Characterization of a cellobiose dehydrogenase from *Humicola insolens*

Charlotte SCHOU*, Margrethe H. CHRISTENSEN† and Martin SCHÜLEIN†[1]

*Løvens kemiske Fabrik, Ballerup, DK-2750, Denmark, and †Novo Nordisk A/S, Bagsvaerd, DK-2880, Denmark

The major cellobiose dehydrogenase (oxidase) (CBDH) secreted by the soft-rot thermophilic fungus *Humicola insolens* during growth on cellulose has been isolated and purified. It was shown to be a haemoflavoprotein with a molecular weight of 92 kDa and a pI of 4.0, capable of oxidizing the anomeric carbon of cellobiose, soluble cellooligosaccharides, lactose, xylobiose and maltose. Possible electron acceptors are 2,6-dichlorophenol-indophenol (DCPIP), Methylene Blue, 3,5-di-t-butyl-1,2-benzoquinone, potassium ferricyanide, cytochrome *c* and molecular oxygen. The oxidation of the prosthetic groups by oxygen was monitored at 449 nm for the flavin group and at 562 nm for the haem group. The curves were very similar to those of the cellobiose dehydrogenase from *Phanerochaete chrysosporium*, suggesting a similar mechanism. The pH-optima for the oxidation varied remarkably depending on the electron acceptor. For the organic electron acceptors, the pH-optima ranged from pH 4 for Methylene Blue to pH 7 for DCPIP and the benzoquinone. In the case of the Fe^{III}-containing electron acceptors, the enzyme displayed alkaline pH-optima, in contrast to the properties of cellobiose dehydrogenases from *Phanerochaete chrysosporium* and *Myceliophthora* (*Sporotrichum*) *thermophila*. The enzyme has optimal activity at 65 °C.

INTRODUCTION

Cellobiose-oxidizing enzymes were first discovered in the extracellular enzyme system of the white-rot fungus *Phanerochaete chrysosporium* [1–3]. The cellobiose-oxidizing enzymes from this fungus have been intensively characterized and are of two kinds: one is a flavoprotein called cellobiose:quinone oxidoreductase (CBQ) suggested to be involved in lignin degradation [1,2], and the other is a haemoflavoprotein called cellobiose dehydrogenase (oxidase) (CBDH) which is proposed to be preferentially involved in cellulose degradation [3]; the gene encoding this enzyme has recently been cloned [4].

Cellobiose dehydrogenases are not restricted to white-rot fungi but have also been found in the brown-rot fungus *Coniophora puteana* [5] and soft-rot fungi such as *Monilia* sp. [6], *Chaetomium cellulolyticum* [7], *Myceliophthora* (*Sporotrichum*) *thermophila* [8] and *Sclerotium rolfsii* [9]. Obviously the function of the cellobiose dehydrogenases from these fungi cannot be linked solely to lignin degradation but rather can play an important role in cellulose degradation.

Humicola insolens is a thermophilic soft-rot fungus and in the present study we show that the CBDH from this fungus is a haemoflavoprotein which in some respects is similar to the well characterized cellobiose dehydrogenases from *Phanerochaete chrysosporium* and *Myceliophthora* (*Sporotrichum*) *thermophila*.

The purified CBDH has been used in a coupled assay for steady-state kinetics measurements of cellulase activity at pH 7.5. The results of the development of this cellulase assay have been published elsewhere [10].

MATERIALS AND METHODS

Enzyme purification

A solution of Celluzyme™ (Novo Nordisk A/S) was used for the purification. It contained many cellulases and two cellobiose dehydrogenases. 21.4 g of protein was applied to a 300 ml arginine Sepharose column (Pharmacia) which had been equilibrated with 50 mM Tris/HCl buffer, pH 7.0. The CBDH was eluted with simultaneous gradients of Tris/HCl, pH 7.0–9.0 and 0–0.2 M NaCl. The eluate containing the cellobiose oxidizing activity from the two enzymes was adjusted to pH 5.0 with 20 % HCl and applied to an S-Sepharose column in 20 mM sodium citrate, pH 5.0. This column bound most of the cellulases whilst the CBDH eluted with the eluent. The CBDH fraction was adjusted to pH 7.0, applied to an anion-exchange column (HPQ-Sepharose) previously equilibrated with 50 mM Tris/HCl, pH 7.0 and the column was eluted with a 0–1 M NaCl gradient. This separated the minor cellobiose dehydrogenase (94 kDa and pI 4.4) from the major one (92 kDa and pI of 4.0). Gel filtration on a Superdex 200 Hiload column eluted the cellobiose dehydrogenase with an apparent molecular mass of 92 kDa, thus separating it from the contaminating cellulases of lower molecular mass.

SDS/PAGE and electrofocusing

Analytical polyacrylamide-gel electrophoresis of the different fractions was carried out on 10 % gel slabs with a Bio-Rad apparatus following the manufacturer's procedure with a Tris/glycine buffer system. Electrofocusing was carried out using an LKB multiphore apparatus and Ampholine precast gels (LKB). CBDH activity after electrofocusing gel was determined using a 1 % agarose overlayer containing cellobiose and DCPIP. The CBDHs appeared as distinct clearing zones.

Spectroscopy

All absorption spectra and kinetic measurements were recorded on a Hewlett–Packard 8452A Diode Array Spectrophotometer in 0.75 ml black cuvettes with 1 cm optical path. The fluorescence spectra were recorded on a Perkin–Elmer LS 50. NMR spectra were recorded on a Bruker ARX500 spectrometer at 300 K. Mass spectra were recorded on a Micromass VG QUATRO II

Abbreviations used: CBQ, cellobiose:quinone oxidoreductase; CBDH, cellobiose dehydrogenase (oxidase); DCPIP, 2,6-dichlorophenol-indophenol. Enzymes: cellobiose:quinone oxidoreductase (EC 1.1.5.1); cellobiose dehydrogenase (oxidase) (EC 1.1.99.18); cellulase (3.2.1.4).

[1] To whom correspondence should be addressed.

mass spectrometer using atmospheric pressure chemical ionization and electrospray ionization.

Buffers

Measurements were performed in 0.1 M sodium phosphate buffer (pH 7.5) at 40 °C unless otherwise stated.

Determination of activity

450 μl of a mixture of 100 μM DCPIP (Merck) and 250 μM cellobiose (Sigma) were mixed with 50 μl of enzyme. One unit of activity corresponds to 1 μmol cellobiose oxidized (DCPIP reduced) per min.

Cellulase assay

The presence of cellulases was determined using cellohexaitol as substrate [10]. 450 μl of 100 μM DCPIP and 250 μM cellohexaitol were mixed with 50 μl of 0.22 μM CBDH. A change in absorbance at 600 nm of less than 0.5×10^{-5} absorption units/s was taken as evidence for the absence of cellulases.

Amino acid composition

The amino acid composition was determined using an Applied Biosystems amino acid apparatus.

Sugar content

The sample (250 pmol) was hydrolysed with 1 M HCl at 100 °C for 4 h. The acid was removed by evaporation *in vacuo* and the identity of the sugars present was established by HPLC with a pulsed electrochemical detector (Dionex Corp. Sunnyvale, U.S.A.) and a Carbopac PA1 microcolumn.

Identification of the prosthetic groups

The spectrum of 500 μl of 6.6 μM CBDH was recorded and 10 μl of 5 mM cellobiose or a few grains of sodium dithionite (Merck) were added to give the reduced CBDH. For detection of the flavin group, fluorescence spectra of 2.3 μM CBDH were recorded. Emission spectra for the exitation at 397 and 443 nm and exitation spectra for emission at 480 nm were recorded.

Absorption coefficients

The absorption coefficient of CBDH ($\epsilon_{280} = 240\,000\ M^{-1} \times cm^{-1}$) was estimated: using the amino acid composition (Table 2).

Two absorption coefficients were measured for DCPIP: in the range pH 2 to 5.5 $\epsilon_{530} = 7500\ M^{-1}\,cm^{-1}$ and pH 5.5 to 10 $\epsilon_{600} = 14\,000\ M^{-1}\,cm^{-1}$. Potassium ferricyanide (Merck): $\epsilon_{420} = 970\ M^{-1}\,cm^{-1}$, 3,5-di-t-butyl-1,2-benzoquinone (Merck): $\epsilon_{410} = 1400\ M^{-1}\,cm^{-1}$, Methylene Blue (Merck): $\epsilon_{610} = 42\,000\ M^{-1}\,cm^{-1}$, cytochrome *c* (Sigma, from horse heart: $\epsilon_{550} = 19\,600\ M^{-1}\,cm^{-1}$ [11].

Identification of oxidation product

Cellobiose (40 mg) and DCPIP (35 mg) were dissolved in water (10 ml) and CBDH was added (5 μl of a 122 μM solution). The mixture was stirred overnight at room temperature. The aqueous solution was extracted twice with ethyl acetate in order to remove the reduced DCPIP and the aqueous phase was freeze dried to give 44 mg of product. The product was analysed by NMR and MS. The assignment of the NMR spectra was done using 2D NMR. ^{1}H NMR (D_2O, reference HDO: δ 4.66) δ: 3.25 (dd, 1H, H-2′), 3.3–3.4 (m, 2H, H-4′, H-5′), 3.41 (t, 1H, H-3′), 3.6–3.83 (m, 4H, H-6, H-6′), 3.84 (m, 1H, H-5), 3.91 (t, 1H, H-4), 4.00 (dd, 1H, H-3), 4.08 (d, 1H, H-2) and 4.54 (d, 1H, H-1′). ^{13}C NMR (D_2O) δ: 63.40 (C-6′), 63.49 (C-6), 72.28 (C-4′), 74.36 (C-3), 74.60 (C-5), 75.11 (C-2), 76.25 (C-2′), 78.42 (C-3′), 78.82 (C-5′), 86.82 (C-4), 105.82 (C-1′) and 181.00 (C-1). MS (ES, acetonitrile/H_2O, 1:1): 357.2 m/z $[M-1]^+$, MS (APCI, acetonitrile/H_2O, 1:1): 357.1 m/z $[M-1]$.

CBDH kinetics

450 μl of 15 μM to 5 mM (depending on K_m) of electron donors and acceptors and 50 μl of 100–300 nM of enzyme depending on k_{cat} were mixed in a total volume of 500 μl. The reactions were monitored for 400 s as changes in absorbency at the appropriate wavelength (see above). The benzoquinone was dissolved in ethanol to a concentration of 10 mM and diluted in phosphate buffer to the appropriate concentration. The catalytic constants (k_{cat}) were expressed as mol of oxidized cellobiose $\cdot\, s^{-1} \cdot mol^{-1}$ of enzyme. One equivalent of DCPIP, Methylene Blue or benzoquinone oxidizes one equivalent of cellobiose whereas two equivalents of cytochrome *c* or ferricyanide oxidizes one equivalent of cellobiose. The kinetic constants were determined using Lineweaver–Burk plots and were the result of dual determinations.

Oxygen as electron acceptor

10 μM Cellobiose and 10 μM CBDH were mixed in 0.1 M aerated sodium phosphate buffer at 40 °C and the reaction monitored at 449 nm and 562 nm.

For determination of hydrogen peroxide, 200 μl of 500 μM cellobiose and 300 μl of 17 μM of CBDH were mixed in a total volume of 500 μl and left overnight for complete reoxidation of the prosthetic groups, 50 μl of the reaction mixture was then mixed with 500 μl of 2 mM diammonium 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonate) and 50 μl of 50 nM *Coprinus cinereus* peroxidase [12]. The absorption at 418 nm was monitored for 3 min.

pH-dependence

All measurements were performed in Britten–Robinson buffer, pH 2.0 to 10.0 at 40 °C with the exception of the experiments with DCPIP as electron acceptor. In this case the buffers were: pH 3.5–6.0 50 mM sodium acetate, pH 6.5–7.5 0.1 M sodium phosphate and pH 7.5–9.0 50 mM Tris/HCl. 225 μl of a 500 μM cellobiose solution and 50 μl of a 0.3 μM of CBDH solution were mixed with a 225 μl solution of one of the electron acceptors in the following concentrations: 100 μM Methylene Blue, 200 μM of DCPIP, ferricyanide and cytochrome *c* and 2 mM of benzoquinone. The change in absorbance at the appropriate wavelength was monitored for 400 s.

Temperature-dependence

Measurements were performed in 50 mM Tris/HCl (pH 7.5) 450 μl of 250 μM cellobiose and 100 μM DCPIP were mixed with 50 μl of 0.4 μM CBDH. The change in absorbance at 600 nm was monitored for 400 s.

RESULTS

Purification of the cellobiose dehydrogenase from *Humicola insolens*

Throughout the purification, the cellobiose dehydrogenase was detected in the fractions by its absorbance at 420 nm and its activity on cellobiose and DCPIP. The first purification step resulted in a mixture of a component of 60 kDa and two CBDHs. The CBDHs were identified by isoelectric focusing and a subsequent overlayer of DCPIP and cellobiose which showed two distinct clearing zones. The amount of component at 60 kDa was reduced by the cation-exchange step. For the separation of the two CBDHs a gel filtration was tried at this point. However, the two CBDHs were indistinguishable on a size column, implying they were both monomers. Alternatively, the minor CBDH (94 kDa and pI 4.4) was separated from the major CBDH (92 kDa and pI 4.0) by anion-exchange chromatography due to their difference in pI (data not shown). After this third step, measurement of cellulase activity on cellohexaitol showed the presence of contaminating cellulases. Since the enzyme was purified with the purpose of using it in a cellulase assay it was very important that the cellobiose dehydrogenase was cellulase free. Therefore the final step, a gel filtration, was necessary since it resulted in a cellulase-free and pure CBDH. Table 1 summarizes the purification scheme. The purity of the enzyme was confirmed by both electrofocusing and SDS/PAGE. The purified enzyme was analysed for N-terminal residue but was found to be blocked.

Amino acid composition and sugar content

The amino acid composition is shown in Table 2. The protein is a glycoprotein with a total sugar content of 2% (w/w). The following sugars were detected: 4 mol of glucosamine, 4 mol mannose and 3 mol of galactose per mol enzyme.

Identification of the haem group

The visible spectrum (Figure 1) of the CBDH is characteristic of a haemoprotein. The oxidized state has an absorption maximum at 420 nm (γ band, 148000 M^{-1} cm^{-1}) while the spectra of the reduced state shows absorption peaks at 564 nm (α band, 44000 M^{-1} cm^{-1}), 534 nm (β band, 33000 M^{-1} cm^{-1}) and 432 nm (γ band, 209000 M^{-1} cm^{-1}). These spectra are typical of a cytochrome *b* with the exception that the absorption coefficients are very high due to the presence of many tryptophan residues.

The flavin group was weakly fluorescent, with an emission maximum at 480 nm and excitation maxima at 397 and 443 nm.

Catalytic properties

Electron donors

The enzyme was shown to be able to oxidize different di-

Table 2 Amino acid composition of CBDH from *Humicola insolens* (this work) compared with *M. thermophila* and *P. chrysosporium*

The values for *H. insolens* enzyme were calculated from the amino acid composition measured after 24 h of hydrolysis. N.D., not done.

Amino acid	CBDH from *H. insolens* (mol/mol)	CBDH from *M. thermophila* [15] (mol/mol)	CBDH from *P. chrysosporium* [4] (mol/mol)
Aspartate/asparagine	87	114	87
Threonine	74	66	77
Serine	63	54	73
Glutamate/glutamine	94	54	48
Proline	49	54	54
Glycine	109	108	76
Alanine	74	75	68
Cysteine	6*	9	4
Valine	53	60	56
Methionine	13	3	8
Isoleucine	34	36	38
Leucine	58	63	56
Tyrosine	31	30	25
Phenylalanine	32	36	36
Lysine	29	36	18
Histidine	8	12	10
Arginine	34	30	19
Tryptophan	35†	N.D.	19

* Under normal hydrolysis conditions cysteine/cystine frequently decompose making quantitation of this amino acid difficult, so the number is a minimum.

† Values determined after 12 h of hydrolysis with 4 *N*-methanesulphonic acid.

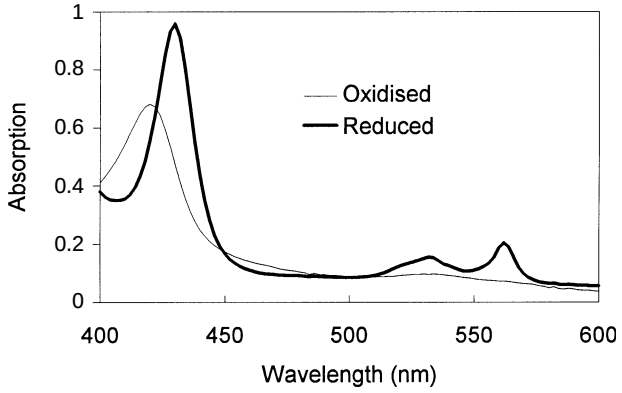

Figure 1 Absorption spectra of CBDH in the oxidized and reduced state

The reduction was performed with either cellobiose or sodium dithionite.

Table 1 Purification of cellobiose dehydrogenase from *Humicola insolens*

	A_{280}/A_{420}	Total protein (mg)	Total activity (μmol/min)	Specific activity ($\mu mol \cdot min^{-1} \cdot mg^{-1}$)	Yield (%)	Purification (-fold)
Celluzyme™	28.7	20588	441000	21.4	100	1
Arginine Sepharose	3.70	552	414000	750	94	35
S-Sepharose	2.86	245	315000	1286	71	60
HPQ-Sepharose	2.16	65	170600	2645	39	124
Superdex	1.63	18	77600	4409	18	206

Table 3 Kinetic constants for electron donors

All measurements were performed in 0.1 M sodium phosphate buffer (pH 7.5) at 40 °C DCPIP (90 μM) was used as electron acceptor. Kinetic constants were determined using Lineweaver–Burk plots.

Electron donor	K_m (μM)	k_{cat} (s^{-1})
Cellobiose	11	14
Cellotriose	19	12
Cellotetraose	21	12
Cellopentaose	17	12
Lactose	51	14
Maltose	11 000	1.2
Xylobiose	7100	3
N,N-Diacetylchitobiose	—	0
N-Acetyllactosamine	—	0
Glucose	—	0

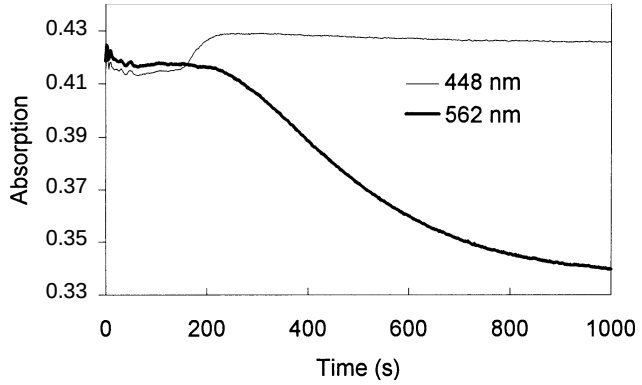

Figure 2 Oxidation of the prosthetic groups by oxygen

10 μM cellobiose and 10 μM CBDH were mixed in aerated 0.1 M sodium phosphate buffer (pH 7.5) at 40 °C. The flavin signal was monitored at 449 nm while that of the haem was monitored at 562 nm.

saccharides and cello-oligosaccharides as listed in Table 3. It was not able to oxidize glucose. The product of the oxidation of cellobiose was identified using ^{1}H and ^{13}C NMR and mass spectrometry. In both the ^{1}H and ^{13}C NMR spectra the peaks corresponding to the α- and β-anomer of the reducing end had disappeared implying oxidation at C-1. The ^{1}H NMR spectrum was identical to a spectrum of cellobionate presented by Higham et al. [13] except for a shift of 0.1 ppm probably due to the use of different internal standards. Additionally, a peak at 181.00 ppm corresponding to the carboxylate of cellobionate was found in the ^{13}C NMR ([14], 180.78 p.p.m.). The mass spectra confirmed that the isolated product was cellobionic acid/cellobionate.

Cellobiose and the cello-oligosaccharides are readily oxidized by the CBDH with approximately the same k_{cat} and K_m independent of the degree of polymerization, as seen in Table 3. Lactose is oxidized at a rate comparable to those of the cellooligosaccharides. Maltose and xylobiose are also substrates. However, these substrates display a significantly weaker binding than that of the cello-oligosaccharides. Glucose, *N*,*N*-diacetylchitobiose and *N*-acetyl-lactosamine were not oxidized.

Table 4 Kinetic constants for electron acceptors

All measurements were performed in 0.1 M sodium phosphate buffer (pH 7.5) at 40 °C. Cellobiose (225 μM) was used as electron donor

Electron acceptor	K_m (μM)	k_{cat} (s^{-1})
DCPIP	26	17
Benzoquinone	132	21
Ferricyanide	12	14
Cytochrome *c*	93	27

Oxygen as electron acceptor

Oxygen can act as electron acceptor. This has been shown by monitoring the oxidation of the prosthetic groups in the presence of aerobic buffer. The haem was monitored at 562 nm where only the haem contributes to the absorption whereas oxidation of the flavin was followed at 449 nm which is isosbestic for the haem while the flavin possesses significant absorption in the oxidized form. The curves for the oxidation of the prosthetic groups are shown in Figure 2.

Cellobiose and CBDH were mixed in aerated 0.1 M phosphate buffer, pH 7.5. The prosthetic groups were immediately reduced by cellobiose (not seen in the figure). Then a steady state appears until the cellobiose is depleted. The steady state is then followed by a period where the oxidation can be observed.

The reaction mixture was tested for hydrogen peroxide using a peroxidase assay. The formation of hydrogen peroxide could, however, not be detected under conditions capable of detecting hydrogen peroxide in concentrations down to 1 μM.

Electron acceptors

The carbohydrates were oxidized with the help of an electron acceptor. The possible electron acceptors are listed in Table 4.

The experiments were all performed at pH 7.5 because of the interest in the CBDH for an assay of cellulase activity in the neutral to alkaline pH range. Most of the electron acceptors showed pH-optima close to pH 7.5 (Figure 5) with the exception of Methylene Blue which showed an acidic pH-optima. This is due to the fact that reduced Methylene Blue is not stable at neutral pH in the presence of oxygen. The K_{cat} for DCPIP was found to be larger than that observed for cellobiose using DCPIP as electron acceptor. This is due to the fact that the extinction coefficient of DCPIP is so high that it is only possible to monitor DCPIP in a concentration of approx. 3.5 times K_m.

pH-dependence

The pH-dependence of the CBDH from *H. insolens* was determined for the five different electron acceptors (Figure 4): DCPIP (a), Methylene Blue (b), 3,5-di-t-butyl-1,2-benzoquinone (c), ferricyanide (d) and cytochrome *c* (e).

Although the CBDH appears active at pH 4 to above 9.5, the reaction of Methylene Blue can only be measured at a pH below 5.0 in the presence of oxygen due to fast autoxidation at higher pH. Reduced DCPIP and reduced benzoquinone are not stable above pH 7.5 in the presence of oxygen. If the pH of the solution with reduced DCPIP was raised to pH 9.0 it was immediately oxidized. The very high absorption coefficient of DCPIP makes this acceptor very useful for an assay of cellulase activity at

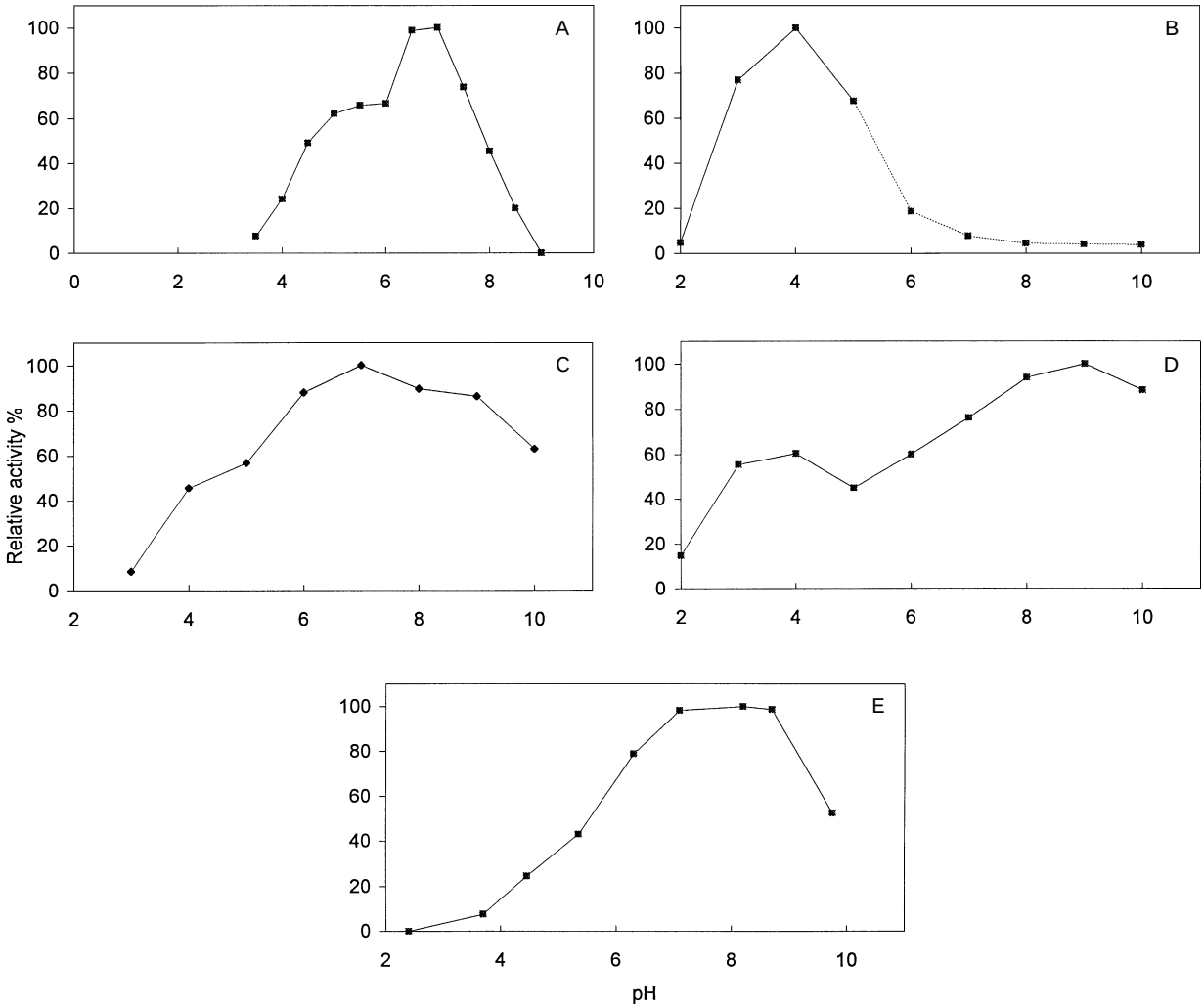

Figure 3 pH-dependence of the CBDH for the five different electron acceptors

225 μM of cellobiose was used as electron donor. The activities are expressed as relative activities. See the Materials and Methods section for details on the buffers. The electron acceptors were (**A**) DCPIP, detection at 530 nm, pH 2–5.5 and 600 nm, pH 5.5–10, (**B**) Methylene Blue, detection at 610 nm, (**C**) 3,5-di-t-butyl-1,2-benzoquinone, detection at 410 nm, (**D**) ferricyanide, detection at 420 nm and (**E**) cytochrome *c*, detection at 550 nm.

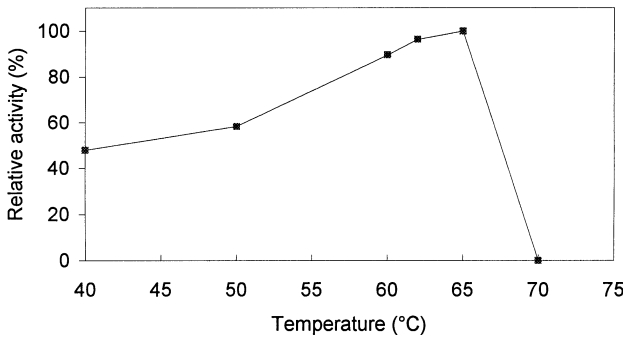

Figure 4 Temperature-dependence

The experiments were performed in Tris/HCl buffer (pH 7.5). The substrates were cellobiose and DCPIP. The activities are expressed as relative activities.

neutral pH [10]. The two Fe^{III}-containing electron acceptors show alkaline pH-optima, making them interesting for cellulase assays at alkaline pH.

Temperature-dependence

The enzyme activity increased up to a temperature of 65 °C, above which activity was completely lost (see Figure 4).

DISCUSSION

Two cellobiose dehydrogenases have been identified in a solution of Celluzyme®. Only the enzyme with a molecular mass of 92 kDa and pI of 4.0 was purified. The two enzymes were found to be monomers of similar molecular mass. This is in contrast with the findings of Canevascini et al. [16] for the two cellobiose dehydrogenases from *M. thermophila* which were a monomer of 91 kDa and a dimer of 192 kDa.

The amino acid compositions of the CBDHs from *H. insolens* and *M. thermophila* [16] and from the cloned *P. chrysosporium* [4] are listed in Table 2. The amino acid composition of the purified CBDH from *H. insolens* was similar to that of the CBDH from *M. thermophila*. The number of cysteines however, varies upon the source of enzyme, with 9, 6 and 4 residues per mol for *M.*

thermophila, *H. insolens* and *P. chrysosporium*, respectively. The *M. thermophila* CBDH was also found to contain a smaller amount of sugars (less than 4%) [16].

The visible spectrum (Figure 1) of the CBDH is characteristic of a haemoprotein and is similar to those of the CBDHs from *P. chrysosporium* [3,15] and *M. thermophila* [16]. Additionally, the same ratio of 1.63 between A_{280} and A_{420} is also found for the CBDHs from *P. chrysosporium* and *M. thermophila*. The presence of a flavin group was confirmed by spectrofluorimetry. It is thus assumed that the CBDH from *H. insolens* is an enzyme similar in type to the CBDHs from *P. chrysosporium* and *M. thermophila*.

One interest in the CBDH derived from the possibility of using it in a coupled assay for the measurement of cellulase kinetics. As seen in Table 3, cellobiose and soluble cello-oligosaccharides are readily oxidized by the CBDH with approximately the same k_{cat} and K_m, independent of the degree of polymerization. Thus, cellulases which are producing cellobiose, cellotriose and cellotetraose when hydrolysing a substrate can readily be monitored using the CBDH in a coupled assay [10]. Glucose, which is sometimes formed with some cellulases, will not be monitored. Because of the interest in testing the potential of the enzyme in kinetic assays for other enzymes, other disaccharides were tried as substrates. Lactose was the only substrate which was oxidized at a rate comparable to that of the cello-oligosaccharides. This property has already been used for the detection of lactose in milk as described by Canevascini et al. [17]. Maltose and xylobiose are also substrates; however, the specificity constants (k_{cat}/K_m) are very small for these substrates and the CBDH would thus have to be added in a large excess if it was to be used in a coupled kinetic assay for amylases or xylanases.

Higham et al. [13] have shown using NMR that the initial product of the oxidation of cellobiose by the CBDH from *P. chrysosporium* is cellobionolactone which is spontaneously converted to cellobionic acid after standing overnight. In the case of the CBDH from *H. insolens*, the product was isolated after reaction overnight and therefore a possible initial formation of cellobionolactone could not be detected.

The *H. insolens* CBDH is able to oxidize cellobiose using different electron acceptors. The oxidation of the prosthetic groups by oxygen was monitored as described by Wilson et al. [18] for the oxidation of the prosthetic groups of *P. chrysosporium*. The curves for the oxidation of the prosthetic groups (Figure 2) are very similar to those presented by Wilson et al. [18] with the exception that the rate of oxidation is considerably slower for the CBDH from *H. insolens*. The slow rate may be due to the experimental conditions which might not be optimal. However, the high similarity between the curves for the oxidation of CBDH from *P. chrysosporium* [18] and that of the CBDH from *H. insolens* suggests that the mechanism of oxidation for the two enzymes is very similar, if not identical.

Morpeth [15] has reported that hydrogen peroxide was formed by the CBDH from *P. chrysosporium*. No hydrogen peroxide was detected by Canevascini et al. [16] for the oxidation by *M. thermophila* and, instead, superoxide radicals were detected. For the CBDH from *H. insolens* the reduced oxygen product could not be identified as hydrogen peroxide under the given experimental conditions. Further experiments may hopefully elucidate the identity of the reduced oxygen species.

All the electron acceptors listed have been reported to be electron acceptors of the CBDH from *M. thermophila* [8,9]. The pH-dependence of the CBDH of *M. thermophila* for the four different electron acceptors DCPIP, Methylene Blue, 3,5-di-t-butyl-1,2-benzoquinone [8] and ferricyanide [19] have been described. DCPIP, Methylene Blue and ferricyanide resulted in slightly acidic pH-optima, whereas the benzoquinone gave a pH-optimum around neutral. For the CBDH from *P. chrysosporium* the pH-optima for DCPIP and cytochrome *c* were found to be around pH 4. As can be seen in Figure 3 the pH-optima of the *H. insolens* benzoquinone and Methylene Blue are similar to those found with the CBDH from *M. thermophila* [8]. The pH-optimum for the CBDH from *H. insolens* is more alkaline than those of the CBDHs from *P. chrysosporium* and *M. thermophila* when DCPIP is used as the electron acceptor.

In the case of the Fe^{III}-containing compounds such as ferricyanide and cytochrome *c*, there is a significant shift to higher pH, compared with the pH-optima for the two other enzymes which are completely inactive above pH 7 with Fe^{III} as electron acceptor. As suggested by Samejima and Eriksson [20], it is the haem group which is responsible for the reduction of cytochrome *c* and possibly other Fe^{III}-containing compounds as well. There might, therefore, very well be a significant difference in the environment of the cytochrome *b*-type haem of the CBDH from *H. insolens* and those of the CBDHs from *P. chrysosporium* and *M. thermophila*.

Kremer and Wood [21] have suggested that the CBDH from *P. chrysosporium* is primarily an Fe^{III} reductase and that the CBDH can cause damage to cellulose by the production of Fenton's reagent [22] under acidic conditions. The cellulases from this fungus also have acidic pH-optima [2] and it is thus possible for the cellulases and the CBDH to act together synergistically to breakdown cellulose. The CBDH from *H. insolens* reduces Fe^{III} under alkaline conditions and it might thus be able to produce Fenton's reagent under these conditions. The cellulases from *H. insolens* have alkaline pH-optima [22] and it could therefore be significant that the CBDH has a similar pH-profile. The fact that it is only the Fe^{III}-containing electron acceptors which give alkaline pH optima suggest that these compounds are especially important as electron acceptors. It could therefore be that the enzyme is a cellobiose:Fe^{III} oxidoreductase as suggested by Kremer and Wood [21] for the CBDH from *P. chrysosporium*.

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