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Cellulose oxidation and bleaching processes based on recombinant *Myriococcum thermophilum* cellobiose dehydrogenase

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ABSTRACT

Myriococcum thermophilum cellobiose dehydrogenase (*Mt*CDH) was expressed in *Pichia pastoris* using the pPICZαA expression vector under the control of methanol inducible AOX promoter. The purified recombinant *Mt*CDH with a specific activity of 3.1 U mg^{-1} was characterized to obtain kinetic constants for various carbohydrate substrates. Additionally, the C1 oxidation of the reducing ends of cellobiose, cellotetraose and maltotriose by MtCDH was verified by HPLC-MS. *Mt*CDH was employed to oxidize several different cellulose-based materials by production of hydrogen peroxide. Based on the obtained results a one-pot enzymatic scouring/bleaching process for cotton fabrics was developed using pectinases as scouring agent and *Mt*CDH to produce H_2O_2 for bleaching. An average increase in whiteness (Berger) ΔE of 26 and an average 95% increase in wettability were observed in all *Mt*CDH treated fabrics. In addition, *Mt*CDH oxidized typical colored cotton flavonoids (morin, rutin, isoquercitrin).

1. Introduction

Cellobiose dehydrogenase (EC 1.1.99.18) is the only currently known extracellular flavocytochrome produced by wood degrading fungi. It oxidizes cellobiose and higher cello-oligosaccharides using a wide range of electron acceptors like quinones, phenoxyradicals, redox dyes, Fe^{3+}, Cu^{2+} and oxygen [1–3]. CDH was first isolated from the basidiomycetes *Phanerochaete chrysosporium* and *Trametes versicolor*. It is expressed under cellulolytic conditions in the presence of cellulose concomitantly with cellulases and hemicellulases [4,5]. Judging from its activity of oxidizing cellobiose and use of many electron acceptors a number of *in vivo* functions have been attributed to CDH. These include preventing inhibition of cellulases by cellobiose due of its involvement in converting cellobiose into cellobiono-δ-lactone as well as reducing quinones and phenoxy radicals generated by lignin peroxidase or laccase during lignin degradation thereby preventing polymerization [2,6]. Recently, a synergistic interaction of CDH and glycoside hydrolase 61 was demonstrated to enhance cellulose depolymerization [7]. CDH has also been shown to oxidize or reduce many industrially interesting molecules such as cellodextrins, maltose, lactose, diphenolic compounds and catecholamines [8,9]. This ability is exploited by biotechnologists for the development of biosensors [10–13] biofuel cell anodes [14,15], for the monitoring of cellulose degradation and for bleaching pulps [2,16]. The production of H_2O_2 is a remarkable property that can extend the use of CDH to applications like cotton bleaching, detergents [17], aseptic packaging of foods, detoxification of cyanide tailings in mines and the destruction of soluble cyanides, sulfides and phenols.

In this study a *Myriococcum thermophilum* cellobiose dehydrogenase (*mtcdh*) gene (EF492052) was codon-optimized for expression in *Pichia pastoris* using the pPICZαA expression vector under the control of the methanol inducible AOX promoter. The ability of the recombinant *M. thermophilum* cellobiose dehydrogenase *Mt*CDH to produce H_2O_2 from different cellulose based substrates for *in situ* cotton bleaching systems was further investigated. Bleaching of cotton is an essential step for achieving a good level of whiteness, especially for material to be dyed in lighter shades since scouring processes do not significantly remove natural colorants from the cotton fiber. The bleaching process is therefore important to remove the yellowish or brown coloration of the cotton fiber caused by protein and flavone pigments of cotton flowers [18]. *Mt*CDH can produce H_2O_2 for bleaching cotton either from cellulose and cellooligomers or from carbohydrates (starch or cellulose derivatives, galactomannans) used for sized cloths, which are liberated in the enzymatic desizing process. In contrast to glucose oxidase, which has previously been suggested for this purpose [19–21] CDH is not limited to glucose as substrates and can use a wide range of mono- and oligosaccharides [17].

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2. Materials and methods

2.1. Materials

All chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA) and were of the highest purity available. The cellooligomers (cellobiose, cellotetraose, hexaose) and maltotetraose and the cellulase (*endo*-β-glucanase) from *Aspegillus niger* were purchased from Megazyme, Ireland. *A. niger* glucose oxidase was purchased from Sigma–Aldrich. The sized fabrics were a kind gift provided by Dr. Jan Marek from Inotex, Dvůr Králové, Czech Republic. Chromatographic material and equipment was from GE Healthcare (Uppsala, Sweden), restriction enzymes from Fermentas (Vilnius, Lithuania) and the Phusion polymerase from New England Biolabs (Ipswich, UK). Synthetic oligonucleotides were synthesized by VBC-Biotech (Vienna, Austria). Strains and plasmids used for protein expression in *P. pastoris* were from the EasySelect Pichia Expression Kit (Invitrogen, CA, USA).

2.2. Heterologous expression of M. thermophilum CDH in P. pastoris and purification of enzyme

The *mtcdh* gene (identifier EF492052) was codon-optimized for expression in *P. pastoris* according to the method described in Abad et al. [22] and synthesized by Gen-script (Piscataway, NJ, USA). cDNA encoding for *Mt*CDH was excised from the plasmid pMTSopt using *Eco*RI and *Not*I and cloned into the expression vector pPICZαA along with its native signal sequence. Correct insertion of the gene and the absence of mutations were checked by DNA sequencing (LGC Genomics, Berlin, Germany). The *Sac*I linearized expression plasmid was transformed into electro-competent X-33 cells and transformants were selected on YPD Zeocin plates (1 mg L^{-1}). A 1 L preculture preincubated in 1 L baffled flasks containing 200 mL BMGY medium without methanol for 18 h was used as inoculum. The inoculum was then transferred into 70-L batch bioreactor (Applikon, Schiedam, The Netherlands) filled with 40 L of production medium (Yeast Basal Medium) and immediately induced with methanol. Cultivation was stopped at day five of methanol induction, cells removed by centrifugation (4000 rpm, 20 min) and the enzyme precipitated with 20% ammonium sulfate. *Mt*CDH was partially purified by a one-step purification. The solution was loaded onto a Phenyl-Sepharose Fast Flow column (8500 mL; flow rate 330 mL min^{-1}) equilibrated with 50 mM phosphate buffer (pH 5.5, 20% $(NH_4)_2SO_4$). After washing the column with 1 column volume of the same buffer, *Mt*CDH was eluted with a linear gradient of starting buffer to 50 mM phosphate buffer (pH 6.5) in 1 column colume and fractions were collected. Fractions were tested for CDH activity with the DCIP assay described below and pooled. Purified enzymes were concentrated and diafiltration in 50 mM citrate buffer, pH 5.5, aliquoted and stored at 4 °C for further use. Protein concentration in the culture filtrate and during purification was determined by using bicinchoninic acid protein assay reagent (Sigma, Pierce, Rockford, IL, USA) using bovine albumin as standard.

2.3. Measurement of cellobiose dehydrogenase activity

The activity of cellobiose dehydrogenase was assayed according to Baminger et al. [23] with slight modifications. Briefly, CDH activity was measured by monitoring the decrease in absorbance of 2,6-dichlorophenolindophenol (DCIP), at 520 nm ($\varepsilon_{520} = 6.8 \times 10^3$ M^{-1} cm^{-1}), pH 4.5 and 30 °C using a Hitachi UV-VIS 2001 spectrophotometer. The reaction mixture (in a total volume of 1 mL) contained the following: 100 μL DCIP (3 mM in water containing 10%, v/v ethanol), 100 μL lactose (300 mM in 100 mM sodium acetate buffer, pH 5.0), and the appropriate amount of the same buffer. After temperature adjustment, the reaction was started by addition of an appropriately diluted CDH sample (50 μL) and the decrease in absorbance was monitored during the first 2 min. For calculating the CDH activity only the linear range of the slope was used. One unit of enzyme activity is defined as the amount of enzyme reducing 1 μmol of DCIP per min under the above reaction conditions.

2.4. Enzyme kinetic measurements

Kinetic constants of *Mt*CDH were measured by monitoring the decrease in absorbance of DCIP, at 520 nm ($\varepsilon_{520} = 6.8 \times 10^3$ M^{-1} cm^{-1}), pH 4.5 and 30 °C. The sugar solutions used for measuring activity and kinetic constants were all prepared in buffer and left to equilibrate for at least several hours. The reaction mixture (in a total volume of 1 mL) contained 100 μL DCIP and varying concentrations of carbohydrates in sodium acetate buffer, pH 4.0. All measurements were performed at 30 °C and the kinetic constants calculated by nonlinear least-squares regression, fitting the observed data to the Michaelis–Menten equation (Sigma Plot 11, Systat Software).

2.5. Production of H_2O_2 in the presence of different substrates

The ability of the *Mt*CDH to produce H_2O_2 was investigated in the presence of different carbohydrates including cello-oligomers, lactose, maltose and glucose as electron donors. The reaction was conducted as described by Pricelius et al. [24]. The reaction mixture contained 555 μL of 50 mM sodium phosphate buffer pH 6.5, 100 μL of 300 mM substrate solution, 100 μL of desferrioxamine mesylate (1 mM) and 0.5 U mL^{-1} of *Mt*CDH (enzymatic activity determined at pH 6.5). Since the activity of CDH interferes with the peroxidase activity, the incubation mixture was boiled for 2 min to denature the CDH, which does not influence the H_2O_2 level to any measurable extent. Then the reaction mixture was incubated at 40 °C for 30 min. The color reaction was initiated by the addition of 50 μL horseradish peroxidase (1 mg mL^{-1}), 50 μL of leuco crystalviolet (1 mM dissolved in 0.06 M HCl) and 500 μL sodium acetate buffer (100 mM, pH 4). The absorbance of oxidized crystal violet was measured at 592 nm with a UV/visible spectrophotometer. The absorbance achieved is linearly correlated to the concentration of H_2O_2. Samples without enzyme were used as blank reactions and the calibration curve was obtained by using H_2O_2 in the concentrations from 0 to 200 μM. Similarly the activity of glucose oxidase was measured by incubating 0.5 U mL^{-1} with D(+)-glucose in 0.1 M acetate buffer pH 5 at 50 °C before measuring the amount of H_2O_2 with horseradish peroxidase as described above.

2.6. Oxidation of cellulose

To investigate the ability of *Mt*CDH to oxidize cellulose, a Whatman No. 1 cellulose filter (1 cm × 6 cm) was immersed in 2 mL buffer as and manually crushed and further incubated with 2.56 U mL^{-1} *Mt*CDH at 40 °C for 60 min in 100 mM acetate buffer pH4. The sample was then washed with double distilled several times and ethanol and then dried in an oven. The sample was then monitored using FTIR in the range 650–1800 cm^{-1}. Another set of sample of Whatman No. 1 cellulose filter (1 cm × 6 cm) was incubated with cellulase (*endo*-β-glucanase) from *A. niger* (final activity of 5 U mL^{-1}) at pH 4.8 and 50 °C for 20 min. The resulting partially hydrolyzed cellulose fragments were further incubated under similar conditions in the presence of 2.56 U mL^{-1} *Mt*CDH. The ability of *Mt*CDH to oxidize the cellulose fragments generated by endoglucanase was analyzed by HPLC-MS using cellotetraose and cellohexaose as standards. A Dionex HPLC-UVD-system equipped with a P580 pump, an ASI-100 autosampler and a PDA-100 photodiode array detector was used to separate hydrolysates using a reversed phase HPLC amine column (Supelcosil™ LC-NH2 5 μm, 250 mm × 4.6 mm, Supelco, USA). An isocratic method using double distillate water and acetonitrile in a 1:4 ratio was used a mobile phase. The MS spectra were acquired with an Agilent Ion Trap SL (Palo Alto, CA, USA) equipped with an electrospray ionization system. Dry gas flow was set to 12 L min^{-1} with a temperature of 350 °C, nebulizer set to 70 psi. Maximum accumulation time was fixed to 300 ms and the loading of the trap was controlled by the instrument with an ICC of 30,000. The electrospray voltage set at +3500 V and the coupling products were measured in negative ion mode.

2.7. In vitro bleaching systems

The ability of the CDH to produce H_2O_2 for cotton bleaching associated with some phenolic compounds present in cotton (morin, rutin, isoquercitrin) was investigated. The reaction mixture contained 5 mM final concentration of phenolic compounds, 50 μL of *Mt*CDH (14 U mL^{-1}), 300 mM cellobiose in 0.1 M sodium acetate buffer pH 4. The reaction mixture was incubated at 60 °C for 1 h. The reaction was stopped by cooling the tubes on ice. The bleaching of the different phenolics was monitored using a UV-VIS spectrophotometer in wavelength scan mode within the range 200–800 nm at time 0 and after 1 h. Samples without *Mt*CDH were prepared and were monitored in the same way.

2.8. Enzymatic desizing and scouring of cotton fabrics

Sized cotton fabrics (60 cm^2) were incubated with amylase from *Bacillus amyloliquefaciens* at 60 °C for 2 h. The reaction mixture contained 2.5 mL of enzyme (2.56 U mL^{-1}), 15 mL and 0.1 M sodium acetate buffer pH 5. After desizing, 0.2 mL pectinase from *Aspergillus aculeatus* (13.7 U mL^{-1}) was added to the reaction solution for scouring. The fabric was incubated at 60 °C for 2 h. For comparison one sample from the desizing solution was scoured *via* boiling in 0.2 M sodium hydroxide solution for half an hour. Both desizing and scouring liquors were mixed and used as substrates for the production of H_2O_2 using *Mt*CDH or glucose oxidase. The desized and scoured fabric was washed and dried and their weight determined before the bleaching step.

2.9. Enzymatic bleaching of cotton fabrics

The desized and scoured fabrics were first weighed and then bleached in the desizing bath at 60 °C. The reaction was started by adding 2.5 mL rec*Mt*CDH or glucose oxidase and desizing liqours. Different experimental conditions were investigated including production of H_2O_2 under optimal enzyme conditions followed by bleaching, simultaneous production of H_2O_2 and bleaching, production of H_2O_2 and bleaching in the presence of metals and of TAED (N,N,N′,N′-tetraacetylethylendiamine). The samples were incubated for 1 h, rinsed with deionized water and dried at 40 °C for 24 h. For monitoring the weight loss of the samples, each sample was weighted before and after treatment. Before measuring the weight, samples were dried at 40 °C for 24 h. The change in color that is whiteness (Berger whiteness), color differences (ΔE) was measured by using a ColorLite Sph850 Spectrophotometer (Colorlite GmbH, Vienna, Austria).