A NOVEL ß-GALACTOSIDASE OR EXO-(1→4)-ß-GALACTANASE FROM THE COTYLEDONS OF GERMINATED *LUPINUS ANGUSTIFOLIUS* L SEEDS, AND ITS ROLE IN POST-GERMINATIVE CELL WALL METABOLISM

by

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DECLARATION

I, Marcos Silveira Buckeridge, declare that I wrote this dissertation by myself and that this study has not been previously accepted for any higher degree. Wherever collaboration has been obtained it was properly acknowledged.

Marcos Silveira Buckeridge

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Chapter 2 - MATERIAL AND METHODS

Abbreviations

BSA	bovine serum albumin
CM	carboxymethyl
CWG	Cell Wall Ghosts
CWSP	Cell Wall Storage Polysaccharide(s)
DAB	Diaminobenzidine
DEAE	diethylaminoethyl
EDTA	ethylene diamine tetra acetic acid
HPAE	High Performance Anion Exchange
kDa	kilodaltons
M_r	molecular weight
NAD	Nicotinamide Adenine Diphosphate
PNPgal	p-nitrophenyl-ß-galactopyranoside
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TLC	Thin Layer Chromatography

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ABSTRACT

The cotyledons of *Lupinus angustifolius* contain large amounts of a polysaccharide composed of (1→4)-ß-linked D-galactose residues, probably in the form of branches attached to a rhamnogalacturonan core molecule .

Parker (1984) documented the ultrastructural changes in cell walls of *L.angustifolius* cotyledons following germination and proposed that these changes correspond to the mobilisation of cell wall polysaccharides. Crawshaw and Reid (1984) showed that the main monosaccharides mobilised from the cotyledons of *L.angustifolius* after germination are galactose and arabinose.

The aim of this thesis was to purify the principal enzymes responsible for the mobilisation of the (1→4)-ß-galactan from the cell walls of *L.angustifolius* cotyledons and perhaps to contribute to an understanding of the relevance of galactan in the overall development of the seedling.

A novel enzyme, capable of hydrolysing (1→4)-ß-linked galactan specifically, was isolated from *L.angustifolius* cotyledons using ion exchange chromatography and affinity chromatography. On SDS-PAGE, one band with M_r=60kDa (major) and another band at M_r=45kDa (minor) were obtained after purification. Antibodies raised in rabbit against the 60kDa band and affinity-purified using the major band (60kDa) still cross-reacted with the minor band. The two bands had exactly the same N-terminal sequence (performed at Unilever Laboratories-Colworth House, by Dr.Amanda Heyler). The exo-(1→4)-ß-galactanase did not hydrolyse ß-linked galactose from any plant cell wall polysaccharides other than from lupin galactan. The related ß-galactosidases without action on ß-galactan were partially purified.

Western immunoblotting and activity measurements showed that exo-(1→4)-ß-galactanase was synthesised *de novo* and that its activity and specific protein varied *pari passu* with cell wall mobilisation.

When isolated storage mesophyll lupin cell walls were treated with pure exo-galactanase the morphological changes observed were remarkably similar to those accompanying *in vivo* mobilisation. *In vitro*, the exo-(1→4)-ß-galactanase was capable of hydrolysing ca.60% of the galactose present in the cell walls.

Exo-(1→4)-ß-galactanase coupled to gold particles was used as a cytochemical probe to detect galactan for the first time in ultrathin sections of *L.angustifolius* mesophyll cotyledonary tissue following germination. Using the gold-probe, the pattern of galactan mobilisation was observed directly in the electron microscope.

The decrease in galactan content after germination (estimated using the exo-galactanase) showed a close correlation with increase in cotyledonary area and changes in plasticity and elasticity of the mesophyll storage tissue of *L.angustifolius* cotyledons.

These results showed clearly that exo(1→4)-ß-galactanase is the main enzyme responsible for degradation of the cell walls *in vivo*. They also suggest that galactan is probably a dual-purpose molecule in *L.angustifolius* cotyledons, serving as a reserve substance and at the same time playing a role in cotyledonary expansion during seedling development.

Chapter 1

GENERAL INTRODUCTION

The Plant Seed

Seed maturation

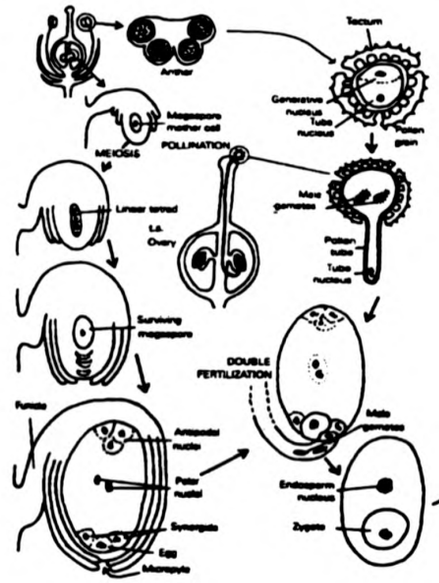
The first Angiosperms probably appeared on earth approximately 65 million years ago, around the end of the Mesozoic and the beginning of the Cenozoic (Beck, 1974).

As part of their adaptation to colonize new territories, the Angiosperms developed new and more sophisticated life cycles. One aspect of these is the development of seeds inside a protective structure, the fruit.

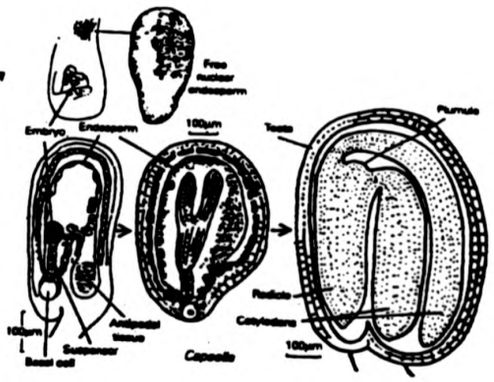
The seed is at the same time, the end and the beginning of a plant's life cycle. For the initiation of the sexual reproductive cycle to occur, flowering plants produce pollen, the male mobile microgametophyte, and ovule(s), the female immobile megagametophyte. The latter is located inside an ovary which, depending on the species, can produce one or many ovules. The ovule is composed of a haploid egg cell or ovum, five other identical haploid cells and a central cell, containing two further identical haploid nuclei named polar nuclei (**Figure 1.1A**). During fertilization, the growing pollen tube reaches the micropyle and penetrates the ovule. The microgametophyte (pollen grain and tube) contains two sperm nuclei (male gametes); one will fuse with the egg cell to form the zygote, which develops into the diploid **embryo** and the other (usually) with the two polar nuclei to form the

Figure 1.1. Summary of the events occurring between gamete formation and the end of seed maturation. **A** - Pollination and double fertilisation in Angiosperms; **B** - embryogenesis exemplified by *Capsella* seed and **C** - Some examples of different seed types and structures [Adapted from Ingroille (1992) and Bewley and Black (1978)].

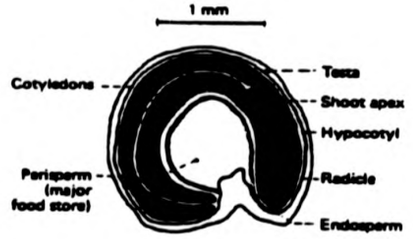
A-Generalised reproductive cycle in Angiosperms



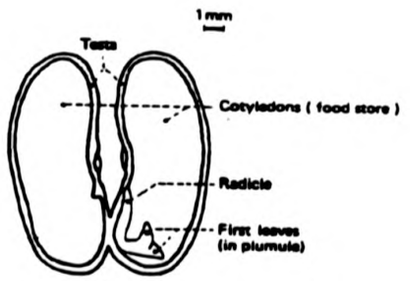
B-Embryogenesis in Capsella



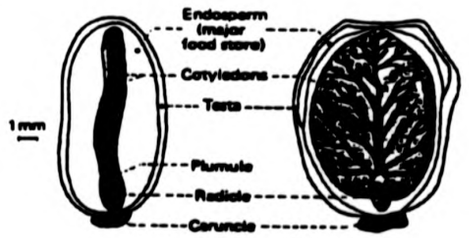
C-Different types of seeds



A. Beta vulgaris (beet)



B. Phaseolus coccineus (runner bean)



C. Ricinus communis (castor bean)

endosperm initial, which develops into the triploid **endosperm.** With the end of the fertilization process,i.e the fusion of the nuclei of the male and female gametes, the development of the diploid embryo starts. After a series of cell divisions followed by a cell extension period, it culminates in the mature embryo (**Figure 1.1B**). At the same time, the (usually) triploid endosperm develops, and storage material may also be deposited in that tissue. This whole process is named seed maturation, and in Angiosperms it occurs inside the fruit. Many different structures, which vary according to the species, may also develop during seed maturation. The embryo is usually composed of 1) a radicle, which will develop into the root tissues; 2) the shoot apex or plumule, which will develop into the leaves and most of the aerial part of the adult plant and 3) one or two cotyledons, which in many dicotyledonous plants are adapted as photosynthetic and/or storage organs (**Figure 1.1B** and **C**). The cotyledons of dicots seem to be very variable tissues, acquiring many different forms and sizes depending on the species. They usually have a relatively short life, being active mainly during the establishment of the seedling.

The endosperm tissue often surrounds the growing embryo (**Figure 1.1B**), and its main function is thought to be as a source of nutrients for the growing embryo. Although the endosperm can be consumed during development, in many species it can persist, eventualy being used as a

source of food reserves during germination and seedling establishment.

In the course of its development, the Angiosperm seed normally produces variable amounts of reserve substances (protein, lipids, carbohydrates, phytin etc) plus many other compounds, such as growth regulators, defence substances etc. These have to be produced and stored in the right proportion to provide the developing embryo with a correct balance of concentrations when germination occurs. This process seems to be genetically and environmentally regulated.

If this beautiful and delicately balanced interaction between environment and biological processes is successful, the seed will dry out, will (via other complex processes) be dispersed and will encounter a variety of conditions that may or may not be optimal for germination and growth.

Germination and seedling establishment

After development and dispersion, the seeds are, in a sense, ready to germinate and develop in new adult plants. However, this will only occur if they meet the right conditions for germination.

As summarised above, a whole range of different strategies have evolved for the successful distribution of key chemical compounds (reserves and growth regulators for

example) throughout the different seed structures, in order to ensure that germination will occur with the correct correspondence between internal (biochemical) and external (environmental) conditions.

The wide range of seed forms and shapes encountered in Nature may be viewed as a consequence of these strategies of adaptation (**Figure 1.1C**).

There are extremes. Some seeds have no reserves at all and therefore depend on interspecific interactions [for example the dust-like seeds of some orchids (Bryant, 1985)]. In other species, the seeds accumulate very large amounts of reserves in the endosperm (e.g. starch in cereals; galactomannan as cell wall storage polysaccharide in some legumes) and/or in the cotyledons (e.g.protein in peas and soy beans; xyloglucan, a cell wall storage polysaccharide, in some legumes). Other species have adopted yet another strategy: they possess relatively low amounts of reserves, and the cotyledons are very well adapted as photosynthetic organs (e.g. *Anadenanthera falcata, Peltogyne gracilipes*).

If satisfactory conditions of temperature, humidity, light and gases are met, the seed will re-start its metabolism and the embryo will develop with a velocity that is directly dependent on its interrelations with its accessory structures (endosperm, cotyledons, seed coat etc).

It has to be noted that the microenvironment of a seed during germination is totally different from the environment in which an adult plant is immersed. Therefore, for the life cycle of a given Angiosperm individual to be complete, the seed has to escape any adverse conditions found in its microenvironment, and develop a different structure, which will be adapted to macroenvironmental variables.

Besides all the physiological and biochemical devices necessary for the development of the embryo itself, support structures such as aril, seed coat, endosperm and cotyledons will have a role on the establishment of the growing seedling. This may be during seed dispersal (aril), protection and maintenance of impermeability (seed coat), in the provision of reserves *sensu strictu* (starchy endosperms and cotyledons in hypogeal germinating seeds), imbibition and reserve (galactomannan-containing endosperms) reserve and/or photosynthesis (cotyledons in epigeal germinating seeds).

Basically, the factor which re-starts or accelerates the minimal metabolism of the dry non-dormant seed is the imbibition of water. The embryo then starts growing, feeding on the reserve structures around it, until it reaches a state in which it can sustain its own autotrophy.[1]

The work reported in this thesis relates to this

[1]Note that this happens if the seed does not have any mechanism of dormancy. Dormancy is a very important phenomenon in seed physiology and biochemistry, but it is beyond the scope of this work. The book by Bewley and Black (1978) is referred to for further details.

interaction between the parts of the embryo that will develop into an adult plant and the support structures containing reserves for seedling establishment.

Reserve tissues and compounds in seeds

The three main group of reserve substances which are stored in seeds are the lipids, the proteins and the carbohydrates. The quantitative distribution of these groups of substances in seeds from different species varies. **Table 1.1** (data from Bewley and Black, 1978) provides an illustrative survey of the main food reserves in various seeds. It is perhaps noteworthy that, using the data of **Table 1.1**, it can be calculated that no correlation exists between the amounts of protein and lipid in a given seed (r^2= -0.16), whereas a clear negative correlation is found between the levels of protein and carbohydrate (r^2= -0.47) and specially between lipids and carbohydrates (r^2=-0.85). It is not clear why carbohydrates and lipids tend to be mutually exclusive but this trend may have evolutionary significance. However, it is important to note that most of the species represented in **Table 1.1** are culturally important and have been manipulated by Man over a long period of time.

Although many seed tissues, like the perisperm and the embryonic axis, can be adapted to reserve storage, the endosperm and the cotyledons are the main structures which

Table 1.1. Principal food reserve in various seeds.

Species	Average % composition (dry wt)			Major storage organ
	Protein	Lipid	N-free extract[1]	
Zea mays (corn)	11	5	75 (starch)	Endosperm
Zea mays (sweet corn)	12	9	70 (starch)	Endosperm
Avena sativa (oat)	13	8	66 (starch)	Endosperm
Triticum aestivum (wheat)	12	2	75 (starch)	Endosperm
Secale cereale (rye)	12	2	76 (starch)	Endosperm
Hordeum vulgare (barley)	12	3	76 (starch)	Endosperm
Vicia faba (broad bean)	23	1	56 (starch)	Cotyledon
Linum usitatissimum (flax)	24	36	24 (starch)	Cotyledon
Pisum arvense (field pea)	24	6	56 (starch)	Cotyledon
Pisum sativum (garden pea)	25	6	52 (starch)	Cotyledon
Arachis hypogea (peanut)	31	48	12 (starch)	Cotyledon
Glycine max (soybean)	37	17	26 (starch)	Cotyledon
Gossypium spp (cotton)	39	33	15	Cotyledon
Brassica napus (rape)	21	48	19 (starch)	Cotyledon
Citrullis vulgaris (watermelon)	38	48	5	Cotyledon
Bertolletia excelsa (Brazil nut)	18	68	6	Radicle/ hypocotyl
Elaeis guineensis (oil palm)	9	49	28	Endosperm
Phytelephas macrocarpa (ivory nut)	5	1	79 (galactomannan)	Endosperm
Phoenyx dactylifera (date)	6	9	58 (galactomannan)	Endosperm
Ricinus communis (castor bean)	18	64	trace	Endosperm
Pinus pinea (pine)	35	48	6	Megagametophyte

[1]Nitrogen free extract consists of material which is not protein, lipid, fibre (including cellulose) or ash (mineral nutrients). Thus, free sugar and dextrins are the usual components. Data from Bewley and Black, 1978.

have this function in Angiosperm seeds (Bewley and Black, 1978; Bryant, 1985; Mayer and Poljakoff-Mayber 1989).

Although some endosperms contain mainly reserve lipids and proteins (e.g. castor beans), most seem to have specialised generically as carbohydrate-storing tissues. Starch is usually present in the cereal endosperm, and galactomannans in legumes. On the other hand, cotyledons are relatively more generalist in their stored substances. They can store carbohydrates (starch and cell wall polysaccharides), along with large amounts of lipids and protein (**Table 1.1**). The "reserve" status of all these compounds is based simply on the observation of their post-germinative mobilisation (Bewley and Black, 1978; Bryant, 1985; Mayer and Poljakoff-Mayber, 1989).

Storage lipid and starch metabolism will be not discussed further, since this work is focused primarily on the cell wall storage polysaccharides and, to a lesser extent on proteins. For further details on the other reserve compounds the reviews by Bewley and Black (1978) and Mayer and Poljakoff-Mayber (1989) are recommended.

Reserve proteins in seeds

Seeds containing protein reserves have been relatively well studied (Bewley and Black, 1978), although most of the work has been done on species from the Leguminosae and Gramineae, which are important in human and animal

nutrition.

Osborne (1924 *In* Bewley and Black, 1978) classified seed proteins on the basis of their solubility. Four groups were defined: 1) *albumins*, which are soluble in water at neutral or slightly acid pH values and are heat coagulable (albumin preparations usually include enzymes); 2) *globulins*, which are insoluble in water but soluble in salt solutions and are not as heat coagulable as the albumins; 3) *glutenins*, which can only be extracted with strongly acidic of alkaline solutions; 4) *prolamins*, which are soluble in 90% ethanol, but not in water.

Gramineae contain prolamins, whereas this group of proteins is not common in other seeds. Cereals are also rich in glutenins, while globulins seem to predominate in dicotyledonous seeds such as legumes (Bewley and Black, 1978).

The reserve proteins are stored in sub-cellular structures called *protein bodies*. These are oval or circular in cross-section, are located in the cytoplasm and are bounded by a lipoprotein membrane (Yatsu, 1965 *In* Bewley and Black, 1978).

After germination, protein reserves are thought to be degraded by proteinases either in one step, in which polypeptides are attacked by amino or carboxypeptidases, releasing free amino acids; or in two steps, in which polypeptides are firstly degraded by endopeptidases to small oligopeptides, which are then degraded by peptide

hydrolases to free amino acids (Bewley and Black, 1978). Electron microscopic studies on the protein bodies of cowpea (*Vigna unguiculata*) suggested that the proteinases may be synthesised in the rough ER and packed into vesicles which fuse with the protein body membrane (Harris and Chrispeels, 1975 *In* Bewley and Black, 1978).

According to Bewley and Black (1978), considerably less is known about the detailed biochemical mechanism of reserve protein degradation than about the mobilisation of carbohydrates or lipids.

Major Cell Wall Storage Polysaccharides (CWSP) in seeds

Cell wall breakdown is probably a common occurrence in seed storage tissues following germination. However, in many species, the cell walls are extremely thick and their polysaccharide constituents form a major source of substrate for the development seedling. In such cases, CWSP may constitute 40% or more of the dry weight of storage (endosperm of cotyledons) tissue, and specialised (thick) cell walls are present containing massive amounts of a single cell wall component. Only such major CWSP are discussed here.

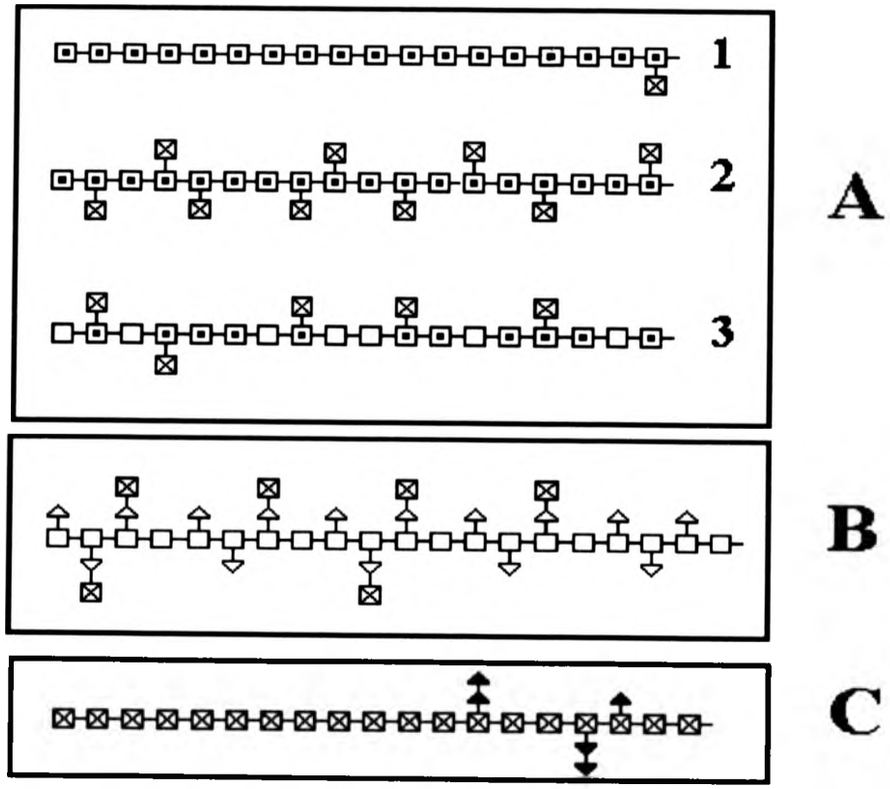
There are three main groups of major CWSP: the **mannan group** (which is composed of glucomannans, galactomannans and pure mannans) (**Figure 1.2A**), **xyloglucans** (**Figure 1.2B**) and **arabinogalactans** (**Figure 1.2C**).

The Mannan group

Galactomannans. Galactomannans are formed from D-mannose residues which are (1→4)-ß-linked to form a linear backbone, and D-galactose residues which are attached to the backbone by (1→6)-**α**-linkages (**Figure 1.2B**). Galactomannans are typical of the legume seed endosperm. In Leguminosae the ratio mannose:galactose in galactomannans, and the statistical distribution of galactosyl residues along the mannan backbone vary from species to species (Reid and Meier, 1970, Bailey, 1971, Dea et al.,1986). Indeed, the three subfamilies of the Leguminosae (Caesalpinioideae, Mimosoideae and Faboideae) can be reasonably distinguished by the mannose/galactose ratio of their seed galactomannan (Bailey, 1971; Buckeridge et al., 1989; Buckeridge, Panegassi, Rocha and Dietrich, unpublished).

The post-germinative metabolism of galactomannans in fenugreek (*Trigonella foenum-graecum*) has been documented in some detail by Reid and collaborators (Reid, 1985 and references therein). In this species the endosperm cells are largely non-living at seed maturity, the space formerly

Figure 1.2. Schematic representation of the structure of the three main groups of cell wall storage polysaccharides in seeds. The reducing end of the molecule is on the right **A** - the mannan group; mannan (1), galactomannan (2) and glucomannan (3). **B** - xyloglucan. **C** - arabinogalactan; this polymer is thought to be covalently linked via the reducing group to a rhamnogalacturonan core molecule.



STRUCTURAL FEATURES OF CELL WALL STORAGE POLYSACCHARIDES

A - Mannan group	
1-pure mannan	Mannose (1→4)-ß
2-galactomannan	Glucose (1→4)-ß
3-glucomannan	Galactose (1→6)-α
B - Xyloglucan	Glucose (1→4)-ß
	Galactose (1→2)-ß
	Xylose (1→6)-α
C - Arabinogalactan	Galactose (1→4)-ß
	Arabinose (1→5)-α

occupied by cytoplasm having been filled by the galactomaannan-containing cell wall. The only living cells of the endosperm are those of the one cell-thick peripheral aleurone layer.

Post-germinative galactomannan degradation starts from the aleurone layer (between endosperm and seed coat) and proceeds inward toward the cotyledons (**Figure 1.3**). After germination, galactomannan is hydrolysed in a concerted fashion by three glycosyl-hydrolases (**Figure 1.4**); α-galactosidase hydrolyses the (1→6)α-linkages of the branching-points and produces free galactose whereas the backbone is hydrolysed by the concerted action of an endo-(1→4)-ß-mannanase and an exo-(1→4)-ß-mannosidase to produce free mannose. The presence of these three enzymes seems to be a general feature in endospermic legume seeds which contain galactomannan (Reid, 1971; Reid and Meier, 1973; Seiler, 1977; McCleary, 1983; Buckeridge, 1988).

Although at least two of the galactomannan hydrolysing enzymes have been demonstrated to be induced in the aleurone layer, the mobilisation of galactomannan in seeds of *Trigonella foenum-graecum* seems not to be controlled by the embryo (Reid and Meier, 1973; Reid, 1985). Instead, an inhibitor (possibly a saponin-like substance) needs to be leaked out of the endosperm for galactomannan mobilisation to begin (Spyropoulus and Reid, 1988, Zambou et al., 1993, Buckeridge, Vieira and Dietrich, unpublished).

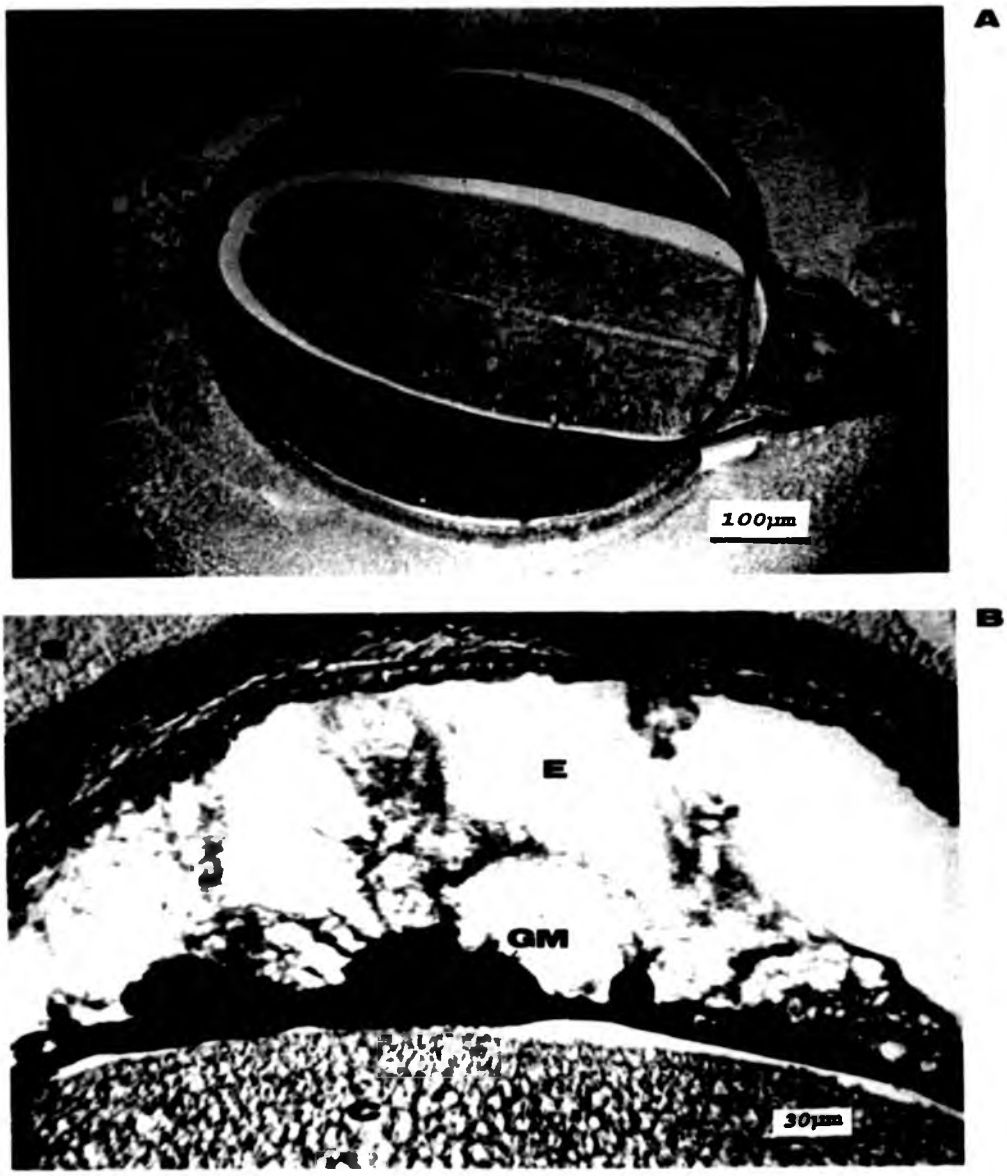


Figure 1.3. Galactomannan degradation in *Trigonella foenum-graecum* seeds. **A** - imbibed ungerminated seed. Aleurone layer (A), endosperm (E) cotyledons (C) and (S) seed coat. **B** - advanced stage of galactomannan (GM) degradation.

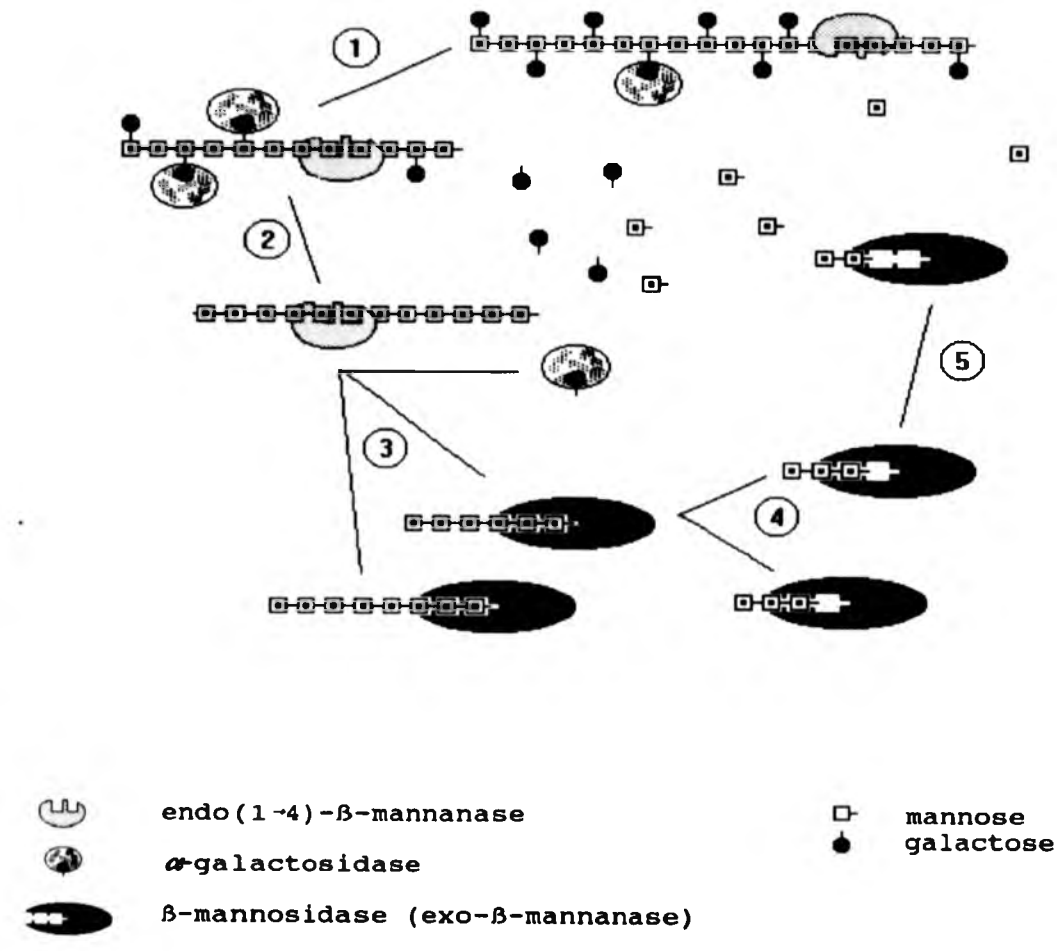


Figure 1.4. Probable enzymatic mechanism of galactomannan break down in endospermic legume seeds after germination. After α-galactosidases hydrolyses the single galactose branches (steps 1 and 2), the main chain [a (1→4)-ß-linked mannan] is made accessible to attack by the endo-(1→4)-ß-mannanase and ß-mannosidase [exo-(1→4)-ß-mannanase] (3-5). Free galactose and mannose are produced.

Besides acting as a post-germinative reserve, galactomannans can also serve as imbibing substances in early stages of germination. The highly branched hydrophillic galactomannan takes up proportionally high amounts of water and distributes it around the embryo (**Figure 1.3A** - Reid and Bewley, 1979). The endosperm, thus imbibed can then protect the embryo against desiccation by buffering it against water-loss during post-imbibition drought (Reid and Bewley, 1979).

As a result, Reid and Bewley (1979) (see also Reid, 1985) proposed that galactomannans are multifunctional molecules, playing a role in the water relations of the seed during germination and serving as a reserve compound during seedling development.

Mannans. "Pure" mannans are composed mainly (90%) of a linear chain of (1→4)-linked mannopyranosyl residues. Up to 10% of the mannose residues are branching points carrying single units of (1→6)-**α**-linked galactoses. The mannans are therefore related structurally to the galactomannans, but have very much fewer galactosyl branch-points. For this reason they are (unlike the galactomannans) water-insoluble and self-interactive to the extent of being to some extent crystalline in the cell wall. Mannans are found in Monocotyledons (e.g. *Phoenix dactylifera* and *Phytelephas macrocarpa*) and in Dicotyledons (*Coffea arabica* and *Carum carvi*) (Reid, 1985). Mannan mobilisation has been studied

in detail only in the Monocotyledonous species *Phoenix dactylifera* (the date palm). *Phoenix dactylifera* seed germination was studied by Sachs (1862) and extended by Keusch (1968). In this seed, a small conical embryo develops slowly and its cotyledon is transformed into a haustorium, which absorbs the products of degradation of the reserves along germination.

De Mason et al. (1983) and De Mason (1985) performed a thorough study on the *Phoenix dactylifera* seed reserve degradation. ß-Mannanase and ß-mannosidase were detected in the dissolution zone near the haustorium. However the authors proposed that the haustorium may possibly not be responsible for the production of the enzymes. They could be activated in the endospermic cells, which are living and metabolically active, by a signal coming from the haustorium. The authors, however, did not rule out the hypothesis that inactive enzymes could have been secreted by the haustorium and activated in the endosperm.

Mannans possibly have other functions apart from being reserve substances. The great hardness of the date seed is due to the presence of this polymer in endosperm cell walls, and mannan-containing seeds are, in general very hard and resistant to mechanical damage.

Glucomannans. Of the CWSP of the mannan group, the glucomannans are the least studied. They can be extracted with alkali from seeds of *Asparagus officinalis* and *Edymion*

nutans (Goldberg, 1969), *Scilla nonscripta* (Thompson and Jones, 1964), *Iris ochroleuca* and *I.sibirica* (Andrews et al.,1953). The structures of glucomannans have been determined by methylation analysis. They have a linear (1→4)-ß-linked backbone containing almost equal numbers of glucopyranosyl and mannopyranosyl residues. Some branching (3-6%) with single (1→6) (probably α-linked) galactopyranosyl residues occurs (Reid, 1985). There is little information available concerning the biochemistry of their post-germinative catabolism.

Xyloglucans

When large amounts of xyloglucan are present in a seed, the cell walls can be stained with iodine and show a distinctive blue colour (Vogel and Schleiden, 1839). Because of this interaction with iodine, xyloglucans are also known as "amyloids" (**Figure 1.5A**). The botanical distribution of amyloids has been surveyed histochemically by Kooiman (1960), who made use of the iodine reagent as described by Vogel and Schleiden (1839).

The substances now known to be xyloglucans were detected in seeds very early. Heinricher (1888) and Reiss (1889) reported the presence and mobilisation following germination, of amyloids in seeds of *Impatiens balsamina, Tropaeolum majus* and *Cyclamen europaeum*.

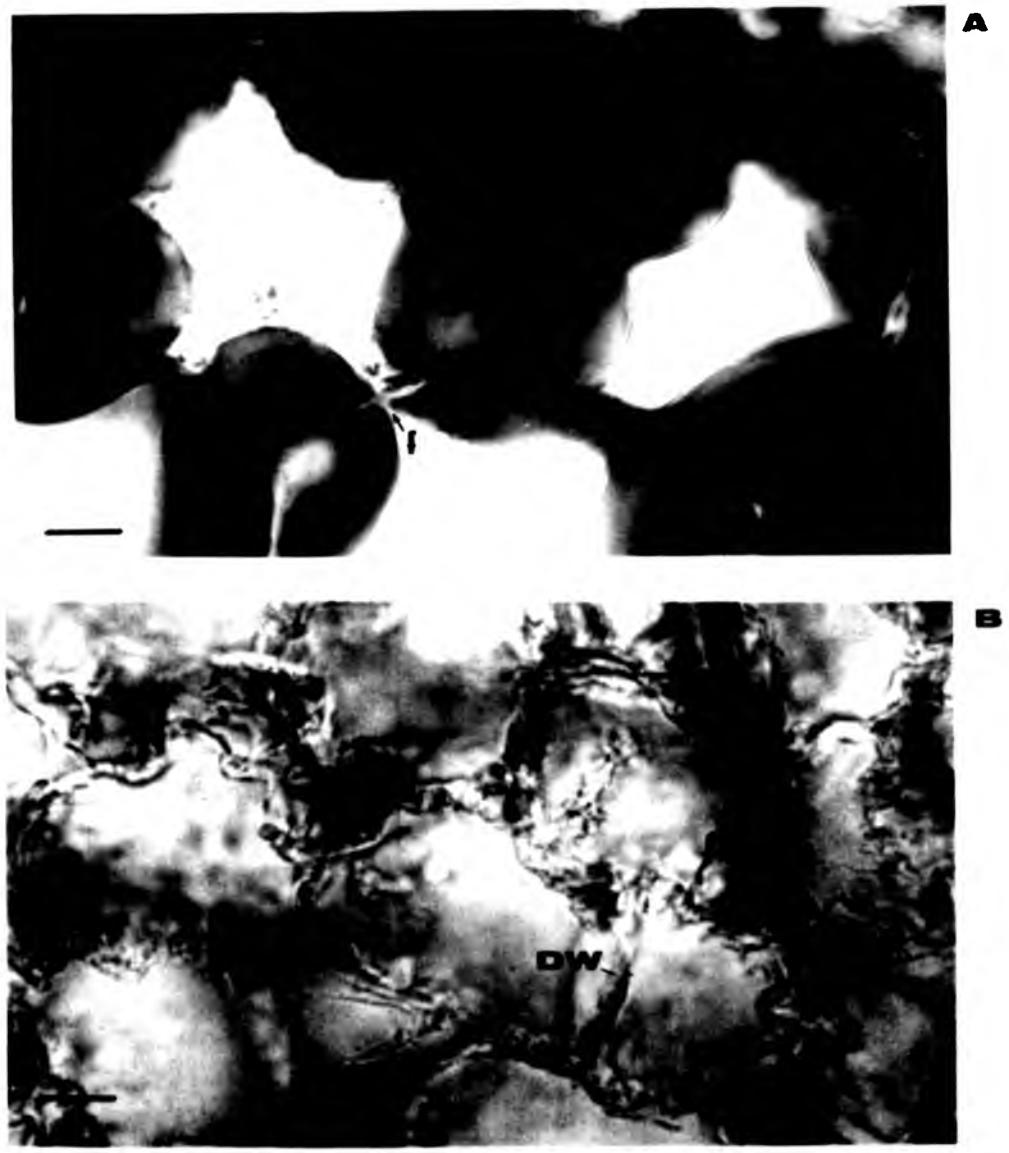
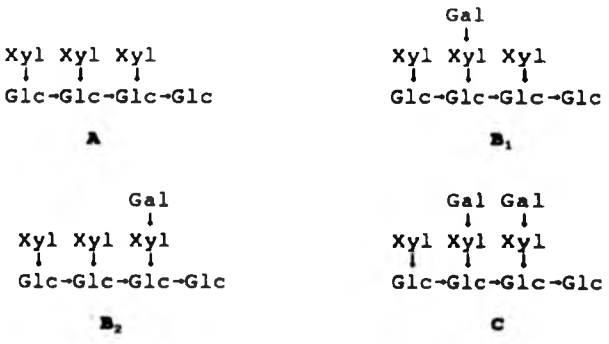


Figure 1.5. Xyloglucan degradation in *Copaifera langsdorfii* seeds.Bars represent 10µm. **A** - imbibed ungerminated seed; cotyledonary cell walls stained with iodine solution. **B** - Cotyledonary cell walls after reserve degradation showing only slight residual staining with iodine. **XW**=Xyloglucan containing wall; **DW**=wall after xyloglucan mobilisation; **f**=pit field. From Buckeridge et al.(1992).

Seed xyloglucans have a cellulose-like (1→4)-ß-glucan backbone to which single-unit **α**-xylopyranosyl substituents are attached (1→6)-**α**. Some xylose residues are further substituted at carbon-2 by ß-D-galactopyranosyl residues (Edwards et al., 1985). The pattern of xylose-substitution is remarkably regular, virtually the whole molecule being composed of (glucose$_4$: xylose$_3$) repetitive units (**A**, below) with variable galactose substitution (**B$_1$**,**B$_2$** and **C**, below) (York et al., 1990)

Recent work using purified xyloglucanases (see below) have demonstrated that the seed xyloglucans from *Tropaeolum majus, Tamarindus indica* and *Copaifera langsdorfii* all have in common the four basic oligosaccharide structure subunit (**A**,**B$_1$**,**B$_2$** and **C**), which are combined in different proportions to give a fine structure that varies according to the species and even populations of the same species (Buckeridge et al.,1992).



The reserve function of xyloglucan in cotyledons of *Tropaeolum majus* seeds was confirmed by Edwards et al.(1985). In this work, the authors showed that xyloglucan mobilisation *in vivo* is accompanied by a rise and fall of the activities of three hydrolases: ß-galactosidase, endo-(1→4)-ß-glucanase, α-xylosidase.

Reid and co-workers (Edwards et al., 1986; Edwards et al., 1988; Fanutti et al., 1991) have isolated the three main enzymes responsible by xyloglucan degradation in *Tropaeolum majus.* A xyloglucan-specific endo-(1→4)-ß-D-glucanase or xyloglucan endotransglycosylase (Edwards et al., 1986, Fanutti et al., 1993), a ß-galactosidase with high specificity towards xyloglucan (Edwards et al., 1988) and a xyloglucan-specific oligosaccharide-specific α-xylosidase or oligoxyloglucan exo-xylohydrolase (Fanutti et al.,1991).

The possible interaction of these enzymes (plus a ß-glucosidase) in the degradation of xyloglucans during reserve mobilisation in seeds is shown in **Figure 1.6**. The four enzymes probably work in a concerted fashion producing free galactose, glucose and xylose. The scheme from **Figure 1.6** is based on what is known about the structure of xyloglucans as well as about the mode of action of the enzymes involved in its degradation in reserve tissues.

Reis et al.(1987) described cytochemically the digestion of the xyloglucan-containing cell walls of *Tamarindus indica* cotyledons. Using the techniques of

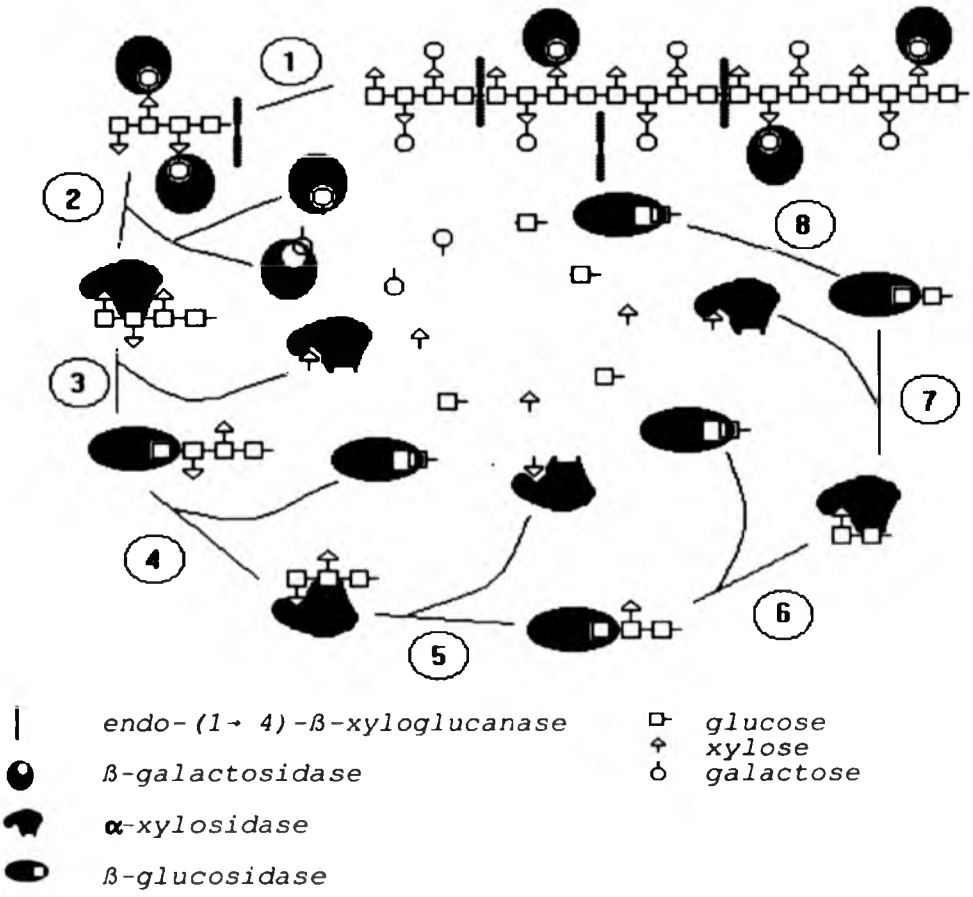


Figure 1.6. Probable enzymatic mechanism of hydrolysis of storage xyloglucans *in vivo*. Xyloglucan specific endo-(1→4)-ß-glucanase breaks down the main chain (step 1) at the same time that ß-galactosidase hydrolyses galactose branches (step 2). α-xylosidase is able to attack only the xylose residues that are linked to the glucose at the non-reducing end of the oligosaccharide (steps 3,5 and 7) and by removing xylose it makes the glucose residues at the non-reducing end accessible to ß-glucosidase (steps 4, 6 and 8). After one cycle of hydrolysis, each of the xyloglucan oligosaccharide blocks (glucose:xylose:galactose - 4:3:1 and 4:3:2 or glucose:xylose 4:3) is completely degraded to free glucose, xylose and galactose.

iodine staining (Kooiman, 1960) and also a gold-probe prepared by complexing *Escherichia coli* ß-galactosidase with gold particles, they were able to study xyloglucan mobilisation in cotyledonary cell wall at the ultrastructural level with greater specificity.

In *Copaifera langsdorfii*, the mobilisation of the thickenings of cotyledonary cell walls has been observed cytochemically (using iodine), physiologically and biochemically (Buckeridge et al., 1992). These authors have studied two different populations, from two distinct phytosociological regions (Forest and Savanna) and they found variations in the fine structure of the polysaccharide. It was proposed that these inter-species and inter-population differences in the amount and pattern of galactose substitution might indicate an environmental influence on the biosynthetic process.

Although no direct evidence has been produced of xyloglucan as a dual-purpose molecule in seeds, this polymer possesses hydrodynamic properties which are similar to those of galactomannans, suggesting a similar function as in imbibition and xeroprotection.

Another important aspect of the presence of xyloglucans in storage cell walls is that there is a high structural similarity between this polysaccharide and the xyloglucans which are present as major hemicellulosic components of dicot and some monocot primary cell walls. The main structural difference between seed and primary

cell wall xyloglucans is the presence in the latter of L-fucosyl residues attached α-(1→6) to galactose (Hayashi, 1989). Thus, the highly specific enzymes isolated from storage xyloglucan-bearing seeds are important structural tools for studying the plant primary cell walls. Furthermore there is increasing evidence that xyloglucan turnover in primary cell walls is brought about by enzymes with specificities and structures similar to the enzymes responsible for xyloglucan mobilisation in seeds (Nishitani and Rumi, 1992, O'Neill et al.,1989). This highlights the importance of the studies on storage cell walls for the understanding of many aspects of primary cell wall metabolism. It also reinforces the hypothesis (Reid, 1985) that there is an evolutionary link between the CWSP of seeds and individual non-cellulosic polysaccharides of non-seed cell walls.

Arabinogalactans

Arabinogalactans in non-seed tissues and in seeds

Arabinogalactans have been found in several tissues of plants (McNeil et al., 1984). They are composed of arabinose and galactose residues, the latter being usually in much higher proportion than the former. They have been classified into two different groups namely Arabinogalactans I and II (Fry, 1988). Arabinogalactan I is a cell-wall polysaccharide formed of a (1→4)-ß-linked

galactosyl backbone which is occasionally substituted through C-3 with arabinosyl and galactosyl side chains (throughout this thesis they will be referred to as "galactans" or "(1→4)-ß-galactans"). Arabinogalactan II is a highly branched polysaccharide containing 3-,6- and 3,6-linked galactosyl residues with variable amounts of arabinosyl, galactosyluronic acid and glucosyluronic acid residues. Since arabinogalactan II has not been reported as a major Cell Wall Storage Polysaccharide, it will not be discussed in detail here and the review by Fincher and Stone (1983) should be referred to for further information.

(1→4)-ß-Galactan was reported to occur in the cell walls of sycamore suspension cells by Talmadge et al.,1973). They presented evidence that the (1→4)-ß-galactan is covalently attached to the rhamnose units of a pectic rhamnogalacturonan. Keegstra et al. (1973) speculated that in primary cell walls of Angiosperms, galactan might bridge the pectin network to the cellulose/xyloglucan network. This was based on the possibility that fragments of xyloglucan might be covalently linked to the non-reducing end of the arabinogalactans (type I) [Talmadge et al.,(1973) and Bauer et al.,(1973)]. In fact, minor amounts of xylose and/or glucose are normally found in (1→4)-ß-galactan preparations from several biological materials (Knee, 1973; Crawshaw and Reid, 1984; Carré et al.,1985; Mankarios et al., 1980; Redgwell and Selvendran, 1986).

Relatively high proportions of (1→4)-ß-linked galactans have been detected in apple fruits (Knee, 1973), tomato fruits (*Lycopersicon esculentum*)(Gross and Wallner, 1979; Pressey, 1983), strawberry fruits (Knee et al., 1977), onion (*Allium cepa*)(Redgwell and Selvendran, 1986).

There is evidence suggesting that the galactans are always covalently linked to rhamnogalacturonan (RGI) in the cell wall (Knee, 1973; Talmadge et al.,1973; McNeil et al.,1984; Carré et al., 1985; Redgewell and Selvendran, 1986; Al-Kaisey and Wilkie, 1992 and others). This is based on the fact that galactan cannot be extracted without contamination with small amounts of an acidic polysaccharide usually containing galacturonic acid and rhamnose.

The presence of galactose-containing material in seed cell walls was first noted in the late 19th century. Schulze and Steiger (1892) identified galactose as the main sugar released on hydrolysis of the thickened cell walls of lupin cotyledonary tissues. Hirst et al.(1947) were the first to identify galactan as a distinct polysaccharide in seeds of the white lupin (*Lupinus albus*). They also proposed that some arabinose, in the form of an arabinan, was associated with galactan in the cell walls. On the basis of chemical structural determinations including methylation analysis they concluded that the polysaccharide was a (1→4)-ß-linked D-galactan.

Tomoda and Kitamura (1967) isolated two complex acidic polysaccharides by alkali extraction followed by ion exchange chromatography from *Lupinus luteus* seeds. These polysaccharides were demonstrated to be composed of galactose and arabinose residues with lesser amounts of galacturonic acid.

Brillouet and Riochet (1983) studied the composition of the cell walls of several species of lupins. They found a high positive correlation (r^2=0.92, n=15) between the ratio galactose/arabinose in the cell wall polysaccharides and the percentage of cell wall material in the seed. They also found a positive correlation (r^2=0.75, n=14) between the amount of cell wall material in lupin cotyledons and the rhamnose/galacturonic acid ratio. These authors attributed this correlation to an increase in sites (rhamnose residues) for anchoring galactan side chains to a pectic core. Thus they proposed that increased cell wall galactan could be due to an increased number of possible branching points on a pectic core rather than to increased galactan chain length. Amongst the species studied by Brillouet and Riochet (1983), *Lupinus angustifolius* had one of the highest yields in cell walls (21.1% of the dry weight of the seed) and proteins (50.4%) and one of the lowest levels of lipids (9.2%).

Recent studies by Al-Kaisey and Wilkie (1992) on methylation of the entire cell wall of *Lupinus angustifolius* cotyledons have added evidence for the predominance of (1→4)-ß-galactosyl linkages, attachment of

the galactan to a rhamnogalacturonan and also the presence of a xyloglucan-similar structure.

The biochemistry of the post-germinative mobilisation of cell wall storage polysaccharides (rich in galactan) from *Lupinus angustifolius* cotyledons has been described by Crawshaw and Reid (1984), and the ultrastructural changes accompanying the mobilisation process have been documented by Parker (1984b). Before germination, the cotyledonary cell wall material is very rich in galactose (71%) and arabinose (20%) residues, the remainder being composed of small amounts of glucose, uronic acid and rhamnose. After germination most of the galactose and arabinose are removed from the cell wall, leaving a residual wall which is enriched in rhamnose, uronic acid and glucose (Crawshaw and Reid, 1984).

Although galactose rich polysaccharides are present in other legume seeds [*Acosmium dasycarpum* (Buckeridge and Dietrich, 1990); *Ormosia sp.* (Brischi, Buckeridge and Dietrich, unpublished)], neither their structures, their metabolism nor their functions have been studied.

The overall function of galactan in lupins has been the subject of a long-standing debate. Reid (1985) described the scientific controversy which took place in the nineteenth century about the function of the cell wall in cotyledons of *Lupinus angustifolius, Lupinus luteus* and *Lupinus albus*. Whereas Nadelman (1890) concluded that the

galactan mobilised after germination was a reserve compound, Elfert (1894) strongly refuted this hypothesis, suggesting that the main function of the galactans would be rather in controlling the expansion of the cotyledons.

Matheson and Saini (1977) and Parker (1984b) described galactan catabolism respectively from the biochemical and anatomical point of views without, however, taking a side. On the other hand, Crawshaw and Reid (1984) pointed out that the galactose and arabinose produced during galactan mobilisation from *Lupinus angustifolius* cotyledonary cell walls were used as a reserve during plantlet development, but without ruling out the possibility that they might play a role in cotyledon expansion as well.

The aim of this thesis was the purification of the principal enzyme(s) responsible for the mobilisation of the (1→4)-ß-galactan from the storage cell walls of *Lupinus angustifolius* cotyledons and to contribute to an understanding of the relevance of galactan in the overall development of the seedling.

We have achieved the first goal, purifying for the first time one of the enzymes that perform the mobilisation of (1→4)-ß-galactan from the cell walls of *Lupinus angustifolius* cotyledons (Chapter 3). The enzyme, named exo-(1→4)-ß-galactanase, was showed to account for most (70%) of the galactose hydrolysed during the period of reserve mobilisation. It was further used as a tool for localising (1→4)-ß-galactan at the ultrastructural level in

cotyledonary cell walls (Chapter 5). Consequently, it was possible to describe galactan degradation with high precision during seedling development. Complementary studies of the physiological and morphological changes accompanying reserve mobilisation and cotyledonary expansion are described (Chapter 6). These results are placed in the context of possible multiple biological functions for CWSP and the evolutionary trends in the Leguminosae as far as reserve polysaccharides are concerned. Also a hypothetical model for the storage cell wall of *Lupinus angustifolius* is proposed (Chapter 7).

The species studied in this thesis was *Lupinus angustifolius*. The genus *Lupinus* L. belongs to the family Leguminosae, subfamily Papilionoideae (Faboideae), tribe Genistae, subtribe Lupininae (Polhill and Raven, 1981). The genus comprehends approximately 200 species, but only 4 are commercially important: *Lupinus albus, Lupinus angustifolius, Lupinus luteus* native to the Mediterranean area, and *Lupinus mutabilis* which originated in the Andes and the Rockies.

Areas where lupins originated

Chapter 2

MATERIAL & METHODS

Material

Seeds of *Lupinus angustifolius* cv. New Zealand Bitter Blue were obtained from Royal Sluis Ltd, Leyland, Preston, UK. The chemicals used in this work were of the highest purity possible.

Methods

General procedures

Germination and growth

Seeds of *Lupinus angustifolius* were soaked for 24h in distilled water at 20°C, transferred to trays (30 x 57 x 3 cm deep; 300 seeds per tray) and allowed to germinate at 20±2°C (photon fluence rate of 200 $\mu mol.m^{-2}.s^{-1}$). The seedlings were grown at 20°C with a light intensity at the cotyledon surface of 850 $\mu mol.m^{-2}.s^{-1}$ (12h day: 12h night).

Hydrolysis of polysaccharides and analysis of monosaccharides

Acid hydrolysis using sulphuric acid. Polysaccharides were hydrolysed to their monosaccharide constituents using 72% H_2SO_4-4% H_2SO_4 (Saeman et al. 1945). 5mg of polysaccharide was incubated for 45min. at 30°C in 72% w/w H_2SO_4 (0.1ml). The sample was diluted to 4% w/w with distilled water and hydrolysed in an autoclave for 1h at 120°C.

Analysis of monosaccharides by Thin Layer Chromatography (TLC). TLC of saccharide mixtures was performed on aluminium foil-backed silicagel layers 0.2 mm in thickness (Merck DC-Alufolien, Kieselgel 60). The solvent mixture used was n-propanol:ethanol:water (7:1:2 v/v/v). For separation of monosaccharides the plate was run twice whereas for separation of oligosaccharides it was run thrice. Carbohydrates were detected by spraying the plates with 5% w/w sulphuric acid in ethanol and heating at 120°C for 5min.

Analysis of monosaccharides by HPAE (High Performance Anion Exchange) chromatography. The neutral monosaccharides in the neutralised (NaOH) hydrolysates were identified by high-performance anion exchange (HPAE) chromatography. Samples were applied into a CarboPac PA1 anion-exchange column (250x4mm; DioneX, Camberley, UK) which was eluted isocratically with a 20mM NaOH solution for 2min. followed by water for 28min. at a flow rate of 1ml/min. Sugars were determined quantitatively using a pulsed amperometric detector (DioneX). Detector responses were determined using the appropriate standards and molar response factors for standard monosaccharides were determined daily.

Determination of uronic acids. The content of uronic acids was determined in isolated galactan from *Lupinus angustifolius* using the procedure described by Blumenkrantz and Asboe-Hansen (1973). After hydrolysis as described

above, aliquots (0.02ml) were taken and mixed with 0.0125M sodium tetraborate in concentrated H_2SO_4 (1.2ml) and boiled for 5 minutes. 20 µl 0.15% meta-hydroxydiphenyl in 0.5% NaOH was added and absorbance was read at 520nm. A standard curve was constructed using galacturonic acid (0-100µg/ml).

Isolation and analysis of galactan from *Lupinus angustifolius* cotyledons

Cotyledons of fully imbibed *Lupinus angustifolius* seeds (120g) were hand-isolated and homogenised in a blender with 10% NaCl (1 L). The mixture was stirred for 3h at room temperature and filtered through cheese cloth. The residue was re-extracted 3 times. The final residue was suspended in 0.2% w/w NaOH, stirred for 12h and filtered. The residue was re-extracted in the same way. The combined filtrates were neutralized with HCl, and boiled for 3h. The resultant thick suspension was centrifuged (15000g, 15° C, 30min.). The supernatant was treated with 3 volumes of ethanol, and the precipitated material was collected by filtration through cheese cloth and dried under reduced pressure. When the dried material was stirred into distilled water it dissolved almost completely. The solution was clarified by centrifugation (15000 · g, 15°C, 30min.) and the supernatant was treated with ethanol (3 volumes). The precipitate was redissolved in water and the ethanol-precipitation procedure repeated three times. The final precipitate was dissolved in distilled water,

dialysed exhaustively against distilled water and freeze-dried. The yield obtained was of 6.5% of the dry weight of the seeds.

Galactose determination using Galactose Dehydrogenase (GDH)

D-galactose was determined using ß-D-galactose dehydrogenase (Sigma Chem. Co.)(Kurz and Wallenfels, 1974). 64µl of the galactose-containing solution up to 1mM was incubated with 0.9 ml of Tris-HCl buffer pH 6.9, 64µl of ß-D-galactose dehydrogenase at 2.5 units.ml^{-1} (dissolved in 1M ammonium sulphate containing 1mM EDTA) and 32 µl of a 16.5mM NAD solution. At 20°C, absorbance at 340nm was constant after 40min. incubation. The absorbance was read at 340nm after one hour at room temperature. A reagent-blank was incubated at the same time and used as reference and a standard curve was constructed using galactose (0-1mM).

Protein Determination

The protein concentration was determined according to the method of Sedmak and Grossberg (1977). To 0.5 ml of each properly diluted sample, 0.5 ml of a Coomassie Blue solution (0.06% solution of Coomassie Blue G250 in 3% perchloric acid, filtered through Whatman No.1 paper) was added and the absorbance at 620nm was recorded after 5min. at room temperature. The absorbance values were used to

calculate the protein concentration by comparison with a standard curve constructed using Bovine Serum Albumin (BSA) between 0 and 50 µg/ml in the same buffer as the samples.

Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Polyacrylamide gel electrophoresis (PAGE) containing sodium dodecyl sulphate (SDS) was performed according to the method described by Laemmli (1970). The following solutions were prepared:

a) Separating gel buffer: 1.5M Tris, 0.4% SDS and the pH was brought to 8.7 with concentrated HCl.

b) Sample buffer: 30ml of 10% SDS, 12.5ml of 0.5M Tris, 10% SDS pH 6.8, 10ml of glycerol and the pH brought to 6.8 with concentrated HCl.

c) Reservoir buffer: 0.125M Tris, 0.960M glycine, 0.5% SDS pH 8.3.

0.75mm thick gels (9x6cm) were prepared containing acrylamide concentrations of 12% unless otherwise stated. In order to avoid lateral streaking of the bands, the design of the slab gels was modified according to Neuhoff et al. (1986), in which the stacking gel is prepared using the reservoir buffer instead of Tris-SDS pH 6.8.

Samples were prepared by adding the equivalent volume of sample buffer plus 5µl of bromophenol blue (0.1%) and ß-mercaptoethanol (0.02%) followed by boiling for 5min. Up to 20µl of sample solution was applied into the gel wells and it was run at a current of 20 mA for about 1 hour, time

necessary for the marker (bromophenol blue) to arrive to the end of the gel.

Staining was performed for 30min. at room temperature with continuous agitation, in Coomassie Brilliant Blue R250 dissolved in acetic acid:methanol:water (2:5:5 - v/v/v) and destained through several washings with a methanol:acetic acid:water (1.3:1:11 - v/v/v) solution.

Detection of proteinase activity in gelatin gels

SDS-PAGE gels were prepared as described below except for the fact that gelatin was incorporated into the resolving gel at a final concentration of 0.2%.

After electrophoresis, the gels were incubated in 2.5% Triton X-100 in water for 30min. and then in 20mM Sodium acetate buffer pH 5.0 for 16h at 30°C. The gels were stained with Coomassie blue R250 as above. After the gel was destained, proteinase activities were revealed as light zones against a dark blue background. The procedure was that of Heussen and Dowdle (1980) and performed in collaboration with Dr.Stuart Wilson.

Determination of Molecular Weight using gel chromatography on Bio-Gel P-100

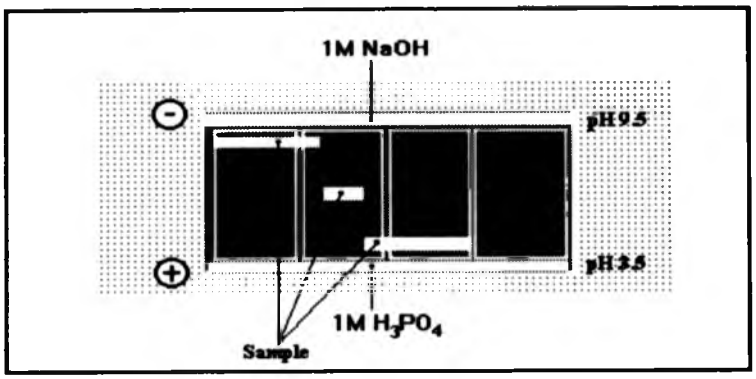
For determination of the molecular weight of the enzymes extracted, a calibrated BioGel P-100 (1 x 39cm) column was used. This column was calibrated using blue dextran (2000 KDa), bovine albumin (66 KDa), egg albumin

(45 KDa), carbonic anhydrase (29 KDa) and cytochrome c from horse heart (12.4 KDa). One millilitre fractions were collected and the elution volume corresponding to the enzyme was obtained using enzymatic activity. The approximate molecular weights of the enzymes were calculated using a calibration curve prepared on the basis of the elution volumes of the standards mentioned above.

Isoelectric Focusing (IEF)

Isoelectric focusing was carried out using an LKB 2117 Multiphor apparatus (Pharmacia LKB Ltd, Uppsala, Sweden). The gels used were from LKB (Ampholine PAGplate) and permitted separation of proteins with pI values between 3.5 and 9.5. 1mm thick gels were cut to have a size of 122x110mm. Samples were applied on three different regions of the gel which was run for 3h. Sample was not applied to one fourth of the gel, which was subsequently used for determination of the range of pH after running. After separation, the gels were cut in four portions as shown in the diagram below.

One of the portions (**St.**) was fixed for 1 hour in a solution containing 34mg/ml sulphosalicylic acid and 115mg/ml trichloroacetic acid solution and stained for 30min. in 1mg/ml Coomassie Brilliant Blue R250 in water:ethanol:acetic acid: (12.5:4:1 - v/v). Apart from the portion used for staining, three of the other portions (**Enz.Act.** and **SDS-PAGE** and **pH**) were cut along the axis of



pH change into 0.5cm slices. The slices of the portion **pH** were used for pH determination along the gel. Each slice of **Enz.Act.** was incubated overnight at 5°C and the levels of ß-galactosidase (PNPGal as substrate) and exo-galactanase (lupin galactan as substrate) activities were determined. After identification of the bands by staining and activity of the enzyme, the slices from the other portion (**SDS-PAGE**), which corresponded to the active polypeptides, were subjected to extraction by homogenisation with 20mM Tris-HCl pH 7.8 containing 0.5% SDS. After centrifugation the supernatant was collected, freeze dried, re-suspended in sample buffer and subjected to analysis by SDS-PAGE.

Western immunoblotting

After separation by SDS-PAGE the proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) for 1h in 0.4M Tris-glycine - 20% methanol buffer pH 8.3 using a constant current of 200 mA. The membranes were blocked for 3h in a solution containing 0.1% gelatin, 1% BSA in phosphate buffer saline (PBS), pH 7.0, containing some drops of Tween 20. The membrane was incubated overnight with either the crude antiserum (diluted 1:1000) or the affinity-purified antiserum (descriptions of how they were obtained will be given latter in this chapter). After incubation with the antiserum (1h) the membrane was washed 5 times with 50mM Tris-HCl, pH 7.4, 0.5% tween 20. The membrane was incubated for 1h with the second antibody [goat anti-rabbit IgG conjugated to horseradish peroxidase (Dynatech, Billingshurst, UK)] and the bands were visualised by the addition of diamino-benzidine (DAB, Sigma) solution (50 mg in 100ml 50mM Tris-HCl, pH 7.4) followed by 100 µl of 0.03% hydrogen peroxide (v/v).

Enzyme assays

ß-galactosidases and/or ß-galactanases were detected in extracts from the cotyledons of *Lupinus angustifolius* using two substrates: p-nitrophenyl-ß-D-galactopyranoside (PNPGal) and isolated lupin galactan.

PNPGal assay. Enzyme extract (50µl) was incubated for 20min. at 30°C in 50mM ammonium acetate buffer pH 5.0 with PNPGal at a final concentration of 25mM. The reaction was stopped by adding 1.0ml of 0.1M Na_2CO_3. The absorbance at 400nm was recorded for estimation of p-nitrophenol released.

Galactanase assay. Enzymatic assay using galactan as substrate was performed using 60µl of a 1% galactan solution, 60µl of enzyme extract and 0.1ml of 50mM ammonium acetate buffer pH 5.0. After incubation for 20min. at 30°C, the reaction was stopped by boiling the solution for 2min. Aliquots (64µl) were used for determination of galactose using GDH.

Time course of ß-galactosidase and exo-galactanase activities

Four replicate samples of 3 cotyledons each from 0 to 20 days old lupin plantlets (or seeds) were homogenised [Omni-Mixer, (Sorvall, Newtown, Conecticut, USA)] with 0.2M sodium phosphate buffer pH 7.2 (50 ml) containing insoluble polyvinylpolypyrrolidone (PVP) (0.3 g). The mixture was stirred for 1h at 5°C and centrifuged (19000g; 5°C; 30min.). Ammonium sulphate was added to the supernatant to give 80% saturation. The precipitate was collected by centrifugation as described above, dissolved in 0.1M NaCl and dialysed overnight against the same solution. The precipitate formed

during dialysis was removed by centrifugation (25000g; 5°C; 20min.), and the supernatant was dialysed exhaustively (16h) against 50mM ammonium acetate buffer pH 5.0. The precipitate formed during dialysis was removed by centrifugation as before. Galactanase and ß-galactosidase assays (see above) were carried out on the supernatants.

SDS-PAGE was performed using aliquots of the samples from 2,6,10,12,14,18 and 20 days of development.

Galactanase Purification

Crude Extract Preparation

A batch of 2,600 cotyledons harvested from 18 days old lupin plantlets was stirred mechanically in 800ml of 0.2M sodium phosphate buffer pH 7.2 containing insoluble polyvinylpolypyrrolidone (PVP) (3 g). The homogenate was centrifuged at 15000g for 40min. at 5°C and the supernatant was filtered through a double layer of cheese cloth.

Ammonium Sulphate Precipitation

Solid ammonium sulphate was added to the filtered solution up to 80% saturation. After stirring for 1h at 5°C, the pellet was collected by centrifugation (20000g; 30min.; 5°C), dissolved in 20mM Tris-HCl buffer pH 7.8 and dialysed against the same buffer overnight with four changes. The precipitate formed during dialysis was separated by

centrifugation at 15000g, 30min. at 5°C and discarded. The supernatant containing galactanase activity was subjected to anion exchange chromatography.

Anion exchange chromatography

The sample was applied onto a 2.5x27 cm DEAE-cellulose (DE-52, Whatman) column which had been equilibrated with 20mM Tris-HCl pH 7.8. Fractions of 9 ml were collected up to the point at which absorbance at 280 nm was constant and no galactanase activity (using galactan as substrate) was detected.

The main fractions containing galactanase activity were pooled and ammonium sulphate (up to 80% saturation) was added. After stirring for 1h at 5°C, the precipitate was collected by centrifugation (20000g; 40min.; 5°C).

The pellet was redissolved in 50mM ammonium acetate pH 5.0 and dialysed overnight against the same buffer.

Cation exchange chromatography

The dialysate obtained after DEAE-cellulose chromatography was applied onto a 2.5x15 cm CM-cellulose (CM-52, Whatman) column which had been pre-equilibrated with 50mM ammonium acetate buffer pH 5.0.

Fractions of 8 ml were collected and absorbance at 280nm, ß-galactosidase (PNPGal assay) and ß-galactanase (GDH assay) activities were monitored.

As all the ß-galactanase was retained by the column, a gradient of NaCl (0-0.5M) was applied to the column and the protein and enzymatic activities were monitored as mentioned above.

The main fractions containing ß-galactanase activity were pooled and precipitated with ammonium sulphate (80% saturation). The pellet was collected by centrifugation (20000g; 30min.;5°C), redissolved in a small volume of 50mM ammonium acetate buffer pH 5.0 and dialysed against the same buffer for 24h with 3 changes.

Affinity Chromatography

Some of the affinity resins which might specifically bind to ß-galactosidases [p-aminobenzyl-1-thio-ß-D-galactopyranoside-agarose, N-(ε-aminocaproyl)-ß-D-galactopyranosylamine-agarose, sepharose 6B-ß-1,4-galactan, lactose-agarose and sepharose-Con A] were tested in order to find one that could purify the exo-(1→4)-ß-galactanase. Aliquots (100 µl) of the CM-52 pooled sample were applied to 1ml columns prepared with each resin. Fractions of 200 µl were collected followed by determination of ß-galactosidase (using PNPGal), galactanase (using lupin galactan) and protein (absorbance at 280nm). Each column had been pre-equilibrated with 50mM acetate buffer pH 5.0 and a standard BSA solution (1 mg/ml) was applied in order to determine the void volume of each column.

Lactose-agarose was the only resin that was able to retain and release galactanase activity. It was observed that, if eluted with 50mM acetate buffer pH 5.0, the galactanase activity would elute as a very broad peak. On the other hand, if eluted with 20mM Tris-HCl pH 8.6, a sharp peak was obtained. A 46 ml lactose-agarose column (1.5 x 26 cm) was used for the separations and the change of pH from 5.0 to 8.6 was timed to make the galactanase activity elute as a sharp peak after the non interactive proteins had already been eluted.

Preparation of the sepharose-galactan resin. Sepharose-galactan resin was prepared strictly as described by Pharmacia Fine Chem. (Affinity Chromatography, principles & methods, 1983 pg.27). 1g of epoxy-activated Sepharose 6B (Sigma) was washed and re-swelled with distilled water (100ml). A 2% buffered solution of isolated lupin galactan (in 0.25M phosphate buffer pH 11.9) was added to the washed epoxy-activated Sepharose 6B and left shaking at 35°C for 16h. After incubation, the resin was washed with distilled water, 0.1M bicarbonate buffer pH 8.0 and 0.1M acetate buffer pH 4.0. Blocking of the excess groups was performed by incubating the galactan-Sepharose resin with 1M ethanolamine overnight. Binding of exo-galactanase occurred when the enzyme was applied to a 1ml column which had been pre-equilibrated with 20mM ammonium acetate buffer pH 5.0. Neither 20mM Tris-HCl pH 8.6 nor 1M NaCl nor 50mM PNPGal were able to elute the enzyme.

Properties of the galactanase

pH Optima and Stabilities were evaluated using 0.2M McIlvaine buffer from pH 2.6 to 7.8 and 0.1M carbonate-bicarbonate buffer for pH 9, 10 and 11. To investigate pH stability, the enzyme was incubated in the absence of substrate at the appropriate pH for 10min. at 30°C and then assayed with PNPGal or lupin galactan. The pH of the assay mixture was adjusted to 5.0 with 0.5M ammonium acetate. Temperature stability was tested by pre-incubating the enzyme solution (pH 5.0) for 10min. at 20, 30, 40, 50, 60 and 70° C followed by assays with PNPGal and lupin galactan.

Specificity and mode of Action on ß-1,4-pentagalactopyranoside

For specificity studies, the following glycosides, oligo and polysaccharides were used (the final concentrations during assay are shown in parentheses): larch wood arabinogalactan (10mg/ml), p-nitrophenyl-α-D-galactopyranoside (10mM), p-nitrophenyl-ß-D-galactopyranoside (10mM), p-nitrophenyl-ß-D-glucopyranoside (10mM) p-nitrophenyl-ß-D-mannopyranoside (10mM), methyl-ß-D-galactopyranoside (10mM) Gal-(1→4)-ß-Glc (lactose-10mM), Gal-(1→6)-ß-Gal (10mM), Gal-(1→3)-ß-Ara (10mM), Gal-(1→4)-ß-Man (10mM) and Gal-(1→4)-ß-Gal-(1→4)-ß-Glc (9mM) (all from Sigma Chem.Co, Poole, Dorset, UK), Gal-(1→4)-ß-Gal (11mM) and Galactopentaose [(1→4)-ß-linked](10mM) (from

Megazyme, Aust. Pty.Ltd.North Rocks), lupin (1→4)-ß-galactan (1mg/ml) prepared as described above, tamarind xyloglucan (10mg/ml) (Glyloid from Dainippon Pharmaceutical Corporation, Osaka, Japan), xyloglucan oligosaccharide (0.24mM), prepared by Cristina Fanutti by enzymatic digestion of tamarind xyloglucan.

The kinetic constants (K_m and V_{max}) were determined after incubation of purified exo-galactanase with varying concentrations of the substrate (each substrate had to be studied separately for determining the optimal range of concentrations). The reciprocal of the values of concentrations of substrate and corresponding activities (velocity) were used to construct a Lineweaver-Burk double-reciprocal plot. K_m was calculated from the 1/substrate concentration intercept and V_{max} from the 1/v intercept. The results (K_m and V_{max}) were confirmed using the software Enzyme Fitter (published by Elsevier-Biosoft, 1987) and these are the values given.

The mode of action of exo-galactanase was studied using (1→4)-ß-galactopentaose. A solution of this oligosaccharide was incubated with the purified exo-(1→4)-ß-galactanase at 30°C at a final concentration of 10mM. Aliquots were taken after 0,2,4,6,8,10,17,21 and 25 hours and separated by TLC. Galactose, (1→4)-ß-galactobiose and (1→4)-ß-galactopentaose (both from Megazyme, Australia) were used as standards. Carbohydrates were visualised by spraying the plate with 5% sulphuric acid in ethanol followed by 5min. at 100°C.

Raising Antibodies Against the 60 Kda Band

Isolation of the 60 kDa Band

Purified galactanase protein (100 µg) was strip-loaded on a 15% SDS polyacrylamide gel and run as above. The gel was stained lightly with Coomassie blue G-250. The major (60 kDa) band, which was cleanly separated from the minor (45 kDa) band, was excised and frozen, and crushed in PBS (1.2 ml) to which 0.55 ml of Freund's complete adjuvant was added. The solution was sonicated twice for 2min. on ice and the resultant suspension was injected into a rabbit (New Zealand White). Further preparations using incomplete rather than complete adjuvant were injected 16 and 38 days later. Blood was collected and the serum tested against the purified enzyme by immunoblotting 16, 38, 48, 51 and 62 days after the first injection. The maximum titre was at 62 days. This was the crude antiserum which was purified further.

Affinity purification of the Antibodies

The method of affinity purification followed the description by Stronach (1991) of the methodology developed by Olmsted (1981).

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The method of affinity purification followed the description by Stronach (1991) of the methodology developed by Olmsted (1981).

The purified enzyme protein (100 µg) was strip-loaded on an SDS gel and the separated proteins were transferred to a nitrocellulose membrane as above. The membrane was stained with 0.1% Ponceau S in 0.1% acetic acid 1.5% trichloroacetic acid to reveal the two separated bands. Destaining was performed by washing with 0.1M Tris-HCl buffer pH 7.5 containing 0.9% NaCl. Each band was excised and incubated with 250 µl of the crude antiserum overnight at 5°C. The strips were then washed thoroughly with blocking solution (0.1% gelatin, 1% BSA in phosphate buffer saline (PBS), pH 7.0, containing some drops of Tween 20) to remove any non-specifically bound antibodies and then with 5 ml glycine buffer pH 2.5 to dissociate the bound antibodies. The glycine buffered antibodies solution was neutralised immediately (0.2M NaOH) and kept at -70°C. This was the affinity-purified antiserum.

Partial purification of other ß-Galactosidases present in Lupin Seeds

ßI enzyme

The proteins that bound to the anion exchanger during purification of the exo-galactanase were eluted using gradient of NaCl (0-0.1M 500ml). This procedure eluted bound ß-galactosidase activities which showed very low activity on lupin galactan, but high activity on PNPGal.

Two separate peaks of activity on PNPGal and on galactan were eluted. The fractions corresponding to each peak of activity were collected separately and named ßI and ßII. The pooled ßI fraction was precipitated with 80% ammonium sulphate and after centrifugation (20000g; 40min.; 5°C) the pellet was collected and redissolved in 50mM sodium acetate buffer pH 5.0. After dialysis for 20h against the same buffer with four changes, the sample was applied to a cation exchanger column (CM-52) with exactly the same dimensions and under the same conditions as described above for exo-galactanase preparation. Activity on PNPGal was eluted from the column as a single peak and no activity on lupin galactan could be detected.

Gel chromatography in Bio-Gel P-60

Fractions containing ß-galactosidase activity were pooled and applied to a BioGel P-60 column (3 x 73cm), which had been pre-equilibrated with 50mM acetate buffer pH 5.0. Fractions of 3ml were collected and a single peak of activity on PNPGal was obtained. The main fractions containing ß-galactosidase activity were analysed by SDS-PAGE and the polypeptide pattern was used to decide what fractions should be pooled.

Properties of ßI enzyme

pH optimum, isoelectric focusing, SDS-PAGE, Western Blottings and assays were performed according to procedures previously described for exo-(1→4)-ß-galactanase. Molecular weight was determined by SDS-PAGE and Bio-Gel P-100 as already described.

ßII enzyme

ßII enzyme (from DE-52 gradient) pooled fractions were precipitated with ammonium sulphate (80% saturation) and the pellet was collected by centrifugation (20000g; 40min.; 5°C). The pellet was re-solubilised in 50mM sodium acetate buffer pH 5.0, dialysed for 20h with four changes and applied onto a cation exchanger column (CM-52). The conditions of elution as well as the size of the column were the same as described for the exo-(1→4)-ß-galactanase. Only one peak of activity was eluted from the column with 50mM sodium acetate buffer pH 5.0. This enzyme was active on PNPGal but had very low activity on lupin galactan. When a gradient of NaCl (0-0.5M - 500ml) was applied to the column a single peak of activity possessing only activity on PNPGal was observed.

The properties of this enzyme were not investigated, except for determination of molecular weight corresponding to ß-galactosidase activity by gel chromatography on a calibrated BioGel P-100 (1 x 39cm) column (conditions described above).

Preparation of the Cell Wall Ghosts (CWGs) and examination by scanning electron microscopy

The procedure described below was performed by Hutcheon and Reid in 1984, and it has not been published before. For this reason, and for the sake of understanding what has been done in this work, a transcription of the procedure used by these authors will be given below.

Sixteen hours imbibed seeds of *Lupinus angustifolius* cv.Unicrop had their testae and embryonic axes removed. The dissected cotyledons were ground in a mortar with 3 volumes (v/w) of 100mM potassium phosphate buffer pH 7.0. The suspension was further homogenised in the Ultraturrax with 4 pulses of 15 seconds each. The homogenate was centrifuged for 5min. in a bench centrifuge and the pellet re-suspended (1:1 v/v) in buffer. The suspension was sieved through a 250 µm pore nylon mesh, the filtrate centrifuged and the pellet re-suspended as before. The resuspended filtrate was sieved through a 53 µm pore nylon mesh. The residue was re-suspended in buffer, centrifuged as before and the pellet was washed 3 times with distilled water. The clean pellet was re-suspended in a minimum volume of water and dialysed against distilled water for 16hs at 4°C and freeze-dried.

For preparation of the CWGs from 14 days old cotyledons, a different procedure was used. Cotyledons were stripped of the epidermis, ground in a mortar with 3 volumes (v/w) of 100mM potassium phosphate buffer pH 7.0

and centrifuged as above. The pellet was re-suspended and sieved through a 140 µm pore nylon mesh. The residue was frozen and thawed, centrifuged and the pellet re-suspended as above and the suspension was ground in glass homogeniser. After sieving (140µm pore nylon mesh) the residue was subjected to the same procedure once more and a last filtration through 53µm pore nylon mesh was performed. The residue was re-suspended in buffer, dialysed and freeze-dried as described for the 16 hours cotyledons (see above).

According to the authors, the procedures described above were optimised for the two developmental stages of the tissue, by following the resultant isolated cell wall material using light microscopy.

In this work, the freeze dried CWGs prepared by Hutcheon and Reid were hydrolysed with sulphuric acid and analysed by TLC and HPAE as described above.

For enzyme assays, the dry CWGs were re-suspended in 50mM ammonium acetate buffer pH 5.0 and incubated for various times at 30°C with or without enzyme. Release of galactose was monitored using the GDH assay. When no more galactose was released (144h), the hydrolysed (and control) CWGs were dialysed against distilled water for 20h (4 changes) and freeze-dried. The dry CWGs were coated with gold (Edwards Supercoater S150) and observed using a scanning electron microscope (ISI 60A).

Light and electron microscopy

Fixation, embedding and cutting were performed mostly by or with the help of Mr. Jim Purvis, who at the time was a technician at the Department of Biological and Molecular Sciences at the University of Stirling.

Cotyledons were fixed using Karnovsky's fixative solution (2% paraformaldehyde; 2.5% glutaraldehyde in 0.1M sodium calcodylate buffer pH 7.4). Cubes of approximately 1 mm^3 from the central part of the cotyledons were dehydrated in an alcohol series up to 100%. The blocks were embedded in LR White resin, following the manufacturer's instructions exactly. Polymerisation of the resin was performed at 60°C overnight. Sections (1µm) were cut in an ultramicrotome (LKB Ultratome III, 8800), from the fixed and embedded blocks.

For light microscopic observations, sections were placed on glass slides and stained with methylene blue. Sections that were fixed, embedded and cut as described above were covered with some drops of 0.1% methylene blue and incubated for ca.1min. at 60°C. The excess of methylene blue was taken by blotting the slide on whatman number 3 paper. The sections were then observed and photographed directly using a camera attached-microscope (Zeiss, Oberkichen, FRG).

For electron microcopy observations, sections were collected on nickel grids and stained with 2% uranyl acetate (20min.), washed several times with distilled water, incubated with 4% lead citrate (10min.), washed

with distilled water as before and observed at the transmission electron microscope (Jeol JEM 100C).

Preparation of the colloidal gold and estimation of the gold number

Fifty millilitres of a 0.01% tetrachloroauric acid solution was boiled for 2-3min and 2ml of 2% trisodium citrate was rapidly added. The solution was maintained in a boiling water bath (ca.5min.) until it went from a very light yellow to purple and then wine red colour. With this procedure, 15nm particles were formed (Roth, 1983).

In order to estimate the amount of pure enzyme necessary to stabilise the colloidal gold sol, 0,5,10,20,30,40 and 50 µl of pure partially denatured (50°C for 5min.) exo-(1→4)-ß-galactanase was incubated in Ependorff test tubes with 250 µl of the gold solution. After 1min., 25µl of 10% NaCl was added to each tube. Blue, through violet up to wine-red colours developed, the wine red solutions being the ones in which the gold particles had been stabilised by the pure enzyme (Roland and Vian, 1991). In this experiment, stable red colour was observed in the solutions which had from 20 (0.35µg of enzyme protein) to 50µl (0.8µg of enzyme protein) of enzyme for each 270 µl of gold solution. Therefore the proportion of approximately 1µg of enzyme protein per ml of gold solution was the minimal for obtaining a stable gold-complex

preparation. For different exo-(1→4)-ß-galactanase preparations, which had different enzyme concentrations, the gold number was estimated again.

For the preparation of the enzyme-gold probe, 200µl (3.5µg of protein) of active, partially denatured (50°C for 10min.) or denatured (boiled for 5min.) enzyme protein solubilised in 20mM Tris-HCl pH 8.6 was mixed with 0.8ml of the colloidal gold solution in an Ependorff tube at room temperature. The mixture was centrifuged in a bench centrifuge (Heraeus Christ bench centrifuge, model Biofuge A for 10min. at 13000rpm) and the red mobile pool was carefully removed with an automatic pipette. This procedure yielded ca.50µl of a dark-red enzyme-gold probe, that was used for localisation of galactan on electron microscopic sections.

Ultrathin sections cut, fixed and embedded in LR-White as described above were collected on nickel grids and incubated with a mixture (1:1) of the enzyme-gold complex and 0.1M ammonium acetate buffer pH 5.0 for 30 minutes in a moist chamber at room temperature. After incubation, the sections were thoroughly washed with distilled water and counter-stained with uranyl acetate and lead citrate as described above. Controls were prepared by dissolving galactan, starch, α-amylase, in ammonium acetate buffer pH 5.0 up to a final concentration of 0.05M.

Measurement of mechanical properties of lupin cotyledonary tissue

Estimation of plasticity and elasticity

Mesophyll tissues from cotyledons at different developmental stages (2,4,6,8,10,12,14 and 16 days after imbibition) were dissected in order to produce cuboid blocks measuring 0.5mm length x 0.2mm width x 0.2 depth. The blocks were immediately frozen by immersion in liquid nitrogen and thawed by immersion in 20mM Tris-HCl buffer pH 8.6 at room temperature.

Cuboids were subjected to two cycles of stress (extension)-relaxation of 10min. each, while immersed in 20mM Tris-HCl pH 7.8 at 21°C. Plastic and elastic extensibilities were obtained by measuring the maximum extension obtained at the end of the second stretching cycle (**Figure 2.1**). An average of six replicates was obtained for each point.

For comparison with the changes in mechanical properties, some physiological and biochemical parameters were recorded using the cotyledons from plantlets growing in exactly the same conditions. Fresh weight, dry weight, cotyledonary area, contents of protein and galactan and ß-galactosidase activity were recorded.

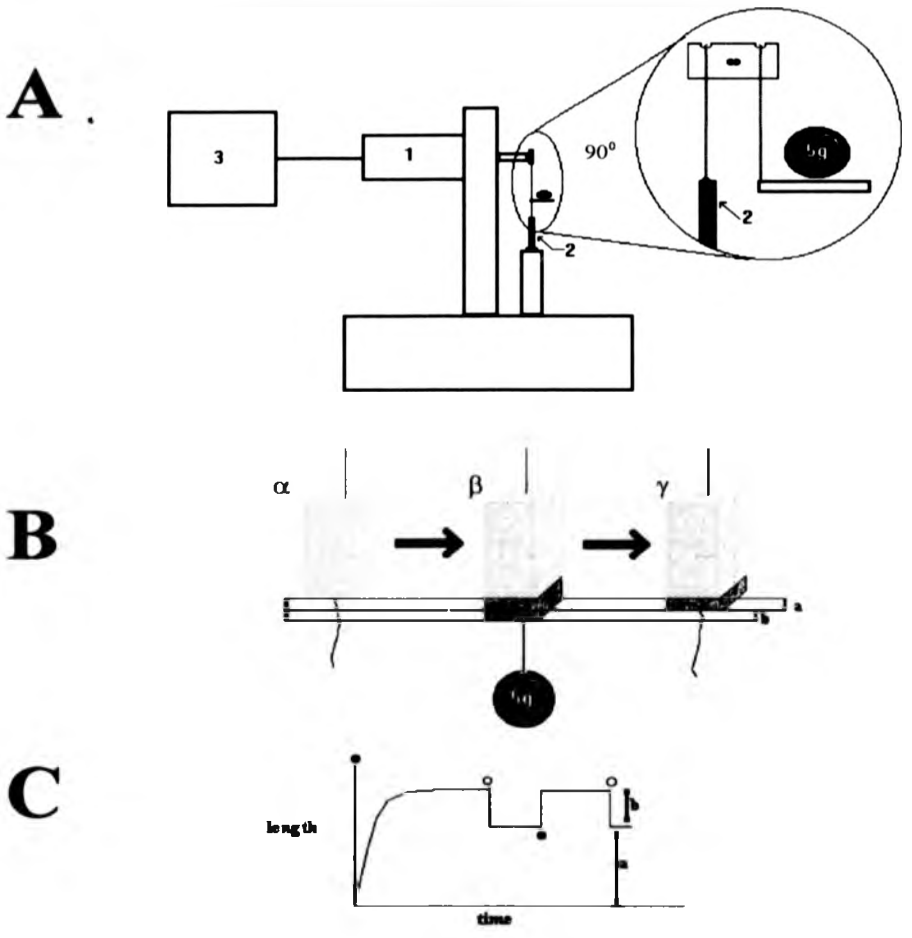


Figure 2.1. Mechanical properties experiment. **A** - Schematic representation of the machine used for creeping experiments. Magnification is rotated 90°.Base and top of a cuboid (0.5x0.2x0.2cm) excised from cotyledons of *Lupinus angustifolius* were attached to the metal structures of the apparatus using super glue. The container was filled with 20mM Tris-HCl pH 7.4 during extension.**1**-transducer out put calibrated to linear movement; **2**-container where the sample is immersed in buffer; **3**-plotter. **B** - Complete cycle of one extension experiment. **α**=cuboid before extension; **β**=cuboid extended (5g load on) for 15min; **γ**=cuboid relaxed (5g load off). Measurements were performed after two complete cycles. Full circles=**load on**; open circle=**load off**; **C** - Typical result obtained. **a**=Plastic component, **b**=Elastic component.

For determination of cotyledonary area, the shapes of the cotyledons were drawn on paper, excised and weighed. The area of each cotyledon was calculated by comparison with the weight of a $1cm^2$ square excised from the same paper. Eight replicates were used.

Determination of galactan content using pure exo-galactanase

Two cotyledons from the time course from the mechanical properties experiments (2,4,6,8,10,12,14 and 16 days after initiation of imbibition) were harvested and subjected to extraction of cell wall polysaccharides. Cotyledons were crushed in 24% KOH and centrifuged (bench centrifuge) for 10min. at room temperature. The supernatant had its pH adjusted to 5.0 with acetic acid and was subsequently precipitated with ethanol (3 vol). After 30min. at 5°C, the precipitate was collected by centrifugation (15000g, 30min.5°C). The pellet was washed 3 times with 75% ethanol, re-solubilised in distilled water and freeze-dried.

For incubation with exo-galactanase, the dry precipitates were re-suspended in distilled water by boiling for 10min. Incubation with exo-galactanase partially purified up to CM-52 step (this extract was demonstrated to possess only the bands corresponding to exo-galactanase by Western immunoblotting standards - Brickell, Buckeridge and Reid, unpublished). After

incubation for 20h (sufficient to hydrolyse all galactan present in the mixture), galactose amounts were determined in the mixture using GDH assay. Knowing the amount of galactose, the content of galactan in each extract could be estimated.

Chapter 3

PURIFICATION AND PROPERTIES OF A NOVEL ß-GALACTOSIDASE OR *EXO*-(1→4)-ß-D-GALACTANASE FROM THE COTYLEDONS OF GERMINATED *LUPINUS ANGUSTIFOLIUS* L. SEEDS.

Introduction

Plant ß-galactosidases were reported for the first time in emulsins of some Rosaceae (almond, peach, apricot and apple trees) by Bourquelot and Herissey in 1903 (*In* Wallenfels and Malhotra, 1961). Since then, several different techniques have been applied in order to produce semi-purified and pure extracts of ß-galactosidases from different sources. Ammonium sulphate precipitation and ion exchange chromatography using CM-cellulose and/or DEAE-cellulose followed by gel chromatography have been the most common methods used in early stages of purification (Wallenfels and Malhotra, 1961; Dekker and Richards, 1976; Dey and Campillo, 1984). However, in order to purify ß-galactosidases to homogeneity, techniques that take into consideration specific features of the enzyme molecule are necessary. Some examples are affinity chromatography (Dey and Campillo, 1984; Kanfer et al., 1973; Simos et al., 1989), interaction with concanavalin A (Matheson and Saini, 1977) and affinity chromatography-HPLC (De Veau et al., 1993).

In most of the plant systems which have been subjected to ß-galactosidase extractions, this enzyme occurs as a group of isoenzymes, normally with a very high degree of similarity concerning their properties, but with rather different specificities (Matheson and Saini, 1977; Pressey, 1983; Giannakouros et al., 1991; De Veau et al., 1993)

Affinity chromatography techniques for isolating ß-galactosidases have frequently been used (for example: Labavitch et al., 1976; Simos et al., 1989; Giannakouros et al., 1991). Probably because of the great variety in specificity of ß-galactosidases, there is not an affinity chromatography resin that can be considered of general use in purifying this class of enzymes. Nevertheless, there seem to exist subclasses of ß-galactosidases which, independently of the biological system from where they originate, bind to a given type of resin. Simos and colleagues (1989) were able to purify ß-galactosidases from seven different plant species using lactosyl-agarose. Chromatofocusing and electrofocusing have been used for successful isolation of the ß-galactosidases from nasturtium (Edwards et al., 1988) and radish (Sekimata et al., 1989).

More recently, ß-galactosidases from carrot (Konno and Katoh, 1992) and kiwi fruit (Ross et al., 1993) have been purified using gel permeation on Sephacryl S-200HR and Superose-12 respectively.

Beta-galactosidases are widely distributed in nature, and several enzymes from microorganisms, animals and plants have been isolated and characterised (Dey and Campillo, 1984). Few galactanases have been reported, none of them from plants. Those which act on (1→4)-ß-galactans include several endo-cleaving enzymes from fungi (Nakano et al., 1988; Lahaye et al., 1991; Van de Vis et al., 1991) and an

exo-(1→4)-ß-galactanase from the bacterium *Bacillus subtilis*. The *Bacillus subtilis exo*-galactanase catalyses the sequential cleavage of galactobiose units from the non-reducing end of galactan substrates (Nakano et al., 1990). Van Etten and Bateman (1969) reported that a (1→4)-ß-galactanase present in crude extracts of *Sclerotium rolfsii* hydrolysed *Lupinus albus* galactan to D-galactose only, which suggested that this might be an exo-enzyme. In plants there are numerous potential substrates for ß-galactosidases, including membrane galactolipids, oligosaccharides and certain cell wall polysaccharides with terminal, non-reducing ß-linked galactopyranosyl residues. These include the arabinogalactans [(1→3)- and (1→6)-linked galactose residues at branch termini], xyloglucans [terminal, non-reducing galactose residues linked (1→2) to xylopyranosyl residues in the side chains] and galactans [linear (1→4)-linked polymers]. The arabinogalactans and the galactans are also potential substrates for *endo*- or *exo*-acting galactanases.

There is evidence that plant ß-galactosidases may be associated with developmental events involving primary cell wall turnover, for example elongation-growth (Nevins, 1970; Tanimoto and Igari, 1976; Dopico et al., 1990), and fruit ripening (Bartley 1974; Wallner and Walker 1975; Pressey 1983; Dick et al., 1990; Ross et al., 1993). Yet, there is no clear indication as to which of the macromolecular components of the primary cell wall are involved. One

ß-galactosidase which has been firmly linked to a substrate in a process of cell wall modification *in vivo* is that from the cotyledons of the germinated nasturtium (*Tropaeolum majus* L.) seed. Although nasturtium cotyledonary extracts contain multiple ß-galactosidase activities, only one of them varies *pari passu* with xyloglucan mobilisation (Edwards et al., 1988). When isolated, this enzyme had the unique capability of hydrolysing the terminal, non-reducing ß-galactosyl residues from the galactosylxylose side-chains of the intact xyloglucan molecule (Edwards et al., 1986) as well as from xyloglucan-derived oligosaccharides.

Matheson and Saini (1977) have distinguished three ß-galactosidase activities in germinated *Lupinus luteus* L. cotyledons, and have suggested that they may be involved in galactan mobilisation. However, ß-galactosidases are commonly present in plant tissues (for example, Li et al., 1975; Kundu et al., 1990; Giannakouros et al., 1991), and it has been difficult to assign them specific physiological roles.

In this chapter, the isolation and characterisation of a hydrolytic enzyme from germinated lupin seed cotyledons is described. It has restricted ß-galactosidase activity, and catalyses the rapid depolymerisation of lupin (1→4)-ß-D-galactan by the successive release of galactose from the non-reducing end of the chain. On the basis of its specificity we have named it an *exo*-(1→4)-ß-D-galactanase. The developmental regulation of this enzyme in lupinseed cotyledons following germination indicates a key role for it in the depolymerisation of (1→4)-ß-galactan *in vivo*.

Results

Isolation of a natural galactan substrate and optimization of galactanase assays

It is becoming increasingly clear that the enzymes involved in the turnover of plant cell wall polysaccharides *in vivo* often possess a very high degree of specificity towards their natural substrates (for example: Edwards et al., 1986; Nishitani and Nevins 1991; Fanutti et al., 1991, 1993). Thus when attempting to detect such enzymes it is important to use the putative natural substrate in the assay system.

The (1→4)-ß-galactan component cannot be removed from the lupinseed cell wall by direct water-extraction. However, alkali-extraction of lupin (*Lupinus albus*) cotyledonary cell wall material results in the solubilisation of a (1→4)-ß-galactan-enriched fraction which, once isolated, redissolves freely in water (Hirst et al., 1947). The equivalent soluble material from *Lupinus angustifolius* [referred to below as "lupin galactan"] was utilized as substrate for the detection and assay of galactan-degrading activities [referred to below as "galactanases"].

The isolated galactan from *Lupinus angustifolius* cotyledons was a white powder and yielded 6.5% of the dry weight of the seed. After hydrolysis using sulphuric acid

(Saeman et al., 1945) and trifluoroacetic acid (TFA) 2N for 2h, the percentages of hydrolysis were 71.1% and 74.5% for sulphuric acid and TFA respectively. When hydrolysates were analysed by TLC, galactose, arabinose, glucose, xylose, rhamnose and galacturonic acid were detected, galactose being by far the main component. On analysis of the monosaccharides by HPAE (**Table 3.1**) the main components were galactose (ca.78%) and arabinose (ca.17%), the other monosaccharides being distributed amongst the remaining 5%.

Comparison of the compositions of the entire cell wall and the extracted galactan, showed that arabinose and galactose-containing polysaccharides are the main cell wall components. The ratio galactose/arabinose decreased from 5.6 in the cell to 4.4 in the extracts. The extract contained less glucose and xylose than the wall material.

In order to establish the general conditions for assay(s) to be used during the purification procedure, crude enzyme extracts were prepared from germinated lupin seeds and incubated with the isolated galactan. Enzymes which cleave linear polysaccharides in an *endo*-fashion are most conveniently assayed viscometrically. The initial viscosity of the lupin galactan preparation was, however, very low, preventing the reliable use of the viscometric method. Incubations were therefore analysed by TLC to detect any breakdown products of the galactan substrate. Only homogenates prepared from cotyledons during or after cell wall mobilisation *in vivo* catalysed any detectable

Table 3.1. Neutral monosaccharides and uronic acid in soluble lupin galactan and in isolated lupin cotyledonary cell walls.

	Monosaccharide Composition (%)					
	Neutral monosaccharides[1]					Uronic Acid[2]
	Rhamnose	Arabinose	Galactose	Glucose	Xylose	
Galactan	2.3	17.6	77.9	--	2.2	5.0
Cotyledonary Cell Walls	2.0	13	74	5.0	5.0	8.0

[1] as % of total neutral monosaccharides

[2] as % of cell wall material

breakdown of the galactan. On TLC galactose and a small amount of arabinose were the only breakdown products observed on the chromatograms. No oligosaccharides were detected even when incubation times were short. These results provided evidence that the extracts contained *exo*-galactanase or ß-galactosidase activity. They suggested also that *endo*-galactanase activity, which would produce galacto-oligosaccharides, was absent. Although the possibility remained that galacto-oligosaccharides might have been converted very rapidly to galactose by excess *exo*-galactanase activity, the release of galactose from galactan was nonetheless used to monitor the purification process, and it led directly to the purification of the *exo*-galactanase described below. No evidence for *endo*-galactanase activity in the cotyledonary extracts was obtained at any stage of the purification procedure.

PNP-ß-galactopyranoside (50mM) and PNP-α-arabinofuranoside (50mM) were also used as substrates and they were hydrolysed showing the presence of at least one ß-galactosidase and one α-arabinosidase (data not shown) in lupin cotyledons.

As the first goal was the purification of the enzymes responsible for the degradation of the lupin galactan, assay conditions for the activities present in the crude extract were optimised using galactan to detect ß-galactanase activity and PNPGal (p-nitrophenyl-ß-galactopyranoside) to detect ß-galactosidases.

Apparent pH optima and kinetic parameters were obtained using both PNPGal and galactan. The pH optima were 4.25 and 4.5 and the Km values 2.2 mM and 0.75 mg/ml respectively. Thus, the conditions for assay were standardised as 20-30 mM and 5-7 mg/ml final concentrations of PNPGal and lupin galactan respectively and pH 4.5 when using both substrates.

Developmental time course of ß-galactanase and ß-galactosidase activities in germinating *Lupinus angustifolius* cotyledons

At 18°C, germination was complete 6 days after the beginning of imbibition, and by day 10 most of the plantlets had already emerged and were showing the first pair of leaves. The expansion of the cotyledons started from day 6 and continued up to day 10, when shrivelling started. It persisted up to approximately 30 days, when the cotyledons started to fall. Thus, although in this work we have used a different cultivar of *Lupinus angustifolius* from Crawshaw and Reid (1984), the morphological changes during plantlet development were essentially the same. Detailed descriptions of the physiological, anatomical and morphological changes in lupin during and after seed germination will be given in Chapters 5 and 6.

Crude cotyledonary extracts were prepared and assayed using both substrates (PNPGal and galactan).

Figure 3.1 shows the variation in ß-galactosidase and ß-galactanase activities in the crude extract during germination and seedling development.

Beta-galactosidase activity was already present on the first day after initiation of imbibition, increasing approximately 5 fold from the 4th to the 8th day and then decreasing slowly up to the day 25.

Beta-galactanase activity could not be detected during the first 6 days, after which it started to increase very quickly, reaching a maximum activity on day 12. A plateau of activity was maintained for nearly 8 days. ß-Galactanase activity then decreased at a rate comparable with ß-galactosidase.

In this time course experiment, ß-galactanase activity increased later than the other ß-galactosidase(s) (**Figure 3.1**). Whereas the activity of PNPGal peaked at day 8 followed by a linear decrease up to day 25, ß-galactanase activity reached its maximum activity on day 12 and it was the dominant activity thereafter. These results show that under the conditions of this experiment, at least two distinct ß-galactoside/ß-galactan hydrolysing enzymes were active in different periods during and after germination. In another time-course experiment, performed

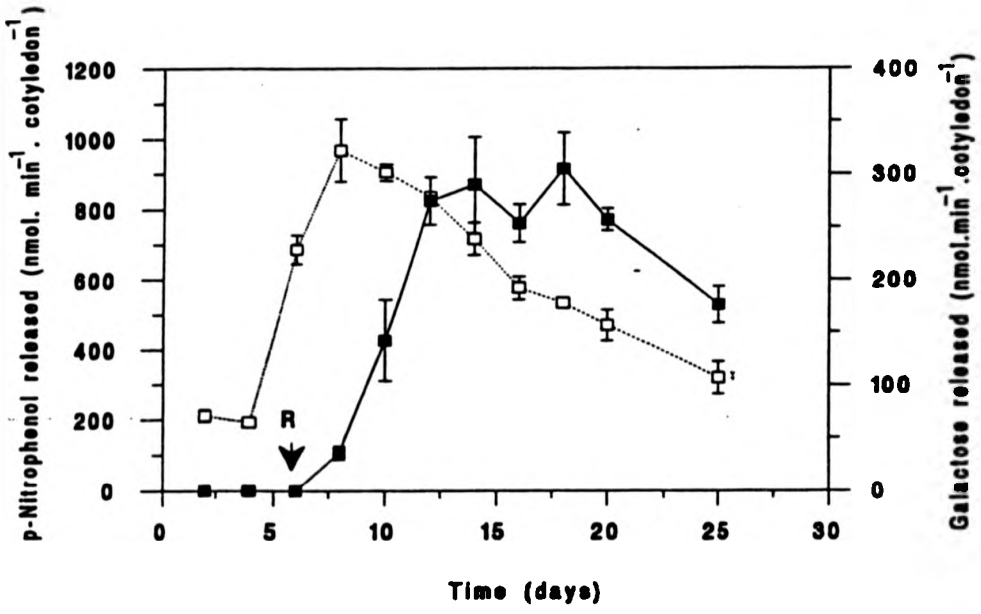


Figure 3.1. Time course of ß-galactosidase and galactanase activities in extracts of *Lupinus angustifolius* cotyledons during germination and seedling development. (□----□, PNP-ß-gal; ■——■, galactan). **R** - radicle protrusion.

at 24°C, radicle protrusion occurred by day 3 and both activities increased together so that the ß-galactanase and ß-galactosidase could not be distinguished at all (data not shown).

Taking these observations into account, it was deemed necessary to attempt large-scale ß-galactanase purifications using cotyledons harvested from 18 day-old plantlets grown at 18°C. This decision was based also on the polypeptide pattern observed on day 18 (**Figure 3.2**). Extraction of proteins from quiescent seeds as well as from 2 days old cotyledons was difficult, probably because seed imbibition had not yet been completed at this time. It is clear from **Figure 3.2** that reserve protein mobilisation occurred from day 6 to day 14. Thus, 18 days old cotyledons were chosen for enzyme preparations because at this time most of the reserve proteins have been mobilised and a much less complex polypeptide pattern was detected.

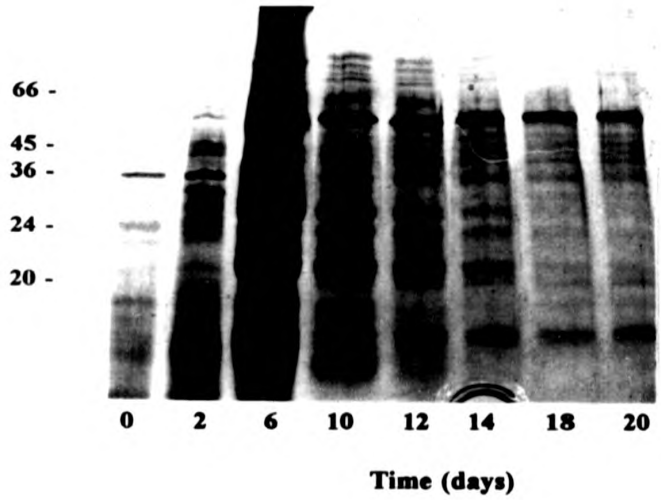
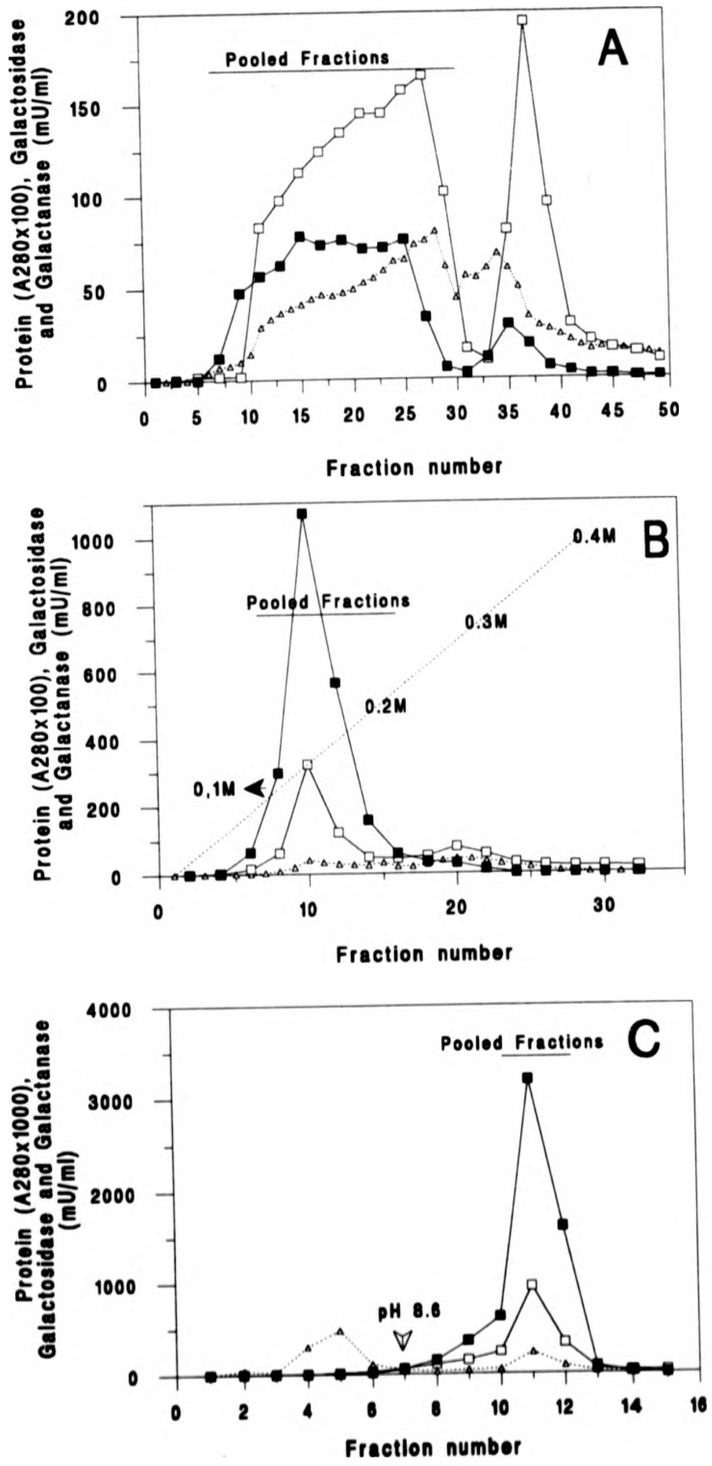


Figure 3.2. Polypeptide pattern in *Lupinus angustifolius* cotyledonary extracts (SDS-PAGE) during germination and seedling development. The numbers on the vertical scale are the molecular weights of standard markers (kDa).

Purification of *exo*-(1→4)-ß-galactanase

The successful purification procedure involved anion exchange chromatography on DEAE-cellulose, cation exchange chromatography on CM-cellulose and an affinity-purification on a lactose-agarose column (**Figure 3.3**). Most of the galactanase activity in the 18 day cotyledonary extract was not retained by the DEAE-cellulose column in dilute buffer at pH 7.8 (**Figure 3.3A**), whereas most of the ß-galactosidase activity remained attached to the column under these conditions (see Chapter 4). The non-bound galactanase and ß-galactosidase activities emerged from the DEAE-cellulose column in two peaks (**Figure 3.3A**). The second peak, which presumably contained those proteins which were slightly retarded, but not retained by the column, contained only a very small proportion of the galactanase, and it was not investigated further. It did, however, contain a significant proportion of the non-bound ß-galactosidase activity. In dilute buffer, pH 5.0, the galactanase was retained on the CM-cellulose column, and was eluted as a single, symmetrical peak in a sodium chloride gradient. The ß-galactosidase activity was eluted in two peaks, the major one coinciding exactly with the peak of galactanase activity (**Figure 3.3B**).

Figure 3.3.Purification procedure for isolation of the exo-ß-1,4-galactanase from lupin cotyledons. **A** - Elution profile of the material not retained on the DEAE-Cellulose (DE-52) column. The column (2.5x33cm) was eluted with 20mM Tris-HCl buffer pH 7.8. The sample (190ml of the redissolved 0-80% ammonium sulphate precipitate) was applied in the same buffer. Fractions of 10ml were collected. **B** - Elution profile of the material eluted from the CM-cellulose (CM-52) column in the NaCl gradient (0-0.4M). A 270ml solution was applied to a column (2.5 x 15cm) and 10ml fractions were collected. Elution was with 50mM NaOH-acetic acid buffer pH 5.0. **C** - Affinity chromatography on Lactose-agarose. Fractions (3ml) were collected. The column (1.5 x 26cm) was eluted with 50mM ammonium acetate buffer pH 5.0 during the 7 first fractions and with 0.2mM TRIS buffer pH 8.6 up to fraction 15.(▲----▲,protein, absorbance at 280nm;□----□,ß-galactosidase (PNP-ß-gal as substrate);■----■, galactanase (galactan as substrate).



Several affinity chromatography resins, to which ß-galactosidases might bind, were tried. They were p-aminobenzyl-ß-D-thiogalactopyranoside-agarose (resin 1), N-(ϵ-aminocaproyl)-ß-D-galactopyranoside-agarose (resin 2), p-aminophenyl-ß-thiogalactopyranoside-agarose (resin 3) and a resin prepared by binding lupin galactan covalently to galactan (sepharose-lupin galactan - resin 4).

The galactanase bound very weakly to resins 1, 2 and 3. When loaded onto small (1ml) columns containing the resins above no clear separation of *exo*-galactanase from the non-enzymatic polypeptides in the mixture could be observed. The galactanase bound very strongly to sepharose-lupin galactan (resin 4) and it could not be eluted with 0.2M Tris-HCl pH 8.6, 50mM PNPGal in 50mM ammonium acetate pH 5.0, 0.5M galactose in 50mM ammonium acetate pH 5.0 or 1M sodium chloride.

As can be seen in **Figure 3.3C**, the lactose-agarose column separated much inactive protein from the galactanase and ß-galactosidase activities, which co-eluted in a single peak. The separation occurred by retardation rather than by strong binding to the column.

Table 3.2 summarises the purification of the *exo*-galactanase. The enzyme was purified ca. 25 times (recovery of 2%) when calculated as ß-galactosidase and 40 times with a recovery of 3%, when calculated as galactanase activity.

The pooled, active fractions from the lactose-agarose column gave a strong band on SDS-PAGE (60kDa), plus a weaker one, at (45kDa)(**Figure 3.4**).

Table 3.2. Purification of the exo-ß(1→4)-galactanase. PNPGal and galactan were both used as substrates during the entire purification and both sets data are given for comparison.

Step	Total Volume (ml)	Protein (mg)	Total Activity µmoles.min.$^{-1}$		Specific Activity µmoles.min.$^{-1}$.mg of prot^{-1}		Purification		Recovery (%)	
			PNP[1]	GAL[2]	PNP	GAL	PNP	GAL	PNP	GAL
Crude	850	1968	1619	1405	0.82	0.71	-	-	100	100
$[NH_4]_2SO_4$	250	1727	1712	1125	0.99	0.65	1.21	0.92	105.74	80.07
DE-52	270	45	383.4	284.9	8.52	6.33	10.39	8.92	23.68	20.28
CM-52	126	6.73	65.4	119.9	9.72	17.82	11.85	25.10	4.04	8.53
LAC-AGA	87	1.53	30.8	42.5	20.13	27.78	24.55	39.13	1.90	3.02

[1]**PNP** - assay using p-nitrophenyl-ß-galactopiranoside as substrate
[2]**GAL** - assay using isolated lupin galactan as substrate

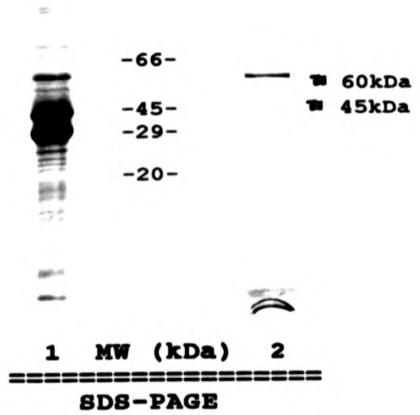


Figure 3.4. SDS-PAGE of the crude extract (**A**) and of the purified exo-ß-1,4-galactanase (**B**).

Relationship between the 60kDa and the 45kDa polypeptides

In order to investigate the relationship between the 60kDa and the 45kDa bands and in particular whether both ß-galactosidase and *exo*-galactanase activities could be attributed to the same polypeptide, a series of experiments was performed which included attempted separations (based on molecular weight, hydrophobic interactions, isoelectric focusing), production of antibodies against the 60kDa band and determination of the N-terminal sequence of the 60kDa and 45kDa bands. In all the separation experiments, PNPGal and lupin galactan were used as substrates.

Taking into consideration the differences in molecular weight between the two bands (60kDa and 45kDa), gel chromatography on BioGel P-60 and P-100 were used to try to separate the two bands. Samples containing the lactose-agarose affinity purified galactanase were applied to columns of BioGel P-60 (3x73cm) and P-100 (1x40cm) which had been calibrated with appropriate standards. After BioGel P-60 chromatography, a single peak of activity was obtained and analysis by SDS-PAGE demonstrated that the two bands were present with intensities corresponding to the levels of activity. The molecular weight estimated using the P-60 column was ca. 30kDa and activity on PNPGal and lupin galactan could not be separated. Similarly, after chromatography on BioGel P-100, a single peak (50-60kDa) of activity was obtained. Activities using either PNPGal or

galactan as substrate coincided exactly. SDS-PAGE showed the two bands present in the main peak (data not shown).

When samples obtained by affinity purification using lactose-agarose were applied to a phenyl-sepharose column (1x5cm) no activity was eluted either with 1.7M ammonium sulphate in 20mM Tris buffer pH 7.8 or in a negative ammonium sulphate gradient. The enzyme activity may have bound very tightly to the column or may have been eluted so gradually that it was not detected.

Isoelectric Focusing. When the non-denatured enzyme preparation was subjected to isoelectric focusing, two protein bands were observed, in approximately the same relative proportions as the two bands on SDS-PAGE. Their pI values were closely similar (major band pI=7.0, minor band pI=6.7). Unstained portions of the isoelectric focusing gel were sliced and a portion of each slice was assayed for both galactanase and ß-galactosidase activities. Both activities coincided exactly with each other and with the protein bands (**Figure 3.5A**). Two gel slices accounted for most of the enzyme activity on the gel (**Figure 3.5A**). The one with the highest activity coincided most closely with the major protein band (pI=7.0) whilst the other (adjacent) slice coincided more closely with the minor protein band (pI=6.7). The resolution obtained on isoelectric focusing was not sufficient to assure complete separation, on the adjacent slices, of the two protein bands. Further portions

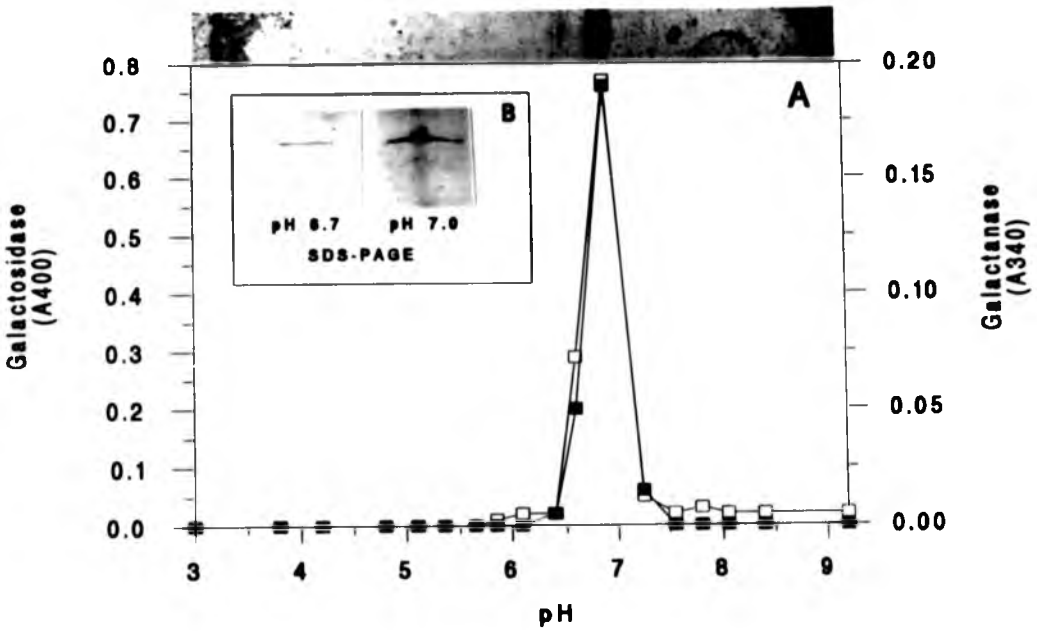


Figure 3.5.Isoelectric focusing of the purified exo-ß-1,4-galactanase.(□—□, ß-galactosidase; ■—■, galactanase). (**A**) enzyme activity; (**B**) SDS-PAGE of the peptides extracted from the gel slices with pH 6.7 and 7.0 respectively.

of these two gel-slices were therefore extracted with buffer containing SDS, and the extracts were subjected to SDS-PAGE (**Figure 3.5B**). Both gave bands corresponding to the 60kDa and the 45kDa polypeptides, but in different proportions. In each case the 60kDa band was the major component, but the slice coinciding more closely with the minor (pI=6.7) protein had a higher proportion of the 45kDa peptide. Thus the two bands observed on isoelectric focusing almost certainly corresponded to the two peptides resolved on SDS-PAGE.

Raising antibodies against the 60kDa band. The major (60kDa) band was purified from gels and a rabbit polyclonal antiserum was raised against it. A weak response was obtained when pre-immune serum was used against the purified *exo*-galactanase. The crude antiserum bound to both the 60kDa and the 45kDa proteins on Western immunoblots of both the purified enzyme preparation and the crude extract. After separation of the 60kDa and 45kDa polypeptides by SDS-PAGE, the proteins in the gel were transferred to a nitrocellulose membrane and the strip containing the isolated 60kDa band was cut and subsequently used for adsorption and desorption of the polyclonal antibodies which bind specifically to this 60kDa band. After this affinity purification procedure using the 60kDa band, the 45kDa band still cross-reacted with the affinity purified antibody. When used to detect the purified exo-galactanase

bands in crude extracts from 18 days old lupin cotyledons, a significantly lower number of bands cross reacted with the affinity purified antibodies as compared with the crude antiserum (**Figure 3.6A**)

Apart from the 60kDa and 45kDa polypeptides (**Figure 3.6D**), the purified antiserum recognised 5 other polypeptides in the crude extract (**Figure 3.6C**). It may therefore be characterised as an oligospecific antibody.

Thus, the immunological approach highlighted not only the possibility of existence of structural domains in common between the 60kDa and 45kDa polypeptides, but also with other peptides in the crude extracts, suggesting the presence of an entire family of ß-galactosidases in the cotyledons after germination.

N-terminal sequencing of the ß-galactanase bands. Although the 60kDa and 45kDa polypeptides clearly had immunological determinants in common, it was possible that they were distinct gene-products.

They were therefore isolated individually after SDS-PAGE and subjected to N-terminal sequencing. This experiment was performed by Dr. Amanda Heyler at Unilever Research Laboratories, Colworth House, Bedford (data not shown).

The N-terminal sequences, extending to 20 amino acids, were identical. This indicated strongly that the minor 45kDa component was breakdown a product of the major 60kDa

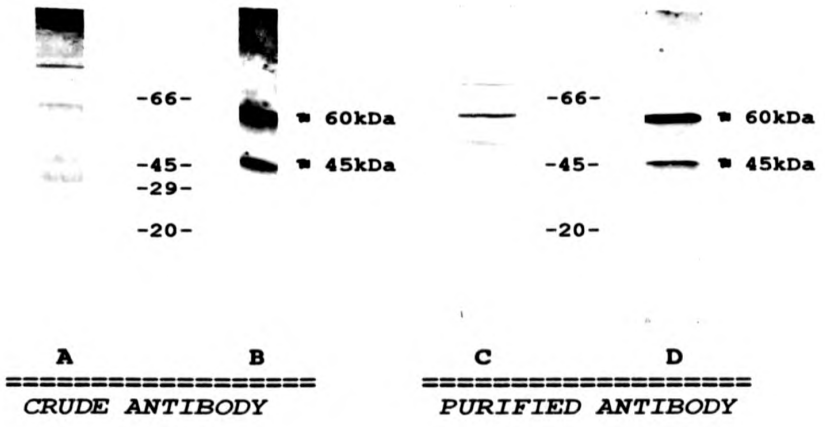


Figure 3.6. Western immunoblotting showing the reaction of the antiserum raised against the 60kDa band with lupin galactanase extracts. **A** - crude antiserum reacting with crude enzyme extract; **B** - crude antiserum reacting with purified exo-ß-1,4-galactanase; **C** - affinity purified antiserum reacting with the crude enzyme extract and **D** - affinity purified antiserum reacting with the purified exo-ß-1,4-galactanase.

polypeptide. Limited proteolysis of the 60kDa polypeptide may have occurred accidentally during the isolation process.

Mechanism of production of the two bands. As proteolytic activity is very high during the initial stages of lupinseed germination due to the fact that the storage protein bodies are being degraded (see Chapter 5 - **Figure 5.6**), the presence of this kind of activity was investigated using gelatin-SDS-PAGE (Heusen and Dowdle, 1980). At least two distinct proteolytic activities were detected under these conditions (**Figure 3.7**). Both activities were separated by SDS-PAGE in a gel containing gelatin and the gels were incubated at pH 5.0, to remove SDS and renature separated proteins at least partially. Finally the gels were incubated at pH 5.0, 30°C and stained with Coomassie blue. Proteinase activities were revealed as light zones against the dark blue background.

In order to check whether a proteinase, present during the early stages of purification, could be the responsible for the production of the 45kDa band, crude extracts were incubated for 1h at 30°C in 20mM Tris pH 7.8 and 50 mM ammonium acetate pH 4.5 and the levels of activity on PNPGal and galactan were measured. No significant changes in activity were observed in either case (data not shown).

The possibility that preparation of samples for SDS-PAGE could be producing the 45kDa band from the 60kDa, by

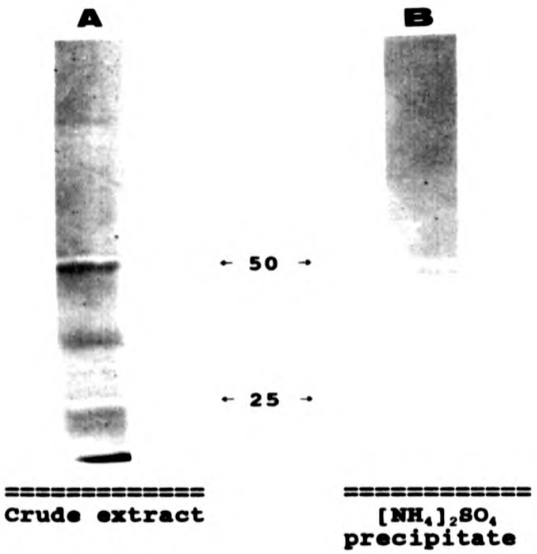


Figure 3.7. Proteolytic activity in the 10th day's crude extract (A) and after ammonium sulphate precipitation (B). Gelatin-SDS-PAGE was run as described in Material and Methods and incubated overnight in 0.1M ammonium acetate buffer pH 4.5 at 30° C.

chemical hydrolysis was ruled out due to the fact that samples (purified with lactose agarose) which had been boiled for 10 minutes resulted in several bands, but not the 45kDa one (data not shown).

Catalytic Properties of the Purified Enzyme

The pH optimum and stability of the enzyme were investigated using both the galactanase assay (lupin galactan as substrate) and the ß-galactosidase assay (PNPGal as substrate). A single set of data are shown in **Figure 3.8**, but our several repetitions the values were closely similar. Identical pH-activity and pH-stability curves were obtained (pH optimum between 4.0 and 4.5 (**Figure 3.8A**) and stability between pH 6 and pH 9) (**Figures 3.8B**).

At the pH optimum in the absence of substrate, the enzyme (assayed with PNPGal and galactan) was stable up to 40° C. Almost all activity was lost on incubating it at 50° C for 10 minutes. Again the galactanase and the ß-galactosidase assays gave almost identical results (**Figure 3.9**).

When studying the substrate specificity of the *exo*-galactanase, the choice of substrates to be used took into consideration two facts: 1) that this enzyme was isolated from a plant tissue, and although other kinds of substrates

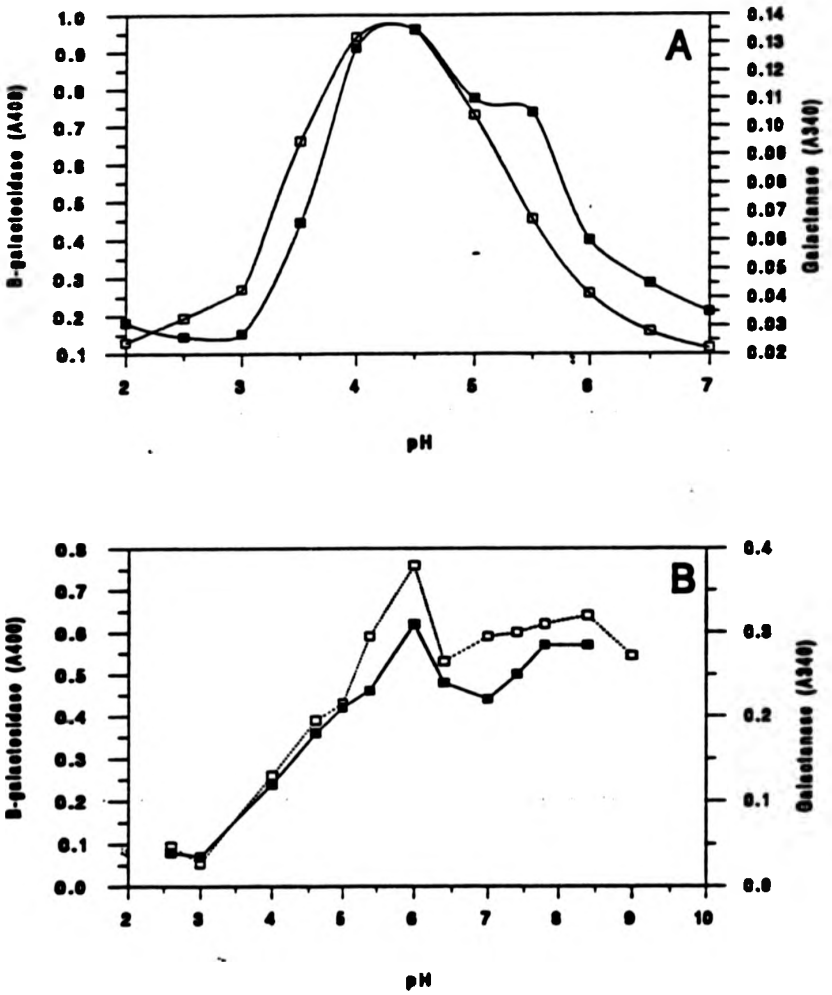


Figure 3.8. pH optima (A) and stability (B).(□----□, β-galactosidase; ■——■, galactanase).

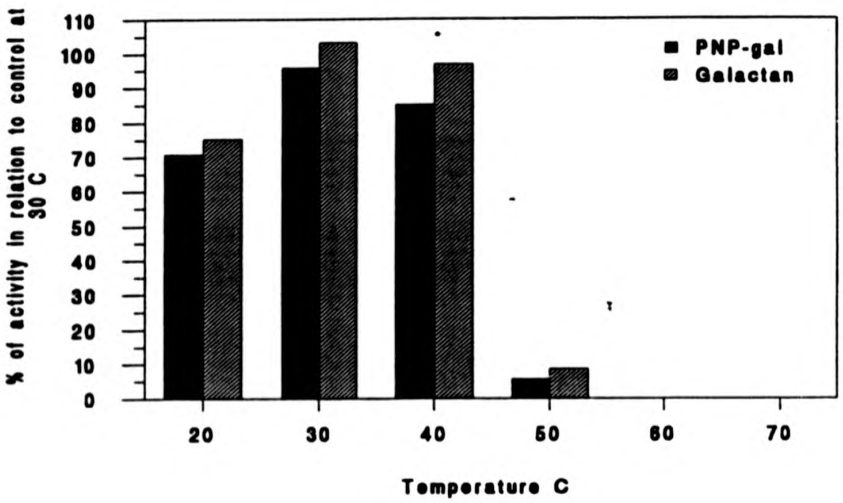


Figure 3.9. Optimal temperatures using PNPGal and galactan as substrates.

might have been used, oligosaccharides and polysacharides chosen were thought to be compatible with a hypothetical function in plant tissues. 2) that the *exo*-galactanase is most probably a cell wall enzyme, so that the polysaccharides chosen as substrates are the ones commonly found in plant cell walls. Thus, although features that might relate the lupin *exo*-galactanase to the ß-galactosidases found in microorganisms or ß-galactosidases in general are important and interesting, this aspect was not followed in this work, the main aim of our study being to understand the biological function of the enzyme in plants.

The substrate specificity of the enzyme was tested (**Table 3.3**) using simple glycosides (synthetic glycosides and disaccharides), with plant cell wall polysaccharides and with oligosaccharides. The enzyme did not catalyse the hydrolysis of any simple ß-linked glucosides or α-linked galactosides. It catalysed the hydrolysis of some, but not all, of the simple ß-galactosides assayed. Of the two synthetic ß-galactosides tested, the *p*-nitrophenyl galactoside was hydrolysed rapidly whereas the methyl galactoside was not hydrolysed at all. The (1→4)- and (1→6)-ß-linked galactobiose disaccharides were both hydrolysed rapidly, whereas lactose (ß-galactopyranosyl-(1→4)-glucose) was hydrolysed only very slowly. This enzyme catalysed a very rapid release of galactose from lupin (1→4)-ß-galactan. On the other hand it did not hydrolyse

Table 3.3. Action of the enzyme on potential substrates. Initial rates are given for specific substrate concentrations, and kinetic parameters when achievable.

Compound	Activity µmol galactose released per min per mg protein. Substrate concentration 10 mM unless indicated otherwise)	Km (mM unless stated otherwise)	Vmax µmol galactose released per min per mg protein
p-nitrophenyl-α-D-galactopyranoside	0	-	-
p-nitrophenyl-ß-D-galactopyranoside	7.3	5.6	12.2
methyl-ß-D-galactopyranoside	0	-	-
p-nitrophenyl-ß-D-glucopyranoside	trace	-	-
p-nitrophenyl-ß-D-mannopyranoside	0	-	-
Gal-(1→4)-ß-Glc (lactose)	trace	-	-
Gal-(1→4)-ß-Gal	5.7 (11 mM)	59.4	34.9
Gal-(1→6)-ß-Gal	22.9	6.4	42.4
Gal-(1→3)-ß-Ara	0	-	-
Gal-(1→4)-ß-Man	0	-	-
Gal-(1→4)-ß-Gal-(1→4)-ß-Glc	2.9 (9 mM)	-[1]	-[1]
Galactopentaose [(1→4)-ß-linked]	4.0	-[1]	-[1]
lupin (1→4)-ß-galactan	19.4 (1.0 mg ml^{-1})	0.71 mg ml^{-1}	37.0
larch wood arabinogalactan	0 (10 mg ml^{-1})	-	-
tamarind xyloglucan[2]	trace[2] (10 mg ml^{-1})	-	-
xyloglucan oligosaccharide: G→G→G→G ↑ ↑ ↑ X X X ↑ ↑ GaGa	0 (0.24mM)	-	-

[1]Large deviations from Michaelis-Menten kinetics

[2]Contains a small amount of (1→4)-ß-linked galactan (Gidley et al. 1991)

the other plant cell wall polysaccharides which are known to have terminal, non-reducing ß-D-galactopyranosyl residues. Neither xyloglucan (terminal non-reducing (1→2) ß-linked galactosyl residues), nor larch arabinogalactan (terminal non-reducing (1→3) and (1→6) linked galactose residues) were hydrolysed.

Kinetic parameters (K_m and V_{max}) were determined for lupin galactan and for the rapidly-hydrolysed simple galactosides, and the values are listed in **Table 3.3**. These substrates all exhibited simple Michaelis-Menten kinetics. Two oligosaccharides with the (1→4)-ß-galactosyl linkage [(1→4)-ß-galactopentaose and the (1→4)-ß-linked trisaccharide Gal→Gal→Glc] were tested as substrates and both were hydrolysed fairly rapidly (**Table 3.3**). In each case deviations from Michaelis-Menten kinetics were observed, which were consistent with strong inhibition by a product of the reaction.

The patterns of action of the enzyme on galactopentaose and on lupin galactan were examined in more detail by TLC analysis of the reaction products at different times during prolonged digestions. With galactopentaose there was a very rapid hydrolysis of the pentasaccharide to give free galactose and the tetrasaccharide, followed by a much slower sequential removal of further galactose residues to give the trisacharide and then the disaccharide (**Figure 3.10**).

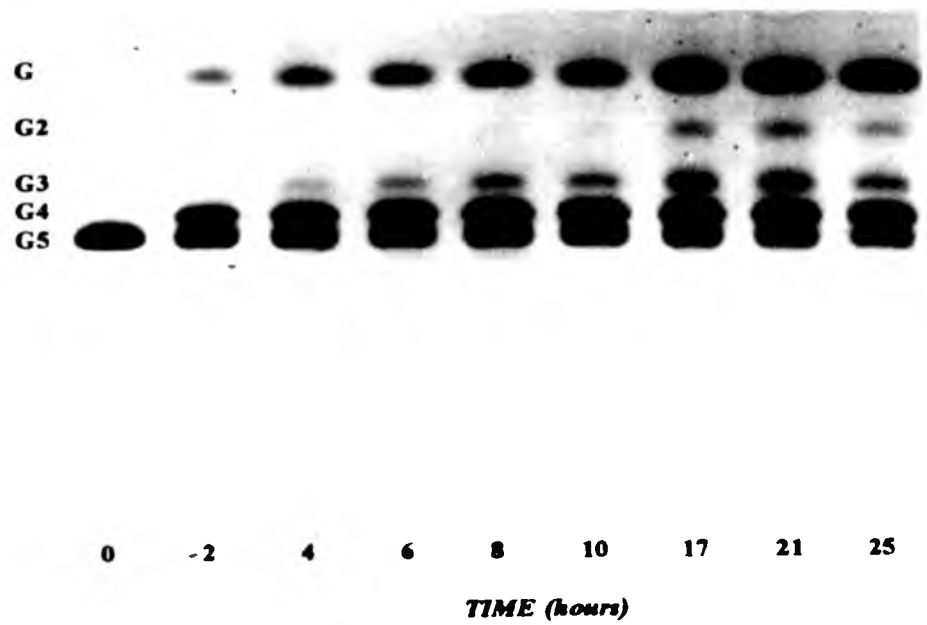


Figure 3.10. Time course of galactose release under incubation of ß-1,4-galactopentaose with exo-galactanase at 30°C. **G**=galactose, **G2**=galactobiose, **G3**-galactotriose, **G4**=galactotetraose and **G5**=galactopentaose.

With lupin galactan, much galactose was released, although polymeric material remained even after prolonged incubation. This material was isolated by ethanol precipitation and its neutral monosaccharide composition was determined for comparison with that of the galactan substrate. The polymer remaining after enzyme digestion had non-galactose sugar residues in the same relative proportions as the galactan substrate, but its galactose-content had been reduced by ca.80% (**Table 3.4**).

The enzyme did not have a divalent metal-ion requirement, although Cu^{+2} was inhibitory (75% at 40 mM). Galactose and γ-galactonolactone were also inhibitory at high concentrations (52% and 100% inhibition respectively at 40 mM)(**Table 3.5**).

Table 3.4. Neutral monosaccharide composition of lupin galactan before and after incubation with the exo-(1→4)-ß-galactanase. Incubations were performed at pH 5.0 at 30 °C

Incubation time	Monosaccharide ratio (relative to rhamnose)			
	Rhamnose	Arabinose	Galactose	Xylose
0 h	1.0	7.7	34	0.9
24 h	1.0	7.4	8.0	0.9
48 h	1.0	7.3	6.2	0.8

Table 3.5. Inhibition of exo-galactanase enzyme by metal ions, galactose and γ-galactonolactone. p-Nitrophenyl galactopyranoside was used as substrate.

INHIBITORS [40mM]	Relative Activity (%)
$CaCl_2$	87.4
$CuCl_2$	24.7
$MgSO_4$	100.0
Galactose	48.0
γ-galactonolactone	0

Changes in enzyme protein levels and their relationship with storage cell wall mobilisation in lupin cotyledons during seedling development

Using our seeds and culture conditions, cotyledonary cell wall digestion, as witnessed by the appearance of zones of degradation which will be described in Chapters 4 and 5, was first detected 6 days after imbibition (coincidentally with radicle protrusion). Although in **Figure 3.1** galactanase activity was not detected until slightly later than this, replicate experiments often gave low galactanase activity at day 6. Variations in the level of enzyme protein over the same time period were obtained using western immunoblotting analysis. The antiserum used had been raised against and affinity purified using the major polypeptide component of the purified enzyme (the 60kDa band). The results (**Figure 3.11**), which were obtained using the same extracts as in **Figure 3.1**, demonstrated clearly that low levels of galactanase protein were present at day 6, and that the variations in galactanase activity documented in **Figure 3.1** were correlated closely with the levels of the enzyme protein (both 60kDa and 45kDa bands). Besides the 60kDa and 45kDa bands, four other bands which cross-reacted with the affinity-purified anti-galactanase serum also showed systematic changes during and after germination (**Figure 3.11**). The two high molecular weight

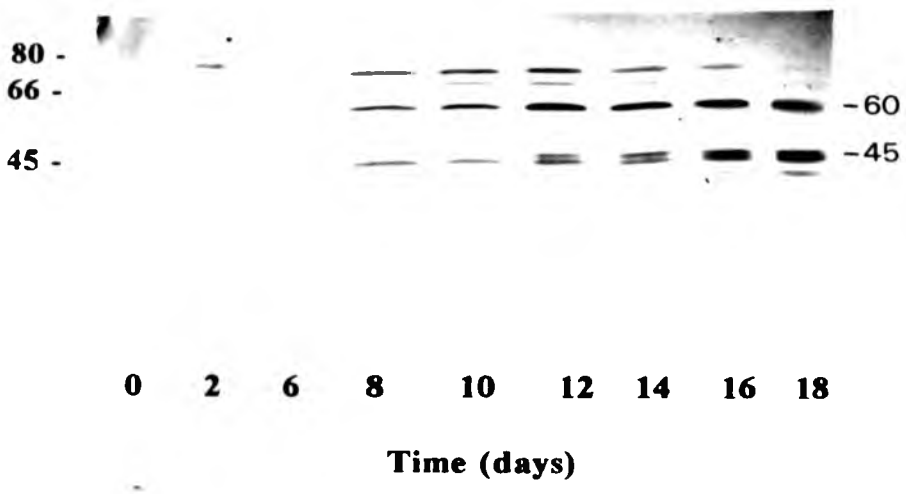


Figure 3.11. Pattern of variation of exo-ß-galactanase protein and other immunologically cross-reacting polypeptides during and following germination in cotyledons of *Lupinus angustifolius*. The affinity-purified antiserum was used for western immunoblotting.

(78 and 75kDa) bands that cross-reacted with the purified antibodies peaked in intensity at day 8. Their pattern of variation recalled that of ß-galactosidase activity in **Figure 3.1.** They could be ß-galactosidases. Another polypeptide with molecular weight 44kDa showed a broad peak as seen by the intensity in western immunoblotting. It has been shown to be a ß-galactosidase distinct from the exo-galactanase (Chapter 4).

The exo-galactanase as well as the other cross-reacting polypeptides seem to be synthesised *de novo* during germination. The specificity of the exo-galactanase towards lupin cell wall galactan together with the pattern of variation of its corresponding polypeptides strongly suggests that this enzyme is involved in galactan mobilisation during plantlet establishment.

Discussion

In order to purify one of the ß-galactosidase isoenzymes to homogeneity from any source, one could start with ammonium sulphate precipitation and ion exchange chromatography (DEAE and/or CM-cellulose) and then investigate what is the best way for finally purifying the enzyme. The final step of purification should take account

of unique properties of the enzyme such as molecular weight, pI, specificity towards substrates and inhibition.

During isolation of the lupin *exo*-galactanase we tried different techniques, including gel permeation on BioGel P-60 and several different affinity chromatography resins. Lactose-agarose proved to be an adequate final step of purification. Instead of binding strongly to the column, the enzyme was retarded, probably due to a weak interaction with the lactose oligosaccharide. This is supported by the evidence that lactose was hydrolysed by purified *exo*-galactanase only very slowly (**Table 3.3**).

The enzyme described in this chapter is a ß-galactosidase in the sense that it hydrolyses some low molecular weight ß-galactosides [*p*-nitrophenyl-ß-galactoside, (1→4)- and (1→6)-linked ß-galactobioses]. Other ß-galactosides which would be expected to be substrates for a general ß-galactosidase are either not hydrolysed (methyl-ß-galactoside) or hydrolysed very slowly (lactose). The lupinseed enzyme catalyses the extensive removal of galactose residues from the (1→4)-ß-galactan-enriched polysaccharide preparation from lupin cotyledonary cell walls (**Table 3.4**). Exhaustive digestion of this material with the enzyme results in the liberation of more than 80% of the galactose residues originally present in the polysaccharide material (56% by weight of the material itself) as free galactose. The material remaining after enzyme digestion is still

polymeric and contains other neutral sugar residues (arabinose, rhamnose and xylose) in exactly the same proportions as before. This supports the hypothesis (Crawshaw and Reid 1984) that the galactan of the lupin cotyledons is a side-chain of a pectic rhamnogalacturonan core polysaccharide. About 20% of the galactose residues in the polysaccharide substrate are resistant to hydrolysis by the *exo*-galactanase (**Table 3.4**). Some of these may be present in branched arabinogalactan side-chains of the pectic core, which would not be susceptible to hydrolysis by this enzyme. Others may be (1→4)-ß-linked, but so close to the backbone as to be inaccessible to the galactanase.

Thus, the catalytic properties of the *exo*-galactanase are such that it could alone catalyse the post-germinative hydrolysis of (1→4)-ß-galactan in lupin seeds. That it does so is indicated by our failure to detect any *endo*-galactanase activity in cotyledonary extracts.

Working with *Lupinus angustifolius* cv. Unicrop, Hutcheon and Reid (unpublished results) showed that crude extracts of germinated cotyledons were able to produce the same changes in the composition of the isolated cell wall storage galactan as observed *in vivo*. In this case, mainly galactose and arabinose are detected as products of hydrolysis and the authors demonstrated that both α-arabinosidase and ß-galactosidase activities were present and that their activity increased after germination.

Matheson and Saini (1977) demonstrated that at least three ß-galactosidases and two α-arabinosidases are present in cotyledonary extracts of *Lupinus luteus*. The ß-galactosidases were named Ia, Ib and II on the basis of partial purification studies and had apparent molecular weights on gel chromatography of 74, 54 and 65kDa respectively. The enzymes Ib and II were active on cell wall galactan whereas enzyme Ia was not. However, no systematic studies were performed in order to investigate the time course of these activities.

We have now confirmed and extended Matheson and Saini's and Hutcheon and Reid's work concerning the ß-galactosidase activities of lupins and our results indicate that at least two distinct ß-galactosidases are present in cotyledons of *Lupinus angustifolius* cv New Zealand Bitter Blue. In cotyledons growing at 18°C, these two activities change independently. Only one of them was able to hydrolyse the isolated galactan and with high efficiency. It was therefore termed a ß-galactanase, rather than a ß-galactosidase.

A key role for the galactanase in the post-germinative mobilisation of galactan is indicated by its pattern of developmental regulation. Although there was high ß-galactosidase activity in cotyledonary extracts prior to galactan mobilisation, galactanase activity could be detected only when cell wall polysaccharide mobilisation began. The activity then increased throughout the "grand

period" of galactan breakdown (as determined by Crawshaw and Reid, 1984), remained constant and declined. Immunoblotting experiments demonstrated clearly that the changes in galactanase activity were correlated with levels of galactanase protein. Thus, the galactanase is synthesised *de novo* in response to a developmental signal as yet unknown, and catalyses the mobilisation of lupin galactan *in vivo*.

Despite affinity-purification on the galactanase protein, the antiserum used in the immunoblotting experiments cross reacted with a limited number of protein components of the cotyledonary extracts. One of these has been purified highly and is a ß-galactosidase with no action on galactan (see Chapter 4). It is possible that the others are also ß-galactosidases with differing substrate specificities.

It is instructive to compare the properties of the lupinseed *exo*-ß-galactanase enzyme and the ß-galactosidase from nasturtium, which is the only other ß-galactosidase known for certain to have a cell-wall polymer as its natural substrate (Edwards et al., 1988). Both have low molecular weight ß-galactoside substrates in common, are immunologically cross reactive (Buckeridge, Fanutti and Reid, unpublished), but act quite differently on cell wall polymers. The nasturtium enzyme shows high specificity towards xyloglucan polymers and oligomers, and hydrolyses lupin galactan only very slowly, whilst the lupin enzyme

hydrolyses lupin galactan rapidly and has no action either on xyloglucan, its derived oligosaccharides or arabinogalactan II. The extent to which the xyloglucan-specific and the galactan-specific ß-galactosidases are similar will become clear on completion of current studies on the corresponding genes. At present it may be speculated that plant tissues contain multiple ß-galactosidases which are directed to their various natural substrates not only by spatial targeting (e.g. to the cell wall or cytosol) but also via major differences in substrate specificity.

Chapter 4

INVESTIGATION OF THE NATURE OF THE OTHER ß-GALACTOSIDASE ACTIVITIES IN LUPIN COTYLEDONS DURING AND AFTER GERMINATION

Introduction

In Chapter 3 the purification of a ß-galactosidase or *exo*-(1→4)-ß-galactanase was described and evidence presented that it is the enzyme responsible for the degradation of the cell wall (1→4)-ß-galactan after germination. In the course of purification of the galactanase, evidence was obtained for the presence in cotyledonary extracts of other distinct ß-galactosidase activities. Also, some polypeptides in cotyledonary extracts cross reacted with an antiserum that had been affinity purified against the 60kDa band of *exo*-(1→4)-ß-galactanase (**Figure 3.6C** - Chapter 3). These results suggested that several ß-galactosidases may be present in lupin cotyledons.

In this chapter, the partial purification of a ß-galactosidase (named ßI) which showed no activity on cell wall (1→4)-ß-galactan, but effectively hydrolysed PNPGal, ß-methyl-galactopyranoside, (1→4)-linked galactobiose, lactose and branched arabinogalactan from larch is described, and the presence of a further distinct ß-galactosidase (named ßII) is indicated. Although the latter enzyme is far from fully characterised, our results indicate that it is probably a third enzyme, since it has higher molecular weight and does not hydrolyse galactan.

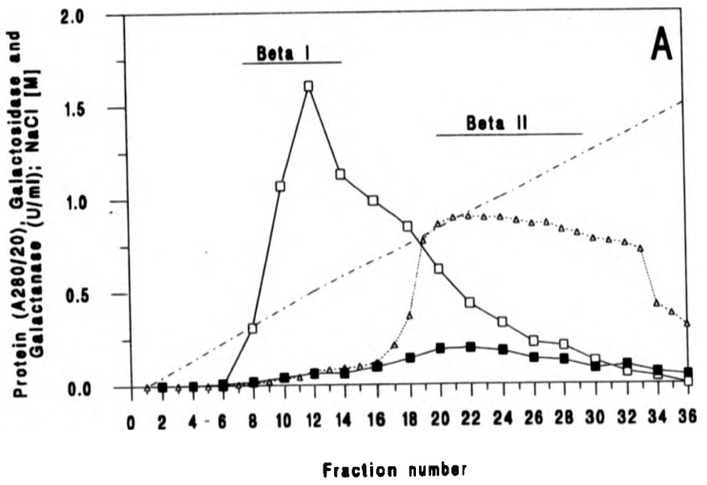
Results

Identification and partial purification of two ß-galactosidases from lupin cotyledons

When a gradient (0-0.15M NaCl) was applied to the same DEAE-cellulose column on which the exo-galactanase was not retained (Chapter 3 - **Figure 3.3A**) a peak containing high ß-galactosidase activity (using PNPGal as substrate) was eluted (**Figure 4.1A**). Only very low galactanase activity (using lupin galactan as substrate) was eluted from the column. Fractions were pooled differentially with the aim of collecting the activities on PNPGal and galactan separately. Throughout this chapter, these two preparations will be referred to as ßI and ßII enzymes respectively as indicated in **Figure 4.1A.**

ßI activity was eluted very early during the gradient (ca. 50 mM NaCl) before most of the protein that bound to the column started to be eluted. As a result, the SDS-PAGE profile of the main fractions showing ßI activity contained relatively few polypeptides. The fractions pooled as ßII eluted in the main protein peak and thus had highly complex SDS-PAGE profile (**Figure 4.1B**).

Further purification of the ßI enzyme was performed using CM-Cellulose. At pH 5.0 the enzyme did not bind to the ion exchanger and the activity emerged from the column



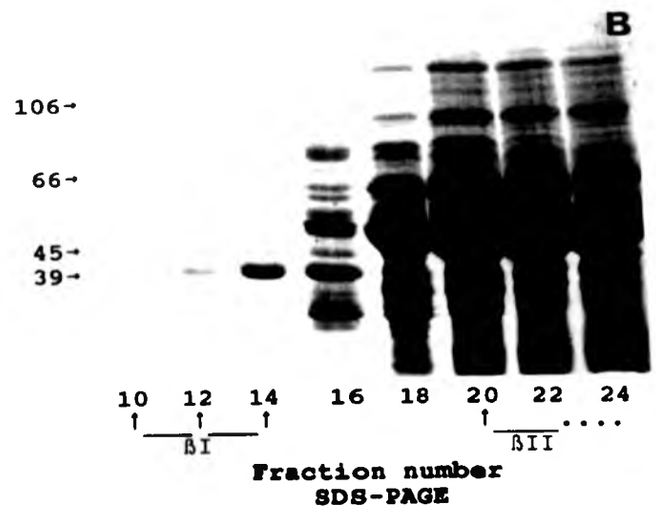
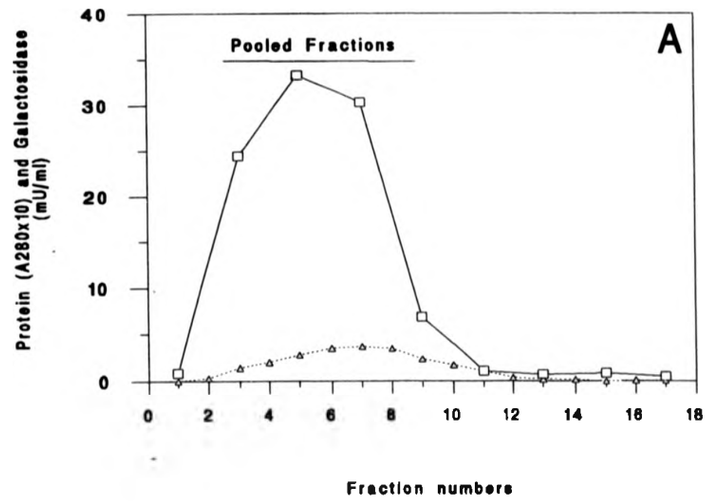


Figure 4.1. Purification of the ßI enzyme. **A.** DEAE-cellulose chromatography. The applied sample was a concentrated (ammonium sulphate-80% saturation) 18-day cotyledonary extract in 20 mM Tris-HCl, pH 7.8. The column was eluted with the same buffer and then eluted with a NaCl gradient in the same buffer. Only the gradient is shown. **B.** SDS-PAGE showing the polypeptide pattern in selected fractions from DEAE-cellulose column possessing ß-galactosidase (p-nitrophenyl-ß-galactoside as substrate= □) and *exo*-galactanase (lupin galactan as substrate = ■). ∇ = protein (A_{280}) ----- = sodium chloride concentration.

as a single peak (**Figure 4.2A**). The SDS-PAGE profile shows that a main band at ca. 44kDa was purified in relation to the other polypeptides in the sample loaded onto the column (**Figure 4.2B**).

After pooling, the fractions with activity (**Figure 4.2A**) were applied to a BioGel P-60 column. A single peak of activity emerged from this column, corresponding to a molecular weight of ca. 44kDa (**Figure 4.3**). Indeed, a main band with molecular weight of 45kDa was visualised after SDS-PAGE analysis (**Figure 4.4A**). The molecular weight corresponding to the enzyme activity was further confirmed by gel chromatography in BioGel P-100 as approximately 48kDa (data not shown). The partial purification of the ßI enzyme is summarised in **Table 4.1**. The P-60 pooled fractions (**Figure 4.3**) were purified c.a. 13 times with a recovery of 0.66%.

When analysed by SDS-PAGE, contaminating polypeptides could still be visualised (**Figure 4.4A**). Thus, ßI enzyme can not be considered to have been purified to homogeneity. However, Western blotting using the antibody affinity purified by adsorption to the 60kDa band of *exo*-(1→4)-ß-galactanase shows clearly that ßI enzyme has been isolated from the other cross reacting polypeptides present in crude extracts. It is also clear (**Figure 4.4B**) that ßI enzyme corresponds to one of the cross reacting bands previously detected in lupin cotyledons crude extracts (see also **Figure 3.6C** - Chapter 3). All the affinity purification



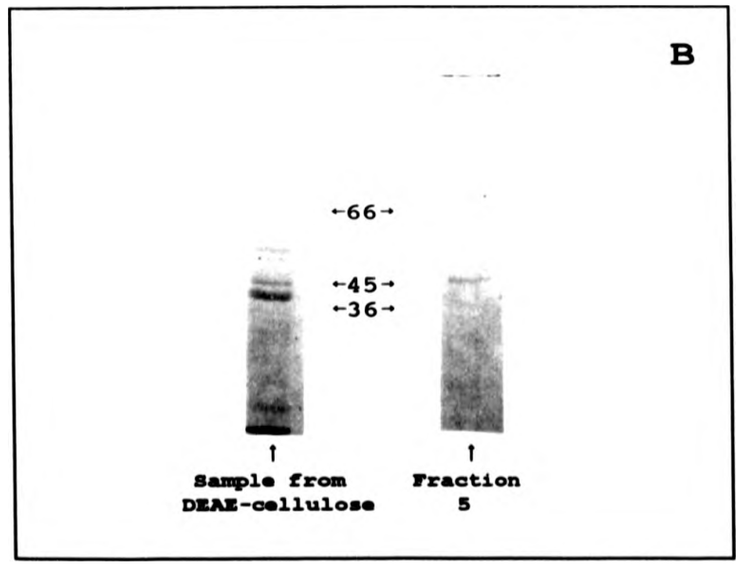


Figure 4.2 Purification of the ßI enzyme. **A.** CM-cellulose chromatography. The pooled, concentrated (ammonium sulphate - 80% saturation) fractions from the DEAE-cellulose column were applied to a CM-cellulose column which had been pre-equilibrated with 50 mM sodium acetate buffer, pH 5.0. The column was eluted with the same buffer. **B.** SDS-PAGE of the sample applied to DEAE-cellulose compared with fraction 5 from the CM-cellulose column. ▽▽▽ = protein (A_{280}). □□□ = ß-galactosidase (p-nitrophenyl-ß-galactoside as substrate) activity .

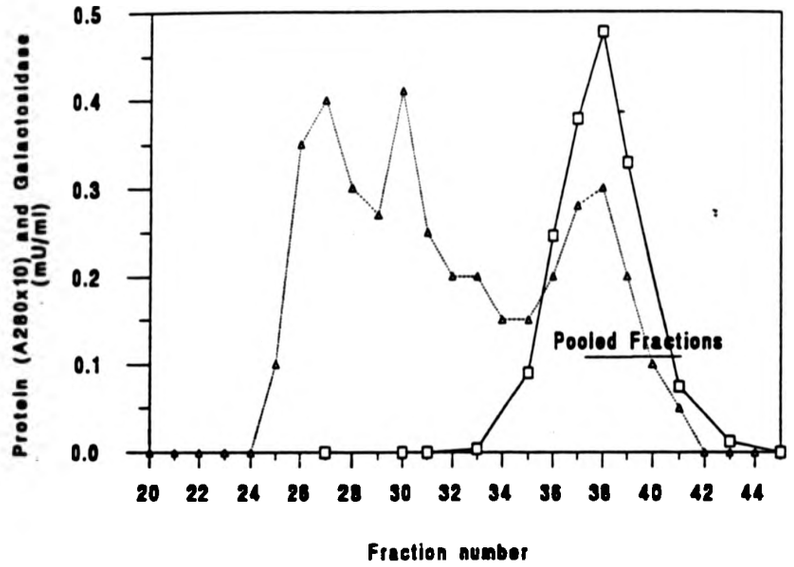
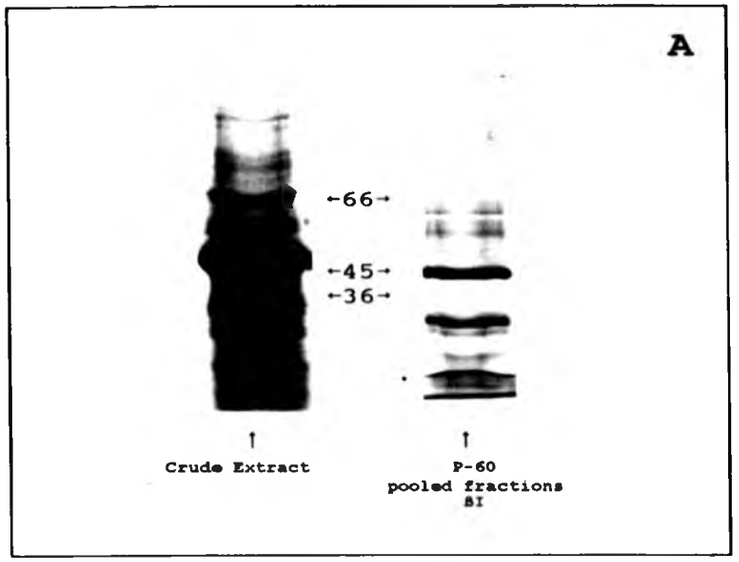


Figure 4.3. Gel chromatography of ßI enzyme on BioGel P-60 (3x73cm). The column was eluted with 50 mM ammonium acetate buffer. ▽▽▽ = protein (A_{280}). □□□ = ß-galactosidase (p-nitrophenyl-ß-galactoside as substrate) activity .



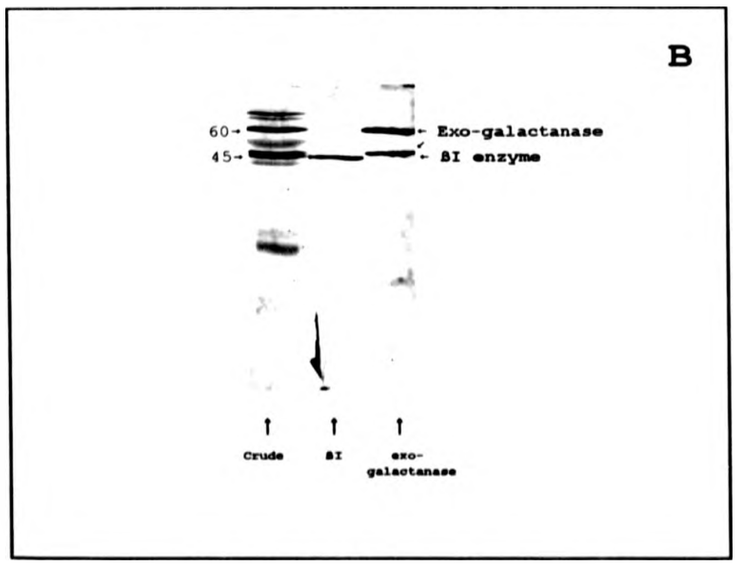


Figure 4.4. SDS-PAGE and Western immunoblotting of the crude extract and partially-purified ßI enzyme. **A** = SDS-PAGE of the crude extract compared with the pooled fractions after gel chromatography (P-60). **B** = Western immunoblotting. The antiserum had been raised against and affinity-purified on the 60kDa band in the purified enzyme.

Table 4.1. Purification of ßI lupin ß-galactosidase.

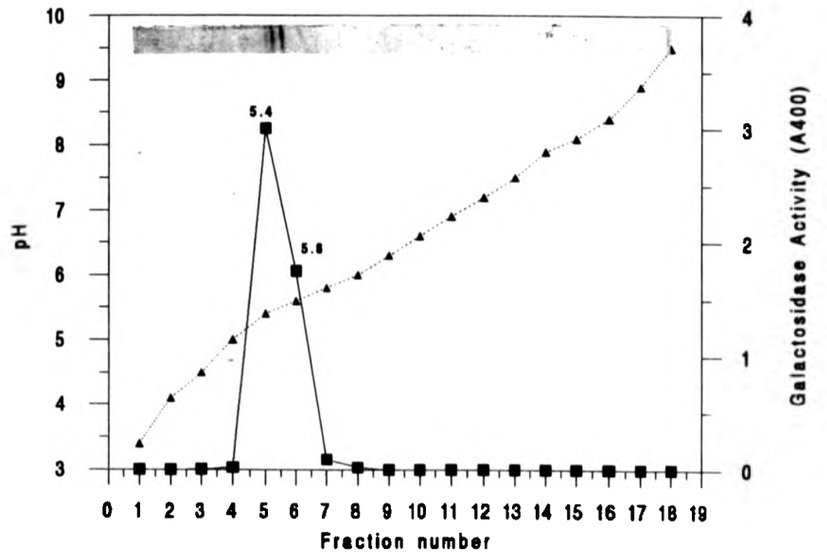
Step	Total Volume (ml)	Protein (mg)	Total Activity µmoles.min.$^{-1}$	Specific Activity µmoles.min.$^{-1}$. mg of prot^{-1}	Purification	Recovery %
Crude	740	200.8	1,399.6	6.97	-	100
$[NH_4]_2SO_4$	110	84.9	369.8	4.36	0.63	26
DE-52	59	6.7	52.8	7.88	1.13	3
CM-52	65	0.61	34.1	55.90	8.02	2
P-60	13.5	0.10	9.3	93.00	13.34	0.66

resins tested for the *exo*-(1→4)-ß-galactanase were also used attempting further purification of ßI. Columns (1ml) were set up using the resins p-aminobenzyl-ß-D-thiogalactopyranoside-agarose, N-(ϵ-aminocaproyl)-beta-D-galactopyranoside-agarose, p-aminophenyl-ß-thiogalactopyranoside-agarose. They were equilibrated with 50mM ammonium acetate buffer pH 4.5. Active ßI samples (0.2ml), which had been previously dialysed against the same buffer, were applied to the columns and in all cases activity was eluted in the void volume, i.e. not retained.

Isoelectric Focusing

When non-denatured ßI was analysed by isoelectric focusing, two main bands with approximately the same intensity were detected. Their pI values were 5.4 and 5.6 respectively (**Figure 4.5**). Following exactly the same procedure used for the *exo*-(1→4)-ß-galactanase, the unstained electrofocusing gel was sliced and portions of the gel were assayed for PNPGal. Activity was detected in both slices corresponding to the two main bands, the higher activity being related to the band with pI 5.4.

Figure 4.5. Isoelectric focusing of the partially-purified ßI enzyme. The gel was stained with Coomassie Blue. Activity and pH were obtained by eluting strips of unstained gel. For pH determination, strips were eluted for 1 h with distilled water. For activity determination, strips were eluted with 50 mM acetate buffer, pH 5.0. ∇ = pH; ■ = ß-galactosidase activity. This range was used to align the photo with the graph.



Properties of the ßI enzyme

The pH optimum of the ßI enzyme was around 4.0. The curve obtained was different from the one obtained for the *exo*-(1→4)-ß-galactanase which had a pH optimum of 4.5-5.0 (**Figure 4.6**).

The substrate specificity of the ßI enzyme was tested using some simple glycosides and some cell wall polysaccharides (**Table 4.2**). Differently from the *exo*-(1→4)-ß-galactanase described in the last chapter, ßI enzyme was not able to hydrolyse lupin cell wall (1→4)-ß-galactan. It catalysed the release of galactose from PNPGal ß-methyl-galactopyranoside, lactose, larch arabinogalactan and also from (1→4)-ß-linked galactobiose. Analysis by TLC showed that only galactose is released after 24h incubation with larch arabinogalactan. The kinetic parameters K_m and V_{max} were calculated for PNPGal (K_m=0.4 mM and V_{max}=27.97 mU) and lactose (K_m=15.6 mM and V_{max}=26.22 mU) only. It is interesting to note the dramatic difference in affinity between these two substrates, while having approximately the same values for V_{max}.

No hydrolysis was observed after incubation with other ß-linked simple glycosides such as PNP-ß-glucopyranoside and PNP-ß-mannopyranoside, α-linked galactosides (PNP-α-galactopyranoside and α-methyl-galactopyranoside) and with melibiose, an α-linked oligosaccharide.

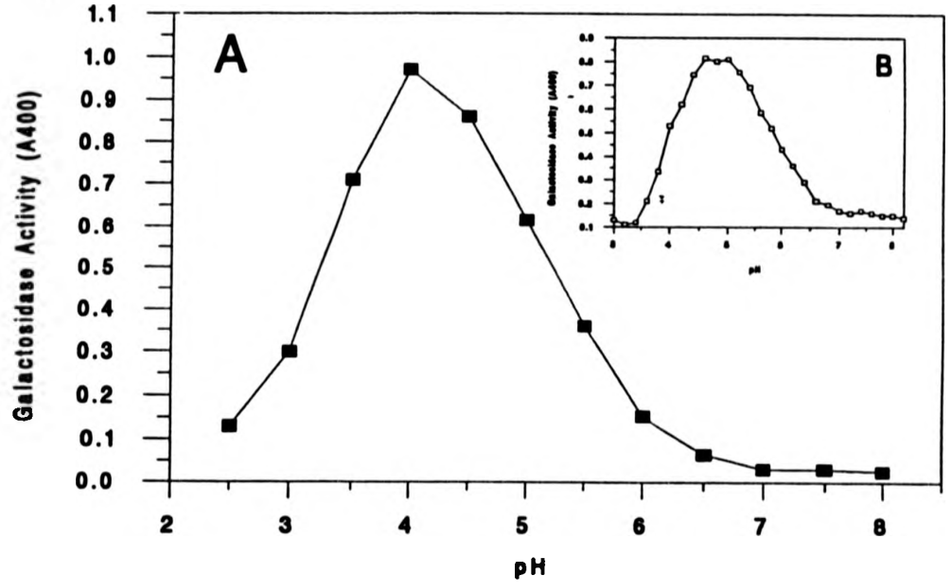


Figure 4.6. Optimum of pH for the ßI enzyme (**A**) compared with the pH optimum for the *exo*-(1→4)-ß-galactanase (**B** - from **Figure 3.9A** - Chapter 3).

Table 4.2. Action of the ßI enzyme on potential substrates. Initial rates are given for specific substrate concentrations.

Substrate	Concentration	Activity (mU)
methyl-α-D-galactopyranoside	7 mM	0
methyl-ß-D-galactopyranoside	7 mM	36.2
p-nitrophenyl-α-D-galactopyranoside	7 mM	0
p-nitrophenyl-ß-D-galactopyranoside	7 mM	176.2
p-nitrophenyl-ß-D-glucopyranoside	7 mM	0
p-nitrophenyl-ß-D-mannopyranoside	7 mM	0
Gal-(1→4)-ß-Glc (lactose)	7 mM	15.1
Gal-(1→4)-ß-Gal (galactobiose)	11 mM	0.8
Gal-(1→6)-ß-glu (melibiose)	7 mM	0
Galactopentaose [(1→4)-ß-linked]	11 mM	0
lupin (1→4)-ß-galactan	1 %	0
larch wood arabinogalactan	1 %	1.3
tamarind xyloglucan	0.1%	0
guar galactomannan	0.1%	0

Although the ßI enzyme was able to hydrolyse the (1→4)-ß-linkages of lactose and galactobiose, it did not attack lupin (1→4)-ß-galactan or (1→4) galactopentaose at all. The release of galactose only, from larch arabinogalactan indicates that (1→3) and/or (1→6) linked galactoses can be hydrolysed.

The ßI enzyme was strongly inhibited by γ-galactonolactone (ca. 90% at 10mM) and galactose (c.a. 60% at 10mM) both common inhibitors of ß-galactosidases. Copper chloride (10mM) caused approximately 60% inhibition (**Table 4.3**).

Evidence for a distinct ßII enzyme

Further investigation was performed on the activity named ßII. When the pooled fractions ßII (**Figure 4.1A**) were applied to CM-cellulose, ß-galactosidase activity did not bind to the column and one single peak of activity was obtained. This peak had high activity of ß-galactosidase, and lower galactanase activity. Although there is the possibility that this peak is due to a ß-galactosidase other than exo-galactanase or ßI, it is also possible that this peak corresponds to a mixture of *exo*-galactanase and ßI enzyme. This was not investigated further.

When an NaCl gradient (0-0.5mM) was applied to the CM-cellulose column, another ß-galactosidase activity was eluted, which contained no significant activity on lupin

Table 4.3. Inhibition of ßI enzyme by metal ions and monosaccharides.

INHIBITORS [10mM]	Relative Activity (%)
$CaCl_2$	69.8
$CuCl_2$	37.7
$MgSO_4$	118.8
Galactose	41.5
γ-galactonolactone	11.3
Glucose	100.0

galactan (**Figure 4.7A**). This extract was further applied onto a BioGel P-100 calibrated column and emerged as a single symmetrical peak corresponding to the void volume of the column. As the exclusion limit of this column was enough to exclude a 66kDa protein (ovalbumin), it could be at least concluded that its molecular weight is greater than 66kDa (**Figure 4.7B**). It must therefore be distinct from both the exo-galactanase and the ßI enzyme.

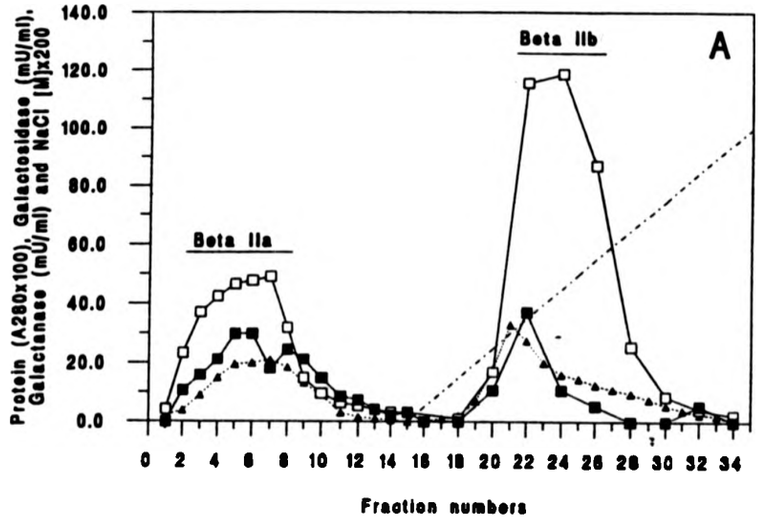
Discussion

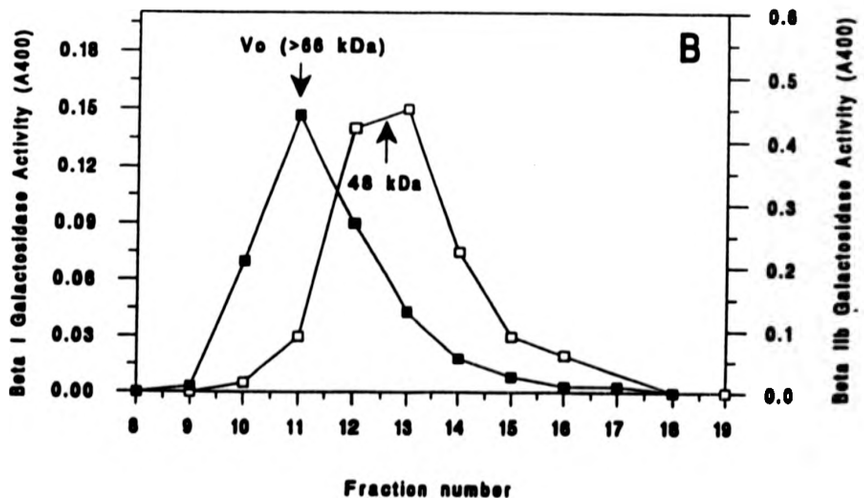
The results described in this Chapter confirm that at least two other ß-galactosidases (ßI and ßII) are present in cotyledons of germinated *Lupinus angustifolius*.

ßI enzyme showed clear cross reaction with the antiserum raised against *exo*-(1→4)-ß-galactanase (**Figure 4.4B**). However, the study of its properties made clear that it is a distinct enzyme.

The enzyme ßI showed some activity on cell wall polysaccharide, since it was able to release galactose from larch wood arabinogalactan. These results suggest that the galactanase activity observed when larch arabinogalactan was used as substrate is due to the hydrolysis of either (1→3) and/or (1→6) linkages. It was also effective in hydrolysing PNPGal, ß-methyl-galactopyranoside and lactose. It is interesting to note that (1→4)-ß-linkages are

Figure 4.7. Purification of the ßII enzyme. **A.** CM-cellulose chromatography. The pooled, concentrated (ammonium sulphate - 80% saturation) fractions (ßII) from the DEAE-cellulose column were applied on a CM-cellulose column pre-equilibrated with 50 mM sodium acetate buffer, pH 5.0. The column was eluted with the same buffer, and then with a linear gradient (0-0.5M) of sodium chloride in the same buffer. **B.** Gel chromatography of ßI and ßIIb in a BioGel P-100 column which had been previously calibrated with Bovine albumin (66kDa), Egg albumin (45kDa), Carbonic anhydrase (29kDa) and Cytochrome C (12.4Kda). ■ = ßII enzyme □ = ßI enzyme. Activities were measured using p-nitrophenyl-ß-galactoside as substrate.





attacked by ßI enzyme, but not if it is part of a polymer i.e it hydrolysed (1→4)-ß-linked galactobiose slowly, but it did not hydrolyse galactopentaose or galactan containing the same type of linkages.

Possible role for ßI enzyme

When plantlets were growing at 18°C, the amount of ßI protein varied in step with the rates of increase of the 60kDa band of the *exo*-galactanase (**Figure 3.12**). This pattern of variation indicates that, like the *exo*-galactanase, this enzyme probably has a transitory function, being used for a specific purpose during the biological process of seedling adaptation. However, attempts to hydrolyse isolated lupin galactan as well as isolated lupin cell walls (Cell Wall Ghosts - see Chapter 5) using ßI enzyme failed to produce free galactose or any oligosaccharides.

The nature of ßII enzyme

The enzyme named ßII was not investigated to the same extent as ßI. However, the results produced suggest that ßII enzyme does not correspond either to the same polypeptide as ßI or to the *exo*-galactanase. The evidence is: 1) ßII had a high apparent molecular weight (>66kDa) as

compared with exo-galactanase (60 and 45kDa) and ßI enzyme (44kDa); 2) ßII bound strongly to DEAE-cellulose at pH 7.8 but not to CM-cellulose at pH 5.0. On the other hand, exo-galactanase (pI 7.0) did not bind to DEAE-cellulose but bound to CM-cellulose at pH 7.8 and ßI enzyme (pI~5.5) bound to DEAE-cellulose at pH 7.8 but did not bind to CM-cellulose at pH 5.0. Thus, ßII enzyme probably has pI intermediate between 5.5 (ßI enzyme) and 7 (exo-galactanase); 3) ßII enzyme showed high activity on PNPGal but very low activity on lupin galactan. Therefore, ßII activity could not be attributed either to the presence of ßI since the later enzyme does not hydrolyse lupin galactan at all or to the presence of *exo*-galactanase, since much higher activity on lupin galactan would be detectable.

Although no immunological data was obtained regarding ßII enzyme, it is possible that it corresponds to one of the two cross-reacting polypeptides with high molecular weight which were detected during the time course of ß-galactosidase activity (**Figure 3.12**)

Further investigations are necessary in order to understand the biological function of the two enzymes described in this chapter. Nevertheless, it is now clear that germinating *Lupinus angustifolius* cotyledons possess at least three different ß-galactosidases (exo-galactanase, ßI and ßII) which are immunologicaly related, but have slightly different molecular properties and major differences in specificity.

Chapter 5

CYTOLOGICAL STUDIES

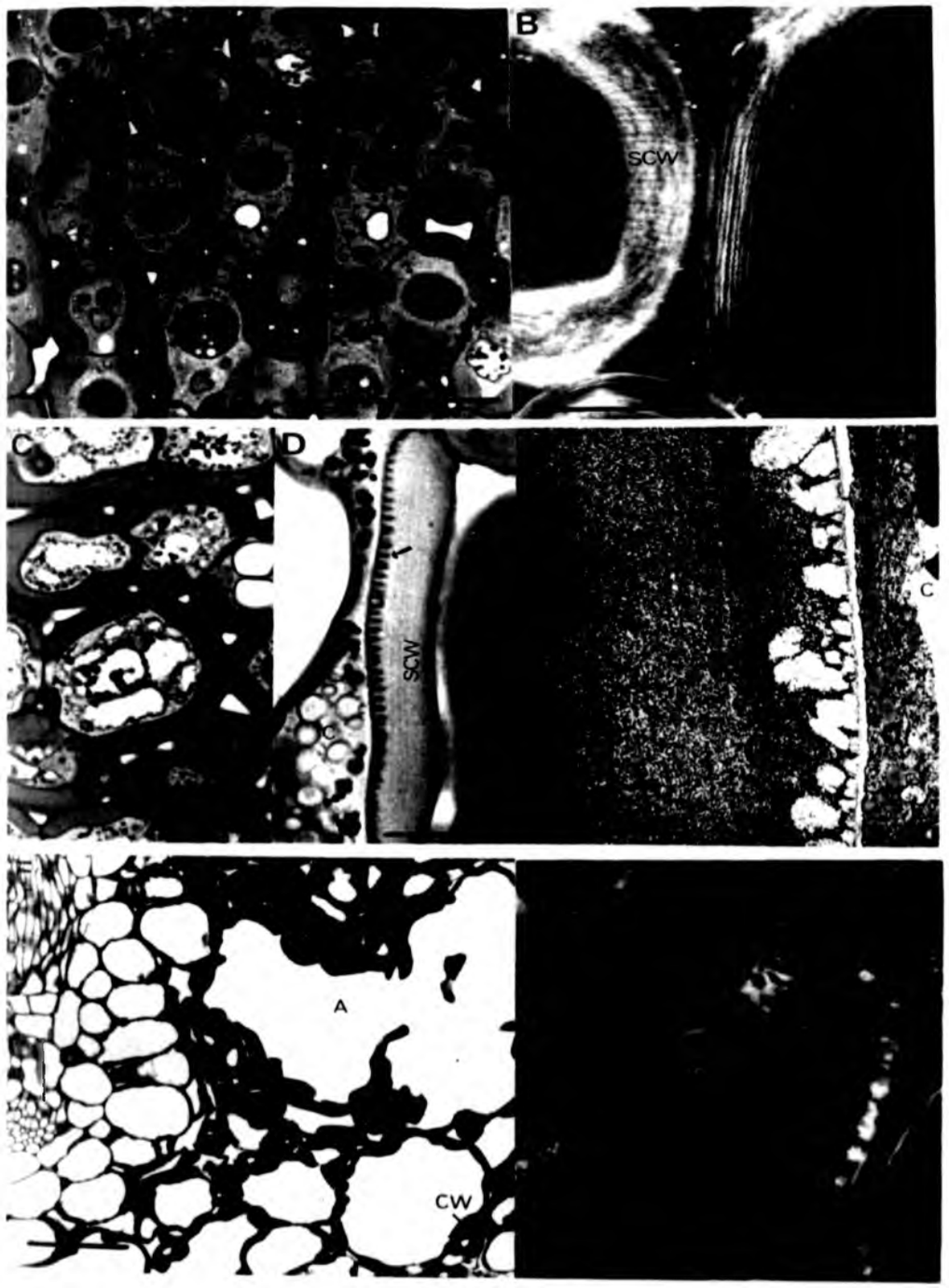
THE FUNCTION OF EXO-GALACTANASE DURING RESERVE MOBILISATION AND ITS ROLE IN THE DEVELOPMENT OF THE COTYLEDONS OF *LUPINUS ANGUSTIFOLIUS* FROM A STORAGE ORGAN INTO A LEAF-LIKE ORGAN

Introduction

The mobilisation of storage cell wall galactans from *Lupinus angustifolius* has been studied by Parker (1984a and b). Using electron microscopy, she described cytochemically the deposition (Parker 1984a) and the mobilisation (Parker, 1984b) of the thickenings of the cotyledonary cell walls of *Lupinus angustifolius* (**Figure 5.1**). Periodic Acid Schiff, which stains periodate-reactive of carbohydrates producing free p-rosaniline which is red and insoluble (**Figure 5.1A**)(Reid, 1989) and calcofluor (**Figures 5.1 B** and **G**, which is thought to react with fibrils of structural polysaccharides (Herth and Schnepf, 1980 *In* Parker, 1984a), were used to visualise transformations in the storage cell walls (**Figure 5.1**). After deposition, regular striations were observed in the thickenings of the storage cell walls and the author attributed this to diurnal/nocturnal alternating cycles during deposition of the cell wall material during seed maturation (Parker, 1984b - **Figure 5.1B**).

In *Lupinus angustifolius* cotyledons, cells possessing storage cell walls are confined to the mesophyll in the centre of the cotyledons (Parker, 1984a and b, see also **Figure 6.3** - Chapter 6). After germination, the cotyledons rapidly become green and reserve degradation starts. The thickenings of the cell wall are then mobilised completely.

Figure 5.1. Storage cell wall mobilisation in cotyledons of *Lupinus angustifolius* 2 days (**A** and **B**), 5 days (**C** to **E**) and 14 days (**F** and **G**) after imbibition (from Parker, 1984b). **A** - two days after imbibition. Bar represents 100µm; **B** - staining with calcofluor. Striations have a peak-to-peak distance of about 1.5µm. Bar represents 25µm; **C** - five days after imbibition. Bar represents 25µm; **D** - Detail of the storage cell wall showing digestion pockets (arrow).Bar represents 20µm; **E** - Detail of the storage cell wall showing digestion pocket.Bar represents 2µm; **F** - 14 days after imbibition.Bar represents 100µm; **G** - Detail of the storage cell wall stained with calcofluor. Bar represents 25µm. *A-aerenchyma; C-cytoplasm; CW-partially digested cell wall; SCW-storage wall; PB-protein body;*



Parker (1984b) observed that pockets of degradation appear near the plasmalemma and that degradation proceeds towards the middle lamella until very thin cell walls are left (**Figure 5.1C,D** and **E**). Parker (1984b) suggested that galactan is the substance that is mobilised from the cell walls after germination, based mainly on the facts that 1) calcofluor-induced fluorescence decreases dramatically after germination (compare **Figures 5.1B** and **G**) and 2) galactose and arabinose are the main monosaccharides mobilised after germination of *Lupinus luteus* (Matheson and Saini, 1977) and *Lupinus angustifolius* (Crawshaw and Reid, 1984).

Apart from cell wall polysaccharides, other reserve substances are also mobilised. After germination, protein bodies are completely degraded, leaving one vacuole which takes up most of the cell lumen (**Figures 5.1A,C** and **F** - Bewley and Black, 1978, Parker, 1976, 1984b). Besides the reserve proteins, glycosidases (α-mannosidase and α-galactosidase) have been reported to be associated with *Lupinus angustifolius* protein bodies (Plant and Moore, 1982). These authors detected α and ß-galactosidase activities in association with protein bodies from cotyledons of lentil and suggested that glycosidases associated with protein bodies might play a role in the modification or degradation of glycoprotein oligosaccharides following germination.

In a lower proportion, transitory starch and lipid bodies are also present in lupin cotyledons (Parker, 1976, 1984).

Both *Lupinus albus* (Parker, 1976) and *Lupinus angustifolius* (Crawshaw and Reid, 1984) mobilise all their reserves during approximately 10-15 days after initiation of imbibition. Consequently, a great loss (>70%) in dry weight of the cotyledons is observed.

After reserve mobilisation, lupin cotyledons rapidly acquire a leaf-form. In certain regions of the cotyledons, mainly between the vascular bundles, collapse of the thinner cell walls occurs, resulting in morphological transformations that resemble leaf development (**Figure 5.1F**). Large air pockets are produced probably as a result of cell wall degradation. A palisade with cylindrical cells containing chloroplasts is present at the adaxial surface of the cotyledons. Vascular bundles are well developed and stomata can be seen at both abaxial and adaxial surfaces (Parker, 1976, 1984b).

In addition to the work presented by Parker (1984b), some of the principal landmarks that lead to the idea that exo-galactanase is playing an important role in cell wall mobilisation can now be summarized: 1) galactose residues form the main component in *Lupinus angustifolius* cotyledonary cell walls; 2) the galactose residues are present mostly as a (1→4)-ß-linked polymer (i.e.galactan) in *Lupinus angustifolius* cotyledons; 3) galactose is the

main monosaccharide residue mobilised from germinating cotyledons of *Lupinus angustifolius* and 4) exo-galactanase shows high specificity for (1→4)-ß-linked galactans and its activity as well as its specific protein levels vary consistently with the grand period of reserve mobilisation in germinating *Lupinus angustifolius* cotyledons. The evidence listed above sums up to build a strong basis to support the hypothesis that "*(1→4)-ß-galactans* are degraded by *exo-(1→4)-ß-galactanase* and consequently mobilised from the *Lupinus angustifolius* cotyledonary *cell walls*" is correct. Nonetheless, the evidence is still circumstantial.

Therefore, in this Chapter experiments are described in which the purified exo-(1→4)-ß-galactanase is used to identify (1→4)-ß-galactan **directly** as the compound that is mobilised from *Lupinus angustifolius* cell walls after germination. To achieve this goal, two different strategies were used: **1)** lupin isolated mesophyll cell walls (Cell Wall Ghosts - CWGs) were incubated with the purified exo-galactanase; before and after incubation their appearance was examined using scanning electron microscopy and their monosaccharide composition was determined; **2)** the purified exo-galactanase was complexed with gold particles and the gold-enzyme complex was used as a probe to follow the events occurring during cell wall mobilisation.

Although observations using light and electron microscopy, together with biochemical experiments, have produced evidence that galactomannans, xyloglucans and

arabinogalactans are present in thickened storage cell walls in endosperms and cotyledons of several plant seeds (see review by Reid, 1985), only few experiments have been directed towards the direct localisation of CWSP in seeds (Reis et al. 1987, Vian et al. 1991).

An introduction to the basis of the two techniques used in this Chapter is given below.

Isolation of plant cell walls that preserve their original shape

Although isolation of entire plant cell wall material have been performed (Fry, 1988 and references therein), the materials extracted are not usually shown to preserve, even partially, the shape of the original cells. The only exception to this seems to be the work carried out by Hayashi and Maclachlan (1984) and Hayashi et al. (1984). These authors were able to isolate cell wall ghosts from pea epicotyls. Hayashi et al. (1984) used 1,4-ß-glucanase to modify, *in vitro,* pea cell wall ghosts, hydrolysing xyloglucan preferentially. The modifications in texture of the cell walls due to enzyme action could be appreciated using fluorescence labelling and calcofluor.

In 1984, Hutcheon and Reid (unpublished) isolated cell walls from *Lupinus angustifolius* cotyledons which had been previously imbibed for 16 hours. The method that they used

was particularly successful in preserving the shapes of the cotyledonary mesophyll cells. This method consists basically of tissue disruption and limited cell breakage by Ultraturrax homogenisation and sequential wet sieving through sieves of decreasing mesh size in parallel with examination of the degree of integrity of the isolated cell walls under the light microscope. Thus, the cytosol is eliminated, leaving the isolated cell walls, from which a "population" with low damage is selected. After freeze drying, the isolated cell walls are a white powder which can be shadowed with gold and visualised by scanning electron microscopy. The isolated cell walls from 16h imbibed cotyledons retain a rounded shape, and pit fields as well as storage thickenings can be clearly distinguished. Because these cell walls probably resemble the original shape of the cell, they are called Cell Wall Ghosts (CGWs). The possibility of visualizing the isolated cell walls at this level of detail suggested that the CWGs could be very valuable tools for probing the transformations in cell walls after incubation with isolated enzymes.

The use of enzyme-gold complexes to localise compounds in biological tissues

The enzyme-gold technique was developed by Bendayan in 1981 to localise nucleic acids in animal cells. Basically, the enzyme-gold technique consists of stabilising a gold sol by adsorbing a purified enzyme to the surface of the gold particles. If the gold-complexed enzyme then preserves, at least partially, the substrate specificity of the native enzyme, it can be used to localise its substrate in ultrathin sections. The main steps for obtaining a specific gold-enzyme probe are summarized below:

1) Gold particles with a definite size, which has to be proportional to the dimensions of the structures that will be visualised at subcellular level as well as to the magnification that will be used during the visualisation under the electron microscope, are prepared. For gold sol preparation, trisodium citrate is rapidly added to a boiling solution of tetrachloroauric acid thus reducing it

to metallic (colloidal) gold. Depending on the concentrations of the reagents, different particle size distributions are obtained (Roth, 1983).

2) To a fixed amount of the gold sol, different amounts of the purified enzyme to be coupled are added. The minimum amount of enzyme that is capable of stabilizing the gold suspension can be determined by adding salt. Stable sols retain their bright red colour when in presence of NaCl; aggregation is marked by a colour-change to purple or blue. The minimum amount that stabilises the gold suspension is called the **gold number**.

3) After determination of the gold number, a probe is made by loading an amount of enzyme slightly higher than at necessary to stabilise the gold sol. After centrifugation, the gold-complex probe is collected in the precipitate (the mobile pool). The gold-complex probe is then incubated with ultrathin sections of the biological tissue under selected conditions and if the enzyme still preserves partial biological activity, its substrate(s) may be visualised under the electron microscope by means of adhering gold particles.

4) Careful controls have to be carried out to certify the specificity of the enzyme-gold complex cytochemical probe. The controls to be used vary according to the enzyme and substrate, but controls such as **i)** using inactivated enzyme; **ii)** digestion of the substrate to be localised previous to gold labelling and **iii)** blocking the sections with excess of substrate previous to or during gold labelling, are essential to be sure that specific labelling has been achieved.

Amongst the glycosidases that have been complexed with colloidal gold aiming to localise their respective substrates are: mannosidase (Londoño and Bendayan, 1987), xylanase (Vian et al. 1983; Ruel and Joseleau, 1984), cellulase (Berg et al., 1988 *In* Bendayan, 1989), exo-glucanase (Benhamou and Ouellete, 1987), pectinase (Benhamou and Ouellete, 1986), ß-galactosidase (Reis et al. 1987, Vian et al., 1991) and xyloglucan endo-(1→4)-ß-glucanase (Vian et al., 1991).

An α-amylase-gold complex was used by Bendayan (1984) to localise glycogen deposits in rat liver and by Benhamou and Ouellette (1987) to localise glycogen in fungal cells.

Escherichia coli ß-galactosidase was complexed with colloidal gold and applied to sections of tamarind (*Tamarindus indica*) cotyledons to localize xyloglucan (Reis et al., 1987). More recently Vian et al., 1991 used purified nasturtium ß-galactosidase and endo-(1→4)-ß-glucanase to localise xyloglucan in tamarind and nasturtium cotyledons. These authors reported for the first time that

heavier staining is obtained when the purified enzyme is heat-deactivated before gold-complexing.

This Chapter describes cytological studies performed during mobilisation of the cell wall storage polysaccharide from cotyledons of growing *Lupinus angustifolius* plantlets after germination.

A series of experiments was performed in which scanning electron microscopy was used to examine isolated cell walls that after extraction still preserve the shape of the cells (referred to here as cell wall ghosts - CWG). Their biochemical and structural features were compared before and after CWSP mobilisation *in vivo*, as well as before and after using the purified exo-(1→4)-ß-galactanase *in vitro*. It was demonstrated that this enzyme alone is able to reproduce a large proportion of the effects brought about by mobilisation *in vivo*.

Purified *exo*-(1→4)-ß-galactanase was complexed to gold particles and the enzyme-complex was used to localise for the first time (1→4)-ß-galactan in mesophyll cell walls of lupin cotyledons. The enzyme-gold complex was also used to investigate the post-germinative mobilisation of the cell wall storage polysaccharide from lupin cotyledons.

These results demonstrated clearly that (1→4)-ß-galactan is the main storage cell wall polysaccharide mobilised after germination and also uphold the propositions made throughout this work that exo-(1→4)-ß-galactanase is the main enzyme responsible for its degradation *in vivo*.

Results

Characteristics of the isolated storage mesophyll cell walls

Through a process of careful tissue/cell breakage using the Ultraturrax homogeniser, followed by wet sieving and freeze drying, Hutcheon and Reid (unpublished results) were able to isolate storage parenchyma cell walls from lupin cotyledons which still preserved a remarkable similarity with the intact cell (Cell Wall Ghosts - CWGs). **Figure 5.2** shows a scanning electron micrograph of this cell wall material isolated from 16h imbibed cotyledons alongside a light microscopic section of the parent cotyledons (**Figure 5.2A**). It was observed that a significant proportion of the isolated cell wall material still preserved the shape of the cells (**Figure 5.2B**). Under higher magnification, it could be seen that most of the CWGs had holes through which the cytoplasmic contents had presumably leaked during isolation (**Figure 5.4A**). The characteristic pit fields can be clearly seen in CGWs as well as under light microscopy.

When CWGs prepared by Hutcheon and Reid (unpublished results) from 16h imbibed lupin cotyledons were subjected to acid hydrolysis followed by analysis by HPAE chromatography the monosaccharide composition was found to

Figure 5.2. Micrograph showing *Lupinus angustifolius* storage mesophyll cells from 16h imbibed cotyledons.**A** - Light microscopy of sections stained with methylene blue. Bar represents 40 µm; **B** - Cell Wall Ghosts seen by scanning electron microscopy x180.
SW-storage wall; F-pit field.

be very similar to that of isolated lupin galactan. The typical composition is given in **Table 3.1** (Chapter 3).

Hutcheon and Reid have also isolated CWGs from 14 days old cotyledons (unpublished). Compositional analysis of the CWGs from 16h and 14 days old cotyledons by Gas Liquid Chromatography (GLC) demonstrated that mainly galactose and arabinose residues had been removed from the cell walls during CWSP mobilisation, leaving polysaccharide(s) rich in uronic acids (**Table 5.1**).

The thick deposits presumably composed of CWSP that are seen in 16h imbibed CWGs (**Figure 5.4A**) are completely removed after mobilisation *in vivo* for 14 days (**Figure 5.4B**).

Table 5.1. Compositions of storage mesophyll cell walls from *Lupinus angustifolius* cotyledons (data from Hutcheon and Reid, unpublished)

	Monosaccharide Composition (%)					
	Neutral monosaccharides[1]					Uronic Acid[2]
	Rhamnose	Arabinose	Galactose	Glucose	Xylose	
Before germination (16h imbibition)	2	12	66	4	15	8
After reserve mobilisation (14days)	11	16	30	23	20	35

[1] as % of total neutral monosaccharides

[2] as % of cell wall material

In vitro treatment of the CWGs from 16h imbibed cotyledons with the purified exo-(1→4)-ß-galactanase

The time course of galactose release from 16h CWGs incubated with *exo*-(1→4)-ß-galactanase is shown in **Figure 5.3**. Most of the galactose susceptible to attack by the *exo*-(1→4)-ß-galactanase was released on incubation for 144h at 30°C, pH 5.0. In this experiment, CWGs were periodically washed with buffer and care was taken to avoid reaching inhibitory concentrations of galactose or contamination with microorganisms.

In a control experiment, CWGs were incubated without the enzyme (exo-galactanase) for the same period and under the same conditions (30°C pH 5.0) as the ones incubated with the enzyme. After 144h incubation, they could not be distinguished from non-incubated 16h CWGs (**Figures 5.4A and 5.4C**).

Incubation with the purified *exo*-(1→4)-ß-galactanase resulted CWGs that were very similar to the ones obtained from 14 days cotyledons i.e. after reserve mobilisation (**Figures 5.4B and 5.4D**). Thus, exo-galactanase was able to reproduce most of the morphological changes observed after reserve mobilisation *in vivo*. It should be noted that the thickenings of the cell walls had completely disappeared after *in vivo* mobilisation (14 days), as well as after *in vitro* incubation with *exo*-(1→4)-ß-galactanase (144h).

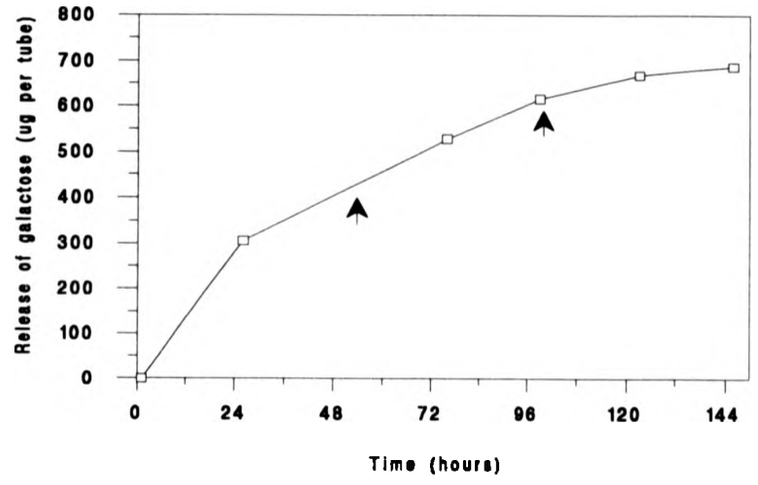
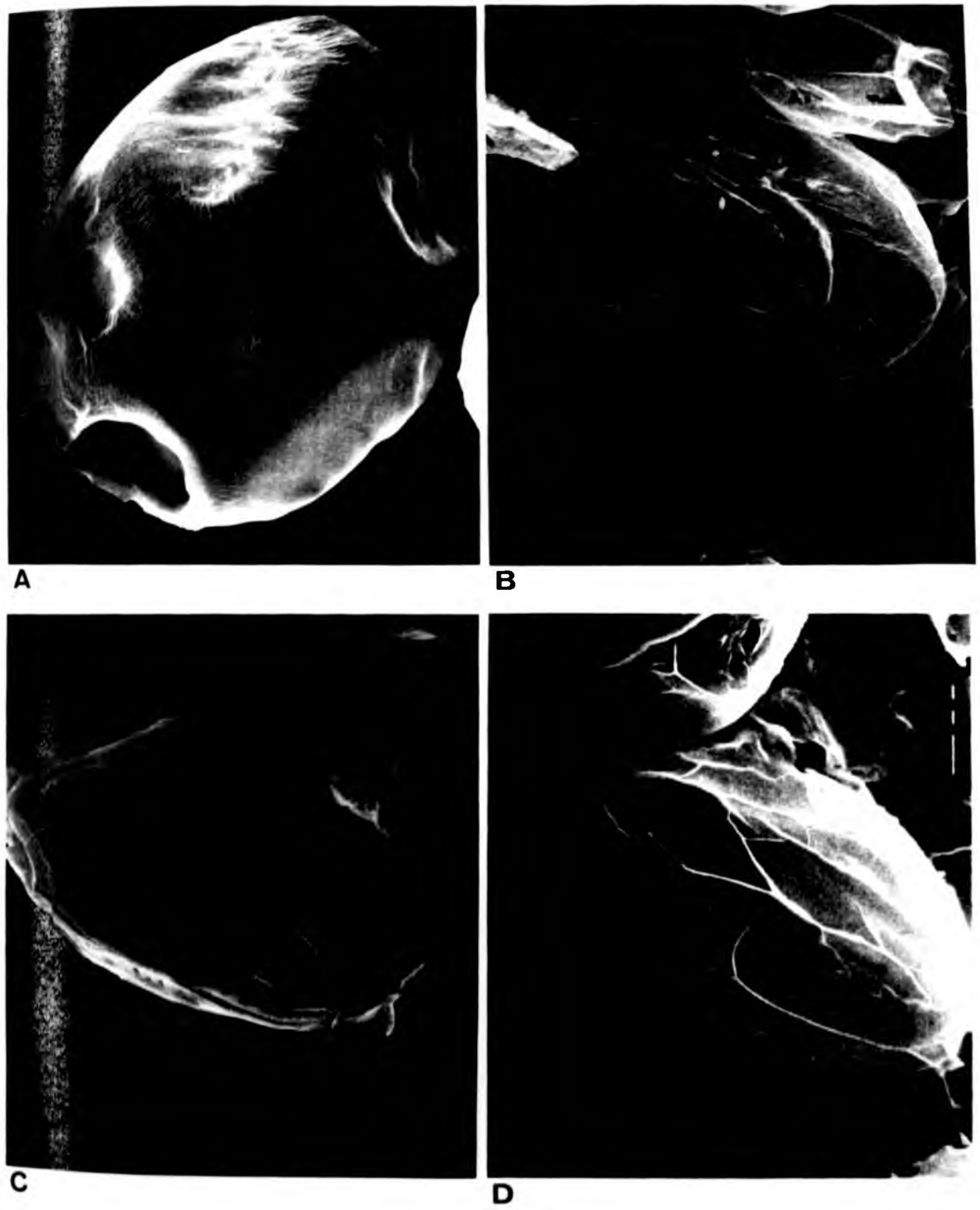


Figure 5.3. Cumulative release of galactose from cell wall ghosts under incubation with pure exo-(1-4)-ß-galactanase at 30°C, pH 5.0. The arrows indicate the times of addition of fresh enzyme after 3 successive washes and centrifugations and collection of the respective supernatants. These supernatants were pooled and analysed by galactose dehydrogenase assay to detect galactose.

Figure 5.4. Appearance of CWGs in scanning electron microscopy before and after transformations of 16h Cell Wall Ghosts after mobilisation *in vivo* and with exo-(1→4)-ß-galactanase *in vitro*. **A** - CWG from 16h imbibed cotyledons x1600; **B** - CWG from 14 days old cotyledons x650; **C** -16h CWG incubated for 144h in 50mM ammonium acetate buffer pH 5.0, 30°C x900; **D** - CWGs incubated with pure exo-(1→4)-ß-galactanase for 144h in 50mM ammonium acetate buffer pH 5.0, 30°C 144h x900. *f-pit field, SW-storage wall.*



The data in **Table 5.2** are derived from Hutcheon and Reid's compositional analyses of CWGs from 16h and 14 days cotyledons, and from new compositional analyses of CWGs before and after incubation with exo-galactanase. It is assumed that glucose residues are not mobilised during CWSP mobilisation. Thus, the percentage of mobilisation of each monosaccharide can be calculated.

Galactose represents ca.66% and arabinose ca.10% of the CWGs. Although the percentage of loss is high for xylose and rhamnose (**Table 5.2**), their mobilisation represent a release of very low amounts of monosaccharides from the cell wall whereas galactose and to a lesser extent arabinose represent a significant amount of the storage cell wall mobilised during germination.

On the basis of this assumption, mobilisation *in vivo* i.e. after 14 days, included the release of small amounts of rhamnose and xylose and large amounts of arabinose, galactose, from the lupin isolated mesophyll cell walls.

Hutcheon and Reid (unpublished) used a crude enzymatic extract, prepared from germinating cotyledons, to modify CWGs and obtained approximately the same composition (**Table 5.2**) as well as the same pictures in scanning electron microscopy as obtained after *in vivo* mobilisation.

Using purified *exo*-(1→4)-ß-galactanase, **only galactose** was released, but in lower proportion (63%) when compared to *in vivo* degradation (92%) or with degradation by a crude enzyme extract from the cotyledons (86%)(**Table 5.2**).

Table 5.2. Comparison of the percentage of loss of each monosaccharide from Cell Wall Ghosts (CWG) after reserve mobilisation *in vivo* (14 days), incubation with cotyledonary enzyme extract (crude enzyme) and with purified exo-(1→4)-ß-galactanase. Our data were combined with Hutcheon & Reid's unpublished data.

Monosaccharide	CWG after 14 days	CWG + crude enzyme	CWG + *exo*-(1→4)-ß-galactanase
Rhamnose	45	10	0
Arabinose	57	56	0
Xylose	45	37	0
Galactose	92	86	63
Glucose	0	0	0

Thus, a large proportion of the galactose present in lupin mesophyll cell walls is mobilised by exo-galactanase only. This enzyme is capable of hydrolysing ca. 70% of the galactose which is hydrolysable *in vivo*.

These results were a clear indication of the major importance of this enzyme for the process of storage cell wall mobilisation *in vivo*.

Specificity of the gold-complexed exo-galactanase as a cytochemical probe.

After fixation and embedding in LR-White, 1µm sections were cut from the storage mesophyll of lupin cotyledons at 16 hours,2,4,6,8,10,12,14,16 and 18 days after initiation of imbibition. The sections were collected on nickel grids, incubated with the gold-probe and stained with uranyl acetate-lead citrate.

The gold number (i.e. optimal concentration of purified exo-galactanase which stabilises the gold sol) was obtained by mixing different volumes of purified exo-galactanase with a fixed volume of gold sol. Since stabilisation of the gold sol depends on the amount of the protein and different batches of enzyme had different concentrations, an individual gold number was obtained for each different enzymatic preparation. Native and partially-denatured (for 10min. at 50°C) exo-galactanase were used for

the preparation of the enzyme-gold complex. Very low activity (ca.10%) is left after denaturation under these conditions (see **Figure 3.9** - Chapter 3). After mixing the gold sol with the appropriate amount of pure enzyme the stable enzyme-gold complex was concentrated by centrifugation, collected and incubated with the lupin cotyledonary sections for 30 min at room temperature. After incubation, sections were washed with distilled water and stained with uranyl acetate-lead citrate.

When sections of 16h imbibed cotyledons were treated with non-denatured exo-galactanase-gold complexes unspecific labelling was obtained (**Figure 5.5A**).

On the other hand, when *exo*-(1-4)-ß-galactanase was partially denatured for 10 minutes at 50°C prior to complexing with the gold particles (**Figures 5.5A, 5.5B and 5.7A**) strong labelling was observed on the lupin cotyledon cell walls, apparently restricted to the thickenings (**Figure 5.5C**). The protein bodies were also labelled with the exo-galactanase-gold complex, although less strongly (**Figure 5.5B**).

In the cell walls, the enzyme gold complex labelled specifically the thickenings associated with the secondary storage cell wall deposits (**Figure 5.5C**). It was also clear that the enzyme-gold complex associated specifically with the electron dense parts of the wall thickenings (**Figure 5.5B**) accentuating the alveolar character of the storage cell wall. In **Figure 5.5B** some enzyme gold particles can be

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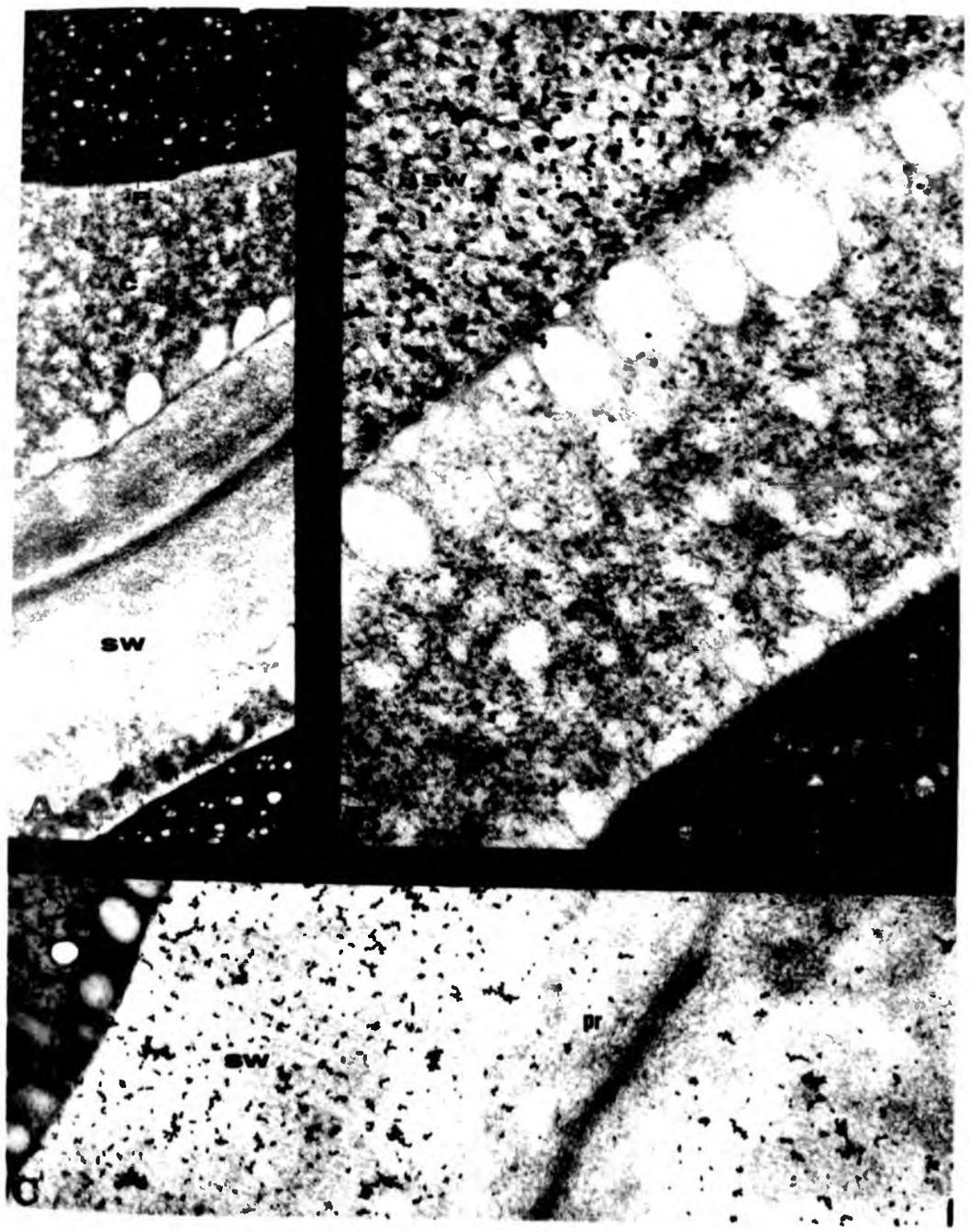
In the cell walls, the enzyme gold complex labelled specifically the thickenings associated with the secondary storage cell wall deposits (**Figure 5.5C**). It was also clear that the enzyme-gold complex associated specifically with the electron dense parts of the wall thickenings (**Figure 5.5B**) accentuating the alveolar character of the storage cell wall. In **Figure 5.5B** some enzyme gold particles can be

Figure 5.5. Labelling of 16h imbibed lupin cotyledonary sections using gold complexes from native and heat-deactivated exo-galactanase. **A** - staining with native exo-(1→4)-ß-galactanase gold complex x22,500; **B** - staining with partially denatured exo-(1→4)-ß-galactanase gold complex x56,000; **C** - wall cross-section showing specific staining of the secondary cell wall thickenings. Labelled with heat-deactivated gold complex x22,500. *C-cytoplasm, m-middle lamella, P-protein body, pr-primary wall, SW-storage wall.*



seen associated with the cytosol. This was probably non specific labelling due to the high concentration of enzyme-gold complex used.

When sections from germinated cotyledons were subjected to enzyme-gold labelling, starch granules labelled strongly. As starch appears later after germination, controls for specificity of labelling had to be performed using 16h old as well as 6 and 8 days old sections. The controls using cotyledonary sections of days 6 and 8 were performed to study interaction of the gold-probe with starch.

The controls performed are described below. Only the heat-deactivated complex was used.

1) ***Controls using sections from 16h old cotyledons:***

a) *blocking with* ***BSA*** *(5mg/ml and 0.1mg/ml) prior to incubation of sections with the enzyme-gold probe.* This control was performed to determine whether or not the externally applied protein would specifically suppress labelling of the protein bodies. Relatively high concentrations of BSA (5mg/ml) completely inhibited the labelling of the section while lower concentrations (50 and 100µg/ml) were not able to prevent labelling of cell walls or protein bodies (data not shown).

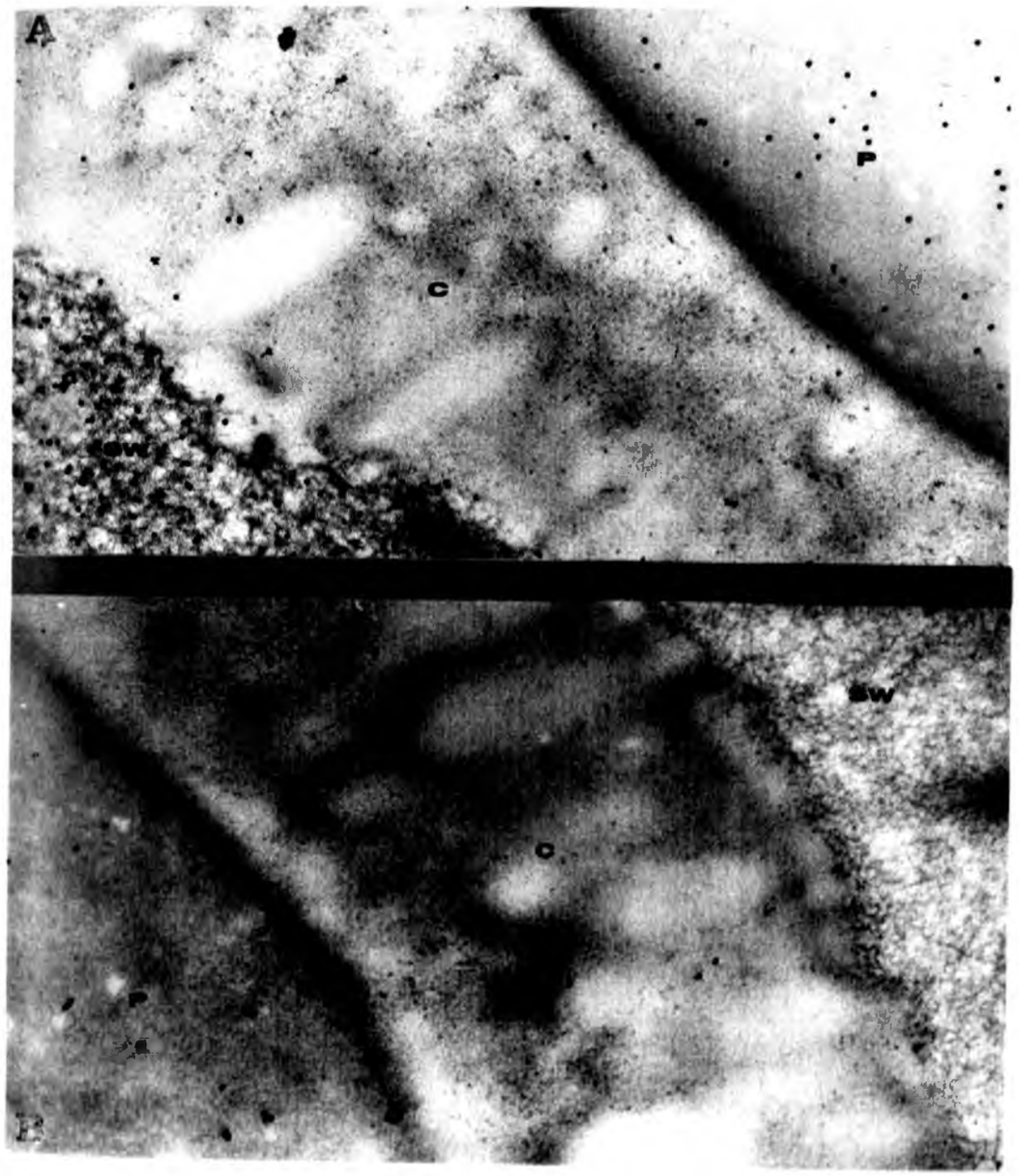
b) *Boiling the enzyme prior to gold complexing.* This treatment did not abolish labelling of the cell walls. In fact, it produced exactly the same results as for the partially-denatured enzyme-complex (**Figure 5.6A**)

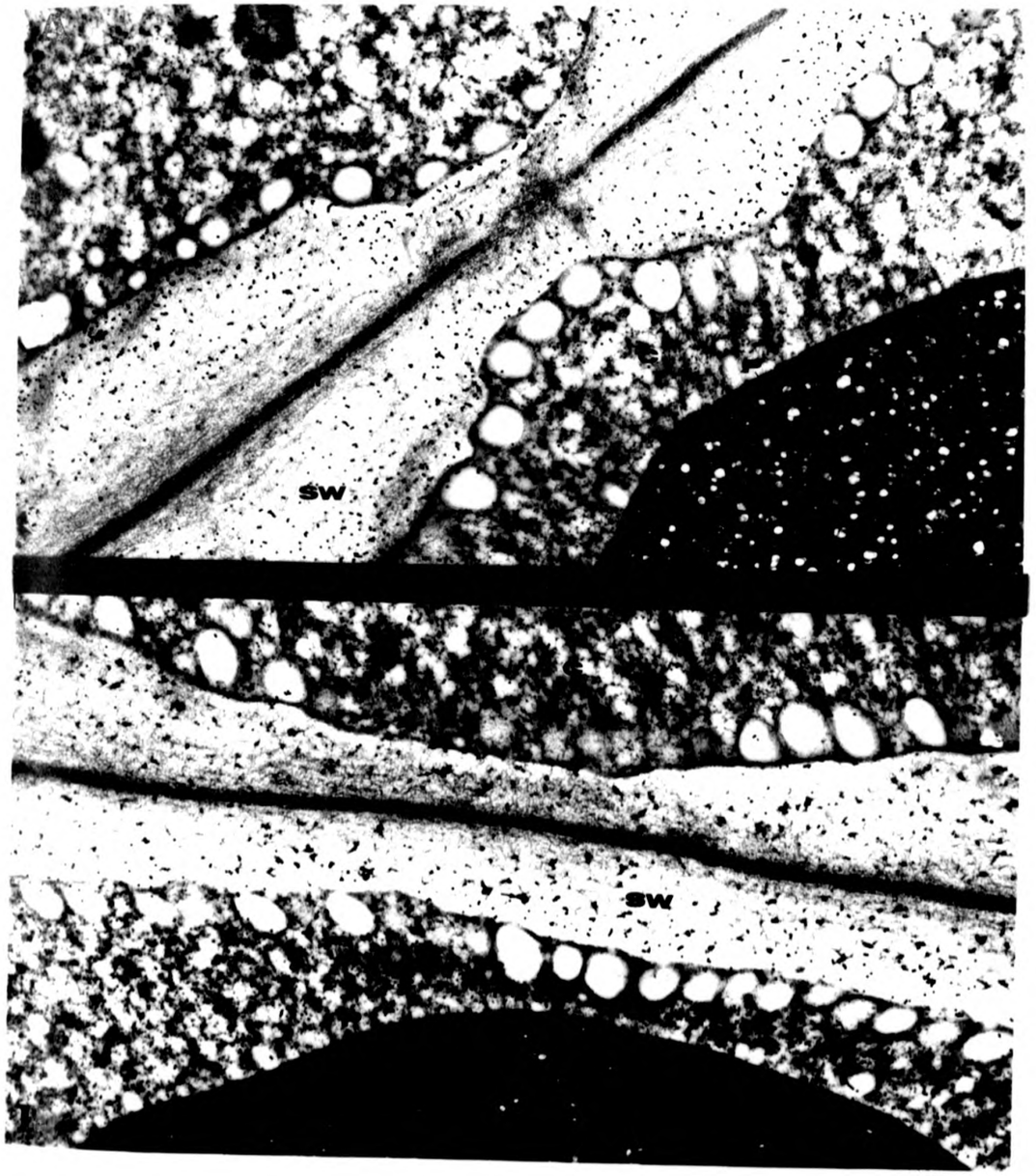
c) *incubation of sections with isolated* ***lupin galactan*** *during incubation with the enzyme-gold complex.* As can be seen in **Figure 5.6B**, pre-treatment with (1→4)-ß-galactan completely prevented labelling of the cell walls, while still maintaining the labelling of the protein bodies. This suggests that exo-galactanase-gold complex might possess higher affinity for its binding site in protein bodies than for the storage cell walls.

d) *incubation with* ***active exo-galactanase*** *prior to incubation with the enzyme-gold probe.* Pre-treatment of sections with *exo*-(1→4)-ß-galactanase greatly reduced labelling of both cell walls and protein bodies (**Figure 5.7B**). In this experiment, a large portion of the storage cell wall seemed to have been degraded. The hydrolysis of galactan chains *in vitro* provoked the appearance of rigid polymers which were not labelled with the gold-enzyme complex (compare with the non-digested storage wall labelled with heat-deactivated gold complex in **Figure 5.7A**). It is also interesting to note that the primary cell wall and the middle lamella did not undergo visible transformations after hydrolysis with exo-galactanase. Some gold particles are present and they are thought to be

Figure 5.6. Opposite. Cytochemical controls using sections of 16h imbibed lupin cotyledons. **A** - enzyme boiled (5min) before gold complexing x44,800; **B** - addition of lupin (1→4)-ß-galactan during staining with the gold complexed enzyme x44,800. *C-cytoplasm, P-protein body, SW-storage wall.*

Figure 5.7. Next page. Cytochemical controls using sections of 16h imbibed lupin cotyledons **A** - labelling with heat-deactivated gold complex x18,500; **B** - pre-incubated with active exo-(1→4)-ß-galactanase before staining with heat-deactivated gold complex. The arrows show artefact produced only after *in vitro* hydrolysis with exo-galactanase x18,500; *C-cytoplasm, P-protein body, SW-storage wall.*




SW
SW

either non specific labelling and/or labelling of scarce galactan chains which have not been hydrolysed (**Figure 5.7B**).

2) ***Controls using sections from 6 and 8 days old lupin cotyledons***:

a) incubation with **starch** at the same time as the enzyme-gold complex was incubated with the ultrathin sections did not prevent labelling at all (**Figure 5.8B** - day 6),

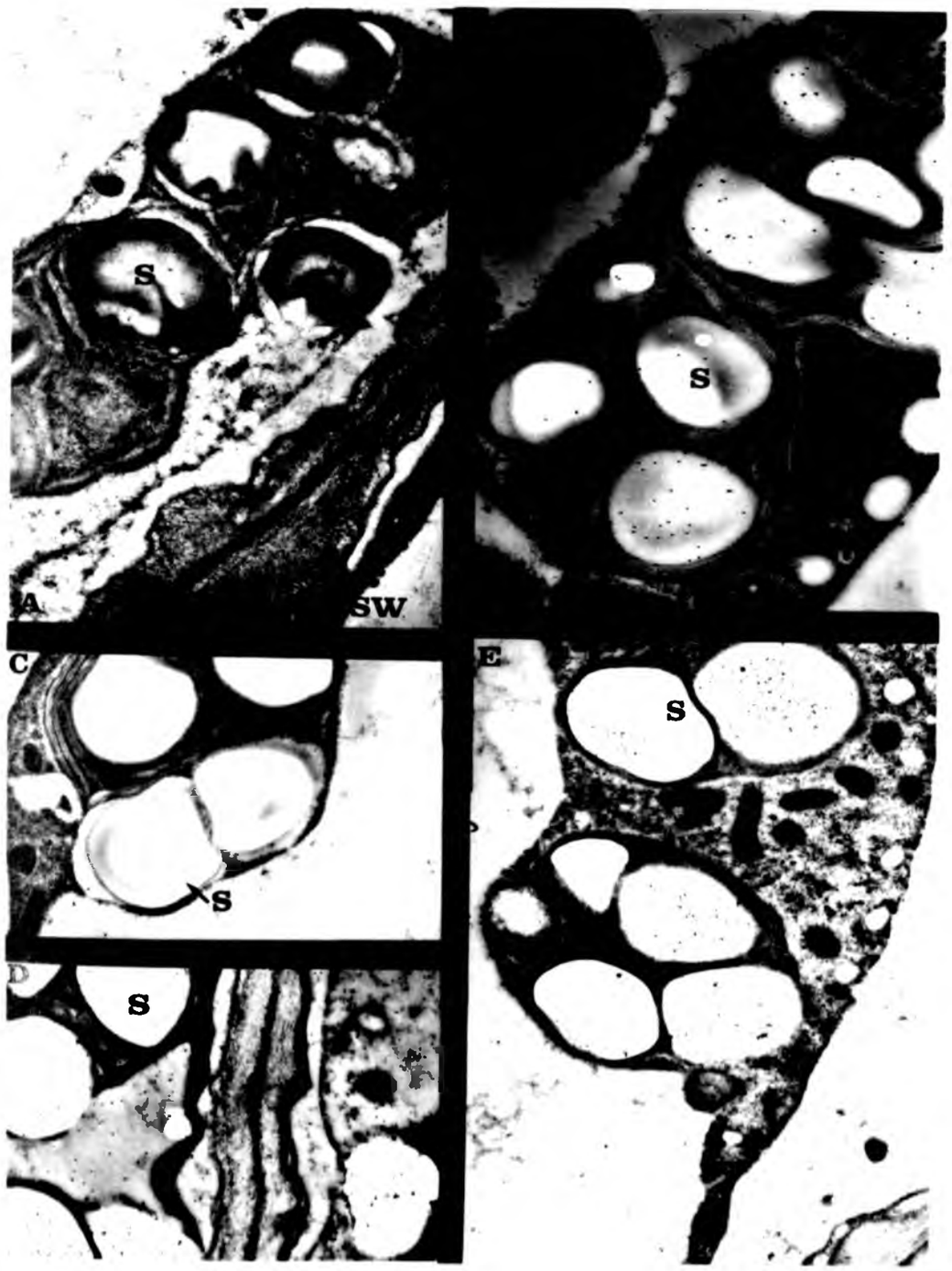
b) the same result as above was obtained when sections from 8 days old lupin cotyledons were incubated with active **α-amylase** prior to incubation with the exo-galactanase-gold complex (**Figure 5.8E**).

c) incubation with **galactan** was very strongly inhibitory (**Figure 5.8C** - day 8) to the binding of exo-galactanase-gold complex to the starch granules.

d) previous treatment with **active exo-(1→4)-ß-galactanase** did not prevent significantly binding of the enzyme-gold probe (**Figure 5.8D** - day 8).

The three controls (a,b and c) above indicate that the specific labelling is not due to binding to starch. It is possible that galactolipids possessing terminal galactose

Figure 5.8. Cytochemical controls using day 6 and 8 lupin cotyledonary sections (x15,000). **A** - control; sections labelled with heat-deactivated gold complex (day 8); **B** - addition of starch prior staining with the heat deactivated gold complex (day 6); **C** - addition of lupin (1→4)-ß-galactan during staining with the gold complexed enzyme (day 8); **D** - cotyledonary sections pre-incubated with active exo-(1→4)-ß-galactanase before staining the heat deactivated gold complex (day 8); **E** - cotyledonary sections pre-incubated with active α-amylase before staining with the heat-deactivated gold complexed enzyme (day 8). *SW-storage wall, S-starch*



residues are associated with the surface of the starch granules and that they are the epitopes to which exo-galactanase binds.

Although substances associated with intracellular organelles (protein bodies and amyloplasts) were labelled with the galactanase-gold probe, the controls used indicated that **in the cell walls** of mesophyll cotyledonary cells from *Lupinus angustifolius* the exo-galactanase-gold complex binds specifically to the storage (1→4)-linked galactan in the cell wall thickenings. The enzyme-gold complex can be therefore considered suitable for visualising **directly** and **specifically** (1→4)-ß-galactan and consequently its mobilisation.

Use of the enzyme-gold complex as a cytochemical probe during reserve mobilisation: description of the events

Using the *exo*-(1→4)-ß-galactanase-gold complex, it was possible to follow changes in the amount/distribution of (1→4)-ß-galactan in cell walls during mobilisation *in vivo*. The general events which occur in parallel with galactan degradation are documented using light microscopy.

In general, the regions of the wall that were selected to be photographed at high magnification were near pit

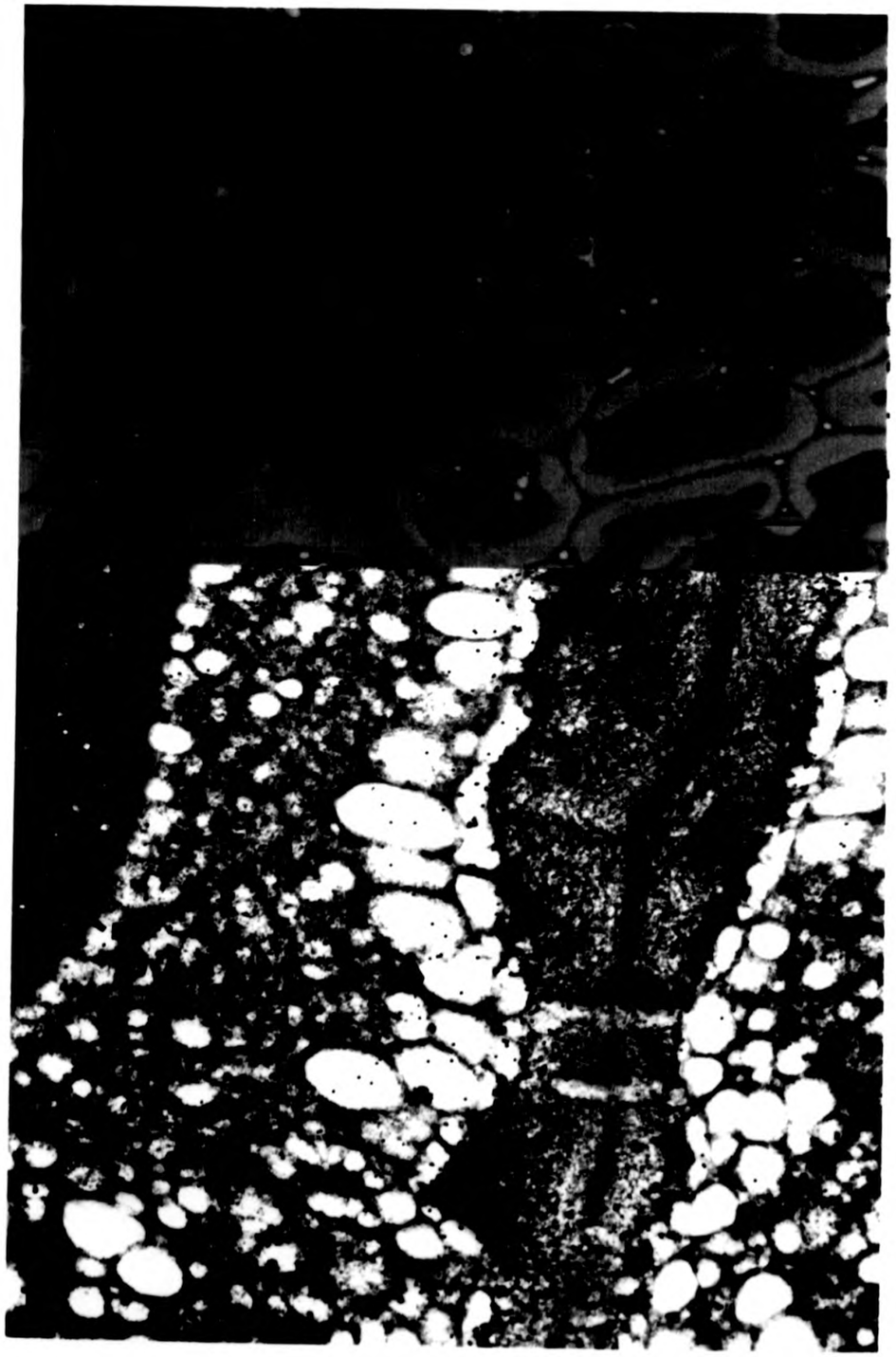
fields. This is due to the fact that in these regions the thickened storage cell walls, the primary cell walls and the middle lamella were clearly visible alongside some of the intracellular organelles (protein bodies and starch granules).

First day (16h). Sixteen hours after the initiation of imbibition the cells still showed signs of incomplete imbibition. The pit fields could not yet be clearly distinguished and few or no vacuoles were observed in the cytoplasm and protein bodies (**Figure 5.9A**). Gold labelling of the storage cell walls showed the characteristic alveolate structure with its electron dense structures specifically labelled by the *exo*-(1→4)-ß-galactanase gold particles. Practically no staining of primary cell wall and middle lamella was observed (**Figure 5.9B**).

Day 2. Imbibition appeared to be complete by day 2. Walls were noticeably thicker and a lower magnification was used in order to show approximately the same field as on first day (**compare Figures 5.9A and 5.10A**). The ultrastructure shows essentially the same features as on the first day, except for a greater number of non-staining bodies at the interface between plasmalemma and cell wall (**compare Figures 5.9B and 5.10B**) Protein bodies were also labelled with the enzyme gold complex (**Figure 5.10B**).

Figure 5.9. Day 0 (16h imbibed seeds) **Opposite. A** - light microscopy of the storage mesophyll tissue of imbibed lupin cotyledons, bar represents 50µm. **Staining with methylene blue; B** - ultrastructural view of a junction between two adjacent mesophyll cells from lupin cotyledons. Arrow indicate non-staining vesicule. x22,500. **Labelling with the heat-deactivated gold-complexed enzyme followed by staining with 2% uranyl acetate and 4% lead citrate.** *C-cytoplasm, f-pit field, m-middle lamella, P-protein body, pr-primary wall, SW-storage wall.*

Figure 5.10. Day 2. **Next page. A** - light microscopy of the storage mesophyll tissue of 2 days old lupin cotyledons. Bar represents 40µm; **B** - ultrastructural view of a junction between two adjacent mesophyll cells from lupin cotyledons. Arrow indicate non-staining vesicule. x22,500; *B-vascular bundle, C-cytoplasm, f-pit field, m-middle lamella, P-protein body*. Staining as in **Figure 5.9.**

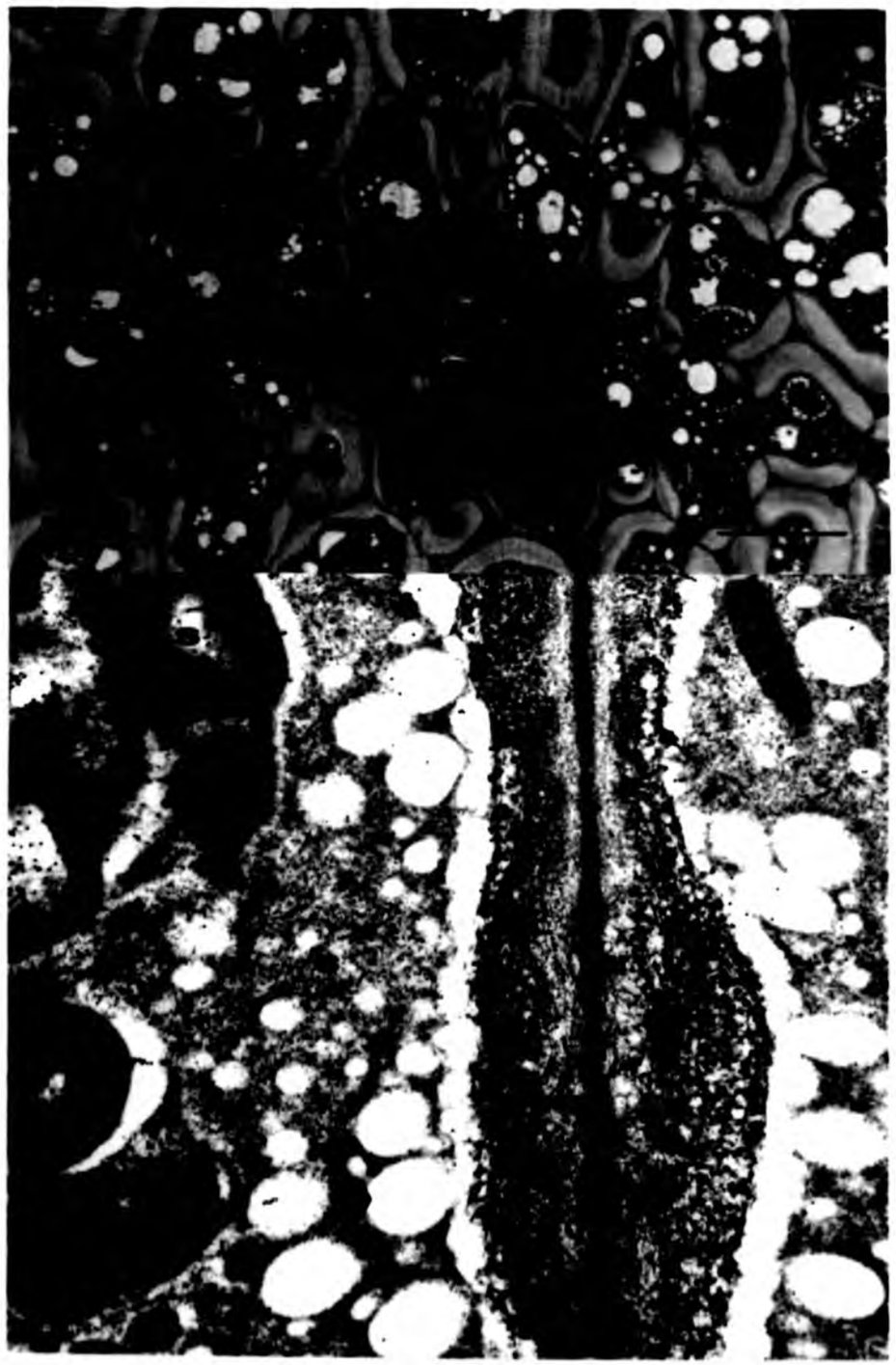


Day 4. At this point, many digestion pockets are present inside the protein bodies of the mesophyll cells (**Figure 5.11A**). This figure shows a developing vascular bundle (phloem can not yet be distinguished), which is surrounded by a sheath of mesophyll cells in which the mobilisation of the protein bodies seem to have been delayed. It is important to note that this delay is probably not a general phenomenon, since degradation was detected very early in sheaths surrounding other vascular bundles in the same cotyledon. All the mesophyll cells, at this stage, had started to accumulate starch granules in the cytoplasm. These granules could be seen in the sheath layer of the vascular bundles (**Figure 5.11A**).

At ultrastructural level, the starch granules were strongly labelled with the enzyme-gold complex (**Figure 5.11B**). Comparing this last figure with **Figure 5.10B** it is possible to notice a great increase in the number of non-staining bodies surrounding the cell walls. The storage cell walls at this stage did not show any change in relation to the previous stages. In **Figures 5.11B,** as well as in **Figure 5.9B,** the primary cell wall can be clearly distinguished and the absence labelling with exo-galactanase-gold complex is noticeable.

Day 6. At day 6 most of the seeds have completed germination (radicle protrusion). At this stage, a great part of the protein bodies have already been degraded or

Figure 5.11. Day 4. **A** - light microscopy of the storage mesophyll tissue of 4 days old lupin cotyledons. Bar represents 40µm; **B** - ultrastructural view of a junction between two adjacent mesophyll cells from lupin cotyledons. Arrow indicate non-staining vesicule. x22,500; *B-vascular bundle, C-cytoplasm, f-pit field, m-middle lamella, P-protein body, pr-primary wall S-starch granule.* Staining as in **Figure 5.9.**



turned into smaller particles which are inside a large vacuole and stain strongly with methylene blue (**Figure 5.12A**). Starch granules are present in the cytoplasm of most of the cells and they can be seen preferentially near the pit field regions. The vascular system has developed and elements of phloem can be seen (**Figure 5.12B**). Striations are seen preferentially in the inner side of the cell wall.

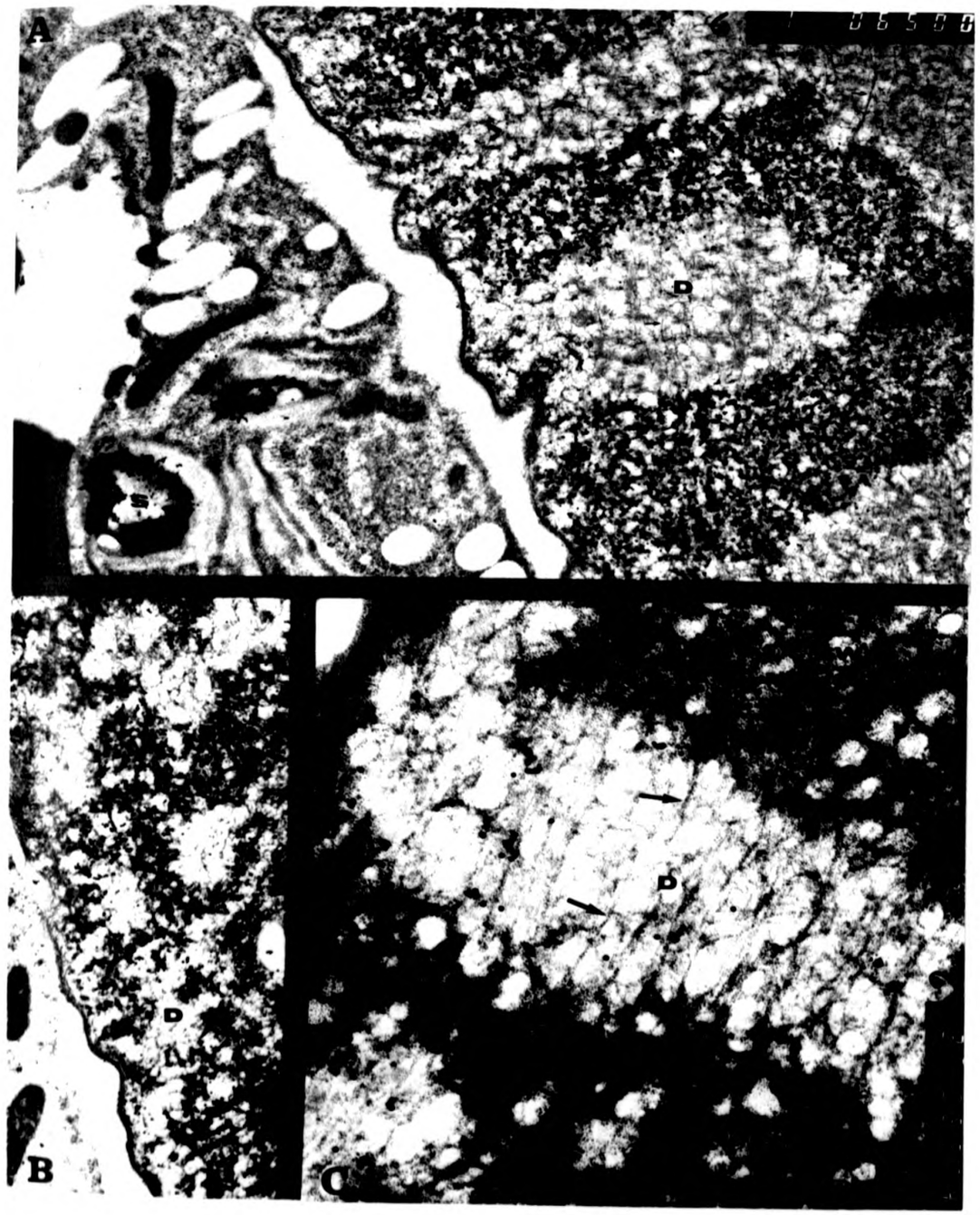
At the ultrastructural level it can be seen that the number of non-staining bodies near the plasmalemma has greatly reduced even in comparison with the first day (**Figure 5.8B**). The starch granules are still present and show strong staining with the enzyme gold complex (**Figure 5.13A**).

In the cell walls, staining was intense with the exception of the large digestion pockets. Inside these pockets a fibrous unreacting structure is left (**Figures 5.13B and 5.13C**). At higher magnification (**Figure 5.13C**) the smaller digestion pockets surrounding a bigger pocket can be seen in detail. There are still a few regions showing positive reaction with the enzyme gold complex inside a large pocket.

The division between the stained portion of the wall and the digestion pockets is sharp and clear. All pockets appeared to begin next to the plasmalemma and extended outwards (**Figure 5.13A**). At this stage, a very thin inner undigested wall which does not label with *exo*-(1→4)-ß-galactanase-gold complex could be distinguished.

Figure 5.12. Day 6. **Opposite.** Aspect of the storage mesophyll of *Lupinus angustifolius* cotyledons 6 days after imbibition. Bars represent 40µm. Staining with methylene blue. **A** - spongy mesophyll storage tissue; **B** - vascular bundle surrounded by storage tissue. *B-vascular bundle, D-digestion pockets, P-protein body, SW-storage wall.*

Figure 5.13. Day 6. **Next page.** Ultrastructural details of cell wall degradation in lupin mesophyll cotyledonary cells 6 days after imbibition. The arrows point to fibrous material (possibly clusters of rhamnogalacturonan core molecules) which appear after degradation of galactan *in vivo.* **A** - detail of cytoplasm-cell wall junction showing digestion pockets (*D*) and starch granule (*S*) x19,200. **B** - region where degradation is disorganised (x19,200) and **C** - detail of a digestion pocket and surroundings x47,800. *D-digestion pocket; SW-storage wall.* Staining as in **Figure 5.9.**


A
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Day 8. At this stage, essentially the same features were observed as compared to day 6, except that degradation of both protein bodies and cell walls are more pronounced and that elements of phloem are clearly formed, providing evidence for transport of digestion products to the growing plantlet (**Figures 5.14A and 5.14B**). The cell walls have large unstained regions with a fibrous appearance (**Figure 5.14C**), but undigested pockets containing (1→4)-ß-galactan remain.

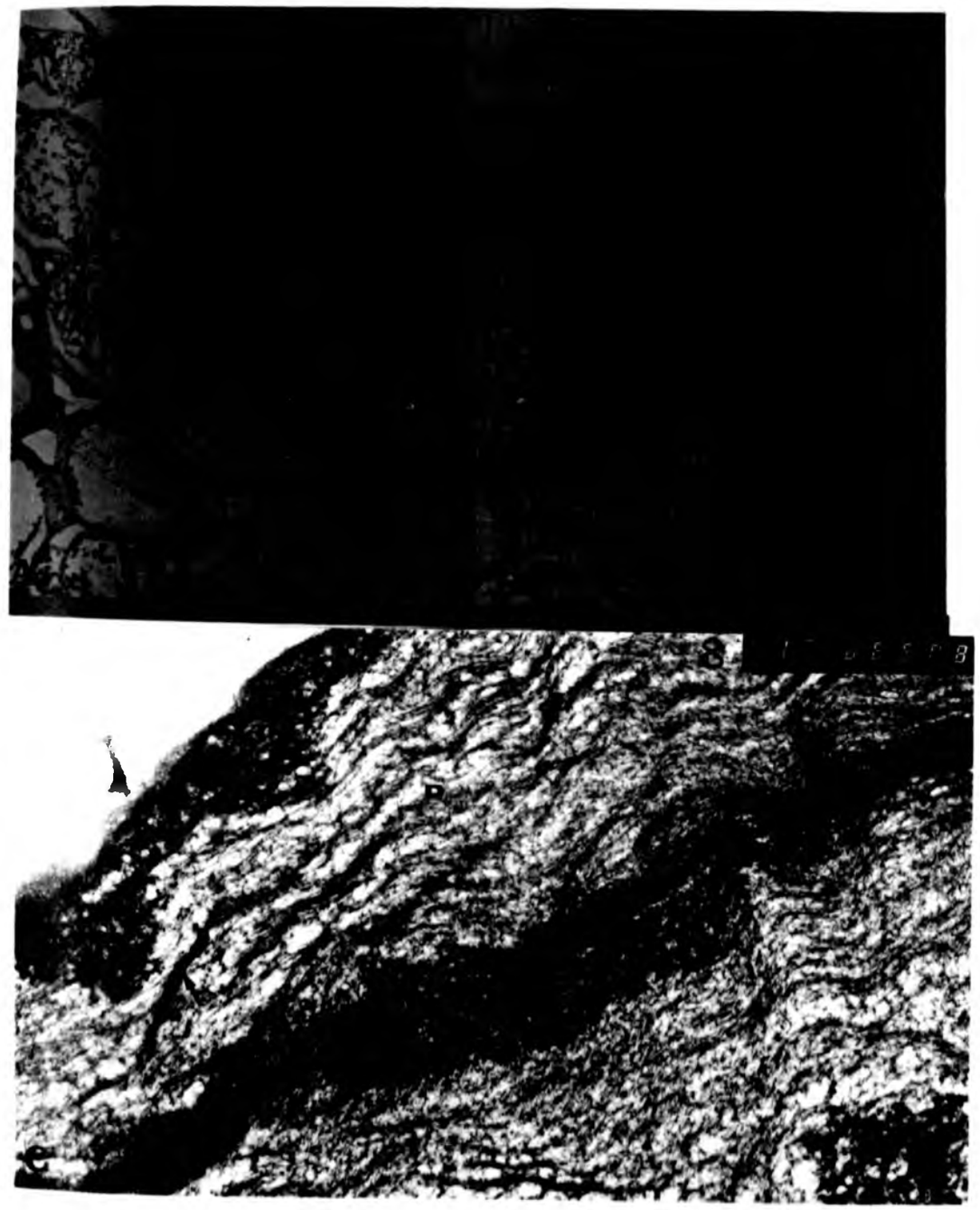
Day 10. At this stage, degradation of the protein bodies has finished completely and most of the cytoplasm is filled with a large vacuole. Mobilisation of the galactan from the cell walls is still not fully complete (**Figure 5.15A**). Regions staining positively with the exo-galactanase-gold complex are still present and can be seen both by light microscopy and at ultrastructural level (**Figure 5.15B**). Chloroplasts are present within the narrow layer of cytoplasm, and some of them contain the enzyme gold complex-labelled starch granules.

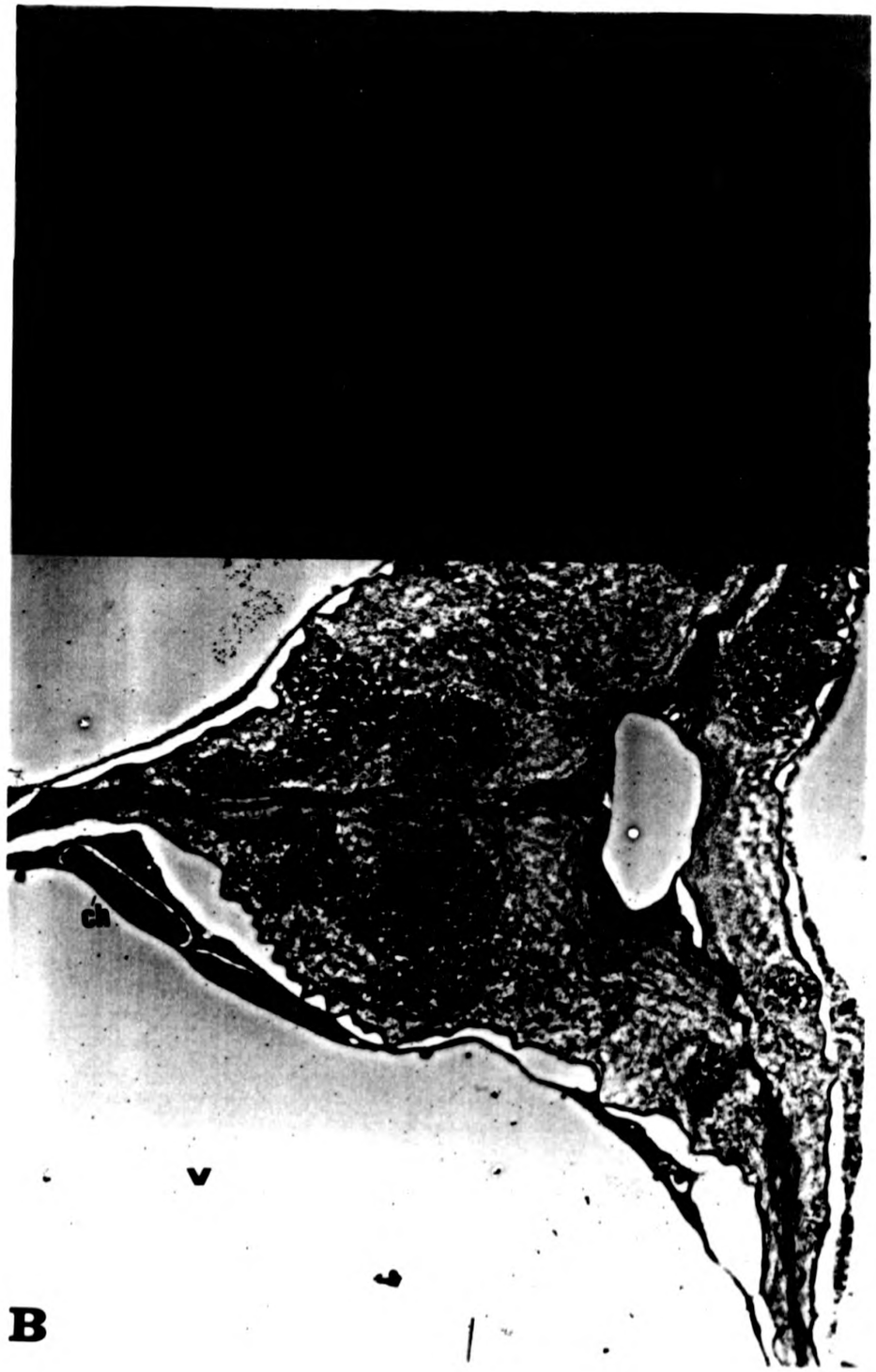
Day 12. Degradation of the cell walls is still not 100% complete. Small areas of staining with exo-galactanase-gold complex are still detected in the cell walls (**Figure 5.16B**). The first evidence of loss of cell adherence was obtained (**Figure 5.16A**).

Figure 5.14. Day 8. **Opposite. A** - lupin storage mesophyll tissue of *Lupinus angustifolius* cotyledons during degradation of storage cell wall and protein body. **B** - detail showing a vascular bundle surrounded by storage tissue. **C** - ultrastructure of the storage cell wall during mobilisation. Arrow points to fibrous material (possibly clusters of rhamnogalacturonan core molecules) which appear after degradation of galactan *in vivo*.x22,500. *B-vascular bundle D-digestion pocket; P-protein body, SW-storage wall, B-vascular bundle*. In **A** and **B** bars represent 40µm. Staining as in **Figure 5.9.**

Figure 5.15. Next page. Day 10. **A** - light microscopy of the storage mesophyll tissue of 10 days old lupin cotyledons. Bar represents 40µm **B** - ultrastructural view of a junction between three adjacent mesophyll cells from lupin cotyledons x6,700; *B-vascular bundle; ch-chloroplasts, D-digestion pocket, V-vacuole*. Staining as in **Figure 5.9.**

Figure 5.16. Day 12. **Following page. A** - light microscopy of the storage mesophyll tissue of 12 days old lupin cotyledons. Bar represents 40µm; **B** - ultrastructural view of a junction between three adjacent mesophyll cells from lupin cotyledons x18,000; *B-vascular bundle; D-digestion pocket, SW-storage wall; V-vacuole*. Staining as in **Figure 5.9.**





B


D
V
B

Day 14. In some places within the storage cell walls, pockets of galactan still remain (**Figure 5.17B**). In many regions the cells have started to lose their form and collapse, forming large air pockets (**Figure 5.17A**). Nevertheless, the tissue still possesses whole cells mainly surrounding the vascular bundles.

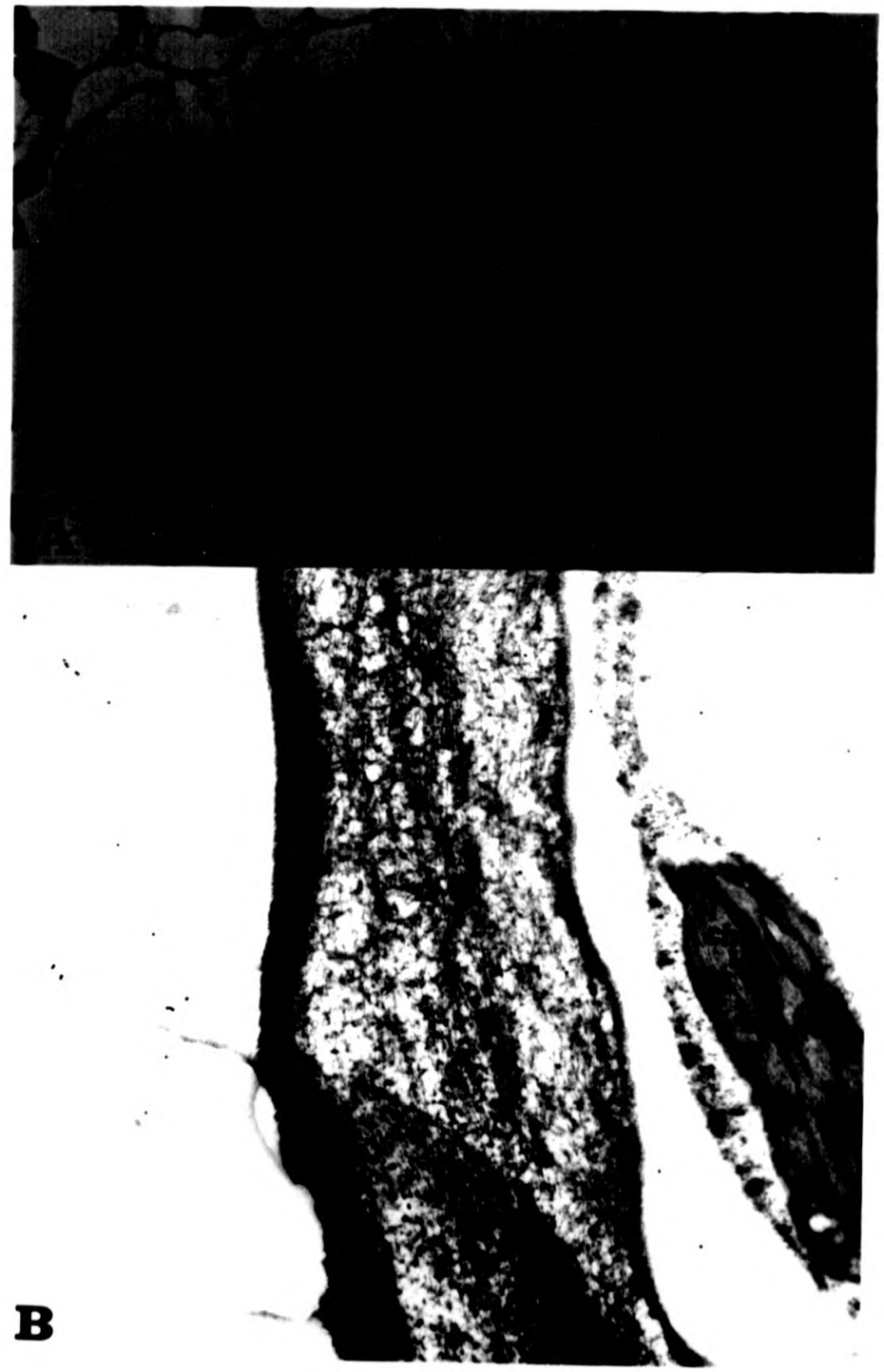
Day 16. At this stage large air pockets have been formed due to the collapse of the cell walls and the tissue as a whole is transformed in a leaf-like structure in which chloroplasts are well developed (**Figures 5.18A and 5.18B**). Most of the galactan degradation is complete and **Figure 5.18B** shows a typical picture of the cell wall after reserve mobilisation.

Day 18. At this stage, the general aspect of the tissue is the same as in day 16, but virtually no galactan is present in mesophyll cell walls, which are very thin (**Figure 5.19C**). Near the vascular bundles transfer cells can be observed (**Figure 5.19A**). These cells contain many mitochondria and are probably metabolically very active. Curiously, the wall extensions in these cells reacted positively with the *exo*-(1→4)-ß-galactanase gold complex (**Figure 5.19B**).

Figure 5.17. Day 14. **Opposite**. **A** - light microscopy of the storage mesophyll tissue of 14 days old lupin cotyledons. Bar represents 40µm; **B** - ultrastructural detail of a mesophyll cells from lupin cotyledons x22,500. *A-aerenchyma, B-vascular bundle, D-digestion pocket, S-starch*. Staining as in **Figure 5.9**.

Figure 5.18. Day 16. **Next page A** - light microscopy of the storage mesophyll cells of 16 days old lupin cotyledons. Bar represents 40µm; **B** - ultrastructural detail of the cell wall of a storage mesophyll cell from lupin cotyledons after reserve mobilisation x22,500. *A-aerenchyma, ch-chloroplast, D-cell wall after galactan mobilisation.* Staining as in **Figure 5.9**.

Figure 5.19. Day 18. **Following page. A** - Transfer cells in the mesophyll of 18 days old lupin cotyledons x11,000; **B** - detail of one of the cell wall-extensions in a transfer cell. Arrows in **A** and **B** point to positive labelling with exo-galactanase gold complex x56,000. **C** - ultrastructural detail of mesophyll cells from lupin cotyledons after germination; *ch-chloroplast; CW- cell wall, mt-mitochondria*. Staining as in **Figure 5.9**.



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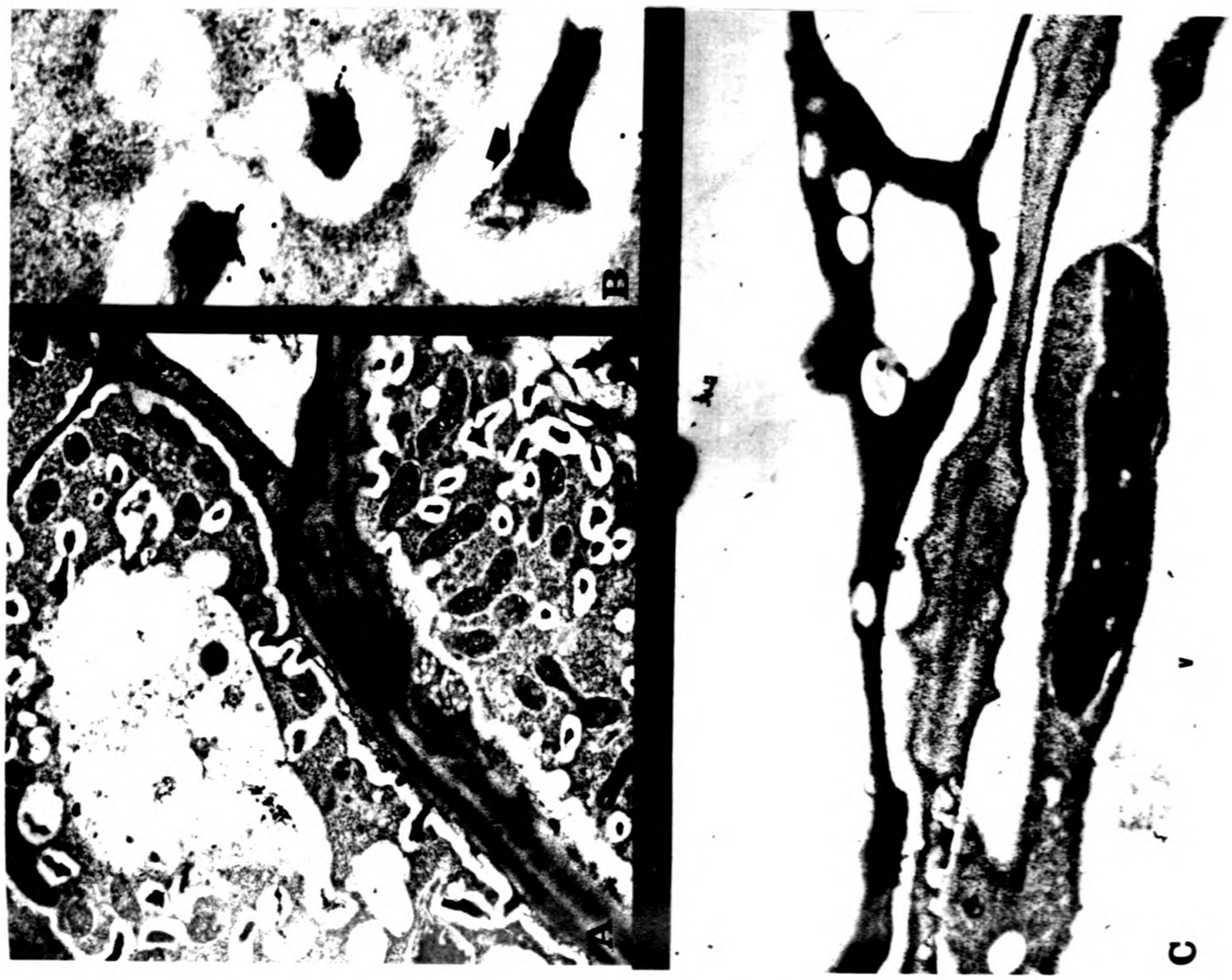
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Discussion

The role of exo-(1→4)-ß-galactanase during reserve mobilisation in lupin cotyledons: modification of the Cell Wall Ghosts

In this Chapter, we have performed analyses using CWGs from 16h and 14 days old cotyledons isolated by Hutcheon and Reid. Their work (unpublished) has been extended by incubating CWGs with purified exo-(1→4)-ß-galactanase, and comparing the modification (structural and compositional) with those accompanying CWSP mobilisation *in vivo*.

After exhaustive incubation of 16h CWGs with exo-(1→4)-ß-galactanase (144h incubation), the appearance of the CWGs in the scanning electron microscope was strikingly similar to the ones extracted from 14 days cotyledons i.e. after mobilisation *in vivo*. Further quantitative analysis showed that exo-(1→4)-ß-galactanase hydrolysed ca.63% of the galactose originally present in the 16h CWGs. CWSP mobilisation *in vivo* (until day 14) resulted in great galactose loss (ca.92%) in relation to the amount originally present. Thus, exo-(1→4)-ß-galactanase was capable of hydrolysing ca.68% of the total amount of galactose hydrolysable *in vivo*. Since galactose residues comprise 66% of the lupin CWGs, this result shows clearly that exo-(1→4)-ß-galactanase is the main enzyme responsible for the mobilisation of the large cell wall thickenings observed by Parker (1984b).

Considering the present knowledge on mobilisation of seed CWSP, the mobilisation of (1→4)-ß-galactan from lupin mesophyll storage cell walls appears to be unique in the sense that in no reserve mobilisation system described is the bulky reserve material mobilised by the action of a single exo-cleaving glycosidase.

Although they represent a very much smaller proportion of the cell wall carbohydrates, arabinose xylose and rhamnose were also mobilised from cell walls after germination. This suggests the presence of other enzymes such as α-arabinosidase, xylosidase and pectinases in lupin cotyledons after germination.

The use of exo-(1→4)-ß-galactanase gold-complex to localise galactan in the cell walls of lupin cotyledons.

In enzyme-gold cytochemistry some controls are necessary to certify that binding is specific to the substrate localised (Bendayan, 1989).

The specificity of the exo-(1→4)-ß-galactanase-gold complex towards (1→4)-ß-galactan in the cell wall was checked by pre-incubation with exo-(1→4)-ß-galactanase or incubation with (1→4)-ß-galactan during staining. Incubations with (1→4)-ß-galactan or exo-(1→4)-ß-galactanase inhibited binding to the cell wall almost completely.

The successful localisation of (1→4)-ß-galactan in lupin cotyledonary cell walls was achieved using heat-activated exo-(1→4)-ß-galactanase rather than active enzyme. The idea that heat-deactivation increases staining ability was confirmed, since gold complexed native enzyme did not stain cell wall galactan. On the other hand, when partially or even completely deactivated exo-(1→4)-ß-galactanase was gold-complexed and used to label (1→4)-ß-galactan from lupin cotyledon cell walls both gave positive labelling. It is possible that the heat-deactivated enzyme still maintains its binding capacity through its catalytic site, not being able, however, to hydrolyse the substrate.

Internal controls

Interestingly, protein bodies and starch granules also stained strongly with the gold-complex. The reasons for this are not known, but it is possible that exo-(1→4)-ß-galactanase is binding to terminal (1→4)-ß-linked galactoses attached to oligosaccharides of glycoproteins. The fact that previous hydrolysis with exo-galactanase reduced labelling of protein bodies points in this direction. Many reserve proteins are known to be glycoproteins and galactose has already been reported to be present in *Phaseolus vulgaris* and *Phaseolus aureus* reserve

globulins [see Bewley and Black (1978) and references therein for details].

As starch granules were specifically stained by the exo-(1→4)-ß-galactanase-gold complex controls were performed by incubating sections of germinated lupin cotyledons with α-amylase and exo-(1→4)-ß-galactanase **prior to** and starch and lupin galactan **during** labelling with the enzyme-gold complex. Only (1→4)-ß-galactan was able to prevent binding. The fact that neither starch nor α-amylase were effective in inhibiting labelling suggests that staining is not related to starch itself. As starch granules are not cut, but either taken out or left complete on the sections, it can be speculated that exo-(1→4)-ß-galactanase-gold complex is binding to galactolipids present at the surface. Indeed, ß-galactosidases associated with surface of the stroma of chloroplasts from mesophyll protoplasts of the primary leaf of wheat were demonstrated to be active on digalactosyl glycerol (Bhalla and Dalling, 1984). If a similar galactolipid is present in association with starch granules this could explain the labelling found. Nevertheless, the lack of inhibitory effect of active exo-(1→4)-ß-galactanase is puzzling and more experiments must be done in order to understand the nature of this binding.

Enzyme-gold cytochemistry during the time-course of galactan mobilisation

This Chapter describes for the first time the use of an specific glycanase [exo-(1→4)-ß-galactanase] complexed to colloidal gold as a cytochemical probe to follow the complete process of *in vivo* mobilisation of its natural substrate [(1→4)-ß-galactan].

In mesophyll cell walls, only the storage cell wall thickenings stained specifically with the enzyme-gold complex. Middle lamella and primary cell wall did not stain. The staining regions were arranged in clusters of particles. The same pattern has been observed by Vian et al. (1991) when studying storage cell wall xyloglucans.

The overall pattern of CWSP mobilisation observed was similar to the one described by Parker, (1984b). Degradation of the CWSP effectively started after germination (by day 6 - **Figures 5.12 and 5.13**) and continued up to day 18 (**Figure 5.19**).

Parker (1984b) observed that mobilisation of the storage cell walls is accompanied by the formation of digestion pockets. She named them "red maculae" because of the strong and distinctive red colour observed when cell walls were stained with the Periodic Acid-Schiff (PAS) reagent. This strong reaction with PAS was attributed to exposure of free hydroxyl groups. Our results showed a similar pattern of appearance of digestion pockets during

storage cell wall mobilisation. Using the gold probe, it was clearly demonstrated that the pockets were areas of (1→4)-ß-galactan mobilisation. It seems that small pockets of degradation first appear close to the inner cell wall and expand until they fuse into bigger pockets (**Figure 5.13C** - Chapter 5) which themselves fuse until all the cell wall (1→4)-ß-galactan has been mobilised.

After mobilisation of the galactan, fibrous structures are left intact which are not stainable with exo-galactanase gold complex (**Figure 5.13C**). The fibrous material is likely to correspond to the rhamnogalacturonan core molecules to which the galactan chains are attached. After mobilisation of the side chains, the core molecules seem to interact forming aggregates which are parallel to the primary cell wall and middle lamella (**Figure 5.14C**).

The results presented in this Chapter concerning galactan degradation are consistent with those presented in Chapter 3 describing the variations in exo-(1→4)-ß-galactanase activity and protein during and following germination. Enzyme activity correlated closely with the pattern of (1→4)-ß-galactan mobilisation using the gold-probe. A small amount of exo-(1→4)-ß-galactanase was already present at day 6 (as witnessed by **Figure 5.13**), although undetected by biochemical assays, and a considerable amount of enzyme is still present in the cotyledons after degradation of (1→4)-ß-galactan had been completed (enzyme protein was detected by western blotting on day 20 - data not shown).

Chapter 6

STORAGE CELL WALL MOBILISATION AND COTYLEDONARY EXPANSION: TWO SIDES OF THE SAME COIN?

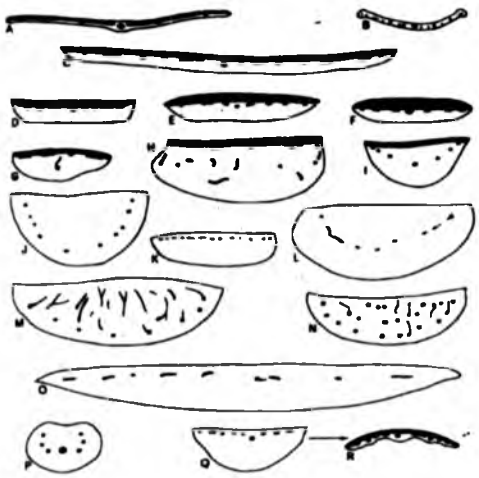
Introduction

The work described in this thesis culminated in the purification of a novel exo-(1→4)-ß-galactanase (Chapter 3), detection of other ß-galactosidases (Chapter 4) and the cytochemical localisation of (1→4)-ß-galactan in the cell walls of cotyledonary mesophyll cells (Chapter 5). These data can be used as evidence for the hypothesis that (1→4)-ß-galactan functions as a cell wall storage polysaccharide.

Nevertheless, the results presented in this thesis, as well as data available in the literature, also indicate that the lupin cotyledon is a dual-purpose organ. In early stages of seedling development it serves as a storage organ; during this period different reserves are catabolized and used as source of energy for the early growth of the plantlet. However, a parallel post-germinative developmental process is the transformation of the cotyledons into photosynthetic organs, with anatomical structures that resemble those of leaves. In this chapter, additional data are presented which suggest that (1→4)-ß-galactan functions primarily as a storage cell wall polysaccharide and that its degradation is necessary to allow cells of the mesophyll to expand.

Classification of lupins in the context of the ratio reserve function/expansion-photosynthesis function in cotyledons during seedling development

In legumes, cotyledon morphology and anatomy are very varied. Thus, in the course of evolution, several different structures resulted from the adaptation of plants to their new environments. According to Smith (1981) cotyledons in Leguminosae can be classified into 4 types (**Figure 6.1**). **Type 1** (e.g *Cassia obtusifolia*) is the most leaf-like structure, a distinction between mibrid and lamina being possible (**Figure 6.1A** and **B**). Cotyledons of this type undergo high degrees of expansion during germination and do not store high amounts of reserve substances. Their main function is to perform photosynthesis until the true leaves take over. **Type 2** (e.g. *Caesalpinia gilliesii*) differs from type 1 in that the adaxial surface of the cotyledon is flat, being therefore less leaf-like. Otherwise, type 2 cotyledons possess approximately the same functional features as type 1 (**Figure 6.1C-F**). **Type 3** (e.g. ***Lupinus angustifolius***) is intermediate, possessing characteristics which resemble those of storage organ i.e. having a fleshy mesophyll, with thick cell walls and enclosing large protein bodies in the cytoplasm (**Figure 6.1G-I**). As the seedling develops, type 3 cotyledons gradually transform into a leaf-like structure, but with little or no



***A**-Cassia obtusifolia; **B**-Petalostylis labicheoides; **C**-Caesalpinia gelliesii; **D**-Sophora japonica; **E**-Retama monosperma; **F**-Chorizema ilicifolia; **G**-Burtonia polyzyga; **H**-**Lupinus angustifolius**; **I**-Caragana microphylla; **J**-Erythrina crista-galli; **K**-Bossiaea aquifolium; **L**-Pisum jomardii; **M**-Pardia discolor; **N**-Lablab purpureus; **O**-Bauhinia purpurea; **P**-Scorpiurus muricatus; **Q**,**R**-Acacia dealbata*

Type 1 - A and B
Type 2 - C to F
Type 3 - G to I
Type 4 - J to R

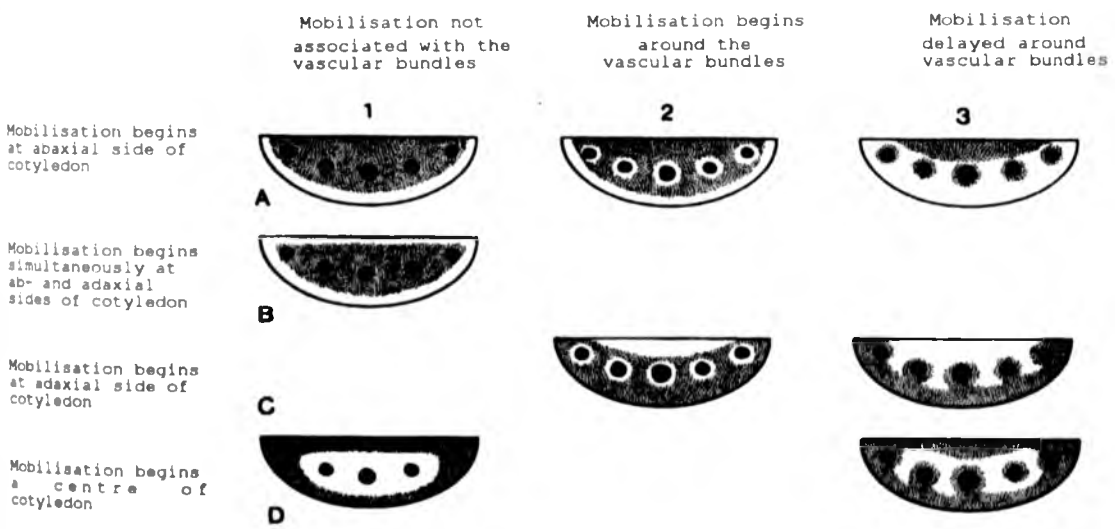


Figure 6.1. Classification of Legume cotyledons according to anatomical features (top) and to the pattern of mobilisation of reserves (bottom). Adapted from Smith (1981)

expansion. **Type 4** (e.g. *Bauhinia purpurea*) is similar in shape to type 3, but no palisade differentiation occurs. Although germination can be either epigeal or hypogeal, type 4 cotyledons act mainly as storage organs, contributing very little to photosynthesis (**Figure 6.1J-R**).

Smith (1981) also classified the patterns of mobilisation in legume cotyledons (**Figure 6.1**). In his classification, *Lupinus angustifolius* was included in the category in which mobilisation begins simultaneously at ab- and adaxial sides of the cotyledon (**Figure 6.1**, type B1) and has no correlation with the vascular bundles.

Lovell and Moore (1970) carried out a comparative study of cotyledons from species whose plantlet development ranges from epigeal to hypogeal. They compared loss in dry weight, area, number of cells, number of stomata, chlorophyll content and CO_2 fixation, in cotyledons of pea (*Pisum sativum*), runner bean (*Phaseolus multiflorus*), french bean (*Phaseolus vulgaris*), blue lupin (*Lupinus angustifolius*), white lupin (*Lupinus albus*), sunflower (*Helianthus annuus*), castor oil plant (*Ricinus communis*), gourd (*Cucurbita pepo*), cucumber (*Cucumis sativus*), clover (*Trifolium pratense*) and mustard (*Sinapis alba*).

Lupinus angustifolius cotyledons did not show a great level of expansion (twice the original area as compared with ca.20 times in mustard cotyledons). In *Lupinus angustifolius* this expansion was assumed to be a result of cell enlargement only, since the number of cells per

cotyledons was found to be constant during the life-span of the cotyledons. Expansion (cotyledonary area) occurred during the first 10 days. Between the 10th and the 20th day, area decreased slightly.

Lupinus angustifolius germination is epigeal, and as the cotyledons rise above the soil they immediately become green, producing chlorophyll. When calculated as chlorophyll per unit of area of the cotyledon, the maximum was reached at day 14 (doubling in relation to day 4) and then decreased. As expected, these last data were consistent with a maximum of incorporation of $^{14}CO_2$ (Lovell and Moore, 1970)

Based on these observations, Lovell and Moore calculated a 'scale of adaptation' ranging from the greatest to the least adaptation as a photosynthetic organ. The scale varied from 2.0 (**epigeal mainly photosynthetic**, e.g.mustard) to 10.2 (**hypogeal mainly reserve**, e.g.french bean) and *Lupinus angustifolius* ranked 7.4, which was considered by the authors as intermediate between the storage cotyledons and the leaf-like ones.

Cotyledonary dry weight decreased continuously after germination, approximately 90% of the dry weight was lost after 20 days (Lovell and Moore, 1970)

In *Lupinus albus* cotyledons, storage protein decreased linearly until approximately day 12 and reserve lipids started to be mobilised only after day 6, coinciding with the increase in chlorophyll (Parker, 1976; Bewley and Black, 1978; Crawshaw and Reid, 1984).

Together with cell wall polysaccharides, proteins are also one of the main reserve substance in lupins. According to Crawshaw and Reid (1984) each of them accounts for 22% of the dry weight of the resting cotyledons. Reserve proteins accumulate in protein bodies (Parker, 1984b) and the ones from *Lupinus angustifolius* have been isolated and characterised by Blagrove and Gillespie (1975). The proteins are mainly globulins and after electrophoresis on acetate strips they were resolved into three groups: α-conglutin, β-conglutin and γ-conglutin. Although the biosynthesis of storage proteins has been studied by Johnson et al. (1985), their degradation seems not to have been studied in detail. An endopeptidase capable of hydrolysing globulin and legumin-like reserve protein from pea *in vitro* was purified from *Lupinus albus* seeds (Duranti and Cerletti, 1989). However its role in storage protein degradation *in vivo* was practically not discussed.

On the other hand, in other works [e.g. Parker (1976, 1984b), Crawshaw and Reid (1984)] reserve proteins were shown to be mobilised after germination.

As already mentioned, Parker (1984b) and Crawshaw and Reid (1984) have described the mobilisation of cell wall storage polysaccharides after germination. This is discussed in Chapters 3 and 5.

Thus, *Lupinus angustifolius* cotyledons are reserve organs, capable of mobilising many different classes of reserve compounds (proteins, lipids, cell wall polysaccharides) in a coordinated fashion.

During and after reserve mobilisation, the cotyledons of *Lupinus angustifolius* undergo a metamorphosis, being transformed into a leaf-like structure, which persists as a photosynthetic organ. In this chapter, **galactan degradation** is taken as a central point and discussed in relation to **morphological** (cotyledon anatomy and morphology of the plantlet), **physiological** (changes in cotyledonary area), **biochemical** (ß-galactosidases activities and protein degradation) and **biophysical** (plasticity and elasticity of the spongy mesophyll tissue of the cotyledons) parameters that may be relevant to the "metamorphosis" of the cotyledons of *Lupinus angustifolius* from a reserve to a leaf-like organ.

Results

Modification of the pace of developmental changes in lupin cotyledons during seed storage

During the approximately 3 years of experiments, the storage of the *Lupinus angustifolius* seeds affected both seedling vigour and percentage of germination. Yet curiously, in later experiments, radicle protrusion of those *Lupinus angustifolius* seeds which germinated occurred 2-3 days after the initiation of the imbibition as opposed to 5-6 days when the seeds were younger. Another important difference noticed at biochemical level was that during the time course of reserve mobilisation the changes in activity of ß-galactosidases (using PNPG as substrate) and galactanase (using lupin galactan as substrate) were compressed to the extent that they could not be distinguished. In earlier time-course experiments (**Figure 3.1** - Chapter 3) there was a clear separation into two distinct peaks of activity. This phenomenon was not investigated systematically because it was beyond the scope of the thesis. Nonetheless it must be borne in mind when comparing rates of seedling development reported here and in Chapters 4 and 5.

Figure 6.3 presents results obtained from the same time course as described in Chapter 5. Nevertheless, the remaining data presented in this chapter were obtained

using identical, but older seeds, which had a faster pace of developmental changes (**Figure 6.2**). Although, the results cannot be compared in a day-by-day basis, they can be compared in terms of the events occurring during different developmental stages.

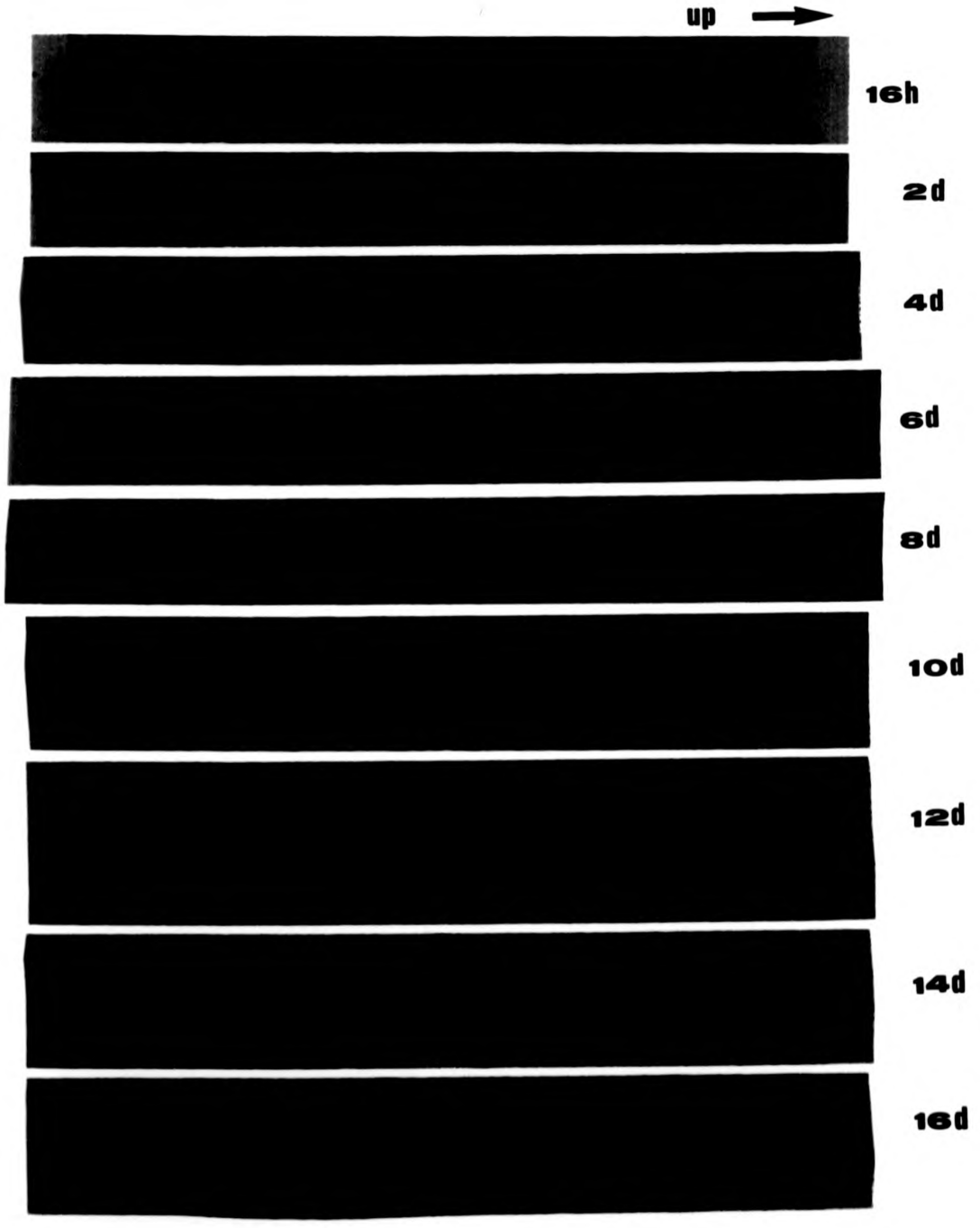
Developmental changes in *Lupinus angustifolius* cotyledons during and after germination.

Gross morphological changes. After germination, the rapid elongation of the hypocotyl raises the cotyledons above the soil and the adaxial (upper) surface of the cotyledons becomes green (**Figure 6.2**). From imbibition up to day 10 both ad- and abaxial surfaces of the cotyledons are quite smooth and the cotyledons reach, in this phase, their **maximal expansion**. On day 14 the first pair of leaves is completely open and the second pair is starting expansion. As the second pair of leaves expand, cotyledons shrivelled and fall approximately by day 25 (**Figure 6.2**).

It is important to stress the point that during the first 10 days, when the cotyledons reach maximum area and are gradually transformed into structures possessing anatomy similar to leaves, the cotyledonary reserves, including galactan, are broken down completely.

Figure 6.2. Opposite. Characteristic morphological features of the aerial portion of plantlets of *Lupinus angustifolius* after 2,4,6,10,14 and 25 days after the beginning of imbibition. Bar represents 3cm.

Figure 6.3. Next page. Anatomical changes in *Lupinus angustifolius* cotyledons during plantlet development. The sections shown are abaxial-adaxial cross sections cut from the centre of the cotyledon.Bars represent 0.2mm.


up
16h
2d
4d
6d
8d
10d
12d
14d
16d

Anatomy. Total imbibition of the cell walls could only be observed at day 2. It should be noted that the proportion (visual) between cell wall and intracellular contents changes dramatically from 16h imbibed cotyledons to 2 days old cotyledons (**Figure 6.3** - see also Chapter 5 - **Figures 5.9** and **5.10**).

It can be clearly seen in **Figure 6.3** (at day 2) that cell wall thickenings are present throughout the abaxial-adaxial cross sectional length of the cotyledons. Nevertheless, thicker cell walls were observed in the cells near the adaxial surface while thinner cell walls are present throughout the 6-7 layers of cells towards the abaxial (lower) surface. On the other hand, protein bodies do not seem to have any differential distribution, being present throughout the cotyledonary tissue in approximately the same proportion (see **Figure 6.3-days 2 and 4**).

In this experiment (the same time-course as in Chapter 5), the main period of protein and cell wall galactan degradation started at days 4 and 6 respectively. The protein bodies were quickly mobilised being barely detectable at day 4 whereas cell wall galactan was mobilised more slowly, until day 10 approximately.

Figure 6.3 permits a clear distinction between the reserve degradation phase and the photosynthetic phase. Although no measurement of expansion was performed in this experiment, the correlation between degradation of the cell walls and the formation of aerenchyma is noteworthy.

Direct estimation of galactan mobilisation using exo-galactanase

Direct estimation of the mobilisation of galactan was performed using alkali extraction of cotyledonary tissue followed by incubation of the extracted polymers with purified exo-galactanase. After exhaustive hydrolysis with exo-galactanase, the amount of galactose released was determined and used to estimate the amount of galactan present in the cotyledons at different times during germination and plantlet growth. In this experiment cotyledons were harvested 2,4,6,8,10,12,14 and 16 days after the beginning of imbibition. After their fresh weight and area were recorded they were divided in three batches: **one** was used for crude enzyme preparation, **two** for measurement of mechanical properties and **three** to determine galactan. Galactan mobilisation started very early (2 days after germination) and proceeded until day 10-12. By day 8, 90% of the galactan present in the cotyledons had been mobilised but a small amount still persisted until day 14 (**Figure 6.4**).

ß-galactosidase activity, protein and galactan mobilisation. In this experiment the activity of exo-galactanase showed unusually high variability. Therefore it was deemed necessary to use ß-galactosidases activity as an approximate estimate of exo-galactanase activity. The

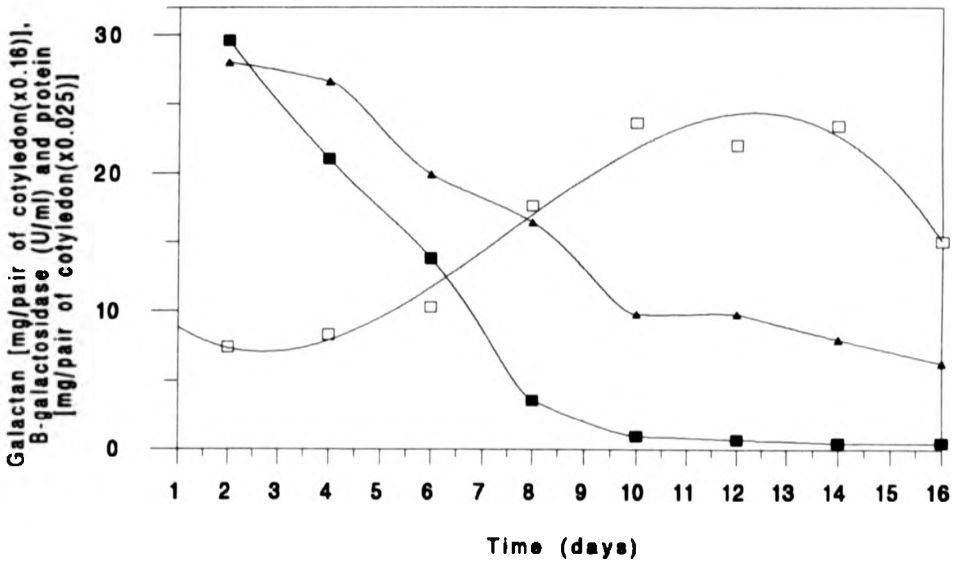


Figure 6.4. Reserve mobilisation (galactan and proteins), and ß-galactosidase in cotyledons of *Lupinus angustifolius* during plantlet development.■ galactan content; □ ß-galactosidase activity; ▲ protein content

presence of exo-galactanase in the extracts was checked using western immunoblotting, and it was possible to show that the changes in intensity of the 60kDa band were consistent with the variation in ß-galactosidase activities as measured by activity on PNPGal (data not shown).

Beta-galactosidase activity rose from day 2 (radicle protrusion) up to day 10, when, almost no (1→4)-ß-galactan could be detected in the cotyledons. A plateau of activity was maintained until day 16 when activity started to decrease (**Figure 6.4**).

Although the aim was not specifically to extract reserve proteins, the method used for extraction of the enzymes (i.e. 0.2M phosphate buffer 0.9% NaCl) was qualitatively appropriate, since the type of storage protein present in lupins (globulins) is easily extracted with NaCl solutions. Therefore, the protein content of each extract in the time course was estimated by measuring protein in the crude enzymatic extracts using the Coomassie Blue G250 (Sedmak and Grossberg, 1977).

Although detectable protein degradation started one day later than the degradation of galactan, their rates of degradation were very similar (**Figure 6.4**). It should be noted that the two reserves can not be compared quantitatively, but only estimated, since no control experiments were carried out to ascertain whether or not extraction of protein or galactan was exhaustive.

Cotyledonary area and galactan degradation. The area of the cotyledons increased approximately in step with galactan degradation, reaching its maximum at day 8, when practically no galactan was left in the cell walls (**Figure 6.5A**). There was a decrease from day 8 to day 10 which is probably due to the formation of aerenchyma. From day 10 to 16 the area was constant. It is noteworthy that cotyledonary expansion, as measured by the increase in cotyledonary area, showed a remarkably close negative correlation (r^2=**-0.99**) with galactan degradation (**Figure 6.5B**). This suggested that galactan might be a factor limiting the expansion.

Changes in the mechanical properties of spongy mesophyll of lupin cotyledons. For measurement of the mechanical properties of the spongy mesophyll of lupin cotyledons, cuboids (5x2x2mm) were dissected from the central part of cotyledons at 2,4,6,8,10,12,14 and 16 days after the beginning of imbibition and subjected to mechanical testing using a "creep-testing" apparatus with the aim of determining the reversible (elastic) and irreversible (plastic) components of wall deformation under constant load. Each cuboid was prepared from the central mesophyll storage tissue of the cotyledons. The cuboids were subjected to two cycles load-unload (5g) of 15 minutes in 20mM Tris-HCl, pH 7.4. Relative measurements of elasticity and plasticity were recorded for a minimum of 5 replicates

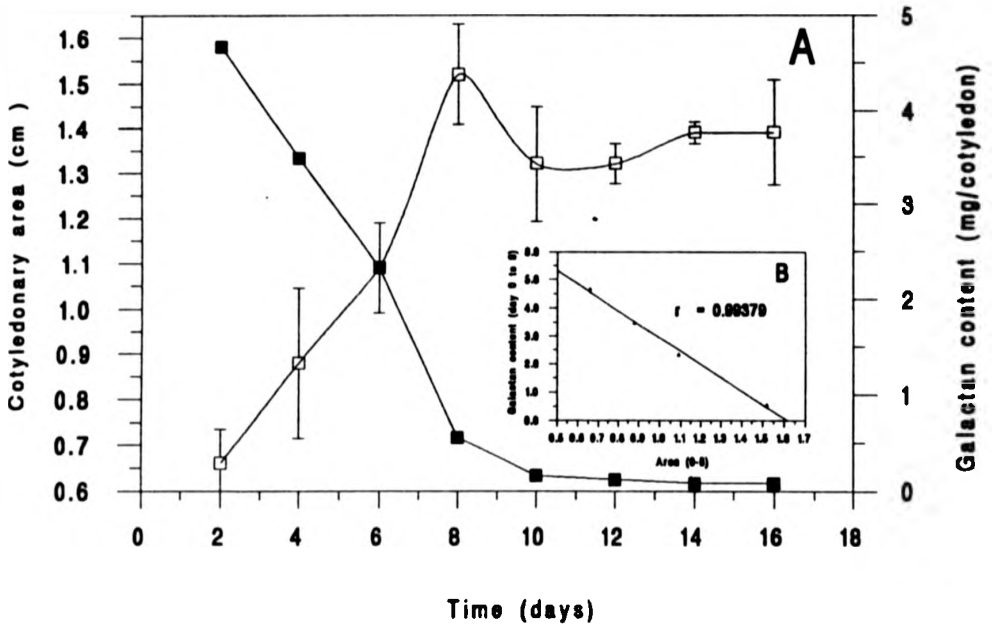


Figure 6.5. A - Changes in expansion of the cotyledons (area) of *Lupinus angustifolius* in contrast with galactan degradation during development. **B** - Linear correlation between galactan mobilisation and expansion during the first 8 days of plantlet development. ■ galactan content; □ cotyledonary area; ▲ galactan/area correlation.

from different plantlets. The basis of the experiment as well as the apparatus used are shown in **Figure 2.1** - Chapter 2).

The initial rates of increase in plasticity and elasticity were very similar to each other until approximately day 12 (**Figure 6.6**) and the total extension increased with time (not shown). Furthermore, the increase in the elastic and plastic components were proportional to the decrease in galactan contents in the cotyledons. During the first 8 days a high correlation was found between galactan and plasticity (r^2=**-0.99**) as well as with elasticity (r^2=**-0.93**).

Although elasticity did not change significantly differently from plasticity up to day 12, the latter continued to increase up to day 14 while the former remained constant. The increase in plasticity from day 12 until day 14 could be related to the formation of the aerenchyma. The formation of large air pockets after reserve mobilisation probably introduces more complex variables into the system, since the tissue becomes heterogeneous in terms of cellular adherence (see **Figure 6.3**, day 12).

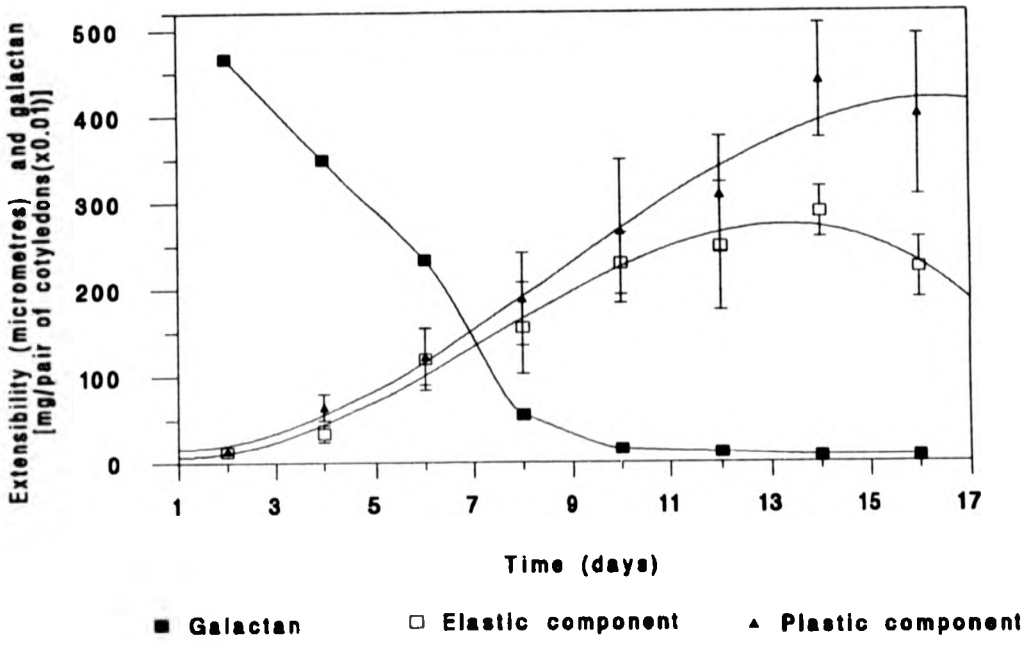


Figure 6.6. Changes in mechanical properties (plasticity and elasticity) of the storage mesophyll tissue of cotyledons of *Lupinus angustifolius* following (1→4)-ß-galactan mobilisation *in vivo*.

Discussion

Taking into consideration the results presented by Parker (1984b) and Crawshaw and Reid (1984) and data presented in this thesis, it can be suggested that the cotyledons of *Lupinus angustifolius* show two different developmental phases as far as function is concerned. The first phase corresponds to the degradation of reserves and the second to photosynthesis. Our results suggest that while degradation is occurring (first phase), the cotyledons are "preparing" for a second phase i.e. to work as a leaf.

The two functional phases of lupin cotyledons development

The **first phase** (from initiation of imbibition until day 10 approximately) corresponds to the grand period of reserve mobilisation. As oligosaccharides and lipids (Crawshaw and Reid, 1984), protein and cell wall polysaccharides are degraded concomitantly with a period of rapid elongation of the hypocotyls, complete expansion of the cotyledons as well as expansion of the first pair of leaves take place.

According to Crawshaw and Reid (1984) as well as data presented in Chapter 5, it is also during this period (the first phase) that maximum amounts of starch accumulate in the cotyledons.

During the first phase of plantlet development all the events occurring at morphological, physiological and biochemical levels are directed mainly towards the mobilisation of reserves. As a loss in dry weight of approximately 90% is observed in this phase (Lovell and Moore, 1970, Crawshaw and Reid, 1984), it is reasonable to assume that by the end of this first phase all or most of the reserve materials have been transferred to the growing plantlet, where it is probably used for respiration as well as elongation of hypocotyl and development of the first leaves.

The **second phase** starts at approximately day 10 and goes on until abscission, which occurs approximately at day 25. The second phase starts with shrivelling of the adaxial surface of the cotyledons, due to the formation of aerenchyma in the spongy mesophyll tissue. After ca. 15 days, a dark green palisade could be observed and the vascular bundles were surrounded by photosynthetic tissue. Thus, by day 15, the anatomy of the cotyledons of *Lupinus angustifolius* is remarkably similar to the ones found in leaves i.e. aerenchyma, stomatas, photosynthetic palisade are well developed (Mauseth, 1988). Under the conditions of our experiments, shrivelling was maximal by day 14-16, coinciding with the stabilisation of the mechanical properties of the mesophyll.

Trying to Look at both sides of the same coin simultaneously

The strong correlation found between cotyledonary area and galactan degradation suggests that mobilisation of the (1→4)-ß-galactan from the cell walls might release all or a large proportion of the constraints to expansion imposed on the cotyledonary tissue. As the cell walls are usually associated with the form of plant cells and tissues, it is reasonable to think that, amongst the parameters cited above, the cell walls would be the most likely to influence cotyledonary expansion.

According to Cosgrove (1993a) the polymeric nature of the cell wall confers on it certain viscoelastic properties. He defines viscoelasticity as referring to materials which exhibit viscous and retarded deformation in response to stress.

Cell expansion and growth are thought to be accompanied by changes in the viscoelasticity of the cell walls. Cell expansion seems to be controlled by the balance between the loosening of the cell walls and turgor pressure (Kutschera, 1991, Cosgrove, 1993a, 1993b). This means that for a cell to expand, turgor has to increase through water uptake, consequently augmenting the hydrostatic pressure that is exerted in the cell wall. If the cell wall does not undergo cleavage of its load-bearing cross-links (i.e. polysaccharide hydrolysis and/or breakage

of hydrogen bonds for example), the hydrostatic pressure equilibrates with the "constraint" exerted by the cell wall structure.

On the other hand, if hydrolysis of chemical bonds occur in the cell wall, the turgor pressure will overcome the cell wall "constraint" and expansion will occur. In this case, the cell wall becomes more plastic i.e. it suffers irreversible extension.

Thus, growth and expansion would normally be associated with changes in the viscoelastic properties of the cell wall. Nevertheless, one has to bear in mind that most of the experiments linking cell wall mechanical properties and expansion or growth are correlative. Cosgrove (1993a) highlights the point that several studies were performed in which no correlation between viscoelasticity and growth behaviour or wall relaxation was found. He concludes that *...growth depends on wall relaxation processes that may or may not be viscoelastic in nature*.

In the case of the expansion of the spongy mesophyll of the cotyledons of *Lupinus angustifolius* our results showed that expansion is accompanied by changes in tissue viscoelasticity. Plasticity and elasticity of the mesophyll tissue increased linearly and with the same rate during the mobilisation of the storage cell walls. Also, the breakdown of galactan in the cotyledons varied *pari passu* with the increase in cotyledonary area.

Thus, although correlative, the data presented in this chapter strongly suggest that the mobilisation of (1→4)-ß-galactan from the storage cell walls of the mesophyll of *Lupinus angustifolius* cotyledons might play a key role in cotyledonary expansion.

The (1→4)-ß-galactan can therefore be seen as a dual purpose molecule, since besides serving as a storage polysaccharide, it is likely to act at the same time as a major constraint to cotyledonary expansion during the first phase of cotyledonary life (0 to 10-15 days).

Thus, the classification of *Lupinus angustifolius* as intermediately adapted between hypogeal (strictly storage and epigeal (strictly photosynthetic) cotyledons proposed by Lovell and Moore (1970) might be explained, at least partially, by the presence of large deposits of (1→4)-ß-linked galactan as storage cell walls.

According to Lovell and Moore (1970) and Crawshaw and Reid (1984) the content of chlorophyll starts to decrease slowly by day 12-14. This means that the actual life span of the cotyledons as photosynthetic organs is remarkably short. By this time, at least two pair of leaves have already been developed and they are likely to account for the necessary CO_2 fixation.

The data gathered and discussed in this chapter suggest that the process of **reserve degradation** in *Lupinus angustifolius* **is at the same time** the process of **metamorphosis** that leads the cotyledons to achieve maximal

expansion. Nevertheless, it is important to state that the data presented in this Chapter are correlative and that no cause and effect relationship between cell wall degradation and expansion was proved.

Chapter 7

GENERAL DISCUSSION

Towards a model for the storage mesophyll cell wall of *Lupinus angustifolius*

Cotyledons of *Lupinus angustifolius* produce a family of immunologically-related ß-galactosidases which increase after germination and decrease after the grand period of reserve mobilisation. The ß-galactosidases are synthesised *de novo*, since no cross-reacting peptides were present in extracts from quiescent lupin seeds. This lupin ß-galactosidase family includes the novel exo-(1→4)-ß-galactanase the purification of which is described in Chapter 3, and the two distinct ß-galactosidases the partial characterisation of which is described in Chapter 4.

The exo-galactanase is clearly a key enzyme in the mobilisation of cell wall storage polysaccharides from *Lupinus angustifolius* cotyledons after germination. It has a very high specificity for and rate of action on (1→4)-ß-galactan, the dominant structural component of the storage cell walls. The levels of galactanase activity and galactanase protein vary in step with CWSP mobilisation. When the pure galactanase is incubated *in vitro* with soluble lupinseed "galactan" it catalyses the release of 82% of the galactose residues in the polysaccharide preparation as free galactose. When similarly incubated with isolated cell walls (Cell Wall Ghosts) from the

storage mesophyll cells of cotyledons of non-germinated lupin seeds, it catalyses the release of 63% of the galactose residues present in the cell walls as free galactose. The enzyme-treated cell wall ghosts are then visually indistinguishable (under scanning electron microscopy) from storage parenchyma cell wall ghosts prepared from lupin cotyledons after the completion of CWSP mobilisation. When coupled to colloidal gold, the exo-galactanase proved to be an effective probe to label (1→4)-ß-galactan in lupin cotyledonary cell walls, and permitted the pattern of post-germinative galactan mobilisation to be followed using the transmission electron microscope (Chapter 5).

Clearly, other hydrolytic enzymes besides the exo-galactanase must be involved in CWSP mobilisation in lupin cotyledonary cell walls. To effect the hydrolysis of the arabinose-containing polysaccharides and the remaining galactose residues, other enzymes must act in concert with the galactanase. The galactose residues which are not hydrolysed on treatment of cell wall ghosts with the galactanase *in vitro* may not be (1→4)-ß-linked, or may be (1→4)-ß-linked but inaccessible to the enzyme either because of lateral branching (Al Kaisey and Wilkie, 1992) or physical entanglements. The first two of these three possibilities are represented schematically in **Figure 7.1.**

In **Figure 7.1** the galactan molecules are depicted as being covalently attached via their reducing termini to a

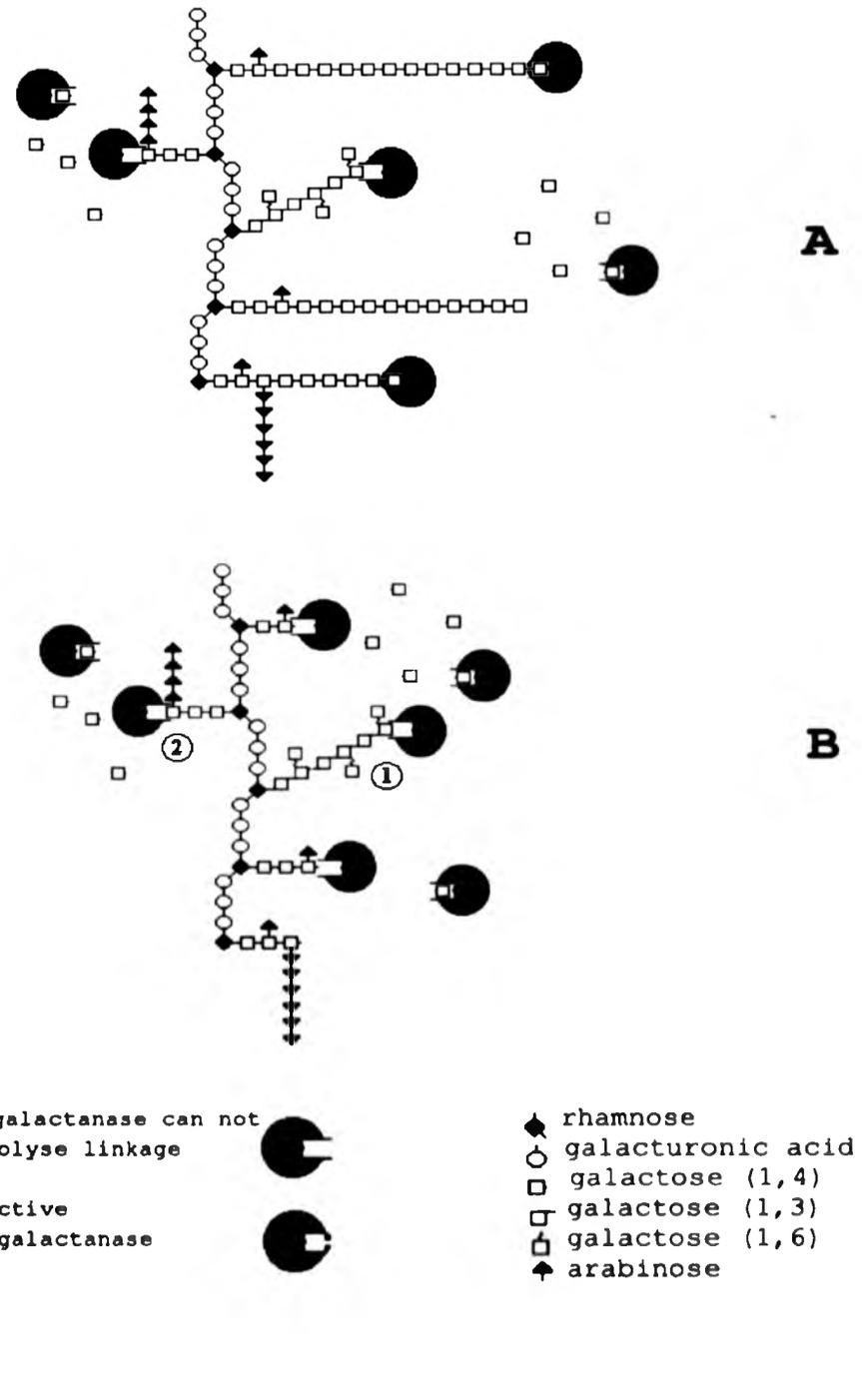


Figure 7.1. Mechanism of degradation of isolated lupin galactan by pure exo-(1→4)-ß-galactanase. **A** - during degradation the enzyme hydrolyses one galactose at a time, from the non-reducing end of the (1→4)-ß-linked galactan chains. **B** - degradation stops possibly when (**1**) only galactose linked through other glycosidic linkages (**non** 1→4-ß) are left and (**2**) when degradation reaches the arabinose or arabinan branches.

pectic rhamnogalacturonan backbone, as has been hypothesised in the past (Crawshaw and Reid, 1984, Brillouet and Riochet, 1984, Al Kaisey and Wilkie, 1992). The data reported in this thesis do not contradict this model. Indeed it is confirmed that after CWSP mobilisation *in vivo* or treatment of cell walls with exo-galactanase *in vitro*, the residual wall material is enriched in galacturonic acid and rhamnose residues. Furthermore, after galactan mobilisation, the cell walls contain a residual fibrous material which does not stain with the galactanase-gold probe. If lupin galactan is present as pectic side-chains, then it is reasonable to view it in the same way as the storage xyloglucans present in seeds of other species, namely as an amplification and adaptation to a storage role of a normal polysaccharide component of the primary cell wall.

Structural studies (Al Kaisey and Wilkie, 1992) indicate that the cotyledonary storage cell wall of *Lupinus angustifolius* do contain small amounts of the "normal" cell wall constituents cellulose and xyloglucan in addition to pectin and overwhelming quantities of the "storage" constituents. The ultrastructural work reported in Chapter 5 of this thesis demonstrates clearly that the (1→4)-ß-galactan "storage" component of the cell wall is restricted to the storage thickenings and is not present in the primary cell wall and middle lamella. On this basis, a tentative and purely schematic model for the storage

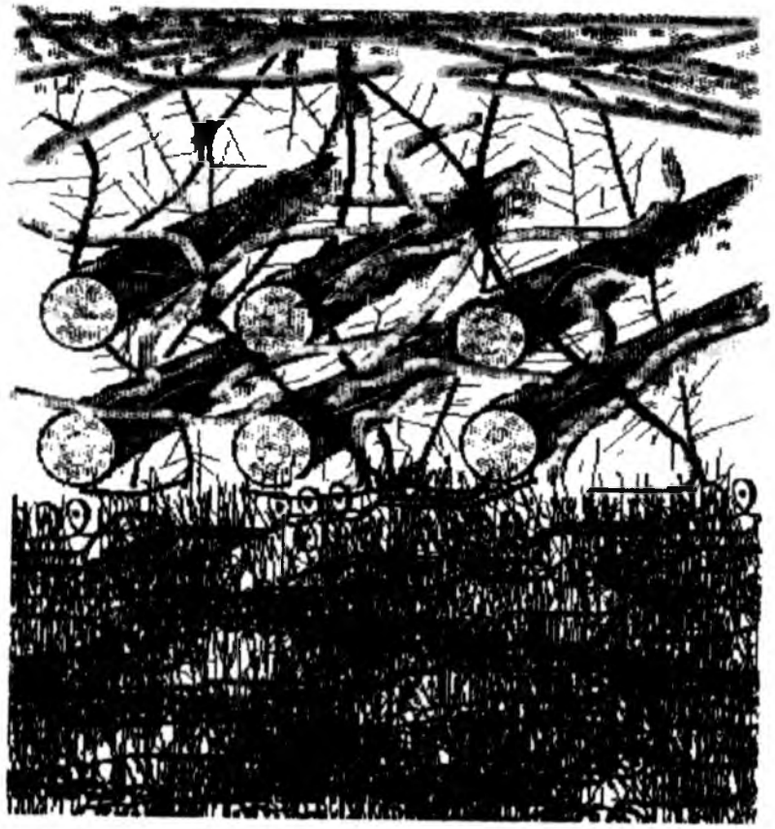
mesophyll cell wall of *Lupinus angustifolius* cotyledons is presented in **Figure 7.2.**

Cell wall proteins are known to be present in lupinseed cell walls (5.6% in *Lupinus albus* according to Carré et al., 1985), but they were not taken into consideration in this tentative model. Arabinans and galactans were considered to be side chains of a core molecule of rhamnogalacturonan and the hypothesis of independent networks was accepted [i.e. there is a primary wall which was assumed to be in the form of the coextensive network architectural model as proposed by McCann and Roberts (1991) and Carpita and Gibeaut (1993)]. It is therefore proposed that a cellulose-xyloglucan network coexists with a pectin network in a "normal" way in the primary cell wall and that a very narrow transition zone separates it from the secondary deposition of a possibly pure pectinic network, containing rhamnogalacturonan heavily branched with (1→4)-ß-galactan chains.

Galactan function in lupin: A storage molecule or a moderator of cotyledonary expansion?

The exo-(1→4)-ß-galactanase seems to be the most important enzyme as far as storage cell wall mobilisation in lupin cotyledons is concerned. However, post-germinative changes in the cotyledonary cell wall as a whole go beyond

Figure 7.2. Model of the storage and primary cell walls and middle lamella from mesophyll cells of *Lupinus angustifolius* cotyledons. The primary cell wall was drawn according to the ideas proposed by McCann and Roberts (1991) and Carpita and Gibeaut (1993). Only part of the alveolate structure of the storage cell wall is shown. The storage cell wall is represented as an extension of the pectinic network in which there is heavy branching with (1→4)-ß-galactan chains. The core rhamnogalacturonan molecule (red) was represented as alternating calcium interaction (green) regions and branched regions (black).



Pectin network: red=rhamnogalacturonan; black=galactan and arabinan chains

Middle lamella.Black and green shades represent calcium interaction

Cellulose microfibril

Xyloglucan molecules interacting with cellulose Yellow=main chains; blue=branching points

Storage Cell Wall. Red=rhamnogalacturonan; black=galactan chains; green=calcium interacion

galactan mobilisation. Galactose residues, which may be present in molecules other than (1→4)-ß-galactan and arabinose-containing polymers are also mobilised after germination. Besides, the collapse of the cell walls in certain regions of the cotyledons with the simultaneous formation of the air pockets after galactan mobilisation implies the degradation of components of the primary cell wall *sensu strictu*. This suggests that pectinases and perhaps cellulases may be active during the period of aerenchyma formation. Whether or not the presence of heavy branching with galactans and arabinans is a major factor in preventing the action of cellulases and pectinases during early stages of germination, it is reasonable to think that these neutral branching points could be controlling, to a certain extent, expansion and morphogenesis of the cotyledons.

Although the galactan in lupin cotyledons is a reserve polysaccharide it is not irrelevant to the process of cotyledonary expansion which follows reserve mobilisation. In Chapter 6, high negative correlation coefficients were found between galactan degradation and increase in cotyledonary area and also with the changes in mechanical properties (plasticity and elasticity) of the mesophyll (galactan storing) cotyledonary tissue. These results are strong indications that galactan is involved in cotyledonary expansion, acting essentially as a constraint. It may be appropriate to characterise galactan as a dual purpose molecule, being not only a reserve substance, but

also a molecular restraint to expanding cotyledons development.

Galactan and other seed cell wall storage polysaccharides: evolutionary trends and ecological meaning

As suggested by Lovell and Moore in 1970, there seems to be a whole range of adaptations concerning the function of the cotyledons as a reserve and/or photosynthetic organ. *Lupinus angustifolius* was classified by these authors as an intermediate stage of adaptation between seeds with hypogeal strictly reserve cotyledons and seeds with leaf-like epigeal strictly photosynthetic cotyledons.

According to Polhill et al. (1981), there is an evolutionary trend in the family Leguminosae towards accumulation of reserves in the cotyledons. In general, the seeds from species belonging to the subfamily Caesalpinioideae, which is considered to be the most primitive one, tend to accumulate large amounts of galactomannan in their endosperm cell walls. Within this subfamily, it is also possible to observe that the most advanced tribes (Amherstieae and Detariae) tend not to have endosperms (they are consumed during maturation),and to accumulate large deposits of xyloglucan in their cotyledonary cell walls (*Copaifera*, *Hymenaea*). Although

germination in these two genera is epigeal, there is nevertheless little expansion of the cotyledons. The subfamily Caesalpinioideae is thought to have originated in the warm and moist tropical regions, but after the Cretaceous there was vast drying and elevation of the lands so that cooler, drier and even deserts evolved, restricting the members of this subfamily to the tropics (Cowan, 1981). The other two subfamilies of the Leguminosae, the Mimosoideae and Faboideae are thought to have developed from the Caesalpinioideae. Species in the Mimosoideae have remained strictly tropical but the more ubiquitous Papilionoideae (or Faboideae) contains species well adapted to tropical regions and species equally well adapted to temperate regions and even the edges of the desert (Polhill et al., 1981).

The subfamily Papilionoideae (Faboideae) is thought to have originated on the planalto of Brazil, the Mexican region, eastern Africa, Madagascar and Sino-Himalayan region. The Mediterranean, Cape and Australia have some species which appear to have radiated from a few basic stocks (Polhill, et al., 1981). In this subfamily it is possible to find species, or entire tribes, with high amounts of galactomannan stored in the cell walls of very thick endosperms (several species in Crotalarieae, Sesbanieae for example). Nevertheless, closely related species store starch in granules which are located in the cytoplasm of the cotyledonary cells.

Thus, the storage function in legume seeds seems to have been transferred from the endosperm (galactomannan) to the cotyledons (xyloglucan and arabinogalactan), but maintaining the storage substance in the cell walls. Indeed, these transference of functions have already been proposed to occur in plants.

Stebbins (1974) suggested that cycles of transference of function have probably occurred during evolution. He proposed a succession of transfers of the function of protection of the embryo sac. This function is believed to have been transferred successively from the megasporangial wall to the ovule integument and then to the cupule wall. With the origin of the Angiosperms, protection of the ovules became the function of the carpel of ovary wall. Each structure cited above, which possibly had a protective function for a certain period, finally adapted to other functions i.e. the megasporangial wall became the nucellus and the cupule wall became the ovular integument.

If a similar cycle of transfers has occurred in the case of the seed CWSP, it would be reasonable to think that the function of carbohydrate reserve for plantlet growth in the Leguminosae was originally performed by the endosperm (galactomannan in Caesalpinioideae, primitive Mimosoideae and some tribes of Papilionoideae). This function may then have been transferred to the cotyledons in advanced Caesalpinioideae (xyloglucan) and some tribes of Papilionoideae (starch in some species of Phaseoleae and galactan in *Lupinus*).

Interestingly, transfers have occurred not only from one tissue to another, but also from one type of cell wall polysaccharide molecule to another i.e. the reserve function has been transferred from the galactomannans to the xyloglucans and galactans.

The concept of cycles of transference of function could explain why some cell wall "reserve" polysaccharides are considered to be multifunctional molecules (Reid and Bewley, 1979). It may be that the cell wall polysaccharide is at an intermediate stage during a cycle of transference, so that it has in fact intermediate functions. Alternatively, secondary functions, such as the imbibing and water-buffering function of galactomannans during germination (Reid and Bewley, 1979) might be purely accidental, simply a consequence of the fact that galactomannans are there, and that they are viscous substances with unique hydrodynamic properties. As proposed by Gould and Lewontin (1979): *one must not confuse the fact that a structure is used in some way....with the primary evolutionary reason for its existence and conformation.*

In fact, the lupins seem to exemplify part of a cycle of transfer of function. According to Parker (1984b) in lupins possessing cotyledons with unthickened walls (e.g. *Lupinus mutabilis* and *Lupinus mecranthus*) these organs show expansion of up to 15 times, whereas in lupins containing thick cell wall deposits (e.g. *Lupinus angustifolius*) the cotyledons expand to twice their initial area. Our results

indicate that the presence of galactan has a role in constraining expansion in *Lupinus angustifolius* cotyledons during development. It is perhaps naive to assume that as the biological function for which galactan has evolved.

There seems to exist little place for contradiction in relation to the function of the cell wall storage galactan in lupin cotyledons. It could be argued, based on evidence presented in this thesis, that its real function is as a reserve compound, being only fortuitously related to cotyledonary expansion. Obviously, expansion can only occur after mobilisation of the wall reserves, as shown by the correlation between galactan degradation and increase in cotyledonary area and mechanical properties of the storage tissue. However, expansion is much higher in the cotyledons of lupin species with little or no galactan deposits on their cotyledonary cell wall. Consequently, if galactan secondary thickenings have any role in expansion they must prevent it. Although not expanding greatly, the cotyledons of *Lupinus angustifolius*, as well as the cotyledons of the other thick-walled-seed species, still have a photosynthetic function which may be very important during plantlet growth. Yet, the main evolutionary trend seems to be towards a reserve function.

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