THE MOLECULAR CELL BIOLOGY OF A XYLOGLUCAN SPECIFIC ENDO 1,4 ß-D-GLUCANASE FROM *TROPAEOLUM MAJUS* L. COTYLEDONS.

by

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ABBREVIATIONS

BSA	bovine serum albumin
CM	carboxy methyl
c.d.	circular dichroism
DEAE	diethylaminoethyl
DDT	dithiothreitol
EDTA	ethylene diamine tetra acetic acid
Endo H	endoglycosidase H
FITC	fluorescein isothyocyanate
GA_3	gibberellic acid
M_r	molecular weight
PAGE	polyacrylamide gel electrophoresis
PMSF	phenylmethylsulphonyl fluoride
PNGase F	n-glycosidase F
RITC	rhodamine isothiocyanate
SDS	sodium dodecyl sulphate
TEMED	N,N,N',N'-tetramethylethylenediamine
TRIC	tetramethylrhodamine isothiocyanate
u.v.	ultra violet
v/v	volume in volume
w/v	weight in volume
2-4-D	2-4-dichlorophenoxyacetic acid

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Acknowledgements, I tend to find,
Are staid and out of line
And so to flout convention
I've put them all in rhyme.

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And last and by no means least
For his patience and his time
Furry cuddles to my pal Steve
My eternal partner in crime.

ABSTRACT

Certain seeds store carbohydrate, not as starch, but as a polysaccharide component of thickened cotyledonary and endosperm cell walls. In nasturtium (*Tropaeolum majus* L.) the storage carbohydrate is xyloglucan. One of the enzymes involved in xyloglucan mobilisation, an endo 1,4 ß-D-glucanase was investigated further.

The purified enzyme was found to be a single molecular species of M_r 29,000 and to have an isoelectric point of 5.1. It was found not to be a glycoprotein and the assembled protein contained 66% ß-sheet, no α-helix and 34% "other structures".

A polyclonal antiserum was raised against the enzyme and used in further studies.

The enzyme was localised in nasturtium cotyledons, over a germination time course, at the light microscope level using an immunofluorescent technique. The enzyme was found to localise initially at the plasma membrane before being distributed throughout the storage wall. It did not co-localise with visible structures thought to be zones of dissolution in the wall, hence it was thought that the endo 1,4 ß-D-glucanase was involved in loosening the xyloglucan for attack by the other enzymes (present in the dissolution zones).

The enzyme was shown to be synthesised *de novo* following germination, the increase in enzyme activity being a direct result of an increase in protein synthesis.

The increase in activity correlates closely with the mobilisation of storage xyloglucan as viewed histochemically (and determined biochemically). The enzyme activity decreased rapidly when the substrate had been mobilised but the level of enzyme protein content does not, suggesting activity denaturation probaly due to the desiccation of the seed. The protein was rapidly degraded at a later stage and this depletion is thought to be a controlled process, possibly via a developmentally regulated protease.

Successful isolation of the messenger RNA from nasturtium was achieved and analysis of the immunoprecipitable protein revealed a single molecular species of M_r 33,000 (as opposed to 29,000 for the pure protein). The difference in M_r is almost certainly due to the presence of a signal peptide on the sequence.

The production of the enzyme was found to require the presence of the embryonic axis. The effect produced by the removal of the axis could not be replaced by GA_3 or 2-4-D.

CHAPTER ONE

CHAPTER 1

INTRODUCTION

1.1 Germination.

The dry seed is characterised by a remarkably low rate of metabolism. This is probably a direct result of the very low level of hydration of the seed, the water content of which is normally between 5% and 10%. Despite this almost complete absence of metabolism it cannot be assumed that the seed lacks the potentiality of metabolism. When dry seeds are broken up, and extracted with buffers, it is possible to demonstrate the existence of a considerable number of active enzyme systems (Bewley and Black, 1978).

Germination of the seeds of the higher plant is regarded as those consecutive events which cause a dry quiescent seed, in response to water uptake, to show a rise in its general metabolic activity and to initiate the formation of a seedling from the embryo. When a viable seed is wetted, water is taken up, respiration, protein synthesis and other metabolic activities begin and after a certain period of time, the embryo emerges from the seed, usually radicle first:- the seed has germinated. Various requirements must first be satisfied before these events can occur, usually including sufficient oxygen and a minimum temperature.

The exact stage at which germination ends and seedling

growth begins is difficult to define as it is normally regarded that the end of germination is signalled by the appearance of either a root or shoot, which in itself is a result of growth! Cell division or cell elongation is thought to cause the piercing of the seed coat by part of the embryo. In some cases it has been shown that cell division occurs first and is followed by elongation however, the reverse has also been observed. Berlyn (1972) studied Pinus lambertiana and showed using thymidine incorporation, that cell division occurred before elongation. This was in contrast to the earlier work of Toole (1924), who noted that the coleorhiza of Zea mays underwent cell enlargement before cell division occurred in the radicle (in Zea mays it is the coleorhiza which first pierces the seed coat).

Haber and Luippold (1960) studied lettuce, using low temperatures and gamma radiation to delay cell division and hypertonic solutions of mannitol to arrest cell elongation. Even though mitosis was prevented by the low temperature and radiation treatment, root protrusion occurred, indicative of germination. However, cell division occurred even when elongation was prevented by hypertonic solutions and no germination was observed. The authors concluded that root protrusion was dependent upon cell elongation, whereas cell division increases the number of cells which can elongate.

Germination, in the early stages, has been observed to be reversible, as in most seeds, after initial water

uptake, the seeds can be dried again without damage. It was thought that germination and growth could be differentiated by the extent of reversibility, however, some seeds (whose seedlings can resist desiccation) may be dried without damage even when the seedling has protruded.

Cell division and elongation only occur once the cells of the seed become activated, so as to permit control by various factors. This activation process precedes growth and may be termed germination. However, it is clear that for normal growth and development of the seedling to occur, both cell division and elongation are required.

Once germination has occurred, growth of the seedling continues, supported by mobilisation of the bulk food reserves.

1.2 Major storage reserves in seeds.

Most seeds contain large amounts of food reserves which support growth and development of the seedling. Reserves include lipid, protein, carbohydrate, organic phosphate and various inorganic compounds. Exceptions to this are certain members of the Orchidaceae, which produce seeds with no food reserves, germination and growth being dependent upon an external supply of organic substances e.g. carbohydrate provided by relationships with saprophytic fungi.

1.2.1 Composition of seed storage reserves.

Seeds can be divided into those whose main storage material is carbohydrate and those whose reserves comprise mainly of lipid. Lipid (in the form of triglyceride) containing seeds are by far the more common of the two. This majority, however, is not reflected among the economically important seeds. Most seeds can store protein alongside carbohydrate and lipid, but protein is not normally the predominant storage material. Soybeans and Machaerium acutifolium (66% protein, Coutinho and Struffaldi, 1972) are exceptions.

For seeds which are regarded as economically important (e.g food or industry) the chemical composition is well documented, while information on that of wild plants is limited. The reserve composition of some seeds and their location (Section 1.2.2) are shown in Table 1.1. (from Bewley and Black, 1978).

The storage components of seeds are determined genetically, but environmental factors can influence the proportion of constituents present. Randall et al. (1979) investigating the effects of sulphur, phosphorus and potassium levels in peas, found that altering the availability of these minerals induced changes in the storage protein content. In soybeans, glycinin and β-conglycinin levels are altered depending on the amount of sulphur (Gayler and Sykes, 1985). Such changes would appear to have little effect on germination as slight changes in seed composition can be tolerated by the germinating seed.

Table 1.1 **Food reserve composition of various seeds.**

Species	Average % composition (dry wt)			Major storage organ
	Protein	Fat	Major CHO	
Corn	11	5	75(Starch)	Endosperm
Sweet corn	12	9	70(Starch)	Endosperm
Oats	13	8	66(Starch)	Endosperm
Wheat	12	2	75(Starch)	Endosperm
Rye	12	2	76(Starch)	Endosperm
Barley	12	3	76(Starch)	Endosperm
Broad bean	23	1	56(Starch)	Cotyledon
Flax	24	36	24(Starch)	Cotyledon
Field pea	24	6	56(Starch)	Cotyledon
Garden pea	25	6	52(Starch)	Cotyledon
Peanut	31	48	12(Starch)	Cotyledon
Soybean	37	17	26(Starch)	Cotyledon
Cotton	39	33	15	Cotyledon
Rape	21	48	19(Starch)	Cotyledon
Watermelon	38	48	5	Cotyledon
Brazil nut	18	68	6	Radicle/ Hypocotyl
Oil palm	9	49	28	Endosperm
Ivory nut	5	1	79(Mannan)	Endosperm
Date	6	9	58(Mannan)	Endosperm
Castor bean	18	64	Trace	Endosperm
Pine	35	48	6	Megagamet-ophyte

Table reproduced from Bewley and Black (1978).

1.2.2 Location of seed storage reserves.

Reserve substances may be stored in the embryo or in extraembryonic tissues (endosperm or more rarely perisperm) or both. Most of the stored carbohydrate (starch) and protein of cereals and other grasses is located in the endosperm, the embryo being relatively small. In some oil-storing seeds, such as the castor bean (Ricinus communis), the endosperm is the storage tissue, in others the cotyledons (e.g. Linum usitatissimum, Arachis hypogaea and Brassica napus). Rarely, the perisperm accumulates reserves as in Yucca where the fat and protein are stored. In many dicotyledonous plants the endosperm is greatly reduced and the reserves are present in the embryo. For example many members of the Leguminosae are non-endospermic and store protein and carbohydrate in their bulky cotyledons. Cotyledons and endosperm may both be involved in storage and may contain reserves of different types (e.g fenugreek and lettuce with lipid and protein in the cotyledons and carbohydrate in the endosperm). The structure of different types of seeds and the location of their food reserves is shown in Figure 1.1. (from Bewley and Black, 1978).

1.3 Cell wall carbohydrate reserves.

One of the most important and well-known reserve carbohydrate in seeds is starch (a polymer of glucose). Starch is stored in the cytoplasm in plastids and constitutes the main storage product of the cereals

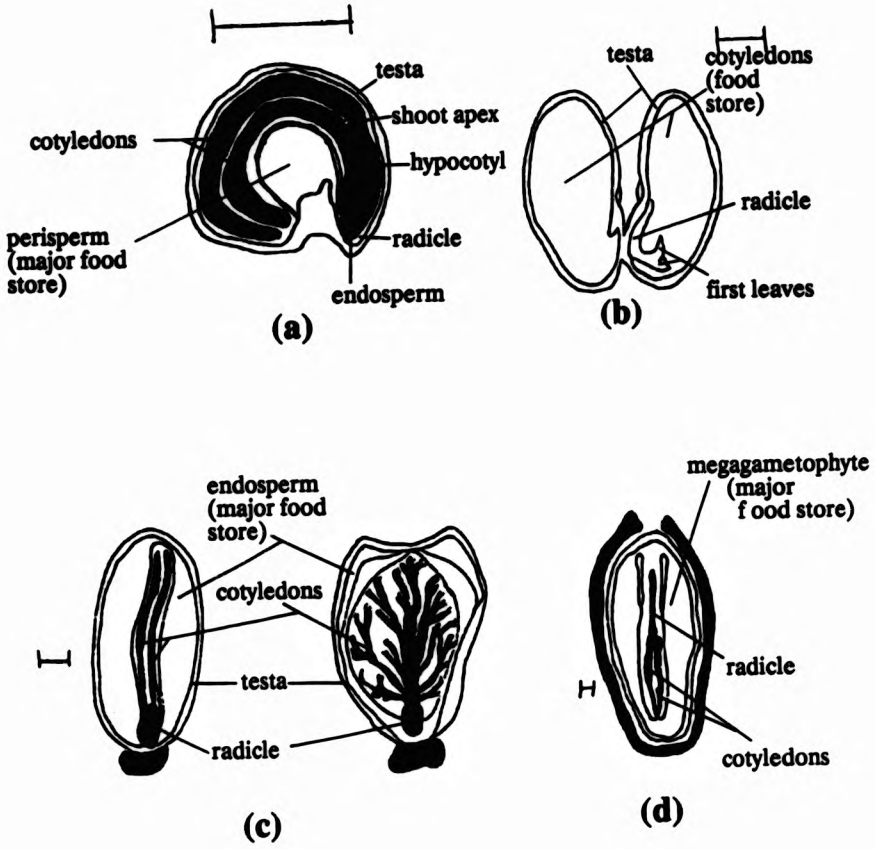


Figure 1.1.

Structure and location of food reserves:

(a) *Beta vulgaris* - beet
(b) *Phaseolus coccineus* - runner bean
(c) *Ricinus communis* - castor bean
(d) *Pinus pinea* - stone pine.

Scale mark in each case = 1mm.

(Gramineae). The structure, metabolism and enzymatic mechanisms of which have been highly researched (Varner et al., 1963 etc.) but it is less well known that seeds store other carbohydrate polymers.

Early microscopic observations in the nineteenth century indicated the presence of major deposits in the cell walls of certain seeds and that they disappeared after germination (Schleiden, 1838; Heinricher, 1888; Reiss, 1889; Tschirch, 1889). The presence in the cell walls of seed endosperm or cotyledons of large amounts of carbohydrate material, and their disappearance following germination led these authors to believe the cell wall materials to be seed reserves. This has been corroborated by later and more precise investigations, for example Reid and Meier (1972) studying galactomannan mobilisation in fenugreek, crimson clover and lucerne and xyloglucan mobilisation in white mustard (Gould et al., 1971) and in nasturtium (Edwards et al., 1985). The mobilisation of these polysaccharides in seeds after germination have shown that their principal role is that of a carbohydrate reserve. Their overall relevance is, however, probably more complex (see section 1.3.2).

1.3.1 Structure of seed cell wall storage carbohydrates.

Although few structural studies have been carried out on seed cell wall storage polysaccharides, it is possible to characterise several main structural types: the mannan group, the galactans (a galactose and arabinose-rich class)

and the xyloglucans (or more correctly the galactoxyloglucans).

The mannans comprise of three structurally related types of polysaccharides: the "pure" mannans, the glucomannans and the galactomannans which are all based to a greater or lesser extent on the (1→4)-ß-D-mannosyl linkage. They are also related in that they are only found in seed endosperms and not in storage cotyledons or axes.

In the case of the "pure" mannans, their structures have been established by studies on the seed endosperms of two palms. The date palm (Phoenix dactylifera) and the ivory nut tree (Phytelephas macrocarpa) yielded pure mannans with a linear 1→4 ß-linked D-mannan backbone with less than 2% of single unit α-D-galactopyranosyl substituents linked 1→6 to mannose (Aspinall et al., 1953; 1958; Meier, 1958). Mannans with structures similar to those of the date and ivory nut have been obtained by alkali extraction from coffee beans (Coffea arabica) (Wolfrom et al., 1961) and from the umbellifer Carum carvi (Hopf and Kandler, 1977). Mannans have been found in large amounts in seed endosperms as Aspinall (1970) reported that the mannan of the ivory nut palm constitutes over 50% of the seed weight. It should also be noted that similarly constituted mannans have been found in non-storage cell walls. Mackie and Preston (1974) reported the presence of pure mannans in certain green seaweeds where they appear to play a structural role.

Glucomannans have been found in the seeds of Asparagus

officinalis and Endymion nutans (Goldberg, 1969). Again similar structures have arisen: a linear 1→4 ß-linked backbone containing equal numbers of D-glucopyranosyl and D-mannopyranosyl residues. Also attached to the backbone is a small percentage (3-6%) of single unit D-galactopyranosyl branches attached 1→6 probably via α linkages (Goldberg, 1969).

Up to 40% of the seed weight of some leguminous species is due to galactomannan, and it has been reported to account for almost all the dry weight of some legume-seed endosperms (Reid, 1971; Leung et al., 1981). Galactomannans are important industrially hence this group have received the most attention. Unlike the pure mannans and the glucomannans they can be extracted with water and form highly viscous solutions. Structurally similar, they all contain a 1→4 ß-linked D-mannan backbone substituted by single unit α-D-galactopyranosyl side chains linked 1→6 to mannose. The degree of substitution varies greatly and is thought to be genetically controlled (Reid and Meier, 1970; Kooiman, 1971).

Halmer (1985) reported that 19 angiosperm families had non-starchy endosperms, containing mannans or galactomannans as reserves in the cell walls. Only one endospermic leguminous seed has been found to contain a storage polysaccharide other than galactomannan. The seed of the judas tree (Cercis siliquastrum: Leguminosae-Caesalpinioideae) contains a galactoglucomannan similar in structure to a galactomannan but with D-glucopyranosyl

residues on the backbone (McCleary et al., 1976).

Again polysaccharides of similar structure have been found in the non-storage cell walls of higher plants. The glucomannans are characterised as hemicelluloses and are the major cell wall components (12-15%) of gymnosperms with residues of D-glucose and D-mannose in the ratio of 1:3, whereas only galactoglucomannans devoid of galactose residues and with glucose and mannose units in the ratio of 1:2 occur in deciduous woods (Aspinall, 1980).

Galactans are of a more restricted occurrence than the other groups, they represent the principal polysaccharide constituents of the thickened cotyledonary cell walls of Lupinus species. They contain a 1→4 ß-linked galactan component and have been shown, on hydrolysis, to release galactose (76%), arabinose (13%), xylose (4%) and uronic acid (7%) (Crawshaw and Reid, 1984). In non-storage walls they are structurally classified as belonging to the pectins. ß 1→4 linked D-galactan chains containing L-arabinofuranose residues in sidechains form a family of structurally related species. ß 1→4 linked D-galactans, devoid of arabinose residues but containing 5-10% of uronic acid (glucuronic and/or galacturonic acid) residues are found in compression wood from a number of gymnosperms (Aspinall, 1980).

Examples of xyloglucan storing seeds are nasturtium (Tropaeolum majus) and tamarind (Tamarindus indica). The polysaccharides are stored in the cotyledonary cell walls and are frequently referred to as "amyloids" and it is this

group which will be discussed in detail.

1.3.2 Biological relevance of seed cell wall carbohydrate reserves.

The function of cell wall polysaccharides in seeds is predominantly that of storage but it has been suggested that they may be involved in protection of the seed. The mannans, galactomannans and glucomannans are all related structurally but differ slightly in their physical properties. The mannans and glucomannans are insoluble in water and Kooiman (1960) suggested that in the cell wall they may be crystalline and hence confer hardness on the seeds preventing extensive softening of the endosperm tissue on imbibition. Indeed Marloth (1883) noted that a hard endosperm with highly thickened cell walls will protect seeds from mechanical damage.

Contrary to this galactomannans and xyloglucans are hard only in the non-imbibed state as they are essentially hydrophillic molecules. Galactomannans are often termed "seed mucilages" and Reid (1985) suggested that the presence of galactosyl residues on galactomannans prevented the self association of the main chain thus preventing the formation of crystalline aggregates. The biological function of galactomannan in fenugreek was investigated by Reid and Bewley (1979). They concluded that during imbibition, galactomannan was responsible for the uptake of copious amounts of water and its distribution around the embryo. During germination it effectively protects the

embryo from desiccation should drought follow imbibition, and following germination the galactomannan acts as a reserve substrate.

1.3.3 Mobilisation of seed cell wall carbohydrates other than xyloglucans.

The only mannan-containing seed in which storage carbohydrate mobilisation has been studied in detail is that of the date palm (Phoenix dactylifera) (Keusch, 1968; De Mason et al., 1985). In this case the distal end of the cotyledon develops into a haustorium after germination. This haustorium grows inwards into the endosperm, absorbing the reserves in a narrow zone in front of it. The cell walls of the date endosperm were completely broken down with the exception of the thin primary walls which accumulated in the dissolution zone. Keusch also showed that the end products of the breakdown process were mannose, manno-oligosaccharides and traces of galactose, which are then absorbed by the haustorium.

Galactomannan mobilisation has received much attention and been studied in a variety of species. The physiology of galactomannan breakdown has been studied in fenugreek (Trigonella foenum-graceum) (Reid and Meier, 1972) and carob (Ceratonia siliqua) (Spyropoulos, 1982). The enzymology of galactomannan degradation has been studied in guar (Cyamopsis tetragonoloba) (M^{c}Cleary, 1983); carob (Ceratonia siliqua L.) (Seiler, 1977) and in fenugreek (Reid and Meier, 1973; Spyropoulos and Reid, 1985; 1988).

In fenugreek the endosperm completely surrounds the embryo and consists of a living peripheral aleurone layer and a mass of galactomannan storage tissue (Reid, 1971). The breakdown of galactomannan in fenugreek endosperm begins 16 hr after germination and is complete by 40 hr after germination (Reid, 1971; Reid and Bewley, 1979). The galactomannan breakdown products are rapidly absorbed by the embryo and converted to sucrose and starch (Reid, 1971). Reid and Meier (1973) proposed that galactomannan breakdown was due to hydrolytic enzymes which were synthesised by the cells of the aleurone layer and secreted into the galactomannan storage tissue. The enzymes involved include an α-galactosidase, a ß-mannanase and a ß-mannosidase (exo ß-mannanase). In fenugreek the first two enzymes are produced by the aleurone layer by de novo synthesis, and secreted into the galactomannan storage tissue (Reid and Meier, 1973; Reid et al., 1977). Their increase in activity have been shown to parallel galactomannan degradation. The ß-mannosidase activity in fenugreek has been shown to increase sixfold (Reid and Meier, 1973), indicating that some activation or synthesis of the enzyme must occur. Similar results were reported by Seiler (1977) for carob seeds. In the guar seed (M^{c}Cleary, 1983) there is no increase in ß-mannosidase activity and when isolated endosperms are incubated in the presence of cycloheximide, no significant change in the level of this enzyme was detected. The author proposed that the enzyme was present in an active state in the resting endosperm.

None of these enzymes has been purified to homogeneity in the fenugreek system but the corresponding enzymes from the guar seed have been purified and their catalytic properties investigated (McCleary, 1982; 1983). The author found that on seed germination endospermic α-galactosidase and ß-mannanase are synthesisied (in the aleurone layer) and catalyse galactomannan degradation to D-galactose and ß-D-manno-oligosaccharides of d.p. 2-5. These manno-oligosaccharides were further hydolysed to mannose by the exo-ß-mannanase already present in the seed endosperm. Some of the mannobiose and mannotriose produced was absorbed by the cotyledons then further hydrolysed by a cotyledonary exo-ß-mannase which appeared to be identical to the endosperm form. The monosaccharides may be further metabolised by the developing embryo.

1.3.3.1 Control of cell wall storage carbohydrate mobilisation.

In certain cereal grains the embryo is known to control the mobilisation of reserves through the production of a hormonal stimulus (Yomo and Varner, 1971). Since the hormonal induction of amylase activity in barley aleurone was described (Varner et al., 1965), there has been a tendency to assume that carbohydrate breakdown is universally induced by gibberellins. The effects of hormones on the metabolism of reserve polysaccharides have been described for many plants (for a review see Bewley and Black, 1978, pp. 245-279). Already mentioned was the

induction of α-amylase in cereals by gibberellin. Other examples include amylase activity induced by cytokinin in dwarf bean cotyledons (van Onckelen et al., 1977) and the control of β-mannanase and α-galactosidase activities in lettuce seeds by gibberellin and red-light (Halmer et al., 1976; Leung and Bewley, 1981).

The breakdown of galactomannans in the endosperm of Trigonella, is less well understood and was reported as not being under the control of the embryo (Reid, 1971). When half endosperms were incubated under "germination" conditions the authors noted that the galactomannan was broken down at the same rate as that in intact seeds. They reasoned that no positive hormonal signal was required from the embryo to allow reserve breakdown. However, more recently, Spyropoulos and Reid (1985) demonstrated that galactomannan mobilisation was in fact under the control of the embryonic axis in fenugreek (Trigonella) seeds. Removal of the axis at any time before the complete mobilisation of the galactomannan caused inhibition and this effect was greater the earlier the axis was removed. The removal of the axis also had a determental effect on the appearance of the enzyme α-galactosidase. This effect was observed to be partially relieved if the excised axes were incubated with the remaining parts of the seed. The authors proposed a dual regulatory role for the embryonic axis. Before germination, the axis is involved in determining the time of appearance of α-galactosidase in the endosperm. In its absence the appearance of the enzyme is delayed. They

speculate that this may be mediated by diffusible factor (possibly benzyladenine and/or gibberellin), which helps to counteract the effect of the endogenous inhibitor in the endosperm. A similar phenomenon was observed by Halmer and Bewley (1979) in their study of the regulation of endo-ß-mannanase activity in the endosperm of the lettuce seed. Neither in fenugreek nor in lettuce has the inhibitor been identified although in both systems abscisic acid inhibits in vitro carbohydrate breakdown.

1.4 Storage xyloglucans in plant seeds.

In the nineteenth century it was observed that the thickened cell walls present in many seeds contained reserve substances. "Reserve celluloses" was the term used by Reiss (1889) to describe the substances which were utilised following germination. This was because they were found in the cell wall and hence mistaken for cellulose. Schleiden (1838) and Vogel and Schleiden (1839) observed that the cotyledonary cell walls of some plant species stained blue on treatment with an iodine solution. The cell wall substance was therefore assumed to be starch-like and was termed "amyloid" by these authors. Kooiman (1960) using the amyloid reaction showed that out of 2700 species tested, at least 237 had amyloid positive seeds. Also of the species tested, no representative xyloglucan was found in 25 families of the monocotyledons. A feature of this study was that amyloid containing seeds were grouped

together taxonomically i.e. entire families were essentially amyloid positive or negative.

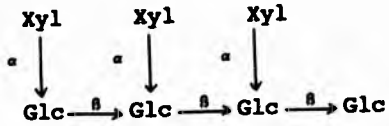
Of the 237 known seed amyloid containing species, only 4 have undergone detailed structural analyses of the amyloids, based on alkali extractions from the seed. These are Tamarindus indica (Kooiman, 1961); Tropaeolum majus (Le Dizet, 1972); Impatiens balsamina (Courtois and Le Dizet, 1974) and Annona muricata (Kooiman, 1967).

1.4.1 Structure of plant seed xyloglucans.

These amyloids are more precisely known as galactoxyloglucans (GXG) as on hydrolysis, galactose, xylose and glucose are released. All have similar structures: a linear 1,4 ß-linked backbone (analogous to that of cellulose) with lateral D-monoxylosyl residues α-linked to the C-6 of the glucan chain. The xylosyl residues are terminal or substituted with ß(1,2)-linked D-galactosyl residues.

The structures of seed amyloids were originally determined using a mixture of methylation analysis, acid hydrolysis and by the use of commercial (impure) enzyme preparations. Myrothecium "cellulase" was used in the analysis of Annona muricata amyloid (Kooiman, 1961); "hemicellulase" was used for tamarind (Srivastava and Singh, 1967); a commercial cellulase known as "Astra" was used on nasturtium along with a ß-glucosidase from buckwheat. These studies established the general features mentioned above.

More recent work has been concentrated on the amyloid of tamarind and has involved chromatographic techniques and enzyme hydrolysis using pure endo 1→4 ß-D-glucanases. The ratio of galactose: xylose: glucose has been shown to be 1:2:3 in xyloglucans isolated from Tamarindus and Tropaeolum seeds; 1:2:4-5 in Impatiens and 1:1:4 in Annona (literature reviewed in Reid, 1985). It should be noted, however, that more recent work involving endo 1,4 ß-D-glucanase hydrolysis (Fanutti et al., 1991) has shown that the bulk of the tamarind molecule is composed of a repeating unit as shown below.



Although seed xyloglucans were the first to be discovered, it is now clear that the plant primary cell wall contains xyloglucans of similar structure which have an important physiological role to play. These plant cell wall xyloglucans are, of course, relevant to the present study and will be discussed in more detail later (c.f 1.5).

1.4.2 Xyloglucan storage.

Reis et al., 1987 investigated the storage cell wall in the cotyledons of the tamarind seed. The thickened walls of the cotyledonary ground tissue (located between the epidermis and procambium) stained bright blue with iodine,

the characteristic reaction of the amyloids. When this region was investigated using cytochemical probes, three domains were found in the cell wall of the ground tissue: 1) a median region which was called the galactoxyloglucan (GXG) storage region; 2) an inner wall layer and 3) an outer wall layer which sandwiched the storage wall.

The median region was found to be the only site where GXG accumulated. This region could be extracted with boiling water and was found to represent 80% of the wall's dry weight. Chemical analysis indicated that the storage compound had a glucose: xylose: galactose ratio similar to that of the amyloids. Developmental studies (on immature seeds) displayed that the outer layer keeps the characteristics of a regular primary wall, whereas the inner layer forms a barrier between the storage area and the cytoplasm.

1.4.3 Xyloglucan formation and deposition.

The formation of xyloglucan during the process of seed ripening was investigated in nasturtium by Hoth et al., (1986) using incorporation of radioactive precursors. Different labelled sugars, as possible precursors of amyloid and starch biosynthesis, were fed to nasturtium seed halves. Feeding of labelled xylose resulted in the incorporation of more radioactivity into starch than into amyloid, thus indicating that exogenously supplied xylose could not be directly activated and used as a precursor of xyloglucan biosynthesis. When seeds were fed with labelled

[U-^{14}C]-glucuronic acid, 90% of the radioactivity that was incorporated into reserve polysaccharides could be found in the xyloglucan fraction. Hence they concluded that the most likely precursors of xyloglucan are UDP-bound monosaccharides.

More recently an ultrastructural investigation of xyloglucan formation in nasturtium was undertaken by Ruel *et al.*, (1990 a,b). They used a polyclonal anti-xyloglucan antibody and an α-D-glucosidase-colloidal gold complex which recognised α-D-xylosyl residues. The latter was developed by the authors.

The storage walls were organised into three concentric layers exhibiting different reactivity towards the stain. The external layer corresponds to the structural primary wall and is thin with a homogeneous structure. Layer two was thick and reported to be rich in polysaccharide material. It contained numerous alveoli which enlarged in diameter towards layer three in which the alveoli were larger. As the cell matured, layer three was reported to progressively disappear. Using an anti-xyloglucan antibody applied on ultrathin sections, the authors noted that layer three was a transient zone and that the xyloglucans were concentrated in layer two. Xyloglucans were first identified in the inner layer three, in the form of randomly distributed deposits, before being accumulated in layer two to be deposited on the inner face of the structural primary wall. They also noted that the transient zone three played an active part in the deposition of the

reserve xyloglucan as the newly synthesised material seemed to accumulate in this zone via numerous vesicles. They concluded that the deposition of the reserve xyloglucan takes place by apposition.

1.4.4 Xyloglucan mobilisation.

Edwards et al., (1985) working on cell wall xyloglucan from nasturtium seeds demonstrated that the xyloglucan diminished during the period when the cotyledon reserves were mobilised. Mobilisation of reserve material occurs following germination and this period also represented xyloglucan depletion. Edwards et al., demonstrated that the mobilisation of reserve polysaccharides is concurrent with the activities of four hydrolytic enzymes; an endo ß-D-glucanase, a ß-galactosidase, an α-xylosidase and a ß-glucosidase. They established that the period when the activities of these enzymes increased corresponded to the period of rapid xyloglucan depletion. It was concluded that mobilisation of xyloglucan in nasturtium was a direct result of the action of these enzymes.

Histochemical and cytochemical studies lead Reis et al., (1987) to determine three main stages in galactoxyloglucan (GXG) mobilisation in tamarind. The first stage (4-5 days) involves complete hydration, vacuolisation and hydration of the protein bodies, corresponding to the axial growth of the seedling. The second stage (from day 5 to about day 25) involves a dramatic decrease in the level of GXG, indicative of mobilisation. It starts when the

cotyledons begin to be lifted above ground level by hypocotyl elongation. During the final stage the GXG remains low and nearly constant but the cotyledons lose about 90% of their initial dry weight. At the organ level, the time course of GXG depletion emphasises the fact that the mobilisation of reserves is sequenced.

However, this is only a very general view on the breakdown of reserves. The attack on GXG appeared as minute and separated spots located on the dorsal region of each cotyledon i.e. in cells farthest from the conductive bundles. The digestion was detectable at days 4 to 5 and progressed laterally cell by cell until the initial and newly formed spots of digestion fused together. When all the GXG had been depleted, the median part of the wall collapsed and the inner and outer layers met, restoring the storage cells to a parenchyma-like state. Transitory pillars were formed between the outer and inner layers which were thought to maintain the wall thickness until an advanced stage of digestion. The persistence of an inner layer was thought to prevent any engulfing of the cytoplasm at the surface, as no direct contact between plasmalemma and the site of GXG lysis was observed. During all steps of mobilisation it was noted that the molar ratio of galactose, xylose and glucose remained constant (1:2:3) and no change in monosaccharide composition was detected. Compared with wall storing endosperm in which wall breakdown corresponds to a collapse of the tissues and cells, the digestion of walls in cotyledons is an original

and controlled wall thinning. Reis *et al.*, also noted that depletion takes place only when the cotyledons are connected to the axis of the seedling. This was observed using free-hand sections stained with iodine. They reported that when the cotyledons were excised between day 1 and days 8-10, their ageing can prolonged by up to 6 to 8 weeks, but their depletion does not occur. More recently, Hensel *et al.*, (1991) reported that excision of the cotyledons from nasturtium seedlings at an early stage of germination prevented mobilisation of major reserves.

Using an agar film technique and a cytochemical approach, Reis *et al.*, indicated that there was a close spatial relationship between the rise of hydrolytic activities and the attack of GXG-storing walls. The pattern and directionality of both are identical thus confirming the conclusion of Edwards *et al.* (1985) that the activities of three hydrolytic enzymes in nasturtium were responsible for the breakdown of xyloglucan in the seed cotyledons.

1.5 Primary cell wall xyloglucans.

The primary cell wall of plants consists of microfibrils of cellulose embedded in a matrix that is composed of polysaccharide, protein and glycoprotein. The polysaccharide components of this matrix can be separated into major classifications based on their solubility properties. Xyloglucans are classified as belonging to the hemicellulose family as they are soluble in dilute alkali.

Although xyloglucans are composed primarily of xylose and glucose, varying amounts of other sugars (hexoses, deoxy hexoses and pentoses) are also present (Aspinall et al., 1969; Bauer et al., 1973 and Darvill et al., 1980). The composition of the xyloglucans isolated from a variety of sources are presented in Table 1.2, (reproduced from Darvill et al., 1980) which indicates the differences between them, for example, all of the cells possessing primary walls produce xyloglucans containing fucosyl residues. A fucosylated xyloglucan was first isolated as a key component of the primary wall from suspension cultured sycamore cells (Aspinall et al., 1969; Bauer et al., 1973). These xyloglucans contain L-fucose in addition to D-glucose, D-xylose and D-galactose, the L-fucose being attached to the 2 position of the terminal galactosyl residues by an α linkage.

Generally xyloglucans comprise as much as 20 to 25% of the primary walls of dicotyledonous plants. Xyloglucans from the primary walls of monocotyledonous species make up about 2-5% of the total polysaccharide in the wall in the grasses, but their overall chemical pattern is similar to that of the dicotyledons (Kato and Matsuda, 1985). The main differences being the presence of fewer xylose chains (e.g in barley seedling xyloglucan Kato et al., 1981, reported that only 36-38% of the glucose residues are xylosylated), less galactose and no terminal fucose. There is, however, an exception to this: Redgwell and Selvendran (1986) analysed the xyloglucan from onion (Allium cepa) and found

Table 1.2 **Glycosyl composition (Mole %) of a variety of Xyloglucans and Amyloids.**

	Glucose	Xylose	Galactose	Fucose
Tamarind	48	36	16	0
Nasturtium	55	27	18	0
Rapeseed I	64	24	12	0
Rapeseed II	48	34	10	7
Extracellular Polysaccharides				
Bean	46	36	10	8
Rose	51	30	10	7
Sycamore	46	37	7	6
Sycamore cell wall	41	36	14	7

Table reproduced from Darvill *et al.* (1980).

that the polysaccharide is composed of terminal fucosyl-galactosyl and terminal galactosyl residues. Also, the relative amounts of xyloglucan present (10%) resembles the quantities found in dicotyledonous species. These differences were explained by Kato and Matsuda (1985) who postulated that xyloglucans in monocotyledons originally contained many substituted xylosyl and galactosyl residues and that the residues are partially deleted during growth. This was supported by Koyama et al., (1981) who detected α-xylosidase and ß-galactosidase activities in soybean cell walls capable of hydrolysing xylosidic and galactosidic linkages of xyloglucan.

1.5.1 Location and function of primary cell wall xyloglucans.

Xyloglucan occurs uniformly across the thickness of the primary wall and also in the middle lamella (Moore et al., 1986). This was established by electron microscopic observation of the binding of gold labelled anti-xyloglucan antibodies to sectioned walls. Hayashi and Maclachlan (1984) used a fluorescence labelled, fucose-specific lectin to show that xyloglucan occurs uniformly throughout a whole cell wall at the light microscopic level.

Xyloglucan is believed to play a key role in the architecture of dicotyledonous cell walls by linking together cellulose microfibrils and possibly other components of the cell wall via hydrogen bonds. In pea stem cell walls, Hayashi and Maclachlan (1984) suggested that

xyloglucan could be hydrogen bonded onto two different cellulose microfibrils in order to achieve structural rigidity. Most cell wall components can be solubilised by hot water, chelating agents or dilute alkali (4% KOH). However, the association between xyloglucan and cellulose is so strong that it requires concentrated alkali (24% KOH) to dissolve the xyloglucan from the xyloglucan/cellulose complex. Hayashi and Maclachlan (1984) showed that xyloglucan was distributed on and between cellulose microfibrils on electron microscopic observations of ghost preparations. Wall ghosts (composed of xyloglucan and cellulose) from elongating regions of pea stems were examined using iodine staining, radiography after labelling with [^{3}H] fucose, fluorescence microscopy using a fluorescein-lectin (a fucose-binding lectin from Ulex europaeus) probe by light microscopy and by electron microscopy after shadowing. Their results indicated that the xyloglucan is located both on and between the cellulose microfibrils. Hayashi et al., (1987) indicated that this was the case in the cell walls of maturing tissues, even when the xyloglucan content of the wall was declining. Thus they concluded that xyloglucan exists in situ in close association with cellulose throughout the wall at all stages of growth.

The structural function of xyloglucan according to Brummell and Maclachlan (1989) is to confer rigidity to the wall to counteract the forces of turgor generated by the protoplasm. Breakdown of the components may be required to

allow the crystalline cellulose microfibrils to move relative to one another and the cell to expand. Hayashi and Maclachlan's (1984) observation that cell wall "ghosts", essentially consisting only of xyloglucan and cellulose, retained the shape of the cell which suggested that xyloglucan not only coated individual cellulose microfibrils but also bonded the microfibrils together. Endohydrolysis of xyloglucan may be essential in order to permit movement of cellulose microfibrils relative to one another and consequently cell wall extension. Many plant cell walls contain enzymes (cellulases) that can cleave xyloglucan (Hayashi et al., 1984), and these enzymes are presumed to be responsible for auxin-induced xyloglucan turnover. A requirement for exposed xyloglucan in growth has been demonstrated by Hoson and Matsuda (1987) using fucose-binding lectins. These lectins bound to the cell wall of azuki bean epicotyl segments and inhibited both auxin-induced cell wall loosening and growth, presumably by protecting xyloglucan from turnover.

1.5.2 Degradation of primary cell wall xyloglucans.

Plant cell walls contain a set of glycosidases and glucanases that hydrolyse xyloglucan into monosaccharides (Koyama et al., 1981; Koyama et al., 1983). Since the backbone of xyloglucan is thought to be composed entirely of glucose residues, a significant breakdown of the molecule is only possible by the action of an endo (1,4) ß-glucanase (cellulase). Many plant cell walls contain

cellulases that can cleave xyloglucan at its non-glycosylated residues (Hayashi et al., 1984) The occurrence of cellulase in plant tissues was first suggested by Ruesink (1969).

The growing cells of pea stems generate two distinct endo 1,4 ß-D-glucanases (Byrne et al., 1975). These were shown to hydrolyse the internal 1,4 ß-glucosyl linkages of xyloglucan as well as a variety of 1,4 ß-glucans, 1,4, 1,3 ß-glucans and cellodextrans (Wong et al., 1977). The action patterns of these pea endo 1,4 ß-D-glucanases are essentially the same as those of fungal cellulases i.e. both hydrolyse internal linkages adjacent to unsubstituted glucose residues (Hayashi and Machlachlan, 1984) thereby introducing a free reducing end-group at these points.

Bal et al. (1976) located the enzymes in ultrathin sections of pea tissues at the electron microscope level using a ferritin conjugate antibody. They showed that an activity with a molecular mass of 70 kD was associated with the inner surface of cell walls, while the other activity with a molecular mass of 15 kD was found to be associated with the rough endoplasmic reticulum.

The primary walls of soybean hypocotyls has also been shown to contain an endo ß (1,4) glucanase activity (Koyama et al., 1981; Koyama et al., 1983). They suggested that this enzyme was responsible for the degradation of the wall-bound xyloglucan at least at the initial stage of degradation. The hydrolysis pattern by soybean enzymes showed a two step degradation of the xyloglucan during

cell elongation. It was first endohydrolysed into large fragments, which were then further broken down into monosaccharides. Koyama et al., suggested that an endo 1,4 ß-glucanase activity was responsible for the first stage of degradation and that the second stage required the activities of an α xylosidase, ß glucosidase, α fucosidase and a ß galactosidase.

1.6 Xyloglucan degrading enzymes.

The changes in storage materials after germination are the result of the activity of enzymes capable of their degradation. These enzymes are not necessarily produced in the same cells in which the storage materials are located, moreover signalling systems exist which regulate their production and interaction between different parts of the seed. For example, the embryonic axis and the cotyledons (pea); the endosperm, embryo and aleurone layer (cereal seeds) or cotyledon, endosperm and embryonic axis (castor bean).

Of the four hydrolytic enzymes involved in xyloglucan mobilisation in nasturtium cotyledons, three have been purified to homogeneity; endo(1,4) ß-D-glucanase (Edwards et al., 1986); ß-galactosidase (Edwards et al., 1988); α-xylosidase (Fanutti et al., 1991). The natural substrate of all of these enzymes was found to be the storage xyloglucan. The endo 1,4 ß-D-glucanase is the key enzyme in the mobilisation of xyloglucan in the nasturtium system. It

hydrolyses the main glucan backbone of the xyloglucan molecule generating fragments which can then be acted upon by the other enzymes. The ß-galactosidase, unlike other ß-galactosidases from other sources, was found to be capable of removing side-chain galactose residues from intact xyloglucan without any prior depolymerisation of the cellulosic backbone (Edwards et al., 1988). The α-xylosidase from nasturtium will not hydrolyse the intact molecule but xylose was released from oligosaccharide fragments produced by hydrolysis of xyloglucan by the endo 1,4 ß-D-glucanase (Fanutti et al., 1991). The authors showed that the endo 1,4 ß-D-glucanase acts on xyloglucan to produce only oligosaccharides which are α-xylosidase substrates i.e. xyloglucan oligosaccharides which have an unsubstituted α-D-xylopyranosyl residue of the "terminal" type attached to D-galactose.

It was found that the endo ß-D-glucanase did not hydrolyse any of the substrates tested except the galactoxyloglucan indicating total specificity towards the substrate. There have been two general hypotheses put forward to explain this specificity: (1) the enzyme recognises certain structural features in the galactoxyloglucan side chains and therefore it may be a true galactoxyloglucanase or (2) it may be an endo (1,4) ß-D-glucanase with a subsite binding requirement that has so far not been found. It was established that the enzyme hydrolysed the galactoxyloglucan from tamarind and nasturtium more extensively than that from mung bean

primary walls, which tends to support the first hypothesis, as the only difference between the two is the presence of some D-fucosyl residues linked to the D-galactosyl residues of the side chains.

Two cellulases (endo (1,4) ß-D-glucanases) have been isolated from elongating pea epicotyls (Byrne et al., 1975) and some of their properties determined (Wong et al., 1977). These enzymes also hydrolyse galactoxyloglucans but unlike the enzyme from nasturtium seed cotyledons they can hydrolyse cellulose and carboxymethyl cellulose. Although the enzyme from nasturtium seed cotyledons is the first which exhibits an unique specificity towards its substrate there may be other enzymes with a similar substrate specificity. It is interesting to note that this xyloglucan specific endo (1,4) ß-D-glucanase would not have been detected if traditional cellulase substrates had been used e.g. carboxymethyl cellulose (Edwards et al., 1986). Similarly, wall-bound α-xylosidase was reported inactive on the most convenient substrate p-nitrophenyl-α-D-xylopyranoside (Koyama et al., 1983). This would serve to illustrate that wall bound enzymes may be more numerous than had been assumed and that many new enzymes involved in wall metabolism might be discovered if wall-derived substrates were employed. Polymers extracted from radiolabelled cell walls would appear to be the most promising potential substrates for the detection of new wall enzymes (Fry, 1988).

1.8 Aims.

The aims of this project included further characterisation of the endo 1,4 ß-D-glucanase from nasturtium cotyledons and to verify earlier results from Edwards *et al.* (1986). Secondly we wished to study the induction of the enzyme by raising a polyclonal antiserum to the enzyme and using this to localise it *in vivo*. The antiserum was used to establish whether the enzyme was synthesised *de novo* during germination. This induction was further investigated at the molecular level by isolating RNA and translating it *in vitro*. The antiserum was used to identify the endo 1,4 ß-D-glucanase amongst the translation products. The physiological role of the embryo and various growth substances on the induction of the enzyme was also investigated.

CHAPTER TWO

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials.

All chemicals and reagents used were of the highest grade available and were purchased from Sigma Chemical Company or BDH Chemicals Ltd. unless otherwise stated.

2.2 General methods.

2.2.1 Determination of protein concentration.

Protein concentration was determined by the method of Sedmak and Grossberg (1977). A protein sample was diluted with distilled water to a volume of 1 ml and to this 1 ml of Coomassie reagent was added. The Coomassie reagent consisted of 0.06% (w/v) Coomassie Brilliant Blue G250 in 3% (v/v) perchloric acid, and was filtered through Whatman No.1 filter paper. The absorbance of the protein solution was measured at 620 nm and 465 nm against an absorbance blank of distilled water. The absorbance ratio 620/465 was calculated and the absorbance ratio of a blank (1 ml distilled water + 1 ml Coomassie reagent) was subtracted.

The value obtained was used to determine the protein concentration from a calibration curve, constructed using concentrations of bovine serum albumin (BSA) between 0 and 40 μg/ml. A BSA standard was included in each set of

protein determinations.

2.2.2 SDS-Polyacrylamide gel electrophoresis.

Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulphate (SDS) was performed by the method of Laemmli (1970). The gels contained acrylamide at a concentration of either 10 or 12% (w/v) and were 1.5 or 0.75 mm in thickness.

The separating gel contained separating gel buffer (1.5 M Tris-HCl, pH 8.7, 0.4% (w/v) SDS), distilled water, acrylamide stock solution (30% (w/v) acrylamide: 0.8% (w/v) methylenebis-acrylamide), TEMED and ammonium persulphate (10% (w/v), made up fresh). The stacking gel contained stacking gel buffer (0.5 M Tris-HCl, pH 6.8, 0.4% (w/v) SDS), distilled water, acrylamide stock solution, acrylamide stock solution (as above), TEMED and ammonium persulphate (as above). Gels were prepared and stored wrapped in "clingfilm" at 4° C for up to 4 days before using.

The reservoir buffer (5 x concentrated) contained 0.125 M Tris, 0.96 M Glycine, 0.5% (w/v) SDS and was diluted 1 in 5 before use.

Samples were prepared by diluting 1:1 with sample buffer 2 x concentrated (30 ml 10% (w/v) SDS, 12.5 ml stacking gel buffer, 10% (v/v) glycerol, pH 6.8 with HCl) containing 2 µl ß-Mercaptoethanol/ml. They were heated in a boiling water bath for 2 min then 5 µl of a 1:1 mixture of 1% (w/v) bromophenol blue with ß-Mercaptoethanol was

added per 100 μl of boiled sample.

Samples were applied to gels using a Hamilton syringe and the gels were run at a constant 20 mA until the marker dye was near the bottom of the separating gel. Gels were stained for protein for 1 hr with Coomassie blue stain (0.1% (w/v) PAGE Blue 83 in acetic acid, methanol and distilled water, 2:5:5 by vol.) and destained with 10% (v/v) acetic acid. Gels which were to be transferred were processed as described in 2.2.3.

A standard protein solution (which is used for determination of the protein M_r) was prepared and used for some gels. Sigma Dalton Mark VII-L was made up as a 2 mg/ml solution in distilled water and stored below 0° C. Standard protein solution was diluted 1:1 with sample buffer (2 x concentrated) and treated as for samples. The standard proteins and their M_r were:- bovine serum albumin (66,000); ovalbumin (45,000); glyceraldehyde 3-phosphate dehydrogenase (36,000); carbonic anhydrase (29,000); trypsinogen (25,000); trypsin inhibitor (21,000); and α-lactalbumin (14,000).

2.2.3 Western immunoblotting.

Separated proteins were electrophoretically transferred onto nitrocellulose paper (0.45 μm, (Schleicher and Schuell) Anderman Ltd.) at 0.25 A for 6 hr in a Transblot apparatus (Bio Rad); or at 0.2 A for 1 hr in a Mini Transblot (Hoefer) according to the method of Towbin et al., (1979). After transfer, the blot was blocked for a

minimum of 3 hr in phosphate buffered saline (PBS; 10 mM potassium phosphate, 150 mM NaCl, pH 7.4) containing 0.2% (w/v) gelatine (Buffer I) to quench non-specific binding of protein. Blocked blots could be stored at this stage at 4°C in the dark.

For immunostaining, blots were incubated in PBS containing 0.2% (w/v) gelatine and 0.1% (v/v) Triton-X-100 (Buffer II), containing the appropriate dilution of primary antibody for 1 hr at room temperature. Blots were washed 4 times for 5 min each in Buffer II minus antibody, then incubated in Buffer II containing 1:1000 dilution of goat anti-rabbit IgG conjugated to horseradish peroxidase (Cappel Laboratories). After a 1 hr incubation in the dark, the blots were washed 4 times for 5 min each in Buffer II and finally in PBS.

Bound horse radish peroxidase was visualised by the addition of diamino-benzidine (DAB, Sigma) solution (50mg/100 ml of 50 mM Tris-HCl, pH 7.4) followed by 0.03% (v/v) hydrogen peroxide (10 μl per 10 ml DAB solution). The reaction was terminated by the rinsing the blots several times in distilled water. Controls were run using pre-immune serum in place of primary antibody. Immunoblots were stored at 4° C in the dark.

2.3 Plant Growth Conditions for nasturtium.

Dry nasturtium (Tropaeolum majus L.) seeds (Royal Sluis, Charvil-Reading, UK) were set out (at time zero) in

trays containing autoclaved wet "Vermiculite" at a depth of 4 cm. The seeds (150 per tray) were placed on the surface of the Vermiculite, covered with a further thin layer of Vermiculite, and watered thoroughly. Trays were placed in a plant growth chamber with a 12 hr day/12 hr night regime: photon fluence rate 850 μmol/m^2 active light at tray level and a temperature of 15-20° C. Trays were covered with a transparent polythene sheeting until shoots appeared. Watering (tap water) was at 2 day intervals. Any seeds which displayed fungal contamination were discarded.

2.4 Purification of an endo 1,4 ß-D-glucanase.

The enzyme was purified essentially as described by Edwards et al. (1986).

2.4.1 Viscometric assay of endo 1,4 ß-D-glucanase.

In the purification of endo 1,4 ß-D-glucanase, the fractions from the DEAE cellulose, CM cellulose and Bio Gel P60 columns, which gave high absorbances at 280 nm, were incubated overnight with glyloid (Tamarind seed xyloglucan, Dai-Nippon Corporation, Japan), 12 mg/ml in McIlvaine phosphate citrate buffer, pH 5.0, at 30° C +/- 1° C. Viscometric flow times were calculated using a graduated pipette (volume 0.1 ml) and the activity determined by 100/viscometric flow time. These "activities" were only used as a means of pooling the appropriate fractions.

2.4.2 Isolation of the enzyme.

Nasturtium (T. majus L.) seedlings were harvested 13 days after planting, when endo ß-D-glucanase activity is at its maximum level in the cotyledons (Edwards et al., 1985). All steps, unless otherwise stated, were carried out at 4°C. Seeds were homogenised for 30 s in a blender in 2 vol. 0.2 M potassium phosphate buffer, pH 7.2 and 2% insoluble polyvinyl polypyrrolidone (PVP). The slurry was then centrifuged (19000 x g, 30 min) and the supernatant filtered through glass wool. Ammonium sulphate was then added to 90% saturation and the precipitate was collected by centrifugation (19000 x g, 30 min). The resultant pellet was re-dissolved in a minimum volume (50 ml) of 20 mM Tris-HCl, pH 7.8 and dialysed overnight against the same solution.

2.4.3 Anion exchange chromatography.

The dialysed supernatant was applied to Whatman DE52 column (2.2 x 20 cm) and the column was washed with 20 mM Tris-HCl, pH 7.8. When the absorbance at 280 nm of the eluate had fallen to a constant value, the column was eluted with a sodium chloride gradient (0-0.5 M, 500 ml) in the same buffer. The fractions containing high endoglucanase activity were pooled and brought to 90% saturation by addition of crystalline ammonium sulphate. The precipitate was collected by centrifugation (19000 x g, 30 min), dissolved in 50 mM sodium acetate, pH 5.0, and dialysed against the same buffer prior to cation exchange

chromatography.

2.4.4 Cation exchange chromatography.

The dialysed supernatant was applied to a CM cellulose column (Whatman CM52, 2.2 x 20 cm). The column was eluted with 50 mM sodium acetate, pH 5.0, until the absorbance at 280 nm of the eluate had fallen to a constant value. A sodium chloride gradient was applied (0-0.5 M, 500 ml) in the same buffer. Fractions containing high endoglucanase activity were pooled and brought to 90% saturation on the addition of ammonium sulphate.

2.4.5 Gel filtration.

The precipitate was collected by centrifugation (19000 x g, 30 min) and dissolved in a minimum volume of 50mM ammonium acetate (5 ml). The sample was then applied directly to a Bio Gel P60 column (3 x 73 cm) and washed with ammonium acetate buffer. The absorbances were read at 280 nm and those fractions containing high enzymic activity were subjected to SDS-PAGE. Fractions which contained a single band of M_r 29000 were pooled and this represented a purified sample and was used in subsequent experiments.

2.5 Characterisation of endo 1,4 ß-D-glucanase.

2.5.1 Determination of isoelectric point.

Analytical isoelectric focusing was carried out using Ampholine PAG plates (LKB Ltd.). They were run and stained with Coomassie Blue according to the manufacturer's instructions.

2.5.2 Circular Dichroism.

A circular dichroism spectrum for endo 1,4 ß-D-glucanase was recorded at 20° C in a Jasco J-600 spectropolarimeter at Stirling University. A spectrum of far-u.v. (260 nm - 200 nm) was recorded in a cell of path length 1 mm.

The mean residual ellipticities of the spectrum were calculated at 5 nm intervals using the equation:

$$[\theta]_\lambda = \frac{m.\theta_\lambda}{10.d.c.}$$

where m is the mean residue weight of an amino acid (112)

θ_λ is the observed ellipticity (degrees)

d is the path length (cm)

c is the protein concentration in g/ml

The units of $[\theta]_\lambda$ are $deg.cm^2dmol^{-1}$.

The data was collected and analysed on a data processor attached to the spectropolarimeter and the spectrum was plotted as $[\theta]_\lambda$ against wavelength (nm).

2.5.3 Investigation of the presence of glycosyl groups on endo 1,4 ß-D-glucanase.

2.5.3.1 Incubation with endo H.

The endo H preparation was isolated from Streptomyces plicatus and purchased from Boehringer Mannheim.

A sample (200 µg) of purified endo 1,4 ß-D-glucanase was denatured by heating with 20% (w/v) SDS, to increase the rate and extent of hydrolysis (Tarentino and Maley, 1974). 100 µg of this denatured sample was incubated with 10 - 50 mU/ml of endo H for 4 hr at 37° C. With substrate glycoproteins at 1 mg/ml and endo H at 30 mU/ml, high mannose oligosaccharide removal is complete in 3 to 6 hours (Tarentino and Maley, 1974; Tarentino et al., 1974). To inhibit potential proteinase activity during extended endo H digestions, 1 mM PMFS was included in the incubation mixture. Activity of endo H was confirmed by incubating 2 parallel reactions containing 10 µg of lipase (a known glycoprotein), one with and one without endo H under the same conditions. 10 µl aliquots of the incubation mixture were subjected to SDS-PAGE and stained as before (2.2.2). Increased mobility of the endo H treated test protein on SDS-acrylamide gels verifies deglycosylation has occurred during the incubation.

2.5.3.2 Incubation with PNGase F.

A sample (200 µg) of purified endo 1,4 ß-D-glucanase was incubated with 1 unit of PNGase F overnight at 37° C.

The reaction mixture also contained 10% (w/v) SDS, to denature the proteins. 10% n-octy-ß-glucoside, a non ionic detergent, was added to the denatured sample before adding the PNGase F in order to avoid inactivation of the enzyme by SDS (Haselbeck and Hösel, 1988). EDTA (0.3 M) and DTT (1M) were also included in the reaction mixture.

Aliquots of the reaction mixture (20 µl) were subjected to SDS-PAGE (2.2.2) and stained as before.

2.5.3.3 **Glycan detection.**

The glycan detection kit was purchased from Boehringer Mannheim. The basic principle of the kit involves the oxidation of adjacent hydroxyl groups in glycoconjugates to aldehyde groups by mild periodate treatment. The spacer linked steroid hapten digoxigenin (DIG) was then covalently attached to these aldehydes via a hydrazide group. Digoxigenin labelled glycoconjugates were subsequently detected in an enzyme immunoassay using an antibody alkaline phosphatase conjugate (<DIG>AP).

The method employed involved oxidation and digoxigenin labelling of glycoproteins prior to analysis by SDS-PAGE and blotting.

10 µg protein was dissolved in 20 µl sodium acetate buffer (0.1 M, pH 5.5). The sample was oxidised by incubation for 20 min in the dark at room temperature in 10 µl sodium metaperiodate (0.03 M). To destroy excess periodate, 10µl sodium disulphite (0.015 M) was added and incubated at room temperature for 5 min. Labelling was

achieved by adding 5 µl DIG-succinyl-Σ-amidocaproic acid hydrazide and incubating at room temperature for 1 hr.

Aliquots were prepared for SDS-PAGE as before (2.2.2) and run on a 10% (w/v) acrylamide gel. The gel was then transferred onto nitrocellulose as described in section 2.2.3.

After immobilisation on nitrocellulose, the proteins can be stained with Ponceau S solution, 0.2% (w/v) in trichloroacetic acid 3% (w/v), which disappears during incubation in the blocking solution. The filter was incubated for at least 30 min in blocking solution (20 ml) (Tris buffered saline (TBS), 0.05 M Tris-HCl, pH 7.5., 1.5M NaCl, containing 0.1% (w/v) gelatine), then washed 3 times for 10 min each time with approx. 50ml TBS, pH 6.5, then incubated in 10 ml of 1:1000 dilution of <DIG>AP conjugate in TBS, pH 6.5 for 1 hr at room temperature, and finally washed 3 times for 10 min each time with approx. 50 ml TBS, pH 6.5. The staining solution was prepared immediately before use and contained:- 10 ml Tris buffer, pH 9.5 (0.1 M Tris-HCl, pH 9.5; 0.05 M $MgCl_2$, 0.1 M NaCl); 37.5 µl 5-bromo-4-chloro-3-indolyl phosphate solution (50mg BCIP dissolved in 1 ml dimethylformamide) and 50 µl nitroblue tetrazolium chloride (NBT) solution (100 mg NBT dissolved in 1.3 ml dimethylformamide, 70% (v/v)). The filter was immersed in the staining solution (without shaking) until a reaction was observed, which was subsequently stopped by rinsing several times with distilled water, then dried and stored at 4° C in the dark.

2.6 Raising of an anti-endo 1,4 ß-D-glucanase antiserum and its characterisation.

2.6.1 Production of antiserum.

Antiserum was produced via injection of a rabbit (New Zealand White) with an equal volume of the antigen preparation (1 ml of a purified sample (100 µg/ml) of endo 1,4 ß-D-glucanase) and the adjuvant (Freund's complete, Sigma) which was vigorously mixed to give a stable emulsion, by repeated aspiration of the mixture into and out of a hypodermic syringe. The antigen preparation was administered at several sites. Multisite injections offer the advantage of presenting the antigen by a variety of routes with the hope of provoking the maximum immune response (Clausen, 1971). Two weeks later a further booster injection was administered in the same manner but this time Freund's incomplete adjuvant was mixed with the antigen. 14 days after the second injection a further booster was administered and blood was collected and thereafter at 14 day intervals.

2.6.1.1 Serum Preparation.

Blood samples were allowed to clot for 1 hr at room temperature. The clot was then cut into several pieces (to allow the serum to separate) and left to settle at 4° C overnight. The serum was then decanted and centrifuged (12000 rpm, 10 min) to remove any clot fragments, then aliquoted and stored at -70° C.

2.7 Characterisation of anti-glucanase antiserum.

2.7.1 Ouchterlony double diffusion assay.

Immunodiffusion analysis was carried out according to Ouchterlony (1968) to identify the number of antigen-antibody systems within a tissue extract and the antiserum.

Microscope slides (cleaned with 100% ethanol) were coated with 3 ml of 1% (w/v) agarose (low melting point, Sigma) solution dissolved in 12 mM Tris, 140 mM NaCl, 1 mM EDTA, pH 7.5. Circular wells were punched into the agarose using a needle of 2 mm bore in an array of five outer wells and one central. The small cylinders of agarose cut out by the needle were removed by suction. The centre well was filled with 10 μl pure antigen (0.5 mg/ml) and the outside wells contained dilutions of the antiserum as follows:- 1:2, 1:4, 1:8, 1:16, 1:32. The slides were placed in a petri dish containing moist filter paper and incubated at 37° C for 12-24 hrs. The agarose slides were then transferred to a tray containing 0.3 M NaCl and gently agitated for 6 hrs and thereafter with 12 mM Tris, 140 mM NaCl, 1 mM EDTA, pH 7.5 for a further 10 hrs to remove non-complexed protein.

2.8 Purification of anti-glucanase antiserum.

2.8.1 Preparation of a 95% IgG fraction.

The method employed was based on a simple salting out procedure used by Harboe and Inglid (1973).

To 2 ml crude serum, solid sodium sulphate was added to give 14% (0.14 g per ml) and allowed to dissolve. A precipitate formed slowly over 5 min. These steps and all the following steps were carried out at 25° C. The solution was centrifuged (14000 x g, 15 min) and the supernatant decanted. The pellet was resuspended in 1 ml of 14% sodium sulphate and spun as before. This step was repeated. The resultant pellet was resuspended in PBS, pH 7.2 (2 ml) containing 0.01% Thimerosal as preservative (Sigma). This preparation was stored at -20° C.

2.8.2 Purification of the antiserum by an affinity technique.

This method was developed by Dr A. Slabas at Unilever Research (Colworth House, U.K.).

Pure protein (100 μg) was applied to a 12% SDS gel and transferred as before (section 2.2.3). The band on the nitrocellulose corresponding to the pure protein was visualised using Ponceau S solution (Boehringer Mannheim) (0.1%) and cut out using a sterile scalpel, then destained in Tris buffered saline (TBS), pH 7.5. The nitrocellulose band was incubated with 500 μl crude serum in 20 ml TBS pH 7.5 containing 1% haemoglobin, overnight with rotation at 4° C. The band was then washed (4 x 10 min) in TBS and the affinity purified serum was eluted with 5 ml 0.2 M glycine/HCl, pH 2.8. The nitrocellulose band was returned to the crude serum/TBS/haemoglobin mixture which could be re-used to obtain more of the affinity purified

preparation. The affinity purified serum was brought back to neutrality on addition of 1 M NaOH (tested using pH papers) and stored in 25 ml TBS/1% haemoglobin at 4° C.

2.9 Light and electron microscopy.

2.9.1 Light microscopy and histochemistry.

Small pieces (1 mm^3) of seed were cut and fixed in 2% (v/v) glutaraldehyde in 25 mM sodium cacodylate buffer, pH 7.2. After fixation, specimens were washed and dehydrated in an ascending series of ethanol: 10 - 100% (v/v) for 1 hr then overnight in 100% at 4° C. The samples were then placed in several changes of JB4 methacrylate (Polysciences, U.K.) resin before being embedded in the same resin in BEEM capsules and allowed to polymerise at room temperature.

The resultant blocks were cut into 4 μm sections with a glass knife using an LKB pyramitome, and heat dried onto glass slides. Sections were stained using 1% (w/v) amido black in 7% (v/v) acetic acid to stain protein (Lillie, 1969); Periodic acid Schiffs reagent (PAS) to stain periodate-reactive carbohydrate polymers (Feder and O'Brien, 1968) and Mitchell's reagent to stain xyloglucan (Kooiman, 1960). Sections were mounted in Histomount on glass slides and photographed using a microscope (Zeiss, Oberkochen, FRG) with camera attachment.

2.9.2 Indirect immunofluorescence.

1 mm^3 pieces of seed were cut and fixed following vacuum infiltration in a solution of 4% (w/v) paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4 for 2-3 hr at room temperature. After fixation, specimens were dehydrated in an ascending series of ethanol and placed in several changes of JB4 resin. The tissue was finally embedded in the same resin in BEEM capsules at 4°C. Thin sections (4 μm) were cut using a glass knife on an LKB pyramitome and placed onto a drop of water on glass microscope slides and dried on a heating block. In this way the sections are "heat fixed" onto the slides. The sections were washed with phosphate buffered saline (PBS: 10 mM KPO_4, pH 7.4 containing 150 mM NaCl) and blocked for 1 hr in PBS/0.2% (w/v) gelatine to reduce non-specific binding. These and the following steps were carried out at room temperature. The sections were incubated with crude antiserum for 1 hr at a dilution of 1:50 in PBS containing 0.5% (w/v) bovine serum albumin (PBSA). After three 10 min washes with PBSA, a further incubation was performed for 1hr with anti-rabbit IgG-fluorescein isothiocyanate (FITC) conjugate (Sigma) at a dilution of 1:50 in PBSA. Following washings (3 x 10 min) in PBSA, the sections were mounted in a mixture of one part PBSA and two parts glycerol and 0.1% (w/v) phenylenediamine (Johnson and de Nogueira Araujo, 1981) and the edges of the coverslip sealed with nail polish. Controls were included either omitting the primary antibody or using pre-immune serum in the incubation mix.

The sections were analysed under a Zeiss (Oberkochen,FRG) microscope equipped for epi-fluorescence with a filter system appropriate for FITC fluorescence.

2.9.2.1 Preparation of immune serum by starch-affinity chromatography.

To 5 g potato starch 2 volumes distilled water was added and the mixture spun (4000 rpm, 10 min) in the bench centrifuge. The water was decanted and the procedure repeated twice. The insoluble starch was packed into a 2 ml disposable syringe plugged with a small piece of glass wool. This column was washed thoroughly with PBS. A 0.5 ml sample of crude/pre-immune antiserum was applied to the column and washed with PBS. The eluent was collected and the volume measured to calculate the dilution factor of the antiserum.

2.9.3 Immunogold labelling.

2.9.3.1 Embedding and fixation of specimens.

1 mm^3 sections of seed were cut and fixed following vacuum infiltration in a mixture of 4% paraformaldehyde and 2.5% glutaraldehyde, to simultaneously preserve antigenicity and structure (Herman, 1988) in 0.1 M sodium cacodylate buffer, pH 7.4 for 2 hr at room temperature. Samples were then dehydrated in a graded series of ethanol at 4° C before infiltration in a 1:1 solution ethanol:LR White resin (Agar Scientific) for at least 1 hr then

overnight in LR White, then polymerised at 65° C for 16 hr in BEEM capsules.

2.9.3.2 Formation of colloidal gold.

The procedure used was that of Roth (1983) to yield gold particles of 15 nm diameter. 0.01% tetrachloroauric acid (100 ml) was boiled in a clean conical flask. When boiling, 4 ml 10% (w/v) trisodium citrate was added and the solution boiled until the suspension turned a deep burgundy colour.

2.9.3.3 Estimation of the minimal amount of protein A needed for stabilisation of colloidal gold.

The gold number is defined as the number of mg of protein added to 10 ml of colloidal gold which just fails to prevent the colour change from red to blue upon the addition of 1 ml of 10% (w/v) NaCl.

The procedure followed was that of Roth *et al.* (1978). To 0.5 ml colloidal gold solution, 0.1 ml sample of serially diluted protein A was added. After 1 min, 0.1 ml of 10% (w/v) aqueous NaCl solution was added and the stabilisation effect was judged visually by the colour of the solution. The lowest protein concentration which prevented a colour change from red to blue after the addition of NaCl was considered to be sufficiently stabilising. 7 μg protein A was found to stabilise 0.5 ml of colloidal gold solution.

2.9.3.4 **Preparation of a crude gold complex.**

To 5 ml of the colloidal gold solution, 140 µl of a 0.5 mg/ml solution of protein A was added. After 2 min, 1 drop of polyethylene glycol (PEG) 20,000 was added as a stabilising agent as it lowers the rate of aggregate formation (Horisberger et al., 1985; Horisberger and Rosset, 1977). To remove any free uncomplexed protein and not fully stabilised gold particles, the mixture was centrifuged (12,000 rpm, 20 min) and the clear supernatant containing any free protein A was discarded. The dark sediment formed by the protein A-gold complex was resuspended in 0.5 ml TBST (TBS: 50 mM Tris-HCl, pH 7.4, 0.15 M NaCl containing 0.1% (v/v) Tween 20) containing 0.02 ml 1% (w/v) PEG 20,000 and stored at 4°C.

2.9.3.5 **Cytochemical labelling.**

Ultrathin sections were cut on an LKB ultramicrotome using a diamond knife by Miss Debbie Wright and collected on nickel grids. The grids were floated on 10 µl of TBS containing 0.2% (w/v) gelatine for 30 min to block non-specific binding. These and the following steps were carried out at room temperature. The grids were washed thoroughly with TBS (2 x 5 min) and incubated on 10 µl aliquots of endoglucanase antiserum diluted 1:50 in TBS containing 0.1% Tween 20 (TBST) for 30 min. After washing (3 x 5 min TBST), the grids were incubated with protein A gold for 30 min, then washed thoroughly with TBS followed by distilled water. The sections were examined without

uranyl acetate staining in the transmission electron microscope.

This procedure was also performed using goat anti-rabbit colloidal gold conjugate (Sigma) in place of the protein A-gold complex.

2.10 Germination Time Course.

To determine at what point in the growth cycle of nasturtium, the enzyme endo 1,4 ß-D-glucanase was produced or if indeed the protein is present throughout growth, a time course was set up. Time zero was regarded as time of planting, and seed germination and growth conditions were as described in Section 2.3. Samples were collected at two day intervals as described below.

2.10.1 Sampling of seeds and seedlings.

Random selection of seeds and seedlings was employed until day 5, thereafter only germinated seeds were used. Duplicate samples of 15 seeds were taken every 2 days until 28 days after planting.

2.10.2 Cotyledon extracts for enzyme assays.

Each cotyledon sample was ground at 4° C in a mortar and pestle with sand (1 g), insoluble PVP (0.15 g) and 0.2M potassium phosphate buffer, pH 7.2 (15 ml). Homogenates were allowed to stand for 1 hr at 4° C, then spun (26,000 x g, 30 min). A sample of the clear liquid between the

pellet and any surface layer of lipid was used for enzyme assays and protein blotting.

2.10.3 Viscometric assay for endo 1,4 ß-D-glucanase.

A 1.2 % (w/v) solution of glyloid (Tamarind seed xyloglucan, 0.6 ml) was immersed in a 30° C +/- 0.1° C water bath. Crude cotyledon extract (Section 2.10.2.), diluted if necessary (0.4 ml) was added. Viscometric flow times were determined using a graduated pipette (volume 0.1 ml). Flow time readings were taken at 5 min intervals, from time of mixing (time zero) until a linear relationship between flow time and elapsed time had been established (approx. 45 min). The activity of the enzyme, in arbitrary units, was calculated from the plot of flow time against elapsed time, extrapolated back to give flow time at time zero. The activity was defined as $100/t_{0.8}$, where $t_{0.8}$ is the elapsed time taken for the flow time to decrease to 0.8 of its value at zero. Provided a linear relationship between elapsed time and flow time was obtained, the activity was directly proportional to the amount of enzyme.

2.10.4 Determination of endo 1,4 ß-D-glucanase protein.

To determine how much protein was present at a specific time, a quantitative method was employed using an absolute standard of endo 1,4 ß-D-glucanase, whose protein content had been determined by amino acid composition analysis by Mr Chris Sidebottom at Unilever Research. This sample was subjected to SDS-PAGE (Section 2.2.2) and then

to Western Immunoblotting (Section 2.2.3). The blot was then scanned using a reflective densitometer and the area under the peak determined and related back to the amount of protein loaded. In this way a standard calibration curve could be set up and any unknown sample could be scanned and the area under the peak calculated as a protein concentration.

2.11 RNA Isolation and Purification.

2.11.1 Total RNA extraction.

The method described here was the most successful of the several procedures tested and was based on Hall et al. (1978).

Cotyledons were excised and stored at -80° C. Frozen material (2 g) was milled in a coffee grinder with solid CO_2 (to ensure the process was kept at a low temperature to prevent RNase activity), then transferred to a sterile mortar and ground to a fine powder in the presence of liquid nitrogen. Homogenisation buffer, 4 ml (0.2 M Tris, pH 9.0, 30 mM EDTA, 1% SDS, 0.4 M NaCl) was added and the frozen mixture was homogenised in the mortar. This step was repeated with an additional 4 ml of homogenisation buffer. Additional liquid nitrogen was added when required to ensure the mixture was kept frozen. The ground mixture was transferred to a sterile corex tube and proteinase K (2.6mg) was added and the mixture incubated at 37° C for 1 hr, which helps to increase the yield of nucleic acids and

maintain their integrity by degrading most of the extracted protein with proteolytic enzymes. 2 M KCl (0.6 ml) was added and the sample chilled on ice for 15 min. The supernatant was collected on centrifugation (12,000 rpm, 15 min) at 4° C, and the nucleic acids were precipitated overnight in 0.5 vol 6 M LiCl (final concentration 2 M) at 4° C.

The pellet was collected by centrifugation (12,000 rpm, 20 min, 4° C) and washed twice in 2 M LiCl to help dissolve any contaminating DNA, low molecular weight RNA's and protein. The pellet was then recovered on centrifugation (12,000 rpm, 20min, 4° C) and dissolved in sterile H_2O. After further centrifugation (12,000 rpm, 20 min, 4° C), the RNA was extracted from the supernatant using phenol/chloroform.

2.11.1.1 **Preparation of a phenol/chloroform solution.**

Phenol solution was prepared from colourless crystalline pure batches only (Sigma). Crystals were melted at 68° C and 8-hydroxyquinoline was added to a final concentration of 0.1%. The phenol solution was extracted with 1.0 M Tris pH 8.0 until the pH of the aqueous phase was not less than 7.8, then stored under 10 mM Tris pH 8.0. The phenol/chloroform solution used was made by mixing equal volumes of phenol and chloroform solutions.

Following addition of an equal volume of the phenol/ chloroform mixture to the RNA solution the phases were mixed by vortex mixing and were separated by centrifugation

at 12,000 rpm for 10 min at room temperature. The aqueous phase was transferred to a clean tube and re-extracted if necessary The RNA was precipitated in an equal volume of 100% (v/v) ethanol overnight at -20° C, then pelleted by centrifugation (10,000 rpm, 15 min, 4° C) and washed twice with 70% (v/v) ethanol. The pellet was dried *in vacuo* and resuspended in 200 µl sterile H_2O. Its concentration was determined from its absorbance at 260 nm assuming an absorbance of 1 was equivalent to 40 µg ml^{-1} RNA (Maniatis *et al.*, 1989). The absorbance ratio of 260 nm/280 nm provided an estimation of the purity of the RNA sample as a pure preparation has an ratio of 2.0 (Maniatis *et al.*, 1989).

2.11.2 Gel electrophoresis of RNA.

A 1.3% (w/v) agarose gel was prepared by boiling 1.3g of agarose in 72.5 ml of distilled water until melted. The gel solution was cooled to 60° C and 10 ml of 10 x MOPS buffer (0.2 M morpholinopropane sulphonic acid, 50 mM sodium acetate, 10 mM EDTA, pH 7.0) was added with 17.3 ml of 37% (w/v) formaldehyde solution. The gel was cast and allowed to set at room temperature. RNA samples were prepared by adding an equal volume of sample buffer, which contained 2.4 x MOPS, 47.6% (v/v) formamide and 10.6% (v/v) formaldehyde. Prior to loading, the samples were heated at 60° C for 5 min then 0.25 vol of loading buffer (0.1 M EDTA, pH 7.0, 25% (w/v) Ficoll type 400, 0.5 µg/ml ethidium bromide) was added. The gel was electrophoresed in 1 x MOPS

containing 6.4% (v/v) formaldehyde for 3 - 4 hr at a constant 40 mA until the dye had almost run off the gel. RNA was visualised by U.V. illumination of the gel which caused fluorescence of RNA bound to ethidium bromide and photographed using a Polaroid instant film (type 667) through a Kodak Wratten filter No. 23A.

2.11.3 Isolation of polyadenylated (poly A^+) RNA.

2.11.3.1 Oligo (dT)-cellulose chromatography.

The method used was that of Aviv and Leder (1972). The oligo(dT) cellulose was equilibrated with 20 mM Tris-HCl, pH 7.6, 0.5 M NaCl, 1 mM EDTA, 0.1% SDS (loading buffer) and poured into a sterile pasteur pipette. The column was washed with three column volumes of sterile H_2O followed by 0.1 M NaOH containing 5 mM EDTA followed by sterile H_2O, then with 5 volumes loading buffer before application of the sample. The sample was heated to 65° C for 5 min, before addition of an equal volume of 2 x loading buffer, and was applied to the column. The flow through was collected and re-applied to the column. The column was then washed with 5-10 column volumes of loading buffer followed by 4 column volumes containing 0.1 M NaCl. The poly A^+ RNA was eluted with 2-3 column volumes of 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.05% SDS. Sodium acetate (3M) was added to give a final concentration of 0.3 M, before precipitation with 2.2 volumes 100% ethanol at -20° C. The pellet was washed in 70% (v/v) ethanol and re-dissolved in sterile H_2O.

2.11.3.2 Isolation of poly A^+ RNA using messenger affinity paper (mAP).

Due to the comparatively low yield of total RNA obtained (<500 µg/2 g fresh tissue), the poly A^+ was recovered using messenger affinity paper (mAP) by a method devised by Wreschner and Herzberg (1984). This alleviates the loss of message incurred by the repeated passage through columns and contamination by salt.

Total RNA samples (concentrated to 10 mg/ml), 200 µg (approx 20 µl) were heated at 65° C for 5 min, chilled on ice and then 4 M NaCl was added to give a final concentration of 0.5 M NaCl. Pieces of mAP (1 cm^3) were placed onto several layers of normal filter paper, pre-soaked with 0.5 M NaCl and left to air dry. The RNA samples were applied to the mAP pieces and allowed to dry, these then were washed twice with 0.5 M NaCl and once with 70% (v/v) ethanol. The poly A^+ RNA was eluted by placing the mAP pieces into sterile water and heating at 70° C for 5 min.

For *in vitro* translation and immunoprecipitation experiments, the poly A^+ RNA was re-precipitated in 100% ethanol at -20° C, washed with cold 70% (v/v) ethanol, dried and re-dissolved in sterile water.

2.11.4 *In vitro* Translation.

Poly A^+ RNA and Total RNA preparations from nasturtium seeds were translated in a standard amino acid incorporation system from wheat (Marcus *et al.*,1974). RNA samples (approx 1 µg poly A^+, 4 µg total in a volume of 4µl)

were incubated in 10 µl of translation mix for 1 hr at 25° C. Translation mix contained 27 µl potassium acetate, 18 µl amino acid mixture minus methionine, 15 µl ^{35}S-methionine and 135 µl wheat germ extract. The incubated sample was divided into two samples, one was used for immunoprecipitation the other for electrophoresis of the translated proteins.

2.11.5 Immunoprecipitation of translated proteins.

A sample of the translation mix (7 µl) was diluted 1:100 in buffer A (1% Triton X-100, 50 mM Tris-HCl, pH8.0, 0.3 M NaCl, 1% SDS), a 2µl sample of anti- endoglucanase antiserum was added and incubated for 3 hr with rotation at room temperature. The incubated mixture was transferred to a clean, sterile eppendorf containing 5mg protein A sepharose and incubated for a further 1 hr at room temperature with rotation. The immunoprecipitate was washed three times in buffer A and once in 0.005 M Tris-HCl, pH 8.0. The sample was prepared for SDS-PAGE (Section 2.2.2) and run using a 10% polyacrylamide gel.

2.11.6 Fluorography and autoradiography of translation and immunoprecipitation gels.

Samples were subjected to SDS-PAGE as described in section 2.2.2. The gel was prepared for fluorography by treatment with 2,5-diphenyloxazole (DPO) dissolved in dimethylsulphoxide (DMSO), prior to drying under a vacuum (Bonner, 1984). Fluorography was performed on a dry gel

using a pre-flashed Kodak X-AR-5 x-ray film and left to develop at -70° C for up to 4 weeks.

2.12 Effect of embryo removal on induction of endo 1,4 ß-D-glucanase.

To discover the effect the embryo/embryonic axis had on the production of the enzyme, the axis was removed at 2 day intervals after planting and growth was allowed to continue until day 15 after planting (after maximum enzyme activity, Edwards et al., 1985). The axis was removed by dissecting the seeds into two halves and the half seeds allowed to continue growing separately, the half seed containing the embryo being maintained as a control. The half seeds were surface sterilised with 70% (v/v) ethanol containing 1% (v/v) Tween 20, then grown on 3 layers of moist filter paper in sandwich boxes and watered (tap water) when necessary. Any seeds exhibiting microbial or fungal infection were removed. Incubation conditions were as described before in Section 2.3.1. Sampling of seeds and cotyledon extracts were as described before (Section 2.10.1, 2.10.2).

2.12.1 Effect of Plant Growth Hormones on induction of endo 1,4 ß-D-glucanase.

To study the effect of plant growth hormones on the induction and the activity of the enzyme, de-embryonated half seeds were used.

The half seeds were surface sterilised with 70% (v/v) ethanol containing 1% (v/v) Tween 20 and samples were placed on 3 layers of filter paper in plastic boxes with lids to prevent evaporation. The filter paper was soaked with either 10^{-4} M, 10^{-5} M, 10^{-6} M GA_3 or 10^{-4} M, 10^{-5} M, 10^{-6} M 2-4-D containing 1 mM $CaCl_2$ and 300 mg/l ampicillin and kept moist throughout the duration of the experimental incubation. The embryos were removed at 2 day intervals after planting and assays were conducted at 3, 6 and 9 days after initial planting. Sampling of seeds (Section 2.10.1) and preparation of cotyledon extracts (Section 2.10.2) were as described before.

CHAPTER THREE

CHAPTER 3

PURIFICATION AND CHARACTERISATION OF AN ENDO 1,4 ß-D-GLUCANASE AND THE RAISING OF A MONOSPECIFIC ANTISERUM AGAINST IT.

This chapter describes the purification and characterisation of an endo 1,4 ß-D-glucanase from nasturtium cotyledons, the use of the pure enzyme to obtain an antiserum against it and the characterisation of this antiserum.

3.1 Purification of endo 1,4 ß-D-glucanase.

Endo 1,4 ß-D-glucanase was purified essentially as described by Edwards *et al.* (1986). Nasturtium seeds were harvested 13 days after planting as this represented the peak level of activity of the enzymes involved in the hydrolysis of galactoxyloglucan (Edwards *et al.*, 1985). Cotyledon extracts were concentrated by ammonium sulphate and subjected to anion exchange chromatography on a Whatman DE-52 column, followed by cation exchange chromatography on a CM cellulose column and finally by size exclusion chromatography on Biogel P-60.

Typical elution profiles of the three chromatographic columns are depicted on Figure 3.1. Protein was detected at 280 nm and activity was assayed viscometrically. The profile from the gel permeation column (Figure 3.1 (c))

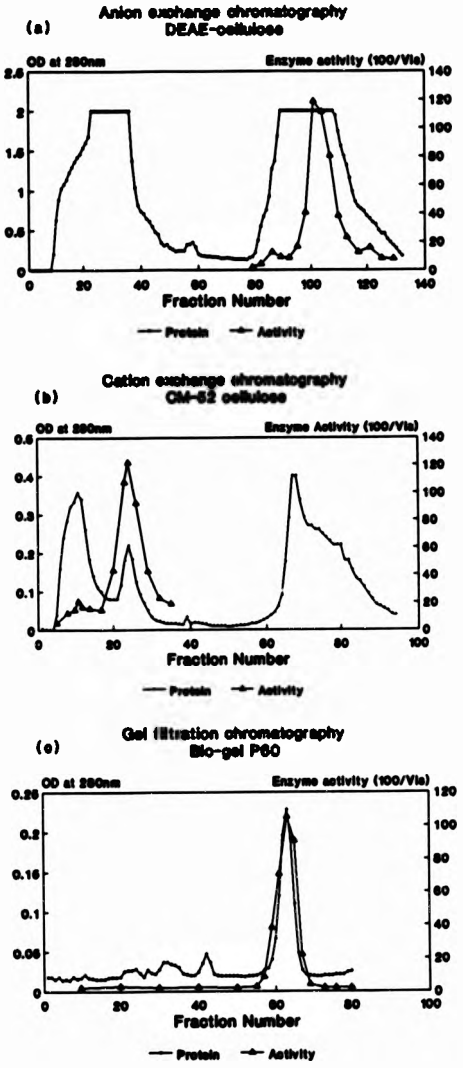


Figure 3.1
Elution profiles of the three chromatographic columns used to purify endo 1,4 ß-D-glucanase

shows a single symmetrical peak. Fractions from this peak were subjected to SDS-PAGE and those samples which gave a single band of apparent molecular weight at 29,000 were pooled, and used in subsequent experiments.

Samples of crude and pure preparations were subjected to SDS-PAGE as shown on Figure 3.2. The purification procedure resulted in a single band of molecular weight 29,000. The purification data shown in Table 3.1 suggested that a 12 fold purification resulted in a homogeneous enzyme. This implies that the enzyme constituted about 9% of the total protein in the cotyledon extract, but from Figure 3.2 it was clear that the enzyme represents a smaller percentage. An overestimation of the specific activity during the earlier stages of purification may have occurred due to synergistic interactions between the endo 1,4 ß-D-glucanase and other xyloglucan degrading enzymes present. Edwards et al. (1986) recorded a 10 fold purification for the endo 1,4 ß-D-glucanase, which again was an underestimation. From about 800 g fresh weight of tissue, approximately 2 mg purified protein was obtained.

3.2 Characterisation of endo 1,4 ß-D-glucanase.

The observation of Edwards et al. (1986) were confirmed with respect to molecular weight (Figure 3.2) and to the determination of the isoelectric point (see section 3.2.1). The authors also reported that a small amount of carbohydrate (< 5%) may have been associated with the

Figure 3.2

SDS-PAGE of the purification of endo 1,4 ß-D-glucanase. Lane A contains a sample (10 µl) of crude extract, lane B a sample (10 µl) of DEAE column exluate, lane C a sample (10 µl) of CM column luate. Lanes D-I contain samples (10 µl) from fractions eluted from the Biogel P-60 column.

Molecular weight markers are:

(1) α-lactalbumin (14,200); (2) soybean trypsin inhibitor (20,100); (3) trypsinogen (24,000); (4) carbonic anhydrase (29,000); (5) glyceraldehyde 3-phosphate-dehydrogenase (36,000); (6) ovalbumin (45,000) and (7) bovine serum albumin (66,000) as shown in lane J.

Table 3.1 **Purification data for endo 1,4 B-D-glucanase**

	Total vol. (ml)	Total protein (mg)	Act.	S.A. units /mg	% recovery
Extract	120	558	42858	76.81	100
DEAE cellulose[a]	169	74.9	10562.5	141.0	25
CM cellulose[b]	5	15.64	8450	540.3	19.7
Biogel P-60	32	3.2	2667	833.3	6.22

Act. Activity in viscometric units

S.A. Specific Activity

a ammonium sulphate concentrated, dialysed against 20 mM Tris-HCL, pH 7.8.

b after concentration of pooled fractions by ammonium sulphate saturation.

enzyme. This was further investigated by the use of specific carbohydrate cleaving enzymes and a carbohydrate detection system which involved the reaction of a labelled antibody with the steroid hapten digoxigenin.

3.2.1 Determination of molecular weight and isoelectric point.

As can be seen from Figure 3.2, the apparent molecular weight of the enzyme is 29,000. Analytical isoelectric focusing was carried out using Ampholine PAG plates (LKB Ltd.). They were run according to the manufacturers instructions and stained with coomassie blue. Figure 3.3 also yielded a single band demonstrating the purity of the preparation with the isoelectric point being 5.0. These observations concur with those reported by Edwards et al. (1986).

3.2.2 Circular dichroism.

The far-u.v. c.d. spectrum obtained for endo 1,4 ß-D-glucanase is shown on Figure 3.4. The analysis indicated that the protein contains no α-helix, 66% ß sheet and 34% other.

3.2.3 Investigation of the presence of glycosyl groups on endo 1,4 ß-D-glucanase.

In recent years glycosidases have become valuable and useful tools for the analysis of glycoproteins (e.g. Steube et al., 1985) and glycopeptides (e.g. Tarentino et al.,

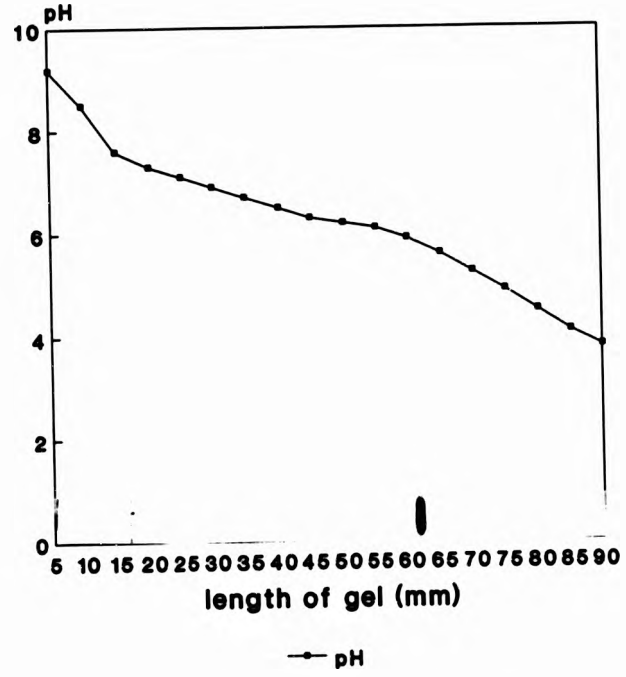


Figure 3.3
Isoelectric focusing for pure endo 1.4 ß-D-glucanase

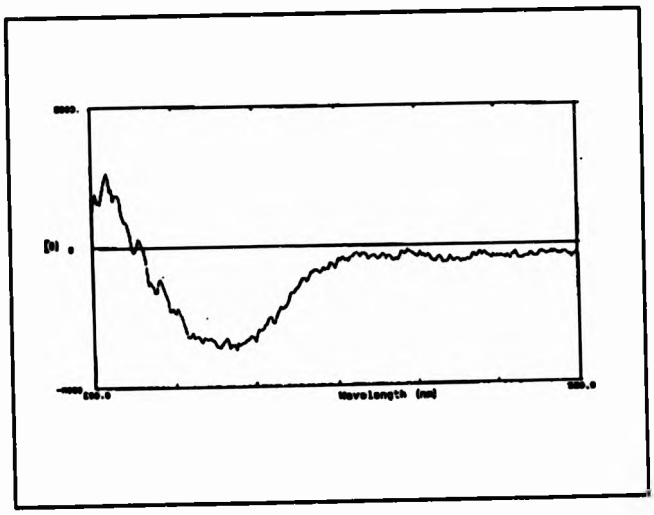


Figure 3.4

Far-u.v. circular dichroism spectrum of endo 1,4 ß-D-glucanase.

1985). Endoglycosidase H (Endo H), Endoglycosidase F (Endo F) and N-Glycosidase F (PNGase F) are among the most important for the hydrolysis of N-glycans.

Endo F and Endo H hydrolyse the diacetylychitobiose linkage of N-linked carbohydrates, whereas PNGase F directly hydrolyses the aspartylglycosylamine linkage (as shown in Table 2 from Tarentino et al., 1985).

Hydrolysis by Endo H (isolated from Streptomyces olicatus) is restricted to high mannose type oligosaccharides (Tarentino et al., 1978). The bond susceptible to enzyme hydrolysis is apparently associated with di-N-acetylchitobiose since on hydrolysis, a single N-acetylglucosamine residue remains with asparagine, whereas the other becomes the reducing end of the oligosaccharide (Tarentino et al., 1974). Tarentino et al. (1974) also noted that treatment with Endo H had no visible effect on enzyme activity or on the c.d. difference patterns, but that carbohydrate depleted proteins migrated somewhat faster on acrylamide gel electrophoresis. This was the parameter used to detect whether endo 1,4 ß-D-glucanase contained any oligosaccharides.

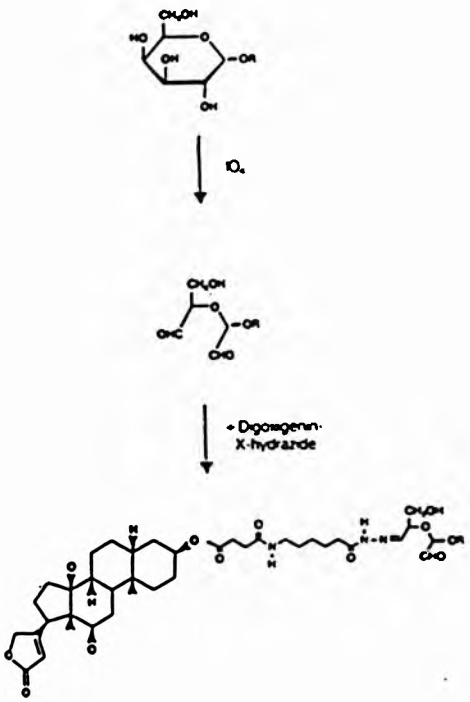
The rate and extent of hydrolysis is enhanced when substrate glycoproteins are denatured by heating (at 100° C) in a 1-2 fold weight excess of SDS for 1-2 minutes (Tarentino and Maley, 1974). The SDS is diluted before the reaction is allowed to proceed. With few exceptions, glycosylation sites, on proteins in their native state, are generally inaccessible to oligosaccharide chain cleaving

enzymes (Chu et al., 1981) and denaturation is necessary to promote complete access to these sites (Tarentino et al., 1978; Tarentino and Plummer, 1982).

Both PNGase F and Endo F hydrolyse high mannose chains, but only the former was effective on all types of complex oligosaccharides (Tarentino et al., 1985). N-Glycosidase F is isolated from the culture filtrate of Flavobacterium meningosepticum and differs from Endo H by cleaving the N-glycan linkage between asparagine and the carbohydrate chain (Tarentino et al., 1985). As with deglycosylation with Endo H, the action of PNGase F is increased when the substrate is denatured. However, Haselbeck and Hösel (1988) noted that ionic detergents, such as SDS, completely inactivate the enzyme. They also noted that non-ionic detergents, such as Triton-X-100, Nonidet P-40 and octyglucoside, have a protecting influence on enzyme activity. It is not known whether non-ionic detergents really stabilise the enzyme or merely prevent the binding of the hydrophobic protein to the walls of the test tube. This inactivation can also be prevented by the addition of protein to the incubation mixture, but this is not as efficient as the non-ionic detergents (Haselbeck and Hösel, 1988). Nonidet P-40 was found to be the most effective detergent, but was also found to interfere with protein separation on SDS gels hence octyglucoside was employed as this did not have to be removed from the incubation mix before SDS-PAGE analysis.

The principle of the carbohydrate detection system is

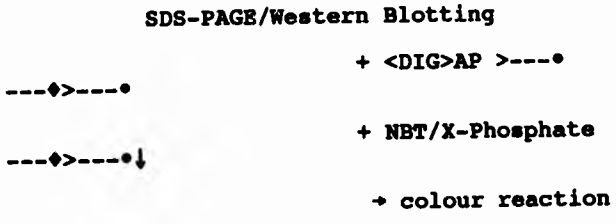
the reaction of a labelled antibody with the steroid hapten digoxigenin. Digoxigenin is introduced into sugars of glyco-conjugates chemically by the reaction outlined below:-



The detection of glycoproteins on electroblots is frequently achieved using lectins as probes (Glass et al., 1981; Clegg, 1982; Gershoni and Palade, 1982). However, since lectins have restricted sugar specificities, their uses as generalised glycoprotein detection reagents are limited. In addition, a complication of this technique is

that the solution used for blocking remaining protein binding sites on the nitrocellulose needs to be devoid of glycoproteins (O'Shannessy et al., 1987). More recently, use has been made of enzyme hydrazides (Gershoni et al., 1985; Keren et al, 1986) to detect aldehydes generated on the oligosaccharide moieties of glycoproteins by mild periodate oxidation.

The spacer linked steroid hapten digoxigenin (DIG) can be covalently attached to these aldehydes via a hydrazide group. Digoxigenin labelled glycoconjugates are subsequently detected in an enzyme immunoassay using an antibody, alkaline phosphatase, conjugate (<DIG>AP).



The glycan detection kit, especially when used with PNGase F is generally suited for the detection of carbohydrates in proteins, as the removal of carbohydrate chains can be monitored by changes in molecular weight in gel electrophoresis as well as by the absence of a carbohydrate reaction.

3.2.3.1 Incubation of endo 1,4 ß-D-glucanase with endo H.

The activity of the Endo H enzyme was confirmed by incubating two parallel reactions, one containing 5-10 µg of a known glycoprotein (in this case lipase was used), the other 5-10 µg of the endo 1,4 ß-D-glucanase. Controls were also included where no Endo H was added.

Figure 3.5 shows the reaction of Endo H on endo 1,4 ß-D-glucanase (lanes 6 and 7) and on lipase (lanes 4 and 5). There is a distinct shift in the molecular weight of lipase, indicating that the Endo H was active, but no apparent shift can be seen for endoglucanase. There are two possible explanations for this:- either the endo 1,4 ß-D-glucanase contains no glycosyl residues (i.e. is not a glycoprotein) or the carbohydrate associated with it is of a more complex nature, as is the case with DNase A and RNase B which remain unaffected by Endo H action (Tarentino et al., 1974).

3.2.3.2 Incubation of endo 1,4 ß-D-glucanase with PNGase F.

The enzyme PNGase F, which will cleave the more complex carbohydrate residues, was then tested on endoglucanase. Again a parallel reaction containing lipase, with and without PNGase F was employed to ensure enzyme activity. The results are depicted on Figure 3.6. Again there is no apparent shift in the molecular weight of endoglucanase (lanes 4 and 6), with the corresponding shift for lipase (lanes 1 and 3) indicative of enzyme activity. These results suggest that the endoglucanase

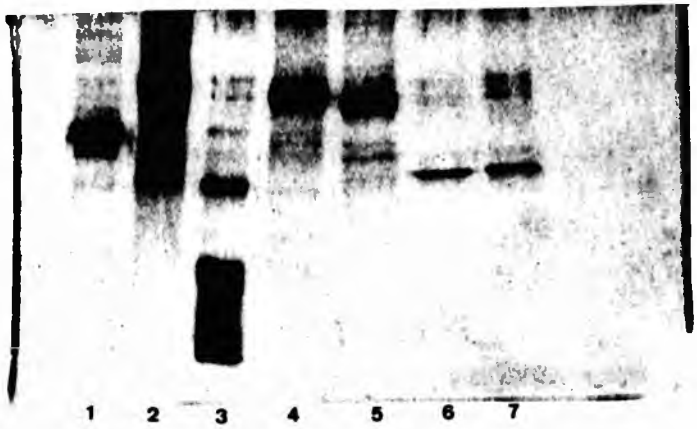


Figure 3.5

SDS-PAGE of endo 1,4 ß-D-glucanase incubated with endo H.

Lanes 1-3: molecular weight markers.

Lane 4: lipase control.

Lane 5: lipase with endo H

Lane 6: endo 1,4 ß-D-glucanase control.

Lane 7: endo 1,4 ß-D-glucanase with endo H.

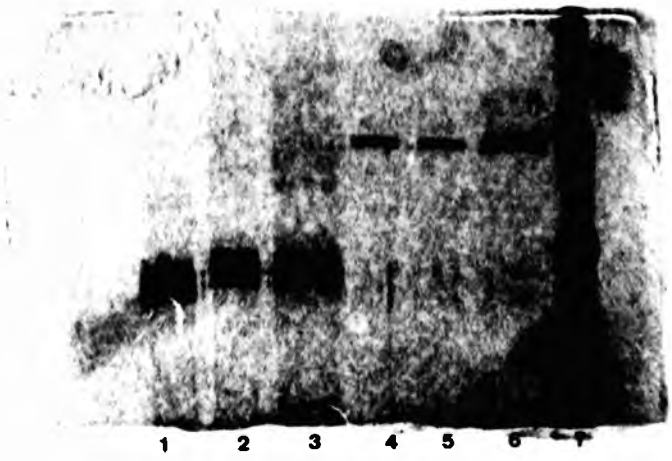


Figure 3.6

SDS-PAGE of endo 1,4 ß-D-glucanase incubated with PNGase F.

Lane 1: lipase with PNGase F.

Lanes 2&3: lipase control.

Lane 4: endo 1,4 ß-D-glucanase with PNGase F.

Lanes 5&6: endo 1,4 ß-D-glucanase control.

Lane 7: molecular weight markers.

does not have either high mannose or complex sugar residues attached to it, giving a strong indication that it is not a glycoprotein.

3.2.3.3 Glycan detection.

To further test this hypothesis, a glycan detection kit was purchased from Boehringer Mannheim. For this experiment both the control glycoprotein (lipase) and the endo 1,4 ß-D-glucanase were incubated with and without PNGase before labelling with digoxigenin. The working instructions provided with the kit mention two procedures for digoxigenin labelling of glycoproteins; method A, where the label is added prior to Western transfer or method B where the label is added after Western transfer. Method A was employed because it allows greater detection sensitivity, but may yield broad or slightly smeared bands due to varying amounts of DIG bound to each glycoprotein molecule and subsequent SDS-PAGE. 10 µg of both enzymes were treated with PNGase F and oxidised in solution and labelled with digoxigenin. The derivatised samples were subsequently resuspended in SDS sample buffer and a 1 µg sample was subjected to SDS-PAGE. The incorporated digoxigenin was then detected on the nitrocellulose in an enzyme immunoassay.

Figure 3.7 shows the results obtained:- lanes 1 and 2 contained treated (with PNGase F) and untreated endoglucanase with lanes 3 and 4 containing treated and untreated lipase. The absence of any visible band for the

Figure 3.7

Immunoblot of glycan detection reaction.

Lane 1: endo 1,4 ß-D-glucanase with PNGase F.

Lane 2: untreated endo 1,4 ß-D-glucanase.

Lane 3: lipase with PNGase F.

Lane 4: untreated lipase.

endoglucanase would tend to suggest that the endoglucanase contains very little or no carbohydrate. The detection limit varies for each glycoprotein, but the kit will detect 10 ng of α-acid glycoprotein.

3.3 Raising of an anti-endo 1,4 ß-D-glucanase antiserum and its characterisation.

Antisera may be produced against a single protein antigen (monospecific antiserum) or against a mixture of protein antigens (polyspecific or multispecific antiserum). It is, of course, desirable for many immunochemical studies on enzymes or proteins to produce a monospecific antiserum (Walker et al., 1976).

It should always be assumed that an antigen which is pure by biochemical criteria (e.g. a single component on SDS-PAGE) is not pure immunologically (Mayer and Walker, 1978). It is well known that an injection of "pure" protein antigens into a number of animals of the same species will not cause the same immune response in all of them. Some animals may form only one specific antibody, while others may form antibodies against other antigens. This "polyspecific" response to a pure antigen may be explained by adsorption of a few foreign antigen molecules onto it (Clausen, 1971).

It should also be noted that enzyme and protein purification procedures give rise to preparations in buffered solutions of different pH and ionic strength,

which can contain a variety of agents which have to be included to protect the protein from inactivation (e.g. dithiothreitol, 2-ß-mercaptoethanol, EDTA, glycerol, stabilizing ions and coenzymes). Work with membrane protein antigens usually means the isolation and purification of a protein in the presence of detergents (often non-ionic) or at very high salt concentrations. These agents may affect the microheterogeneity of antibody subpopulations (e.g. by masking antigenic determinants) but in general there is little evidence that they affect antibody production. Antisera to membrane antigens in detergents (e.g. Triton X-100) are not difficult to produce (e.g. Dennick and Mayer, 1977).

Protein antigens are usually mixed with material that will increase the concentration of circulating antibodies i.e. adjuvants. There are a variety of adjuvants which may themselves be antigenic (e.g. tubercle bacilli) or not (e.g. mineral oil) but which can improve the humoral immune response by:- increasing the number of cells involved in antibody formation, assuring a more efficient processing of antigens and prolonging the duration of the antigen in the immunised animal. The most commonly used adjuvant is Freund's complete adjuvant which consists of killed mycobacteria (e.g. tubercle bacilli), an oil and an emulsifier. In Freund's incomplete adjuvant the mycobacteria are omitted (Clausen, 1971). Many immunisation schedules for proteins have been used but they basically differ in that some authors prefer to use Freund's complete

adjuvant for the first series of injections and subsequently use an incomplete adjuvant (e.g. Mason et al., 1973), whereas others prefer to use complete adjuvant throughout (e.g. Walker et al., 1976). The disadvantage of using complete adjuvant throughout is that sterile abscesses can be produced by subcutaneous injections (Mayer and Walker, 1979). In the production of antiserum to endoglucanase the former schedule was employed.

3.3.1 Raising of the antiserum.

Rabbits were injected initially with a sample of non-denatured endo 1,4 ß-D-glucanase in Freund's complete adjuvant. Subsequent (booster) injections were administered using incomplete adjuvant. Pre-immune serum was collected before the initial injections and subsequent serum preparations were collected at 14 day intervals.

During the production of the antiserum, the increase in titre was tested using Western immunoblotting. The serum was reacted with a sample of pure protein on a nitrocellulose filter. Figure 3.8 shows the increasing titre of the various serum preparations obtained. The times indicated represent days after the initial injection. A control using pre-immune serum was run at each time point, with no reaction being visible (data not shown). Serum from day 68 (approximately 10 weeks after initial injection) gave the highest titre (determined by the intensity of the band obtained in a fixed time) and was used in subsequent experiments. It must be emphasised that sensitivity in

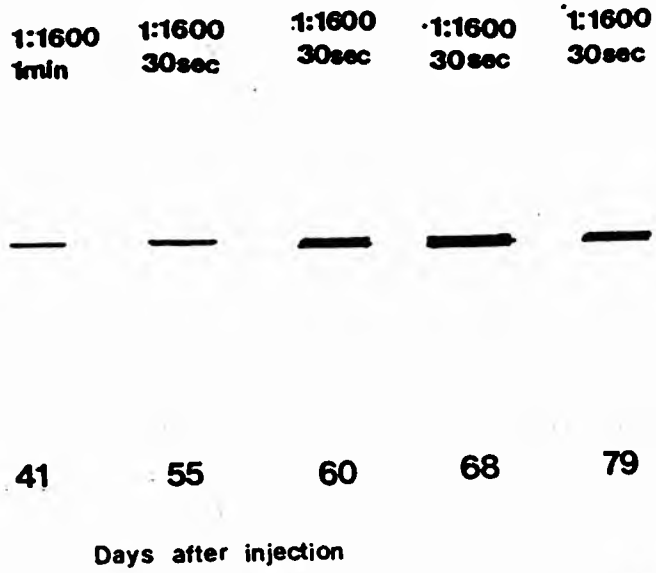


Figure 3.8

Immunoblot of antisera at 1:1600 dilution tested on a nitrocellulose blot of purified endo 1,4 ß-D-glucanase and stained with DAB. The bands appeared within the times stated.

Western blotting is primarily a function of the specific antibody titre of the immune serum being utilised. This serum was used at a dilution of 1:1000 (Figure 3.9).

3.3.2 Characterisation of the antiserum.

The specificity of the antiserum was checked using both immunodiffusion and immunoblotting techniques. The antiserum should be tested against the crudest tissue preparation available. Although this may produce problems of background staining due to non-specific precipitation, it precludes the possibility of missing some antigen reaction with antibodies in the serum.

3.3.2.1 Immunodiffusion analysis.

Double diffusion analyses (Ouchterlony, 1968) can be used to identify the number of antigen-antibody systems within a tissue extract and the antiserum. These analyses are particularly useful for obtaining reactions of identity, comparing a purified antigen (in one well) with components of an antigen mixture (in a second well) by their reaction with an antiserum (in a third well).

Figure 3.10 (a) illustrates the reaction of identity between the pure antigen (well 1), the crude tissue extract (well 2) and the antiserum (well 3). This reaction of identity indicates that the compared antigens are serologically "identical" or they have an antigenic factor in common and are, due to this determinant, precipitated by the antibody component corresponding to this determinant.

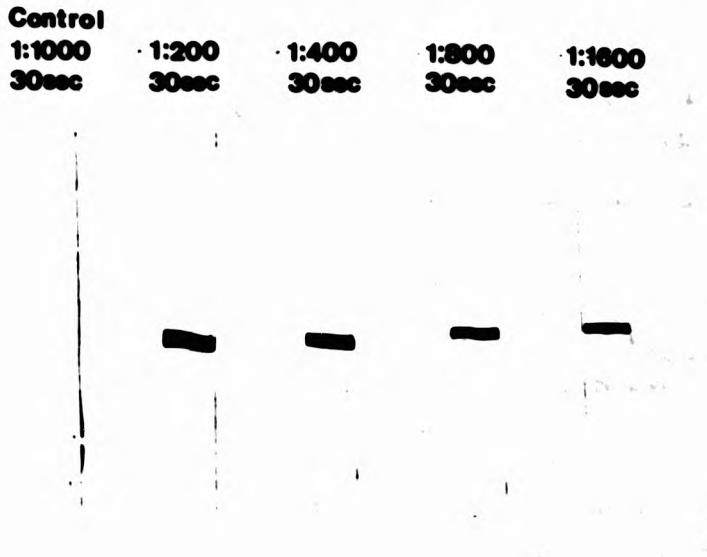


Figure 3.9

Immonoblot of day 68 antiserum tested on a pure sample of endo 1,4 ß-D-glucanase: reaction at various dilutions.

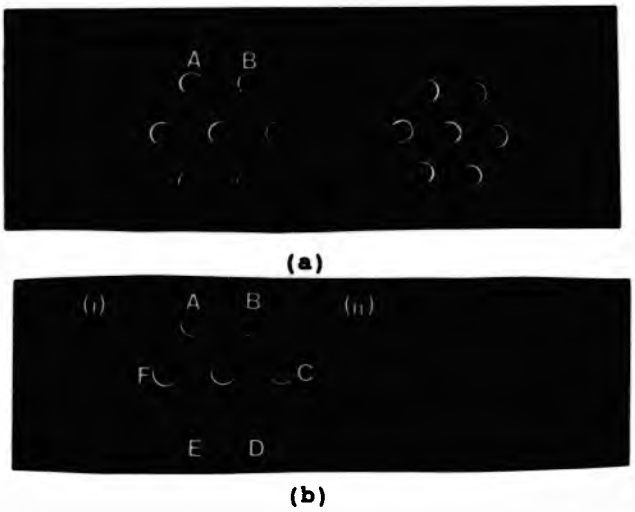


Figure 3.10

Ochterlony double diffusion assay.

(a) Inner wells of a 1% agarose coated slide contained 50 µl antiserum where outer wells A and B contained 50 µl pure antigen (enzyme protein).

(b) Inner wells contained 50 µl antiserum, the outer wells contained serial dilutions of crude homogenate from nasturtium seeds. A: undiluted; B: 1:2 dilution; C: 1:4 dilution; D: 1:8 dilution; E: 1:16 dilution; F: 1:32 dilution.

The concentration of the antigens which are in opposition to an antiserum in an immunodiffusion analysis may be varied by serial dilution e.g. dilution of a tissue extract around a central antiserum-containing well (Ouchterlony, 1968). This is illustrated on Figure 3.10 (b)i where the reaction of an antiserum (centre well) to serial dilutions of the antigen is shown as a precipitation band of decreasing intensity. The same result was obtained when the antigen was challenged by serial dilutions of the antiserum as shown on Figure 3.10 (b)ii.

The advantage of these types of immunodiffusion analyses is that by varying the amounts of the immunoreactants, latent contaminating antigen-antibody systems may be revealed as was found for 6-phosphogluconate dehydrogenase by Piazzi (1969). In general the resolution of immunodiffusion analyses is such that they are only suitable for assessment of antiserum which contain antibodies to a few macromolecular antigens.

3.3.2.2 Western immunoblotting analysis.

Complex mixtures of antigens can be quickly and easily separated by high resolution techniques such as SDS-PAGE. However, once separated it has been difficult to determine which of the separated species reacted with a given antiserum. Several methods have been developed including the overlaid cross immunoelectrophoresis of Soederholm et al. (1975) and the thin section technique of Raamsdonk et al. (1977). However, both of these methods compromised the

resolution of Laemmli SDS-PAGE system. Towbin et al. (1979), overcame this problem by transferring the separated mixture onto nitrocellulose, as had previously been performed with nucleic acids. Once attached to the nitrocellulose, the antigenicity of each of the separated species could be tested by treating the blot with antiserum and the bound antibody detected with either a radiolabelled anti-antibody or with an enzyme conjugated anti-antibody which will produce a colour reaction.

The enzyme conjugate anti-antibody used in these studies contained peroxidase which on reaction with hydrogen peroxide and diaminobenzidine (DAB) produces a coloured product. As can be seen from Figure 3.11 (b) the reaction on a crude tissue extract produces one major and several minor bands. It should be emphasised that the major band picked out by the antiserum corresponds exactly in migration to the endoglucanase and the other bands would appear to be minor when the amido black stain of the crude extract is considered (Figure 3.11 (a)). To determine whether or not these minor bands can be attributed to cross reactions, it was decided to try and purify the antiserum.

3.3.3 Purification of anti-glucanase antiserum.

Immunoglobulins are the most basic globulins of serum. Their solubilities and high isoelectric points relative to other serum proteins form the basis of most purification procedures. Techniques for serum fractionation include ethanol fractionation, salt fractionation, gel filtration

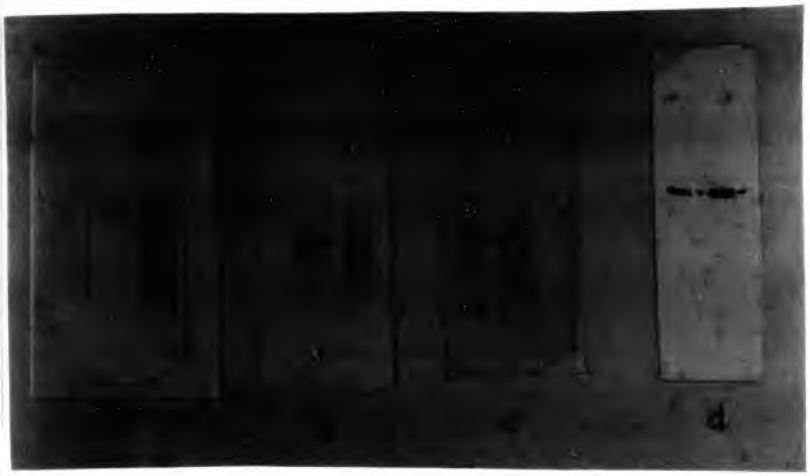


Figure 3.11

Purification of endo 1,4 ß-D-glucanase antiserum.

(a) amido black stain

(b) reaction with crude antiserum

(c) reaction with IgG fraction

(d) reaction with affinity purified antiserum

and ion-exchange chromatography.

Salting out procedures have certain advantages over ethanol fractionation as they are relatively simple and there is only a slight danger of denaturation. The addition of salt and subsequent washings of the precipitate will remove albumin, transferrin, α-proteins including haptoglobin and haemoglobin from a crude serum preparation (Harboe and Inglid, 1973). A simple salt precipitation technique using sodium sulphate was employed to prepare an immunoglobulin G (IgG) fraction from endoglucanase antiserum. As can be seen from Figure 3.11 (c), there was still slight background staining and cross reaction, hence further purification was required.

Antisera can be purified by absorbtion procedures (Kwapiniski, 1972) in which antisera are treated with preparations which are enriched in contaminating antigens. Alternatively, antibodies to the antigen of interest can be purified by the use of preparations which are enriched with or only contain the antigen of interest (Kabat, 1967). Although appealing in principle, the binding of antibodies to some preparations of the purified antigen and its subsequent elution present some problems. Several procedures have been developed whereby purified antigen is bound to a solid support e.g. sepharose (Porath et al., 1973), polyacrylamide (Ternynck and Avrameas, 1976) or nylon (Edelman and Rutishauser, 1974). The immunoadsorbant is then used to bind specific antibodies which can subsequently be eluted. For example, fibrinogen and

ovalbumin have been coupled to sepharose (Bouma and Fuller, 1975) and then used to purify their respective antibodies.

The main problem with the use of immobilised antigen as immunoadsorbant, is the quality of the antigen. If a purified antigen is immunologically impure (Section 3.3) and the same antigen is used to prepare the immunoadsorbant, then contaminating antibodies could be bound to the immobilised antigen. The situation is complex and depends on the degree of purity of the antigen of interest and the contaminants. Binding of antibodies to immunoadsorbants is further complicated by non-specific binding of proteins. This necessitates the use of washing procedures to try to elute these proteins before elution of the antibodies of interest e.g. with 1 M NaCl (Alberts et al., 1975).

For further purification of endoglucanase antiserum, the immunoadsorbant chosen was nitrocellulose as the binding of pure protein onto the membrane is relatively simple. Also the effectiveness of binding can be tested using Ponceau S solution (0.1 % w/v), which will stain the protein on the membrane.

Elution of antibodies of interest can be achieved with a variety of agents. The aim of these treatments is to break the electrostatic and hydrophobic interactions which can bind antigens to antibodies. In this case low pH (e.g. 0.2 M glycine/HCl, pH 2.8) was employed.

The affinity purified preparation was used at a dilution of 1:50 and as can be seen from Figure 3.11 (d),

there appear to be no contaminating bands and considerably less background.

3.4 Discussion.

The purification of endo 1,4 ß-D-glucanase from nasturtium cotyledons produced a homogeneous protein with an apparent molecular weight of 29,000.

The characterisation of the pure enzyme determined that the protein was not a glycoprotein as no major carbohydrate was detected by the methods employed. The methods used were sensitive to 10 ng samples of sugar, hence it can be concluded that if there is any carbohydrate associated with the protein it would constitute less than 3.6% of the total protein sample loaded on the gel.

This chapter also includes the raising of a monospecific antiserum to the enzyme. The polyclonal serum originally produced was tested using an immunodiffusion technique which indicated that the serum contained the specific antibody species to the antigen of interest as it produced the Ouchterlony reaction of "identity".

Western immunoblotting analyses showed a significant amount of contaminating background which could limit our interpretation of future results. Hence it was decided to try to purify the antiserum. The purification of the antiserum yielded both an IgG fraction and a more specific affinity purified preparation. The IgG fraction which isolates part of the crude serum, still gave a positive

result but also produced a significant amount of contaminating background. The affinity preparation which was isolated from the serum by its specific reaction to the pure antigen produced a result on nitrocellulose with no apparent contaminating bands. This was the preparation that was used in subsequent experiments.

CHAPTER FOUR

CHAPTER 4

HISTOCHEMICAL AND IMMUNOCYTOCHEMICAL STUDIES ON ENDO 1,4 ß-D-GLUCANASE PROTEIN IN RELATION TO XYLOGLUCAN MOBILISATION.

This chapter describes the use of histochemical methods to follow the mobilisation of xyloglucan in nasturtium cotyledons from time of imbibition and to correlate this with specific localisation of endo 1,4 ß-D-glucanase using immunocytochemical techniques. Several of the problems encountered during this study are investigated and the conclusions discussed.

4.1 Light microscopy and histochemistry.

It was Raspail who first began to develop the use of chemical tests for light microscopical studies in the late 1820's, and consequently he is regarded as the founder of histochemistry. He used the iodine reaction for starch and the xanthoproteic reaction for protein on plant tissues (Pearse, 1980).

To view plant tissues under the microscope they have to be cut very thinly, as whole tissue is usually very dense and opaque. Cutting thin sections of living tissues is rarely possible, since soft tissue squashes and the sections are not usually thin enough to determine ultrastructural detail. Hence the tissue has to be hardened

by either freezing or embedding in a resin. These procedures usually kill the tissue, and can alter the chemical picture by dissolving out important tissue constituents. This effect can be reduced by fixation, which stabilises particular chemical substances, making them less likely to be washed out by subsequent preparative procedures.

Hopwoood (1973) classified fixatives according to their chemical nature to produce 5 classes:-

1. Aldehydes e.g. formaldehyde, glutaraldehyde, acrolein and glyoxal.
2. Oxidising agents e.g. osmium tetroxide, potassium permanganate and potassium dichromate
3. Protein denaturing agents e.g. acetic acid, ethanol and methanol.
4. Cross-linking agents e.g. carboidimides and dimethyl suberimidate.
5. Fixatives of unknown mechanism e.g. mercuric chloride and picric acid.

The requisite qualities of the ideal fixative were listed by Jones (1973) who stressed, as a prime requirement, preservation from osmotic damage and shrinkage, amounting to avoidance of all consequent changes in morphology. A second, less important, requirement was the retention <u>in situ</u> of all tissue components. Inherent in this provision would be the preservation of all original reactivities, not affecting subsequent reactions of a histochemical nature by destroying functional groups.

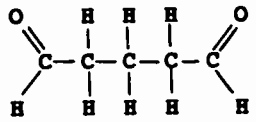
Therefore, for histochemical purposes, a fixative should be chosen which does not attach to or otherwise alter the particular chemical groups under investigation.

Formaldehyde is the best known because of its application in the tanning industry (Pearse, 1980; Hopwood, 1982). It is commonly found as a stable high molecular weight solid known as paraformaldehyde, which on heating generates pure gaseous formaldehyde and, when dissolved in water, reverts to the monomeric form. Formaldehyde exists primarily in the form of a monohydrate methylene glycol, $CH_2(OH)_2$ and as low molecular weight polymeric hydrates or polyoxymethylene glycols. The essential feature of formaldehyde fixation is the formation of cross links between protein end groups (Pearse, 1980). In particular, amino groups may be blocked by a condensation reaction, or cross-linked by methylene bridges. Other groups affected include imino and amido groups, peptide, guanidyl, hydroxyl, carboxyl, sulph-hydryl groups and aromatic rings.

In histological and histochemical work, formaldehyde is nearly always used in buffered solutions at, or above the neutral point and Wolman (1955) suggested that part of the effectiveness was due to the rapid conversion in neutral and alkaline solutions of the polymerised form of HCHO to the monomer.

Glutaraldehyde was first used in the leather industry as a tanning agent (Fein and Filachione, 1957; Selisburger and Sadlier, 1957) and was introduced into histochemical use by Sabatini et al. (1963). Ultraviolet spectroscopy

showed two peaks; the first at 235 nm, contained oligomers, polymers and impurities; the second at 280 nm, contained only the monomeric form.



It is now generally accepted that the pure monomeric glutaraldehyde is the better fixative, and much less inhibitory to enzymes than the mixed monomeric - polymeric product. It cross-links proteins, especially by reacting with amino groups. Glutaraldehyde reacts more quickly than formaldehyde, blocking more groups in the process (Pearse, 1980). Structural preservation is particularly good, hence it tends to be favoured over formaldehyde for these studies.

After fixation, the tissue is embedded in a hard matrix, to prevent distortion during thin sectioning (microtomy). Most embedding media are not soluble in water and consequently fixed specimens are dehydrated by passing them through a sequence of solutions (with increasing concentrations) the last of which is miscible with the embedding medium. The two most widely used dehydrating agents are ethanol and acetone. They give very similar results with the majority of specimens, but ethanol is usually preferred, since acetone readily takes up water and incomplete dehydration may result (Glauert, 1974)

For light microscope work, the fixed tissue sections

were embedded in the methacrylate JB4. Methacrylates are readily soluble in both ethanol and acetone hence no intermediate solvent is required. Once embedded, thin sections (≈ 4 μm) are cut on a microtome and stained specifically. Since the embedding matrix is hydrophillic, it is quite permeable to aqueous stains.

Periodic acid Schiff's (PAS) stain is specific for carbohydrate , in that the periodic acid oxidises 1,2 glycol groups to dialdehydes and the aldehyde groups are then detected specifically by Schiff's reagent (Feder and O'Brien, 1968). Amido black has been widely used for staining protein in paper chromatography (Lillie, 1969) and was used in this case as a specific protein stain. Mitchell's reagent (iodine potassium iodide solution) was used to detect the "amyloid" reaction first observed by Schleiden (1838) and Vogel and Schleiden (1839). Kooiman (1960) used this stain to detect the occurrence of amyloids in a variety of plant species. The solution contained 0.3 g iodine and 1.6 g potassium iodide in 100 ml of water and was employed in this investigation to specifically stain amyloid (xyloglucan).

4.1.1 Time course of xyloglucan mobilisation in the cotyledonary cells.

The time course of xyloglucan mobilisation from the thickened storage cell walls of cotyledonary cells was investigated in seeds at days 1, 3, 7, 9, 13, 17, and 19 after planting. Xyloglucan mobilisation does not appear to have occurred at 3 days after imbibition (Figures 4.1 and 4.2). The storage cell walls are virtually completely stained in both the sections from days 1 and 3, except for the inner margin of the wall which although unstained with Mitchell's reagent, is highly positively stained with PAS (as indicated on Figure 4.1 (b) and 4.2 (b)). At this stage it should also be noted that there is a lack of PAS positive material in the cytoplasm of these sections (no starch). The protein bodies are uniformly stained indicating that at this stage they are still intact.

Between days 3 and 7, changes in the appearance of the cells can be observed. Mobilisation of the cotyledonary reserves has begun by about day 7 (Figure 4.3). Also at this stage, there would appear to be mobilisation of protein, as the protein bodies seem to be partially vacuolated with granular clumps of positively stained material in them. Their appearance would tend to suggest that at least part of their protein content has been mobilised (Figure 4.3 (c)).

Areas of the cell wall have become depleted of xyloglucan, giving an "arched" appearance at the cytoplasmic side of the wall (as indicated by ↑). This

Figure 4.1

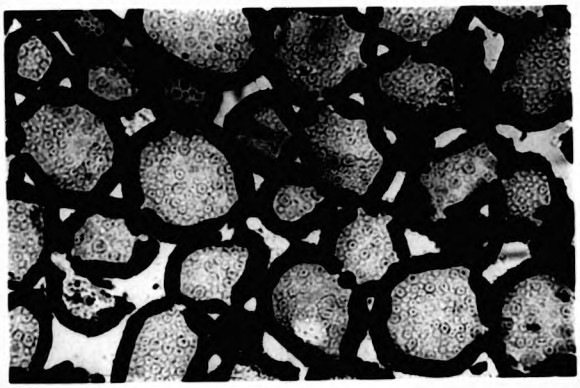
Sections from day 1 after imbibition of nasturtium seeds stained with;

(a) PAS

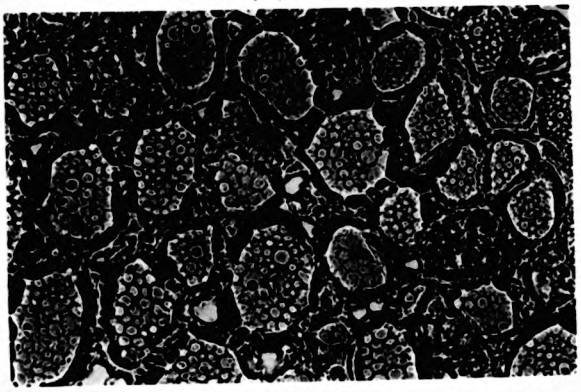
(b) Mitchell's reagent

(c) Amido black

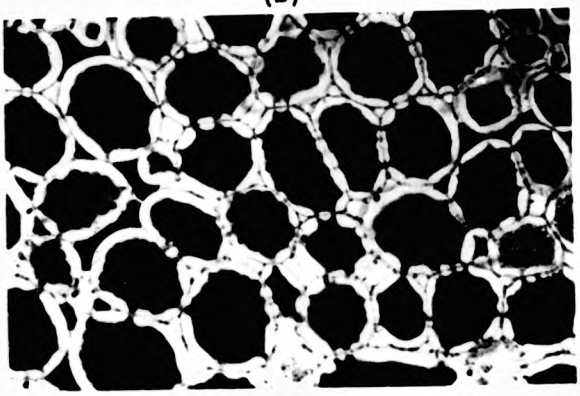
magnification x 300



(a)



(b)



30μm

(c)

Figure 4.2

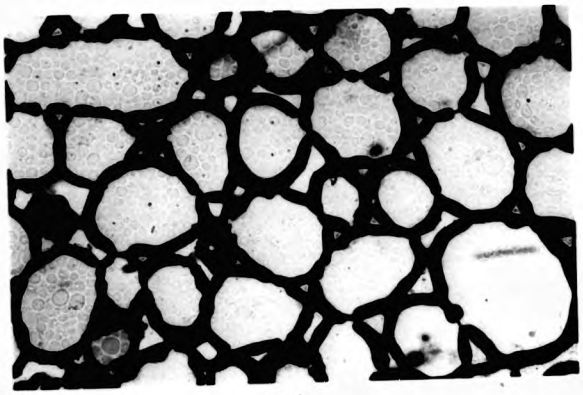
Sections from day 3 after imbibition of nasturtium seed stained with;

(a) PAS

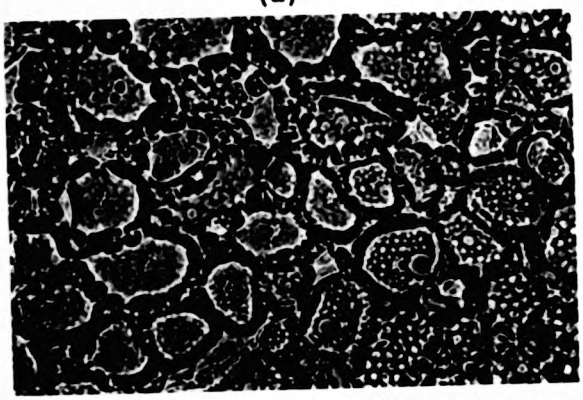
(b) Mitchell's reagent

(c) Amido black

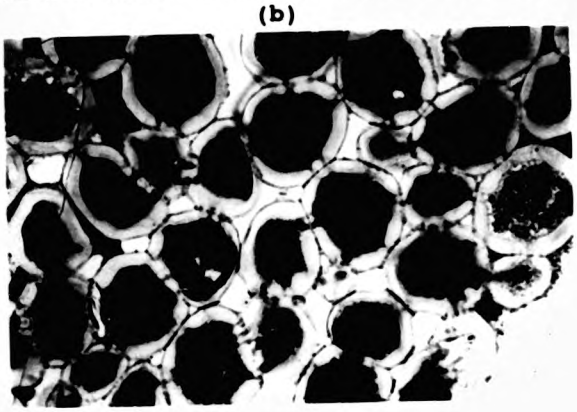
magnification x 300



(a)



(b)



30μm

(c)

Figure 4.3

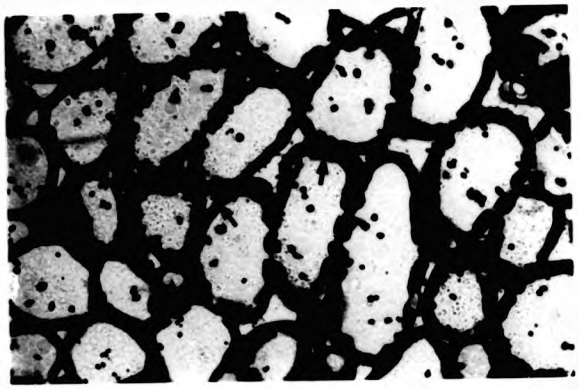
Sections from day 7 after imbibition of nasturtium seeds stained with;

(a) PAS

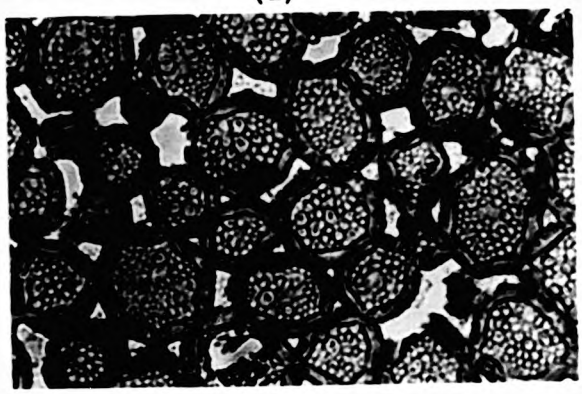
(b) Mitchell's reagent

(c) Amido black

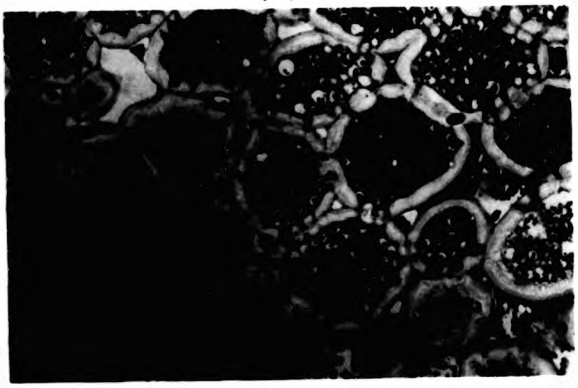
magnification x 300



(a)



(b)



30μm

(c)

would tend to suggest that the enzymes necessary for the degradation of xyloglucan have come from the cytoplasm and are degrading the polysaccharide from the edge of the storage wall out towards the primary cell wall. There are, however, areas of positive staining material at this edge remaining, forming "pillars" giving the appearance of "gothic windows" in the cell wall.

Unfortunately, the embedding procedures remove the lipid reserves, however, it would seem likely that any lipid reserves present in the cotyledonary cells would begin to be mobilised during this stage. Also during this period, positive staining starch deposits appeared in the cytoplasm (Figure 4.3 (a)) as transitory starch formation is common during seed mobilisation.

By day 9 after imbibition, extensive xyloglucan degradation is observed, with the storage walls again exhibiting an "arched" appearance (Figure 4.4 (a), as indicated by ↑). Although at this stage this effect is more marked. There is, however, a fairly large expanse of storage wall adjacent to the primary wall which has not yet been degraded. This is more prominant in the PAS stained section (Figure 4.4 (a)). At this stage the protein bodies appear flocculent containing a few slightly stained granular areas. A few protein bodies have been fully degraded by this time leaving behind negatively staining vacuoles (Figure 4.4 (c)). Again positive PAS staining starch grains are present in the cytoplasm of the cell (Figure 4.4 (a)).

Figure 4.4

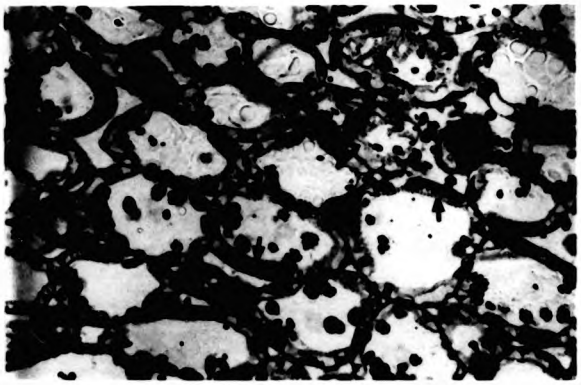
Sections from day 9 after imbibition of nasturtium seeds stained with;

(a) PAS

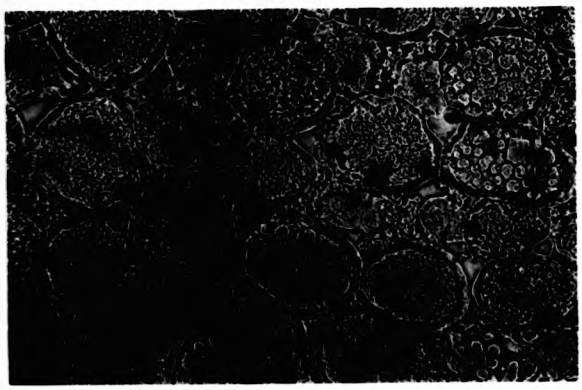
(b) Mitchell's reagent

(c) Amido black

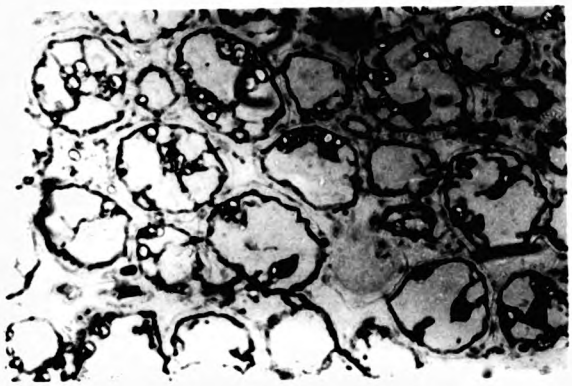
magnificaton x 300



(a)



(b)



30μm

(c)

By day 13 after imbibition, almost all of the xyloglucan has disappeared as judged by the lack of positive PAS staining material in the secondary wall (Figure 4.5 (a)). All that remains are the "pillars" of positively staining material. These pillars which remain in the storage wall may have a supportive function. It is possible that they maintain the wall thickness preventing any engulfing of the cytoplasm as has been suggested to be the case in xyloglucan mobilisation in tamarind cotyledons (Reis et al., 1987). These pillars could maintain the wall thickness until an advanced state of digestion. At this stage the cotyledonary cell wall can be divided into three components. The primary cell wall (incorporating the middle lamella), the thickened storage wall and the narrower inner membrane adjacent to the cytoplasm. Again the presence of starch grains in the cytoplasm which appear to have increased in size and number, but the protein deposits have been completely mobilised, giving rise to large vacuoles (Figure 4.5 (a),(b),(c)).

The appearance of cotyledonary cell walls at 17 and 19 days after imbibition are similar (Figures 4.6 and 4.7). Virtually all of the xyloglucan has been mobilised with fewer isolated "pillars" of positively staining PAS material located in the cell wall (Figure 4.6 (a)). There are occasional starch grains present in the cytoplasm of the day 17 sample (Figure 4.6 (a)), but they have disappeared by day 19 (Figure 4.7 (a)) indicating that they have been utilised by this time. The vacuoles, which are

Figure 4.5

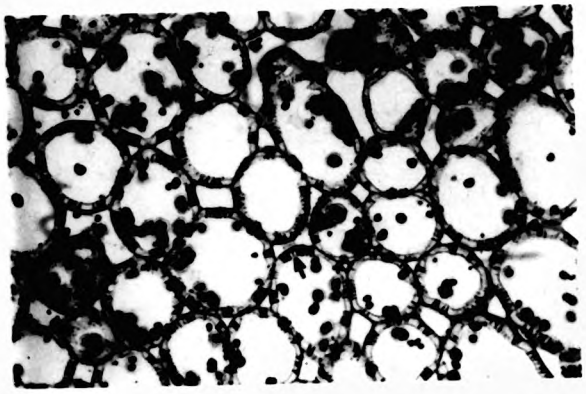
Sections from day 13 after imbibition of nasturtium seeds stained with;

(a) PAS

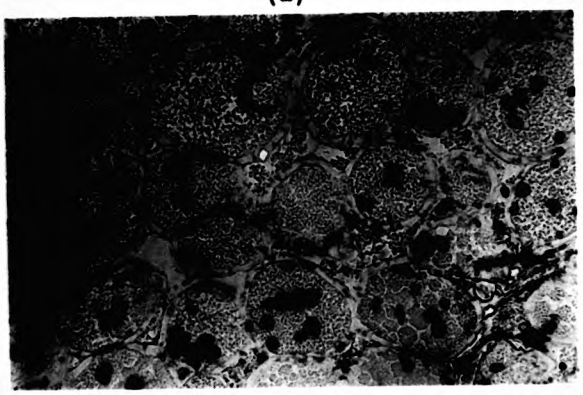
(b) Mitchell's reagent

(c) Amido black

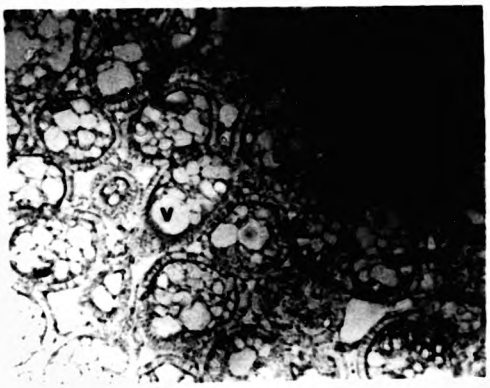
magnification x 300



(a)



(b)



(c)

30μm

Figure 4.6

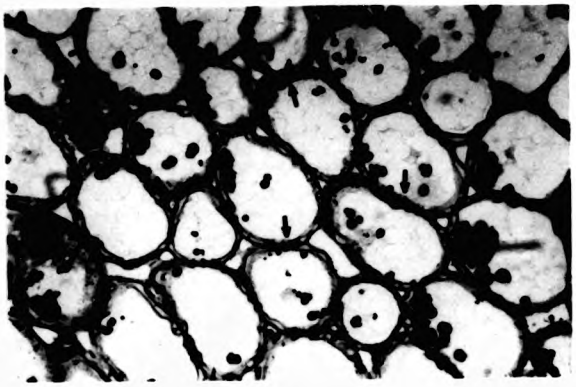
Sections from day 17 after imbibition of nasturtium seeds stained with;

(a) PAS

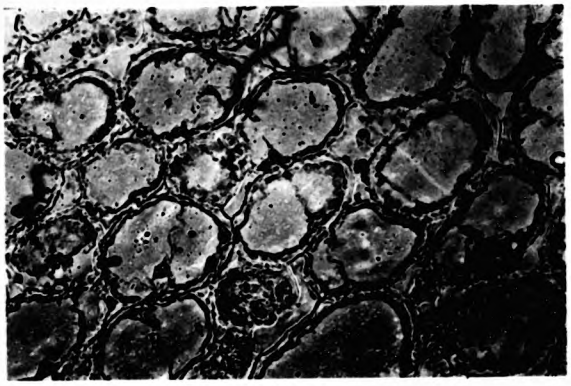
(b) Mitchell's reagent

(c) Amido black

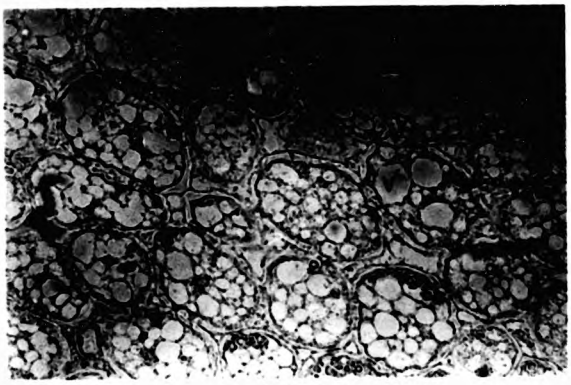
magnification x 300



(a)



(b)



30μm

(c)

Figure 4.7

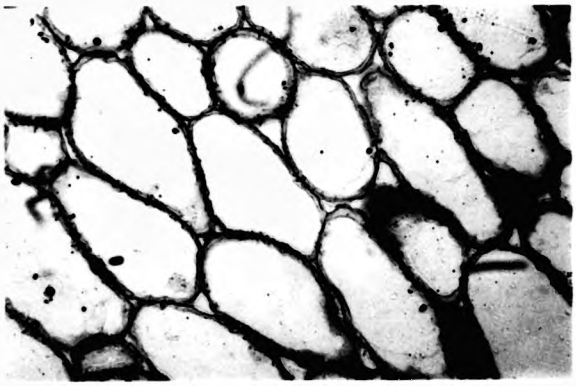
Sections from day 19 after imbibition of nasturtium stained with;

(a) PAS

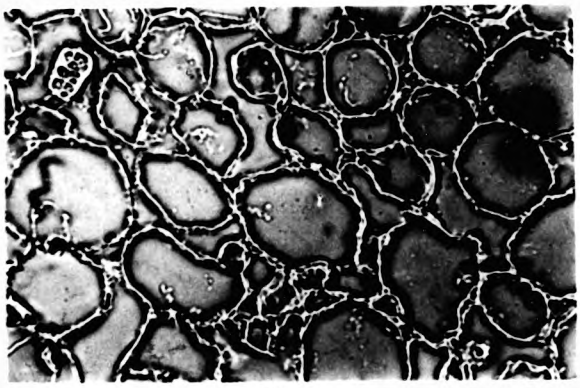
(b) Mitchell's reagent

(c) Amido black

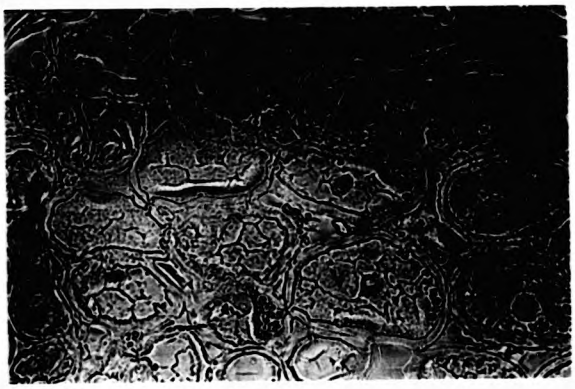
magnification x :



(a)



(b)



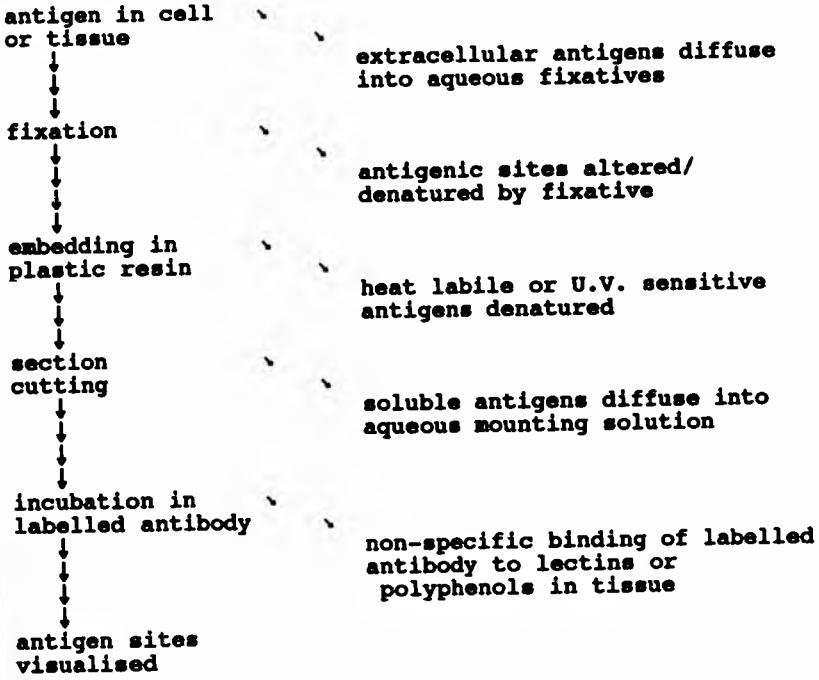
30μm

(c)

the result of protein degradation, have become larger. This expansion and vacuolation of the protein bodies is due to the loss of cytoplasmic lipid and a fusion of vacuoles, as the number of vacuoles per cell decrease as the time course proceeds.

4.2 Immunofluorescence Studies.

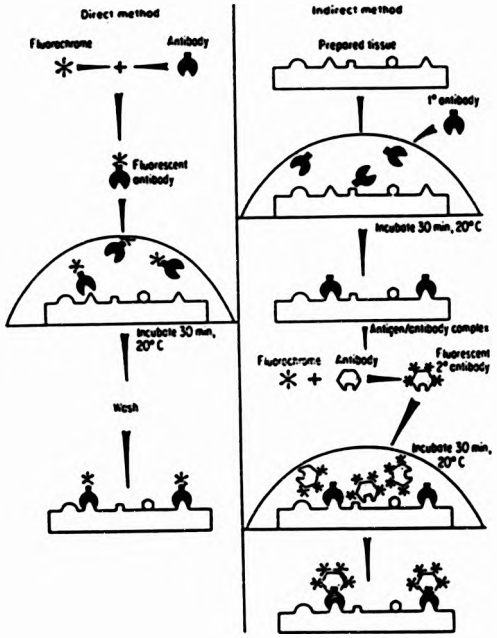
Immunocytochemical methods depend for their success on the integrity of the antigenic determinants in their cellular sites, which bind to the labelled antibody. These methods are basically of two types:- pre-embedding staining procedures, in which whole cells, protoplasts or tissue slices are incubated in labelled antibody; or post-embedding staining procedures in which cells or tissues are fixed, embedded and thin sections are incubated in labelled antibody (Kreahenbuhl *et al.*, 1980). The former method suffers from the disadvantage that the antibody molecules have to diffuse into cut or whole cells through the plasma membrane, giving false negative results. The latter method permits access of antibody molecules to all cut surfaces, but depending on the supporting matrix, binding may occur only at the surface of the section. Some of the problems encountered in using the post embedding procedure are outlined below: from Knox *et al.* (1980).



There are substances with inherent fluorescent capabilities, such as chlorophylls, some oils and waxes etc. They exhibit "primary fluorescence" and therefore can be viewed without staining. Unfortunately, all those microscopic specimens of most biological interest (cells, tissues, cultures etc.) do not possess "primary fluorescence" and only occasionally a limited amount of autofluorescence. To obtain specific fluorescence of a particular area in/on a specimen, staining is required. This can be achieved by the use of fluorescent dyes such as fluorochromes.

Immunofluorescence is a field of fluorescence microscopy used to detect antigens by way of immune

reactions, developed by Coons and Kaplan (1950) to distinguish healthy from diseased patients. Two principal approaches to immunofluorescence microscopy are commonly employed as illustrated below.



In the direct or "fluorescent antibody method", the specific antibody is located by conjugating it directly to a suitable fluorescent dye. The conjugated antibody is incubated with the specimen, and after washing to remove excess antiserum, the bound conjugate is visualised by excitation of the fluorochrome in light of an appropriate wavelength. A disadvantage of this method is that each antiserum must be separately conjugated to the fluorochrome. The specificity and specific activity of the antiserum can be markedly reduced by the process of

conjugation with the fluorochrome, especially if the fluorochrome:protein ratio is high (Kawamura, 1977), resulting in increased non-specific staining.

In the indirect or "sandwich" method (Weller and Coons, 1954), tissue sections are incubated with an unmodified primary antibody. After washing to remove unbound immmunoglobulins, the section is then incubated in a second labelled anti-antibody. The indirect method offers much greater sensitivity than the direct method, due to the fact that for each reactive site on an antigen available for binding a primary antibody, more are available to the secondary antibody, and the fluorescence is therefore increased. However, it should be noted that any non-specific binding will be increased by a corresponding amount.

The most commonly used fluorescent dyes for immunofluorescence are isothyocyanates of fluorescein (FITC) and members of the rhodamine group (RITC, TRIC), chosen for their maximum separation between absorption and fluorescent wavelength maxima, and for high quantum efficiency. FITC has an absorption maximum at 495 nm and fluoresces in the yellow-green at 500 nm. The rhodamines absorb in the blue-green at 540 nm and fluoresce in the orange and red between 570 and 600 nm.

Problems can arise in plant tissue due to autofluorescence, whereas lignified tissues, and tissues with phenolic deposits, fluoresce strongly, competing in intensity with specific fluorescence from FITC (Jeffree et

al., 1982). This autofluorescence can be readily filtered out by the use of green barrier filters, but the closeness of the chlorophyll autofluorescence to the orange-red specific fluorescence from rhodamine makes their separation by filtration difficult, and seriously impairs the usefulness of rhodamines in immunofluorescent studies of photosynthetic plant tissues. The only advantage rhodamines appear to have over fluorescein for work with plant tissues, is their substantially greater resistance to fading in the excitation beam.

4.2.1 Applications of immunofluorescence microscopy to studies of plant cells and tissues.

Methods to localise enzymes in plant cells are numerous and varied, most methods are different and dependent upon the type of tissue used. For example, Murray and Knox (1977) investigated the distribution of urease in the cotyledons of jackbeans (Canavalia enisformis) using direct immunofluorescence. They found that the seeds had to be pre-treated with glycosides to prevent lectins (in the cell wall) non-specifically binding to the immunoglobulins.

Localisation of RuBisCo by Hattersley et al. (1977) using the indirect method, demonstrated that fresh unfixed tissue would not label, whereas ethanol fixation was essential for the penetration of chloroplasts by the antibodies. Difficulty was encountered in recording the results on black and white film because the images obtained often failed to distinguish between specific fluorescence

and autofluorescence of chloroplasts. Such difficulties were, however, obvious from colour photographs.

More recently, in spinach, α 1,4-glucan phosphorylase (Schächtele and Steup, 1986) and chalcone synthases (Beerhues et al., 1988) have been localised using immunofluorescence. In both cases autofluorescence was negligible.

4.2.2 Localisation of endo 1,4 ß-D-glucanase using a fluorescent probe.

As described before, the ideal fixative should preserve ultrastructural features and maintain the antigenicity of the specimen. For general plant ultrastrucural studies, glutaraldehyde is usually the fixative of choice (O'Brien and McCully, 1981). However, glutaraldehyde has two major disadvantages. While maintaining good ultrastructure, it tends to be used at high concentrations to ensure satisfactory fixation, which results in a major loss of antigenicity (Kraehenbul et al., 1980), especially with protein antigens. This can be overcome by using a mixture of glutaraldehyde and paraformaldehyde. The second disadvantage is that glutaraldehyde fixation results in substantial yellow/green autofluorescence of tissues, masking the specific fluorescence of FITC. This effect is illustrated in Figure 4.8 where unlabelled sections were looked at using the FITC filter. As can be seen, there is substantial autofluorescence in both samples; Figure 4.8 (a) was fixed

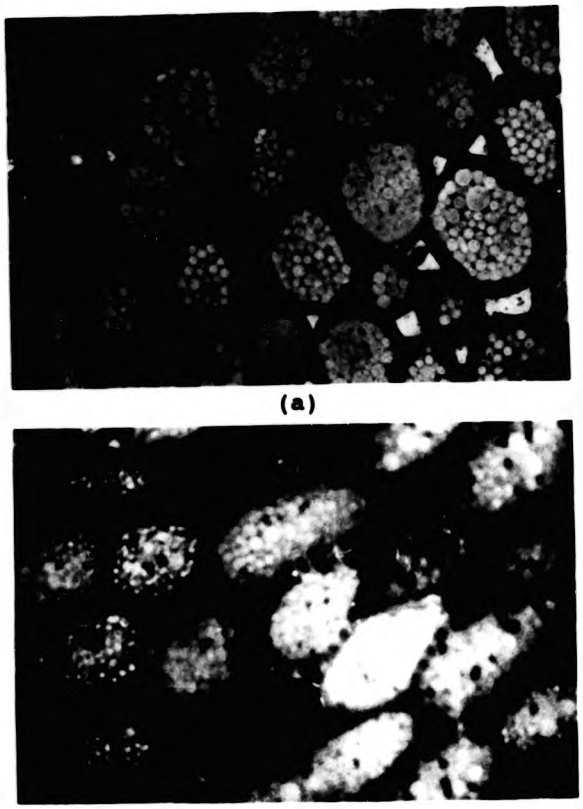


Figure 4.8

Fixation of specimens in different embedding media.

(a) 3% glutaraldehyde

(b) 1% glutaraldehyde/4% paraformaldehyde

magnification x 300

using glutaraldehyde alone and Figure 4.8 (b) fixation was in conjunction with paraformaldehyde. Fulcher and Holland (1971) used acrolein as a fixative as it is a better cross-linker than formaldehyde (Pearse, 1980). However, they found that it was also detrimental to fluorescence staining. Solvent fixatives have also been used, e.g. 95% ethanol was the choice of Hapner and Hapner (1975) on the seeds and roots of Sanfoin (Onobrychis viciifolia), however, this produced autofluorescence at 495 nm, hence they used rhodamine in place of fluorescein as the label. Jeffree et al. (1982) used ethanol for studies on Datura seed tissue but found it caused opacification, resulting in excessive light scattering and loss of resolution. Hence in this study we decided to use formaldehyde alone as fixative.

Animal tissues are typically prepared for immunofluorescence microscopy by freeze-sectioning. Although plant material can be cryosectioned successfully, many soft vacuolate tissues disintegrate when cut thinly and protoplasts may be lost from the cut cells. However, some authors claim sections may be obtained which are as thin as 2 μm. Jacobsen and Knox (1973) successfully obtained thin sections from barley aleurone layers. Attempts were made to cut thin sections from frozen nasturtium tissue which had been fixed in 4% paraformaldehyde in 50 mM sodium cacodylate buffer, pH 7.2, using the method outlined below. Whole sections (2 mm^2) were frozen in liquid nitrogen and held in place on the cryostat

block using an adhesive. The blocks have to be kept frozen or the glue will soften. Sections were cut but they were 15-16 μm thick. Attempts to cut thinner sections proved unsuccessful as the tissue disintegrated. The sections obtained were not very good for observing ultrastructural organisation. Also resolution of the fluorochrome distribution is coarsened in thick sections and penetration of cells by the relatively large antibody molecules may be inhibited, by intact membrane systems in thick sections masking antigens (Jeffree et al., 1982). Hence for this study, it was decided to use resin embedded material as thinner sections can be obtained.

In the present study, a post-embedding staining technique was adopted to overcome the difficulties of antigen mobility in aqueous media. Nasturtium tissue samples (1 mm^3) were obtained at 2 day intervals, fixed in 4% paraformaldehyde, dehydrated in a graded series of ethanol and embedded in JB4 plastic resin (Polysciences, Inc.), a methacrylate, which has the advantage of short infiltration and embedding times at 4° C, and of being anhydrous so that there should be little possibility of the antigens showing diffusion artifacts. Its hydrophillic nature presumably permitted better access of the labelled antibodies to the antigens, and it was known that this method was suitable for the localisation of hydrolytic enzymes in pollen grains (Knox et al., 1980).

Results from immunofluorescent studies are usually recorded on film but it should be noted that there are a

few problems associated with this type of photomicroscopy. Total light intensity is often low, especially when only a small part of the section is immunofluorescent, thereby exposure times have to be extended. However during long exposure times, fluorescence intensity itself fades. Shorter exposure times are possible by using films with a high ASA rating (Pearse, 1980). Furthermore, a well standardised automatic exposure meter is required because light measurement is synchronised with the exposure and any decrease in fluorescence during exposure is automatically compensated for. Fading in general is increased by the presence of oxidising agents and by excitation at short wavelengths. The rate of fading increases with increasing intensity of irradiation. Storage in the dark at low temperatures may induce some, but not total recovery. Fading is due to photochemical reactions i.e. chemical reactions of the excited molecules of the fluorophore usually leading to its decomposition, and to a reduction in quantum efficiency due to heating. These effects can be slowed by the use of phenylenediamine in the mounting medium (Johnson and de Nogueira Arajo, 1981).

4.2.2.1 Use of crude antiserum on tissue sections.

Thin tissue sections from 13 day old nasturtium seeds, fixed in 4% paraformaldehyde and embedded in JB4, were treated with crude antiserum and then with FITC conjugated goat anti-rabbit IgG (Section 2.9.2). From the results of this initial experiment, it was found that the ideal

concentration of both the antiserum and the fluorophore was 1:50 and was used in subsequent experiments. However it was observed that there was a significant amount of cytoplasmic staining appearing on some control sections (pre-immune serum) as well as some challenged with the primary antibody. This effect was not apparent on the control sections where the primary antibody was omitted (Figure 4.9). It was concluded that something in the serum was causing this non-specific fluorescence, and the pattern of the effect suggested that it was due to a non-specific cross-reaction with starch. It is possible that a component in rabbit serum might be immunologically reactive to starch when one considers that starch probably makes up a substantial part of the rabbit's diet. To alleviate this problem two possible solutions were considered. Firstly, to purify the antiserum as before (Section 2.8), however, this is a lengthy process using up a considerable amount of serum as it would have to be used at a lower dilution. Secondly, if the non-specific staining is due to the presence of starch, then the removal of the starch binding product in the serum should alleviate this problem. To test this hypothesis a simple experiment was devised, where the crude antiserum was passed through a small column containing insoluble starch and washed with PBS. The hope was that the component in the serum that was binding non-specifically to the starch grains would be retained by the column. The serum which had been passed through the column was tested using Western immunoblotting to ensure it would

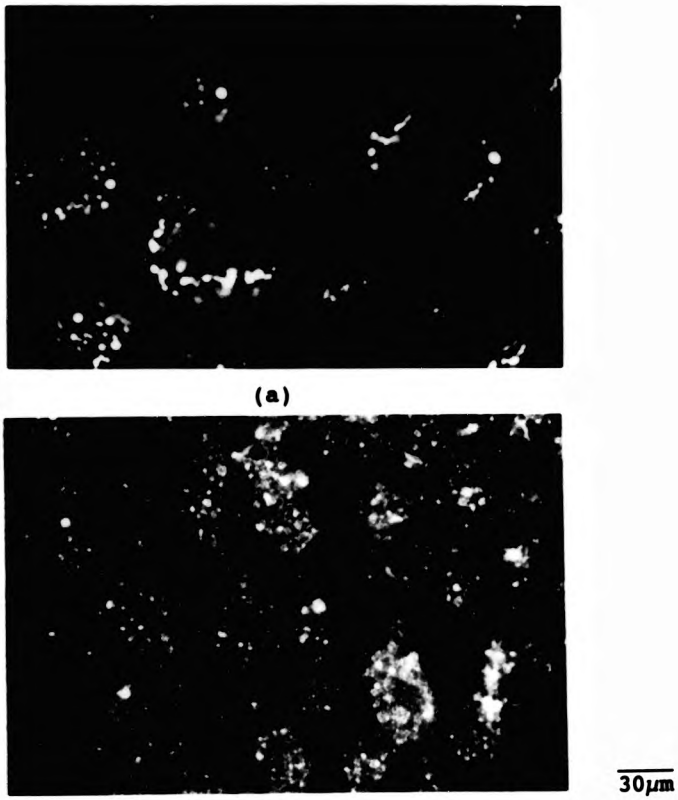


Figure 4.9

Effect of crude antiserum on day 13 tissue sections.

(a) pre-immune serum

(b) primary antibody

magnification x 300

still detect the enzyme (data not shown). When this serum was used on tissue sections, the non-specific fluorescence in the cytoplasm had been removed.

4.2.3 Time course of fluorescence.

Sections from various points throughout the time course of development were incubated first in anti-endoglucanase (which had previously been passed through a starch column) and secondly in a FITC conjugate. The specificity of fluorescence was ensured by using two controls:- pre-immune serum as primary antibody and omission of the primary antibody.

When the fluorescence from the various times of development are compared (Figure 4.10) there is clearly an increase in fluorescence as the time course proceeds with maximum fluorescence being exhibited by the day 13 sample. The control sections exhibited virtually no fluorescence indicating that the fluorescence observed was due to the presence of the enzyme. There was no apparent fluorescence at day 0 suggesting that the enzyme has not yet localised in the wall. Increased magnifications for some of the time points are shown on Figures 4.11 to 4.14. The control sections (b), were incubated with pre-immune serum. The controls where the primary antibody was omitted were virtually identical and therfore not illustrated.

There is a slight amount of fluorescence at day 7 (Figure 4.11) which on some cells would appear to be localised mainly at the inner surface of the cell wall. By

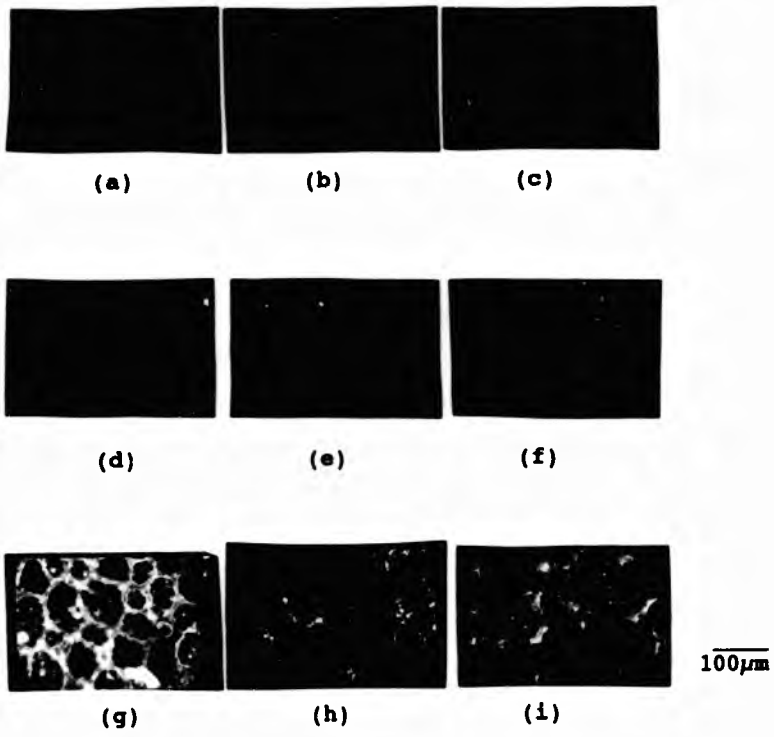


Figure 4.10

Immunofluorescence of endo 1,4 ß-D-glucanase at various stages throughout development.

(a) day 1; (b) day 3; (c) day 5; (d) day 7; (e) day 9; (f) day 11; (g) day 13; (h) day 15; (i) day 17.

magnification x 100

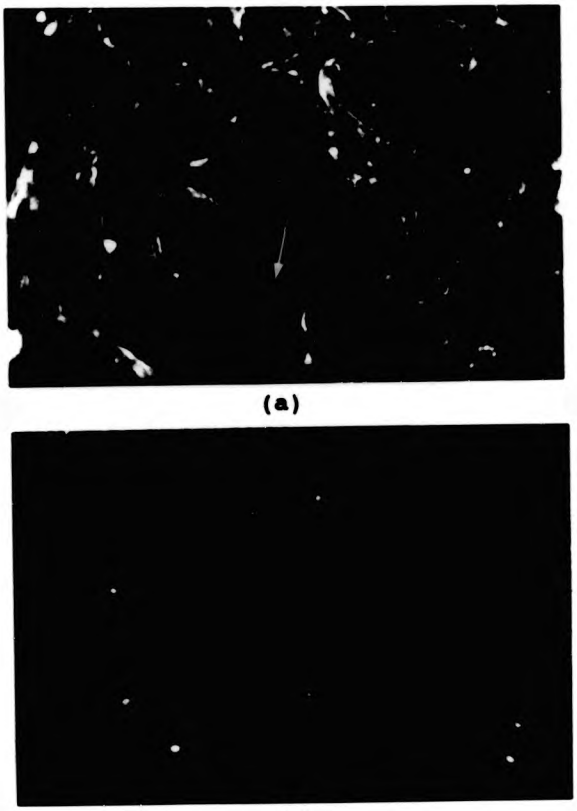


Figure 4.11

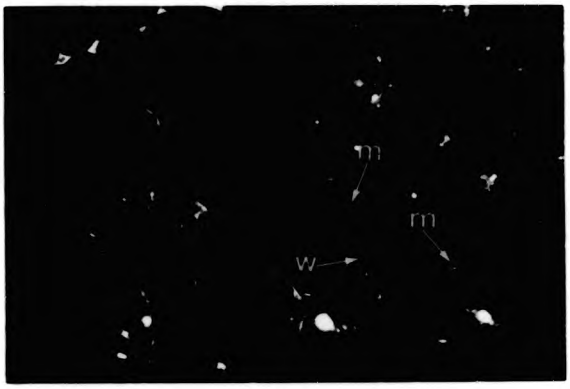
Immunofluorescence on a tissue section from day 7.

(a) primary antibody

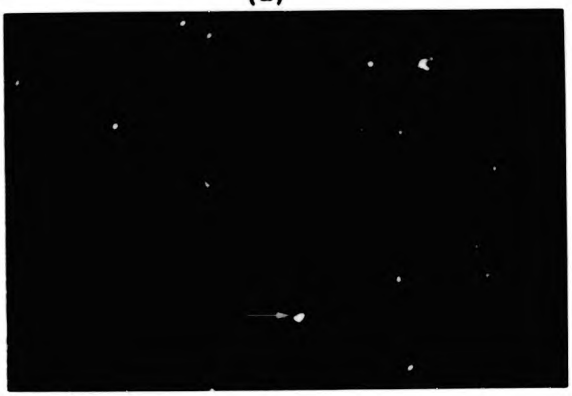
(b) control (pre-immune serum)

magnification x 300

day 9 (Figure 4.12) there is significant labelling and the fluorescence is more apparent around the inner cell wall. As noted before this was the area in which xyloglucan first started to disappear. Also observed at this stage was slight fluorescence located throughout the wall. Slight staining was located in the cytosol but was disregarded because of similar patterns in the control section (Figure 4.12 (b)). By day 11 (Figure 4.13) there is significant fluorescence throughout the wall, but in one cell, in the centre of the photograph, concentrated areas of fluorescence was observed. However, in the majority of cells at this stage, the fluorescence was distributed throughout the wall and not in specific localised areas. By day 13 (Figure 4.14) there was significant fluorescence located throughout the wall, which again would not appear to be concentrated in any particular areas. The enzyme appeared to be localised firstly at the inner surface of the wall, then it gradually migrated throughout the whole of the storage wall. There was no evidence to suggest that its distribution coincided with the "gothic windows" effect observed during xyloglucan breakdown, where specific areas of xyloglucan were mobilised first. Information relating to the migration of the enzyme from its point of synthesis, to its point of action could not be determined from this study, hence it was decided to take this study further using immunogold labelling at the electron microscope level.



(a)



30μm

(b)

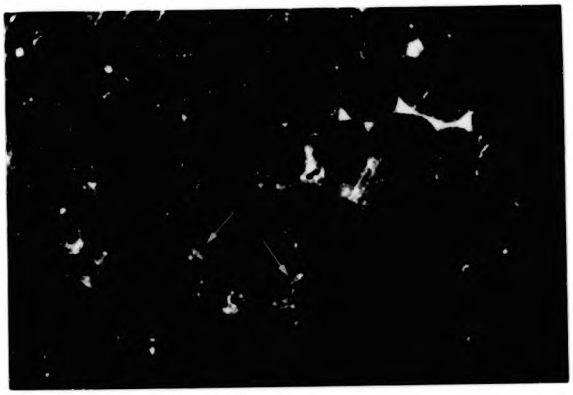
Figure 4.12

Immunofluorescence on a tissue section from day 9.

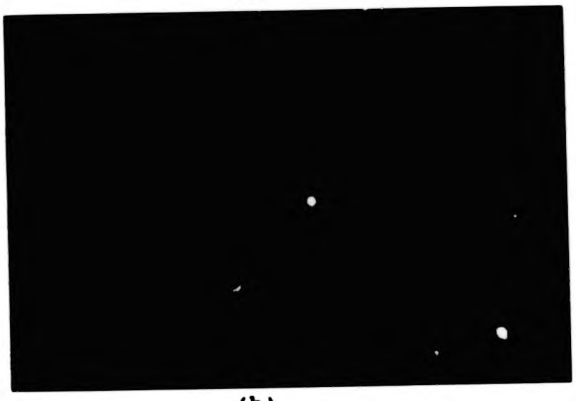
(a) primary antibody

(b) control (pre-immune serum)

magnification x 300



(a)



30μm

(b)

Figure 4.13

Immunofluorescence on a tissue section from day 11.

(a) primary antibody

(b) control (pre-immune serum)

magnification x 300

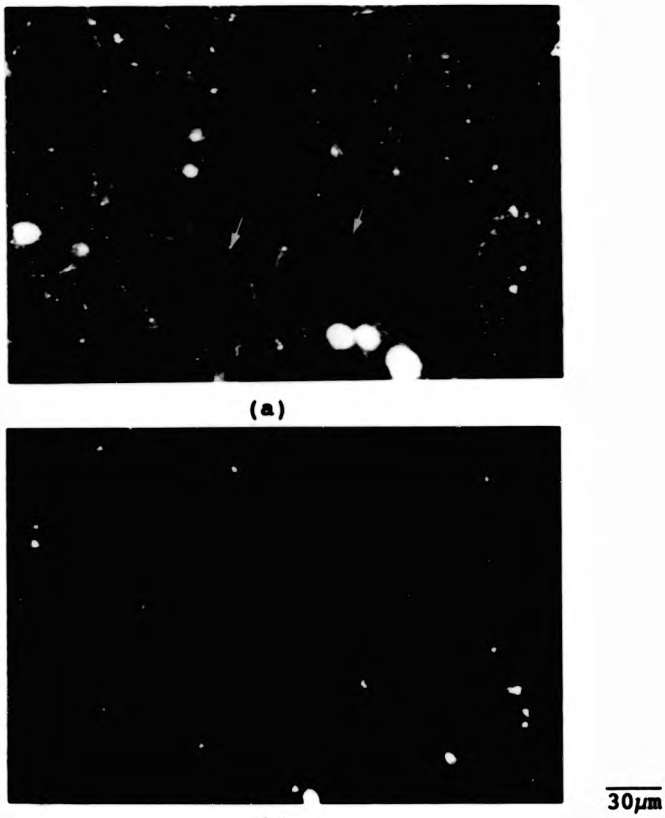


Figure 4.14

Immunofluorescence on a tissue section from day 13.

(a) primary antibody

(b) control (pre-immune serum)

magnification x 300

4.3 Immunogold labelling.

To localise antigens by electron microscopy (E.M.), it is necessary to impart electron density to the bound antibodies. This is generally accomplished using an indirect technique where a secondary label is conjugated to an electron dense material. Second antibodies coupled to ferritin or peroxidase were formerly the methods of choice, but investigators now prefer to use colloidal gold labels.

It was hoped that the same embedded samples as were used for fluorescence labelling could be used for immunogold, however, it was decided to fix the material in a mixture of glutaraldehyde and paraformaldehyde to simultaneously preserve antigenicity and structure (Herman and Shannon, 1984). This type of fixation was not possible for fluorescence studies due to the autofluorescence of glutaraldehyde fixed specimens.

The choice of embedding resin also determines structural preservation and retention of antigenicity. Embedding resins are usually grouped into two categories:- the hydrophobic epoxy resins such as Epon, araldite and Spurrs; and the hydrophillic acrylic resins known as LR White and Lowicryl. Epoxy embedding techniques are no longer the first choice for immunocytochemistry due to the disadvantages that have been found, such as low retention of antigenicity (Craig and Goodchild, 1982) and higher background labelling (Roth et al. 1981).

Lowicryl is a hydrophillic acrylic resin which results

in antigen retention, hence labelling tends to be high density, specific and with low background (due to the low attraction of the background plastic for hydrophillic immunoreagents). However, Craig and Goodchild (1982) and Herman and Shannon (1984) reported difficulties in the labelling of plant tissues, which was thought to be due to pigments which absorb light in the blue range (U.V.), hence interfere with polymerisation of the resin. Complex carbohydrates of plant cells are poorly embedded in Lowicryl, with much of the fibrillar detail of the cell wall being omitted. It has also been noted that starch grains, chloroplasts and membrane organelles are poorly preserved (Herman and Shannon, 1984; Brewin et al., 1985; Shaw and Henwood, 1985).

Many of the problems associated with Lowicryl have been alleviated by the use of LR White, an aromatic acrylic resin. It has been reported to be less extractive of plant tissues than Lowicryl but LR White will often embed tissues which have proven difficult in Lowicryl e.g. bark (Herman, 1987) and tobacco seeds (Hoffman et al., 1987).

Osmium tetroxide treatment irreversibly destroys many antigenic sites which limits its usefulness. However, in common with other acrylic resins, LR White is less lipophilic than epoxides and therefore not as likely to disrupt ultrastructure when post osmification is omitted. The embedding of unosmicated tissue in LR White is accomplished by dehydrating the fixed plant tissue in a graded ethanol series (Newman and Hobot, 1987). While it is

not necessary to completely dehydrate tissue in order to embed in LR White, Herman (1987) noted that a complete dehydration resulted in a more successful embedding. Complete removal of the lipid components (with 100% ethanol) prior to resin infiltration prevents semi polymerisation of tissue samples.

Infiltration times for plant specimens are longer than those for animal tissues. Tissue shrinkage can be a problem, when osmium is omitted, but can be avoided by using intermediate steps of diluted LR White (2:1, then 1:1, LR White:ethanol) before 100% LR White then polymerisation (Newman et al., 1987).

Polymerisation of LR White at 50° C is reported to result in limited crosslinking of the resin and consequent formation of long chain polymers, while polymerisation at 65° C results in extensive crosslinking (Newman and Hobot, 1987). Herman et al. (1988) found that polymerisation at 50° C resulted in soft tissue in the blocks which could not be successfully thin sectioned.

4.3.1 Labelling with colloidal gold-protein A.

15 nm gold conjugate (protein A) was prepared using the procedure of Roth (1982). Colloidal gold solution was prepared by reducing tetrachloroauric acid with sodium citrate according to the method of Frens (1973). This procedure yields monodisperse colloids and depending on the amount of sodium citrate added, monodisperse solutions with particle diameters varying from 15 nm to 150 nm can be

obtained. 100 ml 0.01% tetrachloroauric acid was heated and when boiling, 4 ml 10% (w/v) trisodium citrate was added and the solution boiled gently until a deep burgundy colour was obtained. This colour change indicates the formation of monodisperse spherical particles, the reduction of the gold particles being complete after 5 min. Frens (1973) also noted that neither prolonged heating nor the addition of extra citrate produced any substantial change in the suspension after that period. This combination yields colloidal gold of 15 nm diameter.

To form a stabilised complex with protein A, the "gold number" needs to be determined (Zsigmondy, 1901). The gold number was defined as the number of mg of a protein (or other substance) which when added to 10 ml of colloidal gold just fails to prevent the colour change from red to blue upon addition of 1 ml of 10% (w/v) NaCl. Following the protocol of Roth (1982), serially diluted solutions of protein A (0.1 ml) was added to the colloidal gold solution (0.5 ml). After the addition of NaCl (0.1 ml of 10%), the stabilisation effect was judged visually by the colour of the gold. The lowest protein concentration which prevented a colour change from red to blue, after addition of NaCl, was considered to be sufficiently stabilising.

In our experiments it was found that 7 μg of protein A stabilised 0.5 ml colloidal gold solution. To prepare a crude complex of protein A-gold, the protein solution was added to the gold, then 1% aqueous polyethyleneglycol (PEG) 20,000 was added to act as a further stabilising agent, to

lower the rate of aggregate formation (Horisberger et al., 1975; Horisberger and Rosset, 1977). Romano et al. (1974, 1975) have used BSA as a further stabilising agent and added NaCl (final concentration 1%) to the crude preparations, as this is thought to remove unstabilised or incompletely stabilised gold particles. Attempts to label sections with this complex proved unsuccessful as there was no specificity to the gold labelling. Initially, at higher dilutions of gold (e.g. 1:20) no labelling was detected. When the dilution of the gold label was decreased (concentration increased), the label was deposited throughout the section and would also appear to be in the plastic (e.g. Figure 4.15 (a)).

4.3.2 Labelling with colloidal gold conjugate.

Goat anti-rabbit gold conjugate (15 nm) was purchased from Sigma and used in place of the colloidal gold as secondary antibody at a dilution of 1:50. The grids were examined without uranyl acetate staining, but again no gold labelling was observed (e.g. Figure 4.15 (b)).

Undiluted conjugate was applied but unfortunately this resulted in non-specific labelling with clumps of gold particles being deposited on the sections (data not shown).

Attempts to produce specific labelling were undertaken by Mr Colin Smith at Unilever Research, but as yet no specific labelling of cells has been obtained.The reason for the failure of EM level labelling is unclear given the success of immunofluorescent labelling.

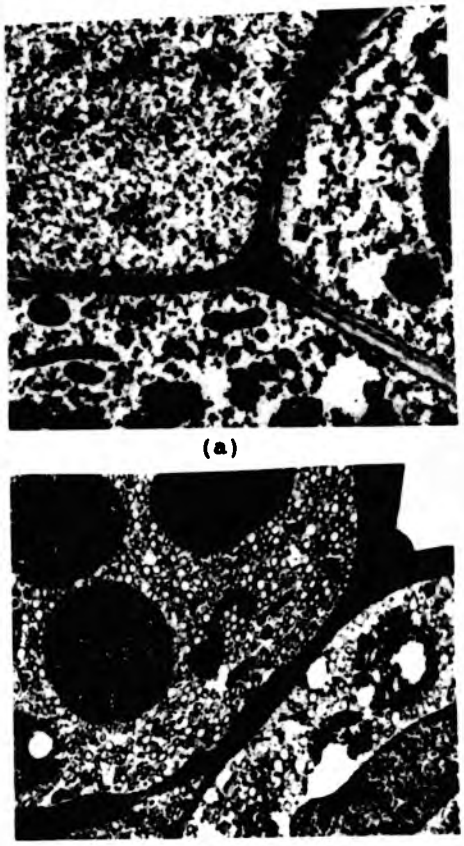


Figure 4.15

Immunogold labelling on sections from day 7.

(a) labelling with colloidal gold-protein A

(b) labelling with colloidal gold conjugate (Sigma)

magnification x 4000

4.4 Discussion.

The cotyledon cells of nasturtium seeds contain xyloglucan (XG) rich cell walls which disappear following germination. This phenomenon has been reported by Heinricher (1888) and Edwards *et al.* (1985) and is examined in more detail here using histochemistry.

Mobilisation of XG from thickened cotyledonary cells was monitored at 2 day intervals. The storage material appeared to begin to be removed by about day 6 after imbibition, with the most rapid depletion being observed between days 9 to 15 and complete mobilisation by about day 20. These results concur with the chemical analysis of XG mobilisation by Edwards *et al.* (1985). By day 20, only isolated pillars of XG would appear to be present. It is thought that these are analogous to those observed in tamarind seeds (whose storage material is also XG) which are considered by Reis *et al.* (1987) to prevent the flattening of the cell wall and the resultant influx of the cytoplasm. As yet we do not understand the reason for the survival of these isolated "pillars" of storage material.

Staining with amido black of the protein bodies throughout this period showed that the protein was being mobilised at about the same time as the XG. By about day 20, all the protein bodies have disappeared leaving behind vacuolated areas.

Initially there is no carbohydrate staining in the cytoplasm, but as the time course proceeds, positive PAS

staining of starch grains was observed. This is assumed to be the formation of transitory starch which is common in seed storage mobilisation.

Edwards et al. (1985) followed the mobilisation of xyloglucan via chemical analysis and found that the amount of xyloglucan was already diminishing by about day 8. Between days 9 and 13, they recorded maximum depletion of xyloglucan and that mobilisation was complete by day 19. However, it may be that the initial depletion is so slight that it does not significantly alter the quantitative amounts detected by the gravimetric analysis of Edwards et al. (1985).

Fluorescence staining was designed to be specific for the detection of the enzyme endo 1,4 ß-D-glucanase. Several problems had to be overcome, the main one being autofluorescence of the tissues. Much of the problem of autofluorescence can be solved by epifluorescence microscopy and the use of appropriate filters.

Specific fluorescence emission is relatively weak compared to the total light necessary for total illumination. Therefore high intensity light as provided by the mercury arc high vacuum lamp is a minimum requirement to obtain bright immunofluorescence. Filters are used between the illuminating light and the specimen to provide the range of light (>300 nm) required and to cause only minimal autofluorescence (Sternberger, 1986).

Another problem was overcome by using formaldehyde alone as fixative in place of glutaraldeyde as the latter

caused non-specific autofluorescence of tissues.

Starch grains which would appear to increase in number at the latter stages of development, also produced a problem in that non-specific fluorescence was exhibited in the control samples in which pre-immune serum was used. This problem would appear to have been alleviated by passing the serum down a small affinity column, thus removing this non-specific binding.

The increase in fluorescence observed as the time course proceeded would appear to be indicative of an increase in the amount of enzyme present at that time. The endo 1,4 ß-D-glucanase first appeared at the inner surface of the storage cell wall and then migrated to become distributed throughout the wall. No correlation between the "arched" (gothic windows) pattern of xyloglucan breakdown and enzyme localisation was observed. It could therefore be proposed that the endo 1,4 ß-D-glucanase serves to loosen the storage wall by attacking the xyloglucan backbone, thus allowing the other hydrolytic enzymes involved in this process access to the wall.

Attempts to clarify the migration pattern of the enzyme from its point of synthesis to its point of action by immunogold labelling proved unsuccessful and the results produced were inconclusive. At higher dilutions of the gold label, no staining was observed whereas at lower dilutions of gold label, the deposition of gold particles appeared to be random. This can be clearly seen, as specific gold labelling should produce uniform deposition with individual

gold particles being identified. The deposition of gold found on our samples was indicative of clumps of gold being precipitated on the surface of the sample. Indeed, it would appear that we successfully labelled the plastic matrix into which the tissue sample was embedded.

The process was tested using different gold labels:- colloidal gold attached to protein A and using a commercial label purchased from Sigma Chemicals Co. However the result would appear to be the same for both.

Various possibilities for the unsuccessful labelling could explain this result. These include the fixation procedure which may have resulted in the loss of antigenicity, the embedding matrix may have been masking the antigens hence the reaction would not occur or that the gold label was not sensitive for this type of system. Some of these problems were investigated by Mr. Colin Smith at Unilever Research. He used 4% formaldehyde / 1% glutaraldehyde as fixative and the gold label was goat anti-rabbit IgG gold conjugate purchased from Janssens Pharmaceuticals who specialise in immuno-cytochemical products. However, as yet, no specific staining has been observed. It could be that there may be an inherent problem in nasturtium tissue which prevents this type of labelling.

CHAPTER FIVE

CHAPTER 5

ENDO 1,4 ß-D-GLUCANASE ACTIVITY AND SYNTHESIS IN RELATION TO THE TIME COURSE OF GERMINATION AND XYLOGLUCAN MOBILISATION.

This chapter describes the use of antiserum to investigate endo 1,4 ß-D-glucanase (endoglucanase) protein in relation to changes in activity.

5.1 Germination time course.

For nasturtium, germination is complete by about day 5 after imbibition (Edwards et al., 1985). After this time the mobilisation of the nasturtium reserve polysaccharide, galactoxyloglucan (amyloid), is dependent upon the activities of 3 hydrolytic enzymes: a ß-galactosidase to remove side chain galactosyl units, an α-xylosidase to hydrolyse side chain xylosyl residues and an endo 1,4 ß-D-glucanase to hydrolyse the internal linkages of the backbone.

Mobilisation of xyloglucan in nasturtium starts 8 days after imbibition and is complete by day 22 (Edwards et al., 1985). Endo 1,4 ß-D-glucanase activity was monitored throughout this period using the viscometric assay and its appearance throughout the time course followed using Western blotting.

5.2 Viscometric Analysis.

The activity of endo 1,4 ß-D-glucanase can be determined using the ferricyanide reductimetric assay (Halliwell and Riaz, 1970), where the concentration of reducing glucose residues released from the substrate (in this case tamarind seed xyloglucan - glyloid) can be determined spectrophotometrically, however, this method can only be applied to purified samples of the enzyme. Crude preparations of protein from cotyledon extracts contain other exo hydrolytic activities which would also hydrolyse the substrate, causing increased reducing power.

A more specific viscometric assay was therefore employed which is especially suitable and sensitive to endo (random splitting) rather than exo (endwise splitting) cleavage of polymers (Almin and Eriksson, 1967). The viscometric assay is extremely sensitive during the initial phase of the reaction, hence activity was calculated from the plot of flow time against elapsed time and extrapolated to give flow time at time zero (time of mixing). The activity was defined as $100/t_{0.8}$, where $t_{0.8}$ is the elapsed time taken for the flow time to decrease to 0.8 of its value at time zero.

A typical assay contained glyloid at a concentration of 10 mg/ml, and viscometric flow times were taken at 2 minute intervals. Because of the temperature dependence of viscometry, the assays were performed in a water bath which was accurate to ± 0.1° C and measurements were taken until

a linear relationship between flow time and elapsed time had been established (usually after 45 min.).

5.2.1 Appearance of endo 1,4 ß-D-glucanase activity as detected by viscometric analysis.

The enzyme activity conferred a steady increase from day 7, reaching a peak at day 13 and declining to a minimum by day 28, as illustrated in Figure 5.1. The activity of this enzyme and others (namely ß-galactosidase and α-xylosidase) have been shown to mediate the mobilisation of the xyloglucan present in nasturtium cotyledons following germination (Edwards *et al.*, 1985). The results shown here confirm the observations of the histochemical analysis, where xyloglucan was observed to become most rapidly depleted between days 9 to 15 and complete mobilisation was shown to have occurred by day 20 (Section 4.1.1). It would appear that there is a link between xyloglucan mobilisation and the activity of the endo 1,4 ß-D-glucanase.

5.3 *De novo* synthesis?

Whether this increase was due to *de novo* synthesis of the enzyme or activation from a latent form was investigated using a Western blotting technique (Towbin *et al.*, 1979). Previous methods for determining *in vivo* protein synthesis include the use of protein synthesis inhibitors. Seeds were incubated with inhibitors (e.g. cyclohexamide) and the effect (if any) on enzyme activity

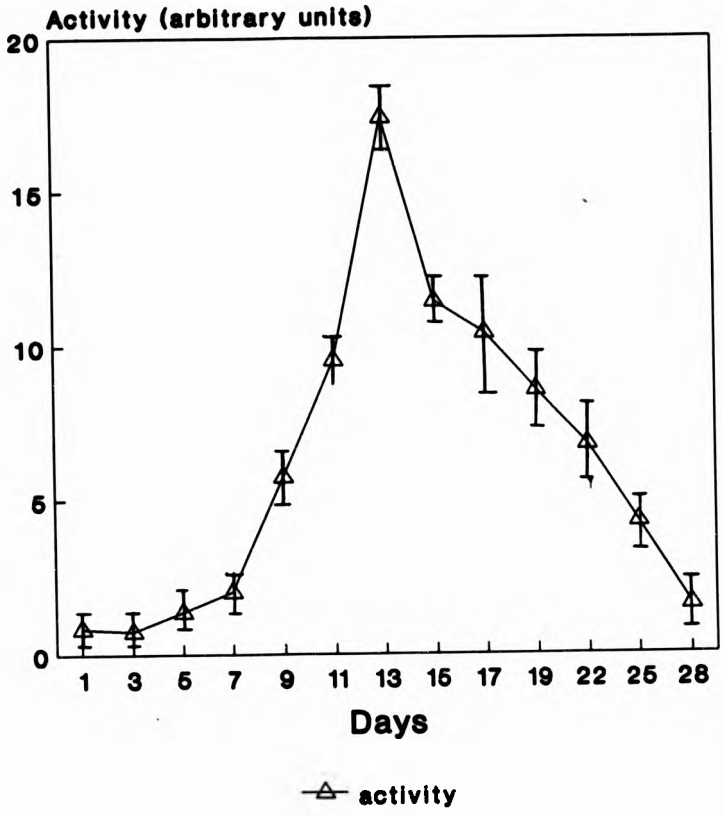


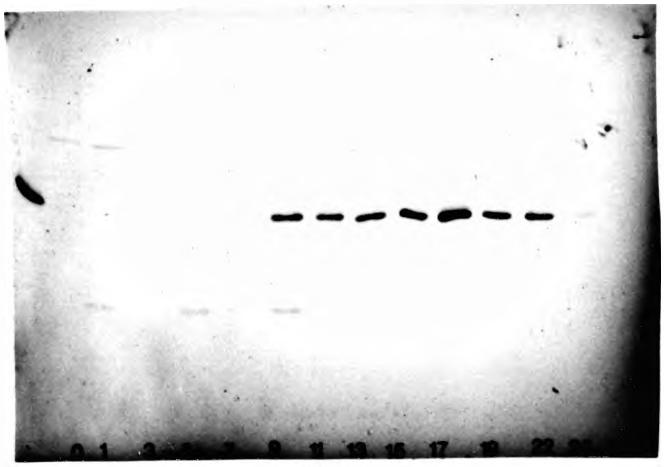
Figure 5.1
Activity per cotyledon pair over a 28 day time course (error ± 2 x S.E.)

determined. A lack of enzyme activity was assumed to be due to the lack of synthesis of the appropriate protein. Another method utilises density labelling where a ^{2}H label is incorporated into a protein synthesising system and newly synthesised proteins can be detected using caesium chloride density gradients. Western immunoblotting was employed for two reasons:- firstly it is more sensitive as it used a specific antibody for the enzyme of study and secondly, the amount of protein present at a particular time can be calculated quantitatively (see section 5.4).

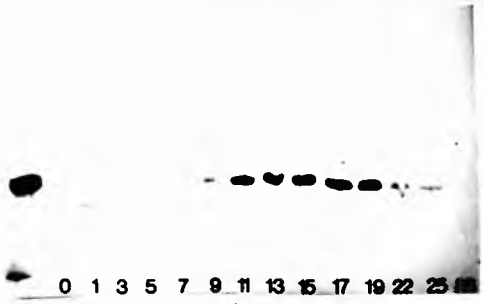
5.3.1 The appearance of endo 1,4 ß-D-glucanase as detected by western immunoblotting.

Seeds were harvested at two day intervals from the start of imbibition and cotyledon samples prepared. Crude cotyledon extracts were prepared as described before (Section 2.10.4) and the resultant "crude" protein sample was applied to a 12% SDS gel, immunoblotted (Section 2.2.4) then challenged with the endoglucanase antibody.

Figure 5.2 (a) illustrates a typical blot of the effect of crude antiserum on a developmental blot. Clearly a strong reaction producing a band of apparent M_r of 29,000 (as determined by the position of the pure endoglucanase sample) first becomes visible at day 5 after imbibition and becomes more prominent indicating an increase in protein content as the time course proceeds. The lack of any visible band prior to this time would suggest that the protein was not present. However two other bands appeared



(a)



(b)

Figure 5.2

Immunoblot of the appearance of endo 1,4 ß-D-glucanase over a 28 day time course.

a) Blot challenged with crude antiserum.

b) Blot challenged with affinity purified antiserum.

on the blot, the first was a slight cross-reacting band of higher molecular weight than 29,000 which was present from day 0 onwards and disappeared at about day 11. The second cross-reaction appeared at a lower molecular weight than 29,000 and was also present from day 0 onwards and disappears around day 11. A similar blot which was challenged with affinity purified serum, was shown in Figure 5.2 (b). The bands of higher and lower molecular weight than the enzyme marker disappeared, hence it was concluded that the bands from Figure 5.2 (a) were not immunologically related species to the purified enzyme.

5.4 Accurate quantification of endo 1,4 ß-D-glucanase protein levels.

To determine exactly how much enzyme protein was present at each stage during the time course, a quantitative method was developed, involving the use of an absolute standard of endo 1,4 ß-D-glucanase the protein content of which had been measured accurately by amino acid analysis. The amino acid composition was determined, using an autoanalyser, by Mr. Chris Sidebottom of Unilever Research Laboratories (Colworth House, Sharnbrook, Bedford). Dilutions of this standard were subjected to SDS-PAGE (Section 2.2.2) and transferred as before (Section 2.2.3). Immunoblotting was performed using the affinity purified preparation of the antiserum, the blot was scanned using a reflective densitometer and the areas under the

peaks determined and related back to the amount of protein loaded in each lane. Figure 5.3 showed that a linear relationship was observed over a 100 fold dilution of antigen. This method is sensitive to measure 1.78 ng of protein.

The amount of endoglucanase protein present over the 28 day time course was determined by scanning the blot shown in Figure 5.2 (b). No endoglucanase protein appeared to be present until day 5, after which the protein content increased dramatically reaching a peak at day 15 of 570 ng (per cotyledon pair) as illustrated in Figure 5.4. This trend parallels the first rising part of the curve of activity versus development (Figure 5.1). However, the amount of protein did not substantially decrease until after day 22, unlike the activity curve which showed a rapid decrease after day 13. This trend was reproduced when another data set of samples was analysed, suggesting that much of the protein present after this stage is inactive although not apparently degraded. There is quite a sharp reduction in protein from 500 ng at day 19 to less than 5 ng at day 22 suggesting that much of the protein is degraded at this time.

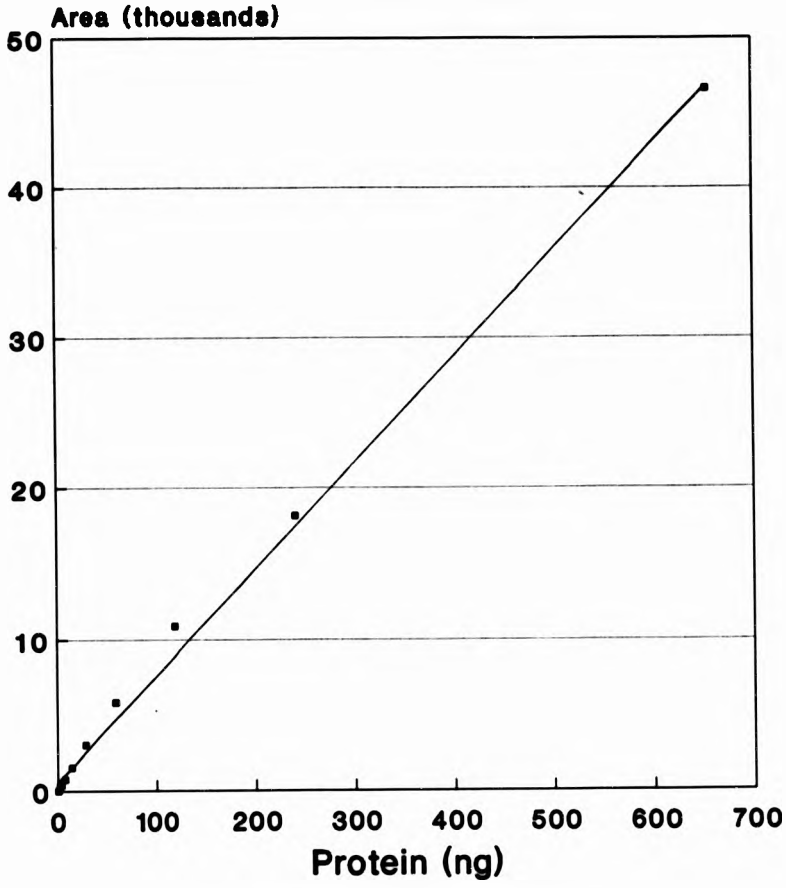


Figure 5.3
Calibration curve demonstrating the relationship between protein concentration and its area measured densitometrically.

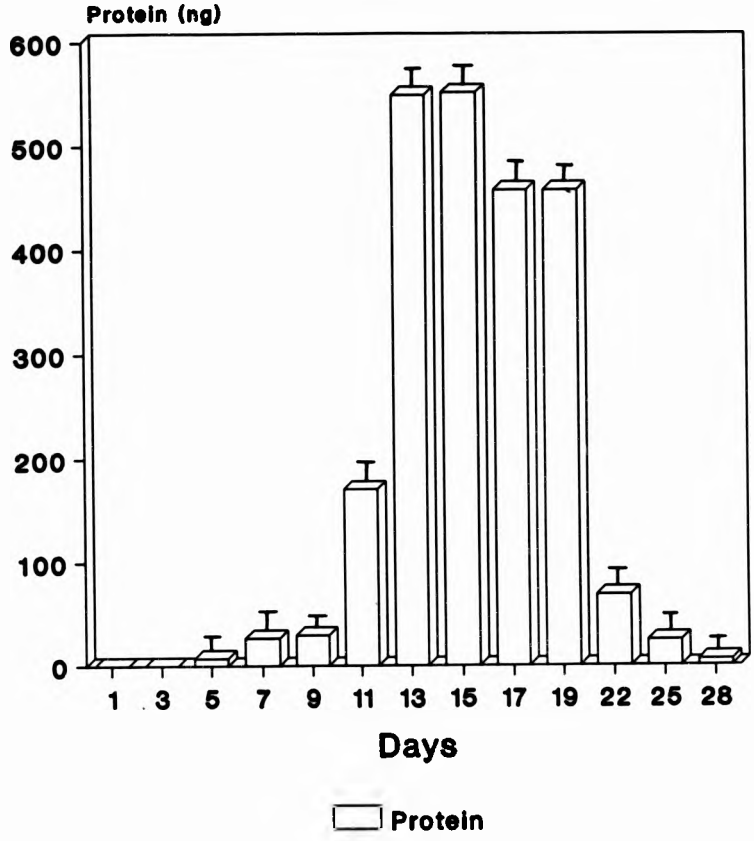


Figure 5.4
Protein per cotyledon pair over a 28 day time course (error • 2 x S.E.)

5.5 Discussion.

Endo 1,4 ß-D-glucanase from nasturtium cotyledons was measured both in terms of activity and protein content. The activity curve of endoglucanase agreed with that of Edwards *et al.* (1985), with an initial sharp increase in activity (36 fold), peaking at day 13 and declining to a minimum by day 28. It was noted that there was a link between enzyme activity and the breakdown of xyloglucan as observed in the previous chapter. The appearance of the enzyme was monitored using the specific antibody to the protein, which indicated that the protein was synthesised *de novo* and not produced via an activation of a pre-formed latent form. The initial immunoblot (Figure 5.2 (a)) demonstrated a band with a higher M_r which was present at the time of planting but disappeared shortly before peak enzyme activity. It was suspected that this band could represent a latent form of the enzyme, the higher M_r representing the presence of a signal peptide which would be cleaved off (post-translational modification) to create the active form of the enzyme. However, when a similar immunoblot was challenged with affinity purified antiserum, this band disappeared suggesting that this band did not represent an immunologically related species, and therefore was considered not to represent a higher molecular weight form of the endo 1,4 ß-D-glucanase.

The amount of endoglucanase protein present was measured quantitatively, revealing a dramatic increase in

the amount of protein synthesised between days 7 to 13. This pattern correlates with that of activity of the enzyme measured viscometrically over this time period (Figure 5.5). The protein, however, does not decrease until after day 19 unlike the activity which decreases after day 13. This would suggest that a substantial amount of protein present at this time was inactive but not degraded. When other data sets were analysed, this trend was also observed. If one considers the state of the cotyledons at this time, these results are not unreasonable. As cotyledonary cells age they lose much of their water content and begin to dry up, hence the enzyme could, at this stage, be denatured due to environmental conditions. The dramatic decrease of enzyme protein between days 19 and 22 was probably due to protein degradation. This degradation must have been rapid and extensive as no cross-reacting bands of a smaller molecular weight were detected on the immunoblot which would have been indictive of gradual protein deterioration. This implies that this degradation is a controlled process, which could be explained by the presence of a protease whose activity/synthesis could be triggered by the conditions produced as the cotyledonary cells age.

Hence it can be concluded that endo 1,4 ß-D-glucanase protein was synthesised <u>de novo</u>, and that this synthesis was under precise control. The rising curves for both activity and protein were similar indicating that the increase in activity was a direct result of the increase in

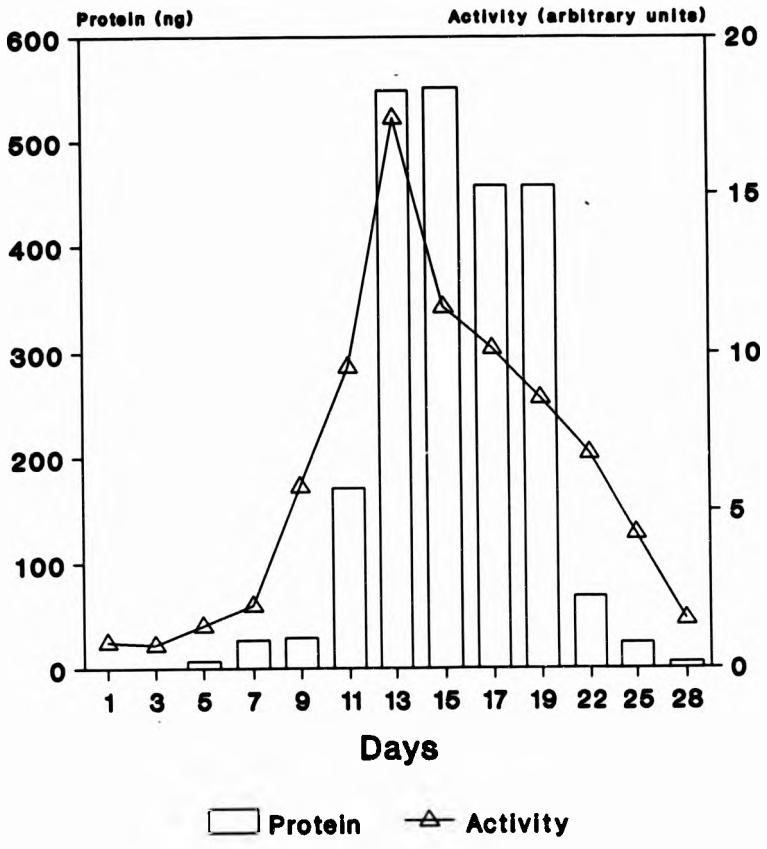


Figure 5.5
Protein/activity per cotyledon pair over a 28 day time course.

protein. The decrease in endo 1,4 ß-D-glucanase activity only occurred after the time of rapid xyloglucan depletion (i.e. after it had completed its function.). This decrease in activity seemed to be determined largely by denaturation, although the decrease in protein content would appear to be a controlled process. The control of the synthesis of the enzyme is examined at the molecular level in Chapter 6, where physiological control and other factors affecting protein production are examined in Chapter 7.

CHAPTER SIX

CHAPTER 6

TRANSLATABLE ENDO 1,4 ß-D-GLUCANASE MESSENGER IN RELATION TO ENDO 1,4 ß-D-GLUCANASE SYNTHESIS.

This chapter involves the isolation of translatable RNA from cotyledons at different stages of development and its translation *in vitro*. The results are compared with the data from the previous chapter. During the isolation of messenger RNA (mRNA), many problems were encountered which are also discussed.

For help with some of those problems I am indebted to Dr. Jacquie de Silva, Mr Carl Jarman and their colleagues at Unilever Research, Colworth House, Sharnbrook for their help and advice.

6.1 The purification of Ribonucleic acid (RNA).

The main objective in the isolation of RNA is to obtain a high yield of undegraded polymer. The techniques used most frequently originated from the work of Kirby (1956). As the RNA of the living cell is usually complexed with protein, the extraction process involves the removal of RNA from protein by disruption of hydrogen bonds. Kirby achieved this by using an aqueous phenol solution containing a detergent, in this case SDS.

Because of the great diversity of cells and tissues there are many different procedures described in the

literature. Most have certain features and requirements in common:

1) the need to separate RNA from protein and DNA in a quantitative a manner as possible

2) to prevent RNase activity both during and after isolation to obtain intact biologically active RNA

3) to separate RNA species from each other.

Several general techniques are commonly employed in specific mRNA isolation, however, modifications may be required in order that these methods become applicable to a particular system. Many problems are also encountered in the isolation of intact message, some of which are discussed below.

The main practical problem is the prevention of ribonuclease (RNase) action. RNases are present both on fingers and in tissues as well as being contaminants in many reagents (Taylor, 1979), hence handling of tissues has to be avoided. An effective inhibitor of RNAse is diethyl pyrocarbonate (DPC) which reacts with the imidazole nitrogens of histidine residues and free amino groups, resulting in loss of enzyme activity (Ehrenberg et al., 1976), thus materials and reagents that cannot be autoclaved can be treated with DPC. The preparation of translationally active mRNA from most tissues requires RNase inhibitors in the tissue homogenates (Taylor, 1979).

Some tissues themselves contain an endogenous RNase inhibitor, such as rat liver (Roth, 1956), Gagnon and de Lamirande (1973) purified this inhibitor and as such has

been used in RNA isolation. Bernheimer and Steele (1955) showed that RNase inhibitors have also been found in plants, where 3 extracts out of 48 exhibited inhibition of RNase:- bayberry leaves (Myrica carolinesis), dogwood leaves (Cornus florida) and lilac leaves (Syringa vulgaris). The first two extracts were found to form insoluble complexes with gelatine, and they prevented RNase activity by co-precipitation along with the gelatine. The lilac however, appeared to inhibit degradation of RNA by reacting with the enzyme. These inhibitors have been added to RNA preparations but Gerlinger et al. (1973) reported that they exhibit tissue specificity which reduced their effectiveness in other systems. Protein denaturants, such as detergents, also provide protection against RNase contamination, Noll and Stutz (1968) reported that SDS was effective in this process.

Another problem is that of obtaining dissociation of proteins from the ribonucleoprotein complex, without degrading the RNA. Kirby (1956) developed the removal of protein from RNA by disruption of the hydrogen bonds using phenol. The mixture is centrifuged to separate the aqueous and organic phases with the nucleic acids partitioning to the aqueous phase and the protein compacting at the interface.

Temperature, salt and pH all affect phenol extraction and these variables have been studied by Brawerman (1973), Brawerman et al. (1972) and Perry et al. (1972). They found that alkaline buffers (pH 9) were required to enhance the

partitioning of RNA to the aqueous phase when cold phenol was used, whereas neutral buffers enhanced the binding of mRNA to the denatured proteins and hence was found at the interface. It was found (Brawerman, 1973) that, in particular, poly A^+ mRNA bound to denatured proteins. However, Perry et al. (1972) found that extraction at room temperature with an equal volume of chloroform, aided in the partitioning of mRNA to the aqueous phase. Phenol extraction can cause aggregation of RNA species, which can be dissociated by heating at 65° C (Rhoads, 1975; Longacre and Rutter, 1977). Alternatively, brief treatment of the cell extract with proteinase K before phenol extraction will reduce the protein interface and improve the RNA yield. Other components include 1% (v/v) isoamylalcohol, to minimise foaming, and 0.1% 8-hydroxyquinoline as an anti-oxidant for the phenol and as a chelating agent to destabilise RNA - protein interactions (Suzuki and Brown, 1972; Mach et al., 1973).

6.1.1 Isolation of polyadenylated (poly A^+) RNA.

Eukaryotic mRNA contains a covalently attached 3'-terminal poly (A) segment of heterogenous length (ranging in size from 20 - 250 bases in length). Poly (A) - containing RNA's can be separated from other cellular RNA's by affinity chromatography on oligo d(T)-cellulose (Aviv and Leder, 1972) or by adsorption onto nitrocellulose filters (Brawerman et al., 1972) or messenger affinity paper (Wrescher and Herzberg, 1984).

Oligo d(T) cellulose consists of 12 - 18 nucleotides covalently attached to microgranular cellulose usually packed into a small column or syringe. The RNA solution is passed over the affinity support, at a slow rate, with the poly A^- RNA's passing through (including ribosomal RNA). The bound RNA (poly A^+) is eluted with a low ionic strength buffer. An intermediate low salt washing step often helps remove non-specifically adsorbed RNA (Aviv and Leder, 1972).

Even using the most efficient chromatography techniques, as much as 20% of the translatable mRNA activity from rat liver (Taylor and Tse, 1976), chicken oviduct (Shapiro and Schimke, 1975) and other tissues has been reported to fail to bind to the affinity ligand. These authors suggested that this lack of binding may be due to very small poly A segments (i.e. less than 10-20 bases in length) or a poly A segment that is blocked in some manner. Also there is the possibility that the mRNA could be lost in the repeated passage through columns or during alcohol precipitations, possibly via RNase presence in one of the vessels. Wreschner and Herzberg (1984) devised a method whereby a diazonium activated paper is inactivated by poly (U), hence giving it a very high affinity for poly (A) sequences. Werner et al. (1984) using this paper developed a technique to isolate mRNA and comparing the two isolation methods, concluded that the messenger affinity method was advantageous because it was quicker and the poly A^+ RNA can be recovered in small volumes which avoids further loss due

to precipitations.

6.1.2 Translation *in vitro* of isolated RNA.

Cell free protein synthesising systems capable of translating exogenous mRNA are useful for monitoring mRNA activity during purification, which generally require specific antibodies to detect particular radioactively labelled protein products by immunoprecipitation. Several of these systems have been used in recent years for the translation of eukaryotic mRNA's. Of these the rabbit reticulocyte lysate and the wheat germ extract have received the most attention.

There are advantages and disadvantages for both systems, but the major disadvantage for the reticulocyte lysate is the presence of high levels of endogenous globin mRNA, which limits the exogenous mRNA detection level unless a sensitive antibody is available. The fact that the system is already saturated with endogenous mRNA means that the added mRNA is only translated to the extent that it can compete with the endogenous mRNA. A further disadvantage is that the added mRNA cannot be assayed by simple radioactive amino acid incorporation methods, but only by analysis of the radioactive products synthesised. This in turn demands that the mRNA under test codes for a protein which is easily resolved from the proteins synthesised on endogenous reticulocyte mRNA. However, Pelham and Jackson (1976) devised a method whereby micrococcal nuclease is used to destroy endogenous mRNA, the nuclease, in turn, is Ca^{+}

dependent, hence can be inactivated by the addition of a chelating agent (EGTA).

Marcus et al. (1974) developed a cell free protein synthesising system from untoasted wheatgerm. This system is advantageous as it is easy to prepare, economical and has a low endogenous background of mRNA activity. Some mRNA's are translated more efficiently by wheatgerm extract than by reticulocyte lysate e.g. cytoplasmic and secreted yeast invertase (Perlman and Halvorson, 1981). However, one of the main disadvantages of the wheatgerm system is the translation efficiency of large molecular weight proteins. The reticulocyte system translates larger molecular weight proteins more efficiently and for smaller molecular weight proteins the wheatgerm system is more proficient. In addition, a number of amino acids whose endogenous levels in the reticulocyte lysates are high, can be more effectively incorporated into translational products by the wheatgerm system.

6.2 Development of a technique to isolate undegraded translatable RNA from nasturtium seeds.

The initial procedure was a simple phenol extraction based on Berger and Birkenmeier (1979). Seeds are very tough tissues, hence they were ground to a fine powder before extraction. To minimise RNase action, this process was carried out at a low temperature - with liquid nitrogen. The sample was then allowed to thaw into lysis

buffer (10 mM Tris, pH 7.8, 150 mM sodium acetate) containing vanadyl ribonucleoside complex (VRC) which is an ribonuclease (RNase) inhibitor. After centrifugation, the sample was extracted in buffer (40 mM Tris, pH 7.8, 40 mM EDTA, 70 mM sodium acetate, 2% SDS) which destabilised the ribonucleoprotein complex. Phenol/Chloroform extractions followed to separate out the denatured proteins and the RNA was precipitated with ethanol. The RNA pellet was collected on centrifugation and dried under nitrogen before being dissolved in 0.5% SDS (1 ml), which resulted in preparation 1 (Table 6.1). The purity of the sample was calculated by the ratio A_{280}/A_{260} where a ratio of 2 is considered to be a pure sample (Maniatis et al., 1989). The concentration of RNA obtained from the sample was found assuming that an O.D. at 260 nm of 1.0 is equivalent to 40 μg RNA (Maniatis et al., 1989). Preparation 1 was considered to be pure but the yield was very poor. Plant seeds contain large amounts of polysaccharides which are known to be extracted into the aqueous phase together with the RNA (Clemens, 1984). This was considered as a distinct possibility for the poor yield obtained given the high xyloglucan content of nasturtium tissue and hence subsequent preparations were prepared by slightly modifying the technique. These modifications included:-

a) Washing the pellet in 3 M sodium acetate pH 6.0 to remove polysaccharide.

b) Keeping the tissue frozen at all times and thawed directly into extraction buffer to reduce RNase

Table 6.1 **Comparison of RNA isolation methods.**

Prep. no.	Starting Material	A_{280}	A_{260}	260/ 280	Dilution Factor	Conc. µg/ml	RNA µg*
1	0.2g	0.065	0.132	2.03	1:10	52.8	26.4
2	0.2g	0.039	0.055	1.41	1:10	22.0	11.0
3	0.2g	0.034	0.029	0.85	1:10	11.6	8.7
4	0.2g	0.016	0.028	1.75	1:100	112	84.0
5	0.2g	0.029	0.050	1.72	1:100	200	100
6	1.0g	0.057	0.081	1.40	1:100	324	32.4
7	1.0g	0.073	0.075	1.03	1:200	600	30.0
8	1.0g	0.027	0.046	1.70	1:200	368	36.8
9	1.0g	0.040	0.072	1.80	1:200	576	28.8
10	1.0g	0.058	0.094	1.62	1:200	752	37.6
11	0.5g	0.023	0.039	1.70	1:100	156	31.2
12	2.0g	0.075	0.137	1.82	1:200	1096	54.8
13	30.0g	0.088	0.142	1.62	1:200	1136	37.9
14	2.0g	0.493	0.885	1.80	1:50	1770	177
15	2.0g	0.824	1.476	1.79	1:50	2952	295
16	2.0g	0.282	0.505	1.79	1:50	1010	101
17	2.4g	0.423	0.881	2.08	1:100	3524	294

* µg of RNA per g starting material

activity.

c) Removing cell debris by centrifugation after initial extraction.

This resulted in preparation 5 (Table 6.1) which had a greatly improved yield. Attempts to translate this preparation proved unsuccessful as there was no incorporation of radioactive label. Lack of translation may be due to the presence of VRC which is known to be a potent inhibitor of protein synthesis initiation (Schmike et al., 1974). Hence a modified isolation procedure was adopted.

Chirgwin et al. (1979) noted that to eliminate nucleolytic degradation of RNA one has to denature all of the cellular proteins including RNase. However, this would only be successful if the rate of denaturation exceeded the rate of RNA hydrolysis by RNase. In recent years the strong reducing properties of guanidine salts have been used as potent chaotropic agents to dissociate ribonucleoprotein complexes and inactivate RNases by breaking the disulphide bonds (which are essential for RNase activity). Other techniques utilise urea-denatured material with lithium chloride (Barlow et al., 1963; Jaquet et al., 1977; Auffray and Rougeon, 1980), however, under those conditions RNases are only partially inhibited.

Cathala et al. (1983) described a technique that combined the advantages of direct lithium chloride (LiCl) precipitation and homogenisation in guanidine monothiocyanate. The lysis buffer, used to homogenise

tissue, contained 5 M guanidine monothiocyanate, 10 mM EDTA, 50 mM Tris-HCl, pH 7.5 and 8% (v/v) ß-mercaptoethanol (filtered to remove any insoluble materials). This homogenisation allows guanidine monothiocyanate to prevent RNA degradation, which otherwise can occur within seconds, and ensures complete cell or tissue solubilisation which minimises DNA contamination and loss of RNA. After complete dissolution (any solid matter remaining was removed via centrifugation) the RNA was precipitated with 7 volumes of 4 M LiCl and left overnight at 4° C. The RNA's, some denatured proteins and contaminating DNA were pelleted by centrifugation, and the supernatant (containing DNA, protein and small RNA's) was discarded. The pellet washed in 2 M LiCl/ 4 M urea, which removed any DNA and protein contamination. After centrifugation the pellet was dissolved in solubilisation buffer (0.1% w/v SDS, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5) and the RNA was extracted using phenol/chloroform (1:1). If the upper aqueous layer appeared cloudy, this step was repeated. The RNA was precipitated overnight at -20° C in 2.5 volumes cold ethanol. After centrifugation, the pellet was washed with 70% (v/v) ethanol (to remove salt) dried down under nitrogen and re-dissolved in sterile water. This procedure resulted in preparation 6. However, attempts to translate this preparation were unsuccessful hence further modifications were made to the isolation procedure. These included:-

a) The tissue was thawed directly into lysis buffer.

b) The number of phenol/chloroform extractions was increased.

c) The volume of lysis buffer was reduced from 7 ml to 3.5 ml (Cathala *et al.*, 1983).

This approach appeared to be successful although the yield was still quite low (Prep. 9, Table 6.1). To try to obtain enough RNA to purify several isolations were performed (Prep. 10 - Prep. 13), which also ensured that the experiment was reproducible. The combined amount of RNA obtained from these preparations was 852 µg. A sample of this preparation was run on a gel and ribosomal bands were clearly observed (Figure 6.1).

6.2.1 Poly A^+ Isolation

Several techniques have been developed to separate polyadenylated (Poly A+) RNA from non-polyadenylated RNA's. The method used was chromatography on oligo (dT) cellulose (Aviv and Leder, 1972) which can be obtained commercially. This method depends upon the annealing of poly A rich mRNA to oligothymidylic acid cellulose columns and its elution with buffers of low ionic strength. From the 852 µg RNA applied to the column, 16.8 µg poly A+ was eluted (≈ 2% total).

6.2.2 *In Vitro* Translation.

Both poly A+ and total RNA were translated using ^{35}S-methionine in the wheatgerm system at the Botany department at Edinburgh University. The translation

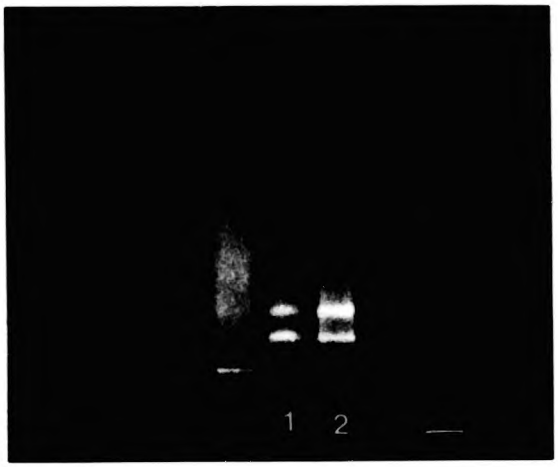


Figure 6.1

Agarose gel of nasturtium RNA showing intact ribosomal bands.

Lane 1: nasturtium RNA (10 μg).

Lane 2: ribosomal markers.

products were run on a 10% SDS gel. The gel was then dried, fixed and exposed to an X-ray film. As can be seen from Figure 6.2 only the poly A+ sample translated, the total RNA sample did not.

In order to obtain translatable RNA it would appear that many preps would be required to obtain enough total RNA to purify, hence an improved method of isolation was required to try to maintain the purity but increase the yield. Unilever Research Laboratories at Colworth House, Sharnbrook very kindly let me use their facilities and share their expertise.

Successful RNA extractions from rape and tobacco seeds (Safford et al., 1988; Safford et al., 1991, unpublished) have been obtained using the method of Hall et al. (1978). This method was modified slightly by including a phenol/chloroform extraction step. The full method is described in Section 2.11.1.

The seeds were ground to a fine powder in liquid nitrogen, then homogenisation buffer was added and the frozen mixture was ground to a fine powder before being allowed to thaw when proteinase K was added. This gave preparation 14 which gave a reasonable purity (a A_{260}/A_{280} of > 1.8 was regarded as reasonable) and a very good yield almost 5 times greater than previously obtained. This preparation was obtained from seeds that still retained their outer testa, hence an extraction was tried using

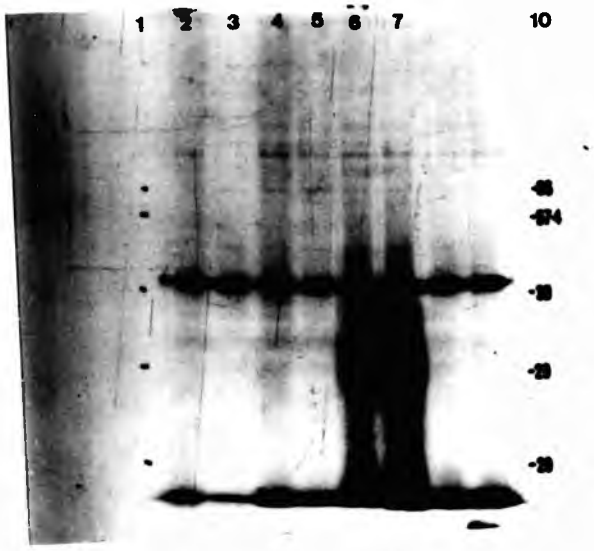


Figure 6.2

SDS-PAGE of ^{35}S-methionine translation products; exposed to an X-ray film for 17 days.

Lane 1: protein markers.

Lane 2: control (no RNA).

Lane 3: total RNA (5 μg).

Lane 4: total RNA (10 μg).

Lane 5: total RNA (20 μg).

Lane 6: poly A^+ RNA (1 μg).

Lane 7: poly A^+ RNA (2 μg).

Lane 10: protein markers.

"peeled seeds" in which the outer coat was removed at harvest using a scalpel blade. This resulted in preparation 15 which did not substantially increase the purity but gave a slightly better yield. This method was repeated using slightly more starting material to ensure the process was reproducible (Prep. 16).

Another method tried was based on the observations of De Vries *et al.* (1988). To omit the use of complicated and expensive RNase inhibitors, they employed a number of techniques, these included:

1) The use of high pH and chelating agents (EDTA).
2) The use of detergents (SDS).
3) Direct thawing of the pulverised material into hot phenol and extraction buffer.

The phenol:RNA extraction buffer (0.1 M LiCl, 1% SDS, 0.1 M Tris-HCl, pH 9.0, 10 mM EDTA) was heated to 90° C. The seed tissue was ground to a fine powder in liquid nitrogen and transferred to a flask at -70° C (ethanol/dry ice bath), then the hot phenol mix was added and the slurry was adjusted to room temperature (25 - 30° C) by occasional heating in the 90° C water bath. Chloroform was then added and the aqueous phase obtained. This aqueous layer was re-extracted with chloroform before precipitation with 8 M LiCl for 16 - 18 hr at 4° C. The RNA pellet was collected upon centrifugation, washed once with 2 M LiCl and twice with 70% ethanol. The pellet was vacuum dried then re-dissolved in sterile water. As can be seen from Table 6.1 this preparation, 17, did not give as high a yield as the

previous method. It was concluded that this method may have been more suited to larger amounts of starting material (i.e. > 5 g), hence it was decided to use the method based on Hall et al. (1978) to isolate RNA from before, during and after germination. A sample of the RNA obtained was run on an agarose gel and intact ribosomal bands were observed (Figure 6.3).

6.3 Isolation of Total RNA from different developmental stages

RNA was isolated from nasturtium tissue at 2 day intervals after imbibition. Samples of the final purified preparations were subjected to a wavelength scan to determine purity and concentration. The results are shown on Table 6.2. The purity for all of the samples was high with slight variations in the concentrations at each stage.

6.3.1 Poly A^+ Isolation

The method utilising messenger affinity paper was used at this stage mainly because of the number of samples obtained. Also smaller concentrations of Total RNA can be applied and obtained avoiding losses due to precipitations (Werner et al., 1984).

The RNA samples from the different developmental stages were concentrated to a concentration of 10 µg/ml (by re-precipitation in ethanol) and 20 µl of each sample (200µg) was applied to the mAP paper. Assuming a minimum of

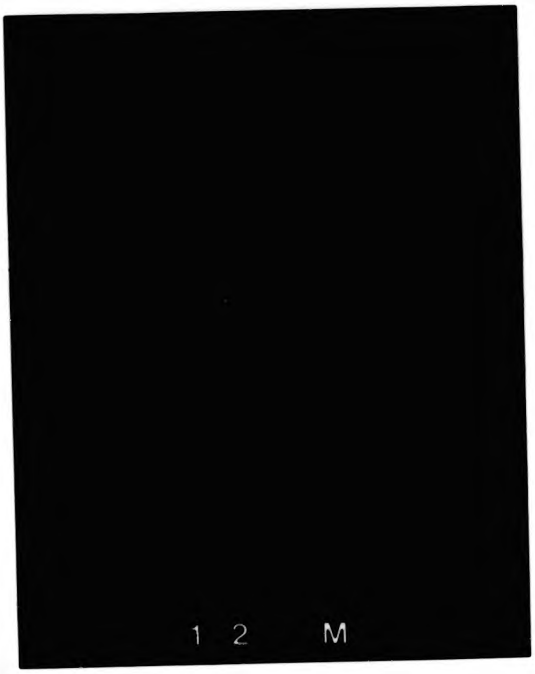


Figure 6.3

Agarose gel of RNA from Preparation 17 showing intact ribosomal bands.

Lane 1: RNA (10 μg).

Lane 2: control RNA from rapeseed (10 μg).

Lane M: ribosomal markers.

Table 6.2 **Purity and concentration of total RNA samples extracted from nasturtium tissue at different developmental stages.**

Days/ Parameter	2	4	6	8	10	12	14
230nm	0.172	0.151	0.153	0.180	0.170	0.296	0.151
240nm	0.209	0.210	0.020	0.024	0.251	0.419	0.233
250nm	0.348	0.367	0.344	0.408	0.435	0.684	0.412
260nm	0.400	0.429	0.400	0.474	0.506	0.786	0.480
270nm	0.316	0.340	0.315	0.378	0.403	0.637	0.380
280nm	0.188	0.202	0.184	0.226	0.236	0.390	0.222
290nm	0.086	0.089	0.080	0.101	0.097	0.168	0.089
300nm	0.014	0.010	0.008	0.014	0.005	0.023	0.000
260/230	2.30	2.84	2.60	2.63	2.97	2.65	3.18
260/280	2.13	2.12	2.17	2.10	2.14	2.01	2.16
conc/ 200µl	240	257	240	284	303	471	288
conc/g material	120	128	120	142	151	196	144

Concentration of RNA is in µg.

1% yield, 2 μg of Poly A^+ should be obtained.

6.3.2 *In vitro* translation of both Total and Poly A^+ RNA from different developmental stages.

Both Poly A^+ and Total RNA were translated using the wheatgerm system and the translation products were applied to a 10% SDS gel and subjected to autoradiography as shown on Figure 6.4. As can be seen from this figure both Poly A^+ and total translated with many protein bands being formed. As the time course proceeds it would appear that the stability of the RNA species alters. The RNA from day 14 would appear to produce less bands than the RNA from earlier stages which could be an indication that it is being degraded at this time.

6.3.3 Immunoprecipitation of Translation products

Both total and Poly A^+ RNA (from day 12) was immunoprecipitated using the endo 1,4 ß-D-glucanase antiserum. Included in the immunoprecipitation mix were varying concentrations of SDS, in order to obtain a single immunoprecipitation band. The results are displayed on Figure 6.5. As shown, concentrations of SDS greater than 0.1% generate a single immunoprecipitation band. When compared to tme protein markers (lane 14), this band would appear to have an apparent molecular weight of 33,000.

Further immunoprecipitation experiments using total RNA from morphologically staged seedlings were carried out at Unilever Research Laboratories by Dr. J. de Silva. It

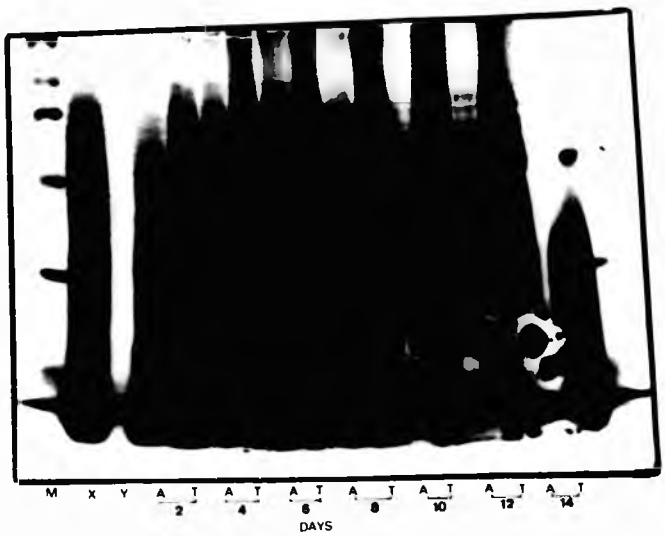


Figure 6.4

SDS-PAGE of ^{35}S-methionine translation products from nasturtium RNA obtained at different stages throughout development.

M = protein markers.

X = positive control (rapeseed RNA).

Y = negative control (no RNA).

A = poly A^+ RNA sample.

T = total RNA sample.

Figure 6.5

SDS-PAGE of immunoprecipitation products.

Effect of SDS concentration on immunoprecipitation.

Lane 1: Total RNA, translation products.

Lane 2: Total RNA, immunoprecipitate control; no antibody, 0% SDS.

Lane 3: Total RNA, immunoprecipitate, 0% SDS.

Lane 4: Total RNA, immunoprecipitate, 0.1% SDS.

Lane 5: Total RNA, immunoprecipitate, 0.5% SDS.

Lane 6: Total RNA, immunoprecipitate, 1.0% SDS.

Lane 7: Total RNA, immunoprecipitate, 2.0% SDS.

Lane 8: Poly A^+ RNA, translation products.

Lane 9: Poly A^+ RNA, immunoprecipitate control; no antibody, 0% SDS.

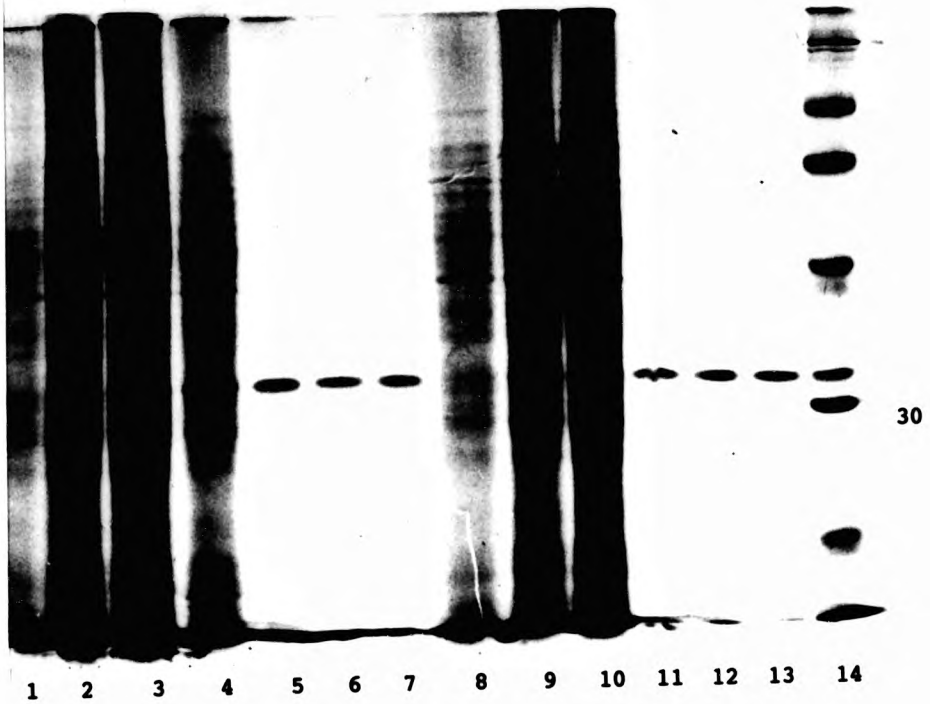
Lane 10: Poly A^+ RNA, immunoprecipitate, 0% SDS.

Lane 11: Poly A^+ RNA, immunoprecipitate, 0.1% SDS.

Lane 12: Poly A^+ RNA, immunoprecipitate, 0.5% SDS.

Lane 13: Poly A^+ RNA, immunoprecipitate, 1.0% SDS.

Lane 14: Poly A^+ RNA, immunoprecipitate, 2.0% SDS, containing ^{14}C protein markers.


30
1 2 3 4 5 6 7 8 9 10 11 12 13 14

was established from these that the 33 kD immunoprecitation band was present throughout development, although in small quantities. An increase in this polypeptide was also observed which reached a peak level with the timing being similar to that of protein production.

6.4 Discussion.

Isolation of translationally active RNA from nasturtium seeds, initially proved difficult. The main problem may have been the presence of large amounts of carbohydrate in the tissue. Various methods were employed and adapted both at Stirling University and at Unilever Research Laboratories. The final extraction procedure being one which yielded both high quality (purity) and sufficient amounts to be obtained for further studies. The quality of the RNA obtained was tested on agarose gels, where the appearance of ribosomal bands was interpretated as an indication of intact, undegraded RNA. However, this was found not to be an accurate measure of active RNA, as this method did not give any information as to whether the RNA would translate. Therefore, RNA samples were translated *in vitro*, and run using SDS-PAGE, with the appearance of protein bands being regarded as indictive of active RNA.

The final RNA extraction procedure yielded both translationally active total and poly A^+ RNA. In the presence of SDS, the endo 1,4 ß-D-glucanase antiserum appeared to specifically precipitate a polypeptide of M_r

33,000. Endo 1,4 ß-D-glucanase protein yields a polypeptide of M_r 29,000, therefore it was concluded that the difference in M_r between these two polypeptides (4,000) represented a signal peptide that could be cleaved off when the final protein was translated.

Further work carried out at the Unilever Research Laboratories yielded information on the presence of active message (mRNA) at variuos stages throughout development. It was found that message was present throughout development but there was also an increase observed which reached a peak level, indicating that transcription must occur. The timing of this peak was found to be similar to that of protein production (Chapter 5), however it was noted that this increase was not as dramatic as either protein or activity levels.

It is clear from these results (and those from Chapter 5) that there is a developmental regulation of endo 1,4 ß-D-glucanase production and that this regulation is probably controlled at both the transcription and translational levels.

CHAPTER SEVEN

CHAPTER 7

PHSIOLOGICAL ASPECTS OF THE CONTROL OF ENDO 1,4 ß-D-GLUCANASE INDUCTION BY THE EMBRYONIC AXIS AND GROWTH HORMONES.

The possible control of endo 1,4 ß-D-glucanase production by the presence of the embryonic axis was investigated and the effect of the axis in other systems is discussed. The removal of the axis was found to have a negative effect on enzyme production, hence the possible reversal of this effect by the growth hormones gibberellin (GA_3) and auxin (2-4-D) was also investigated.

7.1 Control by the embryo/embryonic axis.

In certain cereal grains, the embryo is known to control the mobilisation of endosperm reserves through the production of a hormonal stimulus (Yomo and Varner, 1971). In this well characterised system, the aleurone layer responds to gibberellins emanating from the germinating embryo by synthesising and/or secreting hydrolases into the endosperm to degrade the storage materials.

Food mobilisation in barley, rice and wheat does not occur in endosperms from which the embryo has been removed, demonstrating that the embryo was responsible for the initiation and subsequent control of reserve breakdown. However, the role of the embryo or embryonic axis in the

control of food mobilisation in seeds of dicotyledonous plants is less well understood.

In dicotyledonous plants the mobilisation of reserves has been shown to follow germination and accompany subsequent growth of the embryo or embryonic axis e.g. protein hydrolysis in the storage organs of peas (Chin et al., 1972) castor bean (Stewart and Beevers, 1967) and various members of the Cucurbitaceae (Wiley and Ashton, 1967; Davis and Chapman, 1979 a). The proteolytic products are then transferred to the embryonic axis. A similar effect has been reported for the breakdown of lipid reserves in e.g. castor bean (Muto and Beevers, 1974) and cucumber (Davis and Chapman, 1979 a). Carbohydrate storage products also decline during growth of a seedling e.g. starch in the cotyledons of pea (Juliano and Varner, 1969) and mungbean (Minamikawa, 1979); and galactomannan in fenugreek seeds (Meier and Reid, 1972).

At the onset of reserve mobilisation, there is usually an increase in the activities of the key enzymes involved. For example, protease activity increases in pea (Yomo and Varner, 1973),squash (Wiley and Ashton, 1967) and cotton (Ihle and Dure,1969). Lipase activity increases in castor bean (Muto and Beevers, 1974) and apple (Smolénska and Lewak, 1974). In starch and phosphate metabolism there is also the corresponding increase in the enzymes involved; in pea cotyledons starch phosphorylase, α amylase and phosphatase all increase (Juliano and Varner, 1969; Yomo and Varner, 1973; Young et al., 1960).

Seedling growth and the subsequent mobilisation of reserves appear to be synchronised processes suggesting that the embryo or embryonic axis has an effect on reserve breakdown and enzyme activity in these seeds.

Excision of the axis either prior to or during germination often results in reduced rates of reserve mobilisation in the storage organs. The breakdown of storage protein has been investigated for a variety of species:- peas (Kern and Chrispeels, 1978), squash (Wiley and Ashton, 1967) and cucumber (Davis and Chapman, 1979 a,b). They reported that maximal rates of protein mobilisation depended upon the presence of the axis. Similar results have been reported for lipid breakdown in cucumber cotyledons where reduced rates of triglyceride, diglyceride and free fatty acid utilisation are found when the axis was removed (Slack et al., 1977; Davis and Chapman, 1979 a,b). A similar effect is also found in groundnut (Allfrey and Northcote, 1977). The breakdown of starch reserves is also inhibited in the groundnut and also in mungbean (Minamikawa, 1979).

There are, however, reports that removal of the embryonic axis has no effect on reserve mobilisation e.g. galactomannan breakdown in fenugreek seeds (Meier and Reid, 1972), lipid breakdown in castor bean seeds (Marriott and Northcote, 1975 a) and also protein and starch breakdown in peas (Bain and Mercer, 1966). However, Young et al. (1960) reported that the development of proteinase activity and protein breakdown are prevented by isolating the

cotyledons. This contradiction of results (particulary apparent in pea studies) could be due to the difference in varieties used or non-standardised experimental conditions between laboratories.

The effect of the growing axis on food mobilisation is apparently via the enzyme activities associated with this process. Protease activity in pea cotyledons does not increase in excised cotyledons (Guardiola and Sutcliffe, 1971). Protease activity in mungbeans (Kern and Chrispeels, 1978) and squash (Penner and Ashton, 1967 b) are also reported to be depended upon the axis. Yomo and Varner (1973), studying peas, showed the different behaviour of protease on the one hand, and α and β amylase on the other, during incubation of excised pea cotyledons as compared to their behaviour in attached cotyledons. They reported that the activity of the protease does not increase in excised cotyledons (as compared to a control), that of the amylases does, suggesting differences in the mechanism of regulation of the two kinds of enzyme. In the case of α amylase activity, the consequences of excision of cotyledons include inhibition (Varner et al., 1963; Locker and Ilan, 1975), promotion (Yomo and Varner, 1973) and no appreciable effect whatsoever (Bain and Mercer, 1966)!

Hirasawa (1989) investigated this phenomenon and concluded that the effect of oxygen also had to be taken into consideration. By his analogy to diamine oxidase (Hirasawa, 1988), he concluded that oxygen is essential for the induction of α amylase in pea cotyledons. The supply of

oxygen in attached cotyledons is limited in the early days of germination, hence α amylase activity in attached cotyledons should not be compared with that in detached cotyledons to which adequate amounts of oxygen are available. To investigate the influence of the embryonic axis on its development in pea cotyledons, α amylase activity should be compared between detached cotyledons and cotyledons from which the testa was removed but the axis remained. Hence it was shown that the embryonic axis had a positive effect upon α amylase activity in pea cotyledons after germination.

The enzymes involved in lipid metabolism also show dependence upon the axis e.g. Black and Altschul (1965) showed that lipase activity was suppressed in distal halves of cotton seeds; Gientka-Rychter and Cherry (1968) studying isocitrate lyase in peanut noted that the axis was required in the early stage of germination; and fatty acyl coA dehydrogenase in castor bean (Huang and Beevers, 1974) was also dependent upon the axis.

Other enzymes in which the embryonic axis is required for activity include mannanase in lettuce seeds (Halmer and Bewley, 1979); phosphatase in peas (Young et al., 1960) and amylase activity in bean (Gepstain and Ilan, 1970). Excised cotyledons of Phaseolus vulgaris develop less α amylase than the attached organs, but this effect is manifest only 96 h after excision (Dale, 1969). Thus the initiation of the enzyme can occur without the axis but continued production after 4 days requires its presence.

In contrast, other enzymic activities have been found to be independent of axial control. In mungbean, removal of the axis 7 h after imbibition, slows down the rate of protease development but does not generally decrease the amount of enzyme formed (Minamikawa, 1979). If the axis is excised prior to imbibition, then no protease is found, indicating an axial requirement at the earliest stages of development. Isocitrate lyase development in groundnut (Allfrey and Northcote, 1977); peanut (Marcus and Freely, 1974) and soybean (Tester, 1976) are all independent of the axis. In castor bean seed (Huang and Beevers, 1974) fatty acyl coA dehydrogenase was found to be dependent upon the axis while isocitrate lyase, catalase and cytochrome oxidase were found to be independent. Lipase, isocitrate lyase, malate synthase and fructose 1,6 biphosphatase in cucumber seeds are all found to be independent of the embryonic axis (Slack et al., 1977; Becker et al., 1978; Davis and Chapman, 1979 a).

Studies on seed which store reserves in non-embryonic tissues also yields evidence for and against the concept that the embryo initiates and controls mobilisation. Pinus ponderosa and Pinus sylvestris exhibit a positive role of the embryo on enzymes of the storage tissue. In previously chilled (stratified) P. ponderosa, isocitrate lyase activity in the storage tissue rises during the first 4 days after imbibition, removal of the embryo reduces this level by about 60%; removal of the embryo after 2 days has no such effect (Bilderback, 1974).

It has been reported that misleading results concerning the effect of the axis can be obtained depending upon the incubation conditions used. Many workers use petri dishes as they provide a convenient method for incubation under sterile conditions (especially when test solutions are being applied to excised tissues). When cucumber cotyledons were incubated on moist paper towelling in seed trays, they were found to be autonomous for the production of lipolytic enzymes (Slack et al., 1977; Davis and Chapman, 1979 a). Similar results were obtained when petri dishes were used and the cotyledons placed on moist filter paper. Ford et al., (1976) showed that the apparent axial control was an artefact produced by the experimental techniques used and was due entirely to the physical conditions imposed upon the excised plant parts. They concluded that any excess of water leads to a progressive inhibition of enzyme development creating an illusionary effect of axial enhancement. Similar results were found with squash isocitrate lyase (Penner and Ashton, 1967 a) and pea ribonuclease and phosphatase (Bryant and Haczynski, 1976). Ford et al., (1976) concluded that the probable cause was the availability of oxygen. When cotyledons are partially submerged there is a reduction in the rate of diffusion of molecular oxygen which may be the limiting factor in the production of a particular enzyme.

7.2 Effect of embryo removal on endo 1,4 ß-D-glucanase.

An investigation into the effect of the embryo on production of endo 1,4 ß-D-glucanase was undertaken. The embryo was removed at various times:- 0, 2, 4, 6, 8 and 10 days after planting and growth was allowed to continue until day 15. The excised cotyledonary halves were incubated in plastic trays on moist filter paper; any seeds which exhibited microbial contamination were discarded.

Crude protein samples were made and assayed for activity (Section 5.2). The activities observed when the embryo was removed at various stages were compared to the control and are illustrated graphically in Figure 7.1. The crude protein samples were also analysed using Western immunoblotting and scanned to determine the amount of endo-glucanase protein present (Section 5.4). The amount of enzyme protein present at each stage was represented graphically, again samples are plotted compared to the control (Figure 7.2). As can be seen from Figure 7.2 if the axis was removed at day 0, no endo 1,4 ß-D-glucanase protein can be detected, although enzyme activity was observed, but when compared to the control this would appear to be insignificant. No protein can be detected when the embryo was removed at day 2 and again there was no significant increase in activity when compared to the control. If the axis was removed at 6 days after planting when protein can be detected (Section 5.3.1, Figure 5.2), there was no apparent increase in the activity, as compared

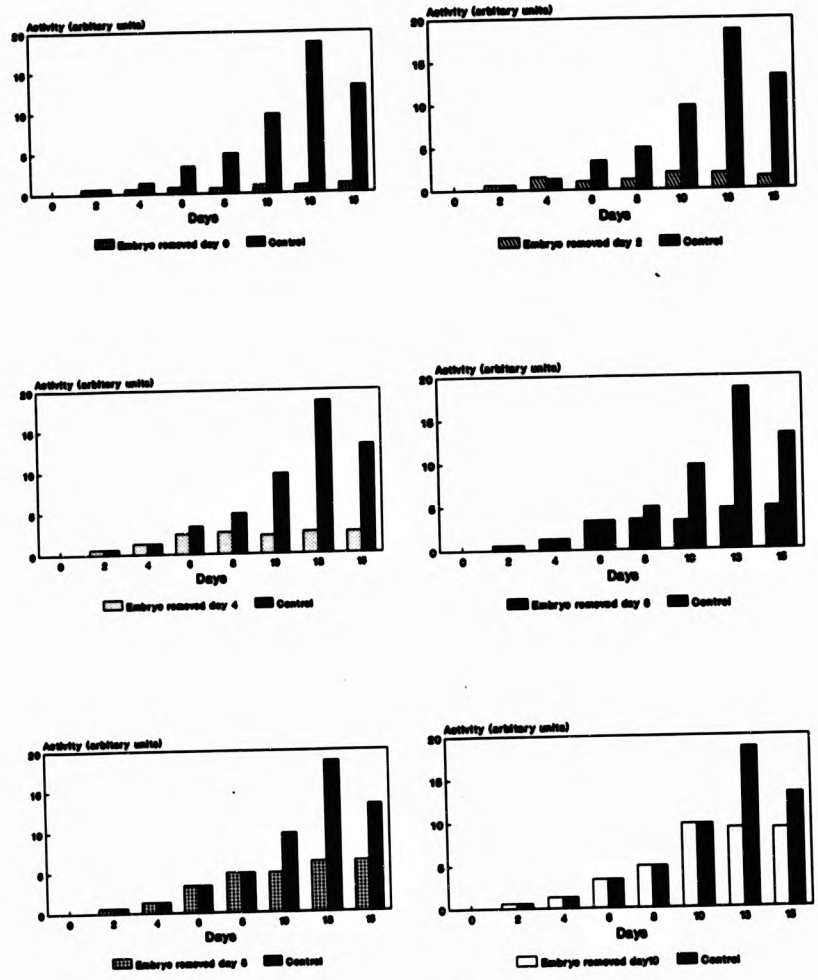


Figure 7.1

Effect of embryo removal at various stages on endo 1,4 ß-D-glucanase activity levels as compared to the control

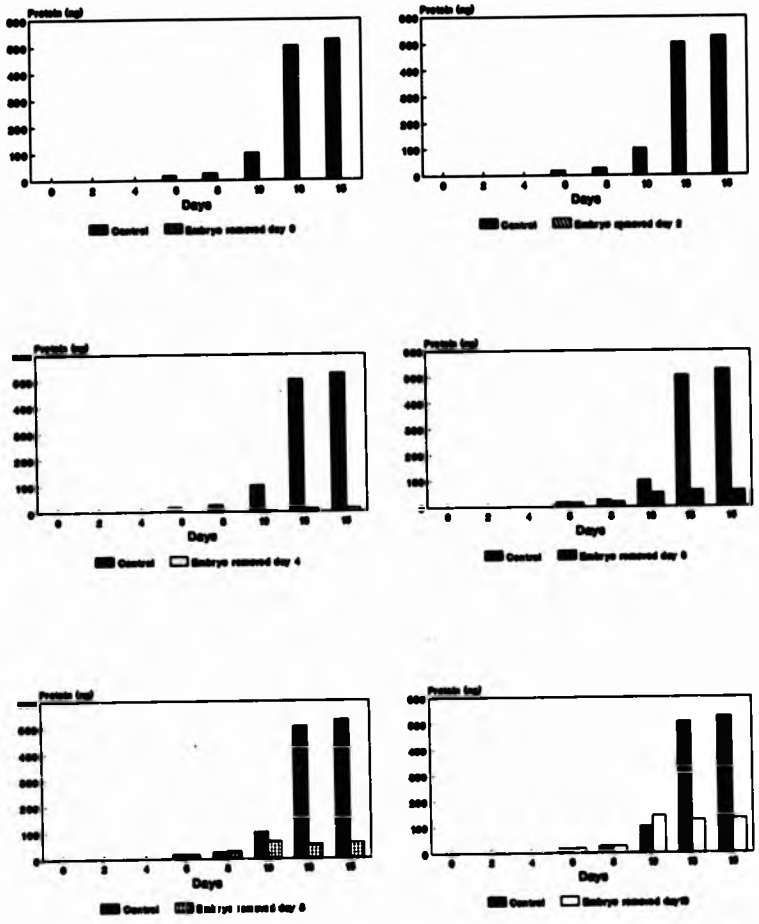


Figure 7.2

Effect of embryo removal at various stages on endo 1,4 ß-D-glucanase protein levels as compared to the control

to the control. There was a slight increase in the amount of protein throughout the time course, but not comparable to the amount that would be present in the intact seed. A similar effect occurs when the axis was removed at days 8 and 10. Thus removal at these later stages of development does have an effect on enzyme production. This effect was also found for α amylase activity in the cotyledons of Phaseolus vulgaris by Dale (1969). Although he concluded that the initial development of α amylase activity in the cotyledons was not dependent upon the presence of the embryo, in the absence of the embryo continued increase in activity of the enzyme does not occur.

An interesting result was obtained when the embryo was removed at day 4, initially no protein, but as the time course proceeds, the protein begins to appear in small amounts. This increase does not equal the amount present in the control and there is no apparent increase in activity.

When the activities at all the different stages are compared (Figure 7.3) to the amount of protein present at each stage (Figure 7.4) there is a correlation. The lack of increase in activity could be attributed to the lack of protein present. At the early stages of development, days 0 and 2, slight activity can be detected but no protein. This phenomenon could be explained by the fact that crude protein samples were used for activity assays and that the viscometric assay (Section 5.2) is not completely specific for endo 1,4 ß-D-glucanase. Other exo hydrolytic activities present in the crude preparation could be causing a slight

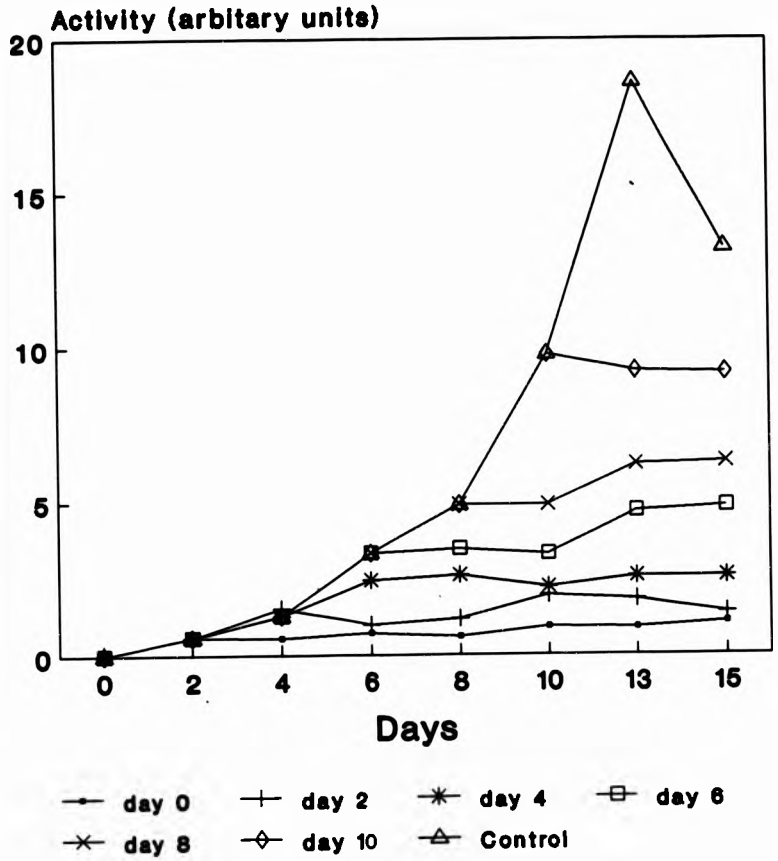


Figure 7.3
Effect of embryo removal on activity of endo 1,4 ß-D-glucanase: Combined data

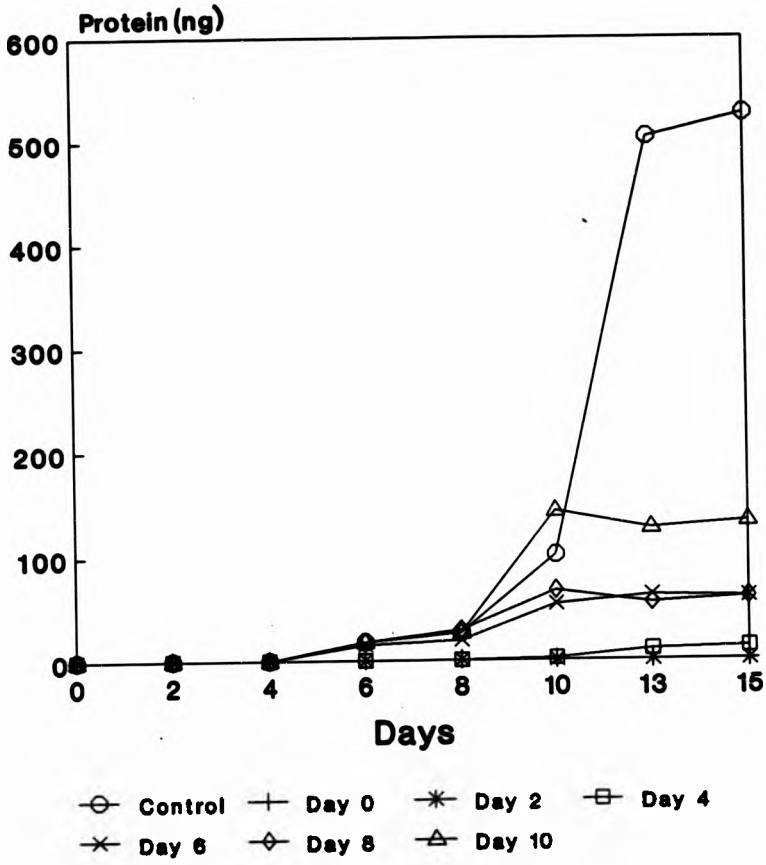


Figure 7.4
Effect of embryo removal on endo 1,4 ß-D-glucanase protein levels : Combined data

decrease in the viscosity of the substrate which could be mistaken as enzyme activity at these early stages of development. It should be noted, however, that these "activities" are negligible when compared to the control and also they remain virtually constant (within error) when there is no protein present hence it could be regarded as background. When protein can be detected (around day 4) a significant increase in activity was found.

Hence from these results it would appear that removal of the embryo does have a negative effect on endo 1,4 ß-D-glucanase presence and activity. Also the embryo is required not only for the initiation of the protein but also for its continued development and the subsequent increase in activity.

There are two possible mechanisms by which the embryonic axis exerts a control over reserve mobilisation and hence the corresponding enzymes involved. In the first, the embryo may produce a factor (hormone) which stimulates the development of the enzyme in the storage organs (hormonal control). In the second, the growing axis, by acting as a metabolic sink, may prevent the accumulation of quantities of the soluble products of reserve degradation, which would prove inhibitory to enzyme development and/or activity i.e. preventing feedback inhibition. The former of these possibilities was investigated further.

7.3 Plant hormone effects.

It has been argued that if a known hormone such as gibberellin or auxin can replace the beneficial effect of the embryo, the possibility that an endogenous hormone might naturally be involved can be raised. Much of the evidence implicating plant hormones in the control of reserve mobilisation comes from studies in which hormones have been exogenously supplied to excised storage organs in an attempt to mimic the effect of the embryonic axis.

In squash cotyledons it was reported that cytokinins increase protein breakdown and subsequently replace the axial requirement for the normal development of proteolytic enzyme activity, but no other growth regulators can replace the effect of the axis (Penner and Ashton, 1967 b). Cytokinins are also reported to enhance the activities of glyoxylate cycle enzymes involved in lipid mobilisation. Again this effect was seen in squash (Penner and Ashton, 1967 b), where 2 day old seedlings contained 2.5 times more isocitrate lyase activity than excised cotyledons of a similar age, when treated with benzyladenine. Similarly, gibberellins have also been reported to be involved in lipid mobilisation. The same enzyme, isocitrate lyase, in hazel cotyledons was shown to be enhanced (9 fold increase) when gibberellin (GA_3) was added (Pinfield, 1968). In cotton seeds, lipase activity was replaced by GA_3 when the embryo was removed, however, the authors noted that other samples of equivalent viability did not exhibit a requirement for

GA_3, which they attributed to differences in conditions of maturation and storage (Black and Altshul, 1965).

Enzymes associated with the mobilisation of carbohydrate reserves can also be affected by the application of hormones. There is a great deal of evidence to support the action of a gibberellin like substance on amylase production in the aleurone layer of cereals, much less is known about starch degradation in the seeds of dicotyledonous plants. Cytokinins are known to enhance α amylase activity in bean cotyledons (Gepstain and Ilan, 1970). In mannanase production by lettuce endosperm, both cytokinins and gibberellins are reported to replace the influence of the embryonic axis (Halmer and Bewley, 1979). In contrast, in the groundnut (Allfrey and Northcote, 1977), ABA was reported to promote both starch breakdown and α amylase activity, whereas gibberellin has no effect. Although such observations suggest a possible role for endogenous hormones, either singly or in conjunction with other factors (e.g. light), anomalies have arisen from this type of experiment. For example, a variety of the enzymes involved in lipid mobilisation, in excised castor bean endosperms, have been found to be enhanced on the addition of gibberellin, even though the axis has apparently little effect (Huang and Beevers, 1974; Marriott and Northcote, 1975 b).

In peas the situation is more confused. Varner et al., (1963) reported that the pea embryo axis effect on α amylase could be replaced by gibberellin and kinetin, but

Sprent (1968) could not demonstrate any promotive influence. Garcia- Luis and Guardiola (1978) reported that gibberellin had an inhibitory effect on α amylase development and they also demonstrated that the hormone had only a minor effect on the hydrolysis of starch reserves. A more recent study from Hirasawa (1989) reported that it was in fact auxin and not gibberellin which induced α amylase activity in peas.

Auxins do not rate highly as a control hormone for the mobilisation of reserve substances and the consequential effect on the enzymes involved. Auxin has mainly been reported as an inhibitory factor, the exception being α amylase in peas (Hirasawa, 1989). Their involvement would appear to be as the controlling factor in stem elongation, by initiating wall breakdown. Reports have been made that auxin enhances the activity of a ß 1,3 glucanase associated with the avena cell wall fraction (Masuda and Yamamoto, 1970). The same authors had also found a similar effect in pea and barley segments.

Gibberellins have also been reported to have an effect on cell wall dissolution. Taiz and Jones (1970) noted that the walls of aleurone cells in barley underwent extensive breakdown and they concluded that this breakdown was accomplished through the action of a glucanase whose secretion, like that of α amylase was under gibberellin control.

A pea endo 1,4 ß-D-glucanase, whose natural substrate is thought to be the endogenous cell wall xyloglucan, was

also found to be induced by auxin (Hayashi and Maclachlan, 1984).

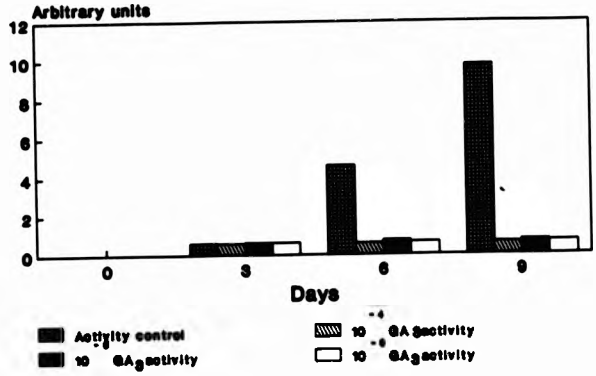
It was suggested by Edwards et al., (1986) that the xyloglucan specific endo 1,4 ß-D-glucanase similar to those from nasturtium may be involved in the turnover of primary cell wall xyloglucans, hence it is possible that auxin may also be the controlling factor for the development of this enzyme.

Both gibberellin (GA_3) and auxin (2-4-D) were tested on nasturtium seeds from which the embryo had been removed.

7.3.1 Effect of gibberellin.

The effect of 3 different concentrations of gibberellin:- 10^{-4} M; 10^{-5} M and 10^{-6} M were tested on nasturtium seeds where the embryo had been removed at days 0, 3, 6. Growth was allowed to continue until day 9 when the embryo was removed at the earlier stages i.e. days 0 and 3, and growth was allowed to continue to day 15 when the embryo was removed at day 6. As can be seen from Figure 7.5, none of the concentrations of GA_3 had a stimulatory effect on the production of enzyme protein if the embryo was removed at day 0. The activity was negligible and no increase could be detected over the time course.

If the embryo was removed at day 3 (figure 7.6), again no enhancement could be detected. It was thought that this case may be analogous to that of Dale (1969) with Phaseolus vulgaris, where α amylase activity can only be enhanced 96 hr after imbibition. Therefore the embryo was removed at



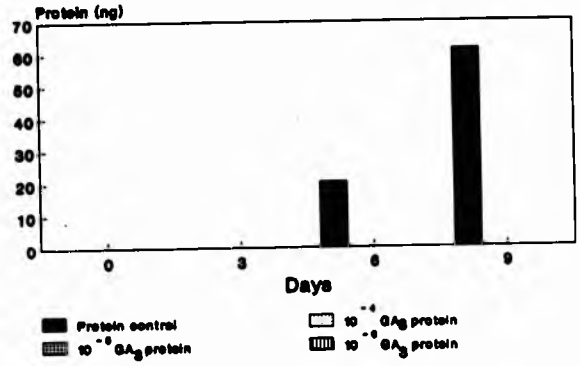


Figure 7.5

Effect of GA_3 on endo activity and protein when the embryo was removed at day 0.

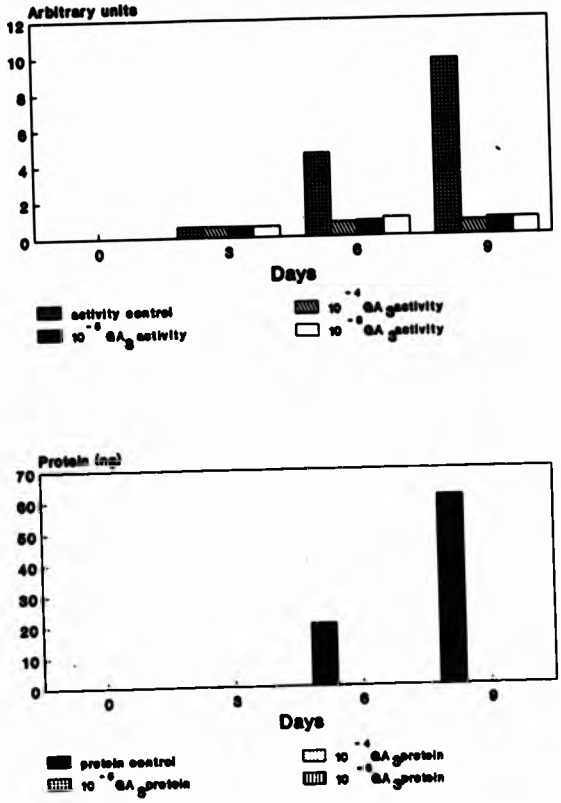


Figure 7.6

Effect of GA $_3$ on endo activity and protein when the embryo was removed at day 3.

a later stage, day 6, when both endo 1,4 ß-D-glucanase activity and protein can be detected. It may be that the hormone is not involved in the initiation of the enzyme, but may be required for continued production of the enzyme. However from Figure 7.7, it can be seen that none of the concentrations of GA_3 enhance activity or protein levels. The protein levels increase slightly at day 8, then decrease as the time course proceeds, but as this change represents less than 1 ng, it was considered to be insignificant.

It is interesting to note that the amount of enzyme protein produced in the presence of GA_3 decreased slightly when compared to the original experiment when the embryo was removed at day 6 (Figure 7.2). Whether this is due to the presence of gibberellin or is simply due to natural variation within a different set of seeds has yet to be determined. It can therefore be concluded that gibberellin has no appreciable enhancement effect on the production of endo 1,4 ß-D-glucanase from nasturtium seeds from which the embryonic axis has been removed.

7.3.2 Effect of auxin.

The auxin used was 2-4-dichlorophenoxyacetic acid (2-4-D). Auxin was applied at 3 different concentrations, 10^{-4} M, 10^{-5} M and 10^{-6} M. It had been noted that one of the major properties of auxins is that they stimulate growth and synthesis at low concentrations (generally 10^{-10} M to 10^{-5} M) and inhibit these processes at higher levels

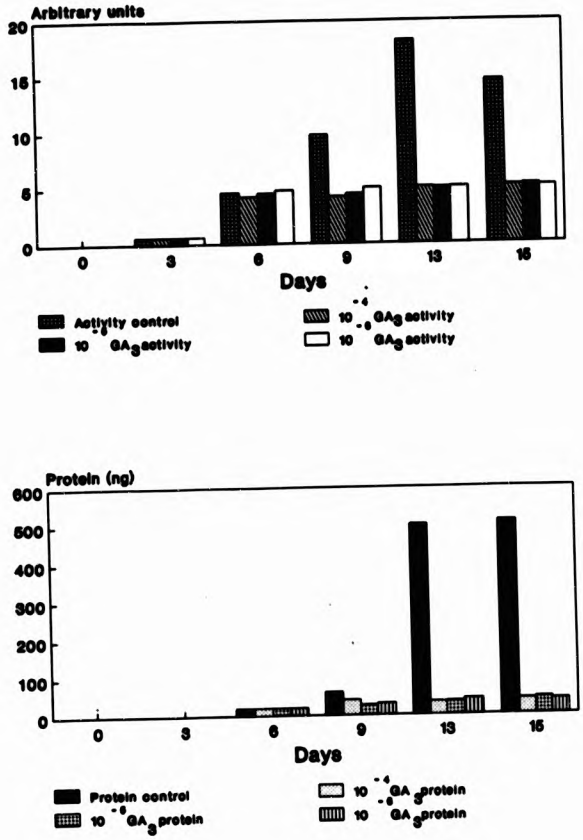


Figure 7.7

Effect of GA_3 on endo activity and protein when the embryo was removed at day 6.

(Hilton, 1966).

The embryo was removed at days 0, and 3 and growth was allowed to continue until day 9. The embryo was also removed at day 6 and growth allowed to continue until day 15. Figures 7.8 and 7.9 show the effect when the embryo was removed at days 0 and 3 respectively. Again there is no significant enhancement of either enzyme activity or protein. The blots used to determine protein content showed no reaction with the enzyme antiserum, hence the blots were restained with amido black (data not shown) to ensure that protein was present on the immunoblot (i.e. to ensure that there was adequate transfer). It would appear that these concentrations of 2-4-D have no apparent effect on endo-glucanase production.

When the embryo was removed at day 6, when both activity and protein can be detected, 2-4-D does not have a significant influence on replacing the effect produced by the embryo (Figure 7.10). Both the protein and activity levels would appear to remain static when the embryo was removed.

These results would appear to reinforce the idea that the embryo is required for both initiation and continued development of endo 1,4 ß-D-glucanase in nasturtium cotyledons, and that these two hormones at the concentrations stated, singly, do not replace the deleterious effect produced by removing the embryonic axis.

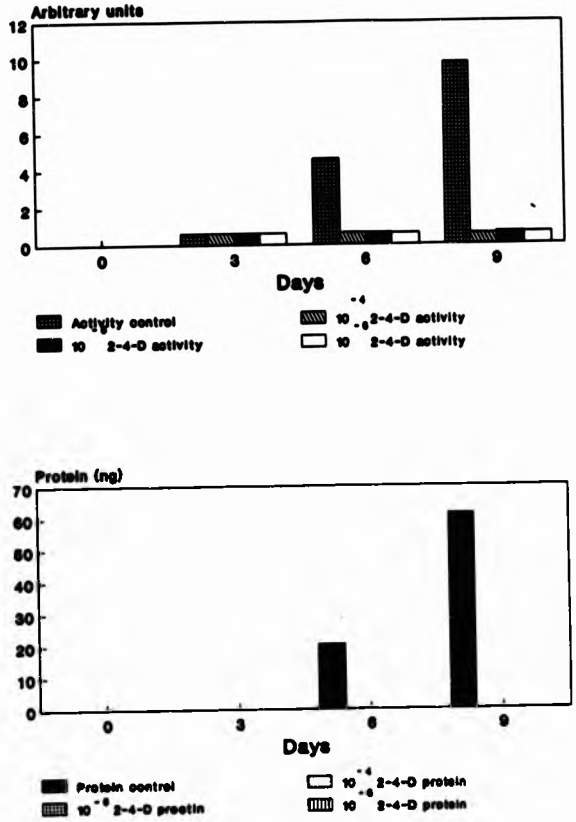


Figure 7.8

Effect of 2-4-D on endo activity and protein when the embryo was removed at day 0.

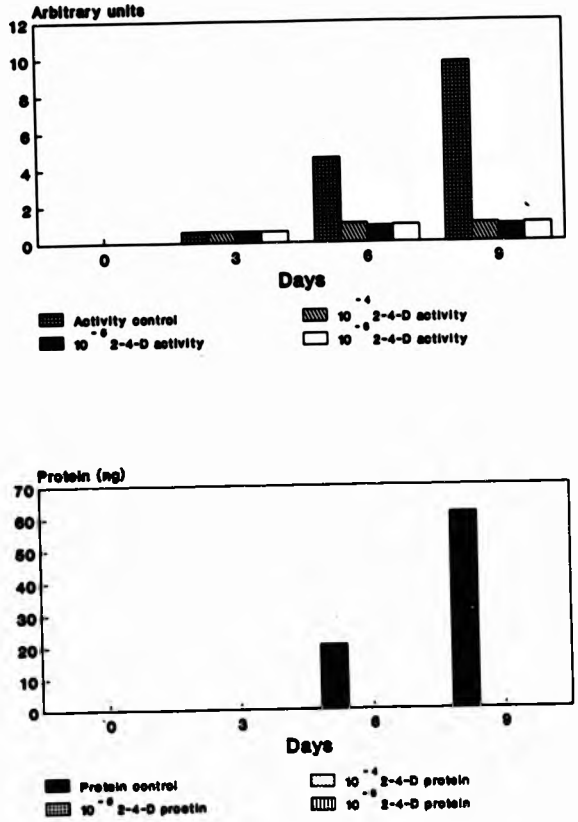


Figure 7.9

Effect of 2-4-D on endo activity and protein when the embryo was removed at day 3.

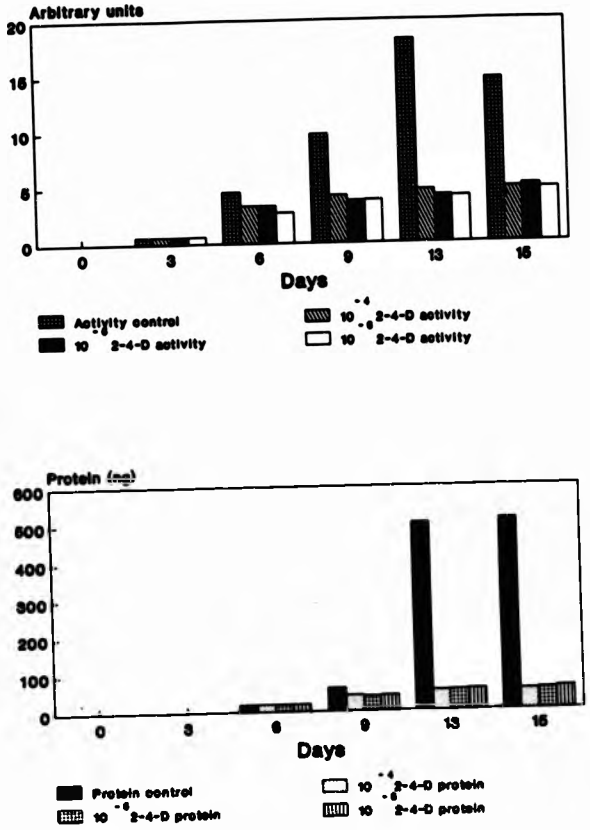


Figure 7.10

Effect of 2-4-D on endo activity and protein when the embryo was removed at day 6.

7.4 Discussion.

It appears that the presence of the embryo is essential for enzyme formation and is also required for the continued production of the enzyme. It is assumed that this lack of enzyme and activity is due to an inhibition of protein synthesis as no enzyme protein can be detected on the blots (>1 ng) when the embryo was removed at early stages of development. At the later stages of embryo removal, i.e. day 6, it would appear that no increase in protein synthesis was occurring as the levels of endo 1,4 ß-D-glucanase protein do not increase significantly when compared to the control. In the intact seed the level of enzyme protein increases 100 fold between days 7 to 13.

The suggestion that the embryo or embryonic axis controls food mobilisation via the production of a hormonal stimulus is usually based on the observation that exogenously applied hormones may partially or completely replace the axial requirement for normal enzyme development (Black and Altschul, 1965; Penner and Ashton, 1967 a; Sze and Ashton, 1971). Exogenously applied gibberellin and auxin had no effect on enzyme production at the concentrations tested. Auxins induce growth, but only under conditions of active oxidative metabolism. Removal of the embryo/embryonic axis would disrupt the normal metabolism of the seed, hence auxin could have an effect, but under the experimental conditions used it was not possible to visualise. It could be that a highly complex pattern of

hormonal control involving auxins, gibberellins, cytokinins and other factors is involved in the mobilisation of food reserves.

Another approach to demonstrate convincingly that the role of the embryonic axis, in enzyme development, is primarily to produce a hormonal stimulus, involves the use of natural diffusates or extracts of embryos to elicit changes in the rates of food mobilisation and in the rates of enzyme activities. Protein degradation in isolated cotyledons of squash is enhanced when they are incubated with excised axes (Wiley and Ashton, 1967), as is fructose 1,6 biphosphatase activity in castor bean endosperm in response to an extract from the embryo (Scala et al., 1969).

Environmental factors are also known to affect seed germination and subsequently reserve mobilisation. Water stress has been found to inhibit carbohydrate breakdown and enzyme production in fenugreek (Spyropoulos and Reid, 1988) and carob (Spyropoulos and Lambiris, 1980) seeds.

The lack of enzyme protein and activity when the embryo was removed at later stages is unusual as one would expect that once initiation of the protein had been accomplished, an increase would follow. The fact that there was no substantial increase in the amount of protein formed would tend to suggest that the embryo still exerts a controlling influence. It may be that this influence is hormonal, or it could be due to the source - sink effect, where enzyme activities and subsequent early seedling

growth are regulated by feed-back effects mediated by reserve breakdown products. In the presence of an axial sink breakdown products are continually removed and reserve breakdown can continue. In the absence of such a sink, reserve breakdown is curtailed. Protein breakdown in cucumber cotyledons is inhibited in the absence of the axis, although proteolytic activity develops normally. However, Davis and Chapman (1980) noted that *in vivo* activities of proteolytic enzymes can be substantially inhibited by free amino acids and peptides:- the breakdown products of proteins. Similarly isocitrate lyase and fructose 1,6 biphosphatase activities in castor bean endosperm are inhibited when the sink (in this case the cotyledons)are removed and this correlates with a 10 fold increase in glucose levels (the end product of lipid metabolism) (Huang and Beevers, 1974). In pea and bean it has been suggested that proteolytic enzyme formation is inhibited when there is an accumulation of amino acids in the detached cotyledons.

The breakdown products of xyloglucan, in primary cell walls, are oligosaccharides which are known to exhibit a regulatory function and inhibit auxin induced growth in pea stems (York *et al.*, 1984). The breakdown products of nasturtium seed xyloglucan are initially oligosaccharides- by the action of endo 1,4 ß-D-glucanase alone (Fanutti *et al.*, 1991), hence it is conceivable that an accumulation of these oligosaccharides may produce an inhibitory effect.

The axial control of endo 1,4 ß-D-glucanase in

nasturtium is probably due a combination hormonal and/or the sink effect. It is possible that initially the embryo is required for initiation of enzyme activity via a hormonal influence and the lack of enzyme protein and activity increase at the later stages is due to an accumulation of end products by removal of the sink. It is clear further work on this topic needs to be done in order that the control mechanism can be determined.

CHAPTER EIGHT

CHAPTER 8

DISCUSSION

The endo 1,4 ß-D-glucanase from nasturtium cotyledons has been purified to homogeneity and some of its characteristics investigated. These included the determination of the isoelectric point, the nature of its folding and the presence of glycosyl groups on the polypeptide. Endo 1,4 ß-D-glucanases have been purified from many other sources (mainly from bacteria and fungi) but including some plants. Examples include Clostridium josui (Ohmiya et al., 1989); Bacillus circulans (Olvera-Trevino et al., 1989); Penicillium pinophilum (Bhat et al., 1989) and from a fungus Y-94 (Yamanobe and Mitsuishi, 1990 a,b). However, none of these exhibit the total xyloglucan-specificity of the nasturtium enzyme.

Examples of plant endo 1,4 ß-D-glucanases (from non-seed sources) have been isolated from peas (Hayashi et al., 1984) and soybeans (Koyama et al., 1981). These have been shown to hydrolyse the internal 1,4 ß-linkages of cellodextrins, cellulose derivatives (e.g. soluble carboxymethyl cellulose), mixed linkage ß-glucans and xyloglucans.

Most endo 1,4 ß-D-glucanases are described in the literature as "cellulases". This is incorrect because most do not hydrolyse crystalline celluloses, although all do, effectively hydrolyse xyloglucan. Hayashi et al. (1984)

discussed the point that in a cell wall containing a xyloglucan:cellulose macromolecular complex, xyloglucan is more accessible to hydrolysis *in vivo*, even though *in vitro* the pea epicotyl endo 1,4 ß-D-glucanase hydrolysed cellodextrins and other cellulose derivatives much more rapidly than xyloglucan.

Our data (unpublished) had shown that the nasturtium endo 1,4 ß-D-glucanase and a cellulase from bean abscission zones (Sexton *et al.*, 1981) were immunologically unrelated. In both Ouchterlony and Western Immunoblotting analysis, no immunological cross-reaction was observed. The endo 1,4 ß-D-glucanase from nasturtium is the only one so far to be isolated from seeds and has been found to be completely specific for xyloglucan. Hence it could be suggested that the enzyme from nasturtium seeds is a xyloglucanase and not a true endoglucanase.

Edwards *et al.* (1986) have pointed out that similar enzymes may exist which have corresponding substrate specificities, but their detection may have been overlooked due to their inability to hydrolyse carboxymethyl cellulose the universal endoglucanase screening substrate.

The enzyme from nasturtium was a single molecular species with an apparent M_r of 29,000 and an isoelectric point of 5.1. The folding of this molecule was investigated by circular dichroism and the enzyme was found to contain no α-helix. This was thought to be rather unusual and when the data from the spectrum was analysed further (following Chou-Fasman rules for secondary structure) it was predicted

that the assembled protein contained 66% ß-sheet and 34% "other structures". The molecular must fold in a highly compact manner since it has been observed that the apparent molecular weight of the non-denatured protein (as detected by gel filtration) is about 15,000 (Edwards et al., 1986).

The nasturtium enzyme was also found to contain less than 10 ng sugar molecules per µg protein, which intimates that the enzyme is not a glycoprotein. This is unusual for an enzyme which is secreted from the cell. The low molecular weight of the endo 1,4 ß-D-glucanase contrasts the other purified enzymes from nasturtium which are involved in xyloglucan breakdown. Both ß-galactosidase and α-xylosidase are high molecular weight glycoproteins, with M_r's of 97,000 for ß-galactosidase (Edwards et al., 1986) and 85,000 for α-xylosidase (Fanutti et al., 1991). This suggests that the endo 1,4 ß-D-glucanase may have a key function in the process of xyloglucan mobilisation. Its compactness would allow it to penetrate the cell wall first, as the pores in the wall have been reported to allow the passage of globular proteins with a M_r's of up to 17,000 (Carpita, 1982; Carpita et al., 1979). Hence it is possible that the endo 1,4 ß-D-glucanase could diffuse into the wall to hydrolyse the xyloglucan backbone, loosening the structure to allow the other enzymes to penetrate.

Visualisation of the endo 1,4 ß-D-glucanase by immunofluorescence confirmed the assumption that the enzyme penetrated the whole of the wall, after being first localised to the inner membrane of the cell wall. The

localisation of the enzyme did not correspond to the lighter staining areas in the wall, which occurred at localised points shaped like "gothic windows" which are assumed to be areas of xyloglucan dissolution. Immunofluorescence showed that the enzyme did not localise at these "gothic windows" but was diffused throughout the wall. It may be that the other enzymes involved in xyloglucan breakdown are found at these zones, and the specific role of the small compact endo 1,4 ß-D-glucanase is to diffuse into the wall generating substrates for the other enzymes. It has been pointed out by Fanutti et al. (1991), that the substrates for the α-xylosidase are xyloglucan oligosaccharides produced by the action of the endoglucanase as this enzyme will not hydrolyse the intact xyloglucan molecule.

Attempts to visualise the enzyme at the electron microscope level all proved unsuccessful as specific labelling could not be obtained. The reason for this is not yet clear, although it could be that the simple logistics of the process have yet to be determined for this system.

The time course of xyloglucan mobilisation as observed histochemically paralleled the increase in enzyme activity (measured viscometrically) and the increase in enzyme protein. The protein was measured both visually in sections and quantitatively using Western Immunoblotting.

This demonstrated that the time of rapid xyloglucan depletion coincided with a dramatic increase in both enzyme activity and protein. The enzyme was found to be

synthesised *de novo* as no protein was detected until day 5 after imbibition. The initial increase in activity was matched by a corresponding increase in protein levels, hence it can be concluded that the increase in enzyme activity was due to an increase in protein synthesis. It was observed in this study that the protein levels did not coincide with the enzyme activity for the latter part of the developmental course after xyloglucan had been depleted. Protein levels did not decrease as dramatically as activity levels implying that much of the protein was inactive, although later there was a rapid depletion of protein probably due to degradation. The lack of smaller molecular weight bands being present on the developmental immunoblot (Figure 5.2), which would be an indication of protein degradation, implies that this process was rapid. This rapid decrease could possibly be due to the appearance of a protease, perhaps triggered by the desiccation of the cotyledonary cells at this time.

It was logical at this stage to examine some further aspects of the control of the initiation of the enzyme. Translation *in vitro* of the messenger RNA would provide further information on the control of synthesis of the protein. Active mRNA was successfully isolated, purified and translated in a wheatgerm translation system. The immunoprecipitation product from this reaction was found to produce a single molecular species of M_r 33,000. This was assumed to represent the enzyme protein with the signal molecule still attached. This signal molecule would be

cleaved when the protein was transferred from its site of synthesis to its site of action and hence the final protein would have a smaller M_r. A study to determine whether this message was present throughout development was carried out at Unilever Research by Dr J. de Silva and Mr C. Jarman, who found that the message was present at the early stages of development. They also found that this message did increase by about 50% from day 8 to day 12 after imbibition, however, this rise was not as dramatic as the increase in protein levels. Although the message was present, it seems evident that the protein was not translated until required and the increase in protein levels also required an increase in message. The regulation mechanism for the production of this enzyme would appear to involve both transcription and translation processes.

The physiological aspects of the control of endo 1,4 ß-D-glucanase were investigated. It was first established that the removal of the embryonic axis, at various stages throughout development, exerted a detrimental effect on the production of the enzyme. Both enzyme protein and activity were affected when the embryo was removed. It was found that when the embryonic axis was removed before day 4, no endoglucanase protein was produced. If the axis was removed after this time, protein was present but did not increase when compared to the control.

In this study it was found that the negative effect produced by removing the embryo could not be reversed by the presence of gibberellic acid, or the auxin derivative

(2-4-D) at the concentrations stated. However, a recent article by Hensel *et al.* (1991), which was published after this study was completed, reported that if the embryo was removed at day 8 after planting and the seed incubated in 10^{-6} M 2-4-D, the storage xyloglucan would decrease rapidly when compared to water controls. The authors also noted a corresponding increase in endoglucanase activity (determined viscometrically), hence they concluded that the enzyme could be induced by auxin at this concentration. They did, however, state that this effect was only apparent when the embryo was removed 7 or 8 days after planting. Nevertheless, by this time the xyloglucan levels had already begun to decline and the enzyme activity was rising, hence it cannot be concluded that this hormone induces the enzyme. The results from this study do, however, confirm our own observations that axial control could not be replaced by auxin or gibberellin if the axis was removed prior to day 8.

It would be interesting to establish whether the axis is required for the production of the messenger RNA which codes for the endo 1,4 ß-D-glucanase, this, of course, would be a fascinating research possibility for the future.

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