Some properties of cellobiose oxidase from the white-rot fungus *Sporotrichum pulverulentum*

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Cellobiose oxidase from the white-rot fungus *Sporotrichum pulverulentum* has been purified to homogeneity by a new procedure. The carbohydrate and amino acid compositions of the enzyme have been determined. Cellobiose oxidase contains FAD and cytochrome *b* prosthetic groups. M_r of the enzyme has been estimated at 74 400 by sedimentation equilibrium. The enzyme is a monomer. Optical, fluorescence and e.p.r. spectra of oxidized and reduced cellobiose oxidase have been determined. A preliminary investigation of the substrate specificity of cellobiose oxidase reveals that disaccharides and even some insoluble polysaccharides are substrates, but not monosaccharides. Strong substrate inhibition is seen at high concentrations of cellobiose. This effect is particularly marked when oxygen is the electron acceptor. Cellobiose oxidase is unusual among flavoproteins, since it stabilizes the red anionic flavin semiquinone and forms a sulphite adduct, yet appears to produce the superoxide anion as its primary reduced oxygen product.

The white-rot fungus *Sporotrichum pulverulentum* (= *Phanerochaete chrysosporium*) is one of the organisms able to degrade the polysaccharides and the lignin present in plant material. There have been many reports over the last few years of *S. pulverulentum* and other white-rot fungi producing extracellular enzymes capable of oxidizing cellobiose to cellobionolactone (Westermarck & Eriksson, 1974a,b; Ayers *et al.*, 1978; Ander & Eriksson, 1977; Morpeth, 1984). In general, brown-rot fungi, which digest only the polysaccharides in wood, do not produce cellobiose oxidoreductase (Ander & Eriksson, 1978; Morpeth, 1984), though there are two documented exceptions to this (Dekker, 1980; Coudray *et al.*, 1982).

One of the cellobiose oxidoreductases present in *S. pulverulentum* has been characterized and named 'cellobiose oxidase' (Ayers *et al.*, 1978). It contains both haem and flavin cofactors and binds irreversibly to concanavalin A–Sephadex, suggesting that it is a glycoprotein. The aim of the present study was to complete the characterization of cellobiose oxidase, since a better understanding of the enzyme and its properties will help in discovering the role of cellobiose oxidase in ligninocellulose biodegradation.

Experimental

Materials

Glass-distilled water was used throughout. All chemicals were of the highest purity available and were purchased from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. FAD was purified by DEAE-cellulose chromatography (Massey & Swoboda, 1963).

Growth of the organism

Sporotrichum pulverulentum (Novobranova) was obtained from the Commonwealth Mycology Institute (CMI 174 727) and grown by subculturing on to a malt-extract/agar slope for 7–10 days at 30°C. One slope was used to inoculate 1000 ml of the modified Norkrans media described by Eriksson & Pettersson (1975) in a 2000 ml flask with absorbent cottonwool as the sole carbon source. The flask was shaken at about 150 rev./min. Enzyme activity was monitored until no further increase was seen, after 10–14 days, and cultivation was stopped. Alternatively, the fungus was grown in static culture with softwood sawdust as carbon source for 28 days. In both cases the extracellular medium was separated by filtration through six layers of cheesecloth.

Purification of cellobiose oxidase

Extracellular medium (32 litres) from cotton-wool growths was concentrated to 2 litres in 4–6 h by ultrafiltration in a Millipore Pellicon system fitted with a 10000- M_r -exclusion-limit polysulphone membrane. Less than 1% of the cellobiose oxidoreductase activity was present in the filtrate. The protein solution was cooled to 4°C and adjusted to pH 7 by the addition of K_2HPO_4 from a 1 M stock solution. The solution was clarified by centrifuging and solid $(NH_4)_2SO_4$ (365 g·l⁻¹) slowly added, the pH being maintained as described above. After the mixture had been stirred for 2 h at 4°C, the resultant precipitate was collected by centrifugation.

The precipitate was suspended in 1 litre of 20 mM-potassium phosphate buffer, pH 7.5, concentrated to 50 ml by ultrafiltration through an Amicon PM 10 membrane and then applied to a column (60 cm × 10 cm) of Bio-Gel P2 equilibrated with 5 mM-potassium buffer, pH 7.5. The coloured protein fractions were pooled, concentrated to 35 ml as described above and then applied to a DEAE-Sephadex column (90 cm × 2.5 cm) equilibrated with 5 mM-potassium phosphate, pH 7.5.

After washing the column with 5 mM-potassium phosphate buffer, pH 7.5, one of the cellobiose oxidoreductases was eluted. This is probably the cellobiose quinone dehydrogenase described previously (Westermarck & Eriksson, 1975; Morpeth, 1984). Cellobiose oxidase and another cellobiose-oxidizing activity were separated by washing the column with a 2-litre linear gradient from 5 mM- to 80 mM-potassium phosphate buffer, pH 7.5 (Morpeth, 1984).

Cellobiose oxidase peak fractions were pooled and precipitated with $(NH_4)_2SO_4$ (285 g·l⁻¹). The precipitate was resuspended in 100 ml of 50 mM-potassium phosphate buffer, pH 6, concentrated to 5 ml by ultrafiltration and loaded on to a Sephacryl S-200 gel-filtration column (110 cm × 5 cm). The column was eluted with 50 mM-potassium phosphate buffer, pH 6, and the peak fractions pooled, concentrated as described above and dialysed against 10 mM-potassium phosphate buffer, pH 6. The enzyme was applied to a column (2.5 cm × 8 cm) of Reactive Red-agarose equilibrated in the dialysis buffer and the column washed with that same buffer. Cellobiose oxidase did not bind to this resin under these conditions, but a number of minor contaminants were removed.

If the enzyme was not pure as assessed by polyacrylamide-gel electrophoresis a further DEAE-Sephadex column (1.5 cm × 15 cm) was performed with a 500 ml total volume gradient under the same conditions as those described above.

Enzyme assay

Cellobiose oxidase activity was monitored at

25°C and 600 nm in 2.5 ml of a solution containing 0.125 mM-potassium phosphate buffer, pH 6, 0.5 μ mol of cellobiose and 60 nmol of 2,6-dichlorophenol-indophenol. Increasing the concentration of cellobiose resulted in a significant inhibition of the assay. One unit of enzyme activity is defined as the consumption of 1 μ mol of cellobiose under the above assay conditions.

Oxygen measurements

The concentration of dissolved oxygen was monitored by means of a Hansatech oxygen electrode (Hansatech, Kings Lynn, Norfolk, U.K.).

Protein determination

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin fraction V as a standard. The concentration of bovine serum albumin was determined by using a value of 6.67 for $A_{279}^{1\%}$ (Janatova *et al.*, 1968). Dry-weight measurements with cellobiose oxidase gave a value of 77% of that obtained from the analysis above.

Flavin constituents

The flavin coenzyme was released from the enzyme by addition of trichloroacetic acid to 5% (w/v) final concentration. The red pellet was removed by centrifugation at 15000g for 10 min and the supernatant extracted three times with cold diethyl ether, before being adjusted to between pH 6 and 7 with 0.01 M- Na_2CO_3 . The solution was evaporated to dryness and redissolved in a small volume of water for t.l.c. analysis on silica-gel plates. Two solvent systems were used: 6% (w/v) Na_2HPO_4 ·12H₂O in water and butan-1-ol/acetic acid/water (4:3:3, by vol.) (Kilgour *et al.*, 1957; Fazekas & Kokai, 1971).

Electrophoresis

Polyacrylamide-gel electrophoresis was performed at 5°C in 7.5% (w/v) gels in 5 mM-Tris/50 mM-glycine buffer on an LKB Multiphor horizontal-electrophoresis unit. Gels were stained for protein with Coomassie Brilliant Blue R-250. Activity was revealed by incubating the gel in a stain, 50 ml of which contains Nitro Blue Tetrazolium (25 mg), phenazine methosulphate (0.5 mg) and cellobiose (40 μ mol) in 50 mM-potassium phosphate buffer, pH 6. If the stain was allowed to develop aerobically the phenazine methosulphate could be omitted.

Absorption and fluorescence measurements

Absorption spectra and enzyme assays were measured on a Cary 210 recording spectrophotometer. Fluorescence spectra were recorded on a Perkin-Elmer LS5 recording fluorimeter.

Anaerobic experiments

Anaerobic spectral experiments were done using a Thunberg-type cell equipped with two side arms. For dithionite titrations an apparatus similar to that previously described was used (Foust *et al.*, 1969). Anaerobiosis was obtained by alternately evacuating the cuvette with a vacuum pump and then admitting N_2/H_2 (99:1) that had been freed of O_2 by passage through a Deoxo catalytic purifier (Engelhard Industries, Newport, Gwent NP6 4YN, Wales, U.K.). N_2 treated in this way contained less than 0.3 p.p.m. of oxygen.

Analytical ultracentrifugation

Sedimentation-equilibrium experiments were performed at 20.3°C in a Beckman model E ultracentrifuge. Rotor speeds of 26000 rev./min were used with an initial enzyme concentration of 0.58 mg/ml in 50 mM-potassium phosphate buffer, pH 6. The enzyme was fully stable for the duration of the experiment. The density of the buffer and the partial specific volume of the enzyme were determined by using a mechanical oscillator densitometer from Stanton-Redcroft, London S.W.17, U.K., which consists of a PAAR DMA 60 density meter and a PAAR DMA 602 density-measuring cell.

Reaction with insoluble polysaccharides

Contaminating soluble carbohydrates were removed from all insoluble polysaccharides by washing four to five times with water and centrifugation.

The reduction of cellobiose oxidase by insoluble polysaccharides was examined by mixing them in an anaerobic cuvette, quickly making it anaerobic and leaving it for several hours, after which the insoluble powder settled and a visible spectrum could be recorded. Rapid reduction of cellobiose oxidase was monitored by observing the maxima of the Soret band.

Amino acid and sugar analysis

Amino acid analyses were performed at the Agricultural Services Unit of this University. Total carbohydrate was determined by the method of Dubois *et al.* (1965). Neutral- and amino-sugar analyses were performed by Dr. J. Kennedy at the Department of Chemistry, University of Birmingham, Birmingham, U.K. Tryptophan was determined as described by Pajot (1976).

E.p.r. procedure

The e.p.r. spectra of frozen samples were measured on a Varian E9 spectrometer in the laboratory of Dr. R. C. Bray, School of Molecular Sciences and Chemistry, University of Sussex, Falmer, Brighton, Sussex, U.K. The flavin semi-

quinone spectra were detected by using the following conditions: temperature, 120 K; microwave power, 5 mW; microwave frequency, 9.3 GHz; modulation amplitude, 0.16 mT. Conditions to detect the signal from the oxidized cytochrome were: temperature, 12.2 K; microwave power, 5 mW; microwave frequency, 9.34 GHz; modulation amplitude, 1 mT.

Results

Growth of the organism

As assessed by activity staining of concentrated extracellular medium run on native polyacrylamide gels, *S. pulverulentum* produces the same three cellobiose-oxidizing isoenzymes under both growth conditions. All three of the cellobiose oxidoreductases appeared in primary growth, at the same time as the various cellulase activities also produced by this organism (F. F. Morpeth, unpublished work).

Purification

Pure cellobiose oxidase shows one diffuse band on electrophoresis. On occasions, up to four very minor protein and activity bands of slightly greater mobility can also be seen. These minor bands account for less than 5% of the total activity and are presumably due to glycolytic or proteolytic action during the growth. Including benzamidine (5 mM) in the growth media to prevent proteinase action stopped fungal growth. A typical purification of cellobiose oxidase is summarized in Table 1. The large decrease in activity after the DEAE-Sephadex column is due to the separation of cellobiose oxidase from the two other cellobiose oxidoreductases found in *S. pulverulentum* (Morpeth, 1984). The final pure product from Table 1 is extremely stable and only slowly loses its activity over several months when stored at 4°C in 50 mM-potassium phosphate buffer, pH 6. Cellobiose oxidase is very stable to extremes of pH; large losses of activity only occur below pH 2 and above pH 11. The purified enzyme loses no activity over 6 months in 0.5 ml lots at a concentration of 3–5 mg·ml⁻¹ when stored in the standard manner. The above purification yields three to six times more pure protein than does that of Ayers *et al.* (1978). Pure cellobiose oxidase has a maximum specific activity of 1.2 units/mg in the standard assay.

In sedimentation-equilibrium experiments linear plots were observed over the whole cell. By using a partial specific volume, determined by densitometry, of 0.741, an M_r of 74400 ± 3800 was found. Evidence that this is an acceptable estimate of the M_r is as follows. Firstly, cellobiose oxidase contains only one tryptophan residue (to the

Table 1. *Purification of cellobiose oxidase from the culture fluid of S. pulverulentum*

Purification step	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (-fold)
Culture filtrate	32000	23000	360	0.0156	100	1
(NH ₄) ₂ SO ₄	33	7500	350	0.047	97	3
DEAE-Sephadex	18	1600	49.5	0.031	13.8	2
(NH ₄) ₂ SO ₄	6.4	975	48.9	0.05	13.6	3.2
Sephacryl S-200	5.4	36*	35.2	0.98	9.8	63
Reactive Red	6	32*	35	1.09	9.7	70

* Based on a Lowry correction factor of 0.77 when bovine serum albumin is the protein standard.

Table 2. *Amino acid composition of cellobiose oxidase*
Unless otherwise specified, each value represents an average of results obtained after 24, 48 and 72 h of hydrolysis. Threonine and serine were estimated by extrapolation to zero time; the value for isoleucine is that obtained after 72 h of hydrolysis, and tryptophan was determined as described in the text; cysteine was determined after formic acid oxidation.

Amino acid	Composition (mol/mol of protein of <i>M_r</i> 74000)
Aspartate	75
Threonine	67
Serine	79
Glutamate	34
Proline	60
Glycine	72
Alanine	54
Valine	42
Methionine	12
Isoleucine	27
Leucine	44
Tyrosine	15
Phenylalanine	28
Lysine	13
Histidine	10
Arginine	15
Tryptophan	1
Cysteine	8

Table 3. *Carbohydrate analysis of cellobiose oxidase*
Each value represents an average of two analyses. There is some doubt about the identity of rhamnose, since the peak was eluted in almost, but not absolutely, the rhamnose position of the standards.

Type of sugar	Component	Abundance (%)
Neutral	Mannose	6.8
	Xylose	1.0
	Glucose	0.9
	Ribose	0.8
Basic	Rhamnose	1.8
	Glucosamine	0.43
	Galactosamine	0.14

Amino acid and sugar analysis

The amino acid composition of cellobiose oxidase is given in Table 2. Particularly noteworthy is the presence of only one tryptophan residue and the low content of basic amino acids.

The sugar content of cellobiose oxidase is shown in Table 3; the bulk of the sugar residues are mannose, which is consistent with the irreversible binding of the enzyme to concanavalin A-Sepharose (Ayers *et al.*, 1978; F. F. Morpeth, unpublished work). The absolute amounts of the sugars indicate that the enzyme has a carbohydrate content of 11.9%, which agrees with the estimate for total carbohydrate of 10%.

Identification of the prosthetic groups

Analysis by t.l.c. of the cofactors of cellobiose oxidase released by precipitation of the enzyme with 5% trichloroacetic acid revealed that the flavin component migrated the same distance as FAD in both solvent systems. The protein pellet was red, and cytochrome was removed by extraction with acidified acetone. The nature of the cytochrome was identified by preparing a pyridine haemochromogen. The band had a maximum absorption band at 557 nm, showing it to be a cytochrome *b*.

The optical spectrum of the neutralized cofactors released by trichloroacetic acid showed on one

nearest integer); from this, a minimum *M_r* of 71000 could be calculated. Secondly, from the dithionite titrations of the enzyme's prosthetic groups, an *M_r* of 72400 may be estimated from the end point of the titration. Gel electrophoresis by the method of Weber & Osborn (1969) in the presence of sodium dodecyl sulphate suggested that the enzyme's *M_r* was 90000. This can, however, be discounted, since (as discussed below) cellobiose oxidase is a glycoprotein. Finally, by using a calibrated Sephacryl S-200 column, an *M_r* of 84200 was estimated for cellobiose oxidase. This high value probably reflects cellobiose oxidoreductase interacting with the resin in a manner that is not only related to its *M_r*.

occasion two extra absorption bands at 430 and 600nm. Chromatography of these cofactors by the method of Mayhew *et al.* (1974) revealed two components: FAD and a green chromophore with an optical spectrum similar to that of 6-hydroxy-FAD. Further characterization of this chromophore has not been possible, because it is only present as a very minor component of cellobiose oxidase except for this one particular preparation.

Absorption and fluorescence spectra

As shown in Fig. 1, the visible absorption spectrum of oxidized cellobiose oxidase is typical of a cytochrome *b*. The absorption maxima (with molar absorption coefficients in parentheses) were 421 ($57200\text{M}^{-1}\cdot\text{cm}^{-1}$) 530 ($5650\text{M}^{-1}\cdot\text{cm}^{-1}$) and 561 ($3900\text{M}^{-1}\cdot\text{cm}^{-1}$)nm. The oxidized enzyme also displays shoulders at 370 ($18260\text{M}^{-1}\cdot\text{cm}^{-1}$), 453 ($11960\text{M}^{-1}\cdot\text{cm}^{-1}$) and 486 ($7390\text{M}^{-1}\cdot\text{cm}^{-1}$)nm. On reduction with either cellobiose or sodium dithionite the spectra maxima were shifted to 429 ($76700\text{M}^{-1}\cdot\text{cm}^{-1}$), 532 ($8550\text{M}^{-1}\cdot\text{cm}^{-1}$) and 562 ($14460\text{M}^{-1}\cdot\text{cm}^{-1}$)nm, with shoulders at 523 ($5050\text{M}^{-1}\cdot\text{cm}^{-1}$) and 542 ($4400\text{M}^{-1}\cdot\text{cm}^{-1}$)nm.

There were no obvious transient changes on reduction that could be readily ascribed to flavin semiquinone (see below), though the strong absorbance of the cytochrome moiety would make such changes difficult to detect. In the u.v. the oxidised enzyme has a peak at 278nm and the

280nm/260nm absorbance ratio of the enzyme is 1.5.

The flavin in cellobiose oxidase was weakly fluorescent, with an emission maxima at 564nm and excitation maxima at 380 and 444nm. The FAD in cellobiose oxidase was approx. 50 times less fluorescent than FAD purified by chromatography (Massey & Swoboda, 1963).

The single tryptophan residue in cellobiose-oxidase had a fluorescence emission maximum at 336nm and an excitation maximum at 280nm, and, on reduction with cellobiose, no significant change occurred.

Adding Na_2SO_3 to cellobiose oxidoreductase caused a spectral change (see Fig. 2). There was little change in the cytochrome absorption, but the decrease in absorbance in the range 440–500nm suggests that the flavin coenzyme had been reduced. This was confirmed by a complete loss of flavin fluorescence in the presence of sulphite; probably the FAD forms a covalent adduct with sulphite, which is typical of the simple flavoprotein oxidases (Massey & Hemmerich, 1980).

Fig. 1. Absorption spectra of oxidized and reduced cellobiose oxidase

Enzyme ($9.2\mu\text{M}$) in 50mM-potassium phosphate buffer, pH6, was made anaerobic and the spectrum was recorded at 20°C (curve 1). The enzyme was titrated with a solution of sodium dithionite that had been standardized by titration with FAD purified by chromatography. The spectrum of the fully reduced enzyme is shown in curve 2.

Fig. 2. Absorption spectrum of the sulphite adduct of cellobiose oxidase

Sodium sulphite (8mM) was added to cellobiose oxidase in 50mM-potassium phosphate buffer, pH6. Spectra were recorded before (curve 1) and after (curve 2) addition of the sulphite at 20°C. The spectrum of the sulphite adduct did not change significantly over the course of 6h, and extra additions of sulphite caused no further spectral changes.

E.p.r. spectra

At liquid-N₂ temperatures, oxidized cellobiose oxidase has no detectable e.p.r. signal. On reduction by cellobiose or dithionite a flavin radical signal appeared with a linewidth of 1.4 mT, characteristic of the anionic or red flavin semiquinone (Massey & Palmer, 1966). An oxidised cytochrome *b* signal was detected at liquid-helium temperatures, which is typical of a low-spin haem and is reduced by addition of substrate. The parameters for the signal are as follows: $g_1 = 3.52$, $g_2 = 2.027$ and g_3 could not be seen, owing to g strain, which results in very large linewidths at low g values.

Dithionite reduction

The enzyme was rapidly reduced by dithionite. In a quantitative titration with a standardized dithionite solution the enzyme's spectrum was converted into that of a typical reduced cytochrome *b*, as shown in Fig. 1. The end of the titration was evident from the cessation of spectral changes. In Fig. 3 A_{429} and A_{465} are plotted against added dithionite. The lower wavelength is the reduced cytochrome *b* peak, whereas the higher is essentially clear of the haem Soret band and will reflect both flavin and cytochrome *b* reduction. Clearly the cytochrome *b* is of a higher potential than either of the flavin half-reactions. During addition of the first electron, when the cytochrome was being predominantly reduced, there were isosbestic points at 348, 423 and 442 nm. However,

these were lost as the titration proceeded and the flavin was also reduced.

Nature of the reduced oxygen product

In an assay carried out at 25°C in 2.5 ml of 50 mM-potassium phosphate buffer, pH 6, containing 30 nmol of cellobiose and cellobiose oxidase, a final decrease in the concentration of dissolved oxygen corresponding to 28 nmol was seen. If catalase was added to the reaction mixture, 12.4 nmol of oxygen was evolved. This stoichiometry was seen with several different preparations of cellobiose oxidase and shows that cellobiose oxidase does produce H₂O₂, an observation in contrast with that of Ayers *et al.* (1978). When cellobiose oxidase (0.14 μ M) was incubated aerobically in 50 mM-potassium phosphate buffer, pH 6, with cellobiose (40 μ M) and cytochrome *c* (20 μ M) at 25°C, the cytochrome *c* was rapidly reduced at a rate corresponding to 0.32 mol·s⁻¹·mol of enzyme⁻¹. In the presence of 9000 units of superoxide dismutase this rate was markedly decreased to a limiting value of 0.026 mol of cytochrome *c* reduced·s⁻¹·mol of enzyme⁻¹. The presence of bovine serum albumin (1 mg) in the aerobic cytochrome *c* assay had no effect on the apparent rate of reduction. The apparent rate of reduction of cytochrome *c* observed under anaerobic conditions was 0.135 mol·s⁻¹·mol of enzyme⁻¹, 42% of the activity seen in the presence of oxygen.

These results suggest that superoxide could be the primary reduced oxygen species produced by cellobiose oxidase. The H₂O₂ detected is possibly formed by the dismutation of the superoxide anion.

Specificity

The results of assays of cellobiose oxidase with various substrates and electron acceptors under standard conditions are shown in Table 4. None of the monosaccharides tested showed any activity at various concentrations between 0.04 and 40 mM with either 2,6-dichlorophenol-indophenol or oxygen as the electron acceptor. Cellobiose had the lowest K_m of the disaccharides examined, followed by lactose and maltose. The slightly higher estimate for V_{max} when 2,6-dichlorophenol-indophenol is varied rather than cellobiose is due to the difficulties in estimating this parameter in the presence of substrate inhibition.

In Fig. 4 a double-reciprocal plot is shown in which cellobiose is varied at a constant concentration of 2,6-dichlorophenol-indophenol. Particularly noticeable is the very strong substrate inhibition seen at high concentrations of cellobiose.

When cellobiose oxidase activity is monitored with an oxygen electrode, the lowest concentration of cellobiose that can be used without infringing

Fig. 3. Anaerobic reduction of cellobiose oxidase by sodium dithionite

Conditions were as for Fig. 2. The changes in absorbance at 429 and 465 nm (corrected for dilution) are plotted as a function of the added dithionite.

Table 4. Apparent Michaelis constants and maximum velocities of various substrates of cellobiose oxidase. Values for K_m and V_{max} were determined from double-reciprocal plots. For the determination of K_m values for sugar substrates, the concentration of 2,6-dichlorophenol-indophenol was held constant at 30 μM . For the K_m values of electron acceptors, the concentration of cellobiose was held constant at 200 μM . The maximum velocities were calculated by assuming an M_r of 74 400 for cellobiose oxidase. All values are for 25°C and pH 6 and were determined in 50 mM-potassium phosphate buffer. No activity was shown towards glucose, galactose, xylose and fructose.

	$K_m(\text{app.})$ (μM)	$V_{max}(\text{app.})$ (s^{-1})
Substrates:		
Cellobiose	42	1.6
Lactose	580	1.6
Maltose	6600	0.22
Electron acceptors:		
2,6-Dichlorophenol-indophenol in the absence of oxygen	17	1.9
2,6-Dichlorophenol-indophenol in the presence of 240 μM -oxygen	12	1.9
Benzyl Viologen	3000	0.048

Fig. 4. Initial-rate measurements of cellobiose oxidase catalysed oxidation of cellobiose by 2,6-dichlorophenol-indophenol

The reciprocal of the initial specific rate (e/v_0 , where e is the molar concentration of cellobiose oxidase and v_0 is the initial rate of cellobiose oxidation) is plotted against the reciprocal of cellobiose concentration. The reaction was carried out in 50 mM-potassium phosphate buffer, pH 6, at 25°C, at a constant concentration of 40 μM -2,6-dichlorophenol-indophenol.

the steady-state assumption is 15 μM . Down to this concentration, cellobiose oxidase activity was increasing with decreasing cellobiose. This was seen both at pH 6 in 50 mM-potassium phosphate buffer and at pH 4 in 20 mM-sodium acetate buffer and suggests that, when oxygen is the electron acceptor, substrate inhibition is particularly marked.

A wide range of polysaccharides were also examined as substrates for cellobiose oxidase. The following polysaccharides were capable of causing an immediate reduction as judged by a shift in the Soret band of the enzyme to 429 nm: cellulose (Sigmacell type 20), chitin (from crab shells), xylan (from oat spelts) and agarose. Mannan (from *Saccharomyces cerevisiae*) will slowly reduce cellobiose oxidase over a number of hours, but starch had no effect on the enzyme.

Two other electron acceptors deserve mention. When potassium ferricyanide is varied at a constant concentration of cellobiose (200 μM), non-linear double-reciprocal plots give a downward curve. This type of behaviour would be expected if potassium ferricyanide were reacting at differing rates with spatially distinct flavin and cytochrome.

When 2,6-dichlorophenol-indophenol was varied at a constant cellobiose concentration, linear double-reciprocal plots are obtained. In the presence of 21% (240 μM) oxygen the K_m for 2,6-dichlorophenol-indophenol is 12 μM ; the anaerobic value is 17 μM . Including 1000 units of superoxide dismutase in the assay mixtures abolished the oxygen activation. The most likely explanation for this phenomenon is that, in the presence of oxygen, both reduced enzyme and the superoxide anion are reducing the dye.

Electron acceptors that had no activity with cellobiose oxidase when assayed anaerobically were riboflavin, FMN and FAD.

Discussion

The role of oxidoreductases in ligninocellulose breakdown is an area of increasing importance. Incorporated here is a simplified preparation of cellobiose oxidase and determination of a number of its properties. From the viewpoint of the

enzyme's biological function, possibly the most important observation is the ability of cellobiose oxidase to oxidize a range of cell-wall polysaccharides to produce the superoxide anion. Activated forms of oxygen have been implicated in lignin depolymerization (Forney *et al.*, 1982), so this could be of significance.

Cellobiose oxidase is a classic flavoprotein oxidase, since it forms a sulphite adduct and stabilizes a red anionic semiquinone. However, unlike other flavoprotein oxidases the superoxide anion rather than H_2O_2 is the primary reduced oxygen product. In this behaviour it is similar to 'Old Yellow Enzyme' (NADPH dehydrogenase, EC 1.6.99.1), which also stabilizes a red radical and yields the superoxide anion (Massey & Hemmerich, 1980). It has been suggested, that this is due to the red radical stabilized by Old Yellow Enzyme being neutral, rather than an anion (Massey & Hemmerich, 1980). However, with cellobiose oxidase the radical was identified by its e.p.r. rather than its optical properties. There is a very considerable difference in the linewidths between the blue-neutral and red-anion forms of the radical (Massey & Palmer, 1966). Thus it is considered unlikely that a red neutral radical would have exactly the same form as the red anion radical. The stabilization of the red semiquinone does, however, suggest that there is a basic residue of the protein in the N1-C2 locus of the flavin (Massey & Hemmerich, 1980).

In free solution it is known that the anionic flavin radical reacts with oxygen to produce superoxide at a rate orders of magnitude greater than that displayed by the neutral radical (Vaish & Tollin, 1972). Thus, if the role of cellobiose oxidase is indeed to produce superoxide, then stabilization of the anionic radical would seem sensible.

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