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Purification and Properties of a Polyol Dehydrogenase from *Cephalosporium chrysogenus*

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A polyol dehydrogenase of broad specificity was purified 178-fold from extracts of the filamentous fungus *Cephalosporium chrysogenum*. The enzyme was found to act as an oxido-reductase in two substrate-coenzyme systems: D-sorbitol (or xylitol)-nicotinamide adenine dinucleotide (NAD) and D-mannitol-nicotinamide adenine dinucleotide phosphate (NADP). The dehydrogenase was composed of five isozymes, which, as a mixture, exhibited these properties: K_m to D-sorbitol and D-mannitol, 7.15×10^{-2} M; pH optimum, 9 to 10; molecular weight, 300,000; subunit weight, 29,000; P_I, 5.8 to 7.5. The NADP-linked activity was labile to treatment with heat or ethylenediaminetetraacetic acid. Mixed substrate assays support the hypothesis that both NAD- and NADP-linked activities are associated with isozymes of a single dehydrogenase.

The oxidation of polyol sugars to ketoses by polyol dehydrogenases has been reported to occur in several bacteria (11, 12, 15), fungi (3), as well as in mammalian tissues (9). The oxidation is catalyzed by pyridine nucleotide-linked dehydrogenases as the second step of an aldose to ketose conversion pathway which was suggested by Hers (9) to be the mechanism of fructose production in seminal vesicle tissue. The reactions involved are as follows:

$$\text{aldose} + \text{NADPH} \rightarrow \text{polyol}$$
$$\text{polyol} + \text{NAD} \rightarrow \text{ketose}.$$

Two types of pyridine nucleotide polyol dehydrogenases have been studied in microorganisms. The first type is specific for a single polyol substrate such as mannitol as in the case of *Lactobacillus brevis* (15), D-arabitol in *Aerobacter aerogenes* (6), or D-sorbitol in *Aspergillus niger* (5). The second type is characterized by broad substrate specificity. Such an enzyme has been found in *Gluconobacter oxydans* (12), *Bacillus subtilis* (11), and *Candida utilis* (2). Both types of enzymes employ either nicotinamide adenine dinucleotide (NAD) or nicotinamide adenine dinucleotide phosphate (NADP) as coenzyme. A given enzyme, however, utilizes only one of the coenzymes.

In this paper, we report the purification and characterization of a third type of polyol dehydrogenase from the fungus *Cephalosporium chrysogenum*. The enzyme is relatively nonspecific as to polyol substrate and also is capable of using either NAD or NADP as its coenzyme.

MATERIALS AND METHODS

Materials. Diethylaminoethyl-Sephadex A-25 and G-200 Sephadex (Pharmacia) were employed according to the recommendations of the manufacturer. Protamine sulfate, "ultrapure" tris(hydroxymethyl)-aminomethane (Tris)-hydrochloride buffer (pH 9.0) and ultrapure sucrose were obtained from Schwarz/Mann, Orangeburg, N.Y. Naphthol black was purchased from Eastman Organic Chemicals, Rochester, N.Y. Coomassie blue and nitroblue tetrazolium were acquired from Cyclo Chemicals Corp., N. Y. NAD, NADP, phenazine methosulfate, bovine serum albumin, D-arabitol, xylitol, adonitol, i-erythritol, i-inositol, and dulcitol were obtained from Sigma Chemical Co., St. Louis, Mo. Reagent grade D-sorbitol and D-mannitol, as well as all inorganic salts, were obtained from Fisher Chemical Co., N.Y.

Growth of fungus and preparation of enzyme extracts. A study of the effects of sugar supplement, salt content, soy-peptone concentration, and time of mycelial harvest of *Cephalosporium chrysogenum* (CBS 401.65) was conducted to maximize both growth and enzymatic yields. It was found that while production of enzyme remained unchanged per unit fungal weight, D-sorbitol as the sugar supplement produced a doubling of the mycelial mass over that obtained when D-glucose was employed at an equal molar concentration. *C. chrysogenum* was grown in 1-liter flasks for 48 h at 29 C. The flasks were placed on a rotary shaker (model G-25, New Brunswick Scientific Co., Inc., New Brunswick, N.J.) which was operated at 240 rpm/min. Each flask contained 200 ml of the following medium: soy peptone (Sheffield Chemical Co., Norwich, N.Y.), 20 g; D-sorbitol, 10 g;

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$NaH_2PO_4 \cdot H_2O$, 890 mg; Na_2SO_4, 443 mg; KCl, 381 mg; $MgCl_2 \cdot 6H_2O$, 334 mg; $FeSO_4 \cdot 7H_2O$, 25 mg; $ZnSO_4 \cdot 7H_2O$, 19 mg; and $CuSO_4 \cdot 5H_2O$, 3 mg; all per liter of glass-distilled water. D-Sorbitol was autoclaved separately and combined with the other components of the medium before use.

Fungal suspensions obtained from the 48-h experimental flasks were centrifuged at 5,000 × *g* for 20 min. The cellular material was washed three times with cold distilled water and centrifuged. Next, 30 to 40 g (wet weight) of fungus was distributed into six 75-ml Duran flasks along with 30 g of glass beads (0.45 to 0.50 mm) and approximately 8 ml of distilled water per flask. Cell disruption was accomplished by the use of a Braun MSK cell homogenizer (Bronwill, West Germany) operated at 4,000 rpm for 2 min with liquid CO_2 as coolant. After disruption, the glass beads and most of the fungal debris were removed by centrifugation at 1,500 × *g* for 20 min. The supernatant fluid was next centrifuged at 20,000 × *g* for 20 min.

Enzyme assays. Polyol dehydrogenase activity was routinely assayed spectrophotometrically by monitoring the change in absorbance at 340 nm. A double-beam recording ultraviolet spectrophotometer (Perkin Elmer, model 202) was used to follow the reaction at 25 C. The reaction mixture (final volume, 3.2 ml) contained 200 μmol of Tris-hydrochloride buffer (pH 9.0), 4.5 μmol of NAD, and 1,200 μmol of D-sorbitol for NAD-linked activity. For NADP-linked activity, 300 μmol of D-mannitol replaced D-sorbitol and 4.5 μmol of NADP was substituted for NAD. For the determination of relative substrate velocities, 300 μmol of each substrate was used in the assay system. The reverse reaction was assayed in a similar manner except that the pH of the buffer was adjusted to 7.0 and 4.5 μmol of the reduced coenzymes were used.

Reaction product. Reaction products were identified by thin-layer chromatography on Silica Gel G (Analtech, N.C.). A solvent system of chloroform-methanol-acetic acid (70:30:15) was used with fructose, glucose, sorbitol, and mannitol as standards. The assay mixture was allowed to react for 10 min at room temperature after which 10-μl aliquots were chromatographed on the thin-layer chromatography plates. Spots were visualized by spraying with acid aniline phthalate reagent (3.25 g of phthalic acid, 2 ml of aniline, 98 ml of water saturated with butanol) followed by heating at 100 C for 5 min.

Enzyme activity. One unit of enzyme activity was defined as the quantity of enzyme which catalyzed the formation of 1 μmol/min of NADH or NADPH in the assay cuvette volume of 3.2 ml at 25 C. An extinction coefficient of 6.2×10^6 cm^2/mol was used to calculate coenzyme production from the change in optical density at 340 nm. The specific activity was expressed as enzyme units per milligram of protein. Protein was determined by the method of Lowry et al. (13) with bovine serum albumin as standard.

Gel electrophoresis. Disc gel electrophoresis was carried out in 7.5% polyacrylamide gels by the method of Davis (4) in glass tubes at 8 C with a current of 2 mA per tube. Sodium dodecyl sulfate gel electrophoresis was performed by the dansylation procedure of Talbot and Yphantis (16). This procedure permits immediate visualization of protein bands without staining by use of ultraviolet light illumination which excites the dansylated proteins to fluorescence. Sodium dodecyl sulfate gel electrophoresis was performed in the presence of mercaptoethanol to maintain the protein in reduced form. Approximately 20 μg of protein was applied to each gel.

Gel isoelectric focusing. Gel isoelectric focusing in 5% acrylamide gels was performed by the procedure of Wrigley (18) with ampholine carrier ampholytes (LKB, Bromma, Sweden) having a pH range of 3 to 10 in both 5 × 65- and 5 × 120-mm glass tubes at 8 C. Approximately 20 μg of protein was applied to each gel.

Isoelectric focusing in sucrose solution. Isoelectric focusing in a sucrose density gradient was performed by the procedure of Haglund (8) in an LKB "Uniphor" 200-ml column, at 0 C for 24 h starting at 500 V for 1 h and then continuing at 1,000 V. Fractions (4 ml) were collected.

Staining of acrylamide gels. Acrylamide gels were fixed and stained in a solution of 0.1% Coomassie blue in 12.5% trichloroacetic acid and destained in 12.5% trichloroacetic acid overnight. Parallel gels were stained for detection of enzymatic activity by use of a modification of the procedure of Gabriel (7). The staining mixture used for the detection of NAD-sorbitol activity consisted of 40 ml of 0.1 M Tris buffer, pH 9.0; 25 mg of nitroblue tetrazolium; 3 mg of phenazine methosulfate; 30 mg of NAD; and 20 ml of 2 M D-sorbitol. The mixture used for NADP-mannitol activity was identical in composition except that 30 mg of NADP was substituted for NAD and 20 ml of 0.5 M D-mannitol replaced the sorbitol. Gels were incubated in appropriate activity staining solution for 15 min, washed in water, and stored in 7% acetic acid.

Purification of polyol dehydrogenase from C. chrysogenum. (i) Protamine sulfate. Protamine sulfate was added with stirring at 4 C at a concentration of 3 mg of extract per ml. After 5 min, the precipitate was removed by centrifugation at 5,000 × *g* for 20 min. The enzyme activity in the supernatant fraction was concentrated approximately fivefold in a model 202 Amicon ultrafiltration cell (Amicon Corp., Lexington, Mass.) equipped with a PM-30 membrane under a nitrogen pressure of 15 lb/in^2. The concentrated extract was subjected to diafiltration by passing four volumes of 0.05 M Tris buffer, pH 9.0, through the cell under a nitrogen pressure of 15 lb/in^2. The liquid passing through the membrane was discarded.

(ii) Diethylaminoethyl-Sephadex chromatography. The enzymatically active material retained by the Amicon membrane in the first step (20 ml) was applied to a diethylaminoethyl-A-25 Sephadex column (2.5 × 65 cm) previously equilibrated with 0.05 M Tris buffer, pH 9.0. The column was eluted by means of a linear gradient of 0.05 to 0.15 M (Cl^-) generated in a two-chamber apparatus: the mixing chamber was filled with 150 ml of 0.05 M Tris buffer, pH 9.0, and the second chamber contained 150 ml of 0.10 M NaCl. The flow rate was adjusted to 25 ml/h and 5-ml fractions were collected. Fractions

containing high-enzymatic activity were combined and concentrated in the Amicon ultrafiltration cell as described above.

(iii) Sephadex G-200. The concentrated enzyme preparation (5 ml) was injected into a G-200 Sephadex column (2.5 × 88 cm) and subjected to upward flow chromatography. The flow rate was 20 ml/h and 5-ml fractions were collected. The protein was eluted with 0.1 M NaCl and 0.1 M Tris buffer, pH 9.0. Fractions of high enzyme activity were pooled and concentrated in the Amicon apparatus as previously described. The resulting enzyme preparation used for subsequent assays contained 0.09 mg of protein per ml.

RESULTS

Enzyme purification. The results of the purification scheme are detailed in Table 1. Assays for D-sorbitol-NAD-linked dehydrogenase activity were used to detect the presence of the enzyme. Active fractions contained both NAD-linked as well as NADP-linked activities. The ratio of D-sorbitol-NAD-linked activity to D-mannitol-NADP-linked activity was 0.4 (±0.1). The use of protamine sulfate appeared to stimulate enzymatic activity resulting in over 100% recovery for this step. The enzyme was eluted from Sephadex G-200 in a single peak. The purification procedure employed resulted in a 178-fold overall purification.

Disc gel electrophoresis. Polyacrylamide gel electrophoresis of the purified-enzyme preparation showed the presence of two protein bands after staining for enzyme activity and after staining for protein on parallel gels. Sorbitol oxidation required NAD as coenzyme, whereas mannitol oxidation required NADP. Activity stains, which were sharpest and most intense with the stain for D-mannitol-NADP activity, indicated that more enzyme, as judged by band intensity, was present at 7 to 8 mm as compared to a faint band at 10 to 11 mm in a 50-mm running gel. Protein staining of parallel gels indicated protein bands of similar intensities at these two positions. No enzymatically inactive bands were detected in this procedure.

Gel electrofocusing. To obtain a better understanding of the gel electrophoresis pattern, isoelectric focusing was carried out in 5% gels using a pH 3 to 10 gradient. Five enzymatically active bands were separated (Fig. 1). Three protein fractions corresponding to the three most intense activity bands appeared on parallel gels. No inactive bands were visualized with the protein stain when 40 to 50 μg of purified enzyme were applied to the gel. The gel shown in Fig. 1 was stained with mannitol-NADP activity stain after being run with acid solution, the anode, and sample in the upper chamber.

Isoelectric focusing of polyol dehydrogenase in a sucrose density gradient. Isoelectric focusing of the purified enzyme preparation in a sucrose density gradient revealed both NAD-linked-D-sorbitol dehydrogenase activity, as well as NADP-linked-D-mannitol activity in the pH 5.8 to 7.5 region (Fig. 2). Several isozymes of polyol dehydrogenase capable of using either NAD or NADP as coenzymes were found to be present in the preparations examined. The latter observation is supported by the relatively broad activity peak obtained in this phase of the investigation and by the observation of five active bands obtained after gel electrofocusing (see above). Further, focused gels were sectioned, the slices were eluted with distilled water, and the pH was determined for each gel fraction. In each active fraction assayed, both NAD-linked as well as NADP-linked activities were present, although the relative reaction velocity ratios differed from fraction to fraction, indicating that either varying concentrations of each isozyme were present or different kinetic characteristics for each enzyme form. Isozymes of broad specificity, such as those of lactate dehydrogenase, have been found to display

TABLE 1. *Purification of polyol dehydrogenase from C. chrysogenum*

Fraction	Vol (ml)	Total protein (mg)	D-Sorbitol dehydrogenase (U)[a]	Recovery (%)	Sp act[b]
Crude extract	92	1,104	40.5	100	0.037
Protamine sulfate supernatant fluid	89	837	45.5	112	0.055
Amicon diafiltration retentate	20	370	34.2	84	0.092
DEAE A-25 Sephadex	17	5.4	24.6	61	4.550
Sephadex G-200	18	1.7	11.2	28	6.588

[a] One unit is the amount of enzyme that catalyzes the formation of 1 μmol/min of reduced coenzyme in the assay volume of 3.2 ml at 25 C. The ratio of D-sorbitol-NAD-linked activity to mannitol-NADP-linked activity when assayed with identical substrate concentrations (300 μmol) was 0.4 (±0.1) in the crude extract, diethylaminoethyl-Sephadex (DEAE), and Sephadex G-200 preparations.

[b] Units per milligram of protein.

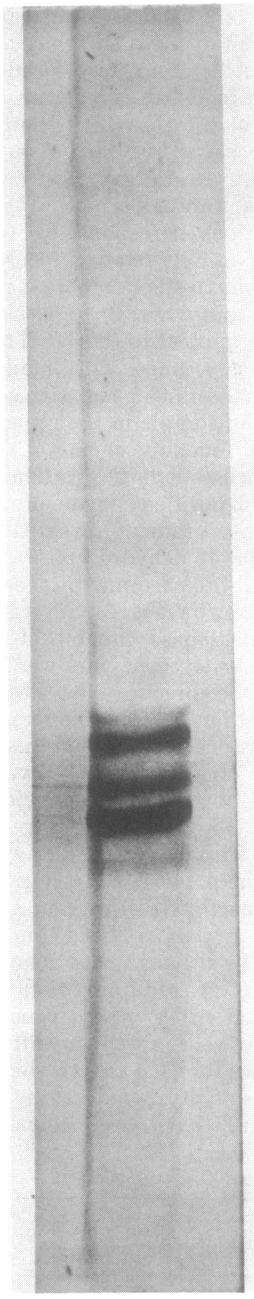

FIG. 1. *Isoelectric focusing of purified polyol dehydrogenase in 5% polyacrylamide gel (65-mm length) with a pH range from 3 to 10.0. The anode and acid solution were in the upper chamber. Gel stained for enzymatic activity (NADP-D-mannitol dehydrogenase).*

different reaction velocities toward the same substrate (14).

Properties of the purified enzyme preparation. (i) Molecular weight. The molecular weight of polyol dehydrogenase was estimated by the Sephadex gel filtration method (1) using apoferritin, catalase, gamma globulin, and aldolase as standards. A value of 300,000 was obtained from the standard calibration curve (Fig. 3).

(ii) Subunit composition. A sample of purified polyol dehydrogenase was treated with sodium dodecyl sulfate and mercaptoethanol before disc gel electrophoresis by the procedure

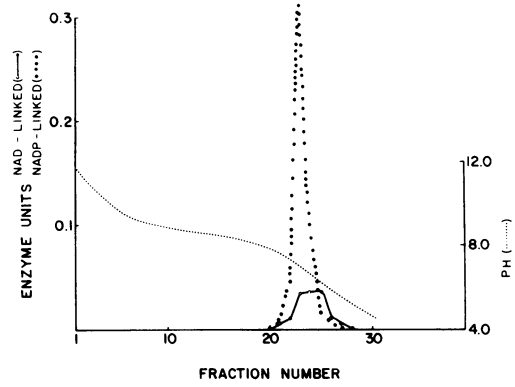

FIG. 2. *Isoelectric focusing of purified polyol dehydrogenase was carried out in a sucrose density gradient loaded with ampholine solution (pH range, 3 to 10) in an LKB "Uniphor" at 0 C for 24 h at 1,000 V. Each fraction (4 ml) was assayed for NAD-D-sorbitol activity as well as for NADP-D-mannitol activity.*

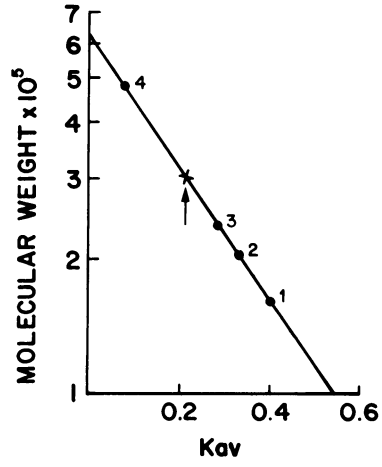

FIG. 3. *Plot of standard proteins and polyol dehydrogenase chromatographed on Sephadex G-200. Standard proteins and their molecular weights were: (1) aldolase, 158,000; (2) gamma globulin, 205,000 according to Andrews (1); (3) catalase, 240,000; (4) apoferritin, 480,000. Arrow indicates position corresponding to* K_{av} ($[V_e - V_o]/[V_t - V_o]$) *of active polyol dehydrogenase.*

of Talbot and Yphantis (16). The results showed a single protein band. The molecular weight of the subunit was determined by employing the previously established molecular weight values for standards under reducing conditions (17). The data yielded an estimate of 29,000 for the subunit (Fig. 4).

(iii) Effect of pH. The oxidative activity of the polyol dehydrogenase in the range of pH 7 to 10.5 is shown in Fig. 5. Both NAD-linked and NADP-linked activities rose steadily as the pH was increased from a value of 7 to a pH of 9. At the latter value, the NADP-linked enzymatic activity reached a plateau and no significant increase in activity was apparent in the higher alkaline range. The NAD-linked activity continued to increase slightly above pH 9.

(iv) Substrate specificity and dependence of activity on substrate concentration. The reaction velocity of an aliquot of enzyme solution with D-sorbitol as substrate and NAD as coenzyme was arbitrarily assigned a value of 100 for NAD-linked activity. Similarly, the velocity observed with a fixed quantity of enzyme oxidizing D-mannitol while reducing NADP was assigned a value of 100 for NADP-linked activity. Table 2 shows the results of a comparative assay of 10 substrates. The order of reactivity found for NAD-linked oxidation is: xylitol > D-sorbitol > adonitol > D-mannitol > D-arabitol. Erythritol, inositol, dulcitol, and, as expected, glucose and fructose were not oxidized at all. NADP-linked oxidative activity in order of relative velocity obtained with listed substrates was: D-mannitol > D-arabitol > D-sorbitol > xylitol. All other substrates tested did not react with NADP.

To ascertain whether two distinct polyol dehydrogenases, NAD-linked as well as NADP-linked, were both present in the preparation, a

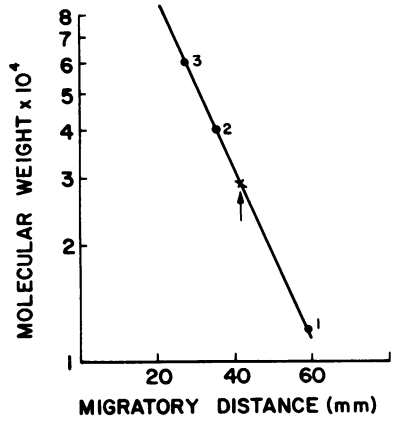

FIG. 4. *Plot of migration distance of standard proteins and polyol dehydrogenase following electrophoresis in sodium dodecyl sulfate-polyacrylamide gels. Proteins were reduced with 2-mercaptoethanol. Standard proteins and their molecular weights under reducing conditions were: (1) cytochrome c, 12,000; (2) aldolase, 40,000; (3) catalase, 60,000. Arrow indicates position of band corresponding to purified, reduced polyol dehydrogenase. Dansylated enzymes and standards were run both separately and then on the same gel, and protein bands were detected by fluorescence immediately after electrophoresis.*

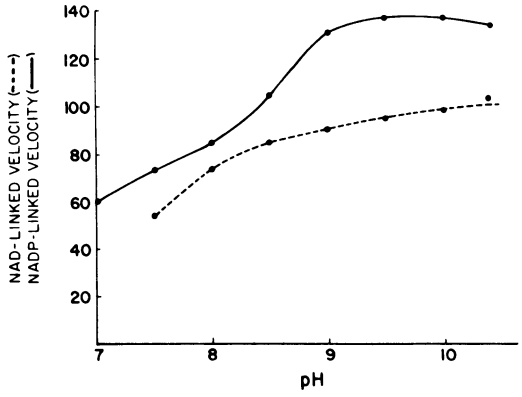

FIG. 5. *Effect of pH on polyol dehydrogenase activity. Both NAD-linked as well as NADP-linked activities were assayed at each pH as indicated using identical aliquots of the purified enzyme preparation. Velocities plotted as micromoles per liter times reciprocal minutes were obtained employing 28.3 μg of the enzyme preparation in the assay system as described in Materials and Methods.*

TABLE 2. *Relative reaction velocities of polyol dehydrogenase with several sugar substrates*

Substrate[a]	NAD-linked activity[b]	NADP-linked activity[c]
Xylitol	115.0	0.5
D-Sorbitol	100.0	15.0
Adonitol	31.0	0
D-Mannitol	13.0	100.0
D-Arabitol	0.2	16.0
i-Erythritol	0	0
i-Inositol	0	0
Dulcitol	0	0
Glucose	0	0
Fructose	0	0

[a] Substrates were employed in a concentration of 300 μmol in the assay system described in Materials and Methods.

[b] Expressed as reaction velocity relative to that obtained with D-sorbitol as a substrate which is arbitrarily given a value of 100.

[c] Expressed as reaction velocity relative to that obtained with D-mannitol as a substrate which is arbitrarily given a value of 100.

study of substrate competition was performed (Table 3). The reaction velocity of reduction of NAD with D-sorbitol and a fixed quantity of enzyme was assigned a value of 100% for NAD-linked activity. Similarly, reduction of NADP by D-mannitol and an enzyme aliquot was given a 100% value for NADP-linked activity. Using this arbitrary scale, D-mannitol was found to support 5% NAD-linked activity while D-sorbitol supported 40% NADP-linked activity. When both mannitol and sorbitol were combined to produce final concentrations in the assay mixture identical to those at which each was assayed individually, the mixed substrate supported 81% NAD-linked activity and 93% NADP-linked activity. If two distinct dehydrogenases were present, the mixed substrate should have produced 105% NAD-linked oxidation and 140% NADP-linked oxidation. Therefore, these results tend to support the conclusion that a single enzyme rather than two distinct dehydrogenases is present in the preparation studied. However, it is possible that inhibitory effects of one enzyme upon a second enzyme could also result in a loss of activity.

Lineweaver-Burke plots were used to calculate the apparent K_m and V_{max} of D-sorbitol and D-mannitol for the forward reaction. With reduced coenzymes and fructose as substrate for the reverse reaction, the appropriate constants were also obtained (Table 4). The K_m values obtained with D-mannitol and D-sorbitol were both 7.15×10^{-2} M indicating that the dehydrogenase, in vitro, has very low affinities for its substrates.

(iv) Reaction product. The reaction product for the forward reaction for both NAD- and NADP-linked oxidations is D-fructose as determined by thin-layer chromatography. The reaction product for the back reactions could not be detected by the methods employed.

(v) Effect of heat treatment. Heating of the enzyme solution in Tris buffer at 50 C for periods of time up to 5 min completely inactivated NADP-linked dehydrogenase activity (Table 5). If NADP was added to the enzyme solution before heat treatment, a significant degree of activity was retained. For example, whereas only 8% of NADP-linked activity was present after 1 min of heat treatment of the enzyme solution, 81% of the activity was retained if NADP was added before heat treatment. All activities were based on assigning as 100% the reaction velocity displayed by a portion of untreated enzyme solution assayed by the method described above. The K_m and V_{max} of NAD-linked-D-sorbitol dehydrogenase activity was unchanged by heat treatment. Hence, NAD-linked activity was found to be heat stable in contrast to NADP-linked activity which appeared to be heat labile.

TABLE 3. *The effect of individual and mixed polyol substrates on polyol dehydrogenase activity*

Substrate (μmol)	NAD-linked activity (%)[a]	NADP-linked activity (%)[a]
D-Sorbitol (600)[b]	100	40
D-Mannitol (150)	5	100
D-Sorbitol (600) + D-mannitol (150)	81	93

[a] Percentages indicate reaction velocity relative to preferred substrate used with its proper coenzyme e.g., D-sorbitol with NAD = 100%.

[b] Numerals indicate quantities of substrates employed in the assay system described in Materials and Methods.

TABLE 4. *Kinetic constants of various substrates of the polyol dehydrogenase from C. chrysogenum*[a]

Substrate	Cofactor	K_m (M)	V_{max} (μmol/liter per min)
D-Sorbitol	NAD	7.15×10^{-2}	91.0
D-Mannitol	NADP	7.15×10^{-2}	250.0
D-Fructose	NADH	2.00×10^{-1}	12.5
D-Fructose	NADPH	1.0×10^{0}	300.0

[a] Kinetic constants were determined employing 28.3 μg of the enzyme preparation.

TABLE 5. *Effect of heat (50 C) on polyol dehydrogenase with D-mannitol as substrate*

Incubation (min)	NADP-linked activity[a]	
	Heated with NADP added (%)	Heated without NADP (%)
1	81	8
2	62	3
3	57	2
5	38	0

[a] Percentage of activity remaining relative to control.

(vi) Effect of EDTA and magnesium salts. Ethylenediaminetetraacetic acid (EDTA) was found to be an inhibiting ion toward NADP-linked dehydrogenase activity when the enzyme solution was incubated at room temperature for 5 min in the presence of the anion in water solution. At a final concentration of 0.008 M, EDTA caused a 76% drop in the oxidation rate of D-mannitol by NADP in the presence of an aliquot of enzyme. If NADP was added to the

enzyme before incubation with EDTA, full enzymatic activity was retained. This finding is similar to the effect of heat inactivation in that the addition of NADP before incubation with EDTA protected enzymatic function from inhibition. NAD-linked dehydrogenase activity was not affected by incubation with EDTA with or without prior addition of coenzyme.

Magnesium (Mg^{2+}) at a final concentration of 0.1 M, was found to inhibit both NAD- and NADP-linked activities, whereas concentrations in the range of 0.001 to 0.01 M proved to slightly stimulatory to both activities.

DISCUSSION

Using the growth medium developed for the present investigation, the polyol dehydrogenase of *C. chrysogenum* was produced in high yield. The enzyme was purified 178-fold and appeared to be free of enzymatically inactive material or extraneous enzymatic activity as determined by the criteria of polyacrylamide gel electrophoresis and electrofocusing. Because the active enzyme has a molecular weight on gel filtration of approximately 300,000, whereas under reduced sodium dodecyl sulfate gel electrophoresis only one band of 29,000 is present, as many as 10 subunits may be present in the native state.

The existence of several active multimeric forms of the enzyme is a possible explanation for the two active bands observed after polyacrylamide gel electrophoresis and the five active bands observed after gel electrofocusing. The pattern of enzymatic activity after electrofocusing in a sucrose density gradient indicates maximal NADP-linked activity focused at a P_I of 6.95 and NAD-linked activity peaked in a slightly broader fashion between pH 6.95 and 6.15. The slight mixing of fractions during removal of the sucrose solution after completion of isoelectric focusing may have contributed to the broadening of this peak. The existence of several active species of the enzyme having P_I values between pH 5.8 to 7.5 was confirmed by cutting focused gels and measuring the pH of eluted ampholytes from the gel section. Determination of whether these different isozymes represent multimeric forms of polyol dehydrogenase awaits further study.

Most of the NAD-linked oxidative properties of the polyol dehydrogenase of *C. chrysogenum*, such as pH optimum, lack of substrate specificity, V_{max} and K_m are similar to the enzyme extracted from *C. utilis* (2). However, the enzyme from *C. chrysogenum* is notably different from the latter polyol dehydrogenase in its ability to use both NAD and NADP as coenzymes. Another difference is that NADP protects NADP-linked oxidative activity from inactivation by EDTA or heat. Chakravorty et al. (2) reported that the polyol dehydrogenase of *C. utilis* is protected by NADH, not NAD, from inactivation by EDTA or heat. The NAD-linked polyol dehydrogenase activity of *C. chrysogenum* is unaffected by such treatments. This evidence suggests the existence of two coenzyme sites on the *C. chrysogenum* polyol dehydrogenase.

The physiological significance of various carbohydrate pathways has been reviewed by Horecker (10). We will not speculate here regarding the significance of the use of both pyridine coenzymes by this fungal enzyme since related enzymes in the nonphosphorylative pathways have not been studied in this species. As hypothesized by Horecker (10), the lack of specificity and low affinity for substrate of polyol dehydrogenases may be indicative of a primitive enzyme system. Concentration of reduced NADH in relation to NADPH may provide a control factor for predominance of either energy producing or biosynthetic pathways.

It was noted that growth of *C. chrysogenum* with D-sorbitol as a sugar supplement produced a doubling of total mycelial yield over that of an equal molar quantity of glucose. Sorbitol may permit elimination of an energy-consuming step such as reduction of glucose with NADPH. This would allow conservation of the reducing power of NADPH for use in amino acid and sugar synthesis elsewhere in the metabolic scheme.

In summary, a polyol dehydrogenase of *C. chrysogenum* has been purified and partially characterized. Although it is very similar to the homologous dehydrogenases described in other microorganisms as well as mammalian tissue, it has the novel property of using either NAD or NADP for substrate oxidation.

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