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Cellobiose dehydrogenase from the ligninolytic basidiomycete *Phlebia lindtneri*

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ABSTRACT

Cellobiose dehydrogenase (CDH), an extracellular flavocytochrome produced by several wood-degrading fungi, was detected in the culture supernatant of the selective delignifier *Phlebia lindtneri* maintained on a cellulose-based liquid medium. Cellobiose dehydrogenase was purified to homogeneity by a rapid procedure, using ammonium sulfate precipitation, ion-exchange chromatography, and chromatofocusing. The enzyme was recovered with a 61.2 fold increased specific activity and a yield of 47.5%. As determined by SDS-PAGE, the molecular mass of the purified enzyme was found to be 104.5 kDa and its isoelectric point was 4.0. The carbohydrate content of the purified enzymes was 22%. In this work, the cellobiose dehydrogenase gene *cdh1* and its corresponding cDNA from fungi *Phlebia lidnteri* were isolated, cloned, and characterized. The 2319 bp full-length cDNA of *cdh1* encoded a mature CDH protein containing 755 amino acids, which was preceded by a signal peptide of 17 amino acids. The deduced protein sequence of *cdh1* shared significant similarity with other known fungal cellobiose dehydrogenase.

1. Introduction

The recent increase in applications of fungal wood decomposing enzymes including cellulolytic, hemicellulolytic and lignolytic complexes [1] has aroused considerable interest in cellobiose dehydrogenase. Its narrow specificity toward electron donors and high sensitivity toward a multitude of electron acceptors has been used in several analytic assays and sensors, waste removal/bioremediation and enzymatic treatment of pulp [2]. The increase in the number of characterized cellobiose dehydrogenases from various producers would allow to take advantage of its potential in new biotechnological applications.

Cellobiose dehydrogenase (CDH; EC1.1.99.18; cellobiose (acceptor) 1-oxidoreductase) is an extracellular flavocytochrome secreted by some white rot and brown rot plant pathogenic and saprotrophic fungi from the dicaryotic phyla of *Basidiomycota* and *Ascomycota* [2,3]. CDHs are involved in the cellulolytic enzyme pathway of these fungi, although their exact physiological role remains rather unclear. Lately, cellobiose dehydrogenase was suggested to enhance the activity of polysaccharide monooxygenase in cellulose degradation [4,5] similarly playing a significant role as feed back enzyme for ligninolytic oxidoreductases [6]. CDHs catalyze the oxidation of cellobiose (Glc-β-1,4-Glc) and other β-1,4-linked disaccharides or oligosaccharides at the C-1 position to the corresponding lactones [7,8]. The best-known CDH are glycoproteins with the molecular weight of 60 up to 130 kDa and a wide pI range from 3 to almost 8 [2,3,9,10].

CDH is a typical oxidoreductase with oxidative and reductive half-reaction activities which occur separately. This implies the ability of CDH to oxidize a saccharide in the C1 position, resulting in conversion of the hemiacetal at this position to a lactone, which hydrolyze spontaneously to a carboxylic acid. The two electrons taken up by the enzyme are further transferred to one two-electron acceptor, or to two one-electron acceptors [11–13]. The proposed electron transfer chain reaction may involve reduction of a ferric electron acceptor (such as cytochrome c) by the CDH ferrous heme, with reoxidation of the enzyme accomplished in two separate FAD–heme b-cytochrome c steps. In contrast, the electron sink mechanism would involve reduction of an electron acceptor by the fully-reduced FAD, followed by transfer of the second electron from the flavin semiquinone to the CDH heme [14].

The goal of the presented work was to isolate and purify the novel extracellular cellobiose dehydrogenase from the basidiomycete fungus *Phlebia lindtneri* and to fully characterize the enzyme, including determination of its physicochemical and kinetic properties. Moreover, we attempted to isolate and sequence the gene and cDNA corresponding to the cellobiose dehydrogenase protein.

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2. Materials and methods

2.1. Microorganism, growth conditions and production of cellobiose dehydrogenase

The white rot fungus *P. lindtneri* strain FCL22 was obtained from the culture collection of the Agriculture Academy in Cracow. The fungus was maintained on 3% (w/v) malt agar plates. To obtain the inocula, pieces of agar plates with the fungus were grown in the Lindenberg and Holm [15] medium in conical flasks for 10 days at 25 °C. Ten-day-old mycelia were homogenized in a disperser homogenizer T18 basic ULTRA-TURRAX (IKA, Staufen, Germany). The fragmented mycelial culture (10%, v/v) was used as a standard inoculum for further studies.

In order to obtain cellobiose dehydrogenase the culture of *P. lindtneri* strain 22 was grown on cellulose-based medium [16] with authors' modifications. The medium had the following composition (1l): 2 g Avicel, 10 g $(NH_4)_2HPO_4$, 1 g KH_2PO_4, 0.3 g $MgSO_4{\cdot}7H_2O_2$, 0.08 g $CaCl_2$, 5 mg $ZnSO_4{\cdot}7H_2O$, 1.5 mg $MnSO_4{\cdot}4H_2O$, 1.5 mg $CoCl_2{\cdot}6H_2O$, 5 mg $FeSO_4{\cdot}7H_2O$, 100 mg yeast extract, and 0.1 mg thiamine. The pH was adjusted to 6.5 with 5 M HCl. After inoculation, the cultures were incubated at 28 °C on a rotary shaker at 120 rpm for 6 days.

2.2. Enzyme activity assay

Cellobiose dehydrogenase activity was measured by following the decrease in absorbance of the electron acceptor 2,6-dichloroindophenol (DCIP) (Sigma Chemical Co., St. Louis, MO, USA) at 520 nm ($\varepsilon_{520} = 6.8\ mM^{-1}\ cm^{-1}$), pH 4.5 and 30 °C [17,18] with a Shimadzu UV-160A (Shimadzu, Tokyo, Japan) spectrophotometer. This assay measures the activity of an intact enzyme as well as of the catalytically active flavin domain. The reaction mixture (1 ml) contained the following: 50 μl of 3 mM DCIP (solution in water containing 10% v/v ethanol), 100 μl lactose (300 mM in 100 mM sodium acetate buffer, pH 4.5), 50 μl NaF (80 mM NaF) in water, and an appropriate amount of the same buffer. After temperature adjustment, the reaction was initiated by addition of diluted CDH sample (100 μl) and the decrease in absorbance was monitored during the first 60 s. The final enzyme activity was expressed as nkat per liter. This assay was used for determination of the activity of native enzyme as well as for the catalytically active flavin domain.

Alternatively, CDH activity was specifically determined by monitoring the reduction of 20 μM cytochrome c (from horse heart), at λ = 550 nm and at 30 °C (Sigma Chemical Co., St. Louis, MO, USA). The reaction was performed in 100 mM sodium acetate buffer, pH 4.5, containing 30 mM lactose and 4 mM NaF. The extinction coefficient (ε) was 19.6 $mM^{-1}\ cm^{-1}$ [19]. This assay determined the activity of the intact protein containing both the flavin and the heme domains.

Protein concentration was determined using the Bradford method [20] with crystalline bovine serum albumin (BSA) as a standard, or by monitoring the ultraviolet (UV) absorbance at 280 nm.

2.3. Enzyme purification and spectral characterization

The extracellular medium (6.2 l) from 6-day-old cultures was centrifuged at 12,000 × *g* on a 6K15 centrifuge (Sigma, Osterode am Harz, Germany) for 30 min. The supernatant was concentrated 10 times on the ultrafiltration system Pellicon 2 Mini holder (Millipore, Bedford, MA) with an Ultracel mini cartridge (10 kDa cut off polysulfonic membrane PM 10) and used as a source of a crude enzyme. The concentrated enzyme solution was precipitated by titration with 30–50% of ammonium sulfate at 0 °C. The ammonium sulfate precipitate was resolved in 100 ml deionized water and desalted using a Sephadex G-50 column. The desalted proteins were concentrated and applied to DEAE-Sepharose (fast flow) column (2.5 × 15), preequilibrated with 50 mM sodium acetate buffer, pH 5.0, connected to chromatographic EconoSystem (Bio-Rad, Richmond, VA) The column was eluted with a linear salt gradient (0–0.5 M NaCl in 50 mM sodium acetate buffer) at a flow rate of 1 ml/min. The active fractions were chromatofocused on an Econo-chromatography column (Bio-Rad, Richmond, VA; 1.5 × 20) with a Polybuffer exchanger PBE 94 equilibrated with 250 ml of 0.025 M imidazole-HCl buffer (pH 7.4). A sample showing CDH activity (5 ml) was injected onto the column and the enzyme was desorbed by elution with 200 ml Polybuffer 74-HCl (pH 2.8) at a flow rate of 0.5 ml/min. The active fractions were pooled out and the purified enzyme solutions were used for kinetic experiments.

The spectrum of homogeneous CDH was recorded from 250 to 650 nm in an oxidized and reduced form using a Shimadzu UV-160A spectrophotometer (Shimadzu, Tokyo, Japan). Oxidized and purified CDH was diluted in 100 mM sodium acetate buffer (pH 4.5) to an absorbance of ~2.5 at 280 nm and measured. The spectrum of the reduced enzyme was recorded 10 s after addition of lactose into the cuvette.

2.4. Electrophoresis and peptide sequencing by LC–MS/MS

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10%), was performed as described by Laemmli [21]. Proteins were visualized by silver staining and Coomassie Blue G250 using PageRuler Prestained Protein Ladder (Fermentas, Glen Burnie, MA, USA). Native PAGE (10%) was performed to identify the enzymatic activities of the proteins. For CDH activities, lanes were stained with 2 mM DCIP in 100 mM sodium acetate buffer (pH 4.5) containing 2 mM of cellobiose [22].

The spectrometric analysis of the polypeptides was carried out in the Environmental Laboratory of Mass Spectrometry, Institute of Biochemistry and Biophysics of the Polish Academy of Sciences in Warsaw (Poland). Protein samples obtained during enzyme purification were separated on SDS-PAGE gels in 10% Tris–HCl as in Walker [23] and visualized by silver staining according to the procedure described by Walker [23]. After the electrophoretic separation of the samples, equal pieces of 2 × 7 mm were cut out from gel lanes. The samples were analyzed by HPLC coupled with tandem mass spectrometry (liquid chromatography/two stage mass spectrometry (LC–MS/MS)) according to Kordan et al. [24]. The output list of precursor and product ions was compared with the protein database of the National Center for Biotechnology (NCBI, USA), using the MASCOT local server.

2.5. Enzyme characteristics

The optimum pH of the purified enzyme was studied over a pH range of 2.5–8.0 in 100 mM McIlvaine buffer using DCIP and cytochrom c as electron acceptors and lactose as a substrate. The pH stability of CDH was determined at 30 °C by incubation in variable pH ranges (Britton–Robinson buffer; pH 2.0–9.0) for 12 h followed by measurement of the residual activities every 30 min.

To determine the optimum temperature at which the CDH reacts, the activity was measured by a standard enzyme assay (100 mM sodium acetate buffer, pH 4.5) at temperatures ranging from 4 to 70 °C. To estimate thermal stability, the enzyme was incubated in buffer at different temperatures (30–60 °C), aliquots were drawn every 30 min for 12 h, and their residual enzyme activities were measured.

Kinetic parameters were determined for various concentrations of CDH substrates (0.1–10 mM) and for DCIP and cytochrome c as electron acceptors. All assays were performed in triplicates. K_m