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# Isolation of Uridine 5'-Pyrophosphate Glucuronic Acid Pyrophosphorylase and Its Assay Using $^{32}$P-Pyrophosphate[1]

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## ABSTRACT

**A procedure was devised to detect and assay uridine 5'-pyrophosphate (UDP)-glucuronic acid pyrophosphorylase in plant extracts. Substrates are UDP-glucuronic acid and $^{32}$P-pyrophosphate, and the $^{32}$P-uridine 5'-triphosphate produced is selectively adsorbed to charcoal. The charcoal adsorption procedure is a modification of that used to determine $^{32}$P-adenosine 5'-triphosphate produced by adenosine 5'-pyrophosphate glucose pyrophosphorylase, and the modification greatly improves the retention of uridine 5'-triphosphate.**

In spite of its importance to plant cell wall formation and growth (8, 9), there are few studies of UDP-glucuronic acid pyrophosphorylase (EC 2.7.7.aa). Work is hampered by difficulties in assaying crude extracts. Crude extracts possess hydrolases which rapidly cleave the labeled D-glucuronate-1-P or UDP-glucuronate used as substrates. Therefore, earlier workers prepared ammonium sulfate fractions as the starting point for assay of this pyrophosphorylase (5, 10, 11, 14), an enzyme that seems unique to higher plants (7, 13).

A procedure which enables UDP-glucuronic acid pyrophosphorylase to be assayed without purification was devised and is described below. Labeled pyrophosphate ($^{32}$P-PP$_i$) is used as substrate, and $^{32}$P-UTP is a product. Enzyme activity was detected and then measured quantitatively in crude extracts of germinated lily pollen, seedlings of mung bean and maize, and developing maize endosperm. UDP-glucose pyrophosphorylase was also assayed using the improved procedure.

## MATERIALS AND METHODS

**Reagents.** Biochemicals were obtained from Sigma Chemical Co., St. Louis, Mo. Ammonium sulfate was enzyme grade from Schwarz/Mann, Orangeburg, N.Y. Labeled sodium pyrophosphate ($Na_4{}^{32}P_2O_7$, about 5 Ci/mmol) was obtained from New England Nuclear. The purity of UDP-glucuronic acid and pyrophosphate, the enzyme substrates, was established by chromatography. The J. T. Baker Co., Philipsburg, N.J., supplied thin layer plates (5 × 20 cm) coated with PEI[4]-cellulose. All reagents were dissolved in glass-distilled $H_2O$.

**Plant Material.** Pollen from Ace lilies (*Lilium longiflorum*) was harvested daily from greenhouse-grown plants, dried under a microbiological hood during the day of harvest, and then stored at 2 C until used. Pollen stored up to 6 months was the source of enzyme for establishing optimum assay conditions. Freshly harvested pollen was germinated using procedures reported earlier (2, 3), and pollen tubes were removed from the medium by suction filtration on Whatman No. 1 paper.

Seeds of *Zea mays* (starchy hybrid WF9×M14) and *Phaseolus aureus* (mung bean) were germinated in darkness in a chamber maintained at 25 C and approximately 100% relative humidity. Maize seeds were placed in glass trays on paper towels moistened with 0.1 mM $CaCl_2$. Mung bean seeds were planted in vermiculite moistened with tap water. At harvest, entire maize seedlings and excised mung bean hypocotyls were rinsed with glass-distilled $H_2O$ and immediately chilled on crushed ice.

Maize endosperm was obtained from ears of the inbred variety WF9 that were hand-pollinated with pollen from plants of the inbred M14. Developing seeds at the milk stage were removed from freshly harvested ears. Endosperms were dissected from these seeds and used immediately as the source of UDP-glucuronic acid pyrophosphorylase. APD-glucose pyrophosphorylase and UDP-glucose pyrophosphorylase were isolated from developing endosperm (22 days postpollination) of the sweet corn variety Illinois 677a.

**Enzyme Isolation.** The isolation medium used for pollen and for seedling tissue contained 10 mM HEPES buffer, 1 mM EDTA, and 1 mM dithiothreitol and was adjusted to pH 7.6. The medium used to isolate UDP-glucuronic acid pyrophosphorylase from maize endosperm resembled that used earlier for other endosperm enzymes (1); it contained 50 mM phosphate buffer, 5 mM $MgCl_2$, 1 mM EDTA, and 1 mM dithiothreitol, all at pH 7.5. A similar medium was used for extraction of ADP-glucose and UDP-glucose pyrophosphorylases except that 10 mM KCl was added and the pH was 7.

Pollen was ground with medium (100 mg pollen/0.5 ml medium) in a mortar for 4 min at 0 C. An equal volume of medium was added, and the homogenate was inspected under low power magnification. Cell breakage was typically about 75%. The enzymes of seedling and starchy maize tissues were extracted as for pollen, using 1 ml of isolation medium/g fresh weight of tissue. Sweet corn endosperms were homogenized 2 min at 0 C in a VirTis 45 homogenizer. Homogenates were clarified by centrifugation for 15 or 20 min at 25,000*g* and 0 C. Pollen UDP-glucuronic acid pyrophosphorylase was partially purified by treatment of a crude extract with 0.18% (w/v) protamine sulfate, recovery of the enzyme from the resulting supernatant fluid as the fraction precipitating between 35 and 50% saturated ammonium sulfate, desalting with an Amicon XM-50 ultrafilter, and adsorption to DEAE-cellulose equilibrated with 5 mM K-phosphate (pH 7.5), followed by elution with 50 mM K-phosphate-4 mM dithiothreitol at the same pH. This fraction was essentially free of phosphatase and was used when reaction

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[4] PEI: polyethyleneimine.

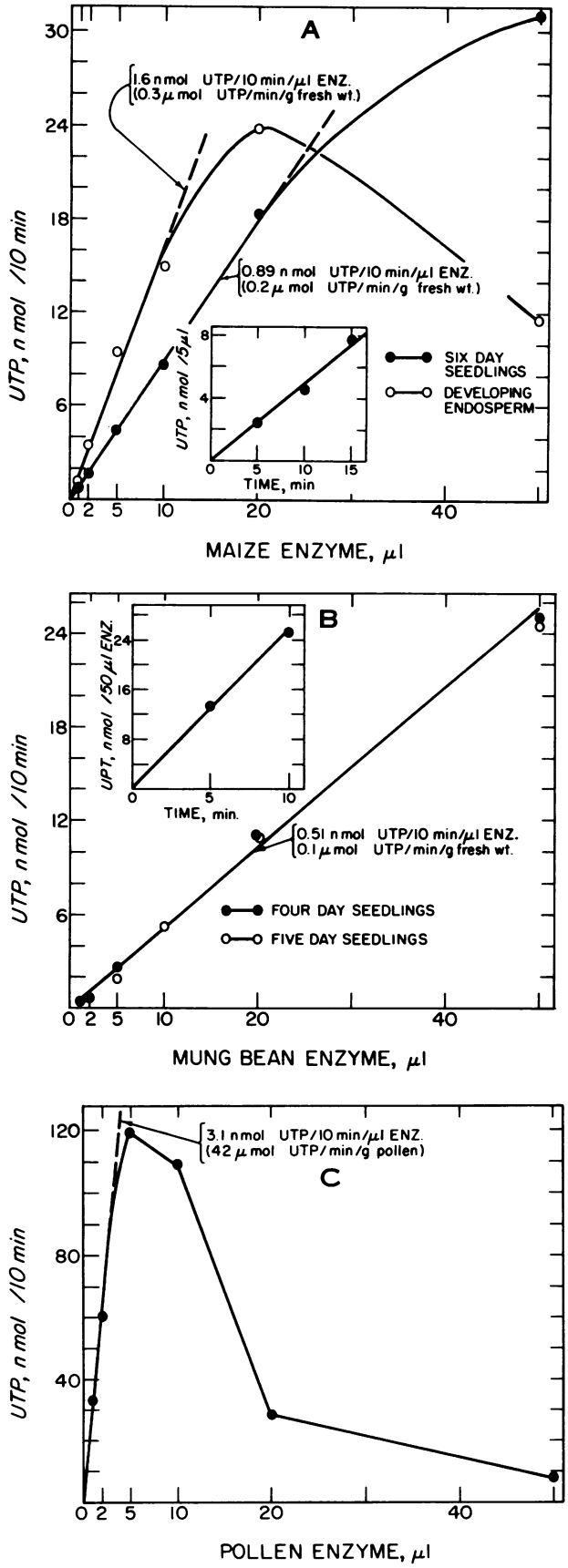


FIG. 1. Assay of UDP-glucuronic acid pyrophosphorylase in crude extracts of various plant tissues. The enzymes were isolated and assayed by production of $^{32}P$-UTP from $^{32}PP_i$ as described in the text. Blank values are given for reaction mixtures that were complete except for omission of UDP-glucuronate. The specific radioactivity is that of $^{32}PP_i$ on day of assay. A: *Zea mays*. For the seedling enzyme, specific radioactivity was 690 cpm/nmol and blank values were 300 to 700 cpm. For the endosperm enzyme, specific radioactivity was 1,234 cpm/nmol and blank values were 500 to 1,400 cpm. B: *Phaseolus aureus* (mung bean) seedling. For 4-day seedlings, specific radioactivity was 961 cpm/nmol and blank values were 400 to 900 cpm. For 5-day seedlings, specific radioactivity was 884 cpm/nmol and blank values were 400 to 800 cpm. C: *Lilium longiflorum* pollen. The pollen was germinated 3 hr before isolation of enzyme. Pollen weight refers to that of the dry pollen prior to addition of culture medium. Specific radioactivity was 854 cpm/nmol and blank values were 500 to 1,400 cpm.

mixtures were chromatographed ascendingly on PEI cellulose thin layers. Development was with 1.6 M LiCl-1 M sodium acetate which gave $R_F$ values of 0.3 and 0.6 for $PP_i$ and UTP, respectively. This procedure is a modification of that used for separation of ATP from $PP_i$ (6).

**Assay of UDP-Glucuronic Pyrophosphorylase.** The standard assay procedure was as follows. Reaction mixtures (0.1 ml final volume) were placed in Kimble culture tubes (10 × 75 mm) and incubated 10 min at 30 C. Each complete reaction mixture contained 2 mM $MgCl_2$, 2 mM UDP-glucuronate, 2 mM $^{32}P$-pyrophosphate (1,000 to 12,000 cpm/nmol), 100 mM tris-HCl buffer (pH 8.1), and enzyme. Reactions were initiated with enzyme. Control tubes were included which lacked enzyme or UDP-glucuronate. Reactions were terminated with 10 μl of 50% (w/v) trichloroacetic acid, and the tubes were immediately placed in ice. One-tenth ml of 0.1 M $Na_4P_2O_7$ (pH 8.2) was added, followed by 0.1 ml of an aqueous suspension containing 10% (w/v) Norit A. After 10 min, a 2-ml portion of 0.01 M trichloroacetic acid (0.16%, w/v) was added, tubes were centrifuged 3 min at top speed, ~ 400*g*, in a Precision Scientific Co. universal centrifuge, and the clear supernatant fluid was withdrawn with a Pasteur pipette. This washing procedure was repeated with two more 2-ml portions of 0.01 M trichloroacetic acid and then with 2 ml of ice-cold water. The washed charcoal was dispersed in 2 ml of 50% (v/v) ethanol containing 0.2% (v/v) $NH_4OH$, and 1 ml of suspension was placed on a planchet. Planchets were dried under a heat lamp, and radioactivity was measured in a Nuclear-Chicago gas flow counter. The quantity of UTP produced by the enzyme was calculated from the specific radioactivity of the $^{32}P$-pyrophosphate substrate which was determined each time that samples of $^{32}P$-UTP were counted.

**Assay of ADP-Glucose Pyrophosphorylase and UDP-Glucose Pyrophosphorylase.** Assay of these enzymes was the same as outlined above for UDP-glucuronic acid pyrophosphorylase with the following exceptions: $Mg^{2+}$ was 15 mM; $^{32}P$-$PP_i$ (specific radioactivity 395–2,135 cpm/nmol) and sugar nucleotide substrates were all 1 mM; the buffer was 150 mM HEPES (pH 7.9); reaction mixtures were 0.2 ml final volume. Reactions were terminated and labeled nucleotides adsorbed to charcoal by the procedure outlined above or that used by Shen and Preiss (12). The latter procedure included the following steps: (*a*) successive additions of 3 ml ice-cold 5% (w/v) trichloroacetic acid to stop the reaction, 0.1 ml 0.1 M $NaPP_i$ at pH 8.1, 0.1 ml Norit A (150 mg solids/ml $H_2O$); (*b*) 3-min centrifugation at 400*g*; (*c*) two rinses of the charcoal with 3-ml portions of 5% trichloroacetic acid and a 3-ml final rinse with distilled $H_2O$; (*d*) dispersal of the washed charcoal in ethanol and determination of radioactivity as described above.

## RESULTS AND DISCUSSION

UDP-glucuronic acid pyrophosphorylase in crude extracts is satisfactorily assayed using UDP-glucuronate and $^{32}P$-$PP_i$ as substrates. The $^{32}P$-UTP produced by the enzyme is selectively adsorbed to charcoal. Interference from hydrolases is minimized

Table I. Assay of ADP-glucose pyrophosphorylase and UDP-glucose pyrophosphorylase in crude extracts of developing maize endosperm

| Enzyme | Assay procedure[a] | Enzyme activity[b] |
|---|---|---|
| 1. ADP-glucose pyrophosphorylase | | |
| a. 3PGA absent | A | 54.4(2) |
| | B | 53.9(2) |
| b. 3PGA present | A | 69.6(2) |
| | B | 73.0(2) |
| 2. UDP-glucose pyrophosphorylase | A | 265. (14) |
| | B | 1,870 (10) |

[a] Procedure A: $^{32}$P-ATP or $^{32}$P-UTP was absorbed to charcoal using the standard procedure of Shen and Preiss(12). Procedure B: modified charcoal absorption procedure as described in text. Concentration of 3PGA was 12.5 mM.

[b] Numbers in parentheses refer to the number of determinations. Activity of ADP-glucose pyrophosphorylase is expressed as nmol ATP/min·ml enzyme. Activity of UDP-glucose pyrophosphorylase is expressed as nmol UTP/min.endosperm. Values for enzyme activities are averages of individual determinations.

by use of: (*a*) small amounts of plant extract (made possible by the great sensitivity of the assay procedure); and (*b*) rather high substrate levels. These conditions ensure that substrate does not become limiting even though some hydrolysis by phosphatase undoubtedly occurs. The phosphorylated substrates may also protect the $^{32}$P-UTP from phosphatase action.

Extensive preliminary studies led to the standard assay procedure that is described in the experimental section. These studies were prompted by an unexpected observation; the charcoal adsorption procedure for ADP-glucose pyrophosphorylase (12) gave much lower values for UDP-glucuronic acid pyrophosphorylase than did replicate assays in which labeled UTP was counted after separation from labeled $PP_i$ on thin layers of PEI-cellulose. The standard assay procedure incorporates the optimum concentration of trichloroacetic acid and the optimum amount of charcoal. These conditions were selected because there was good retention of labeled UTP (at least 70% of UTP formed by the enzyme was retained by the charcoal) and also low radioactivity in the blank assay tubes which lacked enzyme or UDP-glucuronate. The substrate and $Mg^{2+}$ concentrations were also varied, and the concentrations selected for the standard assay represent optimum conditions for the pollen enzyme.

The UDP-glucuronic acid pyrophosphorylase activities of crude enzyme extracts from mung bean, maize, and lily pollen are presented in Figure 1. In each case, activity was proportional to time and to amount of enzyme present in the reaction mixture. The abundant enzyme content of developing endosperm (Fig. 1A) indicates that the inositol oxidation pathway plays a role in cell wall biogenesis of endosperm cells. Lily pollen is a rich source of enzyme (Fig. 1C), even when allowance is made for the fact that the pollen fresh weight includes only about 10% moisture.

Lily pollen resembles other actively growing plant cells (11) in that UDP-glucuronic acid pyrophosphorylase is far more active than is UDP-glucose dehydrogenase; pollen extracts exhibit about 180-fold more activity of the former compared to the latter enzyme (Fig. 1 and ref. 2). Extracts of lily pollen also exhibit considerably more UDP-glucuronic acid pyrophosphorylase activity (about 40-fold more) than glucuronokinase activity (Fig. 1 and ref. 4). This differential between adjacent enzymes of the inositol oxidation pathway points to glucuronokinase as a rate-limiting step.

As would be expected, the improved assay for UDP-glucuronic acid pyrophosphorylase also gives greatly increased activity with UDP-glucose pyrophosphorylase. The latter enzyme was assayed in crude extracts of maize endosperm (Table I). Compared to the procedure devised for assay of ADP-glucose pyrophosphorylase (12), the modified procedure gave a 7-fold increase in retention of labeled UTP. Therefore, the modified charcoal adsorption procedure described in this paper should be used to assay all pyrophosphorylases that produce UTP. In contrast, the two procedures gave similar results when ADP-glucose pyrophosphorylase was assayed (Table I). Hence, ATP is less readily eluted from charcoal with trichloroacetic acid than is UTP.

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