ELSEVIER

www.elsevier.nl/locate/carres

Carbohydrate Research 327 (2000) 401–410

CARBOHYDRATE RESEARCH

Synergy between enzymes from *Aspergillus* involved in the degradation of plant cell wall polysaccharides

Ronald P. de Vries [a], Harry C.M. Kester [a], Charlotte H. Poulsen [b], Jacques A.E. Benen [a], Jaap Visser [a,*]

[a] *Molecular Genetics of Industrial Microorganisms, Wageningen University, Dreijenlaan 2, NL-6703 HA Wageningen, The Netherlands*

[b] *Danisco Ingredients, Edwin Rahrs Vej 38, DK-8220 Brabrand, Denmark*

Received 8 October 1999; accepted 16 February 2000

Abstract

Synergy in the degradation of two plant cell wall polysaccharides, water insoluble pentosan from wheat flour (an arabinoxylan) and sugar beet pectin, was studied using several main-chain cleaving and accessory enzymes. Synergy was observed between most enzymes tested, although not always to the same extent. Degradation of the xylan backbone by endo-xylanase and β-xylosidase was influenced most strongly by the action of α-L-arabinofuranosidase and arabinoxylan arabinofuranohydrolase resulting in a 2.5-fold and twofold increase in release of xylose, respectively. Ferulic acid release by feruloyl esterase A and 4-*O*-methyl glucuronic acid release by α-glucuronidase depended largely on the degradation of the xylan backbone by endo-xylanase but were also influenced by other enzymes. Degradation of the backbone of the pectin hairy regions resulted in a twofold increase in the release of galactose by β-galactosidase and endo-galactanase but did not significantly influence the arabinose release by arabinofuranosidase and endo-arabinase. Ferulic acid release from sugar beet pectin by feruloyl esterase A was affected most strongly by the presence of other accessory enzymes.

Keywords: Xylan; Pectin; Degradation; Xylanolytic enzymes; Pectinolytic enzymes

1. Introduction

Xylan and pectin are the major heteropolysaccharides present in plant cell walls. All xylans contain a Xyl-β-(1→4)-backbone, but large differences exist with respect to the side groups attached to the backbone. The major substituent of wheat arabinoxylan is L-arabinose, but D-galactose, D-4-*O*-methyl-glucuronic acid (MeGluA), and ferulic acid residues are also present [1,2]. In pectin, smooth regions consisting of α-D-(1→4)-linked galacturonic acid residues are interrupted by ramified hairy regions. Side chains consisting mainly of L-arabinose and D-galactose are attached to the α-(1→2)-linked L-rhamnose residues present in the backbone of the hairy regions [3]. Especially sugar beet pectin (SBP) contains high levels of ferulic acid.

Arabinose can be present as single α-(1→3)- or α-(1→2)-linked residues [4] or as short arabino-oligosaccharides in arabinoxylan, but is present as long arabinan and arabinogalactan side chains in pectin. In xylan, galactose

Abbreviations: WIP, water insoluble pentosan from wheat flour; SBP, sugar beet pectin; MeGluA, 4-*O*-methyl glucuronic acid.

* Corresponding author. Tel.: +31-317-484439; fax: +31-317-484011.

E-mail address: office@algemeen.mgim.wau.nl (J. Visser).

0008-6215/00/$ - see front matter
PII: S0008-6215(00)00066-5

has been detected β-(1→5)-linked to single arabinose residues as well as β-(1→4)-linked to xylose in short arabinoxylan side chains [5,6]. Glucuronic acid and its 4-*O*-methyl ether are attached to the xylan main chain via a α-(1→2) linkage [5]. Feruloyl esters are attached to O-5 of the arabinose residues in arabinoxylan [7] and to O-2 of α-(1→5)-linked arabinose residues of the arabinan side chains, or to O-6 of β-(1→4)-linked galactose in the galactan side chains of pectin [8–11].

Due to heterogeneity in the composition and structure of wheat arabinoxylan and pectin, a wide range of enzymes is required for the biodegradation of these polysaccharides. Xylanolytic and pectinolytic enzymes have been purified from many microorganisms, especially from species of the fungus *Aspergillus*. Previously, synergism between endo-xylanases and some accessory enzymes of *Aspergillus* has been demonstrated [12–14]. A combination of different main-chain cleaving enzymes is required for the complete degradation of pectin [15–17]. Degradation of the side chains from the hairy regions requires additional enzymes [12,18–20]. So far, synergy between accessory enzymes involved in the degradation of xylan or pectin has not been studied in detail.

In this paper we have studied 15 xylanolytic and pectinolytic enzymes (Table 1), with respect to their individual activity on wheat arabinoxylan or SBP and the synergy between these enzymes. We have chosen water insoluble pentosan (WIP) from wheat flour as a substrate for our studies, because this polysaccharide contains all the side groups (except acetyl residues) commonly found in xylans. A comparison has been made between incubations with intact WIP and incubations with WIP, which was pre-treated with endoxylanase A to determine whether there was a difference between simultaneous and sequential activity of main-chain cleaving and accessory enzymes.

SBP was chosen because of the high ferulic acid content compared with other pectins and the detailed knowledge, which is available on the structure of this pectin.

2. Results

Analysis of the composition of WIP and SBP.—Xylose and arabinose were the major neutral sugars present in the arabinoxylan sample (Table 2) and only a small amount of galactose, ferulic acid and MeGluA, but no acetyl residues were detected. The isolation of

Table 1
Origin and amounts of the enzymes used in this study

Enzyme	Symbol	Origin	Amount used (μg) [a] (WIP)	Amount used (μg) [b] (SBP)	Reference	Specificity
Arabinofuranosidase B	AbfB	*A. niger*	10.1	22.1	[21]	[22,23]
Arabinoxylan arabino-furanohydrolase A	AxhA	*A. tubingensis*	2.5	–	[24]	[22,23,25–27]
endo-Arabinase	AbnA	*A. niger*	–	20.0	[28]	
endo-Galactanase	GalA	*A. niger*	19.7		megazyme	
endo-Xylanase A	XlnA	*A. tubingensis*	1.0	–	this paper	[4,22,29]
Feruloyl esterase A	FaeA	*A. niger*	0.5	0.9	[12]	[30,31]
α-Galactosidase	AglB	*A. niger*	0.5	–	[32]	
β-Galactosidase	LacA	*A. niger*	2.1	4.2	[32]	
α-Glucuronidase	AguA	*A. tubingensis*	11.0	–	[13]	
Rhamnogalacturonan hydrolase	RhgA	*A. aculeatus*	–	12.0	[33]	
Rhamnogalacturonan acetylesterase	RgaeA	*A. niger*	–	16.7	this paper	
β-Xylosidase	XlnD	*A. niger*	2.5	–	[34]	[25]

[a] As added to 500 μL of 0.5% WIP in the enzyme incubations or pre-treatment.
[b] As added to 500 μL of 1% SBP in the enzyme incubations or pre-treatment.

Table 2
Sugar and ferulic acid analysis of WIP and SBP

Component	% of WIP (mass)	% of SBP (mass)
Xylose	32.1	<0.1
Arabinose	20.7	5.0
Fucose	ND [a]	<0.1
Galactose	2.1	9.6
Glucose	11.2	<0.1
Mannose	ND	<0.1
Rhamnose	ND	2.4
4-*O*-methyl glucuronic acid	6.0	ND
Ferulic acid	0.49	0.63
Cellulose	9.4	ND

[a] ND, not determined.

Table 3
Influence of accessory enzymes on the release of xylose by XlnA and XlnD from WIP [a]

Enzymes	Intact WIP	WIP treated with XlnA
XlnA	4.4	ND
XlnA/AbfB	10.9	6.5 [b]
XlnA/AxhA	8.9	4.8 [b]
XlnA/AbfB/AxhA/FaeA	16.8	ND
XlnA/XlnD	52.4	ND
XlnD	12.6	21.9
XlnD/AbfB	19.6	45.2
XlnD/AxhA	23.3	71.6
XlnA/XlnD/AbfB/AxhA/AguA/AglB/LacA/FaeA	98.7	99.6 [b]

[a] Values are the percentage of the total amount of xylose present in the WIP sample. ND, not determined. Absolute variations between duplicate incubations were all between 0.1 and 0.6%.

[b] XlnA was only used for the pre-treatment but is not present in the incubations.

WIP did not prevent low amounts of glucose and cellulose to be present in the sample. SBP consists predominantly of galacturonic acid but also contains smaller amounts of neutral sugars. Galactose was found to be the major neutral sugar component of sugar beet pectin, followed by arabinose and rhamnose (Table 2). WIP and SBP contain similar amounts of ferulic acid.

Synergistic effects between enzymes involved in the degradation of WIP.—XlnA alone was able to release 4.4% of the total amount of xylose from WIP but in combination with XlnD 52.4% was released (Table 3). AbfB and AxhA acted synergistically with both XlnA and XlnD resulting in increased xylose release. The presence of AglB, LacA, AguA and FaeA resulted in a minor increase in the amount of xylose released by XlnA (data not shown).

The actions of AbfB and AxhA are complementary in the degradation of WIP (Table 4). All other enzymes tested positively influence the release of arabinose by these enzymes. An exception is XlnD, which increases arabinose release by AbfB from intact arabinoxylan, but reduces the amount of arabinose released from pre-treated arabinoxylan (Table 4). XlnD positively influences the action of AxhA on both substrates.

FaeA alone was able to release 14.4% of the total amount of ferulic acid present in wheat arabinoxylan (Table 5). XlnA had the strongest influence on the action of FaeA, whereas AbfB or AxhA only resulted in a minor increase in ferulic acid release. The highest release of ferulic acid was observed when all enzymes acted simultaneously. XlnA also had a strong effect on the release of MeGluA by AguA (Table 6). This enzyme alone released only 0.6% of the total amount of MeGluA from intact WIP and 6.7% from WIP pre-treated with XlnA. When AguA and XlnA were incubated simultaneously 86.7% of the total amount of MeGluA was released from intact WIP. The addition of other accessory enzymes did not significantly increase the amount of MeGluA released.

Synergistic effects between enzymes involved in the degradation of SBP.—The molecular mass distribution of SBP was determined, before and after incubations with RhgA alone and in combination with RgaeA, using size exclusion chromatography. RhgA alone only resulted in a minor decrease in the molecular mass of the pectin polymer (Fig. 1), whereas the combination of RhgA and RgaeA resulted in a large decrease of the molecular mass. These results demonstrate that the removal of acetylesters by RgaeA is essential for the action of RhgA.

Table 4
Arabinose release from WIP by AbfB and AxhA [a]

Additional enzymes	Intact WIP			WIP pre-treated with XlnA		
	AbfB	AxhA	AbfB/AxhA	AbfB	AxhA	AbfB/AxhA
–	40.9	23.7	62.2	54.3	35.6	99.8
XlnA	43.6	37.8	86.4	ND	ND	ND
XlnD	52.0	29.2	83.1	49.8	38.5	92.7
AguA	54.5	29.3	ND	59.4	40.6	ND
AglB	42.9	28.4	ND	58.4	41.5	ND
LacA	46.1	29.8	ND	55.2	40.0	ND
FaeA	52.5	28.3	67.9	58.9	42.1	97.3
XlnA/FaeA	53.9	30.5	91.4	ND	ND	ND

[a] The values are the percentage of the total amount of arabinose present in the WIP sample. ND, not determined. Absolute variations between duplicate incubations were all between 0.1 and 1.0%.

FaeA alone was able to release some ferulic acid from RhgA/RgaeA-treated sugar beet pectin (6.3%), but not from untreated sugar beet pectin (Table 7). Of the other accessory enzymes, LacA had the strongest effect on FaeA activity, raising the amount of ferulic acid released to 14.7%. Incubations with FaeA, AbfB, AbnA, LacA, and GalA resulted in the highest release of ferulic acid from RhgA/RgaeA treated pectin (58%).

Arabinose release by AbnA was higher in untreated pectin than in RhgA/RgaeA treated pectin (Table 8), whereas no significant diffrence was observed using AbfB or a combination of AbnA and AbfB. Addition of LacA and GalA did not result in an increase in the amount of arabinose released by AbfB and AbnA (data not shown). The amount of galactose released by LacA, GalA, and a combination of these enzymes was higher from treated pectin than from untreated pectin (Table 9). FaeA did not influence the release of arabinose or galactose from untreated pectin in incubations using AbfB, AbnA, LacA, and GalA (data not shown). Addition of FaeA to incubations of treated pectin with AbnA alone increased the amount of arabinose twofold, but only a minor effect was observed in incubations with AbfB (Table 8). The release of galactose from pre-treated pectin was increased by the addition of FaeA in all enzyme incubations (Table 9). The highest release of galactose was observed in incubations with all five enzymes.

3. Discussion

So far, synergy in xylan degradation has only been reported between XlnA and AguA [13] and XlnA and AxhA [25,26] and XlnA and FaeA [12,35]. The data in this paper demonstrates that complex synergistic actions exist between all enzymes involved in the degradation of WIP. This correlates well with the coordinated regulation of expression of the genes encoding these enzymes. All genes are expressed in the presence of xylose and all, except *abf*B, are under the control of the xylanolytic transcriptional activator XlnR [36,37].

Table 5
Influence of main chain cleaving enzymes and accessory enzymes on ferulic acid release by FaeA from WIP [a]

Enzymes	Intact WIP	WIP treated with XlnA
FaeA	14.4	40.8
FaeA/AbfB	16.2	44.6
FaeA/AxhA	16.9	43.4
FaeA/AbfB/AxhA	16.6	45.1
FaeA/XlnA	55.0	ND
FaeA/AbfB/AxhA/XlnA	96.9	ND
FaeA/AbfB/AxhA/AguA/XlnD/AglB/LacA	17.2	50.4
FaeA/AbfB/AxhA/AguA/XlnD/AglB/LacA/XlnA	97.5	ND

[a] Values are the percentage of the total amount of ferulic acid present in the WIP sample. ND, not determined. Absolute variations between duplicate incubations were all between 0.1 and 0.9%.

Table 6
Influence of XlnA and accessory enzymes on the release of 4-*O*-methyl glucuronic acid by AguA from WIP [a]

Enzymes	Intact WIP	WIP treated with XlnA
AguA	0.6	6.7
AguA/XlnA	86.7	ND
AguA/AbfB	3.2	16.2
AguA/AxhA	1.6	12.3
AguA/AglB/LacA/AbfB/AxhA/FaeA	3.8	59.2
AguA/AglB/LacA/AbfB/AxhA/FaeA/XlnD	6.2	72.8
AguA/AglB/LacA/AbfB/AxhA/FaeA/XlnD/XlnA	88.9	ND

[a] Values are the percentage of the total amount of 4-*O*-methyl glucuronic acid present in the WIP sample. ND, not determined. Absolute variations between duplicate measurements were all between 0.1 and 0.9%.

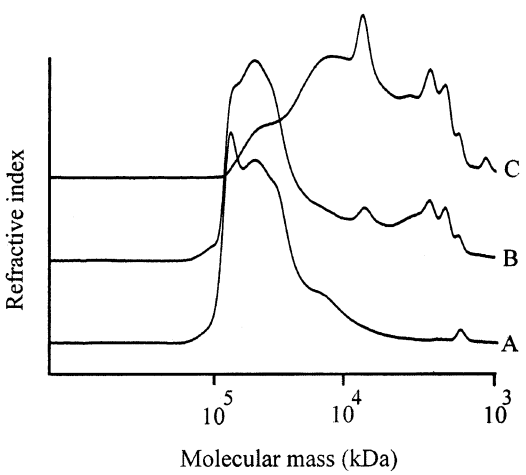

Fig. 1. Molecular mass distribution of sugar beet pectin before (A), and after a treatment with RhgA (B), and a combination of RhgA and RgaeA (C).

All enzymes tested in this paper increased the amount of xylose liberated by XlnA, indicating not only that XlnA prefers unsubstituted regions of xylan as a substrate but also that XlnA is able to cleave the linkage between a terminal xylose residue and the adjacent residue. The stronger synergistic effect between AguA and XlnA compared with a previous study [13] can be attributed to the ability of XlnA to remove terminal xylose residues from xylan, which becomes an important factor when the incubation time is longer (24 h in this study compared with 30 min [13]). The presence of additional enzymes does not significantly increase the amount of MeGluA released from untreated WIP but has a strong positive effect on the release of MeGluA from treated WIP, even though the release does not reach the level of the simultaneous XlnA/AguA treatment. The increase in MeGluA release from treated WIP is most likely an indirect effect of the other enzymes on the activity of XlnD.

Previous studies indicated that AxhA is able to release α-(1→2)- and α-(1→3)-linked arabinose residues from both terminal and non-terminal singly substituted xylose residues, whereas AbfB can only release arabinose from terminal singly substituted xylose residues [4].

Table 7
Influence of the RhgA and RgaeA pre-treatment and the addition of other accessory enzymes on ferulic acid release from SBP by FaeA [a]

Enzyme	Untreated SBP	Treated SBP
FaeA	0	6.3
FaeA/AbfB	2.4	9.4
FaeA/AbnA	0	7.4
FaeA/LacA	0	14.7
FaeA/GalA	0	7.9
FaeA/AbfB/AbnA	4.7	9.7
FaeA/LacA/GalA	0.5	20.1
FaeA/LacA/AbfB	1.4	23.9
FaeA/LacA/AbnA	0	13.4
FaeA/LacA/GalA/AbfB	4.2	38.4
FaeA/LacA/GalA/AbnA	2.6	19.5
FaeA/LacA/AbfB/AbnA	5.5	22.4
FaeA/LacA/GalA/AbfB/AbnA	7.9	58.4

[a] Values are in percentage of the total amount of ferulic acid present in the SBP sample. Absolute variations between duplicate measurements were all between 0.1 and 1.1%.

Table 8
Influence of the RhgA and RgaeA pre-treatment and the addition of FaeA on arabinose release from SBP by AbfB and AbnA [a]

Enzyme	Untreated SBP	Treated SBP	Treated SBP with FaeA
AbfB	64.7	62.4	68.4
AbnA	2.7	5.9	10.4
AbfB/AbnA	68.6	64.8	69.5

[a] Values are in percentage of the total amount of arabinose present in the SBP sample. Absolute variations between duplicate measurements were all between 0.1 and 1.1%.

Table 9
Influence of the RhgA and RgaeA pre-treatment and the addition of FaeA on galactose release from SBP by LacA and GalA [a]

Enzyme	Untreated SBP	Treated SBP	Treated SBP with FaeA
LacA	0.9	8.2	10.4
GalA	8.3	10.0	12.4
LacA/GalA	15.2	30.4	34.9
LacA/GalA/AbfB/AbnA	26.7	44.1	50.4

[a] Values are in percentage of the total amount of galactose present in the SBP sample. Absolute variations between duplicate measurements were all between 0.1 and 0.8%.

The data presented in this paper demonstrates that AbfB is also capable of removing arabinose residues from non-terminal xylose residues, since incubations on intact arabinoxylan with this enzyme result in release of 40% of the total amount of arabinose present. The negative effect of XlnD on AbfB activity suggests that AbfB prefers longer oligosaccharides as substrate, whereas AxhA has a higher activity on shorter oligosaccharides. The addition of AguA and FaeA results in similar relative increases in arabinose released by both AbfB and AxhA, demonstrating that the presence of MeGluA or ferulic acid residues limits the activity of both enzymes.

LacA and AglB alone released only 0.4 and 1.2%, respectively, of the total amount of galactose present in the WIP sample and incubations combining these enzymes with XlnA, XlnD, AbfB, AxhA, AguA and FaeA released 5.6% of the galactose present (data not shown). It is possible that the WIP sample contains a small amount of arabinogalactan, reducing the amount of galactose actually attached to arabinoxylan.

FaeA was shown previously to be active on an arabinose–ferulic acid dimer as well as on small xylo-oligosaccharides containing arabinose linked ferulic acid [30]. Our data indicate that the action of XlnA is a crucial step to allow efficient removal of ferulic acid residues by FaeA, which is then further enhanced by AbfB and AxhA. In the past it has been reported that FaeA is not active against SBP [38,39]. These measurements were performed in the absence of enzymes degrading the rhamnogalacturonan backbone, which are essential for the activity of FaeA on SBP as demonstrated in this paper. FaeA is able to release galactose-linked, but not arabinose-linked ferulic acid from SBP derived oligosaccharides [30]. This is in agreement with the data shown here. Synergy was mainly observed between FaeA and LacA, indicating a preference of the esterase for galactose linked ferulic acid. The addition of AbfB further increases the amount of ferulic acid released, most likely due to the degradation of the arabinan side chains by AbfB resulting in an increased accessibility of the galactose-linked ferulic acid residues. A second feruloyl esterase from *A. niger* (CinnAE) able to release ferulic acid from SBP [20], was shown to be mainly active on arabinose-linked ferulic acid. This enzyme acted synergistically with AbnA in the release of ferulic acid, but no positive interaction was observed with GalA or LacA [20]. This indicates that FaeA and CinnAE have complementary activities.

LacA and GalA acted synergistically in the release of galactose from SBP. Addition of AbfB and AbnA to incubations with LacA and GalA resulted in a further increase in the amount of galactose released. A structure has been proposed for sugar beet pectic side chains in which short galactan side chains connect the longer arabinan chain to the rhamnogalacturonan backbone [40,41]. This is in agreement with the data described above. In such a structure the long arabinan side chains that extend away from the pectin backbone would be accessible for AbfB and AbnA, whereas they would shield the shorter galactan chains. By hydrolysing the rhamnogalacturonan backbone or degrading the arabinan chains with AbfB and AbnA, the galactan chains become more accessible re-

sulting in an increased activity of LacA and GalA. The positive effect of FaeA on galactose release is probably due to the ability of this enzyme to remove ferulic acid residues from the galactanside chains making them more accessible to hydrolysis. CinnAE has a stronger effect on the release of arabinose by AbfB [20]. Adding this enzyme to incubations with the five enzymes used in this study (AbfB, AbnA, LacA, GalA and FaeA) might result in a further increase in the release of both arabinose and ferulic acid from SBP.

This paper presents a detailed study of the synergy between accessory enzymes involved in the degradation of xylan and pectin. The efficient degradation of heteropolysaccharides by *Aspergillus* depends on a complex system of synergistic interactions between enzymes cleaving the different linkages in these structures. The systems for the regulation of gene expression present in *Aspergillus* ensure the simultaneous production of this wide range of cell wall degrading enzymes.

4. Experimental

Sugar and ferulic acid analysis of WIP and SBP.—Water insoluble pentosan (WIP) from wheat flour was isolated by the method of Gruppen and co-workers [42]. The sugar analysis was based on the work of Englyst and Cummings [43] and involved acid hydrolysis of WIP with 1 M H_2SO_4 after which the released monomers were determined by high-performance liquid chromatography (HPLC) using a Carbopac PA1 column (Dionex Corp., Sunnyvale, Ca) and a pulsed electrochemical detector. The ferulic acid content was determined as described previously [44].

Purification of XlnA from A. tubingensis.—Several preparations of partially purified xylanase A from *Aspergillus tubingensis* were pooled with a total volume of 250 mL. This material was rotary evaporated to 50 mL and desalted on Sephadex G25M (50 × 250 mm) in water. This protein pool (120 mL) was separated in three runs on a Tosohaas TSK DEAE 650S ion exchange column (35 × 100 mm, Merck, Darmstadt, Germany) equilibrated in 20 mM Tris, pH 7.5, at a flow of 8 mL/min. XlnA was eluted with a linear gradient from 0 to 0.5 M NaCl in 20 mM Tris, pH 7.5. The resulting pool of 130 mL was separated on a Source 15 PHE (50 × 100 mm) hydrophobic interaction column equilibrated in 50 mM acetate buffer, 1.5 M $(NH_4)_2SO_4$, pH 5.0. XlnA was eluted with a 1800 mL linear gradient from equilibration buffer to 50 mM acetate buffer, pH 5.0 at a flow of 10 mL/min. The enzyme pool was concentrated and desalted into water and the pH was adjusted to 5.0 using acetate buffer. The purity of the preparation was tested on a Mono Q column (Pharmacia, Uppsala, Sweden).

Cloning, sequencing and overexpression of the A. niger rgae*A gene encoding rhamno–galacturonan acetylesterase (RgaeA).*—The *rgae*A gene was cloned from an *A. niger* N400 λ_{EMBL4} genomic library as described previously for the *fae*A gene encoding feruloyl esterase [12] using part of the *A. aculeatus rgae*A gene as a probe. The *A. aculeatus rgae*A cDNA sequence (acc no AJ242854) was used to design two oligonucleotides (5′-ACCGCCGTCTTGCACC-3′ and 5′-CCGTGCATACCACCGCC-3′), which were used in PCR with *A. aculeatus* CBS 101.43 DNA as a template. The resulting 692 bp DNA fragment was sequenced to verify its identity and used as a probe. The sequence of the *A. niger rgae*A gene has been established as described before [12] and has been deposited in the EMBL nucleotide database (acc no AJ242854). Overexpression of the *A. niger rgae*A gene was accomplished using a translational promoter fusion in which the constitutive promoter of the *A. niger pep*E gene, encoding a vacuolar aspartic protease [45]. The fusion of the *pep*E promoter and the *rgae*A gene followed the same strategy as outlined by Benen and co-workers [15] for the fusion of the *pki*-promoter of the pyruvate kinase gene and several *A. niger* endopolygalacturonases encoding genes, and resulted in plasmid pIM3874. This plasmid was used to transform the *A. niger* strain NW188 (*csp*A1, *pyr*A6, *leu*A1, *gox*C17, *prt*F28) as previously described [46]. The *A. niger pep*E–*rgae*A transformant NW188::pIM3874.43, which produced the highest amount of RgaeA, was used for large scale production and purification of the enzyme.

RgaeA purification.—*A. niger pep*E–*rgae*A transformant NW188::pIM3874.43 was cultured using a 2.5 L fermentor in minimal medium [12] with 4 g/L NH_4Cl as N-source instead of $NaNO_3$, supplemented with trace elements [47], 2 g/L yeast extract, 0.5 g/L casamino acids and 90 g/L glucose, as carbon source. The culture medium was inoculated with 10^6 spores/mL and the fermentation was carried out for 20 h at 700 rpm and 30 °C. Relative O_2-saturation was maintained at 30%. The pH was regulated at pH 3.5 by the addition of 5 M NaOH. Culture fluid was collected by filtration, diluted fivefold with water and adjusted to pH 6. Next, 50 g DEAE-Streamline (Pharmacia-Biotech) was added to the filtrate and stirred for 1 h, after which the matrix was collected by filtration. RgaeA was recovered by elution with 10 mM piperazine/HCl buffer pH 6.0, 1 M NaCl, followed by extensive dialysis against the same buffer without the salt. The enzyme was loaded onto a Q-Sepharose Fast Flow column (5×5 cm) (Pharmacia–Biotech) equilibrated in 20 mM piperazine/HCl pH 6.0 and eluted with a linear 1500 mL NaCl gradient (0–1 M). RgaeA containing fractions were pooled and dialysed against 20 mM piperazine/HCl pH 5.2 prior to storage at −20 °C. From 2 L of culture fluid 270 mg RgaeA was purified.

Enzyme assay.—RgaeA activity was routinely assayed at 30 °C in 20 mM piperazine/HCl pH 5.0, using 1% (m/v) sugarbeet pectin as substrate. Samples (100 μL) were taken from the reaction mixture, and the pectin was precipitated by the addition of 100 μL 2-propanol followed by centrifugation. The supernatant was evaporated under vacuum and the residual material was dissolved in 50 μL of water. Acetate release was analysed by HPLC using an Aminex HPX-87H (Biorad) column eluted with 25 mM HCl at 50 °C and using UV (210 nm) and RI detection. Using these standard conditions the activity of RgaeA was 0.3 U/mg (= 5 nkat/mg).

Synergism with rhamnogalacturonan hydrolase (RhgA).—The synergistic effect between RgaeA and *A. aculeatus* RhgA was investigated by incubation of a 500 μL of 1% (m/v) sugar beet pectin solution in 20 mM piperazine/HCl pH 5.0 for 16 h at 30 °C with RgaeA (100 μg) and RgaeA and RhgA (40 μg), respectively. Reactions were stopped by boiling for 5 min. The synergistic effect was monitored by determining the molecular mass distribution of the sugar beet pectin before and after enzymatic treatment. Samples (50 μL) were analysed by high-performance size-exclusion chromatography (HPSEC) as described before [48].

Xylan and pectin incubations.—The amount of enzyme necessary when acting alone to obtain the maximum amount of released monomeric compound after 24 h of incubation was determined by incubating WIP and SBP with the individual enzymes and carrying out a time-dependent analysis of the amount of monomeric compound released. The amounts of the pure enzymes that were applied are indicated in Table 1. Enzyme incubations were all performed in duplicate, using 500 μL of a 0.5% WIP or 1% SBP suspension in 50 mM and 10 mM NaOAc buffer (pH 4.5), respectively, at 30 °C for 24 h. The enzymes were inactivated by heating them in a boiling water bath for 5 min. Undissolved material was precipitated by centrifugation (14,000*g*, 10 min) and the supernatant was transferred to a new tube and stored at −20 °C.

The purity of the enzymes was verified by analysing the time-course incubations of the individual enzymes for the release of all monomeric components of WIP and SBP. All enzymes only released their specific product, indicating that the enzyme samples do not contain small amounts of other enzymes.

Pre-treatment of WIP with XlnA and SBP with RhgA and RgaeA.—To 100 mL of a 0.5% WIP solution in 50 mM NaOAc buffer (pH 4.5) 0.1 mg XlnA was added and 2.4 mg RhgA and 3.4 mg RgaeA were added to 100 mL of a 1% pectin solution in 10 mM NaOAc buffer (pH 4.5). The mixtures were incubated for 72 h at 30 °C to degrade the arabinoxylan and rhamnogalacturonan backbone, after which the enzymes were inactivated by heating the mixtures for 10 min in a boiling water bath.

Sugar and ferulic acid analysis of the incubations.—Ferulic acid concentrations in the incubations were analysed by HPLC with a type RP-8 reverse phase column (Supelco, Belle-

fonte, PA. Separation was achieved by using a linear gradient starting with 32, 0.3, 67.7% (v/v) MeOH, AcOH, water and ending with 64, 0.3, 35.7% (v/v) MeOH, AcOH, water with detection at 325 nm. MeGluA concentrations were also determined by HPLC using a Carbopac PA-100 column (Dionex Corp., Sunnyvale, CA). Separation was achieved by using a linear gradient starting with 100 mM NaOH, 5 mM NaOAc and ending with 100 mM NaOH, 400 mM NaOAc over a 20 min period at a flow of 1 mL/min. Xylose, arabinose and galactose concentrations were determined using a Carbopac MA-1 column (Dionex Corp., Sunnyvale, CA) and isocratic elution with 0.48 M NaOH. Detection of both MeGluA and neutral sugars was carried out using a pulsed amperometric detector (PAD).

Acknowledgements

R.P. de Vries was financed by a grant to J. Visser from Danisco Ingredients, Brabrand, Denmark. Further financial support was obtained from the European Community AIR2 CT94 1345. We thank Karen Marie Søndergaard and Michael Thorsen at Danisco Ingredients for the sugar and ferulic acid analysis of the WIP sample and Troels N. Gravesen and Masoud R. Zargahi at Danisco Ingredients for the purification of XlnA, AxhA and AguA from *A. tubingensis*.

References

[1] J.-M. Brillouet, J.-P. Joseleau, *Carbohydr. Res.*, 159 (1987) 109–126.
[2] M.E.F. Schooneveld-Bergmans, A.M.C.P. Hopman, G. Beldman, A.G.J. Voragen, *Carbohydr. Polym.*, 35 (1998) 39–47.
[3] J.A. de Vries, F.M. Rombouts, A.G.J. Voragen, W. Pilnik, *Carbohydr. Polym.*, 2 25–33.
[4] F.J.M. Kormelink, R.A. Hoffmann, H. Gruppen, A.G.J. Voragen, J.P. Kamerling, J.F.G. Vliegenthart, *Carbohydr. Res.*, 249 (1993) 369–382.
[5] K.C.B. Wilkie, S.-L. Woo, *Carbohydr. Res.*, 57 (1977) 145–162.
[6] A. Ebringerová, Z. Hromádková, E. Petráková, M. Hricovíni, *Carbohydr. Res.*, 198 (1990) 57–66.
[7] W.S. Borneman, D.E. Akinand, W.P. van Eseltine, *Appl. Environ. Microbiol.*, 52 (1986) 1331–1339.
[8] F. Guillon, J.-F. Thibault, *Carbohydr. Res.*, 190 (1989) 85–96.
[9] F. Guillon, J.-F. Thibault, *Carbohydr. Res.*, 190 (1989) 97–108.
[10] M.-C. Ralet, J.-F. Thibault, C.B. Faulds, G. Williamson, *Carbohydr. Res.*, 263 (1994) 227–241.
[11] I.J. Colquhoun, M.-C. Ralet, J.-F. Thibault, C.B. Faulds, G. Williamson, *Carbohydr. Res.*, 263 (1994) 243–256.
[12] R.P. de Vries, B. Michelsen, C.H. Poulsen, P.A. Kroon, R.H.H. van den Heuvel, C.B. Faulds, G. Williamson, J.P.T.W. van den Hombergh, J. Visser, *Appl. Environ. Microbiol.*, 63 (1997) 4638–4644.
[13] R.P. de Vries, C.H. Poulsen, S. Madrid, J. Visser, *J. Bacteriol.*, 180 (1998) 243–249.
[14] T.M. Wood, S.I. McCrae, *Appl. Microbiol. Biotechnol.*, 45 (1996) 538–545.
[15] J.A.E. Benen, H.C.M. Kester, J. Visser, *Eur. J. Biochem.*, 593 (1999) 577–585.
[16] L. Parenicová, J.A.E. Benen, H.C.M. Kester, J. Visser, *Eur. J. Biochem.*, 251 (1998) 72–80.
[17] M. Mutter, I.J. Colquhoun, H.A. Schols, G. Beldman, A.G.J. Voragen, *Plant Physiol.*, 110 (1996) 73–77.
[18] F.M. Rombouts, A.G.J. Voragen, M.F. Searle-van Leeuwen, C.C.J.M. Geraeds, H.A. Schols, W. Pilnik, *Carbohydr. Polym.*, 9 (1988) 25–47.
[19] J.W. van de Vis, M.J.F. Searle-van Leeuwen, H.A. Siliha, F.J.M. Kormelink, A.G.J. Voragen, *Carbohydr. Polym.*, 16 (1991) 167–187.
[20] P.A. Kroon, G. Williamson, *Biotechnol. Appl. Biochem.*, 23 (1996) 263–267.
[21] P. van der Veen, M.J.A. Flipphi, A.G.J. Voragen, J. Visser, *Arch. Microbiol.*, 157 (1991) 23–28.
[22] M.A. Verbruggen, G. Beldman, A.G.J. Voragen, *Carbohydr. Res.*, 306 (1998) 275–282.
[23] F.J.M. Kormelink, H. Gruppen, A.G.J. Voragen, *Carbohydr. Res.*, 249 (1993) 345–353.
[24] M. Scott, I.F. Connerton, G.W. Harris, T.N. Gravesen, S.M. Madrid, J.D. Mikkelsen, *Acta Crystallogr., Sect. D*, 53 (1997) 222–223.
[25] F.J.M. Kormelink, A.G.J. Voragen, *Appl. Microbiol. Biotechnol.*, 38 (1993) 688–695.
[26] M.A. Verbruggen, G. Beldman, A.G.J. Voragen, *Carbohydr. Res.*, 306 (1998) 265–274.
[27] F.J.M. Kormelink, M.J.F. Searle-Van Leeuwen, T.M. Wood, A.G.J. Voragen, *Appl. Microbiol. Biotechnol.*, 35 (1991) 231–232.
[28] D. Ramon, P. van der Veen, J. Visser, *FEMS Microbiol. Lett.*, 113 (1993) 15–22.
[29] F.J.M. Kormelink, H. Gruppen, R.J. Viëtor, A.G.J. Voragen, *Carbohydr. Res.*, 249 (1993) 355–367.
[30] M.-C. Ralet, C.B. Faulds, G. Williamson, J.-F. Thibault, *Carbohydr. Res.*, 263 (1994) 257–269.
[31] G. Williamson, C.B. Faulds, P.A. Kroon, *Biochem. Soc. Trans.*, 26 (1998) 205–209.
[32] P. Manzanares, L.H. de Graaff, J. Visser, *Enzyme Microbiol. Technol.*, 22 (1998) 383–390.
[33] M.E.G. Suykerbuyk, P.J. Schaap, H. Stam, W. Musters, J. Visser, *Appl. Microbiol. Biotechnol.*, 43 (1995) 861–870.
[34] N.N.M.E. van Peij, J. Brinkman, M. Vrsanska, J. Visser, L.H. de Graaff, *Eur. J. Biochem.*, 245 (1997) 164–173.
[35] B. Bartolome, C.B. Faulds, M. Tuohy, G.P. Hazlewood, H.J. Gilbert, G. Williamson, *Biotechnol. Appl. Biochem.*, 22 (1995) 65–73.
[36] N.N.M.E. van Peij, M.M.C. Gielkens, R.P. de Vries, J. Visser, L.H. de Graaff, *Appl. Environ. Microbiol.*, 64 (1998) 3615–3619.
[37] R.P. de Vries, H.C. van den Broeck, E. Dekkers, P. Manzanares, L.H. de Graaff, J. Visser, *Appl. Environ. Microbiol.*, 65 (1999) 2453–2460.

[38] C.B. Faulds, G. Williamson, *Microbiology*, 140 (1994) 779–787.
[39] C. Brézillon, P.A. Kroon, C.B. Faulds, G.M. Brett, G. Williamson, *Appl. Microbiol. Biotechnol.*, 45 (1996) 371–376.
[40] P. Albersheim, *Sci. Am.*, 6 (1975) 81–95.
[41] T. Sakamoto, T. Sakai, *Phytochemistry*, 39 (1995) 821–823.
[42] H. Gruppen, J.P. Marseille, A.G.J. Voragen, R.J. Hamer, W. Pilnik, *J. Cer. Sci.*, 9 (1989) 247–260.
[43] H.N. Englyst, J.H. Cummings, *Analyst*, 109 (1984) 103–112.
[44] C.B. Faulds, P.A. Kroon, L. Saulnier, J.-F. Thibault, G. Williamson, *Carbohydr. Polym.*, 27 (1995) 187–190.
[45] G. Jarai, J.P.T.W. van den Hombergh, F.B. Buxton, *Gene*, 145 (1994) 171–178.
[46] M.A. Kusters-van Someren, J.A.M. Harmsen, H.C.M. Kester, J. Visser, *Curr. Genet.*, 20 (1991) 293–299.
[47] W. Vishniac, M. Santer, *Bacteriol. Rev.*, 21 (1957) 195–213.
[48] H.C.M. Kester, J.A.E. Benen, J. Visser, *Biotechnol. Appl. Biochem.*, 30 (1999) 53–57.