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Purification and Properties of Cellobiose: Quinone Oxidoreductase from *Sporotrichum pulverulentum*

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Cellobiose: quinone oxidoreductase was purified by ammonium sulfate precipitation, SP-Sephadex C-50 chromatography, and hydroxylapatite column chromatography. The purified enzyme is homogeneous by ultracentrifugal and SDS-gel electrophoretic analyses. The enzyme is a flavoprotein with FAD as the prosthetic group and produces cellobiono-δ-lactone as the product of cellobiose oxidation. Cellopentaose is also oxidized but no oxidation of cellulose could be detected. The enzyme oxidizes lactose and 4-β-glucosylmannose but not 4-β-mannosylglucose which implicates the C-2-hydroxyl of the non-reducing end of the disaccharide as important for substrate specificity.

White-rot fungi degrade both polysaccharides and lignin in wood; however, the enzymic mechanism of lignin degradation is still very poorly understood. In two previous papers,[1,2] we reported the discovery and some properties of an extracellular enzyme from the white-rot fungi, *Polyporus versicolor* and *Sporotrichum pulverulentum*, which catalyzes the oxidation of cellobiose with simultaneous reduction of a quinone. The enzyme was given the systematic name cellobiose:quinone oxidoreductase and the trivial name cellobiose dehydrogenase. As the enzyme utilizes a carbohydrate and an oxidized phenol of cellulose and lignin origin, respectively, it was suggested [1,2] that the enzyme may play an important role both in lignin and cellulose degradation by fungi.

The culture solution from *S. pulverulentum* contains, in addition to cellobiose dehydrogenase, a lactonase and large amounts of cellulose degrading enzymes. Therefore to better understand the function and substrate specificity of the enzyme, a pure preparation is needed and this paper reports purification and some properties of cellobiose:quinone oxidoreductase from *S. pulverulentum*.

EXPERIMENTAL

Organism and cultivation. The fungus *Sporotrichum pulverulentum*, Novobranova was formerly called *Chrysosporium lignorum*. Recent investigations have revealed that it is identical with *S. pulverulentum* and the latter name should be used. The origin of the organism and the method of cultivation was described previously.[2]

Enzyme assay. Cellobiose:quinone oxidoreductase was measured spectrophotometrically.[2] One enzyme unit was defined as the amount of enzyme that reduced 1 μmol/min of 3-methoxy-5-*tert*-butyl-benzoquinone-(1,2) at pH 4.5 and 25 °C.

Concentration and ammonium sulfate precipitation. The culture filtrate was concentrated about 30 times by ultrafiltration (membrane 4M-10 Amicon, Cambridge, Mass., U.S.A.). The concentrated solution was centrifuged at 14 600 *g*; then solid ammonium sulfate was added to the supernatant fluid to 60 % saturation. The resulting precipitate was collected by centrifugation at 14 600 *g* for 30 min.

Isoelectric focusing. Isoelectric focusing was carried out as described in the LKB manual (LKB, Bromma, Sweden). The density gradient was formed with 1,2-ethanediol (0 – 75 % w/v) [3] and the carrier ampholyte (pH 3 – 10) was used at a concentration of 1 % in a total volume of 110 ml. After 20 h at 10 °C, focusing was terminated and the column emptied in 2.4 ml fractions. The pH of each fraction was measured at 4 °C.

Desalting and buffer exchange. Gel filtration using a column of Sephadex G-25 (15 × 260 mm) was generally used for desalting and change of buffer system.

Ion exchange chromatography. SP-Sephadex C-50 was used in the first chromatography step. The column (20 × 340 mm) was eluted (36 ml/h) with sodium acetate buffer (0.02 M, pH 4.3) and a linear gradient of NaCl. The total volume of salt eluent was 400 ml with a final concentration of 0.5 M NaCl.

Hydroxylapatite column chromatography. The second chromatography procedure used a column (15 × 110 mm) of Bio-Gel HT (Bio-Rad Lab., Richmond, U.S.A.) eluted (5 ml/h) stepwise initially with phosphate buffer (0.02 M, pH 7.4) and finally with 0.3 M NaCl.

Concanavalin A chromatography. Chromatography with a Concanavalin A Sepharose column (Pharmacia Fine Chemicals, Uppsala, Sweden) (10 × 20 mm) in sodium acetate buffer (0.1 M, pH 5.0) was also employed. Glycoproteins retained by the column were eluted (12 ml/h) with a solution of 10 % α-methyl D-mannoside in starting buffer.

Gel electrophoresis. Polyacrylamide-sodium-dodecylsulfate (SDS) gel electrophoresis [4] were run for 2.5 h at pH 7.1 with a current of 0.2 mA/mm² (6 mA/gel). Proteins were stained with Coomassie Brilliant Blue.[5]

Ultracentrifugation. Determination of the molecular weight was carried out in an ultracentrifuge Spinco Model E by the sedimentation-equilibrium method in an Yphantis doublesector cell. The enzyme was dissolved in phosphate buffer (0.02 M, pH 7.4) and determination carried out at 30 000 rpm for 24 h at 20 °C.

Absorption spectrophotometry. The ultraviolet and visible spectra were determined in phosphate buffer (pH 7.4) in a Cary 118 recording spectrophotometer with a 1 cm light path.

Thin-layer chromatography of flavins. Thin-layer chromatography was performed with 5 % Na_2HPO_4 in water and *t*-butyl alcohol:water 6:4 (v/v) as developing solvents.[6] Flavins were detected by their fluorescence.

Determination of lactones. Lactones were measured colorimetrically [7] as described earlier.[2]

Chemicals. Flavin-adenine dinucleotide (FAD), flavin-adenine mononucleotide (FMN) and α-methyl D-mannoside were purchased from Sigma Chemical Co. Mannodextrins and cellopentaose were gifts (see Acknowledgements).

RESULTS

Purification

Isoelectric focusing. Isoelectric focusing studies on the enzymes from the culture filtrate resolved three peaks of cellobiose dehydrogenase activity with isoelectric points of 4.0, 5.7, and 6.4, respectively. This result as well as data from other fractionation experiments suggests that at least three isoenzymes of cellobiose dehydrogenase are produced by the fungus *S. pulverulentum.*

Purification procedure. A summary of the purification steps is presented in Table 1. The dialysed precipitate from ammonium sulfate fractionation of culture solution (29 l) was first chromatographed on a SP-Sephadex C-50 column at pH 4.3. The bulk of the protein as well as 68 % of the cellobiose dehydrogenase activity were not retarded by the column (Fig. 1 and Table 1). The enzyme that chromatographed on the column was eluted with a salt gradient and was further purified on a hydroxylapatite column. This cellobiose dehydrogenase fraction was readily adsorbed on the hydroxylapatite column while a great deal of contaminating proteins passed unretarded as shown in Fig. 2. A yellow zone became visible on the top of the column and on elution cellobiose dehydrogenase was

Table 1. Purification procedure.

Purification step		Volume ml	Total activity units (U)	Total A_{280}	Specific activity U/A_{280}	Yield %	Fold purification
Culture filtrate		29000	521	6558	0.08	100	1
Ultrafiltrate		930	438	1987	0.220	84	3
$(NH_4)_2SO_4$ precipitation Step 1		17	379	802	0.47	73	6
SP-Sephadex C-50 chromatography	eluate	21	99	83	1.19	19	15
acetate pH 4.3	void	360	256	656	0.39	49	5
Step 2 Hydroxylapatite chromatography		9.8	31.4	2.45	12.8	6	160

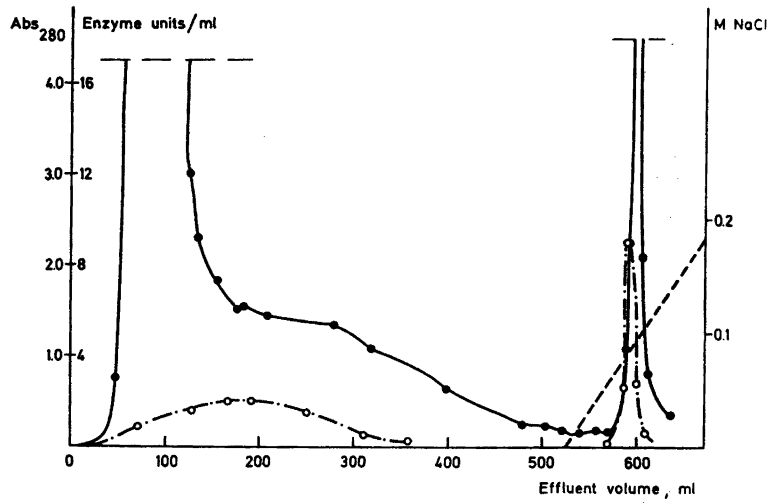

Fig. 1. Chromatography of cellobiose dehydrogenase on SP-Sephadex C-50, pH 4.3. Fractions 9 ml/tube for the starting buffer and 3.5 ml/tube in the gradient. ○, Activity of cellobiose dehy-hydrogenase; ●, absorbance at 280 nm; - - -, concentration of NaCl.

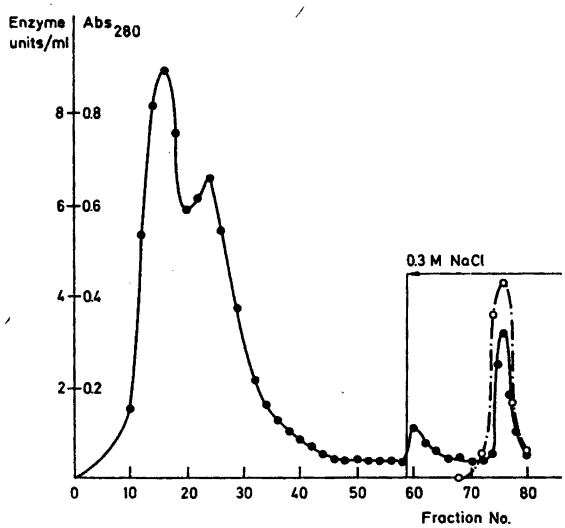

Fig. 2. Adsorption chromatography on hydroxylapatite. Fractions 3.25 ml/tube. ○, Activity of cellobiose dehydrogenase; ●, absorbance at 280 nm.

found in the yellow fractions. It is likely that the purified isoenzyme thus obtained is the most basic of the isoenzymes but no isoelectric focusing was undertaken to show this. This enzyme preparation was inactivated by freezing although crude material could be lyophilized.

Properties of the enzyme

Purity and molecular weight. The enzyme from the hydroxylapatite column showed one single band when run under denaturating conditions on polyacrylamide-SDS gel electrophoresis (Fig.

Fig. 3. SDS-polyacrylamide gel electrophoresis at pH 7.1 of the purified cellobiose dehydrogenase. The enzyme had an absorbance of 0.2 at 280 nm and 0.1 ml was applied.

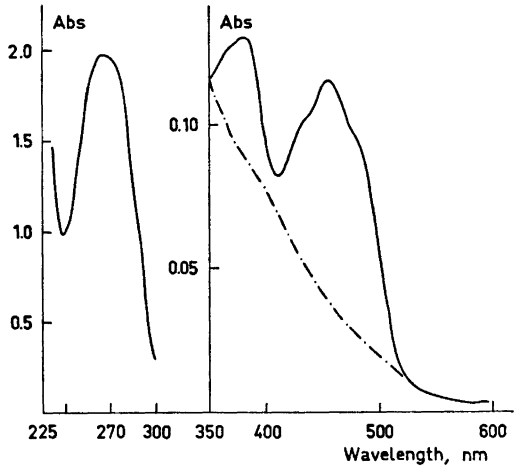

Fig. 4. Ultraviolet and visible spectrum of purified cellobiose dehydrogenase. ———, The spectrum of the oxidized enzyme; —.—, the visible spectrum after addition of the substrate cellobiose.

3). Ultracentrifugation also demonstrated that the enzyme was homogeneous with a molecular weight of 58 000 ± 2 000. An assumed partial specific volume of 0.72 was used in the calculations.

Spectrum of cellobiose dehydrogenase. The visible spectrum of the enzyme (Fig. 4) contains maxima at 375 – 380 and 457 nm and shoulders at about 435 and 475 nm. This type of spectrum is typical for a flavoprotein. The enzyme is visibly yellow and on addition of its substrate cellobiose, the yellow color disappears as well as the absorption maxima in the visible region (Fig. 4). The spectrum after addition of cellobiose is the normal for a reduced flavoprotein, although the absorption at 450 nm is somewhat high.

Identification of the prosthetic group. The prosthetic group was released from the protein by heating the enzyme in a boiling water-bath in darkness for 20 min. The precipitate was removed by centrifugation and the supernatant was analysed by thin-layer chromatography with flavin-adenine dinucleotide (FAD) and flavin-adenine mononucleotide (FMN) as reference substances. The released flavin from cellobiose dehydrogenase had the same R_F as authentic FAD in the two solvent systems used.

Binding to Concanavalin A. The enzymes retarded on the SP-Sephadex C-50 column were applied to a Concanavalin A-Sepharose column. As can be seen (Fig. 5) the cellobiose dehydrogenase enzyme bound to the Concanavalin A-Sepharose column was readily eluted by

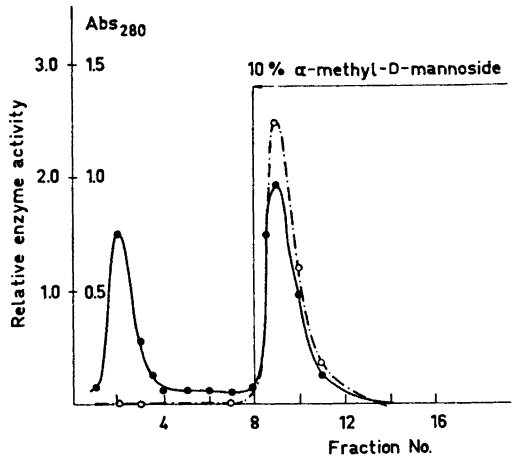

Fig. 5. Concanavalin A Sepharose chromatography of partially purified enzyme. Fractions 1.4 ml/tube. ○, Activity of cellobiose dehydrogenase; ●, absorbance at 280 nm.

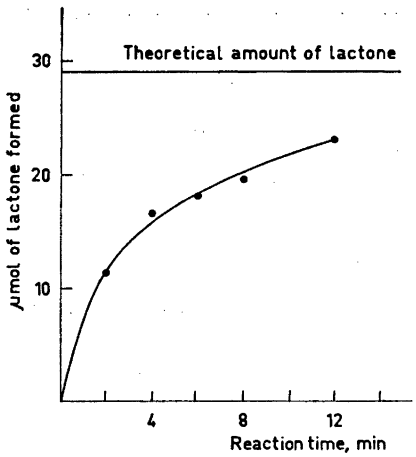

Fig. 6. Formation of cellobiono-δ-lactone. The reaction mixture contained 500 μmol of acetate buffer, pH 5.0, 29 μmol 3 methoxy-5-*tert*-butyl benzoquinone-(1,2), 58 μmol cellobiose, and 6.8 units of cellobiose dehydrogenase in a total volume of 10 ml. The reaction was started by addition of cellobiose.

a 10 % solution of α-methyl D-mannoside. The enzyme yield was about 70 % but no extensive purification occurred as the contaminating proteins were also bound to the column. However, binding to Concanavalin A strongly suggests [8] that the cellobiose dehydrogenase is a glycoprotein which contains terminal α-D-glucosyl and/or α-D-mannosyl or sterically similar residues.

Formation of cellobiono-δ-lactone from oxidation of cellobiose. As described in a previous paper [2] cellobionic acid was identified as a reaction product of the crude cellobiose dehydrogenase preparation. It is, however, more likely that cellobiono-δ-lactone is the first reaction product which then is destroyed by lactonase in the crude preparation. With purified cellobiose dehydrogenase, an accumulation of a lactone in the reaction mixture was demonstrated when the enzyme was incubated with cellobiose and quinone (Fig. 6). When all the quinone was reduced about 80 % of the stoichiometrical amount of lactone was found. The lactone is spontaneously hydrolyzed at pH 5.0 but the short reaction time gave little spontaneous hydrolysis.

Substrate specificity. As described in the previous paper [2] and Table 2 this paper, a number of mono- and disaccharides as well as cellopentaose and cellulose were investigated as possible enzyme substrates. Only cellobiose, cellopentaose, 4-β-glucosylmannose, and lactose were oxidized (Table 2).

DISCUSSION

The function of the different isoenzymes of cellobiose dehydrogenase is unknown; in fact it must still be established that these are true isoenzymes. Purified cellobiose dehydrogenase shows the same substrate specificity as the crude culture filtrate with regard to oxidation of carbohydrates. The same fungus, S. *pulverulentum*, also produces five endo-1,4-β-glucanases which have small but significant structural differences.[9] In particular, they differ in molecular weight (± 4 500) and in isoelectric point (± 1.1 pH unit). All but one are glycoproteins with varying amounts of carbohydrates of dif-

Table 2. Oxidation of different substrates. The reaction mixture contained purified cellobiose dehydrogenase, 300 mmol acetate buffer (pH 4.5), 1 μmol 3-methoxy-5-*tert*-butyl-benzoquinone-(1,2), 2 μmol of sugar in a total reaction volume of 3 ml. The cellulose sample had a degree of polymerization of 150 and about 8 μmol of reducing endgroups.

Substrate	Relative rate of oxidation
Cellobiose	100
Cellopentaose	100
Cellulose	0
Lactose (4-*O*-β-D-galactopyranosyl-D-glucose)	44
4-β-Mannosylglucose (4-*O*-β-D-mannopyranosyl-D-glucose)	0
4-β-Glucosylmannose (4-*O*-β-D-glucopyranosyl-D-mannose)	36
Mannobiose (4-*O*-β-D-mannopyranosyl-D-mannose)	0

ferent composition. There are also differences in their degradative and synthetic activities.[10]

The studied cellobiose dehydrogenase from *S. pulverulentum* has many properties in common with glucose dehydrogenase from *Aspergillus oryzae*.[11] For instance, both enzymes, as do many other sugar dehydrogenases, contain FAD. Glucose dehydrogenase, like cellobiose dehydrogenase, is a glycoprotein which uses certain redox dyes and quinones but not oxygen as a hydrogen acceptor. Glucose dehydrogenase is a soluble intracellular enzyme which is induced by certain quinones;[12] however, small amounts of the enzyme are also excreted extracellularly. The cellobiose dehydrogenase is extracellular and is not induced by quinones but by cellulosic substrates and it has a pronounced specificity for oxidation of degradation products from cellulose while most sugar dehydrogenases can oxidize a number of similar mono- and disaccharides.

From the ability or inability of cellobiose dehydrogenase to oxidize the disaccharides tested (Table 2) some understanding of the enzyme-substrate interaction can be gained. 4-β-Mannosylglucose differs from cellobiose only in the C-2 hydroxyl in the non-reducing end of the substrate yet it cannot be oxidized by cellobiose dehydrogenase. 4-β-Glucosylmannose, on the other hand, which has the C-2 hydroxyl of the reducing sugar unit inverted, is oxidized. However, oxidation of lactose demonstrates that inversion of the hydroxyl at C-4 of the non-reducing end does not prevent oxidation. This may be expected as this hydroxyl is involved in the β-glucosidic bond to the next glucose unit in cellodextrins. Xylobiose which differs from cellobiose by lacking hydroxymethyl groups at both C-5 positions is not oxidized.[2] These results indicate that the C-2 of the non-reducing end of the disaccharide substrates must have the glucose conformation but that inversion may occur at C-4 position. This may explain why monosaccharides are not oxidized. Furthermore, the enzyme needs a hydroxymethyl group at one or both C-5 atoms in the disaccharide. A β-linkage between the two sugar units seems also to be a requirement for activity since maltose is not oxidized.[2] From the substrate specificity and the mode of induction it is obvious that degradation products from cellulose are the proper substrates for the enzyme.

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