Characterization and mode of action of xylanases and some accessory enzymes

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Characterization and mode of action of xylanases and some accessory enzymes

Proefschrift

ter verkrijging van de graad van doctor
in de landbouw- en milieuwetenschappen,
op gezag van de rector magnificus,
dr. H.C. van der Plas,
in het openbaar te verdedigen
op woensdag 9 december 1992
des namiddags te vier uur in de aula
van de Landbouwuniversiteit te
Wageningen.

CIP-DATA KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Kormelink, Felix J.M.

Characterization and mode of action of xylanases and some accessory enzymes / Felix J.M. Kormelink. - [S.1. : s.n.]
Thesis Wageningen. - With ref. - With summary in Dutch.
ISBN 90-5485-052-3
Subject headings: xylanases / enzymes / xylan-degradation.

Abstract

Kormelink, F.J.M. (1992) Characterization and mode of action of xylanases and some accessory enzymes. Ph.D. Thesis, Agricultural University Wageningen (175 pp., English and Dutch summaries).

Three endo-(1,4)-ß-D-xylanases (Endo I, Endo II, and Endo III), a (1,4)-ß-xylosidase and an (1,4)-ß-D-arabinoxylan arabinofuranohydrolase (AXH) were purified from a culture filtrate produced by *Aspergillus awamori* CMI 142717. In addition to these enzymes, an acetyl xylan esterase (AE) was purified from a culture filtrate produced by *Aspergillus niger* DS 16813.

The enzymes were characterized by determining specific activities, molecular weight, iso-electric point, kinetic parameters (K_m, V_{max}), optimum pH and optimum temperature.

Arabinoxylan oligosaccharides were derived from alkali-extracted wheat arabinoxylans by complete digestion with Endo I and III. The structures of unknown oligosaccharides were elucidated by ^{1}H-n.m.r. spectroscopy. From these structures a model was proposed for the mode of action of Endo I and Endo III towards arabinoxylans. The same oligosaccharides were also used to specify the action of (1,4)-ß-xylosidase, AXH and two α-L-arabinofuranosidases towards these arabinofuranosylated arabinoxylan oligosaccharides.

The interaction between the purified enzymes was studied by degradation of xylans from rice bran, oat spelt, wheat-flour, larchwood, and birchwood by single and combined actions of these enzymes on these substrates. The cooperativity was monitored by the amount of reducing sugars and by the types of products released.

Stellingen

1. Nauwkeurige analyse van de gevormde produkten van arabinoxylanen na inwerking van verschillende endo-xylanases zegt meer over een eventuele gelijkenis van deze enzymen dan vergelijking van fysisch-kinetische parameters.
   (Dit proefschrift)

2. Het bestaan van AXH met zijn zeer beperkte substraat-specificiteit doet vermoeden dat endo-xylanases met 2 katalytische aktiviteiten, zoals beschreven in de literatuur, gecontamineerd zijn met een AXH-type enzym.
   (Dit proefschrift)

3. De door Kaji gestelde onderverdeling van α-L-arabinofuranosidases in een *Aspergillus niger* type en een *Streptomyces purpurascens* type voldoet niet.
   Kaji, A., L-Arabinosidases, *Adv. Carbohydr. Chem. Biochem.*, 42 (1984) 383-394.

4. De benaming acetyl xylaan esterase wordt dikwijls ten onrechte gebruikt.
   Mc. Dermid, K.P., Forsberg, C.W., and MacKenzie, C.R., *Appl. Environment. Microbiol.*, 56 (1990) 3805-3810.
   Sundberg, M., and Poutanen, K., *Biotechnol. Appl. Biochem.*, 13 (1991) 1-11.

5. Het begrip "zuiver enzym" komt onder druk te staan door een toenemend gebruik van steeds geavanceerdere technieken.

6. Beoordeling van de kwaliteit van wetenschappelijke publikaties aan de hand van de *Science Citation Index* (SCI) is gebaat bij een beoordeling van de waarde van de citaten.
   Hageman, E., Big Brother meet kwaliteit door te turven en te tellen, *de Volkskrant*, 10 oktober 1992, p. 19.

7. Continuïteit in kennisverwerving en kennisuitwisseling aan de LU is niet alleen gediend bij een groot aantal aio's/oio's, maar ook bij een gedegen post-doc beleid.

8. De maatschappelijke betekenis van de academische titel devalueert.

9. Invoering van kwaliteitssystemen in het bedrijfsleven geeft een extra dimensie aan de onderlinge concurrentie welke ten koste gaat van het bedrijfsleven in ontwikkelingslanden.

10. Een importverbod op nationaal niveau op niet-gekweekte vogels ter voorkoming van uitsterven van bedreigde vogelsoorten dient uitgebreid te worden tot een algeheel handelsverbod.

11. De motivatie van de burger voor het scheiden van afval op huishoudelijk niveau wordt ondermijnd door een inadequate verwerking van gescheiden ingezameld afval.

12. De efficiëntie van werken in het weekend in het laboratorium van de universiteit is gecorreleerd aan het aantal personen dat op dat moment aanwezig.

F.J.M. Kormelink
Characterization and mode of action of xylanases and some accessory enzymes.
Wageningen, 9 december 1992

aan mijn ouders

aan Cyrilla

The research reported in this thesis was supported by a grant from the European Community (contract number EN-3B-0090-NL) and by Gist-brocades (Delft, The Netherlands).

Voorwoord

Na 4 jaar onderzoek is de tijd aangebroken om een dankwoord uit te spreken. Een goed uitgerust lab is niet alleen toereikend om een onderzoek goed te doen verlopen. Naast inzet en apparatuur heb je de steun en ervaring van anderen nodig. Het is dan ook vanzelfsprekend om hier een paar mensen te bedanken zonder wiens steun, bijdrage en geduld het niet zover was gekomen.

Fons Voragen, door jouw overredingskracht ben ik aan dit promotie-onderzoek begonnen. Ik heb gedurende het promotie-onderzoek altijd jouw steun en vertrouwen genoten wat voor mij een extra stimulans was. Ondanks al je tijdrovende werkzaamheden was er altijd nog wel ruimte om onderzoeksgegevens te bespreken. Tijdens deze besprekingen heb ik altijd met bewondering naar jouw enthousiasme gekeken waarmee je mensen aan kon zetten om hun resultaten te publiceren.
Tom Wood from the Rowett Research Institute (Aberdeen, Scotland), thank you for giving me the opportunity to work on the purification of enzymes produced by your microbial strain. I am also thankful for your constructive criticism on some of my manuscripts.
Hans van de Vis en Marjo Searle-Van Leeuwen, met jullie heb ik het grootste deel van de tijd samen doorgebracht op het enzymologisch lab. Ik zou jullie willen bedanken voor de wezenlijke bijdrage aan mijn onderzoek in de vorm van praktisch werk en discussies. Als labgenoot was het prettig om met jullie samen te werken. Doctoraalstudenten Astrid Kemper en Gerbert Kets, bedankt voor jullie bijdrage in mijn onderzoek ook al liep het onderzoek niet altijd even makkelijk.
Harry Gruppen, Remco Viëtor en Eva-Maria Düsterhöft, gezien jullie werkzaamheden op het gebied van arabinoxylanen en andere celwandpolysacchariden, heb ik veel aan jullie bijdrage te danken in de vorm van praktische samenwerking en discussies. Gerhard de Ruiter en Marian Verbruggen wil ik vooral bedanken voor hun bijdrage in het methyleringsgebeuren.
Verder zou ik Henk Schols en Jan Cozijnsen willen bedanken voor de prima staat waarin de HPLC en GLC apparatuur verkeerden en last, but not least, Jean-Paul Vincken, Fred Lahousse en Gerrit Beldman voor de prettige sfeer binnen het Gist-brocades project.
Medewerkers van de fotolokatie en de tekenkamer zou ik verder nog willen bedanken voor het netjes afwerken en vastleggen van mijn resultaten.
Alle medewerkers van de sectie chemie, bedankt voor de zeer prettige sfeer waarin ik met jullie heb mogen samen werken !

Felix Kormelink

List of abbreviations

AE	Acetyl xylan esterase
Ara(*f*)	α-L-Arabinofuranosyl
Arafur	α-L-Arabinofuranosidase
Ara/Xyl	Arabino/Xylose ratio
AUA	Anhydro-uronic acid
AXH	(1,4)-ß-D-arabinoxylan arabinofuranohydrolase
BE1	First $Ba(OH)_2$ extract
BE1-U	DEAE-unbound fraction of BE1
CBB	Coommassie brilliant blue
CM-cellulose	Carboxymethyl-cellulose
Da	Dalton
DEAE	Diethylaminoethyl
DP	Degree of polymerization
DTT	Dithiothreitol
E.C.R.	Effective carbon response
EDTA	Ethylenediamine tetraacetic acid
Endo I	Endo-(1,4)-ß-D-xylanase I
Endo III	Endo-(1,4)-ß-D-xylanase III
FID	Flame ionisation detection
FPLC	Fast protein liquid chromatography
Gal	Galactopyranosyl
GC-MS	Gas chromatography-mass spectroscopy
GLC	Gas liquid chromatography
Glc	Glucopyranosyl
GlcA	Glucopyranosyl uronic acid
^{1}H-n.m.r.	Proton nuclear magnetic resonance
HOHAHA	Homonuclear Hartmann-Hahn spectroscopy
H.p.a.e.c.	High performance anion-exchange chromatography
HPSEC	High performance size exclusion chromatography
HPLC	High performance liquid chromatography
K_m	Michaelis-Menten constant
LMW	Low molecular weight
Man	Mannopyranosyl
MHR	Modified hairy regions
*m*HDP	*m*-Hydroxydiphenyl
M_w	Molecular weight
P.a.d.	Pulsed amperometric detection
PED	Pulsed electrochemical detection
pI	Isoelectric point

PNP	*p*-Nitrophenyl
RI	Refractive index
ROESY	Rotating frame nuclear Overhauser enhancement spectroscopy
R.o.e.	Rotating Overhauser effects
R_t	Retention time
SDS-PAGE	Sodium dodecyl sulphate - polyacryl amide gel electrophoresis
SPS	Soluble soya bean polysaccharide
TFA	Trifluoroacetic acid
U	Unit (μmole/min)
V_{max}	Maximum velocity
WIS	Water insoluble solids
Xyl	Xylopyranosyl
Xyl(*p*)	ß-D-Xylopyranose
X, X_2, X_3...	Xylose, xylobiose, xylotriose...
●	Xyl
◇	α-Ara*f*
●●	β-Xyl*p*-(1,4)-Xyl*p*
●◇	α-Ara*f*-(1,2)-β-Xyl*p*
◇●	α-Ara*f*-(1,3)-β-Xyl*p*

Contents

Chapter 1

General Introduction

Introduction

Energy from biomass: fact or fiction ? Considerable interest has been focussed on the utilization of biomass as a source of fuels and chemicals. The main components of plant biomass are cellulose, hemicelluloses (xylans, arabinans, galactans, glucans, and mannans), pectins, protein and lignin. Xylans are by far the largest group of hemicellulosic polysaccharides present in biomass (Voragen *et al*, 1986).

Xylans are next to cellulose, the most abundant renewable polysaccharide in nature. They occur in appreciable amounts in woody biomass (hardwood and softwood trees), agricultural residues (sugar bagasse, corn crop residues), and a variety of waste products (materials released from wood during pulping and pulp processing, animal waste, and municipal waste; Woodward, 1984; Biely, 1985).

In comparison to cellulose the structure and hydrolysis of xylans have been studied in less detail, not only because the structure of xylan is more complex and varies from plant to plant, but also because the products of xylan breakdown are less valuable than glucose, a breakdown product of cellulose. Since xylan is a heteropolysaccharide, complete breakdown of this hemicellulose into the monomeric constituent sugars requires a combination of enzymes which not only split the (1,4)-linkages between ß-D-xylopyranosyl residues (i.e. endo-(1,4)-ß-xylanases and (1,4)-ß-xylosidases), but also the glycosidic linkages between substituents and xylopyranosyl groups (i.e. accessory enzymes).

Endo-xylanases, which form the largest group of these hydrolytic enzymes, are currently used in four major applications; (i) degradation of agricultural wastes i.e. xylans by endo-xylanases to monomeric sugars enabling the conversion or, where appropriate, fermentation into ethanol, butanol or other useful products (Rosenberg, 1980; Jeffries, 1985); (ii) enzymic treatment of animal feeds resulting in an upgrading of the feed (Bengtsson, 1991); (iii) manufacturing of dissolving pulps yielding cellulose for rayon production (Zamost *et al*, 1991); and (iv) pretreatment of kraft pulp or pulp fibers enhancing the removal of lignin and resulting in modifications of paper properties (Paice *et al*, 1988; Noé *et al*, 1986; Roberts *et al*, 1990).

To improve the overall economics of the bioconversion of biomass it is thus important not only to utilize the cellulose, but also the hemicellulosic part of the plant material. Since xylan is by far the largest group of hemicelluloses, most attention has to be focussed onto this polysaccharide (Woodward, 1984; Biely, 1985). Therefore, a better understanding of multiple enzyme complexes is needed in the process of degradation.

Aim and approach of this study

The aim of this study is to elucidate the mechanism of the enzymic conversion of xylans in the scope of the bioconversion of xylan rich biomass. A study of all enzymes in the enzyme complex of a strongly xylanolytic fungus will be carried out. Other relevant accessory enzymes will be studied in similar fungi strains. Their characteristics, mode of action and cooperativity in the degradation of xylans from various sources will be studied.

The approach involves three parts. The first part consists of a screening and isolation of relevant enzyme activities from *Aspergillus* species. Isolation and purification will involve techniques like conventional liquid chromatography and FPLC. The purified enzymes will be subsequently characterized by determining their physico-chemical and kinetic parameters e.g. molecular weight, isoelectric point, pH and temperature optima, specific activity, K_m and V_{max}. In comparison to the literature, a more detailed investigation will be carried out in relation to the mode of action. The degradation of well characterized substrates will be studied by promising techniques like ^{1}H-n.m.r. spectroscopy and high performance anion-exchange chromatography. Finally the enzymic conversion of [(glucurono)arabino]xylans from various sources can be studied more closely by combined action of well characterized xylan-degrading and accessory enzymes.

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Chapter 2

Xylanases and accessory enzymes: their properties and mode of action

Introduction

Complete breakdown of xylans into their monomeric constituent sugars requires a combination of enzymes which not only split the glycosidic linkages in the main chain, but also the glycosidic linkages between substituents and the main chain. For a better understanding of this process, the structural features of the xylans as well as the characteristics of the enzymes involved have to be known. This chapter mainly describes the characteristics of isolated and purified xylan-degrading enzymes and their mode of action in relation to xylans from various sources.

Structure of xylans

Xylans consist of a backbone of (1,4)-linked ß-D-xylopyranosyl residues. Depending on their origin, i.e. *Gramineae* (grasses and cereals), *Gymnosperms* (softwoods) or *Angiosperms* (hardwoods), the backbone is substituted with α-L-arabinofuranose, α-D-glucopyranosyl uronic acid, or its 4-*O*-methyl derivative, and acetyl groups.
In wood from both groups, the cellulose content is around 43 ± 2%. The dominating hemicellulose in hardwoods is an *O*-acetyl-(4-*O*-methylglucurono)xylan, accompanied by small proportions of a glucomannan. Hardwoods of the temperate zones are chemically distinguished from that of softwoods by their lower content of lignin and glucomannan and their higher content of xylan. Hardwoods from tropical zones, on the other hand, often contain as much lignin as do most softwoods and a correspondingly low proportion of xylan (Timell, 1964). The major softwood hemicellulose is an *O*-acetyl-galactoglucomannan in various structural variations. An arabino-(4-*O*-methylglucurono)xylan forms a smaller but still substantial part of this wood (Timell, 1965). Arabino-(4-*O*-methylglucurono)xylans are also present in grasses and cereals.
The *O*-acetyl-(4-*O*-methylglucurono)xylan has a linear framework of (1,4)-linked ß-D-xylopyranose residues carrying 4-*O*-methyl-α-D-glucuronic acid units attached to its 2-position. Most hardwood xylans contain approximately one monomeric acid side-chain per ten xylose residues, the distribution however is largely unknown. Hardwoods contain *O*-acyl groups (*O*-acetyl and *O*-formyl), usually 3-5% (by weight) of the wood. The acetyl groups are also attached to its 2-position (Timell, 1964; Woodward, 1984).
The arabino-(4-*O*-methylglucurono)xylan has a repeating unit of 13 (1,4)-linked ß-D-xylopyranose residues bearing three (1,2)-linked 4-*O*-methyl-α-D-glucuronic acid residues

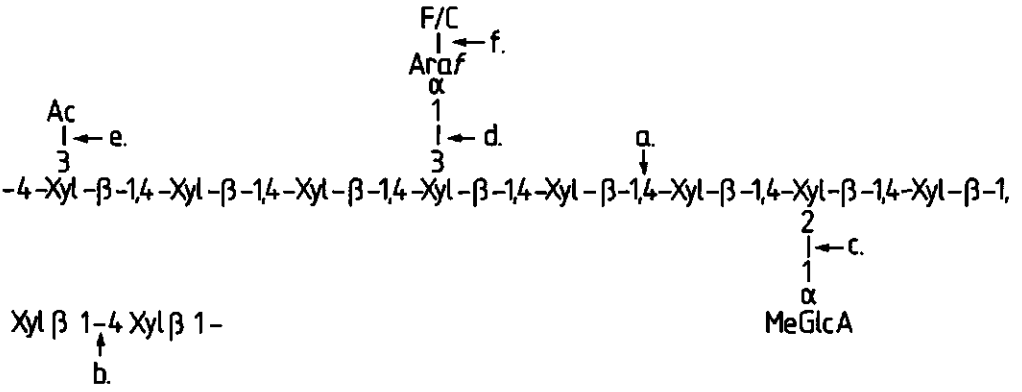
and one (1,3)-linked L-arabinofuranose residues (Timell, 1965; Woodward, 1984).
Arabino-(4-*O*-methylglucurono)xylan from cereals and grasses are commonly divided into non-endospermic hemicellulose (hemicellulose from aerial tissues and organs like bran, cob, hull, husk and leaves), and endospermic hemicelluloses (hemicelluloses associated with cereal starches and flours). In non-endospermic xylans, the various non-acidic side-chains and sugar units are mainly, if not invariably, attached to *O*-3 atoms of xylosyl residues. Glycopyranosyl uronic acid is often found in non-endospermic xylans and, more rarely, in those from the endosperm. In endospermic xylans, many of the xylosyl residues of the xylan chain are singly substituted on *O*-3, or doubly substituted, on *O*-2 and *O*-3, by L-arabinofuranosyl groups (Timell, 1967; Wilkie, 1979).
Some of the arabinosyl groups from graminaceous cell wall arabinoxylans are esterified with *p*-coumaroyl or feruloyl groups (Smith and Hartley, 1983; Kato and Nevins, 1985; Mueller-Harvey *et al*, 1986; Fry, 1988). Dimerization of the esterified phenolic moieties could crosslink the xylan backbone intra- and intermolecularly.

Enzymic degradation of arabino-(4-*O*-methylglucurono)xylan

The enzyme system needed for the degradation of arabino-(4-*O*-methylglucurono)xylan consists of a mixture of endo- and exo-acting enzymes (Fig. 1). The structure of the hemicellulose influences the composition of the xylanolytic enzyme system needed.
Endo-enzymes, i.e. endo-(1,4)-ß-D-xylanases, cleave the ß-(1,4)-glycosidic linkages within the hemicellulose polymer. Oligosaccharides released thereof can be further degraded by (1,4)-ß-xylosidases, which split the xylosidic linkages starting from the non-reducing end. Exo-enzymes, i.e. α-L-arabinofuranosidases, α-glucuronidases, and acetyl (xylan) esterases, remove the arabinofuranosyl, glucopyranosyl uronic acid, or its 4-*O*-methyl derivative, and acetyl substituents, respectively from the polymer (Biely, 1985). It is reported that some endo-xylanases can also split off α-L-arabinofuranosyl substituents (Dekker and Richards, 1976; Wong *et al*, 1988). As xylans *in situ* seem to be linked intra- and intermolecularly by ferulic and coumaric acid, it is also important to study its hydrolysis in a more native form. Desintegration from the cell wall requires the use of a second and third type of esterase, i.e. ferulic acid and coumaric acid esterase.

Endo-(1,4)-ß-xylanases

Endo-(1,4)-ß-xylanases form the largest group of hydrolytic enzymes involved in xylan degradation. They attack the xylan chain in a random manner producing linear and branched xylo-oligosaccharides with a low degree of polymerization (DP). The characteristics, mode of action and hydrolysis products vary according to the source of the enzyme (Table I and II).



a. endo-(1,4)-ß-xylanase (E.C.3.2.1.8)
b. ß-xylosidase (E.C.3.2.1.37)
c. α-glucuronidase (E.C.3.2.1)
d. α-L-arabinofuranosidase (E.C.3.2.1.55)
e. acetyl-esterase (E.C.3.1.1.6) or acetyl xylan esterase
f. ferulic acid or coumaric acid esterase

Figure 1. Hypothetical structure of a plant xylan and the sites of attack by microbial xylanolytic enzymes. Ac, acetyl group; Ara*f*, L-arabinofuranose; MeGlcA, 4-*O*-methyl-D-glucuronic acid; Xyl, D-xylose; F, ferulic acid substituent; C, coumaric acid substituent (adapted from Biely, 1985).

With minor exceptions, all enzymes mentioned in these and other tables were shown to be pure by SDS-gel electrophoresis. Several classification systems have been proposed for endo-xylanases.

Dekker and Richards (1976) divided endo-xylanases into two groups, namely those that do not liberate L-arabinose from arabinoxylans and arabinoglucuronoxylans (Table I), and those that liberate L-arabinose from these substrates (Table II). α-L-Arabinofuranosyl releasing activity was thus claimed as in inherent property of some xylanases. Reilly (1981) subdivided all endo-xylanases in four types. Two types were able to cleave the branch points but differed in the final products formed (xylobiose and xylose or intermediately sized xylo-oligosaccharides), the other two types were not able to cleave the branch points and produced xylo-oligosaccharides larger than xylobiose, or mainly xylobiose and xylose.

Another classification could be made according to the ability of endo-xylanases to degrade insoluble and/or soluble xylan. Since endo-xylanase activity is most often assayed by the release of reducing sugars from soluble xylans, data on activities towards insoluble substrates are available only to a limited extent.

Two endo-xylanases from *Aspergillus niger* (Frederick *et al*, 1985) rapidly but incomplete-

ly hydrolysed soluble larchwood xylan. Both enzymes hydrolysed insoluble larchwood xylan more slowly but to the same extent as soluble xylan. The two endo-xylanases were not active on insoluble xylan when free of branches which suggests the need of branch points for significant attack. A third endo-xylanase from the same micro-organism was active towards insoluble larchwood xylan only when arabinofuranosyl branches had been removed by hydrolysis (Fournier *et al*, 1985). Two endo-xylanases from *Trichoderma reesei* showed highest activity against insoluble birch xylan, oat hulls xylan, and beechwood xylan in decreasing order (Lappalainen, 1986). The endo-xylanases from *Clostridium stercorarium* were able to hydrolyse insoluble larchwood xylan to a reasonable extent (Berenger *et al*, 1985; Sakka *et al*, 1991). Two endo-xylanases purified from *Aspergillus oryzae* by Bailey *et al* (I and II, 1991), hydrolysed partially soluble beech 4-*O*-methylglucuronoxylan equally. However, endo-xylanase II was more effective in the hydrolysis of insoluble beech xylan (DP 30-40) than endo-xylanase I. On the other hand, only endo-xylanase I was capable of hydrolysing insoluble, unsubstituted birch xylan with a DP of $\pm$ 200.

It is difficult to compare all these data, because determination of the endo-xylanase activity can be affected by the type of substrate used (Khan *et al*, 1986).

Wong *et al* (1988) classified endo-xylanases from multiple xylanase systems according to their pI and molecular weight. Low M_w/basic and high M_w/acidic xylanases could be distinguished in *Bacillus* spp., *Clostridium* spp., *Streptomyces* spp., and vaguely also for *Aspergillus* and *Trichoderma* spp. (Table I and II).

Xylanases have been purified from numerous micro-organisms, i.e. bacteria (*Bacillus*, *Clostridium*, and *Streptomyces* sp.), yeasts (*Cryptococcus* sp., *Pichia stipitis*), and fungi (*Aspergillus* and *Trichoderma* sp.). Table I and II show a listing of recently purified endo-xylanases from various sources, including their molecular weight, pI, pH_{opt}, T_{opt}, and reaction products.

Endo-xylanases of fungal origin have molecular weights in the range of 7,000 to 60,000 Da. and are generally most active at pH 3.5-6.0 and 40-60°C. Bacterial endo-xylanases have molecular weights in the range of 15,000 to 85,000 Da., with minor exceptions, and are most active at pH 5.0-8.0 and 50-80°C. Since pulp processing takes place at high temperatures and neutral to alkali conditions, the latter group of enzymes are especially valuable for enzymic treatment of wood pulp.

In general, xylans are degraded by endo-xylanases mainly to xylose (X), xylobiose (X_2), xylotriose (X_3), and a mixture of xylo-oligosaccharides (Table I and II). Along with these xylo-oligosaccharides, oligosaccharides containing L-arabinofuranosyl (Dekker and Richards, 1975 a and b; Richards and Shambe, 1976; Wood and McCrae, 1986; John and Schmidt, 1988; John *et al*, 1979) and D-glucopyranosyl substituents (Nishitani and Nevins, 1991; Comtat, 1983; Khandke *et al*, 1989 b) are released.

The endo-xylanase purified from *Bacillus subtilis* (Nishitani and Nevins, 1988 and 1991) was shown to release a glucopyranosyluronic acid-xylohexaose from maize seedlings xylan only.

Table I. Characteristics of non-arabinose-releasing endo-(1,4)-ß-D-xylanases from various sources.

Source of enzyme	Code	M_w (kDa)	pI	pH_{opt}	T_{opt} (°C)	K_m[1]	Substrate	Reaction product[2]	Reference
Fungi									
Aspergillus niger		20.8*	6.7	5.0	55°		Larchwood	X_2, X_3, X_4	Frederick *et al* (1981)
Aspergillus niger	I	13.0*	8.6	6.0	45°		Larchwood	X_3, X_5, (X_2, X_4, X_6)	Frederick *et al* (1985)
	II	13.0*	9.0	5.5	45°		Larchwood	X_3, X_5, (X_2, X_4, X_6)	
Aspergillus niger		28.0	3.65	5.0	40-45°		Larchwood	X_3, X_5, (X_2, X_4, X_6)	Fournier *et al* (1985)
Aspergillus niger		14.0*	4.5	4.9	45°		Larchwood	X_2, X_3, X_5	Shei *et al* (1985)
Aspergillus niger van Tieghem	I			5.5		X_3-0.7mM X_4-1.0mM X_5-1.8mM	Cotton shell, rice straw	X, X_2	Takenishi and Tsujisaka (1973 and 1975) Fukumoto *et al* (1970)
Aspergillus ochraceus		48.0 50.0*		6.0	50°	1 mM	Larchwood xylan	X_2, X_3	Biswas *et al* (1990)
Aspergillus oryzae	I	28.0*	7.0	5.0			Beechwood glu-	X_n	Bailey *et al* (1991)
VTT-D-85248	II	26.0*	4.9	5.0			curonoxylan	X_n	
Aureobasidium NRRL Y-2311-1		20.0	8.5	4.5	45°				Leathers (1989)
Aureobasidium pullulans		24.0	8.0	4.25	60°		Larchwood	X, X_2, X_3, X_4	Dobberstein and
CBS 58475		14.0*	(7.4/6.75)						Emeis (1989)
Cephalosporium paradoxa	HC-IV	9.55*	6.0				Hemicellu-	X, X_2, X_3, AX_3,	Richards and Shambe
							lose B	AX_4	(1976)
Ceratocystis paradoxa	HC-II		4.50	5.1	80°	0.267	Hemicellu-	X, X_2, X_3, AX_2	Dekker and Richards
							lose B	AX_3	(1975 b)
Chaetomium thermophile var.	I	26.0		5.4-6.0	70°	0.55	Larchwood	X_2, X_3	Ganju *et al* (1989)
coprophile I-110		25.0*							
	II	7.0		4.8-6.4	60°	0.1	Larchwood	X_2	
		6.8*							

Source of enzyme	Code	M_w (kDa)	pI	pH_{opt}	T_{opt} (°C)	K_m	Substrate	Reaction product	Reference
Dichomitus squalens	EXI	39.0	4.6	5.0	60° (PNP-C)		Larchwood,	X_2, X_3	Rouau and Odier
	EXII	36.0	4.5	5.0	60° (PNP-C)		wheat straw	X_2, X_3	(1986)
Fusarium oxysporum SUF 850		21.0		6.0	50-55°				Yoshida *et al* (1989)
Gliocladium virens IFO 8349		7.0*		6.0	45°		Rice straw arabinoxylan	X, X_2, X_3	Takahashi and Kutsumi (1979)
Humicola lanuginosa		21.5 20.0*	4.1	6.0	65°	7.3	Xylan	(X), X_2, X_3	Kitpreechavanich *et al* (1984)
Lentinula edodes		41.0	3.6	4.5-5.0	60°	0.66	Aspen glucuro-noxylan	X, X_2, X_3	Mishra *et al* (1990)
Malbranchea pulchella var. sulfurea No. 48			8.6	6.0-6.5	70°		Hardwood xylan	X, X_2, X_3	Matsuo and Yasui (1985 and 1988 a)
Melanocarpus albomyces 11S-68	I	104.0*			60°	13.3			Chaudhri *et al* (1988)
	II	18.0*			50°	50.0			
Mesophilic fungus strain Y-94	A	51.0 44.0*	5.05	4.9	80°		Larchwood	X, X_2	Mitsuishi *et al* (1987)
	B	48.0	4.40	4.9	80°		Larchwood	X, X_2	
	C	35.0	4.10	4.9	80°		Larchwood	X, X_2	
Paecilomyces varioti IMD RK 032		20.0	5.2	4.0	50°	49.5			Kelly *et al* (1989)
Penicillium herqui (Banier and Sartory)		11.0+8.3	5.1	3.0	50°	4.5	Avena xylan	X_2, X_3	Funaguma *et al* (1991)
Polyporus tulipiferae (Irpex lacteus)	I	38.0		6.0	60°		Larchwood	X, X_2, X_3	Kanda *et al* (1985)
	III	62.0		6.0	70°		Larchwood	X_2, X_3	
Polyporus tulipiferae		38.0	7.6-8.0	4.6-5.2	60°	2.8	Larchwood glucuronoxylan	X, X_2, X_3	Hoebler and Brillouet (1984)

Source of enzyme	Code	M_w (kDa)	pI	pH_{opt}	T_{opt} (°C)	K_m	Substrate	Reaction product	Reference
Robillarda sp. Y-20	I	17.6 18.0*	9.7	4.5-6	50°	12.5	Larchwood	X, X_2, X_3	Koyama *et al* (1990)
	II	59.0 56.5*	3.5	4.5-6	50°	2.0	Larchwood	X, X_2, X_3	
Schizophyllum commune strain # 13 Dellmar (ATCC 38548)	A	21.0	4.5	5.0	50°	8.37	Larchwood xylan	X, X_2	Paice *et al* (1978) Jurasek and Paice (1988)
Schizophyllum radiatum CMI 90347		25.7		4.9	55°	9.5(Oat)	Steamed wheat straw	X_2, X_3, X_4, X_5	Cavazzoni *et al* (1989)
Sporotrichum dimorphosporum	I		7.1 7.2 7.35 7.95						Comtat (1983)
	II	32.0*	3.9	4.5-5	65-70°	5	Birchwood	X, X_2, X_3, X_4, $GlcAX_3$	
		32.0*	4.0	4.5-5	65-70°	5	xylan	X, X_2, X_3, X_4, $GlcAX_3$	
		32.0*	4.4	4.5-5	65-70°	5		X, X_2, X_3, X_4, $GlcAX_3$	
		32.0*	4.7	4.5-5	65-70°	2.6		X, X_2, X_3, X_4, $GlcAX_3$	
		26.0*	5.5	4.5-5	50°	7.8		X, X_2, X_3, X_4, $GlcAX_3$	
Talaromyces byssochlamydoides YH-50	XbI	54.0	3.8	4.5	70°		Xylan	X, X_2	Yoshioka *et al* (1981)
	XbII	45.0	4.0	5.0	70°			X, X_2	Hayashida *et al* (1988)
Thermoascus aurantiacus C436		32.0	7.1	5.1	80°	1.7	Oat spelt	X_2	Tan *et al* (1987 a)
Thermoascus aurantiacus		31.8		5.2	63°		Larchwood	X, X_2, $GlcAX_3$	Khandke *et al* (1989 a and b)
Trichoderma harzianum E58		20.0	9.4	5.0	50°	1.6	Xylan (aspen,	X, X_2, X_3, X_4	Tan *et al* (1985 a
		29.0	9.5	5.0	60-65°	0.66	larch,oat spelt)	X, X_2, X_3, X_4	and b)
Trichoderma harzianum E58		22.0	8.5	4.5-5.0	45-50°				Wong *et al* (1986)

Source of enzyme	Code	M_w (kDa)	pI	pH_{opt}	T_{opt} (°C)	K_m	Substrate	Reaction product	Reference
Trichoderma koningii IMI 73022	2	17.7	7.3	4.9-5.5	50°	4.2 CMX-2.3	Oat straw	(X), X_2, X_3, AX_4	Wood and McCrae (1986)
Trichoderma lignorum	A	21.0	5.1	3.5	45°		Oat spelt arabinoxylan	X, X_2, X_3, AX_4, AX_5	John and Schmidt (1988)
	B	20.0	8.7	6.5	45°			X, X_2, X_3, X_4, AX_4, AX_5	
Trichoderma longibrachiatum Rifai	A	21.5 <5.0*	9.45	5.0	55°	0.15	Larchwood	X_2, X_3	Royer and Nakas (1991)
	B	33.0 13.0*	9.25	5.0	60°	0.19	Larchwood	X, X_2	
Trichoderma reesei	I	32.0	4.1-4.2	4-5			Birchwood	X, X_2	Lappalainen *et al* (1986)
VTT-D-80133	II	23.0	6.4-6.5	4-5			Birchwood	X, X_2	
Trichoderma viride	I	19.9 16.0 (+ 13.1)	8.45 (7.3)	4.5-5	50°	3.3 (rye) 1.3 (larch)	Larchwood	X_2, X_3	Gibson and McCleary (1987)
	II	19.9 16.0 (+ 13.1)	8.45 (7.3)	4.5-5	50°	3.3 (rye) 1.3 (larch)	Larchwood	X_2, X_3	
Trichoderma viride				3.5	50°				Hashimoto *et al* (1971)
Bacteria									
Aureomonas sp. No. 212	L	145.0		7-8	50°		Xylan	X_2, X_3, X_4	Ohkoshi *et al* (1985)
(*ATCC 31085*)	M	37.0		6-8	50°		Xylan	X_2, X_3, X_4	
	S	23.0		5-7	60°		Xylan	X_2, X_3, X_4	
Bacillus circulans WL-12	A	85.0*	4.45	5.5-7.0		8.0	Xylan	X, X_2, X_3, X_4	Esteban *et al* (1982)
	B	15.0*	9.10	5.5-7.0		4.0	Xylan	X_2, X_3, X_4	

Source of enzyme	Code	M_w (kDa)	pI	pH_{opt}	T_{opt} (°C)	K_m	Substrate	Reaction product	Reference
Bacillus coagulans str. 26 (ATCC 8038)		22.0*	10.0	6.0	37°	10.0	Xylan	X, X_2	Esteban *et al* (1983)
Bacillus no. C-59-2			6.3	6.0-8.0	60°		Xylan (Oryza sativa)	X_2, X_3, X_4	Horikoshi and Atsukawa (1973)
Bacillus pumilus IPO		24.0		6.5	40°		Larchwood xylan	X_2, X_3, X_4, X_5	Panbangred *et al* (1983), Okada and Shinmyo (1988)
Bacillus sp. 11-1S		56.0		4.0	80°	1.68	Larchwood xylan	X, X_2, X_3	Uchino and Nakane (1981)
Bacillus sp. no. C-125	A	43.0		6.0-10.0	70°		Xylan	X_2, X_3, X_4	Honda *et al* (1985)
	N	16.0		6.0-7.0	70°			X_2, X_3, X_4	
Bacillus sp. WI	I	21.5	8.3-8.5	6.0	65°	4.5	Larchwood xylan	X_2, X_3, X_4, X_5	Okazaki *et al* (1985)
	II	49.5	3.5-3.7	7.0-9.0	70°	0.95		X, X_2, X_3, X_5	Akiba and Horikoshi (1988)
		48.5*							
Bacillus sp. WII	I	22.5	8.3-8.5	6.0	65°	4.0	Larchwood xylan	X_2, X_3, X_4, X_5	
	II	50.0	3.5-3.7	7.0-9.0	70°	0.57		X, X_2, X_3, X_5	
		51.0*							
Bacillus stearothermophilus str. 21		39.5	4.83	7.0	60°	3.8	Oat spelt xylan	X_2, X_3	Nanmori *et al* (1990)
Bacillus subtilis	A	27.0		4.5-6.0					Nishitani and Nevins (1988 and 1991)
	B	27.0		4.5-6.0					
	C	45.0		6.5-7.0			Maize xylan	$GlcAX_6$	
	D	45.0		6.5-7.0					
	E	45.0		6.5-7.0					
Bacillus subtilis PAP 115		32.0		5.0	50°	1.6	Soluble larchwood xylan	(X), X_2, (X_3)	Bernier *et al* (1983)

Source of enzyme	Code	M_w (kDa)	pI	pH_{opt}	T_{opt} (°C)	K_m	Substrate	Reaction product	Reference
Cellvibrio gilvus		40.0	5.0	6.5	55°	0.64	Larchwood	X, X_2, X_3	Haga *et al* (1991)
Clostridium acetobutylicum	A	65.0	4.45	5.0	50°	6.0	Larchwood	X_2, X_3, X_4, X_5, X_6	Lee *et al* (1987 a)
ATCC 824		63.0*							
	B	29.0	8.50	5.5-6.0	60°	6.7	Larchwood	X_2, X_3	
		29.5*							
Clostridium stercorarium	A	44.0	4.53	5.5-7	75°	3.2	Larchwood	(X), X_2, X_3	Berenger *et al*
	B	72.0	4.43	5.5-7	75°	2.9	xylan	(X), X_2, X_3	(1985)
	C	62.0	4.39	5.5-7	75°	3.7		(X), X_2, X_3	
Clostridium stercorarium	D	53.0	4.5	6.5	75°	1.4	Oat spelt xylan	X_2, X_3	Sakka *et al* (1991)
str. HX-1									
Clostridium thermolacticum	I	39.0	4.9	6.5	80°	0.65	Larchwood glu-	X_2, X_3	Debeire *et al*
str. TC21							curonoxylan		(1990)
Fibrobacter succinogenes S85	2	66.0	8.0	6.3	55°	1.3	Oat spelt xylan	X, X_2, X_3, X_4	Matte and Forsberg
			(7.7/7.4)						(1992)
Nocardiopsis dassonvillei	XI	23.0	4.9	7.0	60°		Oat spelt xylan	X_2, X_3	Tsujibo *et al* (1990)
subsp. alba OPC-18	XII	23.0	5.3	7.0	60°		Oat spelt xylan	X_2, X_3	
	XIII	37.0	4.1	7.0	50°		Oat spelt xylan	X_2, X_3	
Ruminococcus albus 8	1	720.0*	3.8	6.9		0.23	Larchwood		Greve *et al* (1984 b)
	2	260.0*	4.3	6.9		0.28	Larchwood		
Streptomyces lividans 1326		43.0	5.2	6.0	60°	0.78	Oat spelt xylan	(X), X_2	Morosoli *et al*
									(1986 a)
Streptomyces sp. strain 3137	X-I	50.0	7.10	5.5-6.5	60-65°		Hardwood xylan	X, X_2	Marui *et al* (1985)
	X-IIA	25.0	10.06	5.0-6.0	60-65°			X, X_2	Yasui *et al* (1988)
	X-IIB	25.0	10.26	5.0-6.0	60-65°			X, X_2	
Streptomyces sp. strain E-86		40.5	7.3	5.5-6.2	55-60°		Hardwood xylan	(A), X, X_2	Nakajima *et al* (1984)
									Yasui *et al* (1988)

Source of enzyme	Code	M_w (kDa)	pI	pH_{opt}	T_{opt} (°C)	K_m	Substrate	Reaction product	Reference
Streptomyces sp. strain KT-23		44.0 42.0*	6.9	5.5	55°	0.2 X_3-11.7 X_4-1.8 X_5-0.95 X_6-0.66 X_8-0.58	Rice straw xylan	X, X_2	Nakajima *et al* (1984)
Streptomyces T7		21.88 20.5*	7.8	4.5-5.5	60°	10	Larchwood	(X), X_2, X_3, X_4, X_5, X_6	Keskar *et al* (1989)
Streptomyces xylophagus nov. sp.				6.2	55-60°		Xylan	X, X_2	Iizuka and Kawaminami (1965)
Yeasts									
Cryptococcus albidus CCY 17-4-4		48.0	5.7 5.3				Oat spelt xylan	X, X_2	Morosoli *et al* (1986 b)
Cryptococcus albidus CCY 17-4-1	I	26.0*					Hornbeamwood xylan	X_2, X_3	Biely *et al* (1980)
Cryptococcus albidus CCY 17-4-1		48.0 26.0-28.0*	5.0	5.4			Arabinoxylan, glucuronoxylan, linear xylan	X, X_2, X_3	Biely and Vrsanska (1988)
Cryptococcus flavus IFO 0407		25.0 23.0*	10.0	4.5	55°	3.1	Xylan	X, X_2, X_3, X_4	Nakanishi *et al* (1984)
Pichia stipitis str. CBS 5773		43.0 36.0*		5.0	30°				Ozcan *et al* (1991)

* Determined by gel permeation chromatography.

[1] Expressed in mg/mL unless mentioned otherwise.

[2] X, Xylose; A, Arabinose; GlcA, Glucopyranosyl uronic acid.

No activity was observed towards larchwood arabinoxylan and *Rhodymenia* (1,3/4)-ß-D-xylan. The enzyme thus required the presence of glucopyranosyl uronic acid side chains and was therefore designated glucuronoxylan xylanohydrolase.

Similar observations were done for two endo-xylanases from *Aspergillus niger* in relation to L-arabinofuranosyl substituents (Frederick *et al*, 1985). Their low activity on linear xylo-oligosaccharides and the absence of activity towards insoluble linearised xylan, suggested the need of a branch point nearby for significant attack. Their main reaction products were xylotriose and xylopentaose.

Endo-xylanases from fungal as well as bacterial origin, were shown to release L-arabinofuranosyl substituents from arabinoxylan together with xylo-oligosaccharides (Table II). Arabinofuranose was detected in the arabinoxylan digests obtained with endo-xylanases II and III from *Aspergillus niger* van Tieghem (Takenishi and Tsujisaka, 1973), but only endo-xylanase II was able to hydrolyze arabinoxylobiose and arabinoxylotriose to an arabinose and the corresponding xylo-oligosaccharide. All purified endo-xylanases from *Aspergillus niger* (John *et al*, 1979) seemed to be capable of hydrolyzing the (1,3)-α-L-arabinofuranosyl substituents of arabinoxylans, but because of the rather low affinity of these enzymes for arabinoxylo-oligosaccharides, these sugars tend to accumulate in the hydrolyzate. Two hemicellulases (HC-I and III, respectively; Dekker and Richards, 1975 a; Richards and Shambe, 1976) look alike when comparing the end-products released, however, HC-I produces little xylose. HC-I was also shown to degrade arabinoxylobiose, -xylotriose and -xylotetraose to an arabinose and the corresponding xylo-oligosaccharide. One endo-xylanase from *Fibrobacter succinogenes* was shown to be able to release arabinose from both water-soluble oat spelts xylan and rye flour arabinoxylan (Matte and Forsberg, 1992). It was suggested that arabinose was released prior to hydrolysis of the xylan backbone, as substantial arabinose could be observed prior to the detection of xylo-oligosaccharides. Since only small amounts of arabinoxylotriose and arabinoxylotetraose accumulated during xylan hydrolysis, it was also suggested that arabinofuranosyl substituents were cleaved from the xylan backbone by the *Trichoderma koningii* enzyme prior to hydrolysis of the xylan to xylo-oligosaccharides (Wood and McCrae, 1986). The *Trichoderma koningii* enzyme was also able to hydrolyse arabinoxylotriose or arabinoxylotetraose to its corresponding sugars.

Inhibition of endo-xylanases by different components and chemical compounds specific to certain amino-acids, provides information on the structure of the active site and the mechanism of catalysis of endo-xylanases. Endo-xylanases from various sources have shown to be inhibited in their activity by metal ions, like Hg^{2+}, Cu^{2+}, Fe^{3+}, Pb^{2+}, Zn^{2+} and Ag^{+}. Hg^{2+} was found to be the most potent inhibitor, suggesting the presence of thiol-containing amino acids in the active site.

Sulfhydryl reagents like *p*-chloromercuribenzoate, N-bromosuccinimide, iodine, and iodoacetic acid were found to inhibit endo-xylanase activity also, suggesting the requirement of -SH groups. On the basis of the kinetics of inactivation and the chemical modification of the purified enzyme with N-bromosuccinimide, *p*-hydroxymercuribenzoate

Table II. Characteristics of arabinose-releasing endo-(1,4)-ß-D-xylanases from various sources.

Source of enzyme	Code	M_w (kDa)	pI	pH_{opt}	T_{opt} (°C)	K_m[1]	Substrate	Reaction product[2]	Reference
Fungi									
Aspergillus niger van Tieghem	II			5.0		X_3-1.2mM X_4-1.4mM X_5-1.8mM	Arabinoxylan (rice, cotton)	A, X, X_2	Fukumoto *et al* (1970) Takenishi and Tsujisaka (1973 and 1975)
	III			3.5		X_3-0.9mM X_4-1.4mM X_5-1.6mM			
Aspergillus niger no. 11	IIA	31.0*		4.0	50°		Larchwood arabinoxylan	A, X, X_2, AX_n, X_n	John *et al* (1979)
	IIB/C	31.0*		4.0	50°				
	IID	31.0*		6.0-6.5	50°				
	IA	50.0*		4.0-4.5	65-80°				
	IB	50.0*		5.5-6.0	65-80°				
Aspergillus niger str. 14		24.0* 41.0*	4.2	4.2			Wheat chaff arabinoglucuronoxylan	A, X, X_2, X_3	Rodionova *et al* (1977)
Aspergillus niger str. 14		33.0 24.0*	4.2	4.0	50°	CMX-3.0 X_4-1.04 mM	Xylan from various sources	(A), X, X_2, X_3	Gorbacheva and Rodionova (1977 a and b)
Aspergillus sp.		21.9*	6.7	5.0	60°		Spruce wood xylan	A, X, X_2	Sinner and Dietrichs (1975 a and b, 1976)
Cephalosporium sacchari	HC-III	10.7*	9.45				Hemicellulose B (spear grass)	A, X, X_2, X_3, AX_3, AX_4, AX_5	Richards and Shambe (1976)

Source of enzyme	Code	M_w (kDa)	pI	pH_{opt}	T_{opt} (°C)	K_m	Substrate	Reaction product	Reference
Ceratocystis paradoxa	HC-I		9.17	5.5	40°	4.24	Hemicellulose B	A, X, X_2, AX_3, AX_4, AX_5	Dekker and Richards (1975 a)
Oxisporus sp.	A1	38.6*	8.2	5.0	55°		Spruce wood xylan	A, X, X_2	Sinner and Dietrichs (1975 a and b, 1976)
	A2	26.9*	8.6	5.0	55°			A, X, X_2	
	B	37.4*	6.2	5.0	55°			A, X, X_2	
Talaromyces byssochlamydoides YH-50	Xa	76.0	4.3	5.5	75°		Xylan	A, X, X_2, X_3	Yoshioka *et al* (1981) Hayashida *et al* (1988)
Trichoderma koningii IMI 73022	1	29.0	7.24	4.9-5.8	60°	1.4 CMX-0.63	Oat straw arabinoxylan	A, X, X_2, X_3	Wood and McCrae (1986)
Trichoderma viride		17.8*	9.2	4.8	59°		Spruce wood xylan	A, X, X_2	Sinner and Dietrichs (1975 a and b, 1976)
Bacteria									
Fibrobacter succinogenes	1	53.7	8.9	7.0	39°	2.6	Oat spelt xylan	A, X_2, X_3, X_4, X_5	Matte and Forsberg (1992)
Streptomyces roseiscleroticus NRRL B-11019		22.6 5.5*	9.5	6.5-7.0	60°	7.9	Oat spelt xylan	A, X_2, X_3	Grabski and Jeffries (1991)
Streptomyces sp. strain E86		40.5	7.3	5.5-6.2	55-60°		Xylan	(A), X, X_2	Yasui *et al* (1988)

* Determined by gel permeation chromatography.

[1] Expressed in mg/mL unless mentioned otherwise.

[2] X, Xylose; A, Arabinose; GlcA, Glucopyranosyl uronic acid.

and 2-hydroxy-5-nitrobenzyl bromide, evidence was obtained for the involvement of tryptophan and cysteine residues at the active sites of *Streptomyces*, *Bacillus* and *Chainia* xylanases (Keskar *et al*, 1989; Deshpande *et al*, 1990). Carboxy groups were shown to be involved in the mechanism of catalysis of endo-xylanase from *Schizophyllum commune* (Bray and Clarke, 1990).

(1,4)-ß-Xylosidases

ß-Xylosidases have been purified from fungi, bacteria and even from yeast (Table III). They attack short xylo-oligosaccharides from the non-reducing end of the chain and release xylopyranose. In addition to the formation of hydrolysis products, many ß-xylosidases have substantial transferase activity producing oligosaccharides of higher molecular weight than the original substrate.

In comparison to endo-xylanases, ß-xylosidases were found to be high molecular weight compounds. They are most active in the pH range 4.0-7.0 at temperatures ranging from 40-80°C.

ß-Xylosidases are inhibited in their activity by metal ions, like Hg^{2+}, Zn^{2+}, Cu^{2+}, Mn^{2+}, and Ag^{+}, as well as by sulfhydryl reagents e.g. N-succinimide and *p*-chloromercuribenzoate. Xylose, arabinose and even glucose seemed to affect the enzyme activity also (Claeyssens *et al*, 1970 a and b; John *et al*, 1979; John and Schmidt, 1988; Lachke, 1988; Rodionova *et al*, 1983).

Differences between ß-xylosidases can be observed in the K_m values towards *p*-nitrophenyl ß-D-xylopyranoside, xylobiose and higher xylo-oligosaccharides. The ß-xylosidases from *Trichoderma reesei* (Poutanen and Puls, 1988), *Sclerotium rolfsii* (Lachke, 1988) and *Neurospora crassa* (Deshpande *et al*, 1986), and to a lesser extent the ß-xylosidases from *Aspergillus niger* (John *et al*, 1979; Rodionova *et al*, 1983) and *Aureobasidium pullulans* (Dobberstein and Emeis, 1991), are the most active enzymes towards *p*-nitrophenyl ß-D-xylopyranoside.

The affinity of ß-xylosidase towards xylan and xylo-oligosaccharides is, however, more important, because ß-xylosidase is a key enzyme in the overall conversion of biomass to monosaccharides, as endo-xylanases degrade xylan to xylose, xylobiose and higher oligosaccharides. Short, linear xylo-oligosaccharides can be converted completely to xylose by ß-xylosidases. However, the affinity towards xylo-oligosaccharides decreases with increasing chain length.

Shikata and Nisizawa (1975) purified an exo-cellulase from *Trichoderma viride*, which also acted as an exo-xylanase since xylobiose was released from xylan only, after incubation with the enzyme.

The hydrolysis becomes more complicated when xylo-oligosaccharides have arabinofuranosyl or glucopyranosyl uronic acid substituents, because of interferences with the active site.

Table III. Characteristics of (1,4)-ß-xylosidases from various sources.

Source of enzyme	Code	M_w (kDa)	pI	pH_{opt}	T_{opt} (°C)	K_m	Reference
Fungi							
Aspergillus fumigatus		360.0*	5.4	4.5	75°	2.0 mM PNP-X	Kitpreechavanich *et al* (1986)
		90.0				X_2-23.8 mM	
Aspergillus niger		>200.0*	4.6	3.0-4.5		0.34 mM PNP-X	Claeyssens *et al* (1970 a)
Aspergillus niger no. 11		78.0*		6.7-7.0	42°	0.22 mM PNP-X	John *et al* (1979)
Aspergillus niger str. 14		30.0*	4.6	3.0			Rodionova *et al* (1977)
Aspergillus niger str. 15		253.0*	4.9	3.8-4.0	70°	0.23 mM PNP-X	Rodionova *et al* (1983)
		122.0				X_2-0.67 mM	
Aspergillus niger van Tieghem			4.3	3-4			Takenishi *et al* (1973)
Aureobasidium pullulans		224.0	<3.0	4.5	80°	0.43 mM PNP-X	Dobberstein and Emeis (1991)
CBS 58475		(+ 121.0)				X_2-17.5 mM	
Chaetomium trilaterale str. no. 2264				4.5	55°	3.05 mM PNP-X	Kawaminami and Izuka (1970)
Chaetomium trilaterale str. B		240.0*	4.86	4.5		2.8 mM PNP-X	Uziie *et al* (1985)
		118.0				X_2-80.0 mM	Yasui and Matsuo (1988)
Emericella nidulans		240.0*	3.25	4.5-5.0	55°	6.6 mM PNP-X	Matsuo and Yasui (1984 b
		116.0				X_2-1 mM	and 1988 b)
						X_3-1.5 mM	
						X_4-1 mM	
						X_5-1.2 mM	
Malbranchea pulchella var. sulfurea no. 48		26.0*	4.8	6.2-6.8	50°	3.01 mM PNP-X	Matsuo *et al* (1977 a and b)
						X_2-2.86 mM	Matsuo and Yasui (1988 a)
Neurospora crassa 870		83.0	4.3	4.5-5.0	55°	0.047 mM PNP-X	Deshpande *et al* (1986)
Paecilomyces varioti IMD RK 032		67.0	4.0	4.0	60°	5.4 mM PNP-X	Kelly *et al* (1989)

Source of enzyme	Code	M_w (kDa)	pI	pH_{opt}	T_{opt} (°C)	K_m	Reference
Penicillium wortmanni IFO 7237	1	110.0* 115.0	3.7	4-4.5	55°		Matsuo *et al* (1987)
	2	195.0* 105.0	4.28	3-4.0	65°		
	3	210.0* 102.0	4.6	3-4.0	60-65°		
	4	180.0* 100.0	4.8	3-4.0	65°		
Sclerotium rolfsii *(NCIM 1084)*		170-180 170-180*		4.5	50°	0.038 mM PNP-X	Lachke (1988)
Trichoderma lignorum		100.0*	5.9/6.1 7.4	4.5	70°	5.0 mM PNP-X	John and Schmidt (1988)
Trichoderma harzianum E58		75.4	8.3	5.0	50-55°	1.9 mM PNP-X 1.0 mM Salicine 70 μM PNP-G 60 μg/ml Laminarine	Tan *et al* (1987 b)
Trichoderma reesei *VTT-D-80133*		90.0					Lappalainen (1986)
Trichoderma reesei *Rut C30*		100.0	4.7	4.0	60°	0.08 mM PNP-X	Poutanen and Puls (1988)
Trichoderma viride	D1	53.0	5.75	4.5-5.0			Shikata and Nisizawa (1975)
Trichoderma viride		101.0* 102.0	4.86/ 4.45	3.5-4.5	55°	X_2-2.1 mM X_3-1.3 mM X_4-1.0 mM X_5-1.0 mM	Matsuo and Yasui (1984 a and 1988 b)

Source of enzyme	Code	M_w (kDa)	pI	pH_{opt}	T_{opt} (°C)	K_m	Reference
Bacteria							
Bacillus circulans WL-12		85.0*	4.7				Esteban *et al* (1982)
Bacillus coagulans str. 26		190.0*	4.9	6.5	30°		Esteban *et al* (1983)
(ATCC 8038)		26.0					
Bacillus pumilus 12			4.4	7.2		1.43 mM PNP-X	Kersters-Hilderson *et al* (1969) Claeyssens *et al* (1970 b)
Bacillus stearothermophilus str. 21		150.0*	4.13	6.0	70°	1.2 mM PNP-X	Nanmori *et al* (1990)
Clostridium acetobutylicum ATCC 824		224.0* 85.0/63.0	5.85	6.0-6.5	45°	3.7 mM PNP-X	Lee and Forsberg (1987)
Yeast							
Pichia stipitis CBS 5773		34.0* 37.0					Ozcan *et al* (1991)

* Determined by gel permeation chromatography

Takenishi and Tsujisaka (1973) observed the release of a terminal non-reducing xylose from arabinoxylobiose and arabinoxylotriose by a purified *Aspergillus niger* ß-xylosidase. Arabinoxylose and xylose were the end-products. Purified ß-xylosidases from *Aspergillus niger* (John *et al*, 1979) and *Trichoderma lignorum* (John and Schmidt, 1988) were not able to release xylose from arabinoxylotetraose, probably because arabinofuranose was linked to the terminal non-reducing xylopyranosyl residue. Similar observations were done with a commercial ß-xylosidase preparation (Sigma) towards arabinoxylobiose and arabinoxylotriose (Brillouet, 1987). However, the latter enzyme preparation was able to release xylose from glucopyranosyluronic acid xylotriose (Nishitani and Nevins, 1991). Glucopyranosyluronic acid xylobiose and xylose were the end-products. A (4-*O*-methyl)glucopyranosyluronic acid xylotriose incubated with purified ß-glucosidase from *Thermoascus aurantiacus* which also showed ß-xylosidase activity, did not show any release of xylose (Khandke *et al*, 1989 b). As glucuronic acid was attached to the same xylopyranosyl residue as was found for the oligosaccharide purified by Nishitani and Nevins (1991), the lack of ability to split off xylose from the terminal non-reducing end-group could be due to differences in specificity, but also to steric hindrance of the 4-*O*-methyl ester of the glucuronic acid substituent.

α-L-Arabinofuranosidases

α-L-Arabinofuranosidases hydrolyse non-reducing α-L-arabinofuranosyl groups from L-arabinosides of low molecular weight and L-arabino-oligosaccharides. According to Kaji (1984) α-L-arabinofuranosidases can be classified into two groups; (i) the *Aspergillus niger* type of α-L-arabinofuranosidase, and (ii) the *Streptomyces purpurascens* type of α-L-arabinofuranosidase. Beside low molecular weight oligosaccharides, the first group of enzymes is also able to release arabinofuranosyl groups of L-arabinan, arabinoxylan, and arabinogalactan. A more detailed classification is proposed by Beldman *et al* (1992) based on the substrate specificity and mode of action of α-L-arabinofuranosidases.
Of all types only three groups of enzymes are important in the hydrolysis of arabinoxylan, namely α-L-arabinofuranosidases active towards arabinofuranose of arabinoxylan-derived oligosaccharides, α-L-arabinofuranosidases active towards arabinofuranose of arabinoxylan and oligosaccharides derived thereof, and α-L-arabinofuranohydrolases active only towards arabinofuranose of arabinoxylans and oligosaccharides derived thereof.
Non-debranching endo-xylanases and ß-xylosidases are limited in their action against arabino(glucurono)xylans and arabinoxylo-oligosaccharides, respectively, and therefore need the cooperative action of α-L-arabinofuranosidases or α-L-arabinofuranohydrolases.
α-L-Arabinofuranosidases, able to release arabinofuranosyl substituents from arabinoxylan, have been purified from fungi, bateria and yeasts (Table IV). In comparison to endo-xylanases, their molecular weights are relatively high, with pH_{opt} varying in the range 2.5-7.0.

Table IV. Characteristics of α-L-arabinofuranosidases from various sources active on arabinoxylan.

Source of enzyme	Code	M_w (kDa)	pI	pH_{opt}	T_{opt} (°C)	K_m	Substrate	Reference
Fungi								
Aspergillus niger K1		53.0*	3.6	3.8-4.0		4.7 mM PNP-A 0.26 mg/ml beet arabinan 20.4 mg/ml (1,5)-arabinan	PNP-A, L-arabinan, wheat arabinoxylan, gum arabic	Kaji and Tagawa (1970), Tagawa (1970), Tagawa and Kaji (1988)
Aspergillus niger	B	60.0	5.5-6	3.7	60°	0.13 mg/ml PNP-A 3.9 mg/ml UFR-arabinan 7.0 mg/ml UFR (linearised) 17.0 mg/ml (1,5)-arabinan	PNP-A, UFR-arabinan, (1,5)-arabinan, arabino-xylan	Rombouts *et al* (1988)
Corticium rolfsii IFO 4878				2.5		2.86 mM PNP-A 8.47 mg/ml arabinan 28.6 mg/ml (1,5)-arabinan	PNP-A, beet arabinan, (1,5)-arabinan, arabino-xylan	Kaji and Yoshihara (1971)
Dichomitus squalens CBS 432.34		60.0*	5.1	3.5	60°	1.64 mM PNP-A	Sugar beet arabinan, wheat straw, wheat bran and oat spelt xylan	Brillouet *et al* (1985)
Pectinol 59-L							Wheat arabinoxylan	Andrewartha *et al* (1979)
Pectinol R-10				3.8-4.0			PNP-A, sugar beet arabinan, wheat flour arabinoxylan	Neukom *et al* (1967)

Source of enzyme	Code	M_w (kDa)	pI	pH_{opt}	T_{opt} (°C)	K_m	Substrate	Reference
Trichoderma reesei VTT-D-79125		53.0 30.0*	7.5	4.0		1.2 mM PNP-A	Beet arabinan, wheat straw arabinoxylan, arabinoxylan oligosaccharides	Poutanen (1988)
Bacteria								
Bacillus subtilis		65.0	5.3	6.5			PNP-A, arabinan, arabinoxylan	Weinstein and Albersheim (1979)
Ruminococcus albus 8		75.0 310*	3.8	6.9		1.6 mM PNP-A	PNP-A, alfalfa cell walls	Greve *et al* (1984 a)
Streptomyces spp. no. 17-1		92.0	4.4	6.0		3.6 mM PNP-A	PNP-A, beet arabinan, (1,5)-arabinan, arabinoxylan, arabinogalactan	Kaji *et al* (1981)
Yeast								
Rhodotorula flava IFO 0407				2.0		9.1 mM PNP-A	PNP-A, beet arabinan, (1,5)-arabinan, arabinoxylan	Uesaka *et al* (1978)

* Determined by gel permeation chromatography

α-L-Arabinofuranosidase treatment of arabinoxylans may lead to a decrease in the amount of substituents which causes an increase of the insolubility of xylan due to intermolecular aggregation. This results in lowered accessibility to the endo-xylanases which influences the overall conversion to monosaccharides (Andrewartha *et al*, 1979). Pretreatment of arabinoxylan with endo-xylanases devoid of debranching activities, followed by arabinofuranosidase treatment would therefore be a proper alternative to prevent aggregation of long chain aggregates and to obtain the maximum yield of monosaccharides.

Acetyl (xylan) esterases

An important group of enzymes in the hydrolysis of hardwood xylans is formed by the acetyl esterases. These enzymes not only hydrolyse ester linkages from aliphatic and aromatic esters, but some are also able to remove *O*-acetyl groups from the C-2 and C-3 positions of acetylated xylan and/or xylo-oligosaccharides.
Acetyl esterases have been detected in *Schizophyllum commune* (Biely *et al*, 1986; Biely *et al*, 1988), *Trichoderma reesei* (Biely *et al*, 1986; Biely *et al*, 1988; Poutanen *et al*, 1987; Gattinger *et al*, 1990), *Fusarium oxysporum* (Poutanen *et al*, 1987), *Streptomyces* sp. (Poutanen *et al*, 1987; Ball and McCarthy, 1988; Johnson *et al*, 1988), *Rhodotorula mucilaginosa* (Lee *et al*, 1987 b), *Butyrivibrio fibrisolvens* (Hespell and O'Bryan-Shah, 1988), and *Aureobasidium pullulans* (Myburgh *et al*, 1991). The acetyl xylan of the yeast *Rhodotorula mucilaginosa* is of special interest as it is free of xylanolytic activity.
Only few acetyl esterases have been purified from fungal and bacterial sources (Table V). The esterases have molecular weights in the range of 30,000 to 70,000 Da, with a few exceptions. They are most active in the pH range of 5.0-8.0 and at temperatures varying from 40-80°C (all mainly tested towards *p*-nitrophenyl acetate or naphtyl acetate).
Most of the esterases were found to act on aliphatic and aromatic esters. Acetyl esterases of *Aspergillus awamori* (Sundberg *et al*, 1990), *Trichoderma reesei* (Poutanen and Sundberg, 1988; Sundberg and Poutanen, 1991) and *Fibrobacter succinogenes* (McDermid *et al*, 1990 b) have been tested towards acetylated birchwood xylan also, but only the acetyl esterases of the latter two strains showed activity towards this substrate.
The acetyl esterases are important enzymes in the enzymic degradation of acetyl xylan, because the presence of acetyl substituents impede the action of endo-xylanases on this substrate. It was shown that acetyl esterase and endo-xylanase acted cooperatively in hydrolyzing acetyl xylan (Biely *et al*, 1986).
Depending on the molecular weight of the substrate and the specificity of the acetyl esterase, a distinction can be made in acetyl xylan esterase or acetyl esterase. Acetyl xylan esterase is able to release acetic acid from polymeric acetyl xylan without the cooperative action of endo-xylanases whereas acetyl esterase is not able to act on polymeric acetyl xylan and needs an endo-xylanase for the release of acetic acid (Poutanen and Sundberg, 1988; Poutanen *et al*, 1990; Sundberg and Poutanen, 1991).

Table V. Characteristics of acetyl (xylan) esterases from various sources.

Source of enzyme	Code	M_w (kDa)	pI	pH_{opt}	T_{opt} (°C)	K_m	Substrate	Reference
Fungi								
Aspergillus awamori		50.0	3.7-3.8	5.0	75°		Aliphatic and aromatic	Sundberg *et al*
VTT-D-75028		150.0*					esters	(1990)
Aspergillus niger NRRL 337	I	23.0*	3.83	7.0	55°		Aliphatic esters and	Iwai *et al* (1983)
	II	127.0*	3.90	7.5	80°		acetyl esters	Okumura *et al*
	III	29.5*	3.60	5.5	55°			(1983)
	IV	27.0*	3.70	4.5	45°			
Trichoderma reesei		45.0	6.0/6.8	5.5	50°		Naphtyl acetate, glu-	Poutanen and Sund-
Rut C30		67.0*					cose- and xylose-	berg (1988)
							acetate, steamed birchwood	
Trichoderma reesei	AXEI	34.0 (20)*	7.0	5.0-6.0	60-65°		Steamed birchwood xy-	Sundberg and Pou-
Rut C30	AXEII	34.0 (20)*	6.8	5.0-6.0	60-65°		lan, aromatic esters	tanen (1991)
Bacteria								
Bacillus stearothermophilus		47.0		7.0	65°	0.5 mM PNP-Ac	p-Nitrophenyl esters,	Matsunaga *et al*
NCA 2184							m-carboxyl esters	(1974)
Bacillus subtilis NRRL 365	I	36.0		8.0		0.91 mM PNP-Ac	p-Nitrophenyl esters	Meghji *et al* (1990)
	II	110.0* (57.0+48.0)		8.0		0.67 mM PNP-Ac		
Butyrivibrio fibrisolvens		66.0*			39°	0.76 mM NA	Naphtyl-acetate, -buty-	Lanz and Williams
str. 53							rate and -proprionate	(1973)
Fibrobacter succinogenes S85		55.0	4.0	7.0	47°	2.7 mM NA	Naphtyl acetate, birch-	McDermid *et al*
							wood acetyl xylan	(1990 b)
Sulfolobus acidocaldarius		32.0	4.9	7.5-8.5		21.5μM PNP-Ac	p-Nitrophenyl and ali-	Sobek and Görisch
DSM 639							phatic esters	(1988)

The presence of several acetyl esterases in *Trichoderma reseei* indicates the occurence of multiple esterases in this microorganism.

Ferulic acid and coumaric acid esterases

Ferulic and *p*-coumaric acids have been shown to occur esterified to arabinofuranosyl substituents of arabinoxylan chains from graminaceous plants (Smith and Hartley, 1983; Mueller-Harvey *et al*, 1986). Ferulic- and coumaric acid esterases may participate in the overall hydrolysis of cell wall arabinoxylans esterified with ferulic and *p*-coumaric acids.
Ferulic acid and coumaric acid esterase activities have recently been detected in culture filtrates of *Streptomyces olivochromogenes* (MacKenzie *et al*, 1987; Johnson *et al*, 1988), *Fibrobacter succinogenes* (McDermid, *et al*, 1990 a), anaerobic fungi e.g. *Piromyces*, *Neocallimastix*, and *Orpinomyces* sp. (Borneman *et al*, 1990 a), and *Aspergillus awamori*, *Aspergillus phoenicis*, and *Trichoderma reesei* (Smith *et al*, 1991).
MacKenzie and Bilous (1988) have partially purified a ferulic acid esterase from *Schyzophyllum commune* which was only able to release ferulic acid from wheat bran in the presence of an endo-xylanase.
Only two esterases with ferulic acid esterase activity, have been purified to homogeneity (Table VI). The esterases had a low molecular weight and were optimal at a pH around 5.0. The esterase purified from *Aspergillus oryzae* had a wide substrate specificity, liberating ferulic, *p*-coumaric, and acetic acids from steam-extracted wheat straw fragments. The enzyme was similar to the esterase from *S. commune* in that it acted in synergism with endo-xylanase (Tenkanen *et al*, 1991).
The ferulic acid esterase from *Streptomyces olivochromogenes* was active towards methyl ferulate and de-starched wheat bran. This enzyme was not able to release any ferulic acid from de-starched wheat bran, but acted synergistically in the presence of endo-xylanase (Faulds and Williamson, 1991).

α-Glucuronidases

A major substituent of plant xylans is (4-*O*-methyl)glucopyranosyl uronic acid which is α-(1,2)-linked to xylopyranosyl residues. α-Glucuronidases are required in the overall conversion of (arabino)glucuronoxylans to cleave off the terminal (4-*O*-methyl)glucuronic acid side chains.
α-Glucuronidases have been detected in culture filtrates of *Trichoderma reesei* (Dekker, 1983; Poutanen *et al*, 1987), *Streptomyces olivochromogenes* (MacKenzie *et al*, 1987; Johnson *et al*, 1988), *Lentinula edodes* (Mishra *et al*, 1990), *Aspergillus awamori* and *Aspergillus phoenicis* (Smith *et al*, 1991),and *Fibrobacter succinogenes* S85 (Smith and Forsberg, 1991).

Table VI. Characteristics of ferulic acid and coumaric acid esterases from various sources.

Source of enzyme	Code	M_w (kDa)	pI	pH_{opt}	T_{opt} (°C)	K_m	Substrate	Reference
Aspergillus oryzae VTT-D-85248		30.0 19.0*	3.6	4.5-6.0			Acetyl xylo-oligomers, α-naphtyl acetate, steamed wheat straw	Tenkanen *et al* (1991)
Streptomyces olivochromogenes NRCC 2258		29.0	7.9/8.5	5.5	30°	1.86 mM	Methyl ferulate	Faulds and Williamson (1991)

Table VII. Characteristics of α-glucuronidases from various sources.

Source of enzyme	Code	M_w (kDa)	pI	pH_{opt}	T_{opt} (°C)	K_m	Substrate	Reference
Agaricus bisporus str. S. Liese (ATCC 44736)		>450.0* (≈450.0)*		3.3	52°		Birch/beech glucurono-xylan-oligosaccharides	Puls *et al* (1987)
Thermoascus aurantiacus		118.0		4.5	60°	0.145 mM $MeGlcAX_3$	Glucuronoxylan, glucuronoxylan oligosaccharides	Khandke *et al* (1989 c)

* Determined by gel permeation chromatography

Puls *et al* (1987) detected α-glucuronidase activity in *Agaricus bisporus* and *Pleurotus ostreatus*. The α-glucuronidase from *Agaricus bisporus* was partially purified (Table VII) by using 4-*O*-methyl-α-D-glucopyranosyluronic acid-xylobiose. Incubation of native glucuronoxylan with α-glucuronidase did not show the release of glucuronic acid, which indicates the inability of this enzyme to split off glucopyranosyl uronic acid from intact xylan. The α-glucuronidase acted synergistically with endo-xylanase in the breakdown of beechwood xylan. Oligosaccharides were accessible to ß-xylosidase after cleaving off the 4-*O*-methylglucuronic acid.

A second α-glucuronidase was purified from *Thermoascus aurantiacus* (Table VII) using a colorimetric assay based on 4-*O*-methyl-α-D-glucopyranosyluronic acid-xylotriose as substrate. The enzyme hydrolysed 4-*O*-methylglucurono-xylo-oligosaccharides from X_1 to X_7 at rates comparable to those of larchwood xylan. The enzyme is thus different from the *Agaricus bisporus* α-glucuronidase in that it is able to split off glucopyranosyl uronic acid substituents from polymer xylan. Both enzymes had a high molecular weight and were most active at acidic conditions and high temperatures.

Mode of action

(1,4)-ß-D-Xylans, as well as arabino-, glucurono-, arabinoglucurono-, and arabino-4-*O*-methylglucuronoxylans or their acetylated forms, are partly accessible to endo-(1,4)-ß-D-xylanase action yielding xylose, xylo-oligosaccharides, and a range of xylo-oligosaccharides substituted with acetyl groups, arabinofuranose or (4-*O*-methyl)glucuronic acid. The fine structure of arabinoxylans as well as the mode of action of endo-(1,4)-ß-D-xylanases have been studied by examining the structure of substituted oligosaccharides released by endo-xylanase action.

A number of arabinoxylan- (Table VIII), glucuronoxylan- (Table IX), and feruloylated arabinoxylan-derived oligosaccharides (Table X) have been released by enzymic breakdown of the respective substrates and purified to homogeneity.

Most common structures released from arabinoxylans are 3^2-α-L-Ara*f*-Xyl_2, 3^2-α-L-Ara*f*-Xyl_3, and 3^3-α-L-Ara*f*-Xyl_3. The presence of these oligosaccharides in various arabinoxylan hydrolysates could indicate a similar mode of action of the endo-xylanases studied.

Goldschmid and Perlin (1963) suggested the need of two unbranched xylopyranosyl groups in sequence between branched xylopyranosyl residues, as shown in Figure 2. Xylose and xylobiose released by the same enzyme may represent interbranch segments of the xylan main chain.

Hemicellulase II from *Ceratocystis paradoxa* released oligosaccharides with arabinofuranosyl linked to terminal non-reducing xylopyranosyl residues. The enzyme was able to hydrolyse Ara*f*-Xyl_3, Ara*f*-Xyl_4, and Ara*f*-Xyl_5 to Ara*f*-Xyl_2, xylose and xylobiose or xylotriose (Fig. 3). The prefered site of attack is the xylosidic linkage between the third and fourth residue (Dekker and Richards, 1975 b).

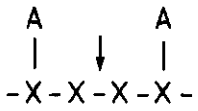


Figure 2. Site of attack (↓) of *Myrothecium verrucaria* endo-xylanase on wheat-flour arabinoxylan (Goldschmid and Perlin, 1963).

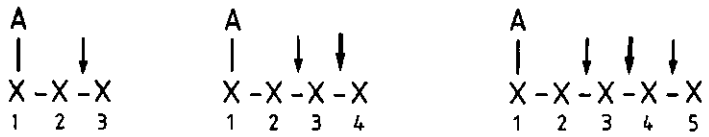


Figure 3. Site of attack (↓, linkage rapidly attacked; ↓, linkage attacked at lower rate) of *Ceratocystis paradoxa* hemicellulase II on hemicellulose B (spear grass) arabinoxylan (Dekker and Richards, 1975 b).

In most cases the glycosidic linkage at the reducing site of the substituent seems to be inaccessible for endo-xylanase action. However, this is not true for endo-xylanase I from *Aspergillus niger* (Takenishi and Tsujisaka, 1973), which released two completely different types of xylo-oligosaccharides, bearing arabinofuranose at the reducing xylopyranosyl residue. It is assumed that this enzyme has a higher affinity to glycosidic linkages adjacent to substituted xylopyranosyl residues than to glycosidic linkages of unsubstituted xylopyranosyl residues in rice straw arabinoxylan (Fig. 4). Two endo-xylanases purified from *Aspergillus niger* by Frederick *et al* (1985) also have affinity to arabinoxylan near branchpoints. It is therefore possible that these enzymes show a similar pattern of action. However, the latter two enzymes differ from the endo-xylanase I purified by Takenishi and Tsujisaka (1973) in that they are not able to release significant amounts of xylose and xylobiose.

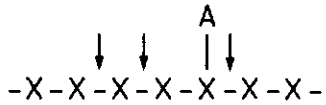


Figure 4. Site of attack (↓) of *Aspergillus niger* endo-xylanase I on rice straw arabinoxylan (Takenishi and Tsujisaka, 1973).

Table VIII. Branched oligosaccharide structures released by endo-(1,4)-ß-xylanases from arabinoxylans.

Source of enzyme	Type	Substrate	Product	Arabinose-releasing	Reference
Myrothecium verrucaria	Cellulase	Wheat straw, cocks-grass, rye flour	3^2-α-L-Ara*f*-Xyl_2	-	Bishop (1956)
Commercial preparation	Hemicellulase		3^2-α-L-Ara*f*-Xyl_2	-	Aspinall *et al* (1960)
Streptomyces QMB 814	Xylanase	Wheat	(A, X), X_2, X_3 3^2-α-L-Ara*f*-Xyl_3	+	Goldschmid and Perlin (1963)
Aspergillus niger	Xylanase I	Rice straw	X, X_2 3^1-α-L-Ara*f*-Xyl_2 3^1-α-L-Ara*f*-Xyl_3	-	Takenishi and Tsujisaka (1973)
Ceratocystis paradoxa	Hemicellulase II	Hemicellulose B (spear grass)	X, X_2, X_3 3^2-α-L-Ara*f*-Xyl_2 3^3-α-L-Ara*f*-Xyl_3	-	Dekker and Richards (1975 c)
Sporotrichum dimorphosporum	Xylanase II	Redwood arabi-noglucuronoxy-lan	X, X_2, X_3 3^2-α-L-Ara*f*-Xyl_2 3^3-α-L-Ara*f*-Xyl_3	+/-	Comtat and Joseleau (1981)
Streptomyces	Xylanase	Rice hull ara-binoxylan	X, X_2, X_3 3^2-α-L-Ara*f*-Xyl_2		Watanabe *et al* (1983)
Streptomyces sp. E86	Xylanase	Corncob ara-binoxylan	3^2-α-L-Ara*f*-Xyl_3 [2-ß-D-Xyl*p*]-3^2-α-L-Ara*f*-Xyl_3 [2-ß-D-Xyl*p*]-3^3-α-L-Ara*f*-Xyl_3		Kusakabe *et al* (1983)
Trichoderma viride (Comm.)	Cellulase	Bagasse LCC	3^2-α-L-Ara*f*-Xyl_3		Kato *et al* (1987)
Polyporus tulipiferae	Xylanase II	Oat spelt ara-binoglucuro-noxylan	X, X_2, (X_3) 3^2-α-L-Ara*f*-Xyl_2 3^3-α-L-Ara*f*-Xyl_3		Brillouet (1987)
Streptomyces sp. E86	Xylanase	Rice straw ara-binoglucurono-xylan	A, X, X_2, X_3 3^2-α-L-Ara*f*-Xyl_2 3^2-α-L-Ara*f*-Xyl_3	+	Yoshida *et al* (1990)

Table IX. Branched oligosaccharide structures released by endo-(1,4)-ß-xylanases from glucuronoxylans.

Source of enzyme	Type	Substrate	Product	Reference
Basidiomycete	Xylanase	Aspen glucuronoxylan	2^3-(4-O-Me-α-D-Glc*p*A)-Xyl_3	Comtat *et al* (1974)
Trametes hirsuta (Wulf) *pilat*	Xylanase	Willow glucuronoxylan	(X_2), X_4, X_6 2^3-(4-O-Me-α-D-Glc*p*A)-Xyl_3 2^4-(4-O-Me-α-D-Glc*p*A)-Xyl_4 2^5-(4-O-Me-α-D-Glc*p*A)-Xyl_5	Kubackova *et al* (1979)
Sporotrichum dimorphosporum	Xylanase II	Redwood arabinoglucuronoxylan	X, X_2, X_3 2^3-(4-O-Me-α-D-Glc*p*A)-Xyl_3 2^4-(4-O-Me-α-D-Glc*p*A)-Xyl_4	Comtat and Joseleau (1981)
Polyporus tulipiferae	Xylanase II	Larchwood glucuronoxylan	X, X_2, (X_3) 2^3-(4-O-Me-α-D-Glc*p*A)-Xyl_3 2^4-(4-O-Me-α-D-Glc*p*A)-Xyl_4	Brillouet (1987)
Thermoascus aurantiacus	Xylanase	Larchwood glucuronoxylan	X, X_2 2^2-(4-O-Me-α-D-Glc*p*A)-Xyl_3	Khandke *et al* (1989 b)
Streptomyces sp. E86	Xylanase	Rice straw arabinoglucuronoxylan	A, X, X_2, X_3 2^3-(4-O-Me-α-D-Glc*p*A)-Xyl_3 2^3-α-D-Glc*p*A-Xyl_3	Yoshida *et al* (1990)
Clostridium thermolacticum	Xylanase I	Larchwood glucuronoxylan	X_2, X_3 $2^1/2^2/2^3$-(4-O-Me-α-D-Glc*p*A)-Xyl_3 $2^3/2^4$-(4-O-Me-α-D-Glc*p*A)-Xyl_4 $2^3/2^4/2^5$-(4-O-Me-α-D-Glc*p*A)-Xyl_5	Debeire *et al* (1990)
Bacillus subtilis	Glucuronoxylanase	Vigna, maize	2^2-α-D-Glc*p*A-Xyl_6	Nishitani and Nevins (1991)

Modes of action which include the influence of α-(1,2) or α-(1,3)-linked arabinofuranose to single substituted xylopyranosyl groups, or the influence of α-(1,2) and α-(1,3)-linked arabinofuranose to double substituted xylopyranosyl groups, can only be examined when structures bearing these substituents are isolated. To preserve these substituted xylo-oligosaccharides in its native form, it is important to use pure enzymes devoid of arabinofuranosidase activity, which is not the case for some of the endo-xylanases used in the past (Table VIII).

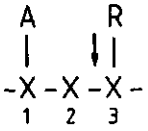


Figure 5. Site of attack (↓) of *Sporotrichum dimorphosporum* endo-xylanase on redwood arabinoglucuronoxylan; R, hydrogen or α-(1,3)-linked arabinofuranose (Comtat and Joseleau, 1981).

Comtat and Joseleau (1981) isolated neutral as well as acidic xylo-oligosaccharides from redwood arabinoglucuronoxylan (Table VIII and IX) by using an endo-xylanase from *Sporotrichum dimorphosporum*. The enzyme required three xylopyranosyl residues unsubstituted at *O*-2 of residue 1 (Fig. 5) and unsubstituted at *O*-2 and *O*-3 of residue 2. Residue 3 can be unsubstituted, substituted at *O*-3 with arabinofuranose, or substituted at *O*-2 with glucopyranosyl uronic acid.

Similar structures were obtained with *Streptomyces* sp. E86 endo-xylanase (Yoshida *et al*, 1990). It seems that the specificity towards arabinofuranosyl substituents is also different from that towards glucopyranosyl uronic acid substituents (Fig. 6). It is, however, not clear whether the difference is due to the distinction between neutral or acidic substituent or to the distinction of α-(1,2) and α-(1,3)-linkages.

Figure 6. Site of attack (↓) of *Streptomyces* sp. E86 endo-xylanase on rice straw arabinoglucuronoxylan (Yoshida *et al*, 1990).

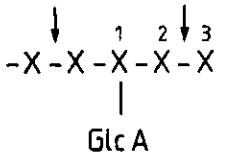


Figure 7. Site of attack (↓) of *Thermoascus aurantiacus* endo-xylanase on larchwood glucuronoxylan (Khandke *et al*, 1989 b).

The endo-xylanase purified from *Thermoascus aurantiacus* (Khandke *et al*, 1989 b) released 2^2-(4-*O*-Me-α-D-Glc*p*A)-Xyl_3 from larchwood glucuronoxylan after extensive hydrolysis. This implies that the enzyme is able to hydrolyse the glycosidic linkage between the second and third residue (Fig. 7), and thereby distinguishes itself from endo-xylanases mentioned above.

The endo-xylanase from *Clostridium thermolacticum* (Debeire *et al*, 1990) was different from all endo-xylanases in that it released xylo-oligosaccharides with glucopyranosyl uronic acid attached to the terminal non-reducing xylopyranosyl residue as well as to the reducing xylopyranosyl residue.

Feruloylated and coumaroylated arabinoxylan-oligosaccharides have also been obtained from cell-wall material (Table X). Commercial enzyme preparations, devoid of ferulic and coumaric acid esterases, were used to obtain these oligosaccharides. The oligosaccharides provide information on the interlinkages of hemicellulosic material in cell-wall material and can be used as substrates for the assay of feruloyl- and *p*-coumaroyl esterase (Borneman *et al*, 1990 b).

The mechanism of endo-xylanases becomes complex when unimolecular hydrolysis of xylo-oligosaccharides is replaced by bimolecular mechanisms i.e. termolecular shifted reaction, transglycosylation, or condensation (Biely *et al*, 1981 b).

More insight in the mechanism of action of endo-xylanases was obtained by determining bond-cleavage frequencies using non-reduced and reduced xylo-oligosaccharides, or by using labeled xylo-oligosaccharides. From these data, indications on the number of subsites and the allocation of the active site were obtained. Estimations for the number of subsites have been made for endo-xylanases from fungi, bacteria as well as yeast, ranging from 4 to 7 subunits (Table XI).

The active site of the *Aspergillus niger* endo-xylanase purified by Biely *et al* (1983) was allocated between the fourth and fifth subsite counting from the non-reducing end, whereas the active site of the *Aspergillus niger* endo-xylanase purified by Meagher *et al* (1988) was allocated between the third and fourth subsite.

Table X. Branched oligosaccharide structures released by endo-(1,4)-ß-xylanases (commercial preparations) from feruloylated xylans.

Source of enzyme	Type	Substrate	Product	Reference
Oxysporus spp. (comm. prep.)	Cellulase	Wheat bran cell-walls	[5-O-(trans-feruloyl)]-2-ß-L-Ara*f*-Xyl	Smith and Hartley (1983)
Basidiomycete	Driselase	Zea shoot cell-walls	[5-O-feruloyl]-3^2-α-L-Ara*f*-Xyl_2	Kato and Nevins (1985)
Oxysporus spp. (comm. prep.)	Cellulase	Barley straw cell-walls	[5-O-(trans-feruloyl)]-3^2-α-L-Ara*f*-Xyl_2 [5-O-(trans-p-coumaroyl)]-3^2-α-L-Ara*f*-Xyl_2	Mueller-Harvey *et al* (1986)
Trichoderma viride (comm. prep.)	Cellulase	Sugar cane bagasse	[5-O-(trans-feruloyl)]-3^2-α-L-Ara*f*-Xyl_3	Kato *et al* (1987)
Basidiomycete	Driselase	Bamboo shoot cell-walls	[5-O-(trans-feruloyl)]-3^2-α-L-Ara*f*-Xyl_2 [5-O-(trans-feruloyl)]-3^2-α-L-Ara*f*-Xyl_3	Ishii and Hiroi (1990)
Basidiomycete	Driselase	Bamboo shoot cell-walls	5,5'-di-O-(diferul-9,9'-diol)-[3^2-α-L-Ara*f*-Xyl_2]	Ishii (1991)

Table XI. Proposed number of subsites of various endo-xylanases obtained by bond-cleavage frequencies.

Source of enzyme	Number of subunits	Active site	Reference
Aspergillus niger	7		Biely *et al* (1983)
Aspergillus niger	5		Meagher *et al* (1988)
Cryptococcus albidus	4		Biely *et al* (1981 a)
Mesophilic fungus str. Y-94			Mitsuishi *et al* (1988)
A	5		
B	5		
C	5		
Trametes hirsuta pilat	5	-	Kubackova *et al* (1979)

The latter enzyme also differed from the former endo-xylanase in that it has no trans-D-xylosylation activity. The inability of these and other endo-xylanases to split xylobiose is probably due to the negative binding-energy of both subsites adjacent to the catalytic site (Biely *et al*, 1983).

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Chapter 3

Purification and characterization of three endo-(1,4)-ß-xylanases and one ß-xylosidase from *Aspergillus awamori*

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J. Biotechnol. in press.

Summary

Three endo-(1,4)-ß-xylanases (endo-xylanase I, II, and III) and one ß-D-xyloside xylohydrolase (ß-xylosidase) were purified from a crude culture filtrate of *Aspergillus awamori* CMI 142717, grown on milled oat straw as carbon source. *Aspergillus awamori* xylanases differ in some characteristics of known xylanases. The optimum pH for the endo-xylanases were between 4.0 and 5.5 and the optimum temperature between 45° and 55°C; ß-xylosidase was optimal around pH 6.5 and 70°C. All endo-xylanases were able to degrade xylan to xylobiose and xylotriose. Endo-xylanase I also produced small amounts of xylose. The molecular weights of endo-xylanase I, II, and III were respectively 39 kDa, 23 kDa, and 26 kDa. The molecular weight of ß-xylosidase was 110 kDa. The specific activities of endo-xylanase I, II, and III towards water-soluble oat spelts arabinoxylan were respectively 69.6 U/mg, 68.6 U/mg, and 16.3 U/mg. The specific activity of ß-xylosidase towards *p*-nitrophenyl-ß-xylopyranoside was 34.1 U/mg. The activity of these enzymes was significantly inhibited by Hg^{2+}, Pb^{2+}, and Ag^{+}.

Introduction

The most abundant type of hemicellulose is xylan, a heteropolysaccharide which can be found in the cell wall of many plants. The total hydrolysis of the backbone of xylan requires endo-(1,4)-ß-xylanases (endo-xylanases; E.C. 3.2.1.8), which cleave the ß-(1,4)-glycosidic linkages between D-xylopyranosyl residues, and ß-D-xyloside xylohydrolases (ß-xylosidases; E.C. 3.2.1.37), which release D-xylopyranosyl from xylooligosaccharides.
Endo-xylanases and ß-xylosidase, major components of xylanolytic systems, are produced by many micro-organisms. Xylanolytic systems can consist of more than one endo-xylanase and more than one ß-xylosidase (see reviews by Dekker and Richards, 1976; Reilly, 1981; Wong *et al*, 1988). Such multi-enzyme complexes degrading xylans have been purified from *Aspergillus* (Rodionova *et al*, 1977; John *et al*, 1978; and Reilly, 1981), *Trichoderma* (Tan, 1985; Lappalainen, 1986), *Sporotrichum dimorphosporum* (Comtat, 1983), and *Neurospora crassa* (Deshpande *et al*, 1986).
In this chapter we discuss the purification and characterization of the xylanolytic system from *Aspergillus awamori* CMI 142717 consisting of three endo-xylanases and one ß-xylosidase.

Materials and methods

Materials
Chemicals used, if not mentioned otherwise, are of analytical grade. Substrates used were oat spelts arabinoxylan (Sigma Chemical Co., USA), water-soluble and water-insoluble oat spelts arabinoxylan fractionated according to the method of Selvendran *et al* (1985) and Blake and Richards (1971), and PNP-ß-xylopyranoside (Koch and Light, Colnbrook, Bucks, England). Alkali extracted wheat and barley arabinoxylan were kindly provided by H. Gruppen and R. Viëtor of the Food Science Department of the Agricultural University (Wageningen, The Netherlands). Water-soluble wheat arabinoxylan was a gift from IGMB-TNO (Wageningen, The Netherlands).
CM-cellulose (Type AF 0305) was obtained from Enka Industrial Colloids (Arnhem, The Netherlands), Avicel was obtained from Serva (Heidelberg, FRG). Sugar beet arabinan was obtained from British Sugar (Norwich, UK) and Koch and Light. PNP-ß-glucopyranoside, PNP-ß-galactopyranoside and PNP-α-arabinofuranoside were purchased from Koch and Light. H_3PO_4-swollen cellulose was prepared according to the method described by Wood (1971). Potatogalactan was isolated at our own laboratory according to the method of Labavitch *et al* (1976). An arabinan rich pectin fraction was isolated from apple juice (MHR; Schols *et al*, 1990).
The *Aspergillus awamori* CMI 142717 culture filtrate was produced at the Rowett Research Institute (Aberdeen, Scotland; Smith *et al*, 1987).

Enzyme purification was carried out at 4°C, using successively Bio-Gel P10 gel filtration medium (85.0 x 2.8 cm), DEAE-Bio-Gel A anion exchanger (2.8 x 18 cm, and 2.0 x 18 cm; Bio-Rad Laboratories, Richmond, California), Ultrogel AcA 54 gel filtration medium (2.5 x 87.5 cm; IBF, Villeneuve-La-Garenne, France) and Mono Q™ anion-exchanger (Pharmacia-LKB Biotechnology, Uppsala, Sweden). Micro-scale runs were done on a Mono Q™ anion exchanger using a FPLC system (Pharmacia LKB Biotechnology). Buffers contained 0.01% (w/v) sodium azide to prevent microbial growth.

Sugar analysis

Sugar analyses were carried out on a Spectra Physics HPLC (San Jose, California, USA). Mono and oligomeric sugars were analysed by injection of the sample on to an Aminex HPX 87P column [eluents: water; flow: 0.5 mL/min; temperature 85°C (Voragen *et al*, 1986)]. Changes in the molecular weight distribution were determined by injection of the sample on to incorporating Biogel TSK 40+30+20 XL columns (High Performance Size Exclusion Chromatography, HPSEC) in series with a TSK guard column (6 x 40 mm) eluting with 0.4M sodium acetate buffer pH 3.0 at 30°C: the flowrate was 0.8 mL/min. Polysaccharides, oligomeric and monomeric sugars were detected by a Shodex SE-61 refractive index detector (Showa Denko K.K., Tokyo, Japan).

Assay for endo-xylanase and ß-xylosidase activities

Endo-xylanase and ß-xylosidase activities were measured according to Beldman *et al* (1985). Enzyme activities were performed in 50mM sodium acetate buffer pH 5.0 at 30°C and calculated from the increase in reducing end groups as measured by the Nelson-Somogyi assay (1952), by the ferricyanide assay in cases experiments were carried out with minor amounts of enzymes or substrates (Rozie *et al*, 1988), or from the release of *p*-nitrophenol from *p*-nitrophenyl-ß-xylopyranoside as measured spectrophotometrically at 400 nm. One unit of activity is defined as the amount of enzyme that catalyzed the release of 1 μmole of reducing end-groups or *p*-nitrophenol from the substrate per minute at 30°C.

SDS-Gel electrophoresis and iso-electric focusing

The molecular weight was determined by SDS-gel electrophoresis on a 10-15% polyacrylamide gel, according to the method described by Laemmli (1970). Electrophoresis was carried out with the PhastSystem (Note 110, PhastSystem, System Guide; Pharmacia LKB Biotechnology, Uppsala, Sweden). A low molecular weight kit (Pharmacia-LKB Biotechnology) from 14.4 up to 94.0 kDa was used for calibration.

The iso-electric point was determined by electrophoresis on a polyacrylamide gel containing ampholytes from pH 3-9 (Note 100, PhastSystem, System Guide). The gels were stained with Coommassie Brilliant Blue or silver (Note 200/210, PhastSystem, System Guide).

Temperature optimum and pH optimum

The optimum temperature for the endo-xylanases was determined by incubation of 0.1% (w/v) water-soluble oat spelts xylan in 50mM sodium acetate buffer pH 5.0 with endo-xylanase I, II, or III at 31°, 37°, 43°, 50°, 55°, 61°, 66°, and 72°C for 1 h. The temperature stability was determined by pre-incubation of endo-xylanase I, II, and III at 40°, 49°, 54°, and 61°C in 50mM sodium acetate buffer pH 5.0 for a set of times. Subsequently water-soluble oat spelts xylan was added to a final concentration of 0.1% (w/v) and the mixtures incubated for 1 h at 30°C.

The optimum pH for the endo-xylanases was determined by pre-incubation of endo-xylanase I, II, and III for 1 h at 30°C in citrate-phosphate buffer in a pH range of 2.5 up to 8.0. After pre-incubation water-soluble oat spelts xylan was added to a final concentration of 0.1% (w/v) and the mixtures incubated for 1 h at 30°C. The enzyme concentrations used were 0.07 μg/mL endo-xylanase I, 0.07 μg/mL endo-xylanase II, and 0.2 μg/mL endo-xylanase III.

The optimum temperature for ß-xylosidase was determined by incubation of 0.02% (w/v) PNP-ß-xylopyranoside in 50mM sodium acetate buffer pH 5.0 with 0.01 μg/mL ß-xylosidase at 32°, 40°, 54°, 60°, 68°, and 78°C for 1 h. The temperature stability was determined by pre-incubation of 0.01 μg/mL ß-xylosidase at 40°, 49°, 54°, 61°C, 70°C and 80°C in 50mM sodium acetate buffer pH 5.0 for a set of times. Subsequently PNP-ß-xylopyranoside was added to a final concentration of 0.02% (w/v) and the mixtures incubated for 1 h at 30°C. The optimum pH for ß-xylosidase was determined in the same way as with the endo-xylanases, however, with a final enzyme concentration of 0.07 μg/mL and a final substrate concentration of 0.02% PNP-ß-xylopyranoside. The activities were measured as mentioned above.

Inhibitory effect of different components

The effect of different components on the activity of the endo-xylanase I, II, III, and ß-xylosidase was tested by incubation of water-soluble oat spelts arabinoxylan with endo-xylanase I, II, or III, and by incubation of PNP-ß-xylopyranoside with ß-xylosidase in the presence of 1mM Cu^{2+}, Ca^{2+}, Pb^{2+}, Mn^{2+}, Ag^{+}, K^{+}, Na^{+}, DTT or EDTA for 1 h at 30°C in 50mM sodium acetate buffer pH 5.0. The release of reducing sugars was tested by the ferricyanide assay; the release of *p*-nitrophenol was assayed at 400 nm.

Time course study

Time course studies of the release of xylose, xylobiose (X_2), and xylotriose (X_3) were carried out over a period of 24 hours, by incubation of 0.2 μg/mL endo-xylanase I, II, or III, with 0.1% (w/v) water-soluble oat spelts arabinoxylan in 50mM sodium acetate buffer pH 5.0. In the case of ß-xylosidase, between 100 and 200 μg/mL X_2, X_3, or xylotetraose (X_4) was incubated with 0.05 μg/mL enzyme over a period of 8 hours. After incubation, the reaction mixtures were inactivated by placing them into a boiling water bath for 5 minutes and then analysed by HPLC.

Enzyme combinations

Water-soluble oat spelts arabinoxylan (0.1% (w/v)) was incubated with crude enzyme preparation (0.025 μg/mL), purified endo-xylanase I (0.01 μg/mL), endo-xylanase II (0.01 μg/mL), endo-xylanase III (0.04 μg/mL) or ß-xylosidase (0.04 μg/mL), and with combinations of these enzymes. After 1 h of incubation at 30°C in 50mM sodium acetate buffer pH 5.0, the reaction was terminated and the release of reducing sugars determined by the ferricyanide method.

Fractionation of xylan-digests

Water-soluble arabinoxylan was incubated with 1.0 μg/mL endo-xylanases I, or III for 24 h at 30°C in 50mM sodium acetate buffer pH 5.0. Enzyme mixtures were inactivated by placing the samples for 5 minutes in a boiling waterbath.

The xylan-digests were freeze dried and subsequently dissolved in 2-3 mL H_2O. The arabinoxylan digests were fractionated by a Bio-Gel P2 column (100 x 2.8 cm) at 50°C according to the method described by Labavitch *et al* (1976). The column was eluted with water, containing 0.02% thiomersal as preserving agent. The collected fractions (2.1 mL) were analysed on an auto-analyser (Skalar Analytical BV, Breda, The Netherlands) for neutral sugar and uronic acid content by the orcinol assay and the phenolsulfuric acid assay, respectively.

Determination of neutral sugars

Neutral sugars were determined by the phenolsulfuric acid assay according to Dubois *et al* (1956) or by an automated orcinol sulfuric acid assay (Tollier and Robin, 1979).

Determination of uronic acids

Uronic acid content was determined by the *m*-hydroxydiphenyl assay according to the method described by Ahmed and Labavitch (1977) and automatically by the *m*-hydroxydiphenyl assay (*m*HDP-assay) according to Thibault (1979). Correction was made for the interference of neutral sugars in the determination of uronic acid.

Determination of the sugar composition

Neutral sugar composition of polymeric and oligomeric substrates was determined by TFA hydrolysis (2M; 1 h at 121°C) and conversion to their alditol acetates as described by Englyst and Cummings (1984). Analysis was carried out on GLC (3% OV 275 on Chrom W HP 80-100 column) at 210°C; carrier gas was H_2 at a pressure of 0.6 kg/cm^3). Detection was performed by FID.

Protein determination

The protein content was measured according to Sedmak and Grossberg (1977) with bovine serum albumin as standard.

Results

Three endo-xylanases (I, II, and III) and one ß-xylosidase have been purified from *Aspergillus awamori* CMI 142717 according to the flowsheet in Figure 1.

Step 1. The enzyme preparation (30 mL of a liquid preparation) was applied on to a Bio-Gel P10 column and eluted with 10mM sodium acetate buffer pH 5.0. Fractions of 6.0 mL were collected (Fig. 2.a). One endo-xylanase (III) was retarded by the column. Thus two separate pools were obtained; fractions 38 till 56, and fractions 62 till 82.

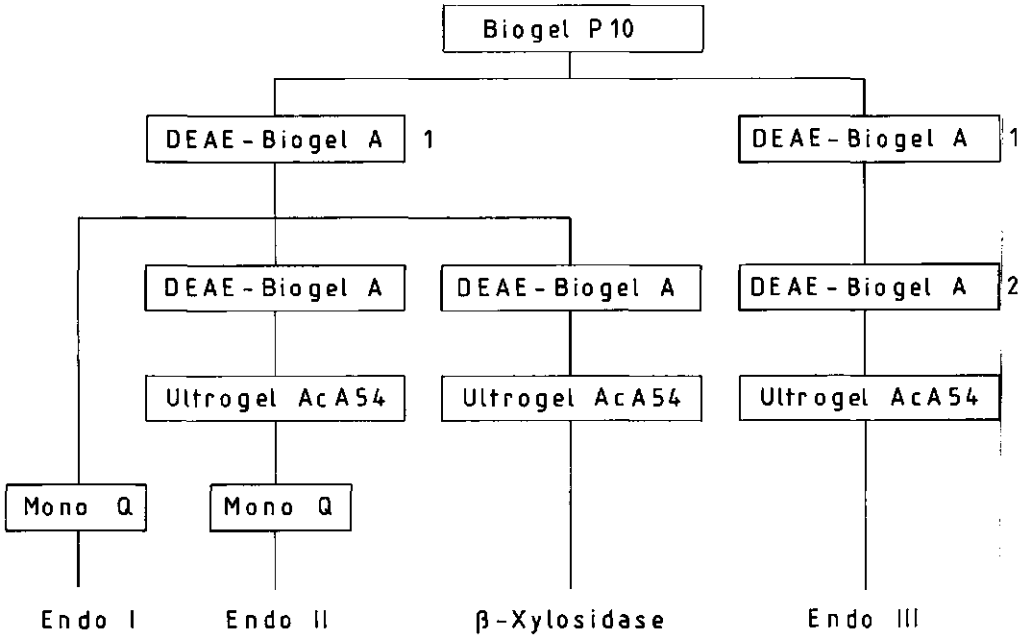

Figure 1. Flowsheet diagram of the purification of three endo-xylanases and one ß-xylosidase from *Aspergillus awamori*; 1, NaCl-gradient 0.0-0.5M; 2, pH-gradient 6.0-3.0.

Step 2. The obtained pools were applied on to a DEAE-Bio-Gel A column (18.0 x 2.8 cm), washed with 10mM sodium acetate buffer pH 5.0, followed by a sodium chloride gradient (10mM sodium acetate buffer pH 5.0 and 50mM sodium acetate buffer pH 5.0 containing 0.5M sodium chloride). Separation of components was maximised by maintaining the composition of the eluent at a fixed value during elution of peaks. Fractions of 9.5 mL were collected. The separation of the first pool (fractions 38 till 56) resulted in two endo-xylanase peaks and one ß-xylosidase peak (Fig. 2.b); one endo-xylanase (I) did not bind to the column, and could be purified to homogeneity by FPLC

using a Mono Q column. The other endo-xylanase (II) and a ß-xylosidase were released from the anion-exchanger by the sodium chloride gradient. The separation of the second pool (fraction 62 till 82) resulted in only one peak (Fig. 2.c).
Step 3. Relevant fractions were pooled and dialysed against 0.05M sodium acetate buffer pH 6.0. Dialysed fractions were applied on to a DEAE-Biogel A column (2.0 x 18.0 cm). Unbound proteins were removed from the column with 0.05M sodium acetate buffer pH 6.0. Adsorbed proteins were eluted with a pH gradient (0.05M sodium acetate buffer pH 6.0 and 0.05M sodium acetate buffer pH 3.0). Fractions of 9.5 mL were collected.
Step 4. Relevant fractions were pooled and subsequently concentrated by ultrafiltration. The concentrated fractions were applied on to an Ultrogel AcA 54 column and eluted with 10mM sodium acetate buffer pH 5.0. Fractions of 2.8 mL were collected.
Step 5. A final purification step was carried out for endo-xylanase I and II on a Mono Q™ anion exchanger in 20mM piperazine buffer pH 9.5 and 5.0, respectively, with a sodium chloride gradient from 0.0-0.5M.

All three endo-xylanases (endo I, II, and III) and the ß-xylosidase showed only one single band on SDS-polyacrylamide gel electrophoresis (SDS-PAGE; Fig. 3).
For the endo-xylanase I, II, and III, the molecular weights, determined by SDS-gel electrophoresis, are, respectively, 39 kDa, 23 kDa, and 26 kDa (Table I); the molecular weight of the ß-xylosidase component is 110 kDa.
The various purified enzymes were tested for their physico-chemical and kinetic characteristics. As Table I shows, the specific activity of endo-xylanase I and II towards water-soluble oat spelts arabinoxylan are 69.6 and 68.6 U/mg, respectively, and these are much higher than the specific activity of endo-xylanase III (16.3 U/mg) towards the same substrate. The specific activity of all enzymes tested was much lower (i.e. in the range of 0.1-2.2 U/mg) when the water-insoluble arabinoxylan fraction was the substrate. The specific activity of ß-xylosidase, using PNP-ß-xylopyranoside as substrate, is 34.1 U/mg.
Other substrates were tested. Notable was the fact that the endo-xylanase II preparation (before purification on Mono Q™) showed some degree of activity on arabinan and PNP-α-arabinofuranoside; this was also noticed for the ß-xylosidase preparation (Kormelink *et al*, 1990).
Of the three endo-xylanases, endo-xylanase I had the highest pI (5.7-6.7); endo-xylanase II and III are isoelectric at pH 3.7 and 3.3-3.5 respectively. ß-Xylosidase had a pI of 4.2.
The optimum pH for the endo-xylanases is between 4.0 and 6.0 and the optimum temperature between 45° and 55°C; the ß-xylosidase activity is optimal at pH around 6.5 and at 70°C.
In relation to the optimum temperature it is important to know the temperature stability of the enzymes. It can be seen from Figure 4 that endo-xylanase I is more stable than endo-xylanase II and III. ß-Xylosidase, however, is more stable than any of the endo-xylanases; at 70°C it is still active whereas the endo-xylanases loose their activity rapidly above 55°C.

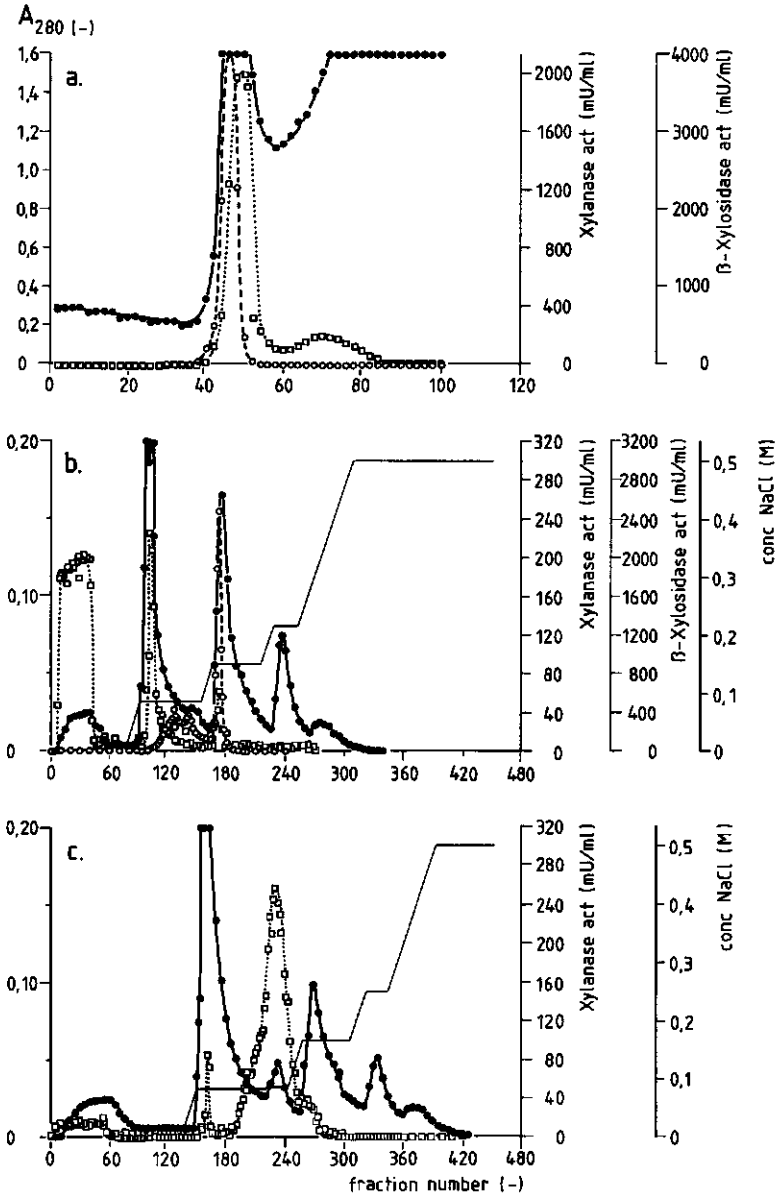

Figure 2. Fractionation patterns of the purification of endo-xylanases and ß-xylosidase from *Aspergillus awamori*; crude enzyme preparation on Bio-Gel P10 (a), fractions 38 till 56 from Fig. 2.a applied on to DEAE-Bio-Gel A (b), fractions 62 till 82 from Fig. 2.a applied on to DEAE-Bio-Gel A (c). Monitoring: —●—●—●—●—●— A_{280}, -□-□-□- endo-xylanase activity, -o-o-o- ß-xylosidase activity.

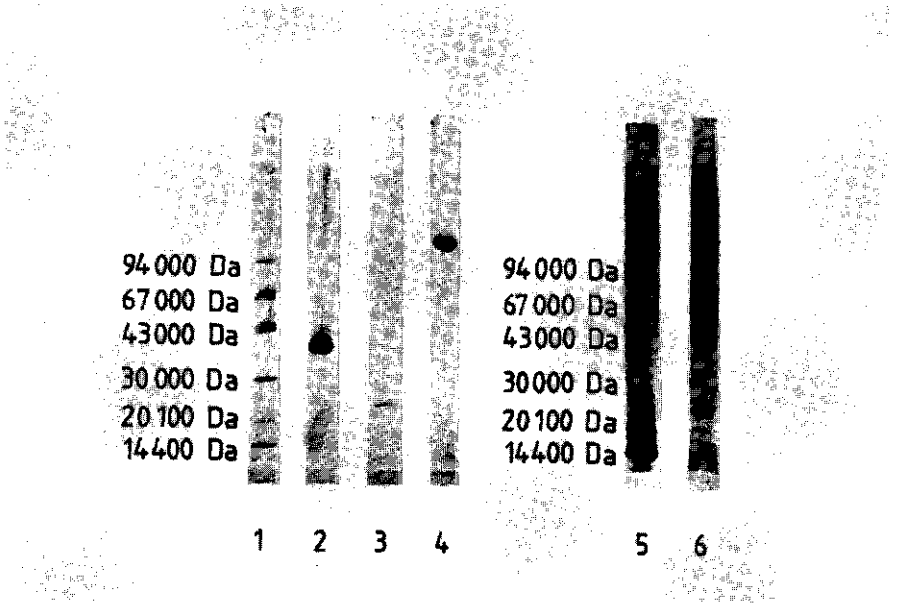

Figure 3. SDS gel-electrophoresis of the three endo-xylanases and ß-xylosidase from *Aspergillus awamori* CMI 142717; Lane 1 and 5, LMW calibration kit; Lane 2, endo-xylanase I; Lane 3, endo-xylanase II; Lane 4, ß-xylosidase; Lane 6, endo-xylanase III; lane 1-4 CBB staining, lane 5-6 silver staining.

Table I. Physico-chemical and kinetic characteristics of the purified endo-xylanase I, II, III, and ß-xylosidase from *Aspergillus awamori* CMI 142717.

Characteristic	Enzyme Endo I	Endo II	Endo III	ß-xylosidase
Specific activity (U/mg)				
- soluble oat spelt xylan	69.6	68.6	16.3	1.7
- insoluble oat spelt xylan	2.2	1.1	1.0	0.1
- PNP-ß-xylopyranoside	0.0	0.0	0.0	34.1
M_r (kD)	39	23	26	110
pI	5.7-6.7	3.7	3.3-3.5	4.2
Optimum pH	5.5-6.0	5.0	4.0	6.5
Optimum T (°C)	55	50	45-50	70
K_m (mg/mL)	1.00	0.33	0.09	0.003
V_{max} (U/mg)	10000	3333	455	476

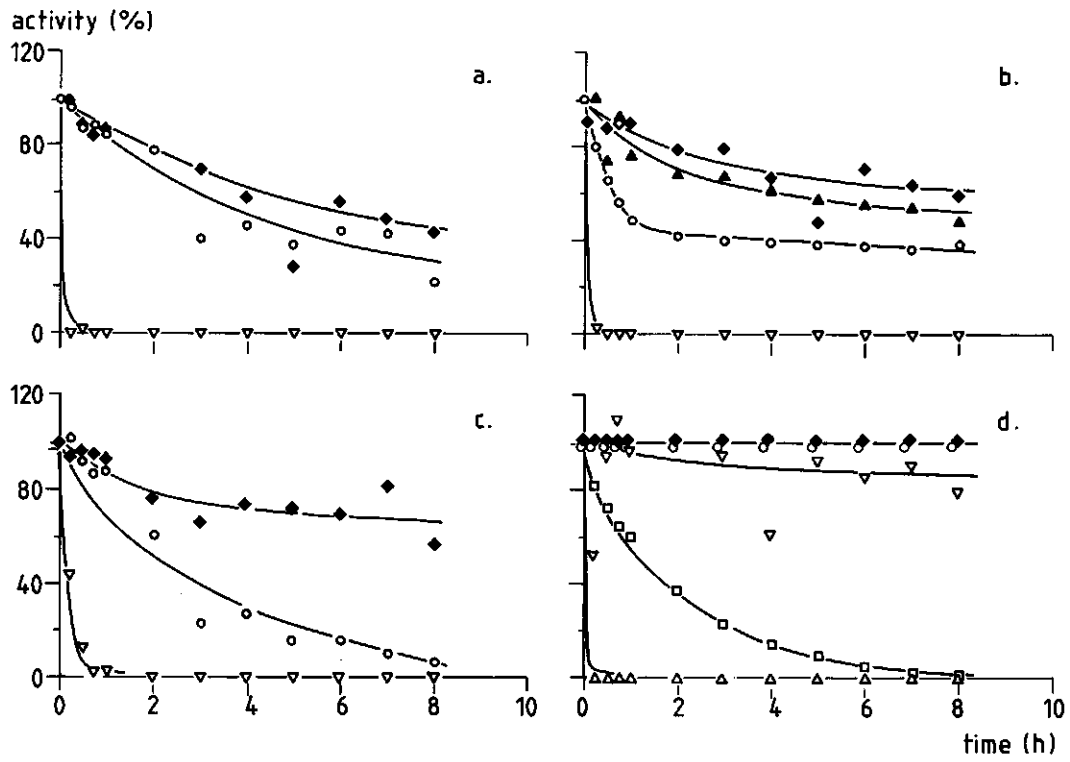

Figure 4. Temperature-stability curves for endo-xylanase I (a), endo-xylanase II (b), endo-xylanase III (c), and ß-xylosidase (d); ◆ 40°C, ▲ 49°C, 54°C, ▽ 61°C, ◻ 70°C, △ 80°C.

Using water-soluble oat spelts arabinoxylan as substrate (Table I), endo-xylanase I had the highest K_m of the three endo-xylanases; it also had the highest V_{max}. All three endo-xylanases were active towards different arabinoxylan fractions of wheat and barley (Table II). It was notable that endo-xylanase III has a low specific activity to the alkali-extractable arabinoxylan from wheat. Neither endo-xylanase is particularly active to an alkali-extractable arabinoxylan from barley. K_m and V_{max} for ß-xylosidase were respectively 0.003 mg/mL and 476 U/mg, determined towards PNP-ß-xylopyranoside. Neither of the polysaccharides was a good substrate for the ß-xylosidase.
The activity of endo-xylanase I, II, and III, and ß-xylosidase towards water-soluble arabinoxylan from oat spelts and PNP-ß-xylopyranoside was determined also in the presence of different additives.

Table II. Substrate specificities (U/mg) of endo-xylanase I, II, III, and ß-xylosidase towards wheat flour and dehusked barley arabinoxylan fractions.

Substrate	Enzyme Endo I	Endo II	Endo III	ß-xylosidase
Wheat:				
- water soluble arabinoxylan	24.2	24.1	21.3	0.7
- alkali extractable arabinoxylan	17.1	19.5	3.3	n.d.
- WIS	11.8	12.2	3.2	0.5
Barley:				
- alkali extractable arabinoxylan	4.5	7.3	3.1	n.d.

n.d. = not determined
WIS = water insoluble solids

Table III. Inhibitory effect of different components on the purified *Aspergillus awamori* enzymes (activities expressed in percent of the original activity).

Enzyme	Additive $CaCL_2$	$Pb(NO_3)_2$	$AgNO_3$	KCl	$HgCl_2$	$MgSO_4$	DTT	EDTA
Endo I	90.4	37.7	84.7	96.4	57.9	89.0	107.3	105.8
Endo II	91.8	58.5	108.0	89.5	71.3	87.2	107.9	122.0
Endo III	67.3	40.1	103.4	98.6	84.3	76.8	92.4	104.8
ß-Xylosidase	97.4	76.4	1.5	95.9	50.2	94.2	104.5	100.0

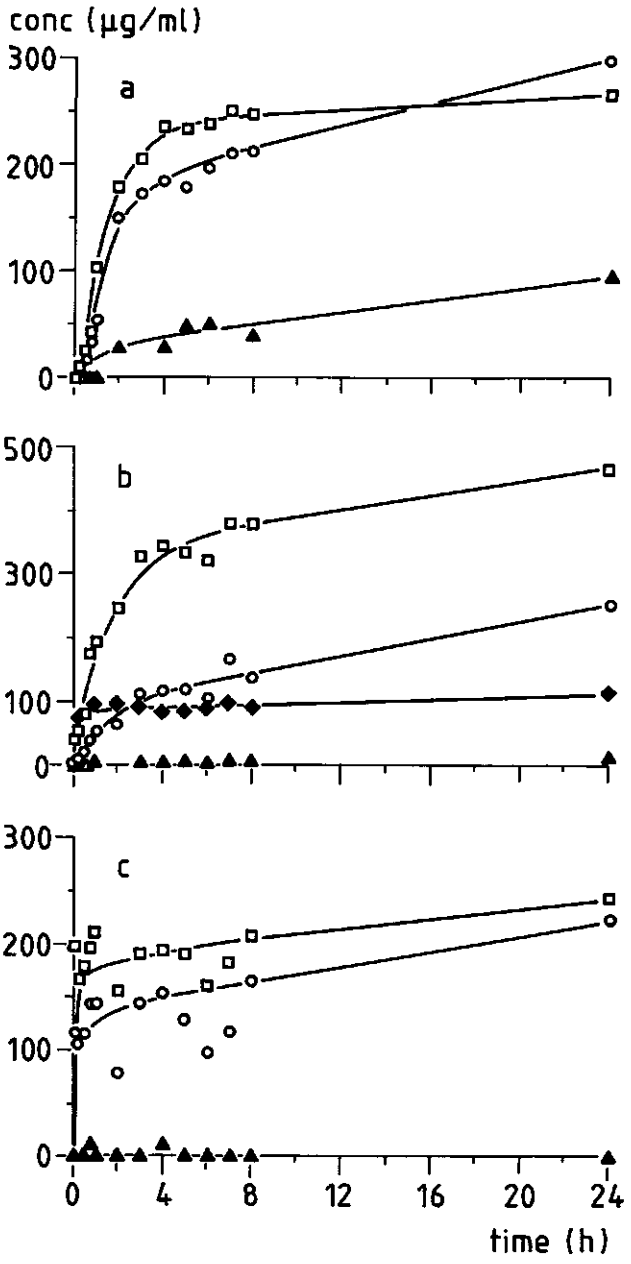

Figure 5. Time-curves for the release of arabinose (◆), xylose (▲), xylobiose (o), and xylotriose (□) from water-soluble oat spelts xylan by endo-xylanase I (a), endo-xylanase II (b), and endo-xylanase III (c).

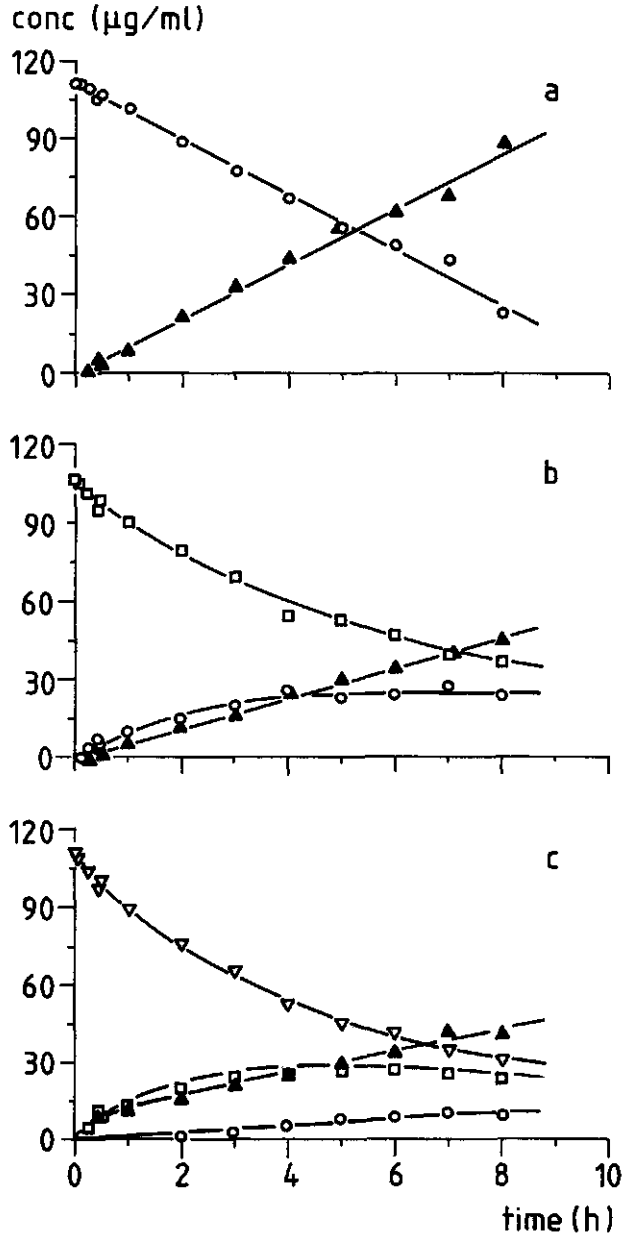

Figure 6. Time-curves for the degradation of xylobiose (a), xylotriose (b), and xylotetraose (c) by ß-xylosidase; Δ X_1, ○ X_2, □ X_3, and ▽ X_4.

It can be seen (Table III) that Pb^{2+} and Hg^{2+} have a strong inhibitory effect on most enzymes; Ag^{+} has a strong inhibitory effect only on the ß-xylosidase.
The pattern of action of endo-xylanase I, II, and III, was studied on water-soluble arabinoxylan from oat spelts (Fig. 5). It can be seen that the endo-xylanases release xylobiose and xylotriose in different ratios but only endo-xylanase I released xylose in small amounts. Arabinose was released only by endo-xylanase II (after purification on Mono Q™); this may be due to the presence of some contaminating (1,4)-ß-D-arabinoxylan arabinofuranohydrolase (Kormelink *et al*, 1991).
The action of ß-xylosidase was further studied using X_2, X_3, and X_4 as substrate (Fig. 6). The conversion of X_2 is linear with time. Xylotriose is converted into xylobiose and xylose, xylotetraose is converted into xylotriose, xylobiose and xylose. Under these circumstances no transferase activity was observed.
Crude culture filtrate supplemented with purified endo-xylanase I, II, III, or ß-xylosidase, has been assayed for the release of reducing sugars from water-soluble oat spelt xylan and the measured values compared to theoretical values obtained by summation of the action of the single enzymes (Fig. 7).

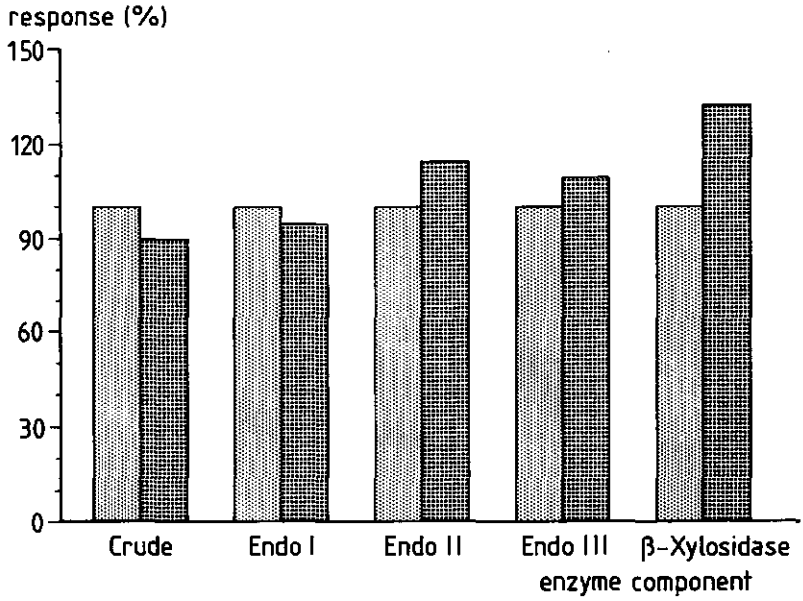

Figure 7. Concerted action of crude culture filtrate of *Aspergillus awamori* supplemented with purified endo-xylanase I, II, III, and ß-xylosidase on water-soluble oat spelt xylan;
theoretical, experimental.

As can be seen from the theoretical and experimental values (expressed in %), a combination of crude culture filtrate with ß-xylosidase increases the release of reducing sugars significantly. This effect can be seen in a smaller extent when endo-xylanase II or III is added. In case of endo-xylanase I or crude culture filtrate, the effect is absent.

Degradation of arabinoxylan was studied more into detail with endo-xylanase I and III. For this purpose water-soluble arabinoxylan was incubated with endo-xylanase I or III for 24 hours. The arabinoxylan digests were fractionated by Bio-Gel P2 (Fig. 8).

One part of the water-soluble arabinoxylan was not degraded after 24 hours: apparently these fragments are not accessible for the xylanase enzyme due to substitution with arabinosyl and glucuronosyl residues. The fragments which are substituted with glucuronic acid can be of polymeric as well as oligomeric size. Because of repulsion between these molecules and the gel filtration medium under aquous conditions, they will all elute in the void of the column.

The respective oligomer peaks were pooled and analysed for neutral sugar content, uronic acid content, and subsequently for their sugar composition (Table IV and V). Differences in the sugar composition of the fractions can be observed, high glucose contents and high AUA contents are seen in the first fractions eluting from the Bio-Gel P2 column. High arabinose content can be observed in the fractions which elute in the front of xylose, xylobiose and xylotriose (pool 4, 5, and 6 from the endo-xylanase I digest; pool 5, 8, and 9 from the endo-xylanase III digest).

In contradiction to endo-xylanase I, the tetramer is absent in the arabinoxylan digest obtained with endo-xylanase III.

High xylose contents can especially be observed in the last three fractions (pool 1, 2, and 3). The differences in oligomer composition further demonstrate the differences in pattern of action of the endo-xylanases. Endo-xylanase I releases next to xylose, xylobiose and xylotriose, also arabino-xylobiose, arabino-xylotriose, and arabino-xylotetraose. Endo-xylanase III, however, does not release arabino-xylobiose or arabino-xylotriose. Next to xylose (small traces), this enzyme releases xylobiose and xylotriose, arabino-xylotetraose and higher arabinoxylan oligomers. ß-Xylosidase was unable to degrade the arabinoxylan oligomers, formed by the endo-xylanases. Xylooligomers, however, were degraded into xylose. All endo-xylanases were unable to convert the arabinoxylan oligomers into arabinose and their complementary xylooligomers. Xylooligomers, higher than trimer were, to a certain extent, hydrolysed into xylose, xylobiose and xylotriose by the endo-xylanases.

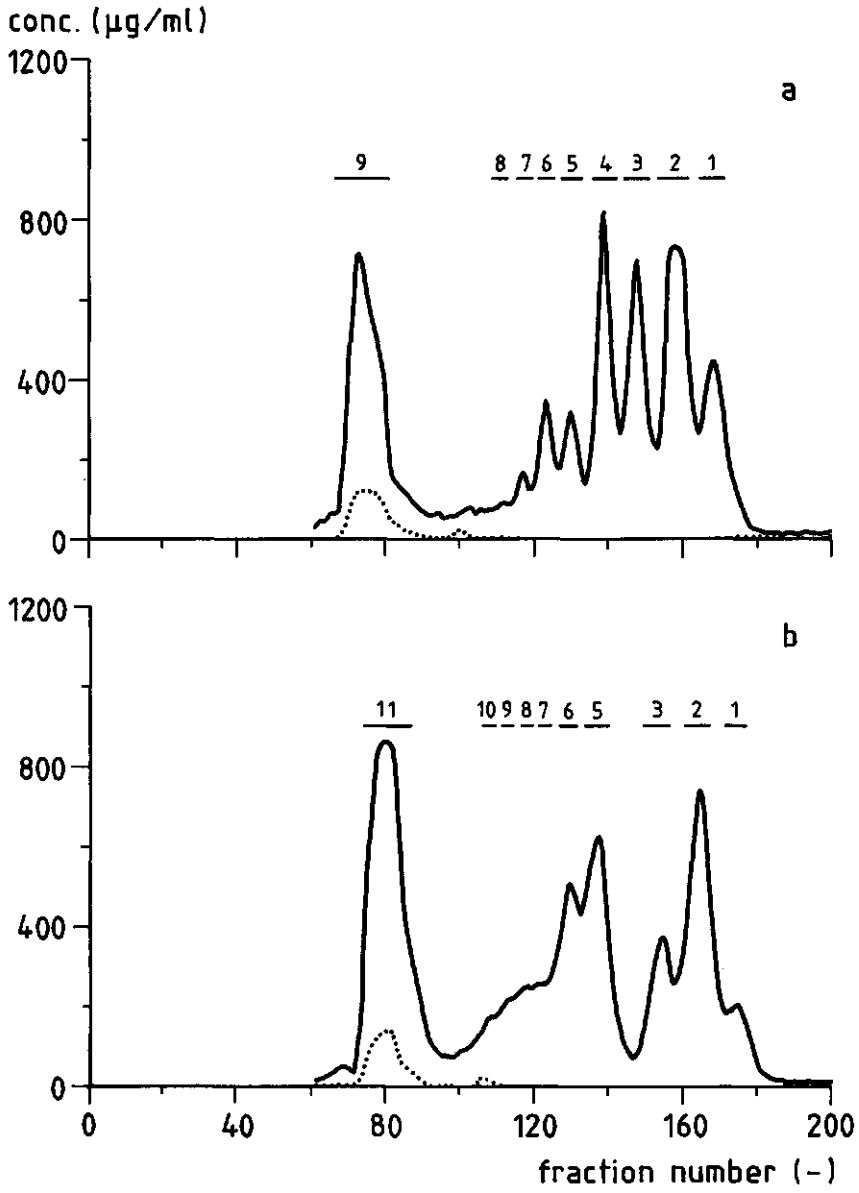

Figure 8. Bio-Gel P2 fractionation pattern of endo-xylanase digests of water-soluble oat spelt xylan; (a) endo-xylanase I, (b) endo-xylanase III; ——— neutral sugars, - - - uronic acids.

Table IV. Sugar composition of Bio-Gel P2 fractions (mole%) from xylan digest by endo-xylanase I.

Pool	Ara	Xyl	Gal	Glu	AUA	A:X	Proposed structure[1]
1	0.00	98.43	0.00	0.00	1.58	<<	X_1
2	0.33	99.40	0.00	0.00	0.28	<<	X_2
3	13.12	86.46	0.00	0.00	0.42	1:6.6	X_3, X_2A
4	23.30	75.92	0.00	0.00	0.79	1:3.3	X_3A
5	20.52	78.11	0.00	0.00	1.38	1:3.8	X_4A
6	18.38	79.71	0.00	0.00	1.91	1:4.3	X_6, X_5A
7	17.03	76.82	2.52	0.00	3.64	1:4.5	
8	17.13	68.91	3.43	1.58	8.96	1:4.0	
9	8.00	59.23	2.94	18.85	10.99	1:7.4	

[1]Gruppen *et al*, 1992.

Table V. Sugar composition of Bio-Gel P2 fractions (mole%) from xylan digest by endo-xylanase III.

Pool	Ara	Xyl	Gal	Glu	AUA	A:X	Proposed structure[1]
1	0.00	96.14	0.00	0.00	3.86	<<	X_1
2	1.34	97.71	0.00	0.00	0.96	<<	X_2
3	0.38	97.08	0.00	1.04	1.51	<<	X_3
5	18.42	80.72	0.00	0.00	0.86	1:4.4	X_5, X_4A
6	15.84	82.75	0.00	0.00	1.42	1:5.2	X_6, X_5A
7	15.63	80.52	0.00	0.00	3.85	1:5.2	X_7, X_6A
8	18.95	77.90	0.31	0.00	2.85	1:4.1	
9	18.73	77.22	0.43	0.00	3.63	1:4.1	
10	16.14	74.61	0.29	0.92	8.03	1:4.6	
11	10.14	64.38	2.26	13.77	9.47	1:6.4	

[1]Gruppen *et al*, 1992.

Discussion

Three endo-xylanases and one ß-xylosidase were purified from a crude culture filtrate of *Aspergillus awamori* CMI 142717. Endo-xylanase I, II, and III, all belong to the group of endo-xylanases which split the glycosidic linkages in the xylan-backbone in an endo fashion. They were unable to cleave L-arabinosyl initiated branch points (Dekker and Richards, 1976; Reilly, 1981). The ß-xylosidase only released limited amounts of xylose

from xylan; it is able to break down short oligosaccharides almost completely to xylose. It seems that the endo-xylanases which have been purified here, can be divided into two groups: endo-xylanase I on one side and endo-xylanase II and III on the other side. Endo-xylanase I has a high pI of 5.7-6.7, a molecular weight of 39 kDa, and releases xylose, xylobiose and xylotriose. Endo-xylanase II and III, however, have a low pI of 3.7 and 3.3-3.5, respectively, a molecular weight of 23 and 26 kDa, respectively, and release xylobiose and xylotriose mainly.

So John *et al* (1978), Reilly (1981), Comtat (1983), Lappalainen (1986) and Tan (1985) fractionated the xylan-degrading enzyme systems produced by different micro-organisms. The former three researchers purified 5 different endo-xylanases, the latter two obtained 3 endo-xylanases. Most of the endo-xylanases purified from *Aspergillus* strains (Reilly, 1981; John *et al*, 1978) had molecular weights lower than 32 kDa. The pI of these endo-xylanases ranged from 3.5 till 6.7 and most of them had optimum temperatures between 40-55°C. John *et al* (1978) obtained two endo-xylanases which had a higher molecular weight, their pI ranged from 8.6 till 9.0. These latter two enzymes were also found to be more heat stable and had a temperature optimum between 65-80°C, similar to the optimum temperature of the endo-xylanases of *Sporotrichum dimorphosporum* (Comtat, 1983).

On the basis of the end-products released, these different endo-xylanases can be divided in endo-xylanases which can or which cannot release xylose, or in endo-xylanases which can or which cannot release arabinose.

Many endo-xylanases are inhibited in their activity by mercury-ions, which is similar to our observation. The inhibition of the endo-enzyme activities is strongest in the presence of Pb^{2+}.

The ß-xylosidase purified from *Aspergillus awamori* is not an exo-type of enzyme since it is not able to release xylose from xylan. Xylooligosaccharides are, however, largely degraded by this enzyme with xylose as main end-product. The enzyme has a molecular weight of 110 kDa. This is in correspondance with the overall idea that the average molecular weight of ß-xylosidases is much higher than the molecular weight of endo-xylanases. ß-Xylosidases with molecular weights in this order of magnitude have been purified from *Aspergillus*: 78 kDa (John *et al*, 1978), 90 kDa (Kitpreechavanich *et al*, 1986), 122 kDa (Rodionova *et al*, 1983), and >200 kDa (Claeyssens *et al*, 1970). Rodionova *et al* (1977) on the other hand, purified a ß-xylosidase from *Aspergillus niger* which had a molecular weight of 30 kDa. The ß-xylosidases mentioned above have a pI between 4.3 and 5.4. The ß-xylosidase from *Aspergillus awamori* perfectly fits in with a pI of 4.2. Its temperature and pH optimum is 70°C and 6.5, respectively. The temperature optimum is in reasonable agreement with values in the range of 60-70°C as found in literature. For the pH, optima have been reported between 3 and 4.5 (Rodionova *et al*, 1977; Rodionova *et al*, 1983; Kitpreechavanich *et al*, 1986; Takenishi *et al*, 1973; Claeyssens *et al*, 1970; Conrad, 1981), but also between 6 and 7 (John *et al*, 1978). The ß-xylosidase purified from *Aspergillus awamori* with its high temperature and pH optimum

thus resembles none of the ß-xylosidases mentioned above.
Different salts have been shown to result in an inhibitory effect; Hg^{2+}, Cu^{2+}, and Fe^{3+}. In the case of the *Aspergillus awamori* ß-xylosidase, Pb^{2+}, Hg^{2+}, and Ag^{+} show an inhibitory effect in increasing order. D-Xylose was not tested here for inhibition.
The diversity in the values for different parameters of the *Aspergillus awamori* endo-xylanases and ß-xylosidase in comparison to the values found in literature, demonstrate the difficulty of classifying these enzymes. At this moment it appears important to gain a better understanding of the pattern of action of these enzymes. This will be the aim of further studies on the *Aspergillus awamori* endo-xylanases.

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Chapter 4

Characterization by ^{1}H-n.m.r. spectroscopy of oligosaccharides derived from alkali-extractable wheat-flour arabinoxylan by digestion with endo-(1,4)-ß-xylanase III from *Aspergillus awamori*

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Submitted for publication.

Summary

Alkali-extractable wheat-flour arabinoxylan, treated with endo-(1,4)-ß-xylanase III from *Aspergillus awamori* CMI 142717, was fractionated by Bio-Gel P-2 size exclusion chromatography at 60°C. Column fractions, corresponding to oligosaccharides with degrees of polymerization from 5 till 10, were collected, and subfractionated by high performance anion-exchange chromatography on CarboPac PA-1.
The structures of the oligosaccharides thus obtained were elucidated by ^{1}H-n.m.r. spectroscopy, showing chains of (1,4)-linked ß-D-xylopyranosyl residues differently substituted at *O*-3 and/or *O*-2,3 with α-L-arabinofuranosyl residues. The structures were different from those obtained by endo-(1,4)-ß-xylanase I of the same xylanolytic enzyme system.

Introduction

In recent years, several groups have aimed at isolating arabinoxylan-derived oligosaccharides from various sources by chemical or enzymic degradation, for structure elucidation[1-7]. Chemical methods are less specific and may give rise to release of side groups giving modified oligosaccharides which are not representative for the arabinoxylan. With enzymes specific glycosidic linkages can be split resulting in unmodified oligosaccharides. Characterization of these oligosaccharides allows conclusions about the structure of the parent arabinoxylans, and about the pattern of action of the enzymes used. In a previous study[8] we have described the structures of arabinoxylan-derived oligosaccharides obtained with endo-(1,4)-ß-xylanase I from *Aspergillus awamori* CMI 142717. Here, structures of arabinoxylan-derived oligosaccharides will be presented, which have been obtained by treatment of alkali-extractable wheat-flour arabinoxylan with endo-(1,4)-ß-xylanase III from the same organism.

Materials and methods

Materials

Chemicals used were of analytical grade. Wheat alkali-extractable arabinoxylan (BE1-U) was prepared according to Gruppen *et al*[9]. Endo-(1,4)-ß-xylanase III was purified[10] from *Aspergillus awamori* CMI 142717.

Preparation of arabinoxylan oligosaccharides

A solution of wheat alkali-extractable arabinoxylan (80 mg) in 50mM sodium acetate buffer (80 mL, pH 5.0) was incubated with endo-(1,4)-ß-xylanase III (0.4 μg/mL) for 24 h at 30°C. After inactivation of the enzyme at 100°C for 10 min, the solution was concentrated to 3 mL by vacuum rotary evaporation, and applied on to a Bio-Gel P-2 column (100 x 2.8 cm, 200-400 mesh, Bio-Rad) at 60°C[11]. The column was eluted with distilled water (17 mL/h) and fractions of 2.4 mL were collected and monitored for total neutral sugar content by the automated sulfuric acid assay[12]. Appropriate fractions were pooled, designated **1** to **14**, and concentrated by vacuum rotary evaporation to 2.0 mL. The Bio-Gel P-2 column was calibrated using a mixture of xylose, maltose, raffinose, stachyose, and Dextran T150 (Pharmacia).

Fractions **5** - **10** were subjected to high-performance anion-exchange chromatography (h.p.a.e.c.) using a Dionex Bio-LC GPM-II quaternary gradient module equipped with a semi-preparative Dionex CarboPac PA-1 column (250 x 9 mm) and a Dionex PED detector in the pulsed amperometric detection (p.a.d.) mode[8]. Samples of 300 μL (5-7 runs) were injected on to the column, and elutions (5 mL/min) involved a linear gradient of sodium acetate in 0.1M NaOH of 50-250mM during 40 min. The eluate was neutralised by M acetic acid.

Corresponding fractions (1.2mL) of each run were pooled, desalted using a Dowex 50W X8 resin column (H^+, 30 x 80 mm) and a AG3 X4A resin column (OH^-, 30 x 80 mm, Bio-Rad) in series, concentrated by vacuum rotary evaporation, and subsequently dried by a constant stream of air.

Neutral sugar composition

Bio-Gel P-2 fractions (100 μg) were hydrolysed by 2M trifluoroacetic acid for 1 h at 121°C. The samples were cooled to room temperature and the trifluoroacetic acid was evaporated under a stream of dried air at 40°C. The residues were reduced in 1.5M ammonia (0.2 mL), containing 50 mg $NaBH_4$/mL, and subsequently converted into their alditol acetates[13]. The monosaccharide composition was determined by GLC[10] using inositol as an internal standard.

Dionex fractions (10 μg) were also hydrolysed with 2M trifluoroacetic acid as described above. The residues were dissolved in water (0.2 mL) and analysed on a CarboPac PA-1 column (250 x 4 mm). Elution (1 mL/min) involved linear gradients of sodium acetate in 0.1M NaOH of 0-100mM during 5 min, then 100-400mM during 35 min.

^{1}H-N.m.r. spectroscopy.

Samples were repeatedly treated with D_2O (99.9 atom% D, MSD isotopes), finally using 99.96 atom% D at pD $\geq$ 7. Resolution-enhanced 600-MHz ^{1}H-n.m.r. spectra were acquired with a Bruker AM-600 spectrometer (SON-hf-NMR facility, NSR-Center, Nijmegen University), operating at a probe temperature of 25°. Chemical shifts are expressed in ppm relative to the signal of internal acetone at δ 2.225 (ref 14), with an accuracy of 0.002 ppm. Full details of the HOHAHA and ROESY experiments have been reported previously[6].

Results and Discussion

Alkali-extractable wheat arabinoxylan was digested by endo-(1,4)-ß-xylanase III from *A. awamori*, and the mixture of oligosaccharides was fractionated by Bio-Gel P-2 size exclusion chromatography (Fig. 1). The sugar composition and yield of the fractions obtained are given in Table I.

The elution pattern showed a large peak eluting at the void of the column. This arabinoxylan fraction was resistent to further enzymic digestion, probably due to the large amount of arabinosyl substituents (Table I). The eluate between 150 and 250 mL did not show significant peaks corresponding to a degree of polymerization (DP) higher than 10.

Between 250 and 350 mL, one small and five large peaks could be observed, corresponding to DP's of 5 up to 10 (fractions **5 - 10**). No large amounts of oligosaccharides corresponding to DP's of 1 up to 4 (fractions **1 - 4**) were released by endo-(1,4)-ß-xylanase III.

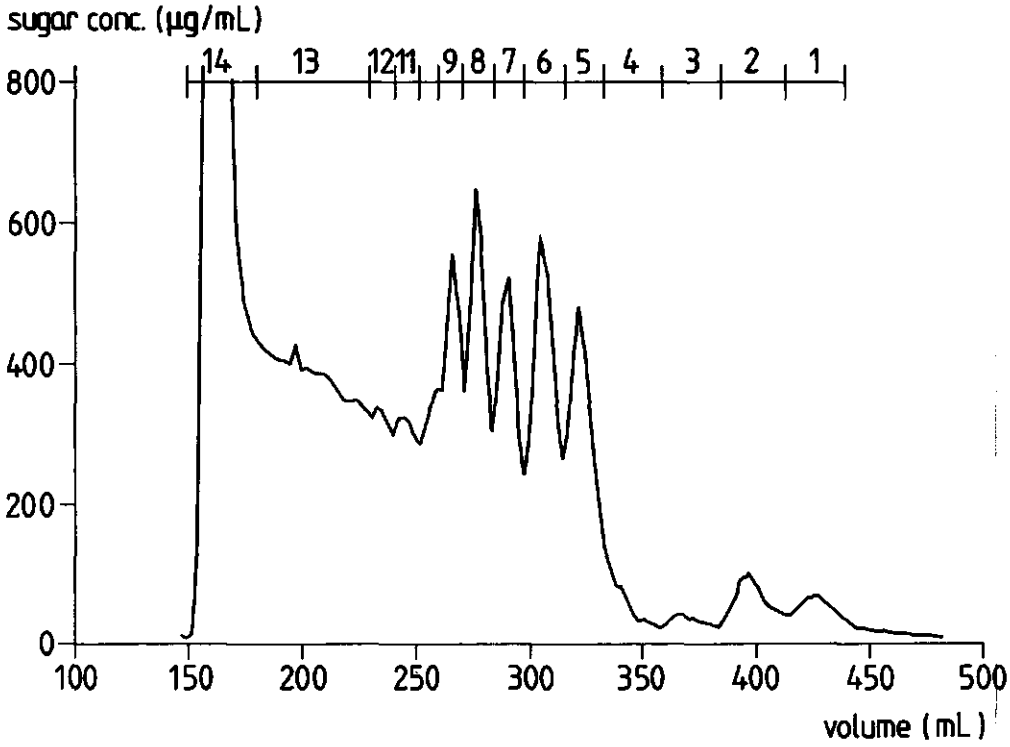


Figure 1. Elution profile of the arabinoxylan digest on Bio-Gel P-2.

The sugar composition of fractions **1** - **3** (Table I) and the presence of a single peak when run on h.p.a.e.c. (results not shown) suggest the presence of xylose, xylobiose, and xylotriose, respectively. Fraction **4** also gave a single peak on h.p.a.e.c., and monosaccharide analysis showed the presence of 25% arabinose and 75% xylose. Fractions **1** to **4**, which represented 7.7% of the total amount of neutral sugars, were not studied further.

Fractions **5** - **10** were subfractionated by h.p.a.e.c. (Fig. 2); the sugar composition and yield of each of the subfractions are given in Table I (the numbering of the subfractions obtained links up with that in previous studies[8,15]). Fraction **5** gave one major component, representing 85% of the p.a.d. response. Fractions **6** and **7** both afforded two major components, together representing 79 and 82% of the total p.a.d. response, respectively. Fractions **8**, **9**, and **10** yielded three or more components. The accumulated peak areas of the collected fractions, represented 79, 77, and 73%, of the total p.a.d. response of **8**, **9**, and **10**, respectively.

For the Ara/Xyl ratio of the oligosaccharides purified, values between 0.18 and 0.66 can be calculated (Table I).

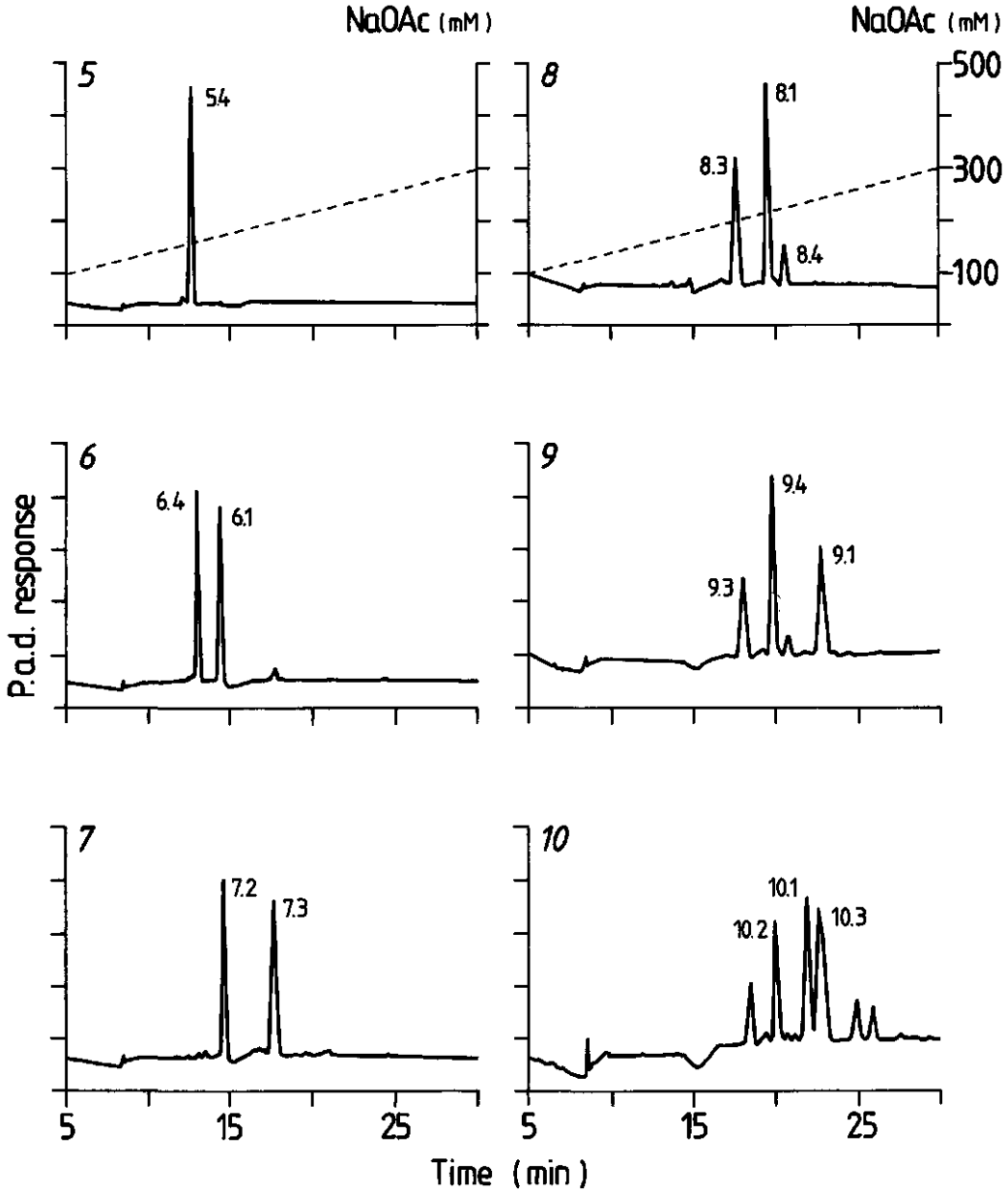


Figure 2. H.p.a.e.c. elution profile of Bio-Gel P-2 fractions **5-10** in Figure 1.

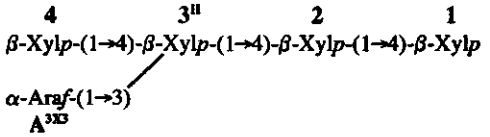
Table I. Data on the fractions obtained by chromatography on Bio-Gel P-2 and CarboPac PA-1 of the products formed by the enzymic degradation of wheat arabinoxylan.

Fraction		Yield (%)		Sugar composition[c]	
Bio-Gel	CarboPac	Bio-Gel[a]	CarboPac[b]	Ara (%)	Xyl (%)
1				0.0	100.0
2				0.0	100.0
3				0.0	100.0
4				25.0	75.0
5		5.7		19.7	80.3
	5.4		85.4	19.2[d]	80.8[d]
6		7.6		27.8	72.2
	6.1		41.9	31.9[d]	68.1[d]
	6.4		37.5	15.1[d]	84.9[d]
7		6.2		29.0	71.0
	7.2		38.9	25.4[d]	74.6[d]
	7.3		42.7	27.0[d]	73.0[d]
8		6.3		33.1	66.9
	8.1		40.5	37.3[d]	62.7[d]
	8.3		30.6	25.7[d]	74.3[d]
	8.4		7.6	36.4[d]	63.6[d]
9		4.9		33.4	66.6
	9.1		25.9	39.8[d]	60.2[d]
	9.3		18.0	22.0[d]	78.0[d]
	9.4		32.8	31.7[d]	68.3[d]
10		3.7		35.7	64.3
	10.1		21.0	38.3[d]	61.7[d]
	10.2		19.5	30.7[d]	69.3[d]
	10.3		32.4	36.6[d]	63.4[d]
11		4.1		36.0	64.0
12		4.2		35.4	64.6
13		20.2		37.9	62.1
14		29.4		44.4	52.1

[a] Yield in % of the total amount of neutral sugars present, determined spectrophotometrically.
[b] Yield in % of the total p.a.d. response from each Bio-Gel P-2 fraction.
[c] Neutral sugar composition of the material in each fraction obtained by GLC analysis.
[d] Neutral sugar composition of the material in each fraction obtained by h.p.a.e.c..

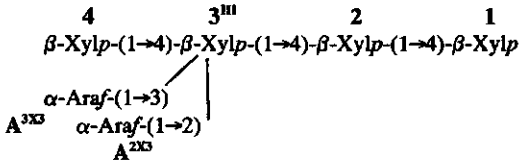
For the identification of the primary structure of the arabinoxylan oligosaccharides isolated by h.p.a.e.c. (Fig. 2), ^{1}H-n.m.r. spectroscopy was applied, and the relevant ^{1}H-n.m.r. data are presented in Tables II or III.

Fraction **5.4**.——The intensities of the H-1 signals in the ^{1}H-n.m.r. spectrum of **5.4** revealed the presence of a single arabinosylxylotetraose. The chemical shift data matched exactly those[6] of **AX-31**, and the chemical shifts of the H-1 resonances are summarised in Table II.



(5.4)

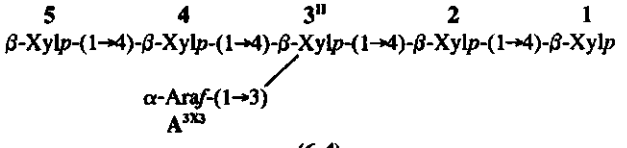
Fraction **6.1**.——The intensities of the H-1 signals in the ^{1}H-n.m.r. spectrum of **6.1** revealed the presence of a single diarabinosylxylotetraose. The chemical shift data matched exactly those[6] of **AX-33**, and the chemical shifts of the H-1 resonances are summarised in Table II.



(6.1)

The same hexasaccharide has been found in the enzymic digest of barley[16] and rye[17] grain.

Fraction **6.4**.——The intensities of the H-1 signals in the ^{1}H-n.m.r. spectrum of **6.4** revealed the presence of a single arabinosylxylopentaose of which the chemical shift data matched exactly those[6] of **AX-32b**, and the chemical shifts of the H-1 resonances are summarised in Table II.



(6.4)

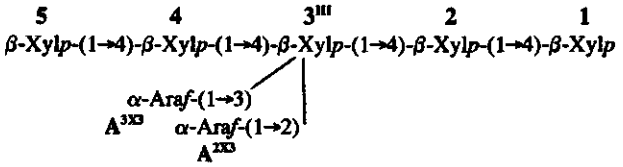
In the ^{1}H-n.m.r. spectrum of **6.4** no indications were found for the presence of the isomer of **6.4** as reported by Hoffmann *et al.*[6] **(AX-32a)**, demonstrating that the digestion was performed to completion.

Table II. Chemical shifts[a] of the H-1 resonances of compounds **5.4**, **6.1**, **6.4**, **7.2**, **7.3**, **8.1**, **8.4**, **9.1**, **9.3^{I}**, **9.3^{II}**, **9.4^{I}**, and **10.1**.

Residue[b]	Compound											
	5.4	**6.1**	**6.4**	**7.2**	**7.3**	**8.1**	**8.4**	**9.1**	**9.3^{I}**	**9.3^{II}**	**9.4^{I}**	**10.1**
α-Xyl*p*-1	5.184	5.183	5.184	5.183	5.184	5.184	5.184	5.184	5.184	5.184	5.184	5.184
β-Xyl*p*-1	4.584	4.584	4.584	4.584	4.584	4.584	4.584	4.584	4.584	4.584	4.584	4.584
β-Xyl*p*-2$_{\alpha}$	4.474	4.465	4.474	4.464	4.474	4.464	4.474	4.464	4.474	4.474	4.464	4.465
β-Xyl*p*-2$_{\beta}$	4.477	4.468	4.477	4.467	4.477	4.467	4.477	4.467	4.477	4.477	4.467	4.468
β-Xyl*p*-3^{II}	4.514	-	4.514	-	4.514	-	4.501	-	4.514	4.514	-	-
β-Xyl*p*-3^{III}	-	4.639	-	4.638	-	4.637	-	4.628	-	-	4.637	4.639
β-Xyl*p*-4	4.442	4.436	4.460	4.454	-	-	-	-	4.459	4.459	-	4.443
β-Xyl*p*-4^{II}	-	-	-	-	4.488	4.480	-	-	-	-	4.480	-
β-Xyl*p*-4^{III}	-	-	-	-	-	-	4.595	4.579	-	-	-	-
β-Xyl*p*-5	-	-	4.448	4.451	4.431	4.435	4.425	4.428	-	4.468	4.453	-
β-Xyl*p*-5^{II}	-	-	-	-	-	-	-	-	4.501	-	-	-
β-Xyl*p*-5^{III}	-	-	-	-	-	-	-	-	-	-	-	4.628
β-Xyl*p*-6	-	-	-	-	-	-	-	-	4.459	-	4.447	4.436
β-Xyl*p*-6^{II}	-	-	-	-	-	-	-	-	-	4.514	-	-
β-Xyl*p*-7	-	-	-	-	-	-	-	-	4.435[c]	4.440[c]	-	-
α-Ara*f*-A^{2X3}	-	5.224	-	5.224	-	5.224	-	5.221	-	-	5.224	5.224
α-Ara*f*-A^{3X3}	5.397	5.274	5.391	5.272	5.388	5.270	5.419	5.293	5.391	5.391	5.270	5.271
α-Ara*f*-A^{2X4}	-	-	-	-	-	-	5.231	5.241	-	-	-	-
α-Ara*f*-A^{3X4}	-	-	-	-	5.398	5.402	5.274	5.281	-	-	5.396	-
α-Ara*f*-A^{2X5}	-	-	-	-	-	-	-	-	-	-	-	5.221
α-Ara*f*-A^{3X5}	-	-	-	-	-	-	-	-	5.391	-	-	5.271
α-Ara*f*-A^{3X6}	-	-	-	-	-	-	-	-	-	5.396	-	-

[a]Measured at 600 MHz on solutions in D_2O at 25° (internal acetone δ 2.225). [b]The Xyl*p* residue in the reducing position is denoted 1, etc.; 2$_{\alpha/\beta}$ means reducing Xyl*p*-1 residue is α/β configuration (anomerisation effect). Ara*f*-A^{2X3} means arabinofuranose linked to O-2 of Xyl*p*-3, etc.; Xyl*p*-3^{I} means Xyl*p*-3 branched at O-2; Xyl*p*-3^{II} means Xyl*p*-3 branched at O-3; Xyl*p*-3^{III} means Xyl*p*-3 branched at O-2,3. [c]Assignments may have to be interchanged.

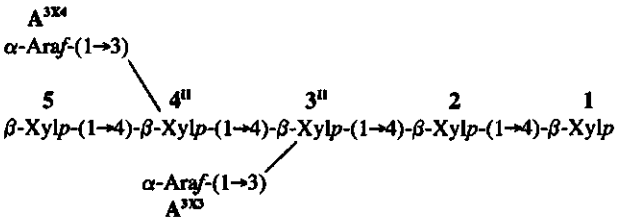
Fraction **7.2.**——The intensities of the H-1 signals in the ^{1}H-n.m.r spectrum of **7.2** revealed the presence of a single diarabinosylxylopentaose. The chemical shift data matched exactly those[6] of **AX-34b**, and the chemical shifts of the H-1 resonances are summarised in Table II.



(7.2)

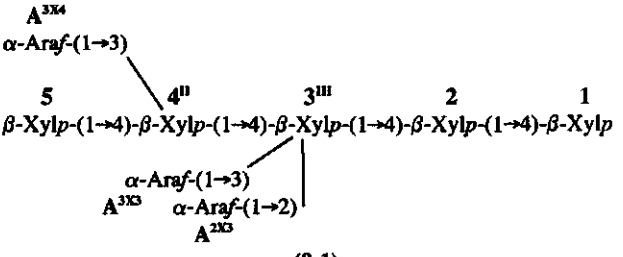
Identical to **6.4**, no indications for the presence of the structural isomer of **7.2** (**AX-34a**)[6] were found in the ^{1}H-n.m.r. spectrum of **7.2**.

Fraction **7.3.**——The intensities of the H-1 signals in the ^{1}H-n.m.r. spectrum of **7.3** revealed the presence of a single diarabinosylxylopentaose. The chemical shift data matched exactly those[7] of **AX-41**, and the chemical shifts of the H-1 resonances are summarised in Table II.



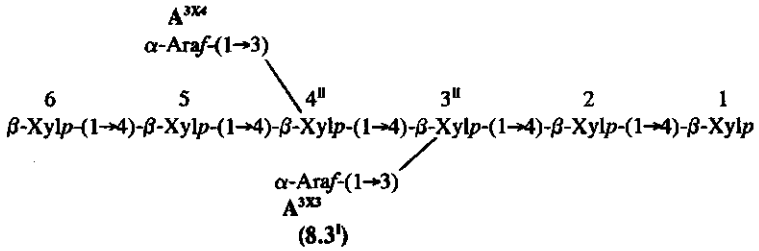
(7.3)

Fraction **8.1.**——The intensities of the H-1 signals in the ^{1}H-n.m.r. spectrum of **8.1** revealed the presence of a single triarabinosylxylopentaose. The chemical shift data matched exactly those[7] of **AX-54c**, and the chemical shifts of the H-1 resonances are summarised in Table II.



(8.1)

Fraction **8.3**.——The Bio-Gel P-2 elution volume of fraction **8**, in conjunction with the Ara/Xyl ratio of 25.7/74.3 for **8.3**, indicate that **8.3** has to contain one or more diarabinosylxylohexaoses. In the region for the anomeric protons in the ^{1}H-n.m.r. spectrum of **8.3** (Fig. 3) the presence of α-Ara*f* H-1 signals at δ 5.39 and β-Xyl*p* H-1 signals at δ 4.52-4.49, in combination with the absence of α-Ara*f* H-1 signals at δ 5.22-5.33 and ß-Xyl*p* H-1 signals at δ 4.59-4.64 (ref 6-8,17), respectively, showed that single 3-substituted, internal β-Xyl*p* residues are the sole branching points. In the region for β-Xyl*p* H-1 signals clearly more than six resonances can be observed, indicating the presence of a mixture of structures. Comparison of the H-1 ^{1}H-n.m.r. data of **8.3** with those of **7.3** showed that the same reducing diarabinosylxylotetraose unit, denoted (α-Ara*f*-**A**^{3X4})β-Xyl*p*-**4**^{II}-(α-Ara*f*-**A**^{3X3})β-Xyl*p*-**3**^{II}-β-Xyl*p*-**2**-Xyl*p*-**1**, was present in one of the diarabinosylxylohexaoses, **8.3**^{I}, of **8.3**. The absence of the non-reducing terminal β-Xylp-**5** H-1 signal at δ 4.431 and the α-Ara*f*-**A**^{3X4} H-1 signal at δ 5.398 of **7.3** indicated a different non-reducing terminal sequence in **8.3**^{I}. In the 2D HOHAHA spectrum (not shown), the total scalar-coupled network for each residue was observed and the data are summarised in Table III. Two non-reducing terminal β-Xyl*p* residues were distinguishable in the HOHAHA spectrum, reflected by the characteristic high-field shifts of their H-3,4,5 resonances[6]. From the r.O.e.s. between H-1 of β-Xyl*p*-(**n**) and H-4,5*eq* of β-Xyl*p*-(**n-1**), observed along the H-1 tracks in the ROESY spectrum (not shown), (Table IV), it was concluded that **8.3**^{I} contained an unbranched xylobiosyl group at the non-reducing end, characterised by the H-1 signals at δ 4.446 and 4.449 for the terminal and penultimate residues, respectively (Table III). The ROESY data show that the H-1 signal at δ 4.449 has a cross-peak with H-4,5*eq* of the 3-branched β-Xyl*p*-**4**^{II} residue of **8.3**^{I}. Thus, this non-reducing xylobiosyl group is 4-linked to β-Xyl*p*-**4**^{II}. The presence of the α-Ara*f*-**A**^{3X3},β-Xyl*p*-**3**^{II} and α-Ara*f*-**A**^{3X4},β-Xyl*p*-**4**^{II} connectivities in the ROESY spectrum completed the structure of **8.3**^{I}. Owing to this non-reducing xylobiosyl extension the H-1 signal of α-Ara*f*-**A**^{3X4} shifts to higher field as compared to H-1 of the same residue in **7.3**.



Identification of a second non-reducing terminal β-Xyl*p* residue in the HOHAHA spectrum of **8.3** pointed to the presence of a second diarabinosylxylohexaose, **8.3**^{II}.

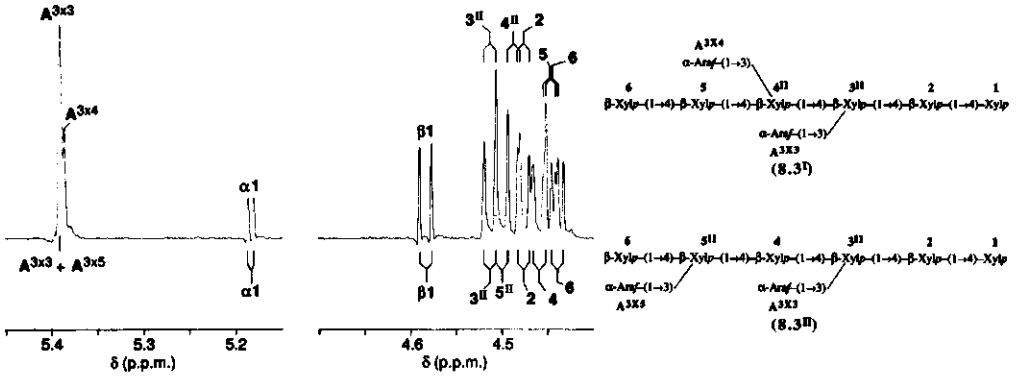


Figure 3. Resolution-enhanced 600-MHz ^{1}H-n.m.r. spectrum of fraction **8.3**. The numbers and letters in the spectrum refer to the corresponding residues in the structures.

Combined HOHAHA and ROESY data (Tables III and IV) identified a non-reducing terminal sequence: β-Xyl*p*-**6**-(α-Ara*f*-$\mathbf{A^{3X5}}$)β-Xyl*p*-$\mathbf{5^{II}}$-β-Xyl*p*-**4**. The characteristic up-field shift of β-Xyl*p*-$\mathbf{5^{II}}$ H-1 from δ 4.514 to 4.501, which is comparable with the up-field shift observed for β-Xyl*p*-$\mathbf{5^{III}}$ H-1 in **10.1 (AX-57a)**[7] from δ 4.639 to 4.628, indicated that this non-reducing terminal sequence had to be linked to a branched β-Xyl*p*. The ROESY data (Table IV) show that the β-Xyl*p*-**4** H-1 signal at δ 4.459 has a cross-peak with H-4,5*eq* of the 3-branched β-Xyl*p*-$\mathbf{3^{II}}$ in $\mathbf{8.3^{II}}$ (see below). Thus, the arabinosylxylotriose unit was 4-linked to the reducing arabinosylxylotriose unit, (α-Ara*f*-$\mathbf{A^{3X3}}$)β-Xyl*p*-$\mathbf{3^{II}}$-β-Xyl*p*-**2**-Xyl*p*-**1**, of which all ^{1}H-n.m.r. signals resonate at identical chemical shift values as those of the corresponding residues in **6.4**. Based on the combined ^{1}H-n.m.r. data the structure of $\mathbf{8.3^{II}}$ is

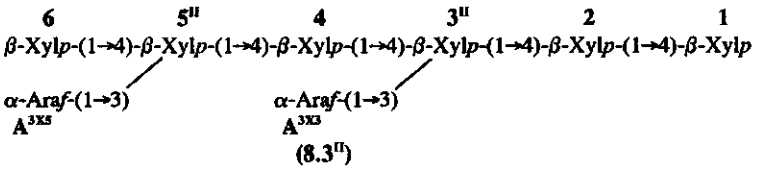
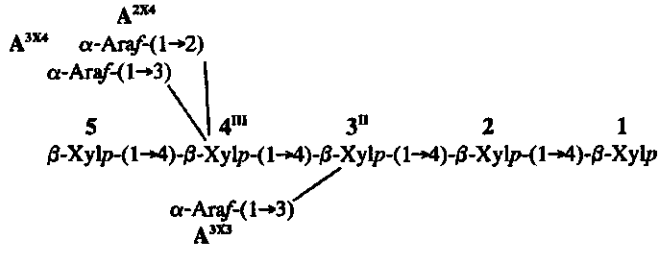


Table III. ^{1}H-N.m.r. data for the diarabinosylxylohexaoses **8.3^I** and **8.3^{II}** derived from enzymically degraded wheat arabinoxylan

Compound[a]	Residue[b]	Chemical shift[c]					
		H-1	H-2	H-3	H-4	H-5*eq*/H-5*proR*	H-5*ax*/H-5*proS*
	α-Xyl*p*-1	5.184	3.544	———	———	3.73—3.82	———
	β-Xyl*p*-1	4.584	3.248	3.546	3.778	4.054	3.377
	β-Xyl*p*-2$_\alpha$	4.474	3.298	3.554	3.792	4.105	3.374
8.3^I	β-Xyl*p*-2$_\beta$	4.477	3.290	3.551	3.792	4.105	3.374
	β-Xyl*p*-3^{II}	4.514	3.440	3.740	3.833	4.121	3.400
	β-Xyl*p*-4^{II}	4.489	3.428	3.729	3.792	4.066	3.364
	β-Xyl*p*-5	4.449	3.268	3.532	3.748	4.044	3.332
	β-Xyl*p*-6	4.446	3.245	3.420	3.619	3.964	3.296
	α-Ara*f*-A^{3X3d}	5.391	4.155	3.900	4.270	3.794	3.713
	α-Ara*f*-A^{3X4}	5.387	4.160	3.909	4.273	3.798	3.719
	α-Xyl*p*-1	5.184	3.544	———	———	3.73—3.82	———
	β-Xyl*p*-1	4.584	3.248	3.546	3.778	4.054	3.377
	β-Xyl*p*-2$_\alpha$	4.474	3.298	3.554	3.792	4.105	3.374
8.3^{II}	β-Xyl*p*-2$_\beta$	4.477	3.290	3.551	3.792	4.105	3.374
	β-Xyl*p*-3^{II}	4.514	3.440	3.744	3.833	4.121	3.400
	β-Xyl*p*-4	4.459	3.278	3.537	3.755	4.049	3.340
	β-Xyl*p*-5^{II}	4.501	3.433	3.744	3.817	4.117	3.390
	β-Xyl*p*-6	4.440	3.243	3.413	3.594	3.911	3.276
	α-Ara*f*-A^{3X3d}	5.391	4.160	3.909	4.273	3.798	3.719
	α-Ara*f*-A^{3X5}	5.391	4.160	3.909	4.273	3.798	3.719

[a]Compounds are represented by short-hand symbolic notation: ●,Xyl; ◇,α-Ara*f*; ●●,β-Xyl*p*-(1→4)-Xyl*p*; ●◇,α-Ara*f*-(1→2)-β-Xyl*p*; ◇●,α-Ara*f*-(1→3)-β-Xyl*p*. [b]See Table II for the key. [c]Measured at 600 MHz on solutions in D_2O at 25° (internal acetone δ 2.225).[d]Assignments may have to be interchanged.

Fraction **8.4**.——The intensities of the H-1 signals in the ^{1}H-n.m.r. spectrum of **8.4** revealed the presence of a triarabinosylxylopentaose as the major structure. The chemical shift data matched exactly those[7] of **AX-56**, and the chemical shifts of the H-1 resonances are summarised in Table II.



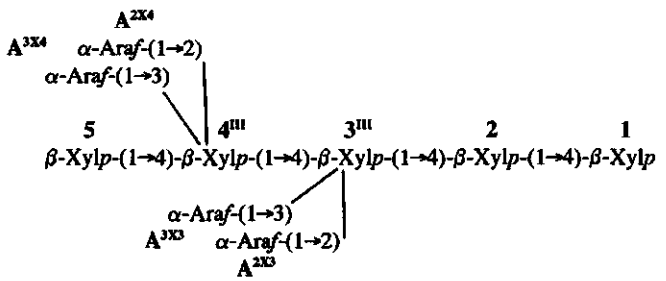
(8.4)

Table IV. Cross-peaks observed at the H-1 track in the ROESY spectra of the diarabinosylxylohexaoses **8.3^{I}** and **8.3^{II}**, measured with a mixing time of 200 ms.

Compound	Residue	N.O.e effect
8.3^{I}	Xyl-2 H-1	Xyl-2 H-3,5*ax*; Xyl-1β H-4,5*eq*; Xyl-1α H-4,5
	Xyl-3^{II} H-1	Xyl-3^{II} H-3,5*ax*; Xyl-2 H-4,5*eq*
	Xyl-4^{II} H-1	Xyl-4^{II} H-3,5*ax*; Xyl-3^{II} H-4,5*eq*
	Xyl-5 H-1	Xyl-5 H-3,5*ax*; Xyl-4^{II} H-4,5*eq*
	Xyl-6 H-1	Xyl-6 H-3,5*ax*; Xyl-5 H-4,5*eq*
	Ara-A^{3X3} H-1	Ara-A^{3X3} H-2; Xyl-3^{II} H-3
	Ara-A^{3X4} H-1	Ara-A^{3X4} H-2; Xyl-4^{II} H-3
8.3^{II}	Xyl-2 H-1	Xyl-2 H-3,5*ax*; Xyl-1β H-4,5*eq*; Xyl-1α H-4,5
	Xyl-3^{II} H-1	Xyl-3^{II} H-3,5*ax*; Xyl-2 H-4,5*eq*
	Xyl-4 H-1	Xyl-4 H-3,5*ax*; Xyl-3^{II} H-4,5*eq*
	Xyl-5^{II} H-1	Xyl-5^{II} H-3,5*ax*; Xyl-4 H-4,5*eq*
	Xyl-6 H-1	Xyl-6 H-3,5*ax*; Xyl-5^{II} H-4,5*eq*
	Ara-A^{3X3} H-1	Ara-A^{3X3} H-2; Xyl-3^{II} H-3
	Ara-A^{3X5} H-1	Ara-A^{3X5} H-2; Xyl-5^{II} H-3

In the ^{1}H-n.m.r. spectrum of **8.4** signals of minor compound(s) (< 10% of the signal intensity) were observed of which the structure could not be unravelled.

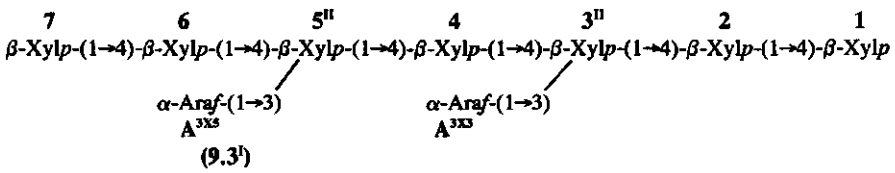
Fraction **9.1**.——The intensities of the H-1 signals in the ^{1}H-n.m.r. spectrum of **9.1** revealed the presence of a tetra-arabinosylxylopentaose as the major structure. The chemical shift data matched exactly those[7] of **AX-59**, and the chemical shifts of the H-1 resonances are summarised in Table II.



(9.1)

In the ^{1}H-n.m.r. spectrum of **9.1** signals were also observed of a minor compound (< 18%) of which the structure could not be unravelled.

Fraction **9.3**.——The Bio-Gel P-2 elution volume of fraction **9**, in conjunction with the Ara/Xyl ratio of 22.0/78.0 for **9.3**, indicate that **9.3** has to contain one or more diarabinosylxyloheptaoses. In the region for the anomeric protons in the ^{1}H-n.m.r. spectrum of **9.3** (Fig. 4) the presence of α-Ara*f* H-1 signals at δ 5.39 and β-Xyl*p* H-1 signals at δ 4.52-4.49, in combination with the absence of α-Ara*f* H-1 signals at δ 5.22-5.33 and β-Xyl*p* H-1 signals at δ 4.59-4.64 (ref 6-8), respectively, showed that single 3-substituted, internal β-Xyl*p* residues are the sole branching points. In the region for β-Xyl*p* H-1 signals more than seven resonances, varying in signal intensities, can be observed, proving the presence of a mixture of structures. Comparison of the H-1 ^{1}H-n.m.r. data of **9.3** with those of **8.3^{II}** showed that the same reducing diarabinosylxylopentaose unit, (α-Ara*f*-A^{3X5})β-Xyl*p*-**5^{II}**-β-Xyl*p*-**4**-(α-Ara*f*-A^{3X3})β-Xyl*p*-**3^{II}**-β-Xyl*p*-**2**-Xyl*p*-**1**, was present in one of the diarabinosylxyloheptaoses, **9.3^{I}**, of **9.3**. The only structure fitting the presented requirements and consistent with the observed enzyme mode-of-action (see also ref 6,7) is **8.3^{II}** extended at the non-reducing end by one unsubstituted β-Xyl*p* residue, giving structure **9.3^{I}**. This non-reducing terminal xylobiosyl unit is characterised by the H-1 resonances at δ 4.459 (see **6.4**) and 4.435 of the penultimate and terminal β-Xyl*p* residue, respectively.



The intensities of the β-Xyl*p* H-1 resonances at δ 5.184/4.584, 4.474/4.477, 4.514, and 4.459, and the intensity of the α-Ara*f* H-1 resonance at δ 5.391 indicated the presence of a second β-Xyl*p*-**4**-(α-Ara*f*-A^{3X3})β-Xyl*p*-**3^{II}**-β-Xyl*p*-**2**-Xyl*p*-**1** reducing unit, belonging to the second diarabinosylxyloheptaose, **9.3^{II}**. The presence of a non-reducing terminal arabinosylxylobiose unit β-Xyl*p*-(α-Ara*f*-A^3)β-Xyl*p* was highlighted by the observed β-Xyl*p* H-1 signals at δ 4.440 and 4.514 in combination with the α-Ara*f* H-1 signal at δ 5.396, resonating at comparable chemical shifts as for the corresponding residues in **5.4**. The typical H-1 signal at δ 4.468 reflects the presence of an internal, unbranched β-Xyl*p* residue, being neither part of an unsubstituted non-reducing terminal xylobiosyl unit (see **6.4**), nor of an unsubstituted reducing xylotriosyl unit[6]. Summarizing the data available, the following structure for **9.3^{II}** is proposed

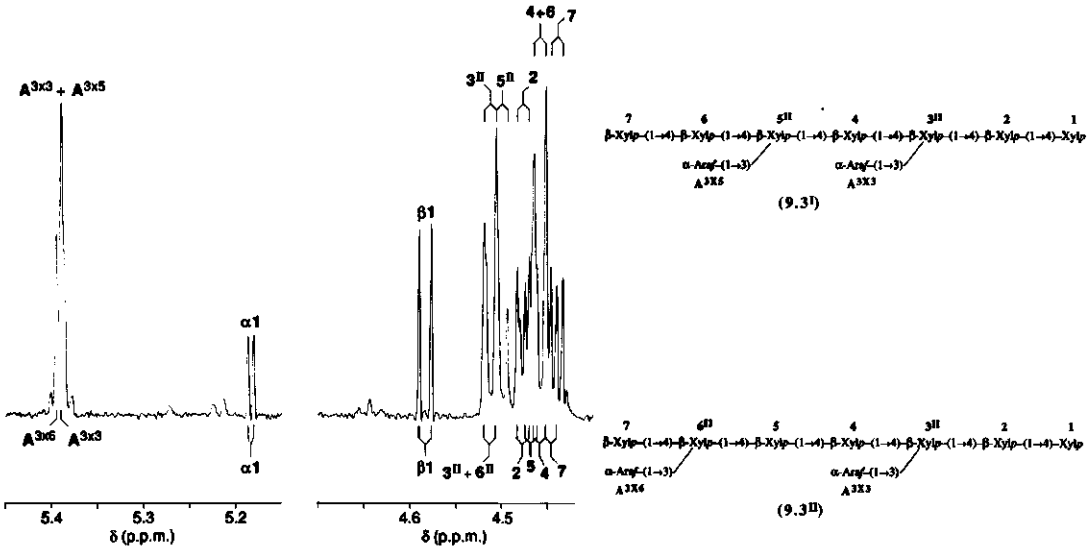
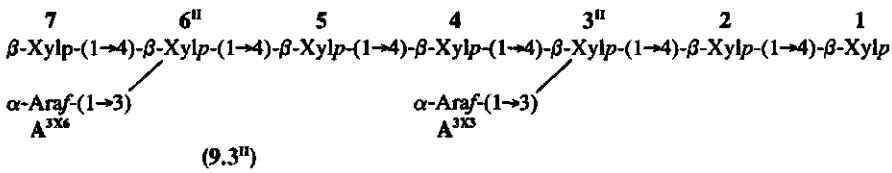
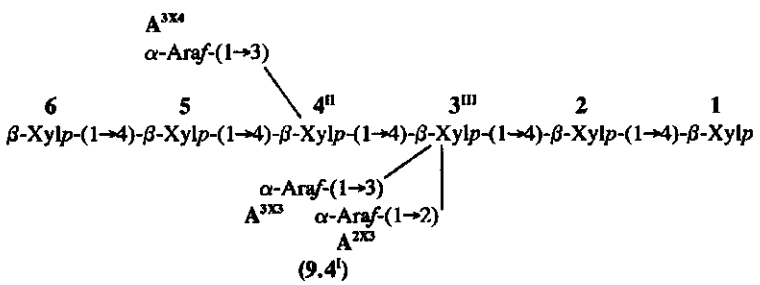


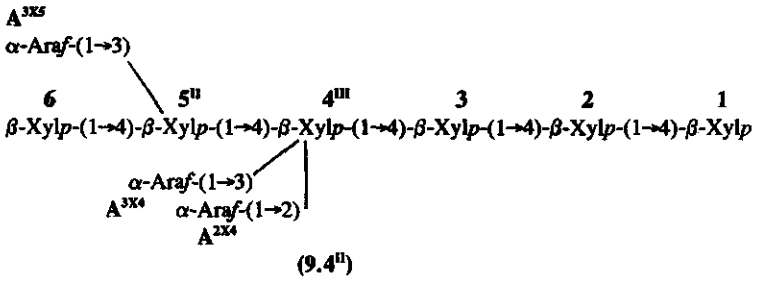
Figure 4. Resolution-enhanced 600-MHz ^{1}H-n.m.r. spectrum of fraction **9.3**. The numbers and letters in the spectrum refer to the corresponding residues in the structures.



Fraction **9.4**.——The intensities of the H-1 signals in the ^{1}H-n.m.r. spectrum of **9.4** revealed the presence of a triarabinosylxylohexaose, **9.4**^{I}, as the major component. The chemical shift data matched exactly those[7] of **AX-54b**, and the chemical shifts of the H-1 resonances are summarised in Table II.



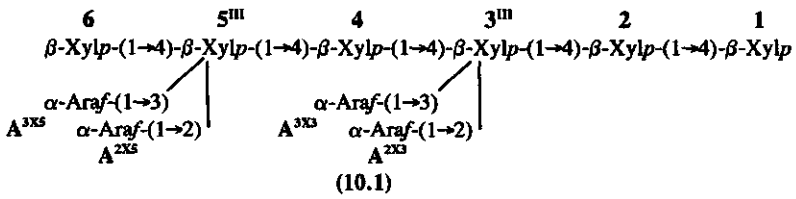
Additional β-Xyl*p* H-1 signals at δ 4.472, 4.435, and a α-Ara*f* H-1 signal at δ 5.402 in the ^{1}H-n.m.r. spectrum of **9.4** marked the presence of a minor compound (17% of the material), **9.4^{II}**, probably the isomer of **9.4^{I}**, as reported by Hoffmann *et al.*[7] (**AX-54a**).



(9.4^{II})

In the ^{1}H-n.m.r. spectrum of **9.4** signals were also observed of a third compound of which the structure could not be unravelled, due to the still lower amount of material and complexity of the spectrum.

Fraction **10.1**.——The intensities of the H-1 signals in the ^{1}H-n.m.r. spectrum of **10.1** revealed the presence of a single tetra-arabinosylxylohexaose. The chemical shift data matched exactly those[7] of **AX-57^e**, and the chemical shifts of the H-1 resonances are summarised in Table II.



(10.1)

Fraction **10.2**.——The ^{1}H-n.m.r. spectrum of **10.2** showed the presence of at least two components of which the structure could not be unravelled, due to the complexity of the spectrum. Further separation of the mixture was not attempted because of the low amount of material.

Fraction **10.3**.——The ^{1}H-n.m.r. spectrum of **10.3** showed the presence of at least two components of which the structure could not be unravelled, due to the complexity of the spectrum. Further separation of the mixture was not attempted.

Concluding remarks

The majority of oligosaccharides purified and characterised in this study were previously described by Hoffmann et al[6,7]. However, some new structures were found which can be added to the existing ones. Oligosaccharides presented in this chapter indicate the presence of these structures in the wheat alkali-extractable arabinoxylan[9].
Most of the oligosaccharides obtained with endo-(1,4)-ß-xylanase III from *Aspergillus awamori* CMI 142717, are different from the oligosaccharides obtained with endo-(1,4)-ß-xylanase I[8] from the same xylanolytic system. The pattern of action of enzymes from the same xylanolytic system will be the subject of future communications.

Acknowledgements

This work was supported also by Unilever Research Vlaardingen, the Dutch Ministry of Economic Affairs (ITP-program), and the Netherlands Foundation for Chemical Research (NWO/SON).

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Chapter 5

Mode of action of the xylan-degrading enzymes from *Aspergillus awamori* on alkali-extractable cereal arabinoxylans

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Submitted for publication.

Summary

Alkali-extractable cereal arabinoxylan and enzymically derived oligosaccharides thereof with known structures were treated with endo-xylanases I and III from *Aspergillus awamori* CMI 142717 and the digests subjected to analysis by high performance anion-exchange chromatography. Clear differences in the mode of action of the two endo-xylanases were observed. At least one respectively two unsubstituted xylopyranosyl residues adjacent to single and double substituted xylopyranosyl residues towards the reducing end can not be removed by endo-xylanase I. At least two unsubstituted xylopyranosyl residues adjacent to single or double substituted xylopyranosyl residues towards the reducing end can not be removed by endo-xylanase III. ß-Xylosidase from the same xylanolytic system was found to be able to remove terminal xylopyranosyl residues from the non-reducing end of branched oligosaccharides only when two contiguous unsubstituted xylopyranosyl residues were present adjacent to single or double substituted xylopyranosyl residues.

Introduction

Endo-xylanases with different physico-chemical parameters, have been purified from various microbial sources[1]. They hydrolyse glycosidic bonds in the xylan chain in a random fashion yielding a series of linear and branched oligosaccharide fragments.
Endo-xylanases differ in their mode of action in that they are restricted in the hydrolysis of glycosidic linkages in the vicinity of branch points which is reflected in many types of heterogeneous oligosaccharides released by these enzymes[2-5]. They also differ in the number of subsites and the location of the active site among the subsites, which plays an important role in xylo-oligosaccharide hydrolysis[6,7]. The elucidation of the mode of action of endo-xylanases may also be hampered by the possibility of termolecular hydrolysis and transglycosylation reactions which can occur with increasing concentrations of xylo-oligosaccharides[8].
In this chapter a model is proposed for the mode of action of endo-xylanase I, III and ß-xylosidase from *Aspergillus awamori* CMI 142717 based on data of the hydrolysis of polymeric arabinoxylan, a whole range of arabinoxylan-derived oligosaccharides and xylo-oligosaccharides.

Materials and methods

Materials
Endo-(1,4)-ß-D-xylanase I and III (Endo I and Endo III, respectively), (1,4)-ß-xylosidase (ß-xylosidase), and (1,4)-ß-D-arabinoxylan arabinofuranohydrolase (AXH) were purified from *Aspergillus awamori* CMI 142717 (ref. 9 and 10). α-L-Arabinofuranosidase A (Arafur A) was purified from *Aspergillus niger*[11]. The preparation and characterization of alkali-extractable wheat-flour arabinoxylan (BE1-U) is described elsewhere[12].
The isolation of oligosaccharides derived from alkali-extractable wheat-flour arabinoxylan by degradation with Endo I and III, by Bio-Gel P-2 gel permeation chromatography and high performance anion-exchange chromatography, and the elucidation of their structures by ^{1}H-n.m.r. spectroscopy were described by Gruppen *et al*[13] and Kormelink *et al*[14]. The elucidation of the structures obtained from alkali-extractable barley arabinoxylan by degradation with Endo I was described by Viëtor *et al*[15].
Xylo-oligosacccharides were derived from steam exploded birchwood xylan which was a kind gift of Dr. Puls (Institute of Wood Chemistry and Chemical Technology of Wood, BFH, Hamburg, FRG).

Enzyme incubations
A solution of alkali-extractable wheat arabinoxylan (80 mg) in 50mM sodium acetate buffer (80 mL, pH 5.0) was incubated with Endo III (0.4 μg/mL) and AXH (0.1 μg/mL) for 24 h at 30°C. After inactivation of the enzyme, the digest was concentrated to 2.0 mL

under reduced pressure and fractionated by Bio-Gel P-2 chromatography.
The release of arabinoxylan oligosaccharides from alkali-extractable wheat arabinoxylan by Endo I or III in time (0-48 h) was studied by incubation of 0.2 % (w/v) alkali-extractable wheat arabinoxylan with 0.1 μg/mL Endo I or III at 30°C in 50mM sodium acetate buffer pH 5.0.
Arabinoxylan-derived oligosaccharides (30 μg/mL) were incubated with 1.0 μg/mL Endo I, Endo III, ß-xylosidase, or a combination of Endo III and Arafur A for 24 h at 30°C in 50mM sodium acetate buffer pH 5.0.
Xylo-oligosaccharides, i.e. X_2, X_3, X_4, X_5 and X_6 (100 μg/mL), were incubated with 1.0 μg/mL Endo I or III for 24 h at 30°C in 50mM sodium acetate buffer pH 5.0.
After incubation and enzyme inactivation (100°C, 10 min), the reaction mixtures were analysed by h.p.a.e.c. using well characterised oligosaccharides[13,14] as standards.

Bio-Gel P-2 gel permeation chromatography
Arabinoxylan-digests and steam exploded birchwood xylan were fractionated by Bio-Gel P-2 gel permeation chromatography (100 x 2.6 cm, 200-400 mesh; Bio-Rad) thermostated at 60°. Water was used as eluent at a flow of 20 mL/min. Thiomersal (0.01% w/v) was added as preserving agent. Fractions of 2.4 mL were collected and monitored for total neutral sugar content[16].

High performance anion-exchange chromatography (h.p.a.e.c.)
Digests containing arabinoxylan oligosaccharides were analysed by h.p.a.e.c. as described previously[13]. 20 μL Samples were injected on to the CarboPac PA1 column. Elution (1 mL/min) involved linear gradients of sodium acetate in 0.1M NaOH of 0-100mM during 5 min, then of 100-400mM during 35 min at 20°C.

Results

Alkali-extractable wheat-flour arabinoxylan, which has an Ara/Xyl ratio of 0.52, was treated with Endo I or III for 24 h and the digest fractionated by Bio-Gel P-2 gel permeation chromatography[13,14]. Endo I converted about 72% of the arabinoxylan into oligosaccharides with a degree of polymerization (DP) lower than 10. Endo III, however, converted only 50% of the arabinoxylan into oligosaccharides with a DP lower than 10. No significant amounts of monomers to tetramers could be observed in the latter case. These observations indicate a difference in the mode of action of Endo I and III towards arabinofuranosyl substituted xylans and apparently reflect the number of subsites available to form an effective enzyme-substrate complex.
Figures 1 and 2 schematically summarizes the structures of oligosaccharides released from wheat arabinoxylan by Endo I and Endo III, respectively. As can be seen from Fig. 1, xylose, xylobiose and xylotriose were only released by Endo I.

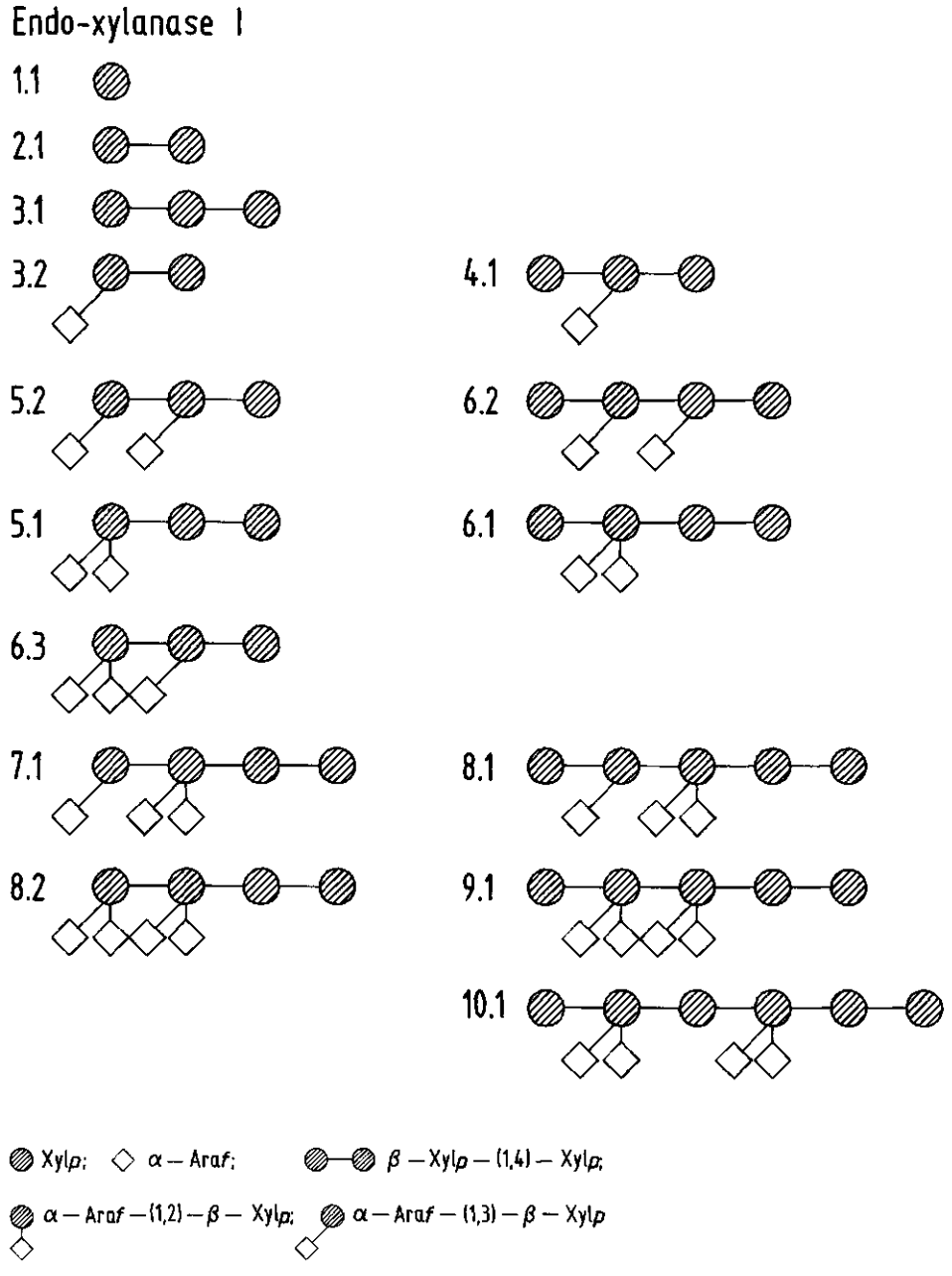


Figure 1. Arabinoxylan-derived oligosaccharides released by Endo I from alkali-extractable wheat-flour arabinoxylan.

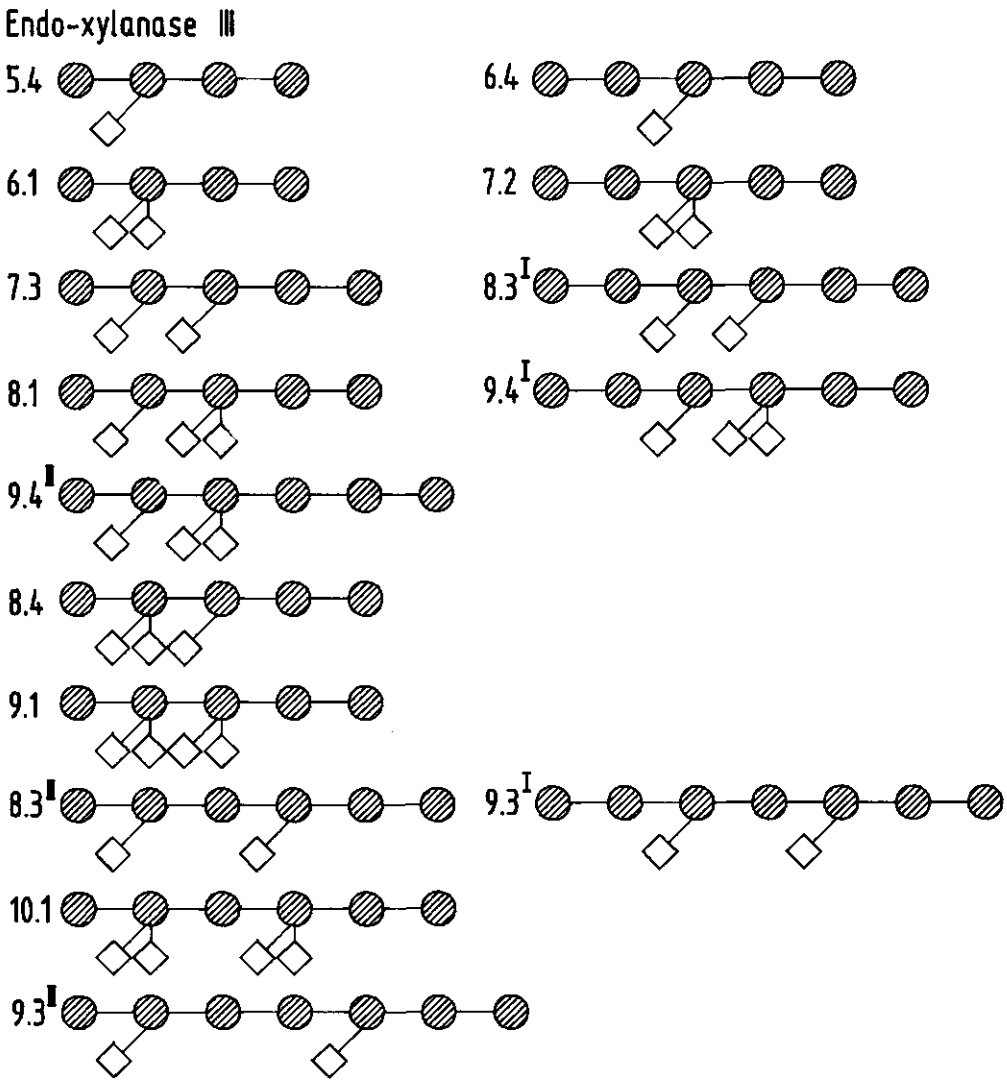


Figure 2. Arabinoxylan-derived oligosaccharides released by Endo III from alkali-extractable wheat-flour arabinoxylan.

Three groups of arabinoxylan-oligosaccharides could be identified, (i) oligosaccharides containing single substituted xylopyranosyl residues only (Endo I: structures **3.2**, **4.1**, **5.2**, and **6.2**; Endo III: structures **5.4**, **6.4**, **7.3**, **8.3^{I}**, **8.3^{II}**, **9.3^{I}**, and **9.3^{II}**), (ii) oligosaccharides containing single and double substituted xylopyranosyl residues (Endo I: structures **6.3**, **7.1**, and **8.1**; Endo III: structures **8.1**, **9.4^{I}**, **9.4^{II}**, and **8.4**), and (iii) oligosaccharides containing double substituted xylopyranosyl residues only (Endo I: structures **5.1**, **6.1**, **8.2**, **9.1**, and **10.1**; Endo III: structures **6.1**, **7.2**, **9.1** and **10.1**).
Close observations of the structures suggest the need for Endo I of at least one and two unsubstituted xylopyranosyl residues at the reducing site adjacent to a single and double substituted xylopyranosyl residue, respectively, and the need for Endo III of at least three unsubstituted xylopyranosyl residues at the reducing site adjacent to a single or double substituted xylopyranosyl residue.
Similar structures as in Figure 1 were also released from barley arabinoxylan by Endo I[15]. The isolation of a tetrasaccharide 2^3-α-L-Ara*f*-Xyl_3 from barley arabinoxylan treated with Endo I[15], could suggest that the hypothesis is only valid for Endo I when arabinofuranosyl substituents are α-(1,3)-linked to xylopyranosyl residues. Endo I is thus more inhibited in its action by α-(1,2)-linked arabinofuranosyl substituents than by α-(1,3)-linked arabinofuranosyl substituents.

The extent of hydrolysis by Endo III could be strongly increased by addition of AXH (Fig. 3), an enzyme able to remove arabinofuranosyl substituents from single substituted xylopyranosyl groups[17]. This results in the formation of new linear sites in the xylopyranosyl backbone and thus in the creation of new subsites for Endo III. After AXH treatment, xylose, xylobiose, and xylotriose could be released (Fig. 3.b) which was not the case by treatment with Endo III alone (Fig. 3.a).
The availability of purified arabinoxylan-derived oligosaccharides as reference compounds made it possible to study more closely the action of Endo I and Endo III by monitoring the release of oligosaccharides from alkali-extractable wheat-flour arabinoxylan (Fig. 4 and 5, respectively). Not only a slower release of oligosaccharides by Endo I can be observed, but also much higher amounts, the latter particularly for xylose to xylotetraose.
Higher oligosaccharides (DP > 4) released by Endo III and not present in the Endo I digests, were incubated with Endo I (Table I). By h.p.a.e.c. analysis it was demonstrated that Endo I was able to release xylose from oligosaccharides containing two unsubstituted xylopyranosyl residues adjacent to a single substituted xylopyranosyl residue towards the reducing site (structures **5.4**, **6.4**, **7.3**, and **8.4**). Xylose could not be released when the xylopyranosyl residue was double substituted (structures **7.2** and **9.4^{I}**).
At the sites towards the non-reducing end, xylobiose was released by Endo I from oligosaccharides containing two unsubstituted xylopyranosyl residues at the non-reducing site adjacent to a single or double substituted xylopyranosyl residue (structures **6.4**, **7.2**, and **9.4^{I}**).

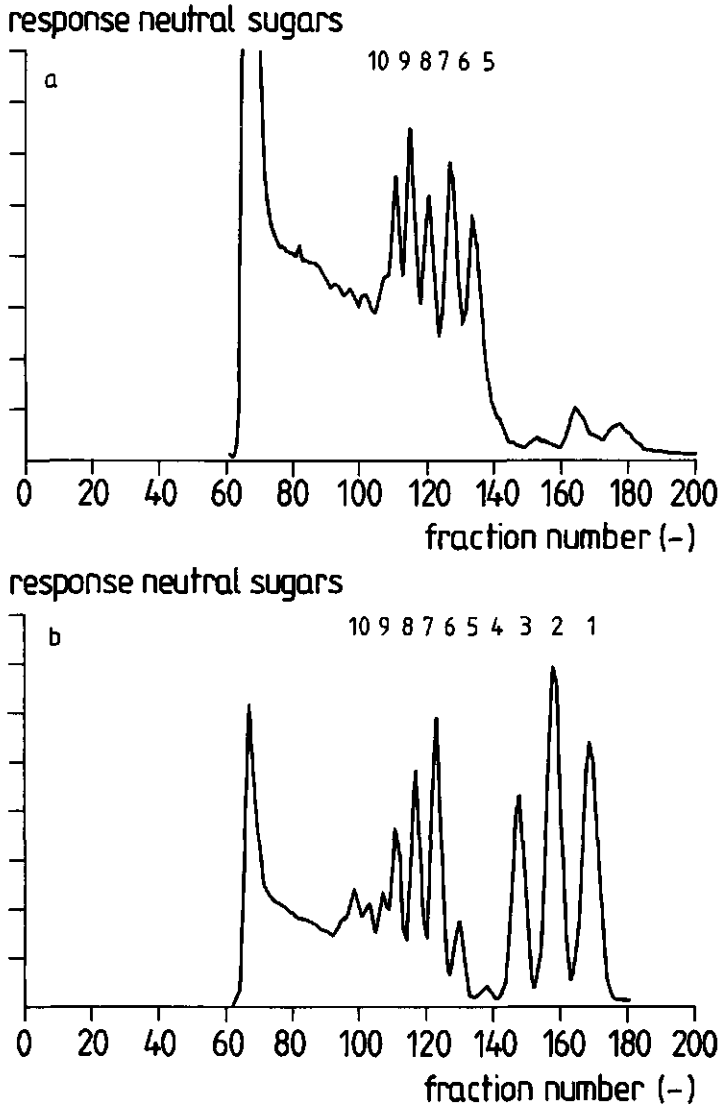


Figure 3. Bio-Gel P-2 elution patterns of arabinoxylan digests obtained with Endo III[14] (a), and a combination of Endo III and AXH (b). The peak number corresponds with the degree of polymerization of the oligosaccharide.

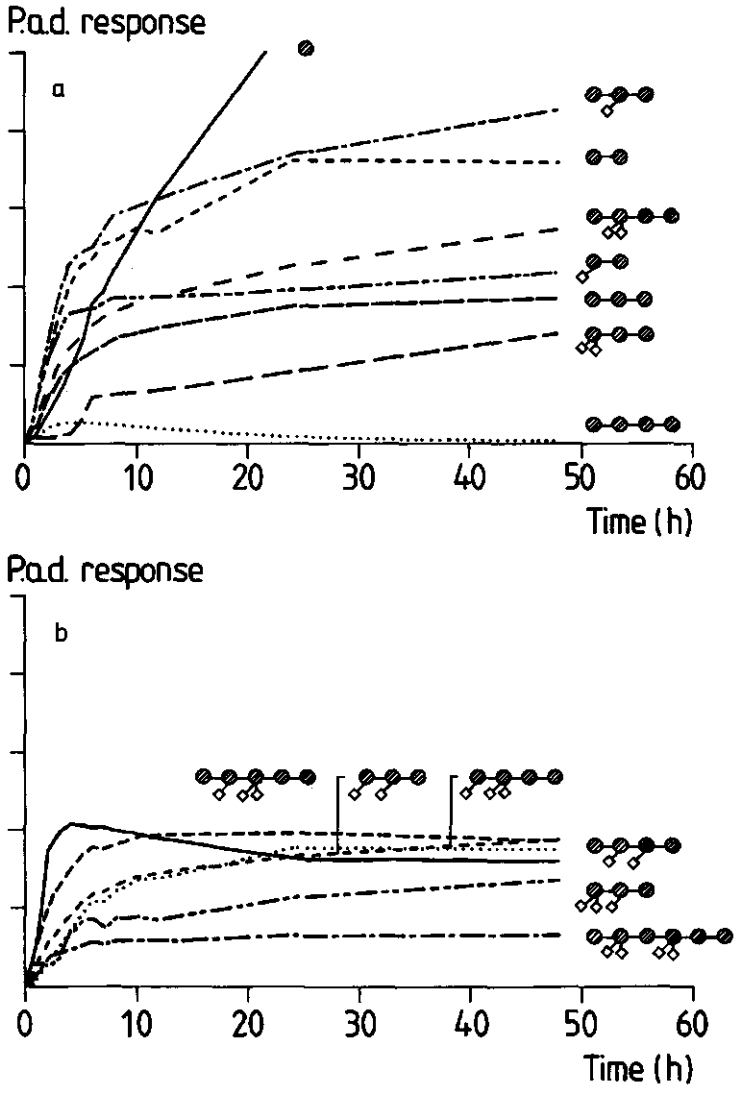


Figure 4. Release of arabinoxylan oligosaccharides by incubation of alkali-extractable wheat-flour arabinoxylan with Endo I as a function of incubation time; a. small oligosaccharides and b. higher oligosaccharides.

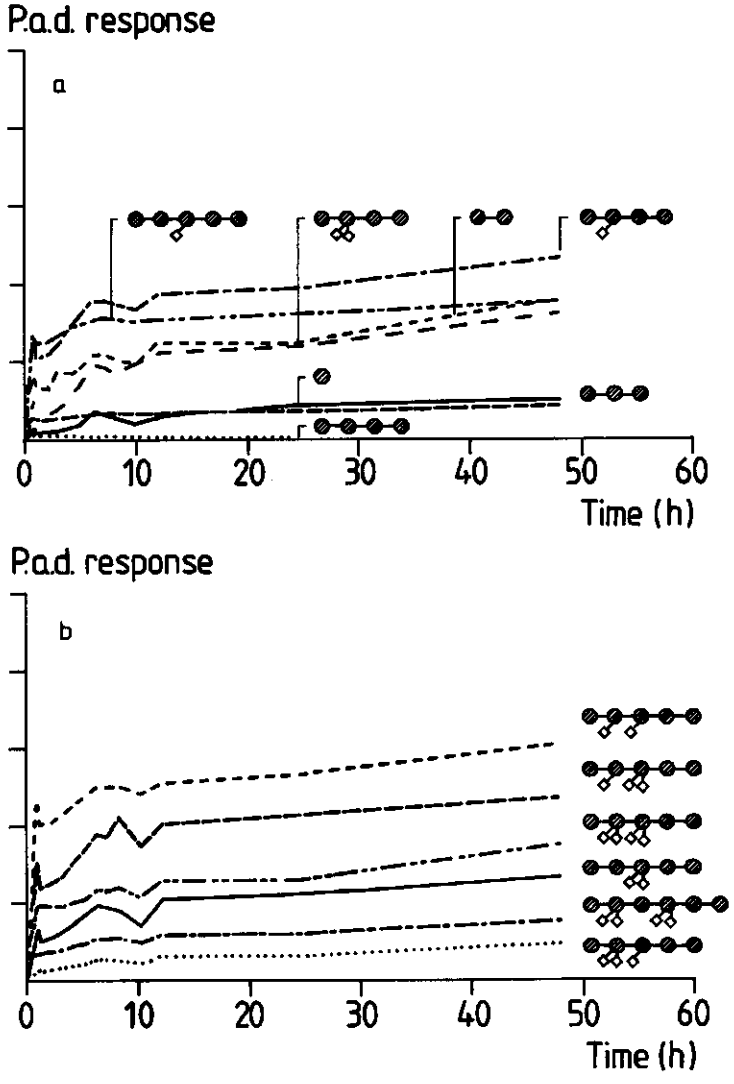
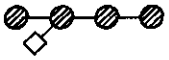
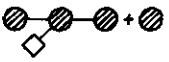
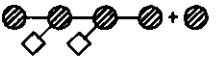
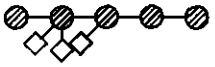
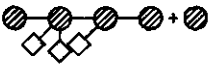
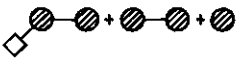
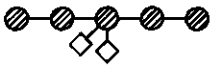
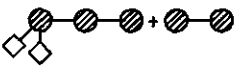
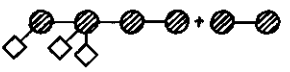


Figure 5. Release of arabinoxylan oligosaccharides by incubation of alkali-extractable wheat-flour arabinoxylan with Endo III as a function of incubation time (a and b, see Fig. 4).

Table I. Hydrolysis of oligosaccharides by Endo I as determined by h.p.a.e.c. using pulsed amperometric detection.

Oligosaccharide	R_t[a] (min)	Hydrolysis products	R_t[a] (min)
	12.35		12.01
	17.04		17.09
	20.21		20.49
	12.59		12.41
	14.15		14.70
	18.93		20.07

[a] R_t = retention time.

Even after prolonged incubation, Endo III was not able to release xylose or xylobiose from structures **5.4** and **6.4** containing one or two unsubstituted xylopyranosyl residues at the non-reducing and reducing site. Concerted action of Arafur A (1 μg/mL) and Endo III (1 μg/mL) on oligosaccharide **8.4** (30 μg/mL) for 24 h, showed the formation of structure **6.1** (results not shown). The release of arabinofuranosyl from the single substituted xylopyranosyl residue by Arafur A (ref. 17), enables Endo III to attack the three unsubstituted xylopyranosyl residues at the reducing site in order to release xylose from this modified oligosaccharide. Thus Endo III is able to release xylose from the reducing site when three unsubstituted xylopyranosyl residues are present next to a substituted xylopyranosyl residue.

Endo I and III have also been compared with respect to their mode of action towards linear xylo-oligosaccharides (Table II). Dimers up to hexamers of xylose were incubated with Endo I and III, respectively for 24 h, and the ratio of the main end-products was determined, i.e. X_3, X_2, and X.

As can be seen from Table II, X_6 is split by Endo I to X_3, X_2 and X in a ratio of 0.6 : 1.5 : 1.0. Endo I splits X_5 also to X_3, X_2 and X, however, with a somewhat higher value for X_2 (2.0). X_4 is split by Endo I to X_2 and X mainly with smaller amounts of X_3. X_3 is only degraded slowly into X_2 and X.

When observing the data for Endo III, it can be seen that Endo III releases X_3 and X_2 mainly with smaller amounts of X from X_6. A similar observation can be done for the degradation of X_5 with even smaller amounts of xylose. A remarkable fact is the degradation of X_4 into mainly X_2. X_3 is almost not degraded into X_2 and X. Both Endo I and III cannot degrade X_2.

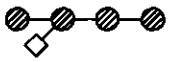
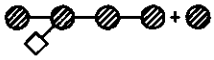
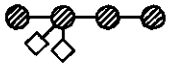
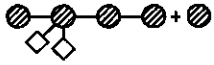
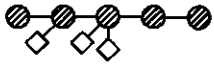
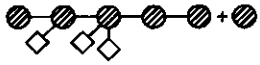
As ß-xylosidase is important in the optimal breakdown of arabinoxylans, digests of the arabinoxylan oligosaccharides with ß-xylosidase were analysed for any breakdown by h.p.a.e.c. (Table III). Data presented in Table III indicate that ß-xylosidase is only able to release xylose from the non-reducing end of arabinoxylan oligosaccharides, when at least two unsubstituted xylopyranosyl residues are present adjacent to a substituted xylopyranosyl residue towards the non-reducing end.

Table II. Ratio's of X_3, X_2, and X, released from linear ß-(1,4)-linked D-oligoxylosides by Endo I or III from *Aspergillus awamori*.

Substrate	Enzyme	X_3 : X_2 : X
X_6	Endo I	0.6 : 1.5 : 1.0
	Endo III	4.9 : 5.1 : 1.0
X_5	Endo I	0.7 : 2.0 : 1.0
	Endo III	6.0 : 6.5 : 1.0
X_4	Endo I	0.3 : 1.5 : 1.0
	Endo III	1.6 : 7.2 : 1.0
X_3	Endo I	0.8 : 0.7 : 1.0
	Endo III	4.8 : 1.2 : 1.0
X_2	Endo I	n.d.[a]
	Endo III	n.d.

[a] n.d. not detected

Table III. Oligosaccharide hydrolysis by ß-xylosidase as determined by h.p.a.e.c. using pulsed amperometric detection.

Oligosaccharide	R_t[a] (min)	Hydrolysis products	R_t[a] (min)
	12.35	n.d.[b]	
	12.59		12.34
	13.98	n.d.	
	14.15		13.95
	19.09	n.d.	
	18.93		18.93

[a] R_t = Retention time
[b] n.d. not detected

Discussion

The endo-xylanases I and III, purified from *Aspergillus awamori* CMI 142717, not only differ in their physico-chemical parameters[10], but were also shown to differ greatly in their mode of action towards arabinoxylan, arabinoxylan oligosaccharides and xylo-oligosaccharides.

Monomers to tetramers were clearly present in the wheat arabinoxylan digest obtained with Endo I, but only in trace amounts in the digest obtained with Endo III (Fig. 1, 2, and 3). Also in the case of oat spelts arabinoxylan, larger amounts of xylotriose, xylobiose and xylose were released by Endo I[10].

Endo III is thus more sensitive to arabinofuranosyl substitution than Endo I, which explains the lower degree of hydrolysis of the arabinoxylan by Endo III. This is also

reflected in the large void fraction in the Bio-Gel P-2 column fractionation[14]. Our results show that the arabinofuranosyl substituents on the xylan backbone clearly inhibit the action of endo-xylanases on glycosidic linkages in the vicinity of the site of substitution.

Endo I is able to split the glycosidic linkage at the non-reducing site of a single or double substituted xylopyranosyl residue. This fact corresponds with observations made by others[18,19,20]. Glycosidic linkages at the reducing site of a xylopyranosyl residue can only be split when the xylopyranosyl residue is unsubstituted and adjacent to an unsubstituted or single substituted xylopyranosyl residue.

Endo III on the other hand is unable to split the glycosidic linkage at the non-reducing site of a single or double substituted xylopyranosyl residue. It can split the glycosidic linkage of the adjacent unsubstituted xylopyranosyl residue or the successive unsubstituted xylopyranosyl residues towards the non-reducing site. The glycosidic linkage at the reducing site of a single or double substituted xylopyranosyl residue can not be split, also not the next glycosidic linkage of the neighbouring unsubstituted xylopyranosyl residues towards the reducing site. Only the glycosidic linkage at the next neighbouring xylopyranosyl residue can be split when the residue is not substituted.

In case of double substituted xylopyranosyl residues, the next two (1,4)-glycosidic linkages towards the reducing site seemed to be resistent to Endo I or III degradation. The same observation was made for α-(1,2)-arabinofuranosyl substituted xylopyranosyl residues in the case of Endo I degradation. Comtat and Joseleau[21] did the same observation with α-(1,2)-linked glucopyranosyl uronic acid substituents, and even suggested that not the type of substituent, but only the position of the substituent is determinative.

It is unknown whether the presence of arabinofuranosyl substituents interfere with the fit of the (1,4)-ß-D-xylan backbone into the binding site. It has been suggested[18,22] that the presence of arabinofuranosyl substituents may not impede enzyme binding to the arabinoxylan in the vicinity of the substituent, because of the rapid hydrolysis of α-L-arabinofuranosyl-xylopentaose and α-L-arabinofuranosyl-xylotetraose by hemicellulase I and II from *Ceratocystis paradoxa*, respectively. However, Brillouet[23] observed that the degree of polymerization of the lowest oligosaccharide undergoing hydrolysis was increased by 1 when an arabinofuranosyl substituent is present at the non-reducing terminal xylopyranosyl residue, and therefore suggests that the presence of these substituents prevents a good fit between substrate and enzyme binding site.

The degradation patterns of xylo-oligosaccharides indicate a difference in the location of the active site among the subsites of Endo I and III. The degradation of xylotetraose shows the most important difference. The main end-products of xylotetraose degradation with Endo I and III were X, X_2, and X_3 in a ratio of 1.0:1.5:0.3 and 1.0:7.2:1.6, respectively. In the latter case the enzyme thus shows a very high affinity towards the glycosidic linkage between the second and the third xylopyranosyl residue counting from the reducing site. In the first case the affinity towards this linkage has to be much lower, resulting in higher concentrations of other products.

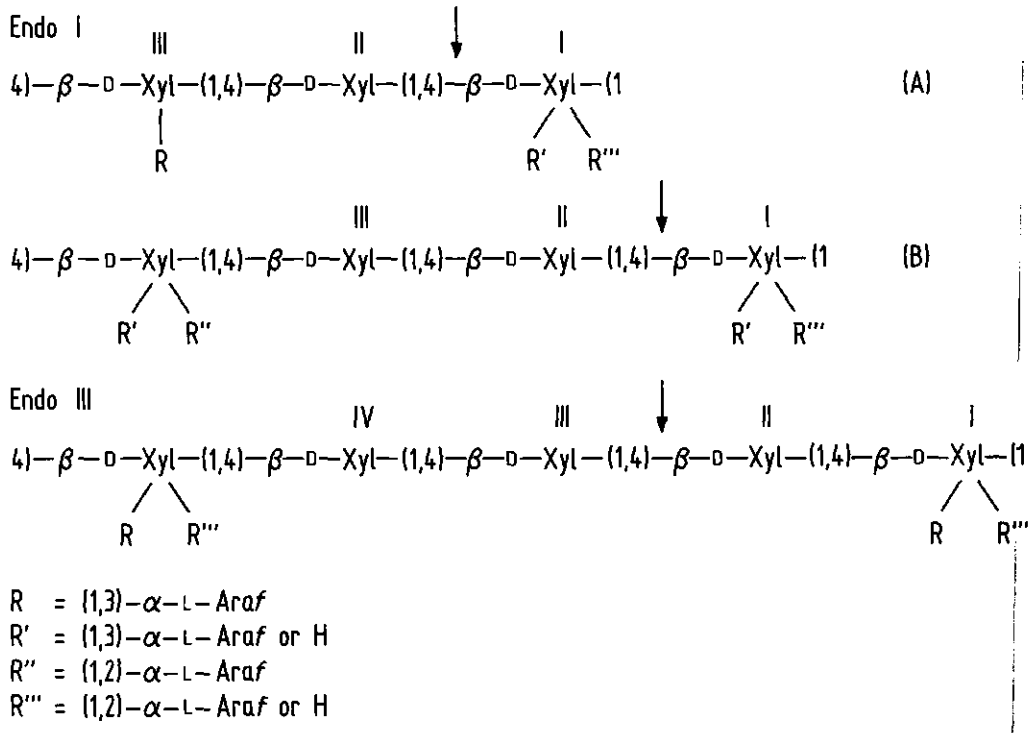


Figure 6. Model for the mode of action of Endo I (A and B) and III from *Aspergillus awamori* towards arabinoxylan.

The model as proposed by Comtat and Joseleau[21] for the mode of action of endo-xylanase II from *Sporotrichum dimorphosporum* appears also to apply for Endo I (Fig. 6 (A)). In our model, also the binding of Endo I requires a region of the xylan chain consisting of three D-xylosyl residues. For cleavage of glycosidic linkages the *O*-2 of residue III, and *O*-2 and *O*-3 of residue II have to be unsubstituted. Residue I may be unsubstituted, single substituted or double substituted. Cleavage takes place between residue I and II. If residue III (Fig. 6 (A)) is single substituted at *O*-2, or double substituted at *O*-2 and *O*-3, two out of three unsubstituted xylopyranosyl residues are needed for cleavage of the glycosidic linkage (Fig. 6 (B)).

Because of the degradation of xylotetraose into xylobiose mainly and the need of at least three contiguous unsubstituted xylopyranosyl residues for cleavage, it is supposed that the binding of Endo III needs a region of the xylan chain consisting of four D-xylosyl residues, in which the *O*-2 and *O*-3 of residue II, III and IV are unsubstituted. Residue I does not have to be unsubstituted. Cleavage takes place between residue II and III (Fig. 6).

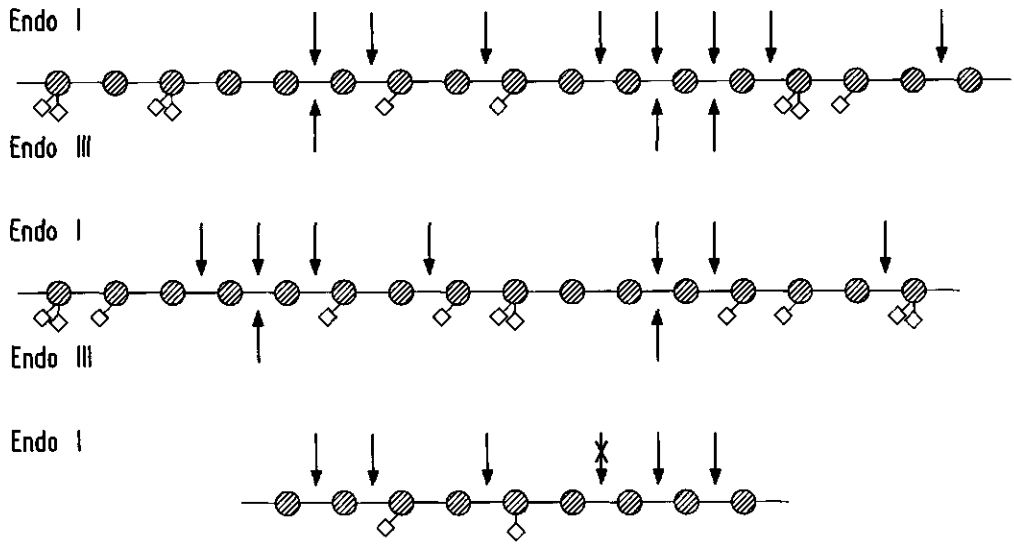


Figure 7. Sites of attack of Endo I and III from *Aspergillus awamori* towards a fictitious arabinoxylan (see Fig. 1 for legend).

Biely *et al*[7] found that the substrate binding site of an *Aspergillus niger* endo-xylanase consisted of seven subsites, whereas Meagher *et al*[24] found a number of five subsites. Conclusions concerning the exact number of subsites can not be made in the case of Endo I and Endo III, because kinetic parameters have not been determined on oligosaccharide-hydrolysis. However, Endo III must have a higher turn-over number on alkali-extractable wheat-flour arabinoxylan than Endo I, because of the higher initial rate in the release of oligosaccharides.

Figure 7 shows an example for the sites of attack of Endo I and III towards a fictitious arabinoxylan, based on all these observations. As can be seen from this Figure, Endo III has much less cleavage-sites than Endo I, which explains the absence of small end-products.

No arabinofuranosyl substituents were linked to the reducing xylopyranosyl residue of oligosaccharides obtained by Endo I or Endo III. Only in the case of hydrolysis of rice straw arabinoxylan with an *Aspergillus niger* xylanase[25], α-L-arabinofuranosyl-(1,4)-ß-D-xylobiose and α-L-arabinofuranosyl-(1,4)-ß-D-xylotriose were produced with the arabinofuranosyl substituent at the reducing residue. The enzyme could thus hydrolyse the (1,4)-glycosidic linkage at the reducing site of a single substituted xylopyranosyl residue and must therefore have a mode of action which differs from Endo I and Endo III.

Structure **3.2** (3^2-α-L-Ara*f*-Xyl_2)[21,23,26-30] and structure **4.1** (3^2-α-L-Ara*f*-Xyl_3)[30,31] have been described before in arabinoxylan digests. Because 1 or 2 oligosaccharides have been

purified in these cases only, no model could be presented as complete as the model proposed here. A similar range of oligosaccharides as obtained by Endo III, was found by enzymic treatment of wheat arabinoxylan by Hoffmann *et al*[32,33]. However, in the latter case the enzyme was not pure as indicated by the presence of free arabinose in the arabinoxylan digest. Also, the degradation was not completed as shown by the presence of structures with three unsubstituted xylopyranosyl residues adjacent to a substituted xylopyranosyl residue at the reducing site.

The structures of the arabinoxylan oligosaccharides obtained by Endo I, Endo III and others[21,23,25-33], indicate the existence of at least three groups of endo-xylanases which differ in their mode of action towards arabinoxylan.

ß-Xylosidase was shown to split off xylopyranosyl residues from the terminal non-reducing site of oligosaccharides, but is limited by arabinofuranosyl substituents. Different observations were made by Takenishi and Tsujisaka[25], who incubated 3^1-α-L-Ara*f*-Xyl_2 with an *Aspergillus niger* ß-xylosidase which resulted in the release of 3-α-L-Ara*f*-Xyl and xylose. Differences can thus be expected also in the mode of action of ß-xylosidases towards substituted xylo-oligosaccharides.

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Chapter 6

Purification and characterization of an (1,4)-ß-D-arabinoxylan arabinofuranohydrolase from *Aspergillus awamori*

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Appl. Microbiol. Biotechnol., 35 (1991) 753-758.

Summary

An enzyme which was able to split off arabinose side-chains from cereal arabinoxylans, was isolated from a cell-free culture filtrate of *A. awamori* CMI 142717 containing milled oat straw as carbon source. The enzyme was highly specific for arabinoxylans and, unlike other α-L-arabinofuranosidases reported in the literature, did not show any activity towards *p*-nitrophenyl α-L-arabinofuranoside, arabinans and arabinogalactans.
This novel enzyme, which can be described as an (1,4)-ß-D-arabinoxylan arabinofuranohydrolase, had a molecular weight of 32000 Dalton when determined by SDS-PAGE and a specific activity of 22 U/mg on wheat arabinoxylan.

Introduction

For complete breakdown of arabinoxylans, combinations of enzymes are needed (Biely, 1985). Enzymes which split off the arabinose from the xylan backbone are important in this context.

Several enzymes able to split off arabinose substituents from arabinoxylans have been described in the literature. Notable in this respect are the endo-(1,4)-ß-xylanases (E.C. 3.2.1.8; Dekker and Richards, 1976) which split the xylan-backbone simultaneously with the release of arabinose side-chains. Such arabinose-releasing endo-(1,4)-ß-xylanases were purified from *Aspergillus* (Takenishi and Tsujisaka, 1973; John *et al*, 1979; Sinner and Dietrichs, 1975), *Trichoderma* (Wood and McCrae, 1986), *Oxisporus* (Sinner and Dietrichs, 1975), *Ceratocystis* (Dekker and Richards, 1975) and *Cephalosporium* (Richards and Shambe, 1976).

Arabinosidases do not degrade the xylan backbone. They comprise of α-L-arabinofuranosidase (E.C. 3.2.1.55) which hydrolyse (terminal) non-reducing α-L-arabinofuranosyl residues from α-L-arabinofuranosides, arabinans, arabinoxylans and arabinogalactans, and endo-(1,5)-α-L-arabinanase (E.C. 3.2.1.99) which hydrolyse α-L-(1,5)-arabinofuranosidic linkages in α-(1,5) linked L-arabinans.

Kaji (1984) subdivides the group of α-L-arabinofuranosidase further into the *A. niger* type of α-L-arabinofuranosidase and the *Streptomyces purpurascens* type of α-L-arabinofuranosidase. The former group of enzymes hydrolyse L-arabinosides of low molecular weight including L-arabino-oligosaccharides, polymeric L-arabinan, L-arabinoxylan and L-arabinogalactan; the latter group can act only on α-L-arabinosides of low molecular weight and α-L-arabino-oligosaccharides.

A. niger type α-L-arabinofuranosidases have been purified from *Aspergillus niger* (Kaji and Tagawa, 1970; Andrewartha *et al*, 1979; Rombouts *et al*, 1988), *Trichoderma reesei* (Poutanen, 1988), *Streptomyces sp.* No. 17-1 (Kaji *et al*, 1981), *Ruminococcus albus* 8 (Greve *et al*, 1984), *Dichomitus squalens* (Brillouet and Moulin, 1985) and *Bacillus subtilis* (Weinstein and Albersheim, 1979).

In this chapter we describe a novel enzyme, which was found during the purification of an endo-(1,4)-ß-xylanase and which falls into neither of the previously described categories (Kormelink *et al*, 1991). The enzyme can best be described as an (1,4)-ß-D-arabinoxylan arabinofuranohydrolase (abbreviated as AXH).

Materials and methods

Materials

Chemicals used were of analytical grade. Substrates used were arabinoxylan ex oat spelts (Sigma Chemical Co., USA) and water soluble arabinoxylan ex oat spelts obtained according to the procedure of Selvendran *et al* (1985). Alkali-extractable wheat ara-

binoxylan was kindly provided by H. Gruppen of the Department of Food Science (Agricultural University, Wageningen, The Netherlands). Water soluble wheat arabinoxylan was a gift from IGMB-TNO (Wageningen, The Netherlands).
Enzyme fractions were tested for activities other than those degrading arabinoxylans using CM-cellulose (Type AF 0305, Enka Industrial Colloids, Arnhem, The Netherlands), H_3PO_4-swollen cellulose (prepared according to the method described by Wood, 1971), Avicel (Serva, FRG); potato arabinogalactan (isolated at our laboratory according to Labavitch *et al*, 1976); citrus galactan and coffee galactan (preparations available in our laboratory); sugar beet arabinan kindly provided by British Sugar (Norwich, UK); larch wood arabino-ß-(1,3/6)-galactan ("stractan", St. Regis Paper Company, Tacoma, Washington, USA); arabinan-rich apple pectin fragments (MHR, Schols *et al*, 1990); linear arabinan kindly provided by Novo Ferment (Basel, Switzerland); and PNP-glycosides (Koch and Light, Colnbrooks, England).
The enzyme preparation used for the purification of AXH was from a culture filtrate of *A. awamori* CMI 142717 prepared at The Rowett Research Institute. α-L-Arabinofuranosidase A and B were purified from *A. niger* by Rombouts *et al* (1988), endo-(1,4)-ß-xylanase III was purified by Kormelink *et al* (1990) also from *A. awamori* CMI 142717, and endo-glucanase IV was purified by Beldman *et al* (1985) from *T. viride*.
Sugar analysis was carried out on a Spectra Physics HPLC (San Jose, California, USA). For analysing mono and oligomeric sugars, samples were pretreated with $Pb(NO_3)_2$ and subsequently injected on an Aminex HPX 87P column (Voragen *et al*, 1986). For determining changes in the molecular weight distribution, samples were injected on to a series of Biogel TSK 40+30+20 XL columns (HPSEC) in combination with a TSK guard column (6 x 40 mm) at 30°C using 0.4M NaAc pH 3.0 as eluent at a flowrate of 0.8 mL/min. Polysaccharides, oligomeric and monomeric sugars were detected by a Shodex SE-61 RI detector (Showa Denko K.K., Tokyo, Japan).
Enzyme purification was carried out on a Bio-Gel P10 gel filtration column and a DEAE-Bio-Gel A anion exchanger (Bio-Rad Laboratories, Richmond, California). Micro-scale runs were carried out on a Mono Q™ anion exchanger and a Mono P™ column by a FPLC system (Pharmacia LKB Biotechnology, Uppsala, Sweden).

Glycanase and glycosidase activities

Glycanase and glycosidase assays were performed according to Beldman *et al* (1985). Enzyme activities were measured in 50mM sodium acetate buffer pH 5.0 at 30°C and calculated from the increase in reducing equivalent, as measured by the method of Nelson-Somogyi (Somogyi, 1952) or by the release of *p*-nitrophenol from the *p*-nitrophenyl glycoside as measured spectrophotometrically at 405 nm. One unit of activity is defined as the amount of enzyme that catalyzes the release of 1 μmole of reducing sugar equivalent or *p*-nitrophenol from the substrate per minute at 30°C.
To determine arabinose-releasing activity, 0.1 % (w/v) solutions of different arabinose-containing substrates were incubated with 0.4 μg/mL of the purified enzyme at 30°C for

2.5 h in 50mM sodium acetate buffer pH 5.0 and analysed by HPLC.

Enzyme purification

Enzyme purification was carried out at 4°C, and involved gel-filtration chromatography on Bio-Gel P10 (85.0 x 2.8 cm) and anion exchange chromatography on DEAE-Bio-Gel A (2.8 x 18 cm, and 2.0 x 18 cm). All buffers contained 0.01% (w/v) sodium azide to prevent microbial growth. Fractions collected were screened for arabinose-releasing activity (by HPLC), xylanase activity (by measuring reducing sugars) and protein content (absorbance at A_{280} nm). Fractions containing these activities were pooled.

Step 1. The enzyme preparation (30 mL of a liquid preparation) was applied on to the Bio-Gel P10 column and eluted with 10mM sodium acetate buffer pH 5.0. Fractions of 6.0 mL were collected.

Step 2. The pooled fractions were applied on to a DEAE-Bio-Gel A column (18.0 x 2.8 cm). After washing the column with 10mM sodium acetate buffer pH 5.0, a sodium chloride gradient with peak control was used (10mM sodium acetate buffer pH 5.0 and 50mM sodium acetate buffer pH 5.0 containing 0.5M sodium chloride). In this way a minimum of contamination is obtained by maintaining the composition of the eluent at a fixed value during elution of peaks. Fractions of 9.5 mL were collected.

Step 3. Pooled fractions were applied on to a DEAE-Bio-Gel A column (2.0 x 18.0 cm). Unbound proteins were removed from the column with 0.05M sodium acetate buffer pH 6.0. Adsorbed protein was eluted with a pH gradient (0.05M sodium acetate buffer pH 6.0 and 0.05M sodium acetate buffer pH 3.0). Fractions of 9.5 mL were collected. The fractions were measured for pH and analysed for arabinose-releasing activity and xylanase activity. Protein content was measured at A_{280}.

A final check for purity was carried out by applying AXH or endo-xylanase II on to a Mono Q™ anion exchanger in 20mM piperazine buffer pH 5.0 by FPLC. The endo-xylanase II fraction needed an extra step on a Mono P™ column for chromatofocusing from 6.3 to 3.0 in 25mM bis-tris-HCl buffer pH 6.3 (1 mL fractions).

SDS-gel electrophoresis

The molecular weight of the enzyme was estimated by electrophoresis (SDS-PAGE) on a 10-15% polyacrylamide gel. This was carried out with the PhastSystem (Note 110, PhastSystem, System Guide; Pharmacia LKB Biotechnology, Uppsala, Sweden).

A low molecular weight kit (Pharmacia) from 94.0 up to 14.4 kDa was used for calibration. The gel was stained with silver (Note 210).

Trifluoroacetic acid treatment

For measuring the extent of release of arabinofuranosyl substituents, a 0.1% (w/v) solution of water-soluble wheat arabinoxylan with a high arabinose/xylose ratio (0.52), and a 0.1% (w/v) solution of water-soluble oat spelts arabinoxylan with a low arabinose/xylose ratio (0.12), were incubated with 0.4 μg/mL AXH or 1.0 μg/mL α-L-arabinofuranosidase

B for 20 h at 30°C. After incubation, the reaction was terminated by boiling the reaction mixtures for 5 minutes. One part of the enzyme-treated arabinoxylan was further hydrolysed by treatment with trifluoroacetic acid (0.1M TFA at 100°C for 1 hour). For comparison with the enzyme-treated/TFA-hydrolysed arabinoxylan, a blank (non-enzyme treated, TFA-hydrolysed arabinoxylan) was included. Samples were analysed for the release of arabinose by HPLC.

Inhibition experiment

AXH and α-L-arabinofuranosidase B were tested for inhibition by L-arabonic acid τ-lactone, a known inhibitor for the latter enzyme. A 0.1% (w/v) wheat arabinoxylan solution was incubated with 0.1 μg/mL AXH, with 5.0 μg/mL α-L-arabinofuranosidase B, and with a combination of these two enzymes. These incubations were carried out for various times in the presence of 10mM L-arabonic acid τ-lactone. The release of arabinose was measured by the Nelson-Somogyi assay.

Synergism studies

Synergism of AXH in combination with α-L-arabinofuranosidase A and B (E.C. 3.2.1.55), endo-(1,4)-ß-xylanase III (E.C. 3.2.1.8) and (1,4)-ß-D-glucan glucanohydrolase (endo-glucanase IV; E.C. 3.2.1.4) was studied by incubating 0.25% (w/v) wheat arabinoxylan solution with 0.4 μg/mL AXH, with 5.8 μg/mL α-L-arabinofuranosidase A or B, with 0.15 μg/mL endo-(1,4)-ß-xylanase III or with 0.27 μg/mL endo-glucanase IV at 30°C in 50mM sodium acetate buffer pH 5.0, separately and in combinations. The reducing sugars released were determined by the Nelson-Somogyi assay.

Protein determination

The protein content was measured according to Sedmak and Grossberg (1977) with bovine serum albumin as standard.

Results

Several crude enzyme preparations were screened for their capability to release arabinose from water-soluble oat spelts arabinoxylan. Release of arabinose was observed in several preparations. The culture liquid of *A. awamori* CMI 142717 was rich in this activity.
During the purification of endo-(1,4)-ß-xylanases from the culture liquid of *A. awamori*, an enzyme fraction was obtained, able to split off arabinose from arabinoxylan (AXH).
The endo-(1,4)-ß-xylanase and AXH were isolated and purified according to the flowsheet diagram as presented by Figure 1. AXH and an endo-(1,4)-ß-xylanase (II) eluted with considerable overlap from the DEAE-column using the pH-gradient. The part of AXH which eluted prior to endo-(1,4)-ß-xylanase II could be further purified in one step on a Mono Q™ column.

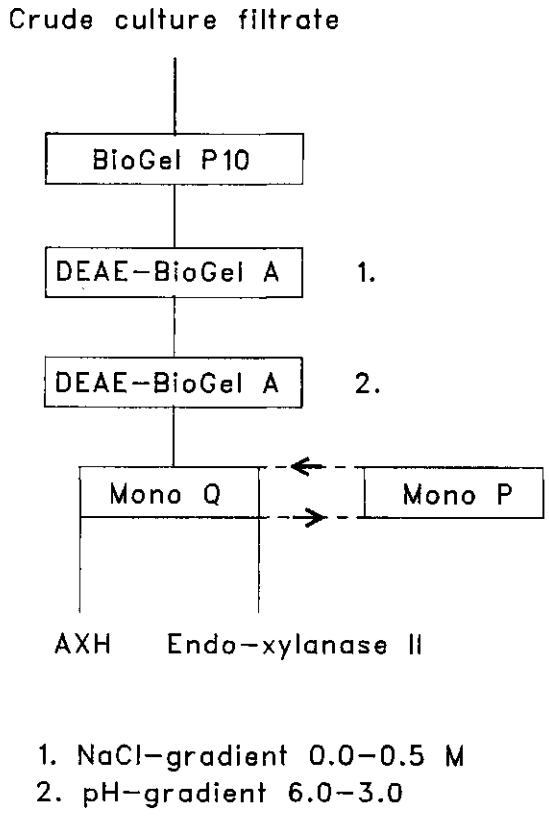

Figure 1. Purification-scheme of AXH; Crude culture filtrate was applied on to a Bio-Gel P10 column. The main endo-xylanase active fractions were applied on to a DEAE-Bio-Gel A column and eluted by a NaCl-gradient in the first case and by a pH-gradient in the second case. The AXH fractions (eluting prior to the endo-xylanase fractions) were applied on to a Mono Q™ column and eluted by a sodium chloride gradient. The endo-(1,4)-ß-xylanase II containing fractions obtained after the pH-gradient, containing AXH activity also, were applied subsequently on to a Mono Q™, a Mono P™ and a Mono Q™ column.

The protein concentrations in these fractions were so low that it hardly can be detected. The AXH which co-eluted with endo-(1,4)-ß-xylanase II could be obtained by fractionation on subsequently a Mono Q™column (sodium-chloride gradient), a Mono P™ column and a Mono Q™ column by FPLC.

AXH was characterized for substrate specificity and physico-chemical parameters (Table I). The enzyme had a specific activity towards alkali-extractable arabinoxylan of 22 U/mg, an optimal activity at pH 5.0 and 50°C, and a molecular weight of 32,000 Da.

AXH releases arabinose only from arabinoxylan and not from other arabinose-containing substrates such as sugar beet arabinan which has an α-(1,5)-linked arabinan backbone with

Table I. Characteristics and kinetic parameters for AXH from *A. awamori*.

Characteristic	AXH
Specific activity	
Wheat alkali-extractable arabinoxylan	22.0 U/mg
M_r	32 kDa
pH-optimum	5.0
Temperature-optimum	± 50°C

Table II. Temperature and pH-stability of AXH. AXH was pre-incubated for a set time at various temperatures and pHs. After the pre-incubation, 0.4 μg/mL of pre-incubated enzyme was added to 0.1% (w/v) wheat arabinoxylan and incubated for 2.5 hours at pH 5.0 and 30°C. The release of arabinose (mg/mL) was determined by HPLC analysis.

Pre-Incubation in 50mM solutions of:	Temp. (°C)	pH	Time of pre-incubation (h) 0.0	0.5	1.0	20.0
Acetic acid						
	30	3.25	0.117	0.143	0.150	0.149
	42	3.33		0.136	0.123	0.095
	50	3.37		0.121	0.060	0.011
Sodium acetate buffer						
	30	5.00	0.136	0.143	0.158	0.168
	42	5.06		0.147	0.137	0.154
	50	5.10		0.140	0.139	0.154
Sodium acetate						
	30	7.25	0.139	0.148	0.161	0.149
	42	7.60		0.151	0.151	0.154
	50	7.57		0.155	0.138	0.038

α-(1,2) and α-(1,3)-linked single unit arabinofuranose substituents, MHR which has a rhamnogalacturonan backbone branched with α-(1,3/5)-linked arabinan chains and ß-(1,4)-linked galactan chains, arabinogalactans which have a ß-(1,4)-D-galactan backbone branched with α-(1,3/5)-linked arabinan chains, or stractan which has a ß-(1,3/6)-D-galactan backbone carrying arabinose side chains. No activity was found on CMC, H_3PO_4-swollen cellulose or Avicel. The enzyme did show some activity on PNP-ß-glucopyranoside and PNP-α-glucopyranoside but this was apparent only after prolonged incubation for 24 hours. Since the purified enzyme was highly specific for arabinoxylans, we have named it a (1,4)-ß-D-arabinoxylan arabinofuranohydrolase (AXH).

The apparent pH and temperature optima must be considered in relation to the pH and temperature stability data presented in Table II. As can be seen from this table, AXH is very stable in a 50mM sodium acetate buffer pH 5.0 at 30°, 42° and 50°C. At a high pH the stability of AXH decreased slowly with increasing temperature and pre-incubation time. However, at a low pH the stability of AXH decreases dramatically.

The pattern of action of the purified enzyme was determined by incubating 0.1% (w/v) water-soluble arabinoxylan ex oat spelts with AXH for 1 and 24 h at 30°C in 50mM sodium acetate buffer pH 5.0. Analysis of these reaction mixtures by HPSEC (Fig. 2.A) show no shift in the molecular weight distribution of the polymeric arabinoxylan. The figure, however, shows a decrease in intensity of the main peak at 25 min. As can be seen from Figure 2.B, only arabinose is released by AXH and no xylose or xylose oligomers could be found, even after 24 h of incubation.

The release of arabinose from the alkali-extractable wheat arabinoxylan by AXH over a 23 h incubation period is shown in Figure 3. It can be seen that after 23 hours of incubation 31-33% of all the arabinose had been released from the arabinoxylan. Addition of fresh enzyme did not increase the amount of arabinose released significantly. The reaction rate was linear in the initial stages of hydrolysis and from this rate a specific activity of 22 U/mg enzyme was calculated; α-L-arabinofuranosidase B, which is also able to release arabinose from arabinoxylan, had a specific activity of only 0.9 U/mg. α-L-Arabinofuranosidase B released 40% of the arabinosyl side-chains from wheat arabinoxylan, but to obtain this result a very high concentration of enzyme was needed.

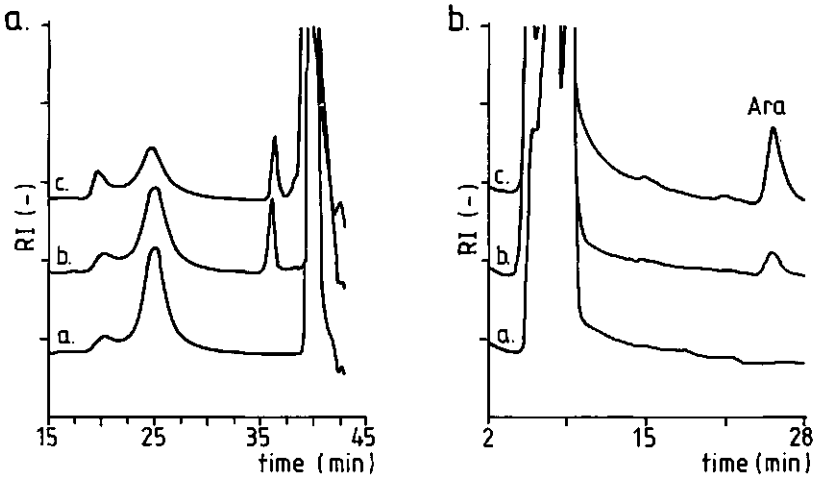

Figure 2. HPLC analysis of the incubation mixtures of arabinoxylan ex oat spelts with AXH; 1.a Analysis of the molecular weight distribution by Biogel TSK 40+30+20 XL, 1.b Analysis of the neutral sugars released by AXH by a CH-Pb column; a. blank arabinoxylan, b. arabinoxylan treated for 1 h with AXH, c. arabinoxylan treated for 24 hours with AXH.

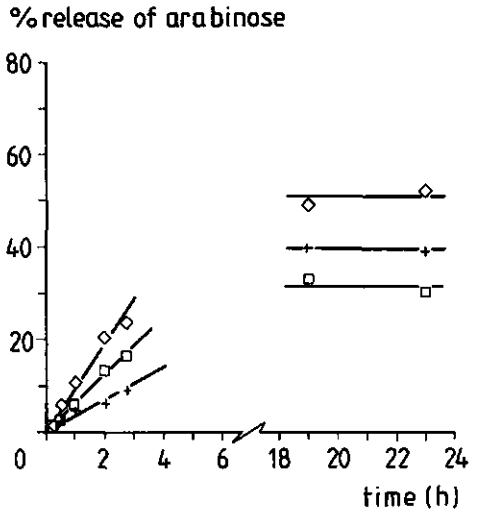

Figure 3. Time curve for AXH and α-L-arabinofuranosidase B. 0.25% (w/v) Wheat arabinoxylan was incubated with 5.8 μg/mL α-arabinofuranosidase B, with 0.4 μg/mL AXH, and with a combination of these two, for 0.25, 0.50, 1.0, 2.0, 2.75, 19.0 and 23.0 hours. Enzyme activities were analysed by the Nelson-Somogyi assay; the amount of arabinose released is expressed in % of the total amount of arabinose linked to the xylan backbone;□, AXH; +, arabinofuranosidase B; ◇ AXH + arabinofuranosidase B.

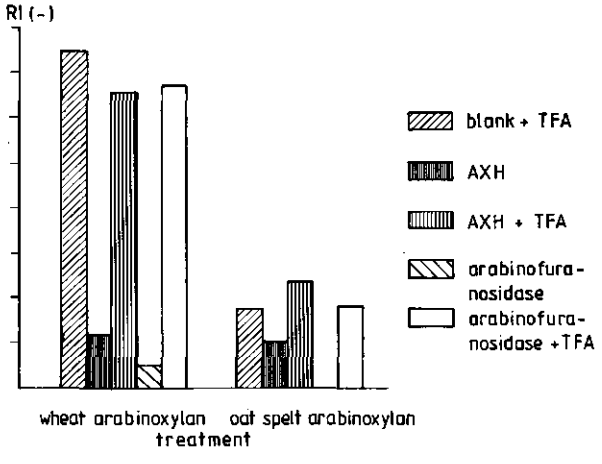

Figure 4. Arabinose content of water-soluble wheat arabinoxylan and water-soluble oat spelts arabinoxylan determined by HPLC after enzymic and chemical treatment with AXH or α-L-arabinofuranosidase B, and TFA (0.1M at 100°C), respectively.

A mixture of AXH with α-L-arabinofuranosidase B resulted in a 52% release of the arabinosyl side-chains after 23 hours. The result (Fig. 3) suggests a difference in the mode of action or affinity of AXH and α-L-arabinofuranosidase B on wheat arabinoxylan. However, as the amount of arabinose released by the combined enzymes was not greater than the summation of the amounts of arabinose released by the two enzymes separately, it was clear that there was no synergistic effect between the two enzymes.

Acid hydrolysis with trifluoroacetic acid was used to determine the total substitution with arabinose. A TFA treatment was carried out before and/or after incubation of arabinoxylan with AXH from *A. awamori* to determine the ultimate amount of arabinose which could be released by the enzyme. Figure 4 shows the amount of arabinose released from the water-soluble wheat arabinoxylan and water-soluble oat spelts arabinoxylan after treatment with enzymes, TFA, and their combinations. It can be seen that AXH released 17.7% and 42.7%, respectively of all arabinosyl substituents in 24 h; α-L-arabinofuranosidase B released only 7.5% and 0.0%, respectively.

The release of arabinose over a period of time from alkali-extractable wheat arabinoxylan was studied using AXH or α-L-arabinofuranosidase B, or combinations of these enzymes in the presence of L-arabonic acid γ-lactone, which is a specific inhibitor of the α-L-arabinofuranosidase. The α-L-arabinofuranosidase B was totally inhibited by the L-arabonic acid γ-lactone; AXH, however, was only slightly inhibited in its arabinose-releasing activity.

The action of AXH was also studied in combination with α-L-arabinofuranosidase A and B, endo-(1,4)-ß-xylanase III and (1,4)-ß-D-glucan glucanohydrolase to test possible synergistic action. Wheat arabinoxylan, which had been extracted with alkali was the substrate.

Table III shows the activities of the combinations of the enzymes, expressed in % of the summations of the activities of the single enzymes which are set at 100%. Table III.A shows that no synergistic effect was observed between AXH and α-L-arabinofuranosidase B, but that some synergism appears to exist between α-L-arabinofuranosidase B and endo-glucanase IV, which is a non-specific cellulase also active on arabinoxylan (Beldman *et al*, 1985). AXH, like α-L-arabinofuranosidase B, showed a strong synergistic effect with the endo-(1,4)-ß-xylanase III.

The effect of the sequential action of AXH and the other xylan-degrading enzymes mentioned above was also tested (Table III.B). In the case of α-L-arabinofuranosidase A, the synergistic effect was clearly present. α-L-Arabinofuranosidase A is able to release arabinose from arabinoxylan after partial degradation of the arabinoxylan by specific or non-specific xylanases. In comparison to the concerted actions, α-L-arabinofuranosidase B or AXH show a less strong synergistic action with endo-(1,4)-ß-xylanase III.

Table III.A. Combined treatment (1 h) of wheat arabinoxylan with endo-(1,4)-ß-xylanase III, endo-glucanase IV, α-arabinofuranosidase B and AXH tested for degradation by the Nelson-Somogyi assay (activities expressed in % of the summations of the activities of the single enzymes which are set at 100%).

Enzyme	AXH	α-arabinofu-ranosidase	Endo-xyla-nase III	Endo-glu-canase IV
+AXH	100	98	133	105
+α-Arafur. B		88	142	121
+Endo-xyl. III			100	86
+Endo-glu. IV				69

Table III.B. Sequential treatment (2 x 0.75 h) of wheat arabinoxylan with endo-(1,4)-ß-xylanase III, endo-glucanase IV, α-arabinofuranosidase B and AXH tested for degradation by the Nelson-Somogyi assay (activities expressed in % of the summations of the activities of the single enzymes which are set at 100%).

Enzyme	First incubation: AXH	α-arabinofu-ranosidase B	Endo-xyla-nase III	Endo-glu-canase IV
Second incubation:				
No enzyme	100	100	100	100
+AXH	110	103	113	120
+α-Arafur. A	N.D.	N.D.	129	142
+α-Arafur. B	98	98	113	130
+Endo-xyl. III	118	115	69	87
+Endo-glu. IV	107	106	89	87

N.D. = Not Determined

Discussion

The results presented in this chapter show clearly that AXH is an enzyme able to split off the arabinose from arabinoxylans without the formation of xylose oligomers.
The values presented here, however, indicate that AXH can only partly release the arabinosyl side-chains. Other workers have reported significant differences in the amount of arabinose released from arabinoxylans using purified enzymes. Thus Andrewartha *et al* (1979), Poutanen (1988) and Kaji and Tagawa (1970) found a release of 18%, 50% and almost 100%, respectively of arabinosyl groups from wheat arabinoxylan. There are various reasons for these differences. For example the molecular heterogeneity of the

arabinoxylan (arabinofuranosyl groups can be glycosidically linked to the *O*-2 and/or *O*-3 of xylopyranosyl residues), the extent of branching (single-substituted or double substituted) or the density of branching (smooth and hairy parts) would be expected to cause variations in the degree of degradation. Prolonged incubation of arabinoxylan with arabinose-releasing enzymes has been shown to result in a progressive loss of arabinose substituents. This progressive loss causes intermolecular aggregation resulting in an increased insolubility of the (arabino)xylan (Andrewartha *et al*, 1979; Brillouet and Moulin, 1985). Incubation of wheat arabinoxylan with our AXH resulted in a haze which precipitated on prolonged incubation. The removal of arabinose by AXH causes aggregation and increases the insolubility of (arabino-)xylan. The increase of the peak at 20 min (Fig. 2.A) is probably the result of this aggregation, which apparently elutes at a higher molecular weight. Similar results were found with α-L-arabinofuranosidases (Andrewartha *et al*, 1979; Kaji and Tagawa, 1970) and endo-(1,4)-ß-xylanases (Dekker and Richards, 1975) on wheat arabinoxylan.

AXH, like α-L-arabinofuranosidase B, showed a strong synergistic effect with endo-(1,4)-ß-xylanase III. These results indicate therefore that a combination of these enzymes enhances the degradation of arabinoxylan. This may suggest that endo-(1,4)-ß-xylanase has a higher affinity towards linearized (arabino-)xylan, or AXH has a higher affinity towards arabino-xylo-oligomers.

Synergism between an α-L-arabinofuranosidase and an endo-(1,4)-ß-xylanase has also been demonstrated on alfalfa cell walls by Greve *et al* (1984), and on wheat straw arabinoxylan by Poutanen (1988).

As with concerted action of the other enzymes, synergism in the release of reducing sugars during sequential action on arabinoxylan was apparent but its degree of synergistic activity observed was much smaller.

AXH was highly specific for arabinoxylans and was not able to cleave any α-(1,2)/α-(1,3)/α-(1,5) glycosidically linked arabinose from arabinans, arabinogalactans or PNP-arabinofuranoside. AXH was only slightly inhibited in its arabinose-releasing activity by L-arabonic acid τ-lactone. These properties show that AXH cannot be classified within the *A. niger* type or *Streptomyces purpurascens* type of α-L-arabinofuranosidase (Kaji, 1984). AXH is able to release arabinose from the arabinoxylan without degrading the backbone into xylose oligomers and this property distinguishes the enzyme from the arabinose-releasing endo-(1,4)-ß-xylanases previously reported. The existence of this enzyme raises the possibility that those endo-(1,4)-ß-xylanases described in literature having two catalytic activities may in fact be contaminated with an AXH-type enzyme.

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Chapter 7

Mode of action of (1,4)-ß-D-arabinoxylan arabinofuranohydrolase (AXH) and α-L-arabinofuranosidases on alkali-extractable wheat-flour arabinoxylan

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Submitted for publication.

Summary

Arabinoxylan-derived oligosaccharides were treated with (1,4)-ß-D-arabinoxylan arabinofuranohydrolase (AXH) and two types of α-L-arabinofuranosidase. Analysis of reaction products by high performance anion-exchange chromatography indicated the removal of arabinofuranosyl substituents from single substituted xylopyranosyl residues. In addition, differences in the specificity of these enzymes towards the various differently substituted oligosaccharides were observed.

^{1}H-N.m.r. spectroscopy and methylation analysis of alkali-extractable wheat-flour arabinoxylan treated with AXH, confirmed the specificity of AXH towards C-3 linked arabinofuranosyl substituents from single substituted xylopyranosyl residues. With these techniques α-L-arabinofuranosidase B was found to cause minor changes in C-2/C-3 linked arabinofuranosyl substituents from double substituted xylopyranosyl residues.

Introduction

Endo-(1,4)-ß-D-xylanases and α-L-arabinofuranosidases have been described which were able to split the α-(1,3)-linkages between arabinofuranosyl and xylopyranosyl residues from arabinoxylans[1,2]. However, no detailed studies have been carried out on the specificity towards the various arabinofuranosyl substituents in arabinoxylan and arabinoxylan-derived oligosaccharides.

In earlier papers we reported on the purification and characterization of a (1,4)-ß-D-arabinoxylan arabinofuranohydrolase (AXH) from *Aspergillus awamori*[3] as well as α-L-arabinofuranosidase A and B from *Aspergillus niger*[4] (Arafur A and Arafur B, respectively). AXH was found to release α-(1,3)-linked arabinofuranosyl substituents from arabinoxylans only and not from other arabinose-containing substrates such as sugar beet arabinan, arabinan-rich apple pectin fragments (MHR)[5], arabinogalactans and stractan[3]. In all these cases, arabinofuranosyl substituents were α-(1,2), α-(1,3) or α-(1,5)-linked. Arafur A was only active towards low oligomeric arabinofuranosides. Arafur B was preferentially active towards L-arabinofuranosyl substituents of arabinan, arabinogalactan, and arabinoxylan[4].

In this chapter specific data are presented concerning the mode of action of AXH, Arafur A and Arafur B towards arabinoxylan and arabinoxylan-derived oligosaccharides.

Materials and methods

Materials

The preparation and characterization of alkali-extractable wheat-flour arabinoxylan (BE-1U) is described elsewhere[6]. Arabinoxylan-derived oligosaccharides were purified by Gruppen *et al*[7]. The tetrameric fragment 2^3-α-Ara*f*-Xyl_3 was isolated from a barley arabinoxylan digest[8]. (1,4)-ß-D-Arabinoxylan arabinofuranohydrolase (AXH) and endo-(1,4)-ß-D-xylanase III (Endo III) were purified from *Aspergillus awamori* CMI 142717 by Kormelink *et al*[3,9]. α-L-Arabinofuranosidase A and B (Arafur A and Arafur B, respectively) were purified from *Aspergillus niger* by Rombouts *et al*[4].

Degradation of arabinoxylan-derived oligosaccharides

Arabinoxylan-derived oligosaccharides (5-10 μg) were dissolved in sodium acetate buffer (50mM, pH 5.0) and incubated with AXH (1.0 μg/mL), Arafur A (1.0 μg/mL), or Arafur B (1.0 μg/mL) for 6 h at 30°C. After incubation, enzymes were inactivated at 100°C for 10 min.

High performance anion-exchange chromatography (h.p.a.e.c.)

Enzyme digests were analysed by h.p.a.e.c. using a Dionex Bio-LC GPM-II quaternary gradient module (Dionex Corporation, Sunnyvale, CA, USA) equipped with a Dionex

CarboPac PA-1 column (250 x 4 mm) and a Dionex PED detector in the pulsed amperometric detection (p.a.d.) mode, as described by Gruppen *et al*[7]. Elution (1 mL/min) involved linear gradients of sodium acetate in 0.1M NaOH of 0-100mM during 5 min, then of 100-400mM during 35 min at 20°.

^{1}H-N.m.r. spectroscopy

In order to enable n.m.r. spectroscopy the viscosity of alkali-extractable wheat-flour arabinoxylan was reduced by dissolving 60 mg in 50mM sodium acetate (12 mL, pH 5.0) and subsequently treated with Endo III (0.25 mU/mL) for 80 minutes at 30°C, followed by enzyme inactivation (10 min, 100°C). After enzyme action the apparent molecular weight, as determined by high performance size exclusion chromatography[9], was 50,000-55,000. Fractions containing 10 mg of the partially degraded arabinoxylan were incubated with AXH (1 μg/mL) and/or Arafur B (5 μg/mL) for 8 h at 30°C. After inactivation at 100°C for 10 minutes, samples were dialysed extensively against distilled water and the retentates were freeze dried. Samples were analysed by a 600MHz Bruker AM 600 spectrometer as described previously[10]. A decrease in the intensity of the H-1 signals representing terminal α-(1,2)- and/or α-(1,3)-linked arabinofuranosyl substituents was studied qualitatively by comparing these signals with the intensity of the signal at 4.50 p.p.m..

Methylation analysis

Methylation was carried out according to a modified Hakamori method[11]. After methylation, samples were dialysed and dried in a stream of air. The methylated samples were subsequently hydrolysed using 2M trifluoroacetic acid (121°C, 1h) and derivatised[12]. The methylated alditol acetates were analysed on a fused silica capillary column (30m x 0.32mm; wall coated with DB1701; 0.25μm) using a Carlo Erba Fractovap 4160 GC equipped with a FID. Glycosidic linkage composition was calculated using effective carbon response (E.C.R.) factors[13]. The identity of compounds was confirmed by GC-MS (Hewlett Packard, MSD 5970-B coupled to a HP 5890), equipped with a fused silica column (CPSIL 19CB, 26m x 0.22mm; 0.18μm). As 2-*O*- and 3-*O*-methylated xylitol acetates co-eluted, their relative amounts were calculated from the relative abundance of the ions at m/z 117 and m/z 129.

Protein determination

The protein content was determined according to Sedmak and Grossberg[14] using bovine serum albumin as standard.

Results and Discussion

In Table I the specificity of AXH, Arafur A and Arafur B towards arabinoxylan

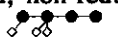
oligosaccharides, together with previous published[3,4] data on polymeric arabinoxylan is given. The results showed remarkable similarities as well as differences in their specificity towards oligomeric and polymeric arabinoxylans with different arabinofuranosyl substitution (Table I). AXH and Arafur B showed activity on arabinoxylan as well as arabinoxylan-derived oligosaccharides. Arafur A, however, only seemed to be active on arabinoxylan-derived oligosaccharides.

Table I. Enzyme specificity of AXH, Arafur A and Arafur B towards polymer and oligomer substrate.

Enzyme	Substrate	
	Arabinoxylan	Arabinoxylan oligomers
AXH[1]	+[3]	+
Arafur A[2]	-	+
Arafur B[2]	+	+

[1] Kormelink *et al*, 1991[3].
[2] Rombouts *et al*, 1988[4].
[3] + active, - not active.

In order to study the differences in specificity towards differently located arabinofuranosyl substituents, arabinoxylan-derived oligosaccharides of known structure were incubated with AXH, Arafur A and Arafur B, and analysed by h.p.a.e.c.. The results are summarized in Table II. As can be seen arabinofuranosyl substituents were only released from single substituted xylopyranosyl residues by AXH regardless whether the substituted xylopyranosyl residue is in a terminal or a non-terminal position. AXH was also found to be able to split the α-(1,2)-linked arabinofuranosyl substituents from 2^3-α-Ara*f*-Xyl_3 (ref. 8).

Arafur A, which acted only on low M_w arabinoxylans (Table I), could split off all α-(1,3)-linked arabinofuranosyl substituents from single substituted xylopyranosyl residues, and therefore acts similarly as AXH. Arafur B on the other hand could only split off arabinofuranosyl substituents from single substituted, non-reducing terminal xylopyranosyl residues (Table II: structures  , and ). An unsubstituted, non-reducing terminal xylopyranosyl residue hinders Arafur B activity.

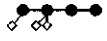
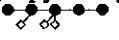
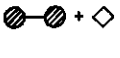
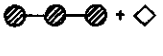
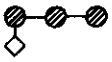
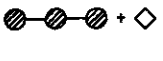
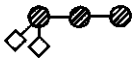
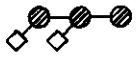
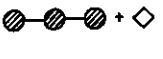
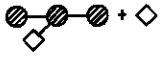
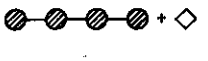
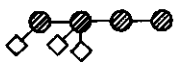
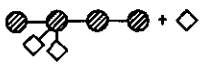
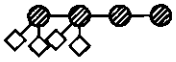
In most cases no complete debranching of arabinoxylan-derived oligosaccharides was observed, because of the presence of double substituted xylopyranosyl residues. However, double substituted xylopyranosyl residues do not inhibit the removal of arabinofuranosyl substituents from the adjacent, single substituted xylopyranosyl residues by AXH, Arafur A or Arafur B (Table II: structures  and ).

Table II. Structure of arabinoxylan-derived oligosaccharides and their degradation products by AXH, Arafur A and Arafur B.

Structure[a]	Enzyme	Product
	AXH Arafur A Arafur B	
	AXH Arafur A	
	Arafur B AXH	N.D.[b] 
	Arafur A Arafur B AXH Arafur A Arafur B	n.d.[c] n.d. N.D. N.D. N.D.
	AXH Arafur A	
	Arafur B	
	AXH Arafur A Arafur B	N.D. N.D. N.D.
	AXH Arafur A	
	Arafur B AXH Arafur A Arafur B	N.D. 

Structure[a]	Enzyme	Product
	AXH ArafurA	
	Arafur B AXH Arafur A Arafur B	N.D. N.D. N.D. N.D.
	AXH Arafur A Arafur B	N.D. N.D. N.D.
	AXH Arafur A Arafur B	N.D. N.D. N.D.

[a] Structures derived from Gruppen *et al*[7].
[b] no degradation.
[c] not determined.

^{1}H-n.m.r. spectroscopy of the endo-xylanase pre-treated arabinoxylan, showed H-1 signals of terminal α-L-arabinofuranosyl substituents at 5.23, 5.28, and 5.39 p.p.m. (Fig. 1). The signal at 5.39 p.p.m. was assigned to anomeric protons of terminal arabinofuranosyl substituents linked to *O*-3 of single substituted xylopyranosyl residues. The signals at 5.23 and 5.28 p.p.m. were assigned to anomeric protons of terminal arabinofuranosyl substituents linked to *O*-2 and *O*-3 of double substituted xylopyranosyl residues[15-17].

Incubation with AXH removed most of the α-(1,3)-linked arabinofuranosyl substituents from single substituted xylopyranosyl residues, as indicated by the almost complete disappearance of the ^{1}H-n.m.r. signal at 5.39 p.p.m. (Fig. 2).

No significant decrease in the two signals at 5.23 and 5.28 p.p.m. was observed. Incubation with Arafur B showed only a small decrease in the intensity of the two signals at 5.23 and 5.28 p.p.m. (Fig. 3), which could indicate a preference for α-(1,3)/α-(1,2)-linked arabinofuranosyl substituents of double substituted xylopyranosyl residues.

A combination of AXH and Arafur B released α-L-arabinofuranosyl substituents from single substituted xylopyranosyl residues as well as from double substituted xylopyranosyl residues, as indicated by a decrease in the intensity of all H-1 signals of α-L-arabinofuranosyl substituents (Fig. 4).

A difference in the mode of debranching of arabinoxylan by AXH and Arafur B was reflected also in the formation of a haze. By the removal of terminal α-(1,3)-linked arabinofuranosyl substituents from single substituted xylopyranosyl residues, a precipitate

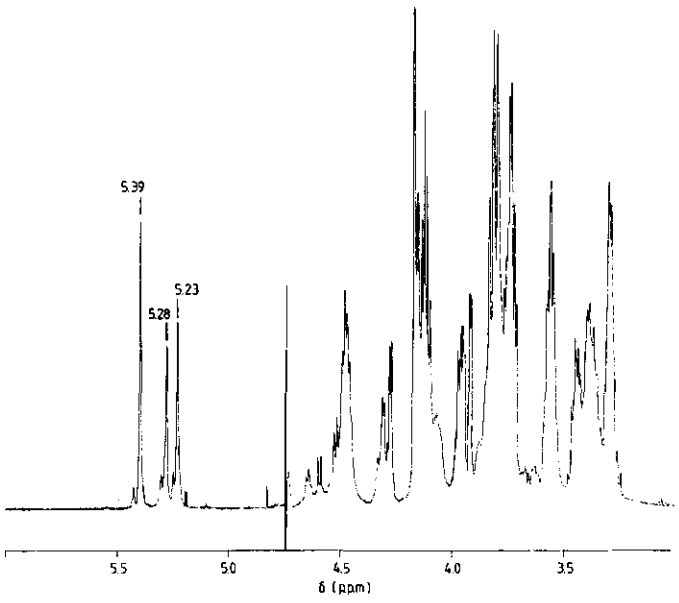


Figure 1. 600.1MHz ^{1}H-N.m.r. spectrum of endo-xylanase-III-pretreated wheat alkali-extractable arabinoxylan (blank).

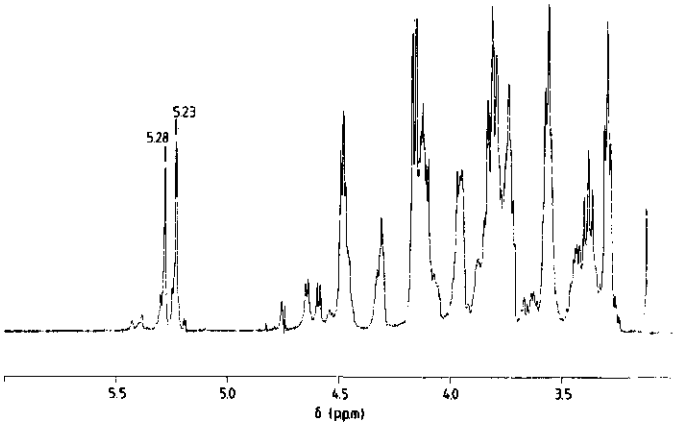


Figure 2. 600.1MHz ^{1}H-N.m.r. spectrum of endo-xylanase-III-pretreated wheat alkali-extractable arabinoxylan after incubation with AXH.

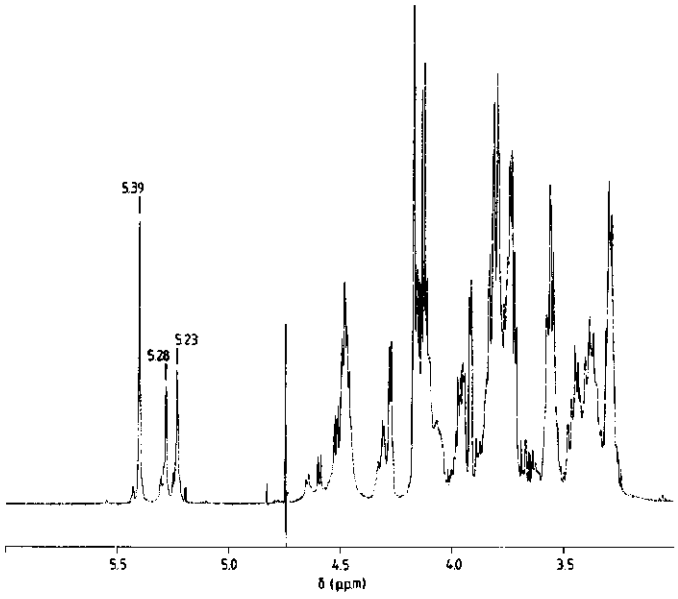


Figure 3. 600.1MHz ^{1}H-N.m.r. spectrum of endo-xylanase-III-pretreated wheat alkali-extractable arabinoxylan after incubation with α-L-arabinofuranosidase B.

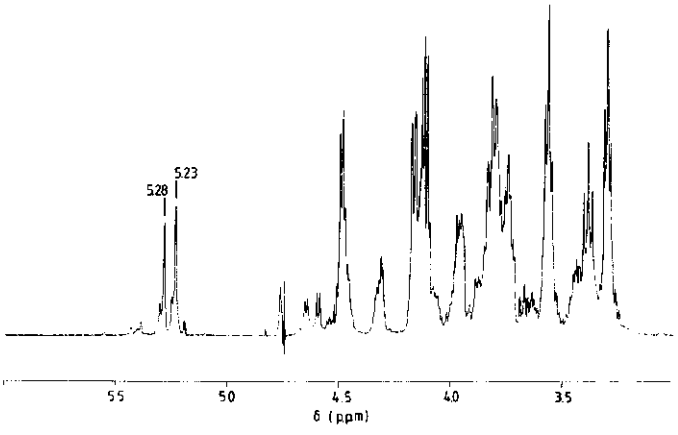


Figure 4. 600.1MHz ^{1}H-N.m.r. spectrum of endo-xylanase-III-pretreated wheat alkali-extractable arabinoxylan after incubation with AXH and α-L-arabinofuranosidase B.

was formed on prolonged incubation with AXH, which was, however, not the case for Arafur B. The formation of a precipitate after incubation of arabinoxylan with arabinose-releasing enzymes, was reported earlier[18,19] and ascribed to aggregation of partly linearised (arabino)xylan chains.

Table III. Glycosidic linkage analysis of alkali-extractable wheat-flour arabinoxylan before and after treatment with AXH, α-L-arabinofuranosidase B, or combinations of both.

Glycosidic linkage	Relative proportions (mole%)[a]							
	Control		AXH		Arafur B		AXH + Arafur B	
2,3,5-Me_3-Ara*f*	33.9		25.5		27.3		21.7	
3,5-Me_2-Ara*f*	0.7		1.4		1.3		1.3	
2,3-Me_2-Ara*f*	3.7		2.2		5.4		1.5	
2,5-Me_2-Ara*f*	0.4		0.4		5.3		0.2	
2,3,4-Me_3-Xyl*p*	2.0	(3.3)[b]	2.9	(4.1)	4.4	(7.3)	4.5	(6.0)
2,3-Me_2-Xyl*p*	39.4	(64.2)	52.8	(74.9)	40.2	(66.1)	57.4	(76.2)
3-Me -Xyl*p*	1.2	(2.0)	0.3	(0.4)	0.7	(1.2)	0.3	(0.4)
2-Me -Xyl*p*	9.7	(15.8)	1.6	(2.3)	9.8	(16.1)	1.5	(2.0)
Xyl*p*	9.1	(14.8)	12.9	(18.3)	5.7	(9.3)	11.6	(15.4)
Ratio terminal/ branching	1.16		0.92		1.25		0.87	

[a] mole% of total arabinofuranose and xylopyranose residues
[b] data between parentheses represent mole% of total xylopyranosyl residues

The results from the methylation analysis are shown in Table III. Incubation with AXH removed most of the arabinofuranosyl substituents from single substituted xylopyranosyl residues, as indicated by a decrease in mole% of single substituted xylopyranose which resulted in an increase of the amount of 2,3-Me_2-Xyl*p* from 39.4 to 52.8 mole%. No decrease in double substituted xylopyranosyl residues was observed.
The data presented here, also suggest that AXH can not cleave all the Ara*f*-α-(1,2)-Xyl*p* and Ara*f*-α-(1,3)-Xyl*p* linkages. 84% Of the 3-*O*-substituted xylopyranose was converted into unsubstituted xylopyranose. However, by comparing the values for unmethylated Xyl*p* before and after enzyme-treatment, an increase could be observed, which was even larger by comparing absolute numbers of methylated xylopyranosyl residues (shown between parentheses). These findings may be partly explained by differences in the extent of methylation.
All these data, however, indicate a preference of AXH for α-(1,2) and α-(1,3)-linked arabinofuranosyl substituents from single substituted xylopyranosyl residues.

From Table III it can also be seen that Arafur B released α-(1,3)/α-(1,2)-linked arabinofuranosyl substituents in only small amounts from double substituted xylopyranosyl residues. No significant amounts of α-(1,2) or α-(1,3)-linked arabinofuranosyl substituents were released from single substituted xylopyranosyl residues.
AXH in combination with Arafur B resulted even in a larger decrease of α-(1,3)-linked arabinofuranosyl substituents down to 21.7 mole% in comparison to treatment with AXH alone (25.5 mole%). The amount of unbranched (1,4)-linked xylopyranosyl residues increased up to 57.4 mole% in comparison to treatment with AXH alone (52.8 mole%). No significant changes can be observed in the mole% of 3-Me-Xyl*p*.
Despite inaccuracy in the mole% of unmethylated Xyl*p*, trends can be recognised from these data. Arafur B seems to be able to release α-(1,3)/α-(1,2)-linked arabinofuranosyl substituents from double substituted xylopyranosyl residues when comparing the data after Arafur B treatment with those from the control or by comparing the data after concerted action of AXH and Arafur B with those after AXH treatment.
Nevertheless, AXH seems to be highly active and specific towards (wheat) arabinoxylan. The amount of arabinofuranosyl substituents released from wheat arabinoxylan can be slightly increased by the concerted action of AXH with Arafur B.
The results obtained from oligosaccharide digestion agree with the findings obtained by ^{1}H-*n.m.r* spectroscopy and methylation analysis of AXH-treated arabinoxylan, but do not confirm the results obtained with Arafur B-treated arabinoxylan. Unfortunately, no data are available from other research groups.

Concluding remarks

AXH, an arabinose-releasing enzyme active on arabinoxylans only[3], was able to split off α-(1,2)-linked and α-(1,3)-linked arabinofuranosyl substituents from single substituted xylopyranosyl residues specifically. The low affinity of AXH towards double substituted xylopyranosyl residues may be explained by the need of a free hydroxyl group at the C-2 or C-3 in the enzyme-substrate complex.
AXH and Arafur A released arabinofuranosyl substituents from terminal as well as non-terminal single substituted xylopyranosyl residues in low M_w oligosaccharides, whereas Arafur B could only release arabinofuranosyl substituents from terminal single substituted xylopyranosyl residues. Arafur A only worked on arabinoxylan oligosaccharides, whereas AXH and, although to a lesser extent, Arafur B were active on both high M_w arabinoxylan as well as oligomeric arabinoxylan structures. Both aspects are important for the optimal breakdown of arabinoxylans by endo-(1,4)-ß-D-xylanases. The ability of endo-(1,4)-ß-D-xylanases to split glycosidic linkages in the main chain next to a branchpoint and thus producing oligosaccharides with terminal substituted xylopyranosyl residues, will enhance the action of AXH or α-L-arabinofuranosidases when these enzymes act together in the degradation of arabinoxylan.

Because AXH released arabinofuranosyl substituents from arabinoxylans only, and not from other arabinofuranose-containing substrates, the enzyme makes requirements towards the surroundings of the types of glycosyl sugar residues. This enzyme could therefore be assigned to the group of enzymes which need two different monosaccharides in the enzyme-substrate complex, like α-glucuronidase[20], or rhamnogalacturonase[21].

Acknowledgements

We thank Mr. Gerhard de Ruiter for performing the methylation analysis, and Gist-brocades for the 600MHz n.m.r. spectroscopy analysis.

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Chapter 8

The purification and characterization of an acetyl xylan esterase from *Aspergillus niger*

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J. Biotechnol., in press.

Summary

An acetyl esterase has been purified from an *Aspergillus niger* culture filtrate based on its activity towards *p*-nitrophenyl acetate. Its molecular weight, determined by SDS-PAGE, was 30480 Da; its iso-electric point was around 3.0-3.2. When tested on steamed birchwood xylan, acetylated apple or sugar beet pectin, only in the case of steamed birchwood xylan, acetic acid could be released by the acetyl esterase. The specific activity of acetyl esterase on steamed birchwood xylan was 32.3 U/mg, the optimum pH and optimum temperature were 5.5-6.0 and 50°C, respectively. The pH stability was very high in the range 3.0-8.0. The activity decreased strongly within 2 hours between 50°C and 55°C.

Three types of endo-xylanase and one ß-xylosidase could not degrade steamed birchwood xylan. In combination with acetyl esterase, however, they released xylose and xylooligomers like X_2, X_3 and X_4. These oligomers were not released until most of the acetyl groups had been removed by the acetyl esterase. Prolonged incubation with acetyl esterase released most of the acetyl groups from the steamed birchwood xylan.

Introduction

Esterases, important for the degradation of esterified substrates, have been localized in animal tissues, plant tissues and micro-organisms. Depending on the site of action, they can be divided into different groups. In the case of biodegradable polysaccharides, the most common ester-groups are methoxyl and acetyl. The presence of these groups emphasizes the important role of methyl esterases and acetyl esterases in the complete breakdown of these polysaccharides.

Methyl esterases, or pectin esterases, are important in the degradation of pectins (Rombouts and Pilnik, 1980). Acetyl esterases are important in the degradation of acetylated polysaccharides, like pectins and xylans (Timell, 1964 and 1965). In this study the emphasis is on acetylated (hardwood) xylan.

For complete degradation of hardwood xylan, which can be highly acetylated (Timell, 1964), an acetyl (xylan) esterase is needed (Biely, 1985 a). To study the effect of acetyl esterase on acetylated xylan, the enzyme has to be purified from any kind of xylanolytic activity. The commonly used assay for acetyl esterase (E.C. 3.1.1.6) is based on the release of *p*-nitrophenol from *p*-nitrophenyl acetate measured spectrophotometrically (Biely *et al*, 1985 b). However, acetyl esterases active on this synthetic substrate are not necessarily active on acetylated polysaccharides. The acetyl (xylan) esterase activity has therefore to be determined by the release of acetic acid from acetyl xylan preparations e.g. birchwood or beechwood.

Many characteristics have been published recently about these acetyl esterases in relation to acetylated (hardwood) xylan. Biely *et al* (1985 b, 1986) reported the presence of acetyl (xylan) esterases in fungal enzyme systems like *Trichoderma reesei* and *Schizophyllum commune*. Because of their specificity towards highly acetylated birchwood xylan, these fungal esterases were termed acetyl xylan esterases.

Three acetyl (xylan) esterases were purified from *Trichoderma reesei* by Poutanen and Sundberg (1988) and Sundberg and Poutanen (1991). Acetyl (xylan) esterases were also purified from *Aspergillus awamori* (Sundberg *et al*, 1990), *Aspergillus oryzae* (Tenkanen *et al*, 1991), and from a rumen bacterium *Fibrobacter succinogenes* S85 (McDermid *et al*, 1990).

Combinations of fungal esterase with endo-xylanase have indicated a cooperative action between these enzymes in hydrolysing acetyl xylan. However, a xylanolytic system is not always necessary for initiation of the deacetylation of acetyl xylan.

In this chapter the purification and characterization of an acetyl esterase from *Aspergillus niger* is described which is able to release most of the acetyl groups from steamed birchwood xylan without the cooperativity of a xylanolytic system.

Materials and methods

Materials

Chemicals, if not mentioned otherwise, are of analytical grade. Substrates used to determine acetyl (xylan) esterase activity are *p*-nitrophenyl acetate (Sigma Chemical Company, St. Louis, USA), and acetylated birchwood xylan kindly provided by Dr. J. Puls (Institute of Wood Chemistry and Chemical Technology of Wood, BFH, Hamburg, FRG). This acetylated xylan was obtained as a nondialyzable fraction of water-soluble polysaccharide produced by steam extraction of birchwood. It contained 70.6 % total sugars, and 10.6 % acetyl (based on dry matter).

Side activities of the acetyl esterase were measured on CM-cellulose type AF 0305 (Enka Industrial Colloids, Arnhem, The Netherlands), Avicel cellulose type SF (Serva, Heidelberg, FRG), H_3PO_4-swollen cellulose prepared according to the method described by Wood (1971), water soluble and water insoluble xylan from oat spelts obtained according to the method described by Selvendran *et al* (1985), arabinogalactan isolated from defatted onion powder according to Labavitch *et al* (1976) and further purified by precipitation in 40% ethanol (Van de Vis *et al*, 1990), an arabinan rich pectin fraction isolated from apple juice (MHR, Schols *et al*, 1990), sugar beet pectin (Lot number X 6938/61-735-0; The Copenhagen Pectin Factory Ltd., Lille Skensved, Denmark), SPS, a soluble soya bean polysaccharide kindly provided by NOVO Ferment (Basel, Switzerland), sugar beet arabinan kindly provided by British Sugar (Norwich, UK), arabinan purchased from Koch-Light Laboratories Ltd. (Colnbrook Bucks, England) and linear arabinan kindly provided by Novo Ferment (Basel, Switzerland).

Enzymes

The acetyl esterase was purified from *Aspergillus niger* DS16813 culture filtrate by a DEAE-trisacryl column (5 x 20 cm; IBF, Villeneuve-la-Garenne, France) and by a semi-preparative Waters DEAE 5 PW column (21.5 mm x 15 cm; Millipore Intertech, Bedford MA, USA). The endo-(1,4)-ß-xylanase I, II, III (E.C. 3.2.1.8) and the ß-xylosidase (E.C. 3.2.1.37) were purified by Kormelink *et al* (1992) from *Aspergillus awamori* CMI 142717.

Acetyl esterase assay

The acetyl esterase assay was carried out according to the procedure of Biely *et al* (1985 b) with *p*-nitrophenyl acetate in 0.2M phosphate buffer pH 6.5. The release of *p*-nitrophenol was followed spectrophotometrically at 400 nm in time. One unit of acetyl esterase is the amount of enzyme which releases 1 μmole *p*-nitrophenol from *p*-nitrophenyl acetate per minute at 22°C.

Acetyl xylan esterase assay

In the acetyl xylan esterase assay 100 μL of a 1% (w/v) steamed birchwood xylan solution

was incubated with 50 μL acetyl esterase (10 μg/mL) and 350 μL 20mM piperazine buffer pH 5.0 at 30°C for 1 hour. The reaction was terminated by placing the incubation mixture in a boiling water bath for 5 minutes. After inactivation, the sample was mixed with isopropanol (1:1), held at ambient temperature for 2 hours, and centrifuged. Acetic acid was determined with a Spectra Physics HPLC (San Jose, California, USA) connected to a Shodex SE-61 refractive index detector (SDK Showa Denko KK, Tokyo, Japan) by injecting the supernatant on to an Aminex HPX 87H column (Bio-Rad Laboratories, Richmond, California) and elution with 0.01N H_2SO_4 at 30°C (Voragen *et al*, 1986 a).
Analogous to this procedure, acetylated substrates like MHR, sugar beet pectin and SPS were tested for their degradability by the acetyl esterase.

Side activity
The acetyl esterase was tested for side activities by incubation of 0.1% (w/v) solutions of cellulose or hemicellulose, or 0.02% (w/v) solutions of PNP-glycopyranosides or glycofuranosides with 1 μg/mL acetyl esterase in 0.05M sodium acetate buffer pH 5.0 for 1 hour at 30°C (Beldman *et al*, 1985). The activity was measured by determining the release of reducing sugars by the Nelson-Somogyi assay (Somogyi, 1952) or by determining the release of *p*-nitrophenol spectrophotometrically at 400 nm. One unit is defined as the amount of enzyme needed to release 1 μmole of reducing endgroups/*p*-nitrophenol from its substrate per minute at 30°C.

Gel electrophoresis
The molecular weight was determined by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) on a 8-25% SDS-gradient gel purchased from Pharmacia. The low molecular weight calibration kit from Pharmacia was used as standard (BSA, 67000 Da; ovalbumine, 43000 Da; carbonic anhydrase, 30000 Da; trypsin inhibitor, 20100 Da and lysozym, 14400 Da). The iso-electric point was deduced from a titration curve on a pH 3-9 iso-electric focusing gel purchased from Pharmacia.The electrophoresis was carried out on a PhastSystem^R (Pharmacia-LKB Biotechnology, Uppsala, Sweden). Gels were stained with Coommassie Brilliant Blue.

HPLC - Neutral sugars
Neutral sugars released by the single and combined action of endo-xylanase I, II, III, ß-xylosidase and acetyl esterase on steamed birchwood xylan were determined by HPLC. Samples were pretreated with $Pb(NO_3)_2$ to remove uronic acid containing fragments and proteins, and injected on to a CH-Pb column (Merck, Darmstadt, FRG) eluted with millipore water (0.4 mL/min) at 85°C (Voragen *et al*, 1986 b). Sugars were detected by a Shodex SE-61 refractive index detector.

pH-Optimum
The optimum pH of acetyl esterase on steamed birchwood xylan was determined

according to the acetyl xylan esterase assay as described above. Only in this case a citrate-phosphate buffer in a pH range from 3.0-8.0 was used.
The pH-stability was determined by pre-incubation of the acetyl esterase in the citrate-phosphate buffer ranging from 3.0-8.0 for 24 hours. After the pre-incubation, substrate was added and incubated with 1.0 μg/mL acetyl esterase for 1 hour at 30°C.

Temperature optimum
The temperature optimum was determined by incubation of 100 μL of a 1% (w/v) steamed birchwood xylan solution with 50 μL 10 μg/mL acetyl esterase and 350 μL 20mM piperazine buffer pH 5.0 for 1 hour at different temperatures. The release of acetic acid was determined by HPLC.
The temperature stability was determined by pre-incubation of a 10 μg/mL acetyl esterase solution at 45°, 50° and 55°C for various times ranging from 0-8 hours. After the pre-incubation, activity was determined according to the acetyl xylan esterase assay.

Inhibition
The acetyl xylan esterase activity was tested for inhibition by different components by assaying its activity in the presence of 1mM $CaCl_2$, $Pb(NO_3)_2$, $AgNO_3$, KCl, $HgCl_2$, $MgSO_4$, $MnCl_2$, $NiCl_2$, $CoCl_2$, $ZnSO_4$, $BaCl_2$, $CuSO_4$, and EDTA or 0.1mM DTT. The resulting activity was expressed as a percentage of the original acetyl xylan esterase activity.

Combined action of acetyl esterase and xylan-degrading enzymes
The release of acetic acid and xylooligomers was determined by HPLC after degradation of steamed birchwood xylan by single or combined action of acetyl esterase and endo-xylanase I, endo-xylanase II, endo-xylanase III and ß-xylosidase. A 0.2% (w/v) steamed birchwood xylan solution in 20mM piperazine buffer pH 5.0 was incubated with 1.0 μg/mL acetyl esterase and 0.1 μg/mL endo-xylanase I, endo-xylanase II, endo-xylanase III or ß-xylosidase at 30°C. The degradation was followed over a time range from 0-8 hours. The reaction was terminated by placing the sample for 5 minutes in a boiling waterbath.

Protein determination
The protein content was determined according to the method of Lowry (1951) and by the method described by Sedmak and Grossberg (1977) with BSA as a standard.

Results

Acetyl esterase was purified from *Aspergillus niger* according to the flowsheet in Figure 1. After growth of the strain, the culture filtrate was centrifuged and the supernatant was concentrated through ultrafiltration. A sample of 73 mL was applied on to a DEAE-

trisacryl column, buffered with Tris-HCl 0.05M pH 7.8, and eluted with a linear gradient of 0.0-1.0M NaCl in the same buffer. Fractions containing acetyl esterase activity were pooled and applied on to a semi-preparative DEAE 5 PW column using HPLC. The column was equilibrated with phosphate buffer 0.05M pH 7.5, and eluted with a linear gradient of 0.0-1.0M NaCl in the same buffer. The second purification step was carried out two times.

The purification scheme is shown in Table I, a 17-fold purification of the acetyl esterase was obtained. The original sample, some intermediate fractions of the acetyl esterase peak after the first step, and the final sample were compared for purity on 8-25% SDS-gel. As can be seen from Figure 2, the final sample shows one single band on gel representing a molecular weight of 30,480 Da.

Most esterases from other sources showed higher molecular weights determined with SDS-PAGE; 66000 Da for an esterase of *Butyrivibrio fibrisolvens* (Lanz and Williams, 1973), 45000 Da for an acetyl esterase from *Trichoderma reesei* (Poutanen and Sundberg, 1988), and 34000 Da for two other acetyl xylan esterases also from *Trichoderma reesei* (Sundberg and Poutanen, 1991). Acetyl esterases from *Aspergillus* species had molecular weights of 50000 Da (Sundberg *et al*, 1990), and 30000 Da (Tenkanen *et al*, 1991). An acetyl esterase from *Fibrobacter succinogenes* S85 (McDermid *et al*, 1990) had a molecular weight of 55000 Da.

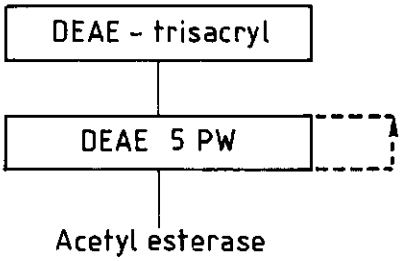

Figure 1. Flowsheet of the purification of acetyl esterase from *Aspergillus niger*.

Table I. Purification-scheme of the acetyl esterase.

Step	Protein content[1] (mg/mL)	Activity (U/mL)	Spec. activity (U/mg)	Purity
Original sample	30	55	1.8	1.0
DEAE-tris-acryl	13.4	260	19.4	10.6
DEAE 5 PW	5.7	180	31.6	17.2

[1] Lowry (1971)

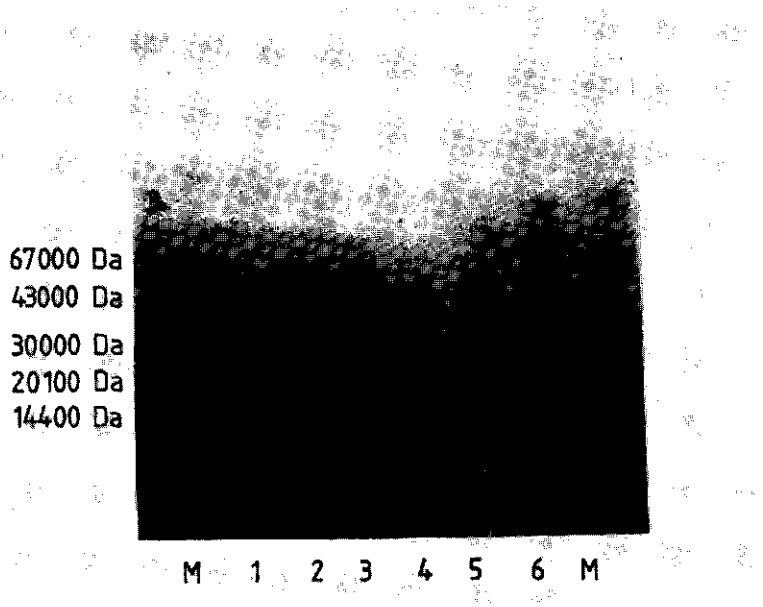

Figure 2. SDS-gel electrophoresis of *Aspergillus niger* acetyl xylan esterase (purified enzyme: lane 1, intermediate fractions: lane 2-5, and starting sample: lane 6). Markers lane M.

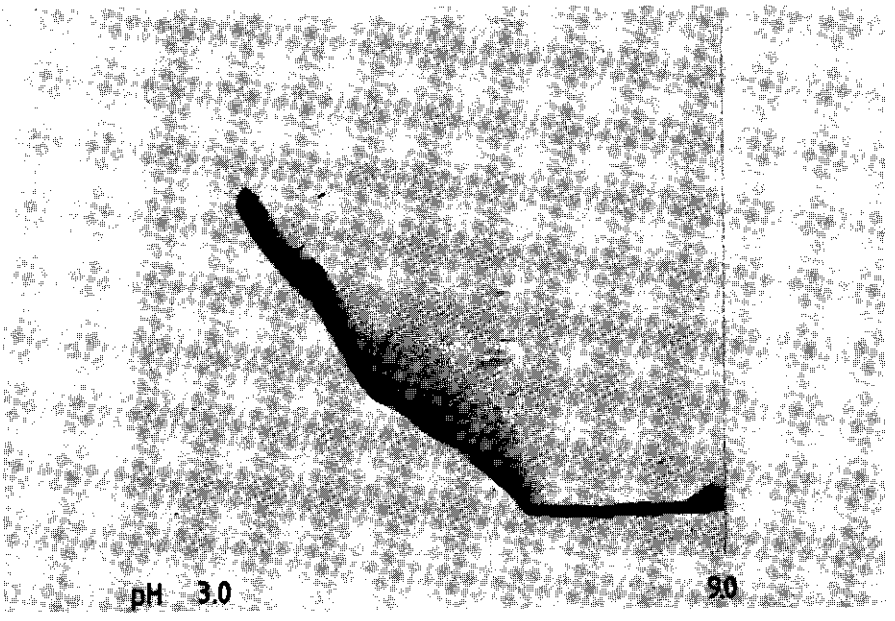

Figure 3. Titration curve of purified acetyl xylan esterase on iso-electric focusing gel (pH 3-9).

Side activities were measured on different substrates after 1 and 24 h (results not shown). Some side activity could be detected after 1 h on PNP-α-arabinofuranoside (3.8 % of the acetyl xylan esterase activity). After 24 h of incubation, small traces of activity could be detected on CMC (1.4 %), water-soluble arabinoxylan (3.6 %), araban (1.0 %) and PNP-ß-xylopyranoside (0.7 %).

A titration curve by iso-electric focusing did not reveal the presence of iso-enzymes (Fig. 3). The iso-electric point was 3.0-3.2, which is close to the iso-electric point of the acetyl esterase from *Aspergillus awamori* (3.7-3.8, Sundberg *et al*, 1990), *Aspergillus oryzae* (3.6, Tenkanen *et al*, 1991), *Fibrobacter succinogenes* S85 (4.0, McDermid *et al*, 1990), and *Schizophyllum commune* (3.4, Biely *et al*, 1987, 1988 a and b). High iso-electric points were found for acetyl esterases from *Trichoderma reesei*, 6.8 and 6.0 (Poutanen and Sundberg, 1988), and 7.0 and 6.8 (Sundberg and Poutanen, 1991), both two iso-enzymes.

The purified acetyl esterase was assayed on different acetylated substrates to test for its substrate specificity. The incubations were carried out for 1 and 24 h at pH 5.0 as well as for 1 h at different pH's. Acetic acid could only be released from the steamed birchwood xylan and not from MHR, SPS or sugar beet pectin, even after 24 hours of incubation (results not shown). Also in the case of different pH's, acetic acid could only be released from steamed birchwood xylan. From these results it can be concluded that this esterase is specific for xylan. The enzyme was not tested for activity on aliphatic substrates.

The commercial available acetyl esterase from citrus (orange peel; Sigma) was also tested on steamed birchwood xylan, SPS and sugar beet pectin at different pH's (3.0-8.0; results not shown), but in neither of these cases acetic acid was released. However, both acetyl esterases from *Aspergillus niger* and citrus do show activity towards PNP-acetate, in a ratio of 12.3 : 1.0.

The characteristics of the purified acetyl esterase have been determined on steamed birchwood xylan, and are shown in Table II. The specific activity is 32.3 U/mg. The optimum pH and temperature were 5.5-6.0 and 50°C, respectively.

Table II. Characteristics of the purified acetyl esterase from *Aspergillus niger*.

	Characteristic
Specific activity	32.3 U/mg
MW	30480 Da
pI	3.0-3.2
pH-optimum	5.5-6.0
T-optimum	50°C

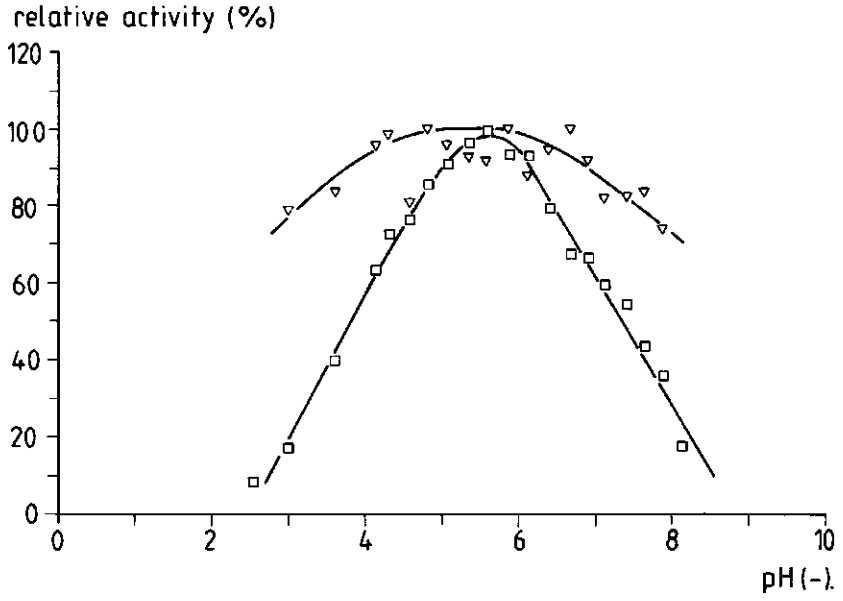

Figure 4. Effect of pH on acetyl xylan esterase activity of *Aspergillus niger*; pH optimum, □ ; pH stability after 24 h of pre-incubation, ▽ (activity in %).

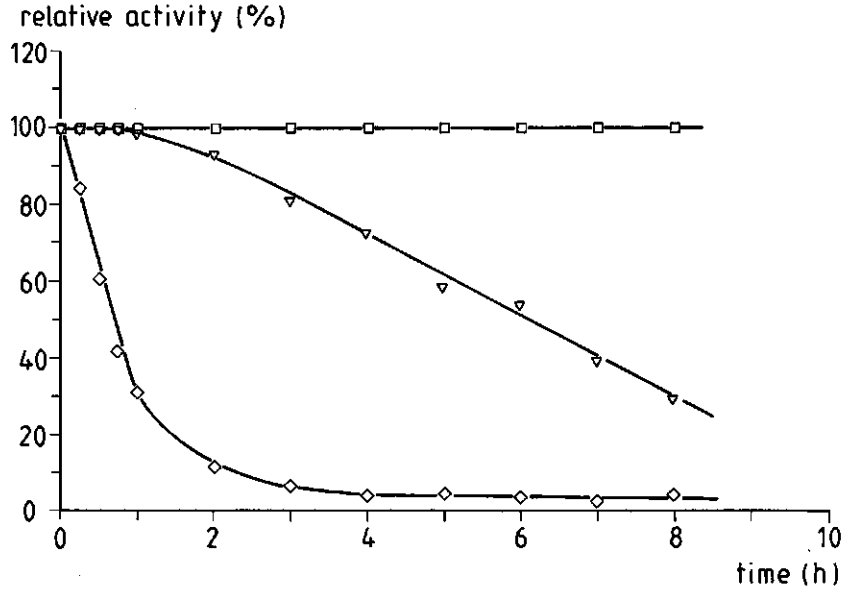

Figure 5. Effect of temperature on acetyl xylan esterase activity of *Aspergillus niger*; 45°C □ 50°C ▽,55°C◇.

The influence of pH on the acetyl xylan esterase activity is shown in Figure 4. The optimum pH was around 5.5-6.0, which is similar to the pH optima found for the acetyl esterases of *Trichoderma reesei* (Poutanen and Sundberg, 1988; Sundberg and Poutanen, 1991), *Aspergillus awamori* (Sundberg *et al*, 1990), and *Aspergillus oryzae* (Tenkanen *et al*, 1991). The pH stability after 24 hours is within the range of 75%-100% of its original activity. The acetyl esterase is therefore very stable over the pH-range 3.0-8.0.

The optimum temperature of the acetyl esterase from *Aspergillus niger* was around 50°C, similar to the acetyl esterase from *Trichoderma reesei* (Poutanen and Sundberg, 1988). The acetyl esterases from *Trichoderma reesei* (Sundberg and Poutanen, 1991), and *Aspergillus awamori* (Sundberg *et al*, 1990) were in the range of 60°-75° and thus more stable to higher temperatures.

The stability curves (Fig. 5) show that the acetyl esterase is even stable at 45°C after 8 hours. However, the acetyl esterase looses 50% of its acetyl xylan esterase activity after 6 hours of pre-incubation at 50°C and within 1 hour of pre-incubation at 55°C. The temperature stability shows almost exactly the same curves for the residual activity, after pre-incubation at 45°, 50° and 55°C, compared to the acetyl esterase from *Trichoderma reesei* (Poutanen and Sundberg, 1988). In the latter case α-naphtyl acetate was used as a substrate.

The acetyl xylan esterase was tested for inhibition by different components as shown in Table III. Only $Pb(NO3)_2$ clearly inhibited the enzyme. Any influence of EDTA, which suggests that metal ions are critical to the catalytic activity, or DTT, which implies the presence of S-S linkages in the active site, was absence.

Table III. Inhibition of the acetyl xylan esterase activity by different components.

Component	Concentration (mMole/L)	Activity (%)
Blank	-	100
$CaCl_2$	1	97
$Pb(NO_3)_2$	1	56
$AgNO_3$	1	99
KCl	1	100
$HgCl_2$	1	92
$MgSO_4$	1	95
$MnCl_2$	1	94
$NiCl_2$	1	92
$CoCl_2$	1	101
$ZnSO_4$	1	103
$BaCl_2$	1	102
$CuSO_4$	1	98
EDTA	1	102
DTT	0.1	98

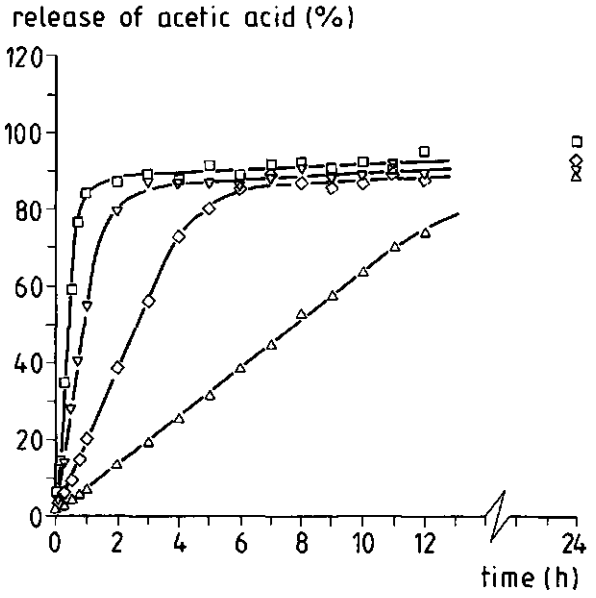

Figure 6. Time curve study of the release of acetic acid from 0.1% (□), 0.2% (▽), 0.4% (◇), and 0.8% (△) steamed birchwood xylan solutions (w/v) by 1.0 μg/mL acetyl xylan esterase of *Aspergillus niger*.

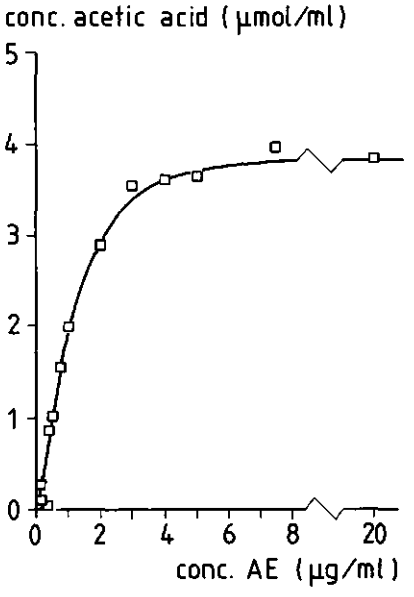

Figure 7. Release of acetic acid from a 0.2% (w/v) steamed birchwood xylan solution vs. increasing concentrations of acetyl xylan esterase of *Aspergillus niger*.

The release of acetic acid in time was studied by incubation of a 0.1%, 0.2%, 0.4% and 0.8% (w/v) steamed birchwood xylan solution with 1 μg/mL acetyl esterase. The release of acetic acid was followed by HPLC, as shown in Figure 6. Prolonged incubation of these steamed birchwood xylan solutions with acetyl xylan esterase finally releases 80-90% of all the acetyl groups.

The release of acetic acid was also studied by incubation of a 0.2% (w/v) steamed birchwood xylan solution with increasing concentrations of acetyl esterase of *Aspergillus niger*.

As Figure 7 shows, higher concentrations than 4.0 μg/mL do not have any influence on the release of more acetic acid within 1 hour, because most of the acetyl groups have already been released.

Michaelis-Menten kinetics did not result in a linear Lineweaver-Burk plot when incubating steamed birchwood xylan, 0.2 up to 3.0% w/v, with acetyl xylan esterase (1 μg/mL) for 1 hour at 30°C. The shape of the curve strongly indicated substrate inhibition on the acetyl xylan esterase activity.

A 0.2% (w/v) steamed birchwood xylan solution was also incubated with acetyl esterase and with combinations of acetyl esterase and endo-xylanase I, endo-xylanase III or ß-xylosidase in time. Figure 8.a, 8.b, and 8.c show the release of acetic acid and neutral sugars released by the combinations of acetyl esterase with endo-xylanase I, III, and ß-xylosidase. As can be seen in these time curves, endo-xylanase I and III start releasing reasonable amounts of xylooligomers (X_2, X_3 and X_4) after most of the acetyl groups have been released. Endo-xylanase I also released monomeric xylose. The acetyl esterase does not release more acetic acid in combination with xylan-degrading enzymes than when used alone. The release of xylose by ß-xylosidase from steamed birchwood xylan is slowly but steady.

Without acetyl esterase, the endo-xylanases and the ß-xylosidase do not degrade the steamed birchwood xylan i.e. they do not release reasonable amounts of X_1, X_2, X_3 and X_4. This indicates that acetyl groups block the enzyme activity of the endo-xylanases or ß-xylosidase activity.

For a closer study of the degradation of steamed birchwood xylan, comparative studies were carried out by incubation of a steamed birchwood xylan for 1 and 24 h with only acetyl esterase, endo-xylanase I, endo-xylanase II, endo-xylanase III or ß-xylosidase, and with combinations of acetyl esterase and these xylan-degrading enzymes. Also pre-incubations with acetyl esterase for 1 h followed by 1 and 24 h incubations with the xylan-degrading enzymes were carried out. Table IV shows the results of the release of acetic acid, xylose, and xylo-oligomers after 1 and 24 hours of incubation.

The acetyl xylan esterase releases 60-65% and 100% (100% = 4.30 μmole/mL) of acetyl groups after 1 and 24 h of incubation respectively. There is no increase in the initial rate for the release of acetic acid by using the combination of xylan-degrading enzymes and acetyl xylan esterase.

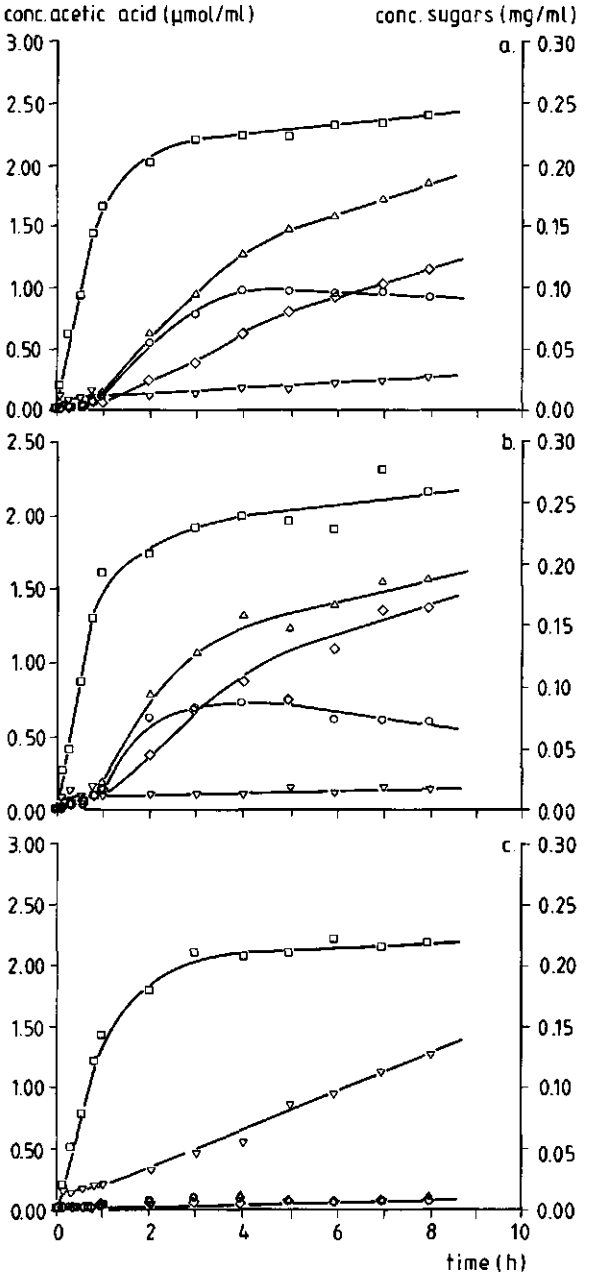

Figure 8. Time curve study of the release of acetic acid from a 0.2% (w/v) steamed birchwood xylan solution by combinations of 1 µg/mL acetyl esterase and 0.1 µg/mL endo-xylanase I (a), endo-xylanase III (b) or ß-xylosidase(c); HAc □; X_1 ▽; X_2 ◇; X_3 △; X_4 ○.

Table IV. Release of acetic acid, xylose and xylooligomers from a 0.2% (w/v) steamed birchwood xylan solution by the single and combined action of 1.0 μg/mL acetyl esterase and 0.1 μg/mL endo-xylanase I, endo-xylanase II, endo-xylanase III or ß-xylosidase.

A. Incubation time: 1 hour.

Type of incubation	Release Acetic acid[a]	X_1[b]	X_2[b]	X_3[b]	X_4[b]
Blank	0.0	11	2	3	0
AE	62.4				
Endo I	0.5	12	3	4	0
Endo II	1.9	9	6	9	0
Endo III	0.0	10	3	5	0
ß-Xylosidase	2.3	23	2	2	0
AE + Endo I	61.0	8	5	13	11
AE + Endo II	65.3	10	23	77	54
AE + Endo III	62.4	8	8	18	11
AE + ß-Xylosidase	63.8	21	2	4	2
AE[c](1 h) + Endo I (1 h)	64.3	10	7	16	11
AE[c](1 h) + Endo II (1 h)	68.1	9	30	69	44
AE[c](1 h) + Endo III (1 h)	58.2	10	13	28	19
AE[c](1 h) + ß-Xylosidase (1 h)	62.9	17	2	4	2

[a] % of maximum release of 4.30 μmole/mL.
[b] μg/mL.
[c] Pre-incubation.

Without acetyl xylan esterase, the endo-xylanases and ß-xylosidase from *Aspergillus awamori* release no or only small traces of xylo-oligomers from steamed birchwood xylan (i.e X_1 or X_1, X_2, and X_3, by ß-xylosidase and endo-xylanase I, respectively). In combination with acetyl xylan esterase, these xylan-degrading enzymes release small amounts of xylo-oligomers after 1 h, and reasonable amounts of xylooligomers after 24 h of incubation.

However, pretreatment of the steamed birchwood xylan with acetyl esterase for only 1 h appears not to result in the same amounts of xylooligomers after 1 and 24 h. The concerted action of acetyl xylan esterase and xylan-degrading enzymes thus releases the highest amount of X_1, X_2, X_3, and X_4.

Discussion

The acetyl esterase purified from *Aspergillus niger* appeared to be specific for xylans.

Table IV. Release of acetic acid, xylose and xylooligomers from a 0.2% (w/v) steamed birchwood xylan solution by the single and combined action of 1.0 μg/mL acetyl esterase and 0.1 μg/mL endo-xylanase I, endo-xylanase II, endo-xylanase III or ß-xylosidase.

B. Incubation time: 24 hours.

Type of incubation	Release Acetic acid[a]	X_1[b]	X_2[b]	X_3[b]	X_4[b]
Blank	0.0	8	2	3	0
AE	100.0				
Endo I	1.4	22	27	79	0
Endo II	2.8	10	11	11	0
Endo III	0.5	10	10	11	0
ß-Xylosidase	3.8	65	0	0	0
AE + Endo I	100.0	43	210	265	48
AE + Endo II	100.0	10	104	252	105
AE + Endo III	97.7	20	209	222	54
AE + ß-Xylosidase	100.0	237	6	7	6
AE[c](1 h) + Endo I (24 h)	61.5	36	149	253	63
AE[c](1 h) + Endo II (24 h)	64.3	10	38	80	45
AE[c](1 h) + Endo III (24 h)	59.2	12	67	77	42
AE[c](1 h) + ß-Xylosidase (24 h)	69.5	113	5	5	0

[a] % of maximum release of 4.30 μmole/mL.
[b] μg/mL.
[c] Pre-incubation.

Only steamed birchwood xylan was deacetylated and no other acetylated polymeric substrates like apple pectin, sugar beet pectin, and SPS. The isolated enzyme had a molecular weight of 30480 Da and a pI of 3.0-3.2.

Purified xylan-degrading enzymes from *Aspergillus awamori* did not degrade the steamed birchwood xylan significantly. This is in agreement with similar findings by Poutanen *et al* (1986) for a crude xylanase preparation of *Aspergillus awamori*.

The degradation of steamed birchwood xylan by xylan-depolymerizing enzymes was strongly increased when acetyl xylan esterase was included in the reaction mixture. This could be concluded from the higher production of xylooligomers over the whole reaction period. Pre-treatment of steamed birchwood xylan with acetyl xylan esterase prior to treatment with xylan-degrading enzymes, also enhanced xylooligomer release, but to a lower degree as was observed in the combined treatment.

It is therefore possible to make oligomers of specific size by degradation of acetylated xylan with endo-xylanase after partial deacetylation with acetyl xylan esterase.

From the results presented here, it is clear that by the initial release of acetyl groups by

the acetyl xylan esterase, new subsites are created on the polysaccharide backbone which can be degraded by endo-xylanase. Poutanen *et al* (1989 and 1990) postulated that xylans are linearized by deacetylation and become prone to aggregation and precipitation and thus less accessible for degradation.

Combinations of acetyl esterase from *Trichoderma reesei* or *Schizophyllum commune*, with endo-xylanase were not only found to act synergistically in hydrolyzing steamed birchwood xylan, but also in the liberation of acetyl groups (Biely *et al*; 1986, 1987, 1988 a and 1988 b). In this study, however, synergism between endo-xylanase or ß-xylosidase and acetyl esterase was only found with respect to xylan depolymerization, not with respect to the release of acetic acid.

The acetyl esterase purified from *Aspergillus niger* differed also from the acetyl esterase from *Trichoderma reesei* (Poutanen and Sundberg, 1988) in its ability to release a considerable proportion of acetic acid from steamed birchwood xylan. The acetyl esterase from *Trichoderma reesei* could only release this amount of acetate from steamed birchwood xylan in combination with endo-xylanase. Similar observations were made for the acetyl esterase of *Schizophyllum commune* purified by Biely *et al* (1987, 1988 a and 1988 b).

Recently Sundberg and Poutanen reported another acetyl xylan esterase isolated from *Trichoderma reesei* (1991), which had the same ability to release acetyl groups from steamed birchwood xylan as the *Aspergillus niger* enzyme described here.

It appears therefore that there exist acetyl xylan esterases more specific for acetyl xylooligomers and acetyl xylan esterases specific for polymeric acetyl xylan. The fact that acetyl xylan esterase was not active on acetylated pectins indicates the existence of specific acetyl pectin esterases.

Acknowledgements

The authors wish to thank M. Vilhelm and J.J. Lecocq (Gist-brocades, France) for their contribution in the purification of acetyl xylan esterase.

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Chapter 9

Degradation of different [(glucurono)arabino]xylans by combination of purified xylan-degrading enzymes

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Appl. Microbiol. Biotechnol., accepted for publication.

Summary

The degradation of [({4-*O*-methyl-}glucurono)arabino]xylans from rice bran, oat spelts, wheat-flour, larchwood, and birchwood with two types of endo-(1,4)-ß-xylanase, (1,4)-ß-xylosidase, (1,4)-ß-D-arabinoxylan arabinofuranohydrolase (AXH), and an acetyl xylan esterase, single and in combinations, was investigated. The endo-(1,4)-ß-xylanases showed highest initial release of reducing endgroups on oat spelts xylan, followed successively by larchwood xylan, wheat-flour xylan, birchwood xylan and rice bran xylan. The extent of degradation governed by degree and pattern of substitution, was highest for oat spelts followed by wheat-flour and larchwood xylan. The extent of hydrolysis for the commercially available birchwood xylan was low, due to the partly insoluble fraction. Rice bran arabinoxylan could only partly be degraded by the combined action of endo-(1,4)-ß-xylanase and AXH. The combination of endo-(1,4)-ß-xylanase I, or III, with (1,4)-ß-xylosidase and AXH, or acetyl xylan esterase, resulted in the highest degree of hydrolysis after 24 hours of incubation.

Introduction

Xylans from different sources i.e. grasses, cereals, softwoods, and hardwoods, differ in their composition. Xylans from grasses and cereals have various amounts of *O*-2 and/or *O*-3 linked arabinofuranosyl substituents and *O*-2 linked glucopyranosyl uronic acid substituents; they may also be acetylated to a low degree (Wilkie, 1979). Xylans from softwoods show resemblances with xylans from grasses and cereals (Timell, 1965). Xylans from hardwoods carry substantial amounts of *O*-2 linked (4-*O*-methyl)glucopyranosyl uronic acid substituents and no or only small amounts of arabinofuranosyl substituents. The latter type of xylan may also be highly acetylated at the *O*-2 or *O*-3 position (Timell, 1964).

For an optimal breakdown of these differently substituted xylans, different combinations of enzymes are required (Biely, 1985).

This chapter describes the degradation of xylans from rice bran, oat spelts, wheat flour, larchwood, and birchwood by two different types of endo-(1,4)-ß-xylanase, a (1,4)-ß-xylosidase, an arabinose-releasing enzyme, and an acetyl xylan esterase, single and in combinations. The degradation was followed by studying the initial release of reducing endgroups, the extent of hydrolysis, and the formation of reaction products.

Materials and methods

Materials

Substrates used were oat spelts xylan (Sigma Chemical Co., St. Louis, USA), larchwood xylan (Sigma Chemical Co.), and birchwood xylan (Roth, Karlsruhe, FRG). Oat spelts and larchwood xylan were fractionated into water-soluble and water-insoluble fractions as described by Kormelink *et al* (1992 a). Alkali-extractable arabinoxylan from wheat flour (BE1-U; Gruppen *et al*, 1992 a) was kindly provided by H. Gruppen from the Department of Food Science (Agricultural University, The Netherlands). Rice bran arabinoxylan (neutral and acidic fraction) and steam exploded birchwood xylan were kindly provided by Dr. Shibuya (National Food Research Institute, Ibaraki, Japan) and Dr. Puls (Institute of Wood Chemistry, Hamburg, FRG), respectively.

Enzymes used were endo-(1,4)-ß-xylanase I and III (Endo I and III), (1,4)-ß-xylosidase (ß-xylosidase; Kormelink *et al*, 1992 a), (1,4)-ß-D-arabinoxylan arabinofuranohydrolase (AXH; Kormelink *et al*, 1991), all purified from *Aspergillus awamori* CMI 142717, and an acetyl xylan esterase (AE; Kormelink *et al*, 1992 b), purified from *Aspergillus niger* DS 16813.

Enzyme incubations

[(Glucurono)arabino]xylan was dissolved in 50mM sodium acetate buffer pH 5.0 (0.1 % w/v) and incubated with Endo I or III (0.1 μg/mL), ß-xylosidase (0.1 μg/mL), AXH (0.1

μg/mL), AE (1.0 μg/mL), or combinations of these enzymes. Incubation mixtures were held at 30°C for 1 or 24 h and subsequently inactivated at 100°C for 10 min.

Reducing sugar assay

The amount of reducing sugars released was determined by the Nelson-Somogyi assay (Somogyi, 1952). Appropriate aliquots (200 μL) were mixed with an equivalent amount of copper reagent, boiled for 10 minutes and cooled down to room temperature. Subsequently 200 μL of arsenic molybdate reagent and 1200 μL H_2O were added and mixed. The extinction was measured at 520 nm after 30 minutes. A calibration curve of xylose was used for calculating the release of reducing sugars.

High performance anion-exchange chromatography analysis (h.p.a.e.c.)

Incubation mixtures of [(glucurono)arabino]xylan with different enzymes were analysed by h.p.a.e.c. as described by Gruppen *et al* (1992 b). 20 μL Samples were injected on to the CarboPac PA1 column. Elution (1 mL/min) involved a linear gradient of 0-100mM sodium acetate in 0.1M NaOH during 5 min, followed by a linear gradient of 100-400mM sodium acetate in 0.1M NaOH during 35 min.

Monosaccharide composition

The monosaccharide composition of the [(glucurono)arabino]xylans was determined by trifluoroacetic acid hydrolysis (2M; 1 h at 121°C) and conversion to their alditol acetates as described by Englyst and Cummings (1984) using inositol as internal standard. Analysis was carried out on GLC (3% OV 275 on Chrom W HP 80-100 column) at 210°C; carrier gas was H_2 at a pressure of 0.6 kg/cm^3. Detection was performed by FID.

The uronic acid content was determined by the *m*-hydroxydiphenyl assay according to the method described by Ahmed and Labavitch (1977).

Methylation analysis

Methylation of xylan samples was carried out according to a modified Hakamori method as described by Sandford and Conrad (1966). Methylation of acidic fractions was preceded by a carboxyl-reduction step according to Taylor and Conrad (1972). After methylation, samples were dialysed and dried in a stream of air. The methylated samples were subsequently hydrolysed using 2M trifluoroacetic acid (121°C, 1h). Derivatisation to alditol acetates was carried out according to Englyst and Cummings (1984).

The methylated alditol acetates were analysed on a fused silica capillary column (30m x 0.32mm; wall coated with DB1701; 0.25μm) using a Carlo Erba Fractovap 4160 GC equipped with a FID.

Glycosidic linkage composition was calculated using effective carbon response (E.C.R.) factors (Sweet *et al*, 1975). The identity of compounds was confirmed by GC-MS (Hewlett Packard, MSD 5970-B coupled to a HP 5890), equipped with a fused silica column (CPSIL 19CB, 26m x 0.22mm; 0.18μm). The 2- and 3-*O*-methylated xylitol

acetates co-eluted. Their relative amounts were calculated from the relative abundance of the ions at m/z 117 and m/z 129.

Results

Sugar and glycosidic linkage composition

The xylans used for enzymic breakdown studies, showed a large variation in their monosaccharide composition which reflects the variation in substitution (Table I). Rice bran arabinoxylans, the neutral as well as acidic fraction, contain a large amount of arabinose. Their Ara/Xyl ratio is 0.98 and 0.90, respectively, which indicates a high degree of branching. Wheat arabinoxylan, larchwood xylan and oat spelts xylan have lower amounts of arabinose, and thus a lower degree of branching (Ara/Xyl ratio's of respectively 0.51, 0.21 and 0.12). Both types of birchwood xylan contain only minor amounts of arabinose.

The rice bran arabinoxylan fractions also contain a rather large amount of galactose, compared with the other types of xylan. Water-soluble larchwood xylan contains a large amount of glucose, probably due to the presence of cellulose.

Another important substituent is glucopyranosyl uronic acid, which is present in large amounts in both types of birchwood xylan and in the acidic fraction of rice bran arabinoxylan. Acetyl groups are only present in steamed birchwood xylan (10.6% based on dry weight; Kormelink *et al*, 1992 b).

Exact data on the glycosidic linkages present, were obtained from methylation analysis (Table II).

Table I. Monosaccharide composition (mole%) of different xylans from various sources.

Source	Rha	Ara	Xyl	Man	Gal	Glc	AUA
Rice bran[1]							
Neutral	n.d.*	44.9	46.0	n.d.	6.1	1.9	1.1
Acidic	0.6	40.9	45.6	n.d.	5.9	n.d.	7.0
Oat spelts	n.d.	9.7	81.4	n.d.	1.1	3.4	4.3
Wheat[2]	n.d.	33.5	65.8	0.1	0.1	0.3	n.d.
Larch	n.d.	11.4	55.6	n.d.	3.1	25.7	4.2
Birchwood (Roth)	n.d.	1.0	89.3	n.d.	n.d.	1.4	8.3
Steamed birchwood	n.d.	1.0	79.2	1.2	3.7	3.6	11.5

[1] Shibuya and Iwasaki (1985)
[2] Gruppen *et al* (1992 a)
* n.d. = not detected

Both rice bran arabinoxylan fractions have 24.6 mole% *O*-2 or *O*-3 single branched xylopyranosyl residues (3-Me-Xyl resp. 2-Me-Xyl), and 6.8-6.9 mole% *O*-2 and *O*-3 double branched xylopyranosyl residues (Xyl). The largest part of these branches consist of single arabinofuranosyl substituents (39.4 and 32.8 mole% 2,3,5-Me_3-Ara, respectively for the neutral and acidic arabinoxylan fraction). The presence of 2,3-Me_2-Ara and 2,5-Me_2-Ara indicate side chains of more than one arabinofuranosyl residue in rice bran arabinoxylan (Shibuya and Iwasaki, 1985). As no glucose was detected in the acidic fraction of rice bran arabinoxylan by GLC analysis of their alditol acetates (Table I), the values shown for glucopyranosyl residues in Table II relate to the glucopyranosyl uronic acid substituents and their linkage types. Most glucopyranosyl uronic acid substituents are terminal. Shibuya and Iwasaki (1985) suggested the presence of single unit side-chains of xylopyranosyl and galactopyranosyl substituents, which thus increases the degree of branches on the main chain.

The glycosidic linkages detected in the neutral fraction of rice bran arabinoxylan, were also found in wheat alkali-extractable arabinoxylan. However, it contained 13.2 mole% single (*O*-2 or *O*-3) substituted xylopyranosyl residues, and 9.5 mole% double (*O*-2 and *O*-3) substituted xylopyranosyl residues. The proportion of single substituted xylopyranosyl residues is thus substantially lower.

Oat spelts and larchwood arabinoxylan contained respectively 11.0 and 12.5 mole% single substituted xylopyranosyl residues, and 1.1 and 1.8 mole% double substituted xylopyranosyl residues, which is much lower than in the wheat or rice bran arabinoxylan. The branches consist of single unit arabinofuranosyl substituents, 6.7 and 9.0 mole% 2,3,5-Me_3-Ara respectively in oat spelts and larchwood, and single unit glucopyranosyl uronic acid substituents, 4.6 and 6.2 mole% 2,3,4,6-Me_4-Glc respectively in oat spelts and larchwood. Water-soluble arabinoxylan from larchwood also contained 19.4 mole% 2,3,6-Me_3-Glc which indicates the presence of cellulose. The amounts of mannose and galactose detected by methylation analysis in oat spelts xylan are in contradiction with amounts analysed as alditol acetates (Table I). We have no explanation for this discrepancy.

Both samples of birchwood xylan seem to contain only single (*O*-2) substituted xylopyranosyl residues, 5.3 and 3.9 mole% respectively for birchwood xylan from Roth, and steamed birchwood xylan (Puls). Most of these substituents are 4-*O*-methyl-glucopyranosyl uronic acid substituents (Puls *et al*, 1985) detected as 2,3,4,6-Me_4-Glc (8.4 and 7.5 mole%, respectively). Again, the amounts found for galactose and mannose cannot be explained.

Enzymic degradation

To study optimal conditions for degradation, xylans from rice bran, oat spelts, wheat, larchwood, and birchwood were incubated with different enzymes. For the purpose of comparing the enzymic degradability of the various xylans, the initial reaction rate towards oat spelts xylan was set at 100, because this xylan was degraded most readily. Differences in the initial reaction rate were in agreement with the degree of substitution,

Table II. Glycosidic linkages present in [(glucurono)arabino]-xylans from various sources (mole%) as determined by methylation analysis.

Residu	Substrate Rice bran Neutral[2]	Rice bran Acid	Oat	Wheat[3]	Larch	Birch (Roth)	(Puls)
2,3,5-Me_3-Ara[1]	39.4	32.8	6.7	33.3	9.0		
3,5-Me_2-Ara	2.9	1.5	1.8	1.0	2.2		
2,3-Me_2-Ara	2.9	1.4					
2,5-Me_2-Ara	4.9	3.4					
2,3,4-Me_3-Xyl	2.9	2.4	4.4	0.6	5.9	1.5	5.5
2,3-Me_2-Xyl	10.8	13.2	57.7	42.3	39.5	73.8	69.8
2-Me-Xyl		16.6	8.5		2.4		
	24.6			13.2			
3-Me-Xyl		8.0	2.5		10.1	5.3	3.9
Xyl	6.9	6.8	1.1	9.5	1.8	0.9	
2,3,4,6-Me_4-Gal	4.6	2.8	0.9		2.0		1.0
3,4,6-Me_3-Gal							0.4
2,3,4-Me_3-Gal							1.5
2,3,6-Me_3-Gal						4.2	
2,4,6-Me_3-Gal			7.8				5.5
2,4-Me_2-Gal		0.9					
2,3,4,6-Me_4-Glc		6.0	4.6		6.2	8.4	7.5
2,3,6-Me_3-Glc		1.9		0.1	19.4		
2,4-Me_2-Glc							0.7
Glc		1.2	1.1		1.6	4.1	2.5
2,3,6-Me_3-Man		1.1	1.3			1.8	
Man			1.7				1.9

[1] 2,3,5-Me_3-Ara = 2,3,5-tri-*O*-methyl-1,4-di-*O*-acetyl-arabinose etc.
[2] Shibuya and Iwasaki (1985)
[3] Gruppen *et al* (1992 a)

except for birchwood xylan. Endo I and III did not show high initial reaction rates towards the neutral and acidic fraction of rice bran arabinoxylan (Table III). This can be explained by the high degree of branching of both fractions.

Somewhat higher values were measured towards wheat and larchwood xylan. As birchwood xylan from Roth was partly insoluble, the initial reaction rate towards this substrate was also low. Steamed birchwood xylan consisted of soluble high oligomeric xylan fragments and was, in comparison to the poorly soluble polymeric substrate, reasonably well degraded.

AXH was able to release arabinose from wheat arabinoxylan with a higher rate than from

Table III. Relative initial reaction rates of Endo I, Endo III, and AXH towards various xylans. The initial reaction rate towards oat spelts arabinoxylan, based on the release of reducing sugars, is set at 100.

Enzyme	Substrate						
	Rice bran		Oat	Wheat	Larch	Birch	
	Neutral	Acid				Roth	Steamed
Endo I	4.2	7.3	100	67.9	74.7	22.8	49.9
Endo III	1.4	2.8	100	45.3	57.0	3.7	41.7
AXH	71.0	55.3	100	111.1	76.9	11.1	N.D.

N.D. = Not Determined

oat spelts xylan, probably because of the higher amounts of arabinofuranosyl substituents present. However, this was not the case for rice bran arabinoxylan which contained even higher amounts of single substituted xylopyranosyl residues than wheat arabinoxylan. Differences in the environment and in the specificity towards *O*-2 or *O*-3 linked arabinofuranosyl substituents of polymeric substrate might be the reason for this.

ß-Xylosidase released no or only minor amounts of reducing sugars from both rice bran arabinoxylan fractions, and from oat spelts xylan (results not shown). As steamed birchwood xylan consisted of a mixture of high oligomeric xylan fragments, ß-xylosidase was able to release significant amounts of reducing sugars from this substrate.

Prolonged incubations of the various xylans with Endo I and III, ß-xylosidase, AXH and AE separately and in combinations did result in remarkable differences in the extent of degradation (Table IV).

Digests of 24 hours of rice bran arabinoxylan (neutral as well as acidic fraction) with Endo I or III, or ß-xylosidase contained no or only small amounts of reducing sugars. AXH, however, released reasonable amounts of arabinose. The combination of AXH with Endo I showed a strong synergistic effect in the degradation of these arabinoxylan fractions, 11.1-12.4 % of glycosidic linkages were hydrolysed. By adding ß-xylosidase, hydrolysis increased to 13.3 %. Similar observations were made with Endo III, degradation levels of 9.4-12.5 % could be realised.

In the case of oat spelts, wheat and larchwood xylan, Endo I and III, and AXH were able to release larger amounts of reducing sugars. The extent of degradation reached its optimum when using combinations of endo-xylanase (I or III), ß-xylosidase, and AXH. Combinations with Endo I reached an optimal breakdown of 39.7, 33.4 and 24.4 % for oat spelts, wheat and larchwood xylan, respectively. Combinations based on Endo III, gave extents of degradation of respectively 42.6, 30.5, and 26.9 %.

Birchwood xylan (Roth) was partly insoluble and resulted therefore in a low degree of hydrolysis. Some reducing sugars were only released by incubation with Endo I. This could be enhanced by the addition of ß-xylosidase.

Table IV. Extent of hydrolysis of xylans* after 24 hours of incubation of xylan with Endo I or III, ß-xylosidase (ß), AXH, AE, or with combinations of these enzymes.

Enzyme	Substrate						
	Rice bran		Oat	Wheat	Larch	Birch	
	Neutral	Acid				Roth	Steamed
Endo I	1.1	1.1	15.7	11.2	9.1	4.9	10.0
Endo III	0.0	0.0	10.3	5.0	5.2	0.2	1.7
ß	0.0	0.0	0.0	0.0	0.6	0.8	4.6
AXH	6.8	6.7	3.0	7.8	2.9	0.6	N.D.
AE	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	2.2
Endo I + ß	1.2	0.8	33.3	15.0	17.1	13.7	17.3
Endo I + AXH	11.1	12.4	21.6	18.0	12.2	4.8	N.D.
Endo I + AE	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	20.3
Endo I + ß + AXH	13.3	13.3	39.7	33.4	24.4	16.0	N.D.
Endo I + ß + AE	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	40.0
Endo III + ß	0.1	0.0	23.6	6.9	12.1	1.4	8.3
Endo III + AXH	9.8	9.0	16.9	16.3	10.0	0.6	N.D.
Endo III + AE	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	16.1
Endo III + ß + AXH	12.5	9.4	42.6	30.5	26.9	1.9	N.D.
Endo III + ß + AE	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	38.2

N.D. Not Determined

* Expressed in % of the amount of glycosidic linkages present

The effect of the addition of AXH was only marginal, as can be expected from the low arabinose content in this xylan (Table I). Endo III gave only 0.2 % hydrolysis; the hydrolysis was enhanced to 1.4 % on the addition of ß-xylosidase and to 1.9 % when AXH was also present. This emphasizes the differences between Endo I and III.

Steamed birchwood xylan consisted of a mixture of acetylated xylo-oligosaccharides. Despite the presence of *O*-2 or *O*-3 linked acetyl groups, incubations with Endo I or ß-xylosidase released some reducing sugars. Endo III, however, released only minor amounts of reducing sugars. Supplementation with AE increased the extent of degradation, especially with Endo III. The extent of degradation could be optimized by the further supplementation of the enzyme mixture with ß-xylosidase, 40.0 % for the mixture based on Endo I and 38.2 % for the mixture based on Endo III.

H.p.a.e.c. analysis

H.p.a.e.c. analysis of the incubation mixtures (Fig. 1, rice bran neutral; 2, rice bran acidic; 3, oat spelts; 4, wheat; 5, larchwood; 6, birchwood; 7, steamed birchwood) shows the oligomeric products formed from the various xylans after incubation with the different enzymes. Arabinose, xylose, xylobiose and higher oligosaccharides, and arabinoxylo- and

glucuronoxylo-oligosaccharides elute at 5, 7, 10-15 and 15-25 minutes, respectively. Incubation of rice bran arabinoxylan (neutral as well as acidic fraction) with AXH and endo-xylanase (I or III) resulted in the first hour only in the release of large amounts of arabinose (Fig. 1.b and 1.d, and Fig. 2.b and 2.d). After 24 h significant amounts of xylotriose, xylobiose and, only in the case of Endo I, reasonable amounts of xylose were also released. No significant amounts of [(glucurono)arabino]xylan oligosaccharides were formed (Fig. 1.c and 1.e, and Fig. 2.c and 2.e).

The hydrolysis of oat spelts (Fig. 3) and larchwood xylan (Fig. 5) showed remarkable similarities. Incubation with Endo I or III for 1 h resulted in a large range of xylo-oligosaccharides as well as [(glucurono)arabino]xylan-oligosaccharides (Fig. 3.b and 3.d, and Fig. 5.b and 5.d). After 24 h the main end-products were xylose, xylobiose, and xylotriose, and in addition a range of [(glucurono)arabino]xylan-oligosaccharides (Fig. 3.c and 3.e, and Fig. 5.c and 5.e).

The same can be observed for wheat xylan. However, lower amounts of xylobiose, xylotriose and arabinoxylan-oligosaccharides were released (Fig. 4). As is the case for rice bran arabinoxylan, only Endo I was able to release xylose from wheat arabinoxylan (Fig. 4.c). Detailed studies of the structural features of arabinoxylan-oligosaccharides released from alkali-extractable wheat arabinoxylan by Endo I (Gruppen *et al*, 1992 b) and Endo III (Kormelink *et al*, 1992 c) did not reveal the presence of any [(glucurono)arabino]xylan oligosaccharides.

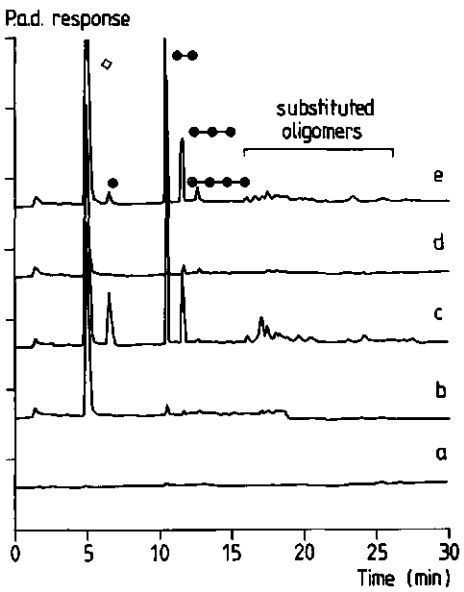

Figure 1. H.p.a.e.c. elution profile of the neutral fraction of rice bran arabinoxylan: blank (a), incubation with AXH and Endo I for 1 and 24 h (resp. b and c), incubation with AXH and Endo III for 1 and 24 h (resp. d and e).

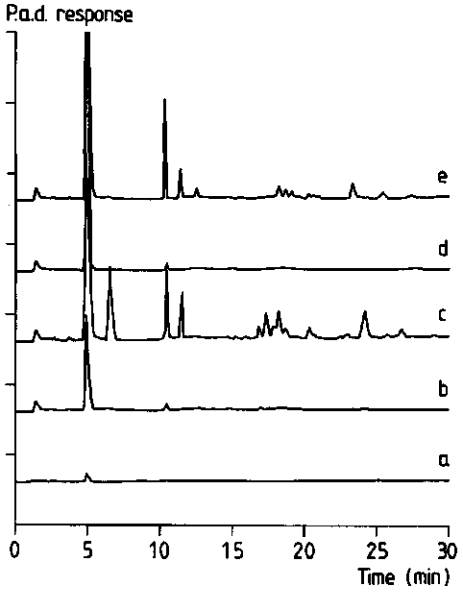

Figure 2. H.p.a.e.c. elution profile of the acidic fraction of rice bran arabinoxylan: blank (a), incubation with AXH and Endo I for 1 and 24 h (resp. b and c), incubation with AXH and Endo III for 1 and 24 h (resp. d and e).

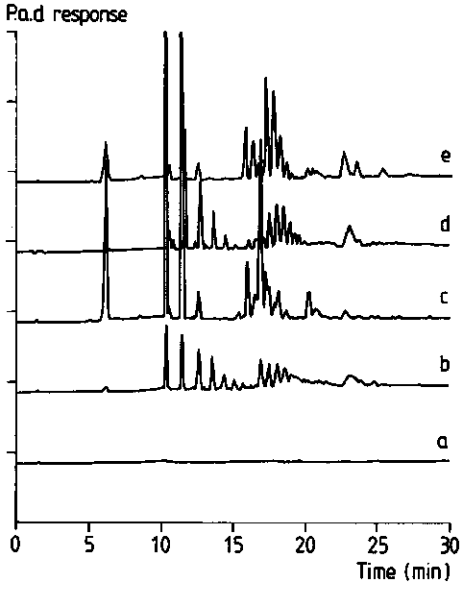

Figure 3. H.p.a.e.c. elution profile of water-soluble oat spelts arabinoxylan: blank (a), incubation with Endo I for 1 and 24 h (resp. b and c), incubation with Endo III for 1 and 24 h (resp. d and e).

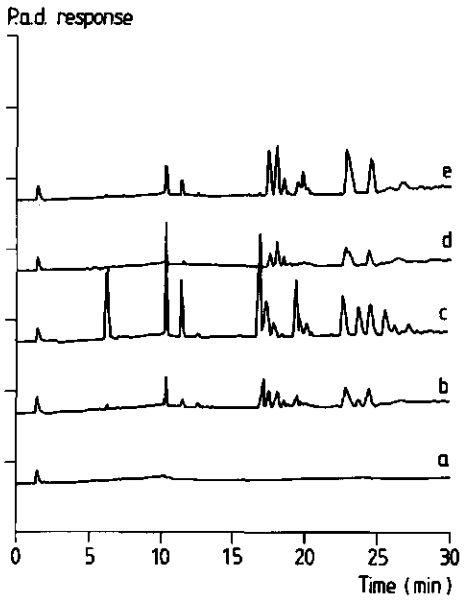

Figure 4. H.p.a.e.c. elution profile of alkali-extractable wheat flour arabinoxylan: blank (a), incubation with Endo I for 1 and 24 h (resp. b and c), incubation with Endo III for 1 and 24 h (resp. d and e).

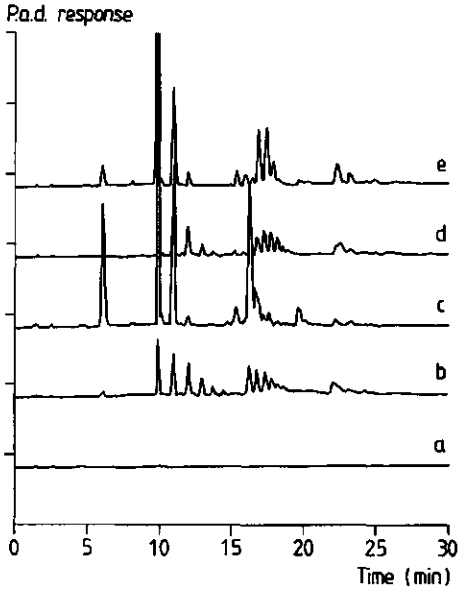

Figure 5. H.p.a.e.c. elution profile of water-soluble larchwood arabinoxylan: blank (a), incubation with Endo I for 1 and 24 h (resp. b and c), incubation with Endo III for 1 and 24 h (resp. d and e).

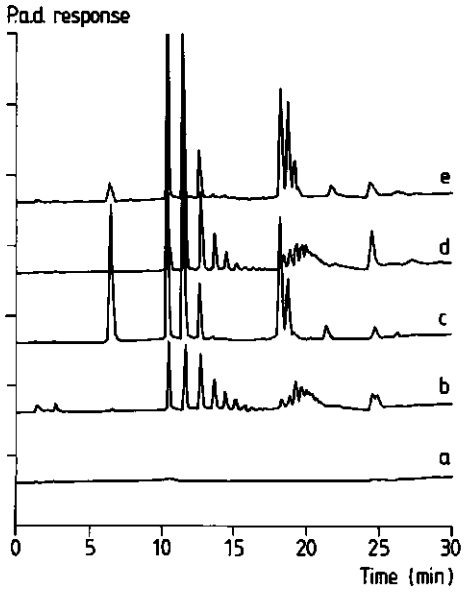

Figure 6. H.p.a.e.c. elution profile of birchwood xylan (Roth): blank (a), incubation with Endo I for 1 and 24 h (resp. b and c), incubation with Endo III for 1 and 24 h (resp. d and e).

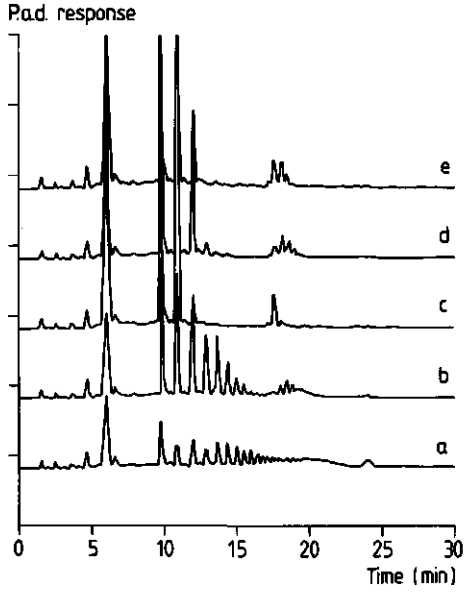

Figure 7. H.p.a.e.c. elution profile of steamed birchwood xylan: blank (a), incubation with AE and Endo I for 1 and 24 h (resp. b and c), incubation with AE and Endo III for 1 and 24 h (resp. d and e).

Endo I and III released a range of xylo-oligosaccharides from birchwood xylan (Roth; Fig. 6.b and 6.d). After 24 h only xylobiose, xylotriose and some xylotetraose could be analysed as end-products (Fig. 6.c and 6.e). This is also true for birchwood xylan, only Endo I was able to release significant amounts of xylose. As the birchwood xylan contained only minor amounts of arabinose, glucuronoxylan-oligosaccharides were expected as end-products.
Steamed birchwood xylan, which contained 10.6 % (w/w) acetyl groups, consisted of a mixture of xylo-oligosaccharides (Fig. 7.a). A high degree of hydrolysis requires an acetyl xylan esterase. As can be seen from Figure 7.c and 7.e, main end-products of Endo I or III-treatment in combination with AE, were xylobiose, and xylotriose (with some smaller amounts of xylotetraose), and glucuronoxylan-oligosaccharides. The latter oligosaccharides were also found in the commercially available birchwood xylan (Roth; Fig. 6.c and 6.e).

Discussion

There is a great heterogeneity in the composition and structure of xylans from various sources (Table I and II). From this heterogeneity it can be expected that depending from the structural features of the xylan different combinations of enzymes are required for maximum degradation (Biely, 1985). Differences in the extent of degradation can also be ascribed to differences in the substrate specificity of enzymes used as is shown for Endo I and Endo III in Table III.
Kormelink *et al* (1992 d) investigated the mode of action of two types of endo-xylanase of *Aspergillus awamori* towards alkali-extractable wheat arabinoxylan. They established that the sites of cleavage and thus the extent of hydrolysis were affected by the content and distribution of arabinofuranosyl substituents. Due to the high degree of branching the extent of hydrolysis of rice bran arabinoxylan with Endo I or III was low.
ß-Xylosidase plays an important role in the overall conversion of xylans and xylan-derived oligosaccharides to xylose. In studies on the hydrolysis of hemicelluloses from bagasse (Dekker, 1983), larchwood xylan (Seeta *et al*, 1989), and steamed birchwood xylan (Poutanen and Puls, 1988) it was found that xylose production correlated more with the level of ß-xylosidase (and esterase) than with that of endo-xylanase. Also in this study, a higher degree of hydrolysis of steamed birchwood xylan was observed (Table IV) by adding ß-xylosidase to the hydrolysis mixture of endo-xylanase. This was also true for the combination of ß-xylosidase, endo-xylanase and esterase as was found by Poutanen and Puls (1988). The xylan-derived oligosaccharides formed by endo-xylanases from wheat arabinoxylan, could only be degraded by ß-xylosidase to a minor extent (Kormelink *et al*, 1992 d).
From Table IV it can be seen that the extent of hydrolysis of xylans by endo-xylanases strongly depends from the degree of substitution with arabinofuranosyl groups. Greve *et al* (1984) observed synergism when α-L-arabinofuranosidase of *Ruminococcus albus* was

used in combination with endo-xylanase in the hydrolysis of alfalfa cell wall. Poutanen (1988) did similar observations with *Trichoderma reesei* enzymes on wheat straw arabinoxylan. Not only arabinose, but also xylose and xylo-oligosaccharides were released in higher amounts. Combinations of AXH and Endo I or III of *Aspergillus awamori* also increased the extent of hydrolysis of rice bran, oat spelts, wheat-flour, and larchwood xylan. Kormelink *et al* (1992 e) showed that AXH, but also α-L-arabinofuranosidase A and B of *Aspergillus niger*, released predominantly arabinofuranosyl substituents from single substituted xylopyranosyl groups and were not able to remove significant amounts of arabinofuranosyl substituents from double substituted xylopyranosyl groups. In rice bran and wheat arabinoxylan the amount of double substituted xylopyranosyl residues is rather large. For a higher degree of hydrolysis it is important to remove these substituents as well. From this point of view it is interesting to know that Neukom *et al* (1967) and Tagawa and Kaji (1969) produced a xylan with only traces of arabinose by incubation of wheat arabinoxylan with a fractionated Pectinol R-10 preparation for prolonged periods.

Hardwood xylan, i.e. steamed birchwood xylan, which contained acetyl substituents located on C-2 and C-3 of the xylopyranosyl residues, could be degraded optimally by a combination of endo-xylanase (either I or III), ß-xylosidase and AXH. Cooperativity between acetyl (xylan) esterase and endo-xylanase as observed by Biely *et al* (1986), Poutanen and Sundberg (1988), and Poutanen *et al* (1990 a and b), was also indicated by an increased glycosidic linkage breakdown and/or the release of acetyl groups from beechwood and birchwood acetyl xylan. Wood and McCrae (1986), however, only observed an increase in the rate, but not in the extent of degradation of chemically deacetylated ryegrass cell walls by endo-xylanases and cellulases of *Trichoderma koningii*.

A crude preparation of *Aspergillus awamori* was found to be unable to hydrolyse glucuronoxylan-oligosaccharides from steamed birchwood xylan (Poutanen *et al*, 1986). Steamed birchwood xylan, however, was degraded to a large extent by Endo I or III in combination with ß-xylosidase and AE (Table IV). In the digests it was shown that oligomers accumulated, eluting at 18 min (Fig. 6 and 7). Similar elution profiles were obtained by Puls *et al* (1991) using beechwood glucuronoxylan. These oligomers are therefore presumably glucuronoxylan oligosaccharides. Concerted or stepwise action of endo-xylanase and AE clearly influenced the composition of hydrolysis products, and thereby the degree of hydrolysis from hardwood xylans also. For optimal breakdown of these xylans, glucopyranosyl uronic acid-xylopyranosyl linkages have to be split, because glucopyranosyl uronic acid substituents seem to protect neighbouring ß-(1,4)-linkages of the xylan backbone from being cleaved by endo-xylanases and/or ß-xylosidases. Puls *et al* (1987) demonstrated synergism between endo-xylanase and α-glucuronidase of *Agaricus bisporus* in the degradation of hardwood xylans. They demonstrated that the xylose yield from beechwood glucuronoxylan, could be optimized by the addition of ß-xylosidase and α-glucuronidase to endo-xylanase.

The presence of large amounts of substituents may hinder the formation of enzyme-substrate complexes and thus impede enzymic hydrolysis. Substituents like acetyl,

arabinofuranosyl and glucopyranosyl uronic acid, but also xylopyranosyl and galactopyranosyl as suggested by Shibuya and Iwasaki (1985) for rice bran arabinoxylan, are important in the degradation of [(glucurono)arabino]xylan. Enzymes able to release these substituents, in particular arabinofuranosyl substituents from double branched xylopyranosyl residues, xylopyranosyl and galactopyranosyl substituents (glycosidases), and acetyl groups (acetyl esterases) are therefore essential in the complete degradation of heteroxylans.

Acknowledgements
We are thankful for the methylation analysis carried out by Miss M. Verbruggen.

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Chapter 10

Concluding remarks

The aim of this investigation was to elucidate the mechanism of the enzymic degradation of xylans in the scope of the overall conversion of biomass to monosaccharides. Enzymes which play an important role in this saccharification process are endo-(1,4)-ß-D-xylanases, ß-xylosidases, arabinose-releasing enzymes, acetyl (xylan) esterases, feruloyl and coumaroyl esterases, and α-glucuronidases (Biely, 1985; Chapter 2).

Purification and characterization of xylan-degrading enzymes

Whereas many enzymes have been isolated and characterized, multiple enzyme complexes of xylan-degrading enzymes have been studied from fungi or bacteria only to a small extent. In a study by the group of Smith *et al* (1991) it was shown that the mesophilic fungus *Aspergillus awamori* CMI 142717 produced a whole mixture of xylan-degrading and debranching enzymes which was most suitable for degradation studies.
The purification of xylan-degrading enzymes from a culture filtrate of *Aspergillus awamori* CMI 142717 resulted in three endo-(1,4)-ß-xylanases (Endo I, II and III; Chapter 3), a (1,4)-ß-D-xylosidase (ß-xylosidase; Chapter 3), and a (1,4)-ß-D-arabinoxylan arabinofuranohydrolase (AXH, Chapter 6). The latter enzyme is a novel enzyme which acts on arabinoxylans only. The enzyme does not belong to the group of endo-xylanases or α-L-arabinofuranosidases which are able to split off arabinose from arabinoxylans. An acetyl xylan esterase (AE) was purified from a culture filtrate of *Aspergillus niger* DS 16813 (Chapter 8).
Enzyme characterization revealed molecular weights in the range of 20-40 kDa except for ß-xylosidase which showed a molecular weight of 110 kDa. Their pH and temperature optima were in the range of 4.0-6.5 and 45-70°C, respectively. These characteristics were in accordance with data as found in the literature.
Concerning the mode of action, not only kinetic parameters were determined but also their action towards different substrates. In comparison to the literature, a more detailed approach was carried out by using techniques like ^{1}H-n.m.r. spectroscopy and high performance anion-exchange chromatography (h.p.a.e.c.). In combination with well characterised substrates, these techniques provided us with a lot of new data on the specificity of these enzymes towards polymeric and oligomeric material.

Mode of action

A model for the mode of action of Endo I and III was proposed based on data of the hydrolysis of polymeric arabinoxylan, of a whole range of well characterised arabinoxylan-oligosaccharides and of xylo-oligosaccharides (Chapter 5). Endo I needs at least one and two unsubstituted xylopyranosyl residues adjacent to single and double substituted xylopyranosyl residues towards the reducing end, respectively, whereas Endo III needs at least three unsubstituted xylopyranosyl residues adjacent to single or double substituted xylopyranosyl residues towards the reducing end. So far our knowledge of the release of glucuronoxylan oligosaccharides from glucuronoxylans is limited but an approach similar to arabinoxylans could enable us to substract also a model for the mode of action towards glucuronoxylans. From these results the effect of glucopyranosyl uronic acid substituents on the mode of action of Endo I and III in comparison to arabinofuranosyl substituents could be determined (Comtat and Joseleau, 1981; Yoshida *et al*, 1990).

ß-Xylosidase has a key function in the overall conversion of xylans to monosaccharides. The enzyme splits off xylopyranosyl groups from the terminal, non-reducing end of xylo-oligosaccharides. The availability of arabinoxylan-oligosaccharides enabled us to study the influence of arabinofuranosyl substituents on the ß-xylosidase activity. The enzyme was only able to split off xylopyranosyl residues from arabinoxylan oligosaccharides when two unsubstituted xylopyranosyl residues were present at the non-reducing, terminal end of the oligosaccharides. The influence of glucopyranosyl uronic acid substituents on the ß-xylosidase activity is yet unknown and needs further study.

AXH and α-L-arabinofuranosidase A and B (Arafur A and Arafur B, respectively; Rombouts *et al*, 1988) were also compared in their mode of action towards arabinoxylan and well characterised oligosaccharides derived thereof (Chapter 7). AXH and Arafur A showed similar action towards α-(1,3)-linked arabinofuranosyl substituents of terminal and non-terminal, single substituted xylopyranosyl residues of oligosaccharides. Arafur B was only active towards α-(1,3)-linked arabinofuranosyl substituents from terminal, single substituted xylopyranosyl residues of oligosaccharides. Only AXH and Arafur B were active towards arabinofuranosyl substituents of polymeric arabinoxylan. AXH was also found to be active towards an oligosaccharide having α-(1,2)-linked arabinofuranosyl substituents of single substituted xylopyranosyl residues. However, more detailed information on the mode of action of these enzymes towards α-(1,2)-linked arabinofuranosyl substituents of polymeric arabinoxylan is desired, because arabinoxylans from other sources may contain considerable amounts of these substituents (Viëtor, 1992).

The acetyl esterase was able to remove all of the acetyl groups of acetylated birchwood xylan. ^{1}H-N.m.r. spectroscopy revealed the release of C-2 as well as C-3 linked acetyl groups from acetylated birchwood xylan (results not shown). The enzyme shows high affinity towards polymeric acetylated xylan (Sundberg and Poutanen, 1991; Chapter 8) and does not need the cooperative action of endo-xylanases. Another group of esterases show high affinity towards oligomeric acetylated xylan (Poutanen and Sundberg, 1988), which

are not able to act on polymeric acetylated xylan without the cooperative action of endo-xylanases. However, no or little information is available on the requirements for the environment of the ester linkages and on the ability of esterases to split different linkages e.g. in acetylated pectin or galactomannan.

Enzymic conversion of xylans

The two major substituents of xylans, arabinofuranosyl and glucopyranosyl uronic acid, have an inhibitory effect on the action of endo-xylanases towards xylans from various sources. Endo III is more inhibited in its action by arabinofuranosyl substituents than Endo I. The effect of glucopyranosyl uronic acid substituents on the two enzymes is yet unknown.

Arabinofuranosyl substituents present in xylans from grasses, cereals, and softwood, occur as single or double branchpoints of xylopyranosyl residues. Arabinose-releasing enzymes like AXH and α-L-arabinofuranosidases of the *Aspergillus niger* type (Kaji, 1984) are needed for the removal of the arabinofuranosyl substituents. However, these enzymes are not able to remove 100% of the arabinofuranosyl substituents. This could be due to their mode of action, as shown for AXH, Arafur A and B (Chapter 7), and to the inability of enzymes to split off arabinofuranosyl substituents from double substituted xylopyranosyl residues. AXH and Arafur B, both active on polymeric substrate, were not tested on arabinoxylan containing high amounts of α-(1,2)-linked arabinofuranosyl substituents. More research has to be done on this aspect. No enzymes exist which can remove all arabinofuranosyl substituents.

Different combinations of endo-xylanases and arabinose-releasing enzymes may affect the overall conversion of xylans to monosaccharides. Arafur A in combination with Endo III would probably result in a higher overall conversion compared to the combination of Arafur B and Endo III, because Arafur B is only able to remove arabinofuranosyl substituents from terminal, non-reducing xylopyranosyl residues of oligosaccharides which are not released by Endo III as the latter enzyme releases arabinoxylan oligosaccharides with arabinofuranosyl substituents linked to non-terminal xylopyranosyl residues only.

Glucopyranosyl uronic acid substituents are present in xylans from grasses, cereals, softwood and hardwoods (Chapter 9). Since glucopyranosyl uronic acid substituents were still linked to oligosaccharides present in different xylan-digests due to the absence of α-glucuronidase activity, the presence of this enzyme could influence the overall conversion of xylans as well. Only two α-glucuronidases have been partly purified up till now (Puls *et al*, 1987; Khandke *et al*, 1989) which have a preference for oligomeric and polymeric glucuronoxylan, respectively. These specificities indicate the existence of two types of α-glucuronidases. However, more studies on the purification and characterization of these enzymes and their cooperative action with endo-xylanases have to be carried out.

Acetyl esterases are important in the bioconversion of hardwood xylan as these xylans may contain a large amount of acetyl substituents.
Results showed that the highest yield of monosaccharides from [(glucurono)arabino]xylans of grasses, cereals and softwoods can be obtained by the combination of an endo-xylanase (I or III), ß-xylosidase and AXH. In case of hardwood xylans, an endo-xylanase, ß-xylosidase and acetyl (xylan) esterase should be used. As the presence of glucopyranosyl uronic acid substituents impedes enzymic degradation of [(glucurono)arabino]xylans, α-glucuronidase should be used in these combinations also.
Arabinoxylans from grasses and cereals were found to be linked intermolecularly by feruloyl and coumaroyl acid (Chapter 2). For a complete disruption of these xylan chains from cell wall particles, these linkages have to be split by feruloyl and coumaroyl esterases. However, no or little information is available on these enzymes and their specificity towards different substituents (Tenkanen *et al*, 1991). Minor traces of galactose or xylose as single side-chains were also found in xylans from grasses and cereals. Glycosidases could be important for the final breakdown of these fragments into monosaccharides.

Enzymic conversion of biomass

As biomass consists partly of insoluble cell wall material, activity towards insoluble xylan could play an important role in the overall conversion. Neither of the enzymes purified were significantly active towards insoluble xylan, and as shown in Chapter 2 only little information is known about this subject. Screening of enzyme preparations for endo-xylanase activity towards insoluble xylan has to be carried out in order to be able to study this aspect into more detail. An improved utilization of biomass and waste materials requires thus a proper combination of enzymes.
The type of application determines the type of strains to be used i.e. fungi, bacteria or yeasts. In the food industry, for example, only food-grade enzyme preparations can be used.
The conditions of the process make demands on the type of enzymes to be used. Pre-treatment of pulp in the paper industry, for example, requires thermostable endo-xylanases with a pH-optimum around neutral, because high temperatures and neutral to alkali conditions are used which could result in a loss of enzyme activity even before they have caused a proper effect on the material.
The aim of the process also determines the type(s) of enzyme(s) to be used. For (simple) modifications only specific enzymes with the proper effect have to be used, for example in the bakery or brewery industry. When a complete degradation to monosaccharides is required for subsequent fermentations, an optimal combination of enzymes has to be used. The types of enzymes in such combinations depend on the composition of the (pre-treated) substrate. Acetyl (xylan) esterases and arabinose-releasing enzymes are not needed for an

overall conversion of alkali-treated and acid-treated biomass, respectively due to the lability of the linkages of acetyl groups and arabinose to the main chain.
The type of process, properties of the enzymes, and aim of the application thus determine the final choice of enzymes to be used.

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Summary

Xylans are heteropolysaccharides consisting of a backbone of (1,4)-linked ß-D-xylopyranosyl residues, bearing arabinofuranosyl, (4-*O*-methyl-)glucopyranosyl uronic acid and acetyl substituents. Xylans occur in appreciable amounts in woody tissues, grasses and cereals. For a complete breakdown of xylans from various sources, a combination of enzymes is needed. The aim of this study was to elucidate the mechanism of enzymic conversion of xylans in the scope of bioconversion of biomass as a renewable source of energy (Chapter 1). The state of the art was presented in a review of data on xylan-degrading and accessory enzymes and their mode of action (Chapter 2).

Three endo-(1,4)-ß-D-xylanases (I, II and III) and one ß-xylosidase have been purified from *Aspergillus awamori* CMI 142717 (Chapter 3). The three endo-xylanases, with molecular weights ranging from 23-39 kDa, were most active at pH 4.0-6.0 and 45-55°C. ß-Xylosidase, which had a molecular weight of 110 kDa, was most active at pH 6.5 and 70°C. Endo-xylanase I released xylotriose, xylobiose and xylose in appreciable amounts from oat spelts xylan, whereas endo-xylanase II and III mainly released xylotriose and xylobiose. ß-Xylosidase cleaved glycosidic linkages from xylo-oligosaccharides and was not able to release significant amounts of xylose from polymeric xylan.

A more detailed study on the release of oligosaccharides from alkali-extractable wheat-flour arabinoxylan by endo-xylanase III has been carried out (Chapter 4). A mixture of arabinoxylan oligosaccharides obtained by endo-xylanase III-treatment of alkali-extractable wheat-flour arabinoxylan, was fractionated by Bio-Gel P2 gel permeation chromatography. Oligosaccharide pools were subsequently purified to homogeneity by high performance anion-exchange chromatography. The structures of the isolated oligosaccharides were then fully elucidated by ^{1}H-n.m.r. spectroscopy. Oligosaccharides with a DP of 5 up till 10 containing single substituted xylopyranosyl residues, single and double substituted xylopyranosyl residues and double substituted xylopyranosyl residues only were identified. From the structures of the arabinoxylan oligosaccharides released by endo-xylanase I it could be derived that at least one, respectively two unsubstituted xylopyranosyl residues adjacent to single and double substituted xylopyranosyl residues towards the reducing end could not be removed by endo-xylanase I. At least two unsubstituted xylopyranosyl residues adjacent to single or double substituted xylopyranosyl residues towards the reducing end could not be removed by endo-xylanase III (Chapter 5). ß-Xylosidase was found to split off xylopyranosyl residues from substituted oligosaccharides only when two unsubstituted xylopyranosyl residues were present at the non-reducing end adjacent to substituted xylopyranosyl residues.

Also an arabinose-releasing enzyme has been purified from the culture filtrate of *Aspergillus awamori*. This enzyme did not belong to the group of α-L-arabinofuranosidases and was able to split of arabinofuranosyl substituents from arabinoxylans only (Chapter 6). The enzyme, assigned (1,4)-ß-D-arabinoxylan arabinofuranohydrolase (AXH), had a molecular weight of 32 kDa and was most active at pH 5.0 and ≈50°C.

The mode of action of AXH and two α-L-arabinofuranosidases (A and B) has been determined by high performance anion-exchange chromatography analysis of arabinoxylan oligosaccharides treated with these enzymes (Chapter 7). AXH and α-L-arabinofuranosidase A were able to split off arabinofuranosyl substituents from terminal as well as non-terminal, single substituted xylopyranosyl residues. α-L-Arabinofuranosidase B was able to split off arabinofuranosyl substituents from terminal non-reducing, single substituted xylopyranosyl residues only. AXH and α-L-arabinofuranosidase B were active towards polymeric as well as oligomeric arabinoxylan, whereas α-L-arabinofuranosidase A was active towards oligomeric arabinoxylan only. ^{1}H-n.m.r. spectroscopy and methylation analysis of polymeric arabinoxylan treated with AXH were in agreement with the results obtained by high performance anion-exchange chromatography analysis of oligosaccharides treated with AXH. However, the results obtained with α-L-arabinofuranosidase B-treated arabinoxylan analysed by ^{1}H-n.m.r. spectroscopy showed some discrepancy with the results obtained from oligosaccharide analysis.

An acetyl xylan esterase has been purified from a culture filtrate of *Aspergillus niger* (Chapter 8). The enzyme had a molecular weight of 30.5 kDa, and was most active at pH 5.5-6.0 and 50°C towards acetylated birchwood xylan. The enzyme was able to release most of the acetyl substituents without the cooperative action of endo-xylanases. Endo-xylanase I, II, III and ß-xylosidase were not able to degrade acetylated birchwood xylan significantly without the interaction of acetyl xylan esterase.

Endo-xylanase I, III, ß-xylosidase, AXH and acetyl xylan esterase have been used to study the optimal breakdown of [(glucurono)arabino]xylans from rice bran, oat spelt, wheat, larch and birchwood. Endo-xylanase I and III were unable to degrade a neutral and acidic fraction of rice bran glucuronoarabinoxylan significantly. Concerted action with AXH was necessary to enable the degradation of this xylan with endo-xylanases. However, the extent of degradation remained low (9.4-13.3%) due to the high degree of substitution of this xylan. Oat spelts arabinoxylan was degraded easily by endo-xylanase I and III, because of the low degree of substitution. Combinations of the endo-xylanases, AXH and ß-xylosidase resulted in the highest degree of hydrolysis (39.7-42.6%). The same was observed for wheat arabinoxylan, however, with a somewhat lower degree of hydrolysis (30.5-33.4%). In the case of larchwood glucuronoarabinoxylan, the glucopyranosyl uronic acid substituents could not be removed due to the absence of α-glucuronidase which probably prevented a high degree of hydrolysis. The maximum degree of hydrolysis was 24.4-26.9%. Polymeric birchwood glucuronoxylan was partly insoluble and resulted therefore in the lowest degree of hydrolysis (16.0%) using endo-xylanase I. Endo-xylanase III was hardly able to degrade this substrate. Steam exploded birchwood xylan, which consisted of a mixture of acetylated xylo-oligosaccharides, could be hydrolysed by endo-xylanase I or III only to a low extent (10.0 and 1.7%, respectively). In combination with acetyl xylan esterase the degradation could be improved significantly. Optimal hydrolysis was obtained by the combination of endo-xylanase (I or III), acetyl xylan esterase and ß-xylosidase (38.2-40.0%).

Samenvatting

Xylanen zijn heterogene polysacchariden opgebouwd uit een hoofdketen van (1,4)-gebonden ß-D-xylopyranosyl eenheden met vertakkingen bestaande uit arabinofuranosyl, (4-*O*-methyl-)glucopyranosyluronzuur en acetyl groepen. Xylanen komen in aanzienlijke hoeveelheden voor in hout, grassen en granen. Om xylanen van verschillende herkomst geheel af te kunnen breken is een combinatie van enzymen nodig. Het doel van dit onderzoek was het ophelderen van het mechanisme van de enzymatische omzetting van xylanen in het kader van de bioconversie van biomassa als alternatieve energiebron (Hoofdstuk 1). Een achtergrond-studie in de vorm van een *State of the Art* van xylaan-afbrekende enzymen, hun karakteristieken en werkingsmechanisme, wordt weergegeven in Hoofdstuk 2.

Drie endo-(1,4)-ß-D-xylanases (I, II en III) en een ß-xylosidase werden gezuiverd uit een cultuurfiltraat van *Aspergillus awamori* CMI 142717 (Hoofdstuk 3). De drie endo-xylanases, met molecuulgewichten in de orde van 23-39 kDa, bleken optimaal actief te zijn bij pH 4.0-6.0 en 45-55°C. ß-Xylosidase, met een molecuulgewicht van 110 kDa, was optimaal actief bij pH 6.5 en 70°C. Endo-xylanase I maakte redelijke hoeveelheden xylotriose, xylobiose en xylose vrij van haver xylaan, terwijl endo-xylanase II en III hoofdzakelijk xylotriose en xylobiose vrijmaakten. ß-Xylosidase splitste glycosidische bindingen van xylo-oligosacchariden en was niet in staat redelijke hoeveelheden xylose vrij te maken van polymeer xylaan. Een gedetailleerde studie werd uitgevoerd naar het vrijkomen van oligosacchariden van tarwe arabinoxylaan met behulp van endo-xylanase III (Hoofdstuk 4). Het hydrolysaat verkregen met endo-xylanase III, werd gefractioneerd met behulp van Bio-Gel P2 gel permeatie chromatografie. De verkregen oligosaccharide pools werden vervolgens gezuiverd met HPLC. De structuren van de gezuiverde oligosacchariden werden opgehelderd met behulp van ^{1}H-n.m.r. spectroscopie. Oligosacchariden met een polymerisatie-graad van 5 tot 10 zijn geïdentificeerd waarin enkel vertakte xylopyranosyl eenheden, enkel en dubbel vertakte xylopyranosyl eenheden, of alleen dubbel vertakte xylopyranosyl eenheden voorkwamen. Uit de structuren van de arabinoxylaan oligosacchariden die zijn vrijgemaakt door endo-xylanase I, kon worden afgeleid dat endo-xylanase I minimaal 1, respectievelijk 2 onvertakte xylopyranosyl eenheden aan het reducerende eind naast enkel en dubbel vertakt xylose niet afsplitst. Zo kon voor endo-xylanase III worden afgeleid dat minimaal 2 onvertakte xylopyranosyl eenheden aan het reducerende eind naast enkel of dubbel vertakt xylose niet worden afgesplitst (Hoofdstuk 5). ß-Xylosidase bleek alleen xylose af te kunnen splitsen van vertakte oligosacchariden indien minimaal twee onvertakte xylopyranosyl eenheden aanwezig waren aan het niet-reducerende uiteinde.

Een arabinose-afsplitsend enzym was tevens gezuiverd uit het cultuur filtraat van *Aspergillus awamori*. Het enzym bleek niet te behoren tot de groep van α-L-arabinofuranosidases en was alleen in staat arabinofuranosyl substituenten af te splitsen van arabinoxylanen (Hoofdstuk 6). Het enzym werd (1,4)-ß-D-arabinoxylaan arabinofuranohydrola-

se (AXH) genoemd. Het had een molecuul gewicht van 32 kDa en was optimaal actief bij pH 5.0 en ≈50°C. De werking van AXH en twee α-L-arabinofuranosidases (A en B) werd bepaald met behulp van HPLC analyse van goed gekarakteriseerde arabinoxylaan oligosacchariden behandeld met bovengenoemde enzymen (Hoofdstuk 7). AXH en α-L-arabinofuranosidase A waren in staat arabinofuranosyl substituenten af te splitsen van zowel de terminale als de niet-terminale, enkel vertakte xylopyranosyl eenheden. α-L-Arabinofuranosidase B bleek alleen in staat te zijn arabinofuranosyl substituenten af te splitsen van terminaal, enkel vertakte xylopyranosyl eenheden. AXH en α-L-arabinofuranosidase B waren actief op zowel polymeer als oligomeer arabinoxylaan, terwijl α-L-arabinofuranosidase A alleen actief was op oligomeer arabinoxylaan. ^{1}H-n.m.r. spectroscopie en methylerings analyse van polymeer arabinoxylaan behandeld met AXH gaven resultaten die in overeenstemming waren met de resultaten verkregen uit HPLC analyse van oligosacchariden behandeld met AXH. In het geval van α-L-arabinofuranosidase B bestaat er echter enige discrepantie tussen de verkregen resultaten.

Een acetyl xylaan esterase werd gezuiverd uit een cultuur filtraat van *Aspergillus niger* (Hoofdstuk 8). Het enzym had een molecuulgewicht van 30.5 kDa, en was optimaal actief op acetyl groepen bevattend berkenhout xylaan bij pH 5.5-6.0 en 50°C. Het enzym bleek in staat te zijn praktisch alle acetyl groepen te verwijderen zonder interactie met endo-xylanases. Endo-xylanase I, II, III, en ß-xylosidase waren niet in staat het berkenhout xylaan in enige mate af te breken zonder de inwerking van een acetyl xylaan esterase.

Combinaties van endo-xylanase I, III, ß-xylosidase, AXH en acetyl xylaan esterase werden gebruikt om de afbraak van [(glucurono)arabino]xylanen uit rijst, haver, tarwe, lariks, en berkenhout te bestuderen. Endo-xylanase I en III bleken niet in staat te zijn een neutrale en zure fractie van rijst arabinoxylaan af te breken. Interactie met AXH maakte de afbraak mogelijk. De mate van afbraak bleef echter nog laag (9.4-13.3 %) als gevolg van de hoge substitutiegraad. Arabinoxylaan van haver werd makkelijk afgebroken door endo-xylanase I en III vanwege de lage substitutiegraad. Een combinatie van endo-xylanase, AXH en ß-xylosidase resulteerde in de hoogste afbraak (39.7-42.6 %). Hetzelfde gold voor tarwe arabinoxylaan maar resulteerde in een iets lagere omzettingsgraad (30.5-33.4 %). In het geval van lariks glucuronoarabinoxylaan verhinderden de glucopyranosyl uronzuur substituenten een hoge omzettingsgraad. Voor hun verwijdering is een α-glucuronidase noodzakelijk welke echter ontbrak in deze studies. Uiteindelijk werd een maximale omzetting van 24.4-26.9 % verkregen. Polymeer berkenhout glucuronoxylaan was gedeeltelijk onoplosbaar en als gevolg hiervan maar in beperkte mate afbreekbaar met endo-xylanase I (16.0 %). Endo-xylanase III bleek amper in staat te zijn dit substraat af te breken. Berkenhout xylaan verkregen met behulp van stoom explosie, bleek te bestaan uit een mengsel van geacetyleerde xylo-oligosacchariden en kon alleen in zeer beperkte mate door endo-xylanase I of III afgebroken worden (10.0 en 1.7 %, respectievelijk). De afbraak kon aanzienlijk verbeterd worden door toevoeging van acetyl xylaan esterase. Een optimale afbraak kon verkregen worden door gebruik te maken van endo-xylanase (I of III), acetyl xylaan esterase en ß-xylosidase (38.2-40.0%).

Curriculum vitae

Felix Jozef Maria Kormelink werd geboren op 5 januari 1964 te Stad Delden. In 1982 behaalde hij het VWO-diploma aan het Twickel College te Hengelo (Ov.). In datzelfde jaar is hij met de studie levensmiddelentechnologie begonnen aan de toenmalige Landbouwhogeschool te Wageningen.

Gedurende de studie lag het accent op organische chemie en biochemie. Het vakkenpakket in de eindfase van de studie omvatte een hoofdvak levensmiddelenchemie en een hoofdvak biochemie. Aansluitend op de hoofdvakken is een stage gelopen aan het Rowett Research Institute te Aberdeen (Schotland) als voorbereiding op het promotie-onderzoek.
Van juli 1987 tot december 1991 was hij werkzaam bij de sectie levensmiddelenchemie, microbiologie en hygiëne van de vakgroep levensmiddelentechnologie aan de Landbouwuniversiteit te Wageningen, alwaar het onderzoek is gedaan dat is beschreven in dit proefschrift.