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Enzymatic Oxidation and Separation of Various Saccharides with Immobilized Glucose Oxidase

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Abstract Glucose oxidase from *Aspergillus niger*, the specific enzyme for β-D-glucose oxidation, can also oxidize other related saccharides at very slow or negligible rates. The present study aimed to compare the kinetics of D-glucose oxidation using immobilized glucose oxidase on bead cellulose for the oxidation of related saccharides using the same biocatalyst. The significant differences were observed between the reaction rates for D-glucose and other saccharides examined. As a result, k_{cat}/K_M ratio for D-glucose was determined to be 42 times higher than D-mannose, 61.6 times higher than D-galactose, 279 times higher than D-xylose, and 254 times higher than for D-fructose and D-cellobiose. On the basis of these differences, the ability of immobilized glucose oxidase to remove D-glucose from D-cellobiose, D-glucose from D-xylose, and D-xylose from D-lyxose was examined. Immobilized catalase on Eupergit and mixed with immobilized glucose oxidase on bead cellulose or co-immobilized with glucose oxidase on bead cellulose was used for elimination of hydrogen peroxide from the reaction mixture. The accelerated elimination of D-glucose and D-xylose in the presence of co-immobilized catalase was observed. The co-immobilized glucose oxidase and catalase were able to decrease D-glucose or D-xylose content to 0–0.005% of their initial concentrations, while a minimum decrease of low oxidized saccharides D-xylose, D-cellobiose, and D-lyxose, respectively, was observed.

Keywords Glucose oxidase · Catalase · Saccharides · Oxidation kinetics · Immobilized enzymes

Introduction

Glucose oxidase (GOD) is an enzyme that catalyzes the oxidation of β-D-glucose to gluconolactone and hydrogen peroxide [1]. These products are subsequently spontaneously

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transformed to gluconic acid and catalytically transformed to water, respectively [2]. GOD from *Aspergillus niger* is a flavin-containing glycoprotein with extreme specificity for the substrate D-glucose. GOD has also been shown to be able to oxidize saccharides other than D-glucose [2–6], but these studies indicated that other saccharides were more slowly oxidized than D-glucose. The differences in oxidation rates of various saccharides indicate that certain structural features of the substrates are important for the enzymatic reaction. Apparently, the pyranose ring in the chair or C1 conformation and equatorially oriented hydroxyl groups at positions 1 and 3 are probably involved in the formation of the enzyme–substrate complex. In addition to derivatives of D-glucose (deoxy-, methyl-, etc.), the ability of GOD to oxidize related saccharides such as D-mannose, D-galactose and D-xylose was estimated [4, 6]. The great difference between the oxidation rate of D-glucose and the other saccharides was observed. The aim of those studies was focused on the estimation of GOD specificity and on the potential way of preparation of various aldonic acids, respectively. The differences in saccharide oxidation rates were utilized in our previous studies for the process of removing the saccharide with the greater oxidation rate from a solution of two saccharides. This technique was used to eliminate D-glucose from a solution with the low-molecular-mass dextran as well as from a solution with D-mannose [7, 8]. The removal of gluconic acid generated during the biotransformation of D-glucose was performed by precipitation with $CaCl_2$ [8]. To enhance enzymatic properties such as reusability, operational stability, recovery, and shelf life, various immobilization techniques were used for GOD [9–12]. The immobilization of GOD on a solid matrix prevents inactivation and increases its thermal and pH stability. Hydrogen peroxide formed during the oxidation reaction can attach the residues of GOD and decrease its catalytic activity [13–15]; to remove it, immobilized catalase (CAT) is usually added to the reaction mixture or is co-immobilized with GOD [15, 16]. The utilization of reactions catalyzed by immobilized GOD plus catalase has increased in recent years in the fields of food processing, chemical processing, analytical practice and medicine [17]. This system allows the complete removal of D-glucose and hydrogen peroxide from the reaction mixture [8, 15].

The goal of the present work was to determine the oxidation kinetics of saccharides other than D-glucose using immobilized GOD on bead cellulose (GOD-TBC). The differences in reaction rates of individual saccharides were evaluated by monitoring the elimination of the more rapidly interacting saccharide (D-glucose, D-xylose) from a mixture with a more slowly reacting saccharide (D-xylose, D-cellobiose) or unreacting D-lyxose. Immobilized GOD on bead cellulose was used in the presence of immobilized CAT-Eupergit (CAT-Eup) to eliminate hydrogen peroxide produced. A simple co-immobilization of GOD together with CAT on the bead cellulose was used to obtain the mixed biocatalyst for efficient biotransformation of saccharide admixtures.

Experimental

Materials

Glucose oxidase GO 2B2 (EC 1.1.3.4) from *A. niger* and catalase (EC 1.11.1.6) from bovine liver (CAT 60640) were obtained from Biozyme Laboratories (Gwent, UK) and from Fluka (Buchs, Switzerland), respectively. Cellulose MT 100 beads were obtained from Iontosorb (Ústí nad Labem, Czech Republic). Eupergit® C was supplied by Degussa Röhm GmbH & Co. KG, Pharma Polymers (Darmstadt, Germany). D-Glucose and D-cellobiose were obtained from Sigma Aldrich (St. Louis, USA), D-ribose and D-fructose from

Lachema (Brno, Czech Republic), and all other saccharides were prepared in the production department of our Institute of Chemistry. A D-glucose test kit was obtained from BioSystems S.A. (Barcelona, Spain). All other chemicals were of analytical grade.

Methods

Preparation of Co-immobilized GOD and CAT on Bead Cellulose

Chlorotriazine bead cellulose (CHTBC) was prepared as described previously [8]. Ten grams of CHTBC was suspended in 25 ml of solution containing 10 mg GOD (activity 189 U/mg) and 1 ml CAT (activity 258 U/μl) in 50 mM acetate buffer (pH 5.8). The suspension was stirred for 18 h at ambient temperature. Afterwards, the co-immobilized biocatalyst (GOD-TBC-CAT) was separated by filtration and washed exhaustively with 50 mM acetate buffer (pH 5.8) to remove unbound enzymes.

A similar procedure was used for the covalent immobilization of GOD alone on bead cellulose. Ten grams of wet CHTBC was added to 10 mg of GOD dissolved in 25 ml of 50 mM sodium acetate buffer, pH 5.8. The suspension was stirred for 3 h, and afterwards, the prepared GOD-TBC was filtered and washed several times with acetate buffer.

The prepared sorbent activity of immobilized GOD was determined by the method described previously [7], and CAT activity was determined according to the method essentially described by Beers and Sizer [18]. The modified assay for immobilized CAT utilized the suspension which consisted of 15 ml of 50 mM sodium phosphate buffer, pH 7.0, containing 59 mM hydrogen peroxide and 10 mg of wet immobilized CAT. One unit of CAT activity corresponds to the decomposition of 1 μmol of hydrogen peroxide, measured as a change in absorbance at 240 nm per minute at 25 °C, under the specified conditions. The amount of the immobilized GOD was determined from the difference between the amount of starting and unbound proteins determined by a Lowry method [19]. When a co-immobilization of GOD and CAT from one stock solution took place, the amount of each protein immobilized was calculated from differences of activities of both enzymes in the stock solution before and after immobilization using specific activities.

Immobilization of CAT on Eupergit

CAT-Eupergit (CAT-Eup) was prepared using the following procedure: 1 g of dry Eupergit C, 0.5 ml of CAT, and 19.5 ml of 0.7 M sodium phosphate buffer, pH 6.0, were intermittently shaken at room temperature over a period of 6 days. Subsequently, the product was filtered and washed with 50 mM sodium phosphate buffer, pH 7.0. The amount of immobilized CAT was determined as proteins using the Lowry method [19] and as activity according to the method described above.

Determination of Oxidation Kinetics of Various Saccharides Using GOD-TBC

Kinetic parameters of saccharides as substrates were measured at 30 °C using the same procedure that was used to determine the activity of immobilized GOD. The assay was based on the horseradish peroxidase-catalyzed oxidation of *o*-dianisidine by hydrogen peroxide described previously [7]. The initial reaction rate was measured within a range of saccharide concentrations from 10 to 200 mM and an oxygen concentration of 20 mg/l.

Biotransformation of D-Glucose to D-Gluconic Acid in D-Xylose or D-Cellobiose Solution

The model solutions contained 0.96% (*w*/*v*) of D-xylose or D-cellobiose and 0.04% (*w*/*v*) D-glucose. A 10-ml aliquot of this solution in 50 mM phosphate buffer (pH 6.0) was stirred with 100 mg GOD-TBC and 100 mg CAT-Eup and continuously aerated with oxygen at ambient temperature. GOD-TBC-CAT (100 and 200 mg, respectively) was used alternatively under the same conditions for the biotransformation of glucose. One-hundred-microliter samples were taken at specific time intervals to determine the glucose content using a glucose test kit. The hydrogen peroxide generated during the previous D-glucose transformation was removed with a CAT solution [8], which was subsequently inactivated. The reaction mixture, before and after 4 h of ongoing biotransformation, was analyzed by gas chromatography (GC) to determine the individual saccharide levels.

Biotransformation of D-Xylose to D-Xylonic Acid in D-Lyxose Solution

The procedure described in "Determination of Oxidation Kinetics of Various Saccharides Using GOD-TBC" was also used for the removal of D-xylose from a solution with D-lyxose. The reaction mixture was aerated with oxygen for 1 h and then was stirred for 1 week at ambient temperature. In 1-day intervals, 150-μl samples were taken and D-xylose and D-lyxose concentrations were determined by GC.

Determination of Saccharides by GC

Trimethylsilyl derivatives prepared from the resulting saccharide samples were analyzed by a Hewlett-Packard Model 5890 A chromatograph using flame ionization detection and separation by a DB-1 column (60 m×0.25 mm; Fison, Loughborough, UK). The following thermal cycle was used: 80 °C for 2 min, followed by stepwise increases in temperature (6 °C/min) up to 250 °C.

Results and Discussion

Kinetic Dependences of Oxidation of Various Saccharides Using GOD-TBC

GOD (from *A. niger*) is a flavin-containing enzyme that catalyzes the oxidation of β-D-glucose by molecular oxygen to gluconic acid. β-D-glucose is the most effective substrate for GOD, but a variety of other saccharides are also capable of acting as substrates. Earlier studies [2, 4–6] indicated that the activity of GOD against other substrates were substantially lower than 2% of its activity against glucose. Those studies focused on the GOD enzyme in solution. Our present work focuses on immobilized GOD.

GOD-TBC was prepared and tested for oxidation of various saccharides. D-Mannose, D-galactose, D,L-xylose, D-fructose, D-cellobiose, D,L-sorbose, D,L-arabinose, D-lyxose, D-ribose, D-allose, and D-tagatose were examined as substrates in enzymatic oxidation reactions with immobilized GOD at 30 °C and pH 6.0. The results of catalytic reaction rates of these saccharides, which served as poor substrates in comparison with β-D-glucose, are shown in Fig. 1. Measurable oxidation reaction rates were obtained with D-mannose, D-galactose, D-xylose, D-fructose, and D-cellobiose (Fig. 1a), but very low reaction rates were measured with D,L-sorbose and D-arabinose (Fig. 1b). No detectable oxidation of the other saccharides was observed with GOD-TBC. Because the oxidation velocity of all

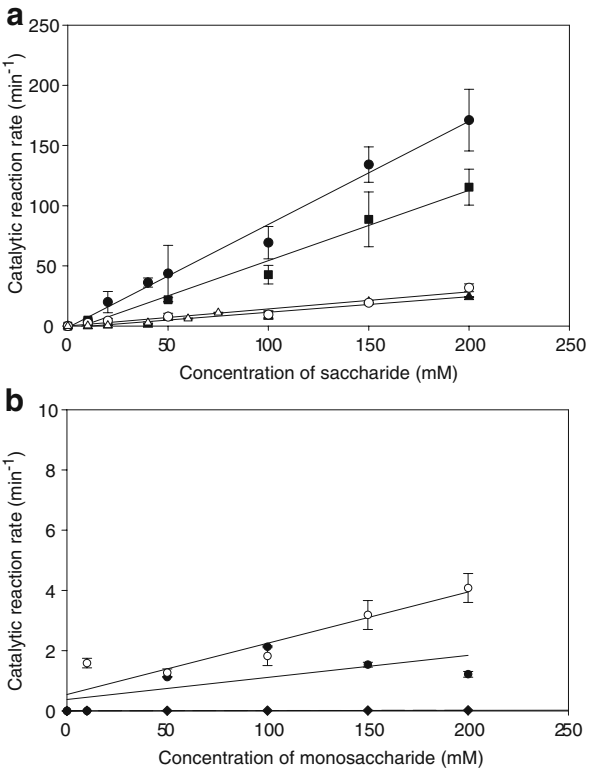

Fig. 1 Kinetics of catalytic oxidation of various saccharides with GOD-TBC. **a** *Closed circle*, D-mannose; *closed square*, D-galactose; *closed triangle*, D-xylose; *empty circle*, D-fructose; *empty triangle*, cellobiose. **b** *Closed circle*, D-sorbose; *empty circle*, L-sorbose; *closed diamond*, D-arabinose

examined saccharides increased linearly in a saccharide concentration-dependent manner, we were only able to calculate the k_{cat}/K_M ratio (efficiency of catalysis), not the individual kinetic constants V_{max} and K_m. The calculated values of k_{cat}/K_M are presented in Table 1. The kinetics of D-glucose, which is the preferred substrate of GOD-TBC, was determined previously; its oxidation follows the Michaelis–Menten equation [8]. Data presented in Table 1 and Fig. 1 show big differences in the oxidation kinetics (k_{cal}/K_M) for individual saccharides as substrates of GOD-TBC in the following order: D-glucose >> D-mannose and D-galactose >> D-xylose, D-fructose and D-cellobiose >> other saccharides. A similar order of decrease of GOD specificity towards these saccharides was described previously

Table 1 Kinetic characteristics of enzymatic oxidation of various saccharides with GOD-TBC.

Saccharide	k_{cat}/K_M [mM^{-1} min^{-1}]	Ratio of glucose/saccharide
D-Glucose	36.0	1
D-Mannose	0.8565	42
D-Galactose	0.5840	61.6
D-Xylose	0.1290	279.0
D-Fructose	0.1416	254.2
D-Cellobiose	0.1415	254.4
D-Sorbose	0.00732	4.92×10^{3}
L-Sorbose	0.0170	2.11×10^{3}
D-Arabinose	8.489×10^{-5}	4.24×10^{5}

with the enzyme in the solution [4–6]. GOD activities towards various saccharides, when expressed as a relative value of an activity towards glucose are: D-mannose 0.2%, D-galactose 0.08%, and D-xylose 0.03% [5].

A significant difference between the rates of D-glucose and D-mannose oxidation was evaluated in our previous study on the removal of glucose admixture from their solution to prepare pure D-mannose [8]. The present work is focused on other possible ways to separate the saccharide with a higher rate of oxidation (D-glucose, D-xylose) from a mixture that includes a saccharide with a much lower oxidation rate (D-cellobiose, D-xylose, and D-lyxose).

Biotransformation Elimination of D-Glucose from a Mixture with D-Xylose

For biotransformation experiments, immobilized GOD on bead cellulose (GOD-TBC) with CAT immobilized on Eupergit (CAT-Eup) or GOD and CAT co-immobilized on bead cellulose (GOD-TBC-CAT) were used. Activities and proteins content of the prepared biocatalysts were determined. The results are summarized in Table 2.

Three types of experiments were performed according to the amount or type of biocatalyst system used:

Experiment A: 100 mg GOD-TBC+100 mg CAT-Eup
Experiment B: 100 mg GOD-TBC-CAT
Experiment C: 200 mg GOD-TBC-CAT

The decrease in D-glucose concentration in the model solution D-glucose + D-xylose during aeration with oxygen is shown in Fig. 2. All of the D-glucose was transformed to its oxidized form within 2 h, as indicated by the results of experiments A and C. A slower oxidation rate of D-glucose was observed during experiment B, in which 100 mg of preparation containing co-immobilized enzymes on bead cellulose was used. There were no significant differences between the oxidation rates using 200 mg of mixed biocatalysts and co-immobilized biocatalysts. In experiment C, the initial and final concentrations of D-glucose and D-xylose were determined by GC analysis. The concentration of D-glucose after 4 h decreased from 400 to 0.0187 mg/l, whereas the content of D-xylose did not change.

Biotransformation Elimination of D-Glucose from a Mixture with D-Cellobiose

The same experiments used for the preparation of pure D-xylose were also performed for a mixture of D-cellobiose with D-glucose. The experiment duration was prolonged to 5 h to obtain complete transformation of D-glucose. Figure 3 shows the courses of D-glucose decreases in the three types of experiments described in "Biotransformation Elimination of

Table 2 Content of proteins and activity of immobilized enzymes.

Biocatalyst	Content of immobilized proteins (mg/g of carrier)	Activity of immobilized enzyme (U/g of carrier)	Specific activity (U/mg of protein)
GOD-TBC	0.91	24.6±0.18	27.0
CAT-Eup	0.37	1,320±70	3,567.5
GOD-TBC-CAT	0.97 (GOD)	17.5±1.60	18.0
	0.17 (CAT)	569±51	3,347.1

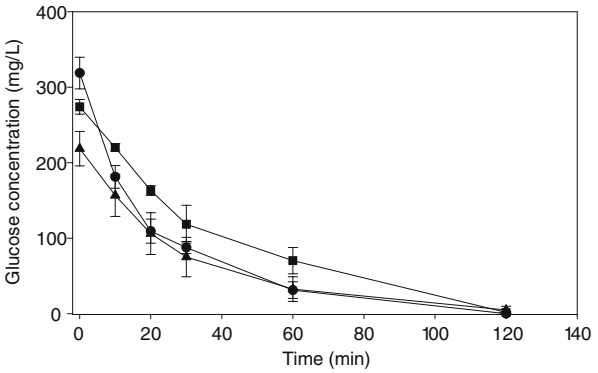

Fig. 2 Decrease of D-glucose in solution with D-xylose using GOD-TBC in the presence of immobilized CAT. *Filled circle*, experiment A (100 mg GOD-TBC+100 mg CAT-Eup); *filled square*, experiment B (100 mg GOD-TBC-CAT); *filled triangle*, experiment C (200 mg GOD-TBC-CAT)

D-Glucose from a Mixture with D-Xylose". All three dependences were similar, but the most complete decrease of D-glucose was achieved using 200 mg of GOD-TBC-CAT. GC analysis of the reaction mixture after the last experiment had proceeded for 4 h determined a final glucose concentration of 0 mg/l, whereas the D-cellobiose concentration did not change.

Biotransformation Elimination of D-Xylose from a Mixture with D-Lyxose

Although D-xylose oxidation with immobilized GOD is approximately 280 times weaker than D-glucose (Table 1), the ability to remove D-xylose from D-lyxose mixture was tested. These two pentoses are distinguished by the position of the hydroxyl group at position 3 C. The results of kinetic experiments ("Kinetic Dependences of Oxidation of Various Saccharides Using GOD-TBC") showed only slight oxidation of D-xylose and no oxidation of D-lyxose. The biotransformation experiment of the solution with D-lyxose and D-xylose in the presence of 200 mg of GOD-TBC-CAT was allowed to proceed for a much longer time period (168 h). The changes in concentration of D-xylose and D-lyxose, determined by

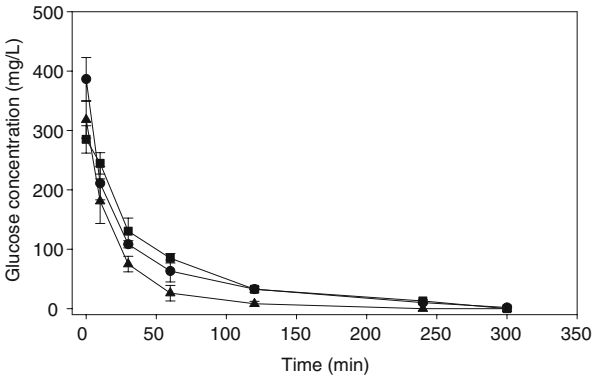

Fig. 3 Decrease in concentration of D-glucose in solution with D-cellobiose using GOD-TBC in the presence of immobilized CAT. *Filled circle*, experiment A (100 mg GOD-TBC+100 mg CAT-Eup); *filled square*, experiment B (100 mg GOD-TBC-CAT); *filled triangle*, experiment C (200 mg GOD-TBC-CAT)

GC during the oxidation process, were presented in Fig. 4. The concentration of D-xylose decreased from 400 to 0.24 mg/l, and the D-lyxose concentration decreased from 9,600 to 9,473 mg/l.

Conclusion

The hypothesis that D-saccharides with a hydroxyl group at position 3, equatorial to the pyranose ring, can serve as substrates of GOD was demonstrated by Pazur [4], Leskovac [5], and Pezzotti [6] by enzymatic reaction in the solution. Our results with immobilized GOD on bead cellulose using saccharide substrates such as D-mannose, D-galactose, D-fructose, and D-xylose and the disaccharide D-cellobiose confirmed this hypothesis. Other tested saccharides either did not function as substrates (D-lyxose, D-ribose, D-allose, D-tagatose) or were oxidized at extremely low rates (D-sorbose, L-sorbose, D-arabinose).

The differences between oxidation rates of saccharides with immobilized GOD have been utilized for the elimination of minor amounts of D-glucose from D-xylose or D-cellobiose (within 2–3 h) and D-xylose from D-lyxose (within 1 week) solution mixtures. The application of immobilized GOD has some advantages because of simple removal of glucose from reaction mixture with improved operational stability and reusability compared to the approach based on a soluble enzyme. The presence of immobilized CAT accelerated elimination of saccharide admixture and decreased the possibility of GOD inhibition with hydrogen peroxide produced. The co-immobilized GOD and CAT was prepared by a simple and fast method to achieve the enhanced efficiency of elimination of saccharide admixtures. The treatment with GOD-TBC-CAT provided a convenient and quantitative means for elimination of residual amount of D-glucose and D-xylose. This method can be helpful for the synthesis of monosaccharides for which the desired saccharide product is contaminated with D-glucose or D-xylose. A complete removal of gluconic (xylonic) acid from a reaction mixture could be achieved by a procedure described in our previous work [8].

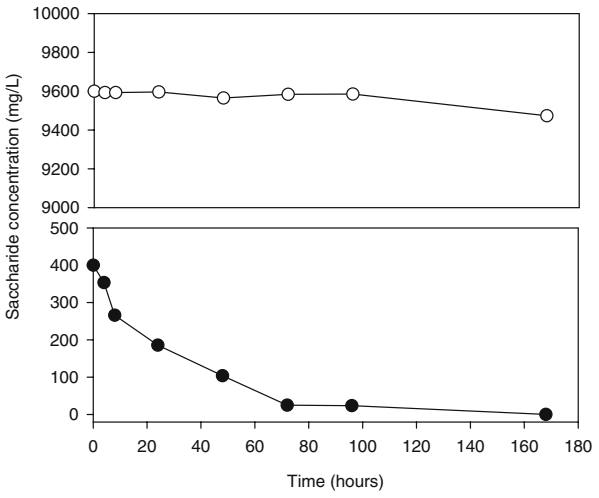

Fig. 4 Biotransformation of D-xylose and D-lyxose with GOD-TBC-CAT. *Filled circle*, D-xylose; *empty circle*, D-lyxose

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