Characterization of cell wall degrading enzymes from *Chrysosporium lucknowense* C1 and their use to degrade sugar beet pulp

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Thesis

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Abstract

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Key words	Pectin, arabinan, biorefinery, mode of action, branched arabinose oligomers, ferulic acid esterase, arabinohydrolase, pretreatment

Sugar beet pulp is the cellulose and pectin-rich debris remaining after sugar extraction from sugar beets. In order to use sugar beet pulp for biorefinery purposes, these carbohydrates need to be degraded to fermentable monosaccharides.

The influence of six mild sulfuric acid or water pretreatments at different temperatures on the enzymatic degradability of sugar beet pulp was examined. An optimal pretreatment of 15 min at 140 °C in water greatly facilitates the subsequent enzymatic cellulose degradation.

Enzymatic degradation of pectin requires an array of different enzyme activities. To this end, cell wall degrading enzymes of the filamentous fungus *Chrysosporium lucknowense* C1 (C1) were purified and characterized. Four arabinohydrolases from C1 were characterized that released up to 80 % of the arabinose present in sugar beet arabinan as monomers. A combination of three arabinohydrolases also released unknown branched arabinose oligomers, which were purified and identified using NMR analysis. With the help of these complex arabinose oligomers the mode of action of four C1 α-L-arabinohydrolases was determined to enable controlled and tailored degradation of arabinan. Complete degradation of sugar beet pulp also requires ferulic acid esterase activity. Three C1 ferulic acid esterases were purified and characterized, of which one could release up to 60 % of ferulic acid from feruloylated sugar beet pectin oligomers, whereas the other two where more active toward feruloylated arabinoxylan.

Contents

Chapter 1

General Introduction

1.1   Preface

Refinery of biomass has received a growing interest over the last decade. Global warming, rising energy demands and enhanced competition towards fossil resources put the use of biomass as renewable energy resource into focus. Another driving force, especially in the EU, comes from the agricultural sector itself: Since crop production is relatively expensive in Europe and the governmental protection of the inner market is decreasing, farmers try to use their resources as efficient as possible. The valorization of agricultural side-streams, mostly bulk biomass like straw, fruit pulp, sugar-beet pulp or other residual material left after crop processing, can be one way to increase competitiveness and sustainability in both ecological and economical terms.

1.1.1   The project

In this research project, the enzymatic hydrolysis of sugar-beet pulp is studied. Sugar-beet pulp is the remaining debris after sugar extraction. It mainly consists of polymeric carbohydrates and, to a minor extent, protein (8 w/w %). Its high remaining carbohydrate content of up to 75 w/w % makes it particulary interesting for the use as feedstock for bioethanol production or other biorefinery processes. Prior to such use, the complex carbohydrate material has to be degraded to monosaccharides in a economical, environmentally effective and controlled way. To date, this can be realized in two ways, either thermochemically by acid hydrolysis at elevated temperatures or enzymatically by the use of cell-wall degrading enzymes.

Enzymes allow the controlled degradation of biomass at mild process conditions, but are, to date, often too inefficient to allow the release of fermentable monosaccharides within reasonable time ranges and quantities. While previous research on sugar-beet pulp has focused mainly on enzymatic liquefaction and viscosity reduction to improve processibility, the current research aims the complete enzymatic hydrolysis of sugar-beet pulp to fermentable monosaccharides.

1.2   Sugar-beet growth in Europe

Sugar beet is the main crop for sugar production in Europe. Its cultivation covers 1.8 % of total arable land and approximately 100 Mtons of sugar beets are harvested annually (`http://appsso.eurostat.ec.europa.eu`). Sugar production in Europe was highly subsidized over the last four decades. In 2006, the European commission decided

to restructure the European sugar production. Uncompetitive sugar producers were encouraged to leave the market and the cultivation area in the EU shrank considerably. As a consequence, the sugar production in Europe decreased by 22 %, but remained constant thereafter (eurostat database).

1.2.1   Availability and use of sugar-beet pulp

Sugar-beet pulp is the cell debris that remains after sugar extraction from the sugar-beet roots. Pressed pulp has a dry matter content of ≈ 20 % and it accounts for 5 w/w % of the fresh weight of unprocessed sugar beets (Coughlan et al., 1986). On dry matter basis, 5 Mtons of sugar-beet pulp are annually available (eurostat database). To date, sugar-beet pulp is mainly used as animal feed and, in areas with no animal production it is used as landfill. Due to its low dry matter content, the transportation costs are relatively high and the net value is often close to zero. It has been attempted to use sugar beet pectin as gelling agent in the food industry to replace commercial citrus pectins. However, sugar beet pectin has a poor gelling behavior.

1.2.2   Future perspectives

The use of sugar-beet pulp for biofuel production could be an opportunity to increase competitiveness of beet sugar. The European biofuels directive of 2003 aims the substitution of 10 % of the fossil fuels by biofuels in 2020. Agricultural side-streams, such as sugar-beet pulp, are considered a valuable biomass source to reach these ambitious goals. A major challenge of using biomass for biofuel production is the heterogeneity of the composition of plant cell walls.

1.3   The plant cell wall

The cells of all plants are surrounded by a rigid network of carbohydrates called the cell wall (McNeil et al., 1984). It fulfills a wide range of physiological functions such as stability, flexibility, water holding capacity, barrier against pathogen attack and intercellular communication (Carpita and Gibeaut, 1993). The composition of the wall is changing not only during different developmental stages of a plant (McCann et al., 1992), but is also different between different parts of the plant, different organs and even within a single cell (Burton et al., 2010).

1.3.1 Morphology of the cell wall

Even though the relative amounts and structures of the cell-wall polysaccharides (CWPS) may vary greatly among species, the fundamental mechanisms of interaction between these CWPS are highly similar (Carpita and McCann, 2000). Morphologically, the cell wall can be distinguished in three different parts (Fig. 1.1). The middle lamella is synthesized as the first layer. It is pectin-rich, has a high water-holding capacity and is a deposit for sugars, water and ions (Reiter, 2002). The middle lamella is very flexible allowing the cells to quickly expand (Verhertbruggen and Knox, 2006). It is the outermost layer of the cell wall and is, in later stages of development, responsible for cell-cell signaling and pathogen-attack response mechanisms (Cosgrove, 2005). After the middle lamella the primary plant cell wall is synthesized. It mainly consists of celluloses and hemicelluloses forming a more rigid but still flexible network (McNeil et al., 1984). When cell growth ceases the secondary cell wall is formed. The hemicellulose-cellulose network is fortified by the secretion and polymerization of phenols, leading to the formation of the stiff, extremely robust lignocellulose network.
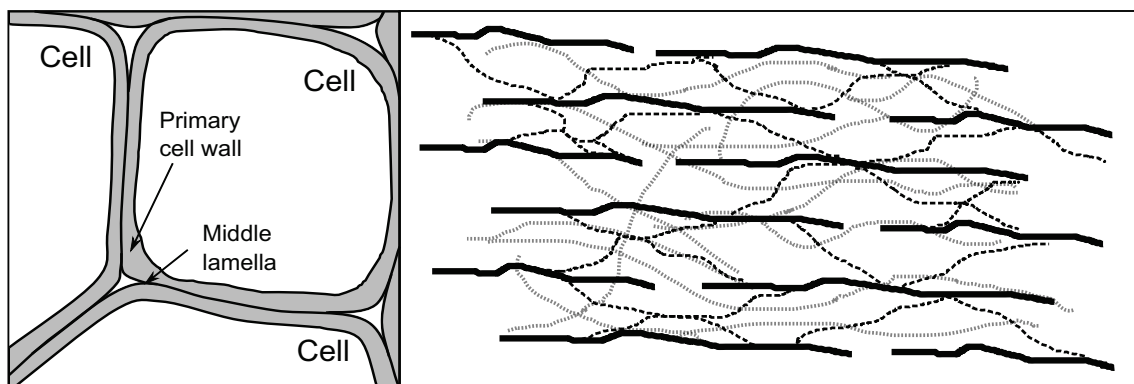

Fig. 1.1: Model of the plant cell wall. Left – schematic view on a single cell surrounded by the primary cell wall; the middle lamelli of the cell walls are thought to connect and interlink neighboring cells (McCann et al., 1992). Right – Simple model of the primary cell wall of flowering plants (Carpita and Gibeaut, 1993). Cellulose microfibrils (black horizontal rods) are linked by hemicellulose chains (dotted black lines). The cellulose-hemicellulose network is embedded in a matrix of pectins (gray dotted lines).

1.3.2 Cellulose

Cellulose is a homopolymer of β-1,4-linked glucose units that is synthesized at the plasma membrane by the cellulase-synthase complex. The nascending chains are released apoplastically, where they group to cellulose microfibrils (Mutwil et al., 2008).

These microfibrils are bundles of 36 cellulose chains of approximately 200 nm length, that provide mechanical strength to the cell wall. It consists of amorphous regions of loosely arranged fibers and crystalline regions that widely persist enzymatic degradation. On average, cellulose constitutes 10–30 w/w % of the total cell wall (McNeil et al., 1984).

1.3.3   Hemicelluloses

Hemicelluloses are a heterogenic group of CWPS that share a β-1,4-linked backbone as a structural motif (Scheller and Ulvskov, 2010). The backbone can be composed of glucose (in xyloglucans and β-glucans), xylose (in xylans, arabinoxylans and glucuronoarabinoxylans), mannose (in mannans and galactomannans) or mannose and glucose (in galactoglucomannans). In contrast to cellulose, the backbone of hemicelluloses is usually substituted. For instance, xylans of the true grasses (Poaceae) can be substituted with arabinose, xylose, glucuronic acid, acetic acid, coumaric acid, ferulic acid or more complex oligomers of these substituents.

Because of their cellulose-like backbone structure, hemicelluloses, like xyloglucan and xylan, can bind non-covalently to cellulose (Levy et al., 1997).

1.3.4   Pectins

Pectins are probably the most complex polysaccharides in nature. The pectin molecule consists of up to 17 different monosaccharides that may be linked in more than 50 ways (Ridley et al., 2001). Pectin is composed of six covalently linked substructures that are arranged to form a backbone, which is substituted with side chains.

Backbone substructures

Homogalacturonan (HG) is a homopolymer of α-1,4-linked D-galacturonic acid (GalA) units. The GalA units can be O–2- and/or O–3-acetylated and/or methyl esterified at their C–6 carboxyl function (Hirst and Jones, 1946; Gee et al., 1959; Rombouts and Thibault, 1986; Ishii, 1997). The number of acetyl groups and methylesters per 100 GalA units expresses the degree of acetylation/ methylesterification (DA/DM). HG chains can cross-link to each other via complexation with calcium ions. Calcium-mediated cross-linking depends on the presence and distribution of methyl esters and may be modulated by the activity of pectin methyl esterase. At least 10 consecutive free carboxyl groups are needed to form calcium complexes (Daas et al., 2001).

Xylogalacturonan and apiogalacturonan have a homogalacturonan backbone and short xylose or apiose side chains (Schols et al., 1995; Ridley et al., 2001). While xylogalacturonan is frequently present in reproductive tissues of a number of flowering dicotyledons (Albersheim et al., 1996), apiogalacturonan has only been described in aquatic monocotyledons (Ridley et al., 2001).

Rhamnogalacturonan I (RG I) consists of repeating units of α-1,4-linked D-galacturonosyl-α-1,2-L-rhamnose (McNeil et al., 1980; Lau et al., 1985). The GalA units may be acetylated on O–2 and/or O–3 (Komalavilas and Mort, 1989; Schols and Voragen, 1994). Some GalA residues have been shown to be substitued with β-1,3-linked glucuronic acid residues (Renard et al., 1999). It is still under discussion, whether GalA units of RG I are methyl-esterified (Rihouey et al., 1995). Commonly, 20–80 % of the rhamnose units are substituted with α-1,4-linked neutral sugar side chains arabinan, galactan and arabinogalactan I (McNeil et al., 1980). Acetylation of the rhamnosyl moieties has been reported in okra (Sengkhamparn et al., 2009).

Side chain substructures

Arabinan is a neutral polysaccharide that is covalently linked to the rhamnose moieties of RG I. It consists of an α-1,5-linked L-arabinofuranose backbone (Hirst and Jones, 1948). The arabinofuranose units of the backbone may be α-O–2 and/or α-O–3-substituted with arabinofuranose monomers or oligomers. The degree of branching and the length of the polymer varies between different plant species (Schols et al., 1990; Beldman et al., 1997).

Galactans can be subdivided into galactan, arabinogalactan I (AG I) and arabinogalactan II (AG II). Galactan and AG I consist of a β-1,4-linked D-galactose backbone. In AG I the backbone may be β-O–6-substitued with D-galactose and/or α-O–3-substituted with L-arabinose (Carpita and Gibeaut, 1993; Ridley et al., 2001). The backbone may be interrupted by α-1,5-linked L-arabinose units (Huisman et al., 2001) or β-1,3-linked D-galactose units (Hinz et al., 2005). AG II has a β-1,3-linked D-galactose backbone and is α-6-substituted with L-arabinosyl-β-1,6-D-galactose. It is mostly present as arabinogalactan proteins (Gaspar et al., 2001). A small fraction of AG II has been shown to be linked to pectin (Immerzeel et al., 2006).

Rhamnogalacturonan II is a highly conserved, complex side chain of homogalacturonan (Darvill et al., 1978). It contains 12 different sugars linked by more than 20 linkages, among them unusual sugars, such as apiose, aceric acid, 3-deoxy-lyxo-2-heptulosaric acid and 3-deoxy-manno-2-octulosonic acid (O'Neill et al., 2004).

1.3.5   Cell-wall models and the role of pectins

It is generally assumed that the cell wall of most plants consists of two independent networks: A hemicellulose–cellulose network and a pectin network (McCann et al., 1992; Carpita and Gibeaut, 1993). Two distinct cell-wall models have been developed for most dicotyledonous flowering plants with xyloglucan as the main hemicellulose (type I cell walls) and for monocotyledonous grasses with arabinoxylans as the dominant hemicellulose (type II cell walls). Type I cell walls generally contain similar amounts of cellulose, xyloglucan and pectins. In type II walls the amounts of the main constituents arabinoxylans and cellulose can vary, but in general they only contain small amounts of xyloglucan and pectin (Carpita and McCann, 2000).

These cell-wall models are generally accepted, but they cannot explain all experimental findings. Some evidence was found for a covalent linkage between pectin and xyloglucan in various angiosperm cell cultures (Fry, 1986; Popper and Fry, 2005, 2008), which indicates that pectin could be an integral part of a single carbohydrate network. Moreover, the type I cell-wall model may not be appropriate for a number of dicotyledonous plants with low xyloglucan contents, such as spinach, potato, celery, sugar beet or cabbage (Stevens and Selvendran, 1980; Zykwinska et al., 2008). Other findings demonstrated the binding of pectic arabinan to cellulose in an *in vitro* model (Zykwinska et al., 2005). Pectins could, therefore, also form a network with cellulose. These data suggests that type I cell walls are structurally more diverse than it was assumed earlier, or that dicotyledons with low xyloglucan content have an independent cell-wall type.

1.3.6   Pectin structural models

Within the type I cell-wall model, different submodels for the pectin network have been developed.

The "rhamnogalacturonan model" was the first detailed pectin model, which describes three main polysaccharides arabinan, galactan and rhamnogalacturonan to be covalently linked to each other (Fig. 1.2A, Talmadge et al., 1973).

The "smooth and hairy regions model" suggests a pectin backbone structure of homogalacturonan (HG) interspersed by RG I, to which the neutral sugar side chains arabinan and galactan are attached (De Vries et al., 1982, refined by Schols and Voragen, 1996). This assembly results in smooth HG stretches and hairy regions of substituted RG I (Fig. 1.2B).

In the side-chain model, all pectin constituents, including HG and XGA, are present as side chains of RG I (Fig. 1.2C, Vincken et al., 2003). This arrangement should solve a
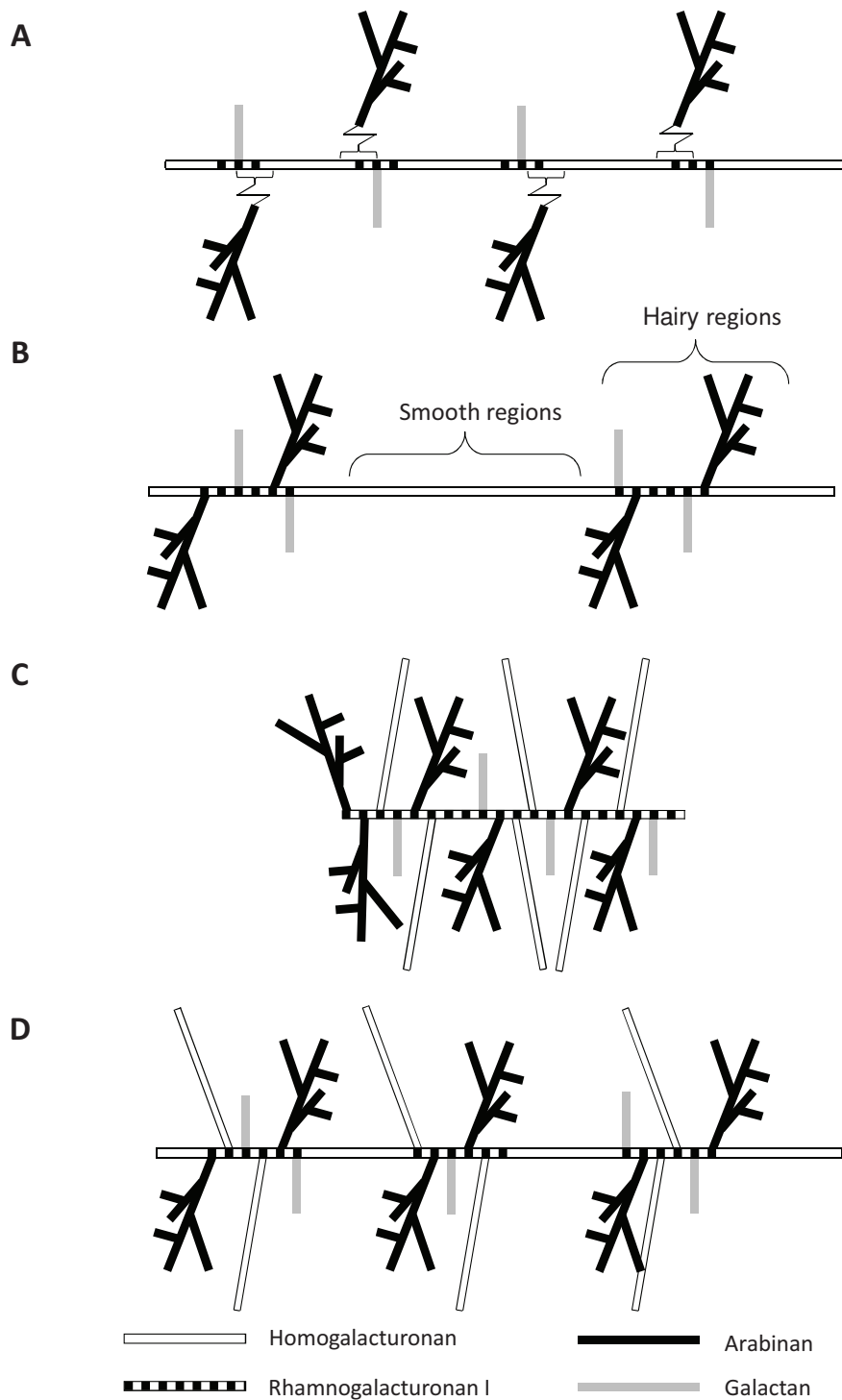
Fig. 1.2: Evolution of the pectin model. A: Rhamnogalacturonan model – precise linkage of arabinan to rhamnogalacturonan unknown (Talmadge et al., 1973), B: Smooth and hairy regions model (De Vries et al., 1982; Schols and Voragen, 1996), C: Side chain model (Vincken et al., 2003), D: Combined side chain – hairy region model (Ralet and Thibault, 2009; Schols et al., 2009).

problem of the smooth and hairy regions model: Pectins do not contain enough rhamnose to explain the degrees of polymerization (DPs) that were observed for HG (DP $\approx$ 90, Thibault et al., 1993; Zhan et al., 1998; Prade et al., 1999) and RG I (up to $\approx$ 250 Rha–GalA dimers, McNeil et al., 1980).

The "smooth and hairy regions model" was supported by data of Coenen and co-workers (2007), who could prove that parts of HG and XGA are linearly connected to the RG I backbone. The conflicts with the side-chain model were solved in a refined "Combined side chain – hairy regions model" (Fig. 1.2D), in which HG is present, together with RG I, in the pectin backbone and as side chains of RG I (Ralet and Thibault, 2009; Schols et al., 2009). This model might also explain the different physicochemical properties of pectins extracted from various plant origins. The model suggests that the importance of the pectin molecule *in muro* and its interactions with other cell-wall polysaccharides is much more complex than it has been assumed earlier.

1.3.7   The cell wall of sugar-beet roots

The cell wall of sugar-beet roots is distinct from normal type I cell walls, because of two reasons: (1) It is almost devoid of hemicelluloses (McCready, 1966) and (2) pectic arabinans and galactans are feruloylated (Guillon et al., 1989; Guillon and Thibault, 1989). The main constituent monosaccharides present in sugar-beet root cell walls are glucose (derived from cellulose), arabinose and galacturonic acid (both derived from pectin). Mannose (1.0–1.5 w/w %) is present as 1,4-linked mannan. Xylose (1.1–1.5 w/w %) is mostly present as xylan (80 w/w %). About 20 w/w % of the xylose is present as terminal xylose that could be associated with xyloglucan (Bertin et al., 1988; Oosterveld et al., 1996).

During saccharose extraction, water-soluble pectins are extracted together with saccharose. The constituent monosaccharide composition of sugar-beet pulp (Fig. 1.3) may, therefore, slightly differ from that of sugar-beet root cell walls. Together with galactose (derived from pectic galactan), arabinose and galacturonic acid make up half of the total mass of sugar-beet pulp and two third of the total sugar content (McCready, 1966). They are organized in a single pectin molecule (Fig. 1.4). Linear galactan and highly branched sugar-beet arabinan are side chains of RG I. Depending on the cultivar, extraction conditions and analysis technique, molecular masses of sugar-beet arabinans have been reported in the range from 4–40 kDa (Hirst and Jones, 1948; Goodban and Owens, 1957; Tomimatsu et al., 1959; Rombouts and Thibault, 1986; Oosterveld et al., 2002).

Ferulic acid may account for 0.5–1.0 w/w % of sugar-beet pulp (Micard et al., 1994; Oosterveld et al., 1997; Saulnier and Thibault, 1999). It can be ester-linked to arabinose in
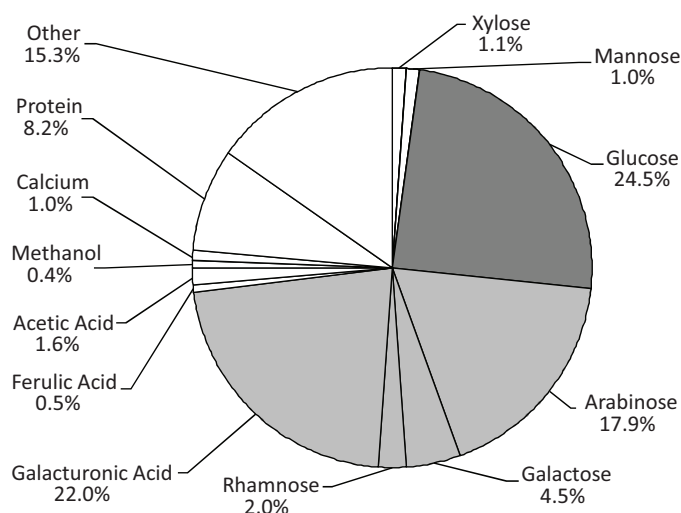
Fig. 1.3: Sugar composition of sugar-beet pulp. Dark gray – glucose mainly derived from cellulose (21.5 %) and residual sucrose (3.0 %), gray – sugars associated with pectin substructures, white – non-carbohydrate compounds.

arabinan (mainly O–2, 60 %) or galactose in galactan (O–6, 40 %), respectively (Colquhoun et al., 1994). Based on the ferulic acid content and the ratio of feruloylation, one out of 40–80 arabinose units and one out of 12–25 galactose units are feruloylated. Sugar-beet pectins can cross-link via the ferulic acid residues under the formation of different dehydrodiferulic acid dimers (Oosterveld et al., 1997; Micard et al., 1997; Ralet et al., 2005).

1.4   Cell-wall degrading enyzmes

Types of cell-wall degrading enzymes

Microorganisms like bacteria and fungi are a rich source of cell-wall degrading enzymes. Generally, cell-wall degradation requires two types of enzymes: Exo-enzymes remove single sugars or small oligomers from the ends of a polymer and hydrolyze oligomers down to monomers, whereas endo-enzymes cleave linkages within a polymer backbone. Endo-action leads to a rapid decrease in average molecular mass of the substrate and to the formation of new ends that may be attacked by exo-enzymes. Most enzymes are rather substrate-specific, which means that each cell-wall polysaccharide requires a specific set of glycosidases for its degradation (exemplified for pectin in Fig. 1.4). In addition to glycosidases, different esterases are required for the removal of non-sugar substituents, like methylesters, acetyl groups and feruloyl groups.

Classification of cell-wall degrading enzymes

Cell-wall degrading enzymes can be classified into three main classes of enzymes: Glycoside hydrolases (E.C. 3.2.1), Polysaccharide lyases (E.C. 4.2.2) and Carbohydrate esterases (E.C. 3.1.1, Webb, 1992). Hydrolases cleave the glycosidic linkage between two sugar moieties with the addition of one water molecule. Lyases cleave the glycosidic linkage by introducing a double bond. The carbohydrate esterases are a heterogeneous group of enzymes, that contain pectin methyl esterases, pectin and rhamnogalacturonan acetyl esterases and hydroxycinnamic acid esterases, like ferulic acid esterase.

An attempt has been made to classify carbohydrate active enzymes based on their enzymatic cleavage mechanism and according to their amino acid sequence. The Carbohydrate-Active enZYme (CAZy) database, therefore, subdivides the enzyme classes glycosyl hydrolase (GH), glycosyl lyase and carbohydrate esterase into different families (Coutinho and Henrissat, 1999). The members of one family have similar structural motifs, but they may have different substrate specificities and modes of action. Analogously, enzymes with the same substrate specificities and modes of action may belong to different families.

A very detailed data collection is provided by the Braunschweig enzyme database (BRENDA, `http://www.brenda-enzymes.info`) that intents to summarize all accessible data of known enzymes (Scheer et al., 2011).

1.4.1 Cellulose-degrading enzymes

Cellulose degradation requires the catalytic action of at least three enzymes: endoglucanase, cellobiohydrolase and β-glucosidase. Cellobiohydrolase is essential for the degradation of crystalline cellulose. Cellulose degradation may also require the presence of non-catalytic proteins, such as expansins, to make cellulose more accessible (amorphous) to enzymes (Cosgrove, 2000).

1.4.2 Hemicellulose-degrading enzymes

Xyloglucan side-chain degradation depends on the activity of α-xylosidases, β-galactosidases, α-fucosidases, α-arabinofuranosidases and acetyl esterases (Hayashi, 1989; De Vries and Visser, 2001). The backbone is degraded either by non-specific endoglucanases or xyloglucan specific endoglucanases and β-glucosidases (Pauly et al., 1999; De Vries and Visser, 2001).

Xylan side-chains are degraded by β-xylosidases, arabinofuranosidases, (4–O-methyl-) glucuronidases and ferulic/coumaric acid esterases (Coughlan and Hazlewood, 1993;

Sunna and Antranikian, 1997). Endo-xylanases and β-xylosidases degrade the xlyan backbone (De Vries and Visser, 2001).

Likewise, **Mannan** degradation requires endomannanase and β-mannosidase for backbone degradation, while β-glucosidase and α-galactosidase activity is needed for the degradation of the side chains (De Vries and Visser, 2001). The backbone of gluco-mannans can be attacked endoglucanases as well.

1.4.3   Pectin-degrading enzymes

Homogalacturonan degradation requires the action of an exo-polygalacaturonase and an endo-polygalacturonase (endoPG). EndoPG is hindered by methyl-esterification, thus pectin methyl esterase (PME) and pectin acetyl esterase (PAE) are also needed for efficient degradation (Fig. 1.4). Pectin and pectate lyases can split the glycosidic linkage between two GalA units by introducing a double bond.

Rhamnogalacturonan I is degraded by RG-lyases or RG-hydrolases (Mutter et al., 1998). Acetyl groups are removed by pectin acetyl esterases.

For the degradation of **galactan and arabinogalactan I**, endo-galactanase, β-galactosi-dase and arabinofuranosidase activities are needed. In Amaranthaceae, like spinach and sugar beet, ferulic acid groups have to be removed by ferulic acid esterases.

Arabinan is degraded by a number of different enzymes that belong to the CAZy glycoside hydrolase (GH) families 3, 27, 43, 51 54, 62 and 93 (Coutinho and Henrissat, 1999, `http://www.cazy.org`).

Endoarabinanases (EC 3.2.1.99, GH family 43) are endo-acting enzymes that hydrolyze the α-1,5-linkages in the linear regions of the arabinan backbone (Rombouts et al., 1988). They are more active on polymers than on oligomers (Dunkel and Amado, 1995).

Exoarabinanases and arabinofuranosidases are exo-enzymes that release their prod-ucts from the non-reducing ends of the substrate (Chávez Montes et al., 2008). Exoarabi-nanases (EC 3.2.1.-, GH families 43 and 93) release specifically arabinose (Ichinose et al., 2008), arabinobiose (Carapito et al., 2009; Sakamoto and Thibault, 2001) or arabinotriose (Kaji and Shimokawa, 1984) from α-1,5-linked arabinan.

α-ʟ-Arabinofuranosidases (E.C. 3.2.1.55; GH families 3, 43, 51, 54 and 62) are subdi-vided into four groups: Arabinofuranosidase (Abf) A, Abf B, bifunctional Abf's and Abf's specifically acting on arabinoxylans (AXH's). Abf A is active toward arabinose oligomers and arabinoxylan oligomers, but is not active on polymers (Matsuo et al., 2000). Abf B is active on beet arabinan polymers and some Abf B also show activity towards arabinoxylan oligomers (De Vries and Visser, 2001).
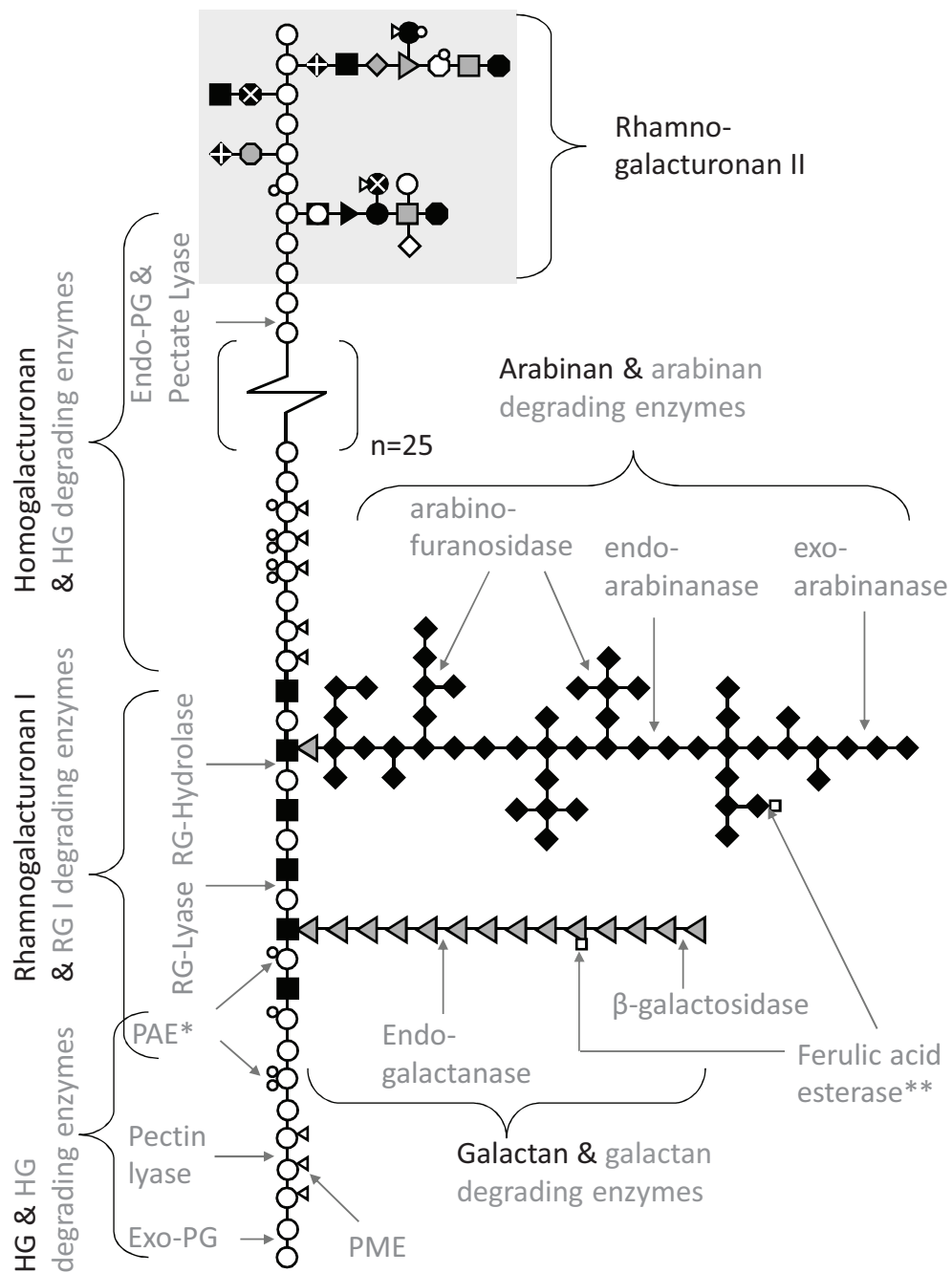
Fig. 1.4: General structure of sugar beet pectin and enzymes needed for its degradation. Relative amounts of sugars were taken into account, except for RG II (gray box), that is only present in minor amounts and is, thus, over-represented in this graph. Black diamonds – α-L-arbinose, gray triangles – β-D-galactose, black squares – α-L-rhamnose, white circles – α-D-galacturonic acid, small white circles – acetyl groups, small white triangles – methyl-ester groups, small white squares – ferulic acid groups. Endo-PG – endopolygalacturonase, exo-PG – exopolygalacturonase, PME – pectin methyl esterase, PAE – Pectin acetyl esterase, * active on HG and RG I, **active on arabinan and galactan.

Table 1.1: Fungal arabinohydrolases described in literature. Data collected from literature as indicated, and from the Brenda database.(Scheer et al., 2011). GH family classification according to the CAZy database (Coutinho and Henrissat, 1999).

Enzyme	Function	Source	GH fam.	pI	Mw	Products	Main substrate	pH	T	Reference
AraA	endo	*Aspergillus aculeatus*	43		45	Ara2+Ara3	LA, BA, potato AG I	5.5		(Beldman et al., 1993)
ArafurB1	AbfB	*Aspergillus aculeatus*			37	Ara	pNP-Araf, LA and BA	3.0-3.5		(Beldman et al., 1993)
ArafurB2	AbfB	*Aspergillus aculeatus*		4.3	37	Ara	pNP-Araf, LA and BA	4.0-4.5		(Beldman et al., 1993)
rARA	endo	*Aspergillus aculeatus*	43		34		LA	5.5	50	(Skjøt et al., 2001)
AwAXH	AXH	*Aspergillus awamori*			32	Ara	AX	5.0	50	(Kormelink et al., 1991)
arafase	Abf	*Aspergillus awamori*		3.6	65	Ara	pNP-Araf	4.6	50	(Wood and McCrae, 1996)
AwabfI	AbfA	*Aspergillus awamori* IFO 4033	51	3.3	81	Ara		4.0	60	(Kaneko et al., 1998; Koseki et al.,2003)
AwabfII	AbfB	*Aspergillus awamori* IFO 4033	54	3.6	62	Ara	pNP-Araf, AXOS, BA	4.0	60	(Kaneko et al., 1998)
AkAbf54	AbfB	*Aspergillus kawachii*	54		62	Ara	BA, LA, AX	4.0	55	(Koseki et al., 2003, 2006)[1]
AkAbfB	AbfB	*Aspergillus kawachii*	54		78	Ara	AX			(Miyanaga et al., 2004)
AkAbf51	AbfA	*Aspergillus kawachii*	51		80	Ara	AX	4.0	55	(Koseki et al., 2003)
	endo	*Aspergillus nidulans*	43	3.25	40		LA	5.5	68	(Ramon et al., 1993)
		Aspergillus nidulans		4.3	36	Ara	pNP-Araf	5.5		(Fernández-Espinar et al., 1994)
		Aspergillus nidulans		3.3	65	Ara	pNP-Araf	4.0	65	(Ramon et al., 1993)
	endo	*Aspergillus niger*	43	2.9	42.5	Ara2+Ara3	LA	4.8		(Dunkel and Amado, 1994, 1995)
ANendoara	endoA	*Aspergillus niger*	43	5	35	Ara2+Ara3	LA	5.0	50	(Rombouts et al., 1988)
ANAbfB1	AbfB	*Aspergillus niger*		3.6	53	Ara	LA and BA	3.9		(Tagawa et al., 1988)[2]
ANAbfB2	AbfB	*Aspergillus niger*		6	60	Ara	pNP-Araf, AOS, AXOS, LA, BA	3.7	60	(Rombouts et al., 1988)
ANAfbA	AbfA	*Aspergillus niger*		6.5	128	Ara	pNP-Araf, AXOS and AOS	4.1	50	(Kormelink et al., 1993)[3]
	endo	*Aspergillus niger* N400		3.0	43	Ara	LA	4.6		(Ramon et al., 1993)[4]
ABFA	AbfA	*Aspergillus niger* N400	51	3.3	83	Ara	pNP-Araf	3.4	60	(Flipphi et al., 1993a)
ABFB	AbfB	*Aspergillus niger* N400	54	3.5	67	Ara	pNP-Araf and BA	3.8	50	(Flipphi et al., 1993b)
	Abf	*Aspergillus oryzae*			116/60	Ara	soy bean polysaccharide	5.5	60	(Hashimoto and Nakata, 2003)
AbfA	Abf	*Aspergillus oryzae*	51		228/55	Ara	pNP-Araf	5.0	50	(Matsumura et al., 2004)
AoAra54A	Abf	*Aspergillus oryzae*	54		61	Ara	BA, AG I, AX	4.0	45	(Yang et al., 2006)
	Abf	*Aspergillus sojae*		3.9	34.3	Ara		5.0	50	(Kimura, 1995)
Abf I	Abf	*Aspergillus terreus*		7.5	39	Ara	AX, BA	3.5-4.5		(Luonteri et al., 1995)
Abf II	Abf	*Aspergillus terreus*		8.3	59	Ara	AX, BA	3.5-4.5		(Luonteri et al., 1995)
Abf III	Abf	*Aspergillus terreus*		8.5	59	Ara	AX, BA	3.5-4.5		(Luonteri et al., 1995)
AbfA	AbfA	*Aureobasidium pullulans*	54		49	Ara	BA	3.5-4.0	55	(De Wet et al., 2008)
	AbfB	*Aureobasidium pullulans*			210/105	Ara	pNP-Araf, LA and BA	4.0-4.5	75	(Saha and Bothast, 1998)
	Abf	*Cochliobolus carbonum*			63	Ara	AX, BA	3.5-4.0	50	(Ransom and Walton, 1997)
CRAbfB	AbfB	*Corticium rolfsii*				Ara	AX, BA	2.5	30	(Kaji and Yoshihara, 1969, 1971)
	Abf	*Cytophaga xylanotica*		6.1	160	Ara	AX, BA	5.8	45	(Renner and Breznak, 1998)
	Abf	*Dichomitus squalens*		5.1	60	Ara	AX, BA	3.5	60	(Brillouet et al., 1985)
	Abf	*Fusarium caeruleum* (Lib.) Sacc.			62	Ara	AG II	3.5		(Sturdy and Cole, 1975)
ABF1	Abf	*Fusarium oxysporum*		6.0	200/66	Ara	BA	6.0	50-60	(Panagiotou et al., 2003)

continued from previous page

Enzyme	Function	Source	GH fam.	pI	Mw	Products	Main substrate	pH	T	Reference
ABF2	Abf	*Fusarium oxysporum*		7.2	180/60	Ara	BA	6.0	50-60	(Panagiotou et al., 2003)
	AbfB	*Fusarium oxysporum* f. sp. *Dianthi*	54		58	Ara	pNP-Araf	4.0	50	(Chacón-Martínez et al., 2004)
Ara I	AbfB	*Geotrichum candidum*		4	80	Ara		4.1		(Golubev et al., 1993)
Ara II	AbfB	*Geotrichum candidum*		3.85	69	Ara		4.1		(Golubev et al., 1993)
Arb93A	exobiase	*Gibberella zeae/Fusarium graminearum*	93		41	Ara2	LA	5.0	40	(Carapito et al., 2009)
	Abf	*Humicola insolens*	43			Abf	AX, AXOS	6.7	53	(Sörensen et al., 2006)
	Abf	*Humicola insolens*	51			Abf	AX, AXOS	6.0	40	(Sörensen et al., 2006)
	Abf	*Meripilus giganteus*	51			Abf	AX, AXOS	6.0	40	(Sörensen et al., 2006)
AFIII	Abf	*Monilinia fructigena*		6.5	40	Ara		5.0		(Kelly et al., 1987)
I	AbfB	*Penicillium capsulatum*		4.15	64.5	Ara	AX, BA	4.0	60	(Filho et al., 1996)
II	AbfB	*Penicillium capsulatum*		4.54	62.7	Ara	AX, BA	4.0	55	(Filho et al., 1996)
Abnc	endo	*Penicillium chrysogenum* C53 31B			35	Ara2+Ara3	LA	6.0-7.0	30-40	(Sakamoto et al., 2003)
AbnX	exoara	*Penicillium chrysogenum*	93		47	Ara2	LA	4.0	40	(Sakamoto and Thibault, 2001)
AFQ1	AbfB	*Penicillium chrysogenum* 31B			79	Ara	AG, AX, BA, AOS	4.0-6.5	50	(Sakamoto and Kawasaki, 2003)
AFS1	AbfB	*Penicillium chrysogenum* 31B			52	Ara	AG, AX, BA, AOS	3.3-5.0	50	(Sakamoto and Kawasaki, 2003)
Abnx	exo	*Penicillium chrysogenum* 31B	93		47/42	Ara2	LA	4.0	40	(Sakamoto et al., 2004)
AXS5	AXH	*Penicillium chrysogenum* 31B	62		35	Ara	pNP-Araf	5.0	40-50	(Sakamoto et al., 2010)
	Abf	*Penicillium funiculosum*				Ara	pNP-Araf	2.5	70	(Karboune et al., 2009)
PfabfB1	AbfB	*Penicillium funiculosum*	54		65	Ara	BA and AX	3.5	60	(Guais et al., 2010)
PfabfB2	AbfB	*Penicillium funiculosum*	54		54	Ara	BA and AX	2.6	50	(Guais et al., 2010)
	AbfB	*Penicillium purpurogenum*	54	6.5	58	Ara	AX	4.0	50	(De Ioannes et al., 2000)
ABF1	Abf	*Penicillium purpurogenum*	54		49.6	Ara	BA, LA, AX			(Carvallo et al., 2003)
ABF2	AbfA	*Penicillium purpurogenum*	51	5.3	70	Ara	AOS, AXOS	5.0	60	(Fritz et al, 2008)
ABF3	Abf/Xyl	*Penicillium purpurogenum*	43		51	Ara+Xyl	XOS, AXOS, AX	5.0	50	(Ravanal et al., 2010)
	AbfB	*Phanerochaete chrysosporium*		7.34	55	Ara	BA, pNP-Araf	2.5		(Coughlan and Hazlewood, 1993)[5]
	Abf	*Phytphthora palmivora* (Butl.)		4.2	88	Ara	AOS, BA, LA	4.0	50	(Akinrefon, 1968)
	Abf	*Rhizomucor pusillus* HHT-1		7.5	63	Ara	AX, BA	4.0	65	(Rahman et al., 2001, 2003)
	Abf	*Sclerotinia sclerotiorum*				Ara		4.0-4.5		(Baker et al., 1979)
ScAra43A	endo	*Streptomyces coelicolor*	43	3.5	40	oligos	LA	6.0	45	(Yang et al., 2006)
	AbfB	*Talaromyces emersonii*			210/105	Ara		3.2		(Tuohy et al., 1994)
	Abf	*Talaromyces thermophilus*			35	Ara	AX, AXOS, pNP-Araf	6.0-7.0	55	(Guerfali et al., 2011)
	Abf	*Thermomyces lanuginosus*				Ara	AX	4.0-4.5	70	(El-Gindy and Saad, 2003)
	Abf	*Thermomyces thermophilus*				Ara	AX	7.0	50	(Guerfali et al., 2010)
	Abf/Xyl	*Trichoderma koningii* G-39	54		50	Ara+Xyl		4.1		(Wan et al, 2007)
	Abf/Xyl	*Trichoderma reesei*		4.7	100	Ara+Xyl	pNP-Araf, pNP-Xylp	4.0	60	(Poutanen, 1988)
abf1	Abf	*Trichoderma reesei*		7.5	53	Ara	AX, GAX, BA, pNP-Araf	4.0	60	(Poutanen, 1988)[6]

Abbreviations: Mw – molecular mass (kDa), pH – pH optimum, T – temperature optimum, Abf – arabinofuranosidase, Abf/Xyl – bifunctional arabinofuranosidase/β-xylosidase, Ara – arabinose, AG I & AG II – arabinogalactan I & II, AOS – arabinose oligomers, AX – arabinoxylan, AXOS – arabinose and xylose containing oligomers from an AX digest, BA – branched arabinan, GAX – glucuronoarabinoxylan, LA – linear arabinan, pNP-Araf – p-nitrophenyl arabinofuranoside, pNP-Xylp – p-nitrophenyl xylopyranoside, Xyl – xylose, XOS – xylose oligomers. [1]see also Miyanaga et al., 2006, [2]see also Kaji and Yoshihara, 1969; Kaji and Tagawa, 1970, [3]see also Rombouts et al., 1988; Pitson et al., 1996, [4]see also Van der Veen et al., 1991, [5]see also Coughlan et al., 1993, [6]see also Margolles-Clark et al., 1996; Nogawa et al., 1999.

Bifunctional Abf's are found in bacteria and they also show β-ᴅ-xylosidase activity (GH family 3, `http://www.cazy.org`). AXH's (GH family 62) have been described to release arabinose specifically from arabinoxylan. Some AXH, however, also release arabinose from arabinan (Kormelink et al., 1991; De Vries and Visser, 2002).

Since substrate specificities can widely differ for the Abf's, the term "arabinohydrolases" will be used from now on instead of arabinan degrading enzymes. A list of fungal arabinohydrolases described in literature is given in Table 1.1. From the substrate specificities it can be seen that the differentiation between exoarabinanase, Abf and AXH is often not clear and often ambiguous. A lack of biochemical data and structural data for a number of enzymes often makes it hard to classify them unambiguously.

GH43 enyzmes are often smaller (35–45 kDa) than GH51 and GH54 enzymes (60–80 kDa). Arabinohydrolases can appear larger *in vivo* than *in vitro*, since some of them can form dimers, trimers or tetramers (Table 1.1). The enzyme have different pI values, ranging from 2.9 (*Aspergillus niger* endoarabinanase) to 8.5 (Abf III from *Aspergillus terreus*). Similarly, pH optima and temperature optima can vary greatly, depending on the origin, but most of the enzymes show optimal activity at pH values in the slightly acidic range and at temperatures between 50–60 °C.

Ferulic acid esterases

Ferulic acid esterases (FAE's) are a rather heterogeneous group of enzymes that release ferulic acid from the cell walls of the Poaceae, sugar beet and spinach (Amaranthaceae). They were functionally classified based on their different activities on synthetic substrates and their ability to degrade dehydrodiferulic acids (Crepin et al., 2004). Some FAE's have been assigned to CAZy carboxyl esterase family 1 (Coutinho and Henrissat, 1999), but most of them are unassigned.

Best described ferulic acid esterases are FaeA (Fae-III) and FaeB (Fae-I) from *Aspergillus niger* (Benoit et al., 2008). FaeA hydrolyzes the O–6 ester linkage to galactose in pectic galactan and the O–5 linkage toward arabinose in arabinoxylans of the Poaceae (De Vries and Visser, 2002). FaeA can release small amounts of dehydrodiferulates from arabinoxylans when co-incubated with xylanase (Kroon et al., 1999). The enzymatic release of dehydrodiferulates from pectic material is not proven up to date. FaeB releases O–5 linked ferulic acid from arabinose units in arabinoxlyans of true grasses and pectic arabinans (Ralet et al., 1994). It is more active on feruloylated pectin and cannot release dehydrodiferulic acid (Kroon et al., 1996).

1.4.4 The enzymatic toolbox of *Chrysosporium lucknowense* C1

Chrysosporium lucknowense is a basidomycetous fungus originally extracted from Indian alkaline soil (Garg, 1966). The strain C1 was isolated in Russia and the production strain was optimized for the secretion of carbohydrate-active enzymes at high yields in a low viscosity fermentation medium (Bukhtojarov et al., 2004; Verdoes et al., 2007). Its genome putatively encodes for 200 carbohydrate active enzymes, of which, 58 are potentially active toward pectin substructures (Hinz et al., 2009).

Among these 58 enzyme, 24 putative cellulases/glucanases, 14 putative arabinohydrolases, six putative galactohydrolase and six putative ferulic acid esterases were identified that could be of relevance for the degradation of sugar-beet pulp (Table 1.2).

The C1 production strain allows high overexpression levels with low background expression of other secretory proteins (Verdoes et al., 2007). C1 is, therefore, a highly interesting alternative to the established strains of the *Trichoderma* and *Aspergillus* gen-

Table 1.2: The enzymatic toolbox of C1. Enzymes likely to be involved in pectin degradation. Multiply mentioned enzymes belong to different GH families. Multiple annotations indicate that the enzyme specificity cannot be deduced from the sequence.

Annotated enzyme	Number of enzymes in		
	C1*	*T. reesei***	*A. niger****
β-Galactosidases	5	2	6
β-Glucosidases / β-xylosidases	11	12	17
Endo-glucanases / galactanases	8	8	10
Polygalacturonases	2	4	21
Arabinanases / arabinofuranosidases / β-xylosidases	10	2	10
Arabinofuranosidases	2	-	2
Galactanases	1	-	2
α-Rhamnosidases	1	-	8
Exo-arabinanases	2	-	-
Ferulic acid esterases	6	-	3
Rhamnogalacturonan acetyl esterases	2	-	2
Pectin methyl esterases	1	-	3
Pectin/ pectate lyases	7	-	8
Total	**58**	**28**	**92**

*from C1 sequencing and annotation data (personal communication with Sandra Hinz, Dyadic Netherlands),
**from the GJI database, ***from the CAZy database

era (Table 1.2) that are well-known for their cellulase secretion and pectinase secretion, respectively.

1.5   Biomass pretreatment for efficient enzymatic liquefaction

Lignocellulosic feedstocks often require a pretreatment prior to enzymatic degradation (Gharpuray et al., 1983). Pretreatments should allow the time and cost efficient degradation of biomass by avoiding chemical decomposition and the formation of compounds that could inhibit subsequent fermentation (Mosier et al., 2005). At the same time high yields of digestible material should be recovered at high concentrations with a minimum effort of both chemicals and energy, that greatly determine the economic feasibility of the process (Alvira et al., 2010). Biomass composition can vary greatly among feedstocks and different pretreatments have been established (extensively reviewed in Gharpuray et al., 1983; Sun and Cheng, 2002; Mosier et al., 2005; Carvalheiro et al., 2008; Alvira et al., 2010; Talebnia et al., 2010).

Pretreatments encompass (thermo-)physical treatments (milling, chopping, microwave and ultrasound treatment, extrusion), (physico-)chemical treatments, such as alkali pretreatments, dilute and concentrated acid pretreatments, organosolv pretreatment, pretreatments with oxidizing agents, hot water pretreatment and a number of steam explosion pretreatments (Mosier et al., 2005). Concentrated alkali pretreatments mainly solubilize lignin and increase cellulose digestibility through swelling, but can also lead to partial pectin degradation through β-elimination (Renard and Thibault, 1996). Dilute alkali pretreatment can remove ester-linked cell-wall substituents and increase hemicellulose and pectin digestibility (Carvalheiro et al., 2008). Acid pretreatments (partially) hydrolyze cell-wall polysaccharides, while leaving lignin uneffected. High acid concentrations and/or high temperatures can lead to chemical decomposition of monosaccharides and to the subsequent formation of toxic compounds (Larsson et al., 1999). Hydrothermal pretreatments, such as pressurized liquid hot water or steam explosion, are relatively mild pretreatments suitable for feedstocks with low lignin content (Carvalheiro et al., 2008).

Saccharification of sugar-beet pulp

Sugar-beet pulp can be saccharified chemically or enzymatically. An overview of different studies is given in Table 1.3. Sugar-beet pulp can be degraded enzymatically up to 90 w/w % without chemical pretreatment. However, high enzymes dosages and long incubation times up to 120 h were necessary.

Table 1.3: Saccharification of sugar-beet pulp described in literature so far.

Reference	Pretreatment	Saccharification	SBP (w/v %)	enzyme/g SBP	time	efficiency
(Ali et al., 1984)	autoclaved (30 min, 121 °C)	*Trichoderma reesei*	2.5%	89 FPU cellulase	48 h	90 % hydrolysis total PS
	50 mM HCl (30 min, 50 °C)	*Trichoderma reesei*	2.5%	89 FPU cellulase	48 h	85 % hydrolysis total PS
	50 mM NaOH (30 min, 50 °C)	*Trichoderma reesei*	2.5%	89 FPU cellulase	48 h	70 % hydrolysis total PS
	Sigma pectinase (920 U/g SBP)	*Trichoderma reesei*	2.5%	89 FPU cellulase	48 h	65 % hydrolysis total PS
(Chamy et al., 1994)	ground; 0.32 M HCl, 2 h, 80 °C		7.5%		2 h	64 % solubilization, 2.2% cellulose hydrolysis
	ground; 0.16 M H_2SO_4, 2 h, 80 °C		7.5%		2 h	54 % solubilization, 1.5% cellulose hydrolysis
	ground; 1.6 M HCl, 2 h, 80 °C		7.5%		2 h	75 % solubilization, 28% cellulose hydrolysis
	ground; 0.8 M H_2SO_4, 2 h, 80 °C		7.5%		2 h	70 % solubilization, 24% cellulose hydrolysis
(Beldman et al., 1984)	ground	Maxazyme CL[1] & Rapidase C80[2]	9.8%	≈58 U[a]	24 h	90 % hydrolysis total PS
(Moloney et al., 1984)	ball-milled	*Talaromyces emersonii*	4%	20.75 U	120 h	up to 35 % hydrolysis total PS, 55 % cellulose
	ball-milled & alkali (4M NaOH, 24 h, 30 °C)	*Talaromyces emersonii*	4%	20.75 U	120 h	up to 35 % hydrolysis total PS, cellulose n. d.
	peracetic acid (20 % peracetic acid, 1 h 100 °C)	*Talaromyces emersonii*	4%	20.75 U	120 h	up to 63 % hydrolysis total PS, 55 % cellulose
	ball-milled & peracetic acid	*Talaromyces emersonii*	4%	20.75 U	120 h	up to 77 % hydrolysis total PS, 63 % cellulose hydrolysis
(Coughlan et al., 1986)	homogenized (blender)	*Talaromyces emersonii*	4%	20.75 U	120 h	≈25 % hydrolysis total PS, 35 % cellulose hydrolysis
	homogenized (blender)	*Talaromyces emersonii*	4%	83 U	120 h	up to 78 % hydrolysis total PS, 83 % cellulose hydrolysis
	Pectinase (288 U/g SBP)	*Talaromyces emersonii* & *Trichoderma reesei*	4%	25 FPU cellulase	120 h	92 % hydrolysis total PS, 89 % cellulose hydrolysis
(Thibault and Rouau, 1990)	ground, particle size below 0.5 mm	SP 249[3]	2.5%*	2 × 60 PGU	120 h	≥ 95 % pectin hydrolysis, 60 % cellulose hydrolysis
	ground, particle size below 0.5 mm	Driselase[4]	2.5%*	2 × 60 PGU	120 h	≥ 95 % pectin hydrolysis, 60 % cellulose hydrolysis
	ground, particle size below 0.5 mm	Onusuka[1]	2.5%*	2 × 60 PGU	120 h	48 % hydrolysis total PS
	ground, hydrogen peroxide/chlorite	Onusuka[1]	2.5%*	2 × 60 PGU	120 h	90 % hydrolysis total PS
(Micard et al., 1996)		Pectinase SP 584[2] & 8 other commercial preparations	1%	10 mg	6-8 h	70 % hydrolysis of neutral sugars, thereof 70–85% as monomers, almost no cellulose hydrolysis
(Micard et al., 1997)	dried & ground, Pectinase SP 584[2]	Celluclast 1.5L[5], Cellulase[1] & 11 other commercial preparations	3.3%	10 mg	96 h	≥ 95 % pectin hydrolysis, up to 20 % cellulose hydrolysis
	dried & ground, Pectinase SP 584[2]	Celluclast 1.5L[5], Cellulase[1] & 11 other commercial preparations	3.3%	50 mg	48 h	up to 35 % cellulose hydrolysis
(Spagnuolo et al., 1997)	ground	Celluclast 1.5L[5], Viscozyme L[7] & 5 other commercial preparations	6%	≈45 U	168 h	up to 80 % hydrolysis total PS
(Doran, 2000)	autoclaved (20 min, 121 °C)	Pectinex Ultra SP[3], Celluclast 1.5L[5]	10%	≈71.2 U[b]	24 h	after 120 h co-fermentation: 0.3 g EtOH/ g SBP
(Foster et al., 2001)		Celluclast 1.5 L FG[5], Novozym 431[6], Viscozyme L[7]	5%	≈94 U[c]	24 h	85 % hydrolysis total PS
	ammonia pressurization/depressurization	Celluclast 1.5 L FG[5], Novozym 431[6], Viscozyme L[7]	5%	≈94 U[c]	24 h	66 % hydrolysis total PS

[1]from *Trichoderma viride*, [2]from *Aspergillus niger*, [3]from *Aspergillus sp.*, [4]from *Irpex lacteus*, [5] from *Trichoderma reesei*, [6]from *Trichoderma longibrachiatum*, [7]from *Aspergillus aculeatus*, U – enzyme units (μmol/min), PGU – polygalacturonase units, PCU – cellulase units, FPU – filter paper units (cellulase activity), PGU – polygalacturonase units. *sugar beet fibre was used as starting material, sugar beet fibre obtained from SBP (Bertin et al., 1988), [a]20 mg Maxazyme CL (≈16 U) and 25 mg Rapidase C80 (≈42 U), 0.85 hemicellulase untis & 60.2 PGU (Celluclast 1.5L), 28.4 cellobiohydrolase units (Novozym 431), [b]5.8 FPU (Celluclast 1.5L) and 65.4 PGU (Pectinex Ultra SP), [c]4.2 FPU (Celluclast 1.5L), 28.4 cellobiohydrolase units & 60.2 PGU (both in Viscozyme L).

Enzyme hydrolysis did not always yield high proportions of fermentable monosaccharides (Coughlan et al., 1986; Thibault and Rouau, 1990). In addition, more enzyme preparations had to be mixed to yield high solubilizations, which might be a drawback for the biotechnological implementation of the process. Chemical saccharification by means of acid hydrolysis (1.6 M HCl) could solubilize 75 % of the sugar-beet pulp in short time, but the process suffers from high amounts of acid waste and hydrolyzes only 28 % of the cellulose (Table 1.3, Chamy et al., 1994). Cellulose degradation is also limited in many enzymatic liquefaction studies (Table 1.3). Thus, it could be beneficial to facilitate the enzymatic degradation of cellulose by a mild pretreatment without the destruction of pectin.

1.6   Aim and outline of the thesis

The aim of this research is the analysis and characterization of the enzymatic toolbox of *Chrysosporium lucknowense* C1 for the hydrolysis of sugar-beet pulp to fermentable monosaccharides.

Enzymatic degradation of biomass often requires long incubation times and high enzyme dosages. The effect of a mild pretreatment on the enzymatic degradability of sugar-beet pulp is described in Chapter 2.

In Chapter 3 we aimed to degrade sugar-beet arabinan to fermentable monosaccharides. For this reason we purified and characterized four arabinohydrolases, among them one endoarabinanase, one exoarabinanase and two arabinofuranosidases. A combination of three arabinohydrolases could only liberate 60 % as monomers and left some material present as oligomers of unknown structure.

The unknown oligomers were purified and structurally identified as branched arabinose oligomers by NMR spectroscopy (Chapter 4).

With the help of the branched arabinose oligomers and reduced arabinose oligomers the modes of action and other biochemical characteristics of the four arabinohydrolases were more closely studied (Chapter 5).

To address the limited degradadability of feruloylated pectins and feruloylated arabinoxylans, we purified and characterized three ferulic acid esterases from C1 (Chapter 6).

The final Chapter discusses the relevance of this research for the use of sugar-beet pulp as a source of renewable energy and the potential of *Chrysosporium lucknowense* C1 for biomass degradation.

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Chapter 2

Aiming the complete utilization of sugar-beet pulp through mild acid pretreatment and enzyme treatment

Abstract

Biomass use for the production of bioethanol or for the production of platform chemicals requires the efficient biomass-breakdown to fermentable monosaccharides. Lignocellulosic feedstocks often require a physicochemical pretreatment prior to enzymatic hydrolysis. Optimal pretreatments can be different for different feedstocks and should not lead to biomass destruction and the formation of toxic products. The influence of six mild sulfuric acid or water pretreatments at different temperatures on the enzymatic degradability of sugar-beet pulp was examined. An optimal pretreatment of 15 min at 140 °C in water can solubilize 60 w/w % of the total carbohydrates present, mainly pectins. Higher severities lead to the destruction of solubilized sugars and to the subsequent production of the sugar degradation products furfural, hydroxy methyl furfural, acetic acid and formic acid. The pretreated samples were enzymatically degraded successfully with an experimental cellulase preparation. Pretreatment of sugar-beet pulp greatly facilitates the subsequent enzymatic degradation within economically feasible times ranges and enzyme dosages. In addition, pretreatment of sugar-beet pulp can be useful to fractionate functional ingredients like arabinans and pectins from cellulose. The optimal combined severity factor to enhance the enzymatic degradation of sugar-beet pulp is between $\text{Log } R'_0 = -2.0$ and $\text{Log } R'_0 = -1.5$. Optimal pretreatment and enzyme treatment solubilized up to 80 % of all sugars present in optimally pretreated sugar-beet pulp, including ≥ 90 % of the cellulose.

2.1 Background

Sugar-beet pulp consists up to 75 w/w % of carbohydrates on dry matter basis. Arabinose, glucose and galacturonic acid are the main sugar moieties present in complex polysaccharide structures (McCready, 1966). The pressed pulp after sugar extraction has a dry matter content of 18–23 w/w %. To date, it is mainly used as animal feed and dumped in landfills in regions with no livestock breeding (Kelly, 1983; Voragen et al., 1997).

The low dry matter content makes combustion of sugar-beet pulp for heat and power production unfavourable. Instead, the low lignin content and high sugar content of sugar-beet pulp makes it an interesting feedstock for biorefinery and/or bioethanol production. The availability of new, pentose fermenting yeast strains allows the efficient use of biomass for bioethanol production (Bettiga et al., 2009). However, no yeast strains are currently available that allow the fermentation of uronic acids to ethanol.

For fermentation the cell wall material needs to be degraded into fermentable monosaccharides. Hereto, lignocellolosic feedstocks are often structurally modified by a mild pretreatment prior to enzymatic release of fermentable monosaccharides (Alvira et al., 2010). A combined severity factor has been introduced as a measure for the harshness of a mild acid pretreatment that takes into account pretreatment time, pretreatment temperature, acid concentration (w/w %) and pH after pretreatment (Chum et al., 1990; Abatzoglou et al., 1992). In general, a pretreatment has to be strong enough to disrupt the cellulose–hemicellulose network and, if present, the cellulose–lignin network. With increasing harshness of the pretreatment, however, more biomass is degraded to non-fermentable products and products toxic to yeast like furfural or hydroxymethyl furfural (HMF) (Palmqvist and Hahn-Hägerdal, 2000b). Along with furfural and HMF release, weak acids, such as acetic acid, may be released that can have a negative impact on yeast growth, viability and fermentation (Banerjee et al., 1981; Rottenberg, 1979). Although yeasts may adapt to moderate levels of furfural and detoxification has been mentioned (Palmqvist and Hahn-Hägerdal, 2000a), it is economically and ecologically favorable to avoid the formation of such compounds.

During the last decades work has been performed on the degradation of sugar-beet pulp that has been shown good yields of solubilized carbohydrates. High dosage treatments with enzymes or chemicals were, however, required (Beldman et al., 1984; Chamy et al., 1994; Martínez et al., 2009; Micard et al., 1997). No data are available for the degradation of sugar-beet pulp in commercially reasonable time ranges and economically feasible enzymes dosages.

In this research the benefit of a hydrothermal pretreatment or mild acid pretreatment of sugar-beet pulp was studied with the aim to enhance enzymatic degradation of sugar-beet pulp. Therefore, a number of pretreatments were performed at different temperatures and pH values. The pretreated samples were characterized and used for an enzymatic saccharification study in order to produce high amounts of fermentable monosaccharides.

2.2   Methods

2.2.1   Starting material

Sugar-beet pulp was obtained as frozen pressed pulp (23 % dry matter content, campaign 2006; Suiker Unie, Dinteloord, The Netherlands). The pulp contained 68 w/w % carbohydrates on dry-weight basis. The constituent sugars were arabinose (Ara, 18 w/w %), glucose (Glc, 22 w/w %), uronic acids (UA, 18 w/w %), galactose (Gal, 5 w/w %), rhamnose (Rha, 2 w/w %), xylose (Xyl, 2 w/w %) and mannose (Man, 1 w/w %), respectively. Sugar-beet pulp contained 4 w/w % residual saccharose. Other components in sugar-beet pulp were protein (8 w/w %, N × 6.25), lipids and salts. Ferulic acid (FA, 0.5 w/w %), acetic acid (1.6 w/w %) and methanol (MeOH, 0.4 w/w %) were present as ester-linked substituents of polysaccharides.

2.2.2   Pretreatment

Pretreatments were carried out with a dry-matter content of 5 w/w %. Therefore, 152 g of pressed pulp was filled up to 700 g with distilled water . The pulp was mixed for five minutes using a blender (type 7011G, Waring Commercial, Torrington, CT, USA) at low speed. Concentrated sulfuric acid (180 µl) was added to the blended pulp, according to the conditions used (Table 2.1). Pretreatment was performed in a PARR 4520 pressure reactor connected to a PARR 4875 Power controller and operated by a PARR 4875 Process controller (PARR instrument company, Moline, Il, USA). The reactor was equipped with an oil bath (200 °C) that made oil flush through a spindle inside the reactor. An electric heating cord was attached on the outside of the reactor to accelerate the average heating rate and to maintain temperature constant after heating up. The oil bath was turned off 20 °C below the desired temperature was reached in the reactor. After 12 minutes, the electric heater was removed and the PARR reactor was cooled with flushing tap water (10 °C) in a specially designed flow-through water bath, giving an overall pretreatment of 15 minutes at the pretreatment temperature aimed. Temperature and pressure were

recorded. The pH of the samples was determined before and after the pretreatment. Directly after the pretreatment, 1 ml of supernatant was taken and filtered through a 0.2 μm round filter (Whatman, Kent, UK) to determine the free amounts of lactic acid, acetic acid, formic acid, furfural and hydroxymethyl furfural.

The severities of the pretreatments were calculated as combined severity factor (Chum et al., 1990; Abatzoglou et al., 1992) R'_0 ($R'_0 = t * [10^{-pH}] * \exp * [(T\text{-}100)/14.75]$) and expressed as Log R'_0 (Kabel et al., 2007).

Table 2.1: Pretreatment conditions of sugar-beet pulp.

Sample	T (°C)	H_2SO_4 [a]	pH [b]	R'_0 [c]	Log R'_0 [d]
0–0	0	0	5.3	-1.77	-6.97
120–0	120	0	4.8	1.76	-3.04
140–0	140	0	4.4	2.35	-2.05
170–0	170	0	4.1	3.24	-0.86
120–1	120	1*	3.9	2.11	-2.14
140–1	140	1*	3.9	2.70	-1.65
170–1	170	1*	3.8	3.62	-0.53

a – sulfuric acid concentration given in w/w % of the dry matter content, b – pH values of the solutions after pretreatment, c – combined severity factor according to Overend et al. (1987), d – decade logarithm of the combined severity factor. *1 w/w % of the dry matter content corresponds to a sulfuric acid concentration of 0.05 w/v %.

2.2.3 Mass balance

The process of sample separation and mass-balance determination is summarized in Fig. 2.1. Nylon panties (15 denier) were used to filter the pretreated slurries. The remaining solids were wrapped in a double layer of cheese cloth and pressed with an IKA hydraulic press (IKA Werke GmbH & Co. KG, Staufen, Germany) at 100 bar until draining ceased. The pressed pellet was soaked three times with 500 ml distilled water (10 min, 60 °C) and pressed. The supernatant was weighted and subsequently combined with the washes. Supernatant and pellets were, both, divided into two and one half (S1 and P1) each was freeze dried for chemical characterization, whereas the second half (S2 and P2) was frozen for enzymatic digestion. The P1 fractions were weighted before and after freeze drying and the dry-matter content was determined as an indicator of the water binding capacity after pretreatment. The S1 supernatants after pressing were centrifuged to remove cell

debris (4 °C, 20 min, 12 320 × g). The clear S1 supernatants were freeze dried, while the debris was added to the P1 pellet fraction. The S2 supernatants were concentrated to their original concentrations and stored in the freezer.
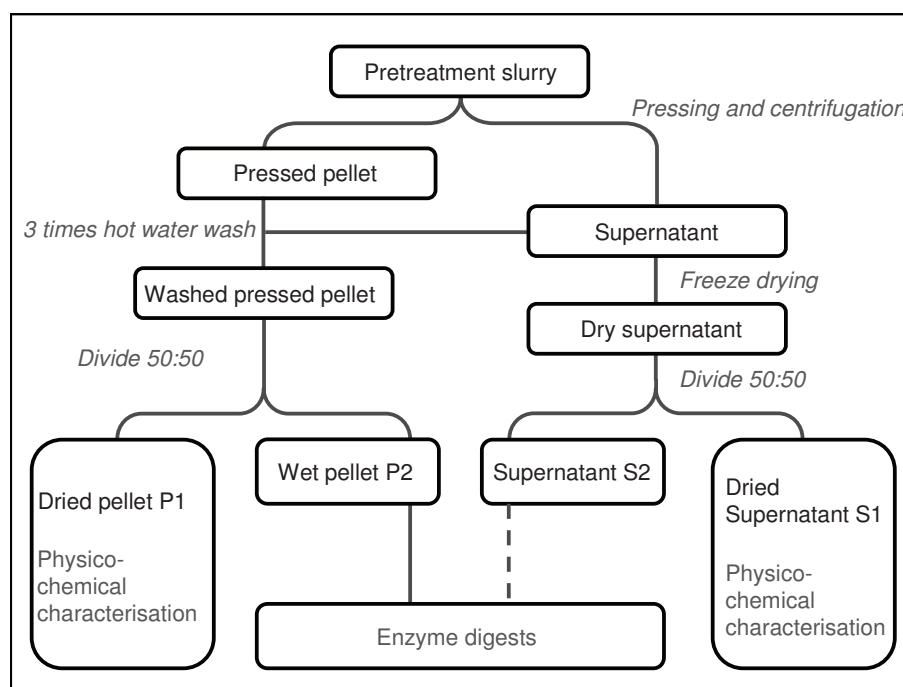

Fig. 2.1: Sample preparation of supernatants and pellets after pretreatment.

2.2.4 Dry matter content

Freeze dried samples (20–40 mg) were weighted into aluminium cups ($\varnothing = 50$ mm) and dried in an oven at 70 °C for up to three days, until the weight was constant.

2.2.5 Chemical characterization of pretreated sugar-beet pulp

After freeze drying the P1 pellet were weighted and ground in a Retsch M2200 swinging mill (Retsch GmbH, Haan, Germany) using 25 ml stainless steel containers with two stainless steel metal balls ($\varnothing = 10$ mm) per container(4 min, 100 Hz). The freeze dried S1 supernatants were treated equally except for the supernatants S1 0–0, S1 170–0 and S1 170–1, which did not crystallize, but formed a syrup. For these fractions, the syrup was dissolved in distilled water and the volume was determined. An aliquot (1 ml) was freeze dried and subsequently oven dried to calculate the dry matter content of the solutions.

2.2.6 Enzyme digestions

Activity assays

Glycosidase activities in the experimental enzyme preparation C1-G1 were measured by determining the hydrolysis of *p*-nitrophenyl-β-ᴅ-glucopyranoside, *p*-nitrophenyl-β-ᴅ-xylopyranoside or *p*-nitrophenyl-α-ʟ-arabinofuranoside at 37 °C after 10 minutes of incubation. The reaction mixture consisted of 25 μl substrate solution (4 mg/ml) and 10 μl of the suitable enzyme concentration in 50 mM sodium acetate buffer (pH 5.0). The reaction was stopped by adding 40 μl of reaction mixture to 60 μl of 250 mM Tris-HCl buffer (pH 8.8). The color formation was measured at 410 nm in a microtiter plate reader. One unit of activity was defined as 1 μmol of glucose, xylose or arabinose liberated per min under the conditions specified. The molar extinction coefficient under these assay conditions was 0.0158 μM^{-1} cm^{-1}.

Xylanase and cellulase activities were determined by measuring the amount of reducing sugars released from the hydrolysis of medium viscosity wheat arabinoxlyan (Megazyme, Bray, Ireland) and phosphoric-acid treated cellulose (Walseth, 1952). The reaction mixture consisted of 50 μl substrate solution (15 mg/ml)and 20 μl of the suitable enzyme concentration in 60 mM sodium acetate buffer (pH 5.0). The reaction mixtures were preheated at 37 °C before adding the enzyme. After an incubation of 10 min, 25 μl of the reaction mixture was used to determine the amount of reducing sugars using a PAHBAH assay (Lever, 1972). Polygalacturonase, rhamnogalacturonase, arabinanase and galactanase activities were determined by measuring the amount of reducing sugars released from the hydrolysis of polygalacturonic acid (Fluka, Sigma-Aldrich, Schnelldorf, Germany), linear arabinan (Megazyme) and potato galactan (Megazyme). The reaction mixture consisted of 180 μl substrate (5 mg/ml) in 50 mM sodium acetate buffer (pH 5.0), to which 20 μl of the suitable enzyme concentration were added. The reaction mixture was preheated at 37 °C before adding the enzyme. After an incubation of 20 minutes, 10 μl of the reaction mixture was used to determine the amount of reducing sugars using the PAHBAH assay (Kühnel et al., 2010). Calibration curves of the different monosaccharides (50–750 μg/ml) were used for quantification.

Digestions of pretreated pulp

Pressed pulp P2 (5 w/w %, 0.2 % of the initial dry matter) was digested with 10 μl of an experimental enzyme mixture (C1-G1; protein content 75 mg/ml, 1 w/w % enzyme dosage; Dyadic Netherlands, Wageningen, The Netherlands) in 100 mM sodium acetate

buffer (pH 5.5) for 2, 5, 10, 24 and 48 h. C1-G1 is an enzyme preparation of the fungus *Chrysosporium lucknowense* C1 (Hinz et al., 2009) that is rich in cellulase and xylanase activities. It also contains arabinanase and galactanase activities (Table 2.4). Ampicillin (100 µg/ml) was added to prevent microbial growth. The reaction took place in 2 ml microcentrifuge tubes placed in an microcentrifuge tube shaker at 40 °C and 800 rpm. Enzymes were inactivated at 100 °C (15 min in a waterbath). The samples were centrifuged (10 min, 20 000 × *g*, 20 °C), and 200 µl of the supernatant was taken for HPSEC and HPAEC analysis. The sugar composition of the remaining pellets was analyzed by GC-FID analysis after alditol-acetates derivatization.

2.2.7   Chromatography

GLC – sugar composition of pretreated sugar-beet pulp.

The neutral-sugar composition was determined analyzing the sugars as their alditol-acetate derivatives by gas chromatography with flame-ionisation detection. A Thermo Focus GC gas chromatograph and a Focus AS 3000 autosampler (Thermo Scientific, Waltham, MA, USA) equipped with a DB-225 column (15 m × 0.53 mm, 1 µm film; J&W Scientific, Santa Clara, Il, USA) was used. To this end, the samples were hydrolyzed stepwise with 72 w/w % sulfuric acid for 1 h at 30 °C followed by 1 M sulfuric acid for 3 h at 100 °C. Subsequently, the free monosaccharides were reduced and derivatized to their corresponding alditol acetates (Englyst and Cummings, 1984). The samples were dissolved in 0.4 ml acetone and 1 µl was injected with 1 s preinjection dwell time and 2 s post injection dwell time and eluted with the following temperature profile: 0–2 min: 180 °C, 2–17 min: a linear gradient from 180–210 °C, 17–22 min: 210 °C.

Uronic acid content was determined colorimetrically (Ahmed and Labavitch, 1978) with 3-phenylphenol using an automated analyzer (Skalar Analytical, Breda, The Netherlands). A galacturonic acid standard curve (12.5–100.0 µg/ml) was used for quantification.

HPLC – organic acids content of pretreated material

Organic acids, hydroxymethyl furfural, furfural and methanol contents were analyzed by HPLC with a Dionex Ultimate 3000 (Dionex, Sunnyvale, CA, USA) equipped with an Aminex HPX-87H Ion exclusion column (300 mm × 7.8 mm; Bio-Rad Laboratories, Hercules, CA, USA) in combination with a self-packed guard column (50 mm × 7.8 mm, packed with AG 50W-X4 Resin; Bio-Rad). Samples were eluted with 5 mM sulfuric acid at a flow rate of 0.6 ml/min at 30 °C. The S1 supernatants were filtered through an 0.2 µm

round filter (Whatman) prior to injection (20 μl). The samples were quantified by mono component standards of known concentrations (0.5 mg/ml). Elution was monitored by refractive index detection (Shodex RI 101; Showa Denko K.K., Kawasaki, Japan).

HPAEC – monomer and oligomer analysis

The monosaccharide and oligosaccharide levels of the digests were analyzed by high performance anion exchange chromatography (HPAEC) with pulsed amperometric detection (PAD) using a ITS-3000 HLPC (Dionex) equipped with a CarboPac PA1 analytical column (2 mm × 250 mm, Dionex) in combination with a CarboPac PA1 guard column (2 mm × 50 mm, Dionex) at a flow rate of 0.3 ml/min. Arabinose, rhamnose, galactose, glucose, xylose, mannose and galacturonic acid monosaccharides in the range from 2–30 μg/ml were used for quantification. Oligomeric standards included arabinose oligomers (DP 2–6, 10 μg/ml; Megazyme), galacturonic acid oligomers (DP 2 and DP 3, 10 μg/ml), a potato galactan digest containing galactose oligomers from DP 2–5 (5 mg/ml potato galactan (Megazyme) partially digested with *Aspergillus niger* endogalactanase, purified in house) and cellodextrins ranging from DP 2–7 (a kind gift of Dr. Vladimir Farkas, Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Slovakia). The samples (10 μl, 50–100 μg/ml) were eluted in post column addition mode with the following elution profile: 0.0–30.0 min: water, 30.0–30.1 min: 0.0–0.1 M NaOH, 30.1–32: 0.1 M NaOH, 32.0–62.5 min: 0.0–0.4 M NaOAc in 0.1 M NaOH, 62.5–67.0 min: 1.0 M NaOAc in 0.1 M NaOH, 67.0–75.0 min: 0.1 M NaOH, 75.1–90.0 min: water (equilibration). Sodium hydroxide (0.5 M, 0.1 ml/min) was added via a post column to allow PAD detection from 0.0–30.1 min and from 68.5–90.0 min.

RP–UHPLC – Ferulic acid analysis

The amount of ester-linked ferulic acid was determined after alkaline hydrolysis and ethylether extraction according to Appeldoorn et al. (2010) on an Accela UHPLC system (Thermo Scientific) equipped with a Hypersyl GOLD column (2.1 mm × 150 mm, 1.9 μm particle size; Thermo Scientific). Elution was monitored with the built-in polydiode array multi-wavelength detector set at 335 nm.

HPSEC – molecular mass distribution of pretreated material

High performance size exclusion chromatography (HPSEC) was performed on a Dionex Ultimate 3000 system (Dionex) equipped with four TSK-Gel superAW columns (Tosoh

Bioscience, Tokyo, Japan) in series: Guard column (6 mm × 40 mm) and separation three columns (4000, 3000 and 2500; 6 mm × 150 mm). The samples (10 μl, 5 mg/ml) were eluted with 0.2 M sodium nitrate at 40 °C at a flow rate of 0.6 ml/min. Molecular masses were estimated with the help of pullulan molecular-mass standards (Polymer Laboratories, Varian Inc., Palo Alto, CA, USA). Elution was monitored by refractive index detection (Shodex RI 101) and UV detection (internal detector) at four wavelengths ($\lambda = 235$ nm, 310 nm, 325 nm and 345 nm). The relative peak-area distribution was calculated by the integration of the peak area in three time increments. The total peak area is the total peak area from 7.0–18.0 min without the negative peak (15.5 min) plus the peak area difference between the negative peak of a water blank and the negative peak of the supernatant sample.

DUMAS – protein content

The protein content of the pretreated samples was determined by combustion (DUMAS method) with a FlashEA 1112 series nitrogen analyzer (Thermo Scientific). The analysis was carried out following the manufacturers manual with methione as a standard and a nitrogen factor of 6.25.

2.3 Results and Discussion

2.3.1 Mass balance of pretreated sugar-beet pulp

The effects of the different pretreatments on the solubilization of sugar-beet pulp were evaluated with the help of a mass balance taking into account the amounts of solubilized material, residual material and decomposed material. The combined severity factors (expressed as R'_0 according to Abatzoglou et al. (1992) and Log R'_0 according to Kabel et al. (2007)) of the pretreatments are given in Table 2.1. This factor is an indicator for the severity of a pretreatment and takes into account the pretreatment temperature, time, acid concentration and final pH. The mass distribution of the samples after pretreatment was plotted against Log R'_0 (Fig. 2.2). It can be seen that a Log R'_0 of around -3.0 to -1.5 is necessary to solubilize substantial parts of the beet pulp. With increasing severity of the pretreatment, an increasing amount of sample is lost, probably by chemical destruction of carbohydrates (e.g. Maillard reaction) and the formation of volatile compounds. Such formation of volatiles was observed during pretreatments with Log $R'_0 \geq -0.86$ (170–1 and 170–0), where a temperature independent pressure increase was monitored after

pretreatment (supplementary Fig. S2.1). A strong color change after pretreatment and a caramel like flavor indicates Maillardation under the conditions used (no further data shown).

Generally, more material is solubilized with increasing pretreatment severity. A mass loss is observed for all samples. These losses can mainly be attributed to the mechanical loss of material during blending and sample handling. When averaging this loss of the first 5 pretreatments the mechanical loss is estimated to account for roughly 8 w/w %. Pretreatments with Log $R'_0 \geq$ -0.86 (140–1) lead to major mass losses, probably due to chemical decomposition, and no further increase of solubles in the supernatant (Fig. 2.2).

The pellet and supernatant curves are both sigmoidal-like shaped, which could imply that the range between solubilization and chemical degradation of the pulp is narrow and, hence, requires a good process control. If higher severities are required for an effective disruption of the cellulose network, solubilized temperature labile compounds should be removed from the reactor to minimize their destruction. Such removal of solubles could be realized in an flow-through reactor setting or an extruder.
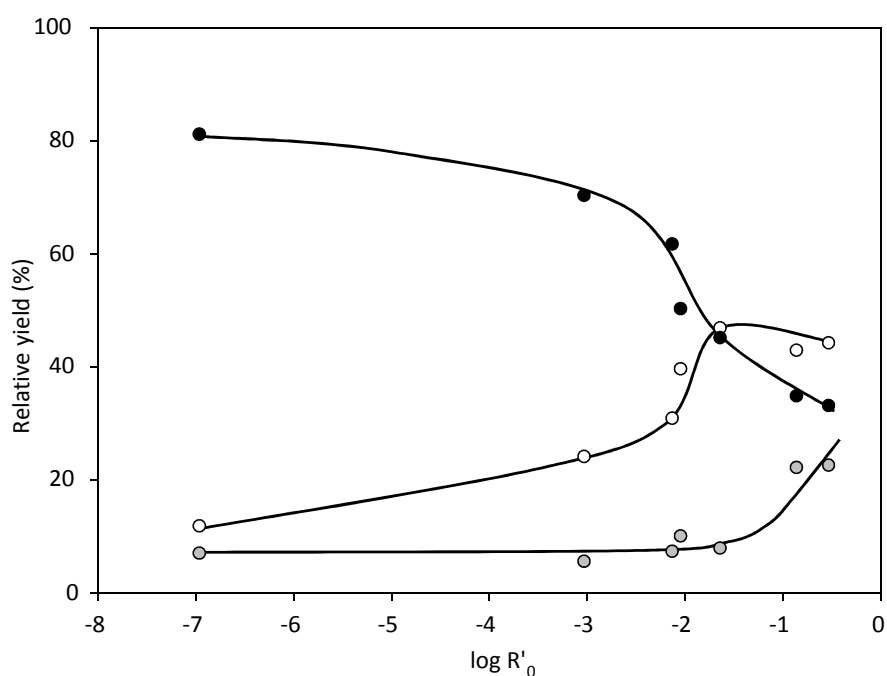

Fig. 2.2: Distribution of pretreated sugar-beet pulp over supernatant fractions, pellet fractions and de-composed material in relation to combined severity factor (Log R'_0). Black dots – pellets, white dots – supernatants, gray dots – mass losses. Lines solely indicate trends.

2.3.2   Sugar composition of released and residual carbohydrate material

Predominantly, the pectin-associated sugars arabinose, galactose and galacturonic acid are released upon pretreatment of sugar-beet pulp, besides some traces of rhamnose derived from the rhamnogalacturonan part of pectin (Fig. 2.3A). In contrast to the increasing levels of solubilized pectin-derived sugars, around 3 w/w % of the glucose is released irrespective of the severity. The glucose released derived most likely from the residual saccharose in the pressed pulp. When pretreated at 170 °C (Log R'_0 ≥ −0.86), the arabinose and galacturonic acid yields in the supernatant fraction decreased whereas the levels of the other sugars remained constant or further increased. Decomposition of 40 % and 90 % was calculated for arabinose and galacturonic acid (Fig. 2.3D), respectively, which suggests that arabinose and galacturonic acid are both heat labile. Pectins have been shown to decarboxylate at elevated temperatures and high levels of acid (Conrad, 1931), leading to the formation of furfural (Dunlop, 1948) and/or arabinose (Zweifel and Deuel, 1956). Therefore, apparent arabinose levels might be a result of arabinose lost during pretreatment and arabinose formed during the decomposition of galacturonic acid.

High solubilization of arabinan and galacturonan from sugar-beet pulp with temperatures from 150–175 °C has been reported previously (Martínez et al., 2009, 2010). Temperatures above 160 °C led to decreasing recoveries of galacturonic acid whereas arabinose recovery was stable up to 167 °C (Martínez et al., 2010). Most of the cellulose remained insoluble under the conditions used. Our data are in line with these observations.

The sugar yields in the pellets continuously decrease with increasing pretreatment severity (Fig. 2.3B). Approximately 30 % of the total sugars present in the starting material are left in the pellet fraction of 170–1. Absolute amounts of glucose remain almost constant, so that the relative glucose content of the pellet fractions increases up to 90 mol % (Supplementary Table S2.1). Even though cellulose is not solubilized during pretreatment, the solubilization of arabinan could also has a positive influence on the enzymatic degradation of cellulose. It has been shown previously that arabinan can absorb to cellulose *in vitro* (Zykwinska et al., 2005). The removal of arabinan is, therefore, expected to lead to higher exposure of the cellulose surface to enzymes.

When adding up the yields of the individual sugars present in the pellet and supernatant fractions (Fig. 2.3C) a decrease is observed for the pretreatments with Log R'_0 ≥ −0.86 (170–0 and 170–1) that is similar to the yield of the total material after pretreatment (Fig. 2.2).
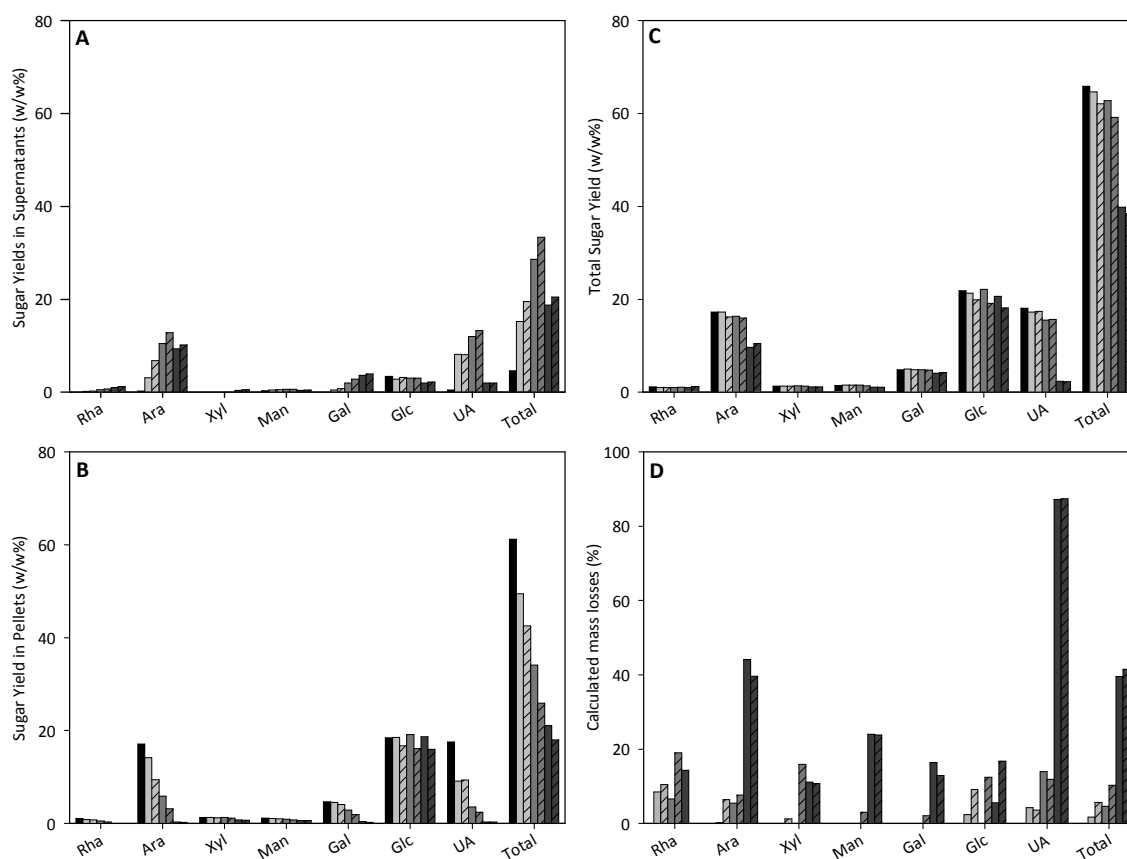
Fig. 2.3: Sugar yields of pretreated sugar-beet pulp fractions. The data are expressed as w/w % based of starting material prior to pretreatment. A: Sugar yields of the supernatant fractions, B: Sugar yields of the pellet fractions, C: Sugar yields of total pretreated material (sum of A and B), D: Calculated individual sugar losses during pretreatment. Black bars – untreated pulp, light gray bars – 120 °C fractions, gray bars – 140 °C fractions, dark gray bars – 170 °C fractions, shaded bars – pretreatments with 1 w/w % sulfuric acid (on a dry matter basis), non-shaded bars – pretreatments in water.

2.3.3 Sugar degradation products formed during pretreatment

Besides lowering the yields, biomass pretreatment carries the risk of forming undesired degradation products that could negatively influence the later use, i.e. yeast fermentation for bio-ethanol production. The formation of formic acid, acetic acid, lactic acid, furfural and hydroxymethyl furfural (HMF) was analyzed (Fig. 2.4). HMF, furfural and formic acid are only observed for pretreatments with Log $R'_0 \geq -0.86$ (samples 170–0 and 170–1). HMF formation in the 170 °C samples may be linked to the thermal decomposition of fructose, which derived from the autohydrolysis of saccharose. Fructose is more acid labile and five times more reactive than glucose (Shallenberger and Mattick, 1983),

probably due to a higher amount of open ring conformation at elevated temperatures (van Dam et al., 1986). HMF formation for the samples 170–0 (0.55 w/w %, 0.04 μmol) and 170–1 (0.71 w/w %, 0.06 μmol) could be derived from the degradation of 33–50 % of the saccharose derived fructose (in SBP: 2 w/w %, 0.12 μmol).

Furfural formation is linked to pentose and uronic acid degradation (Dunlop, 1948; Ulbricht et al., 1984) and only occurs for sample 170–0 (2.1 w/w %, 0.2 μmol) and sample 170–1 (2.6 w/w %, 0.25 μmol). It could explain 14–18 % arabinose degradation (around 1.4 μmol in SBP) or 20–25 % uronic acid degradation (around 1 μmol in SBP). Since 40 % arabinose and 90 % galacturonic acid were decomposed (Fig. 2.3D), it is likely that sugar destruction also occurs via different mechanisms or yields different end-products.
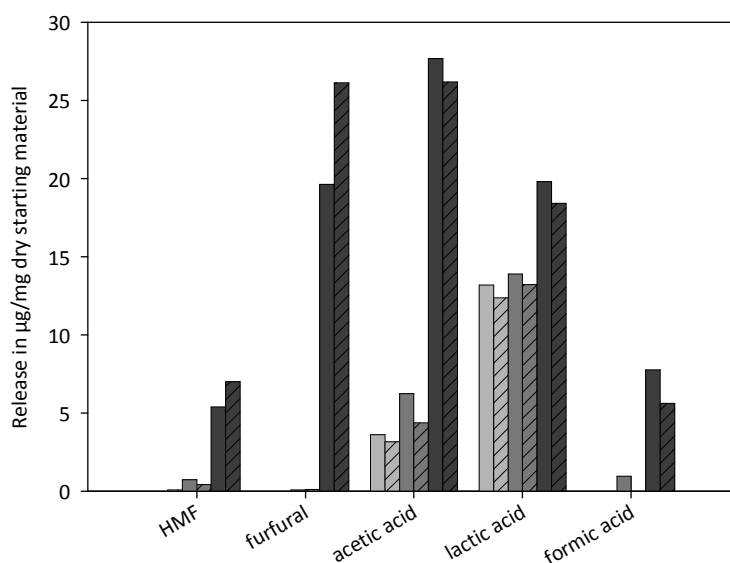
Fig. 2.4: Sugar specific degradation products formed after pretreatment. Light gray bars – 120 °C pretreatment, gray bars – 140 °C pretreatment, dark gray bars – 170 °C pretreatments, shaded bars – pretreatments with 1 w/w % sulfuric acid (on a dry matter basis), non-shaded bars – pretreatments in water.

For example, pectins can undergo β-elimination at high temperatures to form unsaturated galacturonic acid and unsaturated galacturonic acid oligomers (Voragen et al., 1988). A combination of β-elimination and decarboxylation resulting in a pyranose-ring structure of unsaturated 4,5-L-arabinopyranose plus carbon dioxide has been proposed as well (Einhorn-Stoll and Kunzek, 2009). Carbon dioxide could be responsible for the temperature-independent pressure increase that was observed during the 170 °C pretreatments (supplementary Fig. S2.1).

The formation of formic acid may be linked to further degradation of furfural and HMF at higher temperatures (Ulbricht et al., 1984). Furfural and HMF may also polymerize

to form humins under acidic conditions and elevated temperatures (Hurd and Isenhour, 1932). Humin formation can not explain the mass loss observed at Log $R'_0 \geq -0.86$. In addition, it generally requires higher acid concentrations (Hurd and Isenhour, 1932). The presence of acetic acid and lactic acid in all pretreated samples may also indicate the contamination of the sugar-beet pulp starting material with acid-releasing bacteria. The release of acetic acid and lactic acid strongly increases at Log $R'_0 \geq -0.86$. Acetic release (up to 2.5 w/w %) can partly be explained by pectin deacetylation, since sugar-beet pulp contains around 1.6 w/w % ester-linked acetic acid. In addition, acetic and lactic acid are also formed during the Maillard reaction.

The data from these experiments suggest that pretreatments at Log $R'_0 \geq -0.86$ lead to the enhanced destruction of arabinose and galacturonic acid and to the production of toxic compounds that could negatively impact fermentation.

2.3.4 Pressability of pretreated pulp

The pressability was studied as an indicator of the water-binding capacity. The water binding in plant cell walls of dicotyledons and non-graminacious monocotyledons is usually attributed to pectin (Reiter, 2002). The pellets obtained after pretreatment were reduced in pectic arabinan and galacturonan (Fig. 2.3B), but not all of them have a reduced water-binding capacity (Table 2.2). On the opposite, the pellets of the 120 °C and 140 °C samples retain up to 207 % more water than the untreated pulp. The 140 °C pretreated samples have a 50 % higher water content than the untreated pulp, whereas their pectin content is decreased by 70–80 % (Fig. 2.3B). The 170 °C pretreated pellets are almost completely devoid of pectic sugars (Fig. 2.3B), and, after pressing they retain only half of the water compared to the pressed untreated pulp. The swelling behavior sugar-beet pulp, hence, is not only related to its pectin content, but also depends on other parameters, e.g. the cell wall architecture.

Increased swelling and water-holding capacities have been described previously in the pellets of alkali-extracted beet fibres (Rouau et al., 1987; Bertin et al., 1988), even though they were strongly reduced in pectic arabinan and galacturonan. Analogously, it has been reported for cell-wall material of wheat flour that enzymatic removal arabinoxylan increased the water-holding capacity of the residual, cellulose-rich material (Gruppen et al., 1993). As possible explanations for the increased water binding, total polysaccharide content, charge, crystallinity of cellulose, specific surface area and hydrophobic components have been proposed (Rouau et al., 1987).

These results indicate that dry-matter contents higher than 5 % may not possible for the pretreatment and enzymatic digestion of sugar-beet pulp, since the pulp may not be properly pumpable and stirrable. Bioethanol or biogas production requires higher substrate concentrations to make the downstream processing (e.g. by distillation/rectification) economically feasible. To overcome this issue the water-binding capacity could be reduced either enzymatically by the addition of an endopolygalacturonase, thermophysically by a more severe pretreatment or process technologically by a fed-batch setup. Enzymatic degradation would produce galacturonic acid that may be toxic to the yeast, whereas a severe pretreatment would drastically lower the yield by biomass degradation and lead to the release of inhibiting compounds. A fed-batch setup could be a promising alternative to allow high dry-matter concentrations.

Table 2.2: Water binding capacity of pretreated samples.

	Bound water (g/g pellet)	Δ (%)
0–0	2.4	0
120–0	5.5	133
120–1	7.2	207
140–0	3.3	42
140–1	3.6	54
170–0	1.4	-41
170–1	1.2	-47

Pellets were pressed at 100 bar after pretreatment until drainage stopped.

2.3.5   Molecular-mass distribution of solubilized material

The molecular-mass distribution of the solubilized material was determined after pretreatment (Fig. 2.5A). The 0–0 supernatant does not contain any polymeric material. The absence of water soluble pectin in sugar-beet pulp may be explained by the prior industrial extraction of saccharose from sugar-beet pulp that involves a lime treatment to precipitate soluble pectins. Three peaks in the small molecular-mass region could represent residual saccharose, its hydrolysis products glucose and fructose or some salts that were solubilized upon hot water extraction.
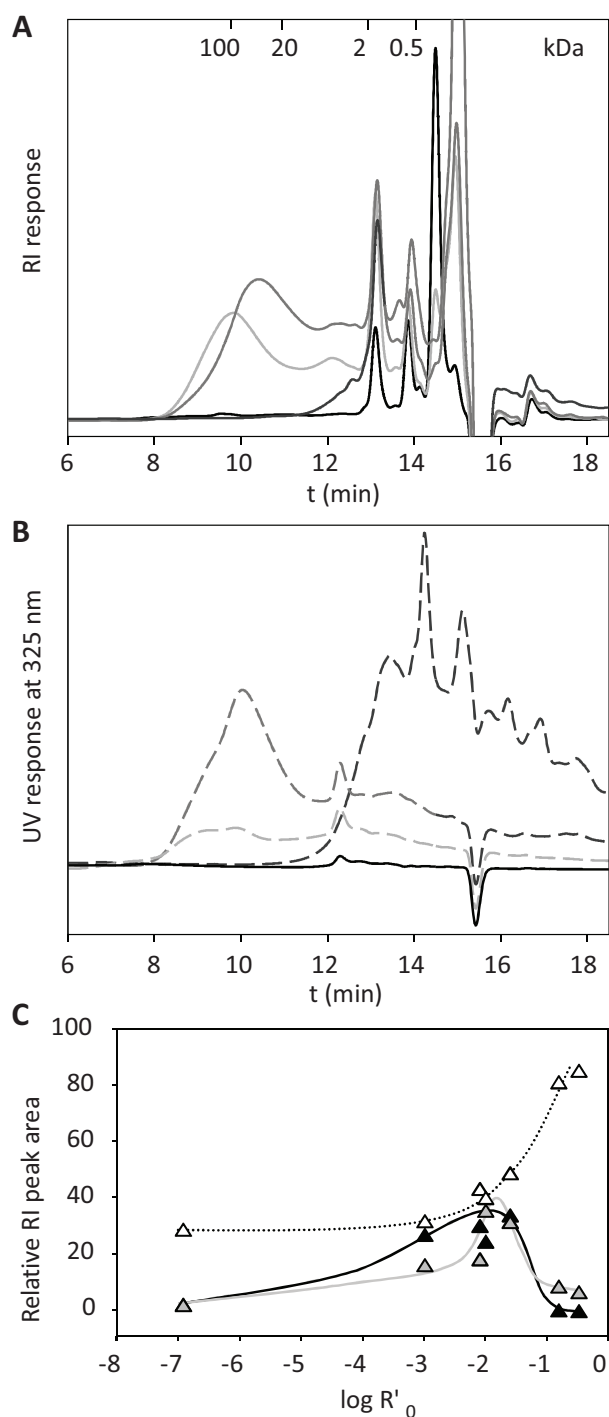
Fig. 2.5: Molecular mass distribution of solubilized material. A and B: HPSEC elution pattern of 0–0 (black), 120–1 (light gray), 140–1 (gray) and 170–1 (dark gray); Solid lines – RI signal (A), dashed lines (B) – UV signal at 325 nm. C: Logarithm of the combined severity factor (Log R'_0) vs. the relative RI peak area. Black triangles – peak area of the time interval 7.0–11.0 min, gray triangles – peak area of the time interval 11.0–13.0 min, white triangles – peak area of the time interval 13.0–15.2 min. Lines show trends.

The 120 °C pretreatments both solubilize a polymeric fraction with an average molecular mass of approximately 100 kDa, based on pullulan molecular mass standards. Samples pretreated with and without sulfuric acid show the same molecular mass distributions as the samples pretreated with water (no further data shown). The average molecular mass of solubilized material decreases with increasing temperature from 50 kDa for the 140 °C pretreatments to oligomeric material ≤ 1 kDa at 170 °C. Along with the solubilization of carbohydrates, an UV signal at 325 nm is detected for these populations that indicates the presence of phenols (Fig. 2.5B). Ferulic acid can be attached to pectic arabinan or galactan (Rombouts and Thibault, 1986; Guillon et al., 1989) and could explain the UV signal in the molecular mass range ≥ 1 kDa. The UV signal in the ≤ 1 kDa molecular mass range could indicate the presence of aromatic degradation products, such as furfural or HMF.

The ferulic acid content of the supernatants was determined separately (Table 2.3) and compared with the HPSEC data presented in Fig. 2.5B. The release of ferulic acid is of relevance because of two reasons. Ferulic acid esters present in arabinan and galactan can inhibit the complete degradation and lower the monosaccharide yields. Secondly, ferulic acid is a high value by-product in itself, since it is a precursor of aroma compounds, such as vanillin (Mathew and Abraham, 2004). With increasing severity of the pretreatment, increasing amounts of ferulic acid were present in the solubilized fractions that could explain the high UV absorption of the 140 °C fractions at 10 min and the UV absorption of the 170 °C samples in the oligomeric range from 13–15 min.

Table 2.3: Yield of ester-linked ferulic acid in solubilized material after pretreatment.

	FA[1] (nmol/mg)	FA[2] (%)
0–0	0	0
120–0	6	6
120–1	9	11
140–0	20	31
140–1	21	38
170–0	36	61
170–1	34	59

1) Absolute amount of solubilized ferulic acid

2) Relative amount of solubilized ferulic acid.

An optimal pretreatment does not necessarily hydrolyze the insoluble material to monosaccharides and oligosaccharides, which are more prone to chemical destruction

than polymers. The molecular-mass distributions of the supernatants indicate that the relative proportions of high and average molecular-mass material increases up to Log $R'_0 = $ -1.65 (sample 140–1), while they strongly decrease for the pretreatments with Log $R'_0 \geq -0.86$ (Fig. 2.5C). In turn, the amount of compounds that elute in the monomeric and oligomeric range increases with increasing severity. These results show, together with the decomposition as presented in Fig. 2.2 and Fig. 2.3D, that pretreatments with Log $R'_0 \geq -0.86$ favor chemical destruction of sugars and are too severe when aiming a maximum yield of fermentable sugars.

2.3.6 Enzymatic degradation of pretreated sugar-beet pulp pellets

For bioethanol production, a broad range of monosaccharides are aimed to be fermented. Besides hexose-fermenting yeast strains (mainly glucose and fructose but also galactose), a number of yeast strains have been described in literature that are able to ferment pentoses (van Maris, 2007). The efficient enzymatic degradation of sugar-beet arabinan has already been demonstrated (Kühnel et al., 2010). In order to verify the influence of the pretreatment on enzymatic digestibility of cellulose, the pretreated sugar-beet pulp pellets were degraded with an experimental enzyme mixture (C1-G1) that is rich in cellulases and xylanases (Table 2.4). It has been designed for the degradation of wheat bran, but also has endogalactanase and endoarabinanase activities (Table 2.4).

Sugar-beet polygalacturonides could be recovered after fermentation and subsequently used to enzymatically produce pectin-derived oligosaccharides.

C1-G1 is almost devoid of polygalacturonase/ pectate lyase activity (Table 2.4). Polygalacturonase deficiency could be beneficial for a subsequential yeast fermentation since

Table 2.4: Activity profile of enzyme mix C1-G1.

Activity	U/mg protein
β-Glucosidase	0.198
β-Xylosidase	0.012
α-Arabinofuranosidase	0.035
Xylanase	1.322
Cellulase	1.400
Arabinanase	0.100
Galactanase	0.142
Polygalacturonase/ Pectate lyase	0.006

high concentrations of weak acids have a negative impact on yeast viability and could, therefore, lower the ethanol production (Rottenberg, 1979).

Pectin-derived oligosaccharides have been shown to have a number of beneficial biologically effects, among them prebiotic activity (Hotchkiss et al., 2003; Mandalari et al., 2007; Gullón et al., 2011). Alternatively, enzymatically released galacturonic acid may be fermented to ethanol by bacteria (Doran, 2000).

The supernatants of the enzyme-digested pellets were analyzed for their monomer sugar content and the yield for each individual sugar was calculated (Table 2.5). Glucose is the main monosaccharide released by C1-G1. Besides glucose, up to 4 % of cellobiose was present after 48h, indicating insufficient β-glucosidase activity (no further data shown). Glucose yields increase from 17 % for the pellet of 0–0 up to 91 % for the pellet of 140–1 and drop to 61–65 % for the pellets of the 170 °C pretreatments. The pattern was confirmed by the sugar composition analysis of the pellets after 48 h enzyme incubation (no further data shown). A comparison of the glucose contents of the supernatants and the pellets after enzymatic degradation with the glucose content of the starting material revealed that no glucose was destructed. The reduced release of glucose from the 170 °C pretreatments could indicate enzyme inhibition or a structural change in the cell-wall complex that made cellulose less accessible.

No galacturonic acid release is observed and only a small release of galactose. Some arabinose monosaccharides are released, reaching up to 29 % yield based on the arabinose originally present in the pellet fraction. Monomer yields of arabinose and galactose may be

Table 2.5: Yields of individual monosaccharides ending up in the supernatant after 48 h enzyme incubation of pretreated pellets with C1-G1 enzyme mixture (1 w/w %).

	Ara	Gal	Glc	Xyl	Man	Total[1]
0–0	6	3	17	6	3	9
120–0	12	4	75	14	20	36
120–1	29	4	83	21	23	52
140–0	24	3	86	21	23	58
140–1	24	2	91	18	26	62
170–0	0	0	61	19	9	54
170–1	0	0	65	22	9	59

Values in % of total amount of individual sugar present in the pellets. 1) Yield of all monosaccharides (% of total neutral sugar present in the pellets).

further increased by the addition of arabinofuranosidases and galactosidases, respectively, since arabinose oligomers up to DP 6 and galactobiose were detected by HPAEC. Taken together, C1-G1 could degrade up to 62 % of all neutral sugars present in the pretreated pellets to monomers including 91 % of glucose originating from cellulose.

The time-dependent enzymatic hydrolysis of cellulose from pretreated pellets is also studied (Fig. 2.6). In general, cellulose hydrolysis is faster for the samples that were pretreated with sulfuric acid. For 120–1 and 140–1 the endpoint is almost reached after 24 h, whereas 120–0 and 140–0 require 48 h for maximal hydrolysis (Fig. 2.6). Ultimately, more sugars are hydrolysed with pretreatment 140–0 and subsequent enzyme digestion, because slightly higher losses occur at 140–1 (Fig. 2.3D). Therefore, pretreatment 140–0 is the optimal pretreatment for the release of glucose.
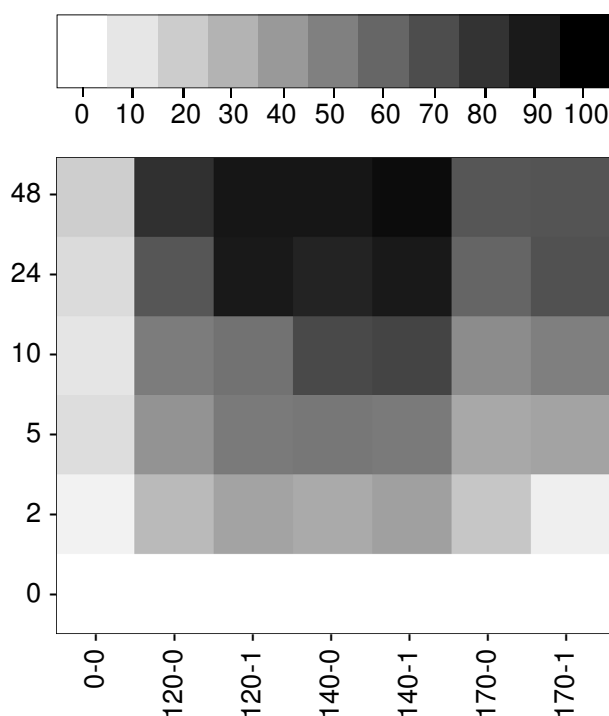
Fig. 2.6: Enzymatic release of glucose and cellobiose from pretreated sugar-beet pulp pellets. Values were transformed into a heatmap ranging from white (0 % solubilization) to black (100 % solubilization). Vertical labels: Incubation times (h), horizontally from left to right: samples in order of increasing severity.

2.3.7 Total solubilization after pretreatment and enzymatic digestion

Upon enzyme digestion of the pretreated pellets, the pellets of all pretreated samples are enriched in protein content from 8 w/w % to up to 25 w/w %, indicating that the main

part of the proteins remains insoluble during pretreatment, whereas their sugar content strongly decreases (no further data shown).

The influence of the pretreatment and enzymatic digestion on the solubility of each individual sugar was analyzed for all samples (Fig. 2.7, supplementary Table S2.2). After pretreatment, the minor constituents rhamnose and galactose are effectively solubilized at 170 °C, whereas the major constituents arabinose and uronic acid (mainly galacturonic acid) are only released to 60 % at 140 °C and are destructed at higher temperatures (Fig. 2.7A).

The total carbohydrate solubilization was calculated as the sum of all material solubilized after pretreatment and the material solubilized from the pellet after enzymatic digestion (Fig. 2.7B). The solubilization is inversely correlated with increasing severity and ranges from 34 % solubilization for the 0–0 digest to 80 % solubilization for the 140–0 digest. The pectin-associated sugars arabinose, galactose and galacturonic acid are solubilized to 85, 70 and 75 %, respectively, for sample 140–1. Mild pretreatments up to
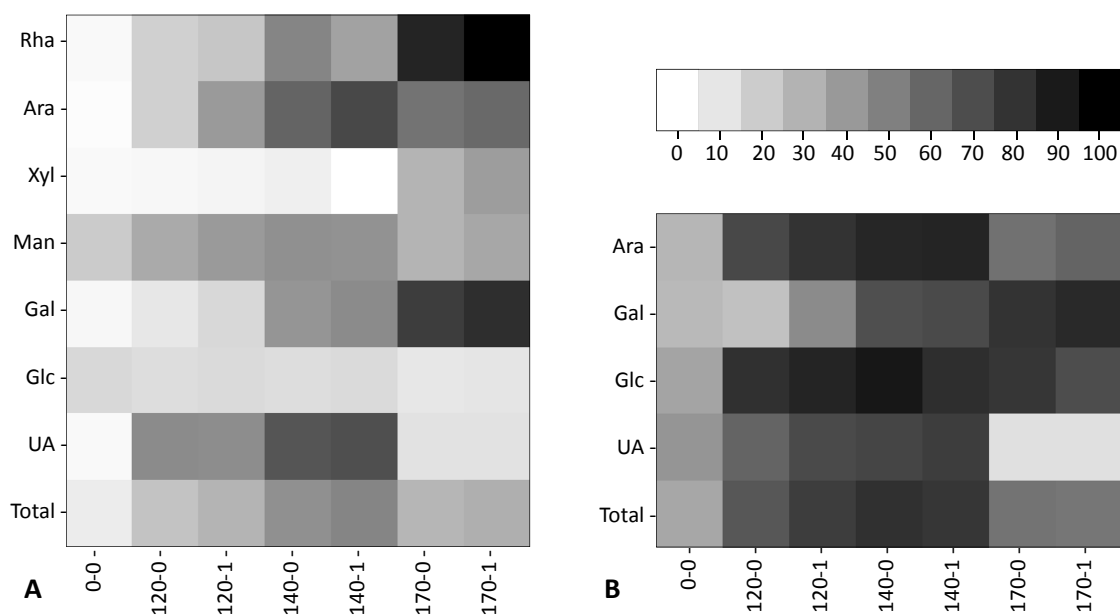
Fig. 2.7: Solubilization of individual sugars after pretreatment and subsequent enzymatic digestion. Values were transformed into a heatmap ranging from white (0 % solubilization) to black (100 % solubilization). A: Solubilization of individual sugars after pretreatment (% from starting material), B: Total yields of the main sugars that were solubilized after pretreatment and 24 h enzyme digestion. Values expressed in w/w % of the sugar content of sugar-beet pulp before pretreatment. Vertical labels: Ara – arabinose, Rha – rhamnose, Xyl – xylose, Man – mannose, Gal – galactose, Glc – glucose, UA – uronic acids, Total – total recovery of all sugars. Horizontally from left to right: samples in order of increasing severity.

Log R'_0 = -1.65 generally support the enzymatic degradation of cellulose from sugar-beet pulp (Fig. 2.7B).

C1-G1 also released high molecular-mass material from the pellets of the samples 0–0, 120–0 and 120–1 (determined by HPSEC analysis, no further data shown). It is likely, that the solubilized material is pectin, since the pellets after 48 h enzyme incubation are strongly reduced in their uronic acid content (no further data shown). The pectin can be solubilized either by the degradation of the cellulose network or by the removal of arabinan side chains that could have linked pectin to cellulose (Zykwinska et al., 2005).

2.4   Conclusions

We could demonstrate that the combination of a mild hydrothermal pretreatment at 140 °C with subsequent enzymatic digestion allows a fast and efficient hydrolysis of sugar-beet pulp. More than 90 % of all cellulose could be hydrolyzed within 24 h, while using lower enzyme dosages than reported in earlier studies. In contrast to chemical pretreatments described in literature, mild pretreatments up to 140 °C neither resulted in a loss of biomass, nor produced any compounds toxic to yeast. If the pretreatment process is implemented in a sugar factory, the pulp is already at 70 °C after saccharose extraction and exhaust heat of the lime ovens could be used in part for the heating up to 140 °C. The mild pretreatments did not lower the water-binding capacity of beet pulp and are, therefore, not suitable to increase the dry-matter content of sugar-beet pulp. Hence, to reduce transportation efforts, pretreated sugar-beet pulp should be fermented in vicinity to the sugar factory. A fed-batch reactor setting could allow reasonable high ethanol concentrations.

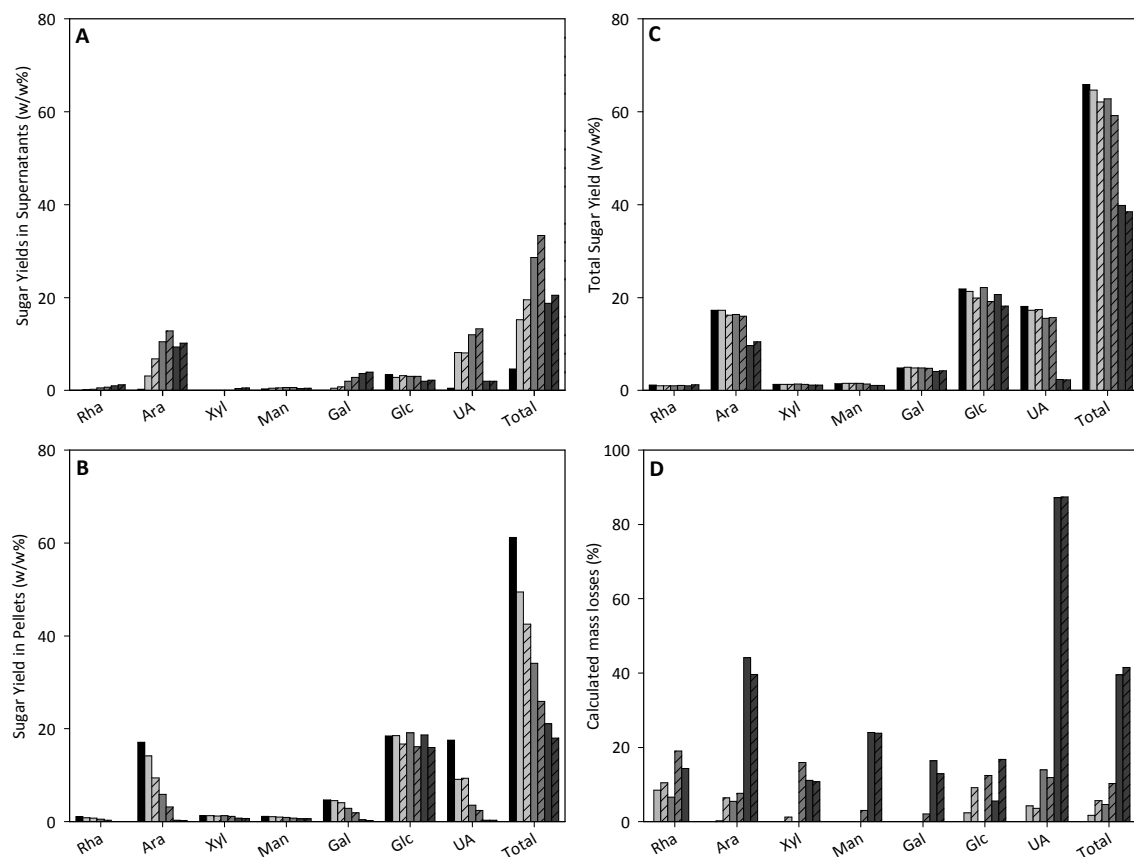## Acknowledgements

2.5 Supplementary data

Figure S2.1: Pressure and temperature curves of a 170 °C pretreatment. Black line – T (°C), gray line – p (bar).

Table S2.1: Molar sugar composition of pretreated samples.

	Rha	Ara	Xyl	Man	Gal	Glc	Uronic acids	Total sugar w/w %
S-Bl	1	6	1	6	3	73	10	39
S-120-0	1	25	0	3	3	18	49	63
S-120-1	1	40	0	3	4	15	63	63
S-140-0	2	43	0	2	7	10	37	72
S-140-1	2	45	0	2	8	8	35	70
S-170-0	5	55	2	2	17	9	9	44
S-170-1	6	55	3	2	17	10	8	46
P-Bl	2	33	2	2	7	29	35	75
P-120-0	2	33	3	2	9	35	16	70
P-120-1	2	26	3	2	9	38	19	69
P-140-0	2	20	4	3	8	54	9	68
P-140-1	1	15	5	3	7	60	8	62
P-170-0	0	2	4	3	2	88	1	60
P-170-1	0	2	4	3	1	88	1	54

Table S2.2: Volumetric concentrations of the main sugars present in sugar-beet pulp obtained after pretreatment and 24 h enzyme digestion.

	Ara	Gal	Glc	UA	Total[1]
Initial[2]	(9.0)	(2.5)	(11.0)	(9.0)	(31.5)
0–0	2.5	0.7	3.9	3.7	10.7
120–0	6.4	0.6	8.8	5.4	21.2
120–1	7.1	1.1	9.4	6.3	23.9
140–0	7.6	1.7	9.9	6.5	25.6
140–1	7.7	1.8	8.9	6.8	25.1
170–0	5.0	2.0	8.6	1.1	16.6
170–1	5.4	2.1	7.6	1.1	16.1

[1]Volumetric concentrations of arabinose (Ara), galactose (Gal), glucose (Glc) and uronic acids (UA) present in the supernatant fraction (g/l), [2]amount of sugars (g) present in 50 g starting material.

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Chapter 3

Chrysosporium lucknowense C1 arabinohydrolases effectively degrade sugar-beet arabinan

Abstract

The filamentous fungus *Chrysosporium lucknowense* C1 (C1) is a rich source of cell-wall degrading enzymes. In the present paper four arabinose-releasing enzymes from C1 were characterized, among them one endoarabinanase, two arabinofuranosidases and one exoarabinanase. Combinations of these enzymes released up to 80 % of the arabinose present in sugar-beet arabinan to fermentable monosugars. Besides the main product arabinobiose, unknown arabinose oligomers are produced from highly branched arabinan when endoarabinanase was combined with exoarabinanase and/or arabinofuranosidase. All described arabinose-releasing enzymes are temperature stable up to 50 °C and have a broad pH stability. This makes C1 arabinohydrolases suitable for many biotechnical applications, like co-fermentation bioethanol production.

3.1 Introduction

Biofuels can partially substitute for fossil fuels (Dale, 2008). Second-generation biofuels use biomass from non-food crops or industrial food side-streams, like wheat bran, wheat straw, corn stover and sugar-beet pulp (Pauly and Keegstra, 2008). In Europe sugar-beet pulp is a major by-product of sugar production that remains after saccharification of the sugar-beet roots. The dried pulp has a carbohydrate content of 75 % of which glucose, arabinose and galacturonic acid are the predominant monosaccharide constituents (McCready, 1966). These sugars are part of the cell-wall polysaccharides cellulose and pectin, respectively.

Pectic sugar-beet arabinans represent 20–25 % of the sugar-beet pulp dry matter. These arabinans are branched molecules with a linear α-1,5-linked arabinose backbone. Arabinose can be single or double substituted with α-1,2-linked or α-1,3-linked arabinose side-chains, which may be further branched themselves (Weinstein and Albersheim, 1979).

A number of different enzymes are known to degrade arabinans. Endoarabinanases are endo-acting enzymes that hydrolyze the linear regions of the arabinan backbone and release a mixture of arabinose and arabinose oligomers (Beldman et al., 1997). All other arabinose-releasing enzymes release arabinose from the non-reducing end of the backbone or the side-chains (Chávez Montes et al., 2008). Exoarabinanases release arabinose (Ichinose et al., 2008), arabinobiose (Carapito et al., 2009; Sakamoto and Thibault, 2001) or arabinotriose (Kaji and Shimokawa, 1984) from linear α-1,5-linked arabinan. Arabinofuranosidases (Abf) subgroup into A and B. Abf A is active towards arabinose oligomers and *p*-nitrophenyl arabinofuranoside (pNP-Ara), but does not act on polymers. It can hydrolyze all kinds of linkages present in arabinan and arabinoxylan oligomers (Matsuo et al., 2000). Abf B is active towards *p*-NP-arabinofuranoside and beet arabinan polymers. It acts mainly on α-1,3-linked arabinose and much less on α-1,5-linkages (Rombouts et al., 1988). Some Abf B also show activity towards arabinoxylan oligomers (De Vries and Visser, 2001). Arabinoxylan arabinofuranohydrolases (AXH) release arabinose specifically from arabinoxylan, however, some AXH also degrade arabinan (De Vries and Visser, 2002; Kormelink et al., 1991).

Sugar-beet pulp became interesting as raw material for bioethanol production with the availability of arabinose-fermenting yeasts (van Maris, 2007). Complete cell-wall degradation is required to efficiently use sugar-beet pulp for bioenergy production. Commercial enzyme preparations can solubilise arabinan from sugar-beet pulp with monomer yields of up to 67 % (Micard et al., 1996). Due to the complex, interwoven structure of

the cell wall, a more efficient release of arabinan may also require cellulase activities, which are lacking in the commercial preparations (Micard et al., 1996). The ascomycete *Chrysosporium lucknowense* C1 is an industrial strain optimized in cellulase and hemicellulase production. The C1 genome has been fully sequenced and annotated. Of the more than 200 candidate genes that have high similarities to carbohydrate active enzymes, 58 pectinolytic enzymes have been putatively identified, among them 14 arabinose-releasing enzymes (Hinz et al., 2009; Verdoes et al., 2007). Therefore, C1 also seems to be a good platform for the degradation of pectin-rich biomass. The aim of this research was the characterization of arabinohydrolases from *Chrysosporium lucknowense* C1 that are necessary for complete degradation of sugar-beet arabinan. Crude overexpressed extracts of the 14 candidate genes were tested towards sugar-beet arabinan. Based on their high activities towards different arabinan substructures, the arabinohydrolases Abn1, Abn2, Abn4 and Abf3 were selected for further characterization.

3.2 Methods

3.2.1 Substrates and Enzymes

Characterization of the C1 arabinohydrolases was performed on linear arabinose oligomers (Megazyme; Bray, Ireland) and linear and branched sugar-beet arabinan (British Sugar; Peterborough, United Kingdom). To determine side activities, purified fractions were tested on konjac glucomannan (Kalys; Bernin, France), arabinogalactan type II (Meyhall Chemical; Thurgau, Switzerland), Tamarind xyloglucan (Dainippon Pharmaceutical; Osaka, Japan), potato galactan and wheat arabinoxylan (both from Megazyme). Other chemicals were from Sigma-Aldrich or Merck.

All enzymes were purified from crude C1 fermentation liquids of homologous overexpressed enzymes. The fermentation liquids were received from Dyadic Netherlands (Wageningen, The Netherlands). Three putative arabinose-releasing enzymes were selected for further characterization. Abn1 has a theoretical molecular mass of 32 kDa, and based on its amino acid sequence it was classified as an endoarabinanases from glycoside hydrolase (GH) family 43. Abn2 has a theoretical molecular mass of 40 kDa and was classified as a GH family 93 exoarabinanase. Abn4 has a theoretical molecular mass of 33 kDa and was classified as a GH43 arabinanase. Abf3 was purified and described to be an arabinoxylan arabinofuranohydrolase by (Hinz et al., 2009) using hydrophobic

interaction chromatography (HIC, SP Sepharose FF) and size exclusion chromatography (SEC, Superdex 200).

3.2.2   Determination of protein concentration

The protein concentrations of the enzyme fractions were determined using the Pierce BCA (bicinchoninic acid) protein assay kit according to the manufacturer's manual. The protein content was calculated based on a standard curve established with bovine serum albumin (5–250 µg/ml). The microtiter plate protocol of the manufacturer was used.

3.2.3   Enzyme purification

All purification steps were performed using an ÄKTA explorer P-900 liquid chromatography system (GE Healthcare, Uppsala, Sweden). Separation was done at room temperature and the fractions were collected on ice with an automated fraction collector. Elution was followed at 214 and 280 nm. The protein composition was verified by sodium dodecyl sulfate - polyacryl amide gel electrophoresis (SDS-PAGE, 3.2.4). Abn1 and Abn2 activities were determined on linear arabinan with the *p*-hydroxybenzoic acid hydrazide assay (PAHBAH, 3.2.6). Abn4 activity was determined using pNP-Ara (3.2.7).

Anion exchange chromatography

All enzymes were subjected to Anion Exchange Chromatography (AEC) on a Source 15 Q column (50/6; GE Healthcare, self-packed) and eluted at 20 ml/min. The samples were dialyzed against 10 mM sodium phosphate buffer (pH 7.0) overnight at 4 °C and 15 ml sample was loaded onto the column at 5 ml/min. All enzymes were eluted using a sodium chloride (NaCl) gradient in 10 mM sodium phosphate buffer (pH 7.0) comprising 5 segments: 0 mM NaCl for 4 column volumes (CV), a gradient of 0-500 mM NaCl over 20 CV, 500 mM NaCl for 4 CV, 1 M NaCl for 5 CV and 0 mM NaCl for 5 CV (equilibration). Abn2 and Abn4 were eluted with 40 mM to 65 mM NaCl and 43 mM to 129 mM NaCl, respectively. Abn1 did not bind to the column. However, a large amount of protein was bound during AEC and the unbound Abn1 was significantly purified. Fractions of 20 ml were collected for all samples.

Hydrophobic interaction chromatography

Abn1 and Abn2 were further purified by HIC using a HiLoad Phenylsepharose HP 26/10 column (GE Healthcare). The active AEC fractions were pooled and mixed 1:1 with

2.4 M ammonium sulfate in 20 mM Bis-Tris/HCl buffer (pH 6.0) and loaded at 5 ml/min. The samples were eluted using a decreasing ammonium sulfate gradient in 10 mM Bis-Tris/HCl buffer (pH 6.0) comprising 4 segments: 1.2 M ammonium sulfate for 5 CV, a gradient of 1.2-0 M ammonium sulfate over 20 CV, 0 M ammonium sulfate for 5 CV and 1.2 M ammonium sulfate for 5 CV (equilibration). Abn1 and Abn2 were both eluted with 0.9 M to 0.72 M ammonium sulfate. Fractions of 20 ml were collected. The Abn2 containing fractions were pooled and dialyzed at 4 °C overnight against the elution buffer containing 50 mM NaCl (V = 5 l) and stored at 4 °C.

Size exclusion chromatography

After HIC, active Abn1 and Abn4 protein fractions were separately pooled and concentrated using an Amicon ultrafiltration device (Billerica, MA, USA) with a 12 kDa cutoff membrane. Concentrated samples (5 ml) were subjected to SEC on a preparative Superdex 75 column (TK 26/100, GE Healthcare) and eluted at 5 ml/min with 10 mM Bis-Tris/HCl (pH 6.0) containing 50 mM NaCl. Fractions of 5 ml were collected. Purified and active fractions were pooled and stored at 4 °C.

3.2.4   SDS-PAGE and isoelectric focusing

SDS-PAGE was performed with the Biorad mini-protean II system and Biorad Powerpac 300 power supply (Hercules, CA, USA). Pierce Tris-Hepes SDS gels (12 %) were used according to the manufacturer's protocol. Coomassie staining was done overnight using the Fermentas PAGE blue stain. Isoelectric focusing with silver staining was performed using the Phast system (GE Healthcare) according to the manufacturer's manual.

3.2.5   Enzyme incubations

All incubations were carried out at 30 °C unless otherwise mentioned. One enzyme unit is defined as 1 µmol (product)/min*mg (enzyme) at 30 °C. For biochemical characterization 0.5 w/v % substrate was used with 0.02 w/w % enzyme on protein basis. Specific activities were determined towards linear arabinan (Abn1 and Abn2) and branched arabinan (Abn4). Substrates were dissolved in buffer at 60 °C. Diluted McIlvaine buffers (20 mM citric acid and 40 mM disodium hydrogen phosphate mixed to give pH 3.0 to pH 8.0) were used to study pH optima and stability. Temperature optima and activity assays were performed in 50 mM sodium acetate buffers (pH 4.5 for Abn2 and pH 5.5 for Abn1 and Abn4) from 20 to 70 °C. To determine stability, the enzymes were incubated for 1 h

at pH 3.0 to 8.0 or T = 20 to 70 °C prior to substrate addition. The subsequent digestion was performed at optimal pH and 30 °C. For end product release linear and branched arabinan (5 mg/ml) were incubated with 0.1 U/ml of the enzymes. Aliquots were taken at 2, 24, 48 and 72 h with 0.1 U/ml additional enzyme at both 24 h and 48 h incubation time. The degradation was followed by high performance size exclusion chromatography (HPSEC). The activity on arabinose monomers and oligomers in the range of DP 2-6 was tested (5 mg/ml; 0.1 U/ml enzyme, t = 2, 24 and 48 h with additional 0.1 U/ml enzyme after 24 h). Products were quantified by high performance anion exchange chromatography (HPAEC) with a calibration curve (2-40 µg/ml) of arabinose monomer and oligomers (DP 2-6).

3.2.6 Determination of reducing ends with PAHBAH assay

PAHBAH reducing-end assay was performed as described by Lever (Lever, 1972). To prepare the working solution, one part of *p*-hydroxybenzoic acid hydrazide (5 w/v %) in 0.5 M HCl was mixed with four parts of 0.5 M NaOH. The sample (10 µl) was mixed with 200 µl working solution and incubated at 70 °C for 30 minutes in microtiter plates covered with aluminum foil. After cooling the microtiter plate was centrifuged at $1000 \times g$ for 2 min and the absorbance was measured at 405 nm. The reducing-end concentration was quantified using an *l*-arabinose calibration curve (5–750 µg/ml).

3.2.7 Activity towards *p*-nitrophenyl-arabinofuranoside

Activity of Abn4 towards pNP-Ara was monitored by the release of *p*-nitrophenol. The sample (10 µl) was incubated with 190 µl pNP-Ara (0.5 mM) in 10 mM sodium acetate buffer (pH 4.5) for one hour at 32 °C. The pH was adjusted to pH 7.4 with 50 µl sodium phosphate buffer (0.25 M, pH 7.4). The absorbance was measured at 405 nm. Arabinose release was quantified indirectly with a *p*-nitrophenol standard curve (10–500 µM).

3.2.8 Sugar composition analysis

Polysaccharides were hydrolysed with aqueous 72 w/w % H_2SO_4 (1 h, 30 °C), followed by hydrolysis with 1 M H_2SO_4 (3 h, 100 °C). Alditol-acetates derivatisation was performed as described by Englyst and Cummings (1984). A Thermo Focus GC gas chromatograph equipped with a Supelco SP 2380 column was used with Helium as inert gas, 24 PSI pressure and a flow rate of 1.1 ml/min. All GC runs were performed using a 2 µl injection volume of sample dissolved in acetone. Uronic-acid content was determined according

to Ahmed and Labavitch (1978) using an autoanalyzer (Skalar Analytical, Breda, The Netherlands). A galacturonic acid standard curve (12.5–100 µg/ml) was used for quantification.

3.2.9 HPSEC

HPSEC was performed on a Thermo Scientific spectra quest HLPC (Thermo Finnigan, Waltham, MA, USA) equipped with a set of 4 TSK-Gel G columns (Tosoh bioscience, Tokyo, Japan) in series: guard column PWXL (6 mm ID x 40 mm) and separation columns 4000 PWXL, 3000 PWXL and 2500 PWXL (7.8 mm ID x 300 mm). Samples (20 µl, 5 mg/ml) were eluted with filtered aqueous 0.2 M sodium nitrate at 40 °C and a flow rate of 0.8 ml/min. Elution was followed by Refractive index detection (Shodex RI 101; Showa Denko K.K., Kawasaki, Japan).

3.2.10 HPAEC

The monomer and oligomer sugar levels of the digests were analyzed by HPAEC according to Albrecht and co-workers (2009). Arabinose and arabinose oligomers (V = 10 µl, c = 50–100 µg/ml) were eluted with an adapted sodium acetate (NaOAc) gradient: 0 mM NaOAc for 5 min, a gradient of 0–500 mM NaOAc over 25 min, 1 M NaOAc for 10 min and 0 M NaOAc for 15 min (equilibration).

3.3 Results and discussion

3.3.1 Characterization of purified arabinohydrolases

The purification required up to three chromatography steps with final activity recoveries up to 50 %. All purified fractions showed a single band on SDS-PAGE displaying the protein of interest (data not shown). The molecular masses of the proteins were determined close to the calculated values for Abn2 (40 kDa) and Abn4 (33 kDa). For Abn1 a molecular mass of 36 kDa was estimated, which is slightly higher than theoretically expected (32 kDa). This difference may reflect glycosylation of the protein. Glycosylation has been reported for enzymes produced in fungi, like the *Aspergillus niger* endoarabinanase AbnA (Flipphi et al., 1993). The specific activities of purified Abn1 and Abn2 towards linear arabinan are 26 U/mg and 7.1 U/mg, respectively. Abn4 has a specific activity of 9.5 U/mg towards branched arabinan. These activities are in the same order of magnitude as reported for many arabinohydrolases from other sources (De Vries et al., 2000; Skjøt

et al., 2001). Purified Abn1, Abn2 and Abn4 did not show activity against oat spelt xylan, wheat arabinoxylan, arabinogalactan type II, potato galactan, konjac glucomannan, polygalacturonic acid, carboxymethyl cellulose and tamarind xyloglucan.

Biochemical characterization

pH and temperature optima

The pH optima determined for Abn1, Abn2 and Abn4 are illustrated in Fig. 3.1A. All enzymes are most active under slightly acidic conditions. Abn1 and Abn4 are most active between pH 5.0 and 6.5 with a maximum at pH 5.5. The Abn2 activity is highest between pH 3.0 and 5.5 with a maximum at pH 4.0. In Fig. 3.1B the temperature optima of Abn1, Abn2 and Abn4 are shown. The temperature optimum is 50 °C for Abn2 and 60 °C for Abn1 and Abn4. The optimum curves for all enzymes are asymmetric with a nearly two fold increase per 10 K temperature increment from 20 °C to 50 °C. Above optimum temperatures the enzyme activities rapidly decrease. For arabinoxylan arabinofuranohydrolase Abf3 optimal reaction rates have been reported at 40 °C and pH 5.0. The enzyme was stable up to 50 °C and completely inactivated above 65 °C (Hinz et al., 2009; Pouvreau et al., 2011).

pH and temperature stabilities

Fig. 3.1C shows the pH stability of Abn1, Abn2 and Abn4. It can be seen that the curves of all enzymes are relatively broad. All enzymes are not stable at pH 3.0 or lower and show different stabilities between pH 4.0 and 8.0. Abn1 is very stable between pH 5.0 and 8.0 and even possesses 70 % of its optimal activity at pH 4.0. Abn2 has similar pH stability as Abn1, but the stability has a more pronounced optimum at pH 6.0 to 7.0. Abn4 is stable in the neutral pH range between pH 6.0 and 8.0, however, the remaining activity is only 80 % indicating that Abn4 is less stable than Abn1 and Abn2. The temperature stabilities of Abn1, Abn2 and Abn4 are presented in Fig. 3.1D. All three enzymes are stable up to 50 °C with Abn2 and Abn4 showing a slightly higher stability up to 55 °C. The remaining activity of Abn1 is 85 % of the optimal activity up to 50 °C and is almost lost 60 °C. Abn2 is the most stable enzyme having 90 % of its initial activity at 55 °C. It is completely inactivated at 70 °C and above. Abn4 behaves similarly with the difference that, even at 20 °C, only 80 % of the initial activity could be recovered. Long-term stability for all enzymes was tested over 24 hours at pH 6.0 and 30 °C. It was found that Abn1 and Abn2 enzymes remain active to more than 90 % and Abn4 still had 80 % of its initial activity (no further
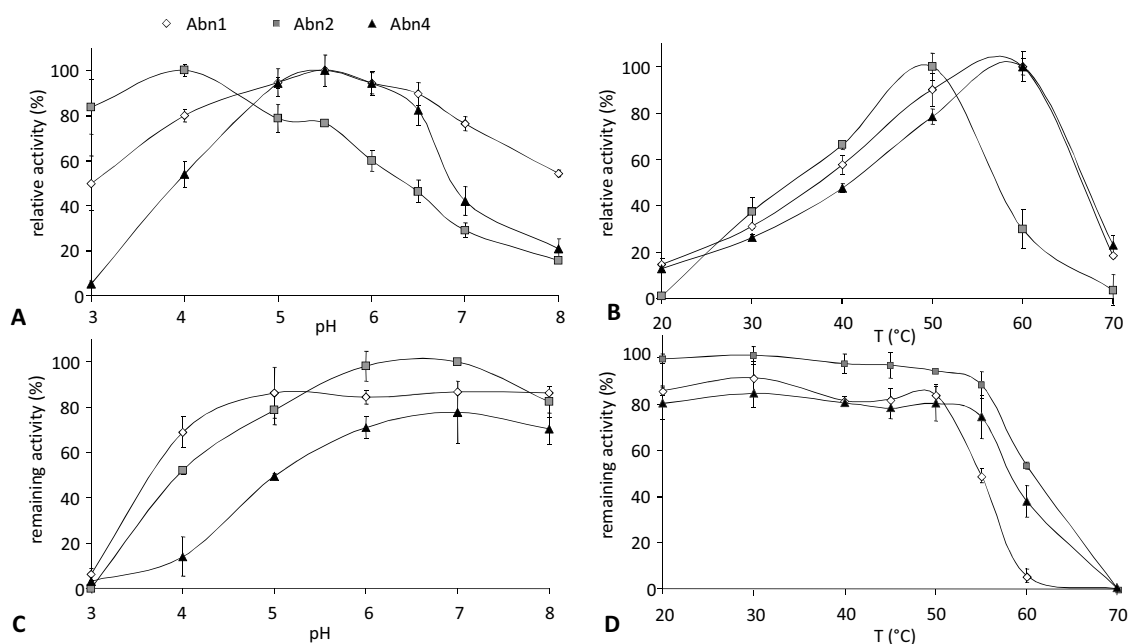
Fig. 3.1: Biochemical characterization of Abn1, Abn2 and Abn4. a) pH optima, b) temperature optima, c) pH stabilities, d) temperature stabilities. Activities are determined on linear arabinan (Abn1 and Abn2) and branched arabinan (Abn4), respectively (n = 3).

data shown). The arabinohydrolases described in this paper have broad pH optima and stabilities and optimal temperatures of around 50 °C. Hence, they can potentially degrade arabinan jointly in a single incubation. The temperature properties are similar to those reported for other arabinohydrolases. In contrast, the C1 arabinohydrolases act at a higher pH and in a broader range than most fungal arabinohydrolases. This may be explained with the origin of C1, which was isolated from Far East alkaline soil (Bukhtojarov et al., 2004). Interestingly, the pH optima of the C1 arabinohydrolases are similar to those of most bacterial arabinohydrolases (Beldman et al., 1997; Saha, 2000). This is probably due to the similar environmental growth conditions of the producing organisms. The pH optima of the arabinohydrolases are also comparable to the pH optimum of typical yeasts, which suggests that the C1 arabinohydrolases can be highly useful in the liquefaction of sugar-beet pulp for bioethanol production.

3.3.2 Enzyme specificity towards arabinose oligomers

The performance of the C1 arabinohydrolases was tested on linear arabinose oligomers ranging from DP 2–6. Arabinose was included in the experiment to rule out that it can serve as a substrate as well, e.g. for transferase activities. Fig. 3.2A shows the action

of Abn1 towards linear arabinose oligomers. After 48 h, Abn1 degraded oligomers in the range from DP 3–6 and produced, on a weight basis, 50–60 % arabinobiose and 20 % arabinose monomers and left 25 % of the oligomers present with DP ≥ 3. No further degradation was observed after prolonged incubation with a fresh ten-fold dose Abn1 added. Recent studies of arabinose oligomers by LC-MS suggest that the linear arabinose oligomers used in the experiments were not completely pure and co-eluted with other arabinose oligomers (Westphal et al., 2010). Arabinotriose was the main product
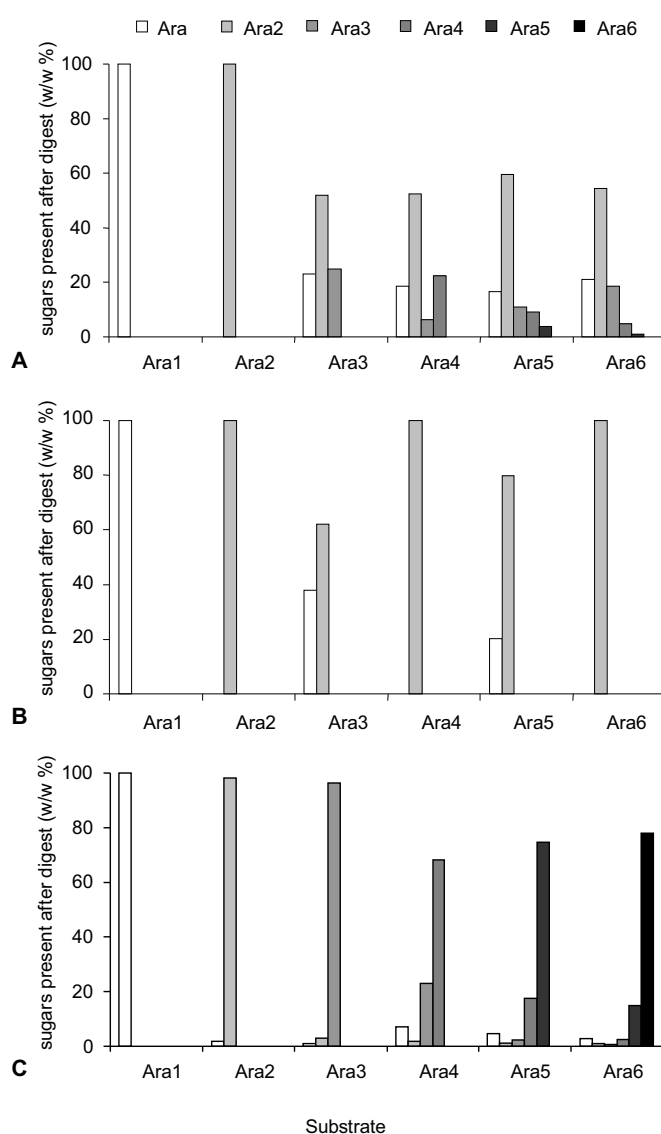
Fig. 3.2: The degradation of linear arabinose oligomers by C1 arabinohydrolases determined by HPAEC. X-axis: Arabinose oligomers DP1–6 used as substrate. a) Abn1, b) Abn2, c) Abn4.

from arabinohexaose after 2 h (data not shown). This pattern can be explained with an endo-mode of action with higher activity on larger oligomers, as also described for *Aspergillus niger* endoarabinanase (Rombouts et al., 1988). Abn2 was active on linear arabinose oligomers starting from arabinotriose (Fig. 3.2B). It split off an arabinobiose unit from the trimer. Arabinotetraose and arabinohexaose were fully converted into arabinobiose. From arabinotriose and arabinopentaose arabinose monomers were left over after releasing dimer from the oligomer. No other oligomers were released at any stage of digestion indicating that Abn2 is an arabinobiose releasing exoarabinanase. Abn4 was poorly active towards arabinobiose and arabinotriose and left more than 90 % of the substrate unaltered (Fig. 3.2C). In contrast, Abn4 removed arabinose monomers from DP 4–6 oligomers. However, this activity was rather low leaving more than 60 % of the oligosaccharides undigested. The poor performance towards arabinose oligomers speaks against a classification as Abf A or B as described by Beldman and co-workers (1997). Based on the obtained results, no clear classification can be done. In contrast to Abn4, the arabinoxylan arabinofuranohydrolase Abf3 acted similar towards linear arabinose oligomers as Abf A and B. It completely degraded linear arabinose oligomers in the range from DP 2–6 to arabinose monomers (data not shown).

3.3.3   Action on linear and branched arabinan

Molecular-mass distribution upon maximal product conversion

In nature, arabinans are covalently bound to the rhamnogalacturonan (RG) I region of the pectin backbone. As shown in Table 3.1, linear and branched arabinans contain considerable amounts of rhamnose, galacturonic acid and galactose (32 and 37 w/w %, respectively) that are likely to be part of RG I.

Table 3.1: Sugar composition (w/w %) of linear and branched arabinan.

Arabinan	Rha	Ara	Gal	Glc	GalA	Total sugar
Branched	3.5	65.7	14.1	4.4	9.8	97.6
Linear	4.2	55.9	18.9	6.9	13.6	99.5

Linear arabinan

The molecular-mass distributions of linear and branched arabinan after different enzyme digestions are presented in Fig. 3.3. When digested with Abn1, the average molecular

mass of the high molecular mass (HMM) fraction between 20 and 25 min shifts from 46 to 30 kDa with a concomitant decrease of the peak area by 60 % (Fig. 3.3A). The 30 kDa peak remains in both, linear and branched, arabinan digestions. It could reflect a rhamnogalacturonan I core structure, to which the arabinan side-chains are bound to. The HMM fraction of a large scale branched-arabinan digest was subjected to sugar composition analysis. The results confirm the presence of rhamnose and galacturonic acid as well as the accumulation of galactose in the HMM fraction (Westphal et al., 2010). Therefore, the 60 % decrease in the HMM peak area suggests that Abn1 can efficiently cut the backbone of linear arabinan and degrade the polymers to small molecular-mass oligomers. Abn2 decreases the peak area of the HMM fraction by 40 %, while it maintains its average molecular mass. This result confirms the exo-mode of action of Abn2. A combined digestion with Abn1 and Abn2 results in the strongest degradation and a 67 % HMM peak area decrease is observed. Abn2 digests contain an additional peak at 29 min derived from ammonium sulfate, which was not fully removed after hydrophobic interaction chromatography.
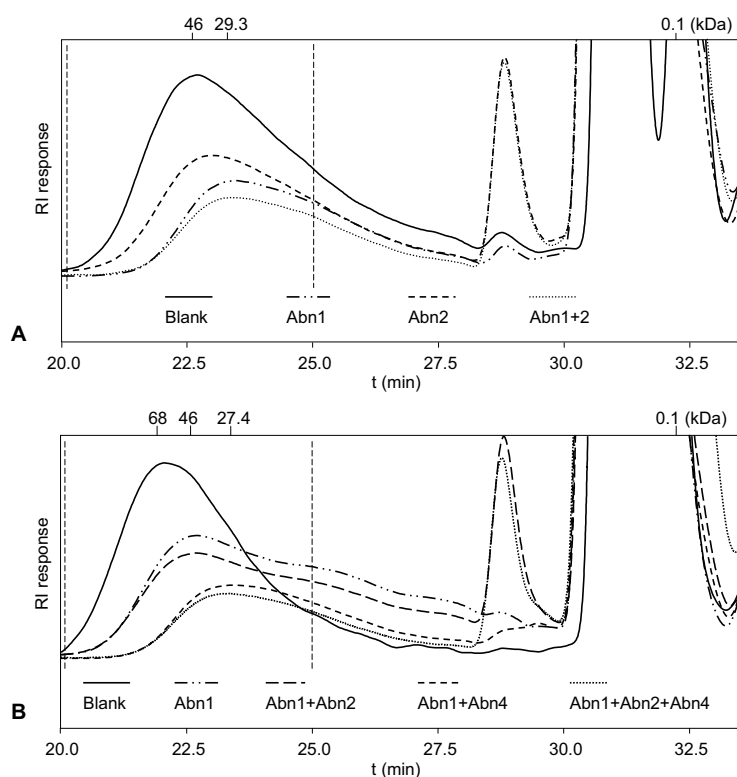

Fig. 3.3: HPSEC elution patterns of linear (a) and branched (b) arabinans digested with different combinations of C1 arabinohydrolases. Elution times of pullulan standards are indicated.

Branched arabinan

When branched arabinan is incubated with Abn1, the average molecular mass of the HMM fraction shifts from 68 to 46 kDa, while its area decreases by 30 % (Fig. 3.3B). Compared to the mass distribution of the linear arabinan after enzyme treatment, the distribution of the branched arabinan treated with Abn1 is much broader. This indicates that Abn1 cleaves the substrate only a few times, suggesting that Abn1 is hindered by arabinose side-chains. A combined digestion with Abn1 and Abn2 results in a similar pattern. However, this combination can degrade 10 % more of the polymeric arabinan than Abn1 alone. This finding suggests that Abn2 can act on parts of the branched arabinan where Abn1 can not act, e.g. Abn2 could be less hindered by arabinose side-chains. Abn4 is active on branched arabinan. However, it only slightly influences the average molecular-mass distribution (not shown). A combination of Abn1 and Abn4 degrades 65 % of the branched arabinan. A combination of all three enzymes degrades 70 % of the arabinan polymer and, as seen for linear arabinan, decreases the remaining average molecular mass to approximately 30 kDa. It can be concluded that effective degradation of linear arabinan only requires Abn1, whereas a combination of Abn1 and Abn4 is needed for the degradation of branched arabinan. Abn2 slightly enhances the degradation of both substrates.

End product release

Linear arabinan

The degradation of linear arabinan by Abn1 was monitored at different times (data not shown). In early stages oligomers in the range of DP 3–15 are produced. These oligomers are mainly broken down to arabinotriose after 24 h and, after 72 h, to arabinobiose and arabinose. A similar pattern was reported for Arabinanase A from *Pseudomonas fluorescens* (McKie et al., 1997). The hydrolysis products were analyzed and quantified after maximal substrate conversion (Fig. 3.4A). Abn1 releases 69 % of the total arabinose as DP 1–4 oligomers, mainly as arabinobiose. Abn2 degrades 40 % of the arabinose present in the polymer to arabinobiose. A combination of Abn1 and Abn2 releases almost 80 % of the arabinose present, mainly as monomers and dimers. Abn4 does not act on the linear arabinan polymer, neither alone nor combined with Abn1 and Abn2. The results confirm that Abn1 is an endoarabinanase. Time-dependent degradation data suggest that Abn1

follows a multiple chain-attack mechanism with preference for larger oligomers. Unlike Abn1, Abn2 does not produce any other oligomers than arabinobiose at any stage of the reaction. It is, therefore, confirmed that Abn2 is an exoarabinanase able the release arabinobiose from the α-1,5-arabinan backbone.
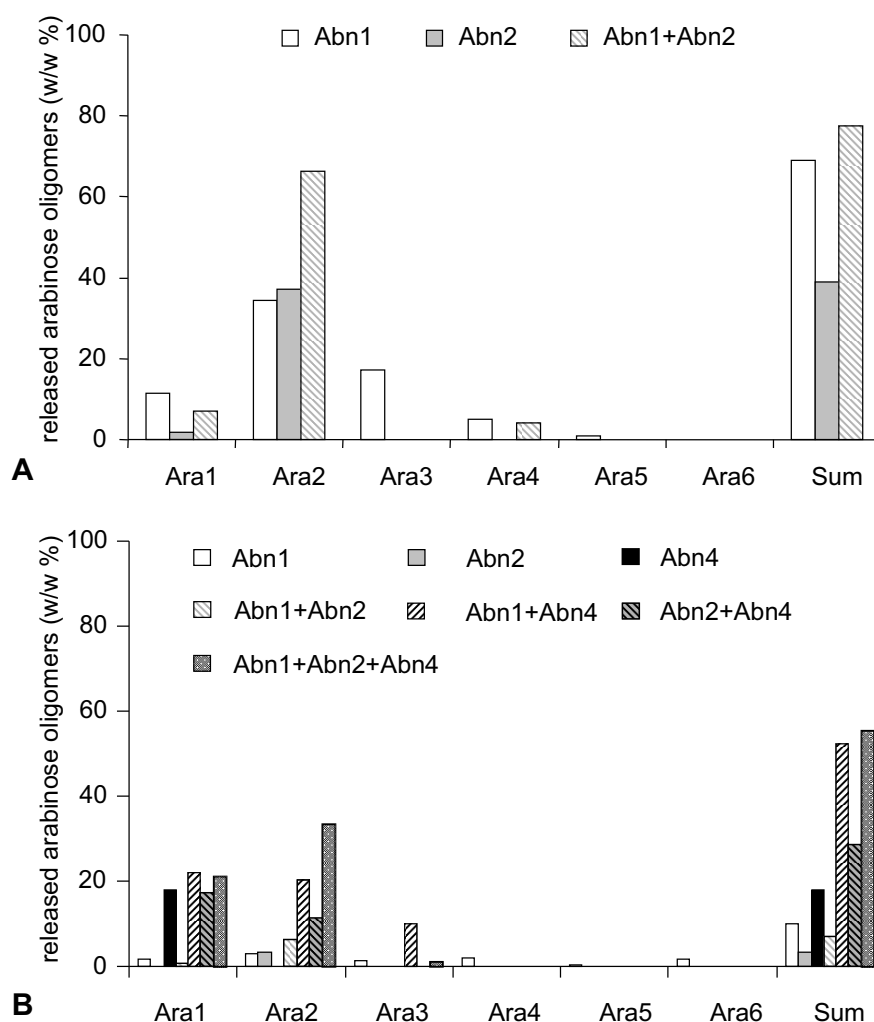

Fig. 3.4: Arabinose oligomers release from linear (a) and branched (b) arabinan with different combinations of C1 arabinohydrolases as determined by HPAEC. X-axis: released monomers and oligomers from DP 2–6.

Branched arabinan

The oligomer release from branched arabinan was quantified after maximal substrate conversion (Fig. 3.4B). Abn1 and Abn2 only released on a weight basis 10 and 3 % of the total arabinose as linear oligomers, respectively. Both enzymes are hindered by the

presence of arabinose side-chains. Abf3 alone did not act on branched arabinan. A combination of Abn1 and Abf3 released, on a weight basis, 25 % of the arabinose present as monomers (no further data shown). This suggests that Abf3 is not active on arabinan polymers, but it can only act on arabinose oligomers. Abn4 could release 18 % of total arabinose as monomers. A combined incubation with Abn1 and Abn4 releases 52 % of total arabinose present as arabinose monomers and linear arabinose oligosaccharides. This indicates that Abn4 is an arabinofuranosidase active on the side-chains of sugar-beet arabinan. The relatively low yield of arabinose monomer and linear oligomers from branched arabinan suggests that Abn4, like *Aspergillus niger* AbfB (Rombouts et al., 1988), cannot hydrolyze all types of linkages present in branched arabinan. More in depth structural analysis of these oligomers is ongoing in order to determine the linkage specificity of Abn4.

Release of non-linear arabinose oligomers

The digest of branched arabinan with Abn1, Abn2 and Abn4 released 56 % of the arabinose present as arabinose monomers and linear oligomers (Fig. 3.3B). A similar amount of arabinose could be released from sugar-beet pulp when digested with a cellulase rich enzyme preparation complemented with Abn1, Abn2 and Abn4 (no further data shown.) The relatively low level of linear arabinose oligomers could be explained by the formation of arabinose isomers that not only comprise α-1,5 linkages. These oligomers could not be quantified due to the lack of standards. Fig. 3.5A shows the HPAEC elution profile of branched arabinan samples treated with C1 arabinohydrolases. It can be seen that Abn2 alone releases small amounts of arabinobiose and two unknown peaks eluting at 10 and 17 min (line a). Abn1 and Abn4 release high amounts of arabinose, arabinobiose and arabinotriose (line b). Besides linear oligomers a number of unknown peaks (marked by asterisks) appear that elute shortly after the linear standard oligomers. A combination of the Abn1, Abn2 and Abn4 (Abn124) produces a more complex mixture of oligomers (line c). It is likely that these peaks represent arabinose oligomers also contain α-1,2 or α-1,3 linked arabinose moieties. To test this hypothesis arabinofuranosidase Abf3 was added to an Abn124 digest. The samples were analysed at higher concentrations (500–1000 µg/ml) with a less steep gradient than normal to achieve higher sensitivity and better separation (0–350 mM NaOAc in 25 min). From Fig. 3.5B it can be seen that, compared to Abn2, even more unidentified peaks can be recognized in the Abn124 digest (line b). When Abf3 is added, the majority of the peaks representing both unknown and linear arabinose oligomers are degraded to monomers (line c). This indicates that the unknown
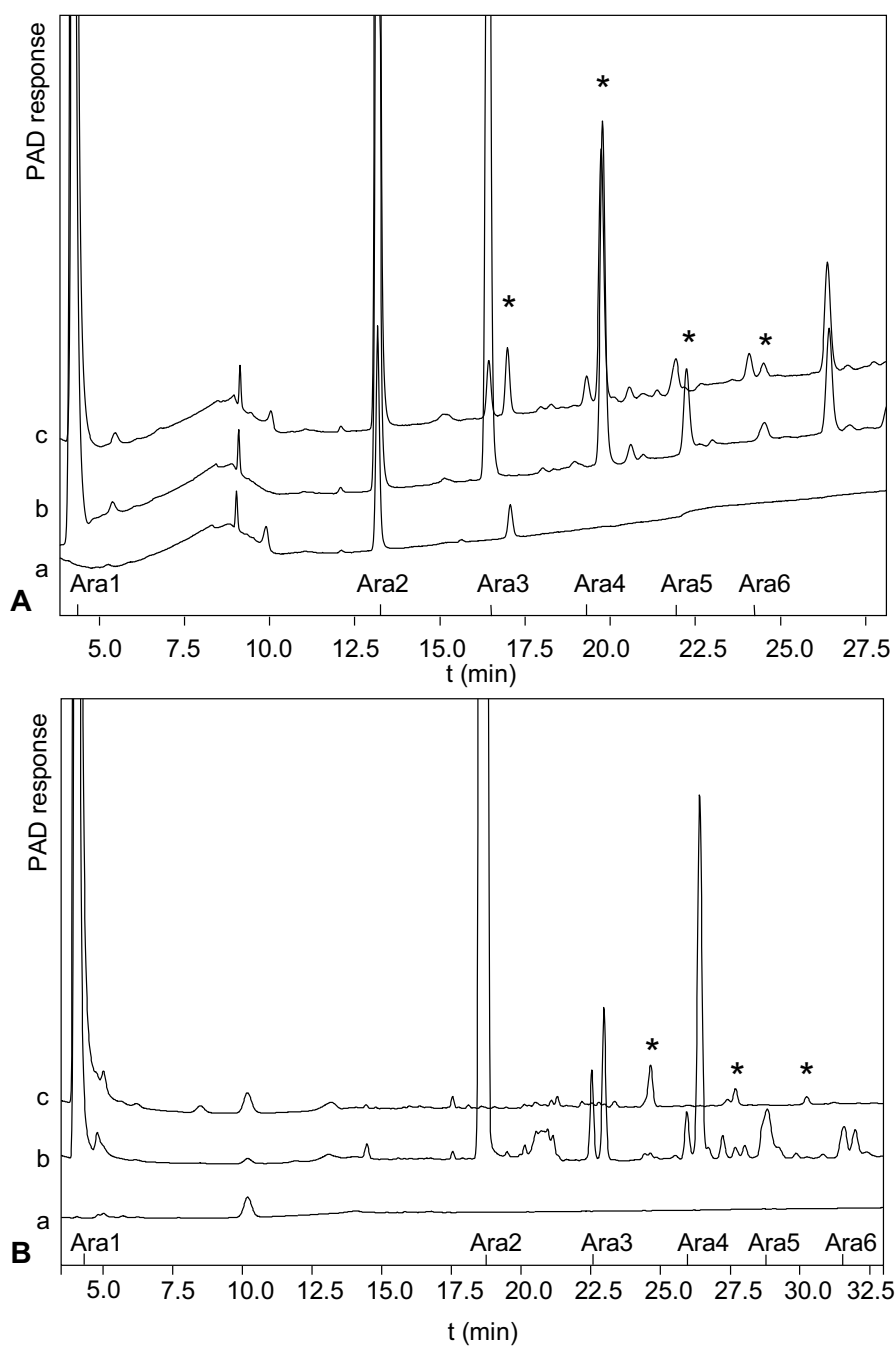
Fig. 3.5: Release of non linear arabinose oligomers from branched arabinan by C1 arabinohydrolases as determined by HPAEC. a) Default HPAEC gradient with total sugar concentrations between 50 and 100 μg/ml. Line a - Abn2; line b - Abn1 and Abn4; line c - Abn1, Abn2 and Abn4. b) Less steep HPAEC gradient with total sugar concentrations of 500–1000 μg/ml. Line a - branched arabinan blank; line b - Abn1, Abn2 and Abn4; line c - Abn1, Abn2, Abn4 and Afb3. Ara1 to Ara6: retention times of linear arabinose oligomers with DP 1–6. Asterisks indicate peaks of unknown structure.

peaks are arabinose oligomers as well. It also strengthens the hypothesis that Abn4 does not act on all types of side-chain linkages. Adding Abf3 also results in a series of unknown peaks (asterisks), probably derived from higher molecular-mass material. The nature of the various isomers formed by the enzymes mixtures were further investigated by Westphal and co-workers (2010). To the authors knowledge the current report is the first one that describes the release of isomeric arabinose oligomers by a combination of an endoarabinanase, an exoarabinanase and an arabinofuranosidase.

3.4 Conclusions

The arabinohydrolases Abn1, Abn2, Abn4 and Abf3 from C1 act together on the degradation of arabinans (Table 3.2). All enzymes are stable in a wide pH range and resist temperatures up to 50 °C, which makes them suitable for arabinan degradation from sugar-beet pulp. A combination of endoarabinanase Abn1, exoarabinanase Abn2 and arabinofuranosidase Abn4 releases arabinose and linear arabinose oligomers more effectively from linear arabinan than from branched arabinan. It was found that branched arabinan digests contain a number of unknown, most likely branched, arabinose oligomers. The arabinofuranosidase Abf3 converts all linear and unknown arabinose oligomers to arabinose monomers.

Table 3.2: Activity of *Chrysosporium lucknowense* C1 arabinohydrolases towards various substrates.

	Linear arabinan	Branched arabinan	Linear Ara oligos	pNP-Ara
Abn1	+++	+/-	+	-
Abn2	+	+/-	+	-
Abn4	-	++	+/-	+
Abf3	-	-	++	+++

Acknowlegdements

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Chapter 4

Branched arabino-oligosaccharides isolated from sugar-beet arabinan

Abstract

Sugar-beet arabinan consists of an α-(1,5)-linked backbone of L-arabinosyl residues, which can be either single or double-substituted with α-(1,2)- and/or α-(1,3)-linked L-arabinosyl residues. Neutral branched arabino-oligosaccharides were isolated from sugar-beet arabinan by enzymatic degradation with mixtures of pure and well-defined arabinohydrolases from *Chrysosporium lucknowense* C1 followed by fractionation based on size and analysis by MALDI-TOF MS and HPAEC. Using NMR analysis, two main series of branched arabino-oligosaccharides have been identified, both having an α-(1,5)-linked backbone of L-arabinosyl residues. One series carries single-substituted α-(1,3)-linked L-arabinosyl residues at the backbone, whereas the other series consists of a double-substituted α-(1,2,3,5)-linked arabinan structure within the molecule. The structures of eight such branched arabino-oligosaccharides were established.

4.1   Introduction

Pectins belong to the main polysaccharides within the primary plant cell wall of dicotyls (Albersheim et al., 1996). Four main pectic components have been identified: homogalacturonan, rhamno-galacturonan-I (RG-I), rhamnogalacturonan-II, and xylogalacturonan, which have been described extensively (Ralet and Thibault, 2002; Ridley et al., 2001; Voragen et al., 1995). RG-I consists of a backbone of repeating units of alternating α-(1,2)-linked rhamnose and α-(1,4)-linked galacturonic acids. Depending on the type of cell wall and tissue, 20-80 % of the rhamnose residues are substituted with side-chains composed of galactose and/or arabinose attached to O–3 and/or O–4. The length of these side-chains can vary from one single sugar residue up to chains of 50 residues (Voragen et al., 1995). The RG-I arabinans are branched molecules with a linear α-(1,5)-linked arabinose backbone, which can be single or double-substituted with α-(1,2)-linked and/or α-(1,3)-linked arabinose side-chains, which again may be further branched (Beldman et al., 1997; Weinstein and Albersheim, 1979). In addition, the arabinan of, for example, sugar-beet cell walls can be feruloylated at the O–2 and/or O–5 position of one of the arabinose moieties (Ishii, 1994; Levigne et al., 2004).

Enzymes able to degrade arabinans of various sources have been reviewed previously (Beldman et al., 1997) and include endo-arabinanases and exo-arabinanases to degrade the linear α-(1,5)-linked arabinan. For the degradation of branched arabinan, arabinofuranosidases (Abf A and B) are necessary to debranch the arabinan and to enable the degradation of the resulting linear α-(1,5)-linked arabinan backbone by the endo- and exoarabinanases (Beldman et al., 1997). Complete insight of the linkage specificity of the arabinofuranosidases has not yet been achieved due to a lack of well-characterized branched arabino-oligosaccharides (AOS). Recently, novel arabinohydrolases from *Chrysosporium lucknowense* C1, Abn1 (endo-arabinanase), Abn2 (exo-arabinanase), and Abn4 (arabinofuranosidase), have been described (Kühnel et al., 2010). Together these enzymes were able to degrade 60 % of the sugar-beet arabinan resulting in the main degradation products arabinose and arabinobiose in the presence of significant amounts of AOS (DP 3-6). Some of these oligosaccharides were hypothesized to be branched AOS (Kühnel et al., 2010). The isolation and identification of these unknown oligomeric arabinan structures would provide essential information for the detailed elucidation of the polymeric arabinan structure. In addition, the further characterization of arabinan-specific enzymes and possible exploration of these AOS for their prebiotic potential, as has been described for linear α-(1,5)-linked AOS (Suzuki et al., 2004), are of great interest. Therefore, this research deals

with the isolation of these novel oligomeric branched arabinan structures derived from sugar-beet arabinan by enzymatic degradation with a mixture of arabinohydrolases from *C. lucknowense* (Abn1, Abn2, and Abn4) and the characterization of purified branched AOS by NMR.

4.2 Material and Methods

4.2.1 Materials

Branched sugar-beet arabinan was obtained from British Sugar (Peterborough, United Kingdom, McCleary (McCleary et al., 1995)). The arabinose content is 66 w/w %, the remaining part consists of hairy regions (rhamnose, galacturonic acid, and galactose) and glucans (glucose) (Kühnel et al., 2010). Linear AOS (DP 2–8) have been purchased from Megazyme International Ltd (Bray, Ireland), and were denoted 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0, respectively. Branched AOS were denoted according to the elution order during HPAEC analysis.

4.2.2 Enzymatic degradation of sugar-beet arabinan

For fractionation and isolation of branched AOS two times 1 g of branched sugar-beet arabinan was digested in 200 ml water set at pH 5 (30 °C) with the arabinohydrolases Abn1, Abn2, and Abn4 (*C. lucknowense* strain C1 (Kühnel et al., 2010)). One arabinan batch (D-100) was incubated for 15 h with an overdose of Abn4 (1.1 U), whereas another batch (D-30) was incubated for 15 h with 0.22 U Abn4 (t = 15 h) resulting in about 30 % of maximal degradation by Abn4. This enzyme dosage was calculated based on the fact that about 18 % of arabinose present can be degraded by Abn4 (Kühnel et al., 2010). The degradation was monitored by arabinose monomer release by HPAEC. After inactivation of the enzyme by boiling for 10 min, both incubations were followed by an end-point degradation of Abn1 and Abn2 (t = 24 h, 4.8 U and 4.4 U, respectively). After enzyme incubation, the samples were freeze-dried.

4.2.3 High performance anion exchange chromatography

Arabinose and AOS were determined by HPAEC with pulsed amperometric detection (PAD). A HPAEC system (ICS-3000, Dionex Corporation, Sunnyvale, CA, USA) was equipped with a CarboPac PA-1 separation column (2 mm ID × 250 mm; Dionex Corporation) and a Carbopac PA-1 guard column (2 mm ID × 25 mm; Dionex Corporation).

A flow of 0.3 ml/min was used and the temperature was kept at 20 °C. AOS (injection volume 10 µl; 10–100 µ/ml) were separated using a gradient with 0.1 M NaOH (A) and 1 M NaOAc in 0.1 M NaOH (B): 0–36 min from 0 % B to 42 % B, 36–42 min at 100 % B, and 42–57 min at 0 % B.

4.2.4   Fractionation based on size: Biogel P2

Freeze-dried material of both digests was each dissolved in 22.5 ml water and subsequently centrifuged. Fractionation of the supernatants was performed on an Äkta Explorer system (Amersham Biosciences, Uppsala, Sweden) equipped with a Bio-Gel P2 column (porous polyacrylamide, 1000 × 26 mm, 200–400 mesh, Bio-Rad Laboratories, Hercules, CA, USA) thermostated at 60 °C and eluted with Millipore water at 1.0 ml/min. Twenty milliliters of each sample (50 mg/ml) were injected. The column efflux was first led through a refractive index detector (Shodex RI72, Showa Denko K.K., Tokyo, Japan) and secondly collected in fractions of 3.5 ml by a fraction collector (Superfrac, GE Amersham, Uppsala, Sweden). Appropriate fractions were pooled and freeze-dried for further analysis.

4.2.5   Determination of neutral sugar and uronic acid content of the Biogel P2 fractions

The total neutral-sugar and uronic-acid content was determined with an automated colorimetric assay analyzer. The total neutral-sugar content was determined by using the orcinol-sulfuric acid color assay with arabinose (25–200 µg/ml) as standard curve (Tollier and Robin, 1979). The uronic-acid content was determined with the m-hydroxy-biphenyl assay based on a standard curve of galacturonic acid (12.5–100.0 µg/ml) (Ahmed and Labavitch, 1978).

4.2.6   MALDI-TOF MS

Each sample was desalted with AG 50W-X8 Resin (Bio-Rad Laboratories, Hercules, CA, USA) and 1 µl of the desalted sample solution was mixed on a MALDI-plate (Bruker Daltonics, Bremen, Germany) with 1 µl matrix solution of 12 mg/ml 2,5-dihydroxy-benzoic acid (Bruker Daltonics) in 30 % acetonitrile and dried under a stream of air (Verhoef et al., 2005). MALDI-TOF MS analysis was performed using an Ultraflex workstation (Bruker Daltonics) equipped with a nitrogen laser of 337 nm and operated in positive mode. After a delayed extraction time of 350 ns, the ions were accelerated to a kinetic energy of 22 000 V

and detected using reflector mode. The lowest laser power required to obtain good spectra was used and 200 spectra were collected for each measurement. The mass spectrometer was calibrated with a mixture of maltodextrins (Avebe, Foxhol, The Netherlands; MD 20; mass range m/z 500–2000). The data were processed using Bruker Daltonics flexAnalysis version 2.2.

4.2.7   NMR analysis

Samples (1–6 mg) have been exchanged with D_2O (99.9 atom % D, Sigma-Aldrich, St. Louis, MO, USA) and subsequently dissolved in 0.5 ml D_2O (99.9 atom % D, Sigma-Aldrich) containing 0.75 % 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid, sodium salt (TMSP, Sigma-Aldrich). NMR spectra were recorded at a probe temperature of 27 °C on a Bruker Avance-III-600 spectrometer, equipped with a cryo-probe located at Biqualys (Wageningen, The Netherlands). Chemical shifts are expressed in parts per million (ppm) relative to internal TMSP at 0.00 ppm. 1D and 2D COSY, TOCSY, HMBC, and HMQC spectra were acquired using standard pulse sequences delivered by Bruker. For the ^{1}H-COSY and -TOCSY spectra, 400 experiments of two scans were recorded, resulting in measuring times of 0.5 h. The mixing time for the TOCSY spectra was 100 ms. For the [^{1}H,^{13}C]-HMBC and -HMQC spectra 800 experiments of 32 scans and 512 experiments of 8 scans, respectively, were recorded, resulting in measuring times of 8.7 h and 2.5 h, respectively.

4.3   Results and Discussion

4.3.1   Enzymatic preparation of AOS from sugar-beet arabinan

Enzymatic degradation of sugar-beet arabinan with a mixture of the arabinohydrolases Abn1, Abn2, and Abn4 (*C. lucknowense*) releases the main degradation products arabinose and arabinobiose, but as well produces various unknown AOS, which elute differently in high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) compared to linear α-(1,5)-linked AOS (Kühnel et al., 2010). To explore the precise structure of various unknown AOS, sugar-beet arabinan was digested with two different treatments of Abn1, Abn2, and Abn4. Although sugar-beet arabinan only contains 66 w/w % arabinose in addition to significant amounts of residual rhamnogalacturonan-I, the use of pure and well-defined arabinohydrolases ensured specific degradation of the arabinan segments for this experiment. To the first digest (D-30) the arabinofuranosidase Abn4 has been added in a concentration that should ensure par-

tial degradation of the side-chains of sugar-beet arabinan resulting in partly debranched backbone. HPAEC results showed that 30 % of the maximal degradation by Abn4 took place. This equals the release of 6 % of all arabinose present (data not shown). To the second digest (D-100) Abn4 has been added in an overdose, thereby allowing Abn4 to cleave all possible linkages by releasing about 18 % of all arabinose present. This results in a heavily debranched arabinan backbone. Both digests were subsequently treated with a mixture of endo-arabinanase Abn1 and exo-arabinanase Abn2 to ensure degradation of the linear part of the arabinan present toward mono-, di-, and oligosaccharides. The HPAEC chromatograms of both enzyme digests, D-30 and D-100, are presented in Fig. 4.1A and B, respectively. In both digests, the main degradation products were arabinose and arabinobiose. Their levels increased with an increase of Abn4 conversion level (D-30 to D-100). This is in good agreement with previous results (Kühnel et al., 2010). In addition to the monomer and dimer, several oligomeric structures were obtained as well. Most of these oligomeric structures do not co-elute with the α-(1,5)-linked AOS standards (Fig. 4.1, suggesting that these peaks are branched AOS as hypothesized already earlier by Kühnel et al (2010). The peak at 19.1 min, which is present in the D-30 digest, and the peak at 22.6 min, which appears in the D-100 digest are the dominant peaks not co-
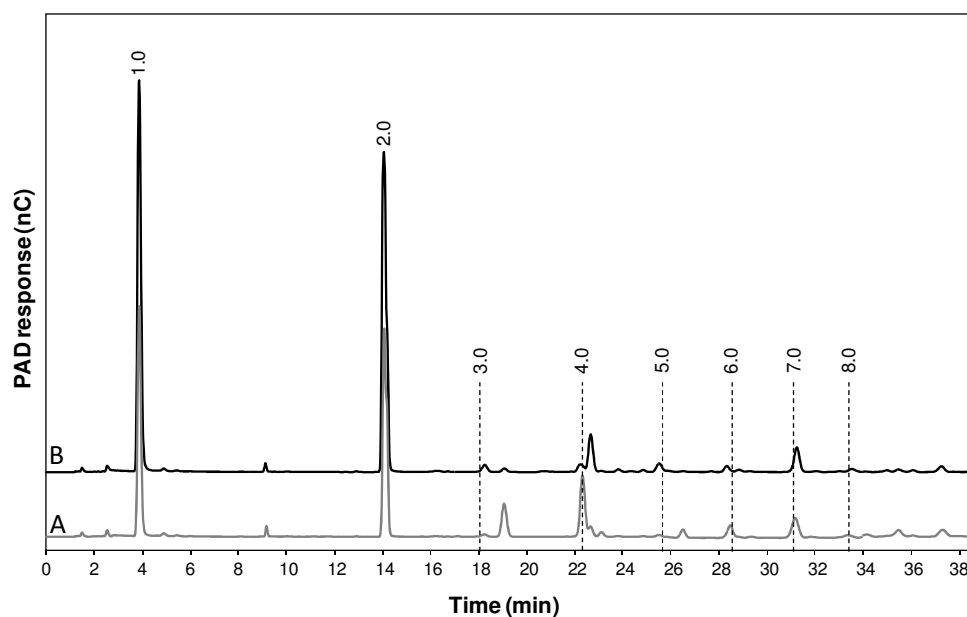

Fig. 4.1: HPAEC elution pattern of AOS after degradation of sugar-beet arabinan with different amounts of Abn4 followed by end-point-incubation with Abn1 and Abn2: D-30 (A), D-100 (B); indication of linear α-(1,5)-linked AOS (DP 1–8; denoted 1.0–8.0).

eluting with linear α-(1,5)-linked AOS. Many more unknown AOS are present in minor quantities in both digests. As HPAEC analysis of both digests indicated the presence of various unknown AOS, both digests were subjected to further analysis.

4.3.2 Fractionation of the AOS derived from sugar-beet arabinan after enzyme digestion

For detailed structural characterization of the AOS, a preparative fractionation based on size of both digests was performed. In Fig. 4.2A-I and B-I the refractive index (RI) patterns of preparative Biogel P2 separations of both digests are given, including the DP as established using MALDI-TOF MS analysis (Fig. 4.2, inset tables). The total-sugar analysis of all fractions taken (3.5 ml each) confirmed the RI patterns of both digests. Significant amounts of uronic acid were only detected in the first 15 fractions of the Biogel P2 separations. Sugar composition of these pools (fractions 1–15) of both digests is given in Table 4.1. It supports the assumption that the main peak in the beginning of the RI patterns in both digests consists of the remaining rhamnogalacturonan-I (RG-I) core structure ('RG-I remnants' in Fig. 4.2A-I and B-I) due to the presence of significant amounts of rhamnose, galacturonic acid, arabinose, and galactose. The arabinose levels (35 and 21 w/w % for D-30 and D-100, respectively) represent 34 and 11 w/w % of the arabinose present in the starting material (crude sugar-beet arabinan), whereas the rest is present as small molecular weight material.

Table 4.1: Sugar composition (w/w %) of the 'RG-I remnants' (Biogel P2 fractions 1–15) of the D-30 and D-100 sugar-beet arabinan digests.

w/w %	Rha	Ara	Gal	Glc	GalA	total sugar
D-30	4	35	17	4	10	70
D-100	5	21	21	6	11	64

Fractions 18–71 from the Biogel P2 separations (3.5 ml each) have been analyzed by HPAEC and MALDI-TOF MS. The fractions have been pooled based on HPAEC analysis aiming at pools with high purity of the compounds present. The pool numbers are indicated as I_{30}–VII_{30} and I_{100}–$VIII_{100}$ in Fig. 4.2A-I and B-I for samples D-30 and D-100, respectively.

HPAEC showed that pools I_{30} and I_{100} consisted of arabinose monomers. In the following part HPAEC, MALDI-TOF MS, and NMR results of the various pools will be

discussed in more detail. Concerning the NMR results of all resolved structures it can be stated that full assignment of both proton and carbon spectra was possible combining the data of the various 2D experiments (Table 4.2). All linkages could be confirmed with HMBC cross-peaks.
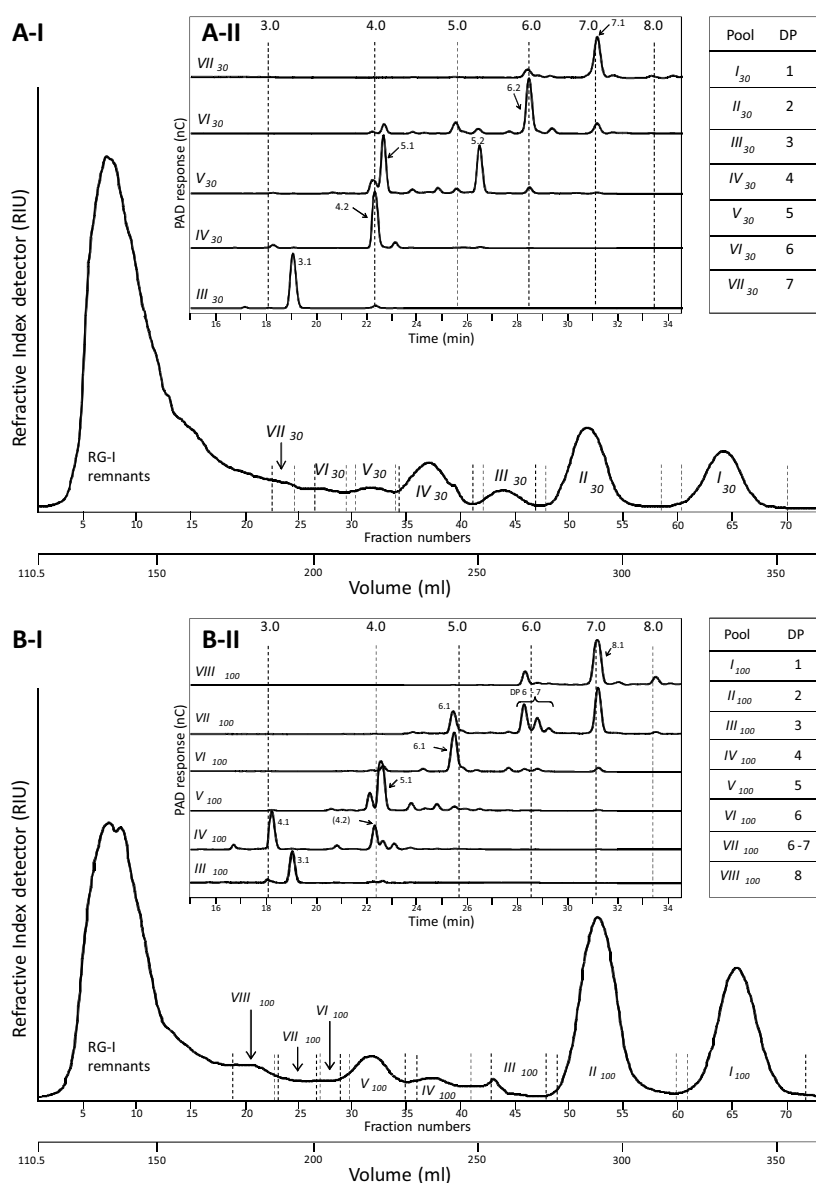

Fig. 4.2: Biogel P2 elution pattern of the D-30 (A) and D-100 (B) digests with indication of the pooled fractions (I); HPAEC elution pattern of pooled fractions (II; insert) with indication of linear α-(1,5)-linked AOS (DP 3–8); tables represent the DP of the pools as analyzed with MALDI-TOF MS.

Table 4.2: ^{1}H and ^{13}C NMR data of arabino-oligosaccharides identified from sugar-beet arabinan.

Pools II$_{30}$ and II$_{100}$	H-1	H-2	H-3	H-4	H5r	H-5s	C-1	C-2	C-3	C-4	C-5
R$_\alpha$	5.265	4.04	4.04	4.24	3.769	3.87	104.01	84.18	78.71	84.23	69.69
R$_\beta$	5.306	4.10	4.10	3.95	3.769	3.86	98.151	78.83	77.19	82.21	71.03
T	5.085	4.132	3.956	4.10	3.72	3.834	110.26	83.7*	79.36	86.8*	64.04
Pool III$_{30}$	**H-1**	**H-2**	**H-3**	**H-4**	**H5r**	**H-5s**	**C-1**	**C-2**	**C-3**	**C-4**	**C-5**
R$_\alpha$	5.257	4.04	4.06	4.236	3.77	3.86	103.98	84.22	78.55	84.10	69.13
R$_\beta$	5.312	4.10	4.10	3.95	3.78	3.85	98.18	78.90	77.16	82.21	70.69
A	5.116	4.293	4.04	4.197	3.768	3.877	110.26	82.03	84.94	86.0*	63.9
T	5.17	4.144	3.948	4.04	3.717	3.842	109.89	84.05	79.38	86.73	63.99
Pool IV$_{30}$	**H-1**	**H-2**	**H-3**	**H-4**	**H5r**	**H-5s**	**C-1**	**C-2**	**C-3**	**C-4**	**C-5**
R$_\alpha$	5.256	4.04	4.06	4.241	3.77	3.86	103.99	84.2	78.55	84.10	69.21
R$_\beta$	5.308	4.10	4.10	3.95	3.78	3.85	98.19	78.88	77.15	82.16	70.73
A	5.122	4.294	4.10	4.319	3.856	3.95	110.29	81.92	85.2	84.52	69.29
T2	5.165	4.14	3.96	4.05	3.713	3.839	109.97	84.05	79.39	86.77	63.99
T5	5.097	4.14	3.96	4.115	3.734	3.839	110.18	83.78	79.39	86.83	69.99
Pool IV$_{100}$	**H-1**	**H-2**	**H-3**	**H-4**	**H5r**	**H-5s**	**C-1**	**C-2**	**C-3**	**C-4**	**C-5**
R$_\alpha$	5.26	4.04	4.06	4.242	3.78	3.86	103.99	84.17	78.58	84.10	69.21
R$_\beta$	5.307	4.10	4.10	3.95	3.78	3.85	98.19	78.86	77.22	84.18	70.73
A	5.253	4.31	4.19	4.19	3.77	3.89	109.14	88.0*	82.9	85.4*	63.54
T2	5.189	4.13	3.97	4.08	3.727	3.84	109.86	84.1	79.31	86.98	63.93
T3	5.165	4.15	3.96	4.05	3.718	3.84	109.7	84.02	79.37	86.83	63.97
Pool V$_{30}$	**H-1**	**H-2**	**H-3**	**H-4**	**H5r**	**H-5s**	**C-1**	**C-2**	**C-3**	**C-4**	**C-5**
R$_\alpha$	5.26	4.04	4.05	4.24	3.77	3.86	103.99	84.21	78.56	84.10	69.22
R$_\beta$	5.3	4.10	4.10	3.96	3.78	3.85	98.17	78.89	77.20	82.20	70.75
A	5.12*	4.29*	4.11	4.32	3.85	3.95	110.2	82.0*	85.18	84.5*	68.71
T3	5.16	4.14	3.96	4.05	3.725	3.84	110.04	84.07	79.40	86.77^a	63.97
B	5.125	4.3	4.04	4.221	3.77	3.88	110.2	82.04	84.95	86.08	63.89
T3	5.164	4.14	3.96	4.05	3.725	3.84	109.86	84.07	79.4	86.85^a	63.97
Pool V$_{100}$	**H-1**	**H-2**	**H-3**	**H-4**	**H5r**	**H-5s**	**C-1**	**C-2**	**C-3**	**C-4**	**C-5**
R$_\alpha$	5.26	4.04	4.05	4.24	3.782	3.86	104.03	84.2	78.62	84.12	69.41
R$_\beta$	5.30	4.10	4.10	3.96	3.782	3.85	98.2	78.9	77.20	82.14	70.96
A	5.260	4.315	4.256	4.32	3.860	3.95	109.2	87.8*	83.12	84.0	68.88
T2	5.189	4.14	3.97	4.08	3.725	3.84	109.8	84.2	79.40	86.92	63.96
T3	5.165	4.15	3.96	4.05	3.725	3.84	109.8	84.0	79.40	86.9	63.96
T5	5.092	4.14	3.96	4.11	3.725	3.84	110.19	83.93	79.44	86.69	63.96
Pool VI$_{30}$	**H-1**	**H-2**	**H-3**	**H-4**	**H5r**	**H-5s**	**C-1**	**C-2**	**C-3**	**C-4**	**C-5**
R$_\alpha$	5.26	4.04	4.06	4.24	3.77	3.85	103.98	84.21	78.56	84.1	69.22
R$_\beta$	5.31	4.10	4.10	3.95	3.77	3.85	98.20	78.88	77.20	84.21	70.80
A	5.21*	4.290	4.12	4.32	3.85	3.96	110.3*	82.09	85.13	84.66	68.72
T3	5.162	4.15	3.96	4.05	3.72	3.84	109.94	84.09	79.40	86.74	63.95$_b$
B	5.133	4.303	4.10	4.346	3.77	3.85	110.21	81.9	85.21	84.66	69.27
T3	5.162	4.15	3.96	4.05	3.72	3.84	109.94	84.09	79.40	86.74	63.95$_b$
T5	5.098	4.14	3.96	4.12	3.73	3.84	110.23	83.8	79.44	86.83	64.00$_b$
Pool VII$_{30}$	**H-1**	**H-2**	**H-3**	**H-4**	**H5r**	**H-5s**	**C-1**	**C-2**	**C-3**	**C-4**	**C-5**
R$_\alpha$	5.26	4.04	4.06	4.24	3.773	3.86	104	84.22	78.55	84.10	69.2
R$_\beta$	5.31	4.1	4.1	3.96	3.773	3.86	98.2	78.87	77.20	82.10	70.69
A	5.12*	4.3	4.1	4.32	3.85	3.96	110.3$_c$	81.94$_d$	85.17	84.51	69.27$_g$
T3	5.164	4.15	3.96	4.05	3.73	3.84	109.96	84.08	79.40	86.74$_f$	63.9$_h$
B	5.09	4.14	4.05	4.23	3.807	3.901	110.3$_c$	83.72$_e$	79.37	85.08	63.07
C	5.127	4.3	4.1	4.32	3.85	3.96	110.27$_c$	81.97$_d$	85.17	84.51	69.38$_g$
T3	5.164	4.15	3.96	4.05	3.73	3.84	109.96	84.08	79.40	86.77$_f$	63.96$_h$
T5	5.097	4.14	3.96	4.12	3.73	3.84	110.2	83.79$_e$	79.44	86.83	64.00$_h$
Pool VIII$_{100}$	**H-1**	**H-2**	**H-3**	**H-4**	**H5r**	**H-5s**	**C-1**	**C-2**	**C-3**	**C-4**	**C-5**
R$_\alpha$	5.26	4.04	4.05	4.24	3.77	3.86	104	84.2	78.60	84.2	69.8
R$_\beta$	5.31	4.09	4.1	3.96	3.77	3.86	98.2	78.9	77.20	82.1	71.2
A	5.10*	4.14	4.04	4.22	3.9	3.807	110.34	84.08$_i$	79.40	85.0*	69.21
B	5.13	4.29	4.12	4.32	3.86	3.96	110.24	82.05	85.12	84.44	68.86
T3	5.162	4.15	3.96	4.05	3.73	3.84	110.02	84.08$_i$	79.40	86.75	63.95
C	5.273	4.33	4.261	4.344	3.86	3.96	109.11	87.63	83.07	84.19	68.86
T2	5.191	4.14	3.97	4.08	3.73	3.85	109.68	84.21	79.40	86.92	63.95
T3	5.162	4.15	3.96	4.05	3.73	3.84	109.78	84.11$_i$	79.40	86.87	63.95
T5	5.092	4.14	3.96	4.12	3.73	3.84	110.26	83.96$_i$	79.47	86.68	63.95

* Signal broadening or splitting due to anomerization effect; superscript values may have to be interchanged.

Dimers

NMR analysis of the pools II_{30} and II_{100} resulted in identical NMR data (Table 4.2). The component present was identified as an α-(1,5)-arabinobiose, which confirms the HPAEC results (data not shown). The NMR data are in agreement with data for α-(1,5)-arabinobiose (Cros et al., 1994).

Trimers

HPAEC analysis of pools III_{30} and III_{100} reveals a major peak in the HPAEC chromatogram at 19.1 min for both samples, not co-eluting with any linear α-(1,5)-linked AOS standard (Fig. 4.2A-II and B-II). MALDI-TOF MS indicates the presence of an oligomer with a degree of polymerization (DP) of 3 for both pools. Apparently, both pools contain the same AOS (denoted 3.1) with a purity of $\geq 90\%$ determined by HPAEC. NMR analysis was carried out with pool III_{30}. The major component (3.1) could be assigned as a dimeric α-(1,5)-linked arabinan backbone with an α-(1,3)-linked arabinose residue at the non-reducing end (Table 4.3; structure 3.1) due to the following NMR characteristics: compared to the data for α-(1,5)-arabinobiose, the α-(1,3)-linkage of a third arabinose residue (Table 4.3, T-residue) is indicated by a cross-peak in the HMBC between H-1 of this T-residue and the C-3 of the A-residue (Fig. 4.3, T1/A3). The downfield shift of 5.6 ppm for C-3 in the arabinose A-residue relative to the C-3 of the T-residue of an α-(1,5)-arabinobiose and the smaller upfield shifts for C-2 and C-4 of 1.4 ppm and 0.8 ppm, respectively, confirm the α-(1,3)-linkage of the arabinose T-residue (Table 4.2, Capek et al., 1983; Dourado et al., 2006; Cardoso et al., 2002). To enable the distinction between the linear α-(1,5)-linked arabinotriose (3.0) and the novel branched arabinotriose, the peak at 19.1 min was denoted 3.1.

Tetramers

Pools IV_{30} and IV_{100} contain oligomers of DP 4 as analyzed with MALDI-TOF MS Fig. 4.2A-II and B-II, inset tables). HPAEC analysis of IV_{30} showed one major peak, which elutes at the retention time of the linear α-(1,5)-linked arabinotetraose (Fig. 4.2A-II; 22.3 min). To investigate if a co-eluting branched AOS is present, pool IV_{30} has been analyzed by NMR. In the 13C-spectra of pool IV_{30} the downfield shift of 5.4 ppm for the C-5 of the A-residue (Tables 4.2 and 4.3; structure 4.2) compared to the A-residue of the component 3.1 in pool III_{30} indicates the presence of an additional α-(1,5)-linked residue. An upfield shift of 1.5 ppm for the C-4 of the A-residue (Table 4.2) and a HMBC cross-peak between H-1 of the
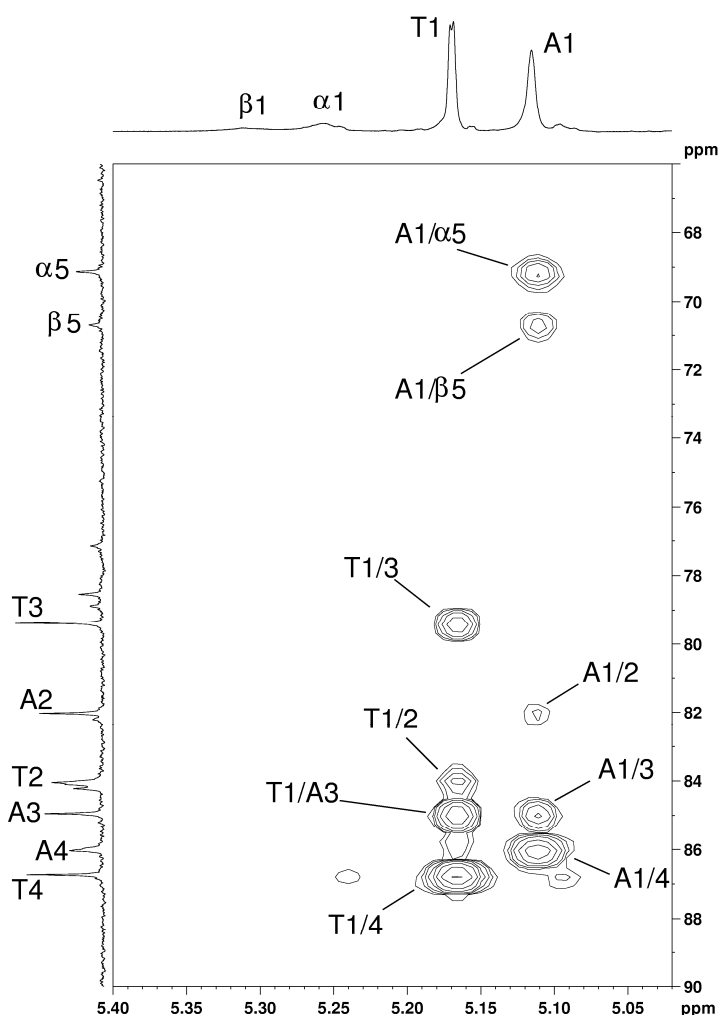
Fig. 4.3: [^{1}H,^{13}C]-HMBC spectrum of pool III$_{30}$ (zoom at 5.40-5.00 ppm (^{1}H) and 66-90 ppm (^{13}C), respectively); T and A as indicated in Tables 4.2 and 4.3.

T5-residue and C-5 of the A-residue confirms the presence of an α-(1,5)-linked T5-residue (Table 4.2). Following these NMR data, the component present in pool IV$_{30}$ (denoted 4.2) could be assigned as a trimeric α-(1,5)-linked arabinan backbone with an α-(1,3)-linked arabinose residue attached at the middle arabinose unit (Table 4.3; structure 4.2). Thus, NMR data reveal that the main tetrameric component in the D-30 digest is a branched AOS (4.2) instead of the linear α-(1,5)-linked AOS (4.0). These two structures are apparently co-eluting in HPAEC with the separation conditions used.

According to HPAEC analysis, the pool IV$_{100}$ contains two major peaks (Fig. 4.2B-II; 18.2 min (denoted 4.1) and 22.3 min (4.0 or 4.2)) next to a number of minor peaks. NMR analysis confirms the presence of two major components. The first component is

identical to the one assigned in pool IV_{30} (4.2). The second component could be identified having an H-1 signal shifted downfield to 5.253 ppm of the A-residue (Tables 4.2 and 4.3; structure 4.1). The HMBC shows a cross-peak with the C-2 of this residue (arabinose-A), and from this C-2 a cross-peak with another H-1 can be found in the HMBC, indicating an α-(1,2)-linkage. Signals for an α-(1,3)-linked T3-residue were also found. Compared to pool III_{30} (3.1), the C-2 of the A-residue is shifted downfield with 6.0 ppm and the C-3 and C-1 are shifted upfield with 2.0 ppm and 1.1 ppm, respectively (Table 4.2), confirming the α-(1,2)-linkage of the T2-residue in pool IV_{100} (4.1). Conclusively, the NMR data reveal the second component (denoted 4.1) as a dimeric α-(1,5)-linked arabinan backbone with an α-(1,2)-linked and an α-(1,3)-linked arabinose residue at the non-reducing end (Table 4.2; structure 4.1).

Pentamers

MALDI-TOF MS reveals that only oligomer(s) with DP 5 are present in both pools (V_{30} and V_{100}, inset Table in Fig. 4.2A and B). According to HPAEC, pool V_{30} consists of two major oligosaccharides present in about equal amounts (Fig. 4.2A-II; denoted 5.1 and 5.2; 22.6 min and 26.5 min, respectively), whereas pool V_{100} showed the presence of only one major peak at 22.6 min (denoted 5.1; Fig. 4.2B-II).

The first component (5.1; 22.6 min) elutes close to the retention time of the linear α-(1,5)-linked arabinotetraose (Fig. 4.2B-II; 4.0; 22.3 min). The second component, present in V_{30}, represents another DP 5-AOS (5.2; 26.5 min; Fig. 4.2B-II) with substantially different retention behavior compared to AOS 5.1, but with a similar retention behavior compared to the linear α-(1,5)-linked arabinopentaose (5.0; 25.6 min). For further characterization of the AOS 5.1, the pool V_{100} was analyzed by NMR as this pool contains the AOS in high purity. In pool V_{100} all signals for the A-residue typical for (1,2), (1,3), and (1,5)-linkages as identified in IV_{30} and IV_{100} are present. Firstly, the H-1 at 5.26 ppm and the C-2 at 87.8 ppm indicate a (1,2)-linkage. Secondly, the chemical shift of C-3 at 83.12 ppm, which results from the combination of a downfield shift due to a (1,3)-linkage and a small upfield shift due to a (1,2)-linkage, indicates a (1,3)-linkage in combination with a (1,2)-linkage. Thirdly, the C-5 at 68.88 ppm indicates a (1,5)-linkage (Table 4.2). These data are in good agreement with Capek et al. (1983). In the HMBC cross-peaks between all three terminal residues (T2, T3, and T5) and the A-residue could be assigned (Fig. 4.4), resulting in a structure as shown in Table 4.3 (structure 5.1). The cross-peaks denoted as X (most likely C-4) and Y are not belonging to the main component as is clear from the ^{13}C-spectrum, in which the signals belonging to this cross-peak are too low. The signals are visible in the
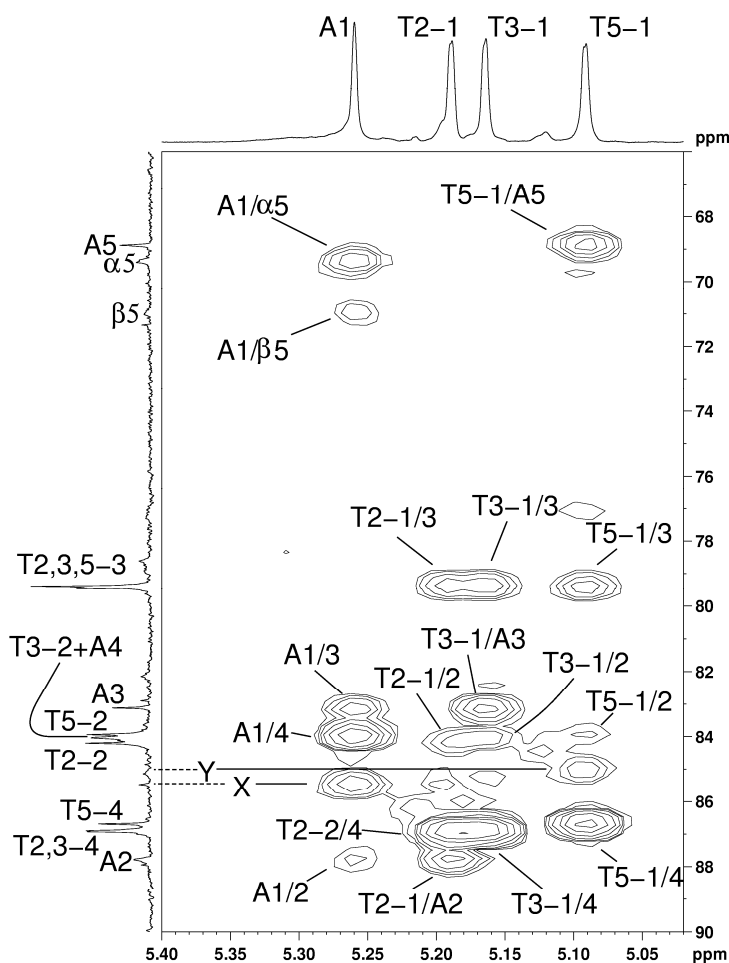
Fig. 4.4: [^{1}H,^{13}C]-HMBC spectrum of pool V_{100} (zoom at 5.40-5.00 (^{1}H) and 66-90 (^{13}C), respectively); T_n, A, B and C as indicated in Tables 4.2 and 4.3; X and Y are explained in the text.

HMBC due to the higher sensitivity of this proton detected 2D experiment and due to the high intensity of cross-peaks between H-1 and C-4 in arabinoses.

Although a mixture of two major compounds was present in pool V_{30}, with component 5.1 (pool V_{100}) as one of them, it was possible to determine the structure of the second compound, because of the presence of three characteristic signals: A signal at 86.08 ppm, assigned as C-4 of the B-residue with only an α-(1,3)-linked arabinose attached to it (compared with pool III_{30} (3.1)), and two signals at 85.18 ppm and 84.95 ppm for the A-residue and B-residue, respectively (Tables 4.2 and 4.3). This is typical for C-3 signals in arabinoses with only α-(1,3)-linked arabinose attached to it. Due to proximity of these two C-3 signals, the almost identical assignments for the two T3-residues and the lower

resolution of the 2D HMBC experiment, only one combined cross-peak in the HMBC confirms the two α-(1,3)-linkages. A cross-peak between H-1 of the B-residue and C-5 of the A-residue connects the two α-(1,3)-substituted arabinoses (data not shown). Cross-peaks between the A-residue and the reducing end arabinose complete the assignment of this structure, resulting in the structure as shown in Table 4.3 for component 5.2.

Hexamers

HPAEC analysis of the pools VI_{30} and VI_{100} reveals the presence of each one major peak at 28.4 min and 25.5 min, respectively (Fig. 4.2A-II and B-II). As MALDI-TOF MS shows the presence of only oligomers of DP 6, these AOS are assigned as 6.2 and 6.1, respectively. NMR analysis shows that the pool VI_{30} is similar to pool V_{30} with respect to the presence of two (1,3)-linked residues as indicated by two signals for C-3 at 85.13 and 85.21 ppm (Table 4.2) together with a combined cross-peak with H-1 of the T3-residues, and a cross-peak between H-1 of the B-residue and C-5 of the A-residue (data not shown). In pool VI_{30} the C-4 signal of the B-residue indicates the presence of an additional (1,5)-linked T5-residue, confirmed by a cross-peak between H-1 of the T5-residue and C-5 of the B-residue in the HMBC (data not shown). Following the NMR data compound 6.2 could be assigned as tetrameric α-(1,5)-linked arabinan backbone with α-(1,3)-substitution of single arabinose residues at the two middle arabinose units as depicted in Table 4.3 (structure 6.2).

In pool VI_{100} signals similar to those in pool IV_{100} could be assigned (Table 4.2), indicating that the same structural element, an arabinose with (1,2)- and (1,3)-linked arabinose residues attached to it, must be present. The two residues between this element and the reducing end, needed to complete the structure could not be assigned due to a large heterogeneity in the spectra. Thus, even though HPAEC showed only one major peak at 25.5 min, NMR analysis revealed that more than one component must be present, indicating insufficient separation of HPAEC for these compounds.

Heptamers

The pool VII_{30} shows one major peak at 31.2 min during HPAEC analysis (Fig. 4.2A-II; denoted 7.1), co-eluting with the linear α-(1,5)-linked AOS (7.0). MALDI-TOF MS analysis shows the presence of an oligomer of DP 7. NMR analysis shows that the pool VII_{30} has all the features of pool VI_{30}: two (1,3)-linked arabinose residues (T3-residues) and one (1,5)-linked T5-residue (Table 4.2). An additional signal at 85.08 ppm, assigned as C-4, indicates the presence of an additional (1,5)-linked arabinose in the backbone. Two positions for

this additional residue are possible: Between the two (1,3)-substituted arabinoses (T3) or between the first (1,3)-substituted arabinose (T3) and the reducing end (Table 4.3; structure 6.2). The first possibility with (1,3)-linked residues on the A-residue and the C-residue, respectively, represents the main component in this pool (7.1). HMBC cross-peaks between the H-1 of the C-residue and the C-5 of the B-residue confirm this assignment (data not shown), resulting in the structure as depicted in Table 4.3 (structure 7.1). The latter possibility would result in slightly different signals for the C-5 of the reducing end and is found for the component in $VIII_{100}$ (see discussion there).

According to HPAEC and MALDI-TOF MS analysis the pool VII_{100} contains a mixture of components with DP 6 and DP 7, thus, no further NMR analysis was done for pool VII_{100}.

Octamers

HPAEC analysis of pool $VIII_{100}$ reveals the presence of one major peak at 31.2 min, co-eluting with the linear α-(1,5)-linked arabinoheptaose (7.0) and component 7.1. MALDI-TOF MS results show the presence of mainly DP 8, thus, the unknown component represents an arabinooctaose (denoted 8.1) with a substantially different retention behavior compared to linear α-(1,5)-linked arabinooctaose (8.0; 33.4 min; Fig. 4.2B-II). NMR analysis shows that in pool $VIII_{100}$ all signals of a triple substituted arabinose are present as were assigned for V_{100} (compare pool V_{100} with $VIII_{100}$ in Table 4.2). In the HMBC at the position of H-1 of the T3-residues two cross-peaks are found with two different C-3 signals: at 83.07 ppm (C-residue, Tables 4.2 and 4.3), characteristic for a (1,3)-linkage in combination with a (1,2)-linkage as mentioned in pool V_{100}, and at 85.12 ppm (B-residue, Tables 4.2 and 4.3), indicating a (1,3)-linkage without (1,2)-substitution at the same residue (similar to IV_{30} (4.2), V_{30} (5.2), VI_{30} (6.2), and VII_{30} (7.1)). As in VII_{30} a C-4 signal at 85.0 ppm indicates the presence of an additional (1,5)-linked arabinose residue, which is located next to the reducing end due to a clear anomerization effect of this signal (A-residue, Tables 4.2 and 4.3). This is, furthermore, substantiated by cross-peaks in the HMBC between the H-1 of this A-residue and the C-5 signals of the reducing end (R α/β). The chemical shifts of these C-5 carbons are slightly different for those of all structures with a (1,3)-substituted A-residue, but resembles the chemical shifts found for α-(1,5)-arabinobiose, confirming the presence of an arabinose residue attached to the reducing end with no (1,3)-substitution (Table 4.2). Following all the NMR data, component 8.1, which is present in $VIII_{100}$, has a structure as depicted in Table 4.3.
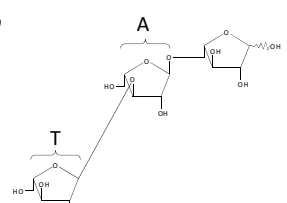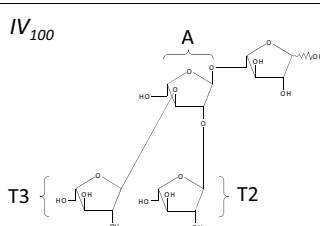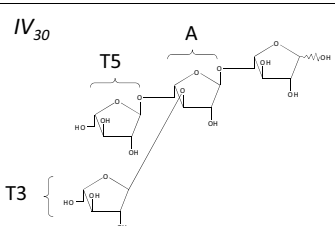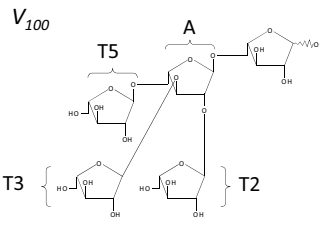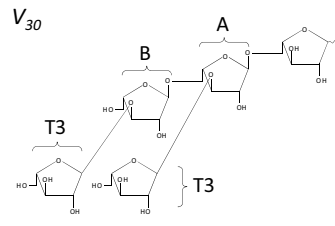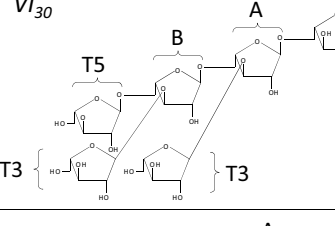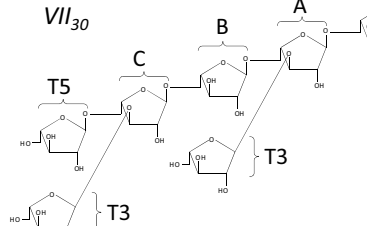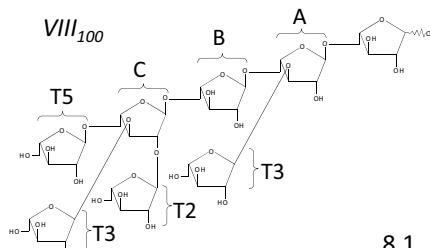
4.3.3   Overview of AOS identified from sugar-beet arabinan

In Table 4.3 an overview of the structures of all identified branched AOS derived from sugar-beet arabinan is given as based on extensive NMR analysis. All of them consist of an α-(1,5)-linked backbone of L-arabinosyl residues. Two main structural features could be identified among all identified AOS, varying in their type of linkages and the degree of substitution. AOS of the first series contain a structure with double-substituted α-(1,2)- and α-(1,3)-linked L-arabinosyl residues (4.1, 5.1, 8.1; Table 4.3, series 1). An additional single-substituted α-(1,3)-linked L-arabinosyl residue may be present within the same molecule as identified in component 8.1 (Table 4.3). AOS of the second series carry single-substituted α-(1,3)-linked arabinose(s) (Table 4.3; series 2). Components with either one or two α-(1,3,5)-linkages were identified (3.1, 4.2 and 5.2, 6.2, 7.1, respectively).

None of the structures identified was substituted at the arabinose at the reducing end, which is in contrast with the synthesized methyl 2–O, methyl 3–O- and methyl 5–O-α-L-arabinofuranosyl-α-L-arabinofuranosides as described by Kaneko et al (Kaneko et al., 1995). The isolated component 3.1 is similar to an earlier described feruloylated arabino-oligosaccharide with an α-L-arabinosyl residue linked at O–3 and a ferulic acid attached at O–2 of the non-reducing end of an α-(1,5)-linked dimeric backbone of L-arabinosyl residues, which has been isolated from spinach leaves (Ishii and Tobita, 1993) and sugar-beet pulp (Colquhoun et al., 1994).

Almost all AOS of the second series (Table 4.3: 3.1, 4.2, 5.2, 6.2, and 7.1) were exclusively present in the D-30 digest, while the three isolated AOS belonging to the first series were mainly present in the D-100 digest. This indicates a different degradability of the structures by the arabinofuranosidase Abn4. Abn4 seems to be able to remove α-(1,3,5)-linked L-arabinosyl residues (present in the AOS of the second series; Table 4.3), whereas a α-(1,2,3,5)-linked double-substituted structure as present in the AOS of the first series (Table 4.3) is not degradable by the arabinofuranosidase Abn4. Further investigation of the precise mode of action and specificity of the arabinohydrolases is currently ongoing.

Table 4.3: Structures of arabino-oligosaccharides identified from sugar-beet arabinan (series 1 and 2) as obtained after degradation of sugar-beet arabinan with the arabinohydrolases Abn1, Abn2, and Abn4 followed by Biogel P2 fractionation (D-30 and D-100, respectively)

	Series 1	Series 2
DP 3		III_{30} 3.1
DP 4	IV_{100} 4.1	IV_{30} 4.2
DP 5	V_{100} 5.1	V_{30} 5.2
DP 6		VI_{30} 6.2
DP 7		VII_{30} 7.1
DP 8	$VIII_{100}$ 8.1	

4.4 Conclusions

Eight novel neutral branched AOS have been isolated from sugar-beet arabinan after enzyme digestion with two different mixtures of the *Chrysosporium lucknowense* C1 arabinohydrolases Abn1, Abn2, and Abn4. NMR analysis revealed two series of branched AOS varying in the type of linkage. To the best of our knowledge, this is the first characterization of such branched AOS. These AOS may now be used for (further) characterization of arabinan-specific enzymes as well as for possible exploration of their prebiotic potential.

Acknowledgments

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Chapter 5

Mode of action of
Chrysosporium lucknowense C1
α-ʟ-arabinohydrolases

Abstract

The mode of action of four *Chrysosporium lucknowense* C1 α-ʟ-arabinohydrolases was determined to enable controlled and effective degradation of arabinan. The active site of endoarabinanase Abn1 has at least six subsites, of which the subsites -1 to +2 have to be occupied for hydrolysis. Abn1 was able to hydrolyze a branched arabinohexaose with a double-substituted arabinose at subsite -2. The exo-acting enzymes Abn2, Abn4 and Abf3 release arabinobiose (Abn2) and arabinose (Abn4 and Abf3) from the non-reducing end of reduced arabinose oligomers. Abn2 binds the two arabinose units only at the subsites -1 and -2. Abf3 prefers small oligomers over large oligomers. It is able to hydrolyze all linkages present in beet arabinan, including the linkages of double-substituted residues. Abn4 is more active toward polymeric substrate and releases arabinose monomers from single-substituted arabinose residues. Depending on the combination of the enzymes, the C1 arabinohydrolases can be used to effectively release branched arabinose oligomers and/or arabinose monomers.

5.1   Introduction

Sugar-beet pulp consists of up to 75 w/w % carbohydrates on dry-matter basis (McCready, 1966). Up to one third of the carbohydrate material is polymeric arabinan, a heavily branched, complex neutral sugar side-chain of pectic rhamnogalacturonan I (Rombouts et al., 1988). Sugar-beet arabinan has a backbone of linear α-1,5-linked L-arabinofuranose units. Monomeric or oligomeric L-arabinofuranose branches are linked to the backbone via α-1,2-linkages and α-1,3-linkages. The oligomer side-chains may even be further branched. A number of researchers report that up to 1 % of the arabinose is present as terminal L-arabinopyranose (Beldman et al., 1997).

Arabinans play a great role in food industry. Polymeric arabinans isolated from beet pulp are able to form spreadable gels and act as fat replacers. They are considered dietary fibers due to their resistance to human digestive enzymes (Cooper et al., 1992; McCleary et al., 1995). Furthermore, arabinose oligomers have been reported to have prebiotic effects on intestinal bacteria in the distal colon (Van Laere et al., 2000; Van Laere and Wissing, 2002). More complex oligomers could have advanced prebiotic properties due to lower digestibility in the proximal colon. *l*-arabinose is considered as a functional ingredient because it inhibits intestinal sucrase, leading to decreased plasma glucose levels (Seri et al., 1996; Osaki et al., 2001).

The degradation of the heavily branched sugar-beet arabinan requires the concerted action of a number of powerful arabinohydrolases. Arabinohydrolases are a group of enzymes that belong to the glycoside hydrolase families GH 3, 27, 43, 51 54, 62 and 93 (`http://www.cazy.org`), of which GH family 3, 43, 51, 54, 62 and 93 encode for α-L-arabinohydrolases. One β-L-arabinopyranohydrolase described so far is grouped into GH family 27 (Ichinose et al., 2009).

Endoarabinanases (EC 3.2.1.99) belong to GH family 43. They hydrolyze the α-1,5-linkages in the unsubstituted regions of the arabinan backbone with an inverting mode of action (Alhassid et al., 2009). Endoarabinanases prefer polymers over oligomers. Their activity decreases with decreasing degrees of polymerization (Dunkel and Amado, 1995). Arabinotriose is the smallest substrate for the enzyme and it is hydrolyzed to arabinose and arabinobiose upon end point hydrolysis (Rombouts et al., 1988). Structural studies of a GH family 43 endoarabinanase from *Geobacillus stearothermophilus* revealed that the substrate binding site is a binding cleft that can harbor at least 5 arabinose units in which arabinotriose occupies the subsites -1 to +2 (Alhassid et al., 2009).

Exoarabinanases belong to GH families 43 and 93 (EC 3.2.1.-) and release arabinose, arabinobiose or arabinotriose from the non-reducing end of the α-1,5-linked arabinan backbone (Kaji and Shimokawa, 1984; Sakamoto and Thibault, 2001; Ichinose et al., 2008). All of these enzymes specifically release only one product and preferably act on linear arabinan. The conformation of the substrate binding site greatly determines the enzymes mode of action. Proctor and co-workers (2005) could change the mode of action of GH family 43 exoarabinanase 43A from *Cellvibrio japonicus* into an endo-mode of action by redesigning its binding site.

Arabinofuranosidases (E.C. 3.2.1.55) active toward arabinan belong to the GH families 3, 43, 51, 54 and 62 (`http://www.cazy.org`). They are able to release arabinose monomers from the non-reducing ends of arabinan. GH family 3 α-ʟ-arabinofuranosidases are bifunctional enzymes that also act as β-ᴅ-xylosidases (`http://www.cazy.org`). GH family 62 arabinofuranosidases are reported to be predominantly active toward arabinoxylan and are, therefore, also called arabinoxylan arabinofuranohydrolases (Beldman et al., 1997). Besides their structural classification, arabinofuranosidases are classified according to their substrate specificity into arabinofuranosidases A & B and arabinofuranohydrolases specific toward arabinoxylans (Beldman et al., 1997). Arabinofuranosidase A (AbfA) and arabinofuranosidase B (AbfB) are both active toward arabinose oligomers and *p*-nitrophenyl arabinofuranoside (*p*NP-Ara*f*). Their activities differ toward polymeric substrates as AbfA is inactive toward linear arabinan and much less active than AbfB toward branched arabinan (Rombouts et al., 1988).

The genome of the ascomycete *Chrysosporium lucknowense* C1 (C1) encodes 14 enzymes that putatively release arabinose or arabinose oligomers from arabinan (Hinz et al., 2009). We have recently purified and characterized four α-ʟ-arabinohydrolases that specifically and synergistically act on the degradation of sugar-beet arabinan (Kühnel et al., 2010). A mixture of linear and branched arabinose oligomers was released when branched arabinan was digested with endoarabinanase Abn1, exoarabinanase Abn2 and arabinofuranosidase Abn4 (Westphal et al., 2010a). No detailed information is available about the mechanism how the C1 arabinohydrolases act in synergy. This information is necessary to ensure a controlled and efficient degradation of the substrate.

Well characterized enzymes are the prerequisite for the directed release of arabinose oligomers. Therefore, in this research branched arabinose oligomers and reduced linear arabinose oligomers were used to determine the substrate specificity and mode of action of the C1 endoarabinanase Abn1, exoarabinanase Abn2 and the two arabinofuranosidoases Abn4 and Abf3.

5.2 Methods

Enzyme substrate binding subsites were named following the nomenclature for sugar-binding subsites in glycosyl hydrolases (Davies et al., 1997). Centrifugation was always performed at room temperature at $15\,000 \times g$ for 10 min.

5.2.1 Material

The C1 enzymes Abn1, Abn2 Abn4 and Abf3 were purified previously by Kühnel and co-workers (2010). Their activities are given in Table 5.1. Linear arabinose oligomers in the range from DP2–7 used for incubations and for the production of reduced arabinose oligomers were purchased from Megazyme (Megazyme; Bray, Ireland). Linear and branched sugar-beet arabinan were from British Sugar (Peterborough, United Kingdom). Pullulan molecular-mass standards were from Polymer Laboratories (Varian Inc., Palo Alto, CA, USA). Branched arabinose oligomers were produced, purified and characterized by Westphal and co-workers (2010a). Other chemicals were from Merck or Sigma–Aldrich.

Table 5.1: Activities of C1 α-L-arabinohydrolases as determined previously (Kühnel et al., 2010). Linear and branched arabinan from sugar beet. 1 U = 1 µmol (product)/ min mg (enzyme).

	GH family	Activity (U/mg)	Substrate
Abn1	GH43	26.0	linear arabinan
Abn2	GH93	7.1	linear arabinan
Abn4	GH43	9.5	branched arabinan
Abf3	GH51	21.4	pNP-Ara$f$

5.2.2 Action of C1 arabinohydrolases toward reduced arabinose oligomers

Production of reduced arabinose oligomers

Arabinose and arabinose oligomers in the range from DP 2–7 (5 mg each) were reduced with 7.5 mg sodium borohydride in 0.2 ml ammonium hydroxide (1.5 M) for 1 h at ambient temperature. The reaction was stopped by the addition of glacial acetic acid until gas formation ceased (pH < 5.0). Methanol (1 ml) was added to remove the sodium borohydride as volatile trimethylborate and the samples were dried under a stream of air.

The methanol washing was repeated two times. Samples were dissolved in water and the reduction was verified by HPAEC (section 5.2.5, Fig. S5.1).

Action of C1 arabinohydrolases toward reduced arabinose oligomers

Pure, reduced arabinose oligomers in the range from DP2–7 (2 mg/ml, 25 µl) were mixed with 25 µl enzyme solution in 100 mM sodium acetate buffer (Abn1 and Abn2: 0.35 mU, Abn4: 0.3 mU and Abf3: 0.4 mU). The samples were incubated for 1, 15 and 20 h (T = 30 °C) with an additional dose of fresh enzyme (4 mU in 10 µl) added after 15 h. After incubation, the samples were diluted 20 times, boiled (t = 5 min) and centrifuged. The supernatant was analyzed by HPAEC (section 5.2.5).

5.2.3   C1 arabinohydrolase activities toward branched arabinose oligomers

Branched arabinose oligomers (0.1 mg) were incubated for 2 h at 30 °C with Abn1 (30 mU), Abn4 (20 mU) or Abf3 (20 mU) in a total volume of 100 µl. After incubation, the samples were diluted with 400 µl water and boiled for 5 min. After centrifugation, the supernatant was analyzed by PGC-HPLC-MS (section 5.2.5).

5.2.4   Product inhibition of C1 arabinohydrolases

Abn1 and Abn2

Product inhibition of Abn1 and Abn2 was studied toward linear arabinan in the presence of arabinobiose as the end product for both enzymes. Abn1 and Abn2 (6 mU) were incubated for 1 h at 30 °C with 0.5 mg linear arabinan and arabinobiose (0, 0.1, 0.5, 1, 2, 3, 4 and 5 mg) in 100 µl total volume. The samples were subsequently boiled (t = 5 min) and centrifuged. The supernatant was transferred into a HPLC vial and subjected to HPSEC analysis (section 5.2.5).

Abn4 and Abf3

The product inhibition of Abn4 and Abf3 was studied toward *p*NP-Ara*f* in the presence of arabinose. Abn4 (0.5 mU) and Abf3 (1 mU) were incubated for 30 min at 30 °C in a microtiter plate in 200 µl total volume, including 10 mM sodium acetate buffer (pH 5.0), 0.5 mM *p*NP-Ara and arabinose monomer concentrations from 0–500 mM. The pH was adjusted to pH 10.0 with 50 µl sodium carbonate (0.5 M) and the amount of free *p*-nitrophenol was determined photospectrometrically at 405 nm. A *p*-nitrophenol standard

curve (10–500 μM in 100 mM sodium carbonate) was used for quantification. Abf3 inhibition kinetics were studied with a slightly modified protocol: t = 15 min, V = 200 μl, 100 μl *p*NP-Ara (0.25–2.5 mM), 50 μl arabinose (2 M) or water, 40 μl sodium acetate buffer (50 mM, pH 5.0) and 10 μl Abf3 (1 mU).

5.2.5 Chromatography

High performance size exclusion chromatography (HPSEC) was performed on a Dionex Ultimate 3000 system (Dionex, Sunnyvale, CA, USA) equipped with a set of four TSK–Gel superAW columns (Tosoh Bioscience, Tokyo, Japan) in series: guard column (6 mm ID × 40 mm) and separation columns 4000, 3000 and 2500 (6 mm ID × 150 mm). The samples (10 μl, 5 mg/ml) were eluted with filtered aqueous 0.2 M sodium nitrate at 40 °C at a flow rate of 0.6 ml/min. Elution was followed by refractive index detection (Shodex RI 101; Showa Denko K.K., Kawasaki, Japan).

The monomer and oligomer carbohydrate levels of the digests were analyzed by high performance anion exchange chromatography (HPAEC) according to Albrecht and co-workers (2009) using a Carbopac PA1 analytical column (2 × 250 mm, Dionex) in combination with a Carbopac PA1 guard column (2 × 50 mm, Dionex). Arabinose and arabinose oligomers (10 μl; 50–100 μg/ml) were eluted in 0.1 M sodium hydroxide with an adapted sodium acetate (NaOAc) elution profile: A gradient of 0–350 mM NaOAc over 30 min, 1 M NaOAc for 10 min and 0 M NaOAc for 15 min (equilibration).

Branched oligomers were separated and identified by PGC-HPLC-ELSD/MS according to Westphal and co-workers (2010b). A Thermo Accela UHPLC system (Waltham, MA, USA) equipped with a Hypercarb column (PGC, 100 × 2.1 mm; 3 μm, Thermo Electron Corporation, San José, CA, USA) was used in combination with a Hypercarb guard column (10 × 2.1 mm; 3 μm, Thermo Electron Corporation). Samples (10 μl; c = 100–400 μg/ml) were eluted at 0.4 ml/min and 70 °C with an elution profile consisting of three eluents: 25 μM lithium acetate (LiAc) in Millipore water (A), 25 μM LiAc in acetonitrile (B) and 25 μM LiAc in 0.2 w/v % aqueous trifluoroacetic acid (C). Elution profile: 0–1 min: 100 % A, 1–15 min: linear gradient from 0–27.5 % B, 15–28 min: linear gradient from 27.5–60 % B and from 0–10 % C, 28–31 min: linear gradient from 60–80 % B and from 10–20 % C, 31–35 min: 80 % B and 20 % C, 35–36 min: gradient from 80 % B and 20 % C to 100 % A, 36–41 min: equilibration with 100 % A. The PGC-column was coupled to a 1:1-splitter (Accurate, Dionex) directing the eluent both to an ELSD85 evaporative light scattering detector (Sedere, Alfortville, France) and to an ESI-MS[n]-detector (LTQ XL MS, ion trap, Thermo Electron Corporation). The drift tube temperature of the ELSD was set

to 50 °C and the gain to 12. MS–detection was performed in positive mode (spray voltage: 4.6 kV, capillary temperature: 260 °C, auto-tuned on arabinopentaose (m/z = 685)). The ion trap was closed either after 10 ms or when intensity of 3×10^5 was reached to enable the detection of minor components and to avoid overload of the MS by abundant components.
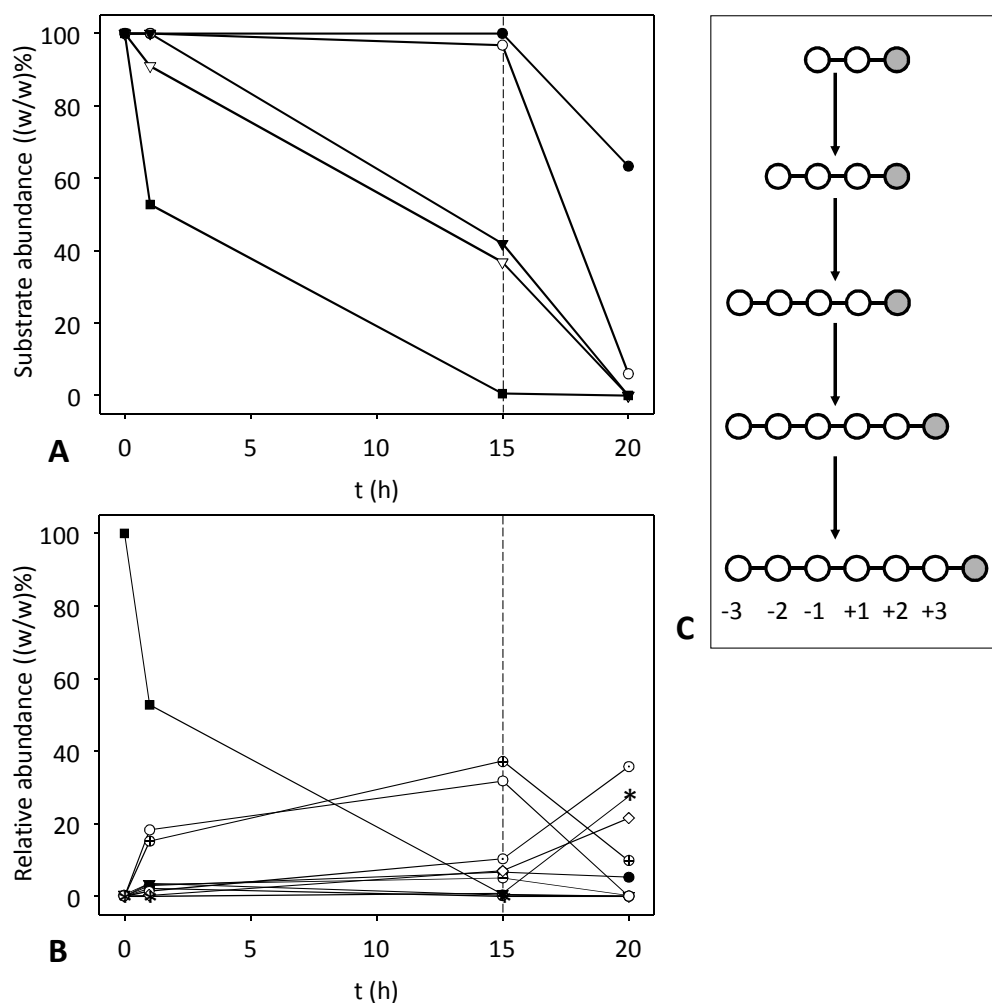

Fig. 5.1: Abn1 action toward reduced arabinose oligomers. A – Time dependent consumption of pure reduced arabinose oligomers in the range from DP3–7, B – Product formation from reduced arabinoheptaose: ◇ – reduced arabinobiose, ● – reduced arabinotriose, ○ – reduced arabinotetraose, ▼ – reduced arabinopentaose, ▽ – reduced arabinohexaose, ■ – reduced arabinoheptaose, ∗ – arabinose, ⊙ – arabinobiose, ⊕ – arabinotriose, ⊖ – arabinotetraose, ⊗ – arabinopentaose, grey dashed line – fresh ten fold enzyme dose added after 15 h incubation. C – Schematic mode of action of Abn1 toward reduced arabinose oligomers and main products formed after first hydrolysis step. White circles – arabinose units, grey circles – reduced arabinose, arrows – primarily hydrolyzed linkage by Abn1, numbers from -3 to +3 – predicted binding subsites of Abn1.

5.3 Results and discussion

5.3.1 Action of C1 arabinohydrolases toward reduced arabinose oligomers

Abn1

The action of α-L-endoarabinanase Abn1 toward reduced arabinose oligomers is shown in Fig. 5.1. Abn1 degraded reduced arabinose oligomers with DP $\geq$ 3 and showed increasing activities with increasing degrees of polymerization (Fig. 5.1A). Reduced arabinobiose was the smallest labeled compound identified in all digests and it was the first product formed in DP3–5 oligomer digests (no further data shown). All produced oligomers with DP $\geq$ 3 were subject to further hydrolysis that yielded arabinose, arabinobiose and reduced arabinobiose as final products. The hydrolytic performance of Abn1 did not linearly increase with increasing DP. It increased stepwise from DP 4 to 5 and from DP 6 to 7, respectively, and remained similar from DP 3 to 4 and from DP 5 to 6, respectively (Fig. 5.1A). The activity increase from DP 4 to DP 5 was connected to the interaction of an additional arabinose with the subsite -3 (Fig. 5.1C). Accordingly, the activity increase from DP 6 to 7 was linked to the interaction of an internal arabinose unit with the subsite +3. In contrast, the occupation of the subsite -2 in the DP 4 oligomer and the +2 subsite in the DP 6 oligomer, respectively, did not alter Abn1 activity. These data imply that the subsite -3 has a higher binding affinity than the subsites -2 and +3.

The reduction of the free aldehyde groups causes structural and conformational changes within the molecule. Nevertheless, Abn1 could degrade reduced arabinoheptaose with the same degree of hydrolysis and specificity as non-reduced arabinoheptaose and was only slightly less active toward reduced arabinotriose when compared to non-reduced arabinotriose (provided as supplementary data, Fig. S5.2). This data suggests that the action of Abn1 is not greatly influenced by the modification of the reducing-end group. The data presented in Fig. 5.1 might further imply that the substrate binding site of Abn1 recognizes at least six arabinose units. The degradation of reduced arabinotriose shows that two non-reducing α-1,5-linked arabinose units covering the subsites -1 and +1 are a prerequisite for hydrolysis.

Abn2

Abn2 degraded reduced arabinose oligomers with DP $\geq$ 3, while enzyme activity increased with increasing DP of the substrate. From Fig. 5.2A it can be seen that Abn2 rapidly degraded reduced arabinoheptaose to reduced arabinopentaose and, further on,
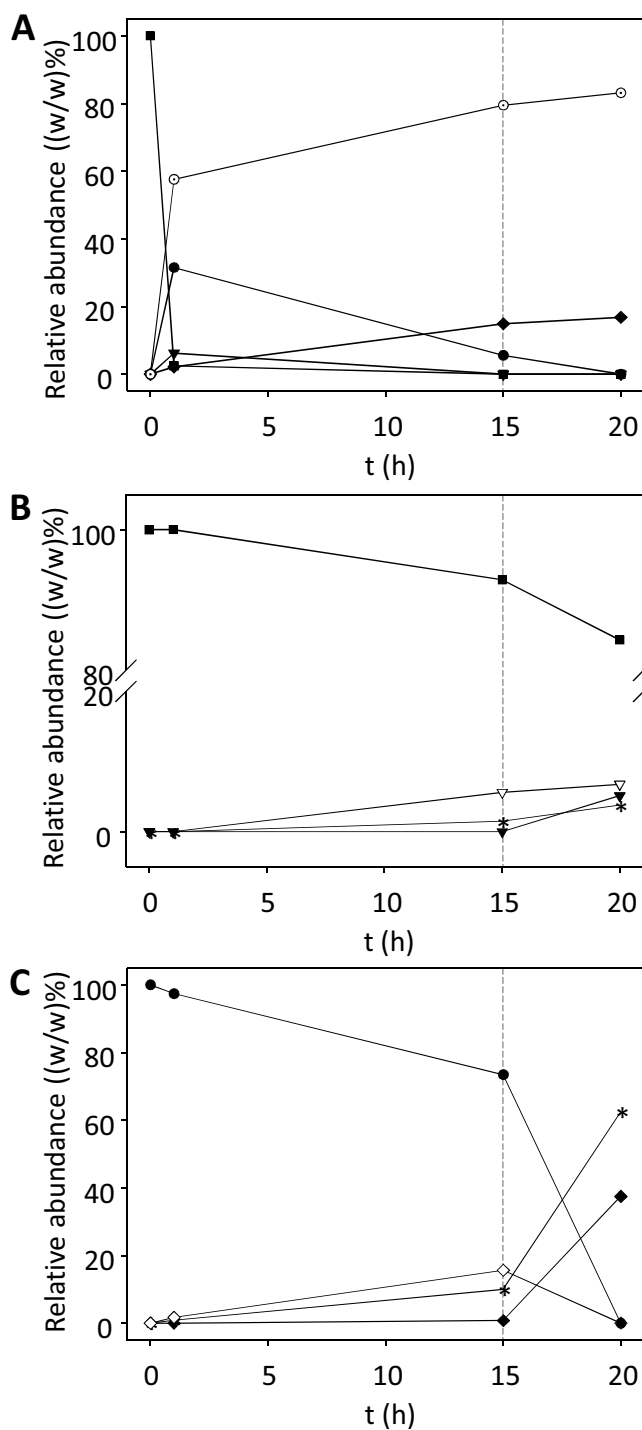
Fig. 5.2: Time curves of C1 arabinohydrolases Abn2, Abn4 and Abf3 toward pure, reduced arabinose oligomers. A – Abn2 toward reduced arabinoheptaose: B – Abn4 toward reduced arabinoheptaose, C – Abf3 toward reduced arabinotriose. ◆ – arabitol, ◇ – reduced arabinobiose, ● – reduced arabinotriose, ▼ – reduced arabinopentaose, ▽ – reduced arabinohexaose, ■ – reduced arabinoheptaose, ∗ – arabinose, ⊙ – arabinobiose , grey line – fresh ten fold enzyme dose added after 15 h incubation.

to arabinotriose by the subsequent removal of arabinobiose from the non-reducing end of the substrate. The data suggest that Abn2 binds the two arabinose units at its substrate binding subsites -1 and -2. The ability of Abn2 to degrade reduced arabinotriose implies that Abn2 can tolerate or does not recognize a reducing end or an alditol at the +1 subsite. Since Abn2 activity decreased with decreasing DP it is likely that Abn2 also binds parts of the substrate on one or more + subsites. Carapito et al. (2009) determined the crystal structure of a GH family 93 arabinobiohydrolase from *Fusarium graminearum*. They describe the substrate binding site as a cleft with a very defined -1 subsite and a more open -2 subsite. toward the reducing end, only the +1 subsite could be identified from where the cleft opens and does not allow any further prediction of additional subsites (Carapito et al., 2009). Abn2 also belongs to GH family 93 and has the same mode of action as the *F. graminearum* arabinobiohydrolase. An Abn2 substrate binding site that is structurally similar to the *F. graminearum* GH family 93 arabinobiohydrolase substrate binding site would be in agreement with the experimental data obtained for Abn2.

Abn4

Abn4 is a GH family 43 arabinofuranosidase that is mainly active toward the side-chains of branched arabinan. It can partly degrade linear arabinose oligomers with DP ≥ 4 in the given time (Kühnel et al., 2010). In contrast, Abn4 was not active toward reduced arabinotetraose and reduced arabinopentaose (no further data shown). Reduced arabinohexaose and reduced arabinoheptaose were partly degraded to yield reduced arabinopentaose and reduced arabinohexaose, respectively (Fig. 5.2B). These data show that Abn4 removes a terminal arabinose unit from the non-reducing end of the molecule. The alditol could decrease the substrate binding to Abn4 and, by this, reduce enzyme action. Alternatively, the alditol could not be recognized by Abn4 and, therefore, Abn4 action toward reduced arabinose oligomers is reduced.

In general, topologies of substrate binding sites are not highly conserved in GH family 43 enzymes (Nurizzo et al., 2002; Vandermarliere et al., 2009). However, these enzymes have a five-bladed β-propeller as a common feature (`http://www.cazy.org/GH43.html`). The binding site of endo enzymes has a cleft topology. In exo enzymes, like *G. stearothermophilus* GH43 β-xylosidase and *Streptomyces avermitilis* exo-1,5-α-arabinofuranosidase, this cleft is blocked or closed on one side and forms a pocket like topology (Brüx et al., 2006; Fujimoto et al., 2010). Low Abn4 activities toward linear arabinose oligomers and higher activities toward polymeric branched arabinan could suggest that Abn4 has a substrate binding site that recognizes a larger part of the substrate. The

binding of the molecule would then occur with an endo-mode of action, whereas the catalytic activity itself would follow an exo-mode of action.

According to this data the arabinofuranosidase Abn4 is not part of the arabinofuranosidase family A or B or arabinoxylan arabinofuranohydrolase as described by Beldman and co-workers (1997). Structural characterization of Abn4 (e.g. by crystallography) could give more insight into the structure-function relationship.

Abf3

Abf3 is a GH family 51 arabinofuranosidase that releases arabinose from arabinoxylan and from linear and branched arabinose oligomers (Hinz et al., 2009; Kühnel et al., 2010). It can be seen from Fig. 5.2C that Abf3 removed single arabinose units from the non-reducing end of reduced arabinotriose. Abf3 activity toward reduced arabinose oligomers was five to ten times lower than Abf3 activity toward *p*NP-Ara*f*. It was most active toward reduced arabinobiose and the activity decreased with increasing DP of the substrate. The clear preference for smaller oligomers and the lack of activity toward polymeric substrate indicates that Abf3 acts toward arabinose oligomers like expected for an arabinofuranosidase A (Beldman et al., 1997; Kühnel et al., 2010).

5.3.2   Product inhibition

Arabinobiose was added to Abn1 and Abn2 digests of linear arabinan since it is the main product that accumulates upon end point conversion. The reaction was followed by HPSEC (Fig. 5.3A+B). The linear arabinan polymer eluted at 9.8 min and was rapidly degraded to intermediate and small molecular-mass materials that eluted from 11.5–15.0 min. The main peak at 14.75 min shows the increasing arabinobiose levels in the samples. The insert represents a zoom into the high molecular-mass region in the range from 8.5–14.0 min.

Abn1

Abn1 was not inhibited in the degradation of linear arabinan even when 50 mg/ml arabinobiose were added to the mixture (Fig. 5.3A). Some variation in the presence of small molecular-mass material was observed. Digests with high concentrations of arabinobiose (20–50 mg/ml) contained more oligomers that eluted at 13.2, 13.5 and 13.9 min than digests with less arabinobiose. Theses changes are mainly considered to be derived from impurities within the arabinobiose preparation that was used in the experiment (as shown

in supplementary data Fig. S5.3). When calculating the molar substrate to product ratios, it can be seen that even a 1700 fold product concentration did not lower Abn1 activity (Table 5.2). The data also correlates with the activities measured toward reduced arabinose oligomers (Fig. 5.1A), in which a decrease in enzyme efficiency was observed with a decrease of the degree of polymerization of the substrate. It suggests that the +2 subsite in Abn1 is not only important for Abn1 activity, but also for substrate binding.

Table 5.2: Product to substrate ratios for linear arabinan and arabinobiose concentrations used in the product inhibition experiment. A molecular mass of 50 kDa is assumed for linear arabinan and 282 Da for arabinobiose. Masses were used in the assay.

	Linear arabinan	Arabinobiose						
Mass (mg)	0.5	0.1	0.5	1	2	3	4	5
Amount of substance (µmol)	0.01	0.35	1.77	3.55	7.09	10.64	14.18	17.73
Ratio	1	35	177	355	709	1064	1418	1773

Abn2

Unlike Abn1, Abn2 was very sensitive to product inhibition (Fig. 5.3B). The addition of 1 mg/ml arabinobiose reduced Abn2 activity to about 75 % and the activity further declined to 20 % and 5 % at concentrations of 5 and 10 mg/ml arabinobiose, respectively, as estimated from the surface area decrease in the range of 8.5–14.0 min. When calculating the molar ratios and assuming only one non-reducing end for a linear arabinan molecule, a 200 fold product concentration inhibited the Abn2 activity to 95 %. Since linear arabinan is produced from branched arabinan, one molecule of linear arabinan may have several non-reducing ends that may be a substrate for Abn2. Therefore, the apparent ratio of inhibition could be even lower. Since the number of non-reducing ends in linear arabinan is not known, no inhibition constant was calculated.

Both enzymes were also incubated with linear arabinan in the presence of 150 mg/ml arabinose. None of the enzymes showed any product inhibition. Therefore, a specific binding of arabinose by Abn1 and Abn2 can be excluded (no further data shown).

Abn4 and Abf3

Exoarabinanase Abn4 did not show any product inhibition when incubated with arabinose (Fig. 5.3C). On the contrary, 50 mM arabinose seemed to stabilize the enzyme and

lead to a 30 % increase in enzyme activity. This increase in activity was also observed when glucose was added to the mixture. However, 50 mM sodium acetate buffer, xylose or inositol did not have any effect on the enzyme activity (no further data shown). It can be concluded that Abn4 is positively affected by the presence of certain sugars.

The Abf3 activity was reduced by 20 % in the presence of 500 mM arabinose (Fig. 5.3C). A linear correlation between product concentration and Abf3 activity was observed. Abf3 digests with different initial substrate concentrations were analyzed to determine the inhibition mechanism. From the Lineweaver-Burke-Plot shown in Fig. 5.3D it can be seen that Abf3 product inhibition followeds a competitive mechanism. The product can bind to the active site with a much lower affinity than the substrate. Abf3 activity is fully recovered when the substrate concentration is increased to 5 mM *p*NP-Ara*f*.
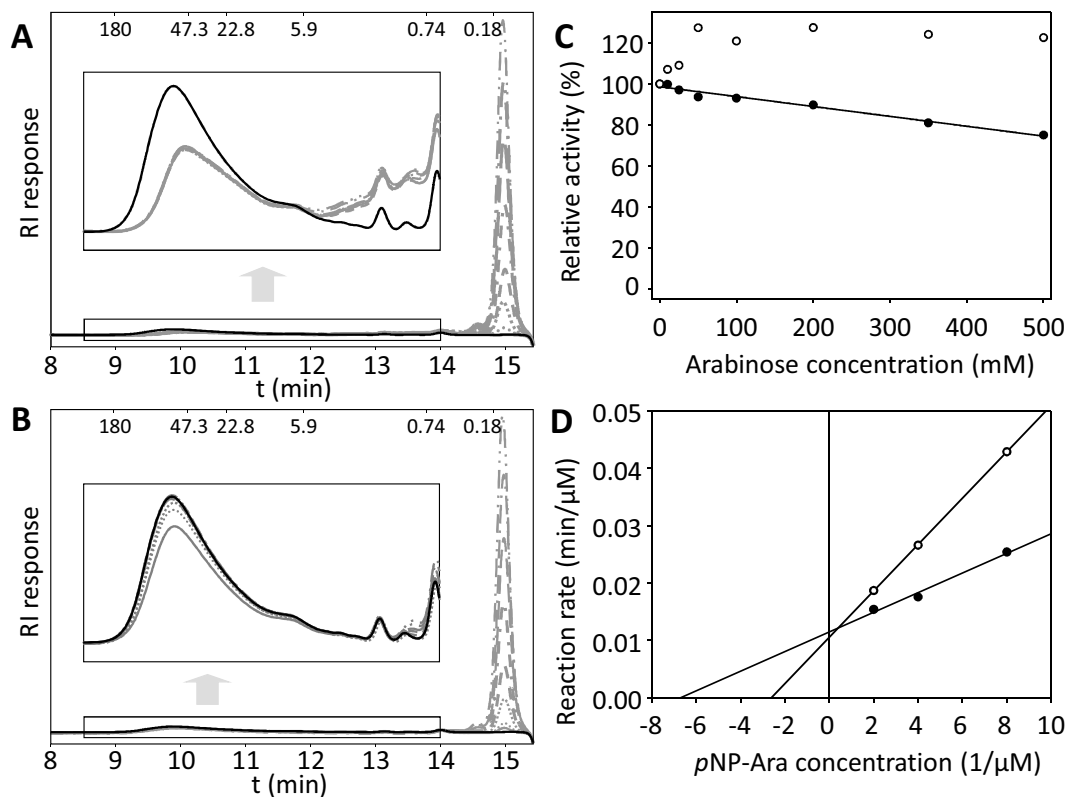
Fig. 5.3: Product inhibition of C1 arabinohydrolases. Linear arabinan degradation by Abn1 (A) and Abn2 (B) in the presence of 0–50 mg/ml arabinobiose (t = 14.75 min), upper X-Axis: Molecular masses of pullulan standards (in kDa), black line – blank, grey line – 0 mg/ml arabinobiose, grey dashed/dotted lines – 1, 5, 10, 20, 30, 40, 50 mg/ml arabinobiose with increasing peak area at 14.75 min. C – Abn4 (○) and Abf3 (●) activity toward *p*NP-Ara*f* (0.5 mM) in the presence of 0–500 mM arabinose. D – Lineweaver-Burke-Plot of Abf3 toward *p*NP-Ara*f* (from 0.125–0.500 mM) with 0 mM arabinose (●) and 500 mM arabinose (○).

Table 5.3: Branched arabinose oligomer structures according to Westphal and co-workers (2010a; 2010b). Horizontal white dots – α-1,5-linked arabinofuranose units, branches attached on the lower left side – α-1,3-linked arabinofuranose side-chain, branches attached on the lower right side – α-1,2-linked arabinofuranose side-chain.

Component	Schematic structure	PGC–HLPC–MS retention time (min)
1.0		< 1.0
2.0		3.5
3.0		7.5
4.0		9.7
5.0		11.4
3.1		7.2
4.1		8.5
4.2		9.4
5.1		10.4
5.2		10.8
6.1b*		11.8
6.2		12.2
7.2**		13.2
8.1		13.9

*predicted structure confirmed by the degradation of 8.1 by Abf3 and Abn1, ** proposed structure based on the degradation of 8.1 by Abn4

5.3.3 Action of C1 arabinohydrolases toward branched arabinose oligomers

Action toward branched arabinopentaose isoforms

The activities of Abn4 and Abf3 toward branched arabinose oligomers were tested to determine the mode of action of the enzymes. The branched pentamers used consist of three α-1,5-linked arabinose units that carry two arabinose side groups. The middle arabinose of the 5.1 isoform is double-substituted with an α-1,2-linked and an α-1,3-linked arabinose, whereas the middle arabinose and the terminal non-reducing arabinose of the 5.2 isomer are single-substituted with two α-1,3-linked arabinose units (Table 5.3). From Fig. 5.4A it can be seen that the branched arabinopentaose mix mainly contained these two structures that were differently degraded by Abn4 and Abf3 (lines II–IV). The evaporative

light scattering (ELS) signal allows the quantification of the compounds present and the formation of arabinose monomers. Fig. 5.4B shows the MS signal of the same samples in which minor compounds are more pronounced (e.g. the 4.2 and 6.2 oligomers, Fig. 5.4B, line I) than in the ELS signal.

Abn4 could only degrade the 5.2 isomer (Fig. 5.4, line II). It released the α-1,3-linked side groups from the pentamer 5.2 which lead to the release of arabinose and arabinotriose as the end products of the reaction. The oligomer 4.2 (Table 5.3) was formed as an intermediate product. This could suggest that the terminal α-1,3-linked arabinose at the end of the molecule is preferred over the α-1,3-linked arabinose attached to the second arabinose. Linear arabinotetraose was recovered from the Abn4 digestion mixture, which was derived from the branched hexamer 6.2 (Fig. 5.4, line II). The pentamer 5.1 could not be degraded by Abn4. It can, therefore, be concluded that Abn4 cannot hydrolyze the linkages of double-substituted arabinose from arabinose oligomers.

In contrast to Abn4, Abf3 could degrade both branched arabinopentaose isomers over time (Fig. 5.4, line III). The amount of enzyme required for the degradation was similar to the amount needed for the degradation of the linear pentamer. The double-substituted isomer 5.1 was converted into 4.1 (t = 8.5 min) by the removal of the terminal α-1,5-linked arabinose residue from the non-reducing end of the molecule. The 4.1 peak accumulated over time and was further degraded to monomer when a 10-fold overdose Abf3 was added to the mixture (Fig. 5.4, line IV). The isomer 5.2 was degraded to the structure 4.2 by the hydrolysis of the terminal α-1,3-linkage at the non-reducing end (Fig. 5.4B, line III, t = 9.4 min). Subsequently, the molecule was further degraded to two trimers 3.0 and 3.1 that eluted at 7.5 min and 7.2 min, respectively. Both trimers were then converted to arabinobiose and subsequently to arabinose as end product (Fig. 5.4, line IV). With respect to Abf3, it can be concluded that it can degrade all linkages present in 5.1 with a clear preference for single-substituted sugars in proximity of the non-reducing end. The double-substituted arabinose side-chains are also degraded. However, the hydrolytic performance is 10-fold lower compared to the hydrolysis of a single α-1,3-linkage or α-1,5-linkage. The absence of any intermediate degradation products smaller than the 4.1 oligomer is in support of this behavior (Fig. 5.4A).

Action toward branched arabinooctaose

The C1 arabinohydrolases Abn1, Abn4 and Abf3 were sequentially tested toward the branched arabinooctaose isomer 8.1 to get more insight in structural limitations of enzymatic degradation. From Fig. 5.5A it can be seen that Abn1 was not active toward 8.1
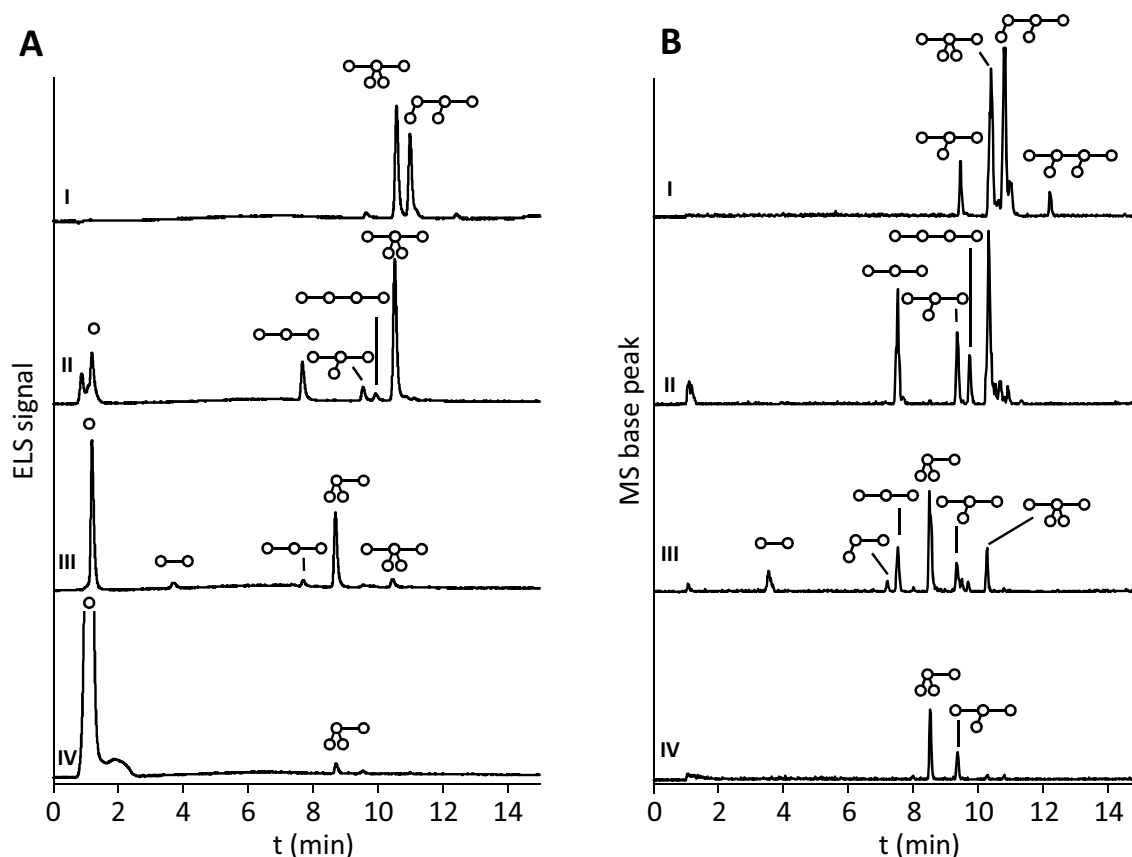
Fig. 5.4: Time dependent degradation of the arabinopentaose isomers by Abf3 and Abn4. A – PGC-HLPC-ELSD retention time profiles of the branched arabinopentaose mix (BAP), B – PGC–HPLC–MS retention time profiles of BAP. I – BAP (blank), II – BAP digested with Abn4, III – BAP digested with Abf3, IV – BAP digested with ten fold concentration Abf3 (t = 3 h).

(line II) whereas Abn4 had a low activity toward 8.1. It removed one arabinose unit and produced an arabinoheptaose isomer that eluted at 13.2 min (line III). This isomer was degraded by Abn1 to the known arabinopentaose isomer 5.1 and arabinobiose (line IV). Therefore, the structure of the arabinoheptaose released by Abn4 was concluded to be as shown in structure 7.2 (Table 5.3). Abn4 released the single-substituted α-1,3-linked arabinose. This release was much slower than the removal of the α-1,3-linked arabinose units from 5.2. It is likely that the neighboring double-substituted arabinose residue sterically hindered the binding of the substrate by Abn4. Small amounts of a hexamer were released upon prolonged incubation with a 50 times overdose of Abn4 (supplementary data Fig. S5.4). Therefore, Abn4 could also hydrolyze the α-1,5-linked arabinose at the non-reducing end. The speed of hydrolysis was very low, probably due to the presence of the neighboring double-substituted arabinose unit.
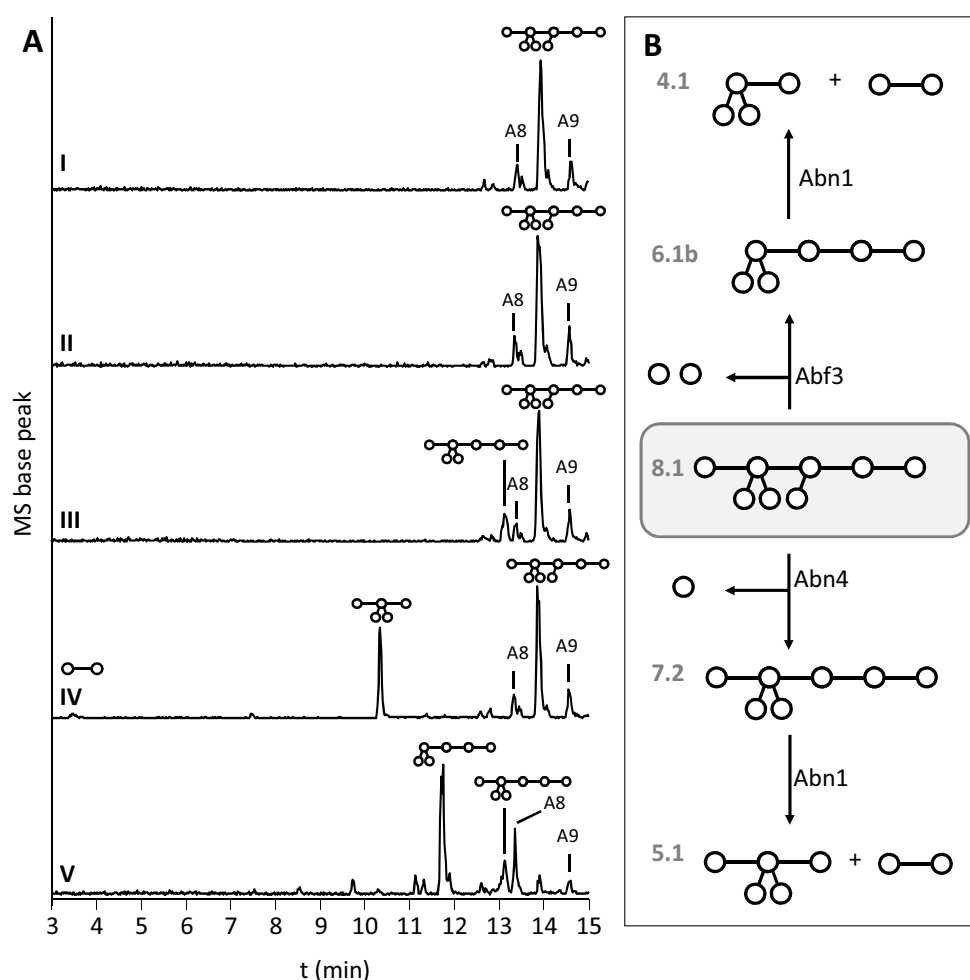
Fig. 5.5: Degradation of branched arabinooctaose 8.1 by C1 arabinohydrolases. A – HPLC–PGC–MS elution profile of 8.1 (I), 8.1 treated with Abn1 (II), 8.1 treated with Abn4 (III), sample III treated with Abn1 (IV) and 8.1 treated with Abf3 (V). Structures are indicated when the component could be identified based on m/z ratio and retention time. Arabinose oligomers of unknown structure and known m/z ratio: A8 – arabinooctaose isomer, A9 – arabinononaose isomer. B – Schematic representation of branched arabinooctaose degradation by Abf3, Abn4 and Abn1.

No statement can be made regarding the performance toward α-1,2-linked arabinose, since no oligomers could be isolated that contain a single-substituted α-1,2-linked arabinose (Westphal et al., 2010a). Nevertheless, it could be conjectured that Abn4 can also hydrolyze this linkage because sugar-beet arabinan contains 4.5–8.0 % α-1,2,5-linked arabinose (Beldman et al., 1993; Kaneko et al., 1998).

The data suggest that Abn1 is side-chain tolerant. Abn1 can hydrolyze the α-1,5-linkage of the backbone despite the presence of a double-substituted arabinose residue at

subsite -2. The topology of the binding cleft of Abn1 allows it to host a double substitution. However, the hydrolytic performance is reduced when compared to the action toward linear arabinose oligomers.

Abf3 degraded 8.1 to the arabinohexaose isomer 6.1b (line V) that could be further degraded by Abn1 to give arabinobiose and 4.1 (no further data shown). It confirms that Abf3 can hydrolyze both, α-1,3-linked and α-1,5-linked arabinose. The appearance of the 7.2 isomer may imply a slight preference of the α-1,3-linked arabinose over the α-1,5-linked arabinose (line V) since this peak was the only arabinoheptaose structure that was released by Abf3. Furthermore, Abf3 digest lead to an increase of the compound A8 eluting at 13.4 min. A8 is an arabinooctaose isomer of unknown structure. The early elution time suggests a highly branched structure that could be produced by the degradation of the arabinononaose isomer (A9) that eluted at 14.6 min.

5.4 Conclusions

The *Chrysosporium lucknowense* C1 arabinohydrolases have different modes of action and substrate specificities. The exoarabinanase Abn2 releases arabinobiose from the non-reducing end of the α-1,5-linked arabinan backbone and prefers, like Abn1, polymers over oligomers. Endoarabinanase Abn1 can hydrolyze substrates that carry a double substitution at the subsite -2 and is therefore considered as side-chain tolerant. The arabinohydrolases Abn4 and Abf3 release arabinose monomers from the non-reducing end of the molecule with different modes of action toward natural substrates. The characterized enzymes allow the controlled and efficient degradation of arabinan to either monomers, or, by partial degradation, to branched arabinose oligomers.
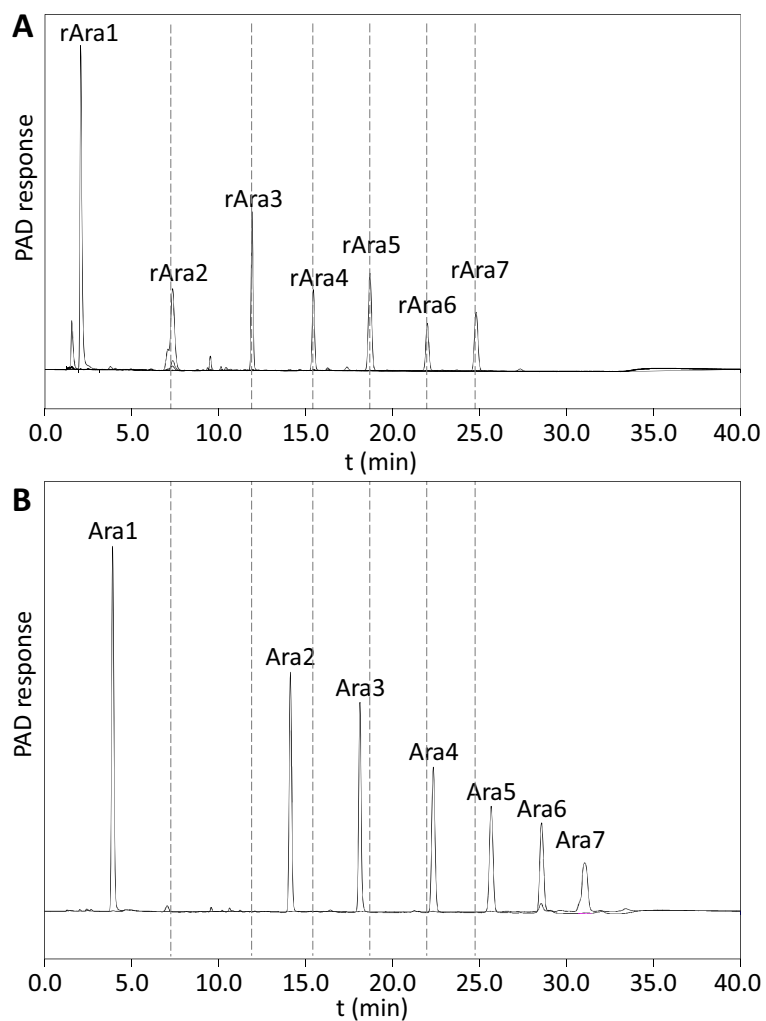
Acknowledgments

5.5   Supplementary data

Figure S5.1: Retention times of reduced and non-reduced arabinose monomer and oligomers in the range from DP 2–7. rAra1–7: reduced arabinose sugar with DP1–7, Ara1–7: arabinose monomer and oligomers from DP1–7.
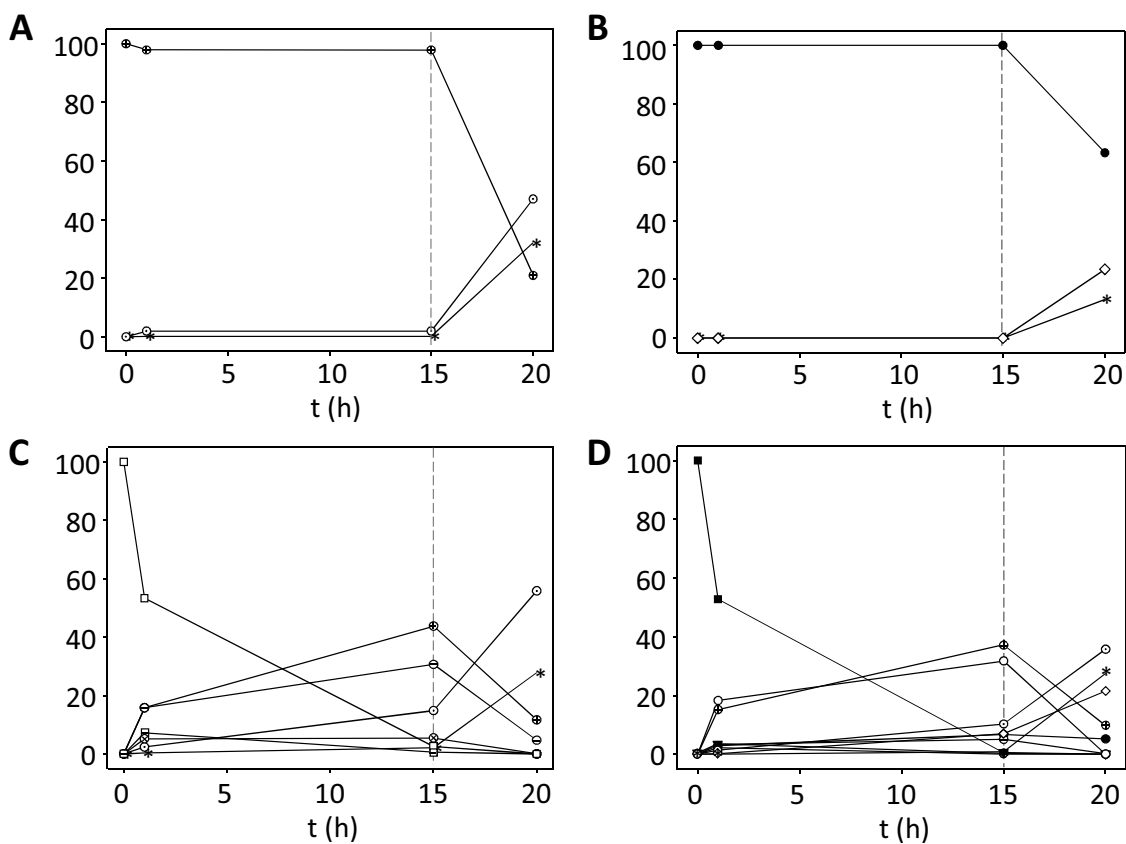
Figure S5.2: Time curves of C1 arabinohydrolase Abn1 toward pure, reduced and non-reduced arabinose oligomers. A – Abn1 toward arabinotriose: B – Abn1 toward reduced arabinotriose, C – Abn1 toward arabinoheptaose, D – Abn1 toward reduced arabinoheptaose. ◆ – arabitol, ◇ – reduced arabinobiose, ● – reduced arabinotriose, ○ – reduced arabinotetraose, ▼ – reduced arabinopentaose, ∇ – reduced arabinohexaose, ■ – reduced arabinoheptaose, ∗ – arabinose, ⊙ – arabinobiose, ⊕ – arabinotriose, ⊖ – arabinotetraose, ⊗ – arabinopentaose, ⊟ – arabinohexaose, □ – arabinoheptaose, grey line – fresh ten fold enzyme dose added after 15 h incubation.
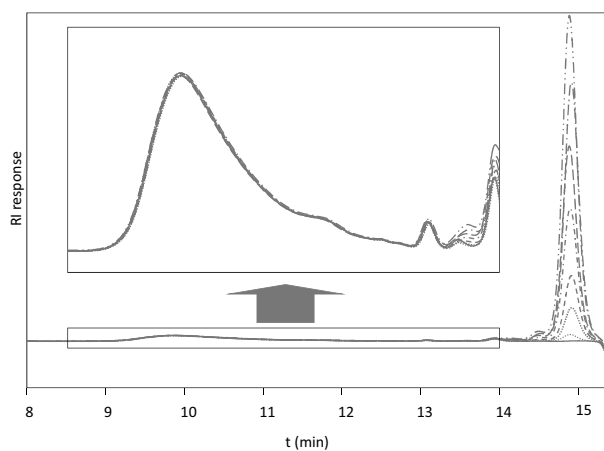
Figure S5.3: HPSEC elution profile of linear arabinan with arabinobiose concentrations in the range from 0–50 mg/ml prior to enzyme incubation.
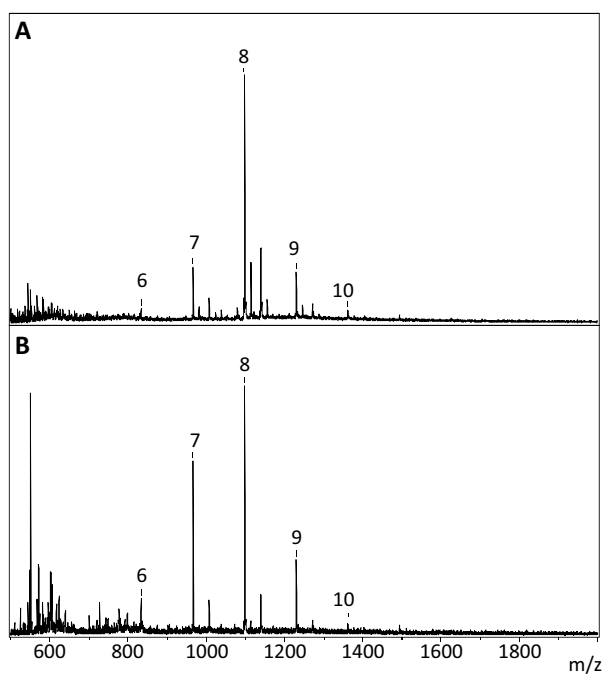

Figure S5.4: MALDI-TOF spectrum of 8-1 digest with Abn4. A: blank spectrum of 8-1, B: 8-1 after extensive incubation with a 50 times overdose of Abn4. Number from 6–10 indicate the DP of the oligomers.

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Chapter 6

The ferulic acid esterases of *Chrysosporium lucknowense* C1: Purification, characterization and their potential application in biorefinery.

Abstract

Three ferulic acid esterases from the filamentous fungus *Chrysosporium lucknowense* C1 were purified and characterized. The enzymes were most active at neutral pH and temperatures up to 45 °C. All enzymes released ferulic acid and *p*-coumaric acid from a soluble corn-fibre fraction. Ferulic acid esterases FaeA1 and FaeA2 could also release complex dehydrodiferulic acids and dehydrotriferulic acids from corn-fibre oligomers, but released only 20 % of all ferulic acid present in sugar-beet pectin oligomers. Ferulic acid esterase FaeB2 released almost no complex ferulic acid oligomers from corn-fibre oligomers, but 60 % of all ferulic acid from sugar-beet pectin oligomers. The ferulic acid esterases were classified based on both, sequence similarity and their acitivities toward synthetic substrates. The type A ferulic acid esterases FaeA1 and FaeA2 are the first members of the phylogenetic subfamily 5 to be biochemically characterized. Type B ferulic acid esterase FaeB2 is a member of subfamily 6.

6.1 Introduction

The use of plant biomass for second generation biofuel production requires the efficient and controlled degradation of their carbohydrate-rich cell walls. Straw and bran from grasses (Poaceae) or sugar-beet pulp are two potential feedstocks for second generation biofuels. The cell walls of grasses contain arabinoxylans that are substituted with hydroxycinnamic acid constituents, such as ferulic acid and coumaric acid (Hatfield et al., 1999). Also, the neutral sugar side-chains of pectins in the cell walls of sugar beet (arabinan and galactan) are feruloylated (Rombouts and Thibault, 1986). Such phenolic substituents have been shown to hinder the enzymatic degradation of sugar-beet pectin (Ralet et al., 1994b) and corn arabinoxylan (Appeldoorn et al., 2010).

Ferulic acid esterases (FAEs, EC 3.1.1.77) are structurally diverse and share sequence similarities with lipases, carbohydrate esterase (CE) family 1 acetyl xylan esterases, chlorogenate esterases and xylanases (Crepin et al., 2004). FAEs are classified into four subgroups (A–D), according to their activities toward synthetic substrates (Fig. 6.1) and dehydrodiferulic acids (Crepin et al., 2004; Topakas et al., 2007). Type A FAEs prefer methyl ferulate

FAE type	p-coumaric acid	caffeic acid	ferulic acid	sinapinic acid	diFerA
A	+	no	++/+++	+++	yes
B	++/+++	++/+++	+	no	no
C	yes	yes	yes	yes	no
D	yes	yes	yes	yes	yes

Fig. 6.1: Chemical structures of hydroxycinnamic acids: R = H – free acid form, R = CH_3 – (Synthetic) methyl-esterified form. Insert table: Classification of ferulic acid esterases based on their activities toward synthetic substrates and their ability to release diferulic acids (diFerA) (Crepin et al., 2004).

and methyl sinapinate over methyl *p*-coumarate and methyl caffeate (Fig. 6.1, Crepin et al., 2004). They hydrolyze the O–6 ester linkage with galactose in pectic galactan and the O–5 linkage with arabinose in arabinoxylan from gramineae (De Vries et al., 2002). They also release dehydrodiferulic acids from arabinoxylans when co-incubated with xylanase (Kroon et al., 1999).

Type B esterases prefer hydroxycinnamic acid esters with free-hydroxyl substituents over methyl ferulate and methyl sinapinate (Fig. 6.1, Crepin et al., 2004). They release ferulic acid that is ester-linked to arabinose of arabinoxylans or pectic arabinans. They also release ferulic acid that is ester-linked to O–6-galactose in pectic galactans (Ralet et al., 1994a), but they are not able to release dehydrodiferulic acids (Kroon et al., 1996).

Type C esterases hydrolyze all hydroxycinnamic acid methyl esters, but do not release dehydrodiferulic acids (Crepin et al., 2004). They are active on arabinoxylans and pectins.

Type D esterases are rather unspecific esterases that hydrolyze all hydroxycinnamic acid methyl esters. They can also release dehydrodiferulic acids and acetic acid from arabinoxylan oligomers (Bartolomé et al., 1997).

Only very few ferulic acid esterases are found in the CAZy database in CE family 1 and GH family 10 (bifunctional esterase/xylanase, Coutinho and Henrissat, 1999). A phylogenetic classification of putative FAEs into subfamilies (SFs) 1–7 was done by Benoit and co-workers (2008). Only SFs 1, 6 and 7 contain biochemically characterized FAEs. Fungal FAEs of the genera *Aspergillus*, *Fusarium*, *Penicillium* and *Talaromyces* have been characterized, but sequence data is not always available to assign them to a single SF. In turn, a multitude of putative FAEs have been identified in genome sequencing projects, but they are rarely biochemically characterized. Type A feruloyl esterase Fae-III and type B feruloyl esterase Fae-I from *Aspergillus niger* have been described in most detail (Benoit et al., 2008).

The genome of the ascomycete *Chrysosporium lucknowense* C1 (C1) comprises six genes that putatively encode for ferulic acid esterases (Hinz et al., 2009). Of these six genes, four were successfully brought to expression and the enzymes were active toward methyl ferulate (Hinz et al., 2009). One enzyme was unstable and was not included in further experiments. In this research we purified and biochemically characterized three ferulic acid esterases from C1.

6.2 Methods

6.2.1 Materials

Methyl caffeate, methyl coumarate, methyl sinapinate and methyl ferulate were obtained from Apin Chemicals Ltd.(Abingdon, Oxon, UK). Alcohol insoluble solids of Brewer's spent grain (BSG), corn cobs (CC) and wheat bran (WB) were present in the laboratory (Kabel et al., 2002). The following commercial enzyme preparations were used: Rapidase® Liq$^+$ (Liq+; DSM, Delft, the Netherlands), Driselase® (Dri; Sigma-Aldrich, St. Louis, MO, USA), Pectinex® Ultra SP (USP; Novozymes, Bagsværd, Denmark) and Rocksoft™MPL (MPL; Dyadic® Netherlands, Wageningen, The Netherlands).

Cell free fermentation supernatants of ferulic acid esterase overexpression lines were obtained from Dyadic®. The ferulic acid esterase candidate genes were identified based on a sequence homology screen with already identified ferulic acid esterases and they were named FaeA1, FaeA2 and FaeB2 (Table 6.1). Due to a lack of biochemical data for the best hits, the second best hit was used for annotation.

Table 6.1: Sequence similarity based preliminary annotation of C1 ferulic acid esterases.

C1 FAE	First hit	similarity	Second hit	similarity
FaeA1 (JF826027*)	*Chaetomium globosum* gene CHGG_06075 (EAQ89456*)	84 %	*Penicillium funicolosum* FAE A (AJ312296*)	52 %
FaeA2 (JF826028*)	*Chaetomium globosum* gene CHGG_06916 (EAQ85663*)	71 %	*Penicillium funicolosum* FAE A (AJ312296*)	45 %
FaeB2 (JF826029*)	*Chaetomium globosum* gene CHGG_10485 (EAQ84081*)	77 %	*Neurospora crassa* FAE B (AJ293029*)	68 %

*GENBANK accession numbers

6.2.2 Preparation and isolation of feruloylated oligomers

A fraction of corn fibre arabinoxylan oligosaccharides, that contains monomeric, dimeric and trimeric ferulic acid constituents linked to the oligosaccharides was used (Appeldoorn et al., 2010, subfraction M–5). Feruloylated oligosaccharides from sugar-beet pulp were obtained from 5 g pretreated sugar-beet pulp (Kühnel et al., 2011, 140–0 supernatant fraction) digested with the C1 enzymes Abn1, Abn4 and Gal1 (Hinz et al., 2009; Kühnel et al., 2010). The feruloylated oligomers were isolated and fractionated by reverse-phase solid phase extraction (Appeldoorn et al., 2010). Samples were eluted with 10 v/v %,

60 v/v % and 100 % methanol. Methanol was removed from the fractions by evaporation in a vacuum rotation evaporator and the fractions were subsequently freeze-dried. The 60 v/v % methanol fraction contained all the ferulic acid, as confirmed by UV spectroscopy at 325 nm (see § *Biochemical properties*).

6.2.3   Enzyme purification

The purification of the enzymes required a single chromatographic step for FaeA1 and two chromatographic steps for FaeA2 and FaeB2. Ferulic acid esterase activity in the purified fractions was monitored by measuring the activity toward methyl ferulate.

FaeA1

FaeA1 was purified by size exclusion chromatography using an ÄKTApurifier 10 analytical system (GE Healthcare, Uppsala, Sweden) equipped with a Superdex™75 analytical column (10/300 GL, 1 column volume (CV) = 23.56 ml; GE Healthcare) at a flow rate of 0.5 ml/min at 20 °C. The sample was manually injected via a 200 µl sample loop and eluted with 50 mM NaCl in 50 mM sodium acetate buffer (pH 5.0). Fractions of 200 µl were collected in microtiter plates using an ÄKTA Frac-950 fraction collector (GE Healthcare).

FaeA2 and FeaB2

FaeA2 and FaeB2 were purified by hydrophobic interaction chromatography followed by anion exchange chromatography using an ÄKTAexplorer 100 preparative system (GE Healthcare). The system was equipped with a self-packed Phenyl Sepharose™HP column (XK16, 58 ml column volume; GE Healthcare) that was equilibrated with five CV of 10 mM sodium acetate buffer (pH 5.0) containing 1.5 M ammonium sulfate (buffer A). The samples (17.5 ml) were mixed 1:1 with 20 mM sodium acetate buffer (pH 5.0) containing 3 M ammonium sulfate. They were cooled on ice for 30 min and subsequently centrifuged (5 min, $5000 \times g$, 4 °C). The supernatants were injected onto the column via a sample pump at a flow rate of 2 ml/min. The samples were eluted at a flow rate of 10 ml/min at 20 °C with 10 mM sodium sulfate buffer (buffer B, pH 5.0): 3 CV 0 % B, 2 CV 0–30 % linear B, 28 CV 30–100 % linear B, 5 CV 100 % B, 5 CV 0 % B (equilibration). Fractions (20 ml) were collected in glass tubes cooled on ice using an ÄKTA Frac-900 fraction collector.

Active fractions (FaeA2: 29.3–33.2 CV, FaeB2: 32.5–34.9 CV) were pooled and extensively dialysed against 10 mM sodium phosphate buffer (pH 6.5) using 12–14 kDa cut-off Visking dialysis tubings (Medicell International Ltd, London, UK; 2 × 24 h, 4 °C). The di-

alyzed fractions were further purified using a self-packed Q Sepharose™anion exchange column (XK26, V = 53 ml, GE Healthcare). Samples were eluted with 10 mM sodium phosphate buffer (pH 6.5) containing 1 M sodium chloride (buffer B) using a linear elution profile: 2 CV 0 % B, 20 CV 0–50 % linear B (22 CV for FaeB2), 1 CV 50–100 % linear B, 2 CV 100 % B, 5 CV 0 % B (equilibration). Active fractions (FaeA2: 7.4–8.9 CV, FaeB2: 13.2–14.3 CV) were pooled and dialyzed against 10 mM sodium phosphate buffer (pH 6.5) containing 50 mM sodium chloride (10-12 kDa cut-off, 24 h, 4 °C).

6.2.4 Enzyme characterization

All activity assays toward synthetic substrates are based on the absorption difference of the free acid and the methyl ester (total concentration: 50 μM) at 335 ± 5 nm (Ralet et al., 1994a). Suitable enzyme concentrations (in stock) were 730 μg/ml (FaeA1), 78 μg/ml (FaeA2) and 36 μg/ml (FaeB2). Sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing were performed as described previously (Kühnel et al., 2010).

Activity toward hydroxycinnamic acid methyl esters

Activities toward the synthetic substrates methyl caffeate, methyl coumarate, methyl ferulate and methyl sinapinate were determined by incubating 10 μl of enzyme in a total volume of 200 μl with 50 μM synthetic substrate in 50 mM sodium chloride and 10 mM BIS-TRIS-HCl (pH 7.0). The reaction took place at 25 °C for up to 2 h in a microtiter plate reader (Tecan infinite F500, Gördig, Austria). The absorbance was measured in 2 min intervals at 340 nm and the activity was determined by calibration curves of the substrates/products (Fig. 6.2).

Biochemical properties

The pH optimum of the enzymes was measured in the range from pH 4–8 using methyl ferulate as substrate. Therefore, 10 μl enzyme was incubated at 25 °C in a final volume of 200 μl with 50 μM methyl ferulate in McIlvaine buffers (40 mM disodium hydrogenphosphate mixed with 20 mM citric acid to give the desired pH). Reaction conditions and quantification were as described in § *Activities toward synthetic substrates*.

Temperature optima were determined using methyl ferulate (50 μM) as substrate at optimal pH values (FaeA1 and FaeB2: 10 mM BIS-TRIS-HCl (pH 7.0) containing 50 mM sodium chloride, FaeA2: 10 mM TRIS-HCl (pH 7.5) containing 50 mM sodium chlo-
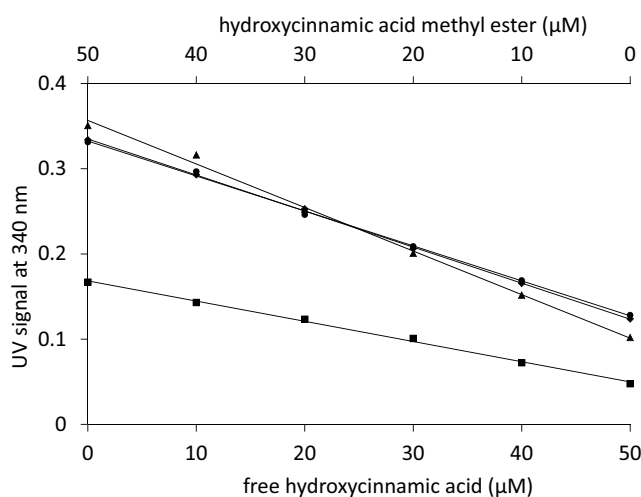
Fig. 6.2: Calibration curves for the quantification of synthetic hydroxycinnamic acids and methyl-esters. All samples contain 50 μM hydroxycinnamic acid in different ratios of free and methyl-esterified form (pH 7.0). Diamonds – caffeic acid & methyl caffeate, squares – coumaric acid & methyl coumarate, triangles - ferulic acid & methyl ferulate, circles - sinapinic acid & methyl sinapinate.

ride).The reaction mixture (990 μl substrate in buffer) was put into a quarz cuvette (1 ml), which was equilibrated at the desired temperature in an UV-1601 photospectrometer equipped with CPS-240A temperature controlled cell positioner (Shimadzu Ltd., Kyoto, Japan). After 15 min, 10 μl of enzyme was added and the sample was mixed by inversion. UV-visible spectra were recorded in the range of 600–200 nm (2 nm resolution) in 2 min intervals over a period of 2 h. The linear range of the time curves of the UV absorption at 336 nm was used to determine the enzyme activities.

Activity toward natural substrates

All samples were incubated for 24 hours in 50 mM sodium acetate buffer (pH 5.0) at 35 °C. The hydrolyses were stopped by boiling at 100 °C for 10 min. Alcohol insoluble solids of brewer's spent grain (BSG), corn cobs (CC), wheat bran (WB) and sugar-beet pulp (SBP) were used as polymeric substrates (5 ml/ml). BSG, CC and WB were first incubated with *Aspergillus awamori* endoxylanases I (xyl-I, GH family 10) or III (xyl-III, GH family 11), both purified according to Kormelink et al. (1993a). SBP (1 g in 100 ml) was digested with Liq+, Dri, USP and MPL. The supernatants obtained after centrifugation (10 min, 10,000 × *g*, 25 °C) were used for ferulic acid esterase activity experiments. All samples were diluted in order to have the same concentrations of ferulic acid constituents. The ferulic acid assays were performed in 50 mM MOPS buffer (pH 7.0) at 35 °C. The amount of FAE was

the same in each experiment (1 µg enzyme/microtiterplate well = 1 µg enzyme/0.3 a.u.). The release of ferulic acid was followed by measuring the absorbance decrease at 340 nm using a microtiterplate reader (Tecan). The release was quantified as described elsewhere (Ralet et al., 1994b).

6.2.5 Hydroxycinnamic acid release from natural substrates

Arabinoxylan oligomers purified from pretreated corn fibre (CF) and sugar-beet pectin oligomers (1 mg/ml each) were incubated with ferulic acid esterases in water at 35 °C for 16 h. The samples were boiled to inactivate the enzymes and centrifuged at room temperature (10 min, 10,000 × g). Hydroxycinnamic acid release from natural substrates was measured by RP-UHPLC-MS analysis in negative ion mode as described earlier (Appeldoorn et al., 2010) on an Accela UHPLC system (Thermo Scientific) equipped with a Hypersyl GOLD column (2.1 mm × 150 mm, 1.9 µm particle size; Thermo Scientific). Elution was monitored with the built-in polydiode array multi-wavelength detector set at 325 nm. The samples were diluted 5 times and 10 µl were injected. Ferulic acid release was quantified with the help of a ferulic acid calibration curve (1.25, 5.00, 12.50, 25.00 µg/ml ferulic acid).

The total amount of ester-linked ferulic acid in corn and sugar-beet oligomers was determined after alkaline hydrolysis and ethylether extraction using the UHPLC method described above.

6.3 Results and discussion

6.3.1 Purification of C1 ferulic acid esterases

The elution profiles of the FAEs after the last purification step are shown in Fig. 6.3. The purity of the pooled fractions was confirmed by SDS-PAGE (Fig. 6.3, insert picture). The size exclusion chromatography (SEC) profile of shows a symmetric peak at 13 ml (grey box), which fractions comprised all feruloyl esterase activity. Colored compounds present in the expression medium elute from 15–20 ml elution volume (Fig. 6.3A).

FaeA2 and FaeB2 were purified by hydrophobic interaction chromatography (HIC) and anion exchange chromatography (AEC). After AEC the FaeA2 active fraction still contained considerable amounts of another protein (Fig. 6.3B, insert picture), but no activity other than FAE activity has been found for the other protein. Therefore, it was
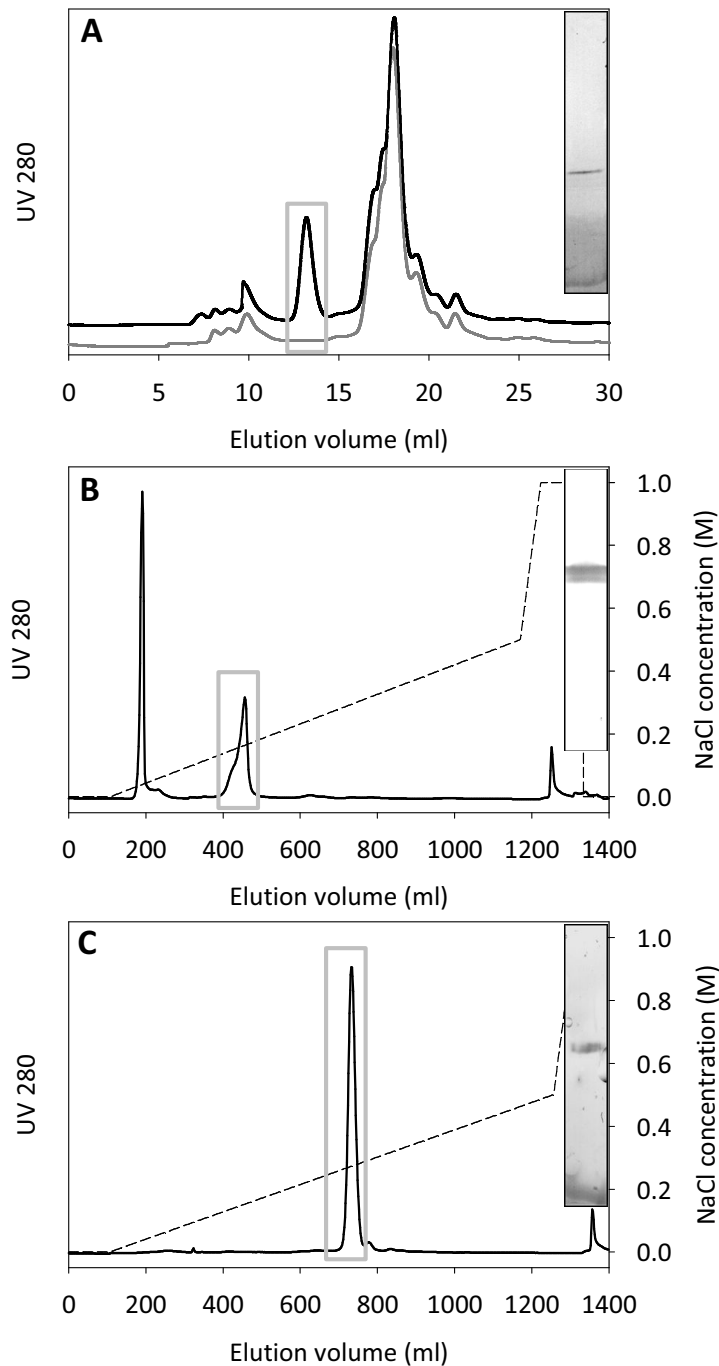
Fig. 6.3: Purification of the C1 ferulic acid esterases FaeA1, FaeA2 and FaeB2. A – SEC elution profile of FaeA1. Grey line: UV signal at 280 nm of the supernatant of the background strain, black line: UV signal at 280 nm of the supernatant of the FaeA1 expression strain; B & C – AEC elution profiles of FaeA2 (B) and FaeB2 (C). Straight lines: UV signals at 280 nm, dashed lines: sodium chloride elution profile. Grey boxes frame the elution volumes of the active fractions. Inserts: SDS-PAGE gels of the purified fractions.

considered as pure enough to allow further characterisation. FaeB2 was did not show impurities after AEC (Fig. 6.3C, insert picture).

Molecular masses of the three C1 ferulic acid esterases were determined experimentally by SDS-PAGE and show good agreement with theoretical values (Table 6.2). Small differences could be due to posttranslational modifications or inaccuracy in the determination. The isoelectric points (pI) are in agreement with calculated values for FeaA1 and FaeA2, whereas the experimental pI of FaeB2 is 1.6 pH units higher than the theoretical value (Table 6.2). A difference of 0.8 pH units has been observed for *Penicillium funiculosum* FaeB from the same SF (Kroon et al., 2000, Table 6.4). That deviation could, thus, be related to the secondary structure of SF 6 enzymes.

Table 6.2: Theoretical and experimental molecular masses and isoelectric points of C1 ferulic acid esterases.

	Molecular mass (kDa)		Isoelectric point	
	theoretical	experimental	theoretical	experimental
FaeA1	29.2	29	5.9	≈ 5.5
FaeA2	31.8	36	5.4	≈ 5.2
FaeB2	31.4	33	4.4	≈ 6.0

Theoretical molecular masses and pI values were calculated with a pI/Mw computing tool (http://expasy.org/cgi-bin/pi_tool).

6.3.2 Biochemical characterisation of C1 ferulic acid esterases

pH optimum

C1 ferulic acid esterases FaeA1 and FaeA2 have a broad pH activity range with more than 60 % of the optimal activity between pH 5.0–7.5. FaeB2 is most active between pH 6.0–8.0 (Fig. 6.4A). FaeA1 is the most pH tolerant of the three esterases: It is more than 50 % active between pH 4.5–8.0 and even more than 90 % activity is retained between pH 5.5–8.0 (Fig. 6.4A). FaeA2 has a more pronounced pH optimum than FaeA1 with minimal 50 % activity between pH 4.8–7.5. As confirmed in individual experiments, FaeA2 activity decreases above pH 7.5. Similar to FaeA1, FaeB2 is most active in at pH $\approx$ 7.0, but its pH optimum range is more within the neutral to slightly alkaline pH range. FaeB2 retains $\geq$ 50 % activity between pH 5.5–8.0 and $\geq$ 70 % activity between pH 6.0–8.0.

The pH optima of FaeA2 and FaeB2 are comparable to the pH optima of other fungal FAEs (mostly pH 6.0–7.0) (Wong, 2006; Benoit et al., 2008; Koseki et al., 2009). The pH

optimum of FaeA1 is much broader than that of other fungal FAEs and is more similar to that of the bacterial FAE_Xyn10A from *Clostridium thermocellum* (Blum et al., 2000).

A number of cell-wall degrading enzymes from C1 have been purified and characterized, among them cellulases, xylanases, arabinohydrolases and acetyl xylan esterases (Bukhtojarov et al., 2004; Gusakov et al., 2005; Ustinov et al., 2008; Hinz et al., 2009; Kühnel et al., 2010; Pouvreau et al., 2011). Most of them show optimal activities between pH 5.0–7.0. C1 FAEs are, therefore, suitable for combined usage with C1 glycosidases to hydrolyze feruloylated biomass.
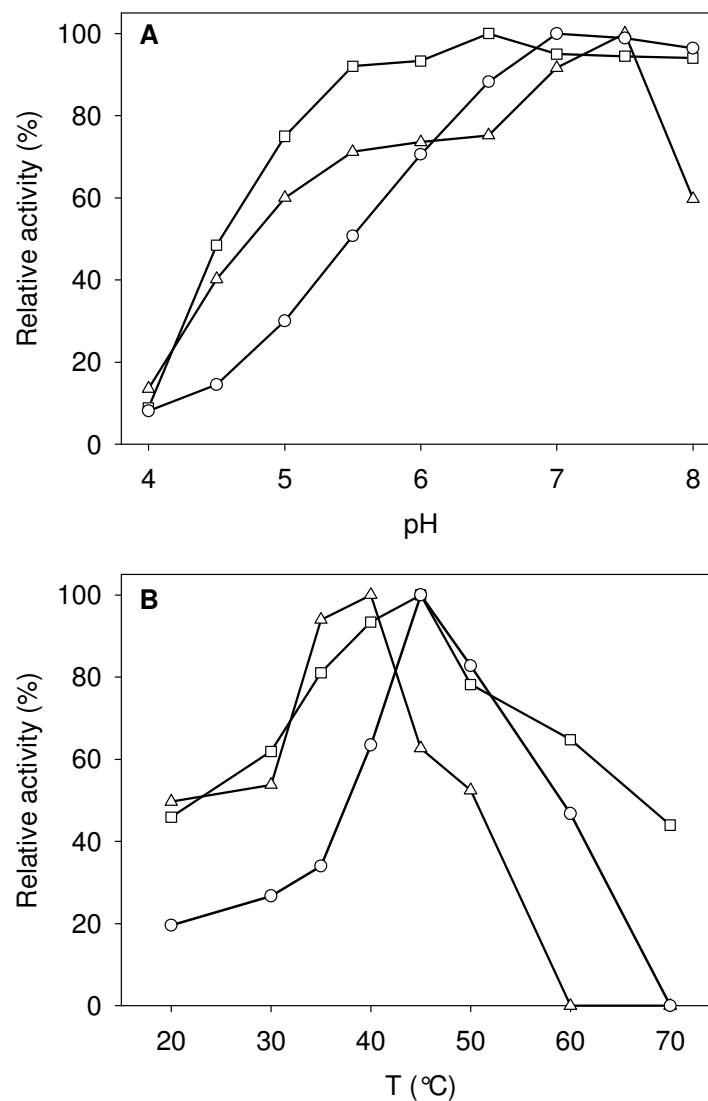
Fig. 6.4: Temperature and pH optima of C1 ferulic acid esterases. A: pH optima (T = 25 °C), B: temperature optima of FaeA1 (□) and FaeB2 (○) at pH 7.0 and of FaeA2 (△) at pH 7.5.

Temperature optimum

FaeA1 and FaeB2 both show optimal activities at 45 °C (Fig. 6.4B). FaeA1 has a broad temperature-optimum range with ≥ 50 % activity between 25–65 °C, whereas FaeB2 shows a rather sharp, defined temperature optimum with ≥ 50 % activity between 40–60 °C and a complete inactivation above 70 °C (Fig. 6.4B). FaeA2 is slightly less stable then FaeA1 and FaeB2, since it starts to denaturate at temperatures above 40 °C and is completely inactive above 60 °C, also in the presence of substrate.

The temperature optima of C1 FAEs are relatively low, since most other fungal FAEs have temperature optima between 50–60 °C (Koseki et al., 2009).

6.3.3 Substrate specificities of C1 ferulic acid esterases

Activity toward hydroxycinnamic acid methyl esters

All three enzymes were tested for their activity toward the hydroxycinnamic acid methyl esters (Table 6.3). FaeA1 is most active on methyl sinapinate and the activities on the other substrates decrease with decreasing degree of substitution: FaeA1 prefers methyl ferulate over methyl caffeate and is least active on methyl coumarate. It has been reported for *Aspergillus niger* that the activity of FAE-III (FaeA) increases with increasing methoxylation of the substrate, whereas CinnAE (FaeB) is more active toward hydroxylated substrates (Kroon et al., 1997). Limited substrate specificty data is available for FaeA from *Penicillium funiculosum*, with which FaeA1 and FaeA2 share high sequence similarities. FaeA1 from C1 has similar substrate specificities on synthetic substrates as *Aspergillus niger* FaeA, with the difference that it is active on methyl caffeate. Nevertheless, FaeA1 is most likely correctly annotated as type A feruloyl esterase.

Table 6.3: Activities of C1 ferulic acid esterases toward hydroxycinnamic acid methyl esters.

	FaeA1	FaeA2	FaeB2
methyl coumarate	30	80	100
methyl caffeate	55	55	65
methyl ferulate	87	100	89
methyl sinapinate	100	17	2

Relative activities (% from the highest activity). The substrates are ordered by increasing degree of substitution.

FaeA2 is most active toward methyl ferulate, while it is 20 % less active on methyl coumarate, 45 % less active on methyl caffeate and 83 % less active on methyl sinapinate. The activity pattern is neither linked to the hydroxylation pattern, nor to the methoxylation pattern of the synthetic substrates. FaeA2 specificity is different than the type A or type B ferulic acid esterase specificities that have been reported previously (Wong, 2006; Benoit et al., 2008). Type A esterases have been reported to be inactive toward methyl caffeate and type B esterase have been reported to be inactive toward methyl sinapinate and to prefer hydroxylated substrates over methoxylated substrates (Crepin et al., 2004). Therefore, more data is needed to confirm the annotation of FaeA2.

FaeB2 is most active toward methyl ferulate and methyl coumarate, but it is almost inactive toward methyl sinapinate. FaeB2 prefers methyl ferulate over methyl caffeate, which suggests a preference of methoxyl-substituents over hydroxyl-substituents. A similar specificity has been described for the type B esterase Fae-1 from *Neurospora crassa* (Crepin et al., 2003) Furthermore, FaeB2 shows high sequence similarities with type B feruloyl esterases from *Neurospora crassa*. Therefore, FaeB2 is most likely a type B esterase.

Activities of C1 ferulic acid esterases toward natural substrates

The C1 ferulic acid esterases are inactive on insoluble cell-wall polysaccharides (no further data shown), but they are active toward feruloylated oligomers (Fig. 6.5). Ferulic acid esterase activity toward complex/insoluble polysaccharides depends on the presence of a carbohydrate-binding module (Koseki et al., 2009). From sequencing data it is known that C1 FAEs do not have a carbohydrate-binding module, which explains the inactivity of the C1 FAEs toward such substrates.

After complete digestion, the FaeA1 releases 90 % of the ferulic acids present in wheat-bran arabinoxylan oligomers released by endoxylanase I (xyl-I WB AXOS) while only 60 % of the ferulic acid released by xyl-III from WB AXOS (Fig. 6.5A&D). FaeA1 is less active toward AXOS from brewer's spent grain (BSG) and corn cobs (CC) than toward WB AXOS. Similar to the activity on WB AXOS, FaeA1 is more active on CC AXOS released by xyl-I ($\approx$ 40 % hydrolysis) than those released by xyl-III ($\approx$ 25 % hydrolysis). The opposite behavior is seen is seen for BSG AXOS, where FaeA1 is more active toward AXOS released by xyl-III ($\approx$ 50 % hydrolysis) than toward those released by xyl-I ($\approx$ 35 % hydrolysis). FaeA1 has no detectable activity on sugar-beet pectin oligomers (SBPOS) released by any of the commercial enzyme preparations (only data for Liq+ shown in Fig. 6.5A).
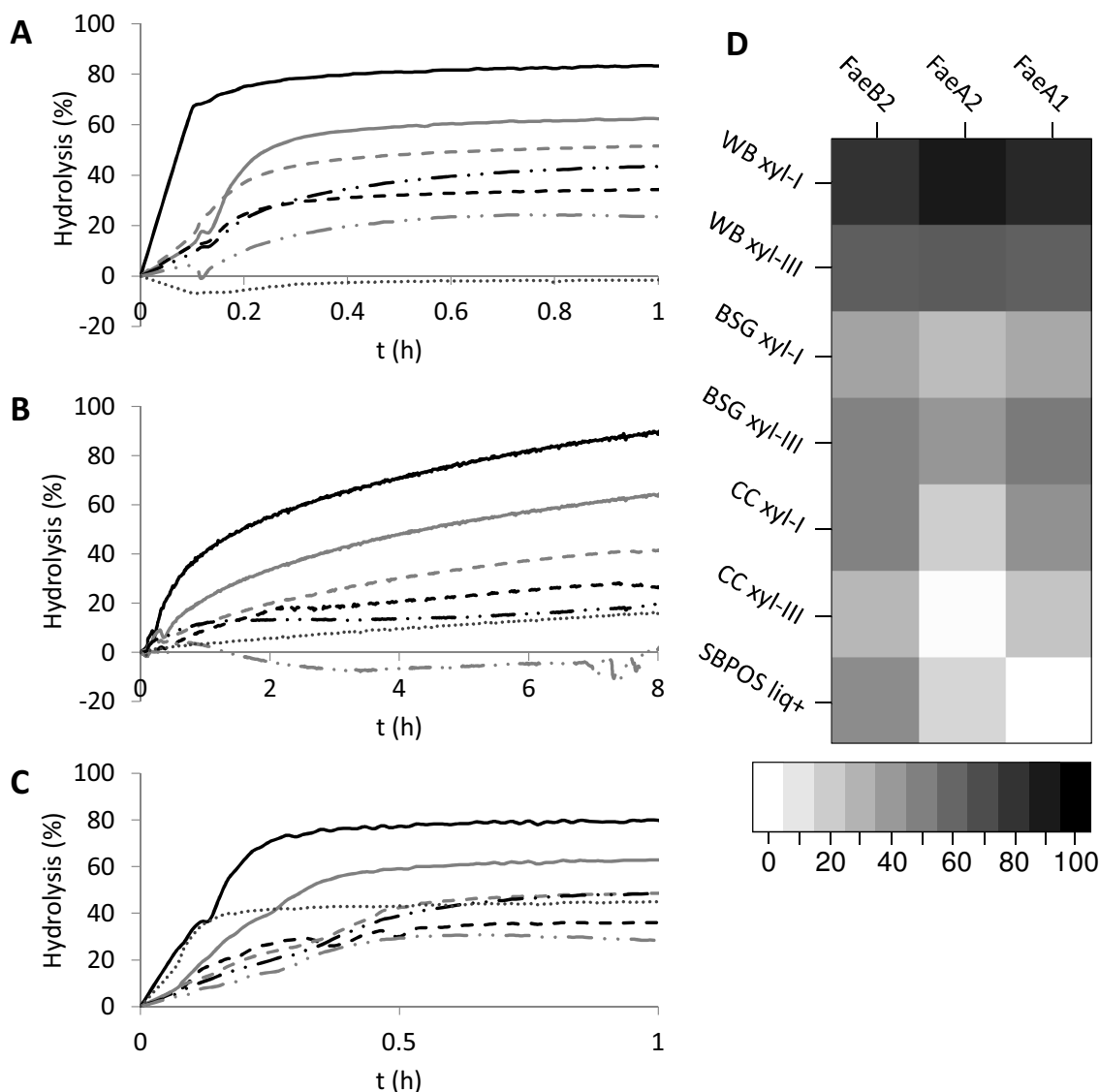
Fig. 6.5: Release of hydroxycinnamic acids by C1 FAEs from feruloylated/coumarylated oligomers from various feedstocks. Hydrolysis rates were calculated from the UV signals at 335 nm (Ralet et al., 1994b). A – FaeA1, B – FaeA2, C – FaeB2. Substrates: Straight line – WB AXOS released by endoxylanase I (black) or III (grey), dashed line – BSG AXOS released by endoxylanase I (black) or III (grey), dashed and dotted line – CC AXOS released by endoxylanase I (black) or III (grey), dotted line – sugar-beet pectin oligomers released by Liq+. D – End-point release of hydroxycinnamic acids (FaeA1 and FaeB2: t = 1 h, FaeA2: t = 8 h). Values were transformed into a heatmap ranging from white (no release) to black (complete release).

FaeA2 is generally less active than FaeA1, but has similar end-points of hydrolysis from WB AXOS and BSG AXOS (Fig. 6.5B&D). In contrast to FaeA1, FaeA2 is inactive toward CC AXOS released by xyl-III, while it is active toward SBPOS released by commercial enzyme preparations with the highest activity toward SBPOS released by Liq+ ($\approx$ 20 % hydrolysis after 8 h).

FaeB2 acts comparably to FaeA1 toward the various AXOS, whereas it is more active on SBPOS than FaeA1 and FaeA2 (Fig. 6.5C&D). FaeB2 has the same initial activities toward xyl-I WB AXOS and SBPOS released by Liq+, but different end-points of reaction ($\approx$ 80 % hydrolysis from AXOS vs. $\approx$ 45 % hydrolysis from SBPOS).

The different end-points of ferulic acid release from AXOS produced by xyl-I and Xyl-III could be caused by the product release pattern of the enzymes. Xyl-I has been shown to release smaller oligomers from alkali-extracted wheat flour arabinoxylan than xyl-III (Kormelink et al., 1993b). It is not known in which way feruloylation influences the performance of xyl-I and xyl-III on arabinoxylans from these feedstocks, or whether xyl-I and xyl-III release oligomers that have differently linked ferulic acid substituents.

Ferulic acid is the main hydroxycinnamic acid in WB arabinoxylan and most of it is linked to O–5-arabinose of the side-chains ($\geq$ 90 %) (Lequart et al., 1999), whereas BSG arabinoxylan contains equal amounts of ferulic acid and *p*-coumaric acid (Bartolomé et al., 2002). Since all C1 FAEs are active on methyl coumarate (Table 6.3), the observed release from BSG AXOS could be both, ferulic acid and coumaric acid release.

Corn arabinoxylans contain up to 2.5 w/w % ferulic acid dehydrodimers (Saulnier and Thibault, 1999). The low end-point releases from CC AXOS indicate that some of these complex phenolic groups cannot be released by the C1 FAEs.

6.3.4 Specific activities of C1 ferulic acid esterases toward feruloylated oligomers from sugar-beet pectin and corn fibre arabinoxylan

In order to study the specific release of hydroxycinnamic acid monomers and oligomers by the C1 FAEs from natural substrates, the product release was determined by UHLPC-MS analysis.

Activity toward feruloylated sugar-beet pectin oligomers

FaeA1 and FaeA2 release $\approx$ 20 % of the ferulic acid present in sugar-beet pectin oligomers (SBPOS), while FeaB2 releases $\approx$ 60 % of the ferulic acid (Fig. 6.6A). Similar end-point releases by FaeA2 and FaeB2 were observed toward SBPOS released by Liq+ (Fig. 6.5B&C),

whereas no ferulic acid was released by FaeA1 (Fig. 6.5A). In the present experiment a ten-fold higher FaeA1 concentration was used than in the experiment described in §6.3.3.2. The different FaeA1 activities in the two experiments could thus indicate a low FaeA1 activity toward SBPOS. The low levels of ferulic acid release from SBPOS show that FaeA1 and FaeA2 cannot hydrolyze all linkages between ferulic acids and the sugar units
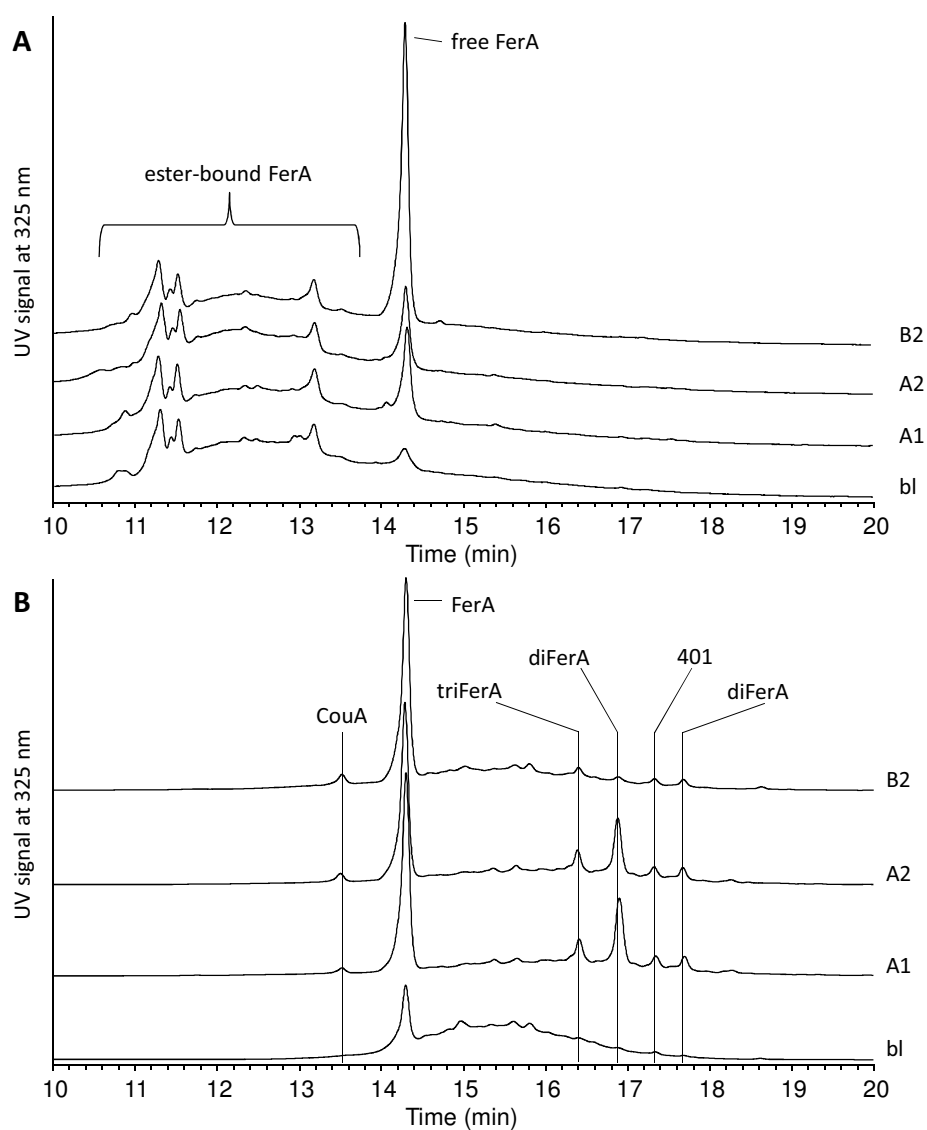

Fig. 6.6: Enzymatic release of hydroxycinnamic acids from sugar-beet and corn oligomers followed by UHLPC analysis upon end-point digestion. A: release of ferulic acid from feruloylated oligosaccharides from sugar-beet pulp; B: release of coumaric acid (CouA), ferulic acid (FerA), dehydrodiferulic acid (diFerA) and triferulic acid (triFerA) from corn oligosaccharides. Bl – substrate blank without enzyme added, A1 - Ferulic acid esterase A1 added, A2 – Ferulic acid esterase A2 added, B2 – Ferulic acid esterase B2 added.

in SBPOS. Type A feruloyl esterases have been described to release ferulic acid linked to O–6-galactose in pectic galactan (Benoit et al., 2008). The ferulic acid released by FaeA1 and FaeA2 could thus derive from feruloylated galactose oligomers. The SBPOS were too large for MALDI-TOF MS analysis, so that no detailed structural characterization was possible within the scope of this research.

The SBPOS contain nearly no dehydrodiferulic acid (no further data shown). Hence, no dehydrodiferulic acid release was observed by the C1 FAEs.

Activity on feruloylated oligomers from corn fibre arabinoxylan

All three ferulic acid esterases were active toward a mixture of corn-fibre arabinoxylan oligomers (CF AXOS, Fig. 6.6B). Besides ferulic acid, all enzymes release some *p*-coumaric acid (Fig. 6.6B, 13.5 min). FaeA1 and FaeA2 also release considerable amounts of ferulic acid dehydrodimers and dehydrotrimers that elute from 16.0–18.5 min, whereas no such release is seen for FaeB2 (Fig. 6.6B).

In order to verify whether the UV signal could be used to quantify the product release, the MS base peak spectra were evaluated (Fig. 6.7). It can be seen that FaeA1 releases, next to *p*-coumaric acid and ferulic acid, four different dehydrodiferulic acids (diFerA-1– 5), three different dehydrotriferulic acids (triFerA-1–3) and three compounds of 402 Da (401-1–3, m/z = 401 in negative mode) that could not be identified (Fig. 6.7B). Not all of the compounds are base-peak separated, so that the UV signal could not be used for quantification (Fig. 6.7A&B).

MS signals obtained for the ferulic acid standard curve showed a linear dependancy on the ferulic acid concentration (no further data shown). Therefore, the MS signals were used, together with MS signals of the saponified CF AXOS, to calculate the hydroxycinnamic acid release (Fig. 6.7C). All FAEs almost completely release all *p*-coumaric acid and ferulic acid present, with the exception of FaeA1 that releases only 80 % of all *p*-coumaric acid present. This reduced release could be due to the lower activity of FaeA1 on methyl coumarate (Table 6.3). FaeA1 and FaeA2 completely release the triFerA-1 isomer and the diFerA-2 isomer, which are most abundant in the corn fibre fraction, whereas only ≈ 50 % or less of the other triFerAs and diFerAs could be released.

FaeB2 shows some activity on CF AXOS that are substituted with dehydrodiferulic acid and dehydrotriferulic acid. It releases small amounts of the diFerA-1 isomer and the triFerA-1 isomer that are minor constituents of CF AXOS (Fig. 6.7B&C). This ability of FaeB2 displays a novel finding among type B ferulic acid esterases.
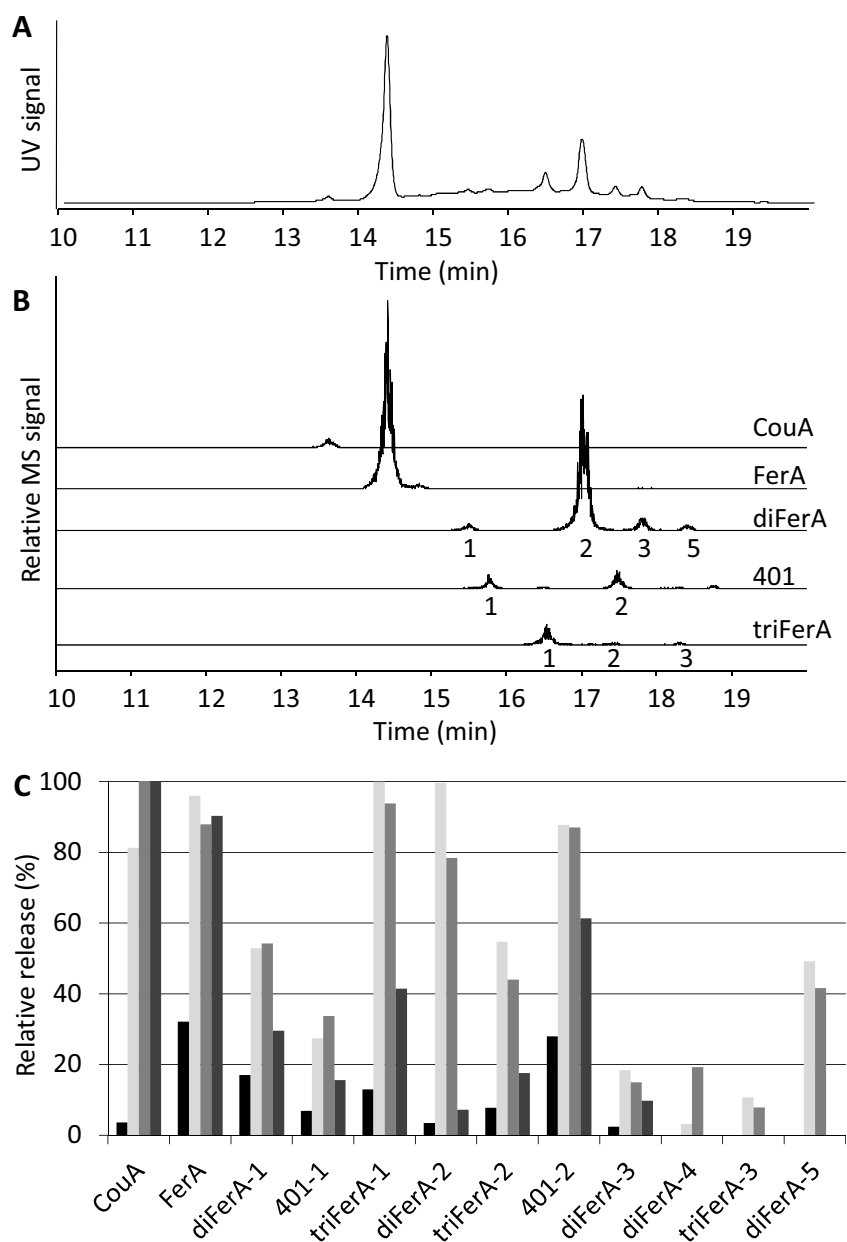
Fig. 6.7: Hydroxycinnamic acid release from CF AXOS by C1 ferulic acid esterases. A – UV elution profile (325 nm) of CFAX digested with FaeA1, B – negative mode, base peak MS elution profiles of CFAX digested with FaeA1: CouA – coumaric acid (m/z = 163), FerA – ferulic acid (m/z = 193), diFerA – dihydrodiferulic acid (m/z = 385), 401 – unknown compound (m/z = 401), triFerA - dehydrotriferulic acids (m/z = 577). C – Quantification of the hydroxycinnamic acid release from CFAX. Relative releases were calculated from the MS elution profiles and the total hydroxycinnamic acid content. Bars: black – blank sample, light grey – FaeA1, grey – FaeA2, dark grey – FaeB2. Isomers of m/z = 401, diFerA and triFerA are annotated according to their elution order.

Different tissues of corn contain different amounts of ferulic acid and *p*-coumaric acid substituents (Eylen et al., 2011). Ferulic acid constituents are the main hydroxycinnamic acid constituents in corn fibre, while corn cobs contains more *p*-coumaric acid substituents (60 %) than ferulic acid substituents (40 %) (Eylen et al., 2011). These differences can explain the different C1 ferulic acid activities determined toward CC AXOS (Fig. 6.5) and CF AXOS.

All FAEs release isomers of an unknown compound of m/z = 401 (402 Da). Based on its mass this compound could be a dehydrodimer composed of ferulic acid and 5-hydroxyferulic acid. 5–Hydroxyferulic acid is involved in the hydroxycinnamic biosynthesis. However, no 5-hydroxyferulic acid (m/z = 209) could be detected during the analysis of saponified CF AXOS, suggesting that this structure is not directly and solely linked to a sugar substituent.

6.3.5 Functional classification of C1 ferulic esterases

FaeA1

FaeA1 has high sequence similarities with feruloyl esterase FaeA from *Penicillium funiculosum*. Phylogenetically, FaeA1 is grouped into subfamily 5 (Hinz et al., 2009), of which no other assigned members have been characterized biochemically.

The activities of the FaeA1 on synthetic and natural substrates are summarized in Table 6.4. FaeA1 prefers methyl sinapinate and methyl ferulate over methyl coumarate and methyl caffeate. It is active toward feruloylated CF AXOS, from which it can release FerA, diFerAs and triFerAs (Fig. 6.7A). Furthermore, FaeA1 can release 20 % of the ferulic acid present in sugar-beet pectin oligomers. The substrate specificity of FaeA1 is very similar to the specificity of type A esterase from *Aspergillus niger* (Kroon et al., 1997) and other fungal type A esterases described (Crepin et al., 2004; Benoit et al., 2008). Based on its activity, FaeA1 is thus annotated as a type A feruloyl esterase.

FaeA2

Like FaeA1, FaeA2 has high sequence similarities with type A esterase FaeA from *Penicillium funiculosum* and is also member of subfamily 5. FaeA2 is most active toward methyl ferulate and methyl coumarate, but far less active toward methyl sinapinate (Table 6.3), which is unusual for type A esterases and fits better with the characteristics of type B feruloyl esterases (Crepin et al., 2004). On the other hand FaeA2 has similar specificities as FaeA1 toward sugar-beet oligomers and corn-fibre oligomers (Fig. 6.6). Hence, it is

concluded that FaeA2 is a type A esterase, which acts like type B ferulic acid esterases on synthetic substrates (A_B). A similar classification was made for type B esterases that acted like type A esterases on synthetic substrates (Koseki et al., 2009).

FaeB2

FaeB2 has high sequence similarities with type B feruloyl esterase from *Neurospora crassa*. Phylogenetically, it is a member of subfamily 6 (Hinz et al., 2009). Other members of this group are *Aspergillus niger* CE family 1 acetyl esterase AceA (Pel et al., 2007), the type B feruloyl esterases FaeB from *Neurospora crassa* (Crepin et al., 2003) and cinnamoyl esterase FAEB from *Penicillium funiculosum* (Kroon et al., 2000; Benoit et al., 2008). FaeB2 is inactive on methyl sinapinate; a pattern that has been described for a number of type B esterases (Crepin et al., 2004). Furthermore, it is active on both arabinoxylan-derived and pectin-derived feruloylated oligomers (Fig. 6.5), which suggests that FaeB2 has a low linkage specificity. Based on the biochemical data it is confirmed that FaeB2 is indeed a type B feruloyl esterase.

Table 6.4: Summary of the substrate specificities of C1 ferulic acid esterases FaeA1, FaeA2 and FaeB2 and their final classification. Activities are indicated from "-" – not active to "+++" – very active.

Activities	FaeA1	FaeA2	FaeB2
methyl caffeate	+	++	+++
methyl coumarate	++	++	++
methyl ferulate	+++	+++	+++
methyl sinapinate	+++	+/-	-
WB oligomers	+++	+/-	+
BGS oligomers	+	+/-	++
CC oligomers	+	+/-	+
CF oligomers	++	++	+
dehydrodiferulic acid release	++	+	+/-
triferulic acid release	++	++	+/-
SBP oligomers	+	+	++
Classification*	type A	type A_B	type B
Subfamily**	SF5	SF5	SF6

*according to Crepin et al. (2004) and Koseki et al. (Koseki et al., 2009),

**according to Benoit et al. (2008), WB – wheat bran, BSG – brewer's

spent grain, CC – corn cobs, CF – corn fibre, SBP – sugar-beet pulp

6.4   Conclusions

Ferulic acid esterases from *Chrysosporium lucknowense* C1 can release a variety of ferulic acids from soluble cell wall material, including more complex oligomeric structures. None of the C1 FAEs is a universally active ferulic acid esterase, so that different types of ferulic acid esterases are required for complete deferuloylation. FaeA1 is suitable for corn fibre and wheat bran deferuloylation, whereas FaeB2 is more suitable for the deferuloylation of pectic biomass. The neutral pH optimum of C1 ferulic acid esterases could limit their applicability in certain biotechnological applications, such as bioethanol production, since the pH of the biomass often decreases during fermentation and pretreatments.

Acknowledgements

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Chapter 7

General discussion

Motivation of the research

sugar-beet pulp (SBP) is a by-product of sugar production in Europe that is rich in carbohydrates, especially pectins. The main monosaccharide constituents are arabinose, glucose, and galacturonic acid. sugar-beet pulp is considered as potential resource of carbohydrates for biorefinery, in particular biofuel production and the production of platform chemicals. Prior to such use the carbohydrates need to be hydrolyzed to their monosaccharide constituents. Harsh hydrolysis conditions are not desired, since they may decompose the biomass, cause large amounts of waste and are energy-demanding.

Enzymatic hydrolysis allows the controlled degradation of biomass under mild process conditions. However, enzymatic hydrolysis rates are in many cases low, because enzymes mixtures comprise not sufficient enzyme activities to degrade complex biomass. Biomass pretreatments are employed to accelerate enzymatic hydrolysis rates. The necessity of such pretreatment for the degradation of SBP was evaluated.

Several fungal strains have been established for the production of cell-wall degrading enzymes. Most of them are either rich in cellulases and hemicellulases or rich in pectinases. The filamentous fungus *Chrysosporium lucknowense* C1 is a well-known producer of hemicellulases and cellulases that also has putative activities toward pectin. Its enzymatic toolbox was investigated for arabinohydrolases and ferulic acid esterases active toward sugar-beet arabinans.

7.1 Pretreatment of SBP

Several mild pretreatments of SBP were performed. An aqueous pretreatment at 140 °C for 15 min was the optimal pretreatment to support enzymatic hydrolysis of cellulose. Higher severities lead to the destruction of solubilized sugars to furfural, hydroxy methyl furfural, acetic acid and formic acid. The pretreated samples were successfully hydrolyzed with an experimental cellulase preparation from the filamentous fungus *Chrysosporium lucknowense* C1 (C1). Compared to earlier saccharification studies (Table 1.3) cellulose degradation required four times less cellulase activities. The aqueous pretreatment did not cause any additional waste streams and did not lead to a considerable loss of biomass. The pretreatment can also be seen as a sterilization step to prevent unwanted microbial growth and mixed fermentation products.

7.1.1 Stability of pectin during pretreatment

Chemical decomposition of galacturonic acid and arabinose constituents was observed during the 170 °C pretreatments of SBP (Fig. 2.3). A pressure increase was observed, going along with an overall loss of biomass in both, supernatant and pellet fraction (Fig. S2.1). The pressure increase could be caused by the decarboxylation of galacturonic acid constituents and would explain 4 w/w % of the total biomass loss. The decarboxylation of galacturonic acid is an endothermic reaction (eq. 7.1) that yields carbon dioxide and arabinose. The thermal decarboxylation of pectin has been reported in the presence of heavy metal ions (Zweifel and Deuel, 1956). The Gibbs free energy, however, is -45.8 kJ/mol, so that the reaction is entropy-driven exergonic and decarboxylation might occur, especially at elevated tempertatures.

$$C_6H_{10}O_7 \xrightarrow{\Delta H_R = 57.6 \text{ kJ/mol}} C_5H_{10}O_5 + CO_2 \tag{7.1}$$
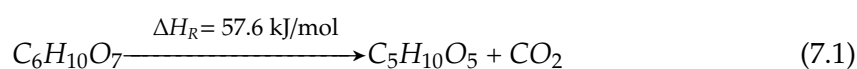
As discussed earlier, the chemical decomposition of SBP lead to a mass loss of 12 % (Fig. 2.2) while no formation of arabinose has been observed during pretreatments. The mass loss can be explained by the further decomposition of the decarboxylated galacturonic acid (Einhorn-Stoll and Kunzek, 2009) and by the decomposition of arabinose to furfural (Fig. 2.3). The addition of heavy metal ions might prevent decomposition of arabinose during the 170 °C pretreatments, but it would also cause additional waste. Therefore, temperatures of 170 °C are not suitable for the pretreatment of sugar-beet pulp.

7.1.2 Energy balance for the pretreatment

Pretreatment of SBP can improve the performance of enzymes toward the degradation of SBP (Chapter 2). However, thermal pretreatments are energy-consuming and negatively effect the greenhouse-gas (GHG) emission savings of bioethanol from SBP. The saccharose refinery process itself is very energy-demanding and produces heat that could be partly used for the pretreatment and to heat the fermentors. Saccharose extraction occurs at 70 °C, so that the pulp needs to be heated by 70 °C to reach the optimal pretreatment temperature of 140 °C. In an ideal case ≈ 300 kJ of energy are required to heat 1 l of water by 70 °C (70×4.184 kJ/cal).

Under the pretreatment conditions used in Chapter 2 (5 w/w % SBP, thereof 70 % carbohydrates), 35 g monosaccharide constituents per litre wet SBP are theoretically available for fermentation. Assuming a hydrolysis efficiency of 90 % and an ethanol fermentation efficiency of 90 %, 28.4 g of monosaccharide constituents (≈ 0.18 mol) per litre wet SBP will be fermented. As described in detail in §7.3, ethanol yields are lower than 2 mol ethanol/mol sugar constituent. Considering the fermentation of 1 mol SBP sugar constituents into 1.8 mol ethanol, 0.32 mol ethanol (1.5 w/v %) with an energy content of 447 kJ ($\Delta H_{C,EtOH} = -1380$ kJ/mol, Table 7.4) are produced per litre of wet SBP. This heating scenario requires $\frac{2}{3}$ of the energy of the final product for the pretreatment.

Ethanol concentrations ≥ 5 w/v % are desired to make fermentation and distillation economically feasible (Vane, 2008). Thus, SBP concentrations of 15 w/w % would be required. This would further implicate that, compared to 5 w/w % SBP, three times the amount of ethanol (0.96 mol) is produced from 1 l wet SBP, reducing the energy impact of the pretreatment to ≈ 22 % of the energy content of the final product.

Sugar-beet pulp is not stirrable or pumpable at concentrations ≥ 10 %, so that other pretreatment techniques are necessary, e.g. extrusion (Rouilly et al., 2006). Even though economically unfavorable, no pretreatment and longer incubation times at higher enzyme dosages could favor the energy efficiency of the process.

Before ethanol can be used as biofuel, it has to be distilled and dehydrated to yield concentrations of 99.5 v/v %. An optimized, heat-integrated process with 5 v/v % ethanol starting concentrations could have an overall energy demand of ≈ 4500 kJ/kg ethanol (Vane, 2008), which equals an energy demand of 207 kJ/mol ethanol or 15 % of the energy content of the ethanol derived from 15 w/w % SBP.

From these basic calculations it can be seen that several energy-demanding processes are involved in the production of fuel ethanol that consume at least 37 % of the energy

available in the ethanol. An optimized overall process design is, hence, crucial for the efficiency of the process.

7.2   C1 arabinan degrading enzymes

A whole array of enzyme activities is required when aiming the utilization of all monosaccharide constituents of SBP (§1.3.7 and §1.4). Arabinan represents ≈ 20 w/w % of SBP. The enzymatic degradation of sugar-beet arabinan depends on endoarabinanase and arabinofuranosidase activities. C1 potentially encodes for 16 arabinohydrolases (Table 7.1), of which one endoarabinanase (Abn1), one exoarabinanase (Abn2) and two arabinofuranosidases (Abn4 and Abf3) have been characterized in the Chapters 3 and 5 of this thesis.

The combination of four arabinohydrolases hydrolyzed up to 80 % of sugar-beet arabinan into arabinose monomers, whereas a combination of Abn1, Abn2 and Abn4 predominantly released arabinose oligomers. These oligomers were isolated and identified as linear and branched arabinose oligomers. Branched oligomers released upon partial and end-point digestion of sugar-beet arabinan were used to determine the mode of action of the four C1 arabinohydrolases.

Abn1 is predominantly active toward linear α-1,5-linked arabinan, although it is active toward branched arabinan as well. Abn1 can hydrolyze the α-1,5-linked arabinan backbone in close vicinity of a double substituted arabinose and requires not more than three consecutive unsubstituted backbone arabinosyl residues.

Abn2 releases arabinobiose from the non-reducing ends of linear α-1,5-linked arabinan. Next to arabinobiose, Abn2 releases small quantities (<1 %) of an arabinotriose isomer of unknown structure.

Abn4 removes α-1,3-linked, and most likely also α-1,2-linked, arabinose moieties from single substituted arabinosyl residues of the arabinan backbone, but not from double substituted arabinosyl residues. It acts synergistically with Abn1 on the degradation of branched arabinan.

Abf3 is most active toward arabinose oligomers and almost inactive toward the arabinan polymer. Abf3 hydrolyzes all linkages present in branched arabinose oligomers and also releases arabinose monomers from double substituted arabinosyl residues of the arabinan backbone .

The activities of Abn1, Abn4 and Abf3 are essential for the release of fermentable monosaccharides; Abn1 and Abn4 activities for polymer degradation and Abf3 activity

for the hydrolysis of the released oligomers. The arabinohydrolases released only 80 % of the arabinose present in sugar-beet arabinan. This might be explained with the relatively high RG I content of sugar-beet arabinan. The arabinohydrolases could be structurally hindered by other RG I substructures.

7.2.1 Specificity of Abn4

Compared to other fungal arabinofuranosidases Abn4 has a high substrate specificity. Abf B from *Aspergillus niger* and *Aspergillus aculeatus* are active toward polymers and oligomers. They release arabinose from single and double substituted arabinosyl residues of the backbone (Rombouts et al., 1988; Beldman et al., 1993). The degradation of sugar-beet arabinan to monomers would, thus, require only one *Aspergillus niger* or *Aspergillus aculeatus* Abf B, but two C1 arabinofuranosidases.

Detailed knowlegde of Abn4 specificity allows the directed production of branched arabinose oligomers. Prebiotic effects have been claimed for linear arabinose oligomers (Van Laere and Wissing, 2002). Linear arabinose oligomers also stimulated growth of *Clostridia* (Van Laere et al., 2000), which are generally considered as potentially harmful (Gibson and Roberfroid, 1995). Double substituted branched arabinose oligomers could more selectively stimulate the growth of *Bifidobacteria* which are able to utilize branched cell-wall derived oligosaccharides (Van Laere et al., 2000; Van Laere and Wissing, 2002). Preliminary data indeed indicate biological activity of branched arabinose oligomers (personal communication Arata Yoneda, RIKEN, Yokohama, Japan).

It is uncertain whether the specificities of arabinofuranosidase Abn4 are unique or whether branched arabinose oligomers were not detected previously. We showed in Chapter 4 that some of the branched arabinose oligomers co-elute with linear arabinose oligomers when using HPAEC, whereas they could be separated by porous graphitized carbon column chromatography (Westphal et al., 2010). It is, therefore, advisable to use multiple analytical techniques, even when reference compounds are available.

7.2.2 Classification of arabinohydrolases

Arabinohydrolases have been functionally and structurally classified. The functional classification of arabinohydrolases is based on different substrate specificities (Abf A and B, AXH and bifunctional arabinofuranosidases/β-xylosidases; Beldman et al. 1996) and modes of action (endoarabinanase and exoarabinanase).

Functional classification of arabinohydrolases

As explained in the previous section, the substrate specificities of arabinofuranosidases can be quite distinct, which is not always reflected in the current nomenclature.

Abf A and Abf B substrate specificities vary within the same subtype (Table 1.1). *Aspergillus niger* Abf A acts toward arabinose oligomers, whereas *Aspergillus kawachii* Abf A acts toward arabinoxylan polymers. Similarily, arabinan-specific and arabinoxylan-specific B-type Abf's exist (Table 1.1). Moreover, AXHs can also have activities toward branched arabinan (Van den Broek et al., 2005). Bifunctional arabinofuranosidases/β-xylosidases should be termed unspecific rather then bifunctional. For example, C1 Abf3 (Abf A) showed minor activities toward *p*NP-xylopyranoside (unpublished results). The subdivision into the different subtypes is, therefore, sometimes confusing and ambiguous.

It would be most helpful to classify the arabinofuranosidases based on linkage specificities, but such data is not available for the majority of the described enzymes. Therefore, it is suggested to use the term Abf A for enzymes with preference toward linear arabinan and/or linear arabinose oligomers, whereas Abf B should comprise all other enzymes with broader linkage and substrate specificities. The term AXH should be abolished or limited to enzymes solely acting toward arabinoxylan.

The term exoarabinanase is currently used for GH family 93 arabinohydrolases that release α-1,5-linked arabinose oligomers (`http://www.cazy.org/GH93.html`). It is sometimes also used for arabinofuranosidases, that mainly act toward α-1,5-linked arabinose moieties (Wong et al., 2008; Lim et al., 2011). We suggest to limit the term exoarabinanase strictly to enzymes with an exo-mode of action that specifically release α-1,5-linked arabinose or α-1,5-linked arabinose oligomers from arabinan.

Structure-based classification

The structural classification is based on amino-acid sequence similarity (Coutinho and Henrissat, 1999). The mode of action of an enzyme is often related with its structure. All endoarabinanases are, for instance, members of the GH family 43 and most exoarabinanases belong to GH family 93 (Table 7.1, Coutinho and Henrissat 1999). The different arabinofuranosidases are generally predominantly present in one GH family, but they are structurally more diverse than arabinanases (Table 7.1). In conclusion, the structural classification not always allows to deduce the mode of action or substrate specificity of an enzyme. It is, thus, not suitable as exclusive classification system.

Table 7.1: Classification of arabinohydrolases in CAZy GH families

Mode of action/ substrate specificity	GH family*
Endoarabinanase	**43**
Exoarabinanase	**93**, 43
Abf A	**51**, 54, 43, 62
Abf B	**54**, 43, 62, 51
AXH	**62**, 54, 51, 43
Abf/Xyl	**3**, 43, 62, 51, 54

*most abundant in bold-typed GH family. All data from the CAZy
database (`www.cazy.org`)

7.3   C1 ferulic acid esterases

The complete enzymatic hydrolysis of sugar-beet pulp is prevented by ferulic acid residues
that are O–2-linked to arabinosyl residues and O–6-linked to galactosyl residues of sugar-
beet pectins. The genome *Chrysporium lucknowense* putatively encodes six ferulic acid
esterases, of which three were purified and characterized as two type A feruloyl esterases
and one type B feruloyl esterase. All three ferulic acid esterases completely released
p-coumaric acid and ferulic acid from corn fibre arabinoxylan oligomers, but only FaeA1
and FaeA2 could release major amounts of complex ferulic acid oligomers. FaeA1 and
FaeA2 released 20 % of all ferulic acid from sugar beet pectin oligomers (SBPOS), while
FaeB2 released 60 %. Type A esterases are known to release ferulic acid O–5-linked to
arabinose in arabinoxylans and O–6-linked to galactose in sugar beet pectin (Crepin et al.,
2004). Assuming that FaeA1 and FaeA2 are true type A feruloyl esterases, they might
release ferulic acid that is O–6-linked to galactose moieties of SBPOS.

Type B esterases are more universal, as they hydrolyze all linkages between ferulic
acid monomers and SBPOS. As type B feruloyl esterase FaeB2 should have been able to
remove all ferulic acid from SBPOS. Since FaeB2 could only release 60 % of all ferulic
acid in SBPOS, they may have another specificity than common type B feruloyl esterases.
Alternatively, the structure of the SBPOS could have prevented complete deferuloylation:
SBPOS were too large for MALDI-TOF analysis and too complex to be further degraded
by Abn4 and Abn1. This suggests a highly branched structure, in which the feruloyl
residues might be less accessible to FaeB2. The partial release of ferulic acid from SBPOS
could, hence, reflect insufficient Abf activity rather than high ferulic acid specificity.

7.3.1   Classification and nomenclature of ferulic acid esterases

Relatively little is known about ferulic acid esterases and their substrate specificities. FAE's were classified in suptypes A–D based on their activities toward synthetic substrates (Crepin et al., 2004), but some FAE's have different activities toward natural substrates (Table 6.4; Koseki et al. 2009). Some FAE's are active toward (insoluble) polymers and this activity is usually linked to the presence of a carbohydrate binding module (Kroon et al., 2000; Koseki et al., 2009).

The CAZy database only assigns FAE's with homology to CE family 1 acetyl-xylan esterases, whereas the majority of the ferulic acid esterases appears not in the CAZy database. Some bifunctional β-xylosidase/ferulic acid esterases are assigned as glycoside hydrolases (`http://www.cazy.org/GH10.html`). The CBMs of some FAE's are assigned to CBM families 1, 2, 6 and 22 with binding affinities toward cellulose, xylans or glucans (`http://www.cazy.org/~CBM`).

The phylogenetic classification approach assigns a variety of putative FAE's to seven subfamilies (Benoit et al., 2008). The members of one subfamily are sometimes of the same subtype based on the biochemical classification by Crepin et al. (2004), but biochemical data is lacking for most of the FAE's.

In conclusion, it can be stated that FAE's are difficult to classify. None of the three classification approaches allows the comprehensive, unambiguous classification of feruloyl esterases. We suggest a classification system based on the linkage specificities of ferulic acid esterases toward natural substrates. The subtype classification of Crepin et al. (2004) coincidently distinguishes linkage specificities as well. Its modification to a linkage-specificity based classification system would also avoid further sub-classification into A_B and B_A sub-subtypes in order to explain all experimental data (Table 6.4, Koseki et al. 2009).

7.4   Potential and performance of *Chrysosporium lucknowense* C1 in biomass saccharification

Fungi are a good source of cell-wall degrading enzymes. Enzyme preparations of the *Aspergillus* genera are well-known for their pectinolytic enzymes, whereas *Trichoderma reesei* and *Talaromyces emersonii* are strong cellulase producers. The complete enzymatic degradation of type II plant cell walls may require the mixture of enzymes from different origins (Table 1.3). The potential of *Chrysosporium lucknowense* C1 to be a universal

source for cellulose, hemicellulose and pectin degrading enzymes was compared with the potential of *Aspergillus niger* and *Trichoderma reesei* (Table 7.2). All fungi encode for a large number of cellulose-degrading enzymes, while *Chrysosporium lucknowense* C1 and *Aspergillus niger* encode for more hemicellulases than *Trichoderma reesei*. In absolute numbers, *Aspergillus niger* is the richest source of annotated cell-wall degrading enzymes, followed by *Chrysosporium lucknowense* C1 and *Trichoderma reesei*.

Table 7.2: Putative cell-wall degrading enzyme activities of selected fungi.

Putative acitvity	C1*	T. reesei**	A. niger***
Arabinofuranosidases/arabinanases/β-xylosidases	16	5	14
Cellulases	21	15	18
Cellobiases	1	3	3
α-ʟ-Fucosidases	0	4	3
α-Galactosidases	3	8	6
β-Galactosidases	6	2	11
Galactanases	1	-	1
GH61 glucanases	26	9	7
β-1,3-Glucanases	12	3	10
Glucanases/galactanases/galactosidase	8	11	13
Glucosidases/β-xylosidases	25	21	41
α-Glucuronidase	2	1	1
Mannanases/mannosidases	21	9	23
Polygalacturonases	2	4	21
Pectin/pectate lyases	6	-	8
Rhamnogalacturonan lyases	1	-	2
α-Rhamnosidases	1	-	8
Xylanases	11	5	5
Xyloglucanase	1	1	1
Acetyl xylan esterases	10	4	16
Ferulic acid esterases/acetyl xylan esterases (CE fam. 1)	9	-	3
Pectin methyl esterases	1	-	3
Rhamnogalacturonanacetyl esterases	2	-	2
SUM	186	105[1]	218

Database analysis performed by Sandra Hinz (Dyadic Netherlands). [1]54 genes of *Trichoderma reesei* are putative glycoside hydrolases of unknown function, *from C1 sequencing and annotation data (Dyadic), **from the GJI database, ***from the CAZy database

7.4.1 Cellulose degradation

C1 potentially encodes for 24 cellulases, 17 endoglucanases / cellobiohydrolases and 11 β-glucosidases / β-xylosidases (Hinz et al., 2009). Of these 55 enzymes, six endoglucanases and four cellobiohydrolases were isolated and characterized (Bukhtojarov et al., 2004; Gusakov et al., 2007). Among the glucanases, all are active toward β-glucan and carboxymethyl cellulose, but only two are also active toward xyloglucan (Bukhtojarov et al., 2004). All enzymes are stable up to 60 °C, some even up to 70 °C, and have optimal activities pH between pH 5.0–5.5 (Bukhtojarov et al., 2004; Gusakov et al., 2005). Different combinations of C1 endoglucanases and cellobiohydrolases hydrolyzed up to 94 % of cotton cellulose, while a more complex enzyme preparation even yielded 98.6 % of the glucose present in cotton cellulose (Gusakov et al., 2007). The complex enzyme preparations could also hydrolyze more than 90 % of pretreated fir-wood cellulose (Gusakov et al., 2007).

7.4.2 Hemicellulose degradation

Arabinoxylan degrading enzymes

In total 82 genes have been identified in C1 that are potentially involved in xylan degradation, among them 11 xylanases, 13 acetyl xylan and/or ferulic acid esterases and 52 exoglycosidases like arbinofuranosidases, galactosidases, glucuronidases and xylosidases (Hinz et al., 2009).

Six C1 endoxylanases (three GH family 10 and three GH family 11) were characterized (Ustinov et al., 2008). Most of them are active between pH 5.0–7.0 and thermostable up to 60 °C.

Abf1 and Abf2 are arabinoxylan-specific arabinofuranosidases from GH family 62. They both release α-1,2-linked and α-1,3-linked arabinose units from single substituted xylosyl residues of arabinoxylan oligomers (AXOS) (Hinz et al., 2009). Both enzymes are most active at pH 5.0 and 40 °C. Abn7 is a GH family 43 arabinofuranosidase active toward the α-1,3-linked arabinose unit of double substituted xylosyl residues in arabinoxylan oligomers (Pouvreau et al., 2011). Abn7 is most active at pH 5.0 and 40 °C (Pouvreau et al., 2011).

The α-glucuronidase Agu1 (GH family 67) releases 4-O-methyl-D-glucuronic acid from the non-reducing ends of wheat-bran AXOS (Hinz et al., 2009).

The acetyl xylan esterases Axe2 (CE family 1) and Axe3 (CE family 5) release all acetyl groups from acetylated AXOS except the one at the non-reducing end (Hinz et al., 2009).

Xyloglucan degrading enzymes

Two endoglucananases were isolated and characterized from C1 that were active toward xyloglucan (Bukhtojarov et al., 2004; Grishutin et al., 2004). Furthermore, C1 putatively encodes for 21 xylosidases, 7 galactosidases, 9 endoglucanases and 9 xyloglucanases (Hinz et al., 2009), which makes C1 a promising source for xyloglucan-degrading enzymes.

7.4.3   Pectin degradation

Galactan degrading enzymes

C1 encodes for six putative β-galactosidases, eight galactosidases/glucanases and at least one endogalactanase (Gal1). Crude overexpressed Gal1 fermentation liquids were tested toward pototo galactan (Fig. 7.1). The enzyme mixture could rapidly decrease the average molecular mass of potato galactan and released 50 % of the sugar constituents as monomers and oligomers up to DP 4 upon endpoint digestion (no further data shown). Gal1 was active at 40 °C and pH 5.0.

Crude overexpressed fermentation liquids of the β-galactosidase Bga2 was positively tested toward potato galactan as well. The oligomers formed by Gal1 could be degraded by Bga2 that is active under the same conditions (no further data shown). Hence, Gal1
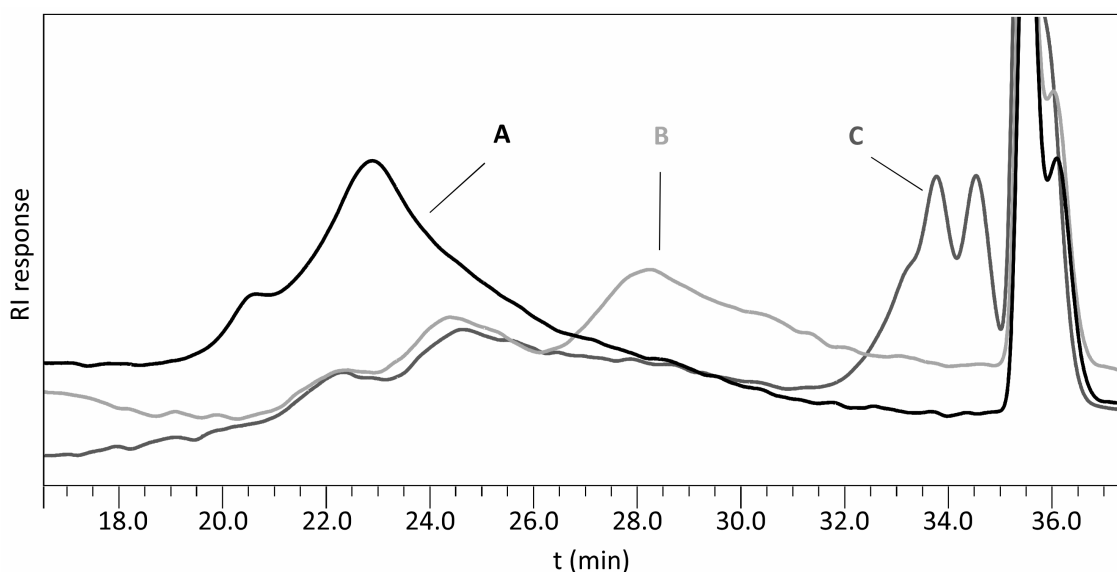

Fig. 7.1: HPSEC elution pattern of potato galactan before (A) and after 1 h (B) or 24 h (C) digestion with Gal1.

and Bga2 can be used together with accessory enzymes to completely degrade sugar-beet galactan and arabinogalactan I.

Galacturonan degrading enzymes

Chrysosporium lucknowense C1 is not a major polygalacturonase producer (Table 7.1). The genome of C1 encodes for only two polygalacturonases, both being exopolygalacturonases. C1 probably degrades polygalacturonan and rhamnogalacturonan with lyases. Most lyases have an endo-mode of action and could act together with exopolygalacturonanases on the degradation of homogalacturonan or with α-rhamnosidases on the degradation of RG I. C1 encodes for seven potential lyases, six pectin or pectate lyases and one rhamnogalacturonan lyase (Table 7.1).

Furthermore, C1 encodes for two rhamnogalacturonan acetyl esterases (RGAEs) and one pectin methyl esterase (PME1). Rhamnogalacturonan acetyl esterase RGA1 and
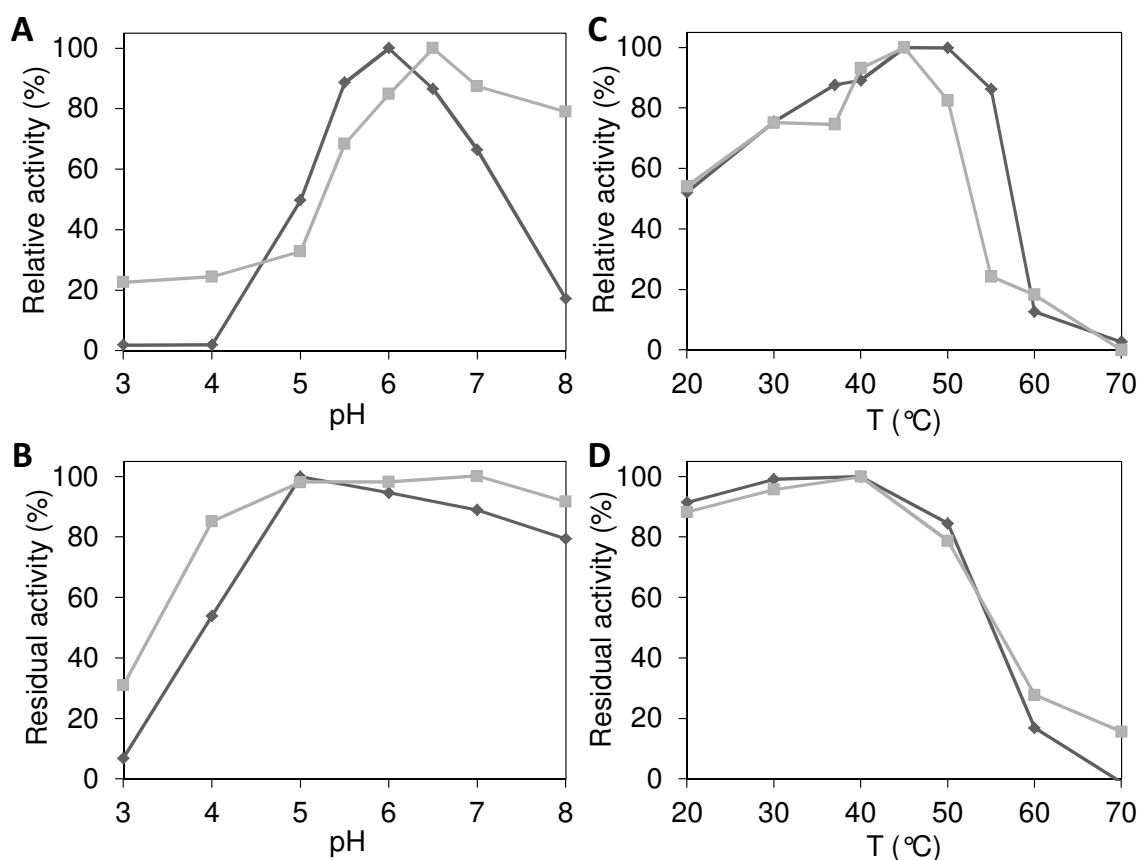

Fig. 7.2: Biochemical properties of PME1 and RGA1. Dark gray – PME1, light gray – RGA1. pH optima (A) and stabilities (B) were determined at 50 °C (PME1) and 45 °C (RGA1). Temperature optima (C) and stabilities (D) were determined at pH 6.0 (PME1) and pH 6.5 (RGA1).

PME1 were purified and preliminary characterized (Fig. 7.2 and 7.3). PME1 is most active between pH 5.5–6.5 and has 50 % activity at pH 5.0 (determined toward DM 70 commercial lemon pectin), whereas RGA1 is most active at pH 6.5 and has 30 % activity at pH 5.0 (determined toward modified apple rhamnogalacturonan, Schols et al. 1990). Both enzymes are stable between pH 5.0–8.0 and 40 °C.

PME1 could strongly increase *Aspergillus niger* polygalacturonase activity toward highly methyl-esterified lemon pectin (Fig. 7.3). After end-point digestion, 60 % of the galacturonic acid substituents could be recovered as monomers, dimers and trimers (no further data shown). In C1, PME1 activity might be essential for pectate lyase activities. PME1 was much less active toward sugar-beet pectin, which indicates that it is hindered by the acetyl groups present.
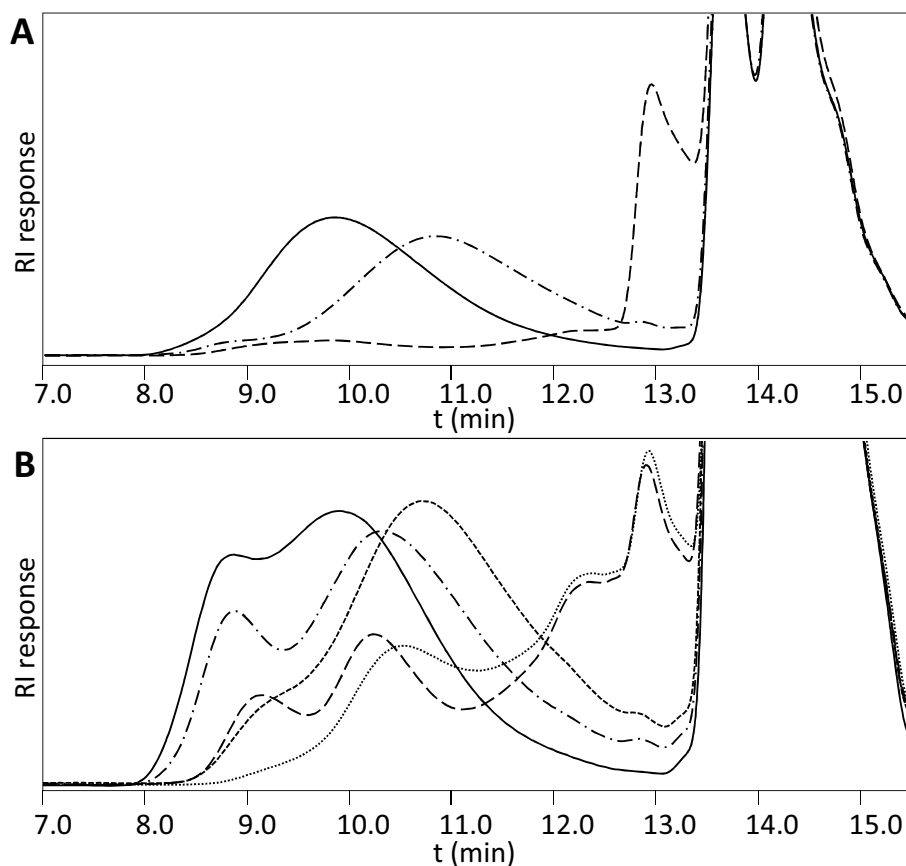

Fig. 7.3: HPSEC elution profiles of pectins digested with endopolygalacturonase and the influence of PME1 and RGA1 on their digestibility. A – high methoxylated lemon pectin (DM 70), B – Sugar beet pectin (DM 57, DA 24). Straight line: substrate blank, dashed & dotted line: substrate digested with *Aspergillus niger* endoPG II, long dashed line: endoPG II & PME1, short dashed line: endoPG II & RGA1, dotted line: endoPG II, PME1 & RGA1.

RGA1 released 42 % of the acetyl groups present in modified apple rhamnogalacturo-nan, which suggests that it is either active on O–2-linked or on O–3-linked acetyl residues (no further data shown). RGA1 was almost inactive toward sugar-beet pectin. It released 3 % of all acetyl groups present (no further data shown). It is, therefore, concluded that RGA1 indeed is a RGAE that is not active toward homogalacturonan. These results are different from previous findings, showing that *Aspergillus aculeatus* RGAE is active toward homogalacturonan, as well (Bonnin et al., 2008).

7.4.4   Conclusions on the performance of C1

Chrysosporium lucknowense C1 is a good source for cellulases, xylanases and xyloglucanases. Most of these enzymes are thermostable up to 60 °C, some even up to 70 °C, and active between pH 5–7. Combinations of C1 cellulases and hemicellulases can be employed to degrade the cellulose/hemicellulose network of type I and type II cell walls. C1 also produces a number of galactan-degrading and arabinan-degrading enzymes active toward the neutral-sugar side chains of pectins. In contrast, C1 encodes for only few galacturonan-degrading enzymes and no endopolygalacturonase activity has been detected. C1 is, therefore, generally less suitable for the degradation of pectin-rich type II cell walls.

7.5   Sugar-beet pulp suitability for bioethanol production

Ethanol production from biomass involves a fermentation step, in which yeasts or bacteria anaerobically convert sugar into ethanol. Common yeasts can only ferment hexoses. Engineered strains have been established that are also able to ferment pentoses (Kuyper et al., 2004; Wisselink et al., 2007; Bettiga et al., 2009). Galacturonic acid fermentation has only been described in bacteria (Doran et al., 2000). This section evaluates the energy losses during fermentation of hexoses, pentoses and uronic acids. Therefore, the reaction enthalpies and Gibbs free energies of each fermentation are calculated from the corresponding standard enthalpies of formation (Table 7.3).

Hexose fermentation

Glucose is the preferred substrate for common and engineered yeasts. During anaerobic fermentation, glucose is converted into two molecules of ethanol and two molecules of CO_2 (eq. 7.2). The Gibbs free energy for the conversion of glucose to ethanol is -208.1 kJ/mol glucose, which equals an energy loss of 7.4 % during fermentation (Table

Table 7.3: Enthalpies of formation and Gibbs free energies of formation for compounds involved in the fermentation of sugar-beet pulp.

Compound name	$\Delta_f H^0$ (kJ/mol)	$\Delta_f G^0$ (kJ/mol)
Glucose[1] (aq.)	-1263.2	-915.9
Arabinose[1] (aq.)	-1043.8	-742.2
Galacturonic acid[2] (aq.)	-1494.4	-1090.4
Water[3] (liquid)	-285.0	-237.0
Water[3] (gaseous)	-242.0	-229.0
Carbon dioxide[3] (gaseous)	-393.0	-393.0
Ethanol[3] (liquid)	-278.3	-168.0*
Acetic acid[3] (liquid)	-486.2	-377.0*

[1]values from Goldberg and Tewari 1989, [2]values from Chin and Janssen 2002,
[3]values from Becker et al. 1996, aq. – aqueous solution, *gaseous phase values

7.4). Based on the combustion enthalpies of glucose and ethanol, however, only 1.54 % of the energy is lost during fermentation (Table 7.4). Hence, glucose fermentation is very efficient, theoretically preserving more than 98 % of the energy content available upon combustion.

$$ C_6H_{12}O_6 \quad \xrightarrow{\Delta G_R = \text{-208.1 kJ/mol}} \quad 2C_2H_5OH + 2CO_2 \tag{7.2} $$
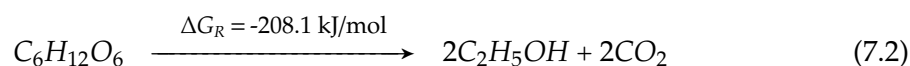
Pentose fermentation

During pentose fermentation, three pentose molecules are converted into five molecules of ethanol. The fermentation of arabinose has a Gibbs free energy of -194.5 kJ/mol and releases $\frac{5}{3}$ mol ethanol/mol arabinose (eq. 7.3).

$$ C_5H_{10}O_5 \quad \xrightarrow{\Delta G_R = \text{-194.5 kJ/mol}} \quad \frac{5}{3}C_2H_5OH + \frac{5}{3}CO_2 \tag{7.3} $$
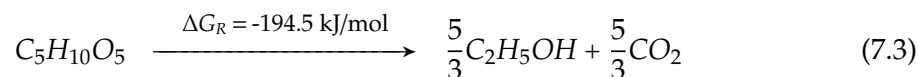
Similar to hexose fermentation, 8.2 % of the energy present in arabinose is used by the yeast (7.4). Based on the combustion enthalpies of arabinose and ethanol, 98.3 % of the energy of glucose is theoretically preserved in ethanol. Pentose fermentation is, thus, as efficient as hexose fermentation.

Galacturonic acid fermentation

Bacteria and fungi have different pathways for the utilisation of galacturonic acid. Assuming a conversion of 1 mol galacturonic acid into 2 mol ethanol (eq. 7.4), the fermentation is endergonic with a Gibbs free energy of 195.4 kJ/mol (Table 7.4). The energy is delivered in the form of two NAD(P)H molecules, which have to be generated by the micro-organism. Because of the endergonic energy balance, galacturonic acid fermentation to ethanol is not likely to occur at high efficiencies under anaerobic conditions.

$$C_6H_{10}O_7 + 2NADPH + 2H^+ \longrightarrow 2NADP^+ + 2C_2H_5OH + 2CO_2 + H_2O \qquad (7.4)$$

In bacteria, anaerobic galacturonic acid fermentation often yields acetic acid and ethanol (Doran et al., 2000). The mixed fermentation of galacturonic acid to ethanol and acetic acid is exergonic with a Gibbs free energy of -242.6 kJ/mol (eq. 7.5), preserving 58 % of the combustion energy of galacturonic acid (Table 7.4).

$$C_6H_{10}O_7 \xrightarrow{\Delta G_R = -242.6 \text{ kJ/mol}} CH_3COOH + C_2H_5OH + 2CO_2 \qquad (7.5)$$

Table 7.4: Reaction enthalpies, Gibbs free energies and combustion enthalpies of the fermentations of glucose, arabinose and galacturonic acid.

		Sugar (kJ/mol)	Ethanol (kJ/mol sugar)	Δ (kJ/mol)	Δ (%)
Glucose	ΔH_R	-2546.8	-2467.4	-79.4	-3.1
	ΔG_R	-2822.1	-2614.0	-208.1	-7.4
	ΔH_C	-2803	-2760	-43	-1.5
Arabinose	ΔH_R	-2131.2	-2056.2	-75.0	-3.5
	ΔG_R	-2372.8	-2178.3	-194.5	-8.2
	ΔH_C	-2338.8	-2300	-38.8	-1.7
Galacturonic acid	ΔH_R	-2073.6	-2467.4	393.8	19.0
	ΔG_R	-2418.6	-2614.0	195.4	8.1
	ΔH_C	-2383	-2760	347	14.6
Galacturonic acid mixed fermentaion	ΔH_R	-2073.6	-2017.5*	-56.1	-2.7
	ΔG_R	-2418.6	-2176.0*	-242.6	-10.0
	ΔH_C	-2383	-2254.5*	-128.5	-5.4

*oxidation enthalpies of ethanol and acetic acid (kJ/mol saccharide constituent. Combustion enthalpies are from Domalski (1972) and Vane (2008) except for galacturonic acid, which was calculated: http://home.fuse.net/clymer/rq/index.html)

In fungi, galacturonic acid is fermented to ethanol and glycerol (Richard and Hilditch, 2009; Hilditch, 2010), resulting in a similar energy balance as the bacterial mixed fermentation (eq. 7.5).

Common yeasts are not able to ferment galacturonic acid. Genetic engineering of yeasts could be one option to overcome this limitation, e.g. by the implementation of other pathways. Moderate levels of galacturonic acid (≈ 5 w/w %) could consume reduction equivalents produced at high glucose fermentation rates and, thus, prevent glycerol production (Wang et al., 2001).

At galacturonic acid concentrations of 20 w/w %, however, the regeneration of reduction equivalents and the toxic effect of galacturonic acid on yeasts (Palmqvist and Hahn-Hägerdal, 2000) remain major problems that make the the commercial fermentation of galacturonic acid by yeasts challenging.

7.6 Use of sugar-beet pulp in biorefinery

Sugar-beet pulp and molasse are the main by-products of the sugar production in Europe. Both are considered as raw material for biorefinery (Fig. 7.4). Prior to such use, SBP needs to be saccharified. As discussed in §7.1, saccharification usually involves a physical pretreatment followed by enzymatic degradation. The resulting sugar solution can be either used for bioethanol production or for the production of biobased products.

The fermentation efficiencies of glucose, arabinose and galacturonic acid were discussed in the previous section. Maximal theoretical ethanol yields from galacturonic acid that are thermodynamically feasible would be close to those of pentose fermentation. Assuming a saccharification efficiency of 90 % and a fermentation efficiency of 90 %, a production of 1.7 Mt bioethanol would be possible from 5 Mt dry SBP that is annually available. Depending on the energy demand of pretreatment and distillation, at least 15–37 % of the energy of bioethanol is consumed during production. In the EU-27 countries, 367.6 Mt of oil equivalent (Mtoe) are yearly consumed as transport fuels, thereof 81.9 % for road transport (eurostat database). Bioethanol from SBP could cover not more than 0.28 % of the total transport energy consumption, and could replace ≈ 0.40 % of the road transport fuels (1 toe = 41.868 GJ, 1 t bioethanol = 30 GJ). The potential of bioethanol from SBP to partly substitute fossils fuels is, thus, negligible.

SBP could be of interest as raw material for a number of biobased products. Arabinose oligomers and galacturonic acid oligomers can be used as prebiotic food additives (§2.3.6 and 7.1.2).
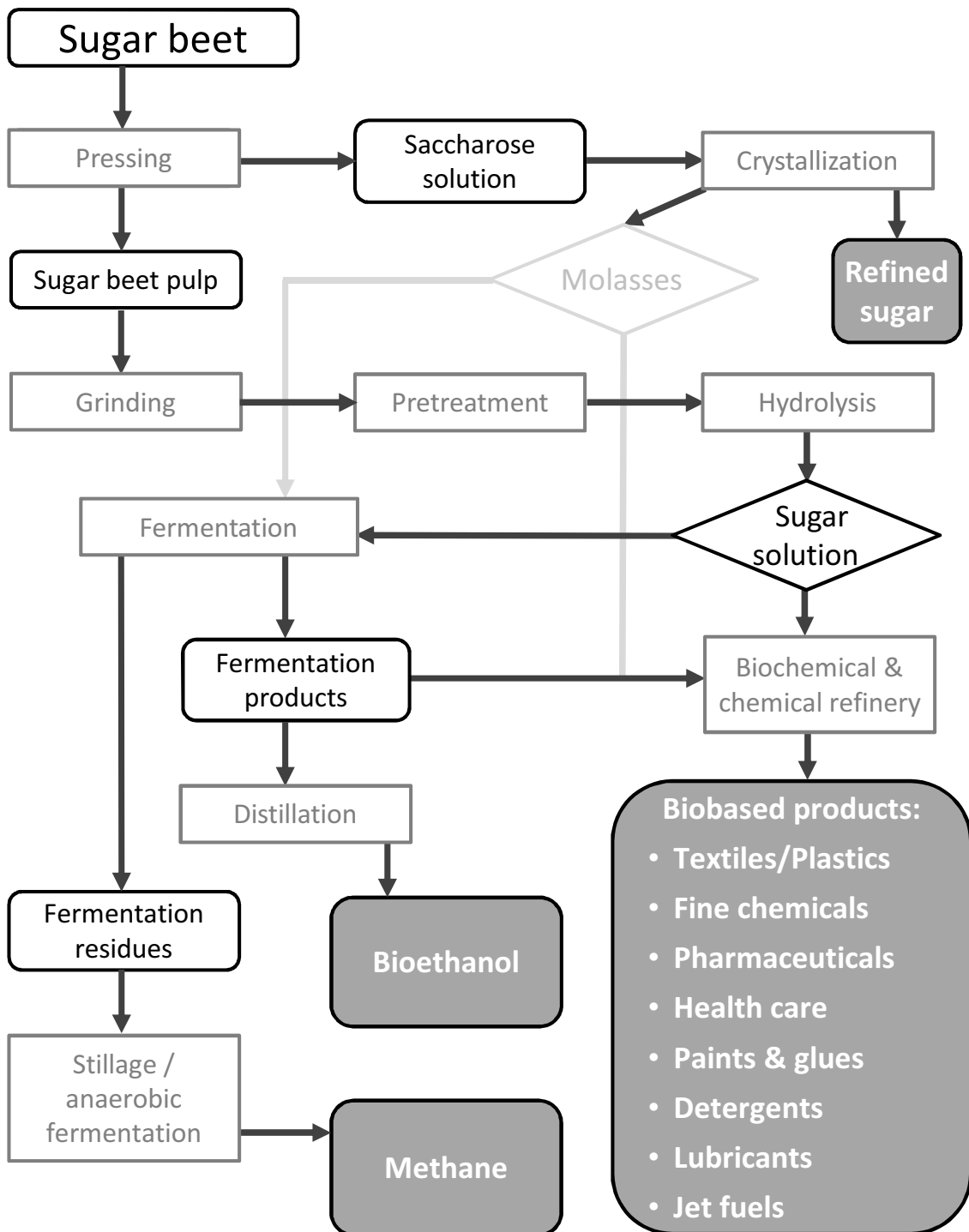
Fig. 7.4: Flowchart of the potential use of sugar-beet pulp in biorefinery processes. Black letters – starting material and intermediate products, grey letters – processing steps, white letters – end-products.

Hitherto petroleum-based products like organic platform chemicals, pharmaceuticals, cosmetics, textiles and plastics can be produced out of the SBP constituent-sugars after saccharification (Sims, 2003; Werpy and Peterson, 2004). Hexoses can be fermented to lactic acid, which can be used as a building block for polylactic acid (John et al., 2007). Ferulic acid can be used to produce aroma compounds (Werpy and Peterson, 2004). Arabinose can be reduced to arabinitol, which can be used as sweetener or as building block for poly-esters (Werpy and Peterson, 2004). It should also be possible to produce furfurals from arabinose and galacturonic acid, as it has been done for xylose (Chheda et al., 2007). From furfural almost any kind of organic platform chemical can be produced (Werpy and Peterson, 2004). Moreover, liquid alkanes produced from biomass are suitable to replace diesel and jet fuels (Huber et al., 2005; Xing et al., 2010).

These examples illustrate that, in the long term, SBP will probably have higher relevance as raw material for biobased products than for bioethanol production.

Advanced techniques like solar-to-fuel (Gray, 2009), wind-to-fuel (Dutton et al., 2000) or genetic modification of hydrocarbons-secreting micro-organisms (Lee et al., 2008) may allow biofuel production at higher quantities. Cyanobacteria may be used to produce ethanol directly from photosynthetically fixed carbon (Angermayr et al., 2009).

7.7   Societal impacts of bioethanol production from SBP

Biofuels raised hopes and concerns in many areas of the society. Biofuel production can support the development of rural areas and lower the dependency on energy imports (Sims, 2003; Hill et al., 2006). But there are also eligible concerns that energy prices and food prices will increase (Demirbas, 2009). Biofuel production may also have a major impact on landscape and fauna, land-use and agriculture (Sims, 2003; Fargione, 2010).

Bioethanol produced from SBP is a second generation biofuel. It does not compete directly for raw materials with food production, but the use of SBP in biorefinery can also effect food prices. SPB is used as fodder and the substitution of SBP could lead to increasing prices of animal feed and meat.

According to the sustainability criteria of the EU, biofuels should allow greenhouse gas (GHG) emission savings of at least 35 % (Schlegel and Kaphengst, 2007). The GHG emission savings potential of SBP bioethanol is difficult to estimate. SBP is an agricultural by-product, so that no negative influence of land-use change on GHG emissions is expected (Searchinger et al., 2008). The production of bioethanol from SBP will probably yield more energy than reported for bioethanol production from corn grain (25 % energy

yield, Hill et al. 2006). The carbon dioxide produced by during fermentation can be easily captured and re-used.

Bioethanol from SBP can minorly contribute to the 20 % reduction of CO_2 emissions aimed by the EU, but it may be a transition technology that explores new areas of how to use and convert biomass. The saccharification of SBP up to 90 % is feasible. Hydrolysis efficiencies close to 100 % remain challenging.

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Summary
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Summary

The aim of this research was the analysis and characterization of the enzymatic toolbox of the filamentous fungus *Chrysosporium lucknowense* C1 (C1) for the hydrolysis of sugar-beet pulp to fermentable monosaccharides. In this framework the necessity and applicability of a mild pretreatment was also evaluated.

Chapter 1 provides general background information on the structure of plant cells and the characteristics of sugar-beet cell walls in particular. Furthermore, cell-wall degrading enzymes are introduced with special attention to enzymes active toward sugar-beet arabinan and the toolbox of C1. Chapter 1 also summarizes previous studies on sugar-beet pulp saccharification.

The influence of a mild pretreatment on the enzymatic degradability of sugar-beet pulp was investigated in Chapter 2. The optimal pretreatment of 15 min at 140 °C in water mainly solubilized pectins. It also improved the enzymatic degradation of the insoluble cellulose. More than 90 % of all cellulose was released after 24 h, while using four times less enzyme than reported in previous studies. The optimal combined severity factor for sugar-beet pulp pretreatment is between $\mathrm{Log\ R'_0} = -2.0$ and $\mathrm{Log\ R'_0} = -1.5$. Higher severities led to the destruction of solubilized sugars to furfural, hydroxy methyl furfural, acetic acid and formic acid.

Chapter 3 describes the purification and characterization of four arabinose-releasing enzymes from C1, among them one endoarabinanase (Abn1), two arabinofuranosidases (Abn4 and Abf3) and one exoarabinanase (Abn2). All enzymes are temperature stable up to 50 °C and have a broad pH stability, which makes them suitable for many biotechnological applications, like co-fermentation in bioethanol production. The four C1 arabinohydrolases released up to 80 % of the arabinose present in sugar-beet arabinan as monomers, while a combination of Abn1, Abn2 and Abn4 also released linear and branched arabinose oligomers.

The branched arabinose oligomers were isolated by fractionation based on size and analyzed (Chapter 4). NMR was used to determine the structure of eight branched arabinose oligomers. They were present in two main series, carrying either one or two arabinosyl substituents per arabinosyl residue of the oligomer backbone.

The branched arabinose oligomers were used to determine the mode of action of the four C1 arabinohydrolases (Chapter 5). The active site of endoarabinanase Abn1 has at least six subsites, of which the subsites -1 to +2 have to be occupied for hydrolysis. Abn1 was able to hydrolyze a branched arabinohexaose with a double substituted

arabinosyl residue at subsite -2 and is therefore considered as side chain tolerant. The exoarabinanase Abn2 releases arabinobiose from the non-reducing end of the α-1,5-linked arabinan backbone and prefers, like Abn1, polymers over oligomers. Abn2 only binds the two arabinosyl residues at the subsites -1 and -2. Abf3 is a universal arabinofuranosidase that hydrolyzes all linkages present in branched arabinose oligomers, but Abf3 is almost inactive toward polymers. Abn4 is more active towards polymeric substrate and releases arabinose monomers from single substituted arabinose residues. The characterized enzymes allow the controlled and efficient degradation of arabinan to either monomers, or, by partial degradation, to branched arabinose oligomers.

Three ferulic acid esterases from C1 were purified and characterized (Chapter 6). The type A ferulic acid esterases FaeA1 and FaeA2 are the first members of the phylogenetic subfamily 5 to be biochemically characterized. Type B ferulic acid esterase FaeB2 is a member of subfamily 6. The enzymes are active at neutral pH and temperatures up to 45 °C. They release ferulic acid and *p*-coumaric acid from corn-fibre arabinoxylan oligomers. FaeA1 and FaeA2 also release major amounts of complex ferulic acid oligomers, e. g. dehydrodiferulic acids and dehydrotriferulic acid, from corn-fibre arabinoxylan oligomers. However, they could only release 20 % of all ferulic acid present in sugar-beet pectin oligomers, while FaeB2 could release 60 %. Therefore, FaeA1 is suitable for corn-fibre and wheat-bran deferuloylation, while FaeB2 is more suitable for pectin deferuloylation.

Chapter 7 discusses the main results of the research. The feasibility and the energy demand of a pretreatment is discussed. Recommendations are made regarding a revised classification system for arabinose-releasing enzymes and ferulic acid esterases. The potential of C1 as enzyme producer is evaluated and unpublished data of C1 enzymes active toward sugar-beet pectin is added. Furthermore, the utilization of sugar-beet pulp for bioethanol production is discussed energetically, with special attention to the microbial ethanol fermentation from galacturonic acid. The impact and potential of bioethanol from sugar-beet pulp is estimated and possible applications in biorefinery are proposed.

Samenvatting

Het doel van dit onderzoek was het analyseren en karakteriseren van de gereedschapskist aan enzymen van de draadzwam *Chrysosporium lucknowense* C1 (C1). Met deze enzymen kan suikerbietenpulp gehydrolyseerd worden tot fermenteerbare monosacchariden. Daarnaast worden de noodzakelijkheid en toepasbaarheid van een milde voorbehandeling geëvalueerd.

Hoofdstuk 1 geeft een overzicht van de structuur van plantecellen en de specifieke eigenschappen van de celwanden van suikerbietcellen. Vervolgens worden de enzymen geïntroduceerd die celwanden kunnen degraderen. De enzymen die arabinanen afbreken worden besproken en de gereedschapskist van C1 wordt geïntroduceerd. Tenslotte worden eerdere onderzoeken op het gebied van suikerbietsaccharificatie samengevat.

Hoofdstuk 2 onderzoekt de invloed van een milde voorbehandeling op de enzymatische afbreekbaarheid van suikerbietenpulp. Tijdens de optimale voorbehandeling, 15 minuten bij 140 °C, werden voornamelijk pectinemoleculen opgelost. Deze voorbehandeling verbeterde ook de enzymatisch afbraak van onoplosbare cellulose. Meer dan 90 % van alle cellulose kwam zo beschikbaar, terwijl er vier keer minder enzym nodig was dan in voorgaande onderzoeken.

The optimale *combined severity* factor voor de voorbehandeling van suikerbietenpulp ligt tussen Log R'_0 = -2.0 en Log R'_0 = -1.5. Hogere *severity* factoren leiden tot de afbraak van opgeloste suikers tot furfural, hydroxy-methyl furfural, azijnzuur en mierenzuur.

Hoofdstuk 3 beschrijft het opwerken en karakteriseren van vier arabinose producerende C1-enzymen, waaronder één endoarabinanase (Abn1), twee arabinofuranosidases (Abn4 and Abf3) en één exoarabinanase (Abn2). Al deze enzymen blijven stabiel tot 50 °C en in een brede pH-range. Dit maakt hen geschikt voor veel biotechnologische toepassingen, waaronder co-fermentatie in bioethanolproductie. De vier C1-arabinohydrolases breken tot 80 % van de in suikerbieten aanwezige arabinose af tot monomeren. Een mix van Abn1, Abn2 and Abn4 breekt de arabinose af tot lineaire en vertakte arabinoseoligomeren. De vertakte arabinoseoligomeren werden vervolgens gescheiden op grootte en geanalyseerd met MALDI-TOF MS en HPAEC.

Hoofdstuk 4 laat zien hoe NMR werd gebruikt om de structuur van acht vertakte arabinoseoligomeren te bepalen. Deze oligomeren bevatten een of twee arabinosylsubstituten per arabinosylresidue.

Hoofdstuk 5 beschrijft hoe de vier C1 arabinohydrolases werken. Het actieve centrum van endoarabinanase Abn1 heeft op zijn mijn minst zes subcentra, waarbij subcentra -1

tot +2 bezet moeten zijn, wil de hydrolyse plaatsvinden. Abn1 kon een vertakte ara-binohexaose hydrolyseren, waarvan een dubbelgesubstitueerd arabinosylresidue zich bevond in subcentrum -2. Dit enzym wordt daarom beschouwd als zijketentolerant. De exoarabinanase Abn2 knipt arabinobiose af van het niet-reducerende uiteinde van het α-1,5-gelinkte arabinan hoofdketen. Net als Abn1 heeft het enzym een voorkeur voor polymeren boven oligomeren. Abn2 bindt alleen aan de twee arabinosylresidueën in de subsites -1 en -2. Abf3 is een universele arabinofuranosidase die alle aanwezige bindin-gen in vertakte arabinoseoligomeren hydrolyseert, maar geen activiteit vertoont met polymeren. Abn4 vertoont een hogere activiteit met polymeren dan met oligomeren en maakt arabinosemonomeren vrij van enkelvoudig gesubstitueerde arabinoseresidueën. De gekarakteriseerde enzymen maken het mogelijk om op een gecontroleerde en efficiënte manier arabinaan af te breken tot vertakte arabinoseoligomeren of monomeren.

Hoofdstuk 6 beschrijft de opwerking en karakterisering van drie ferulazuuresterases van C1. Type A ferulazuuresterases FaeA1 en FaeA2 zijn de eerste leden van de fy-logenetische subfamilie 5 die ooit biochemisch gekarakteriseerd werden. Type B fer-ulazuuresterase FaeB2 is lid van subfamilie 6. Al deze enzymen zijn actief bij een neutrale pH en bij een temperatuur tot 45 °C. Ze converteren arabinoxylaanoligomeren van maisvezel naar ferula- en koumarinezuur. Met ditzelfde substraat produceren FaeA1 en FaeA2 ook grote hoeveelheden complexe ferulazuuroligomeren, bijvoorbeeld dehydro-di-ferulazuren en dehydro-tri-ferulazuren. Ze knippen echter maar 20 % van alle in suikerbietpectine-oligomeren aanwezige ferulazuren los, terwijl FaeB2 60 % omzet. Daarom is FaeA1 geschikt om mais- en tarwevezels te deferuloylatiseren, terwijl FaeB2 meer geschikt is om pectine te deferuloylatiseren.

Hoofdstuk 7 bediscussieert de belangrijkste resultaten van het onderzoek. Zowel de haalbaarheid en het energieverbruik van de voorbehandeling komen ter sprake. Er worden aanbevelingen gedaan voor een verbeterd klassificatiesysteem van arabinose-vrijmakende enzymen en ferulazuuresterases. Het potentieel van C1 als enzymproducent wordt geëvalueerd en ongepubliceerde gegevens van pectine afbrekende C1-enzymen worden besproken. Ook wordt er aandacht besteed aan de energiebalans van het gebruik van suikerbietenpulp in de bioethanolproductie. Speciale aandacht wordt hierbij besteed aan de microbiële ethanolfermentatie met galacturonzuur als substraat. De impact en het potentieel van de productie van bioethanol vanuit suikerbietenpulp word geschat en mogelijke toepassingen voor biorefinery voorgesteld.

Zusammenfassung

Die vorliegende Studie beschreibt die Analyse und Charakterisierung von zellwandabbauenden Enzymen des Fadenpilzes *Chrysosporium lucknowense* C1 (C1) und deren Anwendung zur Hydrolyse von Zuckerrübenpulpe zu fermentierbaren Monosacchariden. In diesem Rahmen wird auch die Notwendigkeit und Anwendbarkeit einer milden physiko-chemischen Vorbehandlung untersucht.

Im ersten Kapitel wird die Struktur von Pflanzenzellen beschrieben und auf den Aufbau der Zuckerrübenzellwand eingegangen. Zellwandabbauende Enzyme werden vorgestellt unter besonderer Berücksichtigung jener Enzyme, welche in der Lage sind, Zuckerrübenarabinan abzubauen. Desweiteren wird das Potential von C1 für den Abbau von Zuckerrübenpulpe beschrieben und frühere Studien zur Verzuckerung von Zuckerrübenpulpe zusammengefasst.

Im zweiten Kapitel wird der Einfluss einer milden physiko-chemischen Vorbehandlung auf den enzymatischen Abbau von Zuckerrübenpulpe beschrieben. Während einer optimalen Vorbehandlung für 15 Minuten bei 140 °C in Wasser werden vor allem Pektine aus dem Zellwandverband gelöst, wodurch sich auch der enzymatische Abbau von unlöslicher Zellulose verbessert. Innerhalb von 24 Stunden konnten bei der Verwendung von nur einem Viertel früher verwendeter Enzymkonzentrationen mehr als 90 % der vorhandenen Zellulose freigesetzt werden. Eine optimale Vorbehandlung von Zuckerrübenpulpe erfolgte bei einem kombinierten Härtegrad zwischen Log R′0 = -2.0 und Log R′0 = -1.5. Noch schwerere Vorbehandlungen führten zur Umsetzung der freigesetzten löslichen Zucker zu Furfural, Hydroxymethylfurfural, Essigsäure und Ameisensäure.

Das dritte Kapitel beschreibt die Gewinnung und Charakterisierung von vier arabinanabbauenden Enzymen aus C1. Hierbei handelt es sich um eine Endoarabinanase (Abn1), zwei Arabinofuranosidasen (Abn4 und Abf3) und eine Exoarabinanase (Abn2). Jedes dieser Enzyme ist stabil bis zu einer Temperatur von 50 °C und über einen weiten pH Bereich. Diese Eigenschaften ermöglichen den Einsatz der Enzyme in vielen verschiedenen biotechnologischen Anwendungen wie z.B. einer kombinierten Hydrolyse und Fermentation in der Bioethanolproduktion. Mit einer Kombination der vier aus C1 gewonnenen Arabinohydrolasen war die Gewinnung von 80 % des in der Zuckerrübenpulpe anwesenden Arabinan in Form von Monomeren möglich. Eine Kombination von Abn1, Abn2 und Abn4 ermöglichte außerdem die Freisetzung von verzweigten Arabinoseoligomeren.

Die verzweigten Arabinoseoligomere wurden der Größe nach getrennt und analysiert (Kapitel 4). Die Struktur von acht dieser verzweigten Arabinoseoligomerfraktionen konnte mit Hilfe von Kernresonanzspektroskopie bestimmt werden. Dabei zeigten sich zwei dominante Strukturen. Das Oligomergerüst trug entweder ein oder zwei Arabinosylsubstituenten pro Arabinosylgruppe des Zuckerrückgrates.

Mit Hilfe der gewonnen verzweigten Arabinoseoligomere wurde die Wirkungsweise der vier C1 Arabinohydrolasen bestimmt (Kapitel 5). Das aktive Zentrum der Endoarabinanase Abn1 erkennt mindestens sechs Zuckermoleküle innerhalb des Zuckerrückgrates. Als Voraussetzung für eine Hydrolyse müssen jedoch nur die drei Zuckermoleküle in den Positionen -1 bis +2 in Bezug auf die zu spaltende Bindung im aktiven Zentrum gebunden sein. Da Abn1 in der Lage ist, verzweigte Arabinohexaosen mit doppelt substituierten Arabinosylresten an Position -2 zu hydrolysieren, kann Abn1 als seitenkettentolerant angesehen werden. Die Exoarabinanase Abn2 setzt Arabinose ausgehend von dem nicht reduzierten Ende des α-1,5-verknüpften Arabinanrückgrates frei und zieht, wie auch Abn1, Polymere den Oligomeren vor. Abn2 bindet nur die zwei Arabinosylreste in den Positionen -1 und -2. Abf3 ist eine universale Arabinofuranosidase, die in der Lage ist, alle Verbindungen innerhalb eines verzweigten Arabinoseoligomeres zu spalten. Allerdings ist sie beinahe inaktiv gegenüber Polymeren. Abn4 besitzt eine größere Aktivität gegenüber polymeren Substraten und ist in der Lage, Arabinosemonomere aus einfach substituierten Arabinoseresten freizusetzen. Die hier beschriebenen Enzyme erlauben einen kontrollierten und effizienten Abbau von Arabinan zu Monomeren oder, durch einen nur partiell ablaufenden Abbau, zu verzweigten Arabinoseoligomeren.

Im sechsten Kapitel werden drei aus C1 gewonnene Ferulasäureesterasen beschrieben. Zum ersten Mal werden hier mit FaeA1 und FaeA2 zwei Ferulasäureesterasen des Typs A biochemisch charakterisiert, welche der phylogenetischen sub-Familie 5 angehören. Die außerdem beschriebene Typ B Ferulasäureesterase FaeB1 ist Mitglied der sub-Familie 6. Die drei beschriebenen Enzyme sind im neutralen pH Bereich aktiv bei Temperaturen bis zu 45 °C. Sie sind in der Lage, Ferulasäure und p-Cumarsäure aus Maisfaserarabinoxylanoligomeren freizusetzen. Zusätzlich setzen FaeA1 und FaeA2 außerdem große Mengen komplexer Ferulasäureoligomeren wie z.B. Dehydrodiferulasäure und Dehydrotriferulasäure frei. Verwendet man jedoch Zuckerrübenpektin als Substrat, können die zwei Typ A Feralsäureesterasen nicht mehr als 20 % der vorhandenen Ferulasäure freisetzen. Die Typ B Ferulasäureesterase FaeB2 hingegen kann bis zu 60 % der vorhandene Ferulasäure freisetzen. Für die Ferulasäurehydrolyse aus Mais- und Weizenkleie kann also

FaeA1 verwendet werden; für die Ferulasäurehydrolyse aus Pektinen eignet sich FaeB2 besser.

Im siebten Kapitel werden die Ergebnisse der gesamten Studie noch einmal zusammengefasst und die Durchführbarkeit und der notwendige Energieaufwand für eine mögliche Vorbehandlung diskutiert. Außerdem wird ein angepasstes Klassifizierungssystem für Arabinose und Ferulasäure freisetzende Enzyme empfohlen. Das Potenzial von C1 als Enzymproduzent wird bewertet, unter anderem mit Hilfe von unveröffentlichten Daten über die Aktivität von aus C1 gewonnenen Enzymen gegenüber Zuckerrübenpektin. Ergänzend wird die Verwendung von Zuckerrübenpulpe für die Bioethanolgewinnung energetisch bewertet, wobei der mikrobiellen Ethanolfermentation mit Galakturonsäure als Ausgangstoff besondere Aufmerksamkeit geschenkt wird. Der Einfluss und das Potenzial des von aus Zuckerrübenpulpe gewonnenen Bioethanols für die Ersetzung konventionell erzeugten Kraftstoffs werden eingeschätzt und mögliche Anwendungsgebiete in der Biotechnologie aufgezeigt.

Epilogue

Vier ereignisreiche Jahre sind an uns vorbeigerauscht und wir reiben uns verwundert die Augen, wo die ganze Zeit geblieben ist. Auch wenn wir uns selten sahen und auch nicht grade jede Woche telefonierten, blieben viele Freundschaften bestehen. Danke für euren Rückhalt, eure Wärme und Kameradschaft. Der Größte Dank geht selbstredend an Kerstin, die sich von meiner Abenteuerlust anstecken ließ und ins kalte Wasser der holländischen Arbeitswelt sprang. Ein ganzes dickes extragroßes Dankeschön für deine enorme Toleranzspanne im letzten Jahr, welches dank Emil und trotz Arbeitsstress ein Kurzweiliges war.

Thank you to all the people I met and with which I spent most of my leisure time: Schwenni, Anne, Anita, Ori, Wibke, Rafa: Chic@s, you made me feel at home. Harold en Rainier, bedankt voor $2\frac{1}{2}$ gezellige jaren in Arnhem met zo fantastische buren, het was steeds leuk met jullie. Maar moeten jullie echt de heg knippen – het ziet er goor uit, Jongens. Jodie en René: klimmen, skiën, BBQen, kroegen of bierfietsen – wij hebben veel samen beleeft. Bedankt voor al deze plezieren en ervaringen (Don't mess with trees!), ik hoop dat wij in contact blijven. A warm cheers goes out to all the people who have been brave enough for the weekly dose of frustation we got applied at the Doctor.

Ph.D. theses can appear as deserts: For months and months you are trying to cultivate the sparse soil of science and to gain the one or the other insight. And then, all of a sudden, in a short period of fertility you have got your hands full to harvest what you sowed. Thanks to Y*w*onne, who has helped me with that.

Yvonne $\frac{V}{W}$, /ku:s/ & /ru:di/ : I has been a wonderful experience to organize the Ph.D. trip to China with you folks. Thanks for afternoons/evenings of long talks, bad food (sometimes), fun (often) and anger (scarce).

During practical supervision I learned a lot about education in the Netherlands. I was surprised by the leisure-like, no-pressure atmosphere in the class room. But hey, I'm

german at last. Thanks to all supervisors, teachers and students that actively participated in the course and made it (most of the time) such a nice experience.

Thanks to all folks who shared room 513 with me during the last four years. It was always a warm and cosy atmosphere, also without enduring conversations. You guys know me a bit, I rather try to get along with the chaos than fighting against entropy. Thank you for tolerating this (or at least complaining behind my back so that I could ignore it ;-). So long, and thanks for all the coffee.

Since I was labhead of 504, it was probably not the most organized place on the fifth floor. Thanks to all the people who have worked with me in the lab and who have been tolerant enough to stand my taste of music.

Henk, I think we know each other a bit and the biggest difference is certainly our handling of stress. We managed to organize our meetings efficiently, sometimes too efficient and despite all assurance we could not really get a routine into it. Which I do not really regret. You accepted me and the way I am and pushed me slightly in the right direction when necessary (not only regarding my (mostly absent) dresscode :-). Thenks. PS: We hebben er nog drie R& O gesprekken aanstaan.

A really big Thank You! goes out to Jolan, Jolanda, Margaret, Edwin, Jan, Mark, Peter and René for their helpfulness and patience and for keeping the engine running.

Mirjam: Onze Wijnexperiment is helaas niet echt gelukt, maar toch was het de moeite helemaal waard. In iederen geval weten wij nu hoe we lekker champagner-sorbée kunnen maken ;-) Ook in het lab hebben wij leuke dingen uitgevonden, bedankt voor raad en daad.

Thanks to the EOS-LT people: I really enjoyed our semi-annual meetings that gave me the feeling to be part of a joint initiative, even though practically, we did not have too much chance to really join forces within the project. Thanks to Sandra, Jaap, Eline and Ton for their inputs on some chapters of this book. Thanks to my opponents and to those reviewers of my articles who helped to improve them by giving constructive comments.

My dear paranymphs Anja and Koos: A big Hug! for all your support, with the preparation of the promotion and the translations of the summary.

And finally, thanks to all cooperative procrastinators a.k.a. Ph.D. students on the fifth floor! Stay as you are (procrastinating?) and where you are (I guess, the only adequate answer here is: In the lab ;-).

Stefan has left the building.

About the author

Curriculum vitae

Stefan Kühnel was born 5th of June, 1980 in Potsdam, Eastern Germany. After finishing secondary school in 2000, he did his civil service at the blood donation centre Potsdam of the German Red Cross. In 2001, Stefan started his biochemistry studies at the University of Potsdam. From 2004 he worked as a student helper in the lab of Markus Pauly at the Max-Planck-Institute for Molecular Plant Physiology, where he also did his MSc thesis on cell wall biosynthesis in the Golgi apparatus. During the course of the MSc thesis, he spent six months in the lab of Ariel Orellana at Andres Bello University, Santiago de Chile, Chile. From May 2007 to September 2011, Stefan did the Ph.D. project described in this thesis in the lab of Food Chemistry at Wageningen University.

List of Publications

Kühnel, S., Hinz, S. W. A., Pouvreau, L., Visser, J., Schols, H. A., Gruppen, H., 2010. *Chrysosporium lucknowense* arabinohydrolases efficiently degrade sugar beet arabinan. Bioresource Technol. 101 (21), 8300–8307.

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Obel, N., Erben, V., Schwarz, T., Kühnel, S., Fodor, A., Pauly, M., 2009. Microanalysis of plant cell wall polysaccharides. Mol. Plant 2 (5), 922–932.

Westphal, Y., Kühnel, S., de Waard, P., Hinz, S. W. A., Schols, H. A., Voragen, A. G. J., Gruppen, H., 2010a. Branched arabino-oligosaccharides isolated from sugar beet arabinan. Carbohyd. Res. 345 (9), 1180–1189.

Westphal, Y., Kühnel, S., Schols, H. A., Voragen, A. G. J., Gruppen, H., 2010b. LC/CE-MS tools for the analysis of complex arabino-oligosaccharides. Carbohyd. Res., 345 (15), 2239–2251.

Conference talks

Arabinanases from *Chrysosporium lucknowense* and their potential in the complete degradation of sugar beet arabinan. Presented at the Cost 928 annual meeting, Krakow, 2009.

Chrysosporium lucknowense arabinohydrolases – Mode of action and substrate specificities. Presented at the Biomass derived pentoses conference, Reims, 2010.

Conference posters

Bioethanol from Sugar Beet Pulp – The Pectin Challenge. Presented at the Pectins and pectinases conference, Wageningen, 2008.

Arabinohydrolases of *Chyrosporium lucknowense* – mode of action and substrate specificity. Presented at the 12th international cell wall meeting, Porto, 2010.

Training activities

General courses

Ph.D. introduction week, Bilthoven, 2007

Ph.D. competence assessment, Wageningen, 2007

Ethics in food science, Wageningen, 2008

Teaching and supervising thesis students, Wageningen, 2009

Basic statistics, Wageningen, 2009

Career orientation, Wageningen, 2011

Discipline specific activities

Conferences and meetings

EOS bietenpulp project meetings, 2007-2011

European biofuels stakeholder meeting, Bruxelles, 2008

Pectins and pectinases, Wageningen, 2008

Cost 928 annual meeting, Krakow, 2009

12th international cell wall meeting, Porto, 2010

Biomass derived pentoses conference, Reims, 2010

Courses

Summer school glycosciences, Wageningen, 2008

Food enzymology, Wageningen, 2008

Waters seminar on ULPC and SFC, Wageningen, 2010

Optional activities

Food chemistry seminars and colloquia

Supervision of M.Sc. thesis students and M.Sc. lab classes

Ph.D. study trip (participant and member of the organizing committee), China, 2008

Ph.D. study trip, Switzerland and Italy, 2010