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RAPID AFFINITY PURIFICATION AND PROPERTIES OF RAT LIVER SORBITOL DEHYDROGENASE

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Summary

A 23-h affinity chromatography purification procedure for sorbitol dehydrogenase (L-iditol:NAD^+ 5-oxidoreductase, EC 1.1.1.14) prepared from freshly excised rat liver has been developed that resulted in an 18% yield of an apparently homogeneous preparation (purification = 439-fold). The molecular weight of the enzyme was approx. 96 000. The enzyme was specific for NAD^+ (NADH), but had no requirement for $NADP^+$ (NADPH). The purified preparation shows significant activity with structurally related polyols and ketoses. K_m values for sorbitol and fructose are 0.35 and 110 mM (at pH 7.1), respectively.

Introduction

Sorbitol dehydrogenase (L-Iditol:NAD^+ 5-oxidoreductase (EC 1.1.1.14)) catalyzes the reversible oxidation of sorbitol to D-fructose in an NAD^+-linked reaction:

sorbitol + NAD^+ ⇌ D-fructose + NADH + H^+

Blakley [1] first described the characteristics of a preparation partially purified from rat liver. The identity of this polyol dehydrogenase as an L-iditol dehydrogenase was confirmed by McCorkindale and Edson [2] using preparation B of Blakley. Subsequently, the enzyme has been prepared from the livers of other animals [3—7], calf lens [8] and ram spermatozoa [9].

The combination of sorbitol dehydrogenase and aldose reductase in concerted action constitutes the sorbitol pathway, an important alternate route for glucose metabolism. The presence of sorbitol and fructose in substantial amounts in tissues has been suggested by Gabbay [10] as evidence for the

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presence of this accessory pathway (which has been associated with several of the adverse metabolic effects accompanying diabetes mellitus).

In this report we present a 1-day isolation and purification sequence of the enzyme from rat liver, based on affinity chromatography. Some physical and kinetic properties of the enzyme are also reported.

Materials

Charles River (CR-CD) male rats (approx. 250 g) were killed using CO_2 anoxia. Animals were routinely housed 3 per cage for at least 10 days and allowed free access to Wayne Lab Blox and water prior to use. Livers. (approx. 10 g) were quickly excised and placed in 10 ml of 20 mM potassium phosphate buffer (pH 7.4, 1 mM, in dithiothreitol) at 4°C. Samples were then subjected to immediate purification, or frozen at −20°C in aliquots (6 ml, representing approx. 3 g of fresh liver) until used. No loss in enzyme activity was observed for these fresh frozen samples when stored, as described, for up to six months.

L-Iditol was prepared by the reduction of L-sorbose [11]. Other polyols were obtained from Pfanstiehl Labs (Waukegan, Ill. 60085). Protein and nucleotide cofactors were purchased from Sigma Chemical Co. (St. Louis, Mo. 63178). Gel (Ultrogel AcA-34) and affinity support media (AGNAD, Types 1 and 3) were obtained from LKB Instruments, Inc. (Hicksville, N.Y. 11801) and PL Biochemicals, Inc. (Milwaukee, Wisc. 53205), respectively. Minocon concentrators were obtained from Amicon Corp. (Lexington, Mass. 02173).

Methods

Protein determination. Protein was determined using the method of Lowry et al. [12] with bovine serum albumin as standard, using the trichloroacetic acid procedure [13].

Enzyme assay. Sorbitol dehydrogenase was determined at 25°C by following the rate of change of absorbance at 340 nm. The assay system (for polyol oxidation) contained 10 μmol (25 μl) sorbitol, 2.3 μmol (25 μl) NAD^+, 50 nM (1.0 ml) Tris · HCl buffer (pH 9.6) and up to 0.1 ml enzyme. A unit of activity was defined as the amount of enzyme which produced 1 μmol NADH per min. A linear correlation between the rate of reaction and enzyme concentration was found for these conditions. Specificity studies for substrates and cofactors were made with 10 μmol substrate and 2.3 μmol NAD^+ or $NADP^+$ in the same system as above. Ketose reduction was monitored in a system containing 50 mM (1.0 ml) MES (2-(*N*-morpholine)ethanesulfonic acid)//NaOH buffer (pH 7.1), 32 nmol (25 μl) NADH or NADPH, 10 μmol (25 μl) substrate and up to 0.1 ml enzyme. Michaelis constants were determined using 50 mM MES/NaOH buffer (pH 7.1) containing 0.54 mM NAD^+ for sorbitol oxidation and 0.058 mM NADH for fructose reduction. Ionic strength was kept at 0.1, using NaCl.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was performed essentially according to the method of Hedrick and Smith [14]. Polyacrylamide gels (4, 7.5 and 12%) were run at 15°C in a cyclohexylaminopropane sulfonic acid/2-amino-2-methyl-1,3-propanediol, buffer (pH 9.5) system (buffer I). The enzymic activity was visualized by staining with sorbitol,

NAD^+, phenazine methosulfate, and NitroBlue Tetrazolium according to Fine and Costello [15]. Protein bands were visualized using Coomassie Brilliant Blue G-250/$HClO_4$ according to Reisner et al. [16].

Gel chromatography. Gel chromatography was performed as described elsewhere [17] using an AcA-34 Ultrogel column (2.6 × 30.5 cm).

Results

Purification of sorbitol dehydrogenase

All procedures were carried out at 0—4°C using 20 mM potassium phosphate buffer (pH 7.4)/1 mM in dithiothreitol.

(1) Crude extract. Rat liver which had been freshly excised was homogenized for 2 min in buffer at 4°C using a Waring blendor. The resulting homogenate was centrifuged at 10 000 × *g* for 30 min.

(2) Protamine sulfate treatment. 18 ml of 2% (w/v) protamine sulfate solution was gradually added to the supernatant (100 ml) of the crude extract with gentle mixing. After standing for 10 min, the solution was centrifuged at 10 000 × *g* for 30 min and the pellet discarded.

(3) $(NH_4)_2SO_4$ fractionation. The supernatant from the protamine sulfate step was treated with $(NH_4)_2SO_4$ by the slow addition of the solid (21 g/100 ml) as a finely ground powder. The suspension was allowed to stand for 15 min, then centrifuged at 10 000 × *g* for 10 min. This supernatant was again treated with $(NH_4)_2SO_4$ by the addition of 83 g/100 ml allowed to stand 30 min, then centrifuged at 10 000 × *g* for 20 min. The pellet was dissolved in buffer, divided into 6-ml aliquots and stored at —20°C or immediately subjected to gel chromatography.

(4) Ultrogel (AcA-34) gel chromatography. The enzyme solution was applied to an AcA-34 Ultrogel column (2.6 × 30.5 cm) previously equilibrated with buffer. Elution was carried out in an upward direction using a flow rate of 9 ml/h. Active fractions were pooled, divided into aliquots and stored at —20°C until used.

(5) AGNAD (Type 1) affinity chromatography. An aliquot (5.5 ml) of enzyme solution applied to an AGNAD (type 1) column (2.2 × 2.6 cm), was then washed with buffer (15—20 ml) followed by a pulse (6 ml) of 10 mM $NADP^+$ solution. After an additional buffer (15—20 ml) wash, 1 mM NAD^+ (20 ml) was passed through the column to elute the enzyme. A final buffer wash (15—20 ml) was then followed by a pulse of 100 mM NAD^+ (8 ml) to remove remaining dehydrogenases/proteins (flow rate throughout = 36 ml/h). 29% of the activity and 98% of the protein applied to the column were recovered in the eluate. To determine the protein content, the pooled active fractions were first concentrated 19-fold using a B15 Minicon concentrator before precipitation with trichloroacetic acid to remove interfering NAD^+. A typical elution profile is shown in Fig. 1.

The enzyme was unstable to the extent of 70% loss of activity within 1 week when stored at —20°C. The addition of 0.1 mg/ml bovine serum albumin to the eluate stabilized the preparation. When enzymic activity was too dilute for kinetic studies, the preparation was concentrated immediately before use (B15 Minicon concentrator).

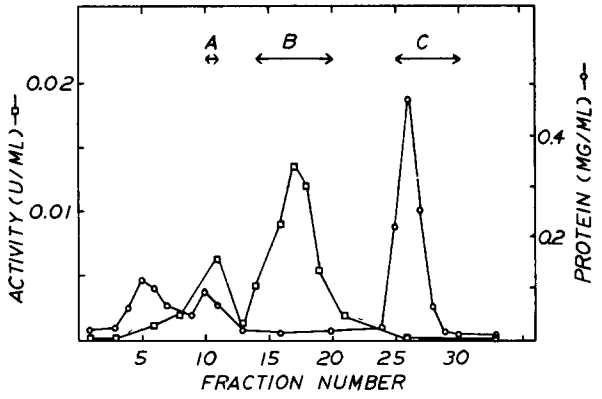

Fig. 1. AGNAD (Type 1) affinity chromatography elution pattern of rat liver sorbitol dehydrogenase. The step 4 fraction (5.5 ml) as described in Results was applied to the column (2.2 × 2.6 cm) equilibrated at 4°C with 0.02 M phosphate buffer, pH 7.4/1 mM dithiothreitol. Elution was carried out with the same buffer system containing: (A) 10 mM $NADP^+$; (b) 1 mM NAD^+; (C) 100 mM NAD^+. Fractions of 3.7 ml were collected at a constant flow rate of 36 ml/h. Protein (○———○) was assayed by a modified Lowry et al. [12] method as described in Materials and Methods. Enzyme activity (□———□) is reported as units/ml.

When AGNAD (Type 3) replaced AGNAD (Type 1) under otherwise identical experimental conditions in step 5, no enzymic acyivity was recovered even in the 100 mM NAD^+ eluant fractions. The five-step purification of sorbitol dehydrogenase required a total of 23 h elapsed time and about 6 h of contact time. A representative purification scheme is shown in Table 1.

Properties of the enzyme

Substrate and cofactor specificity. The rates of polyol oxidation relative to sorbitol and NAD^+ were: xylitol (99); L-iditol (97); D-arabinotol (4); D-mannitol (25); perseitol (0); L-arabinitol (0); galactitol (0); ribitol (50); sorbitol (100). For the reverse reaction the rates of ketose reduction relative to D-fructose and NADH were: D-fructose (100); L-sorbose (64). D-Mannose, D-ribose, D-xylose and L-xylose showed no activity as substrates. Both oxidation and reduction are specific for NAD^+ and NADH, respectively. No activity was found with either $NADP^+$ or $NADPH^+$ as cofactors. These studies indicate that the enzyme is properly classified as L-iditol (sorbitol) dehydrogenase, based on the polyol configurations described by McCorkindale and Edson [2]. Compari-

TABLE I

PURIFICATION SCHEME FOR RAT LIVER SORBITOL DEHYDROGENASE

Step	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg protein)	Recovery (%)	Purification (-fold)	Time (h)
(1) Crude extract	3.1	1.61	93.3	0.017	100	1.0	1
(2) Protamine sulfate	3.1	1.73	47.1	0.037	108	2.1	1
(3) Ammonium sulfate	1.1	1.16	28.8	0.040	72	2.3	2
(4) Ultrogel AcA-34	5.5	0.92	10.4	0.088	57	5.1	16
(5) AGNAD - Type 1	18.0	0.30	0.04	7.47	18	439.0	3

son of the relative rates for polyol oxidation for steps 3, 4 and 5 showed similar ratios of activity for these three enzymatically-active preparations.

Since 10 mM $NADP^+$ partially elutes (i.e., binds) sorbitol dehydrogenase from the AGNAD (Type 1) affinity gel column, it was tested for its possible inhibitory effects. No inhibition was observed when $NADP^+$ was present in the reaction cuvette at 26 times the concentration of NAD^+ (8 mM $NADP^+$ and 0.3 mM NAD^+).

pH vs. activity profile. The influence of pH on the activity of the enzyme was studied in Tris · HCl, MES/NaOH and glycine/NaOH buffer system (50 mM) brought to a constant ionic strength of 0.1 with NaCl. The enzymic activity for the reversible reaction measured over the pH range 4.8—11.3 is shown in Fig. 2. The pH optimum for D-fructose reduction occurred at about 5.9 with sorbitol oxidation occurring over a broad range with maximum activity at pH 8.1—8.5.

Thermal and storage stability. After the $(NH_4)_2SO_4$ fractionation step (Table I, step 3) the preparation remains stable with no apparent loss of activity when stored at —20°C for 1 year. In this form no loss is seen after 5 h at room temperature and 35% activity remains after heating at 60°C for 10 min.

The enzyme remains stable after further purification on Ultrogel AcA-34 with no apparent loss in activity after 5 h at room temperature or 2 months at —20°C. Stability problems are encountered, however, after AGNAD (Type 1) affinity chromatography with 70% loss occurring after storage at —20°C for 1 week. Addition of bovine serum albumin, at a concentration of 0.1 mg/ml to the eluate fractions was found to stabilize the activity both at —20°C and at room temperature. This room-temperature stability allows very small amounts of enzyme to be detected by measuring its activity in an overnight reaction (Tris · HCl buffer, pH 9.6). The identical substrate specificity profile for steps 3, 4 and 5 demonstrates that the albumin does not alter the enzyme activity.

Molecular weight. Standard marker proteins (chymotrypsinogen A, ovalbu-

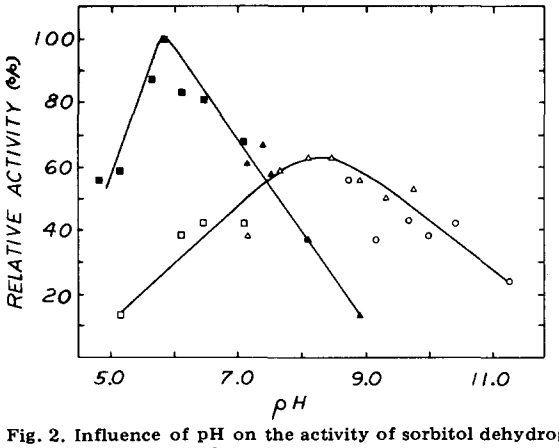

Fig. 2. Influence of pH on the activity of sorbitol dehydrogenase. Enzymic activity for sorbitol oxidation (○, □, △) assayed at 25°C as described in Results was carried out in Glycine/NaOH (○), MES/NaOH (□) and Tris · HCl (△) buffers. Fructose reduction (■, ▲) was assayed in MES/NaOH (■) and Tris · HCl (▲) buffers.

TABLE II

SUMMARY OF LITERATURE DATA FOR SORBITOL DEHYDROGENASE

Source ref.	Date	Fold purity (%)	% Yield	Specific activity (units/mg protein)	M_r [c] ($\times 10^{-3}$)	pH	K_m (mM) for substrate	
							Sorbitol	Fructose
Rat liver (1)	1951	(7) [b]	(29) [b]	—	—	8.0	0.7	—
Sheep liver (3)	1962	379	2	14.4	115 [d]	9.6	1.1	—
						7.0 [h]	5.9 [h]	150 [h]
Calf lens (8)	1963	472	(80) [b]	(0.03) [b]	—	8.0	10	32
Rabbit liver (4)	1973	447	19	15	110 [e]	6.8	—	70
Rat liver (5)	1974	—	—	—	110 [e]	—	—	—
Rat liver (6)	1976	334	7	(0.7) [b]	—	8.3	0.45	—
Horse liver (7)	1976	135	18	0.17	53 [f], 265 [f], 386 [f]	8.8	0.115	325
Rat liver [a]	1978	439	18	7.47	95 [g], 97 [f]	7.1	0.35	110

[a] This paper.
[b] Figures in parentheses represent our best estimate from reported data.
[c] Enzymically active species.
[d] Molecular weight determined using ultracentrifugal analysis assuming partial specific volume of 0.745.
[e] Approximate molecular weights determined using sucrose gradient centrifugation.
[f] Molecular weights determined using gel electrophoresis.
[g] Molecular weight determined using gel permeation chromatography.
[h] In 1975, Christensen et al. [18] using this enzyme preparation reported these K_m values as part of kinetic study.

min, bovine serum albumin and aldolase dissolved at a concentration of 10—15 mg/ml in the preparative buffer system) and the purified sorbitol dehydrogenase were applied to the Ultrogel AcA-34 column and eluted using the experimental conditions described in Methods. The eluate was collected at a constant flow rate of 9 ml/h (4°C). From the plot of the logarithms of the molecular weights against their calculated K_{av} values, the enzyme sorbitol dehydrogenase, present as a single band, was judged to have a molecular weight of 95 000.

Disc gel electrophoresis. The molecular weight of sorbitol dehydrogenase was determined in buffer I (pH 9.5) system using the method of Hedrick and Smith [14]. The following standard marker proteins were used: ovalbumin, monomer and dimer; bovine serum albumin, monomer, dimer and trimer; hemoglobin; and alcohol dehydrogenase. Activity staining of the enzyme revealed a single band with an estimated molecular weight from this standard curve of 97 000.

Michaelis constants. The Micahelis (K_m) constants were determined as 0.35 mM for sorbitol and 110 mM for fructose.

A summary compilation of the physical properties of the enzyme prepared from various sources is shown in Table II.

Discussion

On the basis of the results presented in this paper, the rapid affinity purification of rat liver sorbitol dehydrogenase represents one of the most homogeneous preparations of the enzyme reported to date from this source. By using AGNAD (Type 1) affinity chromatography as the final step, the preparation is

obtained in 1 day with a yield of 18%. The use of a single buffer system throughout the isolation sequence further simplifies the procedure. While the high purity of the final preparation adversely influences the thermal and storage stability of the enzyme, the addition of bovine serum albumin to the final preparation at a concentration of 0.1 mg/ml will counteract the effect without interfering with enzymic activity.

The use of affinity chromatography as the terminal step in the purification sequence gives rise to an approx. 90-fold increase in specific activity. Both AGNAD Type 1 and Type 3 were examined at this point. AGNAD (Type 1) consistently gave the elution profile shown in Fig. 1. However, no activity was recovered in any of the fractions when AGNAD (Type 3) was used. Prolonged (overnight) elution with 2 M KCl generated no additional activity. Mosbach et al. [19] have indicated that, although the Type 1 NAD-agarose linkage is not specifically known, there is evidence that the coenzyme is bound to the spacer (hexanoic acid) through an ester linkage with a ribose moiety of the pyridine nucleotide. In contrast, the Type 3 linkage has a clearly defined matrix [20] containing no unreacted spacer groups (1,6-hexanediamine) with the coenzyme bound to the spacer at the C-8 position of the pyridine nucleotide. While the reason for the total loss of enzymic activity using the type 3 gel is not understood, it is suggested that the location of spacer binding near the site of proton transfer causes the enzyme to bind to the NAD^+ in such a way that its confomation is irreversibly altered, resulting in loss of enzymic activity.

Since $NADP^+$ does cause some elution of the enzyme from the Type 1 column (Fig. 1), it must bind to the enzyme. However, neither $NADP^+$ nor NADPH were enzymatically active with any of the substrates for polyol oxidation or ketose reduction, respectively. This is at variance with Gabbay's statement [10], that both NADH and NADPH can be utilized in the reverse reaction. In addition, it was found that $NADP^+$ at a concentration 26-fold in excess of that of NAD^+ caused no inhibition of enzymic activity. This finding indicates that the locus of $NADP^+$ binding is not operationally adjacent to the active site(s).

While starch and gel electrophoresis of sorbitol dehydrogenase from several sources [7,21—23] were run at pH 8.6 or less, we have consistently found that our preparation did not migrate towards the positive pole up to a pH of 8.9. Migration for molecular weight determination was carried out in pH 9.5 buffer, but the rate of migration was slow relative to standard markers proteins and a commercial preparation of (sheep liver) sorbitol dehydrogenase. Because of this slow migration, gradient gel electrophoresis according to the method of Slater [24] was not carried out, since the method is limited to the extent that the protein must carry enough net charge to move it at an initial rate which is greater than the rate it would have at its "pore-limit". It is possible that the comparatively gentle isolation procedure in a single buffer, described in this paper, gives rise to a form of the enzyme having less overall charge at slightly basic pH values than is generated by conventional purification methods.

It is of interest that large amounts of sorbitol dehydrogenase are found in the freshly excised rat livers, consistent with Murray's [23] finding that near maximal sorbitol dehydrogenase activity is found in normal adult rat livers. Although the combination of this enzyme with aldose reductase has been cited

as prima facie evidence for the presence of the sorbitol (polyol) pathway [10], the full significance of the normal physiological role of this pathway in tissue metabolism remains to be established.

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