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Purification and Properties of Xylitol Dehydrogenase from the Xylose-Fermenting Yeast *Candida shehatae*

VINA W. YANG AND THOMAS W. JEFFRIES*

Institute for Microbial and Biochemical Technology, USDA Forest Service, Forest Products Laboratory, Madison, WI 53705-2398

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ABSTRACT

Xylitol dehydrogenase (EC1.1.1.9) from xylose-grown cells of *Candida shehatae* was purified 215-fold by sequential chromatography on NAD-C8 affinity, Superose-12, and Cibacron blue columns, and a single band was observed by SDS gel electrophoresis. The purified enzyme had a native molecular weight of 82 kDa and a denatured molecular weight of 40 kDa following SDS gel electrophoresis, indicating that it was composed of two subunits. Alcohol dehydrogenase copurified on the NAD-C8 but was substantially removed by Superose-12 and was not detected following Cibacron blue chromatography. The kinetic properties of the *C. shehatae* xylitol dehydrogenase differed considerably from those described previously for the *Pachysolen tannophilus* enzyme. The K_m of the *C. shehatae* enzyme for xylitol was 3.8 times smaller, whereas the K_m for xylulose was 1.7-fold bigger. These factors could account for the lower xylitol production by C. shehatae.

Index Entries: *Candida shehatae;* xylitol dehydrogenase; xylose metabolism; pentose pathway; xylose fermentation.

*Author to whom all correspondence and reprint requests should be addressed.

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INTRODUCTION

D-xylose is the major sugar in the hemicellulose component of various plant materials (*1*), and its efficient utilization is important in processes for the microbial conversion of renewable lignocellulosic resources to liquid fuels. Next to D-glucose, D-xylose in the second most abundant renewable sugar in nature. It comprises up to 25% of the total dry weight of some forestry and agricultural residues (*2*).

Aldopentoses had been considered nonfermentable by yeasts (*3,4*). Since 1981, several reports to the contrary have been published (*5-10*). In these reports, several yeasts, particularly *Pachysolen tannophilus* (*5*), *Candida shehatae* (II), and *Pichia stipitis* (*12*), have been found to produce ethanol from xylose. Of these, *P. tannophilus* has been studied most extensively. *Candida shehatae* can ferment xylose to ethanol at an exceptionally high rate compared to the published data on many other yeasts **(11).**

In yeasts and molds, D-xylose is converted to D-xylulose in two enzymatic steps (*13*). In the oxidoreductive pathway, D-Xylose is first reduced by an NADPH and NADH-linked D-xylose reductase to form xylitol. The reoxidation of xylitol to D-xylulose is effected by another NAD-linked dehydrogenase, xylitol dehydrogenase (XiDH). D-xylulose is then phosphorylated to form a key intermediate, D-xylulose 5 phosphate. Beyond this point, much of the pathway is assumed from biochemical studies with other organisms.

Ethanol and xylitol are the principal products of most yeast fermentations of xylose. However, their ratios vary with the yeast and culture conditions employed. Xylitol accumulates to higher levels with *P. tannophilus* than with *C. shehatae* (*14*). The kinetic properties of XiDH or alcohol dehydrogenase (ADH) could affect the product mixture. The objective of this study was to investigate whether the characteristics of XiDH from *C. shehatae* are different from those of *P. tannophilus.*

MATERIALS AND METHODS

Organism and Cell Growth Conditions

Candida shehatae ATCC 22984 was obtained from the American Type Culture Collection, Beltsville, MD. All stock cultures were maintained on Yeast Malt Agar (YMA, Difco†) and grown for 24 h. Inoculated cells were grown in 50 mL of 0.67% yeast nitrogen base without ammonium sulfate and amino acid (YB, Difco) using urea (2.27 g l^{-1}) and peptone (0.6%) as nitrogen sources and xylose (90 g l^{-1}) as a carbon source. Twenty cultures

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(50 mL each) were set up. Initial cell densities were 1.5–2.0 OD corresponding to 0.2–0.3 mg dry wt cells per mL. Cultures were incubated with shaking at 100 rpm, 30°C. After a 24-h growth period, cells were harvested by centrifugation at 4,000×g for 15 min. Pellets were washed with 0.1M MOPS (3-*N*-morpholino-propane sulfonic acid), pH 6.8. The resulting yeast cell paste was stored at −70°C until ready for preparation of cell free extracts. When stored under these conditions, the activity of XiDH remained essentially unchanged for at least 6 mo.

Preparation of Cell-Free Extracts

For a typical preparation of cell-free extracts, 10–15 g of thawed cell paste was used. Cells were washed and suspended in a minimal volume (8 mL) of 0.1*M* potassium phosphate buffer, pH 7.2, containing 1 mM phenylmethyl sulfonyl fluoride (PMSF) and 33 m*M* 2-mercaptoethanol (Met). Cell slurries (2 mL) were placed in a 13-mm (inside diameter) glass tube containing 2.0 g of 0.5-mm acid-washed glass beads. Six to eight tubes were used. The cell slurries were chilled in ice and blended in a high-speed vortex mixer for two 1-min bursts, after which approx 60–70% cell disruption was obtained (25). After centrifugation at 12,000 g for 20 min, the supernatant was collected as the crude extracts. Crude extracts were dialysed overnight in dialysis tubing against two changes (1 L each) of 10 M*M* potassium phosphate buffer, pH 7.2, containing 2 m*M* 2-mercaptoethanol at 4 °C.

Purification of the Enzyme

Xylitol dehydrogenase was first purified by affinity chromatography (*16*). The matrix used in this step was purchased from Sigma Chemical Co. (St. Louis, MO) and consisted of ß-NAD linked through C-8 by a six-carbon spacer to agarose (designed as NAD-C8). A 1- by 6-cm disposable column holding 2 mL of swollen NAD-C8 was prewashed with 7 mL of 3.4 m*M* NAD in 10 m*M* potassium phosphate buffer, pH 7.2, and equilibrated with 20 mL of the buffer containing fresh 2 m*M* mercaptoethanol. Dialyzed cell-free extracts were loaded onto the column, and phosphate buffer was used to elute the nonspecific binding proteins. XiDH was eluted in 10 mL of 3.4 m*M* NAD in phosphate buffer containing fresh 2 m*M* Met. Fractions containing NAD-eluted dehydrogenase (determined by assay described below) were pooled, concentrated and dialyzed by an Amicon (Lexington, MA) concentrator (10,000 mol wt cutoff) with phosphate buffer.

Gel filtration chromatography was applied by using a Superose-12 column, purchased from Pharmacia (Piscataway, NJ). The column was equilibrated with 10 m*M* phosphate buffer, pH 7.2, and 2 m*M* Met on the FPLC (Pharmacia) system. Positive fractions of XiDH were collected. Dye ligand chromatography was employed by using a Cibacron blue 3 GA agarose (Sigma) column. The column was equilibrated with-the same

buffer containing 2 m*M* Met and was eluted with stepwise NaCl gradient 0.1, 0.2, 0.3, 0.5, 1.0 *N* in phosphate buffer. Xylitol dehydrogenase was detected and collected at 0.2 *N* NaCl buffer elution. The enzyme so purified was used for the investigation. Glycerol was added to a final concentration of 50% to stabilize the enzyme for storage at −20°C. Glycerol was removed by washing preparations with 10 m*M* phosphate buffer using an Amicon or Centricon (Amicon, Lexington, MA) unit.

Enzyme Assays and Determination of Protein Concentration

Xylitol. dehydrogenase (EC 1.1.1.9) activity was measured by following reduction of NAD according to the method of Chakravorty and others (17). One unit is defined as the amount of enzyme catalyzing the reduction of 1 μmol NAD/min at room temperature. Alcohol dehydrogenase (EC 1.1.1.1) activity was determined by following the reduction of NAD according to the method of Vallee and Hoch (*18*). Protein concentration was determined by the Coomasie blue dye binding method of Bradford (*19*). Bovine serum albumin was used as the standard curve. All specific activities are expressed in international units/mg protein (IU mg^{-1}), where an international unit is defined as μmoles substrate consumed per minute.

Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was done by Phast System (Pharmacia, NJ), using 10–15% gradient gels. Gels and SDS buffer strips were purchased from Pharmacia. Gels were stained with Coomassie brilliant blue R-250 by Phast System.

RESULTS

Purification of the Enzyme

The XiDH was purified by sequential chromatography (Table 1). The first step used an NAD-C8 affinity column that had a high binding capacity for XiDH (*16*). When 10 mL of dialyzed cell-free extract was applied to a 2-mL NAD affinity column, very minor XiDH activity was detected in the void volume. High XiDH activity was found in NAD-eluted fractions: Sixty percent of the original XiDH activity was recovered. In these fractions, no NADH/NADPH xylose reductase or xylulokinase activity was detected, yet ADH activity was present (Table 2).

Gel filtration in Superose-12 separated XiDH from ADH (Fig. 1), although minor ADH activity was still detectable (Table 2). Gel filtration chromatography indicated XiDH to have a molecular mass of 82 kDa

Table 1
Purification of Xylitol Dehydrogenase from *Candida shehatae*

Sample	XiDH activity (IU ml^{-1})	Protein. (mg ml^{-1})	XiDH S.A. (IU mg^{-1})	Fold increase
Crude extract	0.107	1.44	0.074	1
NAD affinity column	0.70	0.65	1.075	15
Superose-12	0.43	0.2	2.125	29
Cibacron blue	0.112	0.007	15.95	215

Table 2
Alcohol Dehydrogenase Activity from Column Chromatography

Sample	ADH (IU ml^{-1})	Protein (mg ml^{-1})	ADH specific activity. (IU mg^{-1})
Crude extract	0.062	1.44	0.043
NAD affinity column	1.35	0.65	2076
Superose-12	0.034	0.2	0.17
Cibacron blue	ND*	0.007	ND

*ND, not detectable

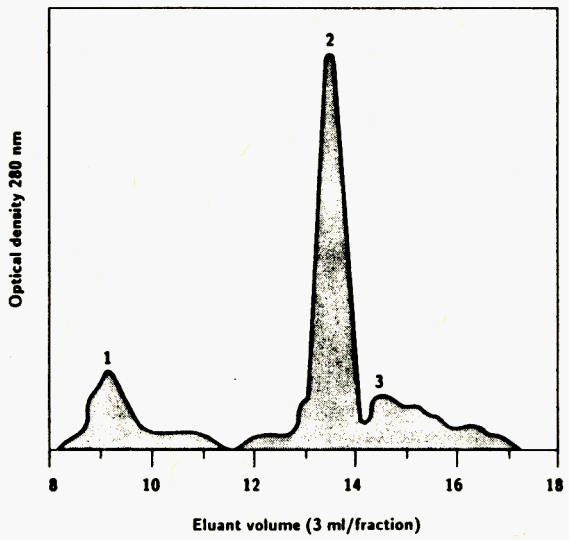

Fig. 1. Protein profile (OD 280) of gel filtration column, Superose-12. The chromatograph was run on an FPLC system. In this profile, 3-mL fractions were collected. Peak 1 contained mainly xylitol dehydrogenase.

(Fig. 2). Cibacron blue dye ligand chromatography separated ADH from XiDH, and XiDH specific activity had increased approximately 215 times from crude extracts (Table 1). SDS-PAGE of this sample yielded only a single protein band with an apparent denatured molecular weight of approximately 40 kDa (Fig. 3).

Properties of the Enzyme

The purified XiDH from *C. shehatae* was studied to determine Michaelis-Menten kinetics with respect to its substrates, xylitol and NAD, and with the substrates of the reverse reaction, xylulose and NADH. The resulting K_m values for xylitol, xylulose, NAD, and NADH are given in Table 3.

Substrate specificity of XiDH is shown in Table 4. Activity was observed with sorbitol (45%) and fructose (3%), but other polyols were not converted. The K_m value for the sorbitol was 0.17 m*M*. XiDH activity was below the detectable limit when NAD was substituted by an equal concentration of NADP for forward reaction and also NADPH for NADH in a reverse reaction.

The effect of pH was studied on XiDH in the forward direction. The rate of the activity at pH 8.6 was four times higher than at pH 7.2 (Fig. 4). At pH 7.2, no XiDH activity was detected when NADP+ was substituted for NAD+ in the forward direction. Very low activity (15%) was detected when NADPH was substituted for NADH in reverse reaction.

DISCUSSION

Xylitol dehydrogenase from C. *shehatae* obtained in this study was apparently composed of two subunits. In this respect, *C. shehatae* XiDH resembles other long-chain dehydrogenases (*24*), including that of *P. stipitis*, which has a native mol wt of 63 kDa and consists of 32-kDa subunits (*23*). The *C. shehatae* enzyme differs from XiDH in *P. tannophilus.* The latter has been reported to have a native mol wt of 172 kDa and consist of four 40-kDa subunits (*16*).

The substrate specificity of XiDH from *C. shehatae* resembles that of the enzyme isolated from *P. tannophilus* (20). The K_m value for xylitol in *C. shehatae,* however, is only about one-fourth of that observed with the *P. tannophilus* enzyme. At the same time, the K_m for xylulose is 1.7 times higher (20). A lower K_m for xylitol coupled with a higher K_m for xylulose would tend to favor the forward reaction. The affinity constants of the *C. shehatae* enzyme bear closer resemblance to the *P. stipitis* enzyme. Xylitol accumulates more in *P. tannophilus* than in strains of *C. shehatae* or *P. stipitis.* (*14*). A lower K_m value of XiDH for xylitol may be responsible for the higher ethanol yields (*14,11*).

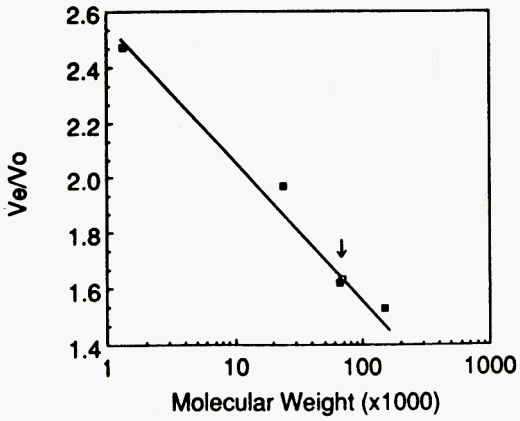

Fig. 2. Determination of native molecular weight by molecular sieve chromatography on Superose-12. Log mol wt of standards are indicated (■). Glucose oxidase (150 kDa), BSA (66 kDa), trypsinogen (24 kDa), vitamin B-12 (1.35 kDa), xylitol dehydrogenase activity (0; 82 kDa).

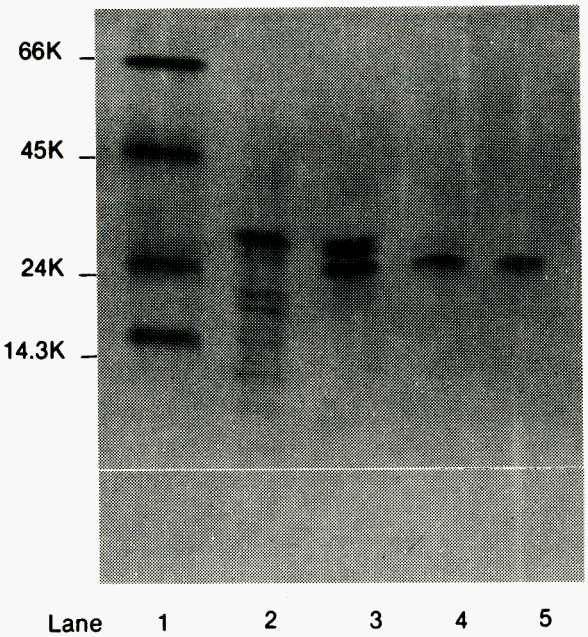

Fig. 3. SDS-PAGE of protein samples taken from stepwise purification. The gel is stained with Coomassie Brilliant Blue R-250. Lane 1 was loaded with the following protein standards: bovine albumin (66 kDa), egg albumin (45 kDa), trypsinogen (24 kDa), lysozyme egg white (14.3 kDa); lane 2, cell free extract; lane 3, NAD elute of the NAD-C8 affinity column; lane 4, XiDH positive peak collected from Superose-12 column; lane 5, XiDH positive from Cibacron blue column.

Table 3
Comparison of Affinity Constants for Xylitol Dehydrogenase of *C. shehatae* with Those of Other Xylose-Fermenting Yeasts

	Kinetic constant (mM)		
	C. shehatae[a]	*P. stipitis*[b]	*P. tannophilus*[c]
Forward reaction			
K_m xylitol	185	26	70
K_m NAD^+	0.24	0.16	0.10
Reverse reaction			
K_m xylulose	13.8	9.6	8.3
K_m NADH + H^+	0.037	0.072	0.013

[a]Present work
[b]Rizzi and others. (1989) (23).
[c]Ditzelmüller and others. (1984) (20).

Table 4
Substrate Specificity of NAD^+ —Xylitol Dehydrogenases of *C. shehatae* and Other Xylose-Fermenting Yeasts

	Relative activity (%)		
Substrate	***C. shehatae***[a]	***P. stipitis***[b]	***P. tannophilus***[c]
NAD^+			
Xylitol	100	100	100
D-sorbitol	45	48	34
Ribitol	–	69	–
Mannitol	1	ND*	ND
Darabitol	ND	ND	ND
Glycerol	ND	ND	ND
$NADH^+$			
D-xylulose	100	100	100
D-fructose	3	17	17

*ND, not detected

The pH has a strong effect on XiDH in *C. shehatae*, *P. stipitis* (*23*) and *P. tannophilus* (*20*). In our report, activity of the enzyme in the forward direction (xylulose forming) is higher at pH 8.6 than at pH 7.2. The K_{eq} value at pH 7.2 calculated from our data (2.3×10^{-8} m*M*) favors the reverse reaction, the accumulation of xylitol and NAD^+, and it is substantially similar to that obtained by Ditzelmüller and others (*20*).

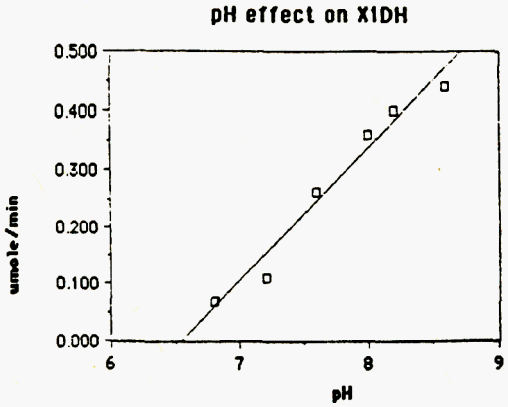

Fig. 4. PH effect on xylitol dehydrogenase activity.

The kinetic properties of XiDH in *C. shehatae* seem to be the basic reason that *C. shehatae* is a better xylose fermenter than *P. tannophilus.* Although other factors undoubtedly affect xylose fermentation, Bruinenberg and others (*21*) have stressed that the catabolism of xylose by yeast results in an accumulation of NADH, the extent of which depends on the degree of aerobiosis. Debus and others (*22*) explained the production of xylitol by *P. tannophilus* in terms of an electron sink of NADPH generated in the pentose phosphate pathway. In this report, we confirm that the high affinity of *C. shehatae* XiDH for xylitol makes it a better xylose fermenter.

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