Metabolism of Uronic Acids in Plant Tissues

PARTIAL PURIFICATION AND PROPERTIES OF URONIC ACID OXIDASE FROM CITRUS LEAVES

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ABSTRACT

A new enzyme, named uronic acid oxidase, was extracted and purified 67-fold by (NH₄)₂SO₄, fractionation and CM-Sephadex column chromatography from ethylene-treated Shamouti orange (Citrus sinensis L. Osbeck) leaves. The enzyme catalyzes the oxidation of D-galacturonic acid and D-glucuronic acid to the corresponding hexaric acids in the presence of molecular oxygen with the production of H₂O₂. The pH optimum for the oxidation of D-galacturonic acid and D-glucuronic acid is between 7 and 8. The enzyme is highly specific for D-galacturonic acid and D-glucuronic acid. It also oxidizes polygalacturonic acid. The apparent Michaelis constant values of the enzyme for D-galacturonic acid and D-glucuronic acid are 0.13 and 0.5 mM, respectively. The molecular weight of the enzyme, as determined by gel filtration, is about 98,000. The enzyme is inhibited by sodium hydrosulfite and other sulfites, indicating that it contains a flavin prosthetic group.

MATERIALS AND METHODS

Plant Material. Six- to 12-month-old leaves of mature Shamouti orange (Citrus sinensis L. Osbeck) trees were cut at the abscission zone and placed in glasses containing a small amount of water. The leaves were treated for 3 days with 30 μl/l of ethylene as previously described (17).

Chemicals. The chemicals used in this work were obtained from the following sources: D-galacturonic acid, D-glucuronic acid, galactaric acid, D-glucaric acid, D-glucuronolactone, polygalacturonic acid, catalase (bovine liver), BSA, Sephadex G-200, CM-Sephadex C-50, and microcrystalline cellulose from Sigma Chemical Co.; D-glucuronic acid-U-¹⁴C, K salt (12.9 mCi/mMole) from The Radiochemical Centre, Amersham, England; peroxidase (horseradish) and Aquacide from Calbiochem; D-d-fumaric acid from Merck AG; a calibration kit for estimation of the mol wt of proteins by gel filtration from Pharmacia Fine Chemicals; and xyluronic acid and arabinuronic acid from Dr. K. O. Larsson. Methyl α-D-galactopyranuronate was synthesized by the method of Wood (22). All other chemicals were reagent grade commercial products.

Enzyme Purification. Sixty g of ethylene-treated leaves were homogenized in 500 ml of 50 mm potassium phosphate buffer, pH 7.4, containing 6% (w/v) (NH₄)₂SO₄, with a Waring Blender and then with an Ultra Turrax homogenizer. Except where noted, this and the subsequent steps were performed at 0 to 4 C. The homogenate was stirred for 30 min, filtered through a nylon fabric, and centrifuged for 10 min at 20,000g.

The supernatant was fractionated with solid (NH₄)₂SO₄, between the limits of 35 to 65% saturation. The precipitate was collected by centrifugation and dissolved in a small volume of 50 mm potassium phosphate buffer, pH 7. The protein solution was dialyzed first against 50 mm potassium phosphate buffer, pH 7 (dialysis I), and then against 30 mm potassium phosphate buffer, pH 6.2 (dialysis II).

The clarified dialyze was applied onto a 1.4 × 20 cm column of CM-Sephadex equilibrated with 30 mm potassium phosphate buffer, pH 6.2. After the column had been washed with 120 ml of the same buffer, the enzyme was eluted with a linear gradient consisting of 130 ml of 30 mm potassium phosphate buffer, pH 6.2, and 130 ml of 30 mm potassium phosphate buffer, pH 6.2, plus 0.27 M NaCl. This step was performed at room temperature. The enzyme was eluted at one point in the gradient. Active fractions were pooled and dialyzed against 50 mm potassium phosphate buffer, pH 7.4.

Enzyme Assays. Three different methods were used for determination of UAO activity according to the enzyme preparations used and the contents of the reaction mixtures.

Method A: Peroxidase-Chromogen Method. The method is

Free uronic acids generated within the plant by hydrolysis of uronic acid-rich polysaccharides or by hydrolysis of nucleotides are quickly metabolized; they are reduced, oxidized, de-carboxylated, or converted to cell wall polysaccharides (10). Tracer studies have demonstrated that plants are capable of oxidizing D-galacturonic acid and D-glucuronic acid to the corresponding hexaric acids, galactaric acid, and D-glucaric acid (9, 11), but the enzymes involved have not yet been investigated. The enzymic conversion of hexaric acids to hexaric acids has been studied in animals (13, 18) and especially in bacteria (3, 5, 23). Bacteria contain an enzyme, hexaric acid dehydrogenase, which catalyzes the NAD-linked oxidation of hexaric acids to hexaric acids. This reaction is the first step in the catabolism of uronic acids in bacteria (2, 4, 6).

Recently, in studying the role of polygalacturonase in abscission of citrus leaf explants (17), we observed that the enzymic preparations used contained an enzyme which oxidized the free galacturonic acid released by the polygalacturonase, as well as the free reducing groups of the substrate, sodium polyacrylate. Based on preliminary studies, the enzyme was named uronic acid oxidase (17). The present work was designed to isolate and characterize the enzyme which catalyzes the oxidation of uronic acids in citrus leaves.

1 This work was supported in part by a grant from the Bat-Sheva de Rothschild Fund for the Advancement of Science and Technology.
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Abbreviation: UAO: uronic acid oxidase.
based on the production of H₂O₂ during the enzymic reaction and its subsequent reaction with peroxidase and o-dianisidine (8). The standard 3-ml reaction mixture contained 3 mM uronic acid, 0.1 M potassium phosphate buffer, pH 7.4, 0.5 mM o-dianisidine (0.1 ml of a solution of 15 mM o-dianisidine in 95% ethanol), and 120 μg of peroxidase. After preincubation for 5 min at 30°C, the reaction was initiated by adding enzyme solution. The reaction mixtures were incubated for 10 min, and the increase in absorbance was then determined at 420 nm on a Zeiss PMQ II spectrophotometer. Zero absorbance was set with a mixture without enzyme and substrate. Reagent blanks and enzyme blanks were included in each assay. The reagent blank contained buffer instead of enzyme, and the enzyme blank contained water instead of substrate. One unit of activity was defined as that amount of enzyme yielding an increase of 1 A₄₁₂ in 1 min under the conditions described above.

**Method B.** This method is based on measuring the decrease in reducing groups due to oxidation of the substrate. The standard reaction mixture in a final volume of 1 ml contained 3 mM uronic acid and 0.1 M potassium phosphate buffer, pH 7.4. After preincubation for 5 min at 30°C, the reaction was initiated by adding enzyme solution. The reaction mixtures were incubated for 30 to 60 min. A reaction mixture to which active enzyme was added at the end of the incubation time served as a blank. The decrease in reducing groups was measured with the dinitrosalicylic acid reagent (16), using D-galacturonic acid or D-glucuronic acid as a standard. This reagent was preferred to the Somogyi-Nelson reagent (19) because it was much less affected by the contents of the reaction mixtures. One unit of activity was defined as that amount of enzyme causing a decrease of 1 μmole uronic acid in 1 hr under the specified reaction conditions.

**Method C: Manometric Method.** The method employed conventional Warburg manometric procedures for the determination of O₂ consumption. The standard reaction mixture in a final volume of 2 ml contained 3 mM uronic acid, 0.1 M potassium phosphate buffer, pH 7.4, and 200 μg of catalase; 0.2 ml of 20% KOH was placed in the center well. The enzyme was added from the side arm after equilibration at 30°C. Results were calculated as μmoles of O₂ consumed per 1 hr.

**Analytical Techniques.** Sugar acids were analyzed by electrophoresis, using thin layer plates coated with 0.25 mm of microcrystalline cellulose. Electrophoresis was carried out in 0.2 M ammonium acetate buffer, pH 5.8, for 70 min at 4°C and 10 V/cm.

Table I. Purification of UAO from Citrus Leaves

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Volume (ml)</th>
<th>Protein (mg)</th>
<th>Total Units¹</th>
<th>Specific Activity</th>
<th>Recovery %</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>475</td>
<td>893</td>
<td>312</td>
<td>0.35</td>
<td>—</td>
<td>1</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>31.5</td>
<td>456</td>
<td>402</td>
<td>0.88</td>
<td>100</td>
<td>2.5</td>
</tr>
<tr>
<td>after dialysis I</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dialysis II</td>
<td>31.5</td>
<td>365</td>
<td>384</td>
<td>1.05</td>
<td>95</td>
<td>3</td>
</tr>
<tr>
<td>CM-Sephadex</td>
<td>40</td>
<td>5.3</td>
<td>125</td>
<td>23.59</td>
<td>31</td>
<td>67</td>
</tr>
</tbody>
</table>

¹ One unit equals a decrease of 1 μmole of D-galacturonic acid in 1 hr.

² Protein and UAO activity in the crude extract were determined in a dialyzed sample.

³ Enzyme activity was found to be relatively low in the crude extract. Calculations of enzyme yield are based on the activity of the (NH₄)₂SO₄ fraction.

Various types of sugars were analyzed by chromatography on thin layer plates coated as above in ethyl acetate-acetic acid-water (2:1:2, v/v) (solvent A) and in ethyl acetate-pyridine-water (2:1:2, v/v) (solvent B).

Sugar analyses were performed with either benidzine (7) or silver nitrate (21) reagents, or both. C-labeled radioactive compounds were located by means of a Packard radiochromatogram scanner, Model 7200.

Molecular weight determination was carried out on a calibrated Sephadex G-200 column (2.5 × 40 cm) prepared according to the directions of Pharmacia Fine Chemicals. The following mol wt standards were used to construct a calibration curve: aldolase; ovalbumin; chymotrypsinogen A; and ribonuclease A. Elution was performed with 50 mM potassium phosphate buffer, pH 7.4, containing 0.1 M NaCl.

Protein was determined by either the method of Lowry et al. (12) for specific activity measurements, or by recording the A₄₁₂ for column chromatography effluents.

**RESULTS**

**Enzyme Purification.** A summary of the data on the partial purification of citrus UAO is presented in Table I. A 67-fold purification of the enzyme with a total recovery of 31% was obtained.

UAO activity in freshly excised citrus leaves was found to be extremely low, but it markedly increased after treatment with ethylene. Therefore, ethylene-treated leaves were used as the source of UAO.

Although we have no evidence that UAO is a bound enzyme, the addition of salt to the extraction buffer was found to be useful. Six percent (NH₄)₂SO₄ in the extraction buffer increased the amount of UAO that was extracted by 30 to 40% as compared to buffer alone.

The procedure used for purification of UAO gave an almost colorless enzyme preparation which was free from catalase activity and thus suitable for studying UAO characteristics by the peroxidase-chromogen method. In all the experiments to be described, purified preparations of the specific activity reported in Table I or comparable activity were used.

**Assay of UAO.** In the course of the present work, we faced several problems in assaying UAO in the various enzyme preparations studied. Some of the inhibitors tested also caused some difficulties by affecting the reagents employed for assaying UAO activity. Therefore, three different methods were used for measuring the activity of the enzyme. Method B was the only method that could be used with all the enzyme preparations studied. However, it was not possible to use this method in the presence of certain inhibitors (KCN and diethylidithiocarbamate) which affected the dinitrosalicylic acid reagent. The manometric method gave variable results with the crude and the (NH₄)₂SO₄ preparations, probably because of the presence of an unknown factor which interfered with the determination of O₂ uptake. This method was used only with purified enzyme preparations to confirm results obtained with other methods or to study the effect of certain inhibitors. The peroxidase-chromogen method was found to be the most sensitive method and it was used whenever possible. The use of this method was, however, limited to purified preparations for the reasons mentioned above, and to each reaction mixture contents which did not affect peroxidase activity or the colored product formed during the reaction.

The dependence of the oxidation of D-galacturonic acid on time and on enzyme concentration was studied with the peroxidase-chromogen method. The reaction was linear for at least 18 min and up to 48 μg of protein in the 3-ml reaction mixture. In all subsequent experiments, rate-limiting quantities of
enzyme were used, and the amount of activity was measured during the first 10 min. Whenever the other assay methods were used, the linearity of the reaction was determined.

Identification of the Reaction Products. Chromatographic analyses suggested that the hexuronic acids are oxidized to the corresponding hexaric acids. Because the two groups of sugar acids have similar R<sub>f</sub> values, the reaction products were further analyzed by thin layer electrophoresis.

Reaction mixtures containing 0.8 µCi D-glucuronic acid-U-<sup>14</sup>C, 3 mM unlabeled D-glucuronic acid, 0.1 M tris-HCl, pH 7.4, and purified enzyme in a total volume of 1 ml were incubated for various periods at 30 C. Ten-µl samples of the above reaction mixtures and 20 µg of D-glucuronic acid and D-glucaric acid were spotted at one side of the thin layer plates and electrophoresed. The plates were then sprayed with the periodate-benzidine reagent and scanned for radioactivity. Figure 1 shows that the oxidation of glucuronic acid yielded a single product which migrated precisely with D-glucaric acid. D-Glucaric acid was the only product which was detected in all the reaction mixtures analyzed. The identity of the product of glucuronic acid oxidation was further confirmed by its reduction with sodium borohydride to the corresponding hexitol according to the procedure of Kessler et al. (9). The radioactive hexitol obtained was identical with D-glucitol upon co-chromatography (solvent B), confirming that the product was D-glucaric acid.

In a similar experiment, unlabeled D-galacturonic acid, 5 mM, was substituted for glucuronic acid in a reaction mixture otherwise identical to the one described above. The reaction was allowed to proceed until all galacturonic acid had been oxidized. Then the products of the reaction were analyzed by thin layer electrophoresis as above. After spraying with the periodate-benzidine reagent, galactaric acid was identified as the product of D-galacturonic acid oxidation by comparing its electrophoretic mobility to that of authentic galactaric acid.

The formation of H<sub>2</sub>O<sub>2</sub> during the reaction was demonstrated by the peroxidase-catalyzed oxidation of o-dianisidine to a colored product.

**ENZYME PROPERTIES**

**Stability.** The (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> fraction can be stored at −16 C for at least 3 weeks without apparent loss of activity. The purified enzyme preparation was somewhat less stable, but retained full activity for at least 3 weeks at −16 C after the addition of 100 µg/ml BSA.

**pH Activity Curve.** Maximum oxidation of D-galacturonic acid and D-glucuronic acid by UAO, assayed by the peroxidase-chromogen method, occurred between pH 7 and 8 (Fig. 2). These results were confirmed by the manometric method, using galacturonic acid as a substrate. A pH of 7.4 was selected as the standard pH for the other experiments.

UAO obtained from 3- to 5-month-old leaves showed two pH optima for the oxidation of both uronic acids; one between 7 and 8 and the other between 9.8 and 11. This may suggest the presence of two UAO isozymes in relatively young leaves.

**Substrate Specificity.** A number of sugars and sugar acids were tested as substrates for UAO with the peroxidase-chromogen method. At substrate concentration of 0.5 mM, only D-galacturonic acid, D-glucuronic acid, and D-glucuronolactone were oxidized at detectable rates (Table II). At this concentration the highest activity was obtained with galacturonic acid, the rates of activity with glucuronic acid and glucuronolactone being 72 and 9%, respectively, the rate of galacturonic acid activity. Polylgalacturonic acid was also oxidized. The polygalacturonic acid was dialyzed before use until chromatographically (solvent A) free from galacturonic acid. No significant activity was obtained with the penturonic acids, arabinuronic acid and xyluronic acid, when assayed at a concentration of 1.5 mM and

![Graph](image-url)

**Fig. 1.** Identification of the reaction products of the enzymic oxidation of D-glucuronic acid-U-<sup>14</sup>C by thin layer electrophoresis. A: analysis of a control reaction mixture incubated for 20 min; B: analysis of a reaction mixture incubated for 20 min. GlcUA: D-glucuronic acid; GlcA: D-glucaric acid.

![Graph](image-url)

**Fig. 2.** Effect of pH on the oxidation of D-galacturonic acid (-----) and D-glucuronic acid (---) by UAO. The enzyme solution was dialyzed before use against 5 mM potassium phosphate buffer, pH 7.4. Activity was assayed by the standard peroxidase-chromogen method, except that the pH of the reaction mixtures were adjusted to the indicated pH. Buffers were: Δ: citrate-phosphate; •: phosphate; ○: boric acid-KCl-NaOH; ▲: phosphate-KOH.
Table II. Substrate Specificity of UAO

All substrates, except polygalacturonic acid, were tested at a concentration of 0.5 mM. The concentration of polygalacturonic acid was 0.2%. Activity was determined with the standard peroxidase-chromogen method. The activity with D-galacturonic acid was 1.85 units/mg protein.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Galacturonic acid</td>
<td>1.00</td>
</tr>
<tr>
<td>D-Glucuronic acid</td>
<td>0.72</td>
</tr>
<tr>
<td>Polygalacturonic acid</td>
<td>0.22</td>
</tr>
<tr>
<td>D-Glucuronolactone</td>
<td>0.09</td>
</tr>
<tr>
<td>Methyl a-D-galactopyranuronate</td>
<td>0</td>
</tr>
<tr>
<td>D-Galactose</td>
<td>0</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>0</td>
</tr>
<tr>
<td>Xyluronic acid</td>
<td>0</td>
</tr>
<tr>
<td>Arabinuronic acid</td>
<td>0</td>
</tr>
<tr>
<td>D-Xylose</td>
<td>0</td>
</tr>
<tr>
<td>D-Arabinose</td>
<td>0</td>
</tr>
</tbody>
</table>

Fig. 3. Effect of D-galacturonic acid concentration on UAO activity. Activity was assayed by the standard peroxidase-chromogen method, except that the concentration of galacturonic acid was changed as indicated. Inset is a Lineweaver-Burk plot of the data.

Fig. 4. Effect of D-glucuronic acid concentration on UAO activity. Activity was assayed by the standard peroxidase-chromogen method, except that the concentration of glucuronic acid was changed as indicated. Inset is a Lineweaver-Burk plot of the data.

Fig. 5. Estimation of the mol wt of UAO on a calibrated Sephadex G-200 column. The enzyme source was an ammonium sulfate fraction concentrated in a dialysis bag against Aquacide. The mol wt standards were: 1: aldolase (158,000); 2: ovalbumin (45,000); 3: chymotrypsinogen A (25,000); 4: ribonuclease A (13,700).

Table III. Inhibition of UAO by Sulfites

UAO activity was assayed by method B under standard conditions, using D-galacturonic acid as a substrate.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₂S₂O₃</td>
<td>μM</td>
<td>%</td>
</tr>
<tr>
<td>Na₂SO₄</td>
<td>20</td>
<td>30</td>
</tr>
<tr>
<td>Na₂SO₃</td>
<td>30</td>
<td>64</td>
</tr>
<tr>
<td>NaHSO₃</td>
<td>100</td>
<td>65</td>
</tr>
<tr>
<td>NaHSO₄</td>
<td>30</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>78</td>
</tr>
</tbody>
</table>

Method, was observed with 1 mM KCN and 1 mM sodium diethylthiocarbamate, ruling out the possibility that the enzyme contains a metal in its active site. Catalase was omitted from the reaction mixtures when the effect of KCN was tested.
DISCUSSION

The present paper reports the presence of a new enzyme, UAO, in citrus leaves. From the identification of the reaction products of UAO, it is concluded that the enzyme catalyzes the oxidation of D-galacturonic acid and D-glucuronic acid to the corresponding hexaric acids, galactaric acid and D-glutaric acid, in the presence of molecular oxygen, which serves as an electron acceptor, with the production of $\text{H}_2\text{O}_2$. The overall reaction can be summarized as follows:

$$\text{hexuronic acid} + \text{H}_2\text{O} + \text{O}_2 \rightarrow \text{hexaric acid} + \text{H}_2\text{O}_2$$

The UAO is highly specific for galacturonic acid and glucuronic acid (Table II) and may be regarded as hexuronic acid oxidase. In bacteria, the oxidation of hexuronic acids is catalyzed by a different enzyme, hexaric acid dehydrogenase, through a NAD-linked reaction (3, 5).

The inhibition of citrus UAO by sulfites (Table III) indicates that the enzyme possesses a flavin prosthetic group. The effects of sodium hydrosulfite (dithionite) on flavoprotein enzymes, such as glucose oxidase, have been thoroughly investigated (14). Hydrosulfite, acting as a reducing compound, brings about the formation of a semiquinoid form of the enzymes which fails to react with the substrate. Sodium sulfite and sodium bisulfite were also found to inhibit flavoprotein oxidases (15, 20), but the inhibitory effect of these sulfites cannot be classed as a simple reduction of the enzymes because reduced enzymes react rapidly with $\text{O}_2$, whereas enzymes bleached with sulfites were found not to react at all with $\text{O}_2$. It has been suggested (15, 20) that sulfite ions are bound at the active site of the enzymes, probably by forming a complex with the flavin prosthetic group. Inhibition by sulfites occurs with flavoprotein oxidases, but not with flavoprotein dehydrogenases (15). Although the nature of the inhibition of citrus UAO by the various sulfites has not yet been investigated, the data in Table III indicate that it is probably a flavoprotein oxidase.

The function of UAO in the metabolism of the plant is yet unclear. It might be that the UAO represents a catabolic enzyme and the oxidation of galacturonic acid and glucuronic acid generated by hydrolysis of polysaccharides is, as in bacteria (2, 4, 6), the first step in the utilization of uronic acids as a source of carbon. Bacteria convert uronic acids to various organic acids, including $\alpha$-ketoglutaric acid (6) and pyruvic acid (4). Since the enzyme was found to oxidize polygalacturonic acid, it might also be that the natural substrate is a polysaccharide containing terminal uronic acid residues. However, the fact that ethylene stimulates the activity of polysaccharide-hydrolyzing enzymes (1, 17), as well as the activity of UAO and the observations that plants contain hexaric acids as natural products (9, 11) support our first suggestion. The physiological role of UAO and the hormonal control of its activity will be further studied.

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LITERATURE CITED