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RIBITOL-5-PHOSPHATE DEHYDROGENASE FROM *LACTOBACILLUS PLANTARUM*

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SUMMARY

The partial purification of a ribitol-5-phosphate dehydrogenase from *Lactobacillus plantarum* (ATCC 8014) is described. The enzyme has been shown to catalyze the reaction

$$\text{D-ribitol 5-phosphate} + \begin{matrix}\text{TPN}^+\\ \text{or}\\ \text{DPN}^+\end{matrix} \rightleftarrows \text{D-ribulose 5-phosphate} + \begin{matrix}\text{TPNH}\\ \text{or}\\ \text{DPNH}\end{matrix} + \text{H}^+$$

INTRODUCTION

In 1954 BADDILEY AND MATHIAS[1] described the isolation of CDP-ribitol and later the presence of ribitol teichoic acids (a family of substituted polyribitol phosphates) in a variety of gram-positive bacteria[2]. The enzymic synthesis of CDP-ribitol from D-ribitol-5-*P* and CTP has been described by SHAW[3]. No enzymes which specifically catalyze the synthesis of D-ribitol-5-*P* have been isolated although an apparently unspecific aldose reductase from silkworm blood will catalyze the reduction of D-ribose-5-*P* to D-ribitol-5-*P* (see ref. 4). We wish to report the partial purification of an enzyme which catalyzes the formation of ribitol-5-*P* from D-ribulose-5-*P*, using either DPNH or TPNH as a hydrogen donor.

EXPERIMENTAL

Materials

Lactobacillus plantarum (ATCC 8014) was grown in a medium containing per l: 4 g of Difco yeast extract, 10 g of Difco nutrient broth, 10 g of sodium acetate, 0.2 g of $MgSO_4 \cdot 7\ H_2O$, 0.01 g of NaCl, 0.01 g of $Fe_2(SO_4)_3 \cdot 9\ H_2O$, 0.01 g of $MnSO_4 \cdot 7\ H_2O$ and 20 g of glucose. An inoculum from a stab was grown in 100 ml of this medium for 12 h, and 6 ml of this culture were used to inoculate 3 l of medium in a 6-l Erlenmeyer flask and the culture allowed to grow for 12 h at 37°. The cells from 18 l of culture medium were harvested by centrifugation and washed twice with 400 ml of 0.05 M Tris–HCl–0.001 M EDTA (pH 8.0). The cells were finally suspended in a minimal volume of 0.05 M Tris–HCl–0.01 M $MgCl_2$–0.001 M EDTA (pH 8.0) and

lyophilized. The lyophilized powder could be stored for at least 3 weeks at −20° without loss of activity.

D-Ribulose-5-*P* was prepared by the method of HORECKER[8]. The 6-phosphogluconic acid dehydrogenases used contained ribose-5-*P* isomerase and the final product, even after column chromatography, was contaminated with some ribose-5-*P*. Pentose was determined by the orcinol method[6], with a 40-min heating period in a volume of 1.5 ml. Ribulose was determined by the cysteine carbazole reaction[7] using authentic ribulose as a standard. Ribulose-5-*P* was determined by the same method after removal of the phosphate with chromatographically purified *Escherichia coli* phosphatase (Worthington Biochemical Corporation), thus permitting the use of free ribulose as a standard in the colorimetric assay. Aldopentoses were determined by the phloroglucinol method[6].

D-Ribulose-*o*-nitrophenylhydrazone was obtained from California Corporation for Biochemical Research. Ribose-5-*P* from Schwartz Laboratories. DPNH and TPNH were obtained from the Sigma Chemical Company. Ribitol-5-*P* was prepared by the method of BADDILEY *et al.*[9].

Ribose-5-*P* isomerase was determined by incubating enzyme with ribose-5-*P* (see ref. 10) under the conditions of the ribitol-5-*P* dehydrogenase assay. At the end of incubation, the sugar phosphates were hydrolyzed with *E. coli* phosphatase and the ribulose formed determined[7]. Protein was determined by the method of WARBURG AND CHRISTIAN[11]. Protamine sulfate was obtained from Sigma Chemical Company. Fresh solutions of protamine sulfate were prepared for the enzyme fractionation and neutralized to pH 7.0 with KOH.

Assay of ribitol-5-P dehydrogenase

The reaction mixture contained: 10 μmoles of Tris–HCl, 0.2 μmole of EDTA, 0.3 μmole of ribulose-5-*P*, 0.1 μmole of TPNH and 500 μmoles of ammonium acetate in a final volume of 1 ml (pH 7.8). Control reaction mixtures contained all the components minus ribulose-5-*P*. Absorbancy measurements were taken at 340 mμ every minute in a Beckman spectrophotometer thermostated at 25°. In all the enzyme fractions except the final pH-5 precipitate (see below) ribose-5-*P* could substitute for ribulose-5-*P* without change of activity.

The enzyme could also be assayed by measuring TPNH formation from ribitol-5-*P* and TPN. The reaction mixture contained 10 μmoles of Tris–HCl, 0.2 μmole of EDTA, 2 μmoles of ribitol-5-*P*, 5 μmoles of TPN and 500 μmoles of ammonium acetate in a final volume of 1 ml (pH 7.8). With the crude extract, values obtained by this assay are low due to the presence of TPNH oxidase. 1 unit is defined as the amount of enzyme required to catalyze a change of 0.01 absorbancy unit/min. The dependence of the activity on enzyme concentration is shown in Fig. 1.

RESULTS

*Purification of ribitol-5-P dehydrogenase**

1 g of dry cells was suspended in 8 ml of 0.05 M Tris–HCl, 0.01 M $MgCl_2$, 0.001 M

* All steps in the enzyme purification were carried out at 3°. All precipitates were collected by centrifugation at 12 000 × *g* for 10 min. Ammonium sulfate was saturated at 3° and neutralized with NH_4OH so that a 1 : 5 dilution had a pH of 7.5.

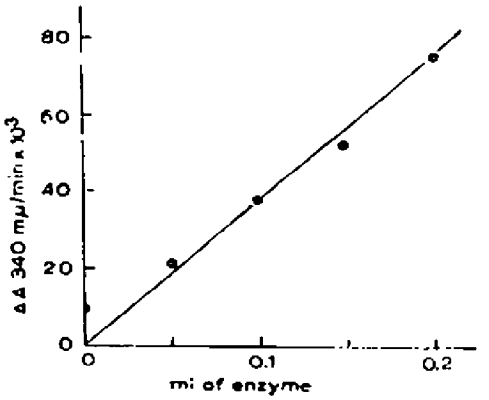

Fig. 1. Dependence of ribitol-5-P dehydrogenase activity on enzyme concentration. Standard assay conditions; the enzyme was ammonium sulphate fraction.

EDTA (pH 8.0), shaken with 8 g of glass beads in a Nossal shaker for three 30-sec periods being cooled in between each 30-sec period in an ice bath. To prevent foaming, a drop of capryl alcohol was added. The glass beads were allowed to settle and the supernatant fluid centrifuged. The beads and precipitate were washed twice with 5 ml of the same buffer.

The pooled supernatant fluid was then fractionated with protamine sulfate. In a typical experiment as shown in Table I, 18.5 ml of 2% protamine sulfate was added to the enzyme and after 10 min the precipitate was collected by centrifugation

TABLE I

PURIFICATION OF RIBITOL-5-P DEHYDROGENASE

	Volume (ml)	*Specific activity (units/mg protein)*	*Total units*
Extract	64	0.91	1480
1 M ammonium acetate extract of protamine precipitate	15	5.45	1200
0–50% ammonium sulphate fraction	12	8.0	720
pH-5.1 precipitate	9	30.0	270

and discarded. To the supernatant fluid an additional 16 ml of 2% protamine sulfate were added, and after 10 min the precipitate was collected by centrifugation and the supernatant fluid discarded.

The precipitate from the second protamine addition was washed with 20 ml 0.2 M ammonium acetate (pH 7.8) and then extracted for 0.5 h with 15 ml of 1 M ammonium acetate (pH 7.8). The insoluble residue was removed by centrifugation and discarded. To the supernatant fluid, containing the enzyme, was added an equal volume of satd. ammonium sulfate, and after 15 min the precipitate was collected by centrifugation and dissolved in 1 M ammonium acetate (pH 7.8).

The enzyme was then dialyzed against 0.15 M acetate buffer (pH 5.1) with

stirring for 4 h. Finally the precipitate was collected by centrifugation and dissolved in 1 M ammonium acetate (pH 7.8).

The enzyme can be kept frozen at all stages in the purification for at least a week without loss of activity. The summary of a typical preparation is shown in Table I. It has been found necessary to pilot the protamine step with every enzyme preparation, since the quantity of protamine which will precipitate most of the nucleic acid but not the enzyme, varies from preparation to preparation.

After the protamine step the enzyme becomes unstable at low ionic strength, and dialysis of this fraction against 0.1 M phosphate (pH 7.5) or 0.1 M Tris (pH 7.5) for 4 h leads to complete loss of activity. Precipitation at pH 5.1 causes a relatively large loss of activity but serves to remove most of the ribose-5-*P* isomerase. Reprecipitation of the enzyme at pH 5.1 does not remove the last traces of isomerase.

In the preparation shown in Table I, the enzyme at the final stage had an activity of 2.9 μmoles/h/ml in the ribitol dehydrogenase assay and the ribose isomerase activity was 0.7 μmole/h/ml of enzyme.

Properties of the enzyme

When the most purified enzyme was tested with D-ribose-5-*P* and D-ribulose-5-*P* as the substrate, the results shown in Fig. 2 were obtained. These results indicate that the substrate of the enzyme is D-ribulose-5-*P* and not D-ribose-5-*P*.

To confirm these observations, enzymic oxidation of ribitol-5-*P* was carried out on a larger scale and the product identified by a colorimetric test and paper chromatography.

The reaction mixture contained 50 μmoles of Tris–HCl, 1 μmole of EDTA, 5 mmoles of ammonium acetate, 24 μmoles of ribitol-5-*P*, 2.5 μmoles of TPN, 100 μmoles of sodium pyruvate, 10 mg of crystalline lactic dehydrogenase and 75 units of enzyme in a volume of 8 ml (pH 7.8). After 1 h at 37°, the solution was deproteinized by the addition of 1.5 ml of 40% trichloroacetic acid to give a pH of 2.0. The precipitate was removed by centrifugation at 3°, and the supernatant fluid neutralized with $Ba(OH)_2$ to pH 6.5. After 1 h at 0°, the precipitate was removed by centrifugation and 4 vols. of ethanol were added. After 4 h the precipitate was collected by centrifugation, washed with 80% ethanol and dried *in vacuo*.

The precipitate was dissolved in 0.03 M glycylglycine–0.003 M $MgCl_2$ (pH 7.5) and digested with 2 mg of *E. coli* phosphatase for 30 min. The solution was deproteinized with perchloric acid and neutralized with KOH. After removal of insoluble $KClO_4$ by centrifugation, the solution was deionized by passage through an Amberlite MB-3 column.

The deionized solution contained 0.20 μmole of ketopentose per ml[7] and 0.145 μmole/ml of aldopentose[8]. The ketopentose color reached its maximum in 10 min, the same as an authentic ribulose standard[13–14]. In the orcinol test the readings at 540 mμ and 670 mμ were consistent with a mixture of ribulose and ribose in the above proportions[15].

Paper chromatography of this solution, using phenol–water (4 : 1) as the solvent, indicated two compounds with the same R_F as authentic ribose and ribulose when sprayed with aniline phosphate[16]. When sprayed with the orcinol reagent only a single yellow spot with the mobility of ribulose was seen which turned red on over-

spraying with aniline phosphate. This reaction is typical of ribulose but not of xylulose[15,16]. These results show that both ribose-5-*P* and ribulose-5-*P* were obtained by the enzymic oxidation of ribitol 5-*P*, but the ratio of ribulose-5-*P* to ribose-5-*P* is 1.3 : 1 and therefore ribulose-5-*P* is the primary product of the enzyme reaction, since at equilibrium in the ribose isomerase reaction this ratio would be 0.32 at 37° (see ref. 10).

To test whether the reduction of ribulose-5-*P* by TPNH was stereospecific, ribulose-5-*P* was reduced with TPNH and the ribitol-5-*P* formed was isolated as the barium salt. The isolated material was treated with *E. coli* phosphatase and chromatographed using pyridine–ethyl acetate–water (2:7:1) as the solvent[3]. The enzymically formed material gave a single spot with the same R_F as authentic ribitol, but distinct from arabitol, ribose and ribulose. Since arabitol is the 2-epimer of ribitol, these results show that the enzymic reduction of ribulose-5-*P* yields only the ribitol configuration at C-2 of the sugar alcohol.

The enzyme will react with DPNH or DPN instead of TPNH or TPN at approx. the same rate. TPNH was used as a substrate, since the apparent oxidase activity in crude extracts is much less for TPNH than for DPNH. After the ammonium sulfate fractionation the enzyme is free of TPNH oxidase activity. The enzyme will not react with glucose-6-*P*, fructose-6-*P*, ribose, ribulose, ribitol or arabitol. The K_m for ribulose-5-*P* is $1.5 \cdot 10^{-4}$ M (Fig. 2). The K_m for TPNH is approx. $9 \cdot 10^{-6}$ M. The enzyme is not stimulated by the addition of Mg^{2+} and has a pH optimum of 7.5.

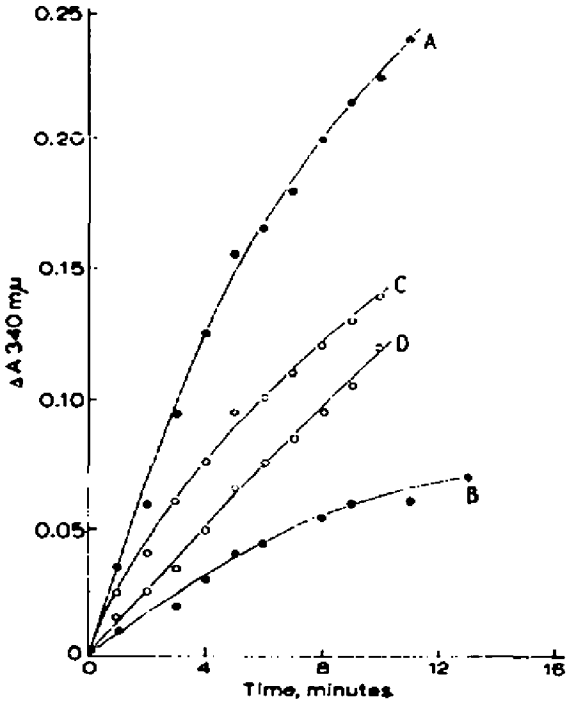

Fig. 2. Substrate specificity of ribitol-5-*P* dehydrogenase. The standard reaction mixture was used with the following substrate additions. Curves A and C: 0.1 μmole of ribulose-5-*P*, the solution contained 0.045 μmole of ribose-5-*P*. Curves B and D: 0.1 μmole of ribose-5-*P*, the solution contained 0.026 μmole of ribulose-5-*P*. All tubes contained ribitol-5-*P* dehydrogenase precipitated at pH 5.1. Curves C and D contained in addition ribose-5-*P* isomerase.

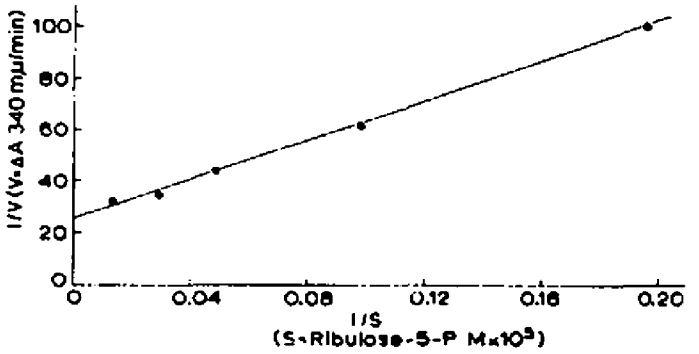

Fig. 3. Activity of ribitol-5-*P* dehydrogenase as a function of ribulose-5-*P* concentration. Standard assay system with substrate concentration as indicated. V = Δ absorbancy units at 340 mμ per min. S = concentration of ribulose-5-*P* (M × 10^4).

DISCUSSION

A large number of polyol dehydrogenases have been described from various natural sources[16], but with one exception[4] all the pentitol dehydrogenases appear to act on the free sugar.

In considering the biosynthesis of ribitol-5-*P* in *Lactobacillus plantarum*, the two obvious possibilities were the reduction of free ribose or ribulose to ribitol, followed by phosphorylation or alternatively reduction of the phosphorylated pentose which is the reaction that has been described. We have been unable to detect a ribitol dehydrogenase in extracts of this organism.

The procedure described for the preparation of ribitol-5-*P* dehydrogenase has been reproducible, but different methods of preparing the cell-free extract, such as the use of a sonic oscillator instead of a Nossal shaker, have given extracts with much lower activity and sometimes completely inactive extracts were obtained.

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