Exo-Arabinanase of *Penicillium chrysogenum* Able To Release Arabinobiose from α-1,5-L-Arabinan

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An exo-arabinanase, designated Abnx, was purified from a culture filtrate of *Penicillium chrysogenum* 31B by ammonium sulfate precipitation, anion-exchange chromatography, and hydrophobic chromatography. Abnx had an apparent molecular mass of 47 kDa. The enzyme released only arabinobiose from the nonreducing terminus of α-1,5-L-arabinan and showed no activity towards *p*-nitrophenyl-α-L-arabinofuranoside and α-1,5-L-arabinofuranobiose. Abnx is the first enzyme with this mode of action.

Arabinose residues are found in arabinans, arabinogalactans, or arabinoxylans in many plant cell walls. In sugar beet arabinan, L-arabinose residues are linked to form α-1,5-L-arabinan to which L-arabinofuranose units are attached mainly at position 3 in the α-configuration as side chains. Soybean arabinogalactans have a linear chain of β-1,4-D-galactan to which α-1,5-L-linked arabinofuranooligosaccharides are bound in side chains. Arabinogalactan in larch wood consists of β-1,3-galactan to which β-1,3-linked arabinooligosaccharides or β-1,6-linked galactooligosaccharides are attached at position 6 as side chains.

Arabinose-containing polymers are degraded by various enzymes, which have been classified into six types depending on their mode of action and substrate specificity by Beldman et al. (3), as follows: (i) α-L-arabinofuranosidase (EC 3.2.1.55), which is not active with polymers (10, 22); (ii) α-L-arabinofuranosidase, which is active with polymers (9, 16); (iii) α-L-arabinofuranohydrolase, which is specific for arabinoxylans (11, 20); (iv) exo-α-L-arabinanase, which is not active with *p*-nitrophenyl-α-L-arabinofuranoside (8, 13); (v) β-L-arabinopyranosidase (4); and (vi) endo-1,5-α-L-arabinanase (EC 3.2.1.99) (7, 21). Of these enzymes, little is known about exo-α-L-arabinanases. This study dealt with isolation and characterization of an exo-arabinanase, designated Abnx, that is produced by *Penicillium chrysogenum* 31B, which was isolated from rotten sugar beet. Interestingly, filtrate from a culture of this microorganism contained at least five different arabinan-degrading enzymes. The work described here was the first step in characterizing these enzymes and should be followed by elucidation of the mode of degradation of sugar beet arabinan by this strain.

Three liters of a liquid medium consisting of 0.2% NH$_4$NO$_3$, 0.1% K$_2$HPO$_4$, 0.05% MgSO$_4 \cdot$ 7H$_2$O, 0.05% KCl, 0.001% FeSO$_4$, 0.1% peptone, 0.1% glucose, and 2% sugar beet pulp (pH 5.0) was inoculated with precultured *P. chrysogenum* 31B and incubated at 30°C for 12 days under static conditions in a 5-liter Erlenmayer flask. The culture filtrate was concentrated by ultrafiltration (10-kDa cutoff), dialyzed against 20 mM acetate buffer (pH 5.0), and used for enzyme purification. A typical assay for arabinan-degrading activity was performed by measuring the release of reducing groups in a reaction mixture containing 195 μl of 0.1% debranched arabinan (Megazyme International Ireland Ltd., Wicklow, Ireland) in 20 mM acetate buffer (pH 5.0) and 5 μl of enzyme sample at 37°C. Reducing sugars were measured by the method of Somogyi (18). One unit of enzyme activity was defined as the amount of enzyme that formed reducing groups corresponding to 1 μmol of L-arabinose in 1 min. For the first step, pulverized crystal ammonium sulfate was added to the enzyme solution to 80% saturation at a rate of approximately 2 g/min. The precipitate was recovered by centrifugation at 6,000 × *g* for 15 min, dissolved in 20 mM acetate buffer (pH 5.0), and dialyzed against the same buffer. The enzyme solution was loaded onto a DEAE-Toyopearl 650M column (4 by 10 cm; Tosoh Corp., Tokyo, Japan) equilibrated with the dialysis buffer. Although 60% of the initial arabinan-degrading activity did not bind to this column, this fraction is not described further here. The bound enzymes were eluted by a 500-ml linear 0 to 0.4 M NaCl gradient in the same buffer. The arabinan-degrading activity eluted from the DEAE-Toyopearl 650M column as two peaks. The peak eluting at a lower NaCl concentration was designated Abnx and purified further. The enzyme solution was then dialyzed against the buffer described above and concentrated under reduced pressure. Pulverized crystal ammonium sulfate was added to the concentrate to 30% saturation, and the enzyme solution was loaded onto a Phenyl Superose HR 5/5 column (Amersham Pharmacia) equilibrated with 20 mM acetate buffer (pH 5.0) containing ammonium sulfate at 30% saturation. The adsorbed proteins were eluted by a linear ammonium sulfate gradient (30 to 0% saturation) at a flow rate of 0.5 ml/min. The arabinanase-containing fractions were pooled, dialyzed against 20 mM acetate buffer (pH 5.0), concentrated, and put on a Mono Q HR 5/5 column (Amersham Pharmacia) equilibrated with the dialysis buffer. The bound proteins were

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TABLE 1. Purification of Abnx from *P. chrysogenum* 31B

Purification step	Protein (mg)	Activity (U)	Sp act (U/mg)	Purification (fold)	Yield (%)
Culture filtrate	473.0	228.0	0.48	1	100
$(NH_4)_2SO_4$	199.0	211.0	1.06	2.2	92.5
DEAE-Toyopearl	31.7	18.9	0.60	1.3	8.3
Phenyl Superose	2.6	5.2	2.00	4.2	2.3
Mono Q	0.6	2.5	4.17	8.7	1.1

eluted by a linear 0 to 0.15 M NaCl gradient at a flow rate of 1 ml/min.

The purification procedure for Abnx is summarized in Table 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the purified enzyme showed that there was a single protein band at a molecular mass of 47 kDa. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by the method of Laemmli (12) with a discontinuous 10% poly-acrylamide gel.

To study the effects of pH and temperature on enzyme activity, the enzyme reaction was performed at various pHs by using 20 mM acetate buffer (pH 3 to 5) and 20 mM phosphate buffer (pH 6 to 7) at 37°C and at various temperatures in 20 mM acetate buffer (pH 5.0). Optimum activity occurred at pH 4.0 and 40°C. Temperature stability was evaluated by measuring the residual activity after 1 h of preincubation of the enzyme (158 µg/ml) at temperatures between 30 and 70°C in 20 mM acetate buffer (pH 5.0). The enzyme was stable at temperatures up to 50°C. pH stability was studied by preincubating the enzyme (75 µg/ml) at 30°C for 16 h at various pHs, using 100 mM HCl-KCl buffer (pH 1 to 2), acetate buffer (pH 3 to 5), phosphate buffer (pH 6 to 8), and Na_2CO_3-$NaHCO_3$ buffer (pH 9 to 11). More than 80% of the initial enzyme activity remained at pH 3 to 8. The sensitivity of Abnx to metals was examined by adding compounds at a concentration of 1 mM to the reaction mixture for the standard arabinanase assay. $HgCl_2$ caused a loss of 70% of the enzyme activity. No effect on activity was detected with the chloride salts of Ba^{2+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Fe^{3+}, K^+, Mg^{2+}, Na^+, Ni^{2+}, and Zn^{2+}, $AgNO_3$, and $CuSO_4$. NaN_3 (1 mM) had no effect on the activity.

To determine the mode of action of the enzyme with linear arabinan, 0.08 mU of Abnx was incubated with 200 µl of 0.2% reduced debranched arabinan in 20 mM acetate buffer (pH 5.0) at 37°C, and after different times the products were analyzed by high-performance anion-exchange chromatography using a Carbopac PA-1 column (Dionex). Sugars were eluted at a flow rate of 1 ml/min with 0.1 M NaOH for 5 min and then with a 30-ml linear 0 to 0.45 M sodium acetate gradient in 0.1 M NaOH. The effluent was monitored with pulsed ampero-metric detection. Only arabinobiose was detected during the early stage of the enzyme reaction. Moreover, no arabino-furanosyl arabitol was formed during degradation of reduced debranched arabinan (Fig. 1). These results indicated that Abnx cleaved α-1,5-L-linked arabinofuranose residues at the nonreducing terminus in an exo manner. Reducing ends of debranched arabinan were reduced by treatment with 10 mM $NaBH_4$ in 25 mM NaOH at 30°C for 4 h (19).

Substrate specificity data for the enzyme are summarized in Table 2. Arabinosidase activity was tested by incubating 5 µl (10 mU) of the enzyme with 195 µl of 0.2% p-nitrophenyl-α-
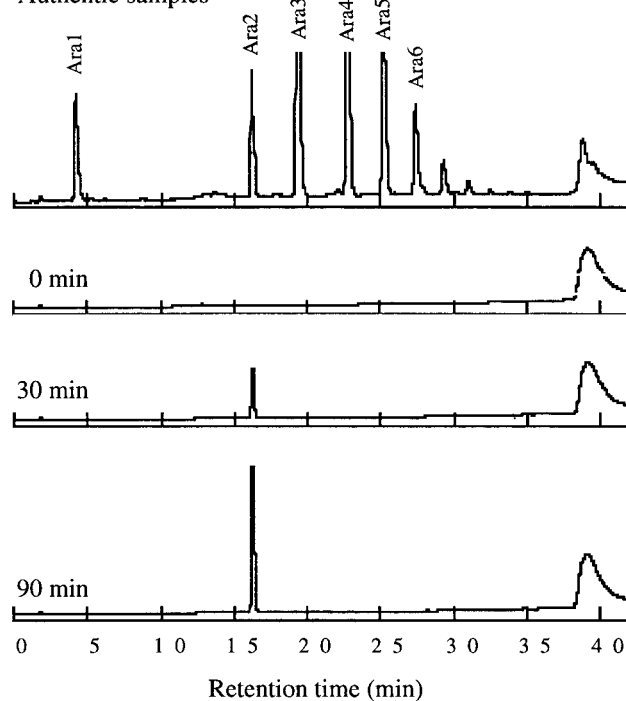

FIG. 1. Analysis of the enzymatic products of reduced debranched arabinan obtained with Abnx. Authentic samples Ara1 to Ara6 represent arabinose to arabinohexaose, respectively.

L-arabinofuranoside or p-nitrophenyl-α-L-arabinopyranoside (Sigma) in 20 mM acetate buffer (pH 5.0) at 37°C overnight. p-Nitrophenol release was monitored spectrophotometrically at 420 nm after 1.8 ml of 0.2 M Na_2CO_3 was added to the reaction mixture. To determine degradation activity with α-1,5-L-arabinofuranobiose (Megazyme), triose (Megazyme), and various polysaccharides, 4 mU of Abnx was incubated with 200 µl of 0.2% substrate in 20 mM acetate buffer (pH 5.0) for 1 h at 37°C, and then the reaction products were analyzed by high-performance anion-exchange chromatography under the conditions described above. The enzyme released significant amounts of arabinobiose from debranched arabinan and α-1,5-L-arabinofuranotriose. Minor activity was detected with sugar beet arabinan (Megazyme) or soybean arabinogalactan, which was prepared as previously described (14). The enzyme did not

TABLE 2. Enzyme activity of Abnx with various substrates

Substrate	Amt of sugar released (µg/ml)[a]
p-Nitrophenyl-α-L-arabinofuranoside	0
p-Nitrophenyl-α-L-arabinopyranoside	0
Sugar beet debranched arabinan	455
Sugar beet arabinan	42
Soybean arabinogalactan	57
Larch wood arabinogalactan	0
α-1,5-L-Arabinofuranobiose	0
α-1,5-L-Arabinofuranotriose	252

[a] Concentration of arabinobiose released into the reaction mixture. Details of the methods used for the enzyme assays are described in the text. The values are averages based on three experiments.

cleave the following substrates: larch wood arabinogalactan (Sigma), *p*-nitrophenyl-α-L-arabinofuranoside, *p*-nitrophenyl-α-L-arabinopyranoside, and α-1,5-L-arabinofuranobiose. Considering the structure of the substrates, these results indicated that Abnx degraded α-1,5-L-linked arabinofuranose residues more than it degraded trimers.

Two exo-arabinanases have been found previously, one in *Erwinia carotovora* IAM 1024 (8) and one in *Pseudomonas fluorescens* subsp. *cellulosa* (13). The former produces only arabinotriose from sugar beet arabinan but not from linear arabinan. In contrast, the latter is not active with sugar beet arabinan, but with linear arabinan it produces arabinotriose. Abnx was similar to the exo-arabinanase from *P. fluorescens* in terms of substrate specificity, except that Abnx produced arabinobiose from linear arabinan. *Enzyme Nomenclature* includes numbers for the following five enzymes that catalyze the release of dimeric sugars from polysaccharides in an exo manner: β-amylase (EC 3.2.1.2) (2), exo-poly-α-galacturonosidase (EC 3.2.1.82) (6), cellulose 1,4-β-cellobiosidase (EC 3.2.1.91) (5), glucan 1,6-α-isomaltosidase (EC 3.2.1.94) (17), and mannan 1,4-β-mannobiosidase (EC 3.2.1.100) (1). Furthermore, Nakano et al. have isolated exo-1,4-β-D-galactanase (which does not have an EC number) from *Bacillus subtilis*; this enzyme releases predominantly β-galactobiose from soybean arabinogalactan (15). No arabinanases which produce arabinobiose from arabinan have been found previously. The Abnx described here may be the first enzyme with its mode of action.

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