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Partial Purification & Kinetic Properties of Nicotinamide Adenine Dinucleotide Phosphate-Dependent Alcohol Dehydrogenase of Phycomyces Blakesleeanus

Thomas Hartz
Western Kentucky University

Hartz,

Thomas K.

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PARTIAL PURIFICATION AND KINETIC PROPERTIES OF NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE-DEPENDENT ALCOHOL DEHYDROGENASE OF *PHYCOMYCES BLAKESLEEANUS*

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PARTIAL PURIFICATION AND KINETIC PROPERTIES OF NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE-DEPENDENT ALCOHOL DEHYDROGENASE OF PHYCOMYCES BLAKESLEEANUS

Recommended August 15, 1975
(Date)

Martin R. Houston
Director of Thesis

Irwin B. Lockwood

James D. Skean

Approved March 15, 1976
(Date)

Elmer Gray
Dean of the Graduate College

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Thomas K. Hartz December, 1975 28 pages
Directed by: M. R. Houston, J. D. Skean, and L. L. Lockwood
Department of Biology Western Kentucky University

$NADP^+$-dependent alcohol dehydrogenase (alcohol:$NADP^+$:oxidoreductase, EC 1.1.1.2.) was partially purified from a cell-free extract of Phycomyces blakesleeanus (+ mating type, Vanderbilt strain). The fungus was grown in shake cultures containing glucose-asparagine broth, and crude cell-free enzyme extracts were prepared. The crude enzyme extracts were partially purified by a combination of ammonium sulfate precipitations and heat precipitation. Purification resulted in a 40% recovery of the enzyme with an increase in specific activity from 0.64 units per milligram of protein to 0.25 units per milligram. The partially-purified enzyme extracts were used to determine the kinetic properties and substrate specificity of the enzyme.

Michaelis-Menten constants (Km) and maximum velocities (Vmax) were calculated by Lineweaver-Burk plots for the primary alcohol series ethyl through octyl alcohol. A decrease in Km value and an increase in Vmax resulted as the carbon chain length of the alcohols were increased to seven carbons. These results indicate that alcohol dehydrogenase of Phycomyces blakesleeanus has a greater affinity for short-chained alcohols than do those alcohol dehydrogenases previously isolated from other sources.

The substrate specificity of this alcohol dehydrogenase was quite high. Employing a variety of alcohols and aldehydes, alcohol dehydrogenase activity was obtained only for primary alcohols and one secondary alcohol, 2-butanol. Formaldehyde also functioned as a substrate for this enzyme in a manner similar to primary alcohols. Stoichiometry studies using disc-gel electrophoresis revealed that alcohol dehydrogenase was the enzyme catalyzing all the reactions reported in this thesis.

INTRODUCTION

Glycolysis and the Krebs' cycle are two major metabolic pathways involved in the oxidation of carbohydrates in most organisms. In the former pathway hexose sugars are oxidized sequentially to the α-keto acid, pyruvic acid. Under anaerobic conditions pyruvic acid may then be decarboxylated to acetaldehyde (21) followed by the reduction of acetaldehyde to ethanol. The enzymes which catalyze the latter reaction are NAD^+-dependent alcohol dehydrogenase (ADH) (alcohol: NAD^+: oxidoreductase, EC 1.1.1.1) or $NADP^+$-dependent ADH (alcohol: $NADP^+$: oxidoreductase, EC 1.1.1.2) (21). However, under aerobic conditions the enzyme may catalyze the oxidation of ethanol to acetaldehyde. In this reaction, ethanol reacts with the specific coenzyme of ADH, either NAD^+ or $NADP^+$, to form a binary complex. Subsequently, the enzyme combines with the binary complex to form a ternary complex after which ethanol is oxidized to acetaldehyde (8). In a similar reaction the acetaldehyde is oxidized to pyruvic acid via aldehyde dehydrogenase or possibly an isozyme of ADH (23).

Although ethanol has been used as the substrate for ADH in most *in vitro* investigations, the enzyme has been shown to exhibit a broad substrate specificity. For example, ADH isolated from such organs of mammals as the brain, eye, stomach, lung, and spleen does not utilize ethanol as a substrate (2, 3, 15, 27). Many types of biogenic alcohols have been shown to be substrates for ADH, such as vitamin A alcohol,

sorbitol, serotonol, and tryptophol along with a wide range of non-biogenic primary and secondary alcohols (25).

Alcohol dehydrogenase activity with secondary alcohols and ketones was reported by Dalziel and Dickinson in 1966 (8). Using horse-liver ADH they confirmed the findings of Sund and Thoerell (23) that the enzyme catalyzes the oxidation of primary, secondary, and cyclic alcohols and also the reduction of several aldehydes and ketones. However, most studies have used ethanol, propanol, butanol, and the corresponding aldehydes as substrates for the enzyme (7). Dalziel and Dickinson (8) confirmed activity of the enzyme with 2-propanol, 2-butanol, and the corresponding aldehydes while studying the mechanisms concerned with the catalytic oxidation of primary and secondary alcohols by ADH.

In 1967 Dickinson and Dalziel (10) attempted to demonstrate the substrate specificities of horse-liver ADH and yeast ADH by using the alcohols 3-pentanol, 4-heptanol, (±) 2-butanol, (±) 3-hexanol, and (±) 2-octanol. Horse-liver ADH was active with all of the preceding alcohols including both isomers of each optically active alcohol. In contrast, the yeast enzyme was completely inactive towards those secondary alcohols where alkyl groups were larger than methyl groups and active with only the (+) isomers of 2-butanol and 2-octanol. The differences between the horse-liver and yeast ADH activity were explained in terms of the structure of the enzymes concerned (11).

In recent years there has been increasing interest in the isozymes of ADH. Dalziel in 1957 (6) isolated two active forms of the enzyme by electrophoresis and chromatography. More recently, isozymes of ADH have been demonstrated which differ considerably in their catalytic properties (20). Dickinson and Dalziel have developed techniques which

have detected up to seven isozymes (11). These isozymes have been shown to be active with specific steroids as well as with ethanol and other alcohols. Moser et al. (18) and von Wartburg et al. (28) have reported that the number of isozymes present in an organism apparently varies with the experimental species.

In 1972 Tassin and Vandecasteele (24) isolated three isozymes from the bacterium Pseudomonas aeruginosa and found that the isozymes had low Km values for long-chained primary alcohols. The Km for octanol was 0.14 mM as compared with 1300 mM for ethanol. They noted that one of the isozymes was specific for NAD^+, and two were $NADP^+$-dependent. Primary and secondary alcohols were more effective as substrates for the bacterial enzyme when the chain length was increased or when secondary alcohols were substituted for the primary alcohols.

Horse-liver and yeast alcohol dehydrogenases have been the most extensively studied. The coenzyme specificity for these enzymes seems to be specifically NAD^+. However, one ADH isozyme from the bacterium Leuconostoc mesenteroides has been shown to utilize either NAD^+ or $NADP^+$ (9). In 1973 Dickinson and Monger (12) studied the kinetics of a yeast NAD^+-dependent enzyme using the alcohols 1-propanol, 1-butanol, and 2-propanol. They showed that these alcohols, and ethanol, were increasingly less effective as substrates for yeast ADH as the chain length increased or when secondary alcohols were substituted. The substrate specificity of ADH from a variety of organisms indicates that the enzyme can catalyze the in vitro oxidation of aldehydes and ketones as well as alcohols with most of the enzymes and isozymes cited being of the NAD^+-dependent type (23).

Alcohol dehydrogenase has not been isolated and studied from the filamentous fungi. The present study was undertaken to determine the substrate specificity and kinetic properties of a partially purified $NADP^+$-dependent ADH isolated from the filamentous fungus Phycomyces blakesleeanus.

MATERIALS AND METHODS

Chemicals

Protamine sulfate, $NADP^+$, phenazine methosulfate, nitro-blue tetrazolium, Trizma-base, and Trizma-HCl were obtained from Sigma Chemical Company. Ultrapure enzyme-grade ammonium sulfate was purchased from Schwartz Mann. Acrylamide (electrophoretic purity), N,N,N',N'-tetramethylethylenediamine (TEMED), N,N'-methylenebisacrylamide, and 2-mercaptoethanol were obtained from Eastman Organic Chemical Company. All other chemicals were of reagent grade and were purchased from Matheson, Coleman and Bell.

Enzyme Assay

Alcohol dehydrogenase activity was assayed by following the reduction of $NADP^+$ at 340 nm at 25 C with either a Bausch and Lomb Spectronic 505 Recording Spectrophotometer or a Cary Model 14 Spectrophotometer. The latter instrument was used only for the kinetic studies. Each assay cuvette contained 0.5 ml of 0.1 M Tris-HCl buffer (pH 9.0) and 0.05 ml of a 15.0 mM aqueous solution of $NADP^+$; water and enzyme were varied to make a total volume of 1.0 ml. The reaction was started by the addition of substrate (alcohol or aldehyde). One unit of enzyme activity was calculated to be that amount of enzyme required to reduce one µmole of $NADP^+$ per minute at 340 nm. Specific activity was expressed as units per milligram of protein.

Protein Determination

Protein concentration was determined using the spectrophotometric method of Waddel as modified by Murphy and Keis (19).

Enzyme Purification

Step I. Source of the enzyme. Stock cultures of Phycomyces blakesleeanus (+ mating type, Vanderbilt strain) were maintained on agar slants of glucose-asparagine medium with the following composition per liter: asparagine, 3.0 g; glucose, 10 g; KH_2PO_4, 1.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; thiamine, 0.2 mg; agar 1.5% and trace elements (added from a stock solution of $CaCl_2$, $FeCl_3$, and $ZnSO_4$): Ca^{+2}, 80.0 mg; Fe^{+3}, 0.3 mg; and Zn^{+2}, 0.4 mg (14). Five-hundred-milliliter quantities of glucose-asparagine broth were inoculated with a heat-shocked spore suspension (3 minutes at 60 C) prepared from the stock cultures. The inoculated media were incubated at 20 C in a New Brunswick Controlled Environmental Incubator adjusted to 100 oscillations per minute. Mycelia were harvested after 5 days by filtration through cheesecloth, washed with distilled water, and frozen in 160-g quantities. Mycelia (160 g) were suspended in 160 ml of 0.1 M potassium phosphate buffer (pH 7.5) containing 20% glycerol and 0.01 mM 2-mercaptoethanol. Twenty grams of alumina were added to this suspension, and the suspension homogenized at 0-5 C using either a Sorvall Omni Mixer or a Waring blender. Homogenation was carried out for 15 seconds at high speed and repeated 4 or 5 times. The homogenate was centrifuged for 10 minutes at 12,000 x g. The supernatant was retained and designated as the crude enzyme extract.

Step II. Protamine Sulfate. A solution of protamine sulfate (0.3 mg of protamine sulfate per mg of protein in 20 ml of distilled water) was added slowly with stirring to the crude extract at 25 C. The white nucleoprotein precipitate was removed by centrifugation at 13,000 x g for 15 minutes.

Step III. Ammonium Sulfate Precipitation, 0-50%. The supernatant from Step II was dialyzed for 4 hours against a solution of saturated ammonium sulfate to reach 50% saturation. The precipitate was removed by centrifugation at 13,000 x g for 20 minutes, and the supernatant was retained.

Step IV. Ammonium Sulfate Precipitation, 50-70%. The supernatant from Step III was dialyzed for 8 hours against a solution of saturated ammonium sulfate to reach 70% saturation. The precipitate formed was removed by centrifugation at 13,000 x g for 20 minutes. The precipitate contained ADH activity and was dissolved in a small amount of 0.1 M potassium phosphate buffer (pH 7.5).

Step V. Heat Precipitation. The enzyme extract from Step IV was heated at 55 C for 30 minutes in order to precipitate additional non-enzymatic proteins. This step resulted in a slight loss in ADH activity but a substantial increase in purification.

Polyacrylamide Disc-Gel Electrophoresis. A stock mixture containing 100 g acrylamide and 3.67 g of N,N'-methylenebisacrylamide was utilized for preparation of gels. From this mixture 7% gels were prepared in glass tubes (0.6 x 9.6 cm) using ammonium persulfate as the catalyst. Electrophoresis was carried out in a Canalco Analytic Disc Gel Electrophoresis Model 6 apparatus.

Two buffer systems were used to prepare the gels: (a) Tris-EDTA-borate and (b) 0.03 M histidine-NaOH. The Tris-EDTA-borate buffer contained 33 mM Tris, 26.9 mM EDTA, 0.03% TEMED, and 20% glycerol; the electrode buffer was composed of 82.5 mM Tris and 2.7 mM EDTA and adjusted to pH 9.0 with saturated boric acid. The 0.03 M histidine-NaOH buffer consisted of 5.4 mM histidine-HCl, 0.03% TEMED, and 20% glycerol and adjusted to pH 9.0 with 1.0 M NaOH. A constant current of 5 mA per tube was used for protein separation employing bromophenol blue as the tracking dye.

After removal from the glass tubes, the gels were stained for either $NADP^+$-dependent ADH or total protein. The $NADP^+$-dependent ADH was stained at 37 C for one hour in a reaction mixture containing 0.1 M potassium phosphate buffer (pH 7.0), 0.08 mM phenazine methosulfate, 2.2 mM nitroblue tetrazolium, 2.0 mM $NADP^+$, and 0.5 mM of substrate. Total protein was detected by staining using the method of Chrambach et al. (4).

Kinetic Properties

The maximum velocity (Vmax) and Michaelis-Menten constant (Km) values of the alcohol series butyl through octyl were calculated using the double reciprocal method, $\frac{1}{v} = \frac{1}{V} + \frac{Km}{V} \cdot \frac{1}{[S]}$, with the initial velocity measured at several substrate concentrations.

Molar Concentration Determination of Primary Alcohols

Molar concentrations of water-insoluble alcohols were determined by gas chromatography using a Varian Aerograph Series 200 Gas Chromatography unit. A Flame Ionization Detector (FID) using hydrogen as the ignition gas was utilized. After ignition of the FID, the carrier gas (nitrogen) was adjusted to a flow rate of 25 cc per minute. The

detector, oven, and ignition temperatures were set at 175 C, 150 C, and 150 C, respectively, and the instrument allowed to equilibrate for 8 to 12 hours. A 10% Carbowax chromatographic column (5' x 1/8") was utilized as the separatory column. Standard concentrations of known alcohols were prepared in chloroform; unknown concentrations were prepared by saturating a 20% glycerol-water solution with individual alcohols. One microliter of water, chloroform, and glycerol was injected separately into the gas chromatograph to determine their respective retention times. After injection of the standard and unknown solutions, the retention time for each alcohol was determined. The resultant area under the respective chromatographic peaks was calculated using the formula $A = H^{I} \frac{.85 + {}^{I}.15}{2}$, where A = area, H = height of the peak, I = width at 85% of the height, and ${}^{I}.15$ = width at 15% of the height.

RESULTS

Purification of the Enzyme

At the onset of this research problem, crude enzyme extracts prepared from Phycomyces blakesleeanus grown under optimal conditions contained relatively little ADH activity. Initially, 5-15 enzyme units were obtained from 160 g (wet weight) of mycelia. The specific activity of the enzyme extracts ranged from 0.0137 to 0.840. Subsequently, the initial specific activity was increased by growing the fungus for 5 days under reduced aeration, by doubling the glucose concentration, and by incorporation into the homogenation buffer (0.1 M phosphate buffer, pH 7.5) 20% glycerol and 0.01 mM 2-mercaptoethanol.

Alcohol dehydrogenase has been reported to be membrane-bound or closely associated with intracellular membranes, specifically, endoplasmic reticulum (13); therefore, Tween 40 was included in the homogenation buffer in an attempt to release any bound enzyme. However, 0.1 M Tween 40 was found not to increase the specific activity of the enzyme in crude extracts.

In attempts to induce the enzyme, which has been accomplished for other alcohol dehydrogenases by Fowler (13), a variety of metabolites was utilized as inducers. Ethanol, pyruvate, and acetate were added to the shake cultures as the only carbon source, but none resulted in an increase in specific activity.

TABLE 1

PARTIAL PURIFICATION OF $NADP^+$-DEPENDENT ALCOHOL DEHYDROGENASE OF PHYCOMYCES BLAKESLEEANUS

	Step	Volume (ml)	Total Protein (mg)	Total Units	Specific Activity	Percent Recovery	Purification
I	Crude	215	602	39	0.064	100	-----
II	0-50% ammonium sulfate	124	347	29	0.082	73	1.3X
III	50-70% ammonium sulfate	9	218	19	0.087	51	1.4X
IV	Heat precipitation	7	61	15	0.252	40	4.0X

Figure 1. Lineweaver-Burk plot for the determination of the Km and Vmax for ethanol.

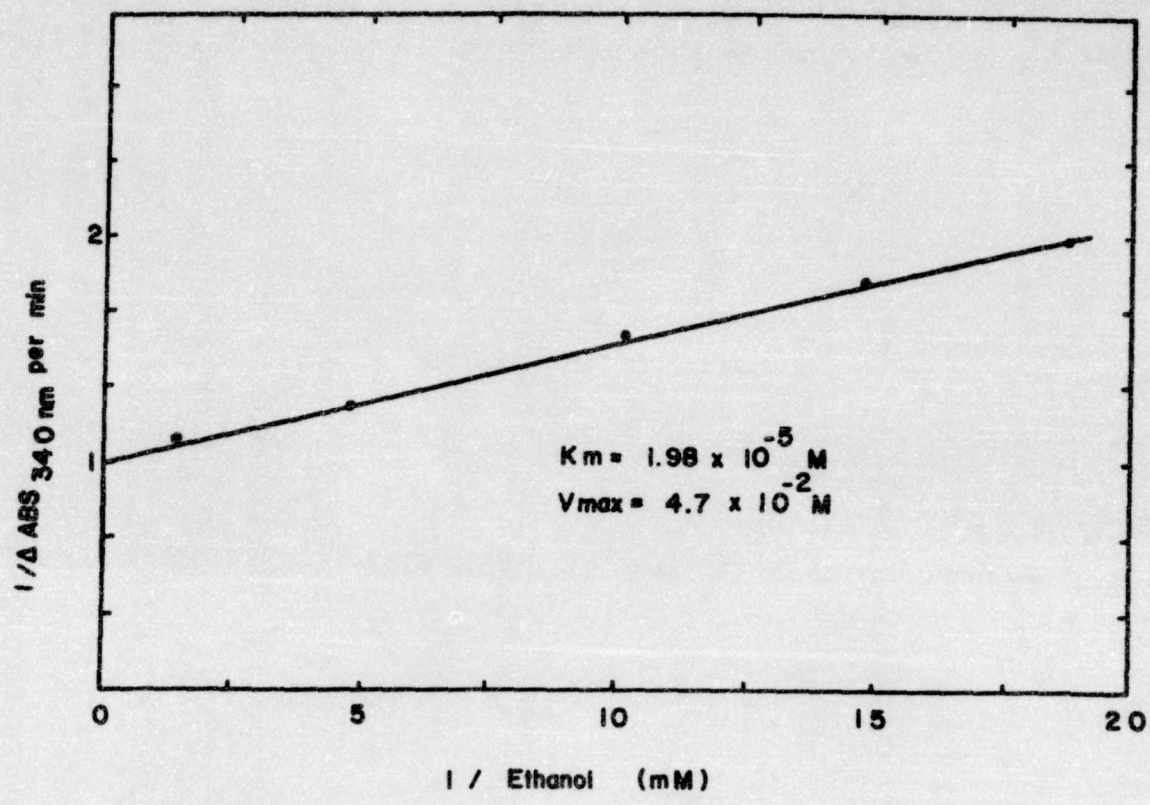
1/Δ ABS 340 nm per min
2
1
0
5
10
15
20
1 / Ethanol (mM)
Km = 1.98 x 10^-5 M
Vmax = 4.7 x 10^-2 M

Figure 2. Lineweaver-Burk plot for the determination of the Km and Vmax for propanol.

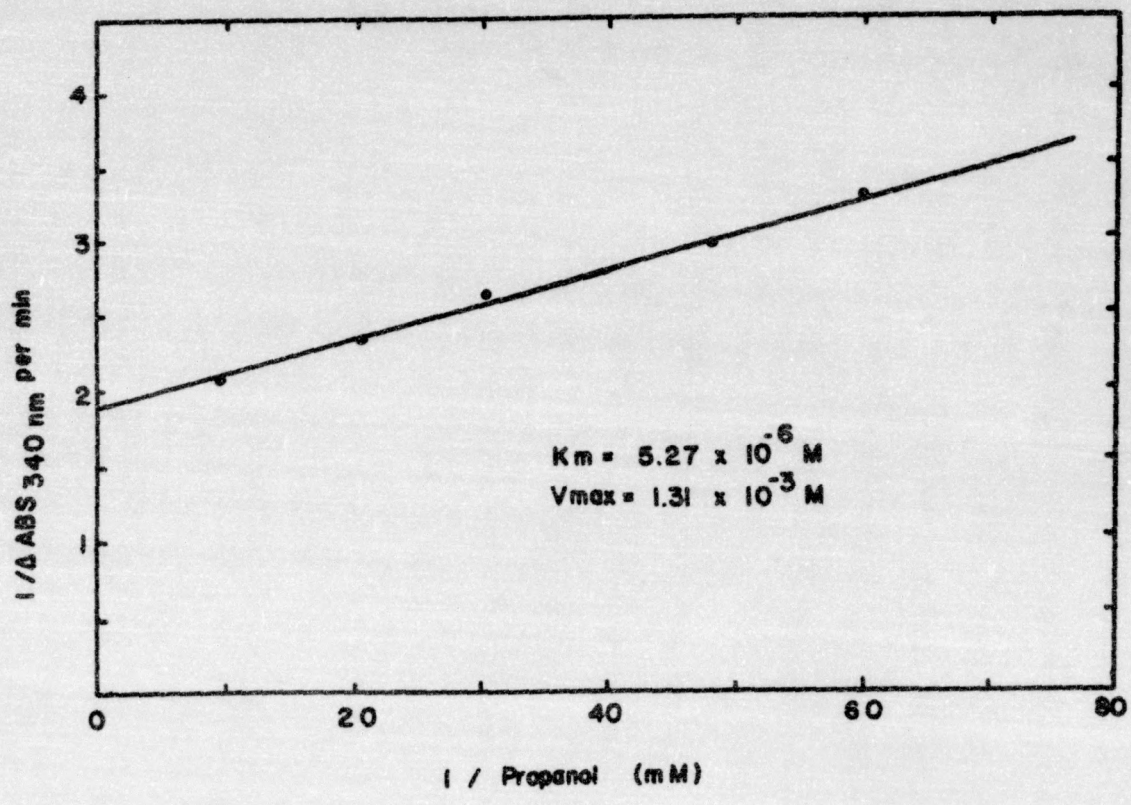
4
3
2
1
0
20
40
60
80
1 / Δ ABS 340 nm per min
1 / Propanol (mM)
Km = 5.27 x 10^-6 M
Vmax = 1.31 x 10^-3 M

Figure 3. Lineweaver-Burk plot for the determination of the Km and Vmax for butanol.

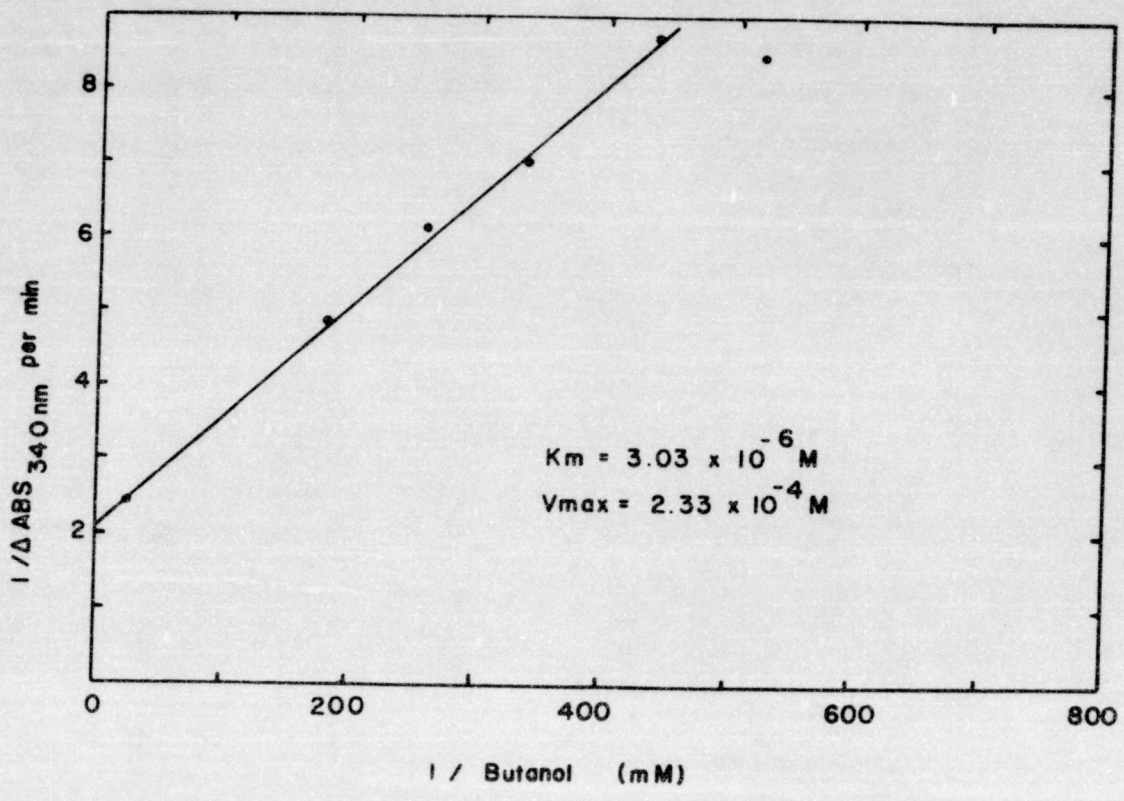
8
6
4
2
0
200
400
600
800
1 / Δ ABS 340 nm per min
1 / Butanol (mM)
Km = 3.03 x 10^-6 M
Vmax = 2.33 x 10^-4 M

Figure 4. Lineweaver-Burk plot for the determination of the Km and Vmax for pentanol.

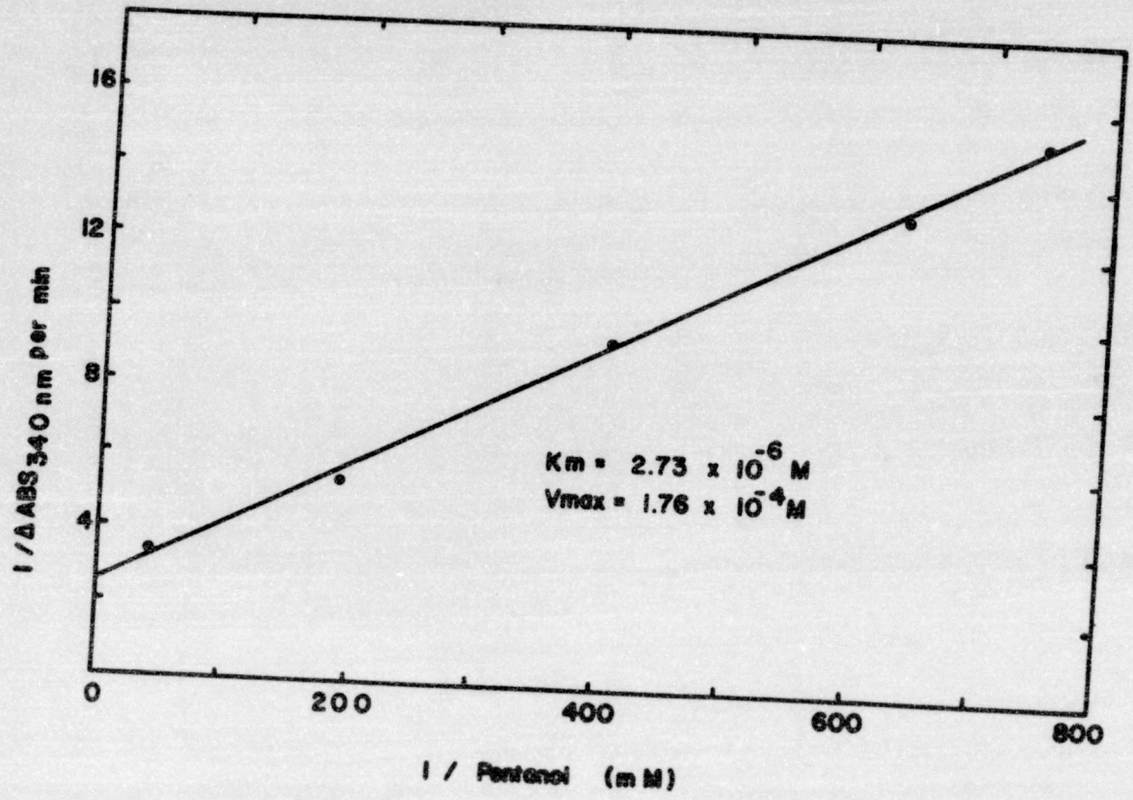
16
12
8
4
0
200
400
600
800
1 / ΔABS340 nm per min
1 / Pentanol (m M)
Km = 2.73 x 10-6 M
Vmax = 1.76 x 10-4 M

Figure 5. Lineweaver-Burk plot for the determination of the Km and Vmax for heptanol.

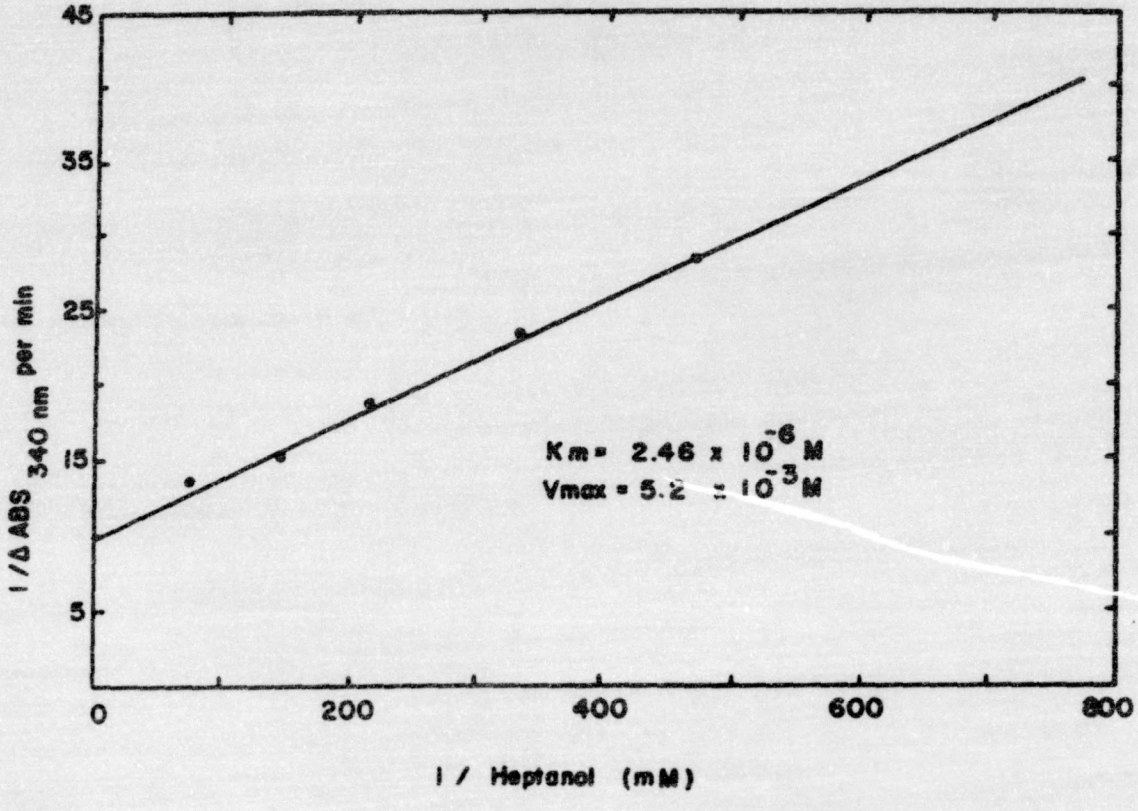
45
35
25
15
5
0
200
400
600
800
1 / Δ ABS 340 nm per min
1 / Heptanol (mM)
Km = 2.46 x 10⁻⁶ M
Vmax = 5.2 x 10⁻³ M

Figure 6. Lineweaver-Burk plot for the determination of the Km and Vmax for octanol.

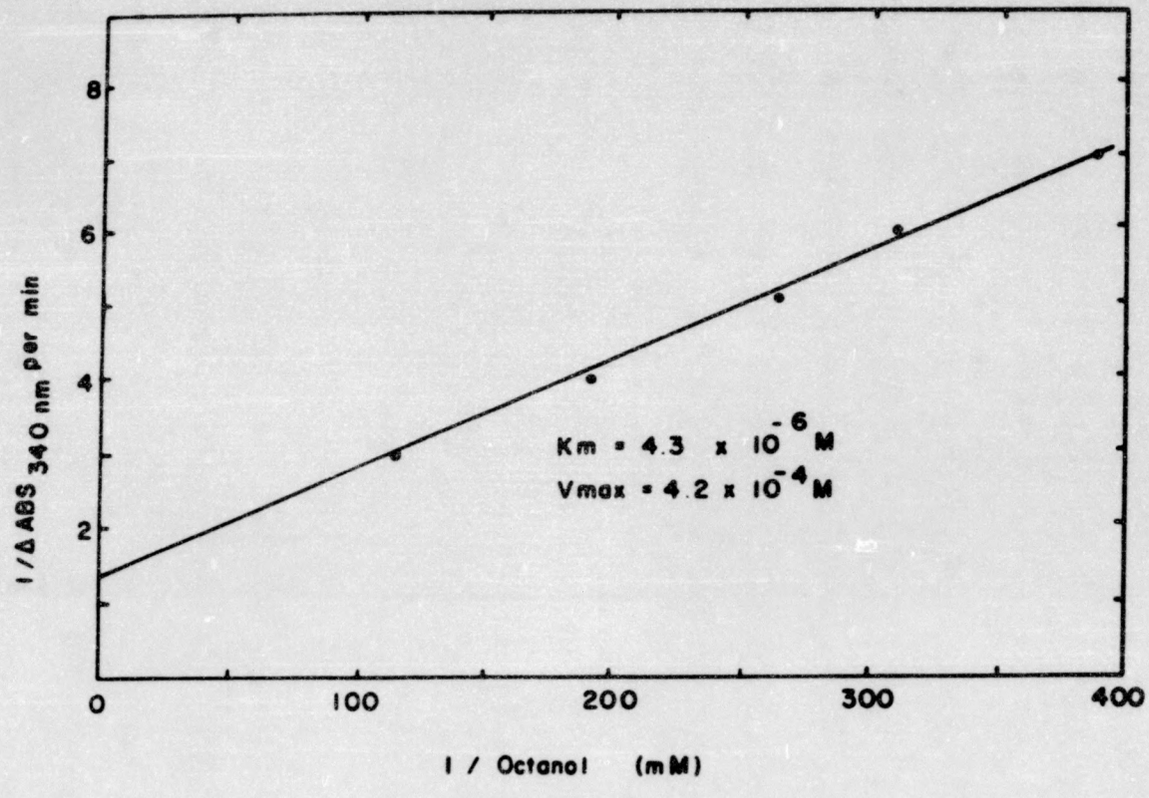
8
6
4
2
0
100
200
300
400
1 / ΔABS 340 nm per min
1 / Octanol (mM)
Km = 4.3 x 10⁻⁶ M
Vmax = 4.2 x 10⁻⁴ M

Figure 7. A comparison of the Lineweaver Burk plots for the determination of the Km values for the alcohol series ethyl alcohol through octyl alcohol.

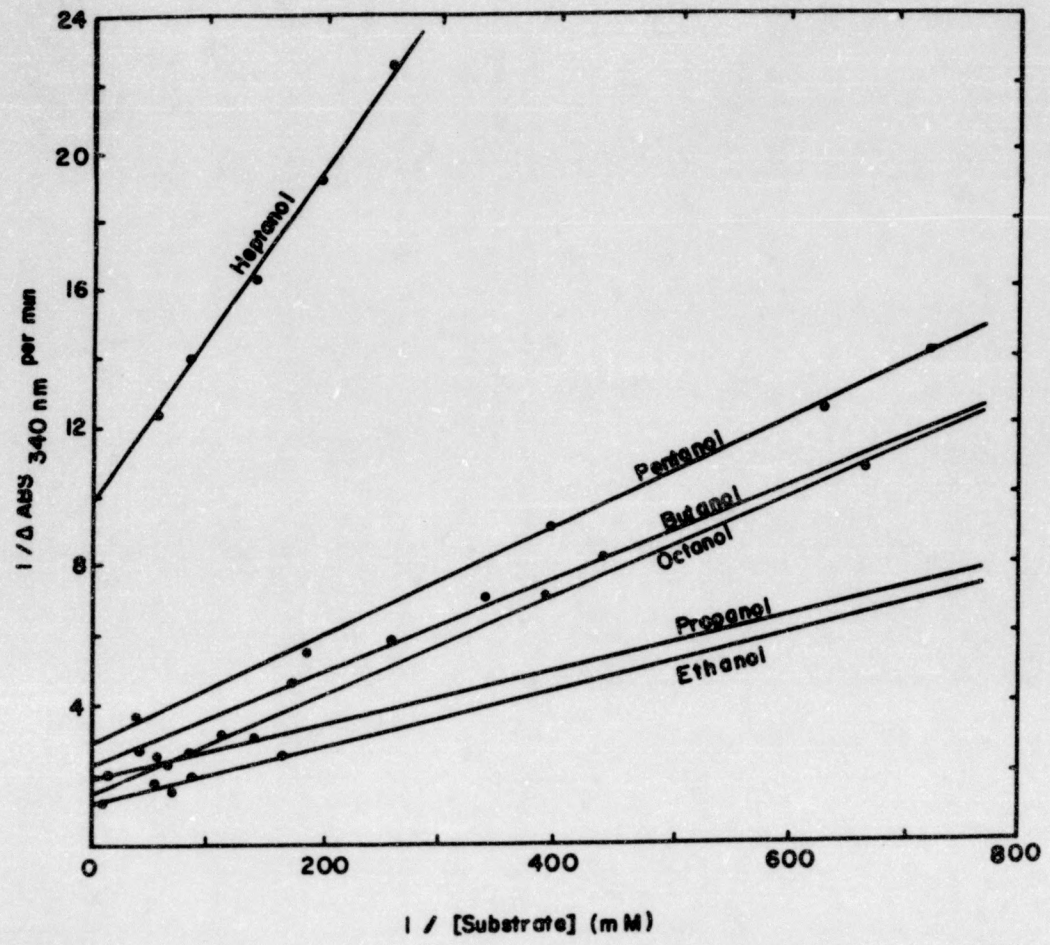
24
20
16
12
8
4
0
200
400
600
800
1 / Δ ABS 340 nm per min
1 / [Substrate] (mM)
Heptanol
Pentanol
Butanol
Octanol
Propanol
Ethanol

TABLE 2

COMPARISON OF Km VALUES OF NAD-DEPENDENT HORSE-LIVER ALCOHOL DEHYDROGENASE (HLADH), YEAST ALCOHOL DEHYDROGENASE (YADH), AND NADP-DEPENDENT ALCOHOL DEHYDROGENASE (PADH) OF PHYCOMYCES BLAKESLEEANUS FOR PRIMARY ALCOHOLS

Alcohol	NAD - dependent HLADH or YADH	Reference	NADP-dependent PADH
Ethanol	5.3×10^{-4}M (HLADH)	8	1.98×10^{-5}M
	1.3×10^{-2}M (YADH)	12, 23	
Propanol	2.3×10^{-4}M (HLADH)	8	5.27×10^{-6}M
	1.7×10^{-2}M (YADH)	12, 23	
Butanol	2.2×10^{-4}M (HLADH)	8	3.03×10^{-6}M
	1.8×10^{-2}M (YADH)	12, 23	
Pentanol	4.0×10^{-2}M (YADH)	12, 23	2.73×10^{-6}M
Hexanol	-------		-------
Heptanol	-------		2.46×10^{-6}M
Octanol	-------		4.32×10^{-6}M

the chain length increased the Km values decreased with heptanol having the lowest value (2.46×10^{-6}M). The Vmax values increased as the chain length increased (Fig. 7).

Substrate Specificity

A wide variety of water-soluble alcohols and aldehydes was compared with ethanol as substrates for ADH. Primary alcohols were found to be the primary substrates for this ADH (Table 3). Only formaldehyde and 2-butanol, having relative activity values of 125% and 17%, respectively, were comparable to the primary alcohols. Thiazole was not utilized as a substrate for this enzyme under the assay conditions used. However, when 0.1 M phosphate buffer at pH 7.5 was substituted for the Tris-HCl assay buffer, thiazole showed a relative activity of 37% when compared with ethanol under the same conditions.

Disc-Gel Electrophoresis

In order to determine whether ADH was catalyzing all of the oxidations reported in this study, discontinuous disc-gel electrophoresis was performed utilizing as substrates those alcohols which showed activity (Table 3). The Rf values calculated for each alcohol revealed that the same enzyme (ADH) was catalyzing the reactions. All substrates tested yielded activity bands except for formaldehyde and thiazole.

TABLE 3

RELATIVE SUBSTRATE SPECIFICITY OF $NADP^+$-DEPENDENT ALCOHOL DEHYDROGENASE OF *PHYCOMYCES BLAKESLEEANUS*

Substrate	Relative Activity (%)
Methanol	0
Ethanol	100
Propanol	129
2-propanol	0
2-butanol	17
Thiazole	0
Acetaldehyde	0
Formaldehyde	125
Furfural	0
Furfurol	46
Butyraldehyde	0
Propionaldehyde	0
t-butanol	0
Allyl alcohol	66
t-amyl alcohol	0

DISCUSSION

The purification procedure described in this thesis resulted in approximately a 40% recovery of partially-purified $NADP^+$-dependent alcohol dehydrogenase. The ADH isolated from Phycomyces blakesleeanus had an average specific activity of 0.25. This low specific activity, as compared to Pseudomonas aeruginosa (specific activity, 2.94) (24) and rat-liver ADH (specific activity, 16.1) (17), introduced a major obstacle in purifying this enzyme to homogeneity.

Protection of the ADH with the reducing agent 2-mercaptoethanol indicated the presence of some sulfhydryl-containing amino acids in the enzyme. The presence of 2-mercaptoethanol possibly prevented the formation of disulfide bridges which would result in the inactivation of the enzyme (5).

This study has revealed an $NADP^+$-dependent ADH with a high affinity for long-chained alcohols. When compared with yeast ADH, the Km values clearly indicated differences between the two types of enzymes. Although both are active with ethanol, the Km value of the $NADP^+$-dependent enzyme from Phycomyces blakesleeanus for this alcohol is quite low. Lower Km values were obtained with alcohols of increasing carbon lengths, whereas for the yeast ADH an opposite relationship has been reported (12). Tassin and Vandecasteele (24) in 1972 noted that low water solubility of long-chained alcohols makes more imperative the necessity of a low Km value for an enzyme utilizing these substrates. They

suggested the presence of a hydrophobic site on the enzyme capable of binding these hydrocarbon chains. Inhibition by excess substrate has been observed, especially for horse-liver ADH, and has partially been explained by the formation of a ternary "abortive complex" (8, 22). Although excess substrate inhibition was not determined in this study, this mechanism, and the one suggested by Tassin and Vandecasteele, could account for the kinetic results obtained in this investigation.

The Km values for the primary alcohol series decreased sequentially from ethanol to heptanol, but the Km increased again when octanol was used as the substrate. These results appear to indicate that the ADH from Phycomyces blakesleeanus has a greater affinity for primary alcohols of somewhat shorter chain length than alcohol dehydrogenase from Pseudomonas aeruginosa and yeast (16, 24). A study of the maximal velocities using ADH isolated from P. blakesleeanus showed that the Vmax increased with increasing chain length of the alcohols. This increase in Vmax is similar to that of yeast ADH (16) and is in contrast to the ADH isozymes found in P. aeruginosa (24) and in yeast described by Dickinson and Monger (12).

Alcohol dehydrogenase from a variety of organisms has been reported to utilize a number of cyclic and secondary alcohols, ketones, and aldehydes (7, 9, 23). In contrast, ADH from Phycomyces blakesleeanus was slightly active with secondary alcohols and aldehydes (Table 3). Formaldehyde, but not its alcohol counterpart, methanol, was a better substrate than ethanol. The fact that aldehydes often exist in the hydrated form could account for this activity. The enzyme may have utilized the hydrated form of the aldehyde as an alcohol since the aldehyde would sterically resemble a primary alcohol (1).

The detection of a $NADP^+$-dependent ADH in Phycomyces blakesleeanus is significant in that $NADP^+$-dependent ADH has only been detected in the bacterium Pseudomonas aeruginosa (24). All other alcohol dehydrogenases reported have been of the NAD^+-dependent type with the exception of an isozyme from Leuconostoc mesenteroides which can utilize either coenzyme (9). To the author's knowledge, this is the first report of a $NADP^+$-dependent ADH found in fungi.

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