

A study of the functional interactions among xylanases purified
from Trichoderma harzianum.

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ABSTRACT

The biodegradation of xylan is achieved by extracellular enzymes produced mainly by fungi and bacteria. The fungus, Trichoderma harzianum, produces at least 5 xylanases and 2 xylosidases. Three of the xylanases have been purified and they show distinct physico-chemical and hydrolytic properties. The 3 enzymes have molecular weights of 20 000, 22 000 and 29 000 daltons and pI's of 9.3, 8.5 and 9.4, respectively. They have different stabilities and temperature optima. They are highly specific xylanases which have little activity on other substrates. None of them releases the arabinose substituent from arabinoxylan or cleaves xylobiose. Other enzymes in the culture filtrate are responsible for the hydrolysis of cellulose, glucans, cellobiose, arabinosyl substituents and xylobiose. The shortest oligosaccharide hydrolyzed by the 20 000, 22 000 and 29 000 dalton xylanase is xylotetraose, xylotriose and xylotriose, respectively. All 3 enzymes are produced by all the clones of T. harzianum which have been examined. Their amino acid compositions indicate that they are distinct gene products.

The importance of this multiplicity in xylanases was examined by studying the hydrolysis of different xylan preparations. Complementation of the characterized enzymes was observed in the hydrolysis of isolated aspen xylans but not oat arabinoxylan. The 3 enzymes together increased the substrate limiting hydrolysis of acetylated aspen xylan and deacetylated aspen xylan. Combinations of any 2 xylanases also increased the hydrolysis of acetylated aspen xylan. Although aspen sawdust was not hydrolyzed, cooperation among the xylanases in the hydrolysis of xylan in aspen holocellulose was considerably greater than that observed in the hydrolysis of isolated xylans. The combination of all 3 xylanases gave the highest hydrolysis of aspen holocellulose. Apparently, xylanase multiplicity is required for effective hydrolysis of xylan in the lignocellulosic complex. Therefore, the characterized xylanases are not redundant enzymes because each enzyme contribute significantly and uniquely to the xylanolytic system.

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LIST OF SOME ABBREVIATIONS AND UNITS

A-X3	arabinosyl-xylotriose
C-2	carbon 2 of a monosaccharide residue
C-3	carbon 3
CM-	carboxymethyl
CMC	carboxymethyl-cellulose
DMSO	dimethyl sulfoxide
equiv.	equivalents
HPLC	high pressure liquid chromatography
IEF	isoelectric focusing
M.W.	molecular weight
SDS	sodium dodecyl sulfate
SDS-PAGE	polyacrylamide gel electrophoresis with SDS
TEMED	N,N,N',N'-Tetramethylethylenediamine
kD	kilodalton (thousand daltons)
p	unit of xylanase activity defined locally for experiments dealing with enzyme limiting hydrolysis
q	unit of xylanase activity defined locally for experiments dealing with substrate limiting hydrolysis
U	activity unit measured at 50 C (μ mol equiv. of products per min)
u	activity unit measured at 28 C

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I. INTRODUCTION

Plants are the major primary producers on earth. They fix carbon which is directly or indirectly exploited as an energy source by almost all other organisms. Much of the carbon is fixed into their cell walls as lignocellulose to produce a major carbon reservoir. Microorganisms, mainly fungi and bacteria, can utilize lignocellulose and thus prevent a permanent loss of carbon from the carbon cycle.

Since lignocellulose is relatively resistant to degradation, it has been used for structural and other purposes. Man has been ingenious in using this biomass resource. We have used lignocellulose for building and making countless artifacts including shelter, clothes, paper, artwork, fire, etc. Today, we are expanding the use of lignocellulose by exploiting the capabilities of microorganisms. Some degradation products from lignocellulose can be used for the production of single-cell protein, gaseous and liquid fuels, and other food and chemical products (Han 1983, Kirk 1983, Ng et al. 1983, Thompson 1983).

Fundamental questions in biomass exploitation, as well as in biomass turnover in nature, include the mechanism of lignocellulose biodegradation. Microorganisms produce a spectrum of enzymes for degrading lignocellulosic materials (Dekker & Richards 1976, Bisaria & Ghose 1981, Eriksson 1981, Janshekar & Fiechter 1983, Kirk 1983, Ladisch et al. 1983). The complexity of the substrate requires these enzymes to act together to

completely break down lignocellulose. Clearly, lignocellulose biodegradation is interesting from the biological, biochemical and biotechnological viewpoints.

Lignocellulose consists of 3 components: cellulose (an insoluble β -1,4 homopolymer of glucose), hemicellulose (non-cellulosic carbohydrates including galactans, glucans, mannans and xylans) and lignin (a complex aromatic polymer). To date, the cellulase system has been studied to the greatest extent. There is also great interest in the xylanolytic system because xylan makes up to 35% of wood (Timell 1967).

Xylan is a heteropolymer consisting mainly of xylose residues. Some degradative organisms produce several enzymes to attack this substrate. I am interested in determining whether this multiplicity of xylanolytic enzymes serve a functional role in enabling these organisms to utilize the substrate more effectively. In this introduction, the complexity of the substrate and the xylanolytic system is discussed as well as possible enzyme-substrate and enzyme-enzyme interactions.

I.1. THE STRUCTURE OF XYLAN

(1 $\rightarrow$ 4)- β -D-xylan is the major non-cellulosic polysaccharide in biomass. It is a heteropolymer identified by its backbone of β -1,4 linked D-xylose residues. The pattern and composition of

branches in an isolated xylan are dependent on its source and the method of its extraction (Timell 1967, Whistler & Richards 1970, Dekker 1979, Thompson 1983, Wilkie 1983). In this section, an introduction to the structure of two wood xylans is presented and the form of xylan in situ and its accessibility to enzyme hydrolysis are discussed.

I.1.1. General structure of the two major forms of wood xylan

Wood xylans are acidic polymers found in two major forms: the O-acetyl-4-O-methylglucurono-xylan of hardwoods and the arabino-4-O-methylglucurono-xylan of softwoods (Timell 1967). Typical segments of these two xylans are shown on Figure 1.1. Xylans from other sources may have different degrees of substitution for each type of substituent, different degrees of polymerization and complex branches containing several residues.

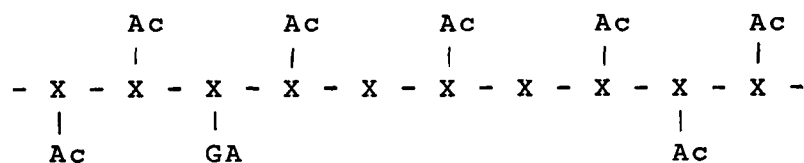
The backbone of hardwood xylan consists of approximately 200 xylose residues and it may not be completely linear.

Approximately 70% of the xylose units are acetylated, usually at the carbon-3 position but also at C-2. Single 4-O-methyl- α -D-glucuronic acid units are distributed randomly along the backbone at a frequency of one per 10 xylose units. These acidic groups are attached to C-2 of nonacetylated xylose residues.

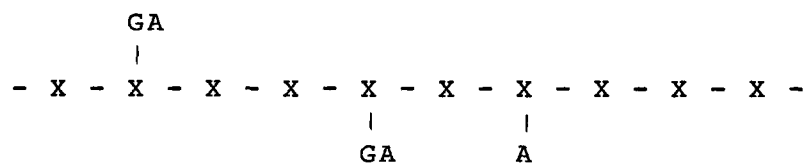
Softwood xylan differs from hardwood xylan in several ways. It is more acidic by having one acidic unit per 5-6 xylose units.

Figure 1.1. Typical segments of a hardwood xylan (a) and a softwood arabinoxylan (b) (Timell 1967).

(a) O-acetyl-4-O-methylglucurono-xylan



(b) arabino-4-O-methylglucurono-xylan



- X - X two xylose residues linked β -1,4
- X - Ac xylose acetylated at C-3 (occasionally at C-2)
- X - GA C-2 of xylose with 4-O-methyl- α -D-glucuronic acid
- X - A C-3 of xylose substituted with arabinose residue

The backbone is not acetylated but instead has L-arabinose substituents. Single arabinose units are linked to C-3 of non-acidic xylose residues and occur at a frequency of one per 8-9 xylose residues.

I.1.2. Integration of xylan in lignocellulose

The composition of lignocellulose varies among species (Fengel & Wegener 1983), among tissues and growth stages of a plant (Wilkie 1979, Brice & Morrison 1982) and across plant cell wall (Timell 1967, Whistler & Richards 1970, Cowling & Kirk 1976). Therefore, the structure of lignocellulose can not be completely defined. It is generally considered as cellulosic fibres cemented together by non-cellulosic polysaccharides linked to lignin.

Xylan is an integral part of lignocellulose and appears to be an important linkage between the polysaccharide and the lignin components. It may be chemically associated with other polysaccharides, particularly pectic materials, because oligosaccharides which contain xylose, rhamnose and galacturonic acid residues have been isolated from some xylans (Shimizu et al. 1976, Ishihara et al. 1978). In mesta fibre (Hibiscus cannabinus), xylan is linked to lignin by ester bonds involving the uronic acid substituents (Das et al. 1984). It is clear that xylan in situ is not an isolated polysaccharide because it is physically and chemically associated with other lignocellulosic

components.

I.1.3. The effect of structure on enzyme hydrolysis

Structure affects xylanase action on lignocellulose at several levels. At the most holistic level, xylan hydrolysis is limited by the association of lignin to carbohydrates. Brice & Morrison (1982) found that with increasing lignin content, lignin-hemicellulose complexes are more resistant to rumen hemicellulase action. Gressel et al. (1983) demonstrated that artificial lignification of cellulose can hinder cellulase action. A similar effect on xylanase action can be expected. The lignin barrier probably protects hydrolysis sites by steric hindrance. Several pretreatment processes have been developed to make carbohydrates in wood more accessible to enzyme hydrolysis (Millett et al. 1976, Chang et al. 1981) and they include the steaming process used at Forintek. In nature, the lignin barrier is disrupted by lignolytic enzymes (Eriksson 1981, Janshekar & Fiechter 1983, Kirk 1983).

Removal of the lignin does not eliminate all the gross physical barriers to enzyme hydrolysis. Since lignocellulose is insoluble, enzymes must penetrate the substrate to reach internal hydrolysis sites. Since enzymes have a physical size, an important factor is the pore sizes of the capillaries which penetrate the substrate (Cowling & Kirk 1976). Grethlein (1985) reported a positive correlation between cellulose hydrolysis and pore sizes.

Pore size should also be a factor in the penetration of xylanases.

The association of the wood carbohydrates can also hinder hydrolysis of specific polysaccharides by their respective hydrolases. Preferential enzyme hydrolysis of xylan in holocellulose has been reported by Boutelje et al. (1971), Sinner et al. (1976, 1979) and Paice & Jurasek (1984), but not all the xylan was hydrolyzed by the purified xylanases. Sinner et al. (1976) found that in beechwood holocellulose, the hydrolysis of cellulose by a cellulase was increased by the addition of a xylanase. A similar response to the addition of a mannanase was reported by Sinner et al. (1979). The hydrolysis of xylan by the xylanase did not increase appreciably with the addition of the cellulase or the mannanase. This information indicates that the hemicellulases act cooperatively with cellulase in the hydrolysis of cellulose in holocellulose.

The fine structures which may prevent complete xylan hydrolysis are the branches of this heteropolymer. I am aware of only one organism which produces xylanases capable of cleaving the acidic group off xylan. Shimizu et al. (1976) and Ishihara et al. (1978) identified free 4-O-methyl-D-glucuronic acid in the hydrolyzate of shirakaba xylan produced by two xylanase from Tyromyces palustris. In other cases, the acidic substituents remain on oligosaccharides which are not cleaved further (Table 1.1). Timell (1962), Kubackova et al. (1979) and Comtat &

Table 1.1. The acidic xylooligosaccharides released by purified xylanases in the hydrolysis of xylan.

reference	organism	enzyme	shortest sugar *	
			neutral	acidic
Sinner & Dietrichs (1976)	<u>Aspergillus niger</u>	-	X1	GA-X3
	<u>Oxiporus sp.</u>	A1	X1	GA-X3
		A2	X1	GA-X3
		B	X1	GA-X3
	<u>Trichoderma viride</u>	-	X1	GA-X2
Shimizu et al. (1976)	<u>Tyromyces palustris</u> #	F1-2	X1	GA-X2
		FII-3-2	X1	GA-X1
Ishihara et al. (1978)				
Kubackova et al. (1979)	<u>Trametes hirsuta</u>	-	X2	GA-X3

* X = xylose, number = degree of polymerization, GA = methyl-glucuronic acid substituent

both xylanases of T. palustris cleave the acidic group off xylan

Joseleau (1981) found that the acidic substituents were always on the non-reducing end of the acidic oligosaccharides isolated from hydrolyzates. Apparently, the xylosidic linkage on the non-reducing side of the substituent is more accessible than that on the reducing side.

The arabinose substituents of arabinoxylan can also protect some xylose linkages. The xylanases have been traditionally grouped into those which can release the arabinose from arabinoxylan and those which can not (Dekker & Richards 1976, Reilly 1981). The latter type of enzyme is more abundant and these xylanases can not cleave the shorter xylooligosaccharides which contain arabinose (Table 1.2). Some arabinose-releasing xylanases also yield arabinose containing oligosaccharides but not the shortest ones (Table 1.2) and therefore appear to prefer removing the arabinose branch before further hydrolysis of short oligosaccharides.

Dekker & Richards (1975b) and Frederick et al. (1985) concluded that substituted regions of xylan contain the preferred hydrolysis sites of a xylanase from Ceratocystis paradoxa and two xylanases from Aspergillus niger respectively, but their data are not totally convincing. Dekker & Richards isolated the insoluble fraction of the xylan hydrolyzate and found it to be less substituted than the original substrate. However, since solubility is positively correlated with the degree of substitution, their conclusion may be premature. Frederick et al. based their conclusion on the observation that enzyme attack

Table 1.2. The shortest neutral xylooligosaccharides released by purified xylanases in the hydrolysis of arabinoxylan.

reference	organism	enzyme	release of arabinose	shortest xylose	sugar * arabinose
Tsujisaka et al. (1971)	<u>Aspergillus</u> <u>niger</u>	I II III	N Y Y	X1 X1 X1	A-X2 A-X5 A-X3
Iwamoto et al. (1973b)	<u>A. niger</u>	I II	Y N	X2 X1	none A-X3
Dekker et al. (1975)	<u>Cephalosporium</u> <u>sacchari</u>	HC-I HC-II	Y N	X1 X1	A-X2 A-X3
	<u>Ceratocystis</u> <u>paradoxa</u>	HC-I HC-II	Y N	X1 X1	A-X4 A-X2
John et al. (1979)	<u>A. niger</u>	IA & IB IIB	Y Y	X1 X1	A-X3 A-X4
Kusakabe et al. (1983)	<u>Streptomyces</u> <u>sp.</u>	-	ns	ns	A-X1

* shortest sugar released excluding monomeric arabinose
(X = xylose, number = degree of polymerization, A =
arabinose substituent)

ns not specified

was lower in a debranched xylan. This substrate was the insoluble fraction of xylan which had its arabinosyl substituents removed by a mild acid hydrolysis (Frederick et al. 1981). Their conclusion is not totally convincing because characterization of this xylan fraction was not provided. Nevertheless, there is adequate information in the literature which indicate that the branches of xylan do affect hydrolysis by some enzymes. It is clear that the xylosidic linkages in xylan are not all equivalent and equally accessible to enzymes.

I.2. XYLANOLYTIC ENZYMES

Enzymes which degrade xylan occur in organisms ranging from bacteria to vertebrates (mucosal cells of gut) to plants (germinating seeds) (Dekker 1985). By far, the most abundant producers of these enzymes are degradative organisms such as fungi, bacteria and yeasts. The xylanolytic system can be subdivided into the xylanases and the β -xylosidases, the hydrolytic ability of the former decreases with decreasing chain-length of xylooligosaccharides and that of the latter increases. I will deal mainly with the xylanases and they have been extensively reviewed by Dekker & Richards (1976), Reilly (1981), Woodward (1984) and Dekker (1985). My purpose here is not to provide a review of existing knowledge, but rather to discuss two interesting points: the substrate specificity of xylanases and the multiplicity of xylanases in microorganisms.

I.2.1. Substrate specificity of xylanases

Cellulases with xylanolytic activity have been reported by Toda et al. (1971), Shikata & Nisizawa (1975) and Kanda et al. (1976). Toda et al. purified a component, from a commercial cellulase preparation of Trichoderma viride, which hydrolyzed carboxymethyl-cellulose (CMC) and xylan. These two activities could not be separated by adsorption on alkali swollen cellulose, adsorption on xylan, gel filtration, polyacrylamide gel electrophoresis, starch-zone electrophoresis or cellulose acetate film electrophoresis. They had similar patterns of stability to temperature and pH. Kinetic analysis indicated that CMC and xylan competed for the same active site. The evidence indicates that the same enzyme is responsible for both the cellulolytic and the xylanolytic activities.

Almost identical results were found in an enzyme isolated from Irpex lacteus by Kanda et al. This enzyme was shown to be homogeneous by 3 types of electrophoresis and ultracentrifugation. Its xylanolytic and cellulolytic activities had the same optimal pH and temperature as well as similar stabilities. Again, CMC and xylan appeared to compete for the same active site. Similar kinetic analysis showed that CMC, xylan and cellobiose compete for the same active site in an enzyme isolated from T. viride by Shikata & Nisizawa.

Table 1.3 lists reports of xylanases active on other substrates. We must be careful when we designate such enzymes as xylanases. Perhaps these enzymes play a critical role in degrading another substrate so that their xylanolytic activity serves as a secondary and redundant function. In such cases, the enzymes do not serve as xylanases for their respective organisms. On the other hand, we must also be concerned with the purity of the enzyme and substrate preparations. It is also a question of degree since we can only assess the importance of secondary activities by their magnitude relative to the primary activity of the enzyme. Table 1.3 shows the cases where the hydrolysis of a secondary substrate was reported to be greater than 10% that of the primary substrate. In fact, the cellulase and xylanase activity are nearly equivalent for enzyme III of Phoma hibernica (Urbanek et al. 1978) and enzyme D-1 of T. viride (Shikata & Nisizawa 1975).

The situation is just as complicated for β -xylosidase. Wide specificity in fungal β -glycosidases has been reported by Deleyn et al. (1978), De Bruyne et al. (1979), Peciarova & Biely (1982), Ishihara & Shimizu (1983), Tavobilov et al. (1984) and Uziie et al. (1985). Another problem arises from the use of p-nitrophenyl- β -xylopyranoside as the substrate for assaying xylobiase. This assay is inadequate because Biely et al. (1980) found an enzyme in Cryptococcus albidus which hydrolyzed p-nitrophenyl- β -xylopyranoside but not xylobiose. As a result, reported β -xylosidase activities may be due to glycosidases with wide specificity which may or may not be able to cleave

Table 1.3. Purified enzymes reported to be active on xylan as well as other substrates.

organism	enzyme	substrates #	reference
<u>Aspergillus niger</u>	IV	mannan, xylan *	Fukumoto et al. (1970)
<u>Aspergillus niger</u>	-	xylan, CMC	Sinner & Dietrichs (1975)
<u>Aspergillus niger</u>	-	glucans, celluloses, xylan	Hurst et al. (1978)
<u>Aspergillus niger</u>	IA & IB	xylan, cellulose, glucan	John et al. (1979)
<u>Aspergillus niger</u>	4	xylan, pectin *, celluloses	Frederick et al. (1981)
	7I & 7II	xylan, celluloses	Frederick et al. (1985)
	8	xylan, CMC *, glucomannan *, galactan, nitrophenyl- glucoside	Fournier et al. (1985)
	9-13B	xylan, celluloses	Shei et al. (1985)
	-	celluloses, xylan *	Uchino & Nakane (1981)
<u>Cephalosporium sacchari</u>	HCI	xylan, glucan	Dekker et al. (1975)
<u>Ceratocystis paradoxa</u>	HCI	xylan, pectin, araban	Dekker & Richards (1975a)
	HCII	xylan, CMC, sucrose	Dekker & Richards (1975b)

(continued)

<u>Fusarium sp.</u>		xylan, glucomannan *	Wankhede et al. (1981)
<u>Gliocladium virens</u>	-	xylan, celluloses, starch	Takahashi & Kutsumi (1979)
<u>Irpex lacteus</u>	F-1	CMC, xylan *	Kanda et al. (1976)
<u>Oxiporus sp.</u>	A1	xylan, mannans	Sinner & Dietrichs (1975)
<u>Phoma hibernica</u>	III	celluloses, xylan *, mannans, glycosides	Urbanek et al. (1978)
<u>Streptomyces exfoliatus</u>	Ib	xylan, cellulose	Sreenath & Joseph (1982)
	Id	xylan, inulin *, pectin *, cellulose *	
<u>Trichoderma harzianum</u>	20 kD & 22 kD	xylan, CMC	Tan et al. (1985b)
<u>Trichoderma reesei</u>	-	cellobiose, xylan *, celluloses	Sprey & Lambert (1983)
<u>Trichoderma viride</u>	F-1 & F-2	CMC, xylan	Toda et al. (1971)
<u>Trichoderma viride</u>	D-1	CMC, xylan *, cellobiose *	Shikata & Nisizawa (1975)
<u>Trichoderma viride</u>	-	xylan, CMC	Sinner & Dietrichs (1975)

the primary substrate is listed first
CMC = carboxymethyl-cellulose

* hydrolysis of secondary substrate was estimated to be greater
than 10% that of the primary substrate

xylobiose.

It is possible that some microorganisms have developed multi-purpose enzymes for biomass utilization. Such enzymes may conserve resources by reducing the number of enzymes required for complete degradation of lignocellulose. Wide specificity may be a strategy used for degrading complex structures including xylan. For example, arabinoxylan could be degraded by a xylanase with some arabinanase activity (Dekker & Richards 1975a).

1.2.2. Multiplicity of xylanases

Xylanases with many different hydrolytic and physico-chemical characteristics have been isolated from many organisms. Some organisms also produce several xylanases and some of these xylanases have been purified (Table 1.4 & 1.5). The interesting biological question is whether this multiplicity of xylanase serves a functional purpose by enabling the organism to utilize the substrate more effectively. Can each xylanase hydrolyze linkages inaccessible to the other xylanases? Are they redundant components of the organism's xylanolytic system which have arisen during the course of evolution? These questions are also relevant in the biotechnological exploitation of these organisms because we need to know whether the entire xylanolytic system is required for effective hydrolysis of biomass.

The multiplicity of xylanases has been studied most extensively

Table 1.4. Multiple xylanases isolated from Aspergillus niger. The data (ns = not specified) include the ability to release arabinose in arabinoxylan hydrolysis (Y = yes, N = no), the smallest xylooligosaccharide hydrolyzed and the smallest xylooligosaccharide released in xylan hydrolysis (number = degree of polymerization) by the enzyme.

reference	enzyme	release of arabinose	smallest sugar cleaved	smallest sugar released
Iwamoto et al. (1973b)	I	Y	X3	X2
	II	N	X3	X1
Tsujisaka et al. (1971)	I	N	X3 *	X1
Takenishi & Tsujisaka	II	Y	X3	X1
(1975)	III	Y	X3	X1
Rodionova et al. (1977)	24 kD	Y	X3	X1
Gorbacheva & Rodionova	41 kD	ns	ns	ns
(1977)				
John et al. (1979)	IA	Y	ns	X1
	IB	Y	ns	X1
	IIA	Y	ns	X1
	IIB	Y	ns	X1
	IID	Y	ns	X1
Frederick et al.	4	N	X3	X1
(1981, 1985),	7I	N	X5	X2
Shei et al. (1985) &	7II	N	X5	X2
Fournier et al. (1985)	8	N	X5	X2
	9-13B	N	X5	X2

* hydrolytic activity on xylotriose by xylanase I is much greater than that by xylanase II and xylanase III

Table 1.5. Multiple xylanases purified from microorganisms other than *Aspergillus niger*. The data (ns = not specified) include the ability to release arabinose in arabinoxylan hydrolysis (Y = yes, N = no) and the smallest xylooligosaccharide hydrolyzed (number equal degree of polymerization) by the xylanase.

organism	enzyme	release of arabinose	smallest sugar cleaved	reference
<u>Bacillus</u> <u>circulans</u>	A	ns	ns	Esteban et al. (1982)
	B	ns	ns	
<u>Bacillus</u> <u>sp.</u>	A	ns	X4	Honda et al. (1985)
	N	ns	X4	
<u>Cephalosporium</u> <u>sacchari</u> * #	HC-III	Y	ns	Richards & Shambe (1976)
	HC-IV	N	ns	
<u>Ceratocystis</u> <u>paradoxa</u> #	HC-I	Y	X4	Dekker & Richards (1975a,b)
	HC-II	N	X3	
<u>Clostridium</u> <u>stercorarium</u>	A	N	ns	Berenger et al. (1985)
	B	N	ns	
	C	N	ns	
<u>Oxiporus</u> <u>sp.</u>	A1	ns	ns	Sinner & Dietrichs (1976)
	A2	N	ns	
	B	ns	ns	
<u>Penicillium</u> <u>janthinellum</u>	I	ns	ns	Takenishi & Tsujioka (1973)
	II	ns	ns	
	III	ns	ns	
<u>Sporotrichum</u> <u>dimorphosporum</u> #	pI 4.4	ns	ns	Comtat (1983)
	pI 4.7	ns	ns	
<u>Streptomyces</u> <u>exfoliatus</u> #	Ib	ns	ns	Sreenath & Joseph (1982)
	Id	ns	ns	
	II	ns	ns	
<u>Talaromyces</u> <u>byssochlamydoides</u>	X-a	Y	ns	Yoshioka et al. (1981)
	X-b-I	N	ns	
	X-b-II	N	ns	
<u>Tyromyces</u> <u>palustris</u> #	F1-2	ns	ns	Shimizu et al. (1976) Ishihara et al. (1978)
	FII-3-2	ns	ns	
<u>Trichoderma</u> <u>harzianum</u>	20 kD	ns	X4	Tan et al. (1985b)
	29 kD	ns	X3	
<u>Trichoderma</u> <u>lignorum</u>	A	N	ns	John et al. (1981)
	B	N	ns	
<u>Trichoderma</u> <u>reesei</u>	1	ns	ns	Dekker (1985)
	2	ns	ns	
	3	ns	ns	
	4	ns	ns	

* designated as HC-I and HC-II in Dekker et al. (1975)

other xylanase fractions reported

in Aspergillus niger. On zymograms of isoelectric focused gels, Biely et al. (1985a) identified 5 major xylanases, and as many as 10 minor xylanolytic enzymes, in the culture filtrate of A. niger strain no. 14. As many as 5 xylanases have been purified to homogeneity from one strain of A. niger (Table 1.4). These enzymes show variance in hydrolytic and physico-chemical properties within each strain and among strains.

Iwamoto et al. (1973a) identified 2 types of xylanase produced by each of 4 strains of A. niger. The 2 purified xylanases reported by Iwamoto et al. (1973b) showed different hydrolytic characteristics. Xylanase I released arabinose from arabinoxytan but it did not release xylose or cleave xylotriase, whereas xylanase II released xylose but not arabinose and it could cleave xylotriase. A combination of the 2 enzymes, but not the enzymes individually, could effectively hydrolyze arabinofuranosyl-xylotriase (A-X3). These characteristics suggested that the two enzymes may cooperate in the hydrolysis of arabinoxytan. This proposed complementary interaction was demonstrated for two A. niger xylanases by Takenishi & Tsujisaka (1975).

Takenishi & Tsujisaka worked with 3 xylanases isolated from A. niger van Tieghem. Xylanase I was not an arabinose-releasing xylanase but it could cleave xylotriase much better than the other two xylanases (Table 1.4). Both xylanase II and III were arabinose-releasing xylanases (Tsujisaka et al. 1971) but xylanase II was more effective by being able to cleave A-X3

(Takenishi & Tsujisaka 1975). Takenishi & Tsujisaka found that xylanase I and II together gave the maximum hydrolysis of rice straw arabinoxylan (Figure 1.2). These two enzymes did not cooperatively hydrolyze cotton xylan which lacks arabinose substituents. The complementation observed in arabinoxylan hydrolysis appeared to be due to the ability of xylanase I to hydrolyze xylotriase effectively and xylanase II to release arabinose. Paper chromatographic analysis of the hydrolyzates indicated that xylanase I was notable for its ability to hydrolyze the higher xylooligosaccharides which remained after hydrolysis by the other 2 xylanases and that xylanase II was notable for its removal of A-X3.

The results of Takenishi & Tsujisaka suggest a functional purpose for the existence of two xylanases in A. niger but the third xylanase appears redundant. An unique function in this third xylanase may be revealed by examining other structural features of the substrate, including its acidic substituents and its association with other lignocellulosic components. These considerations, as well as considerations on substrate specificity, are necessary for examining xylanase systems with even higher degrees of multiplicity.

The 5 xylanases isolated from A. niger no. 11 by John et al. (1979) showed distinctly different mechanisms of hydrolysis. Chromatograms of their respective hydrolyzates quantitatively demonstrated differences in their sugar profiles. However, since all 5 enzymes were arabinose-releasing xylanases and could

Figure 1.2. Hydrolysis of cotton xylan and rice straw arabinoxylan by three xylanases purified from Aspergillus niger van Tiegham (Takenishi & Tsujisaka 1975).

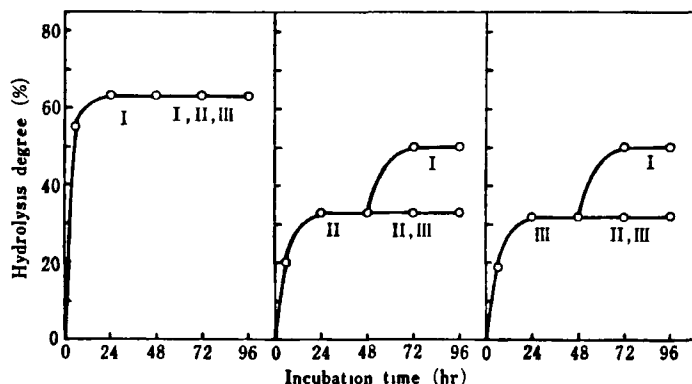


FIG. 6. Time Courses of Hydrolysis of Xylan by Three Xylanases.

Each reaction mixture contained 40 mg of the substrate and 400 units of the enzymes in 40 ml of 0.05 M acetate buffer, pH 4.5, and was incubated at 40°C. An aliquot of the mixture was withdrawn at indicated time and hydrolysis degree was estimated by the method as described in the text. After 48 hr incubation, each reaction mixture was divided into three equal parts and to them were added three xylanases (100 units), respectively, followed further incubation. An aliquot of the mixture was withdrawn at indicated time and hydrolysis degree was estimated by the method as described in text. I: xylanase I, II: xylanase II, III: xylanase III

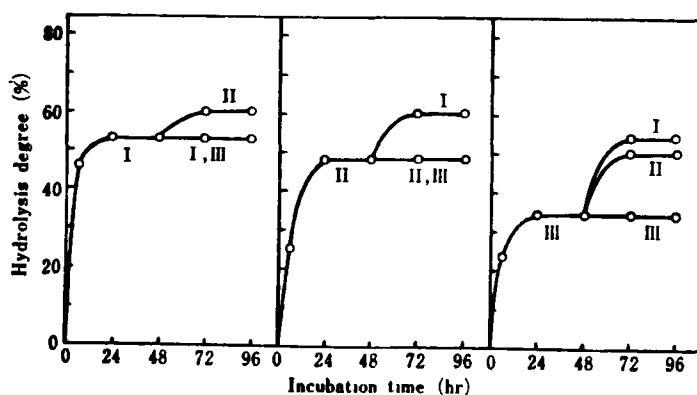


FIG. 7. Time Courses of Hydrolysis of Arabinoxylan by Three Xylanases

The experimental details were same as described in legend of Fig. 6. I: xylanase I, II: xylanase II, III: xylanase III

release xylose, they appear to be redundant enzymes. Reilly and his co-workers (Frederick et al. 1981, 1985, Fournier et al. 1985, Shei et al. 1985) also did not show that all 5 xylanases purified from A. niger Rhozyme HP-150 were essential components of the xylanase system. Differences in the hydrolytic properties of these enzymes were successfully identified using various fractions of larchwood xylan. All 5 enzymes could hydrolyze soluble xylan. Xylanase 4 and 8 could not hydrolyze insoluble xylan unless its substituents have been removed by mild acid hydrolysis whereas xylanase 7I and 7II could only hydrolyze the untreated insoluble xylan. These results emphasize the need to consider substrate structure for differentiating xylanases. Hydrolysis studies using combinations of these enzymes will be required to assess the importance of this multiplicity of xylanases.

Multiplicity of xylanases has been reported for several organisms besides A. niger (Table 1.5). Again, characterizations have shown that these enzymes differ within a species. Yoshioka et al. (1981) performed some hydrolytic studies using enzyme combinations and demonstrated complementation of 3 xylanases from Talaromyces byssochlamydoides. They found that xylanases X-a, X-b-I and X-b-II hydrolyzed xylan to xylobiose to the extent of 38%, 70% and 76% , respectively. Ninety percent hydrolysis was achieved by the combination of all 3 xylanases but hydrolysis by combinations of 2 enzymes was not reported. Sreenath & Joseph (1982) found that the combination of 3 xylanases from

Streptomyces exfoliatus had a specific activity higher than the average calculated from the specific activities of the individual enzymes. This result suggests that the combination of the 3 xylanases is more efficient but it is not clear whether the extent of hydrolysis is improved.

Properties of the xylanase system of other organisms suggest that xylanase complementation may occur. In particular, Ceratocystis paradoxa produces an arabinose-releasing xylanase (HC-I) which can not cleave xylotriase and a xylanase (HC-II) which can. These two xylanases may cooperatively hydrolyze arabinoxylan as demonstrated for two xylanases of A. niger (Takenishi & Tsujisaka 1975). Other possible examples are Cephalosporium sacchari and Trichoderma harzianum from which only one arabinose-releasing xylanase and one xylotriase-cleaving xylanase, respectively, has been isolated. In all these cases, further studies are required to assess the importance of xylanase multiplicity.

I.3. MULTIPLICITY OF XYLANASES IN TRICHODERMA HARZIANUM

The importance of multiple xylanases has been empirically demonstrated in two organisms, A. niger (Takenishi & Tsujisaka 1975) and T. byssochlamydoides (Yoshioka et al. 1981). In both cases, at least 2 xylanases are required to achieve maximum hydrolysis of arabinoxylan. Although some organisms produce more than 2 xylanases, there is no data which clearly indicate that these extra xylanases are essential for xylan hydrolysis.

Two xylanases have been isolated from T. harzianum E58, a strain from the fungal collection of Forintek Canada, Corp. (Tan et al. 1985a). A third xylanase has also been identified. I am interested in addressing the questions concerning the role of xylanase multiplicity. My approach has been to study the hydrolysis of xylan by combinations of xylanases. To consider the possible role of substrate structure, I have worked with different forms of xylan (acetylated xylan and arabinoxylan) and xylan in lignocellulose and holocellulose. In this thesis, I report the preparation of various xylan substrates, the purification of the xylanases and the cooperative interactions among these enzymes in the hydrolysis of the substrates.

II. THE XYLANS USED IN THIS STUDY

Preparation of native xylan from wood is difficult due to its associations with other wood components. High yields of xylan from aspenwood (Populus tremuloides) can be isolated by potassium hydroxide extraction but this alkaline treatment deacetylates the polysaccharide. Hagglund et al. (1956) demonstrated that dimethyl sulfoxide (DMSO) can extract an acetylated xylan from wood and Caregg (1962) found that DMSO does not result in deacetylation or acetyl group migration in acetylated xylopyranosides. Since the chlorine-monoethanolamine method of holocellulose preparation leads to acetyl group migration and elimination, the chlorite treatment of Wise et al. (1946) is recommended for native xylan preparation. However, the chlorite treatment also leads to some modification of native polysaccharides. Lindberg & Rosell (1974) reported some deacetylation and acetyl group migration. The treatment also results in slight depolymerization and some oxidation of reducing ends and 2,3-glycol groups (Whistler & Richards 1970) but the bulk of xylan should remain unchanged since only one reducing end is present and few 2,3-glycol groups exist due to substitutions. The chlorite holocellulose from aspenwood should still have polysaccharides representative of the native forms. In this chapter, the isolation and some characterization of an acetylated xylan from aspenwood are reported.

II.1. MATERIALS AND METHODS

II.1.1. Deacetylated aspen xylan and oat arabinoxylan

The deacetylated form of aspen xylan was prepared at Forintek by Ken West. The method involved potassium hydroxide extraction of aspenwood and had been previously described by Saddler et al. (1983). Oat spelts xylan obtained from Sigma was used without further purification.

II.1.2. Preparation of an acetylated aspen xylan

Extractive-free aspen sawdust (20 mesh and under) had been previously prepared using standard TAPPI (Technical Association of the Pulp and Paper Industry) procedures: 32 h extraction with ethanol:benzene (1:2) followed by 48 h extraction with 95% ethanol. At 75 C, 200 g of sawdust were mixed in 6.4 L of water with 21 mL acetic acid and 60.1 g sodium chlorite (BDH). The reaction mixture was swirled every 5 min for 1h. After a second hour of treatment following the addition of 21 mL acetic acid and 60.1 g sodium chlorite, the reaction was cooled in an ice bath. The residue was separated with a sintered glass filter and washed with at least 4 L of water and then twice with acetone. This two hour chlorite treatment was repeated on the residue after it was air-dried and ground in a Wiley mill with a 1 mm mesh. The resulting holocellulose was made into a 10% (w/v) slurry in dimethyl sulfoxide (DMSO) (Anachemia) and

incubated at 28 C on a shaker overnight. The slurry was then filtered and its residue washed with half volume of DMSO and then with one volume of water. The pooled filtrate was mixed with 5 volumes of 95% ethanol with 0.05% hydrochloric acid. The xylan that precipitated after 24 h was collected, washed with 500 mL ethanol and vacuumed dried at 40 C.

II.1.3. Neutral sugar analysis by high pressure liquid chromatography (HPLC)

Samples for HPLC analysis were prepared in distilled water which had been deionized with a Milli-Q Reagent Water System (Millipore). Insolubles were removed from the samples by 10 min centrifugation with a Model 235A microcentrifuge (Fisher) and then by filtration through Cameo 0.45 μ m nylon filters (MSI). Twenty microliters samples were analyzed on a 7.8 mm X 300 mm Aminex HPX-87P column (Bio-Rad) operating at 85 C. The column was guarded with a 0.45 μ m stainless steel frit, an Anion/OH Micro-Guard cartridge (Bio-Rad) and an Ion Exclusion Micro-Guard cartridge (Bio-Rad). Both Micro-Guards were 4.6 mm X 40 mm columns, the former packed with Aminex A-25 and the latter with Aminex HPX-85H.

Deionized water, which had been degassed under vacuum with heat, was used as the eluent at 0.6 mL/min. Elution was monitored with a Series RI-3 refractive index detector (Varian) and peaks

were integrated by a Vista 401 Chromatography Data System (Varian) which also controlled the Model 5000 liquid chromatograph (Varian). The refractive index detector range was set at 20 and the plotter attenuation was set at 1. Identification and calibration of the peaks were achieved using standards prepared with xylose, arabinose, mannose (Sigma), glucose (Fisher) and xylobiose (Pfanstiehl).

II.1.4. Sugar composition of the xylans

One milligram xylan in 0.4 mL 2 M trifluoroacetic acid was incubated overnight at 95 C in a sealed ampoule. The hydrolyzate was lyophilized, dissolved in 0.5 mL water and re-lyophilized. The sample was then reconstituted with 0.2 mL water and analyzed by HPLC.

II.2. RESULTS AND DISCUSSION

Holocellulose was initially prepared using a 2 h chlorite treatment to minimize modification of xylan. Furthermore, Harwood (1952) had reported significant loss of xylan from wheat straw during the third hour of chlorite treatment. However, this 'crude' aspen holocellulose provided extremely low yield of xylan when extracted with DMSO. Pentosan analysis performed by Ken West (data not shown) indicated that the xylan remained in the crude holocellulose after the chlorite treatment and the

extraction with DMSO. Apparently, aspenwood is more resistant to chlorite treatments than wheat straw. Unextracted crude holocellulose was treated with chlorite for two additional hours and subsequent extraction with DMSO resulted in a final yield of less than 1% acetylated aspen xylan from sawdust (Table 2.1). Since xylan makes up 24% of aspenwood (Timell 1967), it is not clear whether this preparation is a representative sample of native xylan in aspen. On the other hand, the deacetylated xylan preparation contains the bulk of xylan in aspenwood, but it is not in a native form.

Sugar analyses of three xylans are shown on Figure 2.1. The only neutral sugar found in the deacetylated aspen xylan was xylose. The oat spelts xylan was confirmed to be an arabinoxylan. This material appeared to be contaminated by a small amount of glucan. The acetylated aspen xylan preparation was also contaminated by some glucose and arabinose/mannose residues. An estimate of the molar ratio of sugars in each xylan preparation is given on Table 2.2. Further substrate characterization would be required to determine the structural form of the contaminants. The three xylans, aspen sawdust and aspen holocellulose are five different substrates which are used to characterize the xylanase system of T. harzianum.

Table 2.1. The yield of xylan from aspen sawdust by dimethyl sulfoxide extraction of the holocellulose. The crude holocellulose was obtained by a 2 h chlorite treatment (Wise et al. 1946) of the sawdust (20 mesh and under). The holocellulose was prepared with an identical treatment of the crude holocellulose after it had been ground in a Wiley mill to pass through an 1 mm mesh. The procedure for dimethyl sulfoxide extraction was modified from Hagglund et al. (1956).

material	weight (g)	percent
extractive-free aspen sawdust	200.0	100
crude holocellulose	182.5	91.8
holocellulose *	168.1	84.0
acetylated aspen xylan *	1.89	0.94

* results were calculated from a 0.82 scale run

Figure 2.1. HPLC analysis of the monosaccharide constituents of three xylans: deacetylated aspen xylan (i), acetylated aspen xylan (ii) and oat spelts xylan (iii). The retention times for glucose, xylose, arabinose and mannose were 12.2, 13.3, 15.7 and 16.3 min, respectively. A mixture of arabinose and mannose had a retention time of 16.0 min.

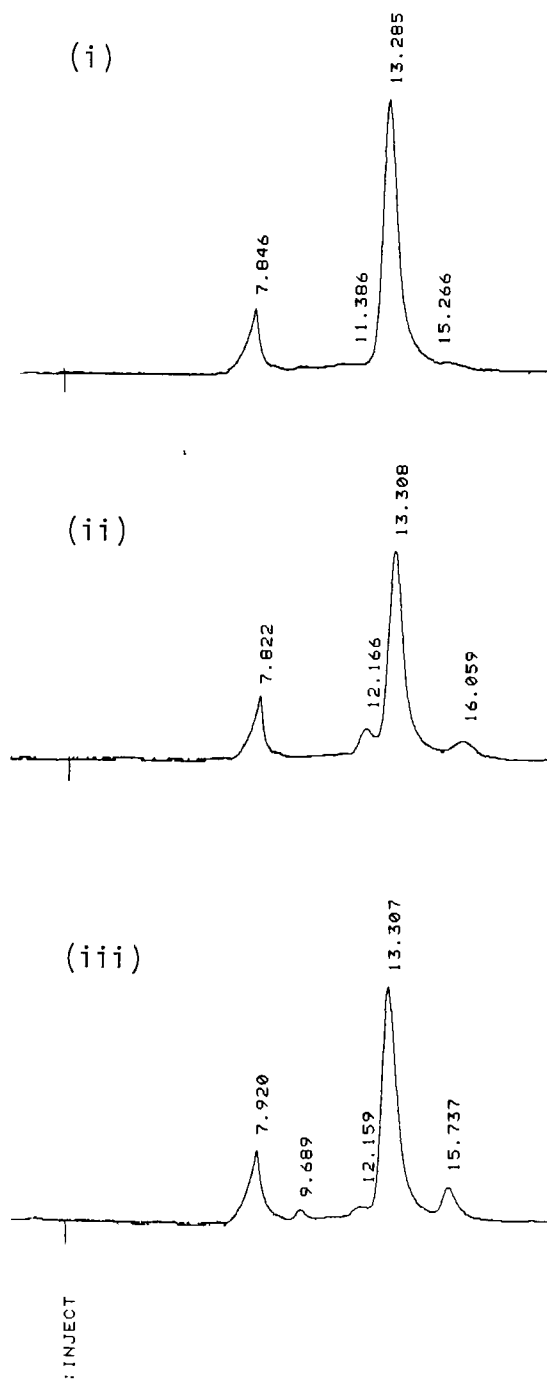


Table 2.2. Neutral sugars of aspen xylan preparations and oat spelts xylan. The polysaccharides had been hydrolyzed with trifluoroacetic acid and the neutral sugars determined by HPLC.

substrate	molar ratio of constituent monosaccharides		
	arabinose	glucose	xylose
oat spelts xylan	6	1	33
deacetylated aspen xylan	0	0	1
acetylated aspen xylan	2 *	1	15

* appears to be a fused peak of arabinose and mannose

III. XYLANASE PREPARATIONS FROM T. HARZIANUM

The 4 day old culture filtrate of Trichoderma harzianum E58, grown on 1% solka floc as described by Saddler et al. (1982), contains high cellulase and xylanase activity. Ultrafiltration using a 10 kD polysulfone membrane is routinely used to concentrate the cellulases. The escape of xylanase activity into the ultra-filtrate had led to the purification of the 2 xylanases reported by Tan et al. (1985a). A third xylanase has been identified in this ultra-filtrate and its purification is reported in this chapter.

III.1. MATERIALS AND METHODS

III.1.1. Assays for xylanase and β -xylosidase

Xylanase activity was assayed by measuring its ability to increase the number of reducing ends in deacetylated aspen xylan (section II.1.1). One milliliter of 50 mM sodium acetate, pH 4.8, containing 10 mg substrate was mixed with 1 mL of enzyme appropriately diluted in the same buffer. The reaction was incubated at 50 C for 10 min and stopped by adding 2 mL of the copper reagent of Somogyi (1952). The reduced copper that formed after 15 min boiling was determined by adding 1 mL of the arsenomolybdate reagent of Nelson (1944), degassing for 5 min under vacuum and subsequently measuring absorbance at 500 nm. One unit of activity (U) was defined as one micromole of

xylose equivalents released per minute. Protein was determined by the Lowry method after precipitation with trichloroacetic acid (Tan et al. 1984), using bovine serum albumin (Sigma) as the standard.

For monitoring column effluents and for locating xylanase on isoelectric focused gels, larchwood xylan (Sigma) was used as the substrate and the reaction was incubated for 30 min. Three millilitres of the dinitrosalicylic acid reagent of Miller (1959) was used to stop the reaction. Reducing sugars were determined spectrometrically at 575 nm after this mixture was boiled for 5 min.

β -xylosidase activity was assayed using 4 mg p-nitrophenol-xylopyranoside (Sigma). The reaction mixture was incubated at 50 C for 30 min. An equal volume of 10% (w/v) sodium carbonate was added and the resulting colour was quantified by measuring absorbance at 410 nm. One unit of activity was defined as one micromole of p-nitrophenol released per minute.

III.1.2. Analytical methods

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (1970) using the solutions given on Table 3.1. Slab gels with 15% (w/v) polyacrylamide at pH 8.8 were used as separation gels and they

Table 3.1. Preparation of gels and solutions for SDS-PAGE.
All electrophoresis grade reagents were obtained from Bio-Rad.

electrode buffer (pH 8.3)

6.0 g Tris
28.8 g glycine
10.0 mL 10% (w/v) sodium dodecyl-sulfate (SDS)
adjust to 1 L with water

15% separation gel

7.1 mL water
7.5 mL 1.5 M Tris-chloride pH 8.8
0.3 mL 10% SDS
15.0 mL 30% (w/v) acrylamide with 0.8% BIS
0.2 mL 10% (w/v) ammonium persulfate
after degassing for 5 min under vacuum, 15 μ L
TEMED was added before the solution was
poured into its cast

3% stacking gel

6.2 mL water
2.5 mL 0.5 M Tris-chloride pH 6.8
0.1 mL 10% SDS
1.0 mL 30%:0.8% acrylamide:BIS
0.2 mL 10% ammonium persulfate
after degassing for 5 min, 10 μ L TEMED was added

sample buffer

4.7 mL water
1.0 mL 0.5 M Tris-chloride pH 6.8
1.0 mL glycerol
1.0 mL 10% SDS
0.2 mL 0.05% (w/v) bromophenol blue
0.1 mL β -mercaptoethanol was added immediately
before mixing with sample

staining solution

0.25% (w/v) Comassie Blue R-250 in 50% (v/v)
methanol and 5% (v/v) acetic acid

destaining solution

1:1:8 (v/v/v) of acetic acid:butanol:water

were overlaid with 3% stacking gel at pH 6.8. Dilute protein preparations for analysis were concentrated by precipitating with cold 7% (w/v) trichloroacetic acid and redissolving in 3% sodium carbonate in 0.1 M sodium hydroxide. Ten or twenty microlitre samples were boiled for 2 min with four part sample buffer before loading into wells casted into the stacking gel. Electrophoresis was carried out at constant voltage of 100 V using Tris-glycine electrode buffer, pH 8.3, and Bio-Rad apparatus and power supply (model 1420A). The separated protein bands were stained with Comassie Blue for an hour and destained overnight. Molecular weight standards (Pharmacia) consisted of α -lactalbumin, soybean trypsin inhibitor, carbonic anhydrase, ovalbumin, bovine serum albumin and phosphorylase b which have molecular weights of 14.4, 20.1, 30, 43, 67 and 94 kD (kilodalton), respectively.

Isoelectric focusing (IEF) was carried out on a FBE-3000 Flat Bed Apparatus (Pharmacia) using methods recommended in the technical manual 'Isogel System' (FMC). Three types of ampholytes have been used during the course of this study: Bio-Lyte 3/10, Bio-Lyte 8/10 (Bio-Rad) and Pharmalyte 8-10.5 (Pharmacia). One and an half millimetre thick gels (containing 1% (w/v) agarose (Pharmacia), 2.5% ampholytes and 10% D-sorbitol) were prepared on GelBond film (Pharmacia) and used to separate protein under constant power. One molar sodium hydroxide was always used as the catholyte whereas 0.2 M sulfuric acid was used as the anolyte for generating a pH gradient between 3 and 10 and 0.25 M L-histidine or HEPES (N-2-hydroxyethylpiperazine-N'-2-

ethanesulfonic acid) (Sigma) was used as the anolyte for pH gradients between 8 and 10.5. The pH gradient was determined by placing 5 mm X 10 mm slices, from 2 tracks of gel, in 0.5 mL water and measuring the pH. Alternatively, the pH was measured at 5 mm intervals with a surface electrode. Xylanase activity across a protein track was determined by assaying 2 mm X 10 mm gel segments. Proteins were visualized after the gel was fixed with cold 10% (w/v) trichloroacetic acid, dried, stained with Comassie Blue for 15 min and destained with 1:3:6 (v/v/v) of acetic acid:methanol:water.

III.1.3. Removal of three xylanases from the culture filtrate

The initial step for the purification of the three xylanases was ultrafiltration of the culture filtrate using a Pellicon Cassette System (Millipore) equipped with 0.47 m² polysulfone membrane with molecular weight cut-off of 10 kD (Tan et al. 1985a). The unexpected escape of the 20 kD, 22 kD and 29 kD xylanases into the filtrate facilitated their purification and enabled the preparation of a retentate free of these xylanases. At 4 °C, 250 mL the initial ultra-retentate was diafiltered using 9 L 10 mM ammonium acetate, pH 5. The process was repeated twice more using buffer containing 0.02% (w/v) sodium azide. The resulting preparation was designated as the purified retentate.

III.1.4. Preparation of the 20 kD and 29 kD xylanases

Purification of the 20 kD and 29 kD xylanases was carried out by the method reported by Tan et al. (1985a). Modifications included the addition of 0.02% sodium azide to all buffers to inhibit microbial growth. Preparation of the 20 kD enzyme was scaled up using a 5 cm X 15 cm phenyl-sepharose (Pharmacia) column. Finally, an extra chromatography with carboxymethyl-sepharose (CM-sepharose)(Pharmacia) was required to purify the 29 kD xylanase because the initial preparation was contaminated by a slightly smaller protein.

III.1.5. Purification of the 22 kD xylanase

In the purification of the 20 kD xylanase on the phenyl-sepharose column, a 22 kD protein was removed because it adsorbed tightly to the column (Tan et al. 1985a). After being used to purify two more batches of 20 kD xylanase, this column was eluted with an increasing linear gradient composed of 2.5 L each of 0 to 90% (v/v) ethylene glycol in 10 mM ammonium acetate, pH 5, with 0.02% sodium azide. The pooled protein fraction was concentrated with an Amicon apparatus fitted with a YM5 membrane (5 kD cutoff). Ethylene glycol was diluted (15 000 fold) out of the preparation by repeated dilution and reconcentration in the acetate buffer.

The 22 kD protein was subsequently purified by chromatofocusing

on a 1 cm X 36 cm column packed with PBE94 (Pharmacia). The column was initially equilibrated with 25 mM ethanolamine-chloride, pH 9.4, which had been degassed under vacuum for an hour. A 6.8 mL sample, containing 84 mg protein in the ethanolamine buffer, was applied on to the column. It was eluted with Pharmalyte 8-10.5 which had been adjusted to pH 8 with hydrochloric acid, diluted 45 folds and degassed. Three millilitre fractions were collected and monitored by their absorbance at 280 nm, their pH and their activity on xylan. The pooled 22 kD xylanase preparation was dialyzed using a Spectrapor 3 (3.5 kD cutoff) membrane (Spectrum) against six changes of the acetate buffer. Final removal of the ampholytes was achieved by two precipitations with 80% (w/v) ammonium sulfate. The precipitate was reconstituted in the acetate buffer and dialyzed against five changes of this buffer. All operations were carried out at 4C.

III.2. RESULTS AND DISCUSSION

Figure 3.1 summarizes the purification scheme for the enzyme preparations to be used in my study. There appears to be a tight association between the 22 kD protein and the 20 kD xylanase since these two proteins were not completely resolved by chromatography on CM-sepharose nor on phenyl-sepharose. Separation was achieved by taking advantage of the difference between their pI's (Table 3.2). Chromatofocusing of the crude 22

Figure 3.1. Purification scheme for the 20 kD, 22 kD and 29 kD xylanases from T. harzianum and an ultra-retentate which lacks these three enzymes.

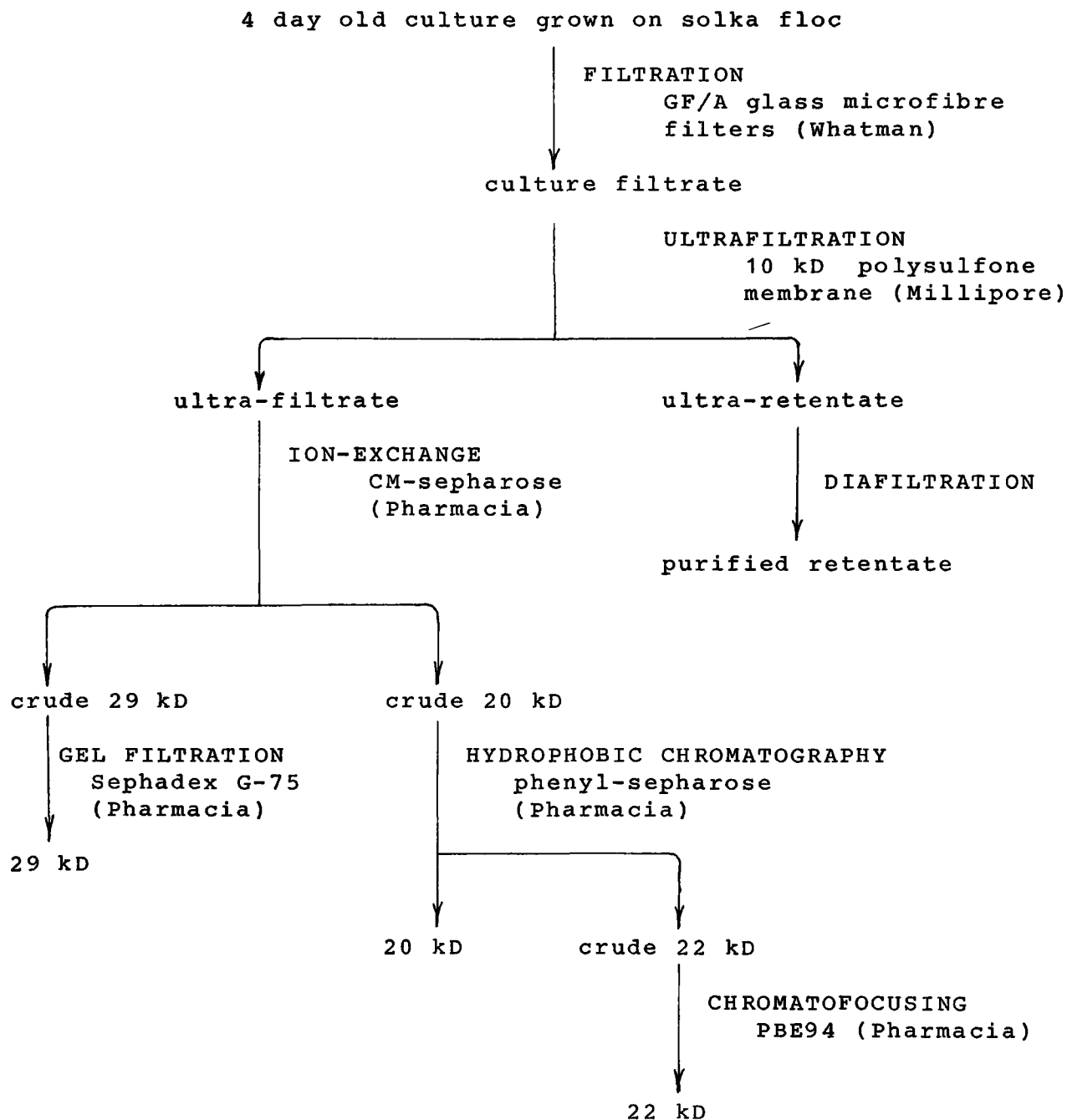


Table 3.2. The pI's of the xylanases and β -xylosidases in the culture filtrate of T. harzianum grown on solka floc for 4 days.

enzyme	type	M.W. (kD)	pI *		
			pI	s.d.	n
xylanase	A	20	9.3	0.4	6
	B	22	8.5	0.4	16
	C	29	9.4	0.5	3
	D	-	5.6	0.2	3
	E	-	4.9	0.1	3
β -xylosidase	F	-	8.3	0.6	2
	G	-	5.8	0.1	2

* each pI value is reported as the mean of n samples with its standard deviation (s.d.)

kD protein from phenyl-sepharose, a by-product from the purification of the 20 kD xylanase, resolved two protein peaks (Figure 3.2). The first peak was the contaminating 20 kD xylanase and it was followed by the pure 22 kD protein. This 22 kD protein was homogeneous according to SDS-PAGE and IEF (Figures 3.3 & 3.4). Upon IEF, the purified 22 kD protein preparation yielded one xylanase peak corresponding to one protein band (Figure 3.5).

The 22 kD xylanase has a specific activity of 0.28 U/mg (Table 3.3) which is considerably lower than the 370 U/mg and 75 U/mg values measured for the 20 kD and 29 kD xylanases, respectively (Tan et al. 1985a). Data in Figure 3.5 as well as Figure 3.2 suggest a considerable loss of activity in the 22 kD xylanase preparation after chromatofocusing. Long-term exposure to the ethanolamine buffer at pH 9.4 had resulted in the appearance of a pI 7.2 protein in the crude 22 kD xylanase from phenyl-sepharose (data not shown). For this reason, the ethanolamine buffer was added to the preparation immediately prior to application on the chromatofocusing column. It is not known whether short-term exposure of the 22 kD xylanase to the ethanolamine buffer had reduced its specific activity. Unless stability studies show otherwise, other chromatofocusing conditions or preparative IEF should be considered for the purification of the 22 kD xylanase in the future. Because the 20 kD xylanase appears closely associated with the 22 kD enzyme, an interesting possibility is that the 20 kD xylanase stabilizes the 22 kD xylanase. Stability studies involving a

Figure 3.2. Chromatofocusing of 84 mg of the crude 22 kD xylanase preparation from phenyl-sepharose on an 1 cm X 36 cm column of PBE94. The column had been initially equilibrated with 25 mM ethanolamine at pH 9.4 and it was eluted with Pharmalyte 8-10.5 at pH 8. Three millilitre fractions were collected and 10 μ L aliquots were assayed for xylanase activity.

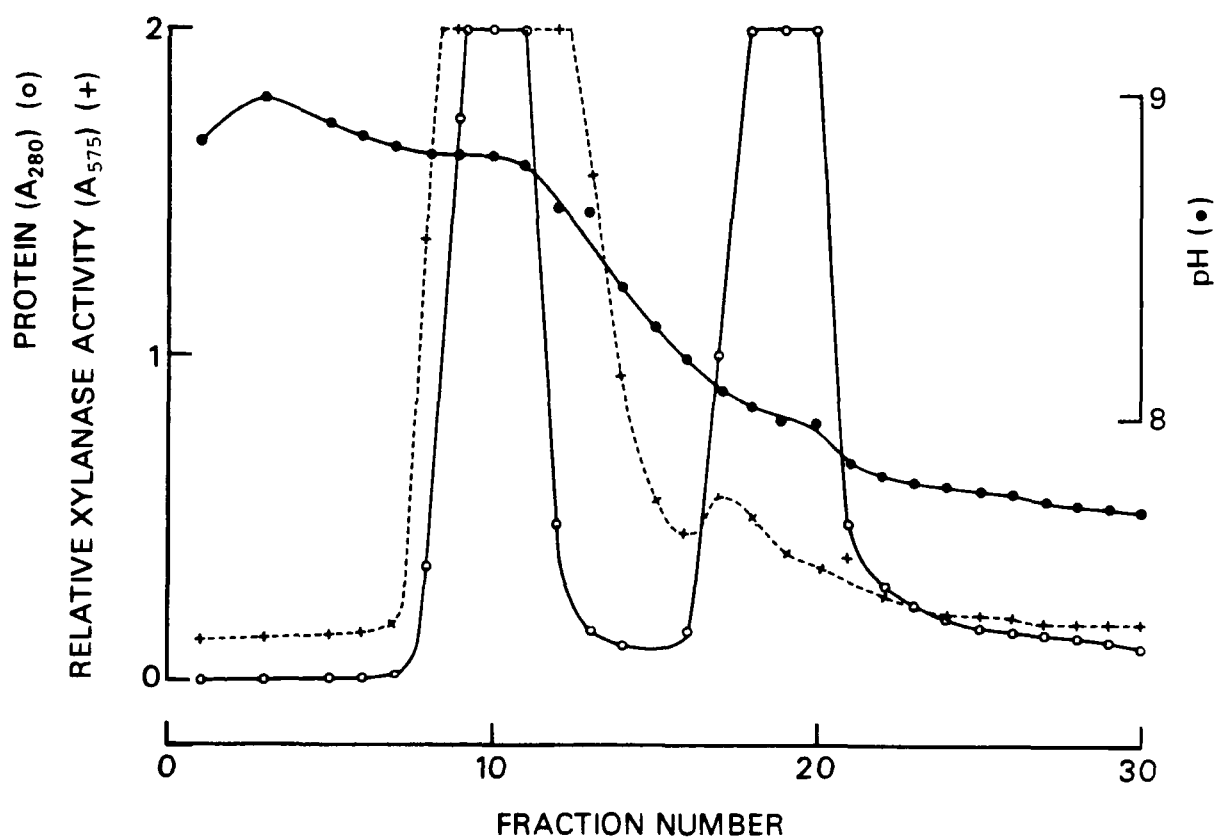


Figure 3.3. SDS-PAGE of the purified xylanases from T. harzianum. It was carried out using 3% stacking gel, 15% separation gel and constant voltage at 100 V. Lane 1: protein standards of 14.4, 20.1, 30, 43, 67 and 94 kD. Lane 2: 75 μ g culture filtrate. Lane 3: 40 μ g of the ultra-filtrate from a 10 kD polysulfone membrane. Lane 4: 4 μ g of the purified 29 kD xylanase. Lane 5: 19 μ g of the 20 kD xylanase from CM-sepharose. Lane 6: 17 μ g of the 20 kD xylanase from phenyl-sepharose. Lane 7: 17 μ g of the 22 kD xylanase from phenyl-sepharose. Lane 8: 14 μ g of the 22 kD xylanase from chromatofocusing after the removal of ampholytes by dialysis and ammonium sulfate precipitation.



Figure 3.4. IEF showing the purification of the 22 kD xylanase from T. harzianum. IEF was carried out on 1% agarose with 2.5% Bio-Lyte 3/10, at constant power of 10 W for 2 h and using 1 M sodium hydroxide and 0.2 M sulfuric acid as the catholyte and anolyte, respectively. Lane 1: 170 μ g crude ultra-retentate obtained from the culture filtrate using a 10 kD polysulfone membrane. Lane 2: 35 μ g ultra-filtrate. Lane 3: 19 μ g of the 20 kD xylanase from CM-sepharose. Lane 4: 17 μ g of the 20 kD xylanase from phenyl-sepharose. Lane 5: 17 μ g of the 22 kD xylanase from phenyl-sepharose. Lane 6: 14 μ g of the 22 kD xylanase from chromatofocusing.

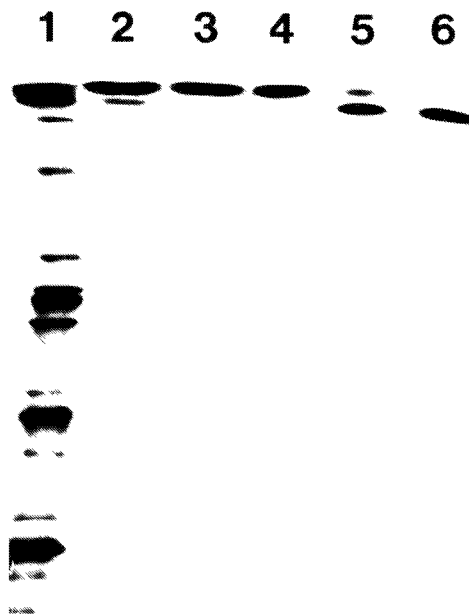


Figure 3.5. IEF analysis of the purified 22 kD xylanase from *T. harzianum*. IEF was carried out on 1% agarose containing 2.5% Pharmalyte 8-10.5 and at constant power of 5 W for 4 h with the voltage limit set at 500 V. One molar sodium hydroxide and 0.25 M HEPES were used as the catholyte and anolyte, respectively. Lane 1: 84 μ g of the 22 kD xylanase from phenyl-sepharose. Lane 2: 140 μ g of the 22 kD purified by chromatofocusing. Duplicate tracks, of crude 22 kD xylanase (+) and pure 22 kD xylanase (o), were assayed for xylanase activity using 2 mm X 10 mm gel segments.

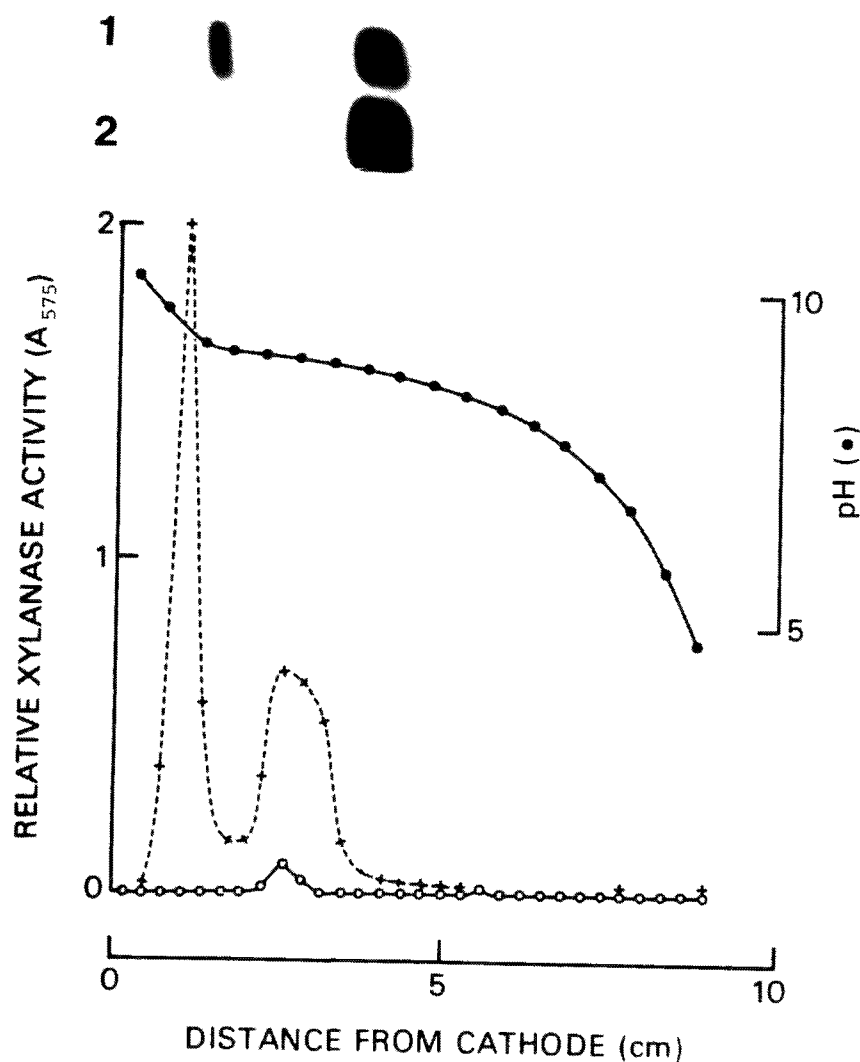


Table 3.3. Purification summary for the 22 kD xylanase.

purification step	protein (mg)	activity (U)	specific
			activity (U/mg)

20 kD xylanase from CM-sepharose *	1140	330 000	290
22 kD xylanase from phenyl-sepharose	101	549	5.44
22 kD xylanase from chromatofocusing #	66.7	16.9	0.253
22 kD xylanase from ammonium sulfate precipitation +	41.5	11.5	0.277

* a 20 kD preparation contaminated with the 22 kD protein

calculated from a 0.83 scale run

+ calculated from a 0.91 scale run

mixture of the two enzymes are difficult because they can not be distinguished by activity alone. This problem may be solved by quantifying activity after IEF.

The ultra-retentate from the culture filtrate is known to contain the cellulase system, the β -xylosidase as well as xylanase. Diafiltration of this crude retentate through the polysulfone membrane removed most of the xylanase activity (Table 3.4). Analysis by IEF indicated that the purified retentate had lost the xylanase activity associated with enzymes in the high pI range (Figure 3.6) and SDS-PAGE confirmed that the 20 kD, 22 kD and 29 kD xylanases were lost (Figure 3.7). It is not clear whether the escape of these enzymes through the 10 kD membrane was due to their compact structure and/or to variance in the pore sizes of the membrane. The residual xylanase activity in the purified retentate was the result of two minor xylanases with pI's of 5.6 and 4.9, respectively.

In contrast, the loss of β -xylosidase activity in the purified retentate was not as drastic. Analysis by IEF suggested that the loss of the pI 5.8 β -xylosidase was more prominent than that of the pI 8.3 enzyme (Figure 3.8). In the crude retentate, minor peaks were resolved on the shoulders of the pI 5.8 β -xylosidase to suggest that there was a cluster of similar enzymes. These minor peaks were not resolved in the purified retentate. Nevertheless, it is apparent that the major components of the xylanolytic system, besides the three purified enzymes, have

Table 3.4. Purification summary of the purified retentate which is free of the three purified xylanases. These xylanases were removed by diafiltration using a 10 kD polysulfone membrane.

retentate	protein (mg)	xylanase activity		β -xylosidase	
		U	U/mg	U	U/mg
crude	1 720	104 000	60.8	478	0.279
purified	978	180	0.184	110	0.112

Figure 3.6. IEF analysis of the purified retentate obtained from the culture filtrate of *T. harzinaum* using a 10 kD polysulfone membrane. IEF conditions were identical to those for the gel shown on Figure 3.4. Lane 1: 140 μ g of the crude ultra-retentate. Lane 2: 17 μ g of the 22 kD xylanase from phenyl-sepharose which is contaminated with the 20 kD enzyme. Lane 3: 170 μ g of the retentate purified by diafiltration. Duplicate tracks of the crude ultra-retentate (+) and the purified retentate (o) were assayed for xylanase activity using 2 mm X 10 mm gel segments.

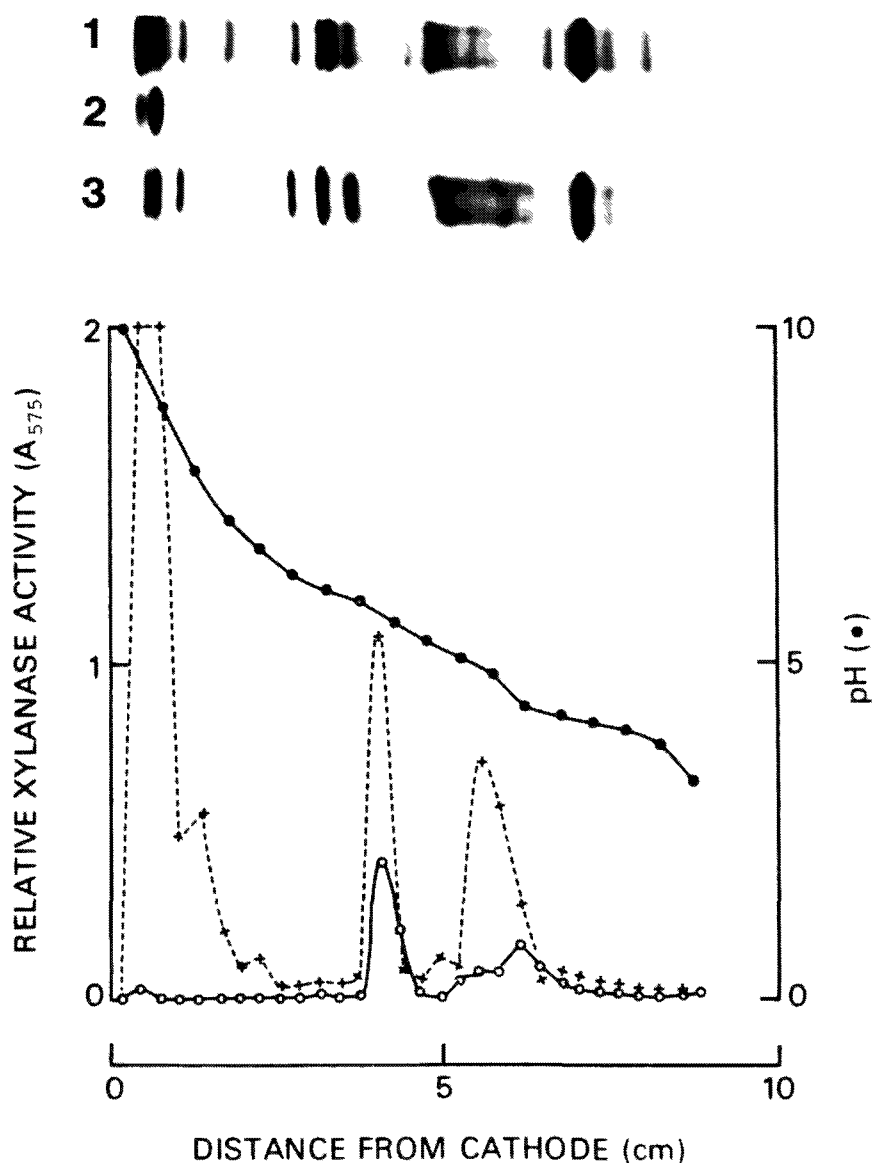


Figure 3.7. SDS-PAGE of the purified retentate obtained from the culture filtrate of *T. harzianum* using a 10 kD polysulfone membrane. Electrophoresis conditions are given on Figure 3.3. Lane 1: molecular weight standards. Lane 2: 75 μ g culture filtrate. Lane 3: 83 μ g of the purified retentate. Lane 4: 40 μ g ultra-filtrate.

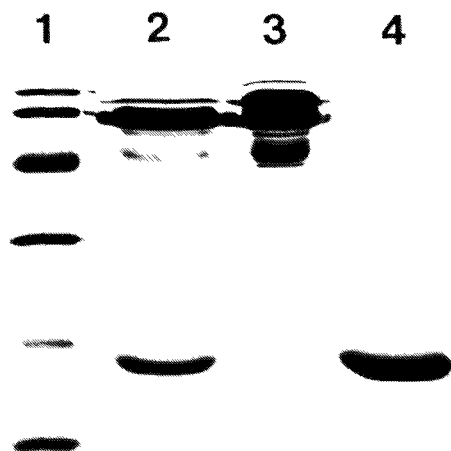
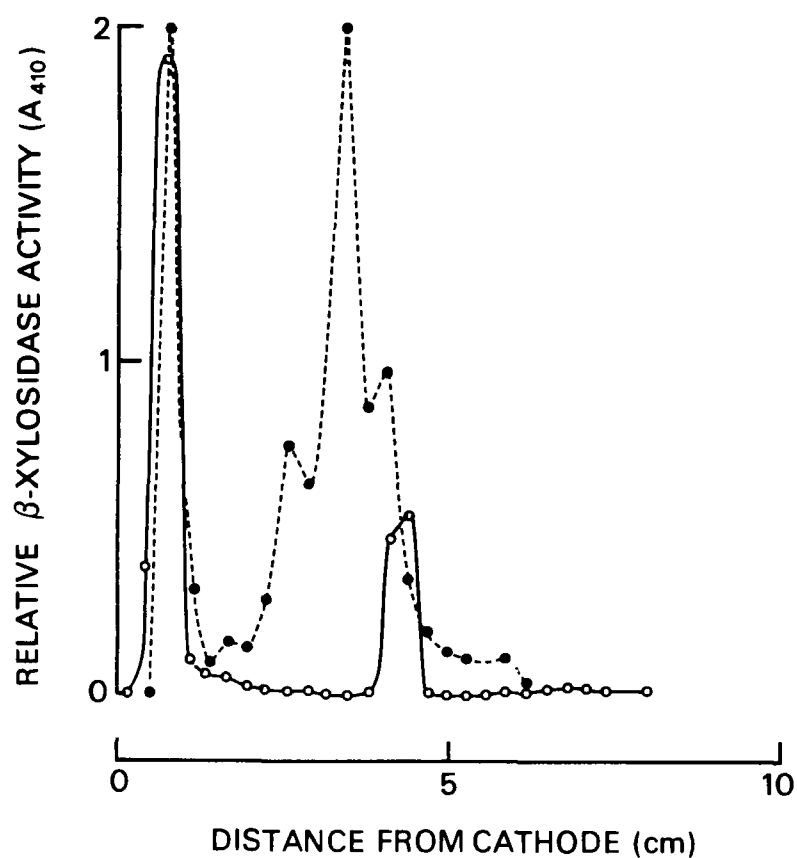


Figure 3.8. β -xylosidase activity of the crude ultra-retentate (●) and the purified retentate (○) after IEF. The retentates were prepared from the culture filtrate of *T. harzianum* using a 10 kD polysulfone membrane. Activity was measured from 2 mm X 10 mm gel segments from duplicate tracks sliced from the gel shown on Figure 3.6.



remained in the retentate. Although these components have been retained differentially, the purified retentate could be treated as a fourth component of the xylanolytic system of T. harzianum which contained the xylanolytic activities not associated with the three purified xylanases. However, it also contained other proteins including the cellulolytic system.

IV. BIOCHEMICAL AND HYDROLYTIC PROPERTIES OF THE XYLANASES

Three xylanases of Trichoderma harzianum have been successfully purified and some of their properties are described in this chapter. The aim of the characterization studies is to identify suitable conditions for hydrolysis studies and to determine whether these enzymes are separate gene products which are components of a distinct xylanolytic system.

IV.1. MATERIALS AND METHODS

IV.1.1. Sugar analysis by paper chromatography

Two solvents have been used to elute Whatman no. 1 paper for the analysis of enzyme and acid hydrolyzates from sugars. The acidic solvent, 2:1:1 (v/v/v) of butanol:acetic acid:water, can resolve acidic residues and it was generally used for hydrolyzates from cellulosic substrates and polygalacturonic acid. The basic solvent, 6:4:3 (v/v/v) of butanol:pyridine:water, can resolve galactose/glucose and arabinose/xylose combinations and it was used to analyze hydrolyzates from hemicelluloses and disaccharides. The paper chromatograms were developed by dipping first in 1.2% (w/v) silver nitrate in acetone, then in 0.5 M sodium hydroxide in ethanol and finally in 10% (w/v) sodium thiosulfate in water. They were air-dried before each dipping.

IV.1.2. Substrates

The polysaccharides and disaccharides were obtained from the sources given on Table 4.1. Sugar constituents of these substrates were determined by acid hydrolysis using trifluoroacetic acid (section II.1.4) and subsequent analysis using paper chromatography. Xylotriose was prepared at Forintek by David Turnbull using paper chromatographic separation of an enzyme hydrolyzate from larchwood xylan. A 10 mL reaction mixture which contained 70 U crude 20 kD xylanase and 0.2 g xylan in 10 mM ammonium acetate, pH 5, was incubated at 50 C overnight. The supernatant obtained after centrifugation was boiled to denature the enzyme, lyophilized to dryness and redissolved in 1 mL water. The sample was applied across a sheet of Whatman 3 mm paper and eluted with the basic solvent. The band which contained xylotriose was cut out and extracted with 20 mL water. Centrifugation and filtration were used to remove the paper fibres in the xylotriose solution before it was lyophilized in water twice to give the final preparation. Purity of the preparation was verified by paper chromatography.

IV.1.3. Optimization and stability studies

pH and temperature optimization studies involved 10 min xylanase assays (section III.1.1), but with 2.5 mg deacetylated

Table 4.1. The sources for the substrates.

substrate	source
deacetylated aspenwood xylan	Forintek
larchwood xylan	Sigma
oat spelts xylan	Sigma
Avicel microcrystalline cellulose	FMC
CF11 cellulose powder	Whatman
CMC (carboxymethylcellulose, medium viscosity)	Sigma
β -glucan from barley	Sigma
laminarin from <u>Laminaria digitata</u>	Sigma
lichenan from <u>Cetraria islandica</u>	Sigma
solka floc (powdered cellulose, BW-300)	Brown
starch	Difco
araban	Koch-Light
arabinogalactan from larchwood	Sigma
galactan	Aldrich
galactan	K & K
mannan from yeast	Sigma
polygalacturonic acid from orange	Sigma
cellobiose	Sigma
3-O- β -D-galactopyranosyl-D-arabinose	Sigma
lactose	Fisher
melibiose	Aldrich
sucrose	Fisher
xylobiose	Bachem or Pfanstiehl

aspen xylan and at different pH's or temperatures. pH optimization was performed at 50 C in 50 mM sodium acetate buffer, for pH 3.5 to 5.5, and in 50 mM MES (2-[N-morpholino]ethanesulphonic acid) (Sigma), for pH 5.5 to 6.5. Temperature optimization was performed in 50 mM sodium acetate, pH 5. The quantity of 20 kD, 22 kD and 29 kD xylanase assayed was 0.2 μ g, 160 μ g and 0.4 μ g respectively.

Stability studies involved incubating the enzyme without the substrate for an hour at various pH's and temperatures. The remaining activity was assayed and reported as the percent of the original activity. pH stability was determined at 50 C while thermal stability was determined in 50 mM sodium acetate, pH 5. The enzymes were tested at concentrations of 50, 160 and 50 μ g/mL for the 20 kD, 22 kD and 29 kD xylanases, respectively.

IV.1.4. Substrate specificity

Enzyme activity on various substrates was determined using 0.05% substrate. The reducing sugars produced was quantified by the method of Nelson and Somogyi (section III.1.1). Conditions for hydrolysis by the 20 kD and 29 kD xylanases were given by Tan et al. (1985a). For the 22 kD xylanase, 0.02 U/mL enzyme was incubated with the substrate at 40 C for 5 h, in 50 mM sodium acetate, pH 5, with 0.02% sodium azide. Reactions with 2 U/mL crude ultra-retentate were incubated at 50 C for 10 min. Hydrolysis of a substrate was considered positive if the

net increase in reducing ends was greater than that observed when xylan was hydrolyzed by 100 fold less enzyme.

Paper chromatography was used to test for hydrolysis of polysaccharides and disaccharides. 0.4 U/mL 22 kD xylanase or 0.7 U/mL crude ultra-retentate was mixed with 0.5% substrate in 50 mM ammonium acetate, pH 5, with 0.02% sodium azide. The former reaction was incubated at 40 C for 18 h and the latter at 45 C for 3 h. Reactions were stopped by 5 min boiling and the hydrolyzates were analyzed by paper chromatography. In both cases, as well as those for the 20 kD and 29 kD xylanases (Tan et al. 1985a), hydrolysis of xylan could be detected with 50 fold less enzyme.

HPLC (section II.1.3) was used to analyze enzyme hydrolyzates from 0.5% xylobiose, 0.5% xylotriose and 1% oat spelts arabinoxylan. The substrates, in 50 mM ammonium acetate pH 5 with 0.02% sodium azide, were incubated with 0.4 U/mL enzyme at 40 C for 24h. The reactions were stopped by 5 min boiling and the samples were subsequently lyophilized, redissolved in water and relyophilized. The samples were redissolved in half their initial volumes for HPLC analysis. An internal standard consisting of 5 μ mol/mL erythritol (Sigma) was added to the arabinoxylan hydrolyzates prior to their concentration.

IV.1.5. Amino acid analysis

The purified xylanases were dialyzed in distilled water with Spectrapor 3 membrane (Spectrum) and subsequently lyophilized to dryness. Amino acid composition was determined by Dr. M. Yaguchi at the National Research Council using the method reported by Paice et al. (1984).

IV.1.6. SDS-PAGE analysis of the culture filtrates from clones of T. harzianum

Clones from T. harzianum have been isolated and mutagenized at Forintek by Paul Meyers and Colette Breuil. Spores from the wild type (defined as the strain from the culture collection) were plated and growing colonies were isolated to give clones of the B and S series. One clone, B20, was sequentially exposed to two mutagenic treatments. Its spores were exposed to ultra-violet radiation and subsequently plated to isolate clones. Spores from one of these clones were then treated with nitrosoguanidine to obtain the clones of the N series. Details on these methods were given by Breuil & Saddler (1985). Four day old culture filtrates, of each clone grown on solka floc, were provided by Colette Breuil. One hundred microlitres of these culture filtrates were analyzed by SDS-PAGE (section III.1.2).

IV.2. RESULTS AND DISCUSSION

IV.2.1. Characterization of xylanases from T. harzianum

Ideally, any study on the interactions among xylanases is carried out under experimental conditions where all enzymes are stable and near their maximal activity. In search of such conditions, optimization and stability studies on the purified xylanases were performed. The 29 kD xylanase appeared to be the most stable of the 3 purified enzymes. It was stable across a relatively broad pH range (Figure 4.1) whereas the 20 kD xylanase was stable in a narrower range near pH 5. At 50 C, the 22 kD xylanase lost most of its activity throughout the entire pH range examined but appeared more stable near pH 5.5. All three xylanases, but particularly the 20 kD and 22 kD enzymes, were less active in the MES buffer.

Thermal stability studies confirmed that the 22 kD xylanase was not stable at 50 C and that the 29 kD xylanase was relatively the most stable (Figure 4.2). The half-life of the enzymes was 1 h at 56 C, 46 C and 60 C for the 20 kD, 22 kD and 29 kD xylanases respectively. These thermal stability characteristics were reflected in the temperature optima of the enzymes which were 50 C, 45-50 C and 60-65 C respectively (Figure 4.3). The stability and temperature optimum data showed that the three enzymes have distinct physico-chemical properties. In contrast, these xylanases were similar by having an optimal pH near 5 (Figure 4.4).

Figure 4.1. pH stability of the purified xylanases. The enzymes were incubated without substrate at 50 C for 1 h. Residual activity are reported in terms of percent of original activity. The 20 kD (+), 22 kD (o) and 29 kD (•) xylanases were incubated at concentration of 50 μ g/mL, 160 μ g/mL and 50 μ g/mL respectively, in 50 mM sodium acetate for pH 3.5 to 5.5 and 50 mM MES for pH 5.5 to 6.

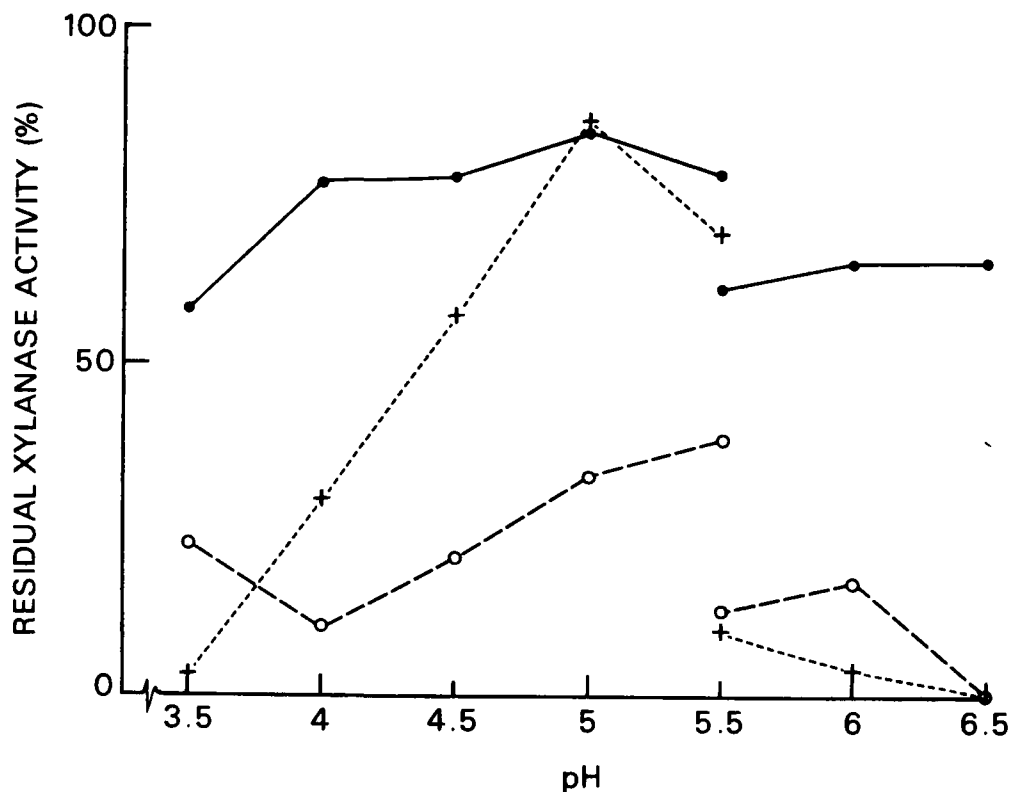


Figure 4.2. Thermal stability of the purified xylanases. The enzymes were incubated in 50 mM sodium acetate, pH 5, without substrate for an hour. Residual activity are reported in terms of percent of original activity. The 20 kD (+), 22 kD (o) and 29 kD (●) xylanases were incubated at concentration of 50 μ g/mL, 160 μ g/mL and 50 μ g/mL respectively.

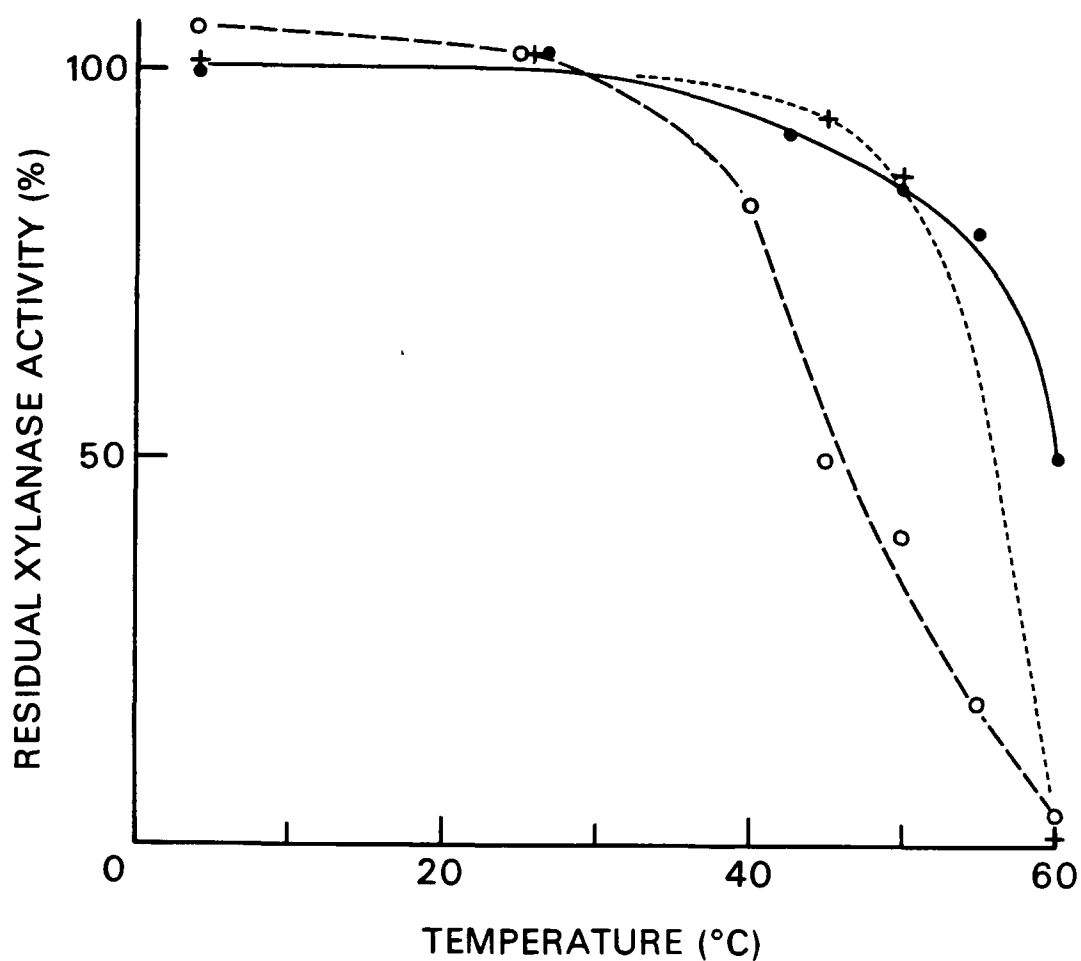


Figure 4.3. Temperature optimum of the purified xylanases. Ten minute assays of 0.2 μ g 20 kD xylanase (+), 160 μ g 22 kD xylanase (o) and 0.4 μ g 29 kD xylanase (•) were performed with 0.25% xylan in 50 mM sodium acetate, pH 5.

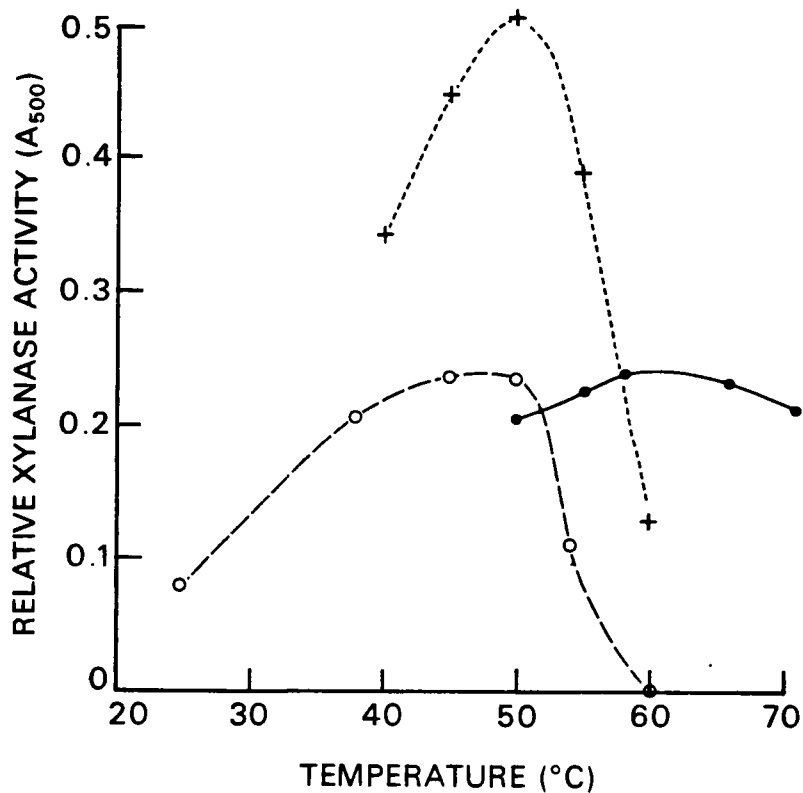
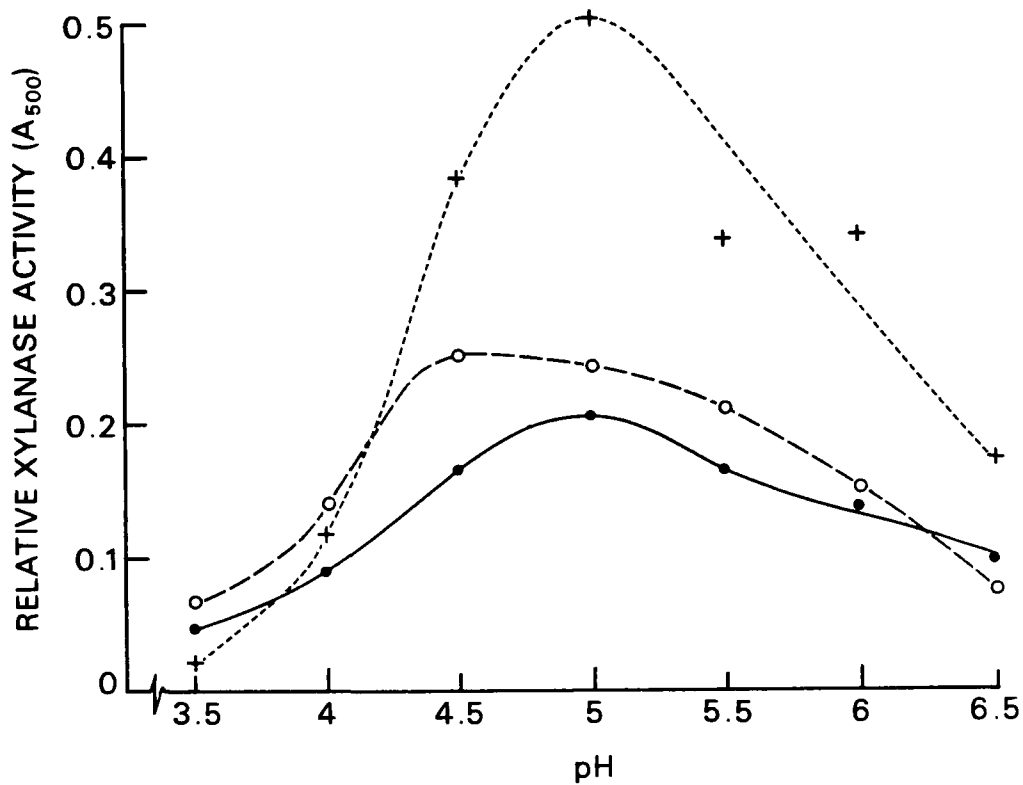


Figure 4.4. pH optimum of the purified xylanases. Ten minute assays of 0.2 μ g 20 kD xylanase (+), 160 μ g 22 kD xylanase (o) and 0.4 μ g 29 kD xylanase (•) were performed with 0.25% xylan at 50 C. The buffers were 50 mM sodium acetate for pH 3.5 to 5.5 and 50 mM MES for pH 5.5 to 6.5.



The acetate buffer at pH 4.8 is well suited for the xylanase system of T. harzianum because it provides the condition for near maximum stability and activity for all 3 purified enzymes. Thermal conditions suitable for studying the system is limited by the poor stability of the 22 kD xylanase. Figure 4.2 suggests that studies should be carried out under 40 C. I will set the temperature at 28 C which is also the temperature at which the fungal culture had been grown. At 28 C, denaturation of the enzymes should be low so that it will not be a factor in the interpretation of results. However, the enzymes will not have their maximal activities so that more enzymes will be required for each experiment.

IV.2.2. A functionally distinct system of xylanases in T. harzianum

To examine the substrate specificity of the 3 purified enzymes, their activity was assayed on numerous polysaccharides and disaccharides (Table 4.1). The identity of each polysaccharides was confirmed by their major sugar constituents (Table 4.2). Only xylans, CMC and solka floc were hydrolyzed by the 20 kD and 29 kD xylanases (Tan et al. 1985b). The activity on CMC was detected only by the reducing sugar assay and that on solka floc yielded xylose and xylobiose in the hydrolyzate. The 22 kD xylanase has similar specificity since it hydrolyzed only xylans

Table 4.2. Paper chromatographic analysis of the acid hydrolyzates from the following polysaccharides.

substrate	monomeric constituents	
	major	minor
aspenwood xylan	xylose	-
larchwood xylan	xylose	glucuronic acid
oat spelts xylan	xylose	arabinose, glucose
avicel	glucose	-
CF11	glucose	-
CMC	glucose	-
glucan	glucose	-
laminarin	glucose	-
lichenan	glucose	galactose
solka floc	glucose	-
starch	glucose	-
araban	arabinose	glucose, galatose
arabinogalactan	galactose	arabinose
galactan (Aldrich)	galactose	-
galactan (K & K)	galactose	arabinose
mannan	mannose	-
polygalacturonic acid	galacturonic acid	-

and solka floc (Table 4.3). It appears that the 3 enzymes are true xylanases since their primary substrate was xylan and they appeared to selectively hydrolyze xylan in solka floc. Although activity detected on CMC suggested weak endoglucanase activity in the 20 kD and 29 kD xylanases, cellulose and other hemicelluloses were not hydrolyzed extensively. Other enzymes in the crude ultra-retentate of T. harzianum were responsible for the hydrolysis of cellulose, glucans, cellobiose and xylobiose (Table 4.3). This fungus also produced enzymes to hydrolyze starch and polygalacturonic acid when grown on these substrates but it did not appear to produce a galactanase (data not shown). It appears that the 3 xylanases are components of a distinct system of enzymes.

The presence of xylobiase activity in the culture filtrate but not in the 3 xylanases was confirmed by HPLC analysis (Figure 4.5). The purified retentate which lacked the 3 xylanases also contained the activity for releasing arabinose from arabinoxylan (Figure 4.6) and the β -xylosidase activity (Figure 3.8). These characteristics of the purified retentate makes it an important component of the xylanolytic system.

Of the purified xylanases, data in Figure 4.6 suggests that only the 29 kD xylanase is capable of releasing xylose from xylan. However, other hydrolysis experiments (Table 4.3, Tan et al. 1985b) indicated that all three xylanases can release xylose from the three types of xylans. Furthermore, the 22 kD xylanase was capable of hydrolyzing xylotriase to release xylose (Figure 4.7)

Table 4.3. Hydrolysis of various substrates by the purified 22 kD xylanase and the crude ultra-retentate from the culture filtrate of *T. harzianum*. Hydrolysis was checked by the Nelson-Somogyi reducing sugars assay (RS) and/or by paper chromatography (PC). A positive result (+) is reported if equivalent hydrolysis of xylan was observed by RS with 100 fold less enzyme or by PC with 50 fold less enzyme. Moderate activity (++) is an intermediate level below the activity observed on xylan (+++).

substrate	crude ultra-retentate		22 kD xylanase	
	RS	PC	RS	PC
aspenwood xylan	+++	+++ *	+++	+++ *
larchwood xylan	+++	+++ *	+++	+++ *
oat spelts xylan	+++	+++ *	+++	+++ *
avicel	-	+ #	-	-
CF11	-	+ #	-	-
CMC	++	+++ #	-	-
glucan	+++	+++ #	-	-
laminarin	+++	+++ #	-	-
lichenan	++	+++ #	-	-
solka floc	+	+++ #	-	+ *
starch	+	-	-	-
araban	-	+ #	-	-
arabinogalactan	+	-	-	-
galactan (Aldrich)	nd	-	nd	-
galactan (K & K)	-	-	-	-
mannan	-	-	-	-
polygalacturonic acid	-	-	-	-
cellobiose	nd	+ #	nd	-
galactopyranosyl- arabinose	nd	-	nd	-
lactose	nd	-	nd	-
melibiose	nd	-	nd	-
sucrose	nd	-	nd	-
xylobiose	nd	+ *	nd	-

* hydrolyzate contained xylose and xylobiose

hydrolyzate contained glucose and cellobiose

nd not determined

Figure 4.5. Hydrolysis of xylobiose by the purified xylanases. (i) Substrate control (no enzyme). The substrate (0.5% xylobiose) was incubated at 40 C for 24 h with 0.4 U/mL 20 kD xylanase (ii), 22 kD xylanase (iii), 29 kD xylanase (iv) and purified retentate (v). The HPLC retention times for xylobiose and xylose were 11.4 and 13.4 min, respectively.

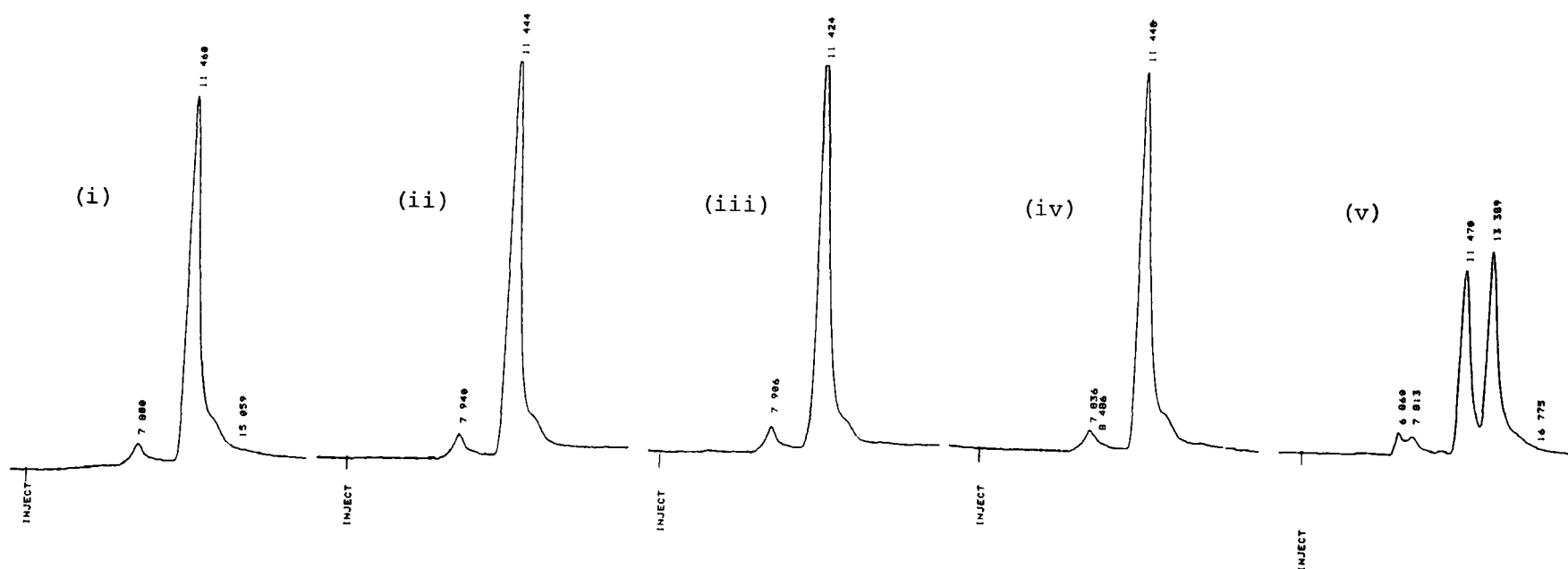


Figure 4.6. Hydrolysis of oat spelts arabinoxylan by the purified xylanases. (i) Substrate control (no enzyme). The substrate (1% xylan) was incubated at 40 C for 24 h with 0.4 U/mL 20 kD xylanase (ii), 22 kD xylanase (iii), 29 kD xylanase (iv) and purified retentate (v). An internal standard of 5 μ mol/mL erythritol was added to the samples before analysis by HPLC. The retention times for xylobiose, xylose, arabinose and erythritol were 11.6, 13.5, 16.0 and 20.2 min, respectively.

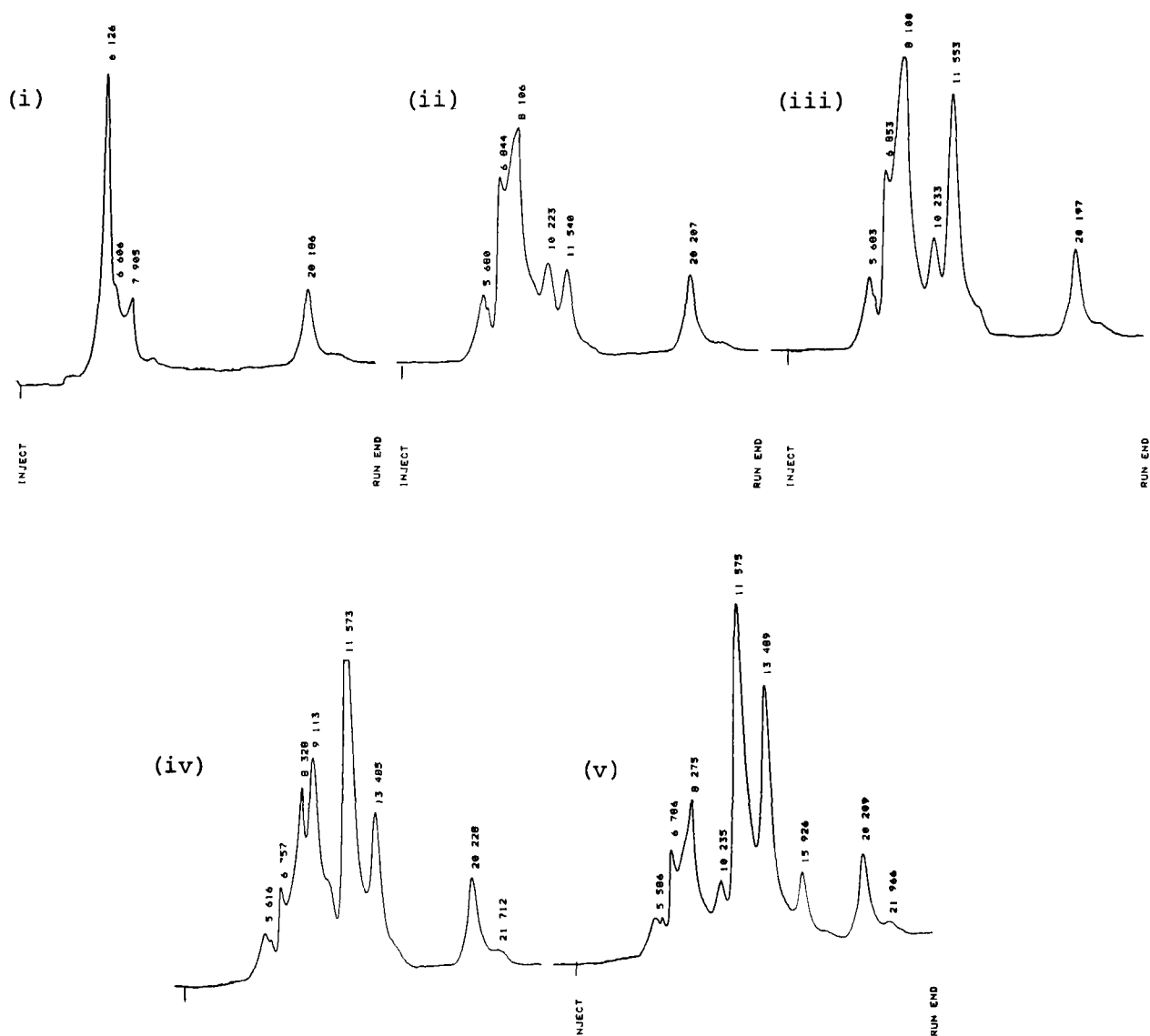
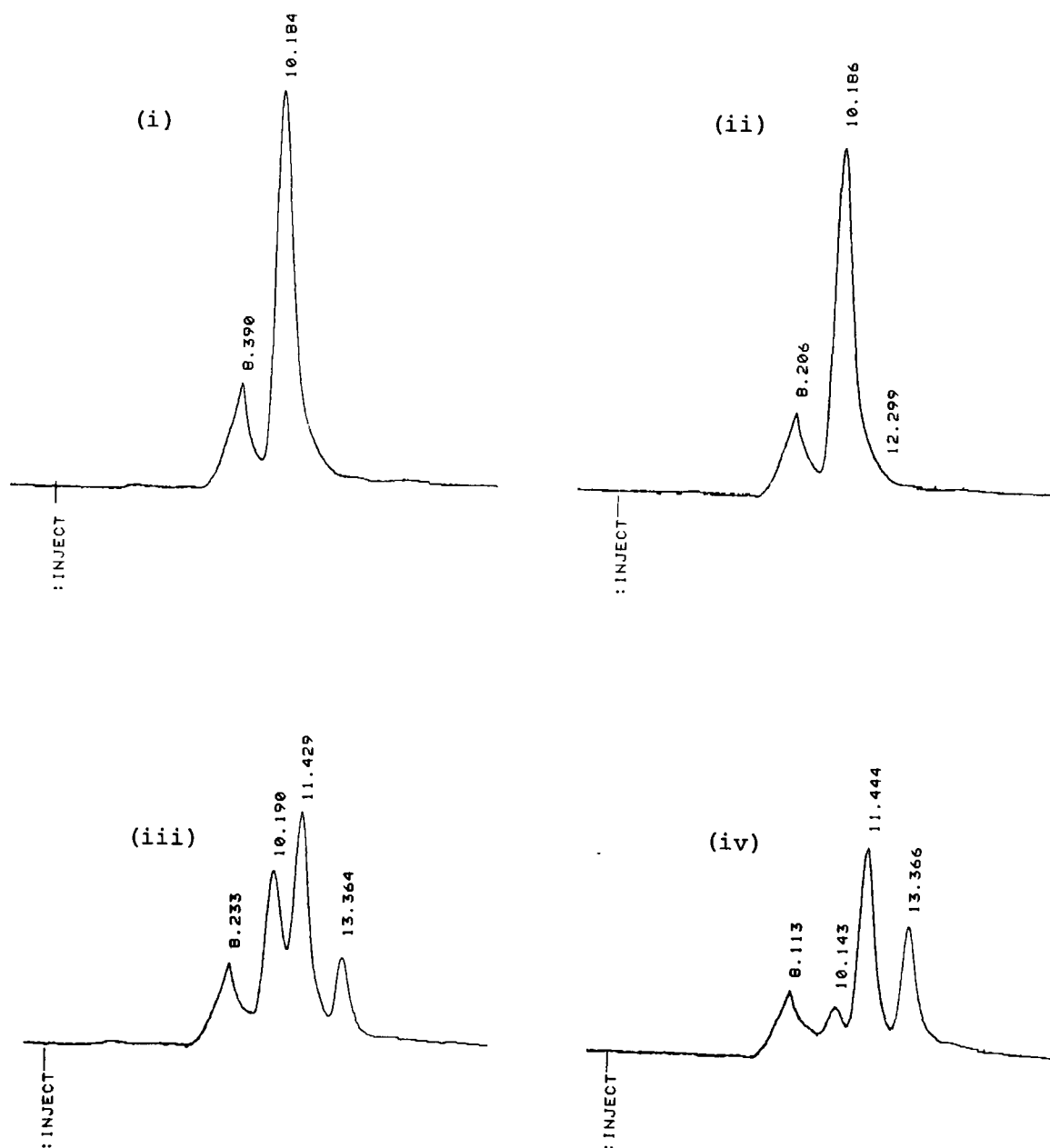


Figure 4.7. Hydrolysis of xylotriose by the purified xylanases. (i) Substrate control (no enzyme). The substrate (0.5% xylotriose) was incubated at 40 C for 24 h with 0.4 U/mL 20 kD xylanase (ii), 22 kD xylanase (iii) and 29 kD xylanase (iv). The HPLC retention times for xylotriose, xylobiose and xylose were 10.2, 11.4 and 13.4 min, respectively.



and the 20 kD xylanase released xylose in the hydrolysis of xylotetraose (Tan et al. 1985b). I believe that the degree of xylose released in the hydrolysis of xylan by the 20 kD and 22 kD xylanases was too low for detection by HPLC analysis under my conditions.

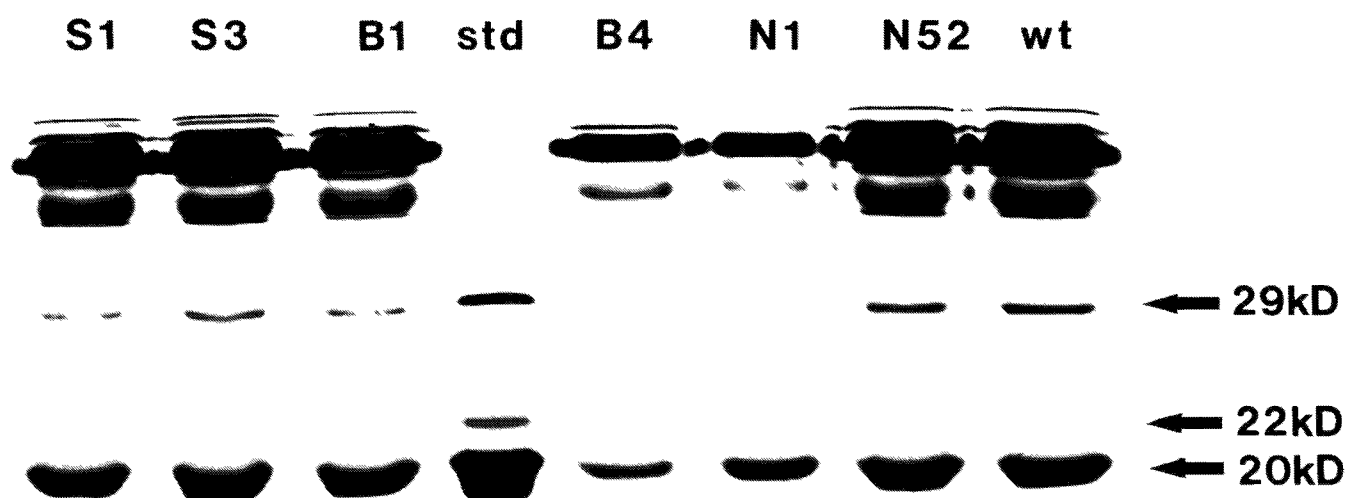
It appears that the three xylanases are different gene products because they have different physico-chemical properties and different abilities to hydrolyze xylan and xylotriose. This suggestion is conclusively demonstrated by the amino acid composition of the enzymes (Table 4.4). Precursor/product and enzyme/proteolytic modification relationships among proteins require that the larger enzymes have all the residues found in the smaller enzymes. However, the 20 kD xylanase has the most threonine and tyrosine and it also has remarkedly more serine and tryptophan than the 22 kD xylanase. Furthermore, the 22 kD xylanase has more glycine, half-cystine and proline than the 29 kD xylanase. This information clearly indicates that the 3 xylanases are distinct gene products. SDS-PAGE analysis of the culture filtrates from 6 clones isolated from T. harzianum showed that they contain the three characterized xylanases (Figure 4.8). A question that remains is whether an essential function can be assigned to each enzyme and thereby demonstrate a functional purpose for the multiplicity of xylanase.

Table 4.4. Amino acid composition of xylanases from T. harzianum.

amino acid	xylanase		
	20 kD	22 kD	29 kD
alanine	8	22	27
arginine	6	5	8
asparagine/aspartic acid ...	20	21	31
half-cystine	0	10	2
glutamine/glutamic acid	9	16	21
glycine	24	26	21
histidine	4	4	6
isoleucine	9	9	12
leucine	5	8	22
lysine	4	5	15
methionine	1	2	2
phenylalanine	7	5	6
proline	6	13	9
serine	20	15	24
threonine	16	9	11
tryptophan	6	0	8
tyrosine	16	11	12
valine	12	16	16
total number	173	197	253
calculated M.W. (kD) *	18.9	20.3	27.7

* assuming 1:1 ratio of asparagine:aspartic acid and glutamine:glutamic acid

Figure 4.8. SDS-PAGE analysis of the culture filtrates from T. harzianum clones. The cultures were 4 days old and 100 μ l samples were applied on the gel. Clones (B1, B4, S1 and S3) were isolated by plating spores from the wild type (wt) and some clones (N1 and N52) had been mutagenized using ultra-violet radiation and nitrosoguanidine. The standards (std) were 38 μ g 20 kD xylanase, 8 μ g 22 kD xylanase and 4 μ g 29 kD xylanase.



V. INTERACTIONS AMONG COMPONENTS OF THE XYLANOLYTIC SYSTEM

Three xylanases have been purified from Trichoderma harzinaum. Since by definition, all xylanases can hydrolyze xylan, the advantage of a multiplicity in xylanases is not immediately obvious. The complexity of the lignocellulosic structure suggests that not all the xylosidic linkages are equally accessible, some may be attacked by all xylanases but others may be hydrolyzed by only a few. Therefore, the importance of xylanase multiplicity may be illustrated by the complementation of these enzymes in the hydrolysis of its complex substrate.

Complementation of the xylanases purified from T. harzianum is studied at two extreme conditions. Under substrate limiting conditions, complementation is examined in terms of the final hydrolysis of the substrate where there is no further net increase in hydrolysis. Substrate hydrolysis may be limited by the total number of accessible linkages and/or product inhibition of the enzymes. In substrate limiting hydrolysis, xylanases complement each other if hydrolysis is greater than the highest level achieved by any single xylanase. Under enzyme limiting conditions, complementation is examined in terms of initial hydrolysis rates and it is therefore biased towards hydrolysis of the more accessible linkages. In enzyme limiting hydrolysis, xylanases complement each other if their overall initial hydrolysis rate is greater than the sum of the individual rates provided by each enzyme.

Complementation under substrate limiting conditions had been established for two xylanases from Aspergillus niger by Takenishi and Tsujisaka (1975). These two xylanases together were able to increase the hydrolysis of an arabinoxylan to a level which was 13% higher than that achieved by the enzymes individually. This was apparently due to the ability of one enzyme to release the arabinose residue and the other to cleave xylotriase. Yoshioka et al. (1981) observed a 20% increase in the substrate limiting hydrolysis of an arabinoxylan by the mixture of 3 xylanases purified from Talaromyces byssochlamydoides. Complementation under enzyme limiting conditions was reported by Sreenath & Joseph (1982) who found that the specific activity of the mixture of 3 xylanases from Streptomyces exfoliatus was 16% higher than the average of their individual specific activities.

For the study of cooperative interaction among purified xylanases, the enzymes may be mixed on the basis of equal protein, of equal xylanolytic activity or of protein ratios found in the culture filtrate. The equal protein strategy is poorly suited for my experiments because there is a large range in the specific activities of my enzyme preparations. The use of the equal activity method is well suited for substrate limiting hydrolysis experiments because conditions can be easily established where the enzymes remain active but are unable to hydrolyze the substrate further. This method would also simplify the data analysis for enzyme limiting hydrolysis experiments because the individual enzyme preparations will provide similar

initial hydrolysis rates. It will be adequate for determining whether positive interaction among the xylanases is possible. I decided to first determine that complementation among the xylanases occurs before considering whether the optimal ratio of xylanases is biologically relevant.

V.1. MATERIALS AND METHODS

V.1.1. Hydrolysis at 28 C

Hydrolysis experiments were carried out at 28 C in a shaking water bath with the agitation set at 100 strokes per minute. The 1 mL sample was in a 1.6 cm X 10 cm test tube (sealed with parafilm for long-term hydrolyses) held at approximately 35° from vertical. Specific activities of the purified retentate and the purified 20 kD, 22 kD and 29 kD xylanases (section III.1.3 to III.1.5) were determined using one hour assays (section III.1.1). To avoid confusion with the activity measured at 50 C, the symbol u was used for activity measured at 28 C.

V.1.2. Time course of xylan hydrolysis

Separate samples containing 2.5 mg deacetylated aspen xylan (section II.1.1) in 1 mL 50 mM sodium acetate, pH 4.8, were incubated with 0.01 u enzyme. At chosen time intervals over a

72 h period, hydrolysis in selected samples was stopped by 5 min boiling. At 48 h, 0.01 u fresh enzyme or 2.5 mg xylan was added directly into selected samples and hydrolysis was allowed to continue. After all the samples were collected, reducing sugars were assayed using the Nelson-Somogyi method (section III.1.1).

V.1.3. Hydrolysis of xylans under substrate limiting conditions

Forty-eight hour hydrolysis of 2.5 mg deacetylated aspen xylan, acetylated aspen xylan (section II.1.2) and oat arabinoxylan (Sigma) by enzyme combinations was carried out in 1 mL 50 mM ammonium acetate, pH 4.8 at 28 C. Each enzyme combination consisted of 1 q of each enzyme preparation in the combination, where 1 q was 0.012 u, 0.011 u, 0.012 u and 0.017 u for the 20 kD xylanase, the 22 kD xylanase, the 29 kD xylanase and the purified retentate respectively. Hydrolysis by all possible combinations of the 4 enzyme preparations was measured in 3 identical trials except that one trial was carried out with 0.6 q purified retentate.

The level of reducing sugars produced by an enzyme combination was compared to the highest level produced by a single enzyme from the combination. For example, the hydrolysis by the combination which consisted of 1 q each of the 20 kD and the 22 kD xylanases was compared to that by 2 q 20 kD xylanase or

that by 2 μ g 22 kD xylanase, whichever was higher. The t-test was used to determine the significance of any differences in these comparisons with the assumption that the variance of the two means were equal.

Part of the hydrolyzates were analyzed by HPLC (section II.1.3) for xylose and xylobiose content. An internal standard of 5 μ mol/mL erythritol was added to these samples before they were lyophilized twice and made up to half their initial volume with water.

V.1.4. Hydrolysis of xylans under enzyme limiting conditions

Triplicate experiments were carried out at 28 C with 2.5 mg substrate in 1 mL 50 mM sodium acetate, pH 4.8. The hour long hydrolysis used a total of 12 μ g enzyme which was made up with equal units of each enzyme preparation in the combination. The enzyme unit μ g was equal to 0.13 mu, 0.09 mu, 0.10 mu and 0.13 mu for the 20 kD, the 22 kD, the 29 kD xylanases and the purified retentate respectively. The hydrolysis by a combination was compared to the arithmetic sum of the hydrolyses by individual enzymes in the combination. For example, the hydrolysis by the combination which consisted of 6 μ g each of the 20 kD and the 22 kD xylanases was compared to the average of the hydrolyses by 12 μ g 20 kD xylanase and that by 12 μ g 22 kD xylanase. The hydrolysis of deacetylated aspen xylan by 12 μ g xylanase proceed at a constant rate over 1 h. Again, the t-

test was used to determine significance of differences but the assumption made was that the variance of the two means were not equal.

V.1.5. Hydrolysis of aspen holocellulose

Hydrolysis of aspen holocellulose was initially carried out with the procedure used for substrate limiting hydrolysis of xylans (section V.1.3) except that the ammonium buffer was replaced by the sodium acetate buffer. A second set of experiments was carried out with combinations of xylanases on an equal protein instead of on an equal activity basis. These experiments used combinations containing 50 μ g of each enzyme and similar data analysis was carried out. The purified retentate was not studied in these experiments.

The stability of the purified xylanases with holocellulose was checked using time course experiments (section V.1.2). 2.5 mg substrate was incubated for 48 h with 0.012 u, 0.011 u and 0.012 u of the 20 kD, the 22 kD and the 29 kD xylanases, respectively. Subsequently, 2.5 mg deacetylated aspen xylan was directly added to the samples and hydrolysis was monitored.

The products of holocellulose hydrolysis were analyzed by HPLC. Hydrolysis of 1% substrate in the ammonium acetate buffer was carried out at 40 C for 24 h using 50 μ g/mL enzyme. After

the hydrolysis was stopped by boiling, an internal standard of 2 $\mu\text{mol/mL}$ erythritol was added to each sample. Before each sample was analyzed, the insoluble residues were removed by centrifugation and the resulting supernatant was lyophilized twice and redissolved in water to one-fifth or one-tenth its initial volume.

V.2. RESULTS AND DISCUSSION

V.2.1. Hydrolysis of isolated xylans

The specific activities of the purified xylanases and the purified retentate at 28 C are given on Table 5.1. Time course studies showed that at 28 C, 48 h hydrolysis of 2.5 mg deacetylated aspen xylan by 0.01 u xylanase was predominately substrate limiting (Figure 5.1). Although further hydrolysis was minimal after 48 h under these conditions, the xylanases remained active and hydrolyzed freshly added substrate. Furthermore, the 3 purified xylanases subsequently hydrolyzed the freshly added substrate to an extent approximately equivalent to the initial 24 h hydrolysis so that product inhibition did not appear to be the cause for the cessation of hydrolysis. These conditions were subsequently used for studying the substrate limiting hydrolysis of xylans by enzyme combinations.

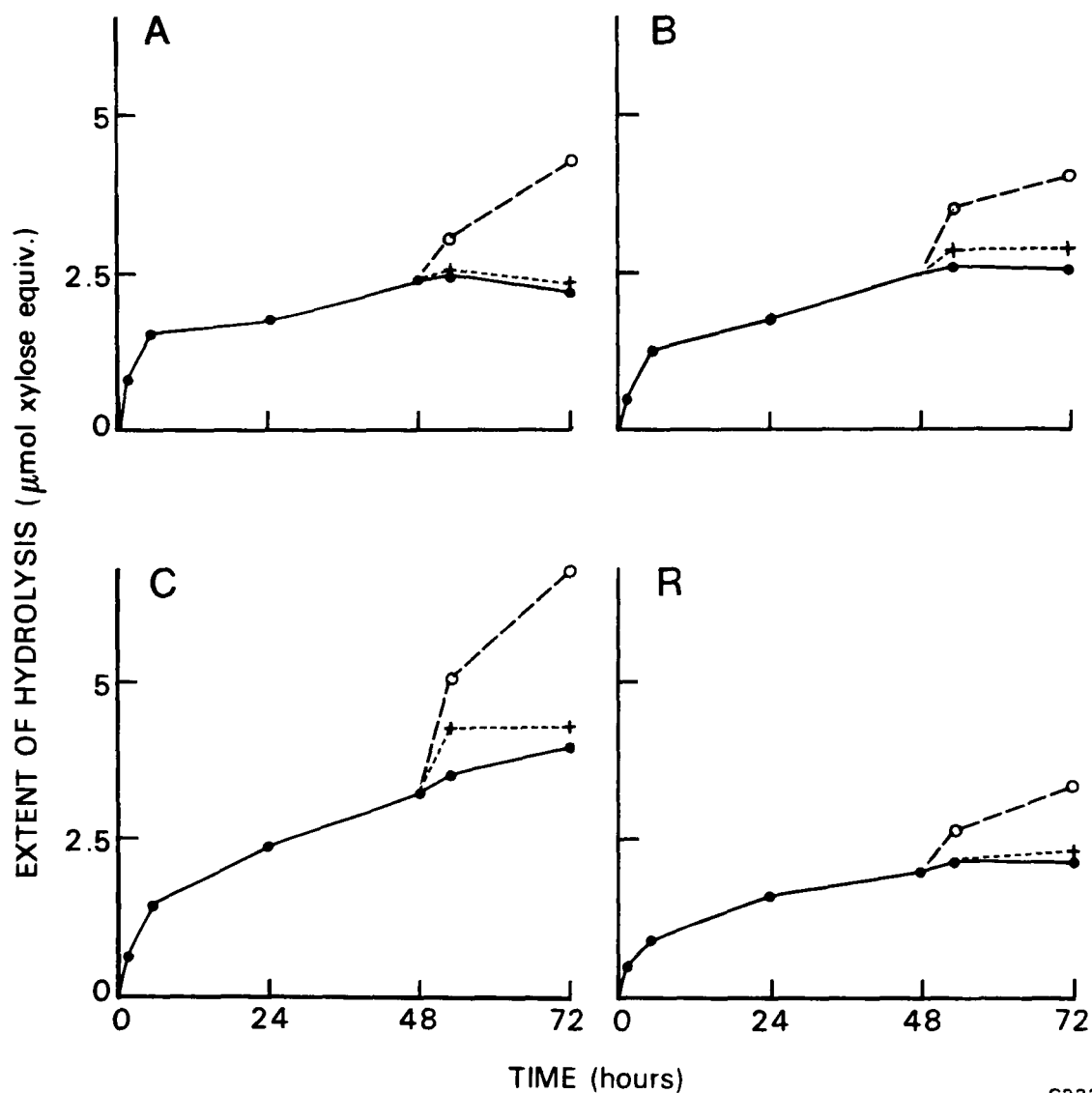
For hydrolysis by single enzyme preparations, predominately substrate limiting conditions had been achieved in most cases

Table 5.1. The enzyme activity of the purified xylanases at 28 C.

assay	enzyme	specific activity (u/mg)
xylanase	20 kD	76
	22 kD	0.16
	29 kD	29
	purified retentate	0.20
β -xylosidase	purified retentate	0.20
CMCase *	purified retentate	1.0

* CMC = carboxymethyl-cellulose
 (assay is identical to xylanase assay except that CMC
 was used as the substrate)

Figure 5.1. The time course of xylan hydrolysis by the 20 kD xylanase (A), the 22 kD xylanase (B), the 29 kD xylanase (C) and the purified retentate (R). 2.5 mg deacetylated aspen xylan was hydrolyzed with 0.01 u enzyme. At 48 h, the hydrolysis was continued with no supplement (●), with the addition of 0.01 u enzyme (+) or with the addition of 2.5 mg xylan (○).



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since there was no great increase in hydrolysis with 4 fold increase in enzyme (Figure 5.2). The exception was the hydrolysis of oat arabinoxylan by the purified retentate. Small increases in the hydrolyses of all 3 xylans by the 20 kD xylanase and the hydrolysis of oat arabinoxylan by the 29 kD xylanase was observed with increasing enzyme. The extent of hydrolysis by the 4 enzyme preparations varied among the 3 xylans. Relative to the other two xylanases, the ability of the 20 kD xylanase to hydrolyze the acetylated aspen xylan and oat arabinoxylan was considerably lower than that on the deacetylated aspen xylan. This result suggests that acetyl and arabinosyl side-groups inhibit the activity of the 20 kD xylanase. It is more difficult to explain the relatively poor capability of the purified retentate in hydrolyzing the deacetylated aspen xylan as compared to the two more complex xylans. It is possible that there was more nonspecific binding between the excess non-xylanolytic enzymes and the substrate which resulted in an inhibition of xylanase activity.

Under substrate limiting conditions, combinations of the purified xylanases did result in statistically significant increases in the hydrolysis of aspen xylans (Table 5.2). The combination of all three xylanases increased the hydrolysis of the deacetylated and acetylated aspen xylans by 18% and 27% respectively (Figure 5.3). It resulted in the maximum hydrolysis by any combinations of these 3 enzymes (Figure 5.4). The combinations with any two xylanases also increased the hydrolysis of the acetylated aspen

Figure 5.2. Substrate limiting hydrolysis of 2.5 mg deacetylated aspen xylan (i), acetylated aspen xylan (ii) and oat arabinoxylan (iii) by the purified xylanases. The enzyme unit q was equal to 0.012 u, 0.011 u, 0.012 u and 0.017 u for the 20 kD xylanase (A), the 22 kD xylanase (B), the 29 kD xylanase (C) and the purified retentate (R) respectively.

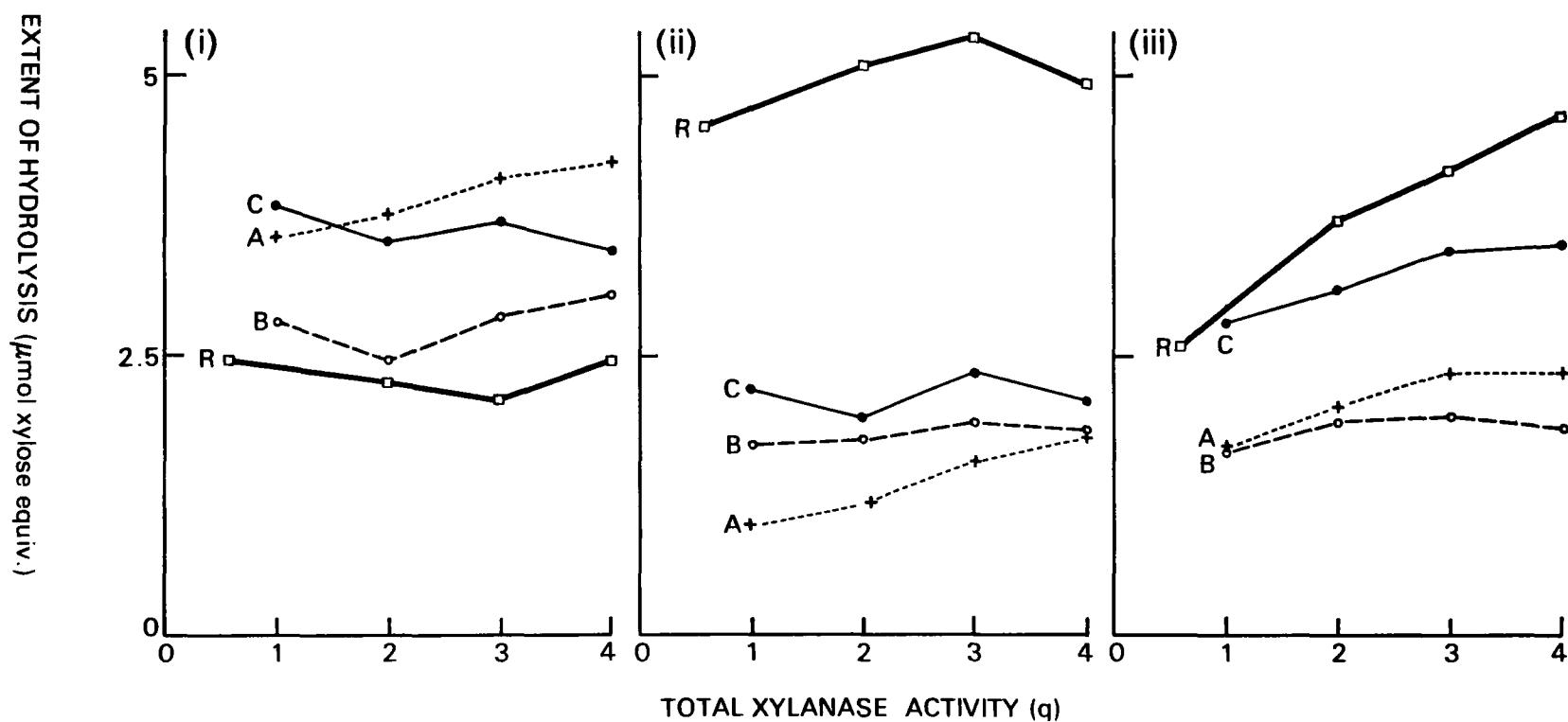


Table 5.2. Substrate limiting hydrolysis of xylans by combinations of the purified xylanases. 2.5 mg deacetylated aspen xylan, acetylated aspen xylan and oat arabinoxylan were hydrolyzed with combinations containing 1 q (see footnote) each of the 20 kD xylanase (A), the 22 kD xylanase (B), the 29 kD xylanase (C) and/or the purified retentate (R) for 48 h at 28 C and pH 4.8.

added enzyme activity (q) *					extent of hydrolysis (umol xylose equiv.) #								
					deacetylated			acetylated			arabinoxylan		
sum	A	B	C	R	mean	sd	sig	mean	sd	sig	mean	sd	sig
1	1	-	-	-	3.6	0.4		0.9	0.1		1.7	0.1	
	-	1	-	-	2.8	0.1		1.7	0.1		1.6	0.1	
	-	-	1	-	3.9	0.3		2.2	0.5		2.8	0.1	
	-	-	-	0.6 @	2.5	0.1		4.6	0.5		2.6	0.1	
2	2	-	-	-	3.8	0.5		1.2	0.1		2.1	0.2	
	-	2	-	-	2.4	0.1		1.8	0.1		1.9	0.1	
	-	-	2	-	3.5	0.2		2.0	0.1		3.1	0.2	
	-	-	-	2	-	-		5.1	0.1		3.7	0.2	
	1	1	-	-	3.6	0.2	nsd	2.2	0.1	+	2.2	0.2	nsd
	1	-	1	-	3.8	0.4	nsd	2.6	0.1	+	3.1	0.2	nsd
	1	-	-	1	-	-		5.0	0.1	nsd	3.4	0.1	nsd
	-	1	1	-	3.2	1.1	nsd	2.5	0.1	+	3.0	0.1	nsd
	-	1	-	1	-	-		3.5	0.1	-	3.4	0.1	nsd
-	-	1	1	-	-		5.6	0.1	+	4.1	0.2	nsd	
3	3	-	-	-	4.1	0.2		1.5	0.2		2.4	0.4	
	-	3	-	-	2.9	0.3		1.9	0.2		2.0	0.1	
	-	-	3	-	3.7	0.3		2.4	0.2		3.4	0.2	
	-	-	-	3	2.1	0.1		5.4	0.3		4.2	0.1	
	1	1	1	-	4.8	0.2	+	3.0	0.3	+	3.3	0.2	nsd
	1	1	-	1	4.1	0.5	nsd	3.8	0.3	-	3.6	0.2	-
	1	-	1	1	3.9	0.5	nsd	5.4	0.1	nsd	4.2	0.2	nsd
	-	1	1	1	2.8	0.2	-	3.9	0.3	-	4.0	0.1	nsd
4	4	-	-	-	4.3	0.3		1.8	0.3		2.4	0.5	
	-	4	-	-	3.0	0.3		1.8	0.2		1.9	0.2	
	-	-	4	-	3.4	0.2		2.1	0.2		3.5	0.2	
	-	-	-	4	2.5	0.1		4.9	0.4		4.6	0.1	
	1	1	1	1	4.8	0.2	+	4.0	0.5	-	4.1	0.2	-

* 1 q was equal to 0.012 u, 0.011 u, 0.012 u and 0.017 u for A, B, C and R respectively

mean from 3 samples with its standard deviation (sd)
 (for combinations with R, data are from 2 samples and a third trial with 0.6 q R does not contradict results)
 significant difference (sig) from expected hydrolysis (i.e. highest hydrolysis by single enzyme from the combination)
 nsd: not significantly different
 + : significantly greater at $P < 0.05$
 - : significantly lower at $P < 0.05$

@ this sample had only 0.6 q R

Figure 5.3. Substrate limiting hydrolysis of xylans by combinations of xylanases expressed as percent of hydrolysis expected if no complementation occurred. Deacetylated aspen xylan (i), acetylated aspen xylan (ii) and oat arabinoxylan (iii) were hydrolyzed with enzyme combinations containing equivalent activity units of the 20 kD xylanase (A), the 22 kD xylanase (B), the 29 kD xylanase (C) and/or the purified retentate (R). When there is no interaction among the enzymes in a combination, the expected hydrolysis is the highest hydrolysis found when the enzyme activity of the combination is made up by a single enzyme preparation from the combination. The single enzyme which provided the highest hydrolysis (100% expected hydrolysis) in each case is underlined on the axis. Significant differences ($P < 0.05$) are marked with *.

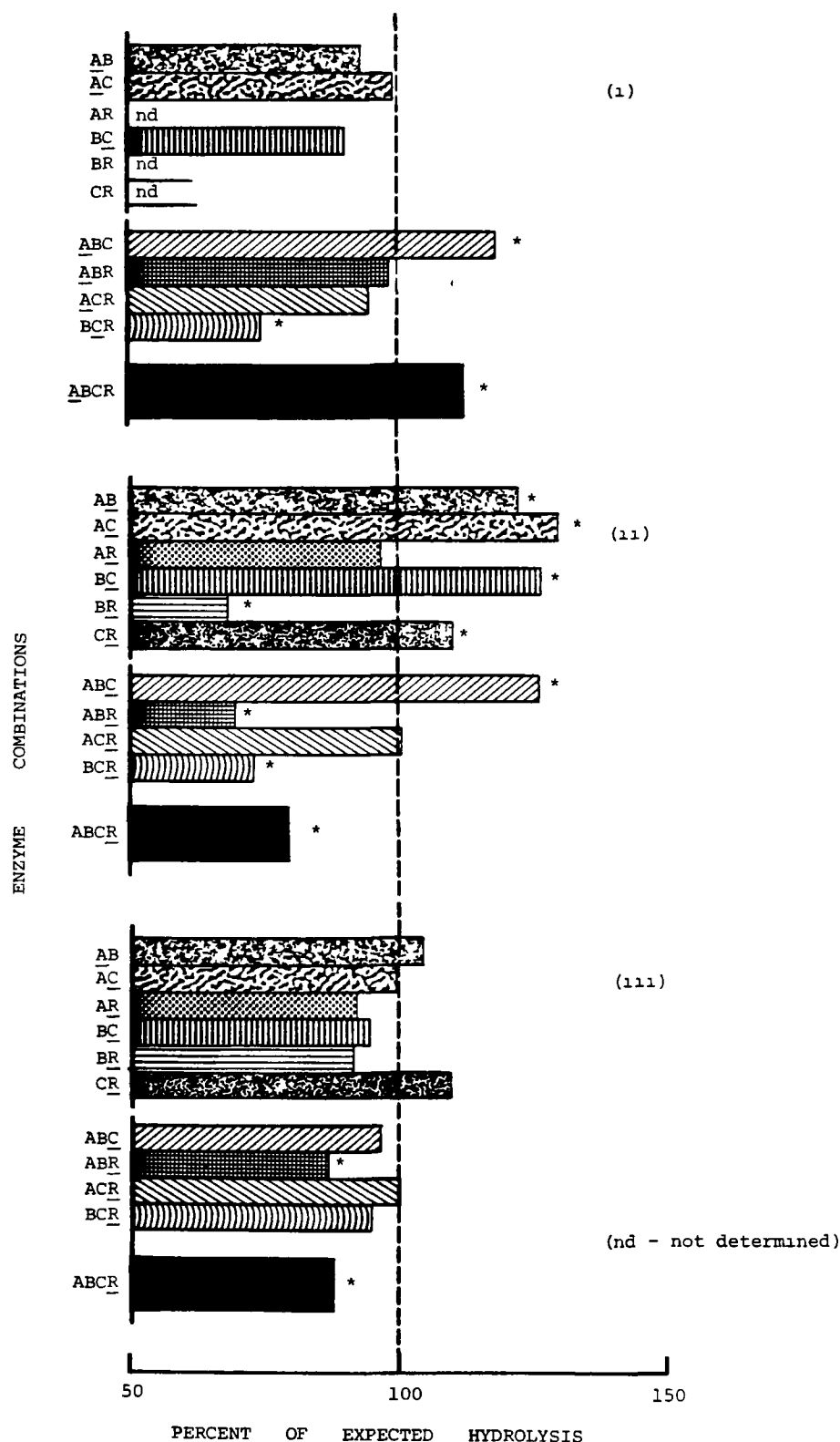
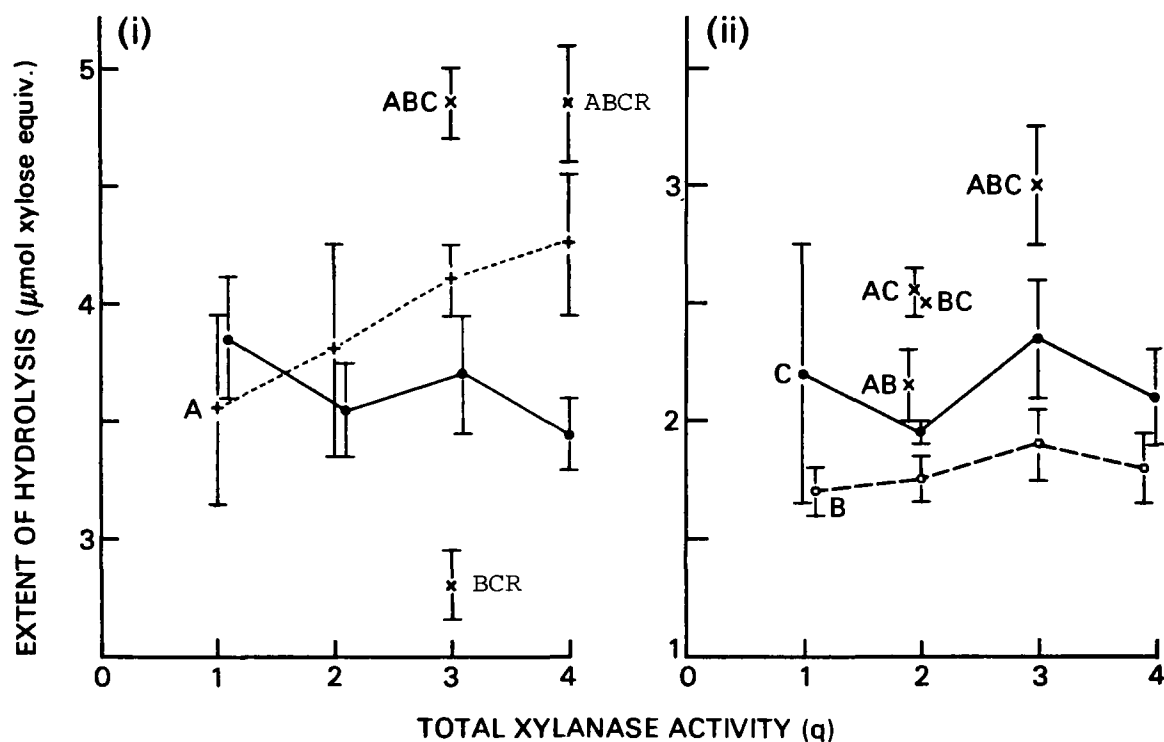


Figure 5.4. Complementation of xylanases in the substrate limiting hydrolysis of 2.5 mg deacetylated (i) and acetylated (ii) aspen xylans. The extent of hydrolysis by enzyme combinations (x) is compared to the highest level achieved with a single enzyme from the combination. Only the highest hydrolyses by single enzymes from the combinations are shown (see Figure 5.2). The enzyme combinations were made up with 1 q each of the 20 kD xylanase (A), the 22 kD xylanase (B), the 29 kD xylanase (C) and/or the purified retentate (R). The enzyme unit q was equal to 0.012 u, 0.011 u, 0.012 u and 0.017 u for A, B, C and R respectively.

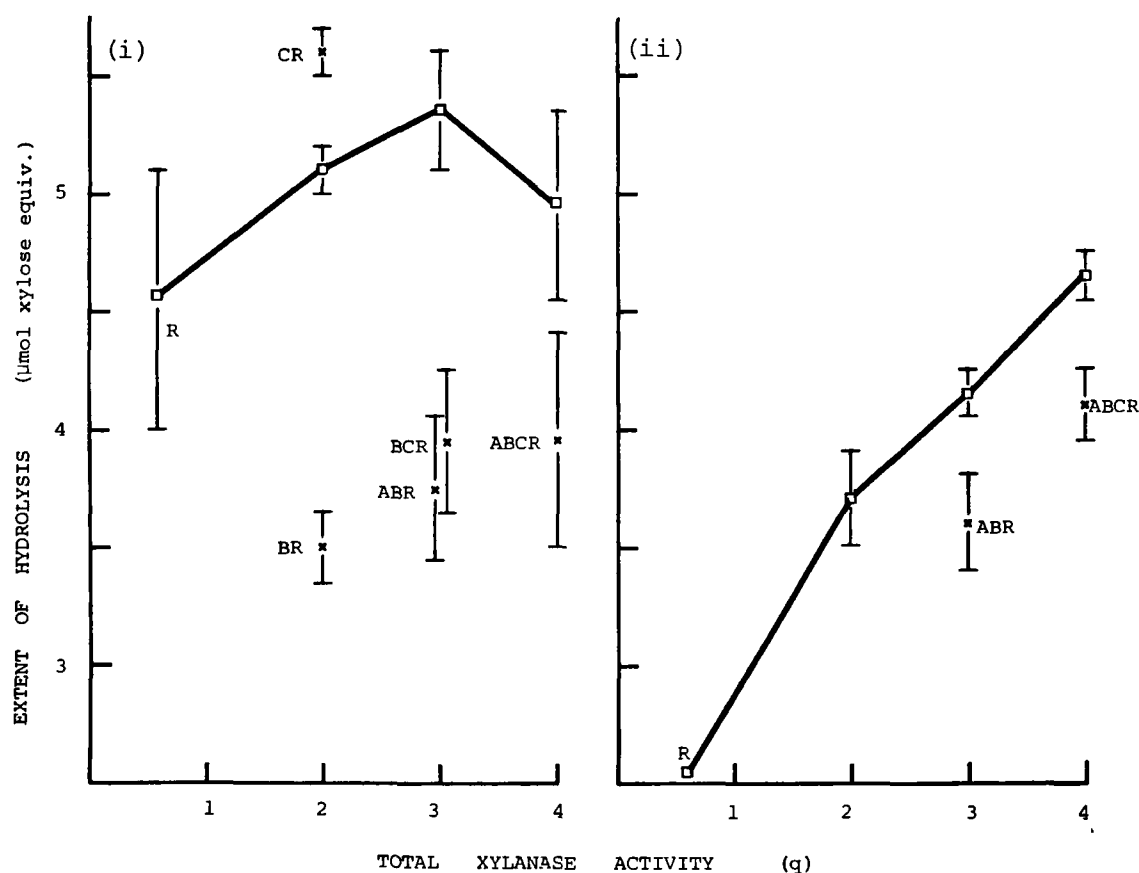


xylan but not to the extent achieved by the combination of all three enzymes. However, since the combination of all 3 xylanases significantly increased the hydrolysis of both aspen xylans, the results do not conclusively demonstrate that acetyl substituents are important factors in the complementation of the 3 xylanases. Complementation between a xylanase and an acetyl xylan esterase in the hydrolysis of an acetylated xylan had been reported by Biely et al. (1985b).

A second noticeable pattern in the data is the inhibition of the purified retentate by the 22 kD xylanase in the hydrolysis of acetylated aspen xylan (Figure 5.5.i). In my experiments, the 22 kD xylanase had been used in copious quantities due to its low activity and may have outcompeted the enzymes in the purified retentate for binding sites. While hydrolyzing the substrate slowly, the 22 kD xylanase may protect hydrolysis sites from more active xylanases in the purified retentate and thus inhibits the overall hydrolysis. This proposed explanation suggests that the inhibition can be negated by increasing hydrolysis time.

Other data in Table 5.2 do not suggest any other conclusive patterns. The combination of the 22 kD xylanase, the 29 kD xylanase and the purified retentate provided unexpected low hydrolysis of deacetylated aspen xylan (Figure 5.4.i) while the 29 kD xylanase appeared to complement the purified retentate in the hydrolysis of acetylated aspen xylan (Figure 5.5.i). Because substrate limiting conditions were not reached in the hydrolysis of oat arabinoxylan, it is difficult to interpret the low

Figure 5.5. Inhibition of the purified retentate in the substrate limiting hydrolysis of 2.5 mg acetylated aspen xylan (i) and oat arabinoxylan (ii). The extent of hydrolysis by enzyme combinations (x) is compared to that by the purified retentate alone (□). The enzyme combinations were made up with 1 q of the purified retentate (R) and 1 q each of the 20 kD xylanase (A), the 22 kD xylanase (B) and/or the 29 kD xylanase (C). The enzyme unit q was equal to 0.012 u, 0.011 u, 0.012 u and 0.017 u for A, B, C and R respectively.



hydrolysis provided by the combinations which contained the purified retentate, the 20 kD and the 22 kD xylanases (Figure 5.5.ii).

The absence of complementation among the 3 xylanases in the hydrolysis of oat arabinoxylan was not unexpected since none of them releases arabinose from arabinoxylan (Figure 4.6). The purified retentate hydrolyzed the oat arabinoxylan more completely (Figure 5.2), probably due to its ability to release the arabinose substituents. Furthermore, it could also hydrolyze the acetylated aspen xylan more completely than the purified xylanases. It would appear that the purified retentate contained the most important components of the xylanolytic system.

However, the high quantity of the characterized xylanases in the culture filtrate indicated that these xylanases could not all be functionally irrelevant. The purified retentate may not have been suited for my complementation studies because it was a crude preparation which contained an excess of non-xylanolytic enzymes besides the xylanolytic enzymes other than the 3 characterized xylanases.

Under enzyme limiting conditions, combinations of xylanases increased overall hydrolysis rates by up to 25% but these increases were not statistically significant (Figure 5.6). A significant increase was observed when deacetylated aspen xylan was hydrolyzed by the combination which contained the 29 kD xylanase and the purified retentate (Table 5.3). This increase

Figure 5.6. Enzyme limiting hydrolysis of xylans by combinations of xylanases expressed as percent of hydrolysis expected if no complementation occurred. Deacetylated aspen xylan (i), acetylated aspen xylan (ii) and oat arabinoxylan (iii) were hydrolyzed with enzyme combinations containing equivalent amounts of the 20 kD xylanase (A), the 22 kD xylanase (B) and/or the 29 kD xylanase (C). When there is no interaction among the enzymes in a combination, the expected hydrolysis is the arithmetic sum of the hydrolysis by the individual enzyme preparations in the combination. Interactions were not significant at $P < 0.05$.

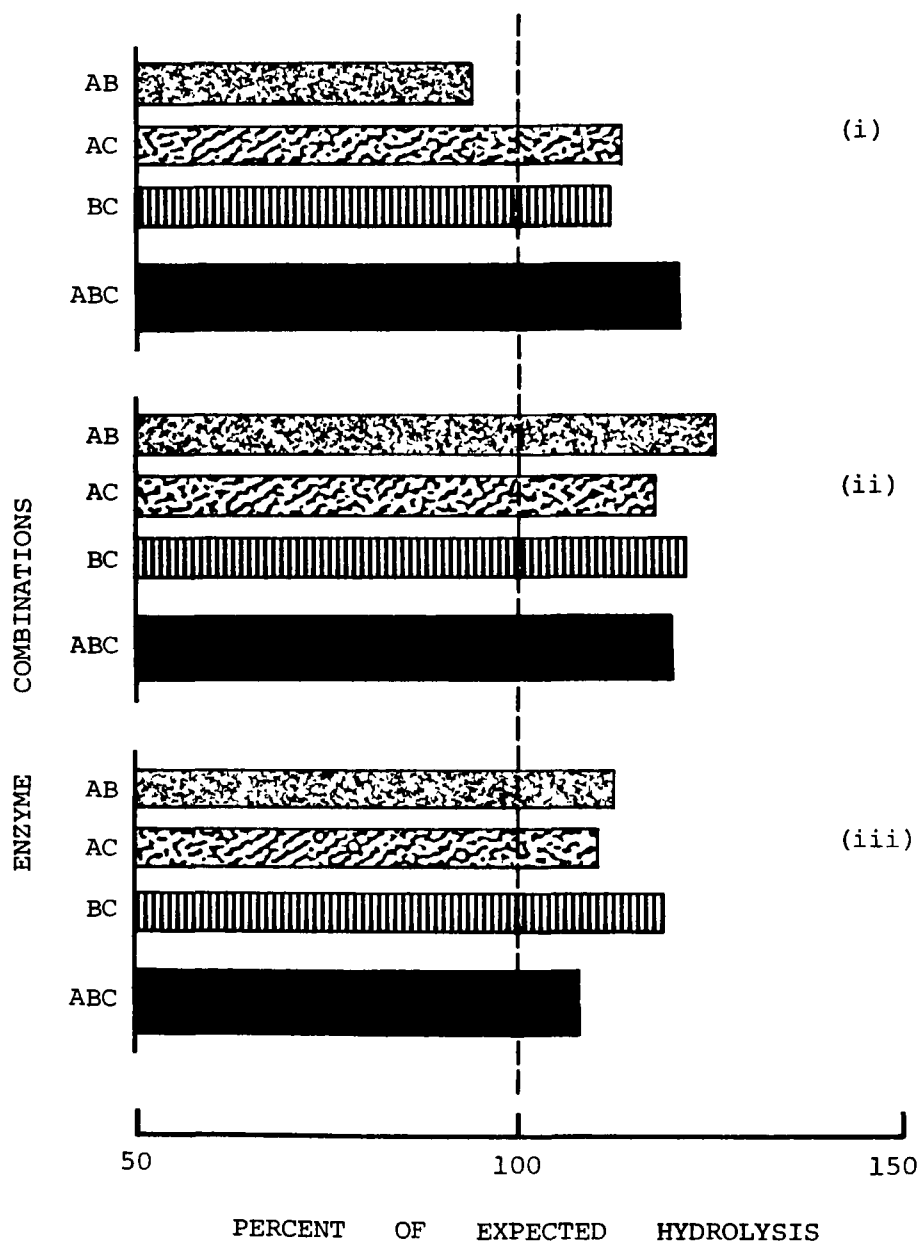


Table 5.3. Enzyme limiting hydrolysis of xylans by combinations of the purified xylanases. 2.5 mg deacetylated aspen xylan, acetylated aspen xylan and oat arabinoxylan were hydrolyzed by combinations containing the 20 kD xylanase (A), the 22 kD xylanase (B), the 29 kD xylanase (C) and the purified retentate (R) for 1 h at 28 C and pH 4.8. The enzyme unit p was equal to 0.13 mu, 0.09 mu, 0.10 mu and 0.13 mu for A, B, C and R respectively. The mean of 3 trials is reported with its standard deviation (sd) and its significance (sig) in difference from the hydrolysis expected if no complementation occurred (nsd: not significantly different, +: significantly greater at P < 0.05). When there is no interaction among the enzymes in a combination, the expected hydrolysis is the arithmetic sum of the hydrolysis by the individual enzyme preparations in the combination.

enzyme activity (p)				extent of hydrolysis (nmol xylose equiv.)								
				deacetylated			acetylated			arabinoxylan		
A	B	C	R	mean	sd	sig	mean	sd	sig	mean	sd	sig
12	-	-	-	85	10		64	10		64	2	
-	12	-	-	44	7		37	9		35	2	
-	-	12	-	46	5		31	5		34	4	
-	-	-	12	80	5		383	25		188	16	
6	6	-	-	60	8	nsd	64	12	nsd	56	6	nsd
6	-	6	-	74	6	nsd	56	8	nsd	54	7	nsd
6	-	-	6	94	7	nsd	290	16	+	157	8	+
-	6	6	-	50	5	nsd	41	9	nsd	41	7	nsd
-	6	-	6	77	14	nsd	311	22	+	143	3	+
-	-	6	6	77	3	+	247	31	nsd	145	2	+
4	4	4	-	70	14	nsd	53	7	nsd	48	3	nsd
4	4	-	4	79	3	nsd	251	17	+	139	12	+
4	-	4	4	82	5	nsd	228	5	+	140	10	+
-	4	4	4	65	8	nsd	247	12	+	131	4	+
3	3	3	3	74	4	nsd	215	19	+	109	6	+

was only 21% over the hydrolysis expected if no complementation occurred. Significant increases were also observed in the enzyme limiting hydrolysis of acetylated aspen xylan and oat arabinoxylan by enzyme combinations which contained the purified retentate. These results are likely artifacts of the contaminating glucan in the substrates (Table 2.2) and glucanases in the retentate (Table 5.1). The high glucanase activity in the retentate may have been responsible for the high initial hydrolysis of these two substrates. The loss in the linearity of hydrolysis rates (data not shown) invalidates the data analysis of the hydrolyses of acetylated aspen xylan and oat arabinoxylan by enzyme combinations which contained the purified retentate.

In summary, the 3 purified xylanases do show some complementation in the hydrolysis of isolated xylans. In combinations, they can increase initial hydrolysis rates slightly and they can significantly increase the substrate limiting hydrolysis of aspen xylans. These increases ranged up to 30% which compares well with previously reported xylanase complementation. However, substrate limiting hydrolysis yielded less than 0.75 mg xylose equivalents from 2.5 mg xylan, which corresponded to an overall hydrolysis of less than 30%.

V.2.2. HPLC analysis of hydrolyzates from substrate limiting hydrolysis of xylans

Complementation of xylanases may occur at a level which results in an increase of xylose or xylobiose content in xylan hydrolyzates. However, HPLC analysis of one set of hydrolyzates did not suggest any remarkable increase of xylose or xylobiose in the hydrolyzates produced by enzyme combinations (data not shown). In general, the relative degree of hydrolysis determined by HPLC agreed with that determined by reducing sugars.

HPLC chromatograms of hydrolyzates produced by single enzyme preparations from the deacetylated aspen xylan, acetylated aspen xylan and oat arabinoxylan are shown on Figure 5.7, 5.8 and 5.9 respectively. Both the purified retentate and the 29 kD xylanase released observable quantities of xylose from all three xylans. The ability of the purified retentate to release arabinose from arabinoxylan was confirmed. The ability of the 20 kD xylanase to release xylobiose from acetylated aspen xylan was noticeably lower than its ability to release xylobiose from the other 2 xylans. Finally, these chromatograms show that any hydrolysis of contaminating glucan in the substrates did not contribute significantly to their substrate limiting hydrolysis by xylanases.

Figure 5.7. HPLC analysis of enzyme hydrolyzates from deacetylated aspen xylan. (i) Substrate control (no enzyme). The substrate (0.25% xylan) was hydrolyzed with 0.024 u/mL 20 kD xylanase (ii), 0.022 u/mL 22 kD xylanase (iii), 0.024 u/mL 29 kD xylanase (iv) and 0.034 u/mL purified retentate (v) at 28 C and pH 4.8, for 48 h. An internal standard of 5 μ mol/mL erythritol was added to the samples after hydrolysis. The retention times for xylobiose, xylose and erythritol were 11.6 min, 13.5 min and 20.2 min, respectively.

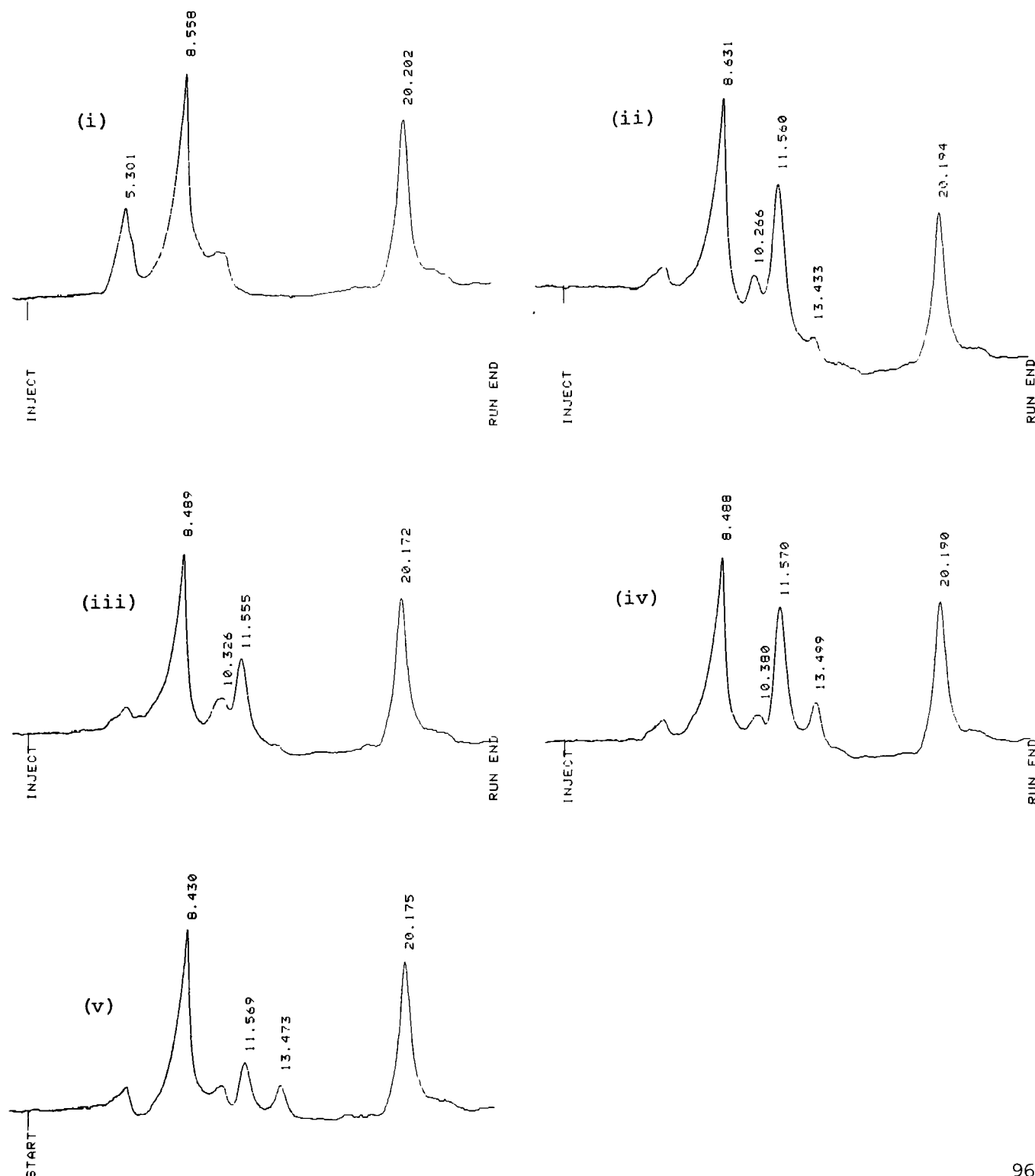


Figure 5.8. HPLC analysis of enzyme hydrolyzates from acetylated aspen xylan. (i) Substrate control (no enzyme). The substrate (0.25% xylan) was hydrolyzed with 0.024 u/mL 20 kD xylanase (ii), 0.022 u/mL 22 kD xylanase (iii), 0.024 u/mL 29 kD xylanase (iv) and 0.034 u/mL purified retentate (v) at 28 C and pH 4.8, for 48 h. An internal standard of 5 μ mol/mL erythritol was added to the samples after hydrolysis. The retention times for xylobiose, xylose and erythritol were 11.5 min, 13.5 min and 20.1 min, respectively.

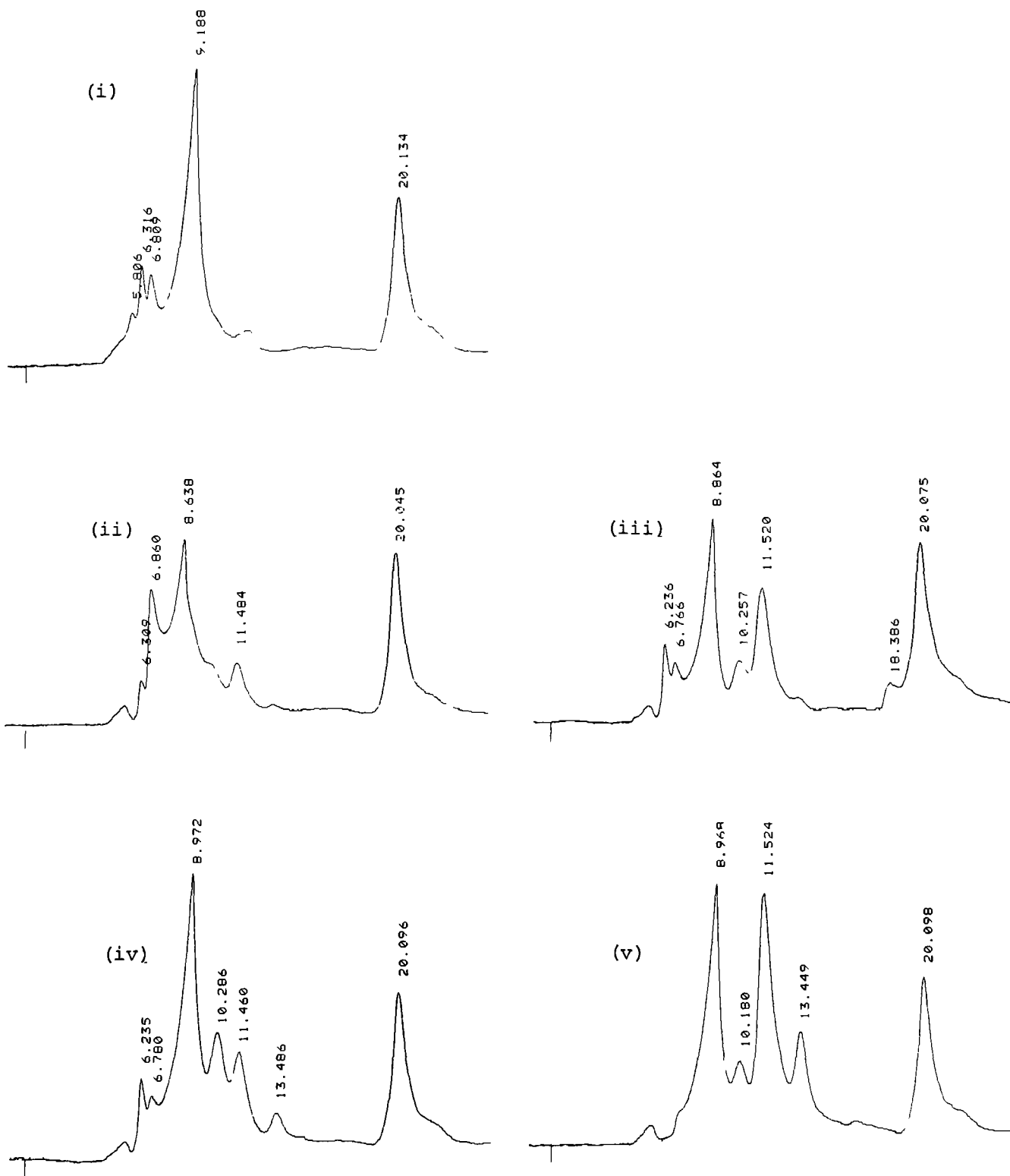
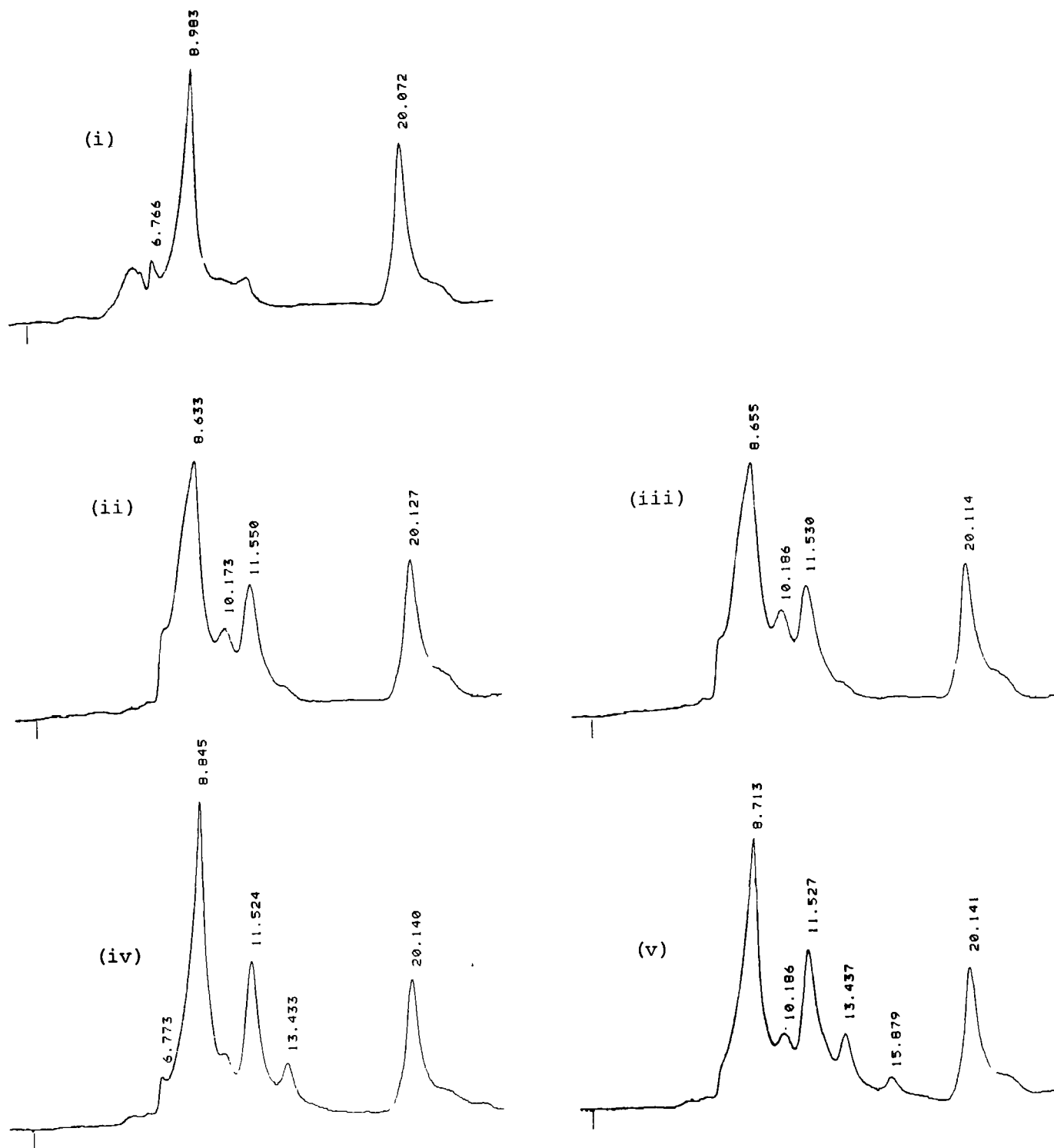


Figure 5.9. HPLC analysis of enzyme hydrolyzates from oat arabinoxylan. (i) Substrate control (no enzyme). The substrate (0.25% xylan) was hydrolyzed with 0.024 u/mL 20 kD xylanase (ii), 0.022 u/mL 22 kD xylanase (iii), 0.024 u/mL 29 kD xylanase (iv) and 0.034 u/mL purified retentate (v) at 28 C and pH 4.8, for 48 h. An internal standard of 5 μ mol/mL erythritol was added to the samples after hydrolysis. The retention times for xylobiose, xylose and erythritol were 11.5 min, 13.4 min and 20.1 min, respectively.

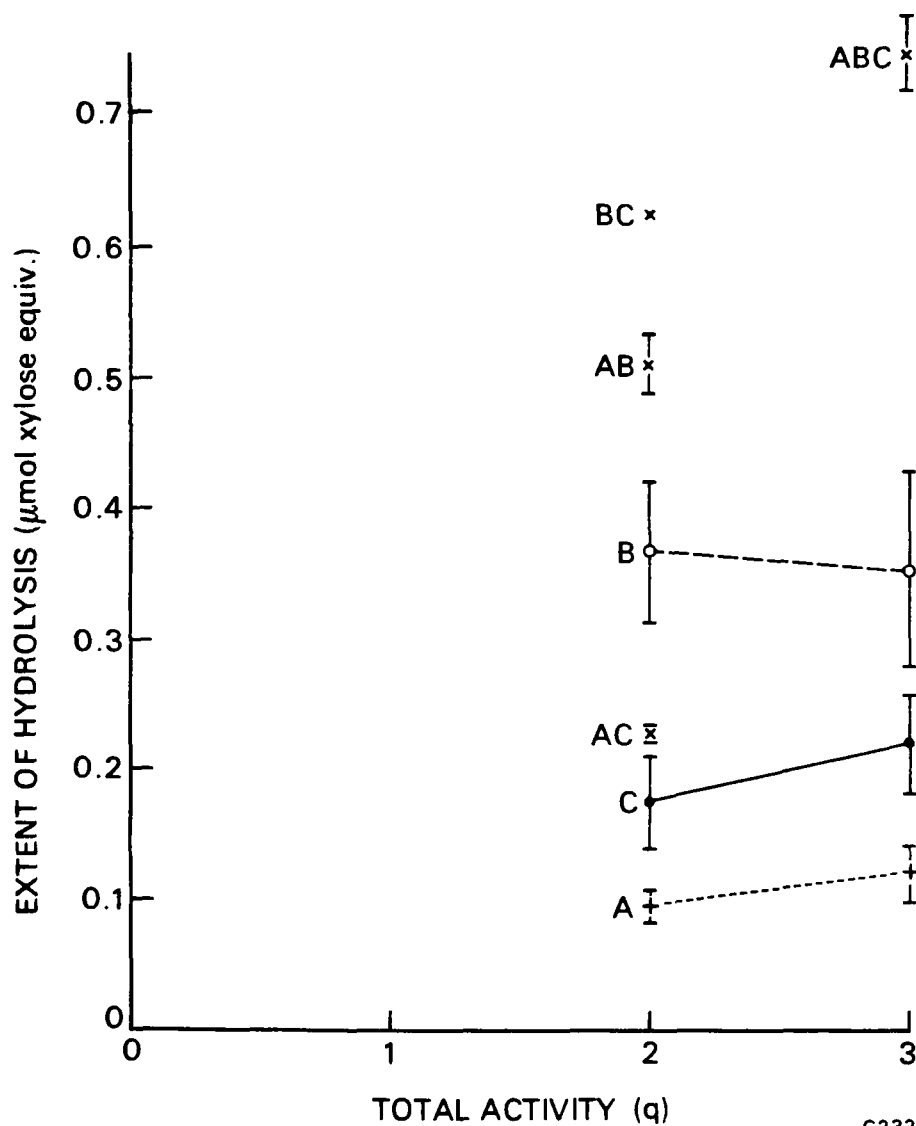


V.2.3. Hydrolysis of holocellulose

Xylan in situ is not an isolated polymer and therefore may be less accessible to enzyme hydrolysis. For this reason, the enzyme hydrolysis of sawdust and holocellulose was studied. Preliminary studies indicated that the 3 purified xylanases, individually or in combination, do not hydrolyze aspen sawdust (data not shown). Although hydrolysis of aspen holocellulose was low, there was a remarkable increase in hydrolysis when combinations of xylanases were used (Figure 5.10). Time course studies showed that the enzymes remain active after incubation with holocellulose (Figure 5.11) so that it is clear that some form of complementation occurred. A second set of experiments was performed to examine complementation of xylanases on an equal protein basis. The results confirmed that there is complementation of xylanases in the hydrolysis of aspen holocellulose (Figure 5.12).

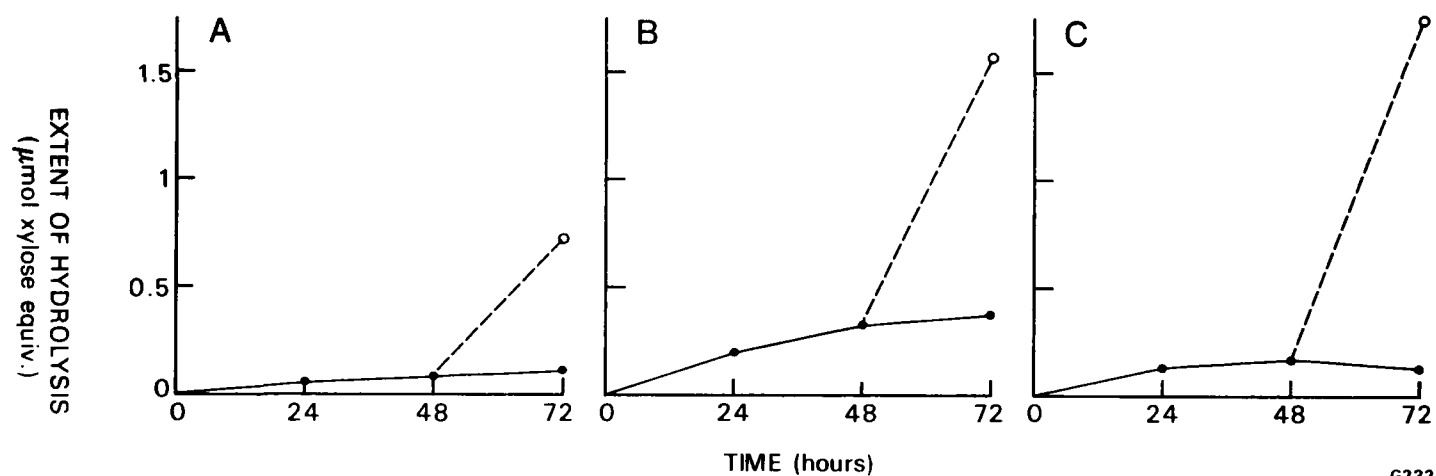
The raw data for these complementation studies are given in Table 5.4. All combinations of the 3 xylanases significantly increased hydrolysis and the combination of all 3 enzymes resulted in the greatest hydrolysis. The increase in hydrolysis ranged from 25% to 110% (Figure 5.13). Some enzyme combinations resulted in hydrolysis greater than the sum of the hydrolyses by individual xylanases. These results indicate that the different xylanases interact synergistically in the hydrolysis of holocellulose.

Figure 5.10. Complementation of xylanases in the hydrolysis of 2.5 mg aspen holocellulose on the basis of equal xylanolytic activity. The extent of hydrolysis by enzyme combinations (x) is compared to that by single enzymes. The enzyme combinations were made up with 1 q each of the 20 kD xylanase (A), the 22 kD xylanase (B) and/or the 29 kD xylanase (C). The enzyme unit q was equal to 0.012 u, 0.011 u and 0.012 u for A, B and C respectively.



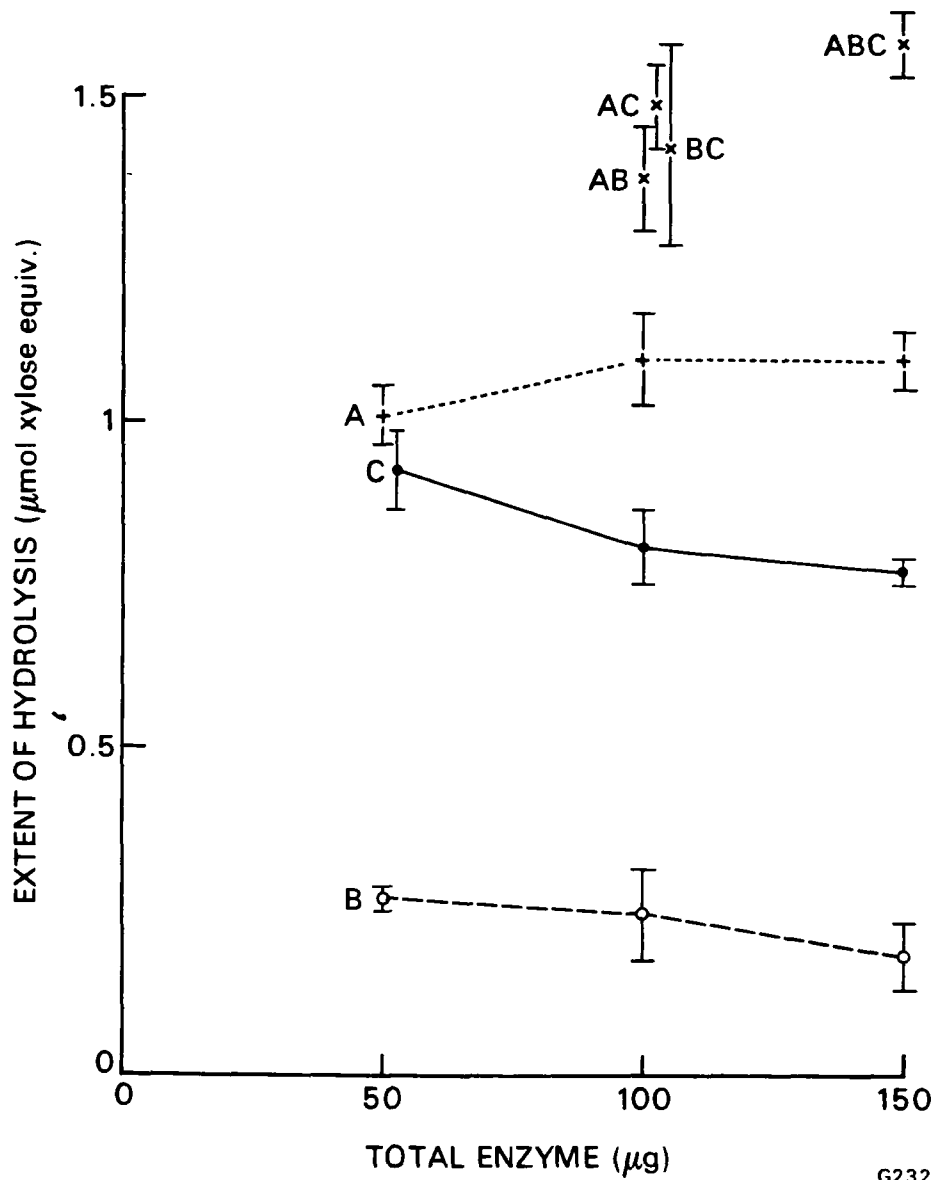
G232

Figure 5.11. The time course of aspen holocellulose hydrolysis by the 20 kD xylanase (A), the 22 kD xylanase (B) and the 29 kD xylanase (C). 2.5 mg holocellulose was hydrolyzed with 0.012 u A, 0.011 u B or 0.012 u C. At 48 h, hydrolysis was continued with no supplement (●) or with the addition of 2.5 mg deacetylated aspen xylan (○).



G232

Figure 5.12. Complementation of xylanases in the hydrolysis of 2.5 mg aspen holocellulose on an equal protein basis. The extent of hydrolysis by enzyme combinations (x) is compared to that by single enzymes. The enzyme combinations were made up with 50 μ g each of the 20 kD xylanase (A), the 22 kD xylanase (B) and/or the 29 kD xylanase (C).



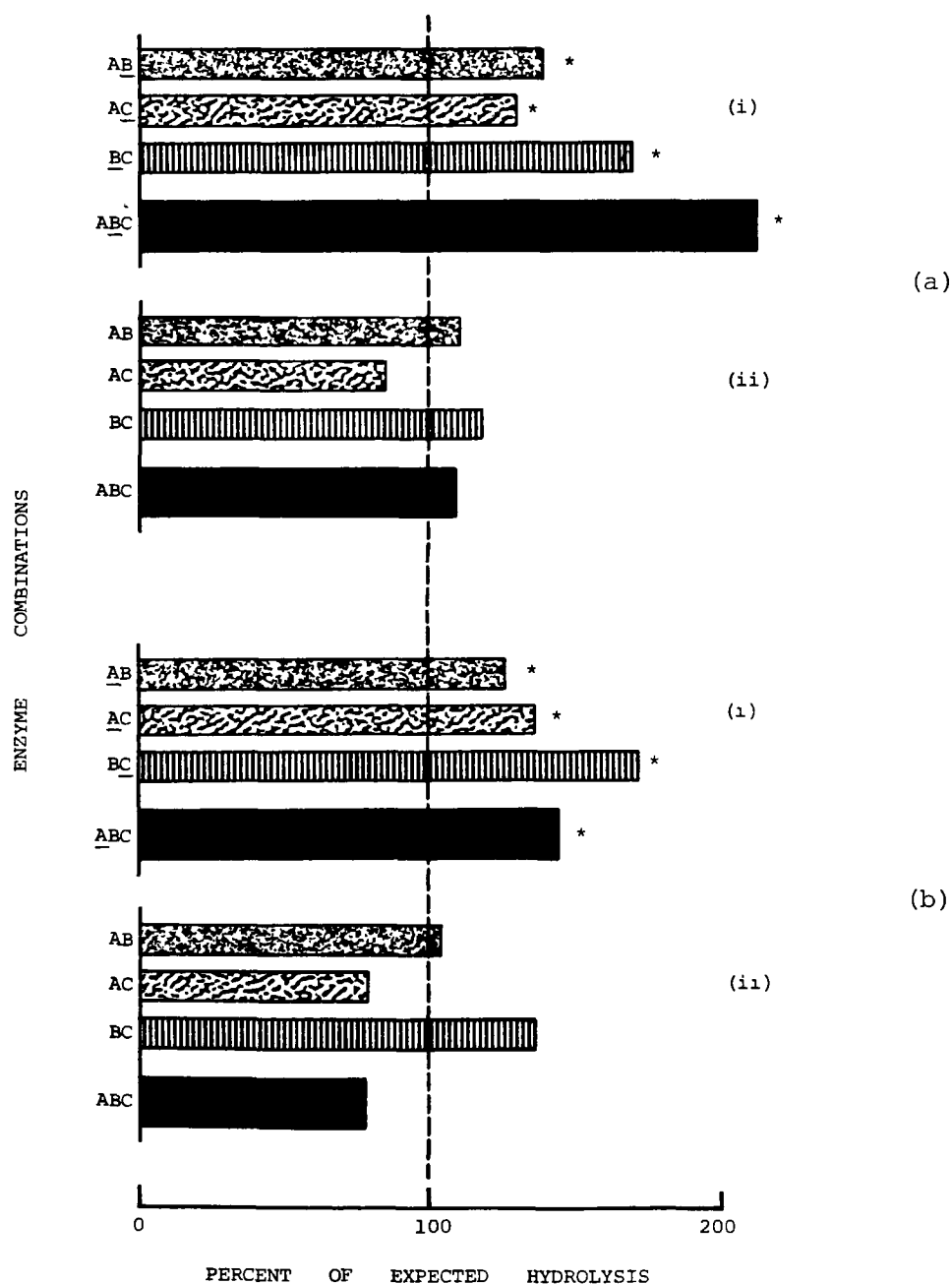
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Table 5.4. Hydrolysis of aspen holocellulose by combinations of the purified xylanases. 2.5 mg holocellulose was hydrolyzed with combinations containing 1 q or 50 µg each of the 20 kD xylanase (A), the 22 kD xylanase (B) and/or the 29 kD xylanase (C) for 48 h at 28 C and pH 4.8. The enzyme unit q was equal to 0.012 u, 0.011 u and 0.012 u for A, B and C respectively.

amount of enzymes				hydrolysis extent (umole xylose equiv.)			
sum	A	B	C	mean	sd #	sig @	sum *
2 q	2	-	-	0.09	0.01		
	-	2	-	0.37	0.05		
	-	-	2	0.17	0.04		
	1	1	-	0.51	0.02	+	+
	1	-	1	0.23	0.01	+	-
	-	1	1	0.63	0.01	+	+
3 q	3	-	-	0.12	0.02		
	-	3	-	0.35	0.07		
	-	-	3	0.22	0.04		
	1	1	1	0.75	0.03	+	+
50 µg	50	-	-	1.00	0.05		
	-	50	-	0.27	0.02		
	-	-	50	0.92	0.06		
100 µg	100	-	-	1.09	0.07		
	-	100	-	0.24	0.07		
	-	-	100	0.81	0.05		
	50	50	-	1.37	0.08	+	+
	50	-	50	1.48	0.07	+	-
	-	50	50	1.42	0.16	+	+
150 µg	150	-	-	1.10	0.04		
	-	150	-	0.18	0.05		
	-	-	150	0.77	0.02		
	50	50	50	1.58	0.05	+	-

sd: the standard deviation of the mean of 3 trials
 @ sig: significant difference from hydrolysis expected if there was no complementation (i.e. highest hydrolysis by single enzyme from the combination)
 nsd: not significantly different
 + : significantly greater (P < 0.05)
 * sum: whether the hydrolysis by the combination was greater than (+) or less than (-) the arithmetic sum of the hydrolyses by the individual enzymes in the combination

Figure 5.13. Hydrolysis of aspen holocellulose by combinations of xylanases expressed as percent of hydrolysis expected if no complementation occurred (i) and as percent of the sum of the hydrolyses by individual enzymes of the combination (ii). Hydrolyses were carried out using combinations containing 1 μ g (a) or 50 μ g (b) each of the 20 kD xylanase (A), the 22 kD xylanase (B) and/or the 29 kD xylanase (C). When there is no interaction among the enzymes in a combination, the expected hydrolysis is the highest hydrolysis found when the enzyme activity in the combination is made up by one enzyme from the combination. The single enzyme which provided the highest hydrolysis (100% expected hydrolysis) in each case is underlined on the axis. Significant complementations ($P < 0.05$) are marked with *.

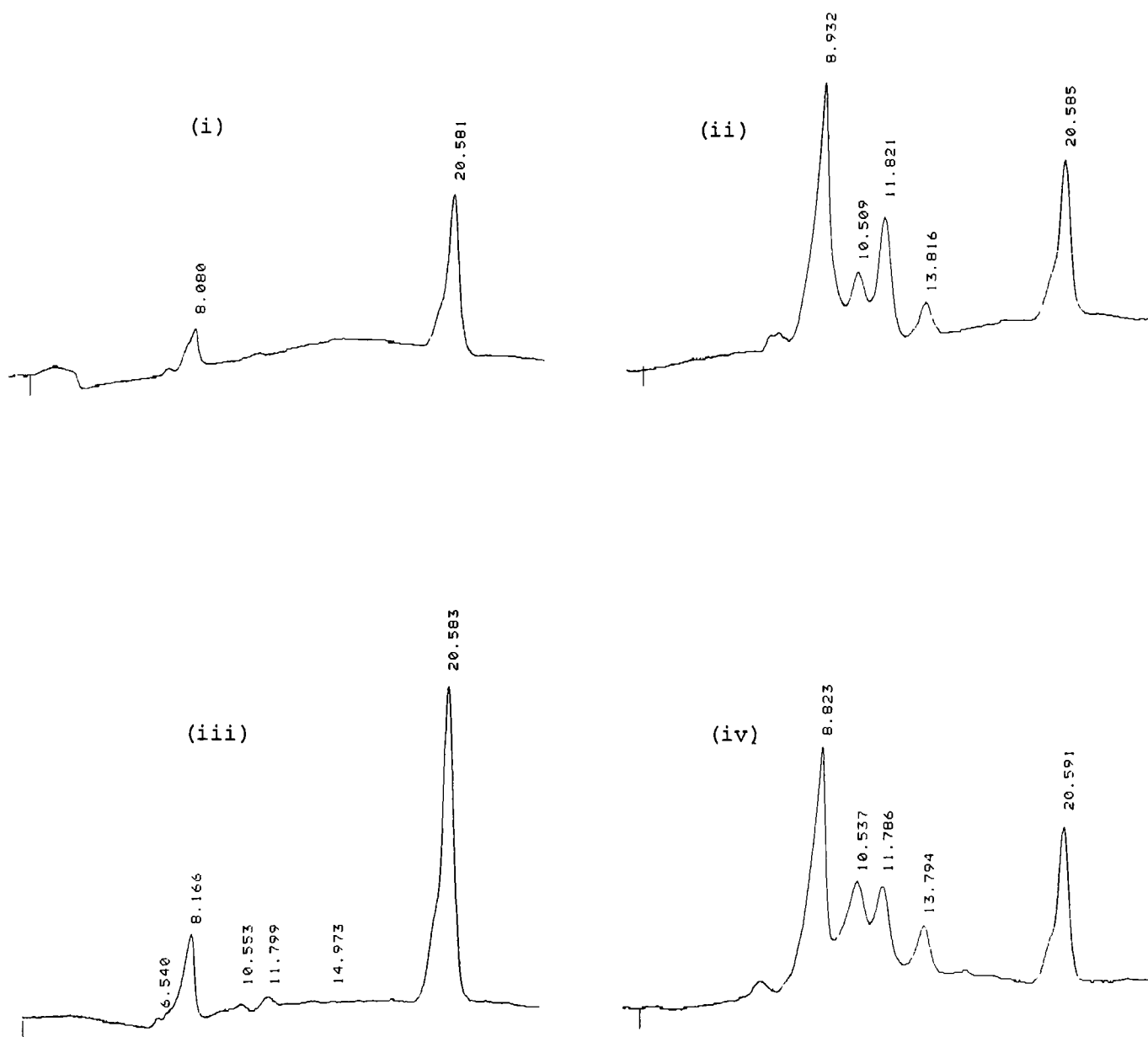


The mechanism of the enzyme interaction is not clear. HPLC analysis of the hydrolyzates did not suggest increased hydrolysis of the cellulose component of holocellulose by xylanase combinations (data not shown). Figure 5.14 shows that the individual xylanases selectively hydrolyze xylan. The synergism among the 3 xylanases may involve the exposition of new hydrolysis sites for each other. These enzymes may have different capabilities in hydrolyzing linkages near various branch points where xylan is integrated into the lignocellulosic structure. This possibility may be examined by identifying the shortest acidic xylooligosaccharide produced by each enzyme.

V.2.4. Functional purpose for the multiplicity of xylanases

The most interesting result of the complementation experiments is the positive interaction among the xylanases demonstrated in aspen holocellulose hydrolysis. It appears that xylan in situ is not accessible to xylanase hydrolysis and that the xylan exposed upon delignification is most completely hydrolyzed by a combination of all 3 xylanases. The observed complementation is relatively much higher than any observed complementation in the hydrolysis of isolated xylans. Approaches for elucidating the mechanism of the synergistic interaction include studying the sequential hydrolysis of holocellulose by the xylanases, the adsorption of xylanases, the structure of the hydrolysis products and the fine structure of holocellulose hydrolyzed by xylanase combinations.

Figure 5.14. HPLC analysis of enzyme hydrolyzates from aspen holocellulose. (i) Substrate control (no enzyme). The substrate (1% holocellulose) was hydrolyzed with 50 $\mu\text{g/mL}$ of the 20 kD (ii), the 22 kD (iii) and the 29 kD (iv) xylanases at 40 C and pH 4.8 for 24 h. An internal standard of 2 $\mu\text{mol/mL}$ erythritol was added to the samples after hydrolysis. The retention times for cellobiose, xylobiose, glucose, xylose, arabinose and erythritol were 10.4, 11.8, 12.6, 13.8, 16.3 and 20.6 min, respectively.



The results demonstrate that the characterized xylanases are not redundant components in the xylanase system of T. harzianum. It is possible that my approach may demonstrate a functional purpose for multiplicity of xylanases in other organisms and for multiplicity of enzymes in other lignocellulolytic systems. For example, the use of complex lignocellulolytic substrates may be useful in determining the importance of the multiplicity of endo-1,4- β -D-glucanases and exo-1,4- β -D-glucanases reported by Gum & Brown (1977), Wood & McCrae (1979) and Henrissat et al. (1985). Wood (1985) reported complementation of two exo-glucanases from Penicillium funiculosum in the hydrolysis of filter paper.

VI. GENERAL DISCUSSION

Trichoderma harzianum E58 produces several xylanolytic enzymes when grown on solka floc. Purification and characterization of 3 xylanases showed that each is a distinct gene product (Table 4.4) and that xylan is their primary substrate (Tan et al. 1985b, Table 4.3). The characterized xylanases do not cleave xylobiose or release arabinose from arabinoxylan (Figure 4.5 & 4.6). A xylobiase and an arabinose-releasing enzyme are present in the 4 day old culture filtrate, which contains at least 5 xylanases and 2 -xylosidases (Figure 3.6 & 3.8). The multi-component nature of the xylanolytic system is not unique to T. harzianum. The multiplicity of xylanases presents the question concerning the contribution of each xylanase to the system. An attempt to answer this question is also an attempt to improve xylanase classification by demonstrating unique roles for each enzyme.

My results demonstrated that the 3 characterized xylanases from T. harzianum are not redundant. These enzymes interact to maximize the hydrolysis of aspen holocellulose (Figure 5.13). An unanswered question is whether all the xylanolytic components of this fungus interact to increase hydrolysis. It would also be interesting to determine whether the ratio of xylanases produced by the fungus is the optimal ratio for hydrolysis. Furthermore, the fungus also produces cellulolytic enzymes which may interact with the xylanolytic system in the hydrolysis of lignocellulose. I have only dealt with a small component of a very complex system of enzymes.

The complementation of xylanases in the hydrolysis of aspen holocellulose is much higher than that observed in the hydrolysis of isolated aspen xylans (Figure 5.3, 5.6 & 5.13). The data therefore suggest that the structure of lignocellulose is a prominent factor in determining xylanase interactions. The complexity of the structure makes it difficult to elucidate the mechanism of xylanase complementation. Studies on the sequential hydrolysis of holocellulose by the xylanases may determine whether the complementation mechanism is a sequential event. The study of holocellulose hydrolysis and adsorption of xylanases with respect to the fine structures of the cell wall may determine whether the hydrolysis by the xylanases is partitioned spatially. It is also important to examine xylanase complementation on holocellulose from other sources. The complexity of lignocellulose has always limited its use in studies on enzyme mechanisms but my results indicate that it needs to be considered and that it can be used to address important biological and biochemical questions.

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