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AN ENZYMIC ASSAY OF L-ARABINOSE, USING β-D-GALACTOSE DEHYDROGENASE: ITS APPLICATION IN THE ASSAY OF α-L-ARABINOFURANOSIDASE

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ABSTRACT

A new spectrophotometric method has been developed for the quantitative determination of L-arabinose, using the NAD-dependent β-D-galactose dehydrogenase from *Pseudomonas fluorescens*. This method has been applied to the determination of L-arabinose produced after degradation of L-arabino-D-xylan by α-L-arabinofuranosidase from *Aspergillus fumigatus*, and provides the basis of a sensitive, accurate, and specific method for assay of this enzyme which is technically straightforward to perform.

INTRODUCTION

Many new methods for the analysis of saccharides have been published in recent years. Monosaccharides may be determined after separation by g.l.c.[1] or h.p.l.c.[2]. The former method suffers from inflexibility, and is not suited to the routine sampling of enzyme digests where the levels of L-arabinose are to be measured. An existing enzymic method for the assay of L-arabinose, using L-arabinose isomerase[3,4], has practical limitations. The enzyme, which occurs intracellularly in *Lactobacillus plantarum*[3] and *L. brevis*[4], has to be separated from a closely related D-xylose isomerase, because the ketoses produced by both enzymes from their respective substrates will be assayed under the analytical conditions. D-Galactose dehydrogenase (EC 1.1.1.48 D-galactose:NAD^+ 1-oxidoreductase) from *Pseudomonas fluorescens* has been reported to oxidise L-arabinose as well as D-galactose[5]. One of the reaction products, NADH, is readily determined spectrophotometrically and this forms the basis of a new assay procedure for L-arabinose, in the absence of D-galactose.

The method has been applied to the assay of α-L-arabinofuranosidase (EC 3.2.1.55, L-arabinofuranoside arabinohydrolase) produced during the growth of *Aspergillus fumigatus*.

α-L-Arabinofuranosidase attacks the (1→3)-linked α-L-arabinofuranosyl side-chains of L-arabino-D-xylans[6–12], and its activity is not limited to the disac-

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charide level, although synthetic, nitrophenyl glycoside substrates are commonly used to assay the enzyme[8,13-16]. Classical assay systems for α-L-arabinofuranosidase involve measurement of reducing power on enzymic degradation of the macromolecular arabinoxylan substrate[7,9,17]. These methods are non-specific, as they do not distinguish between reducing sugars produced by α-L-arabinofuranosidase and by various D-xylolytic enzymes commonly elaborated by cereals[18,19], other terrestrial plants[20], and micro-organisms[21,22]. α-L-Arabinanases can cleave the (1→3)- and (1→5)-linked α-L-arabinofuranosyl residues of sugar-beet arabinan, and also the (1→3)-linked α-L-arabinofuranosyl residues of arabinoxylans; in the former case, they do not act on synthetic, phenyl glycoside substrates[23].

EXPERIMENTAL

Materials. — *Aspergillus fumigatus* S1 was a gift from Dr. B. Flannigan (Heriot-Watt University) and had been isolated from barley husk. Spores were maintained on 2% potato-dextrose agar-slopes at 4°. β-D-Galactose dehydrogenase *(Pseudomonas fluorescens)* and nicotinamide adenine dinucleotide (NAD) were obtained from Boehringer Mannheim.

Barley-straw arabinoxylan for the assay of α-L-arabinofuranosidase was a 5% aqueous KOH extract from a sodium hypochlorite-delignified barley-straw holocellulose[24], and was purified by ammonium sulphate fractionations[25,26]. The resulting arabinoxylan contained only L-arabinosyl and D-xylosyl residues, and was free from D-glucose, D-mannose, and D-galactose. Larch xylan for the arabinoxylan growth-medium was obtained from the Sigma Chemical Co. Ltd. Dried yeast extract was obtained from Difco Yeast Products.

Growth of Aspergillus fumigatus *S1: production of extrallular α-L-arabinofuranosidase.* — *A fumigatus* S1 spores were inoculated aseptically by a dab and streak on ten sterile potato-dextrose (2%) agar-plates and incubated at 37° for 4 days. Spores were removed from plates as suspensions in sterile Tween-80 (0.01%, 10.0 mL per plate), and added aseptically to sterile arabinoxylan growth-medium (1 L) in a 2-L conical flask.

Arabinoxylan growth-medium contained (per litre) larch xylan (2.0 g), $NaNO_3$ (2.0 g), KH_2PO_4 (1.0 g), KCl (0.5 g), $MgSO_4 \cdot 7\ H_2O$ (0.55 g), $FeSO_4 \cdot 7\ H_2O$ (0.01 g), and dried yeast extract (0.05 g). Growth was maintained at 37° in an orbital incubator (120 cycles/min) for 8 days. The mycelial mass was collected by filtration through nylon gauze, and a cell-free extract was obtained by centrifugation (30,000*g* for 30 min). The resulting, clear, supernatant solution (1 L) was dialysed against several changes of distilled water (20 L) at 4° for 96 h. The extract was freeze-dried, to provide the crude enzyme for subsequent experiments

Assay of α-L-arabinofuranosidase. — A mixture of the foregoing, cell-free, culture filtrate (2 mL), 0.1M sodium acetate buffer (pH 5.0, 20.0 mL), aqueous barley-straw arabinoxylan (2.0% w/v, 6.0 mL), and toluene (1.0 mL) was incubated at 30° for 48 h. Samples (1.0 mL) were removed at intervals. After inactivation of en-

zymic activity by heating at 100° for 10 min, the samples were cooled, and any insoluble material was removed by centrifugation. Aliquots (0.05 mL) of the clear supernatant solution were assayed for L-arabinose; 1 unit of enzyme activity releases 1 μmol of L-arabinose/h from the substrate.

Determination of L-*arabinose, using* β-D-*galactose dehydrogenase.* — Spectrophotometric measurements were carried out in a semi-micro (1.0–1.6 mL) quartz cuvette (path length, 1 cm) at 340 nm with a Unicam SP 1800 double-beam spectrophotometer, an external recorder, and an external water re-circulating jacket (thermostatically controlled at 25°). The reaction parameters, namely, pH, NAD concentration, enzyme concentration, and L-arabinose concentration, were optimised. The reaction mixture was pipetted directly into the cuvette and contained 5mM NAD (0.1 mL), β-D-galactose dehydrogenase (5 U/mL, 0.02 mL), and 0.1M Tris-HCl buffer (pH 8.6, 0.83 mL). After temperature equilibration (25°), the absorbance (A_1) of the sample was measured. The reaction was started by the addition, with mixing, of the L-arabinose sample (0.05 mL, 0–20 μg of L-arabinose) to the cuvette. The absorbance was then measured continuously. The production of NADH was measured by (*a*) an end-point assay, in which the absorbance of the sample at 340 nm was measured continuously until no further change was observed (A_2), and the L-arabinose concentration was related to the absorbance change ($A_2 - A_1$) and calculated from the molar extinction coefficient (6.22×10^3 $L \cdot M^{-1} \cdot cm^{-1}$) of NADH at 340 nm; or (*b*) an initial-velocity assay in which the rate (v) at which NADH was produced in the first minute of reaction was measured [ΔA(340 nm)/min]. Identical results were obtained with the two methods. The Michaelis constant (K_m) for the enzyme was calculated from double-reciprocal plots of $1/v$ against $1/s$.

RESULTS AND DISCUSSION

Under the assay conditions used, L-arabinose is specifically oxidised at the C-1 position by β-D-galactose dehydrogenase in the presence of NAD, to yield L-arabinono-1,4-lactone and NADH. At the alkaline pH of the assay, the lactone produced (*1*) is spontaneously and irreversibly hydrolysed to L-arabinonic acid (*2*).

$$\text{L-Arabinose} + \text{NAD} \xrightarrow{\beta\text{-D-Galactose dehydrogenase}} \text{L-arabinono-1,4-lactone} + \text{NADH} + \text{H}^+ \quad (1)$$

$$\text{L-Arabinono-1,4-lactone} + \text{H}_2\text{O} \rightarrow \text{L-arabinonic acid} \quad (2)$$

By using the end-point assay procedure and varying the reaction parameters, optimal conditions for the oxidation of L-arabinose were obtained with respect to NAD concentration (Fig. 1) and pH (Fig. 2). Evidence for slight inhibition of the

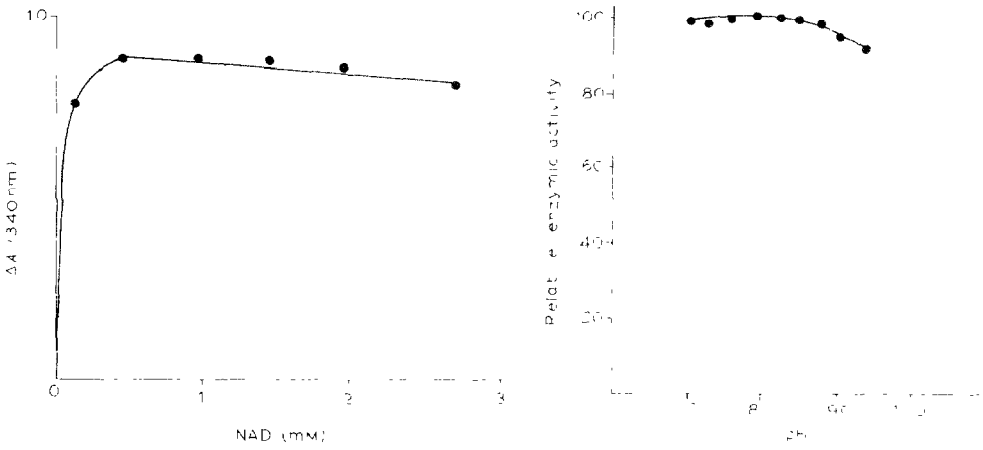

Fig. 1 The oxidation of L-arabinose by D-galactose dehydrogenase, as a function of NAD concentration

Fig. 2 The oxidation of L-arabinose by D-galactose dehydrogenase, as a function of pH. Standard assay conditions were used in 100mM Tris-HCl buffers (pH 7.1–9.5)

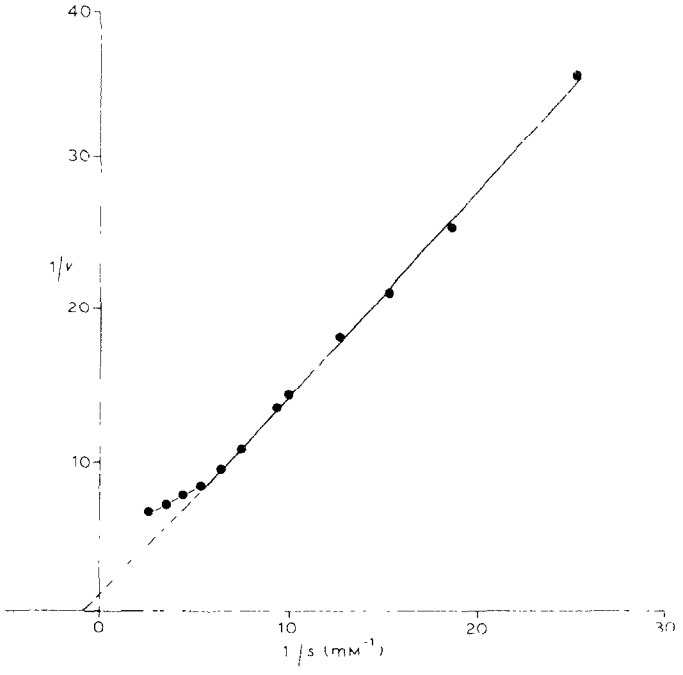

Fig. 3 The Michaelis constant (K_m) for D-galactose dehydrogenase with L-arabinose as substrate. Double-reciprocal plots 1/v against 1/s are used for substrate concentration(s) between 0.013 and 0.33mM.

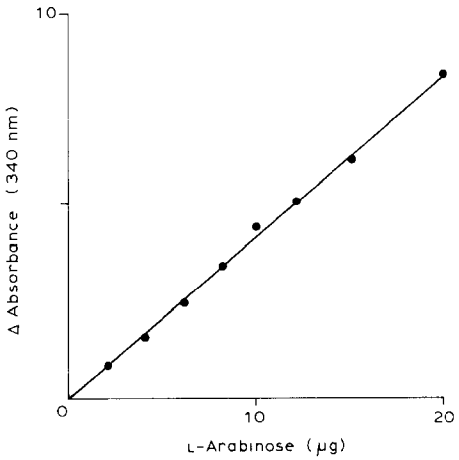

Fig. 4 Determination of L-arabinose by the end-point assay.

reaction at NAD concentrations ≥0.5mM (Fig. 1) and of substrate inhibition at high concentrations of L-arabinose (Fig. 3) are similar to the findings of Blachnitzky *et al.*[27] when D-galactose was used as the substrate. The K_m value for D-galactose dehydrogenase is 1.43mM for L-arabinose, and 0.77mM for D-galactose. These values compare with 10mM and 3.7mM, respectively, for the corresponding enzyme from *P. saccharophila*[28].

Using these optimal reaction conditions, samples of L-arabinose containing 0–0.13 μmol of sugar give a linear calibration graph (Fig. 4). The absorbance values obtained indicate that L-arabinose is quantitatively converted in this procedure. L-Arabinose standards were also assayed under the same conditions, but using an initial-velocity assay procedure in which the change in absorbance (340 nm) was measured during the first minute of reaction. The calibration curve obtained was linear from 0–0.13 μmol of L-arabinose.

The substrate specificity of β-D-galactose dehydrogenase was also investigated by the initial-velocity procedure (Table I). Initial velocities using 0.066 μmol of sugar were recorded, taking the value obtained for 0.066 μmol of D-galactose as 100% and employing the optimal assay conditions for L-arabinose. The results (Table I) indicate that β-D-galactose dehydrogenase is highly specific for L-arabinose and D-galactose. However, a lack of specificity at C-6 of D-galactose is evident, since D-fucose also reacts with D-galactose dehydrogenase[2,7]. The only other sugars that react with D-galactose dehydrogenase, namely, 2-deoxy-D-*lyxo*-hexose and 2-amino-2-deoxy-D-galactose, are not commonly occurring sugars; thus, if D-galactose, D-fucose, and the latter sugars are excluded from assay mixtures, no other practical problems exist in the specific determination of L-arabinose. It is therefore essential that substrates for the assay of the α-L-arabinofuranosidases be free from D-galactose.

TABLE I

SUBSTRATE SPECIFICITY[a] OF D-GALACTOSE DEHYDROGENASE

Sugar	*Relative initial velocity (%)*
D-Galactose	100
L-Arabinose	52.7
D-Fucose[b]	120
2-Deoxy-D-*lyxo*-hexose[b]	63
2-Amino-2-deoxy-D-galactose[b]	1.8

[a]The following sugars were not substrates: D-glucose, D-mannose, L-galactose, D-arabinose, D-ribose, D- and L-xylose, D-lyxose, D-glucuronic acid, D-galacturonic acid, L-fucose, and β-(1→4)-linked D-xylobiose, -triose, and -tetraose. [b]Ref. 27

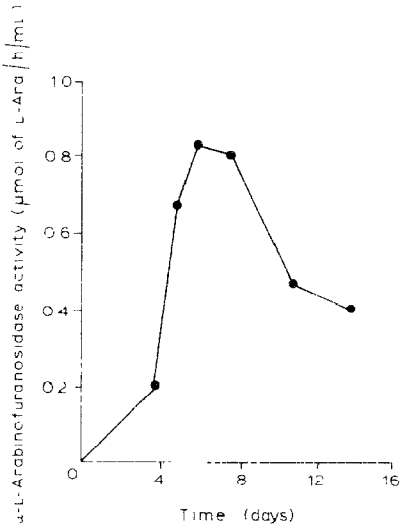

Fig. 5. The elaboration of α-L-arabinofuranosidase by *Aspergillus fumigatus*. Samples (5 mL) were removed at intervals. Cell-free extracts (2 mL) were then used for enzyme assay against barley-straw arabinoxylan.

The elaboration of α-L-arabinofuranosidase by growing cultures of *Aspergillus fumigatus* was investigated (Fig. 5). Maximal enzyme activity was produced by the eighth day of growth, after which lower levels of the enzyme were detected. Also elaborated under the growth conditions were endo-β-D-xylanase and β-D-xylosidase (results not shown), but these enzymes did not appear to interfere in the assay of α-L-arabinofuranosidase. Classical assay procedures[7-9,17] rely on following an increase in reducing power on enzymolysis of arabinoxylan and therefore cannot distinguish between D-xylolytic and L-arabinolytic activities. Thus, this assay procedure may be particularly useful in resolving such enzymic activities.

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