Biochimica et Biophysica Acta, 315 (1973) 37–42

BBA 66931

ARABINOSE (FUCOSE) DEHYDROGENASE FROM PIG LIVER

I. ISOLATION AND CHARACTERIZATION

A. G. MAIJUB, M. A. PECHT, G. R. MILLER AND W. R. CARPER

Departments of Chemistry and Microbiology, Wichita State University, Wichita, Kans., 67208 (U.S.A.)

(Received February 5th, 1973)

SUMMARY

A second arabinose (fucose) dehydrogenase has been isolated from pig liver and highly purified. This enzyme has a molecular weight of 245 000 and a pH maximum of 10 which is similar to that obtained for glucose dehydrogenase from the same source. Unlike glucose dehydrogenase, arabinose (fucose) dehydrogenase is insensitive to high ionic strengths. This enzyme is NAD^+ specific and histidine is involved in the active site.

INTRODUCTION

The existence of a number of sugar dehydrogenases in pig and other mammalian liver was recently reported by Schiwara *et al.*[1,2]. Evidence was given that indicated the presence of both NAD^+- and $NADP^+$-dependent arabinose dehydrogenases. Breusch[3] had previously shown the presence of an arabinose dehydrogenase in cat liver along with other possible dehydrogenases. Metzger and Wick[4] also reported the partial purification of arabinose dehydrogenase from rat liver. In this latter study, the enzyme was seen to be $NADP^+$-dependent, and the reaction product was D-arabinolactone. On the other hand, Palleroni and Douforff[5,6] had previously isolated a NAD^+-dependent enzyme from the bacteria *Pseudomonas saccharophilia*.

More recently, Mobley *et al.*[7] were able to purify a NAD^+-dependent enzyme which utilizes L-fucose or D-arabinose as a substrate, and whose molecular weight is approx. 96 000. Schachter *et al.*[8] have reported the isolation of a similar enzyme from pig liver whose molecular weight is less than 100 000. In this work we report the isolation, purification and characterization of a second arabinose (fucose) dehydrogenase of molecular weight 245 000 from pig liver. This is additional evidence in support of Metzger and Wick's[4] conclusion that there are several enzymes in mammalian liver capable of catalyzing the oxidation of D-arabinose.

MATERIALS AND METHODS

Chemicals

Crystalline deoxyribonuclease (beef pancreas), ribonuclease (beef pancreas) and various sugars were obtained from Nutritional Biochemicals Corp. Sephadex was purchased from Pharmacia and other reagent grade chemicals were obtained from either the California Biochemical Co. or the Sigma Chemical Co.

Assay procedure

The assay procedure was as follows: 2.6 ml 0.05 M NaOH–glycine buffer (pH 10.0), 0.1 ml NAD (1.5 mM), and 0.1 ml enzyme solution were added to two identical cuvettes. To the blank cuvette was added 0.2 ml deionized water and both cuvettes were incubated for 5 min in the chamber of a Gilford DU spectrophotometer connected to a constant temperature bath and circulator set at 30 $\pm$ 0.1 °C. At the end of the incubation period, 0.2 ml of sugar solution (1 M) was added to the sample cuvette. Since the formation of reduced NAD produces an increase in absorbance at 340 nm, the reaction was followed with absorbance at 30 and 90 s after the addition of the sugar solution. All activities were recorded as change in absorbance of 0.001/min. These values were later adjusted to the more recent definition of one unit of activity being the amount of protein causing the formation of 1 μmole of NADH per minute. Protein concentration was determined by 280/260 nm measurements after Warburg and Christian[9].

Enzyme preparation

The following is a modified version of preparations used by Srivastava *et al.*[10] to isolate catalase and by Thompson and Carper[11] to isolate glucose dehydrogenase from pig liver. Pig liver (400 g) fresh from the slaughterhouse was cut into 2 cm cubes, mixed with 2 parts acetone and homogenized in a Waring blender for approximately 1 min. Two parts acetone were added to this suspension and the solution stirred for about 30 min. The suspension was filtered by suction on a large Buchner funnel and the resulting cake was broken up on a paper towel for several minutes.

The moist liver particles were suspended in 8 vol. distilled water and stirred for 30 min at room temperature. The suspension was allowed to settle for about 10 min and the supernatant poured through 4 layers of cheesecloth. The solid material was extracted a second time with water and the extracts combined while the crude precipitate was discarded.

The water extracts were cooled in the cold room, centrifuged, and the precipitate discarded. $(NH_4)_2SO_4$ (28 g/100 ml solution) was slowly added with constant stirring. The stirring was continued for 30 min after the last $(NH_4)_2SO_4$ was added. The solution was allowed to stand overnight and the total volume centrifuged at approx. 8000 $\times$ *g* for 15 min. The collected precipitate was dissolved in distilled water and the protein concentration was adjusted to 6.0 g/100 ml. The resulting suspension was then centrifuged and the insoluble residue discarded.

The pH of the supernatant was lowered to 5.5 with 10% acetic acid. The solution was then stored overnight in the cold room after which it was centrifuged at 11 000 $\times$ *g* for 30 min and the precipitate discarded. At this point, 1 ml of a mixture of deoxyribonuclease and ribonuclease (0.05 mg/ml) was added to the super-

natant and it was then stored at 4 °C for two days. At the end of this period, the solution was centrifuged at 13 000 × *g* for 30 min and the precipitate discarded.

At this point the supernatant was adjusted to 3.0 g/100 ml in protein and then added in 5-ml batches to a 2.5 cm × 45 cm column of Sephadex G-200. The column was eluted with triply distilled and deionized water (pH 7.0). 10-min fractions of 3.1 ml were collected by means of a Gelman Medical Electronics fraction collector. The enzyme was located by the assay method as shown in Fig. 1. The use of either D-arabinose or L-fucose resulted in activity curves with identical maxima and minima as previously noted by other investigators[7,8] for the lower molecular weight enzyme.

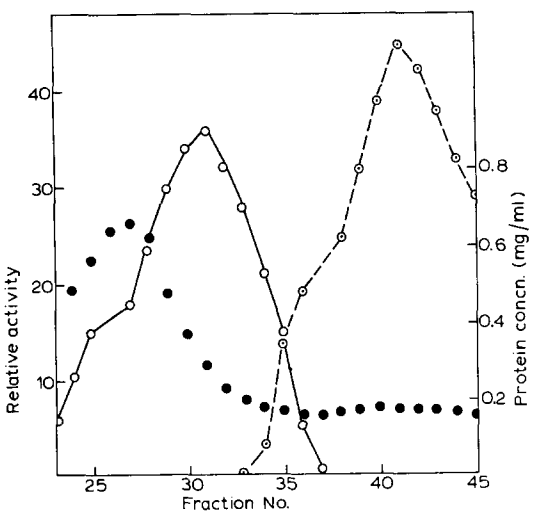

Fig. 1. Elution pattern of protein (● ●), arabinose dehydrogenase (○—○), and glucose dehydrogenase (○—○) activity from Sephadex G-200.

Fig. 1 is a protein concentration and relative activity *vs* fraction number plot for both arabinose (fucose) dehydrogenase and glucose dehydrogenase. The specific activities of the best fractions are 0.432 (D-arabinose) and 0.605 (L-fucose) for arabinose dehydrogenase and 0.613 for glucose dehydrogenase. The latter value may be compared with a maximum value of 0.271 previously obtained by Thompson and Carper[11] after $(NH_4)_2SO_4$ fractionation. It is apparent that the addition of the nucleases to improve the isolation of pig liver catalase by Srivastava *et al.*[10], also results in improved isolation of particular dehydrogenases as well.

RESULTS

Extraction and purification

Table I contains a typical set of values obtained during the purification procedure. We attribute the tremendous increase in purity between the acetic acid fractionation and the Sephadex treatment to the action of the nucleases[10]. A similar preparation without the nucleases yielded a column separation of approximately one-tenth the activity shown in Table I. For purposes of comparison, specific activities

TABLE 1

ENZYMATIC ASSAY DURING PURIFICATION PROCEDURE

The numbers in parentheses refer to D-arabinose as a substrate.

Step	*Specific activity*	*Total protein (mg)*	*Enzyme units*	*A(280 nm)/A(260 nm) ratio*
$(NH_4)_2SO_4$ precipitate	0.0042 (0.0030)	15 000	63.0 (45.0)	0.95
Acetic acid (pH 5.5)	0.0108 (0.0077)	5 200	56.0 (40.0)	1.10
Sephadex	0.605 (0.432)	150	38.9 (27.8)	1.71

for this enzyme from various sources include rat liver, 0.510 (NADP–D-arabinose)[4], sheep liver, 0.190 (NAD–L-fucose)[7], and pig liver, 0.433 (NAD–L-fucose)[8]. All of these enzymes appear to be of molecular weight less than 100 000 as evidenced by their behavior on chromatographic columns.

Attempts to condense and recrystallize arabinose dehydrogenase with $(NH_4)_2SO_4$ as done previously with glucose dehydrogenase[11] were unsuccessful. Colloidon membranes were seen to be ideal for the condensation of arabinose dehydrogenase solutions and were therefore used throughout this investigation.

pH and ionic strength

The inability to recrystallize arabinose dehydrogenase from $(NH_4)_2SO_4$ solutions without destroying its activity suggest that this particular enzyme has unusual properties, and this is verified by the results that follow. First of all, a pH study was completed using Tris–malate and glycine–NaOH buffer systems (0.05 M)

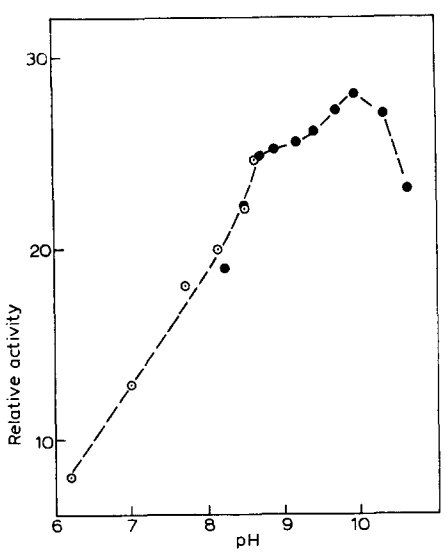

Fig. 2. Enzyme activity as a function of pH using 0.05 M Tris–malate (○—○) and glycine–NaOH (●—●) buffers.

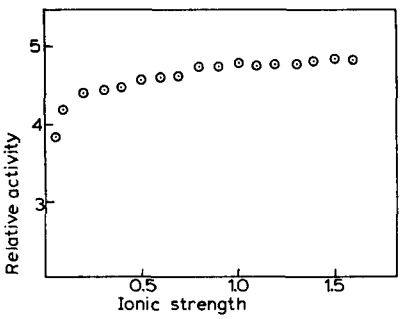

Fig. 3. Enzyme activity as a function of ionic strength in glycine–NaOH buffer (pH 10).

and is shown in Fig. 2. The results are quite similar to those obtained previously for glucose dehydrogenase[11] with a maximum in the vicinity of pH 10. pH curves for this enzyme from various sources[4,7,8] show a maximum varying from pH 9.0 to 10.4.

Fig. 3 contains the ionic strength study at pH 10 and the results are quite startling. For both D-arabinose and L-fucose, this enzyme reaches a maximum velocity at an ionic strength of 0.8 M and then is virtually constant until the enzyme is eventually salted out a very high ionic strengths. These results are contrary to those obtained with glucose dehydrogenase, an enzyme whose maximum velocity peaks at 0.1 M ionic strength. Glucose dehydrogenase is particularly sensitive to metal ions of any kind, whereas preliminary experiments in this laboratory show no such similar relationship with arabinose dehydrogenase. In keeping with our previous work[11] we then proceeded to the active site test that follows.

Histidine reactive site identification

The following is a modification of the diazotization procedure described by Horinishi *et al.*[12]. 1 g of 5-aminotetrazole (Aldrich Chemical Co.) was dissolved in 23 ml of 1.6 M HCl. To this, 0.7 g of $NaNO_2$ in 10 ml water was added slowly at 4 °C. The pH was adjusted to approx. 5.0 with concentrated KOH solution. A sample of diazotetrazole solution was mixed with an excess of histidine in 0.05 M glycine–NaOH buffer at pH 8.8. This solution, after standing at room temperature for 30 min, showed strong absorption at 360 nm indicating that the diazotetrazole solution did react with the histidine. 1 ml of the prepared diazotetrazole solution was mixed with 9 ml of enzyme solution in 0.05 M buffer at pH 8.8. A 9-ml sample of the same enzyme solution was mixed with 1 ml of deionized water as a control. Both control and diazotetrazole–enzyme solutions were allowed to stand at room temperature for 40 min for a complete reaction. The two solutions of 10 ml each were condensed using colloidon membranes after filtering through Whatman No. 42 filter paper. The samples treated with diazotetrazole solution were completely inactive thus indicating that a histidine residue is involved in the active site. Similar results were obtained with glucose dehydrogenase[11] and corroborates the work of Lewin[13] in which histidine was reported to strongly interact with hexoses and pentoses.

Molecular weight and acrylamide gel test

The molecular weight of arabinose (fucose) dehydrogenase was determined to be 245 000 in a manner identical to that reported previously[11]. As Andrews[14] has

pointed out, the function $\log M_r = a + bV_e$ is best for large molecules only.

A sample obtained from a Sephadex G-200 column was condensed and subjected to acrylamide gel electrophoresis as described by Schiwara *et al.*[1,2]. A single dark band was observed, thus indicating the presence of a single arabinose (fucose) dehydrogenase.

Michaelis constants

The apparent K_m values were determined by the method of Lineweaver and Burk[15]. Values of 19 mM for D-arabinose, 5.3 mM for L-fucose and 24 μM for NAD^+–(L-fucose) were obtained. These may be compared to values of 2.1 mM for D-arabinose, 0.32 mM for L-fucose and 20 μM for NAD^+ obtained by Schachter *et al.*[8] for the lower molecular weight pig liver dehydrogenase. K_m values at pH 10.4 for the sheep liver dehydrogenase[7] are 1.5 mM for L-fucose, 7.2 mM for D-arabinose and 190 μM for NAD^+.

CONCLUSION

As has been previously suggested[4], it is now obvious that mammalian liver contains several arabinose (fucose) dehydrogenases. The work of Schiwara *et al.*[1] and Schachter *et al.*[8] when coupled with these results, establish the presence of at least two such NAD^+ requiring enzymes for pig liver. Furthermore, Schiwara's results[1,2] suggest not only multiple NAD^+ requiring enzymes, but also multiple $NADP^+$ requiring arabinose dehydrogenases in a wide variety of mammalian livers. At present we have no explanation of the significance of these enzymes, however, their widespread occurrence suggests a metabolic role of definite importance.

REFERENCES

1 Schiwara, H. W., Domischke, W. and Domagk, G. F. (1968) *Z. Physiol. Chem.* 349, 1571–1581
2 Schiwara, H. W., Domischke, W. and Domagk, G. F. (1968) *Z. Physiol. Chem.* 349, 1582–1585
3 Breusch, F. L. (1943) *Enzymology* 11, 87–91
4 Metzger, R. P. and Wick, A. N. (1967) *Biochem. Biophys. Res. Commun.* 26, 742–747
5 Palleroni, N. J. and Doudoroff, M. (1956) *J. Biol. Chem.* 223, 499–508
6 Palleroni, N. J. and Doudoroff, M. (1957) *J. Bacteriol.* 74, 180–185
7 Mobley, P. W., Metzger, R. P. and Wick, A. N. (1970) *Arch. Biochem. Biophys.* 139, 83–86
8 Schachter, H., Sarney, J., McGuire, E. J. and Roseman, S. (1969) *J. Biol. Chem.* 244, 4785–4792
9 Warburg, O. and Christian, W. (1957) *Methods Enzymol.*, Vol. 3, 454–456
10 Srivastava, R. C., Brundage, C. K., Pecht, M. A. and Carper, W. R. (1971) *Trans. Can. Acad. Sci.* 74, 172–180
11 Thompson, R. E. and Carper, W. R. (1970) *Biochim. Biophys. Acta* 198, 397–406
12 Horinshi, H., Hachimori, Y., Kurihara, K. and Shibata, K. (1964) *Biochim. Biophys. Acta* 86, 477–489
13 Lewin, S. (1956) *Biochem. J.* 63, 5P–6P
14 Andrews, P. (1970) in *Methods in Biochemical Analysis* (Glick, D., ed.), Vol. 18, pp. 1–54, Interscience, New York
15 Lineweaver, H. and Burk, D. (1934) *J. Am. Chem. Soc.* 56, 658–666