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IDENTIFICATION OF URONIC ACID OXIDASE IN PLANT PEROXIDASE PREPARATIONS

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Key Word Index—peroxidase; uronic acid oxidase; glucuronate oxidation.

Abstract—Commercial plant peroxidase preparations contained a uronic acid oxidase, separable from the peroxidase activity by ion exchange chromatography. The partially purified enzyme, devoid of peroxidase, oxidized hexuronic acids, with the greatest activity for D-glucuronic acid, whereas other aldoses were not substrates. The immediate products of reaction of D-glucuronic acid with oxygen were hydrogen peroxide and a D-glucarolactone, which was a very strong inhibitor of β-glucuronidase and believed to be the 1,5-lactone. The sensitivity to sulphite inhibition suggests that the enzyme is a flavoprotein.

INTRODUCTION

D-Glucaric acid is an end product of mammalian metabolism; it is derived from D-glucuronic acid and excreted in the urine [1]. Whilst the free carbohydrate has not been identified in plants, esters of glucaric acid, e.g. cestric acid from leaves of *Cestrum euanthes* [2], have been isolated.

Tracer studies [3] established the direct conversion of glucuronic acid to glucaric acid by plant extracts. No added cofactor was required, as in analogous enzymatic reactions in mammalian [4] and bacterial [5] metabolism, where NAD appears to be obligatory for oxidation of the reducing group of the free sugar.

In model experiments designed to simulate possible peroxisome involvement in glucaric acid formation, it was found that commercial peroxidase preparations from horse-radish (*Armoracia rusticana*) produced glucaric acid from glucuronic acid without the required presence of hydrogen peroxide as an oxidant. It appeared probable that an aerohydrogenase was present, possibly similar to sugar oxidases previously reported in *Citrus sinensis* fruit and leaves [5, 6], and after satisfactory separation from the main peroxidase activity, the general properties of this enzyme and the nature of the reaction were examined.

RESULTS AND DISCUSSION

All the commercial specimens of horse-radish peroxidase which were examined contained some uronic acid oxidase activity, diminishing with the purity of the preparation. Typically, cruder preparations had ratios of enzyme activities (peroxidase:uronic acid oxidase), measured by D-glucaric acid formation, of $5–10 \times 10^3$:1, whereas for the purest peroxidase sample (Boehringer Grade 1) this ratio was 9.3×10^4:1.

Using cation exchange chromatography, it was found possible to separate the peaks of the two enzymes with a minimal overlap (Fig. 1), and preparations of the oxidase were thereby obtained devoid of peroxidase activity. By this procedure, the specific activity of the uronic acid oxidase fraction increased from 0.095 to 0.82 nkat/mg protein, thus achieving a purification of 8.6, with an enzyme recovery of 78%. This partially purified product was used for all subsequent examinations of the properties and mode of action of the enzyme.

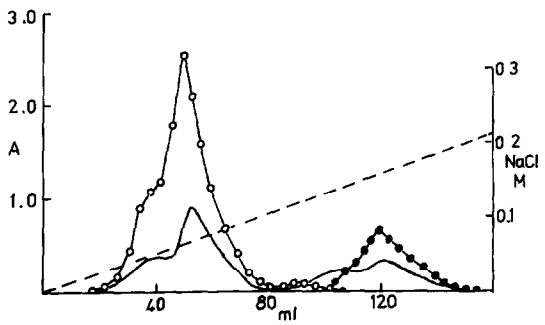

Fig. 1. Separation of peroxidase and uronic acid oxidase by cation exchange chromatography: ○, A_{403} (peroxidase); ●, A_{420} (uronic acid oxidase by assay); ——, A_{280} (protein).

Enzyme properties

Stability. Aqueous solutions of the enzyme at pH 6–8 could be stored at + 3° for 48 hr without apparent loss of activity, but suffered up to 25% loss after 10 days under these conditions.

pH Optimum. Maximal activity with D-glucuronic acid as substrate, assayed by measurement of glucarate production, was at pH 7.9 (with half-maximal activities at pH 5.3 and 9.2), and this value was selected for routine assay at 30°, for the glucarate production, manometric determination of oxygen consumption and chromogen–peroxidase methods. Production of hydrogen peroxide (0–50 μM) at this pH was linear with time for up to 2 hr. A 30 min incubation period and 20 mM substrate concentration were routinely used.

Molecular weight. Gel filtration on a calibrated column gave an M_r of *ca* 42 000. This is similar to that of some isoenzymes of horse-radish peroxidase, and the uronic

acid oxidase in crude peroxidase preparations could not be separated by this technique.

Substrate specificity. Only uronic acids were found to be oxidized (Table 1). D-Glucuronolactone was apparently a substrate, but the enzyme activity towards it, relative to D-glucuronic acid oxidation, decreased sharply when the pH was reduced below 7, i.e. where the lactone was more stable. It is thus probable that this compound only becomes a substrate after hydrolysis to D-glucuronic acid.

Using the conventional double reciprocal plot method [7], values obtained for the K_m were: D-glucuronic acid, 1.7 mM; D-galacturonic acid, 2.2 mM. Insufficient material was available to obtain a K_m value for D-mannuronic acid.

In assays using brief incubation periods (< 10 min) and larger amounts of enzyme, no detectable difference in enzyme activity was found using fresh solutions of β-D-glucuronic acid or those which were kept previously at 20° for 24 hr, when equilibrium had been attained between α- and β-anomers by mutarotation. The enzyme thus showed no observable preference for either anomer.

The following reducing sugars were also tested, at 20 mM concentration, but were found not to be substrates: D-glucose, 2-deoxy-D-glucose, D-galactose, D-mannose, D-glucosamine, *N*-acetyl-D-glucosamine, D-galactosamine, *N*-acetyl-D-galactosamine, D- and L-arabinose, D-xylose, D-lyxose, D- and L-fucose, D-glucoheptose, maltose, cellobiose, sophorose and melibiose.

Inhibition by sulphites. The amount of partially purified enzyme available precluded a direct investigation of possible prosthetic groups or transition metals at the active site, but indirect evidence given by inhibitors strongly suggested that the uronic acid oxidase was a flavoprotein. No inhibition of enzyme activity [determined by assay of glucarate formation, method (i)] was observed in the presence of typical peroxidase inhibitors such as azide, fluoride or cyanide, all at 1 mM concentration. Thus the oxidase almost certainly does not have a haemoprotein structure. However, strong inhibition of glucuronate oxidation, measured by chromogen–peroxidase assay of peroxide formation, occurred in the presence of low concentrations of various sulphites (Table 2). This is typical of flavoprotein oxidases, whereas flavoprotein dehydrogenases are unaffected by sulphites [8].

Stoichiometry of the reaction and nature of the carbohydrate product

Of the reactants involved in the uronic acid oxidase system, three were assayed to determine the overall stoichiometry. Table 3 shows that for each molecule of glucarate generated, there was a concomitant release of one molecule of hydrogen peroxide and a loss of one molecule of oxygen; the stoichiometry of the last two components were substantiated by the effect of adding catalase in the manometric assay. It was also noted that no formation of glucarate or peroxide occurred when the reaction was conducted in an atmosphere of nitrogen.

Oxidation of D-glucuronate was found to proceed in the presence of electron acceptors other than oxygen. Reduction of ferricyanide (0.5 mM) was followed by the change in A at 420 nm, and of dichlorophenolindophenol (0.05 mM) at 600 nm. With 20 mM D-glucuronate as substrate, activities (nmol/min/mg protein) were: utilization of O_2, 46; reduction of ferricyanide, 519; reduction of dichlorophenolindophenol, 117.

Although production of D-glucarate was confirmed by two independent methods of assay (Table 3), this compound was not the immediate oxidation product of D-glucuronate. On addition of an aliquot of the reaction mixture, after incubation and deproteinization but without further treatment, to a β-glucuronidase assay, very strong inhibition of this enzyme was observed. The inhibition was found to be fully competitive [7]. D-Glucuronate is only a weak inhibitor of β-glucuronidase and D-glucarate is non-inhibitory, but the 1,4-lactone of the latter compound is a very specific and powerful inhibitor of this enzyme [9]; its formation from glucarate at low pH is indeed the basis of one method of glucarate assay used here. The immediate formation of a lactone

Table 1. Relative activities of the uronic acid oxidase with different substrates (20 mM) at pH 7.9 and pH 6.0, by determination of H_2O_2 formation

Substrate	Enzyme activity pH 7.9	pH 6.0
D-Glucuronate	100	70
D-Galacturonate	31	23
D-Mannuronate	36	27
D-Glucuronolactone	49	15

Table 2. Inhibition of uronic acid oxidase by sulphites

Inhibitor	Concn (μM)	Inhibition (%)
$S_2O_4^{2-}$	100	97
$S_2O_4^{2-}$	10	59
$S_2O_5^{2-}$	100	98
$S_2O_5^{2-}$	10	63
SO_3^{2-}	100	80
SO_3^{2-}	10	25
Ascorbate	1000	0
Azide	1000	0
Cyanide	1000	0
Sulphide	1000	2

Substrate 20 mM glucuronate at pH 7.9; assay by measurement of D-glucarate [method (ii)].

Table 3. Stoichiometry of the enzymatic D-glucuronic acid (20 mM) oxidation at pH 7.9 and pH 6.0

Parameter	Enzyme activity (nkat/mg protein) pH 7.9	pH 6.0
Glucarate formation [method (i)]	0.85	0.60
Glucarate formation [method (ii)]	0.78	0.54
Hydrogen peroxide formation	0.82	0.58
Lactone formation	0.40	0.55
O_2 consumption	0.76	0.57
O_2 consumption (+catalase)	0.39	0.28

product in the uronic acid oxidase reaction was confirmed by conversion to hydroxamic acid and colorimetric assay. At optimal pH 7.9, the amount of lactone formation (with D-glucaro-1,4-lactone as arbitrary standard) was less than the amount of glucarate determined (Table 3), but this could be predicted as sugar lactones hydrolyse spontaneously above pH 7. Moreover, the chromophore formed might be quantitatively different in colour yield from that produced by the standard. At pH 6.0, formation of lactone was equivalent to that of glucaric acid.

That this product from glucuronic acid was not glucaro-1,4-lactone, however, was demonstrated by the fact that, based on the inhibitory power towards β-glucuronidase, the concentration of the 1,4-lactone required, by comparison with a standard inhibition curve using this compound, would be considerably greater than the measured concentration of either lactone or glucarate produced (Fig. 2). Thus the lactone reaction product had a greater inhibitory power (about 2-fold) than that of D-glucaro-1,4-lactone.

Confirmation of this difference in the lactone product was obtained by thin layer chromatography, based on the visualization of β-glucuronidase inhibitors. The reaction mixture from uronic acid oxidase produced spots for glucuronate (R_f 0.45), glucarate (R_f 0.49) due to trace inhibitory lactone formation in the presence of the pH 4.5 buffer, and a major inhibitory spot at R_f 0.59. A glucaro-1,4-lactone standard gave a single spot at R_f 0.68.

In the absence of direct evidence concerning the structure of this compound, isolation of which would be difficult due to its lability at neutral pH, it can be conjectured that the immediate product of glucuronate oxidation is D-glucaro-1,5-lactone, and that the complete reaction, which would fit the observed stoichiometry, is a 2e oxidation analogous to that of glucose oxidase, as shown in Fig. 3.

The existence of this 1,5-lactone of D-glucaric acid has been deduced in products of glucuronic acid oxidation by oxygen in the presence of a platinum catalyst [10]; again it was found to be a more potent inhibitor of β-glucuronidase than the 1,4-lactone. It was suggested [10] that the former lactone, having a ring structure more analogous to the pyranosiduronic acid structure of β-glucuronidase substrates, was the only true glucaro-lactone inhibitor of this enzyme, being formed from the

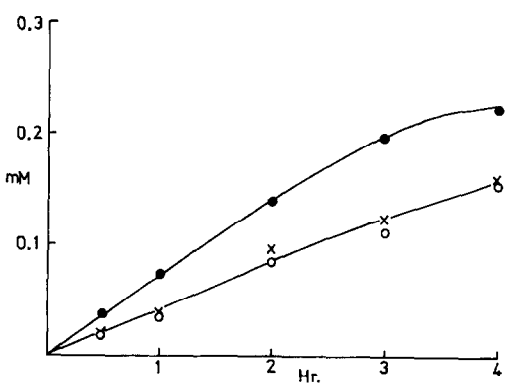

Fig. 2. Products of uronic acid oxidase action on 10 mM D-glucuronate at pH 6 0: ×, D-glucarate; ○, lactone formation; ●, production of β-glucuronidase inhibitor, calculated as equivalent D-glucaro-1,4-lactone concentration.

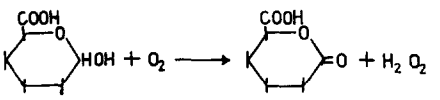

Fig. 3.

1,4-lactone in solution without mandatory initial opening of the lactone ring. Our results do not support this concept; TLC as above indicated that the only inhibitory product on heating D-glucarate solutions at acid pH and 100° was the 1,4-lactone, which presumably has the greater stability. It was noted, however, that unbuffered solutions (pH 4) of potassium hydrogen D-glucarate on standing at 37° for a few hr did produce traces of the hypothetical 1,5-lactone on TLC.

This uronic acid oxidase present in impure commercial plant peroxidase preparations differs from the enzyme obtained from *Citrus sinensis* leaves [6] in having a much smaller M_r and a greater activity towards glucuronate than towards galacturonate; also only the corresponding hexaric acids were identified as products of action of the *Citrus* enzyme. The aldose oxidase [5] from fruits of the same source had no action on uronic acids, although these were strong inhibitors. The physiological function of the horse-radish uronic acid oxidase is unclear; incubation of glucarate with the enzyme resulted in 94% recovery of that compound, but investigations as to whether the lactone product or D-glucarate is further metabolized by whole plant preparations are clearly needed.

EXPERIMENTAL

Peroxidase preparations. Commercial preparations of horse-radish peroxidase were obtained from Sigma (crude, 40 units/mg and Type 1, 80 units/mg, both lyophilized), Boehringer Mannheim (Grade 1, suspension in $AmSO_4$, 250 units/mg protein and Grade 2, lyophilized, 100 units/mg), Calbiochem (lyophilized, 250 units/mg) and British Drug Houses (lyophilized, 80 units/mg). These manufacturers' quoted approximate activities are in Sumner purpurogallin units [11].

β-Glucuronidase (EC 3.2.1 31). The mammalian enzyme was prepared from rat preputial gland (Wistar strain) and partially purified by gel chromatography on Sephadex G-100 [12].

Bacterial enzyme preparation. This contained both glucarate dehydratase (EC 4.2.1.40) and ketodeoxyglucarate aldolase (EC 4.1.2.20), and was prepared from a culture of *E. coli* (strain NCTC 10418) by the procedure of ref. [13].

Substrates β-D-Glucuronic acid, D-glucuronolactone and D-galacturonic acid were obtained from Sigma D-Mannuronic acid was prepared by catalytic oxidation of methyl α-D-mannoside followed by hydrolysis of the mannosiduronic acid [14]. Each of these compounds was tested for purity by paper chromatography. Other sugars were obtained commercially.

D-Glucaro-1,4-lactone monohydrate, mp 89–92°, was prepared from potassium hydrogen D-glucarate (Sigma) by the method of ref. [15].

Uronic acid oxidase assay. Enzyme preparations, freed of peroxidase activity, were incubated with 20 mM uronic acid in 0.05 M Tricine buffer, pH 7.9, at 30° for 30 min (total vol. 5 ml). Shaking was not found to be necessary. Solutions were then chilled in ice for 1 min and immediately deproteinized by centrifugation through a Centriflo membrane cone (Amicon, 25 000 M_r cut-off). Aliquots of the ultrafiltrate were then assayed for the following products.

(a) *Hydrogen peroxide* This was determined spectrophoto-

metrically at 420 nm, using *o*-dianisidine as the chromogen [16]. Purified peroxidase preparations freed of uronic acid oxidase activity by ion-exchange chromatography were used as the catalyst.

(b) *Glucaric acid.* The ultrafiltrate was heated at 100° for 5 min, to convert the lactone product to D-glucaric acid, which was then assayed by two independent methods: (i) by conversion to ketodeoxyglucarate, then to pyruvate, by the bacterial enzyme preparation, followed by assay using lactate dehydrogenase and NADH [17]; (ii) by inhibition of β-glucuronidase after partial conversion to D-glucaro-1,4-lactone by the procedure of ref. [18]. Assays of this enzyme were conducted using 2 mM *p*-nitrophenyl β-D-glucuronide (Merck) as substrate.

(c) *Lactone formation.* By conversion to hydroxamate with hydroxylamine and colorimetric assay of the ferric hydroxamate at 540 nm [19]. D-Glucaro-1,4-lactone was used to construct the standard curve for this estimation.

Peroxidase assay. Determination of peroxidase activity was by the procedure of ref. [20], using guaiacol (Sigma) as the chromogen and determination of the rate of *A* change at 436 nm.

Manometric assay. Measurement of oxygen uptake during the uronic acid oxidase action was made with the Warburg manometer at 30°, with an enzyme concn of 0.5 mg protein/ml and a total flask vol. of 3 ml.

Ion exchange chromatography. Enzyme separation of crude peroxidase preparations (50 mg, Boehringer, Grade 2) was performed on a CM cellulose (Sigma) column (35 × 1.5 cm) equilibrated with 0.05 M cacodylic acid–NaOH buffer, pH 6.3. Elution was with the same buffer plus a salt gradient of 0–0.3 M NaCl, at a flow rate of 6 ml/hr

Gel chromatography. M_r of the uronic acid oxidase was determined by chromatography on a AcA 44 Ultrogel column (82 × 1.6 cm) equilibrated and eluted with Tris–HCl buffer (pH 6.5, $I = 0.05$ M) and calibrated with myoglobin (16 800), carbonic anhydrase (31 000), ovalbumin (44 000) and bovine serum albumin (68 000). The calibration proteins were detected by their *A* at 280 nm and the uronic acid oxidase by assay of its activity.

Thin layer chromatography. Examination of the carbohydrate product of enzymatic glucuronic acid oxidation was made on silica gel (Gelman ITLC Type SG). Before application, the soln was briefly shaken with a little Dowex 50(H) resin to remove cations. Development was with the top layer of the system *n*-BuOH–EtOH–HCOOH–H_2O (4:1:1:5) which had equilibrated at 20° for 48 hr [21]. The dried plates were then sprayed with 4-methylumbelliferone β-D-glucuronide (Merck, 0.1 mg/ml) in 0.02 M acetate buffer, pH 5, then with a β-glucuronidase preparation and incubated at 30° for 5 min. β-Glucuronidase inhibitors were then visualized in an atmosphere of NH_3 vapour under a UV source as dark spots on a blue fluorescent background.

Protein determination, with bovine serum albumin as standard, was by the method of ref. [22].

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REFERENCES

1. Marsh, C. A. (1963) *Biochem. J.* **86**, 77.
2. Nagels, L. U. C., van Dongen, W. and Parmentier, F. (1982) *Phytochemistry* **21**, 743.
3. Kessler, G., Neufeld, E. F., Feingold, D. S. and Hassid, W. Z (1961) *J. Biol. Chem.* **236**, 308.
4. Marsh, C. A. (1963) *Biochem. J.* **89**, 109.
5. Bean, R C., Porter, G G. and Steinberg, B. M. (1961) *J. Biol. Chem* **236**, 1235.
6. Riov, J. (1975) *Plant Physiol.* **55**, 602.
7. Lineweaver, H. and Burk, D. (1934) *J. Am. Chem. Soc.* **56**, 608.
8. Muller, F. and Massey, V. (1971) in *Methods in Enzymology*, Vol. XVIIIB, p. 468.
9. Levvy, G. A. (1952) *Biochem. J.* **52**, 464
10. Conchie, J., Hay, A. J., Strachan, I. and Levvy, G. A. (1967) *Biochem. J.* **102**, 929.
11. Sumner, J. B. and Gjessing, E. C. (1943) *Arch. Biochem.* **2**, 291.
12. Snaith, S. M. and Levvy, G. A. (1967) *Biochim. Biophys. Acta* **146**, 599.
13. Blumenthal, H. J. (1966) in *Methods in Enzymology*, Vol. IX, p. 660.
14. Marsh, C. A. (1952) *J. Chem. Soc.* 1578.
15. Bose, R. J., Hullar, T. L., Lewis, B. A. and Smith, F. (1961) *J. Org. Chem.* **26**, 1300.
16. Bernt, E. and Bergmeyer, H. U. (1974) in *Methods in Enzymatic Analysis* (Bergmeyer, H. U., ed.) 2nd edn, Vol. 4, p. 2246. Academic Press, New York.
17. Marsh, C. A. (1985) *Analyt. Biochem.* **145** (in press).
18. Simmons, C. J., Davis, M., Dordoni, B. and Williams, R. (1974) *Clin. Chim. Acta* **51**, 47.
19. Kawada, M., Takiguchi, H., Kagawa, Y., Suzuki, K. and Shimazono, N. (1962) *J. Biochem. (Tokyo)* **51**, 405.
20. Putter, J. (1974) in *Methods in Enzymatic Analysis* (Bergmeyer, H. U., ed.) 2nd edn, Vol. 2, p. 685. Academic Press, New York.
21. Ishidate, M., Matsui, M. and Okada, M. (1965) *Analyt. Biochem.* **11**, 176.
22. Lowry, O. H., Rosebrough, N. J., Farr, A L. and Randall, R J. (1951) *J. Biol Chem.* **193**, 265.