Journal of Chromatography, 137 (1977) 234–239

CHROM. 10,072

Note

Quantitative thin-layer chromatographic assay for the β-neoagarotetraose hydrolase of *Pseudomonas atlantica**

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(Received July 19th, 1976)

Quantitative densitometry of thin-layer chromatograms has been used to assay sugars[1,2], lipids[3–5] and several other substances[6–10]. Thin-layer chromatography (TLC) also has been used to evaluate the neoagarobiose series of oligosaccharides obtained on enzymic hydrolysis of agarose[11], and as a quantitative assay for intestinal disaccharidases[12] and for the β-neoagarotetrase of *Cytophaga flevensis*[13]. The latter method involved scanning photographs of the chromatograms but the experimental details have not been published.

Agarose is degraded to monomeric sugars, in *Pseudomonas atlantica*, by an extracellular β-agarase and cell-bound oligosaccharidases, β-neoagarotetraose hydrolase and α-neoagarobiose hydrolase. The β-neoagarotetraose hydrolase hydrolyzes neoagarotetraose (O-3,6-anhydro-α-L-galactopyranosyl (1→3)-O-β-D-galactopyranosyl (1→4)-O-3,6-anhydro-α-L-galactopyranosyl (1→3)-D-galactose) by cleaving the central β(1→4) linkage to yield two molecules of neoagarobiose[14] (Fig. 1). Neoagarobiose (O-3,6-anhydro-α-L-galactopyranosyl (1→3)-D-galactose) is further hydrolyzed to the monomers 3,6-anhydro-L-galactose and D-galactose by an α-neoagarobiose hydrolase[15].

Purification and characterization of β-neoagarotetraose hydrolase required development of a sensitive and specific assay method. In crude enzyme preparations, where both β-neoagarotetraose hydrolase and α-neoagarobiose hydrolase are present, the quantitative assay should measure specifically activity of the β-neoagarotetraose hydrolase. Thus a method involving measurement of reducing sugars could not be used since both enzymes increase the reducing power. Activity of β-neoagarotetraose hydrolase was determined by measuring the concentration of neoagarotetraose on thin-layer chromatograms, after visualization of the sugar by charring. In this paper, a technical description of the assay is presented and possible applications discussed.

EXPERIMENTAL

Materials

Neoagarotetraose was prepared as previously described[16].

* This work is based on a thesis submitted by D. Groleau to the faculty of graduate studies, McGill University, in partial fulfillment of the M.Sc. degree requirements.

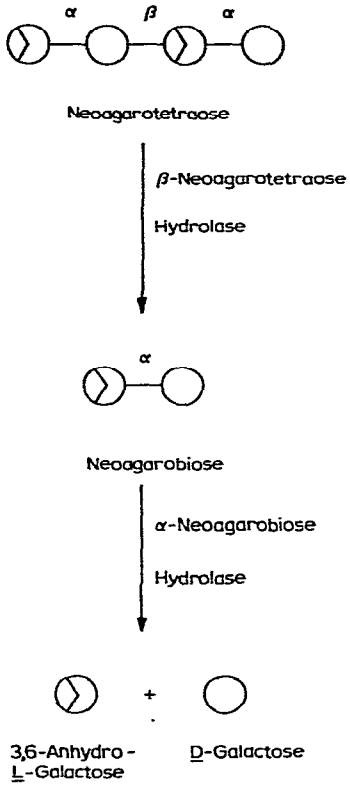

Fig. 1. Hydrolysis of neoagarotetraose by a mixture of β-neoagarotetraose hydrolase plus α-neoagarobiose hydrolase from *Pseudomonas atlantica*.

Silica gel thin-layers. MN-Silica gel N-MR (no binder, Macherey, Nagel & Co., Düren, G.F.R.), 30 g in 80 ml of a 1.0% (w/v) aqueous ammonium bisulphate solution, was layered to a thickness of 0.3 mm on 20 × 20 cm glass plates using a TLC-plate coater (Camag, Muttenz, Switzerland) and then air dried.

Chromatography. The freshly prepared solvent system was: butan-1-ol–ethanol–5% (w/v) aqueous ammonium bisulphate (3:1:1).

Methods

Application of samples. Samples were applied from a 25-μl microsyringe (Hamilton) equipped with a repeating dispenser delivering 0.5 μl. The spots were dried with a current of cold air.

Charring. Following chromatography, the plates were air-dried for about 1 h, heated at 175° for 30 min in a large oven provided with a fan and then cooled at room temperature for 20–30 min. Absorbance was measured within 1–2 h, with no significant change in absorbance after 6 h.

Densitometry. Quantitative densitometry was performed using a Vitatron TLD 100 densitometer (Vitatron, Dieren, The Netherlands). Absorbance of each peak was recorded as a number of pulses. The instrument settings were: transmission

(—log) mode; interference filter no 445; diaphragm, 0.25 mm; integrator, position 7; scanning speed, 0.5 cm/min; recorder speed, 1 cm/min.

Measurement of enzymic activity. Enzyme assays were carried out at room temperature in a serological plate covered with parafilm to prevent evaporation. For a 100 μl assay, the mixture consisted of 50 μl of 1.58×10^{-2} *M* neoagarotetraose (mol.wt. 630); 10 μl of 1 *M* NaCl, 0.01 *M* Tris buffer pH 7.2; and 40 μl of the enzyme preparation. At regular intervals, 2.5 μl of the assay mixture was applied in duplicate to the plate. Enzyme solution, equivalent to the amount present in 2.5 μl of the assay mixture, was applied as control.

RESULTS AND DISCUSSION

The procedure for charring neoagarotetraose was a modification of the technique of Touchstone *et al.*[4] for lipids and steroids. On heating, the oxidizing agent ammonium bisulphate is decomposed to acid that chars the sugars to yield dark-brown spots. Charring of sugars was reported to give quite stable spots[2].

Ammonium bisulphate was not incorporated into the silica gel by dipping the plates into a saturated ethanolic solution of this salt, as originally described, but rather added to the silica gel immediately before spreading the slurry on glass plates. The concentration of ammonium bisulphate used (1.0% w/v) was found to be optimal; lower concentrations yielded pale spots whereas higher concentrations had a deleterious effect on separation of sugars and on shape of the spots. Integration values for charred spots were higher and more reliable when the oxidizing agent was also added to the solvent system. Chromatography was started within 30 min as neoagarotetraose was slightly degraded by the ammonium bisulphate present in the layer. Under the conditions described, a linear relationship between absorbance (expressed as a number of pulses) and concentration of neoagarotetraose was obtained within the 1–22 μg range (Fig. 2).

Products of hydrolysis of neoagarotetraose (Fig. 1) with an enzyme preparation containing β-neoagarotetraose hydrolase and α-neoagarobiose hydrolase are shown

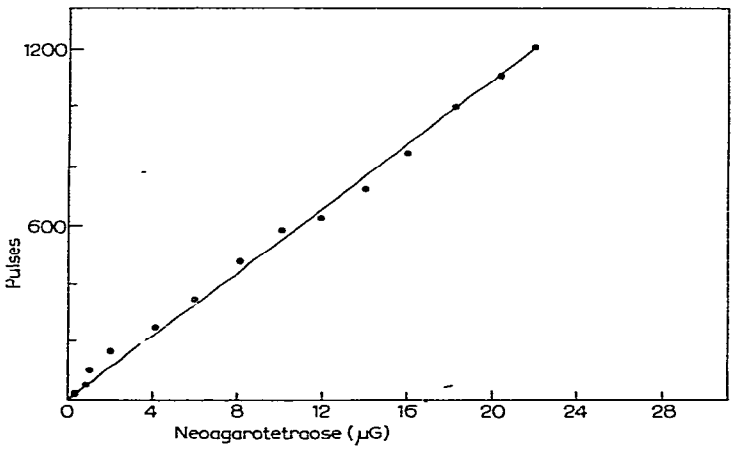

Fig. 2. Calibration curve for neoagarotetraose after charring and densitometry.

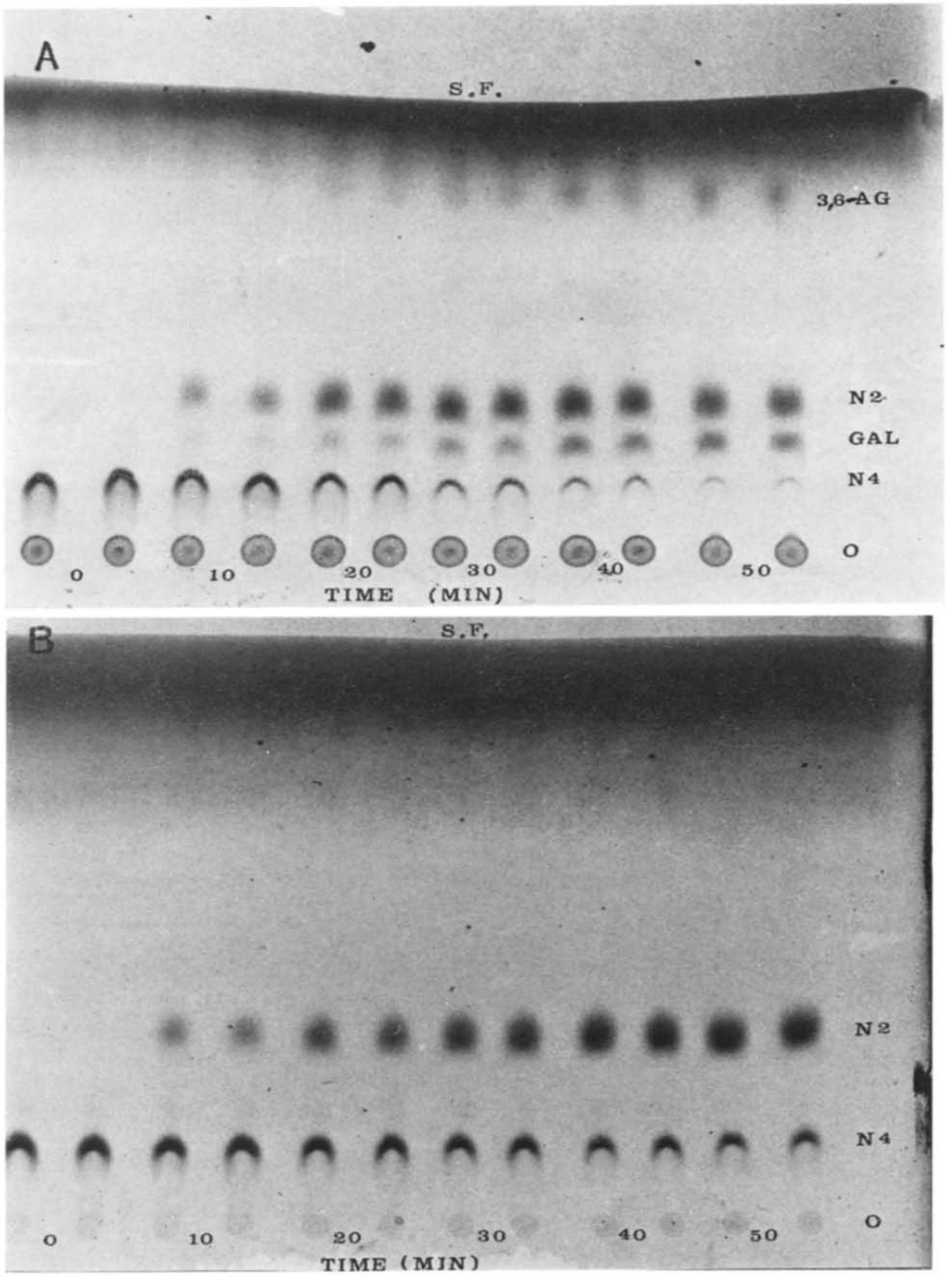

Fig. 3. Products of hydrolysis on TLC obtained on enzymic hydrolysis of neoagarotetraose by a preparation containing β-neoagarotetraose hydrolase and α-neoagarobiose hydrolase (A); and by a purified β-neoagarotetraose hydrolase preparation (B). N2, N4 = Neoagarobiose and -tetraose; GAL = D-galactose; 3,6-AG = 3,6-anhydro-L-galactose; S.F. = solvent front; O = origin.

in Fig. 3A and with a purified β-neoagarotetraose hydrolase preparation in Fig. 3B.

Results of a representative assay are presented in Fig. 4. Aliquots at time 0 contained 12.5 μg of neoagarotetraose and were considered as internal standards. Integration values were corrected on the basis of the value obtained for the internal standard and the concentration of neoagarotetraose determined from the standard curve (Fig. 2). A plot of disappearance of neoagarotetraose *versus* time was used to determine initial velocity of the enzyme (Fig. 4). One unit of β-neoagarotetraose hydrolase was defined as that amount of enzyme hydrolyzing 1 nmole of neoagarotetraose per min at room temperature. The molecular weight of neoagarotetraose was calculated as 630.

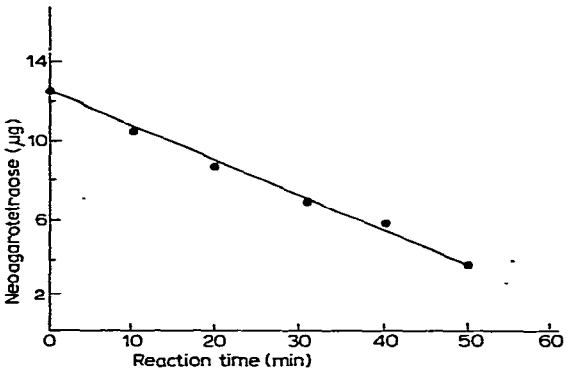

Fig. 4. Hydrolysis of neoagarotetraose by β-neoagarotetraose hydrolase: curve showing the disappearance of neoagarotetraose *versus* time.

Validity of the quantitative assay

The plate to plate variation (22 plates) of the internal standard (12.5 μg of neoagarotetraose) was less than 10% for 17 plates, with a variation within 10–15% for 5 plates. The variation for two identical aliquots on the same plate ranged from 1–7%.

CONCLUSION

The thin-layer chromatographic assay for β-neoagarotetraose hydrolase is accurate and reproducible. The duration of the enzyme assay is of about six hours and three separate assays can be carried out at the same time. The method was used to assay enzyme activity and properties of β-neoagarotetraose hydrolase. It should also be possible to determine the ratio of neoagarobiose to methylated neoagarobiose in different agars by this method, following hydrolysis of the agar samples by a mixture of β-agarase and β-neoagarotetraose hydrolase[17].

ACKNOWLEDGEMENTS

This research was generously supported by a grant from the National Research Council of Canada. We are indebted to Miss Donna Seto for the preparation of the neoagaro-oligosaccharides.

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