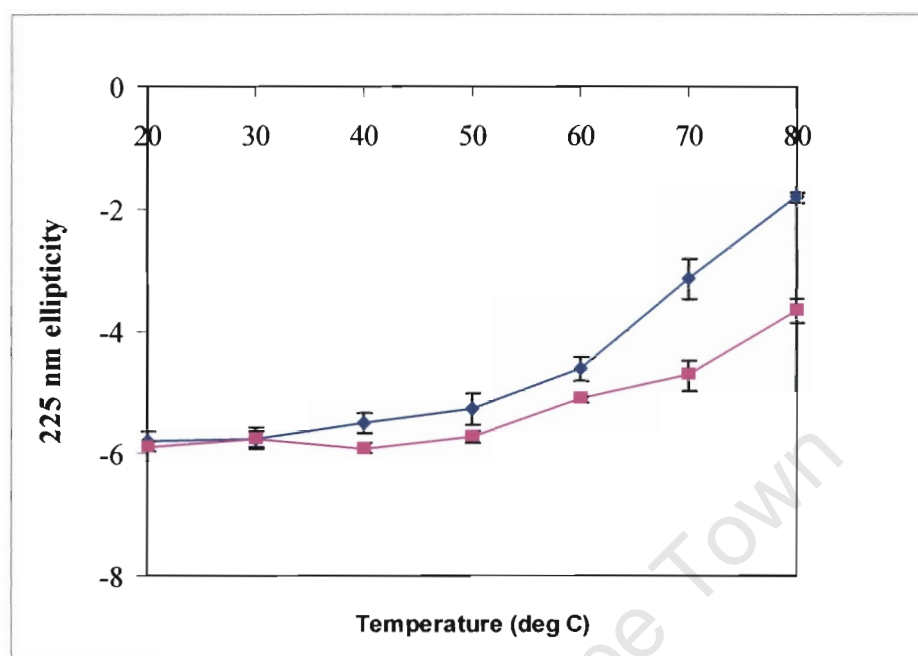


Figure 3.16.2 The effect of ASP 53 on the α -helical content (determined from the ellipticity at 225 nm) of haemoglobin as a function of temperature. — Haemoglobin alone; — in the presence of 0.6 mg/ml ASP 53.

To investigate whether ASP 53 could prevent aggregation of unfolded proteins, we incubated a diluted solution of egg white protein alone or together with increasing concentrations of ASP 53 at temperatures between 20 °C and 90 °C and determined the turbidity of the solution at 350 nm as a function of the temperature. 350 nm was chosen as neither the egg white protein nor ASP 53 absorbed light of this wavelength.

Figure 3.17 shows that the egg white protein solution exhibited no turbidity up to 70 °C, after which it increased markedly reaching a plateau above 80 °C. Addition of increasing concentrations of ASP 53 to the egg white protein solution resulted in a marked decrease in the turbidity observed with a concentration of 1.5 mg/ml ASP 53 completely abolishing protein aggregation. At the same time the T_M of the protein denaturation transition increased from approximately 72 °C to approximately 77 °C.

These results demonstrated that ASP 53 can potentially protect proteins in *A. erioloba* seeds against thermal denaturation. We therefore concluded that ASP 53 has a dual role in these seeds acting as a protective agent as well as a seed storage protein.

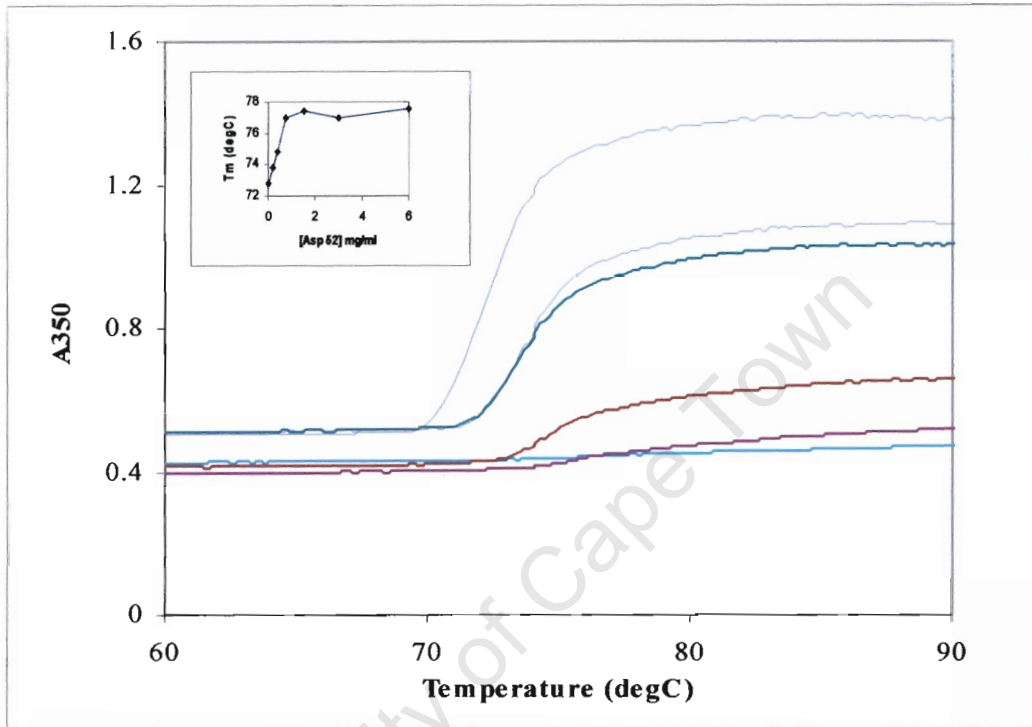


Figure 3.17 The effect of ASP 53 on temperature-induced protein aggregation of total chicken egg white proteins. The turbidity of the protein solution alone — or in the presence of 0.09 mg/ml —; 0.19 mg/ml — ; 0.38 mg/ml — ; 0.75 mg/ml — ; 1.5 mg/ml ASP 53 — at different temperatures. The inset shows the T_M of the main transition of each experiment.

CHAPTER 4

Protein identification

4.1 INTRODUCTION

Protein sequencing is traditionally performed using the Edman degradation, which involves derivatisation of the N-terminus of the protein with Phenyl isothiocyanate (PITC) followed by cleavage of the derivatised amino acid. The cleaved amino acid is identified by comparing its retention time on HPLC to those of amino acid standards. This two-step procedure is repeated, leading to the amino acid sequence of the protein. Recently use of the mass spectrometer has replaced the Edman degradation since it is both faster and more sensitive. A further advantage of sequencing using the mass spectrometer is that the Edman degradation cannot be used to sequence peptides and proteins with blocked N-termini.

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) is an analytical technique that measures the molecular weight of molecules based on their flight in a charged state in an electric field. Charged particles are formed by mixing the analyte with the matrix, a small organic molecule that absorbs light at the wavelength of the laser. The analyte becomes incorporated into the crystal lattice of the matrix, which is then bombarded with a laser, resulting in desorption or ionization of the matrix and analyte by either protonation or deprotonation. Ions produced are then accelerated from the ion source through the flight tube at a constant velocity to the detector. The detector records the time each ion takes to move from the source through the field free region (flight tube) to the detector. It has also been observed

that, after ions have accelerated from the source, they dissociate in the field free region before arriving at the detector. Since this ion fragmentation occurs after acceleration in the field free region, all product ions maintain the velocity of the precursor ion. This results in all the ions arriving at the detector at the same time when the spectrometer is used in a linear Time of Flight (ToF) mode. Since the product ions have a lower mass than the precursor ion, they have a lower kinetic energy. The precursor ion is easily selected for analysis from a complex mixture of ions by using an ion gate. The drawback with a linear reflectron is that it only accommodates a small range of kinetic energy differences, i.e it can only focus ions close in mass to the mass of the precursor ion. To focus ions of lower mass, the voltage on the reflectron has to be dropped sequentially (stepping the reflectron). In a conventional reflectron several different voltages, or segments, are required to focus the entire mass range of the product ions. These segments are then “stitched” together to generate a complete product ion spectrum.

In order to identify peptides using the MALDI-TOF mass spectrometer, the protein is initially digested with trypsin and the masses of the resulting peptide mixture analysed. Since the distribution of arginine and lysine residues differs in each protein, each protein will produce a unique “fingerprint” of peptide masses. These masses can be compared to theoretical peptide masses calculated for each protein in various protein sequence databases. Proteins with a peptide mass fingerprint that closely match the submitted peptide fingerprint are then ranked using various scoring algorithms. In many cases protein identification is definitive, although this is never the case for novel proteins.

In order to identify novel proteins as well as proteins that yielded peptide mass fingerprints that could not be unambiguously identified, the technique of post source decay (PSD) has been used (Spengler, 1997). Peptide fragmentation during PSD results in the production of b-type and y-type ions resulting from peptide cleavage at the N-terminus and C-terminus respectively. These two types of ions are the most prominent ions present, although traces of a-ions resulting from small molecule losses from both b and y ions are also observed. Despite the complex fragmentation patterns, the data can be used to determine the peptide sequence. To reduce the complexity of the data, the tryptic peptides are derivatised in such a manner as to produce only y-type ions. This is performed in two steps. In the first step lysine residues are converted to the more basic homoarginine residues using O-Methylisourea hydrogen sulphate. This derivatisation results in the protection of the ϵ -amino group of the lysine side chain against sulfonation in the second step and increases the mass of the peptide by 42 atomic mass units. The second step introduces a sulfonic acid group at the N-terminus by reaction of the peptide with the chemically assisted fragmentation (CAF) reagent and increases the mass of the peptide by a further 136 atomic mass units. The resulting peptide, modified at the N-terminus with the negatively charged CAF reagent and with a positively charged (homo)arginine residue at the C-terminus, is electrically neutral. This parent peptide is not attracted to the negative quadrupole. PSD is brought about by random ionisation and subsequent fragmentation of the peptide resulting in family of positively charged C-terminal peptides (the y-ions) that are attracted to the negative quadrupole together with a family of neutral CAF-containing peptides (the b-ions) that are not attracted to the

negative quadrupole. The difference in the m/z of the y -ions reflects the amino acid sequence of the peptide derivatised.

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4.2 RESULTS AND DISCUSSION

4.2.1 Peptide derivatisation

Since we were unable to determine any protein sequence data using conventional methodology, we attempted to obtain internal peptide sequence by mass spectrometry after trypsin digestion and derivatisation.

A gel fragment containing ASP 53 was excised from a Coomassie-stained SDS-PAGE gel and the protein reduced and alkylated before in-gel digestion using trypsin. The digest was extracted from the gel by sonication in TFA/ACN solution and analysed by MALDI-TOF mass spectrometry (Figure 4.1a). The peptide masses found were used to determine whether ASP 53 was homologous to known protein; no significant homology was found.

The digest was next derivatised using O-methylisourea-hydrogen sulphate and the CAF reagent in a two-step reaction and the derivatised digest analysed by MALDI-TOF mass spectrometry (Figure 4.1b). Asterisks on peptides in this spectrum indicate tryptic peptides that were successfully derivatised. It is evident from the peptide mass changes (mass changes of 42 and 136 mass units occur on guanidination and sulfonation respectively) that no guanidination of peptides occurred, although extensive sulfonation occurred. Failure to guanidinate has been demonstrated not to affect peptide fragmentation efficiency (Keough *et al.*, 2000).

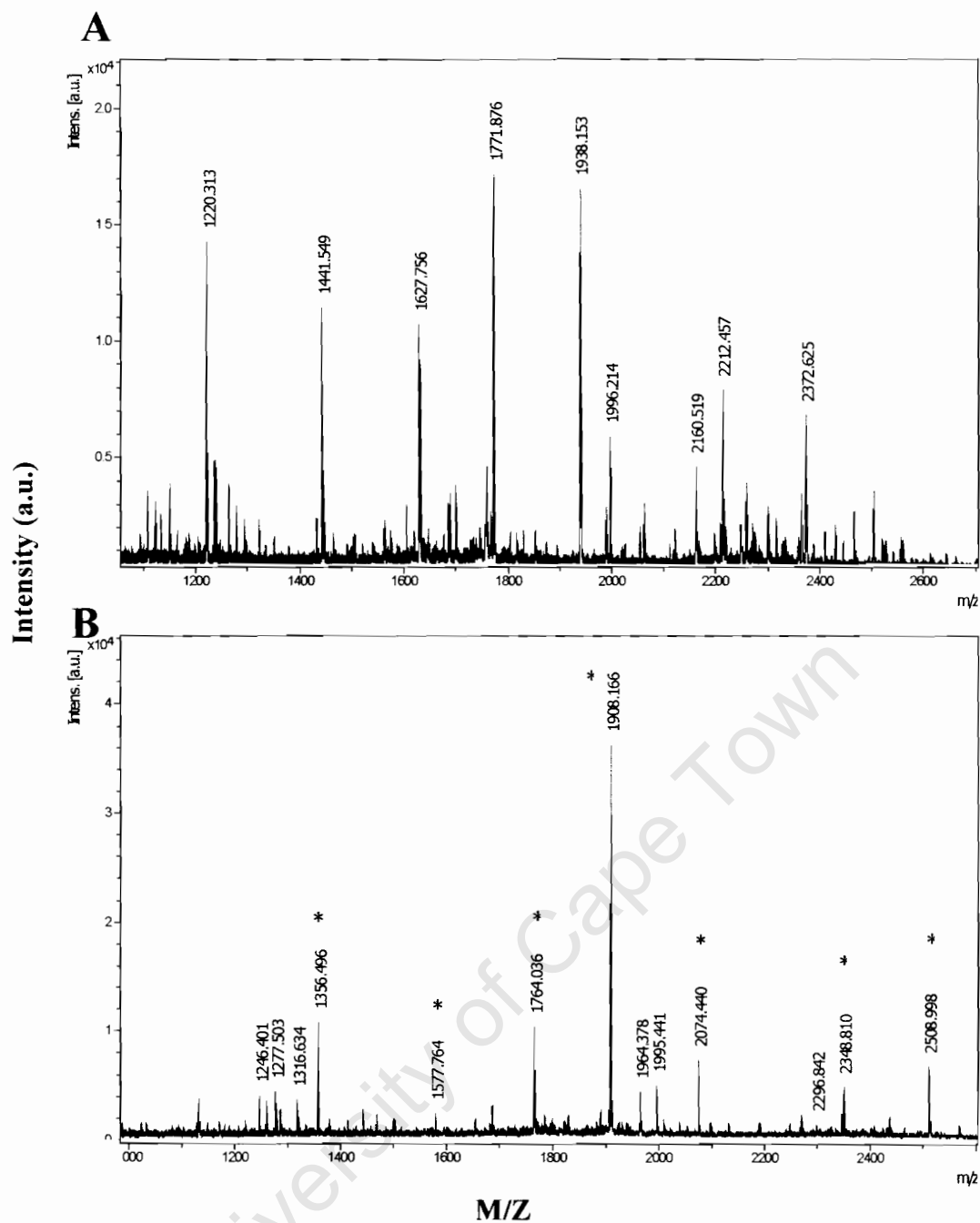


Figure 4.1 A: MALDI-TOF mass spectrum of peptides produced by trypsin digestion of ASP 53. B: MALDI mass spectrum of the same sample after derivatisation. Asterisks indicate peptides with mass changes due to sulfonation of the N-terminal residue.

4.2.2 Post Source decay sequencing of derivatised peptides

Two sulfonated peptides with molecular masses of 1356.5 Da and 1577.7 Da were selected for sequencing by PSD-MALDI-TOF mass spectrometry. The mass spectra (Figure 4.2) obtained from both peptides contained mainly y-type ions as expected. The spectra most importantly showed both the parent ion and an ion with m/z 175. The presence of arginine at the C-terminus of a peptide is inferred from the presence of the y_1 -ion with m/z 175 in the product ion spectrum. Mass differences were determined between adjacent y-ions, starting from arginine at y_1 , to establish the amino acid sequence of the peptide. We concluded that the sequence of the 1356.5 Da peptide was HL(I)QF(Mo)L(I)QDYR and that of the 1577.7 Da peptide was TFVTL(I)NQYDAL(I)R. Leucine and isoleucine cannot be distinguished from one another using this methodology as they have the same residue mass. No homology between these sequences and known proteins was obtained.

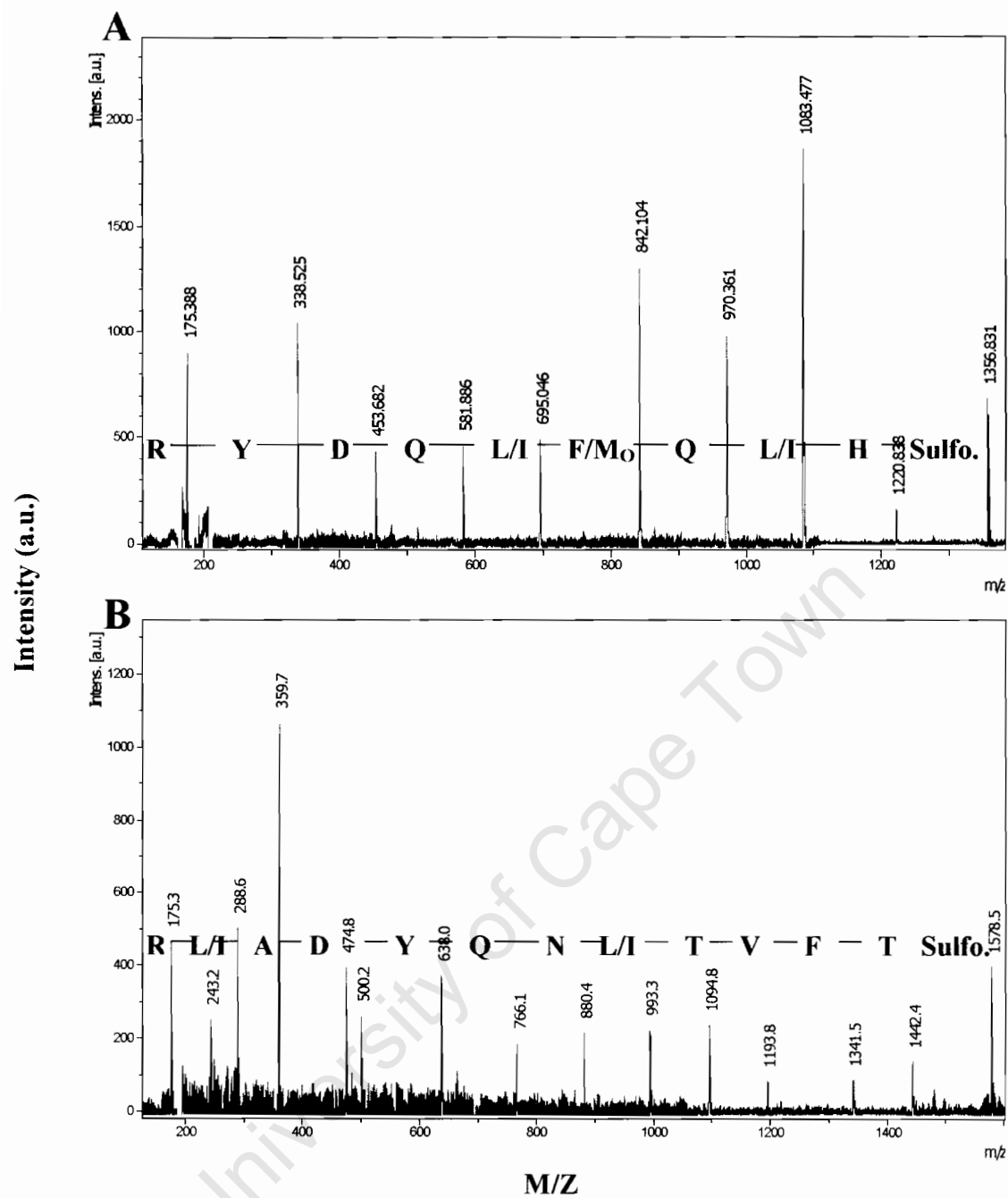


Figure 4.2 PSD MALDI-TOF mass spectra of sulfonated peptides with molecular masses of 1356.5 Da (A) and 1577.7 Da (B). Mo, Oxidised methionine; Sulfo., sulfonation, which adds 136 mass units to the peptide.

4.2.3 RT-PCR

Since the peptide sequences of the tryptic peptides showed no homology with known proteins present on the various protein databases, we next attempted to obtain sequence data by sequencing the gene for ASP 53.

Total RNA was extracted from the mature seeds of *A. erioloba* and treated with DNaseI to minimise any genomic DNA contamination prior to use in RT-PCR. RT-PCR was performed using an oligo dT primer together with a degenerate primer designed from the amino acid sequence of the 1577.7 Da peptide. The PCR product was cloned into pGEM[®]-T Easy and sequenced. Figure 4.3 shows the nucleotide sequence and the translated amino acid sequence of the PCR product. The nucleotide sequence contained sequences of both primers used for its amplification and had an open reading frame (ORF) of 969 bp. This ORF encoded for a protein of 323 amino acids with a molecular weight of 36.1 kDa (approximately 68 % of ASP 53). Although no start codon was found, a stop codon was present, suggesting that this fragment represented the 3' end of the coding region of the gene together with the 3' untranslated region. As a rough guide of whether this PCR product represented the 3' region of the *ASP53* gene, the amino acid sequence encoded by the ORF of the RT-PCR product was analysed using the 'DNAassist' algorithms. The isoelectric point (pI) and the theoretical amino acid composition of the encoded amino acid sequence were compared with the pI values of the native protein (determined by 2-D gel electrophoresis) and the amino acid composition (obtained by acid hydrolysis). The encoded protein, which had a pI of 8.6, had a very similar amino acid composition to the native protein, which had a pI of approximately 9.6. Since these results suggested

that ASP 53 mRNA had indeed been amplified, we next attempted to sequence the 5' end of the *ASP53* gene using 5' RACE.

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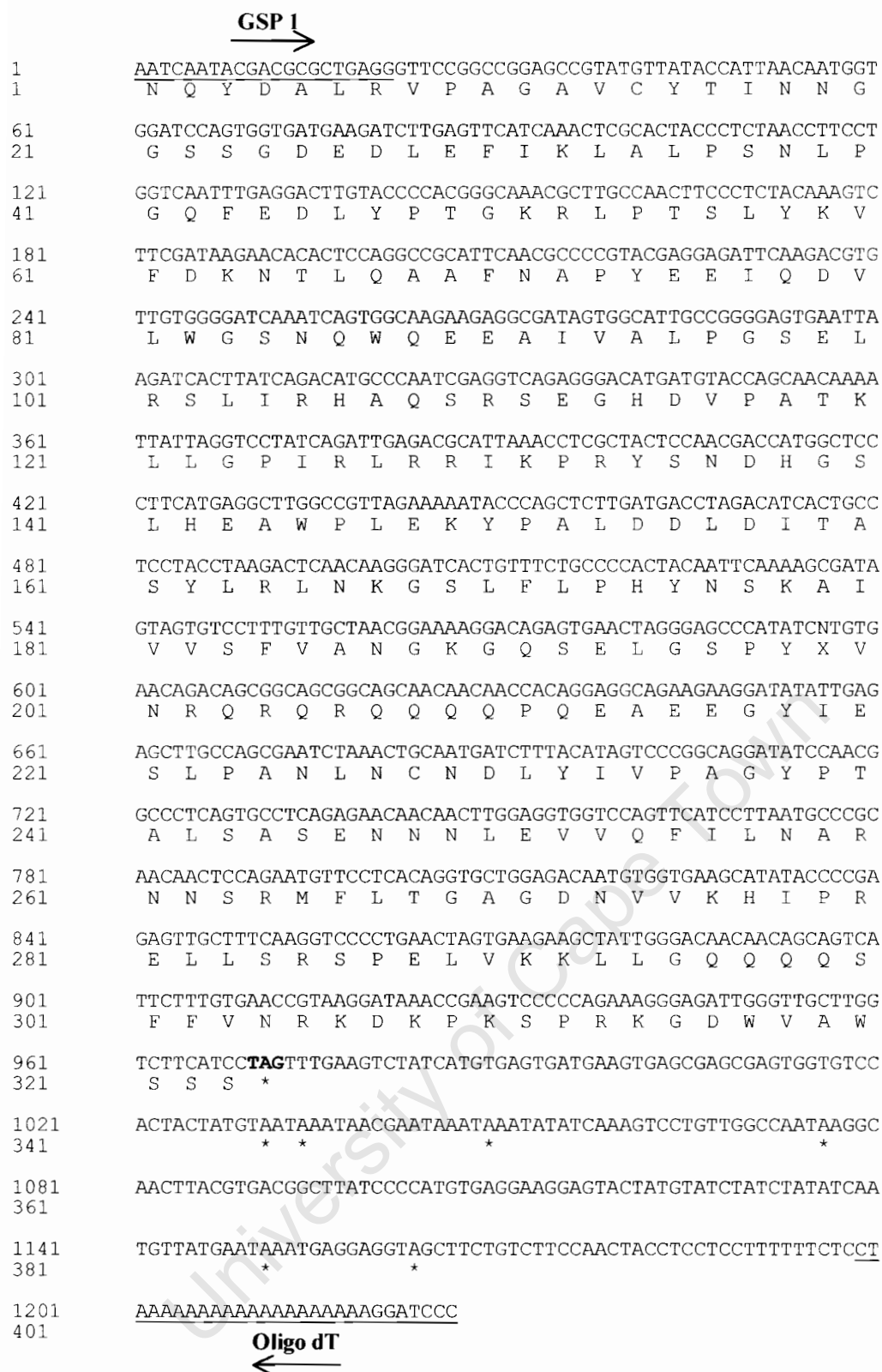


Figure 4.3 Nucleotide and translated amino acid sequences of the RT-PCR product. The sequences of the degenerate gene specific primer (GSP 1) and the oligonucleotide dT primer used to amplify the DNA are underlined. The stop codon is in bold type. No translation of the 3' untranslated region is given except that stop codons are indicated (*).

4.2.4 5' RACE

To determine the sequence of the 5' end of the *ASP53* gene, PCR was performed using a gene specific antisense primer, GSP 2, designed from the DNA sequence of the RT-PCR product, in conjunction with the 5' PCR primer (complementary to the SMART IV oligonucleotide that was ligated to the 3' end of the cDNA). Refer to Figure 2.2 for a schematic diagram of this procedure. The 5' RACE product obtained was cloned into pGEM[®]-T Easy and sequenced. The nucleotide sequence of the product contained the sequences of both the 5' PCR primer and the GSP 2 primer. The sequence of this PCR product overlapped the sequence of the RT-PCR product obtained previously between the 5' end of the latter product and the GSP 2 sequence. The sequence of this region was therefore compared between the two PCR products and found to have an approximate 99.3 % identity. Figure 4.4 shows the nucleotide sequence obtained after merging the two sequences and the translated amino acid sequence is shown in Figure 4.5. The sequence showed an ORF of 1358 bp between positions 80 and 1434 that encodes a protein of 453 amino acids with a molecular weight of 51 kDa. This ORF contains a start codon and a stop codon, indicating that the full-length gene sequence had been obtained. The presence of the sequence of the second peptide (Figure 4.5) sequenced by PSD MALDI-TOF mass spectrometry confirmed that the correct product had indeed been obtained. The amino acid composition derived from the gene sequence was compared to that obtained by acid hydrolysis (Table 4.1). This showed remarkable similarity between these two with the exception of the tryptophan, cysteine and proline content. These residues were not detected after acid hydrolysis as tryptophan and cysteine are hydrolysed by 6 M HCl

and proline, as a secondary amino acid, cannot react with the OPA reagent. The deduced amino acid sequence was used to generate a hydropathy plot using a 7-amino acid window. The plot in Figure 4.6 shows a profile with a pronounced hydrophilicity but with substantial hydrophobic regions. The N-terminus of the protein is highly hydrophobic, suggesting the presence of a signal sequence of approximately 24 amino acids. This suggested that the amino acid sequence of the mature ASP 53 only starts at residue 25, after cleavage of the signal sequence in the endoplasmic reticulum. Removal of these 24 amino acids would reduce the molecular weight of the mature protein to 48.2 kDa. The difference in weight between the hypothesised molecular weight of 48.2 kDa and the actual molecular weight of 53 kDa as determined by MALDI-TOF mass spectrometry might be due to the attachment of carbohydrate residues.

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1      AAGCAGTGGT ATCAACGCAG AGTGGCCATT ACGGGCCGGG GACCATATAC
51     ATCTTCATCG ATTCATTTGA GTGATCAACC ATGAAGCCA GGCTTGCCCT
101    TCTCTGGTTF CTGGGTTCTG TTTTCCTGGC GCGGCCCTCC GTTGGCCTTG
151    CCCGCTCGGA ACAACAAGGA CAAGATGAGC GAACACGGGT TAAACATAAC
201    CCGTATCACA TACACTCGGA TAGGTTCCAA ACCCGATTCC AGAACAAAAA
251    TGGTCAGATC CGGGTCCTCG AACAGTTCGA CAAGCTGTCC CGACACCTTC
301    AGTTTCPCCA AGACTACCGG ATCGTCCAAT ATAAAGCCCG AACCCAAACC
351    ATAGTCCTGC CCCACTATTC CGATGCTGAG TTCCTCCTCA TCATCACCAG
401    AGGAAGCGCC TTCGCTGCTG TTTCGTTTGG TCCGAATGTG GCCAACAGAA
451    CATTCTGCAC CCTCAATCAA TACGACGCGC TGAGGGTTCG GGCCGGAGCC
501    GTATGTTATA CCATTAACAA TGGTGGATCC AGTGGTGATG AAGATCTTGA
551    GTTCATCAAA CTCGCACTAC CCTCTAACCT TCCTGGTCAA TTTGAGGACT
601    TGTACCCAC GGGCAAACGC TTGCCAACTT CCCTCTACAA AGTCTTCGAT
651    AAGAACACAC TCCAGGCCGC ATTCAACGCC CCGTACGAGG AGATTCAAGA
701    CGTGTGTGG GGATCAAATC AGTGGCAAGA AGAGGCGATA GTGGCATTGC
751    CGGGGAGTGA ATTAAGATCA CTTATCAGAC ATGCCCAATC GAGGTCAGAG
801    GGACATGATG TACCAGCAAC AAAATATATTA GGTCCATATCA GATTGAGACG
851    CATTAACCT CGCTACTCCA ACGACCATGG CTCCCTTCAT GAGGCTTGGC
901    CGTTAGAAAA ATACCCAGCT CTTGATGACC TAGACATCAC TGCCTCCTAC
951    CTAAGACTCA ACAAGGGATC ACTGTTTCTG CCCCACTACA ATTCAAAAGC
1001   GATAGTAGTG TCCTTTGTTG CTAACGGAAA AGGACAGAGT GAACTAGGGA
1051   GCCCATATCN TGTGAACAGA CAGCGGCAGC GGCAGCAACA ACAACCACAG
1101   GAGGCAGAAG AAGGATATAT TGAGAGCTTG CCAGCGAATC TAAACTGCAA
1151   TGATCTTTAC ATAGTCCCGG CAGGATATCC AACGGCCCTC AGTGCCTCAG
1201   AGAACACAAA CTTGGAGGTG GTCCAGTTCA TCCTTAATGC CCGCAACAAC
1251   TCCAGAATGT TCCTCACAGG TGCTGGAGAC AATGTGGTGA AGCATATACC
1301   CCGAGAGTTG CTTTCAAGGT CCCCTGAACT AGTGAAGAAG CTATTGGGAC
1351   AACACAGCA GTCATTCTTT GTGAACCGTA AGGATAAACC GAAGTCCCCC
1401   AGAAAGGGAG ATTGGGTTGC TTGGTCTTCA TCCTAGTTTG AAGTCTATCA
1451   TGTGAGTGAT GAAGTGAGCG AGCGAGTGGT GTCCACTACT ATGTAATAAA
1501   TAACGAATAA ATAAATATAT CAAAGTCCTG TTGGCCAATA AGGCAACTTA
1551   CGTGACGGCT TATCCCCATG TGAGGAAGGA GTACTATGTA TCTATCTATA
1601   TCAATGTTAT GAATAAATGA GGAGGTAGCT TCTGTCTTCC AACTACCTCC
1651   TCCTTTTTTC TCCTAAAAAA AAAAAAAAAA AAAGGATCCC

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Figure 4.4 The full-length nucleotide sequence of ASP 53 together with 79 bp of 5' flanking sequence and the 3' untranslated region. The sequences of the gene specific antisense primer (GSP 2) and the 5' PCR primer used to amplify the 5' end of the *ASP53* gene are underlined. The start and stop codons at positions 80 and 1434 are shown in bold type.

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1      MKARLALLWF LGSVFLAAAS VGLARSEQQG QDERTRVKHN PYHIHSDRFQ
51     TRFENKNGQI RVLEQFDKLS RHLQFLQDYR IVQYKARTQT IVLPHYSDAE
101    FLIIITRGSF FAAVSFGPNV ANRTFVTLNQ YDALRVPAGA VCYTINNGGS
151    SGDEDLEFIK LALPSNLPGQ FEDLYPTGKR LPTSLYKVFD KNTLQAAFNA
201    PYEEIQDVLW GSNQWQEEAI VALPGSELRS LIRHAQSRSE GHDVPATKLL
251    GPIRLRRIKP RYSNDHGSLH EAWPLEKYPA LDDLITASY LRLNKGSLFL
301    PHYNKAIVV SFVANGKGQS ELGSPYXVNR QRQRQQQPQ EAEEGYIESL
351    PANLNCNDLY IVPAGYPTAL SASENNLEV VQFILNARNN SRMFLTGAGD
401    NVVKHIPREL LSRPELVKK LLGQQQSFV VNRKDKPKSP RKGDWVWSS
451    S

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Figure 4.5 Amino acid sequence of ASP 53. The sequences of the two peptides sequenced by PSD MALDI-TOF mass spectroscopy are shown in bold type.

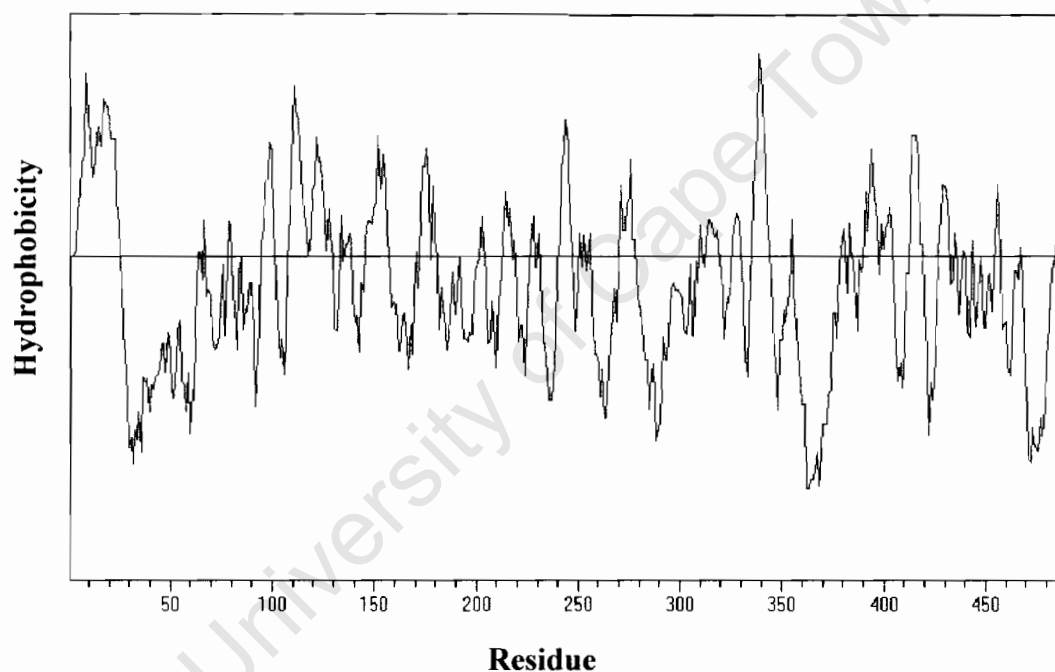


Figure 4.6 Hydropathy plot of ASP 53. Hydropathy plot is according to Kyte and Doolittle (1982) using a window of 7 residues. Negative values indicate hydrophilicity.

Table 4.1 Amino acid content (mole %) of ASP 53. A: from amino acid analysis B: as determined from the amino acid sequence.

Residue	A	B
Glutamic acid (E)	15.0	12.2
Lysine (K)	5.2	4.9
Aspartic acid (D)	13.1	10.6
Glycine (G)	7.1	6.0
Alanine (A)	8.3	8.0
Serine (S)	8.5	8.0
Valine (V)	5.0	6.0
Threonine (T)	3.1	3.3
Leucine (L)	11.7	11.3
Arginine (R)	6.9	6.9
Histidine (H)	3.0	2.4
Tyrosine (Y)	4.0	3.8
Phenylalanine (F)	3.8	4.4
Isoleucine (I)	4.2	4.2
Methionine (M)	0.9	0.4
Cysteine	0.0	0.4
Proline	0.0	5.5
Tryptophan	0.0	1.3

Homology studies using the obtained full length sequence showed that ASP 53 was related to the known glycosylated storage proteins beta-conglycinin, vicilin and canavalin, although the level of amino acid homology between ASP 53 and these proteins were low (Table 4.2). It has been reported (Dunwell *et al.*, 2004) that only low levels of homology exist amongst members of this group of storage proteins, and as a result homology studies amongst these proteins are generally based on comparison of the 3-dimensional structure of the proteins. Members of this group of storage proteins all have a cupin motif present. ASP 53 was found to have two cupin domains between residues 52 and 169 and between residues 253 and 414 suggesting that ASP 53 should be classified as a member of this superfamily. Homology studies showed that beta-conglycinin, vicilin and canavalin had an amino acid homology of 59 %, 53 % and 56 %, respectively with cupin domain I of ASP 53 with 40 %, 35 % and 38 % of identical amino acids. Cupin domain II showed had a homology of 54 %, 53 % and 61 % with beta-conglycinin, vicilin and canavalin, respectively with identical amino acids of 41 % for all three proteins.

Table 4.2 Homology analysis of ASP 53 and other cupin-containing seed storage proteins

Protein	Amino acid homology (%)	Identities (%)
Beta-conglycinin [Glycine max]	55	36
Vicilin	53	35
Canavalin	53	34

CONCLUSION

LEA proteins are characteristically small hydrophilic proteins that remain soluble at high temperature (Baker *et al.*, 1988). They accumulate during late embryogenesis and rapidly disappear upon seed germination. Their expression has been reported to be induced by most environmental stresses that result in water loss. In this thesis we have reported the purification of an unusually large hydrophilic protein from the mature seeds of *A. erioloba*. This 53 kDa protein, ASP 53, exhibited properties typical of LEA proteins. The protein was the most abundant soluble protein constituting approximately 47 % of the total soluble protein in whole seeds. Typical of LEA proteins, it remained soluble at 80 °C, a feature attributed to a high content of hydrophilic amino acids. An unusual feature of ASP 53 was that it remained soluble at 80 °C despite a hydrophobic amino acid content of 34 %. A second unusual feature was that almost half of the structure of ASP 53 was in a β sheet conformation. LEA proteins studied to date, eg the wheat Em protein (Litts *et al.*, 1991) have been reported to assume a largely unordered structure in solution. This unordered structure and the highly hydrophilic nature of these proteins is considered to allow group I LEA proteins to protect cells from desiccation-induced damage by acting as water replacement molecules. The relatively high content of hydrophobic amino acid of ASP 53 together with the presence of defined secondary structure suggested that the solubility of this protein at elevated temperature might be brought about by hydrophobic faces of amphipathic sheets interacting with one another and presenting a hydrophilic face to the environment.

ASP 53 was rapidly degraded during germination and this decline in ASP 53 content was found to coincide with the loss of desiccation tolerance in germinating seedlings. This correlation between the decrease in ASP 53 content and the inability of germinating seedlings to resume growth following water loss suggested possible involvement of the protein in the acquisition of desiccation tolerance in mature seeds. Immunocytochemistry demonstrated the presence of ASP 53 in the vacuoles and cell walls in mature seeds, with the cell wall content decreasing upon germination. We therefore postulate that ASP 53 might be involved in stabilisation of the cell wall and in so doing protecting mature seeds from damage induced by water loss. Association of ASP 53 with nutritional protein storage organelles suggested that the protein might also act as a storage protein. ASP 53 fulfils several characteristics typical of seed storage proteins in that it has a high content of aspartic acid and glutamic acid residues, it is glycosylated and in addition is present in large quantities in mature seeds. A bi-functional role in the cell has been reported previously with some group I LEA proteins postulated to fulfill two different functions (Swire-Clark and Marcotte, 1999). ASP 53 was found to inhibit all three stages of protein thermal denaturation. Thus ASP 53 significantly decreased the rate of loss of alcohol dehydrogenase activity at 55 °C, decreased the rate of temperature-dependent loss of secondary structure of haemoglobin and completely inhibited the temperature-dependent aggregation of egg white protein. Homology studies identified two cupin motifs, with only seed storage proteins exhibiting close sequence similarity. Cupin-containing storage proteins have been reported to be involved in protecting plants against biotic and abiotic stress (Berna and Bernier, 1999).

Future studies.

2-dimensional Nuclear Magnetic Resonance spectroscopy and/or X-ray crystallography would allow the 3-dimensional structure of ASP 53 to be determined now that the full amino acid sequence has been obtained. Site-directed mutagenesis of selected residues might allow the identification of functional groups involved in the thermal protection of target proteins. ASP 53 has potential for use in industrial applications where the application requires activity of an enzyme at a temperature above that which is optimal to that enzyme. The effect of ASP 53 in ameliorating such enzyme activities at required process optima needs to be investigated.

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