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CONVENIENT, LABORATORY PROCEDURE FOR PRODUCING SOLID D-*arabino*-HEXOS-2-ULOSE (D-GLUCOSONE)

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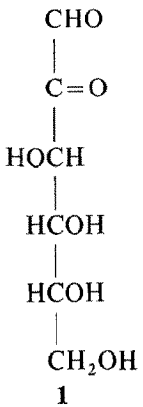
ABSTRACT

The production of solid D-*arabino*-hexos-2-ulose (D-glucosone) from D-glucose by use of an enzyme, pyranose-2-oxidase (EC 1.1.3.10), is described. The enzyme is extracted from the mycelia of *Polyporus obtusus*, partially purified, and then immobilized on activated CH-Sepharose 4B. The enzymic conversion of D-glucose into D-glucosone is simple and convenient, and provides a product free from residual D-glucose. Lyophilization of the filtered reaction-solution yields the product, solid D-glucosone. Assay methods have been developed for monitoring the enzymic reaction and evaluating the purity of the final product.

INTRODUCTION

D-*arabino*-Hexos-2-ulose (D-glucosone, **1**) has been a chemical and biochemical curiosity rather than a commercially promising, chemical compound. However, in 1981, D-glucosone was reported to be the key intermediate in a new process for converting D-glucose into D-fructose[1].



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Compound **1** has been detected in various natural sources, including fungi[2,3], algae[4], and shellfish[5]. Several chemical syntheses are known, but they afford a low

yield of **1** in various degrees of purity[6,7]. We now report the production of pure D-glucosone, free from D-glucose, by using the enzyme pyranose-2-oxidase. We also describe a simple method for isolating D-glucosone in solid form, rather than as the syrupy or gummy material usually obtained. Only one other report of solid D-glucosone is known to us[7]; according to it, only milligram amounts of solid D-glucosone were obtained, after an extensive, purification procedure.

EXPERIMENTAL

Enzyme production and purification. – This method is an adaption of a procedure previously reported[8]. A slant of *Polyporus obtusus* (ATCC No. 26733) was grown for 7 days at 25° on malt-extract–agar. The agar was prepared as follows: malt extract (20 g), D-glucose (20 g), peptone (1 g), and agar (20 g) were added to distilled water (1 L), and the mixture was heated to dissolve the agar, dispensed into tubes, and sterilized for 15 min at 121°.

The slant-grown organism was used to inoculate 5 seed-cultures. The seed-cultures were grown at 250 r.p.m. for 9 days at 25° in sterile yeast–malt extract medium (100 mL of medium/500-mL Erlenmeyer flask). The medium was prepared as follows: yeast extract (3 g), malt extract (3 g), peptone (5 g), and D-glucose (10 g) were added to distilled water (1 L), and the mixture was dispensed into flasks, and sterilized for 15 min at 121°.

The mycelia were separated from the liquid medium by vacuum filtering through Whatman No. 541 filter-paper on a Büchner funnel (9 cm diam.). The mycelia were washed twice with 0.05M potassium phosphate buffer, pH 7.0 (200 mL), and dried as much as possible by vacuum filtration. Mycelia (1 g) and 0.05M potassium phosphate buffer, pH 7.0 (30 mL) were placed in a Waring Blendor, and the contents were homogenized for 35 s; this process was continued until all of the mycelia had been homogenized. The mixture was then centrifuged at 7000 r.p.m. for 20 min, and the supernatant liquor was decanted and placed in a 1-L beaker. Poly(ethylene glycol) (mol. wt. 4000; 10% w/v, final) was slowly added to the supernatant liquor while it was stirred with a magnetic stirrer. The mixture was kept for 30 min at room temperature, centrifuged at 7000 r.p.m. for 20 min, and the supernatant liquor decanted, and discarded. To the precipitate was added 0.2M sodium chloride in 0.05M potassium phosphate buffer, pH 7.0 (36 mL), and the mixture was thoroughly vortexed. The mixture was kept for 30 min, centrifuged at 14,000 r.p.m. for 20 min, and the translucent, amber-yellow solution decanted, and stored at 4° until needed. The cell-free, supernatant liquor (36 mL) contained partially purified, pyranose-2-oxidase (1.3 units/mL). One unit of enzyme is defined as the amount of enzyme that produces 1 μmol of H_2O_2 per min at 25°, as measured by the *o*-dianisidine colorimetric assay[8].

Immobilization of pyranose-2-oxidase. – To partially purified pyranose-2-oxidase (10 mL), dialyzed against distilled water (500 mL) overnight, was added 0.1M sodium hydrogencarbonate (5 mL) at pH 8.0. To this solution was added activated CH-Sepharose 4B (Pharmacia Fine Chemicals, washed, and reswollen on

a sintered-glass filter, using 500 mL of mM HCl) (5 g). Using an end-over-end mixer, the gel suspension was mixed for 1 h at 25°, and then successively washed with 0.1M sodium hydrogencarbonate, pH 8.0 (40 mL), 0.05M Tris buffer, pH 8.0 (40 mL) containing 0.5M sodium chloride, and 0.5M sodium formate buffer, pH 4.0, also containing 0.5M sodium chloride. Immobilized pyranose-2-oxidase (5 g, dry wt.) was thus obtained.

Conversion of D-*glucose into* D-*glucosone.* — β-D-Glucose (2 g), catalase (Sigma Chemical Co.; 200 units, from bovine liver), and immobilized pyranose-2-oxidase (6 g, wet wt.) were added to a 2-L, Erlenmeyer flask containing sterile, distilled water (1 L). The flask was shaken at 200 r.p.m. for 24 h at 25° and the contents were filtered through Whatman No. 541 filter paper on a Büchner funnel (9 cm diam.). The filtrate was then filtered successively through 5-, 1.2-, 0.65-, and 0.22-μm filters, and lyophilized. Solid D-glucosone (1.9 g), free from D-glucose, was thus obtained. This fluffy, pale-yellowish-white solid retains its solid form when stored at 0° over a desiccant.

Analytical methods

Colorimetric assay for D-*glucosone.* This assay method utilized triphenyltetrazolium chloride (TTC), and was based on the differential rate of reduction by sugars. D-Glucosone reduces TTC to a red pigment (a triphenylformazan) ~100 times as fast as an equivalent amount of D-glucose. The use of this method for the assay of D-glucosone has been described in detail[1,2].

Liquid chromatography. D-Glucose and D-glucosone were quantitated by liquid chromatography (l.c.). A Spectra-Physics 8000 liquid chromatograph equipped with a refractive index (r.i.) detector was used. Aqueous samples (10 μL) were injected into a Waters Associates Carbohydrate Analysis column (30 cm × 3.9 mm i.d.) (10 μm mesh). The mobile phase was 20% aqueous acetonitrile containing 3mM (final) potassium phosphate buffer, pH 6.0. The flow was set at 2.0 mL/min, the column temperature, at 25°, and the r.i. detector, at 8X. The use of this assay method for the quantitation of D-glucose and D-glucosone has been described[9].

Thin-layer chromatography. — The oxidation product of D-glucose was co-chromatographed with chemically synthesized D-glucosone in the following thin-layer chromatography (t.l.c.) system: Avicel-coated glass-plates, developed with 4:4:1:2 (v/v) isopropyl alcohol–pyridine–acetic acid–water. The plates were then sprayed either with 1% TTC in 0.5M NaOH, or biphenyl–aniline–phosphoric acid–ethyl acetate (0.15 g/0.8 mL/11 mL/100 mL) reagent.

Optical activity. — A Perkin–Elmer Model 271 polarimeter was used to measure the specific rotations of aqueous solutions.

Mass spectrometry. — The following derivatization procedure was used in order to render the sugar components volatile[2]. The lyophilizate (100 mg) of the reaction solution, 1,1-diphenylhydrazine (110 mg), and 75% aqueous ethanol (1 mL) were placed in a test tube, vortexed, and then kept for 8 h at 25°. To this mixture was added distilled water (3 mL), and the resulting precipitate was collected. To it

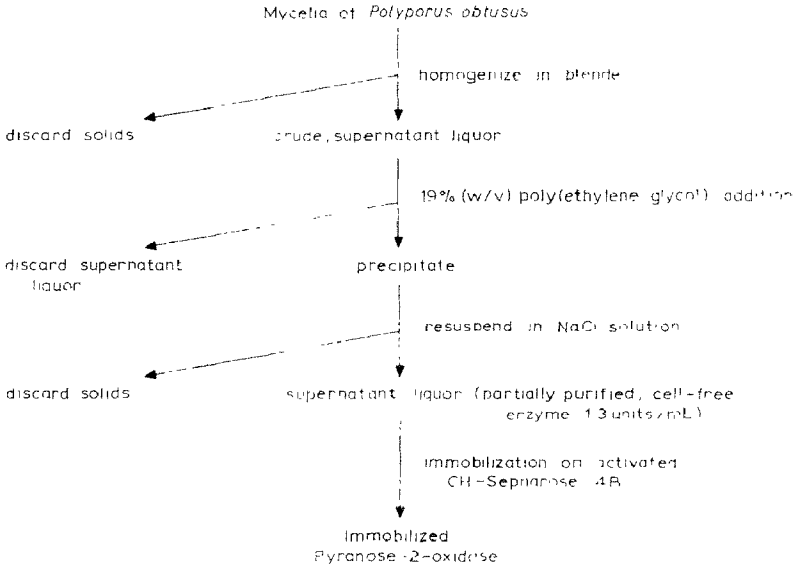
was added 1:1 acetic anhydride–anhydrous pyridine (1 mL), and the mixture was stirred for 20 min at 40 °. Distilled water (2 mL) was added, to stop the reaction, and the resulting mixture was extracted with ethyl ether (two 3-mL portions). The ether layer was dried, and evaporated, to yield a dark-brown syrup which was placed on the probe of a Finnigan 4021 gas chromatograph–mass spectrometer. The mass spectrometer was set at 70 eV (electron-impact ionization) and a probe temperature of 220 °.

Chemically synthesized D-*glucosone.* — D-Glucosone was synthesized by first allowing D-glucose to react with phenylhydrazine, to give D-glucose phenylosazone, and then treating that intermediate with benzaldehyde, to yield D-glucosone[1]. This was a dark-brown, syrupy product.

RESULTS AND DISCUSSION

A summary of the production and immobilization of pyranose-2-oxidase is given in Scheme 1. The method described herein is simple, and most effective as a laboratory process for producing solid D-glucosone.

Pyranose-2-oxidase converts D-glucose into D-glucosone and hydrogen peroxide. The hydrogen peroxide can oxidize certain critical sites on the enzyme molecule, damaging its function. Little is known about the structure of pyranose-2-oxidase, and the specific sites are not yet known, but sulfhydryl groups, or other readily



Scheme 1 Summary of the production and immobilization procedure for pyranose-2-oxidase

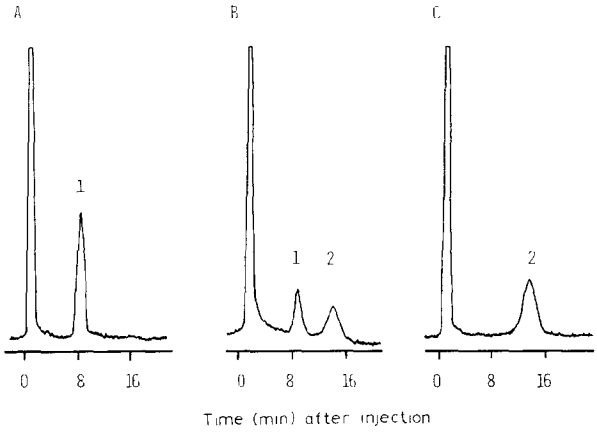


Fig. 1. Conversion of D-glucose into D-glucosone, as monitored by the l.c. assay method, at reaction times of (A) 0, (B) 12, and (C) 20 h. [Assay conditions are described in the text. Peak 1, D-glucose, and peak 2, D-glucosone.]

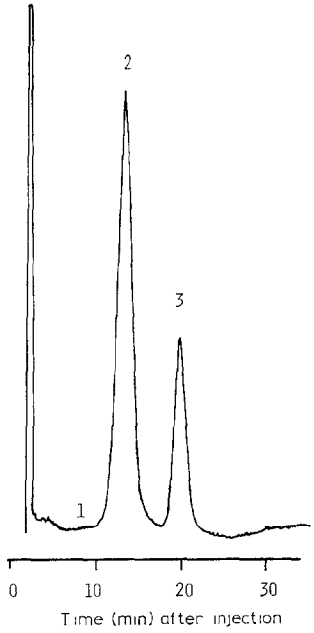


Fig. 2. L.c. measurement of residual D-glucose in the prepared D-glucosone. [Sample: aqueous D-glucosone solution (100 mg/mL) with an internal standard of maltose (30 mg/mL). Assay conditions are described in the text. Peak 1, residual D-glucose (not detected in the sample); 2, D-glucosone; and 3, maltose.]

oxidized groups, are undoubtedly involved. Under the experimental conditions here described, the hydrogen peroxide is decomposed by the catalase to water and oxygen.

$$\text{D-Glucose} \xrightarrow[O_2]{\text{pyranose-2-oxidase}} \text{D-glucosone} + H_2O_2 \xrightarrow{\text{catalase}} H_2O + 1/2\ O_2$$

On lyophilization of the D-glucosone solution, a fluffy, yellowish-white solid is obtained. As this compound is hygroscopic, the solid D-glucosone at room temperature becomes gummy and then syrupy, and its color changes from white to yellow to brown, if it is not kept over a desiccant. However, in over 6 months, solid D-glucosone shows no change in its physical properties when stored over a desiccant at 0 .

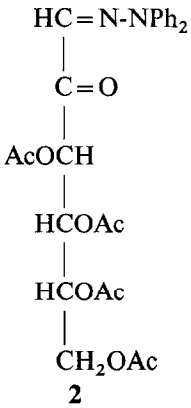
Fig. 1 demonstrates the use of the l.c. assay method for monitoring the conversion of D-glucose into D-glucosone. The absence of residual D-glucose in the solid D-glucosone was readily confirmed by the l.c. assay method (see Fig. 2). By this assay method, 2% of D-glucose in solid D-glucosone would be detected. D-Glucosone produced under the experimental conditions given contained no detectable, residual D-glucose.

Confirmation that the oxidation product of D-glucose was, indeed, D-glucosone was obtained by comparing its optical rotation, and chromatographic and reduction behavior with those of chemically synthesized D-glucosone.

The oxidation product of D-glucose was co-chromatographed with authentic D-glucosone and D-glucose in t.l.c. D-Glucosone gave a streak at R_f 0.40–0.48, and D-glucose, a spot at R_f 0.53. With the triphenyltetrazolium chloride spray-reagent, D-glucosone instantly gave a red spot, whereas D-glucose gave a red spot only on heating for 10 min at 100 . With the biphenyl–aniline–phosphoric acid–ethyl acetate spray-reagent, D-glucosone gave a purple spot, but D-glucose gave a brown spot, on heating for 10 min at 95 . The oxidation product of D-glucose and chemically synthesized D-glucosone showed identical results.

The specific rotation of the oxidation product of D-glucose was similar to that given in the literature[10] for the only other preparation of solid D-glucosone: $[\alpha]_D^{20}$ (degrees): D-glucose (substrate), +52.7; oxidation product of D-glucose, -9.8; literature value[10] for solid D-glucosone, 3.2.

Confirmation of the identity of the product was achieved by mass spectrometry. On appropriate derivatization, D-glucose and D-glucosone yield an acetylated osazone and hydrazone, respectively. That from the oxidation product of D-glucose gave intense mass-ions at m/z 512 (M^+, $C_{20}H_{28}N_2O_9$) and 223 ($C_{14}H_{11}N_2O$; $O{\equiv}C\text{-}CH{=}N\text{-}NPH_2$), diagnostic[2] for derivatized D-glucosone (**2**). A mass ion corresponding to derivatized D-glucose (**3**) at m/z 556 ($C_{28}H_{32}N_2O_{10}$) was not detected.



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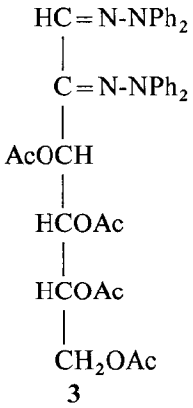

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Final confirmation of the identity of the product was obtained by reducing the product to D-fructose[11], and then comparing the chromatographic behavior of this sugar with that of authentic D-fructose.

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