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Partial Purification and Characterization of D-Ribose-5-phosphate Reductase from *Adonis vernalis* L. Leaves

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ABSTRACT

This study presents evidence for a new enzyme, D-ribose-5-P reductase, which catalyzes the reaction: D-ribose-5-P + NADPH + H^+ → D-ribitol-5-P + $NADP^+$. The enzyme was isolated from *Adonis vernalis* L. leaves in 38% yield and was purified 71-fold. The reductase was NADPH specific and had a pH optimum in the range of 5.5 to 6.0. The Michaelis constant value for D-ribose-5-P reduction was 1.35 millimolar. The enzyme also reduced D-erythrose-4-P, D-erythrose, DL-glyceraldehyde, and the aromatic aldehyde 3-pyridinecarboxaldehyde. Hexoses, hexose phosphates, pentoses, and dihydroxyacetone did not serve as substrates. D-Ribose-5-P reductase is distinct from the other known ribitol synthesizing enzymes detected in bacteria and yeast, and may be responsible for ribitol synthesis in *Adonis vernalis*.

Ribitol, a five-carbon sugar alcohol, is commonly referred to as adonitol which reflects its first definitive characterization by Merck in 1893 from the leaves of *Adonis vernalis* L. (20). The ribitol content of *Adonis* leaves can vary from 0.9 to 5.3% dry weight depending on the species (8, 17) (Chem Abstr 86: 40277c, 1977; Chem Abstr 96: 214320k, 1982). The only other higher plant in which ribitol has been identified is *Bupleurum* (30) (Chem Abstr 64: 6731, 1966), although it has also been reported to occur in fungi, algae, and lichen symbionts (3).

The metabolism of pentoses and their phosphate esters has been intensively investigated in bacteria (23) and to a lesser extent in yeast (2), but in higher plants they have received little attention other than their identification as intermediates in the reductive and oxidative pentose phosphate cycles and as structural components of cell walls and gums (15). To our knowledge, the only research involving ribitol metabolism in higher plants was the observed formation of starch from ribitol in *Adonis* leaves over 70 years ago (29). No enzymes capable of synthesizing or utilizing ribitol have been isolated from *Adonis* although such enzymes have been reported from other sources (10, 11, 19, 24).

A plant containing a relatively large five-carbon pool poses some interesting metabolic questions. To begin this investigation as well as to elucidate further the roles of polyols in higher plants, we have identified an enzyme which may be partially responsible for ribitol synthesis in *Adonis vernalis* L.

MATERIALS AND METHODS

Chemicals. All chemicals were purchased from Sigma. Bovine γ-globulin was purchased from Bio-Rad Laboratories. D-Ribitol-5-P was prepared from D-ribose-5-P by $NaBH_4$ reduction as per Supelco bulletin 774A (28) except that after termination of the reaction with glacial acetic acid, 5 ml of IRA-20 in acid form were added, mixed, and then filtered off. The final concentration of D-ribitol-5-P was determined by GLC.

Partial Purification of D-Ribose-5-Phosphate Reductase. *Adonis vernalis* L. leaves were obtained from plants in Minns Garden, Cornell University. Homogenization and all subsequent steps were carried out in a cold room at 2°C. In a typical preparation, 25 g of leaves were washed in distilled H_2O and homogenized in 250 ml of 100 mM Tris-HCl buffer (pH 7.5) containing 1 mM DTT, 10 mM isoascorbate, and 10 g insoluble PVP which had been prewetted with 100 ml of the extraction buffer 1 h prior to homogenization. Homogenization was conducted with a Waring blendor for two 5-s bursts at full speed, followed by three 15-s bursts at full speed on a Polytron homogenizer equipped with a PTA 20 ST generator. The homogenate was squeezed through polypropylene cloth, 149 μM mesh openings (Spectrum Medical Industries, Los Angeles, CA), and the filtrate was centrifuged at 4,068*g* for 20 min. The supernatant wsa centrifuged at 23,430*g* for 30 min. The resulting supernatant was treated with $(NH_4)_2SO_4$ and the precipitate sedimented at 30 to 70% saturation was dissolved in a minimum volume of buffer and dialyzed overnight against 2 L 10 mM Tris-HCl (pH 7.5) containing 0.1 mM DTT, and 1 mM 2-mercaptoethanol (buffer A). The dialysate was centrifuged at 26,890*g* for 30 min and the supernatant was applied to a Sephadex G-100 column (2.5 × 90 cm) equilibrated with 20 mM Tris-HCl buffer (pH 7.5) containing 0.2 mM DTT (buffer B). The enzyme was eluted with buffer B at a rate of 17 ml/h. Active fractions were pooled and concentrated using an Amicon PM-10 ultrafiltration membrane (Amicon Corp.). An aliquot of the PM-10 concentrate was applied to a Reactive Red 120-Agarose column (1 × 7 cm) previously equilibrated with buffer B. The column was washed with buffer B until no protein was detected in the eluate, washed with 50 ml NADP (1 mM in buffer B), and followed by an additional 50 ml buffer B. The enzyme was then eluted with 1.5 M KCl in buffer B and 1.5-ml fractions were collected. Active fractions were pooled and concentrated using an Amicon PM-10 ultrafiltration membrane and this fraction was dialyzed overnight against 1 L of buffer A.

Enzyme Assays. D-Ribose-5-P reductase was assayed by recording the oxidation of NADPH in the presence of D-ribose-5-P at 340 nm with a Perkin-Elmer model 552 spectrophotometer equipped with a constant temperature cuvette. Unless otherwise indicated, assays were performed at 25°C in a reaction mixture (1 ml) containing 110 μM NADPH, 90 mM Na-phosphate (pH 5.7), 25 μl enzyme (affinity fraction), and 5 mM D-ribose-5-P. Reactions were initiated by the addition of the substrate 2.5 min after the enzyme was incubated with NADPH. Control cuvettes contained all reagents except substrates. NADPH concentration was determined from A_{340} by using a molar extinction coefficient

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of 6.22×10^3 $M^{-1}cm^{-1}$.

An enzyme unit is defined as the amount of enzyme which catalyzes the oxidation of 1 μmol NADPH/min at 25°C under the above assay conditions. Specific activity is expressed as units/mg protein. Protein concentration was determined by the method of Bradford (4), using bovine γ-globulin as a standard.

PAGE was carried out as described by Davis (7). Gels were stained for protein according to the method of Chrambach *et al.* (5). The reductase activity was detected by incubating the gel in the dark at 37°C for 1 h in a mixture which contained 100 mM Tris-HCl (pH 9.0), 20 mM D-ribitol-5-P, 1 mM NADP, 0.04 mg/ml phenazine methosulfate, and 0.4 mg/ml nitroblue tetrazolium.

The product of the reaction mixture, D-ribitol-5-P, was identified by gas chromatography as described by Srivastava *et al.* (27). To further identify the product, the reaction mixture was treated with acid phosphatase (25). Samples were injected into a HP5730A gas chromatograph equipped with 183 × 0.31-cm i.d. stainless steel column packed with 3% SE-30 on Cromosorb WHP, 80 to 100 mesh (Supelco Inc., Bellefonte, PA). The nitrogen flow rate was 30 ml/min and temperature of the injection port was kept at 150°C. After injection, column temperature was increased from 140° to 240°C at 4°C/min.

RESULTS AND DISCUSSION

Table I summarizes the results of a typical purification procedure of D-ribose-5-P reductase from *Adonis vernalis* leaves. The enzyme was purified 71-fold with 38% yield. The enzyme was retained by the affinity material and was not eluted with 1 mM NADP. Polyacrylamide disc gel electrophoresis of the final preparation revealed three closely spaced protein bands. D-Ribose-5-P reductase activity as assayed with D-ribitol-5-P was associated with one of these bands. The dissociation of other aldose reductases on polyacrylamide gel during electrophoresis has been reported by others (1, 18, 26). The possibility still remains that other contaminating protein might be present. The partially purified enzyme gradually lost its activity during storage at 1°C. Seventy-five percent of the activity was lost by the end of the second week. The enzyme was not activated by sulfate ion as has been reported for aldose reductase from calf lens (14). Lithium or ammonium sulfates at 400 mM inhibited the enzyme by 80%. Similar inhibition has been reported for aldose reductases from different sources (1, 26).

To test cofactor requirement and precursor for ribitol synthesis, D-ribose, D-ribulose, D-ribose-5-P, and D-ribulose-5-P were tested at variable concentrations (5 to 200 mM) and over a pH range from 5 to 11 in the presence of either NADH or NADPH. The results indicate that the enzyme is specific for D-ribose-5-P and NADPH (Table II). The product of this reaction mixture was identified by gas chromatography as D-ribitol-5-P (retention time 17.0 min compared with 15.6 min for D-ribose-5-P). Acid phosphatase-treated reaction mixture gave a peak corresponding to D-ribitol (retention time, 8.02 min) which is separated from D-ribose (retention time, 6.33 min). Slight reaction (14%) occurred with D-ribulose-5-P and NADPH (Table II) which could be due to contamination of commercial D-ribulose-5-P with traces of D-ribose-5-P. Incubation of the enzyme with D-ribulose-5-P prior to starting the reaction with NADPH did not change the relative rates of the reaction indicating the absence of D-ribose-5-P isomerase in the final enzyme preparation.

As shown in Figure 1, the enzyme displayed the highest activity, using D-ribose-5-P as a substrate, between pH 5.5 and 6.0 in 100 mM Na-phosphate buffer. Below pH 5, the enzyme was inactivated. Maximum activity was observed at 40°C (5.08 units/mg protein) (Fig. 2).

The K_m value for D-ribose-5-P was 1.35 mM at 25°C in Na-phosphate buffer, pH 5.7 (Fig. 3). The relative activity of the enzyme towards a variety of substrates is presented in Table II. The substrates were tested at 5 mM final concentration and their rate of reduction was compared with D-ribose-5-P. Hexose phosphates, hexoses, and pentoses were not reduced. The enzyme has a greater affinity for D-ribose-5-P than for other pentose phosphates. D-Erythrose-4-P was reduced at a much higher rate than either D-ribose-5-P and DL-glyceraldehyde-3-P. It is interesting to note that even though the enzyme was inactive with β-D-allose and D-ribose, it was active with D-erythrose and DL-glyceraldehyde. These aldoses have similar molecular structures, but it appears that the enzyme has a higher affinity for C_4 and C_3 than C_5 or C_6 aldoses. In addition to the above substrates, the enzyme strongly reduced the aromatic aldehyde, 3-pyridinecarboxaldehyde.

The following is a list of the possible reactions for D-ribitol biosynthesis:

(A) D-Ribulose + NADH
(B) D-Ribulose + NADPH
(C) D-Ribose + NADH → D-Ribitol
(D) D-Ribose + NADPH

(E) D-Ribulose-5-P + NADH
(F) D-Ribulose-5-P + NADPH
(G) D-Ribose-5-P + NADH → D-Ribitol-5-P (Pi) → D-Ribitol
(H) D-Ribose-5-P + NADPH

Route (A) has been identified in bacteria (10). Route (B) has been reported in nematode *Turbatrix aceti* (24). Route (C) is not very common, but Clancy and Coffey (6) detected an NADH polyol dehydrogenase activity in uredospores of rust fungus using D-ribose as substrate. Route (D) has been found in bacteria (31), yeast (19), fungi (22), and bull seminal vesicle (12). Assuming the presence of a phosphatase, route (E) has been detected in bacteria (11). Route (F) has been reported in bacteria (11). Route (G) has been demonstrated in extracts of bean rust uredospores and rust-infected plants (J. W. Hendrix, J. M. Daly, A. Livne 1964 Pyridine nucleotide-linked enzymatic reduction of triose,

Table I. *Summary of Purification of D-Ribose 5-Phosphate Reductase from Adonis vernalis Leaves*

Assay mixture contained 90 mM Na-phosphate (pH 5.7), 50 μl of crude extract or 25 μl of other fractions, 110 μM NADPH, and 20 mM D-ribose-5-P in a final volume of 1 ml. The reaction was initiated by the addition of substrate.

Step	Vol	Total Protein	Total Activity	Specific Activity	Purification	Yield
	ml	*mg*	*units*	*units/mg*	*-fold*	%
Crude extract	240	110.40	2.10	0.019		100
$(NH_4)_2SO_4$ (30–70%)	11.5	94.07	3.89	0.041	2.18	185
Sephadex (G-100)	4.7	15.75	1.64	0.104	5.49	78
Reactive red 120-agarose	3.5	0.59	0.80	1.36	71.58	38

Table II. *D-Ribose-5-Phosphate Reductase Substrate Specificity*

Assays (performed at 25°C) contained 90 mM Na-phosphate buffer (pH 5.7), 110 μM NADPH, 25 μl enzyme (affinity fraction), and 5 mM substrate. Substrate addition initiated the reaction. Activity is expressed as a percentage of the rate with D-ribose-5-P (specific activity, 3.8 units/mg).

Substrate	Relative Activity	Substrate	Relative Activity
	%		%
D-Glucose-6-P	0	D-Glucose	0
D-Galactose-6-P	6	D-Galactose	0
D-Mannose-6-P	0	D-Mannose	0
D-Fructose-6-P	0	D-Allose	0
D-Ribose-5-P	100	D-Ribose	0
D-Arabinose-5-P	5	D-Arabinose	0
D-Xylulose-5-P	20	D-Xylose and L-xylose	0
D-Ribulose-5-P	14	D-Ribulose	0
D-Erythrose-4-P	134	D-Erythrose	37
DL-G yceraldehyde-3-P	20	DL-Glyceraldehyde	29
3-P idinecarboxaldehyde	114	Dihydroxyacetone	0
D-C .acuronic acid	2	Glycoaldehyde	1

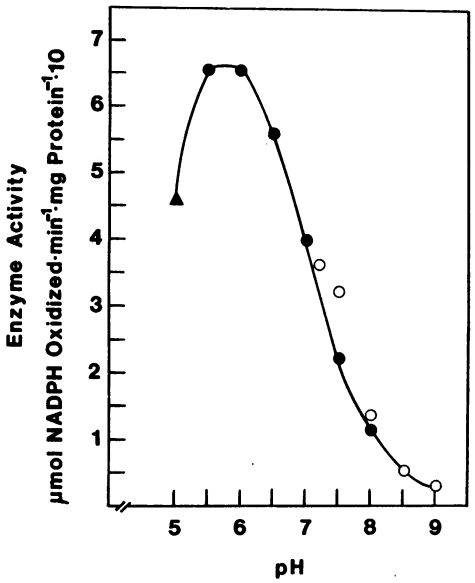

FIG. 1. Effect of pH on D-ribose-5-P reductase activity. Reaction mixtures were as described under "Materials and Methods" except 25 μl of Sephadex G-100 fraction were used. The following buffers (100 mM) were used: (△), Mes; (●), Na-phosphate; (○), Tris-HCl.

pentose, and hexose phosphates associated with rust infection. Phytopathology 54: 895). Route (H) has been reported in silkworm haemolymph (9).

As can be seen from the substrate utilized (Table II), the enzyme isolated from *Adonis vernalis* leaves is distinct from aldose reductase (14), L-hexonate dehydrogenase (21), aldehyde reductase (13), succinic semialdehyde reductase (16), and glycerol dehydrogenase (18) in its reaction with phosphorylated aldoses even though there is an overlapping of some substrates, especially 3-pyridine carboxaldehyde and C_3 and C_4 aldoses. The only other enzyme that reduced D-ribose-5-P in the presence of NADPH is that reported by Faulkner (9) from silkworm blood.

D-Ribose-5-P is a key metabolite in several metabolic pathways such as the oxidative and reductive pentose phosphate cycles and nucleotide synthesis. Furthermore, ribitol is necessary for the formation of riboflavin. The regulation of the various

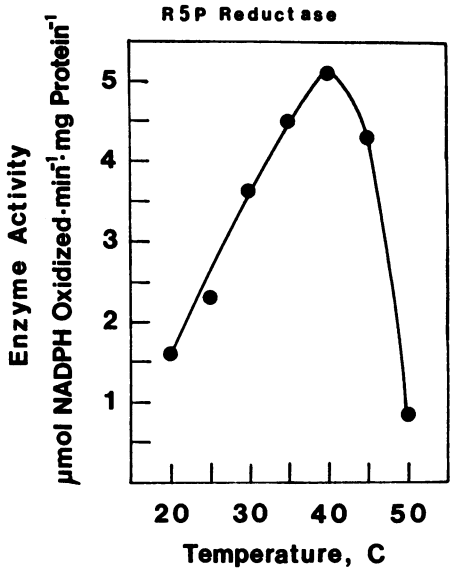

FIG. 2. Effects of temperature on D-ribose-5-P (R5P) reductase activity. Reaction mixtures were as described under "Materials and Methods" except that enzyme (affinity fraction) and NADPH were incubated at each temperature for 5 min prior to addition of substrate.

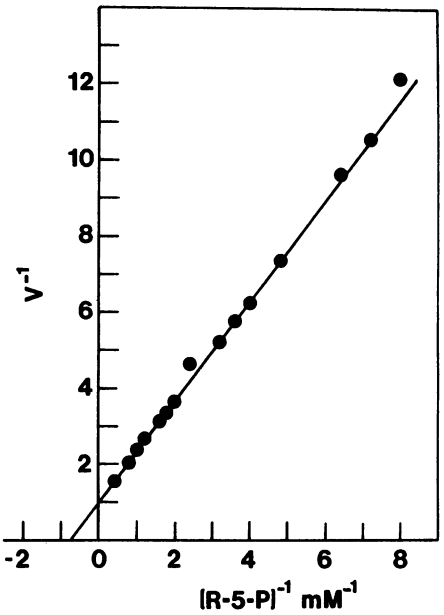

FIG. 3. Lineweaver-Burk plot of D-ribose-5-P reductase (affinity fraction) as a function of D-ribose-5-P concentration. V, μmol NADPH oxidized/min/mg protein. Reaction mixtures were as described under "Materials and Methods" except D-ribose-5-P (R-5-P) concentration was varied as indicated.

enzymes competing for D-ribose-5-P and the localization of D-ribose-5-P reductase should prove of considerable interest.

Based on the information presented above, this enzyme should be named D-ribose-5-P reductase and that D-ribose-5-P is the immediate precursor for ribitol biosynthesis in *Adonis vernalis*.

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