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OXIDATION OF L-GLUCOSE BY A *PSEUDOMONAD*

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Summary

A new enzyme, D-*threo*-aldolse dehydrogenase (2*S*,3*R*-aldose dehydrogenase), found in *Pseudomonas caryophylli*, was capable of oxidizing L-glucose, L-xylose, D-arabinose, and L-fucose in the presence of NAD^+. The enzyme was synthesized constitutively and purified about 120-fold from D-glucose-grown cells. The K_m values for L-glucose, L-xylose, D-arabinose, and L-fucose were $1.5 \cdot 10^{-2}$, $4.5 \cdot 10^{-3}$, $2.8 \cdot 10^{-3}$, and $2.1 \cdot 10^{-3}$, respectively. D-Glucose and other aldoses inhibited the enzyme reaction; this inhibition was competitive with L-glucose as substrate and D-glucose as inhibitor. The optimum pH for the enzyme reaction was 10; the molecular weight of the enzyme was determined by gel filtration to be $7 \cdot 10^4$.

Introduction

Microorganisms capable of utilizing L-glucose were isolated from air, soil, sputum and hay, with the ultimate objective of utilizing formose sugars, which contain L-glucose as a main component, in single-cell protein production. Some of the properties of L-glucose-utilizing microorganisms have been reported previously [1,2].

The metabolic pathways of various rare sugars have been described [3—7], and L-glucose metabolism has been investigated [8—10]; however, these reports indicated that L-glucose was not metabolized. Thus enzymic studies were initiated to clarify the nature of the L-glucose metabolic pathway in *Pseudomonas caryophylli* IFO 13694, which has been isolated and characterized as an

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L-glucose utilizer by this laboratory. During this work, a novel enzyme that oxidized L-glucose in the presence of NAD^+ was found in the cell-free extracts of the strain. The present report describes some properties of this enzyme.

Materials and Methods

Bacteria and culture. The strain of *Pseudomonas* used in this study was isolated originally by Fewkes [1] and found to assimilate L-glucose. The organism since has been identified by Sakane and Banno [11] as *P. caryophylli* and is deposited at the Institute for Fermentation, Osaka, Japan (identification number IFO 13694) and at the American Type Culture Collection (identification number ATCC-29243).

The bacterium was cultured in a medium consisting of 0.5% carbon source, 0.12% $(NH_4)_2SO_4$, 0.7% K_2HPO_4, 0.3% KH_2PO_4, 0.01% $MgSO_4 \cdot 7H_2O$, 0.004% $CaCl_2$, 0.01% NaCl, 7.5 ppm $FeCl_3 \cdot 6H_2O$, 7 ppm $ZnSO_4 \cdot 7H_2O$, 5.5 ppm $MnSO_4 \cdot 4H_2O$, 1 ppm $CuSO_4 \cdot 5H_2O$, 1 ppm $CoSO_4 \cdot 7H_2O$, 1 ppm H_3BO_3, 2.5 ppm $Na_2MoO_4 \cdot 2H_2O$, 2 ppm KI, 0.025 ppm riboflavin, 0.015 ppm folic acid, 0.002 ppm biotin, 0.04 ppm thiamine, 0.0445 ppm pyridoxine, 0.0345 ppm pyridoxamine, 0.00395 ppm calcium pantothenate, 0.027 ppm nicotinic acid, 0.0255 ppm inositol, and 0.008 ppm *p*-aminobenzoic acid. The medium was sterilized with a Millipore filter (0.45 μm), dispensed into 250-ml and 500-ml flasks, inoculated, and incubated at 30°C on a rotary shaker. For large-scale cultures, a 15 l jar fermentor was used (New Brunswick Scientific).

Preparation of cell-free extract. After growth, cells were harvested by centrifugation at 12 000 × *g* for 20 min in a Sorvall centrifuge (rotor type SS-34). Cells were washed once with 0.01 M Tris-HCl buffer (pH 7.5), disrupted by grinding with alumina, and centrifuged again at 27 000 × *g* for 30 min; the resulting supernatant was used as the cell-free extract. For large-scale preparation, a Braun or a Manton-Gaulin homogenizer was used.

Column chromatography. The following column chromatographic procedures were used:

A DEAE-cellulose column (2.0 × 27.8 cm) was equilibrated with 0.01 M Tris-HCl buffer (pH 7.5). After sample application, protein was eluted with 200 ml of 0.01 M Tris-HCl and then with a linear gradient of 0.3 M $(NH_4)_2SO_4$ in 1 l 0.01 M Tris-HCl buffer (pH 7.5). 10-ml portions were collected in tubes.

A Sephadex G-100 column (2.0 × 70 cm) was equilibrated with 0.01 M Tris-HCl buffer (pH 7.5). After sample application, protein was eluted with the same buffer and 5-ml portions were collected.

A DEAE-Sephadex A50 column (1.1 × 30 cm) was equilibrated with 0.01 M Tris-HCl buffer (pH 7.5). After sample application, protein was eluted first with 60 ml of the same buffer and then with a linear gradient of 0—0.3 M $(NH_4)_2SO_4$ in 300 ml of the same buffer. 3-ml portions were collected.

Protein determination. Protein in crude preparations was measured by the method of Lowry et al. [12] and in purified solutions by the method of Warburg and Christian [13]. Bovine serum albumin was the standard protein.

Assay of aldose dehydrogenase activity. The reaction mixture containing 0.5 ml of 0.2 M glycine/NaOH buffer (pH 10), 0.1 ml of 0.6 M aldose solution, 0.1 ml of 0.02 M NAD^+ or $NADP^+$ solution, and a given amount of enzyme

solution in a total volume of 2 ml was preincubated at 30°C for 5 min. The reaction was started by adding enzyme solution.

The change of absorbance at wavelength 340 nm was measured at 30°C with a Gilford multisample absorbance recorder, model 240. The relationship between amount and activity of enzyme was linear up to a change of 9.3 μmol/min. One unit of enzyme activity was defined as the amount of enzyme needed to reduce 1 μmol of NAD^+ or $NADP^+$/min. Specific activity was defined as units of enzyme activity/mg of protein.

Molecular weight determination. Molecular weight was determined by the method of Andrews [14], using Sephadex G-100 gel filtration. Sephadex G-100 was packed in a column (2.2 × 60 cm) to make a gel bed 2.2 × 55.3 cm. Protein (3.3-ml fractions) was eluted with 0.01 M Tris-HCl (pH 7.5) containing 0.1 M KCl.

Chemicals. L-Glucose, NAD^+, $NADP^+$, streptomycin sulfate and alumina (type 305) were purchased from Sigma Chemical Company. DEAE-cellulose, DEAE-Sephadex A50, Sephadex G-100, bovine serum albumin, and standard proteins (ribonucleose, chymotrypsinogen, ovalbumin, and aldolose) for molec-

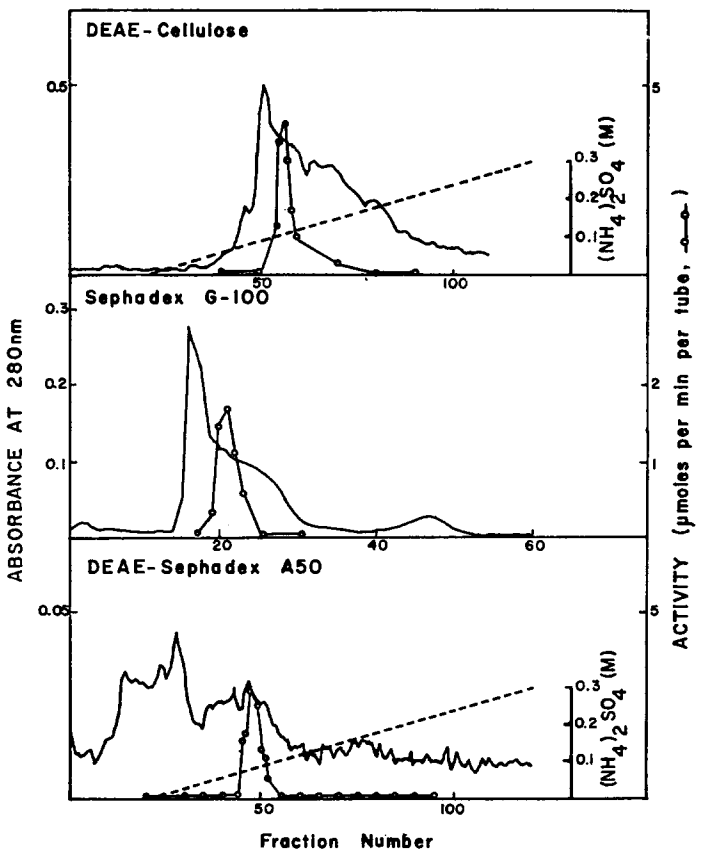

Fig. 1. Summary of column chromatography results used in purification studies. ———, protein; ○, L-glucose dehydrogenase activity. See text for further details.

TABLE I

SUMMARY OF PURIFICATION RESULTS OF L-GLUCOSE DEHYDROGENASE ACTIVITY FROM *P. CARYOPHYLII*

Sample	Total volume (ml)	Protein		Specific activity (μmol/min per mg)	Total activity (μmol/min)	Yield (%)
		Per ml (mg/ml)	Total (mg)			
Crude extract of *P. Caryophylii*	635	1.2	762	0.031	23.6	—
Streptomycin	645	—	—	—	25.8	109
$(NH_4)_2SO_4$	31	0.8	207	0.087	18.1	76
DEAE-cellulose	57	0.29	16.2	0.522	9.9	42
Sephadex G-100	15	0.12	1.78	2.49	4.4	11
DEAE-Sephadex A50	3	0.026	0.78	3.66	0.29	1.2

ular weight determination were purchased from Pharmacia Fine Chemicals.

Enzyme purification. P. caryophylli was grown in 10 l of D-glucose medium. After growth, cells were harvested with a Sharpless centrifuge, disrupted with a Manton-Gaulin homogenizer, and centrifuged at 5860 × *g* for 30 min in a Sorvall centrifuge (rotor type GSA).

To remove nucleic acids, 6.3 ml of 50% streptomycin sulfate solution was added to the supernatant (630 ml) and the precipitate was removed by centrifugation. Solid ammonium sulfate was added to the supernatant to 60% saturation. The precipitated protein was collected by centrifugation and resolved in 30 ml of 0.001 M Tris-HCl buffer (pH 7.5).

After dialysis against 1 l of the same buffer, repeated four times, the enzyme solution was applied to the DEAE-cellulose column. Fractions 54—59 were collected and concentrated with an Amicon Diaflow ultrafilter (PM-10 membrane) to 4.0 ml, which were applied to the Sephadex G-100 column. Fractions 20—22 were collected and applied to the DEAE-Sephadex A50 column. Elution patterns of the column chromatographies are shown in Fig. 1. Purification procedures are summarized in Table I; the enzyme was purified about 120-fold.

Results

L-Glucose oxidation by P. caryophylli cell-free extracts

Cell-free extracts of *P. caryophylli* cells grown on D-glucose oxidized L-glucose in the presence of NAD^+ (Table II). D-Glucose dehydrogenase activity also was found in the extract, as shown in Table II, but this activity was much lower than L-glucose dehydrogenase activity. Specific activities of L-glucose dehydrogenase did not vary in cell-free extracts grown on D-glucose, L-glucose, D-arabinose, and D-arabitol (Table II), indicating that the enzyme was synthesized constitutively.

Properties of the enzyme

Substrate specificity. As shown in Table III, L-xylose, D-arabinose, and L-fucose, as well as L-glucose, were oxidized by the enzyme in the presence of NAD^+. Low activity with D-xylose and D-ribose also was detected. Activity in

TABLE II

L-GLUCOSE DEHYDROGENASE ACTIVITY IN CRUDE, CELL-FREE EXTRACTS OF *P. CARYOPHYLLI* GROWN ON VARIOUS CARBON SOURCES

P. caryophylli IFO 13694 grown on L-glucose was inoculated in each medium and incubated at 30°C until the same cell density resulted in each (1 day in D-glucose, 2 days in DL-glucose, 4 weeks in L-glucose and 2 days in both D-arabinose and D-arabitol), at which time L-glucose dehydrogenase specific activity was determined.

Carbon source	Specific activity	
	NAD^+	$NADP^+$
D-Glucose	0.027	0.001
DL-Glucose	0.028	0.000
L-Glucose *	0.028	0.002
D-Arabinose	0.027	0.003
D-Arabitol	0.029	0.006

* When D-glucose was used as substrate, specific activity with NAD^+ as cofactor was 0.01.

the presence of $NADP^+$ was 10—20% of the activity of the NAD^+-dependent reaction.

Michaelis constants. Of the four main substrates, L-glucose had the least affinity for the enzyme. The K_m was $1.5 \cdot 10^{-2}$ for glucose, $4.5 \cdot 10^{-3}$ for L-xylose, $2.8 \cdot 10^{-3}$ for D-arabinose, and $2.1 \cdot 10^{-3}$ for L-fucose.

Effect of pH. With every substrate, the optimum pH was about 10.

Molecular weight. The molecular weight was estimated to be $7.1 \cdot 10^4$ by Sephadex G-100 gel filtration.

Stability. The crude enzyme preparations were stable, although the purified enzyme was very unstable: it lost 50% of its activity after a month's storage at 4°C. Efforts to find effective stabilizing reagents or conditions were unsuccessful.

TABLE III

SUBSTRATE SPECIFICITY OF D-*threo*-ALDOSE DEHYDROGENASE FROM *P. CARYOPHYLLI*

The purified enzyme solution was used in the experiment and the concentration of the sugars was $6 \cdot 10^{-2}$ M. The relative activity with L-xylose or D-arabinose in the presence of NAD^+ is 100%.

Substrate	Relative activity	
	NAD^+	$NADP^+$
D-Glucose	0	—
L-Glucose	68	6
D-Galactose	0	—
D-Mannose	0	—
D-Arabinose	100	17
L-Arabinose	0	—
D-Xylose	9	—
L-Xylose	100	11
D-Ribose	8	—
L-Fucose	73	18
L-Rhamnose	0	—

TABLE IV

EFFECT OF VARIOUS SUGARS ON D-*threo*-ALDOSE DEHYDROGENASE FROM *P. CARYOPHYLLI*

The enzyme solution partially purified by DEAE-cellulose column chromatography (12-fold) was used in the assay. The concentration of the inhibitors was $6.0 \cdot 10^{-2}$ M.

Sugars	Relative activity		
	L-Glucose	L-Xylose	D-Arabinose
None	100	100	100
D-Glucose	42	64	55
D-Galactose	79	98	91
D-Mannose	100	100	101
L-Arabinose	74	87	76
L-Rhamnose	74	87	88

Inhibition of the enzyme reaction by D-glucose, D-galactose, L-arabinose, and L-rhamnose

D-Glucose, D-galactose, L-arabinose, and L-rhamnose inhibited the enzyme reaction in all cases where L-glucose, L-xylose, and D-arabinose were substrates (Table IV). Inhibition of L-glucose oxidation by D-glucose was studied in more detail: the inhibition mechanism was competitive and the K_i value was $1.3 \cdot 10^{-1}$.

Discussion

These data indicate the presence of L-glucose dehydrogenase activity in *P. caryophylli*, an L-glucose utilizer. The purified enzyme also oxidized L-xylose, D-arabinose, and L-fucose, which have the same C-2 and C-3 configurations as L-glucose. (L-Galactose should also be oxidizable by *P. caryophylli*, but this substrate could not be obtained.) Many NAD^+- and/or $NADP^+$-dependent aldose dehydrogenases have been described [15—32], but L-glucose dehydrogenase activity has never been reported.

An aldose dehydrogenase of a *Pseudomonas* sp. that oxidizes D-glucose, D-galactose, D-xylose, L-arabinose, and D-fucose has been described by Cline and Hu [26]. The substrate aldoses in the present study are DL-isomers of the aldoses oxidized by the Cline and Hu enzyme (L-*threo*-aldose dehydrogenase) and thus should be named D-*threo*-aldose dehydrogenases. To avoid confusion, the chirality of the substrate molecules may be used to describe the enzyme specificity. If chirality is used according to the conventions described by Cahn [33], then the D-*threo*-aldose dehydrogenase becomes 2*S*,3*R*-aldose dehydrogenase, and the L-*threo*-aldose dehydrogenase becomes 2*R*,3*S*-aldose dehydrogenase, as shown in Fig. 2. The symmetrical substrate specificities of these two enzymes (shown in Fig. 2) suggest that differences in their protein structures may be an interesting problem to study.

It seems likely that the enzyme 2*S*,3*R*-aldose dehydrogenase participates in L-glucose metabolism in *P. caryophylli*; the pathway may be similar to the Entner-Doudoroff pathway. In fact, on the basis of paper chromatography R_F values, a byproduct in the culture medium appears to be L-gluconic acid, which is present when the cells are grown with L-glucose as the carbon source.

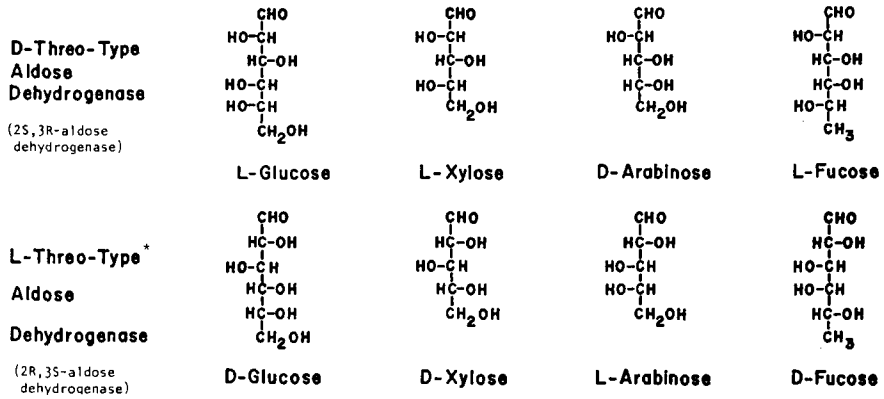

Fig. 2. Substrate specificities of two *Pseudomonas* aldose dehydrogenase. * From Hu and Cline (Ref. 25).

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