Enzymatically Tailored Xyloglucan and its Performance in *in vitro* Assembled Cellulose / Xyloglucan composites

Elaine Wilson

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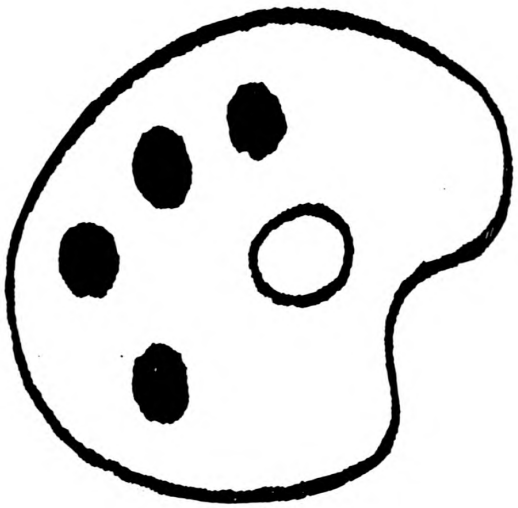

Table of Contents

Table of Contents i
Table of Figures viii
Table of Abbreviations xii
Acknowledgements xiv
Abstract xv

Chapter 1: Introduction 1
1.1 Seed Storage Xyloglucan: Amyloid 2
1.2 Primary Cell Wall Xyloglucans: Extracellular Matrix 2
1.2.1 Cell Walls: A Model Approach 3
1.3 Structure and Composition of Xyloglucans 4
1.4 Mobilisation of Xyloglucan 6
1.4.1 *Endo-β*-D-glucanase / Xyloglucan *endo-β*-D-transglycosylase (NXET) 7
1.4.2 β -D-Galactosidase 9
1.4.3 α -D-Xylosidase 9
1.4.4 β -D-Glucosidase 10
1.5 Conformation of Xyloglucan 11
1.6 Cellulose 12
1.7 The Xyloglucan - Cellulose Interaction 14
1.8 Investigating the Xyloglucan - Cellulose Interaction 16
1.8.1 *Acetobacter xylinus* 17
1.9 Aim of this work 19

Chapter 2: Materials and Methods 21
2.1 Materials 21
2.2 Methods 21
2.2.1 Purification of Enzymes from Nasturtium Seedlings 21
2.2.1.1 *Seed Germination and Growth Conditions* 21
2.2.1.2 *Enzyme Extraction* 22
2.2.1.2.1 Xyloglucan Endo-β -D-glucanase (NXET) 22
2.2.1.2.2 β -D-Galactosidase 23
2.2.1.2.3 β -D-Glucosidase 24
2.2.1.2.4 α -D-Xylosidase 25
2.2.2 Enzyme Activity Assay 26
2.2.2.1 *Preparation of Tamarind Xyloglucan* 26
2.2.2.2 *Enzyme Assays* 27
2.2.2.2.1 Determination of D-Galactose by Galactose Dehydrogenase (GDH) 27
2.2.2.2.2 Determination of Free Pentoses 28
2.2.2.2.3 Viscometric Assay for Endo (1→4)-β -D-glucanase 28
2.2.2.2.4 Nitrophenylglycoside Assay 29
2.2.3 Protein Determination 30
2.2.4 SDS-Polyacrylamide Gel Electrophoresis 30
2.2.5 HPAEC Analysis 31

2.2.5.1 *Oligosaccharide Analysis of Xyloglucan on HPAEC* 31
2.2.5.2 *Monosaccharide Analysis of xyloglucan/cellulose composites on HPAEC* 32
2.2.6 Overexpression and Secretion of NXET in *Pichia pastoris* 33
2.2.6.1 *Basic Protocol for overexpression of NXET in Pichia* 33
2.2.6.2 *Methods for Optimisation of Pichia Protein Expression* 33
2.2.6.2.1 *Proteolysis of Expressed Protein* 33
2.2.6.2.2 *Procedure for Cell Lysis* 35
2.2.7 Preparation of Modified Tamarind Seed Xyloglucans 35
2.2.7.1 *Time Course for the Depletion of Galactose from Xyloglucan* 35
2.2.7.2 *Preparation of Galactose Depleted Xyloglucan (Large Scale)* 36
2.2.7.3 *Preparation of Galactose Depleted 'Gel'* 36
2.2.7.4 *Mild Acid Hydrolysis for the Removal of Xylose from Xyloglucan* 36
2.2.7.5 *Enzymatic Hydrolysis of Xyloglucan with α -Glucosidase* 37
2.2.7.6 *Enzymatic Removal of Xylose using α -xylosidase and NXET* 37
2.2.7.7 *Enzymatic Removal of Xylose using α -xylosidase and TXET* 37
2.2.8 Production of Bacterial Cellulose 38
2.2.8.1 *Revival of Bacterial Stock Cultures* 38
2.2.8.2 *Production of Seed Cultures* 39
2.2.9 Preparation of Composites 39
2.2.10 Biochemical Testing of Composites 40
2.2.10.1 *Sequential Extraction of Xyloglucan with KOH* 40
2.2.10.2 *Alkali Removal of Bound Xyloglucan from Composites* 41
2.2.10.3 *Removal of Xyloglucan from Composites by Agitation Only* 41
2.2.10.4 *Removal of Loosely Bound Xyloglucan by Action of Endoglucanase* 41
2.2.10.5 *Removal of Galactose from Tightly Bound Xyloglucan by Action of β -Galactosidase* 41
2.2.11 Mechanical Testing of Composites
Uniaxial Tensile Testing 42
2.2.12 Deep-Etch, Freeze-Fracture, Transmission Electron Microscopy 41
2.2.13 ^{13}C NMR Spectroscopy 43

Chapter 3: Purification of Four Xyloglucan-Specific Enzymes from Germinated Nasturtium *(Tropaeolum majus* L) Seeds 44
3.1 Introduction 44
3.2 Materials and Methods 46
3.2.1 Purification of Enzymes 46
3.2.2 Preparation of Xyloglucan Solution 46
3.2.3 Determination of Enzyme Activity 46

3.2.4 Protein Determination 46
3.2.5 SDS-Polyacrylamide Gel Electrophoresis 46
3.3 Results **47**
3.3.1 *endo* (1→ 4)β -D-Glucanase 47
3.3.2 β -D-Galactosidase 49
3.3.3 α -D-Xylosidase 50
3.3.4 β -D-Glucosidase 52
3.3.5 SDS-Polyacrylamide Gel Electrophoresis 53
3.4 Discussion **55**

Chapter 4: Overexpression of NXET by the Methylotrophic Yeast *Pichia pastoris* **57**
4.1 Introduction **57**
4.2 Materials and Methods **59**
4.2.1 Investigation of clones 6, 13, 18 60
4.2.2 Optimisation of Conditions for Secretion of NXET 60
4.2.2.1 *To Counteract the Presence of proteases* *60*
4.2.2.2 *Increasing the density of the cells before induction* *61*
4.2.2.3 *Examination of the cell pellet* *61*
4.2.3 Gel Filtration of NXET from *Pichia pastoris* 61
4.2.4 Investigation of Action of NXET on Xyloglucan Solution 61
4.3 Results **63**
4.3.1 Investigation of Clones 6, 13, 18 63
4.3.2 To counteract the Presence of Endogenous Proteases 63
4.3.3 *Increasing the Density of the Cells Before* Induction and the Addition of 1% Casamino Acids to the Induction Media 65
4.3.4 Examination of the Cell Pellet 66
4.3.5 Purification of NXET from *Pichia pastoris* 67
4.3.6 *Action of NXET from Pichia pastoris Compared* to that Isolated from Nasturtium 68
4.4 Discussion **70**

Chapter 5: Modification of Tamarind Seed Xyloglucan by Action of β -Galactosidase **72**
5.1 Introduction **72**
5.2 Materials and Methods **75**
5.2.1 Removal of Galactose with Time (small scale) 75
5.2.2 Production of Modified Xyloglucans (Large Scale) 75
5.2.3 Incubation of Xyloglucan with Nasturtium Seed β -Galactosidase at 50°C 76
5.3.4 ^{13}C NMR Spectroscopy of Aggregated Xyloglucan (~60% galactose depleted) 76
5.3 Results **76**
5.3.1 Removal of Galactose with Time (small scale) 77
5.3.2 Production of Modified Xyloglucans (Large Scale) 76

5.3.3 Incubation of Xyloglucan with Nasturtium Seed β -Galactosidase at 50°C 81
5.3.4 ^{13}C NMR Spectroscopy of Aggregated Xyloglucan (~60% galactose depleted) 81
5.4 Discussion **85**

Chapter 6: Interaction of Galactose Depleted Xyloglucan with Cellulose Produced by *Acetobacter xylinus* **88**
6.1 Introduction **88**
6.2 Materials and Methods **91**
6.2.1 Bacterial Cultures 91
6.2.2 Monosaccharide Analysis of Composite Material 91
6.2.3 Removal of Loosely bound Xyloglucan by Agitation 91
6.2.4 Sequential Extraction of Xyloglucan from Composites Using KOH 91
6.2.5 Enzymatic Digestion of Cellulose / Xyloglucan Composites 92
6.2.6 Incubation of Composites with β -Galactosidase 92
6.2.7 Deep-Etch, Freeze-Fracture, Transmission Electron Microscopy 92
6.2.8 ^{13}C NMR Spectroscopy 93
6.2.9 Uniaxial Tensile Testing 93
6.3 Results **94**
6.3.1 Levels of Xyloglucan Incorporation 94
6.3.2 Removal of Loosely bound Xyloglucan by Agitation 95
6.3.3 Sequential Extraction of Xyloglucan from Composites Using KOH 95
6.3.4 Enzymatic Digestion of Cellulose / Xyloglucan Composites 97
6.3.5 Incubation of Composites with β -Galactosidase 99
6.3.6 Deep-Etch, Freeze-Fracture, Transmission Electron Microscopy 100
6.3.7 ^{13}C NMR Spectroscopy 107
6.3.8 Uniaxial Tensile Testing 113
6.4 Discussion **115**

Chapter 7: Investigation into the Modification of Xyloglucan by Removal of Xylose and *in vitro* Construction of Composites **117**
7.1 Introduction **117**
7.2 Materials and Methods **118**
7.2.1 Removal of Xylose 120
7.2.1.1 *Acid Hydrolysis of a solution of Tamarind seed Xyloglucan* *120*
7.2.1.2 *Incubation of Xyloglucan Solution with α- glucosidase* *120*
7.2.1.3 *Incubation of Xyloglucan with NXET and α -xylosidase* *120*
7.2.1.4 *Incubation of Xyloglucan with Tomato XET and α- xylosidase* *120*
7.2.2 Construction of Cellulose / Xylose-depleted Xyloglucan composites 121

7.2.3 Monosaccharide Analysis of Composite Material 121
7.2.4 Removal of Loosely Bound Xyloglucan by Agitation 121
7.2.5 Sequential Extraction of xyloglucan from Composites Using KOH 121
7.2.6 Enzymatic Digestion of Cellulose / Xyloglucan Composites 122
7.2.7 Deep-Etch, Freeze-Fracture, Transmission Electron Microscopy 122
7.2.8 Uniaxial Tensile Testing 122
7.3 Results **123**
7.3.1 Removal of Xylose from High Molecular Weight Xyloglucan 123
7.3.1.1 *Removal of Xylose from High Molecular Weight Xyloglucan by Mild Acid Hydrolysis* *123*
7.3.1.2 *Removal of Xylose from High Molecular Weight Xyloglucan* by *Hydrolysis with an α -glucosidase* *123*
7.3.1.3 *Removal of Xylose from High Molecular Weight Xyloglucan by Synergistic Action of NXET and α -xylosidase* *124*
7.3.1.4 *Removal of Xylose from High Molecular Weight Xyloglucan by Synergistic Action of tomato XET and α -xylosidase* *124*
7.3.2 Construction and Characterisation of Cellulose/ Xyloglucan composites 125
7.3.2.1 *Levels of Xyloglucan Incorporation* *125*
7.3.2.2 *Removal of Loosely Bound Xyloglucan by Agitation* *125*
7.3.2.3 *Enzymatic Digestion of Cellulose / Xyloglucan Composites* *126*
7.3.2.4 *Sequential Extraction of xyloglucan from Composites Using KOH* *126*
7.3.2.5 *Deep-Etch, Freeze-Fracture, Transmission Electron Microscopy* *127*
7.3.2.6 *Uniaxial Tensile Testing* *130*
7.4 Discussion **132**

Chapter 8: Construction of Cellulose/Xyloglucan composites in the Presence of tomato XET and Xyloglucan Oligosaccharides **134**
8.1 Introduction **134**
8.2 Materials and Methods **136**
8.2.1 Construction of Cellulose / Xyloglucan composites in the presence of tXET and of tXET plus xyloglucan-derived oligosaccharides 136
8.2.2 Monosaccharide Analysis of Composite Material 136
8.2.3 Removal of Loosely Bound Xyloglucan by Agitation 136
8.2.4 Sequential Extraction of xyloglucan from Composites Using KOH 136
8.2.5 Enzymatic Digestion of Cellulose / Xyloglucan Composites 137
8.2.6 Deep-Etch, Freeze-Fracture, Transmission Electron Microscopy 137
8.2.7 Uniaxial Tensile Testing 137

8.3 Results **138**
8.3.1 Levels of Xyloglucan Incorporation 138
8.3.2 Removal of Loosely Bound Xyloglucan by Agitation 139
8.3.3 Sequential Extraction of xyloglucan from Composites Using KOH 140
8.3.4 Enzymatic Digestion of Cellulose / Xyloglucan Composites 141
8.3.5 Deep-Etch, Freeze-Fracture, Transmission Electron Microscopy 142
8.3.6 Uniaxial Tensile Testing 146
8.4 Discussion **149**

Chapter 9: Discussion **151**

Chapter 10: References **156**

Table of Figures

Figure 1.1: Structural features of seed and primary cell wall xyloglucan. Only primary cell wall xyloglucan haas fucosyl residues 5

Figure 1.2: Oligosaccharides resulting from fungal *endo* (1→4)-β-glucanase digestion of tamarind seed xyloglucan 7

Figure 1.3: Substrate subsite recognition of NXET 8

Figure 1.4: The sequential degradation of tamarind seed xyloglucan involves the synergistic action of four hydrolases 11

Figure 1.5: Schematic diagram of the biogenesis of a cellulose microfibril 18

Figure 2.1: Components and Method for BMGY 34

Figure 2.2: Components and Method for BMMY 34

Table 2.1: Components of Hestrin & Schramm Medium 38

Figure 3.1: The Storage Cell Wall Xyloglucan in *Tropaeolum majus* stained with iodine-potassium iodide 45

Figure 3.2a: Anion Exchange Chromatography profile of NXET on DEAE-Cellulose 48

Figure 3.2b: Cation Exchange Chromatography profile of NXET on CM-Cellulose 48

Figure 3.2c: Gel filtration profile of NXET using Biogel P60 48

Figure 3.3a: Anion Exchange Chromatography profile of β-galactosidase on DEAE-Cellulose 49

Figure 3.3b: Cation Exchange Chromatography profile of β-galactosidase on CM-Cellulose 50

Figure 3.3c: Gel filtration profile of β-galactosidase using Biogel P60 50

Figure 3.4a: Xylosidase Purification on DEAE-Cellulose 51

Figure 3.4b: Affinity Chromatography on Concanavalin A 51

Figure 3.4c: Gel filtration profile of α -xylosidase using Biogel P300 52

Figure 3.5a: Anion Exchange Chromatography profile of β-glucosidase on DEAE-Cellulose 52

Figure 3.5b: Cation Exchange Chromatography profile of β-glucosidase on S-Sepharose 53

Figure 3.6: SDS-PAGE of Purified Proteins 54

Figure 4.1: Transglycosylation and Hydrolysis of Tamarind seed Xyloglucan by NXET 58

Figure 4.2: Partial Structure of Seed Xyloglucan Illustrating the Proposed Minimum Requirement for Substrate Recognition 59

Figure 4.3: SDS-PAGE of Clones 6, 13 and 18 64
Table 4.1: Activity of NXET produced by clones 6, 13 and 18 64

Figure 4.4: SDS-PAGE of Protein Produced by Induction with MM 65

Figure 4.5: SDS-PAGE of Protein Produced after Growth of Cells to Higher Density 66

Table 4.2: Activity and Protein Concentration after Growth of Cells to Higher Density 66

Figure 4.6: Gel Filtration Profile of *Pichia* Produced NXET on Biogel P60 67

Figure 4.7: SDS-PAGE of Elution Profile from Biogel P60 68

Table 4.3: Oligosaccharide Composition of High and Low Molecular Weight Xyloglucan as Determined by HPAEC 69

Figure 5.1: Tamarind Seed Xyloglucan Showing 'Crowded' and 'Less Crowded' Sides 73

Figure 5.2: Rate of Removal of Galactose from Xyloglucan with Time 77

Table 5.1: Levels of Galactose Depletion of Isolated Xyloglucans 78

Figure 5.3: Rate of Galactose Removal from 'Crowded' and 'Less Crowded' Sides 79

Figure 5.4: The Volume of Precipitate Increases as Galactose Content Decreases 80

Table 5.2: Data From GDH Assay and Monosaccharide Analysis of Galactose Depleted Xyloglucan 80

Figure 5.5: Precipitated Xyloglucan Consistently showed ~4% more Galactose Removed compared to soluble Xyloglucan 81

Figure 5.6a: SP-MAS and Resolution Enhanced CP-MAS for 60% Galactose Depleted Xyloglucan 82

Figure 5.6b: SP-MAS and CP-MAS for Native Tamarind Seed Xyloglucan (30% Solution) 83

Table 6.1: Initial cellulose:xyloIgucan ratios with Native and Galactose Depleted composites 94

Table 6.2: Cellulose:xyloglucan ratios after agitation of composites 95

Table 6.3: Cellulose:xyloglucan ratios after sequential extraction with KOH 96

Figure 6.1: Galactose content of xyloglucan removed from composites by KOH 97

Table 6.4: Cellulose:xyloglucan ratios after action of *endo*glucanase 98

Figure 6.2: Galactose content of xyloglucan removed from composites by action of *endo*glucanase 99

Figure 6.3a: Composite composed of native xyloglucan and cellulose showing cross-linking and lateral alignment 101

Figure 6.3b: Composite composed of native xyloglucan and cellulose showing xyloglucan/xyloglucan networks 102

Figure 6.4a: Composite constructed in the presence of 30% galactose-depleted xyloglucan showing evidence of xyloglucan/xyloglucan networks 103

Figure 6.4b: Composite constructed in the presence of 30% galactose-depleted xyloglucan showing evidence of cross-linking and lateral alignment 104

Figure 6.5a: Composite constructed in the presence of 60% galactose-depleted xyloglucan showing evidence of xyloglucan/xyloglucan networks 105

Figure 6.5b: Composite constructed in the presence of 60% galactose-depleted xyloglucan showing evidence of minimal interaction with cellulose 106

Figure 6.6: Spectra of hydrated cellulose and xyloglucan 109

Figure 6.7: SP-MAS and CP-MAS spectra of native xyloglucan and bacterial cellulose 110

Figure 6.8: SP-MAS and CP-MAS spectra of 30% galactose-depleted xyloglucan and bacterial cellulose 111

Figure 6.9: SP-MAS and CP-MAS spectra of 60% galactose-depleted xyloglucan and bacterial cellulose 112

Figure 6.10: Uniaxial tensile testing showing substantial differences in the mechanical behaviour 114

Figure 7.1: Minimum substrate for the action of nasturtium and pea α -D-xylosidase 117

Figure 7.2: Structural features of xylose - glucose α- linkage 118

Figure 7.3: Theoretical sequence of events with the synergistic actions of NXET and α -D-xylosidase 119

Table 7.1: Component parts of xyloglucan after mild acid hydrolysis 123

Table 7.2: Biochemical characterisation of xylose-depleted composite 127

Figure 7.4a: Composite constructed with xylose-depleted xyloglucan 128

Figure 7.4b: Higher magnification of xylose-depleted xyloglucan / cellulose composites 129
Figure 7.5a: Uniaxial tensile testing profile of control composite 130

Figure 7.5b: Uniaxial tensile testing profile of composite grown in presence of xylose-depleted xyloglucan 131

Table 8.1: Initial cellulose:xyloglucan ratios obtained from composites produced in the presence of tXET and tXET and oligosaccharides 139

Table 8.2: Cellulose:xyloglucan ratios obtained after agitation of composites 139

Table 8.3: Cellulose:xyloglucan ratios obtained after sequential extraction with KOH 140

Table 8.4: Cellulose:xyloglucan ratios obtained after action of endoglucanase 142

Figure 8.1: Composite composed of native xyloglucan and cellulose showing cross-linking and lateral alignment 143

Figure 8.2: Composite constructed in the presence of tXET illustrating essentially the same features as the control 144

Figure 8.3: Composite constructed in the presence of tXET and oligosaccharides illustrating that no cross-linking or lateral alignment occurs 145

Figure 8.4a: Uniaxial tensile testing of the control composite 147

Figure 8.4b: Uniaxial tensile testing of the composite grown in the presence of tXET 147

Figure 8.4c: Uniaxial tensile testing of the composite grown in the presence of tXET and oligosaccharides 148

Table of Abbreviations

ATCC	American Type Culture Collection
BMGY	Buffered glycerol complex medium
BMMY	*buffered methanol complex medium*
Ca^{2+}	Calcium
C	Carbon
CM Cellulose	Carboxymethyl Cellulose
cm	centimetre
cDNA	Complimentary DNA
CP-MAS	Cross-polarisation magic angle spinning
Da	Dalton
DEAE Cellulose	Diethylaminoethyl Cellulose
DP	Degree of polymerisation
EDTA	Ethylene diamine tetraacetic acid
FAB-MS	Fast atom bombardment mass spectroscopy
Fuc	Fucose
Gal	Galactose
GDH	*Galactose dehydrogenase*
Glc	Glucose
H	Hours
HCl	Hydrochloric acid
H_2SO_4	Sulphuric acid
HPAEC	*High performance anion exchange chromatography*
kb	kilo base
kDa	kilo dalton
KH_2PO_4	Potassium dihydrogen phosphate
K_2HPO_4	Dipotassium hydrogen phosphate
KOH	*Potassium hydroxide*
mA	milliamps
Mg^{2+}	Magnesium
mM	millimolar
MM	Minimal medium
MPa	MegaPascals
Na CH_3COO	Sodium acetate
NaCl	Sodium chloride
NAD	Nicotinamide adenine dinucleotide (oxidised)
NADH	Nicotinamide adenine dinucleotide (reduced)
NaOH	Sodium hydroxide
NaN_3	Sodium azide
Na_2SO_4	Sodium sulphate
nm	nanometers
NMR	Nuclear magnetic resonance
NXET	nasturtium xyloglucan *endo*glucanase / *endo*transglycosylase
O	Oxygen
OD	Optical density
PAD	*Pulsed amperometric detection*
ppm	parts per million
SDS-PAGE	Sodium dodecyl sulphate Polyacrylamide Gel Electrophoresis
SIBIA	Salk Institute Biotechnology / Industry Associates
SP-MAS	*Single pulse magic angle spinning*
TEMED	N,N,N',N',-tetramethylethylenediamine
TFA	Trifluoroacetic acid

TLC	Thin Layer Chromatography
Tris	2-amino-2-[hydroxymethyl]-1,3-propanediol
TXET	Tomato xyloglucan *endo*transglycosylase
U	Units
UDP	Uridine-5'-diphosphate
µg	micrograms
µl	microlitres
XET	Xyloglucan *endo*transglycosylase
XG	Xyloglucan
Xyl	Xylose
XXLG **XLXG** **XLLG** **XXXG**	Please note that this is the shorthand form for the four tamarind seed xyloglucan oligosaccharides resulting from digestion of high molecular weight xyloglucan by *endo*glucanase as put forward by Fry *et al* 1993

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Abstract

Enzymatically Tailored Xyloglucan and its Performance in *in vitro* Assembled Cellulose/Xyloglucan Composites

Xyloglucans are an important class of plant hemicellulosic polysaccharide and fulfil two roles in plants. They are major components of the specialised storage cell walls of some seeds, functioning as energy reserves. They are also present in primary cell walls where they are associated with cellulose in the cellulose/xyloglucan network. This network is considered to play a role in the maintenance of structural integrity and the regulation of cell growth.

To examine the way in which the structural features of xyloglucan affect the xyloglucan/cellulose interaction an *in vitro* system modelling the network has been developed (Whitney SEC, Brigham JE, Darke AH, Reid JSG, Gidley MJ, *The Plant Journal,* 8, 1995). This involves extrusion of cellulose by a Gram negative bacterium *Acetobacter xylinus* (formerly *Acetobacter acetii* ssp. *xylinum* ATCC 53524) into a medium containing xyloglucan resulting in a xyloglucan/cellulose matrix. This thesis examines the interaction between enzymatically tailored tamarind seed xyloglucan and *in vitro* produced cellulose.

Four xyloglucan-specific hydrolases were purified from nasturtium cotyledons. Three of these enzymes, β-galactosidase, nasturtium xyloglucan *endo*glucanase / *endo*transglycosylase (NXET), and α-xylosidase, were then used to obtain structurally modified xyloglucans. The performance of these tailored xyloglucans in the *Acetobacter* model system was then investigated using biochemical techniques, ^{13}C-NMR, Uniaxial Tensile Testing and Deep-Etch Freeze-Fracture Transmission Electron Microscopy.

The β-galactosidase was used to produce xyloglucans with 30% and 60% galactose depletion and characterisation of the resulting composites methods showed that increased removal of galactose from xyloglucan reduced its solubility and caused it to self-aggregate, rendering it unavailable for association with cellulose.

Several approaches to the selective removal of xylose were explored. Xylose-depleted xyloglucan was obtained by the concerted action of α-xylosidase and NXET. Characterisation of the resulting cellulose/xyloglucan composites showed very little association with cellulose due to the lowered molecular weight of the tailored xyloglucan.

Elaine Wilson

Chapter One

1. Introduction

It is necessary for plant cell walls to be rigid enough to give the plant strength and form. Yet, when the situation dictates, the walls must yield freely in order to facilitate growth. The wall protects the plant cell, maintains its shape and prevents excessive uptake of water. Two types of cell walls are found in higher plants: primary cell walls produced by growing cells which facilitate elongation by maintaining an interaction with the living cell, and secondary walls which cannot elongate but impart rigidity and mechanical stability.

Primary cell walls derived from growing plants are composed of complex carbohydrates (approximately 90%) and proteins (10%) which interact with each other during growth (Roelofsen 1959, Rogers and Perkins 1968, Goodwin and Mercer 1974, Preston 1974, Albersheim 1975). The cell wall polysaccharides can be separated into three general fractions: cellulose; the hemicelluloses; and the pectic polysaccharides. In this review the focus will predominantly be on xyloglucan, an important class of plant hemicellulosic polysaccharide, and its interaction with cellulose.

Xyloglucans fulfil two distinct roles in plants. They are major components of specialised storage cell wall in some seeds. In this context they function as energy reserves (Meier *et al* 1982). In association with cellulose, xyloglucans are also present in primary cell walls where their main roles are considered to be the maintenance of structural integrity (Valent and Albersheim 1974) and the regulation of cell growth (Labavitch and Ray 1974).

Xyloglucan oligosaccharides, at extremely low concentrations, have also recently been understood to regulate important cellular processes such as growth (Cutillas-Iturralde and Lorences 1997), development (Vargas-Rechia 1998) and defence (Ozeretskovskaya et al 1995).

1.1 Seed Storage Xyloglucan : Amyloid

During the growth and development of some seeds, polysaccharides accumulate and are stored as thickened cell walls. After germination, these are then utilised intensively via enzyme action to provide monosaccharide metabolites for new growth.

Early studies of these polysaccharides began in the mid-nineteenth century when Vogel and Schleiden (1839) demonstrated the existence of a substance in the thickened cell walls of some seeds which stained blue when treated with iodine reagent. The term 'amyloid' was given to these substances (xyloglucans) as a result of a similar blue colour given by starch when treated in the same way. Kooiman (1960) catalogued the presence or absence of amyloids in over 2600 species of plant seed by developing a method of quantifying the amyloid present in seeds and seed extracts. The seed storage xyloglucan of *Tamarindus indica* (Kooiman 1961& 1967, Gidley *et al* 1991) and *Tropaeolum majus* (Hsu and Reeves 1967, Courtois and Le Dizet 1974, Edwards *et al*, 1985) have been extensively characterised.

In nasturtium seeds (*Tropaeolum majus* L.) xyloglucan disappears during germination (Edwards *et al* 1985) suggesting its function is like that of starch - a reserve carbohydrate (Heinricher, 1888) which is metabolised into the monosaccharides required for growth. The amyloid of tamarind is a galactoxyloglucan with ratios for gal:xyl:glc of 1 : 2.25 : 2.8. That of nasturtium is similar in composition (Courtois and Le Dizet 1972, Gidley *et al* 1991), but there are reports of seed amyloids with different gal:xyl:glc ratios (Kooiman 1967, Siddiqui and Wood 1971, Courtois and Le Dizet 1972, , Buckeridge *et al* 1992).

1.2 Primary Cell Wall Xyloglucans : Extracellular Matrix

Cell expansion involves the depolymerisation and hydrolysis of cell wall polysaccharides. In recognition of this, a term for the primary cell wall was sought which would dispel the idea of a static structure, situated outside the plasma membrane. Thus in recent years the preferred terminology for this dynamic metabolic compartment which can undergo major rearrangements

during growth (McCann 1993) has been the extracellular matrix (Roberts *et al*, 1989). In this review the two terms: primary cell wall and extracellular matrix, will be used interchangeably. With few exceptions plant cells are enclosed within an extracellular matrix. As well as imparting mechanical strength the matrix also defines the shape and size of the cell. Xyloglucans (fucogalactoxyloglucans and arabinoxyloglucan) are quantitatively important non-cellulosic polysaccharides in the primary cell wall of dicotyledonous plants and those monocotyledonous plants that are not grasses (Type I primary cell walls, Carpita and Gibaut 1993). In the cell wall they are noncovalently bonded with cellulose (Bauer *et al* 1973). This network and an independent and coextensive network of pectin together, are believed to influence the mechanical characteristics of the cell wall. Xyloglucans are intimately associated with cellulose microfibrils (concentrated alkali which causes microfibrillar swelling is required to extract xyloglucan from the wall, Chambat *et al* 1984, Edelmann and Fry 1992, Hayashi and MacLachlan 1984) and together (ie XG and cellulose) can comprise up to 50% of dry weight of actively growing dicotyledonous plant cells (Kolpak *et al* 1976, Darvill *et al* 1980). This value is considerably less in grasses (22-25%; Darvill *et al* 1980) where Type II cell walls occur (Carpita and Gibaut 1993).

1.2.1 Cell Walls - A Model Approach

The primary cell wall must facilitate the expansion of the cell, often 10 fold but, on occasion, up to 100 times its original volume (Roelofsen 1959, Rogers and Perkins 1968, Goodwin and Mercer 1974, Preston 1974, Albersheim 1975) while maintaining wall thickness and intermicrofibril distance. New materials must be synthesised to accommodate this expansion and directed to the cell walls. These required conditions would therefore have to be considered for the construction of any cell wall model.

The evolution of models for primary cell walls has progressed concomitantly with technological advances. For example, the use of chemical and enzymatic techniques (York *et al*, 1985) to provide information on the primary structure, has been complimented by high resolution ^{13}C-NMR spectroscopy and Fourier Transform Infra-Red microspectroscopy (McCann *et al*,

1992) which have allowed investigation of the cell wall components within the extracellular matrix.

An early model proposed by Keegstra *et al* (1973) for the primary cell wall incorporated a framework of cellulose microfibrils linked to a matrix by the cellulose-xyloglucan hydrogen bonding. The xyloglucan/pectin/protein matrix was believed to be linked together by covalent linkages. Growth of the cell wall would be facilitated by the movement of xyloglucan chains and cellulose fibres relative to one another, possibly mediated by high hydrogen ion concentration and temperature.

Radical revision of this model by various workers in the intervening years resulted in a more recent model proposed by McCann *et al* (1990) in which the xyloglucan/cellulose network was independent but still interactive with that of the pectin. This model was further extended to include proteins by Carpita and Gibeaut (1993). The xyloglucan/cellulose interaction will be further discussed later in this chapter (section 1.7).

1.3 Structure and Composition of Xyloglucans

Xyloglucans are structurally related to cellulose. They consist of a backbone of β -(1→4)-linked glucose residues as does cellulose but differ in that ~ 80% (Gidley *et al* 1991) of the β -glucopyranosyl residues are substituted at C-6. In endosperms, it is generally substitution with an α -D-xylopyranosyl residue, or a β -D-galactopyranosyl-(1→2)-α -D-xylopyranosyl side chain (Bauer *et al* 1973, McNeill *et al* 1979, Darvill *et al* 1980, figure 1.1). However, reports from Neimann and co-workers (1997) suggest a small amount of oligosaccharides of DP 12 to 18 resulting from the digestion of tamarind seed xyloglucan with fungal *endo*-(1→4)-β-glucanase or 'cellulase' (origin: *Aspergillus niger*). Once purified and characterised some of these oligomers contained β -D-galactosyl-(1→ 5)-α-L-arabinosyl units linked to O-6 of the glucosyl units of the backbone while other L-arabinosyl residues were

located in terminal positions of the xylosyl side chains. The presence of a small amount of arabinose was also observed in the tamarind seed preparation by Gidley *et al* 1991 which, alternatively, was attributed to 1,5-linked arabinan

In the primary cell wall, substitution is with α-D-xylopyranosyl, β-D-galacto-pyranosyl-(1→2)-α-D-xylopyranosyl and α-L-fucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→2) -α-D-xylopyranosyl (Bauer *et al* 1973, McNeill *et al* 1979, Darvill *et al* 1980, figure 1.1) and L-arabinosyl-(1→2)—α-D-xylopyranosyl (in the case of arabinoxylan, Mori *et al* 1980) although the type and degree of substitution of the α-D-xylopyranosyl residue is dependent both on plant species and origin of tissue. As with endosperm xyloglucan, the precise arrangement of substituents along the backbone of the molecule indicates a high degree of metabolic control over the biosynthetic process.

In many cases there is a 'grey area' in defining structural and reserve polysaccharides. Many polymers which are considered to be reserve material, for example in seeds, appear to be very similar to those considered to be primary cell wall polymers. Depolymerisation and hydrolysis of most cell wall polysaccharides occur, to a certain extent, synchronously with tissue growth and the monosaccharides produced by this salvage process re-enter the general 'reserve' of cellular metabolites.

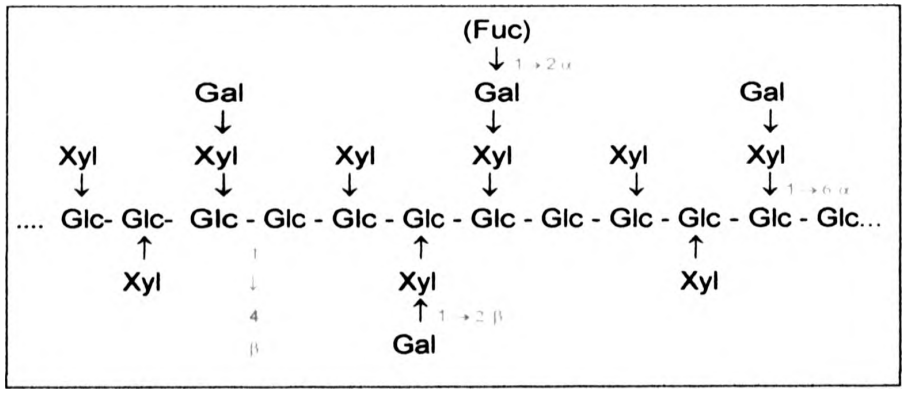


Figure 1.1 Structural features of seed and primary cell wall xyloglucan. Only primary cell wall xyloglucans have fucosyl (Fuc) residues.

Early studies of the chemical structure of seed xyloglucan reported various sugar ratios, and chemical structures differing from that in Figure 1.1 were proposed. It was, however, demonstrated that four oligosaccharides (all heptasaccharides with varying amounts of galactose added) are produced following digestion of seed xyloglucan with *endo*-(1→4)-β -glucanase or 'cellulase' (York *et al,* 1990). This enzyme will cleave the xyloglucan backbone by hydrolysing the glucosyl link formed by an unsubstituted glucosyl residue.

The complete structures (figure 1.2) and relative proportions of the oligosaccharides were determined using 2D ^{1}H-NMR and FAB-MS (York *et al,* 1990).

1.4 Mobilisation of Xyloglucan

Although detailed investigations with seed xyloglucan have been, in the main, directed at defining the structural association between xyloglucan and cellulose microfibrils (Carpita and Gibaut 1993, Fry 1995), the mobilisation of reserve xyloglucan from *Tropaeolum* dicotyledons has been studied extensively by Edwards *et al* (1985, 1986 and 1988). They reported (1985) that reserves were rapidly mobilised between days nine and thirteen after germination. Complete breakdown of the polysaccharide was concluded by day nineteen.

The mobilisation of seed xyloglucan is brought about by the action of at least four enzyme activities acting in synergy. These are,

* *endo*- β- glucanase/xyloglucan *endo*-transglycosylase (NXET) (Edwards *et al* 1986,1988)
* β -D-galactosidase (Edwards *et al* 1985)
* α -D-xylosidase (Fanutti *et al* 1993)
* β -D-glucosidase (Crombie *et al* 1998)

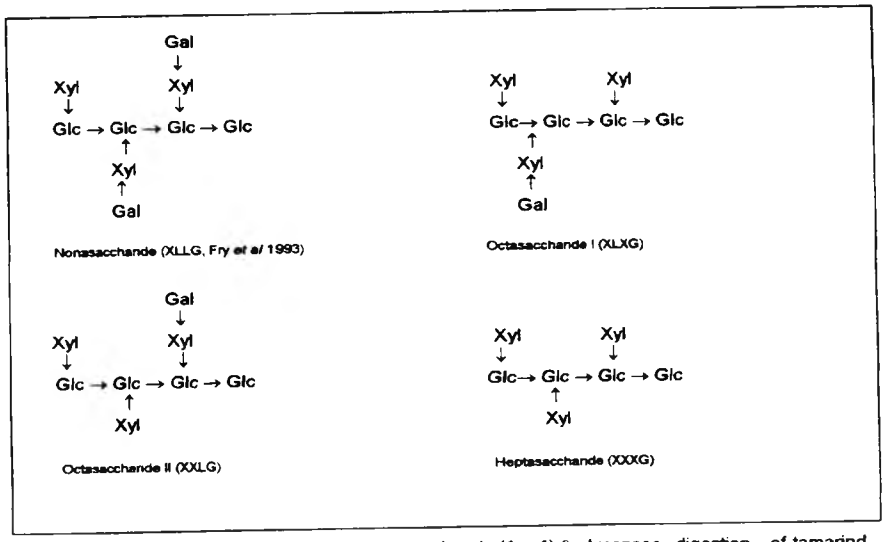


Figure 1.2 Oligosaccharides resulting from fungal *endo*-(1→4)-β-glucanase digestion of tamarind seed xyloglucan

1.4.1 *endo*-β-D-glucanase / xyloglucan *endo*-β -D-transglycosylase (NXET, 31 kDa)

NXET was purified from nasturtium *(Tropaeolum majus)* seed cotyledons by Edwards *et al* (1986). Studies at this time demonstrated that this enzyme was a xyloglucan specific *endo*-(1→4)-β-D-glucanase and did not hydrolyse cellulose, carboxymethylcellulose, or other glucans.

Fanutti *et al* (1991 and 1993) reported that the glucanase enzyme had xyloglucan *endo*-β -D-transglycosylase activity, and that it can only cleave the high molecular weight xyloglucan at the reducing side of glucose residues that are not substituted with either the xylosyl or galactoxylosyl side chains. More recently, a substrate recognition subsite has been identified (Fanutti *et al*, 1996) which as in the xyloglucan sequence shown in figure 1.3, the xylose residues (Xyl) on the backbone glucose (Glc) residues -3, -2,+2 and +3 may be substituted by galactose and the NXET splits the backbone between the unsubstituted glucose at -1 and the xylose-substituted glucose at +1, which is never substituted with a galactose residue. It was

also suggested at this time that NXET action requires a xylose substitution only at glucose residue -3 and does not require that the glucose residues at -4, -2, +1, and +3 are substituted with xylose

Fanutti *et al* (1993) showed that the transglycosylation activity of the enzyme from nasturtium is inhibited by low substrate concentrations when the hydrolytic activity takes over which suggests that this enzyme has an important regulatory role in determining the rate of breakdown of xyloglucan. This is discussed in greater detail in chapter 4.

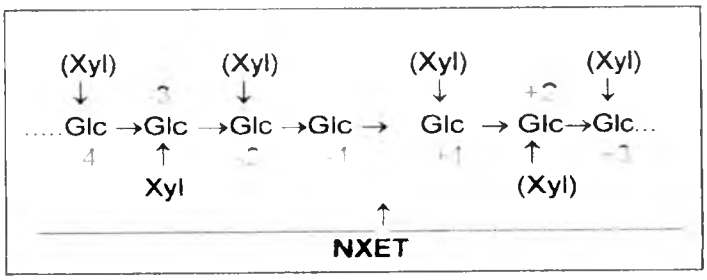


Figure 1.3 Substrate subsite recognition of NXET (Brackets indicating sites of nonessential xylose substitution)

de Silva *et al* (1993) reported the cloning and sequencing of a cDNA encoding the nasturtium enzyme, and demonstrated by means of Northern hybridisation and Western blotting techniques, that the levels of the enzyme and its mRNA correlate with rises in enzyme activity reported by Edwards *et al* (1985).

Recently the 1.5 kb gene for nasturtium XET has been inserted into the methylotropic yeast *Pichia pastoris* (Jennifer Milne, Honours thesis, 1997), and subsequent overexpression in this system has produced an enzyme with a molecular weight of 30 kDa (by SDS PAGE) capable of hydrolysing xyloglucan.

1.4.2 β -D-galactosidase

Purified from germinating nasturtium cotyledons, β-D-galactosidase was found to have a molecular weight of 97 kDa (Edwards *et al* 1988). The enzyme is involved in the hydrolysis of xyloglucan side-chains from which it removes terminal (1 → 2)-β -D-galactopyranosyl residues and is able to attack the native high molecular weight xyloglucan as well as the oligosaccharides generated by hydrolysis of the polysaccharide with cellulase or NXET.

Analysis of the galactose depleted oligosaccharides has shown that the nasturtium β-galactosidase will catalyse the conversion of the nonasaccharide to the octasaccharides (I and II) and the octasaccharides to the heptasaccharide (Fanutti *et al* unpub). It was also demonstrated at this time that the enzyme hydrolyses the two octasaccharide substrates at different rates. This led to the observation that the rate of galactose removal from the xyloglucan side-chain is dependent on the position of the galactose residue, the enzyme preferentially catalysing the hydrolysis of the galactosyl residue located on the less xylosyl-substituted side of the oligosaccharide glucan chain (see Figure 1.1).

1.4.3 α -D-xylosidase

α -D-xylosidase was purified by Fanutti *et al* (1991) from nasturtium and has a molecular weight of 85 kDa. ^{1}H.n.m.r. analysis of α -D-xylosidase action on xyloglucan oligosaccharides showed that the enzyme catalysed the cleavage and release of a single terminal xylose residue from the glucopyranosyl group at the non-reducing end of the oligosaccharide 'backbone'. The α -D-xylosidase will release xylose from the xyloglucan oligosaccharides (figure 1.1) but not from high molecular weight tamarind xyloglucan. This is not surprising given the low concentration of backbone chain-ends in the polymer.

1.4.4 β-D-glucosidase

This enzyme has been purified from nasturtium seed cotyledons and has an apparent molecular weight, by SDS-PAGE analysis (Crombie *et al* 1998) of 80 kDa. As in the case of α-D-xylosidase, this enzyme is not active against high molecular weight xyloglucan but releases a single glucose residue from the non-reducing end of certain xyloglucan oligosaccharides (XXXG and XXLG, figure 1.2). This reaction would only occur, however, after the removal of the terminal xylose residue from the non-reducing end of the oligosaccharide. (Crombie *et al* 1998). Crombie *et al* further reported that β-D-glucosidase exhibited a transglycosylase activity on cello-oligosaccharides producing transiently higher molecular weight products, some with (1→6)-β-linkages.

The mobilisation of seed xyloglucan, therefore, requires the presence and activities of at least these four enzymes acting in a programmed, stepwise manner to produce the monosaccharides required by the young seedling. The sequential reactions required by the four enzymes are summarised below (figure 1.4).

Although these investigations were carried out on storage xyloglucan, the similarity of chemical structure of both amyloid and cell wall polymers could lead to the expectation that almost identical biochemical processes are responsible for the depolymerisation of both.

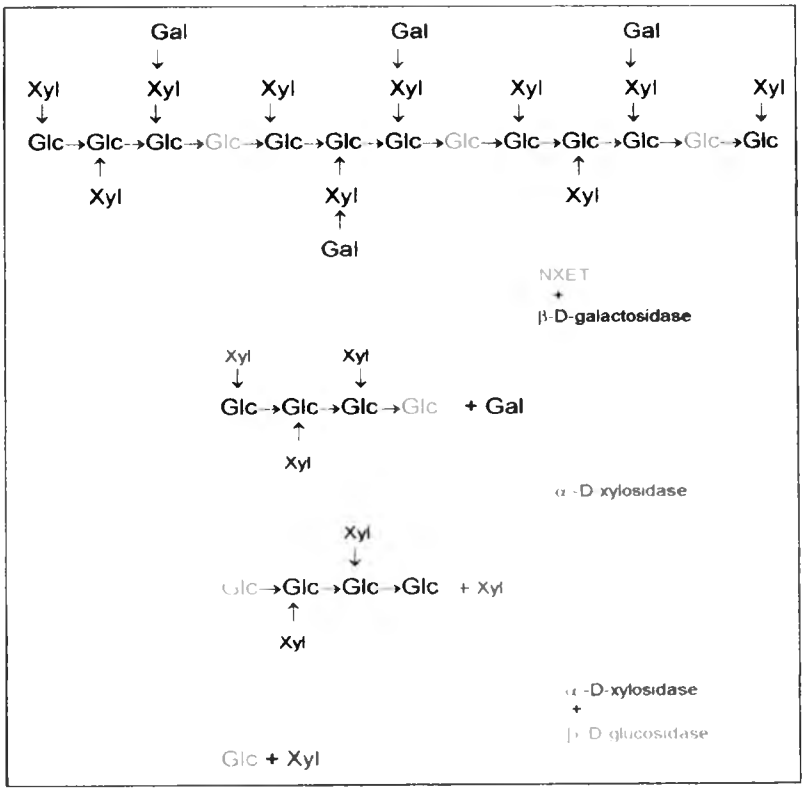


Figure 1.4. The sequential degradation of tamarind seed xyloglucan involves the concerted action of the four hydrolases.

1.5 Conformation of Xyloglucan

Xyloglucan molecules are first assembled in the cisternae of the Golgi apparatus of the plant cell (Camirand, 1986, and 1987, Moore 1991, Driouch 1993) where the glucosyl- and xylosyl-transferases appear tightly coupled (Gordon and Maclachlan, 1989). After attachment of additional sugars in the trans cisternae and secretory vesicles (Gibaut and Carpita 1994) the xyloglucans are then targeted to the plasma membrane in vesicles. These are believed to associate with cellulose fibrils which are synthesised and assembled at the cell surface

(Giddings, 1980, Mueller, 1980, Delmer, 1987, Hotchkiss, 1989). Conformational changes occur when the xyloglucan undergoes a transition from free solution to the bound state and this will be further discussed in section 1.7.

Static light-scattering studies on solubilised xyloglucan indicate a 'stiff' chain with characteristic ratios (C_∞) ~10 times greater than that found for similar polymers, i.e. Carboxymethyl cellulose (CMC, Gidley *et al* 1991). This is ascribed partially to the steric restriction of the motion of the (1 → 4)-β -D-glucan backbone by the extensive substitution which is about 80%.

Interestingly, calculation of the interactions in the backbone glucan indicated that a hydrogen bond was present between O-5 of one and O-3 of the adjoining glucopyranosyl unit (distance = 0.260nm, Tvaroska *et al,* 1978) although no evidence of this was reported by Gidley and co-workers (1991). In addition interactions between the glucan chain and the xylopyranosyl residues are varied by three rotatable bonds between the rigid glucopyranosyl residues, which have torsional potential for rotation around C-5 - C-6, C-6 - O-1, and O-1 - C-1 bonds (Tvaroska *et al* 1978). These results indicate that, in solution, although the conformation of backbone glucan is firmly fixed within the two fold helix, the xylosyl residues have a very flexible structure.

1.6 Cellulose

Cellulose is the most abundant macromolecule on earth and is synthesised by plants, fungi, algae, bacteria and several animals (Hall *et al* 1960, Bold *et al* 1987, Kinimura *et al* 1995, Brown 1996). Cellulose is a linear polymer of D-glucose linked (1→ 4)-β- with a molecular weight of at least 30 - 40 kDa (as defined by Brown 1996), but is more likely to be in the region of 1 x 10^6 Da.

In the intact cell wall cellulose occurs in the form of microfibrils (parallel cellulose macromolecules held together by hydrogen bonds) embedded, as reinforcing rods, in the hemicellulosic and pectic polymers (the 'concrete'). They are synthesised at the cell surface by

cellulose synthase complexes situated in the plasma membrane of the cell (Giddings *et al,*1980, and Mueller *et al,* 1980, Emons, 1991). The microfibrils are not normally arranged at random implying a high degree of control over their biosynthesis and deposition and cellulose synthase complexes from eukaryotes can move in the plane of the plasma membrane under the 'control' of microtubules which confine their movement to channels of specific direction (Robinson *et al,* 1981, Gunning *et al,* 1982, Sugiyama *et al* 1985, Kuga *et al* 1989, Mizuta *et al* 1989 and 1992, Okuda *et al* 1994). There is still debate, however, whether it is the orientation of the microtubular system that predetermines the cellulose deposition patterns or the interaction of the nascent cellulose with other polymers in the cell wall (Jarvis 1992).

Cellulose synthase complexes have been isolated from higher plants (Emons, 1985, Mueller *et al,*1980) however, although cellulose biosynthesis is a major metabolic process in almost all plant cells and given its relatively simple molecular structure, it is all the more surprising that attempts to demonstrate cellulose biosynthesis *in vitro* (Delmer 1987) using enzymes prepared from higher plants had met with limited success. Recent work by Delmer (1999) has, however, led to the discovery that a family of genes existing in higher plants show some similarities to the gene in *Acetobacter xylinus* that encodes the catalytic subunit of cellulose synthase.

In contrast, several types of cellulose synthase complexes or 'rosettes' have been identified from bacteria (Brown *et al,* 1976, Zaar, 1979), and algae (Giddings *et al,* 1980) and it is thought that the nature of these determines the ratio of the two crystalline suballomorphs of cellulose I, the 'native' cellulose synthesised by over 99% of all living organisms (Rånby 1952). These suballomorphs are designated Iα and Iβ (Rånby 1952) and differ in crystallite size and degree of crystallinity. The Iα form can be annealed into the I β form which suggests that the latter is more stable (Debzi *et al* 1991).

Bacterial systems have been largely utilised in the investigation of cellulose biosynthesis and most extensively investigated has been the mechanism of cellulose biosynthesis in the bacterium *Acetobacter xylinus,* ATCC 53524 (formerly *Acetobacter aceti* ssp. *xylinum.*) *A. xylinus* can utilise a variety of substrates for the synthesis of cellulose and can convert as much as 50% of supplied carbon to cellulose. Chemically, cellulose obtained from *A xylinus* is no different than that extracted from plants (White and Brown 1989) but has an altered

crystalline structure. Cellulose isolated from *A xylinus* comprises ca. 60% I α phase (Debzi *et al* 1991). *A xylinus* will be discussed in more detail in section 1.8.

It is now widely believed that the plasma membrane is the site of the interaction between xyloglucan and cellulose (Hayashi 1989), the xyloglucan forming a monolayered coat around the cellulose (Valent and Albersheim 1974, Hayashi *et al* 1987, Vian *et al* 1992, Pauly *et al* 1999), and crosslinking the microfibrils (Fry 1989, Hayashi 1989). Growing plants expand mainly in the plane perpendicular to the orientation of the cellulose microfibrils in the wall and weakening of these crosslinkages would presumably facilitate cell elongation. However little is known about the molecular organisation of xyloglucan in the cell wall.

1.7 The Xyloglucan - Cellulose Interaction

Xyloglucan binds tightly to cellulose crosslinking the microfibrils to form an interwoven xyloglucan - cellulose network (McCann *et al* 1990). Therefore in order to rearrange the cell wall architecture these crosslinks must be cleaved and reconnected. Xyloglucans bind to cellulose through hydrogen bonds (Joseleau *et al* 1981, Hayashi and Maclachlan, 1984); and appear to coat and crosslink the cellulose microfibrils in the walls of growing plants, (Hayashi and Maclachlan 1984).

In vitro reconstitution studies using pea xyloglucan and cellulose from different sources demonstrated that the amount of bound xyloglucan is directly proportional to the cellulose microfibrillar surface area, (Hayashi *et al*, 1987). Evidence also suggested that the binding of xyloglucan to the cell wall and to isolated cellulose was reversible as would be expected for non-covalent hydrogen bonds (Aspinall *et al* 1969, Bauer *et al* 1973).

The xyloglucan-cellulose bond is presumed to be one of the major interconnections of the dicotyledonous primary cell wall and may function to prevent the cellulose microfibrils from forming large aggregates that are characteristic of secondary cell walls (Hayashi *et al* 1987). It has also been suggested that since other glucans do not inhibit the binding of xyloglucan to cellulose the association between the two occurs specifically (Hayashi *et al*, 1986).

Levy *et al* (1991) postulated, by molecular modelling techniques, that the initial steps of the binding of xyloglucan to cellulose were most likely to occur in the vicinity of a fucosylated subunit rather than a non-fucosylated subunit. It was suggested that the presence of a fucose-containing side chain induces a tendency in an adjacent region of the backbone to flatten out, leading to a twisted - flat conformational change in the xyloglucan chain. This flattened region would then bind to cellulose as well as cause propagation of reorientation of other fucosylated side chains and therefore backbone flattening. This model has been recently supported by evidence that fucosylated pea xyloglucan bound to cellulose at a higher rate than nasturtium (non-fucosylated) xyloglucan (Levy *et al*, 1997). Self-association of the xyloglucan macromolecules in solution is thought to be prevented by the low frequency of the formation of these 'nucleation' sites and is maintained in the twisted conformation although in earlier investigations by Reid *et al* 1988, aggregation of tamarind seed xyloglucan was observed after addition of high salt concentration (1M Na_2SO_4). Subsequent studies using mobility-resolved NMR have revealed no change in molecular conformation of xyloglucan to that found in solution (Brooks *et al* unpub).

In contrast to the work by Levy and coworkers (1991& 1997), Finkenstadt *et al* (1995) suggested that binding involving the xyloglucan backbone was sterically restricted. A possible model for binding involved hydrogen bonding between the xylopyranosyl residues and the cellulose surface. It is proposed that xylose residues occupy the lattice spaces on the crystalline surface of the cellulose molecule but that this binding is controlled by the presence of galactose or fucose residues. The ability of a segment of a xyloglucan molecule to bind is suggested to be dependent on the conformation of the (1→6) - linkage so that the presence of galactose or fucose residues facilitate the formation of cross-linkages.

In addition to the suggestion that xyloglucan forms a monolayer coating the surface of the microfibrils it is also proposed to form crosslinkages between them (Fry 1989; Hayashi 1989). Isolated xyloglucan molecules have been measured up to 700 nm in length, long enough to span several microfibrils which have been reported to be 20 - 40 nm apart (McCann and Roberts 1991; McCann *et al* 1990).

Enzyme-catalysed modification of the xyloglucan component of the network is believed to be essential for wall expansion (Talbott and Ray 1992). Pauly *et al* (1999) have recently presented a model which supports the hypothesis that this modification involves the xyloglucan which forms the cross-linkages between microfibrils. Three xyloglucan domains have been identified in the cell walls of higher plants which can be differentially extracted from cell walls. These correspond to that which is removed by the action of a xyloglucan-specific *endo*glucanase, material extracted by the action of 24% KOH after NXET treatment and xyloglucan removed by the action of commercial 'cellulase' on the pre-treated cell walls. Analysis of these three fractions by the authors (Pauly *et al,* 1999) suggests that metabolic activity is confined to the enzyme-susceptible domain.

1.8 Investigating the Xyloglucan/Cellulose Interaction

The nature of the xyloglucan/cellulose interaction and the relevant side-chains involved in binding (if any) are as yet not well understood but one approach to investigating cell wall properties in this context is to use the products of chemical extraction processes. However, although these materials provide information on a molecular (Redgwell *et al,* 1986) and microstructural (McCann *et al,* 1990) level, mechanical, rheological and ultrastructural investigations cannot be carried out due to a lack of continuous materials. Homogenisation of cell walls produce submillimeter-sized pieces which cannot be used in mechanical testing.

A model system developed by Whitney *et al* (1995) to examine the assembly *in vitro* of xyloglucan/cellulose networks can produce centimetre-sized pieces of the composites which mimic cell walls and can be used to investigate (mechanically etc) this interaction further. As previously mentioned this alternative approach utilises the Gram negative bacterium *Acetobacter xylinus*.

1.8.1 *Acetobacter xylinus*

A. xylinus synthesises a twisted ribbon of highly crystalline cellulose I. These ribbons are composed of bundles of microfibrils, smaller crystalline structures which are analogous to the microfibrils of cellulose in plant and some algal cell walls (Brown *et al* 1977) and are extruded parallel to the bacterial cell wall from a single row of discrete cellulose synthase complexes along one of the long axes of the cell (Brown *et al,* 1976, Zaar, 1979). As many as 200,000 glucose molecules per second can be polymerised into the glucan chain by these complexes (Hestrin and Schramm, 1954).

Investigation of the unique cell structure of Gram-negative bacteria and cellulose biogenesis in *A. xylinus* has led to the suggestion that glucan chains are extruded through 'adhesion sites' - sites in the cell envelope where inner (cytoplasmic membrane CM) and outer membranes (OM) adhere (figure 1.5) and through which the transfer of long-chain polymers occurs in other Gram -negative bacteria (Bayer 1979). This model proposes that individual glucan chain synthesis occurs collectively via a cluster of cellulose synthase complexes situated in the adhesion site (Brown *et al* 1983) and that the initial stages of cellulose I crystallisation occurs through van der Waals forces (Cousins and Brown 1995). Hydrogen bonding ensures that, once outside the cell, crystallisation of the cellulose into microfibrils and ribbons takes place. The cellulose ribbons produced intertwine to form a pellicle that floats at the air-liquid interface of static cultures (White and Brown 1989).

The synthesis of cellulose by *A. xylinus* is Mg^{2+}- dependent and UDP-glucose is the substrate (Aloni *et al*, 1983). Biosynthesis and assembly can be disrupted by carboxymethylcellulose (CMC, Haigler *et al* 1980, Haigler and Brown, 1981) and Calcofluor, a fluorescent brightener, (Benziman *et al* 1980. Haigler *et al* 1980), by increasing the rate of polymerisation and altering the ribbon assembly.

First proposed in 1982 by Haigler *et al,* the suggestion of using *A. xylinus* as a model for studying the cellulose/xyloglucan network attracted the attention and further work of Atalla *et al,* (1993), Hackney *et al,* (1994), Yamamoto *et al* (1994) and Uhlin *et al,* (1995).

Figure 1.5 Schematic diagram of the biogenesis of a cellulose microfibril at an 'adhesion site' in the *Acetobacter xylinus* cell envelope (Brown *et al* 1983)

This work was expanded by Whitney *et al* (1995 and PhD Thesis 1996) to encompass the mechanical and ultrastructural aspects of the system thus validating its use as a model for the study of primary cell walls.

Observations using Deep-Etch Freeze-Fracture Transmission Electron Microscopy provides ultrastructural images which are believed to closely resemble the native structures. There is no prerequisite for chemical fixatives or dehydrating agents. This technique was used to visualise that the association of xyloglucan with cellulose at the point of synthesis confers a level of lateral order to the cellulose (Whitney *et al,* 1995 Figure 6.3 a and 6.3 b) and strands of what is thought to be xyloglucan are discerned crosslinking the fibres with cross-bridge lengths similar to those observed for de-pectinated onion cell walls (McCann *et al* 1990).

^{13}C-NMR spectroscopy was used to investigate features of the cellulose/xyloglucan molecular structure resulting from the *A xylinus* system, that could be differentiated on the basis of molecular mobility (Gidley *et al* 1992). Cross-polarisation Magic Angle Spinning (CP-MAS) was found to be most effective in detecting relatively immobile segments of the system in question whereas Single Pulse Magic Angle Spinning (SP-MAS) would detect relatively mobile segments.

Use of these techniques suggested that ca. 80% of associated xyloglucan adopts a 'cellulosic' conformation and is aligned with the cellulose microfibrils whereas that remaining is assigned to the cross-linkages and stays in the twisted conformation found for xyloglucan in solution. The $I\alpha / I\beta$ crystallite ratio for cellulose was also found to have decreased on association with xyloglucan with the non-crystalline content increasing from 18% (without XG) to 47% (with XG, Whitney *et al* 1995). Atalla *et al* (1993) also reported X-ray diffraction data for the disruption of crystallinity within the cellulose component of a cellulose/xyloglucan composite which was similar to data observed for cellulose isolated from higher plants.

Incorporation of the xyloglucan is high with monosaccharide analysis giving a ratio of 1:0.38 (cellulose:xyloglucan) which is similar to the range of ratios found in plant cell walls (Hayashi *et al,* 1984, Redgwell *et al,* 1986). Large deformation uniaxial tensile testing was used to examine the behaviour of the composite under stress as would happen in the cell wall during *in vivo* elongation processes. This regime requires centimeter-sized pieces of material which are produced by the *A. xylinus* system.

The premise that i. the degree of xyloglucan incorporation, ii. the presence and lengths of cross-bridges, and iii. 'ordering' of cellulosic molecular organisation when synthesised in media containing xyloglucan, are similar to those found in plant cell walls suggests a useful model for their investigation. Yet with all *in vitro* experiments, caution has to be exercised when extrapolating results to encompass actual events occurring in the extracellular matrix.

The majority of these experiments use seed xyloglucan, which does not contain fucose, as a model for cell wall xyloglucan, which does. The molecular weight of seed xyloglucan is also much higher than that found in the primary cell wall. The *A xylinus* fermentation system is, furthermore, very dilute compared to that found at the cell walls in plants.

1.9 Aim of this work

The structural rigidity and strength of the plant primary cell wall is thought to depend on the integrity of the cellulose/hemicellulose network yet, although the structural components of this

network are well known, relatively little is known about the method of association of xyloglucan with cellulose from a molecular viewpoint. Various models predict side chain involvement or localised straightening of the glucan backbone in the binding.

Investigations until recently have relied on homogenised extracts from plants which result in very small pieces of material. While useful on a biochemical and microstructural level this material could not be used to examine the network ultrastructurally or mechanically. The development of the *A xylinus* system has enabled the investigator to, now, produce centimeter-sized networks, which have been shown to mimic cell walls, upon which these tests can be carried out.

The aim of this work was to investigate whether the *A xylinus* fermentation system would prove useful in the study of interactions between cellulose and modified xyloglucan thus shedding light on the molecular level of cellulose/xyloglucan interaction. Initially this involved the use of the purified nasturtium enzymes to achieve structural tailoring of xyloglucan. Once isolated, these modified xyloglucans were introduced into the bacterial system and cellulose/xyloglucan composites were formed. The composites were then examined and characterised using various biochemical techniques, ^{13}C-NMR, Large Deformation Uniaxial Tensile Testing and Deep-Etch Freeze-Fracture Electron Microscopy.

Chapter Two

2. Materials and Methods

2.1 Materials

Nasturtium (*Tropaeolum majus* L) seeds were purchased from Royal Sluis, Leyland, Preston, UK (variety: Gleam Hybrids Ref No 04554). Soluble tamarind (*Tamarindus indica*) xyloglucan, Glyloid 3s was obtained from Dianippon Pharmaceutical Corporation, Osaka, Japan.

All preparative and analytical reagents were purchased from Fischer Scientific and Sigma unless otherwise stated in the text.

TXET was kindly supplied by Unilever Research Laboratories.

2.2 Methods

2.2.1 Purification of Enzymes from Nasturtium Seedlings.

The four enzymes used in the modification of tamarind xyloglucan were purified from germinating nasturtium seedlings. The seeds were stored in dry dark conditions. The main proportion of the seeds were coated with an outer covering which softened upon soaking.

2.2.1.1 *Seed Germination and Growth Conditions.*

Dry nasturtium seeds (approximately 200 per tray) were placed in trays (21 x 70 cm) containing 4 cm of damp 'Vermiculite' ('Micafil' fibre-free insulation; Harcros Ltd, Stirling). A thin layer of vermiculite was added to cover the seeds and the trays watered thoroughly with tap water. The trays were placed in a plant growth room with a 12-hr day/12-hr night regime

with light levels of around 200 microeinsteins m^{-2} s^{-1} at tray level and the temperature regulated to 15-20°C. Trays were covered with clear polythene sheeting until shoots appeared (day 5) and were watered at two day intervals. Any seeds which displayed fungal contamination were discarded.

2.2.1.2 *Enzyme Extraction*

All operations were carried out at 4° C unless otherwise stated. Cotyledons were manually removed from seedlings 13 days after sowing (on average around 500g fresh weight with seed coats intact) and washed 2-3 times with distilled water. The seeds were homogenised in a blender (M.S.E. Ltd 'Atomix': 3 x 15 seconds with 15 seconds between each burst) with 600 ml of 0.2 M KH_2PO_4 - K_2HPO_4 buffer pH 7.2 and 6 g insoluble polyvinylpyrrolidone. The suspension was stirred for one hour to allow diffusion of enzyme from the cell walls whereupon the slurry was passed through a double layer of muslin to remove the seed debris. The filtrate was centrifuged at 19000g for 30 minutes. After filtering through glass wool the supernatant was brought to 90% saturation with ammonium sulphate by addition of the crystalline salt and the extract left to stir for one hour. After a further centrifugation (19000g for 30 minutes) the pellets were resuspended in a minimum volume of the appropriate buffer for the subsequent purification of each enzyme.

The procedures for the isolation of each of the enzymes differ in fine detail. These are outlined below.

2.2.1.2.1 *Xyloglucan Endo-β-D-transglycosylase* (NXET)

The pellets from the second centrifugation (as described above) were resuspended in Tris-HCl buffer (20mM) pH 7.8 and dialysed against the same buffer overnight after which the crude enzyme extract was centrifuged for 20 minutes at 26000g to remove any solid material. The supernatant was applied to an anion-exchange chromatography column (DEAE cellulose,

Whatman, column dimensions: 2.2 x 20 cms) using the same Tris-HCl buffer as eluant and fractions of 10 ml collected, under gravity, until the absorbance of the eluate at 280nm had fallen to basal levels. Note that this chromatography step could also be used to collect β-D-galactosidase ie these wash fractions contained the β-D-galactosidase activity.

A sodium chloride gradient (0 - 0.5 M in the same buffer) was applied to the column and again 10 ml fractions were collected, under gravity, until the absorbance of the eluate at 280 nm had fallen to basal levels. The fractions which showed the highest activity (as assayed by viscometric method, section 2.2.2.2.3) were pooled and brought to 90% saturation with ammonium sulphate to precipitate the protein. This was left for 1 hour and then centrifuged at 26000g for 30 minutes. The precipitated enzyme pellet was resuspended in sodium acetate buffer (50 mM, pH 5.0) and dialysed overnight against same. The extract was applied to a cation-exchange column (CM Cellulose, Whatman, column dimensions: 2.2 x 20 cms) where 10 ml fractions were collected, under gravity. Those containing high activity were pooled and the protein precipitated by addition of ammonium sulphate salt (90%), centrifuged and the pellets resuspended in 50mM ammonium acetate buffer pH 5.0 as previously described.

Overnight dialysis against the same buffer was followed by the application of the extract to a gel filtration column (Bio-gel p60, Biorad, column dimensions: 3.0 x 73 cms). Fractions of 4 ml were collected with flow rate controlled to 2 ml/hr and those containing high enzyme activity were pooled and frozen at -20° C.

2.2.1.2.2 *β-D-Galactosidase*

The pellets from the second centrifugation (as described in section 2.2.1.2) were resuspended in Tris- HCl buffer (20mM) pH 7.8 and dialysed against the same buffer overnight after which the crude enzyme extract was centrifuged for 20 minutes at 26000g to remove any solid material. The supernatant was applied to an anion-exchange chromatography column (DEAE cellulose, Whatman, column dimensions: 2.2 x 20 cms) using the same Tris-HCl buffer as

eluant and fractions of 10 ml collected, under gravity, until the absorbance of the eluate at 280nm had fallen to basal levels.

Fractions showing high enzyme activity (as assayed using *p*-nitrophenylgalactopyranoside assay, section 2.2.2.2.4) were pooled and the protein precipitated with 90% ammonium sulphate. After stirring for 1 hour the extract was centrifuged for 20 minutes at 26000g and the pellets resuspended in 50 mM sodium acetate buffer pH 5.0 and dialysed against the same buffer overnight. Note that application of a 0 - 0.5 M sodium chloride gradient to this column would then elute NXET.

The dialysed extract was applied to a CM cellulose cation-exchange column (Whatman, column dimensions: 2.2 x 20 cms) and washed with the same buffer. Fractions of 10 ml were collected, under gravity and the absorbance at 280 nm measured. When the A280 readings reached basal levels, a gradient of sodium chloride (0 - 0.5 M) was applied to the column and fractions collected as before. The fractions showing high activity were pooled and brought to 90% saturation with ammonium sulphate.

After stirring and centrifuging as before the pellets were resuspended in 50 mM ammonium acetate buffer pH 5.0 and dialysed against the same buffer overnight.

The partially purified enzyme was then applied to a gel filtration column (Bio-gel p60, Biorad, column dimensions: 3.0 x 73 cms) and the flow rate controlled to ~2ml per hour. Fractions of 4 ml were collected and these were stored at - 20° C until required.

2.2.1.2.3 *β-D-Glucosidase*

From the initial purification in section 2.2.1.2 the pellets from the centrifugation were resuspended in 20 mM Tris-HCl buffer pH 8.5. The extract was dialysed against the same buffer overnight then applied to the anion-exchange column (DEAE Cellulose, Whatman, column dimensions: 2.2 x 20 cms). Protein was eluted with 20 mM Tris-HCl buffer pH 8.5. Fractions of 10 ml were collected, under gravity and those with high glucosidase activity (as assayed by *p*-nitrophenylglucopyranoside assay, section 2.2.2.2.4) were pooled and the

protein precipitated with 90 % ammonium sulphate. After leaving to stir for 1 hour the crude extract was centrifuged at 26000g as before and the pellets resuspended in 50 mM sodium acetate buffer pH 6.0 + 100 mM sodium chloride. Dialysis was carried out against the same buffer overnight.

After application to a cation-exchange column (S-Sepharose, column dimensions 1 x 30 cms), fractions of 4 ml were collected, under gravity, with 50 mM sodium acetate pH 6.0 + 100 mM sodium chloride as eluant and the A280 measured until basal levels were reached. Application of a sodium chloride gradient (0 - 0.9 M) in the same buffer was carried out and 4 ml fractions again collected as before. Those showing high enzyme activity were pooled and brought to 90% precipitation with ammonium sulphate, left to stir for 1 hour and centrifuged as before. The pellets were resuspended in 50 mM ammonium acetate and dialysed against the same buffer overnight. The resultant purified protein was frozen at -20° until use.

2.2.1.2.4 *α -D-Xylosidase*

The pellets from the second centrifugation (as described in section 2.2.1.2) were resuspended in Tris- HCl buffer (20mM) pH 7.8 and dialysed against the same buffer overnight after which the crude enzyme extract was centrifuged for 20 minutes at 26000g to remove any solid material. The supernatant was applied to an anion-exchange chromatography column (DEAE cellulose, Whatman, column dimensions: 2.2 x 20 cms) using the same Tris-HCl buffer as eluant and fractions of 10 ml collected, under gravity, until the absorbance of the eluate at 280nm had fallen to basal levels. Note that this chromatography step could also be used to collect β-D-galactosidase ie these wash fractions contained the β-D-galactosidase activity.

A sodium chloride gradient (0 - 0.5 M in the same buffer) was applied to the column and again 10 ml fractions were collected, under gravity, until the absorbance of the eluate at 280 nm had fallen to basal levels. The fractions which showed the highest activity (as determined by the method for free pentoses, section 2.2.2.2.2) were pooled and brought to 90% saturation with ammonium sulphate to precipitate the protein. This was left, stirring for 1 hour and then

centrifuged at 26000g for 30 minutes. The precipitated enzyme pellet was resuspended in 5mM sodium acetate buffer pH 5.0 with 1mM calcium chloride, 1mM manganese chloride and 200 mM sodium chloride added.

After overnight dialysis against the same buffer the extract was applied to an affinity column (Concanavalin A-Sepharose, column dimensions 1 x 30 cms) collecting 4 ml fractions, under gravity using the same buffer as eluant. When the values of A280 had reached basal levels, 80 ml of the above buffer plus 10 mM methyl glucopyranoside was applied and fractions collected as before. Those containing the highest activity were pooled and brought to 90% saturation with ammonium sulphate and centrifuged as described before. The pellets were resuspended in 50 mM ammonium acetate buffer pH 5.0. and dialysed overnight against the same buffer.

The extract was applied to a gel filtration column (Bio-gel P300, Biorad, 2.5 x 90 cms) and protein eluted with 50 mM ammonium acetate buffer pH 5.0. Fractions containing the highest enzyme activity were frozen at -20° C until use.

2.2.2 Enzyme Activity Assay

Tamarind xyloglucan was used as a substrate for the determination of nasturtium endo-β-D-glucanase and α -D-xylosidase activities, both in crude extracts and as purified enzymes. It was used as a substrate for the purified β-D-galactosidase only. The activities in β-D-galactosidase and β-D-glucosidase crude extracts were determined using the appropriate *p*-nitrophenylglycoside (Section 2.2.2.2.4).

2.2.2.1 *Preparation of Tamarind Xyloglucan*

As the tamarind powder contained some insoluble particles and approximately 5% free glucose, it was processed as follows: Soluble tamarind seed xyloglucan (0.5%) was stirred

overnight in distilled water at room temperature after which the xyloglucan was precipitated from solution with ethanol (2 volumes ethanol: 1 volume xyloglucan solution). The solution was then centrifuged for 30 minutes at 16000g and the precipitated xyloglucan resuspended in a mixture (2:1) of ethanol and water (250 ml). The solution was brought to 70° C for 20 minutes, allowed to cool and the polymer collected as before. The xyloglucan extraction and precipitation was repeated twice after which the xyloglucan was resuspended in the original volume of water and dialysed overnight against distilled water, initially changing the water every four hours. The resulting solution was frozen at -40° C and freeze dried. The white lyophilised xyloglucan was stored at room temperature.

A 12 mg/ml solution of xyloglucan was prepared by dissolving the freeze-dried material in either phosphate/citrate buffer (McIlvaine buffer, Dawson *et al.*, 1982) pH 5.0 or 50 mM ammonium acetate buffer pH 5.0 by stirring at 80° C in a covered beaker for approximately 1 hour or until most of the polysaccharide had gone into solution. After allowing to cool the solution was centrifuged at 16000g for 20 minutes to removed any undissolved material. This xyloglucan solution was then ready to be used in the enzyme assays.

2.2.2.2 *Enzyme Assays*

2.2.2.2.1 *Determination of D-Galactose by Galactose Dehydrogenase* (Kurz and Wallenfels, 1974)

Prior to use the galactose dehydrogenase enzyme suspension (D-galactose:NAD 1-oxoreductase, EC1.1.1.48. A recombinant protein, expressed in *E coli* using the *Pseudamonas fluorescens* gene, available at 25 U/ml suspended in 3.2M ammonium sulphate) was diluted to a specific activity of 2.5 U/ml with 1 M ammonium sulphate containing 20 mM EDTA. The solution was stored at 4° C.

In a spectrophotometer cuvette (1ml) 904µl of 0.1M Tris-HCl buffer pH 8.6 was mixed, by inversion, with 64 µl sample or standard (galactose, concentrations ranging from 0.1 to 1.0

mM) and 64 µl of galactose dehydrogenase. The absorbance at 340 nm was read (before NAD addition) and to the solution was added 32 µl of NAD^+ (β-nicotinamide adenine dinucleotide: 12.5 mg/ml in water). The absorbance was read again (post NAD addition) and again after 1 hour incubation at room temperature. The concentration of galactose is proportional to the formation of NADH as measured by the change in absorbance at 340 nm as represented by the equation below:

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

The sample was prepared by mixing xyloglucan solution (12mg/ml) with an aliquot of crude or pure enzyme and left for the requisite time. The enzyme activity was then determined by assaying the galactose released during the incubation.

2.2.2.2.2 *Determination of Free Pentoses* (Xylose Assay; essentially method of Roe and Rice, 1948)

This assay was used in determining the activity of the crude and purified α -D-xylosidase. The sample was taken from an overnight digestion of xyloglucan with crude or pure α -D-xylosidase (1.0 ml xyloglucan, 12 mg/ml, plus 200 µl of enzyme extract). In the latter stages of purification of the α -D-xylosidase, 20 µl of cellulase was added to the digest as high molecular weight xyloglucan is not a substrate for the enzyme (Crombie *et al* 1998).

In an microcentrifuge tube, 200 µl of sample or standard (xylose, concentration ranging from 0.1 to 1.0 mM) was mixed with 1 ml of *p*-bromoaniline reagent (prepared by dissolving 2g of *p*-bromoaniline in 100 ml acetic acid pre-saturated with thiourea, approximately 4g) and the solution incubated at 70° C for 10 minutes. After a further incubation at 30° C for 1 hour in the dark (modified from method of Roe and Rice 1948) the absorbance was measured at 520 nm.

This measurement was proportional to the amount of xylose present. At high xyloglucan levels a gel was observed after incubation which interfered with reading. This was removed by centrifugation of the assay mixture using a bench centrifuge (MSE Microcentaur). It is thought that D-galactose at the levels actually present did not interfere with the assay.

2.2.2.2.3 *Viscometric Assay for Endo-(1->4)-β-D-glucanase* (Edwards *et al*, 1985)

It is difficult to designate a specific activity for this enzyme as a transglycosylase reaction is occurring as well as a hydrolysis reaction. This assay is a measure of the hydrolysing power of the enzyme only as measured as a reduction in the viscosity of the xyloglucan solution.

During the purification of *endo*-(1->4)-β-glucanase, aliquots of the fractions (0.2 ml) from the anion and cation exchange and gel filtration columns which gave high absorbances at 280 nm were incubated at 30° C for 2 hours with tamarind seed xyloglucan (1.2% w/v; 1ml). Viscometric flow times i.e. hydrolysis activity were calculated, in arbitrary units, using a graduated pipette (volume 0.1 ml), measuring the elapsed time for 0.1 ml of xyloglucan/enzyme solution to flow through the pipette and using the calculation 100/flow time of sample - 100/flow time of control.

2.2.2.2.4 *Nitrophenylglycoside Assay*

In the initial stages of purification of β-galactosidase and β-glucosidase, this assay using *p*-nitrophenylgalactopyranoside and *p*-nitrophenylglucopyranoside respectively proved to be a quick indication of enzyme activity. The standard assay mixture contained the appropriate *p*-nitrophenylglycoside (100 µl of 50 mM solution in water) mixed with 300µl McIlvaine phosphate/citrate buffer, pH 4.5 for β-galactosidase and pH 5.0 for β-glucosidase and 100 µl

of enzyme extract (appropriately diluted in phosphate/citrate buffer). Incubation at 30° was started on addition of the *p*-nitrophenylglycoside and the reaction was allowed to proceed for 15 minutes when it was stopped by addition of 5 ml 0.1M sodium carbonate.

Absorbances were read at 400nm and activities calculated using the molar extinction coefficient of 1.84×10^4 for *p*-nitrophenol in 0.1 M sodium carbonate.

2.2.3 Protein Determination (Sedmak and Grossberg 1977)

Total protein was assayed by the dye-binding procedure of Sedmak and Grossberg. The Coomassie-Blue reagent was prepared by dissolving 0.06 g Coomassie-Blue G-250 in 3% perchloric acid and filtering through Whatman No 1 Filter paper. A standard calibration curve was constructed using concentrations of bovine serum albumin (BSA) between 0 and 100 mg/ml.

In a small test tube 0.5 ml of Coomassie reagent was mixed with 0.5 ml of protein sample or standard. The absorbance of the resultant blue solution was measured at 620 nm and 465 nm against a blank of distilled water. The absorbance ratio of 620/465 was calculated and the absorbance ratio of a blank (0.5 ml reagent + 0.5 ml distilled water) subtracted.

The value obtained was used to determine the protein concentration from the calibration curve.

2.2.4 SDS-Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) was carried out, essentially according to Laemmli (1970). The gels contained acrylamide at a concentration of 10% (w/v) and were 0.75mm in thickness.

The separating gel contained separating gel buffer (1.5 M Tris-HCl, pH 8.7 and 0.4% (w/v) SDS), distilled water, acrylamide stock solution (Biorad: 30% w/v acrylamide and 0.8% w/v

methylene-bis acrylamide), ammonium persulphate (10% w/v, made fresh) and N,N,N',N',-tetramethylethylenediamine (TEMED). The stacking gel contained stacking gel buffer (0.5 M Tris-HCl, pH 6.8 and 0.4% w/v SDS), distilled water, acrylamide stock solution (as above), ammonium sulphate (as above) and TEMED. Gels were prepared and stored wrapped in 'cling film' at 4° C until use (not more than seven days).

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

Samples were prepared by mixing 3:1 with sample buffer/dithiothreitol reagent (also in a 3:1 ratio) and the solution placed in a boiling water bath for 2-3 minutes.

A molecular weight standard solution was generally used which contained; carbonic anhydrase (29000 Da), ovalbumin (45000 Da) albumin bovine (66000 Da), phosphorylase b (97400 Da), β-galactosidase (116000 Da) and myosin (205000 Da).

Approximately 25 µl of the protein sample was loaded onto the stacking gel and electrophoresis carried out at 20 mA per gel until the marker dye was 0.5 cm from the bottom. Coomassie Blue stain (0.1% w/v R-250 in acetic acid, methanol and distilled water, 2:5:5 by vol) was used to stain the gel (30 minutes with constant gentle motion). It was then destained using a solution of 10% methanol and 7.5% acetic acid in water.

2.2.5 High Performance Anion Exchange Chromatography (HPAEC) Analysis

2.2.5.1 *Oligosaccharide analysis of xyloglucans on HPAEC*

Due to the susceptibility to destruction of xylose using sulphuric acid hydrolysis (Section 2.2.5.2), oligosaccharide composition of the modified xyloglucans, before incorporation and after removal from composites, was determined by precipitation of high molecular weight XG with 3:1 addition of ethanol (3 volumes ethanol: 1 volume sample). After centrifugation the pelleted XG was resuspended in 0.5 ml deionised water and freeze dried. The lyophilised

material was resuspended in 50 µl deionised water after which overnight digestion of the xyloglucan solution with 0.45 U (or 4.35 µ g) *endo*-glucanase (source : *Trichoderma reesei*; Megazyme - prepared by dialysis of the purchased suspension with ammonium acetate, 50 mM pH 5.0 changing tubing every hour until final volume remains constant) produced the oligosaccharides, XXXG, XLXG, XXLG and XLLG. After denaturing the enzyme by boiling, these were applied to the HPAEC (Dionex Bio LC) eluting from a CarboPac PA100 ion-exchange column plus guard column with a linear gradient of 95% 150 mM NaOH (eluant 1) and 5% 150 mM NaOH + 500mM Na CH_3COO (eluant 2) to 85% 150 mM NaOH and 15% 150 mM NaOH + 500 mM Na CH_3COO in 40 minutes. This was followed by a 5 minute wash of the column with 100% of eluant 2. The flow rate was 1ml/min and the oligosaccharides detected with a PAD2 detector. A standard oligosaccharide solution containing a mixture of XXXG, XLXG, XXLG and XLLG was run at the beginning of each day.

2.2.5.2 *Monosaccharide analysis of xyloglucan/cellulose composites on HPAEC*

a. H_2SO_4 Hydrolysis

Monosaccharide composition of xyloglucan/cellulose composites was determined by sulphuric acid hydrolysis. The hydrolysis was carried out in 1ml glass 'Reactivials' (Pierce, Rockford, Illinois, US) where 100 µl of 72% H_2SO_4 (w/w) was added to ~1mg lyophilised sample. This was incubated for 1 hour at 30° C ensuring that all sample was ground up and dissolved in the acid solution using a small glass rod. Upon dilution by 1:10 with deionised H_2O the 'reactivials' were sealed with screw caps and placed in an oven set to 105° C for 3 hours.

The solution was neutralised with 200 mM NaOH before applying to the HPAEC (Dionex Bio LC). Elution from a CarboPac PA1 ion-exchange column plus guard column was with MilliQ water and a 300 mM NaOH post column base was run. After 40 minutes the column was washed for 10 minutes with 400 mM NaOH. The flow rate was 1ml/min and the monosaccharides detected with a PAD2 detector. A standard solution containing myoinositol,

fucose, rhamnose, arabinose, galactose, glucose, xylose and mannose determined the response factors of the detector for the monosaccharides. All calculations for the cellulose : xyloglucan ratios were carried out using xylose as internal standard for xyloglucan with the exception of those where xylose was depleted (Galactose was used in these cases).

b. TFA Hydrolysis

Monosaccharide composition of xyloglucan was determined by hydrolysis with 2M trifluoroacetic acid (TFA). Lyophilised polysaccharide (1 – 2 mg) was dissolved in 200 μl of TFA and heated in the oven at 120 degrees C for 1 hour. (Fry 1988). Insoluble material was removed by centrifugation and the TFA removed by lyophilisation. Monosaccharides were analysed by HPAEC using the method above.

2.2.6 Overexpression and secretion of NXET in *Pichia pastoris*

The production of three viable *pichia*/NXET clones by J Milne (Honours project, University of Stirling, 1997) enabled the overexpression and secretion of NXET in the methylotrophic yeast system. Various attempts were made to optimise conditions for the protein expression from the yeast. All methods were variations on the basic protocol that follows and all were advised in the manual referenced.

2.2.6.1 *Basic protocol for overexpression of NXET in Pichia* (Invitrogen, 1995)

All procedures were carried out aseptically.

A single colony of viable recombinant clone was inoculated into 100 ml buffered Glycerol-complex Medium (BMGY, Figure 2.1) in a 1L baffled flask. This was incubated in an

orbital incubator set to 28° C and 260 rpm for approximately 18 hours or until the OD_{600} reached 2 - 6.

Cells were harvested by centrifugation at 2000g for 5 minutes at room temperature and expression of protein induced by decanting the supernatant and resuspending the pellet in 1/5 to 1/10 the original culture volume of Buffered Methanol-complex Medium (BMMY, figure 2.2). This was placed in a 100 ml baffled flask, covered with sterile gauze and replaced into the incubator under the same conditions as previously mentioned. Methanol (100%) to a final concentration of 0.5% (depending on the volume in flask) was added every 24 H to maintain the production of the induced protein.

When the optimal time, post-induction had elapsed, the protein was harvested by centrifugation in a benchtop centrifuge (Microcentaur MSE) for 2 - 3 minutes at room temperature and the supernatant analysed for *endo*glucanase activity by viscometric assay (section 2.2.2.2.3). The protein was quantified by determination by the method of Sedmak and Grossberg (section 2.2.3) and/or SDS PAGE (section 2.2.4).

BMGY
Peptone (20%) and yeast extract (10%) was dissolved in 700 ml water and autoclaved. After cooling to room temperature, the following filter sterilised or autoclaved solutions were added and mixed well: 100 ml potassium phosphate buffer (1M, pH 6.0), 100 ml yeast nitrogen base (1.34 %),
2 ml biotin (0.02%) and 100 ml glycerol (10%).

Figure 2.1 Components and method for BMGY (Invitrogen, 1995)

BMMY
Peptone (20%) and yeast extract (10%) was dissolved in 700 ml water and autoclaved. After cooling to room temperature, the following filter sterilised or autoclaved solutions were added and mixed well: 100 ml potassium phosphate buffer (1M, pH 6.0), 100 ml yeast nitrogen base (1.34 %), 2 ml biotin (0.02%) and 100 ml methanol (5%).

Figure 2.2 Components and method for BMMY (Invitrogen,1995)

2.2.6.2 *Methods for optimisation of Pichia protein expression* (Invitrogen 1995)

The expected levels for protein production from the system devised by J Milne (Honours project, University of Stirling, 1997) were unknown. Various guidelines were consequently followed in order to optimise conditions.

2.2.6.2.1 *Proteolysis of expressed protein*

The method followed was as for section 2.2.6.1 but unbuffered medium (Minimal Methanol, MM containing yeast nitrogen base, 1.34%, Biotin, 4 x 10^{-5} %, and methanol, 0.5%) replaced BMMY. Also carried out was the addition of 1 % casamino acids added to the buffered medium.

2.2.6.2.2 *Procedure for cell lysis* (J. Nairn, personal communication)

After following the procedure of section 2.2.6.1, 1 ml of overnight yeast culture was centrifuged at low speed (6000 rpm) in bench centrifuge (MSE Microcentaur) for 2 minutes. The supernatant was removed and the cells resuspended in ~70 µl of 50 mM Tris-HCl pH 8.0. Glass beads were added to 1 - 2 mm below the meniscus and the solution cooled on ice for 5 minutes. Vortexing of the solution for 1 minute was followed by cooling on ice for 1 minute, this procedure was repeated five times and the debris pelleted by spinning at high speed (12000 rpm) for 2 minutes in a bench centrifuge. The supernatant was removed and assayed by viscometric analysis (section 2.2.2.2.3).

2.2.7 Preparation of Modified Tamarind Seed Xyloglucans

2.2.7.1 *Time course for the depletion of galactose from xyloglucan (small Scale)*

A solution of native xyloglucan was prepared (12 mg/ml in 50 mM ammonium acetate buffer pH 5.0). β-galactosidase was added (200U/ml of xyloglucan solution, calculated from *para*-nitrophenylglycoside assay section 2.2.2.2.4) and the solution incubated, statically, at 30° C.

Samples were taken at predetermined time points and boiled to denature the enzyme. These samples were analysed by GDH assay (Section 2.2.2.2.1).

2.2.7.2 *Preparation of Galactose Depleted Xyloglucan (Large scale)*

A solution of native xyloglucan was prepared as above (90 ml of a 0.5% solution in filter sterilised Hestrin Schramm medium, Hestrin & Schramm, 1954). β-galactosidase was added 200U/ml of xyloglucan solution) and the solution incubated in a screwtop sterile container at 30° C with constant stirring.

At points determined from the time course the required volume (~30ml) was removed to a sterile glass universal sealed and boiled for ~20 minutes to denature the enzyme and further sterilise the xyloglucan. After 24 H incubation a further 200U/ml aliquot of β-galactosidase added.

2.2.7.3 *Preparation of Galactose-depleted 'Gel'*

A solution of xyloglucan (12mg/ml) was statically incubated with nasturtium β-galactosidase (200U/ml) at 50° C for ca. 3 H. The 'gel' was then heated in a boiling water bath until it returned to liquid state. The solution was allowed to cool back to room temperature.

2.2.7.4 *Mild acid hydrolysis for the Removal of Xylose from Xyloglucan*

A solution of xyloglucan (12mg/ml) was mixed with 0.5M or 1.0M acetic acid and heated to 100° C. The resulting xyloglucan solution was neutralised with 200mM sodium hydroxide and the polysaccharide precipitated and analysed as in section 2.2.5.2

2.2.7.5 *Enzymatic hydrolysis of xyloglucan with α-glucosidase.*

After reconstituting the enzyme in deionised water (to give a solution of 12.5 U/ml), 1 unit or 2 units of α-glucosidase were added to 0.5 ml xyloglucan solution (12 mg/ml). This solution was left to incubate overnight at 30° C after which the enzyme was denatured by boiling for 10 minutes.

Determination of the presence of free xylose was by the Free Pentose assay (section 2.2.2.2.2) performed on the supernatant of the solution. Analysis of the oligosaccharide composition of the xyloglucan was performed as described in section 2.2.5.1.

2.2.7.6 *Enzymatic removal of xylose using α-xylosidase and NXET*

A solution of xyloglucan (24mg/ml, in filter sterilised 50 mM ammonium acetate buffer pH 5.0) was incubated, with constant agitation at 35° C for 72 hours with 32μg/ml α-xylosidase and 50μg/ml NXET (since the apparent activity of α-xylosidase is dependent on the presence of, in this case, NXET which, itself, displays both hydrolysis and transglycosylation actions, a conventional activity quoted in units is not possible, see chapter three). A few drops of chloroform were added into the reaction tube to reduce the occurrence of bacterial growth. The xyloglucan solution was then precipitated and analysed for monosaccharide content as described in section 2.2.5.2. Xyloglucan solutions (24 mg/ml) were incubated independently with NXET and with α-xylosidase to act as controls.

2.2.7.7 *Enzymatic removal of xylose using α-xylosidase and tomato endotransglycosylase (TXET)*

The experimental conditions used above were repeated but with NXET replaced with TXET (50 µg/ml).

2.2.8 Production of Bacterial Cellulose

2.2.8.1 *Revival of Bacterial Stock Cultures*

Acetobacter xylinus was supplied as a freeze-dried culture. This was revived onto glucose/yeast extract agar plates (20 : 10 : 30) and incubated for 5 days at 30 ° C. These plates were then used to inoculate sterile ¼ strength Ringers solution (BDH), to a final cell concentration of 5 X 10^5 cells/ml, as determined by Total Viable Cell counts. 1 ml of this suspension was inoculated onto Cryo-Bead bacterial Preserver (Technical Service Consultants Ltd, UK) and stored at -80° C. When fresh cultures were required, one bead was removed and inoculated onto glucose/yeast extract agar plates as above. Cultures were prepared for long term storage at 4 ° C by inoculating glucose/yeast extract agar slopes with bacteria from the original plates.

Table 2.1 Components of Hestrin & Schramm Medium

Component	g/l
Glucose	20
Bacteriological peptone	5
Yeast extract	5
Sodium phosphate	2.7
Citric acid H_2O	1.15

2.2.8.2 *Production of seed cultures.*

Inoculated, sterile, 1/4 strength Ringers solution (5 ml) was used to inoculate 95 ml Hestrin/Schramm medium (Hestrin & Schramm, 1954, table 2.1) in a 500 ml sterile Roux bottle. After gentle agitation to ensure thorough mixing, the cultures were incubated at 30 ° C for 3 days until a thick pellicle of cellulose was formed. The pellicle was then transferred, aseptically into 50 ml sterilised phosphate buffer (0.02M pH 6.0) and ground using a pre-sterilised Ultra-Turrax T25 (Janke & Kunkel). The cellulose was removed by filtration through sterile Miracloth (Calbiochem Corp., USA) and the bacterial suspension collected in a sterilised shake flask and stored at 4 ° C until further use (S Brooks, personal communication).

2.2.9 Preparation of composites

2.2.9.a A solution (9ml) of 0.5% XG or modified XG in Hestrin-Schramm media was inoculated with 0.5 ml cellulose producing *Acetobacter* culture prepared as above, into a 25 ml canted neck, vented tissue culture flask with 2 μm hydrophobic cap. Cultures were incubated at 30° C for 48 H. After incubation the pellicles were removed aseptically from the culture flasks and washed thoroughly in several changes of sterile deionised water at room temperature. A final wash was carried out in sterile deionised water containing 0.05% NaN_3. Composites were then stored at 4 ° C in a small volume of this solution.

2.2.9.b Three to four colonies of *A. xylinus* from glucose/yeast extract agar plates were inoculated into ~15 ml sterile Ringers solution. After mixing well, 1ml was inoculated into each flask containing 9ml xyloglucan solution as above. The flasks were incubated statically at 30° C for 72 hours after which the composite material was recovered as above.

2.2.9.c These composites were also constructed using the method 2.2.9.b, in the presence of i. 1ml TXET and ii. 1ml TXET plus oligosaccharides (oligosaccharides to a final concentration of 0.5%).

2.2.10 Biochemical Testing of Composites

Once prepared the xyloglucan/cellulose composites were biochemically and mechanically tested. Further analysis with Deep-Etch Freeze Fracture, Transmission Electron Microscopy and ^{13}C- NMR was also carried out.

2.2.10.1 *Sequential Extraction of Xyloglucan with KOH*

Cellulose/xyloglucan composites were deconstructed with increasing concentrations of alkali (Selvendran 1983). Samples of composites (~10mg wet weight) were extracted with orbital agitation at room temperature with 2 x 0.5 M KOH, 2 x 1M KOH, 1 x 4M KOH and 2 x 4M KOH in sequence, (all solutions were nitrogen presaturated and contained 20 mM $NaBH_4$). After each extraction the KOH/XG solution was removed and stored for oligosaccharide analysis, residues were washed with deionised water before the next addition of KOH and the wash added to the KOH/xyloglucan already removed. The KOH/xyloglucan solution was neutralised with acetic acid and the xyloglucan extracted from each stage of the process precipitated with ethanol (2 volumes ethanol: 1 volume extract). After centrifugation (11000 g for 20 minutes) the pellet was washed with deionised water and resuspended in 100 µl water. Digestion with *endo*glucanase (Megazyme) was carried out as described in section 2.2.5 and the resulting oligosaccharides analysed on HPAEC using the same protocol (Section 2.2.5).

Samples of residue were also taken at every stage of the extraction and these were subject to acid hydrolysis with sulphuric acid as per section 2.2.5.2. The monosaccharides were separated on HPAEC under the conditions already mentioned.

2.2.10.2 *Alkali Removal of Bound Xyloglucan From Composites*

Complete, one step removal of xyloglucan from the composites was achieved by the addition of an aliquot of 4M KOH (1 ml) to ~10 mg (wet weight) of the composite. The sample was agitated overnight at room temperature after which the KOH/xyloglucan solution removed was neutralised, precipitated and analysed as described in the previous sections.

2.2.10.3 *Removal of Xyloglucan from Composites by Agitation Only*

Removal of very loosely associated xyloglucan was carried out on the composites. Approximately 10 mg (wet weight) was agitated in 1 ml of ammonium acetate buffer (50 mM pH 5.0) in an orbital incubator at room temperature overnight. Ethanol precipitation of the xyloglucan in 100 µl of the supernatant was carried out and oligosaccharide composition investigated as described above.

2.2.10.4 *Removal of Loosely Bound Xyloglucan by Action of Endoglucanase*

Samples of composites (~10mg wet weight) were added to 0.5ml of ammonium acetate buffer (50 mM pH 5) and incubated with 2.25U of *endo*glucanase (Megazyme) in the orbital incubator at room temperature. After an overnight incubation and denaturation, the supernatant was separated from the composite residue and run on HPAEC as described in section 2.2.5.

2.2.10.5 *Removal of Galactose from Tightly Bound Xyloglucan by Action of β-galactosidase*

The residue remaining from the procedure described in section 2.10 was washed thoroughly with deionised water and placed in 0.5ml ammonium acetate buffer (50mM, pH 5.0).

β-galactosidase was added (100U) and the tube placed in the orbital incubator under the predescribed conditions. After denaturation the monosaccharide composition of the control and sample were determined according to section 2.2.5.

2.2.11 Mechanical Testing of Composites

The rheological behaviour of the composites was investigated using tensile testing. The deformation technique described below will detect differences induced by the presence of xyloglucan and galactose depleted xyloglucans.

Uniaxial Tensile Testing

Rectangular strips with a length/width ration of ~ 10 were cut from the intact, wet composite pellicle (width and thickness of strip were measured with micrometer) and the ends place between vice-grips of a Minimat (Polymer Laboratories, Loughborough, UK). The grips were then moved apart at a constant speed of 10 mm/min using a 20N load beam.

2.2.12 Deep-Etch, Freeze Fracture, Transmission Electron Microscopy

Using a 4mm cork borer, samples were cut from hydrated xyloglucan/cellulose and galactose depleted xyloglucan/cellulose pellicles. These were placed on filter paper attached to an aluminium disc. Metal mirror impact freezing was carried out on a KF80 Cryostation (Leica, UK) using an MM80 Impact Freezer Head. The sample was transferred, after impact, to a CSE-50 Freeze Fracture Unit (Cressington, UK) where fracturing was performed at -179° C. After etching for 8 minutes at -95° C, rotary shadowing at an angle of 45° using 1.5 nm tungsten/tantalum was followed by rotary carbon at 90° to a thickness of 8nm.

Replicas were floated off in distilled water, cleaned in chromic acid overnight and collected on a 450 mesh hexagonal copper grid. Visualisation took place on a Jeol 1200EXII Transmission Electron Microscope and micrographs produced using Kodak Reverse Contrast Paper. The images were analysed using a Quantimet 570 Image Analyser (Leica, UK).

2.2.13 ^{13}C NMR Spectroscopy

Nuclear magnetic resonance spectra were acquired on a Bruker MSL 300 instrument operating at 75.46 MHz for ^{13}C. Dipolar coupling of 67 kHz was used for both SP-MAS and CP-MAS and spin-locking fields for cross-polarization/magic angle spinning (CP/MAS) experiments were ca. 40 kHz. Magic angle spinning speeds were typically 3 kHz and experiments carried out at 303K. Experimental recycle times were 3 - 5 seconds. For resolution enhancement line broadening at -70 Hz and Gaussian multiplication of 0.5 were used.

Chapter Three

3. Purification of Four Xyloglucan-specific Enzymes from Germinated Nasturtium *(T majus* L) Seeds

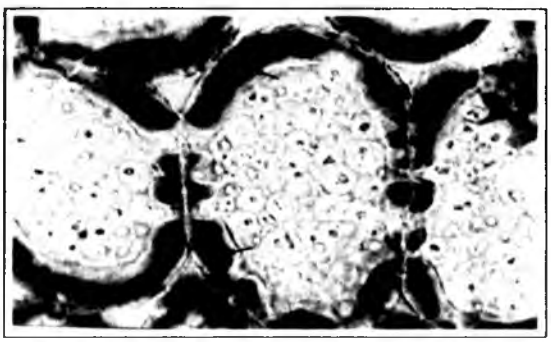
3.1 Introduction

Vogel and Schleiden (1839) described thickenings of the cell walls of cotyledons of certain seeds which were characterised by their ability to stain blue in the presence of iodine/potassium iodide. Microscopic studies on cotyledons in the nineteenth century suggested that amyloids (substances which turn blue when treated with iodine reagent for amylose) disappeared from seeds after germination (Godfrin 1884, Heinricher 1888, Reiss 1889), the assumption being that they were reserve polysaccharides (Figure 3.1). When hydrolysed with acid, extracted amyloid from nasturtium seed released glucose, xylose and galactose (Winterstein 1893).

Xyloglucan also occurs, uniformly, and in association with cellulose, across the thickness of the primary cell walls of most higher plants (Moore *et al*, 1986) and the mechanical properties of these cell walls must change to accommodate turgor driven cell expansion. These changes can be influenced by auxin, Ca^{2+}, changes in pH and cell wall degrading enzymes (Virk and Cleland 1988). Indeed, a vital part in growth regulation is played by endoglucanase (Fry 1989) with xyloglucan as the important substrate (Hayashi, 1989).

As the xyloglucan backbone is composed entirely of glucose residues, significant breakdown of the polymer is only possible by the initial action of an endoglucanase. Edwards *et al* (1985) found that germinating nasturtium seeds mobilise their xyloglucan reserves by the action of an endoglucanase that does not hydrolyse cellulose, carboxymethylcellulose or (1→4) β-D-glucans as do other 'cellulases' (Tomme *et al* 1989). The complete breakdown of the xyloglucan is mediated by the concerted actions of endoglucanase, β-galactosidase, α-xylosidase and β-glucosidase and many plant cell walls also contain these activities

(Koyama et al 1983, O'Neill *et al* 1988). In pea stems these activities are induced by auxin (O'Neill *et al* 1983 and 1989, Hensel *et al* 1991) and Hayashi (1984) reported that pea cellulase preferentially attacked and solubilised xyloglucans from cell wall 'ghosts' (which contain only xyloglucan and cellulose).



Mag X 800

Figure 3.1 The storage cell wall xyloglucan in *Tropaeolum majus* stained with iodine-potassium iodide (section taken 16 H after imbibition. Xyloglucan reserves indicated with arrows). Photograph by Dr R Sexton, University of Stirling.

In order to investigate the role of the structural features of xyloglucan in the binding to cellulose, modification of the tamarind seed xyloglucan must be achieved. Initially this involves the purification of the enzymes known to act in synergy to hydrolyse xyloglucan, thus providing the 'tools' with which to tailor the polysaccharide. In this chapter the isolation of these enzymes is reported.

3.2 Materials and Methods

3.2.1 Purification of Enzymes

Purification methods for all enzymes were carried out as described in Materials and Methods, *section 2.2.1 to 2.2.1.2.4 inclusive.*

3.2.2 Preparation of Xyloglucan Solution

Xyloglucan solution was prepared as described in section 2.2.2.1 in Materials and Methods.

3.2.3 Determination of Enzyme Activity

The activity of the enzymes was determined using both synthetic and natural substrates (in the case of β-galactosidase and β-glucosidase) and natural substrate only (in the case of endoglucanase and α-xylosidase). These were carried out as described in Materials and Methods, section 2.2.2.2.

3.2.4 Protein Determination

Total protein was assayed as described in section 2.2.3 of Materials and Methods.

3.2.5 SDS-Polyacrylamide Gel Electrophoresis

SDS-PAGE was carried out as described in Materials and Methods section 2.2.4.

3.3 Results

The initial purification procedures for all four enzymes gave a very poor yield of active enzyme (data not shown). Following a time course experiment to find the peak of activity of the enzymes in relation to days after sowing (data not shown), where again very little activity was evident, it was discovered that the nasturtium seeds which were some years old were producing less enzyme activity than reported previously (Edwards *et al* 1985 & 1986). A new batch of seeds was received and only the purification data from the new seeds is reported here.

3.3.1 *endo* (1→4)β-D- glucanase

The enzyme was isolated from germinated nasturtium seeds harvested 13 days after sowing, when the activity of the enzyme was found to be high in the cotyledonous tissue. Partial purification of the *endo*(1→4)β-D-glucanase was achieved by anion-exchange chromatography on DEAE-cellulose. The enzyme was eluted in the Na Cl gradient applied to the column (Figure 3.2a). The fractions containing high enzyme activity were applied to cation-exchange chromatography on CM-cellulose where the *endo*glucanase activity was eluted in the wash (Figure 3.2b). The final purification step was accomplished by gel filtration using BioGel P60 (Figure 3.2.c). The purification process was monitored by the enzymes ability to hydrolyse tamarind seed xyloglucan, using the viscometric assay.

A conventional value for the specific activity for this enzyme is not applicable as both hydrolysis and transglycosylation activities are taking place simultaneously, however, a yield of 140 µg/ml pure protein was obtained.

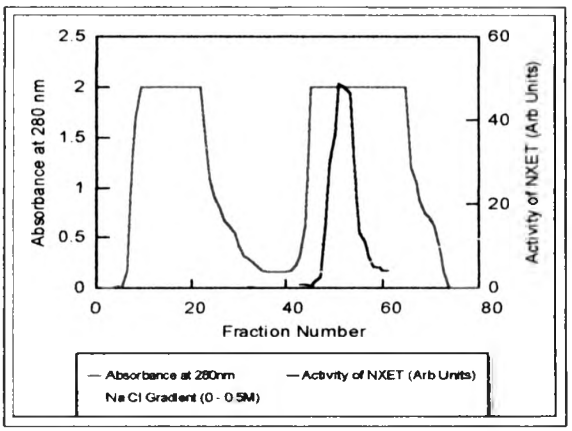


Figure 3.2a Anion-exchange chromatography on DEAE-Cellulose

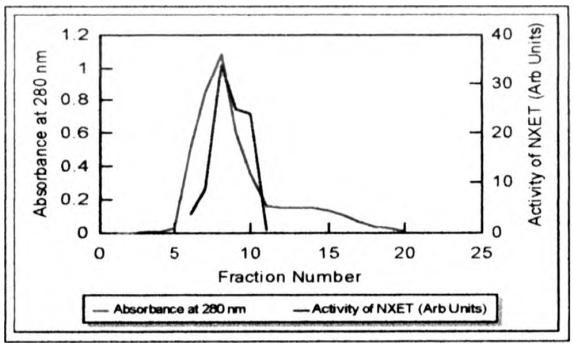


Figure 3.2b Cation-exchange Chromatography on CM-Cellulose

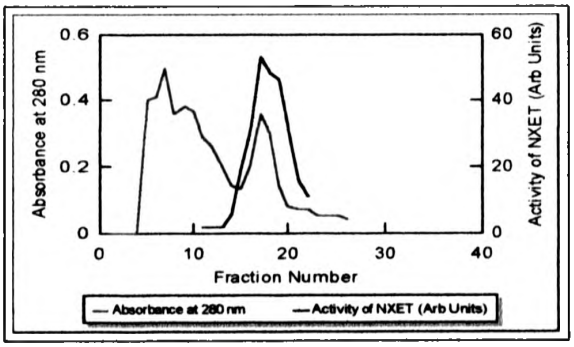


Figure 3.2c Gel Filtration using Biogel P60

3.3.2 β-D-galactosidase

The nasturtium β-D-galactosidase was isolated to the required purity using the procedure described by Edwards *et al* (1988) where partial purification of the protein was achieved by application of the crude extract to a diethylaminoethyl-cellulose (DEAE) column. The unbound enzyme activity was eluted in the wash (figure 3.3a).

After cation-exchange chromatography, where the activity was eluted from carboxymethylcellulose in the sodium chloride gradient (figure 3.3b), further purification was obtained by application of the extract to a Biogel p60 gel filtration column (figure 3.3c). The isolation of β-D-galactosidase was monitored by the nitrophenylgalactoside assay. The specific activity of the isolated enzyme was 2211 nkat.mg^{-1} as determined by the nitrophenylgalactoside assay.

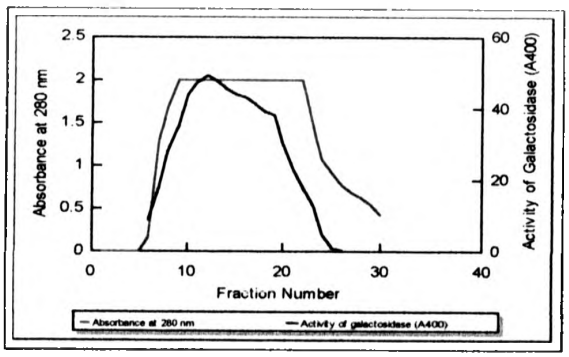


Figure 3.3a Anion-exchange Chromatography on DEAE-Cellulose

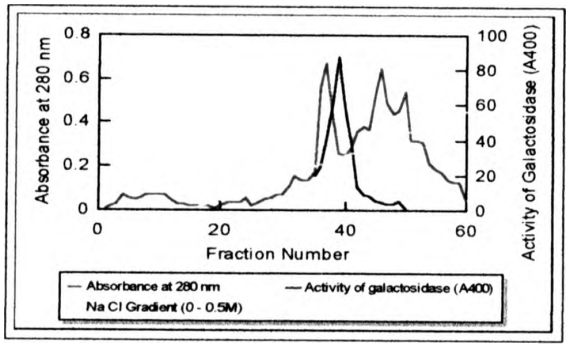


Figure 3.3b Cation-exchange on CM-Cellulose

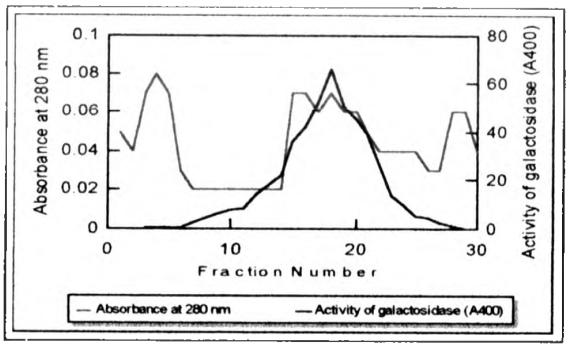


Figure 3.3c Gel filtration on Biogel P60

3.3.3 α-D-xylosidase

Purification from the crude extract was obtained on DEAE-Cellulose where the α-D-xylosidase activity was eluted in the 0 - 0.5 Na Cl gradient (figure 3.4a). Fractions exhibiting high activity were pooled, concentrated and applied to an affinity chromatography column (Concanavalin A figure 3.4b). After elution with 10mM methyl glucoside the extract was further purified by gel filtration on Biogel P300 (figure 3.4c). The progress of the isolation procedure was monitored by the free pentose assay after an overnight incubation with xyloglucan in the presence of cellulase or NXET and is expressed in mM xylose released. A conventional value for the

specific activity of this enzyme is not applicable in this case as the activity of α-D-xylosidase is dependent on the concentration of non-reducing ends of xyloglucan. The substrate has to be xyloglucan oligosaccharides or *endo*glucanase is required to be present. A yield of 160 μg/ml pure protein was obtained.

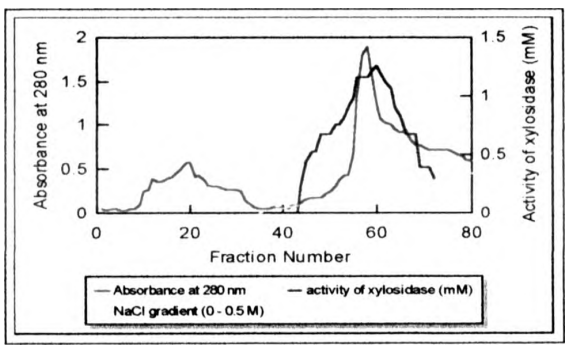


Figure 3.4a Xylosidase purification on DEAE-Cellulose

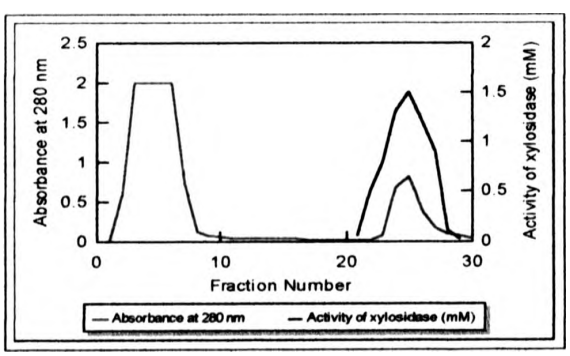


Figure 3.4b Affinity Chromatography on Concanavalin A

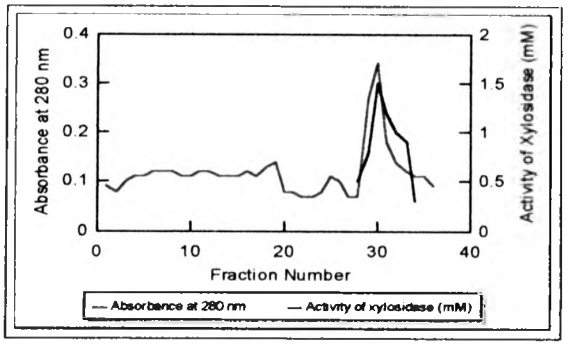


Figure 3.4c Gel Filtration on Biogel P300

3.3.4 β -glucosidase

The isolation of this enzyme was carried out by application of the crude extract to DEAE Cellulose column, the fractions with high activity were eluted in the wash (figure 3.5a). These fraction were pooled, concentrated and subjected to cation-exchange chromatography on S Sepharose (figure 3.5b). The progress of the purification was monitored by the p-nitrophenylglucopyranoside assay The specific activity for this enzyme was found to be 1303 nkat.mg^{-1} as determined by the p-nitrophenylglucopyranoside assay

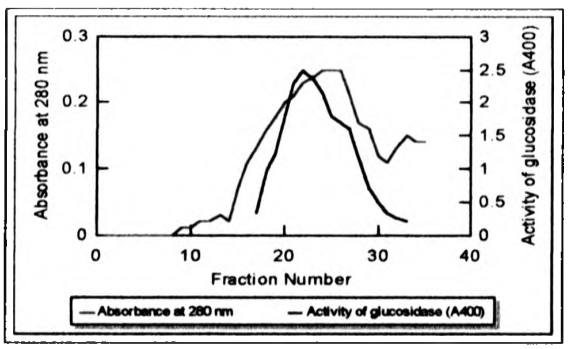


Figure 3.5a Anion exchange Chromatography on DEAE-Cellulose

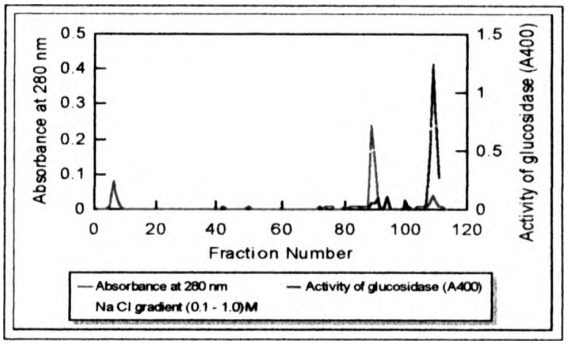


Figure 3.5b Cation-exchange on S Sepharose

The purified enzymes were stored at –20 ° C.

3.3.5 SDS-Polyacrylamide Gel Electrophoresis

The proteins were visualised on SDS-PAGE (figure 3.6) using a molecular weight marker (Myosin 205000; β-galactosidase 116000; phosphorylase 97400; bovine serum albumin 66000; ovalbumin 4500; carbonic anhydrase 29000). All purified proteins were at the positions expected by their respective molecular weights and all were single bands with the exception of β-galactosidase where other components, with lower molecular weights, were present. No contaminating enzyme activities (of interest to this work) were found in any of the preparations of purified enzyme.

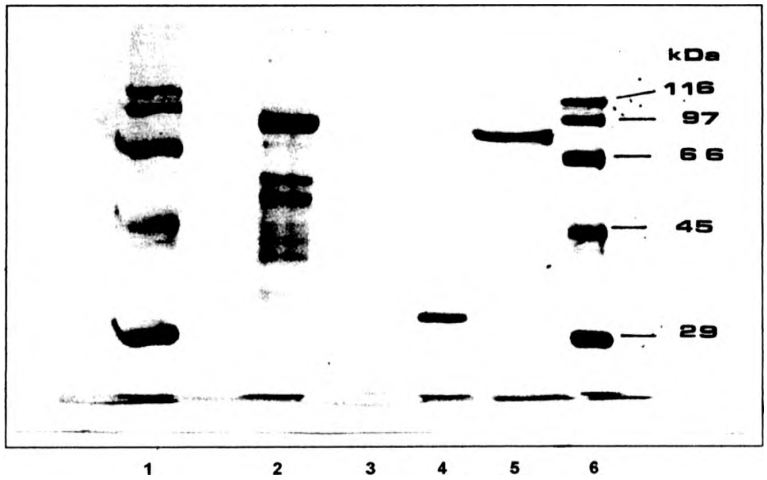


Figure 3.6 SDS PAGE of the purified proteins:- Lane 1+ 6 Molecular weight marker (Myosin 205000; β -galactosidase 116000; phosphorylase 97400; bovine serum albumin 66000; ovalbumin 4500; carbonic anhydrase 29000). Lane 2 Nasturtium β -galactosidase. Lane 3 β -glucosidase. Lane 4 NXET. Lane 5 α-xylosidase.

3.4 Discussion

It has been suggested that xyloglucan breakdown in nasturtium could be regarded as a process of wall turnover (Reid 1985). The striking similarities between nasturtium and pea enzymes, apparently involved in very different physiological processes suggest that the former may be involved in the turnover of primary cell wall xyloglucan which is known to be associated with elongation-growth (Labavitch and Ray 1974).

α-Xylosidase purified from germinating nasturtium cotyledons is very similar in terms of specificity and molecular properties to the enzyme isolated from auxin-treated pea stem (O'Neill *et al* 1989) ie. similar molecular weights and both are glycosylated. A xyloglucan-specific *endo*glucanase has been implicated as a mediator in the cleavage of xyloglucan polysaccharides, with the production of biologically active oligosaccharides, which occurs in the growing regions of the cell wall (Fry 1989). This solubilisation of xyloglucan in primary cell walls is believed to be induced by the action of auxin or the lowering of pH (Labavitch and Ray 1974). Indeed, the similarities between cell wall and seed enzymes suggest that xyloglucan modified with the nasturtium enzymes could mimic cell wall events albeit with a xyloglucan with a molecular weight much higher than that occurring in cell walls.

The purification procedures for all four nasturtium seed enzymes were carried out essentially according to Edwards *et al*: β-galactosidase (1985) and endo -β-glucanase (1986), Fanutti *et al* : α-xylosidase (1993) and Crombie *et al* : β-glucosidase (1998). The isolation of each of these enzymes was considered to be successful as they were either purified to homogeneity, or purified to the stage that no contaminating activities from the other enzymes of interest were found to be present.

The presence of multiple bands of lower molecular weights in the isolated β-galactosidase lane of Figure 3.5 is thought, not to be due to contaminants present in the final product but to breakdown products caused by proteolysis (Edwards *et al* 1988, Fanutti unpub). Fanutti reported that antibodies raised against the major β-galactosidase band cross-reacted with the components of the lower molecular weight as well as with the major band. This is also a

probable explanation for the low activities found for the original β-galactosidase purification procedures carried out (not reported) where the seeds had been stored for a number of years.

In the event that new seeds were not available from the supplier and to investigate a possible alternative source for the enzyme (which did not involve an extensive extraction process), the overexpression of NXET from *Pichia pastoris* was examined and this work is reported in the next chapter.

The activities for all four isolated enzymes from the new seeds were comparable to these obtained previously by the respective researchers and the specificity of the enzymes for xyloglucan could now be utilised in tailoring the polysaccharide for use in the *in vitro Acetobacter* system. This would, in effect, create a 'cell wall' mimic with cellulose and modified xyloglucan.

In the first instance this involved the removal of galactose to form two xyloglucans with different degrees of galactose depletion. This work is reported in chapter 5 with the cellulose / galactose depleted xyloglucan composite construction reported in chapter 6. The removal of xylose from the native tamarind seed xyloglucan was also carried out and is reported in Chapter 7 as is the construction of a xylose depleted xyloglucan / cellulose composite.

Chapter Four

4. Overexpression of NXET by the Methylotrophic Yeast *Pichia pastoris*

4.1 Introduction

Pichia pastoris was developed into an expression system by scientists at Salk Institute Biotechnology / Industry Associates (SIBIA) and Phillips Petroleum for the high level expression of recombinant proteins. *P. pastoris* is a eukaryote and as such has the advantages of higher eukaryotic expression systems. This includes protein processing and folding and post-translational modification while being easy to manipulate. *Pichia* also produces low levels of native proteins. This yeast is also capable of metabolising methanol as its sole source of carbon by the oxidation of methanol to formaldehyde using molecular oxygen, catalysed by alcohol oxidase. This enzyme has a poor affinity for oxygen and the yeast compensates by producing it in large amounts. The promoter regulating the production of alcohol oxidase is the same one used to drive heterologous protein expression in *Pichia*.

Protein produced by *Pichia pastoris* can be either intracellular or secreted, although secretion requires the presence of a signal sequence on the expressed protein to target it to the secretory pathway. The secretion signal sequence used with most success is the α factor prepro peptide from *Saccharomyces cerevisiae* (Gregg 1993, Scorer 1993).

The over-expression system of *Pichia* has been used successfully in the last few years for the production of a wide variety of proteins to varying levels. α Amylase isoenzymes 1 & 2 were secreted to levels of 50 and 1 mg/L respectively (Juge *et al* 1996) and Fierobe *et al* (1997) reported the secretion of 0.4 g/L of an industrially important glucoamylase (from *Aspergillus niger*). A yield of 180 mg/L of procarboxypeptidase has also been reported by Reverted *et al* (1998).

While it was thought that the seeds were producing very little active NXET, it was decided that the over-expression work of J Milne (Honours Thesis, 1997) should be continued. Jennifer Milne and coworkers successfully inserted the cDNA for NXET from a nasturtium library into the *Pichia* expression vector pPIC9. The gene was inserted following the α factor pre pro

secretion signal of *Saccharomyces cerevisiae* and the secretion of a protein with a molecular weight of 30 kDa was achieved. The activity of this protein increased with increasing time post-induction suggesting the continuous expression of NXET during the induction phase. The isolation of three enzyme-producing clones from this work provided the starting material for attempting to increase the yield to levels closer to those reported in the expression manual (≥ 1g/L).

As mentioned briefly in Chapter 1, the action of NXET on xyloglucan observed by Edwards *et al* in 1986 was investigated further by Fanutti *et al* (1993 and 1996). These workers compared the products of hydrolysis of tamarind seed xyloglucan by the nasturtium enzyme with those of an endo-(1→4)-β-D-glucanase of fungal origin. They found that both the nasturtium and fungal enzymes produced the oligosaccharides shown in Chapter 1, figure 1.2. In

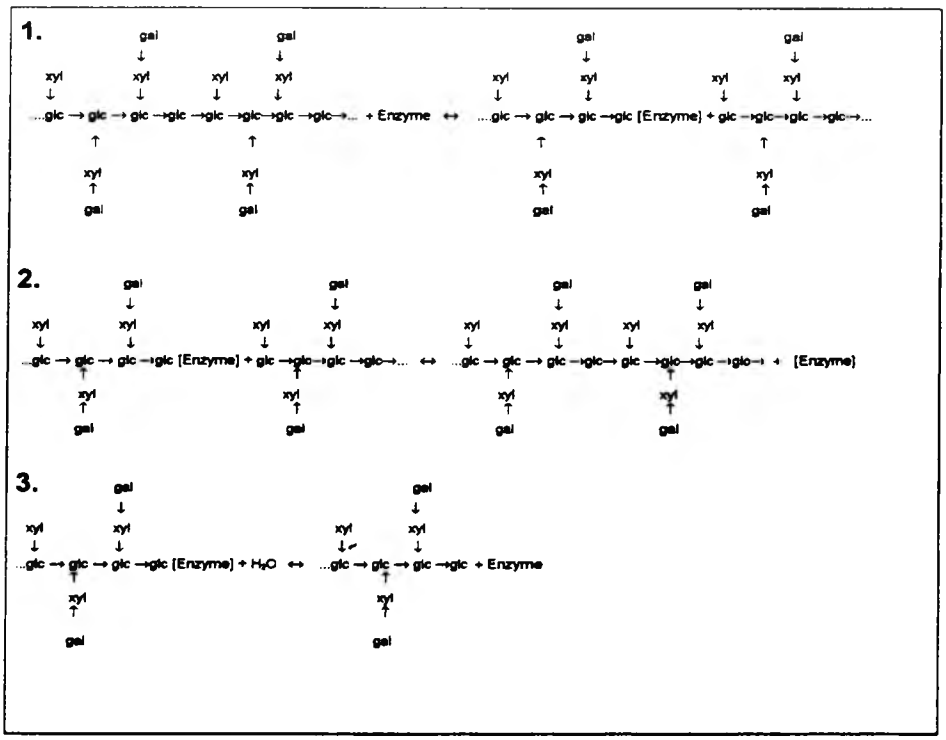


Figure 4.1 Transglycosylation and hydrolysis of tamarind seed xyloglucan by nasturtium specific *endo*glucanase. The portion of the chain towards the reducing end is liberated and the portion towards the non-reducing end is transferred specifically to the non-reducing end of an acceptor xyloglucan (or xyloglucan oligosaccharide) molecule, giving transglycosylation (1. and 2.). When acceptor chain ends are limiting, transfer to water can occur, giving hydrolysis (3.). (Adapted from Fanutti *et al* 1993 and Reid 1996)

addition the nasturtium enzyme produced higher molecular weight products which led to the conclusion that the nasturtium enzyme possessed a powerful transglycosylase action as well as the previously characterised hydrolytic action. The equations shown in figure 4.1 describe the action of the enzyme on tamarind seed xyloglucan and illustrate that the hydrolase action is significant only under the conditions where suitable acceptor chain-end concentrations are limiting, for example at low substrate concentrations or in the first stages of digestion of the polymeric xyloglucan.

Substrate subsite recognition of NXET was also discussed in chapter 1 (section 1.4.1) with Fanutti *et al* (1996) proposing a partial model for the minimum substrate binding requirement (figure 4.2). Such motifs are not found in natural xyloglucans and further work directed at the production of synthetic substrates is required.

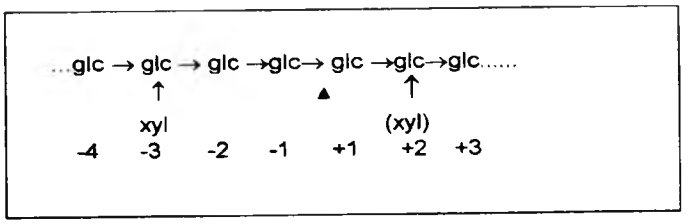


Figure 4.2 Partial structure of seed xyloglucan illustrating the proposed minimum requirement for substrate recognition around the site of chain cleavage (▲). (Adapted from Fanutti *et al* 1996). Brackets denote that xylose could be substituted with galactose.

The work reported in this chapter investigates the optimisation of conditions for the production of NXET from *Pichia pastoris* as well as the possibility of obtaining active, pure protein by a less extensive extraction process as previously reported (chapter 3).

This work also provides a 'validation' of the NXET produced by *Pichia*. This is determined by examining the hydrolytic and transglycosylation action of the yeast produced enzyme on a solution of xyloglucan compared to that purified from nasturtium seed by the conventional method (section 2.2.1.2.1 Chapter 2 and 3.2.1 Chapter 3).

4.2 Methods

4.2.1 Investigation of Clones 6,13 and 18

To determine which of the three clones, resulting from the work of Jennifer Milne, produced the most active enzyme they were removed from the freezer where they had been stored at -70 ° C as glycerol stocks and streaked out on yeast extract peptone dextrose medium plates. After a 48 hour incubation one colony from each of the clones was inoculated into BMGY growth media as described in section 2.2.6.1, chapter two. After reaching an optical density between 2 and 6, the cells were spun, resuspended in BMMY and incubated as described in Methods and Materials (section as before) for 48 hours. The supernatant was then recovered and tested for enzyme activity using the viscometric assay (Section 2.2.2.2.3, chapter two). The proteins were visualised using SDS PAGE and quantified, in this case, using the Gelsys computer program (Gelworks, GDS8000, supplied by UVP).

4.2.2 Optimisation of Conditions for Secretion of NXET

4.2.2.1 *To Counteract the Presence of Proteases*

In order to investigate the occurrence of the overexpressed protein being hydrolysed by the action of endogenous proteases after translation but before secretion, the cells were grown, as before in BMGY but resuspended in MMY (minimal media) as described in section 2.2.6.2.1 of chapter two. As before the proteins were visualised using SDS PAGE, assayed for NXET activity using the viscometric assay and quantified using the method of Sedmak and Grossberg (section 2.2.3, Chapter two)

4.2.2.2 *Increasing the Density of the Cells Before Induction*

The density of the cells was allowed to increase by lengthening the time of the growing phase of the *Pichia* cells before induction. The remainder of the procedure was carried out as previously described but with 1% casamino acids added to the induction media to further minimise the activity of native proteases secreted from the yeast. The quantification, visualisation and activity were determined as previously described in section 4.2.2.1.

4.2.2.3 *Examination of the Cell Pellet*

The cells were lysed after incubation in the induction media, as described in section 2.2.6.2.2 of chapter two. The cell contents were then tested for activity by the viscometric method previously mentioned.

4.2.3 Gel filtration of NXET from *Pichia pastoris*

The enzyme produced from the yeast which was allowed to grow to a higher density before induction (section 4.2.2.2) was applied to a Biogel P60 gel filtration column as described in chapter 2, section 2.2.1.2.1 (final paragraph) and 4 ml fractions collected. Measurement of the absorbance at 280 nm of the eluate indicated the presence of protein and this was visualised on SDS PAGE and the activity monitored by viscometric assay. A protein concentration determination was carried out by the method of Sedmak and Grossberg (1977).

4.2.4 Investigation of Action of NXET on Xyloglucan Solution

A solution of xyloglucan (12mg/ml) was incubated overnight with 12 µg purified NXET (both *Pichia* and nasturtium). This was denatured by boiling for 10 minutes. Ethanol (at a ratio of 3:1

of sample) was added to the solution to precipitate the high molecular weight material which was then collected by centrifugation and both the pellet and supernatant harvested. The ethanol was removed from the supernatant (low molecular weight xyloglucan) by spinning in the Gyrovap (supplier: Howe) for 30 minutes and the remaining solution applied to the HPAEC using the oligosaccharide program described in section 2.2.5.1, chapter two.

After freeze drying, the high molecular weight material was investigated to ascertain the relative proportions of the resulting oligosaccharides present. This was achieved by a further overnight hydrolysis with fungal endoglucanase (4.35 μg). After denaturation the solution was applied to the HPAEC using the oligosaccharide program described above.

4.3 Results

4.3.1 Investigation of Clones 6, 13 and 18

Lanes 2-4 of figure 4.3 contain enzyme produced by the clones 6 (lane 2), 13 (lane 3) and 18 (lane 4) isolated by J Milne *et al* (Hons Thesis 1997). Bands are apparent at approximately 30 kDa for each of these matching the position of those which contained 90 μg/ml (lane 5) and 130μg/ml (lane 6) of the purified nasturtium NXET. Faint bands are also present at approximately 125, 87 and 76 kDa in lanes 2 - 4 of this figure which are absent in lanes 5 and 6.

The Gelsys system for the PC measured the intensity of the 30 kDa bands in lanes 2 - 4 compared to those of known concentration (lanes 5 and 6). Use of this system revealed concentrations of 28.5 μg/ml for clone 6, 33.0 μg/ml for clone 13 and 19.3 μg/ml for clone 18.

All three clones had been handled in the same way over the same time period. It is assumed that the bands of higher molecular weight are either secreted proteins native to *Pichia* or are an artefact of the process.

Assay results can be seen in table 4.1 and, in agreement with the Gelsys results from the SDS PAGE, show that clone 13 is marginally the most productive of active enzyme and as such merited further investigation to optimise the conditions.

4.3.2 To Counteract the Presence of Endogenous Proteases

As the protein was being secreted, there was a possibility that it was being hydrolysed by neutral pH proteases that were also being produced by the *Pichia* cells. Protein synthesis was then induced by the action of unbuffered minimal media (MM) which provides a slightly basic pH.

As figure 4.4 shows, no NXET was visualised on the SDS PAGE. An overnight digest of xyloglucan solution (1 ml of 12 mg/ml in NH_4CH_3COO buffer, 50 mM, pH 5.0) and 0.2 ml *Pichia*

supernatant gave a result consistent with that of the control (1 ml of 12 mg/ml xyloglucan and 0.2 ml buffer).

No NXET was secreted in this case, however there is some evidence of bands at approximately 87kDa and 76 kDa (lanes 2 and 3) as observed in figure 4.3. If, as previously assumed, these bands are proteins associated with *Pichia,* the normal secretory 'machinery' of the yeast was functioning.

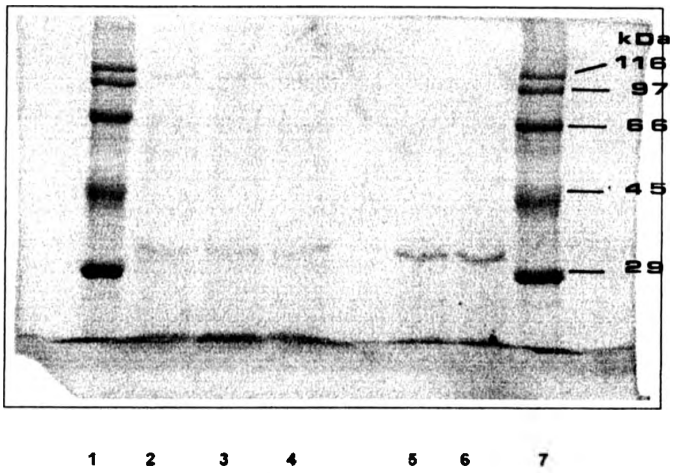


Figure 4.3 SDS PAGE Lane 1+ 7 Molecular weight Marker (Myosin 205000; β-galactosidase 116000; phosphorylase 974000; bovine serum albumin 66000; ovalbumin 45000; carbonic anhydrase 29000). Lane 2 Clone 6. Lane 3 Clone 13. Lane 4 Clone 18. Lane 5 Nasturtium NXET at 90 μg /ml and Lane 6 Nasturtium NXET at 130 μg /ml.

Table 4.1 Activity of NXET produced by clones 6,13, and 18 (measured by viscometric assay in arbitrary units)

Sample	Activity (Arb Units)
Clone 6	5.3
Clone 13	5.9
Clone 18	2.1

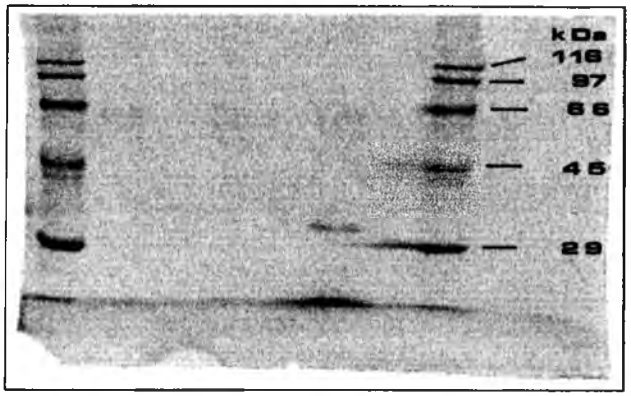


Figure 4.4 SDS PAGE Lane 1+ 5 Molecular weight Marker (Myosin 205000; β -galactosidase 116000; phosphorylase 97400; bovine serum albumin 66000; ovalbumin 45000; carbonic anhydrase 29000). Lane 2 and 3 undilute sample and 1:10 dilution of supernatant resulting from MM 'treatment', respectively. Lane 4 *Pichia* NXET at 90 μg/ml (as determined by Sedmak and Grossberg 1977).

4.3.3 Increasing the Density of the Cells Before Induction and the Addition of 1% Casamino Acids to the Induction Media

Another approach investigated was allowing the cells to achieve a higher density before induction with the methanol containing media containing 1% Casamino acids. In this case the cells were allowed to multiply until an OD of ~9 was obtained before spinning and resuspending in the BMMY. From figure 4.5, the major bands in lanes 2 and 3 corresponds with a molecular weight of 30 kDa and is slightly more intense than previously found. Lane 2 also displays multiple bands of a lower molecular weight than NXET. These bands could be due to the limited breakdown of proteins by a higher rate of cell death caused by the cells growing to a higher density. The presence of other higher molecular weight bands in the lane is consistent with that previously found.

Assay and protein determination results (Table 4.2) confirm that more protein or a protein of higher activity is produced by growing the cells to a higher density.

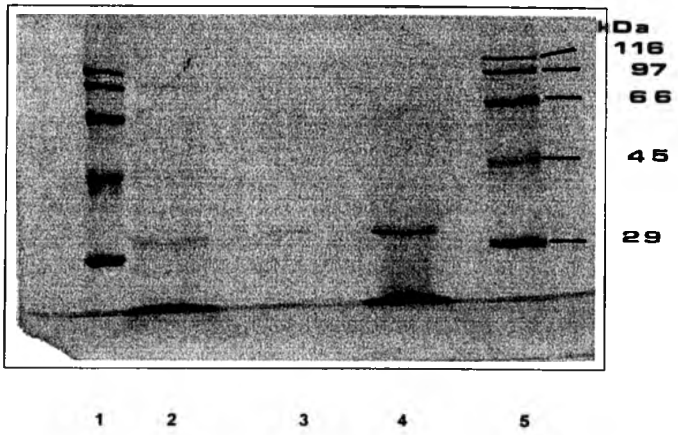


Figure 4.5 SDS PAGE Lane 1+ 5 Molecular weight Marker (Myosin 205000; β -galactosidase 116000; phosphorylase 974000; bovine serum albumin 66000; ovalbumin 45000; carbonic anhydrase 29000). Lane 2 Clone 13 (cells grown to higher density) undilute. Lane 3 As lane 2 but diluted 1:10. Lane 4 *Pichia* NXET at 120 μg/ml (as determined by Sedmak and Grossberg 1977).

Table 4.2 Activity and protein concentration of NXET produced by clone 13 after increasing cell density of growth phase of cells (measured by viscometric method in arbitrary units)

Sample	Activity (Arb units)	Concentration (μg/ml)
Clone 13	5.9	33.0
Clone 13 (HD)[1]	34.6	73.0

[1] (HD) cells allowed to grow to a higher density

4.3.4 Examination of the Cell Pellet

Addressing the possibility of a faulty signal sequence, and therefore unsecreted enzyme was carried out by checking the cell pellet to elucidate the presence of enzyme activity. Assay results showed that no enzyme was being trapped in the cell.

Examination of the cell lysate by SDS PAGE was not applicable in this case as all other native proteins of *Pichia* were present.

4.3.5 Purification of NXET from *Pichia pastoris*

The NXET produced in section 4.3.3 (50 ml of supernatant) was concentrated with ammonium sulphate and dialysed against 50 mM ammonium acetate buffer pH 5.0. The solution was then applied to a Biogel P60 gel filtration column and the elution profile can be seen in figure 4.6. The fractions with the highest protein content (fractions 15 - 23) were tested for NXET activity and those displaying the most activity run on SDS PAGE (figure 4.7). This procedure succeeded in removing the higher molecular weight proteins from fractions 20 - 23, which were assumed to be associated with the yeast itself. As expected the smaller molecular weight bands were still present. After gel filtration, the high activity fractions were pooled and concentrated using 'Vivapore' protein concentrators (Bioscience: final volume 12 ml). The protein concentration was determined as 60 μg/ml.

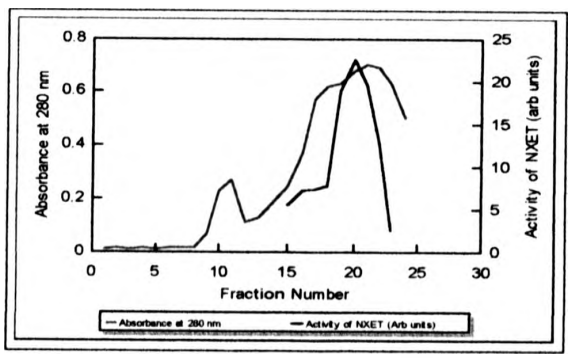


Figure 4.6 Gel filtration profile of *Pichia* produced NXET

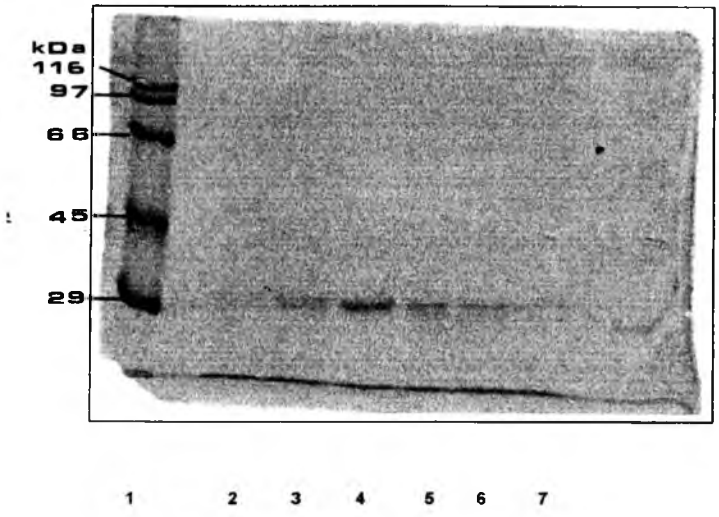


Figure 4.7 SDS PAGE of elution profile of NXET from Biogel P60 gel filtration column. Lane 1 Molecular weight Marker (Myosin 205000; β-galactosidase 116000; phosphorylase 974000; bovine serum albumin 66000; ovalbumin 45000; carbonic anhydrase 29000). Lanes 2 - 7 fractions 18 - 23 .

4.3.6 Action of NXET from *Pichia pastoris* compared to that isolated from Nasturtium.

The relative proportions of the four oligosaccharides, XXXG, XLXG, XXLG and XLLG in the high and low molecular weight xyloglucan digested by NXET from *Pichia* and nasturtium were compared by HPAEC and the results can be seen in Table 4.3. The proportion of free oligosaccharides in the nasturtium digest was distinctly different from that of the endoglucanase digested high molecular weight xyloglucan as was previously found (Fanutti *et al* 1996). The levels of the various oligosaccharides from xyloglucan digested with the *Pichia* produced enzyme was consistent with that found for an equal amount of the nasturtium NXET digest suggesting that the mode of action is the same in each case.

Table 4.3 Oligosaccharide composition of high and low molecular weight xyloglucan as determined by HPAEC

Oligos	Pichia NXET		Nasturtium NXET	
	High Mol Wt	Low Mol Wt	High Mol Wt	Low Mol Wt
XXXG	14.8	19.6	14.4	18.9
XLXG	6.1	11	6.2	11.3
XXLG	29.5	22.5	30.6	23.1
XLLG	49.5	46.9	48.8	46.7

4.4 Discussion

Available data (Invitrogen Expression Manual and references therein) suggests that there is approximately 50 - 70% chance of expressing a protein of interest in *Pichia pastoris* at reasonable levels. The greatest hurdle appears to be generating initial success. This was achieved by Milne (Hons Thesis 1997) who produced three clones from her work that secreted active NXET upon induction. Of the three, clone 13 proved to produce the most concentrated and active enzyme and so was chosen for this limited investigation into the optimisation of *Pichia* protein expression.

The absence of any detectable or non-active NXET after induction in MMY could suggest that the production of protein relies on the presence of buffered media. The methanol was being utilised by the cells as demonstrated by the presence of other bands of higher molecular weight on the gel which were assumed to be native proteins produced by *Pichia*.

A higher concentration of NXET was produced when the cells were allowed to grow to a higher density in the BMGY. This, however, produced more higher molecular weight contaminating bands, the presence which were much reduced by the gel filtration process. The bands of lower molecular weight were thought to be breakdown products of NXET caused by the higher cell density (and consequent higher cell death) and, as expected, were not removed in the purification process. The concentration of protein obtained from this process was higher than that from the original method and the loss of enzyme which occurred during 'handling' was minimal.

Investigation of the contents of the cell pellet showed that the protein produced by the cell had either been secreted, indicating that the secretion signal was functioning, or that it remained intracellularly in a nonactive form.

This limited investigation has concentrated only on the manipulation of the *Pichia* cells at the pre and post induction stages and the subsequent treatment of the protein produced. A further avenue for increasing the levels of expression lies in revisiting the molecular biology involved in producing the clones. Checking for multi-copy recombinants by slot blot or the removal of

the secretion signal so the protein is expressed intracellularly instead of being secreted could be tried.

The *Pichia* expression manual suggested that a yield of 1g/L would not be unusual. The yield obtained from this investigation and that reported by other researchers suggests that this may be the exception rather that the rule. Therefore, whilst providing a simpler method for the production of pure NXET in a laboratory situation, the yield, for equivalent effort, was less than 50% that of produced by extraction of nasturtium seeds (Chapter 3). For commercial reasons this work was taken over by Unilever Research Laboratories for further development.

The similarity observed in the relative proportions of XXXG, XLXG, XXLG and XLLG subunits of the high and low molecular weight fractions of a solution of xyloglucan digested by both nasturtium purified and *Pichia* produced NXET suggests that both have the same mode of transglycolytic and hydrolytic action indicating that these enzymes could be used interchangeably.

Chapter Five

5. Modification of Tamarind Seed Xyloglucan by Action of β-Galactosidase

5.1 Introduction

The hydrolysis reaction to remove galactose from xylose in xyloglucan is catalysed by the enzyme β-galactosidase. Although there are numerous reports of β-galactosidase activities in plant extracts, few have been purified to homogeneity (Li *et al* 1975, Edwards *et al* 1986, Ross *et al* 1993, Kitagawa *et al* 1995). Kitagawa and co-workers reported five β-galactosidase activities from the cell walls of Japanese pear which were separated by hydrophobic and ion-exchange chromatography. All were active, to varying degrees on native cell wall polysaccharides. Several isoforms of the enzyme isolated from ripening kiwi fruit were also reported by Ross *et al* (1993). Similarly, these were found to release galactose from a variety of kiwifruit cell walls polysaccharides although the reported activity of the enzyme *in vitro* was too low to account for the total loss of galactose from cell walls during ripening.

In contrast, the isolation and purification of β-galactosidases from bacteria and yeast have been reported many times (includes Kitamikado 1981, Fukuda 1984, Brady *et al* 1995, Maciunska *et al* 1998). Isolation of a β-galactosidase encoding gene from *Bacillus licheniformis* has recently led to *E. coli* expression , purification and characterisation of the recombinant enzyme (Tran 1998).

Two β-galactosidase activities exist in nasturtium seed and both peak during xyloglucan mobilisation (Edwards *et al* 1986). The major activity reached its maximum immediately prior to the most rapid phase of xyloglucan depletion, the other when mobilisation was almost complete, suggesting that they may have different substrates. The major activity is eluted from the DEAE column during anion-exchange chromatography (see chapter 3) with Tris-HCl buffer pH 7.8. This β-galactosidase is active upon both polymeric xyloglucan and the oligosaccharides generated from this by action of NXET and is a mixture of isoenzymes which

differ in pI but not, detectably, in molecular weight. The other β-galactosidase activity remains bound at this stage of the purification process and is active only on xyloglucan oligosaccharides (Edwards *et al* 1986). The natural substrate of the major activity is suggested to be storage xyloglucan as other commercial preparations of β-galactosidase failed to release galactose from polymeric tamarind seed xyloglucan. It is also significant that the rate of hydrolysis of both tamarind seed, and nasturtium xyloglucan, by the enzyme increases linearly with increasing substrate concentration indicating a 'hydrated solid' is the substrate, reflecting the state of xyloglucan in the cotyledonary cell wall.

β-Galactosidase (source *Aspergillus niger*) digestions carried out on oligosaccharide subunits generated from digestion of polymeric xyloglucan with *endo*glucanase (source *Trichoderma reesei*) have shown selective hydrolysis of the β-D-galactose residues attached to one of the two isomeric octasaccharides present (York *et al* 1993). This observation was also made by Fanutti *et al* (1993) for nasturtium β-galactosidase when it was noted that, in the presence of high salt concentration, the rate of galactose removal from polymeric xyloglucan is dependent on the position of the galactose residue and that the enzyme was preferentially attacking and hydrolysing galactosyl substitutes situated on the less xylosyl-substituted side of the glucan backbone (figure 5.1). This suggested that the structural conformation of xyloglucan in solution was changed to allow easier access to the enzyme in the presence of high salt concentrations. Gelation of tamarind seed xyloglucan on removal of galactose, in the absence of high salt concentration, was also reported at this time confirming reports by Reid *et al* (1988).

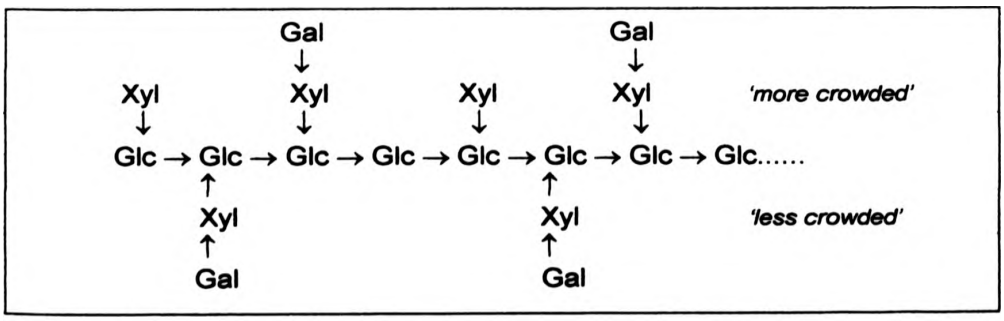


Figure 5.1 Tamarind seed xyloglucan showing 'crowded' and 'less crowded' sides

Recently, tailoring of tamarind seed xyloglucan has been achieved using fungal β-galactosidase (Shirakawa *et al* 1998). It was reported that a temperature-sensitive gel was formed on removal of approximately one third of the galactose. The gel was unique in that it formed only when heated at 40 ° C and reverted to sol when further heated above 80 ° C. This thermo-reversible gelation process has been observed by time-resolved small-angle X-ray scattering (Yamanaka *et al* 1999), who reports the formation of an ordered condensed phase composed of aligned xyloglucan chains in parallel.

The potential for these gels as sustained release vehicles for oral and rectal drug delivery has been reported (Kawasaki *et al* 1999 a. and b.). *In vitro* and *in vivo* studies of administration of aqueous solutions of tamarind seed xyloglucan that has been partially degraded by fungal β-galactosidase (to remove 44% of the galactosyl residues) to form rigid gels, showed sustained release of the drugs indomethicin and diltiazim over 5 hours *in vitro* and 7 hours *in vivo*.

As can be seen, the importance of investigations of this kind impacts on all areas of science, commercial and research, be it food science, pharmaceutical or plant cell wall research. This chapter reports on the action of β-galactosidase from nasturtium seeds on tamarind seed xyloglucan in the absence of Na_2 SO_4, to produce modified xyloglucans for use in the production of xyloglucan/cellulose composites. Also described is a closer investigation into the gelation of tamarind seed xyloglucan when incubated with this enzyme.

5.2 Materials and Methods

5.2.1 Removal of Galactose with Time (small scale)

An investigation of the removal of galactose with time was carried out as described in section 2.2.7.1 of chapter 2. Samples of this static incubation were taken at 0.5, 1, 2, 4, 6, 24 and 48 hours after addition of β-galactosidase. These were boiled for 10 minutes to stop the reaction and analysed for galactose by GDH assay (2.2.2.2.1).

5.2.2 Production of Modified Xyloglucans (large scale)

a. In order to produce sufficient material for use in the construction of the cellulose/xyloglucan composites the initial volumes of xyloglucan and β-galactosidase were scaled up. This was carried out as described in section 2.2.7.2 of chapter 2.

At the desired timepoints the reactions were stopped by boiling for 20 minutes after which small aliquots were taken from each for later analysis. These modified polymers were then freeze-dried. Analysis of the aliquoted samples and the freeze-dried material were carried out by GDH assay (2.2.2.2.1) and oligosaccharide composition determined by HPAEC (2.2.5.1).

b. Production of bulk material was also achieved by the method described in section 2.2.7.2 of chapter 2. After incubation, with constant stirring, for the desired time 30 ml was removed from the reaction vessel, transferred to a sterile container and boiled for 20 minutes. A 1ml aliquot was taken at this point for later analysis.

After a further addition of 200U/ml β-galactosidase the remainder of the reaction mixture was incubated for a further 24 H with constant stirring. After repeating the above procedure to stop the reaction a further 1 ml was removed for analysis. Both samples were analysed by GDH assay (2.2.2.2.1) and by monosaccharide composition as determined by HPAEC (2.2.5.2).

5.2.3 Incubation of Xyloglucan with Nasturtium Seed β-galactosidase at 50° C

An investigation into the formation of 'gel' or network formation with the removal of galactose was carried out as described in section 2.2.7.3

5.3.4 ^{13}C- NMR Spectroscopy of Aggregated Xyloglucan (~60% galactose depleted)

All NMR spectroscopy was carried out as described in section 2.2.12, chapter 2.

5.3 Results

5.3.1 Removal of Galactose with Time (small scale)

Figure 5.2 shows the removal of galactose from Tamarind seed xyloglucan by the action of β-galactosidase purified from nasturtium. The amount of galactose removed was determined by GDH assay.

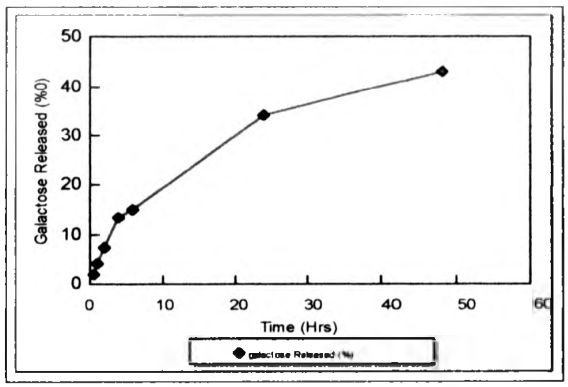


Figure 5.2 Rate of removal of galactose from xyloglucan with time (results the mean of three experiments)

As figure 5.2 shows the rate of reaction slows down after approximately six hours as expected (ie rate of reaction slows down as substrate is used up) but it can also be seen that the enzyme still retains some activity, even after 48 hours incubation. It was noted at this time that the remainder of material in the tube had formed a 'gel' or network.

This timecourse also indicated that in order to isolate modified xyloglucans of ~20% and ~40% galactose depletion, incubation times of 15 and 48 hours, respectively were required.

5.3.2 Production of Modified Xyloglucans (large scale)

a. A larger scale production of modified xyloglucan consequently required larger volumes of starting materials to be used, therefore in order to prevent localised network formation of the kind found in the small scale experiment, the incubations were carried out with constant stirring.

The resulting freeze-dried modified polysaccharides showed that estimates from the timecourse were conservative, with xyloglucans of 26%, 58% and 81% galactose depletion being observed after 15, 24 and 48 hours incubation respectively (as determined by oligosaccharide composition on HPAEC). Results from both the oligosaccharide composition and GDH assay are reported in Table 5.1

Table 5.1 Levels of galactose depletion of isolated xyloglucans as determined by oligosaccharide composition on HPAEC and by GDH assay.

Incubation Time (Hrs)	Galactose Depletion (%)	
	GDH Assay	HPAEC
15	27	26
24	52	56
48	90	81

The higher than expected levels of galactose depletion are thought to be due to the constant stirring which prevented the solution from forming a network. This allowed the enzyme to gain access to a greater surface area of xyloglucan.

From the oligosaccharide compositional data from HPAEC (not shown), the rate of galactose removal from the 'crowded' and 'less crowded' sides of the polymeric xyloglucan molecule were calculated (figure 5.3). As with xyloglucan in the presence of Na_2SO_4 (Fanutti 1993), the galactose from the 'less crowded' side was removed slightly faster than from the 'crowded' side.

Problems arose when trying to re-solubilise the freeze-dried ~50% and ~80% galactose depleted xyloglucans. The lyophilisation process had resulted in a hard 'interwoven' structure for these xyloglucans compared to that of a loose, soft 'cotton wool' structure of the native and 25% galactose depleted xyloglucans. These 'interwoven' structures did not fully break up when attempting to solubilise, even when heated to ~90^0 C with constant stirring. It was therefore decided to abandon this method and use an alternative method for the production of the bulk modified xyloglucans.

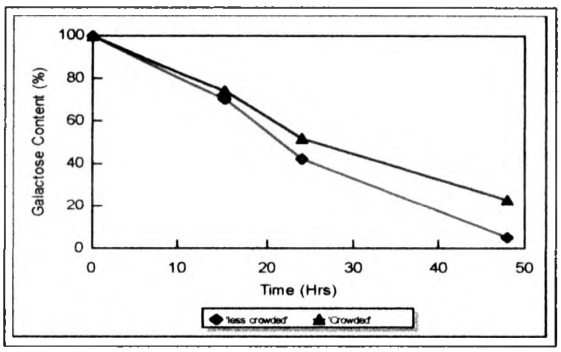


Figure 5.3 Rate of galactose removal from 'crowded' and 'less crowded' sides of the polymeric xyloglucan molecule.

b. The production and isolation of modified xyloglucans, which were then kept in solution until required for composite construction (Chapter six), led to the observation that, as galactose was being removed, the solution was becoming cloudy. On being left to stand a precipitate formed. It was also noted that the volume of precipitate for the 48 hr incubated material was much greater than that of the 24 hr incubated material.

Further investigation of this showed that the volume of precipitate increased as the galactose content of the xyloglucan decreased (figure 5.4) and that the precipitated xyloglucan was consistently approximately 4% more galactose depleted than the soluble xyloglucan (figure 5.5).

Using the method b. above two galactose depleted xyloglucans were isolated. These were of ~30% and ~ 60% galactose depletion (Table 5.2). These solutions had not been freeze-dried but a considerable volume of precipitated xyloglucan was observed in each. It is suggested that

incubation with the enzyme causes a population of xyloglucan molecules with varying degrees of galactose depletion and that the lower galactose content molecules aggregate together and precipitate out of solution.

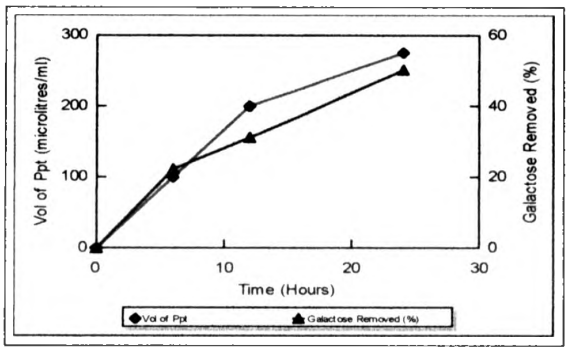


Figure 5.4 The volume of precipitate increases as galactose content decreases.

Table 5.2 Data from GDH assay and monosaccharide analysis of 24 and 48 Hr incubations of Tamarind seed xyloglucan with β -galactosidase.

Incubation Time (Hrs)	Monosaccharide Analysis[1] gal:glu:xyl	GDH Assay (% gal removed)
24	1 : 4.8 : 3.6	32
48	1 : 10.2 : 7.7	60

1 This analysis was carried out on the 'whole' material. The precipitated and soluble xyloglucan were ethanol precipitated and freeze-dried. The material was then analysed for monosaccharide content by H_2SO_4 hydrolysis and HPAEC.

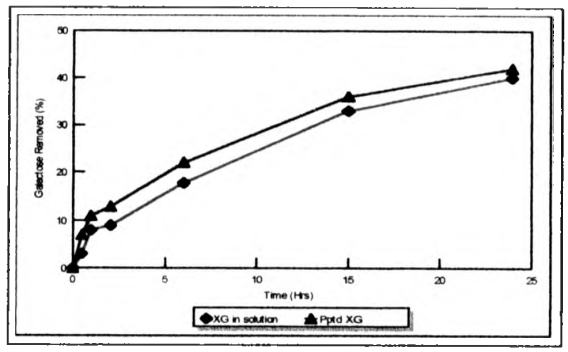


Figure 5.5 Pptd XG consistently showed ~ 4% more galactose removed compared to soluble XG

5.5.3 Incubation of Xyloglucan with Nasturtium Seed β-galactosidase at 50° C

The production of a cloudy 'gel' or network formation was achieved after incubating a 1% solution of Tamarind seed xyloglucan with nasturtium β-galactosidase, statically at 50° C for around 3 hours. When heated to approximately 90° C the network 'broke up' and on cooling did not reform but instead, on standing, separated into precipitated and soluble xyloglucan as had previously been observed. Approximately 40% of the galactose was removed from the xyloglucan for 'gelation' to appear. The solubility of the precipitate was limited as when heated to 100° C only 40% of the material was re-solubilised.

5.3.4 ^{13}C-NMR Spectroscopy of Aggregated Xyloglucan (~60% galactose depleted)

^{13}C-NMR spectroscopy was used to investigate features of molecular structure that could be differentiated on the basis of molecular mobility (Gidley et al 1992). Cross-polarisation Magic Angle Spinning (CP-MAS) is most effective in detecting relatively immobile segments of the

system in question whereas Single Pulse Magic Angle Spinning (SP-MAS) will detect relatively mobile segments.

CP-MAS and SP-MAS spectra of 60% galactose depleted and native xyloglucan are shown in figure 5.6 a and b. Signals at 105 - 106, 103 - 104, and 99 - 100 ppm (all spectra) are assigned to the C-1 of xyloglucan galactosyl, glucosyl and xylosyl residues, respectively (Gidley *et al* 1991).

Comparison of the SP-MAS spectra of the native and galactose depleted xyloglucan show a significant reduction in intensity of the C1 gal and the C1 glc signals at 105 - 106 and 103 - 104 ppm respectively. While the reduction in intensity of the C1 gal reflects the lowered level of galactose residues present in the depleted xyloglucan, the reduced intensity of C1 glc suggests that the glucose is in a relatively less mobile state than that found in native xyloglucan.

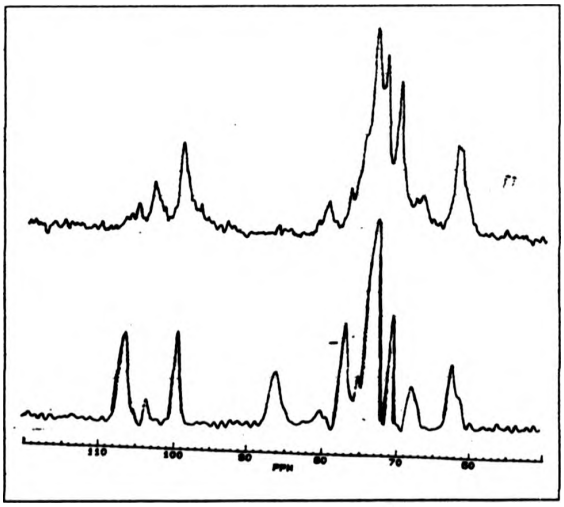


Figure 5.6a SP-MAS (top) and Resolution Enhanced CP-MAS for 60% galactose depleted xyloglucan

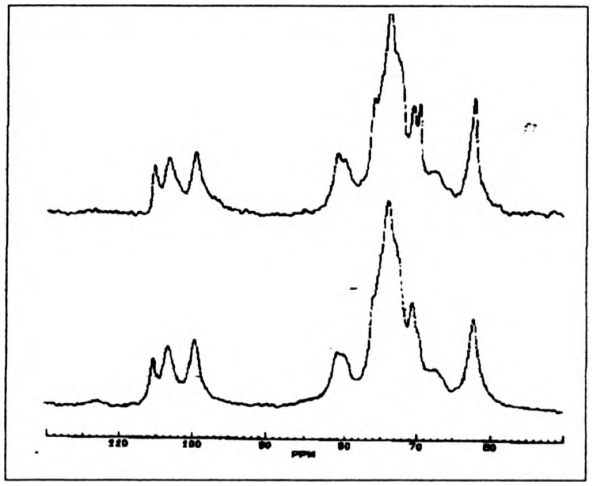


Figure 5.6b SP-MAS (top) and CP-MAS for native Tamarind seed xyloglucan (30% solution).

This is associated with an increase in intensity of the signal for C1 xyl at 99 - 100 ppm. Analysis of the C4 region of the spectra shows that this is essentially identical in both cases. Comparison of the CP-MAS spectra of the two materials show a significant reduction in the intensity of the C1 glc signal at 103 - 104 ppm with an attendant enhancement of the signals at 105 - 106 for C1 gal and at 99 - 100 ppm for C1 xyl. Examination of the C4 region of the spectra shows the appearance of a signal at 84 - 87 ppm in the CP-MAS of the galactose depleted xyloglucan which is not present in either the SP-MAS of this material or the spectra of the native material.

The appearance of the C4 signal at 84 - 87 ppm in the spectra is coincident with that of non-crystalline cellulose (Vanderhart and Atalla 1984 and Isogai *et al* 1989). The altered chemical shift of the C1 glc so that it lies in the same position as the cellulose C1 mirrors the behaviour of the xyloglucan backbone when associated with cellulose (Whitney *et al* 1995). The intensity of the signals at 99 - 100 ppm in both the SP-MAS and CP-MAS spectra of the galactose depleted xyloglucan reflect the relative mobility of the xylosyl residue compared to that of the glucosyl residue which is now fixed within the xyloglucan aggregate.

These data suggest that the lower galactose content molecules self-aggregate by way of the glucan backbone and in doing so change from a 'twisted' formation (found in solution) to a 'cellulosic' formation (found when xyloglucan is associated with cellulose). The absence of any fine peaks at C1 glc (105 - 106 ppm) and of any signal at 89 - 91 ppm on the resolution enhanced spectra for the galactose depleted material suggests that no crystalline features are present.

5.4 Discussion

Tamarind seed xyloglucan formed a gel after 48 hours incubation with nasturtium β-galactosidase. The rate of removal of galactose was, by that time, significantly slower than the initial rate. Formation of the 'gel' network may have played a part in the reduction in reaction rate as the enzyme would not have been free to move around the polysaccharide chain. There is also the consideration that as the chains aligned and aggregated together, there would have been less surface area, and so less galactose residues, upon which the enzyme could act. The introduction of constant stirring into the large scale production of modified xyloglucans gave an unexpected rise in the percentage galactose removal. The network was never allowed to form and as a result aggregated material precipitated out of solution leaving the remaining, higher galactose content molecules, in solution available for hydrolysis.

The faster removal of galactose residues from the 'less crowded' side of the polysaccharide molecule was as expected as there would be less steric hindrance from xylose. It is noted, however, that this difference occurs more noticeably after approximately 15 hours incubation (figure 5.3). At this time there would have been a considerable volume of precipitated xyloglucan (over 200 µl precipitate per ml, figure 5.4), leaving a lower concentration remaining in solution. Higher concentrations of xyloglucan in solution cause gelation, and as such, they are possibly less accessible to the enzyme. This lower concentration could result in more freedom of access by the enzyme to the sites of hydrolysis.

The increased rate of hydrolysis of xyloglucan with β-galactosidase in the presence of 1M Na_2SO_4 is ascribed to the report that the salt 'holds' the polymer in the 'twisted' conformation by increasing the hydrophobic interactions between molecules. This is also the conformation observed when xyloglucan is in solution (Gidley *et al* 1991).

The comparison of the C1 and C4 regions of the CP-MAS spectra of the 60% galactose depleted xyloglucan and cellulose (Isogai *et al* 1989) suggests that the xyloglucan backbone in the aggregated material has similar molecular organisation to non-crystalline cellulose. It is also suggested that the modified xyloglucan backbone is prevented from achieving the tightly

organised packing required for crystallinity by the presence of the xylosyl side-chains (Brooks *et al* unpublished data).

Recent renewed interest in the gelling properties of Tamarind seed xyloglucan, from the aspect of food science and pharmaceuticals, has added to a greater understanding of the events occurring for such a transition to take place. If this xyloglucan is solubilised to a sufficient concentration (>3%) it will form a 'gel' in the absence of Na_2SO_4 and with no removal of galactose. It is however the thermo-reversibility of the gels produced on removal of ~ 40% of the galactose residues by fungal β-galactosidase which renders them remarkable (Shirakawa *et al* 1998; Kawasaki *et al* 1999 a. and b.).

The 'gel network' resulting from hydrolysis of xyloglucan with nasturtium β-galactosidase referred to in this chapter is not to be confused with the 'rigid' and 'handlable' (robust) gels obtained from the work of the above scientists. The nasturtium enzyme induced network had none of the robust qualities of the fungal enzyme induced gel. On disturbing the 'network' of the nasturtium enzyme hydrolysed xyloglucan, aggregated material precipitated out of solution. In both cases, though, an overall galactose depletion of ~40% was observed before 'gelation' or 'networking' took place. The nasturtium β-galactosidase was of a much higher concentration than the fungal enzyme of Shirakawa *et al* (1998) and this may have produced a faster rate of reaction with a polydisperse population of lower galactose content molecules aggregating out of solution rather than a slower reaction resulting in a more homogenous array of molecules.

It is curious to note that galactomannans, like xyloglucans, undergo conformational change in the presence of cellulose (Whitney *et al* 1998). This 'cellulosic' conformation can also be adopted at less than 10% galactose substitution where it occurs in xyloglucan when the glucan backbone is 75% xylose substituted and has some galactose present.

The results reported in this chapter and elsewhere indicate important implications for the functionality of the galactose side chains on xyloglucan. After synthesis in the Golgi apparatus of the cell the soluble polysaccharide is transported, in vesicles, to the plasma membrane where it is available at, or very close to the site of cellulose synthesis. If the galactose residues were not present on the xyloglucan it is very likely that these molecules would

self-aggregate and precipitate out of solution and would therefore be unavailable for association with cellulose.

Chapter Six

6. Interaction of Galactose Depleted Xyloglucan with Cellulose Produced by *Acetobacter xylinus*

6.1 Introduction

The primary cell wall in most dicotyledonous and non-graminaceous monocotyledonous plants has been proposed to be made up of a tightly associated network of cellulose and xyloglucan, embedded in a pectic matrix (McCann and Roberts 1991, Talbot and Ray 1992a, Carpita and Gibaut 1993). Elucidating the nature of the cellulose/xyloglucan interaction has met with limited success. Hayashi (1987), Acebes *et al* (1993) and Baba *et al* (1994) have demonstrated that xyloglucan/cellulose complexes can be produced from component polymers *in vitro* but that the level of incorporation of xyloglucan is much lower than is observed in the cell wall. Recently Hayashi (1994b) identified that a minimum of five consecutive glucosyl residues in a xyloglucan fragment was required for binding to cellulose to occur. The extent of binding was found to increase with increasing glucosyl units (up to dp 12). However, Vincken *et al* (1995) found that , relative to polymeric xyloglucan, adsorption of residues of four glucosyl residues was high suggesting that smaller molecules may more efficiently colonise the small pores in cellulose.

Computer simulations and molecular modelling have predicted the involvement of specific xyloglucan side-chains in the association with cellulose. Levy *et al* (1991, 1997) reported that the fucosylated side-chains caused flattening of the xyloglucan backbone, forcing it into a cellulosic conformation. Hydrogen bonding to cellulose via the mobile xylose residues on the sterically hindered glucan chain has also been suggested (Finkenstadt *et al* 1995) whereby the xylose residues intercalate into the crystal lattice spaces in the cellulose surface. These reports have been questioned by Whitney *et al* (1995) using an experimental approach. A novel model system developed by Whitney and co-workers utilises the Gram negative

bacterium *Acetobacter xylinus* which extrudes microfibrils of cellulose I into medium containing xyloglucan resulting in a cellulose/xyloglucan matrix.

As discussed in chapter one, *A xylinus* is an obligate aerobe (Brown 1976) which synthesises a highly crystalline cellulose I. This is extruded as a twisting ribbon parallel to the cells surface at the media/air interface (Hestrin and Schramm 1954). Fasciation of the cellulose microfibril (3 - 4 nm wide) into ribbons 40 - 60 nm wide can be perturbed by fluorescent brighteners, (Haigler 1980, Kai *et al* 1994), cellulose derivatives such as carboxymethylcellulose, (Ben-Hayim and Ohad 1965, Haigler *et al* 1982) and xyloglucan (Hayashi *et al* 1987, Atalla *et al* 1993, Yamamoto and Horii 1994). *A xylinus* has been demonstrated as a useful system in which to examine cellulose/xyloglucan interactions when xyloglucan is added at the point of cellulose synthesis. When grown statically, the bacterium produces a thick pellicle comprising a mass of continually layered cellulose fibres which supports a growing population of cells at the surface of the culture medium.

Two crystalline suballomorphs of cellulose I, designated $I\alpha$ and $I\beta$, have been proposed (Atalla and Vanderhart 1984, Vanderhart and Atalla 1984) and studies of cellulose from various sources showed that all native celluloses can be classified into two groups (Horii *et al* 1987). Cellulose synthesised be *A xylinus* is rich in the $I\alpha$ component and belongs to the *Valonia* family whereas the cotton-ramie family, which is rich in $I\beta$, includes most higher plant cellulose. The almost total conversion of $I\alpha$ to $I\beta$ can be affected by annealing at high temperatures with saturated steam (Horii *et al* 1997), treating with aqueous solutions (Yamamoto *et al* 1989, Sugiyama *et al* 1990 and 1991b) or culturing *A xylinus* at low temperatures (10 - 36° C, Yamamoto and Horii 1994).

First proposed by Haigler *et al* (1982), use of the *A xylinus* bacterium as a model for studying the cellulose/xyloglucan interaction evolved with the discovery of the agitation tolerant strain ATCC 53524 (Atalla 1993). Development of this system by Whitney and co-workers (1995), demonstrated that the presence of high molecular weight xyloglucan at the point of cellulose synthesis conferred a lateral order to the cellulose similar to that seen for native cellulose/xyloglucan complexes (McCann 1990, McCann and Roberts 1991). Cross-linkages of similar size to that observed in the extracellular matrix were also seen (McCann 1990). The

presence of xyloglucan also decreased (by over half) the Iα component of crystallinity and almost trebled the non-crystalline component. NMR interpretation of the chemical shift of glucosyl C-1 of cellulose-bound xyloglucan (compared to unbound) suggested that xyloglucan is bound to cellulose via its glucan backbone and provided evidence for a transition from 'twisted' to 'flat' conformation upon binding to cellulose. the level of incorporation is comparable to that found in plant cell walls (Regwell and Selvendran 1986, Talbot and Ray 1992) and it is only in the presence of xyloglucan that cellulose order is similar to that found in the cell wall.

The observation that it is the conformation of the xyloglucan itself and not side-chain influence that determines the binding of xyloglucan to cellulose has aroused speculation on the role of xyloglucan-specific enzymes that exist in the cell wall (Whitney 1995); although the chemical basis for the modification is established, the precise function of these enzynes in the cell wall and subsequent effects on the cellulose/xyloglucan interactions has still to be elucidated, This chapter reports the incorporation of specifically tailored xyloglucans into the *A xylinus* system and the characterisation of the molecular, ultrastructural and mechanical changes that result in comparison to the native cellulose/xyloglucan composites.

6.2 Materials and Methods

6.2.1 Bacterial Cultures

Bacterial inoculum was obtained from actively cellulose-synthesising *A xylinus* as described in Materials and Methods (2.2.8.1, 2.2.8.2, and 2.2.9a). incubation was performed statically at 30° C in Hestrin-Schramm media pH 6.2 containing 0.5% native or modified xyloglucan.
After 48 H the resultant pellicle was aseptically removed and washed extensively in sterile deionised water at room temperature.

6.2.2 Monosaccharide Analysis of Composite Material

Monosaccharide analysis was performed on the freeze-dried material as described in section 2.2.6 of Materials and Methods.

6.2.3 Removal of Loosely Bound Xyloglucan by Agitation

Monosaccharide and oligosaccharide analysis (sections 2.2.6 and 2.2.5 respectively) was carried out on the residual material and the xyloglucan that was removed during overnight agitation (section 2.2.10.3).

6.2.4 Sequential Extraction of Xyloglucan from Composites Using KOH

Sequential extraction of xyloglucan from the composites with increasing concentrations of KOH was carried out as described in section 2.2.10.1. For all samples, oligosaccharide analysis of

extracted material was determined by overnight digestion with endoglucanase (source *Trichoderma reesie*; Megazyme Ltd) and HPAEC (section 2.2.5). Monosaccharide analysis by HPAEC of the freeze-dried residual material was carried out after acid hydrolysis (section 2.2.6).

6.2.5 Enzymatic Digestion of Cellulose/Xyloglucan Composites

Approximately 10mg (wet weight) was weighed into an Eppendorf tube containing 0.5ml sodium acetate buffer (pH 5.0, 50 mM). Samples were incubated overnight with 0.45U endoglucanase (Megazyme Ltd) at 30° C with constant agitation. After incubation the enzyme was denatured in a boiling water bath for 15 minutes.

Residual material was collected by centrifugation (Microcentaur, 10 minutes) and freeze-dried for monosaccharide analysis (section 2.2.6). The oligosaccharide composition was analysed by direct injection into the HPAEC using the gradient described in section 2.2.5.

6.2.6 Incubation of Composites with β-Galactosidase

Further removal of galactose from the constructed composites was carried out as described in section 2.2.10.5. Monosaccharide analysis was performed on the residual material after freeze-drying according to section 2.2.6.

6.2.7 Deep-Etch Freeze-Fracture Transmission Electron Microscopy

All microscopy was carried out as described in Materials and Method (chapter two, section 2.2.12)

6.2.8 ^{13}C NMR

All NMR spectroscopy was carried out as described in Materials and Methods, chapter two section 2.2.13.

6.2.9 Uniaxial Tensile Testing

All uniaxial tensile testing was carried out as described in Materials and Methods, chapter 2, section 2.2.11.

6.3 Results

After 48 H static incubation at 30° C the pellicles resulting from composite construction with native polymeric tamarind xyloglucan and 30% and 60% galactose depleted xyloglucan were aseptically removed and washed extensively in sterile deionised water.

6.3.1 Levels of Xyloglucan Incorporation

The levels of incorporation of control and modified xyloglucans (native tamarind xyloglucan; 3.2 : 2.4 : 1, 30% galactose depleted xyloglucan; 4.8 : 3.6 : 1 and 60% galactose depleted xyloglucan; 10.2 : 7.7 : 1 for glc : xyl : gal respectively, see Table 5.2, chapter 5) into the composites are shown in Table 6.1. Xylose was used as an internal marker for xyloglucan. The data for the native unmodified xyloglucan/cellulose composite are in close agreement with those previously reported (Whitney *et al* 1995, 1 : 0.38 when culture flasks were agitated; Hackney *et al* 1994, 1 : 0.43 for a similar system to that of Whitney and co-workers and Hayashi and McLachlan 1984, 1 : 0.7 in pea stem primary walls).

With removal of 30% and 60% galactose from the xyloglucan the cellulose : xyloglucan ratio increased suggesting that either more xyloglucan had associated with the cellulose or that the xyloglucan had self - associated.

Table 6.1 Initial Cellulose : Xyloglucan Ratios; obtained from monosaccharide analysis of the composites using sulphuric acid hydrolysis and HPAEC

Composite	Cell : XG ratio
Cellulose : native XG	1 : 0.50 ± 0.06
Cellulose : 30% galactose-depleted XG	1 : 0.61 ± 0.02
Cellulose : 60% galactose-depleted XG	1 : 0.98 ± 0.15

6.3.2 Removal of Xyloglucan by Agitation Only

Overnight agitation removed approximately 4%, 28%, and 60% of the native, 30% galactose-depleted and 60% galactose depleted xyloglucan (respectively) initially trapped in the composites (Table 6.2) indicating that as galactose is removed, the xyloglucan becomes very loosely associated with the cellulose. This is probably because more xyloglucan is binding to itself as reported in chapter 5 and so less is available to associated with cellulose.

Table 6.2 Cellulose : Xyloglucan Ratios after Agitation; obtained from monosaccharide analysis of the composites using sulphuric acid hydrolysis and HPAEC (results are the mean of three experiments)

Composite	Cell : XG ratio
Cellulose : native XG	1 : 0.48
Cellulose : 30% galactose-depleted XG	1 : 0.44
Cellulose : 60% galactose-depleted XG	1 : 0.39

6.3.3 Sequential Extraction of Xyloglucan from Composites

Treatment of native and galactose depleted cellulose / xyloglucan pellicles with increasing concentrations of KOH showed a progressive reduction in the xyloglucan content as determined by monosaccharide analysis (Table 6.3). Approximately 50% of the native xyloglucan is removed by relatively mild alkali which is consistent with that previously found (Whitney 1996). The 30% galactose depleted xyloglucan / cellulose composite also had 50% of the xyloglucan removed by 0.5M KOH and confirms the suggestion that both native and 30% galactose depleted xyloglucans are either loosely associated to the cellulose moiety of the pellicle or are present as a surface coating and/or crosslinks.

Table 6.3 Cellulose : Xyloglucan Ratios after Sequential Extraction of Xyloglucan with KOH; using acid hydrolysis and HPAEC (results the mean of three experiments)

Alkali Treatment	Cellulose : Xyloglucan Ratio of Composites Remaining		
	Native	30%	60%
0.5 M KOH x2	1 : 0.24	1 : 0.32	1 : 0.11
1.0 M KOH x2	1 : 0.16	1 : 0.25	1 : 0
4.0 M KOH x1	1 : 0	1 : 0	1 : 0
4.0 M KOH x2	1 : 0	1 : 0	1 : 0

Interestingly, only 11% of the initially associated xyloglucan of the 60% galactose depleted XG/cellulose composite remained associated after addition of 0.5M KOH. Moreover, the xyloglucan was removed completely from this composite by treatment of 1.0M KOH. Microfibrillar swelling such as that achieved by addition of 4M KOH was not required to remove all of the XG indicating that no intrafibrillar association takes place when cellulose is synthesised in its presence. This suggest that either galactose is important in the binding of XG to cellulose or as previously mentioned, the galactose depleted xyloglucan is self-associating. The requirement for the addition of 4M KOH to completely remove the native and 30% galactose depleted xyloglucan from cellulose infers that intrafibrillar association similar to that seen in primary cell walls (Chambat *et al* 1984, Edelman and Fry 1992, Hayashi and McLachlan 1984).

NMR spectroscopy has shown perturbed molecular organisation of cellulose in the presence of native xyloglucan which implies intimate intermolecular association between xyloglucan and cellulose. The above results suggested that a similar association might be detected by NMR on the 30% galactose depleted composites.

The oligomeric composition of the xyloglucan extracted from the composites by increasing concentrations of alkali were determined by *endo*glucanase digestion and HPAEC. No

structural or significant galactose content differences were observed in the extracted XG compared to that before incorporation (figure 6.2). This indicated that galactose was not necessary for the association of XG with cellulose as, if that were the case, it would have been expected that the more stringent alkali treatment would result in the removal of XG with a higher galactose content.

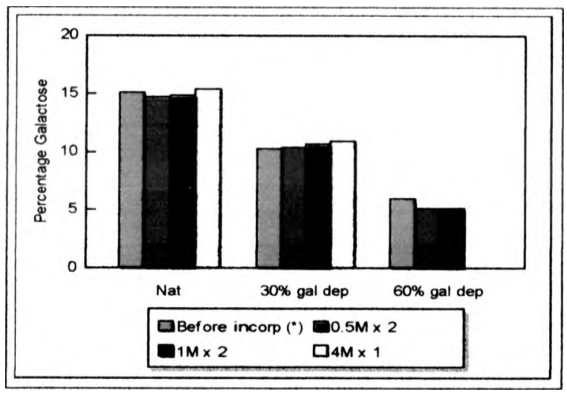


Figure 6.1 Galactose content of XG removed from composites by KOH
(*) figure calculated from GDH assay as lower galactose content XG had precipitated out of solution. See chapter 5 (results the mean of three experiments).

6.3.4 Enzymatic Digestion of Xyloglucan / Cellulose composites

Monosaccharide analysis of the composites after digestion by *endo*glucanase (Table 6.4) showed that consistent levels of XG remained associated in the composites made from native and 30% galactose depleted XG but that more was removed from 60% galactose depleted XG/cellulose composite. Since it was assumed that *endo*glucanase would digest only that xyloglucan which was loosely associated to, or was involved in the cross-linkages between the microfibrils, that remaining associated in each case possible represented a more intimately associated XG which was not accessible to the enzyme.

The levels of removal of xyloglucan from the native XG/cellulose composite was similar to that previously found (Whitney *et al* 1996) and is equal to that removed by 1M KOH, which is not strong enough to cause intrafibrillar swelling (Chambat *et al* 1984, Edelman and Fry 1992, Hayashi and McLachlan 1984).

The level of xyloglucan remaining in the 60% galactose depleted XG/cellulose composite after digestion with the enzyme is consistent with that remaining after treatment with 0.5M KOH again confirming that this XG is only very loosely associated with cellulose component.

Table 6.4 Cellulose : Xyloglucan Ratios of Composites after action of Endoglucanase. Obtained using acid hydrolysis followed by HPAEC. (Results the mean of three experiments)

Composite	Cell : XG ratio
Cellulose : native XG	1 : 0.17
Cellulose : 30% galactose-depleted XG	1 : 0.16
Cellulose : 60% galactose-depleted XG	1 : 0.12

The oligosaccharide composition of the xyloglucan removed from the composites again reflected that which was added to the *A xylinus* system (figure 6.2). Attempts to remove the residual xyloglucan from the composite material left after *endo*glucanase digestion by overnight incubation of the freeze-dried residue with 4M KOH and subsequent hydrolysis by a further aliquot of *endo*glucanase, resulted in no oligosaccharides being detected. Later acid hydrolysis and monosaccharide analysis by HPAEC showed xyloglucan still associated with cellulose. It is possible that the freeze-dried material was not susceptible to the microfibrillar swelling action of the strong alkali but due to lack of material this experiment could not be repeated.

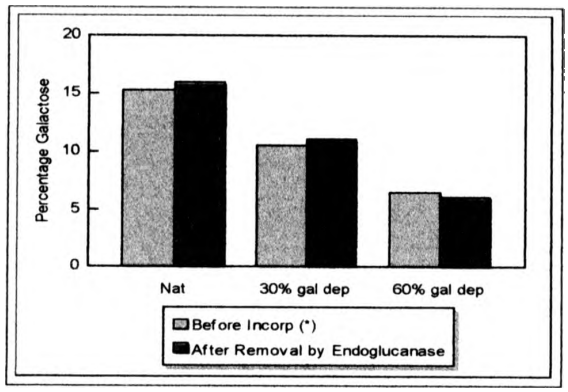


Figure 6.2 Galactose content of xyloglucan removed by action of endoglucanase (*) figure calculated from GDH assay as lower galactose content XG had precipitated out of solution (see chapter 5, results the mean of three experiments).

6.3.5 Incubation of Composite Material with β - Galactosidase

In order to further investigate the role of galactose in associating xyloglucan with cellulose and working on the premise that if galactose were involved in the association, it would not be available to be removed from the composite, the composites were incubated with β-galactosidase. This resulted in the removal of galactose (data not shown) but it was unclear whether it was being removed from the xyloglucan which was dissociated during shaking and was then free in solution to be acted upon by the enzyme.

It was then decided to incubate only the residual material after endoglucanase digestion (only tightly associated XG was thought to remain) with β-galactosidase. Unfortunately the results from this experiment were also inconclusive. The monosaccharide results did reflect lower galactose levels for all three composites after incubation with β-galactosidase (data not shown) but as the initial level of xyloglucan was very low (table 6.3) no conclusion can be drawn.

6.3.6 Deep-Etch Freeze-Fracture Transmission Electron Microscope

Micrographs of tungsten/tantalum/carbon replicas of composites produced by fermentation of *A xylinus* in the presence of native, 30% galactose depleted and 60% galactose depleted xyloglucan are seen in figures 6.3 a and b, 6.4 a and b, and 6.5 a and b respectively.

Figure 6.3 a shows the lateral order achieved by the cellulose when grown in the presence of xyloglucan and the cross-linking that occurs between the cellulose microfibrils. This is consistent with that previously found (Whitney *et al* 1995) and with the role proposed for the network in primary cell wall models (Carpita and Gibaut 1993, McCann and Roberts 1991, Talbot and Ray 1992a).

There is substantial heterogeneity of microstructure across the pellicles and 6.3 b, 6.4 a and 6.5 a all show other areas of the composites which demonstrate XG/XG networks in both the native and modified xyloglucans. It is likely that this XG/XG networking, not observed previously, is a result of the different methods used to prepare the xyloglucan initially. The preparation method cited in this report differs from that of Whitney *et al* 1995 with respect to a 20000 rpm (Sorvall RC5C centrifuge, SS-34 rotor) spin carried out by these authors which was not done at the time of this work. The failure to carry out this spin could result in 'clumps' of xyloglucan, which had not properly solubilised/dispersed during solubilisation, being present in the reaction mixture.

Although Figure 6.4 b again shows areas where alignment and cross-linking of cellulose occurs this appears to be to a lesser extent that that observed for the native XG/cellulose composite. The minimal interaction observed in figure 6.5 b, where a random arrangement of cellulose microfibrils can be seen indicates that the lower the galactose content of the xyloglucan the lower the degree of interaction between it and the synthesising cellulose. Removal of approximately one third of the galactose from xyloglucan only slightly reduces the ability of the XG to cross-link and align the cellulose. In contrast, however, removal of two thirds galactose results in a xyloglucan that has little or no interaction with cellulose indicating that the xyloglucan has networked together to such an extent as to be unavailable to interact with the synthesising cellulose.

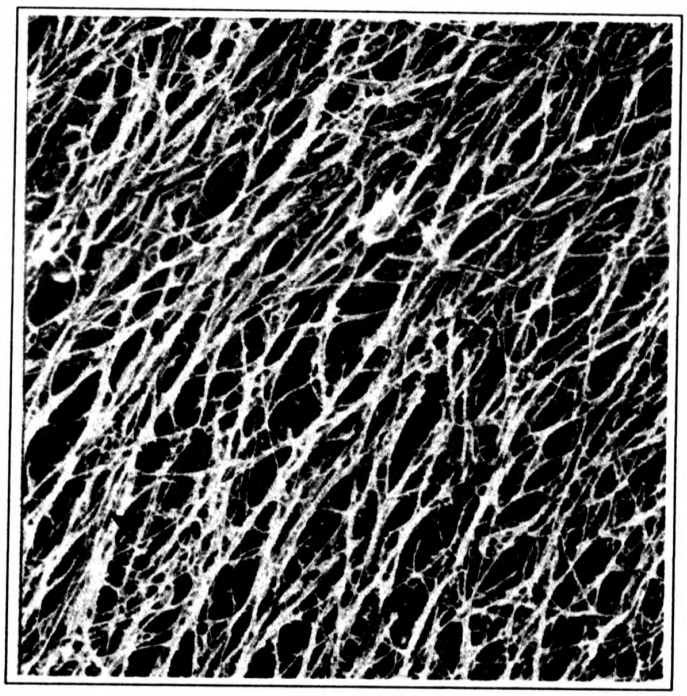


Figure 6.3 a Micrograph of tungstan/tantalum/carbon replica of composite composed by fermentation of *A xylinus* in the presence of native high molecular weight xyloglucan. Areas of the composite illustrate the abundant cross-linking (arrows) and overall lateral alignment of the cellulose ribbons.

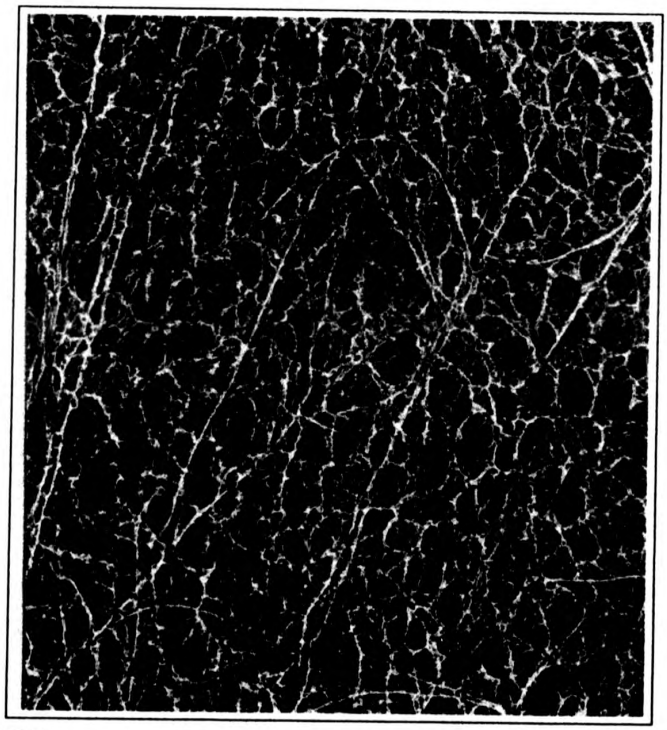
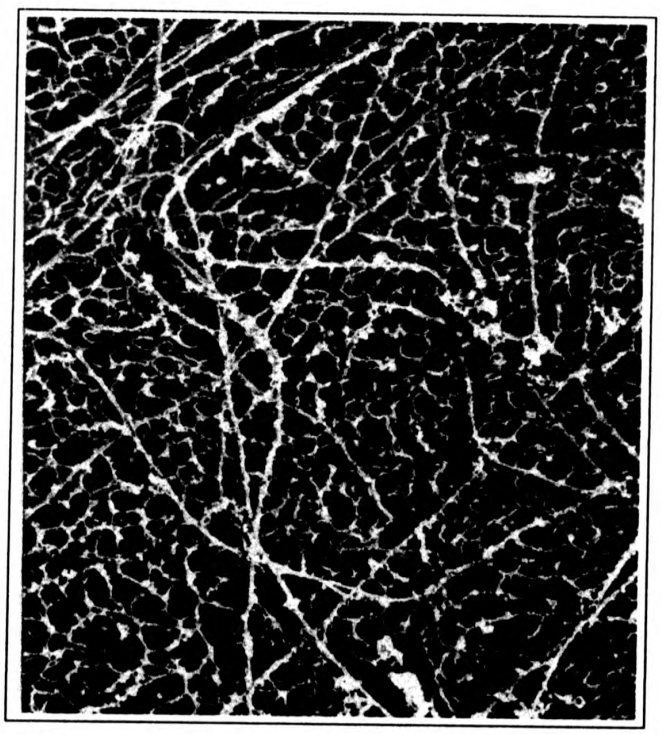
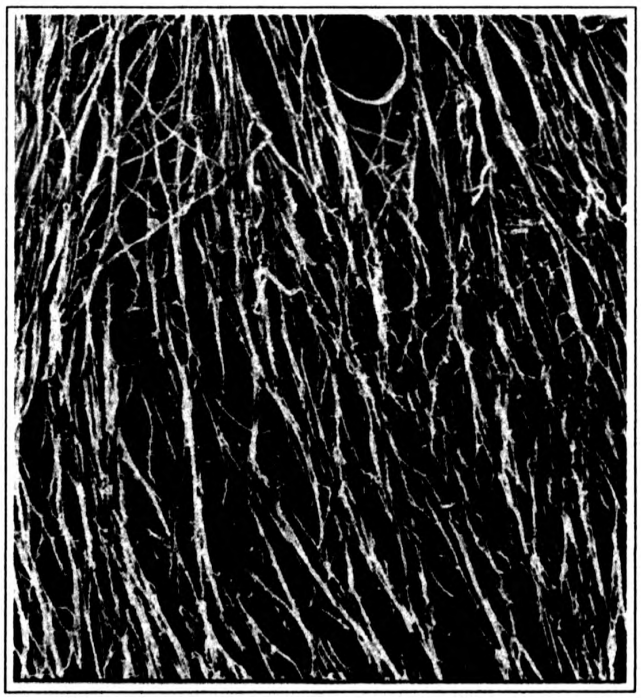


Figure 6.3 b Micrograph of tungstan/tantalum/carbon replica of composite composed by fermentation of *A xylinus* in the presence of native high molecular weight xyloglucan as shown in figure 6.3a. Other areas of the composite show evidence of xyloglucan/ xyloglucan networks. These networks were caused by localised 'clumping' together of xyloglucan which was not removed by centrifugation.



X 20000 ——— 0.5μm

Figure 6.4 a Micrograph of tungstan/tantalum/carbon replica of composite composed by fermentation of *A xylinus* in the presence of 30% galactose-depleted xyloglucan. Areas of this composite also show evidence of xyloglucan/ xyloglucan networks caused by localised aggregations of xyloglucan which would not be available to associate with cellulose.



X 20000 ——— 0.6μm

Figure 6.4 b Micrograph of tungstan/tantalum/carbon replica of composite composed by fermentation of *A xylinus* in the presence of 30% galactose-depleted xyloglucan illustrates that the removal of one third of the galactose content results in a xyloglucan which will, as found previously in figure 6.3 a, cross-link (arrows) and confer lateral alignment on the cellulose.

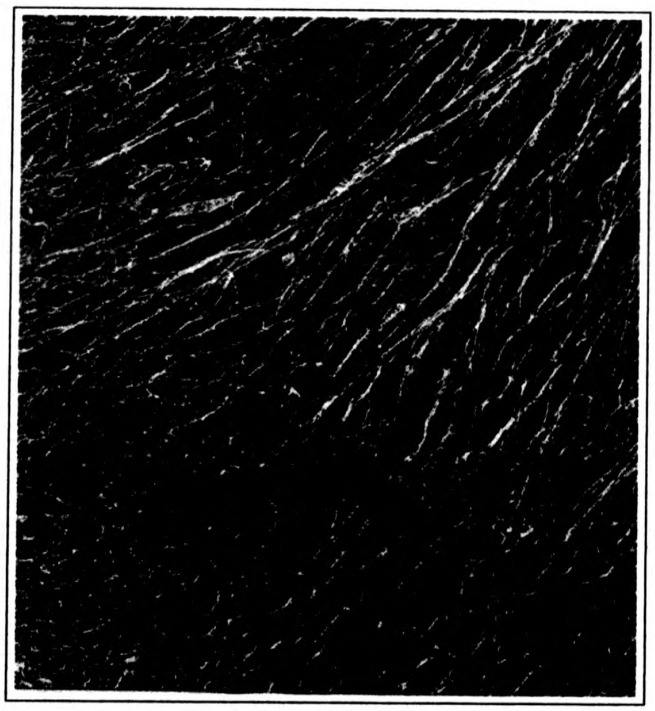


Figure 6.5a Micrograph of tungstan/tantalum/carbon replica of composite composed by fermentation of *A xylinus* in the presence of 60% galactose -depleted xyloglucan again indicate areas of xyloglucan/xyloglucan networks. In this case it is suggested that the removal of galactose has caused the xyloglucan to aggregate and so render it unavailable to associate with cellulose.

Figure 6.5 b Micrograph of tungstan/tantalum/carbon replica of composite composed by fermentation of *A xylinus* in the presence of 60% galactose-depleted xyloglucan illustrated that this xyloglucan has minimal interaction with cellulose. In this case it is suggested that the removal of galactose has caused the xyloglucan to aggregate and so render it unavailable to associate with cellulose.

6.3.7 ^{13}C NMR

^{13}C NMR spectroscopy was used to investigate features of molecular structure that could be differentiated on the basis of molecular mobility (Gidley *et al* 1992). Cross-polarisation Magic Angle Spinning (CP-MAS) detects relatively immobile segments. Where molecular mobility is greater, however, Single Pulse Magic Angle Spinning (SP-MAS) was used.

CP-MAS and SP-MAS spectra of *A xylinus* produced in the presence of native tamarind seed xyloglucan are shown in figure 6.7. Comparison of these to that of cellulose and xyloglucan alone (figure 6.6) show that signals at ca. 99 - 100 ppm and 80 ppm are additional to those found in cellulose alone but appear in the spectrum of xyloglucan (Gidley *et al* 1991, Whitney *et al* 1995). The signal at 105 ppm is coincident with those of glucose and galactose of xyloglucan and of glucose of cellulose (Gidley *et al* 1991).

CP-MAS and SP-MAS spectra of *A xylinus* produced in the presence of 30% galactose depleted xyloglucan is shown in figure 6.8. The appearance of signals at 99 - 100 ppm and 80 - 85 ppm in the CP-MAS spectrum are consistent with that previously found for cellulose produced in native tamarind seed xyloglucan. The signal at 105 ppm again coinciding with that for cellulose C1 / glucose / galactose.

Spectra of native and 30% galactose-depleted xyloglucan in cellulose-based composites are essentially identical. However integration of the two signals at 105 ppm and 99 - 100 ppm show a ratio for cellulose : xyloglucan of 1 : 0.34. As the monosaccharide analysis gave a ratio of 1 : 0.61, only around 50% of the xyloglucan has been detected in the CP-MAS. This contrasts with the native composite where the vast majority was associated with cellulose. In contrast, SP-MAS produced a spectrum which was not significantly different to that of native xyloglucan in solution. Also, signals at 105, 103 and 99.5 ppm correspond to galactose, glucose and xylose respectively and the absence of C1 for cellulose at 105 ppm shown that this xyloglucan was not associated or only very loosely associated with cellulose.

A xylinus cellulose produced in the presence of native tamarind seed xyloglucan undergoes a change in the degree of crystallinity implying an intimate relationship between cellulose and

xyloglucan. From signals at 88 - 91 ppm and 84 - 86 ppm it is estimated that the crystalline content changes from 80 - 85 % (figure 6.7 c) to 50 - 55 % (figure 6.7 d, Debzi *et al* 1991, Whitney *et al* 1995). From comparison of these same signals the presence of 30% galactose depleted xyloglucan is estimated to change the crystalline content to ~ 60%. This is more than change made by native XG but less than cellulose alone.

The presence of the 30% galactose-depleted polymer also resulted in a decrease in the Iα crystalline form of cellulose. From figure 6.8 b it can be seen that signals at 105 ppm, 90.5 ppm and 88.5 ppm, corresponding to the Iα form and those at 106 ppm, 104 ppm, 88.5 ppm and 87.7 ppm, corresponding to the Iβ form show a 40/60 ratio for Iα/Iβ (by comparison of published signal patterns by Debzi et al 1991 and Yamamoto and Horii 1993).

Composites assembled by incorporation of 60% galactose depleted xyloglucan into the xyloglucan/cellulose produced the CP-MAS and SP-MAS spectra shown in figure 6.9a, b and c. The CP-MAS spectrum shows signals at 99 - 100 ppm and at 80 - 85 ppm which are again consistent with that found previously for cellulose produced in the presence of xyloglucan and are additional to those found for cellulose alone(Atalla and Vanderhart 1984). A signal at ca. 105 ppm again coincides with that for C1 of cellulose, glucose and galactose.

Integration of the two signals at 105 ppm and 99 - 100 ppm show an estimated ratio for cellulose : xyloglucan of 1 : 0.36. As the monosaccharide analysis gave a ratio of 1: 0.98, it is possible that ~ 60 % of the 'associated' xyloglucan has not been detected by this method, however although the variables of relatively immobile and mobile xyloglucan have been measured in this case we have a third variable - that of aggregated (immobile) xyloglucan whose spectra is also present (chapter 5).

The SP-MAS spectrum was essentially that of tamarind seed xyloglucan in solution. Signals for C1 of galactose, glucose and xylose were present. The intensity of the signals at 99 - 100 ppm in both the SP-MAS and CP-MAS spectra of the 60% galactose-depleted xyloglucan reflect the relative mobility of the xylose residue compared to that of the glucose residue which is now fixed within the xyloglucan aggregate.

A xylinus cellulose grown in the presence of 60% galactose depleted xyloglucan gave estimated ratios for $I\alpha$ /$I\beta$ to be ca. 70 : 30 which is the same as cellulose alone. This suggests that this modified xyloglucan did not intimately associate with and within the cellulose microfibrils. The probable reason for the lack of association between 60% galactose-depleted xyloglucan and cellulose is that it has self-associated prior to the production of cellulose.

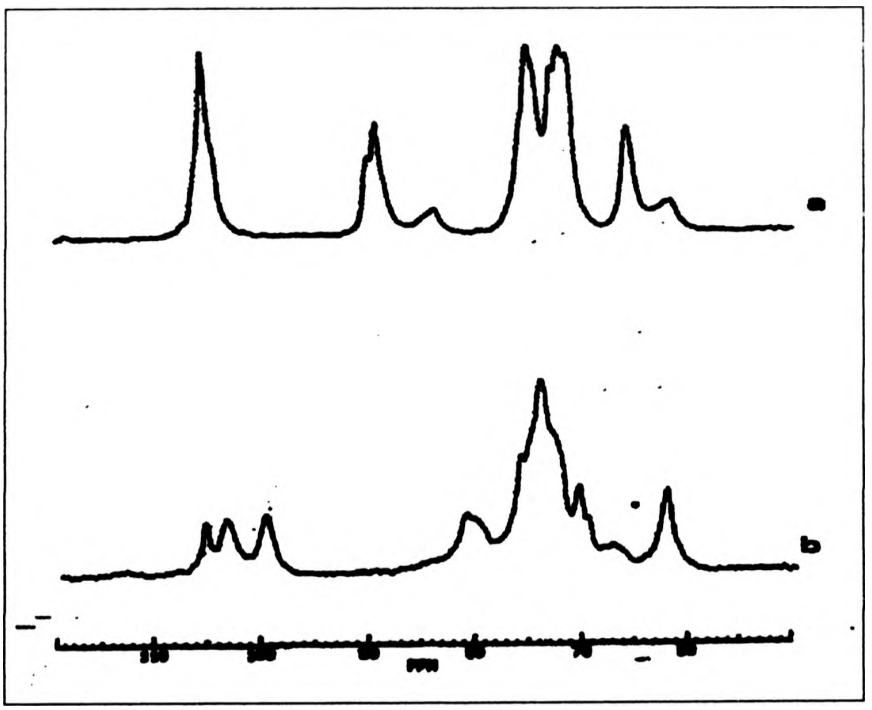


Figure 6.6 a. Hydrated cellulose from Acetobacter xylinus. 30% solution of Tamarind seed xyloglucan (After Whitney et al 1996)

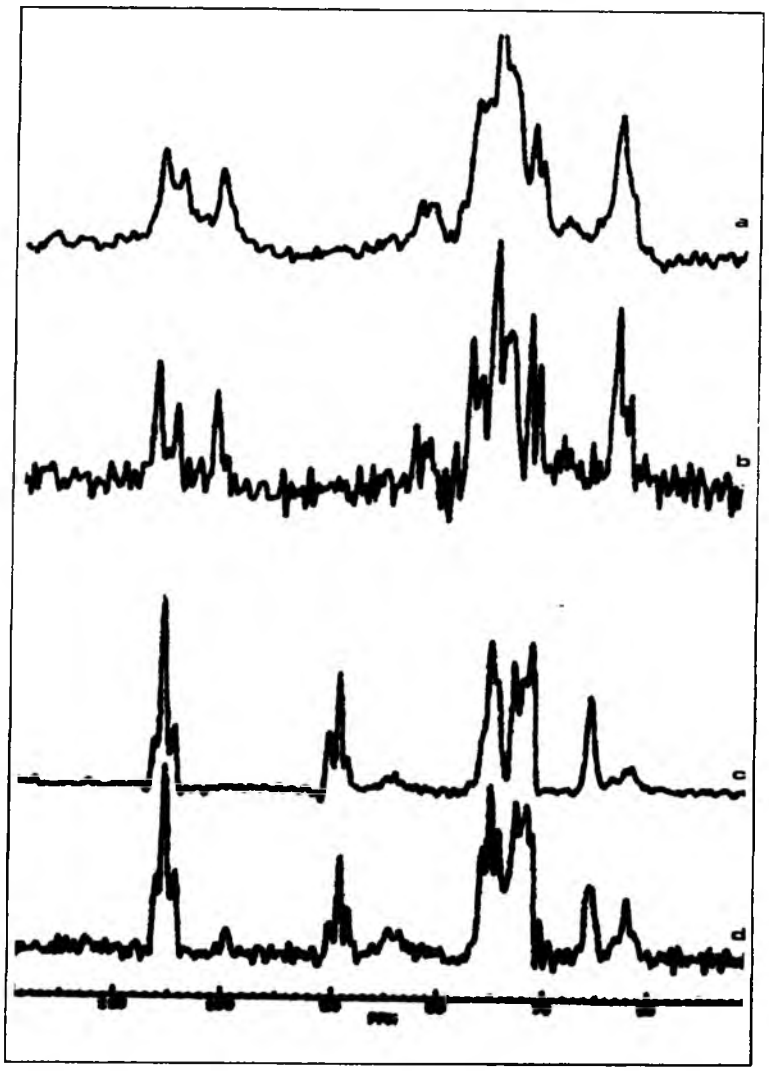


Figure 6.7 (a) ^{13}C SP-MAS spectra of bacterial cellulose/native tamarind seed xyloglucan composites. SP-MAS, (b) as (a) but resolution enhanced, (c) CP-MAS of cellulose produced by A xylinus (d) CP-MAS of bacterial cellulose/native tamarind seed xyloglucan composites. (After Whitney et al 1996)

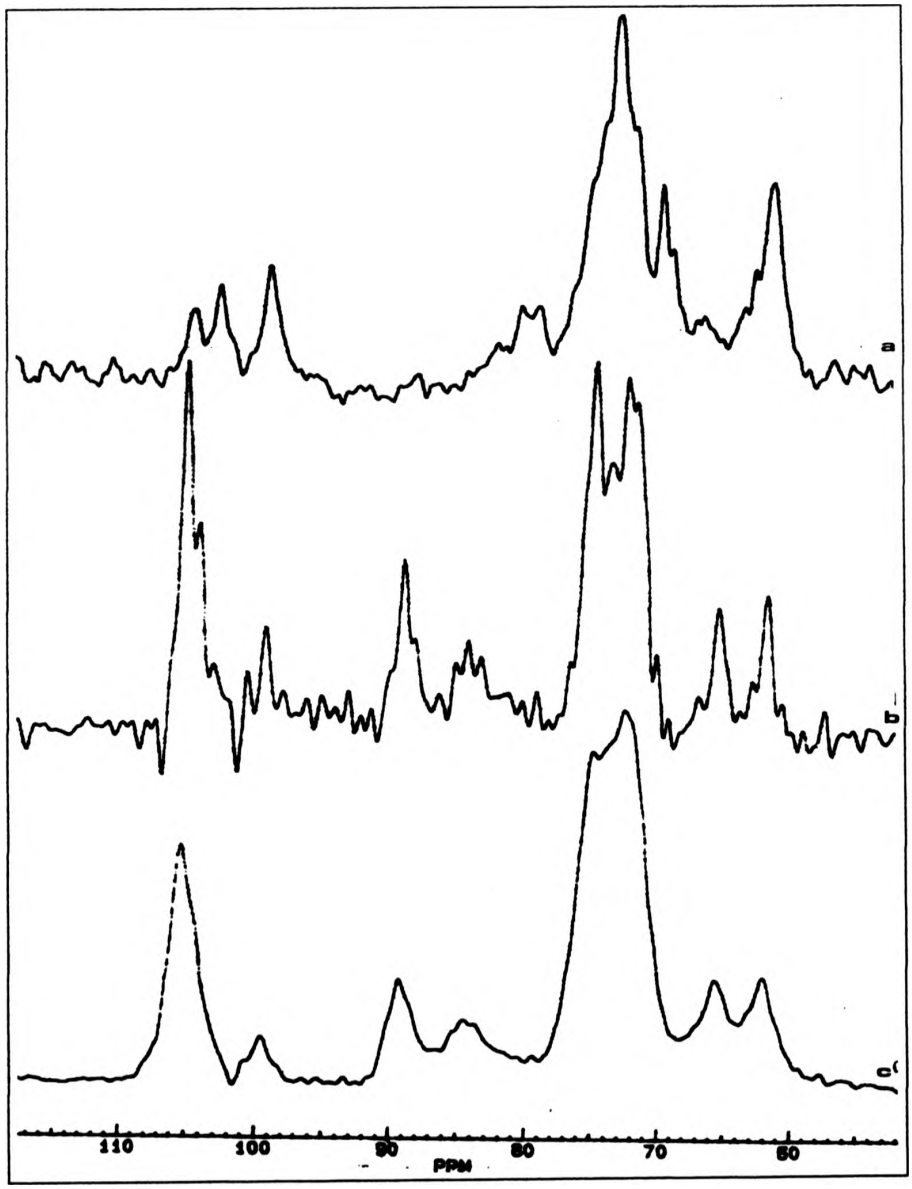


Figure 6.8 ^{13}C SP-MAS and CP-MAS spectra of bacterial cellulose / 30% galactose-depleted xyloglucan composites. (a) SP-MAS (b) CP-MAS (resolution enhanced) and (c) as (b) but not resolution enhanced.

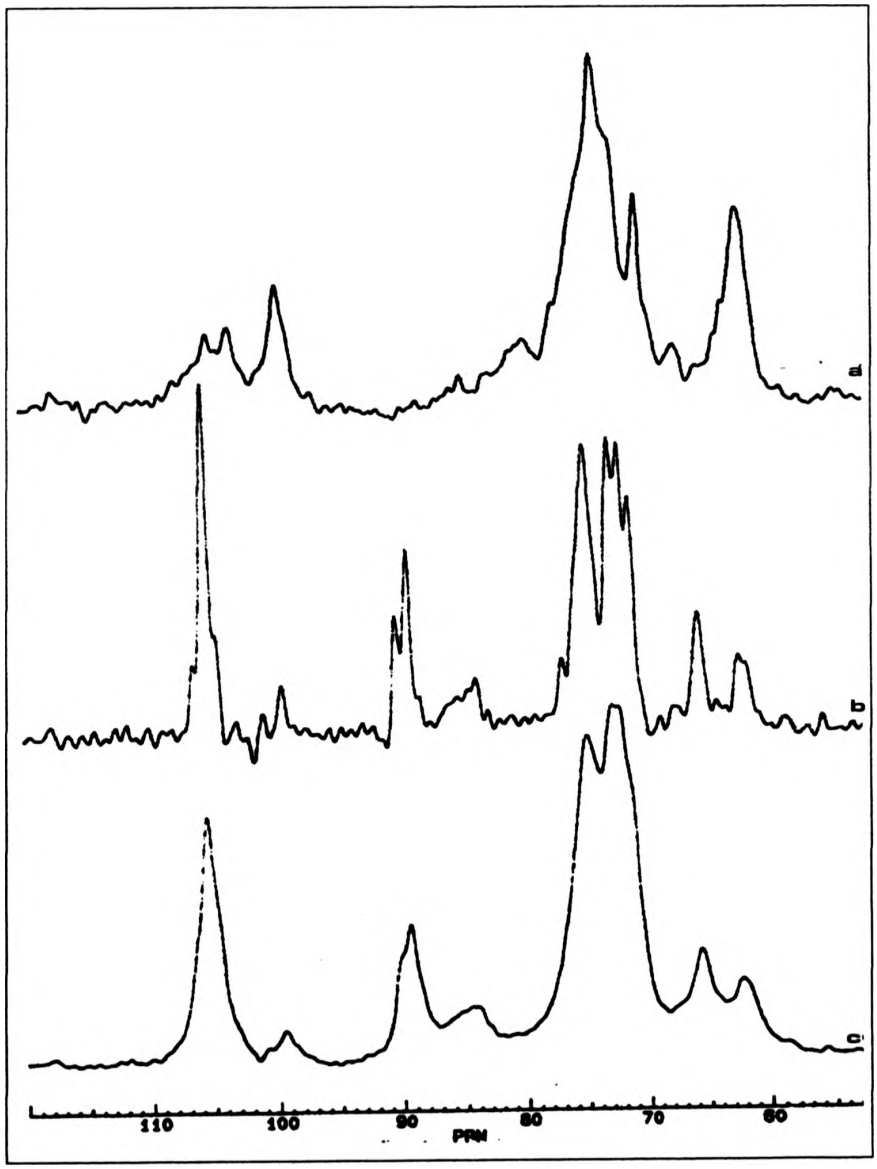


Figure 6.9 ^{13}C SP-MAS and CP-MAS spectra of bacterial cellulose / 60% galactose-depleted xyloglucan composites. (a) SP-MAS (b) CP-MAS (resolution enhanced) and (c) as (b) but not resolution enhanced.

6.3.8 Uniaxial Tensile Testing

Uniaxial tensile testing detected substantial differences in the mechanical behaviour between the different composites (figure 6.10). Cellulose alone appeared to be stronger under tension reaching a stress of 0.35 MPa compared to composites made with native or modified XG which reached approximately 0.18, 0.14 and 0.08 MPa for the native, 30% and 60% galactose-depleted xyloglucan respectively. It also appeared to be more brittle than the native XG/cellulose composite with a nominal strain of ~ 25% required for failure but less brittle that the composite material formed from the modified xyloglucans. The native xyloglucan/cellulose composite, on the other hand, had a breaking strain of ~ 50% indicating a more pliable, stretchable network failing at a strain of only 0.175 MPa.

The initial behaviour of the 30% galactose depleted xyloglucan composite was similar to that observed for native XG/cellulose after which a more 'cellulosic' pattern emerged with failure at ~17 % and 0.08 MPa, much lower than that of the native XG/cellulose composite. In contrast, however, 60 % galactose depleted xyloglucan/cellulose composite behaved very like cellulose alone from the initial stages but again had a much lower breaking strain (~ 15%) than any of the others under test.

From these data it can be seen that cellulosic materials are less pliable than those constructed in the presence of native xyloglucan. It is the cellulose and not the presence of modified xyloglucan which is the major contributor to the mechanical properties observed for the 30% and 60% galactose depleted composites, as self-aggregation of the galactose-depleted XG has made it unavailable for association with cellulose. The observed weakening is thought to be due to a reduced amount of cellulose per unit mass of material.

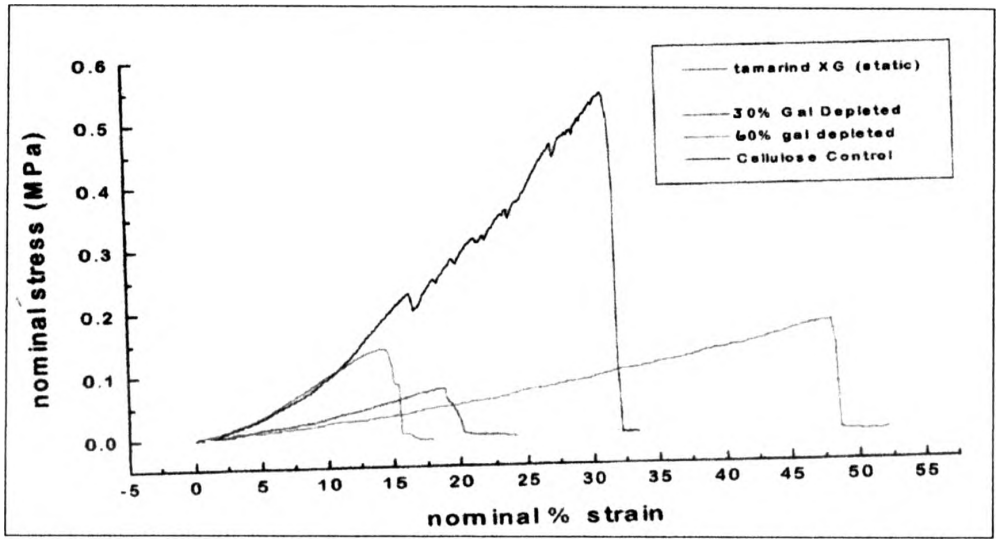


Figure 6.10 Uniaxial Tensile Testing shows substantial differences in the mechanical behaviour (results shown are the mean of ten experiments).

6.4 Discussion

The *Acetobacter* technique provided a useful method for examining the changes that occur when modified xyloglucan is introduced into the growing system. The presence of native tamarind seed xyloglucan confers a lateral arrangement to the microfibrils and forms cross-linkages between them which is consistent with the role proposed for the XG/cellulose network in the primary cell wall (McCann *et al* 1990, McCann and Roberts 1991, Carpita and Gibaut 1993, Talbot and Ray 1992a).

The observation that the conformation of xyloglucan changed when associated with cellulose with no obvious involvement of xyloglucan side-chains (Whitney *et al* 1995) has led, in this work, to an investigation if the changes observed when modified polymeric xyloglucan was present in the media when the bacteria were growing and producing cellulose.

On removal of galactose, xyloglucan changes conformation and effectively binds to itself (chapter 5). The lower the galactose levels the more self-association takes place. This self-binding xyloglucan precipitates out of solution but is still present, as a suspension, in the reaction mixture when cellulose is being produced. The increase in the fundamental cellulose : xyloglucan ratios reflect this in that, in the cases of 30% and 60% galactose depleted polymer, the XG is associating with itself as well as with cellulose thereby giving the appearance of more cellulose-associated xyloglucan.

Agitation was all that was required to remove over half the 60% galactose depleted XG from the composites and only relatively mild KOH, not of sufficient concentration to introduce intrafibrillar swelling, completely removed the xyloglucan. This implies a superficial interaction only. Xyloglucan removed from composites by all extraction methods did not show any structural differences. If galactose were an important factor in the association with cellulose the sequential extraction by alkali would have removed xyloglucans with sequentially greater galactose content (the more intimately associated having the greater galactose content).

The trends observed from the NMR spectra reinforce the conclusion that as galactose is removed and the XG self-binds, less of it is available to be associated with cellulose. Neither of the modified xyloglucans affected the degree of crystallinity of the cellulose to the same extent as the native xyloglucan although that shown by the 30% galactose depleted XG was similar. As less that one third of the galactose had been removed in this case, the behaviour is probably caused by a slight dilution effect (where only the higher galactose content molecules are associating with cellulose). Removal of ca. two thirds of the galactose, however, produced a xyloglucan which did not affect the molecular organisation at all indicating not intimate association.

Changes in mechanical behaviour can be attributed to the probability that in binding to itself XG is unavailable to associate intimately with cellulose. The failure of the composite constructed with 60% galactose depleted xyloglucan to show exactly the behaviour pattern for cellulose alone can be attributed to a dilution effect caused by a decrease in the amount of cellulose per unit mass of composite.

Although the association of xyloglucan to cellulose is not, perhaps, directly influenced by substituent of the glucan backbone (Whitney *et al* 1995), the removal of galactose changes the availability of xyloglucan to cellulose, As previously mentioned, this has important implications for the functionality of the galactose side-chains on the xyloglucan molecule. If galactose were not present, it is very likely that these molecules would self-aggregate and precipitate out of solution.

The introduction of pre-modified xyloglucans into the cellulose/XG reaction mixture has met with limited success and investigations of galactose substitution on cellulose association has been hindered by the fact that removal of galactose side-chains causes self-association. The temporal events of the work ie. the galactose was removed prior to the introduction of the XG into the culture medium, may be a limiting factor as different result would possibly be achieved if β-galactosidase was present with the native XG in the culture medium.

Chapter Seven

7. Investigation into the Modification of Xyloglucan by Removal of Xylose and *in vitro* Construction of Composites

7.1 Introduction

As described previously, the storage xyloglucan of germinating nasturtium seeds is degraded by the concerted actions of NXET, β-galactosidase, α-xylosidase and β-glucosidase (figure 1.4, chapter 1) to produce the monosaccharides, galactose, glucose and xylose (Edwards *et al* 1985, 1986, and 1988, Fanutti *et al* 1993, Crombie *et al* 1998). A xyloglucan oligosaccharide-specific α-xylosidase has been purified to homogeneity and characterised (Fanutti *et al* 1991). The protein was found to be 16 - 20 % glycosylated with an apparent molecular weight of 85kDa on SDS PAGE and a maximum activity at a pI of 6.1. It did not hydrolyse ρ-nitrophenyl-α-D-xylopyranoside, isoprimeverose (α-D-xyl (1→6) D-glucose) or high molecular weight xyloglucan, but had a highly restricted specificity in that it catalysed the hydrolysis of a single unsubstituted terminal xylose residue from the non-reducing end of an oligo-xyloglucan molecule.

α-D-xylosidase activity has also been discovered in the primary cell wall of auxin treated pea stems where the enzyme is thought to regulate the levels of biologically active xyloglucan-derived oligosaccharides (O'Neill *et al* 1989). The mode of action of both nasturtium and pea derived α-D-xylosidase is reported to be identical, with the minimum oligosaccharide which functions as substrate shown in figure 7.1 (Fanutti *et al* 1991).

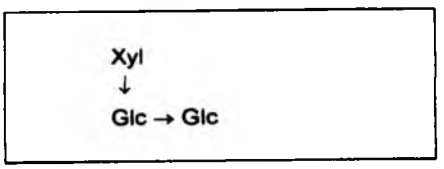


Figure 7.1 Minimum Substrate for the action of nasturtium and pea α-D-xylosidase (after Fanutti *et al* 1991)

After the interesting observations made when galactose was removed from polymeric xyloglucan and cellulose/xyloglucan composites constructed with these 'tailored' molecules, the theme of modification of xyloglucan by removal of side-chains was continued.

As approximately two out of three of the xylopyranosyl residues in Tamarind seed xyloglucan is further substituted with galactose, a maximum of only ~ 33% of the xylose is 'available' for hydrolysis. α-D-xylopyranosyl residues are linked (1→6) to approximately 80% of the glucose sugars of the glucan backbone (figure 7.2, Reid 1984, Gidley *et al* 1991). The xylose side chains are α-linked and as such might be susceptible to selective hydrolysis with strong acids.

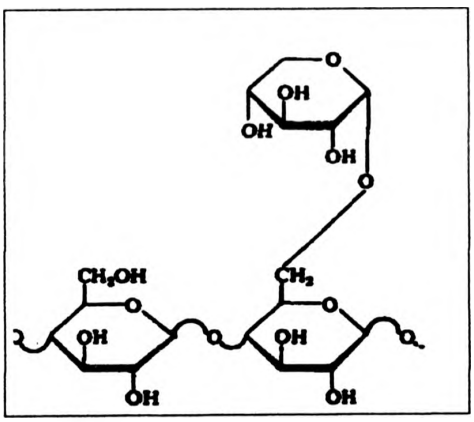


Figure 7.2 Structural features of xylose - glucose α linkage (After Edwards *et al* 1985)

Conformational and configurational homologies between α-D-xylopyranose and α-D-glucopyranose has, in the past, led to an α-D-glucosidase-gold complex having the ability to recognise α-D-xylosyl terminal residues of xyloglucans (Ruel and Joseleau 1989). It was hoped that this homology would be further exploited by attempting to remove xylose residues from the xyloglucan backbone by the action of an α-D-glucosidase.

This chapter describes an investigation into the removal of those available xylose residues by mild acid hydrolysis, an α-glucosidase and by the concerted actions of NXET and α-D-xylosidase upon tamarind seed xyloglucan. The synergistic activities of these two enzyme

should, in theory, provide us with higher molecular weight xyloglucan with two adjacent unsubstituted glucose residues (figure 7.3, see also chapter 4 for NXET action). Also explored and reported is the characterisation of cellulose/xyloglucan composites constructed with these tailored xyloglucans.

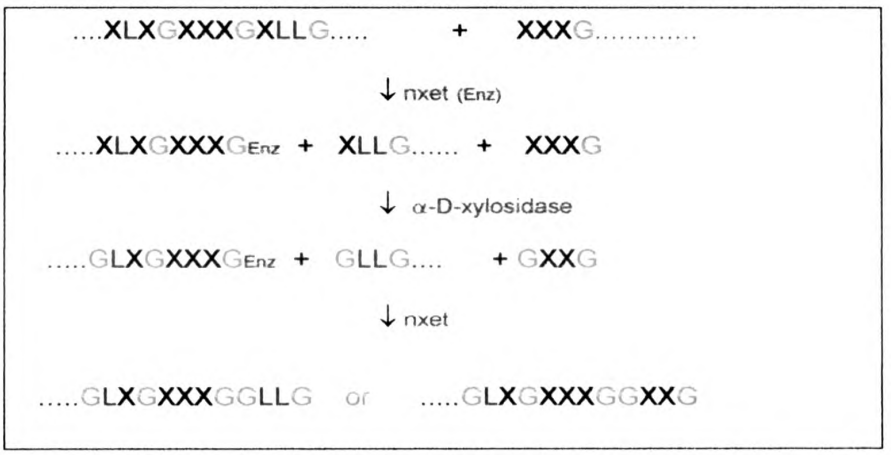


Figure 7.3 Theoretical sequence of events with the synergistic actions of NXET and α-D-xylosidase

7.2 Materials and Methods

7.2.1 Removal of Xylose

7.2.1.1 *Acid Hydrolysis of a Solution of Tamarind Seed Xyloglucan*

Mild acid hydrolysis was with 0.5M or 1.0M acetic acid was carried out as described in section 2.2.7.4 of chapter 2.

7.2.1.2 *Incubation of Xyloglucan Solution with α-glucosidase*

Enzymatic hydrolysis with α-glucosidase (source: Yeast) was carried out as described in section 2.2.7.5.

7.2.1.3 *Incubation of Xyloglucan with NXET and α-xylosidase*

The dual incubation of tamarind seed xyloglucan with both NXET and α-xylosidase was carried out as described in section 2.2.7.6 of chapter 2.

7.2.1.4 *Incubation of Xyloglucan with Tomato XET and α-xylosidase*

The simultaneous incubation of tamarind seed xyloglucan with both tomato XET and α-xylosidase was carried out as described in section 2.2.7.7 of chapter 2 but replacing the NXET with TXET.

7.2.2 Construction of cellulose/xylose-depleted xyloglucan composites

Bacterial inoculum was obtained from actively cellulose-synthesising *A xylinus* as described in Materials and Methods (2.2.8.1 and 2.2.8.2). Incubation was performed statically at 30° C in Hestrin-Schramm media pH 6.2 containing 0.5% native or modified xyloglucan as described in section 2.2.9.b of chapter 2.

7.2.3 Monosaccharide Analysis of Composite Material

Monosaccharide analysis was performed on the freeze-dried material as described in section 2.2.5.2 a and b (H_2SO_4 and TFA hydrolysis respectively) of Materials and Methods.

7.2.4 Removal of Loosely Bound Xyloglucan by Agitation

Monosaccharide and oligosaccharide analysis (sections 2.2.6 and 2.2.5 respectively) was carried out on the residual material and the xyloglucan that was removed during overnight agitation (section 2.2.10.3).

7.2.5 Sequential Extraction Of Xyloglucan From Composites Using KOH

Sequential extraction of xyloglucan from the composites with increasing concentrations of KOH was carried out as described in section 2.2.10.1. For all samples, oligosaccharide analysis of extracted material was determined by overnight digestion with *endo*glucanase (source *Trichoderma reesei* ; Megazyme Ltd) and HPAEC (Section 2.2.5). Monosaccharide analysis

by HPAEC of the freeze-dried residual material was carried out after acid hydrolysis (Section 2.2.6).

7.2.6 Enzymatic Digestion of Cellulose/Xyloglucan Composites

Approximately 10 mg (wet weight) was incubated overnight with 0.45U *endo*glucanase (Megazyme Ltd) at 30° C with constant agitation. After incubation the enzyme was denatured in a boiling water bath for 15 minutes as described in section 2.2.10.4 of chapter 2.
Residual material was collected by centrifugation (Microcentaur, 13,000 rpm, 10 minutes) and freeze-dried for monosaccharide analysis (section 2.2.6). The oligosaccharide composition was analysed by direct injection into the HPAEC using the gradient described in section 2.2.5.

7.2.7 Deep -Etch Freeze Fracture Transmission Electron Microscopy

All microscopy was carried out as described in Materials and Methods (chapter 2, section 2.2.11).

7.2.8 Uniaxial Tensile Testing

All uniaxial tensile testing was carried out as described in Materials and Methods (chapter 2, section 2.2.11).

7.3 Results

7.3.1 Removal of Xylose from High Molecular Weight Xyloglucan

7.3.1.1 *Removal of Xylose from High Molecular Weight Xyloglucan by Mild Acid Hydrolysis.*

Hydrolysis with 0.5M and 1.0M acetic acid proved unsuccessful in the removal of available xylose from the glucan backbone (Table 7.1). A reduction in the viscosity of the 1.0M acid treated sample (data not shown), however, suggested that the acid was of sufficient concentration to hydrolyse the polymer backbone without removing xylose.

Table 7.1 Component parts of Xyloglucan after treatment with 0.5M and 1.0M Acetic Acid. *Monosaccharide analysis carried out by HPAEC after H_2SO_4 hydrolysis.*

Treatment (M Acetic acid)	Xyloglucan		
	glc	gal	xyl
None	48.6	15.7	35.7
0.5M	48.7	15.5	35.8
1.0M	48.6	15.8	35.7

7.3.1.2 *Removal of Xylose from High Molecular Weight Xyloglucan by Hydrolysis with an α-glucosidase*

Hydrolysis with α-glucosidase proved unsuccessful in the removal of xylose from the glucan backbone (data not shown). This is perhaps due to the absence of the $-CH_2OH$ group on the xylose residue of the xyloglucan.

7.3.1.3 *Removal of Xylose from High Molecular Weight Xyloglucan by Synergistic Action of NXET and α-xylosidase.*

The TFA hydrolysis and subsequent monosaccharide analysis on HPAEC showed that a 72 hr incubation of xyloglucan (24 mg/ml) with NXET and α-xylosidase simultaneously resulted in a removal of 25% of the total xylose compared to that of the controls. This represents a removal of ~78% of the available xylose.

The appearance of the resultant freeze-dried material, however, was of a white powder and not an interwoven network observed when native high molecular weight xyloglucan is lyophilised. The viscosity of a solution (12mg/ml) of this material was approximately half that of the control solution which indicated that the xylose depleted xyloglucan was of a lower molecular weight than the native. After running on TLC (data not shown) it was noted that the xylose depleted xyloglucan remained on the base line and so was thought to be of a sufficiently high molecular weight to attempt the production of cellulose/xyloglucan composites.

7.3.1.4 *Removal of Xylose from High Molecular Weight Xyloglucan by Synergistic Action of Tomato XET and Nasturtium α-xylosidase.*

TFA hydrolysis and subsequent monosaccharide analysis by HPAEC showed that a 72 hr incubation of xyloglucan (24 mg/ml) with tomato XET and nasturtium α-xylosidase concurrently resulted in a removal of 28% of the total xylose. This represents a removal of approximately 85% of the available xylose. Lyophilisation of the resulting material produced an interwoven network of fibres similar to that observed when native high molecular weight xyloglucan is freeze-dried. A viscosity consistent with that of the control was determined by viscometric assay indicating that the reduction in molecular weight found previously was almost certainly a result of the action of NXET.

Due to time constraints this material has yet to be used in the assembly of cellulose/xyloglucan networks utilising the *Acetobacter xylinus* system.

7.3.2 Construction and Characterisation of Cellulose/Xyloglucan Composites

After 96 hours static incubation at 30° C the resulting pellicles were aseptically removed from the flasks and washed intensively with deionised water. A slower rate of cellulose production was observed using the method described in section 2.2.9b of chapter 2. The pellicles were very heterogeneous in appearance with many areas of differing thickness.

7.3.2.1 *Levels of Xyloglucan Incorporation.*

The levels of incorporation of control and modified xyloglucan (native and xylose depleted tamarind seed xyloglucan) into the composites were 1 : 0.54 ± 0.10 and 1 : 0.37 ± 0.17 respectively (see Table 7.2). In this case galactose was used as an internal standard for xyloglucan. The cellulose : xyloglucan ratio for the native cellulose/xyloglucan composite is consistent with that previously found in this thesis (1 : 0.50 ± 0.06).

There was a large range in the levels of XG incorporation found for the xylose depleted polymer (1 : 0.19 to 1 : 0.54). This, in conjunction with the observed variation in pellicle thickness, may suggest that an individual pellicle contained areas with both native unmodified xyloglucan and xylose depleted xyloglucan incorporated into it.

With the combined properties of depleted xylose and lower molecular weight, it appears that less xyloglucan becomes associated with the growing cellulose than in the control composites.

7.3.2.2 *Removal of xyloglucan by Agitation only*

Overnight agitation removed approximately 60% of the xyloglucan initially trapped in the composites (Table 7.2) indicating that this is only very loosely associated with the cellulose.

7.3.2.3 *Enzymatic Digestion of the Cellulose/Xyloglucan Composites.*

Monosaccharide analysis of the composites after digestion by *endo*glucanase (Table 7.2) show that, again, ~60% of the associated xyloglucan is removed. As these samples were also agitated during incubation, however, it can only be said that the enzyme cannot remove the remaining 40% of the xyloglucan. It is assumed that *endo*glucanase will digest only that xyloglucan which is loosely bound to, or is involved in cross-linkages between, the microfibrils. This suggests, therefore, that the xyloglucan remaining is more intimately associated with cellulose and is not readily accessible to the enzyme.

That this result is equivalent to that found for agitation only could again suggest that a proportion of the xyloglucan remained unmodified and is more closely linked to the cellulose component within the composite.

7.3.2.4 *Sequential Extraction of Xyloglucan from Composites*

Treatment of control and xylose depleted cellulose/xyloglucan pellicles with increasing concentrations of KOH showed a progressive reduction in the xyloglucan content. In the case of the modified xyloglucan composite, approximately 81% of the total xyloglucan present was removed by 0.5M KOH (Table 7.2). The pattern of reduction of xyloglucan in the control composite was consistent with that previously found (Whitney *et al* 1996).

The fact that such a small xyloglucan moiety remained after the application of 0.5M KOH and that none at all remained after the application of 1.0M KOH indicates that no microfibrillar swelling was required to remove the xyloglucan and so no intrafibrillar association took place when cellulose was synthesised in its presence.

No structural differences were observed in any of the oligosaccharides resulting from xyloglucan removed from the cellulose in the case of all of the above.

Table 7.2 shows the complete biochemical characterisation results.

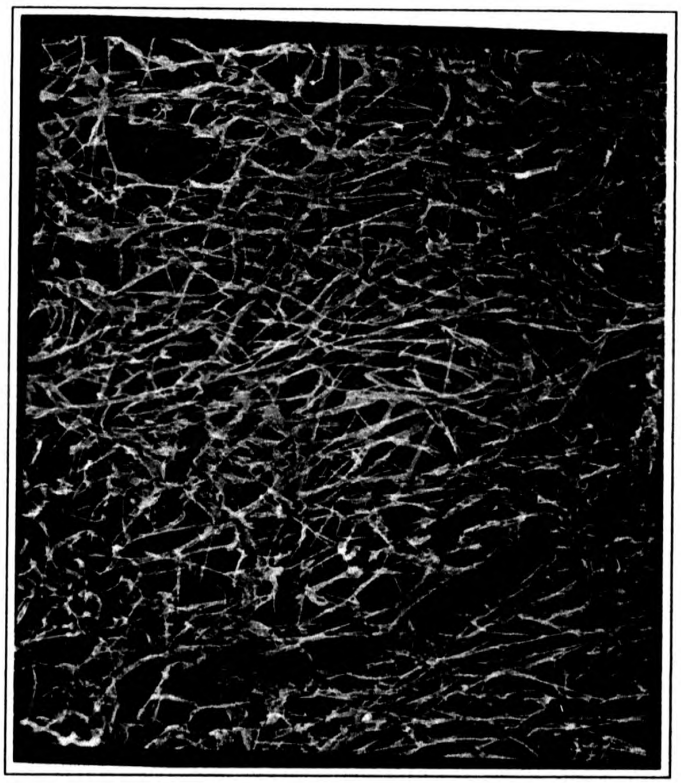
7.3.2.5 *Deep-Etch Freeze-Fracture Transmission Electron Microscope*

The network architecture of pellicles synthesised in the presence of xylose depleted xyloglucan are shown in figures 7.4a and b. No alignment of the microfibrils of cellulose is observed (compared to the control pellicle shown in figure 6.3a) and there is no evidence of cross-linkages being formed.

Comparisons can be made, however, from studies by Whitney et al (1996) of the incorporation of low molecular weight xyloglucan (peak mwt 156000 kDa) where it is postulated that the ability to confer a lateral order on and form cross-linkages between microfibrils, may be a function of molecular weight.

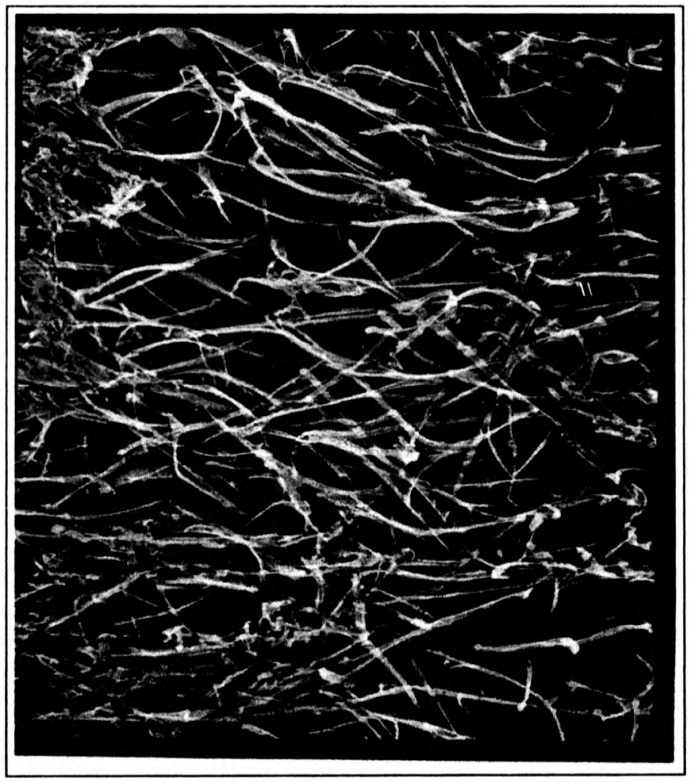
Table 7.2 Biochemical Characterisation results. *Monosaccharide analysis carried out by HPAEC after H_2SO_4 hydrolysis.*

Treatment	Composite	
	Native	xylose depleted
H_2SO_4 hydrolysis	1 : 0.54 ± 0.10	1 : 0.37 ± 0.17
*Endo*glucanase	1 : 0.20	1 : 0.15
Agitation	1 : 0.36	1 : 0.15
0.5M KOH x 2	1 : 0.28	1 : 0.07
1M KOH x 2	1 : 0.10	1 : 0
4M KOH x 1	1 : 0.06	1 : 0
4M KOH x2	1 : 0	1 : 0



X 20000 ——— 0.5μm

Figure 7.4 a Micrograph of tungstan/tantalum/carbon replica of composite composed by fermentation of *A xylinus* in the presence of xylose depleted xyloglucan. No evidence of lateral alignment of cellulose microfibrils or cross-linkages is seen (compared to the composite composed when cellulose was produced in the presence of native high molecular weight xyloglucan figure 6.3a).



X 40000 ——— 0.25μm

Figure 7.4 b Micrograph of tungstan/tantalum/carbon replica of composite composed by fermentation of *A xylinus* in the presence of xylose-depleted xyloglucan (as 7.4a) at higher magnification again showing no evidence of any interaction of cellulose with the xyloglucan.

7.3.2.6 *Uniaxial Tensile Testing*

Limited uniaxial tensile testing showed substantial differences in mechanical behaviour when cellulose was synthesised in the presence of xylose depleted xyloglucan compared to that found when grown in the presence of control xyloglucan (figures 7.5 a and b). The profile of the control xyloglucan/cellulose composite (figure 7.5a) is consistent with that previously found (see chapter 6 figure 6.10) but due to lack of material only three runs of the xylose depleted composite were possible and as such, it was difficult to make assumptions from these.

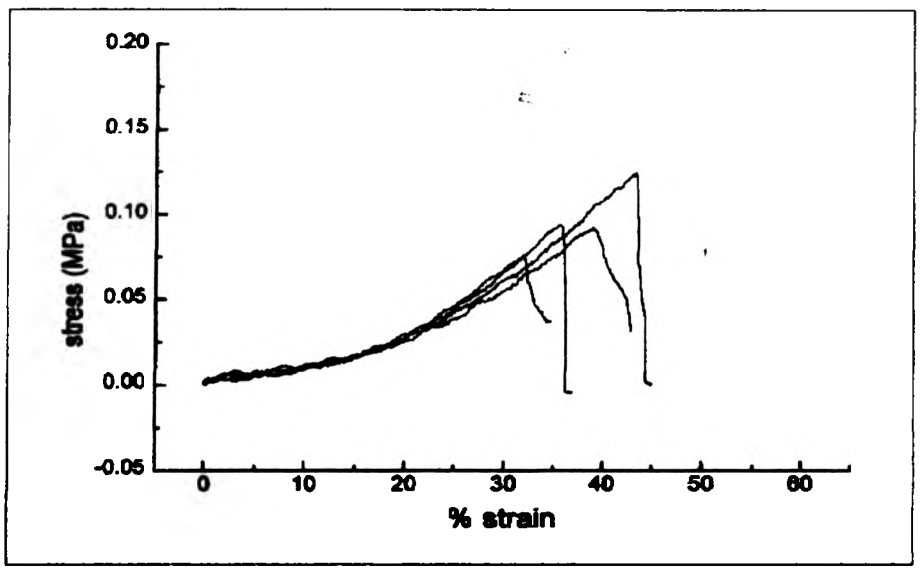


Figure 7.5 a Uniaxial tensile testing profile of control composite (cellulose grown in the presence of native xyloglucan

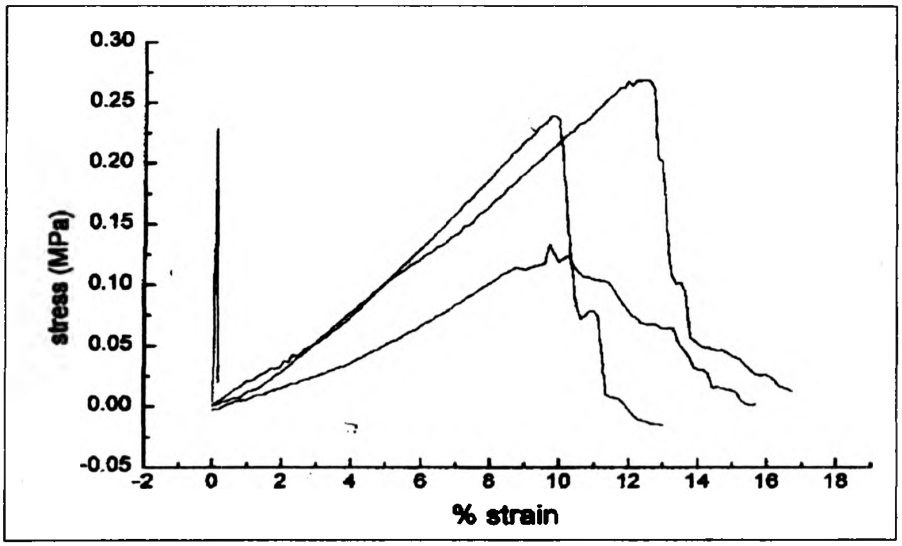


Figure 7.5 b Uniaxial tensile testing profile of composite resulting when cellulose grown in the presence of xylose depleted xyloglucan

7.4 Discussion

Mild acid hydrolysis and α-glucosidase failed to remove any xylose from high molecular weight xyloglucan. This could be due to the steric hindrance of the galactose residues which would be present on the adjacent xylose residues.

The mobilisation of reserve xyloglucan in germinating nasturtium cotyledons by the four enzymes acting 'in concert' could be considered to be a process of cell wall turnover (Reid 1985). The similarities observed between nasturtium and pea α-xylosidase suggest that the nasturtium enzyme also may be involved in the turnover of primary cell wall xyloglucan which is known to be associated with elongation-growth (Labavitch and Ray 1974).

Nasturtium α-xylosidase is associated with hydrolysis of reserve xyloglucan and is active only on the α-linkage of the terminal xylose situated at the non-reducing end of the oligo-xyloglucan molecule (Fanutti *et al* 1991). In germinated nasturtium seeds, the peak of activity occurs slightly after that for NXET which, due to the hydrolytic and transglycolytic actions of this enzyme, suggests that the removal of xylose to produce a 'doublet' of glucose residues possibly exists *in muro*. The juxtaposition of two unsubstituted glucose residues may alter the conformation of the xyloglucan in solution which, in turn, could have been responsible for the reduction in its association with cellulose. In contrast to the removal of galactose, however, xylose depletion did not cause self-aggregation as the presence of galacto-xylosyl side chains continued to provide steric hindrance, which is also observed in native xyloglucan.

The heterogeneity of the pellicle obtained and range of values obtained for levels of xyloglucan incorporation leads to speculation that perhaps two types of molecule existed in the xyloglucan solution within the composite reaction mixture - 'native' xyloglucan, which would have been capable of associating with the newly synthesised cellulose and the molecule containing glucose 'doublets' which, perhaps, would not.

In contrast, the reduction in molecular weight as a result of the action of NXET was possibly the main contributor to the results observed rather than the xylose-depletion. Comparisons can be drawn from the work of Whitney *et al* (1996), who observed similar results when cellulose

was grown in the presence of low molecular weight xyloglucan. The much reduced level of incorporation (compared to that of control xyloglucan) of xylose-depleted xyloglucan into the composites and the ease with which it was removed indicate that very little interaction with cellulose took place. That no intrafibrillar swelling (by KOH) was required to completely remove all xyloglucan also strongly suggests that no intimate association occurred.

In contrast to the higher level of association for residues of four consecutive glucosyl residues, compared to that of polymeric xyloglucan reported by Vincken *et al* (1995), levels for the lower molecular weight XG were below that found for the control. This may be due to the slower rate of cellulose production observed.

Further investigation by NMR would indicate any changes occurring in the crystallinity of the cellulose produced by the *A. xylinus* in the presence of the xylose depleted xyloglucan. This would provide evidence of whether or not a small population of native xyloglucan molecules is available for limited intrafibrillar association within the reaction mixture. Indication of the 'true' levels of incorporation of xylose-depleted xyloglucan into the composite would also be elucidated.

From the data collected, virtually no association between cellulose and xyloglucan was observed in this case and work is in progress to attempt to eliminate the variable of the reduced molecular weight. The concerted actions of α-xylosidase and TXET has produced a xylose-depleted xyloglucan which does not have a reduced molecular weight and the construction of cellulose/xyloglucan composites using this material is being investigated.

As with the exploration with galactose-depleted xyloglucan (chapter 5 and 6), however, the temporal events occurring in the cell wall would possibly have both enzymes present at the site of synthesis of cellulose and its association with xyloglucan. Further investigation of this, therefore, could have the enzymes working simultaneously on xyloglucan whilst in the presence of the growing cellulose.

Chapter Eight

8. Construction of Cellulose/Xyloglucan Composites in the presence of Tomato XET and Xyloglucan Oligosaccharides

8.1 Introduction

Plant cells are enclosed by walls that define the shapes and sizes of cells and mediate cell-to-cell contact. The dynamics of plant growth, morphogenesis and differentiation require concomitant modification of the primary cell wall, both in cell expansion and in developmental events, such as fruit softening, where cell size remains static but where wall loosening is an important feature. Disassembly of the cellulose - xyloglucan network *in situ* probably involves the synergistic action of groups of cell wall modifying proteins, where one family of enzymes possibly mediate the activity of another, providing ordered cell wall restructuring and turnover (Rose and Bennett 1999).

A class of enzymes known as xyloglucan endotransglycosylases have the potential to enzymatically modify wall components by the transference of fragments of cleaved polysaccharide to the C-4 position of non-reducing glucosyl units in other xyloglucan molecules or their oligosaccharide subunits (Farkas and Sulova, 1992, Fanutti *et al* 1993).

This modifying of xyloglucan proceeds predominantly by a transglycosylation mechanism (Farkas and Sulova, 1992 and Fanutti *et al* 1993) probably via the production of a relatively stable glycosyl-enzyme intermediate. This intermediate is stable in water but rapidly decomposes by transfer of its glycosyl moiety to xyloglucan-derived oligosaccharides, whereupon the enzyme is released (Sulova *et al* 1998).

Interest in XETs has been stimulated since it was shown that they may participate in cleaving and reforming xyloglucan molecules during gradual expansion of plant cell walls during growth (Carpita and Gibaut, 1993, Fry 1995, Nishitani 1997) and great effort has been exerted in the purification and characterisation of a variety of XETs (Campbell and Braam 1999a + b, Sulova and Farkas 1999, Garcia-Garrido *et al* 1999, Steele and Fry 1999). Consistent with a role in

growth, levels of XET activity have been shown to correlate with elongation in maize roots (Pritchard *et al* 1993).

An isolated cDNA clone from nasturtium which encodes a seed enzyme with XET activity (Edwards *et al* 1986, Fanutti *et al* 1993, de Silva *et al* 1993) was used to further isolate several cDNA clones from tomato whose encoded polypeptide sequences exhibit around 40% homology with nasturtium XET (de Silva *et al* 1994). Heterologous expression in *E. coli* was used to confirm XET activity with no detectable hydrolytic action (Arrowsmith and de Silva 1995). Overexpression of the enzyme in the yeast system, *Pichia pastoris* has led to increased yields, giving sufficient quantities for use in these and other investigations.

This chapter reports on the construction of xyloglucan/cellulose composites using the *A. xylinus* system where the possible conditions arising at the cell wall can be mimicked. Composites resulting from xyloglucan and newly synthesised cellulose become associated in the presence of tXET, which will have a transglycolytic activity only, and in the presence of tXET and xyloglucan-derived oligosaccharides, which also confers a hydrolytic activity upon the enzyme (Arrowsmith and de Silva 1995).

8.2 Materials and Methods

8.2.1 Construction of cellulose/xyloglucan composites in the presence of tXET and tXET and xyloglucan-derived Oligosaccharides

Bacterial inoculum was obtained from actively cellulose-synthesising *A xylinus* as described in Materials and Methods (2.2.8.1 and 2.2.8.2). Incubation was performed statically at 30° C in Hestrin-Schramm media pH 6.2 containing 0.5% native xyloglucan plus tXET or tXET and xyloglucan-derived oligosaccharides as described in chapter two, section 2.2.9c.

8.2.2 Monosaccharide Analysis of Composite Material

Monosaccharide analysis was performed on the freeze-dried material as described in section 2.2.5.2 a and b (H_2SO_4 and TFA hydrolysis respectively) of Materials and Methods.

8.2.3 Removal of Loosely Bound Xyloglucan by Agitation

Monosaccharide and oligosaccharide analysis (sections 2.2.6 and 2.2.5 respectively) was carried out on the residual material and the xyloglucan that was removed during overnight agitation (section 2.2.10.3).

8.2.4 Sequential Extraction Of Xyloglucan From Composites Using KOH

Sequential extraction of xyloglucan from the composites with increasing concentrations of KOH was carried out as described in section 2.2.10.1. For all samples, oligosaccharide analysis of

extracted material was determined by overnight digestion with *endo*glucanase (source *Trichoderma reesei* ; Megazyme Ltd) and HPAEC (Section 2.2.5). Monosaccharide analysis by HPAEC of the freeze-dried residual material was carried out after acid hydrolysis (Section 2.2.6).

8.2.5 Enzymatic Digestion of Cellulose/Xyloglucan Composites

Approximately 10 mg (wet weight) was incubated overnight with 0.45U *endo*glucanase (Megazyme Ltd) at 30° C with constant agitation. After incubation the enzyme was denatured in a boiling water bath for 15 minutes as described in section 2.2.10.4 of chapter 2.
Residual material was collected by centrifugation (Microcentaur, 10 minutes) and freeze-dried for monosaccharide analysis (section 2.2.6). The oligosaccharide composition was analysed by direct injection into the HPAEC using the gradient described in section 2.2.5.

8.2.6 Deep -Etch Freeze Fracture Transmission Electron Microscopy

All microscopy was carried out as described in Materials and Methods (chapter 2, section 2.2.12).

8.2.7 Uniaxial Tensile Testing

All uniaxial tensile testing was carried out as described in Materials and Methods (chapter 2, section 2.2.11).

8.3 Results

After 96 hours static incubation at 30° C the pellicles resulting from the composite construction with cellulose synthesised by *A xylinus* and tamarind seed xyloglucan in the presence of tXET and tXET plus xyloglucan-derived oligosaccharides were aseptically removed from the flasks and washed extensively with sterile deionised water. Pellicles constructed in the presence of tXET were homogeneous and gave the impression of being more 'robust' and more stiff than the control pellicles. In contrast, those pellicles constructed in the presence of tXET and oligosaccharides were very heterogeneous and were more pliable than those of the control xyloglucan.

8.3.1 Levels of Xyloglucan Incorporation

The levels of incorporation of control xyloglucan, of xyloglucan in the presence of tXET and tXET plus xyloglucan-derived oligosaccharides, into the composites are shown in Table 8.1. Xylose was used as an internal marker for xyloglucan. The data for the native, control composite is consistent with that previously found (Whitney et al 1995, 1 : 0.38 when culture flasks were agitated; Hackney et al 1994, 1 : 0.43 for a similar system to that of Whitney and coworkers and Hayashi and McLachlan 1984, 1 : 0.7 pea stem primary walls).

When composite construction was carried out in the presence of tXET the cellulose to xyloglucan ratio remained similar to that of the control. When composite construction occurred in the presence of tXET and xyloglucan-derived oligosaccharides, however, the level of xyloglucan incorporation decreased by ca. 50% indicating that the presence of the oligosaccharides in the reaction mixture either modifies the xyloglucan in some way (by lowering the molecular weight) or, in causing the xyloglucan to become less concentrated had the effect of increasing the concentration of cellulose in the composite.

Table 8.1 Initial Cellulose : Xyloglucan Ratios Obtained From Monosaccharide Analysis of the Composites Using Acid Hydrolysis and HPAEC (Results from 6 repeats)

Composite Construction Conditions	Cell : Xyl Ratio
Native XG only	1 : 0.54 ± 0.10
XG + tXET	1 : 0.47 ± 0.10
XG + tXET + Oligos	1 : 0.25 ± 0.12

8.3.2 Removal of Xyloglucan by Agitation Only

Overnight agitation of the pellicles in 50mM ammonium acetate buffer pH 5.0 removed ca. 40% of the associated xyloglucan from the pellicle constructed in the presence of tXET and tXET plus oligosaccharides (Table 8.2). Agitation of the control pellicle, however, also dislodged around 30 % of the associated xyloglucan. This is in contrast to the data from chapter six (section 6.3.2) where only 4% of the xyloglucan associated with cellulose was removed. This could be due to the variability ie 'shaking speed' of the equipment used or to the time taken for construction of the composites, which was double that taken for pellicle production in chapter six.

Table 8.2 Cellulose : Xyloglucan Ratios After Agitation (Obtained from monosaccharide analysis of the composites using acid hydrolysis and HPAEC (Mean of 3 results)

Composite Construction Conditions	Cell : Xyl Ratio
Native XG only	1 : 0.36
XG + tXET	1 : 0.29
XG + tXET + Oligos	1 : 0.16

There were no differences found in the oligosaccharide composition of the xyloglucan removed from the composites (data not shown).

8.3.3 Sequential Extraction of Xyloglucan from the Composites

Treatment of native xyloglucan/cellulose pellicles and pellicles grown in the presence of tXET and tXET plus oligosaccharides with increasing concentrations of KOH showed a progressive reduction in the xyloglucan content as determined by monosaccharide analysis (Table 8.3). Approximately 50% of the native xyloglucan was removed by relatively mild alkali, which is consistent with that previously found (Whitney 1996 and chapter 6, this thesis). The composite constructed in the presence of tXET only also had ca. 45% of the xyloglucan removed by 0.5 M KOH as did the pellicle constructed in the presence of tXET and oligosaccharides. This suggests that, although the initial levels of associated xyloglucan differ in the test pellicles, the xyloglucan in each case is either only loosely bound, is present as a surface coating to, or forming cross-linkages between, the cellulose moiety of the pellicle.

Table 8.3 Cellulose : Xyloglucan Ratios After Sequential Extraction of the Xyloglucan with Potassium Hydroxide Using Acid Hydrolysis and HPAEC (Mean of 3 Results)

Alkali Treatment	Cellulose : Xyloglucan Ratio of Composite Remaining and conditions of growth		
	Native	tXET only	tXET and oligos
0.5 M KOH X 2	1 : 0.28	1 : 0.26	1 : 0.11
1.0 M KOH X 2	1 : 0.12	1 : 0.16	1 : 0.08
4.0 M KOH X 1	1 : 0.06	1 : 0.08	1 : 0
4.0 M KOH X 2	1 : 0	1 : 0	1 : 0

Application of 1.0 M KOH to the pellicles further removed ca. 50%, 40% and 30% of the remaining xyloglucan from the control, tXET and tXET and oligosaccharides grown pellicles, respectively, bringing the control and tXET pellicles to similar levels. That 4M KOH was required for the complete removal of xyloglucan from the cellulose, in each case, indicated that microfibrillar swelling was required to remove all of the xyloglucan. The requirement for 4M KOH to completely remove the xyloglucan from the control and test pellicles implies intrafibrillar association similar to that seen in primary cell walls.

Complete removal of xyloglucan from the tXET plus oligosaccharides pellicle was achieved by a single application of 4M KOH suggesting that the association between the xyloglucan and cellulose was not as intimate as observed for the control and tXET xyloglucan.

The oligomeric compositions of the xyloglucans extracted from the composites by increasing concentrations of alkali were determined by *endo*glucanase digestion and HPAEC. No structural differences were observed in the extracted XG compared to that before incorporation (data not shown).

8.3.4 Enzymatic Digestion of Xyloglucan / Cellulose Composites

Monosaccharide analysis of the composites after digestion by endoglucanase (Table 8.4) showed that the levels of xyloglucan remaining in the pellicles of the two test composites were consistent with ca. 75% xyloglucan removed from the tXET pellicle and ca. 60% of the originally associated xyloglucan removed from the tXET plus oligosaccharide pellicle. The removal of ~50% xyloglucan from the control composite is consistent with that previously found (Whitney et al 1996 and this thesis, chapter 6).

As it is believed that endoglucanase will digest only that xyloglucan which is loosely associated to or involved in cross-linkages between the microfibrils, that remaining associated in each case possibly represents a more intimately associated XG which is not accessible to the enzyme. The xyloglucan content remaining after endoglucanase digestion is equal to that remaining after treatment with 1M KOH. This suggests that this remaining

xyloglucan is intimately associated with the cellulose component as intrafibrillar swelling (treatment with 4M KOH) is required for complete removal of the xyloglucan (Chambat *et al* 1984, Edelman and Fry 1992, Hayashi and MacLachlan 1984).

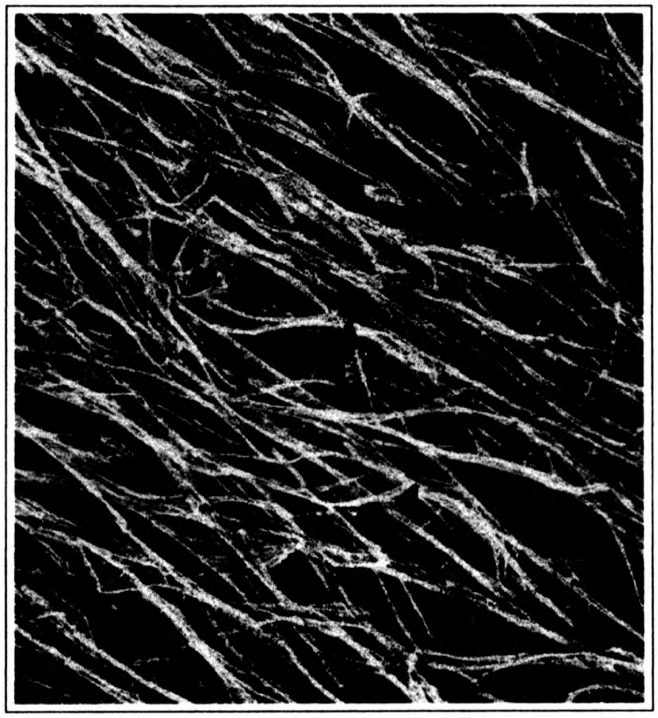
Table 8.4 Cellulose : Xyloglucan Ratios of Composites after action of *Endo*glucanase Obtained using Acid Hydrolysis followed by HPAEC (mean of 3 results)

Composite Construction Conditions	Cellulose : Xyloglucan Remaining
Native XG	1 : 0.20
XG + tXET	1 : 0.11
XG + tXET + Oligos	1 : 0.10

The oligosaccharide composition of the xyloglucan removed from the composites again reflected that which was added to the *A. xylinus* system (data not shown).

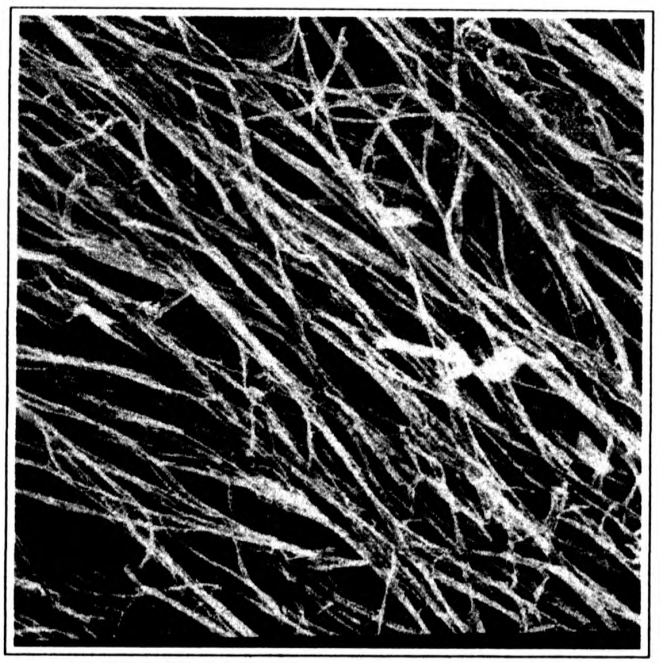
8.3.5 Deep-Etch Freeze-Fracture Transmission Electron Microscopy

Micrographs of tungsten/tantalum/carbon replicas of composites produced by fermentation of *A xylinus* in the presence of native xyloglucan, xyloglucan in the presence of tXET and xyloglucan in the presence of tXET and xyloglucan-derived oligosaccharides are seen in figures 8.1, 8.2 and 8.3, respectively.



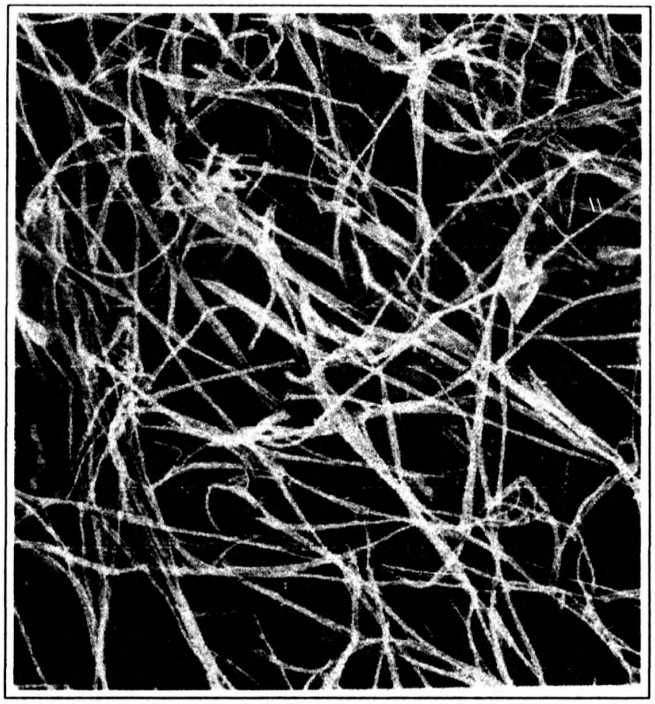
X 40000 ——— 0.26μm

Figure 8.1 Micrograph of tungstan/tantalum/carbon replica of composite composed by fermentation of *A xylinus* in the presence of native high molecular weight xyloglucan illustrating abundant xyloglucan cross-linkages (arrows) and lateral order conferred upon the cellulose microfibrils by the xyloglucan.



X 40000 ——— 0.25µm

Figure 8.2 Micrograph of tungstan/tantalum/carbon replica of composite composed by fermentation of *A xylinus* in the presence of native high molecular weight xyloglucan and tXET. This figure illustrates essentially the same features as the control ie xyloglucan cross-linkages (arrows) and a lateral order conferred upon the cellulose microfibrils by the xyloglucan.



X 40000 ——— 0.25µm

Figure 8.3 Micrograph of tungstan/tantalum/carbon replica of composite composed by fermentation of *A xylinus* in the presence of native high molecular weight xyloglucan and tXET plus xyloglucan-derived oligosaccharides. This figure shows that no lateral order or cross-linking occurs in the presence of oligosaccarides. This is thought to be as a result of the presence of oligosaccharides effectiely lowering the molecular weight of xylglucan .

These figures show different areas of the control and test xyloglucan/cellulose composites at a magnifications of 40000. Figure 8.1 show the lateral order achieved by the cellulose when grown in the presence of xyloglucan and the cross-linking that occurs between the cellulose microfibrils. This is consistent with that found previously (Whitney *et al* 1995 and chapter 6, this thesis) and with the role proposed for the network in primary cell wall models (Carpita and Gibeaut 1993, McCann and Roberts 1991, Talbot and Ray 1992a). Several thin strands assigned to xyloglucan can be observed at both magnifications forming bridges between neighbouring cellulose ribbons.

Figure 8.2 again show areas of the composite (constructed in the presence of tXET) where cross-linking of cellulose occurs to a slightly lesser extent than that observed for the native XG/cellulose composite. The aligned microfibrils, however, appear to be more dense than those of the native XG/cellulose composite which may be due, in part, to the longer incubation time for composite construction.

The minimal interaction observed in figures 8.3, where a random arrangement of cellulose microfibrils can be seen, indicates that the presence of xyloglucan-derived oligosaccharides lowers the degree of interaction between xyloglucan and the synthesising cellulose. It is possible that the oligosaccharides preferentially bound to the newly synthesised cellulose but it could also be possible that the concentration of oligosaccharides in the reaction solution was such that the tXET catalysed the transglycosylation reaction between a high molecular weight xyloglucan and an oligosaccharide, essentially creating a lower molecular weight polymer even although the enzyme possesses no hydrolytic activity *per se*.

8.3.6 Uniaxial Tensile Testing

Limited uniaxial tensile testing detected substantial differences in the mechanical behaviour when composite construction took place under test conditions (figures 8.4 a, b&c). In the presence of xyloglucan under non-modifying conditions (figure 8.4 a), the composites have a

lower stiffness and greater extensibility than cellulose alone and are consistent with that previously reported (see chapter 6, figure 6.8).

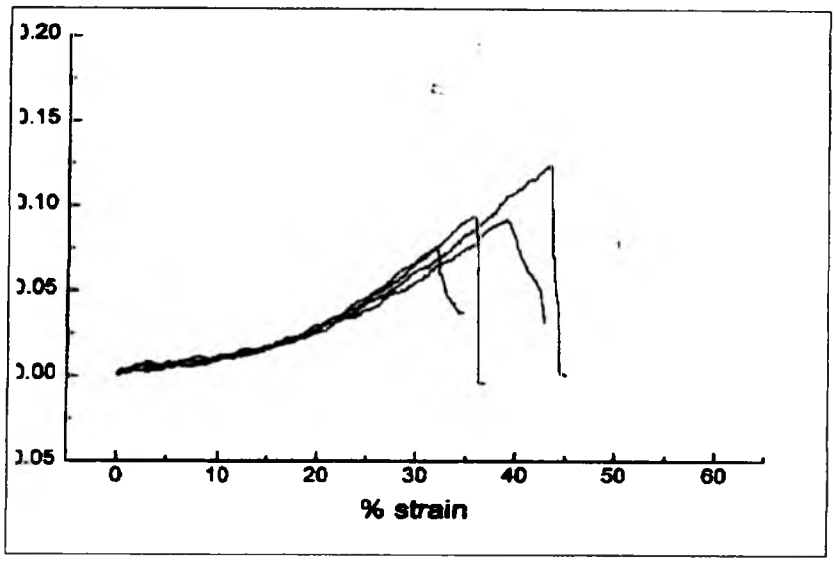


Figure 8.4 a Uniaxial tensile testing of the control composite

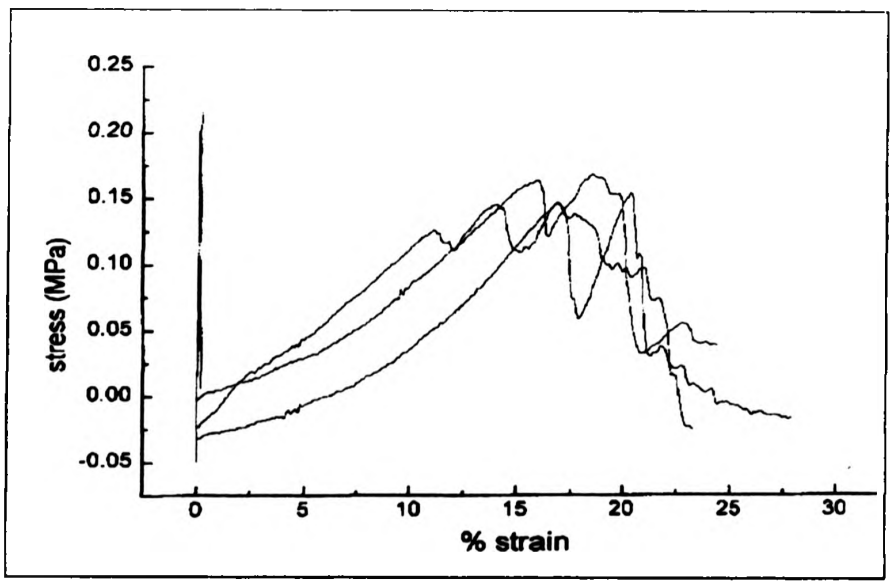


Figure 8.4b Uniaxial tensile testing of the composite grown in the presence of tXET

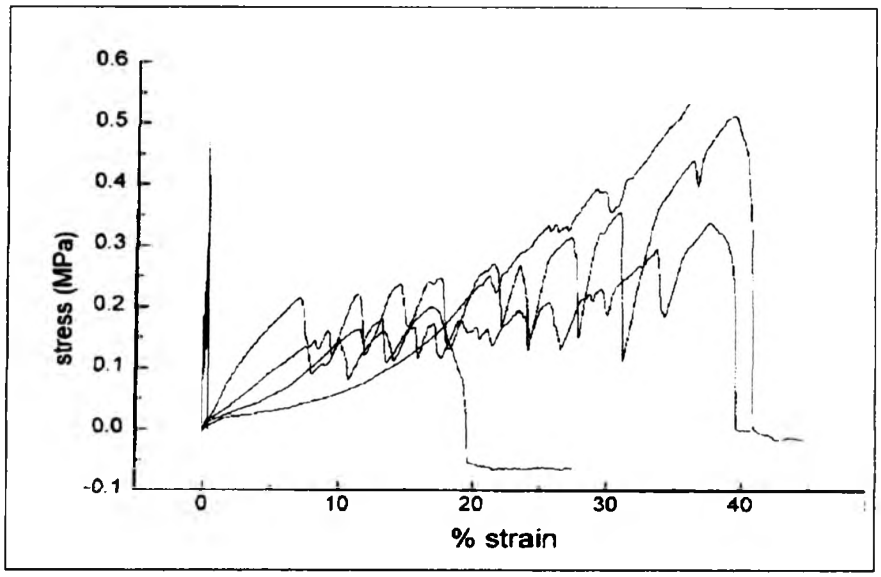


Figure 8.4 c Uniaxial tensile testing of the composite grown in the presence of tXET and xyloglucan-derived oligosaccharides

Due to lack of material only three runs of the composites constructed with TXET and xyloglucan-derived oligosaccharides were possible and as such, it was difficult to make assumptions from these.

8.4 Discussion

The requirements for primary cell walls are high strength and the ability to accommodate turgor-driven cell expansion. For this to take place certain cell wall-modifying enzymes must be present. Xyloglucan endotransglycosylases are a group of enzymes which have been implicated in mediating cell expansion (Rose and Bennett 1999). As a mimic of a cell wall assembly the construction of cellulose /xyloglucan composites was carried out in the presence of modifying conditions that might be expected at the cell wall.

The presence of tXET in the reaction mixture produced a 'robust' pellicle which, in appearance and in the main biochemically, resembled that of the control. The observation of considerable lateral alignment accompanied by cross-linking suggested that the modifying enzyme had, in effect, not changed the molecular integrity of the xyloglucan. Sequential extraction of xyloglucan with increasing strengths of KOH indicated that microfibrillar swelling was required for complete removal of the associated xyloglucan from the composites indicating intimate association essentially identical to the control composite.

Upon digestion with endoglucanase, however, only one quarter of the xyloglucan initially associated with cellulose remained. This observation suggest minimal interaction with cellulose.

The modifying presence of tXET and oligosaccharides produced pellicles which were very heterogeneous in appearance and, in all aspects of testing, were indicative of having little interaction with cellulose, although a degree of microfibrillar swelling (4M KOH x1) was required for complete removal. The presence of oligosaccharides alongside tXET could also have had the effect of lowering the molecular weight of the xyloglucan to a level where little association would occur (Whitney *et al* 1995).

Other variable factors affecting this system were possibly due to the non-standardised conditions involved. Different inoculum levels could have affected the time taken for the bacteria to produce sufficient amounts of cellulose for initiation of xyloglucan association. The inoculum used in the initiation of the composite construction was, in this case, lower than that used previously. Three or four single colonies from a plate were inoculated into Ringers

solution which then had the xyloglucan and modifying conditions added. Time would have been taken for the bacteria to have multiplied to such a density as to produce the cellulose required for association with xyloglucan. In chapter 6 the galactose-depleted xyloglucan was added to actively growing *A. xylinus* thus reducing the time for the xyloglucan to begin associating with cellulose. This time lapse would lead to the enzyme and substrate reacting together in the absence of any cellulose and thus the mimicking of conditions at the cell wall does not occur. These conditions could have led to variable levels of xyloglucan incorporation and probably to a xyloglucan which has already been 'modified' before association begins to take place.

Further investigation by NMR would indicate whether the composites grown in these modifying conditions had altered the crystalline structure of cellulose.

Future work should involve the inoculation of bacteria in the growth phase into the medium containing xyloglucan and the modifying conditions.

Chapter Nine

9. Discussion

Growing cells are surrounded by a metabolically active extra cellular matrix which is capable of expanding (Kerr and Baily 1934). The structural integrity and strength of the wall is thought to depend on the integrity of the cellulose/ hemicellulose (xyloglucan or arabinoxylan) network and enzyme-catalysed modification of the hemicellulosic component of this network is considered to be essential for expansion during turgor-driven growth. (Talbott and Ray 1992).

The structural components of the cellulose/hemicellulose network are well known. Cellulose microfibrils consist of linear molecules of β-1,4-linked D-glucopyranosyl residues, hydrogen-bonded together. The major hemicellulose in non-graminaceous monocotyledonous and dicotyledonous plants is xyloglucan which, like cellulose, has a β-1,4-linked D-glucopyranosyl backbone but which is extensively substituted at C6 with α-D-xylopyranosyl residues and β-D-galactopyranosyl (1,2) α-D-xylopranosyl residues. The structure and molecular distribution of the side chains varies in different plant tissues and species.

A most important feature of xyloglucans is their ability to form non-covalent associations with cellulose (Valent and Albershiem 1974, Hayashi 1989, Hayashi *et al* 1989) and cellulose microfibrils cross-linked with putative xyloglucan 'threads' have been visualised by electron microscopy of rotary shadowed replicas of rapidly frozen, deep-etched cell-wall samples (McCann *et al* 1990, McCann *et al* 1992, Itoh and Ogawa 1997). This method was also used to visualise xyloglucan cross-links apparent in the artificially assembled bacterial cellulose/xyloglucan composites (Whitney *et al* 1995).

As yet, little is known of the actual sites of, and molecular species involved in the interaction of xyloglucan with cellulose. Prevailing hypotheses include: Induction of a 'flattened' (cellulose-like) region of the xyloglucan molecule by adjacent fucosylated side chains. This region would then bond with cellulose and cause propagation of this behaviour by the reorientation of other fucosylated side chains (Levy *et al* 1991 and 1997). Alternatively, Finkenstadt *et al* (1995) postulated that association of the glucan backbone with cellulose was

sterically hindered and suggested that xylopyranosyl residues were the source of the hydrogen bond where lattice spaces on the surface of the cellulose crystal were filled with these residues. The presence of galactose and/or fucose residues modulated binding and the ability, of xyloglucan, to bind (or not) to cellulose was dependent on the conformation of the (1,6)-linkage of the xylose.

The advent of the assembly of cellulose/xyloglucan networks, *in vitro*, using the cellulose-producing *Acetobacter xylinus* and tamarind seed xyloglucan allowed ultrastructural and molecular features of the interaction between cellulose and xyloglucan to be studied (Whitney *et al* 1995). Centimetre-sized networks are obtained from this system allowing mechanical measurements to be made which can be extrapolated to native cell walls.

In this work we proposed to test whether the *A xylinus* system could be used effectively to further clarify the association between xyloglucan and cellulose. Would networks resulting from cellulose synthesised into medium containing modified xyloglucans provide more information about the involvement of side-chains in the intimate relationship? Would the presence of an enzyme, expected to be found *in muro*, produce a composite network which would assist in explaining the role of xyloglucan-modifying enzymes in turgor-driven cell expansion by showing the importance of sidechain / cellulose interactions?

The successful purification and isolation of active enzyme preparations of NXET, β-galactosidase, α-xylosidase and β-glucosidase from germinated nasturtium cotyledons (chapter three) provided, along with xyloglucan, the 'raw materials' required for the tailoring of the polysaccharide prior to its introduction into the *A xylinus* system. Initial problems with old seeds led to additional work being carried out into the optimisation of conditions for the overexpression of NXET in *Pichia pastoris* (chapter four). This work was a continuation of an honours project by Jennifer Milne (1997), where NXET was successfully cloned into the yeast expression system and production of an active enzyme was achieved.

After the initial problems of the nasturtium purified enzyme activity were solved, xyloglucans with differing degrees of galactose depletion were produced (chapter five). The removal of galactose from a static incubation of nasturtium β-galactosidase led, as had been previously noted (Reid *et al* 1988, Fanutti *et al* 1993) to a 'gel' being formed. This gel differed from that

formed when tamarind seed xyloglucan was incubated with β-galactosidase of fungal source (*Aspergillus oryzae* , Shirakawa *et al* 1998). The gel produced (35% galactose depletion) in this case (Shirakawa *et al* 1998) was robust and possessed the unique property of forming a 'handlable' gel on heating and reverting to a solution state on cooling whereas that produced by the nasturtium enzyme resulted in precipitation of the galactose depleted xyloglucan upon perturbation of the networked 'gel'.

Stirred incubations of nasturtium β-galactosidase resulted in aggregation and subsequent precipitation of the lower galactose content molecules with, as expected, faster removal of galactose from the 'less crowded' side of the molecule.

^{13}C NMR evidence indicated that the aggregated xyloglucan assumed a similar molecular organisation to non-crystalline cellulose. This is in contrast to the gel resulting from addition of 1M Na_2SO_4 where the conformation was that found when xyloglucan is in solution.

The functionality of galactose substitution *in planta* is implicated in these results. The data suggests that if galactose were not present, a self-associated insoluble precipitate would result and that this probably would not be available to associate with cellulose thus the basic integral structure would not exist.

Upon the construction of composites using the galactose-depleted molecules (chapter six) it was found that, in the *A xylinus* system, self-associated xyloglucan (60% galactose-depleted) had minimal interaction with the synthesising cellulose. Digestion with *endo*glucanase and sequential extraction of xyloglucan from the composites by KOH indicated only very loose, surface association took place. No change to the crystalline content of the cellulose was observed with the modified polysaccharide as the self-aggregated xyloglucan was not available to 'integrate' into the growing microfibrils of cellulose.

Galactose, indeed, provides steric hindrance in the self-association of xyloglucan but in order to investigate whether it affects the binding of xyloglucan to cellulose in the *A xylinus* system, the time-scale of the experiment would have to be changed. Self-association of xyloglucan is avoided in higher plants where it is synthesised in the Golgi apparatus and transported, with attached galactose residues, in vesicles, to the plasma membrane. There, it is believed, the vesicles fuse with the plasma membrane providing xyloglucan at, or close to, the site of

cellulose synthesis. Pre-modification of xyloglucan, then, does not mimic this system. If we assume that the xyloglucan modifying enzymes are present at the cell wall, then tailoring of the xyloglucan, if it occurs, must be carried out concomitant with the synthesis of cellulose.

The conclusions drawn from the data collected in chapters five and six can also be applied to chapter seven, where a pre-modified xyloglucan of lowered xylose content (and of lowered molecular weight) was present in the medium in which *A xylinus* was synthesising cellulose. Again minimal association with cellulose was observed which was probably a result of the reduced molecular weight (as a consequence of the NXET action) rather than a result of the lowered xylose content. The successful isolation of a higher molecular weight xyloglucan with lower xylose content (as reported in chapter seven) could shed light on the importance of xylose in the association with cellulose when it is introduced into the bacterial system later this year but again, this is a pre-modified polymer and as such, data collected should be reviewed with caution.

Chapter eight represented a change to the pre-modification of the polysaccharide with cellulose/xyloglucan composite construction occurring in the presence of tXET and in the presence of tXET and xyloglucan-derived oligosaccharides. In the case of the former conditions the resultant pellicle produced data similar to that of the control pellicle in all but the *endo*glucanase digestion, which suggested minimal interaction only. Examination on an ultrastructural level revealed lateral alignment of cellulose microfibrils with extensive cross-linking similar to that found for the control composite

The addition of oligosaccharides into this medium had the effect of, again, introducing very little interaction with cellulose. This could have been due, either to the solution of oligos diluting the xyloglucan to the extent that it did not associate with cellulose or that, in the presence of tXET the oligosaccharides effectively lowered the molecular weight of the xyloglucan in the medium.

The method of composite construction differed slightly in the case of the composites constructed in chapters seven and eight inasmuch as the inoculum used in the work for these chapters was from three to four single colonies from a glucose/yeast extract agar plate stored at 4° C, mixed in Ringers solution, whereas that for the galactose-depleted work came from

actively growing *A xylinus*. As a consequence the composites took longer to grow and any action of the enzyme would have already taken place before the bacteria started to produce cellulose.

So what is the functionality of xyloglucan sidechains in the xyloglucan/cellulose interaction in the *A. xylinus* system and can any information elucidated here be ascribed to behaviour *in muro*? Investigations on nasturtium cotyledons showed that the β–galactosidase activity peaks after that of NXET. Thus the higher molecular weight xyloglucan would possibly be hydrolysed into oligosaccharides before galactose residues are removed and so the self-association of xyloglucan would not occur. If this also is the case at the primary cell wall, it seems apparent that the main function of galactose residues on xyloglucan is to provide steric hindrance to stop self-association taking place on the route from the Golgi to the cell wall. The removal of xylose from xyloglucan, on the other hand, does not result in self-association but could, consequentially, lead to a conformational change by the action of NXET and α–xylosidase. As both enzymes are active at the same time, in the same location in the cell wall, it is reasonable to assume the a 'doublet' of unsubstituted glucose residues may result from the synergistic action of the enzymes. Would this conformational change alter the way in which the xyloglucan adopts a 'cellulosic' structure to associate with the cellulose?

The use of the *A xylinus* fermentation system to provide further information about the interaction between cellulose and xyloglucan in the plant cell wall, by the introduction of enzymatically tailored xyloglucans has met with only limited success but has provided the investigator with other avenues to pursue. The addition of pre-modified xyloglucans into the non-standardised system could not effectively mimic naturally occurring events and to this end future work may concentrate on the standardisation of the system ie levels of inoculum and timing of addition of xyloglucan in order to ensure concomitant synthesis of cellulose. The system would then be able to be used, via the introduction of cell wall-modifying enzymes into the growing network to elucidate information which then can be extrapolated to the situation in the cell wall.

Chapter Ten

10. References

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