The Purification and Properties of Cellobiose Dehydrogenase from *Sclerotium rolfii* and its Role in Cellulolysis

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An extracellular cellobiose dehydrogenase has been purified from the culture filtrates of *Sclerotium rolfii*. The purified enzyme is homogeneous as determined by disc gel electrophoresis, with and without SDS, and by analytical isoelectric focusing in polyacrylamide gel. The enzyme is a single-subunit glycoprotein containing 8.9% total carbohydrate; its M_r is 63 000–64 500, and its isoelectric point 5.18. The enzyme oxidized cellobiose, other cellodextrins and lactose whereas other disaccharides tested were not utilized as substrates. The rate of cellodextrin oxidation decreased and the K_m increased with increasing degree of polymerization of the substrate. Cytochrome *c* was reduced though at a considerably lower rate than 2,6-dichlorophenolindophenol. The natural electron acceptor for the enzyme has not been identified.

INTRODUCTION

The degradation of cellulose by cellulolytic micro-organisms proceeds through the concerted action of several hydrolytic enzymes (Wood & McCrae, 1979; Wood *et al.*, 1980), though additional nonhydrolytic, e.g. oxidative (Westermarck & Eriksson, 1974, 1975; Ayers *et al.*, 1978) and phosphorolytic (Sheth & Alexander, 1967; Alexander, 1961, 1968), enzymes have also been reported to be operative in some cases. In *Sporotrichum pulverulentum* an oxidative enzyme, cellobiose oxidase, which oxidizes cellobiose and higher cellodextrins to their corresponding aldonic acids using molecular oxygen, has been implicated in cellulose breakdown (Ayers *et al.*, 1978). The enzyme is a haemoprotein and also contains an FAD group. Vaheri (1982, 1983) reported that an oxidative system is involved in the degradation of cellulose by *Trichoderma reesei*, probably at the first stage in the breakdown of crystalline cellulose. Another oxidative enzyme, cellobiose:quinone oxidoreductase, first discovered by Westermarck & Eriksson (1974, 1975) in the culture filtrates of *Sporotrichum pulverulentum*, has been implicated in the breakdown of cellulose and lignin. A similar oxidative enzyme, cellobiose dehydrogenase, but which does not react with quinones, has been found in a non-lignolytic *Monilia* sp. (Dekker, 1980), and the cellulolytic but non-lignolytic fungus *Sporotrichum thermophile* (Coudray *et al.*, 1982). The enzyme from both *Sporotrichum* species oxidizes cellobiose and other cellodextrins (but not cellulose) by a few oxidizing agents but not with molecular oxygen (Westermarck & Eriksson, 1975; Coudray *et al.*, 1982). The cellobiose dehydrogenase from *Sporotrichum pulverulentum* is a flavoprotein containing FAD as the prosthetic group and produces cellobionolactone from cellobiose in the presence of lignin degradation products like quinones (Westermarck & Eriksson, 1975).

During the course of purification of various cellulolytic enzymes from the culture filtrates of *Sclerotium rolfii*, a cellobiose dehydrogenase was also detected. The enzyme was obtained in a homogeneous state and some of its properties were studied with a view to understanding its possible role in cellulolysis.

Abbreviations: CBDH, cellobiose dehydrogenase; DCPIP, dichlorophenolindophenol.

METHODS

Materials. The sources of the materials used have been described previously (Sadana *et al.*, 1983, 1984; Patil & Sadana, 1984). *p*-Nitrophenyl β -D-cellobioside was kindly supplied by Dr K. Wakabayashi (Shinshu University, Japan). Cellobiitol, laminaribiose and pustulan were kindly supplied by Dr E. T. Reese (US Army Laboratories, USA); cellodextrins (cellotriose to cellohexaose) were supplied by Dr E. T. Reese and by Professor D. A. I. Goring (Pulp and Paper Institute, Canada). Dichlorophenolindophenol (DCPIP) was from BDH; xylose, arabinose, raffinose, NAD⁺, NADP⁺, FMN, FAD, methylene blue, cytochrome *c* (*Candida krusei*), *p*-benzoquinone, ubiquinone Q-10, α -tocopherol and menadione were from Sigma. All other chemicals used were from commercial sources and were of analytical grade.

Analytical methods. Reducing sugars, expressed as D-glucose equivalents, were determined by the *p*-hydroxybenzoic acid hydrazide method (Hurst *et al.*, 1977) or the Nelson-Somogyi method (Nelson, 1944; Somogyi, 1952), and D-glucose by the D-glucose oxidase-peroxidase method using 'Glox' reagent (Bergmeyer, 1974). Protein was determined by the Lowry procedure. In column effluents, protein was estimated by measuring the absorbance at 280 nm. The carbohydrate content of the purified enzyme was determined as glucose equivalents by the anthrone-sulphuric acid method (Updegraff, 1969).

Polyacrylamide gel electrophoresis and isoelectric focusing. Analytical disc gel electrophoresis, preparative polyacrylamide gel electrophoresis and isoelectric focusing in polyacrylamide gel were performed as described previously (Shewale & Sadana, 1981; Patil & Sadana, 1984). The isoelectric point of the pure enzyme was determined in a 110 ml LKB electrofocusing column at 5–7 °C according to the procedure described in the LKB Instruction Manual. Ampholine carrier ampholyte, pH range 4–6, was used.

The M_r of the enzyme was determined by molecular sieve chromatography (Andrews, 1964) and by gel electrophoresis as described by Hedrick & Smith (1968). SDS-gel electrophoresis, in gels containing 7.5% (w/v) total acrylamide monomer and 0.1% SDS was carried out as described previously (Sadana *et al.*, 1984).

Amino acids (Spackman *et al.*, 1958) and amino sugars (Walborg *et al.*, 1963) were analysed in a Beckman model 120-B automatic amino acid analyser. For amino acids, samples of the enzyme (0.5–1.0 mg) were hydrolysed as described previously (Husain & Sadana, 1974); for amino sugars, the enzyme was hydrolysed with 4 M-HCl for 12 h (Gum & Brown, 1976). The tryptophan and tyrosine contents were determined by the spectrophotometric method of Goodwin & Morton (1946).

Enzyme assays. *Cellobiose dehydrogenase (CBDH) activity.* The assay mixture contained 0.05 ml 1.8 mM-DCPIP, 0.9 ml 2.5 mM-cellobiose in 100 mM-phosphate buffer, pH 6.3, and 0.05 ml of suitably diluted enzyme in a glass cuvette at 37 °C in a Shimadzu spectrophotometer equipped with a thermostatically controlled cuvette holder. The decrease in A_{600} of DCPIP was measured. The reduction of DCPIP caused a slight decrease in the pH of the reaction mixture. The decrease in A_{600} on reduction of DCPIP obtained during the first 5 min of the reaction was used to estimate enzyme activity. (To determine the pH optimum, reduction of DCPIP was followed at 520 nm for buffers of pH < 5 and at 600 nm for buffers of pH > 5.) The unit of activity (U) is expressed as $\mu\text{mol DCPIP reduced min}^{-1} (\text{ml enzyme})^{-1}$. The molar absorption coefficient (ϵ_{600}) used for DCPIP was $18.5 \times 10^3 \text{ l mol}^{-1} \text{ cm}^{-1}$ at pH 6.5 (Philips & Langdon, 1962). In experiments using electron acceptors other than DCPIP, the assay procedure was similar (using the various electron acceptors at 1 mg ml⁻¹) and the decrease (or increase) in absorbance was determined at their respective absorption maxima (Peel, 1972).

Other assays. β -Glucosidase, endo- β -glucanase (carboxymethylcellulase), cellobiohydrolase, and cellulase (Avicel-solubilizing activity) activities were determined as described previously (Sadana *et al.*, 1983, 1984; Patil & Sadana, 1984; Sadana & Patil, 1985).

Fungal strain and growth conditions. *Sclerotium rolfsii* CPC 142 was grown for 14 d with Cellulose-123 as the sole carbon source as described previously (Shewale & Sadana, 1978).

RESULTS

Physical properties of purified enzyme. CBDH from *Sclerotium rolfsii* was purified as by Patil & Sadana (1984); the results are summarized in Table 1.

Polyacrylamide gel electrophoresis of the purified enzyme at pH 2.9 and 8.9, with or without SDS treatment, and isoelectric focusing in 7.5% polyacrylamide gel over the pH range 3.5–10, revealed only one protein band (Fig. 1). The M_r for CBDH determined by gel permeation on Bio-Gel P-150 was 63 100 as compared to values of 62 500 and 64 500 obtained by electrophoresis by the slope method and by migration in SDS-polyacrylamide gels, respectively. Carboxyamido-methylation of the reduced form of the enzyme on SDS-gel electrophoresis also gave one protein band with a molecular weight corresponding to the native protein, indicating that the enzyme is composed of a single polypeptide chain. The pI of pure *Sclerotium rolfsii* CBDH was 5.18.

Fig. 1. Polyacrylamide gel electrophoresis and electrofocusing of CBDH from *Sclerotium rolfsii*. (a, b) Disc gel electrophoresis at pH 2.9 (a) and at pH 8.9 (b); (c) SDS-gel electrophoresis (CBDH treated with 1% SDS containing 1% 2-mercaptoethanol and 0.5% iodoacetamide); (d) the glycoprotein band after disc gel electrophoresis at pH 8.9 stained with periodate fuchsin; (e) isoelectric focusing in 7.5% polyacrylamide gel in the pH range 3.5–10.

Table 1. *Purification of CBDH from Sclerotium rolfsii*

Starting from the culture filtrate, the purification of CBDH was done twice. The results obtained were similar; representative results are shown.

Purification step	Total protein (mg)	Total activity (U)	Specific activity [U (mg protein) ⁻¹]	Purification (fold)	Yield (%)
Crude extract	17202	33540	1.95	1.00	100
Ammonium sulphate, 0–90% saturation	13937	31820	2.30	1.18	95
Sephadex G-75 chromatography	7815	26570	3.40	1.74	79
Fraction A					
Ultrafiltration of Fraction A (Amicon PM-10)	6051	21180	3.50	1.79	63
DEAE-Sephadex A-50 chromatography	50	1060	21.20	10.87	3
Preparative polyacrylamide gel electrophoresis	8.5	229	26.90	13.79	0.7

Absorption spectrum. The absorption spectrum of the purified CBDH (1 mg protein ml⁻¹ as determined by Lowry method) showed an absorption maximum at 280 nm. The absence of any peak in the regions 445–455 nm and 405–415 nm indicated that the enzyme did not contain a flavin or haem component. In contrast, the CBDH from *Sporotrichum pulverulentum* is an FAD-containing protein (Westermarck & Eriksson, 1975).

Chemical properties. The CBDH was completely adsorbed on a Concanavalin A-Sepharose column and eluted by 1% methyl α -D-glucopyranoside or methyl α -D-mannopyranoside. Polyacrylamide gels stained with Coomassie brilliant blue and with periodate fuchsin (Zacharius *et al.*, 1969) showed a single coinciding band for CBDH (Fig. 1), indicating that CBDH from *Sclerotium rolfsii* is a glycoprotein. The total carbohydrate content of the purified enzyme was 8.9%.

CBDH from *Sclerotium rolfsii* is high in acidic and low in basic amino acid contents (Table 2). Cystine and half-cystine were not detected with or without performic acid oxidation.

Amino sugar analysis of the acid hydrolysate showed that CBDH contains 5.6 residues of glucosamine per molecule of enzyme; galactosamine was not detected.

Table 2. *Amino acid composition of CBDH from Sclerotium rolfsii*

The results are from a single determination.

Amino acid	No. of residues*	Molar percentage
Aspartic acid	59	9.8
Threonine†	49	8.1
Serine†	39	6.5
Glutamic acid	56	9.3
Proline	46	7.6
Glycine	81	13.4
Alanine	53	8.8
Half-cystine‡	0	0
Valine§	25	4.1
Methionine	7	1.2
Isoleucine§	30	5.0
Leucine§	44	7.3
Tyrosine	26 (24)	4.3
Phenylalanine	28	4.6
Lysine	14	2.3
Histidine	7	1.2
Arginine	8	1.3
Tryptophan	(30)	5.0
Total residues	602	

* Calculated using a molecular weight of 63 100. Values given in parenthesis were determined by the method of Goodwin & Morton (1946).

† Extrapolated to zero time.

‡ Determined as cysteic acid after performic acid oxidation.

§ 72 h value used.

|| Determined as methionine sulphone after performic acid oxidation.

Enzymic properties. The properties of CBDH were tested with cellobiose as electron donor and DCPIP as electron acceptor, unless otherwise indicated. The purified enzyme was stable when stored at -15°C at pH 4.5 and was also stable to repeated freezing and thawing. It was most stable at pH 4.5–5.0. Heating at 70°C for 10 min destroyed 86% of the activity; addition of bovine serum albumin (0.5 mg ml^{-1}) had no protective effect. The enzyme was completely inactivated by heating at 100°C for 2 min.

The pH and temperature optima were 6.3–6.4 and 37°C . The pH optima for CBDH from *Monilia* sp. (Dekker, 1980) and *Sporotrichum pulverulentum* (Westermarck & Eriksson, 1975) were between 4.0 and 4.5. With $0.2\text{ }\mu\text{g}$ enzyme, the rate of reduction of DCPIP was linear with time for the first 5 min. Proportionality between activity and enzyme concentration ($0.1\text{--}0.5\text{ }\mu\text{g}$) was also observed in a 2 min assay. The activation energy, calculated from an Arrhenius plot, was 101.9 J mol^{-1} . There was no inhibition of CBDH activity at high cellobiose concentrations (25 mM). Glucono-1,5-lactone (100 mM) did not inhibit the activity of CBDH.

Stoichiometry of reaction. Two series of experiments were run, one with cellobiose and one with DCPIP as the limiting substrate. The data obtained showed that one mol DCPIP was reduced per mol cellobiose oxidized.

Specificity and kinetics. (a) *With respect to oxidizable substrates.* The apparent K_m and $V_{\max}$ values for the oxidation of cellobiose and other cellodextrins by CBDH with DCPIP as electron acceptor are shown in Table 3. The enzyme oxidized cellobiose, other cellodextrins (cellotriose to cellohexaose) and lactose. The relative rate of lactose oxidation compared to that of cellobiose under similar assay conditions was 40%. The turnover rate of CBDH, as represented by $V_{\max}$, for cellobiose, cellotriose, cellotetraose, cellopentaose and cellohexaose decreased, whereas the empirical K_m value increased, with the chain length of the substrate. The higher K_m values for cellodextrins with higher degree of polymerization support the concept of a preferred order of attack based on molecular size of the substrate. No reaction was observed with glucose, sucrose, maltose, xylose, mannose, melibiose, galactose, arabinose, raffinose, fructose, laminaribiose,

Table 3. *Kinetic constants for CBDH from Sclerotium rolfsii*

Kinetic studies were carried out in the standard assay system at 37 °C, pH 6.3, varying the substrate concentrations. The K_m and V_{max} values were calculated from Lineweaver–Burk plots, which were linear in all cases. The results are representative values from two determinations.

Substrate	K_m (μM)	V_{max} [$\mu\text{mol min}^{-1}$ (mg enzyme) $^{-1}$]
<i>Electron donor</i>		
Cellobiose	41.6	36.0
Cellotriose	62.5	16.6
Cellotetraose	66.6	15.4
Cellopentaose	83.3	13.3
Cellohexaose	100.0	12.5
<i>Electron acceptor</i>		
DCPIP	220	36.0
Cytochrome <i>c</i> *	384	0.017

* The rate of reduction of cytochrome *c* was calculated from the molar absorbance at 550 nm [Δ , reduced minus oxidized = $18.7 \times 10^3 \text{ l mol}^{-1} \text{ cm}^{-1}$ (Phillips & Langdon, 1962)].

trehalose, arbutin, cellobiitol, pustulan, lichenan, glucosamine, galactosamine, xylobiose, glucono-1,5-lactone, *p*-nitrophenyl β -D-glucoside, *p*-nitrophenyl β -D-cellobioside, carboxymethylcellulose, cellulose and H_3PO_4 -swollen cellulose.

The apparent K_m values of CBDH at pH 6.5, 37 °C, for DCPIP (with cellobiose as electron donor) and cellobiose (with DCPIP as electron acceptor) were 220 μM and 41.6 μM , respectively.

(b) *With respect to electron acceptors.* Among the artificial electron acceptors tested, DCPIP was the most effective. Cytochrome *c* and potassium ferricyanide were also reduced, though at a considerably lower rate. With cellobiose as electron donor (2.5 mM) and at equivalent concentrations of electron acceptors, the rates of cytochrome *c* and ferricyanide reduction were 30% and 5% of that of DCPIP. Methylene blue, (at pH 5.0–6.0), *p*-benzoquinone, 9,10-anthraquinone, ubiquinone Q-10, α -tocopherol, menadione, triphenyltetrazolium chloride, phenazine methosulphate, NAD^+ , NADP^+ , FMN, FAD and molecular oxygen failed to accept electrons arising from the enzymic oxidation of cellobiose.

DISCUSSION

The purified CBDH from *Sclerotium rolfsii* was obtained in a homogeneous state and was a glycoprotein. The CBDH from *Sporotrichum pulverulentum* is also a glycoprotein (Westermarck & Eriksson, 1975). The M_r of *Sclerotium rolfsii* CBDH was 63 000–64 500; CBDH from *Monilia* sp. (Dekker, 1980) and *Sporotrichum pulverulentum* (Westermarck & Eriksson, 1975) had M_r values of 48 000 and 58 000, respectively. The pI of CBDH from *Sclerotium rolfsii* was 5.18, and that of CBDH from *Monilia* sp. 5.3–5.5 (Dekker, 1980). The apparent K_m values for *Sclerotium rolfsii* CBDH (pH 6.3, 37 °C) for DCPIP (with cellobiose as electron donor) and cellobiose (with DCPIP as electron acceptor) were 220 μM and 41.6 μM , respectively. The corresponding values for *Monilia* sp. CBDH (pH 6.6, 37 °C) were 80 μM and 12.2 μM , respectively (Dekker, 1980). For *Sporotrichum thermophile* CBDH, the apparent K_m for cellobiose (with DCPIP as oxidant) (pH 7.0, 30 °C) was 6.9 μM (Coudray *et al.*, 1982).

Like the CBDH from *Monilia* sp., the *Sclerotium rolfsii* enzyme does not react with benzoquinones, for which the dehydrogenases from both *Sporotrichum pulverulentum* and *Sporotrichum thermophile* have a high affinity. The natural electron acceptor *in vivo* for CBDH from *Sclerotium rolfsii*, *Monilia* sp. and *Sporotrichum thermophile* has not been indentified. The natural electron acceptor for *Sporotrichum pulverulentum* CBDH has been identified as phenoxy radicals or quinones emanating from the oxidation of phenols derived as lignin degradation products (Eriksson, 1978).

The concerted action of cellobiohydrolase and endo- β -glucanases from *Sclerotium rolfsii* on cellulose produces cello-oligosaccharides, cellobiose and other higher cellodextrins, and small

quantities of glucose (Sadana & Patil, 1985). The *Sclerotium rolfii* CBDH oxidizes cellobiose and other cellodextrins. The function of CBDH in cellulose hydrolysis could be in the metabolism of cellobiose (and other cellodextrins) by oxidation rather than by intracellular hydrolysis. CBDH (Westermarck & Eriksson, 1975) and cellobiose oxidase (Ayers *et al.*, 1978) have been shown to be a feature of the cellulolytic system of *Sporotrichum pulverulentum*. The CBDH may have a regulatory role, possibly on the activity of endo- β -glucanases and cellobiohydrolase, by protecting them from cellobiose inhibition. In most cellulolytic fungi, such a function is usually ascribed to the β -glucosidase component. In *Sporotrichum thermophile*, which contained CBDH, β -glucosidase was totally absent in culture filtrates (see Coudray *et al.*, 1982), and it was suggested that in this organism the metabolism of cellobiose occurs via oxidation rather than by intracellular hydrolysis. *Sclerotium rolfii*, on the other hand, contains both β -glucosidase (Shewale & Sadana, 1981; Sadana *et al.*, 1983) and CBDH, both capable of utilizing cellodextrins (cellobiose to cellohexaose); i.e. cellodextrins in *Sclerotium rolfii* could be metabolized by oxidation or by intracellular hydrolysis.

The K_m values of *Sclerotium rolfii* β -glucosidase decreased, and the V_{max} values increased, with the chain length of the cellodextrins (Sadana *et al.*, 1983); the reverse was true for CBDH. With *Sclerotium rolfii* BG-III β -glucosidase at pH 4.5, 37 °C (optimum conditions for Avicel solubilization: Sadana & Patil, 1985), the K_m values for cellobiose and cellohexaose were 2 mM and 0.5 mM, and those with CBDH were 45.4 μ M and 111 μ M, respectively. The turnover numbers [μ mol substrate utilized (μ mol enzyme) $^{-1}$ min $^{-1}$] of BG-III β -glucosidase and CBDH for cellobiose and cellohexaose were 2597 and 5035, and 806 and 323, respectively; i.e. even though the K_m values of cellodextrins for CBDH were lower (4.5 to 44 times) than those for β -glucosidase, the turnover numbers of the pure β -glucosidase for cellodextrins were much higher (3 to 16 times) as compared with CBDH. This suggests that the major route of cellodextrin metabolism in *Sclerotium rolfii* is through the hydrolytic pathway via β -glucosidase.

The absolute CBDH and β -glucosidase activities of *Sclerotium rolfii* culture filtrate under *in vivo* conditions [pH 3.0, 30 °C (Shewale & Sadana, 1978) during growth from the 3rd to the 14th day] were 0.42 and 1.18 μ mol cellobiose utilized min $^{-1}$ ml $^{-1}$, respectively. Thus, under *in vivo* conditions, about 30% of cellobiose could be metabolized via oxidation through CBDH. Under optimum conditions of Avicel solubilization, the absolute CBDH and β -glucosidase activities were 2.6 and 2.65 μ mol cellobiose utilized min $^{-1}$ (ml culture filtrate) $^{-1}$, respectively (Sadana & Patil, 1985). Although the absolute CBDH and β -glucosidase activities were about the same, the synergistic effect observed in reconstitution studies with pure cellulolytic enzymes (cellobiohydrolase and endo- β -glucanases) for solubilization of Avicel was much greater with β -glucosidases than with CBDH (Sadana & Patil, 1985). All the data suggested that CBDH has only a minor role in the overall hydrolysis of crystalline cellulose to glucose when *Sclerotium rolfii* is grown on cellulose. However, when the organism is growing on lignocellulosic materials, CBDH may play a more important role in the degradation of both cellulose and lignin, as has been shown for *Sporotrichum pulverulentum* (Westermarck & Eriksson, 1975), where CBDH has been postulated to be involved in the reduction of lignin degradation products through a reaction coupled with the oxidation of cellobiose.

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