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PRODUCTION AND CHARACTERIZATION OF CELLULASES AND XYLANASES FROM THE THERMOPHILIC ASCOMYCETE *Thielavia terrestris* 255B

by

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ABSTRACT:

Enzymatic conversion of lignocellulosic substrates offers opportunities to convert these vast and renewable resources into useful products such as fuel, chemical feedstocks and paper products. Most of the current knowledge on cellulases and xylanases is based on enzymes isolated from mesophilic sources such as *Trichoderma* and *Aspergillus* spp. Thermophilic enzymes could offer many potential advantages in bioconversion processes such as rapid kinetics and reduced risks of contamination. We initiated a study of the cellulases and xylanases produced by *Thielavia terrestris* 255B because this organism was previously shown to produce large amounts of thermostable enzymes (Breuil et al. 1986).

Initially, we studied the production of cellulases and xylanases after growth of *T. terrestris* 255B on various substrates in order to obtain maximum production of these enzymes. We used non-denaturing electrophoretic techniques to compare the profile of enzymes produced on the various substrates. We found that *T. terrestris* 255B produced two major and at least 5 minor endoglucanase components. We discussed the possibility that some of these components might exist as multi-enzyme complexes that were difficult to isolate in an intact form. We purified two cellobiohydrolases and one β-glucosidase which were all partially characterized. One of the cellobiohydrolases (CBHII) seemed to be a major component of the cellulase system as it accounted for about 40.8% of the observed activity when crystalline cellulose was used as the substrate.

T. terrestris 255B produced two major forms of xylanases with pI's of 4.6 (xylanase I) and 6.1 (xylanase II). The latter enzyme could be purified to >99% homogeneity using anion-exchange chromatography and gel filtration. Xylanase II had a molecular mass of 25.7 kDa (SDS-PAGE) and optimal pH and temperature of 3.6-4.0 and 60-65°C, respectively. The activity of xylanase II was very specific

towards the hydrolysis of xylan and had extremely low activity on cellulose. Thus, as it had potential application for the pre-bleaching of kraft pulps, the characterization of this enzyme became the main focus of the work.

We used amino acid composition and partial amino acid sequencing to demonstrate that xylanase II belonged to a family of low molecular weight xylanases which had been previously designated the G family by Gilkes et al. (1991b). Currently, xylanase II is the only thermophilic xylanase that has been shown to belong to the G family of β-1,4-glycanases. Xylanase II has one disulfide bridge and it is possible that this might account for its higher thermostability when compared to the other members of the G family.

We studied the mode of action of xylanase II and compared it with a 32-kDa xylanase derived from *Thermoascus crustaceus*. This latter enzyme is a thermophilic xylanase which appears to belong to the F family of β-1,4-glucanases. Xylanase II was more efficient at solubilizing insoluble xylan and yielded hydrolysis products with higher degrees of polymerization than the 32-kDa xylanase. Xylanase II could not cleave xylotriose although it cleaved xylotetraose to xylobiose and xylotriose using a process involving transxylosidation. The 32-kDa xylanase could cleave both xylotriose and xylotetraose to xylobiose and xylose. Xylose was a major product of xylan hydrolysis by the 32-kDa xylanase while it was only a minor product when the hydrolysis was performed with xylanase II.

Although xylanase II and the 32-kDa xylanase seem to belong to different families of xylanases and had different modes of action, they did not show any cooperative hydrolysis action when they were added to xylans from various sources (cereal, hardwoods and softwoods). The profile of products obtained when the two thermophilic xylanases were used together was almost identical to the profile obtained when the 32-kDa xylanase acted alone.

Previously, it has been shown that xylanase pre-treatment could reduce the amount of chlorine required to bleach kraft pulp and/or help develop chlorine-free bleaching processes (Viikari et al. 1986). The two thermophilic xylanases were able to remove between 8 to 20% of the residual xylan in a poplar kraft pulp. This amount of xylan removal is considered to be sufficient to significantly decrease the chemical loadings required to bleach kraft pulp and consequently decrease the amount of chloro-organics detected in pulp mill effluents. We discuss the possibility that these two thermophilic xylanases could be used in the development of high-temperature processes for the pre-bleaching of kraft pulps.

RÉSUMÉ:

La conversion de substrats lignocellulosiques à l'aide d'enzymes offre l'opportunité de convertir ces ressources vastes et renouvelables en des produits utiles tels que des carburants de remplacement, des produits chimiques et des produits du papier. La majorité des connaissances actuelles sur les cellulases et les xylanases a été obtenue par l'étude d'enzymes provenant d'organismes mésophiles tels que diverses souches de champignons des genres *Trichoderma* et *Aspergillus*. Des enzymes thermophiliques offriraient de nombreux avantages potentiels tels que des vitesses de réaction plus rapides et des risques réduits de contamination. Nous avons initié une étude des cellulases et des xylanases produites par *T. terrestris* 255B parce que des travaux précédents ont démontré que cet organisme produisait des quantités abondantes d'enzymes thermostables (Breuil et al. 1986).

Nous avons d'abord étudié la production de cellulases et de xylanases lorsque *T. terrestris* 255B est poussé sur divers substrats de façon à obtenir une production maximale de ces enzymes. Nous avons utilisé des techniques d'électrophorèse non-dénaturantes pour comparer les profils d'enzymes produites sur les divers substrats. Nous avons trouvé que *T. terrestris* 255B produisait deux endoglucanases majeures et au moins 5 endoglucanases mineures. Nous discutons de la possibilité que certaines endoglucanases puissent exister sous forme de complexes qui seraient difficiles à isoler de façon intacte. Nous avons purifié deux cellobiohydrolases et une β-glucosidase que nous avons aussi partiellement caractérisées. Une des cellobiohydrolases (la CBHII) semblait être la composante majeure du système cellulasique car elle était responsable d'environ 40.8% de l'activité sur la cellulose cristalline.

T. terrestris 255B produit 2 formes majeures de xylanases qui ont des pI's de

4.6 (xylanase I) et de 6.1 (xylanase II). Nous avons purifié cette dernière enzyme à un niveau d'homogénéité supérieur à 99% par l'utilisation de chromatographie sur échangeur d'ions et par filtration sur gel. La xylanase II possède un poids moléculaire de 25.7 kDa (par SDS-PAGE) et un pH et une température optima respectivement de 3.6-4.0 et 60-65°C. La xylanase II est très spécifique pour l'hydrolyse du xylane et possède très peu d'activité sur la cellulose. Donc, la xylanase II pourrait être utile pour le pré-blanchiment des pâtes de papier kraft et cette enzyme est devenue l'objet principal de nos travaux subséquents.

Nous avons déterminé la composition en acides aminés et partiellement la structure primaire de la xylanase II afin de démontrer que cette enzyme appartenait à une famille de xylanases de faible poids moléculaire désignée "famille G" par Gilkes et al. (1991b). Présentement, la xylanase II est l'unique xylanase thermophilique dont l'appartenance à la famille G a été clairement démontrée. La xylanase II possède un pont disulfure et nous discutons de la possibilité que cet élément structural soit responsable de la thermostabilité supérieure de la xylanase II comparativement aux autres membres de la famille G.

Nous avons étudié le mode d'action de la xylanase II et nous l'avons comparé avec celui d'une xylanase de 32 kDa de *Thermoascus crustaceus*, qui est une xylanase appartenant à la famille F. La xylanase II est plus efficace pour solubiliser le xylane insoluble et donne des produits finaux avec des degrés de polymérisation supérieurs que la xylanase de 32 kDa. La xylanase II ne peut couper le xylotriose mais coupe le xylotétraose en xylotriose et xylobiose en utilisant un mécanisme implicant la transxylosidation. La xylanase de 32 kDa est capable de couper le xylotriose et le xylotétraose en xylobiose et xylose. Le xylose est un produit majeur de l'hydrolyse du

xylane par la xylanase de 32 kDa alors qu'il est un produit mineur lorsque l'hydrolyse est faite avec la xylanase II.

Bien que la xylanase II et la xylanase de 32 kDa appartiennent à des familles différentes d'enzymes et qu'elles aient des modes d'action différents, nous n'avons pas observé d'effet synergétique ou coopératif lors de l'hydrolyse de xylanes provenant de diverses sources (céréales, bois mous et bois francs). Lorsque les deux xylanases thermophiliques ont été utilisées ensemble, le profil des produits obtenus était presqu'identique à celui obtenu lorsque la xylanase de 32-kDa agissait seule.

Le pré-traitement par des xylanases pourrait permettre de réduire la quantité de chlore requise pour blanchir les pâtes kraft et/ou d'aider à développer des procédés de blanchiment sans utilisation du chlore (Viikari et al. 1986). Les deux xylanases thermophiliques ont été capables d'enlever de 8 à 20% du xylane résiduel dans une pâte kraft provenant du peuplier. L'enlèvement de telles quantités de xylane résiduel est considéré comme suffisant pour réduire de manière significative les quantités de produits chimiques nécessaires pour blanchir les pâtes kraft et ainsi réduire les émissions de produits organo-chlorés dans les effluents des industries. Nous discutons de la possibilité que les deux xylanases thermophiliques soient utilisées pour développer des procédés de bioblanchiment des pâtes kraft à des températures élevées.

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LIST OF ABBREVIATIONS:

A.a.	Amino acid
A_{280}	Absorbance (280 nm)
CAPS	3-(cyclohexylamino)-1-propanesulfonic acid
CBH	Cellobiohydrolase
CMC	Carboxymethylcellulose
CNBr	Cyanogen bromide
Da	Dalton
DNS	3,5-Dinitrosalicylic acid
DTT	Dithiothreitol
EG	Endoglucanase
EM	Electron microscopy
FP	Filter paper
HMW	High molecular weight
HPLC	High performance liquid chromatography
IEF	Isoelectrofocusing
kDa	Kilodalton
KP	Kraft pulp
LMW	Low molecular weight
MW	Molecular weight
O.D.	Optical density
PAGE	Polyacrylamide gel electrophoresis

PAS	Periodic acid-Schiff
pI	Isoelectric point
PKP	Poplar kraft pulp
PMSF	Phenylmethylsulfonyl fluoride
pNPC	para-nitrophenyl-cellobioside
pNPX	para-nitrophenyl-xylopyranoside
PTH	Phenyl thiohydantoin
PVDF	Poly(vinylidine difluoride)
R_f	Relative mobility
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SN	Somogyi-Nelson
TLC	Thin layer chromatography

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1.0 INTRODUCTION:

1.1 BACKGROUND:

Cellulose and hemicelluloses are the most abundant renewable resources and these substrates represent a vast potential as a source of food, fuel and chemical feedstocks. Enzymatic conversion of lignocellulosic substrates may achieve better yields than chemical processes, thus offering opportunities to turn a major portion of the agricultural and forestry wastes into useful by-products. Enzymes specific for the hydrolysis of xylans have also been suggested as potential bleaching agents in the pulp and paper industry (Viikari et al. 1986).

No economically viable process yet exists for the use of enzymes in the wood processing industries. It is generally accepted that the mechanisms involved in wood-degradation are poorly understood and that the identity of all the factors (enzymatic and non-enzymatic) involved in wood-degradation and their mode of action is still not well defined. Research in this field is further complicated by the heterogenous nature of the process, where an insoluble substrate is attacked by soluble enzymes that give products that are both soluble and insoluble. Gaining a better understanding of wood-degradation processes has been identified as one of the key factors in developing efficient bioconversion strategies.

Other industrial applications of enzymes, such as those in the starch-industry, have progressively increased the use of thermophilic enzymes. Thermostable enzymes offer many potential advantages which include rapid kinetics, reduced risks of contamination and increased opportunity for enzyme recycling. However, there is little information available in the literature on thermophilic cellulases and xylanases when compared to the large amount of work that has been carried out on enzymes from mesophilic fungi such as *Trichoderma* spp. and *Aspergillus* spp.

The thermophilic ascomycete *Thielavia terrestris* 255B was previously shown to

produce large amounts of thermostable cellulases and xylanases (Breuil et al. 1986). Initially, we studied the production of cellulases and xylanases by this organism when it was grown on various substrates. We used electrophoretic techniques to identify the xylanolytic and cellulolytic enzymes produced and the major enzyme components were purified in order to study their properties and their modes of action. After an initial comparison of some of the enzymes, we concentrated most of our efforts on the purification and characterization of one of the xylanase components. This thermophilic xylanase was then assessed for its action on various substrates.

1.2 STRUCTURE AND CHEMICAL COMPOSITION OF WOOD CELL-WALL:

The structure and chemical composition of wood has a significant influence on its degradation by microorganisms and the type of cell, chemical composition and cell-wall morphology may all influence the type of degradation that can occur. Wood is composed of three main organic materials including cellulose (41-45%), hemicelluloses (23-30%) and lignin (19-33%) while other components which are present include extractives (approx. 5%) and inorganic materials (approx. 0.3%). On a dry weight basis, wood contains less than 0.1% nitrogen (Barton and Brownell 1981). Differences in the chemical composition occur among various wood species and also among different types of cells within one kind of wood.

Within the layers of wood cell-wall, the cellulose molecules are aggregated into bundles called microfibrils. The fibrillar arrangement of cellulose molecules gives tensile strength and flexibility to the fibres. Lignin interpenetrates the fibrils and strengthens the cell-wall while hemicellulose is thought to provide the intimate interlacing and bonding between the lignin and cellulose.

1.2.1 CHEMICAL AND PHYSICAL STRUCTURE OF CELLULOSE:

Cellulose is a linear polymer of up to 15,000 anhydroglucose residues with glucose units in the chair configuration held together by β-1,4 linkages. Each residue

is rotated 180° with respect to its adjacent residue on the polymer (Coughlan 1985) and as a result, the basic repeated unit is cellobiose. The degree of polymerization of cellulose chains varies depending on its source and its mode of preparation. The degree of polymerization for cellulose from wood has been reported to vary from 7000 to 10300 from wood (Fengel and Wegener 1983) while cotton has a degree of polymerization of 15300. Cellulose chains are oriented in a parallel fashion and they associate to form insoluble fibrils with hydrogen bonding occurring between successive and adjacent glucose residues. The hydrogen bonding network gives rigidity to the cellulose chains and cellulose fibrils have been shown to have areas that are in a crystalline state and other areas that are less well-ordered (amorphous).

In wood, cellulose microfibrils are generally 3-5 nm in diameter (Fig. 1) and are cemented together by hemicelluloses and lignin (Fengel, 1971). The wood needs to be pretreated in order to expose the cellulose to enzymes for hydrolysis. Relatively harsh chemical treatments are necessary to separate the cellulose microfibrils from the bonding agents and these treatments can cause significant degradation of the cellulose microfibrils. Thus, it is difficult to study both the ultrastructure of native microfibrils in wood as well as the action of cellulases on these microfibrils at the microscopic level.

Some workers have used cellulose from bacterial and algal origins as models for the study of the cellulose ultrastructure. The microfibrils from these sources are generally larger and less strongly associated with other polymers than those from wood. Some *Acetobacter* spp. secrete 3-6 nm wide cellulose microfibrils that aggregate into ribbons (Kuga and Brown 1988). These ribbons can be disrupted by mechanical and acid treatments to release the individual microfibrils. Microfibrils of 20-30 nm diameter and over 200 nm length can be prepared from the cell-wall of the unicellular alga *Valonia ventricosa* (Bourret et al. 1972). Each microfibril is considered to consist of a single microcrystal with no obvious sub-structural

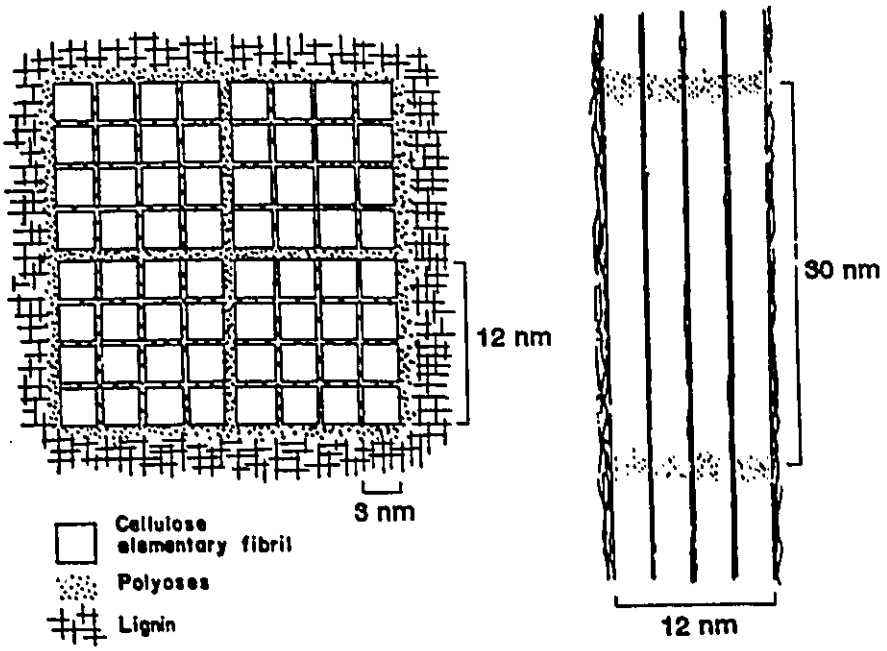


FIGURE 1: Model of the ultrastructural organization of the cell wall components of wood (From Fengel, 1971).

organization. The crystallinity of isolated microfibrils was considered to be close to 100% (Bourret et al. 1972). The size and crystalline perfection of the cellulose microfibrils from *Valonia ventricosa* make them suitable subtrates for studying cellulose degradation using electron microscopy.

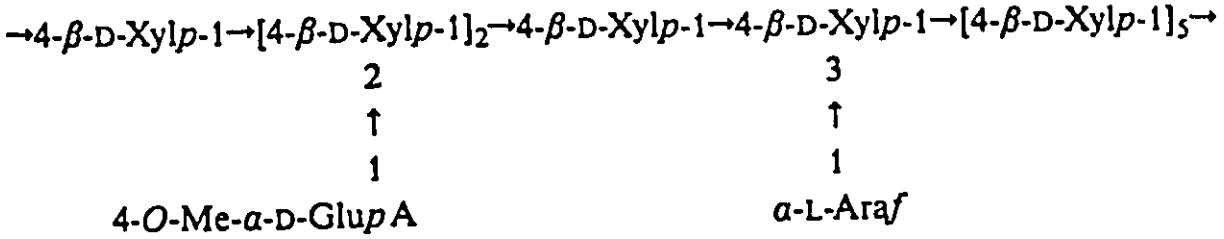
1.2.2 CHEMICAL AND PHYSICAL STRUCTURE OF HEMICELLULOSES:

The hemicelluloses are the major non-cellulosic polysaccharides of the cell-wall and they constitute about 23-30% of the dry weight of hardwoods and softwoods (Barton and Brownell 1981). The hemicelluloses are formed from a number of sugars, the most important being glucose, galactose, mannose, xylose and arabinose. They are structurally more complex than cellulose and generally occur in an amorphous form. Softwoods contain two major types of hemicelluloses: galactoglucomannan (12-18% of the wood) and xylan (7-14% of the wood). Hardwood hemicelluloses are mostly xylans (19-26% of the wood) although glucomannan is also present (3-5% of the wood).

When looking at the hemicellulose degrading enzymes from *T. terrestris* 255B, we limited our study to the degradation of xylans. Xylans are heterogeneous polysaccharides consisting mainly of linear chains of β-1,4 linked D-xylosyl residues. Depending on the source, substituents such as α-L-arabinofuranosyl, 4-O-methyl-glucuronosyl and acetyl groups can be present at various frequencies. Esterification of arabinosyl substituents with ferulic and coumaric acids have been shown in some hemicelluloses (Mueller-Harvey et al. 1986). It has also been suggested that xylans in plant cell-walls may be cross-linked with lignin by diferulate bridges (Hartley and Jones 1976).

Hardwood xylans are classified as acetylated glucuronoxylans (Fig. 2) and seven out of ten xylose units in the backbone are generally substituted with an acetyl group at the C-2 or C-3 carbons. One 4-O-methyl-α-D-glucuronic acid residue is 1,2

Arabinoglucuronoxylan



Glucuronoxylan

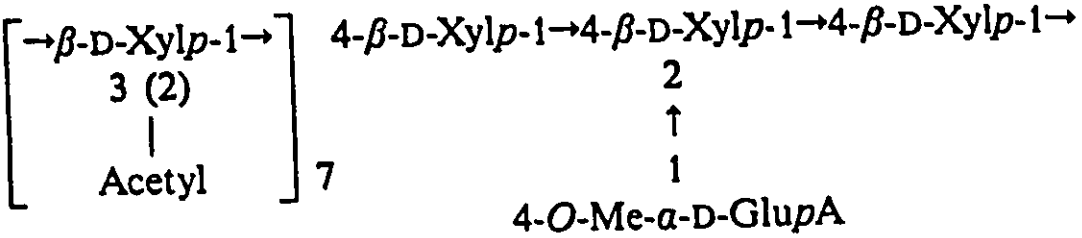


FIGURE 2: Structure of wood xylans. Arabinoglucuronoxylans are the major xylans in softwoods while acetylated glucuronoxylans are the major xylans in hardwoods. (From Erikkson et al. 1990)

linked to the xylan backbone approximately every 10 xylose residues (Eriksson et al. 1990). Softwood xylans are classified as arabinoglucuronoxylans and the xylan backbone is substituted at C-2 with 4-O-methyl-α-D-glucuronic acid groups with approximately two such units per ten xylose residues. The xylan backbone is also substituted with α-L-arabinopyranosyl residues approximately every 10 residues. The xylans in grasses and cereals are generally arabinoxylans (Wilkie 1979).

1.3 BIODEGRADATION OF WOOD:

Wood is relatively resistant to biodegradation because of the crystallinity of cellulose and the recalcitrance of lignin. In nature, microbial degradation will often follow mechanical damage or injury to the tree. Relatively few types of micro-organisms can cause extensive decay of wood. In most cases, a combination of microorganisms will have to act to achieve complete degradation of wood.

Fungi are the major decomposers of wood because of the penetrating effect of their hyphae. The type of wood-decay caused by fungi is generally classified into three groups: white-rot, brown-rot and soft-rot. White-rot and brown-rot decays are generally caused by basidiomycetes while soft-rot decay is caused by either ascomycetes or deuteromycetes. *Thielavia terrestris* belongs to the group of soft-rot fungi which cause a decay characterized by the soft appearance of the wood surface. Soft-rot decay tends to occur on wood that has a high moisture content. The soft-rot fungi attack mainly the polysaccharides and moderately the lignin. Their mycelia develop along the cellulose microfibrils in the S_2 layer of the wood cell-walls and cause cavities resulting from the solubilization of cellulose.

Both aerobic and anaerobic bacteria have been shown to degrade lignocellulosic substrates under conditions of high humidity such as those which occur in soil litter or wood stored under water. Bacteria probably play important roles in degradation processes caused by consortia of microorganisms.

1.3.1 ENZYMATIC DEGRADATION OF CELLULOSE:

Cellulose is chemically very simple as it is composed of an unbranched polymer of glucose units. However, it is physically complex and exists under different amorphous and crystalline states. Hydrolysis of cellulose involves an insoluble substrate which is attacked by soluble enzymes to yield products that are both soluble and insoluble. Many distinct enzymes are necessary to achieve efficient hydrolysis of cellulose and the complexity of the cellulolytic enzyme systems is probably a reflection of the complexity of the mechanisms required to hydrolyze cellulose.

1.3.1.1 Characterization of the cellulolytic enzyme components:

The detection and definition of the enzymes involved in cellulose hydrolysis have been influenced by the substrates available to assay cellulases (Table 1). The 3 major types of enzymes that are thought to be involved in cellulose hydrolysis are:

- Endoglucanases (1,4-β-D-glucan 4 glucanhydrolase, EC 3.2.1.4): cleave β-1,4 linkages inside cellulose chains.
- Cellobiohydrolases (1,4-β-D-glucan cellobiohydrolase EC 3.2.1.9): release cellobiose units from non-reducing ends in an exo-type mechanism (often called exoglucanase).
- Cellobiase or β-glucosidase (β-D-glucoside glucohydrolase EC 3.2.1.21): cleave cellobiose and soluble oligomers to glucose.

The filter-paper assay has been used as a method for quantifying overall hydrolytic activity. The hydrolysis products can be quantified by assaying the reducing sugars by colorimetric methods or by HPLC analysis of the sugars. Considerable effort has been devoted to the standardization of the conditions of this assay in order to allow comparison of cellulase activities between laboratories (Esterbauer et al. 1992, Ghose 1987, Mandels et al. 1976). It was determined that both rigorous standardization of the assay conditions and HPLC analysis of the products are necessary to obtain values which represent the hydrolytic potential of a cellulase

TABLE 1: Various assays for hydrolytic enzymes involved in cellulose degradation (Coughlan, 1985):

Enzyme assayed	Substrate used	Product or property measured
Complete cellulase	Filter paper Avicel Solka Floc	Release of reducing sugars Loss of weight Decrease in turbidity of solutions
	Cotton	Decrease in tensile strength of fibers
	Dyed cellulose	Release of dye
	Cellulose-agar	Clarification of agar
Endoglucanase	Carboxymethyl cellulose Hydroxyethyl cellulose Cello-oligosaccharides	Release of reducing sugars Decrease in viscosity
	Dyed cellulose derivatives	Release of dye
	CMC-agar	Clarification of agar
Cellobiohydrolase	Crystalline cellulose Amorphous cellulose	Reducing sugars Cellobiose
	p-nitrophenyl-β-cellobioside p-nitrophenyl-β-lactoside	p-nitrophenol
β-Glucosidase	Cellobiose Cello-oligosaccharides	Glucose
	Salicin	Reducing sugars
	p-nitrophenyl-β-glucoside	p-nitrophenol
	4-methylumbelliferyl-β-glucoside	4-methylumbelliferone

system (Schwald et al. 1988).

Endoglucanases can be assayed using cellulose derivatives such as carboxy-methyl-cellulose (CMC). Endoglucanases can cleave between unsubstituted residues inside the cellulose chain and will cause depolymerisation of CMC with the release of reducing sugars. The carboxymethyl groups will prevent any extensive action of cellobiohydrolase which can only act on unsubstituted residues from the non-reducing ends of the polymer.

Cellobiohydrolases (CBH) can be assayed by measuring the production of cellobiose from crystalline cellulose or from soluble oligomers. It can also be assayed by measuring the release of chromophoric groups from cellobioside derivatives. However, all these substrates are susceptible to attack by the other cellulolytic enzymes which are probably also present in complex cellulase mixtures. Thus, direct measurement of CBH activity in unpurified preparations is not possible. Some researchers have turned to the use of monoclonal antibodies to quantify CBH in crude mixtures (Kolbe and Kubicek, 1990).

The β-glucosidases can be assayed by measuring the release of glucose from cellobiose or from aryl-glucoside derivatives. Chromophoric or fluorescent groups have also been coupled to glucose for assays where their release by β-glucosidases is monitored.

Other enzymes that are thought to be involved in cellulose hydrolysis are cellobiose oxidases (EC 1.1.99.18), cellobiose dehydrogenases (EC 1.1.5.1), cellobiose phosphorylases (EC 2.4.1.20) and lactonases (EC 3.1.1.17). These enzymes have been characterized in only some cellulolytic systems (Eriksson et al. 1990, Coughlan 1985). Their roles have not been clarified and these enzymes could simply be part of alternative pathways used to metabolize cellobiose. However, some authors believe

that oxidative activity is needed to disrupt the crystallinity of cellulose (Eriksson et al. 1974) while others believe that the oxidative enzymes could transform hydrolysis products to more potent inducers (Iyayi et al. 1989).

1.3.1.2 Microorganisms producing cellulases:

A large number of microorganisms can degrade cellulose in nature. As mentioned previously, white-rot, brown-rot and soft-rot fungi have long been known to produce cellulases. Anaerobic rumen-inhabiting fungi have more recently been shown to be highly cellulolytic (Wood et al. 1986). Cellulase production by aerobic and anaerobic bacteria have also been widely reported (Eriksson et al. 1990). However, many microorganisms secrete an "incomplete" cellulase system which can only hydrolyze amorphous or soluble derivatives of cellulose. In nature these organisms are probably associated with other microorganisms which can achieve degradation of crystalline cellulose.

The white-rot fungus most commonly studied for cellulase production is *Phanerochaete chrysosporium*. Among brown-rot fungi, cellulases from *Poria placenta* have been the most extensively studied (Highley et al. 1981). Numerous studies on cellulase production by soft-rot fungi have been carried out. Some of the most studied organisms include *Trichoderma* spp., *Fusarium solani* and *Penicillium funiculosum* which are all known to produce complete cellulase systems. Most of the current concepts on regulation, production and structure-function relationships of cellulases were derived from studies on cellulases from *Trichoderma reesei*. This organism was originally isolated from a rotting cartridge belt in the jungles of New Guinea. This study was initiated because the U.S. army was concerned about the rapid decay of uniforms during the Second World War (Coughlan 1985). Since its isolation, *Trichoderma reesei* has been subjected to series of mutation programs and several hyperproducing mutants have been obtained. Secretion of up to 2% extracellular protein and up to 25 filter paper IU/mL have been reported (Hendy et

al. 1984). Because of their industrial potential and their production in large amounts, the cellulases from *Trichoderma* spp. have been studied very extensively. In fact, some authors have expressed concerns that the majority of the detailed description of fungal cellulases are strongly biased because the cellulases of only a few producers (generally of industrial interest) have been studied in any detail (Goyal et al. 1991).

Cellulases of bacterial origins have been recently reviewed by Gilkes et al. (1991a), Eriksson et al. (1990) and Robson and Chambliss (1989). Bacteria generally produce less cellulases than fungi but their specific activity (U/mg protein) is generally comparable to that of fungal cellulases (Saddler 1986, Gilkes et al. 1991a). The most studied bacterial producers of cellulases are from the genera *Clostridium, Cellulomonas, Bacillus, Streptomyces, Thermonospora and Bacteroides.*

1.3.1.3 Regulation of cellulase production:

Cellulases are inducible enzymes with cellulose, cellulose derivatives, cellobiose, cellobiolactone, sophorose (2-O-β-D-glucopyranosyl-D-glucose) and lactose known to act as inducers in some organisms (Bisaria and Mishra 1989, Eriksson et al. 1990). In virtually all of the microorganisms examined so far, growth on cellulose resulted in the best production of cellulases while glucose repressed cellulase synthesis. Due to its insoluble nature, cellulose can not directly induce cellulase production and it is therefore generally believed that low constitutive levels of cellulases initiate the attack on cellulose and hydrolysis products would then act as inducers. Some groups have suggested that membrane bound β-glucosidases transform hydrolysis products into inducers through a transglycosylation action (Umile and Kubicek 1986). However, there is still no clear indication of what molecules are responsible for the induction of cellulases.

Soluble molecules such as sophorose and lactose do not induce cellulase production in every organism (Mandels 1982). In organisms where these soluble

sugars can act as inducers, the levels of production are generally lower than those obtained when cellulose is used as the inducer. When present at high concentrations, these substrates may also cause catabolite repression because they are relatively easy to metabolize. It is probable that cellulase production is regulated by differences between the amount of inducing molecules and the availability of easily metabolizable substrates.

1.3.1.4 Mechanism of cellulose degradation:

As mentioned earlier, the mechanisms by which the different cellulases can hydrolyze crystalline cellulose are still unresolved (Goyal et al. 1991, Shimada and Takahashi 1991). The initial hypothesis was that an amorphogenesis factor ("C_1" factor) would initiate the attack by disrupting the crystalline organization of the cellulose chains (Reese et al 1950). This would allow endo-type cellulases to cleave the exposed chains and give rise to soluble products. Different enzymatic and non-enzymatic factors have been suggested as being responsible for the amorphogenesis step (Krull et al. 1988, Vaheri 1983, Koenigs 1974, Eriksson 1969). However, nobody has clearly demonstrated the role of these factors in the hydrolysis of crystalline cellulose.

A generalized scheme for cellulose hydrolysis is shown in Figure 3. Endoglucanases initiate the attack on amorphous regions of the cellulose substrate and the action by endoglucanases would create multiple sites for the CBHs which then remove cellobiose units from the non-reducing ends. The combined action of endoglucanases and CBHs produces soluble oligomers and cellobiose which are cleaved to glucose by β-glucosidases. Synergism between endoglucanases and CBHs has been observed by many authors (Coughlan 1985, Eriksson and Wood 1985, Finch and Roberts 1985). Synergistic action is necessary because intact cellulose has very few sites which would be accessible to individual endoglucanase and CBH action. By cleaving inside the cellulose chains, endoglucanases produce more non-reducing ends

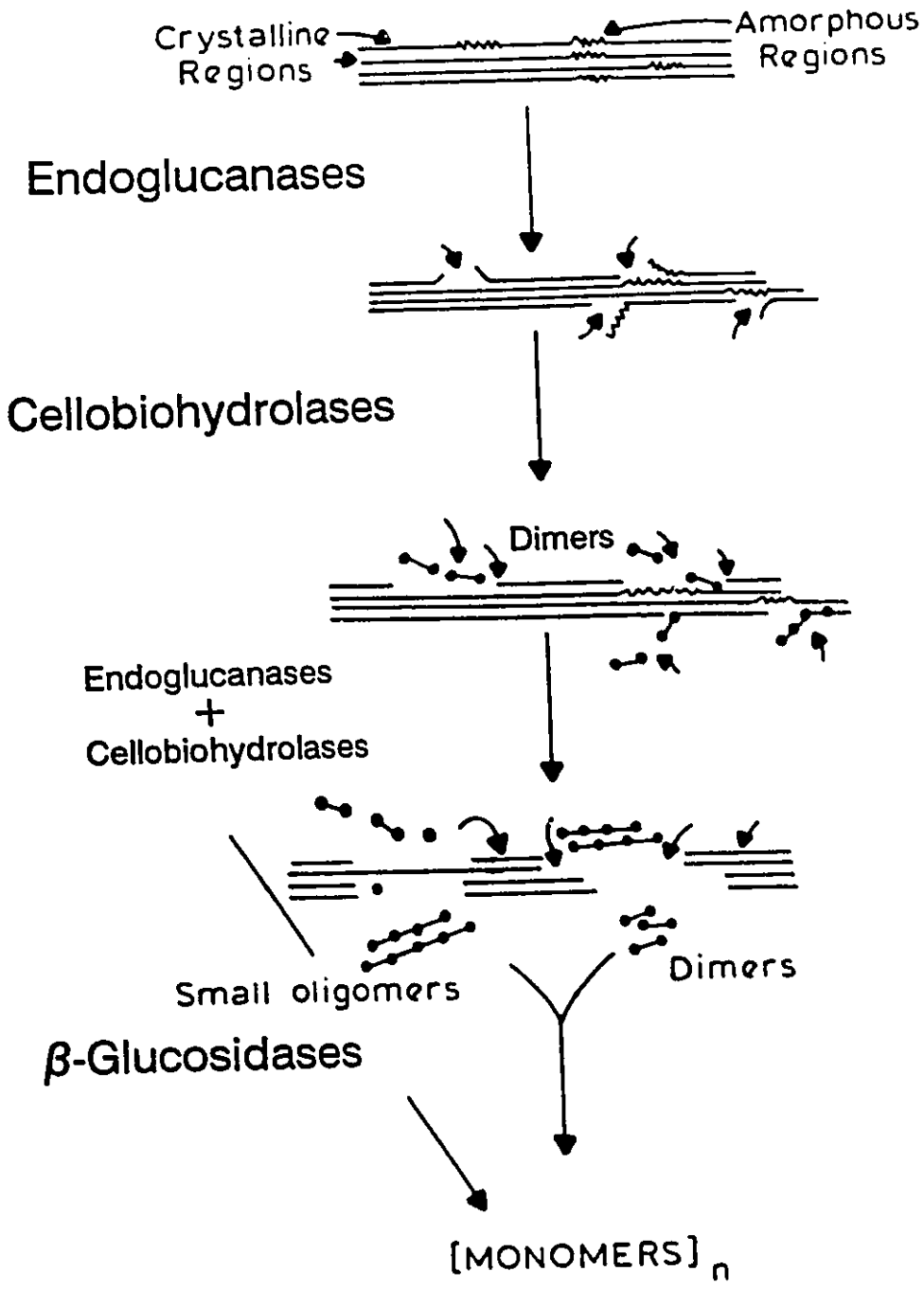


FIGURE 3: Generalized scheme of cellulose hydrolysis by the different hydrolytic enzymes (from Montenecourt and Eveleigh, 1983):

consequently increasing the available sites for CBH action. By removing cellobiose units from the non-reducing ends, CBHs uncover more sites for endoglucanase action.

Although consistent with most observations, this model is still considered unsatisfactory. The endo- and exo- definition of cellulases is largely based on the use of model soluble substrates. Many authors question the validity of these substrates to predict the role of the enzymes involved in the hydrolysis of crystalline cellulose (Enari and Niku-Paavola 1987). When used individually, only CBHs can attack crystalline cellulose significantly (Chanzy et al. 1983, Enari and Niku-Pavola 1987). However, Wood et al. (1989) found that the addition of very little endoglucanase activity was enough to substantially increase the activity of CBH on crystalline cellulose.

The functional significance of the multiplicity of cellulase components is still unclear. Most fungal cellulolytic systems are comprised of at least 2 cellobiohydrolases and 5 endoglucanases (Goyal et al. 1991). Theoretically, two types of both CBH and endoglucanase would be necessary because of the two possible stereoconfigurations of the cellobiosyl units in the cellulose chains (Goyal et al. 1991). However, it does not satisfactorily explain the different mechanism of action of the two CBHs (CBHI and CBHII) from *Trichoderma reesei* (Enari and Niku-Paavola 1987), which are the best characterized CBHs to date. Also, it does not account for the high number of endoglucanases that are usually detected. Other proposed reasons for the multiplicity of cellulases include: proteolytic degradation (Nakayama et al. 1976), different extents of glycosylation (Messner and Kubicek 1988), enzyme/substrate complexes (Alurralde and Ellenrieder 1984) and enzyme/enzyme complexes (Sprey and Lambert 1983). However, fungal cellulases are generally believed to exist as loose components.

In some bacteria, especially anaerobes, the cellulases are aggregated together. The best characterized system of this type is the cellulosome of *Clostridium thermocellum* (Lamed and Bayer 1988, Wu and Demain 1988). The cellulosome is a discrete structure with a diameter of about 18 nm which comprises at least 15 different polypeptides. According to the current model, the cellulosome would mediate attachment between bacterial cells and the substrate. Some cellulosome components cleave cellulose to cellobiose which would be directed to the bacterial cell wall.

1.3.1.5 Molecular structure of cellulases:

Proteolytic cleavage of cellulases into separate catalytic and cellulose-binding fragments was the first evidence that cellulases were comprised of specific domains. (Van Tilbeurgh et al. 1986). Based on small angle X-ray scattering studies of native CBH (Schmuk et al. 1986) and amino acid sequences analysis, Knowles et al. (1987) suggested that cellulases consisted of a catalytic domain linked by a flexible "hinge" to a "tail" responsible for binding to cellulose. The linker ("hinge" region) is rich in threonine/glycine residues and is heavily O-glycosylated.

High resolution structural data has only been obtained for two cellulases, the CBH II of *Trichoderma reesei* (Rouvinen et al. 1990) and the endoglucanase CelD of *Clostridium thermocellum* (Juy et al. 1992). Crystallization of the intact CBH II was unsuccessful presumably because of the flexibility of the linker between the catalytic and the binding domains. However, the catalytic domain could be crystallized and the three-dimensional structure was determined by X-ray diffraction. This domain appears to have a tunnel in which two aspartic acid residues presumed to be involved in catalysis are located. The three-dimensional structure of the binding domain was determined by two-dimensional nuclear magnetic resonance (Kraulis et al. 1989) and it was shown to be compact and wedge-shaped. Its shape suggested a role in displacing cellulose chains from the crystalline cellulose. The displaced cellulose

chains would thread into the enclosed tunnel within the catalytic domain.

The three-dimensional structure of the endoglucanase CelD from *Clostridium thermocellum* was determined by X-ray diffraction (Juy et al. 1992). It was also comprised of two domains with the larger one being recognized as the catalytic domain. The smaller domain was located at the N-terminal and its role was not elucidated. Although endoglucanase CelD is a thermostable enzyme, the authors did not suggest any structural feature that might be responsible for its thermostability.

Gilkes et al. (1991b) compared the amino acid sequence of cellulose-binding domains of cellulases from bacterial and fungal origins. The cellulose-binding domains were located at either the N or the C terminal of the cellulases. Bacterial cellulose-binding domains are about 100 amino acid long while the fungal ones are about 35 amino acid long. It is not clear if bacterial and fungal cellulose-binding domains have similar mechanisms of action. Din et al. (1991) showed very clearly that the isolated cellulose-binding domain of a bacterial endoglucanase could disrupt crystalline cellulose to release small fragments. Although cellulose disrupting activity of fungal cellulases has been suggested by structural data, it has not yet been clearly demonstrated.

Based on the sequence homology of various catalytic domains, Gilkes et al. (1991b) suggested the classification of cellulases and xylanases into nine β-1,4-glycanases families (Table 2). Family C contains only fungal enzymes; family D contains only bacterial enzymes; families A, B, F, G and H contain fungal and bacterial enzymes while family E contains bacterial and plant enzymes and a factor involved in the germination of slime mold spores. As more data becomes available, enzymes from different sources might be added to families presently containing enzymes from only one source. There is much more sequence data available on cellulases from bacteria than other sources, probably because it is easier to clone

TABLE 2: Families of cellulases and xylanases: the thermophilic organisms are denoted by bold characters (from Gilkes et al. 1991b):

Family	Organism	Enzyme	Activity
A	Bacillus sp.	EG1, CelA, CelB, CelC	
	Bacillus lautus	CelB	
	Bacillus polymyxa	Eg1	
	Bacillus subtilis	Eg1	
	Bacteroides ruminicola	Eg1	
	Butyrivibrio fibrisolvens	CelA, End1	
	Caldocellum saccharolyticum	CelB	
	Clostridium acetobutylicum	Eg1	All endoglucanases
	Clostridium cellulolyticum	CelA	
	Clostridium thermocellum	CelB, CelC, CelE, CelH	
	Erwinia chrysanthemi	CelZ	
	Fibrobacter succinogenes	Egl3	
	Robillarda sp.	Egl	
	Ruminococcus albus	Egl1, CelA, CelB	
	Trichoderma reesei	EglIII	
	Xanthomonas campestris	EngXCA	
B	Cellulomonanas fimi	CenA	Endoglucanase
	Microbispora bispora	CelA	Endoglucanase
	Streptomyces sp. strain KSM9	CasA	Endoglucanase
	Trichoderma resei	CbhII	Exoglucanase
C	**Humicola grisea**	CbhI	Exoglucanase
	Phanerochaete chrysosporium	CbhI	Exoglucanase
	Trichoderma reesei	CbhI	Exoglucanase
	Trichoderma reesei	EgII	Endoglucanase
	Trichoderma viride	Cbh	Exoglucanase
D	Bacillus circulans	Bgc	
	Cellulomonas uda	Egl	All endoglucanases
	Clostridium thermocellum	CelA	
	Erwinia chrysanthemi	CelY	
E	Butyrivibrio fibrisolvens	CedI	Cellodextrinase
	Cellulomonas fimi	CenB and CenC	Endoglucanase
	Clostridium thermocellum	CelD	Endoglucanase
	Clostridium stercorarium	CelZ	Endoglucanase
	Dictyostelium discoideum	SGSP270-6	Spore germination
	Persea americana	Egl, Cel1 and Cel2	Endoglucanase
	Pseudomonas fluorescens	Egl	Endoglucanase

Continued on following page

Table 2: *Continued*

F	Bacillus sp. strain 125	XynA	Xylanase
	Butyrivibrio fibrisolvens	XynA	Xylanase
	Caldocellum saccharolyticum	CelB	Exoglucanase
	Caldocellum saccharolyticum	XynA	Xylanase
	Caldocellum saccharolyticum	ORF 4	Unknown
	Cellulomonas fimi	Cex	Exoglucanase
	Clostridium thermocellum	XynZ	Xylanase
	Cryptococcus albidus	Xyn	Xylanase
	Pseudomonas fluorescens	XynA and XynB	Xylanase
	Streptomyces lividans	XynA	Xylanase
	Thermoascus aurantiacus	Xyn	Xylanase
G	Bacillus circulans	Xyn	All xylenases
	Bacillus pumilus	XynA	
	Bacillus subtilis	Xyn	
	Clostridium acetobutylicum	XynB	
	Schizophyllum commune	XynA	
	Streptomyces lividans	XynB and XynC	
	Trichoderma harzianum	20-kDa Xyn	
H	Aspergillus aculeatus	Egl	Endoglucanase
	Erwinia carotovora	CelS	Endoglucanase
I	Ruminococcus flavefaciens	CelA	Cellodextrinase

enzymes from these sources. Although the classification of Gilkes et al. (1991b) provides a useful basis for comparing the different cellulases and xylanases, there is still a large gap to fill between the sequencing data available and the characterization of the mode of action of these enzymes. Very little biochemical characterization has been done for many of the enzymes appearing in this classification. Conversely, some cellulases and xylanases that have been characterized extensively using biochemical techniques do not appear in this classification because their complete sequence is not available.

It should be noted that the known CBHs do not all fall in the same family. Thus, they sometimes show more homology with some endoglucanases than with other CBHs. Gilkes et al. (1991b) suggested that CBH versus endoglucanase activity might be a consequence of fine details in the three-dimensional structure rather than in the overall conformation. More information on both types of enzymes is needed in order to clarify the mode of action of cellulases.

1.3.2 ENZYMATIC DEGRADATION OF XYLANS:

Xylan is chemically more complex than cellulose because the xylan backbone is substituted by various groups. Xylans are generally amorphous and the xylanases do not have to overcome a physical barrier such as the tight packing that occurs in the chains of crystalline cellulose. Although the xylan chains are physically more accessible, the diversity of side-chains means that various debranching enzymes are required in order to obtain complete hydrolysis.

1.3.2.1 Characterization of the xylanolytic enzyme components:

The two major types of enzymes involved in xylan hydrolysis are the endo-β-1,4-D-xylanases (EC 3.2.1.8) and the β-xylosidases (EC 3.2.1.37). The endo-β-1,4-D-xylanases cleave β-1,4 linkages inside the xylan chains while the β-xylosidases cleave xylo-oligosaccharides to xylose. It is generally believed that exo-xylanases would not

be very effective on xylan because the substituents would drastically limit the action of such enzymes (Eriksson et al. 1990). Reilly (1981) reported that some enzymes had exo-xylanase activity. These enzymes were mostly active on xylo-oligosaccharides and might actually belong to a specific group of β-xylosidases.

Diverse debranching enzymes (often called "accessory enzymes") are necessary to remove the substituents on the xylan backbone (Johnson et al. 1989). The α-L-arabinofuranosidases (EC 3.2.1.55) hydrolyze the α-L-arabinofuranosyl residues while the α-D-glucuronidases remove the 4-O-methylglucuronic acid substituents (Fig. 4). Finally, esterases are required to remove esterified acids such as acetic acid and feruloyl acid. The mechanisms proposed in Figure 4 suggest that endo-β-1,4-D-xylanases and the accessory enzymes listed above would be sufficient to achieve complete hydrolysis of xylans. However, the real scenario is almost certainly more complex and as appropriate substrates are developed, new types of enzymes will probably be identified. Nishitani and Nevins (1991) described a new type of endo-β-1,4-D-xylanase that cleaved xylans only when glucuronopyranosyl moieties are present as side chains. Kormelink et al. (1991) isolated a specific arabinofuranohydrolase that released arabinose residues from only arabinoxylans. This is in contrast with most of the α-L-arabinofuranosidases which are active mainly on arabinose-substituted xylo-oligomers and on aryl-α-arabinofuranosides (Poutanen 1988). The development of appropriate substrates will be necessary before the identity and the characteristics of all the enzymes involved in xylan hydrolysis can be defined.

Because of the complexity of xylanolytic systems, we focussed our work on the study of endo-β-1,4-D-xylanases produced by *T. terrestris* 255B. The endo-β-1,4-D-xylanases can be conveniently assayed using commercially available xylans and the hydrolysis products can be quantified using a reducing sugar assay or by HPLC analysis.

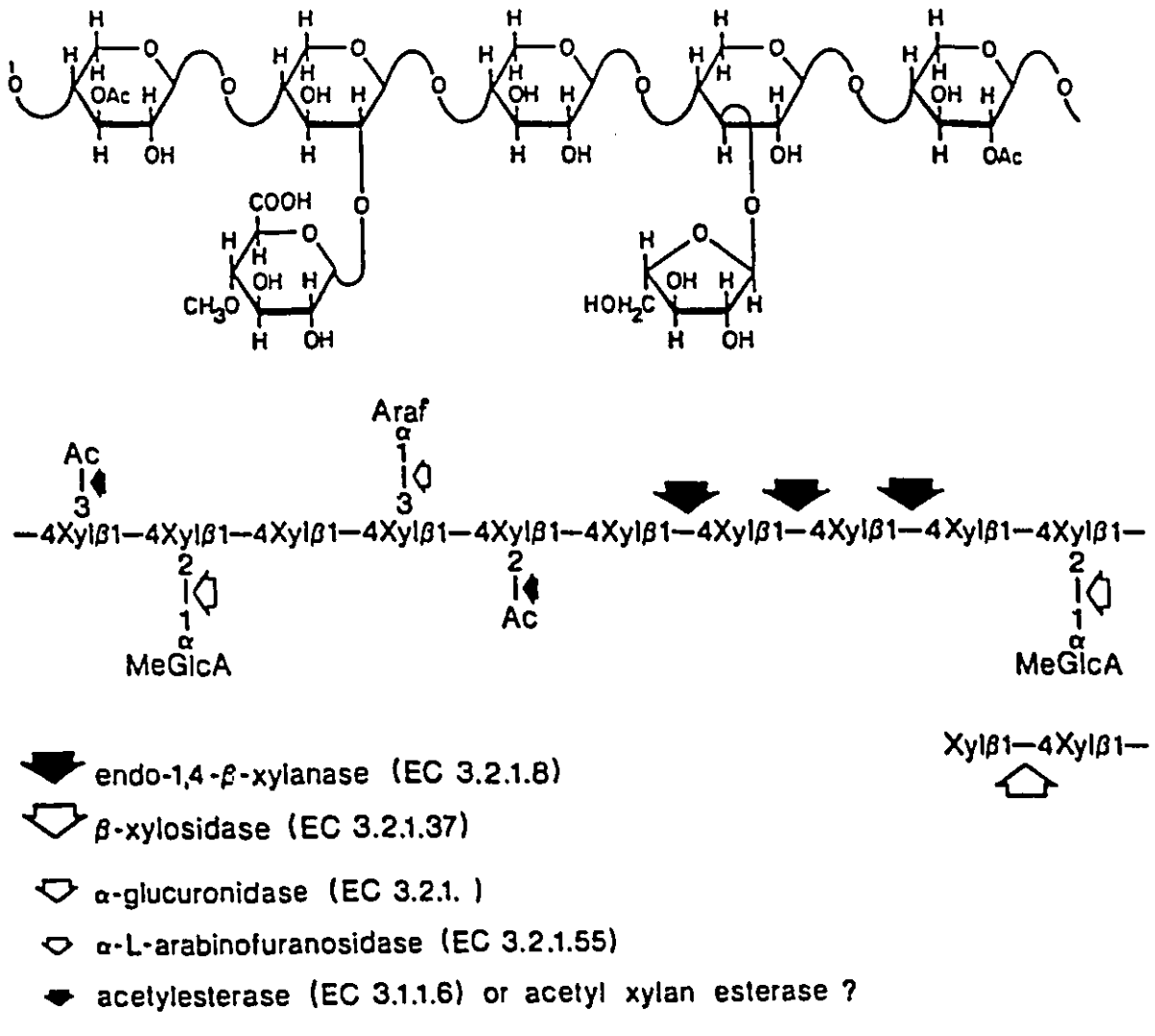


FIGURE 4: Sites of attack of xylan by different microbial xylanolytic enzymes (from Biely 1985):

1.3.2.2 Production and regulation of xylanases:

Xylanases are produced by both fungi and bacteria, although fungal xylanases have generally been the most extensively studied because of their higher levels of production. The best characterized fungal xylanases are those from *Aspergillus* spp. (Eriksson et al. 1990, Poutanen et al. 1987) and *Trichoderma* spp. (Wong and Saddler 1992). Xylanase production by yeasts has been reported (Özcan et al. 1991, Biely et al. 1980), however, the levels of production are generally lower than those obtained with filamentous fungi. The most extensively studied bacterial xylanases are from species of *Streptomyces* (Sharek et al. 1991) and from *Bacillus* species (Esteban et al. 1983). Some anaerobic bacteria such as *Clostridium* spp. (Lemmel et al. 1986) and *Thermoanaerobacter ethanoliticus* (Wiegel and Ljungdahl 1986) have also been shown to produce xylanases.

Xylanases are generally considered to be inducible (Eriksson et al. 1990) and the most commonly used inducers are xylan, soluble fragments of xylan, xylobiose, 4-thiocellobiose and methyl-β-D-xylopyranoside (Eriksson et al. 1990). As was described in the case of cellulase induction, the exact molecule responsible for xylanase induction has not yet been identified. Biely et al. (1980, 1984) and Hrmová et al. (1991) used synthesized positional isomers of xylobiose to induce xylanases. They found that 1,3-β-xylobiose, 1,2-β-xylobiose and 1,4-β-xylobiose were all good inducer of xylanases in *Cryptococcus albicus*, *Trichosporon cutaneum* and *Aspergillus terreus*.

It is still not clear if xylanases and cellulases are under separate regulatory control or to what extent the regulatory systems overlap. These studies are complicated by the fact that many cellulases and xylanases show some substrate cross-specificity. Hrmová et al. (1991) used various synthetic homo- and heterosaccharides to study the regulation of xylanases and cellulases in fungi. Xylose dissaccharides induced xylanases while glucose dissaccharides induced cellulases. Mixed

disaccharides induced both types of enzymes in *Aspergillus niger* but did not act as an inducer in *Trichoderma reesei*. Although these data suggest that xylanases and cellulases are under separate regulatory controls, it does not seem that these conclusions can be extended to all microorganisms.

1.3.2.3 Families and multiplicity of xylanases:

Various criteria have been used to classify xylanases. These criteria included amino acid sequence homology (Gilkes et al. 1991b), the ability to release L-arabinose (Dekker 1985, Matte and Forsberg 1992), the ability to solubilize xylan (Frederick et al. 1985), the presence of transferase activity (Gorbacheva and Radionova 1977) and the formation of xylose or only xylo-oligosaccharides as hydrolysis products (Lee et al 1987).

The various properties of xylanases provide a basis for cooperation between these enzymes during the hydrolysis of complex substrates. Wong et al. (1988) have discussed the functional significance of the multiplicity of xylanases from one microorganism. Different specificities might be necessary to hydrolyze different types of linkages in lignocellulosic substrates and a range of distinct endo-β-1,4-D-xylanases might allow the organism to use a wider range of substrates. Wong et al. (1986) showed that three distinct xylanases from *Trichoderma harzianum* had high level of cooperativity in the hydrolysis of xylan in aspen holocellulose.

As mentioned earlier, Gilkes et al. (1991b) classified β-1,4-glycanases into 9 families. Endo-β-1,4-D-xylanases were found in two families (F and G, Table 2). The G family contains low molecular mass xylanases (20-31 kDa) which are specific for xylan hydrolysis. The F family contains xylanases of higher molecular masses (approx. 30 to 110 kDa). This family contains enzymes with mixed substrate specificity. Endoglucanase activity (Tan et al. 1987a, Lüthi et al. 1990) and aryl-β-D-cellobiosidase activity (Kellet et al. 1990, Lüthi et al. 1990, Grépinet et al. 1988) have been

shown for some of the xylanases within the F family. In fact, the F family contains some enzymes described as exoglucanases (Saul et al. 1989, O'Neil et al. 1986), although these enzymes were also shown to have xylanase activity.

In the original paper by Gilkes et al. (1991b), all the endo-β-1,4-D-xylanases from the same organism were found in the same family. However, recent data suggests that the different xylanases produced by an organism may fall into different families. Sharek et al. (1991) sequenced 3 distinct xylanases from *Streptomyces lividans*. Xylanase A was homologous to the xylanases of the F family while xylanases B and C were specific of the G family.

1.3.2.4 Structure of xylanases:

So far, high resolution structural data has only been reported for xylanase A from *Bacillus pumilus* (Katsube et al. 1990). Xylanase A belongs to the G family and is specific for the hydrolysis of xylan. The three-dimensional structure obtained showed that it has an ellipsoidal-shape of approximately 40Å×35Å×35Å (Fig. 5). The enzyme has a well-defined deep cleft running down one side of the ellipsoidal molecule. The substrate is presumed to bind to that cleft. The overall structure is predominantly characterized by three β-sheets (I, II and II) which are formed by 57% of the total residues in the protein. Katsube et al. (1990) used site-directed mutagenesis to change residues that were possibly involved in catalysis according to the three-dimensional structure. They found that residues Glu^{93} and Glu^{182} were essential for activity and they suggested that these two residues were involved in a general acid-base catalysis analogous to that of hen egg-white lysozyme. They plan to confirm this model by determining the structure of a complex between the enzyme and its substrate or an analogue of the substrate.

No three-dimensional structure is presently available for a xylanase from family F. Using circular dichroism, Goodenough et al. (1991) estimated that xylanase A

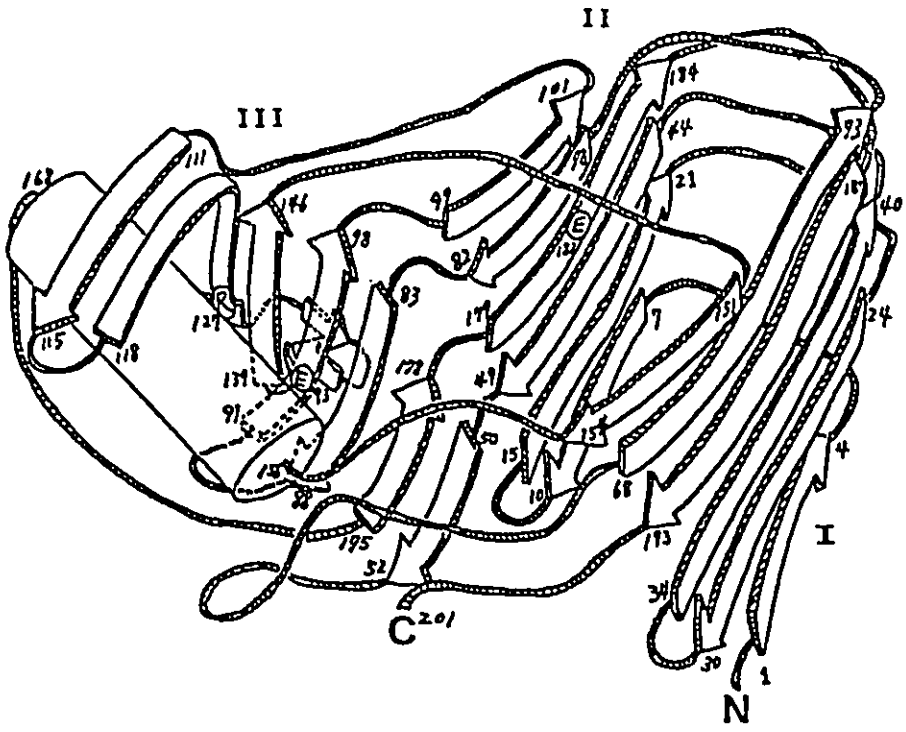


FIGURE 5: Schematic representation of the amino acid backbone structure of xylanase A from *Bacillus pumilus* (from Katsube 1990):

from *Pseudomonas fluorescens* subsp. *cellulosa* had 65.9% β-structure. This value is similar to the one found for xylanase A from *Bacillus pumilus* (57%). However, a more detailed structure of xylanase A from *P. fluorescens* is necessary before we can evaluate whether these two xylanases share significant structural homology.

1.4 POTENTIAL APPLICATIONS OF XYLANASES AND CELLULASES:

Cellulose and hemicelluloses are the most abundant renewable organic molecules. There are about 7×10^{11} tons of cellulose in existence and about 4×10^{10} tons are synthesized annually as a result of photosynthesis (Coughlan 1985). Hemicelluloses are slightly less abundant with about 6×10^{11} metric tons in existence and about 3×10^{10} metric tons synthesized annually (Wilkie 1983). Thus, these substrates represent a vast potential as a source of food, fuel and chemical feedstocks.

One major application of wood-degrading enzymes is in the conversion of lignocellulosic substrates to fermentable sugars which can be used to make products such as ethanol. Bio-ethanol represents a promising alternative to fossil fuels as its utilization does not constitute a net addition of carbon dioxide to the atmosphere and thus, does not promote the greenhouse effect. Lynd et al. (1991) estimated that ethanol from cellulosic biomass could be a cost-competitive process by the end of this decade. They considered that the energy balance, feedstock supply and environmental impacts are not significant barriers to the wide-spread use of fuel ethanol derived from biomass. However, they considered that research is still needed to develop cellulosic energy crops and to improve the conversion process. Improvements in the conversion process will require a better understanding of the mechanism of cellulose degradation.

The efficient exploitation of the lignocellulosic substrates requires the use of all the wood components. Thus, considerable effort has been devoted to finding enzymes

that could degrade major non-cellulosic polysaccharides such as xylans. Xylanases can be used to convert xylans to fermentable products. Because some xylanases have no activity towards cellulose, they also have potential applications in the pulp and paper industry (Wong and Saddler, 1992). Xylanases could be used to selectively remove residual xylan in order to obtain highly purified cellulose pulps suitable for the preparation of cellulose derivatives. Also, Viikari et al. (1986) found that xylanase pre-treatment of kraft pulp reduces the requirement for chlorine during pulp bleaching. This results in lower chemical costs and lower amounts of organochlorine compounds released in the effluents. Xylanase pretreatment could also be used in chlorine-free bleaching sequences with chemicals such as hydrogen peroxide (Kantelinen et al. 1991). Because of environmental pressure and new legal regulations, there is considerable effort currently underway to develop chlorine-free bleaching processes.

1.5 UTILIZATION AND ADVANTAGES OF THERMOSTABLE ENZYMES:

1.5.1 Advantages of thermostable enzymes:

Thermostability is a desirable property for enzymes used in industrial processes (Zamost et al. 1991, Bergquist et al. 1987, Margaritis and Merchant 1986a, Sonnleitner and Fiechter 1983). The advantages of using thermophilic enzymes are listed in Table 3 and they are particularly relevant to processes such as cellulose and xylan hydrolysis. Hydrolysis of these substrates is a heterogenous process because xylan is partially soluble and native cellulose is insoluble. Carrying out hydrolysis at higher temperatures allow better mass transfer which should improve reaction rates. Microbial contamination can be a major problem in cellulose hydrolysis because the final product is glucose, a substrate utilised by many microorganisms. This problem could be greatly reduced by using a process which operates at higher temperature. Thermostable enzymes could also be useful in the pulp and paper industry. In the Kraft pulping process, the temperature of the pulp entering the bleach plant is around 70°C. Thermostable xylanases could be more effective for treating this

Table 3: Advantages and limitations of thermostable enzymes in industrial processes:

Advantages: -Increased reaction rates -Reduced risks of contamination -Increased opportunity for enzyme recycling -Facilitated mass transfers -Reduced viscosity -Higher solubility of substrates and/or products -Decreased oxygen solubility (anaerobic processes) -Higher resistance to denaturating agents -Reduced cooling expenses -Facilitated recovery of volatile products
Limitations: -Greater stresses on material and equipment -Extensive evaporation -Decreased oxygen solubility (aerobic processes) -Thermolability of substrates and/or products

incoming pulp because they are less sensitive to inactivation.

Currently, the most successful use of thermophilic enzymes is in the starch industry. The liquefaction of starch is carried out at temperatures ranging from 80 to 110°C using bacterial α-amylases. The use of very high temperatures breaks up the starch granules (gelification) which greatly increases the accessibility of the substrate. Also, it prevents microbial contamination of the sugar syrup.

Another successful application of thermostable enzymes is the DNA polymerase from *Thermus aquaticus*. This enzyme is used to amplify DNA in polymerase chain reaction (PCR) methods. Its high thermostability allows the running of repeated cycles of thermal denaturation/renaturation of DNA without the need to add fresh enzyme.

1.5.2 Sources of thermostable enzymes:

Many strategies are available to obtain thermostable enzymes. The thermostability of mesophilic enzymes can be increased by protein engineering (Nosoh and Sekiguchi 1990), by cross-linking, by immobilization or by addition of substances such as glycerol (Gupta 1991). Currently, considerable effort is devoted to find enzymes that are intrinsically thermostable. These enzymes are expected to be produced by thermophilic organisms (Kristjansson 1989). There are no precise rules for the classification of an organism as thermophilic. For fungi, those with an optimal growth temperature above 40°C are generally considered thermophilic (Crisan 1973). The highest temperature that a fungus can tolerate for growth seems to be 62°C (Brock 1985). For bacteria, those with an optimal growth temperature between 45-65°C are considered thermophiles while those that grow optimally at >70°C are called extreme thermophiles (Brock 1985). The most extremely

thermophilic organisms reported to date are archaebacteria with a maximum growth temperature of 110°C (Stetter 1986).

Initially, wood-degrading thermophilic bacteria and fungi were isolated from self-heating habitats such as compost and wood-chip piles. The temperature of these habitats can go as high as 65-70°C (Johri 1980, Ofosu-Asiedu and Smith 1973) and allows enrichment of thermotolerant organisms. The activity of enzymes from these sources generally had optimal temperatures ranging from 60 to 80°C (Yu et al. 1987, McCarthy et al. 1985, Durand et al. 1984).

More recently, cellulase and xylanase production has been studied in extremely thermophilic bacteria isolated from hot springs (Bragger et al. 1989, Tucker et al. 1989, Donnison et al. 1989). A cellobiohydrolase (Ruttersmith and Daniel 1991) and a xylanase (Simpson et al. 1991) isolated from a *Thermotoga* sp. were both optimally active at 105°C. Clearly, the most thermostable enzymes are likely to be those produced by the extremely thermophilic bacteria. However, enzyme productivity from these organisms is much lower than that of thermophilic fungi.

1.6 PREVIOUS WORK WITH *Thielavia terrestris*:

Thielavia terrestris was first isolated and described by Apinis (1963) during his studies on thermophilic fungi in alluvial soils in Great Britain. He gave the name *Allescheria terrestris* to this organism. Malloch and Cain (1973) and Arx (1975) considered that the fungus described as *Allescheria terrestris* actually belonged to the genus of *Thielavia*. *T. terrestris* is a cleistothecial ascomycete which is classified in the order of Sphaeriales. It is characterized by dark-brown globose ascocarps that are 100-200 μm in diameter. Its ascospores are ovoid, 4-6 X 3-4 μm and dark brown and

the conidia are ovoid to pyriform and 3-5 X 1.5-2.5 μm (Malloch and Cain 1973).

T. terrestris is a thermophilic fungus which can grow at temperatures ranging from about 20°C to 55°C (Lundström 1974) and strains have been isolated from coal spoil tips, air, soil, compost and wood chip piles (Samson et al. 1977). The strain used in this study (*T. terrestris* 255B) was isolated from wood-chip piles.

Early physiological studies of *T. terrestris* included the determination of pH preference, temperature tolerance and requirements for nitrogen, carbohydrates and vitamins (Lundström 1974). When degrading wood, *T. terrestris* caused the formation of cavities in the secondary cell walls of wood fibres and an erosion of the cell walls which started from the cell lumen (Nilsson 1973). Based on these observations, *T. terrestris* was considered to cause a soft-rot type of wood-degradation. When growing on wood, *T. terrestris* caused weight losses ranging from 3% to 28%, depending on the incubation time and the type of wood (Eslyn et al. 1975, Ofosu-Asiedu and Smith 1973). Glucan, xylan and mannan were the major structural components which were degraded but lignin was also significantly degraded (Eslyn et al. 1975). It is well recognized that some microorganisms can show *in vitro* activity on modified cellulose but fail to significantly degrade natural lignocellulosic substrates. The ability of *T. terrestris* to degrade unmodified lignocellulosic substrates demonstrated its potential for various bioconversion applications.

Durand et al. (1984) compared the production and thermostability of cellulases and hemicellulases from *T. terrestris* NRRL 8126 with those from various mesophilic fungi. They found that *T. terrestris* produced enzymes with better stability for all of the tested activities but it produced much less enzymes than the mesophilic strains.

They also disproved the claim by Skinner and Tokuyama (1978) that *T. terrestris* NRRL 8126 produced cellulases that could be boiled for 3 hours and still retain over 20% of original activity. *T. terrestris* enzymes were 10°C-30°C more thermostable than enzymes from mesophilic sources; however, enzymatic activity was rapidly lost above 70°C (Durand et al. 1984). In other work, using *T. terrestris* ATCC 26917, Margaritis et al. (1986b and 1983) and Merchant et al. (1988) varied the fermentation conditions (pH, temperature, agitation, aeration rate and substrate concentrations) in order to obtain improved productivity of cellulases and hemicellulases. They could get production levels comparable to those obtained with mesophilic wild strains. In addition to cellulases and xylanases, *T. terrestris* also produces thermostable mannanases, galactanases and arabinanases (Araujo and Ward, 1990a, 1990b, Karimi and Ward, 1989).

The strain used in this work (*T. terrestris* 255B) was selected by Wojtczak et al. (1987) as a good producer of thermostable β-glucosidase and cellulases following a screening of a culture collection of wood-inhabiting fungi (Forintek Canada Corp.). They found that the extracellular β-glucosidase, endoglucanase and filter paper activities had half-lives at 60°C of 10.8, 96 and 32 hours, respectively. They also observed that the cell-associated β-glucosidase activity (half-life of 24 hours at 65°C) was significantly more thermostable than the extracellular activity.

1.7 OBJECTIVES OF THIS RESEARCH:

As mentioned in sections 1.3.1.5 and 1.3.2.3, multiple enzyme components have been extensively studied for cellulases and xylanases from mesophilic organisms. However, there is much less information on the individual enzyme components that are produced by thermophilic organisms. Except for one publication on mannanases (Araujo and Ward, 1990b), all of the studies on the enzymes produced by *T. terrestris* have used crude preparations. Although different activities were assayed, each of the activities probably reflected the action of many enzyme components. Presumably, multiple forms of enzymes with similar activities would be necessary to deal with the different physico-chemical variations found in heterogeneous substrates such as lignocellulosics. In this work, we studied the thermophilic cellulases and xylanases produced by *T. terrestris* 255B in order to compare their properties with the equivalent components produced by mesophilic organisms. We also hoped to identify enzyme components with potential application in the bioconversion of wood products. In achieving these goals we tackled the following objectives:

- To study the production of cellulases and xylanases after growth on various substrates in order to obtain maximum production of these enzymes.

- To compare the profile of the proteins produced after growth on the various substrates by using non-denaturing electrophoretic techniques.

- To identify the major components that are involved in cellulose and xylan degradation using activity staining methods after electrophoresis.

- To purify certain enzyme components in order to study their properties and compare them to their mesophilic counterparts.

The following objectives were established after we decided to concentrate our

effort on the major cellulase-free xylanase component (xylanase II) which was of particular interest because of its potential application in the pre-bleaching of kraft pulps:

- To perform structural studies on xylanase II in order to compare its properties with mesophilic xylanases.

- To attempt to identify structural features responsible for the higher thermostability of xylanase II.

- To study the mode of action of xylanase II and compare it with other characterized xylanases.

- To determine if xylanase II displays synergistic or cooperative interaction with a thermophilic xylanase from another family of enzymes.

- To determine if xylanase II is effective in removing residual xylan from kraft pulps in order to act as a pre-bleaching agent.

2.0 MATERIALS AND METHODS:

The "Materials and Methods" section is divided in six sub-sections corresponding to the six sub-sections of "Results and Discussion". Some specific techniques are described in more than one sub-section in order to report accurately the modifications that were made to adapt these techniques to the different experiments performed.

2.1 PRODUCTION AND CHARACTERIZATION OF CELLULASES AND XYLANASES:

2.1.1 Organism and culture conditions:

Thielavia terrestris 255B was obtained from the Forintek Canada Corp. culture collection. It was kept on malt agar medium (2% Difco Bacto malt extract and 2% Difco Bacto agar) at room temperature. Inoculum for production studies was prepared by growing *T. terrestris* 255B in rich medium (per litre: glucose, 20 g; Difco yeast extract, 2 g; Difco peptone, 1 g; $MgSO_4*7H_2O$, 0.2 g; $CaCl_2$, 0.1 g) for 48 hours at 45°C. The mycelium was centrifuged at 15,000 *g* for 20 min at 4°C and the pellet washed in 100 mL distilled H_2O and centrifuged again. The mycelium was resuspended in distilled water (approx. 7 mg dry weight per mL) and used at a concentration of 5% (v/v) to inoculate the medium used for the production of enzymes. The medium for production of enzymes has been described by Breuil et al. (1986). It contained (per litre): Bacto peptone, 1 g; Bacto yeast extract, 1 g; $(NH_4)_2SO_4$, 2 g; KH_2PO_4, 2.72 g; Potassium Hydrogen Phthalate, 3 g; $CaCl_2*H_2O$, 0.395 g; $MgSO_4*7H_2O$, 0.3 g; $FeSO_4*7H_2O$, 5 mg; $MnSO_4*H_2O$, 21 mg; $ZnSO_4*7H_2O$, 14 mg; $CoCl_2*6H_2O$, 37 mg. The pH of the medium was adjusted to 5.8 with NaOH. The following carbon sources were used at a 1% concentration: glucose, cellobiose, acid-swollen cellulose, Solka Floc BW300 (James River Co., Hackensack, N.J.) and oat-spelts xylan (Sigma Chem. Co., St-Louis, Mo.). Solka Floc is the trade name for a type of purified cellulose manufactured from wood pulp. Acid-swollen cellulose is an

cellulose manufactured from wood pulp. Acid-swollen cellulose is an amorphous cellulose obtained by treating cellulose (Avicel PH101) with 85% H_3PO_4. It was prepared according to Wood (1988). Duplicate cultures were grown on each substrate in two replicate batches.

2.1.2 Sample preparation:

Samples of 15 mL of the fungal culture were collected every day for a total of six days. The samples were centrifuged at 8000 *g* for 10 min at 4°C. The supernatants were filtered through glass fibre filter (#GF/A, Whatman Paper Ltd, Maidstone, England) and NaN_3 was added to give a final concentration of 0.02%. For enzymatic assays, the samples were desalted on BioRad Econo-Pac 10DG columns (BioRad Lab., Richmond, CA) using 50 mM sodium acetate buffer (pH 4.8) as eluant. For biomass determination, the mycelium was resuspended in 10 mL distilled water.

2.1.3 Biomass determination:

One mL of 3 N NaOH was added to 2 mL of mycelium suspension. The mixture was boiled for 5 min and cooled in running water. The total protein was determined according to Herbert et al. (1971) using the Biuret method. One mL of Benedicts reagent was added to each sample and the O.D. at 555 nm was measured after 10 min. of incubation at room temperature. The total biomass was calculated with the assumption that the average protein content of the mycelia was 17% (Wojtczak 1989).

2.1.4 Enzymatic assays:

Protein in the culture filtrate was determined by the Bradford method using a Protein assay kit according to the manufacturer's instructions (BioRad Lab.) with bovine γ-globulin as the standard. The various enzymatic assays were performed in 50 mM sodium acetate buffer (pH 4.8), except for the protease assay which was carried out in 100 mM MES buffer (pH 6.5). Filter paper activity was

for 60 min at 65°C. The soluble sugars released in the assay were determined by HPLC analysis (Schwald et al., 1988). A unit of filter paper activity was defined as the amount of enzyme catalyzing the release of 1 μmol reducing power per minute.

β-Glucosidase was determined in a 30 min assay at 70°C using 0.5% cellobiose (w/v) as substrate. The glucose released was measured with a YSI glucose analyzer Model 27 (Yellow Springs Instr. Co., Yellow Springs, Ohio). A unit of β-glucosidase activity was defined as the amount of enzyme catalyzing the cleavage of 1 μmol of cellobiose per minute. Xylanase and endoglucanase activities were assayed for 30 min at 65°C, using 0.5% (w/v) oat-spelts xylan (#X-0376, Sigma Chem. Co.) and carboxymethylcellulose (#C-4888, Sigma Chem. Co.), respectively as the substrates. Reducing sugars released during incubation were quantified by the 3,5-dinitrosalicylic acid (DNS) method of Miller (1959). Unit activity for xylanase and endoglucanase was defined as the amount of enzyme catalyzing the release of the equivalent of 1 μmol per minute of xylose and glucose, respectively. Proteolytic activity was determined by incubating 1 mL of appropriately diluted enzyme with 10 mg Hide Powder Azure (#H-6268, Sigma Chem. Co.) for 60 min at 60°C. The reaction was stopped by adding 0.5 mL of 15% (w/v) trichloroacetic acid and the absorbance of the solution at 595 nm was read after centrifugation of the unhydrolyzed substrate. A unit of protease activity was defined as the amount of enzyme that causes an increase of 1.0 absorbance unit at 595 nm in 1 hour. The various enzymatic activities in duplicate cultures showed less than 10% variation from the mean. A maximum of 20% variation from the mean was observed between corresponding cultures from different replicate batches.

2.1.5 Electrophoresis:

For electrophoresis, the samples were concentrated 20-fold using Centricon microconcentrators (Amicon, Danvers, MA) fitted with a 10 000 MW cut-off membrane. The samples were subjected to native-PAGE using the automated

"Phastsystem" (Pharmacia, Uppsala, Sweden). The samples (1 µL) were loaded onto a gradient gel (Phastgel 8-25) and the separation was performed at a constant current of 10 mA for 280 accumulated volthours. The temperature of the cooling bed was set at 15°C. The molecular weight markers used were from the Pharmacia high molecular weight calibration kit. For IEF, the samples were rediluted (5-fold) and loaded onto a Phastgel IEF 3-9. The separation was performed at a constant current of 2.5 mA for 500 accumulated volthours. The pI markers were from the Pharmacia Broad pI Calibration Kit, pH 3-10. The gels were developed by silver staining using the Phastgel Silver kit according to the manufacturer's manual (Pharmacia).

2.1.6 Enzymatic activities in gels:

Prior to silver staining, the separation gel was briefly washed in sodium acetate buffer and overlaid on a 2% agarose gel containing either 0.1% carboxymethylcellulose or 0.1 % xylan (in 50 mM sodium acetate, pH 4.8) in order to detect endoglucanase and xylanase activities, respectively (Coughlan 1988). The substrate gels were cast between two sheets of Gelbond (Pharmacia) with the gel sandwiched between the hydrophobic and hydrophilic sides of the sheets. In order to obtain thin gels of uniform thickness, we used a LKB Ultramould 221720 with a 0.3 mm spacer. The separation and overlaid gels were incubated at 65°C for 5-10 min (IEF) or 10-20 min. (native-PAGE). After incubation, the substrate gels were briefly washed in 1 M NaCl and stained for 30 min (zymogram with native-PAGE) or 60 min (zymogram with IEF) in 0.1% Congo red. After staining, the substrate gels were incubated in 1 M NaCl until clearing zones were visible. To increase the contrast, the gels were put in 5% (v/v) acetic acid (the background turns from red to dark blue).

2.1.7 Electron microscopic observation of the hydrolysis of *Valonia* microcrystals:

Valonia ventricosa vesicles were a gift from Dr. H. Chanzy (Centre de Recherches sur les Macromolécules Végétales, Grenoble, France). Microcrystals from *Valonia* were prepared by an acid treatment according to Chanzy et al. (1983)

except that HCl was used instead of acetyl chloride. *Valonia* microcrystals were suspended in 50 mM sodium acetate buffer (pH 4.8) at a concentration of 1 mg/mL. A *T. terrestris* 255B cellulase preparation (obtained after 4 days of growth on Solka Floc) was used at a final concentration of 0.02 filter paper unit per mL. The hydrolysis was carried out at 65°C for 24 hours with gentle agitation provided by a gyratory shaker. After incubation, the microcrystals were recovered by centrifugation for 5 min in a microcentrifuge. The soluble sugars present in the supernatant were analyzed by HPLC using an Aminex 87H column (BioRad Lab.).

In preparation for electron microscopy (EM), the microcrystals were washed twice with 0.1 M NaOH (for 5 min) and four times with distilled water (brief washes). Aqueous suspensions were deposited for 15 min on Formvar coated grids. The specimen were then negatively stained with 3% uranyl acetate for 1 min. Electron microscopy was performed with a Philips EM 201C electron microscope operating at 60 kV. The specimens were examined at a plate magnification of 28 500 on Kodak EM film #100 50.163 (Kodak Canada Ltd, Toronto, Canada).

2.2 PURIFICATION AND INTERACTION OF THE CELLULASES:

2.2.1 Enzyme production:

T. terrestris 255B was grown as described in 2.1.1 with 1% Solka Floc BW300 as carbon source. The culture was harvested after 48 h of growth at 45°C and filtered through two layers of glass fibre filter (#934 AH, Whatman Paper Ltd). The filtrate (2.4 L) was the source of extracellular enzymes. It was concentrated to 21 mL using an Amicon stirred cell fitted with a 10 000 MW cut-off membrane and then to 9.3 mL using Centricon microconcentrators (Amicon). The final volume of the retentate contained 203 mg of total protein.

2.2.2 Enzyme fractionation:

The enzymes were fractionated using a Pharmacia FPLC chromatography system

(Pharmacia LKB Biotechnology, Upssala, Sweden). Protein elution was followed by measuring the A_{280} with a UV-1 monitor (Pharmacia LKB Biotechnology). Samples of 500 μL (11 mg protein) were repeatedly injected on a gel filtration column with dimensions of 10 X 300 mm (Superose 12 HR 10/30). The column was eluted using 50 mM sodium acetate (0.02% NaN_3, pH 4.8) at a flow rate of 0.5 mL/min. Fractions of 0.5 mL were collected and analyzed for various activities using a shortened assay procedure (10 min, 65°C) in order to analyze large numbers of fractions. β-Glucosidase was assayed using 0.5% salicin (#S-0625, Sigma Chem. Co.) and the glucose released determined using the DNS assay (Miller 1959). Substrates used for determining endoglucanase and xylanase activities were described in section 2.1.4.

Activity against crystalline cellulose was assayed in selected fractions representing the major protein peaks identified by A_{280}. Aliquotes of these fractions were incubated with 1% Avicel PH-101 (Fluka Chem. Corp. Ronkonkoma, NY) for 2 h at 65°C with agitation. The hydrolysis was stopped by boiling the mixture for 5 min. and the sugars released analyzed by HPLC using a Aminex 87H column (BioRad Lab.).

2.2.3 Calibration of the gel filtration column:

In order to estimate the molecular weight of the fractionated enzymes under native conditions, we calibrated the Superose 12 column with different standard proteins (Pharmacia LKB Biotechnology). These standards included thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and ribonuclease (13.7 kDa).

2.2.4 Purification of the major cellobiohydrolase:

Fractions #36-37 from repeated runs on the Superose 12 column were pooled. Attempts were made to complete the purification of the major cellobiohydrolase by

anion-exchange chromatography as described in section 2.2.6. However low recovery yields and incomplete purification were obtained with the conditions used for this step. Running the fractions #36-37 for a second time on the Superose 12 column gave better recovery yields and a preparation that contained the cellobiohydrolase purified to at least 95% homogeneity as judged by SDS-PAGE.

2.2.5 Purification of a β-glucosidase:

Fractions #24 to #26 from repeated runs on the Superose 12 column were pooled. They were loaded on a cation-exchange column (Pharmacia Mono S HR 5/5) with dimensions of 5 mm X 50 mm. The column was rinsed with 3 mL (3 column volumes) of the start buffer (50 mM sodium acetate, 0.02% NaN_3, pH 4.8). The column was eluted with a gradient of 0 to 0.4 M NaCl in the start buffer over a total volume of 20 mL. The flow rate was 1 mL and fractions of 0.5 mL were collected. β-Glucosidase activity was detected using the shortened assay described in section 2.2.2.

2.2.6 Purification of β-1,3-glucanase:

Fractions #20 to #22 from repeated runs on the Superose 12 column were pooled. The buffer was exchanged to 20 mM Bis-Tris (0.02% NaN_3, pH 6.5) using a desalting column (BioRad Econo-Column 10 DG). The material was loaded on an anion-exchange column (Pharmacia Mono Q HR 5/5) with dimensions of 5 mm X 50 mm. The column was rinsed with 3 mL (3 column volumes) of the start buffer. The column was eluted with a gradient of 0 to 0.4 M NaCl in the start buffer over a total volume of 20 mL. The flow rate was 1 mL/min and 0.5 mL fractions were collected. One major protein peak was observed and analysis by native-PAGE showed that it contained a HMW protein purified to at least 99% homogeneity. This protein had weak activity against laminarin, a polymer of β-1,3-linked glucose units. This assay was performed using 0.5% laminarin (Sigma Chem. Co. #L-9634) and the conditions described in section 2.1.4.

2.2.7 Protein assay:

The protein content of the purified enzyme preparation was assayed using the bicinchoninic acid method (Redinbaugh and Turley 1986). The reagent were from the #BCA-1 kit for total protein determination (Sigma Chem. Co.). The assay was carried out in a microtiter plate and read at 550 nm using a microplate autoreader EL-310 (Bio-Tek Instr. Inc., Burlington, VT).

2.2.8 Electrophoresis:

Native-PAGE and isoelectrofocusing were performed as described in section 2.1.5 except that the gels were developed by the coomassie blue method using PhastGel Blue R (Pharmacia LKB Biotechnology) according to the manufacturer's instructions. SDS-PAGE was run using PhastGel Gradient 10-15 and PhastGel SDS buffer strips. Before loading, the samples were boiled for 3 min in 10 mM Tris-HCl (pH 8.0), 1mM EDTA, 2.5% SDS, 100 mM DTT and 0.01% bromophenol blue. Protein separation was performed at constant current of 10.0 mA for 80 accumulated volthours. The gels were stained with PhastGel Blue R according to the manufacturer's instructions (Pharmacia LKB Biotechnology). For glycoprotein detection, a SDS-PAGE gel was stained using the PAS (periodic acid, Schiff's reagent method) method as described in the booklet "Polyacrylamide Gel Electrophoresis" (Pharmacia LKB Biotechnology). The Schiff' reagent was purchased from Sigma Chem. Co. (#S-5133).

2.3 PURIFICATION OF THE MAJOR XYLANASE COMPONENT:

2.3.1 Production of enzymes:

T. terrestris 255B was grown as described in section 2.1.1 except that the inoculum obtained after growth on glucose was used to inoculate a 16 L fermentor (Model SF116, New Brunswick Sc. Comp. Inc., Edison, NJ) operated with a total volume of 12 L. Oat-spelts xylan (Sigma Chem. Co.) at a concentration of 0.5% was used as the carbon source. After 48 hours, the culture was harvested and filtered

through 2 glass fiber filters (#934-AH, Whatman Paper Ltd). Sodium azide (0.02% final concentration) was added as a preservative to the culture filtrate.

2.3.2 Purification procedure:

The culture filtrate was concentrated using a Pellicon ultrafiltration unit (Millipore, Bedford, MA) fitted with a 10 000 MW cut-off membrane. The concentrated material was applied to a Q-Sepharose column (5 cm X 10.5 cm) equilibrated at pH 7.2 (20 mM Bis-tris, 0.02% NaN_3). The column was washed with a column volume (200 mL) of the equilibration buffer. The bound material was eluted with a linear gradient of 0 to 0.5 M NaCl in the equilibration buffer. Fractions of 10 mL were collected. Protein elution was followed using the bicinchoninic acid assay. When the conditions described above were used, most of the xylanase activity did not bind to the column and was eluted with the equilibration buffer. The unbound material was applied to a Superose 12 column (10 mm X 30 cm, Pharmacia LKB Biotechnology) using a FPLC system and a UV-1 detector. The column was eluted using 50 mM sodium acetate (pH 4.8). The peak which eluted last contained xylanase II purified to at least 99% homogeneity.

2.3.3 Electrophoresis:

Isoelectrofocusing and native-PAGE were performed as described in section 2.1.5. SDS-PAGE was run according to Laemmli (1970). A 14% gel was cast and run at 100 volts for 40 min using the Mini-Protean II slab cell (Bio-Rad Lab., Richmond, CA). The gel was fixed, stained and destained according to the Bio-Rad instruction manual using coomassie blue R-250. The molecular markers were from the Pharmacia low molecular weight calibration kit (Pharmacia LKB Biotechnology).

2.3.4 Protein and enzymatic assays:

Protein was determined by the bicinchoninic acid method as described in section 2.2.7. The xylanase assay was performed as described in section 2.1.4. The standard

assay for xylanase determination was carried out at 65°C for 30 min while chromatographic fractions were analyzed using a 10 min assay.

2.4 STRUCTURAL STUDIES OF XYLANASE II:

2.4.1 Amino acid analysis:

Amino acid analysis of xylanase II was performed in collaboration with the Protein Structure and Mutagenesis Group of the Institute for Biological Sciences (National Research Council of Canada, Ottawa). Dr. M. Yaguchi is a research officer in this group and Mrs P. Lanthier operates the amino acid analyzer. Xylanase II was hydrolyzed with 6 M HCl for 24 hours at 110 °C. The first amino acid analysis was performed using a Durrum D-500 amino acid analyzer (Dionex Co., Sunnyvale, CA). This system was replaced by a "Derivatizer-Analyzer Sytem" (Applied Biosystems, Foster City, CA). Repetitions of the amino acid analysis were performed on this system. This system included a 420A Hydrolyzer-Derivatizer, a 130A HPLC Separation System and a 920A Data Analysis Module. Xylanase II was hydrolyzed with 6 M HCl for 75 min at 156°C. For cysteine content, samples were first incubated with performic acid at 4°C overnight. This procedure oxidized the cysteine (and cystin) residues to the more stable cysteic acid (Hirs 1967). Samples were then hydrolyzed and analyzed as described above. For tryptophan content, the HCl hydrolysis was performed in the presence of dodecanethiol in order to improve the recovery of tryptophan residues (Bozzini et al. 1991). Lysozyme was analyzed in parallel in order to calculate the recovery yield of tryptophan.

2.4.2 Cleavage with cyanogen bromide at methionine residues:

Cleavage of peptide bonds on the carboxyl side of methionine residues was performed using CNBr in the presence of formic acid according to the method of Gross (1967). A sample of 1.5 mg of xylanase II was dissolved in 500 µL of 88% (v/v) formic acid. Solid CNBr (5 mg) was added and the reaction tube was flushed with argon. The tube was sealed and incubated in the dark at room temperature for 24

h. The reagent and solvent were removed under a stream of N_2. The remaining material was resuspended in 1 mL of Milli-Q water (Millipore, Bedford, MA) and freeze-dried. The last step was repeated in order to remove the last traces of reagent.

The cleavage products were dissolved by boiling for 2 min in a buffer containing: 10 mM H_3PO_4 (pH adjusted to 6.8 with Tris base), 8 M urea, 2.5% SDS, 5% β-mercaptoethanol and 0.02% bromophenol blue. The cleavage products were separated using the Swank and Munkres (1971) electrophoresis system which includes the use of 8 M urea in the gel in order to increase the resolution and the solubility of peptides. The gel was cast and run using a Mini-Protean II slab cell (BioRad Lab.). Following separation, the gel was fixed (1 h) in 25% isopropanol and 10% acetic acid and stained (1 h) in 0.02% coomassie blue R-250 in 7% acetic acid. The gel was destained in 7% acetic acid until the polypeptide bands were clearly visible against the background (approx. 30-60 min). The polypeptide standards were a mixture of the partial CNBr cleavage products from sperm whale myoglobin (Calibration kit from Pharmacia LKB Biotechnology).

For amino acid sequencing, the polypeptides were transferred from an unstained gel to a PVDF membrane (Immobilon, IPVH0010, Millipore). Following electrophoresis, the gel was equilibrated in the transfer buffer which contained 10 mM CAPS (pH 11) and 10% methanol. The polypeptides were transferred to a PVDF membrane using a Mini Trans-Blot Electrophoretic Transfer Cell (BioRad Lab.) at 100 volts for 1 hour. They were detected after the membrane was stained (10-15 min) with 0.2% coomassie blue in 50% methanol and 10% acetic acid. The membrane was destained with 40% methanol and 5% acetic acid. The membrane was rinsed extensively with distilled water and the polypeptide bands cut out and introduced in the cartridge of the gas-phase sequencer described in section 2.4.3.

2.4.3 Amino acid sequencing:

The amino acid sequencing was performed in collaboration with the Protein Structure and Mutagenesis Group of the Institute for Biological Sciences. Mr D. C. Watson operates the amino acid sequencer. The N-terminus of the intact xylanase II was sequenced by automated Edman degradation using an Applied Biosytems (Foster City, CA) 475A protein sequencing system incorporating a model 470A gas phase sequencer equipped with an on-line model 120A PTH analyzer under the control of a model 900A control/data module.

2.4.4 Isolation and study of a preparation containing a nicked xylanase II:

The purification procedure described in section 2.3.2 was used with the following modifications. The material that did not bind to the anion-exchange column was passed through a column eluate concentrator CEC1 (Amicon, Danvers, MA) fitted with a 10 000 MW cut-off membrane. Over 70% of the xylanase activity was found in the filtrate. This material was loaded on a 2.5 cm X 58 cm column packed with Sephacryl S-300 gel (Pharmacia LKB Biotechnology). Xylanase II interacted with the Sephacryl S-300 as it eluted in a volume approximately equivalent to the total volume of the column (200 mL). This enriched preparation was applied to the Superose 12 column which was eluted as described in section 2.3.2 except that the flow rate was 0.15 mL/min. Two major protein peaks were observed. The peak which eluted last contained xylanase II purified to at least 99% homogeneity according to SDS-PAGE. The other peak eluted from the Superose 12 column contained what appeared to be a nicked xylanase II. Analysis by SDS-PAGE revealed two bands. The two bands were transferred to a PVDF membrane as described in section 2.4.2 except that 0.1% SDS was added to the transfer buffer and methanol was omitted. The N-terminal sequence of the transferred bands was determined as described in section 2.4.3.

2.4.5 Evidence for the presence of a disulfide bridge:

Xylanase II was boiled for 3 min in a sample buffer containing 10 mM Tris-HCl

(pH 8.0), 1 mM EDTA, 2.5% SDS, 0.01% bromophenol blue and 100 mM DTT. A sample was also prepared without DTT. Electrophoresis was performed using the automated Phastsystem (Pharmacia LKB Biotechnology) and pre-cast PhastGels containing 20% acrylamide. The gels were stained using PhastGel Blue R according to the manufacturer's instruction. The shift in mobility observed in the presence of DTT indicated the presence of a disulfide bridge in xylanase II.

2.5 PROPERTIES AND MODE OF ACTION OF XYLANASE II:

2.5.1 Xylanase assay:

The standard xylanase assay consisted of incubating a sample of appropriately diluted xylanase with 0.5% oat-spelts xylan (#X-0376, Sigma Chem. Co.) in a total volume of 2 mL (50 mM sodium acetate, 0.02% NaN_3, pH 4.8). The mixture was incubated 30 min at 65°C. The reducing sugars were quantified using the DNS reagent (Miller 1959). The Somogyi Nelson method (Somogyi 1952, Nelson 1944) was also used for the determination of reducing sugars. A unit of xylanase activity was defined as the amount of enzyme catalyzing the release of the equivalent of 1 µmol of xylose per minute.

The time dependance of the standard assay was tested over 60 min using 90 ng of purified xylanase. The enzyme concentration dependance of the standard assay was tested over a range of 50 to 300 ng of purified xylanase.

2.5.2 Substrate specificity:

The substrate specificity of xylanase II was studied with the following substrates: 0.5% oat-spelts xylan (#X-0376, Sigma Chem. Co.), 0.5% larch wood xylan (#X-3875, Sigma Chem. Co.), 0.5% carboxymethylcellulose (#C-4888, Sigma Chem. Co.), 0.5% acid-swollen cellulose (Wood 1988), 0.5% salicin (#S-0625, Sigma Chem. Co.), 5 mM p-nitrophenyl β-D-xylopyranoside (#N-2132, Sigma Chem. Co.) and Whatman #1 filter paper (70 mg in a final volume of 2 mL). All the substrates, other

than filter paper, were incubated with the enzyme for 30 min at 65°C. The filter paper assay was carried out for 60 min at 65°C. Between 50-100 ng of xylanase II was added to each of the xylan rich substrates. The cellulosic substrates had either 50 or 2500 ng of xylanase II added to them to determine if hydrolysis could occur over a wide range of enzyme concentrations. Reducing sugars were assayed using the DNS method (Miller 1959). Release of p-nitrophenol from p-nitrophenyl xylopyranoside was determined by absorbance measurements at 410 nm following the addition of 1 mL of 1 M Na_2CO_3 to the 1.1 mL assay volume.

2.5.3 Optimal pH and optimal temperature determination:

The optimal pH for activity of xylanase II was determined by the standard xylanase assay using either 50 mM sodium acetate or 50 mM sodium citrate at various pH's. The temperature optimum of xylanase II was determined by incubating 0.5% oat-spelts xylan (in 50 mM sodium acetate, pH 4.8) for 30 min at temperatures ranging from 45°C to 80°C. Thermal stability was determined by incubating xylanase II (0.4 mg/mL) at temperatures ranging from 50 to 65°C for 10 hours. Aliquots were taken at various times and their activity was compared to the activity of a control sample incubated at 4°C.

2.5.4 Effect of inorganic salts on xylanase II:

The effect of the following inorganic salts was tested at a concentration of 2 mM: $CaSO_4$, $CaCl_2$, $CuSO_4$, $MgSO_4$, Na_2SO_4, NaCl, lead acetate, $ZnSO_4$. Disodium EDTA was also tested at a concentration of 10 mM. The standard xylanase assay (30 min at 65°C) was performed with and without the salts listed above.

2.5.5 Determination of K_m and of k_{cat}:

Kinetics parameters were determined in triplicate using oat-spelts xylan (#X-0376, Sigma Chem. Co.), larch wood xylan (#X-3875, Sigma Chem. Co.) and 4-O-methyl-D-glucurono-D-xylan (#M-5144, Sigma Chem. Co.). Xylanase II (220 ng) was

incubated at 60°C for 10 min with 0.25 to 10 mg/mL of the substrates mentioned above. The reducing sugars were determined using the Somogyi Nelson method. The kinetics parameters (K_m and k_{cat}) were calculated using a non-linear regression method with the software "Enzfitter" (R.J. Leatherbarrow, Elsevier-BIOSOFT, Cambridge, UK). K_m is the Michaelis-Menten constant and is defined as the concentration of a specific substrate at which a given enzyme yields one-half of its maximum velocity. The turnover number (k_{cat}) of an enzyme represents the maximum number of substrate molecules converted to products per active site per unit time.

2.5.6 The 32-kDa xylanase of *Thermoascus crustaceus*:

The mode of action of xylanase II was compared with another thermostable xylanase, the 32-kDa xylanase from *Thermoascus crustaceus*. This xylanase was purified by Dr. Larry U. L. Tan (Tan et al. 1987a). About 2 mg of the 32-kDa xylanase were available for this project. The purity of the material was checked by SDS-PAGE and IEF.

2.5.7 Analysis of the products of oat-spelts xylan hydrolysis:

Twenty mg of oat-spelts xylan (in 2 mL of 50 mM ammonium acetate, pH 4.8) were incubated with 1 U of xylanase II or 32-kDa xylanase. Samples were taken after various times and analyzed by TLC and for reducing sugars (Somogyi Nelson method). Samples for TLC analysis were mixed with acetonitrile (25% final concentration) to inactivate the enzyme. They were air-dried and rehydrated (in 25% acetonitrile) using various volumes in order to obtain aliquots of equivalent reducing sugar concentrations. They were then spotted in the preadsorbent zone of high performance TLC plates (10 X 10 cm, #LHP-KF, Whatman Paper Ltd). The plates were developed (approx. 1 h) using ethyl acetate:H_2O:methanol (40:15:20). Following development, the plates were dried and immersed in a solution of silver nitrate (1 mL aqueous saturated $AgNO_3$ in 200 mL acetone). The plates were then dried and sprayed with 0.5 N NaOH in ethanol. This method allows the detection of reducing

sugars (Trevelyan et al. 1950) as black spots over a clear background. The background could be reduced by brief immersion in 5% sodium thiosulfate followed by washing in distilled water.

2.5.8 Hydrolysis of xylo-oligosaccharides:

Xylo-oligosaccharides were kindly provided by Dr. W. Wakarchuk (Protein Structure and Mutagenesis Group, National Research Council of Canada, Ottawa). Xylotriose and xylotetraose were used at a concentration of 10 mM in 50 mM ammonium acetate (pH 4.8). Xylanase II or the 32-kDa xylanase from *T. crustaceus* were added at a final concentration of 2 U/mL. The mixtures were incubated at 60°C for up to 120 min. Samples were withdrawn after various times and the enzyme inactivated by the addition of acetonitrile (25% final concentration). The hydrolysis products were analyzed by TLC on 10 X 10 cm high performance TLC plates as described in section 2.5.7.

2.5.9 Solubilization assay:

The insoluble part of oat-spelts xylan (#X-0376, Sigma Chem. Co.) was prepared as follows. Oat-spelts xylan (10-15 g) was added to 200 mL of distilled water and the mixture was brought to 100°C and stirred for 5 min. The mixture was cooled down to room temperature and centrifuged for 10 min (3000 *g*). The pellet was washed 3 times with hot (60-70°C) distilled water and the insoluble material recovered and freeze-dried. The insoluble material represented approximately 25-30% of the starting material.

For the solubilization assay, the insoluble xylan was dispersed in 50 mM sodium acetate (pH 4.8) using a mortar. The final concentration was 1 mg/mL. Xylanase II or the 32-kDa xylanase from *T. crustaceus* were added at a concentration of 0.25 U per mL. The mixtures were incubated at 60 °C with agitation (gyrotory shaker, 200 rpm) for up to 120 min. The solubilization of xylan was followed by measuring the

decrease of absorbance (light scattering) at 620 nm.

2.6 HYDROLYSIS OF VARIOUS XYLANS BY THE TWO THERMOPHILIC XYLANASES:

2.6.1 Substrates:

Oat-spelts xylan (#X-0376), larch wood xylan (#X-3875) and 4-O-methyl-D-glucurono-D-xylan (#M-5144) were purchased from Sigma Chem. Co. Insoluble xylan was prepared as described in section 2.5.9. Poplar kraft pulp (PKP) and spruce kraft pulp (SKP) were gifts from M. Paice (Pulp and Paper Research Institute of Canada, Pointe-Claire). Xylans were extracted from PKP and SKP using 18% (w/v) sodium hydroxyde by Dr. K. K. Y. Wong (Chair of Forest Products Biotechnology, U.B.C.). All the substrates were suspended in a 50 mM sodium acetate buffer (pH 4.8) which contained 0.02% NaN_3 as an antimicrobial agent. When chromatography analysis was to be performed, ammonium acetate replaced sodium acetate.

2.6.2 Enzyme limiting hydrolysis:

7.5 mg of each xylan substrate (in 0.75 mL of sodium acetate buffer) was incubated with 6 mU of xylanase II or 32-kDa xylanase from *Thermoascus crustaceus*. Ten mU of each enzyme was used when insoluble xylan was the substrate. The two xylanases were used individually and together in the enzyme limiting hydrolysis which were performed under static conditions at 60°C for 30 min. This experiment was performed 3 times. The reducing sugars were quantified using the Somogyi Nelson method as described in section 2.5.1.

2.6.3 Substrate limiting hydrolysis:

Twenty mg of each xylan substrate (in 2 mL of ammonium acetate buffer) were incubated with 1 U of xylanase II or 32-kDa xylanase. The two xylanases were used individually and together in substrate limiting hydrolysis of 24 h at 60 °C. The hydrolyses were performed 3 times. The substrate was kept in suspension by agitation

(200 rpm) in a G24 incubator shaker (New Brunswick Scientific Instr. Co., Edison, N.J.). Samples were taken after various times and the reducing sugars quantified by the Somogyi Nelson method. After 24 hours, the mixtures were boiled for 3 min to inactivate the enzyme. Five mg of erythritol were added to be used as an internal standard for HPLC analysis. Insoluble material was removed by centrifugation in a microcentrifuge. The soluble material was dried using a SpeedVac concentrator (Savant Instruments Inc., Hicksville, N.Y.) and kept frozen and dessicated until analysis.

2.6.4 Hydrolysis of residual xylan in poplar kraft pulp:

Fifty mg of poplar kraft pulp (in 5 mL of ammonium acetate buffer) were incubated with 1 U of xylanase II or 32-kDa xylanase. The two enzymes were used individually and together for 3 h at 60°C. This experiment was performed 3 times. The substrate was kept in suspension by agitation (200 rpm) in a G24 incubator shaker. After 3 h, the enzymes were inactivated by boiling for 3 min. One mg of erythritol was added to be used as an internal standard for HPLC analysis. The pulp was sedimented by centrifugation (10 000 *g*, 10 min). The reducing sugars were quantified in the supernatant by the Somogyi Nelson method. Samples to be used for HPLC analysis were dried using a Speed Vac concentrator. These samples were kept frozen and dessicated until the analysis was performed.

2.6.5 HPLC analysis:

The samples were rehydrated with distilled water and analyzed by HPLC using a Waters 625 LC System (Millipore Co., Waters Chromatography Division, Milford, MA). The samples (15 μL) were injected using a WISP 700 autosampler. The neutral xylo-oligosaccharides were separated using BioRad columns HPX-87P and HPX42A installed in series (Senior et al. 1988). The detector was a Waters differential refractometer model 410. Mr R. Lenz (Chair of Forest Products Biotechnology, U.B.C.) operated the HPLC system.

3.0 RESULTS AND DISCUSSION:

3.1 Production and Characterization of the Cellulases and Xylanases Secreted by *Thielavia terrestris* 255B:

3.1.1 Introduction:

The ascomycete *Thielavia terrestris* 255B was selected as a good producer of thermostable wood-degrading enzymes following a screening of a culture collection of wood-inhabiting fungi (Wojtczak et al. 1987). Because of the thermostability of these enzymes and their relatively high production levels, *T. terrestris* 255B appeared to be a favourable alternative to the mesophilic fungi such as *Trichoderma* spp. and *Aspergillus* spp. typically used in bioconversion processes.

Because of the potential of *T. terrestris* 255B, we decided to study the cellulases and the xylanases produced by this organism in more details. Initially, we pursued the objective of studying the production of these enzymes on various substrates in order to obtain maximum production of cellulases and xylanases. We also compared the profile of the enzymes produced on the various substrates using non-denaturing electrophoretic techniques in order to identify the major components that are involved in cellulose and xylan degradation.

3.1.2 Biomass production:

In order to obtain maximum production of cellulases and xylanases, we first evaluated the growth of *T. terrestris* 255B on a number of soluble and insoluble substrates. Fungal biomass was measured in order to correlate the growth of the organism with enzyme secretion. For all the substrates used, maximal fungal biomass was obtained after 2 days of growth (Fig. 6). A faster growth rate was observed with glucose and cellobiose which are soluble and easily metabolizable substrates. After

more than 2 days, a decline in biomass was observed during growth on all of the substrates.

Concomitant with the decline in fungal biomass, extracellular protease activity was detected after more than two days of growth (Fig. 7). These observations suggest that depletion of the substrates occurred within 2 days of growth, followed by autolysis of the mycelium. The levels of protease present in the culture filtrates varied considerably depending on the substrate used for growth (Fig. 7). The two cellulosic substrates gave low protease levels while the two soluble substrates (glucose and cellobiose) gave relatively high levels of protease activity. The highest levels of protease activity were obtained after growth on xylan. The onset of high protease levels after two days of growth on xylan must be considered if this substrate is to be used as carbon source for enzyme production.

3.1.3 Enzyme production:

Growth on glucose and cellobiose media resulted in the secretion of low levels of protein that increased slowly after more than two days of growth (Fig. 8). As glucose and cellobiose are soluble and can be transported inside the cell, it is understandable that minimal secretion of extracellular protein occurred. These low levels of protein corresponded to the low amounts of the different cellulolytic and xylanolytic enzymes that were obtained when *T. terrestris* 255B was grown on either glucose or cellobiose (Fig. 9-12). These low levels of cellulases and xylanases were probably released as a consequence of autolysis because these activities appeared when a decrease in biomass was observed (Fig. 6). Growing *T. terrestris* 255B on cellobiose did not yielded high levels of extracellular β-glucosidase. The β-glucosidase probably remained cell-associated because moderate levels were observed only when autolysis occurred.

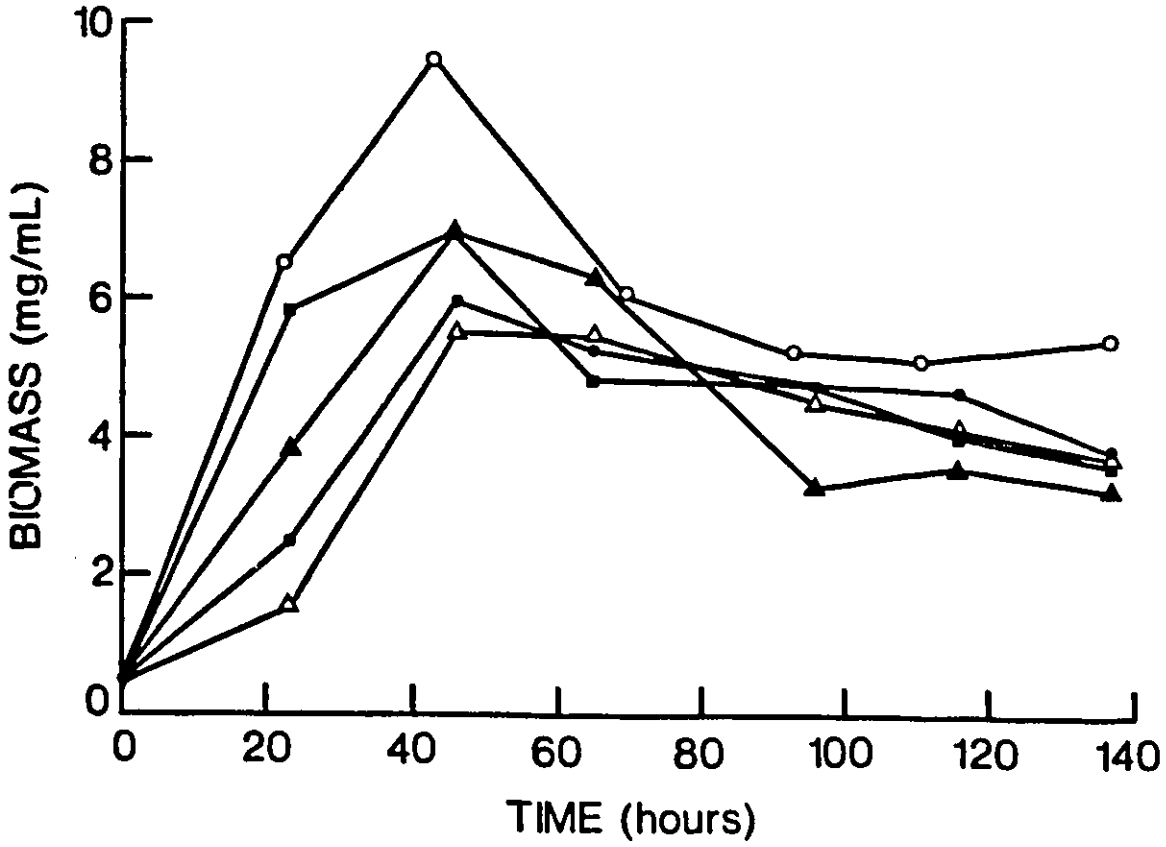


Figure 6: Determination of fungal biomass (based on total mycelial protein) in cultures of *T. terrestris* 255B after growth on glucose (■), cellobiose (○), Solka Floc BW300 (●), acid-swollen cellulose (△) and oat-spelts xylan (▲).

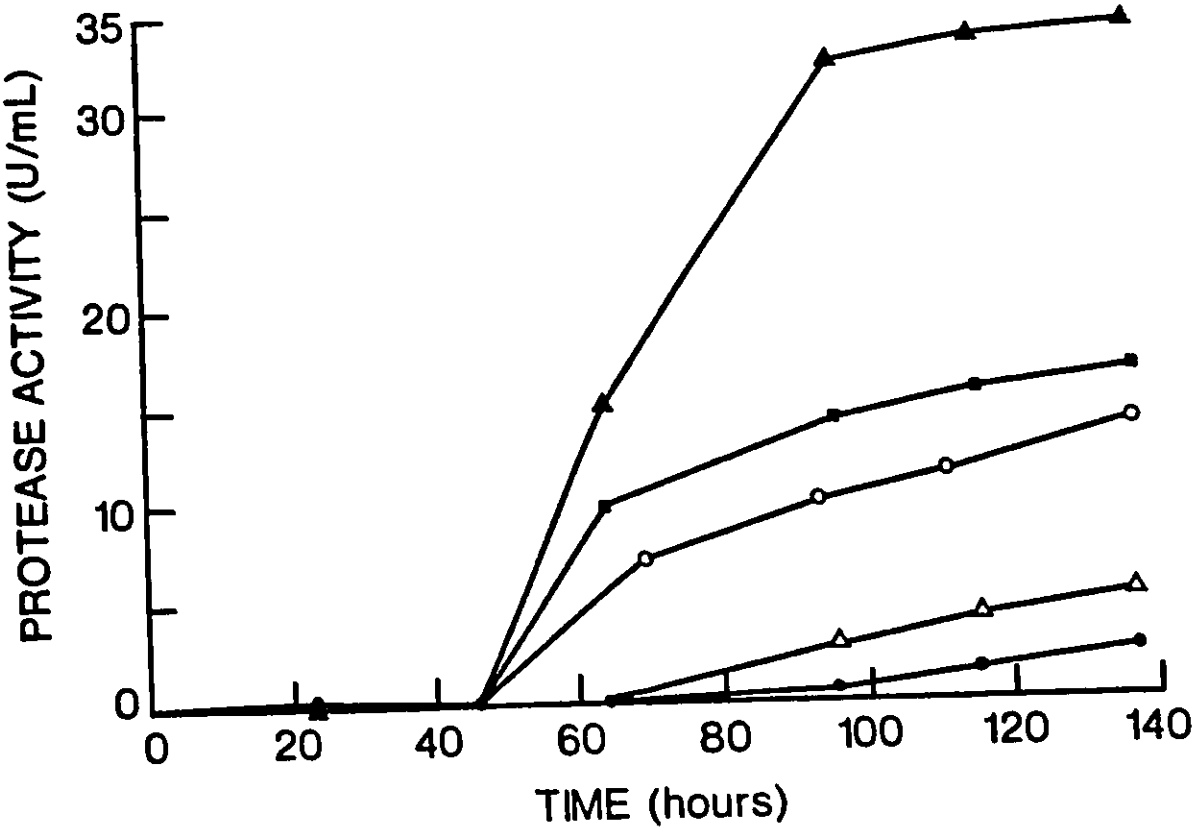


Figure 7: Determination of the protease activity in the culture filtrates of *T. terrestris* 255B after growth on glucose (■), cellobiose (○), Solka Floc BW300 (●), acid-swollen cellulose (△) and oat-spelts xylan (▲).

High levels of filter-paper activity were detected within two days of growth on Solka Floc BW300 (Fig. 9). Although filter-paper activity was relatively stable between day 2-6, the proportion of the different cellulase components probably varied because endoglucanase activity rose two fold and β-glucosidase dropped three fold during this period. The decrease in β-glucosidase activity was possibly a consequence of inactivation of this enzyme by low pH (McHale and Coughlan, 1981) as the pH dropped to around 4.0 between day 2 and 3. Although samples from days 2 and 6 showed comparable filter paper activities, it is obvious that the two preparations differed in their compositions and therefore differed in their ability to carry out long-term hydrolysis.

Growth on acid-swollen cellulose resulted in only low levels of endoglucanase and filter-paper activities (Fig. 9-10). However, significant levels of β-glucosidase activity were obtained (Fig. 11). Acid-swollen cellulose is very amorphous and the ease of its hydrolysis to soluble products may partially repress the production of cellulases.

The highest levels of xylanase activity were obtained after three days of growth on oat-spelts xylan (Fig. 12), levels which declined over the period of day 4 to 6 of growth on oat-spelts xylan. The high levels of protease activity detected at that time (Fig. 7) were probably responsible for the decline of xylanase activity. Relatively high levels of xylanase activity were obtained after 3 to 6 days of growth on Solka Floc BW300. The manufacturer (James River Co., Hackensack, NJ) of this substrate claims that it is at least 99.5% cellulose. However, Solka Floc BW300 was shown to contain approximately 10% xylan (Senior et al. 1989). This small amount of contaminating xylan probably caused the induction of xylanase when *T. terrestris* 255B was grown on Solka Floc BW300.

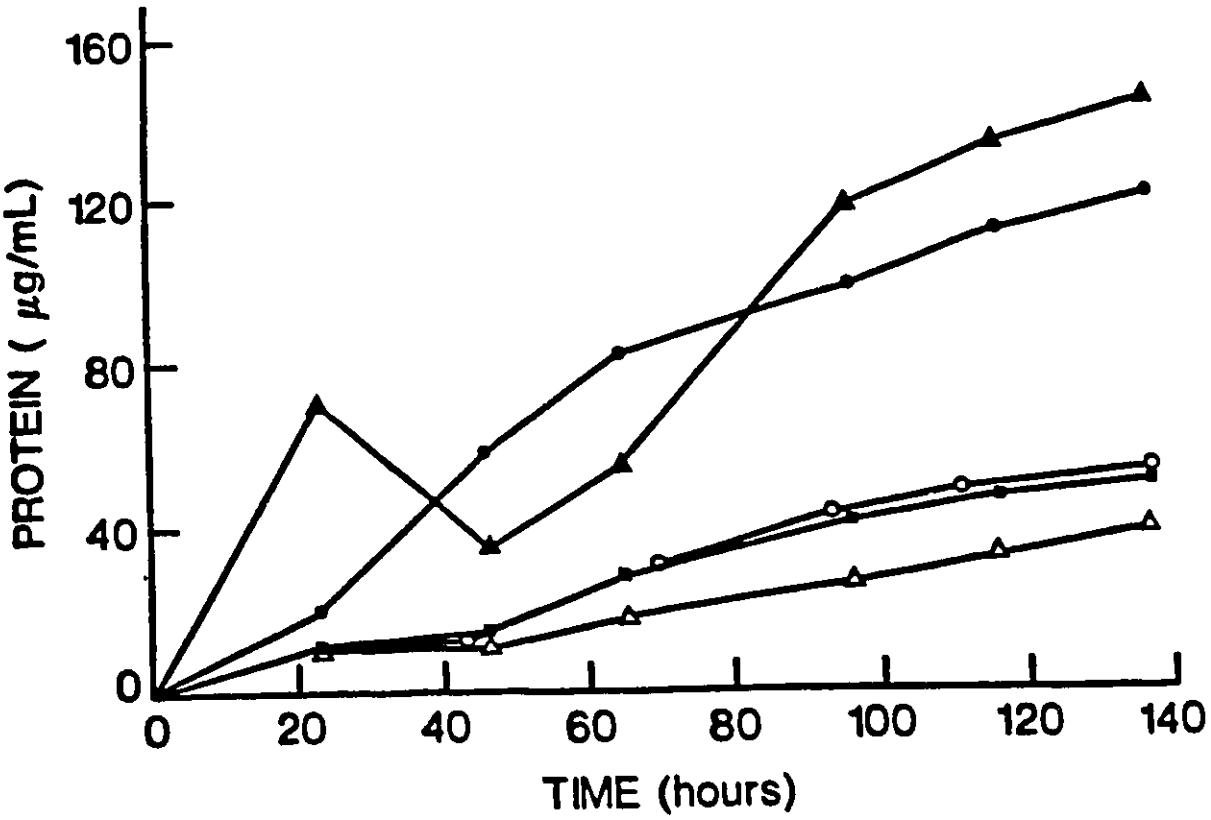


Figure 8: Determination of extracellular protein in the culture filtrates of *T. terrestris* 255B after growth on glucose (■), cellobiose (○), Solka Floc BW300 (●), acid-swollen cellulose (△) and oat-spelts xylan (▲).

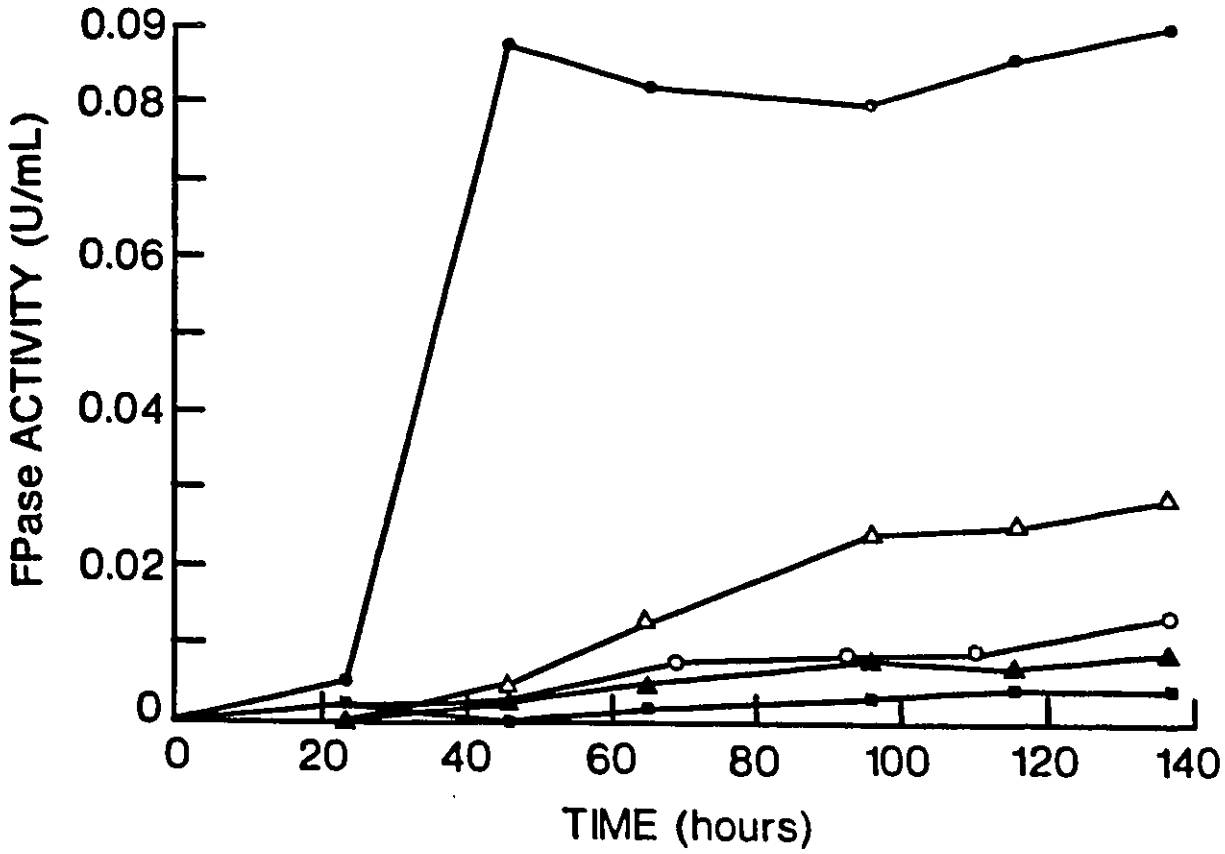


Figure 9: Determination of filter paper activity in the culture filtrates of *T. terrestris* 255B after growth on glucose (■), cellobiose (○), Solka Floc BW300 (●), acid-swollen cellulose (△) and oat-spelts xylan (▲).

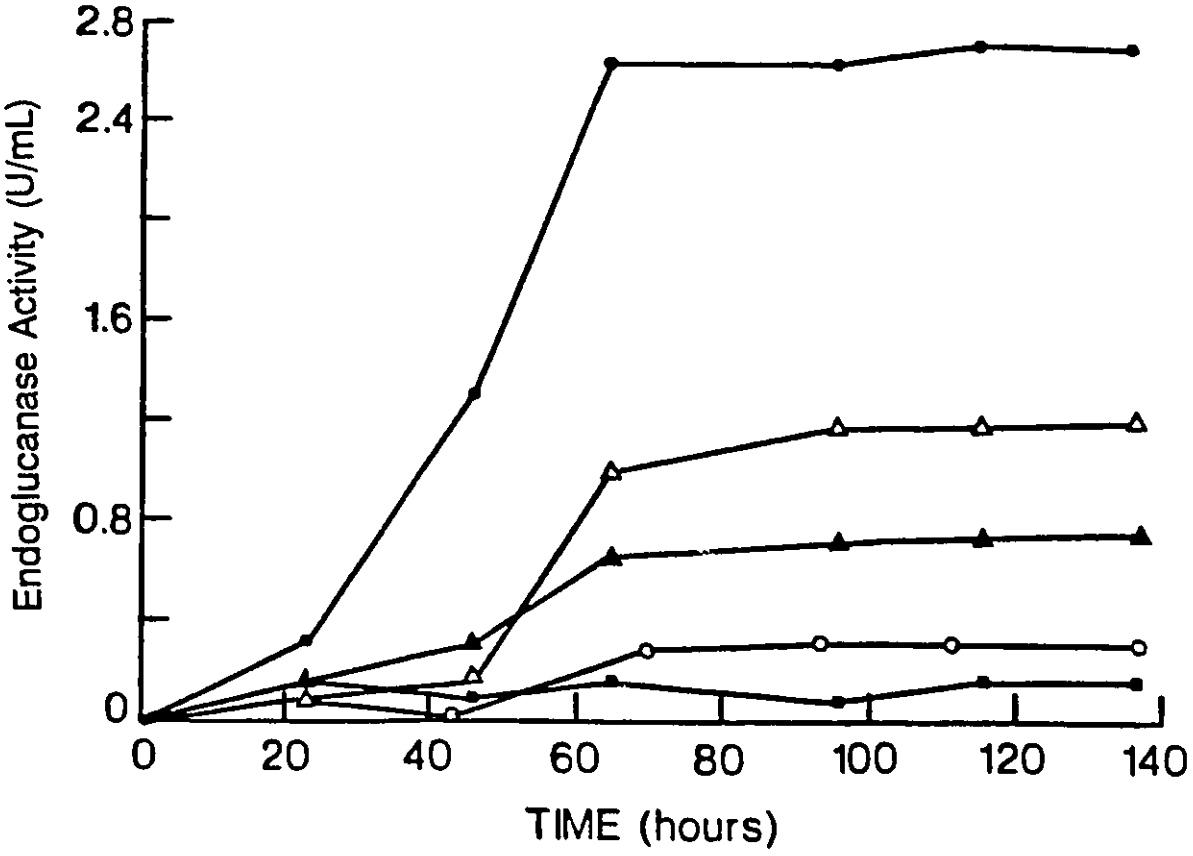


Figure 10: Determination of endoglucanase activity in the culture filtrates of *T. terrestris* 255B after growth on glucose (■), cellobiose (○), Solka Floc BW300 (●), acid-swollen cellulose (△) and oat-spelts xylan (▲).

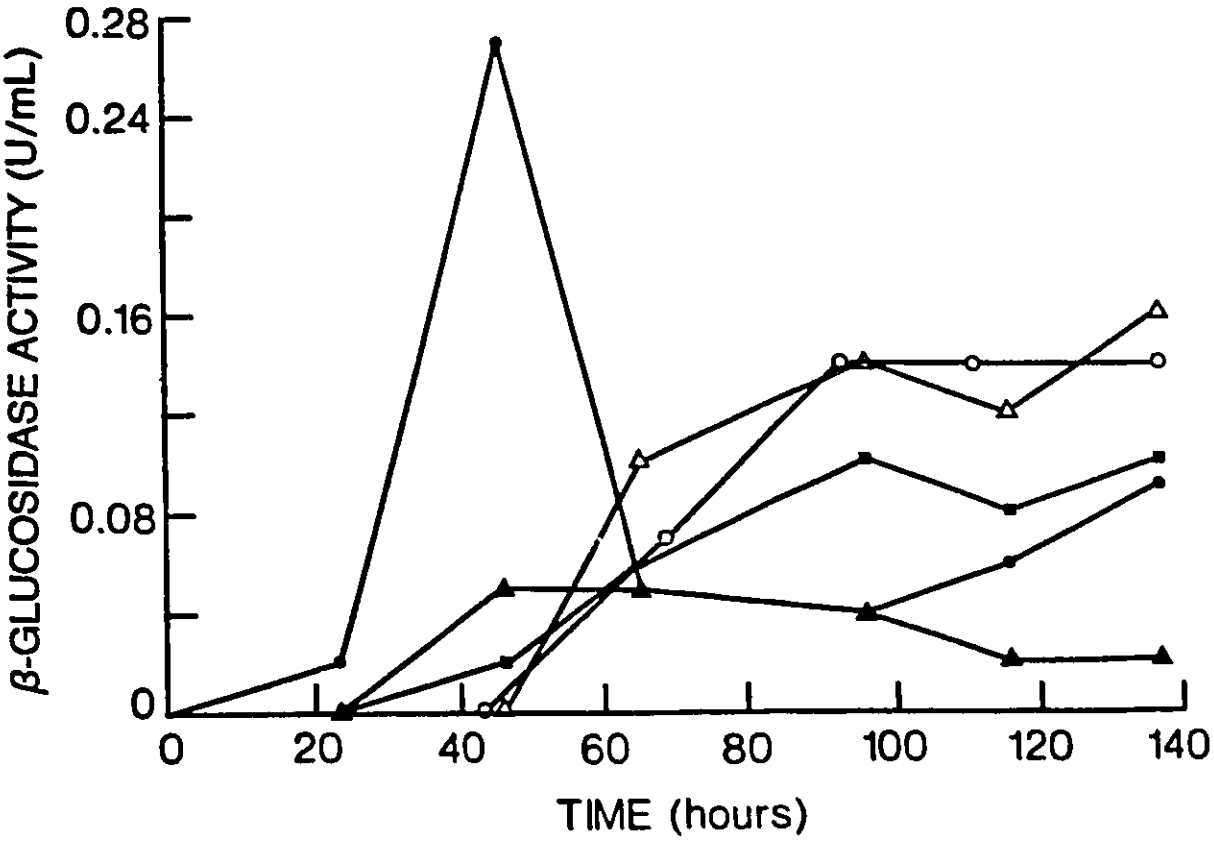


Figure 11: Determination of β-glucosidase activity in the culture filtrates of *T. terrestris* 255B after growth on glucose (■), cellobiose (○), Solka Floc BW300 (●), acid-swollen cellulose (△) and oat-spelts xylan (▲).

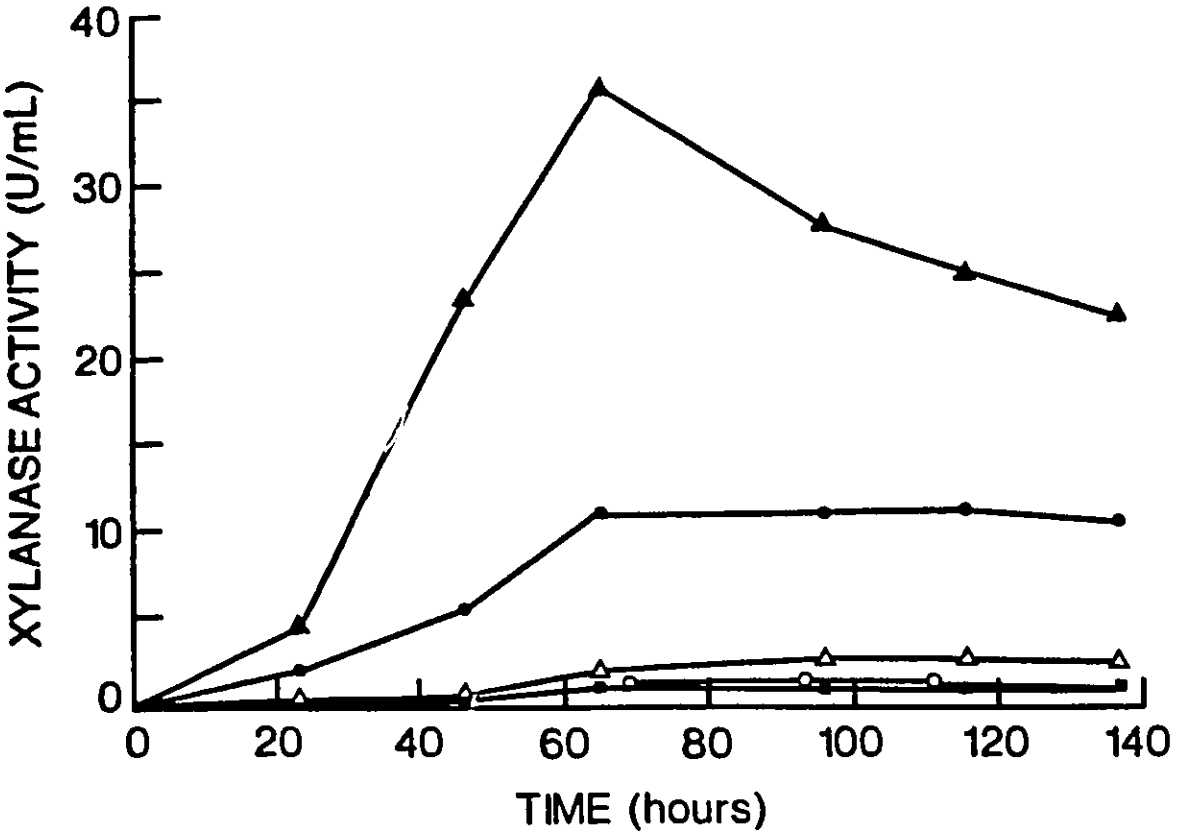


Figure 12: Determination of xylanase activity in the culture filtrates of *T. terrestris* 255B after growth on glucose (■), cellobiose (○), Solka Floc BW300 (●), acid-swollen cellulose (△) and oat-spelts xylan (▲).

The detection of extracellular enzymes following growth on insoluble substrates is influenced by many factors such as, induction by soluble molecules derived from the substrate, increased secretion of enzymes, adsorption of the enzymes to the insoluble substrate, inactivation of the enzymes through various processes, inhibition of the secreted enzymes by their products and repression of further enzyme synthesis by easily metabolizable products.

Thus, the profile of enzymes detected at any particular moment during the growth cycle is determined by a combination of factors. As *T. terrestris* 255B achieved maximal biomass production within two days of growth (Fig. 6), we can assume that extracellular cellulases and xylanases were produced during that time to depolymerize the insoluble substrate. However, we detected maximal cellulase and xylanase activities after more than two days of growth (Fig. 9-12). It is probable that the enzymes present during the first two days of growth were adsorbed to the residual substrate and could not be detected in the culture filtrate. Once the substrate was depleted, these enzymes were liberated in the culture filtrate. Some additional enzymes were probably also liberated due to autolysis of the cells. These two factors could explain why peak enzyme activities were observed after maximum biomass production was detected.

3.1.4 Electrophoretic analysis of the produced enzymes:

As well as assaying the culture filtrates for cellulolytic activity and protein concentration, we also compared the protein profiles using non-denaturing polyacrylamide gel electrophoresis on a gradient (gradient native-PAGE). Bands with endoglucanase and xylanase activities were detected using overlay gels containing carboxymethylcellulose and xylan, respectively.

No protein bands could be detected in culture filtrates from *T. terrestris* 255B grown on glucose or cellobiose after less than two days of growth (Fig. 13). After three to six days of growth, one major and 5 minor protein bands were detected with silver staining. The carboxymethylcellulose overlay (not shown) did not reveal any bands with endoglucanase activity. The xylan overlay revealed one xylanase band after growth on the glucose medium and two xylanase bands after growth on the cellobiose medium. However, these bands were very weak and it was not possible to conclude if there was differential induction of the second xylanase or if we were unable to detect a weak band.

When culture filtrates from *T. terrestris* 255B grown on Solka Floc BW300 were subjected to gradient native-PAGE, proteins of lower mobilities initially predominated (Fig. 14). Two major endoglucanase bands and one major xylanase band were observed after 2 days of growth on this substrate. As the culture grew older, we observed additional bands which had higher mobilities. The trend towards higher mobility bands is consistent with reports suggesting that proteolytic action is partially responsible for the multiplicity of cellulases (Nakayama et al. 1976). Dominguez et al. (1992) reported that purified endoglucanase I from *Trichoderma reesei* had a tendency to self-aggregate under some conditions and resulted in the detection of multiple bands when run on native-PAGE. The dissociation of such aggregates could lead to an increase in bands with higher mobility. We also suggest that distinct components might associate into multi-enzyme complexes which would dissociate as the culture grows older (section 3.1.5).

When *T. terrestris* 255B was grown on acid-swollen cellulose (Fig. 15), the same two major endoglucanase bands obtained after growth on Solka Floc were also

detected but with lowered intensities. It is possible that the acid-swollen cellulose induces essentially the same enzymes as Solka Floc but the faster hydrolysis of the former substrate may partially repress the production of cellulases. Alternatively, acid-swollen cellulose may induce an incomplete cellulase system as fewer components may be required to hydrolyze this amorphous substrate. The smaller number of bands observed when acid-swollen cellulose was used as the substrate (compared with Solka Floc) supports this view. However, it is not clear how the organism could differen-tiate between these two chemically similar substrates. Until the inducing molecules are identified, this issue is likely to remain unresolved.

The protein and enzyme profiles obtained after growth on xylan are shown in Figure 16. Little clearing was observed with the CMC overlay which is consistent with the low activity detected by enzymatic assay (Fig. 10). The xylan overlay showed two major clearing bands over the first three days of growth. Another major xylanase band which appeared after days 4 to 6 was possibly a proteolytic product of one of the original bands. The two major xylanase bands obtained on xylan had the same mobility as those obtained after growth on Solka Floc.

Isoelectrofocusing was also used as a non-denaturing separation method (Fig. 17 and 18). This technique was found to be more sensitive than native-PAGE for both protein and enzymatic detection. For example, after three days of growth on glucose, six protein bands were detected by native-PAGE and 11 were detected by IEF. After growth on cellobiose, no endoglucanase bands could be detected by the CMC overlay on native-PAGE but at least 5 endoglucanase bands could be detected by overlay on an IEF gel. It was difficult to resolve the enzymes produced during growth on Solka Floc and the large clearing zones obtained were probably due to

Figure 13: Analysis, by native-PAGE on a gradient gel (8-25%), of the extracellular protein obtained after growth of *T. terrestris* 255B on glucose or cellobiose (1 μL of culture filtrate concentrated 20-fold was applied to each lane):

Lane 1: MW standards ($\times 10^3$).

Lanes 2-5: Glucose medium;

Lane 2: day 1 (silver stain)
Lane 3: day 4 (silver stain)
Lane 4: day 6 (silver stain)
Lane 5: day 4 (xylanase zymogram)

Lanes 6-9: Cellobiose medium;

Lane 6: day 1 (silver stain)
Lane 7: day 4 (silver stain)
Lane 8: day 6 (silver stain)
Lane 9: day 4 (xylanase zymogram)

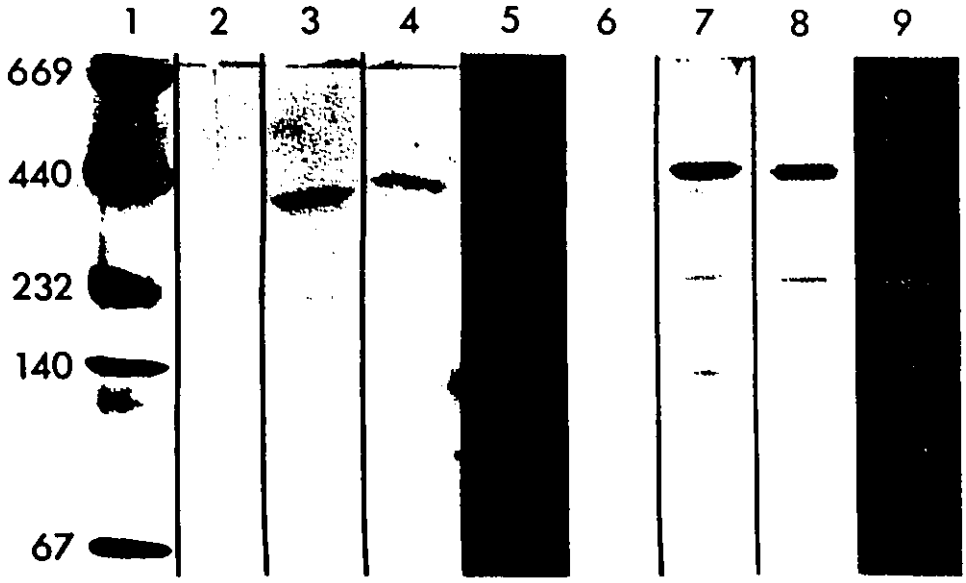


Fig.13

Figure 14: Analysis, by native-PAGE on a gradient gel (8-25%), of the extracellular protein obtained after growth of *T. terrestris* 255B on Solka Floc BW300 (1 μL of culture filtrate concentrated 20-fold was applied to each lane):

Lane 1: MW standards ($\times 10^3$).

Lanes 2-5: Silver stain;

Lane 2: day 1
Lane 3: day 2
Lane 4: day 4
Lane 5: day 6

Lanes 6-8: Endoglucanase zymogram;

Lane 6: day 2
Lane 7: day 4
Lane 8: day 6

Lanes 9-10: Xylanase zymogram;

Lane 9: day 2
Lane 10: day 6

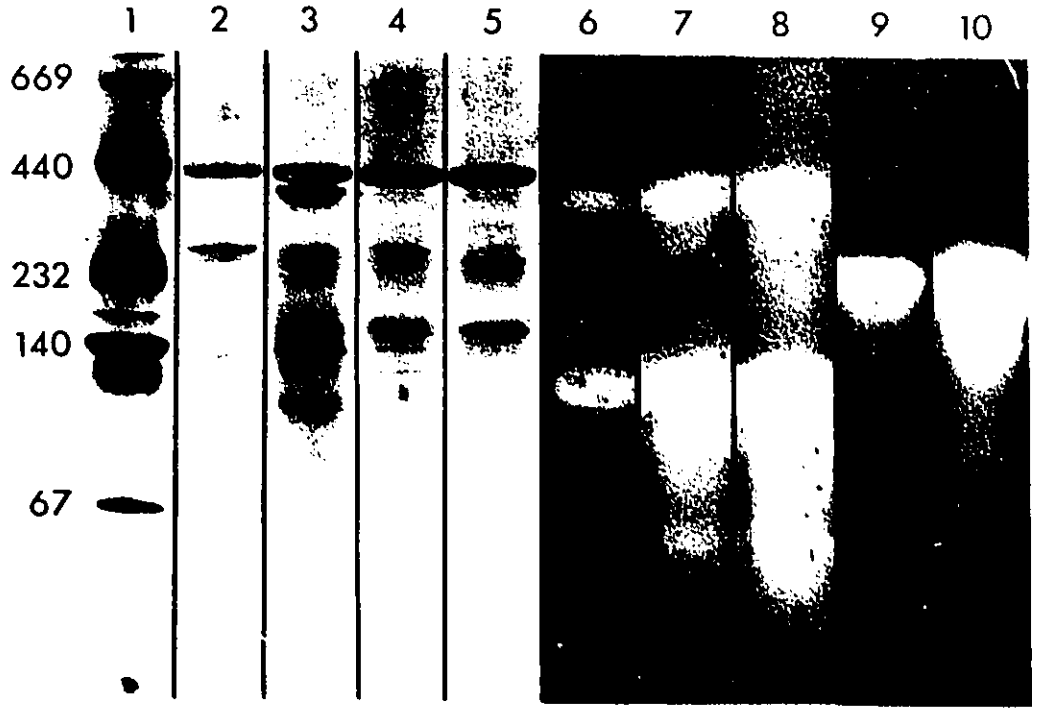

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Figure 15: Analysis, by native-PAGE on a gradient gel (8-25%), of the extracellular protein obtained after growth of *T. terrestris* 255B on acid-swollen cellulose (1 µL of culture filtrate concentrated 20-fold was applied to each lane):

Lane 1: MW standards ($\times 10^3$).

Lanes 2-5: Silver stain;

Lane 2: day 1
Lane 3: day 3
Lane 4: day 4
Lane 5: day 6

Lanes 6-7: Endoglucanase zymogram;

Lane 6: day 3
Lane 7: day 6

Lanes 8-9: Xylanase zymogram;

Lane 8: day 3
Lane 9: day 6

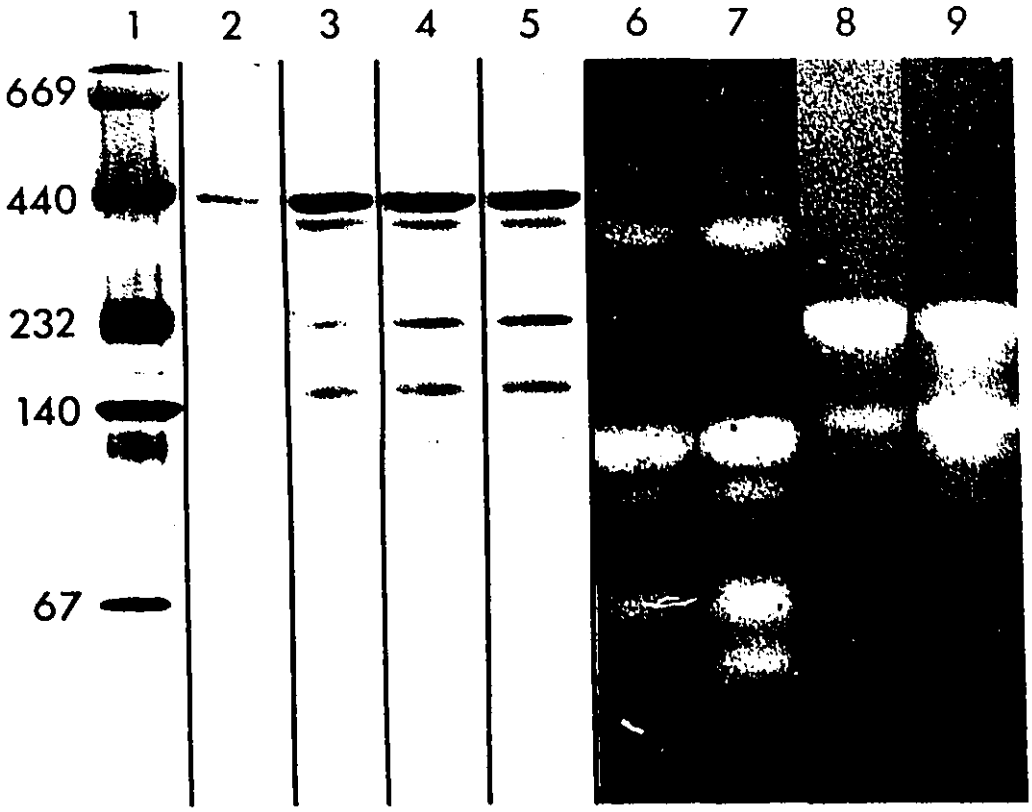
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Figure 16: Analysis, by native-PAGE on a gradient gel (8-25%), of the extracellular protein obtained after growth of *T. terrestris* 255B on oat-spelts xylan (1 μL of culture filtrate concentrated 20-fold was applied to each lane):

Lane 1: MW standards ($\times 10^3$).

Lanes 2-5: Silver stain;

Lane 2: day 1
Lane 3: day 2
Lane 4: day 4
Lane 5: day 6

Lanes 6-7: Endoglucanase zymogram;

Lane 6: day 2
Lane 7: day 6

Lanes 8-9: Xylanase zymogram;

Lane 8: day 2
Lane 9: day 6

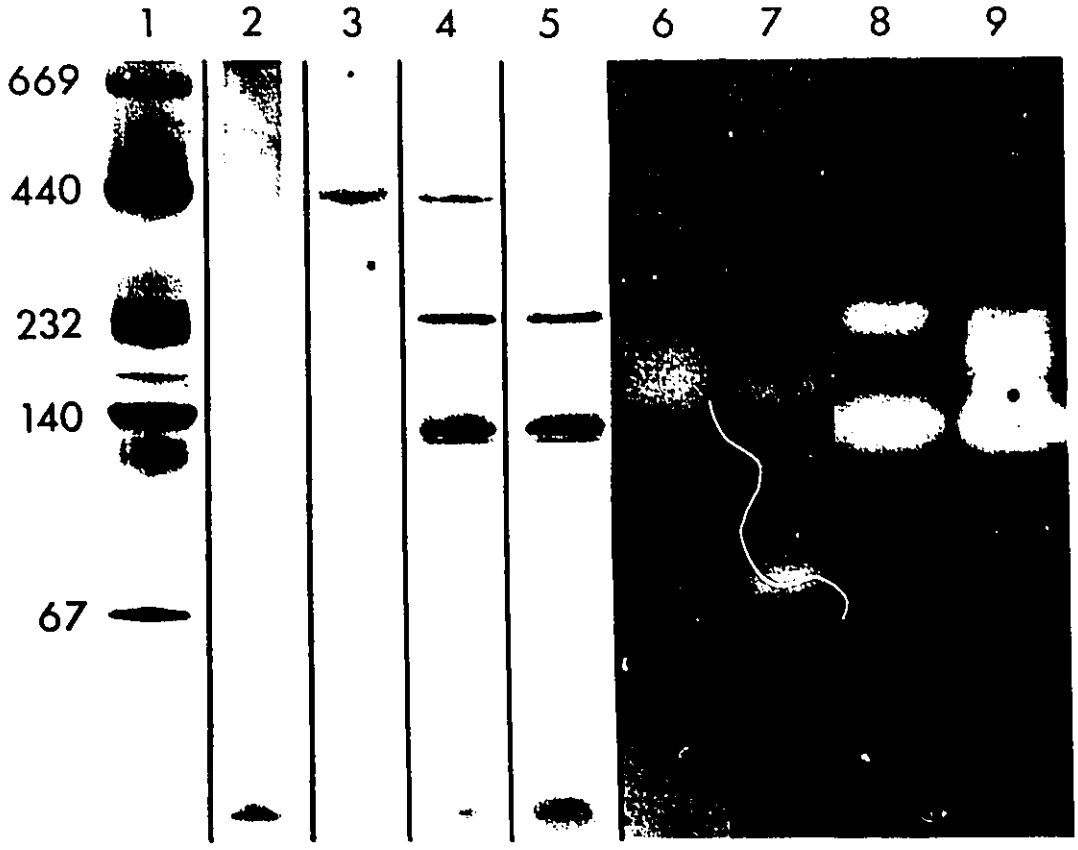
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Figure 17: Analysis by IEF of the extracellular protein obtained after growth of *T. terrestris* 255B for 3 days on various substrates (1 µL of culture filtrate concentrated 4-fold was applied to each lane):

Lane 1: pI standards

Lanes 2-6: Silver stain;

Lane 2: Glucose medium
Lane 3: Cellobiose medium
Lane 4: Solka Floc medium
Lane 5: Acid-swollen cellulose medium
Lane 6: Xylan medium

Lanes 7-10: Endoglucanase zymogram;

Lane 7: Cellobiose medium
Lane 8: Solka Floc medium
Lane 9: Acid-swollen cellulose medium
Lane 10: xylan medium.

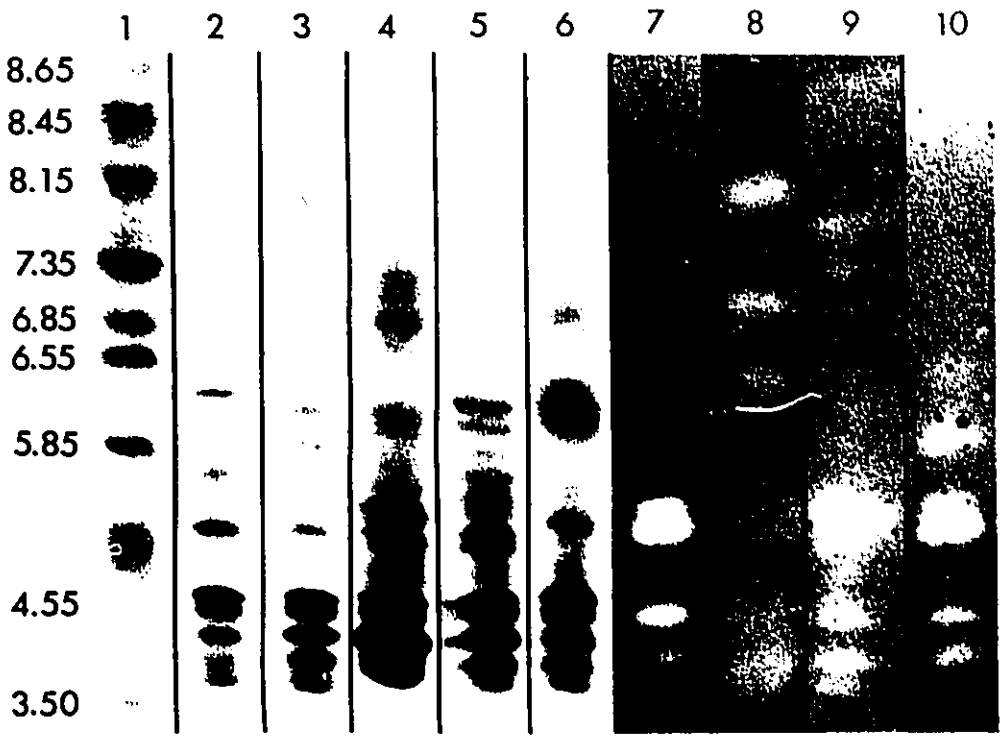

1
2
3
4
5
6
7
8
9
10
8.65
8.45
8.15
7.35
6.85
6.55
5.85
4.55
3.50

Figure 18: Detection of the bands with xylanase activity following separation by IEF of the protein obtained after growth on various substrates for three days (1 μL of culture filtrate concentrated 4-fold was applied to each lane):

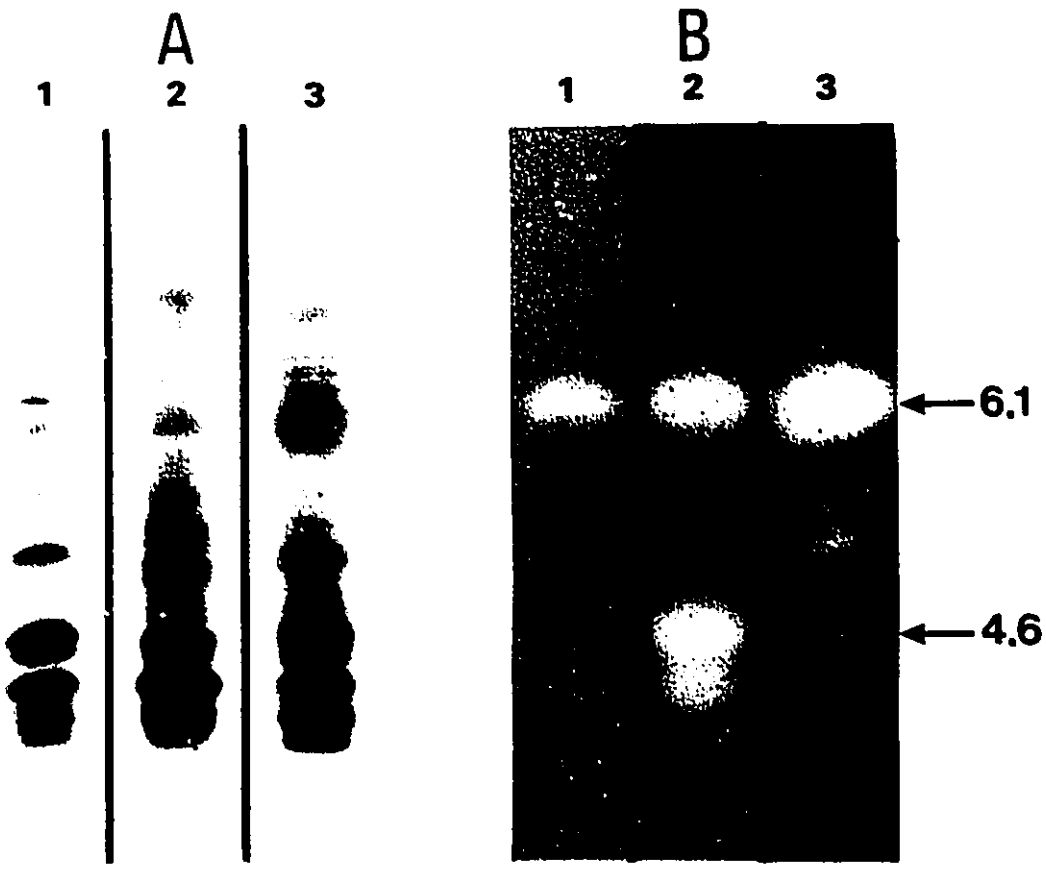
Panel A: Silver staining of the gel.

Panel B: Overlay with gel containing oat-spelts xylan.

Lane 1: Growth on glucose.

Lane 2: Growth on Solka Floc BW300.

Lane 3: Growth on oat-spelts xylan.


A
1
2
3
B
1
2
3
6.1
4.6

bands that focussed closely to each other. The higher sensitivity of IEF is probably due to the low concentration of polyacrylamide (5%) in the gel which allows better transfer of enzymes to the overlay. In addition to providing higher sensitivity, IEF also showed that most of the enzymes in the cellulase system of *T. terrestris* 255B had acidic pI's (below 5.).

IEF revealed that *T. terrestris* 255B produced two major forms of xylanases (Fig. 18) which we designated xylanase I (pI=4.6) and xylanase II (pI=6.1) following the International Enzyme Commission recommendation of numbering isoenzymes according to their pI values. These two xylanase bands were also observed when the extracellular protein were separated by native-PAGE (Fig. 13, 14, 15, 16). Xylanase I was the band with the lower mobility (best observed on Fig. 14, lane 9) and xylanase II was the band with the higher mobility (best observed on Fig. 16, lane 8). We also confirmed that the band with a pI of 6.1 on the IEF gel and the xylanase band with the higher mobility on native-PAGE were the same by purifying this enzyme and running the eluted protein on the corresponding gels (not shown).

Only xylanase II was detected after growth on glucose which apparently failed to completely repress the production of this form of xylanase (Fig. 18). Weak bands of both xylanases I and II were detected after growth on cellobiose or acid-swollen cellulose (not shown). Xylanase II largely predominated after growth on oat-spelts xylan (Fig. 18). The induction of xylanase II was probably responsible for the higher levels of xylanase activity found after growth on oat-spelts xylan (Fig. 12). Both xylanase I and II were detected with comparable clearing zone intensities after growth on Solka Floc BW300. A third clearing zone was also detected at a pI of approximately 4.3 but the fuzzy definition of this band suggested that it might have

been caused by more than one protein. Solka Floc BW300 contains contaminating xylan which was probably responsible for the induction of relatively high xylanase activity after *T. terrestris* 255B was grown on this substrate (Fig. 12). However, the production of xylanase I was probably also influenced by the presence of cellulose because the intensity of the clearing zone suggested that, relative to xylanase II, more of xylanase I was produced after growth on Solka Floc BW300 than after growth on oat-spelts xylan. Both the cellulolytic and xylanolytic systems comprise many components and it is possible that the close association of their substrates in nature has led to enzyme regulation that is inter-related.

3.1.5 Estimation of the molecular weight of the major endoglucanases:

The combined use of non-denaturating electrophoretic techniques and overlay methods enabled us to compare the protein and enzyme profiles obtained after growth of *T. terrestris* 255B on various substrates. By using gradient native-PAGE, we also wanted to determine whether large molecular weight complexes were present in the cellulase system of *T. terrestris* 255B. Although extracellular cellulases produced by fungi are generally considered to exist as individual entities, there are a few reports which suggested the existence of fungal cellulase complexes (Sprey and Lambert 1983, Wilson and Wood 1992). Furthermore, Goyal et al. (1991) have pointed out that most work on fungal cellulase have focussed on the purification of single components without taking into account the possibility that these components might be part of functional complexes *in vivo*.

Non-denaturing separation techniques are necessary to demonstrate the presence of multi-component complexes which are expected to have large molecular weights. Native-PAGE using a gel with a gradient of acrylamide concentration (8 to

25% in our case) theoretically provides the separation of the proteins according to their size (Margolis and Wrigley 1975). In this technique (also called pore gradient electrophoresis), the increasing acrylamide concentration causes a progressive decrease in the mobility of the proteins. If electrophoresis is continued until protein migration has virtually stopped (pore-size limit), proteins of low-charge density will "catch up" to proteins of a similar size but higher charge density.

The molecular weight markers indicated in Figures 13-16 were from a kit (Pharmacia HMW calibration kit) designed to be used for the determination of protein molecular weights using pore gradient electrophoresis. The markers were well separated according to their molecular weights (MW's) and a plot of log(MW) against mobility yielded a correlation coefficient (r) of -0.997. We used these markers to calculate the apparent MW's of the components with the lower mobilities following gradient native-PAGE of the protein obtained after growth on Solka Floc (Figure 14). The protein band with the lower mobility had an apparent MW of 405,000. The endoglucanase and xylanase bands with the lower mobilities had apparent MW's of 370,000 and 230,000, respectively. Most fungal cellulases and xylanases were reported to have MW's below 100,000 (Coughlan and Ljungdahl 1988). Thus, it is possible that the bands with the lower mobilities correspond to complexes of different enzyme components. Complex formation could provide optimal spatial organization of the components for efficient cellulose hydrolysis. However, it would be important to confirm the MWs using another technique (such as gel filtration) and to purify the proteins with apparent high MW to study their composition. These attempts are presented in section 3.2.2.

3.1.6 Hydrolysis of *Valonia microcrystals:*

The cellulase systems produced by various microorganisms have been roughly classified as "complete" or "incomplete" based on whether or not they could degrade crystalline cellulose (Coughlan 1985). The ability to degrade crystalline cellulose has generally been tested using commercially available substrates such as Avicel-PH101 which is a microcrystalline cellulose. The cellulase system of *T. terrestris* could degrade this substrate but we further confirmed that *T. terrestris* 255B produced a complete cellulase system by showing that it could attack *Valonia ventricosa* microcrystals. This latter substrate is considered to be one of the most crystalline celluloses, with a crystallinity index close to 100% (Bourret et al. 1972). The crystalline perfection of *Valonia* microcrystals also makes them suitable substrate for electron microscopy studies (Chanzy et al. 1983).

Unhydrolyzed *Valonia* microcrystals appeared as smooth and very long microfibrils, having lateral dimensions in the order of 20 nm (Fig. 19). We incubated these microcrystals with a crude preparation of cellulases obtained by growing *T. terrestris* 255B for 4 days on Solka Floc BW300. After 4 hours of hydrolysis, we could already observe that the microcrystals were disrupted to microfibrils with narrower widths ranging from about 4 to 20 nm (Fig. 20). As reported by Chanzy and Henrissat (1983), the hydrolysis process is very heterogeneous and a percentage of the intact microcrystals can still be found after extended hydrolysis (Fig. 20 D). As suggested by Chanzy et al. (1983), it appears that the microcrystals are sub-fibrillated in smaller elements with narrower widths but with roughly equivalent lengths. The edges of the hydrolyzed microcrystals do not appear as smooth as the edges of the initial substrate. This is probably a consequence of the enzymatic attack that caused an "erosion" of the microcrystals. HPLC analysis of the soluble products showed that

Figure 19: *Valonia* microcrystals incubated for 26 hours in 50 mM sodium acetate (0.02% NaN_3, pH 4.8) and negatively stained with uranyl acetate. Bar= 200 nm.

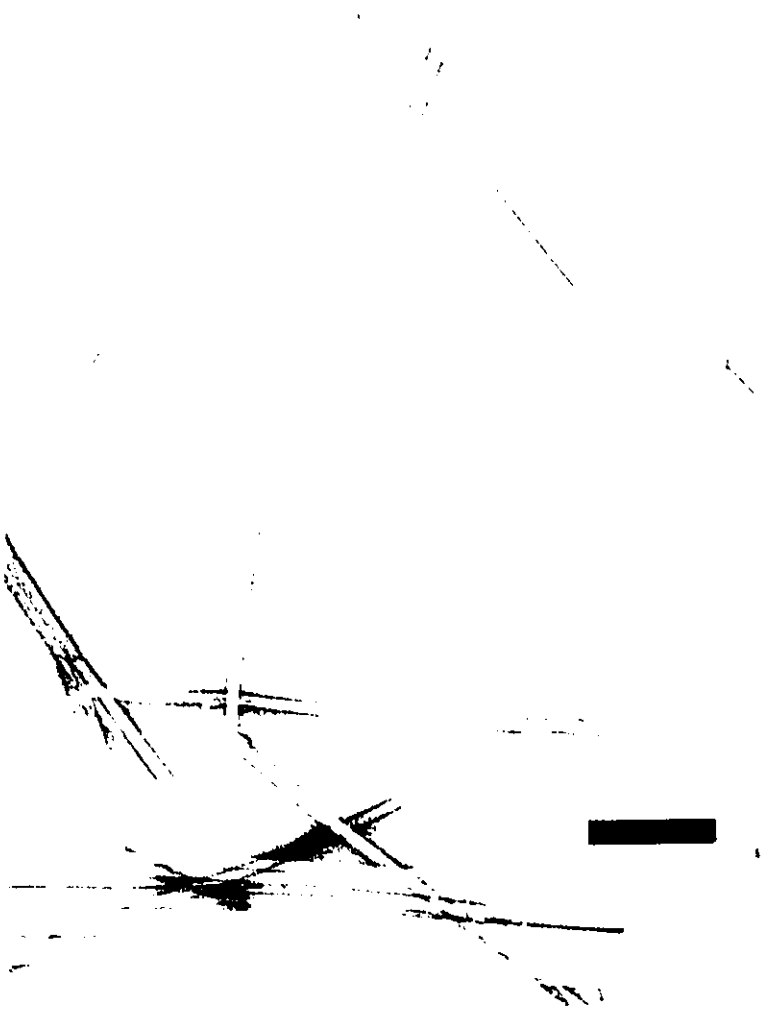
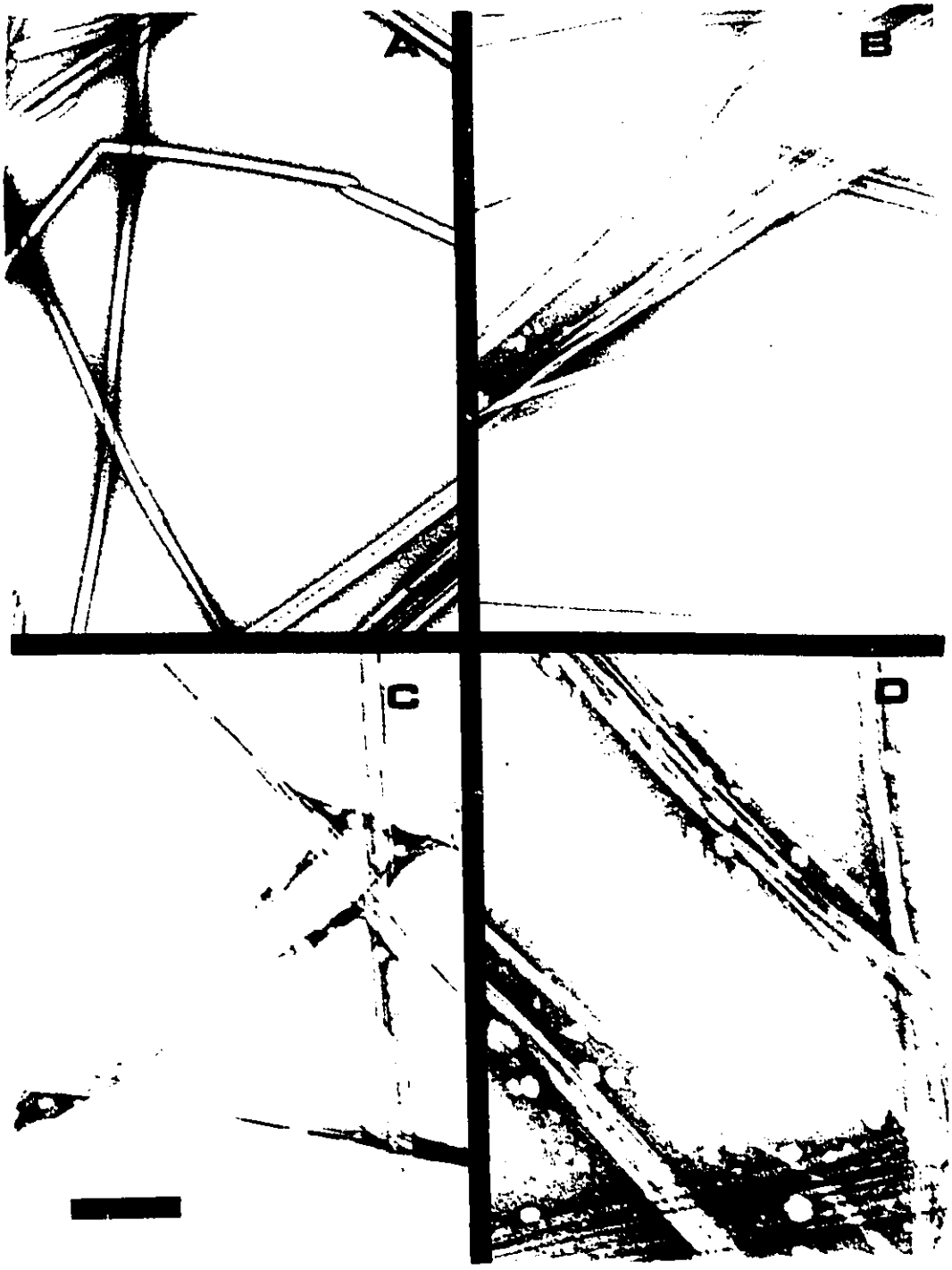


Figure 20: *Valonia* microcrystals incubated with *T. terrestris* 255B cellulolytic system. The microcrystals were treated with NaOH and negatively stained with uranyl acetate. (A) No incubation with cellulases; (B) Incubation of 4 hours; (C) Incubation of 10 hours; (D) Incubation of 26 hours. Bar= 200 nm.


A
B
C
D

23-27% of the microcrystals were solubilized after 26 hours of incubation at 65°C. The hydrolysis yield could probably be improved by increasing the enzyme loading and the incubation time.

The total magnification used in this experiment (87 000X) was theoretically high enough to allow the observation of molecules such as cellulase enzymes. We did observe "circular spots" that seem to be bound to the cellulose microfibrils (Fig. 20 B, C and D). However, the presence and the size of these elements were not consistently observed among replicates of this experiment. These spots might have been preparation artifacts. The direct observation of the enzymes on the cellulose microcrystals would probably require special preparation techniques in order to preserve the enzyme structure. However, it was clear that *T. terrestris* 255B cellulases could degrade *Valonia* microcrystals which confirms that it is a "complete" cellulase system. Also, the degradation of *Valonia* microcrystals by cellulases from *T. terrestris* 255B provides the only independant confirmation of the work reported by Chanzy et al. (1983) and Henrissat and Chanzy (1986) on the EM observations of the hydrolysis of *Valonia* microcrystals by cellulases. The limited availability of this substrate seems to have restricted the ability of other groups to reproduce this work.

3.2 Purification and Characterization of some of the Components of the Cellulolytic System of *Thielavia terrestris* 255B:

3.2.1 Introduction:

Thielavia terrestris 255B produced large amounts of cellulases and xylanases when it was grown on cellulose and xylan substrates. We undertook to purify the individual enzyme components in order to study their properties and compare them with their mesophilic counterparts. In this section we present the purification and characterization of some of the major components of the cellulolytic system of *T. terrestris* 255B.

Previously we have shown that maximum production of cellulase and β-glucosidase was obtained after 2 days of growth on Solka Floc BW300. After this period, the cellulase activity was stable for at least 4 days, but the β-glucosidase declined, presumably because of its sensitivity to the low pH of the culture. We used a two day old culture of *T. terrestris* 255B grown on Solka Floc BW300 as the source for the purification of individual enzyme components. Native-PAGE followed by an overlay technique suggested that some components with endoglucanase activity were of high molecular weight and were possibly arranged as enzyme complexes. However, this observation required confirmation by a different separation technique, such as gel filtration, which is also based on molecular weight. We chose this technique as a first step in the purification of the major cellulase components because it could provide a way of confirming the high molecular nature of some of the components.

3.2.2 Enzyme fractionation:

The enzymes secreted by *T. terrestris* 255B after growth on cellulose were separated by gel filtration on a Superose 12 column (Fig. 21). Seven protein peaks were partially resolved. The elution times of these peaks were compared with the elution times of MW markers (indicated by arrows in Fig. 21). Peaks #1 and #2 had

apparent MW's over 150,000. However, we did not detect enzymatic activity in peak #1. Peak #2 had very high β-glucosidase activity. There was no evidence of cellulases with high MW's and most of the cellulolytic activity (Fig. 21b and table 5) eluted in peaks #4 to #6 which had apparent MW's of 14,000 and 500, respectively. These unusually low apparent MW's suggested that some form of interaction took place between the enzyme components and the gel filtration matrix.

Golovchenko et al. (1992) have studied the chromatographic behaviour of different endoglucanases on gel filtration matrices. They showed that hydrophobic interaction was the probable cause for the late elution of the endoglucanases. They also showed that there was a correlation between the hydrophobicity of some cellulases and their ability to bind to cellulose. Klyosov (1990) suggested that the adsorption ability of endoglucanases played the principal role in the hydrolysis of cellulose. He found that an increase in the adsorption capability of 6 purified endoglucanases correlated with increased action on crystalline cellulose.

The separation of the enzyme components on the Superose 12 column was a result of both the MW's of the components and their ability to interact with the gel filtration matrix. It seemed that the interaction with the matrix was the predominating factor because of the very late elution of the cellulases. Thus, we could not confirm if some cellulases exist as high MW complexes as suggested by the native-PAGE/zymogram technique. We used the latter technique to analyze the endoglucanase bands present in the different peaks eluted from the Superose 12 column (data not shown). The endoglucanase band with an apparent MW of 370,000 (according to native-PAGE) was present in peaks #3, #4 and #6. These peaks and peak #5 contained many other endoglucanase bands with some of them being present in more than one peak. Therefore, none of the endoglucanase components was well resolved from the others by gel filtration under the conditions used in this work.

FIGURE 21: Gel filtration of the enzymes secreted by *T. terrestris* 255B after two days of growth on Solka Floc BW300. The sample was applied to a Superose 12 column and eluted at 0.5 mL/min with 50 mM sodium acetate (pH 4.8):

A: the elution of protein was monitored by measuring the O.D. (280 nm).

B: fractions were assayed for endoglucanase, xylanase and β-glucosidase activity as described in materials and methods.

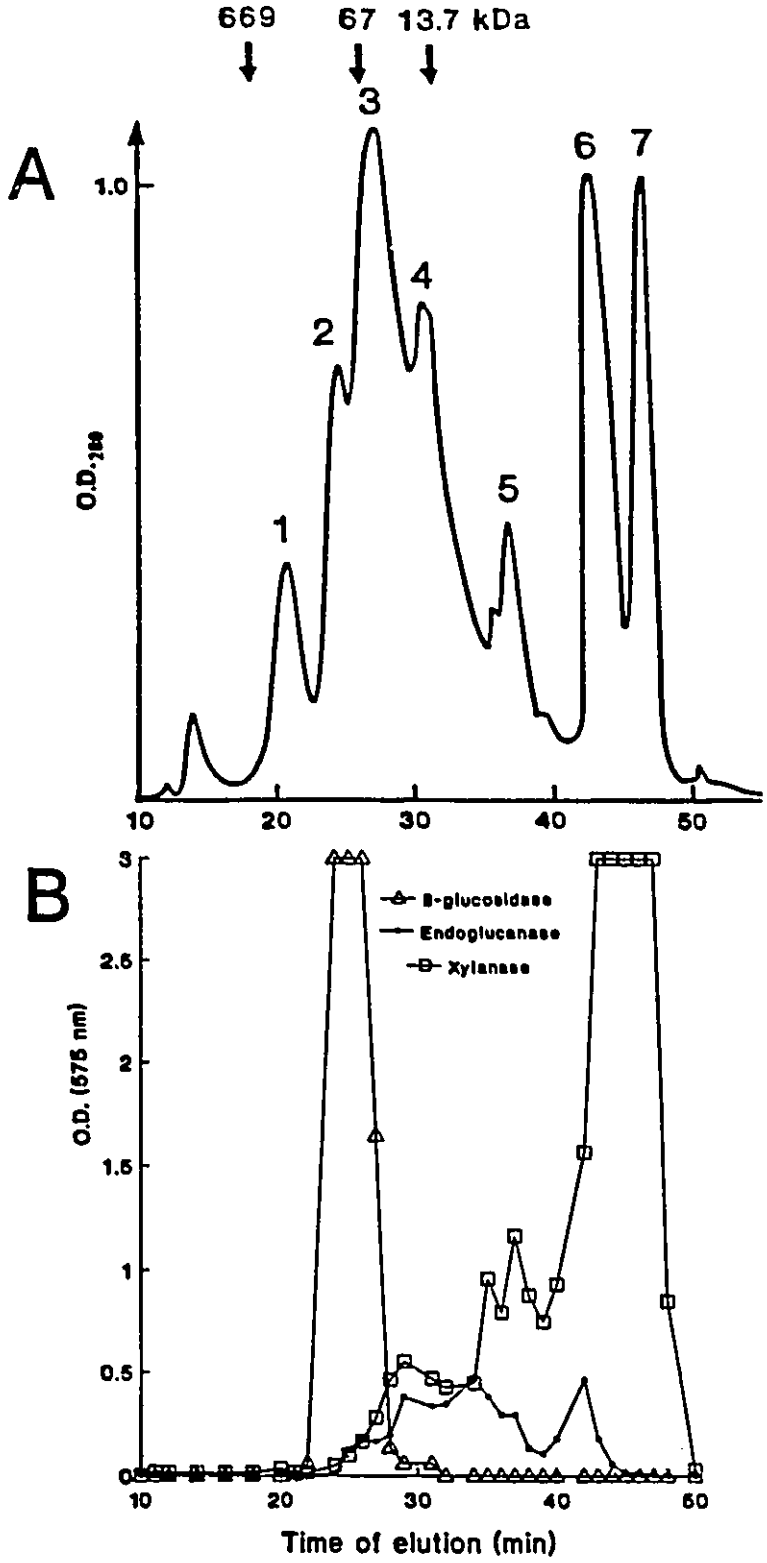

669
67
13.7 kDa
A
1.0
O.D.280
1
2
3
4
5
6
7
10
20
30
40
50
B
β-glucosidase
Endoglucanase
Xylanase
O.D. (575 nm)
3
2.5
2
1.5
1
0.5
0
10
20
30
40
50
Time of elution (min)

Fig.21

We have observed by IEF that *T. terrestris* 255B secreted over 10 components with endoglucanase activity (Fig. 17). Some of these components might interact to form complexes as suggested by native-PAGE (Fig. 14). These components might also interact with the matrix when separated by gel filtration. These two types of interaction probably explain why we obtained a complex elution pattern for the endoglucanase components when we used gel filtration. It is also possible that fungal multi-component complexes are loosely associated and consequently they would be extremely difficult to purify as a discrete unit. This is in contrast to the cellulases from anaerobic bacteria that are associated in complexes which are so stable that the purification of individual components is difficult (Robson and Chambliss, 1989).

3.2.3 Activity on crystalline cellulose:

We used short-term hydrolysis of Avicel-PH101 as a method to determine which peaks contained enzymes that could attack microcrystalline cellulose (Table 4). Cellobiose and glucose were the only products identified by HPLC. Peak #3 and #4 contained 17.3% and 19.2%, respectively, of the total hydrolytic activity. Peak #5 contained the enzymes that were the most active on crystalline cellulose and provided 40.8% of the total hydrolytic activity. The sugars produced by the fractions acting individually added up to 86% of the amount of sugars obtained when all the fractions were used together. Synergism is a common characteristic of the enzymatic cellulase systems that can degrade crystalline cellulose (Eriksson et al., 1990, Coughlan, 1985). Synergism and cooperation between the enzymes probably explain why the pooled fractions produced more soluble sugars than the sum obtained by the fractions acting individually. In this case, we observed a low degree of synergism among the peaks (less than 15%). However, less than 5% of the substrate was hydrolyzed in these short term assays. Synergism may become more obvious only when the substrate becomes more recalcitrant after more extensive hydrolysis. Furthermore, the peaks contained enzymes that were only partially purified and synergism is better observed when extensively purified enzymes are used (Wood et

Table 4: Short-term hydrolysis of crystalline cellulose (Avicel-PH101) by fractions of the peaks obtained after gel filtration of the enzymes secreted by *Thielavia terrestris* 255B:

Peak	Soluble Sugars (µg)	Standard deviation	% of total activity[a]	G_1:G_2 (%:%)[b]
1	0	0	0	-
2	35.2	1.1	7.2	100:0
3	84.2	10.5	17.3	91:9
4	90.5	17.7	19.2	36:64
5	191	8.1	40.8	17:83
6	0	0	0	-
7	7.1	4.5	1.5	0:100
Pooled[c]	490	9.6	100	100:0
Sum[d]	401	22.6	86	36:64

Rem.: products analyzed by HPLC.

a: compared to the activity of the pooled fractions. Calculated using the anhydro-forms of the sugars produced.
b: Relative proportion of glucose (G_1) and cellobiose (G_2) .
c: The hydrolysis was performed with pooled fractions from peaks 1 to 7
d: Sum of the sugars produced by the fractions used individually.

al. 1989).

The separation by gel filtration of the various activities is summarized in Table 5. As mentioned earlier, the endoglucanase activity seemed to be spread over many peaks which is probably due to the number of endoglucanase components and to their interaction with the matrix and possibly between each other. The β-glucosidase, cellobiohydrolase, and xylanase activities were found in specific peaks. The cellobiohydrolase activity is difficult to assay because there is no specific substrate available. Generally the presence of cellobiohydrolase is inferred if cellobiose is the main product obtained when an enzyme fraction is incubated with either crystalline or amorphous cellulose. However, the presence of β-glucosidase or endoglucanase can give rise to a mixture of products which will obscure the picture. Peak #3 may possibly contained cellobiohydrolase but it was probably contaminated with β-glucosidase (from peak #2) which would have converted most of the cellobiose to glucose. Peak #4 probably contained a cellobiohydrolase as 64% of the products were cellobiose. Peak #5 contains the major cellobiohydrolase because it gave the highest activity on crystalline cellulose and cellobiose was the major identified product (over 83%). It interacted with the gel filtration matrix as it eluted with an apparent MW of 3,500. This result is in keeping with the suggestion of Golovchenko et al. (1992) that the most active cellulases have hydrophobic properties which retard their elution on gel filtration matrices.

3.2.4 Purification of the major cellobiohydrolase:

We attempted to further purify the cellobiohydrolase from peak #5 by anion-exchange chromatography. For unknown reasons, we obtained poor yield by this method. Instead, we rechromatographed peak #5 on the gel filtration column. Approximately 100-200 μg of cellobiohydrolase could be obtained from 10 mg of crude preparation. The cellobiohydrolase was purified to at least 95% homogeneity as judged by SDS-PAGE, IEF and native-PAGE (Fig. 24, 25 and 26). Wood et al.

Table 5: Summary of the enzymatic activities detected in the peaks obtained after gel filtration of the enzymes secreted by *Thielavia terrestris* 255B:

Peak	β-glucosidase	Endogluca-nase	Cellobio-hydrolase	Xylanase
1	-	-	-	-
2	+++	+	-	-
3	+	++	+	+
4	-	++	+	+
5	-	+	+++	+
6	-	++	-	+++
7	-	-	-	+++

(1989) have previously discussed the difficulty in trying to obtain extensively purified cellulases. They suggested that cellulases have a tendency to aggregate together or form complexes that are not well characterized. These workers pointed out that trace contamination of endoglucanase activity will have a great effect on the action of a cellobiohydrolase. Thus, misleading results could be obtained if one studies the mode of action of a cellobiohydrolase using an incompletely purified preparation.

The cellobiohydrolase from peak #5 had a pI of 4.2 and MW of 77,100 according to IEF and SDS-PAGE, respectively. This enzyme is glycosylated as it was stained by the PAS-reagent (Fig. 24B). This cellobiohydrolase was designated CBH II because we had already purified another cellobiohydrolase from *T. terrestris* 255B which was designated CBH I. CBH I was purified previously using anion-exchange chromatography, hydrophobic chromatography and gel filtration. CBH I had a pI of 3.9 and a MW of 63,200 according to SDS-PAGE (Table 6). CBH I also differs from CBH II in its behaviour on the Superose 12 column. It did not interact with the gel filtration matrix and eluted as a protein with an apparent MW of 76,200. The discrepancy between this value and the MW obtained by SDS-PAGE could have been caused by the different separation principles of the two techniques.

The mesophilic cellulases which have been the most extensively studied to date have been obtained from *Trichoderma reesei* (Goyal et al. 1991, Eriksson et al. 1990). This cellulase system also included two distinct cellobiohydrolases which were designated CBH I (MW: 65,000, pI: 3.5-4.2) and CBH II (MW: 58,000, pI: 5.0-6.3). There is insufficient data to determine if the CBH I and CBH II from *T. terrestris* 255B are the thermophilic counterparts of the CBH I and CBH II of *Trichoderma reesei*. However, it seems that the thermophilic system also requires multiple forms of cellobiohydrolases. Fägerstam and Pettersson (1980) have suggested that two CBHs with different stereospecificities would be necessary to attack the two types of non-reducing ends that can be found in the substrate (due to the 180° rotation of

every second glucose unit in the cellulose chain). However, to date, no experimental data has confirmed this hypothesis. Also, some researchers have suggested that CBHII from *Trichoderma reesei* has endoglucanase activity (Kyriacou et al. 1987). Thus, it is still not clear what the exact roles of the different forms of cellobiohydrolases are in the various cellulase systems.

3.2.5 Purification of a β-glucosidase:

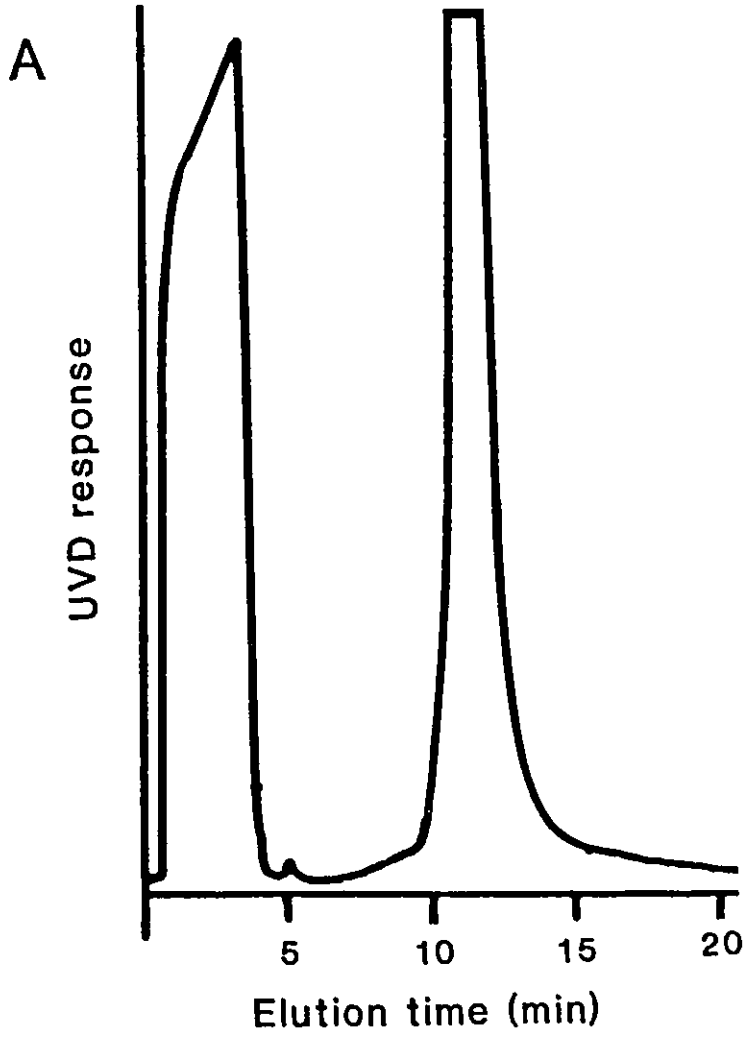
Peak #2 obtained after gel filtration using Superose 12 contained strong β-glucosidase activity. This enzyme was further purified by cation-exchange chromatography (Fig. 22). Most of the β-glucosidase activity bound to the cation-exchange column and was eluted in a single peak when a salt gradient was applied. This peak contained a β-glucosidase which was purified to at least 99% homogeneity as judged by SDS-PAGE and native-PAGE (Fig. 24-25). The protein was glycosylated as determined by staining with the PAS reagent (Fig. 24B). A major band with a pI of 6.3 was obtained by IEF (Fig. 26) but a smear spreading from pI 5.6 to 6.5 was also observed. The microheterogeneity detected by IEF could also be due to either the heterogeneity of the protein conformation, the heterogeneity of the glycosidic part of the enzyme or to interactions between the carrier ampholytes and the protein. McHale and Coughlan (1982) also observed smearing when they analyzed by IEF a glycosylated β-glucosidase from *Talaromyces emersonii*. This enzyme had a carbohydrate content of 51% and they suggested that heterogeneity of the glycosidic part was responsible for the smearing observed by IEF.

The β-glucosidase from *T. terrestris* 255B is an oligomeric protein with a subunit MW of 99,300 kDA according to SDS-PAGE and a MW of 250,000 according to gel filtration. However, it is still not clear if two or three subunits are present. β-Glucosidases with relatively high subunit MW and complex subunit structure have been isolated from certain other fungi, such as *Coriolus versicolor*, *Botryodiplodia theobromae*, and *Macrophomina phaseolina* (Coughlan 1985). However,

Figure 22: Purification of a β-glucosidase from *T. terrestris* 255B. Peak #2 (from gel filtration using Superose 12, Fig. 21) was applied to a cation-exchange column (Mono-S, 5 mm X 50 mm). A linear gradient of 0-0.3 NaCl (in 50 mM sodium acetate, 0.02% NaN_3, pH 4.8) was applied at the time indicated.

A: The elution of protein was monitered by using a UV detector (280 nm).

B: β-glucosidase activity of the fractions, each collected at every 0.5 min with a flow rate of 1 mL/min.


A
UVD response
5
10
15
20
Elution time (min)

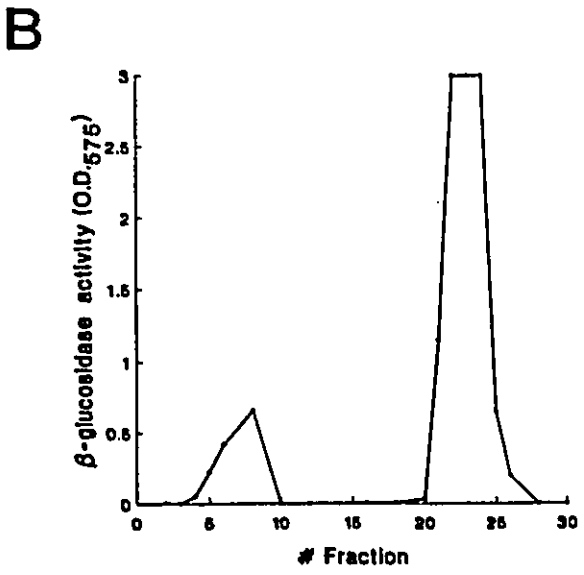

B
β-glucosidase activity (O.D.575)
3
2.5
2
1.5
1
0.5
0
0
5
10
15
20
25
30
Fraction

Fig. 22

β-glucosidases are not always oligomeric. For instance, the β-glucosidases from the thermophilic fungi *Thermoascus aurantiacus* (Tong et al. 1980) and *Talaromyces emersonii* (McHale and Coughlan 1982) have single sub-unit structure.

Woodward and Wiseman (1982) defined β-glucosidases as enzymes that can catalyze the hydrolysis of alkyl and aryl-β-D-glucosides as well as glycosides containing only carbohydrates such as cellobiose. Depending on their source and their physiological function, β-glucosidases were found to show large variations in affinity for the different glycosides. Aryl-β-D-glucosides such as salicin (2-[hydroxy-methyl]phenyl β-D-glucopyranoside) are often used to follow purification of β-glucosidases because they are easy to assay. For an application such as the bioconversion of cellulose, it is important to verify that the isolated enzyme is also active on cellobiose. The β-glucosidase from *T. terrestris* 255B had specific activities of (335.9 U/mg enz.) and (237 U/mg enz.) on salicin and cellobiose, respectively. Thus, this enzyme is a β-glucosidase which has activity on both aryl-β-D-glucosides and cellobiose. This enzyme was the only β-glucosidase that we identified in the two-day old culture filtrate of *T. terrestris* 255B grown on Solka Floc BW300. It was present in a sufficiently high level since glucose was the only product identified when crystalline cellulose was hydrolyzed in its presence (Table 5). However, *T. terrestris* 255B might also possess distinct cell-associated β-glucosidases as observed with many other fungi (for review, Eriksson et al. 1990).

3.2.6 Purification of a β-1,3-glucanase:

Peak #1 from gel filtration (Fig. 21) contained a HMW protein (493,000 by gel filtration) which turned out to be the easiest component to purify from the enzymatic system of *T. terrestris* 255B. A single major protein peak was obtained when peak #1 (from gel filtration using Superose 12) was applied to an anion-exchange column and eluted with a salt gradient (Fig. 23). Analysis by SDS-PAGE, IEF and native-PAGE (Fig. 24, 25 and 26) showed a single band. The HMW protein

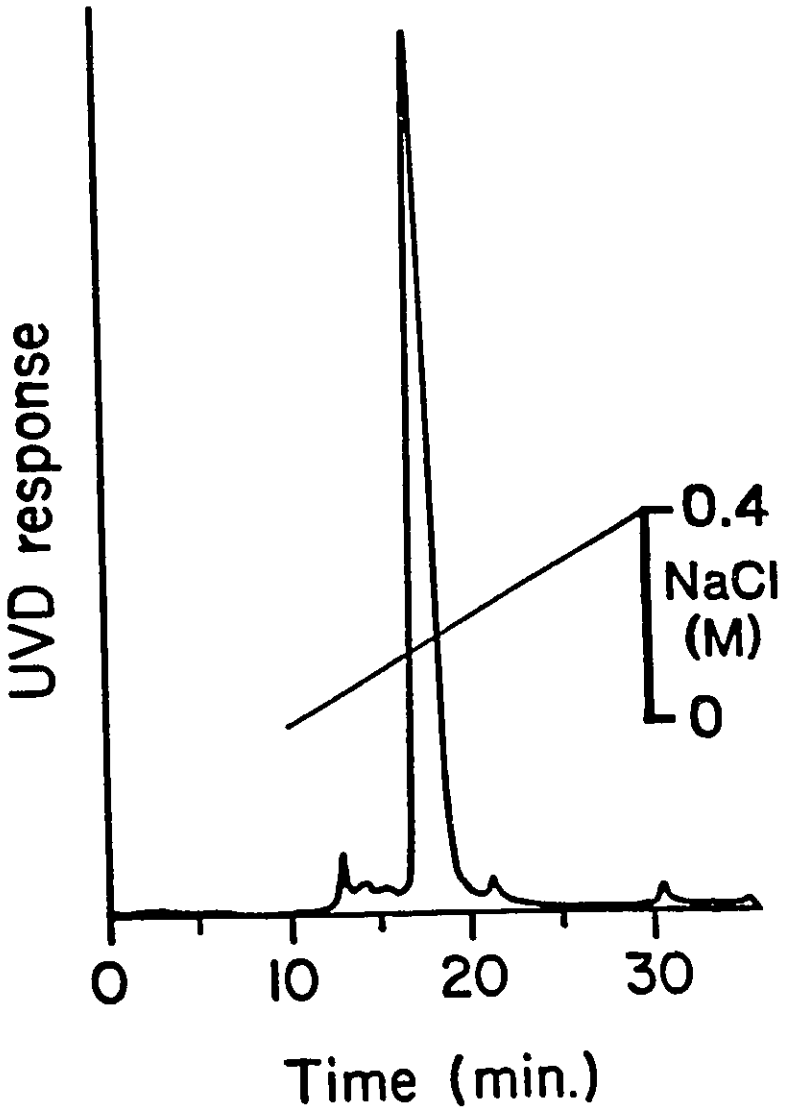


Figure 23: Purification of a β-1,3-glucanase secreted by *T. terrestris* 255B. Peak #1 (from gel filtration using Superose 12, Fig. 21) was applied to an anion-exchange column (Mono Q, 5 mm X 50 mm). A linear gradient of 0-0.4 NaCl (in 20 mM BisTris, 0.02% NaN_3, pH 6.5) was applied at the time indicated. The elution of protein was monitored by using a UV detector (280 nm)

had a pI of 4.4 and a subunit MW of 82,700 (SDS-PAGE). It had a MW of 493,000 according to gel filtration and a MW of 405,000 according native-PAGE. In this case, native-PAGE correctly predicted a large size for the protein, although there was some discrepancy between the values obtained by the two techniques. The protein is clearly oligomeric, having 6 subunits according to the MW calculated by gel filtration. It is a glycoprotein as it was stained by the PAS-reagent (Fig. 24B).

The HMW protein had no activity when various substrates were used to assay for a range of cellulase activities. We then assayed this protein for activity on various β-glucans and found that it had low (2.6 U/mg enz.) but significant activity on laminarin, a type of β-1,3-glucan. This β-1,3-glucanase seems to be a major component of the *T. terrestris* 255B system as judged by the native-PAGE profiles obtained when the organism was grown on various substrates (section 3.1.4). It was the major protein band which migrated the slowest on the gradient gel (Fig. 13, 14, 15 and 16) and it was detected from day one onwards when *T. terrestris* 255B was grown on acid-swollen cellulose or Solka Floc BW300 (Fig. 14 and 15). It appeared after more than two days of culture when *T. terrestris* 255B was grown on glucose, cellobiose or xylan (Fig. 13 and 16). Its appearance coincided with the appearance of low cellulolytic activity in the filtrates of the cultures grown on glucose or cellobiose. In general it seemed to be present whenever cellulolytic activity was detected.

The role of the β-1,3-glucanase might be related to the secretion of enzymes. Kubicek (1982) found that β-1,3-glucanase activity correlated well with the excretion of β-glucosidase by *Trichoderma pseudokoningii*. He suggested that cell wall modifying enzymes were necessary for the secretion of β-glucosidase. On the other hand, Ruel and Joseleau (1991) found that an extracellular β-1,3-1,6-D-glucan sheath was secreted by *Phanerochaete chrysosporium* during degradation of wood. They suggested that this sheath provided a junction between the hyphae and the wood and

Table 6: Summary of the physico-chemical properties of the major components of the cellulolytic system of *T. terrestris* 255B:

Component	MW ($\times 10^3$) by gel filtr.	MW ($\times 10^3$) by SDS-PAGE	pI	Comments
β-1,3-glucanase	493	82.7	4.4	Glycosylated[a]
Cellobio-hydrolase I[b]	76.2	63.2	3.9	
Cellobio-hydrolase II	-	77.1	4.2	Glycosylated[a]
β-glucosidase	250	99.3	6.3	Glycosylated[a]
Endo-glucanases	-	-	-	2 major components, up to 10 minor components

a: Determined by SDS-PAGE and PAS staining (see Fig. 24).

b: This component was purified in another project (unpublished).

Figure 24: Analysis of the purified enzyme components by SDS-PAGE on a gradient gel (10-15%):

Panel A: Coomassie blue staining.

Panel B: PAS staining for glycoproteins.

Lane 1: Carboxypeptidase (0.6 μg: positive control for PAS staining).

Lane 2: Xylanase II (2 μg: see Chapter 3.3).

Lane 3: Cellobiohydrolase II (1 μg).

Lane 4: β-Glucosidase (1.2 μg).

Lane 5: β-1,3-Glucanase (1.2 μg).

Lane 6: Molecular weight markers ($\times 10^3$).

A

B

Figure 25: Analysis of the purified enzyme components by native-PAGE on a gradient gel (8-25%):

Lane 1: Molecular weight markers ($\times 10^3$).

Lane 2: β-Glucosidase (2 μg).

Lane 3: β-1,3-Glucanase (1.9 μg).

Lane 4: Cellobiohydrolase II (1.5 μg).

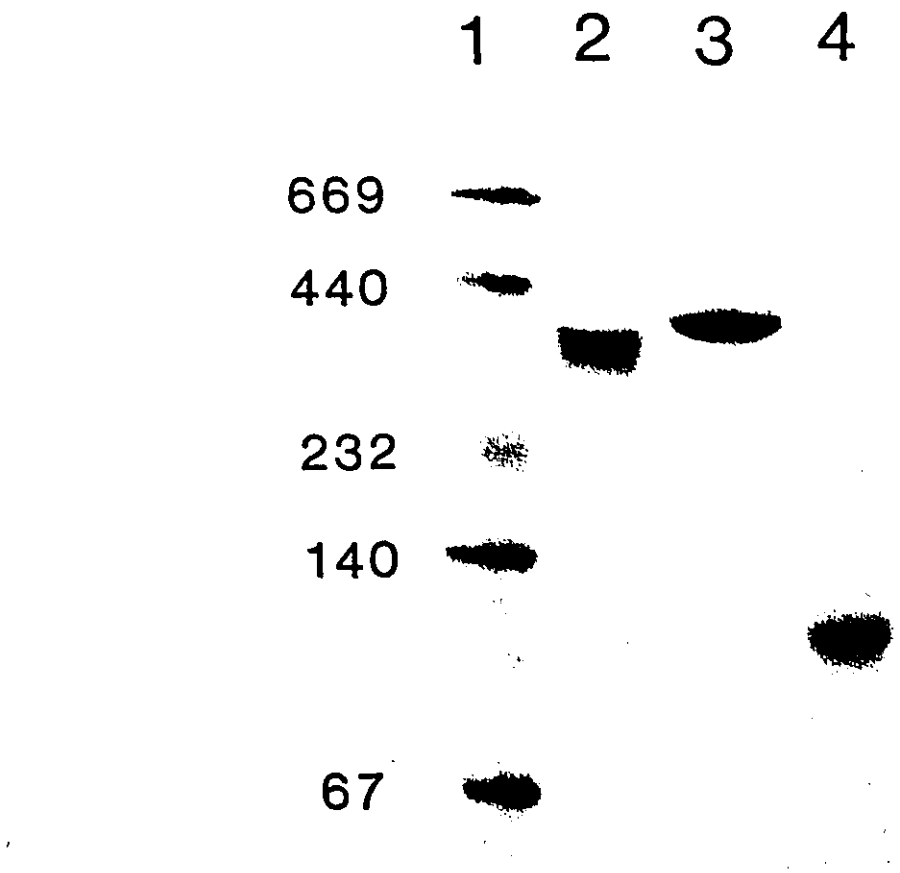

1
2
3
4
669
440
232
140
67

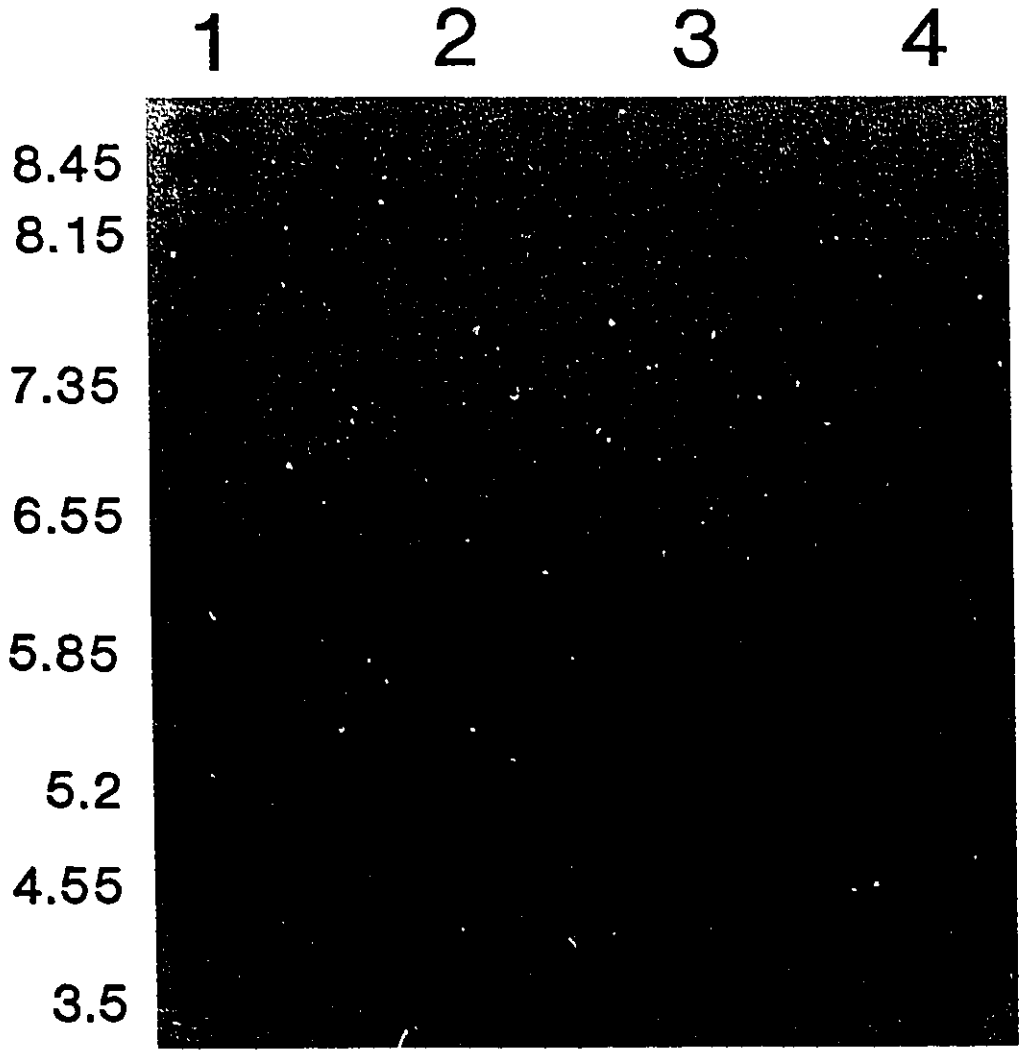
Figure 26: Analysis of the purified enzyme components by IEF (pI range 3-9):

Lane 1: pI markers.

Lane 2: β-Glucosidase (2 μg).

Lane 3: β-1,3-Glucanase (1.9 μg).

Lane 4: Cellobiohydrolase II (1.5 μg).


1
2
3
4
8.45
8.15
7.35
6.55
5.85
5.2
4.55
3.5

consequently convey the enzymes to the site of wood degradation. The glucan sheath would have a transient existence and it would need to be modified and/or degraded to allow the enzymes to attack at other sites. The modification of such β-1,3-1,6-D-glucan sheaths is another possible role for the β-1,3-glucanase from *T. terrestris* 255B.

3.2.7 Peaks with xylanase activity:

Most of the xylanase activity was found in peaks #6 and #7 following separation by gel filtration (Fig. 21). Peaks #6 and #7 contained enzymes that strongly interacted with the matrix because they eluted as proteins with apparent MWs of 500 and 300, respectively. The relative intensities of peak #6 and #7 varied considerably between runs of different loadings or cultures (data not shown). In general, peak #6 would be relatively bigger when more protein was applied to the column. Comparatively, the relative intensities of the other peaks (#1 to #5) was more reproducible between different preparations and over a wide range of protein loadings (3 to 19 mg), although the resolution was better with low protein loadings. The late elution of peaks #6 and #7 was probably influenced by both protein-matrix and protein-protein interaction. Thus, various loadings or various preparations could result in a different elution profile for these two peaks depending on which interaction is predominant.

Peak #6 contained both xylanase and endoglucanase activities while peak #7 contained only xylanase activity (Fig 21, Table 5). IEF followed by overlay techniques for endoglucanase and xylanase detection was used to analyze peaks #6 and #7. The two peaks contained the same xylanase band with a pI of 6.1 (designated xylanase II in section 3.1.4). Peak #6 also contained many endoglucanase and protein bands. These bands were very faint or absent in peak #7. Apparently peak #7 contained xylanase II purified almost to homogeneity while peak #6 contained the xylanase II associated with other components. Xylanase II was shown to be the major xylanase component produced by *T. terrestris* 255B (section 3.1.4) and because it could be isolated relatively easily, without any contaminating cellulase activity, it became the major focus of the next part of the thesis.

3.3 PURIFICATION OF THE MAJOR XYLANASE COMPONENT SECRETED BY *Thielavia terrestris* 255B:

3.3.1 INTRODUCTION:

Thermophilic xylanases could have considerable value as pulp biobleaching agents, provided there is no contaminating cellulase activity present. A cellulase-free xylanase preparation could be obtained when we separated the enzymes produced by *T. terrestris* 255B by gel filtration (Fig. 21 and section 3.2.7). We found that this enzyme preparation contained xylanase II which was identified as the major xylanase component produced by *T. terrestris* 255B (Fig. 16 and 18). We wanted to purify this enzyme in order to study its mode of action as well as compare its structure with xylanases from mesophilic sources. In this section, we present the strategy used to purify large amounts of this xylanase.

3.3.2 Source of xylanase II:

We have determined that maximum xylanase activity was obtained after three days growth on oat-spelts xylan (Fig. 12). However, proteases were also detected in the culture filtrate after 3 to 6 days growth (Fig. 7). This was probably a consequence of autolysis because the cell biomass decreased over the same period of time (Fig. 6). We therefore decided to use a two-day old culture of *T. terrestris* 255B grown on oat-spelts xylan as the source of enzyme for the purification of xylanase II.

3.3.3 Purification of xylanase II:

The extracellular protein produced by *T. terrestris* 255B was concentrated using a Pellicon ultrafiltration unit (Millipore) fitted with a 10 000 MW cut-off membrane. On average 63% (SD= 4%, 6 experiments) of the xylanase activity was recovered in the retentate with a concentration factor of 25-fold. The ultrafiltrate contained 18% (SD= 6%) of the original activity. The passage of most of the xylanase activity through an ultrafiltration membrane with a MW cut-off of 10 000 has been reported

for the enzymes produced by *Trichoderma harzianum* (Tan et al., 1987b). It was not clear if the ability of the enzyme to pass through the membrane was due to the shape of the xylanase molecules or to the greater permeability of the membrane. Approximately 20% of the original activity was lost during the ultrafiltration process, possibly through denaturation or precipitation in the retentate.

The concentrated material was applied to an anion-exchange column under conditions (20 mM Bis-Tris, 0.02% NaN_3, pH 7.2) that should normally have bound a protein with a pI of 6.1 such as xylanase II. However, most of the xylanase activity was eluted with the equilibration buffer (Fig. 27). A small xylanase peak containing less than 5% of the applied activity eluted early after the application of the salt gradient (0-0.5 NaCl). This fraction was not further characterized. Although the net charge of xylanase II was probably negative at the pH used (7.2), the negative groups could have been prevented from interacting with the anion exchanger. This effect might be the result of the distribution of charges on the enzyme itself or to interactions with other molecules present in the crude material, such as polysaccharides or other proteins. Nevertheless, the anion-exchange step achieved purification with an increase in specific activity from 176 U/mg for the Pellicon retentate to 462 U/mg for the material that did not bind to the anion-exchanger (Table 7).

The material that did not bind to the anion-exchange column was subjected to gel filtration on a Superose 12 column (Fig. 28). Two xylanase peaks (designated peak A and B) were observed. Peak A eluted first and contained 25-30% of the recovered activity. It was not further characterized. The peak that eluted last was designated peak B and contained xylanase II. Comparison with elution volumes of standard proteins showed that xylanase II must have interacted with the gel matrix. An apparent MW below 500 was calculated for that protein. Unusually high gel filtration elution volumes have also been reported during the purification of

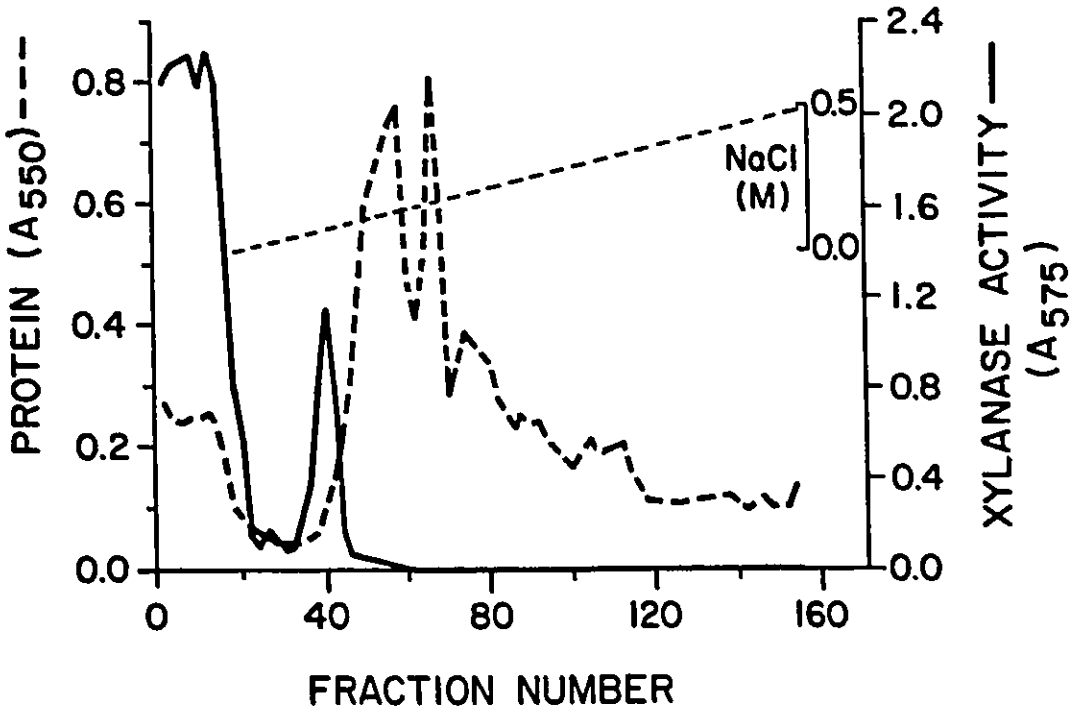


Figure 27: Anion exchange on a Q-Sepharose column of the Pellicon retentate containing xylanase from *T. terrestris* 255B. A linear gradient of 0-0.5 M NaCl (in 20 mM Bis-Tris, 0.02% NaN_3, pH 7.2) was applied from fraction 21 to 160.

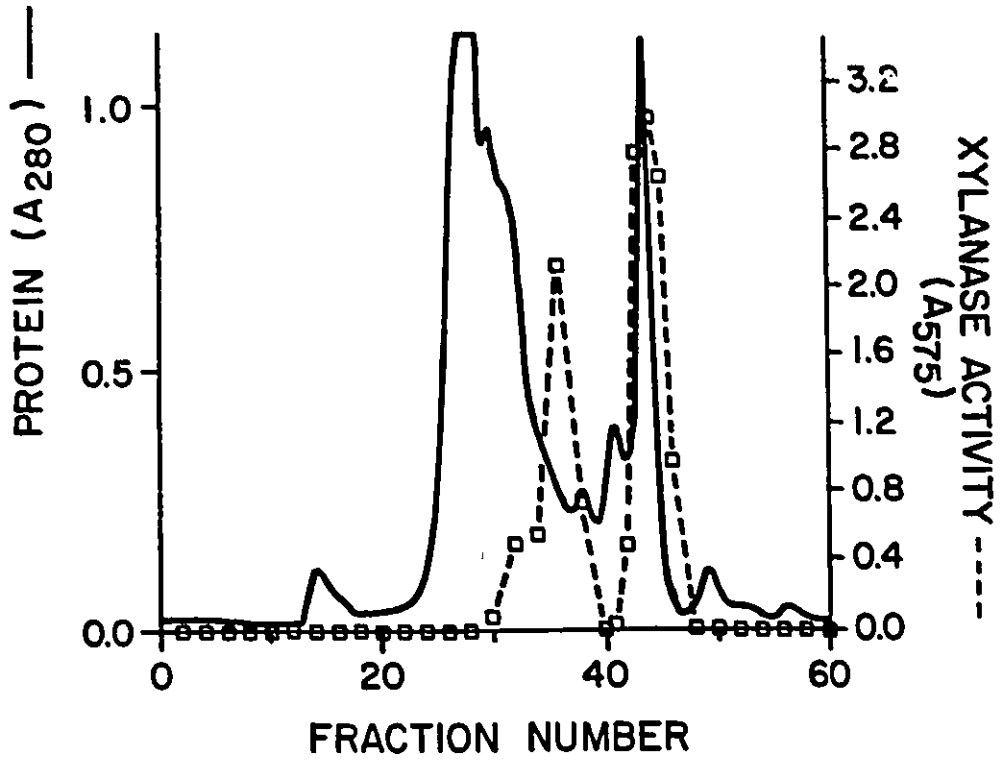


Figure 28: Gel filtration on a Superose 12 column of the xylanase from *T. terrestris* 255B that did not bind to the anion-exchange column. Sodium acetate (50 mM, pH 4.8) was used as the elution buffer.

xylanases from other microorganisms (Dean and Anderson 1991, Wood and McCrae 1986, Baker et al. 1977). It has been suggested that the high tyrosine content could be responsible for the interaction with the gel filtration matrix (Dean et al. 1991).

No direct relationship exists between the xylanase peaks A and B obtained from this separation (Fig. 28) and the xylanase peaks #6 and #7 described in section 3.2.7 (Fig. 21). Peaks #6 and #7 were observed when the culture filtrate from *T. terrestris* 255B was directly applied to the Superose 12 column (Fig. 21) and they both contained xylanase II. Peaks A and B were observed when a fraction (enriched in xylanase II) from the anion-exchange column was applied to the Superose 12 column (Fig. 28). In this case, only peak B contained xylanase II while peak A contained another xylanase which was not further characterized.

Analysis of peak B by SDS-PAGE and IEF (Fig. 29) showed that xylanase II was purified to at least 99% homogeneity. Although the interaction with the gel filtration matrix was not fully determined, it acted as an efficient final purification step.

A purification summary is showed in Table 7. Typically, 2.5 to 5 mg of purified xylanase II could be obtained from a 12 L culture with a recovery yield of 4 to 6%. On average, the specific activity of the purified material was 1782 U/mg (SD=790, 3 purifications). The wide variation in specific activity is probably related to the sensitivity of xylanase II to proteolytic degradation. We harvested the culture before the onset of detectable proteases. However, low levels of proteolytic activity could still have affected the xylanase during the purification procedure. Xylanase II seems to have a site hypersensitive to protease attack. In section 3.4.6, we will discuss the fact that the two resultant fragments could remain associated and would therefore co-purified with the intact xylanase II. This would result in a preparation with a reduced specific activity.

Table 7: Steps involved in the purification of xylanase II from culture filtrate of *T. terrestris* 255B:

Purification step	Protein (mg)	Activity (U)	Specific activity (U/mg)	Purification (Fold)	Yield (%)
Culture filtrate	2,806	128,600	46	1.0	100
Pellicon Retentate	445	78,400	176	3.8	61
Anion exchange	140	64,500	462	10.0	50
Gel Filtration	2.8	6,032	2,154	46.8	4.7

Figure 29: Analysis of the purified xylanase II:

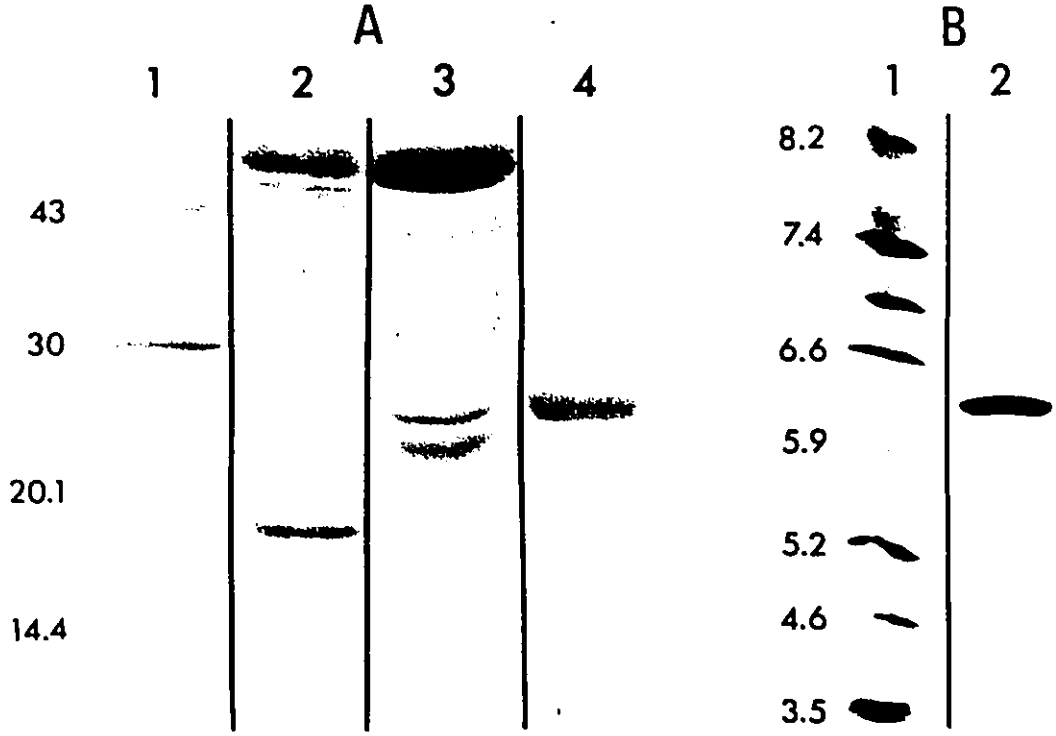
Panel A: SDS-PAGE on 14% acrylamide gel;

Lane 1: Molecular mass standards (kDa).
Lane 2: Crude xylanase (6.2 μg).
Lane 3: Material that did not bind to the anion-exchange column (25 μg).
Lane 4: Xylanase II (1 μg) after gel filtration.

Panel B: IEF gel (pH range 3-9);

Lane 1: pI standards.

Lane 2: Xylanase II (0.7 μg) after gel filtration.


A
1
2
3
4
43
30
20.1
14.4
B
1
2
8.2
7.4
6.6
5.9
5.2
4.6
3.5

3.4: STRUCTURAL STUDIES OF XYLANASE II:

3.4.1 Introduction:

Thielavia terrestris 255B produced two major forms of xylanases with pI values of 4.6 (xylanase I) and 6.1 (xylanase II). Xylanase II was the major xylanase component produced after growth on oat-spelts xylan. We purified xylanase II to >99% homogeneity using anion-exchange chromatography and gel filtration. Xylanase II was very active against xylan and had very low activity towards cellulosic substrates, indicating a possible application of this thermophilic enzyme in the pulp and paper industry. The characterization and potential application of xylanase II became the major focus of the remaining work.

The physico-chemical properties of xylanase II suggested that it belonged to a family of low-molecular weight xylanases. This family was designated the "G" family by Gilkes et al. (1991b) in their classification of β-1,4-glycanases. We used amino acid composition and partial amino acid sequencing to confirm that xylanase II was related to the other xylanases within the G family. Most of the xylanases within the G family which have been characterized to date, have been isolated from mesophilic fungi. We obtained information on the structure of xylanase II to see if we could identify structure-function relationships responsible for the enzyme's higher thermostability.

3.4.2 Physico-chemical properties:

Xylanase II had a molecular mass of 25.7 kDa as determined by SDS-PAGE (Fig. 29). Xylanase II was not stained by the PAS reagent (Fig. 24B) which suggests that it is not glycosylated. Xylanase II had a pI of 6.1 as determined by IEF (Fig. 29). Wong et al. (1988) observed that most xylanases are characterized by either low-MW/high-pI or high-MW/low-pI. This relationship between MW and pI was particularly obvious for the different xylanases isolated from *Clostridium* and *Streptomyces* spp. Xylanase II seems to be an exception to this classification as it had

a low MW and a slightly acidic pI.

Based on amino acid sequence homology, Gilkes et al. (1991b) suggested classifying β-1,4-glycanases into 9 families. Xylanases were classified into the F and the G families. The G family contained low molecular mass xylanases (20-31 kDa). The F family was more heterogeneous with the molecular masses of the enzymes ranging from 31 kDa to 110 kDa, although the homology was mostly restricted to the catalytic domains (estimated to range from 30 kDa to 38 kDa) of these enzymes. With its molecular mass of 25.7 kDa, xylanase II is within the range of the G family (Table 8). The pI values of the enzymes in the G family are predominately basic, however, xylanase A from *Schizophyllum commune* is acidic and xylanase II from *T. terrestris* 255B is another exception. The xylanases which have been classified into the F and G families were the ones for which complete amino acid sequences were available. We therefore wanted to obtain the amino acid composition and partial amino acid sequence of xylanase II in order to confirm its relatedness to other xylanases within the G family.

3.4.3 Amino acid composition:

The amino acid composition of xylanase II was calculated based on a content of 5 arginine residues (Table 9). A molecular mass of 25.2 kDa was calculated from the deduced amino acid composition. This value is in good agreement with the molecular mass of 25.7 kDa which was estimated by SDS-PAGE (Fig. 29). The relative amino acid content of xylanase II was compared with the values determined with the xylanases from the G family and with the frequency of occurence of each amino acid in unrelated proteins (Table 10). Xylanase II and the xylanases from the G family all exhibit a high threonine, serine, tyrosine, tryptophan and glycine content while they are all poor in leucine and lysine. These common properties support the suggestion that xylanase II should be grouped within the G family.

Table 8: Physico-chemical properties of xylanase II and of other xylanases within the G family:

	Xyl.II[1]	Xyl.A[2]	20-kDa[3]	Xln B[4]	Xln C[5]	Xyn[6]	Xyn[7]	Xyn[8]	Xyn B[9]
Species	*Thielavia terrestris*	*Schizophyllum commune*	*Trichoderma harzianum*	*Streptomyces lividans*	*Streptomyces lividans*	*Streptomyces sp. No.36A*	*Bacillus pumilus*	*Bacillus circulans*	*Clostridium acetobutylicum*
pI	6.1	4.5	9.4	8.4	>10.25	N.R.[a]	N.R.	9.0	10
MW (kDa)	25.7	20.9	20.6	31	20.7	20.7	22.4	20.5	28
pH opt.	3.6-4.0	5.0	5.0	6.5	6.0	N.R.	6.5	6.0	6.0
T opt. (°C)	60-65	50	50	55	57	N.R.	40	50	37-43
Thermostability[b]	50% 10 h 60°C	N.R.	80% 1 h 50°C	45% 6 h 60°C	0% 2 h 60°C	N.R.	N.R.	100% 3h 50°C	50% 10 min 50°C

a: N.R.: Not reported.

b: activity recovered after incubation for the indicated time and temperature.

1: Gilbert et al. 1992b.
2: Jurasek and Paice 1988.
3: Tan et al. 1985b.
4: Kluepfel et al. 1990.
5: Kluepfel et al. 1992.
6: Nagashima et al. 1989.
7: Panbangred et al. 1983.
8: Yang et al. 1989 and W. Wakarchuk (unpublished).
9: Zappe et al. 1987.

Table 9: Amino acid composition of xylanase II.

Amino acid	Estimated Residues (per molecule)
Asx	23
Glx	19
Gly	29
Ala	21
Val	18
Leu	5
Ile	6
Pro	4
Met	2
Cys	3
Thr	28
Ser	25
Tyr	20
Phe	12
Trp	9
His	2
Lys	2
Arg	5
total	**233**

Table 10: Comparison of the amino acid content (mole %) of xylanase II with xylanases from the G family and with the average amino acid contents of unrelated proteins:

A. a.	Xyl.II *T. t.*	Xyl.A *S. c.*	20-kDa *T. h.*	Xln B *S. l.*	Xln C *S. l.*	Xyl. *S. sp.*	Xyl. *B. p.*	Xyl. *B. c.*	Xyn. B *C. a.*	Unrelated proteins
Asx	9.9	10.7	12.1	12	11	10.6	11.5	13.5	11.6	9.9
Glx	8.2	6.1	5.3	5.1	5.8	4.2	7.0	3.8	8.2	10.1
Gly	12.5	15.2	14.7	14.7	16.8	15.9	11.5	13.5	10.3	7.5
Ala	9.0	6.1	4.2	7.9	3.7	5.8	5.5	4.9	4.3	9.0
Val	7.7	4.6	6.8	7.2	5.8	5.3	4.0	7.6	4.3	6.9
Leu	2.2	3.6	2.6	2.7	1.6	1.6	4.0	2.2	3.9	7.5
Ile	2.6	4.1	5.3	2.1	3.1	3.2	5.0	3.2	6.0	4.6
Pro	1.7	3.6	3.2	3.4	2.1	2.7	2.5	3.2	3.0	4.6
Met	0.9	0.5	0.5	2.1	3.1	3.2	3.0	1.1	1.7	1.7
Cys	1.3	1.0	0	0.3	1.1	1.1	0.5	0	1.3	2.8
Thr	12.0	9.6	9.0	12.6	13.6	12.7	9.5	13.5	10.7	6.0
Ser	10.7	14.7	12.6	10.6	10.5	9.5	10.0	9.7	10.7	7.1
Tyr	8.6	8.6	9.5	6.5	9.4	9.0	8.0	8.1	6.9	3.5
Phe	5.2	1.5	3.7	2.1	3.1	3.2	4.5	2.2	3.4	3.5
Trp	3.9	4.1	3.2	4.4	3.7	4.2	3.0	6.0	3.0	1.1
His	0.9	1.5	2.1	0.3	0.5	1.1	2.0	1.1	0.9	2.1
Lys	0.9	2.5	2.1	2.4	2.1	1.6	4.5	2.7	7.3	7.0
Arg	2.2	2.0	3.2	3.8	3.1	5.3	4.0	3.8	2.6	4.7

T.t.: *Thielavia terrestris* (Gilbert et al. 1992b),
T. h.: *Trichoderma harzianum* (Yaguchi et al. 1992),
S. sp.: *Streptomyces* sp. (Nagashima et al. 1989),
B.c.: *Bacillus circulans* (Yang et al. 1988),
S.c.: *Schizophyllum commune* (Oku et al., unpublished),
S.l.: *Streptomyces lividans* (Shareck et al. 1991),
B.p.: *Bacillus pumilus* (Fukusaki et al. 1984),
C.a.: *Clostridium acetobutylicum* (Zappe et al. 1990).

Unrelated proteins: Average contents in a.a. of 207 unrelated proteins (Klapper 1977).

3.4.4 N-terminal amino acid sequence:

The strategy used to obtain partial amino acid sequence is depicted in Figure 30. The N-terminal sequence could be obtained from the native xylanase II. Additional sequence data was collected from fragments obtained by CNBr cleavage and from a preparation containing a nicked xylanase II.

Determination of a single N-terminal sequence by automated Edman degradation (Fig. 31) confirmed the purity of the isolated enzyme. Only marginal sequence identity was found between the N-terminal sequence of xylanase II and the xylanases from the G family. Comparison of complete sequences showed that higher sequence identity is found in the carboxy-terminal two-thirds of the xylanases from the G family (Shareck et al. 1991, Paice et al. 1986). Thus, we can not confirm the relatedness of xylanase II to the G family based on the N-terminal sequences.

However, the N-terminal of xylanase II had a high level of sequence identity with the xylanase from *Aureobasidium pullulans* (Fig. 31). The *A. pullulans* xylanase is a small xylanase with a molecular mass of 20 kDa. Its complete sequence has not been reported so that its relationship to members of the G family has not been confirmed. The high level of sequence identity between xylanase II and the 20-kDa xylanase from *A. pullulans* is not easily explained in terms of phylogenic closeness between the two organisms. *T. terrestris* 255B is a thermophilic ascomycete causing soft-rot decay. *T. terrestris* 255B is actually more closely related to *Trichoderma harzianum* (a mesophilic deuteromycete causing soft-rot decay) than it is to *A. pullulans* (a mesophilic yeastlike deuteromycete that is not considered to be a wood-decay fungus). However, the first 30 amino acid residues at the N-terminal of xylanase II showed only 4 identical residues with the N-terminal of the 20-kDa xylanase from *Trichoderma harzianum* while it showed 22 identical residues with the 20-kDa xylanase from *A. pullulans*. The genes of these xylanases probably have a common ancestor, but it is not clear why the amino acid sequence was better

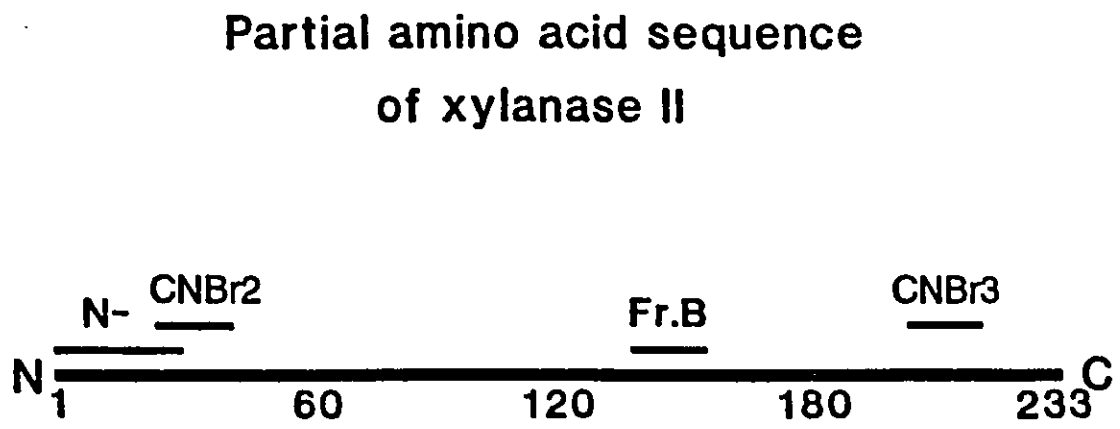


Figure 30: Strategy for partial amino acid sequencing of xylanase II. The N-terminal sequence ("N-") was obtained from the native xylanase II. The sequences "CNBr2" and "CNBr3" were obtained from CNBr fragments. The sequence "Fr.B" was obtained from a preparation containing a "nicked" xylanase II. This representation assumes that xylanase II has 233 residues. The position of the fragments is approximate and based on the homology with xylanases from the G family for which complete sequences are available.

Figure 31: Comparison of the N-terminal sequence of xylanase II with the 20-kDa xylanase from *Aureobasidium pullulans* and with xylanases from the G family (based on alignment of the xylanases from the G family by Shareck et al. 1991):

TT: *T. terrestris* 255B xylanase II.

AP: *Aureobasidium pullulans* 20-kDa xyl. (Leathers et al. 1988).

TH: *Trichoderma harzianum* 20-kDa xyl. (Yaguchi et al. 1992).

SC: *Schizophyllum commune* Xyl.A (Oku et al., unpublished).

SLB: *Streptomyces lividans* Xyl. B (Shareck et al. 1991).

SLC: *Streptomyces lividans* Xyl. C (Shareck et al. 1991).

SS: *Streptomyces* sp. No.36A Xyl. (Nagashima et al. 1989).

BP: *Bacillus pumilus* Xyl. (Fukusaki et al. 1984).

BC: *Bacillus circulans* Xyl. (Yang et al. 1988).

CA: *Clostridium acetobutylicum* XynB (Zappe et al. 1990).

Rem.: Residues identical to xylanase II are underlined and boldfaced.

TT	(1-17)			Ala	Ala	Gly	Ile	Asn	Tyr	Val	Gln	Asn	Tyr	Asn	Gly	Asn	Leu	Gly	Tyr
AP	(1-19)	Ala	Gly	Pro	Gly	Gly	Ile	Asp	Tyr	Val	Gln	Asn	Tyr	Asn	Gly	Asn	Leu	Gly	Gln
TH	(1-14)				Gln	Thr	Ile	-	Gly	Pro	Gly	Thr	Gly	Tyr	Ser	Asn	Gly	Tyr	Tyr
SC	(1-14)					Ser	Gly	Thr	Pro	Ser	Ser	Thr	Gly	Thr	Asp	Gly	Gly	-	Tyr
SLB	(1-18)		Asp	Thr	Val	Val	Thr	Thr	Asn	Gln	Glu	Gly	Thr	Asn	Asn	Gly	Tyr	Tyr	Tyr
SLC	(1-17)			Ala	Thr	Thr	Ile	Thr	Thr	Asn	Gln	Thr	Gly	Thr	Asp	Gly	Met	Tyr	Tyr
SS	(1-17)			Ala	Thr	Thr	Ile	Thr	Thr	Asn	Gln	Thr	Gly	Tyr	Asp	Gly	Met	Tyr	Tyr
BP	(1-16)					Arg	Thr	Ile	Thr	Asn	Asn	Glu	Met	Gly	Asn	His	Ser	Gly	Tyr	Asp
BC	(1-5)														Ala	Ser	Thr	Asp	Tyr
CA	(31-47)				Pro	Lys	Thr	Ile	Thr	Ser	Asn	Glu	Ile	Gly	Val	Asn	Gly	Gly	Tyr	Asp

															A.a. identical to TT:
TT	(18-30)	Phe	Thr	Tyr	Asn	Glu	Gly	Ala	Gly	Gln	Phe	Ser	Met	Tyr	Thr	30
AP	(20-32)	Phe	Thr	Tyr	Asn	Glu	Asn	Ala	Gly	Thr	Tyr	Ser	Met	Tyr	Trp	22
TH	(15-27)	-	Tyr	Ser	Tyr	Trp	Asn	Asp	Gly	His	Ala	Gly	Val	Thr	Tyr	4
SC	(15-27)	Tyr	Tyr	Ser	Trp	Trp	Thr	Asp	Gly	Ala	Gly	Asp	Ala	Thr	Tyr	2
SLB	(19-31)	Ser	Phe	Trp	Thr	Asp	Ser	Gln	Gly	Thr	Val	Ser	Met	Asn	Met	5
SLC	(18-30)	Ser	Phe	Trp	Thr	Asp	Gly	Gly	Gly	Ser	Val	Ser	Met	Thr	Leu	8
SS	(18-30)	Ser	Phe	Trp	Thr	Asp	Gly	Gly	Gly	Ser	Val	Ser	Met	Thr	Leu	7
BP	(17-29)	Tyr	Glu	Leu	Trp	Lys	Asp	Tyr	Gly	Asn	Thr	Ser	Met	Thr	Leu	5
BC	(6-18)	-	Trp	Gln	Asn	Trp	Thr	Asp	Gly	Gly	Gly	Ile	Val	Asn	Ala	3
CA	(48-60)	Tyr	Glu	Leu	Trp	Lys	Asp	Tyr	Gly	Asn	Thr	Ser	Met	Thr	Leu	4

Fig. 31

conserved between *T. terrestris* 255B and *A. pullulans*. The xylanase gene might have been transferred between these two organisms but direct evidence for this occurence is lacking.

3.4.5 Sequencing of CNBr fragments:

Xylanase II contained 2 methionine residues (Table 9). We used CNBr to cleave specifically at these sites in order to obtain additional sequence informations. Three fragments were produced and separated by SDS-PAGE in presence of urea. Their measured molecular masses (15.8, 8.1 and 6.5 kDa) added up to 30.4 kDa which is significantly higher than the 25.7 kDa value obtained for the native enzyme. However, small peptides often display mobility that deviate considerably from standard curves (Schägger and von Jagow, 1987) and this can account for the observed discrepancy between the molecular mass of the native enzyme and the sum of the molecular masses of the fragments.

The N-terminal of the native xylanase II revealed a methionine at position 28. Cleavage at this site should have produced a fragment with a molecular mass of approx. 3.1 kDa. We presumed that the smallest fragment (6.5 kDa, designated CNBr1) generated by CNBr cleavage corresponds to this N-terminal peptide (a.a. 1-27). However, this fragment could not be transferred to a medium suitable for sequencing following separation by SDS-PAGE in presence of urea, thus we could not confirm its identity as the peptide containing the amino acids 1 to 27. Nevertheless, this is probably the case because the sequences of the two other fragments are consistent with this interpretation (see below).

The 15.8-kDa fragment was designated CNBr2. Its N-terminal corresponded to amino acids 28 to 46 of xylanase II. This assignation is suggested by the overlap of the residues Met-Tyr-Thr (a.a. 28-30) of the native xylanase II (Fig. 31) with the first 3 amino acids (Met-Tyr-X) of the CNBr2 fragment (a.a. 28-46). The "X"

Figure 32: N-terminal sequence of the CNBr2 fragment of xylanase II from *T. terrestris*. Comparison with sequences of the 20-kDa xylanase from *Aureobasidium pullulans* and with xylanases from the G family. The sequences are aligned according to Shareck et al. (1991).

TT: *T. terrestris* 255B xylanase II (CNBr2 fragment).

AP: *Aureobasidium pullulans* 20-kDa xyl. (Leathers 1988).

TH: *Trichoderma harzianum* 20-kDa xyl. (Yaguchi et al. 1992).

SC: *Schizophyllum commune* Xyl.A (Oku et al., unpublished).

SLB: *Streptomyces lividans* Xyl. B (Shareck et al. 1991).

SLC: *Streptomyces lividans* Xyl. C (Shareck et al. 1991).

SS: *Streptomyces* sp. No.36A Xyl. (Nagashima et al. 1989).

BP: *Bacillus pumilus* Xyl. (Fukusaki et al. 1984).

BC: *Bacillus circulans* Xyl. (Yang et al. 1988).

CA: *Clostridium acetobutylicum* XynB (Zappe et al. 1990).

Rem.:

-Residues identical to xylanase II are underlined and boldfaced.

-"X" denotes unidentified residues. A sequencing cycle can fail to identify a residue for various reasons:

- Oxidation of Cys can occur and the products are difficult to sequence.
- Thr, Ser, Gln, Asn and Trp yield unstable PTH-derivatives which are not easily detected using microsequencing techniques.
- Various types of post-translational modifications of the residues can interfere with the sequencing.

TT	(28-46)	**Met**	**Tyr**	X	Gln	Gln	**Gly**	**Val**	**Ser**	Asn	**Asp**	X	**Val**	**Val**	X	**Leu**	**Gly**	X	Thr	Thr
AP	(30-45)	**Met**	**Tyr**	Trp	Asn	Asn	**Gly**	**Val**	Asn	Gly	**Asp**	Phe	**Val**	**Val**	Gly	**Leu**	**Gly**			
TH	(26-44)	Thr	**Tyr**	Thr	Asn	Gly	**Gly**	Gly	Gly	Ser	Phe	Thr	**Val**	Asn	Trp	Ser	Asn	Ser	Gly	Asn
SC	(26-44)	Thr	**Tyr**	Gln	Asn	Asn	**Gly**	Gly	Gly	Ser	Tyr	Thr	Leu	Thr	Trp	Ser	**Gly**	Asn	Asn	Gly
SLB	(29-47)	**Met**	Asn	Met	Gly	Ser	**Gly**	Gly	Gln	Tyr	Ser	Thr	Ser	Trp	Arg	Asn	Thr	Gly	Asn	Phe
SLC	(28-46)	**Met**	Thr	Leu	Asn	Gly	**Gly**	Gly	**Ser**	Tyr	Ser	Thr	Gln	Trp	Thr	Asn	Cys	Gly	Asn	Phe
SS	(28-46)	**Met**	Thr	Leu	Asn	Gly	**Gly**	Gly	**Ser**	Tyr	Ser	Thr	Arg	Trp	Thr	Asn	Cys	Gly	Asn	Phe
BP	(27-45)	**Met**	Thr	Leu	Asn	Asn	**Gly**	Gly	Ala	Phe	Ser	Ala	Gly	Trp	Asn	Asn	Ile	Gly	Asn	Ala
BC	(18-36)	Ala	Val	Asn	Gly	Ser	**Gly**	Gly	Asn	Tyr	Ser	Val	Asn	Trp	Ser	Asn	Thr	Gly	Asn	Phe
CA	(58-76)	**Met**	Thr	Leu	Lys	Asn	**Gly**	Gly	Ala	Phe	Ser	Cys	Gln	Trp	Ser	Asn	Ile	Gly	Asn	Ala

A. a. identical to TT:

AP: 9/13 SS: 3/15
TH: 3/15 BP: 2/15
SC: 3/15 BC: 1/15
SLB: 2/15 CA: 2/15
SLC: 3/15

Fig. 12

Figure 33: N-terminal sequence of the CNBr3 fragment of xylanase II from *T. terrestris* 255B. Comparison with sequences of xylanases from the G family. The sequences were aligned according to Shareck et al. (1991). "X" denotes unidentified residues.

TT: *T. terrestris* 255B xylanase II (CNBr3 fragment).

TH: *Trichoderma harzianum* 20-kDa xyl. (Yaguchi et al. 1992).

SC: *Schizophyllum commune* Xyl.A (Oku et al., unpublished).

SLB: *Streptomyces lividans* Xyl. B (Shareck et al. 1991).

SLC: *Streptomyces lividans* Xyl. C (Shareck et al. 1991).

SS: *Streptomyces* sp. No.36A Xyl. (Nagashima et al. 1989).

BP: *Bacillus pumilus* Xyl. (Fukusaki et al. 1984).

BC: *Bacillus circulans* Xyl. (Yang et al. 1988).

CA: *Clostridium acetobutylicum* XynB (Zappe et al. 1990).

Rem.: Residues identical to xylanase II are underlined and boldfaced.

			A.a. identical to TT:
TT	CNBr3	**Met Ala Val Glu** Ala X Ser Gly **Ser Gly** Tyr **Ala Thr Val Thr** Ile	15
TH	(174-189)	Val **Ala Val Glu** Gly Tyr Phe Ser **Ser Gly** Ser **Ala** Ser Ile **Thr** Val	6
SC	(181-196)	Val **Ala** Thr **Glu** Gly Tyr Gln Ser **Ser Gly** Thr **Ala Thr** Ile **Thr** Val	7
SLB	(174-189)	**Met Ala** Thr **Glu** Gly Tyr Gln Ser **Ser Gly** Thr Ser Ser Ile Asn Val	5
SLC	(174-189)	**Met Ala** Thr **Glu** Gly Tyr Gln Ser **Ser Gly** Ser Ser Asn Ile **Thr** Val	6
SS	(175-190)	**Met Ala** Thr **Glu** Gly Tyr Gln Ser **Ser Gly** Ser Ser **Thr** Ile **Thr** Val	7
BP	(179-194)	Phe Thr **Val Glu** Gly Tyr Gln Ser **Ser Gly** Ser **Ala** Asn **Val** Met Thr	6
BC	(169-184)	**Met Ala** Thr **Glu** Gly Tyr Gln Ser **Ser Gly** Ser Ser Asn **Val Thr** Val	7
CA	(211-226)	Phe Asn Ile **Glu** Gly Tyr Gln Ser **Ser Gly** Lys **Ala** Asp **Val** Asn Ser	5

Fig. 33

corresponds to a sequencing cycle for which no residue could be identified (see Fig. 32). The assignation of the CNBr2 fragment to residues 28-46 is also suggested by its high level of sequence identity with residues 30-45 of the 20-kDa xylanase from *Aureobasidium pullulans* (Fig. 32). Nine out of the 13 identified residues were identical between the *Aureobasidium* xylanase and xylanase II, reiterating the high homology between the N-terminal sequences of these two xylanases.

The 8.1-kDa CNBr fragment was designated CNBr3. It was clearly homologous with a region corresponding to the residues 179 to 194 of the *Bacillus pumilus* xylanase (Fig. 33). Between 5 to 7 residues within the xylanases from the G family were identical to the 15 residues identified in CNBr3. This alignment was done without inserting any gaps. Three residues were conserved for all the xylanases, including the residue Glu^{182} which was suggested to participate in the catalysis (Wakarchuk et al. 1992, Katsube et al. 1990). The presence of this residue at a similar position within the a.a. sequence of xylanase II provides additional support to this suggestion.

3.4.6 Isolation and study of a preparation containing a nicked xylanase:

We had noticed that the specific activity of purified xylanase II varied considerably from one purification run to the other (see section 3.3.3). There was also the occurence of one extra band when certain preparations of purified xylanase II were subjected to native-PAGE (Fig. 34A) and two extra bands when the samples were subjected to SDS-PAGE (Fig. 34B). The amount in each of the additional bands varied between purification runs and tended to increase as the preparation aged. The two extra bands were designated fragment A and fragment B and had molecular masses of 14.8 and 11.2 kDa, respectively. We suspected that proteolytic degradation was causing the appearance of the extra bands as their combined molecular masses added up to 26 kDa which is close to the molecular mass of the intact xylanase (25.7 kDa). Following separation by SDS-PAGE, we transferred

fragments A and B to a PVDF membrane suitable for microsequencing. Fragment A had the same N-terminal as the intact xylanase II (data not shown). Fragment B had a N-terminal sequence homologous to a region corresponding to residues 137 to 151 of *Bacillus pumilus* xylanase (Fig. 35). Between 5 to 8 of the residues within the xylanases from the G family were identical to tl. 11 identified residues of Fragment B. Four residues from this region were conserved for all the xylanases. This area is one of the best conserved regions of the xylanases from the G family (Shareck et al. 1991). According to the size of fragments A and B, the sequenced region would correspond to residues 130 to 144 (considering that xylanase II has 233 residues). This assignation is consistent with the location of this region in the xylanases of the G family (Fig. 35).

The proteolytic degradation of xylanase II was unexpected as we had took the precaution of harvesting the enzymes before the onset of detectable proteolytic activity in the culture filtrate (section 3.3.2). However, low levels of proteases might still have been present. We added protease inhibitors (PMSF, leupeptin, EDTA and pepstatin) in order to avoid the degradation of xylanase II into fragments A and B. In spite of all the precautions taken during purification, degradation products still appeared in some of the preparations. We suspect that xylanase II was nicked by proteases before we collected the culture filtrates used as the sources of enzymes. The intact and the nicked xylanase II would then co-purify because the two fragments of the nicked xylanase II would remain associated and behave in the same manner as the intact xylanase II.

The difficulty in separating the intact from the nicked xylanase II is illustrated by the results obtained with a preparation containing xylanase II contaminated by large amounts of the fragments A and B (Fig. 34). When this preparation was separated by native-PAGE, two bands were detected (Fig. 34A). An overlay technique showed that the slower migrating band (Low R_f) had xylanase activity

Figure 34: Separation by electrophoresis of two forms of xylanase II (intact and nicked):

A: A mixture containing the two forms of xylanase II (Mix) was subjected to native-PAGE on a gradient gel (8-25%). Two bands (High R_f and Low R_f) of a replica lane were transferred for analysis by SDS-PAGE gel (adjacent gel).

B: Separation by SDS-PAGE (on a 20% gel) of a mixture containing two forms of xylanase II (Mix). The other lanes show the polypeptides found in the two bands (High R_f and Low R_f) obtained by native-PAGE. Int.: intact xylanase II, RO-Int.: re-oxidized intact xylanase II, Fr.A: fragment A, Fr.B: fragment B.

Rem.: The light band identified as "RO-Int." was observed when β-mercaptoethanol was used as the reducing agent in the sample buffer. It probably corresponded to a re-oxidized xylanase II as it was absent when a stronger reducing agent (DTT) was used (Fig. 37).

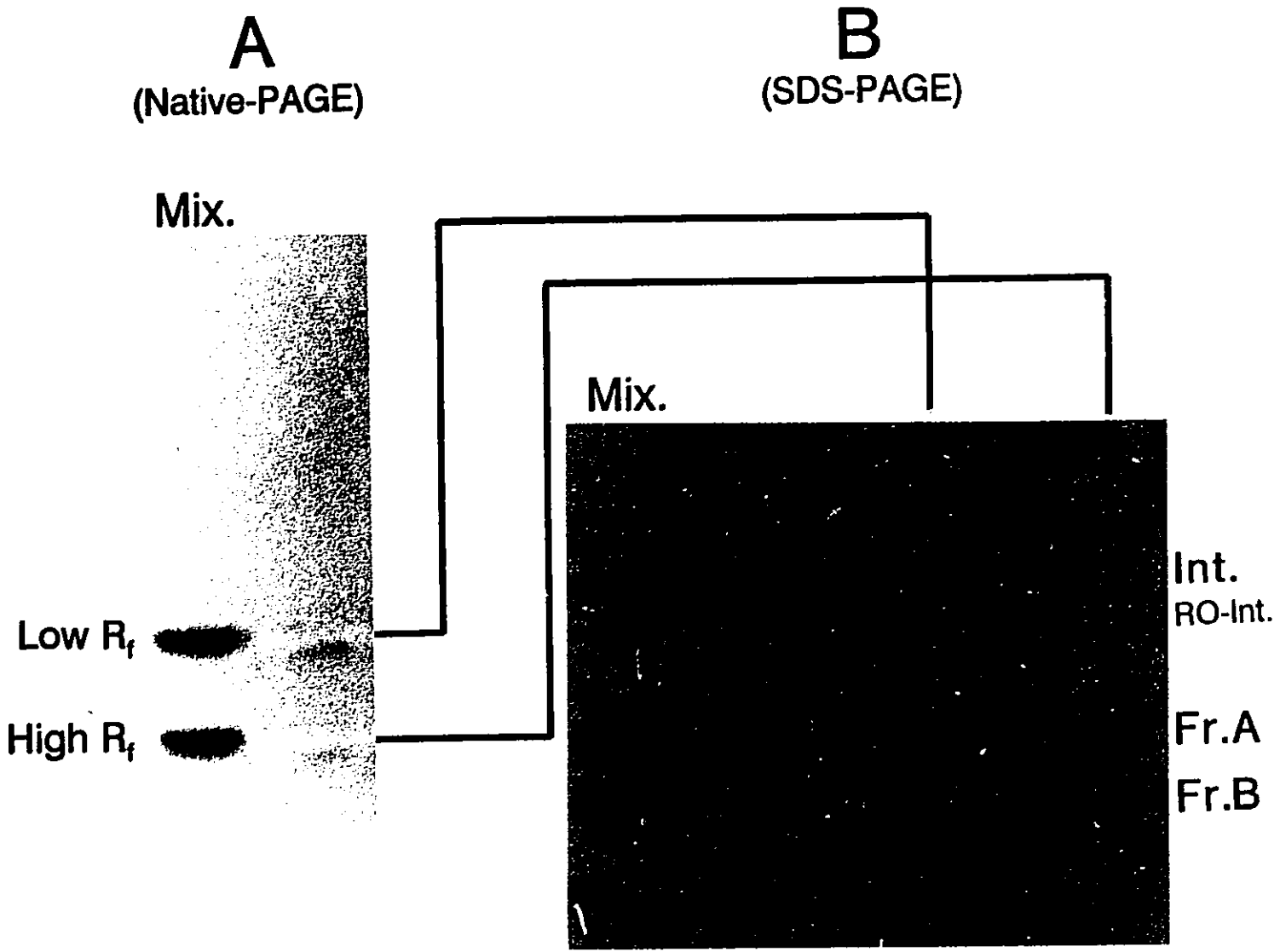

A
(Native-PAGE)
B
(SDS-PAGE)
Mix.
Mix.
Low R_f
High R_f
Int.
RO-Int.
Fr.A
Fr.B

Figure 35: N-terminal sequence of fragment B from a preparation containing a nicked xylanase II (see Fig. 34). Comparison with sequences of xylanases from the G family. The sequences are aligned according to Shareck et al. (1991). "X" denotes unidentified residues.

TT: *T. terrestris* 255B xylanase II (CNBr3 fragment).

TH: *Trichoderma harzianum* 20-kDa xyl. (Yaguchi et al. 1992).

SC: *Schizophyllum commune* Xyl.A (Oku et al., unpublished).

SLB: *Streptomyces lividans* Xyl. B (Shareck et al. 1991).

SLC: *Streptomyces lividans* Xyl. C (Shareck et al. 1991).

SS: *Streptomyces* sp. No.36A Xyl. (Nagashima et al. 1989).

BP: *Bacillus pumilus* Xyl. (Fukusaki et al. 1984).

BC: *Bacillus circulans* Xyl. (Yang et al. 1988).

CA: *Clostridium acetobutylicum* XynB (Zappe et al. 1990).

Rem.: Residues identical to xylanase II are underlined and boldfaced.

			A.a. identical to TT:
TT	Frag.B	Ser Thr Phe X Gln Phe X Ser Val X Gln Ser Thr X Thr	11
TH	(132-146)	Ala Thr Phe Tyr Gln Tyr Trp Ser Val Arg Arg Asn His Arg Ser	5
SC	(133-147)	Gln Thr Phe Glu Gln Phe Trp Ser Val Arg Asn Pro Lys Lys Ala	6
SLB	(131-145)	Arg Thr Phe Asp Gln Tyr Trp Ser Val Arg Gln Ser Lys Arg Thr	8
SLC	(129-143)	Lys Thr Phe Gln Gln Tyr Trp Ser Val Arg Gln Ser Lys Val Thr	8
SS	(130-144)	Ala Ala Phe Asp Gln Tyr Trp Ser Val Arg Gln Ser Lys Val Thr	7
BP	(137-151)	Ala Thr Phe Lys Gln Tyr Trp Ser Val Arg Gln Thr Lys Arg Thr	7
BC	(123-137)	Thr Thr Phe Thr Gln Tyr Trp Ser Val Arg Gln Ser Lys Arg Pro	7
CA	(169-183)	Thr Thr Phe Lys Gln Tyr Trp Ser Val Arg Arg Thr Lys Arg Thr	5

Fig. 35

while the faster migrating band (High R_f) was inactive (data not shown). Following coomassie blue staining, the two bands were extracted and applied to a SDS-PAGE gel (fig. 34B). The inactive band (High R_f) yielded fragments A and B, while the active band (Low R_f) gave the intact xylanase II plus minor bands of fragments A and B upon SDS-PAGE. It seems that when xylanase II is nicked by a protease, the two fragments remain associated and the overall structure stays temporarily unperturbed (see Fig. 36). This type of nicked xylanase II would behave similarly to the intact xylanase II when subjected to separation under native conditions. Upon storage and manipulation, the overall structure of the nicked xylanase II would collapsed to a more compact and inactive protein (observed as a faster migrating band under native-PAGE). This process would be possible because of the additional flexibility provided by the nick in the polypeptide chain and it could occur slowly over time. If the cleavage has occurred before purification, it would explain why precautions against proteolysis appear to fail to prevent the degradation of xylanase II.

Loss in activity has also been observed while purifying a xylanase from *Cellulomonas flavigena* (Pembroke et al. 1991). Xylanase preparations with higher stability could be obtained only when a protease negative mutant of *Cellulomonas flavigena* was used as enzyme producer. This strategy could be applied to *T. terrestris* although the genetics of this organism are not well known. The fact that xylanase II is cleaved into only two fragments suggests that it contains a specific site that is hypersensitive to proteolytic attack. Modifying this site by genetic engineering is another strategy which could be used to obtain a more stable enzyme.

We tried another strategy to purify the intact xylanase II free from the nicked xylanase II (see section 2.4.4). A preparation containing both the intact and the nicked xylanase II was applied to a Superose 12 column and eluted at a slow rate (<0.15 mL/min). Two protein peaks were partially resolved (data not shown) and

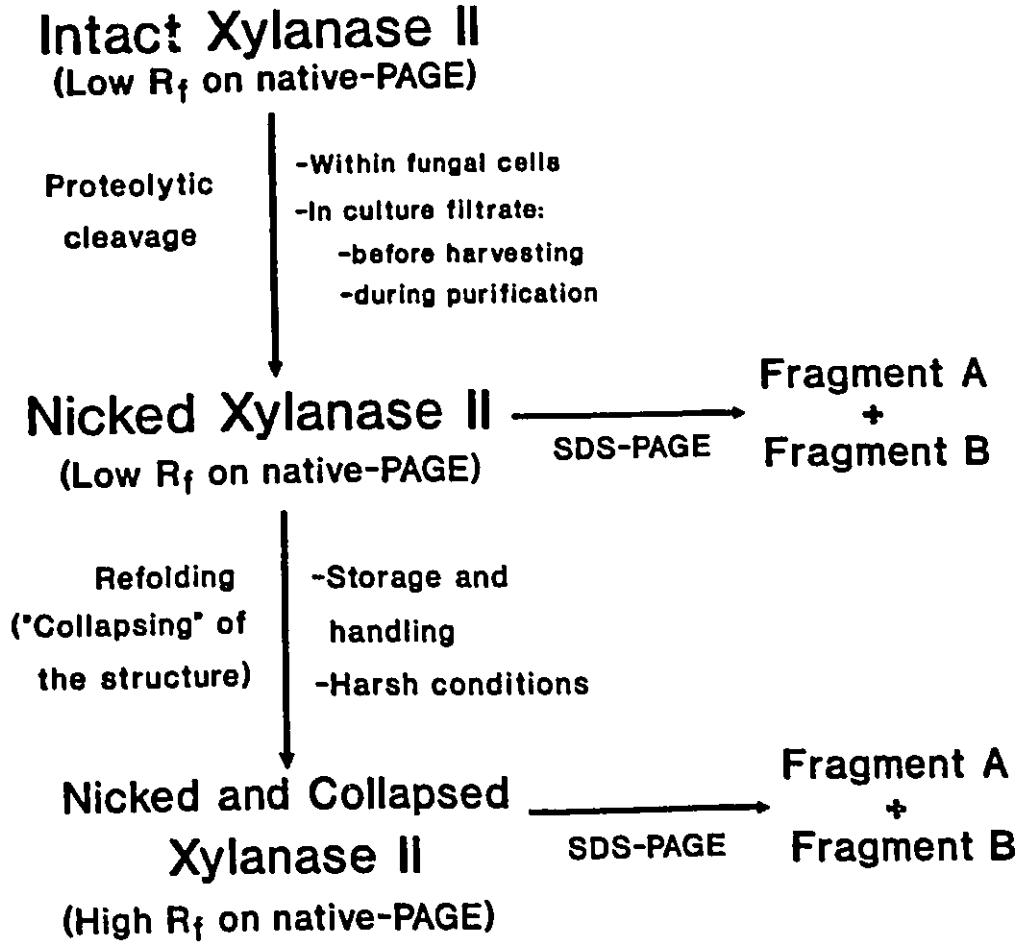


Figure 36: Proposed mechanism for the origin of the two forms of xylanase II.

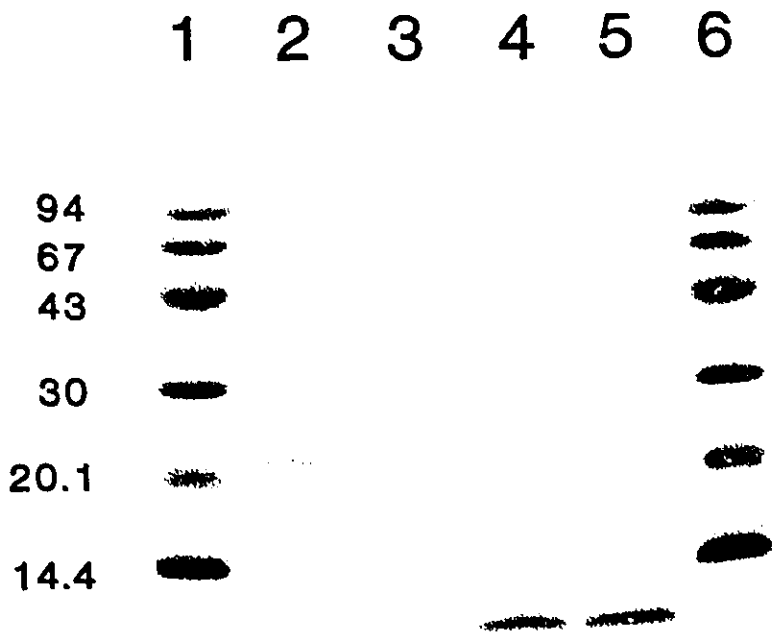
analysis by SDS-PAGE showed that the nicked xylanase II was in the peak that eluted first while the intact xylanase II was in the second peak. By pooling early fractions of the first peak and late fractions of the second peak, we obtained homogeneous preparations containing the nicked and the intact xylanase II's respectively (Fig. 37). The amino acid compositions of the two preparations were identical, supporting the assertion that fragments A and B come from the intact xylanase II. Although this procedure was effective in separating the nicked from intact xylanase II, the yields were low because we could only recover some of the fractions.

3.4.7 Presence of a disulfide bridge:

The amino acid composition of xylanase II revealed the presence of three cysteines (Table 9). We showed that two of these residues were involved in a disulfide bridge as a shift in mobility is observed when xylanase II is treated with a reducing agent before running on SDS-PAGE (Fig. 37). We could not localize the exact position of the disulfide bridge because we did not have the complete amino acid sequence. However, a shift in mobility is also observed for fragment A from a preparation of nicked xylanase II (Fig. 37). Thus, the disulfide bridge occurs in a region between residues 1 and 130. As we know that there is no cysteine in the first 38 residues at the N-terminal (Fig. 31 and 32), we can narrow the region where the disulfide bridge occurs down to residue 39 to 130.

Xylanase II is the most thermostable of all the xylanases that were shown to belong to the G family (Table 8). The presence of a disulfide bridge might be responsible for this higher thermostability. The xylanases from *Bacillus pumilus, Bacillus circulans, Streptomyces lividans* (xylanase B) and *Trichoderma harzianum* have one or no cysteine and consequently they do not have disulfide bridges. The xylanases from *Streptomyces lividans* (xylanase C), *Streptomyces* sp. No.36a and *Schizophyllum commune* have two cysteines while the xylanase fron *Clostridium*

Figure 37: Demonstration of the presence of a disulfide bridge in xylanase II. The intact (2 μg in each of lanes 2 and 3) and nicked (3.6 μg in each of lanes 4 and 5) xylanases II were subjected to SDS-PAGE. The samples applied to lanes 3 and 5 were treated with 100 mM DTT before electrophoresis. Lanes 1 and 6: molecular mass markers (kDa).


1 2 3 4 5 6
94
67
43
30
20.1
14.4

acetobutylicum has three cysteines (Table 10). It has not been reported if these residues are involved in disulfide bridges. If this is the case, it could mean that the disulfide bridge in xylanase II is providing more stability than the disulfide bridges in these other xylanases. The position of the disulfide bridge could be the critical factor determining stabilizing effects.

The role of a disulfide bridge has been shown to be important in the thermal stability of a xylanase from *Humicola lanuginosa* (Tatu et al. 1990). Thermal denaturation studies using circular dichroism and UV spectroscopy showed that the presence of a disulfide cross-link offers resistance against enzyme unfolding at extremes of temperature and pH. The xylanase from *H. lanuginosa* showed thermal properties similar to xylanase II from *T. terrestris* 255B with a temperature optimum of 65 °C and stability for at least 1 hour in the range of 30-60 °C. This xylanase might belong to the G family as it has a LMW and it is rich in tyrosine and tryptophan residues (Anand et al. 1990). However, its relatedness to the G family has not been confirmed by sequencing.

Other intrinsic factors that can cause protein stabilization include additional hydrogen bonding, ionic interactions (salt bridges) and hydrophobic interactions (Nosoh and Sekiguchi, 1990). However, three-dimensional structural data of both mesophilic and thermophilic xylanases would be necessary in order to demonstrate the role of any one of these factors.

3.5 PROPERTIES AND MODE OF ACTION OF XYLANASE II:

3.5.1 Introduction:

Following the earlier structural studies, we characterized various properties and kinetic parameters of xylanase II. The mode of action of xylanase II was compared with the action of the 32-kDa xylanase from *Thermoascus crustaceus* (isolated by Tan et al. 1987a) which appeared to belong to the F family. We hoped to demonstrate that these two thermophilic xylanases had different modes of action and verify if they could complement each other in the hydrolysis of different xylans.

3.5.2 Determination of xylanase activity:

Accurate quantification of xylanase activity is difficult to obtain because the substrate used for its determination is polymeric (often partially soluble) and is modified during the assay. The products of the initial attack represent new types of substrate for which the enzyme might have different affinities. Bailey et al. (1992) suggested standard assay conditions in order to obtain comparable values between laboratories in spite of the inadequacy of the substrate. They recommended the use of the dinitrosalicylic acid (DNS) method (Miller 1959) for the quantification of the reducing sugars produced during the assay because this method was widely used and is easy to perform. We initially used this method to quantify xylanase activity in crude preparations and to monitor the purification of xylanase II.

The method of Somogyi Nelson (Somogyi 1952, Nelson 1944) is less popular because it is more time-consuming and the reagent is more toxic than the DNS reagent. However it gives more consistent reducing sugar values between equimolar quantities of monomers and various oligosaccharides produced during xylanase or cellulase assays (Bailey et al. 1992, Robyt and Whelan 1972). For instance, equimolar amounts of xylose and xylotetraose gave relative absorbances of 1/2.63 (xylose/xylotetraose) with the DNS method and 1/0.69 with the Somogyi Nelson (SN) method. The DNS method causes partial hydrolysis of oligosaccharides, leading to

an overestimation of the number of free reducing ends (Bailey et al. 1992). The SN method slightly underestimates oligosaccharides by giving a slightly lower response with increasing chain length. As xylanase II produced mostly long chained oligosaccharides (Fig. 41) a large difference was observed between the specific activities obtained with the DNS (1782 U/mg enzyme, SD=790) and the SN (214 U/mg enzyme, SD=49) methods. This kind of discrepancy has been observed with other xylanases (Bailey et al. 1992, Tan et al. 1985a) and is explained by the different dependence of the methods on the chain length of the oligosaccharides. Because the SN method gave a more uniform response for the various xylo-oligosaccharides, we used this method for the remaining work.

We tested the time-dependence of our standard xylanase assay procedure with purified xylanase II. At 65°C, the production of reducing sugars was almost linear over a period of 30 minutes (not shown). The rate after 30 minutes was over 85% of the one observed after 5 minutes. Carrying out the assay at 60°C or at 55°C gave the same results indicating that the slight reduction in rate was not primarily due to thermal inactivation. Substrate modification was probably the cause of the reduction in the rate although it must have been a minor effect as a near linear relationship with time was observed.

Using an assay time of 10 minutes, we observed a linear dependence of the assay on enzyme concentration in the range of 50 to 300 ng of purified xylanase II. A linear response of the xylanase activity over this range of enzyme concentrations was observed using both the DNS and SN methods. The linear response observed with both time and enzyme concentration indicated that the assay conditions were appropriate for quantifying the activity of xylanase II.

3.5.3 Substrate specificity:

Xylanase II was shown to be highly specific in cleaving linkages in only β-1,4

xylans (Table 11). Xylanase II showed very high activity towards oat-spelts xylan (175 U/mg enz.) and larch wood xylan (196 U/mg). It did not cleave p-nitrophenyl xylopyranoside, xylobiose or xylotriose and, therefore, did not exhibit β-xylosidase activity. Absence of activity on salicin indicated it had no β-glucosidase activity either. The enzyme showed no activity on filter paper or acid-swollen cellulose, and very low activity on carboxymethylcellulose (<2 U/mg enz.). The high specificity for xylan hydrolysis is another characteristic of the xylanases from the G family (Gilkes et al. 1991b) and confirms the relatedness of xylanase II to this group of enzymes.

3.5.4 Determination of optimal pH for activity:

The optimal pH of xylanase II was determined in different buffers. The McIlvaine buffer (a citric acid-Na_2PO_4 buffer) has been widely used to determine pH optimum of xylanases because it covers the range of pH 2.6-7.6. However, we found that buffers containing phosphate interferred with both of the reducing sugar assay methods (DNS and SN). Using citrate buffer, we obtained a symmetrical relative activity curve with an optimal pH at 4.0 (Fig. 38). With acetate buffer, a slightly more acidic optimal pH at 3.6 was obtained. The decline of activity on the acidic side could not be fully documented with this buffer because it is only effective down to about pH 3.5.

3.5.5 Temperature optimum and thermostability:

The optimal temperature for hydrolysis over a 30 min assay was 60-65°C (Fig. 39A). Xylanase II was stable for at least 10 hours when incubated at temperatures of up to 50°C. However, xylanase II was found to be very unstable when incubated at temperatures above 60°C (Fig. 39B) in the absence of substrate. Less than 15% of the original activity was recovered after 30 minutes of incubation at 65°C (Fig. 39B). On the other hand, over 80% of the activity was still present when the enzyme was incubated under the same conditions in the presence of substrate (0.5% oat-spelts xylan). Binding to the substrate probably stabilizes the structure of xylanase II.

Table 11: Substrate specificities of xylanase II:

Substrate	Activity (U/mg enzyme)
Oat-spelts xylan	175
Larch wood xylan	196
Xylobiose	not degraded[1]
Xylotriose	not degraded[1]
p-Nitrophenyl xylopyranoside	0[2]
Filter paper	0
Acid-swollen cellulose	0
Carboxymethylcellulose	<2
Salicin	0

Activity determined using a reducing sugar assay (Somogyi Nelson) except:

1: analysis of products by TLC.

2: determination of nitrophenol by measuring A_{420}.

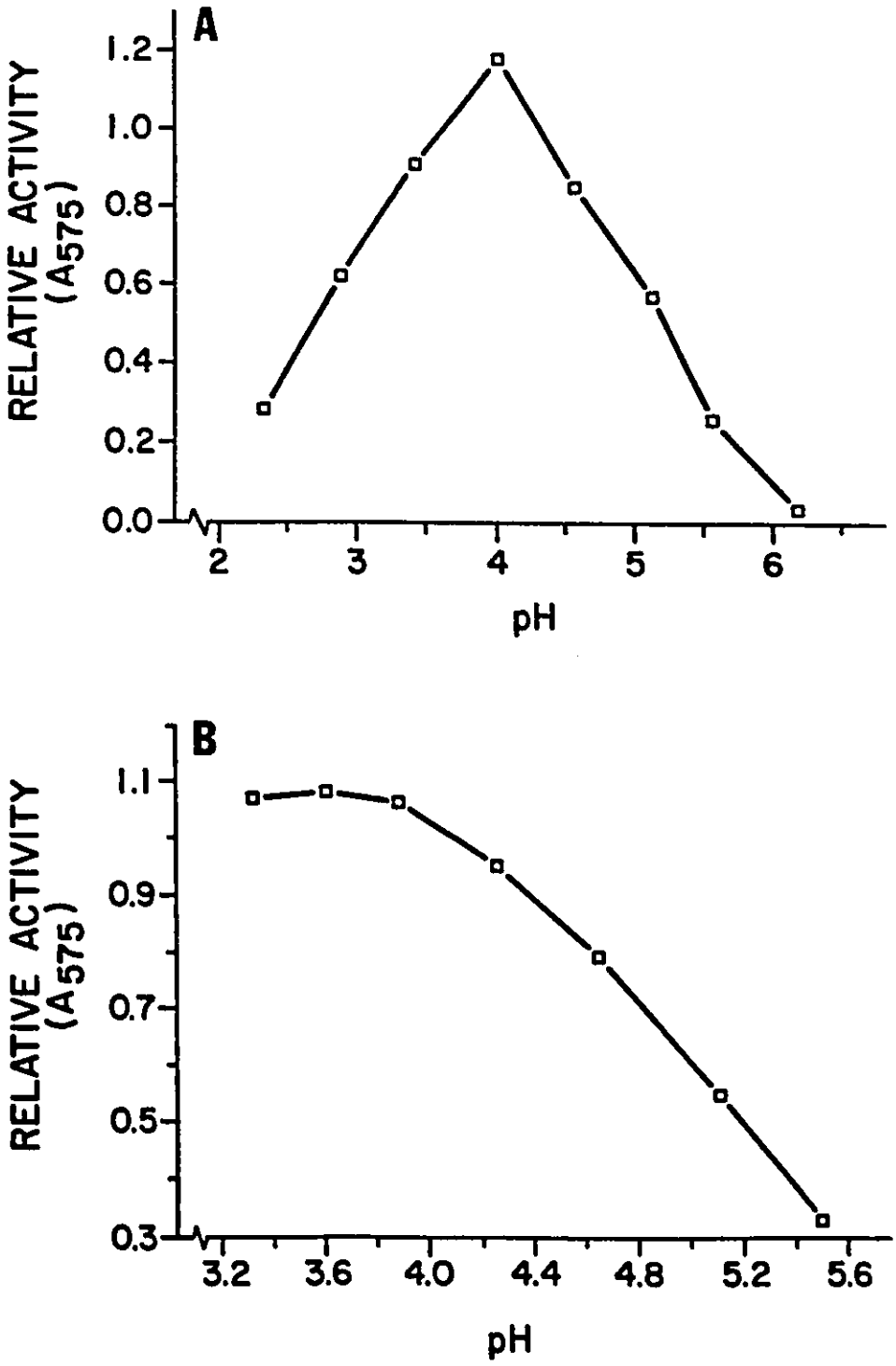


Figure 38: Determination of the optimal pH of xylanase II using 50 mM of citrate buffer (A) and 50 mM of acetate buffer (B).

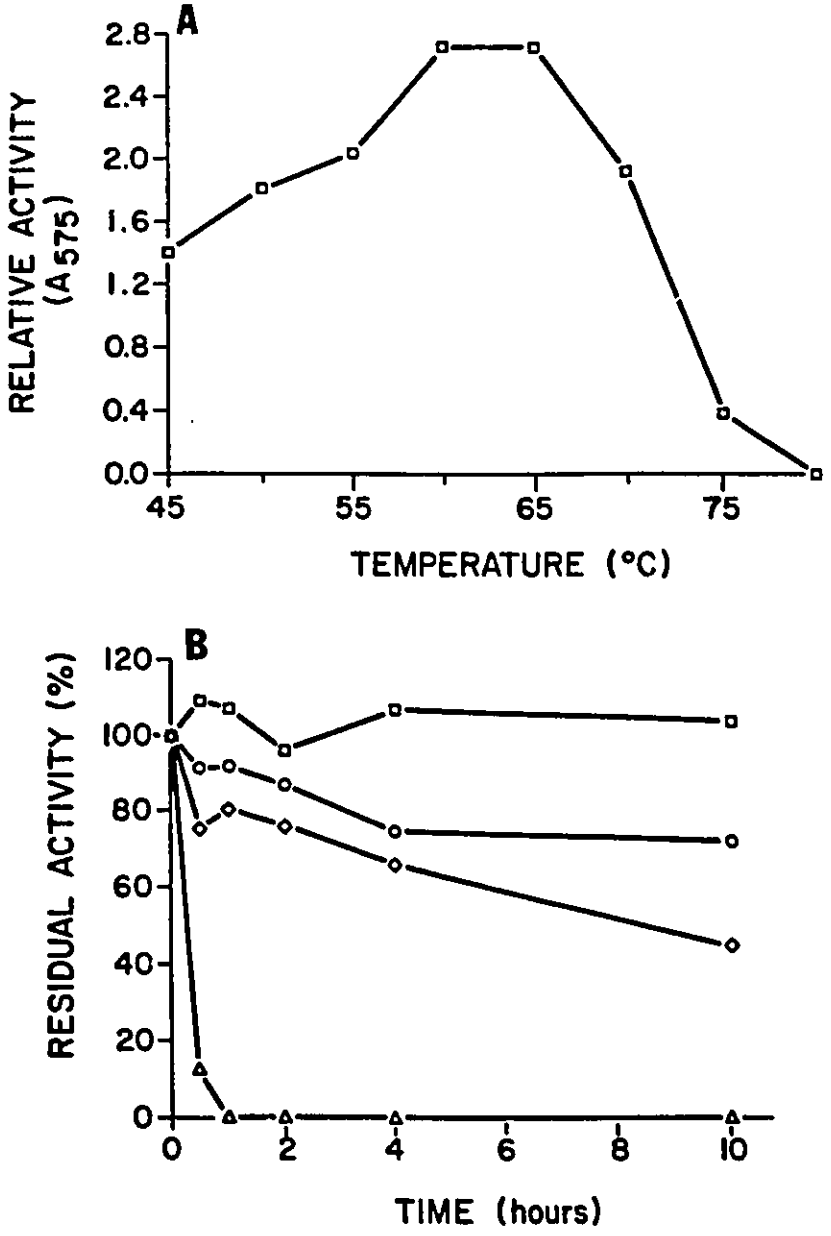


Figure 39: Thermostability of purified xylanase II. Panel A: Relative activities of xylanase II were determined using 30-min assays with 0.5% oat-spelts xylan at various temperatures. Panel B: Thermostability of xylanase II incubated in the absence of substrate at 50°C (□), 55°C (○), 60°C (◇) and 65°C (△). After various durations of incubation, aliquots were assayed using the 30 min xylanase assay at 65°C.

Xylanase II from *T. terrestris* 255B is the most thermostable xylanase that has been clearly shown to belong to the G family (Table 8). Other thermophilic xylanases that might also belong to the G family include a 33-kDa xylanase from *Streptomyces thermoviolaceus* (Tsujibo et al. 1992), a 25.6-kDa xylanase from *Humicola grisea* (Monti et al. 1991), a 22.5-kDa xylanase from *Humicola lanuginosa* (Anand et al. 1990), a 25-kDa xylanase from *Clostridium thermocellum* (MacKenzie et al. 1989), a 26-kDa xylanase from *Chaetomium thermophile* (Ganju et al. 1989), a 25-kDa xylanase from *Paecilomyces varioti* (Krishnamurthy and Vithayathil, 1989), a 20.5-kDa xylanase from *Streptomyces* T_7 (Keskar et al. 1989) and a 22.5-kDa xylanase from *Bacillus* spp (Okazaki et al. 1985). All these xylanases have some properties that suggest that they belong to the G family, but sequencing data is not available to confirm their potential relationship to this group of enzymes. They have temperature optima ($T_{opt.}$) in the range of 60-65°C except for the 26-kDa xylanase from *Chaetomium thermophile* and the 25.6-kDa from *Humicola grisea* which have $T_{opt.}$ of 70°C. Similarly to xylanase II, these two xylanases were relatively unstable when incubated in the absence of substrate at temperatures above 60°C. No $T_{opt.}$ was reported for the 25-kDa xylanase from *Clostridium thermocellum*, but this enzyme is likely to be the most thermostable of this list because it had a half-life of 24h at 70°C. If this xylanase turns out to belong to the G family, it would be the only one reported to date to be a member of this family and significantly more thermostable than xylanase II from *T. terrestris* 255B.

Other characterized thermophilic xylanases include the 32-kDa xylanase from *Thermoascus aurantiacus* (Khandke et al. 1989a) and the 40.5-kDa xylanase from *Caldocellum saccharolyticum* (Lüthi et al. 1990) which were shown to belong to the F family and had thermostability properties similar to the 25-kDa xylanase from *Clostridium thermocellum*. The most thermostable xylanase reported to date is the 31-kDa xylanase from *Thermotoga* sp. strain FjSS3-B.1 which has a $T_{opt.}$ of 105°C (Simpson et al. 1991). However, the data was insufficient to classify this xylanase into

the F or the G family. Other extremely thermostable xylanases are likely to be purified and characterized in the future as many workers have started to look at habitats such as hot-springs and deep-sea vents as sources for thermophilic microorganisms and thermostable enzymes.

3.5.6 Effect of various ions on xylanase II:

The effect of various ions on xylanase activity was tested using 2 mM of Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+} and Pb^{2+}. Only Cu^{2+} and Pb^{2+} affected xylanase II and reduced the original activity by 50% and 40%, respectively. It was not possible to determine if the inhibiting effect was due to a specific interaction but it is known that Pb^{2+} ions tend to interact with cysteine residues while Cu^{2+} ions tend to interact with cysteine and histidine groups (Creighton 1983).

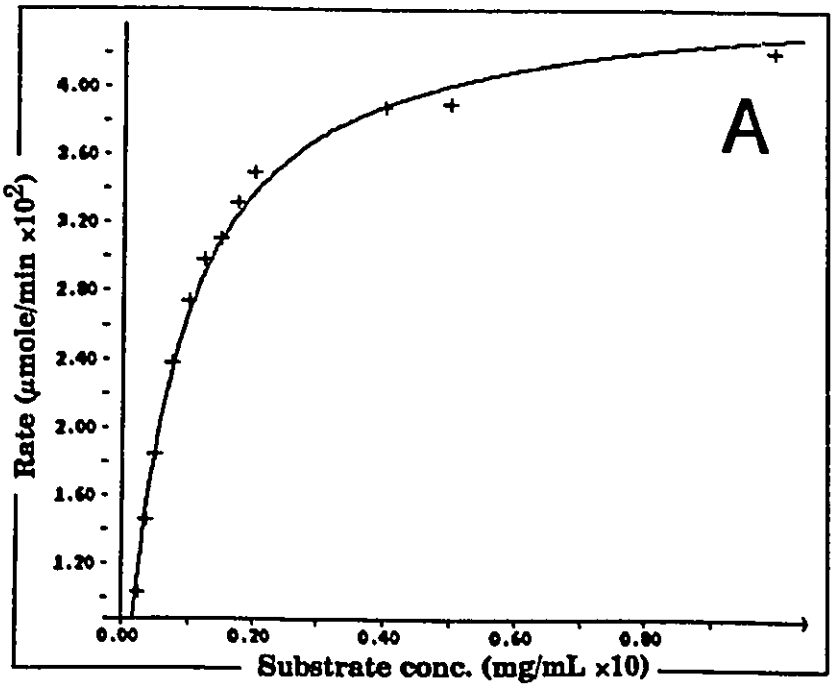
The xylanase activity was also tested in the presence of 10 mM disodium EDTA, a chelating agent. The absence of effect by EDTA further confirmed that xylanase II did not have a requirement for divalent cations.

3.5.7 Kinetic parameters of xylanase II:

Michaelis-Menten constants were determined using larch wood xylan, 4-O-methyl-D-glucuronoxylan-D-xylan (from birch wood) and oat-spelts xylan. For all substrates, the increase in velocity followed saturation kinetics with respect to increasing substrate concentration (Fig. 40 A, C and E). The data were approximately linear when transformed to Eadie-Hofstee plots (Fig. 40 B, D and F). Deviation from linearity was more prominent in the lower and higher concentration ranges. Consequently the parameters were calculated by using only the data in the range from 0.5 to 2 mg/mL of substrate.

The K_m values of xylanase II (Table 12) were within the range reported for other xylanases (0.15-49.5 mg/mL). These K_m values have to be considered as

Figure 40: Determination of kinetics parameters of xylanase II using larch wood xylan (A and B), birch wood xylan (C and D) and oat-spelts xylan (E and F). The curves of the rates versus substrate concentrations (A, C and E) were linearized using Eadie-Hofstie plots (B, D and F).


A
Rate (μmole/min ×10²)
4.00
3.60
3.20
2.80
2.40
2.00
1.60
1.20
0.00
0.20
0.40
0.60
0.80
Substrate conc. (mg/mL ×10)

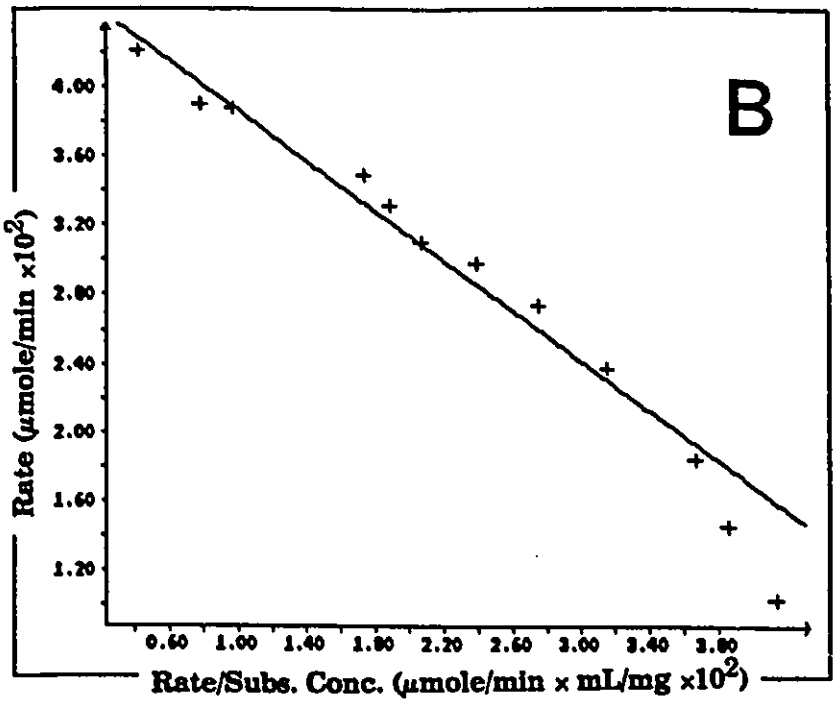
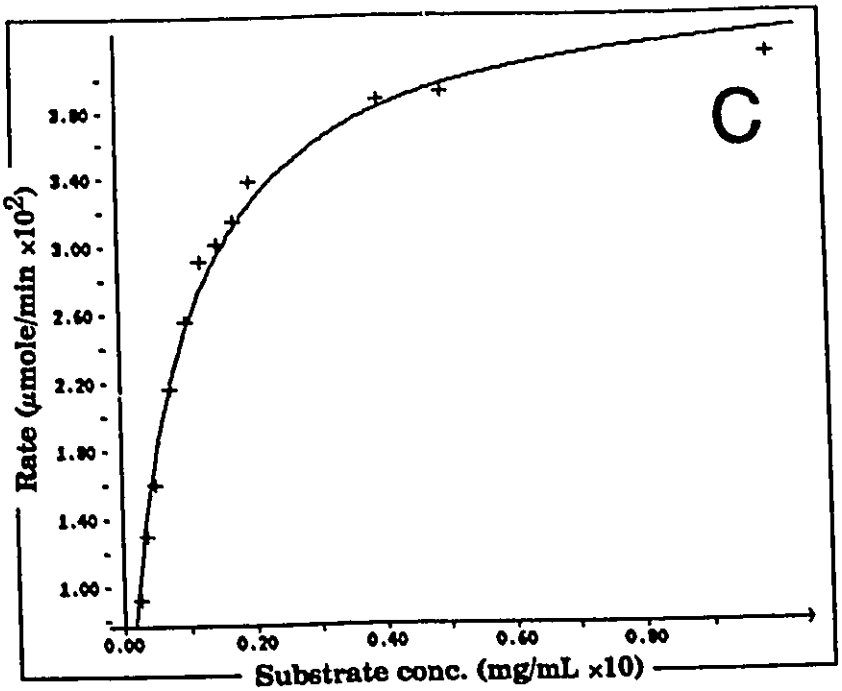

B
Rate (μmole/min ×10²)
4.00
3.60
3.20
2.80
2.40
2.00
1.60
1.20
0.60
1.00
1.40
1.80
2.20
2.60
3.00
3.40
3.80
Rate/Subs. Conc. (μmole/min × mL/mg ×10²)

Fig. 40


C
Rate (μmole/min ×10²)
3.80
3.40
3.00
2.60
2.20
1.80
1.40
1.00
0.00
0.20
0.40
0.60
0.80
Substrate conc. (mg/mL ×10)

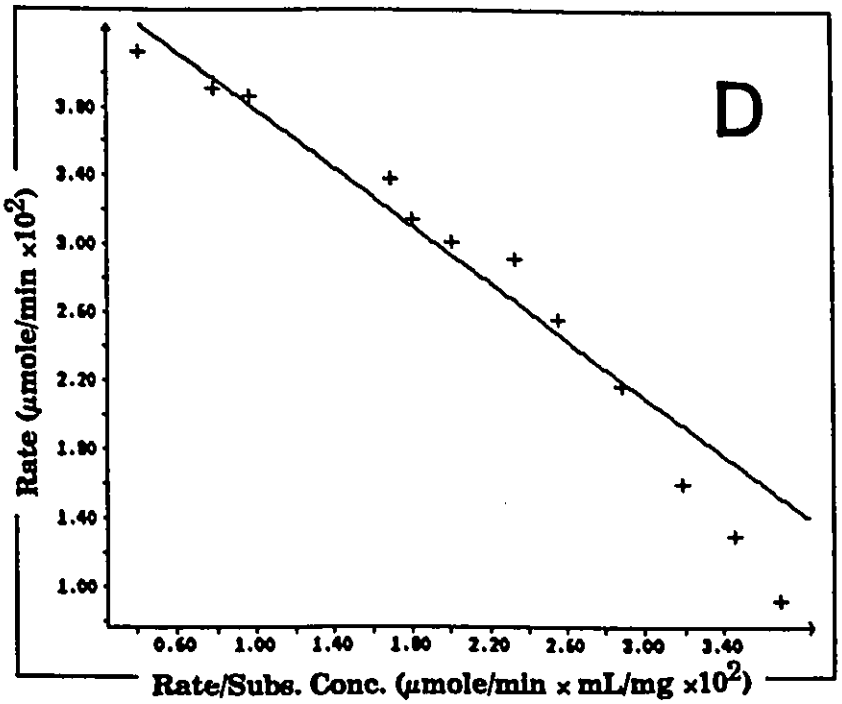
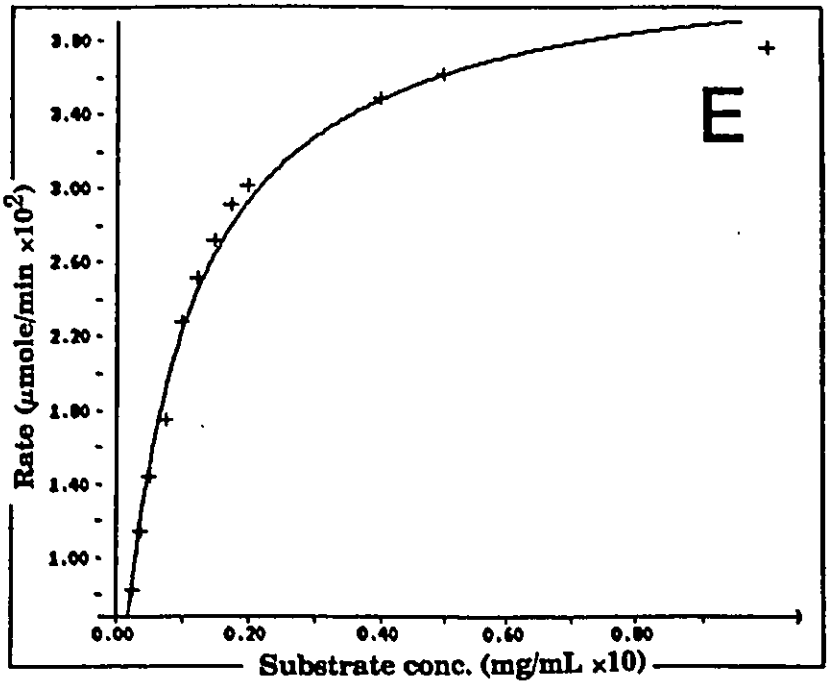

D
Rate (μmole/min ×10²)
3.80
3.40
3.00
2.60
2.20
1.80
1.40
1.00
0.60
1.00
1.40
1.80
2.20
2.60
3.00
3.40
Rate/Subs. Conc. (μmole/min × mL/mg ×10²)

Fig. 40


E
Rate (μmole/min ×10²)
3.80
3.40
3.00
2.60
2.20
1.80
1.40
1.00
0.00
0.20
0.40
0.60
0.80
Substrate conc. (mg/mL ×10)

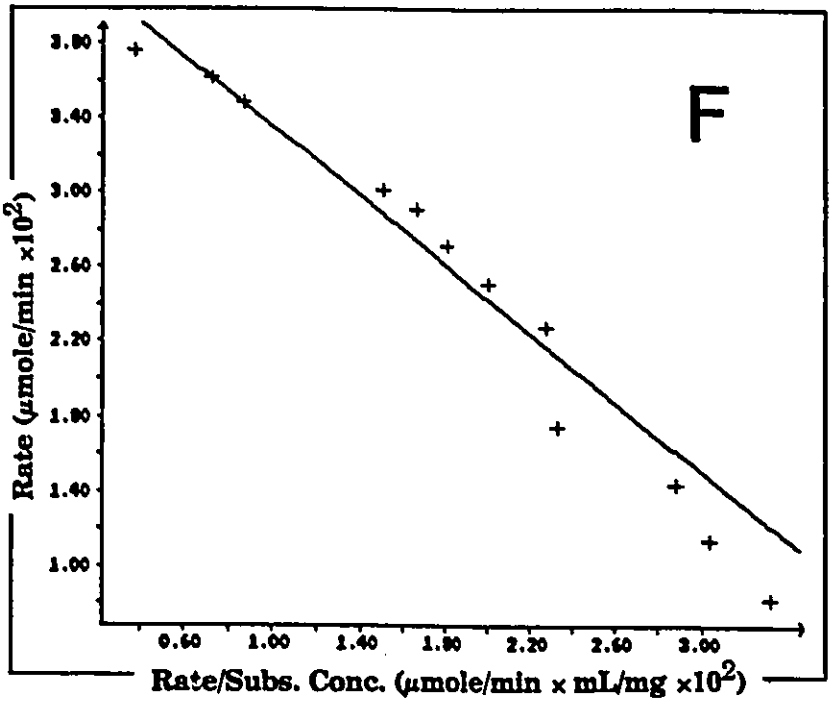

F
Rate (μmole/min ×10²)
3.80
3.40
3.00
2.60
2.20
1.80
1.40
1.00
0.60
1.00
1.40
1.80
2.20
2.60
3.00
Rate/Subs. Conc. (μmole/min × mL/mg ×10²)

Fig. 40

Table 12: Comparison of kinetic parameters obtained for xylanase II with different xylans:

Type of xylan	K_m (mg/mL)	k_{cat} (sec^{-1})	k_{cat}/K_m (mL/mg×sec)
Larch wood	0.80 (±0.03)	205.6 (±1.7)	259 (±9.9)
Birch wood	1.04 (±0.1)	221.0 (±14.1)	213 (±24.6)
Oat-spelts	1.23 (±0.04)	206.5 (±1.7)	168 (±0.19)

apparent values because xylans are complexed substrates. Also, products of xylan hydrolysis themselves become substrates for further hydrolysis which make the apparent K_m an average approximation of the K_m's for a number of substrates.

The k_{cat}/K_m values indicated that xylanase II had higher activity on larch wood xylan and lower activity on oat-spelts xylan. The presence and/or position of substituents on the xylan backbone are possible causes for the preference of xylanase II for one or the other types of xylan. Substituents can cause steric hindrance which limits the hydrolysis (Poutanen et al. 1988). On the other hand, some substituents increase the solubility of the substrate which lead to higher hydrolysis rates (Tenkanen et al 1992). All the substrates used were substituted with either arabino-furanosyl groups (oat-spelts xylan), 4-O-methyl-glucuronosyl groups (birch wood xylan) or both of these groups (larch wood xylan). Detailed information was not available on the amounts of substituents present in the substrates used. However, larch wood xylan was the most soluble xylan while oat-spelts xylan was the least soluble xylan. Thus, the differential solubility of the substrates seems to have been a major factor that resulted in the preference of xylanase II for larch wood xylan.

3.5.8 Xylanase from *Thermoascus crustaceus*:

Previously, Tan et al. (1987a) had purified a 32-kDa xylanase from *Thermoascus aurantiacus*. This organism was later re-identified as *Thermoascus crustaceus* (unpublished data). Khandke et al. (1989a) purified a 31.8-kDa xylanase from *Thermoascus aurantiacus* which had properties similar to the 32-kDa xylanase purified by Tan et al. (1987a). The 31.8-kDa xylanase from *Thermoascus aurantiacus* was later completely sequenced and shown to belong to the F family (Srinivasa et al 1991). Given the close relatedness between the two microorganisms and the similarities between the two enzymes, it is probable that the 32-kDa xylanase purified by Tan et al. (1987a) also belongs to the F family. We compared the mode of action of this xylanase with xylanase II in order to determine if enzymes from the two families

could attack xylan by different mechanisms.

3.5.9 Mode of hydrolysis of oat-spelts xylan:

TLC analysis was used to analyze the products obtained when xylanase II or the 32-kDa xylanase hydrolyzed oat-spelts xylan (Fig. 41). With xylanase II, the early (5 min) hydrolysis products were xylobiose, xylotriose, xylotetraose and unresolved oligomers with higher degrees of polymerization. After extended hydrolysis (24 hours), the most intense products were xylobiose and xylotriose. These data indicated that xylanase II had an endo-type mechanism with the end products being xylobiose and xylotriose.

Tan et al. (1987a) reported an endo-type mechanism for the 32-kDa xylanase. Our data are consistent with this report as xylobiose, xylotriose and xylotetraose were the early hydrolysis products (after 5 min) and xylose and xylobiose were the main limit products obtained after extended hydrolysis (24 hours). The 32-kDa xylanase differed from xylanase II in two aspects: 1) the intensity of the unresolved (higher degree of polymerization) oligosaccharides was slightly lower for the 32-kDa xylanase, 2) the 32-kDa xylanase released xylose as one of the major final products.

3.5.10 Hydrolysis of xylo-oligosaccharides:

Xylanase II produced xylobiose and xylotriose as the main limit hydrolysis products obtained from oat-spelts xylan. This data suggested that xylanase II could not cleave xylobiose or xylotriose to xylose. Incubation of xylanase II with 10 mM xylotriose confirmed that it could not cleave this substrate (data not shown). The same confirmation could not be obtained with xylobiose because we did not have enough of this substrate available. However, xylobiose is a major limit product after extended hydrolysis of xylan and this observation strongly suggests that xylanase II can not cleave xylobiose.

Figure 41: TLC analysis of the products obtained after the hydrolysis of oat-spelts xylan by xylanase II or by the 32-kDa xylanase from *Thermoascus crustaceus*. The samples were diluted to contain equivalent amount of reducing sugars as determined by the Somogyi Nelson assay:

Lane 1: Standards

Lanes 2 to 5: Incubation with xylanase II for 5 min, 15 min, 60 min and 24 h respectively.

Lanes 6 to 9: Incubation with the 32-kDa xylanase for 5 min, 15 min, 60 min and 24 h respectively.

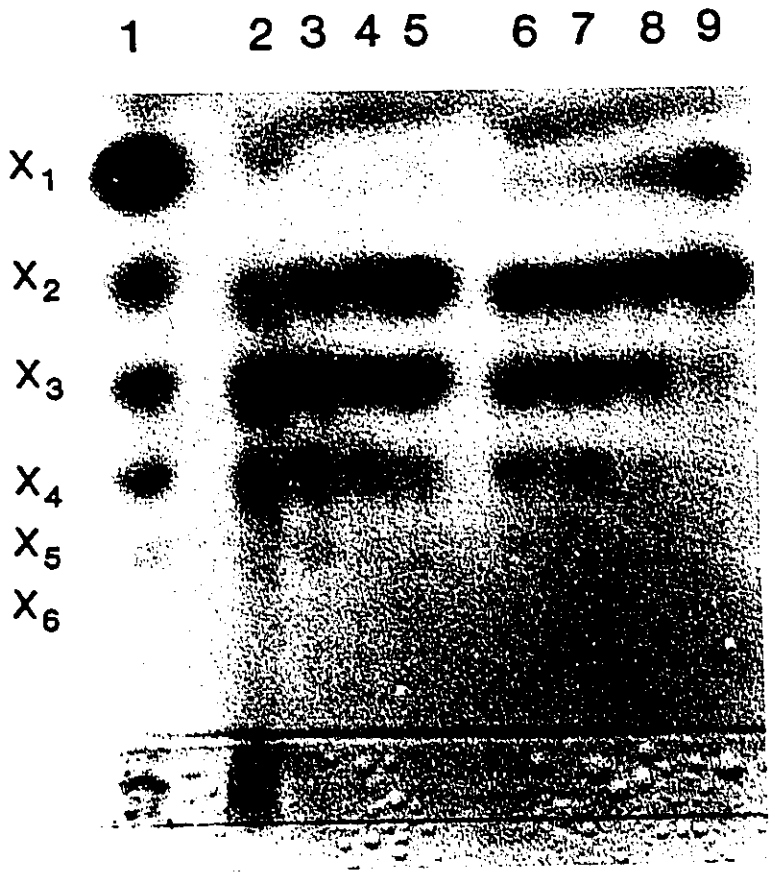
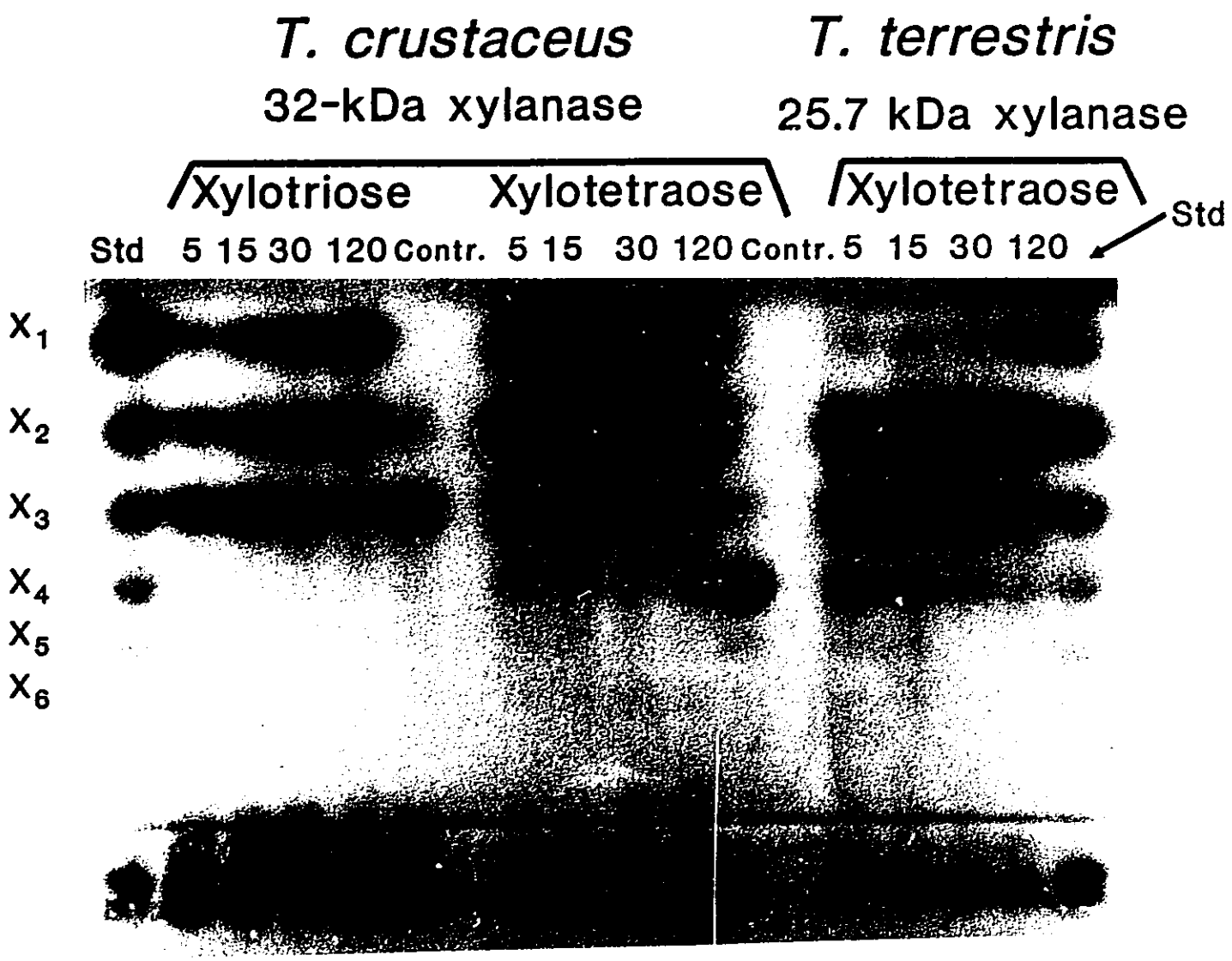
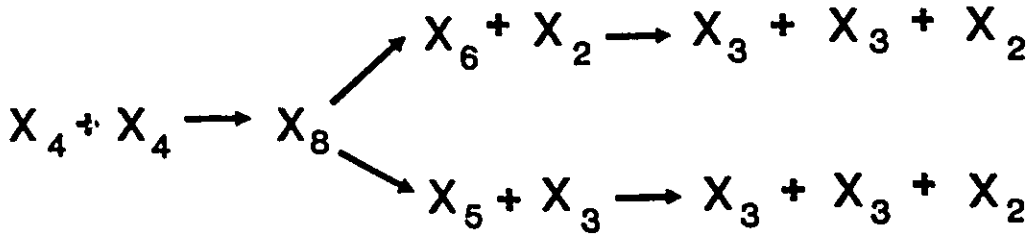

1 2 3 4 5 6 7 8 9
X1
X2
X3
X4
X5
X6

Figure 42: TLC analysis of the hydrolysis products of xylo-oligosaccharides. Xylotriose and xylotetraose (10 mM) were incubated with either the 32-kDa xylanase or xylanase II (not shown with xylotriose). Incubation times are in min. Contr.: Substrate control (no enzyme), Std: xylo-oligosaccharides standards.


T. crustaceus
32-kDa xylanase
T. terrestris
25.7 kDa xylanase
Xylotriose
Xylotetraose
Xylotetraose
Std
Std 5 15 30 120 Contr. 5 15 30 120 Contr. 5 15 30 120
X1
X2
X3
X4
X5
X6
Incubation time is in minutes

of xylotetraose by xylanase II (Fig. 42). Xylose was observed as a minor product after extended hydrolysis. The relative intensities of the TLC spots suggested that the main cleavage event resulted in the release of two xylobiose units. A less frequent cleavage event would have resulted in the release xylose and xylotriose. However, the relative intensities of the TLC spots suggested that there was more xylotriose produced than xylose. The additional xylotriose could have been produced by a process involving transxylosidation. Transxylosidase activity has been reported for other xylanases (Tan et al. 1985b). A possible scheme for the cleavage of xylotetraose by a mechanism involving transxylosylation is:



Confirmation of this model would require the demonstration of the occurence of the intermediates (X_8, X_6 and X_5). As none of these products were observed on the TLC plates, this suggests that the intermediates (X_8, X_6 and X_5) might remain bound to the enzyme until the two cleavage events have occurred.

The 32-kDa xylanase from *Thermoascus crustaceus* could cleave xylotriose to xylobiose and xylose (Fig. 42). It cleaved xylotetraose to xylobiose and xylose. Xylotriose was an intermediate product in early hydrolysis and as a minor product after 2 hours of hydrolysis of xylotetraose. Thus, the 32-kDa xylanase can remove one xylose unit at a time from xylotetraose. A second cleavage event would then produce an additional xylose unit and a xylobiose unit. However such a mechanism would yield two xylose units and one xylobiose unit for each hydrolysed xylotetraose. If this was the only mechanism, the TLC spot for xylose should be more intense than the one for xylobiose as the staining method is based on the detection of reducing sugars. Since this is not detected on the chromatogram, it is probable that the 32-kDa

Since this is not detected on the chromatogram, it is probable that the 32-kDa xylanase can also cleave xylotetraose directly to 2 xylobiose units. Khandke et al. (1989b) observed that the latter process was the major one through which the 31.8-kDa from *Thermoascus aurantiacus* hydrolyzed xylotetraose.

3.5.11 Solubilization assay:

Xylanases have been reported to vary in their ability to attack insoluble xylan (Bailey at al. 1991). We tested the ability of xylanase II and of the 32-kDa xylanase to attack insoluble xylan by using an assay based on light scattering by insoluble xylan (Tan et al. 1985b). A suspension of insoluble xylan was incubated with equivalent amounts (based on the standard xylanase assay) of either enzyme. The two xylanases showed very different rates of solubilization of insoluble xylan (Fig. 43). Xylanase II broke down insoluble xylan very efficiently causing a rapid decrease in O.D. (620 nm) in the first 30 minutes of incubation. Following the initial attack, the remaining xylan was solubilized at a much slower rate.

The 32-kDa xylanase caused a slower solubilization of insoluble xylan than xylanase II. However, the rate remained more stable over the incubation period. During extended incubation periods (up to 24 h, not shown), the solubilization caused by the 32-kDa xylanase eventually proved to be as extensive as the solubilization caused by xylanase II. Thus, it appears that the 32-kDa xylanase had the same ability as xylanase II to solubilize xylan but it proceeded at a slower rate. With both xylanases, residual material remained after extended hydrolysis (24 hours). The residual material had an O.D. (620 nm) corresponding to approximately 30% of the control O.D. (620 nm). This material was not analyzed but it could have been either refractory xylan or non-xylan polymers which often contaminate commercial xylan preparations.

The solubilization data suggests that xylanase II proceeded through a typical

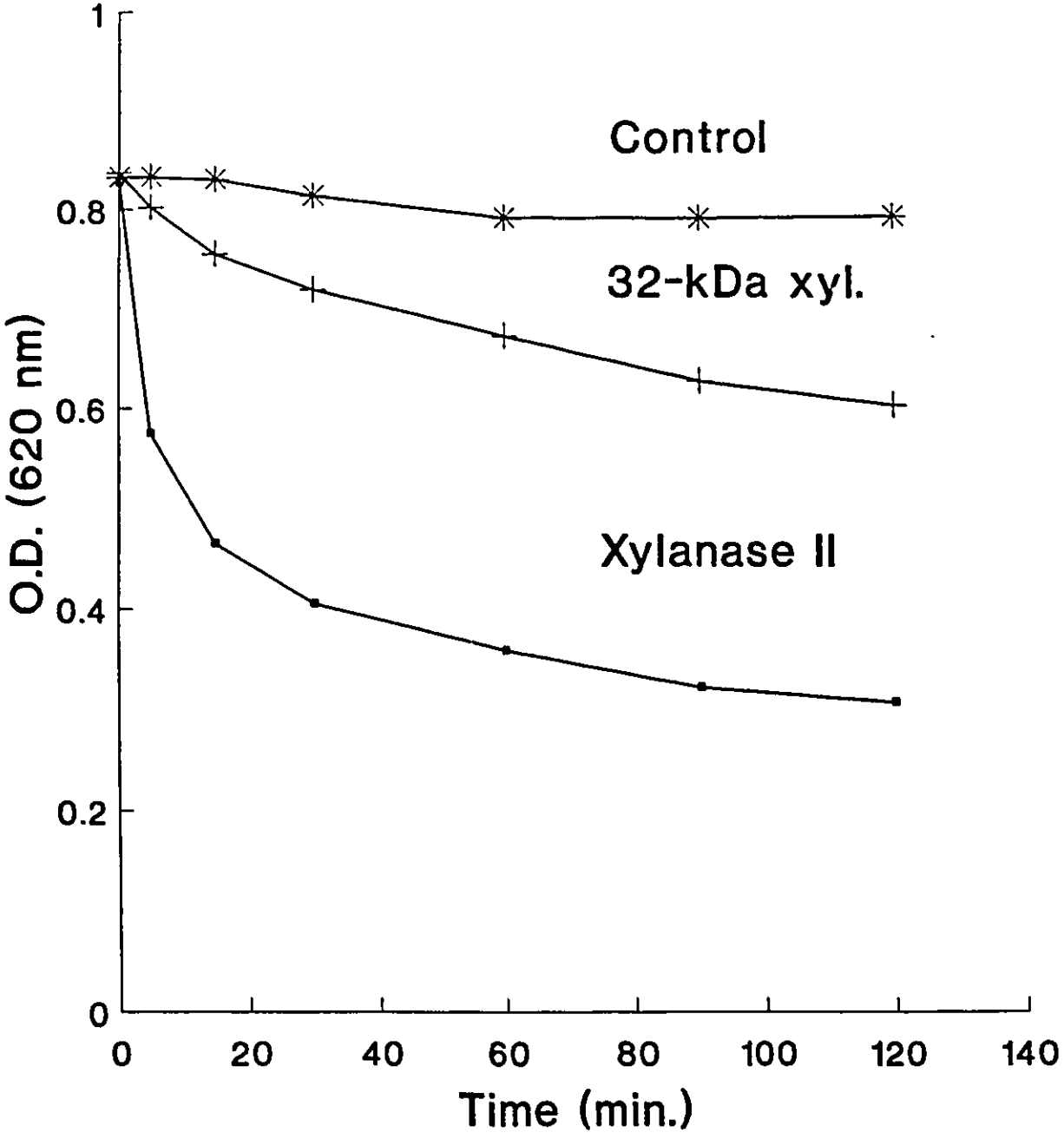


Figure 43: Solubilization of the insoluble part of oat-spelts xylan by the two thermostable xylanases (each enzyme was used at 0.5 U/2 mg substrate). The solubilization was followed by measuring the reduction of O.D. at 620 nm.

endo-mode with the initial attack breaking the insoluble (high degree of polymerization) xylan down to soluble xylan fragments. The rate of solubilization rapidly decreases as progressively less insoluble xylan is available for the enzyme. The solubilization data obtained with the 32-kDa xylanase could suggest that it proceeds through an exo-type mechanism which tends to show a slower but steady rate of solubilization. However, the hydrolysis products (Fig. 41) and the published data (Khandke et al. 1989b, Tan et al. 1987a) indicated that it had an endo-type mechanism. Furthermore, no evidence has yet been reported for exo-xylanases equivalent to the exo-cellobiohydrolases present in the cellulase systems of fungi. Thus, we can assume that the two xylanases under study are both endo-xylanases.

Various criteria have been recommended as a way of classifying xylanases including the ability to release arabinose substituents from arabinoxylans (Dekker, 1985), solubilizing capability (Frederick et al. 1985), release of xylose as a end-product of xylan hydrolysis (Lee et al. 1987) and the ability to act on cellulosic substrates (Lee et al. 1987). The ability of some xylanase preparations to release arabinose has been recently suggested to be due to insufficient purification (Kormelink et al. 1991). As summarized in Table 13, all the other criteria can be used to contrast xylanase II and the 32-kDa xylanase. In general, xylanase II was more efficient in solubilizing insoluble xylan and resulted in end-products with higher degrees of polymerization while the 32-kDa xylanase was more efficient in cleaving small xylo-oligomers with xylose being a major end-product.

Table 13: Comparison of the modes of action of xylanase II and of the 32-kDa xylanase:

Xylanase II	32-kDa xylanase
Xylose is not a major product of hydrolysis of xylan	Xylose is a major product of the hydrolysis of xylan
Can not cleave xylotriose	Cleaves xylotriose to xylobiose and xylose
Rapidly solubilized insoluble xylan	Low activity on insoluble xylan
No activity on cellulosic substrates	Low activity on cellulosic substrates

3.6 HYDROLYSIS OF VARIOUS XYLANS BY A COMBINATION OF TWO THERMOPHILIC XYLANASES:

3.6.1 INTRODUCTION:

Xylanase II from *T. terrestris* 255B and the 32-kDa xylanase from *Thermoascus crustaceus* appear to belong to different families of xylanases. These two thermophilic xylanases also had different modes of action with xylanase II being more effective in solubilizing xylan and with the 32-kDa xylanase being able to cleave xylan to xylose. Having demonstrated that these two enzymes have different mechanisms, we next wanted to determine if their combined action would be more effective in the degradation of xylan.

A major potential application of xylanases is to achieve complete hydrolysis of xylan to fermentable products. We tested if the two thermophilic xylanases would show increased ability to hydrolyze various types of xylans when they are used together. A positive interaction between the two xylanases would be apparent if an increase in the rate and/or in the extent of hydrolysis was observed.

Another application of xylanases is in the removal of residual xylans in kraft pulp in order to enhance the extractibility of the lignin which cause the dark color of the pulp. Most of the enzymes tested for the latter application have been derived from mesophilic organisms. In this work, we examined the hydrolysis of xylans in pulps by the two thermophilic xylanases used individually and in combination.

3.6.2 Hydrolysis of various xylans by xylanase II:

We first examined the ability of xylanase II to degrade various types of xylans (Fig. 44). The substrates used in this study included oat-spelts xylan (a cereal arabinoxylan), insoluble xylan (the insoluble part of oat-spelts xylan), birch wood xylan (a hardwood xylan), larch wood xylan (a softwood xylan), spruce kraft pulp (KP) xylan (a xylan extracted from a softwood KP) and poplar KP xylan (a xylan

extracted from a hardwood KP). For all the substrates, the initial release of products (measured by the SN method) was rapid with a plateau reached within 3 hours of incubation (Fig. 44). Incubation for up to 24 hours did not release significantly more products. End-product inhibition was probably not a major cause of inhibition as the initial concentration of substrate (1%) was relatively low, resulting in the release of only low concentrations of sugars.

Complete data on the composition and structure of the xylans used in this work were not available from either the literature or the manufacturers. Thus, it was difficult to measure the extent of xylan degradation achieved after the plateau in product release was reached (Fig. 44). We estimated the extent of xylan hydrolysis by performing HPLC analysis of the products released after 24 hours of incubation and using the substrate compositions reported in Appendix 1 (from Dr. K. K. Y. Wong, Chair of Forest Product Biotechnology, UBC). However the calculated extents of hydrolysis (Table 14) are only estimates because the xylan contents reported in Appendix 1 were probably underestimated due to limitations in the analysis method (see Appendix 1, Rem.). Also, the HPLC method used in this work allowed us to quantify only the xylose, xylobiose, xylotriose and xylotetraose. The substituted xylo-oligosaccharides and the oligosaccharides with a degree of polymerization higher than 4 units were not quantified because of the limitations of the HPLC method. When the HPLC data were converted to reducing sugar equivalents, we found that these values were lower than the ones obtained with the Somogyi Nelson method for all the substrates used (Table 14). This is another indication that the HPLC analysis underestimated the amounts of products released during hydrolysis by xylanase II.

We estimated that between 36% and 69% of the different xylans were converted to soluble xylo-oligosaccharides by xylanase II (Table 14). It appeared that xylanase II could not achieve complete degradation of the xylan present in the

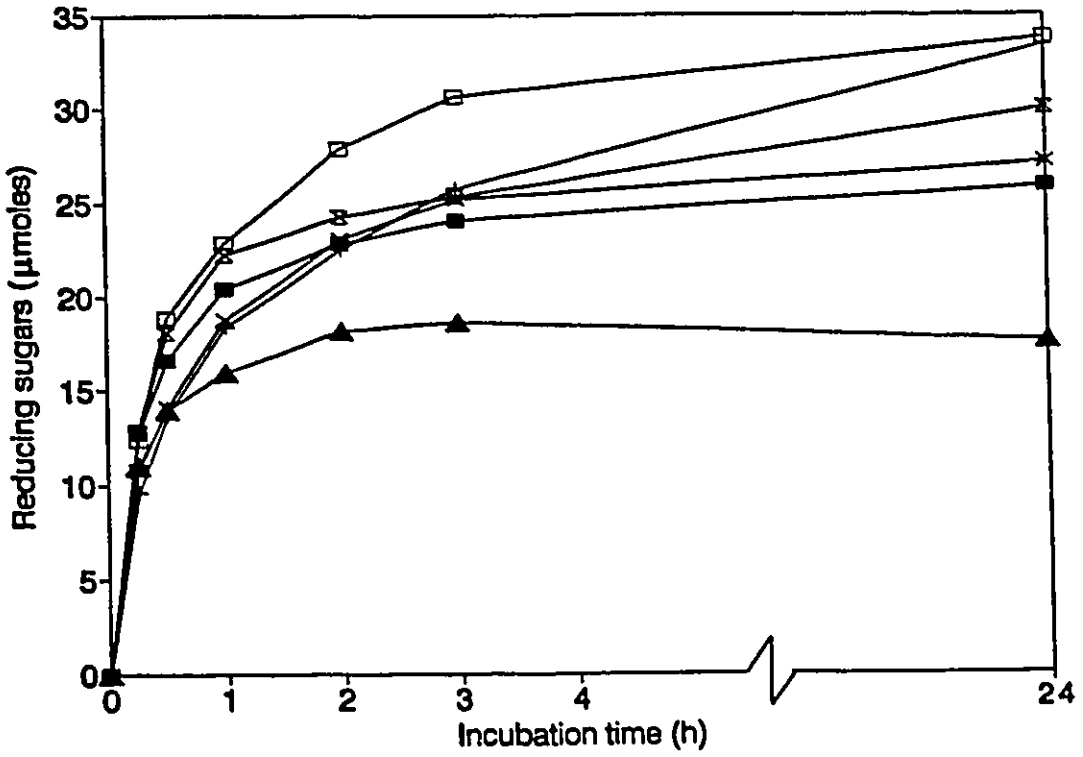


Figure 44: Hydrolysis of different xylans by xylanase II. Twenty mg of each substrate was incubated with 1 U of xylanase II at 60°C for up to 24 hours. The reducing sugars were quantified using the Somogyi Nelson method. Spruce KP xylan (▲), birch wood xylan (■), poplar KP xylan (×), larch wood xylan (I), insoluble xylan (+) and oat-spelts xylan (□).

Table 14: Hydrolysis of 20 mg of various xylans by 1 U of xylanase II:

Substrate (20 mg)	Reducing sugars by S.N. (µmole)	Reducing Sugars by HPLC (µmole)	% Xylan solubilized[a]
Oat-spelts xylan	33.7 (±3.1)	25.7 (±2.3)	68.6 (±6.3)
Insoluble xylan	33.3 (±0.9)	18.5 (±6.7)	37.4 (±14.4)
Birch wood xylan	25.9 (±2.8)	15.1 (±5.9)	43.4 (±17.4)
Larch wood xylan	30.0 (±1.4)	17.7 (±4.0)	49.9 (±12.6)
Poplar KP xylan	27.1 (±1.0)	19.0 (±1.4)	41.8 (±4.2)
Spruce KP xylan	17.6 (±0.6)	9.1 (±2.0)	35.9 (±9.3)

a: calculated using the HPLC data and the substrate compositions reported in appendix 1.

substrates used (Table 14). Incomplete conversion (sometimes below 20%) has also been reported for the hydrolysis of xylans by xylanases from *Trichoderma reesei* (Tenkanen et al. 1992). The inability of purified xylanases to achieve complete substrate degradation could be due to the heterogeneity of the xylans. Xylans are partially soluble indicating heterogeneity in the degree of polymerization of the chains. Also, the nature and position of substituents could hinder the action of the xylanases.

Because of incomplete information on the substrate composition, it was difficult to correlate the various yields with structural characteristics of the substrates. The low yield obtained with insoluble xylan can be explained by the lower accessibility of the chains in that substrate. The xylan extracted from spruce kraft pulp is associated with relatively high amounts of lignin (8.3%) and mannan (21.8%). These materials might have limited the access of xylanase II to the xylan, thus partially explaining the low yield obtained with this substrate.

The highest yield was obtained with oat-spelts xylan which is known to be highly substituted in position 3 with arabinofuranosyl units. Arabinose substituents give a gel-like structure to xylan by preventing the xylan chains from interacting together as cellulose chains are known to do (Andrewartha et al. 1979). Thus, arabinoxylan offers increased accessibility to endo-xylanases while apparently still allowing cleavage next to the substituents. However, the insoluble xylan is also highly substituted with arabinose units. In this case, the degree of polymerization of the chains might be higher which consequently decreases the solubility of the chains.

The substituents of birch wood xylan, larch wood xylan and poplar KP xylan were not identified but are probably 4-O-methylglucuronosyl groups which result in a high degree of solubility for these substrates. Thus, it is not clear why a lower yield was obtained with these substrates than with oat-spelts xylan. The 4-O-methylglu-

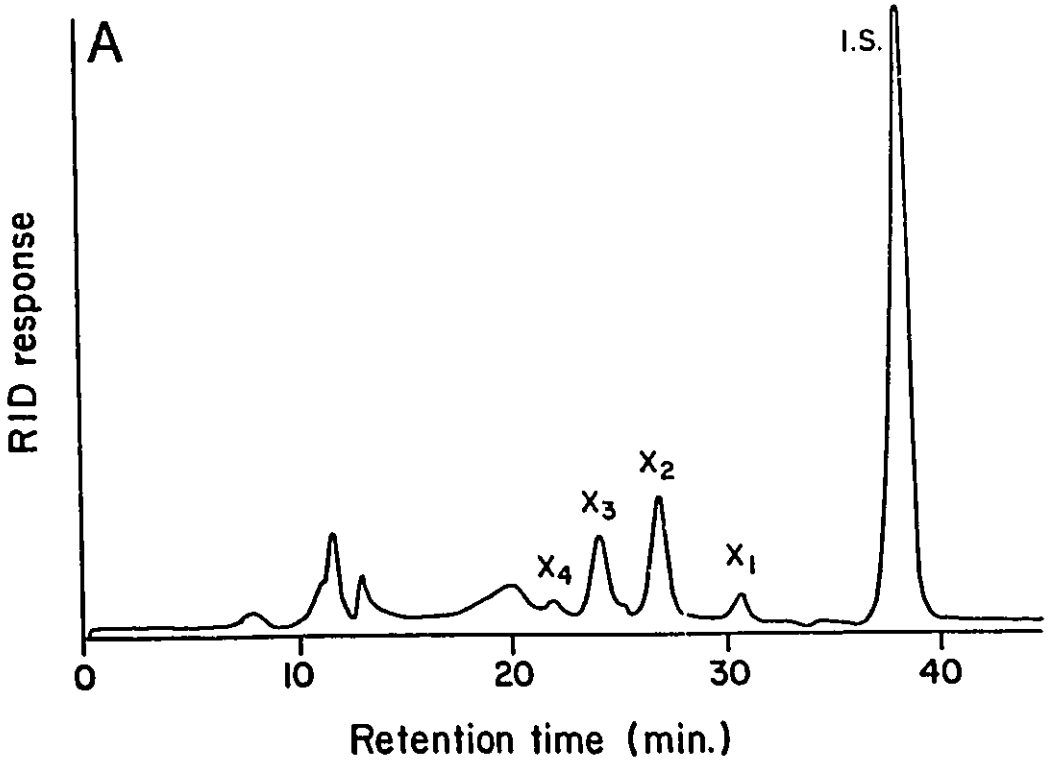
curonosyl groups are charged and larger than the arabinofuranosyl groups and thus they might be more restrictive with respect to the action of xylanase II. However, it is important to note that one xylanase has been reported to require the presence of a 4-O-glucuronosyl sustituent in order to cleave between adjacent xylose residues (Nishitani and Nevins, 1991). Since this xylanase does not interact in the same way with arabinosyl substituents, it re-affirms differences in enzyme interactions with different substituents.

The finding that the highest yield was obtained with oat-spelts xylan is somewhat contradictory with the kinetic parameters (k_{cat}/K_m) which indicated that xylanase II was more active on larch wood xylan and less active on oat-spelts xylans. However, the kinetic parameters were derived from initial rates which involved very little conversion of the substrates. Thus, these data might have been more influenced by the solubility of the substrate which was higher for larch wood xylan. In the case of extended hydrolysis, the yields are probably more influenced by the total number of bonds which can be cleaved by xylanase II.

According to HPLC analysis of the hydrolysis products, xylanase II did not release arabinose from oat-spelts xylan. Some xylanases have been reported to remove arabinose substituents (Matte et al. 1992, Wood and McCrae 1986) and this property has sometimes been used as a classification criteria. However, Kormelink et al. (1991) argued that the arabinofuranohydrolase activity of xylanase preparations was probably due to incomplete purification. Our observation that the purified xylanase II does not release arabinose supports the suggestion that homogeneous xylanase preparations have no activity on the arabinose substituents.

HPLC analysis revealed some differences in the profiles of the products obtained after the hydrolysis of the various substrates (Fig. 45). For all the substrates, the major products of hydrolysis were xylobiose and xylotriose in ratios (X_2/X_3)

Figure 45: HPLC analysis of the products obtained after incubating 20 mg of each substrate with 1 U of xylanase II for 24 hours. A) Spruce KP xylan, B) Birch wood xylan, C) Poplar KP xylan, D)Larch wood xylan, E) Insoluble xylan, F) Oat-spelts xylan. RID response = refractive index detector response. I.S.= Internal Standard (erythritol).


A
I.S.
RID response
X2
X3
X4
X1
0
10
20
30
40
Retention time (min.)

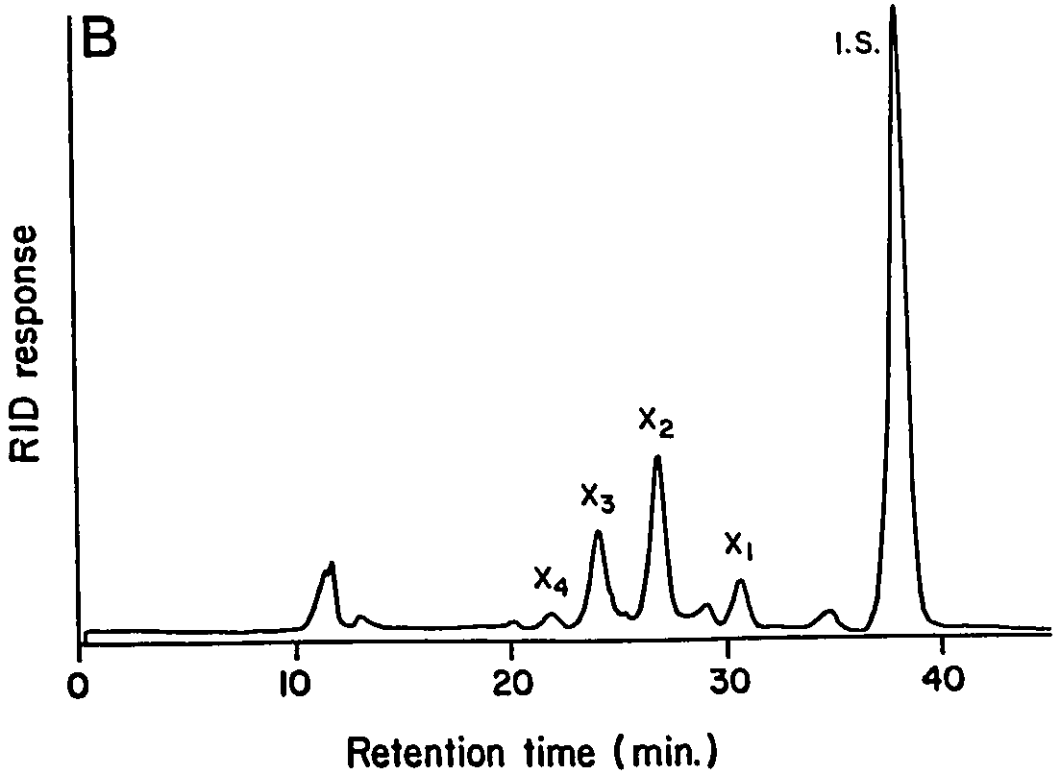
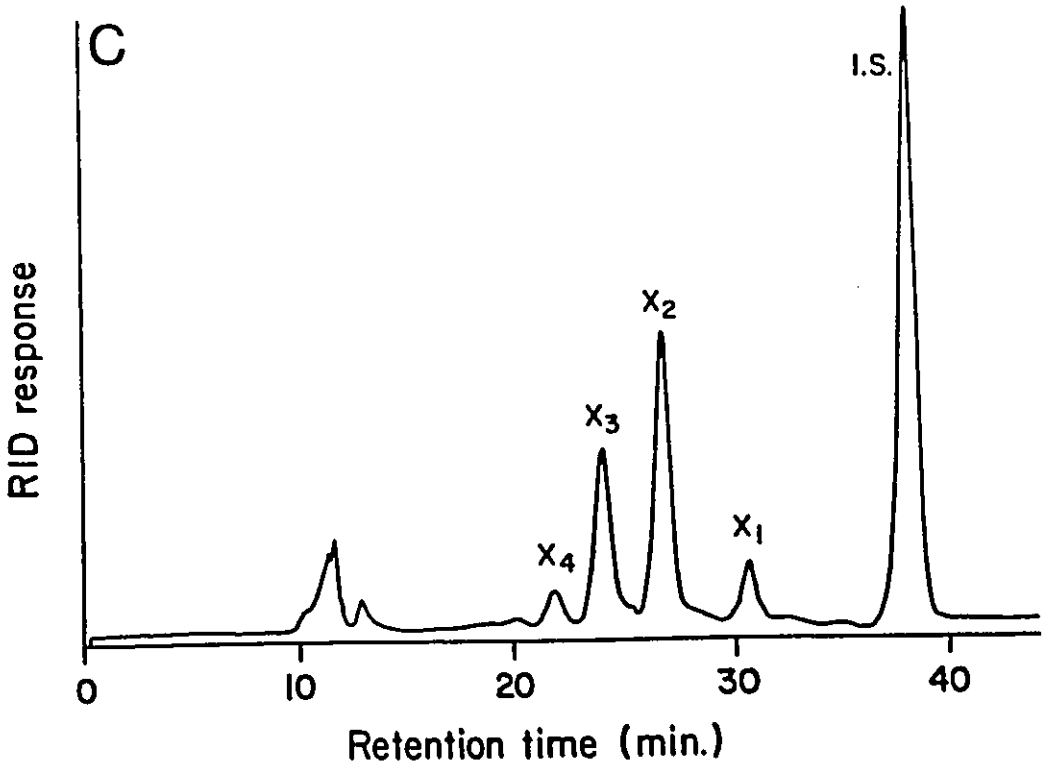

B
I.S.
RID response
X2
X3
X4
X1
0
10
20
30
40
Retention time (min.)

Fig.45


C
I.S.
X2
X3
X1
X4
RID response
0
10
20
30
40
Retention time (min.)

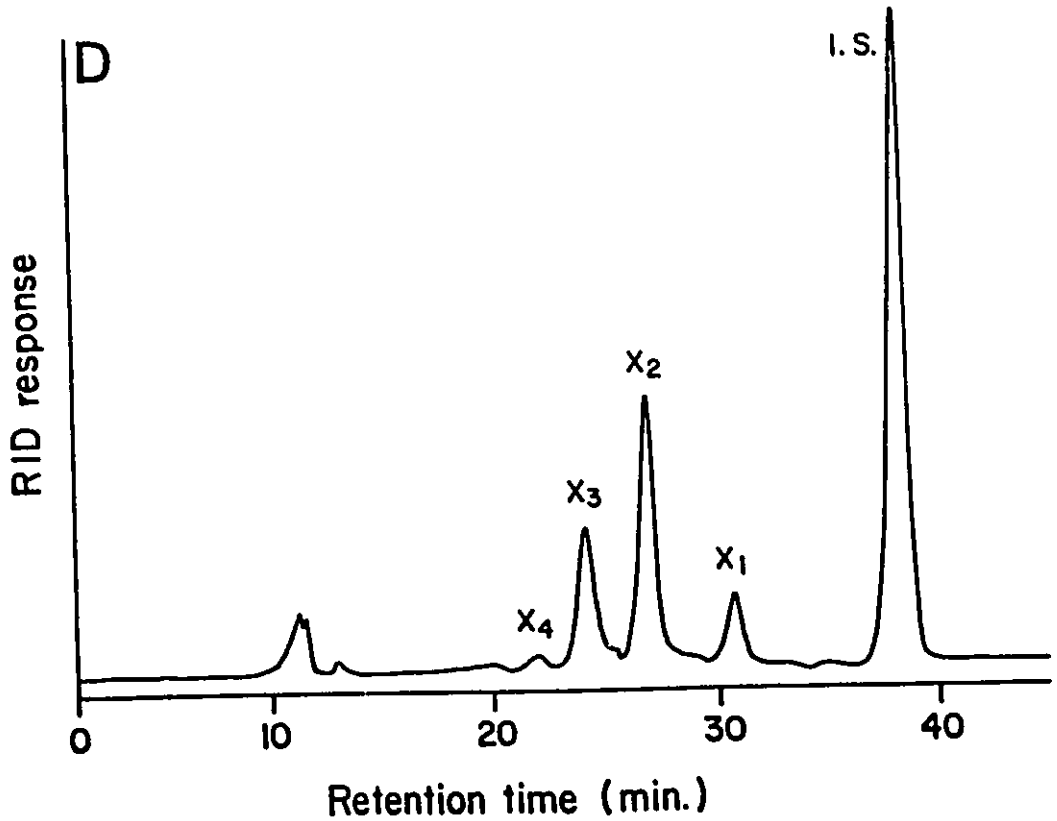

D
I.S.
X2
X3
X1
X4
RID response
0
10
20
30
40
Retention time (min.)

Fig.45

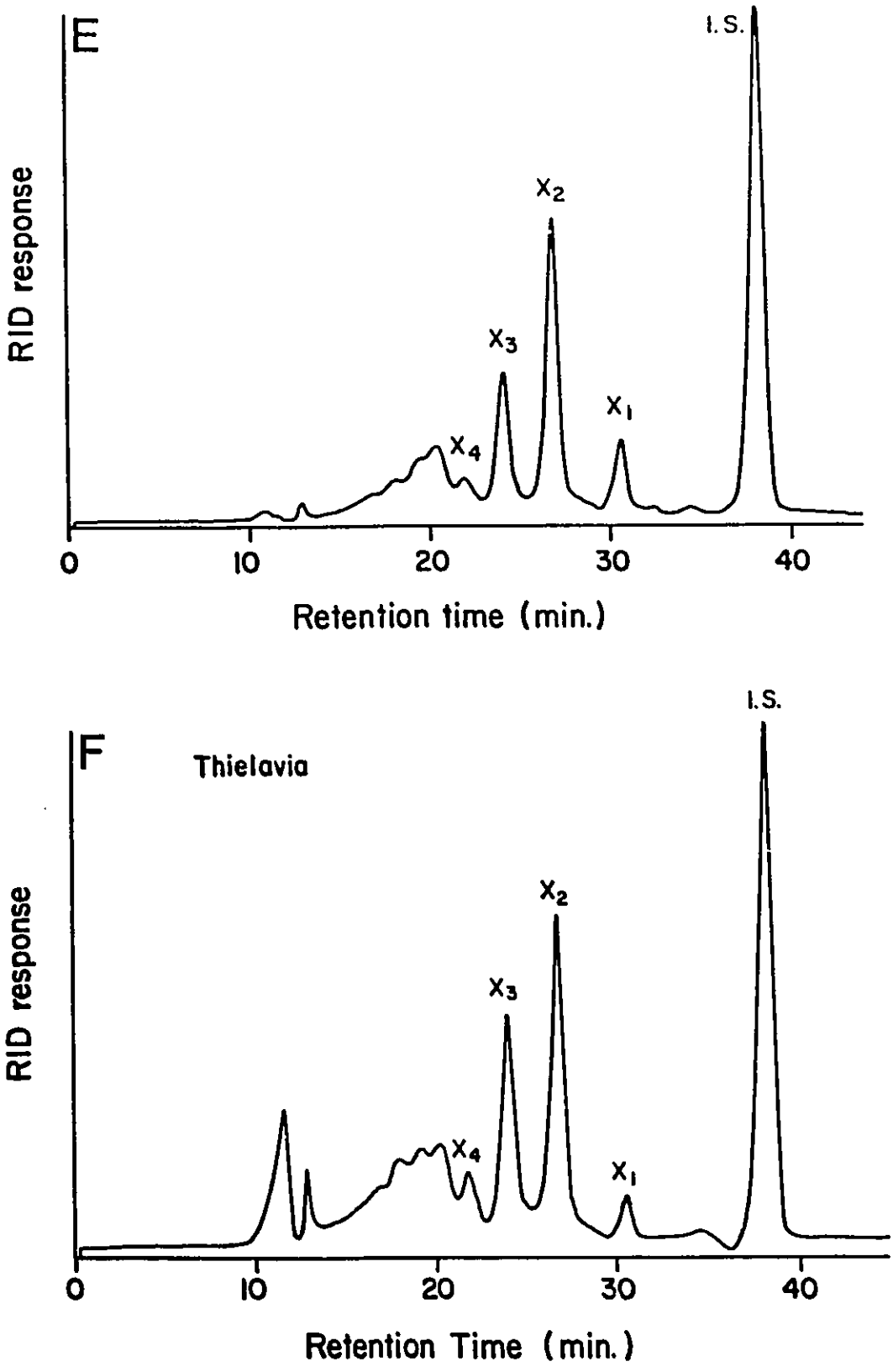

E
I.S.
X2
X3
X1
X4
RID response
0
10
20
30
40
Retention time (min.)
F
Thielavia
I.S.
X2
X3
X1
X4
RID response
0
10
20
30
40
Retention Time (min.)

Fig.45

ranging from 1.3 to 1.7. Xylose and xylotetraose were minor products which were present for all the substrates. The peaks eluting after approximately 10 to 13 minutes varied between the different xylans and corresponded to unhydrolysed substrate, as these peaks were also present in controls incubated without enzymes (not shown).

The peaks eluting between 15 and 20 minutes (just before xylotetraose) were particularly abundant in the hydrolysis of oat-spelts xylan and insoluble xylan. These unresolved peaks may have contained arabinose-substituted xylo-oligosaccharides as oat-spelts and insoluble xylans are highly substituted with arabinose. These peaks could have also contained long-chain unsubstituted xylo-oligosaccharides. These two types of xylo-oligosaccharides are very difficult to resolve by HPLC and standards are not commercially available. Their presence demonstrated a distinctive characteristic of the hydrolysis products obtained when xylanase acted on oat-spelts and insoluble xylans.

3.6.3 Short-term hydrolysis of various xylans by xylanase II and the 32-kDa xylanase:

We have shown that xylanase II from *T. terrestris* 255B and the 32-kDa xylanase from *Thermoascus crustaceus* have different modes of action (sections 3.5.9 to 3.5.11). This data suggested that these two thermophilic xylanases could display synergistic interaction when used together. An enhancement of the rate of hydrolysis would be best demonstrated under conditions where there is an ample supply of substrate and the rate is governed mostly by the enzyme activity present. Such conditions were described as enzyme-limiting conditions by Wong et al. (1986) and could be met under short-term hydrolysis conditions.

Short-term hydrolysis (30 minutes) of various xylans revealed differences between the two enzymes (Table 15). When converted to specific activity, these data showed that the two thermophilic xylanases had approximately the same specific

Table 15: Hydrolysis of various xylans by two thermophilic xylanases: measurement of initial rates (30 min) of hydrolysis (reported in specific activities):

Substrate (1%)	Xylanase II (U/mg enz.)	32-kDa Xylanase (U/mg enz.)
Oat-spelts xylan	207 (±7.4)	198 (±12)
Insoluble xylan	138 (±6.0)	67 (±4.2)
Larch wood xylan	249 (±16)	264 (±4.2)
Birch wood xylan	232 (±2.1)	239 (±3.3)
Spruce KP pulp	134 (±25)	153 (±29)
Poplar KP xylan	162 (±8.6)	172 (±15)

activity on oat-spelts xylan, birch wood xylan and poplar KP xylan while the 32-kDa xylanase had slightly higher activity on larch wood xylan and spruce KP xylan. Xylanase II had a specific activity which was twice as high as the 32-kDa xylanase on insoluble xylan. This agrees with the results of the solubilization assay (Fig. 43). However, no synergism was observed when the two thermophilic xylanases were used together in the short-term hydrolysis of the various xylans (data not shown). The rate observed when the two enzymes were present was equivalent to the arithmetic sum of the rates of the enzymes acting individually. Using enzyme-limiting conditions, Wong et al. (1986) also did not observed any significant synergism between three distinct xylanases from *Trichoderma harzianum*.

3.6.4 Long-term hydrolysis of oat-spelts xylan and poplar KP xylan by xylanase II and the 32-kDa xylanase:

Another type of positive interaction between distinct xylanases would be a cooperation to increase the extent of hydrolysis obtained after long-term hydrolysis with high enzyme loadings. These conditions have been described as substrate-limiting conditions by Wong et al. (1986) and are used to demonstrate if the combined action of the enzymes can lead to the hydrolysis of substrate that would be inaccessible if the enzymes were acting individually. We selected two substrates to determine if the combined action of the two thermophilic xylanases would cause more extensive hydrolysis than any of the two enzymes acting individually. Oat-spelts xylan was selected because it is widely available and poplar KP xylan because of its significance to the potential application of pulp pre-bleaching.

After an incubation period of 24 hours, xylanase II solubilized 68.9% of oat-spelts xylan into xylo-oligosaccharides (X_1 to X_4) while the 32-kDa xylanase solubilized 95.6% of that substrate (Table 16). Xylanase II had a larger proportion of unidentified peaks between 15 and 20 minutes of elution time (Fig. 46). Therefore, the extent of hydrolysis was significantly underestimated in the case of xylanase II.

Table 16: Long-term hydrolysis of oat-spelts xylan (20 mg) by two thermophilic xylanases:

Enzyme	Reducing sugars by S.N. (µmole)	Reducing Sugars by HPLC (µmole)	% Xylan solubilized[a]
Xylanase II (1 U)	33.7 (±3.1)	25.7 (±2.3)	68.6 (±6.3)
32-kDa xyl. (1 U)	51.8 (±2.0)	42.7 (±3.8)	95.6 (±7.7)
Xylanase II + 32-kDa xyl. (1 U + 1 U)	55.5 (±1.4)	45.9 (±2.3)	104.6 (±5.7)

Table 17: Long-term hydrolysis of poplar KP xylan (20 mg) by two thermophilic xylanases:

Enzyme	Reducing sugars by S.N. (µmole)	Reducing Sugars by HPLC (µmole)	% Xylan solubilized[a]
Xylanase II (1 U)	27.1 (±1.0)	19.0 (±1.4)	41.8 (±4.2)
32-kDa xyl. (1 U)	44.2 (±1.9)	26.6 (±3.7)	43.5 (±6.2)
Xylanase II + 32-kDa xyl. (1 U + 1 U)	48.7 (±1.3)	28.1 (±4.1)	46.1 (±6.9)

a: calculated using the HPLC data and the substrate compositions reported in appendix 1.

The solubilization of oat-spelts xylan seemed practically complete (104.6 % ±5.7) when the two enzymes were present. Although this value represented a slight improvement over the individual enzymes, it was only slightly superior to the value obtained when the 32-kDa xylanase acted alone (95.6, ±7.7). It seemed that the 32-kDa xylanase alone was essentially able to achieve complete solubilization of the oat-spelts xylan. Thus, there was no advantage in using the two xylanases together in the hydrolysis of oat-spelts xylan.

When xylanase II was used, the main hydrolysis products of oat-spelts xylan were xylobiose and xylotriose while the 32-kDa xylanase gave xylose and xylobiose as the main products (Fig. 46). The HPLC profile obtained when the two enzymes acted together was essentially the same as the one obtained when the 32-kDa xylanase acted alone. As already observed with the hydrolysis of xylo-oligosaccharides (section 3.5.10), the 32-kDa xylanase was able to cleave the substrate to smaller products than xylanase II. Apparently, the main effect of using the two enzymes together was that the 32-kDa xylanase would cleave the oligosaccharides produced by xylanase II down to smaller products.

Long-term hydrolysis of poplar KP xylan resulted in lower conversion yields than oat-spelts xylan. Xylanase II and the 32 kDa-xylanase solubilized 41.8% (±4.2) and 43.5% (±6.2), respectively, of the poplar KP xylan after a 24 hour hydrolysis (Table 17). A very slight increase in conversion yield (46.1%, ±6.9) was observed when the two thermophilic xylanases were used together. The main hydrolysis products with xylanase II were xylobiose and xylotriose (Fig. 17). Small amounts of xylose and xylotetraose were also present. The HPLC profile of the products obtained for the combined enzymes was similar to the one obtained when the 32-kDa xylanase was acting alone (Fig. 47) with xylose and xylobiose being the main products. As observed for oat-spelts xylan, the main effect of the combined action was that the products of xylanase II were further cleaved to smaller products by the

Figure 46: HPLC analysis of the products obtained from the hydrolysis of 20 mg of oat-spelts xylan by the two thermophilic xylanases: A) 1U of xylanase II, B) 1U of the 32-kDa xylanase, C) 1 U of each of the two thermophilic xylanases. I.S.= Internal Standard (erythritol).

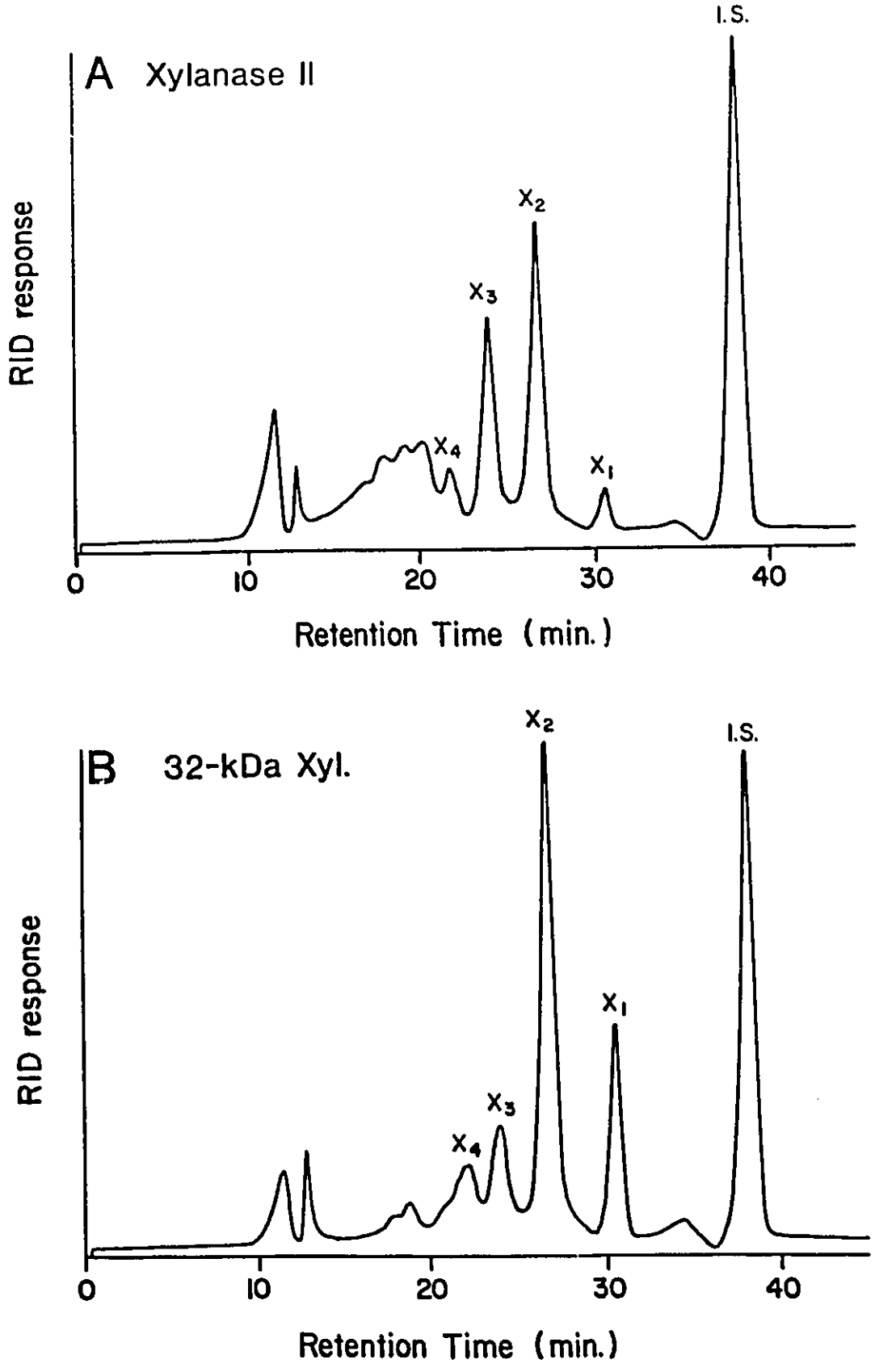

A Xylanase II
I.S.
X2
X3
X4
X1
RID response
0
10
20
30
40
Retention Time (min.)
B 32-kDa Xyl.
X2
I.S.
X1
X3
X4
RID response
0
10
20
30
40
Retention Time (min.)

Fig.46

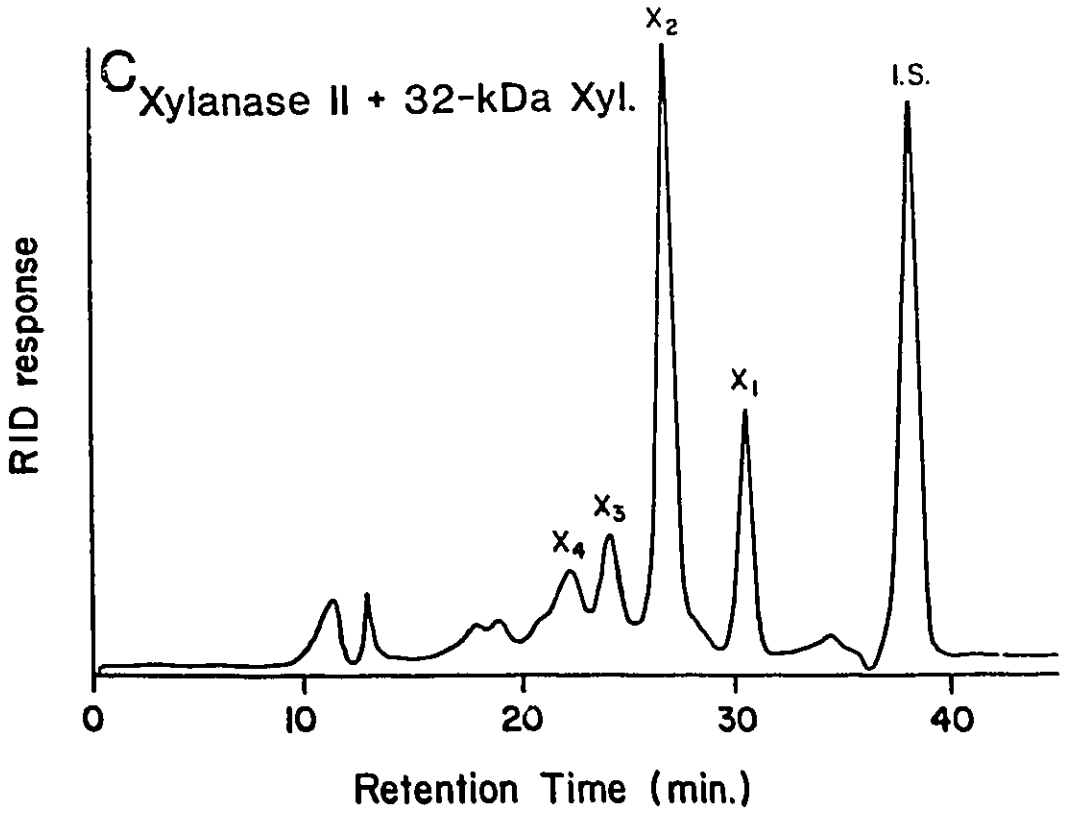

C
Xylanase II + 32-kDa Xyl.
X2
I.S.
X1
X3
X4
RID response
0
10
20
30
40
Retention Time (min.)

Fig.46

Figure 47: HPLC analysis of the products obtained from the hydrolysis of 20 mg of poplar KP xylan by the two thermophilic xylanases: A) 1 U of xylanase II, B) 1 U of the 32-kDa xylanase, C) 1 U of each of the two thermophilic xylanases. I.S.= Internal Standard (erythritol).

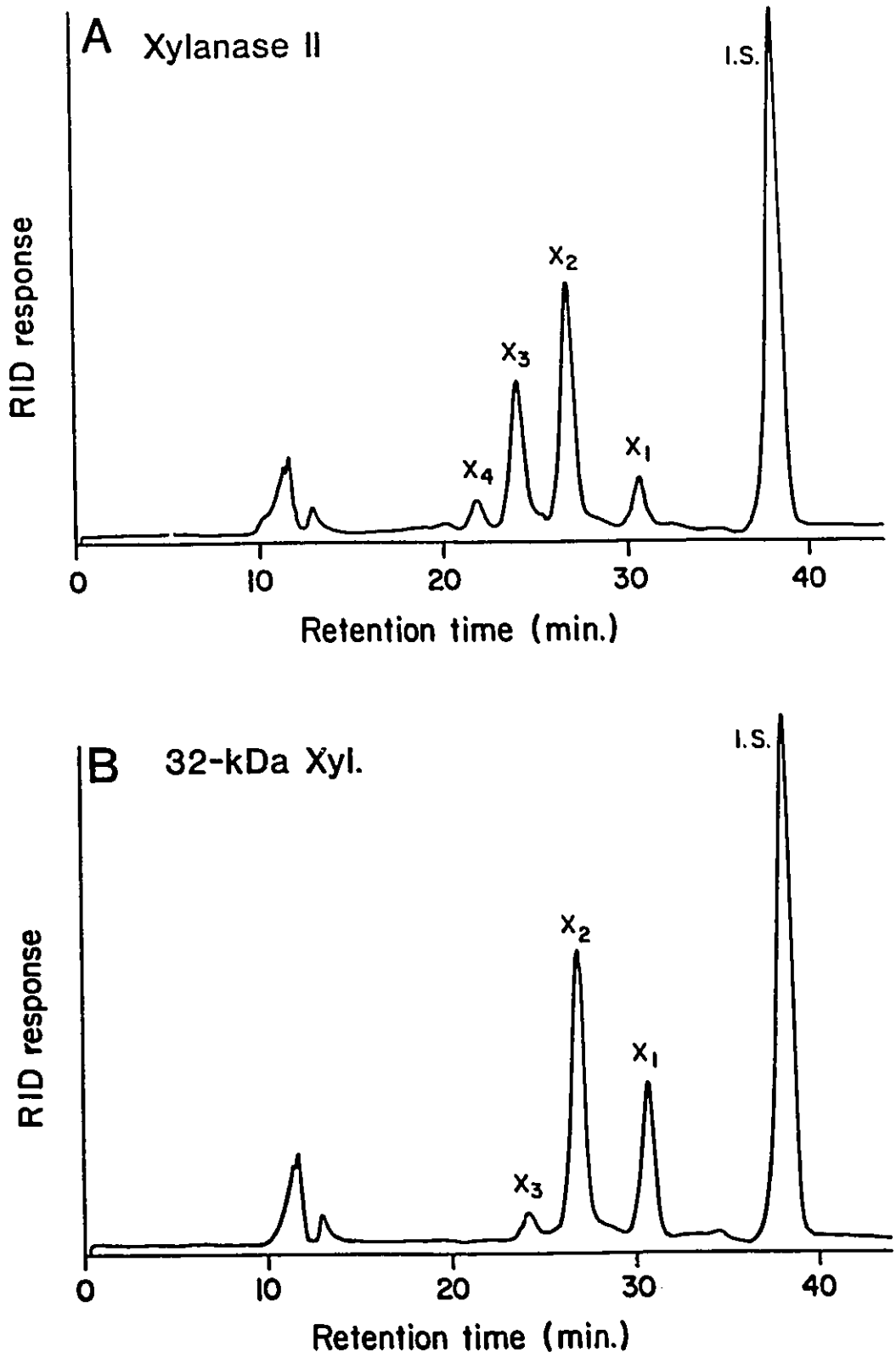

A Xylanase II
I.S.
X2
X3
X4
X1
RID response
0
10
20
30
40
Retention time (min.)
B 32-kDa Xyl.
I.S.
X2
X1
X3
RID response
0
10
20
30
40
Retention time (min.)

Fig.47

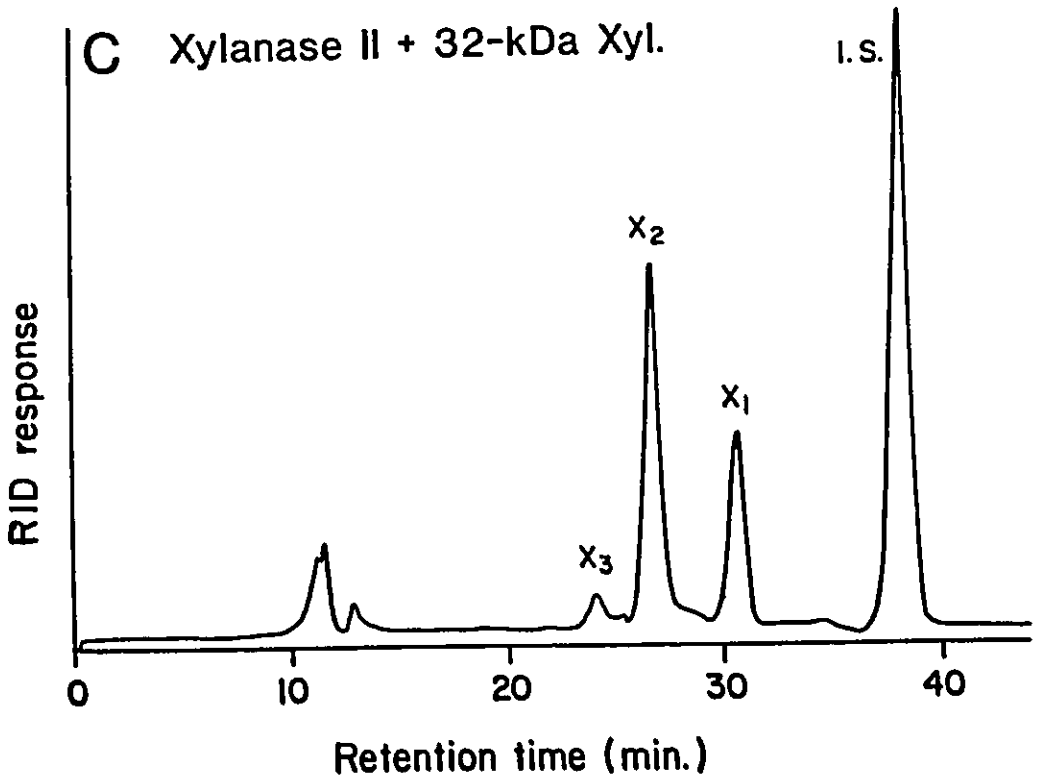

C Xylanase II + 32-kDa Xyl.
I.S.
X2
X1
X3
RID response
0
10
20
30
40
Retention time (min.)

fig.47

32-kDa xylanase.

Several groups have looked at the hydrolysis of xylan by multiple xylanases from one microorganism, however only Kluepfel et al. (1992) have shown that the enzymes in the mixture were from different families of enzymes. They reported that xylanase A (family F) from *Streptomyces lividans* No.66 cooperated with xylanase B or C (family G) only if the enzymes were added in a specific sequence. They did not report any hydrolysis yields but only HPLC profiles of the hydrolysis products. Essentially, it seems that they obtained results similar to the results we obtained with the two thermophilic xylanases i.e. that the xylanase from the F family gave hydrolysis products of lower degrees of polymerization than the xylanase from the G family. They also observed that mixtures of xylanases from different families gave similar results to the ones obtained when the xylanase from the F family was used alone.

3.6.5 Hydrolysis of residual xylan in poplar kraft pulp:

It has been observed that xylanase treatment of kraft pulps resulted in decreased chemical loadings during the bleaching of pulps to a target brightness (Viikari et al. 1986). Xylanase pre-treatment of kraft pulps could reduce the release of chloro-organics in mill effluents and could be used to develop chlorine-free bleaching processes. Most of the xylanases tested for that purpose have been isolated from mesophilic microorganisms. Zamost et al. (1991) suggested that thermostable enzymes would be more suitable for this application and suggested the use of a thermophilic xylanase from *Myceliophtera thermophila*. Perrolaz et al. (1991) reported the use of a thermostable xylanase from *Thermonospora fusca* in the pre-bleaching of birch wood kraft pulp. In this work, we tested if xylanase II and the 32-kDa xylanase were able to remove residual xylan from poplar kraft pulp.

The two thermophilic xylanases were used to treat 50 mg of poplar kraft for

3 hours, a typical residence time for a step in a bleaching sequence. Xylanase II released between 8.7 to 20.5% of the residual xylan when used at loading ranging from 0.5 U to 10 U/50 mg pulp (Fig. 48). A saturation effect was observed when the enzyme loading was increased from 1.0 U to 10.0 U as it resulted in less than a two-fold increase in products released.

Because of its limited supply, the 32-kDa was used at a concentration of only 0.5 and 1.0 U which removed 10.6% and 12.3%, respectively, of the residual xylan (Table 18). Using the two thermophilic xylanases together, we obtained slightly better removal of residual xylan than when using each of the enzymes individually (Table 18). However, the demonstration of a definite advantage when the two enzymes are used together would have required evidence that higher loadings of the individual enzymes can not compensate for the increased yield observed when the two enzymes were used together.

The HPLC analysis showed that the main hydrolysis products obtained with xylanase II were xylobiose and xylotriose with the latter sugar giving the higher peak (Fig. 49). With the 32-kDa xylanase, the main products were also xylobiose and xylotriose but in this case, xylobiose was more prominent. The profile of the products obtained with the two enzymes was similar to the profile obtained with the 32-kDa xylanase alone as observed with oat-spelts xylan and poplar KP xylan (Fig. 46 and 47). Again, the major effect of the combined action seemed to have been that the 32-kDa xylanase converted the products from xylanase II to smaller products.

No glucose was detected by HPLC analysis of the products released by the two thermophilic xylanases. Thus, both xylanases did not cause extensive attack of the cellulosic portion of the pulp which is a pre-requisite for using an enzyme in the pre-treatment of pulps. However, the integrity of the fibers would still need to be tested in order to determine if the pulp retained good hand-sheet properties following

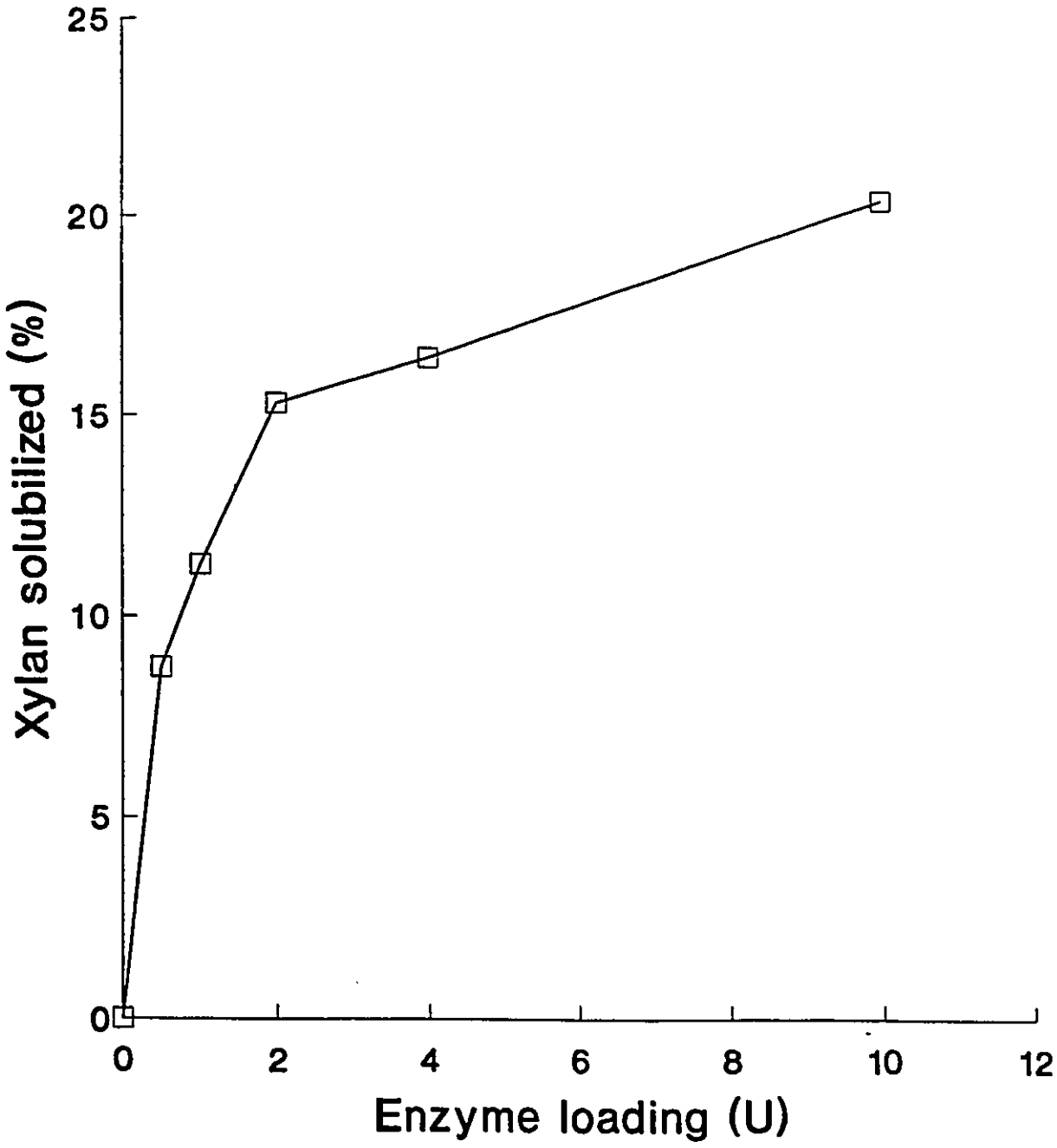


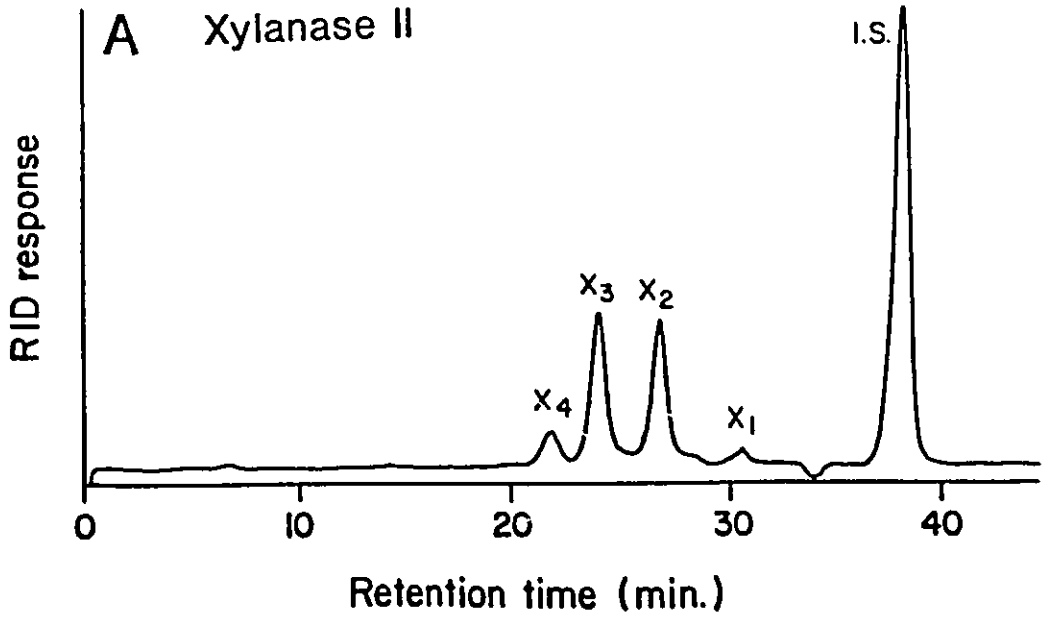
Figure 48: Hydrolysis of the residual xylan of poplar kraft pulp by increasing loadings of xylanase II. The products were quantified by HPLC and the extent of hydrolysis is expressed as the percentage of residual xylan that was solubilized.

Table 18: Removal of residual xylan of poplar kraft pulp by two thermophilic xylanases:

Enzyme		Red. Sugars by S.N. (μmole)	Red. Sugars by HPLC (μmole)	% Xylan solubilized[a]
Xylanase II	0.5 U	3.79 (±0.05)	2.03 (±0.41)	8.72 (±1.44)
	1.0 U	4.46 (±0.17)	2.79 (±0.27)	11.29 (±1.21)
	2.0 U	5.5 (±0.15)	3.93 (±0.8)	15.31 (±3.46)
32-kDa xylanase	0.5 U	4.67 (±0.06)	2.93 (±0.72)	10.63 (±2.73)
	1.0 U	5.92 (±0.24)	3.57 (±0.25)	12.29 (±0.96)
Xylanase II + 32-kDa xylanase	0.5 U + 0.5 U	5.75 (±0.2)	3.94 (±0.94)	14.24 (±3.45)
	1.0 U + 1.0 U	7.09 (±0.09)	4.83 (±0.74)	16.54 (±2.42)

a: calculated considering a residual xylan content of 15.8% in poplar kraft pulp (Dr. K. K. Y. Wong, UBC).

Figure 49: HPLC analysis of the products obtained after the treatment of 50 mg of poplar kraft for 3 hours at 60°C. A) 1 U of xylanase II, B) 1 U of the 32-kDa xylanase, C) 1 U of each of the two thermophilic xylanases. I.S.= Internal Standard (erythritol).


A
Xylanase II
I.S.
RID response
X3
X2
X4
X1
0
10
20
30
40
Retention time (min.)

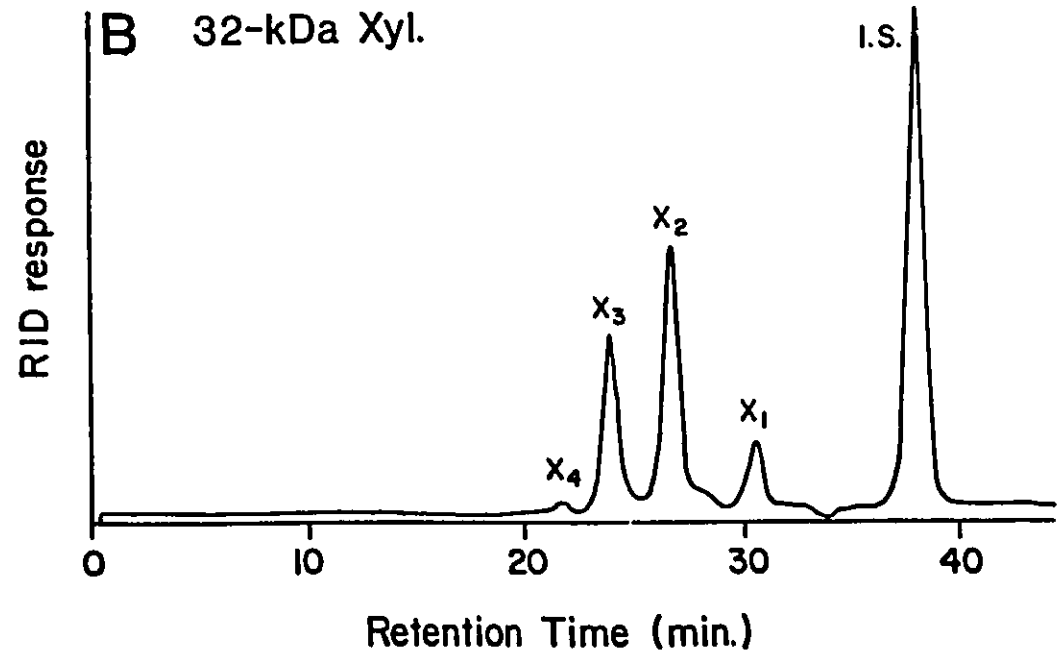

B
32-kDa Xyl.
I.S.
RID response
X2
X3
X1
X4
0
10
20
30
40
Retention Time (min.)

Fig.49

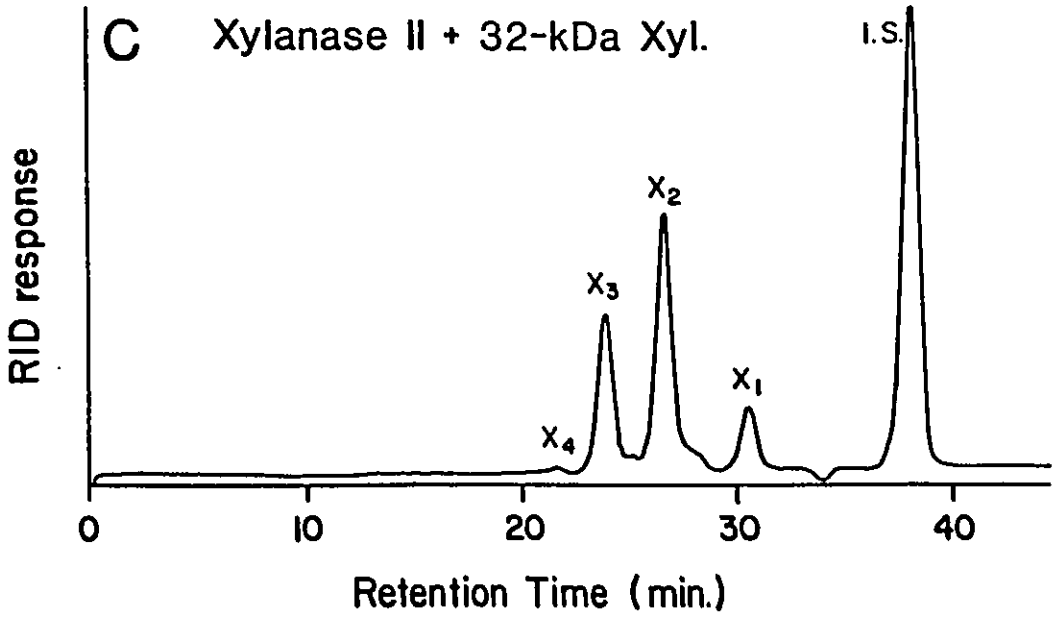

C
Xylanase II + 32-kDa Xyl.
I.S.
X2
X3
X1
X4
RID response
0
10
20
30
40
Retention Time (min.)

Fig.49

enzymatic treatment.

Although the two thermophilic xylanases showed only a low level of cooperation, their use together or individually achieved significant removal of residual xylan from poplar kraft pulp (8.7 to 20.5%). Other workers have shown that the removal of 10 to 20% of the residual xylan may be sufficient to decrease the requirement for chlorine and chlorine dioxide in pulp bleaching. Paice et al. (1992) have suggested that the xylanase pre-bleaching effect resulted primarily from depolymerization, but not necessarily solubilization, of xylan. According to their hypothesis, a decrease in xylan chain-length would result in facilitated diffusion of trapped lignin and/or in easier extractability of carbohydrate-lignin complexes. Complete solubilization of xylans is not desirable as it would unacceptably reduce pulp yields and have a detrimental effect on paper properties. Both xylanase II and the 32-kDa resulted in solubilization yields comparable to those obtained with the mesophilic xylanases currently assessed for biobleaching of pulps at the pilot-scale level.

Although xylanase II removed less residual xylan than the 32-kDa xylanase, it could still be more effective for the purpose of pre-bleaching because its higher activity on insoluble xylan (Fig. 43) suggests that it could be more effective in depolymerizing the xylan deposited in the pulp fibers. Also, xylanase II has extremely low activity on cellulosic substrates which is preferable in order to preserve the structure of the pulp fibers. The 32-kDa xylanase has low but significant activity on cellulosic substrate which could be detrimental to the properties of the pulp. However, it is more thermostable than xylanase II and it could probably be used at temperatures up to 75°C while xylanase II could not be used at temperatures above 65°C. The two thermophilic xylanases have potential advantages for use in high-temperature pre-bleaching of kraft pulps. Ultimately, their viability as pre-bleaching agents will depend on their ability to allow chemical savings while the integrity of the treated fibers is preserved in the process.

4.0 CONCLUSIONS:

We initially selected *T. terrestris* 255B because earlier work showed that it had a good combination of high enzyme productivity and thermostability (Wojtczak et al. 1987). However, several microorganisms showing increased enzyme productivity and/or thermostability have been isolated or developed since we first started our work on *T. terrestris* 255B. The amounts of enzymes produced by *T. terrestris* 255B are comparable with the levels obtained with mesophilic wild strains, however hyper-producing mutants of mesophilic fungi such as *Trichoderma* spp. have recently been developed and they secrete much higher levels of cellulases and hemicellulases. Higher thermostability can partially compensate for low productivity by allowing more efficient recycling of the enzymes. The cellulases and xylanases from *T. terrestris* 255B are 15°C to 20°C more thermostable than the enzymes produced by the hyper-producing strains that have been developed for commercial applications.

Cellulases and xylanases with very high thermostability have been obtained from extremely thermophilic bacteria (Hudson et al. 1991, Simpson et al., 1991, Bragger et al. 1989). These enzymes have optimal temperatures ranging from 80°C to 105°C and are more thermostable than any enzyme from fungal sources. However, the productivity of extremely thermophilic bacteria is generally very low. Currently, fungal strains such as *T. terrestris* 255B are probably easier to modify through mutation programs if large amounts of enzymes are required to be stable in the range from 60°C to 75°C for any potential commercial applications.

The *T. terrestris* 255B cellulase system contained multiple forms of both cellobiohydrolases and endoglucanases which were difficult to purify and characterized because of possible enzyme-enzyme interactions. Many distinct enzymes are needed to achieve efficient cellulose hydrolysis and the complexity of cellulase systems is probably a reflection of the complexity of the mechanism required to hydrolyze cellulose. We found that the *T. terrestris* 255B cellulase system contains at

least 2 distinct cellobiohydrolases. We also observed that *T. terrestris* 255B secreted two major and about 5 minor endoglucanase components according to the use of native-PAGE (Fig. 14). Over 10 endoglucanase bands were detected using IEF (Fig. 17). Various factors such as proteolysis and different extents of glycosylation have been suggested as causes for the multiplicity of endoglucanases (Goyal et al. 1991). Native-PAGE on gradient gels showed that some endoglucanases had apparent high molecular weights suggesting a possible organization of the components in multi-enzyme complexes. Some of the minor components might correspond to dissociated sub-units but the data available is insufficient to confirm this suggestion.

The structure and mode of action of xylanase II became the major focus of our work after we observed that this enzyme could be isolated relatively easily without any contaminating cellulase activity. The high specificity of xylanase II for xylan hydrolysis, its low molecular mass (25.7 kDa) and its high content in certain amino acids suggested that it belonged to a family of LMW xylanases designated the G family by Gilkes et al. (1991b). Partial amino acid sequencing confirmed the relationship of xylanase II to the G family, with high homology being observed for two fragments (fragment B and CNBr3) and the conservation of a Glu residue that was suggested to participate in the catalysis carried out by this group of enzymes (Wakarchuk et al. 1992, Katsube et al. 1990).

Although xylanase II from *T. terrestris* 255B is not the most thermostable xylanase which has been reported to date, it was still significantly (10-20 °C) more thermostable than the mesophilic xylanases shown to belong to the G family (Table 8). Thus, it could serve as a model to demonstrate which structural features can increase the thermostability of a protein. We showed that xylanase II had a disulfide bond and have discussed the possibility that it might be responsible for the higher thermostability of xylanase II. Other possible factors include additional hydrogen bonding, extra salt bridges and hydrophobic interactions. However, three-dimensional

structural data would be required to demonstrate the involvement of these structural features.

According to its physico-chemical properties and to its similarity to the enzyme sequenced by Srinivasa et al. (1991), the 32-kDa xylanase from *Thermoascus crustaceus* appeared to belong to the F family of β-1,4-glycanases defined by Gilkes et al. (1991b). We showed that xylanase II belonged to the G family and thus it seems that the two thermophilic xylanases belong to different categories of enzymes based on both their structure and their mode of action. Wong et al. (1988) suggested that different types of xylanases might allow a microorganism to use a wider range of substrates in nature. In this work we studied two thermophilic xylanases from different microorganisms, however it is possible that *T. terrestris* 255B also possesses a xylanase that belongs to the F family as we showed that *T. terrestris* 255B produced at least one other major xylanase component (Fig. 18).

Under the experimental conditions used in this work, no strong synergism or cooperation was observed between xylanase II and the 32-kDa xylanase. This data could imply that these two thermophilic xylanases play similar roles in their respective organism. However, enzymes from both the F and the G families have been shown to be produced by the same organism (Kluepfel et al. 1992) and it suggests that these enzymes have specific roles. In nature, the composition and accessibility of xylans probably display more diversity than the isolated xylans used in this study. For instance, hardwood xylans are often acetylated at the C2 or C3 positions of the xylose units. The xylans used in this work were deacetylated during their preparation and thus we could not determine if the two thermophilic xylanases differed in their ability to attack acetylated xylans. Other conditions that we could not reproduce were the various types of associations that xylans could have with other polymers in plant cell walls. Thus, it is still possible that xylanases from the F and the G families cooperate in nature to efficiently attack a wider range of

substrates.

Previously, it has been shown that xylanase pre-treatment could reduce the amounts of chlorine required to bleach kraft pulp and/or help to develop chlorine-free bleaching processes. This would result in the release of less chloro-organics in mill effluents. We showed that both xylanase II and the 32-kDa xylanase were able to remove significant amounts of residual xylan in a poplar kraft pulp. Thus, the two thermophilic xylanases are potential candidates for use in high-temperature pre-bleaching of pulps. However, the effects on the integrity of the treated pulps and the chemical savings will first have to be determined at the laboratory scale before their use in industrial processes can be considered. Other work that could be performed would include studies of the mechanisms of hydrolysis of the residual xylan in pulps and how it leads to enhanced extractibility of the lignin-carbohydrate complexes. This would involve extensive characterization of the xylan structure and the determination of the actual sites attacked by the xylanases.

Additional fundamental work that could be done on xylanase II would include the cloning of its gene in order to obtain the complete sequence and compare it with the mesophilic low molecular mass xylanases. Also it would be interesting to crystallize this enzyme in order to obtain the complete three-dimensional structure. This data would allow a more comprehensive analysis of the structural elements that may confer a higher thermostability to xylanase II.

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APPENDIX 1:

Sugar composition of the xylans used in this work:

Type of Xylan	Glucose (%)	Xylose (%)	Galac-tose (%)	Arabi-nose (%)	Mannose (%)	Total (%)
Larch wood	0.1	47.2	0.7	0	1.5	49.5
Oat-spelts	0.4	56.9	0.2	7.2	0	64.7
Insoluble (oat-spelts)	0.3	67.7	0	7.6	0	75.6
4-O-methyl-glucuronoxylan (from birch wood)	0.9	47.8	0.9	0	2.2	51.7
Xylan from Poplar Kraft Pulp	0.9	64.7	0.2	0	2.6	68.2
Xylan from Spruce Kraft Pulp	6.0	35.8	1.0	Trace	21.0	68.9

Rem.: - Data provided by Dr. K. K. Y. Wong (Chair of Forest Products Biotechnology, UBC).

- The residual lignin was not measured and this factor partially accounts for a recovery yield which is less than 100%.

- The analysis of these substrates involved a hydrolysis step in presence of 4 N trifluoroacetic acid. This step probably caused sugar decomposition and this factor could not be corrected because no standards were available. Consequently the xylan content in the substrates were probably underestimated.

APPENDIX 2:

The following publications were based on the work presented in this thesis:

Gilbert, M., Breuil, C. and J. N. Saddler 1992. Characterization of the enzymes present in the cellulase system of *Thielavia terrestris* 255B. Biores. Technol. 39:147-154.

Gilbert, M., Breuil, C., Yaguchi, M. and J. N. Saddler 1992. Purification and characterization of a xylanase from the thermophilic ascomycete *Thielavia terrestris* 255B. Appl. Biochem. Biotechnol. 34:247-259.

Manuscript in preparation:

Gilbert, M., Wong, K. K. Y., Breuil, C. and J. N. Saddler. Hydrolysis of various xylans by a combination of two thermophilic xylanases. To submit to the journal: Applied Microbiology and Biotechnology.