



Chryso sporium lucknowense arabinohydrolases effectively degrade sugar beet arabinan

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ABSTRACT

The filamentous fungus *Chryso sporium lucknowense* (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 bio-ethanol production.

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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 (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 (*p*-NP-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 *Chryso sporium lucknowense* C1 is an industrial strain optimized in cellulase and hemicellulase production. The C1 genome has been fully sequenced and

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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 *C. lucknowense* 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.

2. Methods

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 endoarabinanase 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).

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.

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–polyacrylamide gel electrophoresis (SDS–PAGE, Section 2.4). Abn1 and Abn2 activities were determined on linear arabinan with the *p*-hydroxybenzoic acid hydrazide assay (PAHBAH, Section 2.6). Abn4 activity was determined using *p*-NP-Ara (Section 2.7).

2.3.1. Anion exchange chromatography

All enzymes were subjected to Anion Exchange Chromatography (AEC) on a Source 15Q 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 five 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 40–65 mM NaCl and 43–129 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.

2.3.2. 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 four 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.

2.3.3. 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 Superdex75 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.

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.

2.5. Enzyme incubations

All incubations were carried out at 30 °C unless otherwise mentioned. One enzyme unit is defined as 1 µmol (product) $\text{min}^{-1} \text{mg}^{-1}$ (enzyme) at 30 °C. For biochemical characterization 0.5% (w/v) substrate was used with 0.02% (w/w on protein basis) enzyme. 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 3.0–8.0) 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 3.0–8.0 or $T = 20$ –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 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 $\mu\text{g/ml}$) of arabinose monomer and oligomers (DP 2–6).

2.6. Determination of reducing ends with PAHBAH assay

PAHBAH reducing end assay was performed as described by 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 min in microtiter plates covered with aluminum foil. After cooling the microtiter plate was centrifuged at 1000g for 2 min and the absorbance was measured at 405 nm. The reducing end concentration was quantified using an α -arabinose calibration curve (5–750 $\mu\text{g/ml}$).

2.7. Activity towards *p*-nitrophenyl-arabinofuranoside

Activity of Abn4 towards *p*-NP-Ara was monitored by the release of *p*-nitrophenol. The sample (10 μl) was incubated with 190 μl *p*-NP-Ara (0.5 mM) in 10 mM sodium acetate buffer (pH 4.5) for 1 h 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).

2.8. Sugar composition analysis

Polysaccharides were hydrolyzed 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 $\mu\text{g/ml}$) was used for quantification.

2.9. HPSEC

HPSEC was performed on a Thermo Scientific spectra quest HPLC (Thermo Finnigan, Waltham, MA, USA) equipped with a set of four TSK-Gel G columns (Tosoh Bioscience, Tokyo, Japan) in series: guard column PWXL (6 mm ID \times 40 mm) and separation columns 4000 PWXL, 3000 PWXL and 2500 PWXL (7.8 mm ID \times 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).

2.10. HPAEC

The monomer and oligomer sugar levels of the digests were analyzed by HPAEC according to Albrecht et al. (2009). Arabinose and arabinose oligomers ($V = 10 \mu\text{l}$; $c = 50\text{--}100 \mu\text{g/ml}$) 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. Results and discussion

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.

3.1.1. Biochemical characterization

3.1.1.1. pH and temperature optima. The pH optima determined for Abn1, Abn2 and Abn4 are illustrated in Fig. 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. 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 2-fold increase per 10 K temperature increment from 20 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., submitted for publication).

3.1.1.2. pH and temperature stabilities. Fig. 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 6.0–7.0. 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. 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 h 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 data

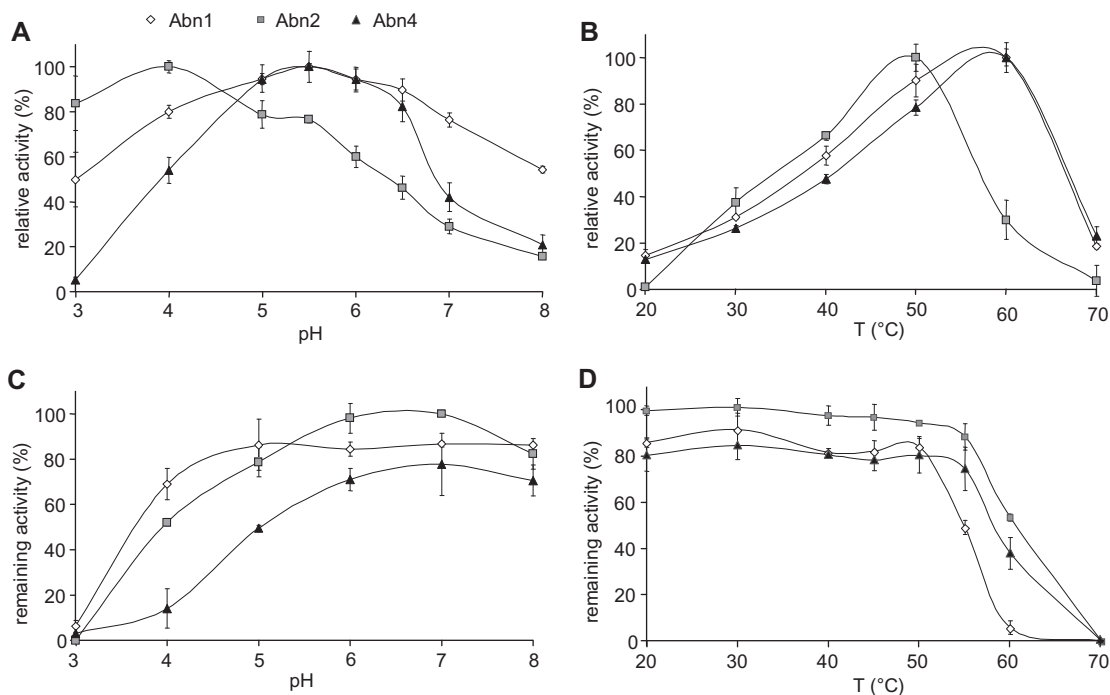


Fig. 1. Biochemical characterization of Abn1, Abn2 and Abn4. (A) pH optima, (B) temperature optima, (C) pH stabilities, and (D) temperature stabilities. Activities are determined on linear arabinan (Abn1 and Abn2) and branched arabinan (Abn4), respectively ($n = 3$).

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 evolutionary adaptation to 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.

3.2. 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. 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 10-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 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. 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. 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 et al. (1997). Based on the obtained results, no clear classification can be done. In contrast to Abn4, the arabinoxyylan 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. Action on linear and branched arabinan

3.3.1. 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 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.

3.3.1.1. Linear arabinan. The molecular mass distributions of linear and branched arabinan after different enzyme digestions are presented in Fig. 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. 3A). The 30 kDa peak remains in both, linear and branched, arabinan digestions. It could reflect a

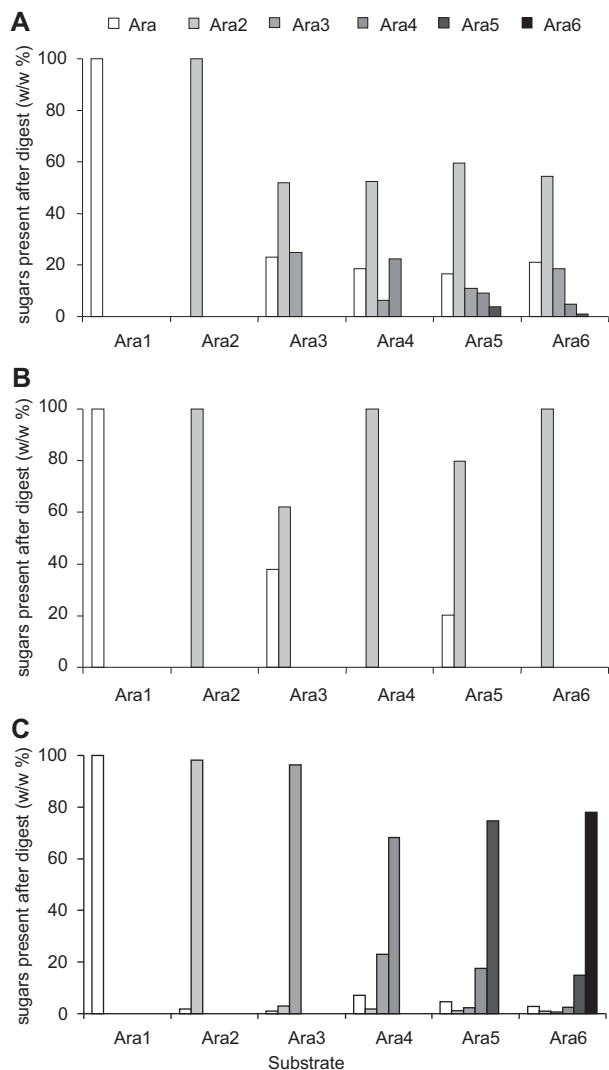


Fig. 2. The degradation of linear arabinose oligomers by C1 arabinohydrolases determined by HPAEC. X-axis: arabinose oligomers DP 1–6 used as substrate. (A) Abn1, (B) Abn2, and (C) Abn4.

Table 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

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.

3.3.1.2. 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. 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 cannot 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.

3.3.2. End product release

3.3.2.1. 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. 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 to release arabinobiose from the α -1,5-arabinan backbone.

3.3.2.2. Branched arabinan. The oligomer release from branched arabinan was quantified after maximal substrate conversion (Fig. 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.

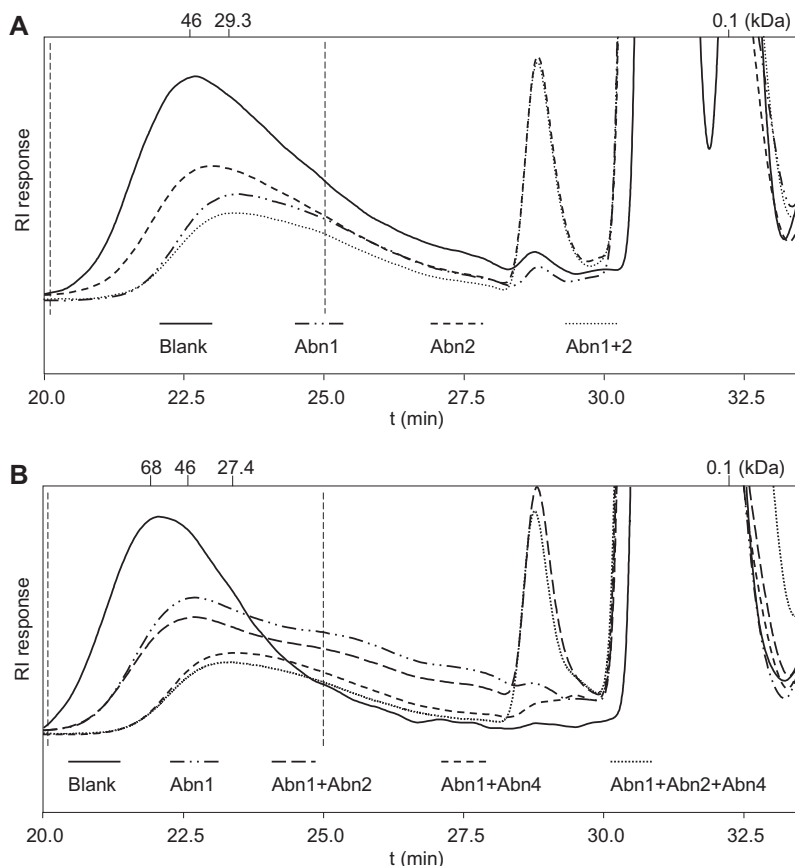


Fig. 3. HPSEC elution patterns of linear (A) and branched (B) arabinans digested with different combinations of C1 arabinohydrolases. Elution times of pullulan standards are indicated.

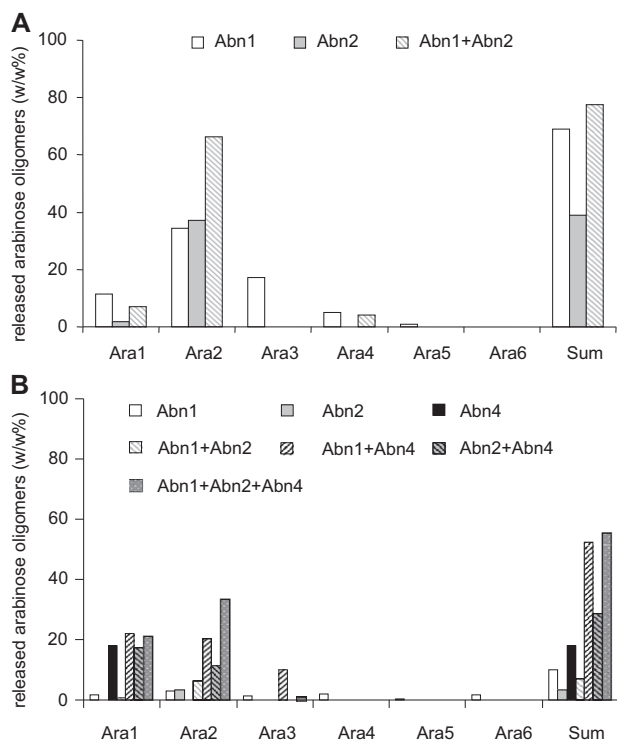


Fig. 4. Arabinose oligomers release from linear (A) and branched (B) arabinan with different combinations of C1 arabinohydrolases as determined by HPAEC. X-axis: released monomers and oligomers from DP 2–6.

3.3.3. 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. 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. 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 analyzed at higher concentrations (500–1000 μ g/ml) with a less steep gradient than normal to achieve higher sensitivity and better separation (0–350 mM NaOAc in 25 min). From Fig. 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 peaks are arabinose oligosaccharides as well. It also strengthens

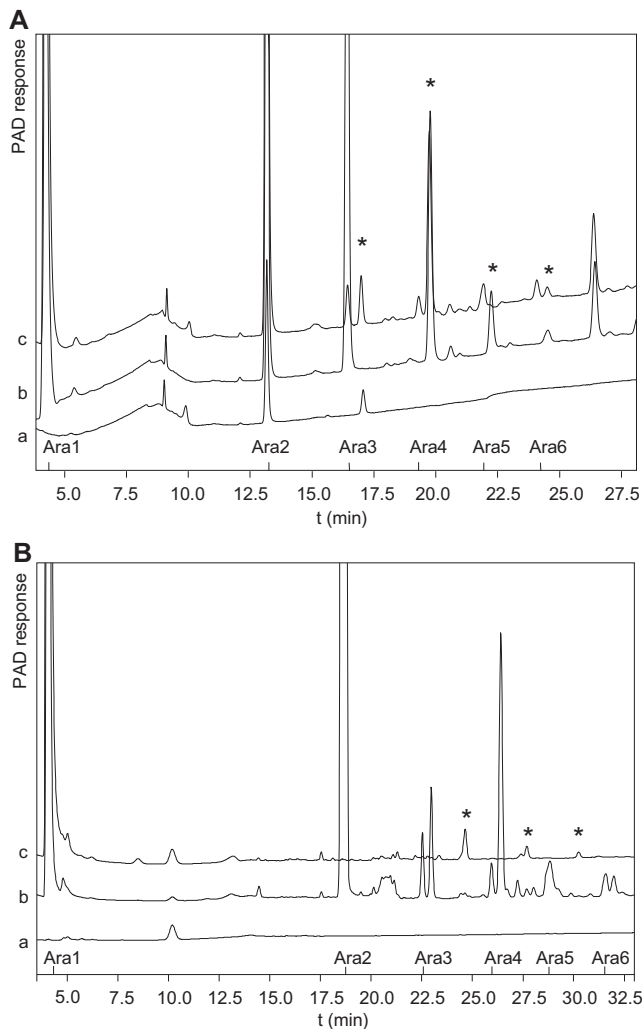


Fig. 5. Release of non-linear arabinose oligomers from branched arabinan by C1 arabinohydrolases as determined by HPAEC. (A) Default HPAEC gradient with total sugar concentrations between 50 and 100 µg/ml. Line a – Abn2; line b – Abn1 and Abn4; line c – Abn1, Abn2 and Abn4. (B) Less steep HPAEC gradient 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 Abf3. Ara1 to Ara6: retention times of linear arabinose oligomers with DP 1–6. Asterisks indicate peaks of unknown structure.

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 weight material. The nature of the various isomers formed by the enzymes mixtures were further investigated by Westphal et al. (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.

4. Conclusions

The arabinohydrolases Abn1, Abn2, Abn4 and Abf3 from C1 act together on the degradation of arabinans (Table 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,

Table 2

Activity of *Chrysosporium lucknowense* arabinohydrolases towards various substrates.

	Linear arabinan	Branched arabinan	Linear ara oligos	p-NP-Ara
Abn1	+++	+/-	+	-
Abn2	+	+/-	+	-
Abn4	-	++	+/-	+
Abf3	-	-	++	+++

arabinose oligomers. The arabinofuranosidase Abf3 converts all linear and unknown arabinose oligomers to arabinose monomers.

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