Oxidation of Uronic Acids by a Large Excess of Glucose Oxidase Preparations

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Three commercial enzyme preparations of glucose oxidase (GOD) showed an oxidizing ability of glucuronic acid and galacturonic acid to form sugar acids. The optimum conditions of GOD reaction were at pH 8.0 and 50°C, whereas those of uronic acid oxidase (UOD) reaction were at pH 3.5 and 40°C. In spite of extensive attempts to separate GOD from UOD, the isolation of each activity was unsuccessful, and UOD reaction resulted from the wide substrate specificity of GOD reaction. More than 6-fold higher K_m values for glucuronic acid and galacturonic acid than for glucose might suggest that UOD reaction was the alternative reaction of GOD enzyme. However, a more definite conclusion on UOD activity should be drawn from further studies. The reaction products from uronic acids were analyzed by HPLC and paper chromatography, and some products were shown to be identical with sugar acids.

Beet pulp contained a large amount of uronic acids, which were found not only in the pectic fraction, but also in the hemicellulose and cellulose fractions.¹⁾ Although the effective saccharification of beet pulp might increase biomass content about 1.4-fold for the ethanol production by yeast fermentation, uronic acids could not be used as carbon sources. The chemical conversion of uronic acids by water-soluble carbodiimide increased the yield of glucose and galactose in beet pulp fraction on enzymic hydrolysis with pectinases and hemicellulases.²⁾ However, no papers were found concerning the enzymic reduction of uronic acids to neutral sugars. We have been screened the enzyme that converted uronic acids to neutral sugar. In contrast to our object, no enzyme concerning the production of neutral sugar from acidic sugar was obtained, but reaction catalyzing a further oxidation of uronic acids was observed. In the present paper, we describe the

oxidation of uronic acids with glucose oxidase (GOD) preparation from commercial sources.

MATERIALS AND METHODS

Materials. Three preparations of glucose oxidase were obtained from Toyobo (GOD^T: 165 units/mg, isolated from *Aspergillus* species), Wako Pure Chemical Industries Ltd. (150-250 units/mg, isolated from *Aspergillus niger*), and Oriental Yeast Co. (348 units/mg, isolated from microorganism). The glucose was measured by the glucose oxidase kit (Glucose B-Test Wako). Horseradish peroxidase (100 units/mg) was purchased from Wako Pure Chemical Industries Ltd. Most sugars, including sugar acids, were commercial preparations purchased from Wako.

Assay of uronic acid oxidizing activity. The reaction mixture contained enzyme solution (20 μ L), substrate solution (10 μ L), and buffer solution including additional components (20 μ L). After incubation at 30°C for 20 min, the reaction was stopped by the addition of 1 M NaOH (25 μ L). The mixture was neutralized

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with 1 M HCl (25 μ L) and subjected to a UOD assay, which was essentially based on the GOD assay kit of B-Test Wako. For the UOD assay, a cocktail from the GOD assay kit was treated at 100°C for 5 min for the inactivation of GOD; that is, phenol and 4-aminophenazone in the GOD cocktail were used. The peroxidase of horseradish (0.2 mg/mL 67 mM phosphate buffer, pH 7.6) was supplemented for the UOD assay. Inactivated GOD cocktail (100 μ L) and peroxidase solution (25 μ L) were added to the above reaction mixture and incubated at 30°C for 20 min. After dilution with water (375 μ L), absorbance was measured at 505 nm. One unit each of GOD and UOD activity was defined as the amount of enzyme, which converted 1 μ mol glucose/min at pH 7.5 (50 mM TEMED-HCl buffer) and 1 nmol glucuronic acid/min at pH 3.5 (50 mM Tris-acetate buffer), respectively, at 30°C incubations. Calibration curves with standard sugars were obtained by the above methods, including alkaline inactivation and subsequent neutralization steps that required 2 or 3 min.

HPLC analysis. HPLC gel filtration analysis was done with Tosoh G3000SW column (7.8 $\times$ 300 mm), which was equilibrated with 17 mM phosphate buffer (pH 7.6)–0.2 M NaCl–0.02% NaN₃. GOT^T was eluted from the column at 0.6 mL/min, and fractions were collected every 30 s to measure the enzyme activity and protein by a UV detector (Jasco UV-870). The product sugars of UOD reaction were separated by the column of Aminex carbohydrate HPX-87C (7.8 $\times$ 300 mm). The reaction mixture was eluted from the column with water (0.6 mL/min), and sugars were detected by an RI detector (Jasco RI-830).

Paper chromatography (PPC). A product analysis of the UOD reaction was done with filter paper (20 $\times$ 20 cm, Advantec Toyo, No. 50). The development was done at room temperature overnight with 65% 1-propanol, and sugars were detected by the silver nitrate dip procedure.

RESULTS

Screening of UOD activity.

About 40 commercial enzyme preparations, including some oxidases, were tested for the oxidase activity of glucuronic acid and galacturonic acid conversion. UOD activity was assayed for the production of H₂O₂ with the color development reaction coupled with peroxidase, phenol, and 4-aminophenazone. As shown in Table 1, three GOD preparations gave UOD activity. No remarkable UOD activity was detected from the remaining 37 enzyme preparations. Since GOD of Toyobo (GOD^T) had the highest activity for galacturonic acid and was available in a large quantity at a low price, further experiments were done with this enzyme.

Characteristic of UOD reaction.

The general properties of the GOD^T preparation in the reaction with galacturonic acid (UOD activity) were compared with those in the reaction with glucose (GOD activity). As shown in Fig. 1a, pH optimum for GOD activity was at pH 8.0, and higher activity was obtained at neutral to alkaline pH ranges. In contrast, the maximum activity of UOD reaction was obtained at pH 3.5; higher activity occurred at the acidic pH regions. A comparison of optimum temperatures also revealed a marked difference between the GOD and UOD activities. The former activity was maximum at 40 to 60°C, and the UOD reaction was maximum at 30 to 50°C; about 60% of the UOD activities were detected at the low temperature of 10°C, at

Table 1. The comparison of uronate oxidizing activity among the GOD preparations.^a

Enzymes	GlcUA	GalUA
GOD/Toyobo	38	230
GOD/Wako	27	13
GOD/Oriental	637	112

^aThe reaction mixture (300 μ L) contained enzyme (10 mg/mL, 100 μ L), 10% substrate sugar (100 μ L), and 0.2 M TEMED-HCl buffer (pH 7.5, 100 μ L). After incubation at 30°C for 20 h, the enzyme activity was measured as described in the text.

Fig. 1. Optimum conditions of GOD and UOD reactions.

(a) Optimum pH. (b) Optimum temperature. The reaction mixture (100 μ L) for GOD activity (○) contained GOD^T (10 μ g/mL, 25 μ L), 0.1 M glucose (25 μ L), and 0.2 M TEMED-HCl buffer (pH 3.75–11.0, 50 μ L). After incubation at 30°C for 10 min, the enzyme activity was measured as described in the text. The optimum temperature was measured at pH 8.0. The reaction mixture (100 μ L) for UOD activity (●) contained GOD^T (10 mg/mL, 25 μ L), 10% galacturonic acid (25 μ L), and 0.2 M Tris-acetate buffer (pH 3.1–8.5, 50 μ L). After incubation at 30°C for 20 min, the enzyme activity was measured as above. The optimum temperature was measured at pH 3.5.

which GOD activity was less than 10% (Fig. 1b).

The carbohydrate oxidizing activity of commercial GOD^T preparation was examined with neutral and acidic sugars (Table 2). Although glucose and xylose gave significantly high activity, galactose and mannose also served as good substrates. Galacturonic acid and glucuronic acid were classified into the group of good substrates together with arabinose and glucosamine. Besides these sugars, glucurono γ -lactone, maltose, and sucrose were good substrates.

A comparison of kinetic constants for UOD and GOD reactions showed further differences

Table 2. The carbohydrate oxidizing activity of commercial GOD^T preparation.

Substrate	Activity (U)
Arabinose	619
Galactose	1168
Glucose	1413
Mannose	1095
Xylose	1572
Galacturonate	743
Glucuronate	430
Glucosamine	507
Lactose	92
Maltose	665
Sucrose	464
Gluconate	9
Glucono δ -lactone	13
Glucurono γ -lactone	252
Gulono γ -lactone	8
None	6

^aThe reaction mixture (80 μ L) contained enzyme (10 mg/mL, 10 μ L), 1% substrate sugar (20 μ L), and 50 mM HCl-acetate buffer (pH 3.5, 50 μ L). After incubation at 30°C for 20 h, the enzyme activity was measured as described in the text.

as shown in Figs. 2a and b. The GOD^T preparation gave biphasic lines for glucuronic acid and galacturonic acid; two extrapolated K_m values, 99 and 329 mM for glucuronic acid; and 86 and 235 mM for galacturonic acid. In contrast, the K_m value for glucose was 13.2 mM at pH 7.5, whereas the K_m value was 25.5 mM at pH 3.5. A comparison of the k_0 values showed a large difference in the UOD and GOD reactions. The k_0 values for galacturonic acid (1.2–2.7 min⁻¹) were about 1000-fold smaller than those for glucose (1.2 and 2.9×10^3 min⁻¹ at pH 7.5 and pH 3.5, respectively).

Separation of UOD from GOD activity.

To determine whether UOD activity was identical with GOD activity, a separation of UOD and GOD activities was examined by various column chromatography methods, including a con A-Sepharose column for the separation of glycoproteins. Most results gave no clear separation of the two activities. That is, as shown in Fig. 3, the activities and the protein peak had identical behaviors on the gel filtration chromatography. Moreover, on the PAGE separation, the major peak of UOD activity

Fig. 2. Double reciprocal plots for UOD and GOD reactions.

(a) UOD reaction with glucuronic acid (●) and galacturonic acid (○). The reaction mixture (50 μ L) contained GOD^T (10 mg/mL, 25 μ L) and uronic acids (50–250 mM) dissolved in 0.2 M Tris-acetate buffer (pH 3.5, 25 μ L). Incubation was done at 30°C for 20 min. (b) GOD reaction with glucose at pH 3.5 (●) and pH 7.5 (○). The reaction mixture (50 μ L) contained GOD^T (10 μ g/mL, 25 μ L) and glucose (5–50 mM) dissolved in 0.2 M TEMED-HCl buffer (pH 3.5 or 7.5, 25 μ L). Incubation was done at 30°C for 10 min. V values in y axis were represented by the GOD unit of glucose μ mol and thus multiplied to 1000/6 times for the UOD reaction in (a). Therefore K_m and V_{max} values were calculated as follows: (a) UOD with glucuronic acid, 99 and 0.044; 329 and 0.080, and with galacturonic acid, 86 and 0.037; 235 mM and 0.064 nmol as glucuronic acid/min/mg enzyme, respectively; (b) GOD with glucose at pH 3.5, 25.5 and 34.5, and at pH 7.5, 13.2 mM and 83.3 μ mol glucose/min/mg enzyme, respectively.

corresponded to that of GOD activity (relative mobility; R_m 0.53) and gave no indication of a separation of UOD and GOD, which was measured with the sliced gel segments from PAGE and SDS-PAGE (data not shown). From all these results we might conclude that UOD and GOD reactions were catalyzed by the identical enzyme molecule of GOD. However, a more definite conclusion for the identity of UOD and GOD enzymes should be drawn from further studies.

Analysis of the products.

Based on the reaction mechanism of GOD activity, the reaction products from uronic acids and UOD reaction were assumed to be the oxidized form of the aldehyde group at the C-1 position. Namely, galactaric acid and glucaric acid were produced from galacturonic acid and glucuronic acid, respectively. Therefore a

comparison of R_T (relative retention time) and R_{Glc} (relative mobility to glucose) values of reaction products with the standard sugars was made by HPLC and paper chromatography (PPC), as shown in Table 3. HPLC analysis revealed two main product peaks of R_T 8.5 and 9.8 min, besides the substrate glucuronic acid. A major product of R_T 7.3 min was obtained from the substrate galacturonic acid. Sample peaks at 8.5 and 7.3 min were almost identical to peaks 8.4 and 7.2 min of standard glucaric acid and galactaric acid, respectively. Three lactones had R_T values of more than 10 min and higher R_{Glc} values (1.08–1.29) than glucose (R_{Glc} 1.00) on the PPC analysis. The reaction products from glucuronic acid had R_{Glc} 0.81 and 0.60, and product from galacturonic acid had R_{Glc} 0.88 (Table 3). Standard glucaric acid and galactaric acid gave three spots (R_{Glc} 0.80, 0.63, and 0.49) and two spots (R_{Glc} 0.78 and 0.63),

Fig. 3. HPLC gel filtration pattern of GOD^T preparation.

The enzyme solution (10 mg/mL, 20 μ L) was subjected to the analysis as described in the text. Protein (○), GOD activity (●), and UOD activity (▼) were measured for the isolated fractions (300 μ L/tube). GOD activity was represented by glucose (μ g) equivalent value, and UOD activity was represented by the same value multiplied 1000-fold after the conversion of the UOD unit into the GOD unit, where the GOD 1.0 unit corresponded with the UOD 0.006 unit.

Table 3. The comparison of R_T and R_{Glc} values of reaction products with standard sugars.

Sugars	HPLC (R_T , min) ^a	PPC (R_{Glc}) ^a
Markers		
Glucose	9.8	1.00
Galacturonate	8.1	0.75
Glucuronate	7.5	0.72
Glucono δ -lactone	11.4	1.29
Glucurono γ -lactone	12.7	1.13
Gulono γ -lactone	12.0	1.08
Galactonate	—	0.66
Gluconate	11.2	0.67
Galactarate	7.2	0.78, 0.63
Glucarate	8.4, 7.4	0.80, 0.63, 0.49
Products from		
Galacturonate	(8.1), 7.3	0.88, (0.74)
Glucuronate	9.8, 8.5, (7.7)	0.81, (0.72), 0.60

^aValues in parentheses corresponded to the peaks or spots detected in the reaction mixture at zero time.

respectively. Because large amounts of residual substrates in the reaction mixture greatly affected the R_T and R_{Glc} values of product sugars, some deviations of values from the authentic sugars were observed. Moreover, it was already known that sugar acid had several isomers, such as 1,4-lactone, 3,6-lactone, and di-lactone forms. Therefore the spots of

reaction products could not be firmly identified as authentic sugar acids in both cases of glucuronic acid and galacturonic acid.

DISCUSSION

During the screening of enzyme, which catalyzes the reduction of uronic acids to neutral sugars, commercial preparations of glucose oxidase were found to catalyze the oxidation of uronic acids (Table 1). In these experiments, high concentrations of enzyme (final 0.25%) and substrate uronic acids (final 2.5%) were adopted. It has already been noticed that GOD catalyzed the oxidation of several neutral sugars besides glucose.^{3,4)} However, usually, no examination of the substrate specificity with acidic sugars was reported. Under the above conditions of a particular high enzyme-substrate system, UOD reaction could be detected with the colorimetric determination method, though reaction with an ordinary level of GOD concentration gave no noticeable amount of reaction product. The GOD preparation had high activity to glucuronic acid, galacturonic acid, and glucosamine besides neutral monosaccharides other than glucose (Table 2). Therefore we focused on this uronic acid oxidizing activity and examined the characteristics of UOD in detail. Although glucurono γ -lactone was susceptible to UOD, other lactones tested had no reaction with it. Moreover, some disaccharides such as lactose, maltose, and sucrose were oxidized with this enzyme. In the earlier paper, an oxidation of lactose, maltose, and cellobiose by GOD was observed.⁵⁾ Different from the above three disaccharides, sucrose has no reducing end group, and the action of UOD is limited to positions other than C-1. Thus the first possibility of high value with sucrose shown in Table 2 may be attributable to the acidic hydrolysis of sucrose at pH 3.5 and the subsequent oxidation of resulting glucose with GOD. An enzymic cleavage of sucrose molecule indicates a second possibility, where the oxidation of sucrose might be ascribed to the cooperation with some other contaminated enzymes such as invertase with GOD yielding the oxidation of glucose and fructose. In

this consequence, biphasic kinetics shown in Fig. 2a could be explained by the action of a contaminated enzyme. Alternatively, other factors such as a change of pH or an increase of product inhibition, which might occur because the high concentration of acidic sugar substrates and the reaction mechanism yielding H_2O_2 would lead to biphasic lines. Biphasic lines were also reported on the kinetic experiments done with purified enzymes of dextranucrase.^{6,7)} The third possibility may be attributable to contaminated enzymes such as the other type of oxidase having specificity to positions other than C-1, which catalyzed the direct oxidation of sucrose molecules. Moreover, it was interesting to note that the oxidation of galactose was catalyzed by two types of galactose oxidases, the predominant type oxidized at the C-6 position⁸⁾ and the minor type oxidized at the C-1 position of galactose.⁹⁾

Since the optimum conditions of GOD and UOD reactions differed greatly (Fig. 1a, b), the separation of UOD from GOD was extensively tested. However, no separation of the two activities was attained, as illustrated by Fig. 3, and we concluded that the UOD activity was ascribed to the wide substrate specificity of GOD reaction. A large increase in the enzyme and substrate concentrations made it possible to detect current UOD activity. A difference in optimum pH and temperature shown in Fig. 1 might be related to the formation of the dissociated free-acid form of acidic sugars other than the salt form, although further evidence should be required to verify this idea. Based on the results in Fig. 1, at first we considered that the UOD enzyme was different from GOD and made extensive trials to separate the two, but no experiments provided evidence for differentiation of the two enzymes. Therefore we should suggest the identity of UOD activity with GOD activity. However, the identity of these activities should be confirmed by further studies on enzyme characteristics. Although some recent studies reported the enzymic oxidation of neutral sugars,^{10,11)} the oxidation of acidic sugars would be another useful method for the production of carboxylated sugars. In most cases, sugar acids such as glucaric acid

and galactaric acid were currently produced by the chemical reactions catalyzed by monochloroacetic acid.^{12,13)}

The action of UOD on glucuronic acid and galacturonic acid produced one or two sugars,^{14,15)} which were analyzed by HPLC and PPC methods (Table 3). Although no definite assignment of products was completed, the substrates were oxidized at the C-1 position of the aldehyde group, and the product had a dicarboxylated form. One piece of additional evidence was provided by the measurement of reducing power by the Nelson-Somogyi method during the reaction of UOD with galacturonic acid (data not shown). As presented in Fig. 1a, UOD activity was higher at acidic pH than at alkaline pH, and the decrease in reducing power of galacturonic acid was also higher at acidic pH. A loss of reducing power during the UOD reaction would be explained by the oxidation of the aldehyde group to form the carboxyl group at C-1 position. More definite evidence will be provided for the isolated products by further analysis with such as mass spectrometry.

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高濃度のグルコースオキシダーゼによる ウロン酸の酸化

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3種類の市販品酵素のグルコースオキシダーゼ (GOD) はグルクロン酸, ガラクツロン酸に作用して糖酸を生成する作用を有することが認められた。GODの最適反応条件はpH 8.0, 50℃であったが, ウロン酸酸化酵素 (UOD) 活性の最適条件はpH 3.5, 40℃であった。GOD活性とUOD活性を分離することができなかったため, UOD反応はGODの基質特異性の一部に帰因するものと推定された。ウロン酸に対する K_m がグルコースの6倍も高いことも, UOD反応がGODの副反応であることを示している。しかしながら, UODがGODとは別の酵素である可能性も否定できないため, 両酵素の異同性に関してはさらに検討が必要である。ウロン酸からの反応生成物をHPLC, ペーパークロマトグラフィーで分析した結果, 糖酸の標準品と一致する挙動を呈する成分の存在が確認された。