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Note

The purification of commercially available endo-α-L-arabinanases and α-L-arabinosidase for use in the structural analysis of pectic polysaccharides

Patrice Lerouge [1], Malcolm A. O'Neill, Alan G. Darvill and Peter Albersheim *

Complex Carbohydrate Research Center, The University of Georgia, 220 Riverbend Road, Athens, GA 30602-4712 (USA)

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Commercially available glycanases have considerable potential for use in the structural analysis of complex carbohydrates[1,2]. However, these preparations often contain mixtures of both endo- and exo-glycanases that can limit their use in structural studies. For example, we have found that arabinosyl-containing oligosaccharides, released by treating rhamnogalacturonan I (RG-I), a plant cell wall pectic polysaccharide, with a commercially available fungal endo-α-(1 → 5)-L-arabinanase, were hydrolyzed by a contaminating a-L-arabinofuranosidase[3].

Fungal arabinases have been purified to apparent homogeneity by fast protein liquid chromatography (FPLC)[4] although their use for the fragmentation of plant cell wall polysaccharides was not reported. We now report that both an endo-arabinanase and an α-L-arabinosidase can be obtained, by FPLC, in a level of purity suitable for the structural analysis of RG-I and arabinans. The activities of the endo-arabinanase and arabinosidase were monitored colorimetrically and by using a method based on high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE–PAD).

The endo-arabinanase and arabinosidase used in this investigation were both obtained from commercial preparations purified from the enzymes secreted by the fungus *Aspergillus niger*. The partially purified endo-arabinanase fragmented a linear (1 → 5)-α-L-arabinan to give a mixture of arabinose oligomers and free arabinose. The presence of free arabinose suggested that the endo-arabinanase preparation contained arabinosidase activity. The commercial preparation also released small amounts of free galactose, xylose, and galacturonic acid from sycamore RG-I. Thus, in addition to arabinosidase, the endo-arabinanase prepara-

[1] Present address: LPTF-CNRS 118, route de Narbonne, 31062 Toulouse, France.
* Corresponding author.

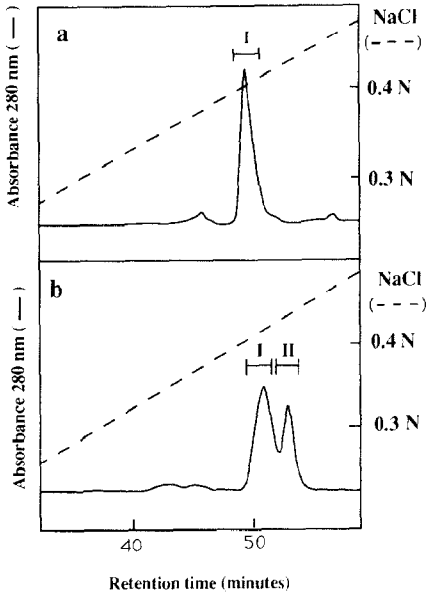

Fig. 1. Purification, by FPLC on a Mono-Q column, of (a) the commercial endo-arabinanase and (b) the commercial arabinosidase. The enzymes were eluted from the column with a linear gradient of 0–0.5 M NaCl, and the eluant was monitored by absorption at 280 nm. The individual fractions were pooled as shown by the bars.

tion also appeared to contain galactosidase, xylosidase, and galacturonosidase.

The endo-arabinanase was resolved into one major and several minor peaks by FPLC on a Mono-Q column (Fig. 1a). Only the major peak (Fraction I, see Fig. 1a), which gave a single bond when analyzed by SDS-PAGE, fragmented a linear (1 → 5)-α-L-arabinan. The arabinan was partially fragmented within 2 min into oligoarabinosides with degrees of polymerization (dp's) between 2 and 20 (Fig. 2a). (We have assumed that oligosaccharides composed of (1 → 5)-linked α-L-arabinosyl residues are eluted from the CarboPac column in order of increasing molecular weight.) Oligoarabinosides with dp's between 2 and 5 were the major products detected after a 15-min incubation (Fig. 2b). This oligosaccharide profile was not altered after even prolonged periods of incubation with the purified endo-arabinanase. Most importantly, only trace amounts of free arabinose were detected in the enzymic digest, which established that the endo-arabinanase was essentially free of arabinosidase. The purified endo-arabinanase released, from sycamore RG-I, $< 1\%$ of the galactosyl residues and $\approx 20\%$ of the xylosyl residues indicating that the galactosidase and xylosidase were not completely removed. Galactosyl and xylosyl residues account for ≈ 31 and ≈ 1 mol%, respectively, of sycamore RG-I. Thus, these contaminating enzymes were present at levels sufficiently low to allow us to use the endo-arabinanase to generate arabinosyl-containing oligosaccharides from RG-I[3].

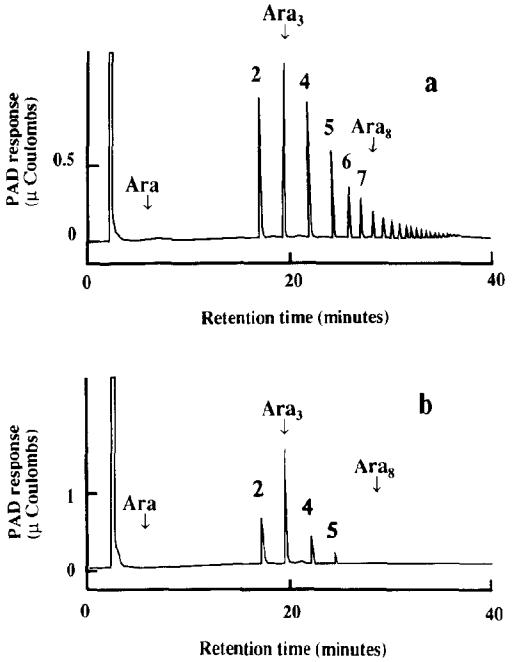

Fig. 2. HPAE–PAD analysis of the products released by treating a linear (1 → 5)-linked α-L-arabinan with the purified endo-arabinanase for (a) 2 and (b) 15 min. The CarboPac PA-1 column was calibrated with authentic arabinose (Ara), (1 → 5)-linked α-L-arabinotriose (Ara_3), and α-L-arabinooctaose (Ara_8). The numbers above each peak correspond to the assumed dp of the oligoarabinoside in that peak.

The commercial α-L-arabinosidase preparation hydrolyzed the *p*-nitrophenyl derivative of α-L-arabinofuranose. In addition the enzyme preparation released arabinose from arabinan. Thus, the commercial preparation contains both arabinosidase and exo-arabinanase activities. The enzyme mixture was resolved into two major peaks by FPLC on a Mono-Q column (Fractions I and II, see Fig. 1b). Fractions across each peak were assayed for their ability to release arabinose from (1 → 5)-α-L-arabinofuranotriose and from a linear (1 → 5)-linked α-L-arabinan. Fractions I and II both hydrolyzed the (1 → 5)-α-L-arabinofuranotriose to give a mixture of mono-, di-, and tri-arabinosides. This result is consistent with a previous study[4] showing that *A. Niger* secretes two α-L-arabinosidases. Neither of the two fractions released arabinose from the linear arabinan. Thus, the contaminating exo-arabinanase activity was eliminated using a one-step FPLC procedure. The two active arabinosidase fractions were separately pooled and re-chromatographed on the Mono-Q column under the same conditions. Fraction I contained the most α-L-arabinosidase activity. This arabinosidase hydrolyzed (1 → 5)-α-L-arabinotriose, after 3 h of incubation, to free arabinose and small amounts of arabinobiose and arabinotriose (Fig. 3). This purified arabinosidase, in combination with the purified endo-arabinanase, has been used to release 95% of the arabinosyl residues from RG-I[3].

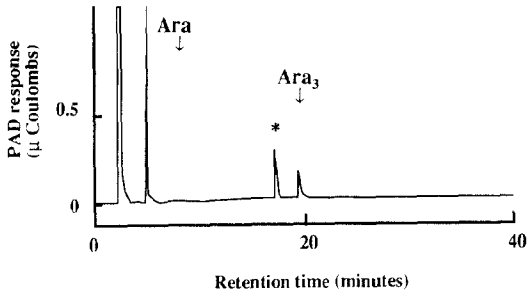

Fig. 3. HPAE–PAD analysis of the products released by treating (1 → 5)-linked α-L-arabinotriose with the purified arabinosidase (Fraction I, see Fig. 1b). The CarboPac PA-1 column was calibrated with authentic arabinose (Ara) and (1 → 5)-linked α-L-arabinotriose (Ara_3). The component eluting at 17.1 min (*) is assumed to be (1 → 5)-linked α-L-arabinobiose.

The ability to rapidly purify glycanases and to monitor their hydrolytic activities has been improved by the development of FPLC and HPAE–PAD. The endo-arabinanase, in particular, has considerable potential for the structural analysis of plant cell wall pectic polysaccharides and arabinans. HPAE–PAD has become a powerful technique for monitoring the activities of glycanases[5,6]. We have used HPAE–PAD as a sensitive procedure for monitoring the activities of the endo-arabinanase and exo-arabinosidase using authentic oligo- and poly-saccharides as well as a plant cell wall polysaccharide as substrates. HPAE–PAD can resolve oligosaccharides composed of up to 20 (1 → 5)-linked α-L-arabinosyl residues. Thus, this method is a considerable improvement over a previously reported HPLC procedure[7] in which oligomers larger than arabinotetraose could not be separated. Furthermore, the HPAE–PAD procedure has been used to assist in the isolation of some of the endo-arabinanase-generated products of RG-I in amounts sufficient for structural characterization[3].

EXPERIMENTAL

Materials.—The endo-arabinanase and arabinosidase from *A. niger*, linear (1 → 5)-α-L-arabinan, (1 → 5)-α-L-arabinotriose, and (1 → 5)-α-L-arabinooctaose were all purchased from Megazyme (North Rocks, Australia). The enzymes were dialyzed (1000 molecular weight cut-off tubing) against 25 mM Bis Tris · HCl, pH 6, at 4°C to remove ammonium sulfate prior to purification.

Purification of the enzymes by FPLC.—The endo-α-(1 → 5)-arabinanase and α-L-arabinosidase were separately purified on a Mono-Q column using a Pharmacia FPLC system. The column was equilibrated in 25 mM Bis Tris · HCl, pH 6. The enzyme (~ 1 mg protein) in 25 mM Bis Tris · HCl, pH 6 (1 mL), was loaded and eluted at 0.5 mL/min with a linear 60-min gradient from 0 to 0.5 M NaCl in 25 mM Bis Tris · HCl, pH 6. The column eluant was monitored by absorption at 280 nm, and fractions (250 μL) were collected.

Arabinosidase assay.—Solutions of (1 → 5)-α-L-arabinotriose (100 μg) in 50 mM NH_4OAc, pH 4 (500 μL), were separately treated with either the dialyzed arabinosidase (0.2 Units; 1 Unit of enzyme releases 1 μmol of arabinose reducing-sugar equivalents per minute from 5 mM *p*-nitrophenyl-α-L-arabinofuranoside at pH 4 and 40°C) or the individual FPLC fractions (10 μL) and incubated at 40°C. Aliquots (10 μL) of the digest were taken after 1 and 2 h, mixed with 10 μL of 0.2 M NaOH, and analyzed by HPAE–PAD.

Endo-arabinanase assay.—An aliquot (25 μL) of each FPLC fraction was incubated with arabinan (500 μg) in 100 mM NaOAc, pH 4 (100 μL), for 15 min. The amount of reducing sugar liberated was determined colorimetrically[8].

Analysis of the products released by endo-arabinanase treatment of a linear (1 → 5)-linked-α-L-arabinan.—Solutions of arabinan (1 mg) in 50 mM NH_4OAc, pH 4 (1 mL), were separately incubated at 40°C with either the dialyzed endo-arabinanase (32 mUnits; 1 Unit of endo-arabinanase releases 1 μmol of arabinose reducing-sugar equivalents per minute from a 0.6% solution of linear arabinan at pH 4 and 40°C) or FPLC purified endo-arabinanase (10 μL). After 2 and 15 min, aliquots (10 μL) of each digest were separately mixed with 10 μL of 0.2 M NaOH and analyzed by HPAE–PAD. The dp of the arabinose-containing oligomers released by enzymic hydrolysis was determined by using a CarboPac PA-1 column calibrated with authentic arabinotriose and arabinooctaose.

HPAE–PAD.—HPAE–PAD was performed with a Dionex metal-free BioLc interfaced to an AutoIon Series 400 data station. Carbohydrates were separated on a Carbopac PA-1 column and detected using a pulsed electrochemical detector equipped with a gold working electrode. To facilitate the detection of carbohydrates, 400 mM NaOH was added post-column at a flow-rate of 0.6 mL/min. Carbohydrates were eluted at 1 mL/min with 100 mM NaOH for 5 min then with a 40-min linear gradient to 600 mM NaOAc in 100 mM NaOH.

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