Isolation of Uridine 5' -Pyrophosphate Glucuronic Acid Pyrophosphorylase and Its Assay Using 32P-Pyrophosphate

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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 32P-pyrophosphate, and the 32P-uridine 5'-triphosphate produced is selectively adsorbed to charcoal. The charcoal adsorption procedure is a modification of that used to determine 32P-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 hydrolyases which rapidly cleave the labeled glucurionate-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 (32P-PF) is used as substrate, and 32P-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 (Na232P2O7, about 5 Ci/mmol) was obtained from New England Nuclear. The purity of UDP-glucuronic acid and pyrophosphate, enzymatic substrates, was established by chromatography. The J. T. Baker Co., Phillipsburg, N.J., supplied thin layer plates (5 x 20 cm) coated with PEI-cellulose. All reagents were dissolved in glass-distilled H2O.

Plant Material. Pollen from Ace lilies (Lilium longiflorum)

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3 Present address: Standard Fruit Co., La Ceiba, Honduras.
4 PEI: polyethylenimine.
mixtures were chromatographed ascendingly on PEI cellulose thin layers. Development was with 1.6 M LiCl-1 M sodium acetate which gave Rf values of 0.3 and 0.6 for PP and UTP, respectively. This procedure is a modification of that used for separation of ATP from PP (6).

Assay of UDP-Glucuronic Acid Pyrophosphorylase. The standard assay procedure was as follows. Reaction mixtures (0.1 ml final volume) were placed in Kimble culture tubes (10 x 75 mm) and incubated 10 min at 30° C. Each complete reaction mixture contained 2 ml MgCl2, 2 mM UDP-glucuronic acid, 2 mM 32P-

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-glucuronic acid. Reactions were terminated with 10 μl of 50% (v/v) trichloroacetic acid, and the tubes were immediately placed in ice. One-tenth ml of 0.1 M Na2HPO4 (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, ~400g, 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) NH4OH, 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 32P-pyrophosphate substrate which was determined each time that samples of 32P-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: Mg2+ was 15 mM; 32P-PPi (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 NaPPi, pH 8.1; 0.1 ml Norit A (150 mg solids/ml H2O); (b) 3-min centrifugation at 400g; (c) two rinses of charcoal with 3-ml portions of 5% trichloroacetic acid and a 3-ml final rinse with distilled H2O; (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-glucuronic and 32P-PPi as substrates. The 32P-UTP produced by the enzyme is selectively adsorbed to charcoal. Interference from hydrolyses is minimized

values are given for reaction mixtures that were complete except for omission of UDP-glucuronic acid. The specific radioactivity is that of 32PPP 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.
Table I. Assay of ADP-glucose pyrophosphorylase and UDP-glucose pyrophosphorylase in crude extracts of developing maize endosperm

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Assay procedurea</th>
<th>Enzyme activityb</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. ADP-glucose pyrophosphorylase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. 3PGA absent</td>
<td>A</td>
<td>56.4(2)</td>
</tr>
<tr>
<td>b. 3PGA present</td>
<td>B</td>
<td>53.9(2)</td>
</tr>
<tr>
<td>2. UDP-glucose pyrophosphorylase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a.</td>
<td>A</td>
<td>265. (14)</td>
</tr>
<tr>
<td>b.</td>
<td>B</td>
<td>1,870 (10)</td>
</tr>
</tbody>
</table>

a) Procedure A: 32P-ATP or 32P-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-mg 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 phosphorlated substrates may also protect the 32P-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 UDP-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 PPi, 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 Mg2+ 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 glucurononokinase 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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