THE SELECTIVE HYDROLYSIS OF TAMARIND SEED XYLOGLUCAN (TAMARIND GUM) USING ENZYMES ISOLATED FROM GERMINATED NASTURTIUM (*Tropaeolum majus* L.) COTYLEDONS.

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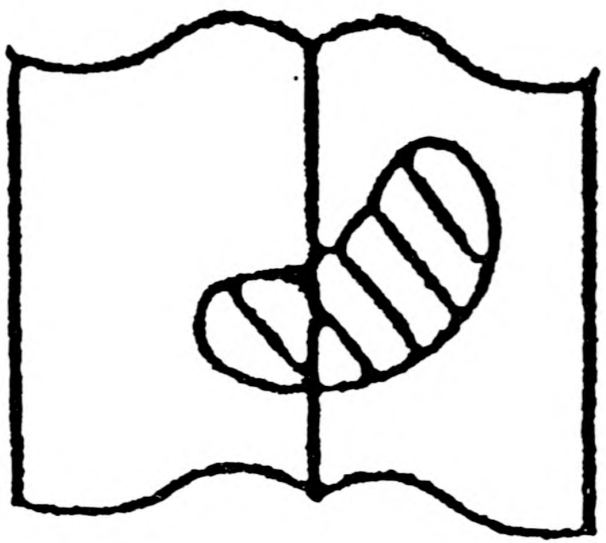

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ABSTRACT.

"The selective hydrolysis of tamarind seed xyloglucan (Tamarind gum) using enzymes isolated from germinated nasturtium (*Tropaeolum majus* L.) seeds".

Background.

Tamarind seed xyloglucan is a storage cell wall polysaccharide which consists of a rigid cellulose-like backbone of (1→4)-ß-D-linked glucopyranosyl units carrying both D-xylopyranosyl residues and D-galactopyranosyl-ß-(1→2)-xylopyranosyl residues. The xylosyl residue is linked α-(1→6) on to the main glucan chain. The xyloglucan polysaccharide of the primary cell wall of growing plants has very similar composition and structure with the only exception that some terminal galactosyl residues carry L-fucosyl substituents attached α-(1→2) (Fry, 1989; Hayashi, 1989). Mobilization of storage xyloglucan following germination in *Tropaeolum* is brought about by the synergistic action of at least four enzymes, namely *endo*-(1→4)-ß-D-glucanase, ß-galactosidase, α-xylosidase and ß-glucosidase (Edwards *et al.*, 1985). The aims of the project were the purification and characterization of xyloglucan-specific hydrolases from germinated nasturtium cotyledons.

Results and discussion.

(A) The enzyme α-xylosidase was purified to homogeneity from germinated nasturtium cotyledons and the molecular properties were investigated. The enzyme is a glycoprotein (approx. Mr 85000, on SDS-PAGE) able to remove a single unsubstituted xylosyl residue attached to the backbone glucose at the non-reducing end of the xyloglucan oligosaccharide molecule (Fanutti *et al.*, 1991).

(B) The nasturtium *endo*glucanase (Edwards *et al.*, 1986) was shown to be a novel type of transferase (xyloglucan *endo*-transferase) able to catalyze the transglycosylation of xyloglucan molecules (Fanutti *et al.*, 1993). Enzymes with the same specificity of the nasturtium *endo*glucanase have been correlated with primary cell wall elongation (Albersheim, 1976).

(C) The nasturtium ß-galactosidase (Edwards *et al.*, 1988) catalyzes the selective removal of galactose residues from polymeric xyloglucan as well as xyloglucan oligosaccharides (Edwards *et al.*, 1988). The rheological properties of modified xyloglucan were studied. Galactose-removal led to increased chain-chain interactions, and these were enhanced in the presence of Na_2SO_4. Galactose residues at different positions in xyloglucan oligosaccharides were hydrolysed at different rates.

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TABLE OF CONTENTS.

ABBREVIATIONS.

Ara	L-(+) Arabinose
ATP	adenosine-5'-triphosphate
BSA	Bovine serum albumin
DP	degree of polymerization
EDTA	[ethylenedinitrilo]tetraacetic acid
Fuc	L(-)-Fucose (6-Deoxy-L-galactose)
Gal	D-(+)-Galactose
GalN	D-(+)-Galactosamine
GalNac	N-acetyl-D-galactosamine
Glc	D-(+)-Glucose
GlcN	D-(+)-Glucosamine
GlcNac	N-acetyl-D-glucosamine
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HPLC	high-performance liquid chromatography
Man	D-(+)-Mannose
Mr	relative molecular mass
NAD^+	ß-nicotinamide adenine dinucleotide
NADP	ß-nicotinamide adenine dinucleotide phosphate
pI	isoelectric point
PBS	phosphate buffered saline
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
Temed	N,N,N',N'-tetramethylethylendiamine
TCA	trichloroacetic acid
TFA	trifluoroacetic acid

TFMS	anhydrous trifluoromethanesulfonic acid
TLC	thin layer chromatography
Tris	2-amino-2-[hydroxymethyl]-1,3-propanediol
Xyl	D-(+)-Xylose

CHAPTER ONE

INTRODUCTION.

1.1. CELL WALL STORAGE POLYSACCHARIDES: XYLOGLUCANS.

Certain seeds are known to store polysaccharides (other than starch) as reserve material which are mobilized and utilized by the embryo during the early stages of plant development. The polysaccharides stored in the seeds are diverse in composition and chemical structure. The deposition of these polysaccharides in special areas of the seed (endosperm or storage cotyledons) also varies between species. All, however, are stored in the cell wall and are known as cell wall storage polysaccharides (Reid, 1985). Comprehensive reviews have been written by Meier and Reid (1982), Reid (1985) and Halmer (1985), and a general classification of storage polysaccharide can be found in Table 1.1..

Vogel and Schleiden (1839) first described the occurrence of the material now known as storage xyloglucan. They described thickenings of the cotyledonary cell wall of certain seeds which were characterized by their ability to stain blue in the presence of iodine/potassium iodide (Fig.1.1). Since this technique had previously been used to detect starch granules in seeds, the blue-staining cell wall material was named "amyloid" (from Latin amylum = starch).

galacto-(1→4)-ß-mannans	stored in the endosperm cell walls of many legumes including carob, fenugreek guar and three non-leguminous species. The degree of galactose substitution of the mannan chain varies from 20% to nearly 100%.
"pure" (1→4)-ß-mannans	stored in the thickened cell walls of the endosperm of *Palmae* where they confer the characteristic hardness, in the endosperm of *Umbelliferae*, in coffee beans (*Coffea arabica*).
galacto-glucomannans	stored in the endosperm cell walls of some species in the *Liliaceae* and *Iridaceae*.
xyloglucans	stored in the thickened endosperm cell wall of 7 dicot families and in the thickened cotyledonary cell walls of 10 other dicot families.
arabino-(1→4)-ß-galactans	stored in the cotyledonary cell wall of soybean and some species of *Lupinus*.
(1→5)-arabinans	storage reserve of mustard cotyledons.
(1→3),(1→4)-ß-glucans	stored as a major reserves in the endosperm cell walls of barley, oat and rye.
arabino-(1→4)-ß-xylans	stored in the endosperm and in the aleurone layer cell walls of barley and wheat.

References:

Dea and Morrison (1975)
Halmer (1985)
Meier and Reid (1982)
Reid (1985)

Table 1.1. Storage polysaccharides (other than starch).

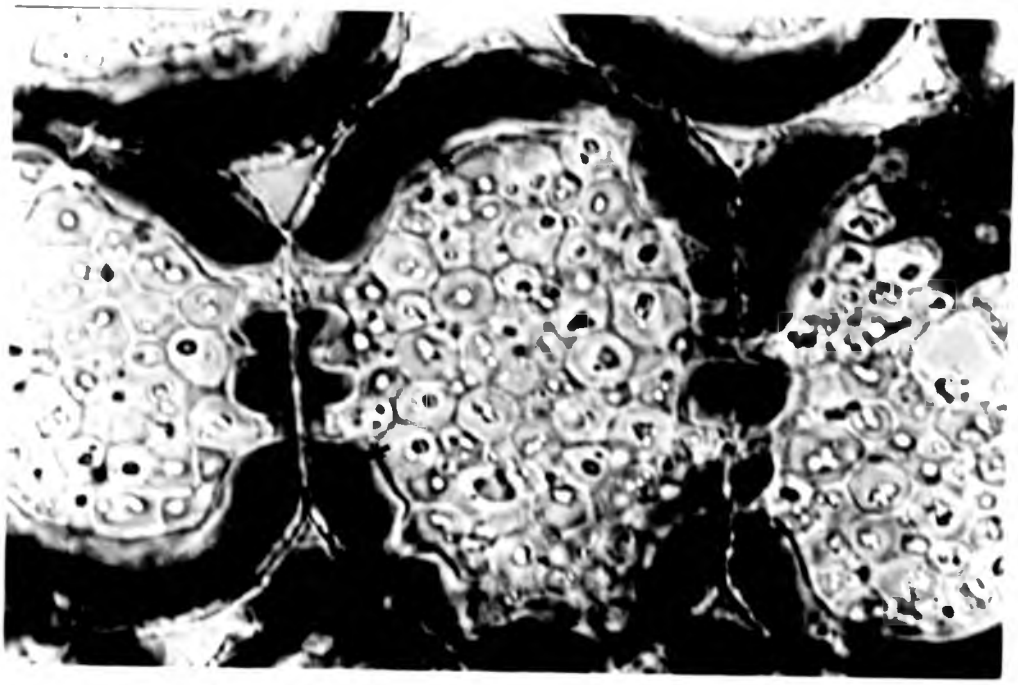


Fig.1.1. The storage cell wall xyloglucan in *Tropaeolum majus* stained with iodine-potassium iodide. The section was taken 16 h after imbibition. The xyloglucan reserves are indicated with arrows (magnification 800 x). The photograph was kindly supplied by Dr. R. Sexton, University of Stirling.

Cell wall storage xyloglucans (amyloids) are abundant, representing 33% of the seed dry-weight in *Tropaeolum majus* cotyledons (Edwards *et al.*, 1985) and 40% in *Copaifera langsdorfii* (Buckeridge *et al.*, 1992). Xyloglucans are hydrophilic and water soluble like cell wall storage galactomannans which have been shown to protect seed structures from desiccation (Reid and Bewley, 1979). It is probable that the xyloglucans have a similar role.

The cell walls of non-seed tissues are generally classified as primary and secondary. The primary cell walls of growing plants are thin and generally non-lignified. Secondary walls, deposited after cessation of growth, are generally thicker and mainly lignified. The xyloglucan-containing walls of seed tissue are, however, both thick and non-lignified. In this thesis they will be referred to as storage cell walls.

1.2. BOTANICAL DISTRIBUTION OF XYLOGLUCAN IN SEEDS.

Using the iodine-staining procedure of Vogel and Schleiden (1839), Kooiman (1960) identified over 2600 species containing storage xyloglucan in the seeds. On the basis of this investigation he concluded that amyloids were contained in the cell wall of either cotyledon or endosperm in the following plants:

"(1) All genera of the tribe *Cynometrea Amherstieae-Schlerolobiae* of the *Leguminosae-Cesalpinioideae* with the exception of two groups of genera; in the other tribes of this subfamily no amyloid has been found.

(2) All investigated species of the *Primulales*, the *Annonaceae*, *Limnanthaceae*, *Melianthaceae*, *Pedaliaceae*, *Thunbergiaceae* and *Tropaeolaceae*.

(3) A number of species of *Balsaminaceae*, *Anthaceae*, *Leguminosae-Papilionatae*, *Linaceae*, *Ranunculaceae*, *Sapindaceae* and *Sapotaceae*." (Kooiman, 1960 from Meier and Reid, 1982). Iodine/potassium iodide staining has also been used to detect and investigate xyloglucans from the cotyledonary cell walls of 8 species of the genus *Copaifera* (Buckeridge *et al.*, 1992).

There are no reports on the occurrence of xyloglucan as a reserve polysaccharide in monocotyledon seeds.

1.3. COMPOSITION AND CHEMICAL STRUCTURE OF SEED XYLOGLUCAN.

In the late nineteen century amyloids were shown to release D-glucose, D-xylose and D-galactose upon acid hydrolysis (Reid, 1985) but it is only more recently that they have been referred to as xyloglucans or galactoxyloglucans.

The molar ratio Glc:Xyl:Gal for *Tamarindus indica* xyloglucan has been reported to be 3:2:1 by Rao (1959), 4.0:2.99:1.31 by Kooiman (1961), and 4:2:1 by Srivastava and Singh (1967). More recently Gidley *et al.* (1991) have studied tamarind seed xyloglucan from various commercial sources and obtained the ratio Glc:Xyl:Gal of 2.8:2.25:1. The molar ratio Glc:Xyl:Gal has been found to be 3:2:1 in *Tropaeolum majus* (Hsu and Reeves, 1967; Le Dizet, 1972); 4-5:2:1 in *Impatiens*

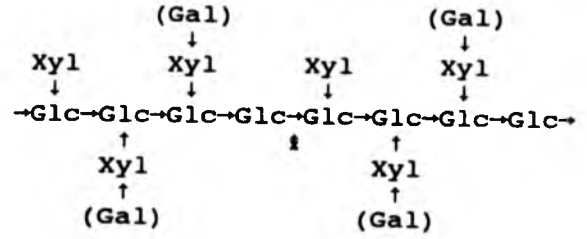
balsamina (Courtois and Le Dizet, 1972); 4:1.2:0.9 in *Annona muricata* (Kooiman, 1967) and 5.3:2:1 in *Brassica campestris* (Siddiqui and Wood, 1971). The xyloglucans from different populations of *Copaifera langsdorfii* differ slighly but significantly in their Glc:Xyl:Gal ratios (4.0:2.9:1.5 and 4.0:2.8:1.7 from savanna and from forest population, respectively; Buckeridge *et al.*, 1992).

Investigations on the chemical structure of seed xyloglucans have been carried out on the alkali-extracted polysaccharides of *Tamarindus indica*, *Annona muricata* (Kooiman, 1961 and 1967), *Tropaeolum majus* (Le Dizet, 1972) and *Impatiens balsamina* (Curtois and Le Dizet, 1974). All are structurally similar. They consist of a cellulose-like backbone of (1→4)-ß-D-linked glucopyranosyl units carrying both D-xylopyranosyl units and D-galactopyranosyl-ß-(1→2)-xylopyranosyl units. The xylosyl residue is linked α-(1→6) on to the main glucan chain.

It was observed in an early investigation (Kooiman, 1967) that enzyme hydrolysis of tamarind xyloglucan with a fungal *endo*-(1→4)-ß-glucanase (cellulase) yielded heptasaccharide units with composition Glc_4Xyl_3, octasaccharide units with composition Glc_4Xyl_3Gal and nonasaccharide units with composition $Glc_4Xyl_3Gal_2$ suggesting that tamarind xyloglucans may be composed by "repeating" units (Kooiman, 1967). This finding was confirmed by York *et al.* (1990) who purified and characterized by means of ^{1}H-NMR the oligosaccharides produced by enzymic digestion of xyloglucan with the *Trichoderma viride* cellulase. The main structural features of seed xyloglucan are illustrated in Fig.1.2.a.

The xyloglucan polysaccharides of the primary cell walls of growing plants have very similar compositions and chemical structures (Fig.1.2.b). The main difference between seed and primary cell wall xyloglucan is that some terminal galactosyl

(a)



(b)

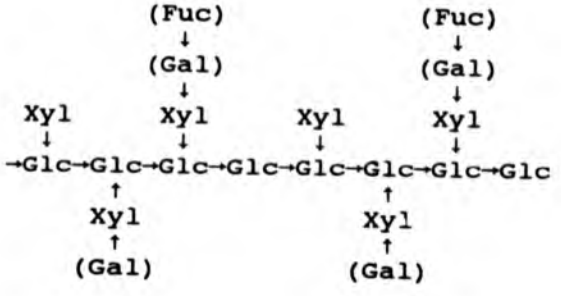


Fig.1.2. Structural features of storage and primary cell wall xyloglucan.

(a): Seed xyloglucan. The molecule consists largely of Glc_4Xyl_3 repeating units which may be galactose-substituted at the positions indicated. The degree of galactose-substitution and its distribution vary according to species. The arrow (↟) indicates the site of cleavage by *endo*-(1→4)-β-D-glucanase (cellulase).

(b): Structural features of primary cell wall xyloglucan.

residues in the latter carry L-fucosyl substituents attached α-(1→2) (Fig.1.2.b). The only seed xyloglucan which has so far been reported to contain fucose residues is that of white mustard (Gould *et al.*, 1971).

The chemical structure and the function of primary cell wall xyloglucan have been extensively investigated and comprehensive reviews have been published (Fry, 1989; Hayashi, 1989).

In the primary cell wall xyloglucan of dicotyledons most of the molecule appears to consist of Glc_4Xyl_3 repeating units which carry galactosyl and fucosyl-galactosyl side chains as indicated in Fig.1.2.b (Darvill *et al.*, 1980). Thus if each glucosyl residue in the backbone -in the preferred conformation- is rotated at 180° relative to the next, it follows that one side of the xyloglucan molecule will carry twice as many xylose substituents as the other (Fry, 1989). This has some implication in the physiological role of the polysaccharide during wall turnover and will be discussed later in this thesis.

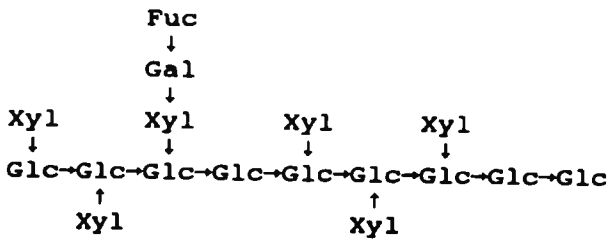
Arabinose has often been mentioned as a possible component of seed xyloglucans but its position within the xyloglucan molecule is controversial (Srivastava and Singh, 1967). Gidley *et al.* (1991) have found that the arabinose present in hydrolysates of tamarind xyloglucan is derived from low levels (2-3%) of a branched (1→5)-α-arabinofuranan which co-purifies with the xyloglucan. The only example of a seed arabinoxyloglucan is provided by Mabusela *et al.* (1990). This polysaccharide, however, cannot be classified as a cell wall polysaccharide since it is located on the surface of *Helipterum eximium* seeds. On complete hydrolysis this material gave glucose, galactose, xylose and arabinose in equimolar ratios. This highly substituted glucan contains arabinosyl residues attached α-(1→2) and

ß-galactosyl-(1→2)-xylosyl units attached α-(1→6) onto the same glucose residue. Xyloglucans extracted from the seed cell walls are generally water soluble and give very viscous solutions. The solubility of of xyloglucan in aqueous solution has been suggested to depend on the degree of substitution of the glucan backbone (Kooiman, 1967). Xyloglucan extracted from *Annona muricata* has been shown to be water-insoluble due to the lower degree of substitution of the glucan backbone (Kooiman, 1967). With increasing substitution of the cellulosic main chain the polysaccharide has been shown to be more water-soluble suggesting that the presence of side-chains may prevent non-covalent association between xyloglucan molecules (Gidley *et al.*, 1991).

1.4. CONFORMATION OF XYLOGLUCANS.

The conformation of seed xyloglucan isolated from tamarind and primary cell wall xyloglucan isolated from pea stem has been investigated by X-ray fibre diffraction. Tamarind xyloglucan occurs as two-fold helix with periodicity (or pitch) of 2.06 nm, twice that observed for cellulose (Taylor and Atkins, 1985). The helices of the (1→4)-ß-D-linked glucosyl residues in the preferred C_1 conformation have been reported to generate a flat ribbon-like structure (Taylor and Atkins, 1985). The observed periodicity of the tamarind xyloglucan chain can probably be attributed to the repeating oligosaccharide units which are based on a 4 main-chain glucose residues (Glc_4Xyl_3Gal). Conformational calculations for primary cell wall

xyloglucan show that the (1→6)-α linkage allows some flexibility of the side chain xylose residues relative to the rigid backbone (Ogawa *et al.*, 1990). Molecular dynamics studies have been carried out to investigate the preferred side-chain conformation of fucosylated xyloglucan oligosaccharides (Levy *et al.*, 1991). Two possible backbone conformations "flat" cellulose-like and the "twisted" cellobiose-like have been considered. In the former (cellulose-like conformation) it was shown that the fucosylated side-chain folds onto only one surface of the backbone (the fucosylated region of the backbone). In this conformation the region located on the opposite side is more exposed. Levy *et al.* (1991) have suggested that this less substituted region may be responsible for the binding of the xyloglucan with cellulose which is known to occur in the primary cell wall. The twisted conformation corresponds, however, to the most probable state as calculated for an oligosaccharide with DP = 17 and with molecular structure reported below:



In this conformation (cellobiose-like) the presence of side-chains on both sides of the xyloglucan molecule may prevent strong xyloglucan-xyloglucan or xyloglucan-cellulose interaction (Levy *et al.*, 1991).

1.5. CELL WALL DEPOSITION OF SEED XYLOGLUCAN.

Electron microscopic xyloglucan cytochemistry has been carried out using specific anti-xyloglucan antibodies with gold-labelled secondary antibodies (Ruel *et al.*, 1990). Xyloglucan has also been detected by enzyme-gold cytochemistry at the electron microscope level. In the absence of commercially available α-xylosidase enzymes and exploiting the conformational and configurational similtarity between α-D-glucopyranosyl and α-D-xylopyranosyl residues, a commercial α-glucosidase-gold complex has been used for the cytochemical localization of xylose side-chains of seed xyloglucan (Ruel and Joseleau, 1990). Both the immunogold and enzyme-gold techiques indicated that during deposition of xyloglucan in the cell wall in *Tropaeolum majus* the polysaccharide is localised in three very distinctive concentric layers: a very thin external structural xyloglucan layer, associated with the primary cell wall, which persists after mobilization of xyloglucan reserves, a very thick intermediate layer containing the bulk of xyloglucan reserves and an internal one characterized by the presence of large "alveoli". These structures may contain newly synthesized xyloglucan directed towards the intermediate layer and have been observed to disappear at the end of the deposition process (Ruel *et al.*, 1990).

Athough the biosynthesis of primary cell wall xyloglucan has been extensively studied *in vitro* (Hayashi *et al.*, 1988; Hayashi, 1989; Brummel *et al.*, 1990), the biosynthesis of cell wall storage xyloglucan in seeds remains still unclear. It has been shown that exogenous [U-^{14}C]-labelled xylose applied to ripening seeds is

preferentially transformed to glucose residues in starch[1] rather than to xyloglucan polysaccharide (Hoth *et al.*, 1986). Thus it has been concluded that xylose is not directly activated and incorporated in to xyloglucan (Hoth *et al.*, 1986). Moreover it has been found that radioactivity from exogenous [U-^{14}C]-labelled glucuronic acid applied to ripening seeds was incorporated in to the xyloglucan molecule and [U-^{14}C]-labelled xylose was clearly detected in the xyloglucan side-chains (Hoth *et al.*, 1986). This observation is supported by a similar finding during *in vitro* investigations of the biosynthesis of primary cell wall xyloglucan in the rapidly expanding regions of soybean (*Glycine max*) seedlings (Hayashi *et al.*, 1988). It has therefore been suggested that xylose may derive from oxidation of UDP-glucose, catalysed by an UDP-glucose dehydrogenase, and subsequent [C-6] decarboxylation of UDP-glucuronic acid, catalysed by an UDP-glucuronate decarboxylase (Hayashi *et al.*, 1988). Hayashi *et al.* (1988) were able to demonstrate that the enzyme UDP-glucuronate decarboxylase co-migrated on a linear sucrose gradient with Golgi vescicles, where the biosynthesis of the polysaccharide xyloglucan has been shown to occur.

1.6. MOBILIZATION OF SEED XYLOGLUCAN.

The mobilization of amyloids following germination was first described in the late

[1] transitory synthesis of starch occurs in the early stages of xyloglucan biosynthesis. Starch then disappears in the final steps of seed maturation.

19th century suggesting that they were reserve polysaccharides (Heinricher, 1888). A fucosylated xyloglucan from mustard seed has been observed to disappear after germination indicating that it had been mobilized (Gould *et al.*, 1971). However detailed investigations on xyloglucan mobilization have been carried out only more recently at a biochemical level in *Tropaeolum* cotyledons (Edwards *et al.*, 1985) and at a histochemical and cytochemical level in *Tamarindus* cotyledons (Reis *et al.*, 1987).

The mobilization of xyloglucan reserves in nasturtium cotyledons has been monitored during water-imbibition, germination and early seedling development. Water-imbibition was complete by day 3 and the radicle protrusion occurred between days 3 and 5 (Edwards *et al.*, 1985). The xyloglucan reserves were mobilized rapidly between day 9 and 13. The breakdown of the polysaccharides was complete by day 19 (Edwards *et al.*, 1985). The enzyme activities required for the complete breakdown of xyloglucan reserves in the cotyledons were assayed using both synthetic (*p*-nitrophenyl-glycoside) and natural (xyloglucan) substrates and they were determined during depletion of xyloglucan reserves to establish the correlation between the enzyme activities and the rate of polysaccharide mobilization. These enzymes were an *endo*-(1→4)-ß-D-glucanase, responsible for the cleavage of ß-(1→4)-linked glucan backbone, a ß-D-galactosidase and an α-D-xylosidase, responsible for the removal of galactosyl and xylosyl residues from xyloglucan side-chains and a ß-D-glucosidase, responsible for the cleavage of cello-oligosaccharides to glucose. The levels of these enzymes with the exception of ß-glucosidase, were found to increase in step with the rate of mobilization of the polysaccharide (Edwards *et al.*, 1985). At day 0 the activities of α-xylosidase,

ß-galactosidase and *endo*-glucanase were very low. After a peak of enzyme activity corresponding with the rapid mobilization of xyloglucan, the levels of these enzymes decreased rapidly to 0 activity by day 25 (Edwards *et al.*, 1985). The activity of the *endo*-glucanase, assayed viscometrically using tamarind xyloglucan as substrate, increased 17-fold with a maximum at day 13. This enzyme has been purified to homogeneity and has been shown to be specific towards xyloglucan and to be unable to hydrolyze cellulose or its derivatives (Edwards *et al.*, 1986). The activity of the enzyme ß-galactosidase was monitored by determining the galactose released from xyloglucan and by using *p*-nitrophenylgalactoside. When xyloglucan was used as substrate the enzyme activity increased 7-fold with a maximum at day 14. However, the ß-galactosidase activity monitored with nitrophenylgalactoside showed a sharp increase with a maximum at day 10, indicating that multiple ß-galactosidases with different degrees of specificity towards xyloglucans may be present in the nasturtium cotyledons (Edwards *et al.*, 1985). This has been confirmed by Edwards *et al.* (1988) and the ß-galactosidase which catalyzes the hydrolysis of galactose residues from the native xyloglucan has been purified to homogeneity. The activity of the enzyme α-xylosidase was assayed by determining the xylose released from xyloglucan. It was shown that this enzyme was unable to hydrolyze *p*-nitrophenylxyloside. The enzyme activity showed a 8-fold increase with a maximum at day 12 (Edwards *et al.*, 1985).

It has been suggested that mobilization of xyloglucan may be due to the synergistic interaction of the three enzyme activities in nasturtium cotyledons, *endo*-(1→4)-ß-D-glucanase, α-D-xylosidase, ß-D-galactosidase together with ß-glucosidase (Edwards *et al.*, 1985).

It has been observed that some enzyme activities are present prior to xyloglucan mobilization, suggesting that they may be products of synthesis of long-lived mRNA, stored in the mature seeds. Evidence has been provided for the utilization of stored mRNAs in the early stages of seed germination (Payne, 1976; Goldberg *et al.*, 1989), but the question whether or not mRNAs serve as templates for the enzymes involved in the breakdown of xyloglucan reserves in nasturtium cotyledons is unclear. An investigation was recently carried out to establish whether or not the enzyme *endo*-glucanase was synthesised *de novo* in nasturtium cotyledons using western blotting techniques and a polyclonal antibody raised against this enzyme. It was concluded that the synthesis of the *endo*-glucanase coincided with the mobilization of xyloglucan (Stronach, 1991; de Silva *et al.*, 1993).

Histochemical and cytochemical studies have been performed on the xyloglucan-containing cotyledons of tamarind seeds before germination (pre-mobilization stage), during germination and seed development using a periodate-based staining procedure for the polysaccharide and a gold-complex of the *Escherichia coli* ß-galactosidase (Reis *et al.*, 1987). The mobilization of the xyloglucan reserves in the cotyledonary cell walls occurred between days 5 to 25 (Reis *et al.*, 1987). It was preceded by seed hydration, hydrolysis of protein bodies and vacuolisation (Reis *et al.*, 1987). Xyloglucan mobilization progressed, describing a wave moving from the outer layer of the cotyledon inwards. The digestion of xyloglucan molecules appeared to occur in precise areas or "digestion pockets" which extended progressively within the wall. Individual "pockets" were separated by transitory columns of undigested material between the outer and the inner layers (Reis *et*

al., 1987). A very similar digestion pattern has been observed during the mobilization of xyloglucans in *Copaifera langsdorfii* (Buckeridge *et al.*, 1992). During the mobilization of xyloglucan reserves in *Tamarindus indica* the molar ratio Glc:Xyl:Gal (3:2:1) has been reported to be maintained constant (Reis *et al.*, 1987). The breakdown of the xyloglucan reserves is not, however, complete and about 5% of the polysaccharide is not mobilized (Reis *et al.*, 1987). It is possible that the unsolubilized xyloglucan may be a component of the primary cell walls of cotyledonary cell types which do not have a storage function.

The sequence of events that characterizes the mobilization of a reserve polysaccharide (i.e. its histological pattern) has been suggested to be under genetic control (Halmer, 1985). A regular pattern of enzyme digestion has been described in the mobilization of galactans in *Lupinus angustifolium* (Parker, 1984). Galactomannan mobilization in fenugreek (*Trigonella foenum graecum*) endosperm has been reported to initiate at the aleurone layer and to proceed inwards towards the cotyledons (Meier and Reid, 1982). Endosperm reserve mobilization has been shown to begin in proximity of the embryo and expand radially in celery (*Apium graveolens*) seeds (Jacobsen and Pressman, 1979). The histochemical investigation on sequential events characterizing intramural digestion in *Tamarindus* indicated that the mobilization of xyloglucan reserves occurs in precise areas of the wall (Reis *et al.*, 1987). It has been speculated that the hydrolases responsible for the xyloglucan breakdown may be produced and secreted in the wall as "inactive precursors" unable to attack cell walls without prior activation (Fincher and Stone, 1981). This mechanism of enzyme activation would ensure the cooperative interaction between different enzymes and the

complete hydrolysis of the polysaccharide.

It has been observed that starch is often synthesized transitorily during reserve polysaccharide mobilization. This process has been observed also in tamarind, celery, fenugreek, *Lupinus*, and it has been suggested that starch biosynthesis occurs to overcome temporary over-supply of carbohydrate directed to the embryo during the early stages of plant growth (Halmer, 1985).

In tamarind the transport of monosaccharides (or oligosaccharides) produced by xyloglucan digestion is carried out by special "transfer" cells in which the breakdown of xyloglucan reserves is delayed (Reis *et al.*, 1987). The mechanism underlying the transport of sugars through the thick walls of these cells is not known.

1.7. HORMONAL REGULATION OF XYLOGLUCAN BREAKDOWN.

The main carbohydrate storage material of certain cereals is starch and its mobilization is initiated by the action of an α-amylase synthezised *de novo* in the aleurone layer, under the control of embryo-produced giberellins (Halmer, 1985). The hormonal regulation of starch breakdown in dicotyledons is apparently more complex and varies according to the species.

In excised cotyledons of nasturtium and tamarind incubated under germination control it has been shown that the mobilization of xyloglucan is inhibited (Reis *et al.*, 1987; Hensel *et al.*, 1991). This observation suggests that the rate of

mobilization of reserve polysaccharides may depend on the demand of the embryo which may act as a "natural sink" (Chapman and Davies, 1983). In the case of excised nasturtium, however, it was found that the breakdown was restored by addition of a synthetic auxin (2,4-dichlorophenoxyacetic acid) (Hensel *et al.*, 1991). It was speculated that auxin may enhance xyloglucan hydrolysis by activating the xyloglucan-specific *endo*glucanase, responsible for the cleavage of the glucan backbone (Hensel *et al.*, 1991). The auxin-induced stimulation of xyloglucan mobilization occurred only after day 8 from imbibition, indicating that a potential receptor (which is not present in the early stage of nasturtium seed germination) may be involved in the auxin control mechanism (Hensel *et al.*, 1991).

1.8. PRIMARY CELL WALL XYLOGLUCAN.

The primary cell walls of higher land plants generally contain cellulose, as the major component (20-30% dry weight). Also present in large amounts are the hemicelluloses, represented principally by xylans and ß-(1→3,1→4)-glucans in grasses, and xyloglucans in dicotyledons and certain non-graminaceous monocotyledon (10-20% dry weight). Pectic polysaccharides (principally homogalacturonans and rhamnogalacturonans) are also quantitatively important in the dicotyledons. The structural proteins are represented by the group of the extensins, hydroxyproline-rich glycoproteins, which are the most abundant proteins in dicotyledonary cell walls (for review, Varner and Lin, 1989; McCann and

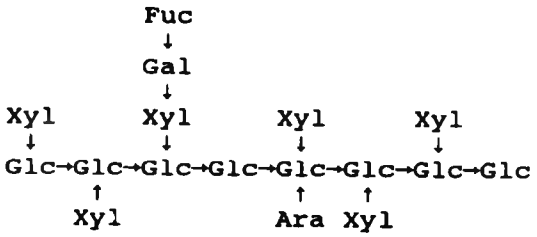
Roberts, 1991; Carpita and Gibeaut, 1993).

Xyloglucans extracted from the primary cell walls of dicotyledons contain D-glucose, D-xylose, D-galactose and L-fucose. The chemical structure of this polysaccharide is very similar to storage cell wall xyloglucan in that they both consist of a backbone of (1→4)-ß-D-linked glucopyranosyl residues substituted with D-galactopyranosyl-(1→2)-ß-xylopyranosyl or with single D-xylopyranosyl residues. The xylose in both cases is linked α-(1→6). The major difference between primary and storage cell walls is the presence of L-fucopyranosyl residues attached α-(1→2) onto galactosyl residues in the former (Darvill *et al.*, 1980). The structure of primary cell wall xyloglucan is represented in Fig.1.2.b.

Xyloglucan represents only 2-5% of the dry weight in the primary cell wall of grasses (Kato *et al.*, 1981; Kato and Masuda, 1985). It contains less xylose and galactose than xyloglucan extracted from dicotyledons (Hayashi, 1989) and lacks fucosyl residues. The xyloglucan of onion (*Allium cepa*), a non-graminaceous monocotyledons, is structurally similar to the xyloglucans of dicotyledon primary cell walls (Mankarios *et al.*, 1980; Redgwell and Selvendran, 1986).

The monosaccharide arabinose has often been reported as a component of fucosylated xyloglucans. An arabinoxyloglucan isolated from tobacco (*Nicotiana tabacum*) leaves was reported to contain arabinosyl residues attached α-(1→6) to the glucan backbone (Mori *et al.*, 1979). The cell walls of *Solanum tuberosum* have been reported to contain an arabinoxyloglucan which is highly substituted at position 6 with α-D-xylopyranosyl residues carrying either L-arabinofuranosyl or ß-D-galactopyranosyl residues as substituents at position 2 (Ring and Selvendran, 1981). The presence of arabinosyl substitution in primary cell wall xyloglucan was

also reported more recently by Kiefer and co-workers (1990) who carried out the characterization of a xyloglucan heptadecasaccharide secreted into the medium of suspension-cultured sycamore cells. The saccharide was composed of a nonasaccharide ($Glc_4Xyl_3GalFuc$) and an octasaccharide (Glc_4Xyl_3Ara) units and the arabinosyl residue being linked at O-2 of the ß-glucosyl residue at the non reducing end of the octasaccharide. The structural features of this heptadecasaccharide are depicted below:



The presence of arabinose prevented enzymic attack from fungal *endo*-(1→4)-ß-glucanases (Kiefer *et al.*, 1990). Xyloglucan isolated from suspension-cultured sycamore cells has been shown to be substituted by O-acetyl groups (York *et al.*, 1988). A similar finding has been obtained for xyloglucan extracted from suspension-cultured *Rubus fructicosus* cells (Chambat *et al.*, 1990). These substituents may affect the rheological properties of xyloglucans and may also alter the susceptibility to enzyme cleavage (Kiefer *et al.*, 1989).

Enzyme digestion of mung bean (*Vigna radiata*), suspension-cultured sycamore cell wall and rape-seed (*Brassica campestris*) hull xyloglucans with a fungal cellulase yielded three major oligosaccharides: a heptasaccharide Glc_4Xyl_3, a

nonasaccharide $Glc_4Xyl_3GalFuc$ and a decasaccharide $Glc_4Xyl_3Gal_2Fuc$ (Hayashi and Maclachlan, 1984; York *et al.*, 1990). An octasaccharide with composition Glc_4Xyl_3Gal was also obtained by digestion of cotton fiber xyloglucan with *Streptomyces* cellulase (Hayashi and Delmer, 1988). The structure of these oligosaccharides has been determined by ^{1}H-NMR and f.a.b. mass spectrometry by York and co-workers (1990). The structure of these oligosaccharides is illustrated in Fig.1.3..

Primary cell wall xyloglucan is tightly bound to cellulose in the primary cell wall (Bauer *et al.*, 1973; Valent and Albersheim, 1974; Joseleau and Chambat, 1984; Hayashi and Maclachlan, 1984; Hayashi *et al.*, 1987). Xyloglucan was first shown to exhibit association with cellulose by Aspinall *et al.* (1969), who used cellulose to affinity-purify xyloglucan from other charged cell wall polysaccharides. The interaction between xyloglucan and cellulose was shown to be non-covalent type *in vitro* (Bauer *et al.*, 1973; Joseleau and Chambat, 1984) and *in vivo* (Hayashi *et al.*, 1987). The binding is specific since a 10-fold excess of (1→2)-ß-glucans, (1→3)-ß-glucans, (1→6)-ß-glucans and mixed (1→3,1→4)-ß-glucans did not affect the ability of xyloglucans to bind to cellulose (Hayashi *et al.*, 1987).

Xyloglucan molecules are distributed between/on cellulose fibers and, as the contour length of each xyloglucan molecule is 40 to 400 times greater than the diameter of cellulose microfibrils, they may cross-link adjacent cellulose fibrils. This could contribute to the mechanical strength of the cell wall (Fry and Miller, 1989; Hayashi, 1989). The non-covalent nature of the association between xyloglucan and cellulose can only partially explain the difficulty experienced extracting xyloglucan from the primary cell walls with 4 M KOH and with

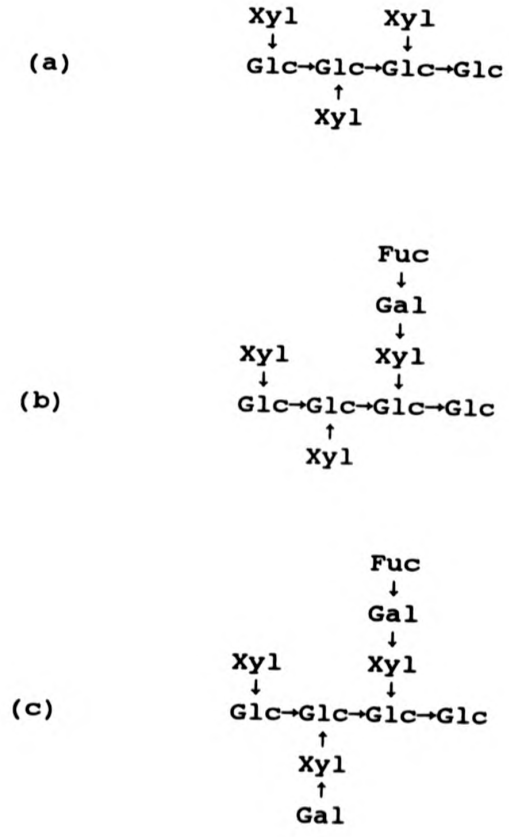


Fig.1.4. Structural features of the major oligosaccharides obtained by digestion of primary cell wall xyloglucan with a fungal *endo*-(1→4)-ß-D-glucanase (cellulase). (a): heptasaccharide Glc_4Xyl_3; (b): nonasaccharide $Glc_4Xyl_3GalFuc$. (c): decasaccharide $Glc_4Xyl_3Gal_2Fuc$.

chaotropic agents such as 8 M urea. It has been suggested that ester bonds may contribute to the cross-linking of xyloglucans in the wall as well as hydrogen bonds (Fry, 1989). This hypothesis is supported by the recent isolation of a feruloylated xyloglucan disaccharide from the primary cell wall of bamboo (*Phyllostachys edulis*) shoots (Ishii *et al.*, 1990).

Auxins and acidification of the cell wall are known to promote wall loosening and extension (Cleland, 1971; Labavitch and Ray, 1974; Labavitch, 1981; Fry, 1989). The auxin-induced wall extension is believed to be an enzyme-mediated process (Johnson *et al.*, 1974; Byrne *et al.*, 1976; Hayashi *et al.*, 1984). The cleavage of load-bearing bonds between xyloglucan and cellulose by a specific enzyme could enable cellulose microfibrils to move apart or along relative to one another thus allowing plastic (= irreversibile) extension. It has been observed that several enzymes including an *endo*-(1→4)-ß-glucanase (Hayashi *et al.*, 1984), a ß-galactosidase and an α-xylosidase (O'Neil *et al.*, 1989) are activated by auxin in the rapidly elongating tissues of pea stem. Since the enzymic hydrolysis of xyloglucan molecules would cause a loss of wall strength and the solubilization of xyloglucan, it has been postulated that a mechanism of transglycosylation may be activated in order to restore the linkages broken within xyloglucan chains (Albersheim *et al.*, 1976; Fry, 1989; Nishitani and Tominaga, 1991; Farkas *et al.*, 1992; Fry *et al.*, 1992). Xyloglucan-specific transglycosylase activity has been found in suspension-cultured *Spinacia* cell walls (Smith and Fry, 1991) and more recently a novel xyloglucan *endo*-transglycosylase (XET) was isolated from auxin-treated cell walls of *Vigna angularis* (Nishitani and Tominaga, 1992).

1.9. REGULATORY ROLE OF XYLOGLUCAN OLIGOSACCHARIDES.

Biologically-active oligosaccharides have been found to play an important role in disease resistance, in differentiation and morphogenesis and in growth control (for a review see Darvill *et al.*, 1992). Cell wall fragments able to regulate plant metabolism have been termed "oligosaccharins". Auxin-induced cell wall elongation has been shown to be inhibited by a fucosylated xyloglucan nonasaccharide with composition $Glc_4Xyl_3GalFuc$ (York *et al.*, 1984; Mc Dougall and Fry, 1988; Emmerling and Seitz, 1990). The concentration required for inhibition is 10^{-9}-10^{-8} M, and higher concentrations of the nonasaccharide had no effect. An identical saccharide was isolated from the culture media of suspension-cultured *Spinacia* cells (Fry, 1986). The fucosylated nonasaccharide also inhibited acid-induced cell wall elongation suggesting that auxin and lowering of pH may operate through similar mechanisms (Lorences *et al.*, 1990). Several xyloglucan oligosaccharides, including the trisaccharide (GlcGalFuc) which is present in human milk have been tested for their ability to suppress auxin-induced cell elongation using excised pea stem segments. Only fucosylated oligosaccharides showed anti-auxin activity (McDougall and Fry, 1989). On the other hand non-fucosylated xyloglucan octa- and nonasaccharides at a concentration of 10^{-6} M were found to promote growth, in the pea-stem bio-assay, and to stimulate the activity *in vitro* of a cellulase extract from *Phaseolus vulgaris* (McDougall and Fry, 1990). It has been suggested that the anti-auxin inhibition may be controlled at the level of a highly discriminating receptor and a feed-back mechanism has been

proposed where low concentrations of the fucosylated nonasaccharide (1 nM) may inhibit auxin-induced growth but higher concentrations of the saccharide may restore growth (McDougall and Fry, 1988). A similar mode of action has been suggested to explain the process of apical dominance by York *et al.* (1984).

Fucosylated and non-fucosylated xyloglucan oligosaccharides have been found to increase the activity of the nasturtium xyloglucan-specific *endo*glucanase assayed *in vitro* (Farkas and Maclachlan, 1988).

Athough xyloglucan oligosaccharides have not been observed during the mobilization of the storage xyloglucan in germinated nasturtium cotyledons (Edwards *et al.*, 1985), the possibility that biologically active oligosaccharides may contribute to the control of mobilization cannot be completely ruled out.

1.10. AIMS OF THIS THESIS.

It has been shown that storage and primary cell wall xyloglucans present many similarities at a compositional and structural level. The post-germinative mobilization of storage xyloglucan in nasturtium cotyledons is brought about by the cooperative interaction of very specific enzymes, which catalyze the complete hydrolysis of xyloglucan by cleaving specific glycosyl residues (Edwards *et al.*, 1985, 1986 and 1988). Enzymes with very similar molecular properties and specificity have been purified from the primary cell wall of growing plants (O'Neill *et al.*, 1989; Nishitani and Tominaga, 1992). It seems therefore important to draw some

comparisons between the xyloglucan metabolism of the growing primary cell wall and the enzymatic mobilization of xyloglucan reserves in germinated seeds. It has been suggested that the mobilization of the polysaccharide can be regarded as a process of turnover, where the high levels of the enzyme activities promote the complete hydrolysis of the xyloglucan reserves (Edwards *et al.*, 1985). By contrast xyloglucan turnover in the primary cell wall, under the hormonal control, is finely regulated at enzymic level and does not lead to complete degradation.

The principal aim of this Thesis was to characterize some enzymes involved in the post-germinative mobilization of xyloglucan reserves in nasturtium cotyledons. This involves:

(i) the purification and characterization of the nasturtium α-xylosidase (Chapter Three).

(ii) studies of the mode of action of the xyloglucan-specific *endo*-glucanase with the conclusion that this enzyme should be re-classified as xyloglucan *endo*-transglycosylase (Chapter Four).

(iii) studies of the action of the nasturtium ß-galactosidase on tamarind xyloglucan (Chapter Five).

(iv) The cooperative action of these enzymes in xyloglucan turnover is discussed in Chapter Six.

CHAPTER TWO

MATERIALS AND METHODS.

2.1. MATERIALS.

Nasturtium (*Tropaeolum majus*) seeds were purchased from Royal Sluis, Leyland, Preston, UK. Soluble tamarind (*Tamarindus indica*) xyloglucan (Glyloid 3S) was obtained from Dainippon Pharmaceutical Corporation, Osaka, Japan. The powder contained some insoluble particles and about 5% free glucose, which were removed before use (section 2.2.1.). The mixture of manno-oligosaccharides used as TLC reference standards was suppied by M. Edwards. Enzymes for analytical purposes were purchased from Sigma, except for hexokinase which was from Boehringer, Mannheim, FRG.

General chemicals and buffer salts were of analytical grade or better and were purchased from Sigma unless otherwise stated.

2.2. METHODS.

2.2.1. ENZYME ACTIVITY DETERMINATION.

Tamarind xyloglucan was used as a substrate for the determination of enzyme activities. The soluble tamarind seed polysaccharide (ca. 0.5%) was dissolved overnight in distilled water at room temperature. The solution was centrifuged for 30 min at 19000 g and dialysed extensively against water. The solution was then frozen and freeze-dried. The white lyophilized material was stored at room temperature in a dry place.

A 1.2% (w/v) solution of xyloglucan was prepared by dissolving the polysaccharide in either phosphate/citrate buffer (Mc Ilvaine buffer, Dawson *et al.*, 1982) pH 5.0 or 50 mM ammonium acetate buffer pH 5.0 by stirring overnight at room temperature. The xyloglucan solution (500 μl) was mixed with 200 μl of pure enzyme or crude enzyme extract and 500 μl of Mc Ilvaine buffer pH 5.0 (or 50 mM ammonium acetate buffer pH 5.0). It was generally found that at pH 5.0 most of the hydrolytic enzymes were stable since this pH corresponds to the physiological pH of the plant cell wall (Johnson *et al.*, 1974). Aliquots of the enzyme-polysaccharide incubation mixture were taken at regular intervals (i.e. 30 min/1 hour) and the enzyme reaction was stopped by heat-denaturation using a boiling water bath. The enzyme activity was then determined by assaying the monosaccharides released during the incubation or by monitoring the reducing

power. Controls containing only the substrate solution were run in parallel.

Pea (*Pisum sativum*) xyloglucan was used as substrate for the determination of α-fucosidase activity since tamarind xyloglucan does not contain terminal fucosyl residues on the xyloglucan side-chain. Pea xyloglucan (prepared using the method of Edwards *et al.*, 1986; by Dr J.S.G. Reid) or mung bean (*Phaseolus aureus*) xyloglucan prepared by A. Downie were used. The polysaccharide was dissolved in Mc Ilvaine buffer pH 5.0 or 50 mM ammonium acetate buffer pH 5.0 and all enzyme incubations were carried out as stated previously.

2.2.1.1. DETERMINATION OF D-GALACTOSE BY GALACTOSE DEHYDROGENASE (Kurz and Wallenfels, 1974).

The galactose dehydrogenase (D-galactose:NAD 1-oxidoreductase, EC 1.1.1.48) enzyme suspension was diluted with 1 M ammonium sulphate containing 20 mM EDTA to a final concentration of 2.5 U/ml. The solution was stored at 4°C and was stable for a few months. A solution of NAD^+ (ß-nicotinamide adenine dinucleotide) was prepared by dissolving 12.5 mg/ml of the coenzyme in water. This solution was aliquoted and stored at -20°C.

In a spectrophotometer cuvette 904 µl of 0.1 M Tris-HCl buffer pH 8.6 were mixed with 64 µl of sample or standard (galactose, concentration ranging from 0.1 to 1.0 mM) and 64 µl of galactose dehydrogenase. The extinction was read at 340 nm (E_1). To this solution 32 µl of NAD^+ were added and extinction (E_2) was read

after 1 hour incubation at room temperature. The concentration of galactose was proportional to the formation of NADH as measured by the change in extinction at 340 nm. The enzyme reaction is represented below:

$$\text{ß-D-galactose} + NAD^+ \xrightarrow{\text{Gal-DH}} \text{D-Galactono-}\delta\text{-lactone} + NADH + H^+$$

The D-galactono-δ-lactone which is formed is either hydrolysed to D-galactonate and/or rearranged to D-galactono-γ-lactone according to the conditions.

2.2.1.2. DETERMINATION OF D-GLUCOSE WITH HEXOKINASE AND GLUCOSE-6-PHOSPHATE DEHYDROGENASE (Bergmeyer *et al.*, 1974).

A solution of 150 mM ATP (adenosine-5'-triphosphate) and 12 mM $NADP^+$ (nicotinamide adenine dinucleotide phosphate) was prepared using 0.3 M triethanolamine buffer, pH 7.5 containing 30 mM $MgSO_4$. The stock solution was stored at -20°C in small aliquotes.

The commercial suspension of glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate:$NADP^+$ 1-oxidoreductase, EC 1.1.1.49) was diluted with 3.2 M ammonium sulphate to a final suspension containing 100 U/ml. The hexokinase (ATP:D-hexose-6-phosphotransferase. EC 2.7.1.1.) stock suspension was diluted with 3.2 M ammonium sulphate solution to a final suspension containing 100

U/ml. These enzyme solutions were stored at 4°C and were stable for several months.

In a spectrophotometer cuvette 830 μl of 0.3 M triethanolamine buffer pH 7.5 containing 30 mM $MgSO_4$ were mixed with 70 μl of sample or standard (glucose, concentration ranging from 0.1 to 1.0 mM), 10 μl of glucose-6-phoshate dehydrogenase and 70 μl of ATP/$NADP^+$. The extinction was read at 340 nm after 2 minutes (E_1). To this solution were added 10 μl of hexokinase. The extinction was read at 340 nm after 7 minutes (E_2). The formation of NADPH, as measured by the change in extinction at 340 nm, was proportional to the amount of glucose present ($E_2 - E_1 = \delta E$ glucose). The schematic reaction is represented below:

$$\text{reaction (1)}\quad \text{D-glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{G-6-P} + \text{ADP}$$

$$\text{reaction (2)}\quad \text{G-6-P} + \text{NADP}^+ \xrightarrow{\text{G6P-DH}} \text{6-phosphogluconate} + \text{NADPH} + \text{H}^+$$

2.2.1.3. DETERMINATION OF FREE PENTOSES (XYLOSE ASSAY) (Roe and Rice, 1948).

The *p*-bromoaniline reagent was prepared by dissolving 2 g of *p*-bromoaniline in 100 ml of acetic acid saturated with thiourea.

In a glass test tube 1 ml of sample or standard (xylose concentration ranging from

0.1 to 1.0 mM) was mixed with 5 ml of reagent (when possible the amounts of reagent and sample required for the assay were scaled down to 0.5-1.0 ml volume and the reaction was carried out in microcentrifuge tubes). The solution was first incubated 10 min at 70°C in a shaking thermostated bath and then 1 hour at 30°C in a thermostated bath in the dark. The pink colour developed during this time and was stable for 30 minutes thereafter at room temperature. The extinction was measured at 520 nm and it was proportional to the amount of xylose present. High xyloglucan concentrations were found to interfere with the extinction readings at 520 nm since the polysaccharide precipitates during the incubation step. The assay mixture was therefore centrifuged after incubations using a bench centrifuge (MSE).

2.2.1.4. DETERMINATION OF L-FUCOSE BY L-FUCOSE DEHYDROGENASE ASSAY.

The commercial fucose dehydrogenase (6-deoxy-L-galactose:NAD^+ 1-oxidoreductase, EC 1.1.1.122) was diluted with Tris-HCl buffer pH 8.0 to give a final concentration of 2.5 U/ml. The solution was stored at -20°C and was stable for a few months. A solution of NAD^+ (ß-nicotinamide adenine dinucleotide) was prepared by dissolving 12.5 mg/ml of the coenzyme in water. This solution was stocked in small aliquots and stored at -20°C.

In a spectrophotometer cuvette, 904 μl of Tris-HCl pH 8.0 were mixed with 64

μl of each sample or standard (L-fucose, concentrations ranging from 0.1 to 1.0 mM) and 64 μl of fucose-dehydrogenase. The extinction was read at 340 nm (E_1). To this solution 32 μl of NAD^+ were added and extinction (E_2) was read after 1 hour incubation at room temperature. The formation of NADH, as measured by the change in extinction at 340 nm was proportional to the amount of L-fucose present. The enzyme reaction is represented below:

$$\alpha\text{-L-6-deoxy-galactose} + NAD^+ \xrightarrow{\text{Fuc-DH}} \text{L-6-deoxy-galactono lactone} + NADH + H^+$$

2.2.1.5. VISCOMETRIC ASSAY FOR *ENDO*-(1→4)-β-D-GLUCANASE (by the method of Edwards *et al.*, 1985).

A 1.2% (w/v) solution of tamarind xyloglucan was used for monitoring changes in viscosity brought about by the action of *endo*glucanase. The polysaccharide (2 ml) was placed in glass test tubes immersed in a thermostated bath at 30°C. The enzyme (0.4 ml) was added to the xyloglucan solution and mixed. The viscosity measurements were carried out at 30°C using a graduated pipette (0.1 ml volume). The flow-time readings were taken at 2-5 min intervals at first and at 15-30 min intervals when the changes in viscosity were markedly reduced. The activity of the enzyme, in arbitrary units, was calculated from the plot of flow-time against time and the flow-time value corresponding to time 0 (time of the mixing)

was extrapolated. The enzyme 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 0.

2.2.1.6. REDUCING POWER ASSAY DETERMINED BY THE FERRICYANIDE METHOD (Halliwell and Riaz, 1970).

In a small glass tube (or a microcentrifuge tube) 125 μl of saccharide solution were mixed with 125 μl of 0.1 M sodium carbonate. To this solution were added 250 μl of a solution containing 0.064% (w/v) potassium cyanide and 0.52% (w/v) sodium carbonate and 500 μl of a solution containing 0.05% (w/v) potassium ferricyanide. A glucose solution with concentrations ranging between 0.1 and 0.5 mM was prepared freshly as standard solution. The mixture was then placed in a boiling water bath at 100°C for 25 min. During this step the yellow colour decreased. After cooling the exctinction was read at 420 nm.

In this assay the oxidation of the reducing carbon of the carbohydrate is accompanied by the reduction of the Fe^{3+} to Fe^{2+}, indicated by the disappearance of yellow colour which is proportional to the amount of the saccharide present in the reaction mixture.

2.2.1.7. NITROPHENYLGLYCOSIDE ASSAY.

A solution containing the appropriate *p*-nitrophenylglycoside at a concentration of 50 mM was prepared by dissolving the *p*-nitrophenylglycoside in water. This solution was stocked in small aliquots and stored at -20°C.

Small glass tubes containing 300 μl of Mc Ilvaine buffer pH 4.5 were immersed in thermostated bath at 30°C. Appropriate dilutions of the purified enzyme or crude enzyme extract (100 μl) were placed in the tube, to which 100 μl of *p*-nitrophenylglycoside were added. The mixture was incubated for 15 minutes at 30°C. The reaction was stopped by adding 5 ml of 0.1 M sodium carbonate and the extinction was read at 400 nm. The enzyme activity was calculated using the molar extinction coefficient of 1.84×10^4 for *p*-nitrophenol in 0.1 M Na_2CO_3.

2.2.2. PROTEIN DETERMINATION (by the Coomassie-Blue method of Sedmak and Grossberg, 1977).

The reagent was prepared by dissolving 0.06% Coomassie blue G-250 in 3% perchloric acid. In a spectrophotometer cuvette 0.5 ml of a protein solution containing 10 to 60 μg of protein/ml were mixed with 0.5 ml of reagent. A standard solution of bovine serum albumin was also prepared by dissolving the BSA in a solution to same composition as the one containing the protein to be

determined. The exctinction was measured at 620 nm and at 465 nm. The amount of protein was extrapolated from the plot of the BSA solution concentrations against the following extinction ratio:

$$\frac{E_{620}\ \text{protein}}{E_{465}\ \text{protein}} - \frac{E_{620}\ \text{blank}}{E_{465}\ \text{blank}}$$

2.2.3. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS.

Sodium dodecyl sulphate polyacrylamide gel elecrophoresis was carried out essentially according to Laemmli (1970) in a vertical slab-gel system (Bio-Rad mini slab-gel). A maximum of 10 mini-gels (0.75 x 82 x 60 mm high, not including stacking gel) were prepared at one time and kept at 4°C for no longer than one week in a fridge.

The separating gel buffer contained 1.5 M Tris and 0.4% SDS, pH 8.7 with HCl.

The stacking gel buffer contained 0.5 M Tris and 0.4% SDS, pH 6.8 with HCl.

The sample buffer, 2 x concentrated, contained 30 ml of 10% SDS, 12.5 ml of stacking gel buffer, 10 ml glycerol and 4.0 M urea, pH 6.8.

The reservoir buffer, 5 x concentrated, contained 0.125 M Tris, 0.96 M glycine and 0.5% SDS, pH 8.3 with HCl. All the solutions listed above were stored at room temperature and were stable for a few months.

A 10% solution of ammonium persulphate was prepared freshly.

The acrylamide stock solution contained 30% acrylamide and 0.8% bis-acrylamide and was prepared using separating gel buffer. This solution was kept in a dark bottle at 4°C and was stable for 2 weeks.

To prepare 9% acrylamide gels, 30 ml of acrylamide stock solution, 45 ml of water and 25 ml of separating gel buffer were placed in a flask. The solution was de-aerated for few minutes. To this solution 57 μl of Temed (N,N,N',N'-tetramethylethylendiamine) and 0.7 ml of ammonium persulphate solution were added and the solution was poured between the glasses and the aluminium oxide plates previously assembled in the mini gel casting apparatus (Hoefer, Newcastle-under-Lyme, UK). When the acrylamide-bisacrylamide solution was polymerized, the individual gels, sandwiched between glass and aluminia plates, were removed, rinsed with distilled water and wrapped in cling foil.

To prepare 4% stacking gel 1.25 ml of stacking gel buffer were mixed with 3.0 ml of water and 0.67 ml of acrylamide stock solution. The solution was de-aerated for a few minutes. Temed (5 μl) and 50 μl of ammonium persulphate solution were gently mixed. This solution was poured on the top of the running gel, the slot-forming comb was inserted, and the gel allowed to polymerize.

Samples were prepared by mixing equal volumes of protein solution (50 μl) and sample buffer, 2 x concentrated. This solution was placed in a boiling water bath for two minutes and 10 μl of a solution made by mixing equal volumes of 1% bromophenol-blue and ß-mercaptoethanol was added to the denatured protein. Molecular weight markers were α-lactalbumin (14200 Da), soybean trypsin inhibitor (20100 Da), trypsinogen (24000 Da), carbonic-anhydrase (29000 Da), glyceraldehyde-3-phosphate dehydrogenase (36000 Da), ovalbumin (45000 Da)

and bovin serum albumin (66000 Da). A mixture of the proteins listed above was purchased from Sigma (Dalton Mark VII-L). Alternatively a solution containing the following proteins (high molecular weight standard) was used: carbonic-anhydrase (29000 Da), ovalbumin (45000 Da), bovine albumin (66000 Da), phosphorylase b (97400 Da), ß-galactosidase (116000 Da) and myosin (205000 Da). The unknown molecular weight of a protein X was interpolated from the plot of the log molecular weight of the protein standards listed above and d, the distance in cm covered by the protein standards from the top of the running gel.

About 25 μl of protein solution containing 1-5 μg of protein was applied to the stacking gel. Elecrophoresis was carried-out at 20 mA per gel for 1 hour. At the end of a run the polyacrylamide gel was stained for proteins with a solution containing 0.1% Coomassie Blue R-250 dissolved in a solution of 10% acetic acid, 40% methanol in water. The gel was then destained in a solution of 10% methanol, 7.5% acetic acid in water.

2.2.4. WESTERN BLOTTING.

Proteins were transferred from polyacrylamide gels on to nitrocellulose membranes in a Hoefer (Newcastle-under-Lyme, UK) mini transfer apparatus. Western blotting was essentially carried out using the Towbin's method (1979). The SDS-polyacryamide gel was prepared as before. At the end of the run, the gel was removed from the electrophoresis apparatus and separated from the

aluminia and glass plates. It was then placed between a nitrocellulose membrane (Schleicher and Schuell, Dassel, FRG) and a filter paper layer (Whatman 3 MM). A further filter paper layer was placed on the nitrocellulose membrane and the assembled parts were put in the cassette of the transfer cell. The cassette was submerged in transfer buffer containing 6 g of Tris, 28.8 g glycine, 400 ml of methanol in a final volume of 2 liters. The transfer run was carried out for 1 hour at 200 mA. The cooling system was activated before and during the transfer.

At the end of the run a small strip was cut from the nitrocellulose membrane and stained for proteins with amido black. The staining solution was prepared by dissolving 100 mg of amido black in 100 ml of destaining solution. The destaining solution contained a solution of 10% glacial acetic acid and 40% methanol in water. The nitrocellulose membrane strip was placed in staining solution for few minutes and quickly destained. It was then washed with distilled water and dried on filter paper (Whatman n°1).

The remainder of the nitrocellulose membrane was placed overnight in a solution of 0.2% gelatine (w/v) and 0.1% Triton X 100 in PBS (phosphate buffer saline) and shaken gently overnight. Any protein binding sites on the nitrocellulose membrane were blocked in this way to minimize non-specific binding of the specific ligand (lectin or antibody).

The PBS buffer (phosphate buffer saline) was prepared by adding about 400 ml of 10 mM potassium-dihydrogen orthophosphate to a 2 l solution of 10 mM dipotassium-hydrogen orthophosphate to give a pH = 7.4. Sodium chloride at a concentration of 0.15 M was then added to this solution. A 5 x concentrated PBS stock solution was prepared and stored at room temperature. This solutions was

stable for few months.

2.2.5. ANALYTICAL ISOELECTRIC FOCUSING.

Analytical isoelectric focusing was carried out using commercial Ampholine polyacrylamide plates (LKB, Bromma, Sweden). Thin layers of 5% polyacrylamide gel (dimensions 0.75 x 11 x 25 cm) containing 2.2% ampholine for analytical electrofocusing were supported by a special thin plastic foil to which the gel adhered firmly.

The pH range used was either 5.5-8.5 or 3.5-9.5 and the Multiphor LKB 2117 System designed for the horizontal electrophoretic technique was used.

The electrode solutions were 0.4 M HEPES for the anode and 0.1 M NaOH for the cathode when the pH range 5.5-8.5 was used, and 1 M H_3PO_4 for the anode and 1 M NaOH for the cathode when the pH range 3.5-9.5 was employed. Samples containing 10-20 μg of protein were placed on small strips of glass paper and placed on the gel slab. The power was set at 25 W, corresponding to a current of 50 mA and the time of run set to 2.5 hours. At the end of a run, part of the gel was cut in to small strips and used to determine the pH gradient between anode and cathode. Each strip (0.5 cm wide x 2 cm long) was placed in a test tube containing 1 ml of distilled water and allowed to stand for 1 h at room temperature to allow ampholine diffusion. The pH of the solution was then measured and plotted against gel length.

Another part of the gel was used for enzyme activity determination. In this case each strip of gel (0.5 cm wide x 2 cm long) was placed in test tubes containing 1 ml Mc Ilvaine buffer pH 5.0 and left to diffuse for two hours at 4°C. The enzyme activity was then determined using the appropriate method (see previous sections). The remaining part of the gel was stained for proteins.

For protein staining, the gel was placed in fixing solution containing 57.5 g of trichloroacetic acid and 17.25 g of sulfosalicylic acid in 500 ml of distilled water for 50-60 min. In this step proteins were precipitated by trichloroacetic acid and ampholines were solubilized and removed from the gel by sulphosalicylic acid. The gel was then placed for a few minutes in a solution of 25% ethanol and 8% acetic acid in water. The gel was then placed in the staining solution, prepared by dissolving 460 mg of Coomassie brillant blue R-250 in 400 ml of destaining solution. This step was carried out in a thermostated bath at 60°C for 10-20 min. The gel was destained with 25% ethanol and 8% acetic acid solution.

2.2.6. COLORIMETRIC ASSAYS FOR CARBOHYDRATES.

In general the development of colours was based upon the formation of furfural derivatives (see also pentose determination section 2.2.1.3.) obtained by heating the saccharide dissoved in aqueous solution in presence of a strong acid. The development of a colour is attributed to the complex action of the furfural derivative with an appropriate organic compound. Two procedures were used for

carbohydrate determination: the phenol sulphuric acid reaction and the anthrone assay.

(1) THE PHENOL-SULPHURIC ACID REACTION FOR CARBOHYDRATES.

This assay was carried out as described by Dubois *et al.* (1956). The specific organic colour-developing agent used was 80% phenol (w/w). The reagent contained concentrated sulphuric acid, reagent grade (specific gravity 1.84).
In a thick glass tube 0.4 ml of the saccharide solution, appropriately diluted, were mixed with 10 μl of phenol/water solution and 1 ml of concentrated sulphuric acid. A standard solution of monosaccharides or oligosaccharides (1 to 20 μM) was prepared to determine the carbohydrate concentration. Since the heat required for color development was provided by the exothermic dilution of sulphuric acid in water, this step was done using a fast flow-time pipette. The extinction was read at 485 nm.

(2) ANTHRONE ASSAY.

This assay was carried out as described by Dische (1962).
The reagent contained 0.2% anthrone in concentrated sulphuric acid.
In a thick glass test tube 0.5 ml of aqueous saccharide solution (5-50 μg carbohydrate) were mixed with 1 ml of the anthrone-sulphuric acid reagent. The tubes were placed in a boiling water bath for 5 min at 100°C. They were cooled at room temperature and the extinction was read at 620 nm. The colour

developed depended on the nature of the saccharides. For instance, hexoses and 6-deoxyhexoses gave a blue colour, whereas other saccharides gave a green colour. The standard carbohydrate solution was always prepared freshly using the appropriate monosaccharide or a mixture of monosaccharides (as for the determination of xyloglucan oligosaccharide).

2.2.7. PREPARATION OF ISOPRIMEVEROSE.

Isoprimeverose (α-D-xylopyranosyl-(1→6)-D-glucose) was prepared by hydrolysis of tamarind xyloglucan with Driselase (Sigma, Poole, Dorset, UK). Driselase is a fungal enzyme preparation from *Basidiomycetes* containing *endo-* and *exo-* enzymes including laminarinases, xylanases and cellulases. Driselase is capable of cleaving all the glycosyl linkages in the xyloglucan polysaccharide except the α-1,6-D-xylosyl linkage. A limit Driselase digest of xyloglucan therefore contains monosaccharides plus the disaccharide isoprimeverose (Fry, 1988).

Tamarind xyloglucan (288 mg, final concentration 10 mg/ml) in 50 mM ammonium acetate buffer pH 5.0 was mixed with Driselase (600 mg) and the solution was incubated for 24 hours at 30°C. The progress of the reaction was monitored by TLC. When the reaction was completed the enzyme was heat-denatured at 100°C for 10 min and centrifuged (26000 x g for 20 min). The supernatant was freeze-dried, dissolved in 3 to 4 ml of water and applied to a Bio Gel P2 column (100 x 2.2 cm) equilibrated with water or 50 mM ammonium

acetate buffer pH 5.0. Fractions of 2.5 ml were collected and assayed for carbohydrates using the phenol-sulphuric acid method and carbohydrate-containing fractions were analysed by TLC. Only the fractions containing the disaccharide isoprimeverose were pooled and freeze-dried to give a white lyophilized powder (96 mg). The identity of the material was checked by acid hydrolysis and quantitative determination of the sugar released by gas-liquid chromatography of the corresponding alditol acetates. Only xylose and glucose were detected, in equimolar amounts.

2.2.8. CARBOHYDRATE HYDROLYSIS AND GLC DETERMINATION OF MONOSACCHARIDES.

(1) CARBOHYDRATE HYDROLYSIS.

In a conical glass centrifuge tube 100 μl of 72% w/w H_2SO_4 were mixed with 0.5 ml of 1% myoinositol solution (added as internal standard), 1.2 ml of water and about 5 mg of the carbohydrate. The tube was covered with a glass marble and heated at 120°C for 1 hour in an autoclave. During this step the saccharides were hydrolyzed. The tubes were then placed in ice and 3.2 ml of water were added to the mixture. The sulphuric acid was neutralized by adding solid barium carbonate. Congo red paper was used to check the neutralization. About 3.0 ml of water were added to the hydrolysate and solution was cleared from undissolved material

by centrifugation at 1000 x g in a bench centrifuge. The supernatant was transferred to a small flask and concentrated using a vacuum rotary evaporator at 45°C.

(2) BOROHYDRIDE REDUCTION OF CARBOHYDRATES.

Sodium borohyride solution was prepared freshly when required by dissolving 100 mg of the white powder in 5 ml of 1 M ammonia solution. In a flask, the carbohydrates were dissolved in 0.5 ml of sodium borohydride solution and mixture was allowed to stand for 1 hour at room temperature. The reaction was then neutralized by adding few drops of acetic acid (H_2 is evolved during this step). The solution was evaporated to dryness using vacuum rotary evaporation. The borate was removed by evaporation of the volatile methyl-borate; this was achieved by addition of methanol (HPLC grade) and evaporation to dryness. This step was repeated 5 times.

(3) PREPARATION AND SEPARATION OF ALDITOL ACETATES.

In a small ground-glass necked flask 0.5 ml of acetic anhydride were mixed with the alditols. Flasks were stoppered and placed in a oil bath at 130°C for 3 hours. They were then removed from the oil bath, cooled to room temperature and analysed by GLC. About 100 μl were injected onto the GLC column.

Separations of monosaccharide alditol acetates by GLC were carried out using a Pye Series 104 chromatograph (Pye-Unicam, Cambridge, UK) with twin columns

and flame-ionisation detectors. The columns (3% polyester ECNSS-M on Gas Chrom Q, dimensions 200 x 0.4 cm) were operated isothermally at 180°C to 190°C.

2.2.9. THIN LAYER CHROMATOGRAPHY OF MONO- AND OLIGOSACCHARIDES.

Thin layer chromatography was carried out on aluminium foil-backed silica-gel layers 0.2 mm in thickness (Merck DC-Alufolien, Kieselgel 60). Samples (20-50 μg) were applied to the plate which was placed in a glass chamber pre-equilibrated and saturated with the appropriate irrigant. An aliquot (5 μl) of a 0.1% solution of monosaccharide(s) standard dissolved in ethanol was applied to the TLC plate. Monosaccharides or small oligosaccharides (DP up to 5) were run twice in a solution containing n-propanol:ethanol:water in the proportions 7:1:2. Higher molecular weight saccharides were run three times in a solution containing n-propanol:nitromethane:water in the proportions 5:2:3. All the reagents were AR grade and they were purchased from Aldrich (Gillingham, Dorset, UK).

After each run plates were blow-dried completely. Staining was achieved by spraying saccharides with a solution of 5% sulphuric acid in ethanol and plates were placed in a dark oven at 120°C for a few minutes. Carbohydrates appeared as a dark zone on a light background.

Monosaccharides, especially pentoses, were not revealed sensitively by this

procedure. Sensitive staining of xylose-containing samples was achieved by using a solution containing 1.232 g of *p*-anisidine and 1.66 g of phthalic acid in 100 ml of ethanol. The solution was sprayed and plate was placed in a dark oven at 105-110°C for 5 min. This staining procedure was specific for pentoses and hexoses (Kirchner, 1967).

2.2.10. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC DIONEX).

HPLC analysis of oligosaccharides was carried out using a Dionex BioLC chromatograph equipped with CarboPac PAI ion-exchange column, pulsed amperometric detector and microinjector system.

The sample (2-10 μg) was applied to the column and separated by gradient elution as described below. A solution of 100 mM sodium hydroxide and a gradient of sodium acetate in 100 mM sodium hydroxide were used in the separation of oligosaccharides. A reverse gradient of sodium hydroxide was used in the separation of monosaccharides. All solutions were prepared using carbonate-free de-ionized water.

(1) GRADIENT FOR XYLOGLUCAN OLIGOSACCHARIDES DP<9.

The column was equilibrated with a solution of 50 mM sodium acetate in 100 mM sodium hydroxide. The sample was applied to the column (time = 1 min), and eluted with a linear gradient 50 mM to 100 mM sodium acetate in 100 mM sodium hydroxide starting after 5 min and up to 60 min.

(2) GRADIENT FOR XYLOGLUCAN OLIGOSACCHARIDES DP>9.

The column was equilibrated with a solution of 75 mM sodium acetate in 100 mM sodium hydroxide. The sample was injected and the column washed for 5 min with the starting solution. At time = 5 min a linear gradient from 75 to 100 mM sodium acetate in sodium hydroxide was applied to the column up to time = 20 min, followed by a linear gradient 100-300 mM sodium acetate in sodium hydroxide up to time = 45 min. Higher molecular weight material was eluted with a linear gradient of 0.5 M sodium acetate in 100 mM sodium hydroxide up to time = 60 min.

(3) GRADIENT FOR MONOSACCHARIDES.

The column was equilibrated with 20 mM sodium hydroxide and monosaccharides were eluted with a reverse gradient to de-ionized water starting 2 min after injection (20 mM NaOH to water for 30 min). A standard solution of myoinositol, fucose, rhamnose, arabinose, galactose, glucose, xylose and mannose was used to

determine the molar response factors of the pulsed amperometric detector.

2.2.11. REDUCTION OF OLIGOSACCHARIDES TO ALDITOLS.

Saccharides (1-2 mg) were dissoved directly in 0.5-1 ml of a solution of 0.5 M sodium borohydride in 1 M ammonia and left at room temperature for 2 h. The solution was acidified with glacial acetic acid (30-40 μl) to decompose excess of borohydride.

The solution was then applied to a small column (5 ml bed volume) packed with Amberlite IR 120 (H^+) and eluted with de-ionized water. Fractions (1 ml) containing the saccharide alditol were collected and concentrated by vacuum rotary evaporation. The borate was removed by evaporation of the volatile methyl-borate. This was achieved by addition of methanol (HPLC grade) and successive evaporation. This step was repeated 5 times. The sample was then re-suspended in water and freeze-dried.

2.2.12. ACID HYDROLYSIS OF OLIGOSACCHARIDES (by TFA).

Saccharides (1-2 mg) were dissolved directly in 2 M trifluoroacetic acid (TFA) and heated for 1 h at 120°C (Fry, 1988). Insoluble material was removed by

centrifugation (12000 x g for 10 min) and TFA was evaporated by freeze-drying. Monosaccharides were analysed by HPLC or GLC. Acid hydrolysis of saccharides in sulphuric acid is described in section 2.2.8..

2.2.13. PURIFICATION OF ENZYMES FROM NASTURTIUM SEEDLINGS.

The growth conditions of nasturtium seedling, the harvest, the homogeneization and the anion-exchange chromatography of the crude extracts are almost identical in the purification of ß-galactosidase, *endo*-(1→4)-ß-D-glucanase and α-xylosidase.

2.2.13.1. SEED GERMINATION AND GROWTH CONDITIONS.

The nasturtium seeds had an average germination of 84%. The germination average was as high as to 93% when batches of fresh seeds were used and slowly decreased with time yielding a germination average of 79% when 4 year old seeds were used. Seeds were kept in the dark and in a dry place. A low proportion of "coated" seeds were present in the mixture and the fruit-coats had to be removed manually before homogenization.

Dry seeds were placed in trays (21 x 70 cm) containing 3-4 cm of "vermiculite" ("Micafill" fibre-free insulation; Dupre Vermiculite Ltd., Hertford, Herts, UK).

The seeds (350 to tray) were placed on the surface of the vermiculite layer, covered with a further layer of vermiculite and watered thoroughly. Trays were then placed in a plant growth chamber with 12-h day/12-h night regime. Photon fluence rate was 850 μmol m^{-2} s^{-1} at tray level and the temperature was kept between 15-20°C. Trays were covered with a transparent polyethene sheet until shoots appeared and seeds watered every 1-2 days with warm tap water.

2.2.13.2. ENZYME EXTRACTION.

All operations were carried out at 4°C unless otherwise stated. Cotyledons were removed manually from the seedlings at day 13 after sowing. They were washed 2-3 times with distilled water and divided in lots of about 200 g, which were individually homogeneized for 30 sec in a blender (M.S.E. Ltd "Atomix") with 300 ml of cold 0.2 M KH_2PO_4-K_2HPO_4 buffer pH 7.2 and 2% insoluble polyvinylpyrrolidone (Sigma, Poole, Dorset, UK). Homogenates were then centrifuged at 19000 g for 30 min. A thin layer of lipid was present on the surface of the tubes after centrifugation and was removed by absorbing it in a paper tissue. The supernatant was collected and the pellets were re-suspended in the same buffer and subjected to a further centrifugation at 26000 g for 30 min. Since the enzyme activity in the pellet accounted for only 10-15% of the total enzyme activity this step was omitted when large enzyme preparations were carried out. The supernatants were pooled and brought to 90% saturation with ammonium

sulphate by addition of the crystalline salt. The suspension was left for 1 h at 4°C and centrifuged at 19000 g for 30 min. Pellets were re-suspended in the minimum volume of 20 mM Tris-HCl buffer pH 7.8 and dialysed against the same buffer (250 ml of enzyme extract were dialysed against 2 x 5 l Tris-HCl buffer pH 7.8). The enzyme extract was centrifuged at 26000 g for 20 min to remove undissolved material and was then applied to an anion-exchange chromatography column. Further stages in the purification of α-xylosidase, *endo*-(1→4)-ß-D-glucanase and ß-galactosidase are described in the appropriate chapters.

CHAPTER THREE

PURIFICATION AND CHARACTERIZATION OF AN α-XYLOSIDASE FROM GERMINATED NASTURTIUM (*Tropaeolum majus L.*) SEEDS

3.1. INTRODUCTION

The distribution of α-D-xylosyl linkages in plant polysaccharides is restricted to the xyloglucan type (see Chapter 1) and the literature is sparse concerning the hydrolysis of such linkages by specific enzymes. α-D-xylosidases able to cleave the α-(1→6)-xylosidic linkage have been reported to be involved in the depletion of xyloglucan side-chains during the mobilization of this polysaccharide in nasturtium cotyledons (Edwards *et al.*, 1985). More recently α-xylosidase activity has been found in the primary cell wall of auxin-treated pea stems, where this enzyme may regulate the levels of biologically active xyloglucan-derived oligosaccharides (O'Neill *et al.*, 1989).

Enzymes able to attack and cleave α-(1→6)-linkages have also been reported in the basidiomycete *Armillaria mellea* (Molodtsov *et al.*, 1974), in the culture filtrate of *Aspergillus niger* strain AMS 4111 (Matsushita *et al.*, 1985; Kato *et al.*, 1985; Matsushita *et al.*, 1987), from which two different components of the enzyme have been purified and characterized, and from *Bacillus* sp. N 693-1 (Zong and Yasui,

1989; Zong *et al.*, 1989). The fungal and bacterial α-xylosidase were in all cases monitored by the release of *p*-nitrophenol from *p*-nitrophenyl-α-D-xylopyranoside or by the determination of xylose and glucose released by the hydrolysis of the disaccharide isoprimeverose (6-O-α-D-xylopyranosyl-D-glucose). The *Bacillus* α-xylosidase was also able to cleave (1→3) and (1→4)-α-xylobioses. By contrast the purified enzyme α-xylosidase from the auxin-treated pea (*Pisum sativum*) seedlings (O'Neill *et al.*, 1989) and an enzyme extract obtained from immature soybean tissue (Koyama *et al.*, 1983) have been found to be unable to hydrolyse either *p*-nitrophenyl-α-D-xylopyranoside or the disaccharide isoprimeverose. In this respect they do not resemble the bacterial or fungal hydrolases.

The α-xylosidase in extracts of nasturtium cotyledons has also been shown to be inactive with *p*-nitrophenyl-glycoside (Edwards *et al.*, 1985).

In this Chapter the purification to homogeneity of α-xylosidase from germinated nasturtium cotyledons is reported together with the molecular properties of this enzyme. The specificity of this enzyme towards the native and the enzymically digested xyloglucan substrate is investigated and compared with other known α-xylosidases.

The molecular mode of action of this enzyme on xyloglucan and its derived oligosaccharides sheds light on the *in vivo* role of the enzyme during the post-germinative mobilization of nasturtium xyloglucan and indicates a possible involvement of similar activities in the regulatory mechanism of primary cell-wall xyloglucan solubilization during cell-wall turnover (see Chapter 6).

3.2. MATERIALS AND METHODS

Chromatofocusing was carried out using poly-buffer exchanger PBE 94 and Polybuffer 74. Both were purchased from Pharmacia, Uppsala, Sweden. Hydrophobic interaction chromatography was carried out using Phenyl Sepharose CL 4B purchased from Pharmacia, Uppsala, Sweden. Gel filtration was carried out using Bio Gel P60 and P300, from BioRad, Watford, Herts, UK. The deglycosylation kit was purchased from Oxford GlycoSystems, Abingdon, Oxford, UK. The enzyme *endo*-(1→4)-ß-D-glucanase purified from *Trichoderma viride* was purchased from MegaZyme, North Rocks N.S.W., Australia. Other materials and methods are described in Chapter 2.

3.2.1. ENZYME ASSAYS.

The activities of ß-galactosidase and ß-glucosidase were determined by the appropriate *p*-nitrophenylglycoside as substrate. Galactose was determined by the D-galactose dehydrogenase assay (Kurz and Wallenfels, 1974) and glucose by the hexokinase/glucose-6-phosphate-dehydrogenase (Bergmeyer *et al.*, 1974). Protein was determined quantitatively by the procedure of Sedmak and Grossberg (1977), using bovine albumin as standard. The total carbohydrate was determined by the phenol-sulphuric acid (Dubois *et al.*, 1956) or by the anthrone assay (Dische,

1962) The reducing power of saccharide solutions was determined by the procedure of Halliwell and Riaz (1970). All these assay procedures are described in Chapter 2.

3.2.2. ROUTINE ASSAY FOR α-D-XYLOSIDASE ACTIVITY.

The activity of α-xylosidase was routinely assayed at pH 5.0 and 30°C using either 50 mM ammonium acetate buffer or McIlvaine phosphate-citrate buffer. The ammonium acetate system was preferentially used for TLC analysis as it is volatile and did not affect the mobility of saccharides.

The activity of the enzyme was monitored by the determination by the *p*-bromoaniline procedure of Roe and Rice (1948) of free pentoses released upon hydrolysis of substrate.

Preliminary investigations showed that only crude enzyme extracts and those partially purified preparations which still contained the xyloglucan-specific *endo*-(1→4)-glucanase activity contained xylose as well as other products of hydrolysis of xyloglucan. It was therefore concluded that the native xyloglucan was not itself a substrate for α-xylosidase and that substrates were generated by the action of the nasturtium *endo*-glucanase on the xyloglucan.

The α-D-xylosidase activity was therefore monitored in column fractions by mixing 0.2 ml of individual column fractions with 0.5 ml of a solution of 24 mg/ml tamarind xyloglucan in acetate or Mc Ilvaine buffer pH 5.0 (final concentration

of 1% w/v) and 0.1 ml of the pure nasturtium *endo*-(1→4)-glucanase, in a final volume of 1.2 ml. Controls containing only xyloglucan and xyloglucan/*endo*glucanase were run in parallel. The mixtures were incubated overnight at 30°C and the enzyme(s) inactivated by heat-denaturation. The xylose released during the incubation was then determined using the spectrophotometric assay for pentoses.

3.2.3. GENERAL SEPARATION METHODS.

SDS-polyacrylamide gel electrophoresis, western blotting, analytical isoelectric focusing, analytical and preparative thin layer chromatography, and high performance liquid chromatography (HPLC) were carried out as described in Chapter 2 and 3.

3.2.4. PURIFICATION OF α-XYLOSIDASE.

Seedling growth conditions, homogenization and enzyme extraction were as described in Chapter 2. Cotyledons from 13 day old seedlings were harvested and homogenized in 0.2 M potassium phosphate buffer pH 7.2. The insoluble material was removed by centrifugation (19000 g x 30 min) and supernatant brought to

90% saturation with ammonium sulphate by addition of the crystalline salt. The precipitate was collected after centrifugation (26000 g x 30 min), dissolved in 20 mM Tris-HCl buffer pH 7.8 and dialysed against the same buffer.

3.2.4.1. ANION-EXCHANGE CHROMATOGRAPHY.

The dialysed supernatant (250 ml) was applied to a column (2.2 x 40 cm) of DEAE-cellulose (Whatman DE52) and the column was washed with the 20 mM Tris-HCl buffer 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 in the same buffer over 7-8 bed volumes). The column flow-rate was 80 ml h^{-1} and 10 ml fractions were collected. The activity of α-xylosidase in column fractions was assayed as described above. Fractions containing high enzyme activity were pooled and concentrated by protein precipitation, achieved by adding ammonium sulphate up to 90% saturation. The suspension was left a 4°C for 1 h and centrifuged at 26000 for 20 min. The pellet was dissolved in and dialyzed against 50 mM sodium acetate buffer pH 5.0 and subjected to cation-exchange chromatography.

3.2.4.2. CATION-EXCHANGE CROMATOGRAPHY.

The enzyme extract (about 100 ml) was applied to a carboxymethyl-cellulose (Whatman CM-52) column (2.2 x 30 cm), equilibrated with the same buffer. The flow-time of the eluant was 50 ml/h and 10 ml fractions were collected. The column was washed with 50 mM sodium acetate buffer pH 5.0 until absorbance at 280 nm was constant. The column was then eluted with a sodium chloride gradient (0-0.5 M in the same buffer over 8 bed volumes). The α-xylosidase activity was eluted early in the sodium chloride gradient. Fractions containing high enzyme activity were pooled and concentrated by the addition of ammonium sulphate (90% saturation). The protein was collected by centrifugation at 26000 g for 30 min, dissoved in 25 mM imidazole-HCl buffer pH 7.4 and dialyzed against the same buffer.

3.2.4.3. CHROMATOFOCUSING.

A column (20 x 0.5 cm) was packed with PBE- (polybuffer-exchanger) 94, and eluted with polybuffer 74 (pH range pH 7.4-5.0). The gel, which was degassed prior to use, was washed with 1 M NaCl (200 ml) and then with 200 ml of the starting buffer (25 mM imidazole buffer pH 7.4). The sample was applied to the column and eluted with a gradient of polybuffer-HCl (200 ml). The eluant was

prepared according to manufacture's instructions by diluting polybuffer-74 1:8 with water and adjusting the pH to 5.0 with HCl. The flow-time was reduced to 15 ml/h and 3 ml fractions were collected for protein and enzyme determination. Fractions were analysed also by SDS-polyacrylamide gel electrophoresis. Fractions containing high enzyme activity were pooled and concentrated by the addition of ammonium sulphate (90% saturation). The protein was collected by centrifugation at 26000 g for 30 min and dissolved in a minimum volume (3-4 ml) of 50 mM ammonium acetate buffer pH 5.0, prior to gel filtration.

3.2.4.4. GEL FILTRATION.

The sample was applied to a Bio-gel P-60 column (3.0 x 70 cm) and eluted with 50 mM ammonium acetate buffer pH 5.0. The flow-time was 2.0 ml/h and 4 ml fractions were collected from the column. The fractions containing high enzyme activity were pooled, aliquoted and stored at -20°C.

The purification of the enzyme achieved using cation-exchange chromatography followed by gel filtration gave a poor yield of the enzyme. Following the finding that the enzyme α-xylosidase was a glycoprotein an alternative and more efficient method was designed, using lectin-binding affinity chromatography as described in 3.2.4.6.

3.2.4.5. HYDROPHOBIC INTERACTION CHROMATOGRAPHY.

This technique was used in an attempt to improve the yield of enzyme obtained on purification. The sample applied to the phenyl Sepharose column was a crude extract partially purified by cation exchange chromatography.

The hydrophobic interaction column (15 x 1.0 cm) was packed with phenyl Sepharose CL 4B and chromatography was carried out according to the manufacturers' instructions.

The column was washed with 200 ml of distilled water, with 200 ml of 50 mM ammonium acetate pH 5.0, and then pre-equilibrated with 200 ml of 2 M ammonium sulphate in 50 mM ammonium acetate buffer pH 5.0. The protein sample, dissolved in 50 mM ammonium acetate buffer pH 5.0, was prepared by adding crystalline ammonium sulphate salt up to a final concentration of 2 M and was applied to the column. The flow-time was reduced to 15 ml/h using a peristaltic pump and 2 ml fractions were collected. A linear gradient from 2.0 to 0 M ammonium sulphate in 50 mM ammonium acetate buffer pH 5.0 (50 + 50 ml) was first applied, followed by a linear gradient from 0 to 50% ethylene glycol in 50 mm ammonium acetate buffer pH 5.0 (50 + 50 ml). Proteins are generally eluted in the first or in the second gradient.

As no enzyme was eluted with the eluants used the column was re-equilibrated with 100 ml of 50 mM ammonium acetate pH 5.0 and an additional linear gradient from 0 to 100% ethanol (50 + 50 ml) was applied to the column. The column was then eluted with 2% Tween-20 (50 ml) and washed with 20% ethanol.

3.2.4.6. AFFINITY CHROMATOGRAPHY ON CONCANAVALIN-A SEPHAROSE 4B.

The fractions from anion-exchange chromatography with high enzyme activity were pooled and precipitated by the addition of ammonium sulphate (90% saturation). The suspension was spun and the pellet was dissolved in and dialyzed against 5 mM sodium acetate buffer pH 5.0 containing 1mM $CaCl_2$, 1 mM $MnCl_2$ and 200 mM NaCl and subjected to affinity chromatography on Concanavalin-A Sepharose 4B. The column (25 cm x 1.0 cm) was packed with Concanavalin-A Sepharose 4B and pre-equilibrated with starting buffer (5 mM sodium acetate pH 5.0, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 200 mM NaCl). The flow-time was decreased to 10 ml/h using a peristaltic pump. The sample (about 50 ml) was applied to the column and 4 ml fractions were collected. The unbound material was eluted with 5 mM sodium acetate buffer pH 5.0 containing 1mM $CaCl_2$, 1 mM $MnCl_2$ and 200 mM NaCl and column was washed until the absorbance at 280 nm had fallen to a constant value. The column was then eluted step-wise with methyl-α-D-glucopyranoside (80 ml each of 0, 10, 20 and 100 mM) in the same buffer. Fractions were analysed by SDS-polyacrylamide gel electrophoresis and assayed for α-xylosidase activity. The fractions from 10- and 20- mM elution steps which contained α-xylosidase activity and only a single protein band on SDS-PAGE were pooled. The protein was concentrated by ammonium sulphate (90% saturation) and the precipitate collected by centrifugation (26000 x g for 20 min). The pellet

was dissolved in 5 mM sodium acetate buffer pH 5, 1 mM $CaCl_2$, 1 mM $MnCl_2$ and 200 mM NaCl, dialysed against the same buffer and applied to a second affinity column of Concanavalin-A Sepharose 4B. The re-chromatography of the enzyme was performed to purify the enzyme further from minor protein contaminants and to concentrate the enzyme. This time the α-xylosidase was eluted with a single step-gradient of 100 mM α-methyl-glucopyranoside. The α-xylosidase was eluted in a sharp peak and the enzyme-containing fractions were pooled and stored at -20°C in small aliquots.

At the end of each run the Concanavalin A Sepharose 4B column was washed with cold 0.1 M HCl (200 ml) and then with a solution of 25 mM sodium acetate containing 5 mM $CaCl_2$, 5 mM $MnCl_2$, 5 mM methyl-α-D-glucopyranoside (200 ml) containing 0.05% thimerosal as preservative.

Some preparations contained a minor protein contaminant, ß-galactosidase, with a higher electrophoretic mobility on SDS-PAGE. In such cases the α-xylosidase preparation was brought to 90% saturation with ammonium sulphate and left at 4°C overnight. The precipitate was collected by centrifugation (26000 x g for 30 min) and pellet was re-suspended in 2-3 ml of 50 mM ammonium acetate buffer pH 5.0. This solution was applied to a Bio Gel P300 column (70 x 2.2 cm) pre-equilibrated with the same buffer. Fractions of 2 ml were collected and analysed for enzyme activity and by SDS-polyacrylamide gel electrophoresis. The contaminating band was separated clearly from the main α-xylosidase peak.

3.2.5. DETECTION OF GLYCOPROTEIN IN THE SDS-POLYACRYLAMIDE GEL BY THE PERIODIC ACID/SCHIFF'S STAINING (by a modification of Neville and Glossmann, 1974).

SDS-polyacrylamide gel electrophoresis was carried out as described in Chapter 2 and the gel cut in half: one part was stained for proteins with the Coomassie blue solution and the remaining part was stained for glycoproteins according to the following procedure. The gel was placed overnight in a solution containing 40% methanol and 7% acetic acid. It was then washed twice with distilled water and placed in an oxidizing solution prepared by dissolving 1 g of periodic acid in 100 ml of 7% acetic acid. The gel was incubated for 1 hour at 4°C in the dark, shaking occasionally. The excess of periodate was removed by washing the gel with 7% acetic acid. The gel was then left in 7% acetic acid solution and after 2 hours transferred to a solution containing Schiff's reagent (Sigma). This step was carried out for 1 hour at 4°C in the dark. The gel was then placed in a solution of sodium metabisulphite prepared by dissolving 5 g $Na_2S_2O_5$ in 500 ml of water containing 4.125 ml of concentrated HCl.

In this step the maximum colour developed and rose-pink bands appeared which corresponded to glycoprotein bands. Athough this procedure was very specific for glycoproteins it was not very sensitive and required higher loading of protein on the SDS-PAGE gel.

3.2.6. A SENSITIVE ENZYME-LINKED LOCALIZATION OF THE α-XYLOSIDASE PROTEIN ON ELECTROBLOTS (modification of Clegg's. 1982. procedure).

Protein transfer onto nitrocellulose membranes (western blotting) was carried out as described in Chapter 2. Concanavalin A type IV from jack bean (*Canavalia ensiformis*), which binds to α-mannosyl and α-glucosyl residues, was used for the specific detection of glycoproteins.

The nitrocellulose membrane containing the purified α-xylosidase or a crude enzyme extract was incubated with 5-10 ml of a solution of Concanavalin-A prepared by dissolving 1 mg of lectin in 100 ml of PBS buffer containing 2% gelatine and 0.5% Triton X-100. This step was carried out for 1 hour at room temperature, shaking gently. The unbound lectin was removed by washing the membrane with the PBS buffer described above for 10-15 minutes. The washes were repeated three times. The nitrocellulose membrane was then incubated with a 0.05% solution of horseradish peroxidase in the PBS buffer described above. This step was carried out at room temperature for 1 hour. The presence of 10 μM $MgCl_2$, $MnCl_2$, $CaCl_2$ did not affect the binding of peroxidase to Concanavalin A and it was therefore omitted. Three further washes of 10-15 minutes each were carried out as before to remove excess horseradish peroxidase. The nitrocellulose membrane was then placed in a solution of 0.05% diaminobenzidine in 50 mM Tris-HCl buffer pH 8.0, freshly prepared. The detection of peroxidase activity was achieved by the addition of 5 μl of hydrogen peroxide (30% v/v). As the staining

appeared, the membrane was quickly washed with water and then with 2% SDS. It was then dried on filter paper and stored in a dark and dry place. A standard solution containing several proteins including albumin from egg (a glycoprotein) was used as control.

3.2.7. CARBOHYDRATE ANALYSIS OF THE GLYCOSYLATED ENZYME.

A solution of the purified enzyme α-xylosidase was divided into three lots containing 20-50 μg of protein each, which were placed in microcentrifuge tubes. The protein was lyophilized. The first lot was treated with 100-200 μl of 2 M trifluoroacetic acid and hydrolysis carried out at 80°C for 1 h to remove sialic acid from the protein. The second and third lot were treated with 100-200 μl of 2 M TFA at 120°C for 2.5 h and at 120°C for 4 h to remove neutral monosaccharides and aminosugars, respectively. The procedure used was that suggested by Dionex (Glycoprotein Workshop, 1989; Dionex, Sunnyvale California, US). The monosaccharides released upon acid hydrolysis were analysed by HPLC using the monosaccharide program as described in section 2.2.10. and the following gradients:

(i) The column was equilibrated with 10 mM sodium hydroxide. The sample was injected and eluted with water (starting at time = 4 min).

Retention times for monosaccharide standards: Fuc 5.43±0.1min; Ara 13.5±0.2min; Gal 18.63±0.2min; Glc 22.04±0.4min; Xyl 26.72±0.4min; Man

30.02±0.4min. GalN 14.04±0.5min; GlcN 19.11±0.5min; Glc(Gal)Nac 29.16±0.5min.

(ii) The column was equilibrated with 15 mM sodium hydroxide. The sample was injected and after 4 min the column was eluted with 5 mM sodium hydroxide up to time = 12.8 min. A linear gradient from 5 to 0 mM sodium hydroxide was then applied up to time = 14.7 min. The column was then washed with water for 1 min and re-equilibrated with 15 mM sodium hydroxide.

Retention times for monosaccharide standards: Fuc 4.35±0.2min; Ara and GalN 8.25±0.4; GlcN 10.55±0.2; Gal 11.22±0.3; Glc 12.83±0.4; Xyl and Glc(Gal)Nac 15.0±0.4; Man 16.99±1 min.

(iii) Sialic acid (N-acetyl-neuraminic acid) was injected in the column pre-equilibrated with 50 mM sodium acetate in 100 mM sodium hydroxide and sample was eluted with the same solution after 9 ± 0.1 min.

3.2.8. DEGLYCOSYLATION OF THE ENZYME.

The procedure and the materials used for the deglycosylation of α-xylosidase were included in the deglycosylation kit purchased from Oxford Glycosystems.

The procedure was that of Sojar and Bahl (1987) with some modifications. The kit included the following reagents: anhydrous trifluoromethanesulphonic acid (TFMS), toluene and pyridine.

TFMS has been found to cleave N- and O-linked glycans non-selectively from

glycoproteins, leaving the primary structure of the protein intact. The mechanism of deglycosylation is not yet completely understood. Since the reaction occurs only under anhydrous conditions, it has been suggested that deglycosylation may be achieved by an acid-catalysed dehydration.

About 6 ml of a solution containing 300 μg of purified enzyme were concentrated by rotary-evaporation and de-salted by gel filtration on a Sephadex G-25 column (25 x 1.0 cm) and eluted with double distilled water. The protein was then re-concentrated by vacuum rotary evaporation, transferred to the glass reaction vial provided with the kit and freeze-dried overnight. Standards including ovalbumin from egg (about 400 μg) and horseradish peroxidase (about 200 μg) were also freeze-dried, each in separate vials.

About 60 μl of toluene were transferred to the ampoule of trifluoromethanesulfonic acid using a dry glass syringe and mixed very gently. The vials containing the protein samples were placed in an ethanol-dry ice bath for about 30 seconds. Very slowly 50 μl of the TFMS-toluene mixture were added to the samples which were allowed to stand in the ethanol-dry ice bath for further 10-20 seconds. They were then placed in a -20°C freezer. After 5-10 min they were removed from the freezer and shaken gently to aid melting. They were left at -20°C for about 4 hours, to allow complete deglycosylation of the glycoproteins. The vials were removed from the freezer and placed in the ethanol-dry ice bath for 30 seconds. Using a syringe, 150 μl of pyridine were added to them very slowly. The vials were left in the ethanol-dry ice bath for further 20 seconds, transferred to a dry ice bath for 5 min and then to a wet ice bath for further 15 min. A solution of 0.5% ammonium bicarbonate (400 μl) was added to the de-

glycosylated samples and mixed gently. During this step protein aggregation occurred, and the samples were left at 4°C for 45 min. The suspension was transferred to microcentrifuge tubes and the precipitates were removed by centrifugation. The supernatants were retained. The pellets were washed 4 times with 4 x 200 μl of 0.5% ammonium bicarbonate and the supernatants and washings were combined, placed in a dialysis bag and dialysed against 0.5% ammonium bicarbonate (500 ml x 2). Supernatants and pellets were then freeze-dried. The lyophilized material was re-suspended in 100 μl of distilled water and analysed by SDS-PAGE gel electrophoresis. The precipitated proteins did not dissolve very easily in water and they were therefore boiled with the SDS sample buffer for 15 min at the end of which the solutions appeared clear. The unknown molecular weight of the de-glycosylated protein X was extrapolated from the plot of the log molecular weight of the protein standards and d, the distance in cm covered by the protein standards from the top of the running gel (section 2.2.3.).

3.2.9. CROSS-LINKING METHOD FOR THE DETECTION OF OLIGOMERIC PROTEINS.

The cross-linking reaction was carried out according to Davies and Stark (1970), in 0.2 M triethanolamine hydrochloride buffer pH 8.5. The cross-linking reagent dimethyl suberimidate was freshly prepared in the same buffer to give a final concentration of 40 mM in a volume of 50 μl. The enzyme α-xylosidase was

dialyzed against triethanolamine buffer pH 8.5. The concentrations of the enzyme ranged from 20-100 μg/ml. The reagent was dissolved in the triethanolamine buffer pH 8.5 to give a final concentration of 0.4-1 mg/ml in a final volume of 50 μl. The reaction was carried out at room temperature for 3 hours. Before SDS-PAGE the proteins were denatured with 1% SDS and 1% ß-mercaptoethanol at 37°C for 2 hours. About 10 μl of a solution containing 50% glycerol and 0.01% bromophenol blue were added to the denatured proteins and electrophoresis was carried out using 5% and 12% polyacrylamide gels.

3.2.10. GEL FILTRATION ON BIO GEL P4: PREPARATION AND SEPARATION OF TAMARIND XYLOGLUCAN OLIGOSACCHARIDES.

Tamarind xyloglucan (final concentration 1% w/v) was incubated with the pure nasturtium *endo*-(1→4)-ß-D-glucanase (final concentration 8 μg of enzyme/ml) and the mixture placed at 30 °C in a thermostated bath overnight. After this time the reducing power did not change, and the enzyme was heat-denatured. Precipitated material was removed by centrifugation (26000 x g for 10 min) and two volumes of ethanol were added to the supernatant. The high molecular weight material which precipitated was removed by a further centrifugation step (26000 x g for 10 min). The supernatant was retained, concentrated by vacuum rotary evaporation and applied to a gel filtration Bio Gel P4 column (2.2 x 100 cm) pre-equilibrated with 50 mM ammonium acetate buffer pH 5.0. Fractions (2.5

ml) were collected and analysed by the phenol/sulphuric acid assay (section 2.2.6.). Fractions containing carbohydrates were retained and small aliquots spotted on TLC plate. Fractions which contained two or more components were subjected to a further purification step using preparative TLC.

3.2.11. PREPARATIVE THIN LAYER CHROMATOGRAPHY.

Tamarind xyloglucan oligosaccharides partially purified from gel filtration on Bio Gel P4 were purified by preparative thin layer chromatography.

TLC plates were strip-loaded with saccharides (10-15 mg) and run three times as described in section 2.2.9.. At the end of the runs two strips from the each side and one strip from the central part were cut from the plate and stained for carbohydrates. They were used to localize the saccharide(s). The separated saccharide bands were removed from the plate by scraping off the silica gel, placing it in a test-tube and suspending it in distilled water. The tubes were incubated at room temperature for 2 hours and the silica removed by centrifugation (26000 x g for 20 min). The supernatant was retained, and the silica gel re-suspended in water. The silica was removed with a second centrifugation and supernatant combined with the previous one. The saccharide-containing solutions were then concentrated by vacuum rotary evaporation or by freeze-drying and saccharide concentration determined by the phenol-sulphuric acid assay or by the anthrone assay as described in section 2.2.6..

3.2.12. ENZYME DIGESTION OF XYLOGLUCAN OLIGOSACCHARIDES.

The *endo*-(1→4)-ß-glucanase enzyme preparation purified from *T. viride* was de-salted as described in section 4.2.6..

Purified saccharides or saccharide mixtures (0.2-1.0 mg) were dissolved in 50 mM ammonium acetate pH 5.0 in a final volume of 50-100 μl and incubated with the fungal cellulase (0.2-1.0 U) or with the nasturtium *endo*glucanase (0.2-1.0 μg protein). Incubations of xyloglucan oligosaccharides with the nasturtium ß-galactosidase were carried out treating the saccharides (0.2-1.0 mg) with 0.5-2.0 μg of enzyme in a final volume of 20-50 μl. Incubations of xyloglucan oligosaccharides with ß-glucosidase from almonds (50 U/ml) were carried out by treating the saccharides (0.2-1.0 mg) with 1.0-2.0 U of enzyme in a final volume of 20-50 μl. Incubations of xyloglucan oligosaccharides with Driselase were carried out by treating the saccharides (0.2-1.0 mg) with 0.2-0.5 mg of enzyme preparation in a final volume of 20-50 μl. All incubations were carried out overnight at 30°C using 50 mM ammonium acetate buffer pH 5.0 and stopped by heat-denaturation of enzyme(s).

3.2.13. ^{1}H-NMR ANALYSIS.

The ^{1}H-NMR analysis was carried out by Dr M.J. Gidley at Unilever Laboratories, Colworth House, Bedford. Saccharides (0.5-1.0 mg) or their corresponding alditols were dissolved in deuterated water and proton NMR recorded at 85-90°C on a model AM 200 spectrometer (Bruker Analytische Messtechnik, Rheinstetten, FRG) operating at 200.13 MHz. To ensure full signal responses for accurate integration, a 10-s recycle time was employed between successive 90° pulses. Samples were lyophilized once or twice from deuterated water prior to analysis to reduce interference from residual protiated solvent. Chemical shifts are referenced to external tetramethylsilane.

3.3. RESULTS

3.3.1. PURIFICATION OF THE α-XYLOSIDASE.

The α-D-xylosidase was purified from an extract prepared from nasturtium seedlings 13 d after sowing, when the enzyme activity in the cotyledonary extracts had reached its maximum. Partial purification of α-D-xylosidase was achieved by anion-exchange chromatography on DEAE-cellulose column. The enzyme was

eluted in the sodium chloride gradient applied to the column as shown in Fig.3.1.a. The fractions containing high enzyme activity were subjected to cation-exchange chromatography using CM-cellulose. The α-xylosidase activity was eluted in the sodium chloride gradient applied to the column (Fig.3.1.b). Partially purified α-xylosidase was subjected to chromatofocusing on PBE 94 pH range 7.4 to 5.0. The column fractions containing high enzyme activity spanned the pH range 6.8 to 5.0 indicating that α-xylosidase contained several isoenzymes (Fig.3.1.c). Some of these fractions also contained ß-galactosidase activity. The column fractions containing α-xylosidase were pooled and purified further.

Gel filtration on Bio Gel P60 led to a successful purification, but in very low yield (Fig.3.1.d). The enzyme was eluted from the column in a highly asymmetric "tailing peak" indicating that it had a high degree of structural heterogeneity. Only the tail end of the enzyme peak did not overlap with contaminating proteins and contained a single protein band as monitored by SDS-PAGE gel electrophoresis. The yield of purified enzyme was therefore poor and this method was later abandoned.

Hydrophobic-interaction chromatography was carried out as an alternative but the enzyme activity could not be recovered from the column. This indicated that the enzyme was highly hydrophobic.

It was considered possible that the enzyme was a glycoprotein, since it apparently exibited poly-dispersity during gel filtration. SDS-polyacrylamide gels containing the pure protein and a crude enzyme extract were therefore stained with the periodate-Schiff reagent, which is specific for carbohydrates. The purified α-xylosidase gave a single rose-pink band, and several bands in the enzyme extract

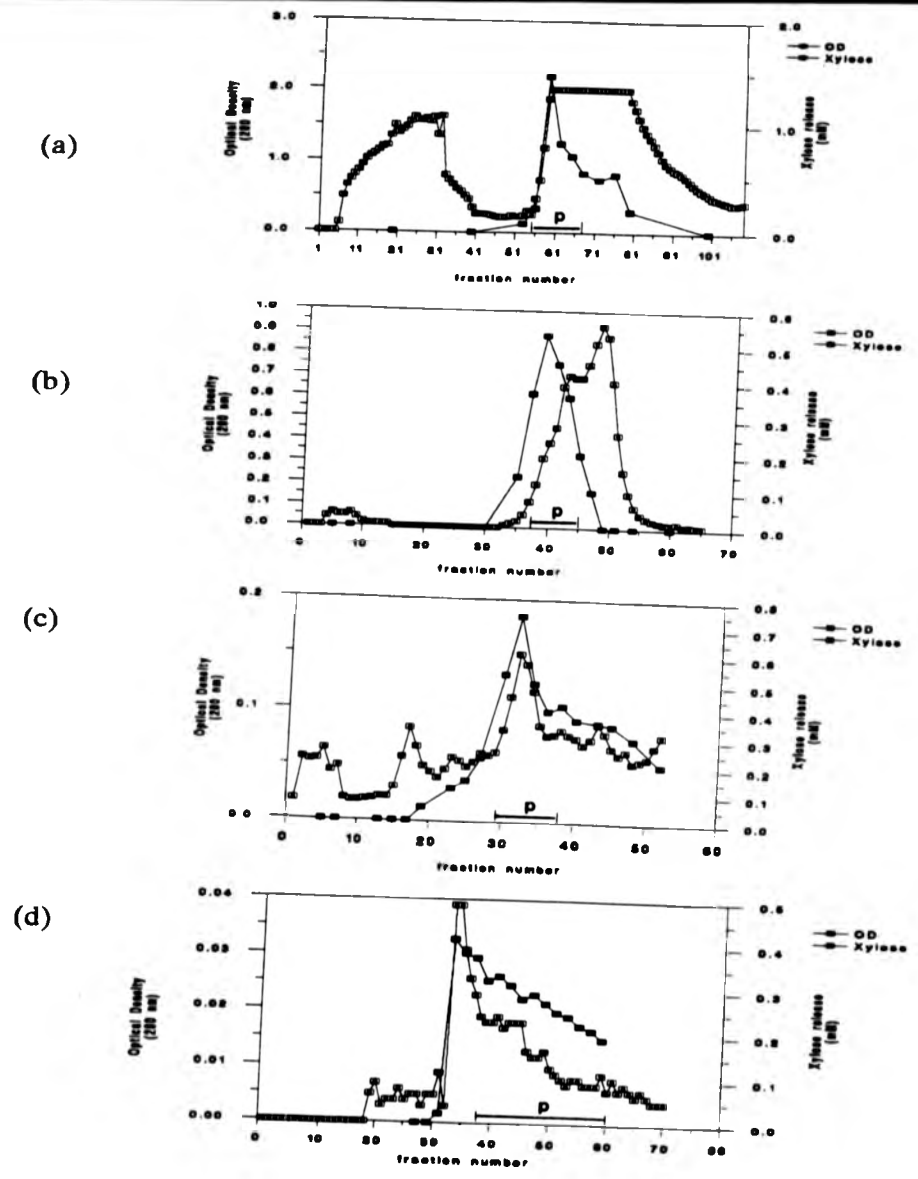


Fig.3.1. Purification of the α-xylosidase. (a): Anion-exchange chromatography on DEAE-cellulose of a crude enzyme extract from nasturtium cotyledons; (b): Cation-exchange chromatography on CM-cellulose; (c): Chromatofocusing; (d): Gel filtration using Bio-gel P60. Column fractions were analysed for the release of xylose from tamarind xyloglucan in the presence of the nasturtium *endo*-(1→4)-ß-D-glucanase. P = pooled fractions.

were stained, including one with same electrophoretic mobility as the purified α-xylosidase (Fig.3.2.). Although this technique was specific for carbohydrate it was not very sensitive and a more sensitive staining procedure was developed, involving enzyme-linked localization of α-D-xylosidase on electroblots. Crude extracts and purified enzyme preparations were transferred on to nitrocellulose membranes and incubated with Concanavalin-A, which has high affinity for α-mannose and α-glucose residues and binds to the mannose-rich determinants of glycoproteins. Western blots were then treated with horseradish peroxidase (which is a glycoprotein) and stained for peroxidase activity with the diaminobenzidine technique. This procedure was more efficient and more sensitive than the previous periodate-Schiff method and clearly demonstrated that α-D-xylosidase was a glycoprotein (Fig.3.3.).

Following this finding, a simple and efficient purification procedure based on lectin-affinity chromatography was developed for the purification to homogeneity of this enzyme. The α-xylosidase-containing fractions eluted from DEAE-cellulose column were pooled and re-chromatographed on a Concanavalin-A Sepharose 4B column. The bulk of enzyme was eluted as a symmetrical peak using 10 mM methyl-α-D-glucopyranoside and the remainder was eluted with higher concentrations of the glucoside (Fig.3.4.a). It was found that there was essentially no difference in the pattern of enzyme elution when methyl-α-D-glucopyranoside was replaced with methyl-α-D-mannopyranoside. A further affinity-chromatography step on the same Concanavalin-A Sepharose 4B support was carried out to concentrate the enzyme activity. In this case the enzyme was eluted in a narrow peak with a single step-gradient of 100 mM methyl-α-glucoside. Some

(a)

(b)

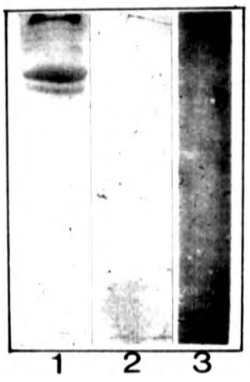
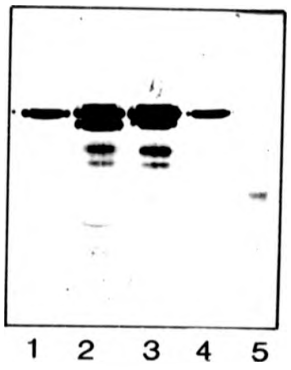


Fig.3.2. SDS-polyacrylamide gel electrophoresis of the purified α-xylosidase and partially purified enzyme extracts. (a): Gel stained with Coomassie Blue; (b): Gel stained to reveal glycoproteins with the periodate/Schiff procedure.

Lane 1: enzyme extract after DEAE-cellulose chromatography. *Lane 2*: purified α-xylosidase. *Lane 3*: Molecular weight markers, Myosin (Mr 205 KDa), ß-galactosidase (Mr 116 KDa), phosphorylase B (Mr 97.4 KDa), bovine serum albumin (a glycoprotein, Mr 66 KDa), ovalbumin (Mr 45 KDa), carbonic anhydrase (Mr 29 KDa).

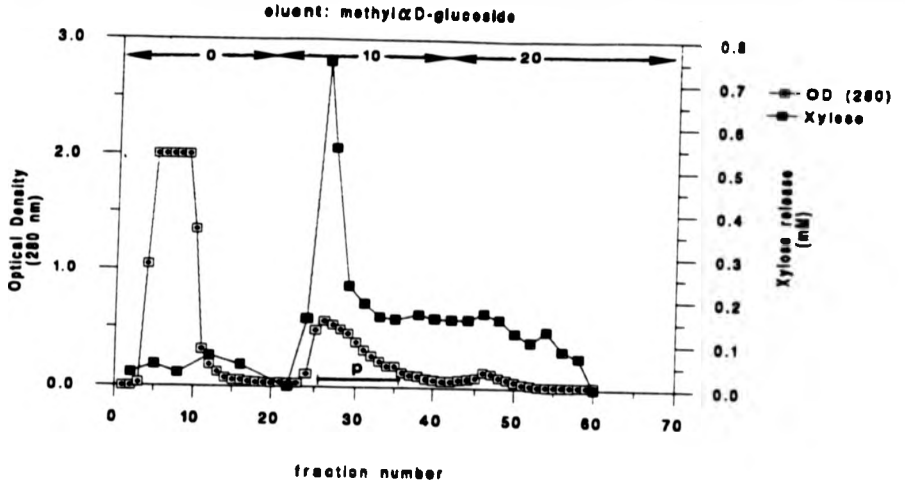
(a)



(b)

Fig.3.3. Western blot of the purified nasturtium α-xylosidase and the partially purified seed extracts (a) stained to reveal glycoproteins with Concanavalin A/horseradish peroxidase and (b) stained for proteins with Coomassie Blue. *Lanes 1,4*: purified α-xylosidase. *Lane 2*: seed extract after ammonium sulphate precipitation of the proteins. *Lane 3*: enzyme extract after anion-exchange chromatography. *Lane 5*: Molecular weight markers, Myosin (Mr 205 KDa), ß-galactosidase (Mr 116 KDa), phosphorylase B (Mr 97.4 KDa), bovine serum albumin (a glycoprotein, Mr 66 KDa), ovalbumin (Mr 45 KDa), carbonic anhydrase (Mr 29 KDa).

(a)



(b)

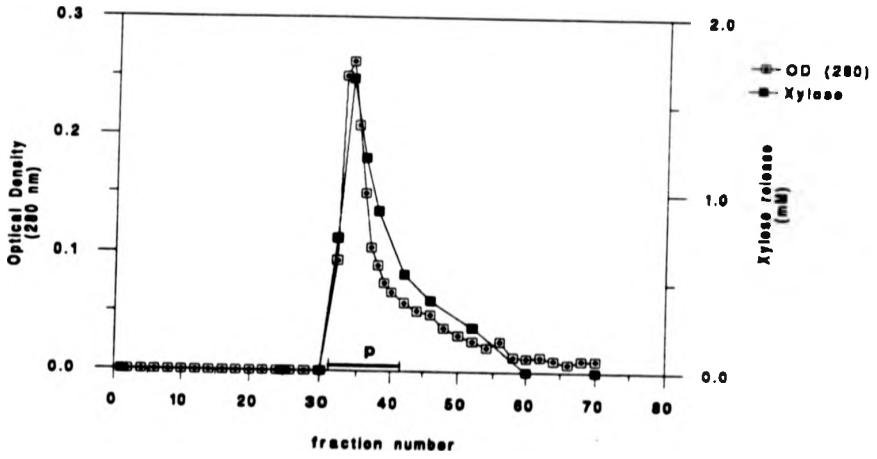


Fig.3.4. (a) Affinity chromatography on Concanavalin-A Sepharose 4B of the nasturtium seed enzyme extract after anion-exchange chromatography and (b) gel filtration on Bio Gel P300. The activity of α-xylosidase is expressed as millimolar xylose released from the incubation of the xyloglucan with the pure/crude enzyme plus an aliquot of the nasturtium xyloglucan-specific *endo*glucanase. P = pooled fractions.

purified enzyme preparations still contained a very small protein contamination of higher electrophoretic mobility (Mr 63000). It was probably a ß-galactosidase since such preparations contained low levels of ß-galactosidase activity. The contaminating protein and the ß-galactosidase activity were removed by gel-filtration on Bio Gel P-300. It was observed that during this gel filtration procedure the enzyme activity was concentrated in a major peak and the "tailing" effect mentioned earlier was reduced (Fig.3.4.b) It is possible that during the affinity purification step only those enzyme molecules with a similar degree of glycosylation were collected and that those molecules containing lower or higher carbohydrate substituents were rejected.

The purified α-D-xylosidase had an apparent molecular weight of 85 KDa on SDS-PAGE (Fig.3.3.b). The molecular weight of the native enzyme obtained by gel-filtration on Bio Gel P300 was 400 KDa, using a protein standard mixture which included apoferritin (Mr 443 KDa), ß-amylase (Mr 200 KDa), alcohol dehydrogenase (Mr 150 KDa), bovine serum albumin (Mr 66 KDa). This finding suggested that the α-xylosidase may be composed by oligomeric sub-units of Mr 100 KDa. Cross-linking studies using dimethylsuberimidate showed that this enzyme was a monomer as no higher protein bands were present in the enzyme-reagent mixture analysed by SDS-PAGE. The discrepancy between the relative molecular size of the native and the denatured enzyme was not further investigated.

It was not possible to construct a conventional purification table for the α-D-xylosidase enzyme, since its apparent activity was affected by the presence of other glycosidases and glycanases (notably *endo*-ß-D-glucanases). The degree of

purification achieved can be appreciated qualitatively by comparing crude and partially purified enzyme extracts with the purified α-D-xylosidase in Fig.3.3.b. No other enzyme activity was found to be present in the purified enzyme.

Isoelectric focusing of the purified α-D-xylosidase indicated that there were several protein bands spanning between 5.0 and 7.1 pI values. The enzyme activity, determined over the same pI range, showed that the maximum enzyme activity was associated with a protein component corresponding to pI = 6.1 (Fig.3.5.). The crude enzyme extract contained high levels of ß-galactosidase activity covering the pI range 6.0-7.5.

3.3.2. CARBOHYDRATE ANALYSIS OF THE GLYCOSYL SUBSTITUENTS OF THE PURE α-XYLOSIDASE.

The purified α-xylosidase was hydrolysed with trifluoroacetic acid and the monosaccharides released were analysed by HPLC. The components were identified and determined quantitatively by reference to appropriate mixtures of monosaccharide standards. The data obtained is reported in the Table 3.1..

The carbohydrate moiety corresponded to 16-20% (w/w) of the total glycoprotein. High levels of the monosaccharide glucose which accounted for ca 40% of the total carbohydrate were found to be present in the glycosyl moiety. There was no sialic acid.

Relatively low values of GlcNac or GalNac and mannose suggested that this

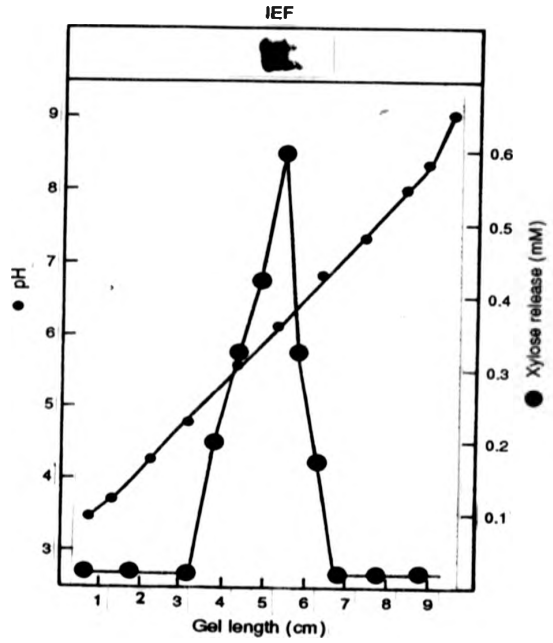


Fig.3.5. Isoelectric focusing (IEF) of the purified α-xylosidase enzyme.

Monosaccharide	Percentage
Fuc	3.6 ± 0.8
GalN	2.8 ± 0.5
Gal	12.3 ± 1.0
GlcN	11.3 ± 0.9
Glc	39.3 ± 3.7
Xyl	8.5 ± 0.5
Glc(Gal)Nac	22.3 ± 3.5

Table 3.1. Monosaccharide composition (± standard deviations) of the α-xylosidase glycosyl moiety.

glycoprotein could not be included in the "high mannose glycoprotein" group.

3.3.3. DEGLYCOSYLATION OF THE α-XYLOSIDASE GLYCOPROTEIN.

Given the unusual glycosylation pattern of the enzyme it seemed unlikely that the use of any commercial deglycosylating enzyme would succed. Furthermore preliminary attempts by Dr S. Chengappa to achieve enzymatic deglycosylation had been unsuccessful. Deglycosylation of the pure α-xylosidase was therefore carried out using anhydrous trifluoromethanesulphonic acid. This reagent is known to bring about the removal of both N- and O-linked glycosyl residues from the proteins, without degradation of the protein moiety (Sojar and Bahl, 1987). Glycosylated protein standards, including horseradish peroxidase and ovalbumin, were used as controls. The deglycosylation of α-xylosidase and the protein standards was monitored by SDS-PAGE. The apparent molecular weight of the α-xylosidase decreased from 85 KDa to 75 KDa after deglycosylation indicating that at least some glycosyl substituents had been removed (Fig.3.6.a). Transfer of SDS-polyacrylamide gels onto nitrocellulose followed by treatment with Concanavalin A and horseradish peroxidase showed that the de-glycosylation of ovalbumin and horseradish peroxidase was complete, whereas the α-xylosidase protein was still containing some glycosyl substituents (Fig.3.6.b).

The precipitation of the partially de-glycosylated α-xylosidase protein during the deglycosylation procedure suggests that the glycosyl substitution may contribute

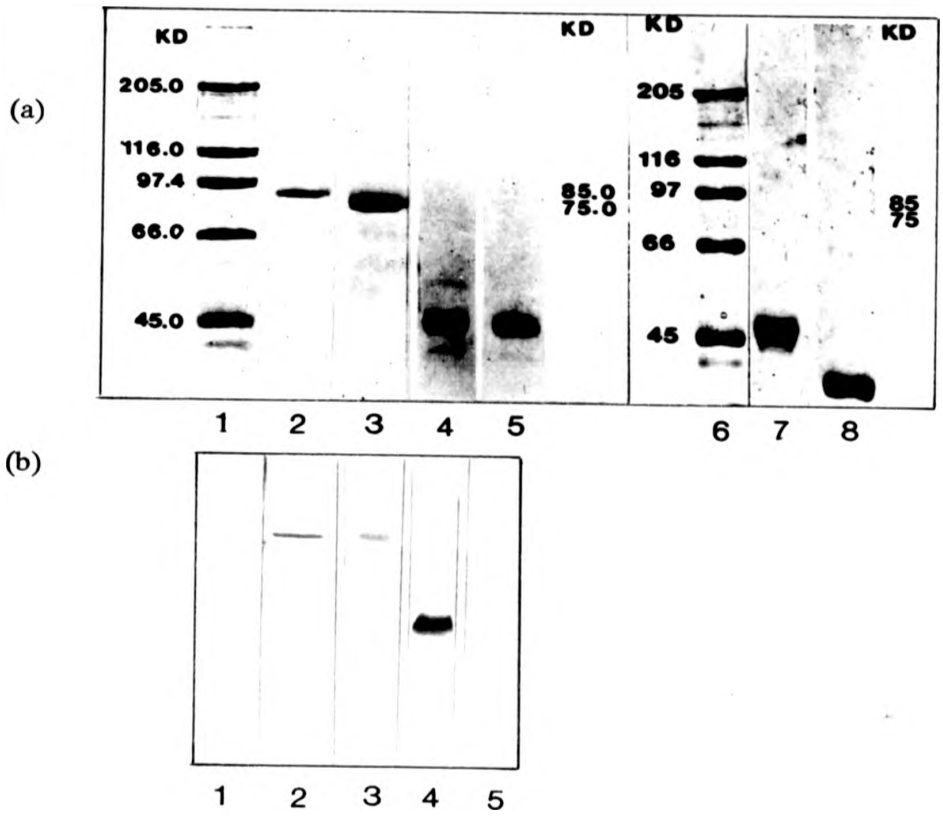


Fig.3.6. (a): SDS-polyacylamide gel electrophoresis of the intact and the de-glycosylated glycoproteins. (b): Western blotting of the intact and the de-glycosylated glycoproteins.

Lanes 2 and 3: Intact and de-glycosylated α-xylosidase, respectively; *Lanes 4 and 5*: Intact and de-glycosylated ovalbumin, respectively; *Lanes 7 and 8*: Intact and de-glycosylated horseradish peroxidase, respectively; *Lanes 1 and 6*: Molecular weight markers, Myosin (Mr 205 KDa), ß-galactosidase (Mr 116 KDa), phosphorylase B (Mr 97.4 KDa), bovine serum albumin (Mr 66 KDa), ovalbumin (Mr 45 KDa), carbonic anhydrase (Mr 29 KDa). Nitrocellulose membrane was stained for carbohydrates using the Concanavalin A/horseradish peroxidase procedure.

to its solubility. It has been observed (S. Chengappa, personal communication) that the protein core has a high percentage of hydrophobic residues. Furthermore the enzyme bound strongly to a hydrophobic interaction column (see section 3.3.1.). It is possible that the glycosyl substituents prevent self-aggregation by hydrophobic interaction.

3.3.4. SUBSTRATE SPECIFICITY OF THE ENZYME.

Early attempts to purify the enzyme showed clearly that it would release xylose from tamarind xyloglucan only in the presence of the xyloglucan-specific *endo*-glucanase. Thus tamarind xyloglucan was not clearly a substrate, whereas its *endo*-glucanase digestion products contained one or more substrates.

The products of digestion of tamarind xyloglucan with commercial *endo*-(1→4)-ß-D-glucanase preparations from *Trichoderma viride* or *Penicillium funiculosum* also contained substrate(s) for this enzyme. Isoprimeverose (α-D-xylopyranosyl-(1→6)-D-glucose) however was not hydrolysed by the α-D-xylosidase even on prolonged incubation with the enzyme.

This indicated that the specificity of this enzyme was not that of a general α-xylosidase, in that only certain (1→6)-α-D-xylosyl linkages were susceptible to its action. To identify and characterize the substrate(s) for the α-xylosidase, a solution of tamarind xyloglucan was digested with the nasturtium *endo*-(1→4)-glucanase. The reaction was allowed to continue until the reducing power of the

incubation mixture was constant. The enzyme digest of xyloglucan with the nasturtium *endo*glucanase contained a mixture of saccharides differing in molecular weight and contained no free glucose as indicated by analytical thin layer chromatography (Fig.3.7., see also Fig.4.4.). The saccharides of high molecular weight (lower TLC mobility) were removed by ethanol precipitation and retained separately. The ethanol-soluble fraction containing the lower molecular weight saccharides was subjected to fractionation on a Bio Gel P4 column. Individual saccharide-containing fractions from the P4 column were collected, concentrated by freeze-drying, dissolved to give a final concentration of 1% w/v carbohydrate and incubated with the purified α-D-xylosidase. The enzyme clearly removed xylose from all the fractions tested, including the ethanol-insoluble fraction. This indicated that all fractions contained substrate(s) for the α-D-xylosidase. The release of xylose was monitored by analytical TLC. Since the general sulphuric acid/ethanol staining procedure did not stain xylose efficiently, the upper part of the TLC plate was stained separately using *p*-anisidine phthalate, a reagent which is useful for the sensitive detection of pentoses. Saccharide-containing fractions digestion with the α-D-xylosidase are shown in Fig.3.8.. Although xylose was released in every case, it was observed that there was no significant change in the TLC mobilities of the saccharides after treatment with α-xylosidase. This suggested that the structural features of the intact and their corresponding xylose-depleted saccharides were probably very similar.

Since the depolymerized xyloglucan contained substrates for the α-D-xylosidase whereas the intact xyloglucan was not itself a substrate for this enzyme, the purification and structural characterization of the oligosaccharide substrates was

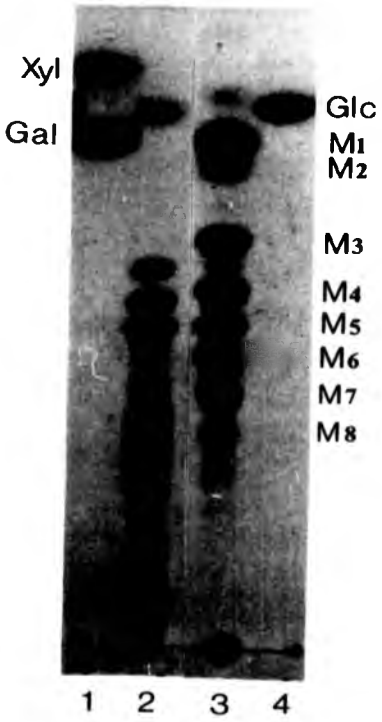


Fig.3.7. Thin layer chromatography of an enzyme digest of xyloglucan with the nsturtium *endo*-(1→4)-ß-D-glucanase. The polysaccharide preparation used as substrate had not been dialysed fully free of glucose. For a digest of a glucose-free sample, see Fig.4.4..

Lane 2: enzyme digest; *Lane 3*: manno-oligosaccharide standard (M_1 to M_8). *Lane 1*: monosaccharides D-xylose and D-galactose. *Lane 4*: monosaccharide D-glucose. The plate was stained with *p*-anisidine phthalate in methanol.

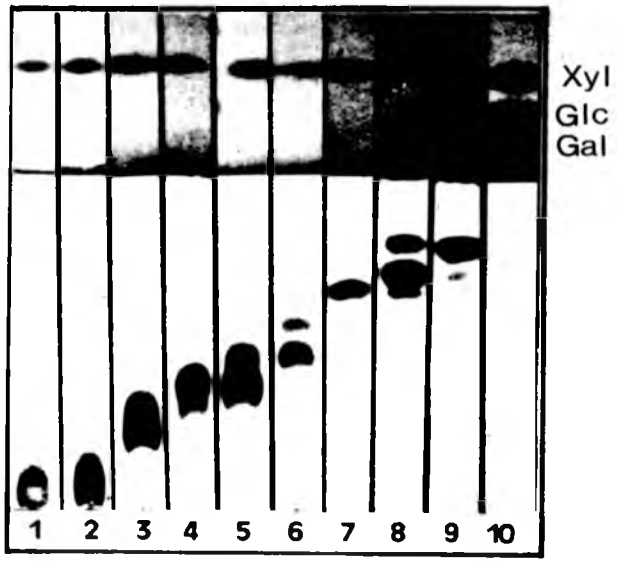


Fig.3.8. Thin layer chromatography of α-xylosidase digest of xyloglucan oligosaccharide fractions obtained by digestion of tamarind xyloglucan with the nasturtium *endo*-(1→4)-ß-D-glucanase.

Lanes 1 to 9: fractions individually treated with the nasturtium α-xylosidase. *Lane 10*: monosaccharide standards, D-xylose (uppermost spot), D-glucose and D-galactose. The plate was cut in two parts: the top part was stained with *p*-anisidine phthalate in methanol, for the sensitive detection of pentoses, and the lower part was stained with sulphuric acid in ethanol.

identified as an important prerequisite for the investigation on the molecular mode of action of α-D-xylosidase.

3.3.5. CHARACTERIZATION OF THE *ENDO*-(1→4)-ß-D-GLUCANASE DIGEST.

A 1% solution of tamarind xyloglucan was incubated with the xyloglucan-specific *endo*glucanase and the hydrolysis was monitored by reducing power, TLC and the viscometric assay. The hydrolysis of tamarind xyloglucan was considered complete when reducing power and viscosity remained constant, i.e. after 20 hours. The reaction was stopped and the enzyme heat-denatured in a boiling water bath. The higher saccharides were precipitated in 70% ethanol, and the undissolved material was removed by centrifugation. TLC analysis of the 70% ethanol-soluble fraction showed that the enzyme digest consisted of a mixture of three series of saccharides (discernible in Fig.4.4.), each containing several components of similar mobility on TLC. The upper group (higher TLC mobility) contained three components with an apparent DP<6, based on the standard manno-oligosaccharide mixture (Fig.3.7.). There was a second group of five saccharides with intermediate TLC mobility and a poorly resolved group of even lower TLC mobility (Fig.3.7.). The three saccharides in the group of higher TLC mobility were purified to apparent homogeneity by a combination of gel filtration on Bio Gel P4 and preparative TLC. The three purified saccharides A (highest TLC mobility), B and C (lowest TLC mobility) are shown in Fig.3.9..

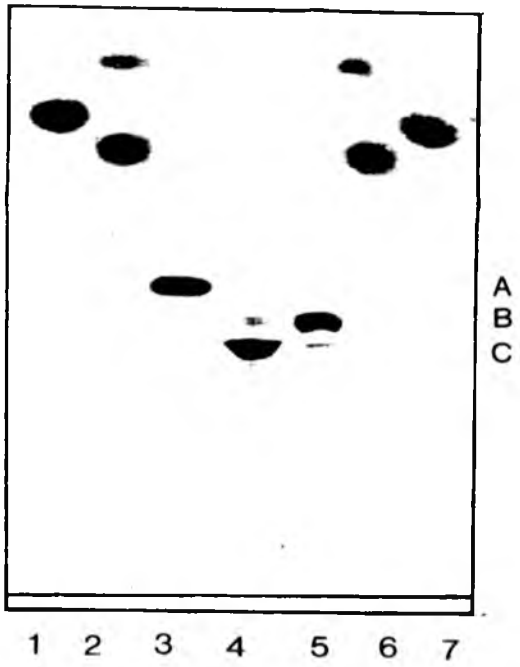


Fig.3.9. Thin layer chromatography of the purified low molecular weight xyloglucan oligosaccharides A, B and C.

Lane 3: oligosaccharide A; *Lane 4*: oligosaccharide C; *Lane 5*: oligosaccharide B; *Lanes 1,7* monosaccharide standard D-glucose; *Lanes 2,6*: monosaccharide standards D-xylose (uppermost spot) and D-galactose.

3.3.6. CHARACTERIZATION OF LOWER MOLECULAR WEIGHT OLIGOSACCHARIDES.

Oligosaccharides A , B and C were investigated using enzymes able to remove specific glycosyl residues and by means of proton NMR. The former was carried out entirely in Stirling, whereas the ^{1}H-NMR analysis was carried out by Dr M.J. Gidley at the Unilever Research Laboratory, Colworth House, Sharnbrook, Bedford, UK.

3.3.6.1. STRUCTURE OF OLIGOSACCHARIDE A.

This oligosaccharide was essentially homogeneous by TLC and gave a symmetrical peak on HPLC (Fig.3.10.a). Digestion of this oligosaccharide with the commercial enzyme Driselase (a fungal enzyme preparation containing various *endo-* and *exo*-enzymes able to hydrolyze several glycosyl linkages, but not the (1→6)-α-D-xylosyl linkage) produced only glucose and isoprimeverose (α-D-xylopyranosyl-(1→6)-D-glucose) (Fig.3.10.b). Since the monosaccharide galactose and the disaccharide isoprimeverose had identical TLC mobilities, their separation was achieved by running the plates twice in a solution containing n-propanol:ethanol:water (7:1:2). An aliquot of the Driselase-oligosaccharide mixture was analysed by TLC using this solution. This confirmed that the

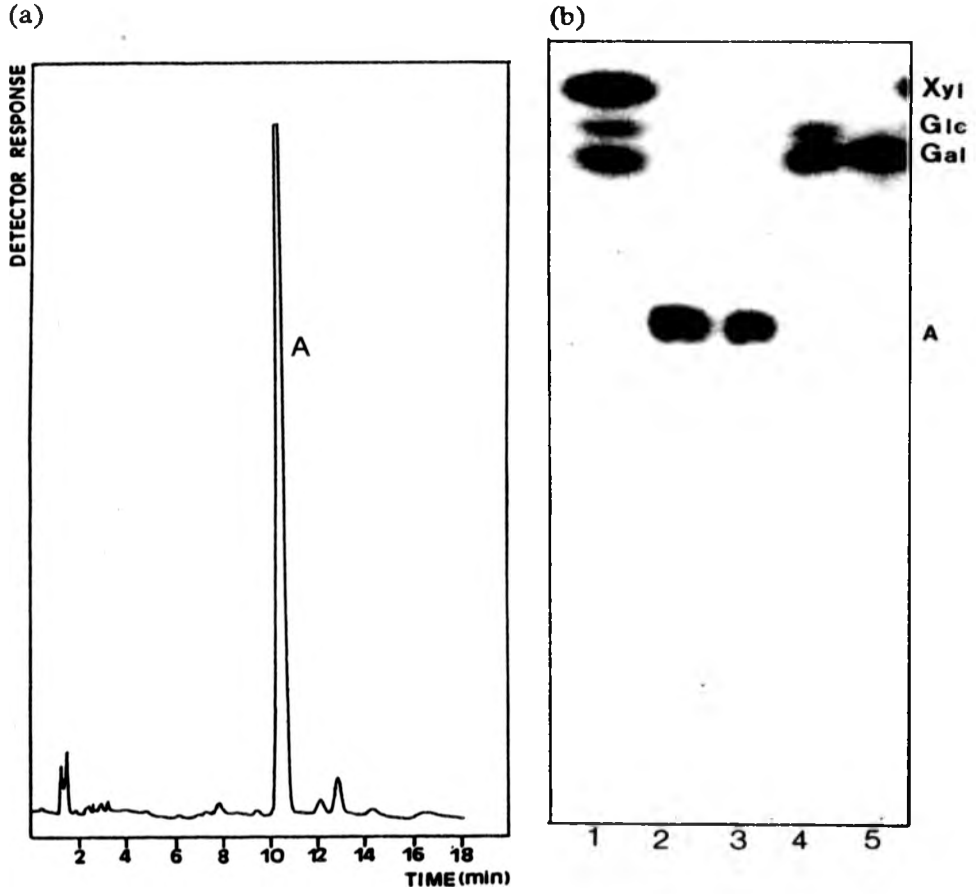
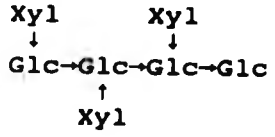


Fig.3.10. HPLC and TLC analysis of enzyme digests of oligosaccharide A. (a): HPLC of purified oligosaccharide A. (b): Thin layer chromatography of enzyme digests of oligosaccharide A treated with Driselase and with the nasturtium ß-galactosidase. *Lane 2*: purified oligosaccharide A; *Lane 3*: oligosaccharide A treated with ß-galactosidase; *Lane 4*: oligosaccharide A treated with Driselase. *Lane 1*: monosaccharide standard D-xylose (uppermost spot), D-glucose and D-galactose (lower spot); *Lane 5*: the disaccharide isoprimeverose.

products of the Driselase digestion of oligosaccharide B contained both saccharides, galactose and isoprimeverose (result not shown).The separation of the monosaccharide galactose and the disaccharide isoprimeverose on TLC plates (result not shown). No galactose was produced when this oligosaccharide was treated with the purified ß-galactosidase (Edwards *et al.*, 1988)from nasturtium. Anomeric proton NMR gave the composition glc_4 xyl_3. The corresponding alditol of oligosaccharide A was prepared by borohydride reduction, and the NMR spectrum of the alditol was compared with that of the original saccharide. It was observed initially that the chemical shift associated with one of the three xylosyl residues changed significantly on borohydride reduction, indicating that it might be attached to the glucose residue at the reducing end of the saccharide. However, it was shown later that this was an artefact caused by the complexation of residual borate from the reduction reaction to the glucitol residue at the former reducing terminus. When the last traces of borate were removed from the alditol, it was found that the proton resonances associated with the xylosyl residue were almost identical in the alditol and in the parent oligosaccharide (Table 3.2.). Furthermore the spectrum was quantitatively and qualitatively identical with the published spectrum of one of the three oligosaccharides released from tamarind xyloglucan by the action of a fungal *endo*-(1→4)-ß-D-glucanase (York *et al.*, 1990). This oligosaccharide (XG7, 2R) had been shown to have the structure depicted below. Oligosaccharide A was clearly identical.



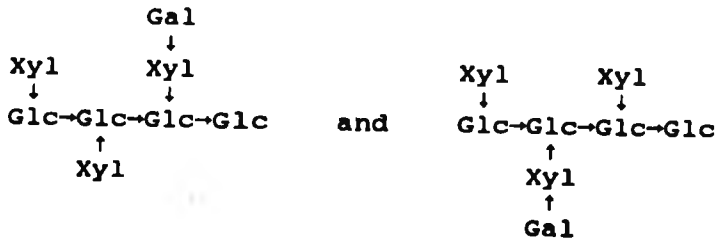
Assignment	Oligosaccharide A	Oligosaccharide A reduced
"Terminal" 1-linked α-xylose	4.945 (1 H)	4.945 (1 H)
"Internal" 1-linked α-xylose	4.961 (2 H)	4.961 (2 H)
Reducing 4-linked α-glucose	5.245 (1 H)	absent
Reducing 4-linked ß-glucose	4.677	absent
Non-reducing 1-linked ß-glucose	4.561 (1 H)	4.559 (1 H)
1,4-linked ß-glucose	4.586 (2 H)	4.598 (1 H)
		4.655 (1 H)

Table 3.2. Anomeric signals of oligosaccharide A and its alditol by ^{1}H-NMR spectra.

Chemical shifts downfield from standard ppm. relative integrals shown in parenthesis H-1, H-2 coupling constant for α and ß residues were all in the ranges 3.4-3.8 Hz, respectively.

3.3.6.2. STRUCTURE OF OLIGOSACCHARIDE B.

This oligosaccharide was essentially homogeneous by TLC, but was resolved into two peaks on HPLC, indicating that this oligosaccharide was a mixture of structural isomers (Fig.3.11.a). Upon treatment with Driselase the oligosaccharide gave galactose, glucose and isoprimeverose (Fig.3.11.b). Since galactose and the disaccharide isoprimeverose had the same TLC mobilities, an aliquot of the Driselase-oligosaccharide mixture was also analysed by TLC using the irrigant described in section 3.3.6.1.. This confirmed that the products of the Driselase digestion of oligosaccharide B contained both saccharides (result not shown). When the oligosaccharide was incubated with the purified nasturtium ß-galactosidase, galactose was released and the galactose-depleted oligosaccharide co-migrated with oligosaccharide A on TLC (Fig.3.11.b). ^{1}H-NMR analysis gave the composition glc_4 xyl_3 gal. Proton NMR spctra of the two isomers B_1 and B_2 were identical with the published spectra of the oligosaccharides 3R and 4R, produced by digestion of tamarind xyloglucan with the fungal *endo*-(1→4)-ß-D-glucanase (York *et al.*, 1990). These oligosaccharides (3R and 4R) were shown to have the following structures:



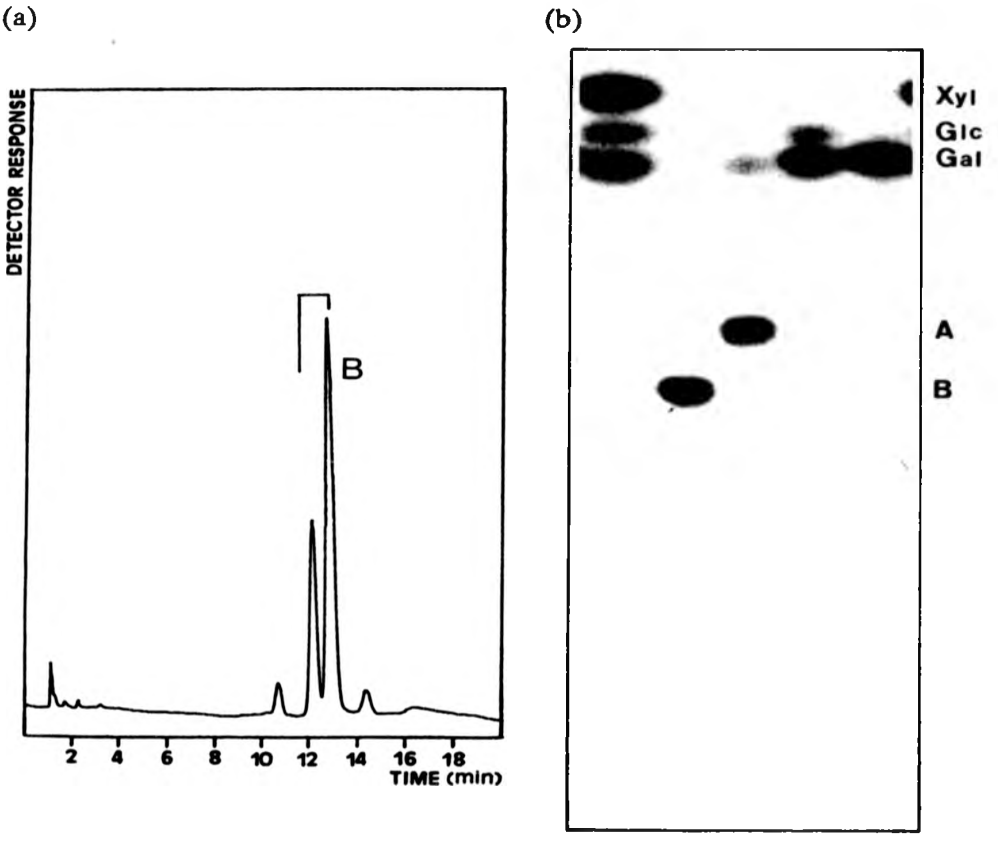
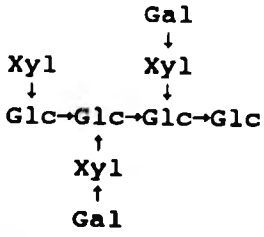


Fig.3.11. HPLC and TLC analysis of enzyme digests of oligosaccharide B. (a): HPLC of purified oligosaccharide B. (b): Thin layer chromatography of enzyme digests of oligosaccharide B treated with the nasturtium ß-galactosidase and with Driselase. *Lane 2*: purified oligosaccharide B; *Lane 3*: oligosaccharide B treated with ß-galactosidase; *Lane 4*: oligosaccharide B treated with Driselase. *Lane 1*: monosaccharide standard D-xylose (uppermost spot), D-glucose and D-galactose (lower spot); *Lane 5*: the disaccharide isoprimeverose.

3.3.6.3. STRUCTURE OF OLIGOSACCHARIDE C.

This oligosaccharide was homogeneous by TLC and gave a peak on HPLC (Fig.3.12.a). Digestion of this oligosaccharide with Driselase generated glucose, galactose and isoprimeverose (Fig.3.12.b). Since galactose and the disaccharide isoprimeverose co-migrated on TLC, an aliquot of the Driselase-oligosaccharide mixture was also analysed by running the plate in the solvent described in section 3.3.6.1.. This confirmed that the products of the Driselase digestion of oligosaccharide C contained both saccharides, galactose and isoprimeverose (result not shown). On incubation this oligosaccharide with the nasturtium ß-galactosidase galactose was produced and the mobility on TLC of the galactose-depleted oligosaccharide corresponded to that of octasaccharide A (Fig.3.12.b). ^{1}H-NMR analysis gave the composition glc_4 xyl_3 gal_2. Again the ^{1}H-NMR spectrum of this oligosaccharide and its corresponding alditol were identical with those of one of the oligosaccharides (XG9, 5R) released from tamarind xyloglucan by a fungal *endo*-glucanase (York *et al.*, 1990). XG9 (5R) and oligosaccharide C were identical and C was attributed the structure:



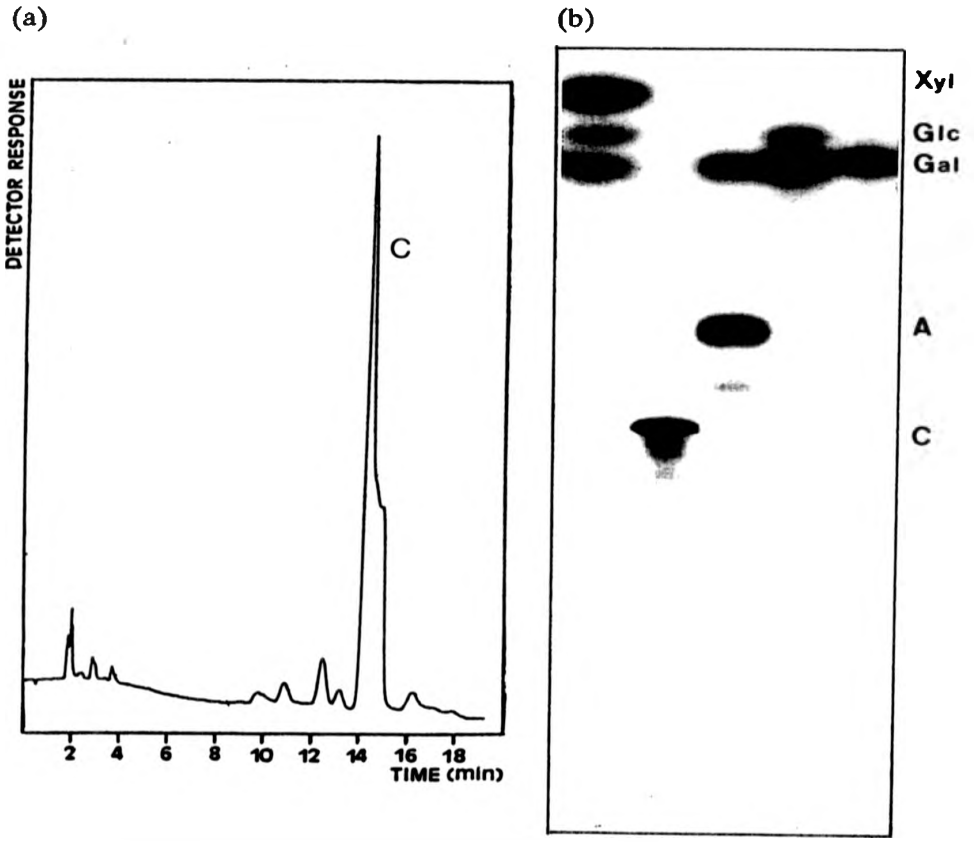


Fig.3.12. HPLC and TLC analysis of enzyme digests of oligosaccharide C. (a): HPLC of purified oligosaccharide C. (b): Thin layer chromatography of enzyme digests of oligosaccharide C treated with Driselase and with the nasturtium ß-galactosidase. *Lane 2*: purified oligosaccharide C; *Lane 3*: oligosaccharide C treated with ß-galactosidase; *Lane 4*: oligosaccharide C treated with Driselase. *Lane 1*: monosaccharide standard D-xylose (uppermost spot), D-glucose and D-galactose (lower spot); *Lane 5*: the disaccharide isoprimeverose.

3.3.7. ^{1}H-NMR SPECTRA OF OLIGOSACCHARIDES A, B AND C.

The proton NMR spectra of oligosaccharides A, B and C are shown in Fig.3.13., with the assignment of resonance to the anomeric protons of reducing glucose residues (RG), to galactose-substituted xylose residues (GalX), to unsubstituted xylose residues (X), and to galactose plus glucose residues (Gal+G). The resolution was sufficient to allow the calculation of the number of sugar residues in these molecules (all residues/RG), and the sugar residue composition. The number of xylose residues was equal to GalX+X/RG. The number of galactose residues (only, attached to xylose in xyloglucan) was GalX/RG. The number of glucose residues was obtained by difference. The interpretation of the spectra is explained in Table 3.3..

It should be noted that the spectra of oligosaccharides C has a single doublet in the unsubstituted xylose region. This resonance must originate from a single unsubstituted xylose attached to the non-reducing terminal glucose. It is therefore referred to as a "terminal" xylose resonance. The unsubstituted xylose region of oligosaccharides B and A are more complex, being composed of two overlapping doublets, one of them corresponding closely to the terminal xylose doublet of oligosaccharide C. The second doublet is more intense in oligosaccharide A than in oligosaccharide B, and it is due to the presence of non-terminal or "internal" non-susbstituted xylose.

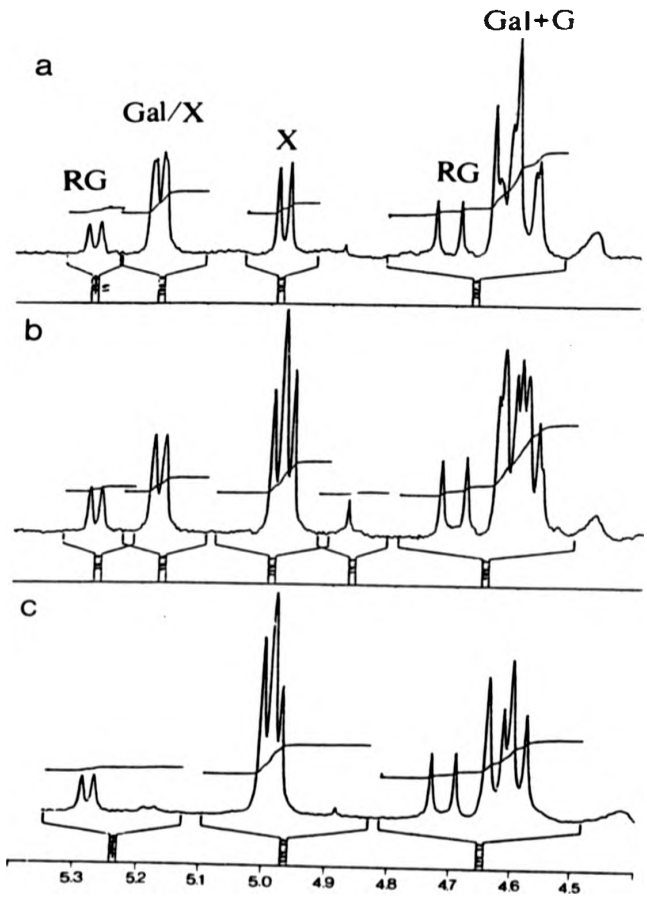


Fig.3.13. The anomeric region of the ^{1}H-NMR spectra of oligosaccharides A, B and C. (a): C; (b): B; (c): A.

RG = reducing glucose residues; GalX = galactosyl-substituted xylose residues; X = unsubstituted xylose residues; Gal + G = galactose + glucose residues.

Signal[a]	Assignment	Relative integral		
		A	B	C
5.143 (3.5)	1,2-linked α-xylose	0	1	2
4.961 (3.5)	"Internal" 1-linked α-xylose	2	1	0
4.994 (3.6)	"Terminal" 1-linked α-xylose	1	1	1
5.245 (3.8)	Reducing 4-linked α-glucose			
4.676 (7.8)	Reducing 4-linked ß-glucose	1	1	1
4.54-4.60 (7.7-7.9)	1,4-linked ß-glucose			
4.53-4.60[b]	1-linked ß-galactose	3	4	5

Table 3.3. Analysis by ^{1}H-NMR of anomeric protons in saccharides A, B and C.

a. Chemical shifts in ppm downfield from standard, with H-1, H-2 coupling constants (Hz) in parenthesis.

b. Unresolved coupling.

3.3.8. MOLECULAR MODE OF ACTION OF α-D-XYLOSIDASE.

The observation that intact tamarind xyloglucan is not a substrate for α-D-xylosidase whereas the depolymerized xyloglucan contains substrate(s) indicated that some of the xylose residues within the xyloglucan molecule were made accessible to the α-xylosidase by the cleavage of the polysaccharide by the *endo*-(1→4)-ß-D-glucanase. A ^{1}H-NMR study was carried out to compare the spectra of the intact and the *endo*-glucanase digested tamarind xyloglucan molecule. The anomeric region of the native and the hydrolysed xyloglucan is shown in Fig.3.14. Apart from the appearance of reducing glucose resonances in the digested tamarind polysaccharide the only difference between the two ^{1}H-NMR spectra is in the area region between 5.0 and 4.9 ppm, comprising the resonances associated with the terminal unsubstituted side-chain xylose residue. The spectrum of the depolymerized xyloglucan contains a peak in this region which is not present in the spectrum of the native polysaccharide. Since the action of *endo*-glucanase is to create new chain-ends it seemed plausible to assume that the new peak was due to unsubstituted residues at the newly created chain-ends. This observation suggested that those newly created chain-ends may also represent the recognition and cleavage site of the α-xylosidase, thus explaining the inability of the enzyme to catalyze the removal of xylose from native xyloglucan.

The determination of the mode of action of the enzyme on hydrolysed xyloglucan was performed on the lower molecular weight saccharides produced by the nasturtium *endo*-(1→4)-ß-D-glucanase digestion of tamarind xyloglucan, namely

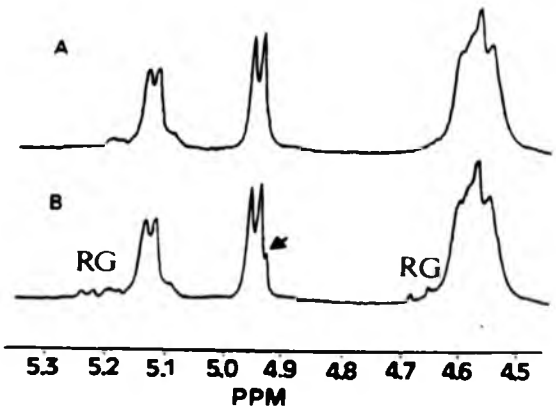


Fig.3.14. The anomeric region of the ^{1}H-NMR spectra of the native tamarind xyloglucan (A) and the tamarind xyloglucan treated with the nasturtium *endo*-(1→4)-ß-D-glucanase (B). RG = resonances associated with reducing glucose residues. The arrow indicates the new peak in the region of the spectrum associated with unsubstituted xylose residues.

oligosaccharides A, B and C.

Oligosaccharide C has only one potentially available cleavage site for the enzyme α-D-xylosidase since it contains only one unsubstituted terminal xylosyl residue. The other two xylosyl residues are substituted at position 2 with D-galactose. Oligosaccharide A, on the other hand, has three terminal xylosyl residues, all potential sites of cleavage for the enzyme. Oligosaccharides A and C were therefore chosen for the determination of the mode of action of the α-xylosidase.

3.3.8.1. ACTION OF THE ENZYME ON OLIGOSACCHARIDE C.

A solution (0.64 mM) of oligosaccharide C was digested with the α-D-xylosidase and the progression of the enzyme reaction was followed by TLC (Fig.3.15.a), by HPLC (Fig.3.15.b) and the by the quantitative determination of the pentose released (Fig.3.15.c).The quantitative pentose assay indicated that a single xylose residue was removed by the action of the enzyme (Fig.3.15.c). HPLC analysis showed that the appearance of xylose was accompanied by the disappearance of oligosaccharide C and the production of a new compound with a retention time close to that of an octasaccharide. The enzyme α-D-xylosidase clearly catalyses the removal of a single non-substituted xylose from oligosaccharide C to give an octasaccharide with the composition $glc_4xyl_2gal_2$.

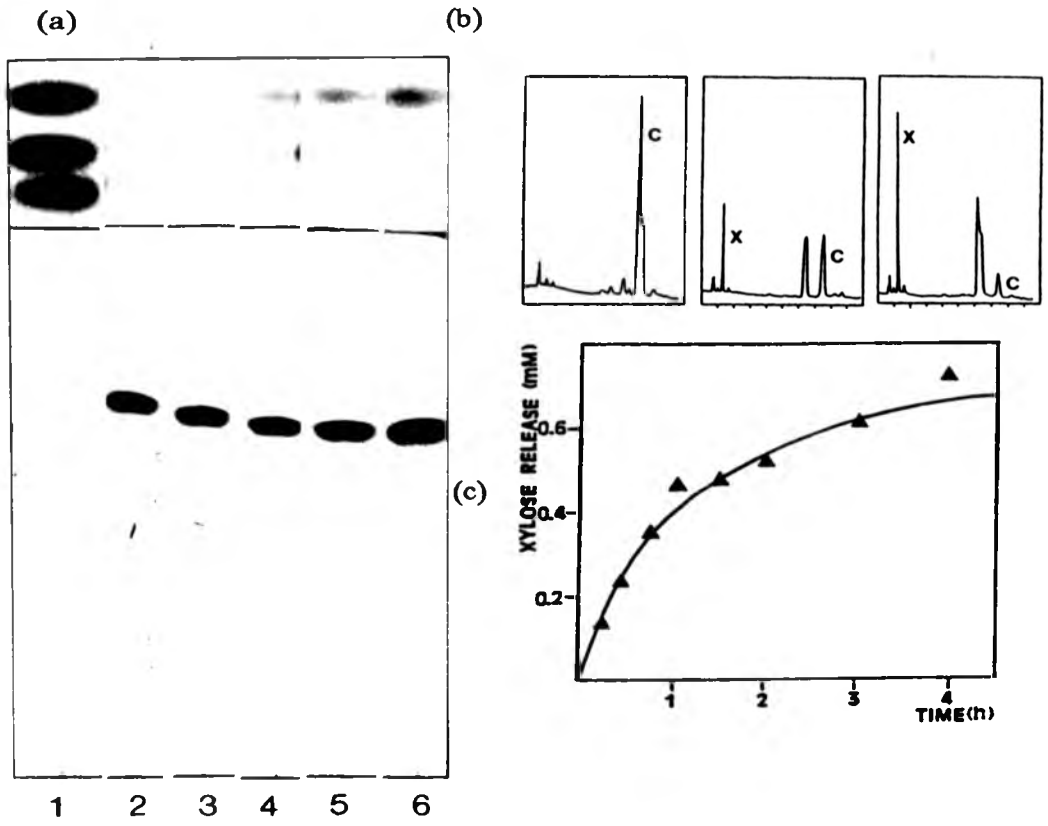


Fig.3.15. α-xylosidase digestion of oligosaccharide C.

(a): Thin layer chromatography of oligosaccharide C incubated with α-xylosidase. *Lanes 2 to 6*: incubation mixture after 0, 0.5, 1, 2, 20 h. *Lanes 1,7*: monosaccharide standards D-xylose (uppermost spot), D-glucose and D-galactose (lower spot). The upper part of the plate is stained with *p*-anisidine phthalate and the lower part with H_2SO_4/ethanol. (b): HPLC analysis of the digest after 30 s (1), 30 min (2), 120 min (3), C: oligosaccharide C; X: xylose. (c): Incubation of oligosaccharide C (0.64 mM) with α-xylosidase (165 μg/ml) and xylose determination by the pentose assay.

3.3.8.2. ACTION OF THE ENZYME ON OLIGOSACCHARIDE A.

Oligosaccharide A has three terminal non-reducing α-D-xylopyranosyl residues. The progress curve of the digestion of oligosaccharide A at a concentration of 0.625 mM with α-D-xylosidase was plotted by estimating quantitatively the D-xylose released (Fig.3.16.c). Thin layer chromatography (Fig.3.16.a) showed that there was no change in saccharide mobility before and after treatment with the enzyme α-xylosidase. HPLC Dionex analysis (Fig.3.16.b) of the saccharide before and after α-D-xylosidase incubation clearly indicated that a single xylose was cleaved from the heptasaccharide even upon prolonged incubation of the enzyme with the oligosaccharide. HPLC analysis of the enzyme-saccharide mixture showed the progressive appearance of a new compound with a retention time lower than the one corresponding for oligosaccharide A. It was deduced to be a hexasaccharide glc_4xyl_2. Since all three monoxylosyl residues in oligosaccharide A are potential sites of cleavage by the enzyme, structural analyses were performed on the hexasaccharide and its corresponding alditol by ^{1}H-NMR spectroscopy. A solution containing the oligosaccharide A was extensively digested with the enzyme α-D-xylosidase and the hexasaccharide produced was purified by gel filtration on Bio Gel P4. The ^{1}H-NMR spectra obtained for the hexasaccharide and its corresponding alditol were compared against spectra obtained for oligosaccharides A, B and C and their alditols. A comparison of A, A_{itol} and the xylose-depleted oligosaccharide A are given in Fig.3.17.. Clearly the xylose-depleted alditol has lost its "terminal" unsubstituted xylose residue.

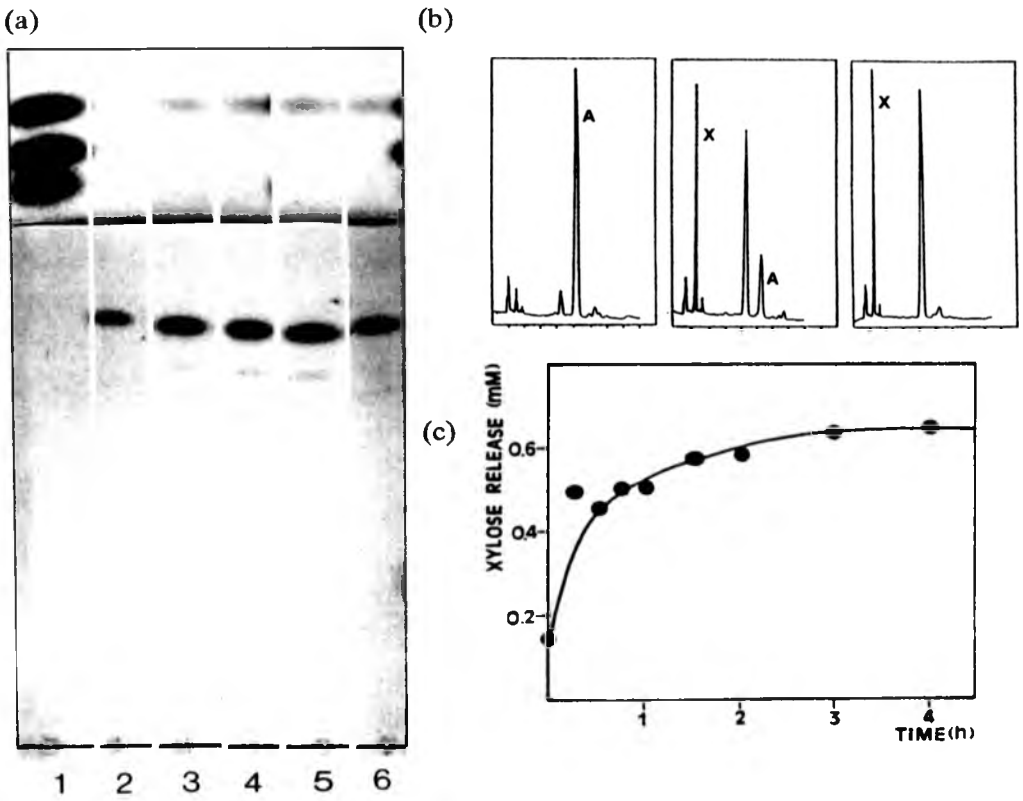


Fig.3.16. α-xylosidase digestion of oligosaccharide A.

(a): Thin layer chromatography of oligosaccharide A incubated with α-xylosidase. *Lanes 2 to 6*: incubation mixture after 0, 0.5, 1, 2, 20 h. *Lanes 1,7*: monosaccharide standards D-xylose (uppermost spot), D-glucose and D-galactose (lower spot). The upper part of the plate is stained with *p*-anisidine phthalate and the lower part with H_2SO_4/ethanol. (b): HPLC analysis of the digest after 30 s (1), 30 min (2), 120 min (3), A: oligosaccharide A; X: xylose. (c): Incubation of oligosaccharide A (0.625 mM) with α-xylosidase (165 μg/ml) and xylose determination by the pentose assay.

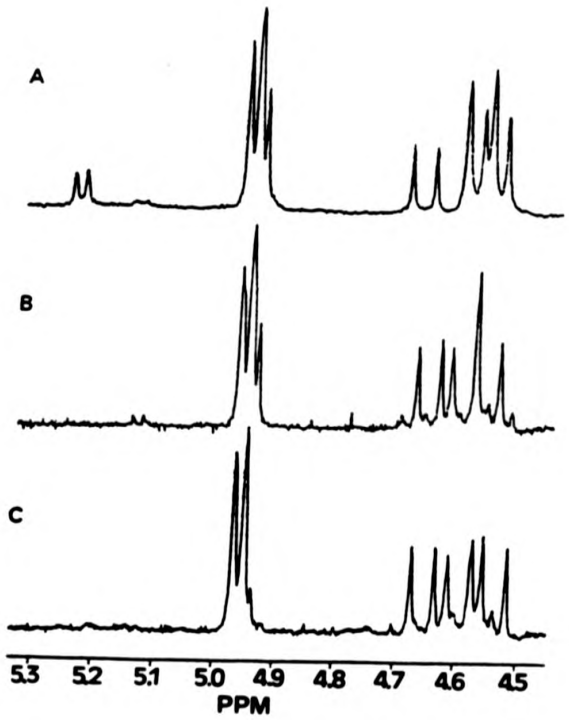
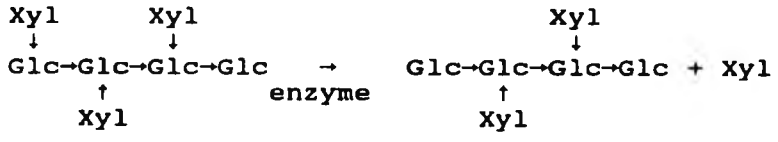


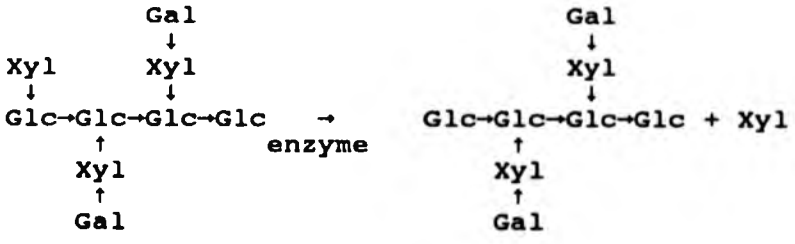
Fig.3.17. (A): The anomeric region of the ^{1}H-NMR spectra of oligosaccharide A; (B): oligosaccharide A reduced by sodium borohydride; (C): xylose-depleted oligosaccharide A reduced by sodium borohydride.

The ^{1}H-NMR spectra of the hexasaccharide and its reduced form were also compared with that of cellotetraose (Table 3.4.). The ^{1}H-NMR spectra showed that identical chemical shifts arose from the reducing and the terminal non-reducing glucose residue in both oligosaccharides, whereas the chemical shifts associated with the internal glucoses were significantly different. It was clear that the enzyme selectively cleaved the monoxylosyl residue attached to the glucose residue remote from the non reducing end of the molecule. The action of the enzyme α-xylosidase with xyloglucan oligosaccharides A and C is summarized below.

Xylose-depletion of oligosaccharide A:



Xylose-depletion of oligosaccharide C:



Assignment	Xylose-depleted oligosaccharide A	Xylose-depleted oligosaccharide A reduced	Cellotetraose
"Terminal" 1-linked α-xylose	Absent	Absent	Absent
"Internal" 1-linked α-xylose	4.961 (2 H)	4.961 (2 H)	Absent
Reducing 4-linked α-glucose	5.246 (1 H)	Absent	5.246 (1 H)
Reducing 4-linked ß-glucose	4.677	Absent	4.676
Non-reducing 1-linked ß-glucose	4.537 (1 H)	4.537 (1 H)	4.536 (1 H)
1,4-linked ß-glucose	4.580 (2 H)	4.594 (1 H)	4.564 (2 H)
		4.655 (1 H)	

Table 3.4. Anomeric signals of oligosaccharide A, reduced oligosaccharide A and cellotetraose by ^{1}H-NMR.

Chemical shifts downfield from standard ppm. relative integrals shown in parenthesis H-1, H-2 coupling constants for α and ß residues were all in the ranges 3.4-3.8 Hz, respectively.

3.3.8.3. MINIMUM SUBSTRATE RECOGNITION REQUIREMENT.

The oligosaccharides A, B and C, which were substrates for the α-D-xylosidase, all had in common a single unsubstituted xylosyl residue linked α-(1→6) to the glucose residue furthest from the reducing end of the saccharide molecule. It was however known that the disaccharide isoprimeverose was not hydrolysed by the enzyme. To investigate the minimum size of a saccharide having substrate properties, a number of xyloglucan oligosaccharides were produced by partial digestion of xyloglucan with driselase and cellulase. These oligosaccharides were prepared by Dr E. Lorences at Edinburgh University, and their purification was monitored by HPLC. The experiment was initially designed to identify the molecular structure of the two oligosaccharides. The retention times on HPLC of these saccharides indicated that they were a pentasaccharide with composition Glc_3Xyl_2 and a trisaccharide with composition Glc_2Xyl. They were incubated with the nasturtium α-xylosidase to establish whether or not these saccharides had a xylose residue attached to the terminal non-reducing glucose residue. The enzyme reactions were monitored by HPLC. Both oligosaccharides were hydrolyzed by the α-xylosidase, confirming that the structure of the oligosaccharides were as following:

Glc_3Xyl_2 Glc_2Xyl

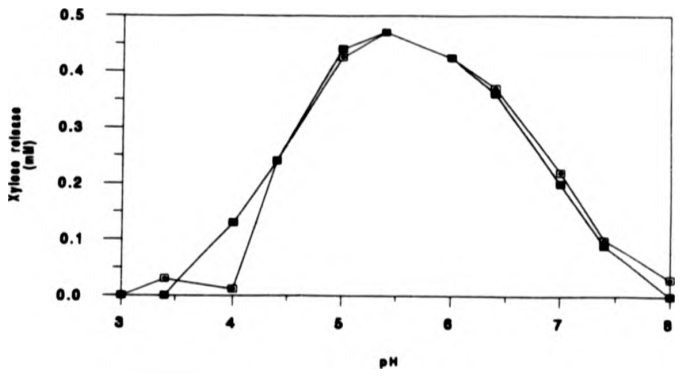
The pentasaccharide was converted to a tetrasaccharide Glc_3Xyl and xylose as shown by the decrease in retention time of the xylose-depleted oligosaccharide on HPLC. The trisaccharide Glc_2Xyl was converted to cellobiose and xylose as shown by HPLC analysis. This experiment showed additionally that the trisaccharide Glc_2Xyl is the smallest oligosaccharide to have substrate properties for the enzyme α-xylosidase.

3.3.9. SOME CATALYTIC PROPERTIES OF THE α-D-XYLOSIDASE.

3.3.9.1. pH OPTIMUM.

The oligosaccharide C was used to determine the pH optimum for the enzyme α-D-xylosidase. The buffer system used was the phosphate-citrate (McIlvaine) buffer ranging from pH 2.6 to pH 7.6. Fig.3.18.a shows that (i) the enzyme was inactive below pH 3.2 and above pH 7.4; (ii) that pH 4.0 and 6.5 corresponded to half-maximal enzyme activity; (iii) that the pH optimum using the substrate C was pH 5.0.

(a)



(b)

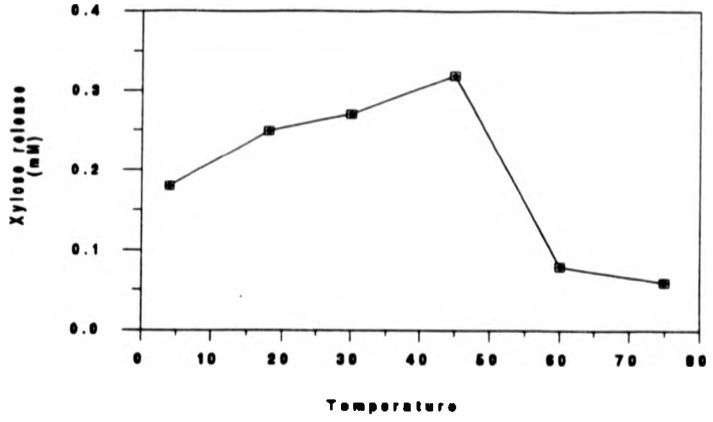


Fig.3.18. pH and temperature optima.

(a): The pH optimum was determined using oligosaccharide C and McIlvaine phosphate-citrate buffers spanning the range pH 2.6 to pH 7.6. (The two curves represent two different experiments).

(b): The temperature optimum was determined using oligosaccharide C and ammonium acetate buffer pH 5.0.

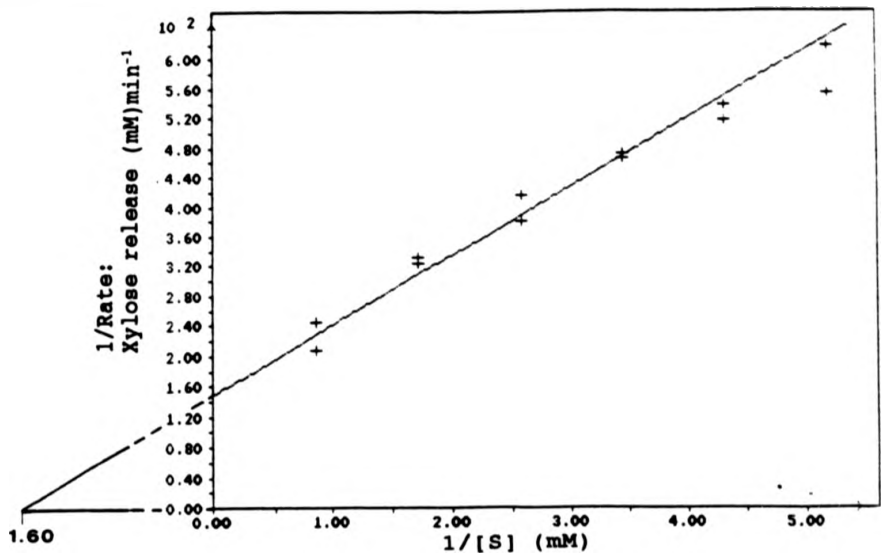
3.3.9.2. TEMPERATURE OPTIMUM.

A solution of oligosaccharide C dissolved in the 50 mM ammonium acetate buffer pH 5.0 was used to determine the temperature optimum. The enzyme-oligosaccharide mixture was incubated 100 min at 4°, 25°, 30°, 45°,60° and 75°C. Fig.3.10.b shows the results of this experiment: the temperature optimum, corresponding to the highest enzyme activity, was found to be 45°C. Temperature values above the temperature optimum were found to inactivate the enzyme.

3.3.9.3. ENZYME KINETICS.

The Km and Vmax values were determined using both oligosaccharides A and C. Lineweaver Burk (1/v vs 1/[S]) plots are shown in Fig.3.19.a and b. The results showed that: (i) when oligosaccharide A was used as substrate the K_m calculated was 0.62 mM and the V_{max} was 14 nkat mg protein^{-1}; (ii) when oligosaccharide C was used as substrate K_m was 0.32 mM and the V_{max} was 7.6 nkat mg protein^{-1}.

(a)



(b)

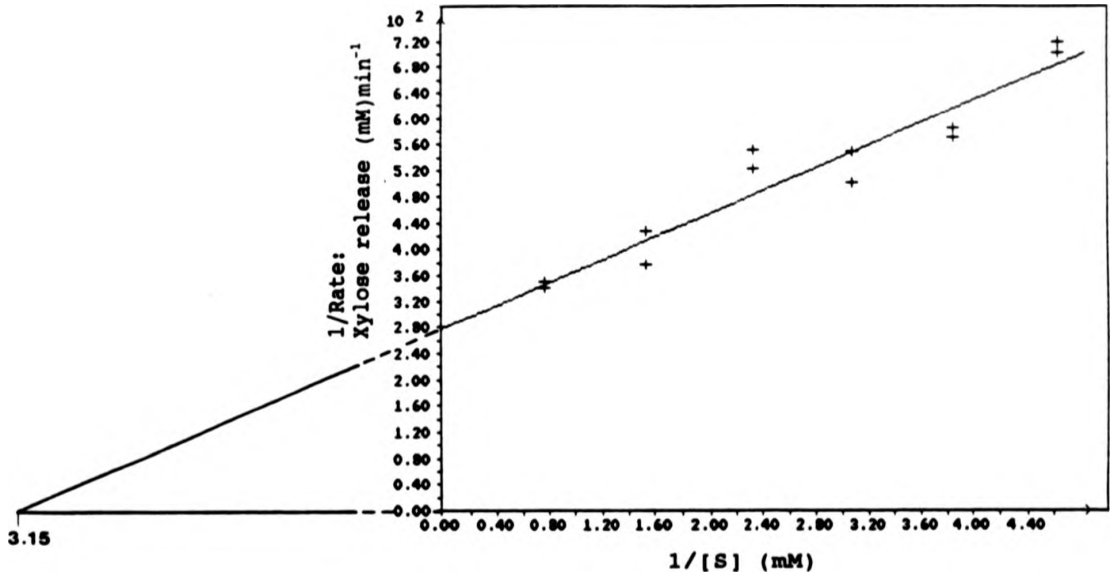


Fig.3.19. Lineweaver-Burk plots (1/V versus 1/[S]).

(a): oligosaccharide A as substrate.

(b): oligosaccharide C as substrate.

Kinetic parameters (K_m and V_{max}) were obtained by statistical analysis of initial velocity/substrate concentration using the computer programme of Cleland (1979).

3.4. DISCUSSION.

3.4.1. MOLECULAR PROPERTIES OF THE NASTURTIUM α-XYLOSIDASE.

The enzyme α-xylosidase was purified to homogeneity from germinated nasturtium cotyledons. The protein is a monomer with an apparent Mr of 85 KDa on SDS-PAGE. Isoelectric focusing of the purified enzyme shows that it contains closely related molecular species covering the pI range 5.0 to 7.1, with a peak of enzyme activity corresponding to pI = 6.1. The pH and temperature optima are pH 5.0 and 45°C, respectively, using a xyloglucan nonasaccharide as substrate.

Few α-xylosidases have been purified to homogeneity. The enzymes purified from *Bacillus* (Zong and Yasui, 1989) and *Aspergillus* (Matsushita *et al.*, 1985) have different pH optima (7.5 and 2.2-3.0, respectively), different pI values (4.25 and 5.6, respectively) and also different molecular weights (subunit Mr 82 KDa and 123 KDa, respectively). These two enzymes are able to catalyze the hydrolysis of *p*-nitrophenyl-α-D-xylopyranoside and they hydrolyze the disaccharide isoprimeverose to glucose and xylose. The specificity of these enzymes and their molecular properties indicate that they are not related to the nasturtium enzyme. The α-xylosidase enzyme purified from germinated nasturtium cotyledons is, however, very similar in terms of specificity and molecular properties to the enzyme isolated from auxin-treated pea stem (O'Neill *et al.*, 1989). The molecular weight of the pea stem α-xylosidase is 85 KDa on SDS-PAGE and it contains

isoenzymes covering the pI range 7.35 to 7.7. The difference in pI between the pea and the nasturtium enzymes may arise from the different method used to obtain pure enzyme. The pea α-xylosidase was isolated using a complex procedure which involved ion-exchange, gel filtration, hydrophobic interaction and chromatofusing. It is possible that only a part of a spectrum of isoenzymes, which may not cover the full native pI range, was isolated.

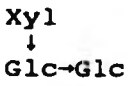
The nasturtium protein is glycosylated to the extent of 16-20% and treatment of the enzyme with anhydrous trifluoromethanesulphonic acid gives a protein with an apparent molecular weight of 75 KDa on SDS-PAGE. Carbohydrate analysis of the glycosyl moiety indicates that glucose accounts for 40% of the glycosyl residues, followed by Glc(or Gal)NAc (20%). Analysis of the partial cDNA clone obtained for this enzyme by Dr Chengappa at the Unilever Research Laboratory, showed that there are few putative N-glycosylation sites characterized by the sequence Asn-X-Ser(or Thr). There are however several Ser and Thr residues which could be potential sites for O-type of glycosylation. The relatively low levels of Man and GlcNac residues appear to confirm this view. The enzyme from auxin-treated pea stem is also glycosylated, and carbohydrate analysis is in progress (Dr Christofer Augur, personal communication).

The failure to reveal any activity of the nasturtium enzyme from the phenyl-sepharose column (section 3.3.1.) suggests that this protein may be highly hydrophobic. The partial sequence deduced by Dr. Chengappa confirms this. Furthermore the observed precipitation of the partially de-glycosylated protein (section 3.3.3.) suggests that the presence of carbohydrates on the α-xylosidase protein may contribute to the solubility of the enzyme in aqueous media.

3.4.2. SPECIFICITY OF α-XYLOSIDASE.

Evidence is presented in this Chapter that the enzyme α-xylosidase purified from germinated nasturtium cotyledons is xyloglucan specific. This study confirms the observations of Edwards *et al.* (1985) that this enzyme does not hydrolyze *p*-nitrophenyl-α-D-xylopyranose. The disaccharide isoprimeverose (α-D-xylopyranosyl-(1→6)-D-glucose) is not hydrolyzed by this enzyme. The nasturtium α-xylosidase has clearly a highly restricted specificity, in that it catalyzes the hydrolysis of a single unsubstituted terminal xylosyl residue from the non reducing end of an oligoxyloglucan molecule. In this respect it would be more appropriate to term this enzyme "*exo*-xyloglucan-α-xylohydrolase". The molecular mode of action of α-xylosidase from pea stem and from nasturtium are identical: both enzymes have the same highly restricted specificity, and both these enzymes are *exo*-xyloglucan-α-xylohydrolases.

The minimum oligosaccharide size which functions as substrate is the trisaccharide Glc_2Xyl with the following structure:



The enzymes from germinated nasturtium cotyledons and from auxin-treated pea stem are probably related structurally. This will become clear when the sequences are available.

The nasturtium α-xylosidase is associated with the hydrolysis of xyloglucan reserves in germinated cotyledons as documented by Edwards *et al.* (1985). It has been suggested that xyloglucan breakdown in nasturtium could be regarded a process of wall turnover (Reid, 1985). The striking similarities between the nasturtium and the pea enzymes apparently involved in very different physiological processes suggest that the former may be involved also in the turnover of xyloglucan, possibly the turnover of primary cell wall xyloglucan which is known to be associated with elongation-growth (Labavitch and Ray, 1974).

3.4.3. GLYCOSYLATION AND CELL SORTING TO THE CELL WALL.

A number of hydrolytic enzymes are found both in the cell wall and in the vacuolar compartment of the cell (Driovich *et al.*, 1989). The sorting system required to direct proteins to the cell wall from the ER compartment is mediated by the Golgi complex. A sorting system should ensure that some proteins are directed to the cell wall, presumably transported by secretory vescicles, and some proteins are directed to vacuoles. Athough it has been speculated that a carbohydrate signal located on the protein may be responsible for the sorting system, directing the proteins to the correct target, more recent results have shown that de-glycosylation of the glycoprotein does not prevent transport of the proteins to the wall (Faye *et al.*, 1886; Driovich *et al.*, 1989). The available evidence now favours the view that in yeast cells and in mammalian cells the

transport to the vacuole or to the lysosomes requires positive sorting information (glycosyl substitution), while secretion does not. Secretion is, therefore, categorized as a default pathway. The glycosylation may contain targeting information or play some other role in transport. For instance, glycosylation may play an important role in the protection against degradation during transport or after secretion in the medium.

CHAPTER FOUR

THE SPECIFICITY OF THE *ENDO*-(1→4)-ß-D-GLUCANASE.

4.1. INTRODUCTION.

During the post-germinative mobilization of xyloglucan reserves in nasturtium cotyledons the levels of a novel xyloglucan-specific *endo*-(1→4)-ß-D-glucanase have been found to increase and decrease following the rate of mobilization of the polysaccharide (Edwards *et al.*, 1985). This enzyme has been purified to homogeneity and has an apparent molecular weight of 29 KDa by SDS-gel electrophoresis (Edwards *et al.*, 1986).

Other *endo*-(1→4)-ß-glucanase enzymes have been purified from microorganisms such as *Trichoderma viride*, *Trichoderma reesei* and *Penicillium funiculosum* and they have been partially characterized. These enzymes are better known as cellulases as they are, unlike the nasturtium enzyme, capable of hydrolyzing soluble (1→4)-ß-D-glucans, carboxymethyl and hydroxyethyl celluloses, mixed-linkage (1→4,1→3)-ß-glucans, and hemicelluloses such as xyloglucans. The specificity of these enzymes has been reviewed (Tomme *at al.*, 1989).

Computer analysis of 28 different cellulase aminoacid sequences has indicated that the putative active site of at least half of them contains an aminoacid

sequence of -Asn-Glu-Pro- (Baird *et al.*, 1990). Moreover the mutation of Glu→Gln by site-directed mutagenesis performed on two apparently highly divergent cellulases from *Bacillus subtilis* and from *Bacillus polymyxa* resulted in the total loss of enzyme activity. A similar result was obtained when site directed mutagenesis was performed on the presumed catalytic site of *Trichoderma reesei endo*glucanase I (Mitsuishi *et al.*, 1990).

The occurrence of cellulases in the animal kingdom has also been reported (Anzai *et al.*, 1984). A cellulase purified from the gastric teeth of *Dolabella auricularia*, a mollusc belonging to *Ophisthobranchia*, (Mr 44 KDa) has been shown to cleave swollen cellulose, carboxymethycellulose and cello-oligosaccharides. The limit-recognition size was found to be a cellotetraose as the enzyme did not attack oligosaccharides lower than Glc_4 (Anzai *et al.*, 1984).

There are not many examples of pure *endo*-(1→4)-ß-glucanases or cellulases from the plant kingdom, and the few enzymes known include an *endo*glucanase from ripening avocado fruit, two from auxin-treated pea stem, a cellulase from the abscission zone of bean leaves, and two forms of anther cellulase. They have been purified and in some cases characterized. Avocado (*Persea americana* Mill cv. Fuerte) cellulase has been purified to homogeneity from the ripening fruits where it has been associated with cell wall modification leading to fruit softening. It has an apparent molecular weight 49 KDa based on SDS-PAGE (Awad and Lewis, 1980) and it catalyzes the hydrolysis of cellulose, carboxymethycellulose and mixed-linkage (1→4,1→3)-ß-D-glucans. Investigations on the molecular mode of action of this enzyme using cello-oligosaccharides and *Avena* mixed mixed-linkage (1→4,1→3)-ß-D-glucans have shown that this enzyme has a limit-recognition site

of four ß-D-linked glucosyl residues. Hatfield and Nevins (1986) have also shown that avocado cellulase is not active towards the cellulosic component of isolated fruit cell wall.

The cellulases purified from auxin-treated pea (*Pisum sativum*) epicotyls (Mr 20 and 70 KDa) catalyze the *endo*-hydrolysis of cellulose powder, carboxymethylcellulose, higher cellodextrins and mixed linkage ß-glucans (Byrne *et al.*, 1975; Wong *et al.*, 1977). More recent *in vitro* studies have showed that pea cellulase preferentially attacks and solubilizes xyloglucan from cell wall "ghosts" which contain only the cellulose-xyloglucan wall component (Hayashi *et al.*, 1984).

A cellulase has been purified from bean leaf abscission zones (*Phaseolus vulgaris* cv Red Kidney) and its expression has been found to be induced by ethylene and suppressed by auxins (Tucker *et al.*, 1988). The specificity of this enzyme (AZ cellulase, Mr 50 KDa) has not been yet investigated. It is known that bean cellulase has significant sequence homology with the avocado fruit cellulase and it is therefore possible that these enzyme have similar specificities.

Two forms of anther cellulases, purified from sweet pea (*Lathyrus odoratus*), have been shown to be related to the bean abscission cellulase, as they could be immunoprecipitated by the antibodies raised against bean AZ enzyme and they also had a similar molecular weight, approx. 50 KDa (Sexton *et al.*, 1990). The activity of the two cellulases was assayed using carboxymethyl cellulose as substrate, but their specificities have not yet been investigated.

The presence of various cellulases and xyloglucan-specific *endo*glucanases has been recently reported in ripening tomato (*Lycopersicon esculentum* var. Sunny) fruit (Maclachlan and Brady, 1992), but such enzymes have not been purified to

homogeneity.

There are only two enzymes yet known able to specifically attack and cleave in an *endo*-fashion the glucan backbone of xyloglucan polysaccharides. These include the xyloglucan-specific *endo*glucanase purified from germinated nasturtium seeds (Edwards *et al.*, 1986) and a novel xyloglucan *endo*-transglycosylase recently purified from the extracellular space of *Vigna angularis* epicotyls by Nishitani and Tominaga (1992). The latter enzyme is a glycoprotein with an apparent molecular weight of 33 KDa which has been shown to be able to transfer fragments of xyloglucan-donors onto xyloglucan acceptors by a mechanism of transglycosylation. The authors have postulated a possible involvement of this unusual enzyme in the extension of primary cell walls.

The turnover of xyloglucan in germinated cotyledons and in primary cell walls has been well documented (see introduction, Chapter 1). In particular, solubilization of xyloglucan in primary cell wall is believed to be induced by the action of auxin or the lowering of pH (Labavith and Ray, 1974). In both cases, it has been suggested that cleavage of xyloglucan polysaccharides, with the production of biologically active oligosaccharides may occur in the growing regions of the cell wall as an enzyme-mediated mechanism (Fry, 1989) and that this enzyme could be a xyloglucan-specific *endo*glucanase (Farkas *et al.*, 1992; Fry *et al.*,1992; Nishitani and Tominaga, 1992).

In this Chapter the specificity of nasturtium *endo*glucanase is investigated and compared with that of an *endo*-(1→4)-ß-D-glucanase (cellulase) purified from *Trichoderma viride* using tamarind, nasturtium and other xyloglucans as substrates. It is concluded that the nasturtium enzyme has a powerful and specific

transglycosylase action. The specificity and the conditions for transglycosylation of the nasturtium enzyme are investigated in relation to the proposed *in vivo* mobilization and turnover of cell wall xyloglucan in Chapter 6.

4.2. MATERIALS AND METHODS.

Chemicals and buffer salts were of analytical grade or better, and were purchased from Sigma. The *endo*-(1→4)-ß-D-glucanase purified from *Trichoderma viride* was purchased from MegaZyme, North Rocks N.S.W., Australia. The enzyme ß-glucosidase was partially purified from germinated nasturtium cotyledons. Barley xyloglucan was a gift of Dr Y. Kato, Laboratory of Food Science, Faculty of Education, Hirosaki University, Bunkyo-cho Hirosaki, Aomori 036, Japan. Gel filtration was carried out using Bio Gel P, BioRad, Watford, Herts, UK.

4.2.1. ENZYME ASSAYS.

Tamarind xyloglucan was used as substrate for the assay of the nasturtium enzyme *endo*-(1→4)-ß-glucanase; the activity was monitored by the viscometric assay. The reducing power assay was performed only with the purified enzyme, since this method is not specific.

The ß-glucosidase and ß-galactosidase activities were monitored using the appropriate *p*-nitrophenyl-glycosides and by the release of D-glucose and D-galactose as monitored by the hexokinase/glucose-6-phosphate dehydrogenase assay (Bergmeyer *et al.*, 1974) and by the galactose dehydrogenase assay (Kurtz and Wallenfels, 1974), respectively. Pentose was assayed by the method of Roe and Rice (1948). The determination of total carbohydrate was carried out with the phenol/sulphuric acid method or with the anthrone technique. Protein dermination was carried out using the dye-binding procedure of Sedmak and Grossberg (1977) and using bovine serum albumin as standard. All enzyme assays and general methods are described in Chapter 2.

4.2.2. GENERAL SEPARATION TECHNIQUES.

SDS-polyacrylamide gel electrophoresis and western blotting were carried out according to Laemmli (1970) and Towbin *et al.* (1979), respectively. Immunoblotting and anti-serum incubations with the *endo*glucanase antibody (Stronach, 1991) were carried out as described in section 5.2.5.1. Analytical isoelectric focusing was carried out using Ampholine PAGplates (LKB Ltd) according to the manufactures' instructions. All procedures are described in Chapter 2. Analytical and preparative thin layer chromatography and high performance liquid chromatography (HPLC) were carried out as described in Chapters 2 and 3.

4.2.3. PURIFICATION OF THE ENZYME.

Seeds, germination and growth were as described in Chapter 2. Cotyledons from 13 day old seedlings were harvested and homogenized in 0.2 M potassium phosphate buffer pH 7.2. The insoluble material was removed by centrifugation (19000 g x 30 min) and the supernatant brought to 90% saturation with ammonium sulphate by addition of the crystalline salt. The precipitate was collected after centrifugation (26000 g x 30 min), dissolved in 20 mM Tris-HCl buffer pH 7.8 and dialysed against the same buffer. The crude enzyme extract was subjected to anion-exchange chromatography.

4.2.3.1. ANION-EXCHANGE CHROMATOGRAPHY.

The dialysed supernatant (250 ml) was applied to a column (2.2 x 40 cm) of DEAE-cellulose (Whatman DE52) and the column was washed with the 20 mM Tris-HCl buffer 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 in the same buffer over 7-8 bed volumes). The column flow-rate was 80 ml h^{-1}, and 10 ml fractions were collected. The enzyme *endo*-glucanase was eluted from the column in the sodium-chloride gradient. Fractions containing high *endo*-

(1→4)-ß-glucanase activity were pooled and concentrated by ammonium sulphate precipitation (90% saturation). The precipitated protein was collected by centrifugation (26000 g x 30 min), dissolved in 50 mM sodium acetate buffer pH 5.0 and dialysed against the same buffer.

4.2.3.2. CATION-EXCHANGE CROMATOGRAPHY.

The concentrated and dialysed sample as above (about 100 ml) was applied to a carboxymethyl-cellulose (Whatman CM-52) column (2.2 x 30 cm), equilibrated with the same buffer. The flow-time of the eluant was ca. 50 ml/h and 10 ml fractions were collected and analysed for absorbance at 280 nm. *Endo*glucanase activity was eluted in the wash. Fractions containing high *endo*glucanase activity were pooled and concentrated by the addition of ammonium sulphate (90% saturation). The protein was collected by centrifugation at 26000 g for 30 min and dissolved in a minimum volume (3-4 ml) of 50 mM ammonium acetate buffer pH 5.0.

4.2.3.3. GEL FILTRATION.

The sample was applied to a Bio-gel P-60 column (3.0 x 70 cm) and eluted with 50 mM ammonium acetate buffer pH 5.0. The flow-time was 2.0 ml/h and 4 ml fractions were collected from the column. The fractions containing high enzyme activity were pooled, aliquoted and stored at -20°C. Protein standards were used for molecular weight determination in SDS-PAGE and they are listed in methods, Chapter 2.

4.2.4. PARTIAL PURIFICATION OF ß-GLUCOSIDASE.

Seeds, germination and growth were as described in Chapter 2. Preparation of the crude enzyme extract and anion exchange chromatography were carried out as described in section 4.2.3. and 4.2.3.1., respectively. The enzyme activity was monitored by the release of *p*-nitrophenol from *p*-nitrophenyl-ß-D-glucopyranoside.

The ß-glucosidase activity was eluted in the wash from anion-exchange chromatography carried out using DEAE. Fractions containing high enzyme activity were pooled and brought to 90% saturation by the addition of crystalline ammonium sulphate. The precipitate was collected after centrifugation (26000 g x 30 min), dissolved in 50 mM sodium acetate buffer pH 5.0 and dialyzed against

the same buffer. The enzyme extract was subjected to cation-exchange chromatography as described in section 4.2.3.2.. The enzyme activity was eluted in the sodium chloride gradient. Fractions containing high enzyme activity were pooled and concentrated by the addition of ammonium sulphate as before. The precipitate collected by centrifugation (26000 g x 30 min) was re-suspended in the minimum volume of 50 mM ammonium actetate buffer pH 5.0 and subjected to gel filtration on Bio Gel P60. Fractions containing high enzyme activity were pooled and stored at -20°C. Although the enzyme preparation contained several protein components when analysed by SDS-PAGE, only ß-glucosidase activity was detected in this enzyme extract. The ß-glucosidase preparation was able to release glucose from cellotetraose, cellobiose and xyloglucan oligosaccharides treated with α-xylosidase (Fanutti *et al.*, 1991).

4.2.5. CRUDE ENZYME EXTRACTS FROM TOMATO, TOBACCO AND NASTURTIUM TISSUES.

Germination and growth of nasturtium seeds were as described in Chapter 2. Leaves and stems from 13 day old seedlings were removed manually, washed gently with water and homogenized with 0.2 M potassium phosphate buffer pH 7.2. Tomato (*Lycopersicon esculentum*) and tobacco (*Nicotiana tabacum*) seeds were grown in greenhouses and were harvested when the seedlings reached approx. 15 cm. The stems and leaves of 21-day old tomato seedlings and the stem

and leaves of 15 day old tobacco seedlings were removed manually, washed with water and homogenized with 0.2 M phosphate buffer pH 7.2. Only stem material above the first internode was taken.

The fresh tissue was weighed and homogenized with the appropriate volume of phosphate buffer pH 7.2, based on the enzyme extraction of nasturtium cotyledons described in section 2.2.13.2..

The insoluble material was removed by centrifugation (19000 g x 30 min) and the supernatant brought to 90% saturation with ammonium sulphate by addition of the crystalline salt. The precipitate was collected after centrifugation (26000 g x 30 min), dissolved in the minimum amount of 20 mM Tris-HCl buffer pH 7.8 and dialysed against the same buffer (2 x 1 l). Protein determination indicated that: nasturtium stem extract contained 1 mg protein/ml; nasturtium leaf extract contained 6 mg protein/ml; tobacco stems extract contained 1.5 mg protein/ml; tobacco leaf extract contained 4.8.mg protein/ml; tomato stem extract contained 0.6 mg protein/ml; tomato leaves extract contained 3.3 mg protein/ml. The nasturtium seed extract used as control contained 2.2 mg protein/ml.

4.2.6. PURIFICATION OF XYLOGLUCAN OLIGOSACCHARIDES.

A solution of tamarind xyloglucan (final concentration 1% w/v) was digested with the purified nasturtium *endo*glucanase and saccharides purified by gel filtration on Bio Gel P4 and preparative TLC as described in section 3.2.10. and 3.2.11..

The purified saccharides were analysed by HPLC and TLC and their concentrations were determined by the phenol/sulphuric acid or by the anthrone assay, using standard mixtures of glucose, xylose and galactose in the correct proportions.

4.2.7. SACCHARIDE REDUCTION WITH SODIUM BOROHYDRIDE AND PURIFICATION OF THE ALDITOLS.

The procedure was carried out as described in Chapter 2 with the following modification: the excess of borate was removed as the volatile methyl borate by repeated co-evaporation with methanol and the sample was concentrated by vacuum rotary evaporation. The sample was re-suspended in 100-200 μl of 0.5 M ammonia and placed in a micro centrifuge tube containing Dowex 50W ion-exchange resin (500 μl) which had been converted from the H^+ form to the NH_4^+ form by repeated washing with 0.5 M ammonia. The mixture was centrifuged (12000 x g for 5 min) and the supernatant subjected to a further ion-exchange using fresh Dowex 50W pre-equilibrated with 0.5 M ammonia. This step was repeated 4-5 times to ensure that sodium ions were removed from the sample. The supernatant was freeze-dried and dissolved in water or buffer.

4.2.8. ENZYMIC DIGESTION OF OLIGOSACCHARIDES D TO H AND THEIR ALDITOLS.

The *endo*-(1→4)-ß-glucanase enzyme preparation purified from *T. viride* was supplied as a suspension in ammonium sulphate. The enzyme (500 U) was brought into solution by diluting in 50 mM ammonium acetate buffer pH 5.0, and de-salted by gel filtration using Sephadex G25. The column (40 x 1 cm) was eluted with 50 mM ammonium acetate buffer pH 5.0. The activity of the enzyme was determined by the reducing power assay using tamarind xyloglucan as substrate before and after chromatography. The final enzyme preparation contained 50 U/ml.

The purified saccharides and their respective alditols (0.2-1.0 mg) were dissolved in 50 mM ammonium acetate pH 5.0 in a final volume of 50-100 μl and incubated with the fungal cellulase (0.2-1.0 U) or with the nasturtium *endo*glucanase (0.2-1.0 μg protein). The reaction was carried out at 30°C, stopped after 20 h and the enzyme heat denatured. The enzyme reaction was monitored by HPLC, analytical TLC or by the reducing power assay when sufficient saccharide material was available.

Incubations of xyloglucan oligosaccharides with the nasturtium ß-galactosidase were carried out by treating the saccharides (0.2-1.0 mg) with 0.5-2.0 μg of enzyme in a final volume of 20-100 μl. Incubations of xyloglucan oligosaccharides with the nasturtium α-xylosidase were carried out by treating the saccharides (0.2-1.0 mg) with 0.2-1.0 μg enzyme protein in a final volume of 20-100 μl. Incubations

of oligosaccharides with the nasturtium ß-glucosidase enzyme extract were carried out by treating the saccharides (0.2-1.0 mg) with 0.5-2.0 μg of enzyme extract in a final volume of 20-100 μl. All incubations were carried out overnight at 30°C using 50 mM ammonium acetate buffer pH 5.0 and stopped by heat-denaturation of enzyme(s).

4.3. RESULTS.

4.3.1. PURIFICATION AND CHARACTERIZATION OF THE ENZYME.

The procedure of Edwards *et al.* (1986) with some modifications was used to purify to homogeneity the enzyme *endo*-(1→4)-ß-D-glucanase. The enzyme was isolated from germinated nasturtium seeds harvested 13 days after sowing, when the activities of endoglucanase and α-xylosidase were found to be very high in the cotyledonary tissue. Partial purification of the *endo*glucanase was achieved by anion-exchange chromatography on DEAE-cellulose column. The enzyme was eluted in the sodium chloride gradient applied to the column (Fig.4.1.a). The fractions containing high enzyme activity were subjected to cation-exchange chromatography using CM-cellulose. The *endo*glucanase activity was eluted in the wash, i.e. it was not bound to the column (Fig.4.1.b). The final purification of the enzyme was achieved by gel filtration using Bio Gel P60 (Fig.4.1.c). The enzyme

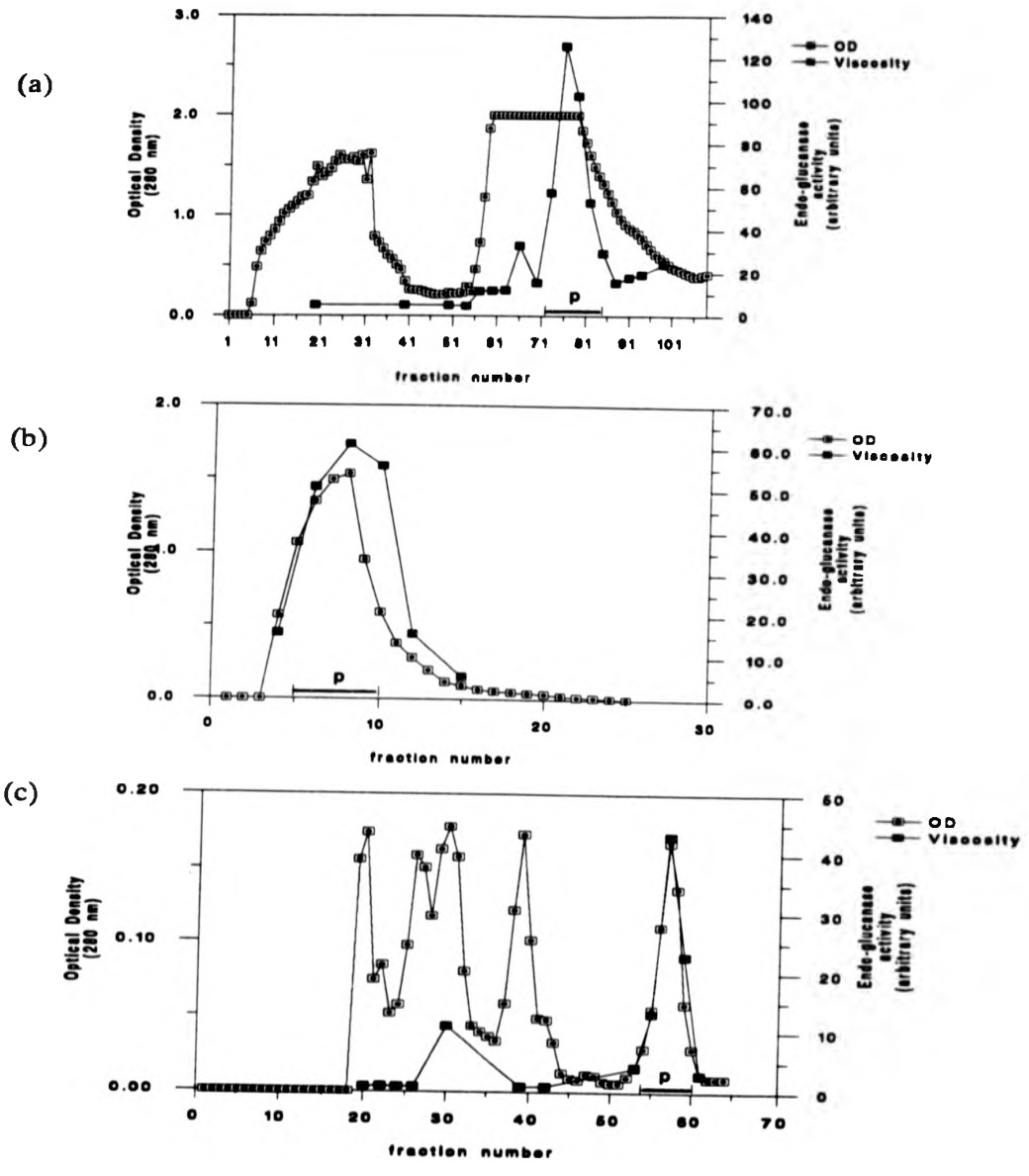


Fig.4.1. Purification of *endo*-(1→4)-ß-D-glucanase. (a): Anion-exchange chromatography on DEAE-cellulose. (b): Cation exchange chromatography on CM-52. (c): Gel filtration on Bio Gel P60.

The activity of *endo*-(1→4)-ß-D-glucanase is expressed as 100/viscometric flow time - 100/viscometric flow time of the control. P = pooled fractions.

purification was monitored by its ability to hydrolyze tamarind xyloglucan using the viscometric assay or the reducing power assay.

The molecular weight of the denatured enzyme was found to be 29 KDa by SDS-polyacrylamide gel (Fig.4.2.), whereas the non-denatured protein gave a molecular weight of 13 KDa by gel filtration on Bio Gel P60 (Edwards *et al.*, 1986). The difference in molecular weight between the denatured and the non-denatured forms of the enzyme could be explained by the disruption of a highly compact molecular structure of the native protein in the presence of denaturing agents (Stronach, 1991).

The pH optimum of this enzyme was found to be 4.5-5.0 (Edwards *et al.*, 1986) and the isoelectric point was 5.0 (Fig.4.3.).

4.3.2. SUBSTRATE SPECIFICITY.

The nasturtium *endo*glucanase had a highly restricted specificity since only xyloglucans extracted from both primary cell walls (fucosylated xyloglucan) or from the thickened cell walls of nasturtium or tamarind seeds were substrates for the enzyme (Edwards *et al.*, 1986). Other (1→4)-ß-linked polysaccharides which are substrates for the fungal *endo*glucanases (cellulases) were not hydrolyzed by the nasturtium enzyme (Edwards *et al.*, 1986).

To investigate the basis of the nasturtium enzyme specificity a limit substrate digest was obtained using tamarind xyloglucan and the products of enzyme

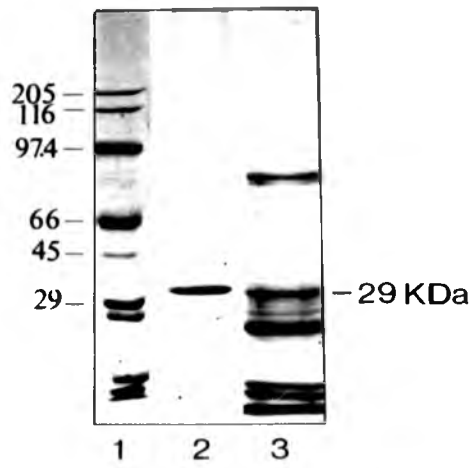


Fig.4.2. SDS-polyacrylamide gel electrophoresis of the purified nasturtium *endo*-(1→4)-ß-D-glucanase and a partially purified seed extract.

Lane 2: purified *endo*-(1→4)-ß-D-glucanase; *Lane 3*: enzyme extract after anion-exchange chromatography; *Lane 1*: Molecular weight markers, Myosin (Mr 205 KDa), ß-galactosidase (Mr 116 KDa), phosphorylase B (Mr 97.4 KDa), bovine serum albumin (Mr 66 KDa), ovalbumin (Mr 45 KDa), carbonic anhydrase (Mr 29 KDa).

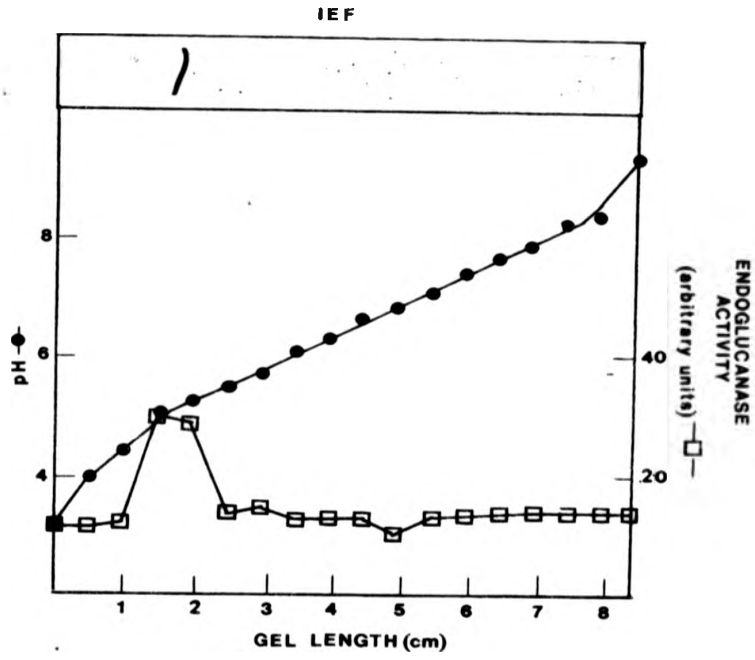


Fig.4.3. Isoelectric focusing (IEF) of the purified *endo*-(1→4)-ß-D-glucanase enzyme. The activity of *endo*-(1→4)-ß-D-glucanase is expressed as 100/viscometric flow time - 100/viscometric flow time of the control.

digestion characterized.

4.3.2.1. CHARACTERIZATION OF THE ENZYME DIGEST.

Tamarind xyloglucan was incubated with the xyloglucan-specific *endo*glucanase to constant reducing power, and some of the products of hydrolysis were purified and analysed enzymatically and by means of ^{1}H-NMR, as described in Chapter 3. The specific viscosity of the incubation mixture decreased very quickly in the early stages of the enzyme reaction, whereas the reducing power increased more slowly over a longer period of time. This behaviour during hydrolysis (slow increase of reducing power and rapid decrease of viscosity) is characteristic of an *endo*-type of enzyme cleavage. The hydrolysis of tamarind xyloglucan was considered complete when reducing power and viscosity remained constant, i.e. after 20 hours. The reaction was stopped by heat-denaturion of the enzyme in a boiling water bath. Two volumes of ethanol were added to the saccharide mixture and the ethanol-insoluble material was removed by centrifugation. An aliquot of the ethanol-soluble fraction was then applied to a TLC plate and saccharides separated. Analysis of the TLC plate showed that the enzyme digest consisted of a mixture of oligosaccharides differing in molecular weight. No monosaccharides were present (Fig.4.4.). The ethanol-soluble fraction accounted for only 20% by weight of the starting material.

TLC analysis of the ethanol-soluble fraction showed three series of saccharides,

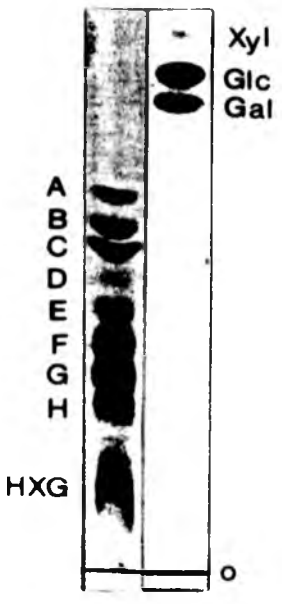
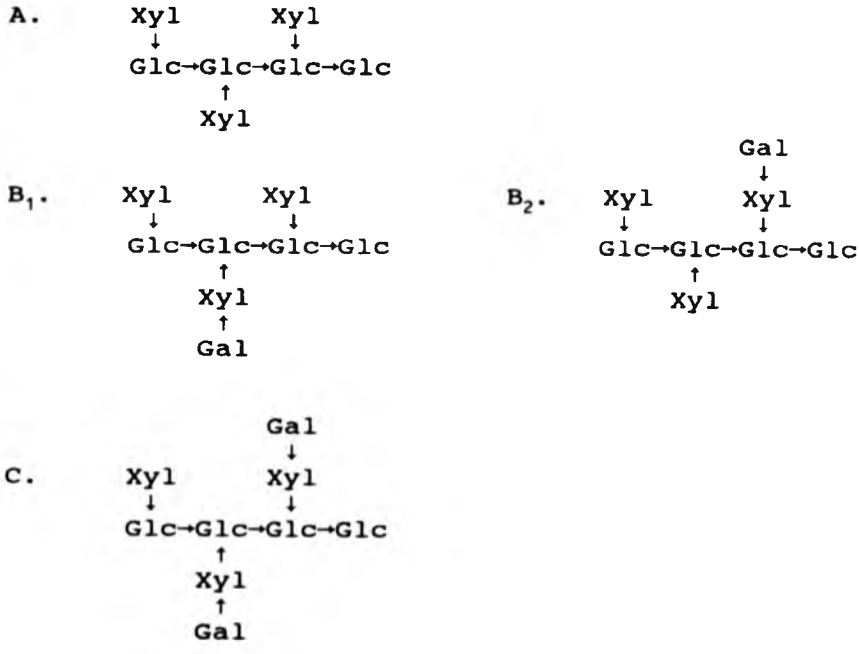


Fig.4.4. Thin layer chromatography of an enzyme digest of tamarind xyloglucan with the nasturtium *endo*-(1→4)-ß-D-glucanase. The high molecular weight material was removed by precipitation with 70% ethanol prior to TLC analysis. The oligosaccharides A, B and C were identified previously as a hepta- an octa- and a nonasaccharide, respectively (Chapter 3, Fanutti *et al.*, 1991).

Lane 1: enzyme digest; *Lane 2*: monosaccharide standard, D-xylose, D-glucose and D-galactose.

each containing several components of similar mobility. A group of three lower molecular weight saccharides were identified as a heptasaccharide A, an octasaccharide B (which consisted of a mixture of two isomeric forms B_1 and B_2) and a nonasaccharide C (Fanutti *et al.*, 1991; Chapter 3) with the following structures:



The second group consisted of five saccharides D, E, F, G and H (in order of decreasing TLC mobility) with intermediate molecular weight (apparent DP>5, based on manno-oligosaccharides standards, see Fig.3.7.) and the third group contained several higher molecular weight saccharides (lower mobility), which were not well resolved. A pure fungal *endo*-(1→4)-ß-D-glucanase (cellulase) from

Trichoderma viride catalyzed the hydrolysis of tamarind xyloglucan to give only three oligosaccharides with the same TLC mobilities of oligosaccharides A, B and C. No higher saccharides were produced. Analysis by HPLC confirmed that the three oligosaccharides in the cellulase digestion of tamarind xyloglucan were the heptsaccharide A, the octasaccharide B (B_1+B_2) and the nonasaccharide C (Fanutti *et al.*, 1991). A minor band corresponding to a lower molecular weight saccharide with a mobility higher than A but lower than glucose was also visible when a large amount of cellulase digest was applied to a TLC plate (Fig.4.5.). The presence of such an oligosaccharide has not been reported before, but it was tentatively identified by its HPLC retention time as a pentasaccharide with the composition glc_3xyl_2.

To understand the mode of action of the nasturtium endoglucanase and the reason for its inability to hydrolyze tamarind xyloglucan completely it was necessary to determine the structures of oligosaccharide group next in size to A, B and C.

4.3.2.2. CHARACTERIZATION OF THE OLIGOSACCHARIDES D TO H.

The saccharides D to H (see Fig.4.4.) were resistant to *endo*glucanase hydrolysis. The observation that the enzyme was unable to cleave these oligosaccharide suggested that they might contain some structural feature (e.g. galactosyl substitution) operating as a "blocking group" to the enzyme action. Fractionation

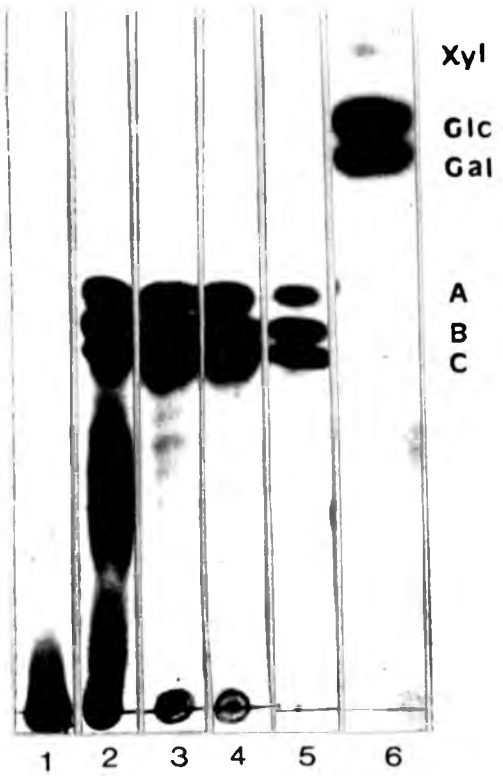


Fig.4.5. Thin layer chromatography of an enzyme digest of tamarind xyloglucan with the pure fungal *endo*-(1→4)-ß-D-glucanase (cellulase) from *T. viride*.

Lanes 1 to 4: Incubation mixture after 2 min, 10 min, 5 h and 20 h, respectively. *Lane 5*: Oligosaccharides A, B and C. *Lane 6*: monosaccharide standard, D-xylose, D-glucose and D-galactose.

on Bio Gel P4 column and preparative TLC plate were carried out, and the saccharides D to H were purified as single components. Some preparations contained minor contaminations of the saccharide(s) next in size (Fig.4.6.). The degree of purification was monitored by TLC and HPLC analysis. Each saccharide was essentially homogeneous on TLC analysis but was resolved into more than one component when subjected to HPLC analysis (Fig.4.7.). The only exceptions were saccharides D and H which were eluted each as a symmetrical peak on HPLC (Fig.4.11.). This indicated that saccharides E, F and G were mixtures of structural isomers.

During the structural characterizations described below, saccharides E, F and G were analysed as single compounds.

Individual oligosaccharide fractions D to H were treated with the nasturtium ß-galactosidase (Edwards *et al.*, 1988) and the products of digestion analysed by TLC. They all released galactose with the exception of oligosaccharide D (Fig.4.8.), indicating that only this oligosaccharide did not contain galactose.

Individual saccharide compounds D to H were hydrolysed to their monosaccharide constituents by TFA and the monosaccharides released were analysed by HPLC. Only glucose, xylose and galactose were detected.

The quantitative results indicated that the relative numbers of glucosyl and xylosyl residues were almost identical for all five oligosaccharides D, E, F, G and H. When the values obtained were scaled by setting glucose = 8.0, the relative values for xylose were calculated to be 6.0 and the relative values of galactose were calculated to be 0, 1, 2, 3 and 4, respectively (Table 4.1.). These results were confirmed by compositional analysis carried out using ^{1}H-NMR (Table 4.1.).

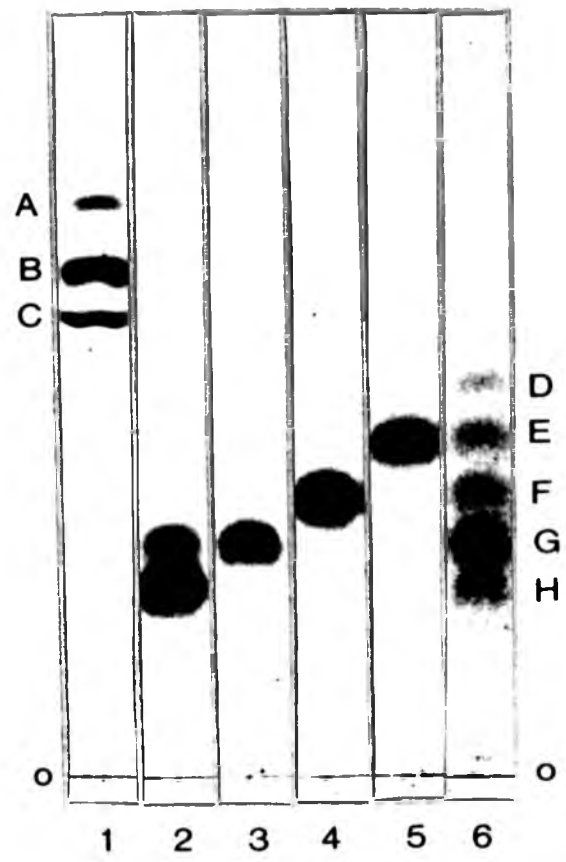


Fig.4.6. TLC analysis of the purified xyloglucan oligosaccharides E to H.

Lanes 2 to 5: purified oligosaccharides H, G, F and E. *Lane 6*: oligosaccharide mixture D to H. *Lane 1*: Oligosaccharides A, B and C. (The purified oligosaccharide D is shown in Fig.4.17.).

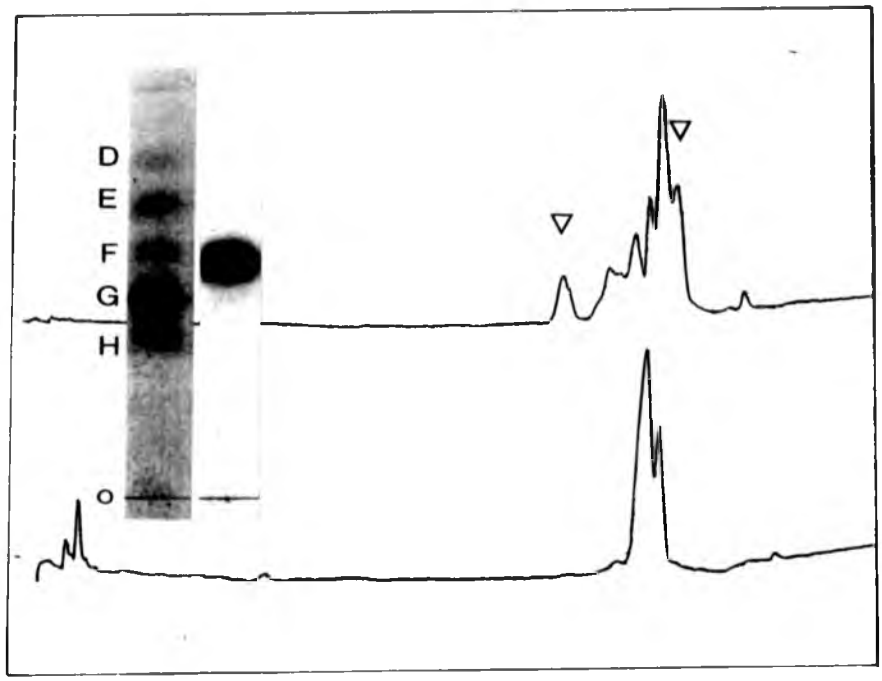


Fig.4.7. HPLC and thin layer chromatography of oligosaccharides D to H and the purified oligosaccharide F. In the upper part oligosaccharides D to H are partially separated on HPLC; Oligosaccharides D and H are indicated by arrows. In the lower part, the oligosaccharide F is eluted using the same gradient.

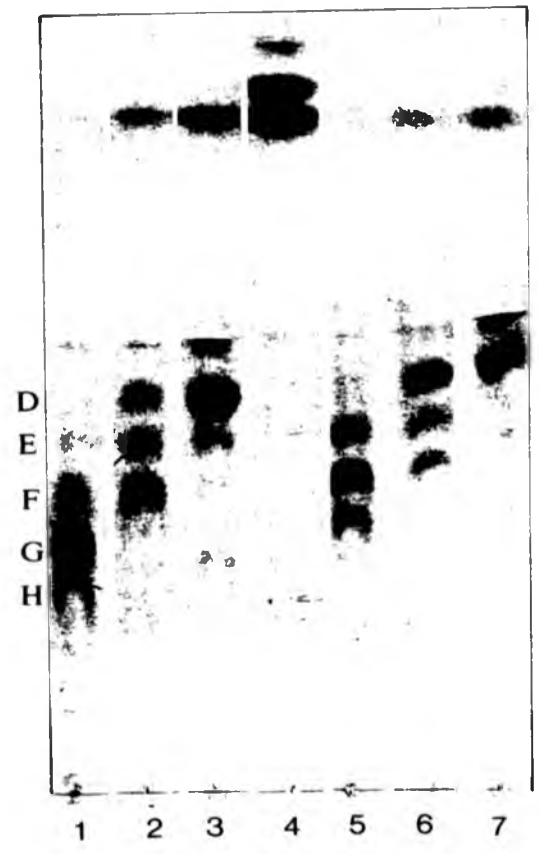


Fig.4.8. Thin layer chromatography of oligosaccharides D to H before and after treatment with the nasturtium ß-galactosidase.

Lane 1: Oligosaccharides F,G and H; *Lane 2 and 3*: Oligosaccharides F,G and H treated with the nasturtium ß-galactosidase after 2 h and 19 h, respectively; *Lane 5*: Oligosaccharides D, E, F and G; *Lane 6 and 7*: Oligosaccharides D, E, F and G treated with the nasturtium ß-galactosidase after 2 h and 19 h, respectively; *Lane 4*: monosaccharide standard, D-xylose (uppermost spot), D-glucose and D-galactose (lower spot).

Compound	Composition by Hydrolysis[a]			Composition by ^{1}H-NMR[b]		
	Glc	Xyl	Gal	Glc	Xyl	Gal
D	8.0	5.9	0.2	8.3	6.4	0.3
E	8.0	6.2	1.1	7.8	6.3	1.1
F	8.0	6.0	1.9	7.7	5.9	1.9
G	8.0	6.2	2.7	8.2	5.8	2.7
[c]H	8.0	6.1	3.5	7.9	5.8	3.3

Table 4.1. Monosaccharide composition of purified oligosaccharides D to H as determined by TFA hydrolysis and by ^{1}H-NMR.

a. Scaled by setting Glc = 8.0

b. Absolute values (assuming a single reducing glucose per molecule).

c. This sample contained significant amounts of oligosaccharide G (see Fig.4.7.).

Oligosaccharide fractions D to H were, respectively, tetradeca- to octadecasaccharides with the compositions $glc_8xyl_6gal_{0to4}$.

Each saccharide fraction D to H was incubated with the pure *endo*(1→4)-ß-D-glucanase from *Trichoderma viride*. The fungal *endo*glucanase catalysed the hydrolysis of each of these oligosaccharides, generating one or more of the oligosaccharides A to C. The products of extensive enzyme digestions were analysed by HPLC and the ratios of the oligosaccharides $A:B_1:B_2:C$ determined. It was found that the complete digestion of saccharide D produced mainly oligosaccharide A (82%) and the complete hydrolysis of saccharide H generated mainly oligosaccharide C (86%). Oligosaccharides E, F and G gave different proportions of oligosaccharides A, B and C. The results which are reported in Table 4.2., indicated that oligosaccharides A to H were all dimers of A, B (B_1 + B_2) and C. Thus H = C + C; G = B + C; F = B + B and A + C; E = B + A; D = A + A.

Enzyme digestion with cellulase gave information on the composition of each saccharide compound D to H, but did not provide information on the sequence of each oligosaccharide A, B and C in the dimers.

Sequence analysis of the xyloglucan oligosaccharides D to H was achieved by treating each individual fraction with sodium borohydride, and digesting the reduced oligosaccharide totally with the fungal cellulase. Each digest was analysed by HPLC and was resolved in a number of peaks corresponding to the oligosaccharides A, B and C and their respective reduced oligosaccharides A_{-itol}, B_{-itol} and C_{-itol}, where the reduced oligosaccharides indicated the reducing-end of the original saccharide molecule. For instance, if oligosaccharide G had the

Compound	Oligosaccharides Released (%)			
	A	B_1	B_2	C
D	82.3	5.0	7.4	5.3
E	50.9	9.5	34.6	5.0
F	28.0	7.8	35.6	28.6
G	3.3	8.4	39.1	49.2
H	4.6	2.0	7.6	85.8

Table 4.2. HPLC analysis of oligosaccharides D to H digested with the fungal cellulase.

following composition C→B_2, reduction with sodium borohydride would produce an oligosaccharide C→$B_{2\text{-itol}}$, which generates the following oligosaccharides by cellulase digestion C + $B_{2\text{-itol}}$. Separation and integration of the peaks corresponding to oligosaccharides C and B_{2itol} carried out by HPLC would then determine the molecular structure of saccharide G = C→B_{2itol}.

Following this reasoning, cellulase digests of reduced saccharides D to H were analysed by HPLC and the relative amounts of oligosaccharides A, B_1, B_2, C and the alditols $A_{\text{-itol}}$, $B_{1\text{-itol}}$, $B_{2\text{-itol}}$ and $C_{\text{-itol}}$ were calculated (Table 4.3.). The oligosaccharides D (tetradecasaccharide) and H (octadecasaccharide) were mainly composed by dimers of oligosaccharides A + $A_{\text{-itol}}$ and C + $C_{\text{-itol}}$, respectively. The deduced molecular structures for saccharides D and H were A→A and C→C, respectively. The determination of the structure of oligosaccharides E, F and G was in some cases more difficult and it was achieved by a more complex process of reasoning.

The compound G was mainly composed by two oligosaccharides, C and B_2 (only traces of B_1) by HPLC analysis of a cellulase digest of this saccharide. Acid hydrolysis by TFA showed that it contained 3 galactosyl substituents. When saccharide G was reduced and digested by cellulase the quantity of oligosaccharide C was found to be only half of the amount of the reduced oligosaccharide $C_{\text{-itol}}$, and the oligosaccharide B_2 was approximately double that of its reduced form $B_{2\text{-itol}}$. This indicated that the amounts of oligosaccharides B_2 and $C_{\text{-itol}}$, and those of oligosaccharides C and $B_{2\text{-itol}}$ were roughly equimolar. Oligosaccharide G therefore consisted of two major structures, B_2→C, accounting for approximately 2/3, and C→B_2 accounting for approximately 1/3 of the total

Compound	Oligosaccharides and Alditols Released (%)							
	A	B_1	B_2	C	A_{-itol}	$B_{1\text{-}itol}$	$B_{2\text{-}itol}$	C_{-itol}
D	47.0	-	-	-	53.0	-	-	-
E	28.5	2.5	22.0	-	22.5	8.0	14.0	2.5
F	14.5	1.0	20.5	11.0	13.5	7.0	16.0	16.5
G	3.0	3.0	25.0	16.0	2.0	3.0	16.0	32.0
H	-	0.5	2.0	46.0	-	-	2.0	49.5

Table 4.3. Cellulase digestion of oligosaccharides D to H and their respective alditols.

molecular structures. Other less abundant structures were also present.

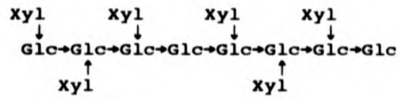
Compound F gave oligosaccharides A, B (B_1+B_2) and C when HPLC analysis of the oligosaccharides released by cellulase hydrolysis was carried out. Since compound F contained only 2 galactosyl residues it was assumed that it consisted of A + C and B + B oligosaccharide dimers. Reduction of compound F and HPLC analysis of the enzyme digest showed that (i) the amounts of oligosaccharides B_1 + B_2 and $B_{1\text{-itol}}$ + $B_{2\text{-itol}}$ were comparable (21.5% and 23% respectively). Since the amount of B_1 was very low (only 1%) the major molecular structures were composed by dimers $B_2 \rightarrow B_{1\text{-itol}}$ (ca 15%) and $B_2 \rightarrow B_{2\text{-itol}}$ (ca 30%). (ii) The amounts of oligosaccharides $A_{\text{-itol}}$ + C_{itol} and A + C were roughly equimolar (30% and 25.5%). The major molecular structures were therefore represented by dimers $A \rightarrow C_{\text{-itol}}$ (ca 30%) and $C \rightarrow A_{\text{-itol}}$ (ca 20%). The presence of minor contaminants (E and G) in the preparation of compound F is indicated by the presence of structures A→A or C→C which, however, accounted only for 5%.

Compound E generated mainly oligosaccharides A and B by digestion with the fungal *endo*glucanase. Acid hydrolysis of this compound with TFA showed that it contained a single galactosyl substitution. This indicated that it consisted of a mixture of A + B oligosaccharide dimers. The amounts of oligosaccharides $A_{\text{-itol}}$ + $B_{\text{-itol}}$ and A + B were comparable (44.5% and 53%). The major molecular structures were represented by dimers $A \rightarrow B_{2\text{-itol}}$ (ca 30%), $A \rightarrow B_{1\text{-itol}}$ (ca 16%), and $B_2 \rightarrow A$ (ca 45%). The presence of minor contaminants (oligosaccharides F and D) in the preparation of compound E is indicated by the presence of structures A→A, A→C and B→B, which accounted for 9%.

On the basis of these results the structures of oligosaccharides D to H are shown

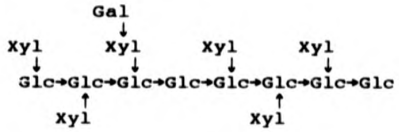
below:

OLIGOSACCHARIDE D:

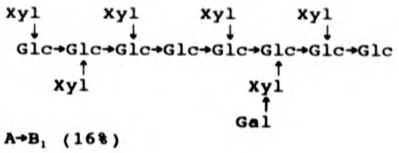


A→A (100%)

OLIGOSACCHARIDE E:

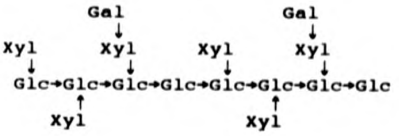


B_2→A (45%)

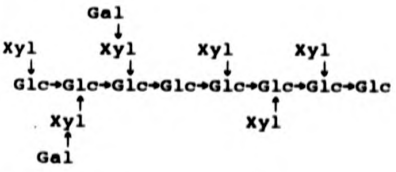


A→B_1 (16%)

OLIGOSACCHARIDE F:

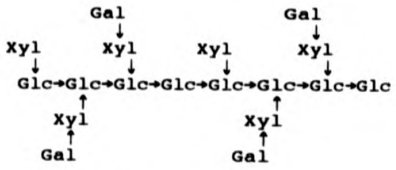


B_2→B_2 (30%)

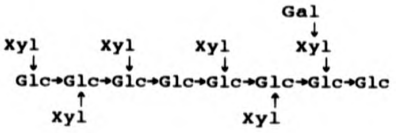


C→A (20%)

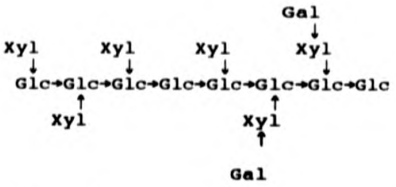
OLIGOSACCHARIDE H:



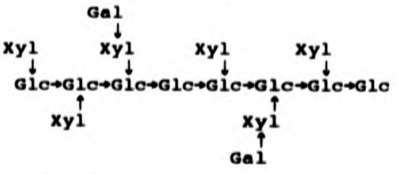
C→C (100%)



A→B_2 (30%)

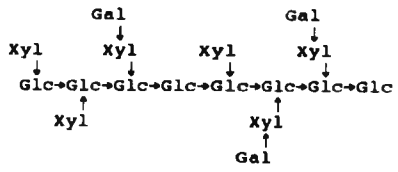


A→C (30%)

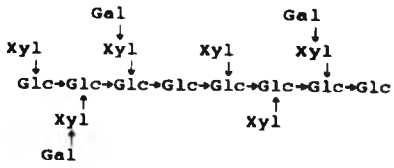


B_2→B_1 (15%)

OLIGOSACCHARIDE G:



B_1→C (66%)



C→B_1 (33%)

4.3.2.3. DIGESTION OF BARLEY XYLOGLUCAN WITH THE NASTURTIUM *ENDO*GLUCANASE.

Monocotyledonous xyloglucans are less substituted than the xyloglucans extracted from dicotyledonous cell walls (Kato *et al.*, 1981). A sample of barley xyloglucan (kindly supplied by Dr Y. Kato) was hydrolysed and the monosaccharides released were analysed by HPLC. The molar ratio Gal:Xyl:Glc was 0.09:1:2.3.

Barley xyloglucan was digested with the nasturtium *endo*glucanase following the same procedure used to hydrolyze tamarind xyloglucan. A large amount of material was precipitated with 70% ethanol, indicating that only a small proportion of the polysaccharide was hydrolyzed by the nasturtium enzyme. Cellulase digestion of barley xyloglucan was extensive and indicated that glucose and a product with a TLC mobility higher than A, but lower than isoprimeverose were produced in large amounts by the fungal enzyme. From an earlier investigation on the structure of barley xyloglucan carried out by Kato *et al.* (1981) it was found that large amounts of a pentasaccharide with composition Glc_3Xyl_2 were produced by digestion of barley xyloglucan with cellulase (Fig.4.9.).

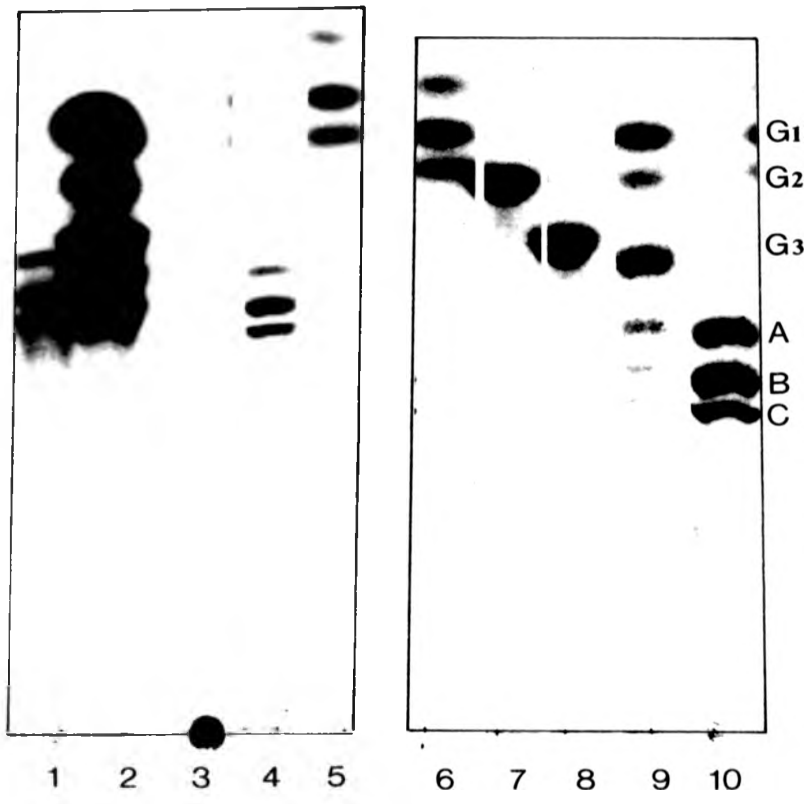


Fig.4.9. Thin layer chromatography of an enzyme digest of barley xyloglucan with the nasturtium *endo*-(1→4)-ß-D-glucanase and with the fungal cellulase.

Lane 1: barley xyloglucan treated with the nasturtium endoglucanase (20 h). *Lanes 2,9*: barley xyloglucan treated with the fungal cellulase (20 h). *Lane 3*: barley xyloglucan (control). *Lane 4*: purified xyloglucan oligosaccharides A, B and C. *Lanes 5,6*: monosaccharide standard, D-xylose, D-glucose (G_1) and D-galactose. *Lane 7*: cellobiose (G_2); *Lane 8*: cellotriose (G_3); *Lane 10*: cellulase digest of tamarind xyloglucan.

The presence of high levels of pentasaccharide in the incubation mixture confirmed the findings of Kato *et al.* (1981). Digestion of barley xyloglucan with the nasturtium enzyme gave mainly three compounds with the same TLC mobilities as the oligosaccharides A, B and C. Higher molecular weight saccharides were also produced and their TLC mobilities suggested that they were probably dimers of the lower oligosaccharides A, B and C. The nasturtium *endo*glucanase, however, was unable to produce oligosaccharides lower than A (Fig.4.9.).

4.3.2.4. FUNGAL CELLULASE AND NASTURTIUM *ENDO*GLUCANASE DIGESTION OF TAMARIND AND NASTURTIUM XYLOGLUCANS: A COMPARISON OF THE LOWER SACCHARIDES RATIO.

Samples of tamarind xyloglucan were digested extensively with the nasturtium *endo*-glucanase and with the purified cellulase. The digests were treated with ethanol. No precipitate was obtained from the cellulase digest and a heavy precipitate was obtained from the nasturtium *endo*glucanase digest. The ethanol soluble fraction obtained by digestion of tamarind xyloglucan with the nasturtium *endo*glucanase was freed from ethanol by vacuum rotary evaporation and re-dissolved in water.

Tamarind xyloglucan was completely converted to oligosaccharides A, B and C by the fungal cellulase. Only about 20% of the xyloglucan was converted to ethanol-soluble saccharides by treating the polysaccharide with the nasturtium

*endo*glucanase. Densitometry of TLC plates containing the enzyme digest indicated that oligosaccharides A, B and C accounted only for ca 25-30% of the ethanol-soluble fraction. By comparing the proportions of oligosaccharides A, B_1, B_2 and C obtained on digestion of xyloglucan with the nasturtium *endo*glucanase against those obtained by the complete cellulase hydrolysis of xyloglucan, it was possible to make deductions concerning the specificity of the nasturtium *endo*glucanase. The ratio A:B_1:B_2:C obtained by digestion of tamarind xyloglucan with the fungal cellulase was found to be 15.2:5.5:31.1:48.2. By comparison the ratio A:B_1:B_2:C obtained by digestion of xyloglucan with the nasturtium enzyme was found to be 20.2:10.4:22.2:47.2. It was observed that in both cases the molecular species B_2 was more abundant than B_1, indicating that the galactosyl distribution on both side of the xyloglucan backbone was different. The ratio B_1:B_2 was, however, not the same in both digests. Cellulase digestion of tamarind xyloglucan gave a B_1:B_2 ratio of 1:5 whereas nasturtium *endo*glucanase digestion gave a ratio of only 1:2. If the cleavage of the xyloglucan backbone by the nasturtium *endo*glucanase was random, one would expect the B_1:B_2 ratio to be mantained constant (i.e. to a value of 1:5) after the polysaccharide hydrolysis. The fact that the ratio B_1:B_2 was altered when the tamarind xyloglucan was hydrolysed with the nasturtium *endo*glucanase indicated that the cleavage was not random. This suggested that certain linkages in the xyloglucan backbone were more susceptible to cleavage by the nasturtium enzyme.

Nasturtium xyloglucan was also used as substrate in an identical series of experiments. Enzyme digestions were carried out as before and the ethanol-soluble fraction produced by the nasturtium enzyme was subjected to HPLC analysis. The oligosaccharide A:B_1:B_2:C ratio was compared against that obtained

by complete digestion of the polysaccharide with the fungal cellulase. In the cellulase digest of nasturtium xyloglucan the ratio B_1:B_2 was quite different to the extent that oligosaccharide B_1 was more abundant than oligosaccharide B_2. The A:B_1:B_2:C ratio of nasturtium xyloglucan digestion with the nasturtium *endo*glucanase was found to be 10.7:27.4:4.4:57.9. The ratio calculated by cellulase digestion of the nasturtium polysaccharide was found to be 4.1:11.5:3.4:81.0. The data relative to the digestion of nasturtium xyloglucan confirmed that the specificities of the two enzymes, nasturtium *endo*glucanase and fungal cellulase, were different and the *endo*-cleavage operated by the nasturtium enzyme was not random. In particular it was deduced that: (i) the values calculated by integration of oligosaccharide peaks A and B_1 were higher than the ones expected for a random type of attack and cleavage; (ii) the values obtained by integration of oligosaccharide peaks B_2 and C were lower. Since oligosaccharides A, B_1, B_2 and C all have in common the same xylosyl-glucose backbone with different degree of galactosylation, it was considered possible that the distribution of galactosyl residues in structures B_2 and C might affect the ability of the enzyme to bind the saccharide molecule either by masking the binding site or by destabilizing the enzyme-substrate complex.

Digestion of tamarind xyloglucan with the nasturtium enzyme always produced a mixture of low and high molecular weight saccharides. The hydrolysate pattern did not change even after prolonged incubation or by increasing the enzyme concentration. Characterization of compounds D, E, F, G and H indicated that these oligosaccharides presented no common pattern of xylosyl and galactosyl-substitution around the central ß-glucosyl linkage which could explain their resistance to hydrolysis.

When the ethanol-insoluble fraction produced by digestion of tamarind xyloglucan with the nasturtium *endo*glucanase was re-incubated with the enzyme, further lower molecular weight oligosaccharides were released. Ethanol precipitation, and HPLC analysis of the lower molecular weight oligosaccharides, showed that the ratio $A:B_1:B_2:C$ was very similar to ratio obtained in the first digestion. This indicated that higher molecular weight saccharides were not totally resistant to hydrolysis but could be further degraded when re-treated with the enzyme. This observation suggested that the enzyme's mode of action was not that of a simple hydrolase.

4.3.2.5. TRANSGLYCOSYLATION OF XYLOGLUCAN OLIGOSACCHARIDES BY THE NASTURTIUM *ENDO*GLUCANASE.

A solution containing purified xyloglucan oligosaccharides E, F, G and H was used to determine whether or not any further hydrolysis would occur on prolonged incubations with the enzyme. The saccharide mixture was re-incubated with the nasturtium *endo*glucanase and the enzyme reaction was monitored by TLC analysis and reducing power. The latter did not change significantly during the enzyme reaction indicating that the same number of reducing saccharides were present in the incubation mixture before and after the enzyme reaction and indicating the absence of hydrolysis (Fig.4.10.a). However, during the incubation, the progressive appearance of oligosaccharides with higher TLC mobility than compounds D, E, F and G plus saccharides with lower TLC mobility was observed

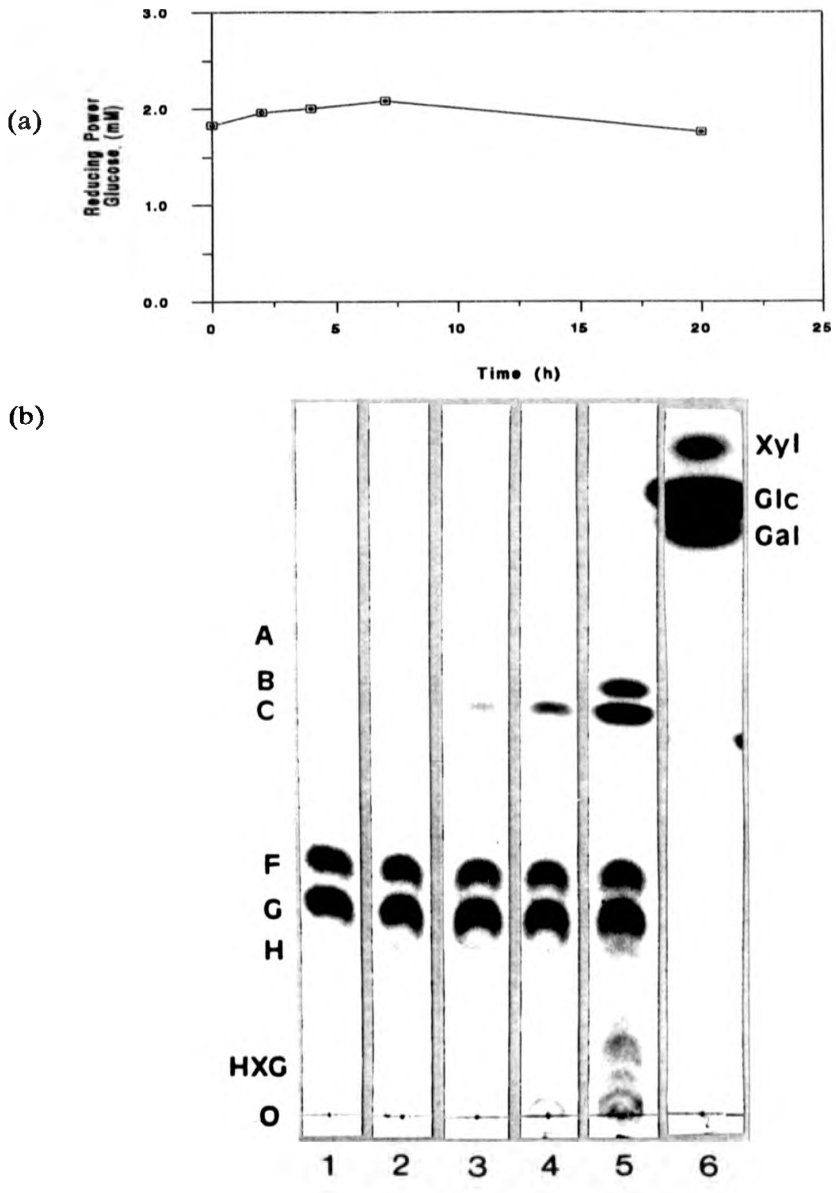


Fig.4.10. Thin layer chromatography (b) and reducing power (a) of an enzyme digest of xyloglucan oligosaccharides F to H treated with the nasturtium *endo*-(1→4)-ß-D-glucanase. *Lane 1 to 5*: oligosaccharides F to H (2 mg in 200 μl) treated with the nasturtium *endo*glucanase (2 μg) for 0, 2, 5, 7 and 20 h, respectively. *Lane 6:* monosaccharide standards, D-xylose, D-glucose and D-galactose. HXG = higher xyloglucan oligosaccharides.

(Fig.4.10.b). The TLC mobilities of the lower molecular weight products generated by the nasturtium *endo*glucanase corresponded to those of oligosaccharides A, B($B_1 + B_2$) and C and their identities were confirmed by HPLC analysis. The appearance of higher and lower saccharides simultaneously indicated that the nasturtium *endo*glucanase was able to catalyze a reaction of tranglycosylation. This enzyme property was not detected during the digestion of high polymeric xyloglucan, since, under those conditions, it was not possible to discriminate between hydrolysis and transglycosylation products. Treatment of oligosaccharides D to H either as a mixture or as purified compounds with the nasturtium enzyme indicated that the nasturtium *endo*glucanase was not a simple hydrolase, capable of cleaving the xyloglucan into fragments, but a true transglycosylase able to catalyse the synthesis of large oligosaccharides by transfer of smaller saccharide unit onto xyloglucan fragments acting as acceptors. The fungal *endo*glucanase was not able to transfer oligosaccharides onto xyloglucan acceptors and oligosaccharides larger than A, B and C were quickly hydrolyzed. The transglycosylation of purified oligosaccharides D, E, F, G and H with the nasturtium *endo*glucanase monitored by HPLC indicated that all these oligosaccharides were substrates for transglycosylation (Fig.4.11.). In all cases the observations were consistent with the central glucose-glucose linkage of the oligosaccharide donor (D to H) being cleaved, and the oligosaccharide fragment originating from the non-reducing end of the molecule being transferred to the non-reducing terminal of another oligosaccharide acting as glycosyl-acceptor. For instance, by transglycosylation of an oligosaccharide D with composition A→A with the nasturtium enzyme the following products would be expected:

$$A{\rightarrow}A + A{\rightarrow}A \leftrightarrow A{\rightarrow}A{\rightarrow}A + A$$

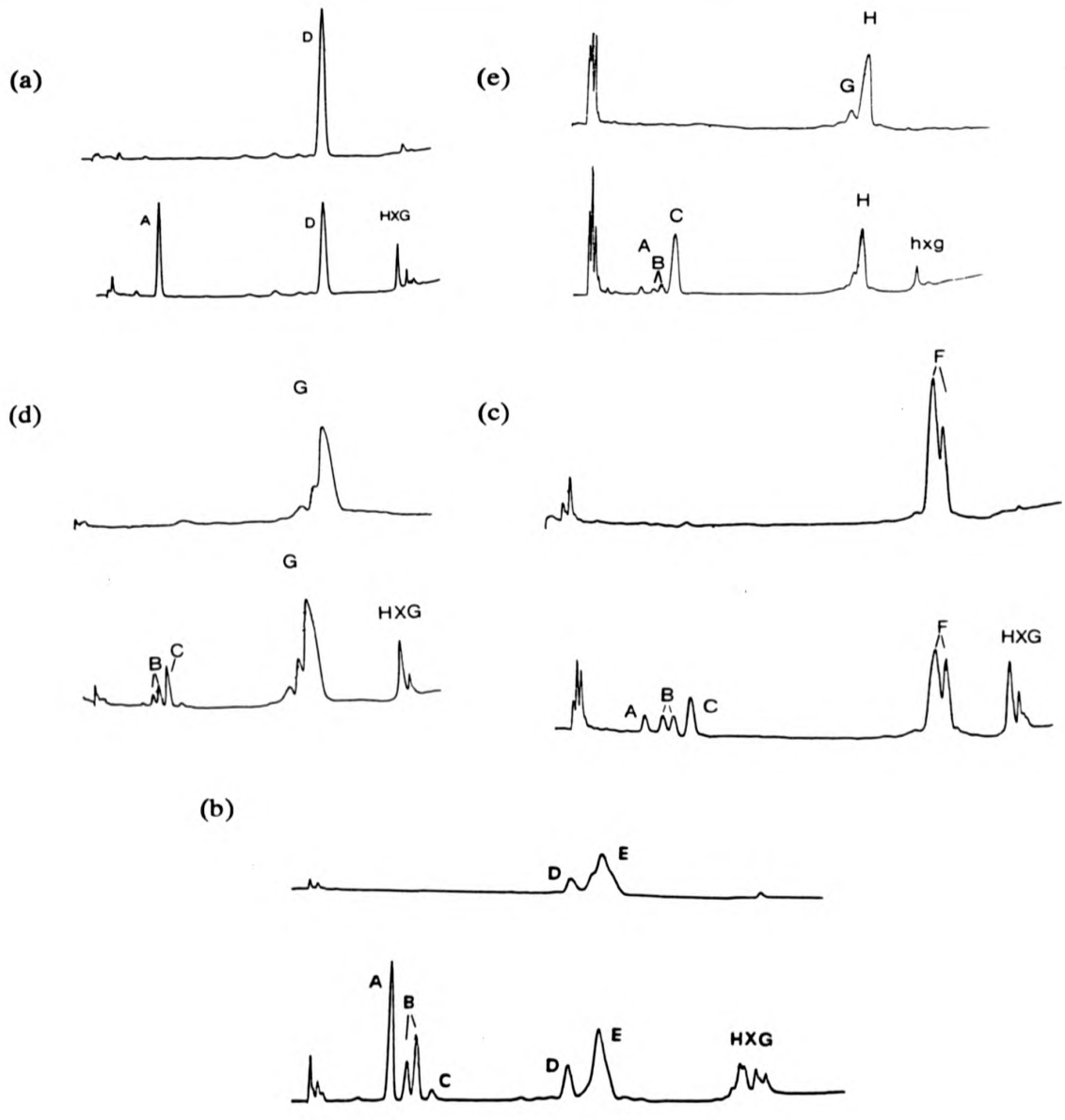
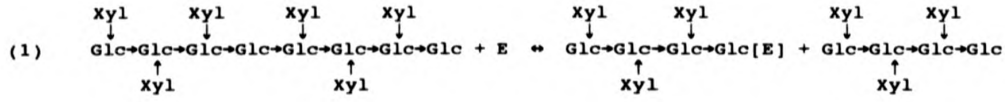
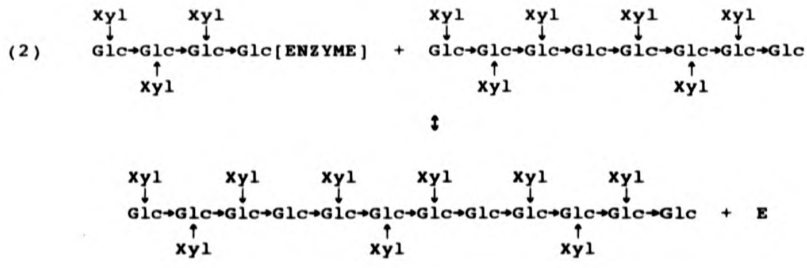


Fig.4.11. HPLC of purified oligosaccharides treated with the nasturtium *endo*-transglycosylase. (a): Oligosaccharide 1.6 mM D; (b): 2.02 mM E; (c): 2.15 mM F; (d): 6.0 mM G; (e): 0.4 mM H; before and after incubation with the enzyme (0.25 μg/25-50 μl of saccharide solution). The incubation time was in all cases 19 h. A = oligosaccharide A; B = oligosaccharide B; C = oligosaccharide C; HXG = higher xyloglucan oligosaccharides.

This was confirmed by incubating the pure oligosaccharide D with the nasturtium *endo*glucanase. The heptasaccharide A and a product with a retention time higher than D were produced as indicated by HPLC analysis. This saccharide was identified as a trimer of oligosaccharide A. The schematic representation of the postulated transglycosylation action of the nasturtium *endo*glucanase with the oligosaccharide D is shown below:





No larger products were detected when the enzyme was incubated in presence of oligosaccharides A, B and C as a mixture or as purified compounds. However when the purified oligosaccharide D was treated with the nasturtium *endo*glucanase in the presence of the purified nonasaccharide C a new oligosaccharide with the same retention time of oligosaccharide F (Fig.4.12.) was produced by the enzyme. The schematic enzyme reaction is the following:

$$A\rightarrow A + C \leftrightarrow A + A\rightarrow C$$

This indicated that the nonasaccharide C was acting as glycosyl-acceptor and that

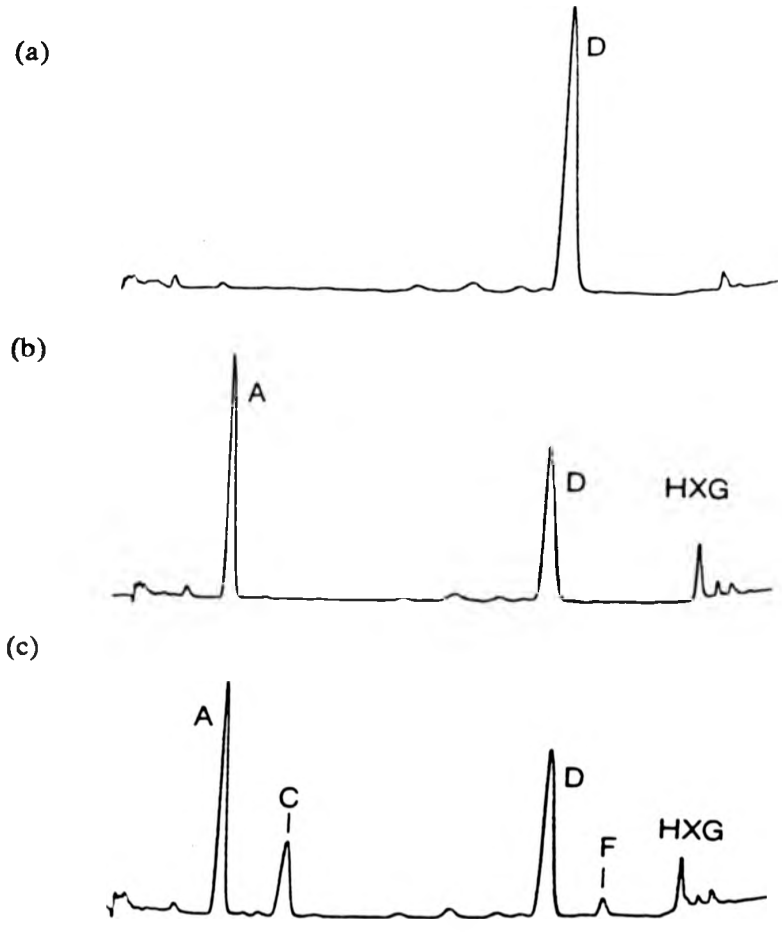


Fig.4.12. Action of the nasturtium xyloglucan *endo*-transglycosylase with xyloglucan oligosaccharides. (a): Oligosaccharide D alone; (b): Oligosaccharide D (0.8 mM, final vol. 25 μl) treated with the enzyme (0.25 μg); (c): Oligosaccharide D (0.8 mM, final volume 25 μl) treated with the enzyme (0.25 μg) in the presence of oligosaccharide C (0.2 mM). A = oligosaccharide A; HXG = higher xyloglucan oligosaccharides.

the energy source for the formation of the A→C link was the breakage of the A→A linkage. The results confirmed the nasturtium *endo*glucanase was a transglycosylase and that xyloglucan oligosaccharides could act as glycosyl-donors and/or glycosyl-acceptors. Cellobiose and cellotetraose were not glycosyl acceptors for transglycosylation, when incubated with oligosaccharides D or G (Fig.4.13.a and b) confirming that xylosyl substitution of the glucose backbone was a requirement for acceptors.

4.3.2.5.1. EFFECT OF SUBSTRATE CONCENTRATION.

Different concentrations of an oligosaccharide mixture (E,F,G and H) were chosen ranging from 3 mM to 0.2 mM and incubations with the enzyme were set up in order to establish the dependence of the transglycosylation reaction on the concentrations of the starting material. TLC analysis (Fig.4.14.) monitored by densitometry (results not shown) showed that higher substrate concentrations generally promoted transglycosylation, whereas lower concentrations promoted hydrolysis. Densitometry of the sample with lower concentration (0.2 mM) (Fig.4.14., lane 6) showed that transglycosylation products were present in the early stages of the reaction of tamarind oligosaccharides with the nasturtium enzyme, but disappeared again after a prolonged incubation with the enzyme. This result is in agreement with the general principle that transglycosylation reactions of this type occur at high substrate concentrations, where transfer of oligosaccharide-donor on to water molecules (=hydrolysis) is overtaken by

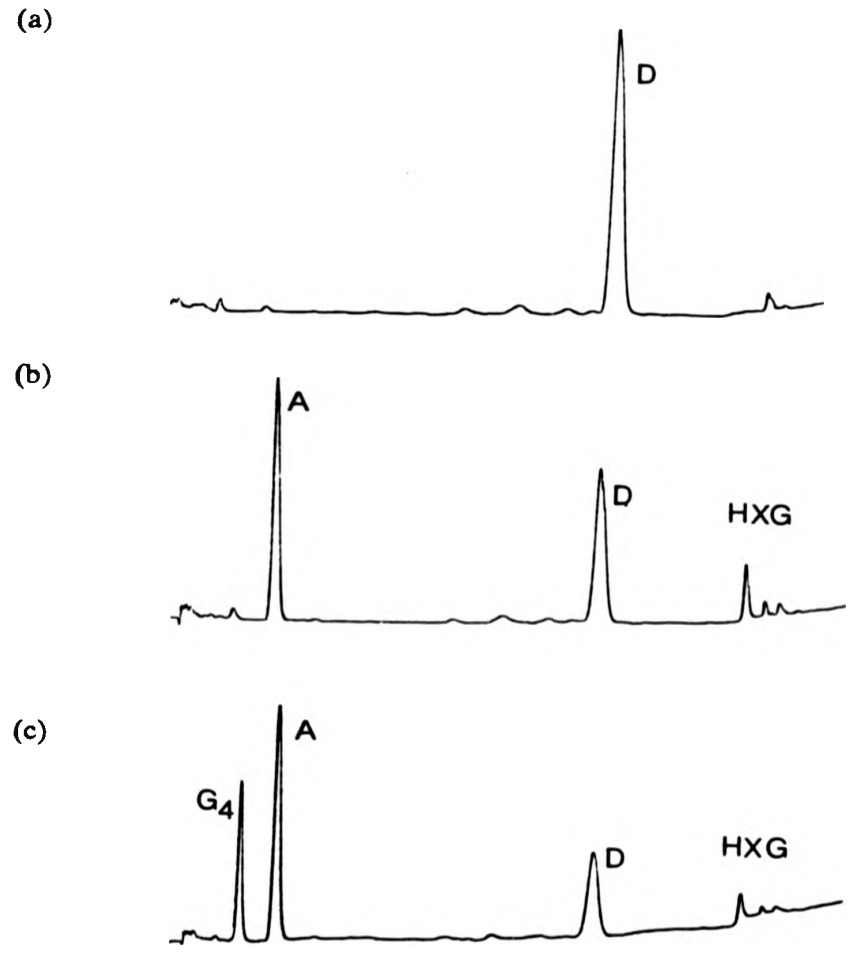


Fig.4.13. Incubation of the nasturtium xyloglucan *endo*-transglycosylase with xyloglucan oligosaccharides and cello-oligosaccharides. (a): Oligosaccharide D alone; (b): Oligosaccharide D (0.8 mM, final volume 25 μl) treated with the enzyme (0.25 μg); (c): Oligosaccharide D (0.6 mM) treated with the enzyme (0.25 μg) in the presence of cellotetraose (G_4, 0.32 mM). A = oligosaccharide A. HXG = higher xyloglucan oligosaccharides.

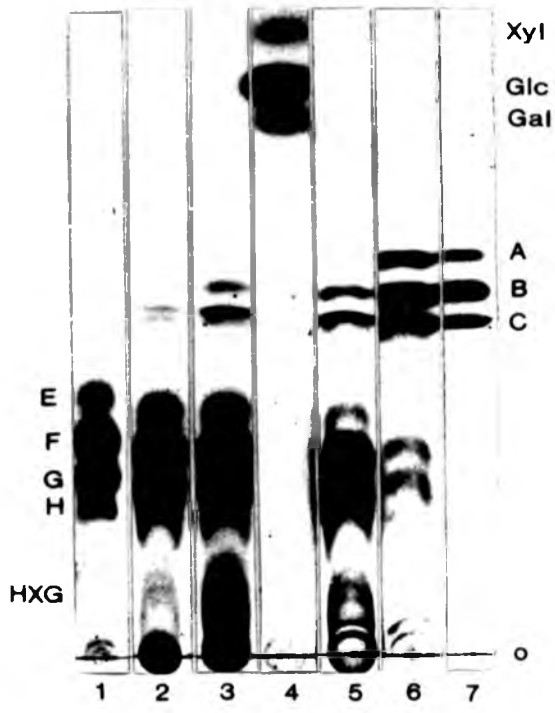
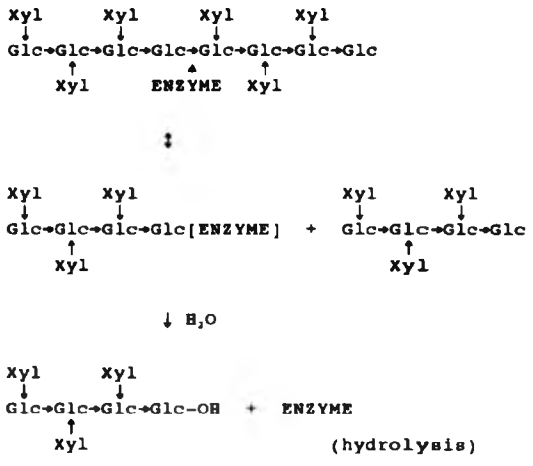


Fig.4.14. TLC analysis of the action of the nasturtium xyloglucan *endo*-transglycosylase on xyloglucan oligosaccharides: effect of substrate concentration. *Lane 1:* Xyloglucan oligosaccharides E, F, G and H (6 mM). *Lanes 2 and 3*: Oligosaccharides E to H (final concentration 3.0 mM), incubated 2 and 19 h, respectively, with the enzyme. *Lanes 5 and 6*: Oligosaccharides E to H (final concentration 0.2 mM), incubated 2 and 19 h, respectively, with the enzyme. *Lane 7*: xyloglucan oligosaccharides A, B and C. *Lane 1*: monosaccharide standards, D-xylose, D-glucose and D-galactose. HXG = higher xyloglucan oligosaccharides.

transfer on to hydroxyl groups of the carbohydrate molecules acting as glycosyl-acceptors (=transglycosylation). The reaction of hydrolysis catalysed by the nasturtium enzyme is represented below:



The action of the nasturtium endoglucanase was then examined by treating the native tamarind xyloglucan at different concentrations ranging from 0.5 % to 0.01% with the enzyme. The enzyme-substrate incubations were carried out as described before and aliquots of each sample were spotted on a TLC plate. In this case the large oligosaccharides were not precipitated by ethanol, and the whole sample was analysed by thin layer chromatography. Although increasing proportions of oligosaccharides A, B and C were produced by the enzyme following the decrease of substrate concentration, a considerable amount of high molecular weight material was still present in the enzyme digest of xyloglucan, even at the lowest substrate concentration (Fig.4.15.).

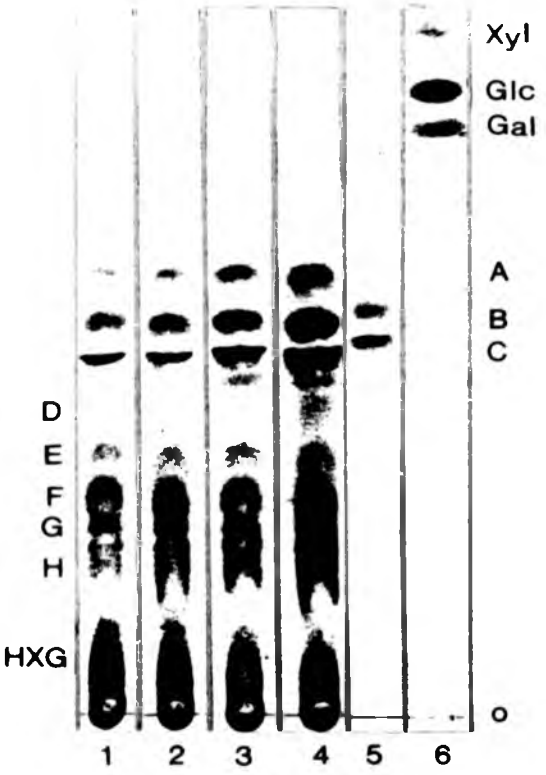


Fig.4.15. TLC analysis of the action of the nasturtium xyloglucan *endo*-transglycosylase on tamarind xyloglucan: effect of substrate concentration.

Lanes 1 to 4: xyloglucan polysaccharide at a final concentration of 5 mg/ml, 2.5 mg/ml, 0.25 mg/ml, 0.1 mg/ml, respectively, treated with the nasturtium enzyme (19 h). *Lane 5*: xyloglucan oligosaccharides A, B and C. *Lane 6*: monosaccharide standards, D-xylose, D-glucose and D-galactose. HXG = higher xyloglucan oligosaccharides.

4.3.2.6. XYLOSE REMOVAL AND ITS EFFECT ON TRANSGLYCOSYLATION.

Treatment of xyloglucan saccharides with the nasturtium α-xylosidase was carried out to determine whether the xylosyl residue situated on the non-reducing end of a xyloglucan sacccharide molecule was an essential requirement for transglycosylation. Oligosaccharide D was treated with the enzyme α-xylosidase and the removal of xylose was monitored by HPLC analysis, and pentose determination when higher amounts of substrate were available. The enzyme α-xylosidase catalyzed the selective removal of a single xylose furthest from the reducing end of the oligosaccharide molecule (Fanutti *et al.*, 1991; Chapter 3). The xylose-depleted oligosaccharide D had a lower retention time on HPLC and it was thus separated from the intact oligosaccharide D (Fig.4.16.a and b). Oligosaccharide D and the xylose-depleted oligosaccharide D' were individually incubated with the nasturtium *endo*transglycosylase and the enzyme reaction was monitored by HPLC. Oligosaccharide D was converted to the heptasaccharide A and the saccharide A→A→A and higher oligomers as shown before. The xylose-depleted oligosaccharide D' incubated with the nasturtium enzyme generated several compounds with lower and higher retention times than oligosaccharide D' (Fig.4.16.d). An oligosaccharide with retention time lower than A, identified as the hexasaccharide A' with composition Glc_4Xyl_2, released by hydrolysis of oligosaccharide D', was produced in addition to heptasaccharide A. Several saccharides with retention times higher than D' were also present in the final reaction mixture as indicated by HPLC, suggesting that they were

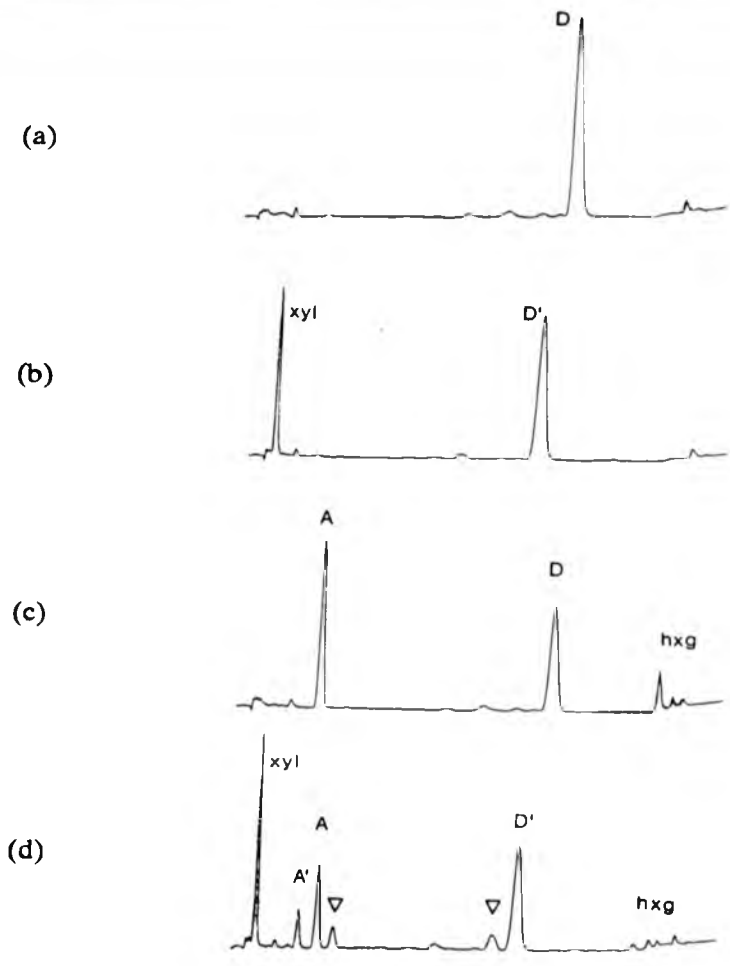


Fig.4.16. HPLC analysis of the action of the nasturtium xyloglucan *endo*-transglycosylase with xyloglucan oligosaccharides: effect of selective removal of the xylosyl residue attached to the glucose furthest from the reducing end of the oligosaccharide. (a): Oligosaccharide D alone; (b): Oligosaccharide D (0.8 mM) treated with the nasturtium α-xylosidase. (c): Oligosaccharide D (0.8 mM) treated with the *endo*-transglycosylase (0.25 μg/25 μl of saccharide solution); (d): Xylose-depleted oligosaccharide D' (0.8 mM) treated with the *endo*-transglycosylase. A = oligosaccharide A; A' = xylose-depleted A; hxg = higher xyloglucan oligosaccharides. The two compounds with unknown molecular structure are indicated by arrows.

transglycosylation products. Two new oligosaccharides were also present in the reaction mixture with retention times greater than A and lower than D', respectively. These saccharides could not derive from hydrolysis of D' itself since this would generate only heptasaccharide A and hexasaccharide A'. Their molecular structures were investigated by using specific hydrolases and by HPLC and TLC analysis of the enzyme digestion products.

(i) It was shown in Chapter 3 that treatment of a xyloglucan oligosaccharide DP = n with the nasturtium α-xylosidase produced an oligosaccharide with decreased molecular weight DP = n-1 but with the same TLC mobility. It was also shown that removal of one galactosyl or one glucosyl residue from the oligosaccharide DP = n determined a shift in the TLC mobility of the compound DP = n-1. The oligosaccharide mixture produced by treatment of D' with the nasturtium *endo*glucanase was analysed by TLC (Fig.4.17.). The results showed that hexasaccharide A' co-migrated with heptasaccharide A and tri-decasaccharide D' co-migrated with tetra-decasaccharide D. Two new compounds were also present in the reaction mixture, one with lower TLC mobility than heptasaccharide A (DP>A) and the other with higher mobility than tetradecasaccharide D (DP<D). Since these oligosaccharides contained only glucose and xylose it was deduced that the difference in mobility was caused by decrease/increase of one or more glucosyl residues, probably one given the small mobility changes. This suggested that the former (DP>A) contained an extra glucosyl residue with respect to heptasaccharide A and the latter (DP<D) contained one less glucosyl residue than tetradecasaccharide D. Higher molecular weight saccharides were present and had even lower TLC mobilities.

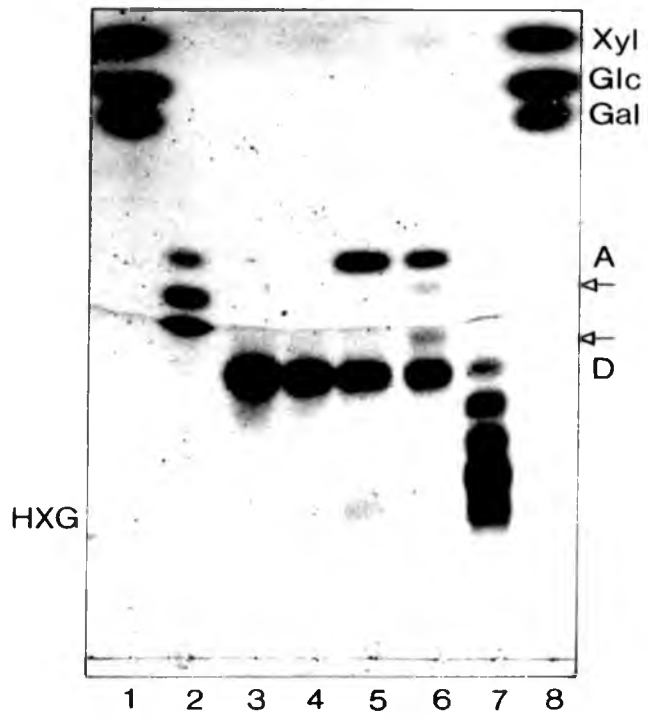


Fig.4.17. TLC analysis of incubations of oligosaccharide D and xylose-depleted oligosaccharide D' (0.8 mM) with the nasturtium enzyme.

Lanes 1, 8: monosaccharide standards glucose, galactose and xylose; *Lane 2*: oligosaccharides A, B and C; *Lane 3*: tetradecasaccharide D; *Lane 4*: xylose-depleted oligosaccharide D'; *Lane 5*: incubation of oligosaccharide D with the nasturtium enzyme; *Lane 6*: incubation of oligosaccharide D' with the nasturtium enzyme; *Lane 7*: saccharides D, E, F, G and H. HXG = higher xyloglucan oligosaccharides. The two compounds with unknown molecular structure are indicated by arrows.

(ii) The saccharide mixture obtained by digestion of xylose-depleted tetradecasaccharide D' with the nasturtium *endo*glucanase was treated with α-xylosidase and the products of the enzyme reaction were compared against the untreated material by HPLC analysis (compare Fig.4.16. and Fig.4.18.a). It was observed that heptasaccharide A had disappeared following treatment with α-xylosidase and the peak previously identified as the hexasaccharide Glc_4Xyl_2 became higher, indicating that all xylosyl residues had been removed from the heptasaccharide A. The retention time of the saccharide with DP>A had not decreased indicating that it did not contain xylosyl residues on the non reducing end of the molecule. The peak corresponding to saccharide DP<D had moved as indicated by the different retention time of the new compound suggesting that it contained a xylosyl residue on the non reducing end of the molecule. As expected there was no change in the retention time of the xylose-depleted saccharide D'.

(iii) The saccharide mixture obtained by digestion of xylose-depleted tetradecasaccharide D' with the nasturtium *endo*glucanase was treated with a nasturtium ß-glucosidase preparation and the enzyme reaction was monitored by HPLC (compare Fig.4.16. and Fig.4.18.b).

The hexasaccharide A' was a substrate for ß-glucosidase since it contained an unsubstituted non-reducing glucose. The product of the enzyme reaction was a pentasaccharide Glc_3Xyl_2 eluted with a retention time lower than A' (A'g, Fig.4.18.). The disappearance of the peak corresponding to saccharide D' was accompanied by the appearance of a new compound (D'g, Fig.4.18.) with lower retention time indicating that an unsubstituted glucosyl residue had

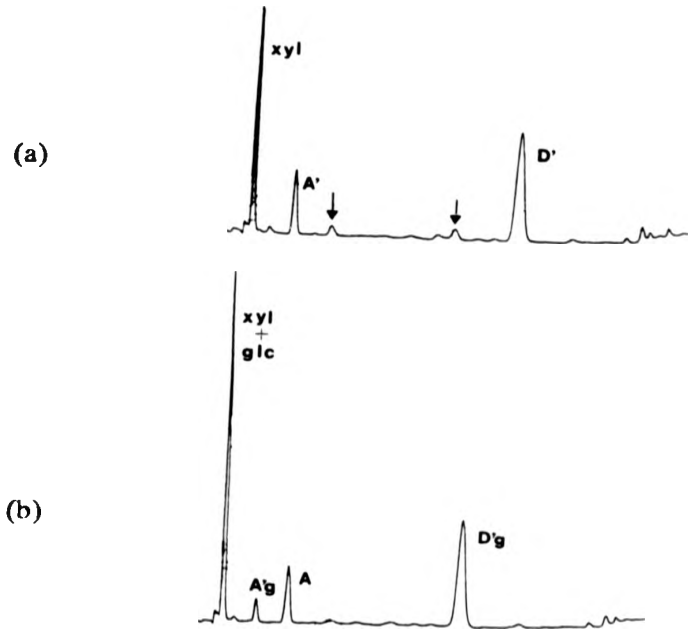
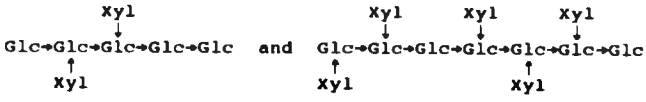


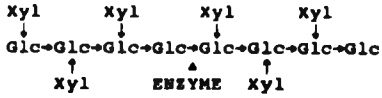
Fig.4.18. HPLC analysis of enzyme treatment of the products obtained on incubating xylose-depleted oligosaccharide D' with the nasturtium *endo*glucanase. (a): Treatment with the nasturtium α-xylosidase; (b) Treatment with the nasturtium ß-glucosidase. A = oligosaccharide A; A' = xylose-depleted oligosaccharide A; A'g = glucose-depleted oligosaccharide A'; D'g = glucose-depleted oligosaccharide D'. The two unknown saccharides are indicated with arrows. Starting mixture is shown in Fig.4.16.d.

been removed from the non reducing end of the saccharide molecule. Heptasaccharide A was not a substrate for ß-glucosidase and the retention time was unchanged. The peak corresponding to saccharide DP>A had disappeared indicating that it contained a non-reducing unsubstituted glucose and it was co-eluted with heptasaccharide A. The peak for saccharide DP<D was not identified and it was probably co-eluted with glucose-depleted saccharide D'.

These observations were entirely consistent with the following structures for the two unknown compounds. The structures of these oligosaccharides is reported below:



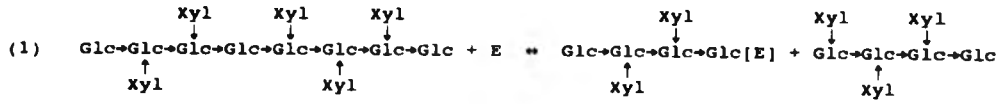
The formation of these two oligosaccharides was rationalised as follows. The nasturtium enzyme cleaves xyloglucan oligosaccharides, as illustrated below, for oligosaccharide D:

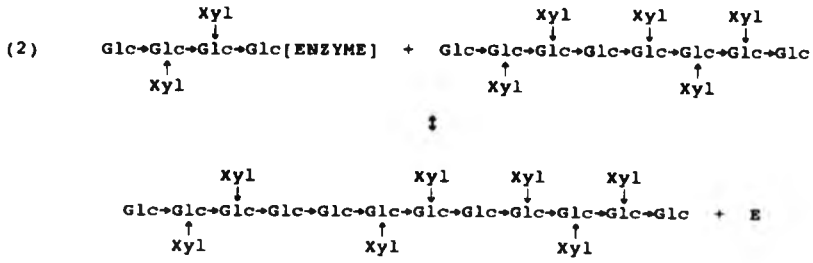


The continued transglycosylation of xylose-depleted oligosaccharide D' indicated that xylosyl residues located on the non-reducing end of a saccharide molecule were not an essential requirement for transglycosylation.

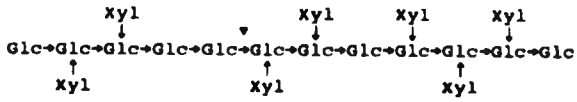
The transglycosylation of oligosaccharide D' would thus generate a trimer

A'→A'→A and a heptasaccharide A in the reaction as shown below:

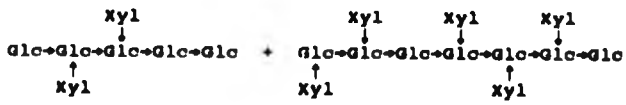




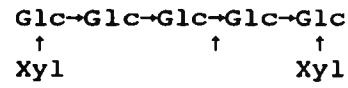
The appearance of compounds with 5 and 7 glucose residues (structures above) would then arise by postulating that the trimer product in the transglycosylation reaction had a new cleavage site for the hydrolysis, not present in the D' molecule. This site is clearly identifiable and is on the opposite side of the molecule:



Hydrolysis at the new site would generate the following saccharides (the structures of which were deduced above):



These results provide clear evidence that the transglycosylation action of the enzyme is not prevented when the glycosyl-acceptor does not carry a xylosyl substituent on the terminal non-reducing glucose. They are also very strongly suggestive that the substrate subsite recognition pattern for the enzyme is the sequence:



with cleavage as indicated. The implications of these observations are considered in the Discussion.

4.3.3. THE PRESENCE OF XYLOGLUCAN-SPECIFIC TRANSGLYCOSYLASES IN OTHER NASTURTIUM TISSUES AND IN OTHER PLANTS.

The nasturtium *endo*glucanase was able to catalyse the *in vitro* transglycosylation as well as the hydrolysis of xyloglucan. The possibility that *endo*glucanases with the same specificity as the nasturtium enzyme may be present in different tissues of the nasturtium plant (leaves and stems) or in different plants (tomato, tobacco) was investigated.

Crude enzyme extracts were prepared essentially with the same procedure used for homogeneization of germinated nasturtium cotyledons. The following plant tissues were used: 13 day-old nasturtium stems and leaves (and seeds, as control); 15 day-old tobacco stems and leaves; 21 day-old tomato stem and leaves. Crude enzyme extracts were analysed by SDS-polyacrylamide gel electrophoresis and

transferred on to nitrocellulose membranes. The polyclonal antibody raised against the nasturtium *endo*glucanase protein (Stronach, 1991) was used to localize the protein in the western blots. The results obtained showed that a protein band with a molecular weight corresponding to 29 KDa (the same molecular weight of the enzyme protein) was recognized by the antibody (Fig.4.19.). Other protein bands were also picked up by the anti-*endo*glucanase antibody which may derive from non-specific binding of the polyclonal serum with other protein determinants present in the crude enzyme extract. In almost all the enzyme extracts from nasturtium, tomato and tobacco tissues the same protein band was recognized by the nasturtium endoglucanase antibody. These findings do not prove that enzymes of the same specificity are present. They are, however, indicative that xyloglucan-specific *endo*glucanases like the nasturtium seed enzyme may play a role in the metabolism of xyloglucan and its turnover in stems and leaves during the plant growth and development (see conclusions, Chapter 6).

4.4. DISCUSSION.

4.4.1. SPECIFICITY OF THE NASTURTIUM *ENDO*-(1→4)-ß-D-GLUCANASE.

The nasturtium *endo*-(1→4)-ß-D-glucanase specifically attacks and cleaves xyloglucan-type of molecules. No other polysaccharides which are generally substrates for *endo*-(1→4)-ß-D-glucanases (cellulases) are hydrolysed by the

Fig.4.19. Immuno-localization using the specific anti-[xyloglucan-*endo*-transglycosylase] antibody (M. Stronach, 1991). Western blots of the purified *endo*transglycosylase from nasturtium (E) and crude extracts from nasturtium stems (St), leaves (L) and seeds (Se); tobacco stems (Tob S) and leaves (Tob L); tomato stem (Tom S) and leaves (Tom L).

nasturtium enzyme. These include cellulose, carboxymethylcellulose and hydroxyethylcellulose, ß-(1→3,1→4)-glucans and cello-oligosaccharides (Edwards *et al.*, 1986). The nasturtium enzyme clearly acts in an *endo*glucanase fashion cleaving internal ß-(1→4) bonds between D-glucopyranosyl residues. Digestion of xyloglucan with the nasturtium *endo*glucanase gives a mixture of oligosaccharides differing in molecular weight. Three oligosaccharides A, B and C have been identified as a heptasaccharide Glc_4Xyl_3, an octasaccharide Glc_4Xyl_3Gal and a nonasaccharide $Glc_4Xyl_3Gal_2$, respectively (Fanutti *et al.*, 1991; Chapter 3). The higher molecular weight saccharides D, E, F, G and H are tetra- to octa-decasaccharide dimers of oligosaccharides A, B and C. Digestion of tamarind xyloglucan with the fungal cellulase gives only three oligosaccharides A, B and C indicating that tamarind xyloglucan is composed essentially of repeating oligosaccharidic units. This observation confirms the data of York *et al.* (1990) and indicates that the fungal cellulase cleaves the xyloglucan backbone after each unsubstituted glucose residue (York *et al.*, 1990).

Although *T. viride* cellulase and nasturtium *endo*-(1→4)-ß-D-glucanase cleave xyloglucan at common cleavage sites, it is clear that the two enzymes must have different specificities, since (1) xyloglucan is completely hydrolysed by the fungal cellulase and only partially hydrolyzed by the nasturtium enzyme; (2) the fungal cellulase is able to attack cellulose as well as xyloglucan, whereas the nasturtium *endo*glucanase is xyloglucan-specific.

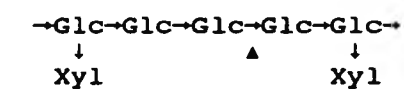
From current data it seems clear that the presence of xylosyl-substituted glucoses is required in order for the nasturtium enzyme to recognize and cleave the xyloglucan backbone. In the (1→4)-ß-D-glucopyranose glucan backbone every glucosyl residue -in the preferred conformation- is rotated relative to the next by

180°; it follows then that one side of the molecule will carry twice as many xylosyl substituents than the other. The xyloglucan molecule with xylosyl substituents located above and below the the glucan backbone is depicted below:

By analogy with other *endo*-acting enzymes such as lysozyme, which requires a binding site of 6 residues (Stryer L., 1988), or *endo*-mannanase, with a binding subsite of 5 residues (McCleary and Matheson, 1983) it is reasonable to postulate that the fungal cellulase may be able to approach the xyloglucan molecule on the less xylosyl-substituted side of the molecule recognising cellotriose sequences and cleaving as indicated above (the arrow represents the cleavage site of the fungal cellulase and the nasturtium enzyme). Computer modelling of the xyloglucan molecule (Levy *et al.*, 1991) shows that in the conformations corresponding to a minimum energy, the potential binding site -G-G-G- is exposed in the less xylosyl substituted region of the molecule. Since xylosyl-residues constitute a necessary requirement for the enzyme activity, the binding site for this enzyme on the xyloglucan molecule must also include the xylose residue(s).

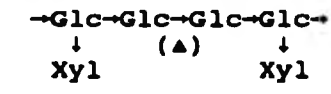
If xylosyl substituents located on the more crowded side of the xyloglucan molecule were involved in the binding with the nasturtium enzyme, then one might expect a complete suppression of transglycosylation activity when xylose-depleted oligosaccharides are used as substrate. The results obtained clearly demonstrated that removal of a single unsubstituted xylosyl residue from the non-reducing end of a tetra-decasaccharide molecule does not prevent transglycosylation, indicating that this xylose is not necessary for enzyme

recognition and cleavage. Moreover the removal of xylose from the non-reducing end and subsequent transglycosylation of the tetra-decasaccharide by the nasturtium enzyme creates a new cleavage site on the xyloglucan molecule which is identical to the heptasaccharide motif depicted below:



Thus it is likely that one or both of the xylosyl residues flanking the cleavage site are recognized by the enzyme.

Tamarind xyloglucan is not a perfect regular structure composed by $Glc_4Xyl_3Gal_{0to2}$ repeating units. It is interrupted by the presence of low amounts of a pentasaccharide Glc_3Xyl_2. This compound is however found only when tamarind xyloglucan is digested with the fungal cellulase. The absence of such oligosaccharide when tamarind xyloglucan is incubated with the nasturtium *endo*glucanase indicates that the nasturtium enzyme is unable to catalyze the release of oligosaccharides with a glucose backbone lower than cellotetraose residues. It could be argued that the amounts of the pentasaccharide are too low to be detected by TLC or HPLC analysis. However *endo*glucanase digestion of barley xyloglucan, which contains large proportions of pentasaccharide (Kato *et al.*, 1981), confirms the absence of this compound in the reaction mixture. This indicates that the potential recognition site on the barley xyloglucan may not accommodate the binding domain of the enzyme and the cleavage site is not accessible:



This indicates that structural "domains" in the xyloglucan molecule which might give pentasaccharide on cellulase hydrolysis are not adjacent to potential cleavage sites for the nasturtium enzyme. They may be in relatively unsubstituted areas of the molecule.

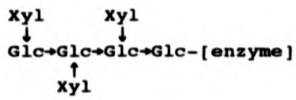
The HPLC analysis of oligosaccharide A, B_1, B_2 and C released by digesting tamarind xyloglucan with the nasturtium *endo*glucanse shows very clearly that the cleavage of the xyloglucan backbone is non-random, and there is a striking discrepancy between the A:B_1:B_2:C ratio obtained by treatment with the nasturtium enzyme and that expected for a complete hydrolysis of the polysaccharide (using the fungal cellulase). Since the only structural difference of oligosaccharides A, B_1, B_2 and C is the presence of galactose on the side chains of the oligosaccharides B and C it follows that galactosylation of xylosyl residues affects the enzyme recognition and cleavage. The galactosylation of xylosyl residues has been shown to induce a sharp kink at the 1→2 linkage and the galactose residue folds around the backbone of the xyloglucan molecule (Fry, 1989; Levy *et al.*, 1991). This suggests that certain galactosyl residues may prevent the enzyme from cleaving by masking the binding site on the xyloglucan molecule. If galactosylation is a general discouraging feature, this may explain the presence of higher oligosaccharides B_2 and C (with galactose attached to the xylosyl residue next to the unsubstituted glucose) in the ethanol-insoluble fraction of the *endo*glucanase digest. The appearance of higher amounts of oligosaccharides A and B_1 in the incubation of xyloglucan with the nasturtium enzyme indicated that the presence of these oligosaccharides (with a single galactose attached to xylosyl residues located below the xyloglucan chain or with no galactosyl substitution) is a feature which may promote cleavage. The relatively low amounts of

oligosaccharides B_2 and C with galactosyl substitution of both xylosyl residues next to the reducing end of the molecule appears to be a discouraging feature.

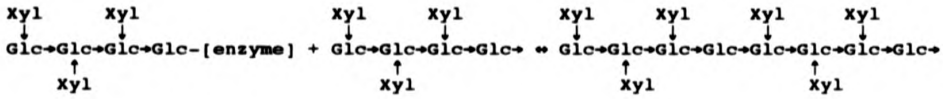
4.4.2. TRANSGLYCOSYLATION OF THE XYLOGLUCAN OLIGOSACCHARIDES BY THE NASTURTIUM *ENDO*GLUCANASE.

From this investigation it is clear that the nasturtium *endo*glucanase is a transglycosylase. Many plant and animal hydrolases have been reported to be able to catalyze the "inverse" hydrolysis reaction by transferring a saccharide donor on to a saccharide acceptor when high substrate concentrations are present in the reaction mixture (Cote and Tao, 1990). Transglycosylation can be regarded as an alternative to hydrolysis (Fry, 1988) in which the enzyme catalyses the cleavage of the glycosidic bond and transfers the saccharide donor to another sugar (= transglycosylation) instead of transferring it to water (= hydrolysis). High substrate concentrations are generally required in order to obtain transglycosylation, since low substrate concentration increases the probability of a glycosyl-transfer onto water acceptors. However the present investigation demonstrates that the nasturtium enzyme is unable to catalyze the complete hydrolysis of xyloglucan even when very low substrate concentrations (up to 0.8 μM) were treated with the enzyme making it impossible to calculate the K_{hyd}. It is therefore more appropriate to term this enzyme *endo*transglycosylase rather than *endo*glucanase. To explain the molecular mode of action of this enzyme it can be postulated that the hydrolysis reaction may simply represent the

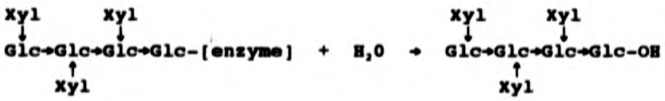
dissociation of an enzyme bound intermediate species of the type:



before transfer to the specific glycosyl-acceptor has taken place. Therefore it may be possible that the **life-time** of the oligosaccharide-[enzyme] intermediate is the factor determining hydrolysis (= transfer to water molecules) or transglycosylation (= transfer on to xyloglucan acceptor) and not the concentration of water molecules in the system (Fanutti *et al.*, 1993). In this case the transfer onto a glycosyl-acceptor of the oligosaccharide-[enzyme] intermediate would be a fast reaction:



and the transfer onto water molecules would be a slow reaction. The increased hydrolysis observed in the first stages of the enzyme reaction on high polymeric xyloglucan could be explained by the low concentration of glycosyl-acceptors available. In this condition the decomposition of the intermediate complex (= hydrolysis) would be able to compete with the more rapid transglycosylation reaction:



The nasturtium xyloglucan-specific *endo*glucanase is the only example yet known

together with the xyloglucan *endo*-transglycosylase purified from *Vigna* (Nishitani and Tominaga, 1992) of enzymes able to attack and cleave specifically the backbone of xyloglucan types of polysaccharides and able to synthesize larger saccharides by transglycosylation of xyloglucan fragments. From this and other investigations (York *et al.*, 1990) it is clear that the fungal *endo*glucanase purified from *T. viride* catalyzes the total hydrolysis of tamarind and primary cell wall xyloglucan indicating that this enzyme does not catalyze the transglycosylation of xyloglucan oligosaccharides. The presence of side-chains seems to be an essential requirement for the transfer of oligosaccharide units on to xyloglucan fragments since the nasturtium enzyme is unable to transfer xyloglucan saccharides on to cello-oligosaccharides Glc_2 and Glc_4 acceptors. This observation confirms the specificity of the nasturtium transglycosylase towards xyloglucan substrates. Galactosylation of xyloglucan side-chains is also an important feature for the action of the enzyme. Characterization of tetra- to octa-decasaccharides products of digestion with the nasturtium *endo*transglycosylase has shown that monomers A and B_1 are preferentially found on the reducing end of the molecule, whereas monomers of the type B_2 and C are found predominantly in the non-reducing end of the oligoxyloglucan molecules. This indicates that oligosaccharides A and B_1 are good glycosyl-acceptors and poor glycosyl-donors in the transglycosylation reaction and indicates that B_2 and C are very good glycosyl-donors and poor glycosyl-acceptors.

The unusual enzyme specificity of the nasturtium enzyme is highlighted by the recent finding that there is essentially no sequence homology between the nasturtium enzyme and plant, bacterial or fungal cellulases (de Silva *et al.*, 1993). Computer matches of cDNA of this and other plant proteins have however found

some homology (about 50%) with a gene (meri-5) found in *Arabidopsis thaliana* the expression of which seems to be high in rapidly dividing tissues such as apical meristem. At the present time its function is still unknown (de Silva *et al.*, 1993). Proteins with similar structure to the nasturtium enzyme were sought in crude enzyme extracts from nasturtium stem and leaves, tomato stem and leaves and tobacco stem and leaves. Western blotting of the extracted proteins and successive serial incubations with the specific antibody raised against the nasturtium *endo*glucanase protein indicates that a cross-reacting protein band with identical molecular weight (29 KDa) was present in other tissues of nasturtium seedlings and also in other plant species. The antibody cross-reacted with other proteins. To demonstrate clearly that protein(s) similar to the xyloglucan-specific *endo*glucanase are present in vegetative tissues of nasturtium or in different plants systems, more substantial evidence must be collected and the future of the research will be discussed in Chapter 6.

The interest in *endo*glucanases with the same specificity of the nasturtium enzyme is determined by the recent evidence of a possible involvement of a xyloglucan *endo*-transglycosylase in the turnover and in the elongation of the primary cell wall (Cleland, 1971; Albersheim, 1976; Heyn, 1981; Hayashi, 1989; Smith and Fry, 1991; Farkas *et al.*, 1992; Fry *et al.*, 1992; Maclachlan and Brady, 1992; Nishitani and Tominaga, 1992) and this will also be discussed in Chapter 6.

CHAPTER FIVE

ENZYMIC MODIFICATION OF TAMARIND XYLOGLUCAN USING ß-D-GALACTOSIDASE PURIFIED FROM GERMINATED NASTURTIUM COTYLEDONS.

5.1. INTRODUCTION.

The activity of the enzyme ß-galactosidase was found to increase and decrease with the rate of the mobilization of the xyloglucan (Edwards *et al.*, 1985) and it was therefore correlated with the hydrolysis of the polysaccharide after germination in nasturtium cotyledons.

This enzyme is involved in the hydrolysis of xyloglucan side-chains from which it removes terminal (1→2)-ß-D-galactopyranosyl residues. Xyloglucan was shown to be the natural substrate for the nasturtium ß-galactosidase (Edwards *et al.*, 1988). This enzyme is able to attack the native xyloglucan as well as the oligosaccharides generated by hydrolysis of the polysaccharide with the nasturtium xyloglucan-specific *endo*-transglycosylase or with the fungal cellulases (Edwards *et al.*, 1988). It was also observed that the nasturtium ß-galactosidase is able to cleave to a limited extent the (1→4)-ß-D-galactopyranosyl residues of galactans (Edwards *et al.*, 1988).

Athough there are several reports on ß-galactosidases from plant tissues, only few enzymes have been purified to homogeneity and characterized. Several authors have suggested that ß-galactosidases are involved in the turnover of pectic substances in the primary cell wall, as some of these enzymes catalyze the hydrolysis of (1→4)-ß-D-galactans (Dopico *et al.*, 1990; Konno *et al.*, 1986; Taiz, 1984). Other ß-galactosidases responsible for the solubilization of ß-galactose from galactans have been associated with the process of fruit ripening (Pressey, 1983; Fischer and Bennet, 1991). The finding that increased levels of ß-galactosidase activity were obtained in auxin-treated pea-stems, indicated the possible involvement of such enzymes in the metabolism of primary cell wall hemicelluloses (Tanimoto and Igari, 1976; O'Neil *et al.*, 1988).

Multiple forms of ß-galactosidases have been found in the germinating seeds of mung beans (*Vigna radiata* L.), including an enzyme with an unusually high molecular weight (162 KDa) isolated from the dry seeds (Dey, 1984). Their substrate specificities have not been yet investigated, and the role of these different ß-galactosidases is still unclear (Kundu *et al.*, 1990).

In an earlier investigation (Reid *et al.*, 1988) it was observed that aggregation of tamarind xyloglucan polysaccharide occurred after addition of high concentrations (i.e., 1 M) of sodium sulphate. The mechanism underlying this spontaneous process was possibly explained by the increase of intermolecular association of xyloglucan molecules (hydrophobic interactions) in presence of high salt concentrations.

In this Chapter the purified enzyme has been used to prepare galactose-depleted tamarind xyloglucan in the presence and in the absence of sodium sulphate and

the rheological and interactive properties of the modified xyloglucans have been investigated. The molecular mode of action of the nasturtium ß-galactosidase is also investigated with the view to understanding the nature of the intermolecular interactions observed during galactose removal from xyloglucan side-chains. The implications of the findings and the *in vivo* role of the enzyme ß-galactosidase during xyloglucan breakdown are discussed in Chapter 6.

5.2. MATERIALS AND METHODS.

Chemicals and buffer salts were of analytical grade or better. The second antibody, goat anti-rabbit IgG, was purchased from North-East, Biomedical Laboratories Ltd., Uxbridge, UK.

5.2.1. ENZYME ASSAYS.

The activity of the enzyme was monitored by the determination of *p*-nitrophenol released from *p*-nitrophenyl-ß-D-galactopyranoside, or by the determination of the galactose released during incubation of the enzyme with tamarind xyloglucan (or xyloglucan oligosaccharides) by the D-galactose dehydrogenase assay (Kurz and Wallenfels, 1974). The enzyme activity was routinely assayed at pH 5.0 and 30°C

using either 50 mM ammonium acetate buffer or McIlvaine phosphate-citrate buffer as described in Chapter 2.

The activity of the nasturtium α-xylosidase was monitored by the determination of free pentoses released upon hydrolysis of enzyme-depolymerized tamarind xyloglucan by the *p*-bromoaniline procedure of Roe and Rice (1948). The activity of ß-glucosidase was determined by the release of *p*-nitrophenol from *p*-nitrophenylglucoside, or by the determination of glucose released from oligosaccharide substrates by the hexokinase/glucose-6-phosphate-dehydrogenase procedure (Bergmeyer *et al.*, 1974). Protein was determined quantitatively by the procedure of Sedmak and Grossberg (1977), using bovine albumin as standard. Total carbohydrate was determined by the phenol-sulphuric acid (Dubois *et al.*, 1956) or by the anthrone assay (Dische, 1962). The reducing power of saccharide solutions was determined by the procedure of Halliwell and Riaz (1970). All the assay procedures are described in Chapter 2.

5.2.2. SEPARATION TECHNIQUES.

SDS-polyacrylamide gel electrophoresis and western blotting were carried out according to Laemmli (1970) and Towbin *et al.* (1979), respectively. Isoelectric focusing was carried out using Ampholine PAGplates (LKB Ltd) and carried out according to manufacturers' instructions. Analytical and preparative thin layer chromatography and high performance liquid chromatography were carried out

as described in Chapters 2 and 3.

5.2.3. PURIFICATION OF THE ENZYME.

Seeds, growth conditions and germination are described in Chapter 2. Cotyledons from 9-10 d seedlings were removed manually and homogenized in 0.2 M potassium phosphate buffer pH 7.2 and insoluble polyvinylpyrrolidone as before (sections 2.2.13.1. and 2.2.13.2.). The homogenate was freed of the undissolved material by centrifugation (19000 g for 30 min) and brought to 90% saturation with ammonium sulphate. The precipitate was collected after centrifugation (19000 g for 30 min) and re-dissolved in 20 mM Tris-HCl buffer pH 7.8. The enzyme extract was dialysed against the same buffer and was clarified by a further centrifugation (26000 g for 20 min) before anion-exchange chromatography.

5.2.3.1. ANION-EXCHANGE CHROMATOGRAPHY.

The crude enzyme extract was applied to a diethylaminoethyl-cellulose column (2.2 x 40 cm) and the column washed with the Tris-HCl buffer. The ß-galactosidase was eluted from the column in the wash containing unbound material.

Fractions containing high ß-galactosidase activity were pooled and concentrated by ammonium sulphate precipitation (90% saturation). The precipitated protein was collected by centrifugation (26000 g x 30 min). The pellet was dissolved in 50 mM sodium acetate buffer pH 5.0 and dialysed against the same buffer.

5.2.3.2. CATION-EXCHANGE CHROMATOGRAPHY.

The dialysed fractions from the previous step, which contained high ß-D-galactosidase activity, were applied to a column (2.2 x 30 cm) of CM-cellulose (Whatman CM52). The column was washed with the same buffer until absorbance at 280 nm was constant. The column was then eluted with a sodium chloride gradient (0-0.5 M in the same buffer over 8 bed volumes). the flow-rate was 50 ml h^{-1} and 10 ml fractions were collected. Fractions containing high ß-D-galactosidase activity (in the sodium chloride gradient) were pooled and protein precipitated by addition of ammonium sulphate up to 90% saturation. The suspension was left for 1-2 h at 4°C and spun at 26000 g for 20 min. The pellet was dissoved in a minimum volume (2-3 ml) of 50 mM ammonium acetate buffer pH 5.0 and applied to a Bio gel P60 column.

5.2.3.3. GEL FILTRATION.

The column (2.5 x 70 cm) was eluted with 50 mM ammonium acetate buffer pH 5.0. The flow rate was 2.0 ml h^{-1} and 4 ml fractions were collected. Fractions containing ß-D-galactosidase activity were pooled, aliquoted and frozen at -20°C.

5.2.4. CHROMATOFOCUSING.

The purified ß-galactosidase was concentrated by addition of ammonium sulphate (90% saturation) and the precipitate collected after centrifugation (19000 x g). The pellet was resuspended in a small volume (2-3 ml) of 25 mM Tris-acetate buffer pH 8.3 and dialyzed against the same buffer.

The column (40 x 1 cm) was packed with PBE- (polybuffer-exchanger) 94, pH range 8.0-6.0. The gel was degassed prior use. It was washed with 1 M NaCl (250 ml) and then with the starting buffer, 25 mM Tris-acetate pH 8.3 (250 ml). The sample was applied to the column and eluted with a gradient of polybuffer 96-acetate (325 ml). The eluant was prepared according to manufacturer's instructions by diluting polybuffer-96 1:13 with water and adjusting the pH to 6.0 with HCl. The flow-time was reduced to 15 ml/h and 3 ml fractions were collected and used for protein and enzyme determination. IEF (described in Chapter 2) was

performed on the sample before and after chromatofocusing. Fraction were also analysed by SDS-polyacrylamide gel electrophoresis.

5.2.5. ANTIBODIES TO ß-GALACTOSIDASE.

Preparative SDS-gel electrophoresis was carried out using the purified ß-galactosidase (150-200 μg). The gel was stained for few minutes with Coomassie Blue and the top band, corresponding to the enzyme, was cut out of the gel and placed at -20°C for 2 h. The ice was shaken off and the gel homogenized in the minimum volume of PBS (1 ml). The initial injection of the rabbit (New Zealand White) contained 100 μg of purified enzyme (1 ml) and Freund's complete adjuvant (1 ml). The injection was administered subcutaneously. Two weeks later a booster injection containing the same amount of protein and 1.25 ml of Freund's incomplete adjuvant was administered in the same way. A third injection was performed two weeks later with protein and incomplete Freund's adjuvant as before. Blood was collected before the immunization (control for pre-immune serum) and thereafter at 14 day intervals.

The blood was allowed to clot for 1 h at room temperature and was then placed at 4°C overnight. The precipitate was then removed by centrifugation (12000 rpm x 10 min), and the serum aliquoted and stored at -70°C.

5.2.5.1. IMMUNOBLOTTING AND ANTIBODY LOCALIZATION OF ß-GALACTOSIDASE.

Protein transfer onto nitrocellulose membranes (western blotting) was carried out as described in Chapter 2. The nitrocellulose membrane containing the purified ß-galactosidase or a crude enzyme extract was washed a few times with the starting buffer (0.2% gelatin in PBS buffer containing Triton X 100, as described in 2.2.4.) and then incubated with the antiserum diluted using the starting buffer. Typical serum dilution used ranged from 1:200 to 1:2000. The incubation was carried out for 1 hour at room temperature, shaking gently. The unbound antibody was removed by three consecutive washes with starting buffer only. The treatment with the second antibody was carried out by incubating the nitrocellulose membrane with the goat anti-rabbit antibody (fraction IgG) diluted 1:1000 with starting buffer. The unbound antibody was removed by three consecutive washes with starting buffer. The membrane was then incubated a few minutes with 0.05% diaminobenzidine in 50 mM Tris-HCl pH 8.0, and staining was achieved by addition of 5 μl hydrogen peroxide (30% v/v). As the staining appeared, the immnunoblot was quickly washed with water and then with 2% SDS. It was then dried in filter paper and stored in a dark and dry place.

5.2.6. ENZYMIC DIGESTION OF OLIGOSACCHARIDES.

The purified saccharides (0.2-1.0 mg) were dissolved in 50 mM ammonium acetate pH 5.0 in a final volume of 50-100 μl and incubated with the fungal cellulase (0.2-1.0 U) or with the nasturtium *endo*glucanase (0.2-1.0 μg protein). Incubations of xyloglucan oligosaccharides with the nasturtium α-xylosidase were carried out by treating the saccharides (0.2-1.0 mg) with 0.2-1.0 μg enzyme protein in a final volume of 20-100 μl. Incubations of oligosaccharides with the nasturtium ß-glucosidase enzyme extract were carried out by treating the saccharides (0.2-1.0 mg) with 0.5-2.0 μg of enzyme extract in a final volume of 20-100 μl. All incubations were carried out overnight at 30°C using 50 mM ammonium acetate buffer pH 5.0, and stopped by heat-denaturation of enzyme(s).

5.2.7. RHEOLOGICAL MEASUREMENTS.

The Bohlin rheometer (Bohlin Reology, UK Ltd., Herts, UK) was equipped with a chamber containing the mixture of enzyme and xyloglucan, which was subjected to small oscillatory deformations. The measurements were taken under the supervision of Dr M. J. Gidley and Mr D. Cook in Unilever Laboratory, Colworth House, Sharnbrook, Bedford. The parameters referred to the changes in viscosity (η) over time, storage and loss components (G' and G'', respectively) and tan δ

defined as the ratio G''/G'. Gelation was monitored by the increase of G'and the decrease or steady status of G''.

5.2.7.1. STRESS AND DEFORMATION IN DYNAMIC TEST.

The deformation produced by a force acting on a surface is defined as stress and it is measured in Pascals (1 Pa = 1 N m^{-2}). The type of deformation induced is variable and depends on the nature of the material and it is referred as strain. Unlike stress, strain has not a standard unit of measurement as it depends on the type of deformation produced. For instance, a deformation involving a sliding movement of a layer contained in a plane respect to an adjacent layer parallel to the first one is described as simple shear strain and it is expressed as δ l/l. In pratice, many rheometers vary the strain and measure the force (stress) produced in the sample (Whorlow, 1980).

If the stress is removed after deformation has occurred, the strain may or may not return to zero. A solution of xyloglucan is referred as a viscoelastic material in that the recovery is not immediate. Immediate recovery occurs in elastic materials, i.e. ideal solids. Generally "thick" materials respond as ideal solids only for very small strains, the magnitude of which depend on the nature of the material. In the case of liquids the stress depends upon the rate of change of deformation rather than the amount of deformation. It is therefore important to define the concept of viscosity which describes the ratio of stress to rate of strain. Units of viscosity

are Pascal seconds (Pas) or Poises (P), (1 Pas = 10 P).

In reality all materials can behave as either solids or liquids, under appropriate conditions and water can behave as a solid at very short times (i.e. 10^{-8} sec). Viscoelastic materials can be regarded as liquids or as solids because they possess both viscous (fluid) and elastic (solid) components.

The relationship between stress, shear and time for a particular sample is the subject of rheology. Most of rheological measurements fall in one of the following three classes. In the first one, material flows steadily through a tube or between rotating surfaces. This type of measurement is used for the monitoring of changes of viscosity operated by *endo*-type of glycosidases during the hydrolysis of large polysaccharide molecules.

In the second class the deformation is not steady and either the variation of strain or the variation of stress are measured. Creep and stress and relaxation tests performed on living tissues or large polymers belong to this class.

In the third class, oscillatory forces are applied to the material and the response measured (Whorlow, 1980). The type of analysis (dynamic measurements) performed on the galactose-depleted xyloglucan belongs to this last class and the increase of chain-chain interaction, induced by the gradual galactose removal on xyloglucan side-chains, is analysed in relation to changes in the "liquid-like" and "solid-like" properties of the material.

In the simple case shown in Fig.5.1.a, a harmonic simple shear stress of amplitude (σ_0) and frequency ($\omega/2\pi$) is applied to the upper face of a thin block of material. The shear stress (σ) expressed in the equation:

(i) $$\sigma = \sigma_0 \cos\omega t$$

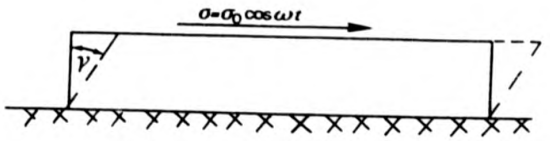
produces a strain (γ):

(ii) $$\gamma = \gamma_0 \cos(\omega t - \delta)$$

where the phase lag δ of strain relative to stress and the amplitude ratio γ_0/σ_0 depend on the material under investigation. An assumption is made that the material deforms in a linear manner: in particular a harmonical stress results in a harmonical strain of amplitude proportional to the stress amplitude and with a phase lag relative to the stress independent of amplitude. The amplitude of the component of strain can be in phase with the stress and $\delta = 0$ is an ideal elastic solid. When the amplitude of the component of strain is 90° out of phase (δ = 90°) the strain and stress are said to be in quadrature. The ratio in phase strain amplitude/stress amplitude is called storage compliance (J') and the ratio quadrature strain amplitude/stress amplitude is called loss compliance (J'') (Whorlow, 1980). In the ideal elastic solid $\delta = 0$, J''= 0 and J' is related to the elastic energy stored in the material, no energy being lost. Fig.5.1.b shows the corresponding stress (σ) and strain (γ) waveforms.

If a sinusoidal strain wave is applied to a viscous liquid, the stress will be exactly 90°out of phase with the imposed deformation because it will have its maximum when the rate of change of strain with time is at its maximum value. The ratio of in-phase stress to strain is known as the storage modulus; the ratio of the 90° out of phase stress to strain is the loss modulus. The storage modulus G' represents the "solid-like" component of the material, whereas the loss modulus G'' represents the "fluid-like" component of the material under investigation. Another parameter used in the deformation of viscoelastic materials is tan of the phase angle δ defined as the ratio G''/G' (also equivalent to the ratio J''/J'). The

(a)



(b)

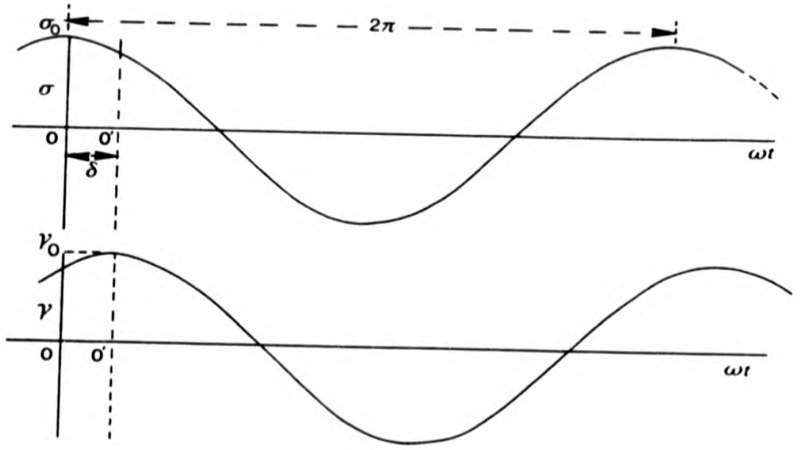


Fig.5.1. Dynamic measurements. (a) A harmonic simple shear stress of amplitude (σ_0) and frequency ($\omega/2\pi$) is applied to the upper face of a thin block of material and (b) the corresponding stress (σ) and strain (γ) waveforms.

parameters G' and G'' can be measured from the overall stress wave using electronic equipment.

Another important parameter is the dynamic viscosity (η^*) defined as the ratio of total stress to frequency of oscillation (omega, radians s^{-1}) related to omega, G' and G''by G^*:

(iii) $$G^* = (G'^2 + G''^2)^{1/2}$$

(iv) $$\eta^* = G^*/\omega$$

In the case of viscoelastic liquids, such as xyloglucan solutions, the material is generally confined between cylinders, cones or plates. The Bohlin rheometer is equipped with a cone-plate system, where θ_0 is the cone-plate angle (which is usually less than 5°). In this system the material rotates through an angle θ under the action of a harmonic torque moving with a frequence of 1000 Hz. The measurement of G' and G''are expressed in pascal. The monitoring of the parameters G' (solid-like) and G''(liquid-like) during the incubation of tamarind xyloglucan with the purified enzyme allows the determination of the gelation point.

5.3. RESULTS.

5.3.1. PURIFICATION OF THE ß-GALACTOSIDASE.

The nasturtium ß-galactosidase was purified to homogeneity using the procedure described by Edwards *et al.* (1988). The enzyme extract contained at least two ß-galactosidase activities which could be separated by ion-exchange chromatography. The cation-exchange chromatography prophile (Fig.5.2.a) shows the ß-galactosidase activities, as determined by the *p*-nitrophenyl-ß-D-galactopyranoside assay: the major activity was eluted in the first peak, indicating that there was no interaction with the exchanger gel, whereas the second was eluted at the front of the peak eluted with a linear sodium chloride gradient. The second ß-galactosidase activity was often found associated with the α-xylosidase activity (see Chapter 3). Fig.5.2.b and c show cation-exchange chromatography (the enzyme eluted in a sodium chloride gradient step) and gel filtration chromatography using a Bio Gel P60 column, respectively. The purified enzyme was stored at -20°C.
On SDS-polyacrylamide gel the enzyme gave a major band of 97 KDa, but minor components were also present with lower molecular weight (Fig.5.3.). The lower protein bands have been reasoned to be breakdown products of the same enzyme molecule (Edwards *et al.*, 1988). To confirm this, an antibody was raised against the major protein band corresponding to the purified enzyme. The SDS-polyacrylamide gel of the purified enzyme was lightly stained with Coomassie Blue

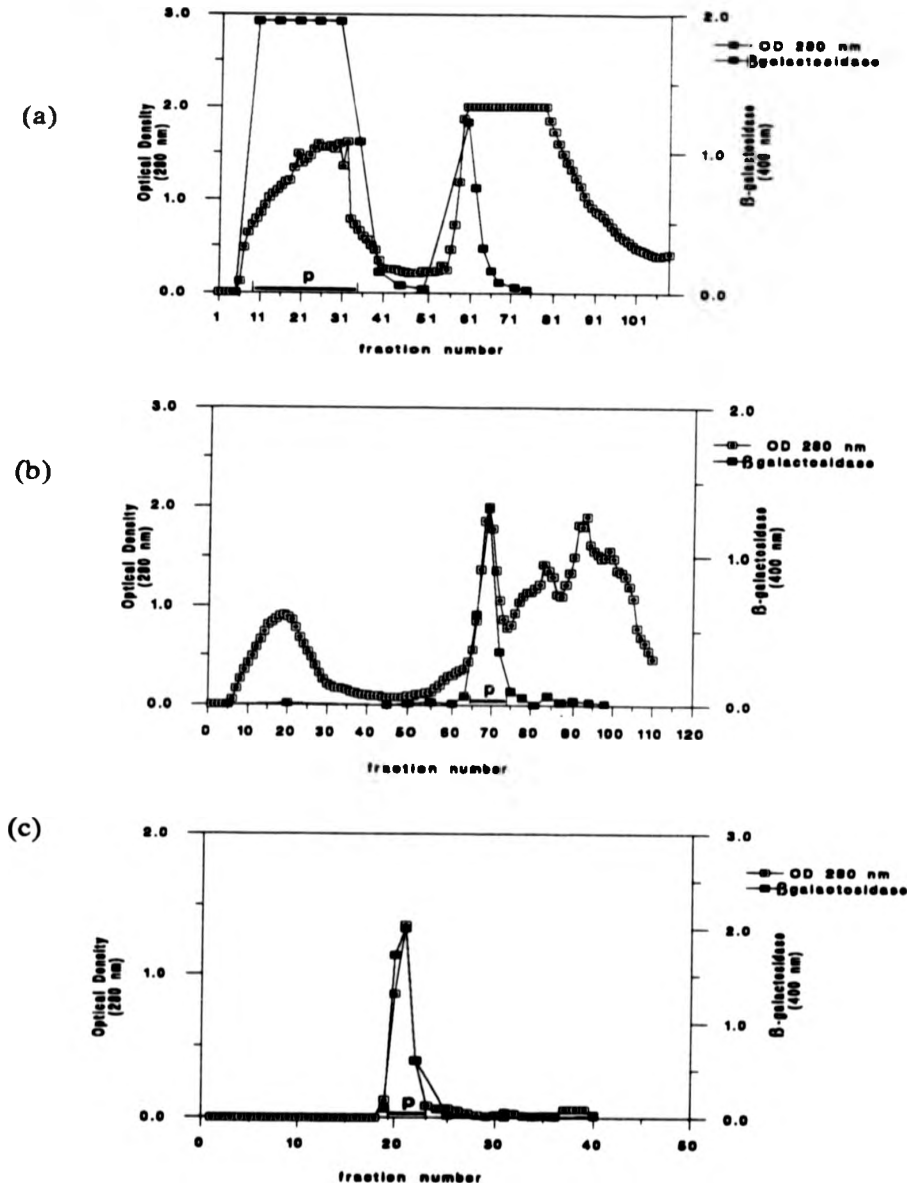


Fig.5.2. Purification of the nasturtium ß-galactosidase. (a): Anion-exchange chromatography on DEAE-cellulose of a crude enzyme extract from nasturtium cotyledons. (b): Cation-exchange chromatography on CM-cellulose. (c): Gel filtration using Bio-gel P60. The activity of the ß-galctosidase was determined as the *p*-nitrophenol released from *p*-nitrophenyl-ß-D-galactopyranoside.

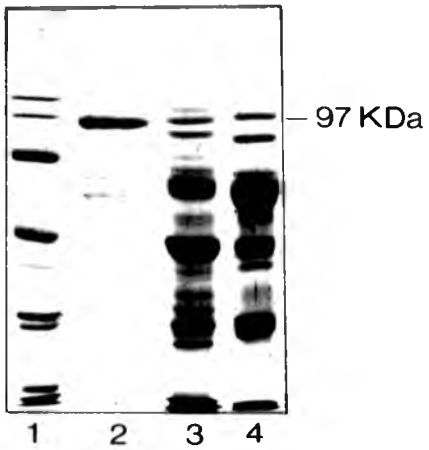


Fig.5.3. SDS-acrylamide gel electrophoresis of the purified nasturtium ß-D-galactosidase and the partially purified seed extracts.

Lane 2: purified ß-D-galactosidase; *Lane 3*: seed extract after ammonium sulphate precipitation of the proteins; *Lane 4*: enzyme extract after anion-exchange chromatography; *Lane 1*: Molecular weight markers, Myosin (Mr 205 KDa), ß-galactosidase (Mr 116 KDa), phosphorylase B (Mr 97.4 KDa), bovine serum albumin (Mr 66 KDa), ovalbumin (Mr 45 KDa), carbonic anhydrase (Mr 29 KDa).

and the top protein band was cut out and used as immunogen. The rabbit antiserum cross-reacted with the minor components as well as with the major protein band. The pre-immune serum used as control did not cross-react with any protein components (Fig.5.4.). This result indicated that the "minor components" and the major protein band were closely related species, reinforcing the suggestion of Edwards *et al.* (1988) that the lower molecular weight protein bands were breakdown products of the same protein, possibly arising during the denaturation of the enzyme before electrophoretic analysis.

Isoelectric focusing of the purified enzyme showed a number of closely related molecular species spanning the range pI = 6.6 to pI = 7.1, with three major components corresponding to pI = 6.6, 6.9 and 7.1 (Fig.5.5.). The enzyme activity was monitored by incubating gel strips corresponding to each protein component with the citrate/phosphate buffer pH 5.0 and by determining the *p*-nitrophenol released from *p*-nitrophenyl-ß-D-galactopyranoside. All protein bands showed enzyme activity (results not shown). Purification of single components was achieved by using the chromatofocusing technique with polybuffer exchanger 94 and Polybuffer 96 which covered a pH gradient from pH 8.7 to pH 6.0 (Fig.5.6.a). The ß-galactosidase activity was eluted in this pH gradient as a series of overlapping peaks. Different fractions collected from the chromatofocusing column gave a single band on IEF (Fig.5.6.b). On SDS-PAGE all contained the same band with an apparent molecular weight of 97 KDa (results not shown). The native enzyme was found to have an apparent molecular weight of 70 KDa on gel-filtration with Bio Gel P300 (Edwards *et al.*, 1988).

The enzyme has a pH optimum of 4-5 and a temperature optimum of 50°C

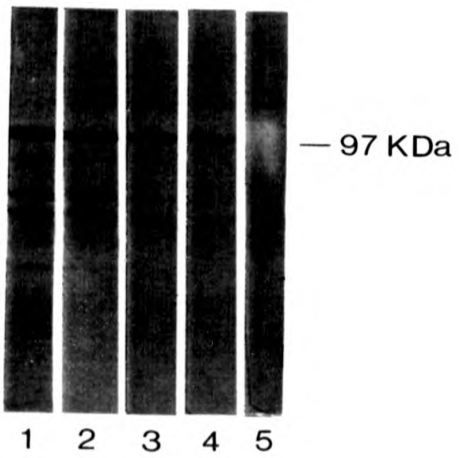


Fig.5.4. Western blot of the purified nasturtium ß-galactosidase incubated with the antibody raised against the purified ß-galactosidase protein (top band). ***Lanes 1 to 4***: the antiserum dilutions of 1:100, 1:200, 1:400, 1:800 were used; ***Lane 5***: control (pre-immune serum).

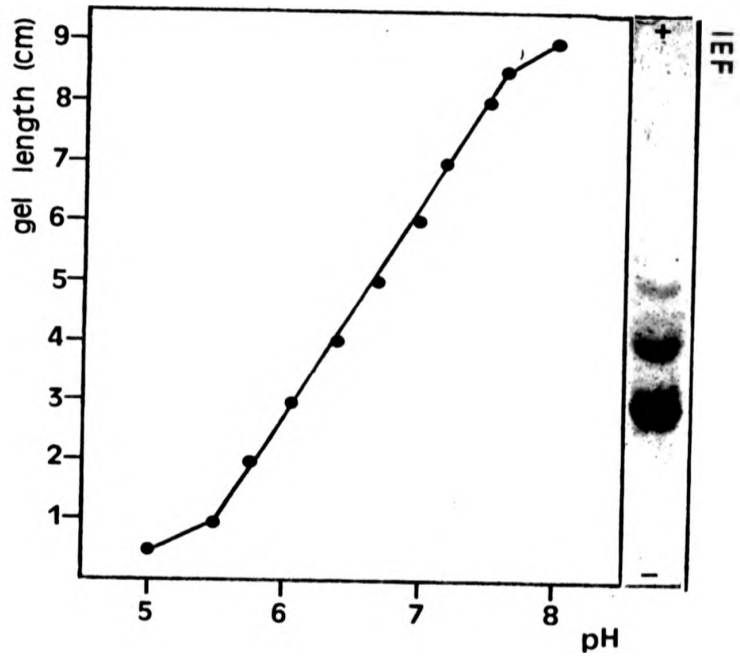
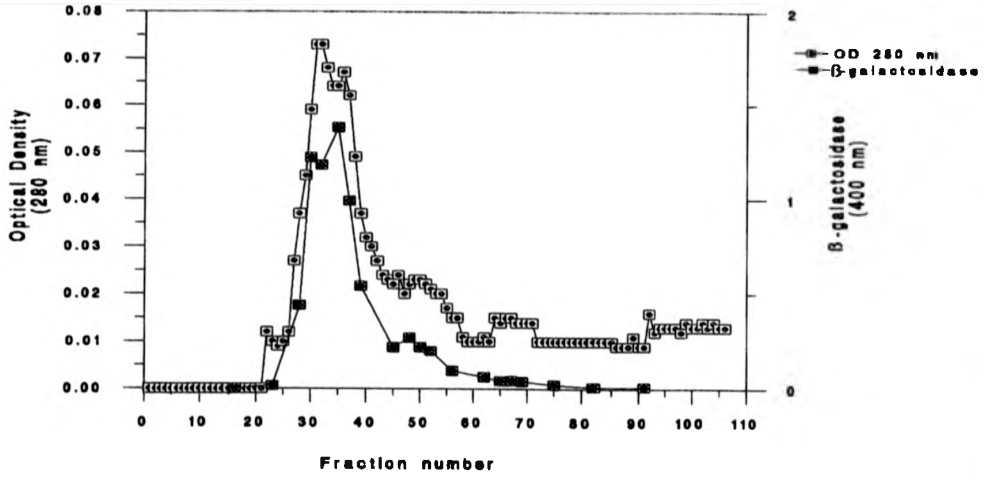


Fig.5.5. Isoelectric focusing (IEF) of the purified ß-D-galactosidase enzyme.

(a)



(b)

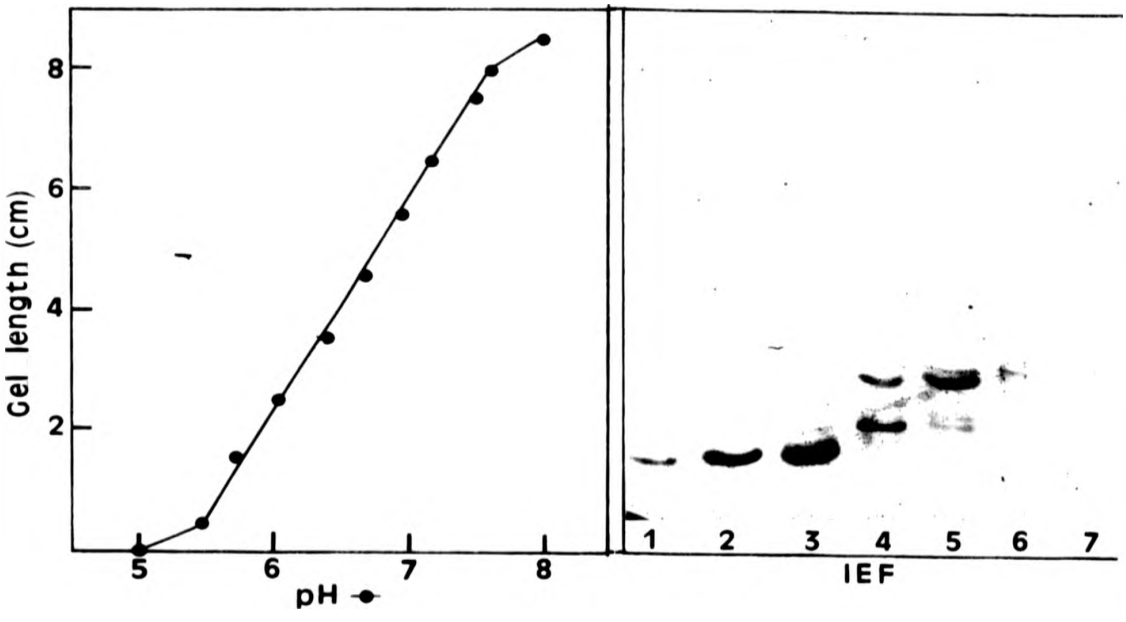


Fig.5.6. (a): Chromatofocusing of the purified ß-galactosidase. (b): IEF of the protein fractions eluted from chromatofocusing column.

Lanes 1 to 7: Fractions 28, 30, 32, 35, 37, 39 and 41 collected from the chromatofocusing column. The activity of the enzyme ß-galctosidase was determined the *p*-nitrophenol released from *p*-nitrophenyl-ß-D-galactopyranoside.

(Edwards *et al.*, 1988).

All the experiments performed in this investigation were carried out using the mixture of isoenzymes.

5.3.2. RHEOLOGICAL PROPERTIES OF TAMARIND XYLOGLUCAN.

The aggregation of xyloglucan molecules observed following the addition of high salt concentration (i.e. 1 M Na_2SO_4) was investigated by Reid *et al.* (1988). They also observed that the rate of galactose removal by the nasturtium ß-galactosidase from xyloglucan side-chains was greatly enhanced when Na_2SO_4 was added to the ß-galactosidase/xyloglucan mixture. In Fig.5.7. tamarind xyloglucan (12 mg, final volume 1.2 ml) was incubated with the purified ß-galactosidase (3.6 μg) in the absence and in the presence of 1 M sodium sulphate. The galactose release in presence of sodium sulphate was increased by 6-fold over 150 min (Fig.5.7.). The reason for this unusual behaviour is still unclear.

The observed increase of interactive properties of xyloglucan molecules treated with the nasturtium ß-galactosidase in the presence of sodium sulphate was further investigated by means of rheological techniques.

To understand the molecular mode of action of the enzyme *in vivo*, the removal of galactose residue from xyloglucan side-chains was also carried out in absence of sodium sulphate. The course of the enzyme reaction and the increase of intermolecular association were monitored by determination of galactose release

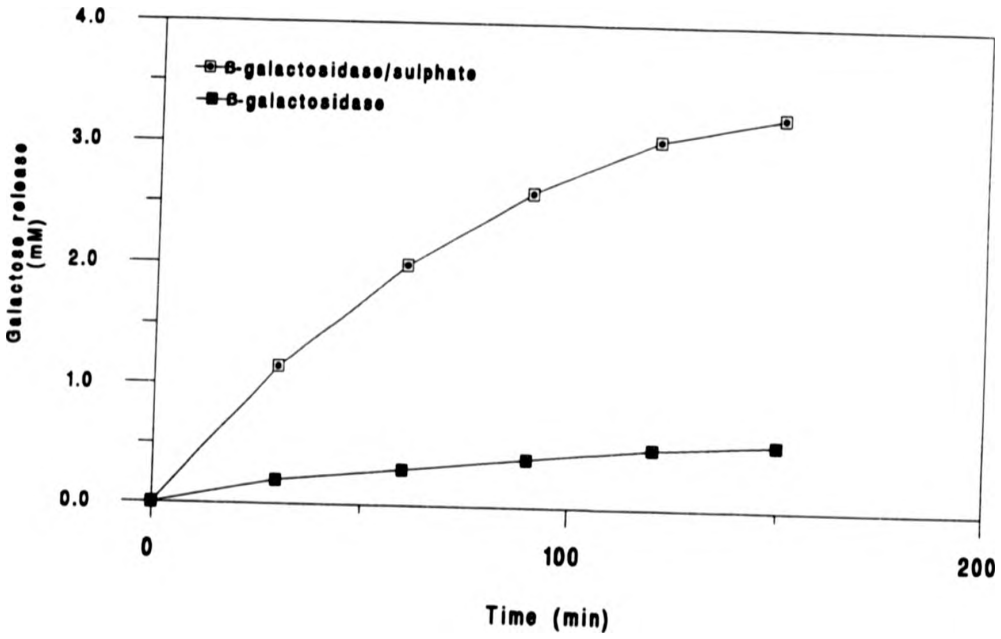


Fig.5.7. Tamarind xyloglucan incubated with ß-galactosidase in presence and in absence of 1 M sodium sulphate.

and by rheological measurements.

5.3.2.1. GALACTOSE REMOVAL FROM TAMARIND XYLOGLUCAN AND THE EFFECT OF SODIUM SULPHATE.

Tamarind xyloglucan (24 mg, final volume 2.4 ml) was incubated with the nasturtium ß-galactosidase (7.2 μg) in the presence of 1 M Na_2SO_4. The enzyme digestion was carried out at 30°C, and aliquots were taken, boiled for 8-10 min and used for the determination of galactose released. It was observed that the gradual removal of galactose as monitored by the galactose dehydrogenase assay was accompanied by a dramatic increase of the viscosity of the solution as measured by the flow-time through a capillary viscometer. Fig.5.8. shows the plot of viscometric flow/time versus galactose release from xyloglucan. The partial removal of galactose side-chains due to the action of the enzyme clearly promoted the increase of intermolecular chain-chain interaction and led to the formation of a thermo-irreversible gel.

The same experiment was repeated with the enzyme-xyloglucan mixture in the Bohlin rheometer: a solution of 1% tamarind xyloglucan was incubated with the purified enzyme in presence of 1 M sodium sulphate, and G', G'' and tan δ were measured as a function of time. The parameters G' and G'' indicate the storage modulus (or the solid-like component) and the the loss modulus (or the liquid-like component), respectively, whereas the phase angle delta (tan δ) is defined as

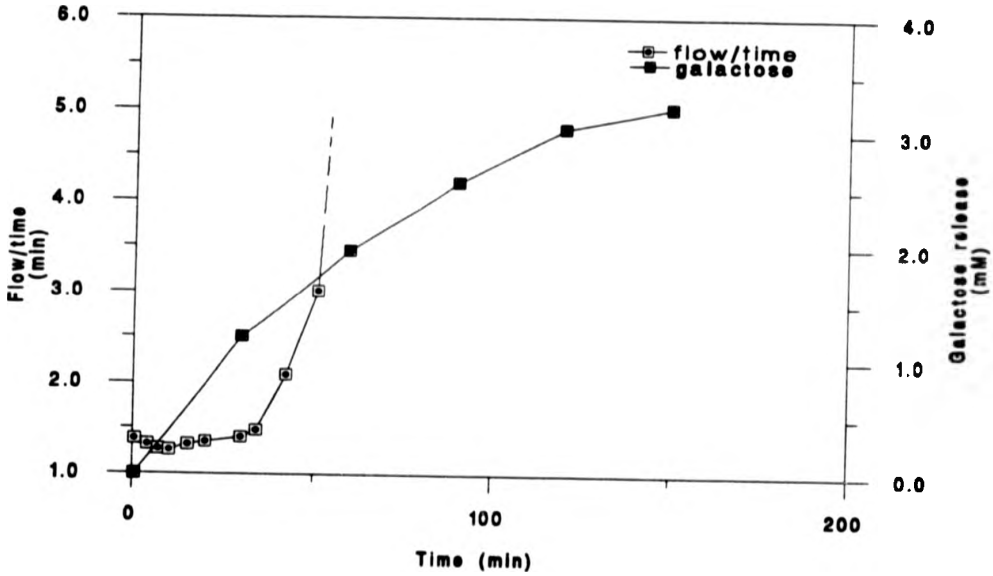


Fig.5.8. Galactose released from tamarind xyloglucan and solutions viscosity (as flow-time through a capillary viscometer).

be expected for a viscoelastic solution. An increase in G', which describes the increase of aggregation in the tamarind polysaccharide occurred after about 40 min and a storage modulus of ca 500 Pa was reached after 2 h incubation. Fig.5.9. describes the results observed in the oscillation test. The gelation of xyloglucan polysaccharide, represented by the intersection of G' with G'', occurred at time = 40 min. Galactose dehydrogenase determination of the galactose removed, and GLC analysis of the monosaccharides released upon acid hydrolysis of enzyme-polysaccharide mixtures, showed that gelation occured when about 50% of the galactose was removed from the xyloglucan side chains. This finding confirmed the observation of Reid *et al.* (1988) that the presence of galactose residues prevents intermolecular interaction of xyloglucan chains, and that the xylose-substituted glucan backbone is essentially an interactive one .

When tamarind xyloglucan was incubated with 1 M sodium sulphate, in the absence of ß-galactosidase, some aggregation of the polysaccharide was observed after 6-7 hours, as indicated by the increase in viscosity. However the oscillation test performed on the sample showed the gradual increase of chain-chain interaction and the progressive formation of a very weak gel. The storage modulus (G') increased slowly up to 1.7 Pa after 17 h and the intersection of G' with G'' occurred at time = 6 h (Fig.5.10.). This findings confirmed essentially the observations of Reid *et al.* (1988). The aggregation observed in the presence of sodium sulphate (and in absence of ß-galactosidase) may be explained as an increase of hydrophobic interactions between xyloglucan molecules induced by high salt concentrations.

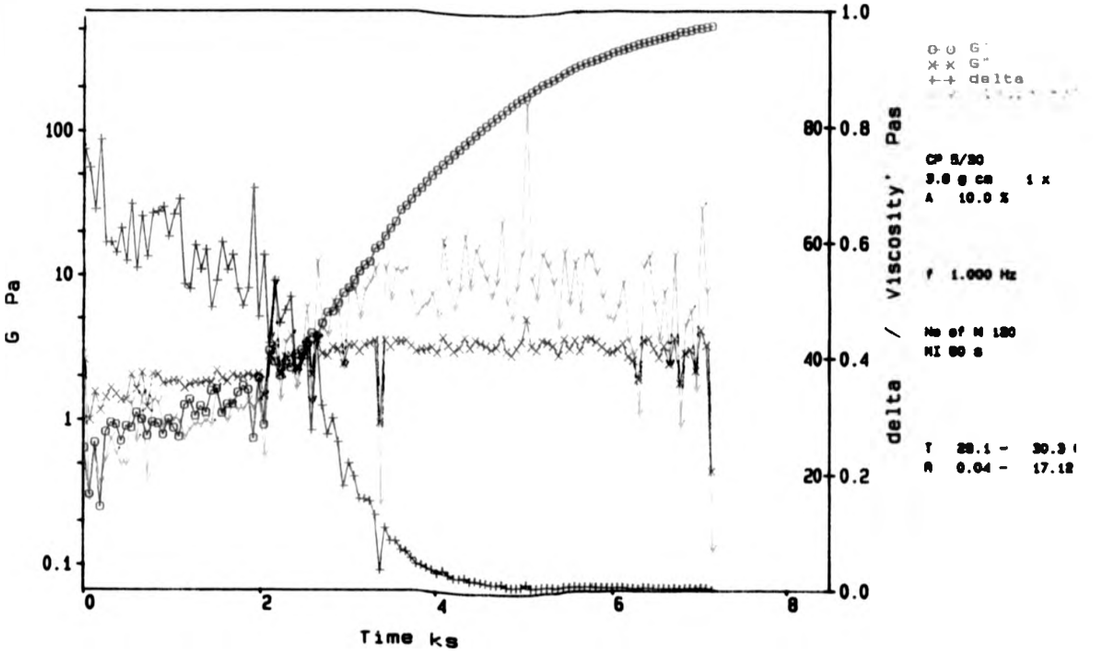


Fig.5.9. Rheological monitoring of galactose removal by the nasturtium ß-galactosidase from tamarind xyloglucan in presence of 1 M Na_2SO_4. The parameters G'(which describes the solid-like component) and G"(which describes the liquid-like component) are measured in Pa.

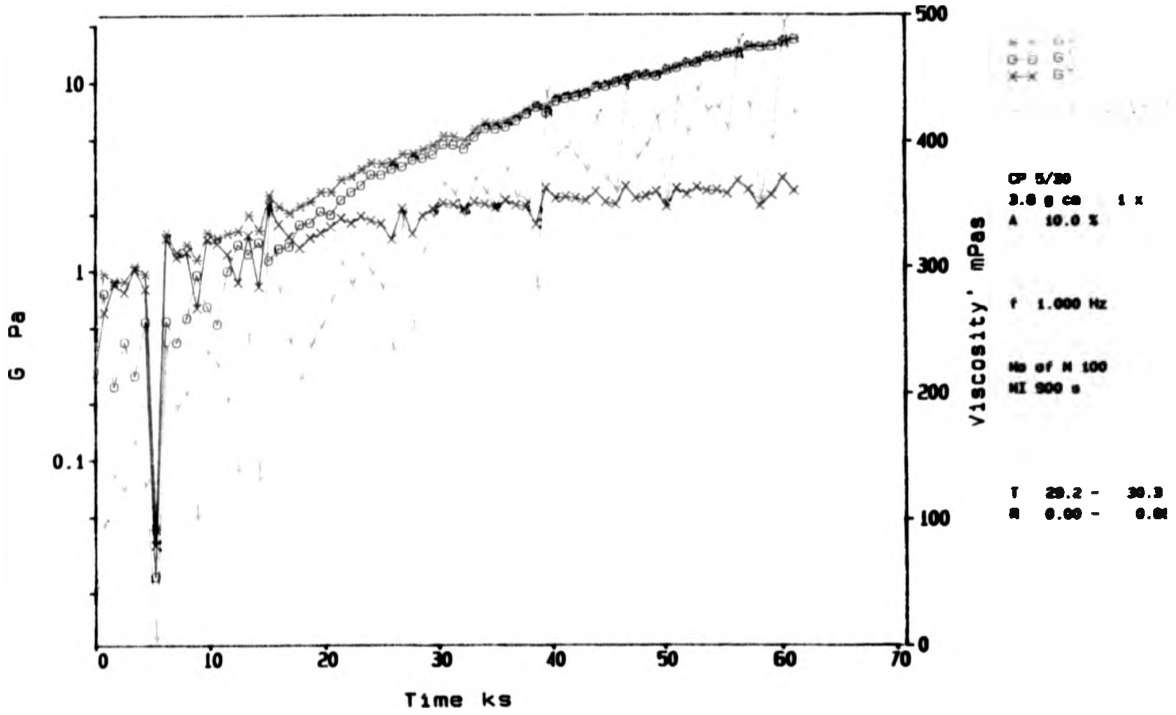
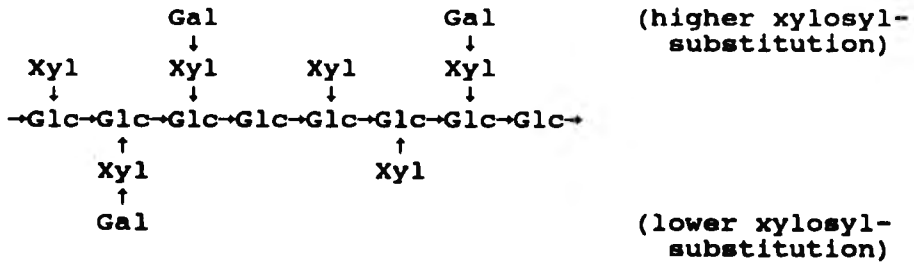


Fig.5.10. Rheological monitoring of tamarind xyloglucan in presence of 1 M Na_2SO_4 alone. The parameters G'(which describes the solid-like component) and G"(which describes the liquid-like component) are measured in Pa.

5.3.2.2. HPLC ANALYSIS OF THE MODIFIED XYLOGLUCAN SAMPLES DIGESTED BY THE FUNGAL CELLULASE.

Tamarind xyloglucan was incubated with the nasturtium ß-galactosidase in the presence of 1 M Na_2SO_4 as described earlier and the enzyme reaction was stopped by heat-denaturation after 30 min, 1 hour and 2 hours. The samples were exhaustively dialyzed against 50 mM ammonium acetate buffer pH 5.0 to remove free galactose and the galactose-depleted polysaccharide were digested overnight with the fungal *endo*-(1→4)-ß-D-glucanase (cellulase) from *T. viride*. The oligosaccharides released by total hydrolysis were analysed by HPLC. Since galactose substitution can occur on only two of three xylose residues of the Glc_4Xyl_3 repeating motif of xyloglucan, it is evident that the ratio of oligosaccharides A:B_1:B_2:C will indicate the galactose distribution on the more xylosyl-substituted and less xylosyl-substituted side of the glucan backbone, as shown below:



HPLC analysis of xyloglucan treated with ß-galactosidase and digested with the fungal cellulase provided information on the overall distribution of galactosyl

residues on the molecule during the course of the enzyme reaction. The determination of the galactose released and the GLC analysis of the modified samples indicated that the degrees of modification were respectively 16%, 40% and 50%. Gelation was, however, observed only when about 50% of the total galactose was enzymically removed from the xyloglucan side-chains.

HPLC analysis of the oligosaccharides obtained by cellulase digestion of the galactose-depleted xyloglucan samples and the ratio $A:B_1:B_2:C$ are in Table 5.1.. The nasturtium ß-galactosidase catalyzed the conversion of oligosaccharide C to B (B_1, B_2) and A, as observed from the decrease of oligosaccharide C during the xyloglucan incubation with the enzyme, accompanied by the increase of oligosaccharide A. The levels of oligosaccharide B_1 decreased rapidly in the early stages of the enzyme reaction (up to 60 min) and more slowly thereafter. It was observed that the levels of oligosaccharide B_2 increased after 60 min incubation with the ß-galactosidase from 29.6% (control) to 39.1% and decreased only very slightly after 2 h incubation to 38.4%. Oligosaccharide B_2 derives by hydrolysis of the galactose residue attached onto the xylose nearest to the reducing glucose oligosaccharide C. This galactose is located in the higher xylosyl-substituted side of the glucan backbone.

The transitory accumulation of oligosaccharide B_2 indicated that the removal of galactose from polymeric xyloglucan by the nasturtium enzyme in the presence of Na_2SO_4 is achieved more slowly probably due to a lower rate of enzyme activity. This observation indicated that the rate of galactose removal from the xyloglucan side-chain is dependent on the position of the galactose residue and suggested

	A	B_1	B_2	C	Glc	Xyl	Gal	Modification (%)
30 min	25.7	5.1	32.3	36.9	4.0	3.0	1.11	16.5
60 min	36.9	3.7	39.1	20.3	4.0	3.0	0.83	37.3
120 min	45.6	3.0	38.4	13.0	4.0	3.0	0.67	49.3
control	15.6	6.3	29.6	48.5	4.0	3.0	1.33	(0)

Table 5.1. Tamarind xyloglucan/ß-D-galactosidase/Na_2SO_4: cellulase digestion of modified tamarind xyloglucan samples.

that the enzyme ß-galactosidase was preferentially attacking and cleaving galactosyl substituents located on the less xylosyl-substituted xyloglucan backbone.

5.3.2.3. RHEOLOGICAL PROPERTIES OF THE GALACTOSE-DEPLETED TAMARIND XYLOGLUCAN (IN ABSENCE OF Na_2SO_4).

The nasturtium ß-galactosidase was used to remove terminal (1→2)-ß-D-galactopyranosyl residues from native tamarind xyloglucan and the enzyme reaction was followed in the Bohlin rheometer. This time the enzyme reaction was carried out in absence of Na_2SO_4, but otherwise using the same experimental conditions described in the previous sections. The solution was placed in the Bohlin rheometer and the solution properties analysed. The release of galactose was not accompanied by an increase of intermolecular association even on prolonged incubation. The investigation was therefore repeated using higher concentrations of enzyme. The polysaccharide (24 mg, final volume 2.4 ml) was incubated with 72 μg of nasturtium ß-galactosidase and the monitoring of intermolecular association was investigated by the increase of G'(solid-like component) and steady-status of G''(liquid-like component). The removal of galactose was determined by the galactose dehydrogenase assay.

Aggregation of the xyloglucan and gelation were observed in absence of sodium sulphate (Fig.5.11.). The increase of G' which describes the increase of aggregation in the tamarind polysaccharide occurred after about 17 h,

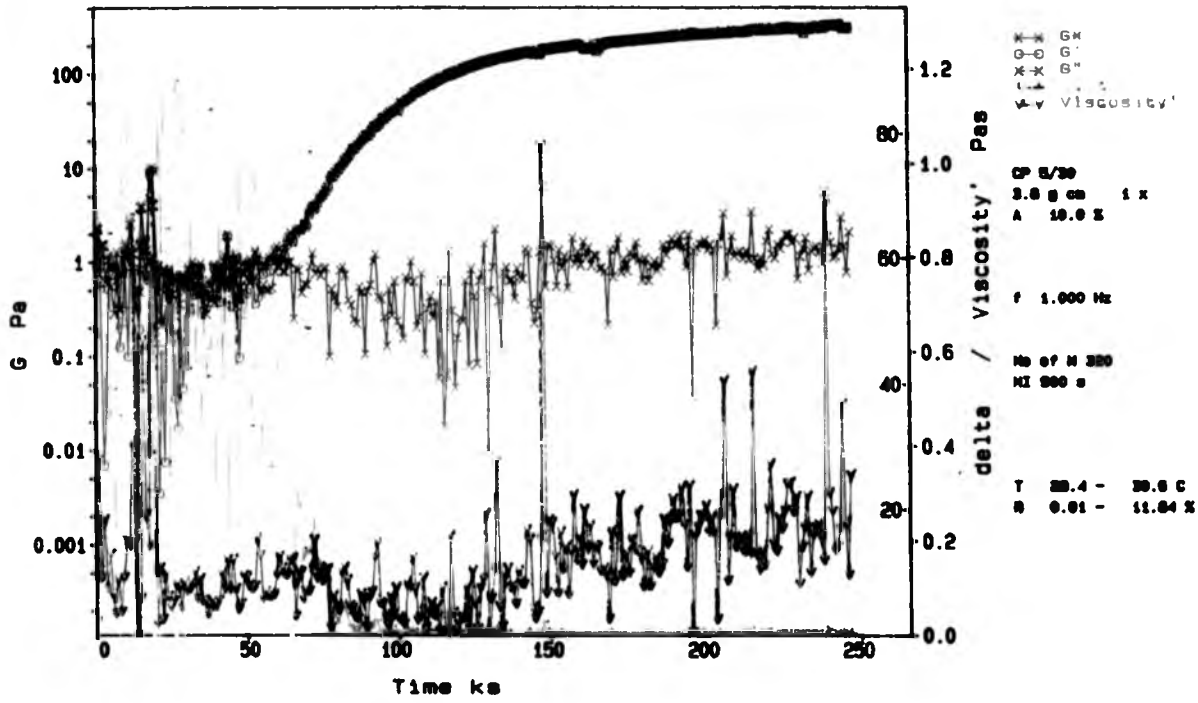


Fig.5.11. Rheological monitoring of galactose removal by the nasturtium ß-galactosidase from tamarind xyloglucan. The parameters G'(which describes the solid-like component) and G"(which describes the liquid-like component) are measured in Pa.

corresponding to a modification degree of ca 50%, and the storage modulus (G') of ca 300 Pa reached after 30 h did not change appreciably after 70 hours.

The formation of the gel occurred more slowly (17 h) with respect to the sample incubated with the enzyme in presence of Na_2SO_4 (only 40 min) and the strength of the gel as described by the storage modulus was weaker.

These results confirmed the previous observations that the presence of terminal galactosyl residues may prevent intermolecular association of xyloglucan molecules contributing to the solubility of xyloglucan in aqueous solutions. The partial removal of these galactosyl residues causes aggregation of xyloglucan chains, altering the solubitity properties of the polysaccharide in aqueous medium.

5.3.2.4. HPLC ANALYSIS OF GALACTOSE-DEPLETED XYLOGLUCAN SAMPLES DIGESTED BY THE FUNGAL CELLULASE.

The tamarind xyloglucan samples which had been treated with the nasturtium ß-galactosidase in the absence of Na_2SO_4, were digested with the fungal cellulase and the oligosaccharides released analysed by HPLC. The oligosaccharide ratio A:B_1:B_2:C obtained for each sample, and the degree of modification expressed as the amount of galactose removed by the nasturtium ß-galactosidase are reported in Table 5.2.. The nasturtium ß-galactosidase catalyzed the conversion of oligosaccharide C to B_1, B_2 and A, as observed from the decrease of oligosaccharide C during the xyloglucan incubation with the enzyme. The

corresponding to a modification degree of ca 50%, and the storage modulus (G') of ca 300 Pa reached after 30 h did not change appreciably after 70 hours.

The formation of the gel occurred more slowly (17 h) with respect to the sample incubated with the enzyme in presence of Na_2SO_4 (only 40 min) and the strength of the gel as described by the storage modulus was weaker.

These results confirmed the previous observations that the presence of terminal galactosyl residues may prevent intermolecular association of xyloglucan molecules contributing to the solubility of xyloglucan in aqueous solutions. The partial removal of these galactosyl residues causes aggregation of xyloglucan chains, altering the solubitity properties of the polysaccharide in aqueous medium.

5.3.2.4. HPLC ANALYSIS OF GALACTOSE-DEPLETED XYLOGLUCAN SAMPLES DIGESTED BY THE FUNGAL CELLULASE.

The tamarind xyloglucan samples which had been treated with the nasturtium ß-galactosidase in the absence of Na_2SO_4, were digested with the fungal cellulase and the oligosaccharides released analysed by HPLC. The oligosaccharide ratio A:B_1:B_2:C obtained for each sample, and the degree of modification expressed as the amount of galactose removed by the nasturtium ß-galactosidase are reported in Table 5.2.. The nasturtium ß-galactosidase catalyzed the conversion of oligosaccharide C to B_1, B_2 and A, as observed from the decrease of oligosaccharide C during the xyloglucan incubation with the enzyme. The

	A	B_1	B_2	C	Glc	Xyl	Gal	Modification (%)
2 h	34.5	5.1	30.4	30.0	4.0	3.0	0.95	29.6
4 h	41.6	4.2	27.1	27.1	4.0	3.0	0.85	37.0
19 h	64.1	2.6	17.4	15.9	4.0	3.0	0.52	61.5
25 h	66.3	2.8	15.3	15.6	4.0	3.0	0.49	63.7
Control	14.1	5.9	31.3	48.7	4.0	3.0	1.35	(0)

Table 5.2. Tamarind xyloglucan/ß-D-galactosidase: cellulase digestion of modified tamarind xyloglucan samples.

appearance of higher levels of oligosaccharide A was accompanied by the disappearance of the oligosaccharide C. Oligosaccharides B_1 and B_2 were also found to decrease with an increase of oligosaccharide A. It was however observed that under these experimental conditions the oligosaccharide B_2 did not increase in the early stages of the enzyme reaction. It was previously shown that the rate of galactose release is stimulated by the presence of high concentrations of Na_2SO_4 and it was also shown that oligosaccharide B_1 was converted to oligosaccharide A more rapidly than oligosaccharide B_2, causing a transitory accumulation of oligosaccharide B_2. In absence of sodium sulphate the rate of galactose released is decreased, and only 29.6% of the galactose is removed after 120 min, compared with 50% of galactose removed in the presence of sodium sulphate after 120 min. Although higher concentrations of enzyme (10 x concentrated) were used, the degree of modification of 61.5% was achieved only after 19 h of incubation of xyloglucan with the nasturtium enzyme and did not change significantly after 25 h (63.7%).

The molecular mode of the enzyme action appears to be affected by the presence/absence of sodium sulphate. The gradual removal of galactose from the polysaccharide in the absence of Na_2SO_4 appears to occur evenly on both sides of the xyloglucan molecule (with respect to the xylosyl substitution), as described by the ratio B_1+C/B_2+C. This ratio describes the occurrence of galactosyl substitution on both sides of the molecule at any given time. The ratio was found to decrease only very slightly during the course of the enzyme reaction, indicating that the distribution of galactosyl substituents was even on each side of the xyloglucan molecule. In the presence of sodium sulphate the ratio B_1+C/B_2+C

was found to decrease, and after 120 min treatment with the enzyme it was found to be halved. This finding suggests that the presence of sodium sulphate may induce a conformational change of the polymeric xyloglucan molecule which may expose "certain" galactosyl residues to the attack of the enzyme. These galactosyl residues are those located on the less xylosyl-substituted side of xyloglucan. In these conditions, aggregation and the formation of a strong gel occurs following higher rates of galactose removal. The lower rates of galactose removal in the absence of sodium sulphate allows the enzyme to attack and cleave both sides of the xyloglucan molecule. The increase of intermolecular association between xyloglucan chains is achieved more slowly and the investigation of properties of the modified polysaccharide indicates that the gel is weaker and not thermo-irreversible.

5.3.3. MOLECULAR MODE OF ACTION OF ß-GALACTOSIDASE WITH THE NATIVE TAMARIND XYLOGLUCAN.

It was shown that a plot of the rate of galactose removal from tamarind xyloglucan in the presence of ß-galactosidase versus substrate concentration was linear (Edwards *et al.*, 1988). The Lineweaver-Burk plot 1/v versus 1/[S] passed through the origin and it was not possible to determine the K_m and V_{max} using xyloglucan as substrate (Edwards *et al.*, 1988). It was speculated that the substrate concentrations chosen were lower than the substrate-saturation of the enzyme.

This was explained by assuming that the substrate-saturation of the enzyme occurred at very high substrate concentrations (i.e., hydrated solid rather than solutions).

To investigate further the rates of the enzyme catalysis and the mode of action of the nasturtium ß-galactosidase, the lower molecular weight oligosaccharides C, B_1 and B_2 were used as substrates.

5.3.3.1. ENZYME KINETICS WITH THE PURIFIED XYLOGLUCAN OLIGOSACCHARIDES.

Oligosaccharide C was purified by gel filtration on Bio Gel P4 and preparative TLC. Oligosaccharide C (0.5 mM, final volume 54 μl) was incubated with the purified ß-galactosidase (4 ng) and the enzyme reaction was monitored by HPLC (Fig.5.12.). Oligosaccharide C contains two galactose residues, one attached onto the xylosyl substituent nearest to the reducing-end of the molecule and the other attached on to the adjacent xylose residue, located on the less substituted side of the molecule. Both galactosyl substituents are hydrolysed by the nasturtium ß-galactosidase as previously shown by TLC analysis (Chapter 3). However HPLC analysis of the products of the enzyme reaction showed that galactose removal was accompanied by the decrease of oligosaccharide C and the gradual appearance of oligosaccharide B_2 and A (Fig.5.12.). The scheme of the enzyme reaction is depicted below:

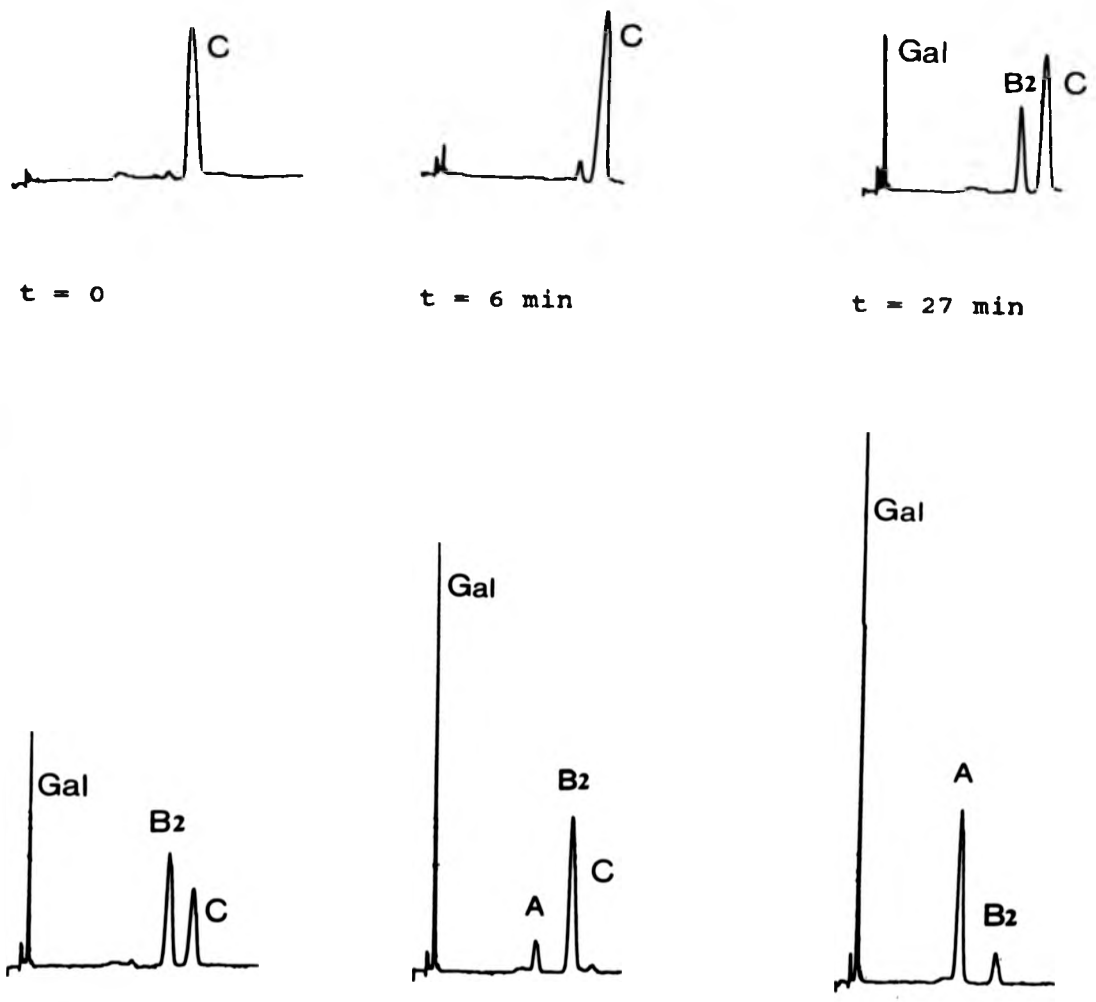
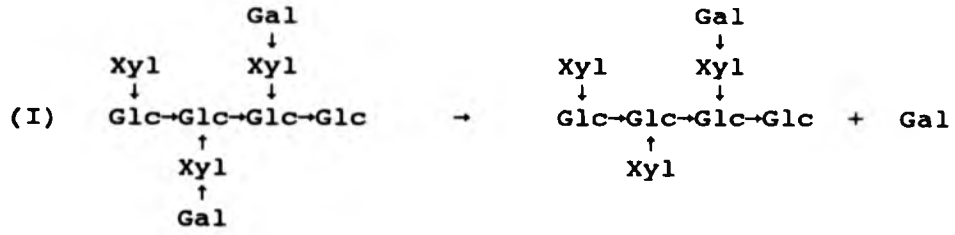
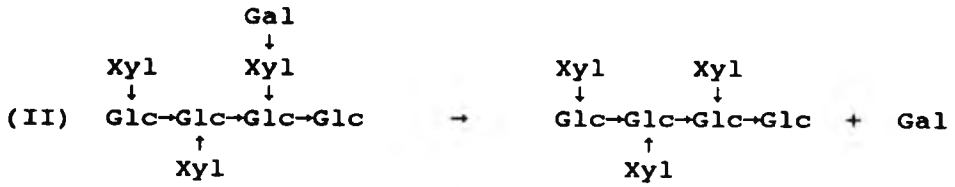


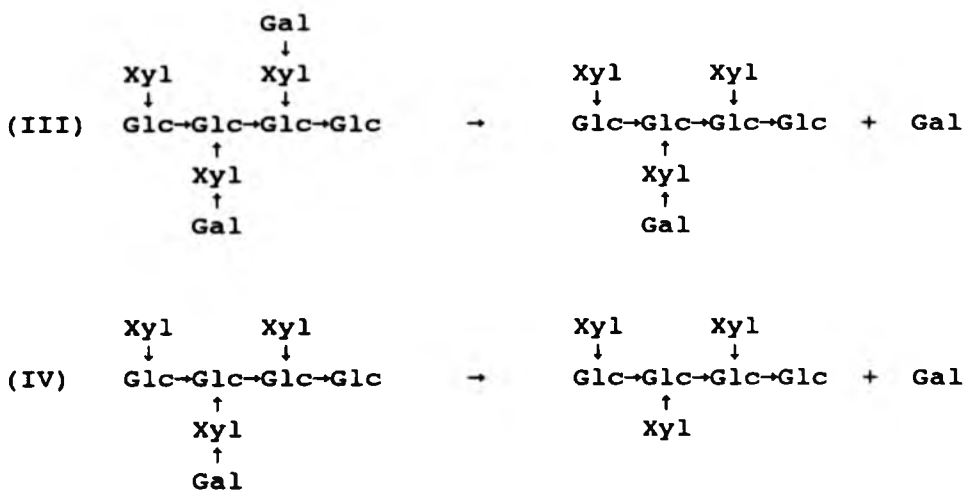
Fig.5.12. Oligosaccharide C is treated with the purified ß-galactosidase; time course of the incubation.



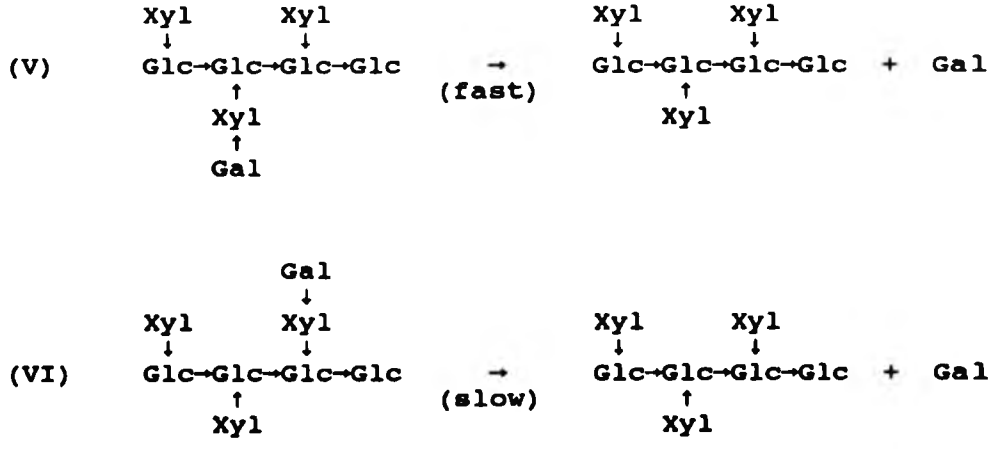


The analysis of the enzyme reaction by HPLC showed that oligosaccharide B_1 was not produced during galactose removal from oligosaccharide C. This could only be explained by assuming that the nasturtium enzyme hydrolyzes first the galactose residue located "below" the plane containing the glucan backbone. This side of the xyloglucan molecule is characterized by a lower xylosyl substitution and it may be more accessible to the enzyme. The oligosaccharide A is produced when most of oligosaccharide C has been converted to B_2 and galactose.

The conversion of C to B_1 was never observed during the enzyme reaction monitored by HPLC analysis. Therefore reaction (III) followed by reaction (IV) did not occur in these conditions:



Oligosaccharide B containing the mixture of isomers $B_1 + B_2$ (3 mM, final volume 250 μl) was incubated with the purified ß-galactosidase (0.2 μg) and the enzyme reaction was monitored by HPLC. Fig.5.13. shows that the conversion of oligosaccharide B_1 to A was much faster than that of B_2 to oligosaccharide A. The enzyme reaction is depicted below:



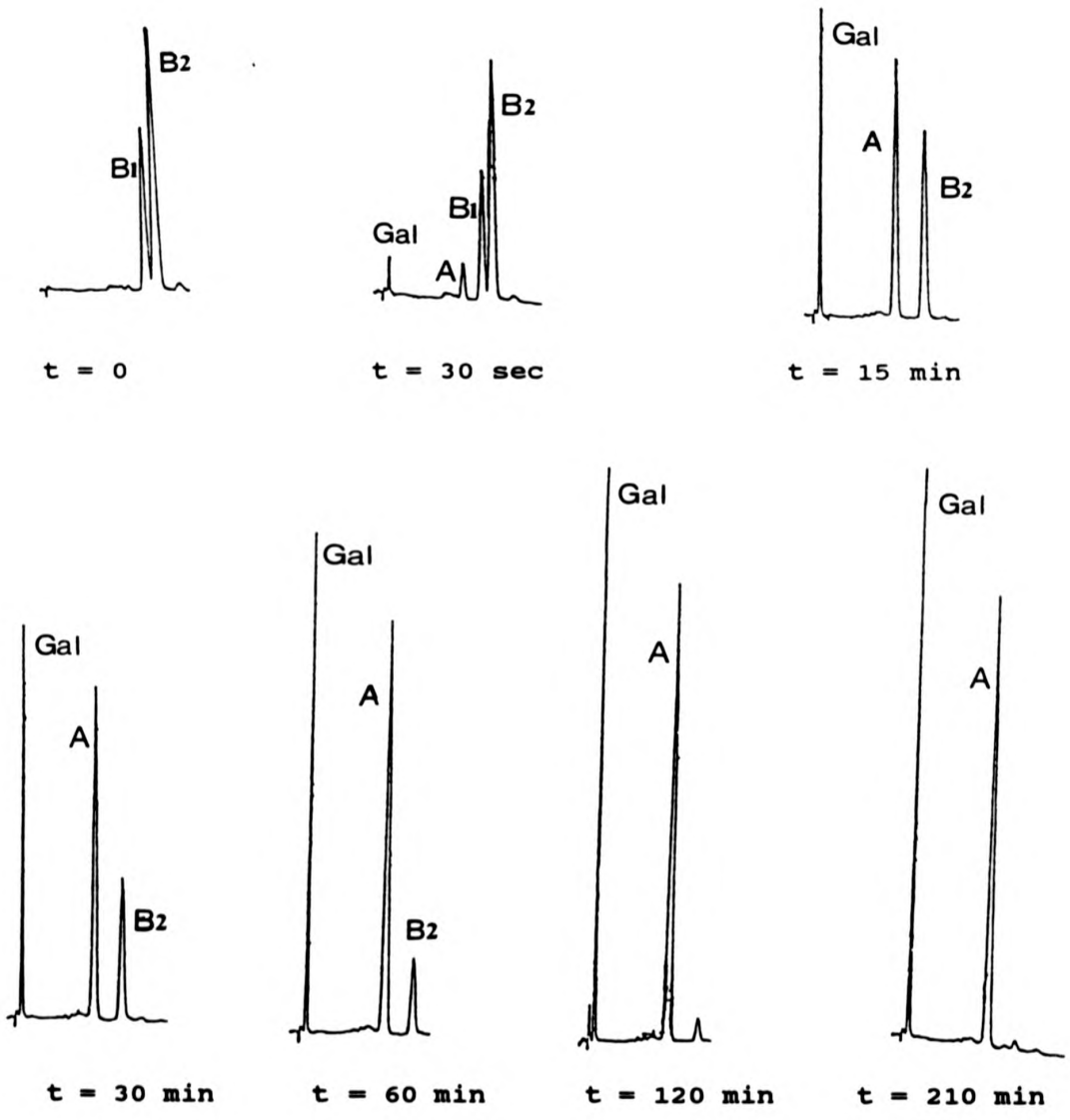


Fig.5.13. Oligosaccharide B (B_1+B_2) is treated with the purified ß-galactosidase; time course of the incubation.

This finding confirmed that the rates of hydrolysis of oligosaccharides B_1 and B_2 are considerably different, and depend on the position of the galactosyl residue in the saccharide molecule.

5.3.3.2. SPECIFICITY OF ß-GALACTOSIDASE USING THE XYLOSE-DEPLETED XYLOGLUCAN NONASACCHARIDE.

To establish whether the xylosyl residue located on the non reducing end of the saccharide molecule affected the rate of galactose removal from both sides of the saccharide ("above" and "below") by the nasturtium ß-galactosidase the nonasaccharide C (0.5 mM, final volume 50 μl) was treated with the nasturtium α-xylosidase (1 μg) and the oligosaccharide obtained was incubated with the enzyme ß-galactosidase (50 ng). The rate of xylose removal was monitored by HPLC analysis. The removal of xylose from the non-reducing end of the saccharide was complete (Fig.5.14.a). The octasaccharide C' was eluted as a symmetrical peak and its retention time was lower than the nonasaccharide. Since the octasaccharide C' has one galactosyl-xylose on each side of the oligosaccharide, it was speculated that the rate of galactosyl removal might be identical from both sides of the xylose-depleted saccharide C'. The octasaccharide C' was incubated with the nasturtium ß-galactosidase, which catalyzed the total hydrolysis of galactose generating a hexasaccharide Glc_4Xyl_2 (Fig.5.14.b). HPLC analysis showed that the disappearance of octasaccharide C' was accompanied by

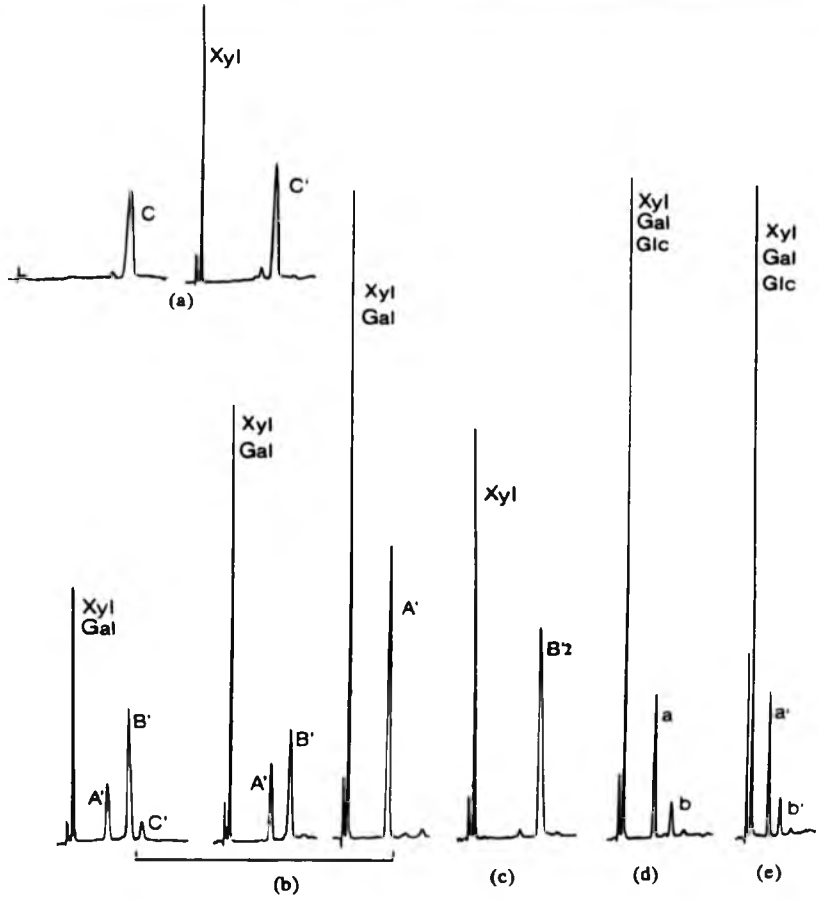


Fig.5.14. Mode of action of ß-galactosidase with the xylose-depleted oligosaccharide C'.

(a) Nonasaccharide C and the xylose-depleted octasaccharide C'.

(b) Incubation of saccharide C' with ß-galactosidase after 15 min, 45 min and 20 h.

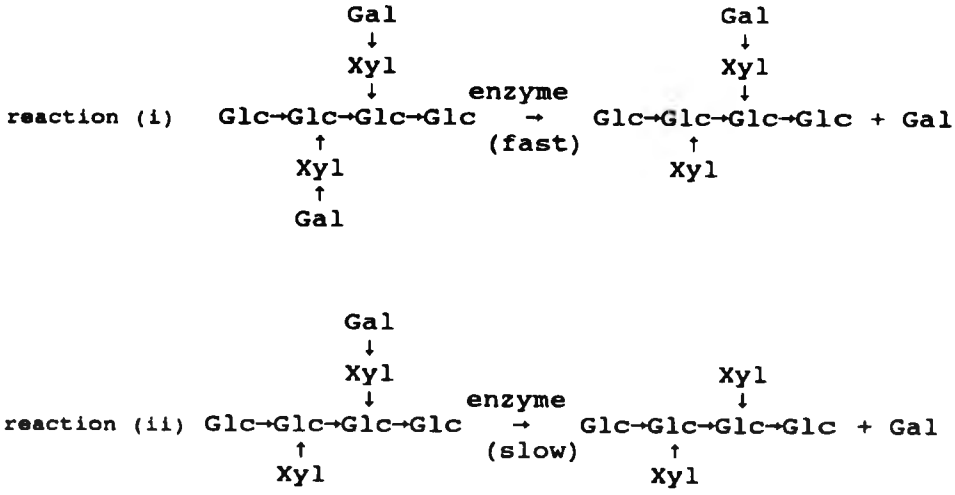
(c) Xylose-depleted oligosaccharide B_2.

(d) Octasaccharide C', partially digested with ß-galactosidase, treated with the nasturtium ß-glucosidase.

(e) The digest in (d) was subsequently treated with the nasturtium α-xylosidase. a = pentasaccharide Glc_3Xyl_2; b = hexasaccharide Glc_3Xyl_2Gal; a' = xylose-depleted pentasaccharide a; b' = xylose-depleted hexasaccharide b.

the appearance of a heptasaccharide Glc_4Xyl_2Gal which was subsequently converted to the hexasaccharide and galactose. This indicated that the enzyme attacked and cleaved preferentially galactosyl residues on one side of the octasaccharide molecule suggesting that the rate of galactose release was dependent on the position of the galactose in the molecule. To identify the structure of the oligosaccharide products of the ß-galactosidase reaction, the oligosaccharide B_2 was treated with the nasturtium α-xylosidase to generate a heptasaccharide Glc_4Xyl_2Gal. This compound was eluted as a symmetrical peak and had a retention time close to the product of partial hydrolysis of saccharide C' with the nasturtium ß-galactosidase (Fig.5.14.c). This suggested that the galactosyl residue hydrolyzed by the enzyme was attached onto the xylose residue nearest to the non-reducing end of the saccharide C'. To confirm this observation, the oligosaccharides produced by partial hydrolysis of saccharide C' with ß-galactosidase (oligosaccharides A' and B') were digested with the nasturtium ß-glucosidase enzyme extract, and analysed by HPLC (Fig.5.14.d). Both oligosaccharides A' and B' were substrates for the ß-glucosidase enzyme preparation, since the xylosyl residue attached onto the non-reducing glucose end of the molecule had previously been removed, and the retention times of the new compounds (a and b) were lower. A further incubation of these two oligosaccharides (a and b) with the nasturtium α-xylosidase showed that they were both substrates for the α-xylosidase, since the retention times of the two compounds (a' and b') were lower (Fig.5.14.e). This confirmed that the products of the partial hydrolysis of saccharide C' with ß-galactosidase were a hexasaccharide Glc_4Xyl_2 and a heptasaccharide Glc_4Xyl_2Gal, containing a

galactosyl residue linked to the xylose residue nearest to the reducing-end of the saccharide. The ß-galactosidase enzyme reaction is depicted below:



These results showed clearly that xylosyl substitution of the non-reducing glucose does not alter the rates of hydrolysis of galactosyl substituents from both sides of the molecule. As shown for the intact oligosaccharide C the nasturtium ß-galactosidase preferentially attacked and cleaved galactosyl residues located further from the reducing end of the saccharide, and the removal of an unsubstituted xylose on the non-reducing end of the molecule did not alter this mechanism of enzyme action. This indicated that the nasturtium enzyme is probably approaching the oligosaccharide molecule from the non-reducing end of the molecule, from which it hydrolyzes the galactosyl residue in its proximity. This mechanism of enzyme action would also explain the different rates of galactose removal observed with oligosaccharides B_2 and C.

5.3.3.3. INHIBITION OF THE ENZYME ACTIVITY BY GALACTOSE.

The nasturtium ß-galactosidase was found to catalyze the hydrolysis of ca 50% of the total galactose present in the xyloglucan molecule (approx. 10mM). The reasons for the inability of the nasturtium ß-galactosidase to catalyze the complete hydrolysis of galactose side-chains was investigated. It was speculated that the increase of aggregation caused by partial removal of galactose might prevent the enzyme from cleaving further galactosyl residues situated in those regions of the xyloglucan molecule in which the inter-chain interaction occurs. However it was also possible that a feed-back mechanism (i.e., inhibition by the product) could prevent the enzyme from cleaving further galactose residues.

The action of the ß-galactosidase on tamarind xyloglucan was re-investigated. The polysaccharide-enzyme mixture was placed in dialysis tubing to allow the continuous removal of the monosaccharide galactose gradually produced by the enzyme. The galactose-depleted xyloglucan was then digested overnight by the fungal cellulase. The ratios $A:B_1:B_2:C$ are reported in Table 5.3.a and b. The data obtained by HPLC analysis confirmed that the monosaccharide galactose exerts an inhibitory effect on the enzyme ß-galactosidase. This is clear by comparing the products of cellulase digestion of enzyme incubation carried out in a "closed" system (where galactose is retained) against the same incubation mixture carried out in a continuous "open" system (where galactose is gradually removed from the incubation mixture). The maximum galactose removal corresponded to 70% of the

	A	B_1	B_2	C	Glc	Xyl	Gal	Modification (%)
270 min	23.0	5.8	29.3	41.9	4.0	3.0	1.19	11.8
24 h	66.6	2.5	18.7	12.2	4.0	3.0	0.46	66.2
32 h	70.7	1.9	15.3	12.1	4.0	3.0	0.41	69.3
44 h	71.5	2.0	14.5	12.0	4.0	3.0	0.40	69.8
Control	14.1	5.9	31.3	48.7	4.0	3.0	1.35	(0)

Table 5.3.a Tamarind xyloglucan/ß-D-galactosidase: cellulase digestion of modified tamarind xyloglucan samples. Enzyme reaction in "open" system.

	A	B_1	B_2	C	Glc	Xyl	Gal	Modification (%)
270 min	20.1	5.4	30.1	44.4	4.0	3.0	1.24	8.1
24 h	33.0	5.0	27.0	35.0	4.0	3.0	1.02	24.4
32 h	37.6	4.8	26.0	31.6	4.0	3.0	0.94	30.4
44 h	41.0	4.5	25.2	29.3	4.0	3.0	0.88	34.6
Control	14.1	5.9	31.3	48.7	4.0	3.0	1.35	(0)

Table 5.3.b Tamarind xyloglucan/ß-D-galactosidase: cellulase digestion of modified tamarind xyloglucan samples. Enzyme reaction in "closed" system.

total galactose. The complete removal of galactose from the xyloglucan side-chains was not achieved even on prolonged incubations of tamarind xyloglucan with the nasturtium enzyme. This indicated that the increased aggregation of the polysaccharide prevented the enzyme from removing those galactosyl residues which may not be accessible.

5.4. DISCUSSION.

The xyloglucan-active ß-galactosidase was purified from germinated nasturtium cotyledons by a simple procedure involving ion-exchange chromatography and gel filtration according to Edwards *et al.*(1988). The purified protein has a molecular weight of 97 KDa on SDS-polyacrylamide gel and contains several minor components. Western blots of the purified protein treated with an antiserum raised against the major protein band show cross-reactivity with the lower molecular weight components. This indicates that the lower molecular weight protein components are breakdown products of the same protein possibly due to limited proteolysis, confirming the observation of Edwards *et al.* (1988). Chromatofocusing of the purified enzyme shows that ß-galactosidase consists of a number of closely related molecular species spanning the range pI 6.6 to pI 7.1 as observed by Edwards *et al.* (1988).

This enzyme catalyzes the hydrolysis of terminal galactosyl residues linked ß-(1→2) on to the xylosyl side-chain of xyloglucans. Other purified ß-galactosidases

have not been reported to hydrolyze galactosyl side-chains of xyloglucans. Three purified ß-galactosidases (from *E. coli*, yeast and Jack bean) were used to hydrolyze galactose from xyloglucan but were found to be unable to attack galactose side-chains (Edwards *et al.*, 1988).

Enzyme kinetics using oligosaccharide B containing the isomers B_1 and B_2 indicate that oligosaccharide B_1 is quickly hydrolyzed to oligosaccharide A and galactose and oligosaccharide B_2 is hydrolyzed more slowly to oligosaccharide A and galactose. This finding suggests that the position of the galactose residue affects the rate of the enzyme catalysis. The galactose preferentially attacked and cleaved is located on the region of the xyloglucan molecule which is less substituted by xylosyl residues. However studies carried out to establish whether the xylosyl substitution affects the rate of hydrolysis of galactose residues located near the reducing and/or the non-reducing end of the molecule have shown that the the nasturtium ß-galactosidase cleaves preferentially galactosyl substituents located in proximity to the non-reducing end of a xylose-depleted nonasaccharide. This can be explained by postulating that the nasturtium enzyme approaches the oligosaccharide molecule from the non-reducing end. It is likely that galactosyl substitution of the xylose adjacent to the non-reducing glucose is more accessible to the enzyme and this would also explain the observed "higher" and "lower" rates of catalysis of oligosaccharides B_1 and B_2.

Studies of the action of the enzyme on the polymeric xyloglucan substrate have shown that the presence of sodium sulphate induces an increase of hydrophobic interactions of xyloglucan molecules confirming the findings of Reid *et al.* (1988). Moreover in the presence of sodium sulphate the rate of the enzyme reaction is

enhanced and a thermo-irreversible gel is obtained in a relatively short time (2 hours). The digestion of modified xyloglucan with the fungal cellulase clearly shows that in the presence of sodium sulphate the nasturtium ß-galactosidase preferentially removes galactose residues located on the less xylosyl-substituted region of the xyloglucan (B_1 type of substrate). Using similar experimental conditions in the absence of sodium sulphate the nasturtium enzyme was shown to attack both sides of the xyloglucan molecule cleaving galactose residues located both "above" and "below" the plane containing the glucan backbone. Aggregation of galactose-depleted xyloglucan molecules is achieved more slowly and the gel obtained is weaker than that obtained in the presence of sodium sulphate. It can be speculated that the presence of sodium sulphate may induce a conformational change of the polymeric xyloglucan molecule which may expose those galactose residues located on the less xylosyl-substituted side of xyloglucan to the enzyme attack and cleavage. This observation may explain the differences in the gel properties caused by galactose removal.

X-ray fibre diffraction of xyloglucans from both primary and storage cell walls, have shown that xyloglucan molecules are characterized by a certain degree of regularity corresponding to the repeating units A, B and C (the nonasaccharide is fucosylated in the primary cell walls) and that they form two-fold helices (Taylor and Atkins, 1985; Ogawa *et al.*, 1990). The helices of (1→4)-ß-linked equatorial glucoses have been found to generate a flat ribbon like structure. If this structure is present in solution the removal of galactose residues could enhance the aggregation of the xyloglucan chains. X-ray diffraction of xyloglucan samples treated with the nasturtium ß-galactosidase, carried out by Millane and Narasaiah

at Purdue University showed that some regions of the polysaccharide had highly ordered fibre arrangements. These "crystalline" regions probably derive from the galactose-depleted areas of the polysaccharide molecule which are responsible for the observed gelation (Millane R.P. and Narasaiah T.V., unpublished work) formation of a thermo-irreversible gel is due to the rapid increase of chain-chain interactions between adjacent xyloglucan molecules. The gradual removal of galactose side-chains catalyzed by the ß-galactosidase induces a self-association of xyloglucan chains which may occur through those regions of the polysaccharide characterized by a lower degree of xylose substitution. This is made possible by cleaving off the galactosyl residues (in the presence of sodium sulphate the enzyme ß-galactosidase attacks preferentially this side of the xyloglucan molecule) determining aggregation between the xyloglucan chains. It follows that the presence of xylosyl substitution does not affect the inter-molecular association between xyloglucan chains, whereas galactose prevents aggregation and contributes to the solubilization of the polysaccharide in aqueous solutions.

In this respect the xyloglucan aggregation appears to be in contrast with that of other polysaccharide such as cellulose and galactomannan, where the intermolecular association is caused by the backbone-backbone interaction. Addition of the xyloglucan-specific *endo*glucanase to the xyloglucan/ß-galactosidase mixture prevents the formation of a gel, indicating the production of the gel is a function of the high molecular weight of xyloglucans (Reid *et al.*, 1988).

CHAPTER SIX

CONCLUSIONS.

6.1. MOBILIZATION OF XYLOGLUCAN.

The results presented in the previous Chapters concerned the purification and the molecular properties of specific hydrolases involved in the mobilization of xyloglucan reserves in seeds. In particular, in Chapter three, the enzyme α-xylosidase was purified to homogeneity from germinated nasturtium seeds and it was shown to catalyze the removal of a single unsubstituted xylosyl residue attached to the backbone glucose at the non-reducing end of the xyloglucan molecule. The nasturtium *endo*glucanase, in Chapter four, was shown to be a novel type of transferase (XET) able to catalyze the transglycosylation of oligoxyloglucan-donors onto xyloglucan-acceptors. The study of the action of the enzyme ß-galactosidase on tamarind xyloglucan showed that this enzyme catalyzed the selective removal of galactose residues from xyloglucan side-chains, bringing about an increase of chain-chain interaction and progressive gelation was described in Chapter five. The data presented here were however obtained from *in vitro* experiments carried out by treating the xyloglucan polysaccharide with the pure enzymes and subsequent analysis of the breakdown products.

The molecular mode of action of these hydrolases was investigated to gain an insight into the physiological role of the enzymes in the post-germinative mobilization of xyloglucan. By analogy with the enzymic breakdown of other polysaccharides, for instance cellulose, it was proposed that the mobilization of xyloglucan reserves in *Tropaeolum* was carried out by several enzymes acting "in concert" (Edwards *et al.*, 1985). In the case of cellulose, three enzymes are required for its breakdown, an *endo*-(1→4)-ß-D-glucanase, an exo-cellobiohydrolase and a ß-D-glucosidase (Walker and Wilson, 1991). Similarly the hydrolysis of the xyloglucan requires the action of at least four enzymes an *endo*-(1→4)-ß-D-glucanase, a ß-galactosidase, an α-xylosidase and ß-glucosidase. During the hydrolysis the enzyme *endo*-glucanase (*endo*-transglycosylase) attacks and cleaves the glucan backbone, creating a number of newly accessible non reducing chain ends that are available for attack by other hydrolases. The enzyme α-xylosidase does not attack the native polysaccharide, but hydrolyzes a monoxylosyl residue from the non-reducing end of the xyloglucan fragments generated by the *endo*glucanase. The xylose-depleted oligosaccharides produced by the α-xylosidase represent substrates for the enzyme ß-glucosidase, which cleaves a single glucosyl residue from the non reducing end of the molecule. The synergistic action of ß-glucosidase and α-xylosidase will complete the breakdown of xyloglucan oligosaccharides producing glucose and xylose. The enzyme ß-galactosidase removes ß-galactosyl residues from the native polysaccharide and from the oligosaccharides produced by *endo*glucanase. The mechanism of xyloglucan breakdown is summarized in Fig.6.1.

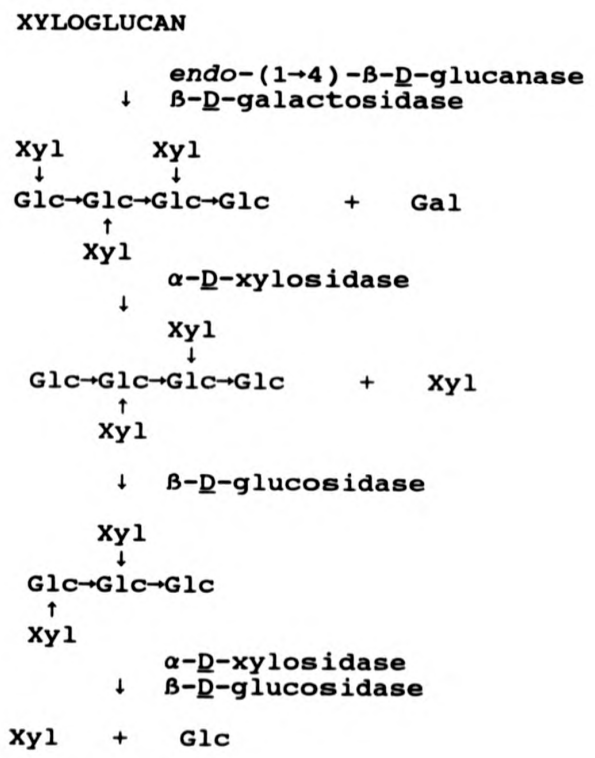


Fig.6.1. The mechanism of xyloglucan mobilization involves the synergistic interaction of specific hydrolases.

The involvement of a transglycosylase (and not a conventional *endo*glucanase) in the mechanism of xyloglucan break-down represents perhaps the most interesting aspect of the mobilization. The process of xyloglucan mobilization consists of the "dismantling" of the polysaccharide into smaller saccharides which will be taken up by the embryo.

It was observed that, *in vivo*, xyloglucan oligosaccharides do not accumulate (Edwards *et al.*, 1985; Brummel and Maclachlan, 1991) and the final products of xyloglucan hydrolysis are the monosaccharides xylose, galactose and glucose. If the nasturtium *endo*-transglycosylase was a conventional *endo*-(1→4)-ß-D-glucanase the levels of this enzyme and its activity would determine the rate of enzymic release of oligosaccharides. The fact that this enzyme is a transglycosylase allows that the rate of xyloglucan breakdown to be determined by the levels of other hydrolases. If the xyloglucan oligosaccharides are quickly mobilized by the other hydrolases, the "equilibrium" hydrolysis-transglycosylation (where hydrolysis is considered as the transfer of oligosaccharide donors to H_2O molecules acting as acceptors) will shift towards hydrolysis and more fragments will be released by the *endo*-transglycosylase. In Chapter Four it was shown that lower substrate concentration encourages hydrolysis but does not abolish transglycosylation completely. However the oligosaccharide "re-shuffle" will eventually cease when the substrate concentration is zero.

A number of factors and variables will affect the mechanism of xyloglucan mobilization and they must be taken in to account. For instance, the levels of each enzyme vary during xyloglucan mobilization and the maximum activity of each enzyme is therefore reached at different times (days). The enzyme ß-

galactosidase appears to be synthesized before other enzyme activities, whereas the enzyme ß-glucosidase appears to be present before the enzymic mobilization of xyloglucan begins, and its levels do not change appreciably (Edwards *et al.*, 1985). Moreover the enzyme ß-galactosidase hydrolyzes substrates B_1 and B_2 at different rates (Chapter 5) and it is inhibited by galactose. The persistence of galactose on the non-reducing terminal xylose will prevent the α-xylosidase (and the ß-glucosidase) from attack and cleavage.

Hensel and co-workers (1991) have recently provided evidence for a hormonal control of xyloglucan mobilization in germinated nasturtium seeds. They observed that in excised cotyledons the mobilization ceases but it is restored by auxins. They suggested that auxin may, directly or through a specific receptor, activate the xyloglucan-specific *endo*-transglycosylase. This is the first report on auxin-control of *endo*-transglycosylase activity during the mobilization of xyloglucan reserves of nasturtium cotyledons.

Other variables such as temperature, pH and water intake will affect the breakdown of xyloglucan reserves, but the effects of these factors on the seed germination and on reserve mobilization go beyond the aims of this thesis.

The mechanism of transglycosylation of xyloglucan oligosaccharides and the enzymic control of the rate at which small xyloglucan fragments are produced and further degraded appears to be in agreement with the "natural sink" theory of Chapman and Davies (1983). It has been suggested that the rate of mobilization of reserve polysaccharides may vary according to the demand of the embryo. In absence of such "natural sink", i.e. excision of the axis from the cotyledons, the mobilization may be inhibited by a simple machanism of feed-back regulation of

break-down products. This would overcome the problem of oversupply of food reserves necessary for the development of the young seedling (Halmer, 1985).

An interesting finding recently reported by Farkas *et al.* (1991) shows that nasturtium seedlings contain high levels of the enzyme α-fucosidase. This result was confirmed by treating a cellulase digest of fucosylated xyloglucan, purified from pea stems or mung bean hypocotyls, with the crude enzyme extract prepared from 11-d nasturtium cotyledons and observing the released of fucose. The enzyme α-fucosidase was co-eluted with the *endo*-transglycosylase during anion- and cation-exchange chromatography (results not shown). The reason for the presence of this enzyme is obscure as the storage cell wall xyloglucan is not fucosylated. It may be speculated that a number of genes, which encode various cell wall hydrolases, may be activated through a common mechanism (i.e., induced by auxin). It may be plausible that the presence of α-fucosidase inactivates potentially biologically active oligosaccharides, such as the fucosylated nonasaccharide mentioned in the introduction, which could derive from the hydrolysis of xyloglucan associated with the primary cell wall.

In certain leguminous seeds, during the synthesis of storage cell wall galactomannan, low levels of an α-galactosidase have been found (Edwards *et al.*, 1992). This enzyme is responsible the final "trimming" of some of the galactose side-chains of the polysaccharide. An analogous situation may occur during the deposition of xyloglucan in nasturtium seeds. This does not explain, however, the high levels of this enzyme during xyloglucan mobilization. It may be that some m-RNA, stored in the seed, is translated following germination.

6.2. XYLOGLUCAN TURNOVER IN THE PRIMARY CELL WALL.

The primary cell walls of plants contain several polysaccharides, differing in structure and function (see Chapter 1). Cellulose, the major component of the cell wall in both monocotyledons and dicotyledons, hemicelluloses and pectins represent the three major extracellular matrix polysaccharides. Cellulose and xyloglucan are hydrogen bonded together in the primary cell wall (Bauer *et al.*, 1973; Joseleau and Chambat, 1984; Hayashi *et al.*, 1987), and it has been suggested that each xyloglucan molecule may cross-link several cellulose microfibrils with parts of the molecule suspended between microfibrils (Fry and Miller, 1989). Other types of interaction, covalent and non-covalent, between matrix polysaccharides and proteins contribute to the integrity of the cell wall (McCann and Roberts, 1991).

There is a great deal of indirect evidence that turgor-driven cell expansion in dicots is due to an enzymic cleavage of the xyloglucan molecule, which loosens the interaction between xyloglucan and cellulose microfibrils. Under turgor stress the cellulose microfibrils are free to move along or apart from each other, leading to irreversible or plastic extension of the wall. To prevent weakening of the wall, which would cause an irreversible damage of the mechanical properties of the wall matrix, the linkages between xyloglucan "ends" must be restored. It has been proposed that a specific enzyme, a xyloglucan-specific *endo*-transglycosylase (XET), may play an important part in the mechanism of bond synthesis between

two adjacent xyloglucan chains (Albersheim, 1976; Fry, 1989). A mechanism of transglycosylation of xyloglucan molecules could explain the observed wall loosening and tightening ensuring that the cell wall retains the mechanical properties (Farkas *et al.*, 1992; Fry *et al.*, 1992)(Fig.6.2.).

Several glycosidases and *endo*glycanases are able to catalyze transglycosylation as well as hydrolysis and they are common in plant systems (Cote and Tao, 1990). *Endo*-ß-mannanase from fenugreek has been found to transglycosylate mannoligosaccharides *in vitro* (McCleary and Matheson, 1983); an auxin-induced dextranase (α-(1→6)-D-glucan-6-glucanohydrolase, EC3.2.1.11) from *Avena sativa* coleoptiles has been found to transglycosylate low molecular weight dextran at a concentration of 0.1% (Heyn, 1981); a D-enzyme (D = disproportionation) from potato has been reported to synthesize maltodextrin oligosaccharides by transglycosylation of maltodextrin and glucose (Walker and Whelan, 1959). Xyloglucan-specific *endo*-transglycosylases represent a new type of enzyme. It was recently shown that an exogenous ^{3}H-xyloglucan nonasaccharide was incorporated into endogenous cell wall xyloglucan of suspension-cultured *Spinacia* cells (Smith and Fry, 1991). This finding provided evidence for an enzyme able to transfer xyloglucan fragments in to a polymeric xyloglucan molecule *in vivo* and to participate in xyloglucan turnover in primary cell walls. A transglycosylation mechanism was also proponed to explain the *in vitro* formation of high molecular weight xyloglucan in the presence of an enzyme extract obtained from *Vigna angularis* epicotyls (Nishitani and Tominaga, 1991). This enzyme has been purified very recently from the rapidly elongating regions of *Vigna* cell walls (Nishitani and Tominaga, 1992).

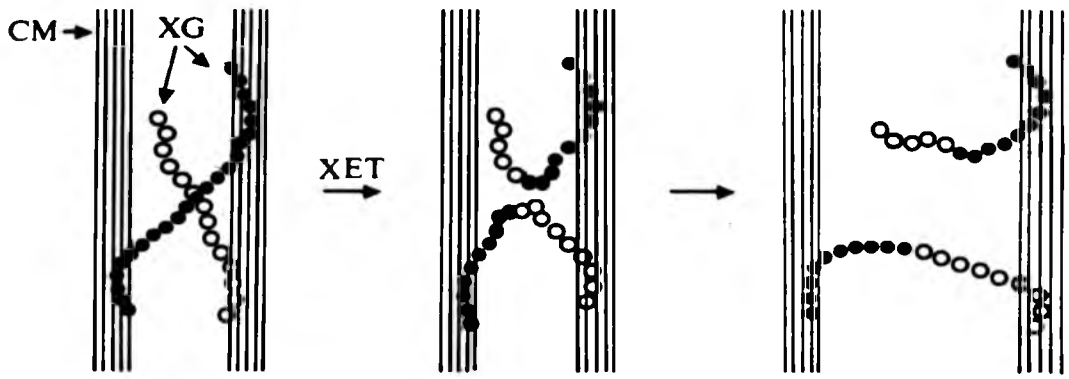


Fig.6.2. Proposed model for cell wall loosening mediated by the xyloglucan *endo*-transglycosylase (XET).

The recombination reaction between xyloglucan chains (XG) causes cell wall loosening followed by cell wall extension. CM = cellulose microfibrils.

(Re-drawn after Nishitani; 1992).

The molecular properties (molecular weight, pH optimum) of this enzyme indicates that it and the nasturtium *endo*-transglycosylase are closely related proteins. The most remarkable similarity between this novel XET enzyme and the nasturtium *endo*-transglycosylase consists in the high sequence homology between the cDNA of these proteins and a gene isolated from *Arabidopsis* (meri-5), highly expressed in rapidly dividing tissues, the function of which is still unknown (Nishitani K., personal communication; de Silva *et al.*, 1993).

Cell wall extension is accompanied by the deposition of newly synthesised wall material. By analogy with xylans, the major hemicellulosic component in woods, it has been proposed that newly synthesised xyloglucan may interact and associate with cellulose microfibrils after secretion in the cell wall (Vian *et al.*, 1986). Newly synthesised xyloglucan must be, however, re-arranged in the wall. A xyloglucan-specific *endo*-transglycosylase would be able to catalyze the incorporation of newly synthesized xyloglucan into the extracellular matrix xyloglucan.

6.2.1. FUNCTION AND PHYSIOLOGICAL IMPLICATION OF XYLOGLUCAN-SPECIFIC HYDROLASES.

Auxins and other growth hormones, including synthetic ones, have been shown to promote wall loosening and cell wall extension (Cleland, 1971). The basis of this mechanism is not entirely understood. It is known that auxins bring about an acidification of the cell wall (Jacobs and Ray, 1975; Keegstra *et al.*, 1973; Lorences

et al., 1990) and promote a solubilization of xyloglucans (Labavitch and Ray, 1974; Terry *et al.*, 1981; Hoson, 1990). Several investigations have shown clearly that specific hydrolases involved in xyloglucan metabolism are "activated" by auxin. They include an *endo*-(1→4)-ß-D-glucanase (Hayashi *et al.*, 1984), an α-xylosidase (O'Neil *et al.*, 1989), a ß-galactosidase (Tanimoto and Igari, 1976) from pea-stem and, very recently, a xyloglucan *endo*-transglycosylase (XET), from mung beans (Nishitani and Tominaga, 1992). The strong similarities between the auxin-treated pea stem α-xylosidase and the nasturtium α-xylosidase, and the strong similarities between the XET and the nasturtium xyloglucan *endo*-transglycosylase in terms of specificity and molecular properties suggest that these proteins may be encoded by a similar gene(s), the expression of which is developmentally regulated. This would explain the presence of high levels of similar hydrolases in the cotyledons and in the rapidly expanding regions of the plant.

In Chapter 4 it was shown that the specific anti-*endo*transglycosylase antibody cross-reacted with some protein components present in crude enzyme extracts of nasturtium, tomato and tobacco stem. This provides some evidence for the presence of enzymes such as the nasturtium xyloglucan-specific *endo*-transglycosylase in the elongating regions of plant tissues other than nasturtium. The gene encoding nasturtium XET has recently been isolated and transferred to a target plant (de Silva *et al.*, 1993). The up-and-down regulation of different levels of this enzyme in the transgenics will hopefully give more detailed information on its role in *in vivo* xyloglucan turnover.

Another interesting aspect of the physiological role of hydrolases able to modify xyloglucans concerns the rheological properties of the cell wall matrix. It has been

shown that xyloglucan and cellulose are tightly bound in the primary cell wall and it has been speculated that this interaction between xyloglucan and cellulose interaction may decrease the self-association of cellulose microfibrils. It has also been shown that the nasturtium ß-galactosidase is able to hydrolyze galactose residues from the xyloglucan side-chain causing a dramatic increase of inter-molecular association of xyloglucan chains. The rapid increase of viscosity was accompanied by the formation of a gel, as shown in Chapter 5. It is possible that enzymes with same specificity as the nasturtium ß-galactosidase may be involved in xyloglucan turnover. Activation of an auxin-induced ß-galactosidase has been reported by Tanimoto and Igari (1976). The interaction of xyloglucan with cellulose could therefore be regulated by an enzymatic mechanism: by removing galactosyl residues an increased asssociation between xyloglucan and cellulose (or other xyloglucan molecules) may occur leading to a modification of the mechanical properties of the cell wall. The isolation of the gene(s) of the enzyme ß-galactosidase from nasturtium is in progress. The regulation of the levels of this enzyme in a transgenic plants may give more information on the physiological function of this and other xyloglucan-specific hydrolases and on the assembly of wall polysaccharides in the extracellular matrix.

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