The specificity of recombinant *Microdochium nivale* carbohydrate oxidase

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The kinetic parameters of oxidation of carbohydrates catalyzed by recombinant *Microdochium nivale* carbohydrate oxidase (rMnO) were determined. The cation radical of phenoxazine 10-propionic acid (PAPX) was used as an artificial electron acceptor. Among the carbohydrates studied, value of apparent reductive constant (k_{red}) related to the specificity of the enzyme was the highest for cellobiose. The carbohydrates according to k_{red} decrease ranked as follows: cellobiose (100%), cellotriose (21%), cellotetraose (15%), maltotetraose (0.39%), maltotriose (0.22%), maltose (0.18 %) and glucose (0.06 %). A comparison of the specificity of cello-oligosaccharides showed that the central factor of k_{red} decrease was related to a decrease of the catalytical constant. The decrease of the activity of maltooligosaccharides was determined primarily by a decrease of the bimolecular constant of enzyme interaction with carbohydrate.

Key words: carbohydrate oxidase, *Microdochium nivale*, kinetics, carbohydrate, substrate specificity

INTRODUCTION

Carbohydrate oxidases containing flavin as a cofactor may catalyze the oxidation of monohydrates in the 1st or 2nd position. Glucose oxidase (β-D-glucose oxidase: oxygen 1-oxidoreductase, EC 1.1.3.4) from fungal sources such as *Aspergillus niger*, *Penicillium notatum* and *Penicillium vitale* belongs to the first type of oxidases catalyzing the oxidation of certain aldoses to the corresponding lactones with highest activity towards β-D-glucose [1-5]. Other flavoprotein oxidases catalyze the oxidation of D-glucose and some other carbohydrates at carbon 2 in their pyranose forms. Glucose 2-oxidase (pyranose oxidase, pyranose:oxygen-2-oxidoreductase, EC 1.1.3.10) has been synthesized by a number of basidiomycete fungi such as *Polyporus obtusus*, *Phanerochaete chrysosporium*, *Peniophora gigantea*, *0udemansiella mucida* and *Coriolus versicolor* [6–8]. The oxidation of D-glucose in the presence of glucose 2-oxidase results in the formation of D-glucosone (2-keto-D-glucose; D-arabino-2-hexosulose) and hydrogen peroxide [6]. Glucose 2-oxidases exhibit a broad specificity showing the highest activity towards D-glucose, D-xylose and L-sorbose [6]. During turnover the both types of oxidases produce reduced flavin, which reacts with different electron acceptors [1–8].

A novel flavin containing carbohydrate oxidase from *Microdochium nivale* (rMnO) has been identified, cloned and heterologously expressed in *Aspergillus oryzae* [9, 10]. The gene encoding the protein showed a sequence with low homology (≤ 25% identity or 65% similarity) to other known flavins containing carbohydrate oxidases [10]. The rMnO showed a broad substrate specificity towards carbohydrates reacting with aldoses in the 1-position and it was attributed to the first type of oxidases [11]. The rMnO oxidized β-form of D-glucose and the product of D-glucose oxidation was D-gluconic acid [11]. The mechanism of carbohydrate oxidation by oxygen and artificial electron acceptors has been described by a ping-pong scheme [11]. Compared to *Aspergillus niger* glucose oxidase (GO), the reactivity of rMnO at pH 7.0 was significantly lower; k_{cat} was 20, k_{ox} 11 and k_{red} 22 times less, using oxygen as electron acceptor. The reactivity was also low with artificial two electron acceptors. However, compared to oxygen, rMnO showed a much higher activity towards single electron acceptors.

The aim of this investigation was to determine the kinetic parameters of rMnO-catalyzed oxidation of oligoglucosides with a special emphasis on the specificity of 1,4-α- and 1,4-β-oligoglucosides.

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EXPERIMENTAL

Materials and methods

Recombinant *Microdochium nivale* carbohydrate oxidase (rMnO) as a solution in 20 mM Tris-HCl buffer solution containing 0.1 M NaCl, pH 7.2, recombinant *Coprinus cinereus* peroxidase (rCiP) and *Aspergillus niger* catalase were received from Novozymes A/S (Denmark). The concentration of rMnO was determined spectrophotometrically at 450 nm, using the differential extinction coefficient of the flavin cofactor of 1.31×10^4 $M^{-1}cm^{-1}$ [3]. Phenoxazine 10-propionic acid (PAPX) was synthesized at the Institute of Biochemistry, Vilnius. Nitrosonium-tetrafluorborate was received from Aldrich. Glucose (α, β-D-Glc) and maltose (α-D-Glc-[1 → 4]-D-Glc) were from Reachim, Russia. Cellotriose ([β-D-Glc-(1 → 4)]$_2$-D-Glc), cellotetraose ([β-D-Glc-(1 → 4)]$_3$-D-Glc) were from Merck. Cellobiose (β-D-Glc-[1 → 4]- D-Glc), maltotriose ([α-D-Glc-(1 → 4)]$_2$-D-Glc), maltotetraose ([α-D-Glc-(1 → 4)]$_3$-D-Glc) were from Sigma. Solutions of carbohydrates were made in distilled water and allowed to mutarotate 1 day before use. All the concentrations of carbohydrates and calculated kinetic parameters are expressed in the terms of total amount of α and β anomers. Buffer reagents and other chemicals were of analytical grade.

To prepare the cation radical of PAPX by the enzymatic method, 50 µl of PAPX solution in methanol (14 mM) was added to 1 ml of 50 mM phosphate buffer solution, pH 7.2, containing 20 µl of rCiP (1 µM) and 2 × 50 µl of hydrogen peroxide (20 mM). The reaction was terminated after 5 min of incubation by addition 20 µl of catalase (132 000 CIU/ml). The final concentration of catalase was 100 nM. To prepare the cation radical by the chemical method, 202 mg of PAPX was dissolved in 1 ml of methanol. Nitrosonium-tetrafluorborate (107 mg) was also dissolved in 1 ml of methanol. Both solutions were cooled on ice. $NOBF_4$ solution was added dropwise to PAPX solution. Methanol was evaporated under vacuum, the residue was washed twice with heptane (20 ml) and dried in a vacuum desiccator.

Kinetic measurements and calculations

The reduction of cation radical of PAPX was monitored spectrophotometrically, using a computer-assisted Gilford Instrument 2600 spectrophotometer in 1 cm optical length quartz cells at 25 °C and aerobic conditions and pH 7.2. The kinetic curves were recorded at 530 nm, *i.e.* at maximum absorbance of $PAPX^{+\cdot}$. The concentration of the PAPX cation radical was calculated using its experimentally determined extinction coefficient 1.6×10^4 M^{-1} cm^{-1}. The reaction rate was calculated by fitting the kinetic curves to a third-order polynomial function, and the dependence of the initial rate on the substrate concentration was used for further calculations. The rate dependence on substrate (cation radical and carbohydrate) concentrations was analyzed by applying the ping-pong scheme of the enzyme action [3]:

$$E_{ox} + S \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} E_{ox} - S \xrightarrow{k_2} E_{red} + P, \quad (1)$$

$$E_{red} + M_{ox} \xrightarrow{k_3} E_{ox} + M_{red}. \quad (2)$$

Following this scheme, the dependence of the reaction rate on substrate concentration was expressed:

$$[E]_t/v = (1/k_{cat}) + 1/(k_{red} \cdot [S]_0) + 1/(k_{ox} \cdot [M]_0), \quad (3)$$

where $k_{cat} = k_2$, $k_{red} = k_1 \cdot k_2/(k_{-1} + k_2)$ and k_{ox} $k_{ox} = k_3$ are catalytic, apparent reductive and oxidative constants, respectively, $[M]_0$ and $[S]_0$ are the initial acceptor ($PAPX^{+\cdot}$) and carbohydrate concentration, respectively, $[E]_t$ is the total enzyme concentration.

At a saturated concentration of one of the substrates the rate was dependent on the concentration of the other substrate. When the concentrations of both substrates were infinite, the rate was limited by the catalytic constant (k_{cat}).

Apparent $K_{m(S)}$ and $K_{m(M)}$ for carbohydrate and acceptor were calculated according to equations 4 and 5, respectively:

$$K_{m(S)} = k_{cat}/k_{red}, \quad (4)$$

$$K_{m(M)} = k_{cat}/k_{ox}. \quad (5)$$

At a low carbohydrate concentration, when $[S] < K_{m(S)}$, and a saturated concentration of electron acceptor the initial rate is linearly proportional to the carbohydrate concentration:

$$V = k_{red} \cdot [E]_t \cdot [S]_0. \quad (6)$$

The relative rate estimated in these conditions and a fixed substrate concentration correspond to the ratio of reductive constants and has been called the "specificity" of carbohydrates.

RESULTS AND DISCUSSION

rMnO reaction with PAPX cation radical

The solution of PAPX cation radical showed maximal absorbance at 530 nm. At pH 7.2, in the pre-

sence of 0.1 M of glucose and 2.2 nM rMnO, the cation radical was reduced and the color bleached during 2–3 minutes. The kinetic parameters were calculated from the initial rate of the cation radical reduction. The apparent bimolecular constant of PAPX cation radical reduction (k_{ox}) rMnO was 3.4 · 10^7 $M^{-1}s^{-1}$. At a $PAPX^{+\cdot}$ concentration higher than 10–15 μM the reduction rate practically was independent on its concentration and limited by the enzyme turnover number (k_{cat}). The calculated catalytic constant of the enzyme was 44 s^{-1} and the apparent $K_{m(M)}$ of PAPX cation radical was 1.3 μM. The low apparent $K_{m(M)}$ and high k_{ox} enabled the use of PAPX cation radical as an artificial electron acceptor in the investigations of rMnO reaction with different substrates.

rMnO catalyzed oxidation of different carbohydrates

The kinetics of PAPX cation radical reduction at different concentrations of cellotriose is shown in Fig. 1. The kinetic parameters calculated from the initial rate are listed in Table. However, application of the calculated parameters to full kinetic curves revealed a large divergence from the experiment (Fig. 1). To explain this divergence, a suggestion was made that only the β-form of carbohydrates had been oxidized. The β-anomeric specificity has been reported previously for glucose [11]. The concentration of β anomer may be part of total carbohydrate concentration. The suggestion was confirmed when the oxidation of carbohydrates was carried out at different enzyme concentrations (Fig. 2). It was shown that the initial reaction rate was dependent on enzyme concentration. However, the rate became practically independent on enzyme concentration at a longer time. Possibly at this stage the rate was limited by carbohydrate mutarotation.

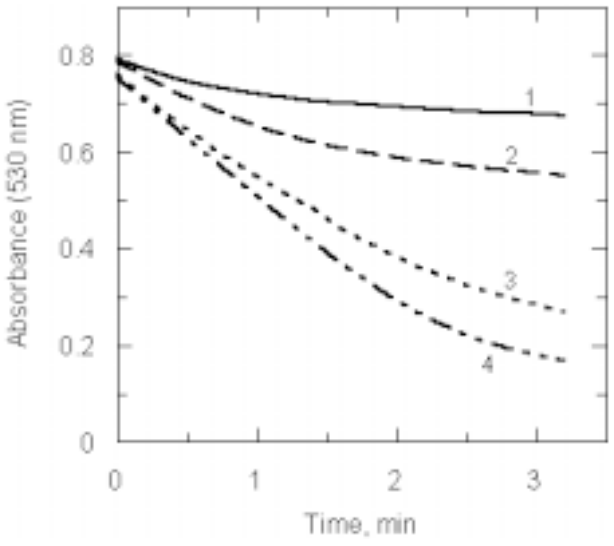

Fig. 1. Decrease of $PAPX^{+\cdot}$ absorbance in presence of different cellotriose concentrations and rMnO. Cellotriose concentration 34 (1), 68 (2), 136 (3), 272 (4) μM, rMnO 15 nM

Table 1. **Kinetic parameters of carbohydrate oxidase *M. nivale* at pH 7.2, 25 °C, and of *Phanerochaete chrysosporium* cellobiose dehydrogenase (k_{CDH}) at pH 5.0, 35 °C** [(a)]

Substrate	k_{cat} s^{-1}	$K_{m(S)}$ M	k_{red} $M^{-1}s^{-1}$	k_{CDH} $M^{-1}s^{-1}$
Glucose	–	–	$5.0 \cdot 10^2$	1.6
Cellobiose	43.9	$5.1 \cdot 10^{-5}$	$8.5 \cdot 10^5$	$1.4 \cdot 10^5$
Cellotriose	10.5	$5.9 \cdot 10^{-5}$	$1.8 \cdot 10^5$	$9 \cdot 10^4$
Cellotetraose	8.5	$6.6 \cdot 10^{-5}$	$1.3 \cdot 10^5$	$5 \cdot 10^4$
Maltose	17.7	$1.2 \cdot 10^{-2}$	$1.5 \cdot 10^3$	4.8
Maltotriose	–	–	$1.9 \cdot 10^3$	–
Maltotetraose	–	–	$3.3 \cdot 10^3$	–

[(a)] The kinetic constants of CDH from [12].

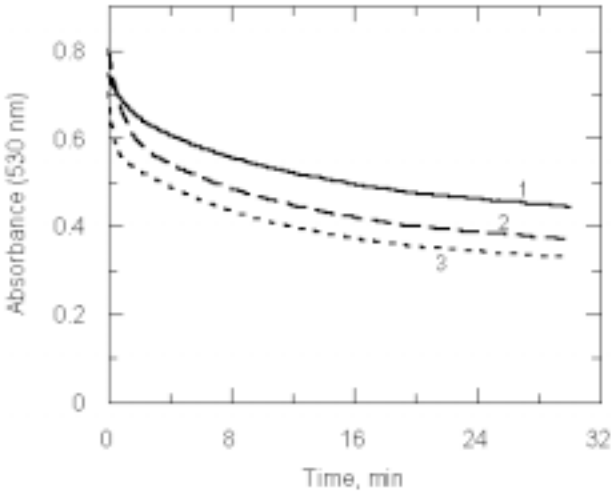

Fig. 2. Decrease of $PAPX^{+\cdot}$ absorbance in presence of cellotriose and different rMnO concentrations. Cellotriose concentration 72 μM, rMnO 7 nM (1), 14 nM (2), 28 nM (3)

The specificity of rMnO was investigated using malto- and cellooligosaccharides. They contained a glucose molecule as a monomer unit, but in different anomeric forms. Maltooligosaccharides contained glucose in α-form and cellooligosaccharides in β-form. All the carbohydrates tested contained glucosyl units linked (1 → 4) by glycoside bonds. Among the carbohydrates studied, the k_{red} value was highest for cellobiose (Table). k_{red} for cellotriose and cellotetraose decreased in comparison to cellobiose. k_{red} of maltooligosaccharides increased as the number of glycosyl units increased, but did not exceed the values of cellooligosaccharides.

The specificity of carbohydrates, as is shown above, correlated with k_{red}, which was dependent on k_1, k_{-1} and k_{cat} (eq. 1, 2). If k_{-1} changed little for diffe-

rent carbohydrates, k_{red} change was associated with k_1 and k_{cat} change. A comparison of the specificity of cellooligosaccharides showed that the main factor of specificity decrease was related to k_{cat} decrease. However, the decrease of k_{red} of maltooligosaccharides was determined mainly by an apparent decrease of the bimolecular constant (k_1).

It is worth noting that the specificity of rMnO is similar to that of cellobiose dehydrogenase (CDH) (Table). The preferred substrates for CDH are di- or polysaccharides, whereas monosaccharides such as glucose are poor substrates. The discrimination against glucose by CDH is strong, as indicated by an 87000-fold higher specificity constant (k_{cat}/K_M) for cellobiose compared to glucose [12]. Investigations of the crystal structure of the flavoprotein domain of CDH allowed to hypothesize that the catalytic site of the enzyme has two hexose binding subsides [13]. The difference of the constants for glucose and cellobiose suggested that the subside binding the nonreducing end of cellobiose is the specificity-determining site in CDH. The constants of rMnO may indicate a similar structure of the active center of the carbohydrate oxidase studied.

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REKOMBINANTINĖS *Microdochium nivale* ANGLIAVANDENIŲ OKSIDAZĖS SPECIFIŠKUMAS

S a n t r a u k a

Nustatyti *Microdochium nivale* angliavandenių oksidazės (rMnO), katalizuojamos angliavandenių oksidacijos, kinetiniai parametrai. PAPX katjonas-radikalas buvo naudojamas kaip dirbtinis elektronų akceptorius redukuotai rMnO. Tarp tirtų angliavandenių didžiausia tariamosios redukcinės konstantos (k_{red}), kuri atitinka reakcijos greitį esant mažoms substrato koncentracijoms ir fermento specifiškumą, reikšmė nustatyta celobiozei. Angliavandenių aktyvumas k_{red} mažėjimo tvarka buvo toks: celobiozė (100%), celotriozė (21%), celotetraozė (15%), maltotetraozė (0,39%), maltotriozė (0,22%), maltozė (0,18%) ir gliukozė (0,06%). Palyginus celooligosacharidų specifiškumą matyti, kad pagrindinis k_{red} mažėjimo veiksnys yra susijęs su katalitinės konstantos sumažėjimu. Maltooligosacharidų aktyvumo mažėjimą daugiausia nulėmė bimolekulinės fermento ir angliavandenio sąveikos konstantos mažėjimas.