

Fungal Exo-acting Enzymes with Novel Catalytic Properties

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Tatsuji Sakamoto^{1,*}

¹*Division of Applied Life Sciences, Graduate School of Life and Environmental Sciences, Osaka Prefecture University (1-1, Gakuen-cho, Osaka 599-8531, Japan)*

Abstract: This article deals with characterizations of two *Aspergillus niger* exo-polygalacturonases (exo-PGs; EC 3.2.1.67) and a *Penicillium chrysogenum* exo-arabinanase (no EC number). Two exo-PGs, termed exo-PG1 and -PG2, purified from a commercial *A. niger* enzyme preparation (Pectinex AR) had molar masses of 82 and 56 kDa, respectively. Exo-PG1 was stable over wider pH and temperature ranges than exo-PG2. Exo-PG1 had a broad specificity towards oligogalacturonates with different DPs, while digalacturonate was the most favorable substrate for exo-PG2. Both enzymes degraded xylogalacturonan from pea hull in an exo manner to produce galacturonic acid (GalA) and Xyl-GalA disaccharide, as identified by electrospray ion trap mass spectrometry (ESI-ITMS). Moreover, exo-PGs split acetylated homogalacturonan in an exo manner, producing GalA and acetylated GalA, as shown by ESI-ITMS. An exo-arabinanase, termed Abnx, was purified from a culture filtrate of *P. chrysogenum* 31B. The enzyme released only arabinobiose from the non-reducing terminus of α -1,5-L-arabinan and showed no activity towards *p*-nitrophenyl α -L-arabinofuranoside or α -1,5-L-arabinofuranobiose. The nucleotide sequence of the *abnx* cDNA gene, which encodes Abnx, was determined. Abnx was found to be structurally distinct from known arabinan-degrading enzymes based on its amino acid sequence and a hydrophobic cluster analysis (HCA). The *abnx* cDNA gene product expressed in *Escherichia coli* catalyzed the release of arabinobiose from α -1,5-L-arabinan. The activity of the recombinant Abnx towards a series of arabino-oligosaccharides, as expressed by k_{cat}/K_m value, was greatest with arabinohexaose. The recombinant enzyme was found to possess trans-arabinobiosylation activity on various acceptors, such as aliphatic alcohols, sugars and sugar alcohols. The transfer product of glycerol was identified as *O*- α -L-arabinosyl-(1 \rightarrow 5)-*O*- α -L-arabinosyl-(1 \rightarrow 1)-glycerol on the basis of the spectral data, fast atom bombardment-mass (FAB-MS) and ¹H- and ¹³C-NMR. Unlike endo-arabinanases, Abnx catalyzed the hydrolysis of linear arabinan without inverting the anomeric configuration.

Key words: exo-polygalacturonase, xylogalacturonan, acetylated homogalacturonan, exo-arabinanase, arabinobiose

Pectins are acidic polysaccharides of high molar mass and one of the main components of the primary cell walls of dicotyledons. Pectins consist of homogalacturonic or "smooth" regions, and rhamnogalacturonic or "hairy" regions carrying neutral sugar side-chains. GalA residues may be substituted by different compounds such as methanol, acetic acid and xylose, in the "smooth" as well as in the "hairy" region. The carboxyl groups of pectin are partially esterified with methanol, and the hydroxyl groups at position 2 or/and 3 of GalA residues are sometimes acetylated. Xylogalacturonan, an α -1,4-D-galacturonan to which D-xylopyranose units are attached at position O-3 in the β -configuration as side chains, can be found in cell walls such as Japanese radish,¹⁾ carrot,²⁾ soy³⁾ and apple.⁴⁾ In pea hull xylogalacturonan, the degree of substitution of GalA by xylose residues is 72%. GalA

residues carry mainly single terminal xylose residues attached on O-3 and a few short side chains of xylosyl residues linked on O-2 or sometimes on O-3.⁵⁾ In contrast, arabinans and arabinogalactans are most frequently found in hairy regions of pectins. Sugar beet and apple pectins are characterized as containing high amounts of branched L-arabinan, a highly branched α -1,5-L-arabinan possessing 1,2 or 1,3- α -linked L-arabinofuranosyl residues as side-chains. Arabinogalactan, which has a linear chain of β -1,4-D-galactan to which α -1,5-L-linked arabinofuranooligosaccharides are bound in side-chains, is rich in soybean pectin. Extensive degradation of pectic polysaccharides is important in the processing of plant materials such as the clarification of fruit juices.⁶⁾ Enzymatic degradation of pectic polysaccharides is also efficient for their structural analysis since enzymes have strict substrate specificities. Exo-acting enzymes are especially useful in this field because they can split polysaccharides from the terminus sequentially.

This paper deals with characterizations of exo-acting enzymes with novel catalytic properties, which include two *A. niger* exo-PGs and a *P. chrysogenum* exo-arabinanase.

* Tel. +81-72-254-9456, Fax. +81-72-254-9921, E-mail: sakamoto@biochem.osakafu-u.ac.jp.

Abbreviations: AHG, acetylated homogalacturonan; DAc, degree of acetylation; DM, degree of methylation; ESI-ITMS, electrospray ion trap mass spectrometry; Exo-PG, exo-polygalacturonase; FAB-MS, fast atom bombardment-mass spectrometry; GalA, galacturonic acid; HCA, hydrophobic cluster analysis; HG, homogalacturonan; HPAEC, high-performance anion-exchange chromatography; MBP, maltose binding protein; PAD, pulsed amperometric detection.

Two *exo*-PGs from *A. niger* able to degrade xylogalacturonan and acetylated homogalacturonan.⁷⁾

Two *exo*-PGs were isolated from the commercial *A. niger* enzyme preparation Pectinex AR (Novozymes, France). SDS-PAGE analysis of the purified enzymes showed a single protein band at a molar mass of 82 and 56 kDa for *exo*-PG1 and *exo*-PG2, respectively. *Exo*-PG1 was stable over a wider pH range and over a wider temperature range than *exo*-PG2. Addition of 0.01 mM HgCl₂ increased *exo*-PG2 activity by 3.4 times and had no effect on *exo*-PG1 activity. *Exo*-PG2 was deduced to be similar to the *A. niger* *exo*-PGs reported by Mill⁸⁾ and Hara *et al.*⁹⁾ which were also stimulated by Hg²⁺. The *A. tubingen-sis* *exo*-PG is an enzyme with a molar mass of 78,000, broad specificity towards oligogalacturonates (mentioned below), and non-sensitive to Hg²⁺.¹⁰⁾ Taking into account these properties, *exo*-PG1 seems to be similar to this enzyme.

Table 1 shows the specificities of the purified *exo*-PGs on galacturonates with different DPs at a 0.1 mM concentration. Digalacturonate was the favorite substrate for the action of *exo*-PG2, which seemed to prefer oligogalacturonates to homogalacturonan (HG). On the other hand, *exo*-PG1 had a broad specificity towards galacturonates with different DPs compared to *exo*-PG2 and was also active on the polymer. Degradation of various plant cell wall polysaccharides by *exo*-PGs was also investigated (Table 2). The enzymes exhibited the highest activity towards HG. Sugar beet pectin was the poorest substrate for both enzymes. The activities on de-esterified sugar beet pectin were higher than those on sugar beet pectin in both enzymes. These substrates had similar quantities of "hairy" regions but different degree of methylation (DM) and degree of acetylation (DAc). The differences of sub-

Table 1. Enzyme activity of the *exo*-PGs towards galacturonates with different DPs.

Substrate	Relative activity	
	<i>Exo</i> -PG1	<i>Exo</i> -PG2
DP 2	100	100
DP 3	86	40
DP 4	72	16
DP 5	63	13
DP 149	42	5

The enzyme was incubated with 100 µL of 0.1 mM the substrate in 50 mM sodium acetate-HCl buffer (pH 3.6) at 40°C for 10 min. Concentration of GalA released was determined by HPAEC.

Table 2. Sugar compositions and enzyme activity of *exo*-PGs on various substrates.

Substrate	Sugar composition (mg/g)					DAc (%)	DM (%)	Amount of GalA released (µg/mL)	
	Rha	Ara	Xyl	Gal	AUA			<i>Exo</i> -PG1	<i>Exo</i> -PG2
SBP	30	213	3	53	414	49	51	1	1
DE-SBP	32	202	3	54	461	26	15	38	65
HG	2	0	0	0	804	<1	<3	106	186
AHG	35	53	2	53	586	45	24	23	38
E81	14	3	–	44	877	–	81	8	19
XG	–	–	282	–	413	3	0	14	8

SBP, sugar beet pectin; DE-SBP, de-esterified sugar beet pectin; HG, homogalacturonan; AHG, acetylated homogalacturonan; E81, highly methylated pectin; XG, xylogalacturonan; AUA, anhydro uronic acid; DAc (%), degree of acetylation; DM (%), degree of methylation.

strate specificity seemed to be due to DM or DAc. Highly methylated pectin (Grinsted™ Pectin URS 1200; E81) was also considerably resistant to the enzymes, indicating that methyl ester groups interfered with the enzyme activities. An *A. niger* *exo*-PG has been reported to be able to release free GalA from the nonreducing terminus only when the following GalA is not methyl-esterified.¹¹⁾ Acetylated homogalacturonan (AHG) could be degraded to some extent by the enzymes although most GalA residues in this substrate are esterified with acetate (45% of DAc) or methanol (24% of DM). This result suggests that the *exo*-PGs released acetylated GalA. Enzyme activities of the *exo*-PGs on xylogalacturonan were low. High-performance anion-exchange chromatography (HPAEC) profiles of the reaction products of the substrate with each enzyme revealed that both enzymes released GalA as the main product accompanied with an unknown compound. Figure 1 shows an example for *exo*-PG2.

To study the mode of action of *exo*-PGs on xylogalacturonan, the unknown compound released from the substrate by *exo*-PG2 was isolated on a CarboPac PA-1 column (Dionex) and analyzed by both positive and negative ESI-ITMS and by acid hydrolysis. In positive ESI-ITMS, two major ions with molar masses of 349 and 675 were observed, which were assigned to be the sodiated molecules [M+Na]⁺ and [2M+Na]⁺, respectively. Negative ESI-ITMS gave an intensive peak at 325 corresponding to

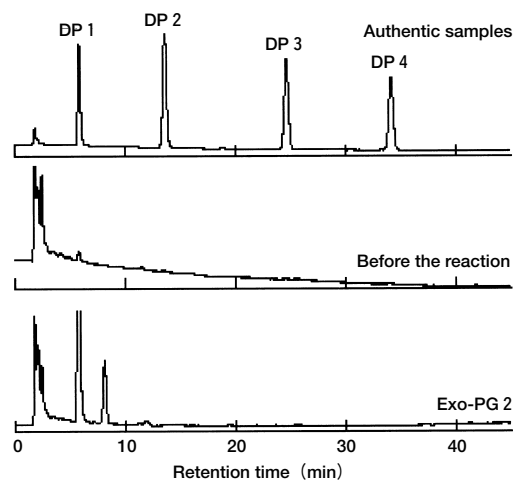


Fig. 1. HPAEC analysis of the enzymatic products of pea hull xylogalacturonan obtained with *exo*-PG2.

Authentic samples DP 1 to DP 4 represent GalA to tetragalacturonate, respectively.

the deprotonated $[M-H]^-$. These results indicated that the molar mass of the compound was 326. Moreover, acid hydrolysis of the compound and its alditol acetate derivative gave a peak corresponding to xylose in gas liquid chromatography. It could then be concluded that the unknown product was the disaccharide (Xyl-GalA) composed of xylose ($M_r=150$) and GalA ($M_r=194$). Furthermore, positive and negative ESI-MS/MS gave intensive daughter ions at 217 ($194+23$) and 175 ($194-18-1$), respectively, when molecular ions of Xyl-GalA were selected to be trapped. Cleavages of glycosidic linkages occur preferentially at the nonreducing and reducing side of the glycosidic oxygen in positive and negative ESI-MS/MS, respectively.^{12,13} Therefore, it can be concluded that the compound was Xyl1→GalA. Considering the structure of xylogalacturonan, it is likely that this disaccharide is Xyl1→3GalA. These results demonstrated that both enzymes degraded xylogalacturonan in an exo manner, producing GalA and Xyl-GalA. The progression of products released by exo-PG2 on xylogalacturonan is shown in Fig. 2. During the early stage of the reaction, the enzyme released GalA more easily than the disaccharide. When the incubation was prolonged, the enzyme gradually produced the disaccharide. This data suggested that the enzyme had higher affinity for the non-substituted GalA residues than for those substituted with xylose. Our previous results about the fine structure of xylogalacturonan demonstrated that 28%, 53% and 19% of the total GalA residues were free, substituted with a single xylose, and with dixylosides, respectively.⁵ When the percentages and sugar content of the substrate were taken into account (Table 2), concentrations of GalA, Xyl-GalA and Xyl-Xyl-GalA units in xylogalacturonan of the reaction mixture were calculated to be 96, 319 and 163 $\mu\text{g/mL}$, respectively. Exo-PG2 released 40.1 $\mu\text{g/mL}$ of GalA and 60.2 $\mu\text{g/mL}$ of Xyl-GalA after 48 h of reaction, demonstrating that 42% and 19% of free GalA residues and Xyl-GalA units were released and that 22% of total glycosidic link-

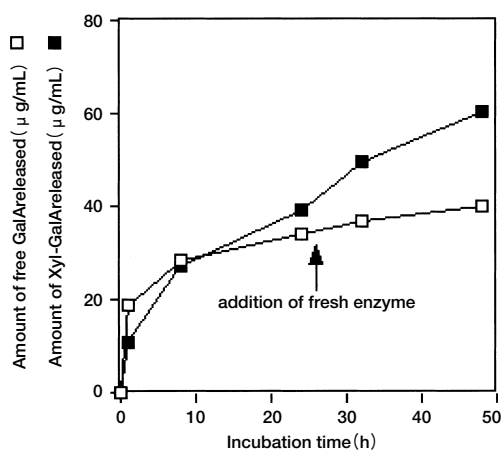


Fig. 2. Progression of products released by exo-PG2 on pea hull xylogalacturonan.

A reaction mixture containing 0.3 mL of 0.1% xylogalacturonan in 50 mM sodium acetate-HCl buffer (pH 3.6) and 60 μL of the enzyme (50 mU) was incubated at 40°C at times shown. The fresh enzyme (25 mU) was added to the mixture at 24 h after the reaction started. The products released into the mixture were quantified by HPAEC.

ages between GalA were cleaved. The high percentage of free GalA released may indicate that xylogalacturonan had HG regions without xylose substitution. During degradation of xylogalacturonan, GalA and Xyl-GalA were predominantly formed and little quantities of two other compounds, which have not been identified, were also produced. Even though 19% of the total GalA is substituted by dixylosides, there was not an intensive peak corresponding to Xyl-Xyl-GalA. This suggested that substitution with dixylosides interfered with the activity of exo-PG2.

As mentioned above, exo-PGs seemed to degrade AHG extracted from sugar beet pectin, which is characterized by a high DAC at O-2 and/or O-3 of the GalA in the backbone. Taking into account the ability of exo-PGs to split xylogalacturonan and its chemical structure, our interest was focused on the degradation of AHG by these enzymes. To confirm the release of acetylated GalA from AHG with exo-PGs, we tried to separate acetylated and free GalA by reverse phase chromatography. Reaction products of AHG with two exo-PGs were analyzed using a Superspher column (Merck), which gave an unknown compound in addition to GalA (data not shown). The compound was then purified with the same column and treated with 0.1 M sodium hydroxide for 2 h at room temperature. The unknown compound disappeared and GalA and acetic acid appeared, which strongly suggested that GalA was esterified with acetic acid. Then, the unknown product was analyzed by ESI-ITMS, which gave a molar mass of 259 in positive ESI-ITMS and 235 in negative ESI-ITMS, respectively. From these results, it can be concluded that the unknown compound was acetylated GalA.

In conclusion, both exo-PGs isolated here degraded pea hull xylogalacturonan in an exo manner to produce GalA and Xyl-GalA, which was identified by analyses of HPAEC, ESI-ITMS, and acid hydrolysis. So far, exo-PGs produced by both *A. aculeatus*³ and *A. tubingensis*¹⁴ have been reported to be able to split the saponified soy and apple xylogalacturonan, respectively. Moreover, one enzyme termed endo-xylogalacturonan hydrolase found in *A. tubingensis*,¹⁵ was able to cleave a xylose-substituted GalA backbone but not HG. These enzymes would be useful for structural analysis of plant cell walls as well as the processing of xylogalacturonan-rich materials. Degradation activity on AHG was also a new feature of *A. niger* exo-PGs. GalA and acetylated GalA as the reaction products could be separated by using a C18 column. To our knowledge, it is the first report on the degradation of AHG by an exo-PG. Exo-PGs could be applied for degradation of sugar beet pectin, which has a high DAC, in combination with other pectolytic enzymes and also for analysis of acetylation pattern in HG.

*Exo-arabinanase of P. chrysogenum able to release arabinobiose from α -1,5-L-arabinan.*¹⁶⁻¹⁸

1. Purification and properties of an exo-arabinanase.

The *P. chrysogenum* 31B strain has been found to produce at least six different enzymes which degrade arabinose-containing polymers. These include an exo-1,5- α -L-arabinanase (no EC number),¹⁶ two α -L-arabinofuranosidase (EC 3.2.1.55),¹⁹ an arabinoxylan arabinofura-

nohydrolase (EC 3.2.1.55), and two endo-1,5- α -L-arabinanses (EC 3.2.1.99).²⁰⁾ To the best of my knowledge, no article dealing with an exo-arabinanase has been published. Characteristics of an exo-arabinanase, termed Abnx, produced by this strain are described here.

Abnx was purified from a culture filtrate of *P. chrysogenum* 31B and had an apparent molecular mass of 47 kDa. For determination of the mode of the enzyme action towards linear arabinan, 0.08 mU of Abnx was incubated with 200 μ L of 0.2% reduced debranched arabinan in 20 mM acetate buffer (pH 5.0) at 37°C and the products from different times of reaction were analyzed by HPAEC. Only arabinobiose was detected from the early stage of enzyme reaction. Moreover, no arabinofuranosyl arabitol was formed in the course of degradation of reduced debranched arabinan (Fig. 3). These results indicated that Abnx cleaved α -1,5-L-linked arabinofuranose residues at their non-reducing terminus in an exo manner.

Enzyme activity towards arabinose-containing substrates was examined. The enzyme significantly released arabinobiose from debranched arabinan. Minor activity was detected on soybean arabinogalactan and branched L-arabinan. The enzyme did not cleave the following substrates: *p*-nitrophenyl α -L-arabinofuranoside, *p*-nitrophenyl α -L-arabinopyranoside, larch wood arabinogalactan, or α -1,5-L-arabinofuranobiose. The enzyme activity was 10 times higher on debranched arabinan than on branched L-arabinan. Considering the structure of the two substrates, these results indicated that activity of Abnx was interfered by arabinose side chains attached to arabinan backbones.

2. Molecular cloning and overexpression of an exo-arabinanase gene.

For determination of the nucleotide sequence of the *abnx* gene, which encodes Abnx, cassette-ligation mediated PCR was performed using primers constructed on the basis of the N-terminal and partial internal amino acid sequences of Abnx. A 1695-bp sequence from the putative ATG start codon of the *abnx* gene was determined (GenBank accession number AB096108). To clone the full-length *abnx* cDNA, two primers were synthesized based on the sequencing data of genomic DNA. The two primers were used for PCR amplification using the cDNA that

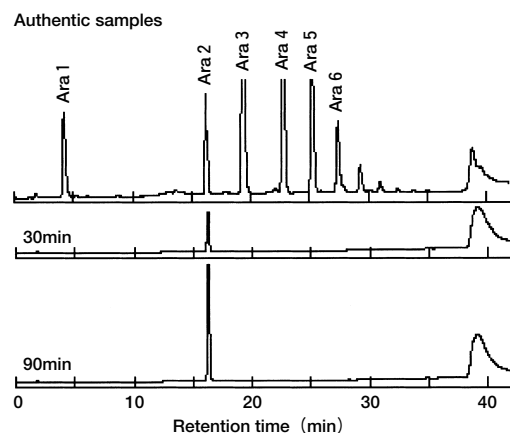


Fig. 3. Analysis of the enzymatic products of reduced debranched arabinan with Abnx.

Authentic samples: Ara1 to Ara6 represent arabinose to arabinohexaose, respectively.

was reverse transcribed from total RNA of *P. chrysogenum* 31B as the template. The coding sequence of the *abnx* gene contained 1134 bp which encodes a protein of 378 amino acids with a calculated molecular mass of 41,636 Da and a calculated isoelectric point of 5.11.

Arabinan-degrading enzymes have been mainly grouped into four glycosyl hydrolase (GH) families based on their amino acid sequence (GH 43, GH 51, GH 54 and GH 62) (<http://afmb.cnrs-mrs.fr/CAZY/index.html>). Four *A. niger* enzymes that are examples of each of these respective families are endo-1,5- α -L-arabinanase (*abnA*),²¹⁾ α -L-arabinofuranosidase (*abfA*),²²⁾ α -L-arabinofuranosidase (*abfB*)²³⁾ and 1,4- β -D-arabinoxylan arabinofuranohydrolase (*axhA*).²⁴⁾ However, the deduced amino acid sequence of Abnx has no similarity to the deduced sequences of enzymes belonging to these GH families. Although the identities of these amino acid sequences were low, comparisons of primary structures of proteins are generally not a reliable way to detect structural similarities. Therefore, a HCA was performed to more precisely classify Abnx. HCA allows detection of conserved secondary structure elements in proteins which show low sequence identity.^{25,26)} Abnx was compared with the mentioned four *A. niger* arabinan-degrading enzymes. The secondary structures were also predicted by PHDsec on the Internet (<http://cubic.bioc.columbia.edu/pp/>). No structural similarity was found between Abnx and the four *A. niger* enzymes (data not shown), demonstrating that Abnx is distinct from known arabinan-degrading enzymes in terms of protein structure. Recently, Abnx has been grouped into GH 93 with six hypothetical proteins (two from *A. nidulans*, two from *Neurospora crassa*, one from *Bacteroides thetaiotaomicron* and one from *Magnaporthe grisea*) and a *Streptomyces coelicolor* putative bifunctional protein.

In order to obtain the mature Abnx, the region amplified by PCR was ligated to the pMAL-c2X vector (New England BioLabs), forming a new plasmid termed pMAL-AX. Because the PCR product was inserted into a pMAL-c2X downstream from the *malE* gene, which encodes maltose binding protein (MBP), the recombinant Abnx expressed from pMAL-AX was expected to be a MBP-fusion Abnx. The recombinant protein in the cell-free extract of *E. coli* DH5 α transformed with the plasmid was purified using an Amylose Resin column (New England BioLabs).

3. Characteristics of the recombinant Abnx.

The kinetic parameters of the recombinant Abnx towards arabino-oligosaccharides with different DPs (DP 2

Table 3. Kinetic parameters of the recombinant Abnx towards arabino-oligosaccharides with different DPs.

Substrate	K_m (mM)	k_{cat}/K_m ($s^{-1} \cdot mM^{-1}$)
DP3	1.01	3.01
DP4	0.85	11.53
DP5	0.63	17.80
DP6	0.33	39.22
DP7	0.36	32.62

In a typical assay, 0.168 μ g of the enzyme was incubated with various concentrations of arabino-oligosaccharide as the substrate. The data were analyzed by Lineweaver-Burk plots using a least-squares linear regression.

to DP 7) were examined at pH 5.0 and 37°C. Table 3 shows that k_{cat}/K_m increased with increasing of DP, reaching the highest value at DP 6.

In order to study the action pattern of the recombinant Abnx, product formation at different reaction times was monitored with HPAEC using α -1,5-L-arabinoheptaose as the substrate. Based on the mode of action of Abnx, the substrate α -1,5-L-arabinoheptaose would be expected to yield arabinopentaose, arabinotriose, arabinobiose and arabinose. During the early stage of the enzyme reaction, arabinobiose and arabinopentaose, but not arabinotriose, were formed. During later stages of the reaction, arabinobiose and arabinotriose accumulated and a small amount of arabinose was detected (Fig. 4). Generally enzymes degrade substrates in either multi-chain or single-chain attack mode. The data obtained here suggest that Abnx acts in a multi-chain attack mode and that arabinotriose is more resistant to the enzyme than larger oligomers as demonstrated in Table 3.

Acceptor specificity of Abnx was examined by incubat-

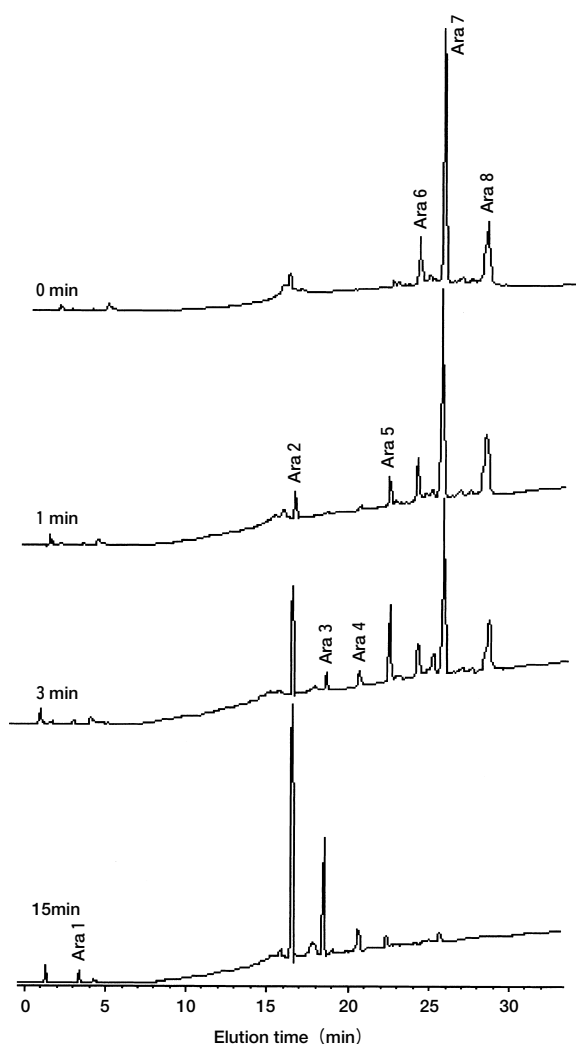


Fig. 4. Analysis of the enzymatic products of α -1,5-L-arabinoheptaose with the recombinant MBP-fusion Abnx.

A reaction mixture containing 7 μ g of the enzyme and 100 μ L of 5 mM arabinoheptaose in 20 mM acetate buffer (pH 5.0) was incubated at 37°C for the times shown. Aliquots were taken at intervals and inactivated by boiling for 5 min, followed by analysis of the reaction products with HPAEC. Ara1 to Ara8 represent arabinose to arabinooctaose, respectively.

ing acceptors including aliphatic alcohols, sugars and sugar alcohols, with the recombinant Abnx in the presence of debranched arabinan. The transglycosylation activities of the enzyme towards various compounds are summarized in Table 4. Abnx was able to synthesize transfer products in the presence of most alcohols tested. Comparison of the transglycosylation activities towards C4 alcohols can help to determine specificity of the enzyme. Monohydroxybutanes such as 1-butanol and 2-butanol were not good acceptors. Abnx efficiently transferred arabinobiose to 1,2-butanediol and 1,3-butanediol but not to 2,3-butanediol. These results suggest that the enzyme preferred the primary hydroxyl group in polyhydroxybutanes as acceptors. When sugars were used as acceptors, two or three transfer products were detected. The transfer activity of Abnx was higher towards sugar alcohols than towards sugars. Among the tested compounds, glycerol was the best acceptor for transglycosylation with Abnx.

Large-scale synthesis of transfer products was done to identify the structure of the transfer products of glycerol. The reaction was carried out at pH 4 and 40°C in the presence of 20% glycerol and 1% debranched arabinan. Two transfer products were synthesized at a ratio of 11:1 (Table 4). In this study, only the major product, termed GA, was isolated by a Sep-Pak Vac 20 cc (NH_2 cartridges; Waters) and a CarboPac PA-1 column. FAB-MS spectra of GA gave a significant molecular ion peak at m/z 357 $[\text{M}+\text{H}]^+$. Adding NaI solution, an adduct ion peak m/z 379 $[\text{M}+\text{Na}]^+$ was observed. This data was consistent with a molecular formula of $\text{C}_{13}\text{H}_{24}\text{O}_{11}$ (molar mass: 356) and the structure of arabinobiosyl glycerol. The structure of GA was confirmed by ^1H - and ^{13}C -NMR spectroscopy.

Table 4. Transglycosylation activity of Abnx on various compounds.

Acceptor	Area of product		
	Arabinobiose	Major transfer product	2nd-major transfer product
None	214	—	—
Methanol	125	25	—
Ethanol	113	6	—
1-Propanol	18	3	—
1,2-Propanediol	96	56	5
1,3-Propanediol	64	83	—
1-Butanol	43	6	—
2-Butanol	53	—	—
1,2-Butanediol	47	44	6
1,3-Butanediol	77	60	3
1,4-Butanediol	72	18	—
2,3-Butanediol	119	10	—
1-Pentanol	85	4	—
Glycerol	70	182	17
Sorbitol	160	104	—
Xylitol	139	141	4
Arabinose	126	33	30
Fructose	163	57	20
Galactose	141	26	24
Glucose	118	49	25
Rhamnose	141	29	—
Xylose	105	31	6

Areas of the products released into the mixture were determined by HPAEC. The area ratios given are not true product ratios although PAD responses are often approximately equimolar.

All signals were assigned by ^1H - and ^{13}C -NMR, DEPT (distortionless enhancement by polarization transfer), and two dimensional NMR methods. The ^1H - and ^{13}C -NMR spectral data of GA are shown in Table 5. Four sets of carbon signals from hydroxymethyl groups were detected at δC 63.1, 64.3 (64.3), 68.2 and 70.2 (70.25). These signals were consistent with the provisional structure, which would give signals from C-5 and C-5'' of the two arabinosyl residues, and also from the two glyceryl terminal carbons C-1' and C-3' (made non-equivalent by arabinosylation) (Fig. 5). In addition, arabinosylation would make the C-2' of the glyceryl residue asymmetric, leading to formation of two diastereomeric arabinobiosyl glycerol molecules, and two signals for both C-1' and C-3' (data in parentheses above for one diastereomer). The signal at δC 68.2 was correlated to the anomeric proton C-1'' at δH 4.93 by a COLOC (correlation spectroscopy via long-

Table 5. ^1H - and ^{13}C -NMR spectral data for the transfer product (GA).

	Position	^{13}C -NMR δC	^1H -NMR δH (integral, mult., J Hz)
Glycerol	1'	70.20 ^a	3.43 (1H, m) 3.77 (1H, m)
		70.25 ^a	3.50 (1H, dd, $J = 4.3, 10.4$ Hz) 3.69 (1H, dd, $J = 6.4, 10.4$ Hz)
	2'	72.11 ^b	3.78 (1H, m)
		72.20 ^b	3.76 (1H, m)
	3'	64.27 ^c	3.53 (1H, dd, $J = 7.0, 11.3$ Hz) 3.58 (1H, dd, $J = 1.8, 11.3$ Hz)
		64.32 ^c	3.52 (1H, dd, $J = 7.0, 11.3$ Hz) 3.57 (1H, dd, $J = 1.8, 11.3$ Hz)
68.21			3.65 (1H, dd, $J = 4.0, 11.0$ Hz) 3.85 (1H, dd, $J = 5.2, 11.0$ Hz)
Arabinose (5-linked)	1	109.80 ^d	4.88 (1H, d, $J = 1.5$ Hz)
		109.96 ^d	4.88 (1H, d, $J = 1.5$ Hz)
	83.23	3.98 (1H, dd, $J = 1.5, 3.1$ Hz)	
	79.18	3.90 (1H, dd, $J = 3.1, 5.8$ Hz)	
	84.22 ^e	4.05 (1H, m)	
Arabinose (non-reducing end)	1''	109.63	4.93 (1H, d, $J = 1.5$ Hz)
		83.14	3.99 (1H, dd, $J = 1.5, 3.1$ Hz)
	78.71	3.83 (1H, dd, $J = 3.1, 5.8$ Hz)	
	85.86	3.97 (1H, dd, $J = 3.4, 5.8$ Hz)	
	63.05	3.63 (1H, dd, $J = 5.5, 11.9$ Hz) 3.74 (1H, dd, $J = 3.4, 11.9$ Hz)	

The spectra were measured at 500 MHz for ^1H and at 125 MHz for ^{13}C . ^{a-c} Superscripted data are for pairs of diastereotopic carbons (see text).

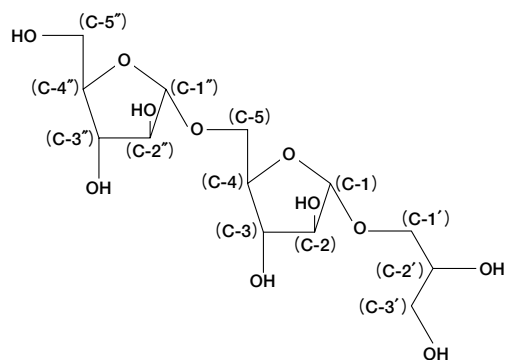


Fig. 5. The postulated structure of GA.

The numbers in the parentheses show the carbon assignments used in the text.

range coupling) spectrum. Likewise the signal at δC 70.20 (70.25) was correlated to other anomeric proton C-1 at δH 4.88 in the spectrum. These data indicated that the arabinobiosyl residue was linked to a primary hydroxyl of glycerol. Three peaks appeared in the region for anomeric carbons of the arabinose residues: the signal at δC 109.6 was assigned to the non-reducing end arabinose and the other two peaks (at δC 109.8 and 110.0) were assigned to the 5-linked arabinose residues. The integrated areas of the latter two ^{13}C -NMR peaks were similar, indicating that the two diastereomers were formed in almost equal amounts. The two glycosidic linkages in GA were determined to be the α -configuration on the basis of the coupling constants ($J = 1.5$ Hz) of the anomeric protons. Accordingly, the structure of GA was elucidated to be *O*- α -L-arabinosyl-(1 \rightarrow 5)-*O*- α -L-arabinosyl-(1 \rightarrow 1)-glycerol, demonstrating that the anomeric configuration of arabinose is conserved in the hydrolysis of linear arabinan by Abnx.

In conclusion, Abnx secreted by *P. chrysogenum* 31B is an unusual arabinan-degrading enzyme with respect to both its mode of action and protein structure. The enzyme catalyzes the hydrolysis of linear arabinan without inverting the anomeric configuration.

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ン酸残基は部分的にメタノールや酢酸、キシロースで修飾されている。本研究では *A. niger* より 2 種の Exo-PG を単離し、種々のタイプのペクチン質に対する特異性を検討した。その結果、両酵素ともにキシロガラクトクロナンおよびアセチル化ホモガラクトクロナンを末端から切断し、キシロガラクトクロン酸およびアセチル化ガラクトクロン酸を生成することを ESI-ITMS 分析等により明らかにした。すなわち、Exo-PG はホモガラクトクロナンに結合するキシロースや酢酸によって反応が妨害されないことが判明し、ペクチン中の修飾基分布の解析に有効であることが示唆された。(2) *Penicillium chrysogenum* 由来のエキソアラビナーゼの機能解析：直鎖状還元型アラビナンを基質として、本酵素の反応産物を HPAEC 分析した結果、本酵素は非還元末端よりピオース単位で切断することが明らかとなった。DP 7 を基質として、酵素反応産物の経時変化を分析することにより、本酵素は“multi-chain attack mode”で基質に作用することを明らかにした。種々のアルコール、糖の存在下で直鎖型アラビナンに本酵素を作用させると、アラビノピオース転移能を示した。グリセリンを受容体とした場合の主要転移産物を単離し、その構造を NMR 等により *O*- α -L-arabinosyl-(1 \rightarrow 5)-*O*- α -L-arabinosyl-(1 \rightarrow 1)-glycerol と決定した。このことから本酵素はアノマー保持型酵素であることが証明された。さらに本酵素遺伝子の塩基配列を決定し、アミノ酸配列相同性検索と疎水性クラスター解析の結果から、本酵素は既報のアラビナン分解酵素とは異なるタンパク構造を有していることが判明し、7 種の機能未知タンパク質とともに新たに GH ファミリー 93 が形成された。

【質問】

信州大 北畑

Fig. 2 において、エキソポリガラクトクロナーゼにより生成するガラクトクロン酸とキシロガラクトクロン酸の生成比率は基質のキシロガラクトクロナンの構造と矛盾しないですか。

【答】

基質に用いたキシロガラクトクロナン中の全 GalA 残基のうち 28% がフリー、53% がキシロース、19% がキシロピオースで修飾されています。この数値と反応液組成を考慮すると、酵素反応に使用した基質中には GalA, GalA-Xyl, GalA-Xyl2 の濃度はそれぞれ 96, 319, 163 $\mu\text{g}/\text{mL}$ と計算できます。Exo-PG により 48 時間後に遊離された GalA と GalA-Xyl の濃度はそれぞれ 40.1 と 60.2 $\mu\text{g}/\text{mL}$ であり、基質中の 42% の GalA と 19% の GalA-Xyl が遊離されたこととなります。基質全体としては 22% の α -1,4-GalA 結合が切断された計算となります。また、Fig. 2 において反応前半では GalA の生成比率が高いことから、基質中のキシロースによる修飾は均一ではなく、部分的に集中している領域が存在しているのではないかと考えて

カビの生産する新規機能を有する エキソ型酵素に関する研究

阪本龍司¹

¹ 大阪府立大学大学院生命環境科学研究科

(599-8531 堺市学園町 1-1)

(1) *Aspergillus niger* 由来のエキソポリガラクトクロナーゼ (Exo-PG) の機能解析：ペクチン中のガラクトクロ

おります。

【質問】

石川県大 谷口

Abnx をアラビノースヘプタオースに作用させたとき、アラビノースは生成しないのでしょうか？また、アラビノトリオースに作用させたとき、アラビノビオースは多量に生成するのでしょうか？

【答】

DP 3-7 のアラビノオリゴ糖に対する特異性を調べた結

果、本酵素は重合度に比例して活性は高くなり、DP 6 で最大活性を示し、DP 3 に対しては微弱であります (Table 3)。また、本酵素にはアラビノビオース分解活性はありません。したがって、アラビノヘプタオースを基質とした場合には主要反応産物としてアラビノビオースとアラビノトリオースを蓄積します (Fig. 4)。しかし、過剰の酵素反応を行いますと、アラビノトリオースはアラビノースとアラビノビオースに分解します。