TWO THERMOSTABLE HEMICELLULASES AND THEIR EFFECTS ON WOOD PULPS

A thesis submitted in partial fulfilment
of the requirements for the Degree
of
Doctor of Philosophy
in
Biological Sciences
at the University of Waikato
by

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1992

ABSTRACT

Two hemicellulases, cloned from a thermophilic anaerobe (isolate TP8 6.3.3.1) were partially purified and characterised. The ability of these enzymes to hydrolyse the hemicellulose components of various types of pulps and their effects on the bleachability of radiata pine kraft pulp were examined. The xylanase (two preparations) and mannanase had broad pH optima (6-7.5). The xylanases had temperature optima of 70°C (10 min assay) and the mannanase had an optimum of 80°C (10 min assay). All the cloned enzymes were thermostable with more than 95% of the enzyme activity remaining after 24 hours at 70°C, in an unbuffered solution. Buffering affected the thermostability of the enzymes. The mannanase had a half life of 48 min at 85°C. The xylanases were able to hydrolyse xylans but also had a pH-dependent CM cellulase activity, but this was never more than 10% of the xylanase activity. The mannanase hydrolysed mannan, glucomannans and galactomannans. The glucose content of the glucomannans had little or no effect on mannanase activity but the galactose substituents of the galactomannans severely inhibited the hydrolysis of these substrates.

The thermostable xylanase hydrolysed more than 75% of soluble radiata pine xylan to low molecular weight (<DP10) xylo-oligomers. These included; xylose, xylobiose, xylotriose, arabinose-substituted xylobiose and xylotriose and 4-O-methyl glucuronic acid substituted xylotrioses. The arabinose substituents were bound to the terminal, non-reducing xylose residue; the glucuronic acid substituents may be bound to the non-reducing or central xylose residues of the substituted xylotriose. The thermostable mannanase also hydrolysed more than 75% of soluble radiata glucomannan, producing mannose, mannobiose, mannotriose, glucomannose and glucomannobiose.

The hemicellulases were used to treat (at 70°C, 24 hr) a variety of wood pulps, including kraft (softwood and hardwood), radiata NSAQ pulp, radiata CTMP and radiata TMP. Both enzymes solubilised their respective substrates from these pulps. The chemical pulps were more susceptible to enzymatic attack than the mechanical pulps, possibly due to the greater fibre porosity of the chemical pulps. The xylanase solubilised ~4% of the xylan and the mannanase solubilised 10% of the glucomannan from radiata kraft pulp. In comparison with other xylanases, the

thermostable xylanase was less effective in solubilising xylan from kraft pulps. The hemicelluloses of kraft pulps that had been enzyme treated were extracted. The extracted xylan from the xylanase-treated pulp was lower in average DP than the xylan extracted from untreated pulps. The average DP of the extracted glucomannan from the mannanase-treated pulps was no different than that of glucomannans extracted from untreated pulps. The extracted xylan and glucomannan from untreated kraft pulps were readily hydrolysed by the xylanase and the mannanase respectively, suggesting that enzyme inaccessibility to the substrate within the fibre wall was the main cause of their resistance to enzymatic attack.

The effect of these enzymes on the bleachability of radiata kraft pulps was studied by measuring the reduction in kappa number (κ), relative to untreated controls, after a standard D/C E prebleaching sequence. The xylanase and the mannanase had little or no effect on the bleachability of radiata kraft pulps. Xylanase treated kraft pulp required a protease treatment after the hemicellulase treatment but before bleaching, in order to elicit any improvement in bleachability (~7% drop in κ relative to untreated control pulps). Mannanase treated kraft pulp showed no improvement in bleachability. For comparison commercial xylanases (including a thermostable xylanase) were used to treat radiata kraft pulps and did improve bleachability significantly. These enzymes also solubilised three to four times more xylan than the cloned, thermostable xylanase. Therefore, the ineffectiveness of the cloned xylanase may be due to its very limited ability to hydrolyse intra-fibril xylan in kraft pulp fibres.

ACKNOWLEDGEMENTS

I would like to gratefully acknowledge my supervisory committee Dr. Tom Clark, Dr. Hugh Morgan, Dr. Keith Mackie and Dr. Roy Daniel for their support, advice and encouragement. In particular I would like to express my appreciation for their support during the hectic "final days".

I also wish to acknowledge several individuals;

Roger Meder and Suzanne Gallagher for the NMR analysis,
Mary Bruce for instruction on pulp bleaching and analysis,
Diane Steward for help with sugar and pulp analysis, HPLC and GC,
Colin Monk and Lynne Parker for laboratory assistance at Waikato,
Steve Wrathall for viscosity and brightness analysis,
Dell Bawden for water retention values,
Jurgen Puls for anion exchange chromatography.

I also wish to thank colleagues, friends and flatmates from Hamilton and Rotorua for the rewarding time I have spent in New Zealand.

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CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

Wood pulps require bleaching before they can be used in the manufacture of fine papers. This is particularly true for pulps that are produced by alkaline pulping processes such as the kraft process, which is the most commonly used method of producing chemical pulps. Historically, pulps have been bleached with elemental chlorine. This inexpensive chemical is an effective bleaching agent but results in the production and discharge of chlorinated organic compounds which may have detrimental effects on aquatic environments. These environmental concerns have compelled industry to reduce or eliminate chlorine in the bleaching stages of pulp manufacturing. One option is the use of hemicellulose degrading enzymes as bleaching enhancers.

Hemicellulase assisted bleaching was first reported in 1986 when it was found that enzyme-treated pulps required 35% less chlorine than untreated pulps to bleach to high brightness. The effectiveness and simplicity of enzymatic bleach enhancement has attracted the interest of the pulp and paper industry and many commercial hemicellulases have been developed. Ascertaining how these enzymes affect wood pulps is essential for the improvement of enzyme assisted bleaching. The search continues for superior enzymes that are more effective, stable under a variety of conditions and inexpensive. Thermostability in particular has been identified as a desirable property for hemicellulases used in enzyme assisted bleaching.

The geothermal regions of New Zealand are a source of thermophilic micro-organsisms that produce a range of enzymes stable at relatively high temperatures. Many of these thermostable enzymes have been produced and purified by genetic engineering. The thermostability of these enzymes can also be exploited to assist in their purification. This thesis describes the production and characterisation of two cloned, thermostable hemicellulases (a xylanase and a mannanase) and their effects on several types of wood pulps.

1.1 WOOD CHEMISTRY AND COMPOSITION

1.1.1 Wood Chemistry

Chemically, cellulose is a linear homopolysaccharide composed of D-glucopyrannose residues linked by ß-(1,4) glycosidic bonds (Aspinall 1970). The ß-linkage "twists" every other glucose residue about the longitudinal axis so that cellobiose (a gluco-disaccharide) is the actual repeating unit in the polysaccharide (Fig 1.1). As with the monomer, each cellulose chain has a reducing hydroxyl at the free C1 carbon and a non-reducing hydroxyl at the free C4 carbon.

In nature, individual cellulose chains run in parallel, with reducing ends oriented in the same direction (Phillips 1989). This type of configuration, known as cellulose I, is found in the microfibrils of the plant cell wall. Microfibrils are cellulose aggregates that form ribbon-like structures of indefinite length and cross-sectional dimensions of approximately 5nm by 10 nm (Mackie 1974). However, fibrils of smaller dimensions have been recognised (Fengel and Wegener 1984). Microfibrils form the structural framework of the plant cell wall and dictate many cell wall properties such as porosity and strength (Phillips 1989; Fengel and Wegener 1984; Harada and Cote 1984; Mackie 1974). Cellulose orientation within microfibrils is not clear but crystalline and amorphous regions are recognised, with wood having a crystallinity index of between 60 and 70% (Fengel and Wegener 1984).

Cellulose crystallinity is governed by a complex of intra-molecular and inter-molecular hydrogen bonds (H-bonds) (Mackie 1974; Phillips 1989; Okamura 1991). In cellulose I, intra-molecular H-bonds, such as those between C3 hydroxyls and C5 oxygens of adjacent glucose residues, stabilise individual cellulose strands (Okamura 1991; Phillips 1989; Mackie 1974). Inter-molecular H-bonds between C6 hydroxyls of one cellulose chain and C3 hydroxyls of neighbouring chains aid in holding chains in the microfibrillar configuration (Okamura 1991). Cellulose I can be irreversibly converted to the more stable cellulose II by mercerisation (treatment with alkali) (Aspinall 1970). In cellulose II, individual chains run in an anti-parallel manner enabling other types of H-bonding (Okamura 1991; Phillips 1989). Other types of cellulose can be prepared from these forms by treating with liquid ammonia (cellulose III) and heated glycerol (cellulose IV) (Okamura 1991).

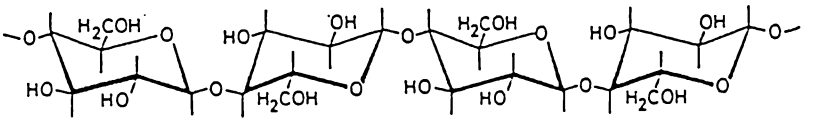

Fig 1.1 Chemical formula of cellulose
(Fengel and Wegener 1984;pg 69)

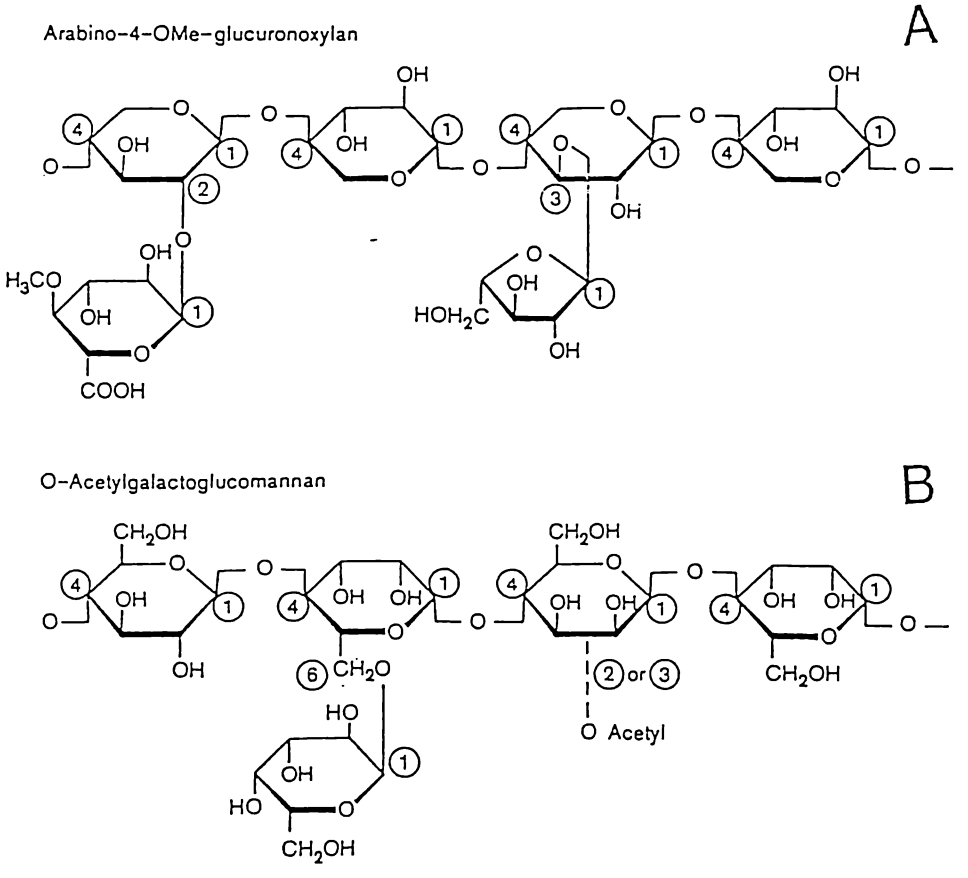

Fig 1.2 Chemical structure of softwood (A) arabino-4-OMe-glucuronoxylan and softwood (B) O-Acetylgalacto-glucomannan

Therefore, naturally occurring cellulose I is not the only possible arrangement of cellulose.

The length of the average cellulose molecule also affects various microfibril properties. Polysaccharide lengths are described by the degree of polymerisation (DP) which is defined as the ratio of the molecular weights of polysaccharide to that of the backbone monomer (Fengel and Wegener 1984). The degree of polymerisation accounts for some of the properties of celluloses such as solubility and viscosity of solutions (Aspinall 1970). In general, celluloses isolated from woods have DP of 4000 to 10,000 (Fengel and Wegener 1984).

Hemicellulose is the term used for the structural, non-cellulose polysaccharides found in plant cell wall (Wilkie 1983). In woods the two main hemicelluloses are xylans and glucomannans. The former is predominant in hardwoods, the latter in softwoods. These heteropolysacchrides, in association with lignin, form a matrix that surrounds cellulose microfibrils (Fry 1986).

Xylans are acidic, linear polysaccharides of ß-1,4 linked xylopyrannose residues (Aspinall 1970). The acidic moiety found in all wood xylans is due to 4-O-methylglucuronic acid side groups linked to the backbone by α-1,2 glycosidic bonds (Timell 1967). Hardwood xylans are acetylated in the O-2 or O-3 positions of the xylopyrannose residues (Timell 1967). These alkali-labile ester bonds are often lost during extraction procedures. Softwood xylans are not acetylated but possess L-arabinofuranose side groups joined to the backbone by α-1,3 glycosidic bonds (Fig 1.2A).

The structure of glucomannans are patterned on those previously described, namely a polysaccharide of mannopyrannose and glucopyrannose residues joined by ß-1,4 glycosidic bonds (Aspinall 1970). Mannose to glucose ratios of 3:1 are typical of most woods with the glucose residues distributed randomly throughout the polysaccharide (Timell 1967)(Fig 1.2B). Mannose residues of softwood glucomannans can be acetylated in O-2 or O-3 positions (Timell 1967). Glucomannans may possess α-1,6 galactose side-groups (Timell 1967; Aspinall, 1970). These highly substituted biopolymers are known as galactoglucomannans (Aspinall, 1970).

The carbohydrate fraction of wood is known as holocellulose and comprises not

only the cellulose and hemicellulose fractions but also lesser amounts of other polysaccharides such as arabinans, galactans and galacturonans, non-cellulosic glucans (starch, ß-1,3-glucans) and others (Fengel and Wegener 1985). The amounts and types of these compounds can vary with wood species and growth conditions.

The non-carbohydrate fraction of extracted wood is called lignin (Lewis and Yamamoto 1990). This substance is a high molecular weight, aromatic biopolymer formed by condensation reactions of certain phenylpropanoid monomers such as p-coumaryl, coniferyl and sinapyl alcohols (Higuchi 1990; Lewis and Yamamoto 1990). The aromatic moieties of these compounds are refered to as p-hydroxyphenyl, guaiacyl and syringyl, respectively. Their structure and system of nomenclature is presented in Fig 1.3.

Polymeric lignin is formed by the apparently random combinations of the various phenylpropanoid monomers linked by certain types of bonds (Higuchi 1990; Lewis and Yamamoto 1990). These include ß-O-4 ether linkages, the predominant bond type, as well as other types of ether linkages and phenyl-phenyl bonds (Higuchi 1990; Lewis and Yamamoto 1990; Higuchi 1985).

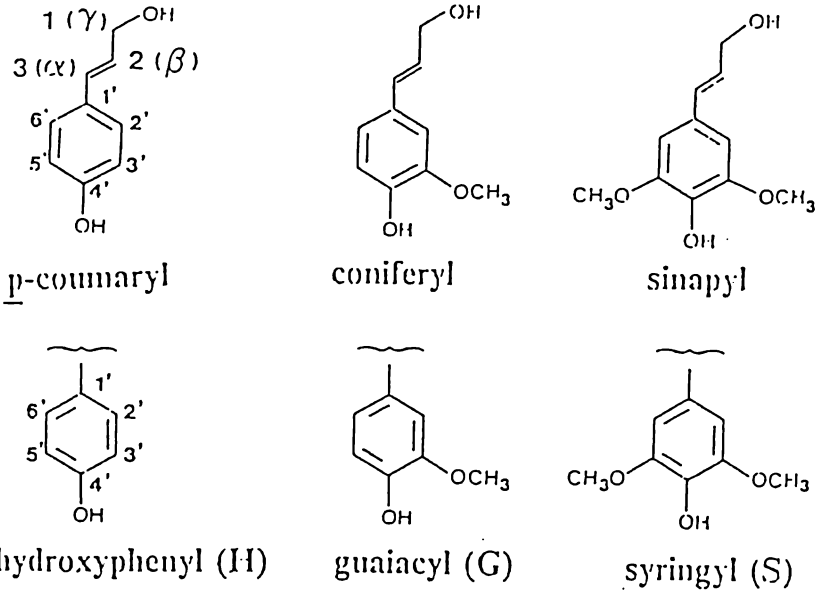

Fig 1.3 Lignin precursors and aromatic constituents (Lewis and Yamamoto 1990)

1.1.2 Wood Structure

Wood is a complex aggregation of plant cell wall material. In gymnosperms (softwoods), the main structural cells are the tracheid type. These are long, thin tubular cells with closed ends that are organised in a radial manner but run parallel to the stem axis (Fengel and Wegener 1984). Typical tracheid dimensions range from 3 to 5 mm in length and 20 to 80 μm in diameter (Harada and Cote 1985). Within individual trees, seasonal differences affect tracheid growth so that in spring large, thin-walled cells are formed (earlywood) while in late summer smaller, thick-walled cells develop (latewood) (Fengel and Wegener 1984). This differentiation results in annual growth rings being visible in cross section.

Angiosperms (hardwoods) are morphologically more complex than gymnosperms. Tissue strength is given by associations of libriform fibres and fibre tracheids which are structurally similar to softwood tracheids but smaller in size (Fengel and Wegener 1984). Typically, hardwood fibres and tracheids are 0.5 to 2.0 mm in length and have diameters of 10 to 40 μm (Fengel and Wegener 1984). Nutrient transport is by means of vessel elements, large open-ended tubes dispersed through the tissue.

Tracheid and fibre cell walls account for 80% to 90% of the mass of extracted wood and are composed of the three polymeric substances cellulose, hemicellulose and lignin (Fengel and Wegener 1984). The residual material is a mixture of starches, pectins and ash (Fengel and Wegener 1984). Microscopic examination of tracheid (or fibre) cross sections readily show that the cell wall consists of several, concentric layers (Fengel and Wegener 1984). A schematic of a model tracheid cell wall is given in Fig 1.4.

The outermost layer is designated the primary wall (P layer) and is formed during cell growth. The secondary wall (S layer) is constructed of several sub-layers and is formed during cell wall thickening (Saka 1991). A thin tertiary wall (T) may be present close to the cell lumen while the luminal surface itself may be covered by a thin warty layer (Fengel and Wegener 1984; Saka 1991). The layers are distingushed from each other by differences in composition and microfibril orientation.

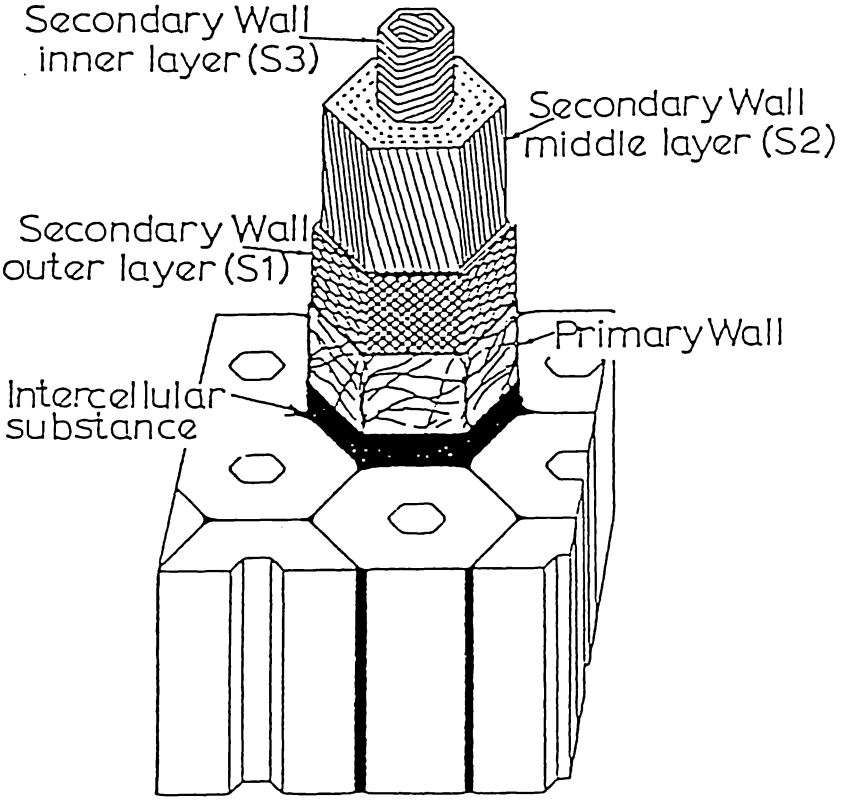

Fig 1.4 Schematic representation of plant cell wall (Mackie 1974)

General descriptions of the layers can be applied to most types of normal woods but it should be noted that variances between species, growth conditions or tension woods complicate cell wall ultrastructure. A useful method of describing cell wall composition is to compare the levels of individual components in the various layers of the cell wall with the "average" composition of the wood. In this way relative distributions in the various layers are emphasised. The relative proportions of cellulose, hemicellulose, lignins and other components varies between tree species as well as between individual trees within the same species. With this variation in mind, the average compostion of some hardwoods amd softwoods are presented in Table 1.1.

The primary wall is the thinnest of all the layers and has a high concentration of lignin. Cellulose concentrations in the P layer are lower than those of the S layers and microfibrils are oriented in a loose, indiscriminate manner (Fengel and Wegener 1984). Other polysaccharides present include xylans and pectic substances but levels of glucomannans are relatively low (Meier 1984; Saka 1991). The S_1 layer has near average levels of cellulose, glucomannans and xylans but little of the pectins found in the P layer. It is three to five times thicker than the P layer and can take up almost 15% of the total cell wall volume (Fengel and Wegener 1984). Microfibrils are arranged in a series of lamina in which the fibrils spiral in shallow angles around the tracheid or fibre. Lignin concentrations are lower than that of the P layer. The thickest layer (S_2) can occupy up to 75% of the total cell wall volume. It has average concentrations of cellulose and glucomannans and near average levels of xylans but no pectins (Saka 1991; Fengel and Wegener 1984; Meier 1984). The cellulose microfibrils, again in a laminar pattern, are aligned at an angle oblique to the longitudinal axis of the fibre (Fengel and Wegener 1984). Lignin concentrations of the S_2 are less than average but due to its large volume, most of the lignin is found in this layer (Saka and Goring 1984). The S_3 layer is similar in composition to the S_2 but microfibril arrangement is not as ordered. The same pattern is seen in tracheids that have tertiary cell wall layers (T layer). The warty layer found on the luminal surface is mainly galactoglucomannan with some xylan present (Saka 1991; Fengel and Wegener 1984). Takabe *et al.* (1988) have generalised softwood tracheids as having an outer S_2 layer rich in cellulose with the

Table 1.1 Chemical compositions of five extracted hardwoods and six extracted softwoods (from Timell 1967).

	% (w/w) Cell Wall Component				
	Cellulose	Lignin	Hemicellulose		Other[#]
			Xylan[x]	Glucomannan[g]	
Hardwood					
Acer rubrum (red maple)	45	24	25	4	2
Betula papyrifera (white birch)	42	19	35	3	1
Fagus grandifolia (beech)	45	22	26	3	4
Populus tremuloides (aspen)	48	21	24	3	4
Ulmus americana (white elm)	51	24	19	4	2
Softwood					
Abies balsamea (balsam fir)	42	29	9	18	2
Picea glauca (white spruce)	41	27	13	18	3
Pinus strobus (yellow pine)	41	29	9	18	3
Pinus radiata (radiata pine)*	38	25	9	18	5
Tsuga canadensis (eastern hemlock)	41	33	7	16	3
Thuja occidentalis (white cedar)	41	31	14	12	2

x in hardwoods - glucuronoxylan; in softwoods - glucuronoarabinoxylan

g in hardwoods - glucomannan; in softwoods - glucomannan and galactoglucomannan

\# pectins, starches and other non-cellulosic glucans

* from Smelstorius 1971 and 1974 (only 95% of yield recovered)

S_3 layer having higher xylan levels. The glucomannan concentration steadily increased from the S_1 to the lumen. In hardwood fibres, the S_1 and outer S_2 were richest in cellulose while the inner S_2 and S_3 layers were richest in xylan.

The inter-fibre space, known as the middle lamella, has a lignin concentration two to three times that of all the S layers. However, due to the relatively low volume of the middle lamella, less than 20% of the total lignin is found in these areas (Saka and Goring 1984). The middle lamella is virtually all lignin with some pectic substances but little or no hemicelluloses or cellulose. The term "compound middle lamella" includes the two P layers of adjacent fibres as well as the inter-fibre space. It is used when the P layers are not readily distinguishable from the true middle lamella.

Unlike the chemical or ultrastructural nature of wood, the exact nature of cellulose/hemicellulose/lignin interactions are still subject to much debate. It has been proposed that a network of cellulose microfibrils acts as the structural framework of the cell wall while hemicelluloses form a gelatinous matrix between microfibrils (Fry 1986). Lignin acts as an encrusting adhesive (Harada and Cote 1984). This representation suggests close associations between the various polymers including covalent bonds, hydrogen bonds, and ionic bonds as well as physical entanglement (Fengel and Wegener 1984; Fry 1986). Glycosidic lignin/xylan bonds have been identified in *P. radiata* with other types of lignin/polysaccharide bonds also possible (Smelstorius 1974). Ester linkages between the α-C of the phenylpropane residues and the uronic acid residues of xylans, ether linkages between the α-C and the arabinose residues of xylans as well as glycosidic linkages between galactose side-groups and the phenol hydroxyl of the phenylpropanoid have all been proposed (Fig 1.5) (Fengel and Wegener 1984). From microscopic examinations it is also apparent that the cell wall has a lamellar nature with microfibrils associated in thin plates (Kerr and Goring 1975; Scallan 1978). This type of microfibril orientation was evident after delignification and fibre swelling (Kerr and Goring 1975).

A model of the cell wall proposed by Kerr and Goring (1975) incorporates many of these observations (Fig 1.6). Cellulose microfibrils are arranged as lamella or plates surrounded by a hemicellulose layer. These plates are set within the

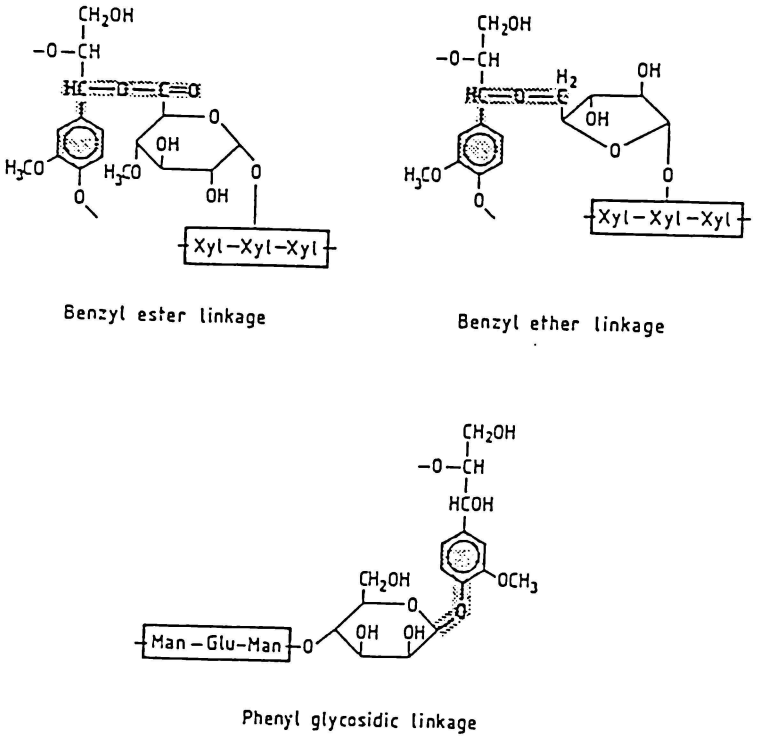

Fig 1.5 Possible lignin-polysaccharide linkages in wood (Fengel and Wegener 1984, pg 172)

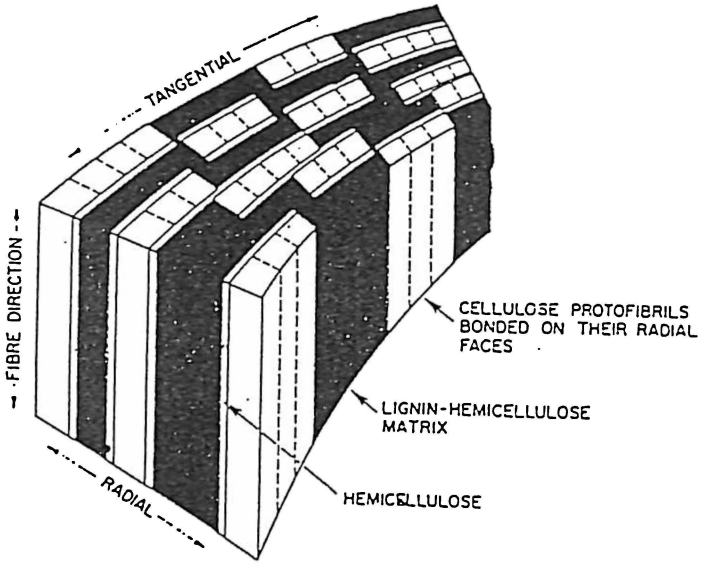

Fig 1.6 Interrupted lamella model proposed by Kerr and Goring (1975) for the ultrastructural arrangement of lignin, cellulose and hemicellulose in a fibril cell wall

porous hemicellulose/lignin gel matrix. During delignification, the porosity of the cell wall increases as the inter-lamellar matrix is removed (Kerr and Goring 1975; Scallan 1978). This serves as a reasonable working model of the cell wall.

1.2 PAPER MANUFACTURING

Paper manufacturing can be divided into two separate stages. The first is the pulping stage in which solid wood is disrupted into a fibrous mass (pulp); the second is the actual papermaking stage in which the pulp is drained, felted and dried into paper (Smook 1987). Prior to papermaking, pulps are often further worked to improve their physical properties. Examples of this are the bleaching stages of pulping in which residual lignin is removed or rendered colourless and pulp beating where mechanical forces render the pulp more suitable to paper manufacturing. Wood pulp that is used as a source of cellulose for chemical conversion to other products is known as dissolving pulp.

Wood can be pulped by mechanical, chemical or thermal processes. Pulping technologies that combine these processes have also been developed. The type of pulping process employed depends on the desired pulp properties and the economics of the individual processes (Scott 1984). In the following sections brief outlines of the more common pulping technologies are presented.

1.2.1 Mechanical Pulping

Mechanical pulping uses physical forces to disrupt wood into individual fibres. There are two main types of mechanical pulping; stone groundwood (SGW) and refiner mechanical pulping (RMP). Various modifications have been reported, particularly with RMP (Smook 1987). Stone groundwood pulping involves pressing blocks or bolts of wood into a rapidly rotating, abrasive stone surface (Smook 1987). The wood, pressed longitudinally into the stone to release intact fibres, is pulped by the rapid compression/decompression that occurs at the stone surface. The high temperatures (150°C to 190°C) generated by friction at the surface facilitate the pulping process by softening the lignin of the middle lammela (Fengel and Wegener 1984; Smook 1987). The fibres can then be washed from the stone with a shower of hot water (Smook 1987).

In refiner mechanical pulping, wood chips are fed into the narrow space between two large disks that are rapidly rotating in opposite directions (Smook 1987). Some designs involve one stationary disk and one rotating disk or one rotating disk between two stationary disks. The shear forces generated in the refiner serve to shred the chips into individual fibres. This shearing effect is assisted by "breaker bars"; thin wedges which run radially along the face of the refiner plate(s). These bars are widely separated at the centre of the plate (where chips are largest) but spaced closer towards the plate periphery (where chips have been broken down to fibres). Centrifugal force moves the fibres towards the edge of the refiner plate were they escape from the refiner. Water can be added at the centre of a plate to maintain pulp consistency (Smook 1987).

Thermomechanical pulping (TMP) is a modified RMP process where wood chips are steamed (under pressure) before refiner pulping (also under pressure). Steaming serves to soften the lignin of the middle lamellae thus facilitating the refiner pulping stage. Chemithermomechanical pulping (CTMP) includes a chemical and steam pretreatment before refiner pulping (Fengel and Wegener 1984). These pulping techniques produce a stronger pulp than simple RMP by reducing fibre damage and shive content (Smook 1987).

1.2.2 Chemical pulping

Two types of chemical pulping are prevalent. Together these two pulping techniques account for more than 60% of the annual global pulp output. The main form of chemical pulping is the sulphate, or kraft, process. The older sulphite process, though still of commercial significance, is declining in importance.

In the sulphite process wood chips are infiltrated with the cooking liquor composed of aqueous forms of sulphur dioxide (SO_2), in the presence of a cationic base. The chips are cooked under pressure for 2-12 hours at temperatures of from 120 to 150°C. These parameters vary with the quality and yield of pulp desired, the type of wood being pulped and the specific sulphite process being used (Smook 1987). Delignification occurs when sulphurous acid (H_2SO_3) reacts with lignin to produce lignosulphonic acids. These compounds are relatively insoluble but form soluble salts with the cationic base. The lignosulphonates can then undergo further

degradation to smaller fragments. Without the cationic base, the lignosulphonic acid re-deposits on the surface of the fibres giving them a darkened or "burnt" appearance. After the cook, the chips and liquor (still under pressure) are "blown" from the digestor into a blowtank. This rapid eruptive drop in pressure disintegrates the delignified chips. The resulting pulp can be drained of cooking liquor and washed before progressing through the plant. Unlike kraft pulping, chemical recovery is not common.

Traditionally, calcium was used as the cationic base because of its availability and cost. However, the low solubility of the resulting calcium sulphite necessitated maintaining the cooking liquor below pH 3 to ensure the calcium sulphite remained in solution (Fengel and Wegener 1984). In the 1950s other cations were recognised as useful bases for sulphite pulping. The sulphite salts of these bases were more soluble than calcium sulphite allowing for higher pH during the cooks and in some cases allowing limited chemical recovery. Today, cations such as sodium, ammonia and magnesium are commonly used as bases (Smook 1987).

The alkaline sulphate, or kraft process, is the most common form of commercial pulping. The advantages of kraft pulping such as feedstock versatility, relatively short cooking times, economic spent liquor recovery and superior pulp strength offset the environmental problems and high capital investment associated with most kraft mills. Pulps produced by the kraft process tend to be dark in colour and may require a subsequent bleach (Fengel and Wegener 1984).

In the kraft process, wood chips saturated with fresh cooking liquor (white liquor) are cooked for 4 to 6 hours at 160°C to 180°C (Kleppe 1970). Cook times and temperatures may be varied to suit specific purposes. White liquor contains sodium hydroxide (NaOH) and sodium sulphide (Na_2S) as the active agents. Both these chemicals completely dissociate in solution with the sulphide ion (S^{2-}) further converted to hydrosulphide (HS^-) at cooking temperatures according to the following:

$$S^{2-} + H_2O \rightarrow HS^- + OH^- \qquad \text{(Smook 1987).}$$

The conversion of HS^- to H_2S is much slower at high temperatures than that of S^{2-} to HS^-, ensuring that loss of the hydrosulphide ion is minimal. Delignification is accomplished by the free hydroxyl (OH^-) and hydrosulphide (HS^-) ions which

fragment the polymeric lignin predominantly by nucleophilic reactions (Bryce 1980). The resulting lignin fragments are solubilised in the liquor (Kleppe 1970). After the cook the digestor contents are discharged into a blow tank where the spent liquor (black liquor) is drained from the pulp which is then washed and bleached (if desired) (Smook 1987). The drained black liquor (weak) is concentrated from ~15% solids to over 50% solids (strong black liquor) and then incinerated thus providing energy for the process while the inorganic furnace smelt is reclaimed by dissolving in water (green liquor). This green liquor is converted to white liquor for re-use in the process (Smook 1987; Fengel and Wegener 1984).

In kraft pulping, the rate of delignification occurs in three distinct phases. In the first phase, extractable lignin is readily removed (20-30% of total lignin in wood), in the second phase or bulk phase delignification proceeds at a slower but even pace, while in the third phase delignification slows even further (Kleppe 1970). In this manner, the lignin content of wood can be reduced by up to 90% with only minor deleterious effects on pulp strength and yield (Smook 1987). However, further delignification can result in unacceptable levels of holocellulose degradation resulting in poor paper qualities. This residual lignin gives unbleached pulp its typical brown colour making it unsuitable for many uses. A subsequent bleaching stage is often required after pulping to brighten the pulp (Lorås 1980).

1.2.3 Bleaching

Bleaching is the process by which wood pulps are whitened before papermaking. White pulps are often required for the production of high quality printing papers, specialty papers (ie: photographic papers) or dissolving pulps. Commercial bleaching processes involve the chemical oxidation of residual lignin in the pulp (Lorås 1980). As holocellulose is white in colour, this residual lignin is thought to be the main chromophore in wood pulps (Smook 1987). Oxidation can either disintegrate the lignin into extractable fragments (delignification) or convert the chromophore to a colourless form (brightening) (Smook 1987). In the former process pulp yields are lowered, in the latter the resulting papers often yellow with age. Bleaching without lignin removal is not efficient and most commercial bleaching operations involve an initial delignification stage with one or more

ensuing brightening stages (Lorås 1980).

Delignification can be monitored by the reduction in the kappa number (κ) of a pulp before and after a bleaching sequence. The kappa number is defined as the amount of 0.1 N potassium permanganate consumed within 10 minutes at 25°C by one gram of pulp (Lorås 1980; APPITA P201-86; TAPPI 236 cm-85). It is directly related to lignin concentration by the relationship:

$$\% \text{ Lignin (w/w)} = 0.147 \times \kappa$$ (Lorås 1980).

Other estimates of lignin content are available but the κ method gives a linear response over a wide range of pulps and is routinely used in many mills, although the factor relating κ to lignin can vary slightly with the wood species being pulped (Lorås 1980).

Pulp brightness is measured by comparing the intensity of light reflected from the sample pulp to that reflected from a standard, often a plate of magnesium oxide, under set conditions (Lorås 1980). This type of brightness measurement is expressed as a percentage of the standard and is applicable to that of final papers.

Commercial bleaching operations are multistage, continuous processes that include chemical treatments, extractions and washes in a set sequence. Abbreviations used to describe the steps within a sequence are given in Table 1.2. In a typical CEDED bleaching sequence, delignification predominates in the CE phases while brightening occurs during the DED phases (Smook 1987). Water washes to remove soluble lignin fragments as well as unconsumed chemicals occur between each stage unless otherwise indicated (Smook 1987).

Chlorine and chlorine derivatives (eg chlorine dioxide) are common bleaching agents. Under acidic conditions (~pH 2), chlorine (dissolved in water) reacts rapidly with lignin by substitution, oxidation and addition type reactions (Lorås 1980). These reactions are summarised as follows:

Substitution

$$R\text{-}H + Cl2 \rightarrow R\text{-}Cl + HCl$$

Oxidation

$$R\text{-}H + Cl2 + H_2O \rightarrow R\text{-}OH + 2HCl$$

Addition

$$R\text{-}CH{=}CH\text{-}R + Cl2 + H_2O \rightarrow R\text{-}CHCl\text{-}CH(OH)\text{-}R + HCl.$$

Substitution reactions predominate resulting in demethylation and chlorination,

especially of the aromatic moieties. This increases the solubility of lignin fragments and facilitates their extraction. Oxidation reactions form acidic groups which also aids in solubilisation while addition reactions may assist in the removal of the propane group from the phenylpropanoid units (Lorås 1980). The overall result of these reactions is a massive disruption of lignin structure with the formation of soluble lignin fragments.

Chlorination is often followed by an alkali extraction with sodium hydroxide used as the caustic agent. Caustic extraction serves to neutralise acidic compounds and remove solubilised lignin fragments (Smook 1987).

Chlorine dioxide (ClO_2) is an important chlorine derivative used in many bleaching operations. It has 2.5 times the reducing equivalent of chlorine on a mole basis (2.63 on a weight basis)(Smook 1987). However, the main advantage of chlorine dioxide is its high reactivity against lignin without any adverse effects on the polysaccharides (Reeve *et al.* 1991; Smook 1987; Lorås 1980). Environmentally, chlorine dioxide bleaching results in lower levels of chloro-organics as compared to chlorine bleaching thus lowering the environmental impact (Heimburger *et al.* 1988a). Chlorine dioxide is often used in conjunction with chlorine in delignification stages and alone in brightening stages of high brightness bleaching operations (Smook 1987, Table 1.2). Other bleaching agents include hydrogen peroxide and molecular oxygen (Smook 1987). Peroxide brightens without lignin removal by oxidising conjugated double bonds thus decreasing their chromophoric potential (Lorås 1980). Oxygen bleaching is also an oxidative process which operates in a similar manner. Both these agents brighten without delignification and are used in to brighten pulps.

1.3 ENZYMATIC HYDROLYSIS OF LIGNOCELLULOSE

1.3.1 Cellulose Degrading Activity

The study of the microbial degradation of lignocellulose has focused most extensively on the hydrolysis of cellulose by fungi and bacteria. The result of this endeavour is that the mechanism of some cellulase systems are well documented and serve as models for overall cellulolytic activity. The term "cellulase" refers to a collection of extracellular enzymes that depolymerise cellulose strands to low

Table 1.2 Some typical bleaching treatments (Smook 1987, p154)

Chlorination	(C_c) -	use of elemental chlorine under acidic conditions for delignification and brightening (subscript refers to proportion of C in a D/C mixture).
Alkaline extraction	(E) -	solubilisation of lignin fragments in NaOH.
Hypochlorite	(H) -	reaction with HOCl under alkaline conditions.
Chlorine dioxide	(D_D) -	reaction with ClO_2 under acidic conditions (subscript refers to proportion of D in a D/C mixture)
Peroxide	(P) -	reaction with peroxides under alkaline conditions.
Oxygen	(O) -	reaction with elemental oxygen at high pressure under alkaline conditions.
Hemicellulase	(X) -	enzymatic treatment under mild conditions.

molecular weight oligosaccharides which can be assimilated and metabolised (Walker and Wilson 1991). Cellulase systems exhibit one or more of the following hydrolytic activities: endo-1,4-ß-glucanase (EC 3.2.1.4), exo-glucanase (EC 3.2.1.91) and ß-glucosidase (EC 3.2.1.21) (Walker and Wilson 1991; Eriksson and Wood 1985). Other related activities, such as cellobiases or cellodextrinases, may be present at minor levels.

Endoglucanases randomly hydrolyse internal ß-1,4- glucosidic linkages, thus producing a range of low molecular weight oligosaccharides and increasing the concentration of reducing ends (Eriksson and Wood 1985; Sharrock 1988). Such enzymes are typically assayed by measuring the rate at which reducing equivalents are produced by the enzyme on soluble cellulose derivatives such as carboxymethyl cellulose (CMC) (Sharrock 1988, Ghose 1987). Exoglucanases (eg: cellobio-hydrolases, exo-cellulases) hydrolyse cellobiose from the non-reducing end of cellulose strands and shorter cellulo-oligomers with the product undergoing inversion during hydrolysis (Walker and Wilson 1991; Sharrock 1988; Eriksson and Wood 1985). Because of their low activity towards CM-cellulose, exoglucanases are

commonly assayed on artificial chromophoric substrates such as methylumbelliferyl-ß-D-cellobiose (MUC) or *p*-nitrophenyl-ß-D-cellobiose (pNPC) (Sharrock 1988). ß-Glucosidases, which hydrolyse cellobiose (and some cellodextrins) to glucose, are routinely assayed with *p*-nitrophenyl-ß-D-glucose as a substrate (Walker and Wilson 1991; Sharrock 1988).

No one enzyme has been found that can completely degrade cellulose to glucose. Rather the enzymes previously described, along with others, act synergistically to hydrolyse the crystalline polysaccharide. The currently accepted "cellulase" model involves the action of endo-glucanases on cellulose fibrils thereby reducing the average DP of the substrate and increasing the abundance of reducing and non-reducing ends on the surface of the fibril. The non-reducing ends are further hydrolysed by exoglucanases to produce cellobiose which is either metabolised directly or hydrolysed by ß-glucosidases to glucose and then metabolised (Walker and Wilson 1991; Woodward 1991; Eriksson and Wood 1985). The overall scheme, as outlined in Fig 1.7, is found in bacterial and fungal cellulase systems (Gilkes *et al.* 1991; Goyal *et al.* 1991). This model is complicated by several factors such as end-product inhibition, the multitude of iso-enzymes found within cellulase systems and the aggregation of these iso-ezymes, particularly in bacteria (Walker and Wilson 1991; Woodward 1991; Eriksson and Wood 1985).

1.3.2 "Ligninase" Activity

The enzymology of lignin degradation has been the focus of much interest and controversy since the identification of an extracellular lignin peroxidase (LiP) in *Phanaerochaete chrysosporium*, a white rot fungus (Tien and Kirk 1983; Glenn *et al.* 1983). This enzyme, a H_2O_2 dependant peroxidase, catalyses the one electron oxidation of nucleophilic aromatic rings in polymeric lignins and lignin model compounds, thus producing unstable radical cations within the substrate. These radicals can spontaneously react resulting in the cleavage of ß-O-4 ether linkages, Cα-Cß bonds and other inter-phenylpropane linkages (Kirk and Farrell 1987; Schoemaker and Leisola 1990).

The enzymology of LiP, as reviewed by Schoemaker and Leisola 1990, involves the oxidation of native LiP by H_2O_2 to compound I (Fig 1.8). In turn, compound I

oxidises the substrate (lignin) by a one-electron transfer mechanism resulting in a new LiP form (compound II) which can be reduced still further by veratryl alcohol (a secondary metabolite of the fungus) to the native enzyme thus completing the cycle. Compound II, however, can also react with H_2O_2 to produce an inactive form of the enzyme (compound III). Other postulated roles for veratryl alcohol include an oxidising mediator between LiP and reaction sites on remote lignins (Harvey and Palmer 1990) as well as a possible compound III regenerating agent (Schoemaker and Leisola 1990). The lignolytic scheme presented (outlined Fig 1.8) requires the direct involvement of the fungus as a source of veratryl alcohol and for the production of H_2O_2 (Schoemaker and Leisola 1990). This is evident from *in vitro* experiments where partially purified LiP, in the presence of H_2O_2, caused further lignin polymerisation (Lewis and Yamamoto 1990; Kirk and Farrell 1987). This observation has suggested that LiP may only modify polymeric lignin thus making it susceptible to an as yet unknown lignolytic mechanism (Lewis and Yamamoto 1990). However, direct lignin degradation by LiP has been identified by the production of low molecular weight compounds from lignin, decreases in the average molecular weight of the polymeric substrate and the mineralization of ^{14}C-lignin (in the presence of washed *P. chrysosporium*) (Schoemaker and Leisola 1990; Kirk and Farrell 1987; Tien and Kirk 1983, Higuchi 1990). Depolymerisation is thought to require an active LiP redox system as well as degradation product removal by fungal metabolism (Schoemaker and Leisola 1990) or the spatial segregation of LiP from the substrate by a cation mediator ie: veratryl alcohol (Harvey and Palmer 1990).

As well as LiP, *P. chrysosporium* also produces a manganese-dependent peroxidase (MnP) (Schoemaker and Leisola 1990). This enzyme oxidises Mn (II) to Mn (III) which in turn oxidises phenolic compounds. Laccase is a peroxidase produced by some fungi that also specifically attacks phenols (Kirk and Farrell 1987). The exact role these enzymes play in lignolytic degradation is not fully understood but it is thought to act synergistically with LiP in the oxidation of phenolic compounds (Schoemaker and Leisola 1990).

To date, most research on lignin degradation has focused on the aerobic systems of the white rot fungi (Zimmerman 1991; Lewis and Yamamoto 1990; Vicuna 1988;

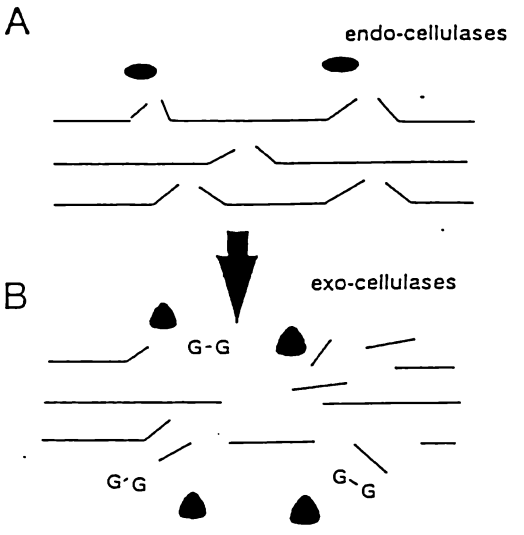

Fig 1.7 A: Random endo-cellulase attack on cellulose microfibril producing new reducing and non-reducing ends B: Exo-cellulase attack on the non-reducing ends produced by previous enzymatic attack releases cellobiose and cello-oligomers

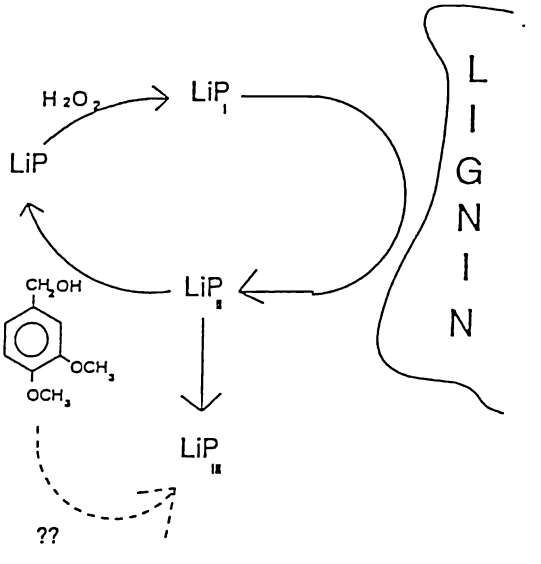

Fig 1.8 Schematic of lignin peroxidase (LiP) system of *P. chrysosporium* as described by Schoemaker and Leisola (1991). The role of veratryl alcohol in regenerating LiP from LiP_{III} has not been established.

Kirk and Farrell 1987). Aerobic degradation of low molecular weight lignin model compounds (LMC) has been reported by pseudomonads and other Gram negative bacteria as well as by actinomycetes (Vicuna 1988; Zimmermann 1990). Bacterial lignin degradation has been reported in pure and mixed cultures (Zimmermann 1990) and appears to be more specific than fungal lignolytic activity (Vicuna 1988). Examples of such enzymes include pathways that break biphenyl linkages (Vicuna 1988), benzyaldehyde lyase which cleaves 1,2-diarylethane (an LMC) (Zimmermann 1990) and a ß-aryl etherase which cleaves the ß-O-4 ether linkages between phenylpropanes (Masai *et al.* 1991). Under such a strategy, it is unlikely that one bacterial species could possess all the necessary enzymes required for total lignin biodegradation. Therefore, in nature complete lignin degradation is probably achieved by microbial consortia (Zimmermann 1990; Vicuna 1988). It should be stressed that degradation of lignin model compounds alone does not necessarily ensure polymeric lignin degradation.

Anaerobic lignin degradation by bacteria has been reported but at very low rates and on low molecular weight fractions (Vizcuna 1988; Kirk and Farrell 1987). The long incubation periods required for anaerobic (several months) as compared to aerobic (on the order of weeks) lignin biodegradation has lead to suggestions that LMC, leached from the polymeric material over time, are metabolised by the bacteria (Kirk and Farrell 1987). However, reports of anaerobic lignin biodegradation support some type of anoxic lignin biomodification. Some of the highest rates of anaerobic lignin mineralisation have been reported in mixed enrichment cultures under thermophilic conditions (at 55°C) where rates of mineralisation of 15% to 20% within 60 days have been reported (Brenner and Hodson 1985). This suggests that high temperatures may facilitate lignin biodegradation under anaerobic conditions.

Single cell bacteria that invade the actual cell wall of tracheids and fibrils (originally thought to be a fungal mechanism) through cavitation and tunnelling have been recently discovered (Daniel *et al.* 1987). These bacteria, observed by electron microscopy but as yet not isolated, exemplify the perplexing nature of lignin biodegradation.

1.4 HEMICELLULOLYTIC ACTIVITY

Hemicellulases have been reported in aerobic and anaerobic eubacteria, filamentous and unicellular fungi, protozoa, insects, molloscs, snails, crusteaceans and the germinating seeds of terrestrial plants but not in vertebrates who presumably rely on intestinal microflora for hemicellulose digestion (Dekker and Richards 1976). Many hemicellulolytic fungi and bacteria are also cellulolytic suggesting a cooperative effect between the two activities. Notable exceptions to this are some xylanolytic yeasts such as *Cryptococcus albidus* (Biely and Vršanská 1988) and *Pichia stipitis* (Lee *et al.* 1986) which are not cellulolytic. In general, hemicellulases are extracellular with some being cell associated (Dekker 1985; Dekker and Richards 1976). However, intracellular hemicellulolytic activities have been reported (Biely 1985; Dekker 1985). Both constitutive and inducible hemicellulases have been reported with many species having separate regulatory mechanisms for both xylan and mannan degradation.

Xylans and glucomannans are hydrolysed by separate enzyme systems. Although dedicated to their individual substrates, these enzyme systems function in a parallel manner. This involves the action of "endo"-ß-1,4-glycohydrolases (xylanases and mannanases) that depolymerise the substrate to an array of oligosaccharides which are further degraded by "exo"-ß-glycohydrolases (ß-xylosidases and ß-mannosidases) to metabolisable monosaccharides (Dekker 1985; Dekker and Richards 1976). "De-branching" glycohydrolyases (ie: α-L-arabinofuranosides, α-glucuronidases and α-galactosidases) and acetyl esterases remove side-groups which sterically hinder the endo- and exo- glycohydrolases (Puls and Poutanen 1989; M^{c}Cleary 1988; Beily 1985; Dekker 1985). Complete xylan and glucomannan degradation requires the concerted activity of these enzymes.

Dekker (1985 and 1989) has proposed two scenarios for the enzymatic degradation of hemicelluloses. In the first, de-branching enzymes remove inhibitory side groups. Once these substituents are cleaved, the polysaccharide can be hydrolysed by endo- and exo- glycohydrolases to metabolisable oligosaccharides. The second scheme has endo-hemicellulases attacking available sites on the polymer producing a range of branched oligosaccharides that cannot be degraded further by endo-glycohydrolases alone. These oligomers are de-branched by the appropriate

enzymes and then hydrolysed by endo- and exo-hemicellulases. Evidence such as the synergism between specific de-branching enzymes and xylanases (Puls *et al.* 1991; Puls and Poutanen 1989; Biely *et al.* 1986), similar synergies between α-galactosidases and mannanases (M^{c}Cleary 1988) and the relatively high activity some de-branching enzymes show towards substituted oligmers (Puls and Poutanen 1989) favour the second scheme.

1.4.1 "Endo" Hemicellulases

Endo-ß-1,4-xylanase (EC 3.2.1.8) and endo-ß-1,4-mannanases (EC 3.2.1.78) hydrolyse the ß-1,4 inter-glycosidic linkages of xylan and glucomannans respectively, producing oligosaccharides and decreasing the average DP of the substrate (Beily 1985; Dekker 1985; Reilly 1981; Dekker and Richards 1976). Endo-hemicellulases are routinely assayed by incubating the enzyme with an appropriate soluble polysaccharide as substrate and monitoring the increase of reducing equivalents over time (Ghose and Bisaria 1987; Dekker and Richards 1976). However, substrate source and method of preparation should be detailed as hydrolase activities often vary with these parameters. Molar ratios of the monosaccharide composition (ie: xyl:arab and mann:gluc:galact) are often sufficient for comparative purposes. Other techniques of estimating endo-hemicellulase activity include monitoring the decrease of the DP by viscometric or nephelometric assays (Puls and Poutanen 1989) and by the release of dye from derivatised xylans (ie: Remazol brilliant blue-xylan)(Biely 1985) or galactomannans (ie: Remazol brilliant blue-carobgalacto-mannan) (M^{c}Cleary 1988). The dye-based assay techniques are especially useful for screening cultures and gels for xylanase activity (Biely *et al.* 1985b).

Numerous xylanases have been successfully purified from a diverse array of fungal and bacterial sources (Wong *et al.* 1988; Dekker and Richards 1976), many xylanase-genes have been cloned (Bhalerao *et al.* 1990; Luthi *et al.* 1990; Sakka *et al.* 1990; Ghangas *et al.* 1989). One survey reported the cloning of fourteen different xylanases from bacteria alone (Bhalerao *et al.* 1990). Mannanases have been purified from similar sources but, to date, only two have been cloned, the first from an alkalophilic *Bacillus* sp. (Akino *et al.* 1989), the second from a cellulolytic thermophile (isolate TP8 6.3.3.1) tentatively named "Caldocellum saccharolyticum"

(Luthi *et al.* 1991).

Characterisation and classification of these enzymes has focused on enzymatic properties and reaction end-products (Reilly 1981; Wong *et al.* 1988; Dekker 1985). Xylanases studied to date are small to medium sized enzymes (molecular weight range 10,000-80,000 Da) with pH optima in the neutral to slightly acidic region (4.5-7.5). Wong *et al.* (1988) has noted an inverse correlation between molecular weight (MW) and isoelectric point (pI) in xylanases (ie: high MW/low pI or low MW/high pI). This relationship seems to be more consistent in bacteria than in fungi suggesting a difference in xylanolytic stratagem, however fungal xylanolytic systems have been studied to a greater extent than those of bacteria therefore conclusions in this respect may be premature. Mannanases tend to be larger than xylanases (molecular weight range 22,000-73,000 Da) with a broader pH range (4.5-9.0). They are also acidic in nature with pIs lower than 6.0 (Torries *et al.* 1990), but some exceptions have been reported. Yamaura *et al.* (1990) have purified a mannanase with a pI 9.4 and two mannanases from snail gut with pIs of 7.7 and 7.0 have also been reported (M^{c}Cleary 1979). Mannanases tend to be highly specific hydrolysing only mannans, glucomannans and some galactomannans that are not excessively substituted (McCleary and Matheson 1983; Talbot and Sygusch 1990). Some highly purified xylanases, however, appear to have some CM cellulase activity (Casimir *et al.* 1991; Uchino and Nakane 1981; Viet *et al.* 1991; Wong and Saddler 1991; Wong *et al.* 1988). Some cloned xylanases also exhibit this, suggesting that CM cellulase cross reactivity may be a characteristic of some xylanases (Sakka *et al.* 1991). Temperature optima and thermostabilities of these enzymes tend to reflect the temperature extremes of the organsims from which they were isolated. Mannanases tend to be more thermostable than xylanases when isolated from similar organisms. For example a mannanase from *Bacillus stearothermophilus* was stable for 24 hr at 70°C (Talbot and Sygusch 1990) while a xylanase purified from a bacterium identified as the same species was only stable at 60°C for 1 hr (Nanmori *et al.* 1990). Temperature optima and thermostabilities of several xylanases and mannanases of microbial origin are summarised in Table 1.3.

Some purified xylanases and mannanases are glycosylated but the nature and function of these carbohydrate moieties has not been investigated but may be

involved in thermostability or specificity of the enzymes (Araujo and Ward 1990; Biely and Vršanská 1988; Hayashida 1988; Berenger *et al.* 1985). Many micro-organisms produce a multitude of distinct xylanases (some as many as five!) implying that xylanolytic activity is more complex than originally thought (Wong *et al.* 1988).

Mannanase multiplicity has also been shown in two *Bacillus* species (Aruajo and Ward 1990; Akino *et al.* 1988), *Trichoderma harzianum* (Torrie *et al.* 1990), *Polyporus versicolor* (Johnson and Ross 1990) and *Thielavia terrestris* (Araujo and Ward 1990a). Other organisms do not readily exhibit mannanase multiplicity (M^{c}Cleary 1988) but do so with xylanases. The regulation of these two enzyme systems appears to be under independent control in many organisms but the regulation and function of the multiple enzyme forms, particularly in xylanases, is yet to be explained in detail.

1.4.2 Mode Of Action Of "Endo" Hemicellulases

The hydrolysis of xylans and glucomannans, by their respective enzymes, is affected by the extent and order of substitution (M^{c}Cleary 1988; Wong *et al.* 1988; Dekker 1985; Biely 1985; M^{c}Cleary and Matheson 1983). That of glucomannans is further complicated by the distribution of glucose within the polysaccharide backbone (M^{c}Cleary and Matheson 1983).

Classification of xylanases by end-product arose from observations that some, highly purified xylanases release L-arabinose from arabinoxylan while others do not (Dekker and Richards 1976). Both types of enzymes attack substituted and unsubstituted xylans, but xylo-oligomers produced by non-arabinose releasing xylanases may have arabinofuranoside branches (Dekker 1985, Dekker and Richards 1976). Methyl glucuronic acid substituted xylo-oligomers are produced by the action of both types of xylanases on glucuronoxylans. Arabinose releasing xylanases appear to be less prevalent in bacteria than in fungi however the reader is reminded of the caveat mentioned earlier regarding the study of fungal and bacterial xylanases. Indeed several fungi have been found to produce both types of xylanases simultaneously suggesting a synergy between the two types of activities (Wong *et al.* 1988; Dekker 1985).

Table 1.3 Thermostabilities and temperature optima of some xylanases and mannanases of microbial origin.

Species	Temperature Optima °C (assay time)	Half Life	Reference
	Xylanase		
Aeromonas caviae	50 (10 min)	60 min at 55°C	(Viet *et al.* 1991)
Streptomyces lividans	55 (30 min)	4.2 hr at 80°C	(Kluepfel *et al.* 1990)
Streptomyces roseiscleroticus	60 (10 min)	Not reported	(Grabski and Jeffries 1991)
Bacillus stearothermophilus	60 (30 min)	60 min at 70°C	(Nanmori *et al.* 1990)
Aureobasidium pullulans	60 (10 min)	~30 min at 60°C	(Dobberstein and Emeis 1989)
Humicola grisea	70 (30 min)	30 min at 60°C	(Monti *et al.* 1991)
Bacillus spp. alkalophile	70 (10 min)	no loss 10 min/60°C	(Okazaki *et al.* 1984)
Clostridium stercorarium	75 (10 min)	90 min at 81°C	(Berenger *et al.* 1985)
Clostridium thermolacticum	80 (15 min)	10% loss 4 days/60°C	(Debeire *et al.* 1990)
Talaromyces emersonii	80 (30 min)	4.2 hr at 80°C	(Tuohy and Coughlan 1992)
Thermomonospora fusca	80 (30 min)	no loss 3 days/6O°C	(Casimir *et al.* 1990)
Bacillus sp. acidophile	80 (10 min)	15 min 70-75°C	(Uchino and Nakane 1981)

Table 1.3 Thermostabilities and temperature optima of some xylanases and mannanases of microbial origin (continued).

Species		Temperature Optima °C (assay time)	Half Life	Reference
		Mannanase		
Bacillus stearothermophilus		70 (5 min)	30 min at 75°C	(Talbot and Sygusch 1990)
Bacillus spp. cloned		60 (10 min)	stable 30 min/60°C	(Akino *et al.* 1989)
Bacillus pumilus	A	60 (30 min)	60 min 70°C	(Arujo and Ward 1990a)
	B	70 (30 min)	21 min 70°C	
Thielavia terrestris		75 (30 min)	1.5 hr at 70°C	(Arujo and Ward 1990b)

The xylo-oligosaccharides produced are typically within the DP1-DP8 range with xylobiose (DP2) and xylotriose (DP3) being the most abundant oligomers after prolonged incubation (Reilly 1981; Dekker 1985). From specificity data (V_{max}/K_m) and bond cleavage frequencies of various oligomers (DP2-DP6), active site models of various xylanases have been proposed (Debeire *et al.* 1990; Dekker 1985). Active sites which bind four, five and seven xylose residues have been reported (Dekker 1985; Debeire *et al.* 1990). Catalytic sites are usually located between residues that produce xylobiose and another xylo-oligomer (DP2-DP5). Substitution of certain xylose residues of the binding motif prevents binding of the substrate to the active site while other substituted residues within the motif do not affect either binding or catalytic activity (Debeire *et al.* 1990; Dekker 1985). This is exemplified in the model of the active site of a xylanase from *Clostridium thermolacticum* (MW 39 kDa, pI 4.9, non-arabinose releasing) (Debeire *et al.* 1990). This enzyme has a substrate binding site of five xylose residues (non-reducing A to reducing E) with cleavage occurring between residues B and C. Xylobiose, xylotriose and a mixture of glucuronoxylo-oligomers (DP5 or less) were produced from larchwood xylan. Glucuronic acid substitution of residues D or E did not affect catalytic activity

while the substitution of residues A,B and C prevented binding of the substrate to the enzyme (Fig 1.9). Therefore, higher xylo-oligosaccharides substituted at the A,B or C residues were resistant to hydrolysis.

Mannanases have active sites analogous to those of many xylanases. The active site of an *Aspergillus niger* mannanase has a binding motif of five hexose residues (non-reducing A to reducing end E), cleavage is between the C and D residues (Fig 1.9). Interactions between the hydroxymethyl moieties (C6) of the pyrannose ring and the enzyme occur at residues B and D while residues C and E bind to the enzyme through equatorial hydroxyls at the C2 and C3 positions of the pyrannose ring (M^{c}Cleary and Matheson 1983). Equatorial hydroxyls at the C3 position are also imperative for the binding of the A residue but the orientation of the C2 hydroxyl is not as stringent. From this information, the specificity of the mannanase from *A. niger* can be characterised as follows (Fig 1.9):

A residue can be either mannose or glucose. The hexoses must be unacetylated but can be galactosylated,
B residue can be either a mannose or glucose. The hexose must not be galactosylated but can be acetylated,
C residue must be a non-acetylated mannose,
D residue as with B residue,
E residue as with C residue.

Similar data has been obtained from five other mannanases which differ only in the substitution of the C residue (M^{c}Cleary and Matheson 1983). A major deduction from these rules is that an enzymatically produced glucomanno-oligosaccharide cannot have a reducing glucose. If such oligomers are produced then either other enzymes have modified the oligomer or a different mannanase mechanism has been used.

1.4.3 "Exo" Hemicellulases

Oligosaccharides produced by the action of xylanases and mannanases on their corresponding substrates are further degraded by specific ß-glycosidases (Dekker 1989; Puls and Poutanen 1989). These enzymes which include ß-xylosidases (EC 3.2.1.37) and ß-mannosidases (EC 3.2.1.25), hydrolyse monosaccharides from the non-reducing end of oligosaccharides (Puls and Poutanen 1989, Dobberstein and Emeis 1989). The anomeric configuration of the product is conserved (Dekker

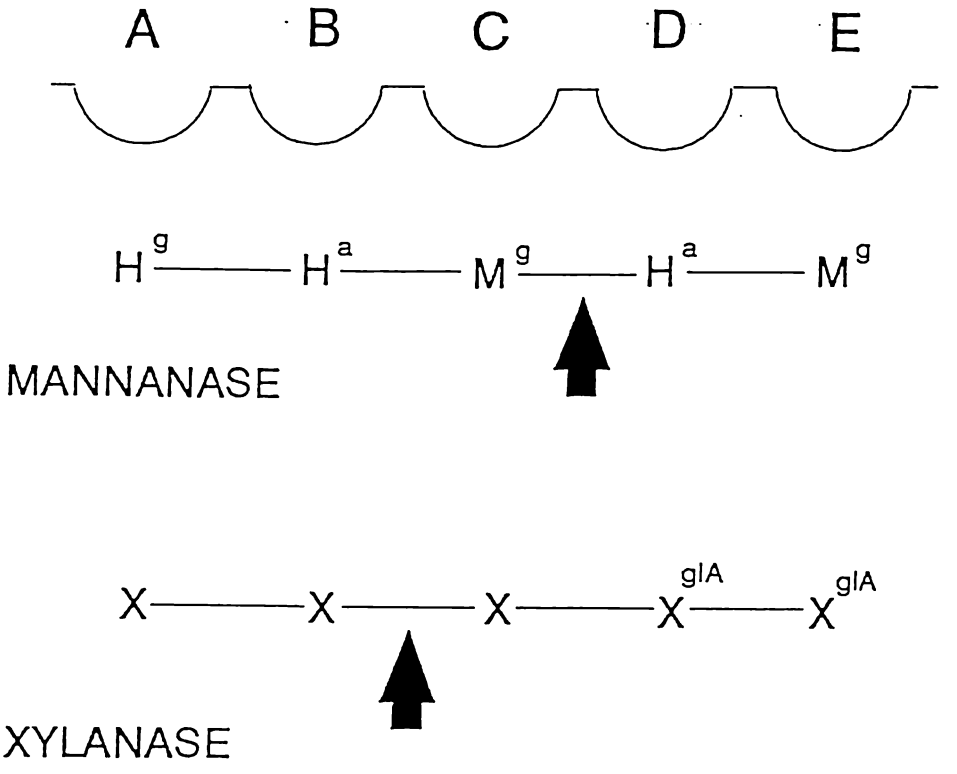

Fig 1.9 Five residue binding site model for the *A. niger* mannanase (M°Cleary and Matheson 1983) and *Clostridium thermolacticum* xylanase (Debeire *et al.* 1990)
- arrows represent bond hydrolysed by the enzymes
- H can be either mannose or glucose
- M must be mannose
- X must be xylose
- superscripts denote possible substituents that would not interfere with catalytic activity (g galactose; a acetyl ester; glA methyl glucuronic acid)

1985) but relatively high transferase activities are characteristic of these enzymes (Reilly 1981). ß-Xylosidases and ß-mannosidases are found in most microbial hemicellulolytic systems and are usually extracellular or cell wall associated (Matsuo and Yasui 1988, Beily 1985). Some cystolic ß-xylosidases have been described implying intracellular xylo-oligomer metabolism in some microbes (Biely 1985).

Glycosidase activities are routinely assayed by the release of the chromophores from synthetic substrates such as methylumbelliferyl-ß-glycopyrannose or nitrophenol-ß-glycopyranoside (either ß-mannopyrannose or ß-xylopyrannose) (Puls and Poutanen 1989). However, to determine the true nature of glycosidase activity, the degradation of higher molecular weight oligo-saccharides (DP2-DP5) is essential. For example, many ß-xylosidases preferentially hydrolyse xylobiose while others tend to attack higher molecular weight xylo-oligmers (Puls and Poutanen 1989). The former are often refered to as xylobiases. ß-Mannosidases may show similar behaviour. A ß-mannosidases purified from snail gut (*Helix pomatia*) hydrolysed individual manno-oligomers (DP2-DP5) at similar rates (M^{c}Cleary 1988c).

The existence of hemicellulolytic exo-hydrolyases analogous to exo-cellulases had been postulated (Dekker 1985; Reilly 1981) but no such exo-xylanases have been found (Dekker 1989) and only two "exo"-mannanases have been described (Araki and Kitamikado 1988; M^{c}Cleary 1988a). These types of enzymes are severely inhibited by non-reducing end substitution (Reilly 1981). Therefore, the role of such exo-hydrolyases in hemicellulolytic activity is not considered to be of significance (Dekker 1989; Wong *et al.* 1988).

1.4.4 "De-Branching" Enzymes

Due to the heterogeneity of most xylans and glucomannans, other activities are needed to remove side-groups that may sterically hinder depolymerisation by the previously mentioned glycohydrolases. These "de-branching" enzymes include exo-glycosidases such as α-L-arabinofuranosidases, methyl-glucuronidases and α-galactosidases as well as acetyl esterases and ferulic acid esterases (Dekker 1989; Puls and Poutanen 1989).

α-L-Arabinofuranosidases (EC 3.2.1.55) remove terminal, non-reducing arabinose residues from xylans but do not cleave glycosidic bonds between xylose residues (Kaji 1984). Extracellular α-L-arabinosidases are easily assayed with the appropriate p-nitrophenol derivatives (Kaji 1984). An α-L-arabinosidase purified from *Trichoderma reesei* was active against both arabinose substituted xylo-oligomers and polymeric arabinoxylan but was more specific towards the former (Poutanen 1988).

α-Glucuronidases cleave methylglucuronic branches from xylans (Puls *et al.* 1987). Unlike other xylanolytic enzymes, no simple procedure exists for assaying α-glucuronidase activity, therefore this important activity has not received the attention that others have. First identified by Puls *et al.* (1987) in xylan-grown culture filtrates of the fungi *Agaricus bisporus* and *Pleurotus ostreatus*, α-glucuronidase activity is determined by measuring the release of free glucuronic and methylglucuronic acids from 2-O-(4-O-methyl-α-glucopyranosyluronic acid)-xylobiose by anion exchange HPLC. The substrate was prepared by the acid hydrolysis of beechwood (4-O-methyl-D-glucurono)-D-xylan (Puls *et al.* 1988). Further study of the *A. bisporus* α-glucuronidase showed that the enzyme cleaved methylglucuronic acid from substituted xylobiose, xylotriose and xylotetrose in decreasing order, but not from substituted xylose or methylglucuronoxylan (Puls and Poutanen 1989).

α-Galactosidases (EC 3.2.1.22) cleave α-1,6-galactose substituents from glucomannanan polymers. Nitrophenol α-galactoside derivatives (either *ortho-* or *para-*) are assay substrates for this enzymes. Some α-galactosidases are active against the non-reducing, α-galactose residues of a diversity of di- and trisaccharides (eg: melobiose, raffinose, stachyose) (M^{c}Cleary 1988d). In a species of *Bacillus stearothermophilus* grown on galactomannan, the α-galactosidases produced had greater activity towards lower molecular weight oligosaccharides than towards polymeric galactomannans (Talbot and Sygusch 1990).

Acetyl esterases hydrolyse acetyl groups from the C2 and C3 positions of acetylated xylans in hardwoods (Biely *et al.* 1985c). Acetylxylan esterases and xylanases act synergistically in the hydrolysis of acetylated xylans by fungi (Biely *et al.* 1986). Similar esterase activities may also be required for the de-acetylation

of softwood glucomannans. Nitrophenyl acetate assay for esterase activities are available but true esterase activity must be assayed on an acetylxylan substrate (Khan *et al.* 1990). Fungal acetyl esterases have been assayed on acetylated xylan and nitrophenol acetate (Khan *et al.* 1990, Biely *et al.* 1985c). The esterases showed greater activity against nitrophenol acetate again implying that this debranching enzyme has a greater specificity towards low molecular weight substrates rather than acetylated polymers.

1.5 BIOBLEACHING

Chlorine is commonly used to bleach kraft pulps but the resulting effluents are relatively high in chlorinated organic compounds (Heimburger *et al.* 1988a). The chloro-organic fraction of mill effluents, often described as absorbable organic halides (AOX), contains dioxins and chlorophenolics (Heimburger *et al.* 1988a). The recalcitrant nature of these compounds and their toxicity to many types of aquatic life has focused public attention on this issue. Unlike kraft black liquors, these effluents are high in chloride which prohibits their disposal in the recovery furnaces (Smook 1987). Current trends to cut the AOX levels of mill effluents involve reducing or replacing elemental chlorine in the delignification stage of the bleaching sequences and improving waste treatment processes (Heimburger *et al.* 1988a, 1988b). As prevention is preferable to remediation industry has shown much interest in the former approach.

Effluent AOX levels are directly related to the chlorine dosage used during the delignification stages of bleaching sequences (Axegard 1989; Heimburger *et al.* 1988a). Chlorine dosages can be minimised by removing as much residual lignin, prior to bleaching, as possible. This approach, known as extended delignification, involves such techniques as oxygen delignification, exhaustive washing of unbleached pulp or modifications to the kraft cook regime (Heimburger *et al.* 1988a).

A recent innovation in extended delignification is biobleaching which uses certain micro-organisms, enzymes or other biological material to facilitate the removal of residual lignin from pulp. *Phanerochaete chrysosoporium* and *Trametes versicolor* have been used to delignify pulps under aerobic conditions (Reid 1989;

Trotter 1990). The latter organism has been shown to increase pulp brightness by 15 points in liquid culture (Paice *et al.* 1989) and in an immobilised form (Kirkpatrick *et al.* 1990) demonstrating that fungal contact with the pulp is not necessary. Presumably, fungal delignification results from the action of either peroxidases (ligninase, laccase etc.) or oxidising intermediates secreted by the fungi.

Yet fungal brightening of pulps has some drawbacks. Reaction times are long, on the order of days, and cultures require additional nutrients to minimise cellulose hydrolysis by the fungus and maximise lignolytic activity (Paice *et al.* 1991; Reid 1989; Trotter 1990). Other recent developments in biobleaching include the use of porphyrins as oxidising agents to act directly against lignin (Eriksson 1990). This type of bleaching can decrease κ of pine and birch kraft pulps by up to 30% within a 24 hr period. Some decrease in viscosity was noted, but this may be alleviated by closely monitoring dosage (Eriksson 1990). This type of "biomimetic" bleaching is still at the laboratory stage and requires further optimisation but shows great promise as a means of improving pulp brightness without the use of chlorine compounds. With the discovery of lignin peroxidases in *Phanerochaete chrysosoporium*, many thought that the enzymatic bleaching of pulps would soon follow. However, enzymatic lignolysis is a complex activity and appears to require the direct involvement of viable mycelium to generate H_2O_2 and other intermediates, replenish necessary enzymes and consume lignin fragments (Kirk and Farrell 1987). To date no report of sustainable enzymatic lignolytic activity without direct fungal involvement has been reported. The most promising biotechnological application in the bleaching of pulps appears to be the use of hemicellulases, particularly xylanases, to facilitate the bleaching of kraft pulps.

1.5.1 Enzyme Assisted Bleaching

The first report of enzyme assisted bleaching used crude hemicellulase preparations (high xylanase, low cellulase) from *Aspergillus awamori* and *Streptomyces olivochromogens* to treat birch and pine kraft pulps (Viikaru *et al.* 1986). Treated pulps and non-treated controls were then subjected to peroxide delignification. Both types of pulps had κ that were from 20 to 30% lower than their respective controls. Similar results obtained with chlorine delignified pine kraft

pulps translated into a 25% reduction in the required chlorine charge. Interestingly, two fungal "ligninases" had no effect on the lignin content of peroxyacid birch or pine pulps (Viiraki *et al.* 1986). Subsequent studies by Viiraki *et al.* (1987) with similar hemicellulase preparations, including one from *Bacillus subtilis*, expanded on previous findings. Hemicellulase treated pine kraft pulps had κ that were up to 15% lower than those of untreated control pulps after both had undergone a full (DC)EDED bleach. Pulp properties were virtually identical between the treated and untreated pulps. Again cell-free "ligninase" preparations, this time from *P. chrysosporium* and *Phlebia radiata* had no effect on the κ or peroxide delignification efficiency of birch or pine kraft pulps. Nor was there any apparent synergy between the hemicellulases and ligninases. These initial reports sparked a number of papers that gave some insight into enzyme assisted bleaching.

Carbohydrate analysis of filtrates from hemicellulase treated pulps showed that large proportions of xylan associated monosaccharides were released, suggesting xylanases contributed to the bleaching effect. Experiments with purified xylanases (Chauvet *et al.* 1987; Kantelinen *et al.* 1988) and cloned xylanases (Paice *et al.* 1988), confirmed that this activity is primarily responsible for bleaching assistance. Xylanases from either fungal and bacterial sources can cause the effect but this does not imply that all xylanases improve bleachability.

Whether other hemicellulase activities contribute to the bleaching assistance effect is still unclear. Preliminary data suggested that xylanase preparations lacking arabinosidase and xylosidase activities were less effective at bleaching assistance than those that possessed these activities (Viikari *et al.* 1987). Nominal improvements in bleachability were found with xylanases augmented with arabinosidase or glucuronidase over xylanase alone (Kantelinen *et al.* 1988). Chemically deacetylated aspen TMP pulp was 10 fold more susceptible to xylanase activity than acetylated TMP (Jeffries and Lins 1989) suggesting that enzymatic deacetylation may also improve the xylanase susceptibility of TMP pulps. However, acetyl esterases have no effect on the delignification of kraft pulps which are deacetylated during cooking. Side-groups sterically hinder xylanases from completely hydrolysing soluble xylans (SECT 1.4.2), however within the cell wall xylanase activity is probably hindered to a greater extent by physical inaccessibility.

Most likely, de-branching enzymes remove side-groups from substituted xylo-oligomers solubilised by the xylanase.

The role of mannanases in bleaching assistance is also confused. Clark *et al.* (1990) have reported improved bleachability of pine (*Pinus radiata*) kraft pulps by a *Bacillus subtilis* mannanase alone. A 25% savings in the chlorine dosage of a (DC)E delignification was achieved. In similar experiments Viikari *et al.* (1990) used a *B. subtilis* mannanase to treat pine (*Pinus silvestris*) kraft pulps yet reported no enhancement of bleachability after peroxide delignification. Both studies had comparable glucomannan release and small but significant amounts of xylan solubilisation. The solubilised xylan was polymeric and presumably leached from the pulp (Clark *et al.* 1990). A fungal mannanase that did not solubilise any xylan had no effect on pulp bleaching despite solubilising twice the amount of glucomannan that the bacterial enzyme solubilised (Clark *et al.* 1990). The two studies may reflect differences between the two delignification procedures. However, both peroxide and chlorine based delignifications are effective with xylanase treated pulps. Therefore, the conflicting results may reflect differences between the pulps or the enzyme preparations. Mannanase augmented xylanase preparations that improve (Kantelinen *et al.* 1988) and that do not improve (Kantelinen *et al.* 1991) bleachability when compared to the xylanase alone have been reported.

Cellulase impurities in enzyme preparations were found to cause dramatic decreases in pulp viscosities (Paice and Jurasek 1984). Their elimination alleviates this problem (Senior *et al.* 1988). Indeed, pulps treated with a cloned xylanase (cellulase free) had higher viscosities due to the removal of low molecular weight xylans (Paice *et al.* 1988). Cellulase cross reactivity of some highly purified xylanases has not been reported to adversely affect pulp properties. The effects of xylanase treatments on pulp properties were minimal with only minor changes in tear and tensile strength reported (Clark *et al.* 1991; Paice *et al.* 1988).

Bacterial and fungal xylanases have been used to improve the bleachability of kraft pulps and the effect has been shown with both softwoods and hardwoods. An extraction stage is required to detect a minor drop in κ between enzyme treated and untreated pulps (Chauvet *et al.* 1987) but this effect is magnified by either a

peroxide or chlorine delignification stage. Chlorine savings of up to 25% have been reported for xylanase treated softwood kraft pulps (Clark *et al.* 1991; Viikari *et al.* 1986) with typical incubation times of 12 to 24 hr. Chlorine savings of 35% have been reported for xylanase treated hardwoods (Pedersen 1990). Recently, a commercial xylanase preparation that reduces elemental chlorine consumption by as much as 80% in a standard (CD)EDED bleaching sequence, while still producing a high brightness (90% ISO) pulp, has been described (du Manoir *et al.* 1991). Another commercial xylanase has been successfully incorporated into a (EOP)D(EOP)D bleaching sequence resulting in a savings in a 50% savings of ClO_2 consumed (Skerker *et al.* 1991). Pulps were bleached to 86% ISO brightness. The enzyme had no adverse effects on pulp properties and was effective against both hardwood and softwood pulps.

Reductions in bleach plant effluent AOX have been linked to lower Cl_2 in the bleaching regime (Axegard 1989). By using xylanases to reduce the Cl_2 required for bleaching, decreases in effluent AOX have been reported. Mill trials have shown up to 50% reductions in the chlorine consumed and, more importantly, consequential reduction in the effluent AOX (Vaheri *et al.* 1989). A 25% to 30% reduction in the extracted AOX, along with improvements in other effluent parameters, has been reported for another commercial xylanase preparation (Senior and Hamilton 1991). A summary of the literature on xylanase assisted bleaching and its effects on various pulps is presented in Table 1.4.

Enzyme assisted bleaching has rapidly become a viable economic option for many mills that are faced with stringent environmental regulations regarding the release of chloro-organics. Oxygen delignification, peroxide bleaching and extended delignification require major alterations to pulping and bleaching regimes requiring large capital investment (Heimburger *et al.* 1988a, b). Xylanase assisted bleaching can be easily incorporated into most chlorine bleaching sequences with minimal capital investment. The enzyme can be metered into the brownstock storage chest and incubated with the pulp for the recommended time period (typically about 3 hr for commercial xylanase preparations) (du Manoir *et al.* 1990). Of course, incubation temperature and pH must be compatible with the enzyme.

The main cost in xylanase assisted bleaching is the enzyme itself. For xylanase

assisted bleaching to be economically viable it has been estimated that enzyme cost can be no higher than $5(US) per tonne of pulp (Layman 1990). To achieve this, production costs must be minimised by growing high xylanase yielding organisms on cheap feedstocks, and curbing downstream processing costs. The enzymes that are used must be highly specific and stable. High specific activity lowers the required xylanase dosages while stability facilitates enzyme recycling.

1.5.2 Mode Of Action

Before the mechanism of enzyme assisted bleaching can be understood, the nature of the hemicelluloses in the fibre wall and their relation to residual lignins must be examined. Residual lignin is the 5% to 10% of the lignin remaining in kraft pulp after cooking. This lignin cannot be removed without prolonged cooking under conditions that would hydrolyse cellulose and thus lower yields and adversely affect pulp properties.

Residual lignin compositions fall between those of milled wood lignin (MWL) and kraft lignin, except that residual lignins have higher molecular weights than the other two types (Isogai *et al.* 1987; Iversen and Wannstrom 1986; Jiang *et al.* 1987). This indicates that residual lignins are not degraded to the same extent during cooking as kraft lignins. The solubility of isolated residual lignin in alkaline solutions has also been demonstrated indicating that incompatibility with the solvent is not the cause of its resistance to cooking (Isogai 1987; Isogai *et al.* 1987; Yamasaki *et al.* 1981). Rather the recalcitrance of residual lignins to kraft pulping is thought to be due to direct covalent linkages between the lignin and hemicellulosic components of the pulp (Isogai 1987; Isogai *et al.* 1987; Jiang *et al.* 1987; Minor 1986; Yamasaki *et al.* 1981). Carbohydrate/lignin bonding would impede the solubilisation of both components (Aurell and Hartler 1965). Hemicellulose derived monosaccharides have been found directly linked to residual lignins by alkali stable bonds (Yamasaki *et al.* 1981). From the methylation analysis of carbohydrates obtained from cellulase digested pine kraft pulps, it has been suggested that residual lignins are bound to galactans, arabinans, glucomannans and arabinoxylans (Isogai 1987; Minor 1986). Similar linkages to cellulose may also be possible (Isogai 1987; Iversen and Wannstrom 1986; Jiang *et al.* 1987).

Table 1.4 Effects of various xylanases on improving the bleachability of kraft pulps. (N.B. Conditions vary between treatments)

Source of Enzyme	Pulp	Post-enzyme Treatment	Δ Kappa# (%)	Reference
Aspergillus awamori (crude prep)	Birch	peroxide	34	(Viikari *et al.* 1986)
	Pine	peroxide	19	
Streptomyces olivochromogens (crude prep)	Pine	(DC)E	27	(Viikari *et al.* 1986)
Aspergillus awamori (crude prep)	Birch	peroxide	15	(Viikari *et al.* 1987)
	Pine	peroxide	16	
Streptomyces olivochromogens (crude prep)	Birch	peroxide	22	(Viikari *et al.* 1987)
	Pine	peroxide	15	
Trichoderma harzianum	Pine	(DC)E	25	(Clark *et al.* 1991)
Bacillus subtilis (crude prep)	Birch	peroxide	3	(Viikari *et al.* 1987)
	Pine	peroxide	9	
Sporotricum dimorphosporum (purified)	Softwood	CE	25	(Chauvet *et al.* 1987)
	Poplar	CE	31	
Streptomyces olivochromogens (crude prep)	Pine	peroxide	14	(Kantelinen *et al.* 1988)
Aspergillus awamori (crude prep)	Pine	peroxide	14	(Kantelinen *et al.* 1988)

Difference in κ between xylanase treated and non-treated pulps as a percentage of the original

Table 1.4 continued

Source of Enzyme	Pulp	Post-enzyme Treatment	Δ Kappa# (%)	Reference
Streptomyces olivochromogens (crude prep)	Pine	peroxide	14	(Kantelinen *et al.* 1988)
Aspergillus niger ("Pectinase" prep)	Pine	peroxide	17	(Kantelinen *et al.* 1988)
Xylanase (cloned)	Hardwood	CED	24	(Paice *et al.* 1988)
Streptomyces lividans (cloned)	Hardwood	(CD)EDED	80% Cl_2 saved	(Senior and Hamilton 1991)
Pulpzyme Xylanase (alkaline stable)				
Bacterial	Pine	(DC)E	9.5	(Pedersen and Elm 1991)
Fungal	Pine	(DC)E	20	
Carterzyme HS Xylanase	Hardwood	X(EO)-CD(EO)D	15	(Skerker *et al.* 1991)
Iogen Commercial Xylanase	Softwood	(CD)E	25	(Tolan and Canovas 1991)

Difference in κ between xylanase treated and non-treated pulps as a percentage of the original

Hexosans and pentosans are thought to be ether linked by the C-6 oxygen or the C-5 oxygen, respectively, to the α-C of the propane moiety of polymeric lignins (Jiang *et al.* 1987; Minor 1986; Yamasaki *et al.* 1981) and possibly to the ß-C and γ-C as well (Iversen and Wannstrom 1986). These ether linkages may be either through backbone or side-groups residues. Other types of bonding (ie: C-C) may also be possible (Minor 1986) but alkali labile ester linkages are not thought to

survive cooking (Iversen and Wannstrom 1986; Yamasaki *et al.* 1981). Isogai *et al.* (1987) has suggested three possible types of associations between residual lignin, cellulose and hemicelluloses (Fig 1.10); (A) lignin linked to cellulose via hemicellulose, (B) hemicellulose linked to cellulose via lignin, (C) a combination of the two previous models. Presumably pectic substances such as galactans and arabinans would behave in a manner similar to the hemicelluloses.

Xylan in kraft pulps exists predominantly in a redeposited form known as "retake" xylan (Meller 1965). This xylan arises from the solubilisation of the pentosan during the kraft cook. Under hot alkali conditions, the xylan is rapidly deacetylated (hardwoods) and may be partially depolymerised by the well known "peeling" reaction (Meller 1965). Arabinose substitution at the C-3 hydroxyl of a xylose residue may prevent excessive depolymerisation (Aurell and Hartler 1965). Some, but not all, of the uronic acid substituents are also removed (Aurell and Hartler 1965; Meller 1965). As the cook progresses, the alkalinity of the liquor drops and the solubility of the xylan decreases. The xylan thus precipitates on the fibre surface, possibly as discrete amorphous aggregates (Meller 1965; Mora *et al.* 1986b). This redeposited xylan is difficult to remove by alkali extraction (Kantelinen *et al.* 1991) and is thought to be held on the fibre surface by strong hydrogen bonding to cellulose (Mora *et al.* 1986b). However, alkali extraction should disrupt these bonds and solubilise the deposited xylan unless it is physically held within microcavities within the fibre wall (Scott 1984). The redeposited xylan may also be crystallised to cellulose or covalently linked by transglycosylation (Meller 1965). Crystallisation may be more probable with hardwood xylans as the uronic acid substituents are readily removed during cooking (Meller 1965). However, with pine kraft pulps many of the arabinose substituents may remain bound to the xylan backbone (Aurell and Hartler 1965) which would prevent any crystallisation of the pentosan. This precipitated xylan influences interfibrel bonding and paper strength properties. It has been estimated that as much as 50% of the xylan in pine kraft pulps is redeposited while the redeposited xylan in birchwood kraft pulps is much less (Meller 1965).

The association of redeposited xylan with lignin is unclear; lignin/carbohydrate bonding has been postulated (Meller 1965). Some papers have shown that new

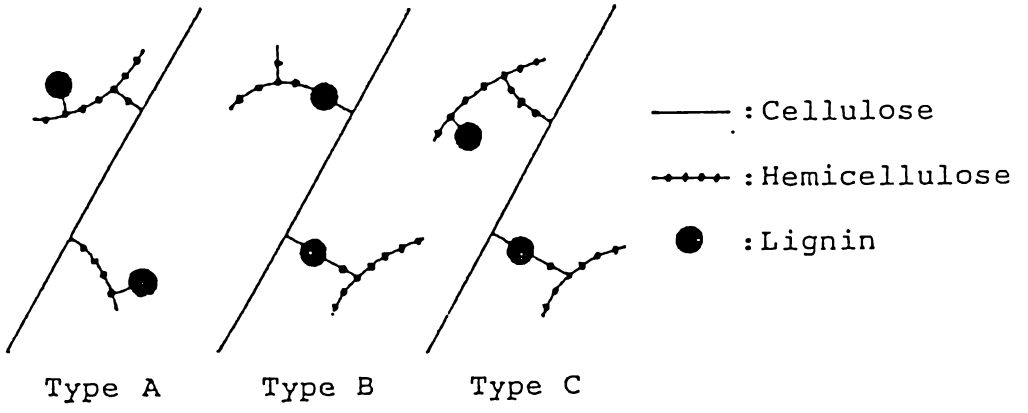

Fig 1.10 Three possible arrangements for residual lignin, residual hemicellulose and cellulose in kraft fibre wall (Isogai *et al.* 1987)

bonds could be formed (Iversen and Wannstrom 1986) while recent work has shown that no new ether bonds are formed between lignin and hemicellulose during cooking (Minor 1986). Unless new covalent bonds are formed to hold lignin fragments to the fibre surface then any association between lignin and redeposited xylan would be weak. Some of the xylan is not dissolved and remains in its original locale. As residual lignin is retained within the cell wall, rather than on the surface, it is probable that native xylan and residual lignin maintain the same association previously described.

Two general mechanisms of enzyme assisted bleaching are thought to occur. In the first, enzymes hydrolyse xylans that are directly associated with residual lignin; in the second xylanases modify cell wall properties, particularly porosity, by the enzymatic removal of specific xylans. Both types of mechanisms could occur simultaneously.

Xylanase treatment affects hemicelluloses associated with residual lignins by either cleaving xylans that link cellulose to lignin (Fig 1.10 model A), removing xylans that physically shield lignin from bleaching chemicals (Fig 1.10 model B) or both (Fig 1.10 model C). The first type of scheme should allow the simple alkaline extraction of residual lignin (Yamasaki *et al.* 1981). Post-enzymatic alkaline extraction alone removed a modest amount of lignin from xylanase treated hardwood kraft pulps (Paice *et al.* 1988). This suggests cleavage of xylan bridges between lignin and cellulose may be occurring to a moderate degree. However, chemical delignification of enzyme treated pulps results in much greater attack of the residual lignin (Kantelinen *et al.* 1988; Viikari *et al.* 1987; Viikari *et al.* 1986) suggesting that the enzymes also remove xylans that may be shielding lignins from bleaching chemicals.

Residual lignins are directly associated with glucomannans and pectins as well as xylans. If the removal of hemicelluloses shielding residual lignins was the main mechanism of enzyme assisted bleaching, then other hemicellulases should be as effective as xylanases in improving bleachability. This would be evident with softwoods which have a much higher glucomannan content than hardwoods. Data from various experiments are inconclusive in this respect. A pectinase that improved pulp bleachability contained a major xylanase component which could

have been solely responsible for this effect (Kantelinen *et al.* 1988). A mannanase purified from *Bacillus subtilis* did improve the bleachability of a pine kraft pulp but a significant proportion of the solubilised carbohydrate was xylan suggesting that the release of this polymer may have boosted bleaching (Clark *et al.* 1990). In the same study, a purified *Aspergillus niger* mannanase that solubilised 11% of the glucomannan within the fibre did not improve bleachability. In another study, a purified *B. subtilis* mannanase had no effect on the bleachability of softwood kraft pulp despite removing some glucomannan (Viikari *et al.* 1990). A radiata pine kraft pulp treated simultaneously with a xylanase and a mannanase purified from *Trichoderma harzianum* showed a moderate improvement in bleachability over pulps treated with both enzymes separately (Clark *et al.* 1991). The inconsistent effects seen with mannanase treatment may reflect differences between enzyme preparations or the degree of substitution of the glucomannan substrate of the various pulps. Mannanases that do improve bleachability appear to remove galacto-glucomannan rather than glucomannan (Clark *et al.* 1989).

In general, xylanases appear to be more effective bleach boosting agents than mannanases. Also, if the removal of carbohydrates directly associated with residual lignins were the main mechanism of enzymatic bleach boosting then the more of these carbohydrate solubilised the greater the improvement in bleachability that would be expected. No such correlation has been found (Clark *et al.* 1990, 1991). It would seem that the enzymatic hydrolysis of hemicelluloses associated with residual lignin is not the only mechanism of enzyme assisted bleaching. Hemicellulases, particularly xylanases, are known to have an effect on overall cell wall properties. These types of modifications may also influence chemical delignification.

Xylanase treatment of aspen pulps has been shown to increase fibre swelling as determined by increases in water retention values (WRV), however average pore size and total pore volume were drastically reduced. (Mora *et al.* 1986a). Similar increases in WRV, an indicator of fibre porosity (Scallan and Caries 1972), have been cited for an unbleached birchwood kraft pulp and a spruce sulphite pulp (Noe *et al.* 1986). Improved swelling would facilitate lignin diffusion through the fibre wall (Favis *et al.* 1981; Lagstrom-Nasi *et al.* 1987). Therefore, enzymatic xylan

removal from within the wall may increase the porosity of the fibre resulting in a greater rate of diffusion of lignin fragments, produced during bleaching. This assumes that the xylanase has reasonable access to xylans throughout the fibre wall. Fibre saturation point data indicates that from 30% to 50% of the total pore volume of a radiata pine kraft fibre pulp has a diameter large enough to accommodate an enzyme-sized molecule (Clark *et al.* 1990). Electron microscopy data has shown that these pores are distributed throughout the entire fibre wall (Screbotnik and Messner 1990; Screbotnik *et al.* 1990). This suggests that xylanase treatment could indeed affect a large part of the total fibre volume. This would mean that xylanase related fibre swelling could affect the entire fibre.

Redeposited surface xylan may also inhibit diffusion of lignin from the fibre. Birch wood kraft pulps with high or low levels of retake xylan were prepared (Kantelinen *et al.* 1991). Non-enzymatic carbohydrate diffusion was greater with the low retake xylan pulps than with those that had higher levels of redeposited xylan. It was proposed that the redeposited xylan prevented diffusion of carbohydrates from the fibre (Kantelinen *et al.* 1991). This type of non-specific carbohydrate leaching has been shown in other pulps and, like lignins, is temperature dependent (Lagstrom-Nasi *et al.*1987).

These high and low redeposited xylan pulps were then treated with a xylanase (Kantelinen *et al.* 1991). The enzyme hydrolysed more xylan from the pulp with the higher amounts of retake xylan than from the low retake xylan pulps. This was interpreted as hydrolysis of the retake xylan from the fibre surface and suggests that redeposited xylan was susceptible to enzymatic hydrolysis. Peroxide delignification of the two types of xylanase treated pulps showed that greater improvements in bleachability were obtained with pulps containing high amounts of redeposited xylan. However, the pulps with the low amounts of retake xylan were delignified to a greater extent. This was interpreted as meaning that the removal of redeposited xylan had a greater effect on bleachability than the removal of native xylan (Kantelinen *et al.* 1991). This has lead to the proposal by Kantelinen *et al.* (1991) that surface deposited, retake xylan physically blocks the diffusion of residual lignin from the fibre. Xylanases act to increase the surface area over which this diffusion can occur, by removing the xylan barrier. The effect of xylanases on

native xylan, as measured by the bleach boosting effect of the xylanase on the pulps with the low amounts of retake, was several fold lower than the effect of the xylanases on the pulps with the high levels of retake xylan (Kantelinen *et al.* 1991).

Several mechanisms that explain enzyme assisted bleaching have been proposed. The only mechanism that has been shown to have a direct effect on bleachability is the enzymatic removal of redeposited xylan (Kantelinen et al. 1991). Other mechanisms that have been proposed require confirmation to establish to what degree they affect enzyme assisted bleaching, if at all. This can be achieved by examining the effect that the enzymes have on pulps and correlating this with any improvments in bleachability. Precisely determining how xylanases, and possibly other hemicellulases, improve bleachability will be essential for optimising the effect.

1.6 HEMICELLULASE THERMOSTABILITY

Ultimately cost will dictate whether hemicellulases are adopted in pulp bleaching. A key factor to minimising enzyme cost is its stability. Enzymes stable to environmental extremes, such as temperature, may be less expensive to produce and purify (Ward and Moo-Young 1988). As brownstock is washed with hot water (Smook 1987), thermostable enzymes may be more suitable to many established pulping regimes. Also, increased enzyme thermostability would allow longer incubation periods (lowering required dosages) with the possibility of recycling enzyme from wash waters.

Enzymatic hydrolysis at higher temperatures may expedite delignification. Non-specific leaching of lignin from kraft pulps is a diffusion controlled phenomenon (Favis *et al.* 1981). As such, the diffusion coefficient of lignin from the fibre wall is temperature dependent (Favis and Goring 1983). However, the diffusion rate of lignin rises dramatically at temperatures of 70°C and higher (Favis and Goring 1983). This rapid rise in diffusion, much faster than predicted by the Stokes-Einstein equation (Favis and Goring 1983), is thought to be due to temperature mediated changes within the fibre wall (Goring 1985). Enzyme treating at these temperatures may increase delignification by leaching over the course of the incubation.

Under conditions where a protein demonstrates catalytic activity, secondary and tertiary structure necessary for catalytic activity are maintained by a number of non-covalent forces between various amino acids (Daniel *et al.* 1989; Ward and Moo-Young 1988). Briefly these include;

- hydrophobic/hydrophilic interactions which tend to arrange polar, hydrophilic amino acids on the exterior and hydrophobic amino acids in the interior of the protein molecule,

- hydrogen bonding, especially in the stabilisation of intramolecular polar atoms buried within the hydrophilic interior of the protein (all interior polar atoms are hydrogen bonded) (Chothia 1975),

- ionic interactions, which are governed by the pKa of individual amino acids,

- van der Waals interactions.

Disulphide bonds are not thought to contribute significantly to protein secondary and tertiary structure (Ponnuswamy *et al.* 1982). If the sum of these stabilising forces is greater than extraneous destabilising forces (ie: free energy increase caused by a rise in temperature), then protein structure and catalytic activity is maintained. If not then denaturation occurs; protein structure is altered (coagulation, precipitation) and catalytic activity is lost (Daniel *et al.* 1989).

Intra-molecular stabilising forces are dictated by the primary structure of the protein (ie: amino acid sequence of the peptide) (Daniel *et al.* 1989; Ward and Moo-Young 1988). Extraneous factors such as polyols, cofactors, prosthetic groups and metal ions may enhance thermostability but ultimately this is a property of the protein (Ward and Moo-Young 1988). Purified enzymes tend to keep their thermostability. Genes coding for these enzymes cloned into mesophilic organisms produce thermostable enzymes (Bergquist *et al.* 1987, Daniel *et al.* 1989). Purification of thermostable enzymes from a milieu of thermally unstable proteins can be achieved by raising the temperature to a point where the latter are denatured while the former are not (Patchett *et al.* 1989; Schofield *et al.* 1988). This type of purification protocol may be a rapid and useful method for the large scale purification of thermostable hemicellulases. Genetic engineering has been used to produce cellulase-free xylanases that have successfully bleached kraft pulps (Paice *et al.* 1988). Therefore, purification by combined genetic manipulation and heat

treatment would seem to be an ideal way of purifying thermostable xylanases.

Thermostable xylanases have been purified from eubacteria (Hudson *et al.* 1991; Sharrock *et al.* 1983), actinomycetes (Holtz *et al.* 1991) and fungi (Table 1.3). A thermostable xylanase purified from *Thermomonospora fusca* has been used to treat a kraft hardwood pulp for 3 hr at 80°C (Perrolaz *et al.* 1991). The xylanase treated pulps that were delignified by a peroxide soda extraction had κ that were 24% lower than untreated controls while the κ of delignified control pulps were lowered by only 15%. This was not as large a reduction in κ as reported by others (Table 1.4) for pulps treated with xylanases from mesophiles but the latter were incubated for longer periods of time (Perrolaz *et al.* 1991). Therefore, thermostable xylanases at temperatures as high as 80°C are also effective bleach boosting agents.

Other organisms may also produce thermostable xylanases that are of research interest and may be effective bleach boosting agents. This thesis examines a thermostable xylanase and other thermostable hemicellulases from one such organism.

A thermophilic, cellulolytic anaerobe, designated isolate TP8 6.3.3.1, was isolated from the geothermal areas in the Lake Taupo region of New Zealand (Reynolds *et al.* 1986; Sisson *et al.* 1987). A gene library of this organism has been constructed (Love and Streiff 1987). Several genes coding for enzymes pertaining to cellulose and hemicellulose degradation have been cloned into *Escherichia coli* thus facilitating their purification and study (Patchett *et al.* 1989). These include endo- and exo-cellulases (Schofield *et al.* 1988),a ß-glucosidase (Love and Streiff 1987; Plant *et al.* 1988), a xylanase (Luthi *et al.* 1990b, c) a ß-xylosidase (Luthi and Bergquist 1990, Hudson *et al.* 1991) and an acetyl xylan esterase (Luthi *et al.* 1990a). All of the enzymes cloned to date from this organism show strong thermostability. The organism may well produce other hemicellulases. In this investigation the cloned xylanase and other cloned hemicellulases are evaluated for their effects on softwood pulps and as bleach boosting agents.

CHAPTER TWO

MATERIALS AND METHODS

2.1 GROWTH OF BACTERIA AND ENZYME PREPARATION

2.1.1 Isolate TP8 6.3.3.1 ("Caldocellum saccharolyticum")

TP8 6.3.3.1 was obtained from the culture collection of the Thermophile and Microbial Biochemistry and Biotechnology Unit, University of Waikato, Hamilton, New Zealand. A basal salts medium with the following composition (as per F. Rainey University of Waikato) was used for growth and maintenance of the thermophile: 2.0 gL^{-1} tryptone, 1.0 gL^{-1} yeast extract, 3.3 gL^{-1} KH_2PO_4, 0.2 gL^{-1} $MgSO_4.7H_2O$, 12 mgL^{-1} $CoCl_2.6H_2O$, 1.0 gL^{-1} cysteine HCl and 10 mlL^{-1} of a trace elements solution (Appendix 1). The medium was buffered with 10 gL^{-1} MOPS (3-[N morpholino] propane sulphonic acid) and the pH adjusted to 7.2. The pH of the medium at 70°C was 6.9. Resazurin (0.1 mgL^{-1}) was added as a redox indicator. The basal salts medium was prepared as a concentrate (90% of final volume) and dispensed into 28 ml Bellco tubes (9ml of medium) or 125 ml serum bottles (90 ml of medium). The medium was boiled and vessels capped with butyl rubber stoppers. The vessels were evacuated and flushed with O_2-free N_2 (three times), then autoclaved (121°C, 15-20 min). Concentrated carbohydrate solutions (10X) were boiled, degassed and sterilized in a similar manner. Sterile carbohydrate solutions (10 ml/90ml of basal medium) and filter sterilised Wolins vitamins solution (10 mlL^{-1}) (Appendix 1, Wolin *et al.* 1963) were aseptically added to the basal medium. Media vessels were again flushed with sterile, O_2-free N_2 (three times). Once inoculated, media vessels were incubated at 70°C. Samples were withdrawn with sterile 1 ml tuberculin syringes. Growth was monitored by microscopic examination. Cultures of TP8 6.3.3.1 were maintained on the basal medium with cellobiose (1 gL^{-1}) as the carbon source.

2.1.2 *Escherichia coli* Clones

Three *E. coli* clones containing DNA fragments from TP8.6.3.3.1 were prepared

at the Centre for Gene Technology, University of Auckland, Auckland NZ. Clones have been deposited with the culture collection of the Thermophile and Microbial Biochemistry and Biotechnology Unit, University of Waikato, Hamilton NZ and are described in Table 2.1. Clones were grown on Luria broth (yeast extract, trypticase peptone and NaCl 5 gL^{-1} of each) which had been augmented with the appropriate filter (0.45 μm pore size) sterilized antibiotic to a final concentration of 100μgL^{-1}. Inoculated media was incubated at 37°C for 18 to 24 hr under aerobic conditions. Aliquots of actively growing cultures were lysed by sonication (in an ice bath, 30 sec on/30 sec off) and assayed for specific enzyme activities. Clones were maintained on Luria agar (15 gL-1) plates supplemented with antibiotic as described. After growth at 37°C for 24 hr, plates were sealed with Parafilm and kept at 4°C. Cultures were transferred approximately every two weeks.

Table 2.1 Description of three *Escherichia coli* clones used in this study.

	Clone (Plasmid Identification)		
	pNZ1417	pNZ1435	pNZ1019
Enzyme Activity of cloned DNA	Xylanase	Xylanase	Mannanase
Size of TP8 DNA fragment in plasmid (gene cloned)	~2 kb (*xynA*)	1.3 kb (*xynA*)	~5 kb (*manA*)
Antibiotic Resistence marker	Chloram-phenicol	Ampicillin	Ampicillin
Reference (clone preparation, gene sequence)	Luthi *et al.* 1990b#	Luthi *et al.* 1990b	Luthi *et al.* 1991

deletion derivative of pNZ1076

2.1.3 Large Scale Growth And Harvesting Of Clones

Clones were grown on a large scale (10 L) in a 14 litre fermenter with a fed batch system as described below (P. Janssen, Waikato University, pers. comm.). Fermenter medium was prepared by dissolving the following in 9.5 L of deionized water: 126 g glycerol, 100 g trypticase peptone, 20 g yeast extract, 32.2 g NH_4Cl, 10.5 g KH_2PO_4, 1.35 g $MgSO_4.7H_20$, 0.43 g K_2SO_4, 0.02 g $CaCl_2.2H_20$, 0.03 g $FeSO_4.7H_20$, 50 g MOPS and 10 ml of the trace elements solution (Appendix 1). The pH was adjusted to 7.0 and the medium autoclaved (121°C, 60 min) in the fermentation vessel. The fed batch concentrate was prepared by dissolving the above components in 500 ml of water and autoclaving as above. Filter sterilised antibiotic and autoclaved antifoam (Bevaloid 5901, Bevaloid Chemicals Ltd.) were aseptically added to the cooled fermenter. The final antibiotic concentration was 100 μgml^{-1}. The fermenter was inoculated with an 18 hr *E. coli* culture, grown on 100 ml Luria broth (5% w/v of each: yeast extract, tryptone, NaCl) and incubated at 37°C with vigorous agitation and aeration. The fed batch concentrate was added to the fermenter after 12 to 18 hr growth. Cultures were incubated for a further 6 to 8 hours and then harvested. With some runs a pH of 7 was maintained by the manual addition of 1 M NaOH. Growth was monitored by measuring OD_{600} of appropriately diluted culture aliquots. Ten litres of *E. coli* culture were concentrated to 1 L by filtration through an Amicon (Beverly Mass., USA) hollow fibre system with a pore size of 0.5 μm. The resulting slurry was centrifuged (9000g for 20 min) and the cell pellet collected.

2.1.4 Cell Lysis And Enzyme Purification

E. coli pellets were lysed by the detergent-osmotic shock-lysozyme method specified by Patchett *et al.* (1989). Cell pellets were slurried with glycerol (33% of pellet weight), Triton X-100 (1% v/wet pellet weight, added as a 10% solution) and 2-mercaptoethanol (0.1% v/wet pellet weight). Slurries were refrigerated for 2.5-3.5 hour with agitation. After osmotic shock, the slurry was diluted with approximately six volumes of extraction buffer at pH 7. This buffer was composed of MOPS (50 mM), 2-mercaptoethanol (7.5 mM), Na_2EDTA (7.5 mM), NaN_3 (0.02% w/v) and lysozyme (0.02% w/v). After mixing, the slurries (~2-3 l) were incubated at 35°C

with agitation for 2 hr. Once at temperature(~20 min), 0.02% (w/v) DNAase (Sigma, St. Louis Mo., USA) and 0.06% (w/wet pellet weight) phenylmethane-sulphonyl fluoride were added, the latter as a 1% (w/v) acetone solution.

After lysis, enzyme solutions were partially purified by heat treatment (Patchett *et al.* 1989; Schofield *et al.* 1988). Solutions were incubated at 70°C in a gyratory shaker (150 rpm) for 1.5 hours. After incubation, enzyme solutions were rapidly cooled to room temperature (ice bath) and centrifuged (9000g for 20 min). Pellets were discarded and the enzyme containing supernatants were centrifuged again (16,000g for 15 min). Supernatants were decanted and concentrated to approximately $1/10^{th}$ their original volume by ultrafiltration through a YMS (Amicon) polysulfonate membrane with a molecular weight cutoff of 10,000 Da. Enzyme solutions were washed with distilled water, concentrated and freeze-dried. Enzyme powders were stored under vacuum at 4°C.

Further purification of the freeze-dried mannanase was on a smaller scale. A 325 mg sample of this powder was dissolved in 50 mM citrate buffer at pH 5.5 (1.5 mg of protein per ml). Biocryl 2100 anion exchange resin (TosoHaas, Philadelphia, PA USA) was added to the protein solution (25 μl of resin per ml of solution), mixed well and left to stand for 15 minutes at room temperature. The Biocryl 2100 (TosoHaas, PA USA) was pelleted by centrifugation and the supernatant concentrated by ultrafiltration. The enzyme concentrate was injected (2.4 mls) onto a TSK Preparative Gel Permeation Chromatography (GPC) column at room temperature, eluted with 10 mM citrate buffer (pH 6, flow 5ml min^{-1}) with a UV detecter. Fractions (12.5 ml) were collected and the mannanase activity assayed. Six fractions with the highest mannnase activity were pooled. Three such injections were made and the pooled fractions concentrated to ~7 ml.

2.2 ENZYME AND PROTEIN ASSAYS

2.2.1 Screening For Endo-Hemicellulases

Screening for enzymatic depolymeristion was done by a modification of the Congo Red diffusion plate technique described by Teather and Wood (1982, Wood *et al.* 1988). Polysaccharides (5 gl^{-1}) and agar (15 gl^{-1}) were dissolved in hot buffer (100 mM MOPS, pH 7) and autoclaved (121°C, 15 min). Petri plates were poured

(~20 ml per plate) and left to solidify. Excess condensate was blotted from the lids and the plates inverted for storage (refrigerated). When required, wells were cut into the agar plates with a cork borer (9 mm diameter). No more than six, evenly spaced wells were cut per plate. Wells were filled with 100 μl of enzyme solution (ie: culture supernatants or control solutions). Plates were sealed with Parafilm and incubated at 70°C for 8 hr. To visualise areas of polysaccharide hydrolysis, plates were flooded with approximately 15 ml of 1% (w/v) Congo Red solution and left to stand for 20 min. After this time, the dye was poured off and the plates flooded with 1M NaCl (15-20 min). The salt solution was poured off and the dye/salt protocol repeated. Areas high in polysaccharides were stained a deep red colour by the dye, zones where the polysaccharide had been depolymerised were not stained so that a zone of clearing ("halo") was evident. These halos were centered around wells containing enzymes that hydrolysed the dissolved polysaccharide. Halo diameters were measured.

Two enzymes were used as controls; a thermostable xylanase obtained from a sonicated culture of *E. coli* clone pNZ14 (heat treated supernatant) and a commercially available mannanase from *Bacillus subtilis* (Megazyme, North Rock Australia).

2.2.2 Endo-Glycohydrolases

Endo-xylanase and endo-mannanase activities were assayed by incubating the enzyme with the appropriate substrate and monitoring the increase in reducing sugar concentration over time (Sharrock 1988). Reducing sugars were measured by the p-aminobenzoic acid hydrazide (PAHBAH) technique of Lever (1973). The reagent for this technique was prepared daily as follows:

- 10 ml each of (0.5 M Na_3citrate.$2H_2O$, 0.1 M $Na_2SO_3.7H_2O$, 0.2 M $CaCl_2.2H_2O$ and 5 M NaOH) are mixed, in order,
- 50 ml of water is added,
- 1.52 g of *para*-aminobenzoic acid hydride (Sigma) is dissolved in the solution,
- the volume of the solution is adjusted to 100 ml with water.

The PAHBAH reagent (freshly prepared, daily) was kept refrigerated and in the dark until required.

Assay protocols for the xylanase and the mannanase were identical except that with the former oat spelt xylan (0.25% w/v) was the substrate while the latter used locust bean gum (0.5% w/v). Enzymes were routinely assayed at 70°C and pH 6.0 (50 mM citrate) unless otherwise indicated.

Endo-glycohydrolase assay protocol involved pre-incubating 400 μl of substrate in a 1.8 ml polypropylene eppendorf tube at the required temperature (~5 min). The assay was started by the addition of 100 μl of an appropriately diluted enzyme solution. Eppendorf tubes were rapidly capped and inverted to mix the reactants. After exactly 10 min, the reaction was stopped by the addition of 1 ml cooled PAHBAH reagent. The high alkalinity of the reagent effectively denatured the enzyme and stopped the reaction. The reaction solution was heated in a boiling water bath for 5 min, cooled to room temperature and the optical density at 420 nm was determined. Reducing sugar concentrations were estimated by reference to a standard curve of either xylose or mannose standards. One unit (U) of activity was defined as 1 μmole of reducing sugar equivalent released per minute.

Endo-cellulase activity was assayed as described except that 0.5% (w/v) CMC was used as substrate and glucose was used to establish the PAHBAH standard.

All assays were done in triplicate and corrected for substrate/enzyme controls. These were run parallel with the assay but enzyme solution was not added to the substrate until after the PAHBAH reagent, thus any increase in Ab_{420} was not attributed to enzymatic activity. This background absorbance was subtracted from those of enzyme-containing samples. Typical standard curves for xylose and mannose are presented in Fig 2.1.

2.2.3 Exo-Glycohydrolases

Exo-glycohydrolase activities, such as ß-xylosidase, ß-mannosidase, α-arabinosidase and α-galactosidase, were assayed using the appropriate p-nitrophenolglycoside derivative as substrate (5 mM of substrate in 50 mM citrate buffer, pH 6). Assay protocols were as described above except that 0.5 ml of cold 1M Na_2CO_3 was used to stop the reaction. The optical density (400 nm) was read and the concentration of *p*-nitrophenol released determined by using the Beer Lambert equation (extinction coefficient of *p*-nitrophenol = 18,500 $M^{-1}cm^{1}$)

(Schofield, DPhil University of Waikato 1990). Activity was expressed as μmoles of *p*-nitrophenol released per minute by one ml of enzyme solution. Assays were done in triplicate and absorbances were corrected for background readings as previously described.

Exo-cellulase activity was determined using methylumbelliferyl-ß-D-cellobiose (MUC)(Schofield, DPhil University of Waikato 1990). The protocol was identical to that used with other exo-glycohydrolases except that the reaction was stopped by the addition of 3.5 ml of 0.5M glycine/NaOH buffer (pH 10.4) and the extinction coefficient of methylumbelliferone was 18,357 $M^{-1}cm^{-1}$. Assays were done in triplicate and corrected for separate substrate and enzyme controls. MUCase activity was defined as μmoles of methylumbelliferone released per minute by one ml of enzyme solution.

2.2.4 Protein

Protein was quantified by the modified Lowry method of Petersen (1977) with bovine serum albumin as a standard (10 mgL^{-1} to 150 mgL^{-1}). Fresh Lowry reagent buffer was prepared by adding 1 ml of solution A (0.5% $CuSO_4.5H_2O$/1% Na_3citrate) to 50 ml of solution B (2.0% Na_2CO_3/0.4% NaOH). Folin Ciocalteau reagent (BDH, Poole UK) was diluted 1:1 with water. Sample or standard (200 μl) was added to one ml of Lowry reagent buffer, vortexed and allowed to stand for 10 min. Diluted Folin Ciocalteau reagent (100 μl) was added to each tube, which was then immediately mixed and allowed to stand at ambient temperature for 1 hr. The Ab_{750} was measured after the incubation period and protein concentrations were obtained by reference to a standard curve established with BSA (Sigma). A standard curve is presented in Fig 2.2. All samples were analysed in triplicate.

2.2.5 Molecular Weight Determination Of Enzymes

The molecular weight of the mannanase was determined by analytical GPC on the highly purified aliquot using an analytical TSK Gel Permeation Chromatography (GPC) column at room temperature, eluted with 10 mM citrate buffer (pH 6, flow 0.8 ml min^{-1}) with a UV detecter (280 nm). A 25 μl sample of the mannanase solution (~0.5 U) was injected, fractions were collected (3ml) and assayed for

protein and mannanase activity. The molecular weight of the mannanase was estimated by comparison of retention times with those of standards (Pierce Standards, Rockford Ill.): cytochrome C 12,500 Da, chymotrypsin A 25,000 Da, bovine serum albumin 67,000 Da, rabbit muscle aldolase 158,000 Da).

2.3 CARBOHYDRATE ANALYSIS

2.3.1 Polysaccharides

An assortment of xylans and mannans were utilised in this study. Oat spelt xylan, larchwood xylan, locust bean gum, guar gum and carboxy methyl cellulose (CMC) were purchased from Sigma (St. Louis, Mo. USA). Ivory nut mannan was purchased from Megazyme Ltd (Australia). Konjac gum, *Pinus radiata* xylan and *Pinus radiata* glucomannan were kindly provided Dr. T. Clark, Forest Research Institute, Rotorua NZ. Total carbohydrate and neutral sugar composition of the various polysaccharides are summarised in Table 2.2.

The radiata hemicelluloses were purified by a two-step cation/anion exhange process. The cation exchange procedure used a glass column (50 cm X 1 cm) packed with 40 ml of standard grade Amberlite IR $120H^+$ cation exchange resin (BDH, Poole UK). A 0.25% radiata glucomannan solution was passed through the column at a flow of 0.25 bed volumes per min. The eluent was collected and the pH was monitored (~pH 4). The eluent was then passed through a (20cm X 1.5 cm) bed of Dowex 1X8 anion exchange resin (BDH) in the acetate form at the same flow rate. The eluent was concentrated by roto-evaporation and freeze dried. Radiata xylan was purified in a similar manner except that the anion exchange step was omitted. The carbohydrate contents of the radiata glucomannan and xylan were 94% and 89%, repectively. Both purified hemicelluloses had the same % monosaccharide composition as reported in Table 2.2.

An acetylated softwood glucomannan, an acetylated birchwood xylan and an unacetylated birchwood xylan were kindly provided by Dr. J. Puls (BFH, Hamburg, Germany).

TABLE 2.2 Carbohydrate composition of the polysaccharides used in this study.

Polysaccharide	%Carbohydrate Content	%Monosaccharide composition				
		Glc	Xyl	Man	Gal	Ara
oat spelt[#] xylan	76	2.9	83.0	0.0	2.4	11.6
larchwood[#] xylan	71	1.1	96.8	0.6	0.0	1.3
radiata[#] xylan	42	2.9	77.1	1.2	5.2	14.3
guar gum[*] galactomannan	ND	0	0	67	33	0
locust bean[#] gum galactomannan	81	1.7	0	78.4	20	0
konjac gum[#] glucomannan	83	36.5	0	62.9	0	0
radiata pine glucomannan[#]	50	20	3.4	72.8	3.2	0.6
ivory nut mannan[$]	99	0	0	100	0	0

as determined by D. Steward (FRI)
* from Aspinall (1971)
$ supplier's specification (Megazyme, North Rocks, Australia)

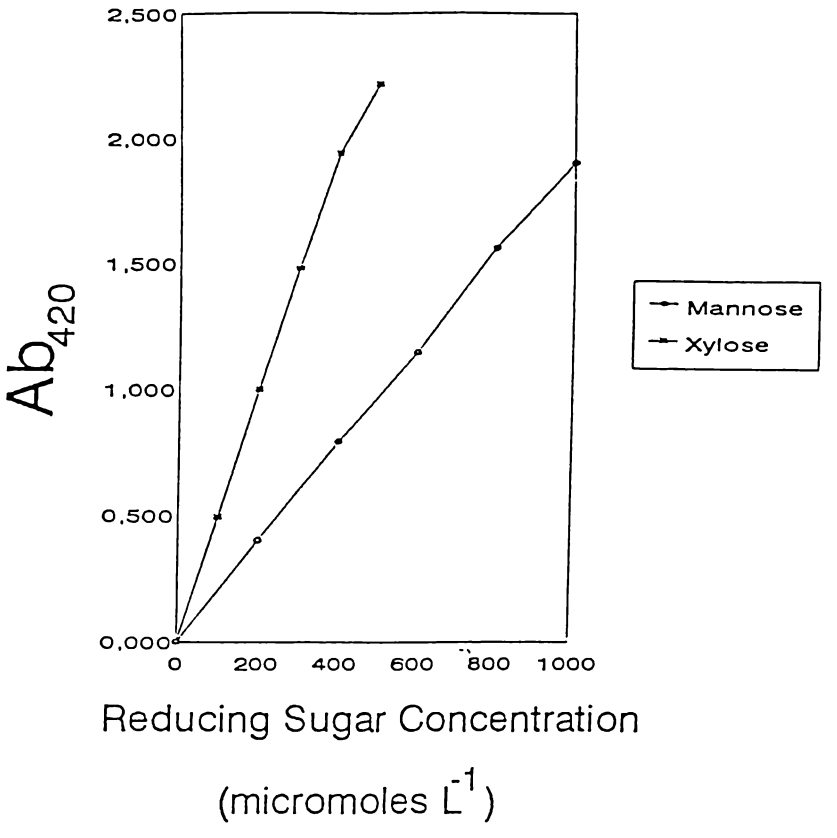

Fig 2.1 PAHBAH standard curve for xylose and mannose

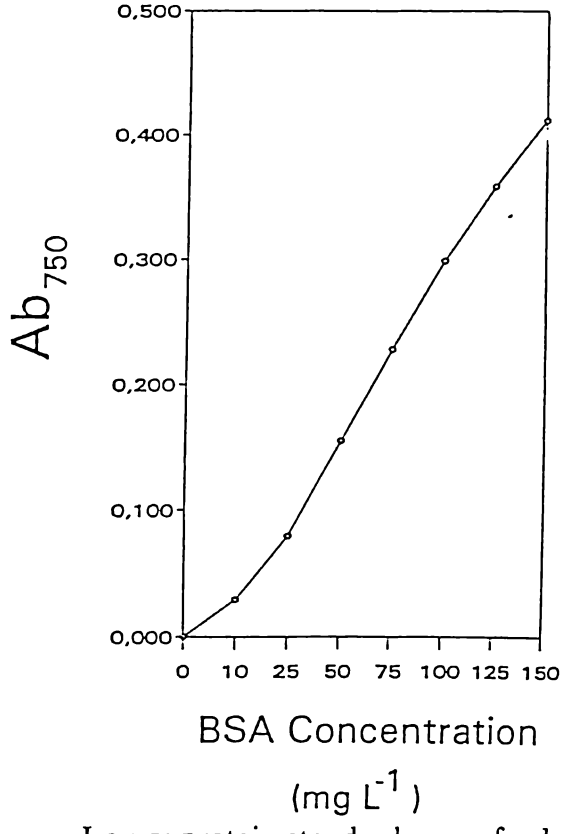

Fig 2.2 Lowry protein standard curve for bovine serum albumin (BSA)

2.3.2. Oligosaccharide Analysis

2.3.2.1 Xylo-oligosaccharide ion exchange fractionation

Xylo-oligosaccharides produced by the enzymatic hydrolysis of xylans were separated into neutral and acidic fractions by a cation/anion exchange chromatography procedure akin to that of Fontana *et al.* (1988). Hydrolysate solution containing 100-200 mg carbohydrate was passed through a syringe packed with 10 ml of standard grade Amberlite IR 120 (H^+ form) cation exhange resin. The resin was washed with 50 ml of distilled water. Eluant and washings were collected, pooled and applied to a glass column (2 cm X 25 cm) packed with 40 ml of Dowex 1-X4 anion exchange resin (100-200 mesh) in the acetate form. The column was eluted with 500 ml distilled water at 0.25 bed volumes per min. The eluant was collected and designated the neutral fraction. The anion exchange column was then eluted with 500 ml of 5 M acetic acid. The eluant was collected and designated the acidic fraction. Both fractions were roto-evaporated to dryness and subjected to further analysis (total carbohydrate, gel permeation chromatography and monosaccharide analysis).

2.3.2.2 Gel permeation of neutral oligosaccharides

Manno-oligosaccharide and neutral xylo-oligosaccharide solutions were fractionated into oligomers of DP1-10 by gel permeation chromatography (GPC). Samples (10-100 mg in 0.5 ml of water) were applied to the GPC system which included two, 1 meter columns (1.6 cm i.d.) in series packed with Biogel P2 (Pharmacia Ltd.). The columns were kept at 60°C and eluted with degassed MilliQ water at 0.5 ml min^{-1}. Detection was by monitoring refractive index (Waters, Milford Mass., USA). The system was calibrated using a standard containing Dextran Blue (210 min retention, excluded peak), cellobiose (550 min retention) and glucose (600 min retention). Fractions (10 ml) were collected, pooled and roto-evaporated before further analysis.

2.3.2.3 Manno-oligosaccharide isomer resolution

Glucomanno-isomers from the DP2 and DP3 GPC fractions were resolved by HPLC (A. M^{ac}Donald FRI, unpublished results) or preparative paper

chromatography (Kusakabe *et al.* 1988). In the HPLC method, a dextro-PAK radial compressed column (Waters, Bedford, Mass., USA) was used. The column was kept at room temperature and eluted with filtered MilliQ water (0.6 ml/min). Detection was by refractive index (HP 1040 RI detector). Fractions were collected as required and roto-evaporated to dryness.

In the paper chromatography method used, 10 mg of sample was spotted in a thin line onto 3 mm Whatman No. 1 chromatography paper (45 cm X 60 cm, run length 30 cm). The paper was eluted with n-butanol:pyridine:water (8:3:3) for 72 hr at ambient temperature in a descending manner. Thin strips (1.5 cm wide) cut from the edges of the paper were used to visualise sample components. Detection was by the silver nitrate dip method described by Chaplin (1986) except that 5% (w/v) $Na_2S_2O_3$ was used as a fixative. Areas of the paper that contained the components of interest were cut and the oligosaccharides eluted from the paper with water. The collected eluant was roto-evaporated to dryness before subsequent analysis.

2.3.2.4 Acid hydrolysis of oligosaccharides

Oligosaccharides and polysaccharides were hydrolysed to their monomeric constituents by acid hydrolysis. This was routinely done by refluxing 2 to 10 mg of carbohydrate in 10 ml of 2 M trifluoroacetic acid (TFA) for 3 hours. TFA was removed by roto-evaporation, the sample washed with water (3-5 times) and taken to dryness.

2.3.2.5 Whole hydrolysate analysis

Enzyme hydrolysed hemicelluloses were analysed by J. Puls (BFH, Hamburg, Germany) using anion exchange chromatography with a AS6 HPLC column and a pulse amperometric detector (Dionex Corp, Sunnyvale CA USA). Samples were eluted with a two phase, linear gradient (Puls *et al.* 1991). The linear gradient commenced with 5% eluant A (100 mM NaOH + 1 mM Na acetate) and 95% eluant B (100 mM NaOH) and finished (20 min) with 21% eluant A and 79% eluant B. The total run time was 30 min. A chromatogram showing the resolution of monosaccharides and xylo-oligomers is presented in Fig 2.3.

Identification of specific xylo-oligosaccharides was achieved by sequential enzymatic hydrolysis with various exo-hydrolases and re-analysis of the oligomers as outlined in Appendix 2. The following enzymes were used: acetylxylan esterase (Poutanen *et al.* 1990, Puls *et al.* 1991), α-glucuronidase from *Agaricus bisporus* (Puls *et al.* 1987) and α-L-arabinofuranosidase (Poutanen 1988). Other enzymes used in this procedure were purified and were of a mesophilic nature.

2.3.3 Monosaccharide Analysis

Monosaccharides were identified and quantified by either HPLC of underivatised sugars or capillary gas chromatography (GC) of acetylated sugar alcohols. The former system was used for hydrolysates obtained by TFA hydrolysis only, while the latter was used for TFA hydrolysates and H_2SO_4 hydrolysed pulps.

The HPLC system consisted of two Biorad HPX-87P columns in series at 85°C linked to a Hewlett Packard Refractive Index (model HP 10474) detector. The eluent was MilliQ water (degassed) at a flow of 0.4 ml/min. Erythritol (5 mg/ml) was added as an internal standard at a concentration of 1 ml standard to 10 ml of sample. Prior to injection (20 μl), monosaccharide solutions were de-ionized through an anion/cation exchange resin mixture (~ 1 ml of a 1:1 Serdolite AW14:Amberlite CG-120 H^+ mixture) and filtered (0.45 μm pore size). Base-line resolution of glucose, xylose, galactose, arabinose and mannose was obtained and erythritol was used as an internal standard. A chromatogram of these standards is presented in Fig 2.4.

Alditol acetate derivitisation and capillary gas chromatography (GC) was by the method of Theander as recommended by the IEA Bioenergy Agreement-Voluntary Standards Activity (IEA, 1991). Hydrolysate aliquots (200 μl) were made alkaline by adding of 20-40 μl of concentrated NH_4OH. Aldoses were reduced by the addition of 100 μl of 2.8M KBH_4 (dissolved in 3M NH_4OH) to the alkaline solutions and incubating for 90 min at 40°C. The reduction reaction was stopped by mixing 100 μl of glacial acetic acid into the solutions. Acetylation of the sugar alcohols was done by adding 500 μl 1-methylimidazole

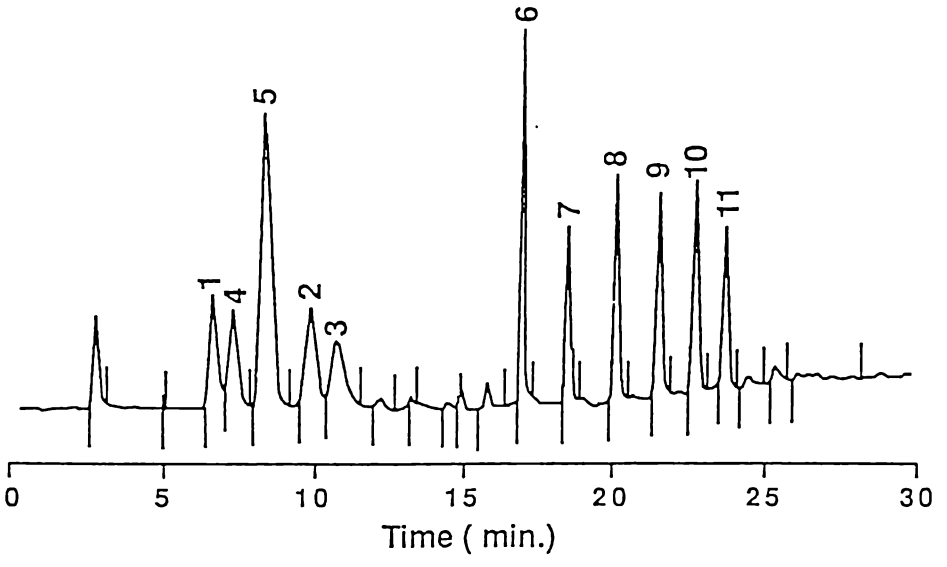

Fig 2.3 Chromatogram of arabinose, galactose, glucose, xylose, mannose and a range of xylo-oligomers (X_2-X_7) as resolved by anion exchange chromatography (Dionex system, SECT 2.3.2.5)

carbohydrate	μg injected
1 arabinose	0.08
4 galactose	0.08
5 glucose	0.30
2 xylose	0.10
3 mannose	0.10
6 xylobiose (X_2)	0.20
7 xylotriose (X_3)	0.25
8 X_4	0.30
9 X_5	0.35
10 X_6	0.40
11 X_7	0.40

and 2 ml acetic anhydride to samples and vortex mixing. After a 10 min reaction time, water (5 ml) was added to the samples to decompose excess acetic anhydride. Dichloromethane (2 ml) was vortexed into samples. The phases were allowed to separate. Approximately 1.5 ml of the dichloromethane phase was transferred into a GC vial and securely capped.

An HP Gas Chromatograph (model 5890) with a DB-225 capillary column (30 m x 0.25 mm i.d.) (JW, Folsom CA) was used for the analysis of the alditol acetate derivatives. Helium was the carrier gas (30 cm/sec, split injection 1:25) and detection was by flame ionisation. The column was maintained at 220°C throughout the run. Injector and detector temperatures were 240°C. The injection volume was 2 μL and run time was 30 min. A typical chromatogram of the five standard monosaccharides (ara, xyl, gal, man and glc) and the internal standard (inositol) is presented in Fig 2.5.

On occasion, descending paper chromatography was used to identify neutral monosaccharides. Solutions containing monosaccharides were spotted onto Whatman No 1 paper (45 cm X 60 cm, 30 cm run length, medium flow, 0.16mm thick). Samples were eluted with n-butanol:pyridine:water (8:3:3) for 24 hr at ambient temperature. Component detection was by the silver nitrate method described previously. Sugar identification was by comparisons of R_f values with those of known standards.

2.3.4 Total Carbohydrate

Total carbohydrate was determined by a modification of the orcinol/sulphuric acid method described by White and Kennedy (1986). Mannose, glucose or xylose (10 mgL^{-1} to 120 mgL^{-1}) was used as a standard. Sample or standard (1 ml) was dispensed into acid washed, glass test tubes and the tubes immersed in an ice bath for 15 min. Two ml of freshly prepared orcinol reagent (0.2% w/v orcinol dissolved in conc H_2SO_4) was rapidly added to each tube which was vortexed and incubated in a boiling water bath for exactly 15 min. Tubes were then cooled in an ice bath and their Ab_{540} determined. Total carbohydrate was determined by reference to an appropriate standard curve (xylose, glucose or mannose). Samples were analysed in triplicate. A typical standard curve is presented in Fig 2.6.

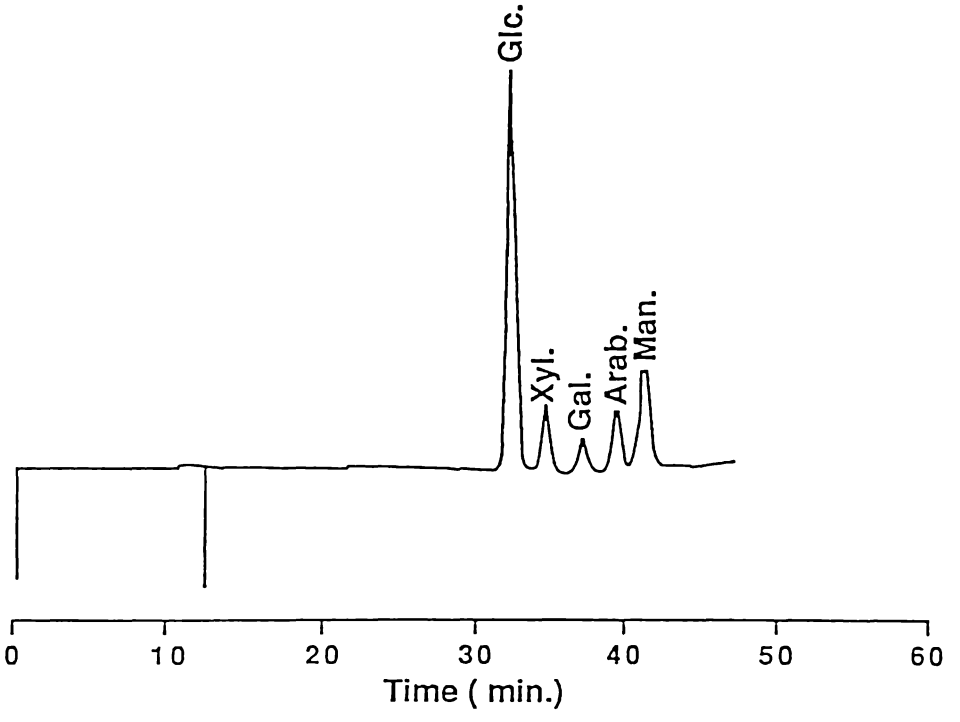

Fig 2. Chromatogram of five monosaccharides as resolved by HPLC (Biorad system, SECT 2.3.3). Erythritol (not shown) had a retention time of 62.9 min and was used as an internal standard.

carbohydrate	mg injected
glucose	0.41
xylose	0.20
galactose	0.09
arabinose	0.07
mannose	0.18

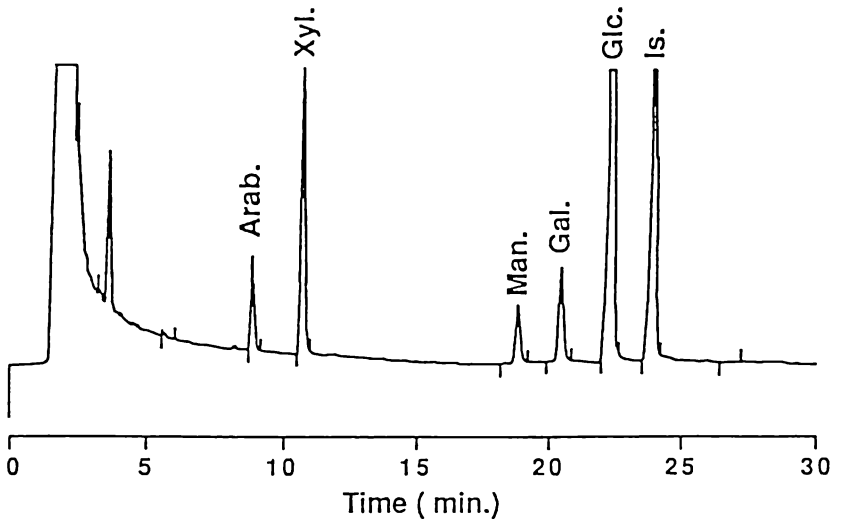

Fig 2 Chromatogram of five alditol acetates, derived from the correponding monosaccharides, as resolved by capillary GC (SECT 2.3.3).

carbohydrate	μg injected
arabinose	0.5
xylose	3.0
mannose	0.6
galactose	0.9
glucose	9.5
inositol (IS)	2.4

The Molisch test was used for carbohydrate detection in fractions, supernatants and other solutions. Two drops of Molisch reagent (10% w/v α-naphthol in absolute ethanol) was added to 0.5 ml of sample solution. One ml of concentrated H_2SO_4 was then gently introduced into the sample. The appearance of a red/pink colour within a few minutes was indicative of the presence of carbohydrates in the sample.

2.3.5 NMR Spectroscopy

Proton and ^{13}C NMR spectra of oligosaccharides were acquired on a Bruker AC-200 spectrometer using a 5 mm probehead. Samples were dissolved in D_2O (~3 $mgml^{-1}$).

Proton spectra were aquired at 313°K using a presaturation sequence to suppress the residual HOD resonance. At this temperature the residual HOD signal was shifted from the anomeric proton region to ~4.6 ppm where it could be safely irradiated. Presaturation occurred during the relaxation delay of 5 sec. A 90° excitation pulse was used. ^{13}C NMR spectra were acquired using a power gated composite pulse decoupling sequence at 300°K. Exponential line broadening was applied. All spectra were referenced to external acetone (δ_H 2.17 ppm, δ_C 29.8 ppm).

2.4 PULP ANALYSIS AND BLEACHING

2.4.1 Source Of Pulps

Two unbleached *Pinus radiata* pulps, a kraft and a NSAQ (neutral sulphite anthroquinone) pulp, were obtained from a local mill. An unbleached hardwood (predominantly tawa) kraft pulp was also obtained. Several kilograms of each pulp were obtained to provide a consistent stock for the duration of this study. Pulps were washed by agitation in water at 2% consistency at ambient temperature for 1 hr. The pulps were then screened and hand pressed to approximately 25% consistency and the wash repeated. Pulps were then screened, pressed and fluffed. Pulps were equilibrated in sealed containers for several days and then divided into small lots of 25 g to 250 g wet weight and sealed in plastic bags. Pulps

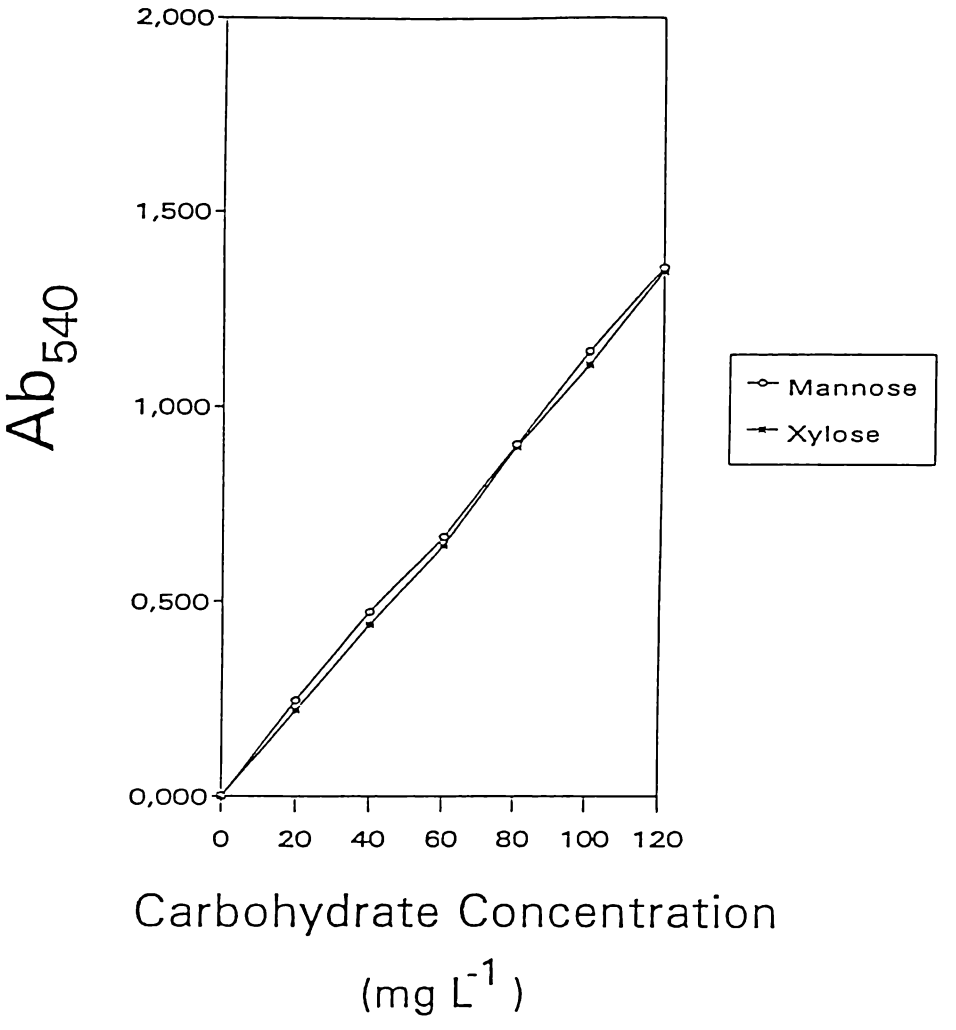

Fig 2.6 Total carbohydrate standard curve (orcinol procedure) for xylose and mannose (SECT 2.3.4)

were kept refrigerated (4°C) and used as required.

Two unbleached radiata mechanical pulps, a CTMP pulp and a TMP pulp, were obtained from the pilot plant facility of PAPRO, FRI (Rotorua NZ). Both mechanical pulps were divided into two lots. The first lot was deacetylated by suspending the pulp in 0.32% (w/v) NaOH at ~2% consistency. The second lot (acetylated) was treated as the first except that water was subsituted for the NaOH solution. Pulps were treated for 24 hr at ambient temperature, then filtered through terylene cloth and hand pressed. The mechanical pulps were washed at room temperature by suspending in water (~3% consistency) for 1 hr with agitation. Pulps were drained and pressed as described. Both types of mechanical pulps were kept frozen (-20°C) until required. Washed unbleached kraft aspen pulp was obtained from Dr. T. Clark (FRI).

2.4.2 Chemical Analysis Of Pulps

2.4.2.1 Determination of pulp dry weight content

Samples of wet pulps were weighed in clean, tared weighing jars and oven dried overnight (~18 hr) at 105°C. After drying, samples were allowed to cool in a dessicator and the dry weight of the pulps was determined. Pulp consistencies were calculated as the percentage ratio of the dry pulp weight to wet pulp weight (ie: g of OD pulp/100g of wet pulp). All samples were done in triplicate.

2.4.2.2 H_2SO_4 hydrolysis of pulps

Pulps were analysed for Klason lignin, acid soluble lignin and carbohydrate composition by the method of Theander as recommended by the IEA Bioenergy Agreement-Voluntary Standards Activity (IEA 1991). The first stage of this procedure is the acid hydrolysis of the carbohydrate fraction of the pulp. Oven dried pulps were accurately weighed into glass test tubes (16 mm X 100 mm). Three ml of 72% H_2SO_4 (60 ml of conc H_2SO_4 diluted with 90 ml distilled water) was added to these tubes. These were incubated for 1 hr at 30°C and mixed occasionally with a glass rod. The glass rod was not removed from the tubes to minimise sample loss. After the incubation period, 79 ml of water was used to transfer each sample to a 500 ml Erlenmeyer flask for secondary hydrolysis. If the carbohydrate

composition was to be determined by the alditol acetate method of SECT 2.3.3, then 5 ml of internal standard solution (myo-inositol, 20 mg/ml) was added to each flask. Flask mouths were covered with inverted 50 ml beakers and the flasks heated for 1 hr at 125°C. Standards were subjected to the secondary hydrolysis procedure to account for carbohydrate loss during this stage.

Klason lignin is defined as the insoluble fraction of acid hydrolysed wood or pulp. This was determined by filtering the hydrolysed pulp samples, under vacuum, through dried, tared asbestos-lined gooch crucibles. The sample filtrates were used for carbohydrate and acid soluble lignin analysis. The acid insoluble material in the gooch crucibles was washed with distilled water (25-40 ml). The crucibles were oven dried overnight, cooled in a desiccator and weighed. Dry weight of insoluble material was defined as Klason lignin and was expressed as a percentage of the pulp dry weight.

Acid soluble lignin was determined by measuring the Ab_{205} of the previously described hydrolysed pulp filtrates within six hr of the hydrolysis. The absorbance was measured on a Philips PU 8740 UV/VIS spectrophotometer using a quartz cell (1 cm path length). Dilute H_2SO_4 (3 ml of acid diluted with 84 ml water) was used to zero the spectrophotometer and also tc dilute samples, if needed, so that absorbances fell below 1.000 Ab Units. The acid soluble content was expressed as a percentage weight on pulp and calculated as follws:

$$\% \text{ acid soluble lignin (g/100 g of OD pulp)} = (Ab_{205} \times 0.084\ L)/\epsilon_{205}$$

$$(\epsilon_{205} = 110\ Lg^{-1}cm^{-1}).$$

The concentration of individual sugars in the acidic lignin-filtrate solutions was determined by either gas chromatography of alditol acetates or HPLC (SECT 2.3.3). In instances where the latter was used, the following modifications to the hydrolysis procedure were required; no myo-inositol was added, 84 ml of water was used in the secondary hydrolysis instead of 79 ml. Samples were then analysed by the HPLC procedure previously outlined.

2.4.2.3 Enzyme treatment of pulps

Enzyme treatment of pulps were carried out in pre-weighed plastic jars with screw top lids or mason jars. Pulps were suspended in distilled water (2% consistency) and the entire system was weighed prior to incubation or enzyme addition. The relevant enzyme(s) were then mixed into the pulp suspension, the vessel sealed and incubated at 70°C for 24 hr. All experiments included a control treatment in which no enzyme was added but was otherwise performed in exactly the same way as the enzyme treatments.

After the incubation period, the pulps were cooled to room temperature and weighed. Any losses in weight were deemed to be due to evaporation and these losses were taken into consideration during total sugar calculations. The pulp suspensions were filtered through terylene cloth to recover the pulp and collect the liquor. The filtrate which contained the solubilised carbohydrates, protein and particulate matter, was retained for subsequent analysis. Before further analysis, the drained water was filtered through Whatman glass microfibre filter (GF/C) to remove particulates. Filtrates were kept frozen until required for analysis.

If pulps were to be bleached after enzymatic treatment they were washed (twice) by resuspending the drained, hand pressed pulps in hot tap water (2% consistency) and mixing for 15 min. Pulps were then drained, hand pressed, torn into fragments and equilibrated overnight in sealed plastic bags.

2.4.2.4 Extraction of hemicelluloses from pulps (Hamilton procedure)

Xylans, glucomannans and galactoglucomannans were extracted from radiata kraft pulps by the modified Hamilton procedure as described by Beelik *et al.* (1967). All extractions were carried out at ambient temperature. To remove extraction liquors from pulps, slurries were filtered under vacuum through Whatman glass microfibre filters (GF/C). Pulp washes involved suspending pulp in solvent for ~15 min with rigorous mixing and then filtering as described.

The extraction protocol is summarised in Fig 2.7. Pulps were suspended in 4.4% (w/v) $Ba(OH)_2.H_2O$ at 6% consistency. After 1 hr with occasional stirring, concentrated NaOH (18.5%w/v) was added so that the $Ba(OH)_2$ and NaOH concentrations of the slurries were 2.0% (w/v) and 10% (w/v), respectively. The

pulp consistency was altered to 2.8%. After a further hour with intermittent mixing, the pulp slurries were drained through a glass microfibre filter. The pulps were washed with fresh $Ba(OH)_2$/NaOH solvent as mentioned above (pulp consistency 2%). Pulps were drained and the filtrates and corresponding washings pooled. The filtrates were rich in extracted xylans, the pulps (known as holocellulose A) were washed with water (~2% consistency) six times. After the washes, holocellulose A was slurried in a 5% (w/v) acetic acid solution overnight at ambient temperature and then washed until the pH was approximately 6.5.

Washed holocellulose A was air dried and slurried in 1% (w/v) NaOH (~3% pulp consistency) for 30 min. Pulps were drained and washed with fresh 1% (w/v) NaOH (~5% consistency). Filtrates and corresponding washes were pooled. These were rich in extracted galactoglucomannan. The residual pulp material, known as holocellulose B, was washed to neutrality with water and air dried.

Holocellulose B was further extracted with 15% (w/v) NaOH (~3% consistency) for 1 hr with intermittent mixing, then filtered and washed with fresh 15% (w/v) NaOH. Matched filtrates and washes were rich in glucomannan. Pulp residues (holocellulose C) were water washed to neutrality and air dried.

Samples of the various holocelluloses were collected throughout the process. These samples were dispersed in acetone (~2% consistency) for 2 hr, then filtered and sealed in plastic bags. The holocelluloses were analysed for sugar composition and lignin content as previously described (SECT 2.4.2.2).

2.4.2.5 Isolation of extracted hemicelluloses

Xylan and galactoglucomannan rich liquors were concentrated in a similar manner (Fig 2.7). Liquors were acidified to pH 5 with glacial acetic acid. Absolute ethanol (150-200 ml) was added to the hemicellulose liquors to precipitate the polysaccharides. The liquors were centrifuged (15 min, 10,000 g) and the supernatant decanted. Molisch tests of supernatants confirmed that carbohydrates were precipitated from the solution. Carbohydrate rich pellets were redissolved in water (~20 ml) and added, drop-wise, to absolute ethanol (100-150 ml). Pellets were again recovered by centrifugation as described above. The ethanol precipitation was repeated. The resulting pellets were dissolved in water (~20 ml) and

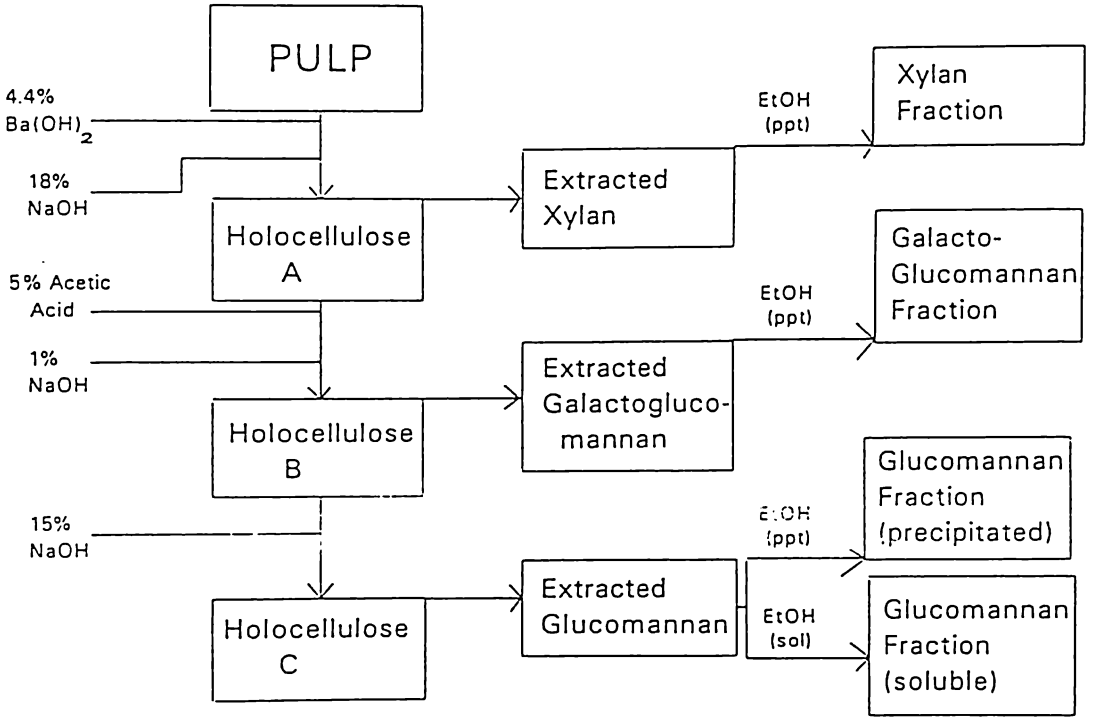

Fig 2.7 Outline of the Hamilton procedure for the extraction of hemicelluloses from pulp (SECT 2.4.2.4 and SECT 2.4.2.5)

freeze-dried.

Glucomannan rich liquors (~100 ml) were treated with fresh 4.4% (w/v) $Ba(OH)_2.8H_2O$ overnight at ambient temperature. The liquors were then centrifuged (15 min, 10,000 g). Barium treated supernatants were decanted and kept. Pellets were dissolved in 10 ml water and added drop-wise to 90 ml of absolute ethanol. The ethanol solution was centrifuged as described above. Ethanol precipitation was repeated. Pellets were dissolved in 10 ml of water and freeze dried. This fraction was identified as the "precipitated" glucomannan in Fig 2.7.

The barium treated supernatants described above were found to have considerable amounts of polysaccharides (Molisch test). The supernatants were concentrated to approximately 20 ml by roto-evaporation. These concentrates were treated with 25 ml of 4N acetic acid overnight under ambient conditions. The liquors were ethanol precipitated (80 ml) and centrifuged as previously described. The ethanol precipitation was repeated and the pellets were collected, dissolved in 10 ml water and freeze dried. This fraction was designated the "soluble" glucomannan (Fig 2.7).

2.4.2.6 Molecular weight distribution of extracted hemicelluloses

The molecular weight distribution of the extracted hemicelluloses was determined using gel permeation chromatography (GPC). The GPC system consisted of a column (100 cm X 1.6 cm) packed under moderate pressure with Toyopearl HW-55 (superfine) as instructed by the manufacturer (Tosoh Corp, Tokyo Japan). The column was eluted with 100 mM sodium phosphate buffer (pH 7.2, filtered and degassed) and maintained at 60°C. The system was regularly calibrated with dextrans (Pharmacia, Uppsala Sweden) of known sizes (Mw range 9,000 to 73,000). Dextran Blue (Mw $>2X10^6$) (Sigma) was used to determine the exclusion volume of the column. Peaks were monitored using an HP 10474 refractive index detector (Waters Associates, Milford Mass.).

Average molecular weight (M_w) and average molecular number (M_n) were determined by the integral weight distribution method. Specifically, the molecular weight distribution was integrated and the retention time of the median was

compared to a standard curve prepared using the Dextran standards (Fig 2.8). Average degree of polymerisation (DP) was calculated by dividing the M_n by the molecular weight of the appropriate anhydrous monomer residue (mannose 162; xylose 132). Polydisperisty was calculated as the ratio of M_w/M_n.

2.5 PULP BLEACHING

2.5.1 Pulp Bleaching

Laboratory scale pulp bleaching methodology was as outlined by Pulp and Paper Research Organisation (PAPRO, FRI, Rotorua, NZ). Pulps were bleached with a $D_{35}C_{65}E$ sequence.

Total elemental chlorine required was calculated as follows:

g of Cl required/100 g dried pulp = (κ X Chlorine multiple),

where chlorine multiple, a dosage factor, was between 0.14 and 0.20.

Chlorine was added in two forms, chlorine dioxide (35% of elemental Cl required) and Cl_2 solution (65% of elemental Cl required). Chlorine water and chlorine dioxide solutions were obtained from PAPRO and tested for active chlorine on a daily basis (Appendix 3).

Bleaching (DC stage) commenced when chlorine dioxide was stirred into pulps suspended in make-up water. After 1 min, the required chlorine water was added and stirred vigoursly for 30 sec. Final pulp consistency was 2.5%. Beakers were sealed and left to stand at ambient temperature for exactly 1 hr. after the chlorine stage of the (DC) E sequence, the pulps were drained through terylene and the pH of the liquor was determined. The residual chlorine was determined by titration as described in Appendix 3. Pulps were washed (15 min in hot tap water at 0.5% consistency) and drained. Excess water was squeezed from pulps by hand. Pulps were then fragmented by hand and caustic extracted.

Caustic extractions (E stage) were carried out by mixing pulps with an NaOH solution in sealed plastic bags and incubating for 70 min at 70°C. Final pulp consistency was 10%. The NaOH charge (g of NaOH/100 g dried pulp) was half the chlorine charge calculated above. In some cases pulps were rapidly heated in

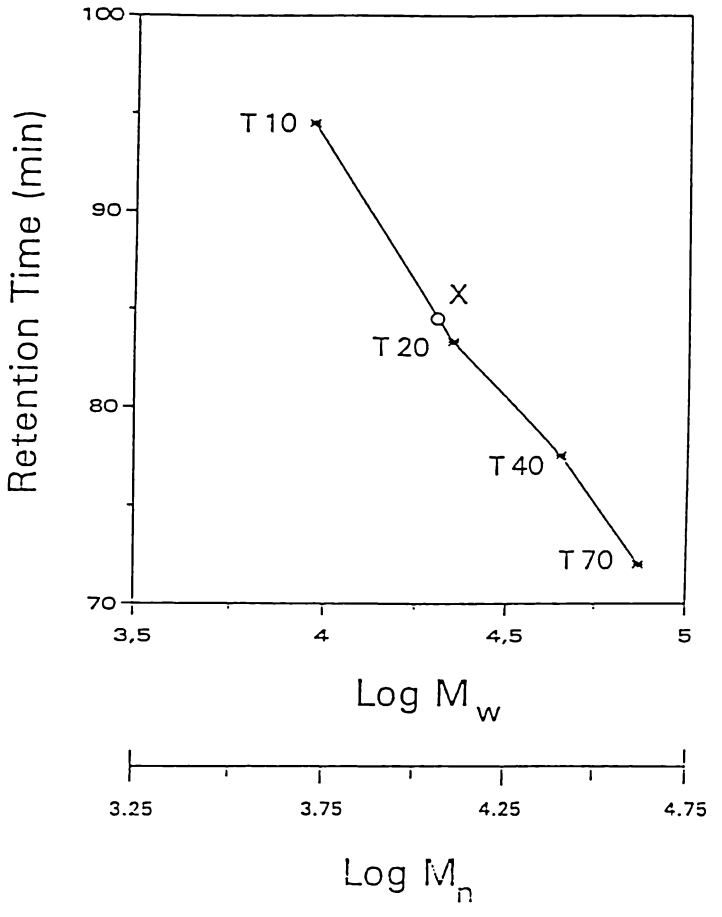

Fig 2.8 Log M_w and Log M_n vs GPC retention time.
(SECT 2.4.2.6)
Dextran standards used
Dextran T70 (M_w 73,400; M_n 42,400);
Dextran T40 (M_w 44,400; M_n 28,900);
Dextran T20 (M_w 22,300; M_n 15,000);
Dextran T10 (M_w 9,300; M_n 5,700)
X Average Retention Time of standard mixture
- 5 replicates, average 84.5 min (stdev +/- 1.07, % CV 1.3%)

a microwave oven and then incubated at temperature for 60 min. After the incubation, pulps were drained through terylene and the extraction liquors were analysed for pH and Ab_{280}. Pulps were washed as before, excess water was hand squeezed from the pulps which were then hand fluffed and allowed to equilibrate overnight in sealed plastic bags. Dry weights and κ were then determined on the bleached pulps.

2.5.2 Kappa Number (κ)

Kappa numbers were determined by the TAPPI microkappa method (useful method 246). Water (~60 ml) was added to a weighed, wet pulp sample (~1-4 g) of known consistency. The pulp was dispersed with a magnetic stirrer. Ten ml of 4 N H_2SO_4 and 4 ml of 0.25 N $KMnO_4$ were combined in a beaker. The assay was started when this solution was added to the dispersed pulp. The reaction was allowed to proceed, with stirring, for exactly 10 min. The temperature of the assay solution was taken 5 min into the reaction. The reaction was stopped by the addition of the 2 ml of 1 N KI. The free iodine was titrated to a 1% (w/v) starch endpoint with a standard 0.10 N $Na_2S_2O_3$ solution. The exact normality of the $Na_2S_2O_3$ solution was determined daily by titration against 0.1N KIO_3 (Appendix 2). Assay blanks were prepared daily in a similar manner except that no pulp was used.

Kappa number is related to the percent $KMnO_4$ consumed during the 10 min assay period. This was calculated as follows:

$$p = (b-a)n/0.1$$

where

b is the titre (ml of $Na_2S_2O_3$) of a no-pulp blank,
a is the titre (ml of $Na_2S_2O_3$) of the pulp being assayed,
n is the exact normality of the $Na_2S_2O_3$.

$$\%\ KMnO_4 \text{ consumed} = (p/\text{ml of } KMnO_4 \text{ added}) * 100\%$$

The % $KMnO_4$ consumed was used to determine the "f" factor, which corrects the p value to 50% $KMnO_4$ consumed. The f factor and temperature factor (T_f) corrected for deviations from standard temperature (25°C) were obtained from Tables in TAPPI (useful method 246). Therefore, κ were calculated according to the equation:

$$\kappa = (p \text{ X } f/\text{dry wt of pulp}) \text{ X } T_f$$

Pulps were assayed at least in triplicate and reported κ are averages of these determinations with a coefficent of variance of 2% or less. Control pulps of known kappa number were assayed daily to ensure reproducibility.

2.5.3 Nitrogen Content Of Pulps

The % (w/w) nitrogen content of pulps was determined by Jean Prince (Soils Section, FRI) using the indophenol blue method on a H_2SO_4/H_2O_2 digest of the pulp being assayed. The % (w/w) protein content of the pulps were estimated by multiplying the % nitrogen content by 6.25 (Haschemeyer and Haschemeyer 1973).

2.5.4 Pulp Viscosity

Pulp viscosities were determined by Steven Wrathall (PAPRO, FRI) using a modified TAPPI standard (230) method. Washed pulps of known consistency were dispersed in 25 ml of water by vigorous shaking with glass beads. Once dispersed, 25 ml of cupriethylenediamine (CED) reagent was added to each pulp suspension (under N_2) and mixed for approximately 20 min. After bubbles had dispersed, 15 ml of the CED solutions were drawn into calibrated capillary viscometers. The apparatus was equilibrated at 25°C for 5 min. The CED solution was drawn to the top mark on the viscometer and the time required for the solution to flow from the top to the bottom mark was accurately measured.

The viscosity of the pulp solution was calculated using the following formula:

$$V = Ctd$$

where

V is viscosity of the CED solution and dissolved pulp in mPa.s (cp) at 25.0°C,
C is a viscometer constant found by calibration,
t is the efflux time (sec),
d is the density of the pulp solution (set as 1.052).

Samples were done in duplicate with the viscosity of each duplicate read twice. The value reported is the average of the four determinations.

2.5.4 Pulp Brightness

Pulp brightness was determined by Steven Wrathall (PAPRO, FRI) using a modified ISO 3688-1977 method. Brightness pads were made adding ethylene diamine tetra-acid tetrasodium (EDTA) to a pulp suspension so that the final chelator concentration was 0.4% on OD pulp and the pulp consistency was 0.5%. The pH was adjusted to approximately 5 with acid. Pads were formed by draining the pulp slurry onto a Buchner funnel. No air was drawn through the pad. The pad was pressed and dried at constant humidity and the reflectance on the top surface measured using a R457 filter.

2.5.5 Water Retention Value Of Pulps

The water retention value (WRV) of pulps was determined by Dell Bawden (PAPRO, FRI) using the method of Penniman (1981). In this procedure, a sample of wet pulp was placed in a stainless steel sleeve (see Penniman 1981) with dimensions of 40 mm X 25.4 mm. A 20 mesh screen was soldered to one end of the tube and supported a finer (100 mesh) screen. The wet pulp was centrifuged (900g, 30 min) to remove water from between fibres and from the cell lumen. Any water remaining within the pulp was retained in the fibre wall. The weight of the centrifuged, wet pulp is determined (W1) in a sealed, tared glass jar and the pulp is oven dried (105°C) overnight (~18 hr). The dry pulp and jar are cooled in a dessicator and re-weighed. The weight of dry pulp was determined (W2). The WRV is the ratio of the wet to dry pulp weights (W1/W2) and is expressed as a percentage. Samples were done in duplicate.

CHAPTER THREE

HEMICELLULOLYTIC ACTIVITY OF ISOLATE TP8 6.3.3.1

3.1 INTRODUCTION

The geothermal regions of New Zealand have been recognised as sources of unique thermophiles. Novel, cellulolytic bacteria that grow readily at temperatures greater than 65°C have been isolated from these environments (Hudson *et al.* 1991; Reynolds *et al.* 1986). From a survey of 47 natural thermal sites in the central North Island, eight actively cellulolytic cultures that grew at 75°C were isolated (Sharrock *et al.* 1983; Sisson *et al.* 1987). Some of these were also found to be xylanolytic at these temperatures (Sisson *et al.* 1987). One of these isolates, designated TP8 6.3.3.1, was chosen for further study because of its relative active and thermostable cellulolytic activity (Donnison *et al.* 1988; Sisson *et al.* 1987). It was of biotechnological interest because of its capability to hydrolyse a variety of lignocellulosic substrates including crystalline cellulose, steam exploded SO_2 radiata pine, steam exploded hardwood and a bleached wood pulp (Donnison *et al.* 1988).

Initial studies (Reynolds *et al.* 1986) characterised TP8 6.3.3.1 as an anaerobic, non-sporulating, thermophilic eubacterium that grows optimally at temperatures of 70-72°C. Cellular morphology was described as non-flagellated, long rods (0.6 μm X 4 μm) with oval ends. Despite having gram positive cell wall ultrastructure (by electron microscopy), it stains gram negative (Reynolds *et al.* 1986). It can grow on cellulose, starch, xylan and cellobiose producing acetate, propionate, CO_2 and H_2. Cellulolytic activity is bound to the cellulose but bacterial cells do not (Reynolds *et al.* 1986). Metabolically, TP8 6.3.3.1 has been compared to *Clostridium stercorarium* but G+C ratios of the two species show that they are distinct and separate species (Sisson *et al.* 1987). DNA-DNA hybridisation studies with genomic DNA of TP8 and *Clostridium thermocellum*, 16S RNA sequencing (F. Rainey Thermophile Research Unit, University of Waikato, pers. comm.) of the two and the inability of TP8 to sporulate, clearly show that these two organisms belong to different species and possibly different genera (Donnison *et al.* 1988; Sisson *et al.*

1987). The name *"Caldocellum saccharolyticum"* has been proposed for isolate TP8 6.3.3.1 (Donnison *et al.* 1988) and some papers refer to it as such.

The cellulolytic activity of TP8 6.3.3.1 cell free supernatants was found to be stable at temperatures of 70°C and higher (Sisson *et al.* 1987). This is consistent with the theory that thermostability is a property of the native enzymes (Bergquist *et al.* 1987). A gene from TP8 coding for a ß-glucosidase was cloned and expressed in two mesophiles, *E.coli* and *Bacillus subtilis* (Love and Streiff 1987). The cloned enzyme retained its thermostability (Love and Streiff 1987; Plant *et al.* 1988), in agreement with the idea that this property is related to the primary structure of the protein (Bergquist *et al.* 1987; Love and Streiff 1987). Other enzymes affiliated with the cellulolytic and xylanolytic activities of TP8 have been cloned into *E.coli.* These include, as well as the ß-glucosidase, endo-and exo-cellulases (McCleary 1983; Schofield *et al.* 1988), a xylanase (Luthi *et al.* 1990c; McCleary 1983), a ß-xylosidase (Luthi and Bergquist 1990, Hudson et al. 1991) and an acetylxylan esterase (Luthi *et al.* 1990a). All these cloned enzymes are thermostable.

The difference between the thermostabilities of cloned thermophilic enzymes and the mesophilic enzymes of the host was exploited by Plant *et al.* (1988) in the partial purification of the ß-glucosidase. The technique has been scaled up to 10 L scale and has been applied with many of the listed cloned thermostable enzymes (Patchett *et al.* 1989; Schofield *et al.* 1988).

TP8 is both cellulolytic and xylanolytic at temperatures of 70°C and greater. These activities, as well as its ability to grow on softwood pulps (Donnison *et al.* 1988) imply that this micro-organism may also produce a mannanase. This chapter describes the screening of this thermophile for such an activity. The thermophile has demonstrated an acetylxylan esterase suggesting that it can de-branch various hemicelluloses. Other de-branching enzymes may be produced. Accordingly, the organism was also screened for thermostable α-galactosidase and α-L-arabinosidase activities.

3.2 METHODOLOGY

TP8 6.3.3.1 was grown on the basal media described (SECT 2.1.1) with various carbohydrates as carbon sources, as specified. Growth was under anaerobic

conditions at 70°C for approximately 48 hr. Whole culture or culture supernatants were screened for hemicellulase activities. After the growth period, cell free culture supernatants were prepared by centrifuging at 40,000 g for 5 to 10 min. The supernatants were assayed for various hemicellulase activities.

Mannanase and xylanase activities were assayed by the PAHBAH protocol outlined in SECT 2.2.2. α-Galactosidase activity was assayed by using either *p*-nitrophenol-α-D-galactopyranoside or *o*-nitrophenol-α-D-galactopyranoside as substrate (5 mM concentration) as outlined SECT 2.2.3. The Congo Red plate assay (SECT 2.2.1) was used to assay cultures for polysaccharide depolymerisation. All assays were carried out at 70°C.

3.3 RESULTS AND DISCUSSION

3.3.1 Evaluation of the Congo Red plate technique

The Congo Red technique has been successfully used to screen cellulolytic and xylanolytic bacterial cultures and enzyme solutions (Sazci *et al.* 1986, Wood *et al.* 1988). However, it has been reported that this technique does not stain mannans (Williams 1983). This was not the case with this study. The Congo Red diffusion plate technique was able to distinguish between most hydrolysed (clear zones) and unhydrolysed mannans (Table 3.1). Only the guar gum did not stain consistently, and was possibly due to poor dispersal of the galactomannan. Refrigeration of plates for several hours after enzyme hydrolysis enhanced the dye/polysaccharide staining thus improving the contrast between stained and unstained regions. This was particularly evident with the locust bean gum. For this reason, plates were routinely refrigerated after staining. The incubation of the plates at high temperatures had no adverse affect on the technique. The specificity of the enzyme controls showed that the method is suitable for screening cultures for mannanase activity.

3.3.2 Growth of TP8 6.3.3.1 on various carbon sources

As previously reported (Sisson *et al.* 1987), TP8 grew anaerobically within 48 hr on the basal salts media with either cellobiose or oat spelt xylan as sole carbon source at 70°C. The thermophile was also found to utilize locust bean gum and

Table 3.1 Evaluation of Congo Red diffusion plate technique for detecting the enzymatic depolymerisation of mannans.

Polysaccharide	Diameter of unstained zones (mm)[#]		
	Buffer	Xylanase[@]	Mannanase[$]
oat spelt xylan	-	+ +	-
larchwood xylan	-	+ +	-
locust bean gum	-	-	+ + +
konjac glucomannan	-	-	+ + +
radiata glucomannan	-	-	+ +
guar gum	-	-	-

- no unstained zones (well diameter 9 mm)
\+ diameter of unstained zones from 9-10 mm (weak)
\+ + diameter of unstained zones from 11-13 mm
\+ + + diameter of unstained zones from 14-16 mm
@ Thermostable xylanase (~1 U/ml)
$ *Bacillus subtilis* mannanase (~3 U/ml)

melibiose as sole carbon sources, under similar conditions. Due to medium turbidity caused by some of the substrates, growth was monitored by periodic microscopic examination during which only one cell type was evident; the long, single rods with rounded ends indicative of TP8 6.3.3.1 (Reynolds *et al.* 1986).

Growth on locust bean gum, a galactomannan, suggested that a mannanase was produced. This activity would be required to hydrolyse the polysaccharide to metabolisable oligomers. Yet melibiose utilization denoted α-galactosidase activity which suggested that the galactose branches of the galactomannan, rather than the mannan-backbone, were supporting growth. To confirm whether a mannanase was indeed produced, the cell free supernatant of the galactomannan-grown TP8 6.3.3.1 culture was evaluated for its ability to depolymerise a galactomannan and several glucomannans.

3.3.3 Endo-hemicellulases in TP8 culture supernatants

Screening supernatants (Congo Red technique)

Cell free supernatants of TP8 6.3.3.1 cultures which were grown on basal salts with 0.1% (w/v) cellobiose/0.5% (w/v) locust bean gum as carbon sources,

depolymerised locust bean gum, konjac glucomannan and radiata glucomannan as determined by the modified Congo Red diffusion plate technique (Table 3.2). The depolymerisation of the galactomannan was more extensive than that of the glucomannans. Whether this was due to differences in enzyme specificity or an artefact of the assay system was not evident. Similar experiments were performed with culture supernatants of TP8 grown on oat spelt xylan/cellobiose medium (Table 3.2). As expected, xylanase activity was found in these supernatants. Mannanase depolymerisation was also detected but this activity was weak and inconsistent. This could indicate low levels of mannanase induction by the xylan or cross reactivity of the xylanase. Data presented in Table 3.2 suggests that TP8 produced an extra-cellular mannanase when grown on a galactomannan.

Cellobiose grown cultures of TP8 did not depolymerise either mannans or xylans despite good growth of the culture (Table 3.2). Cellobiose was added to the medium to ensure prolific growth of TP8 and presumably was rapidly consumed during the inital stages of growth. As cellobiose alone did not induce either the xylanase or the mannanase, it was concluded that in the mixed cellobiose/polysaccharides medium, the polysaccharides induced the hemicellulases. The results were inconclusive with respect to the possible repression of these hemicellulases by cellobiose. The disaccharide was probably consumed very early during growth and any repressive effects would not be evident after the 48 hr growth period.

3.3.4 Assay of supernatants for hemicellulases

To simulate a lignocellulosic substrate, TP8 was grown at 70°C for 48 hr on a mixture of oat spelt xylan, locust bean gum and cellobiose (0.1% (w/v) of each). Culture supernatants were assayed for xylanase and mannanase activities by the PAHBAH reducing sugar method. As this polysaccharide mixture might also induce de-branching enzymes, assays for α-galactosidase and α-L-arabinofuranosidase were also performed using the appropriate *p*-nitrophenol glycosides as substrate. The levels of four hemicellulases are presented in Table 3.3. The results show that xylan-hydrolysing and mannan-hydrolysing enzymes are produced concurrently when TP8 is grown on the mixed polysaccharide media. This included two de-branching

Table 3.2 Comparison of relative polysaccharide depolymerisation by cell-free supernatants of TP8 6.3.3.1 grown on different carbon sources

	Carbon source for growth		
	Cellobiose alone	Oat spelt xylan + cellobiose	Locust bean gum + cellobiose
	Relative diameter of unstained zone[#]		
Assayed polysaccharide			
oat spelt xylan	-	+ +	-
locust bean gum	-	+	+ + +
konjac gum	-	+	+ + +
radiata glucomannan	-	-	+ +

- no unstained zones (well diameter 9 mm)
\+ diameter of unstained zones from 9-10 mm (weak)
\+ + diameter of unstained zones from 11-13 mm
\+ + + diameter of unstained zones from 14-16 mm

Table 3.3 Hemicellulolytic activity of TP8 6.3.3.1 grown on a xylan/galactomannan/cellobiose medium.

	U/ml*	
Mannanase	15.6	(0.6)[+]
Xylanase	4.7	(0.9)[+]
α-Galactosidase	0.031	(0.003)[+]
L-arabinosidase	0.011	

\+ average of two independent determinations (std. dev.)
* mannanase and xylanase 1 U is the amount of enzyme that produces 1 μmole of reducing sugar equivalent per min (man or xyl)
* for galactosidase and arabinosidase 1U is the amount of enzyme that releases 1 μmole of *p*-nitrophenol per min
(see SECT 2.2.1 and SECT 2.2.2)

activities, α-galactosidase and an α-arabinosidase, which could act synergistically with the mannanase and the xylanase, respectively. This suggests that the two hemicellulolytic systems are not mutually repressive. Instead their induction may be either simultaneously independant or concurrent.

The levels of mannanase activity in the TP8 culture supernatants (Table 3.3) are comparable to those of other bacteria grown on locust bean gum or similar mannans. Mannanase activities of 15 U/ml and 14 U/ml were reported for a *Bacillus subtilis* culture filtrate (Ratto and Poutanen 1988) and an alkalophilic *Bacillus* species (Akino *et al.* 1987), respectively. A gene encoding mannanase activity has been cloned from the latter (Akino *et al.* 1989). Others, however, have reported low levels of free mannanase in organisms that grow readily on locust bean gum. Talbot and Sygusch (1990) listed mannanase levels of 0.9 U/ml for *Bacillus stearothermophilus* while Torrie *et al.* (1990) described a *Trichoderma harzianum* strain that produced maximum mannanase activities of 0.6 U/ml, yet grew well on locust bean gum and other mannans. Two other fungi, *Aspergillus awamori* and *Trichoderma reesei*, also possessed relatively low mannanase activities (2 U/ml and 1.4 U/ml respectively) despite good growth (Ratto and Poutanen 1988). The varying levels of extra-cellular mannanase activity reflect differences in enzyme regulation between the various organisms. Therefore, definitive proof of mannan degrading activity is given by growth on mannan type substrates but not necessarily extra-cellular mannanase concentrations as these may be misleadingly low.

The α-arabinosidase activity had not been previously recorded in TP8, although its occurrence is no surprise. Although it is believed that α-arabinosidase and xylanase activies are separate peptides, it is conceivable that these activities could be attributed to the xylanase alone. Study of the cloned enzyme should clarify this point.

Production of an α-galactosidase was implied by the growth of TP8 on melibiose (a gluco-galactose disaccharide). Its production by the organism during growth on the polysaccharide mixture alludes to a role for this enzyme in the hydrolysis of galactomannans, most probably in side-group cleavage of the galactomannan. Bacteria grown on locust bean gum tend to have much lower extra-

cellular concentrations of α-galactosidase than mannanase. This is seen with *Bacillus stearothermophilus* (Talbot and Sygusch 1990) where mannanase levels were 5 fold higher than those of α-galactosidase. It was also evident with *B. subtilis* and *Streptomycese olivochromogenes* (Ratto and Poutanen 1988). TP8 showed similar patterns of enzyme distribution with α-galactosidase activity being several orders of magnitude lower than mannanase activity (Table 3.3). An analogous pattern is also evident with the xylanase and arabinosidase activities in TP8 (Table 3.3).

This type of de-branching enzyme induction is consistent with the hemicellulolytic scheme proposed by Dekker (1989). In this scheme, endo-hydrolases act to produce substituted oligosaccharides which are attacked by de-branching enzymes. Thus high levels of endo-hydrolyases (mannanase and xylanase) are induced first, followed by de-branching enzyme induction as substituted oligomers accumulate. There is also the possiblity that some of the de-branching enzymes may be cell associated and not detected in the culture supernatants.

3.3.5 Induction of the α-galactosidase

To examine the induction of α-galactosidase, TP8 was grown on four different carbon sources; cellobiose alone, melibiose alone, cellobiose/locust bean gum and cellobiose/xylan. Culture supernatants were assayed for α-galactosidase activity with either *p*-nitrophenol-α-D-galactopyranoside or *o*-nitrophenol-α-D-galacto-pyranoside as substrate (Table 3.4). α-Galactosidase activity in TP8 was more active against the *para-* isomer rather than the *ortho-* isomer of the nitrophenol derivatives, which may reflect an enzymatic specificity. This isomer specificity is the opposite of that found with the cloned TP8 ß-glucosidase (Plant *et al.* 1988), where the enzyme is more specific for the *ortho-* rather than the *para-* isomer.

Cellobiose and oat spelt xylan were poor inducers of α-galactosidase activity in TP8 (Table 3.4). The low levels of activity in cellobiose grown cells may represent constitutive α-galactosidases. Melibiose also appears to be a poor inducer of extracellular α-galactosidase activity in TP8. However, most of the α-galactosidase may be cell associated when the thermophile is grown on this disaccharide. This is exemplified by *B. stearothermophilus* which produces intra-cellular α-galactosidases

Table 3.4. The α-galactosidase activity of TP8 6.3.3.1 grown on different substrates

Growth substrate	α-Galactosidase activity (U/ml) Nitrophenol-α-D-Galactopyranoside *para-*	*ortho-*
1% (w/v) cellobiose	0.001	>0.001
1% (w/v) melibiose	0.003	0.002
0.1% (w/v) cellobiose + 0.2% (w/v) locust bean gum	0.033	0.010
0.1% (w/v) cellobiose + 0.2% (w/v) oat spelt xylan	>0.001	>0.001

when grown on melibiose (Pederson and Goodman 1980) but extra-cellular activities are detected when grown on locust bean gum (Talbot and Sygusch 1990). Indeed supernatants from locust bean gum grown TP8 cultures have free α-galactosidase activities that are ten fold higher than melibiose grown cultures. This parallels the *B. stearothermophilus* situation. It is also possible that two α-galactosidases are produced, one is cell associated and is specific for disaccharides with an α-galactose linkage while the second is secreted and compliments mannanase hydrolysis of galactoglucomannans.

3.4 CONCLUSIONS

TP8 6.3.3.1 grows on locust bean gum. Culture supernatants depolymerised various types of mannans. This establishes that the organism produces an extra-cellular mannanase that functions at 70°C. TP8 also grows on melibiose. Subsequent assays of melibiose and locust bean gum grown TP8 confirmed the presence of an α-galactosidase in both these supernatants. Neither the mannanase nor the α-galactosidase were repressed by cellobiose or xylan. TP8 also produced an α-L-

arabinofuransoidase when grown on xylan.

The TP8 gene library was screened for a mannanase activity by E. Luthi of the Centre for Gene Technology, University of Auckand (Auckland NZ) using the Congo Red diffusion technique. A gene coding for a mannan depolymerising enzyme was found and cloned into *E.coli*. The cloning and sequencing of this gene is reported by Luthi et al. (1991). This is the second report of a cloned mannanase following that of Akino et al. (1989).

Before the mannanase could be appraised as a bleaching aide, properties such as thermostability and specificity towards various substrates must be examined. Studies on the production and characterisation of this and the xylanase cloned from TP8 are presented in following chapters.

CHAPTER FOUR

CHARACTERISATION OF CLONED HEMICELLULASES

4.1 INTRODUCTION

Before the comprehensive evaluation of the cloned xylanase and mannanase as bleaching aides could be initiated two criteria needed to be met; sufficient enzyme had to be available and the enzymes characterised to an extent that would allow proper experimental design. The latter included determining the effectiveness of the hemicellulases against their intended substrates. This chapter describes the production of three TP8 hemicellulases cloned into *E. coli* (two xylanases, one mannanase), the purification of the cloned enzymes and their subsequent characterisation.

A 6 kb DNA fragment from TP8 was cloned into *E. coli* (host strain TG1) as described by Luthi *et al.* (1990). The clone containing plasmid pNZ1400 produced a xylanase active at 70°C. The DNA fragment was sequenced and found to contain five open reading frames (OR 1-5). A sixth ran off the fragment. Subcloning from the 6 kb fragment identified OR1 as the *xynA* gene which codes for a xylanase with a weak xylosidase activity, OR2 as the *xynC* gene which codes for an acetylesterase (Luthi *et al.* 1990a) and OR5 (*xynB* gene) which codes for an aryl xylosidase (Luthi and Bergquist 1990) as characterised by Hudson *et al.* (1991). Peptides from OR3 and OR4 have not been identified. Two clones containing *xynA* were used in this study. The first, containing plasmid pNZ1417 (a pCGN566 construct) used chloramphenicol resistance as a marker; the second contained plasmid pNZ1435 (a pBS- construct with a 1.3 kb DNA fragment from TP8) and had an ampicillin resistance marker (Luthi *et al.* 1990b, d). The five open reading frames and the subclones are represented in Fig 4.1.

The cloned xylanase has been purified and well characterised by Schofield (DPhil, University of Waikato 1990), augmented with data from the gene sequence (Luthi *et al.* 1990c). The cloned xylanase has a molecular weight of 40,455 Da

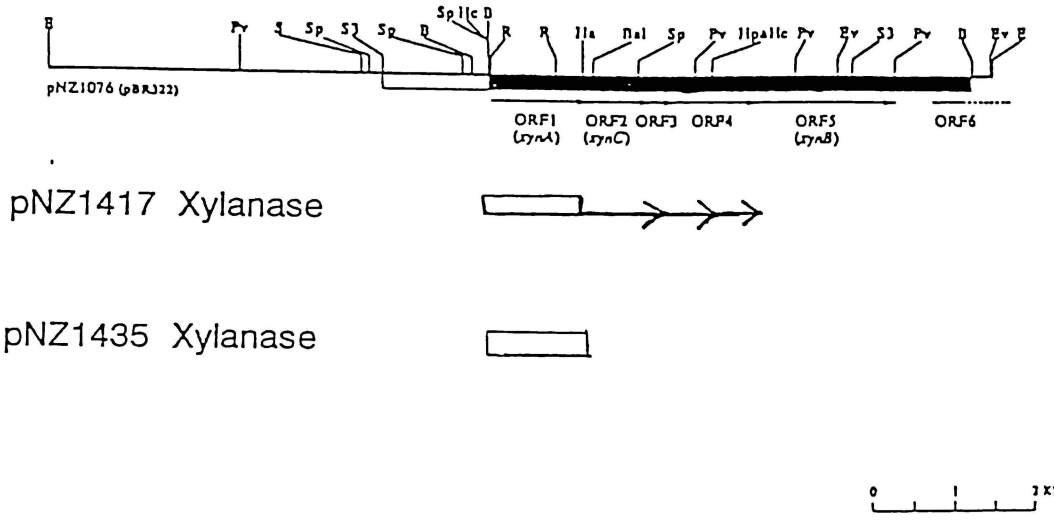

Fig 4.1 Restriction maps of the xylanase gene cluster (A), the *manA* gene (B) and the cloned TP8 DNA fragments in the pNZ1417, pNZ1435 and pNZ1019 plasmids.
(from Luthi *et al.* 1990b, 1990c)

Restriction enzyme abbreviations

B	*Bam*HI	Hc	*Hin*cII
Bal	*Bal*I	Pv	*Pvu*II
E	*Eco*RI	R	*Rsa*I
Ev	*Eco*RV	S	*Sal*I
Ha	*Hae*III	S3	*Sau*3AI
Hp	*Hpa*I	Sp	*Sph*I

as determined from the gene sequence of Luthi *et al.* (1990), although a molecular weight of 42,000 Da was determined by SDS-PAGE (Schofield, DPhil, University of Waikato 1990). The pI of the cloned enzyme was estimated as approximately 5 (Schofield DPhil, University of Waikato 1990) which agrees with the acidic pI\high molecular weight relationship proposed by Wong *et al.* (1988). The xylanase had a temperature optimum of 70°C in a 10 min assay and a broad pH optimum of 5-7.7 (Schofield DPhil, University of Waikato, 1990). The thermostability of the cloned enzyme (pNZ1435 clone) has not been satisfactorily determined; Schofield (DPhil, Univertsity of Waikato 1990) has given a half life ($t_{1/2}$) of 20 min at 75°C while Luthi *et al.* (1990b) have reported a $t_{1/2}$ of approximately 9 hr at 70°C but only 3 min at 80°C. Compared with other enzymes cloned from TP8, the thermostability of the xylanase is low. For example, the $t_{1/2}$ of the cloned acetyl esterase at 70°C is 64 hr (Luthi *et al.* 1990a) and that of the cloned ß-glucosidase is 15 hr at 80°C (Plant *et al.* 1988). More data on the cloned xylanase will be provided throughout this chapter.

Cloning of the *manA* gene into *E. coli* (host strain TG1) was done by using pNZ1019, a pBR322 construct which contained a 5 kb *Bam*HI DNA fragment from TP8 (Luthi *et al.* 1991). The marker in pNZ1019 is for ampicillin resistance. The *manA* gene which codes for a mannanase activity in TP8, was sequenced and found to be 2.1 kb in length (Luthi *et al.* 1991). The *manA* gene map is presented in Fig 4.1.

Relatively little is known about the cloned mannanase. The gene sequence provides an accurate estimate of the molecular weight of the enzyme (38,904 Da) (Luthi *et al.* 1991). The mannanase was active against various mannan type substrates but not against CM cellulose or oat spelt xylan. It had temperature and pH optima of 80°C and 6, respectively (Luthi *et al.* 1991). The temperature optimum is amongst the highest of any of the TP8 cloned enzymes with only those of the ß-glucosidase (85°C) (Love and Streiff 1987) and the *celB* cellulase (85°C) being higher (Saul *et al.* 1990). This may imply a higher thermostability. The enzyme has been described as thermostable (no loss in activity after 1 hr at 80°C) but further studies are required to confirm this (Luthi *et al.* 1991).

From their respective gene sequences it has been shown that both *xynA* and *manA* have signal sequences which indicate that the native peptides are to be found outside the cell (Luthi *et al.* 1991,1990c). Results from Chapter 3 (SECT 3.3.3) show that at least some of these hemicellulolytic activities are produced by TP8 as free enzymes. Indirect evidence suggests that a membrane associated form of the xylanase may also exist. The *xynA* signal sequence is an uncommon type that has a region rich in charged or low molecular weight, hydrophobic amino acids (Luthi *et al.* 1990c). The sequence has homology with a TP8 cellulase (*celB* gene), a *xynA* gene from an alkalophilus *Bacillus*, a *xynA* gene from *Bacillus pumilus* and a penicillinase gene from *Bacillus licheniformis* (Luthi *et al.* 1990c). The latter is a membrane bound enzyme suggesting that the TP8 xylanase may also exist as such.

Comparisons of the overall sequences have shown that *xynA* has significant homology with five other genes; *celB* gene from TP8 which codes for a bifunctional exo/endo cellulase (Saul *et al.* 1990), the *xynA* gene from an alkalophilus *Bacillus* sp., the C-terminal domain of the *xynZ* gene from *Clostridium thermocellum*, the *cex* (exocellulase) gene from *Cellulomonas fimi* and the OR4 of the TP8 xylanase gene cluster (Luthi *et al.* 1990c). The *manA* gene shows regions of strong homology with the *celB* gene from TP8 suggesting that the two genes may be modified versions of each other (Luthi *et al.* 1991).

The results presented in this chapter will verify and expand on the information about the xylanase and mannanase cloned from TP8. In particular the effects of the cloned enzymes on soluble radiata hemicelluloses will be examined and the end-products from this will be identified. This will confirm the activity of the enzymes against their respective substrates as well as indicate whether any regions within the hemicelluloses are resistant to enzyme attack and why.

4.2 METHODOLOGY

Ten litres of each *E. coli* clone (pNZ1417, pNZ1435, pNZ1019) were grown in the fed-batch fermentor system described in SECT 2.1.3. Cells were harvested by hollow fibre ultrafiltration and centrifugation to give a wet cell pellet. Cell lysis was by the lysozyme/osmotic shock procedure of Patchett *et al.* (1989) as outlined in SECT 2.1.4. Once lysed, the enzyme suspensions were heat treated for 1 hr at 70°C,

centrifuged to remove denatured protein and the supernatant washed and concentrated by ultrafiltration. Enzyme concentrates were then freeze dried and used in further experiments. The appropriate enzyme activities and protein concentrations were followed throughout the lysis and purification procedure. A small aliquot of the mannanase was purified further by using a combination of ion exchange and gel permeation chromatography (SECT 2.1.4).

Substrate specificities, temperature and pH optima were assayed as described in SECT 2.2. Enzymatic polysaccharide hydrolysis was performed by adding the appropriate enzyme to a 2% (w/v) polysaccharide solution (in water) at a dosage of 100U/g of carbohydrate and incubating for 1 hr at 70°C. The reaction was stopped by heating in a boiling water bath for approximately 15 min. Hydrolysis conditions vary where indicated. Radiata xylan, hydrolysed by the xylanase, was analysed by either fractionation into neutrals and acidics by ion exchange (SECT 2.3.2.1) or directly by anion exhange chromatography with pulse amperometric detection (SECT 2.3.2.5). In the former procedure, neutrals were further fractionated by GPC (SECT 2.3.2.4), TFA hydrolysed (SECT 2.3.2.4) and the monomers identified by HPLC (SECT 2.3.3), the acidics were analysed by HPLC (Aminex anion exchange, SECT 2.3.2.6).

Xylo-oligomers from the radiata xylan hydrolysate were identified by a sequential treatment with various purified hemicellulases and analysis of the resulting oligomers performed using anion exchange (Dionex system, SECT 2.3.2.5). This involved analysing the total, hydrolysed carbohydrate fraction. Oligomer identification was by co-elution with standards or by subsequent treatment with either a mesophilic ß-xylosidase (to remove terminal, non-reducing xylose residues from the oligosaccharides), L-arabinofuranosidase (to remove arabinose side-groups) or an α-glucuronidase (to remove methyl glucuronic acid side-groups). Anion exchange chromatography was repeated after treatment with the mesophilic enzymes. From changes in the oligosaccharide profile, the original oligomers (produced by the cloned xylanase) could be deduced.

The oligomers produced by the cloned mannanase on various mannans were fractionated on the basis of DP by GPC (SECT 2.3.2.2.). Oligomers of similar DP were separated by HPLC (SECT 2.3.2.3) or paper chromatography (SECT 2.3.2.3).

Oligosaccharides were identified by their monomer composition, as determined by TFA hydrolysis and HPLC (see above) and by NMR spectroscopy (SECT 2.3.5).

4.3 ENZYME PRODUCTION

4.3.1 Xylanase from *E. coli* clone (pNZ1417)

E. coli (pNZ1417) was grown for 18 hr in the fed batch system. No external pH control was implemented. Final OD_{600} and pH were 7.2 and 5.04, respectively, with 280 g of wet cells harvested from the 10 L fermenter. As *E. coli* growth decreases as pH values approach 4.5, higher cell yields might have been possible with pH control.

The xylanase was released from the pellet by cell lysis and purified by heat treatment and ultrafiltration. The filtrate was also assayed. The efficiency of each purification step is summarised in Table 4.1.

Xylanase from the pNZ1417 clone was purified three fold with a 52% recovery of the enzyme. This was in the form of a freeze dried powder (7.21 g) which was 94% protein. The final specific activity of the freeze dried preparation was 1.10 U (xylanase) per mg of protein.

4.3.2 Mannanase from *E. coli* clone (pNZ 1019)

The pNZ1019 clone was grown under similar conditions as the pNZ1417 clone except that manual pH control was used in the final stages of growth. The pH of aliquots removed every hour after the addition of the fed batch concentrate (12 hr) was checked. If this was below 6.5 the culture was neutralised by the slow addition of sterile 4M NaOH (50 ml). In this way, a final OD_{600} of 27 was reached within 20 hr (final pH 6.9). The 10 L culture yielded 452 g of a wet cell pellet.

Mannanase purification during lysis, heat treatment and ultrafiltration is summarised in Table 4.2. Heat treatment and ultrafiltration effectively increased enzyme purity by three fold with a 50% yield. However, freeze drying resulted in a dramatic loss in enzyme activity with no corresponding decrease in protein, indicating enzyme denaturation during the process. Final mannanase activity was 0.61 U mg^{-1} (less than that of the cell pellet!) in 11.23 g of freeze dried protein.

The mannanase preparation was too impure for an accurate molecular weight determination by GPC. Therefore, an aliquot of the freeze dried preparation was purified further. This involved anion exchange using a soluble resin (Biocryl 2100) which removed contaminating proteins (Table 4.2). The most effective purification step was fractionation by preparative GPC which increased purity twelve fold (Table 4.2).

4.3.3 Xylanase from *E. coli* clone (pNZ1435)

Growth of *E. coli* clone (pNZ1435) followed the procedure described for the pNZ1019 clone with manual pH control commencing after 12 hr (pH 5.04, OD_{600} 6.0). The culture pH was maintained above 5.5. The fermentation was stopped after 19 hr. Final culture pH and OD_{600} were 5.71 and 9.2, respectively, with 162 g of wet cell pellet harvested. The low cell yield was attributed to a partial block of the aeration system during the fermentor run. Poor aeration shifts *E. coli* from oxidative to fermentative growth which results in lower biomass yields and high acidity. Physiological differences between the pNZ1435 clone and the others may also account for the poor growth. However, total xylanase activity produced by the pNZ1435 clone was higher than that of the pNZ1417 clone, despite the lower biomass yield. This reflects the improved enzyme expression by the pNZ1435 clone.

Xylanase purification (Table 4.3) was as described for the pNZ1417 clone except that two centrifugation steps were introduced prior to ultrafiltration. These included a "low spin" (9000g/15 min) and a "high spin" (16000g/15 min) to remove pellet cellular debris and denatured proteins, respectively. These proteins fouled the ultrafiltration system and their removal alleviated this problem to some extent. Data for the ultrafiltration concentrate and filtrate fractions are presented (Table 4.3). The xylanase from *E. coli* clone pNZ1435 was purified ten fold with almost 50% recovery, a significant improvement over the pNZ1417 xylanase preparation. The xylanase specific activity of the freeze dried powder (1.80g, 61% protein) was 7.4 U per mg of protein.

In general, the lysozyme/osmotic shock procedure was considered an efficient means of cell disruption at the scale of operation used. This is evidenced by

Table 4.1 Purification of pNZ1417 xylanase (280 g wet cell pellet)

Purification Step#	Vol (ml)	Xylanase Activity Total (U)	Specific (U/mg)	Total Protein (mg)	Yield %	Purity
Crude prep	10000	14400	0.36	40000	100	1.0
Cell lysis	3200	11520	0.34	33920	80	0.9
Heat treat.	2000	12200	0.49	24720	85	1.4
U.f. conc.	1000	9600	0.77	12470	67	2.1
Freeze Dried	7.21g	7460	1.10	6780	52	3.1
Filtrate	1000	2400	0.25	9300	17	0.7

Crude prep - total activity of the fermenter at harvest (from sonicated aliquot)
Cell lysis - activity after lysozyme/osmotic shock
Heat treat.- activity after heat treatment/centrifugation
U.F. conc. - activity ultrafiltration concentrate prior to freeze-drying
Freeze Dried-activity of reconstituted freeze dried powder
Filtrate - activity of permeate from ultrafiltration

Table 4.2 Purification of pNZ1019 mannanase (452 g wet cell pellet)

Purification Step#	Vol. (ml)	Mannanase Activity Total (U)	Specific (U/mg)	Total Protein (mg)	Yield %	Purity
Crude prep	10000	82200	1.22	67220	100	1.0
Cell lysis	2500	98000	1.48	66250	119	1.2
Heat treat.	2500	81500	3.26	25000	99	2.7
U.f. conc.	300	40800	3.49	11700	50	2.9
Freeze Dried	11.23g	6850	0.61	11230	8	0.5
Filtrate	2200	0	0.00	250		
Further purification						
Freeze dried powder	50	210	0.65	325	100	1.0
Biocryl 2100	50	204	1.40	146	97	1.8
U.f. conc.	5	204	1.46	140	97	1.8
GPC (prep)/ ultrafilt.	10	198	7.86	25	94	12.1

Crude prep - total activity of the fermenter at harvest (from sonicated aliquot)
Cell lysis - activity after lysozyme/osmotic shock
Heat treat.- activity after heat treatment/centrifugation
U.F. conc. - activity ultrafiltration concentrate prior to freeze-drying
Freeze Dried-activity of reconstituted freeze dried powder
Filtrate - activity of permeate from ultrafiltration
Biocryl - activity after anion exchange
U.f. conc. - activity of ultrafiltration concentrate
GPC (prep)/ ultrafilt. - activity of ultrafiltration concentrate of GPC fractions

Table 4.3 Purification of pNZ1435 xylanase (162 g wet cell pellet)

Purification Step#	Vol. (ml)	Xylanase Activity Total (U)	Specific (U/mg)	Total Protein (mg)	Yield %	Purity
Crude prep	10000	17470	0.72	24300	100	1.0
Cell lysis	1600	13280	0.64	20800	76	0.9
Heat treat.	1600	14720	0.76	19480	84	1.1
Low spin	1500	13200	1.53	8655	76	2.1
High spin	1450	11310	1.50	7540	65	2.1
U.f. conc.	140	8960	4.57	1960	51	6.4
Freeze Dried	1.81g	8140	7.40	1100	47	10.3
Filtrate	5000	25	0.25	100	0	0.3

Crude prep - total activity of the fermenter at harvest (from sonicated aliquot)
Cell lysis - activity after lysozyme/osmotic shock
Heat treat.- activity after heat treatment/centrifugation
lo/high spin-activity after respective centrifugations (SECT 4.3.3)
U.F. conc. - activity ultrafiltration concentrate prior to freeze-drying
Freeze Dried-activity of reconstituted freeze dried powder
Filtrate - activity of permeate from ultrafiltration

between 75-100% of the estimated enzyme activity in the 10 L cultures being recovered after cell lysis in all three clones (Tables 4.1, 4.2, 4.3).

Heat treatment is also considered an effective means of thermostable enzyme purification. Again, results for all three enzymes gave a maximum loss of only 10% enzyme yield following the heating stage. During this procedure, mesophilic *E. coli* proteins were denatured, thus facilitating their subsequent removal by a secondary separation method (ie: ultrafiltration or centrifugation). Also, heat treatment increased overall preparation stability by inactivating mesophilic proteases and killing any remaining viable mesophilic cells (Patchett *et al.* 1989). The latter point is of significance when dealing with genetically engineered organisms.

Major losses in enzyme yield occurred during secondary separation of denatured proteins. In the purification regimes where only ultrafiltration was used (Table 4.2 and Table 4.3), 30 to 50% of the cloned enzyme was lost during these steps. Analysis of the filtrates confirmed that filter failure was not the cause of these losses (Table 4.1 and Table 4.3). Enzyme stability and mesophilic protease denaturation during heat treatment suggest that enzyme loss was not due to inactivation. Rather it was noted that during ultrafiltration, fouling of the filter membrane occurred. This prevented complete recovery of the protein concentrate, presumably resulting in the reported losses especially between ultrafiltration and freeze drying.

To reduce the initial protein load on the ultrafiltration system, two centrifugation steps were used to remove particulates. Centrifugation has been successfully used in earlier work with cloned thermostable proteins to achieve over 5 fold purification with only 20% yield losses (Schofield *et al.* 1988). This was not deemed practical with the relatively large volumes used in this study. Results with the pNZ1435 xylanase preparation show that the first spin (9000, 15 min), doubled the specific activity of the enzyme with less than a 10% loss in yield. Subsequent centrifugation did not improve enzyme purity (Table 4.3). Subsequent ultrafiltration showed a significant rise in specific activity.

Freeze drying was an acceptable way of de-watering the two xylanase preparations. However, enzyme denaturation occurred with the mannanase preparation. Flask (100 ml) scale experiments with clone pNZ1019 grown on Luria

broth, sonicated, heat treated and freeze dried did not give similar losses in enzyme activity (data not shown). The cause of the denaturation in the large scale preparation remains unclear.

Other techniques also show promise for the large scale purification of the cloned enzymes. In particular ion exchange with a series of soluble resins (Biocryl) may accelerate denatured protein precipitation. Also, preparative GPC, which gave the greatest increase in purity with no loss in yield, is worthy of further investigation.

4.4 CHARACTERISATION OF THE CLONED HEMICELLULASES

4.4.1 Xylanases (pNZ1417 and pNZ1435)

4.4.1.1 Xylanase specificity

Both xylanase preparations were assayed on several different substrates with the relative activities presented in Table 4.4. These data confirm the xylanolytic nature of the two enzyme preparations. Activity profiles of the two clones are similiar with respect to the substrates tested. Both xylanases show only trace activity against *p*-nitrophenol-xylopyranoside which is in agreement with the results of Schofield (DPhil, University of Waikato 1990). Luthi *et al.* (1990d) reported an *o*-nitrophenol-xylosidase activity for this enzyme but no values were given, so comparisons are not possible. Schofield recognised this apparent ß-xylosidase activity as a transferase activity where the xylose moiety of the *p*-nitrophenol-xylopyranoside is transfered, presumably by a reversal of the hydrolysis reaction, to another *p*-nitrophenol-xylopyranoside. The result of this reaction is free *p*-nitrophenol which is chromogenic and *p*-nitrophenol-xylobiose which can undergo further transferase type reactions thus releasing more chromogen.

Earlier studies showed that neither a pNZ1435 xylanase preparation nor a xylanase purified from the cloned TP8 xylanase prepared by Schofield (DPhil, University of Waikato 1990) hydrolysed *p*-nitrophenol-arabinopyranoside. This study expanded on these results and showed that the cloned TP8 xylanases (pNZ1435, pNZ1417 and that purifed by Schofield) do not hydrolyse *p*-nitrophenol-arabinopyranoside and nor do they hydrolyse *p*-nitrophenol-arabinofuranoside isomer (Table 4.4). This suggests that the enzyme does not cleave arabinose side-

Table 4.4 Relative activity of the two xylanase preparations on various substrates

Substrate	% Relative Activity pNZ1417	pNZ1435
oat spelt xylan	100*	100*
larchwood xylan	90	84
radiata xylan	83	86
CM cellulose	8.0	12.0
locust bean gum	<0.1	<0.1
konjac glucomannan	<0.1	<0.1
MUC	<0.1	<0.1
pNP-xylopyranoside	0.9	0.8
pNP-arabinopyranoside	<0.1	<0.1
pNP-arabinofuranoside	<0.1	<0.1

* 100% activity = 0.1 U/assay

groups from xylan. In radiata arabinoxylan, arabinose side-groups are in the furanose form (Harwood 1972) and may hinder the enzymatic hydrolysis of this polysaccharide (Dekker 1985). De-branching enzymes, such as L-arabinosidase, are required for complete hydrolysis. Arabinosidase activity against the *p*-nitrophenol-arabinofuranoside isomer has been identified in the parent organism (SECT 3.3.4). The aryl ß-xylosidase cloned from TP8 is active against the pyranose form but not furanose form of *p*-nitrophenol-arabinose (Hudson *et al.* 1991). Therefore, TP8 must produce a separate enzyme coding for an L-arabinofuranosidase.

The xylanase does not hydrolyse mannans. However, cross reactivity with CM cellulose is seen with both xylanase clones. Trace exo-cellulase (MUCase) cross reactivity was noted with both xylanase preparations. Schofield (DPhil, University of Waikato 1990) reported similar endo- and exo- cellulase activities with the cloned TP8 xylanase but no activity against crystalline cellulose (Avicel). CM cellulase cross reactivity is seen with some other xylanases (Wong *et al.* 1988). In most purifications from culture filtrates CM cellulase activity could be a contaminating enzyme, however with the TP8 cloned xylanse it is recognised as a property of the enzyme. Cellulase activity has the potential to adversely affect pulp quality by reducing the average DP of interfibril cellulose thus lowering strength

properties (Paice and Jurasek 1984). However, the lack of activity against crystalline cellulose previously reported suggests that this may not be a significant problem with the cloned xylanase.

Stereochemistry of C2 and C3 hydroxyls are similar in xylan and CM cellulose (assuming no substitution in the latter) but varies about the C5 carbon. Stereochemistry of the C2 hydroxyls of mannans differs from those of xylans and CM cellulose. Therefore, it is proposed that stereospecificity about the C2 hydroxyl is essential for xylanase binding or catalytic activity. Orientation of this group appears crucial, when compared to the orientation and substitution of the C5 carbon.

4.4.1.2 Temperature optimum of the cloned xylanase

Both xylanase preparations had similar temperature profiles with temperature optima of 70°C within 10 min assays (Fig 4.2). This agrees with results obtained by others with similar clones (Luthi *et al.* 1990d; Schofield DPhil, University of Waikato 1990). The temperature optimum of the cloned xylanase is relatively high but not the highest reported to date (Wong *et al.* 1988). A xylanase purified from an acidophilic thermophilic *Bacillus* sp. with a temperature optimum of 80°C (10 min assay) has been described (Uchino and Nakane 1981). A xylanase with an optimum of 78°C (60 min assay) has also been reported, but this enzyme was not purified (Gruninger and Fiechter 1986). Three xylanases purified from *Clostridium stercorarium* have temperature optima of 75°C (assay period not specified) (Berenger *et al.* 1985) and a purified *Cl. thermolacticum* xylanase has a temperature optimum of 80°C within a 1 hr assay period (Berenger *et al.* 1985). Nine xylanase encoding genes cloned from *Cl. stercorarium* strain F-9 code for xylanases with temperature optima of between 60-80°C within a 10 min assay period (Sakka *et al.* 1990). Xylanases from several actinomycetes also have temperature optima in the 70-80°C range (Holtz *et al.* 1991).

Temperature profile data for both TP8 cloned xylanase preparations, when presented as Arrhenius plots (1/T vs ln v) (Fig 4.3), were linear from 30-70°C.

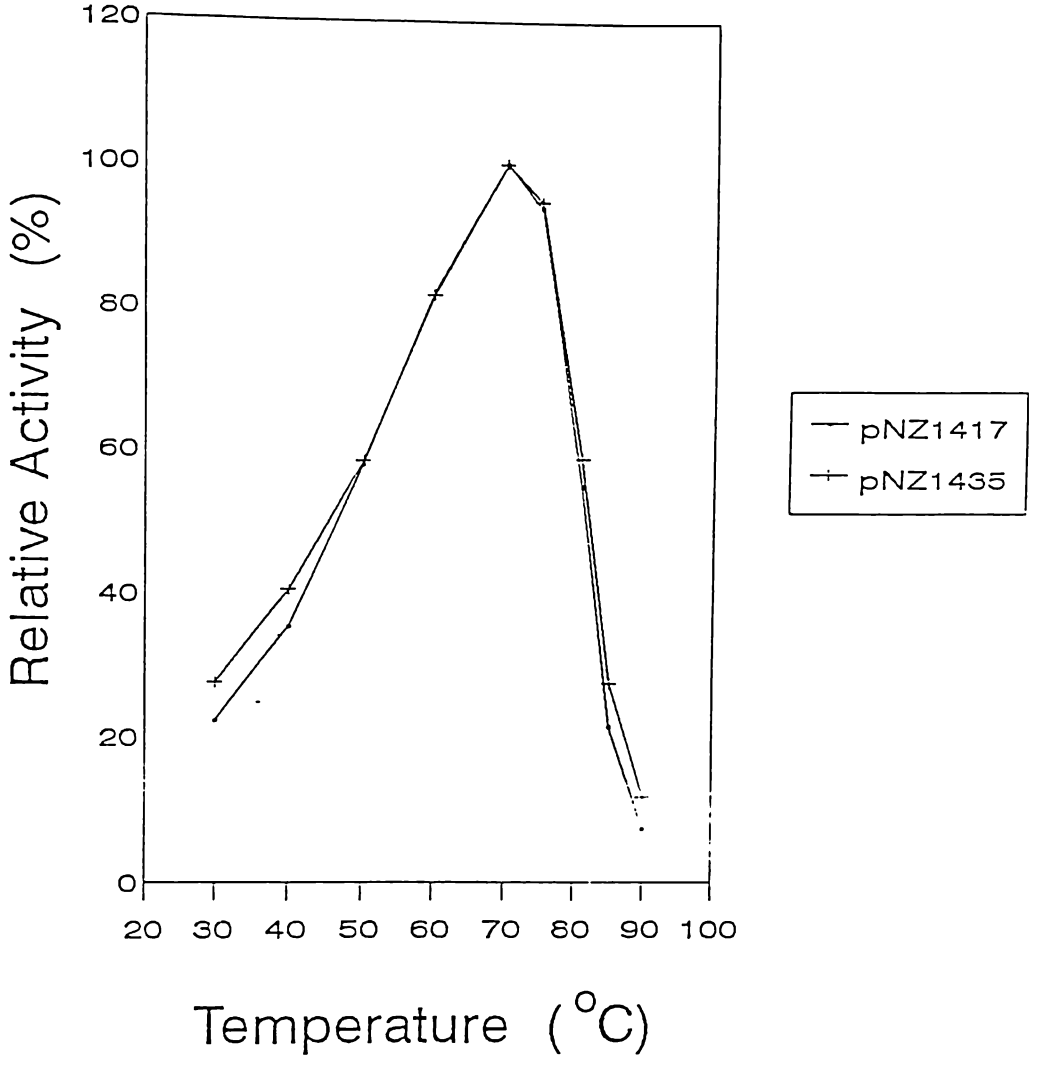

Fig 4.2 Assay temperature (°C) vs % relative xylanase activity for the pNZ1417 and pNZ1435 xylanase preparations.
100% activity = 1 U (pH 6)

Beyond this range the log reaction rate was no longer inversely proportional to temperature, presumably because of excessive denaturation. Activation energies (E_a) of 36 $kJmol^{-1}$ and 30 $kJmol^{-1}$ were calculated for the pNZ1417 and pNZ1435 xylanases, respectively. These values were significantly lower than the E_a of 44 $kJmol^{-1}$ reported by Schofield (DPhil, University of Waikato 1990) for the cloned TP8 xylanase and lower than those reported for the three *Cl. stercorarium* xylanases (36-44 $kJmol^{-1}$) (Berenger *et al.* 1985) and the *Cl. thermolacticum* xylanase (38.8 $kJmol^{-1}$) (Berenger *et al.* 1985). The reason for these discrepancies in E_a is not readily apparent but may reflect differences in assay protocols; others used buffered assay systems near the optimum pH of the enzyme, while in this study the temperature profile was determined in an unbuffered system to approximate biobleaching conditions. This may affect the stability of the enzymes.

Buffering does affect the thermostability of the cloned TP8 xylanase. The pNZ1417 xylanase lost less than 5% of its activity after a 24 hr incubation in water at 70°C. The enzyme was assayed under standard conditions specified in SECT 2.2. Yet after incubation in a buffered system (50 mM MES, pH 6.5), the $t_{1/2}$ of the xylanase was 3.7 hr with only 15% activity remaining after 24 hr at 70°C. When sodium citrate/citric acid buffer (50 mM. pH 6.0) was used, the $t_{1/2}$ of the xylanase was approximately 17 hr, with 35% of the activity remaining after 24 hr at 70°C.

This buffering effect may explain the anomaly regarding the thermostability of the cloned xylanase. Schofield (DPhil, University of Waikato, 1990), using a 50 mM MES/NaOH buffer (pH6), reported a $t_{1/2}$ for the purified, cloned TP8 xylanase of 4 min at 70°C. Increasing the protein concentration during the incubation by the addition of BSA (0.5 gl^{-1}) increased the $t_{1/2}$ from 4 to 30 min. Luthi (1990b) used 50 mM citrate buffer (pH 6) and reported a $t_{1/2}$ of approximately 9 hr at 70°C for the pNZ1435 xylanase. The increased thermostability of the preparations used in this study may be due to the high protein concentrations used during the thermostability incubations (~5 gl^{-1}).

The buffering effect was not investigated further. For the purposes of this study, it was determined that the xylanase was stable (in water) at 70°C for up to 24 hr.

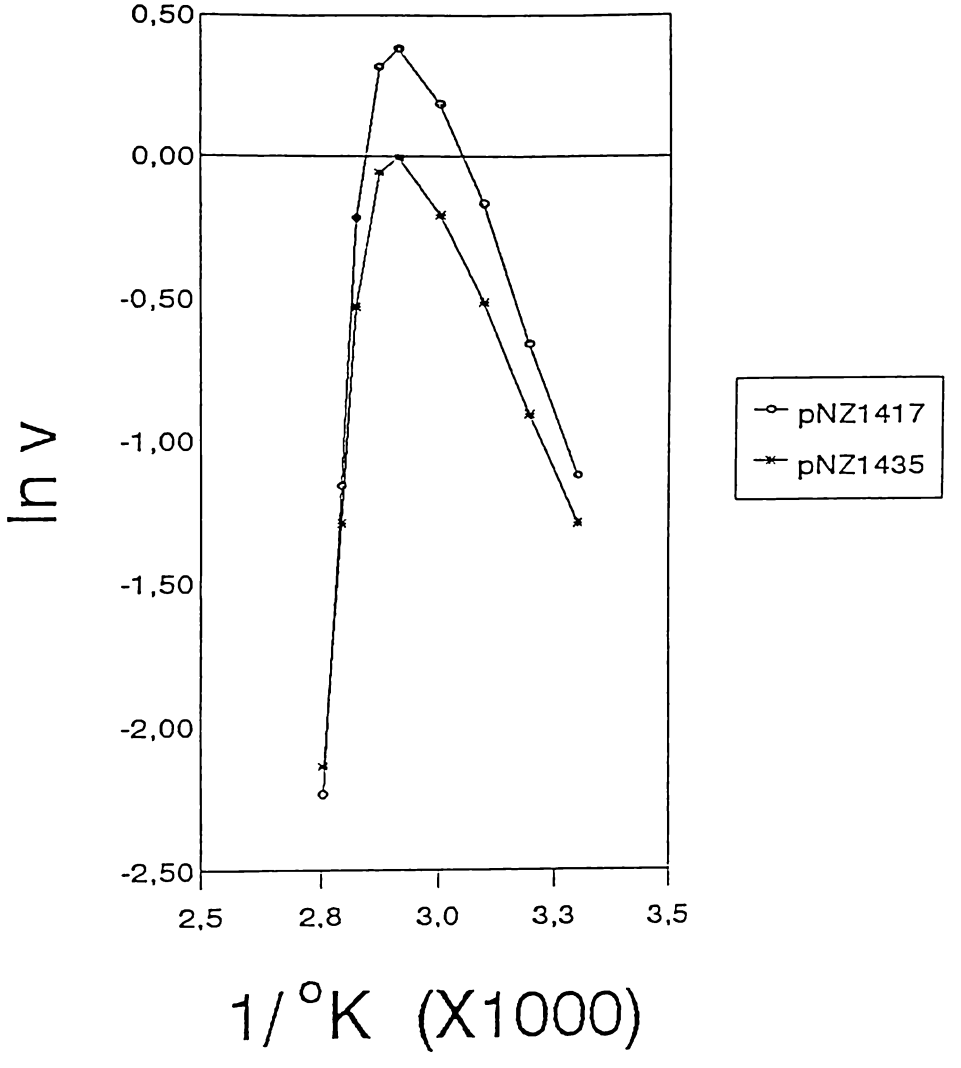

Fig 4.3 Arrhenius plot of ln v against the reciprocal of temperature (°K) for the pNZ1417 and pNZ1435 xylanase preparations.
(Based on the data from Fig 4.2)

4.4.1.3 Kinetics of the cloned xylanase

Both pNZ1417 and pNZ1435 xylanase preparations followed Michaelis-Menten kinetics on oat spelt xylan and purified radiata xylan. The V_{max} and K_m parameters for the two xylanase preparations on these substrates, as determined by a Hanes plot ([S] vs [S]/v) (Dixon and Webb 1967), are presented in Table 4.5. Kinetic parameters of the two preparations are similar on the corresponding substrate. This was expected as the two xylanases are coded by the same gene. However, the parameters do differ from those of Schofield (DPhil, University of Waikato 1990) who reported a K_m of 0.26 mg/ml and a V_{max} of 147.5 U per mg of protein for the purified, cloned TP8 xylanase on oat spelt xylan. The kinetic parameters are comparable to those of other xylanases purified from thermophilic bacteria (Table 4.5).

Both cloned xylanase preparations exhibited a relatively high K_m on radiata xylan, indicative of low enzyme affinity. This substrate had been purified by ion exchange to 90% total carbohydrate (SECT 2.3.1), therefore enzyme inhibition by inorganic contaminants is not considered likely. Rather the relatively low affinity of the cloned xylanase towards radiata xylan is a consequence of the comparatively high degree of substitution of this substrate. The arabinose:xylose molar ratio of the purified radiata xylan is approximately 1:5, that of oat spelt xylan is 1:7 (from data in Table 2.2). Methyl glucuronic acid substitution of the two xylans has been reported as approximately 1 for 8 xylose residues for oat spelt xylan (Sigma catalogue, 1991) and 1 for every 6 xylose residues for radiata xylan (Harwood 1972). Neither xylan is acetylated. The high degree of substitution of radiata xylan may inhibit binding of the xylanase to the xylan backbone. Steric hindrance of this type is manifest as a high K_m. This does not necessarily denote that radiata xylan is resistant to hydrolysis by the xylanase but rather that hydrolysis is slowed. The effect that substitution has on radiata xylan hydrolysis and the extent to which these substituents protect the backbone from enzymatic attack will be examined further in this chapter.

Table 4.5 Michaelis-Menten parameters (V_{max} and K_m) for several xylanases of thermophilic origin

Enzyme Source and Substrate		Parameters# K_m	V_{max}	V_{max}/K_m	Reference
pNZ1417 (TP8)					
Oat spelt xylan		0.76	14.2	18.7	this study
Radiata xylan		3.52	24.7	7.0	
pNZ1435 (TP8)					
Oat spelt xylan		0.69	10.0	14.5	this study
Radiata xylan		2.54	18.3	7.0	
Clostridium stercorarium (three xylanases A,B,C)					
larchwood xylan	A	3.2	5500*	1719	Berenger *et al.* 1985
	B	2.9	3500*	1207	
	C	3.7	4000*	1081	
Clostridium thermolacticum					Debeire *et al.* 1990
larchwood xylan		0.65	ND	ND	
Bacillus sp (acidophilic thermophile)					Uchino and Nakane 1981
larchwood xylan		1.68	8.92	5.3	

K_m mg of soluble substrate/ml
V_{max} μM of reducing sugar produced/min/mg of protein
* V_{max} of highly purified enzyme preparation
ND not done

4.4.1.4 pH optimum of the cloned xylanase

The pH profile of the cloned xylanase (pNZ1417) is given in Fig 4.4. The xylanase activity of the enzyme has a pH range of 4-10 with a broad maximum between pH 6-7.5. Again these results are in agreement with those of Schofield (DPhil, University of Waikato 1990). Appreciable activity (~50%) still remains at a pH of 8.5. This may be of interest as alkaline conditions are often used to swell pulps and thus may facilitate enzyme diffusion into pulp fibres.

The CM cellulase cross reactivity of the xylanase was also pH dependent, with a pH range of 4-9. However, the CM cellulase activity had a sharp pH maximum at pH 5 which quickly fell away (Fig 4.4). At neutral pH, the CM cellulase activity of the enzyme is halved, relative to the activity at pH 5; at a pH of 7.5 it is reduced to a third. It should be emphasised that even at its pH maximum the CM cellulase activity of the xylanase is only approximately 10% of the xylanase activity. The pH profile of the pNZ1435 preparation was assumed to be similar to that of the pNZ1417 preparation.

Whether this pH dependent change in substrate specificity is due to conformational changes in the protein or to some other effect is not certain. However, pH modulation may be a means of minimising the cross reactivity of the xylanase if this is found to be a problem in biobleaching studies.

4.4.2 Mannanase (pNZ1019)

4.4.2.1 Molecular weight of the cloned mannanase

The molecular weight of the mannanase was determined by analytical GPC (TSK, SECT 2.1.5) of the high purity preparation (Table 4.2). The retention time of known standards was plotted against log molecular weight (Fig 4.5). From the mannanase retention time, the molecular weight of the enzyme was estimated at approximately 39,000 Da. This compares very well with the molecular weight determined from the gene sequence of 38,905 Da (Luthi *et al.* 1990c).

4.4.2.2 Mannanase specificity

The pNZ1019 enzyme preparation hydrolysed mannans, glucomannans and galactomannans but not xylans or gluco-polysaccharides (CM cellulose or starch) (Table 4.6). This establishes the enzyme as a mannanase. No α-galactosidase activity was detected but, in a manner akin to the xylanase, a small ß-mannosidase activity was detected. The cloned DNA fragment produces only one peptide (Luthi *et al.* 1990c). As the host *E. coli* strain (RR28) produces no mannanase and all

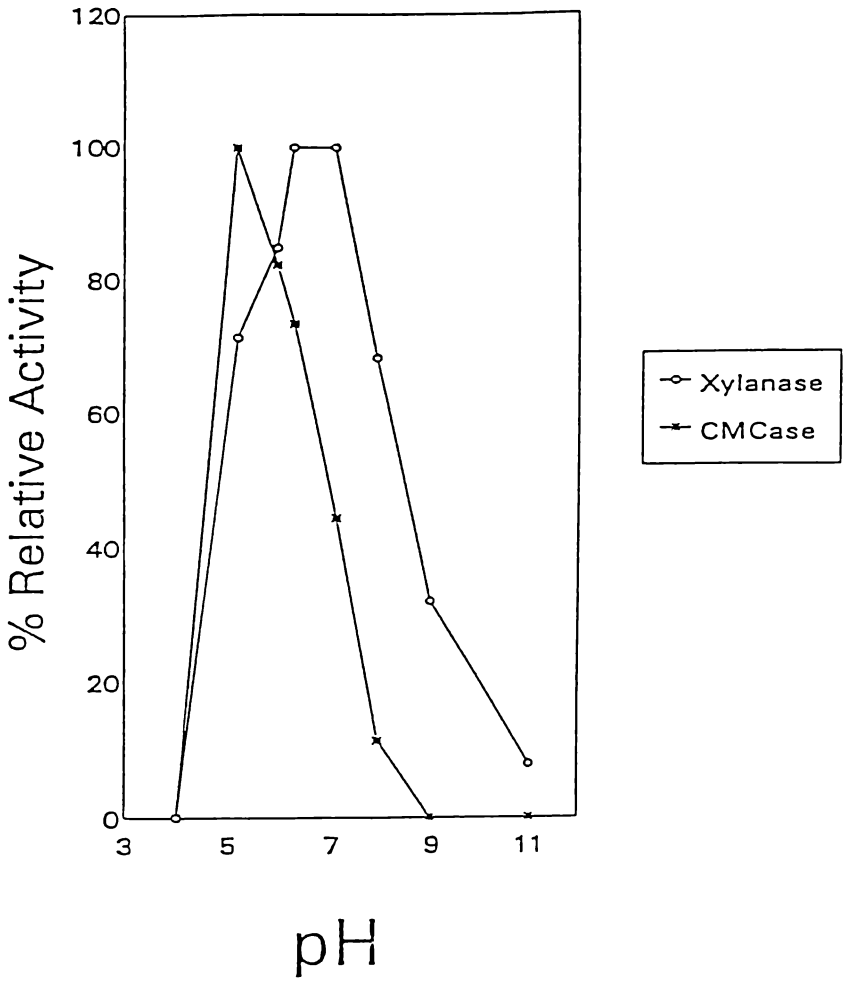

Fig 4.4 % Relative xylanase and endo-cellulase (CMCase) activities of the pNZ1417 xylanase vs pH of the assay. Activity measured under standard assay conditions except for buffers which were modified as follows (all buffers at 50 mM):
pH 4.0-6.0, Nacitrate/citrate buffer; pH 7.1, 3-[N-morpholino] propanesulphonic acid (MOPS);pH 7.9, N-[2-hydroxyethyl]-piperazine-N-[3-propanesulponic acid] (EPPS);pH 9.0, 2-[N-cyclohexylamino]-ethanesulphonic acid (CHES); pH 10, 3-[cyclohexylamino]-1-propane - sulphonic acid (CAPS).
All buffer pHs were set at 70°C, 100% activity = 1 U (70°C)

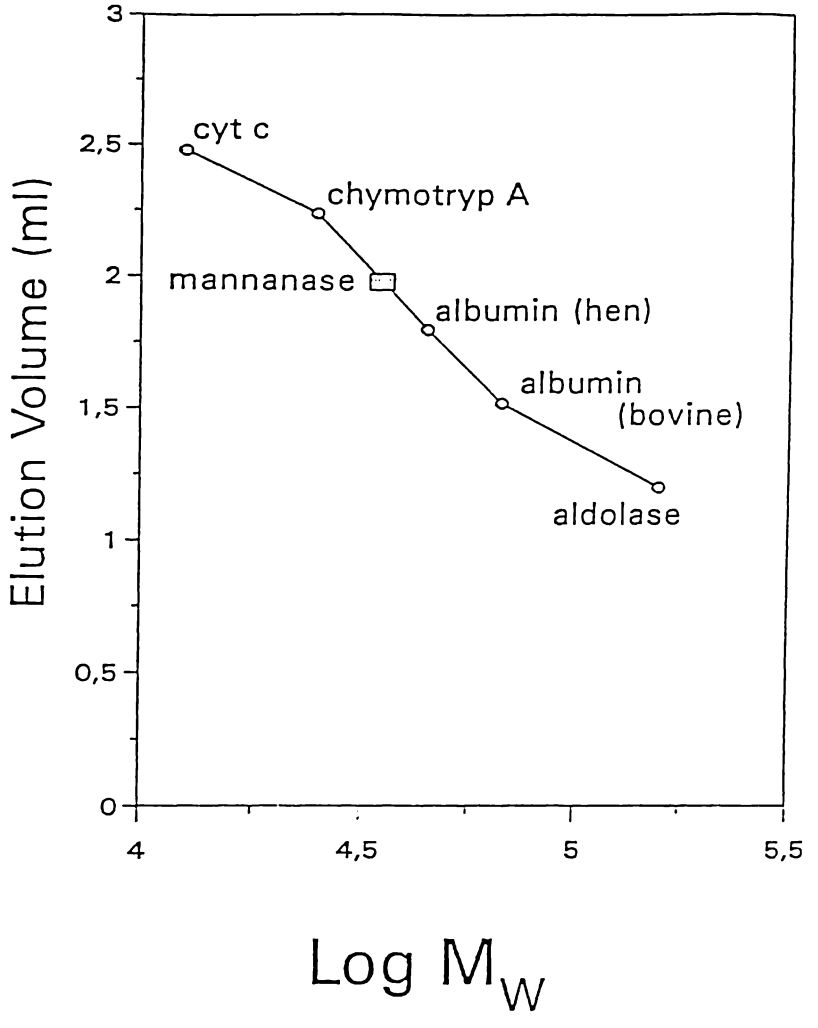

Fig 4.5 Molecular weight determination of mannanase by GPC (SECT 2.2.5) Standards cytochrome c 12,500 Da; chymotrypsin A 25,000 Da; hen egg albumin 45,000 Da; bovine serum albumin 67,000 Da; rabbit muscle aldolase 145,000 Da.

mesophilic proteins are denatured during heat treatment the mannanase activity is attributed to this single peptide.

Mannanase activity varies with the type of mannan substrate (Table 4.6); activity is highest with locust bean gum and lowest with radiata glucomannan. Yet enzyme activity against konjac glucomannan, which is similar to the radiata pine polysaccharide in structure and composition, is relatively high. Differences in relative activities may reflect properties of the enzyme (specificity, steric hinderance) or the substrate (solubility, crystallinity). The nature of this difference will be the subject of further investigation in this chapter.

Establishing that the mannanase hydrolyses radiata glucomannan, albeit at a slower rate than other mannans, is of paramount significance to the biobleaching aspect of this study. The mannanase should selectively attack some or all of the glucomannan within the fibre wall. The effect this has on bleaching can be evaluated and the study of residual glucomannan may provide information on its nature within the fibre wall.

Table 4.6 Relative activity of the pNZ1019 mannanase on various substrates.

Substrate	Relative Activity (as a percentage)
Locust bean gum	100%*
Konjac glucomannan	82%
Ivory nut mannan	89%
Guar galactomannan	49%
Radiata glucomannan	39%
Oat spelt xylan	0%
Larchwood xylan	0%
Radiata xylan	0%
CM cellulose	0%
Starch	0%
pNP-galactoside	<0.1%
pNP-mannoside	5%

* 100% activity = 0.08 Units per assay

4.4.2.3 Kinetic characterisation of the mannanase

Further characterisation of the mannanase was done by determining the Michaelis-Menten parameters (K_m and V_{max}) of the enzyme on the soluble fraction of mannan type substrates. Kinetic parameters were determined from a Hanes plot ([S] vs. [S]/v) where slope is equivalent to $1/V_{max}$ and y-intercept to K_m/V_{max} (Dixon and Webb 1967). The Hanes plots were linear over the substarte concentration used (0.05 -1.5 gL^{-1}).

The mannanase displayed Michaelis-Menten kinetics on the five substrates with the K_m, V_{max} and the specificity parameter (V_{max}/K_m) presented in Table 4.7. The K_m and V_{max} values determined for the cloned mannanase were comparable to those reported for five different mannanases purified from various sources and assayed by monitoring decreases in substrate viscosity (McCleary 1979). However, four mannanases purified from *Polyporus versicolor* had K_m values that were several hundred fold higher than those of the cloned, TP8 mannanase (Johnson and Ross 1990). The V_{max} of these enzymes were also higher. A thermostable mannanase purified from *Bacillus stearothermophilus* had a K_m of 1.5 mg/ml and a V_{max} of

Table 4.7 Michaelis-Menton parameters (V_{max} and K_m) of the pNZ1019 mannanase on various substrates.

Substrate	K_m*	V_{max}#	V_{max}/K_m
Konjac Gum	0.030	31.0	1043.3
Radiata glucomannan	0.053	47.4	894.3
Ivory Nut Mannan	0.062	43.8	706.5
Locust Bean Gum	0.127	63.2	497.6
Guar Gum	0.383	2.5	6.5

* mg of soluble substrate/ml
\# μmoles of reducing sugar produced/min/mg of protein

455 U/mg on locust bean gum (Talbot and Sygusch 1990), both being ten fold higher than those of the cloned mannanase. Major differences in Michaelis-Menten parameters such as those observed between the *Polyporus versicolor* mannanases, the *Bacillus stearothermophilus* mannanase and the cloned TP8 enzyme may reflect variations in the assay protocol or enzyme purity rather than properties of the enzymes. Kinetic data for the first two mannanases was obtained on entire mannan suspensions, which may not represent a homogeneous substrate. Kinetic parameters of the cloned mannanase were assayed on the soluble fraction of the substrate. By removing the insoluble fraction, errors due to substrate inconsistencies were minimised.

The cloned mannanase had its lowest K_m on the konjac glucomannan while the highest was on guar gum, a galactomannan (Table 4.7). Highest and lowest V_{max} values were found with the locust bean gum and guar gum (the two galactomannans) respectively. While those of the ivory nut mannan and glucomannans (konjac and radiata) were midway between the two extremes. The kinetic data suggests that the mannanase has a higher affinity (as indicated by a low K_m) for non-galactose substituted mannans and glucomannans than galactomannans. This was mirrored in the enzyme specificity (Table 4.7) where the V_{max}/K_m values of the non-galactose substituted mannan and glucomannans are higher than those of locust bean gum and guar gum. The V_{max} values obtained may be influenced by substrate solubility, viscosity and enzyme purity. This might explain the dissimilarity between the viscous guar gum and the less viscous locust bean gum. Inhibition of some mannanases by excessive galactose substitution of the mannan backbone, presumably by sterically hindering the enzyme, has been documented (McCleary and Matheson 1983; Ratto and Poutanen 1988). Analysis of the oligosaccharides produced by the action of the mannanase on galactomannans will clarify the effect of galactose side-groups.

The mannanase exhibited essentially similar kinetics on the pure mannan (Ivory nut) as on the glucomannans (radiata and konjac) as manifest by the K_m and V_{max} values (Table 4.7). As glucose residues are randomly distributed throughout the substrate (Aspinall 1970), their presence appears to have no effect on the

mannanase. Again, oligosaccharide analysis of mannanase hydrolysed glucomannans will elucidate the effect that glucose residues have on enzyme activity.

4.4.2.4 The effect of glucose and galatose content on mannanase activity

The mannanase was used to hydrolyze guar gum, locust bean gum, konjac gum, radiata glucomannan and ivory nut mannan in a manner analogous to the xylanase hydrolysis experiments. The extent of hydrolysis was monitored by GPC (Table 4.8). Relatively poor hydrolysis was indicated by high levels of high molecular weight oligomers which presumably were resistant to enzyme attack. From this information the inhibitory effect, if any, of galactose side-groups and glucose residues within the substrate could be elucidated. The carbohydrate content of each fraction was confirmed by either the Molisch test or the orcinol assay (SECT 2.3.4).

Table 4.8 Relative abundance of oligosaccharides produced from various mannans hydrolyzed by the pNZ1019 mannanase.

	% Relative Abundance[@]				
	Ivory Nut Mannan	Konjac Gum	Radiata Gluco-mannan	Locust Bean Gum	Guar Gum
Galactose	0%	0%	3%	17%	33%
Glucose	0%	35%	20%	0%	0%
Oligosaccharide[*]					
DP >10	12.9	18.2	24.4	15.1	80.0
DP 8	-	-	0.5	2.7	5.1
DP 7	-	0.1	1.1	13.7	5.9
DP 6	-	0.9	2.4	13.6	-
DP 5	-	1.0	4.5	12.5	0.8
DP 4	6.1	4.7	9.9	11.7	0.5
DP 3	39.2	27.1	26.9	1.0	1.7
DP 2	35.4	34.6	24.9	20.8	3.0
DP 1[#]	6.3	13.8	4.8	9.4	0.4

* expressed as degree of polymerization (ie: DP1 = monosaccharide, DP 2 = disaccharide, DP 3 = trisaccharide, etc.).
\- not detected
\# DP1 fractions contained mannose only
@ wt of fraction/total weight

Ivory nut mannan, a true mannan, was readily depolymerised with more than 80% of the polysaccharide hydrolysed to oligomers of DP1-4 (Table 4.8). The monosaccharide in the DP1 fraction was identified as mannose by HPLC (SECT 2.3.2.6). Mannose was also the only monosaccharide found in the TFA hydrolysed DP, DP3 and DP4 fractions. ^{1}H-NMR and ^{13}C-NMR, as outlined in SECT 2.3.5. and Appendix 4, were used to confirm the structure of the DP2 and DP3 fractions as ß-D-mannopyranosyl-(1,4)-ß-D-mannopyranose (mannobiose; M-M) and ß-D-manno-pyranosyl-(1,4)-ß-D-mannopyranosyl-(1,4)-ß-D-mannopyranose (mannotriose; M-M-M), respectively. By inference, the tetra-oligomer is assumed to be manno-tetrose. Mannobiose and mannotriose were the main end products of hydrolysis comprising almost 75% of the total carbohydrate. The range of end-products are indicative of an endo- type mode of action.

As the glucose content of the substrate increases (ie: radiata and konjac glucomannans, Table 4.8), higher molecular weight oligomers (DP 5 and higher) accumulate (Table 4.8). But the di- and tri- oligomers still predominate and account for more than 50% of the hydrolysed glucomannans. This suggests that glucose content has a minor effect on hydrolytic activity of the mannanase. Even konjac gum which has a mannose:glucose molar ratio of 2:1 is still readily hydrolysed to low DP oligomers. Also, the glucose residues within the konjac glucomannan appear to be evenly distributed throughout the polysaccharide. If this were not so the size distribution of the oligomers produced would be different to that of ivory nut mannan.

Comparing the kinetics of the mannanase on various substrates (Table 4.7) shows that the enzyme tends to have a higher affinity (ie: lower K_m), higher V_{max} and higher specificity (as determined by V_{max}/K_m) towards the konjac and radiata glucomannans. This suggests that enzyme binding is not hindered by the glucose content of the substrate. Indeed the glucose residues increase the solubility of the glucomannans thus facilitating enzyme activity. Therefore, a glucose content as high as 35% within a glucomannan does not inhibit the rate of mannanase hydrolysis and only affects the extent of hydrolysis to a minor degree.

Unlike the glucomannans, galactomannans are resistant to hydrolysis by the mannanase as indicated by the low proportion of DP 1-5 oligosaccharides (Table

4.8). Guar gum was very resistant with only 6% of the polysaccharide converted to low molecular weight oligosaccharides. Locust bean gum was depolymerized to a greater extent than guar but the high levels of oligosaccharides in the DP4-DP7 range imply incomplete hydrolysis. Mannose was the only monosaccharide produced which is consistent with the trace mannosidase and the absence of α-galactosidase activities (Table 4.6). As the mannanase has a relatively low affinity (ie: high K_m) for the two galactomannans (Table 4.7), it would appear that galactose inhibition of the enzyme is by steric hindrance as described by M^{c}Cleary and Matheson (1983).

The inhibitory effect of galactose side-groups was confirmed by hydrolysing locust bean gum with the cloned mannanase. This hydrolysate was further treated with a mesophilic α-galactosidase (at 45°C for 18 hr). After this treatment, more mannanase was added to the reaction mixture and a further incubation followed (70°C for 12 hr). Gel permeation chromatograms of hydrolysate after the first and final treatments are presented (Fig 4.6). Mannose was the sole monosaccharide present after the first mannanase treatment, while galactose (major) and mannose (minor) were present after the mannanase/galactosidase treatment. Mannose and galactose were present in similar proportions after the final treatment. It is evident that once the galactose is removed from the mannan backbone by the α-galactosidase, the mannanase can degrade locust bean gum to oligosaccharides of DP1-DP3 in a manner analogous to ungalactosylated substrates.

As more than 75% of the soluble radiata glucomannan is hydrolysed by the mannanase (Table 4.8), the enzyme should be useful in evaluating the affect of glucomannan removal in biobleaching.

4.4.2.5 pH optimum of the cloned mannanase

The pH profile of the mannanase paralleled that of the xylanase. The mannanase was active over a wide pH range (3-11) with an optimum between 6.5 to 7.0 (Fig 4.7). The TP8 enzyme retained 50% of its activity at pH 5 and 9. These pH values were comparable to those previously reported for the enzyme (Luthi *et al.* 1990c). In general, pH optima of mannanases are similar to those of the parent organism. This is best illustrated by the alkalophilic *Bacillus* species isolated by

Akino *et al.* (1987). This bacterium has a pH optimum between 8.5 and 9.5; two mannanases purified from the bacterium had pH optima between 8.5 and 9 (Akino *et al.* 1988); a gene cloned from the organism produced two mannanases with pH optima between 8.5 and 9 (Akino *et al.* 1989). This pattern is evident with TP8 and its cloned mannanase.

4.4.2.6 Temperature optimum and thermostability of the cloned mannanase

As with the xylanase, the mannanase was routinely assayed at 70°C which implies reasonable thermostability at this temperature. The temperature profile of the mannanase is given in Fig 4.8 (inset). The enzyme was active over a wide temperature range (30°C to 90°C) with a temperature optimum of 80°C within the 10 min assay period. This is in agreement with the temperature optimum of 80°C reported by Luthi *et al.* (1990c) for the cloned mannanase. The temperature profile data, when presented as an Arrhenius plot (1/T vs ln v; Fig 4.8), was used to calculate an E_a of 28 $kJ.mol^{-1}{}^{o}K^{-1}$ over the 30-75°C temperature range. Beyond this range enzyme denaturation was excessive and the reaction rate no longer increased with temperature.

The mannanase was extremely stable at 70°C with no loss in activity after 24 hr (Table 4.9). After the same time period at 75°C, 20% of the enzyme activity was lost. The half life ($t_{1/2}$) of the mannanase at 85°C was 48 min.

In comparison with other enzymes the cloned TP8 mannanase shows remarkable thermostability. The mannanase cloned from a *Bacillus* sp. was stable at 60°C for 30 min (Akino *et al.* 1989). The $t_{1/2}$ of a purified *Bacillus pumilus* mannanase at 70°C is 60 min (Araujo and Ward 1990); that of a purified *Bacillus stearothermophilus* mannanase at 75°C is 30 min (Talbot and Sygusch 1990). These are among the most thermostable mannanases reported, yet they are more sensitive to thermal inactivation than the cloned TP8 6.3.3.1 mannanase. The mannanase also appears to be one of the more thermostable hydrolytic enzymes cloned from TP8 (Luthi *et al.* 1990c).

Table 4.9 pNZ1019 Mannanase activity remaining after a 24 hour incubation at temperature.

Incubation Temperature	Remaining Activity	Half Life
70°C	98%	>> 24 hrs
75°C	76%	> 24 hrs
85°C	0%	48 min.

4.5 ENZYMATIC HYDROLYSIS OF RADIATA PINE HEMICELLULOSES

It has been shown that the cloned xylanase and mannanase hydrolyse their respective radiata pine hemicelluloses. Further investigation is required to determine the effectiveness of these enzymes against their respective radiata hemicellulose substrates. The cloned hemicellulases were used to hydrolyse soluble radiata xylan and radiata glucomannan by incubating the enzyme and substrate for 24 hr at 70°C. Such a prolonged incubation period ensured thorough substrate hydrolysis, therefore any oligomers present after hydrolysis must be resistant to enzymatic cleavage.

The carbohydrate content and monosaccharide composition of the soluble radiata hemicelluloses are presented in Table 2.2. Crude polysaccharide extracts were used in some hydrolysis experiments while purified substrates were used in others.

A xylose:arabinose molar ratio of 5.4:1 was obtained for the purified radiata xylan which compares favourably with the 5.3:1 ratio previously reported (Harwood 1972). As reported by Harwood (1972), radiata pine xylan has 1 methylglucuronic acid substituent for approximately every 6 xylose residues. Glucuronic acid content was not determined in this study but was assumed to be the same as that measured by Harwood. Therefore, radiata xylan has two substituents, an arbinofuranoside and a methylglucuronic acid, for approximately every 6 xylose residues. A mannose:glucose: galactose molar ratio of 3.6:1:0.2 was obtained for the purified radiata glucomannan. Again, this agrees very well with the previously reported ratio of 3.7:1:0.1 (Harwood 1973).

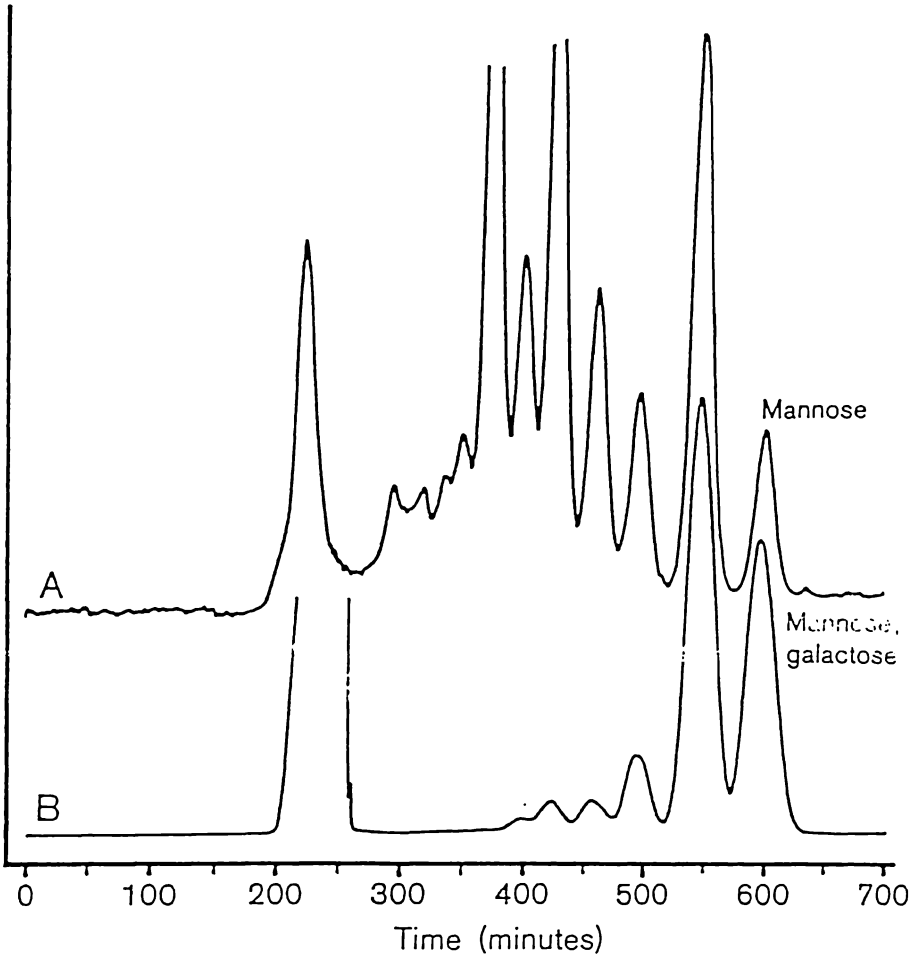

Fig 4.6 GPC chromatograms of hydrolysed locust bean gum (See Results section).
A - locust bean gum hydrolysed by the mannanase alone. DP1-mannose only.
B - locust bean gum hydrolysed by the mannanase (as in "A") followed by an incubation with a mesophilic α-galactosidase and a further incubation with the mannanase. DP1- mannose and galactose (similar amounts) Note: Peak at ~200 min in Fig. 7B is disproportionately large due to the elution of proteins.

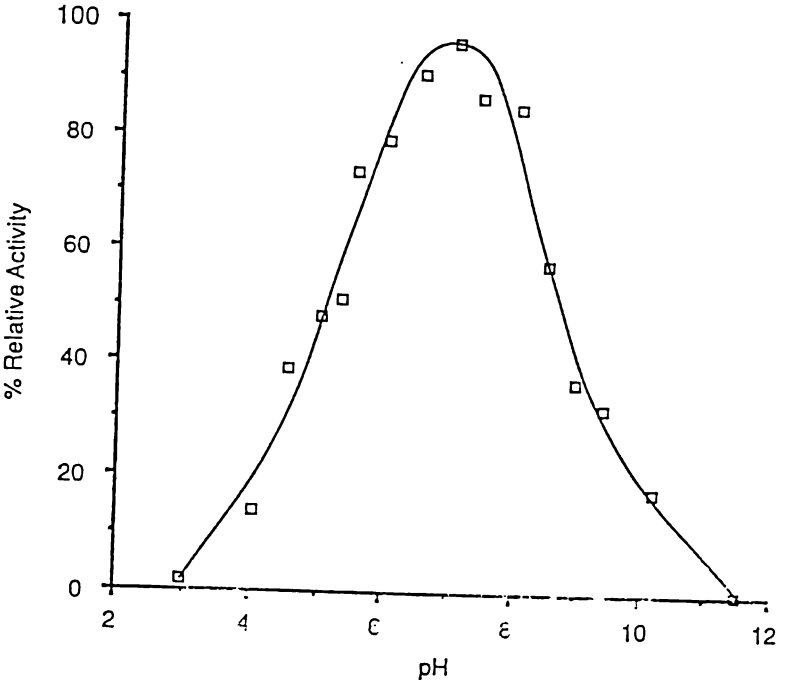

Fig 4.7 pH vs % relative mannanase activity. Activity measured on Locust Bean Gum (10 minute assay at 70°C). Buffers used (all at 50 mM): pH 3.0-6.0, Nacitrate; pH 6.0-7.5, dimethylglutaric acid; pH 8-8.5, N,N-bis[2-hydroxyethyl]-glycine (bicine); pH 9.0-9.5, 2-[N-cyclohexylamino]-ethanesulfonic acid (CHES); pH 10.0-11.0, 3-[cyclohexyl-amino]-2-hydroxy-1-propanesulfonic acid (CAPS).
100% mannanase activity = 0.10 U (pH 7.0, 70°C).

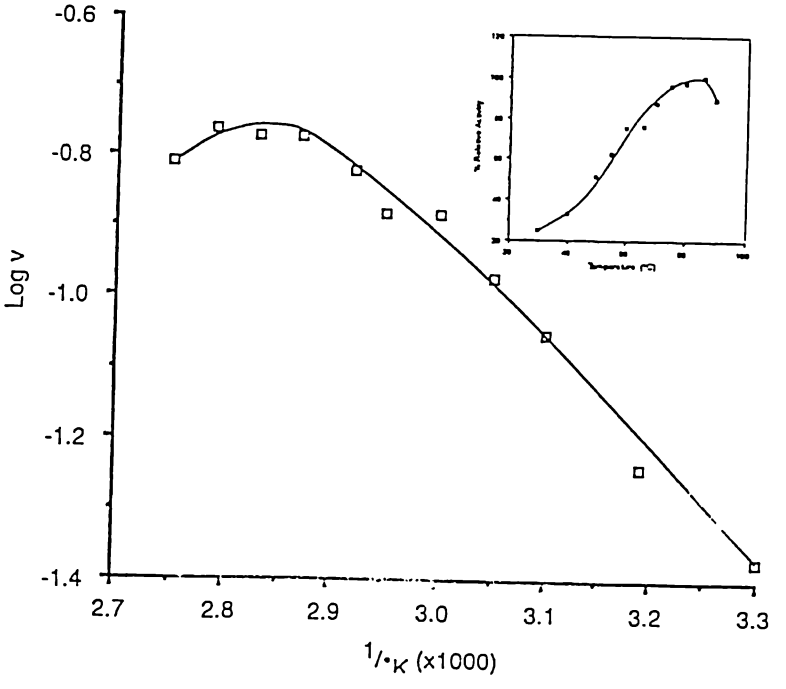

Fig 4.8 Arrhenius plot Log v vs 1/T (°K) and (Inset:) Assay temperature (°C) vs % relative mannanase activity of mannanase (pNZ1019)
100% mannanase activity = 0.09 U (at 80°C, pH 6).

The molecular weight distributions of the purified radiata xylan and glucomannan were determined by GPC (SECT 2.4.2.6). The average DP of the purified xylan was estimated at 220 and that of the purified glucomannan was 140. These results are almost three fold higher than the 78 and 46 previously reported for radiata xylan (Harwood 1972) and radiata glucomannan (Harwood 1972), respectively. This inconsistency may be due to differences in methodology for determining DP (ie: GPC vs methylation analysis) or to the use of ethanol as a means of precipitating solubilised hemicelluloses. Ethanol is most effective in precipitating high molecular weight oligosaccharides. Therefore, using this technique several times, as was done during ion exchange, may have concentrated the high DP fraction of the hemicelluloses. This should not have an effect on the enzymatic hydrolysis of the polysaccharides. The glucomannan was deacetylated during alkali extraction.

Monosaccharide analysis of the two polysaccharides showed that each was contaminated with a small but significant amount of the other. These contaminants were carried through the ion exhange and ethanol precipitation procedures in similar proportions. This suggests that these contaminants are either of a DP greater than 10 which would be required for ethanol precipitation (Aspinall 1970) or they are small oligomers linked to the larger polysaccharides of the fraction. Similar cross contamination has been reported with radiata glucomannan and radiata xylan preparations (Harwood 1972, 1973).

4.5.1 Xylanase hydrolysis of radiata glucuronoarabinoxylan

The pNZ1417 xylanase preparation was used to hydrolyse the crude radiata xylan as described in the methodology (SECT 4.2). The thermostability of the enzyme was again demonstrated when 100% enzyme activity was recovered from the hydrolysate after the incubation period.

The hydrolysate was separated into acidic and neutral fractions by ion exchange (SECT 2.3.2.1). Carbohydrate analysis of these showed that 59% of the original polysaccharide was present as acidic and 27% as neutral oligomers (86% recovery).

4.5.1.1 Neutral oligosaccharide fraction

Neutral oligosaccharides were fractionated by GPC using the Biogel P2 system (SECT 2.3.2.2). Aliquots corresponding to the five regions delineated in the chromatogram (Fig 4.9) were collected. These aliquots contained the monosaccharides (DP1), disaccharides (DP2), trisaccharides (DP3), higher oligomers (DP4 to 8) and the excluded peak. The Molisch test confirmed the presence of carbohydrate in all the fractions except that of the excluded peak. This fraction was not examined further. The weight distribution and monosaccharide composition of the four remaining fractions is summarised in Table 4.10. The monosaccharide composition of each fraction were determined by TFA hydrolysis (SECT 2.3.2.4) followed by HPLC (SECT 2.3.2.5), except for the DP1 fraction where the hydrolysis step was omitted. Unhydrolysed radiata xylan that had been subjected to ion exchange eluted at the void volume when analysed by GPC, thus confirming its polymeric nature.

Neutrals were completely soluble in water with more than 80% of the fraction composed of low molecular weight (DP 1-3) sugars. Arabinose was the main free monosaccharide in fraction A (Table 4.10). Interestingly, neither xylanase preparation demonstrated L-arabinosidase activity against *p*-nitrophenol-

Table 4.10 Monosaccharide composition of xylanase hydrolysed radiata xylan: Neutral fraction

Fraction	DP	% of Neutrals Fraction	% Composition		
			Xylose	Arabinose	Glucose
A	1	7.0	trace	~100	N.D.
B	2	26.9	100	N.D.	N.D.
C	3	51.5	100	N.D.	N.D.
D	4-8	14.6	100	N.D.	trace

N.D. = not detected

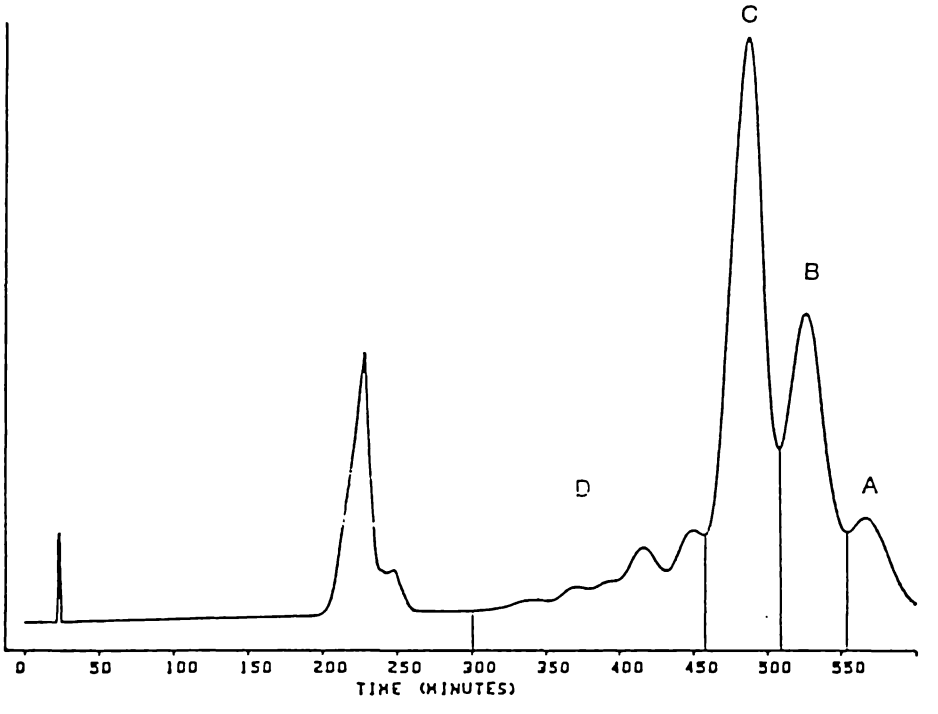

Fig 4.9 Gel permeation chromatogram (Biogel system) of the neutral fraction of radiata xylan hydrolysed by the pNZ1417 xylanase
Four fractions collected (A,B,C and D) described in Table 4.10.

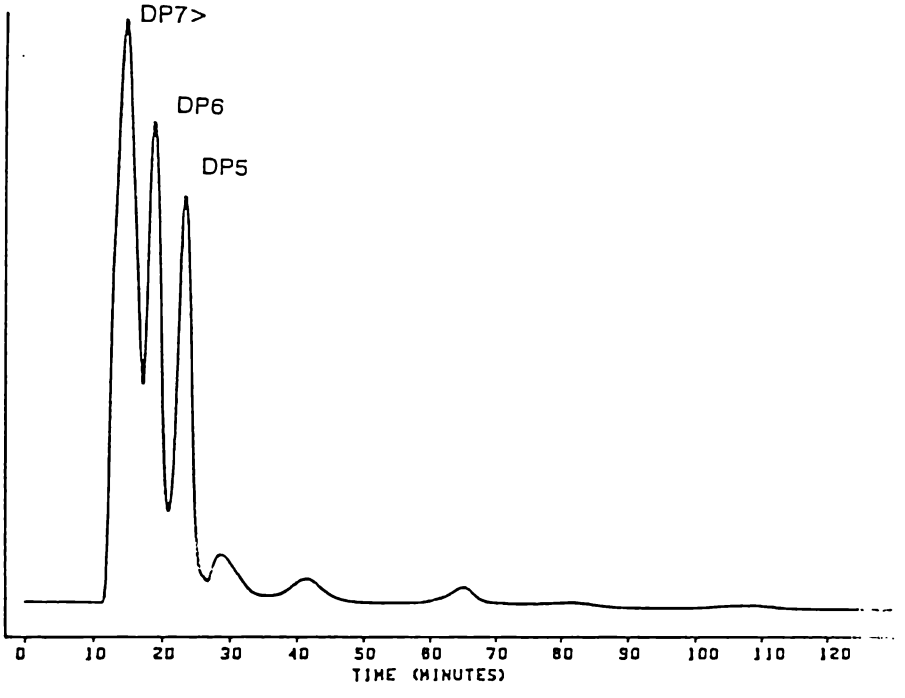

Fig 4.10 HPLC chromatogram (Aminex A-25 system) of the acidic fraction of radiata xylan hydrolysed by the pNZ1417 xylanase

arabinofuranoside (Table 4.4). Arabinose release is thought to be an artefact of the ion exchange procedure rather than a consequence of any arabinosidase activity. No free arabinose was found in hydrolysed radiata xylans that had not undergone ion exchange (see below SECT 4.5.1.4). Therefore, it is concluded that the thermostable xylanase does not exhibit L-arabinofuranosidase activity.

Puls *et al.* (1988) and Fontana *et al.* (1988) have described non-specific arabinose release from xylans during anion exchange, possibly due to the acid labile nature of these linkages. Yet little or no arabinose was cleaved from unhydrolysed xylan subjected to the same ion exchange fractionation step. Also, the free arabinose recovered in the neutral fraction accounted for only 20% of the total arabinose in the polysaccharide. This suggests that some of the arabinose is still linked to xylo-oligomers either in the acidic fraction or the unrecovered portion of the hydrolysate. It may be that some arabinose linkages are more susceptible to acid hydrolysis than others.

Small amounts of xylose, as compared to xylobiose and xylotriose, are due to the transglycosylation activity of the xylanase. As xylose was the predominant monosaccharide in the DP2 and DP3 fractions (Table 4.10), these oligomers were tentatively identified as xylobiose and xylotriose, respectively. These oligomers accounted for most of the neutral fraction but less than 20% of the total polysaccharide, indicating that the remaining 80% is resistant to complete hydrolytic attack by the enzyme. This is probably due to steric hindrance caused by various side-groups.

The presence of higher DP xylo-oligomers is common in endo-xylanase hydrolysates and could result from transglycosylation reactions (Dekker 1985) or steric hindrance due to low levels of branching which have been detected in radiata xylan (Harwood 1972). But for perspective, fraction D in its entirety, accounted for less than 3% of the total xylan.

4.5.1.2 Acidic oligosaccharide fraction

The acidic fraction resulting from xylanase hydrolysis was analysed by ion exchange HPLC (Aminex A-25, SECT 2.3.2.6) which resolved methyl glucuronic acid substituted xylo-oligosaccharides on the basis of DP. A small but significant

portion of the acidic fraction was insoluble (~5%). A chromatogram of the soluble portion (Fig 4.10) clearly shows that more than 90% of the acidics were high DP oligosaccharides (DP 5-7).

The greatest proportion of the soluble acidic fraction occured as aldoheptauronic acid (methyl glucuronic acid substituted xylo-heptose) and higher DP oligomers. These uronic acid substituted xylo-oligosaccharides accounted for 40% of the soluble acidic fraction. Higher uronic acid (DP 7-5) concentrations decreased in a regular manner until the aldopentauronic acids after which levels drop dramatically. This notable difference between the proportion of high DP acidic oligomers (DP5 and greater) as compared to the low DP acidics (DP2-DP4) suggests that the former are resistant to further enzymatic hydrolysis. This resistance may be due to a higher level of substitution (ie: 2 substituents per 6 xylose residues). At least one of the substituents in these xylo-oligomers must be a methyl glucuronic acid to satisfy the acid nature of this fraction.

4.5.1.3 Xylanase treated radiata xylan: unfractionated analysis

The pNZ1435 xylanase was used to hydrolyse purified radiata xylan as previously described. Total hydrolysates were analysed for oligosaccharides by ion exchange chromatography (Dionex system, SECT 4.2). This procedure can separate neutral and acid oligomers in a single chromatographic run.

The end-product profile of the pNZ1435 hydrolysed, radiata xylan (Fig 4.11) differed slightly from that of the pNZ1417 xylanase. As the pNZ1435 hydrolysed xylan was not fractionated by ion exchange, no free arabinose was found in this hydrolysate. This is conclusive evidence that the cloned xylanase does not have any arabinosidase activity and that the free arabinose seen in the pNZ1417 xylanase hydrolysate was an artefact of the acidic/neutral ion exchange procedure. In the pNZ1435 hydrolysate xylose, xylobiose and xylotriose accounted for 28.5% of the total carbohydrate which compares favourably with the neutral fraction of the pNZ1417 hydrolysate. Differences in the ratios of the DP1-DP3 xylo-oligomers between the two xylanase preparations could be due to non-specific arabinose release.

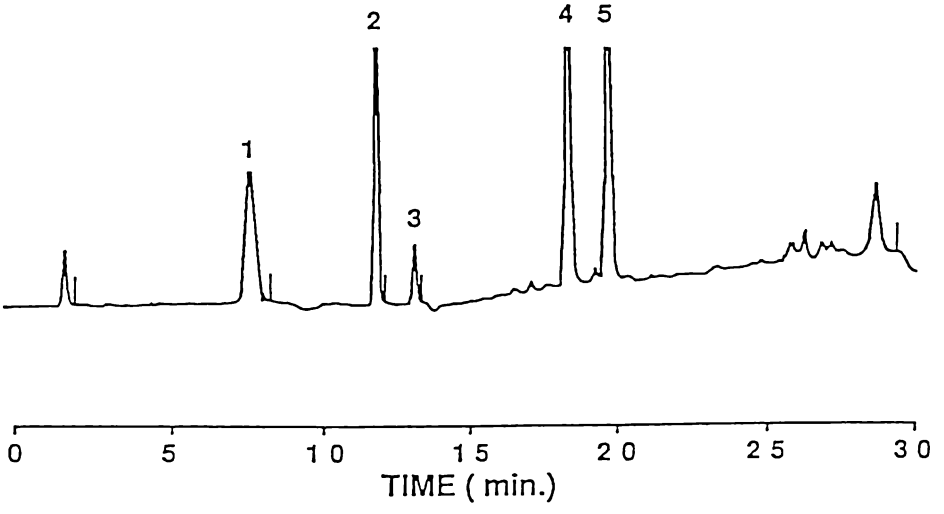

Fig 4.11 HPLC chromatogram (Dionex anion exchange system) of unfractionated, purified radiata xylan hydrolysed by the pNZ1435 xylanase.

1 xylose
2 xylobiose
3 xylotriose
4 arabino-xylobiose (X_A-X)
5 arabino-xylotriose (X_A-X-X) and small amount of glucuronic acid substituted xylotriose (X-X_{GlcA}-X, $_{GlcA}$-X-X-X)
(See Appendix 2 for identification of peaks)

More than 70% of the carbohydrate in the pNZ1435 hydrolysed radiata xylan was found as soluble oligomers (DP5-7) that did not elute as xylo-oligosaccharides (Fig 4.11). Two major peaks (peaks 4 and 5, Fig 4.11) accounted for more than 50% of the total carbohydrate. To identify these unknowns, the hydrolysate was sequentially treated with various mesophilic enzymes (SECT 2.3.2.5). The identification of these xylo-oligomers is outlined in Appendix 2. The first unknown (peak 4) was identified as α-L-arabinofuranosyl-(1,3)-ß-D-xylopyranosyl-(1,4)-ß-D-xylopyranose (arabino-xylobiose; X_A-X). The second unknown (peak 5) contained two types of substituted xylotrioses; the major component was identified as α-L-arabinofuranosyl-(1,3)-ß-D-xylopyranosyl-(1,4)-ß-D-xylopyranose-(1,4)-ß-D-xylopyranose (arabino-xylotriose; X_A-X-X) and the minor as 4-O-methylglucuronic acid-(1,2)-ß-D-xylopyranosyl-(1,4)-ß-D-xylopyranose-(1,4)-ß-D-xylopyranose (aldotetra uronic acid; X-X_{GlcA}-X and $_{GlcA}$-X-X). It should be noted that the arbinofuranosyl substitutent was found on the terminal, non-reducing xylose residue while the methylglucuronic acid substitutent may be on the central xylose residue. The range of xylo-oligomers produced during enzyme hydrolysis clearly demonstrates the endo-hydrolytic nature of the two cloned, xylanase preparations (Dekker 1984).

A difference between the two xylanases preparations was in the relative proportion of acidic xylo-oligomers. The pNZ1435 xylanase seemed to produce a lower proportion of acidic oligomers than the pNZ1417 xylanase. This may reflect incomplete hydrolysis of the acidic oligosaccharides by the latter. Whether this reflects differences in the xylanase preparations or the type of radiata xylan used (ie: unpurified vs purified) is uncertain. However, both xylanase preparations effectively hydrolysed more than 90% of the radiata xylan to low molecular weight, water soluble, substituted and unsubstituted xylo-oligosaccharides. The major end-products directly attributable to xylanase activity include xylose, xylobiose, xylotriose, arabino-xylobiose, arabinoxylotriose, aldotetrauronic acids and higher xylo-uronic acid oligomers; more than 25% of the substrate hydrolysed to soluble, neutral oligosaccharides of DP1-DP3. Schofield (DPhil, University of Waikato 1990) also showed xylobiose and xylotriose were major end-products of oat spelt xylan hydrolysis by the thermostable xylanase. As the enzyme is active against soluble

radiata xylan, any differences in activity in radiata kraft pulps must be attributed to steric effects; either enzyme inaccessability to the substrate or shielding of the substrate by other polymers (ie: lignin, cellulose).

4.6 Mannanase hydrolysis of radiata glucomannan

Radiata glucomannan was hydrolysed with the mannanase as described in SECT 4.2. The unfractionated hydrolysate was analysed by the Dionex HPLC system (SECT 2.3.2.5) with the chromatogram given in Fig 4.12. Again, mannose was the sole monosaccharide produced. A peak eluting at 14.2 min which accounted for more than 20% of the total carbohydrate was thought to be mannobiose, based on relative retention times. Two trisaccharides eluting at 15.9 min (possibly mannotriose) and 16.4 min contained almost 18% of the total carbohydrate, while higher DP oligomers had retention times of 17 min and longer.

The radiata glucomannan hydrolysate was analysed by GPC and seven fractions were collected as outlined in Fig 4.13 and concentrated by roto-evaporation. The monomer composition of the seven fractions after TFA hydrolysis (except the DP1 fraction) and HPLC analysis is presented in Table 4.11. These results confirm that the DP1 fraction is mannose. Also, galactose was only found in the higher DP fractions. This is in accordance with the inhibitory effect these side groups have on the mannanase. Glucose residues of the backbone are evenly distributed in oligomers of DP3 and higher, and are present to a lower extent in the DP2 fraction.

The Dionex chromatogram suggested that various isomers were present in the DP2 and DP3 fractions. These were resolved by HPLC using the dextro-PAK system (SECT 2.3.2.3). The DP2 fraction contained three different disaccharides (Fig 4.14), and the DP3 fraction contained at least six peaks (Fig 4.14). The DP2 fraction was analysed by descending paper chromatography (SECT 2.3.2.3) which showed only two compounds present, presumably the two major peaks in Fig 4.14. Paper chromatography did not resolve any of the peaks in the DP3 fraction as trisaccharides migrate at a much slower rate than the disaccharides.

The DP2 fraction was further fractionated as outlined in Fig 4.14 into $DP2_a$, the first peak which accounted for over 72% of the carbohydrate in the entire DP2

Table 4.11 Monosaccharide composition of mannanase hydrolysed radiata glucomannan

Fraction	DP	% of Total Polysaccharide#	% Composition Man	Glc	Gal
A	1	5.5	99.8	trace	N.D.
B	2	23.7	91.4	8.6	N.D.
C	3	21.0	74.2	25.8	N.D.
D*	4	5.8	14.3	13.7	N.D.
E	5	4.3	70.8	28.3	0.1
F	6-9	5.0	59.7	21.0	19.3
G	>10	27.6	66.6	29.6	3.8

N.D. not detected
93% recovery of the total carbohydrate
* more than 70% of this fraction was xylose (SECT 4.6)

fraction, and $DP2_b$ which contained the two remaining peaks. The major peak in the $DP2_b$ fraction contained 20% of the carbohydrate in the entire DP2 fraction while the smaller peak contained 8%. Proton and ^{13}C NMR spectra of the $DP2_a$ fraction were identical to those of the DP2 fraction of the ivory nut mannan hydrolysate, confirming the former as mannobiose (Appendix 4).

As a mixture, the $DP2_b$ fraction was not as readily identified by NMR. Yet, assuming the major peaks in the proton and ^{13}C NMR spectra (Appendix 4) correspond with the main peak in the $DP2_b$ fraction, the NMR spectra identify this component as ß-D-glucopyranosyl-(1,4)-ß-D-mannopyrannose (G-M). The minor peak may be cellobiose or mannose-glucose although its elution in the dextro-Pak chromatogram was not indicative of either disaccharide but rather its elution time was indicative of a trisaccharide. Also, paper chromatography suggests it could be a trisaccharide contaminant, however to comply with a mannose:glucose ratio of 10:1 (Table 4.11) the contaminant must be mannotriose. The DP2 fraction is composed of 72% mannobiose, 20% glucomannose and 8% of an unknown glucomanno-oligomer.

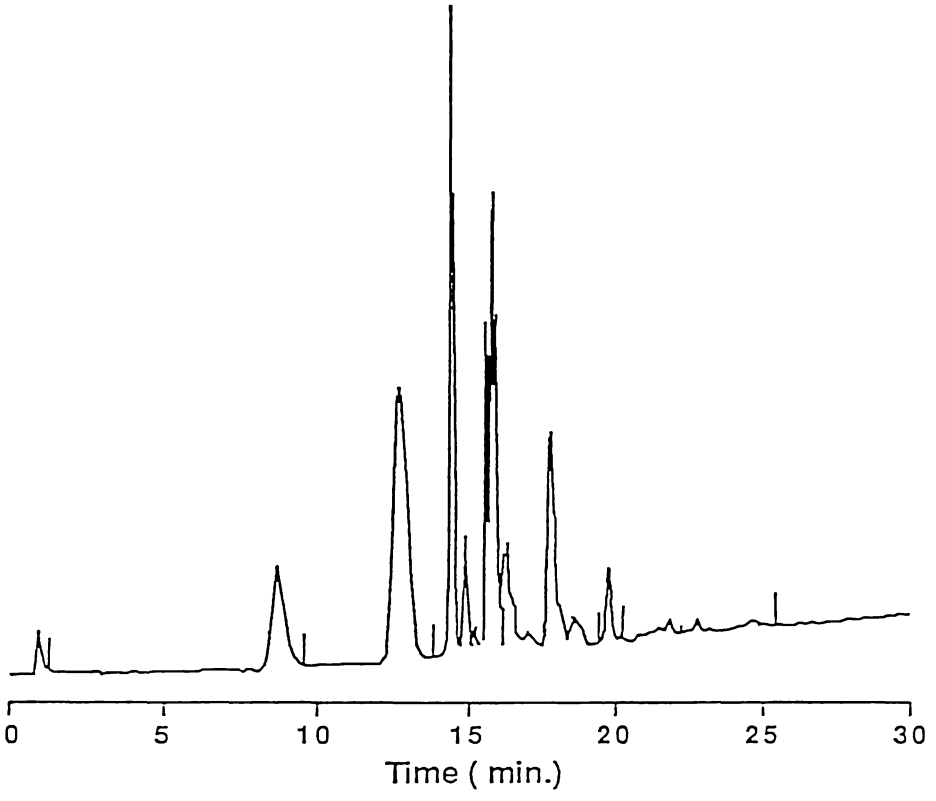

Fig 4.12 HPLC chromatogram (Dionex anion exchange system) of unfractionated, purified radiata glucomannan hydrolysed by the pNZ1019 mannanase

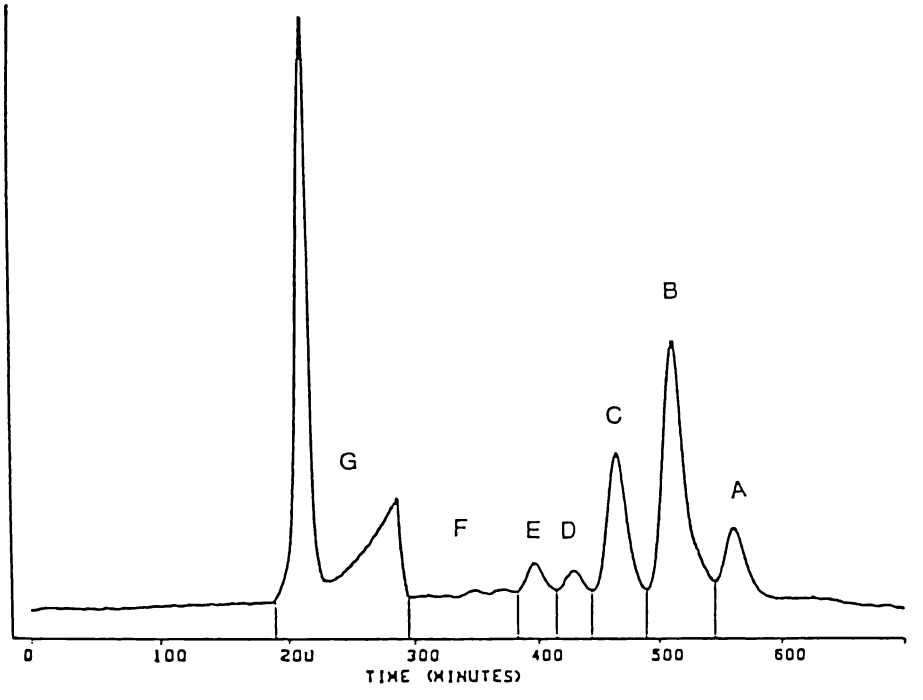

Fig 4.13 Gel permeation chromatogram (Biogel system) of radiata glucomannan hydrolysed by the pNZ1019 mannanase. Seven fractions (A-E) were collected. The composition of these fractions is described in Table 4.11.

The DP3 fraction was also further fractionated by dextro-Pak HPLC as outlined in Fig 4.15. Two fractions obtained were the $DP3_a$ which included the peak eluting at 4.6 min and the $DP3_b$ fraction which included the 5.5 min peak. $DP3_a$ accounted for 35% of carbohydrate in the entire DP3 fraction while $DP3_b$ for 45% with the remaining 20% distributed throughout the smaller peaks (retention time 7-10 min, Fig 4.15). These were not collected. Comparisons of the $DP3_a$ proton and ^{13}C spectra with those of the DP3 from ivory nut hydrolysate again confirmed the former as mannotriose. The $DP3_b$ was identified as ß-D-glucopyranosyl-(1,4)-ß-D-mannopyranosyl-(1,4)-ß-D-mannopyranose or gluco-mannobiose (G-M-M) by NMR (Appendix 4).

A small amount of xylose present in the radiata glucomannan as a contaminant (Table 4.11) eluted in the DP4 fraction, thus demonstrating its polymeric nature and its resistance to mannanase hydrolysis. Whether the xylose is present as a xylo-tetraoligomer, is bound to glucomanno-oligomers or both is not certain. The high proportion of xylose in the DP4 fraction suggests that the pentose is in a polymeric form. If this polymeric xylose were an artefact of the extraction procedure, xylose contamination of other fractions would be expected. Others have also noted a xylan component during the extraction of radiata glucomannan (Harwood 1973). This may indicate the presence of a glucomannan-xylan linkage. Many types of associations between various hemicelluloses and pectins are prevalent (Fry and Miller 1989). These types of hemicellulosic associations are of interest in the study of wood structure and warrant further investigation. The cloned mannanase may be useful in the selective isolation of such hemicellulose linkages.

The major end products of the hydrolysis of radiata glucomannan by the mannanase (mannose, mannobiose, gluco-mannose, mannotriose and gluco-mannobiose) conform to those predicted by the model for the catalytic site of the *Aspergillus niger* mannanase (McCleary and Matheson 1983). This model (described in SECT 1.4.2) does not result in the production of glucomanno-oligomers with a reducing glucose residue. This holds true for the major oligomers produced by the mannanase. Further identification of the minor oligomers produced is required before a model for the binding and catalytic site of the cloned mannanase can be proposed.

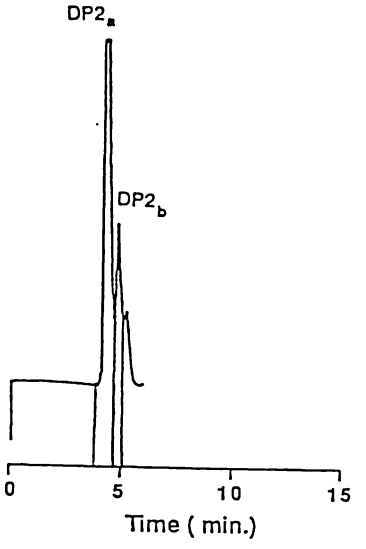

Fig 4.14 Chromatogram (dextro-Pak system) of the DP2 peak of radiata glucomannan hydrolysed by the pNZ1019 mannanase (eluent flow 0.6 ml min^{-1}). Two fractions collected ($DP2_a$ and $DP2_b$).

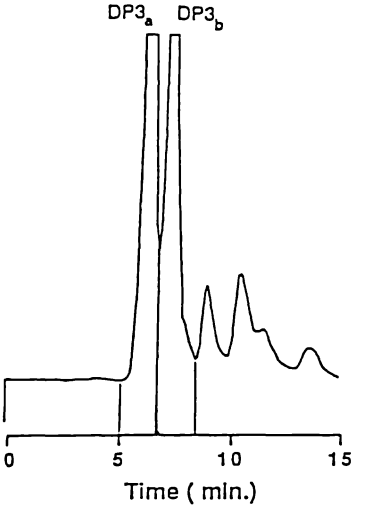

Fig 4.15 Chromatogram (dextro-Pak system) of the DP3 peak of radiata glucomannan hydrolysed by the pNZ1019 mannanase (eluent flow 0.7 ml min^{-1}). Two fractions collected ($DP3_a$ and $DP3_b$).

The cloned mannanase is active against radiata mannan hydrolysing 80% of the substrate to low molecular weight oligomers. Therefore like the cloned xylanase, the mannanase should also hydrolyse its substrate from radiata pulps unless sterically hindered.

4.7 CONCLUSIONS

Sufficient cloned xylanase and mannanase was produced and purified to allow their evaluation as bleaching aides. These enzymes have also been characterised to the point where their pH and temperature optima have been confirmed, their thermostability has been established, their effectiveness against their corresponding radiata hemicellulose has been determined and the major end-products in these hydrolysates have been identified. The characteristics of the cloned hemicellulases are summarised in Table 4.12.

From an applied standpoint, the thermostability of the cloned hemicellulases is significant in two respects. First, their high thermostability reinforces heat treating as a purification technique, even permitting higher temperatures for longer periods in the case of the mannase. Secondly, the thermostability data defines the temperature upper limits for these enzymes in pulp bleaching. The pH and temperature optima of the cloned hemicellulases are compatible allowing pulp treatments with two enzymes simultaneoulsy.

Neither cloned hemicellulase could debranch their respective substrate. However, this did not appear to prevent the partial hydrolysis of either the radiata xylan or glucomannan. Similar hydrolysis patterns would be expected in radiata kraft pulps unless the enzymes are sterically hindered with the substrates either inaccessible or protected by other polymers (ie: lignin). Further investigation is required before an accurate model of the catalytic site of either the xylanase or the mannanase can be developed.

Table 4.12 Summary of the characterisation of the cloned xylanase and mannanase preparations

	pNZ1417 xylanase	pNZ1435 xylanase	pNZ1019 mannanase
pH range	5-10	5-10	4-11
pH optima	6-7.5	N.D.	6-7.5
Temperature range	30-90°C	30-90°C	30-90°C
Temperature optima	70°C	70°C	80°C
Residual activity after 24 at 70°C	95%	100%	98%
Activation energy (E_a) kJ mol^{-1}	36	30	28
Michaelis-Menten parameters on respective soluble radiata hemicellulose			
K_m (g L^{-1})	3.52	2.54	0.053
V_{max} (U mg^{-1} prot)	24.7	18.3	47.4
% of respective radiata hemicellulose hydrolysed (<DP10)	82%	80%	76%
Major end-products of hydrolysis on respective soluble radiata hemicellulose	X, X_2, X_3, acidic X_{3-7}	X, X_2, X_3, X_AX, X_AXX,	M, M_2, M_3, GM, GMM,

CHAPTER FIVE

EFFECTS OF HEMICELLULASES ON WOOD PULPS

5.1 INTRODUCTION

The hemicellulases cloned from TP8 have demonstrated the ability to thoroughly hydrolyse soluble radiata xylan and glucomannan. In this chapter the effectiveness with which these enzymes hydrolyse intra-fibril polysaccharides is examined. Residual xylans occur as retake or native xylan. Their resistance to enzymatic hydrolysis may be attributed to several factors. The retake xylan may be hydrogen bonded to cellulose (Mora *et al.* 1986b) or modified to an enzymatically resistant form (ie: crystalline) (Meller 1965). Native xylan (and glucomannans) may be physically inaccessible due to either shielding by other polymers or entrapment within microfibrils (Scott 1984). Both redeposited (retake) and native xylans, as well as glucomannans, may undergo partial enzymatic cleavage without solubilisation. Only by examining residual hemicelluloses can the extent of enzymatic activity be fully elucidated. The aim of this chapter is to determine how the cloned TP8 hemicellulases affect their respective substrates in the cell wall and to correlate this with any improvements in bleachability arising as a consequence of the activity of the cloned enzymes. Two approaches were used to do this; 1) carbohydrates solubilised from pulps by the enzymes were examined and 2) the hemicelluloses remaining within the pulp after enzyme treatment were extracted and characterised.

Studies of the soluble enzyme degradation products of xylanase treated pulps have shown that xylan can be selectively hydrolysed from hardwood and softwood kraft pulps (Clark *et al.* 1989; Jeffries and Lins 1989; Mora *et al.* 1986a; Senior *et al.* 1988, Viikari *et al.* 1990). Selective enzymatic solubilisation of mannans from pulps has also been shown (Clark *et al.* 1989, 1991; Viikari *et al.* 1990).

Clark *et al.* (1989, 1991) have examined the carbohydrates solubilised from radiata kraft pulps by a fungal xylanase, a fungal mannanase and a bacterial mannanase. None of these enzymes were thermostable. The xylanase solubilised from 10-20% of the available xylan and gave a 25% saving in chlorine consumption during bleaching. The bacterial mannanase solubilised 5% of the glucomannan and

3% of the xylan with a similar improvement in bleachability. An *Aspergillus niger* mannanase hydrolysed more than 11% of the glucomannan but no xylan and gave no improvement in bleachability (Clark *et al.* 1990). The end-products of radiata glucomannan hydrolysis differed between the two mannanases which may indicate differences in their mode of action (Clark *et al.* 1990). The cloned TP8 mannanase appears to function in a similar manner to the *A. niger* enzyme.

Carbohydrate solubilisation by hemicellulases is a common method of determining the distribution of hemicellulases and their possible functions within a pulp or delignified wood (Mora *et al.* 1986a; Sinner *et al.* 1979) as well as assessing enzyme activity and specificity. However, no correlation is evident between the amount of hemicellulose solubilised and improved bleachability (Clark *et al.* 1990; Viikari *et al.* 1990; Clark *et al.* 1991). It appears that only low levels of carbohydrate solubilisation are required to elicit an improvement in bleachability.

5.2 METHODOLOGY

Two cloned hemicellulases were used to hydrolyse various types of pulps with the effects monitored by the amount and composition of the solubilised carbohydrate. These were the pNZ1435 xylanase preparation described in Table 4.3 and the pNZ1019 mannanase preparation of Table 4.1. These enzymes have been characterised on their respective soluble radiata hemicelluloses (Chapter 4). Several pulps were used in this chapter. Pulp sources and analytical methods for determining composition are given in SECT 2.4.1 and 2.4.2 respectively. Pulp compositions are presented below.

The protocol followed for the enzymatic treatment of the various pulps is given in SECT 2.4.2.3. Briefly, pulp to be treated was dispersed in distilled water (at 2% consist.), the required enzyme dosage was added as U of activity per gram of oven dried pulp (U/g OD pulp) and the pulps incubated at 70°C for the specified time. There was no pH control. Time course and enzyme dosage experiments using this protocol were performed with either the mannanase or the xylanase on radiata kraft pulp.

In one series of experiments, enzyme treated pulps were drained (SECT 2.4.2.3) and the liquor analysed for carbohydrate composition by TFA hydrolysis followed by HPLC (SECT 2.3.2.4 and 2.3.3 respectively). In further experiments, the xylan and glucomannans were extracted by the Hamilton procedure (SECT 2.4.2.4, Fig 2.7). The carbohydrate compositions of the treatment liquors, the extracted hemicelluloses and extracted pulps were determined by capillary GC of the alditol acetates as presented in SECT 2.3.3. The molecular weight distribution of the extracted polysaccharides were determined by gel permeation chromatography (SECT 2.4.2.6). An extracted radiata kraft pulp was enzymatically treated and the liquor composition determined as described.

Analytical methods are presented with each series of experiments. In all experiments, pulps with no added enzyme (controls) were run in parallel to ensure that differences between enzyme treatments and controls were indeed attributable to enzyme activity. Results may be expressed as percentage differences between enzyme treatments and no enzyme controls.

5.3 CHARACTERISATION OF PULPS

Five unbleached pulps were used in this study. Four were different radiata pine pulps and included a kraft pulp, a neutral sulphite anthraquinone pulp (NSAQ), a chemimechanical pulp (CTMP) and a thermomechanical pulp (TMP). The fifth was a hardwood (tawa) kraft pulp. The carbohydrate composition and lignin content (Klason and acid soluble) of the pulps were determined as outlined in SECT 2.4.2. The monosaccharide and lignin composition of each is presented in Table 5.1.

The two mechanical pulps (TMP and CTMP) were also subjected to a weak alkali treatment (SECT 2.4.1). Recent studies have shown that an alkali treatment of a hardwood TMP dramatically increased xylanolytic carbohydrate solubilisation (Jeffries and Lins 1989). The mechanical pulps used in this study were alkali treated in a similar manner to determine whether this improved carbohydrate solubilisation by the thermostable hemicellulases.

Carbohydrate compositions and lignin contents of the radiata kraft, radiata NSAQ and hardwood kraft pulps are comparable to those reported in the literature (Bryce 1980; Aurell and Hartler 1965; Clark *et al.* 1991). The CTMP and TMP

Table 5.1 Carbohydrate composition and lignin content of four types of unbleached radiata pulps (kraft, NSAQ, CTMP, TMP), two alkali treated radiata mechanical pulps and an unbleached hardwood kraft pulp

	Pulp						
	Radiata Kraft	Radiata NSAQ	Hardwood Kraft	Radiata CTMP#		Radiata TMP#	
				Con	Alk	Con	Alk
% (w/w) Sugar							
Glucose	80.1	70.3	82.0	64.6	48.3	39.6	41.0
Xylose	9.7	6.0	16.9	4.5	1.9	3.5	1.9
Galactose	<0.1	<0.1	<0.1	0.7	<0.1	0.6	<0.1
Arabinose	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Mannose	4.9	6.9	<0.1	10.0	9.1	6.4	6.7
Sub-total	94.7	83.3	98.9	79.7	59.3	50.1	49.6
% (w/w) Lignin							
Klason	2.7	7.1	2.3	19.7	26.8	25.3	29.5
Acid sol.	0.6	1.9	0.0	0.0	0.0	0.0	0.0
Sub-total	3.3	9.0	2.3	19.7	26.8	25.3	29.5
% Total	98.0	92.3	101.1	99.4	86.2	75.5	79.1

Con - control, Alk- alkali treated

pulps had higher lignin contents than the chemical pulps as would be expected (Table 5.1). The alkali treatment had the greatest effect on the CTMP pulp with an apparent decrease in xylan and cellulose content resulting in an increase in the Klason lignin content. The total lignin and carbohydrate was lower than expected for mechanical pulps. The only significant effect the alkali treatment had on the TMP pulp was a drop in the xylan content. This contrasts with experiments where a similar, mild alkali treatment had no effect on the carbohydrate or lignin contents of a hardwood TMP (Jeffries and Lins 1989); only the acetyl content of the pulp was reduced.

5.4 ENZYME TREATMENT OF PULPS

5.4.1 Enzyme dosage

To economise on enzyme, optimum xylanase and mannanase dosages that would solubilise the greatest amount of carbohydrate within a 24 hr period were determined. This was done by determining the effect of enzyme dosage on total carbohydrate solubilised from a radiata kraft pulp at 70°C within a 24 hr incubation (Fig 5.1). Solubilised carbohydrate increased linearly with the enzyme dose up to about 100 U per g of OD pulp with both enzymes. Beyond this level the rate of increase in carbohydrate release began decreasing.

For exhaustive carbohydrate solubilisation, high hemicellulase dosages are warranted but published studies have shown that low xylanase dosages can still elicit the bleach boosting effect. Enzyme dosages in the 100-1000 U/g OD pulp have been used by many to maximise carbohydrate hydrolysis within pulps (Clark *et al.* 1989; Sinner *et al.* 1979), to assist pulp bleaching (du Manoir *et al.* 1990; Pedersen and Elm 1991) and to improve viscosity (Paice *et al.* 1988). Yet, xylanase dosages as low as 20 U/g of OD pulp (Perrolaz *et al.* 1991) and 5 U/ g of OD pulp (Skerker et al. 1991) have been used effectively to improve pulp bleachability. Commercial applications of xylanase preparations may require however high enzyme dosages to reduce incubation periods and compensate for enzyme inactivation.

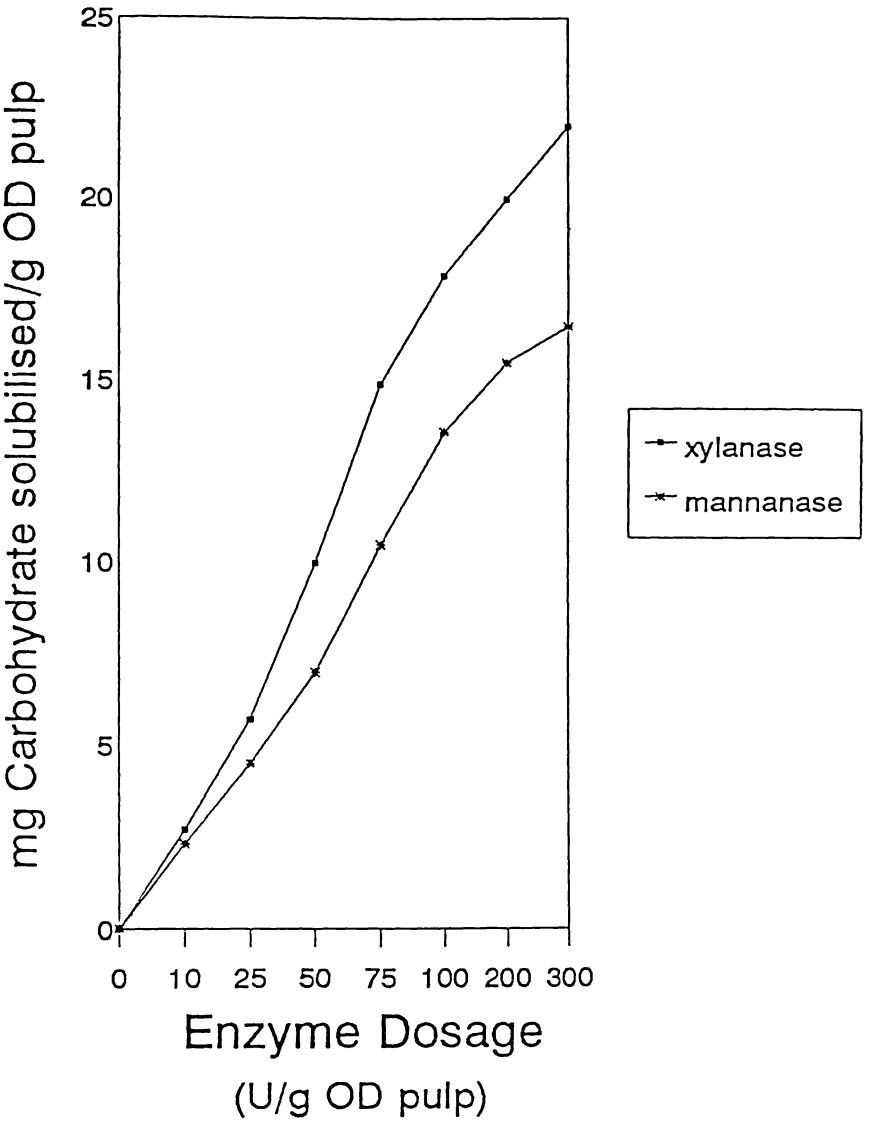

Fig 5.1 Total carbohydrate solubilised (mg CHO/g OD pulp) vs xylanase (pNZ1435) or mannanase (pNZ1019) dosage (2% consistency kraft pulp in water at 70°C for 24 hr)

To try and maximise the effects of the cloned enzymes on pulps, a relatively high dosage (100 U/ g OD pulp) was selected in this study. This dosage was considered optimal for both enzymes as large amounts of carbohydrate (~65% of the maximum for each enzyme) were solubilised at one third of the maximum enzyme dose.

5.4.2 Time course

Radiata kraft pulp was treated with either the cloned xylanase or the cloned mannanase (100 U per gram OD pulp, 2% pulp consistency) at 70°C for up to 24 hr (SECT 2.4.2.). Filtered pulp liquors were monitored over time for dissolved carbohydrate by the orcinol method (SECT 2.3.4). Time courses for xylanase and mannanase treatment of kraft pulp are given in Fig 5.2. Controls, without enzyme were run in parallel to determine the rate at which the carbohydrate leached from the pulp.

The rate of carbohydrate leaching from the control pulp was approximately constant over a 24 hr period with only 0.2% of the OD pulp solubilised. Lignin was also detected in the pulp liquor (Ab_{280} method as described by Favis *et al.* 1981) with 1.7 mg of lignin leached per gram of OD pulp after 24 hr at 70°C. This phenomenon of leachable lignin from kraft pulps has been noted in other studies and is temperature dependent, but the rate of lignin release is too low to be of practical significance (Favis and Goring 1983; Lagstrom-Nasi *et al.* 1987).

In contrast, rate of carbohydrate solubilisation in the mannanase and xylanase treated pulps were much faster (Fig 5.2). The two hemicellulases differed in their initial rates of carbohydrate solubilisation, with that of the mannanase being almost twice that of the xylanase (Fig 5.2). This initial rate rapidly reduced after 5 hr with the mannanase, but persisted for up to 20 hr with the xylanase. Enzyme inactivation was not responsible for the decreasing rate of carbohydrate solubilisation, as the treatment liquors contained over 80% of the initial hemicellulolytic activities after the 24 hr incubation period. Therefore the availability of hydrolysable substrate must decrease over time.

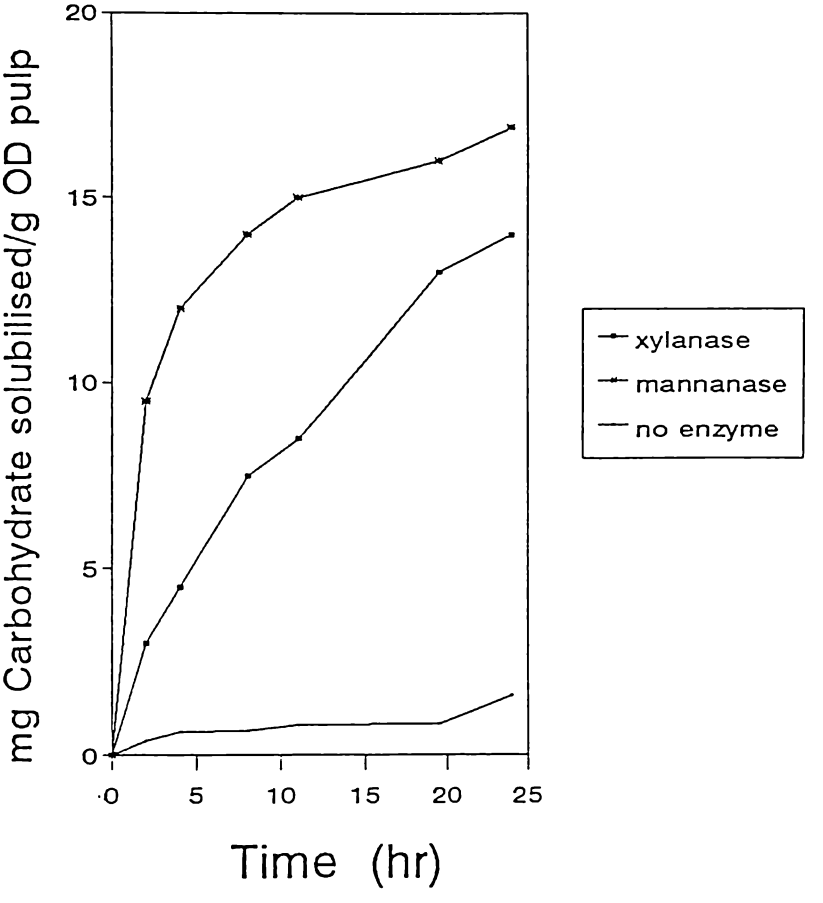

Fig 5.2 Time course of total carbohydrate (mg CHO/g OD pulp) leached (no enzyme) and solubilised by xylanase (pNZ1435) or mannanase (pNZ1019)
(2% consistency kraft pulp in water at 70°C, 100U/g OD pulp)

The carbohydrate solubilised by the respective hemicellulases accounted for only 30% of the total glucomannan and 15% of the total xylan in the original pulp. Hemicellulose distribution in radiata kraft pulp fibres is such that approximately 30% of the total glucomannan and 66% of the total xylan can be found in the P and outer S_1 layers of the cell wall (Kibblewhite and Brookes 1976). Therefore, assuming that both polysaccharides are equally accessible to their respective hemicellulases, it would be expected that xylan would be hydrolysed faster than the glucomannan. This would certainly be so if surface xylans were attacked. As this was not the case, up to 50% of the xylan in the outer layers must be in a form that is not readily hydrolysed by the xylanase.

Again, for the purpose of maximising enzymatic carbohydrate solubilisation, incubation periods of 20-24 hr at a temperature of 70°C were employed throughout the remainder of this study unless otherwise qualified.

5.4.3 Effects of the hemicellulases on different pulps

The seven pulps described in Table 5.1 were hydrolysed with either the cloned xylanase, the cloned mannanase or a combination of the two enzymes (100 U of each enzyme/g OD pulp), under the conditions previously specified. After incubation, the monosaccharide composition of the filtered pulp liquors was determined by HPLC (Biorad system) using TFA hydrolysed liquors (SECT 2.3.3 and SECT 2.3.2.4).

The sugar compositions of the carbohydrates solubilised by the enzyme treatments are presented in Fig 5.3. In general, relatively little carbohydrate leached from the pulps in the control treatments. Xylan was the main polysaccharide removed from the two kraft pulps. Xylan and glucomannan readily leached from the NSAQ pulp, while only glucomannan leached from the mechanical pulps. Alkali treatment had a minor effect on the mechanical pulps with xylan being the main polysaccharide leached from the CTMP and TMP pulps. Little or no cellulose leached from the pulps; most or all of the glucose from the CTMP pulp appeared to have arisen from solubilised glucomannan. Polysaccharides, especially hemicelluloses, as well as lignins are known to leach from pulps after prolonged

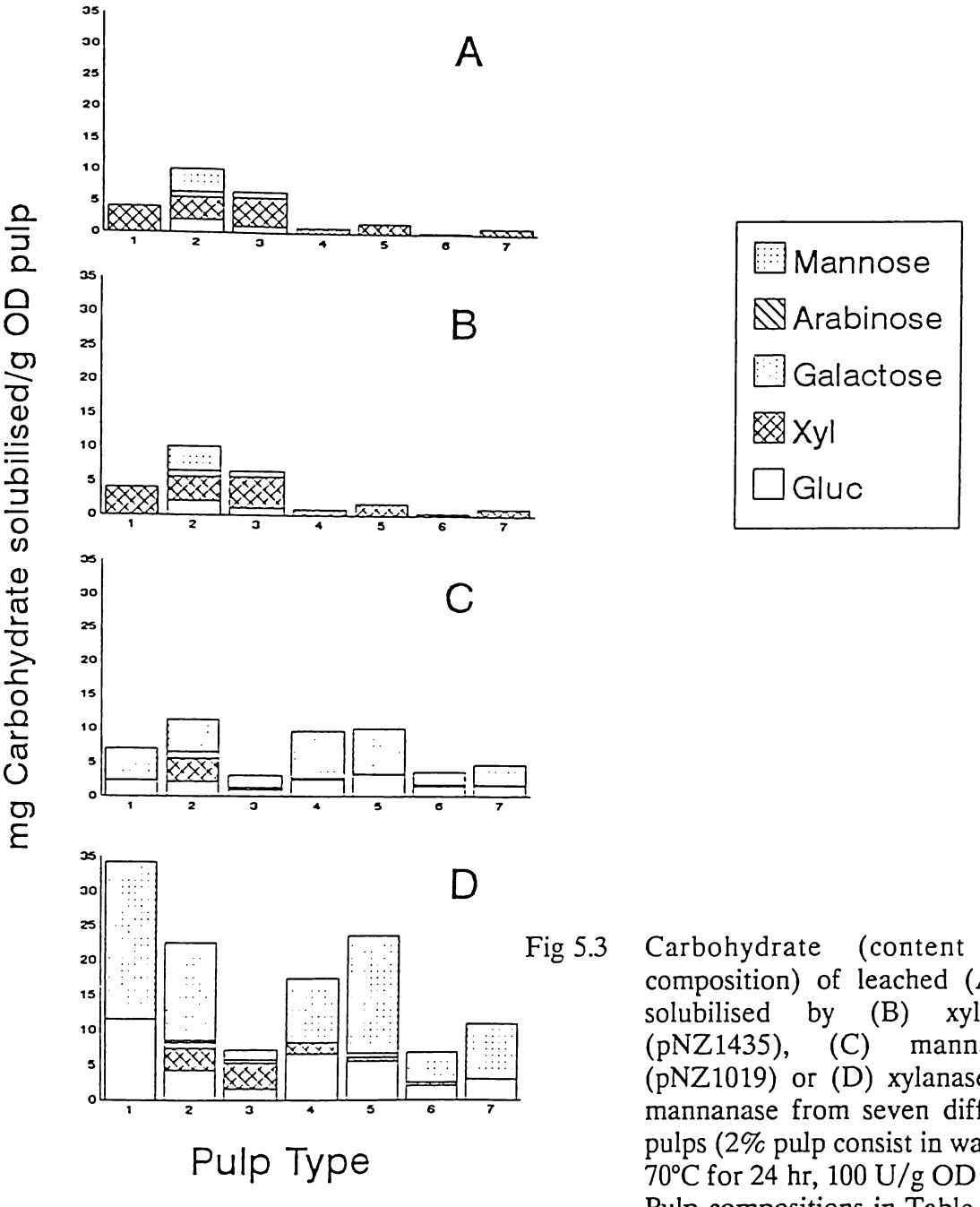

Fig 5.3 Carbohydrate (content and composition) of leached (A) or solubilised by (B) xylanase (pNZ1435), (C) mannanase (pNZ1019) or (D) xylanase and mannanase from seven different pulps (2% pulp consist in water at 70°C for 24 hr, 100 U/g OD pulp) Pulp compositions in Table 5.1.
1 Radiata kraft; 2 Radiata NSAQ; 3 Hardwood Kraft; 4 Radiata CTMP; 5 Radiata CTMP (alkali); 6 Radiata TMP; 7 Radiata TMP (alkali)

incubation at elevated temperatures (60-80°C) (Lagstrom-Nasi et al. 1987). Overall, the low levels of leached carbohydrate are typical of well washed chemical and mechanical pulps.

Xylanase and mannanase treatments affected the amount and the type of carbohydrate released from the pulps (Fig 5.3 B,C). With most of the pulps, carbohydrates in the liquors were composed of the sugars associated with the hemicellulose expected to be hydrolysed by the corresponding enzyme. However, the total carbohydrate solubilised by either enzyme was never more than 2% of the total pulp weight. Glucose release was usually associated with mannose in a ratio indicative of glucomannan hydrolysis. Therefore, neither the xylanase nor the mannanase appear to exhibit any cellulolytic activity.

The substrate specificity of the cloned hemicellulases when treating pulps is best exemplified by the radiata kraft pulp, where the xylanase treatment solubilised only xylan and the mannanase treatment yielded only glucomannan. Yet, despite their specificity, the cloned hemicellulases were not very effective at removing their substrates; the xylanase solubilised only 4% of the available xylan while the mannanase solubilised only 10% of the glucomannan (based on mannose). As a comparison, a purified *Trichoderma harzianum* xylanase preparation was able to solubilise from 12% to 20% of the xylan in a radiata kraft pulp while a purified *A. niger* mannanase released 10% of the mannan in the pulp, primarily as galactoglucomannan (Clark *et al.* 1989, 1991). Since the radiata kraft pulps used in both studies are likely to be similar in composition, pore size distribution and other physical properties, then it appears that the cloned mannanase and the *A. niger* mannanase are behaving in a similar manner with regard to interfibril carbohydrate attack. However, the cloned xylanase solubilised less than a third of the xylan solubilised by a purified *T. harzianum* xylanase preparation. Two explanations for this are possible; i) the fungal xylanase may have access to more of the substrate or ii) the xylan may be in a form that is resistant to hydrolysis by the cloned xylanase but not the fungal enzyme. As the TP8 xylanase has been shown to effectively hydrolyse soluble radiata xylan, the latter is not considered likely. However, the *T. harzianum* enzyme preparation contains three distinct xylanases, all of which have smaller molecular weights (20-29 kDa) (Wong and Saddler 1991)

than the cloned xylanase (43 kDa)(SECT 4.1). The smaller enzymes may hydrolyse xylans in areas of the fibre wall that are inaccessible to the larger enzyme. If this type of size restriction is occurring, it may be a convenient way of enzymatically fractionating xylans associated with different pore sizes.

The xylanase hydrolysed approximately 3% of the available xylan from the hardwood (tawa) kraft pulp (Fig 5.3B) and, surprisingly, some glucomannan was released by the mannanase. No mannan was detected in the original pulp (Table 5.1). The mannose that was solubilised by the mannanase accounted for less than 0.15% of the total pulp. Such a level of mannose is very close to the detection limits of the HPLC system and may not have been detected in the analysis of the original pulp.

The fact that the mannanase was able to solubilise what little glucomannan was present in the hardwood pulp suggests that this polysaccharide was readily accessible to the enzyme. However, the xylanase solubilised only a fraction of the xylan in the pulp. Repeating the enzymatic hydrolysis and determining the sugar composition of the pulp liquor by the more sensitive GC method (SECT 2.3.3) after TFA hydrolysis showed a slight increase in the xylan solubilised to 6% solubilised xylan. A similar experiment with an aspen kraft pulp also showed a low level of xylan hydrolysis comparable to that of the tawa (Table 5.2). For comparison, treatment of the same pulp with a xylanase from *T. harzianum* solubilised ~5% of the xylan from the aspen pulp (T. Clark, unpublished results) under similar incubation conditions (with the exception that the incubation temperature was 50°C). Therefore, it would appear that the thermostable xylanase is as effective as the fungal xylanase in solubilising xylan from the two hardwood pulps examined.

Assuming that the xylans in these pulps are susceptible to enzymatic hydrolysis, two possibilities may be occurring. Either most of the xylans were physically inaccessible to the enzymes or partial hydrolysis of the xylan may be occurring but the polysaccharide was retained within the fibre. There is evidence in the literature to support both these hypotheses. Puls *et al.* (1990) have shown that a fungal xylanase removes only a fraction of the xylan in a beechwood ASAM pulp while the remainder of the xylan is physically shielded from further attack by cellulose. In molecular weight distribution studies, hemicelluloses from hardwood and

Table 5.2 The solubilisation of xylans from two hardwood pulps by the cloned TP8 xylanase.

Kraft Pulps (% xylose content)	Xylose Solubilised (mg/g OD pulp)	% Total Xylose Solubilised
Controls		
Tawa (16.9%)	0.00	0.00%
Aspen (21.4%)	0.00	0.00%
TP8 Xylanase		
Tawa	9.91	5.9%
Aspen	9.65	4.5%

softwood kraft pulps that were enzyme treated had lower average DP than hemicelluloses from untreated controls (Miller *et al.* 1991). Yet these partially hydrolysed xylans had not been solubilised during the incubation period (24 hr) suggesting either physical retention or chemical linkages to other pulp components. It should be noted that differences between pulps and enzymes make these types of comparisons difficult.

A noteworthy exception to the specificity of the two cloned hemicellulases occurred with the radiata NSAQ pulp for which similar amounts of both xylan and glucomannan were solubilised regardless of the cloned TP8 hemicellulase employed (Fig 5.3B,C). As xylan and glucomannan are uniformly distributed throughout the cell wall of radiata sulphite pulps (Kibblewhite and Brookes 1976), it would be expected that similar proportions of each hemicellulose would be accessible to the respective hemicellulases. However, the concurrent solubilisation of both the xylan and the glucomannan suggests that a fraction of the two polysaccharides is associated in such a manner that the hydrolysis of one results in the solubilisation of the other. Electron microscopy has been successfully used to show that the pore distribution of sulphite pulps enables proteins similar in size to the cloned hemicellulases to diffuse throughout the cell wall (Screbotnik and Messner 1990).

Therefore, the solubilisation of a constant proportion of xylan and glucomannan may indicate that their association may be consistent throughout the fibre.

The two hemicellulases again demonstrated selective, but limited, hydrolysis of their respective substrates with the untreated mechanical pulps (Fig 5.3B,C). The CTMP pulp was more susceptible to the mannanase than the TMP pulp. Alkali pre-treatment increased the yield of glucomannan solubilised, especially from the CTMP pulp. The xylanase had a minimal effect on both mechanical pulps, whether alkali treated or not. Acetylation of the substrate is inhibitory to many xylanases (Puls *et al.* 1990, Biely 1985) and mannanases (McCleary and Matheson 1983). Alkali deacetylation should alleviate any inhibition by these substituents. As softwood xylans are unacetylated the effect of the alkali treatment on xylan susceptibility to enzymatic attack was negligible. Softwood glucomannans are acetylated but again the alkali treatment had little or no effect on the susceptibility of the glucomannan to enzymatic hydrolysis.

In general, the combined xylanase and mannanase treatments solubilised more carbohydrate than expected by summing yields for the enzymes when used separately, indicating a synergy between the two (Fig 5.3D). However, in most cases only glucomannan hydrolysis increased and this may indicate the shielding of this polysaccharide by xylan. Radiata kraft pulps that have had all or part of the xylan removed by either caustic extraction or enzyme hydrolysis have been shown to be more susceptible to mannanase hydrolysis (Clark *et al.* 1991; Sinner *et al.* 1979). The hardwood kraft pulp which was extremely low in glucomannan was not significantly affected by the combined hemicellulase treatment as compared to the xylanase alone. The combined hemicellulase treatment of the radiata NSAQ pulp solubilised more than 22% of the mannan yet only 5% of the xylan.

The results obtained clearly show that the extent of intra-fibril hemicellulose hydrolysis by these enzymes is influenced by the pulping process. Enzymatic susceptibility is affected by such parameters as hemicellulose distribution (Clark *et al.* 1991), fibre swelling (Screbotnik and Messner 1990), substrate shielding (Puls *et al.* 1990) and possibly chemical modification of the substrate. Fibre swelling may explain some differences in hemicellulase hydrolysis. For example, sulphite pulps tend to have higher fibre saturation points (ie: greater swelling) than kraft pulps at

the same yield, presumably facilitating greater enzyme infiltration into the cell wall. This may account for the greater enzymatic carbohydrate solubilisation observed with the NSAQ pulp relative to the kraft pulp. TMP pulps have a relatively low degree of swelling, therefore enzyme attack would not be as great as with chemical pulps. This was evident when comparing the amount of carbohydrate enzymatically solubilised from the chemical pulps with that solubilised from the TMP pulp (Fig 5.3).

However, swelling does not account for all the effects. Hemicellulose distribution must be considered when comparing the relative solubilisation of xylans and glucomannans from the CTMP and TMP pulps. The xylans must be located in regions that are not accessible to the xylanase while a greater proportion of the glucomannan is accessible to the mannanase. Shielding of the xylan by cellulose or lignin may also account for its relative recalcitrance. No one factor stands out as the sole reason for the differences noted in hemicellulose susceptibility of various pulps. All the factors probably have a cumulative effect on overall hemicellulase susceptibility. Altering fibre properties by different pulping procedures may prove to be a useful approach to evaluating their effect on the enzyme susceptibility of the hemicelluloses in the fibre wall.

Water saturated hemicelluloses and water saturated lignins undergo glass transitions (T_g) at about 55°C (Goring 1985; Goring 1963). As the temperature of these polymers rise above the T_g, their viscoelasticity changes from a rigid and glassy form to a more pliable or "rubbery" type. The viscoelastic transition of lignin in wood is thought to occur over a higher temperature range (Irvine 1985) and to be a factor in the rapid rise in lignin leaching at temperatures of 70°C and above (Favis and Goring 1983; Goring 1985). This has been interpreted as an increase in the fibre porosity (Goring 1985). This increase in porosity may enable further penetration of enzymes into the wall thus allowing greater hemicellulose hydrolysis. Of course the enzymes must be stable and active at these temperatures. This type of thermal "softening" of lignins is exploited in thermomechanical pulping (Smook 1987; Fengel and Wegener 1985). Hemicelluloses within the cell wall may also experience a similar "softening" and it has been suggested that this facilitates lignin diffusion from the fibre (Favis and Goring 1983; Goring 1985).

The cloned thermostable hemicellulases used in this study appear to be no more efficient at solubilising hemicelluloses at 70°C than hemicellulases at lower temperatures used in other studies (Clark *et al.* 1991). Assuming that the thermostable and mesophilic hemicellulases act in a similar manner, then increasing temperature above the T_g has not increased the enzymatic solubilisation of hemicelluloses. This implies that the transition of hemicelluloses does not necessarily increase their susceptibility to enzymatic hydrolysis. Other factors may overshadow this effect in enzymatic hydrolysis of pulps.

5.5 EXTRACTED HEMICELLULOSE FROM TREATED PULPS

5.5.1 Characterisation of hemicellulase treated pulps and liquors

Radiata kraft pulps were incubated at 70°C for 24 hr in water (2% consistency) without enzyme (Treatment A), with 100 U of the cloned xylanase per gram of OD pulp (Treatment B), with 100 U of the cloned mannanase per gram of OD pulp (Treatment C) or with a combination of the two enzymes at the dosages previously specified (Treatment D). After treatment, various hemicelluloses were extracted according to the modified Hamilton procedure (SECT 2.4.2.4). The treated pulps, pulp liquors, extracted hemicellulose fractions and residual pulps (holocelluloses), as specified in Fig 2.4, were analysed for total carbohydrate content and composition by the orcinol assay (SECT 2.3.4) and the individual monomeric sugar composition analysed by the GC of their alditol acetates (SECT 2.3.3). Pulps were also analysed for lignin content (SECT 2.4.2.2).

The compositions of the treated pulps and liquors are presented in Fig 5.4 (Bottom and Top, respectively). The carbohydrate content and composition of the treatment liquors are comparable to those of the previous experiments (Fig 5.3). As would be expected, the composition of the control pulps (no enzyme) is similar to that of the untreated radiata kraft pulp (Fig 5.1). Minor, but significant, differences between the enzyme treated pulps and the control pulp were observed. These included a loss of arabinose and galactose and slight increases in the Klason lignin content, the latter being possibly due to non specific binding of protein to the lignin (Hortling *et al.* 1990). Hemicellulase treatment alone did not decrease the lignin content of kraft radiata pulps (Fig 5.4B). This is in concurrence with other studies

on hemicellulase treatment of radiata kraft pulps which show a need for a post-enzymatic extraction or chemical treatment to remove residual lignin (Clark *et al.* 1991; Kantelinen *et al.* 1988). In some cases, xylanase treatment alone has given a modest drop in κ but certainly not enough to fully account for the chlorine savings in enzymatic bleach enhancement (Chauvet *et al.* 1987; Paice *et al.* 1988).

The xylan content of the xylanase treated pulp was 9% lower than for the control pulp. The difference in total xylose content (98 mg) was in close agreement with the xylose in the pulp liquor (100 mg). The mannose content of the mannanase treated pulp was 78% of that of the control pulp with the balance accounted for in the pulp treatment liquor. The pulp treated with both enzymes showed a drop in both the xylose and mannose content, relative to the control pulp, of 12% and 19%, respectively. Again this demonstrates that the glucomannan was more susceptible to enzyme hydrolysis than the xylan.

5.5.2 Hemicellulose extraction by the Hamilton procedure

Both cloned hemicellulases have a significant and specific, albeit small, effect on radiata kraft pulp composition. However, the enzymes may affect intra-fibril hemicelluloses that are not solubilised. To determine if the enzymes do indeed affect residual hemicelluloses the xylan, glucomannan and galactomannan of the four pulps (A,B,C and D) were extracted by the Hamilton procedure (Beelik *et al.* 1967). Carbohydrate mass balances and the carbohydrate compositions of the recovered hemicellulose fractions are presented in Table 5.3 and Table 5.4 respectively. The molecular weight distributions of the extracted hemicelluloses from each pulp treatment are given in Appendix 4. The median molecular weights, molecular numbers, degrees of polymerisation and polydispersities of the extracted fractions are summarised in Table 5.5. If the cloned enzymes did affect the residual hemicelluloses then this should be manifest as lower degrees of polymerisation or changes in the carbohydrate composition of the extracted polysaccharides.

The carbohydrate content of the extracts from the four pulps are presented in Table 5.4. Ethanol supernatants were tested for unprecipitated carbohydrate by the Molisch reaction in order to detect unprecipitated polysaccharides and hence

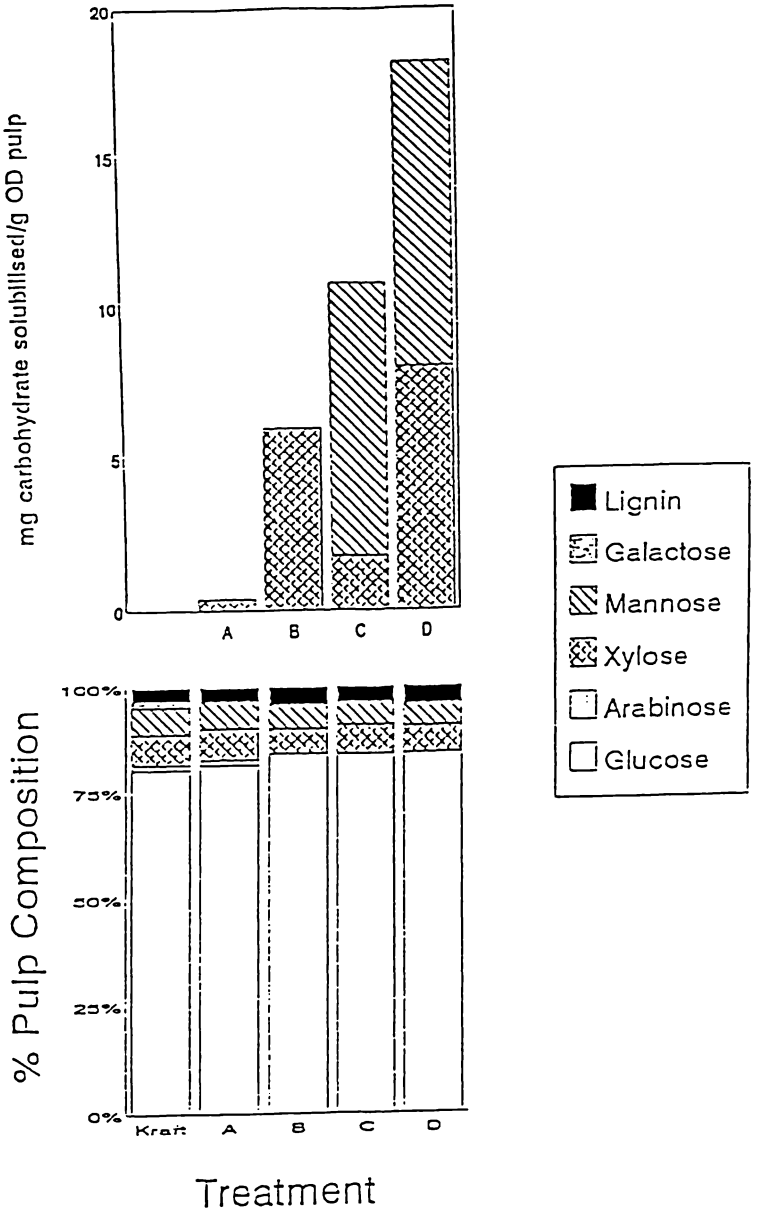

Fig 5.4 Composition of radiata kraft pulps that have been leached (A), treated with xylanase (pNZ1435), treated with mannanase (pNZ1019) or (D) treated with xylanase and mannanase. Kraft pulp composition was obtained from Table 5.1 and included for comparison.

Top - Carbohydrate content and composition of pulp liquors
Bottom - normalised composition of treated pulps and untreated kraft pulp

Table 5.3 Carbohydrate content and yield of the hemicelluloses extracted from the four treated pulps during the Hamilton extraction procedure

	Dry weight mg	Carbohydrate %	Carbohydrate mg
A Treatment#	2460.0*	97%	2376.4
xylan	101.9	94%	95.6
glucomannan (ppt)	45.9	100%	45.9
glucomannan (sol)	78.2	71%	55.5
galactomannan	1.8	100%	1.8
extracted pulp	2046.7	98%	2003.7
	2274.5		2202.5
% Recovered[x]	93		93
B Treatment#	2500.0*	96%	2397.5
xylan	112.7	100%	112.7
glucomannan (ppt)	31.3	100%	31.3
glucomannan (sol)	57.0	36%	20.5
galactomannan	1.3	100%	1.3
extracted pulp	1961.1	100%	1961.1
	2163.4		2126.9
% Recovered[x]	87		89
C Treatment#	2460.0*	96%	2369.0
xylan	87.5	94%	82.6
glucomannan (ppt)	53.1	100%	53.1
glucomannan (sol)	92.5	26%	24.1
galactomannan	1.7	100%	1.7
extracted pulp	1958.5	100%	1958.5
	2193.3		2120.0
% Recovered[x]	89		90
D Treatment#	2530.0*	96%	2426.3
xylan	100.6	100%	100.6
glucomannan (ppt)	51.7	100%	51.6
glucomannan (sol)	62.3	44%	27.3
galactomannan	1.4	100%	1.8
extracted pulp	1917.6	100%	1917.6
	2133.0		2098.9
% Recovered[x]	84		87

* Initial OD weight of pulp to be extracted

\# A treatment - no enzyme control; B treatment - xylanase treated

\# C treatment - mannanase treated; D treatment - xylanase+mannanase

x % Recovery = (Sum of carbohydrates/carbohydrate content of initial pulp)

Table 5.4 Carbohydrate content and yield of the hemicelluloses extracted from the four treated pulps

	Carbohydrate mg	% Composition Ara.	Xyl.	Man.	Gal.	Glu.
A Treatment#						
xylan	95.6	9.2	90.8	0.0	0.0	0.0
glucomannan (ppt)	45.9	2.3	22.4	47.9	4.4	23.0
glucomannan (sol)	55.5	0.0	20.6	62.7	0.0	16.8
extracted pulp	2003.7	0.0	2.9	3.7	0.0	93.2
	2200.7					
% Extracted Carbohydrate Recovered[x]			60	>100		
Total Recovered %	93	31	92	79	6	92
B Treatment#						
xylan	112.7	8.4	91.6	0.0	0.0	0.0
glucomannan (ppt)	31.3	0.0	0.0	100.0	0.0	0.0
glucomannan (sol)	20.5	0.0	46.3	38.9	0.0	14.8
extracted pulp	1961.1	0.0	0.0	3.1	0.0	95.4
	2125.6					
% Extracted Carbohydrate Recovered[x]			60	64		
Total Recovered %	89	29	95	60	0.0	90
C Treatment#						
xylan	82.6	8.3	91.6	0.0	0.0	0.0
glucomannan (ppt)	53.1	0.0	11.5	69.0	0.0	19.6
glucomannan (sol)	24.1	0.0	53.7	46.3	0.0	0.0
extracted pulp	1958.5	0.0	2.6	2.7	0.0	94.7
	2118.3					
% Extracted Carbohydrate Recovered[x]			51	71		
Total Recovered %	89	21	84	88	0.0	93
D Treatment#						
xylan	100.6	8.4	91.6	0.0	0.0	0.0
glucomannan (ppt)	51.6	0.0	0.0	79.0	0.0	21.0
glucomannan (sol)	27.3	0.0	54.6	28.4	0.0	17.1
extracted pulp	1917.6	0.0	1.3	2.2	0.0	96.5
	2097.1					
% Extracted Carbohydrate Recovered[x]			49	59		
Total Recovered %	86	72	100	83	0.0	90

as in Table 5.3; x % Extracted Carbohydrate Recovered (xyl or mann) = 100% X (total mg xyl extracted)/(mg xyl in initial pulp - mg xyl in extracted pulp) where the composition of the initial pulp is obtained from Table 5.1; Total Recovered = 100% X (total CHO recovered)/(CHO initial pulp)

minimise polysaccharide losses during the extraction. In this way 85-90% of the carbohydrates were accounted for in the carbohydrate balance (Table 5.3).

The xylans were readily precipitated by ethanol from the 4.4% $Ba(OH)_2.8H_2O$ extract and recovered as a single fraction. However, the glucomannan components of the four pulps were not as simply recovered. Addition of ethanol to the extraction liquor precipitated a part of the carbohydrate but a significant amount was left in solution. This second, soluble glucomannan fraction was concentrated by rotary evaporation. These fractions are referred to as the precipitated glucomannan (GM-ppt) and the soluble glucomannan (GM-sol), respectively. Their carbohydrate contents reflect the fractionation method used; the precipitated glucomannans were pure carbohydrate while the soluble glucomannans were from 25 to 70% carbohydrate with the balance being inorganic material (Table 5.3). The cellulose rich pulp residues remaining after extraction, called the extracted pulps, were found to have Klason lignin contents between 2.8 and 3.1%, regardless of the type of hemicellulase treatment.

The relative sugar composition of each carbohydrate fraction is presented in Table 5.4 which shows that the methodology employed accurately accounted for the xylose, mannose and glucose. Mass balances on the carbohydrates demonstrate that more than 90% of the xylose and glucose were recovered from the original pulp (~ 5 OD pulp) in the various fractions. The recoveries of the arabinose and the galactose were low, usually less than 40%. Although some of the galactose would be present in the galactoglucomannan fraction, which was not subjected to sugar analysis, the amount of carbohydrate in these fractions was never greater than 2 mg. Nevertheless, the xylose and mannose contents of the various fractions do accurately reflect the xylan and glucomannan content of the pulp. The recovery of the xylan extracted from each pulp treatment is given as a percentage in Table 5.4. This was calculated as the ratio of the total xylose recovered (in all fractions) to the amount of xylose extracted. The extracted xylose was defined as the difference between the xylose content of the radiata kraft pulp before extraction and after extraction. Similar calculations were performed for the mannose (Table 5.4). Between 50 and 60% of the extracted xylose was recovered in the polysaccharide fractions, while from 60 to 100% of the extracted mannose was recovered.

The extraction rate of hemicelluloses by strong alkali is directly related to the location of the polysaccharides within the cell wall (Scott 1984). Hemicelluloses that are closer to the cell wall surface are extracted faster than those further within the S_2 layers. Therefore, the extracted xylan and glucomannan fractions described below should contain the hemicelluloses found in the outer layers of the cell wall. Hemicelluloses that are not removed by alkaline extraction are most probably embedded within the fibre (Scott 1984).

5.5.3 Xylan Fractions

The extracted xylan fractions from the four pulps (A, B, C and D) contained only xylose and arabinose in ratios indicative of native xylan (Table 5.4). The xylose in these fractions accounted for between 40 and 50% of the total xylan in the treated pulps with the remainder distributed throughout the other fractions or retained in the extracted pulp (Table 5.4). These fractions should contain the xylans from the outer wall layers.

The average molecular weight (molecular weight at 50% total weight) of the extracted xylan fractions was used to calculate an average DP (average molecular weight/molecular weight of an anhydrous xylose residue) (Table 5.5). The average DP of the xylans as determined by this procedure were between 210 and 360. These are substantially higher than the average DP of 78 estimated by Harwood (1972) or 93 estimated by Haselmore (PhD Thesis, University of Otago 1973) for extracted radiata pine xylans. Both these estimates were based on the ratio of reducing end to total xylose residues and are deemed an accurate representation of the average DP of radiata pine xylan. Obviously the procedure used in this study overestimates the average DP of xylans. This may be due to differences between the behaviour of the dextran standards used to calibrate the GPC columns (Fig 2.5). However, differences in the average DP of the various xylan fractions as estimated by the GPC techniques used in this study are believed to reflect the relative changes in the average DP due to enzymatic hydrolysis.

The average DP of the fractions are compared in Fig 5.5. Extracted xylans from pulps that were exposed to the cloned xylanase (B and D) had lower average DP values than xylan from pulps exposed to the cloned mannanase or not exposed to

Table 5.5 The median molecular weight (M_w), molecular number (M_n), average deggree of polymerisation (DP) and polydispersities of extracted hemicelluloses* obtained by the Hamilton extraction procedure. (as determined in SECT 2.4.2.6)

Enzyme treatment	M_w	M_n	DP	Polydispersity
Xylan Fractions				
A (control pulp)	48,000	32,000	360	1.5
B (xylanase pulp)	28,000	18,000	210	1.6
C (mannanase pulp)	40,000	27,000	300	1.5
D (Xyl+Mann pulp)	36,000	24,000	270	1.5
Glucomannan (precipitated)				
A (control pulp)	24,000	15,000	150	1.6
B (xylanase pulp)	21,000	13,000	130	1.6
C (mannanase pulp)	24,000	15,000	150	1.6
D (Xyl+Mann pulp)	17,000	10,000	100	1.7
Glucomannan (soluble)				
A (control pulp)	14,000	8,500	86	1.6
B (xylanase pulp)	9,500	5,600	60	1.7
C (mannanase pulp)	13,500	8,000	83	1.7
D (Xyl+Mann pulp)	8,000	4,500	50	1.8

* GPC Traces in Appendix 5

any enzyme. The xylan fraction of the xylanase treated pulp had an average DP of 210 while that from the control pulp had a value of 360. This suggests that the extracted xylan from the xylanase treated pulp had undergone limited enzyme hydrolysis that lowered the average xylan DP by 60%. Similar results were obtained for the combined enzyme treated pulp (D pulp) except that hydrolysis was even less extensive than with the xylanase-only treatment (B pulp) as seen by the higher average DP (270) (Fig 5.5). Similar polydispersities demonstrated that the extent of xylan depolymerisation was uniform throughout the molecular weight distribution of each fraction (Table 5.7).

Unhydrolysed regions in the polysaccharide may be shielded from enzyme attack by other polymers or by side groups. To ascertain whether steric hindrance was indeed a major factor in limiting the enzymatic hydrolysis of the extracted pulp, the extracted xylans were treated with the thermostable xylanase under the same conditions as the pulps. All the extracted xylans were readily hydrolysed by the xylanase with up to 50% of the glycosidic bonds cleaved (Fig 5.6). This clearly established that steric hindrance is not a major factor in preventing enzymatic hydrolysis or solubilisation of residual xylans. Physical inaccessibility appears to be the most probable cause.

It has been estimated than between 30% to 50% of the pore volume within a radiata kraft fibre is accessible to enzyme-sized molecules (Clark *et al.* 1989; Scallan 1978). The xylanase solubilised only a small fraction of the total xylan available. Of the xylan that was not solubilised, approximately half appears to have been partially hydrolysed but not solubilised by the enzyme. Partial shielding of the xylan by lignin or cellulose would explain the limited hydrolysis and retention of xylan within the fibre. The enzyme would hydrolyse only the accessible fraction of the xylan (~4%) and leave the shorter, protected fraction intact.

5.5.4 Glucomannan fractions

The precipitated glucomannan fraction from the control and the mannanase treated pulps contained not only mannose and glucose but also some xylose (Table 5.4) while this fraction from the xylanase and combined enzyme treated pulps contained only mannose and glucose. The mannose in the precipitated glucomannan fractions represents between 20% and 30% of the total mannose in the pulp (Table 5.4).

The soluble glucomannan fraction contained as much xylose as mannose (Table 5.4). The difficulty in separating these hemicelluloses by alkali extraction is characteristic of the separation procedure (Beelik *et al.* 1967; Scott 1984). The mannose content in the soluble glucomannan fractions accounted for less than 10% of the total mannose from the enzyme treated pulps (B,C and D) but 27% of the mannose from the untreated pulp (A).

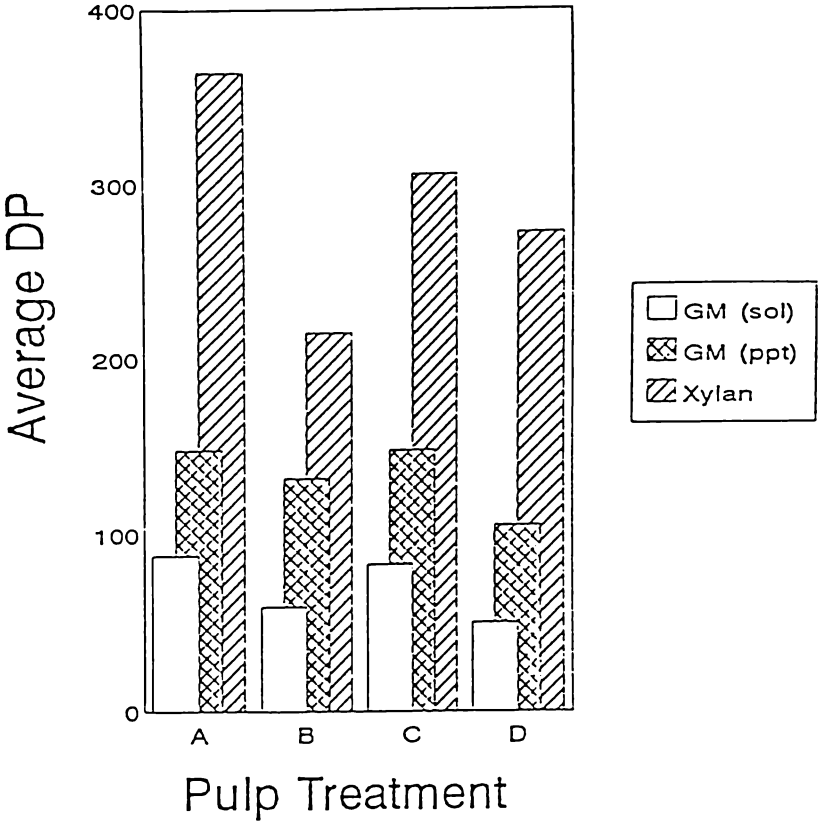

Fig 5.5 Average degree of polymerisation (DP) of the extracted xylan and extracted glucomannan (precipitated and soluble) fractions from four treated kraft pulps
(A) leached, (B) xylanase (pNZ1435), (C) mannanase (pNZ1019) and (D) both xylanase and mannanase

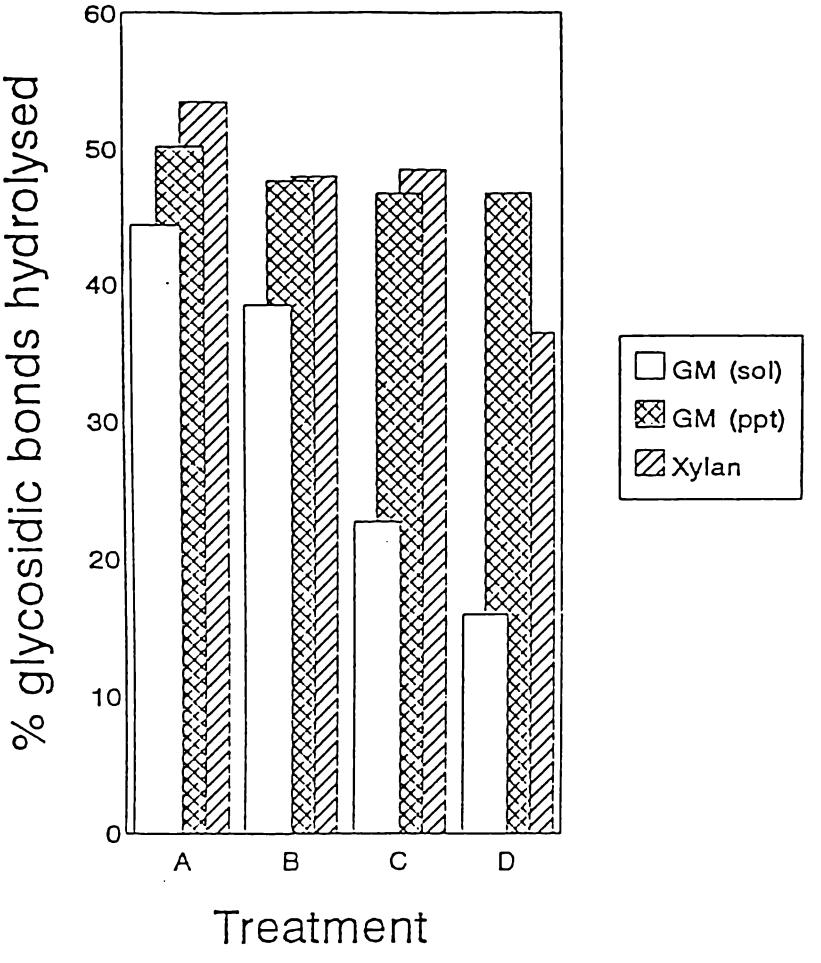

Fig 5.6 Extent of enzymatic hydrolysis of the extracted xylan and glucomannan (precipitated and soluble) fractions from four treated kraft pulps (A,B,C and D as in Fig 5.5). Xylan fractions hydrolysed by the xylanase (pNZ1435); glucomannan fractions hydrolysed by the mannanase (pNZ1019) (1 U/mg of polysaccharide, 70°C, 18 hr)

The glucomannan retained in the extracted pulps, like the xylan, must be well embedded within microfibrils to resist extraction by strong alkali. It seems likely, therefore, that these hemicelluloses would be totally inaccessible to enzymes. Yet pulps treated with the mannanase (C and D) retained between 30% and 35% of the original mannan while the untreated pulps (A and B) retained a higher proportion of the mannan (55%-60%).

The molecular weight distributions of the precipitated glucomannans and the soluble glucomannans are summarised in Table 5.5. The average DP of the precipitated glucomannans ranged from 100 to 150 . Again these appear as overestimates of the DP, when compared with the average DP of 45 estimated for extracted radiata pine glucomannan by Harwood (1973). Again, relative differences in average DPs are however considered to reflect any polysaccharide depolymerisation.

As would be expected the average DP of the precipitated glucomannan was almost twice as high as that of the soluble glucomannan. There was little difference in the average DP of the precipitated glucomannans extracted from the control pulp (A), the xylanase treated pulp (B) or the mannanase treated pulp (C) (Fig 5.5). This must represent a fraction of the glucomannan that is either inaccessible or resistant to enzymatic hydrolysis. At least part of this recalcitrance may be due to some shielding by xylan as the average DP of the precipitated glucomannan from the pulp treated with both hemicellulases (D pulp) was 65% that of the control. The precipitated glucomannan fractions from each pulp were incubated with the mannanase to confirm that they were not resistant to hydrolysis. The extent of hydrolysis is presented in Fig 5.6. Again the polysaccharides were thoroughly hydrolysed which suggests enzyme inaccessibility rather than steric hindrance as the major reason for the lack of enzyme attack on the majority of the glucomannan (70-80%).

The average DP of the soluble glucomannan fractions varied from between 50 to 86 (Fig 5.5). The highest was found with the control (A) and the mannanase treated (C) pulps. Hydrolysis by the mannanase was not as complete with the soluble glucomannans as with the precipitated fractions (Fig 5.6). The soluble glucomannan fractions contained a large proportion of xylan (Table 5.4) which

would be resistant to hydrolysis by the mannanase. After hydrolysis with the mannanase, the soluble glucomannan fraction was treated with the xylanase which hydrolysed the xylan that was present in these fractions.

5.5.6 Hemicellulase treatment of extracted pulp

A significant portion of the hemicelluloses remained in the extracted pulp as exemplified by the control pulp (A) where 35% of the xylose and over 55% of the mannose were not extracted (Table 5.4). Also, an extracted kraft pulp had a κ of 16.8 (2.5% lignin). This is approximately 75% of the residual lignin that was in the original radiata kraft pulp (Table 5.1). It has been proposed that this fraction of the hemicellulose is entrapped within cellulose microfibrils (Scott 1984). There may also be direct bonding between the residual lignin and that fraction of the xylan and glucomannan that was not extracted.

If physical entrapment is retaining the unextracted hemicelluloses then they should be inaccessible to enzyme hydrolysis. If the hemicelluloses are retained by chemical linkages to lignin then some of the hemicelluloses may be hydolysed enzymatically. As the extracted pulps are extremely porous, the enzymes should have access to a larger proportion of the fibre than in the unextracted kraft pulp.

The washed, extracted pulp was treated with the xylanase (50 U/g of OD pulp) and the mannanase (250 U/ g OD pulp) concurrently, as described in previous experiments (2% consistency, 24 hr at 70°C). The solubilised carbohydrates, identified and quantified by HPLC (Biorad system) following TFA hydrolysis, are presented in Fig 5.7.

In the absence of the enzyme, only a trace amount of glucose leached from the extracted pulp; hemicelluloses were not released. Also, trace levels of lignin (less than 2% of the pulp lignin) were detected in the supernatant (Ab_{280}) which shows that the lignin, too, was retained. The cloned hemicellulases were able to solubilise 6.8 mg of carbohydrate from one gram of OD pulp. This was composed primarily of mannose and glucose in a molar ratio of 3.6:1, indicative of glucomannan (Fig 5.7). This represented approximately 9% of the glucomannan in the extracted pulp. Only a trace amount of xylose was detected.

Most of the residual lignin in the unbleached radiata kraft pulp was not extracted by alkali while most of the hemicelluloses were. The remaining hemicelluloses may be linked to the residual lignin via alkali stable covalent bonds as proposed by Minor (1986) and Jiang and Chang (1987), thus stabilising both polymers against further extraction. It was the enzymatic hydrolysis of these types of hemicellulose linkages that was first proposed as a possible mechanism for hemicellulase assisted bleaching (Paice *et al.* 1988; Viikari *et al.* 1987; Viikari *et al.* 1986). If residual lignin is associated with these unextractable hemicelluloses, then the principal mechanism of enzyme assisted bleaching cannot be the hydrolysis of lignin linked hemicelluloses as these hemicelluloses are not readily accessible to enzymatic hydrolysis.

5.6 CONCLUSIONS

The cloned xylanase and mannanase can solubilise small fractions of their respective substrates from several types of pulps. The residual glucomannan remaining in the fibre after mannanase treatment appears not to be affected by the enzyme as indicated by the similar average DPs of glucomannan extracts from mannanase treated and untreated radiata kraft pulps. As these extracted glucomannans are readily hydrolysed by the mannanase, it is apparent that the enzyme is unable to reach the majority of the substrate (70-80% of the total glucomannan) because of limited penetration into the fibre wall or shielding by other polymers. Indeed indirect evidence that xylans may shield a part of the glucomannan has been shown but cellulose is probably the main shielding agent (Puls *et al.* 1990). The portion of the glucomannan reached by the mannanase is completely hydrolysed and solubilised.

The xylanase did not solubilise as much of its substrate as would be expected, with only 5-10% of the total xylan removed. But the reduced average DP of extracted xylans from xylanase treated pulps (relative to untreated pulps) showed that the xylanase did partially attack intra-fibril xylans but that these were not solubilised. Upon extraction, the xylans were thoroughly hydrolysed by the enzyme again indicating physical inaccessibility as the probable reason for the incomplete hydrolysis of the intra-fibril polysaccharide. Two schemes for this limited attack on

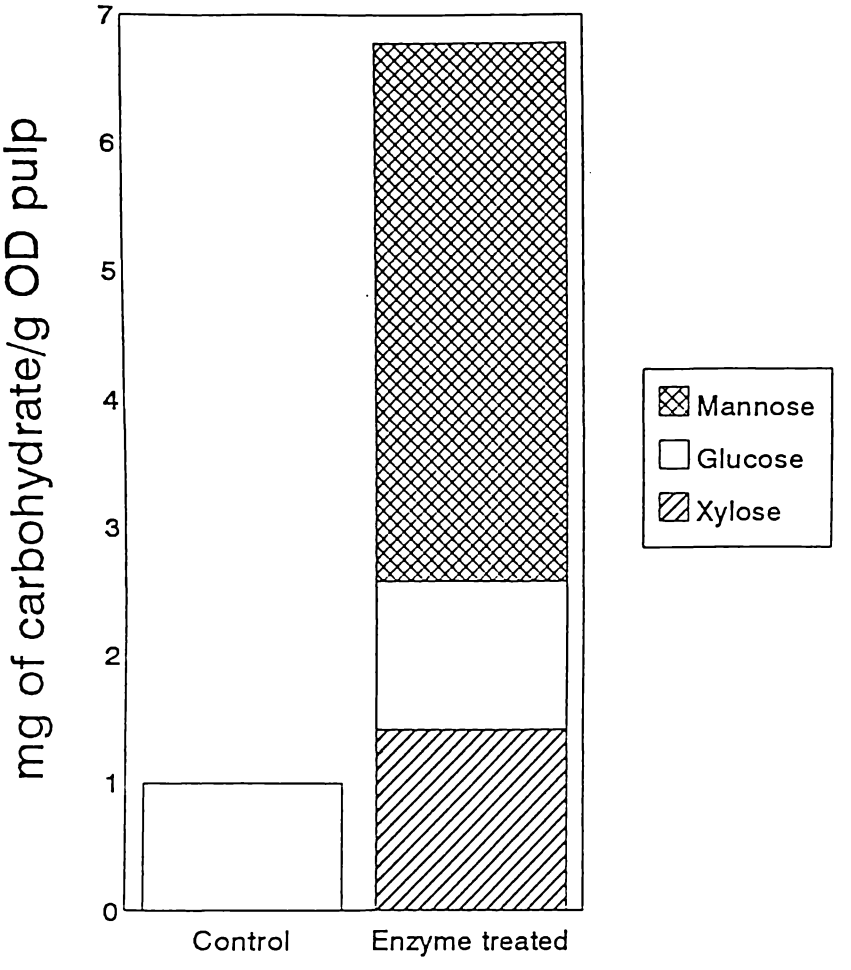

Fig 5.7 Carbohydrate solubilised from an alkali extracted radiata kraft pulp by the xylanase and mannanase (2% pulp in water at 70°C for 24 hr, 100 U/g OD pulp)

the fibril xylan are possible. In the first, native xylan is partially shielded, or embedded, in the microfibril matrix. Exposed regions of the xylan backbone are hydrolysed by the xylanase. Upon extraction, this xylan would have a smaller average DP than extracts from untreated controls. The second scheme refers to retake xylan hydrolysis. This xylan is thought to be held strongly to the fibre surface via hydrogen bonding (Meller 1965; Mora *et al.* 1986b) or possibly covalent bonds (Kantelinen *et al.* 1991). Crystalline regions in this deposited xylan may result from the removal of side-groups but this is still unclear (Meller 1965). It is possible that enzyme hydrolysis may be limited to certain regions (possibly amorphous) with hydrogen bonding preventing the solubilisation of the shorter xylans. Upon extraction, this partially hydrolysed xylan would have a lower average DP than extracts from pulps not treated by the xylanase. The comparable polydispersities of the extracted xylans suggests that all the xylans in the pulp were equally affected by the xylanase. Therefore, both schemes may act simultaneously.

A large portion of the hemicelluloses were not extracted by the Hamilton procedure. These may be firmly embedded within the fibre, probably located as deep as the S_2 layer (Scott 1984). Also, almost 80% of the residual lignin in the unbleached radiata kraft was not removed by alkali extraction. The unextracted hemicelluloses may be covalently bonded to this lignin as has been proposed by some (Jiang *et al.* 1987; Minor 1986). The cloned hemicellulases had a limited effect on these polysaccharides. Therefore, the enzymatic cleavage of hemicellulose-lignin linkages is not considered likely, especially in an intact pulp fibre.

CHAPTER SIX

BLEACHING OF HEMICELLULASE-TREATED WOOD PULPS

6.1 INTRODUCTION

The effects of the cloned hemicellulases on their respective substrates , within the fibre wall, have been described in the previous chapter. The cloned xylanase solubilised a small fraction of the total xylan but also appeared to partially depolymerise other intra-fibril xylans. The mannanase solubilised approximately 11% of the total mannan present in the pulp as either glucomannan or galactoglucomannan. The thermostable mannanase appeared to function in a similar manner to the mannanase produced by *Aspergillus niger*, in terms of its effects on the carbohydrates in radiata kraft pulp (Clark *et al.* 1990). Bleach boosting using crude and purified xylanase preparations has been reported to save from 10-26% of the chlorine required to bleach softwood pulps (Clark *et al.* 1990, 1991; Pedersen 1990). A cloned xylanase has also been successfully used to assist the bleaching of a kraft hardwood pulp with a 50% saving in chlorine (Paice *et al.* 1988). Thermostable xylanase preparations have also shown great promise as bleach boosting agents. A thermostable xylanase from *Thermomonospora fusca* (Casimir *et al.* 1991) has been successfully used at 80°C in a chlorine-free bleaching regime (Perrolaz *et al.* 1991) and a commercial thermostable xylanase is currently being marketed (Skerker *et al.* 1991).

In this chapter the bleach boosting capabilities of the cloned xylanase and mannanase are examined and compared to those of several commercial preparations. These preliminary bleaching results, in conjunction with data from the enzymatic hydrolysis of pulps and soluble hemicelluloses, are discussed in relation to the mode of action of enzyme assisted bleaching.

6.2 METHODOLOGY

Hemicellulase-induced improvements in bleachability were assessed by comparing the κ of enzyme treated pulps with that of untreated controls after a D/C E pre-bleaching sequence. Radiata kraft pulps were treated with the cloned

enzymes for 24 hr at 70°C, in water at 2% consistency, as previously described (SECT 2.4.2.3). Enzyme dosages were 100 U/g of OD pulp unless otherwise specified. Control pulps were also treated as above except that no enzyme was added. After treatment, the pulps were drained and washed, bleached (SECT 2.5.1) and the κ determined (SECT 2.5.2).

Pulp nitrogen contents were determined by the indophenol nitrogen assay of H_2SO_4/H_2O_2 pulp digests (SECT 2.5.3). Pulp protein contents were estimated from the total nitrogen by assuming that the measured nitrogen was derived from proteinaceous material with a nitrogen content of 16% (Haschemeyer and Haschemeyer 1973).

In some instances pulps were "de-proteinised" after hemicellulase treatment, but prior to bleaching, by either a caustic extraction, a protease treatment or a combination of the two. The caustic extraction was performed identically to the E stage of the pre-bleaching protocol for a chlorine multiple of 0.16 (SECT 2.5.3). The protease treatment involved incubating washed pulps with Proteinase K (Sigma catalogue number P 0390) at 5 U of protease activity/g of OD pulp at 2% consistency for 6 hr at 55°C. Pulps were then washed as described after hemicellulase treatment and could be bleached if necessary.

For comparative purposes three commercial xylanases were used to treat radiata kraft pulps. These were Pulpzyme HA produced by Novo Enzymes, the first xylanase specifically marketed as a bleach enhancing agent (Pedersen 1990); Cartazyme HT, a thermostable xylanase preparation sold by Sandoz (Sandoz Products Ltd., Horsforth UK)); and a purified *Trichoderma viride* xylanase sold by Biocon (Biocon, Boronia AUST). Pulps were treated with these enzymes as described above with the following exception; the pulps treated with the Biocon and Pulpzyme xylanases were incubated at 50°C, as these enzymes are not thermostable. The cloned TP8 hemicelluases used were the pNZ1435 xylanase (Table 4.3) and the pNZ1019 mannanase (Table 4.2).

The D/C E κ reported are the averages of at least four determinations on a single pulp, with a coefficient of variation of no greater than 3%. For accurate comparisons, enzyme treated and untreated pulps were bleached in parallel. The

D/C E κ (at the same chlorine multiple) may have varied from day to day but reproducibility within a given bleaching run was always good.

6.3 EFFECT OF THE CLONED HEMICELLULASES ON PULP BLEACHING

Radiata kraft pulps were treated with the cloned xylanase, the cloned mannanase or a combination of the two enzymes. The κ of the enzyme treated pulps and the appropriate control pulps at two Cl-multiples (0.14 and 0.16) are presented in Fig 6.1.

Comparing the κ of the untreated control pulp with those of the enzyme treated pulps shows that the xylanase had no significant effect on κ of the pulps after the D/C E pre-bleaching sequence. The κ of pulps treated with the mannanase or the two cloned enzymes were 10%-20% higher than the untreated controls, suggesting a detrimental effect on pulp bleaching.

To confirm that the pulps used were indeed amenable to enzymatic bleach boosting, the radiata kraft pulp was treated with a commercially available xylanase (Pulpzyme) marketed specifically for this purpose (Pedersen and Elm 1991). The pulps were treated as described above except that the Pulpzyme treatment was incubated at 50°C, since this enzyme is not as thermostable as the cloned enzymes. Also, the recommended dosage of Pulpzyme (1 U/g OD pulp) was used. The κ of the pre-bleached (Cl-multiple 0.14), Pulpzyme-treated pulp was 24% lower than that of the pre-bleached control, confirming that the bleachability of the radiata kraft pulp could be improved by xylanase treatment (Fig 6.2). Respective controls and enzyme treated pulps were treated and bleached in parallel, on the same day, to ensure direct comparability of D/C E κ. However, some variability was apparent in the repeatability of bleaching on different days. This can be seen in the differences in D/C E κ between the two control treatments shown in Fig 6.2, as each series was bleached on a different day.

The apparent inability of the cloned hemicellulases to improve pulp bleaching could be attributed to two main factors; differences in the enzymatic properties of the cloned and commercial enzymes rendering the former less effective; or

exogenous factors in the enzyme preparations which interfere with the pre-bleaching procedure or the κ determination.

A major difference between the cloned enzymes and the commercial enzyme treatments was the amount of protein added. The specific activity of the cloned hemicellulases was relatively low (Table 4.2 and 4.3). At the enzyme dosages used, the total protein added was between 20 to 25 mg of protein/g of OD pulp. For the combined hemicellulase treatments it was as high as 45 mg of protein/g of OD pulp, which was greater than the amount of lignin in the pulp (33 mg of lignin/g of OD pulp). With Pulpzyme, high specific activity and the low dosage meant that the amount of added protein was approximately 10,000 fold lower than that cited for the cloned enzymes. Therefore excess protein may have consumed bleaching chemical, affected pulp bleachability or affected the κ determination and masked any hemicellulolytic bleach boosting effects of the cloned, thermostable enzymes.

Treatments with lower dosages of the cloned xylanase (5-20 U/ g of OD pulp) had no improvement on the pulp κ after pre-bleaching (Fig 6.3). The mannanase, at a dose of 20 U/g of OD pulp, still caused an increase in κ after pre-bleaching compared to untreated pulps. No carbohydrate was solubilised at these dosages. Therefore it was not evident whether any adverse effects on bleaching were due to hemicellulolytic activity or some form of protein interference. To establish whether the increase in κ was due to ineffectiveness of the enzymes or protein interference, it was necessary to treat the pulps under conditions known to affect fibre-wall hemicelluloses and then attempt to remove any bound proteins. If this improved bleachability then a non-enzymatic factor in the preparations was responsible for the poor bleaching effect previously observed. If it did not then the cloned hemicellulases cannot be considered effective in bleach boosting of radiata pine kraft pulp.

6.3.1 The effect of excess protein and its removal

If protein had an effect on the bleaching of the kraft pulps as described above, pulp protein content should be measurably higher in the enzyme treated pulps than in the untreated controls. The protein content of unbleached, radiata kraft pulps

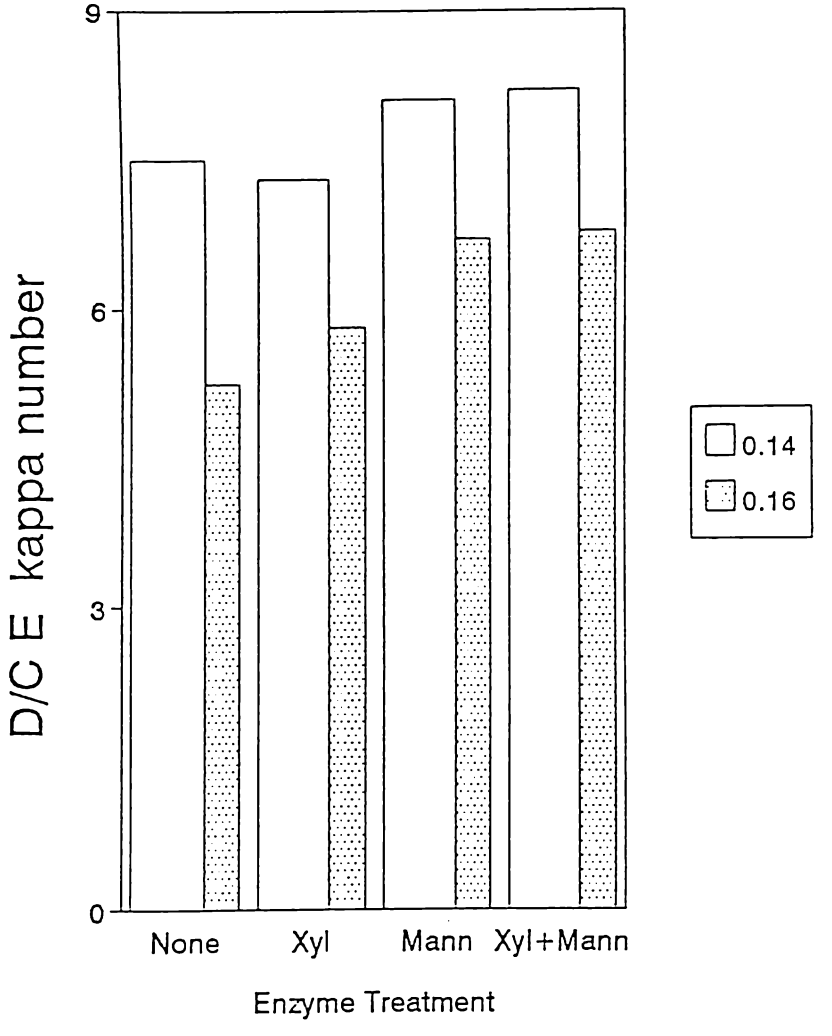

Fig 6.1 Effect of xylanase treatment (pNZ1435), mannanase treatment (pNZ1019) and xylanase/mannanase treatment on the κ of radiata kraft pulp after D/C E bleaching at two different chlorine multiples (0.14 and 0.16)

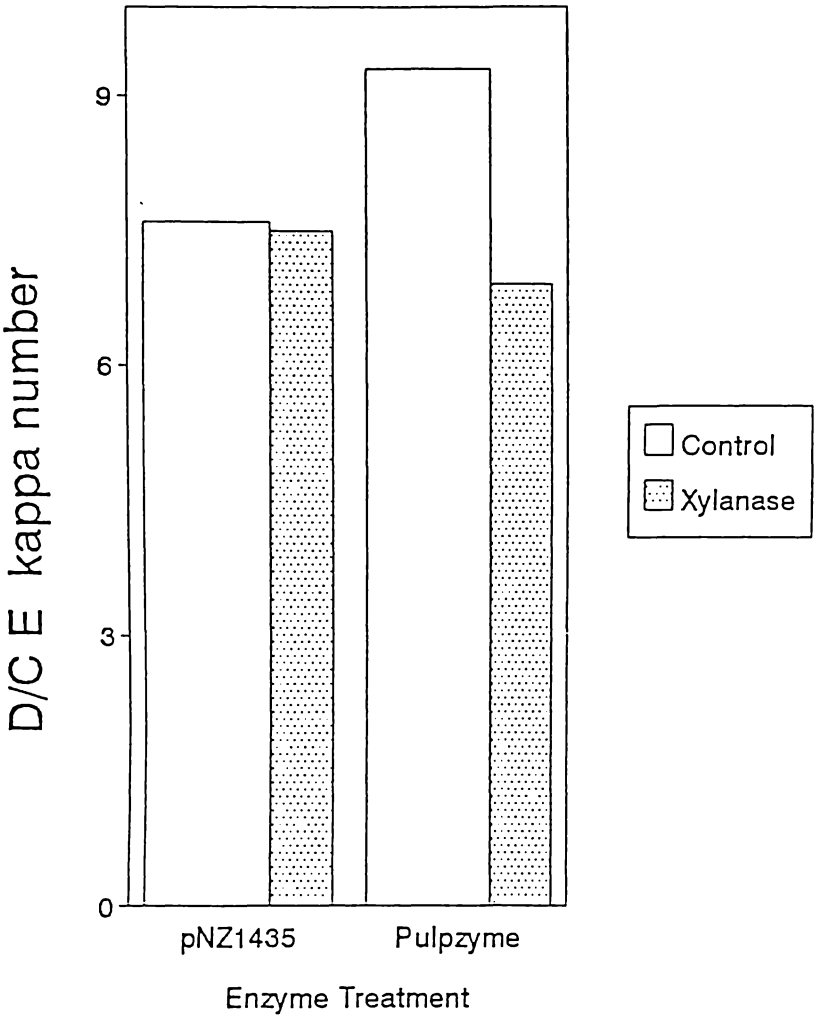

Fig 6.2 Comparison of κ after D/C E bleaching (Chlorine multiple 0.14 radiata kraft pulps i) TP8 xylanase (pNZ1435) treated pulp compa with control pulp (no enzyme) ii) Pulpzyme treated pulp compa control pulp (no enzyme)

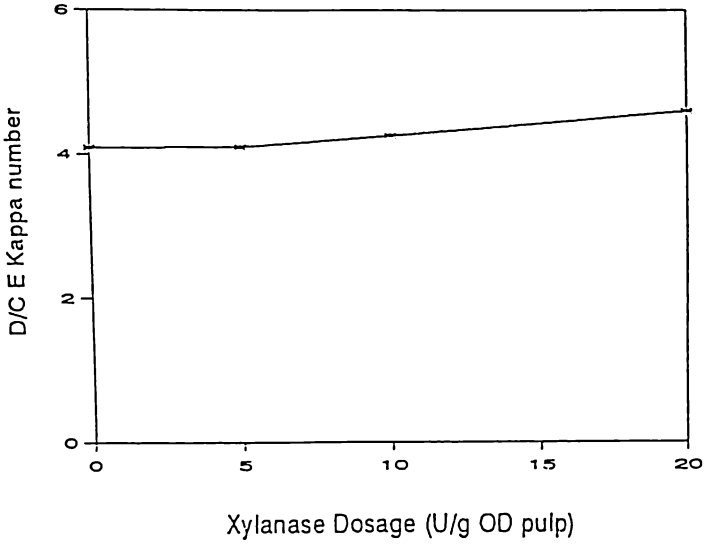

Fig 6.3 Effect of xylanase dosage (pNZ1435) (O-20 U/g OD pulp) in treatment of radiata kraft pulp on the D/C E κ

that had undergone treatment with the cloned xylanase and mannanase at dosages of 100 U/g of OD pulp were estimated. Before nitrogen quantification, the pulps were washed (SECT 2.4.2.3) to remove any unbound protein.

The protein level of the enzyme treated, washed pulps were approximately two to three fold higher than the untreated control pulp levels. But, generally, the protein levels of washed pulps (enzyme treated and control) were low (less than 0.2% on pulp). It seems unlikely that protein would have an effect on pre-bleaching at such low levels. If such low protein levels did have an effect their removal should alleviate any interference. Two such "de-proteinisation" procedures were used to treat radiata kraft pulps after xylanase treatment (100 U/ g of OD pulp) but prior to pre-bleaching. These included a caustic extraction and a protease treatment as described in the methodology section. The κ of pre-bleached (O.16 Cl-multiple), xylanase treated pulps were higher than those of the untreated controls (Fig 6.4). However, xylanase treated pulps that had been de-proteinised by caustic extraction had κ that were 7% lower than xylanase treated pulps that had not been de-proteinised. Xylanase treated pulps that were protease treated had κ that were 10% lower than pulps not enzymatically de-proteinised (Fig 6.4). This suggests that these de-proteinisation procedures may be an effective means of relieving any interference caused by exogenous proteins in the hemicellulase preparations, although further enzyme purification would have been the preferred option had time permitted.

It would seem that, even at low concentrations, exogenous proteins may still interfere with the bleach boosting effect. Caustic extraction and protease treatment were used separately and in combination to determine whether their effects may be cumulative and to determine if bound protein was indeed removed from the pulps. Radiata kraft pulps were treated with either the cloned xylanase or the cloned mannanase (20 U/g OD pulp) and incubated under the conditions described. Control pulps, with no added hemicellulase, were incubated in a similar manner. After the incubation, pulps were drained and washed before de-proteinisation treatments. There was no significant difference between the κ of these pulps (Fig 6.5) which concurs with previous findings that hemicellulase treatment alone does not affect the κ of the pulp (Clark *et al.* 1991). The protein levels of the

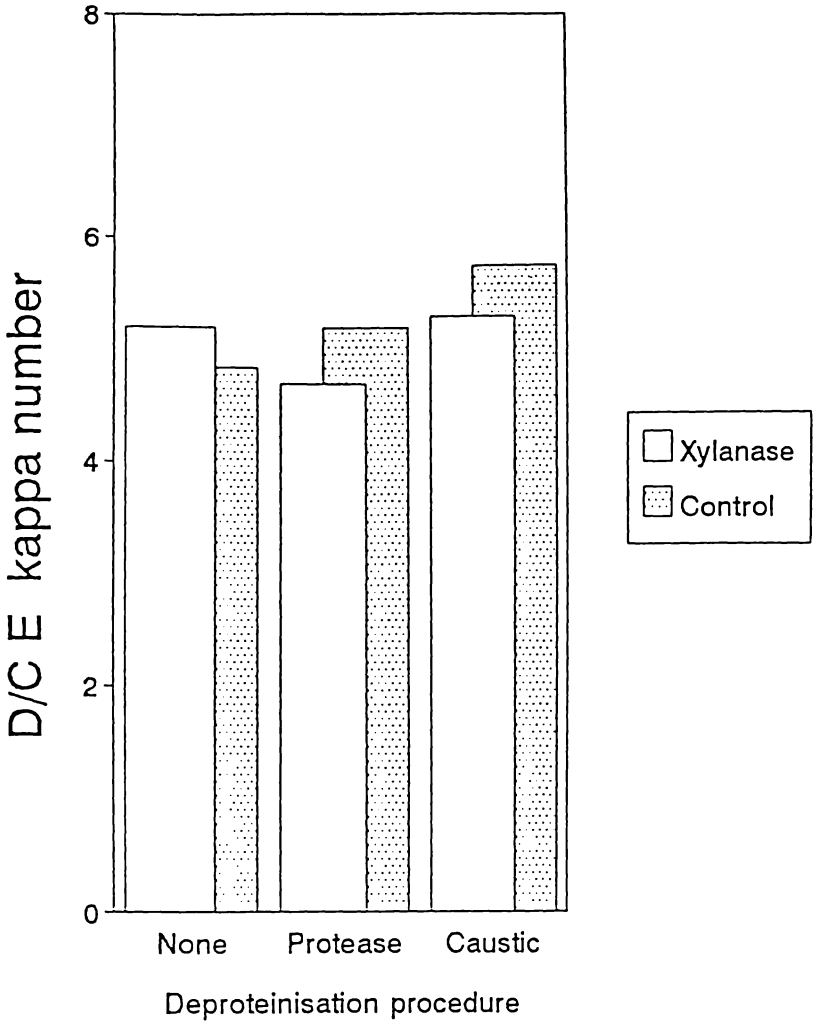

Fig 6.4 κ after D/C E bleaching of radiata kraft pulps (Chlorine multiple 0.16):

i) xylanase (pNZ1435) treated pulp compared with control pulp (no enzyme)

ii) xylanase (pNZ1435) treated pulp compared with control pulp (no enzyme) - protease treatment prior to bleaching

iii) xylanase (pNZ1435) treated pulp compared with control pulp (no enzyme) - caustic treatment prior to bleaching

N.B. each series i), ii) and iii) were treated and bleached on different days.

hemicellulase treated pulps were two-three fold higher than the control pulp. But, again, protein levels were extremely low. Therefore, protein, at the levels found in the enzyme treated pulps, did not appear to affect the κ assay.

The pulps described above were divided in half with one half subjected to protease treatment (in water at 2% consistency for 8 hr at 55°C, 5 U of Protease K/g of OD pulp). The other half of the pulp was incubated in the same way except that the protease was omitted. After the incubation the pulps were washed and analysed for κ and protein content (Fig 6.5). The protein contents of the protease treated pulps tended to be higher than their non-protease treated counterparts. Again protein levels were never more than 0.2% of the pulp weight and no correlation was apparent between these levels and the κ. All the pulps were caustic extracted, washed and analysed for κ and protein content (Fig 6.5). Caustic extraction lowered the κ of all the pulps, whether protease treated or not, by approximately 5%. The effect, also noted previously (Fig 6.4), was probably a general lignin extraction step rather than a de-proteinisation.

Upon bleaching, the κ of the hemicellulase treated pulps that were only caustic extracted were higher than the corresponding controls. This suggests that caustic extraction alone had no beneficial effect on the bleachability of the hemicellulase treated pulps. These results mirrored those of pulps that had not been caustic extracted after hemicellulase treatment (Fig 6.5). With the bleached, protease/caustic extracted pulps the κ of the xylanase treated pulp was significantly lower than that of the control (95% confidence interval, student's t test). This is in agreement with earlier experiments which showed that protease treatment of xylanase treated pulps did improve pulp bleachability to a similar extent (Fig 6.4). There was no significant difference between the mannanase treated pulp and the control (Fig 6.5).

The low protein levels do not appear to have a detrimental effect on the κ assay as evident from the comparisons of protein content and κ (Fig 6.5). There was also no correlation between pulp protein levels and bleachability. However, the improvement in bleachability that was seen with the xylanase treated pulps that had been enzymatically de-proteinised suggests that a compound of a proteinaceous

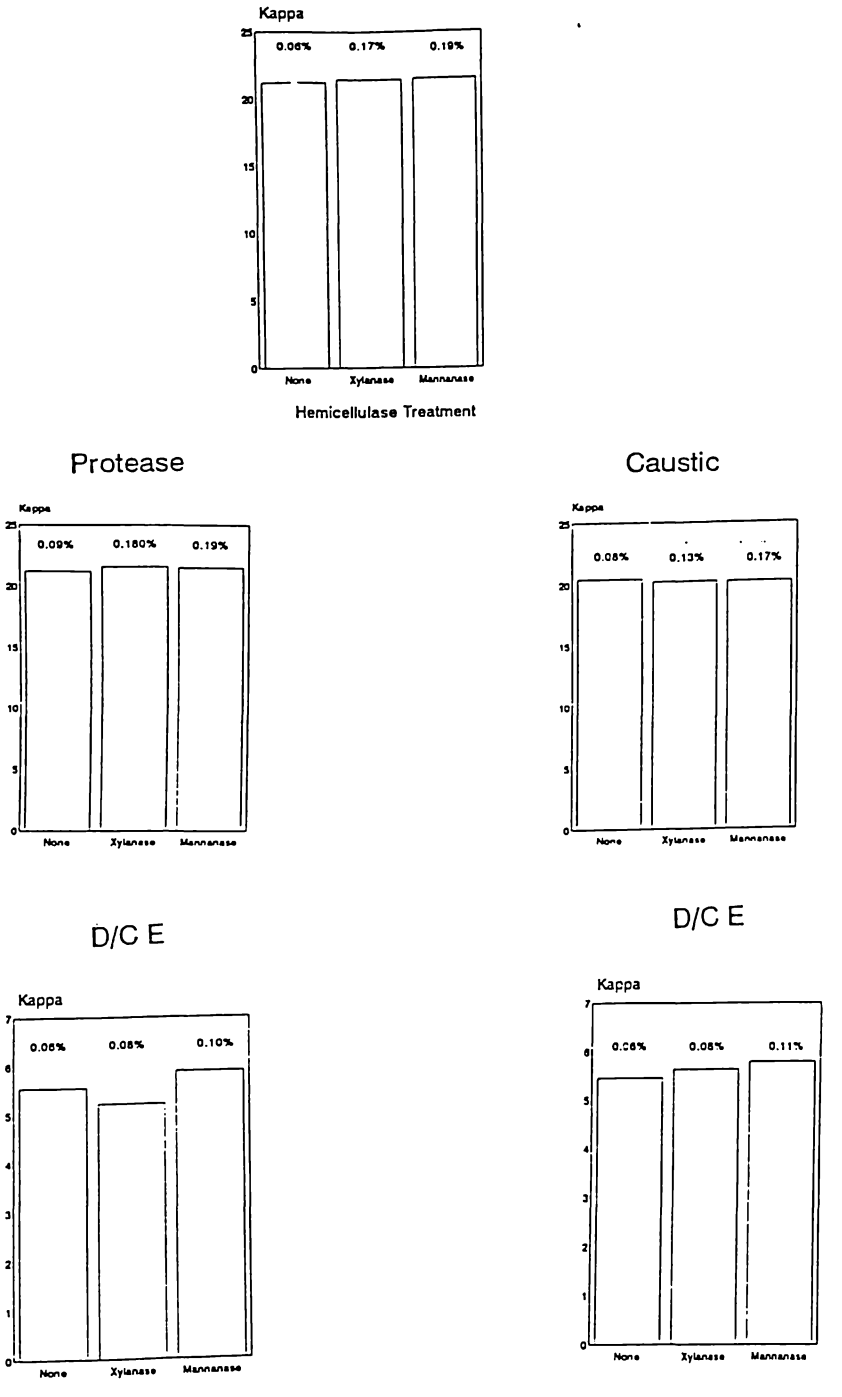

Fig 6.5

Top row: κ and % protein content (number above bar) of radiata kraft pulps treated with no enzyme (control), xylanase (pNZ1435) or mannanase (pNZ1019)

Middle : κ and % protein content of pulps from top row that were de-proteinised by either protease or caustic treatment

Bottom : κ and % protein content of pulps from middle row that were D/C E bleached

N.B. κ and % protein are comparable within each row

nature is interfering with pulp bleaching. This compound is resistant to mild caustic extraction, since such a procedure did not alleviate its effect. It is not known whether the compound affects the bleaching directly by consuming chemicals or alters lignin making it less susceptible to bleaching. Also, it is conceivable that the xylanase and the protease interact to cause a bleach boosting effect while neither has an effect on its own.

Once this bleach-affecting factor was removed by protease treatment, an improvement in the bleachability of pulps treated with the cloned xylanase was evident. This was reproducible with the xylanase treatment lowering the average κ by 7.6% (standard deviation 1.9). In contrast, the κ of Pulpzyme treated pulps was 25% lower than that of untreated controls after bleaching (Fig 6.2). No such improvement was seen with the de-proteinised mannanase treated pulps (Fig 6.5). This suggests that the xylanase does improve pulp bleachability to a small extent but that the mannanase does not.

6.3.2 Comparisons of the cloned hemicellulases with commercial xylanases

The cloned mannanase does not elicit an improvement in the bleachability of radiata kraft pulps while that invoked by the xylanase does not appear to be substantial. Yet both cloned enzymes solubilise their respective substrates from radiata kraft pulps in amounts comparable to those from other studies (Clark *et al.* 1990, 1991). A xylanase that solubilised 12% of the xylan from a radiata kraft pulp lowered the κ of the pulp by approximately 30% after pre-bleaching which resulted in a 25% saving in chlorine (Clark *et al.* 1990).

The effect of the cloned hemicellulases on some selected properties of radiata kraft pulps were determined and compared to those of pulps treated with three commercial xylanases; Pulpzyme HA, Cartazyme HT and Biocon (SECT 6.2). The Pulpzyme HA is a crude xylanase preparation that has a recommended temperature optimum of 50°C and a pH optimum near neutrality (Pedersen 1990). Savings in chlorine of 18% have been reported for kraft birch pulps. The Cartazyme xylanase is a thermostable preparation. The Biocon xylanase is a highly purified enzyme of low molecular weight (20,000). All hemicellulase treated pulps and untreated controls were subjected to a protease treatment, as described (SECT 6.3.1), prior

to the D/C E bleaching sequence. It should be emphasized that this step was specifically incorporated to bring about the bleach boosting effect in the cloned xylanase which is otherwise masked by presumably, an unidentified proteinaceous component of the enzyme preparation. None of the commercial xylanases require this step for bleach boosting.

The D/C E κ , at three chlorine multiples (0.16-0.20), of the enzyme treated pulps and untreated controls are presented in Fig 6.6. The composition of the solubilised carbohydrates are given in Fig 6.7. The κ of the pulps treated with the cloned mannanase were consistently higher than the corresponding controls, despite the mannanase solubilising 11% of the glucomannan. The mannanase behaved in a manner similar to that of *A. niger* mannanase on radiata kraft pulp (Clark *et al.* 1990). Both mannanases specifically solubilise approximately 11% of the glucomannan within the pulp but not any xylan, yet neither enzyme improved pulp bleachability.

The cloned xylanase also had little or no effect on pulp bleachability, regardless of the chlorine multiple, yet it solubilised approximately 4% of the pulp xylan (Fig 6.6, Fig 6.7, Table 6.1). Pulps treated with the three commercial xylanases showed significant improvements in pulp bleachability at the three chlorine multiples, although the data did not allow precise assessments of the savings in applied chlorine. It would appear that these pulps were not adversely affected by the protease treatment. The κ of the bleached, Biocon xylanase treated pulps were consistently 10% lower than the corresponding values from the control pulps. This was translated into a savings in applied chlorine of about 11% when bleaching for the same κ. This enzyme also solubilised the greatest amount of xylan of all the xylanases, almost 19% (Table 6.1). Yet the other commercial xylanases gave higher savings in applied chlorine.

The lowest κ (2.63) was obtained with Pulpzyme treated pulps at a chlorine multiple of 0.20. (Fig 6.6). This resulted in a chlorine savings of approximately 12%. Cartazyme treated pulps had a κ of 3.85 at a chlorine multiple as low as 0.16, which translates into a 20% savings in applied chlorine (Fig 6.6). Both enzymes solubilised similar amounts of xylan but differed in their bleach boosting capabilities. Cartazyme, which solubilised 14% of the total xylan (Fig 6.7), gave a savings in

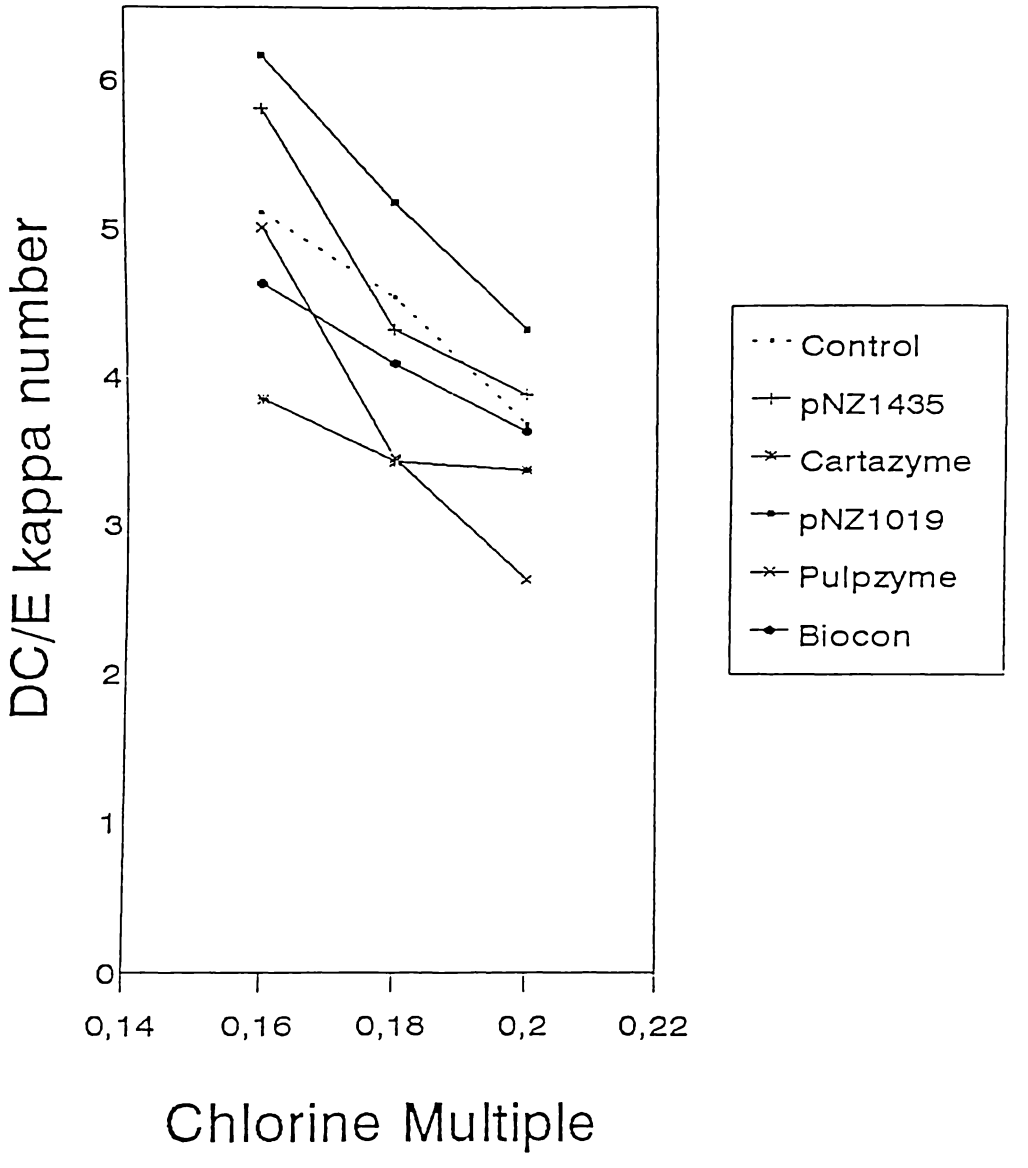

Fig 6.6 κ of enzyme treated radiata kraft pulps after a D/C E bleach at one of three chlorine multiples (O.16-0.20)
- pulps were treated with one of the following enzymes as specified in the text: xylanase (pNZ1435), mannanase (pNZ1019), Cartazyme, Pulpzyme or Biocon xylanase. A control pulp incubated without enzyme was also bleached

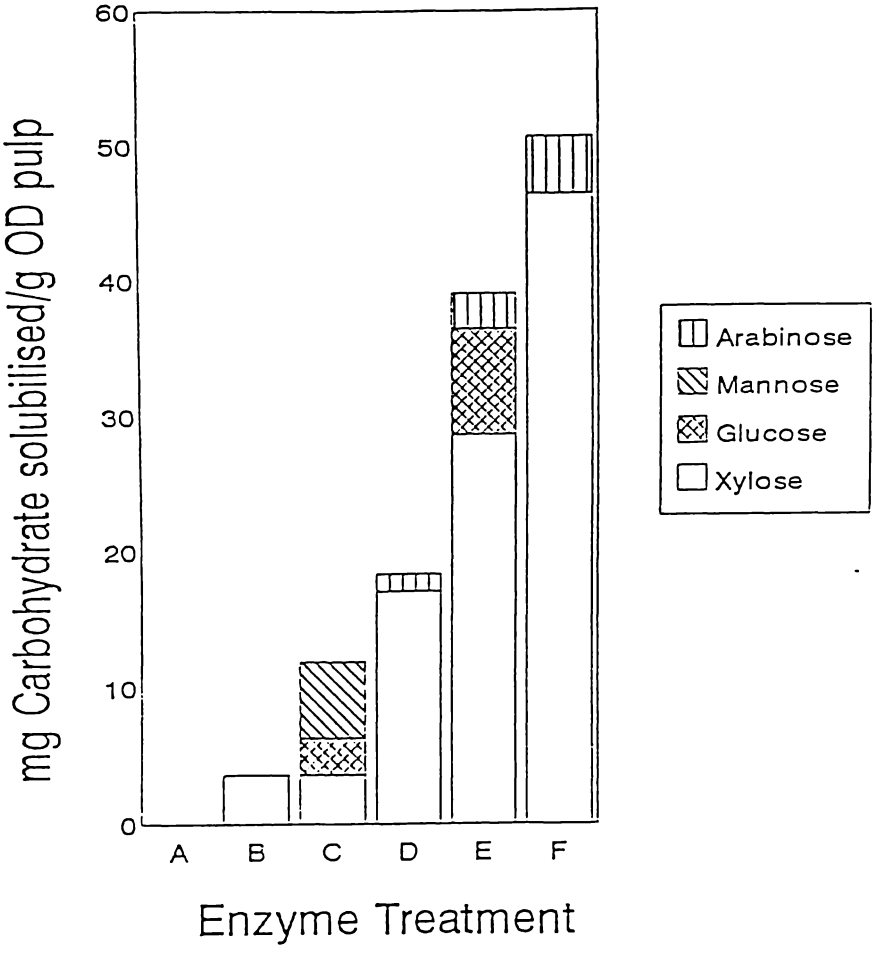

Fig 6.7 Composition of the carbohydrates solubilised from radiata kraft pulp by: A) control treatment (no enzyme); B) xylanase (pNZ1435); C) mannanase (pNZ1019); D) Cartazyme; E) Pulpzyme or F) Biocon xylanase.

Table 6.1 The effects of various enzymatic treatments on the water retention value, viscosity and brightness of radiata kraft pulps.

Enzyme Treatment	% Xylan* Solubilised	% Water# Retention	Viscosity (mPa.s)	Pulpˣ Brightness (% ISO)
Control	0.0	180 ($^{+/-}$3.0)	22.30	38.6
Xylanases				
TP8 cloned	3.8	186 ($^{+/-}$3.0)	31.10	35.1
Cartazyme	14.0	191 ($^{+/-}$4.2)	25.40	39.6
Pulpzyme	12.0	180 ($^{+/-}$3.7)	N.D.	N.D.
Biocon	19.0	180 ($^{+/-}$2.8)	32.90	39.9
Mannanase				
TP8 cloned	13.5^{+}	186 ($^{+/-}$3.0)	31.10	35.1

* 96 mg of xylose/g of OD radiata kraft pulp (See Table 5.1)
\# Average ($^{+/-}$ standard deviation)
x Pulp bleached at a chlorine multiple of 0.16
\+ 49 mg of mannose/g of OD radiata kraft pulp (See Table 5.1)
N.D. not determined

applied chlorine of about 20% while the Pulpzyme solubilised 12% of the xylan and gave only an approximate chlorine savings of 12%. It should be noted that Pulpzyme also has a significant cellulase component as is evident from the solubilised glucose (Fig 6.7). No obvious relationship exists between the amount of enzymatically solubilised xylan and the savings in chlorine. This is in accord with the conclusions of Clark *et al.* (1991) who observed that improvements in bleachability are independent of the amount of solubilised carbohydrate. Yet the fact that all xylanases that improved bleachability solubilised more xylan than the TP8 xylanase suggests that at least a minimum amount of xylan removal is required to enhance bleaching. It appears that the TP8 xylanase did not remove sufficient xylan to accomplish this.

The effects which the various enzymes had on selected pulp properties are presented in Table 6.1. The water retention value is the ratio of the weight of water held in the fibre wall to that of the dry weight of pulp, expressed as a percentage

(Penniman 1981). This parameter is related to the fibre saturation point (Scallan and Caries 1972) and thus is an estimate of the intra-fibril pore volume which, in saturated fibres, should be occupied by water. Therefore, the higher the water retention value the higher the intra-fibril pore volume. Increases in the water retention value would be indicative of an increase in this volume (ie: fibre swelling). None of the hemicellulases significantly affected fibre swelling (95% confidence level, students test) even though moderate amounts of hemicelluloses were solubilised. This is surprising as fibre swelling is thought to be a possible mechanism of enzymatic bleach boosting (Clark *et al.* 1991). However, it is possible that improved fibre swelling following enzyme treatments may occur during the alkaline E stage where fragmented lignins are extracted.

All the enzyme treated pulps had significantly higher viscosities than the untreated control (Table 6.1). This increase, along with the lack of cellulose associated glucose in the soluble carbohydrates, confirms that the hemicellulases do not depolymerise intra-fibril cellulose. Such increases in pulp viscosities are thought to result from the enzymatic removal of low molecular weight xylans (Paice *et al.* 1988). This phenomenon has been demonstrated in xylanase treated radiata kraft pulps but mannanase treated pulps showed a decrease in viscosity (Clark *et al.* 1990). Pulp viscosities were determined after pre-bleaching which may have facilitated the extraction of low molecular weight polysaccharides from the enzyme treated pulps. This may account for the greater viscosities found in the enzyme treated pulps as compared to the non-enzyme treated control. The viscosity of the control pulp was considered lower than usual for a radiata kraft pulp but still well within the normal range for a pulp of this type. It should be noted that glucose was found in the carbohydrate solubilised by Pulpzyme HA suggesting that some cellulose was attacked by this enzyme.

In this study, mannanase treated pulps showed as large an increase in viscosity as the xylanase treated pulps. Pulps treated with the three commercial xylanases showed an improvement in bleachability with lower κ and slight increases in brightness relative to the control pulp (Table 6.1). The pulps treated with the cloned enzymes showed significant decreases in brightness. This suggests that some chromophore present in the protein preparations may be affecting the brightness

of the pulp. This chromophore is probably proteinaceous in nature as evident from the improvements, albeit minor, in bleachability brought on by the protease treatment. The low levels of protein found in pulps after enzyme treatment (and several washes) as well as after bleaching indicate that the chromophore is very recalcitrant.

6.4 CONCLUSIONS

The cloned mannanase has not demonstrated any ability to improve the bleachability of kraft pulps. This is in contrast to work by Clark *et al.* (1990, 1991) who have shown that a purified *Bacillus substilis* mannanase does improve pulp bleachability. However, the cloned mannanase was similar in behaviour to an *A. niger* mannanase, also studied by Clark *et al.* (1990) in terms of the carbohydrates solubilised from radiata kraft pulp (glucomannan and galactoglucomannan but no xylan) and the end-product profile (Clark *et al.* 1990). This fungal mannanase also did not improve pulp bleachability.

The cloned xylanase did not improve the bleachability of radiata kraft pulp to any great extent, certainly not as well as the commercial xylanases employed in this study or that of *Trichoderma harzianum* xylanase used in previous studies (Clark *et al.* 1990, 1991). The various xylanases solubilised up to 19% of the available xylan but the relative amount of solubilisation did not correlate with the extent of delignification (ie: D/C E κ) or any savings in chlorine. The cloned xylanase solubilised less than 4% of the xylan in the pulp and exhibited little or no bleach boosting. Clearly xylanases that do improve bleachability must perform some function that the cloned xylanase does not do. Of the commercial xylanases evaluated, the Cartazyme xylanase gave the greatest savings in chlorine (~20%).

Hemicellulase treatment did not significantly affect the fibre swelling of any of the pulps examined, regardless of the extent of bleach boosting or the amount of hemicellulose solubilised. This is interesting as fibre swelling is thought to be one of the mechanisms by which enzyme assisted bleaching may operate (Clark *et al.* 1991). The results obtained only demonstrate that whatever effect these enzymes have on fibre swelling was not demonstrable under the conditions at which the

water retention values were determined. Xylanolytic activity may facilitate swelling under other conditions (ie: during bleaching).

None of the hemicellulases tested appeared to depolymerise intra-fibril cellulose. This was concluded from two experiments. First, cellulosic glucose appeared to be released by only one enzyme, the Pulpzyme HA. The other xylanases solubilised only xylans. The glucose in the solubilised carbohydrate from the mannanase treated pulp was associated with mannose in a ratio indicative of glucomannan. Second, the bleached enzyme treated pulps had higher viscosities than the bleached controls suggesting that cellulose DP was not affected by the enzymes and that lower molecular weight polysaccharides were removed thus increasing the average DP. This effect had been noted by others (Paice *et al.* 1988) but not to the extent presented here suggesting that bleaching removed significant amounts of low molecular weight polysaccharides.

Both cloned hemicellulase preparations contained a contaminant, possibly proteinaceous, that either discoloured the pulps or protected lignin from bleaching chemicals. This effect remained after a mild caustic extraction but was alleviated by a protease treatment prior to bleaching. Further purification of the cloned enzyme preparations to reduce the amount of added protein should minimise this masking effect to the point where a protease treatment is no longer required.

CHAPTER SEVEN
SUMMARY AND CONCLUSIONS

This study characterised two thermostable hemicellulases, a xylanase (two clones) and a mannanase, that had been cloned from a thermophilic anaerobe. This organism (isolate TP8 6.3.3.1; proposed name *"Caldocellum saccharolyticum"*) was previously isolated from the geothermal hot pools of New Zealand and is amenable to gene engineering. It has been shown by previous workers that several of the genes coding for enzymes involved in lignocellulose hydrolysis (in TP8 6.3.3.1) can be cloned into *E. coli*. The enzymes cloned are thermostable at 70°C and higher, some with half-lives ($t_{1/2}$) of several hours at this temperature. The thermostable nature of these cloned enzymes has been exploited in their purification from other proteins.

The recent discovery that unbleached kraft pulps treated with xylanases could be more easily bleached has created a demand for suitable enzymes which can be used on an industrial scale in the pulp and paper industry. Chlorine savings during bleaching of up to 25% have been reported with a subsequent decrease in the chlorinated organic content of the bleach effluent. Thermostability has been identified as a desirable property in hemicellulases used to enhance pulp bleachability. Thermophilic organisms, such as TP8 6.3.3.1, are a potential source of these types of hemicellulases.

In this study TP8 6.3.3.1 (which was known to produce a number of enzymes involved in the hydrolysis of cellulose and xylan) was screened for the presence of other enzymes involved in the breakdown of wood hemicelluloses. α-Galactosidase, α-L-arabinofuranosidase and mannanase activities were found in culture supernatants of TP8 6.3.3.1 grown on galactomannan and xylan. The organism thus possesses the enzymatic capability to hydrolyse the total carbohydrate fraction of wood. The mannanase and xylanase were of particular interest as possible bleach enhancing agents. The gene coding for the mannanase activity was cloned by workers at the Centre for Gene Technology, University of Auckland (Auckland

NZ) (Luthi *et al.* 1991). A xylanase gene from TP8 had previously been cloned by the same workers.

The thermostable xylanase and mannanase could thus be produced in laboratory scale quantities and partially purified. The cloned xylanase had previously been partially characterised, focusing on the thermostability of the enzyme (Schofield, DPhil, University of Waikato 1990). In this study, two cloned xylanase (pNZ1417 and pNZ1435) preparations were comprehensively characterised. These were found to have similar properties. Both xylanase preparations had temperature optima of 70°C (10 min) and were stable at these temperatures with more than 90% of the activity remaining after 24 hours (in water). Thermostability in buffer was lower. The xylanase preparations thoroughly hydrolysed radiata pine soluble arabinoglucuronoxylan to low molecular weight xylo-oligomers. These included xylobiose, xylotriose, xylose, arabinose substituted xylobiose and xylotriose and glucuronic acid substituted xylotriose.

The cloned mannanase was also characterised and had a molecular weight of 39,000 Da, a broad pH optimum (6-7.5) and a temperature optimum of 70°C (10 min assay). The mannanase was also very thermostable with no loss in activity after 24 hr at 70°C and a $t_{1/2}$ of 48 min at 85°C, making this one of the most thermostable mannanases reported in the literature to date. The mannanase was active against a range of mannans, glucomannans and galactomannans, including radiata pine glucomannan, but not xylans, carboxy-methyl cellulose or starch. The glucose content of the glucomannan had a minor effect on the enzyme's activity. However, the mannanase's activity was inhibited by high levels of galactose substitution of the galactomannan substrates. Soluble radiata glucomannan was thoroughly hydrolysed by the mannanase to mannobiose, mannotriose, mannose, gluco-mannose and gluco-mannobiose.

The xylanase (pNZ1435) and the mannanase were evaluated for their ability to hydrolyse xylan and glucomannan from a variety of pulps including several radiata pine pulps (kraft, NSAQ, CTMP and TMP). The mannanase solubilised 10% of the available glucomannan and the xylanase 4% of the available xylan from an unbleached, radiata kraft pulp. Both enzymes were specific for their respective substrates and did not hydrolyse cellulose from the pulp. The cloned, TP8 xylanase

was less effective in solubilising xylan from kraft pulps than other xylanases reported in the literature. Co-treatment with the xylanase and mannanase improved carbohydrate solubilisation by only a small amount. The type of pulp had a significant effect on the amount of carbohydrate solubilised. Chemical pulps were more susceptible to hydrolytic attack than mechanical pulps, possibly due to their greater fibre wall porosity.

Following treatment of radiata kraft pulps with the two enzymes, the residual xylans and glucomannans remaining in the fibre wall were extracted by the Hamilton procedure. The average DP of the xylan extracted from the xylanase-treated pulp was significantly lower than that of the xylan extracted from untreated pulp, suggesting that the enzyme had partially hydrolysed but not solubilised the polysaccharide from the cell wall. The average DP of extracted glucomannans from mannanase-treated pulps were comparable to those of glucomannan extracted from untreated pulps, suggesting that the residual glucomannan in the fibre wall was totally inaccessible to the mannanase. The extracted hemicelluloses were readily hydrolysed by their respective enzymes, which is in agreement with earlier results on soluble radiata hemicelluloses. Therefore, it appears that the poor hydrolytic activity of the xylanase and the mannanase on radiata kraft pulp is due to the inaccessibility of the enzymes to their respective substrates. There is evidence that suggests that this inaccessibility may be due to shielding of the hemicelluloses by cellulose but other factors, such as enzyme size, the affinity of the cloned enzymes for intra-fibril hemicelluloses or the lignin content of the fibre may also be involved.

The thermostable mannanase when used to treat kraft pulps, did not improve their bleachability. Only a few mannanases effective in enhancing pulp bleachability, have been reported in the literature with most studies finding mannanases to be ineffective. Therefore, the result obtained with the thermostable mannanase is consistent with this trend. More surprising was the fact that the xylanase had little or no effect on the bleachability of kraft pulp. Most xylanases reported in the literature can effect significant savings in chlorine when used prior to bleaching. The cloned hemicellulases were only partially purified with relatively large amounts of non-enzymatic protein. A compound in the enzyme preparations

was found to interfere with the bleaching of xylanase- and mannanase-treated pulps resulting in higher D/C E kappa numbers (κ) than in untreated pulps. This effect could be alleviated by applying either a caustic wash or a protease treatment to the pulp after hemicellulase treatment. Improved bleachability of xylanase-treated pulps (~ 7% decrease in κ relative to untreated controls) was only evident after such a treatment. This finding suggests that in future work, enzymes of a higher level of purity should be used in evaluating enzyme assisted bleaching. The cloned hemicellulases did not affect pulp viscosity or swelling.

In spite of their thermostability, it would seem that the cloned xylanase and mannanase from TP8 are not useful for improving the bleachability of kraft pulps. This may be due to the limited accessibility of the enzymes to the substrates. Screening for thermostable enzymes of lower molecular weight may minimise this inaccessibility as smaller enzymes may diffuse further into the porous cell wall than larger enzymes. As the mechanism of enzyme assisted bleaching is poorly understood, it is still not possible to define the properties required in an effective enzyme. Therefore a simple screening procedure has not been developed. Pulps must be treated with enzyme and bleached to evaluate the effect. This can be a laborious process, especially when enzyme production and purification are taken into consideration. However, it is the only option available at the present. As the geothermal regions of New Zealand are a source of many unique cellulolytic and hemicellulolytic thermophiles, a continued search of these environments for organisms that produce novel xylanases is warranted. Thermostability remains a desirable property of enzymes which may be used in the pulp and paper industry. Therefore, further testing of hemicellulases from new thermophilic isolates will be the only way of identifying commercially useful enzymes.

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APPENDIX 1

Vitamin solution (as per Wolin *et al.* 1963)

	mg L^{-1}
Biotin	2
Folic acid	2
Pyridoxine-HCl	10
Thiamine	5
Nicotinic acid	5
Ca-pantothenate	5
Vit B_{12}	0.1
ß-Aminobenzoic acid	5
Lipoic acid	5
Riboflavin	5

- vitamin solution was filter sterilised (0.45μm pore size)
- added to cooled, sterile media (10 ml vitamin solution L^{-1}

Trace elements solution (as per J. Hudson, University of Waikato)

	mg L^{-1}
$ZnCl_2$	70
$MnCl_2.4H_2O$	100
H_3BO_2	6
$CoCl_2.6H_2O$	130
$CuCl_2.2H_2O$	2
$NiCl_2.6H_2O$	24
Na_2MoO_4	36

APPENDIX 2

Purified radiata xylan (1% w/v) was hydrolysed with the thermostable xylanase (pNZ1435) (100 U/g carbohydrate) for 24 hr at 70°C. The enzyme reaction was stopped by heating the solution in a boiling water bath for 15 min. A no enzyme control and a no substrate control were run in parallel. The carbohydrate solutions were freeze-dried.

The following analysis were done by Jurgen Puls of the BFH (Hamburg, Germany). Freeze-dried samples were reconstituted and analyzed by anion exchange chromatography (SECT 2.3.2.5). Chromatograms of the no enzyme and no substrate controls showed no peaks (not shown). A chromatogram of the xylanase hydrolysed radiata xylan is presented in Fig A.

From a comparison of chromatogram A with that of a series of standards (Fig 2.3) peak 1 is identified as xylose, peak 2 as xylobiose and peak 3 as xylotriose. The identity of the compounds in peaks 4 and 5 were unknown.

The hydrolysate was treated with a purified ß-xylosidase (Fig B). Peaks 2 and The hydrolysate was treated with purified ß-xylosidase. This exo-acting ß-xylosidase cleaves unsubstituted, non-reducing terminal xylose residues from xylo-oligomers. After the incubation, the enzyme was denatured by heating. The chromatogram of the ß-xylosidase-treated hydrolysate is presented in Fig B. The peaks labelled in Fig A as 2 and 3 were hydrolysed to xylose by the xylosidase thus confirming these peaks as unsubstituted xylobiose and xylotriose. The two unknown peaks were unaffected by the enzyme. Assuming the unknowns are substituted xylo-oligomers, their resistance towards the xylosidase denotes substitution of the non-reducing, terminal xylose residue.

The ß-xylosidase treated hydrolysate was further treated with an α-L-arabinofuranosidase which cleaves non-reducing arabinose substituents from xylo-oligomers. In the chromatogram of this hydrolysate (Fig C) free arabinose appears (6 min). With the removal of the arabinose side-groups, unknown peak 4 disappeared and the area of unknown peak 5 was substantially reduced. Two new peaks with retention times similar to xylobiose and xylotriose appeared. The former was the larger of the two (Fig C). This suggests that unknown peak 4 was an arabinose substituted xylobiose. The smaller of the two new peaks had a retention

time comparable to xylotriose. This peak originated from the release of an arabinose substituent(s)from a substituted xylotriose (peak 5). Resistance to the ß-xylosidase suggests that the arabinose substituent must have been on the non-reducing, terminal xylose residue. Unknown peak 5 contained other compounds that were not affected by either the ß-xylosidase or the α-L-arabinosidase (Fig C).

The hydrolysate was again treated with the ß-xylosidase (Fig D). This hydrolysed the two new peaks to xylose. Thus confirming their identity as xylobiose and xylotriose originating from peaks 4 and 5 respectively. Therefore, in the original pNZ1435 hydrolysate unknown peak 4 was identified as an arabinose substituted xylobiose while peak 5 contained an arabinose substituted xylotriose. The arabinose substituents must have been on the non-reducing, terminal xylose residue in order to confer resistance to the ß-xylosidase.

Further treatment with a ß-xylosidase and an α-arabinosidase, concurrently, was performed. A chromatogram is given in Fig D. The peak at 21 min was resistant to the action of both enzymes suggesting that this component was methyl glucuronic acid substituted. Furthermore, the disappearance of the "shoulder" from this peak after the second xylosidase treatment suggests that the original substituent (ie: the "shoulder") was a trisaccharide substituted at a central xylose residue. The xylosidase then removed the non-reducing, terminal xylose residue and the still substituted xylo-oligomer merged with the larger peak (24 min).

The hydrolysate was treated with an α-glucuronidase (Fig E). This enzyme released a small amount of xylobiose (16 min, Fig E). The ß-xylosidase hydrolysed this small peak to xylose (not shown). The appearance of xylobiose after the α-glucoronidase confirms that the larger peak (24 min, Fig E) is a methyl glucuronic acid substituted xylobiose.

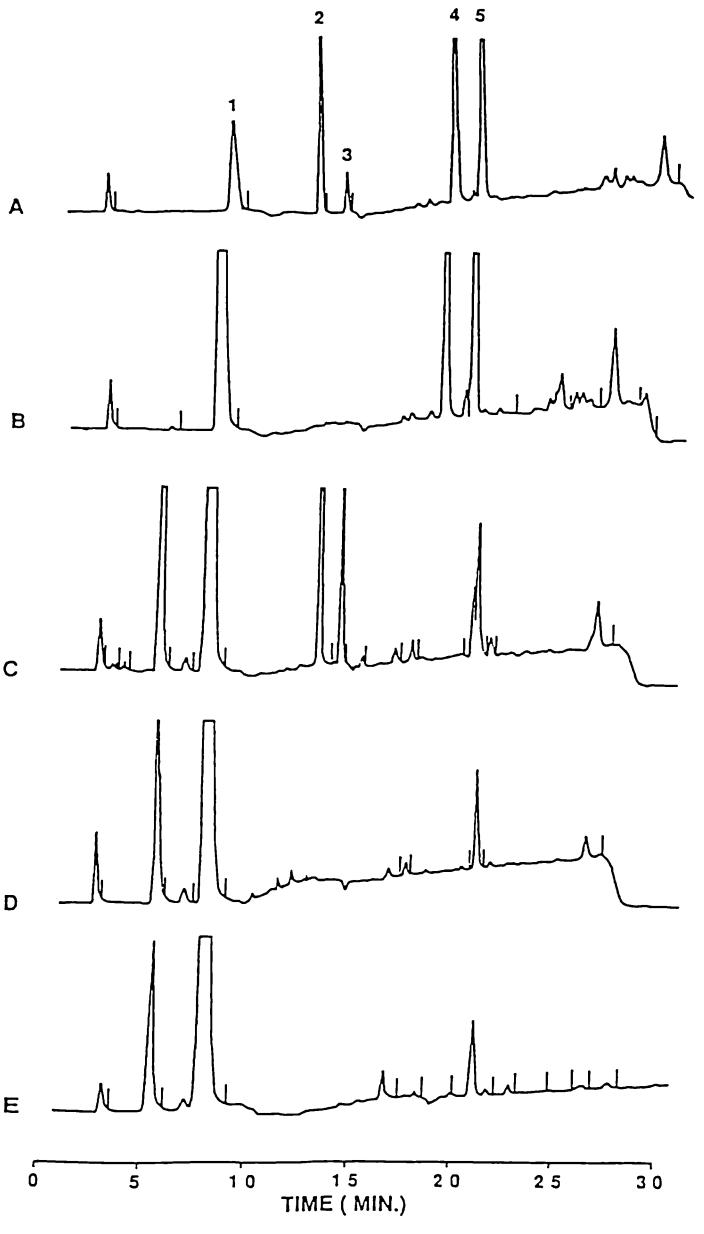


Fig A-E AS DESCRIBED IN TEXT OF APPENDIX 2

Xylo-oligomers identified in pNZ1435 xylanase treated radiata xylan

peak 1 xylose

peak 2 xylobiose

peak 3 xylotriose

peak 4 α-L-arabinofuranosyl-(1,3)-ß-D-xylopyranosyl-(1,4)-ß-D-xylopyranose
(arabino-xylobiose; X_A-X)

peak 5 (major) α-L-arabinofuranosyl-(1,3)-ß-D-xylopyranosyl-(1,4)-ß-D-xylopyranose-(1,4)-ß-D-xylopyranose
(arabino-xylotriose; X_A-X-X)

(minor) 4-O-methylglucuronic acid-(1,2)-ß-D-xylopyranosyl-(1,4)-ß-D-xylopyranose-(1,4)-ß-D-xylopyranose
(aldotetrauronic acid; X-X_{GlcA}-X).

(minor) 4-O-methylglucuronic acid-(1,2)-ß-D-xylopyranosyl-(1,4)-ßD-xylopyranose
(aldotriuronic acid; X_{GlcA}-X).

APPENDIX 3

Chlorine titration calculations

From PAPRO (FRI, Rotorua) laboratory manual (DEC 1988)

Chlorine Water Analysis

- to 100 ml of stirring water add 10 ml of 2% KI, 10 ml of 10% acetic acid and 5 ml of sample.
- titrate to a starch (1%) end point with 0.1N $Na_2S_2O_3$

$$\text{g of } Cl_2 \text{ per } L^{-1} = \frac{\text{titre X 35.36 X N of } Na_2S_2O_3}{\text{5 ml (sample)}}$$

Chlorine Dioxide Analysis

- add 2 ml of sample to 100 ml distilled water and titrate as above to a clear end point (not yellow). Record titre (M) which measures the free chlorine.
- add 10% acetic acid, continue titration to starch end point. Record total tite (T) which measures total chhlorine.

$$\text{g of Free } Cl_2 \text{ per } L^{-1} = \frac{\text{(T-M) X N of } Na_2S_2O_3 \text{ X 67.46}}{\text{4 X 2 ml (sample)}}$$

$$\text{g of Available } Cl_2 \text{ per } L^{-1} \text{ in } ClO_2 = \frac{\text{(M-A/4) X N of } Na_2S_2O_3 \text{ X 35.46}}{\text{2 ml (sample)}}$$

where A = T-M

$$\text{g of Total } Cl_2 \text{ per } L^{-1} = (2.63 \text{ X } ClO_2) + \text{g of Free } Cl_2$$

The total chlorine required set by the chlorine multiple
(ie: total chlorine required = κ X chlorine multiple).

Residual Chlorine

- used to determine residual chlorine in pulp liquor after bleaching.
- as for chlorine water above except that 100 ml of liquor is used.

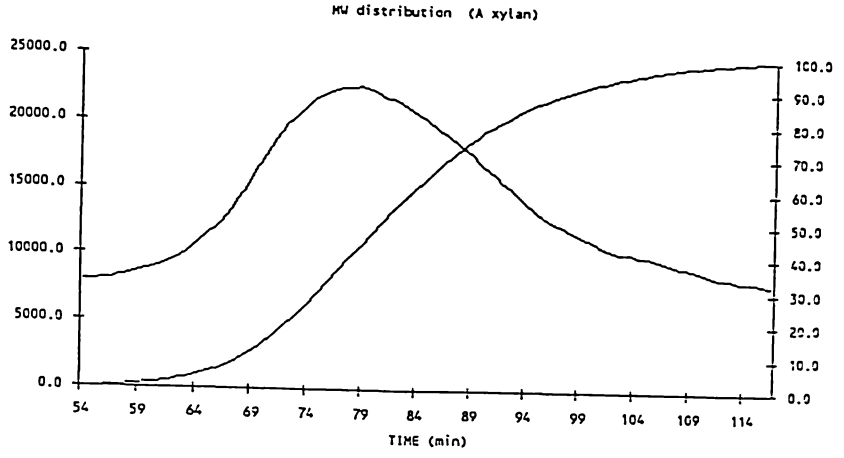
APPENDIX 4

Gel Permeation Chromatograms Of Extracted Hemicelluloses

GPC of hemicelluloses extracted by the Hamilton procedure

A - untreated radiata kraft pulp (xylan, glucomannan ppt and sol)
B - xylanase treated radiata kraft pulp (glucomannan ppt and sol)
C - mannanase radiata kraft pulp (xylan, glucomannan ppt and sol)
D - xylanase + mannanase treated radiata kraft pulp
(xylan, glucomannan ppt and sol)

Chromatograms include molecular weight distribution and % cumulative weight.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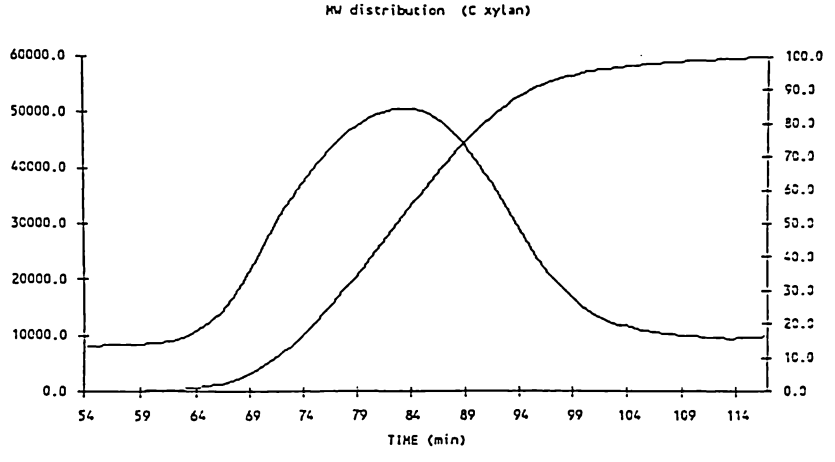
0.0
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
54 59 64 69 74 79 84 89 94 99 104 109 114
TIME (min)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100.0
90.0
80.0
70.0
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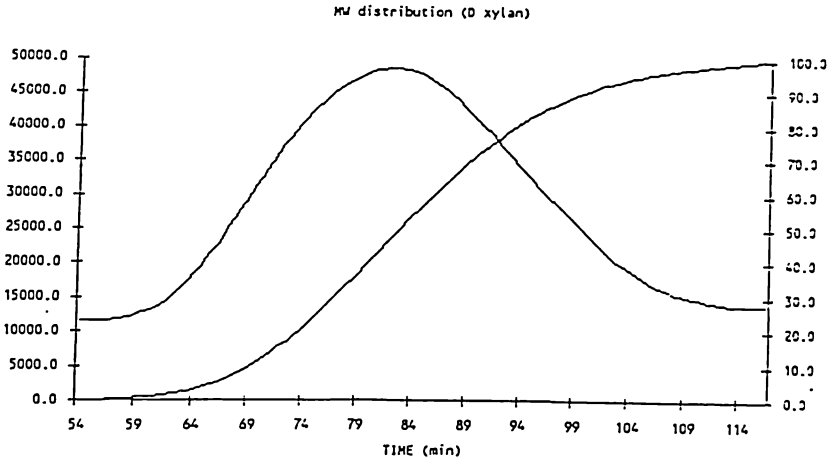
40.0
30.0
20.0
10.0
0.0
54 59 64 69 74 79 84 89 94 99 104 109 114
TIME (min)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100.0
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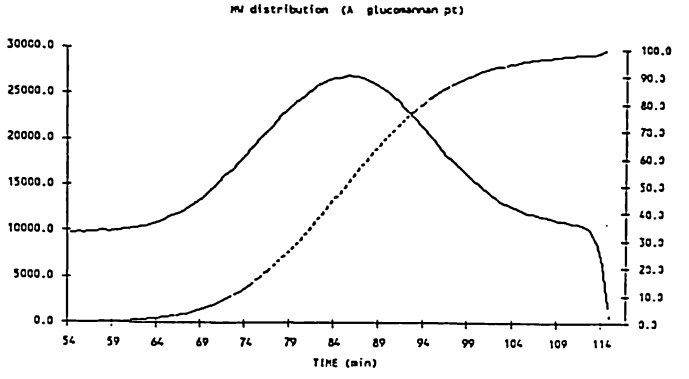
TIME (min)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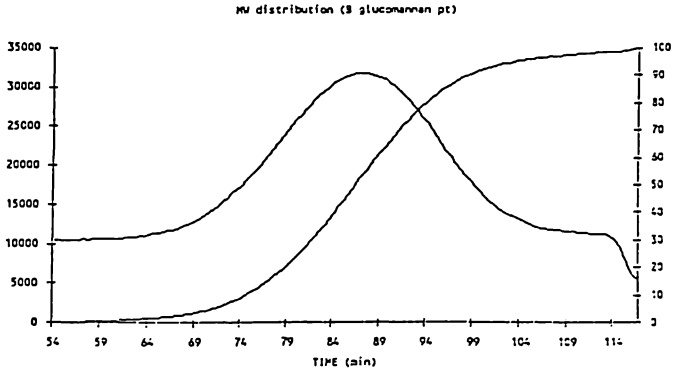
10.0
0.0
54 59 64 69 74 79 84 89 94 99 104 109 114
TIME (min)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90
80
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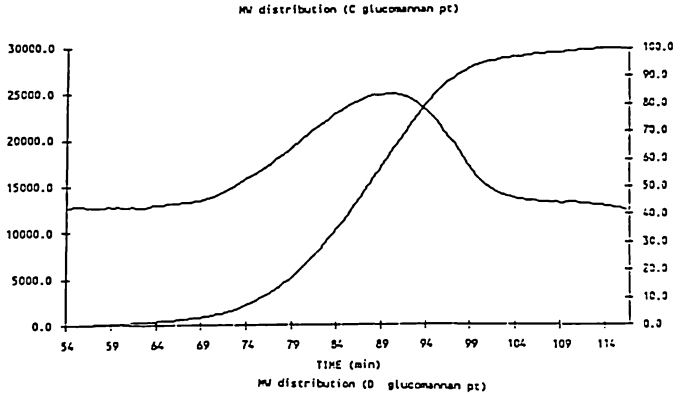
60
50
40
30
20
10
0
54 59 64 69 74 79 84 89 94 99 104 109 114
TIME (min)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10.0
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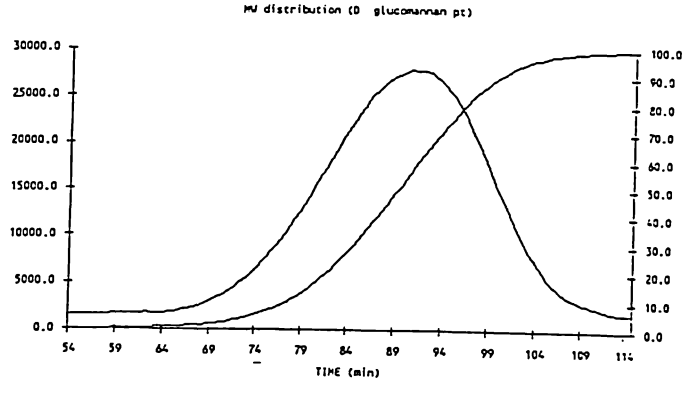
TIME (min)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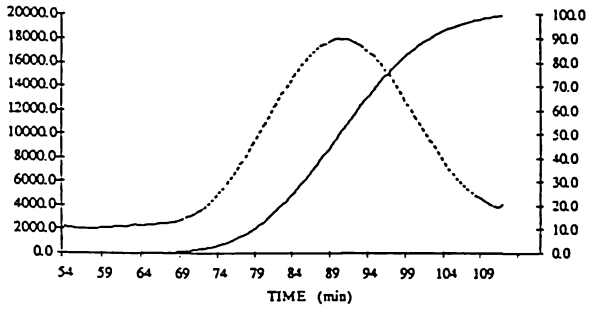
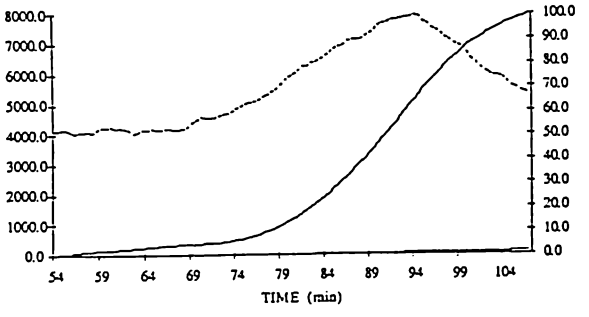
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
54 59 64 69 74 79 84 89 94 99 104 109 114
TIME (min)

MW distribution (A non-ppt glucomannan)



MW distribution (B non-ppt glucomannan)



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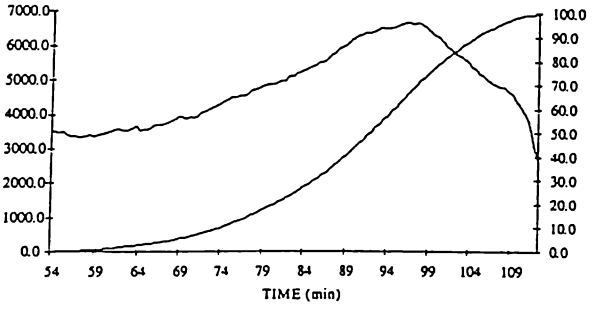
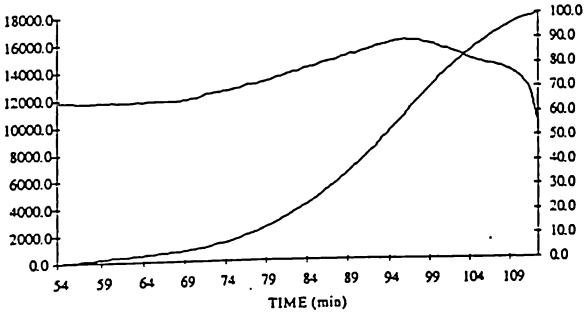


MW distribution (D non-ppt glucomannan)



APPENDIX 5

Identification of manno-oligomers by NMR

manuscript submitted to Carbohydrate Research

^{1}H- and ^{13}C-n.m.r. study of the products of enzymatic treatment of Ivory nut mannan and *Pinus radiata* glucomannan

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(Received ; accepted for publication)

ABSTRACT

Proton and ^{13}C-n.m.r. spectroscopy has been used to characterise the predominant oligosaccharides produced on hydrolysis of Ivory nut mannan and *Pinus radiata* glucomannan by a cloned thermostable mannanase. The oligosaccharides observed are: D-Man*p* (**1**), β-D-Man*p*-(1→4)-D-Man*p* (**2**), β-D-Man*p*-(1→4)-β-D-Man*p*-(1→4)-D-Man*p* (**3**), β-D-Glc*p*-(1→4)-D-Man*p* (**4**) and β-D-Glc*p*-(1→4)-β-D-Man*p*-(1→4)-D-Man*p* (**5**).

INTRODUCTION

There is currently interest in the use of hemicellulases[1] as aids for pulping. To date interest has concentrated on xylanases, which have been shown to improve bleachability resulting in significant savings in bleaching chemical requirements[2]. A thermostable mannanase cloned from "Caldocellum saccharolyticum"[3] has been used to treat Ivory nut mannan and *P. radiata* glucomannan in order to characterise its enzymatic activity[4]. The predominant disaccharides and trisaccharides have been characterised by ^{1}H- and ^{13}C-n.m.r.

RESULTS AND DISCUSSION

Following enzymatic hydrolysis of *P. radiata* glucomannan, the oligosaccharides were separated on the basis of size by gel permeation chromatography. Fractions

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representing trimeric ($R_{glc.}$ 0.93), dimeric ($R_{glc.}$ 0.84) and monomeric ($R_{glc.}$ 1.00) oligosaccharides were collected (Figure 1). The tetrameric fraction was not analysed. The first fraction contained two trimers (**3** and **5**) and the second fraction two dimers (**2** and **4**). They were separated into pure oligomers by h.p.l.c. and the structures assigned by ^{1}H and ^{13}C n.m.r. The third fraction contained only one monomer. Enzymatic hydrolysis of Ivory nut mannan yielded only oligomers **1**, **2** and **3**.

Bring in figure 1 here

Monosaccharide ***1***. - The only monomeric fraction of both hydrolyses showed proton anomeric resonances at 5.20 p.p.m. (H-1α) and 4.92 p.p.m. (H-1β) in a ratio of 2:1. Anomeric ^{13}C-n.m.r. resonances were observed at 95.2 p.p.m. (C-1α) and 94.8 p.p.m. (C-1β). This fraction was found to be mannose[5,6].

Disaccharides ***2*** *and* ***4***. - Disaccharide **2** was obtained from enzymatic hydrolysis of both Ivory nut mannan and radiata glucomannan, where disaccharide **4** was obtained only from hydrolysis of radiata glucomannan.

The ^{1}H-n.m.r. spectrum of fraction **2** shows reducing anomeric resonances at 5.21 (H-1α) and 4.93 p.p.m. (H-1β) in the ratio 2:1 and a non reducing anomeric resonance at 4.76 p.p.m. From the COSY spectrum it is very clear to follow the off-diagonal coupling patterns to obtain assignments for the non-reducing protons, and all but two assignments for the reducing α anomer, which can be assigned from the HOHAHA spectrum. The ^{13}C-n.m.r. spectrum shows 18 signals as expected for a disaccharide with an α, β ratio of 2:1 for the reducing unit. This fraction was determined to be β-D-mannobiose by comparison with other work[7,8]. Proton and ^{13}C chemical shift data are given in Tables 1 and 2.

Bring in Tables 1 and 2 here

The ^{1}H-n.m.r. spectrum of disaccharide **4** shows reducing anomeric resonances at 5.21 (H-1α) and 4.94 p.p.m. (H-1β) which are attributed to mannose. Due to a low signal to noise ratio in the ^{13}C-n.m.r. spectrum only the reducing α signal is observed at 95.3 p.p.m. Non reducing anomeric signals are observed at 4.78 and 4.52 p.p.m. (^{1}H) and 104.2 p.p.m. (^{13}C). This is consistent with a non reducing glucose unit. Fraction **4** is determined to be glucomannose.

Trisaccharides 3 and 5. - As before trisaccharide **3** was obtained from both enzymatic hydrolysis of Ivory nut mannan and radiata glucomannan whereas trisaccharide **5** was obtained only from hydrolysis of the glucomannan.

Fraction **3** shows 23 resolved ^{13}C n.m.r. signals at 100 MHz indicating a trisaccharide although initially the proton spectrum of the mannotriose from *P. radiata* glucomannan showed more anomeric resonances than were first expected. Both HMQC one bond and HMBC long range heteronuclear correlation spectra were acquired in an attempt to explain this observance. The HMQC correlation spectrum shows all four anomeric ^{1}H resonances correlating with C-1' and C-1". The HMBC long range correlation spectrum (Figure 2) shows the two ^{1}H anomeric resonances at higher frequency (4.80 and 4.77 p.p.m.) correlating with C-2' (71.4 p.p.m.) and the two ^{1}H anomeric resonances at lower frequency (4.76 and 4.73 p.p.m.) correlating with C-2" (72.0 p.p.m.).

Bring in Figure 2 here

Reduction of the *P. radiata* fraction **3** with sodium borohydride collapsed the four non-reducing anomeric resonances in the ^{1}H-n.m.r. spectrum to two single resonances (H-1' 4.75 p.p.m. and H-1" 4.68 p.p.m.). This suggests the extra unexplained peaks results from increased shielding of the reducing protons. During extraction barium hydroxide was used to precipitate glucomannan from *P. radiata* and may not have been fully removed from the extracted glucomannan. Barium is known to complex with the hydroxyls of carbohydrates[11]. The C-2 and C-3 hydroxyls of mannose are suitably oriented for complexation to occur. This would result in a high field shift of the reducing anomeric protons. This is further evidenced by addition of barium to the mannotriose from Ivory nut mannan. The barium complex formed produces a ^{1}H-n.m.r. spectrum similar to that initially observed from *P. radiata*. The ^{1}H-n.m.r. spectrum of fraction **3** isolated from the hydrolysis of Ivory nut mannan shows the expected mannotriose anomeric signals at 5.21 (H-1α), 4.94 (H-1β), 4.80 (H-1') and 4.76 p.p.m. (H-1").

Fraction **5**, the trisaccharide resulting from hydrolysis of radiata glucomannan, has a ^{1}H-n.m.r. spectrum showing a reducing mannose (H-1α 5.22 p.p.m., H-1β 4.95 p.p.m.), a non-reducing mannose (4.80 p.p.m.) and a non-reducing glucose (4.53 p.p.m., 7.9 Hz). Comparison of the non-reducing shifts with other work suggests this fraction to be $GlcMan_2$[12]. The ^{13}C-n.m.r. spectrum supports this. Two reducing anomeric signals are seen at 95.0 and 94.9 p.p.m. in an α, β ratio 2:1 and are assigned as the reducing mannose signals. Two non-reducing anomeric signals are seen at 101.2 p.p.m. assigned to C-1 of

mannose in a central position and 104.1 p.p.m. assigned to C-1 of glucose in a terminal position.

EXPERIMENTAL

Extraction of P. radiata glucomannan. - Unbleached radiata kraft pulp was obtained from a local mill. Glucomannan was extracted from the pulp using a modification of the Hamilton procedure[13]. In this process, the pulp is slurried in a 4.4% (w/v) $Ba(OH)_2H_2O$ solution (4% consistency) for 30 minutes. The barium complexes with the glucomannan, allowing caustic extraction of the xylan from the pulp (10% NaOH/2.2% $Ba(OH)_2H_2O$, 2% pulp consistency). After a further 30 minute incubation period, the pulp was filtered and washed with approximately 200 ml of fresh NaOH/$Ba(OH)_2H_2O$ solution. The filtrate contained the xylan component of the pulp.

The xylan-free pulp was washed with water to a final pH of 7. The pulp was slurried in 100 ml of 5% acetic acid (18 hours) which destroyed the Ba-glucomannan complex. The pulp was then washed and extracted with 1% NaOH (150 ml, 30 minutes), filtered and washed with 100 ml of 1% NaOH. This filtrate contained the galactoglucomannan component of the pulp.

The remaining pulp was extracted with 15% NaOH (2% consistency, 30 min), then filtered and washed with 100 ml of fresh 15% NaOH. This filtrate was rich in the glucomannan component of the pulp.

The glucomannan was further purified by the addition of a concentrated $Ba(OH)_2H_2O$ solution (4% final concentration). After a 30 minute incubation period, the solution was centrifuged (15 min, 5000 g) and the pelleted glucomannan dissolved in 100 ml of 2N acetic acid (18 hours). The polysaccharide was precipitated with absolute ethanol (200 ml) and collected by centrifugation. The pellet was dissolved in 20 ml of water and added, drop-wise, to 100 ml of absolute ethanol. The pellet was collected by centrifugation. The previous ethanol precipitation was repeated and the glucomannan was freeze-dried.

The acid hydrolysed polysaccharide had a mannose:glucose ratio of 3.6:1 as compared to 3.9:1 reported by Harwood[14]. Other monosaccharides obtained were galactose (1.6%) and xylose (1.7%) indicating than xylan and galactoglucomannan are present as minor contaminants.

Enzymatic hydrolysis. - Ivory nut mannan (Megazyme, Australia, 98% mannose) was dissolved in 5% (w/v) NaOH and the pH adjusted to 7 with glacial acetic acid. The *P. radiata*

glucomannan was dissolved in water. Both solutions were made up to 0.5% (w/v) carbohydrate. The purified mannanase[2] was added at a dose rate of 200 Units per gram of carbohydrate, and incubated at 70°C for 24 hours. One Unit is defined as the amount of enzyme that produces 1 μmole of reducing sugar per minute. The reaction was stopped by heating for 15 minutes in a boiling water bath.

Gel permeation chromatography. - The oligosaccharides were separated on two 1 m x 1.6 cm i.d. columns packed with Biogel P2 (Pharmacia Ltd). The water jacketed columns were maintained at 60°C and eluted with MilliQ water at 0.5 mL/min. Fractions were detected using a Waters refractive index detector, collected as required, and concentrated under reduced pressure. Relative retention times were compared to the retention time of glucose (600 min.).

H.p.l.c. - The dimeric and trimeric fractions of radiata glucomannan obtained from g.p.c. were further separated using a Waters Dextrapak column. The column was maintained at ambient temperature and eluted with MilliQ water at 0.6 ml/min. Fractions were detected by refractive index, collected and concentrated under reduced pressure.

N.m.r. spectroscopy. - N.m.r. spectra were acquired on Bruker AC-200, AC-300P and AM-400 spectrometers using 5 mm probeheads. Samples were made up in D_2O (ca. 3 mg/mL). Proton spectra were acquired at 313 K, using a presaturation sequence to suppress the residual HOD resonance. The increased temperature moved the HOD resonance from the anomeric proton region to ~4.6 p.p.m. where it could be safely irradiated. Presaturation occurred during the relaxation delay of 5s. A 90° excitation pulse was used. Exponential line broadening of 0.1 Hz was applied. The ^{13}C-n.m.r. spectra were acquired using a power gated composite pulse decoupling sequence at 300 K. Exponential line broadening of 1.0 Hz was applied. All spectra were referenced to external acetone (δ_H 2.170 p.p.m., δ_C 29.8 p.p.m.). Solvent-suppressed double-quantum-filtered phase-sensitive COSY spectra[15,16] were recorded using the (90°)-(t_1)-(90°)-(FID, t_2) sequence (COSYPDHG) as supplied by Bruker. A spectral width of 1506 Hz was used with a data size of 1024 points in F2 and 512 increments recorded. Data in F1 was zero-filled to 1K before Fourier transformation. Homonuclear Hartmann-Hahn (HOHAHA) spectra were acquired with presaturation[17] using the stand HOHAWATR sequence. The MLEV-16 spin lock period was varied between 150 and 220 ms to obtain long range transfers. A spectral width of 1000 Hz with an F2 data size of 2048 points was used. In F1, 256 increments were recorded and zero-filled to 1K before Fourier transformation. HMQC[18] and HMBC[19] heteronuclear correlation spectra (1K x 512 W) were acquired using solvent suppression and a bilinear-rotation sequence to help reduce the intensity of the ^1H-^{12}C signals. The ^{13}C decoupling was achieved using a GARP-1 composite

pulse sequence delivered with Bruker's BFX-5 fast-switching amplifier. Adequate signal-to-noise was achieved using 128 transients for 256 increments.

ACKNOWLEDGEMENTS

The authors are grateful for comments and suggestions from Dr Keith Mackie (FRI) and to Armando McDonald (FRI) for preparation of the *P. radiata* substrate. Thanks are also expressed to Dr Jan Coddington (University of Auckland) for use of the AM-400.

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TABLE 2: ^{13}C chemical shifts (p.p.m.) at 27°C

	C-1	C-2	C-3	C-4	C-5	C-6
mannose α	95.2	71.9	71.4	68.0	73.6	62.1
β	94.8	72.4	74.2	67.8	77.3	62.1
mannobiose						
reducing α	95.1	71.3	70.1	77.6	72.1	62.1[a]
β	94.9	71.8	72.9	77.7	75.9	62.1[a]
non reducing	101.4	71.7	74.0	67.9	78.0	61.7[a]
mannotriose						
reducing α	95.0	71.1	70.1	77.6	72.1	62.2[a]
β	94.9	71.8	72.8	77.6	75.9	62.2[a]
central non reducing	101.3[b]	71.4	72.7	77.9	76.5	61.8[a]
terminal non reducing	101.4[b]	72.0	73.9	67.8	77.9	61.7[a]
glucomannose						
reducing α	95.3	71.8	70.7	78.5	75.2	62.0[a]
β	95.3	72.0	73.5	78.5	76.3	62.0[a]
non reducing	104.2	74.6	77.4	71.2	77.1	61.6[a]
glucomannobiose						
reducing α	95.0	71.6	70.7	78.2	72.3	62.0[a]
β	94.9	72.1	73.1	77.9	76.2	62.0[a]
central non reducing	101.2	71.5	72.7	77.7	76.6	61.9[a]
terminal non reducing	104.1	74.4	77.4	70.1	77.2	61.7[a]

a,b Interchangeable assignments.

FIGURE CAPTIONS

Figure 1. Gel chromatogram of the products of enzymatic hydrolysis of *P. radiata* glucomannan. Unhydrolysed carbohydrate is seen at 220 min.

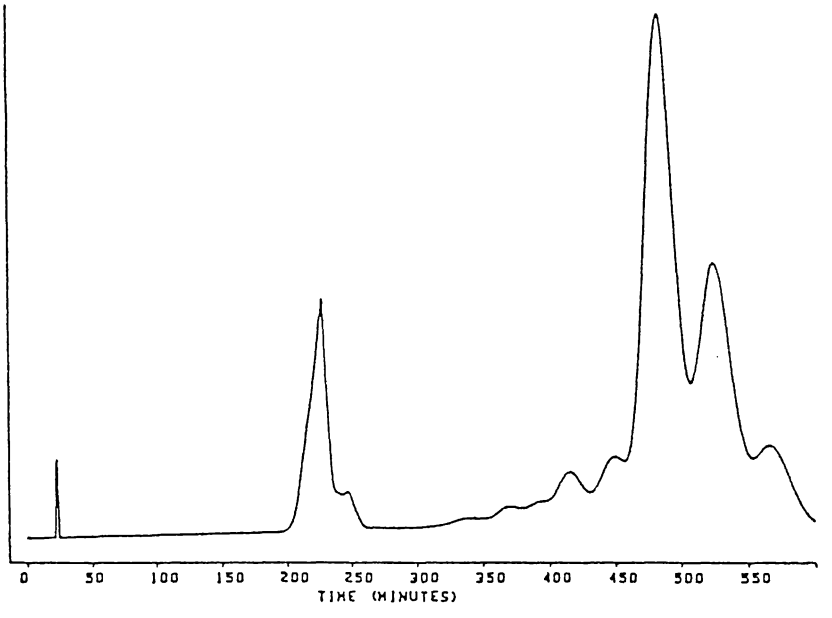

Fig 2 HMBC long range spectrum of mannotriose