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PURIFICATION OF GUANOSINE 5'-DIPHOSPHATE D-MANNOSE OXIDOREDUCTASE FROM *PHASEOLUS VULGARIS*

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SUMMARY

An enzyme characterized as GDP-D-mannose oxidoreductase has been extracted from seedlings of *Phaseolus vulgaris* and purified to apparent homogeneity by $(NH_4)_2SO_4$ precipitation and chromatography on columns of DEAE-cellulose, Sephadex G-100 and hydroxylapatite. Only one protein band could be detected upon sedimentation velocity ultracentrifugation or disc gel electrophoresis of the enzyme preparation. The molecular weight of the enzyme was estimated at 120 000 by sedimentation equilibrium analysis. No requirement for a pyridine nucleotide or any other cofactor could be demonstrated in the reaction catalyzed by the purified enzyme.

INTRODUCTION

In green plants L-fucose has been found as a constituent of heteropolysaccharides[1] and of glycoproteins[2]. It is usually a relatively minor constituent of those polymers, but nonetheless it appears to be distributed widely throughout the plant kingdom[3].

The synthesis of L-fucose in plants presumably takes place, as it does in mammals[4] and certain bacteria[5,6] by the conversion of GDP-D-mannose to GDP-4-keto-6-deoxy-D-mannose and the reduction and epimerization of that intermediate compound to GDP-L-fucose[7,8]. Cell-free enzyme extracts of some plants will also catalyze the stereospecific reduction of the intermediate, without concomitant epimerization, to GDP-D-rhamnose[7]. The role of that compound, if any, in higher plants is unknown; in one species of bacteria, however, it has been shown to serve as a donor of D-rhamnosyl units in the formation of a capsular polysaccharide containing that sugar[9].

This report will be concerned with the purification to apparent homogeneity of an enzyme from the green bean that brings about the dehydration of GDP-D-mannose to GDP-4-keto-6-deoxy-D-mannose. To our knowledge, this is the most highly purified preparation obtained so far from any source.

MATERIALS AND METHODS

Substrates, cofactors and reagents

Guanosine 5'-diphosphate D-mannose labeled uniformly with ^{14}C in the D-mannosyl moiety (100 μCi/μmole) was purchased from the New England Nuclear Co. Unlabeled nucleotides and other cofactors were obtained from Sigma Chemical Co. or Calbiochem. Reagents were bought from the usual commercial sources.

Paper chromatography and electrophoresis

Partition chromatography on paper was carried out on Schleicher and Schuell No. 589 Blue Ribbon paper in the following solvent systems: Solvent I, *n*-propanol–ethyl acetate–water (7:1:2, by vol.); Solvent II, isobutyric acid–1 M NH_4OH (10:6, by vol.); Solvent III, 2-butanone–acetic acid–saturated boric acid solution (9:1:1, by vol.); Solvent IV, pyridine–ethyl acetate–water (1:3.6:1.15, by vol.); Solvent V, 2-butanone–acetic acid–water (8:1:1, by vol.).

Electrophoresis was performed on Schleicher and Schuell No. 589 Orange Ribbon paper with a flat plate apparatus in 0.1 M ammonium formate buffer, pH 3.7, at about 35 V/cm.

Column chromatography of enzyme extracts

DEAE-cellulose (Sigma) in a 2.5 cm × 25 cm column was equilibrated with 0.05 M Tris–HCl–0.02 M mercaptoethanol buffer, pH 7.6.

Sephadex G-100 (Pharmacia) in a 2.5 cm × 50 cm column was equilibrated with 0.025 M sodium phosphate buffer, pH 7.0, overnight.

Hydroxylapatite (Bio-Rad) in a 1 cm × 10 cm column was washed extensively with 0.025 M potassium phosphate buffer, pH 7.0.

Gel electrophoresis

Analytical disc gel electrophoresis was carried out on the enzyme extract after each purification step with a Bio-Rad Model 150 apparatus at 100 V. A gel made up of 7.5% Cyanogum-41 (95% acrylamide and 5% bisacrylamide), 0.1% *N,N,N',N'*-tetramethylethylenediamine and 0.1% ammonium persulfate in 0.4 M Tris–glycine buffer, pH 9.4, was used. Proteins were stained with Amido black and destained in 7% acetic acid.

Analytical ultracentrifugation

A Spinco Model E analytical ultracentrifuge equipped with a schlieren optical system was employed. Sedimentation velocity experiments were conducted in 12-mm path length double sector cells in the An-D rotor at 48 000 rev./min. The temperature was maintained at 20 °C.

Sedimentation equilibrium studies were done by the method of Yphantis[10] in 30-mm optical path length double sector cells at 14 000 rev./min. Temperature was maintained at 20 °C. The Rayleigh patterns were recorded on Kodak spectrographic plates emulsion type IIG.

Analytical methods

Protein concentration in enzyme extracts was estimated by the method of Lowry *et al.*[11].

Radioactive materials were located on paper by exposure to X-ray film (Kodak, no-screen).

Unlabeled sugars were made visible on paper with the *p*-anisidine phosphate reagent[12].

Plant material

Seeds of *Phaseolus vulgaris* (Burpee's "Stringless Green-Pod") were germinated and allowed to grow in the dark for 5 days at 23 °C in an aerosol of water.

Assay of GDP-D-mannose oxidoreductase

Reactions were carried out in thin-walled glass capillaries (1 mm internal diameter). The standard reaction mixture used for estimating the activity of the enzyme consisted of $2 \cdot 10^{-3}$ μmole (5 nCi) GDP-D-mannose labeled uniformly with ^{14}C in the D-mannosyl moiety, 20 μl of the enzyme preparation (about 8 μg protein) and 0.5 μmole sodium phosphate buffer, pH 7.0, in a total volume of 30 μl. The mixture was incubated at 37 °C for 60 min and was then transferred to a test tube containing 1 μmole $NaBH_4$. The reaction was allowed to proceed at room temperature (23 °C) for 10 min. After reduction, 20 μl of 2 M trifluoroacetic acid was added to the mixture, it was heated at 100 °C for 10 min, and the hydrolysate was chromatographed on paper in Solvent I. Radioactive compounds with the R_F values of rhamnose, 6-deoxytalose and mannose were generally the only ^{14}C-labeled substances detected. Those areas of the paper were cut out, and each was placed in a scintillation vial containing 10 ml of scintillation fluid (4 g of PPO and 0.1 g of dimethyl-POPOP in 1 l of toluene). Radioactivity was estimated with a Packard Tri-Carb liquid scintillation spectrometer. The extent of conversion of GDP-D-mannose to GDP-4-keto-6-deoxy-D-mannose was expressed as the percentage of the total radioactive material in the hydrolysate that migrated like rhamnose and 6-deoxytalose.

RESULTS

Preparation of the enzyme

Green bean seedlings were washed, chilled in ice and ground in a mechanical homogenizer (Osterizer) with an equal weight of cold 0.1 M Tris–HCl–0.02 M β-mercaptoethanol buffer, pH 7.6. All subsequent operations were conducted in the cold. In a typical preparation 150 g of the seedlings were ground in 150 ml of buffer. The homogenate was squeezed through two layers of cheesecloth and centrifuged at 30 000 × *g* for 30 min to remove particulate material. The supernatant solution was made 30% saturated with $(NH_4)_2SO_4$ (Mann, specially purified) and the precipitate was discarded. The supernatant solution was then made 50% saturated with the salt, and the precipitate was collected by centrifugation. It was suspended in a minimal volume of 0.025 M Tris–HCl–0.02 M β-mercaptoethanol buffer, pH 7.6, and dialyzed overnight against two 1 l volumes of the same buffer.

A 2.5 cm × 25 cm column of DEAE-cellulose was charged with 50 ml of the dialyzed 30–50% $(NH_4)_2SO_4$ fraction (2.25 g protein) and washed with 0.05 M Tris–HCl–0.02 M β-mercaptoethanol buffer, pH 7.6, until the eluate had an absorbance below 0.200 at 280 nm. Protein was fractionated by elution of the column with increasing concentrations of NaCl in the Tris–HCl–β-mercaptoethanol buffer. A linear

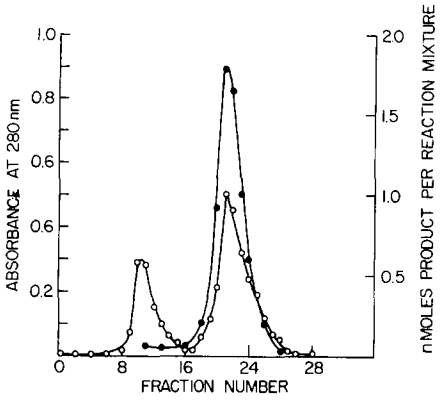


Fig. 1. Final purification of the oxidoreductase on a column of hydroxylapatite. Fractions of 20 ml were collected. Enzyme activity is represented by ●—● and protein by ○—○.

gradient was formed with 200 ml of 1 M NaCl in buffer in the reservoir and 200 ml of buffer in the mixing flask. Two protein peaks appeared in the eluate one of which contained all the enzyme activity. The appropriate enzyme-containing fractions were combined, concentrated to a small volume by ultrafiltration and brought to 70% $(NH_4)_2SO_4$ saturation with the solid salt. After 1 h, the precipitate was collected by centrifugation, dissolved in a minimal volume of 0.025 M Tris–HCl–0.02 M β-mercapthoethanol buffer, pH 7.6, and dialyzed overnight against two 1-l volumes of the same buffer. The total volume of the dialysate was 10 ml (430 mg protein).

A portion of the DEAE-cellulose fraction (5 ml) was applied to a 2.5 cm × 50 cm column of Sephadex G-100 and was eluted with 0.025 M sodium phosphate buffer, pH 7.0. Fractions with enzyme activity were combined and reduced in volume to 2 ml by ultrafiltration.

The Sephadex G-100 fraction was applied to a 1 cm × 10 cm column of hydroxylapatite. Protein was eluted from the column with increasing concentrations of potassium phosphate buffer, pH 7.0. Enzyme activity appeared in the protein eluted with 0.3 M phosphate. These results are shown in Fig. 1. Fractions 20–23 were pooled and represent the purified enzyme.

A summary of the purification procedure is given in Table I.

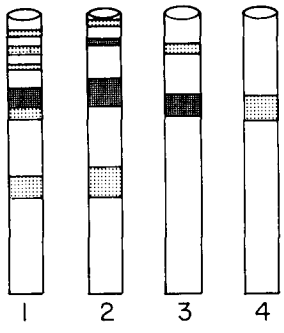
Proof of homogeneity of the enzyme preparation

Analytical disc gel electrophoresis of the enzyme preparations after each purification step is shown in Fig. 2. After elution from the hydroxylapatite column, only

TABLE I

SUMMARY OF PURIFICATION OF GDP-D-MANNOSE OXIDOREDUCTASE FROM *phaseolus vulgaris*

Stage of purification	*Total protein (mg)*	*Total activity (units)*	*Specific activity (units/mg)*
30–50% $(NH_4)_2SO_4$ fraction	2250	8000	3.5
DEAE-cellulose fraction	432	6480	15.0
Sephadex G-100 fraction	23	1680	72.6
Hydroxylapatite fraction	3.6	585	162.2



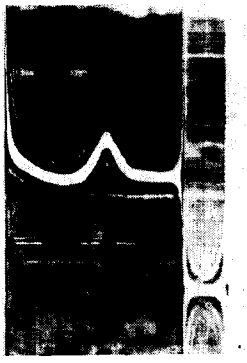


Fig. 2. Diagrammatic representation of the protein patterns obtained upon disc gel electrophoresis of the oxidoreductase preparation at each stage of purification. Electrophoresis was carried out in 7.5% Cyanogum-41 gel at pH 9.4, 100 V. Proteins were stained with amido black. 1, 30–50% $(NH_4)_2SO_4$; 2, DEAE-cellulose eluate; 3, Sephadex G-100 eluate; 4, hydroxylapatite eluate.

Fig. 3. Ultracentrifugal sedimentation of the purified oxidoreductase.

one protein band was visible in the preparation electrophoresed either in 0.1 M phosphate buffer, pH 7.0, or 0.4 M Tris–glycine buffer, pH 9.4.

A sample of the GDP-D-mannose oxidoreductase eluted from hydroxylapatite (3.5 mg protein) was dissolved in 1 ml 0.05 M sodium phosphate–0.1 M NaCl buffer, pH 7.0, and dialyzed overnight against 1 l of the same buffer. The dialysate was subjected to centrifugation at 48 000 rev./min in 12-mm length double sector cells in the An-D rotor of the analytical ultracentrifuge. The enzyme sedimented as a single symmetrical band as is shown in Fig. 3. A value of 6.47 was calculated for the sedimentation coefficient[13].

A 0.05% solution of the purified enzyme was centrifuged at 14 000 rev./min in the ultracentrifuge for 24 h. When the concentration distribution was plotted against the radial distance squared, a straight line was obtained. This constitutes evidence for a homogeneous protein preparation[10]. Assuming a value of 0.72 for the specific volume of the protein, a molecular weight of about 120 000 was calculated from the slope of the line by the Svedberg equation[13].

Properties of the reaction catalyzed by the purified enzyme

Characterization of the product

A sample of the product sufficient for characterization was prepared as follows. A mixture containing 20 μl of the purified oxidoreductase (8 μg protein) in 0.025 M sodium phosphate buffer, pH 7.0, and 0.002 μmole (0.015 μCi) GDP-D-mannose labeled with ^{14}C in the D-mannose portion in a total volume of 30 μl was incubated at 37 °C for 60 min. Unlabeled GDP-D-mannose (0.2 μmole) was added to the mixture, which was then subjected to electrophoresis on paper at pH 3.7. The product of the reaction was characterized as GDP-4-keto-6-deoxy-D-mannose by the following criteria:

(1) It migrated upon paper electrophoresis at pH 3.7 like GDP-D-mannose.

(2) When it was subjected to mild acid hydrolysis (pH 2.0, 10 min at 100 °C), and the hydrolysate was chromatographed on paper with Solvent I, most of the radio-

activity disappeared. (Such lability has been reported to be characteristic of this compound[6,7]).

(3) When the radioactive nucleotide derivative was incubated with the crude green bean GDP-L-fucose synthetase[8], and the product was purified by electrophoresis, hydrolyzed and chromatographed on paper with Solvent I, a radioactive compound indistinguishable from authentic L-fucose appeared.

(4) The 4-keto compound was reduced chemically with $NaBH_4$ (1 μmole borohydride in 20 μl of 0.05 M sodium phosphate buffer, pH 7.0, for 15 min at 23 °C). It was then electrophoresed on paper at pH 3.7 where it was indistinguishable from authentic GDP-D-mannose. It was eluted from the electrophoretogram and chromatographed on paper with Solvent II for 48 h at 23 °C. Two radioactive compounds were detected both of which migrated more rapidly than GDP-D-mannose. They were eluted, hydrolyzed and chromatographed on paper with the solvent systems shown in Table II. The ^{14}C-labeled compounds were indistinguishable from D-rhamnose (or L-rhamnose) and 6-deoxy-D-talose.

TABLE II

PAPER CHROMATOGRAPHIC MOBILITY OF THE 6-DEOXYHEXOSES FORMED BY CHEMICAL REDUCTION OF THE ENZYMICALLY SYNTHESIZED INTERMEDIATE

Details of the methods used to produce these compounds are given in the text. The mobilities of the various sugars are given relative to L-rhamnose (R_{Rham}). The figures in parentheses are taken from the data of Ginsburg[6].

Sugar	*Migration relative to* L-*rhamnose*		
	Solvent III	*Solvent IV*	*Solvent V*
L-Fucose	0.5	0.6	0.7
6-Deoxy-D-glucose	0.8	0.8	0.9
6-Deoxy-D-talose	(2.1)	(1.6)	(1.4)
L-Rhamnose	1.0	1.0	1.0
^{14}C-labeled hydrolysis product A	1.0	1.0	1.0
^{14}C-labeled hydrolysis product B	2.0	1.6	1.5

Kinetics of the reaction

The reaction rate was proportional to time (up to 60 min) and to enzyme concentration (to a maximum of 8 μg protein/30 μl reaction mixture).

The dependence of the reaction rate on substrate concentration was found to follow classic Michaelis–Menten kinetics. A Lineweaver–Burk plot[14] with the substrate, GDP-D-mannose, yielded a straight line. The K_m for that substrate was calculated to be $2.8 \cdot 10^{-5}$ M and the V $1.16 \cdot 10^{-3}$ μmole/30 min. The K_i for GTP was $1.86 \cdot 10^{-5}$ M and for GDP-D-glucose was $8.4 \cdot 10^{-5}$ M. V of the inhibited reaction was the same as that of the uninhibited reaction.

Substrates, cofactors and inhibitors

It has been shown that dTDP-D-glucose oxidoreductase from *Pseudomonas aeruginosa*[15] and CDP-D-glucose oxidoreductase from *Salmonella typhimurium*[16] require the addition of catalytic quantities of NAD^+ for activity even when relatively crude enzyme extracts are used. The synthesis of GDP-L-fucose from GDP-D-mannose by extracts of *Aerobacter aerogenes* also is stimulated by NAD^+ (ref. 5), but with plant extracts we have never been able to show any effct of that pyridine nucleotide[7,8].

TABLE III

THE EFFECT OF PYRIDINE NUCLEOTIDE ON THE ACTIVITY OF GDP-D-MANNOSE OXIDOREDUCTASE

The reaction mixtures each contained 20 μl of purified oxidoreductase (8 μg protein) in 0.025 M sodium phosphate buffer, pH 7.0, 2.0 nmoles (5 nCi) GDP-D-[^{14}C]mannose and 0.1 μmole of the indicated pyridine nucleotide in a total volume of 30 μl. The mixtures were incubated at 37 °C for 60 min. The extent of GDP-4-keto-6-deoxy-D-mannose formation was estimated as described in the text.

Pyridine nucleotide	*% GDP-D-mannose converted to GDP-4-keto-6-deoxy-D-mannose*
None	52
NAD^+	54
$NADP^+$	57
NADPH	52

Similarly, in the course of these experiments with the purified plant oxidoreductase neither NAD^+ or $NADP^+$ increased the rate or extent of the reaction. The results are shown in Table III.

The effect of various other nucleotides on the rate of the oxidoreductase reaction is shown in Table IV. There was no stimulation of the rate of formation of GDP-4-keto-6-deoxy-D-mannose by the addition of ATP, UTP or CTP ($3.3 \cdot 10^{-3}$ M) while either GTP or GMP at a concentration of $3.3 \cdot 10^{-3}$ M caused a 90% inhibition. ITP, a structural analogue of GTP, also inhibited the rate of the reaction (82%) at the same concentration. The kinetics of the process indicate that GTP acts as a competitive inhibitor.

When any one of the sugar nucleotides UDP-D-glucose, ADP-D-glucose or dTDP-D-glucose ($3.3 \cdot 10^{-3}$ M) was added to the reaction mixture, there was no stimulation or inhibition of the rate. GDP-D-glucose at the same concentration brought about an 84% inhibition. D-Mannose ($3.3 \cdot 10^{-3}$ M) and α-D-mannose 1-phosphate ($3.3 \cdot 10^{-3}$ M) were without effect.

TABLE IV

THE EFFECT OF NUCLEOTIDES ON THE ACTIVITY OF GDP-D-MANNOSE OXIDOREDUCTASE

The reaction mixtures each contained 20 μl of purified oxidoreductase (8 μg protein) in 0.025 M sodium phosphate buffer, pH 7.0, 2.0 nmoles (5 nCi) GDP-D-[^{14}C]mannose and a nucleotide in the concentration indicated in a total volume of 30 μl. The mixtures were incubated at 37 °C for 60 min. The extent of GDP-4-keto-6-deoxy-D-mannose formation was estimated as described in the text.

Nucleotide	*Concentration (M)*	*% GDP-D-mannose converted to GDP-4-keto-6-deoxy-D-mannose*
None	—	68
ITP	$3.3 \cdot 10^{-3}$	13
ATP	$3.3 \cdot 10^{-3}$	57
UTP	$3.3 \cdot 10^{-3}$	54
CTP	$3.3 \cdot 10^{-3}$	65
GTP	$3.3 \cdot 10^{-3}$	2
GTP	$6.6 \cdot 10^{-5}$	40
GTP	$3.3 \cdot 10^{-5}$	51
GTP	$1.6 \cdot 10^{-5}$	54
GMP	$3.3 \cdot 10^{-3}$	5

The effect of various metal ions was investigated. These included $MgCl_2$, $CaCl_2$, $MnCl_2$, $NiCl_2$ and $CoCl_2$. $MnCl_2$ at $1.6 \cdot 10^{-2}$ M and $CoCl_2$ and $NiCl_2$ at $8 \cdot 10^{-3}$ M inhibited the reaction rate 90%, $CaCl_2$ at $1.6 \cdot 10^{-2}$ M inhibited the rate 50% while $MgCl_2$ was without effect.

The oxidoreductase reaction catalyzed by the purified plant enzyme was completely inhibited by *p*-hydroxymercuribenzoate ($1 \cdot 10^{-2}$ M) after 30 min at 23 °C. Essentially all the activity was restored by the addition of mercaptoethanol (0.2 M). This suggests that sulfhydryl groups are essential for enzyme activity[17].

pH optimum

A pH optimum was observed from pH 6.6 to pH 7.1 (0.16 M sodium phosphate buffer). Activity was 58% of the maximum at pH 9.2 (0.16 M Tris–HCl buffer) and 40% of the maximum at pH 5.0 (0.16 M sodium acetate buffer). The enzyme was apparently inhibited by Tris–HCl buffer since there was a greater rate in phosphate buffer (16% at pH 7.6) than in Tris–HCl, and the addition of phosphate to the reaction mixture in Tris–HCl did not restore the rate.

Stability of the enzyme

The purified enzyme was unstable on storage. It lost essentially all its activity on freezing and thawing or after storage at 4 °C for more than 72 h. However, if the protein was stored in a 70% saturated $(NH_4)_2SO_4$ solution, it retained its activity for at least several weeks at −15 °C. The purified preparations were therefore ordinarily made 70% with $(NH_4)_2SO_4$, kept at −15 °C until needed and dialyzed for a few hours immediately before use.

DISCUSSION

Two separate communications have appeared recently[17,18] describing the purification and properties of dTDP-D-glucose oxidoreductase from *Escherichia coli*. It has been shown to be a protein of molecular weight 88 000 containing 1 mole of firmly bound NAD^+ per mole of enzyme. Muscle glyceraldehyde phosphate dehydrogenase[19] and yeast UDP-D-glucose epimerase[20] also contain bound NAD^+. It can be released from those enzymes by treatment with *p*-chloromercuribenzoate. The enzymes were inactivated by that treatment, but activity could be restored with NAD^+ and cysteine. When the plant oxidoreductase was treated similarly with *p*-hydroxymercuribenzoate, another compound that has been shown to bind NAD^+ (ref. 19), activity was restored by the addition of cysteine alone; NAD^+ was without effect. Thus it cannot be stated with any certainty that NAD^+ is required in the dehydration of GDP-D-mannose by the plant enzyme. In fact, the reaction may proceed by an entirely different route.

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