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Partial purification and properties of an L-arabinose dehydrogenase from *Azospirillum brasiliense*[1]

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NOVICK, N. J., and M. E. TYLER. Partial purification and properties of an L-arabinose dehydrogenase from *Azospirillum brasiliense*. Can. J. Microbiol. **29**: 242–246.

An L-arabino-aldose dehydrogenase responsible for the oxidation of L-arabinose to L-arabino-γ-lactone has been purified 59-fold from L-arabinose grown cells of *Azospirillum brasiliense*. The dehydrogenase was found to be specific for substrates with the L-arabino-configuration at carbons 2, 3, and 4. K_m values for L-arabinose of 75 and 140 μ*M* were found with NADP and NAD as coenzymes, respectively. The enzyme had a pH optimum of 9.5 in glycine buffer and was stable when heated to 55°C for 5 min. No enhancement of activity in the presence of any divalent cation or reducing agent tested was found. L-Arabinose dehydrogenase had a molecular weight of 175 000 as measured by the gel filtration technique.

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Une L-arabino-aldose déshydrogénase obtenue à partir de cellules d'*Azospirillum brasiliense* cultivées sur de la L-arabinose a été purifiée 59 fois; cette enzyme est responsable de l'oxydation de la L-arabinose en L-arabino-γ-lactone. La déshydrogénase est spécifique pour les substrats ayant une configuration L-arabino aux carbones 2, 3 et 4. Avec la NADP et la NAD comme coenzymes, les valeurs K_m pour la L-arabinose sont de 75 et 140 μ*M*, respectivement. Dans un tampon glycine, l'enzyme a un pH optimum de 9,5 et elle est stable lorsque chauffée à 55°C durant 5 min. Aucune augmentation de l'activité a été observée au présence des cations bivalents ou des agents réducteurs que nous avons testés. Le poids moléculaire de la L-arabinose deshydrogénase déterminé par la technique de filtration sur gel est de 175 000.

[Traduit par le journal]

Introduction

In order for a nitrogen-fixing rhizosphere bacterium, such as *Azospirillum brasiliense*, to be of benefit to a plant it must find rhizosphere conditions favorable to high population densities and to high rates of nitrogen fixation. Favorable conditions would include low O_2 concentration at the site of nitrogenase activity and the presence of large quantities of available carbon substrate. It has been reported that *A. brasiliense* has been found inside *Digitaria* and maize roots (Neyra and Dobereiner 1977). Evidence of large numbers of *Azospirillum* inside roots, though, has come indirectly from counts of reportedly surface-sterilized roots (van Berkum and Bohlool 1980). It seems more likely that *A. brasiliense* does not have a protected site inside the root but rather must compete with many other rhizosphere organisms in the root zone for available substrate. Our previous work (Novick and Tyler 1982) has shown a rapid oxidative pathway by which L-arabinose, a compound plentiful in cell walls (Albersheim 1975), was metabolized to α-ketoglutarate in crude extracts of *A. brasiliense*. The α-ketoglutarate may then be further metabolized by an active tricarboxylic acid (TCA) cycle (Okon et al. 1976) or be used as a substrate for nitrogen incorporation through glutamine synthetase – glutamate synthase activity.

Azospirillum brasiliense can metabolize L-arabinose by the following series of reactions (Novick and Tyler 1982): L-arabinose $\xrightarrow{NAD(P)}$ L-arabino-γ-lactone $\xrightarrow{+H_2O}$ L-arabonic acid $\xrightarrow{-H_2O}$ L-2-keto-3-deoxyarabonic acid $\xrightarrow{-H_2O}$ α-ketoglutaric semialdehyde $\xrightarrow{NAD}$ α-ketoglutaric acid. This pathway has also been demonstrated in *Pseudomonas fragi* (Dagley and Trudgill 1965; Weimberg 1961) and the fast growing rhizobia, *R. meliloti*, *R. trifolii*, *R. phaeseoli*, and *R. leguminosarum* (Duncan 1979; Duncan and Fraenkel 1979). A second pathway which carries through the first three steps of the α-ketoglutarate pathway but cleaves L-2-keto-3-deoxyarabonate to glycolaldehyde and pyruvate has been found in pseudomonad MSU-1 (Dahms and Anderson 1969) and the slow growing rhizobia, *R. japonicum* (Duncan 1979; Pedrosa and Zancan 1974) and *Rhizobium sp.* strain 32H1 (Duncan 1979).

If *A. brasiliense* is to be successfully used to supplement the nitrogen requirements of grasses and other plants, factors that give it a competitive edge in the rhizosphere and which may allow it to reach high population densities there must be understood. The

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systems may then be manipulated through genetic engineering techniques to enhance the beneficial activities.

The present paper describes the purification and characterization of L-arabino-aldose dehydrogenase, the first enzyme in the oxidation of L-arabinose by *A. brasiliense*.

Materials and methods

Bacterial strain

Azospirillum brasiliense (ATCC 29145) was obtained from the American Type Culture Collection. For daily use cultures were maintained on trypticase soy agar (BBL) slants with biweekly transfer. Stock cultures were frozen in liquid nitrogen.

Partial enzyme purification

The organism was grown on L-arabinose as previously described (Novick and Tyler 1982). Crude extracts were prepared in the following manner. Cells near the middle or end of exponential growth were collected by centrifugation, washed twice in 0.1 *M* sodium–potassium phosphate buffer (pH 7.5), and resuspended to ca. 0.25 g/mL in the same buffer. Cells were then passed twice through an Aminco French pressure cell at 20 000 lb/in^2 (1 lb/in^2 = 703.0696 kg/m^2) and centrifuged at 10 000 × *g* for 30 min. The pellet was discarded. The crude extract could be stored for several months at −80°C without significant loss of enzymatic activity.

Nucleic acid precipitation

A 2% solution of protamine sulfate (Sigma, Grade II) in 0.1 *M* sodium–potassium phosphate buffer, pH 7.5, was slowly added to the crude extract to give a final concentration of 0.33%, the suspension was stirred in the cold for 30 min, and centrifuged at 20 000 × *g* for 1 h. The pellet containing the nucleic acids was discarded.

Ammonium sulfate fractionation

Solid ammonium sulfate (Sigma, Grade III) was slowly added to the protamine sulfate supernatant to 40% saturation while maintaining the pH at 7.5 with 0.1 *N* NaOH. The sample was stirred in the cold for 1 h, then centrifuged at 20 000 × *g* for 2 h. Additional ammonium sulfate was then added to the supernatant to reach 50% saturation and the pellet collected as above. The pellets were suspended in 0.1 *M* sodium–potassium phosphate buffer, pH 7.5. The 40% ammonium sulfate pellet contained less than 10% of the total dehydrogenase activity and was discarded. The 50% ammonium sulfate pellet contained 89% of the total dehydrogenase activity and was used in further purification steps.

Heat treatment

The resuspended 50% ammonium sulfate fraction was heated to 55°C for 5 min, immediately cooled in ice to 4°C, and centrifuged at 20 000 × *g* for 1 h. The pellet was discarded.

Gel filtration

The heat-treated sample was added to a 2 × 150 cm Biogel A 1.5 *M* column equilibrated with 25 m*M* sodium–potassium phosphate buffer, pH 7.4. The flow rate was 25 mL/h and 20-mL fractions were collected.

DEAE-cellulose chromatography

A 2 × 10 cm DEAE-cellulose (fine) column was equilibrated with 3 column volumes of 12 m*M* sodium–potassium phosphate buffer, pH 7.4. Active, pooled Biogel fractions were concentrated fivefold to approximately 10 mL in an Amicon pressure cell with a UM 30 filter at 50 lb/in^2. The sample was diluted 10-fold with 12-m*M* column buffer and concentrated in the pressure cell to 5–10 mL. The sample was then washed into the column with 40 mL of 12 m*M* column buffer. The enzyme was eluted with a linear gradient consisting of 150 mL each of column buffer and buffer with 0.40 *M* NaCl. Fractions of 6.5 mL each were collected. Active DEAE fractions were immediately desalted on a 2 × 15 cm G-25 column equilibrated with 12 m*M* sodium–potassium phosphate buffer, pH 7.4. The flow rate was 50 mL/h and 20-mL fractions were collected. Active fractions were concentrated by lyophilization.

Protein determination

Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as the standard.

Enzyme assay

L-Arabinose dehydrogenase activity was determined by following the reduction of NAD or NADP at 340 nm. The reaction mixture contained 125 μmol of glycine–NaOH buffer (pH 9.0), 0.5 μmol of L-arabinose, 0.5 μmol NAD(P), and enzyme in a total volume of 1 mL. One unit of dehydrogenase activity was the amount of enzyme which reduced 1 μmole of NAD(P) per min at 25°C.

Product identification

The product of the dehydrogenation reaction was identified by paper chromatography with the following solvents: (*i*) pyridine – 1-butanol – water (6:4:3); (*ii*) 1-propanol – formic acid – water (6:3:1); and (*iii*) 1-butanol – 1-propanol – water (10:7:5). Compounds were detected with alkaline silver nitrate reagent (Nordmann and Nordmann 1960). The reaction product was chromatographed against L-arabino-γ-lactone, which was produced by boiling potassium arabonate in 0.2 *N* HCl for 5 min. Potassium arabonate was prepared by the hypoiodite-in-methanol oxidation of L-arabinose (Moore and Link 1940).

Molecular weight determination

The 2 × 150 cm Biogel A column was equilibrated with 25 m*M* sodium–potassium phosphate buffer, pH 7.4, and 2 mg of each standard (Boehringer Calibration Proteins II), catalase (240 000 daltons), aldolase (158 000 daltons), albumin (68 000 daltons), and albumin (45 000 daltons), were dissolved in 20 mL of buffer and placed on the column. The column was run at 25 mL/h and 7.5-mL fractions were collected. Standard peaks were located by the change in optical density at 280 nm. L-Arabinose dehydrogenase was located by enzyme activity. A plot of the molecular weights of the standards versus the logarithm of the volume of the half-length of the leading edge of each compound was made. The

TABLE 1. Purification of L-arabinose dehydrogenase

Fraction	Protein (mg)	Specific activity units/mg protein	Total activity units	Purification	Recovery (%)
Crude	1484	0.07	103.8	0	100
Protamine sulfate supernatant	1280	0.07	89.6	0	86
Ammonium sulfate (50% precipitate)	733	0.08	58.6	1.1	56
Heat treatment	288	0.20	57.6	2.9	55
Biogel A 1.5 *M* (fractions pooled)	20.6	1.43	29.4	20.4	28
DEAE; G-25 washed concentrated 10×	0.3	4.12	1.2	58.9	1.2

standards generated a straight line from which the unknown molecular weight was determined.

Results

The purification of L-arabinose dehydrogenase is outlined in Table 1. The dehydrogenase was purified 58.9-fold with 1.2% yield. Attempts were made to improve the purification and yield of L-arabinose dehydrogenase. The utilization of calcium phosphate gels, affinity chromatography (Affi-gel Blue), and DEAE–Sephadex ion exchange columns failed to improve the procedure as outlined here. The elution profiles of L-arabinose dehydrogenase from Biogel A 1.5 *M* and DEAE-cellulose columns are shown in Figs. 1 and 2, respectively. The peak of dehydrogenase activity eluted from the DEAE-cellulose column at 0.12 *M* NaCl.

L-Arabinose dehydrogenase showed no enhancement of activity in the presence of any of the divalent cations listed in Table 2. The presence of $CaCl_2$, $FeSO_4$, $MnCl_2$, and $CoCl_2$ severely inhibited enzyme activity. The presence of EDTA had a slight negative effect on activity as did the presence of the reducing agents glutathione, dithiothreitol, and 2-mercaptoethanol.

The dehydrogenase was specific for substrates with the L-arabino-configuration at carbons 2, 3, and 4 (i.e., L-arabinose, D-galactose, D-fucose) (Table 3). Lineweaver–Burk plots showed a K_m value for L-arabinose of 0.140 m*M* with 10 m*M* NAD as coenzyme and 0.075 m*M* with 10 m*M* NADP as coenzyme. The V_{max} values were 4.0 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein with NAD as coenzyme and 2.6 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein with NADP. The heat-stable dehydrogenase (no loss of activity when heated to 55°C for 5 min) had a pH optimum of 9.5 in glycine–NaOH buffer. L-Arabinose dehydrogenase had a molecular weight of 175 000 according to gel filtration determinations (Fig. 3).

At pH 6.6 the product of the dehydrogenase reaction was L-arabono-γ-lactone, although small amounts of L-arabonic acid were found. At pH 9.0, L-arabonic acid was the major product found owing to spontaneous lysis of the lactone at this high pH.

Discussion

L-Arabinose dehydrogenase from *Azospirillum brasiliense* apparently metabolizes only substrates having the same configuration around carbon 2, 3, and 4 as L-arabinose. Other aldose dehydrogenases are generally not specific for this configuration (Dahms and Anderson 1972*a*). In the process of enzyme purification we found no indication that there was more than one dehydrogenase capable of oxidizing L-arabinose. Dahms and Anderson (1972*a*, 1972*b*) have found two different D-fucose dehydrogenases in pseudomonad MSU-1.

L-Arabinose dehydrogenase from *A. brasiliense* reduced both NAD and NADP. L-Arabinose dehydro-

TABLE 2. Effect of divalent cations and reducing agents on enzyme activity

Reagent	Concentration	Relative activity (%)
None		100
$MgSO_4$	10 m*M*	100
NH_4SO_4	10 m*M*	100
$CaCl_2$	10 m*M*	70
$FeSO_4$	10 m*M*	0
$MnCl_2$	10 m*M*	55
$CoCl_2$	10 m*M*	46
EDTA	2.5 m*M*	92
2-mercaptoethanol	1 m*M*	95
Glutathione (reduced)	1 m*M*	93
Dithiothreitol	1 m*M*	90

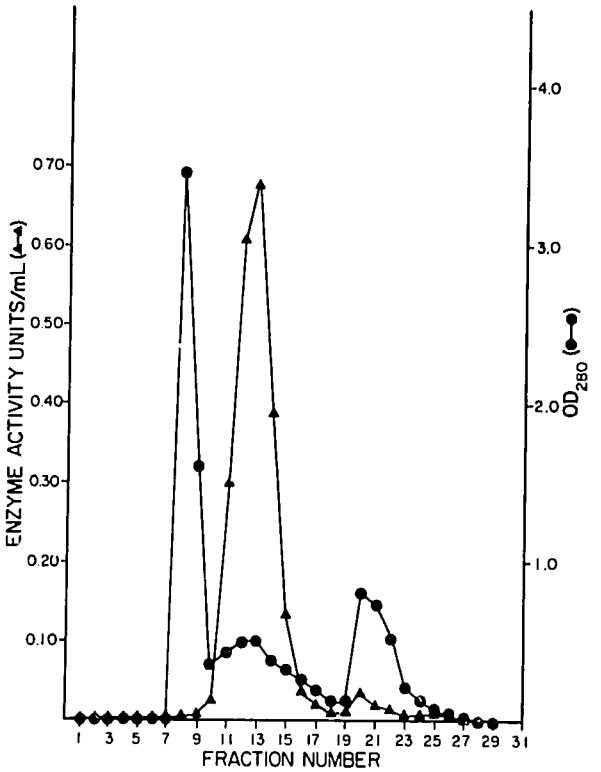

FIG. 1. Elution pattern of L-arabinose dehydrogenase on Biogel A 1.5 *M* at 4°C. Seventeen millilitres of the heat-treatment supernate were loaded on top of the 2.5 × 80 cm column. The column was prepared and run as stated in Materials and methods. Enzyme activity (▲) was determined by following reduction of NAD at 340 nm. Protein (●) was followed by the change in optical density at 280 nm. The void volume was 170 mL.

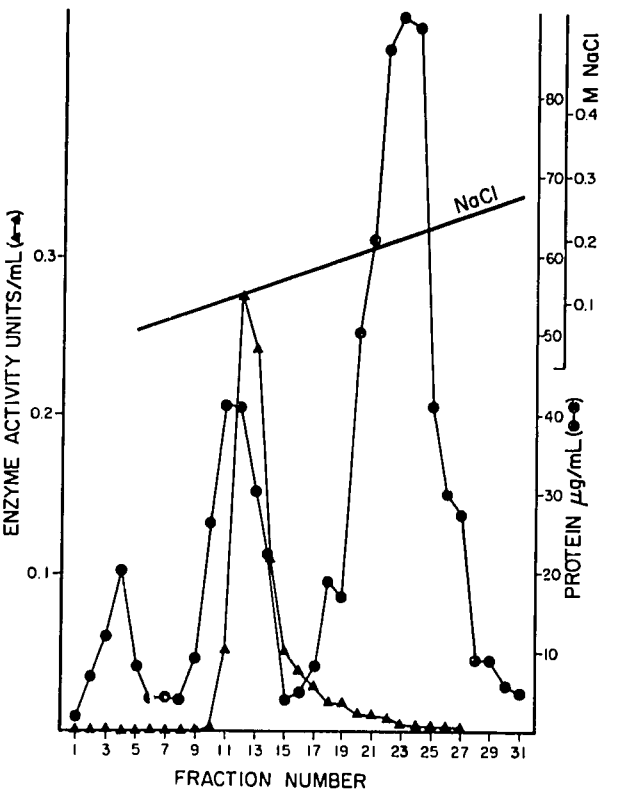

FIG. 2. Chromatography of L-arabinose dehydrogenase on a DEAE-cellulose column (2 × 10 cm) at 4°C. The column was prepared and run as given in Materials and methods. Enzyme activity (▲) was determined by following the reduction of NAD at 340 nm. Protein (●) was determined by the method of Lowry et al. (1951).

genase from *R. japonicum* and L-arabino-aldose dehydrogenase from pseudomonad MSU-1 are both also capable of reducing NAD and NADP (Pedrosa and Zancan 1974; Dahms and Anderson 1972*a*). L-Arabinose dehydrogenase from *Ps. saccharophila* is reported to utilize only NAD as coenzyme (Weimberg and Doudoroff 1955). The K_m values indicated that the dehydrogenase from *A. brasiliense* had a greater affinity for L-arabinose with NADP as coenzyme than with NAD. Despite this fact, the V_{max} was almost twice as great with NAD than with NADP. Reported K_m values from other bacteria are higher than that found in *A. brasiliense*. A value of 920 μ*M* was reported in *Ps. saccharophila* at pH 8.6 with NAD as coenzyme (Doudoroff 1962) and a value of 520 μ*M* (pH 9.4) was found with NADP as coenzyme in pseudomonad MSU-1 (Dahms and Anderson 1972*a*). Differences in K_m values, though, may well be due to differences in the type and pH of the buffer used in the assay and the assay temperature.

L-Arabino-aldose dehydrogenase from pseudomonad MSU-1 is quite similar to the dehydrogenase from *A. brasiliense* in its lack of divalent cation requirement, lack of reducing agent requirement, heat stability, and pH optimum (Dahms and Anderson 1972*a*). The dehydrogenase from *A. brasiliense* showed no difference in its substrate specificity for L-arabinose and D-fucose whereas in pseudomonad MSU-1 the rate of substrate

TABLE 3. Substrate specificity of L-arabinose dehydrogenase

Substrate 10 m*M*	Relative velocity (%)
L-Arabinose	100
D-Ribose	0
D-Galactose	63
D-Xylose	4
D-Fucose	100
L-Fucose	0
L-Rhamnose	0
D-Mannose	0
D-Glucose	0

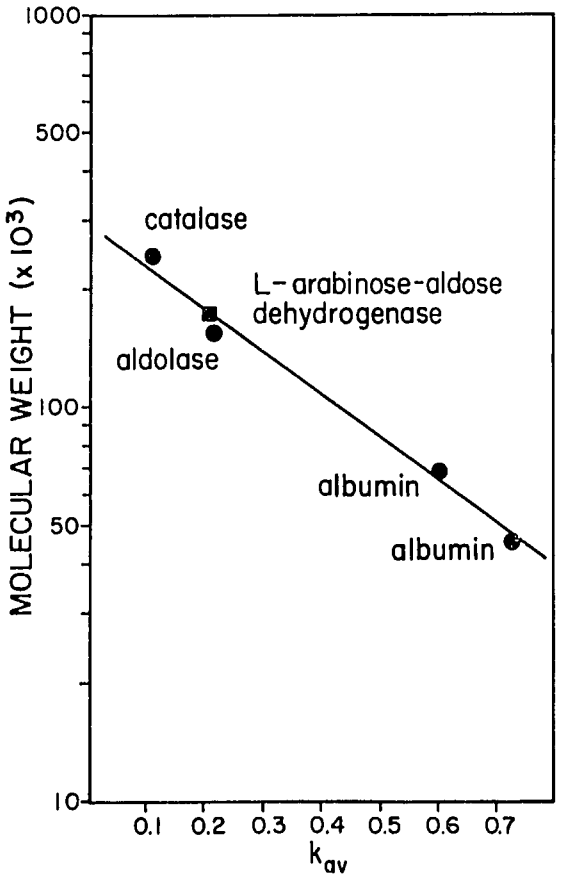

FIG. 3. Determination of the molecular weight of L-arabinose dehydrogenase by the gel filtration technique. The column was prepared and standards and samples run as given in Materials and methods.

oxidation was greater with D-fucose as substrate than with L-arabinose (Dahms and Anderson 1972*a*).

Dehydrogenase activity in crude extracts of *A. brasiliense* (70 nmol·min^{-1}·mg protein^{-1}) are comparable to that found in the fast-growing rhizobia (14–184 nmol·min^{-1}·mg protein^{-1}) and slow-growing rhizobia (6–34 nmol·min^{-1}·mg protein^{-1}) (Duncan 1979). The fact that L-arabinose oxidative pathways have been found in rhizosphere bacteria may indicate that the ability to rapidly metabolize L-arabinose is beneficial in the rhizosphere environment. A likely source for L-arabinose as well as D-fucose and D-galactose would be from root exudates and from the decomposition of root cell walls (Albersheim 1975). Thus, the enzymes involved in the L-arabinose oxidative pathways may be important in maintaining high population densities in the rhizosphere of plants by allowing the bacteria to rapidly metabolize a readily available carbon substrate.

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