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Communication

Purification and Characterization of Cytosolic 6-Phosphogluconate Dehydrogenase Isozymes from Maize[1]

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ABSTRACT

Cytosolic isozymes of 6-phosphogluconate dehydrogenase were purified from roots of maize (*Zea mays* L.). The final preparation contained two 55-kD proteins. Affinity-purified dehydrogenases from a maize line that is null for both cytosolic 6-phosphogluconate dehydrogenase isozymes (*Pgd1-null, Pgd2-null*) lacked the 55-kD proteins. The substrate kinetics of the purified enzyme were determined.

The oxidative pentose phosphate pathway operates in both the cytoplasm and plastids of higher plants (2, 4). The first step is catalyzed by G6PDH[2] (EC 1.1.1.49), which converts glucose-6-P to 6-PG and generates a molecule of NADPH. Subsequently, 6-PGD stimulates the decarboxylation of 6-PG to ribulose-5-P and CO_2 and generates a molecule of NADPH. This reaction is a key step in the oxidative pentose phosphate pathway because it is unidirectional in most organisms. In maize two loci *Pgd1* and *Pgd2*, located on duplicated chromosomal segments, encode cytosolic 6-PGD isozymes (11, 12). The products of these loci form intergenic and interallelic isozyme dimers (PGD1·PGD1, PGD1·PGD2, PGD2·PGD2), which are present at specific levels in extracts from all tissues examined, including pollen (3, 11).

Alleles of both *Pgd1* and *Pgd2* that produce no detectable isozyme on native polyacrylamide gels have been found in separate maize lines. These mutants were used to generate plants homozygous for null alleles at both loci. The 6-PGD double null homozygote (*Pgd1-null, Pgd2-null*) is reproductively viable under normal greenhouse and field conditions, indicating that wild-type levels of cytosolic 6-PGD are not required for development (3). In this communication, we report on the kinetic and physical properties of 6-PGD isozymes purified from seedling roots of maize. The 55-kD proteins identified as 6-PGD are not detected in extracts from leaves of the 6-PGD double-null homozygote.

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[2] Abbreviations: G6PDH, glucose-6-phosphate dehydrogenase; 6-PGD, 6-phosphogluconate dehydrogenase; 6-PG, 6-phosphogluconate.

MATERIALS AND METHODS

Genetic Material

Maize stocks were from Pioneer HiBred International line B73 (*Pgd1-3.8, Pgd2-5*); 6-PGD double-null homozygote (*Pgd1-null, Pgd2-125null*) was generated as described elsewhere (3).

Chemicals

6-PG, $NADP^+$, NADPH, $(NH_4)_2SO_4$, 2′,5′-ADP agarose, protein mol wt markers, and buffers were obtained from Sigma Chemical Co. DE52-cellulose was from Pharmacia Biochemicals. Protein Concentration Reagent was from United States Biochemical Co.

6-PGD Purification from Roots

Five-day-old roots from etiolated seedlings were harvested into liquid nitrogen and stored at −70°C. All procedures were carried out at 4°C. The roots were pulverized, hydrated in two volumes of extraction buffer (100 mM Tris-HCl [pH 7.5], 10 mM H_3BO_4, 30 mM DTT, 15% [v/v] glycerol), and allowed to thaw. The homogenate was filtered through four layers of cheesecloth and two layers of Miracloth (Cal Biochem) and centrifuged twice at 7300*g* for 10 min to remove cell debris. The supernatant was brought to 20% saturation by the addition of $(NH_4)_2SO_4$ crystals during a 20-min period with constant stirring. The suspension was centrifuged at 7300*g* for 10 min, and the pellet was discarded. The supernatant was raised to 70% saturation by further addition of $(NH_4)_2SO_4$ crystals. The suspension was centrifuged for 10 min at 7300*g*, and the pellet was resuspended in 2 to 4 mL of extraction buffer. The sample was desalted by dialysis against 500 mL of 5 mM NaH_2PO_4/$NaHPO_4$ (pH 7.1) for 18 to 20 h, with one buffer change.

The extract was clarified by centrifugation for 5 min at 16,000*g* in a microcentrifuge and applied to a DE52-cellulose column (12 × 2.5 cm) equilibrated in 5 mM NaH_2PO_4/$NaHPO_4$ (pH 7.1) buffer. The column was washed with three volumes of buffer and developed with a linear, 80-mL gradient of 5 to 200 mM NaH_2PO_4/$NaHPO_4$ (pH 7.1). The fractions with 6-PGD activity were pooled and immediately applied to a 1-mL 2′,5′-ADP agarose column equilibrated with 100 mM NaH_2PO_4/$NaHPO_4$ (pH 7.1) buffer. The column was washed with 15 mL of buffer, and 6-PGD was eluted

with 5 mL of 2 mM $NADP^+$ in column buffer. The active fractions were pooled and dialyzed against 50 mM NaH_2PO_4/$NaHPO_4$ (pH 7.1), 20% (v/v) glycerol, 0.05% (v/v) 2-mercaptoethanol, and stored at 4°C. Protein Concentration Reagent was used to assay protein concentration immediately after dialysis, with BSA used as the protein standard. The purified 6-PGD was still active after 1 month of storage at 4°C.

Partial Purification of 6-PGD from Leaves

One-month-old leaves were harvested at midday, frozen in liquid N_2, and stored at −70°C. 6-PGD was partially purified from 25 g of leaves by the procedure followed for roots, except the DE52-cellulose column was omitted. The proteins of the 20 to 70% $(NH_4)_2SO_4$ fraction were passed over the 2′,5′-ADP agarose column and eluted with 2 mM $NADP^+$.

Assay of 6-PGD Activity

6-PGD activity was assayed in a Perkin-Elmer Cetus, Lambda 3B spectrophotometer. The standard 250-μL reaction mixture contained 50 mM Tris-HCl (pH 7.5), 2 mM 6-PG, and 250 μM $NADP^+$. The reaction was initiated by the addition of 10 to 50 uL of extract. The reduction of $NADP^+$ was measured every 0.5 s for 1 min, at 340 nm visible light and room temperature. Activity was only determined if reactions were linear for at least 2 min. One unit of enzyme activity is defined as that amount that reduces 1 μmol of $NADP^+$ per min. The specific activity is the units of enzyme activity per mg of protein.

Substrate Kinetic Analysis

The Michaelis-Menten constants for 6-PG and $NADP^+$ were determined by varying the concentration of substrate or cofactor, at fixed concentrations of the other components of the reaction mixture. A Hanes-Woolf plot of $[S]$ versus $[S] \cdot V^{-1}$ was used to determine K_m values.

Gel Electrophoresis

Proteins (0.5–100 μg) were suspended in SDS loading buffer (10 mM Tris-HCl [pH 6.8], 1% [v/v] 2-mercaptoethanol, 2.5% [v/v] glycerol, 4% [w/v] SDS, 0.1% [w/v] bromophenol blue), boiled for 5 min, and separated by electrophoresis on a 12% (w/v) polyacrylamide, 0.1% (w/v) SDS gel (6).

RESULTS

Purification of 6-PGD from Maize Roots

6-PGD was purified from seedling roots by $(NH_4)_2SO_4$ precipitation, DE52-cellulose ion exchange chromatography, and 2′,5′-ADP agarose affinity chromatography. The activity was enriched 3-fold by $(NH_4)_2SO_4$ precipitation and desalting. The 20 to 70% $(NH_4)_2SO_4$ fraction was loaded onto a DE52-cellulose column and eluted with a gradient of 5 to 200 mM NaH_2PO_4/$NaHPO_4$ (pH 7.1). A single peak of activity was released at about 80 to 120 mM NaH_2PO_4/$NaHPO_4$. The peak fractions were pooled and yielded about an 8-fold enrichment in 6-PGD activity. A 2′,5′-ADP agarose affinity column was used as a second chromatography step. 6-PGD activity was absorbed by the column and rapidly eluted upon the addition of 2 mM $NADP^+$ to the column buffer. This procedure resulted in an overall 265-fold purification of 6-PGD (Table I).

SDS-PAGE Analysis of Purified 6-PGD and Confirmation of 6-PGD Purification

SDS-PAGE analysis revealed that two proteins with apparent molecular masses of 55 kD were the major proteins purified by this procedure. The first protein that eluted from the 2′,5′-ADP agarose column by addition of $NADP^+$ had a slightly slower electrophoretic mobility than the second protein (Fig. 1, lanes A–C). Native-PAGE analysis demonstrated that the PGD2·PGD2 homodimer eluted first, followed by the PGD1·PGD2 heterodimer and, finally, the PGD1·PGD1 homodimer (data not shown).

A maize line that is homozygous null for both cytosolic 6-PGD isozymes (*Pgd1-null, Pgd2-null*) was used to confirm the purification of cytosolic 6-PGD. A partial purification of 6-PGD activity was carried out with leaves of the B73 inbred and the 6-PGD double-null homozygote. Activity was concentrated from leaf extracts by precipitation in 20 to 70% $(NH_4)_2SO_4$ and desalted by dialysis. The extracts were individually applied to a 2′,5′-ADP agarose affinity column, and 6-PGD activity was eluted with $NADP^+$. SDS-PAGE analysis revealed that the affinity column elutants contained numerous polypeptides, presumably leaf $NADP^+$-binding proteins. The major 55-kD protein of the root 6-PGD preparation was

Table I. *Summary of 6-PGD Purification from Maize Roots*

Purification of 6-PGD activity from 63-g of 5-d-old seedling roots. The data are from one of four experiments.

Fraction	Total Protein	Total Activity	Specific Activity	Yield	Fold Purification
	mg	*units*	*units·mg⁻¹*	%	
Crude extract	240.0	18.3	0.078	100.00	1
20–70% $(NH_4)_2SO_4$	48.8	12.0	0.25	65.5	3.2
DE52-cellulose peak	14.3	6.3	0.44	34.4	5.7
2′,5′-ADP Agarose	0.25	5.2	20.8	28.4	265

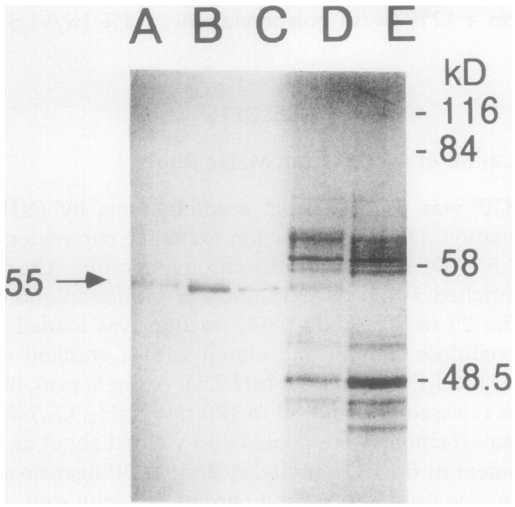

Figure 1. SDS-PAGE (12% [w/v]) fractionation and Coomassie blue visualization of proteins. Lanes A through C, Peak elutant fractions of highly purified 6-PGD from B73 roots. Lane D, Affinity-purified proteins from B73 leaves. Lane E, Affinity-purified proteins from 6-PGD double-null homozygote leaves. The molecular masses (kD) of standard proteins and purified 6-PGD are indicated.

visible in the fraction from B73 leaves (Fig. 1D). The level of the 55-kD protein was dramatically reduced in the fraction from the 6-PGD double-null homozygote leaves (Fig. 1E). This result strongly supports the conclusion that *Pgd1* and *Pgd2* encode a 55-kD protein.

Substrate Kinetics of Purified 6-PGD

The purified 6-PGD had an optimal enzymic activity at pH 7.5 and showed typical Michaelis-Menten saturation kinetics for both $NADP^+$ and 6-PG. Kinetic determinations (Table II) were made using the Hanes-Woolf plot of $[S]$ versus $[S] \cdot V^{-1}$ and were linear (data not shown). A K_m value of 42.2 μM for $NADP^+$ was determined by assaying for 6-PGD activity in the presence of 2 mM 6-PG at variable concentrations of $NADP^+$. A K_m value of 0.32 mM for 6-PG was determined by assaying in the presence of 250 μM $NADP^+$ at variable concentrations of 6-PG. K_m values were about 2-fold lower for both 6-PG and $NADP^+$ when reactions were carried out in the presence of 7.5 mM $MgCl_2$ or 1 mM $MnCl_2$.

NADPH is a known inhibitor of 6-PGD activity (7). The inhibition of 6-PGD activity by 10 μM NADPH was competitive toward $NADP^+$ and noncompetitive toward 6-PG (Table II). In contrast, 6-PGD activity was not inhibited by the presence of 250 μM NADH in the reaction mixture (data not shown).

DISCUSSION

The enzymic reactions of the oxidative pentose phosphate pathway are necessary for generation of NADPH for biosynthetic reactions such as nucleic acid and fatty acid biosynthesis. Oxidative pentose phosphate pathway enzyme activities are elevated in nonphotosynthetic tissues during germination and in the dark (2, 9). The first enzyme in the pathway, G6PDH, regulates the entry of glucose-6-P from the glycolytic pathway and generates 6-PG. The subsequent conversion of 6-PG to 5-ribulose-P and CO_2 is catalyzed by 6-PGD. In maize, active 6-PGD is a homo- or heterodimer of the products of the genes *Pgd1* and *Pgd2* (11). A maize line (*Pgd1-null, Pgd2-null*) with no detectable cytosolic 6-PGD isozymes in roots, leaves, or scutella on activity-stained gels is viable under normal growth conditions (3). We have used the 6-PGD double-null homozygote to confirm the purification of 6-PGD from a maize line with wild-type alleles of *Pgd1* and *Pgd2*.

6-PGD was purified from seedling roots by approximately 250-fold after two chromatographic fractionation steps. The purified protein has an apparent molecular mass of 55 kD. Polypeptides of 55 kD were present at dramatically reduced levels in leaf extracts from the 6-PGD double-null homozygote. Thus, the subunit molecular mass of cytosolic 6-PGD from maize is similar to that of enzymes purified from other plants (1, 8, 10), fungi, animals, and bacteria (7). The analysis also demonstrated minor differences in the amounts of a few other proteins purified from leaves of the two genotypes. In future investigations, we will examine whether the reduction in cytosolic 6-PGD levels in the 6-PGD double-null homozygote affects the level of G6PDH and other NADPH-generating enzymes.

The kinetic properties of purified 6-PGD from maize were similar to those observed from the cytosolic and proplastid 6-PGD from castor bean endosperm. Purified 6-PGD from castor bean showed optimum activity at pH 7.8 to 8.0 and was stimulated by $MgCl_2$ (10). We observed that maize 6-PGD activity increased slightly when either 7.5 mM $MgCl_2$ or 1 mM $MnCl_2$ was included in the reaction mixture. The stimulation of 6-PGD by these divalent cations is thought to be important in the modulation of enzyme activity in response to intercellular levels of ATP (5). As observed for 6-PGD of yeast and bacteria (7), maize 6-PGD activity was inhibited by NADPH in apparent competition for binding of $NADP^+$ but not 6-PG. Thus, the intracellular ratio of $NADP^+$ to NADPH may play a role in the regulation of metabolism through the oxidative pentose phosphate pathway.

The purified 6-PGD has been used to produce a specific polyclonal antiserum in mice (J. Bailey-Serres, unpublished

Table II. *Kinetic Analysis of 6-PGD Purified from Maize Roots*

Enzyme reactions were carried out in the standard reaction mixture with varying concentrations of either $NADP^+$ or 6-PG, in the presence or absence of a divalent cation, or in the presence of NADPH. K_m was calculated as the negative x intercept of a plot of $[S]$ versus $[S] \cdot V^{-1}$.

Addition to Standard Reaction Mixture	K_m	
	$NADP^+$	6-PG
	μM	mM
None	42.2	0.32
7.5 mM $MgCl_2$	23.0	0.14
1 mM $MnCl_2$	ND[a]	0.15
10 μM NADPH	250.0	0.39

[a] ND, Not determined.

results). Our future objective is to use this antiserum to facilitate the identification of 6-PGD genes from maize. Our long-term goal is to use 6-PGD mutants to study the environmental and developmental regulation of metabolic flux through the oxidative pentose phosphate pathway in the cytoplasm and plastids of higher plants.

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