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Note

Determination of inulase isoenzymes on polyacrylamide gels by activity staining

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Fructofuranosidases, which are able to hydrolyze inulin, are commonly found in bacteria[1–5], yeast[6–11], moulds[12–15] and plants[16–20]. Three principal patterns of action of inulases have been described. The most widespread is that of the *exo*-fructan-fructano hydrolase, *exo*-inulase, which liberates single fructose molecules from the polysaccharide chain. It is found in all the sources mentioned above. The second type are the *endo*-fructan hydrolases, which hydrolyze inulin to reducing fructo-oligomers, the inulo-oligooses. The occurrence of this enzyme type has been described only in moulds[15]. The third type, a fructotransferase, found only in bacteria and plants[3–5], is quite different from the usual catabolic carbohydratases. It splits difructose anhydride from the inulin chain and anhydratizes the two fructoses, leading to a difructose dianhydride.

The common analytical procedures for the characterization of inulase isoenzymes confirm only the affinity towards DEAE-cellulose or other ion exchangers[8–11,16–18]. Rocket immunoelectrophoresis[15] was able to detect two isoenzymes, one *endo*- and one *exo*-type enzyme, in a commercial inulase preparation from an Aspergillus species.

In this article we describe a non-denaturating separation of inulase isoenzymes by polyacrylamide gel electrophoresis (PAGE) and visualization by an activity-staining procedure. Further characterization of the type of action of the isolated isoenzymes was done by high-performance thin-layer chromatography (HPTLC).

MATERIALS AND METHODS

PAGE was carried out as described elsewhere[21]: in a block gel apparatus, a separation gel with 10% acrylamide (Merck, Darmstadt, F.R.G.) cross-linked 1:100 with bismethyleneacrylamide (Merck), pH 8.9, containing 6% inulin (Laevosan Gesellschaft, Linz, Austria) was used. The electrophoresis buffer was a Tris–glycine system. For an experiment lasting 2 h, a voltage of 300 V and a starting current of 120 mA were used. The apparatus was cooled with a flow of tap-water.

A 5-μl volume of crude enzyme preparation (Novo Industri, Bagsvaerd, Denmark) was separated. To visualize the zones of enzymatic activity, the gels were immersed in 100 ml of a 0.1 *M* acetate buffer (pH 5.0) and kept at 37°C for 1 h. Then

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the gels were incubated overnight in 100 ml of methanol–acetic acid–water (5:1:5) in a refrigerator. The inulin in the gel precipitates and the zones of enzymatic activity appear as transparent bands.

For the inhibition tests, the gels were incubated in 10^{-3} *M* solutions of Fe^{3+}, Mn^{2+}, EDTA, I_2 or aniline in acetate buffer pH 5.0 for 1 h.

HPTLC was performed on Whatman precoated silica plates, 200 μm, (LHP-KF). The solvent was *n*-butanol–*n*-propanol–ethanol (96%)–water (40:10:30:20). Thymol–sulphuric acid spray reagent was used to visualize the carbohydrates[22]. As a reference, non-reducing inulo-oligosaccharides were isolated from Jerusalem artichoke tubers[23].

RESULTS AND DISCUSSION

As shown in Fig. 1, four enzymes able to hydrolyze inulin are detectable with this electrophoretic method. The three more anodic enzymes (A,B,C) are inhibited with 10^{-3} *M* I_2 (lane 2). The more cathodic, very active enzyme band (D), however, is not inhibited by 10^{-3} *M* I_2. On testing the inulase inhibitors described by Zittan[15], *i.e.*, Fe^{3+}, Mn^{2+}, and EDTA, no inhibition of enzymatic activity was detected.

In one of the first articles about inulases, Schlubach and Grehn[24] describes aniline as a potential inhibitor of fructofuranosidases, both invertases and inulases. As seen in Fig. 1, lane 3, the activity of the more cathodic enzyme band (D) is clearly reduced by 10^{-3} *M* aniline. Regarding the results of Schlubach and Grehn[24], the more

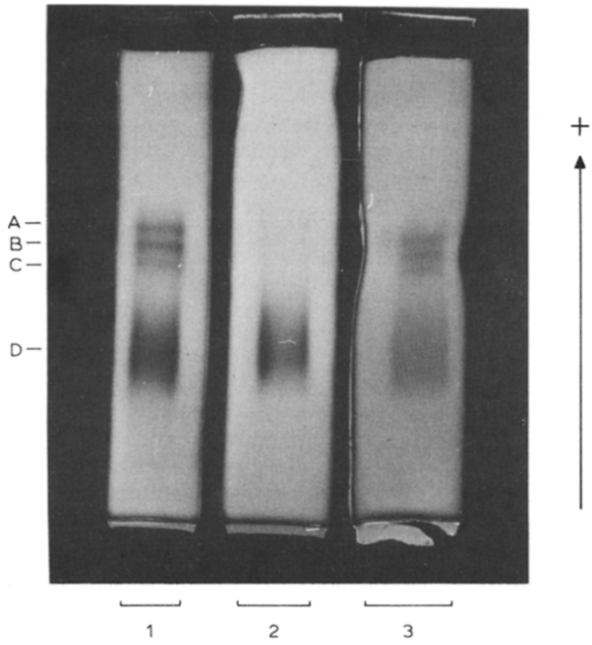

Fig. 1. Zymogram of Aspergillus inulase. 1, 0.1 *M* Acetate buffer pH 5.0, 37°C, 1 h; 2, 0.1 *M* acetate buffer pH 5.0, 10^{-3} *M* I_2, 37°C, 1 h; 3, 0.1 *M* acetate buffer pH 5.0, 10^{-3} *M* aniline, 37°C, 1 h. Each lane contains 5 μl of crude Aspergillus inulase. For staining procedure see Materials and methods.

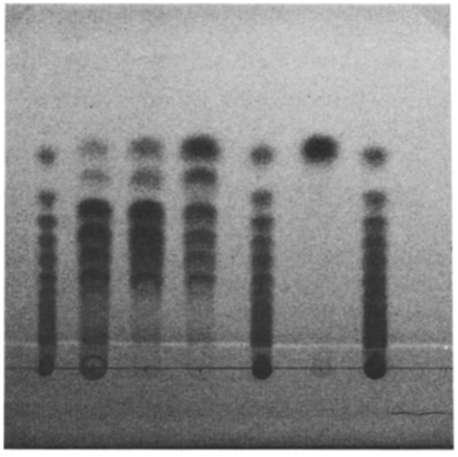

Fig. 2. HPTLC of the digestion products. I = Inulo-oligosaccharides isolated from Jerusalem artichoke tubers; A = digestion products from enzyme A on inulin; B = digestion products from enzyme B on inulin; C = digestion products from enzyme C on inulin; D = digestion products of enzyme D on inulin; after spraying with thymol–sulphuric acid, the plate was heated at 105°C for 5 min. For chromatographic conditions see Materials and methods.

cathodic enzyme should be a fructofuranosidase able to hydrolyze inulin, and therefore be an *exo*-inulase.

To identify and distinguish between the type of action exhibited by the four different inulases, a gel strip was cut into sufficiently small pieces each containing only one isoenzyme band. The gel pieces were then incubated in acetate buffer (pH 5.0) at 37°C for 1 h. The products of the enzyme digestion were analyzed by HPTLC. Fig. 2 shows the chromatogram of the digestion products. The cathodic enzyme (D) produces only monosaccharides from inulin. The three more anodic enzymes (A–C) hydrolyze inulin into reducing fructo-oligosaccharides. Even at very long incubation times, more than 24 h, the composition of the degradation products does not change. The three enzymes A–C are therefore identified as *endo*-inulase isoenzymes.

The migration behaviour of the enzymatically produced fructo-oligosaccharides differs from that of the non-reducing oligosaccharides of the inulin series because of their lack of a terminal glucose.

This new method of electrophoretic identification of inulase isoenzymes is easier and more specific than the time-consuming immunoelectrophoresis.

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