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Note

A simple, rapid, and semiquantitative assay for the determination of *endo*-(1 → 5)-α-L-arabinanase (EC 3.2.1.99) activity in column chromatography eluate fractions

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Column chromatographic (CC) purification procedures of polysaccharide degrading enzymes often entail a large number of determinations of enzymatic activity. These determinations have primarily been performed by hydrolysis of a specific substrate using a reducing sugar colorimetric assay [1]. To speed up the analysis of such a large number of samples, the use of gel diffusion (cup-plate) assays [2], in which radial diffusion of enzymes into a substrate-bearing gel allows the rapid detection of hydrolase activity, is a suitable alternative.

Herein we describe the elaboration of a simple, rapid, and reliable semiquantitative assay for monitoring CC eluate fractions with regard to *endo*-(1 → 5)-α-L-arabinanase (EC 3.2.1.99) (*endo*-ABA) activity. The assay allows the handling of many samples with minimum effort and is applicable to small sample volumes and enzymatic activities. Under standard conditions, the system enables the clear detection of as little as 1.7 nkat/mL of enzymatic activity. It takes advantage of a dye-labelled linear arabinan and the simplicity of cup-plate enzyme diffusion analysis.

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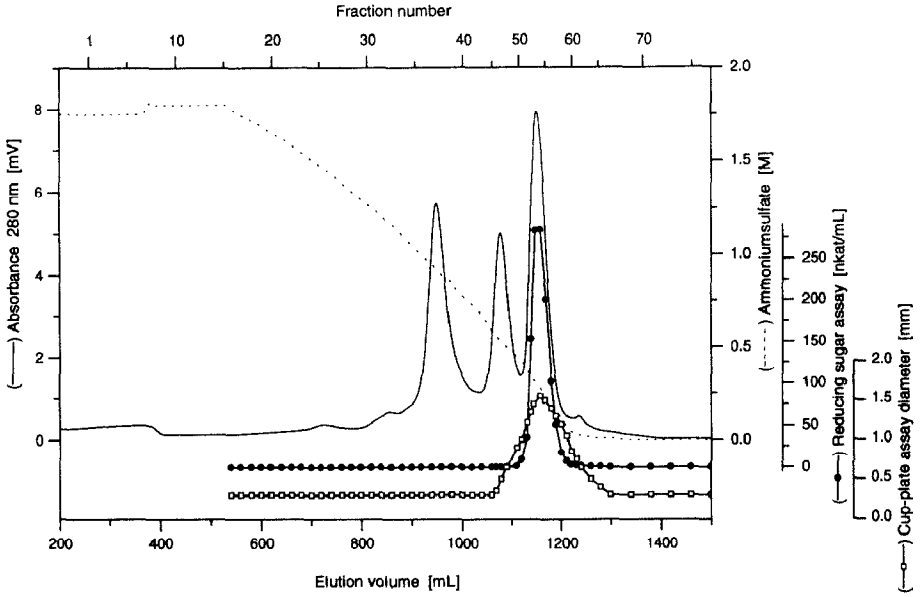

Fig. 1. Correlation between a hydrophobic interaction chromatography eluate profile and *endo*-(1 → 5)-α-L-arabinanase activity. Chromatographic parameters: (1) sample, partially purified *endo*-ABA from Pectinex AR KPG 027; (2) gel, Phenyl Sepharose 6 Fast Flow (low sub); equilibration with 1.8 M ammonium sulfate, 50 mM NaOAc buffer, pH 4.5; elution with 150 mL of equilibration buffer followed by a linear, decreasing ammonium sulfate gradient (1.8 to 0 M) in 50 mM NaOAc, pH 4.5, applied over 700 mL. The gel and all chromatographic components were from Pharmacia Biosystems (Uppsala, Sweden). Buffers contained 0.02% w/v NaN_3 as bacteriostat and the separation was performed at room temperature. Reducing sugars and cup-plate measurement were performed as described in the Experimental section.

A CC separation profile of a partially purified *endo*-ABA preparation is shown in Fig. 1. Quantitative determination of *endo*-ABA activity in CC eluate fractions was performed with linear (1 → 5)-α-L-arabinan by using the Nelson–Somogyi colorimetric reducing sugar assay [3]. Semiquantitative screening of *endo*-ABA activity in CC eluate fractions was performed with a cup-plate assay using a dye-labelled, enzymatically debranched arabinan as substrate. This red substrate was incorporated into an agarose gel and the rates of release of soluble, dye-labelled fragments on hydrolysis, were brought into direct correlation with *endo*-ABA activity [4].

Analysis of all relevant CC eluate fractions resulting from the CC separation in Fig. 1 were performed at one time with only one Petri dish as shown in Fig. 2. The diameters of the cleared zones that appeared in the cup-plate assay were highly correlated to *endo*-ABA activity. Enzyme and buffer controls showed no clearance of the gel and the largest clearing spots (well numbers 44 and 45) corresponded exactly to the maximum activity measured with the quantitative reducing sugar method (Fig. 1). Reasonably linear relationships between cleared zone diameters

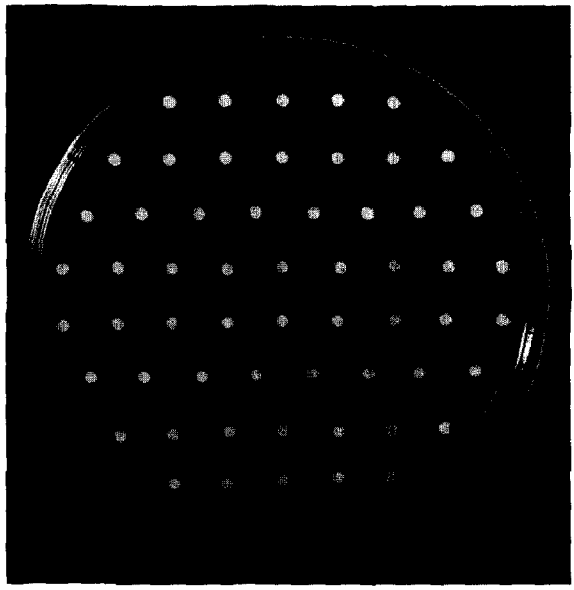

Fig. 2. Cup-plate assays for semiquantitative determination of *endo*-(1 → 5)-α-L-arabinanase activity in column chromatography eluate fractions. Assay conditions: gel, 1% w/v agarose; 0.6% w/v red arabinan in 50 mM NaOAc buffer, pH 4.8. Incubation for 3 h at 40°C. Fraction numbers refer to Fig. 1. Sample volume, 10 μL. Well numbers 1–5 contained heat inactivated aliquots of eluate fractions 50–54, respectively. Well 6 contained 50 mM NaOAc buffer pH 4.5, whereas well 7 contained 1.8 M ammonium sulfate, 50 mM NaOAc buffer, pH 4.5. Wells 8–58 contained untreated aliquots from eluate fractions 16–66. Maximum activity was detected in the wells 44 and 45, which corresponded to eluate fractions 52 and 53, respectively.

and log *endo*-ABA concentrations were observed at enzyme concentrations above 5 nkat/mL. Due to the low sensitivity of the assay to activity changes, fluctuations in pH and salt concentration did not significantly affect the assay. Optimum hydrolysis rates were obtained after 3 h incubation at 40°C (pH 4.8; 0.2% w/v red arabinan).

Consequently, the cup-plate assay presented here proved to be very useful in the determination of *endo*-ABA activity for the large number of eluate fractions typically obtained after CC separation. It may therefore be considered as a major improvement in the optimisation of *endo*-ABA purification procedures, as it was a suitable and advantageous alternative to classical colorimetric reducing sugar assays.

1. Experimental

Substrates, enzyme preparation, and chemicals.—Linear (1 → 5)-α-L-arabinan [98.6 mol% arabinose, 0.7 mol% rhamnose/fucose and 0.7 mol% galacturonic

acid; 91% of the arabinose residues being (1 → 5)-α-L-linked], from apple juice concentrate, was a gift from Novo Nordisk Ferment AG (Dittingen, Switzerland). Red debranched arabinan [5] (88 w/w% arabinose, 6 w/w% galacturonic acid, 4 w/w% galactose and 2 w/w% rhamnose/fucose [5]) from sugar beet was purchased from MegaZyme Pty. Ltd. (Sydney, NSW, Australia). Pectinex AR KPG 027, an enzyme preparation from *Aspergillus niger*, was kindly provided by Novo Nordisk Ferment AG (Dittingen, Switzerland). All buffers contained 0.02% w/v NaN_3 as bacteriostat.

Carbohydrate analysis of substrates.—Galacturonic acid residues in the substrates were determined according to Blumenkrantz and Asboe-Hansen [6] after acidic prehydrolysis [7]. The neutral sugar composition of the substrates was determined by gas chromatography of the hydrolysates [8]. Glycosidic-linkage analysis of arabinose residues was performed by methylation analysis [9,10]. The partially methylated alditol acetates were analysed by GLC–MS, using a Fisons GC 8065 gas chromatograph (Carlo Erba, Milano, Italy) equipped with an SP 2380 column from Supelco Inc. (Bellefonte PA, USA), connected to a MAT SSQ 710 mass spectrometer from Finnigan (San Jose, CA, USA).

Enzyme assays.—(a) Quantitative endo-*(1 → 5)-α-L-arabinanase activity measurement by determination of reducing end groups.* The substrate solution [0.2% w/v linear (1 → 5)-α-L-arabinan in 50 mM NaOAc buffer, pH 4.8] was heated at 85°C for 20 min and allowed to cool to room temperature. *Endo*-ABA activity was determined in a mixture containing 200 μL substrate solution and 25 μL of appropriately diluted enzyme solution in 50 mM NaOAc buffer, pH 4.8. Incubation was performed at 40°C for 20 min. The concentration of reducing end groups was determined according to the Nelson–Somogyi method [3], with the following modifications: 225 μL of incubated enzyme–substrate solution was mixed with 400 μL of alkaline copper reagent and boiled for 20 min. After cooling to room temperature, 400 μL of arsenomolybdate reagent were added. The absorbance was recorded at 520 nm (Uvikon 940 spectrophotometer, Kontron Instruments, Zürich, Switzerland) after 35 min. L-(+)-Arabinose was used as standard. Enzymatic activities were expressed in nkat/mL.

(b) Semiquantitative endo-*(1 → 5)-α-L-arabinanase activity measurement with dye-labelled substrate on a cup-plate assay.* Red debranched arabinan was dissolved in 50 mM NaOAc buffer, pH 4.8, and heated for 30 min at 70°C [5]. Agarose (electrophoresis purity reagent, standard low M_r, Bio-Rad Laboratories, Richmond, CA, USA) was dissolved in the same NaOAc buffer and heated at 90°C for 30 min, allowed to cool down to 70°C and then mixed with the substrate solution. The agarose–substrate solution (0.2% w/v red arabinan, 1% w/v agarose) was poured into disposable Petri dishes and wells were cut into the solidified gel (thickness 1.5–2.0 mm), using a fine stainless steel tube with cutting edges attached to a trap and vacuum pump. After incubation with enzyme solutions (applied as 10 μL-aliquots), rings of clearing in a red background around the wells indicated areas of substrate hydrolysis. For semiquantitative estimation of *endo*-ABA activity the diameter of the cleared zones was measured.

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