CELLOBIOSE DEHYDROGENASE — A NOVEL HEMOFLAVOENZYME FROM THE LIGNOCELLULOSE-DEGRADING BASIDIOMYCETE *PHANEROCHAETE CHRYSOSPORIUM*

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The dissertation "Cellobiose Dehydrogenase — A Novel Hemoflavoenzyme from the Lignocellulose-degrading Basidiomycete *Phanerochaete chrysosporium*" by Wenjun Bao has been examined and approved by the following Examination Committee:

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DEDICATION

To

My husband

Dr. Yu Lu

and

My parents

Profs. Yuanzhen Xiang & Chenglan Bao

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ABSTRACT

CELLOBIOSE DEHYDROGENASE — A NOVEL HEMOFLAVOENZYME FROM THE LIGNOCELLULOSE-DEGRADING BASIDIOMYCETE *PHANEROCHAETE CHRYSOSPORIUM*

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Cellobiose dehydrogenase (CDH) from the extracellular medium of *Phanerochaete chrysosporium* was purified and characterized in detail. Optimal culture conditions for CDH were investigated. The role of CDH in cellulose degradation by cellulases was studied.

Cellobiose dehydrogenase oxidizes cellobiose to cellobionolactone. It is a hemoflavoenzyme which contains a heme *b* and a FAD on a monomer. It was purified to homogeneity by ammonium sulfate precipitation, DEAE-Sephadex, Phenyl Sepharose, Sephacryl S-200 chromatographies and FPLC using a Mono Q column. It is a glycoprotein with a neutral carbohydrate content of 9.4%. The molecular weight is estimated as 90,000 by SDS-PAGE. In addition to cellobiose, cellotriose, cellotetraose, cellopentaose and lactose serve as substrates for CDH. Cytochrome *c*, dichlorophenol-indophenol, Mn^{3+}, ferricyanide and benzoquinone function as electron acceptors to CDH. In the absence of these electron acceptors, oxygen serves as a poor electron acceptor and is reduced to H_2O_2. CDH is very stable in the pH range 3-10 and in temperatures up to 50°C. At lower pH and higher temperature, CDH is inactivated due to the release of flavin from the active site. Native CDH has absorption maxima at 420, 529 and 570 nm. Upon addition of cellobiose, CDH is

reduced and the absorption maxima shift to 429, 534 and 564 nm. The heme of CDH appears to be hexacoordinate.

The effect of different culture conditions on the production of lignocellulose-degrading enzymes by cellulose-degrading cultures of the white-rot fungus *Phanerochaete chrysosporium* was examined. Culture conditions examined included the initial pH of the culture medium, the type of cellulose substrates, and the concentrations of carbon and nitrogen. Succinate medium with an initial pH of 4.5 yields the highest levels of CDH and β-glucosidase and the lowest levels of cellobiose:quinone oxidoreductase. In the presence of low levels of nitrogen, cellulose-degrading cultures of *P. chrysosporium* produce lignin and manganese peroxidases.

The role of CDH in cellulose degradation was studied by CDH addition to *Trichoderma* cellulases. At lower concentration, CDH enhances microcrystalline cellulose degradation by cellulases. Cellulose weight loss increases up to 19%, but CDH has no significant effect on cotton linter, filter paper, and acid-treated cellulose degradation by cellulases.

CHAPTER 1
INTRODUCTION

Wood is the most abundant biomass available on the earth. The major components of wood include cellulose (50-60%), hemicellulose (5-15%) and lignin (20-35%) [Wise & Ratliff, 1947]. Lignocellulose can be converted to gaseous or liquid fuel and chemicals by physical, chemical and biological processes [Philips & Humphrey, 1983] (Fig. 1-1).

1.1 CHEMICAL STRUCTURE OF LIGNOCELLULOSE

In 1838, French chemist Anselme Payen reported that wood lost weight by treatment with nitric acid. He named the solid fiber product cellulose. The dissolved material was later called lignin by Schulze [1857].

1.1.1 Cellulose Structure

Cellulose is a linear, unbranched monopolymer of glucose residues which are linked by β-1,4 linkages (Fig. 1-2). The degree of cellulose polymerization is up to 14,000 glucose units [Coughlan, 1985]. Each glucose residue is related to the next one by a rotation of 180°. Cellobiose is the basic structure of cellulose (Fig. 1-2). Inter- and intramolecular hydrogen bonds are easily formed between glucose residues. This hydrogen bond network makes cellulose highly insoluble in water and provides tensile strength to the fiber (Fig. 1-2). In plants, cellulose fibers are embedded in a matrix of hemicellulose [Fan et al., 1980; Shafizadeh & McGinnis, 1971; Wenzl, 1970]. Cellulose fiber consists of crystalline and amorphous regions [Béguin & Aubert, 1994] (Fig. 1-2). The degree of cellulose crystallinity depends on its origin. In nature, cellulose molecules are highly ordered or crystalline. Cellulose from

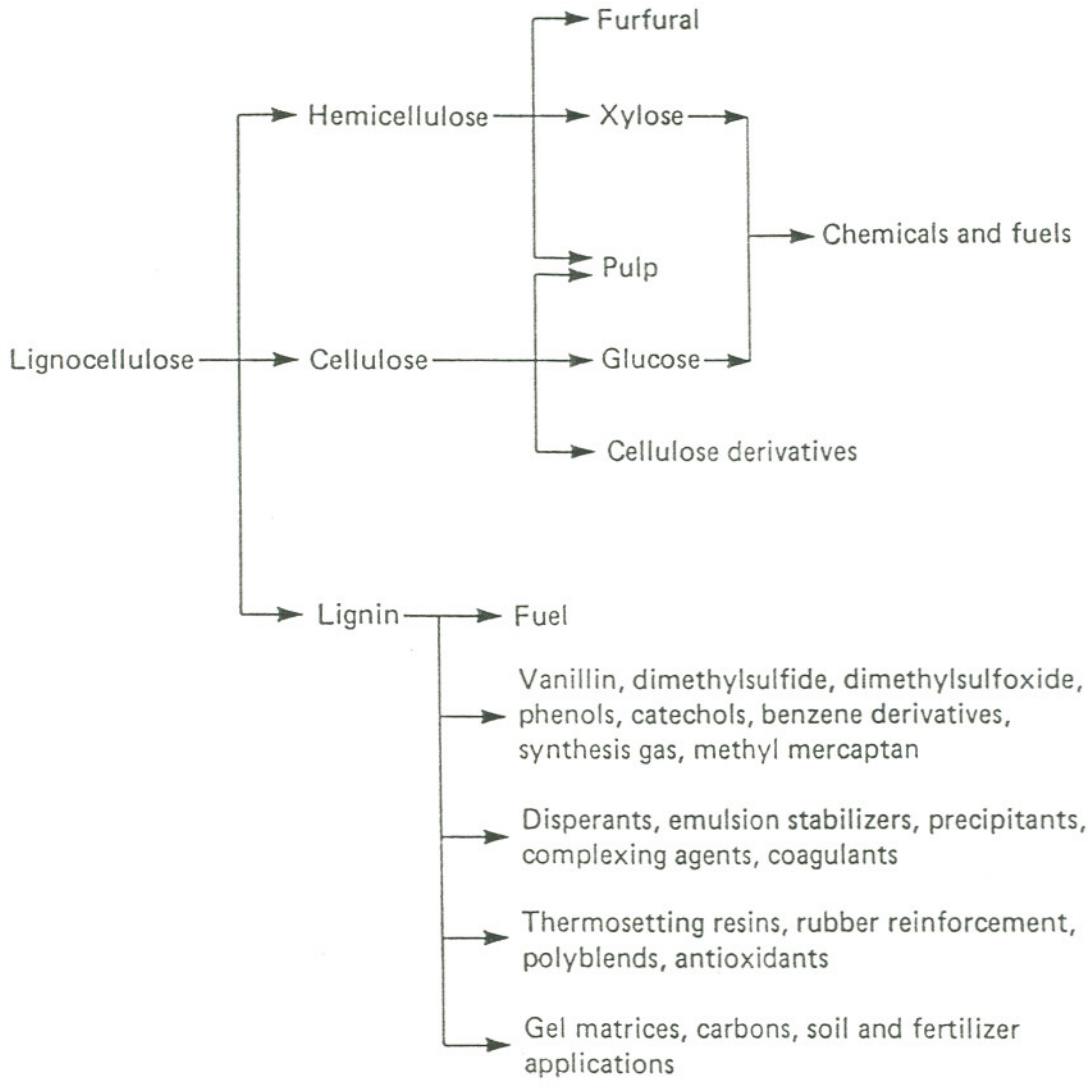
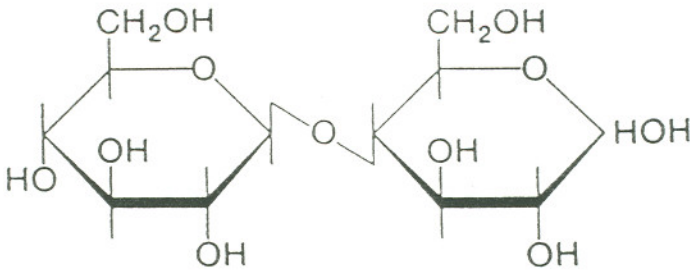


Fig. 1-1 Lignocellulose conversion into chemicals and fuels [Philips & Humphrey, 1983].

A)



Cellobiose: glucose-β-1,4-glucose

B)

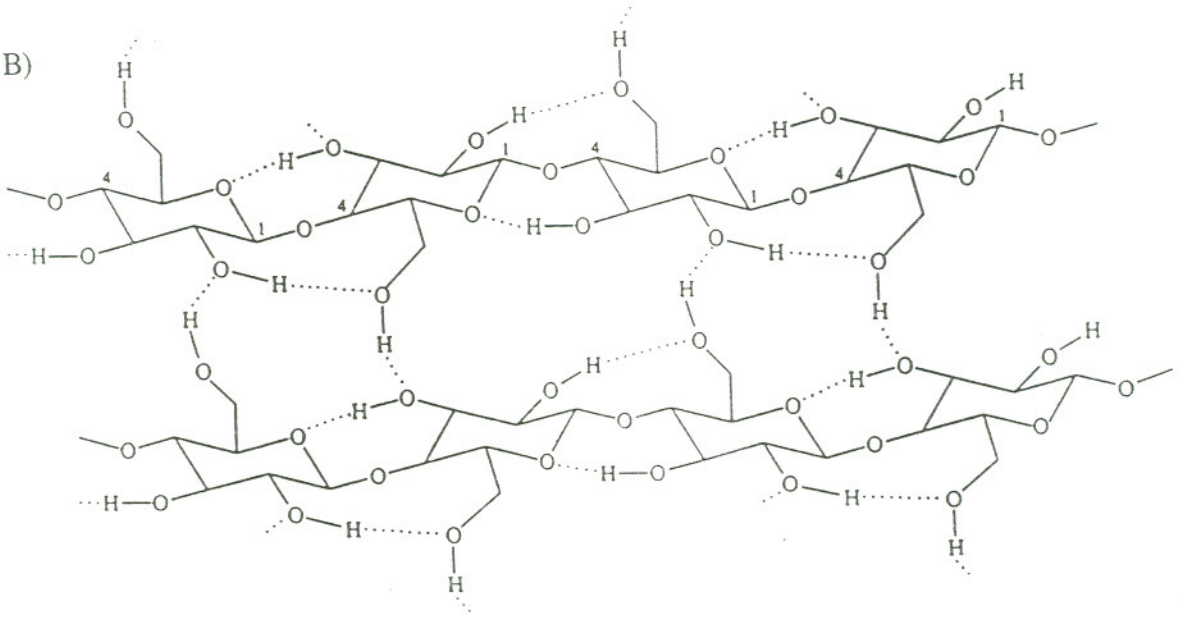


Fig. 1-2 Structure of cellulose. (A) Structure of cellobiose. (B) The hydrogen bond network of cellulose.

cotton is about 70% crystalline [Wood, 1988]. The crystalline structure of cellulose is mainly studied by X-ray diffraction. Other spectral techniques—such as infrared, Raman and ^{13}C NMR magic angle spinning spectroscopy—are used to obtain information about the conformation of cellulose [French, 1985; Blackwell, 1982; Atalla & van der Hart, 1984].

Crystalline cellulose is classified into four forms: cellulose I, II, III and IV. Most natural cellulose is cellulose I. Cellulose II can be obtained from cellulose I by repeated mercerization, i.e., swelling of cellulose I in NaOH. The transition from cellulose I to cellulose II is irreversible. Treatment of cellulose I or II with liquid ammonia forms cellulose III. When soaked in water, cellulose III can revert to the parent form. Cellulose IV can be made from cellulose I, II or III by treating them with glycerol at high temperature. Cellulose IV can revert to the parent form like cellulose III. These four types of crystalline cellulose structure are compared in Fig. 1-3 [Woodcock & Sarko, 1980; Stipanovic & Sarko, 1976; Sarko et al., 1976; Gardiner & Sarko, 1985].

1.1.2 Hemicellulose Structure

The term hemicellulose was coined by Schulze [1891]. He thought that hemicellulose was an intermediate in cellulose biosynthesis or biodegradation which we know now is not true. Hemicellulose and cellulose belong to distinct polysaccharide groups. Hemicellulose is a short, branched heteropolymer of pentose sugars, such as D-xylose, L-arabinose, D-mannose, and D-galactose. Generally, the degree of polymerization of hemicellulose is less than 200 [Enari & Niku-Paavola, 1987]. The composition of hemicellulose in softwood and hardwood is different. The hemicellulose of softwood consists mainly of mannose, while the hemicellulose of hardwood is predominantly made of xylose [Timell, 1964; Wenzl, 1970; Whistler & Richards, 1970].

1.1.3 Lignin Structure

Lignin is a complex heteropolymer which contains phenylpropane units linked by ether and carbon-carbon bonds (Fig. 1-4) [Adler, 1977; Reddy & Forney, 1978].

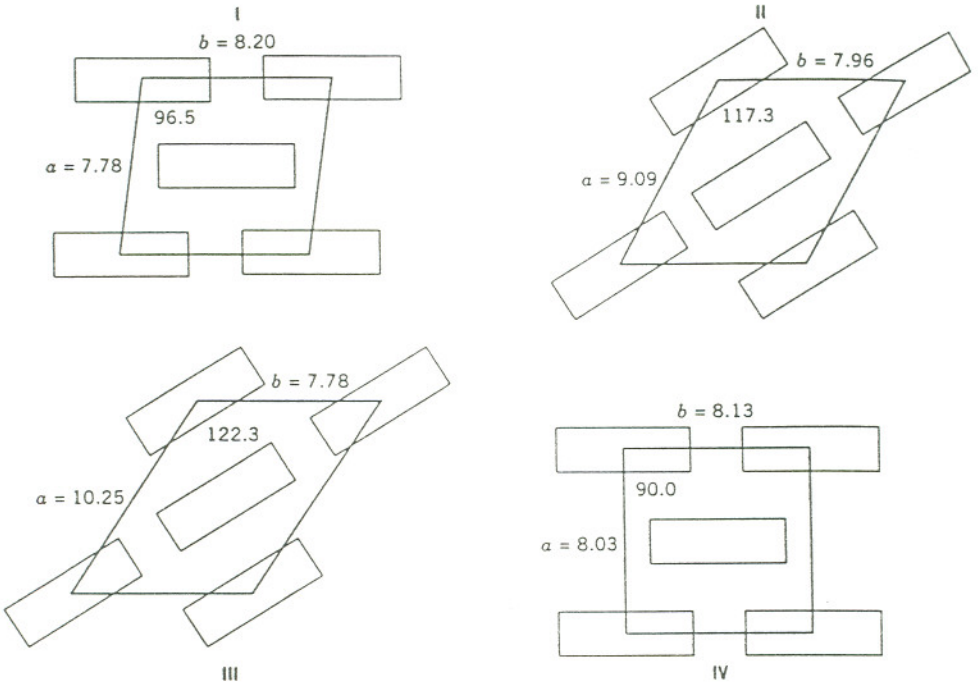


Fig. 1-3 Comparison of the unit cells proposed for cellulose I-IV.

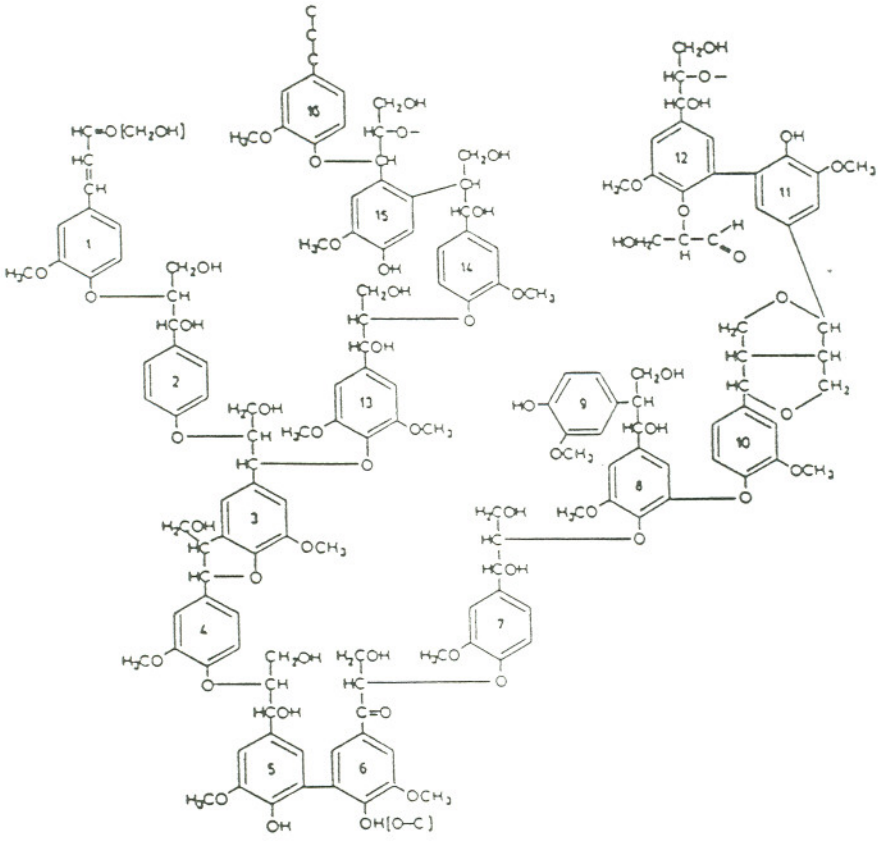


Fig. 1-4 A schematic structure of softwood lignin [Adler, 1977].

Lignin not only encrusts cellulose and hemicellulose but is also physically and chemically bound to them [Wilson et al., 1971; Cote, 1977; Higuchi, 1980]. Lignin coating of polysaccharides seems to protect plants against pathogenic organisms since lignin is resistant to biodegradation [Vance et al., 1980]. Another important physiological role of lignin is to provide mechanical support to the plant.

1.2 CELLULOSE SOURCES AND PRODUCTS

Cellulose is the major component of higher plant cell walls. It is also synthesized by some algae, fungi and bacteria [Hestrin, 1962; Preston, 1974; Brown, 1979]. Cellulose is made into diverse products including lumber, paper, membranes, explosives, textiles and dietary fiber [Nevell & Zeronian, 1985]. It is also modified into cellulose derivatives for various purposes. Water-soluble cellulose ethers, such as carboxymethylcellulose (CMC), hydroxyethylcellulose (HEC), methylcellulose (MC) and ethylcellulose, are the most widely used cellulose derivatives in food, pharmaceutics, construction, paper and cosmetics industries [Durso, 1978].

1.3 MICROBIAL DEGRADATION OF CELLULOSE

Most of the cellulose in nature is degraded by microorganisms, mainly by fungi and bacteria [Coughlan & Ljungdahl, 1988; Ljungdahl & Eriksson, 1985]. Fungi are classified into two groups: the "higher fungi" which include Ascomycotina, Basidiomycotina, Deuteromycotina, and the "lower fungi" which are relatively simple and contain Mastigomycotina and Zygomycotina. Wood-rot fungi which can degrade lignocellulose belong to "higher fungi" [Ingold & Hudson, 1993]. Wood-rot fungi have been divided into three classes: white-rot fungi, brown-rot fungi and soft-rot fungi. Besides fungi, anaerobic and aerobic bacteria also can degrade lignocellulose [Kirk & Cowling, 1984; Eriksson et al., 1990].

1.3.1 Wood-Rot Fungi

White-rot fungi can completely degrade lignin, cellulose and hemicellulose [Gottlieb & Pelczar, 1951; Eriksson et al., 1990]. When lignin is removed by white-rot fungi, the decayed wood becomes bleached white. Most of the white-rot fungi belong to the genus Basidiomycotina [Kirk, 1971] and are commonly found in fields and woods [Ingold & Hudson, 1993].

Unlike white-rot fungi, brown-rot fungi are not capable of degrading lignin to CO_2 and water. However, they can modify lignin giving a brownish color to decayed wood. Brown-rot fungi selectively remove carbohydrates from wood [Ander & Eriksson, 1978; Kirk, 1975]. Most brown-rot fungi are also classified as Basidiomycotina.

Wood decayed by soft-rot fungi has a soft and moist surface. Most soft-rot fungi are classified as ascomycetes or deuteromycetes [Ander & Eriksson, 1978; Corbett, 1965]. In hardwood, the carbohydrate is removed faster than lignin, while in softwood lignin seems to be depleted faster than carbohydrate by soft-rot fungi [Eslyn & Highley, 1976]. This difference in degradation rate is attributed to the different composition of hemicellulose in hardwood and softwood.

1.3.2 Cellulose Degradation by Fungi

A few white-rot and soft-rot fungi produce a complete cellulolytic system to hydrolyze crystalline cellulose. The best studied cellulase systems are those of soft-rot fungi *Trichoderma viride* [Gritzali & Brown, 1979], *Trichoderma reesei* (formerly *Trichoderma viride* QM 6a) [Mandels & Reese, 1964; Bhikhabhai et al., 1985], *Trichoderma koningii* [Halliwell & Vincent, 1981; Wood & McCrae, 1978] and that of the white-rot fungus *Phanerochaete chrysosporium* [Eriksson & Pettersson, 1975a, 1975b; Eriksson, 1978; Eriksson & Wood, 1985]. All of these fungi which hydrolyze crystalline cellulose produce cellulases including endo-1,4-β-glucanases, cellobiohydrolases (also known as exo-1,4-β-glucanase) and β-glucosidases.

Cellulose degradation by brown-rot fungi has not been well studied. Endoglucanase is the only enzyme isolated from most brown-rot fungi cultures [Eriksson et al., 1990]. But recently, both cellobiohydrolase and cellobiose

dehydrogenase were purified and characterized from the brown-rot fungi *Coniophora puteana* [Schmidhalter & Canevascini, 1993a, 1993b].

1.3.3 Cellulases of White-Rot and Soft-Rot Fungi

Generally endoglucanase cleaves β-glucosidic linkages of amorphous cellulose and soluble cellulose derivatives such as carboxymethylcellulose (CMC) and hydroxyethylcellulose (HEC). Endoglucanase alone cannot hydrolyze crystalline cellulose [Eriksson & Wood, 1985]. The activity of endoglucanase is determined by the reduction of the viscosity of CMC or HEC or estimation of the reducing sugar released from cellodextrins (Table 1-1). Multiple forms of endoglucanases differing in molecular weight and carbohydrate content (Table 1-2) have been isolated from *Trichoderma* sp. and *P. chrysosporium* [Wood & McCrae, 1978; Eriksson et al., 1975a, 1975b].

Cellobiohydrolase (or exoglucanase) degrades amorphous cellulose by removing cellobiose residues from the non-reducing end of cellulose which is cleaved by endoglucanase. Cellobiohydrolase itself cannot hydrolyze carboxymethylcellulose (CMC) and crystalline cellulose [Wood, 1989]. Cellobiohydrolase is the major component of *T. reesei* cellulases and exists in two distinct forms (Table 1-3).

β-glucosidase converts cellobiose to glucose and thus completes the hydrolysis of cellulose. β-Glucosidase activity is normally measured by the release of *p*-nitro-phenol from *p*-nitro-phenyl-β-glucoside (Table 1-1). The molecular weights of β-glucosidases from different organisms vary from 39,800 to 172,000 (Table 1-4).

Endoglucanase and cellobiohydrolase cooperate together to degrade crystalline cellulose to yield cellobiose. Then β-glucosidase hydrolyzes cellobiose to glucose. White-rot or soft-rot fungi which cannot degrade crystalline cellulose appear to lack cellobiohydrolase activity [Wood, 1989]. Glucose and cellobiose competitively inhibit β-glucosidase and cellobiohydrolase, respectively. The inhibition of cellulases by glucose and cellobiose appears to regulate cellulose hydrolysis [Enari & Niku-Paavola, 1987].

The mechanism of cellulase action on crystalline cellulose was first proposed by Reese et al. [1950] forty years ago [Mandels & Reese, 1964]. They postulated

Table 1-1 Cellulase activity assay methods [Eriksson et al., 1990]

Enzymes	**Substrates**	**Products or measured effect**
Mixture of cellulases	Insoluble celluloses Dyed celluloses [U-^{14}C]-cellulose	Weight loss Release of reducing sugars Release of dye Clarification of agar Water-soluble ^{14}C-product
Endoglucanases	Cellulose derivatives Cellodextrins	Decrease in viscosity Release of reducing sugar
Cellobiohydrolases	Amorphous cellulose Cellodextrins *p*-Nitro-phenyl-β-cellobioside *p*-Nitro-phenyl-β-lactoside Carboxymethyl-substituted cellodextrins	Reducing sugars Cellobiose *p*-Nitro-phenol
β-Glucosidases	*p*-Nitro-phenyl-β-glucoside Cellobiose Cellodextrins	Reducing sugars Glucose *p*-Nitro-phenol

Table 1-2 Endoglucanases from fungi [Enari & Niku-Paavola, 1987]

Organism	Molecular weight	pI	Carbohydrate content (%)
P. chrysosporium	32,300 36,700 28,300 37,500 37,500	5.32 4.72 4.4 4.65 4.2	10.5 0 7.8 4.7 2.2
T. reesei	55,000 48,000 20,000 43,000 56,000 67,000	4.5 5.5 7.5 4.0 5.0 6.5	10.0 6 0 2-7
T. viride	37,000 52,000 49,000		4.5 15 15
T. koningii	13,000 48,000 48,000 31,000	4.7 4.3 4.3 5.09	

Table 1-3 Cellobiohydrolases from fungi [Enari & Niku-Paavola, 1987]

Organism	**Molecular weight**	**pI**	**Carbohydrate content (%)**
P. chrysosporium	48,600	4.3	0
T. reesei	65,000 58,000	3.6-4.2 6.3	10 8
T. viride	41,800	3.8	9.2
T. koningii	62,000	3.8 3.95	33.0 9

Table 1-4 β-Glucosidases from fungi [Enari & Niku-Paavola, 1987]

Organism	Molecular weight	pI	Carbohydrate content (%)
P. chrysosporium	165,000 172,000	4.8 4.5	
T. reesei	73,000		10
T. veride	47,400	5.7	0
T. koningii	39,800 39,800	5.53 5.85	0 2

that two components of cellulases, C_1 and C_x, are necessary to hydrolyze crystalline cellulose. The C_1-enzyme, a kind of a "hydrogen bondase," cleaves the hydrogen bonds of cellulose first. Then C_x, an enzyme with endoglucanase activity, hydrolyzes the modified cellulose to cellobiose. Finally, β-glucosidase converts cellobiose to glucose. Further, it was suggested that in the absence of the C_1-enzyme, fungi cannot degrade crystalline cellulose.

Although "hydrogen bondase" was never identified in the fungal cultures, endoglucanases and cellobiohydrolases (exoglucanases) were later isolated [Berghem & Pettersson, 1975; Halliwell & Griffin, 1973; Wood & Phillips, 1969]. In 1969, Eriksson et al. studied cellulases of *P. chrysosporium* and revised the cellulose-degradation mechanism proposed by Mandels and Reese [1964]. According to Eriksson et al., the C_x component initiated the attack on cellulose followed by the C_1 component which split off cellobiose units from cellulose. The C_1 and C_x components were later identified as cellobiohydrolase (exoglucanase) and endoglucanase, respectively. The synergistic action of these cellulases was demonstrated to degrade crystalline cellulose [Mandels & Reese, 1964; Li et al., 1965; Selby & Maitland, 1967; Eriksson, 1969; Wood & McCrae, 1972]. An example of the synergistic action of cellulases from *T. koningii* is shown in Table 1-5 [Wood & McCrae, 1979]. White and Brown [1981] found by electron microscopy that endoglucanase and cellobiohydrolase must be simultaneously present to hydrolyze crystalline cellulose. This provides physiological evidence that synergistic actions of cellulases are necessary to degrade crystalline cellulose.

The current model of cellulose degradation (Fig. 1-5) [Béguin & Aubert, 1994] by cellulase is that endoglucanase randomly cleaves the amorphous areas of cellulose first, then cellobiohydrolase removes cellobiose or cellodextrins from the non-reducing chain ends. β-Glucosidase then converts cellobiose to glucose (Fig. 1-6) [Wood & McCrae, 1972; Reese, 1977; Ryu et al., 1984; Wood & McCrae, 1986a; Enari & Niku-Paavola, 1987]. Although this is a widely accepted model, many details of the mechanism of cellulose hydrolysis need further study [Wood, 1989; Coughlan & Ljungdahl, 1988].

Table 1-5 Synergistic action of cellulases from *T. koningii* [Wood & McCrae, 1979]

Enzymes	**Cotton Solubilization (%)[a]**
Cellobiohydrolases (CBH)	1
Endoglucanases (EG)	1
β-Glucosidase (GCD)	1
CBH + EG	53
CBH + GCD	20
CBH + EG + GCD	72
Original culture filtrate	71

[a] All enzymes were present in the same proportions in which they were present in 1 ml of original cell-free culture filtrates. Incubations were for 7 days at 37°C.

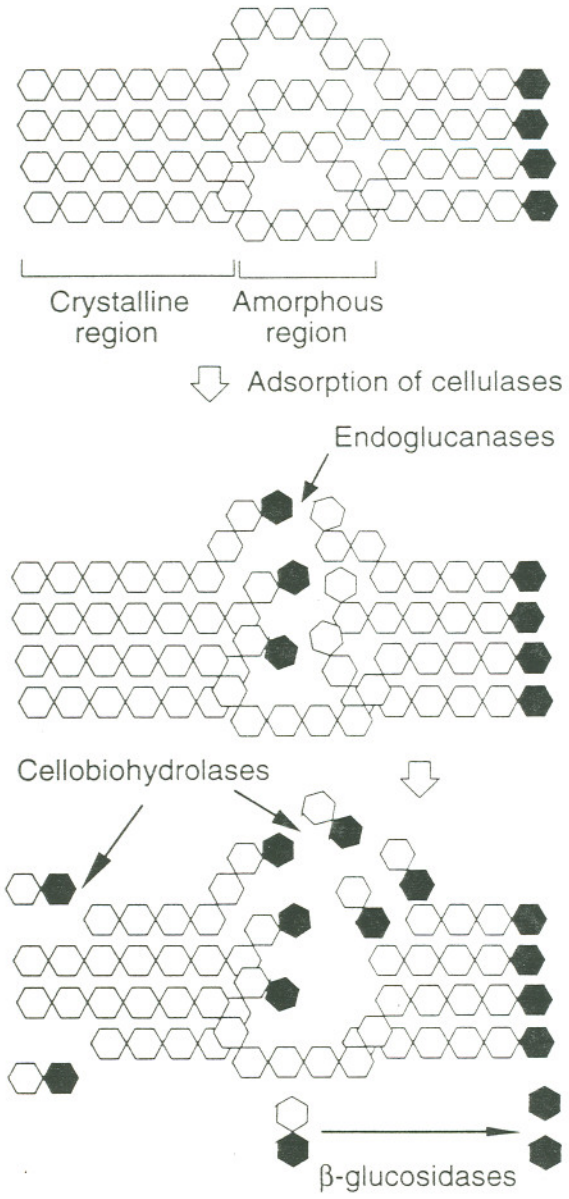


Fig. 1-5 Mechanism of cellulose degradation by soft-rot and white-rot fungal cellulases. Glucose residues are indicated by hexagons; reducing ends are shown in solid hexagons [Béguin & Aubert, 1994].

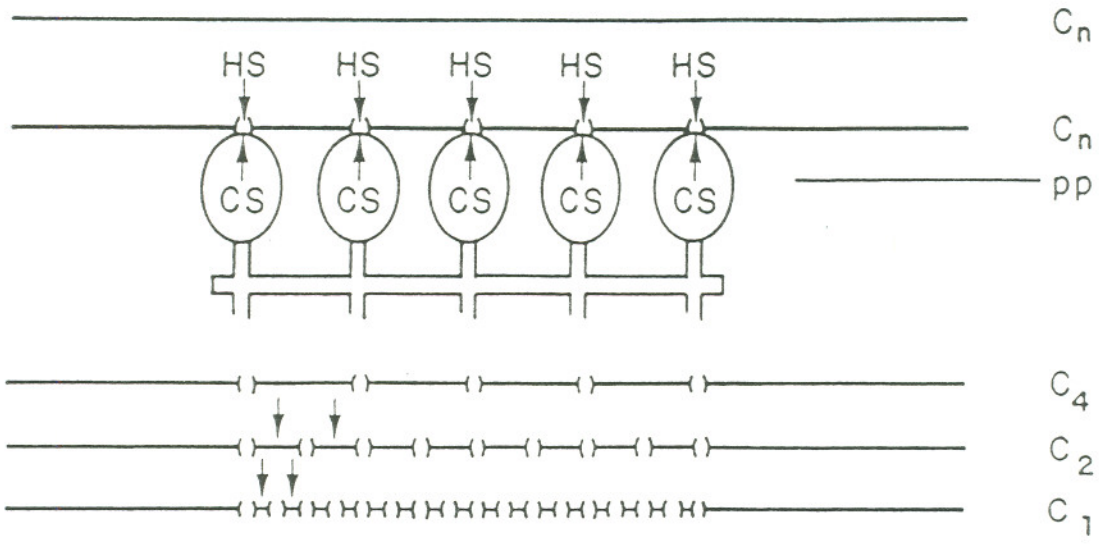


Fig. 1-6 Mechanism of cellulose degradation by *C. thermocellum* cellulosome. **PP**, polypeptides of cellulosome; **CS**, catalytic site on cellulosome; **HS**, hydrolysis site on cellulose; $\mathbf{C_n}$, cellulose; $\mathbf{C_4}$, four units of cellobiose; $\mathbf{C_2}$, two units of cellobiose; $\mathbf{C_1}$, cellobiose.

1.3.4 Cellulases of Brown-Rot Fungi

In vivo, brown-rot fungi drastically reduce the degree of cellulose polymerization, but the cellulose weight loss is relatively low [Highley, 1977]. Endoglucanase activity, but no cellobiohydrolase activity, has been detected in most brown-rot fungi. It seems that the endoglucanase-cellobiohydrolase synergistic theory proposed for white-rot or soft-rot fungi cellulolytic systems is not applicable for brown-rot fungal cellulose degradation. Cellulose-degrading cultures of brown-rot fungi produce H_2O_2 [Highley, 1987]. Koenigs [1974a, 1974b] suggested that hydroxyl radical generated through Fenton reaction ($Fe^{2+} + H_2O_2 \Rightarrow OH^- + \cdot OH + Fe^{3+}$), could be involved in cellulose degradation by brown-rot fungi. Hydroxyl radical, a powerful oxidant, has been suggested to oxidatively cleave polysaccharides [Halliwell, 1978]. In support of this hypothesis, Highley [1982] found hydroxyl radical in brown-rot fungal cultures. Schmidt et al. [1981] demonstrated that oxalic acid, which was found in liquid cultures of brown fungi [Takao, 1965], can reduce Fe^{3+} to Fe^{2+} to provide Fe^{2+} for Fenton reaction. Flournoy et al. [1991] reported that the diameter of the pore on the wood decayed by brown-rot fungi ranges from 15 Å to 38 Å. Very small protein like ribonuclease with a molecular weight of about 13,000 has a diameter of 30 Å [Tyn & Gusek, 1990]. Since the molecular weight of cellulases from brown-rot fungi range from 30,000 to 50,000 [Highley, 1975; Highley & Wolter, 1982; Green et al., 1989], these pores could not have been created by brown-rot fungal cellulases. It supports the theory that nonenzymatic factors, especially hydroxyl radical, are involved in cellulose degradation by brown-rot fungi.

1.3.5 Cellulose Degradation by Bacteria

Bacteria cannot degrade lignin but can hydrolyze polysaccharides in wood [Schmidt & Liese, 1982; Holt & Jones, 1978; Schmidt et al., 1987]. Both aerobic and anaerobic bacteria degrade crystalline cellulose. The best characterized cellulolytic systems are those of anaerobic bacteria. They include *Clostridium thermocellum* [Johnson et al., 1982; Hon-Nami et al., 1986], *Acetivibrio cellulolyticus* [Saddler & Khan, 1981; MacKenzie et al., 1985], *Bacteroides cellulosolvens*

[Giuliano & Khan, 1984], *Bacteroides succinogenes* [Groleau & Forsberg, 1981] and *Ruminococcus albus* [Wood & Wilson, 1984].

While fungal cellulases are excreted into the extracellular medium, bacterial cellulase complexes are not excreted into the medium but are bound to the cell surface [Stack et al., 1983; Groleau & Forsberg, 1983; Latham et al., 1978; Lamed et al., 1983a]. In general, cell-free supernatants from bacterial cellulolytic cultures do not hydrolyze crystalline cellulose although the cellulose is efficiently degraded by the whole culture. It seems that the bacterial cells have to attach to cellulose to hydrolyze them [Ljungdahl, 1989]. The cellulase complex from *C. thermocellum* is known as cellulosome and is the best studied among bacterial cellulolytic systems [Lamed et al., 1983a]. Cellulosome from *C. thermocellum* is a complex which contains about 35 polypeptides with a molecular weight ranging from 20 to 200 kDa [Mayer et al., 1987]. The polypeptides of cellulosome exhibit only endoglucanase activity but no cellobiohydrolase activity [Hon-Nami et al., 1986; Lamed et al., 1983b]. Cellulolytic bacteria also produce cell-bound β-glucosidase and/or cellobiose phosphorylase [Giuliano & Khan, 1984; Alexander, 1986; Latham et al., 1978]. In addition to cellulases, *C. thermocellum* [Ljungdahl et al., 1983] and *A. cellulolyticus* [Patel et al., 1980] produce a water-insoluble yellow pigment called yellow affinity substance (YAS) which has an affinity for both cellulose and cellulases. This increases the affinity of cellulases for cellulose [Ljungdahl et al., 1983; Lamed et al., 1985]. Calcium or magnesium and dithiothreitol are necessary for cellulase complex activity [Coughlan & Ljungdahl, 1988].

The mechanism of cellulose hydrolysis by anaerobic bacteria is mainly inferred from the structure of *C. thermocellum* cellulosome [Mayer et al., 1987]. Under an electron microscope, cellulosome appears as globular particles attached to cellulose. Several polypeptides of cellulosome which attach to cellulose are arranged side-by-side with a defined orientation. The distance between the adjacent polypeptides is about four cellobiose units [Mayer et al., 1987] (Fig 1-7). The polypeptides of the cellulosome cut the cellulose at multiple points to form several such four cellobiose units. The four cellobiose units are then cut into one or more cellobiose units (Fig. 1-7). This model corresponds well with the fact that the major product of cellulose

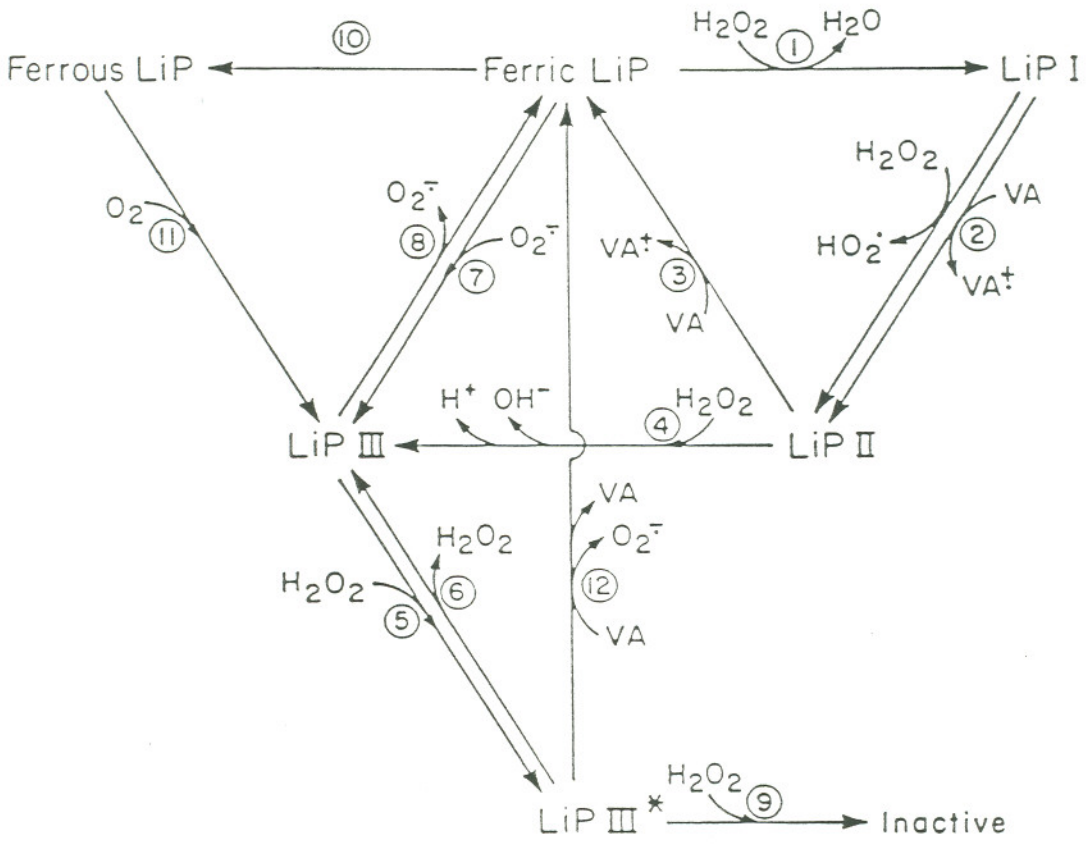


Fig. 1-7 The electron transfer cycle of LiP [Wariishi & Gold, 1990].

degradation by *C. thermocellum* is cellobiose instead of glucose [Johnson et al., 1982; Hon-Nami et al., 1986].

1.4 ENZYMES OF THE WHITE-ROT FUNGUS *P. CHRYSOSPORIUM*

White-rot fungi are the only known microorganisms that can decompose lignocellulose completely [Kirk & Farrell, 1987; Gold et al., 1989; Eriksson et al., 1990]. The best studied white-rot fungus is *P. chrysosporium*. This fungus was first isolated from pine chips and named *Chrysosporium lignorum*. Then the name was changed to *Sporotrichum pulverulentum*. Now *P. chrysosporium* is the widely accepted name for this fungus [Eriksson et al., 1990]. *P. chrysosporium* excretes extracellular cellulases to degrade the cellulose of wood during the primary metabolism [Eriksson & Pettersson 1975a, 1975b; Deshpande et al., 1978]. It produces extracellular enzymes to degrade lignin during the secondary metabolism [Kirk & Farrell, 1987; Gold et al., 1989].

1.4.1 Cellulases of *P. chrysosporium*

P. chrysosporium produces a complete cellulase system to hydrolyze crystalline cellulose. A cellulose-degrading culture of *P. chrysosporium* produces five endo-1,4-β-glucanases [Eriksson & Pettersson, 1975b] (Table 1-2), one exo-1,4-β-glucanase (cellobiohydrolase) [Eriksson & Pettersson, 1975a] and two β-glucosidases [Deshpande et al., 1978]. Unlike most exoglucanases from other fungi, the exoglucanase from *P. chrysosporium* is not a glycoprotein (Table 1-3). The five endoglucanases and the exoglucanase have a strong synergistic effect when the hydrolysis substrate is crystalline cellulose (cotton and Avicel) [Streamer et al., 1975]. In addition to cellulases, *P. chrysosporium* produces two extracellular cellobiose-oxidizing enzymes: cellobiose dehydrogenase (CDH) (also known as cellobiose oxidase, CBO) and cellobiose:quinone oxidoreductase (CBQase). Both enzymes oxidize cellobiose to cellobionolactone [Westermark & Eriksson, 1975; Ayers et al., 1978; Morpeth, 1985; Bao et al., 1993].

1.4.2 Lignin-Degrading Enzymes of *P. chrysosporium*

P. chrysosporium degrades lignin only in the secondary metabolic stage in response to nitrogen, carbon and sulfate limitation [Keyser et al., 1978; Fenn et al., 1981; Jeffries et al., 1981; Reid, 1983]. During the secondary metabolism, *P. chrysosporium* produces at least two extracellular peroxidases—lignin peroxidase (LiP) and manganese peroxidase (MnP)—and H_2O_2-generating enzymes such as glyoxal oxidase. These enzymes are involved in lignin degradation [Kirk & Farrell, 1987; Gold et al., 1989].

1.4.1.1 Lignin and manganese peroxidases

In 1983, lignin peroxidase (LiP) was purified from the extracellular medium of *P. chrysosporium* [Glenn et al., 1983; Tien & Kirk, 1983]. LiP is a heme glycoprotein with a molecular weight of 42,000 and requires H_2O_2 for activity [Kuwahara et al., 1984; Tien & Kirk, 1984]. LiP catalyzes the oxidation of nonphenolic lignin dimeric model compounds and aromatic pollutants in the presence of H_2O_2 [Buswell & Odier, 1987; Kirk & Farrell, 1987; Gold et al., 1989; Enoki & Gold, 1982; Higuchi, 1986; Tien, 1987; Valli et al., 1992; Valli & Gold, 1991]. LiP exists as a series of isozymes with pIs ranging from 3.2 to 4.0. The optimal pH of LiP is about 3 which is unusually low [Kuwahara et al., 1984; Kirk et al., 1986; Leisola et al., 1987].

As for the LiP mechanism, H_2O_2 oxidizes native LiP to form LiP I, a ferryl-oxo species with a porphyrin cation radical character. LiP I reaction with H_2O_2 or a substrate such as veratryl alcohol yields LiP II. LiP II still contains ferryl iron ($Fe^{IV}=O$) but no longer contains the porphyrin cation radical structure. Veratryl alcohol reduces LiP II to native enzyme. In the absence of a reducing substrate, LiP II reacts with H_2O_2 to form LiP III. However, in the presence of veratryl alcohol, LiP III returns to native state with the release of superoxide. In the absence of veratryl alcohol, LiP III is inactivated with the loss of heme (Fig 1-8) [Wariishi & Gold, 1990; Renganathan & Gold, 1986; Marquez et al., 1988; Wariishi & Gold, 1989; Cai & Tien, 1992]. The LiP catalytic cycle is the same as that of horseradish peroxidase (HRP).

Manganese peroxidase (MnP) was discovered by Professor Michael H. Gold and coworkers at the Oregon Graduate Institute [Kuwahara et al., 1984]. MnP is a unique enzyme since it can oxidize phenols, amines and dyes only in the presence of Mn(II) [Kuwahara et al., 1984; Glenn & Gold, 1985; Paszczymski et al., 1986]. Mn(II) is also necessary to induce MnP in the culture medium. In the absence of Mn(II), MnP was not detected in either intracellular extracts or in the extracellular media of lignin-degrading cultures [Brown et al., 1990].

MnP is a glycoprotein and contains an iron protoporphyrin IX prosthetic group. MnP exists as a series of isozymes with pIs ranging from 4.2 to 4.9 and a molecular weight of about 46,000. All isozymes require H_2O_2 and Mn(II) for activity [Kuwahara et al., 1984; Kirk et al., 1986; Leisola et al., 1987; Glenn & Gold, 1985]. The MnP catalytic cycle is similar to that of LiP [Wariishi et al., 1988].

1.4.1.2 H_2O_2-generating enzymes

Extracellular peroxidases need H_2O_2 for activity. Glyoxal oxidase, a H_2O_2-generating enzyme, is present in the extracellular medium of *P. chrysosporium* [Kersten & Kirk, 1987; Kersten, 1990]. Glyoxal oxidase is a copper-dependant enzyme and oxidizes glyoxal to glyoxylic acid. MnP also can generate H_2O_2 in the presence of NAD(P)H [Asada et al., 1986]. In addition, several intracellular H_2O_2-generating enzymes, such as glucose-1-oxidase [Kelley & Reddy, 1988], pyranose-2-oxidase [Volc & Eriksson, 1988] and fatty acyl-CoA oxidase [Greene & Gould, 1984] have been reported in *P. chrysosporium* cultures, but the exact role of these oxidases in lignin degradation is not clear [Gold & Alic, 1993].

1.5 HEMOFLAVOENZYMES

Hemoflavoenzymes are a group of enzymes which contain one heme and one flavin on a single subunit. Besides cellobiose dehydrogenase from *P. chrysosporium*, only a few enzymes belong to this class. The best characterized hemoflavoenzyme is flavocytochrome b_2 (also known as L-lactate dehydrogenase, L-lactate:cytochrome *c* oxidoreductase) from *Saccharomyces cerevisiae* and *Hansenula anomala* [Xia et al.,

1987; Celerier et al., 1989]. The other hemoflavoenzymes include nitric oxide synthetase from mammals [Marletta, 1993], spermidine dehydrogenase from *Serratia marcescens* [Tabor & Kellogg, 1970], rubredoxin oxidase from bacteria [Chen et al., 1993], L-mandelate dehydrogenase from yeast [Yasub & Fewson, 1993], and N-methylglutamate dehydrogenase from bacteria [Boulton et al., 1980].

1.5.1 Flavocytochrome b_2

L-Lactate dehydrogenase (flavocytochrome b_2) is located in the mitochondrial intermembrane of baker's yeast (*S. cerevisiae* or *H. anomala*). It catalyzes the oxidation of L-lactate to pyruvate. D-Lactate is neither a substrate nor an inhibitor of L-lactate dehydrogenase [Hasegawa, 1962]. L-Lactate dehydrogenase is a tetrameric enzyme (molecular weight = 240,000). Each subunit (molecular weight = 57,000) contains one flavin mononucleotide (FMN) and one protoporphyrin IX as cofactors. Both cofactors are noncovalently bound to the protein [Pajot & Claisse, 1974; Daum et al., 1982]. The heme iron is hexacoordinate, and histidine residues 43 and 66 serve as the fifth and sixth ligands [Xia et al., 1987; Desbois et al., 1989]. Six amino acid residues are believed to be at the active site. They are Arg^{376}, Tyr^{143}, His^{373}, Tyr^{254}, Lys^{349} and Ala^{306} [Lederer & Mathews, 1987; Urban & Lederer, 1985; Pompon et al., 1980; Dubois et al., 1990; Reid et al., 1988].

Flavocytochrome b_2 is a component of the respiratory chain [Pajot & Claisse, 1974]. Substrate L-lactate donates two electrons to the flavin group of flavocytochrome b_2 first. The reduced flavin then transfers two electrons to the heme group in two single steps. The reduced heme in turn transfers its electron to ferricytochrome *c*. Ultimately the electron passes down to O_2 [Labeyrie, 1982]. The electron transfer pathway is shown below:

L(+)-Lactate $\Rightarrow$Flavocytochrome b_2 $\Rightarrow$ Cytochrome *c* $\Rightarrow$ Cytochrome *a* + a_3 $\Rightarrow$ O_2

Each subunit of flavocytochrome b_2 is folded into two functional domains which can be cleaved by proteases: a cytochrome core domain and a flavodehydrogenase domain [Celerier et al., 1989; Gervais et al., 1977; Xia et al., 1987]. The cytochrome core domain is located at the N-terminal and transfers the

electron to cytochrome *c* or ferricyanide [Capeillére-Blandin, 1991]. The flavodehydrogenase domain is located close to the C-terminal and it contains lactate-binding site [Celerier et al., 1989]. The flavodehydrogenase domain can transfer an electron to ferricyanide but not to cytochrome *c* in the presence of lactate. Flavocytochrome b_2 has been studied for more than thirty years and could serve as a model for cellobiose dehydrogenase from *P. chrysosporium*.

1.5.2 Nitric Oxide Synthase

Nitric oxide synthase (NOS) has been isolated from brain cells of rats [Bredt & Snyder, 1990; Schmidt et al., 1991], porcine cerebellum cells [Mayer et al., 1990] and from the membrane fraction of bovine aortic endothelial cells [Pollock et al., 1991]. This enzyme oxidizes L-arginine to form citrulline and nitric oxide in the presence of NADPH and O_2 [Marletta, 1993]. Nitric oxide synthases are dimers and their molecular weights range from 135,000 to 160,000. One subunit of NOS contains one FAD and one iron-protoporphyrin IX, and another subunit contains one FMN and one iron-protoporphyrin IX [Hevel et al., 1991]. The iron-protoporphyrin of NOS is very similar to that of cytochrome P-450. The native NOS has a maximum absorbance at 397 nm, a shoulder at 420 nm and broad bands at 538 nm and 650 nm (Table 1-7). The reduced NOS has maximum absorbance at 448 nm and 550 nm [Stuehr & Ikeda-Saito, 1992]. Ca^{2+} and calmodulin are required for NOS activity [Bredt & Snyder, 1990].

1.5.3 Other Hemoflavoenzymes

Rubredoxin oxidase, L-mandelate dehydrogenase (MDH) and N-methylglutamate dehydrogenase are hemoflavoenzymes which are not well characterized. They were isolated from different sources and have different molecular weight (Table 1-6). Rubredoxin oxidase is a dimer and each subunit contains two FAD and one heme [Chen et al., 1993]. L(+)-mandelate dehydrogenase (MDH) from the yeast *Rhodotorula gramimis* is very similar to that of flavocytochrome b_2 [Yasub & Fewson, 1993]. MDH is a tetramer and each subunit contains one heme *b* and one flavin mononucleotide (FMN). MDH oxidizes only L-mandelate; D-

Table 1-6 Comparison of hemoflavoenzyme properties

Name	Sources	Heme	Flavin	M_r	Subunit	Reference
Cellobiose dehydrogenase	*Phanerochaete chrysosporium*	heme *b*	FAD	90,000	monomer	Morpeth, 1985; Samejima & Eriksson, 1992; Bao et al., 1993
Flavocytochrome b_2	*Hansenula anomala*	heme *b*	FMN	240,000	tetramer	Appleby & Morton, 1959; Labeyrie et al., 1978
Nitric oxide synthase	Mammal	heme *b*	FAD & FMN	135,000 - 160,000	dimer	Marletta, 1993
Mandelate dehydrogenase	*Rhodotorula gramimis*	heme *b*	FMN	239,900	tetramer	Yasub & Fewson, 1993
N-methylglutamate dehydrogenase	Bacterium AT_2	heme *c*	FAD	100,000	monomer	Boulton et al., 1980
Spermidine dehydrogenase	*Serratia marcescens*	heme *b*	FAD	76,000	monomer	Tabor & Kellogg, 1970

mandelate is a competitive inhibitor of MDH. N-methylglutamate dehydrogenase is a monomer and contains a cytochrome *c* and an FAD [Boulton et al., 1980]. The enzyme is active with several N-methyl amino acids. Unlike other hemo-flavoenzymes, cytochrome *c* and ferricyanide do not function as electron acceptors for this enzyme. The spectral properties of these enzymes are compared in Table 1-7.

1.6 CELLOBIOSE OXIDIZING ENZYMES

Cellobiose oxidizing enzymes were first discovered in the lignocellulose-degrading cultures of *P. chrysosporium*. This fungus produces two cellobiose oxidizing enzymes: cellobiose dehydrogenase (CDH) and cellobiose:quinone oxidoreductase (CBQase) [Ayers et al., 1978; Westermark & Eriksson, 1975]. Cellobiose dehydrogenase has also been found in the cellulose-degrading cultures of *Sclerotium rolfsii* [Sadana & Patil, 1985], *Sporotrichum thermophile* [Coudray et al., 1982; Canevascini et al., 1991] and *Chaetomium cellulolyticum* [Fahnrich & Irrgang, 1982]. Recently, the first brown-rot fungal cellobiose dehydrogenase was purified from *C. puteana* [Schmidhalter & Canevascini, 1993b]. All of these cellobiose dehydrogenases are glycoproteins and oxidize cellobiose to cellobionolactone. Molecular weights of these enzymes, however, vary from 48,000 to 192,000 kDa. They have different pI (3.5-6.4) which tend to be in the acidic range (Table 1-8). Cellobiose dehydrogenases from *P. chrysosporium* [Ayers et al., 1978; Morpeth, 1985; Bao et al., 1993], *S. thermophile* [Coudray et al., 1982; Canevascini et al., 1991] and *C. puteana* [Schmidhalter & Canevascini, 1993b] are hemoflavoenzymes which contain heme *b* and flavin on the same subunit. Two cellobiose dehydrogenases have been purified from *S. thermophile* having similar catalytic properties but with distinct molecular masses (Table 1-8). Only CBQase from *P. chrysosporium* is a flavoprotein which is believed to be a proteolytic product of CDH [Henriksson et al., 1991; Wood & Wood, 1992] (Table 1-8). Cellobiose dehydrogenase from *S. rolfsii* contains neither heme nor flavin. Cellobiose dehydrogenase from *C. cellulolytium* is not characterized.

Table 1-7 Spectral properties of hemoflavoenzymes

Enzyme	**Oxidized (nm)**	**Reduced (nm)**	**Heme type**	**Reference**
Cellobiose dehydrogenase	420, 529, 570	428, 534, 564	heme *b*	Bao et al., 1993
L-Lactate dehydrogenase	413, 530, 560	423, 528, 557	heme *b*	Appleby & Morton 1954; Labeyrie et al., 1978
Nitric oxide synthase	397, 538, 650	448, 550	heme *b*	Stuehr & Ikeda-Saito, 1992
Mandelate dehydrogenase	413, 530, 560	423, 528, 557	heme *b*	Yasub & Fewson, 1993
Spermidine dehydrogenase	414, 530, 560	427, 530, 560	heme *b*	Tabor & Kellogg, 1970
N-methylglutumate dehydrogenase	409	415, 516, 552	heme *c*	Boulton et al., 1980

Table 1-8 Cellobiose oxidizing enzymes from different fungi

Enzyme	Sources	Prosthetic group	pI	Molecular weight	Subunit	Reference
Cellobiose dehydrogenase	*Phanerochaete chrysosporium*	heme *b*, FAD	4.2	90,000	monomer	Bao et al., 1993; Henriksson et al., 1991
Cellobiose:quinone oxidoreductase	*Phanerochaete chrysosporium*	FAD	6.4 5.7 4.0	60,000	monomer	Westermark & Eriksson, 1975
Cellobiose dehydrogenase	*Sclerotium rolfsii*	not known	5.2	64,000	monomer	Sadana & Patil, 1985
Cellobiose dehydrogenase	*Sporotrichum thermophile*	heme *b*, FAD heme *b*, FAD	4.1 3.5	91,000 192,000	monomer homodimer	Canevascini et al., 1991
Cellobiose dehydrogenase	*Coniophora puteana*	heme *b*, flavin	3.9	111,000	monomer	Schmidhalter & Canevascini, 1993b

1.7 CELLOBIOSE DEHYDROGENASE FROM *P. CHRYSOSPORIUM*

1.7.1 History of CDH

In 1974, Westermark and Eriksson [1974] demonstrated that cell-free cultures of *P. chrysosporium* degraded cellulose twice as fast in an O_2 compared to N_2 atmosphere. By that time, endoglucanases, exoglucanase and cellobiose:quinone oxidoreductase had been isolated from *P. chrysosporium*. All of these extracellular enzymes are hydrolases and do not require O_2 for activity. This led to the suggestion that oxidative enzymes which require O_2 for activity must be involved in cellulose degradation by *P. chrysosporium*. This led to the discovery of cellobiose oxidase (later renamed cellobiose dehydrogenase) by Eriksson and coworkers [Ayers et al., 1978]. They identified cellobiose oxidase as a hemoflavoenzyme containing a heme and a flavin on a monomeric subunit. The heme was identified as a *b*-type which noncovalently bound to the enzyme. The flavin was later identified as a FAD by Morpeth [1985]. In addition to oxidizing cellobiose to cellobionolactone, CDH also oxidizes cellodextrins and lactose. The molecular weight of CDH was estimated as 93,000 kDa. The enzyme binds tightly to concanavalin A sepharose indicating that it is a glycoprotein [Ayers et al., 1978]. Morpeth [1985] reported an improved procedure for the purification of CDH, but the molecular weight of CDH was estimated at 74,400 by sedimentation equilibrium. Morpeth also found that this enzyme is a monomer. Both Ayers et al. [1978] and Morpeth [1985] proposed that superoxide radical is the primary reduced-oxygen species formed in the CDH reaction. Superoxide radical is unstable and dismutates to produce H_2O_2; however, formation of H_2O_2 was not detected. Jones and Wilson [1988] reported a rapid kinetic study of the reduction of CDH. They found that under anaerobic conditions the flavin of CDH is reduced rapidly, then the heme is reduced slowly by cellobiose. A kinetic titration study indicated that three cellobiose molecules (electron donors) reduced two cellobiose dehydrogenase molecules (electron acceptors) [Jones & Wilson, 1988].

When we started this project, only three papers relating to CDH mentioned in above paragraph had been published. The purification procedures reported previously

yielded a non-homogeneous CDH with low specific activity. A detailed study of the structure, function, mechanism, and physiology of CDH and its role in cellulose degradation by cellulase were hampered by poor yield and low specific activity of the enzyme.

1.7.2 Thesis Outline

Ayers et al. (1978) and Morpeth (1985) have reported two different procedures for the purification of CDH from *P. chrysosporium*. In each method, *P. chrysosporium* was cultured in a phosphate-containing medium at an initial pH of 5.8 with powder cellulose as the carbon source. However, CDH produced and purified by these procedures was obtained in low yield and low specific activity. Our preliminary experiments suggested that the yield of cellobiose-oxidizing enzymes could be increased by adjusting the initial pH of the medium to 7 and by using cotton linters as the carbon source. We further developed a five-step purification procedure involving ammonium sulfate precipitation, DEAE-sephadex, Phenyl-Sepharose, Sephacryl S-200 and FPLC (Mono Q column) chromatographies. Our procedure provided CDH with high specific activity and yield [Bao et al., 1993; see also Chapter 2]. CDH purified by our procedure was homogeneous as judged by SDS-PAGE. CDH is a hemoflavoenzyme containing one heme and one flavin per monomer. It is a glycoprotein with a neutral carbohydrate content of 9.4%. Its molecular weight is 90,000. Cytochrome *c*, dichlorophenol indophenol, Mn^{3+}, and benzoquinones serve as electron acceptors for CDH. In the absence of these electron acceptors, oxygen serves as a poor electron acceptor and the reduction product of oxygen was identified as H_2O_2. CDH was earlier called cellobiose oxidase; however, we found that O_2 is a poor electron acceptor for CDH and it actually prefers other electron acceptors. In general, O_2 is the preferred electron acceptor for oxidases and the least preferred electron acceptor for dehydrogenases. These findings led us to rename this enzyme cellobiose dehydrogenase. CDH is a very stable enzyme. At lower pH ($\leq$pH 3) or elevated temperature ($\geq 60°C$), CDH is inactivated due to the release of FAD from the active site. The heme remains in the active site under these conditions. The heme iron of CDH is probably hexacoordinate since the ferric

enzyme does not bind azide or cyanide. CDH oxidizes cellobiose to cellobionolactone and the latter is a mixed-type inhibitor of CDH [Bao et al., 1993; see also Chapter 2].

We also examined the effect of culture conditions including the initial pH of the culture medium, cellulose substrate, and different nitrogen and carbon concentrations on the production of cellobiose dehydrogenase (CDH), cellobiose:quinone oxidoreductase (CBQase), β-glucosidase, lignin peroxidase and manganese peroxidase. The initial pH we studied ranged from 4.5 to 8.0. The examined cellulose substrates included cotton linters, microcrystalline cellulose, filter paper and acid-treated cellulose. Proper selection of the initial pH appears to be important for the production of CDH as well as β-glucosidase. CDH was produced most often in the succinate medium with an initial pH of 4.5 containing 25 mM ammonium phosphate as the nitrogen source and 1% cotton linter. *P. chrysosporium* culture mediums containing phosphate at an initial pH of 5.8 used by Ayers et al. [1978] and Morpeth [1985]. This condition yielded very low amounts of CDH. In this culture, the medium pH remained very acidic. Such an acidic pH could release the flavin from the CDH active site and, thus, could inactivate the CDH [Bao et al., 1993, Chapter 2]. CBQase levels appear to be proportional to the extracellular protease levels. This finding supports the suggestion that CBQase could be a CDH proteolytic cleavage product [Henriksson et al., 1991; Wood & Wood, 1992]. Nitrogen limitation (1.2 mM) triggers a secondary metabolism in *P. chrysosporium*. Cellulose-degrading cultures containing low nitrogen (1.2 mM) produced CDH, CBQase, LiP and MnP simultaneously. This result suggested that lignin- and cellulose-degrading enzymes could be produced simultaneously. CDH and CBQase have been suggested to be involved in the metabolism of quinones produced from lignin degradation. Our findings further support this hypothesis.

The optimized culture conditions enabled us to obtain milligram quantities of CDH with high specific activity. This, in turn, helped in performing a detailed study of the role of CDH in cellulose hydrolysis by cellulases [Bao et al., 1992; Chapter 4]. We studied the effect of CDH on microcrystalline cellulose hydrolysis by *T. viride* cellulases. CDH used in these experiments was purified from *P. chrysosporium* cultures containing a phosphate medium (CDH_{phos}) at an initial pH of 7.0. The

addition of low concentrations of CDH (10 μg/ml) to cellulase from *T. viride* enhanced glucose and cellobiose production from cellulose hydrolysis by 10% and 48%, respectively. Also CDH (10 μg/ml) addition increased microcrystalline cellulose weight loss by 19%. Cellobiose is a competitive inhibitor of cellulases [Gritzali & Brown, 1979]. CDH may be relieving this inhibition by oxidizing cellobiose to cellobionolactone. This could be the role of CDH in crystalline cellulose degradation by cellulases.

In Chapter 5, the effect of CDH on cellulase-dependent hydrolysis of different cellulosic substrates, such as microcrystalline cellulose, cotton linter, filter paper and acid-treated cellulose is presented. CDH was purified from *P. chrysosporium* cultures containing succinate (CDH_{succ}) at an initial pH of 4.5. Differences in the properties of CDH_{phos} and CDH_{succ} are discussed in Chapter 5. In contrast to CDH_{phos}, addition of even very low concentrations of CDH_{succ} (0.5 μg/ml) to cellulases from *T. viride* enhances microcrystalline cellulose hydrolysis significantly. CDH_{succ} (0.5 μg/ml) enhances microcrystalline cellulose weight loss about 16%, and glucose and cellobiose production by 10% and 36% respectively. However, CDH_{succ} only slightly increases cotton linter, filter paper and acid-treated cellulose degradation by cellulases. Cellulose binding studies showed that CDH_{succ} bind tightly and completely only to microcrystalline cellulose. It partially binds to cotton linter and filter paper, and does not bind to acid-treated cellulose. It appears that tight binding of CDH to cellulose is necessary for CDH-dependent enhancement of cellulose degradation. It also appears that CDH is designed to enhance the degradation of the crystalline region of cellulose.

Cellobiose:quinone oxidoreductase (CBQase) is another cellobiose oxidizing enzyme from *P. chrysosporium*. CBQase is a flavoenzyme and has a molecular mass of 60,000. It is purified to homogeneity from the extracellular medium by a five-step procedure including ammonium sulfate precipitation, DEAE-Sephadex, Phenyl Sepharose, Sephacryl S-200 and FPLC (Mono-Q) chromatographies [Bao & Renganathan, 1991]. It appears that CBQase exists as a series of isozymes. CBQase reduces quinone to hydroquinone in the presence of cellobiose [Westermark & Eriksson, 1975]. Since quinone is a product of lignin degradation, CBQase has been proposed as a link between lignin and cellulose degradation. CBQase inhibits

peroxidase-catalyzed reactions such as decarboxylation of vanillic acid, oxidation of 3,4-dimethoxybenzyl alcohol, and polymerization of kraft lignin [Ander et al., 1990]. In Chapter 6, CBQase inhibition of peroxidase-catalyzed oxidation of iodide to triiodide is presented [also Bao & Renganathan, 1991]. A probable mechanism for triiodide reduction by CBQase is also suggested.

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CHAPTER 2
PURIFICATION AND CHARACTERIZATION OF CELLOBIOSE DEHYDROGENASE, A NOVEL EXTRACELLULAR HEMOFLAVOENZYME FROM THE WHITE-ROT FUNGUS *PHANEROCHAETE CHRYSOSPORIUM*

2.1 INTRODUCTION

Many cellulolytic fungi, including *Phanerochaete chrysosporium* [Westermark & Eriksson, 1975; Ayers et al., 1978], *Sporotrichum (Chrysosporium) thermophile* [Canevascini et al., 1991], *Monilia sp.* [Dekker, 1980], and *Sclerotium rolfsii* [Sadana & Patil, 1985], produce extracellular cellobiose-dehydrogenating enzymes, in addition to cellulases. *P. chrysosporium* produces two cellobiose-dehydrogenating enzymes: the hemoflavoenzyme, cellobiose dehydrogenase (previously known as cellobiose oxidase) (CDH) [Ayers et al., 1978], and the flavoenzyme, cellobiose:quinone oxidoreductase (CBQase) [Westermark & Eriksson, 1975]. Both enzymes oxidize cellobiose to cellobionolactone. Cellobiose dehydrogenase from *S. thermophile* has recently been demonstrated to be a hemoflavoenzyme [Canevascini et al., 1991]. In contrast, cellobiose dehydrogenase from *S. rolfsii* does not appear to contain flavin or heme [Sadana & Patil, 1985]. The dehydrogenase from *Monilia sp.* has not been purified and characterized [Dekker, 1985].

Eriksson and coworkers [Ayers et al., 1978] first discovered CDH and demonstrated it to be a hemoflavoenzyme containing non-covalently bound heme *b* and flavin. Morpeth later identified the flavin to be flavin adenine dinucleotide (FAD) [1985]. CDH oxidizes cello-oligosaccharides in addition to cellobiose [Ayers et al., 1978]. Cytochrome *c* [Bao & Renganathan, 1992, Samejima et al., 1992],

dichlorophenol-indophenol (DCPIP) [Morpeth, 1985; Renganathan et al., 1990], benzoquinones [Henriksson et al., 1991], and even Fe^{3+} [Kremer & Wood, 1992a] function as electron acceptors in this reaction. CDH was described as an oxidase because it consumed oxygen during cellobiose oxidation [Ayers et al., 1978]. However, the nature of the reduced oxygen species formed in the CDH reaction is not yet known. CDH appears to be organized into two domains: a heme-binding domain and a flavin-binding domain with cellobiose-oxidizing activity [Henriksson et al., 1991]. Preliminary studies suggest that CDH may also have a specific cellulose-binding domain [Renganathan et al., 1990; Henriksson et al., 1991].

Enzymes containing a heme and a flavin are unusual. Apart from cellobiose-dehydrogenating enzymes from *P. chrysosporium* [Ayers et al., 1978] and *S. thermophile* [Canevascini et al., 1991], only a few enzymes—flavocytochrome b_2 of *Saccharomyces cerevisiae* [Xia et al., 1987], nitric oxide synthase from mammals [Marletta, 1993], rubredoxin oxidase [Chen et al., 1993], L-mandelate dehydrogenase from yeast [Yasub & Fewson, 1993], N-methylglutamate dehydrogenase from bacteria [Boulton et al., 1980] and spermidine dehydrogenase of *Serratia marcescens* [Tabor & Kellogg, 1970]—have been demonstrated to be hemoflavoenzymes. Among these, only flavocytochrome b_2 which dehydrogenates lactate to pyruvate is well characterized [Xia et al., 1987; Celerier et al., 1989; Labeyrie et al., 1978]. In contrast to flavocytochrome b_2, little is known about the structure, function and mechanism of CDH. Progress in understanding CDH has been hampered by the lack of procedures which provide homogeneous CDH with high specific activity and yield. Herein, we have developed a new purification procedure which allows the isolation of CDH with high activity and yield compared to previous purification methods [Ayers et al., 1978; Morpeth, 1985; Samejima et al., 1992; Henriksson et al., 1991]. Detailed characterization of CDH is also reported.

2.2 MATERIALS AND METHODS

2.2.1 Organism

Stock cultures of *P. chrysosporium* OGC 101 [Gold & Cheng, 1978] were maintained on slants of Vogel medium N supplemented with 3% malt extract and 0.25% yeast extract [Alic et al., 1987].

2.2.2 Culture Conditions

The medium composition used for culturing *P. chrysosporium* on cellulose was as reported previously [Eriksson & Pettersson, 1975] except that yeast extract (100 mg) were added and the initial pH of the medium was adjusted to 7.

The mineral salts medium (20 ml in a 250-ml Erlenmeyer flask) containing glucose (0.4 g) was inoculated with the conidia and incubated at 37°C without shaking. After 48-72 h, four such cultures were blended and added to a 2-liter Erlenmeyer flask containing 1 liter of mineral salts medium and 10 g of cotton linters. The cultures were incubated at 30°C with shaking (150 rpm).

2.2.3 Purification of CDH

The extracellular medium (4 liters) from 11 to 12 day-old *P. chrysosporium* cultures was filtered through glass wool to remove mycelial fragments. EDTA (5 mM) and phenylmethylsulfonyl fluoride (0.5 mM) were added to inhibit the extracellular proteases. The medium pH was adjusted to 7 with 2 M K_2HPO_4, and the medium was then concentrated to 10% of its initial volume by ultrafiltration using a hollow fiber cartridge (Amicon, Beverly, MA). The extracellular proteins were precipitated by the slow addition of ammonium sulfate (410 g/l) at 4°C. During this process, the medium pH was maintained at 7 by addition of 2 M K_2HPO_4. The ammonium sulfate precipitate was dialyzed against 5 mM phosphate, pH 7.5, concentrated, and applied to a DEAE-Sephadex A-50 column (12 cm × 4 cm) equilibrated with the same buffer. CBQase did not bind to the column under these conditions. CDH which bound to the column was eluted with 300 ml of buffer containing 0.5 M NaCl. CDH obtained from this procedure was dialyzed against 20

mM phosphate, pH 6; NaCl was added to the dialyzed CDH to 1 M concentration. DEAE-Sephadex-purified CDH was then loaded onto a phenyl-Sepharose column (20 cm × 5 cm) equilibrated with 20 mM phosphate, pH 6, containing 1 M NaCl. CDH was eluted with 250 ml of 20 mM phosphate containing 50% ethylene glycol (v/v) and was further fractionated on a Sephacryl S-200 column (65 cm × 2.5 cm) equilibrated with 50 mM phosphate, pH 6. Finally, CDH was purified to homogeneity by FPLC using a Mono-Q HR 5/5 column (Pharmacia Fine Chemicals, Piscataway, NJ). FPLC separations were performed in 10 mM TRIS-HCl, pH 8, and CDH was eluted with 1 M NaCl gradient (20 ml).

2.2.4 SDS-Polyacrylamide Electrophoresis (SDS-PAGE)

SDS-PAGE was performed with a 10% gel according to Laemmli [1970] by using a Mini-Protean System (Biorad Laboratories, Richmond, CA). The gel was stained with 0.1% Coomassie brilliant blue in 50% methanol and 7.5% acetic acid, and destained with 30% methanol and 7.5% acetic acid.

2.2.5 Enzyme Assays

CDH was routinely assayed by reduction of cytochrome *c* (12.5 μM) in 20 mM succinate, pH 4.5, in the presence of cellobiose (100 μM). Cytochrome *c* reduction was followed by monitoring the increase in absorbance at 550 nm (ϵ = 28 mM^{-1} cm^{-1}) (Fig. 2-1). 2,6-Dichlorophenolindophenol (DCPIP, 25 μM) reduction was monitored by the decrease in absorbance at 600 nm (11.8 mM^{-1} cm^{-1}) in 20 mM phosphate, pH 6, in the presence of cellobiose (100 μM) [Morpeth, 1985; Renganathan et al., 1990]. 3,5-Di-tert-butyl-1,2-benzoquinone reduction was determined by the decrease in absorbance at 420 nm in 20 mM succinate, pH 4.5 [Henriksson et al., 1991]. All assays were performed at room temperature except benzoquinone reduction, which was determined at 56°C as reported by Henriksson et al. [1991].

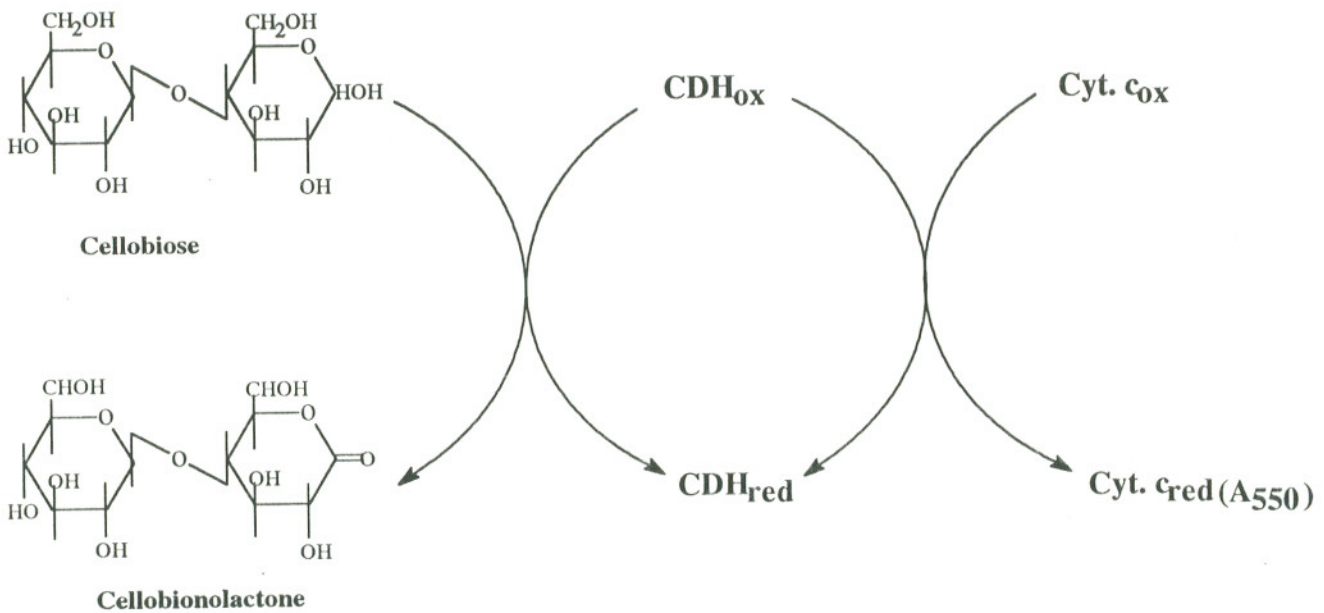

Fig. 2-1 Cellobiose dehydrogenase assay by cytochrome *c* method.

2.2.6 Carbohydrate and Protein Measurements

The neutral carbohydrate content of CDH was determined by the phenol-sulfuric acid method [Dubois et al., 1956]. The amount of sugar released was calculated from a standard curve for glucose. Protein concentrations were determined by the bicinchoninic acid method [Smith et al., 1985].

2.2.7 Estimation of Heme and Flavin

Heme was estimated by the pyridine-hemochromogen method [Appleby & Morton, 1959] and its concentration was calculated using horseradish peroxidase (HRP) as a standard. For estimating flavin, homogeneous CDH was treated with trichloroacetic acid; denatured protein was removed by centrifugation and the supernatant was estimated for flavin at 450 nm. The amount of flavin released was calculated from a standard curve for FAD.

2.2.8 Effect of pH on CDH Stability

CDH stability was tested from pH 2 to 10.5. A fresh sample of CDH (0.2 mg) was incubated in an appropriate buffer (1 ml, 20 mM) at room temperature. An aliquot was removed at regular intervals and CDH activity was monitored by cytochrome *c* assay at pH 4.5. The buffers or solutions and their pH range used in this experiment were as follows: KCl-HCl (pH 2); glycine-HCl (pH 2.5); potassium citrate-potassium phosphate (pH 3); sodium acetate (pH 4 and 5); sodium succinate (pH 5.5); potassium phosphate (pH 6-7); and TRIS-HCl (pH 8 and 9).

2.2.9 Thermostability of CDH

Stability against heat inactivation was determined by incubating a fresh sample of CDH (0.2 mg) at various temperatures in 1 ml of 20 mM phosphate, pH 6. An aliquot was withdrawn and the activity remaining was estimated by cytochrome *c* assay at room temperature over the course of incubation.

2.2.10 H_2O_2 Estimation

Production of H_2O_2 in the CDH reaction was estimated by peroxidase assay [Thomas et al., 1970]. The reaction mixture (50 μl) was added to 1 ml of 20 mM TRIS buffer, pH 8, containing HRP (1 μg) and guaiacol (100 μM). The color end product formation was monitored at 420 nm. The concentration of H_2O_2 was determined from the 420-nm absorbance.

2.2.11 Kinetics

K_m and V_{max} were determined for the various CDH substrates and electron acceptors. The kinetics of substrate oxidation were determined at 550 nm with cytochrome *c* (12.5 μM) as the electron acceptor in 20 mM succinate, pH 4.5. The kinetics of electron acceptors were determined at pH 4.5 with cellobiose (100 μM) as the electron donor.

2.2.12 Chemicals

All chemicals were reagent grade. DEAE-Sephadex, phenyl Sepharose, ethylene glycol, cellobiose, cellotriose, cellotetraose, cellopentaose, ammonium sulfate, DCPIP, HRP (Type VI-A) and cytochrome *c* were purchased from Sigma Chemical Company, St. Louis, MO. Sephacryl S-200 was obtained from Pharmacia LKB Biotechnology, Alameda, CA. Cotton linters were purchased from Fluka Chemical Corporation, Ronkonkoma, NY. Cellobionolactone was prepared from calcium cellobionate as previously described [Isbell & Frush, 1963]. Calcium cellobionate was obtained from ICN Pharmaceuticals, Cleveland, OH.

2.3 RESULTS

2.3.1 CDH Purification

CDH purification from the extracellular medium of cellulolytic cultures by ammonium sulfate fractionation, DEAE-Sephadex chromatography, phenyl Sepharose chromatography, Sephacryl S-200 column, and a final separation by FPLC using a Mono Q column yielded a homogeneous enzyme as judged by SDS-PAGE. This

procedure provided approximately 60-fold purified enzyme with a specific activity of 10.3 μmol min^{-1} mg^{-1} for cytochrome *c* reduction (Table 2-1). The relative molecular mass of CDH as determined by the SDS-PAGE analysis was 90,000 (Fig. 2-2). A molecular mass of 96,000 was determined by gel exclusion chromatography using Sephacryl S-200. The heme *b*:FAD:protein ratio for CDH was 1:1:1. The neutral carbohydrate content of CDH was 9.4% by the phenol-sulfuric acid method [Dubois et al., 1956].

2.3.2 Spectral Properties

The UV-visible spectrum of native, ferric CDH showed absorptions at 420, 529, and 570 nm, and the corresponding extinction coefficients were 99.8, 6.8, and 4.9 mM^{-1} cm^{-1} (Fig. 2-3). The ferrous form of CDH obtained through the addition of either cellobiose or dithionite to the ferric enzyme absorbed at 428, 534, and 564 nm and the calculated extinction coefficients for these absorptions were 139.5, 12.7, and 22.7 mM^{-1} cm^{-1}, respectively (Fig. 2-3). The ferric form of CDH did not bind cyanide or azide. Similarly, the ferrous form did not bind carbon monoxide.

The FAD of CDH was weakly fluorescent with an emission maximum at 535 nm and excitation maxima at 384 and 450 nm. With the addition of cellobiose, the fluorescence was bleached suggesting flavin is reduced during the CDH reaction.

2.3.3 Stability of CDH

CDH was very stable below 50°C in 20 mM phosphate, pH 6 (Fig. 2-4b). At 50°C, 20% activity was lost after 12 h. At 60°C, 80% activity was lost in 1 h. At 70°C, all activity was lost within 10 min. CDH was also very stable in the pH range of 3 to 10.5 at 22°C for 24 h (Fig. 2-4a). However, at pH 2, it was almost completely inactivated within 2 h.

The visible spectrum of pH- or temperature-inactivated CDH showed absorptions at 420, 530, and 570 nm. These spectral features are very similar to those of the native enzyme (Fig. 2-3). Since the visible absorption spectrum of CDH is dominated by heme absorptions, this finding suggests that, even in the inactive CDH, the heme is bound intact to the apoprotein as in the native enzyme. Therefore,

Table 2-1 Purification of cellobiose dehydrogenase

Step	Total protein (mg)	Total activity (U)	Specific activity[a] (U/mg)	Yield (%)	Fold purification
$(NH_4)_2SO_4$ precipitate	1517	264	0.17	100	1.0
DEAE-Sephadex	1280	238	0.19	90	1.1
Phenyl-Sepharose	320	194	0.6	73.5	3.5
Sephacryl S-200	40	150	3.8	56.8	22.4
Mono-Q (FPLC)	11	113	10.3	42.8	60.6

[a] CDH activity was followed at 550 nm with cytochrome *c* as the electron acceptor.

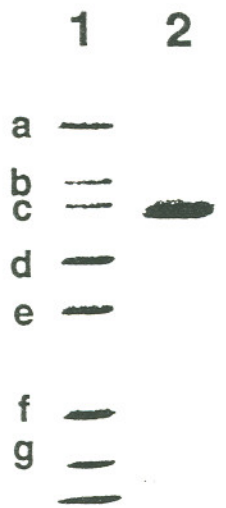

Fig. 2-2 SDS-polyacrylamide gel electrophoresis of purified CDH. Lane 1: molecular weight markers, myosin (200,000) (**a**); β-galactosidase (116,000) (**b**); phosphorylase *b* (97,400) (**c**); bovine serum albumin (66,300) (**d**); glutamatic dehydrogenase (55,400) (**e**); lactate dehydrogenase (36,500) (**f**); and carbonic anhydrase (31,000) (**g**). Lane 2: FPLC-purified CDH.

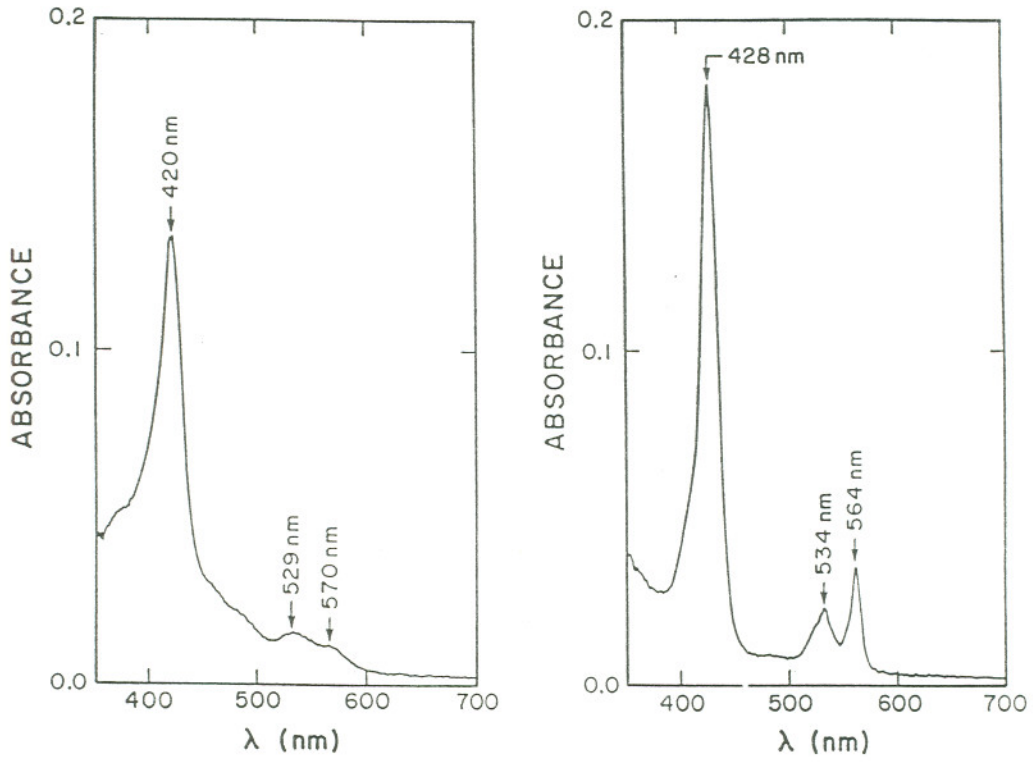

Fig. 2-3 UV-visible spectra of oxidized (**a**) and reduced (**b**) CDH. Reduced CDH was generated by the addition of cellobiose (50 μM) to the oxidized form.

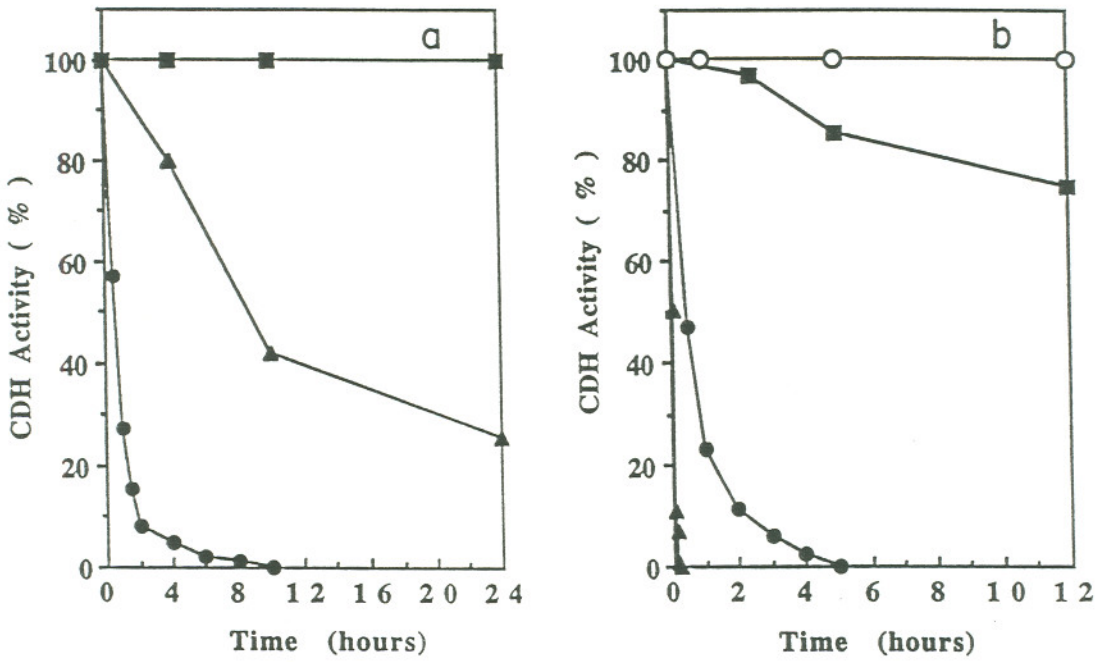

Fig. 2-4 **(a)** pH stability of CDH. CDH (0.2 mg) was incubated in an appropriate buffer (1 ml, 20 mM) at room temperature and the activity was monitored by cytochrome *c* assay. The different buffers used in this experiment are described in the Materials and Methods section. pH 2 (●), pH 2.5 (▲), and pH 3-10.5 (■). **(b)** Thermostability of CDH. CDH (0.2 mg) was incubated at various temperatures in 1 ml of 20 mM phosphate, pH 6, and the activity remaining was assayed by cytochrome *c* reduction. 40°C (o), 50°C (■), 60°C (●), and 70°C (▲).

inactivation was suspected to be due to the release of FAD from the active site. To further understand this inactivation process, low molecular weight compounds of inactivated CDH were separated from proteins by ultrafiltration using a Centricon Microconcentrator (Amicon Corporation, Beverly, MA). The filtrate and retentate were analyzed spectrophotometrically. The filtrate showed absorbances at 370 and 450 nm, characteristic of FAD (Fig. 2-5b).

Estimation of the FAD concentration by its 450-nm absorbance suggested that more than 80% of FAD at the active site was released during inactivation. The retentate containing the higher molecular weight fraction had the same heme spectrum as the native enzyme (Fig. 2-5a).

2.3.4 Specificity for Substrates and Electron Acceptors

The ability of CDH to oxidize various sugars was examined in 20 mM sodium succinate, pH 4.5, with cytochrome *c* as the electron acceptor (Table 2-2). None of the monosaccharides tested were oxidized. Among the disaccharides tested, only cellobiose and lactose served as substrates (Table 2-2). Maltose, isomaltose, and gentiobiose, in which two glucose units are linked by α-1,4, α-1,6, and α-1,6, respectively, were not oxidized by CDH. Cello-oligosaccharides such as cellotriose, cellotetraose, and cellopentaose, which are also cellulose-degradation products, functioned as substrates for CDH as previously described [Ayers et al., 1978; Gritzali & Brown, 1979] (Table 2-2). DCPIP, ferricyanide, benzoquinone, cytochrome *c*, and Mn^{3+} served as electron acceptors in these reactions (Table 2-2). Mn^{3+} is generated extracellularly by the lignin-degrading cultures of *P. chrysosporium* via a manganese peroxidase reaction [Glenn & Gold, 1985].

The substrate specificity of an enzyme is represented by k_{cat}/K_m [Fersht, 1985]. This kinetic parameter was determined for sugar substrates and electron acceptors for CDH. Cellobiose is the best substrate with a k_{cat}/K_m value of 960 mM^{-1} sec^{-1} and lactose was the least-preferred substrate with a k_{cat}/K_m of 46 mM^{-1} sec^{-1} (Table 2-2). Cellotriose, cellotetraose and cellopentaose exhibited k_{cat}/K_m values of 387, 339, and 391 mM^{-1} sec^{-1} and these substrates were preferred to a similar extent. The differences in the k_{cat}/K_m appear to be primarily due to the differences in the K_m

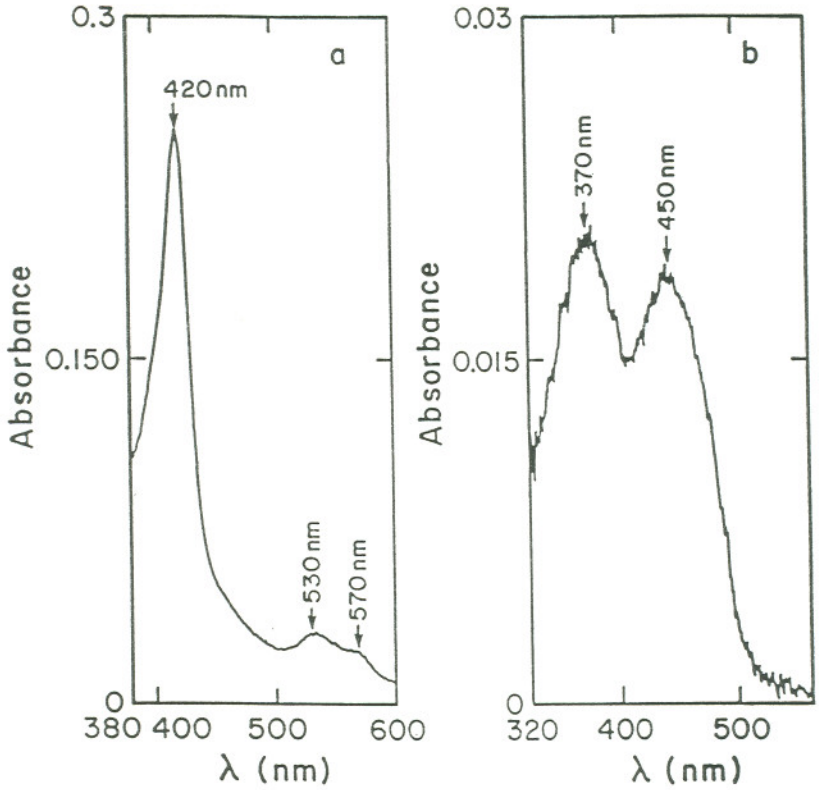

Fig. 2-5 UV-visible spectra of pH-inactivated CDH after ultrafiltration **(a)**, and the corresponding ultrafiltrate **(b)**. CDH (220 μg in 1 ml) was inactivated by incubation in 20 mM KCl-HCl, pH 2, for 2 h. The inactive CDH was ultrafiltered using a Centricon Microconcentrator (Amicon Corp., Beverly, MA). One ml of 20 mM KCl-HCl, pH 2, was added to the retentate and the solution was ultrafiltered. The ultrafiltrates were pooled and analyzed for flavin. The retentate was dissolved in 1 ml of 20 mM succinate, pH 4.5, and analyzed spectrophotometrically.

Table 2-2 Specificity of CDH for substrates and electron acceptors

	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)
Substrates[a]			
Cellobiose	25×10^{-3}	24	960
Cellotriose	62×10^{-3}	24	387
Cellotetraose	61×10^{-3}	20.7	339
Cellopentaose	55×10^{-3}	21.5	391
Lactose	630×10^{-3}	28.8	46
Electron Acceptors[b]			
Cytochrome *c*	1.2×10^{-3}	20.5	17,083
DCPIP	3.6×10^{-3}	33.0	9,166
Ferricyanide	5.2×10^{-3}	22.0	4,230
DTBQ[c]	1.2×10^{-2}	27.0	2,250
Mn^{3+}-malonate[d]	1.5×10^{-1}	14.0	93

Note: Kinetic experiments were performed in 20 mM succinate buffer, pH 4.5, unless specified otherwise.

[a] Cytochrome *c* (12.5 μM) was used as the electron acceptor.

[b] Cellobiose (100 μM) was used as the electron donor. Reduction of cytochrome *c*, DCPIP, ferricyanide, 3,5-DTBQ and Mn^{3+}-malonate was observed at 550 (28), 515 (6.8), 420 (1.15), 420 (1.75) and 270 (9.83) nm, respectively. The numbers in parentheses are the millimolar extinction coefficient values used in the calculation of k_{cat}.

[c] DTBQ, 3.5-Di-tert-butyl-1,2-benzoquinone.

[d] Mn^{3+}-malonate reduction reaction was performed in 50 mM sodium malonate, pH 4.5.

values for the substrates which ranged from 25 to 630 μM (Table 2-2). Among the electron acceptors, cytochrome *c* appeared to be the best acceptor with a k_{cat}/K_m of 17083 mM^{-1} sec^{-1} followed by DCPIP, ferricyanide, 3,5-di-tert-butylbenzoquinone, and Mn^{3+}-malonate, in that order of preference.

Inhibition of cellobiose dehydrogenation by cellobionolactone, glucanolactone, and glucose was determined. Among these, only cellobionolactone inhibited cellobiose dehydrogenation. The kinetics of the CDH reaction were determined at fixed concentrations (0.1, 0.2, 0.4 mM) of cellobionolactone. A Lineweaver-Burke plot of the kinetic values suggests that the inhibition is of the mixed type (Fig. 2-6). A K_i value of 0.59 mM has been calculated from a replot of cellobionolactone concentration versus the slope of reciprocal plots.

2.3.5 CDH Reduction of Colored Peroxidase Degradation Products

H_2O_2 production in an enzyme reaction is assayed using HRP. In this assay, the enzyme is incubated with its substrate, HRP, and a peroxidase substrate such as guaiacol or *o*-dianisidine [Ayers et al., 1978; Thomas et al., 1970]. The amount of color generated is equivalent to H_2O_2 levels. Although CDH in the presence of cellobiose is known to consume oxygen, the reduction product of oxygen has not yet been characterized. Ayers et al. [1978] investigated the H_2O_2 formation by peroxidase assay using *o*-dianisidine as the chromogenic substrate. Color formation was not observed suggesting that H_2O_2 is not generated in the CDH reaction [Ayers et al., 1978]. We speculated that CDH, in the presence of cellobiose, may be reducing the colored peroxidation products. To test this speculation, the effect of CDH and cellobiose on HRP-catalyzed oxidation of guaiacol and *o*-dianisidine was determined. HRP oxidation of guaiacol and *o*-dianisidine was monitored at 420 nm. Addition of CDH (2.5 μg/ ml) and cellobiose (100 μM) to the guaiacol oxidation product caused a 90% decrease in the 420-nm absorbance (Fig. 2-7). When CDH and cellobiose were included in the initial assay mixture, the 420-nm absorbance due to guaiacol oxidation increased and then decreased rapidly (Fig. 2-6). Addition of CDH (2.5 μg /ml) and cellobiose (100 μM) to the *o*-dianisidine oxidation product produced only a 20% decrease in the 420-nm absorbance [data not shown]. However, when CDH (2.5-7.5

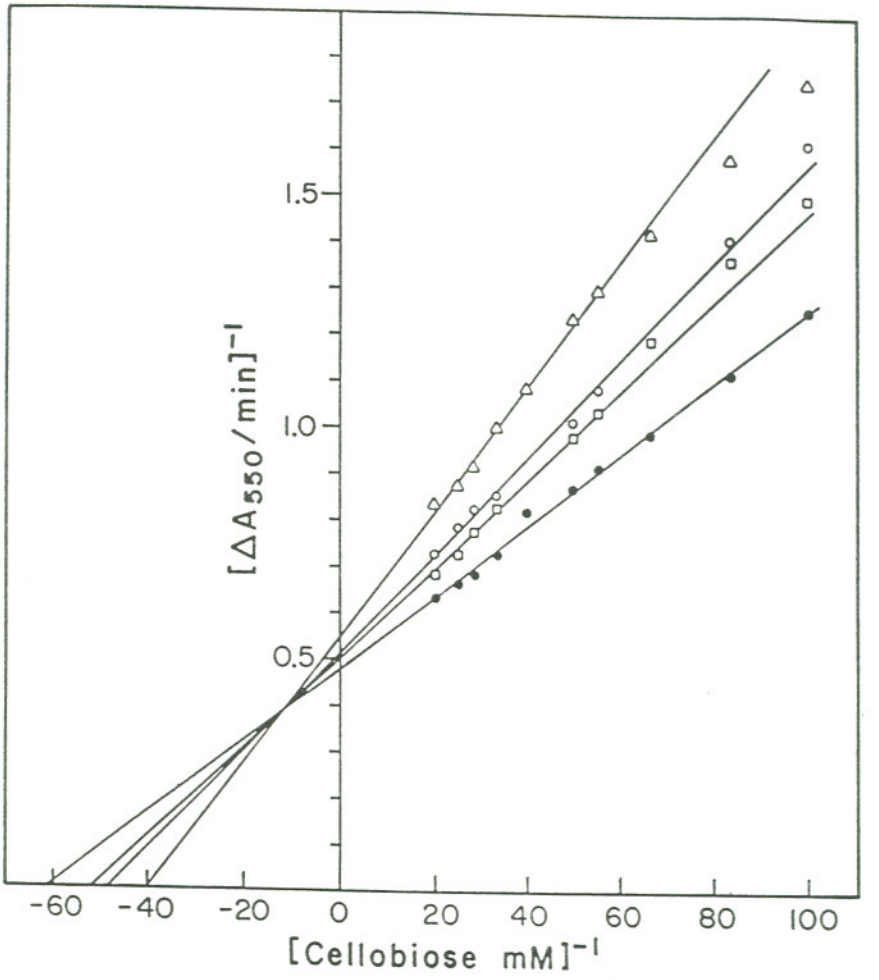

Fig. 2-6 Mixed-type inhibition of cellobiose oxidation by cellobionolactone. Cellobiose oxidation was assayed at 550 nm with cytochrome *c* as the electron acceptor. CDH activity was determined at varying concentrations of cellobiose and in the presence of fixed concentrations of cellobionolactone. 0 mM (●), 0.1 mM (□), 0.2 mM (o), and 0.4 mM (△) cellobionolactone.

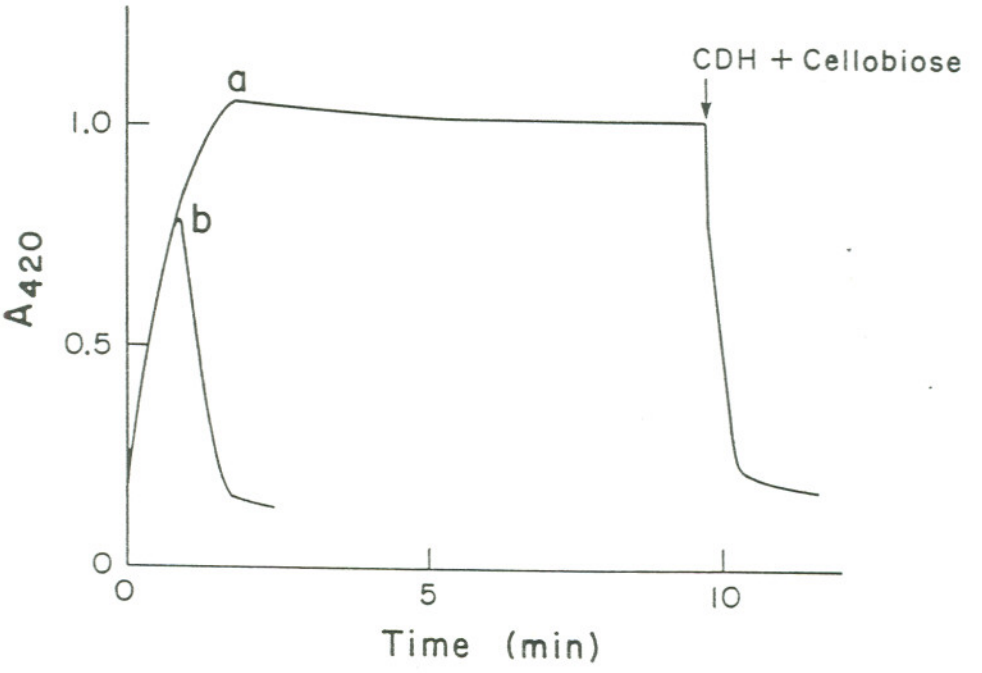

Fig. 2-7 Decolorization of the HRP oxidation product of guaiacol by CDH. **(a)** HRP (2.5 μg) + guaiacol (100 μM) + H_2O_2 (100 μM). CDH (2.5 μg) and cellobiose (100 μM) were added after 10 min. **(b)** Same as (a) but also contained CDH (2.5 μg) and cellobiose (100 μM). Guaiacol oxidation was monitored at 420 nm.

μg) and cellobiose (100 μM) were included in the initial assay mixture, the final 420-nm absorbance decreased progressively depending on the concentration of CDH (Fig. 2-8).

2.3.6 Production of H_2O_2 in the CDH Reaction

The addition of cellobiose to CDH generated a reduced form which was stable under anaerobic conditions but unstable under aerobic conditions (Fig. 2-9). In the presence of O_2, the reduced form was slowly oxidized back to the native form. This transformation could be conveniently followed at 564 nm. For estimating H_2O_2, cellobiose (50 μM) was incubated with a fresh sample of CDH (1.1 μM) and the absorbance at 564 nm was monitored (Fig. 2-8). Loss of absorbance at 564 nm indicated that all of the added cellobiose had been oxidized. At this stage, the reaction mixture was analyzed for H_2O_2 by peroxidase assay with guaiacol as the chromogenic substrate. The ratio of cellobiose oxidized to H_2O_2 produced was determined to be 1:1.

2.4 DISCUSSION

CDH, an extracellular hemoflavoenzyme produced by the cellulose-degrading cultures of *P. chrysosporium*, oxidizes cellobiose to cellobionolactone [Ayers et al., 1978]. In this study, a method for purifying CDH to homogeneity is developed. This procedure provides CDH with high specific activity and yield (Tables 2-1, 2-3). The relative molecular mass of homogeneous CDH is 90,000 as judged by SDS-PAGE and is in agreement with the earlier reports (Fig. 2-2) [Ayers et al., 1978; Morpeth, 1985]. While CDH from *P. chrysosporium* is a monomer, CDH from *S. thermophile* appears to be a dimer [Canevascini et al., 1991]. CDH is a glycoprotein with a neutral carbohydrate content of 9.4%. The molar ratio of heme *b*:FAD:protein is 1:1:1. This ratio has not been previously determined for CDH from *P. chrysosporium* [Ayers et al., 1978; Sadana & Patil, 1985; Samejima et al., 1992; Henriksson et al., 1991]. CDH is very stable between pH 3 and 10.5 and below 50°C. However, below pH 2 or above 50°C, the activity is lost due to release of

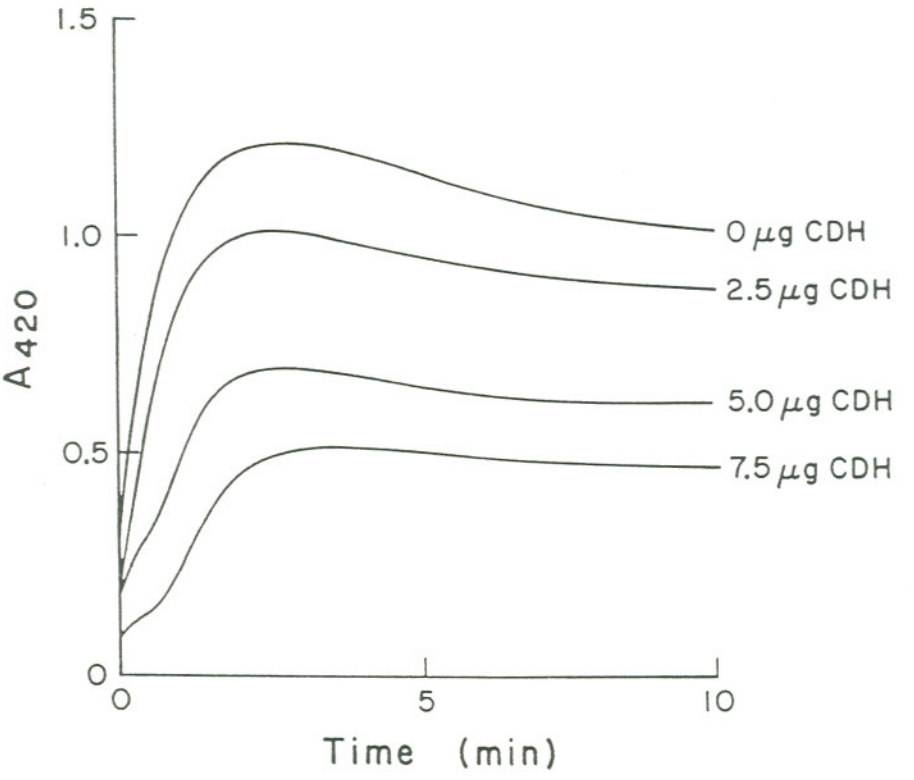

Fig. 2-8 Decolorization of the HRP oxidation product of *o*-dianisidine by CDH. The reaction mixture contained HRP (50 ng), *o*-dianisidine (100 μM), H_2O_2 (100 μM), cellobiose (100 μM) and CDH (0-7.5 μg). Reactions were initiated by the addition of H_2O_2 and the product formation was determined at 420 nm.

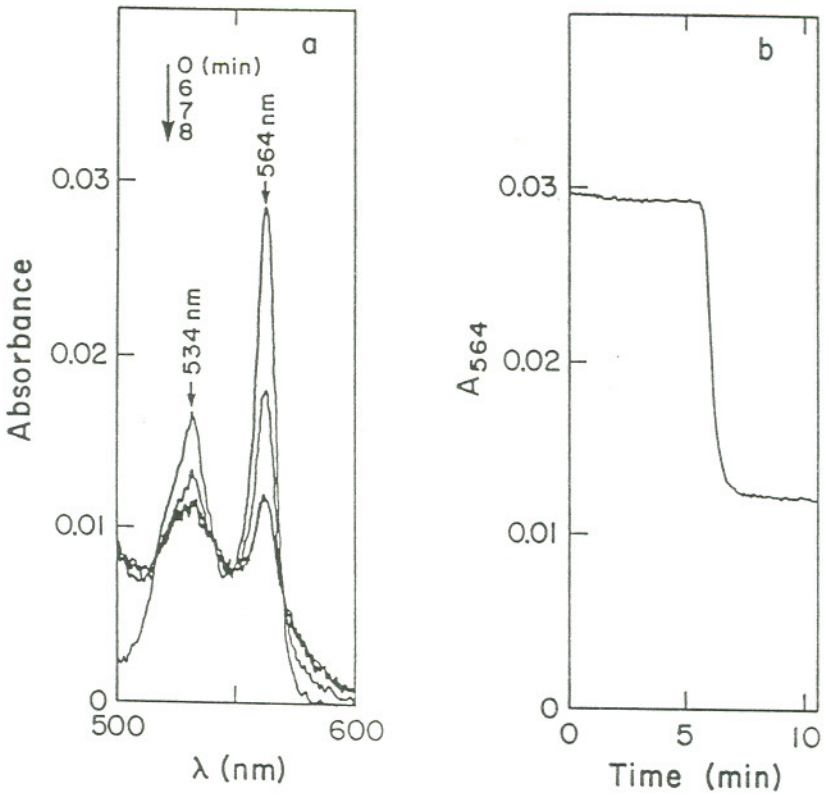

Fig. 2-9 Spectral changes occurring between 500 and 600 nm in the conversion of reduced CDH to oxidized CDH under aerobic conditions (**a**). Time course for the absorbance change at 564 nm of reduced CDH (**b**).

Table 2-3 Comparison of CDH purification methods

Reference	Cytochrome *c* reduction[a] (U/mg)	DCPIP reduction[b] (U/mg)	DTBQ reduction[c] (A_{420}/min/mg)	Yield[d] (mg/g)
This work	10.3	9.0	30.0	5.2
Ayers et al., 1978	-	-	-	0.32
Morpeth, 1985	-	1.1	-	1.39
Henriksson et al., 1991	-	-	1.25	1.32
Samejima et al., 1992	[e]	-	-	2.2

[a] Cytochrome *c* (12.5 μM) reduction was monitored by the increase in absorbance at 550 nm in 20 mM succinate, pH 4.5.

[b] DCPIP (25 μM) reduction was determined by the decrease in absorbance at 600 nm in 20 mM phosphate, pH 6.0.

[c] 3, 5-di-tert-butyl-1,2-benzoquinone (DTBQ) reduction was followed by the decrease in absorbance at 420 nm in 20 mM succinate, pH 4.5, at 56°C.

[d] Milligrams of CDH obtained per gram of extracellular protein.

[e] CDH was assayed using cytochrome *c*; however, the specific activity of purified CDH has not been reported.

flavin from the active site, and the heme appears to be tightly bound to the apoprotein even under these conditions (Fig. 2-5). Deflavinated CDH does not appear to reconstitute readily with FAD. The UV-visible spectrum of oxidized CDH shows absorbances at 420, 529, and 570 nm as reported previously [Ayers et al., 1978; Morpeth, 1985]; with the addition of cellobiose, absorbances shift to 428, 534, and 564 nm (Fig. 2-3). These spectral characteristics of CDH are very similar to the corresponding forms of flavocytochrome b_2 and spermidine dehydrogenase, which suggests that all of these hemoflavoenzymes may have a similar heme environment (Fig. 2-3; Table 2-4). Ferric CDH does not bind azide or cyanide suggesting a hexacoordination for the heme iron as in flavocytochrome b_2 [Xia et al., 1987].

Among the various sugar substrates examined for CDH, only cellobiose, cellotriose, cellotetraose, cellopentaose and lactose served as substrates for CDH (Table 2-2) suggesting specificity for β-1,4-linkage. The determination of k_{cat}/K_m for CDH-catalyzed sugar oxidations suggests that cellobiose is the preferred substrate, whereas lactose is the least preferred substrate (Table 2-2). A similar comparison of k_{cat}/K_m for the electron acceptors of cytochrome *c*, DCPIP, 3,5-di-tert-butyl-1,2-benzoquinone, and Mn^{3+}-malonate, indicates that cytochrome *c* is the preferred electron acceptor (Table 2-2). Recently, Samejima and Eriksson [1992] determined that the K_m and the k_{cat} values for cytochrome *c* in the CDH reaction were 13 μM and 19.1 sec^{-1}, respectively. The K_m for cytochrome *c* reported here is 10-fold lower than the value reported earlier [Samejima & Eriksson, 1992]. CDH oxidizes cellulose very slowly [Sadana & Patil, 1985; Kremer & Wood, 1992b]; however, the physiological relevance of cellulose oxidation by CDH is not yet clear. CDH is susceptible to inhibition by cellobionolactone and the inhibition appears to be of the mixed type (Fig. 2-6). Since cellobionolactone is not a substrate for CDH, it may inhibit cellobiose dehydrogenation by binding to the oxidized and reduced forms of CDH.

Among the several electron acceptors identified for CDH reaction, DCPIP is an artificial electron acceptor; cytochrome *c* is an intracellular protein; and benzoquinone and Mn^{3+} are generated by *P. chrysosporium* only when it degrades lignin or lignocellulose [Kirk & Farrell, 1987]. In the absence of these acceptors, O_2

Table 2-4 Spectral properties of hemoflavoenzymes

Enzyme	Oxidized (nm)	Reduced (nm)	Reference
Cellobiose dehydrogenase	420, 529, 570	428, 534, 564	This work
Flavocytochrome b_2	413, 530, 560	423, 528, 557	Labeyrie et al., 1978; Appleby & Morton, 1959
Spermidine dehydrogenase	414, 530, 560	427, 530, 562	Tabor & Kellogg, 1970

can function as an electron acceptor and can oxidize the reduced CDH to its native form (Fig. 2-9). In this process, O_2 would be reduced either by two electrons to generate H_2O_2 or by a single electron to produce a superoxide radical. The latter will readily disproportionate to yield H_2O_2 and O_2 [Fridovoch, 1989]. H_2O_2 production in the CDH reaction using peroxidase assay was not detected earlier [Ayers et al., 1978; Canevascini et al., 1991] because the colored peroxidation products served as electron acceptors for the CDH reaction resulting in partial or total color loss (Figs. 2-7, 2-8). However, we found that the color is stable in the absence of cellobiose. This finding suggests that all of the cellobiose added to the reaction has to be oxidized before H_2O_2 estimation. We monitored the 564-nm absorbance of reduced CDH as a measure of cellobiose concentration and estimated H_2O_2 after a final decrease at the 564-nm absorbance (Fig. 2-9). By this procedure, the ratio of cellobiose oxidized to H_2O_2 generated was estimated to be 1:1.

Superoxide radical is assayed by its ability to reduce cytochrome *c* and tetranitromethane [Rosen et al., 1982]. Superoxide dismutase and anaerobic conditions inhibit this reduction. Morpeth [1985] reported a 58% decrease in the rate of cytochrome *c* reduction by CDH under anaerobic conditions. However, we did not observe any decrease in the rate of cytochrome *c* reduction by CDH in the presence of superoxide dismutase or in the absence of oxygen. We conclude that these reductions are not mediated by superoxide radical. Samejima and Eriksson [1992] reported a similar observation.

CDH was earlier classified as an oxidase because it consumed O_2 during cellobiose oxidation [Ayers et al., 1978]. However, our findings reported here and other recent studies suggest that CDH prefers to transfer electrons to acceptors, such as cytochrome *c* and DCPIP, rather than to O_2 (Table 2-2) [Morpeth, 1985; Bao et al., 1993; Samejima & Eriksson, 1992]. This, in fact, is characteristic of a dehydrogenase rather than an oxidase. Hence, we have renamed cellobiose oxidase cellobiose dehydrogenase; however, the physiological electron acceptor for CDH is not yet known.

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CHAPTER 3
PRODUCTION OF CELLOBIOSE DEHYDROGENASE AND β-GLUCOSIDASE BY CELLULOSE-DEGRADING CULTURES OF *PHANEROCHAETE CHRYSOSPORIUM*

3.1 INTRODUCTION

White-rot fungi are the only microorganisms known to completely degrade lignocellulose to CO_2 [Gold et al., 1989; Kirk & Farrell, 1987]. Cellulose-degrading cultures of the white-rot fungus *Phanerochaete chrysosporium* produce two extracellular cellobiose-oxidizing enzymes: cellobiose dehydrogenase (CDH, also known as cellobiose oxidase) and cellobiose:quinone oxidoreductase (CBQase), in addition to cellulases [Westermark & Eriksson, 1975; Ayers et al., 1978; Morpeth, 1985; Bao et al., 1993]. CDH is a hemoflavoenzyme, whereas CBQase is a flavoenzyme. These enzymes oxidize cellobiose to cellobionolactone in the presence of electron acceptors such as cytochrome *c*, quinones, and dichlorophenol-indophenol (DCPIP) [Morpeth, 1985; Henriksson et al., 1991; Samajima & Eriksson, 1992; Bao et al., 1993]. CDH is a glycoprotein with an approximate molecular mass of 90,000, and it contains one heme *b* and one FAD per monomer [Ayers et al., 1978; Bao et al., 1993]. The heme and the flavin of CDH are bound by different domains [Henriksson et al., 1991; Wood & Wood, 1992]. Papain can cleave CDH into two peptide fragments: a larger peptide containing the flavin and a smaller peptide containing the heme [Henriksson et al., 1991]. The molecular mass of the flavodomain is similar to that of CBQase; also, this domain can oxidize cellobiose to cellobionolactone. These observations have led to the proposal that CBQase is formed from CDH via proteolytic hydrolysis [Henriksson et al., 1991; Wood & Wood, 1992]. Both CDH and CBQase bind to microcrystalline cellulose, a

characteristic generally found only in cellulases [Renganathan et al., 1990; Henriksson et al., 1991]. We observed that CDH can enhance crystalline cellulose hydrolysis by cellulases and suggested that this may be one of its physiological functions [Bao & Renganathan, 1992].

In this report, production of cellobiose-oxidizing enzymes by cellulose-degrading cultures of *P. chrysosporium* is investigated. In addition, production of extracellular β-glucosidase and protease is examined. β-Glucosidase activity is monitored because it competes with cellobiose-oxidizing enzymes for cellobiose. Protease activity is examined because protease(s) degrades CDH to CBQase [Henriksson et al., 1991; Wood & Wood, 1992]. Also, identification of culture conditions with low protease activity would help in augmenting the yield of CDH in the culture medium. The effect of the initial pH of the medium and of different cellulosic substrates on the production of CDH, CBQase, protease, and β-glucosidase in the extracellular medium is examined.

P. chrysosporium degrades lignin under nitrogen-limiting, secondary metabolic conditions [Gold et al., 1989; Kirk & Farrell, 1987]. Lignin-degrading cultures of *P. chrysosporium* produce two extracellular peroxidases: lignin peroxidase (LiP) and manganese peroxidase (MnP) [Tien & Kirk, 1984; Glenn & Gold, 1985; Kirk & Farrell, 1987; Gold et al., 1989]. These peroxidases, along with an extracellular H_2O_2-generating system [Kersten & Kirk, 1987], seem to constitute the major components of the lignin-degradative system of *P. chrysosporium*. CDH and CBQase inhibit a variety of peroxidase-catalyzed reactions [Ander et al., 1990; Samajima & Eriksson, 1992; Bao et al., 1993]. Our recent results suggest that CDH can also transfer electrons to the H_2O_2-oxidized intermediates of LiP. Such interactions between CDH, CBQase, and lignin-degrading enzymes are possible only if all of these enzymes are produced simultaneously. For this reason, the production of LiP and MnP in cellulose-degrading cultures with low levels of nutrient nitrogen is examined.

3.2 MATERIALS AND METHODS

3.2.1 Organism

Stock cultures of *P. chrysosporium* OGC 101 were maintained on slants of Vogel medium supplemented with 3% malt extract and 0.25% yeast extract [Gold & Cheng, 1978; Alic et al., 1987].

3.2.2 Medium Composition

The medium composition for stationary cultures of *P. chrysosporium* was as previously described [Eriksson & Pettersson, 1975], except that ferric citrate was replaced with ferric nitrate (15 mg/ml). The composition of the phosphate medium with an initial pH of 5.8 was as previously described [Eriksson & Pettersson, 1975]. The phosphate medium with an initial pH of 7 or 8 contained 25 mM potassium dihydrogen phosphate, 20 mM ammonium phosphate, yeast extract (100 mg/ml), ferric nitrate (15 mg/ml), thiamine hydrochloride (0.1 mg/l), and other mineral salts as described [Eriksson & Pettersson, 1975]. The composition of the medium with an initial pH of 4.5 was the same as the pH 7 and 8 media, except that 25 mM potassium dihydrogen phosphate was replaced with either 25 mM sodium succinate or 25 mM sodium acetate. The medium pH was adjusted after addition of all ingredients. The phosphate medium was adjusted to pH 7 or 8. Succinate and acetate media were adjusted to pH 4.5.

3.2.3 Culture Conditions

A stationary culture medium (20 ml in a 250-ml Erlenmeyer flask) supplemented with 2% glucose and 0.5% yeast extract was inoculated with stock cultures of *P. chrysosporium* and incubated at 37°C for 65 h. Two 65-h stationary cultures were homogenized in a blender for 20 s and inoculated into 500 ml of an appropriate shake culture medium in a 1-l Erlenmeyer flask containing 5 gm cellulose. The cultures were incubated at 28°C on a rotary shaker (150 rpm) for 14 days. The extracellular medium from these cultures was assayed for various enzyme activities at regular intervals. Four samples were used in each experiment.

3.2.4 Enzyme Assays

CDH activity was assayed specifically by the reduction of cytochrome *c* (12.5 μM) in 20 mM succinate, pH 4.5, in the presence of cellobiose (100 μM) [Bao et al., 1993]. Cytochrome *c* reduction was followed by monitoring the increase in absorbance at 550 nm (ϵ = 28 mM^{-1} cm^{-1}).

2,6-Dichlorophenol-indophenol (DCPIP) serves as an electron acceptor for both CDH and CBQase [Morpeth 1985; Renganathan et al., 1990]. The specific activity of homogeneous CDH for cytochrome *c* and DCPIP reduction was determined to be 11.8 U/mg and 18.2 U/mg, respectively [Bao et al., 1993]. Thus, 1 U of cytochrome *c* activity of CDH is equivalent to 1.54 U of DCPIP activity. The DCPIP reduction activity of CDH was calculated from its cytochrome *c* activity. The DCPIP activity of CBQase was determined by subtracting the calculated DCPIP activity due to CDH from the total DCPIP activity. DCPIP (25 μM) reduction was monitored by the decrease in absorbance at 515 nm (ϵ = 6.8 mM^{-1} cm^{-1}) in 20 mM succinate, pH 4.5, in the presence of cellobiose (100 μM) [Renganathan et al., 1990; Bao et al., 1993].

β-Glucosidase activity was assayed by monitoring the release of *p*-nitrophenol from *p*-nitrophenyl-β-glucoside hydrolysis. The culture medium (40 μl) was incubated with 1 mM *p*-nitrophenyl-β-glucoside in 1 ml 50 mM phosphate, pH 4.5, at room temperature for 5 min. The enzyme reaction was arrested by the addition of 0.2 ml 1 M sodium carbonate. Release of *p*-nitrophenol was monitored at 400 nm (ϵ = 18.3 mM^{-1} cm^{-1}).

Protease was assayed with azocoll by modification of a previously described method [Eriksson & Pettersson, 1982; Chavin et al., 1984]. Azocoll (5 mg) was shaken vigorously with 1 ml 20 mM sodium acetate, pH 5, at 37°C for 2 h to remove any unbound dye. Then the azocoll suspension was centrifuged and the supernatant was discarded. The precipitate was suspended in 1 ml 20 mM acetate, pH 5, and incubated with the extracellular medium (40 μl) at 37°C for 1 h. The reaction mixture was centrifuged and the supernatant was filtered through glass wool. The absorbance of the filtrate at 520 nm was determined as a measure of protease activity.

LiP was assayed by oxidation of veratryl alcohol (0.5 mM) in 20 mM succinate, pH 3, in the presence of 0.1 mM H_2O_2 [Tien & Kirk, 1984]. Veratraldehyde formation was monitored by the increase in absorbance at 310 nm (ϵ = 9.3 mM^{-1} cm^{-1}). MnP activity was determined by the increase in absorbance at 270 nm due to the formation of Mn^{3+}-malonate complex (ϵ = 11.59 mM^{-1} cm^{-1}) as described [Wariishi et al., 1992]. The assay mixture contained manganese sulphate (0.5 mM) and H_2O_2 (0.1 mM) in 50 mM malonate, pH 4.5.

All enzyme activities, except protease activity, are expressed in units, defined as the amount of enzyme producing 1 μmole of product per minute. Protein was determined by the bicinchoninic acid method [Smith et al., 1985], and 50 μl of culture medium was used for this estimation. pH was determined using a pH meter (Model SA 720, Orion Research Inc., Boston, MA).

3.2.5 Preparation of Acid-Treated Cellulose

Microcrystalline cellulose (Sigmacell Type 50, 50 gm) was added in small portions to a cold phosphoric acid solution (85%, 675 ml) with vigorous stirring. Addition of deionized water to this mixture precipitated the cellulose. The pH of the cellulose precipitate was increased to 7 by washing with deionized water and by equilibration with 50 mM phosphate, pH 7, buffer. The decrystallized cellulose was isolated by filtration and dehydrated to a powder by washing with acetone.

3.2.6 Biomass Dry Weight Determinations

The biomass from four cellulose-degrading cultures was separated by filtration and dried in an oven maintained at 60°C. The samples were cooled to ambient temperature before weighing, and were dried until two consecutive weighings provided the same values.

3.2.7 Chemicals and Biochemicals

Cellobiose was purchased from Aldrich Chemical Co., Milwaukee, WI. Cytochrome *c*, DCPIP, *p*-nitrophenyl-β-glucoside, azocoll, microcrystalline cellulose (Sigmacell Type 50), and bicinchoninic acid reagent were obtained from Sigma

Chemical Co., St. Louis, MO. Cotton linters (cellulose powder) were purchased from Fluka Chemical Co., Ronkonkoma, NY.

3.3 RESULTS

3.3.1 Effect of Initial pH on Extracellular Protein Production

The highest protein concentration (1.4 mg/ml) was found in cultures containing phosphate at an initial pH of 8 (Fig. 3-1). When the initial pH of the phosphate medium was decreased to 7 or 5.8, protein production declined significantly. In fact, the phosphate pH 5.8 culture produced the least amount of extracellular protein. The total protein (1.1 mg/ml) produced by cultures with succinate at an initial pH of 4.5 was comparable to that of the phosphate pH 8 culture. The protein concentration in the succinate culture was at least two-fold higher than the acetate culture adjusted to an initial pH of 4.5 (Fig. 3-1, Table 3-1).

3.3.2 Effect of Initial pH on CDH Production

The appearance of CDH activity varied considerably among the different cultures. In the case of the succinate pH 4.5 culture, CDH activity appeared after 6 days, producing the maximum amount of CDH (141 U/l) (Table 3-1). The acetate pH 4.5 and phosphate pH 5.8 cultures produced the least amounts of CDH (20 and 6 U/l, respectively). CDH activity in phosphate pH 7 and 8 cultures was 50-65% less than in the succinate culture (Fig. 3-2, Table 3-1).

3.3.3 Effect of Initial pH on CBQase Production

CBQase levels in 14-day old cultures decreased in the following order: acetate pH 4.5 > phosphate pH 8 > phosphate pH 5.8 > phosphate pH 7 > succinate pH 4.5. Succinate cultures, which produced the highest levels of CDH, produced only the lowest levels of CBQase (Fig. 3-3, Table 3-1).

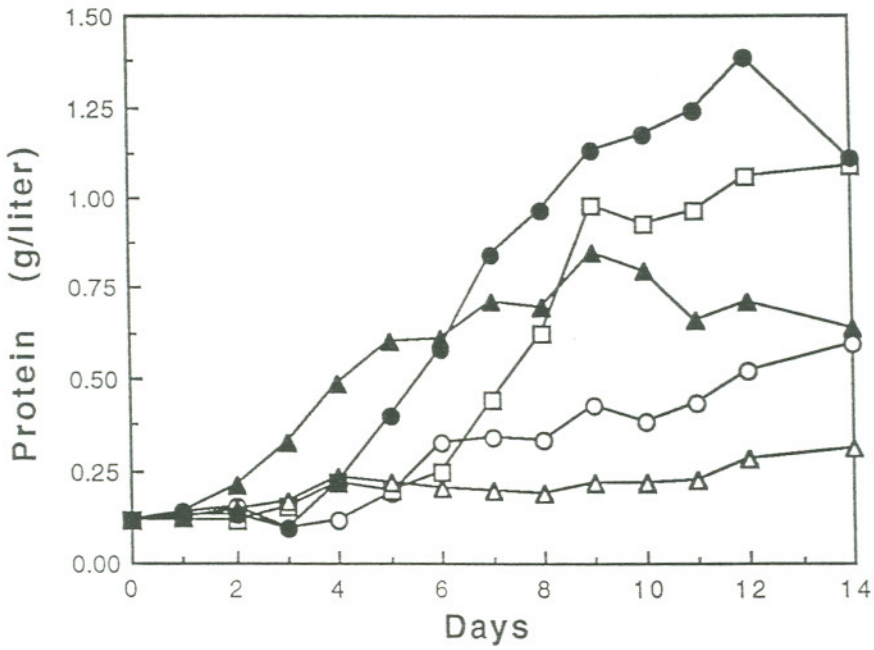

Fig. 3-1 Effect of the initial pH of the culture medium on the production of extracellular protein. The *P. chrysosporium* culture medium was buffered with succinate pH 4.5 (□), acetate pH 4.5 (o), phosphate pH 8 (●), phosphate pH 7 (▲), phosphate pH 5.8 (△).

Table 3-1 Production of CDH, CBQase, β-glucosidase, protease and total protein under different initial pH conditions[a]

Buffer	Initial pH	Protein[a,b] (g/l)	CDH[a,c] (U/l)	CBQase[a,d] (U/l)	β-Glu[e,f] (U/l)	Protease[f,g] (A_{520}/ml)
Succinate	4.5	1.1 ± 0.14	141 ± 16	53 ± 17	237 ± 21 (14)	0.4 ± 0.1 (8)
Acetate	4.5	0.6 ± 0.02	20 ± 1	184 ± 20	23 ± 1 (6)	1.6 ± 0.1
Phosphate	5.8	0.3 ± 0.01	6 ± 1	75 ± 15	7 ± 1 (3)	0.9 ± 0.1
Phosphate	7.0	0.6 ± 0.17	58 ± 6	58 ± 2	45 ± 9 (3)	1.6 ± 0.8
Phosphate	8.0	1.4 ± 0.29	77 ± 3	129 ± 20	81 ± 6 (9)	2.8 ± 0.2

[a] Total extracellular protein and the activities of CDH and CBQase were determined on the 14th day of culture growth.

[b] Protein was determined by bicinchoninic acid.

[c] CDH activity was assayed by the reduction of cytochrome *c*.

[d] CBQase activity refers to the DCPIP reduction activity.

[e] β-Glucosidase activity was determined by the hydrolysis of *p*-nitrophenyl-β-D-glucoside.

[f] The enzyme activity values are the maximum observed values. The numbers in parentheses refer to the day on which the maximum enzyme activity was observed.

[g] Protease was assayed with azocoll.

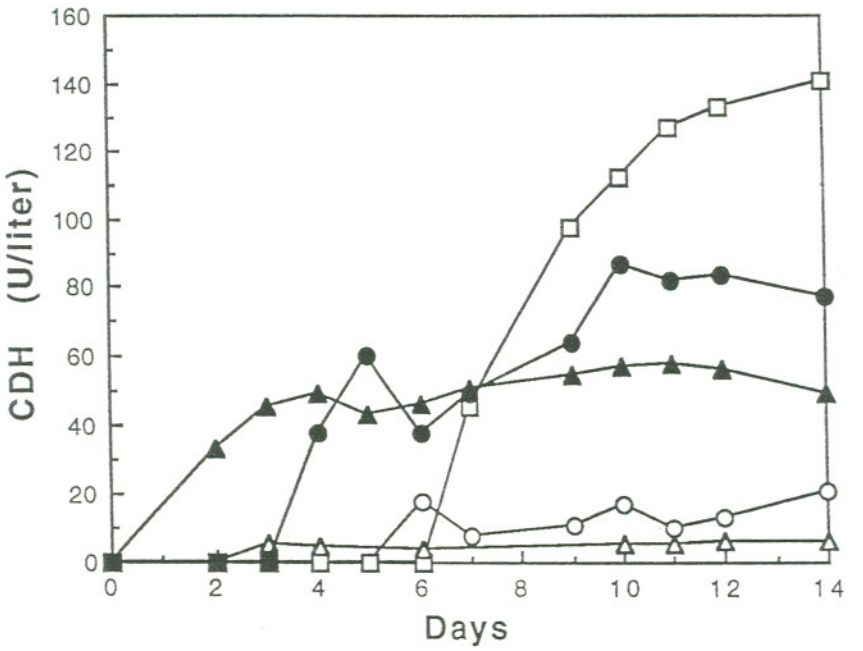

Fig. 3-2 Effect of the initial pH of the culture medium on the production of CDH. The *P. chrysosporium* culture medium was buffered with succinate pH 4.5 (□), acetate pH 4.5 (o), phosphate pH 8 (●), phosphate pH 7 (▲), phosphate pH 5.8 (△).

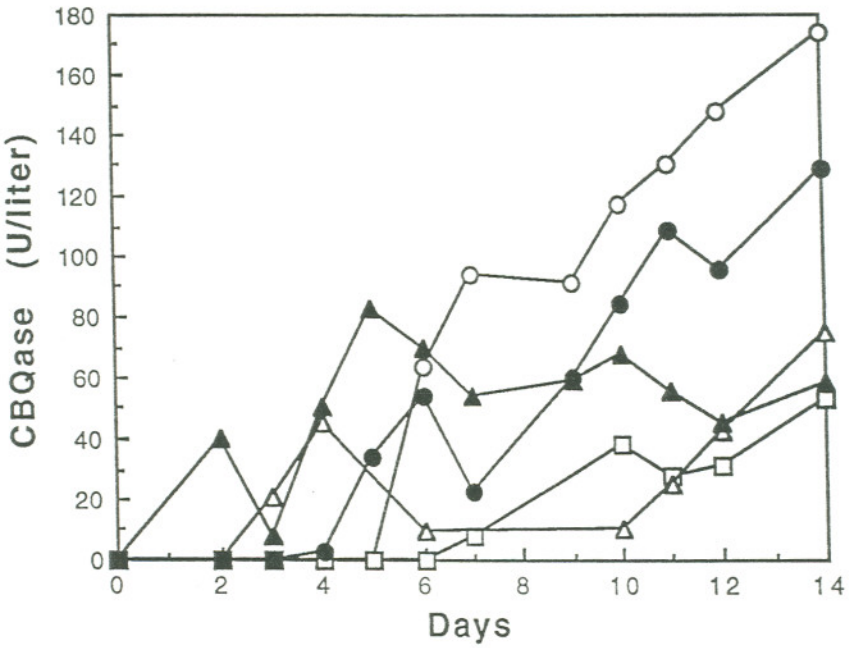

Fig. 3-3 Effect of the initial pH of the culture medium on the production of CBQase. The *P. chrysosporium* culture medium was buffered with succinate pH 4.5 (□), acetate pH 4.5 (o), phosphate pH 8 (●), phosphate pH 7 (▲), phosphate pH 5.8 (△).

3.3.4 Effect of Initial pH on β-Glucosidase Production

The succinate pH 4.5 culture produced the highest levels of β-glucosidase (237 U/l) (Table 3-1). β-Glucosidase activity in phosphate pH 7 and 8 cultures were six-fold and three-fold, respectively, lower than the succinate pH 4.5 culture. Acetate pH 4.5 and phosphate pH 5.8 cultures yielded extremely low levels of β-glucosidase (Fig. 3-4, Table 3-1).

3.3.5 Effect of Initial pH on Protease Activity

The phosphate pH 8 culture produced the highest levels of protease, whereas the succinate pH 4.5 culture produced the lowest levels of protease. Protease activity under other initial pH conditions was moderate (Fig. 3-5, Table 3-1).

3.3.6 pH Variation during Culture Growth

In the case of phosphate pH 7 and 8 cultures, the medium pH initially decreased below 4.5 and then slowly increased to 6 and 5, respectively. In the case of the phosphate pH 5.8 culture, the medium pH rapidly decreased to 2.5 in the initial phase; however, unlike other cultures, the pH of this culture remained very acidic. The succinate pH 4.5 culture maintained between pH 4 and 4.5 initially, and then the pH increased to 6. In contrast, in the acetate pH 4.5 culture, the pH stayed between 4 and 4.5 for four days and then decreased to below 3 (Fig. 3-6).

3.3.7 Effect of Cellulose Substrate on Enzyme Production

The effect of different cellulosic substrates on the production of CDH, CBQase, β-glucosidase, protease, and total extracellular protein was examined. Cotton linters, microcrystalline cellulose (Sigmacell Type 50), filter paper (Whatman No. 1), and phosphoric acid-treated cellulose were used in this experiment. At the end of each experiment, the dry weight of the total biomass produced was also determined. Results of these experiments are presented in Table 3-2. Among the different cellulosic substrates examined, cotton linters yielded the highest levels of total protein, CDH, and β-glucosidase (Table 3-2). Acid-treated cellulose-supplemented cultures yielded the lowest levels of enzymes and protein. All cultures

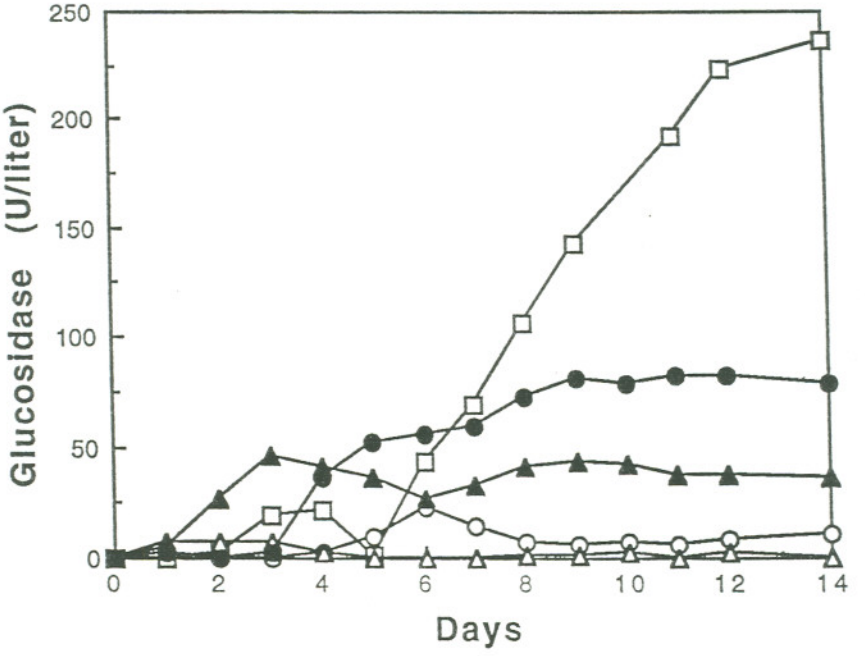

Fig. 3-4 Effect of the initial pH of the culture medium on the production of β-glucosidase by *P. chrysosporium*. The *P. chrysosporium* culture medium was buffered with succinate pH 4.5 (□), acetate pH 4.5 (o), phosphate pH 8 (●), phosphate pH 7 (▲), phosphate pH 5.8 (△).

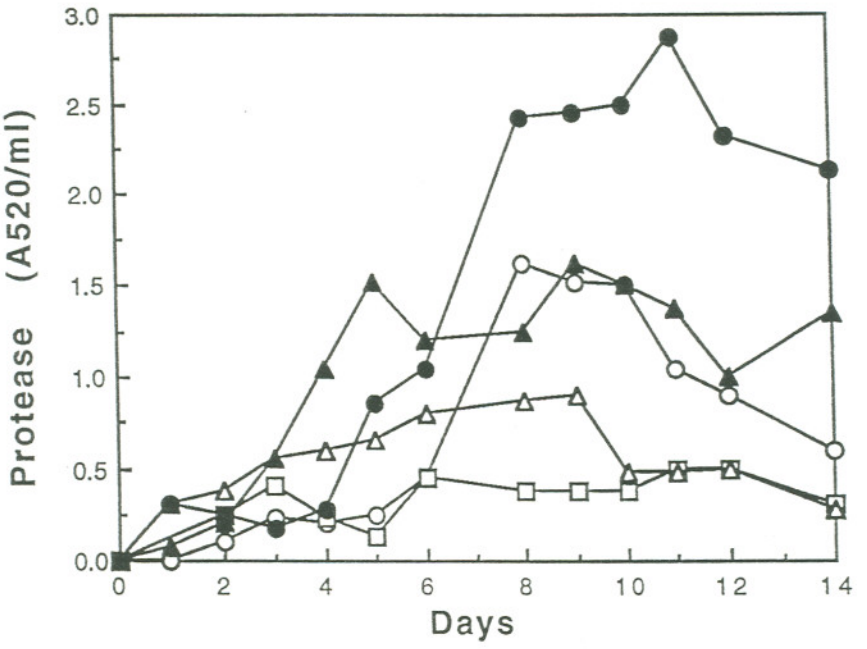

Fig. 3-5 Effect of the initial pH of the culture medium on the production of protease by *P. chrysosporium*. The *P. chrysosporium* culture medium was buffered with succinate pH 4.5 (□), acetate pH 4.5 (o), phosphate pH 8 (●), phosphate pH 7 (▲), phosphate pH 5.8 (△).

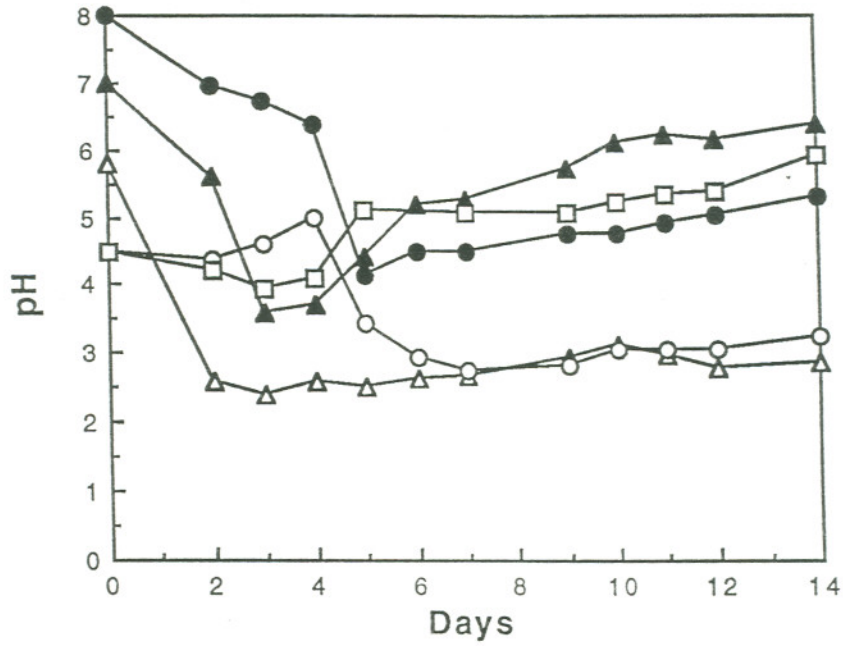

Fig. 3-6 pH changes in cellulose-degrading cultures of *P. chrysosporium*. The *P. chrysosporium* culture medium was buffered with succinate pH 4.5 (□), acetate pH 4.5 (o), phosphate pH 8 (●), phosphate pH 7 (▲), phosphate pH 5.8 (△).

Table 3-2 Effect of cellulosic substrates on enzyme activities, total protein and biomass production

Enzyme activity/ protein/biomass[a]	CL[b]	MCC[b]	FP[b]	AC[b]
CDH (U/l)	138 ± 5	75 ± 9	29 ± 5	35 ± 4
CBQase (U/l)	74 ± 10	90 ± 7	51 ± 12	9 ± 3
β-glucosidase (U/l)	237 ± 21	82 ± 16	155 ± 11	16 ± 3
Protein (mg/l)	939 ± 25	694 ± 54	583 ± 26	421 ± 39
biomass[c] (g)	1.4	1.4	2.0	1.1

[a] Values determined on the 14th day of culture growth.

[b] CL, cotton linter; MCC, microcrystalline cellulose; FP, filter paper; AC, acid-treated cellulose

[c] Biomass was derived from four cultures.

yielded similar amounts of biomass with the exception of filter paper-supplemented cultures. Filter paper cultures did not appear to have degraded all of the added substrate, and the increased biomass may have been due to undegraded cellulose.

3.3.8 Production of Lignin-Degrading Enzymes

The production of lignin-degrading enzymes, LiP and MnP, by cellulose-degrading cultures in the presence of varying amounts of cellulose and nutrient nitrogen was determined (Table 3-3). Three different culture conditions—high carbon-high nitrogen (HCHN, 1% cellulose + 12 mM dihydrogen ammonium phosphate), high carbon-low nitrogen (HCLN, 1% cellulose + 1.2 mM dihydrogen ammonium phosphate) (Tables 3-1, 3-2), and low carbon-low nitrogen (LCLN, 0.1% cellulose + 1.2 mM dihydrogen ammonium phosphate)—were investigated (Table 3-3). In these experiments, cotton linters were the carbon source and the medium contained 25 mM succinate, pH 4.5. HCHN cultures produced only CDH and CBQase. HCLN cultures produced CBQase, LiP, and MnP activities; but CDH activity was not detectable (Table 3-3). LCLN cultures, on the other hand, produced all the enzyme activities and the levels were also generally higher compared to HCLN cultures. Cellobiose-oxidizing enzyme activities were observable from day 5 to day 12 of culture growth. LiP and MnP activities were present from day 3 to day 7.

3.4 DISCUSSION

Many cellulolytic fungi, including the brown-rot fungus *Coniophora puteana*, produce extracellular cellobiose-oxidizing enzymes which oxidize cellobiose to cellobionolactone [Westermark & Eriksson, 1975; Ayers et al., 1978; Dekker 1980; Fahnrich & Irrgang, 1982; Sadana & Patil, 1985; Canevascini et al., 1991; Schmidhalter & Canevascini, 1993]. Among these, only CDH and CBQase produced by the lignocellulose-degrading white-rot basidiomycete *P. chrysosporium* are being studied in detail [Westermark & Eriksson, 1975; Ayers et al., 1978; Morpeth, 1985; Renganathan et al., 1990; Ander et al., 1990; Henriksson et al., 1991; Bao & Renganathan, 1992; Samajima & Eriksson, 1992; Wood & Wood, 1992; Bao et al.,

Table 3-3 Production of LiP, MnP, CDH and CBQase by HCLN and LCLN cultures of *P. chrysosporium*

Days	H C L N				L C L N			
	LiP (u/l)	MnP (u/l)	CDH (u/l)	CBQase (u/l)	LiP (u/l)	MnP (u/l)	CDH (u/l)	CBQase (u/l)
3	0	47 ± 23	0	0	73 ± 13	263 ± 117	0	0
4	0	17 ± 3	0	0	55 ± 32	66 ± 54	0	0
5	25 ± 3	74 ± 16	0	6 ± 6	14 ± 4	8 ± 2	8 ± 5	8 ± 3
6	9 ± 2	33 ± 13	0	7 ± 6	23 ± 13	24 ± 7	0	15 ± 4
7	0	10 ± 1	0	6 ± 6	0	5 ± 2	10 ± 4	5 ± 3
9	0	0	0	5 ± 1.5	0	0	6 ± 2	8 ± 1
10	0	0	0	0	0	0	4 ± 2	8 ± 4

[a] HCLN, high carbon-low nitrogen cultures.
[b] Lignin peroxidase activity was assayed with veratryl alcohol (0.5 mM) and H_2O_2 (0.1 mM) in 20 mM succinate at pH 3.
[c] Manganese peroxidase activity was determined by monitoring the formation of a Mn(III)-malonate complex at 270 nm in the presence of manganese sulfate (0.5 mM) in 50 mM malonate at pH 4.5.
[d] Cytochrome *c* reduction was monitored at 550 nm. Assays were performed in 20 mM succinate, pH 4.5, containing ferricytochrome *c* (12.5 μM) and cellobiose (100 μM).
[e] CBQase activity was determined by substraction of the DCPIP reduction activity due to CDH from the total DCPIP activity as described in Materials and Methods. DCPIP reduction was monitored by the decrease in absorbance at 515 nm. Reactions were performed in 20 mM succinate, pH 4.5, in the presence of DCPIP (25 μM) and cellobiose (100 μM).
[f] LCLN, low carbon-low nitrogen cultures.

1993]. In this report, the physiology of the production of cellobiose-oxidizing enzymes and β-glucosidase by *P. chrysosporium* is investigated. Specifically, the effects of various culture conditions—such as the initial pH, the type of cellulosic substrate, and the nitrogen concentration on the production of CDH, CBQase, protease, β-glucosidase, LiP, and MnP—are examined.

The initial pH and the buffering capacity of the culture medium greatly influence the production of various enzymes (Figs. 3-1—3-6). Cultures with a succinate buffer at an initial pH of 4.5 yield the highest amounts of CDH and β-glucosidase and the lowest levels of CBQase and protease (Figs. 3-1—3-6). Low levels of protease in the succinate cultures may be decreasing the hydrolysis of CDH to CBQase. Also, the pH of these cultures does not decrease below 4 during culture growth (Fig. 3-6). The medium composition used by various laboratories for the production of cellobiose-oxidizing enzymes has a phosphate buffer with an unadjusted pH of 5.8 [Ayers et al., 1978; Morpeth, 1985; Henriksson et al., 1991; Uzcategui et al., 1991]. Our study shows that phosphate pH 5.8 medium is unsuitable for cellulose-degrading enzyme production (Figs. 3-1—3-6, Table 3-1). Under these culture conditions, the medium pH decreases below 3, perhaps inhibiting the production of extracellular enzymes (Fig. 3-6). We have demonstrated previously that CDH is unstable below pH 3 and is converted to the inactive form with the loss of the flavin but not the heme cofactor [Bao et al., 1993]. CDH purified from phosphate pH 5.8 culture may be partially inactive due to the loss of the flavin [Ayers et al., 1978; Morpeth, 1985; Henriksson et al., 1991]. We recently reported the purification of CDH from phosphate pH 7 cultures [Bao et al., 1993]. The specific activity of CDH prepared from these cultures is several-fold higher than the values described previously [Henriksson et al., 1991; Wood & Wood, 1992]. The present study suggests that the method of CDH production, rather than the method of purification, is responsible for the low CDH-specific activity reported earlier [Morpeth, 1985; Henriksson et al., 1991].

A comparison of protease and CBQase production under different culture conditions suggests that the CBQase levels are proportional to the extracellular protease levels (Table 3-1). Succinate pH 4.5 cultures, which produce the lowest

levels of protease, have the highest levels of CDH and the lowest levels of CBQase. Under all other initial pH conditions examined, protease levels are two- to seven-fold higher than in the succinate pH 4.5 culture, and CBQase activity is also elevated in those cultures (Table 3-1). It has been proposed that CBQase is formed via proteolytic hydrolysis of CDH [Henriksson et al., 1991; Wood & Wood, 1992] and our observations further support this.

The effects of cotton linters, microcrystalline cellulose, filter paper, and acid-treated cellulose on CDH production also were examined. The cellulose structure of cotton linters and microcrystalline cellulose is predominantly crystalline, whereas the structure of acid-treated cellulose is completely amorphous [Hoshino et al., 1992]. Amorphous acid-treated cellulose produces the lowest amounts of extracellular protein and enzymes (Table 3-2). Cotton linter cultures produce the most protein and enzymes, and microcrystalline and filter paper-supplemented cultures yield intermediate amounts. All but the filter paper culture yield similar amounts of biomass (Table 3-2). Thus, the observed differences in the production of cellulose-degrading enzymes in cultures containing various cellulosic substrates may reflect the ability of various substrates to induce these enzymes.

The lignin-degrading enzymes, LiP and MnP, are produced by *P. chrysosporium* under nitrogen-limiting conditions [Glenn & Gold, 1985; Kirk & Farrell, 1987; Gold et al., 1989; Wariishi et al., 1992]. Reactions of these peroxidases are inhibited by CDH and CBQase in the presence of cellobiose [Ander et al., 1990; Samajima & Eriksson, 1992]. A proposed inhibition mechanism involves the reduction of free radical intermediates formed from peroxidase reactions by cellobiose-oxidizing enzymes [Ander et al., 1990; Samajima & Eriksson, 1992]. The generation of LiP and MnP by cellulolytic cultures in the presence of varying nitrogen concentrations is examined here. HCHN cultures produce high levels of cellobiose-oxidizing enzymes; however, LiP and MnP activities are not detectable [data not shown]. HCLN and LCLN cultures produce low levels of both lignin-degrading and cellobiose-oxidizing enzymes. However, LCLN cultures express higher levels of these enzymes than HCLN cultures (Table 3-3). Thus, this study demonstrates that LiP, MnP, CDH, and CBQase can be expressed simultaneously by *P. chrysosporium*

under nutrient nitrogen-limiting conditions. This provides the physiological evidence that CDH could be involved in lignin degradation. Earlier, Kelleher et al. [1987] reported the production of low levels of CBQase by HCLN cultures of *P. chrysosporium*. Recently, Costa-Ferreira et al. [1992] observed the production of low levels of CBQase and lignin-degrading peroxidases by HCHN cultures of *P. chrysosporium* ME-446 in the presence of Tween-80.

In summary, this study demonstrates that a succinate medium with an initial pH of 4.5 and with cotton linters as the cellulose source, is most suitable for production of CDH and β-glucosidase from *P. chrysosporium*. Physiological conditions which favor CDH synthesis also appear to favor β-glucosidase production, suggesting that expression of these two enzymes may be coordinately controlled. The optimized culture conditions identified here should permit isolation of milligram quantities of CDH and β-glucosidase from *P. chrysosporium*. Furthermore, this study shows that the culture conditions favoring protease production decrease CDH yield but increase CBQase yield. This observation strongly suggests that CBQase is generated through the proteolytic hydrolysis of CDH.

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CHAPTER 4

CELLOBIOSE DEHYDROGENASE OF *PHANEROCHAETE CHRYSOSPORIUM* ENHANCES MICROCRYSTALLINE DEGRADATION BY CELLULASES

4.1 INTRODUCTION

Cellulose constitutes 40-60% of woody plant cell walls, and its bioconversion into fuels and chemicals is of great interest [Philips & Humphrey, 1983]. The cellulase systems of several fungi have been studied intensively [Klyosov, 1990]. Some cellulolytic fungi produce extracellular cellobiose-oxidizing enzymes in addition to cellulases [Ayers et al., 1978; Canevascini et al., 1991; Dekker, 1980; Morpeth, 1985; Sadana & Patil, 1985; Westermark & Eriksson, 1975]. However, the role of these enzymes in cellulose-degradation is not understood. *Phanerochaete chrysosporium* produces two cellobiose-oxidizing enzymes: cellobiose dehydrogenase (CDH) and cellobiose:quinone oxidoreductase (CBQase) [Ayers et al., 1978; Westermark & Eriksson, 1975]. CDH is a hemoflavoprotein. In the absence of suitable electron acceptors, oxygen serves as a poor electron acceptor for CDH [Bao et al., 1993], whereas CBQase is a flavoprotein and requires a quinone for activity [Westermark & Eriksson, 1975]. Both enzymes oxidize cellobiose to cellobionolactone. Recently, cellobiose dehydrogenase from *Sporotrichum (chrysosporium) thermophile* has also been demonstrated to be a hemoflavoenzyme [Canevascini et al., 1991]. We demonstrated that CDH binds to microcrystalline cellulose and suggested that it may be organized into two domains: a cellulose-binding domain and a catalytic domain [Renganathan et al., 1990]. Previously only cellulases were known to have this structural organization [Tomme et al., 1988; Gilkes et al., 1988]. In this study, the effect of CDH addition on *Trichoderma viride* and *Trichoderma reesei* cellulase-catalyzed hydrolysis of microcrystalline cellulose is

investigated. Our findings suggest that CDH enhances crystalline cellulose hydrolysis by these cellulases.

4.2 MATERIALS AND METHODS

4.2.1 Cellobiose Dehydrogenase

P. chrysosporium strain OGC 101 [Alic et al., 1987] was grown with cotton linters (10 g/l) in agitated cultures as described previously [Renganathan et al., 1990]. CDH was purified to homogeneity from the extracellular medium by a procedure involving ammonium sulfate precipitation and DEAE-Sephadex, phenyl Sepharose, Sephacryl S-200 and Mono-Q (FPLC) chromatography [Bao et al., 1993]. The specific activity of the purified enzyme, as determined with ferricytochrome *c* as the electron acceptor, was 9.7 U/mg.

4.2.2 Estimation of Glucose, Cellobiose and Cellobionolactone

Glucose was estimated by glucose-6-phosphate dehydrogenase assay [Schachter, 1975]. The sample containing glucose (10 μl) was incubated with ATP (1 μmole), $MgCl_2$ (1 μmole), hexokinase (0.3 U) and TRIS-HCl (25 μmole) in a total volume of 0.3 ml at 37°C for 15 min. The reaction was arrested by incubating the sample at 100°C for 2 min. The heat-treated sample was then incubated with NADP (0.5 μmole), $MgCl_2$ (20 μmole), TRIS-HCl (100 μmole) and glucose-6-phosphate dehydrogenase (0.5 U) in a total volume of 2 ml at 37°C for 30 min. The amount of NADPH formed was determined spectrophotometrically at 340 nm. The concentration of glucose was determined from a standard curve.

Cellobiose concentration was estimated using a CDH assay. A sample (5 μl) containing cellobiose was incubated with CBO (1 μg, 0.01 U) and cytochrome *c* (37.5 μM) in 20 mM succinate, pH 4.5, at room temperature for 30 min. The amount of reduced cytochrome *c* formed was determined at 550 nm. Cellobiose concentration was determined from a standard curve.

Cellobionolactone was estimated by modification of a previously reported method [Jones et al., 1975]. The lactone-containing solution (50 μl) was reacted with

25 mM periodate in 0.35% H_2SO_4 solution (0.1 ml) at room temperature for 30 min. Sodium metabisulfite (1 M, 50 μl) was then added to decompose excess periodate. After 5 min, 0.1 ml of 2,3,4-trihydroxybenzoic acid (10 mg/m) was added, followed by 2.7 ml of concentrated H_2SO_4. Finally, 20 μl each of ammonium sulfate (0.5 g/ml) and ferric nitrate (10 mg/ml) was added and the solution was heated at 40°C for 30 min. The blue color which developed was measured at 590 nm.

4.2.3 Microcrystalline Cellulose Hydrolysis by *Trichiderma* Cellulases—Effect of CDH

Microcrystalline cellulose (20 mg, Sigmacell Type 50) was incubated with crude, dialyzed cellulase from *T. viride* or *T. reesei* (0.5 mg) in 2.5 ml of 50 mM acetate buffer, pH 5, at 28°C with shaking (150 rpm) for 18 h. Tetracycline (10 μg/ml) was included in these incubations to inhibit bacterial growth. At the end of the incubation, the reaction was stopped by heating the reaction mixture at 100°C for 2 min, and the reaction mixture was then centrifuged at 12,000×g. The amounts of glucose, cellobiose and cellobionolactone in the supernatant were determined. The conditions for determining the effect of CDH on cellulose hydrolysis were identical except that 5-80 μg CDH were added.

For determining the cellulose weight loss, cellulose (600 mg) was incubated with crude *T. viride* cellulase (6 mg) and 0, 0.3 or 1.8 mg CDH in 30 ml 50 mM acetate, pH 5, as described above. The catalase concentration, when added to these incubations, was 0.15 mg. At the end of the incubation, the reaction mixture was filtered through a pre-weighed Buchner funnel with a medium porosity, fritted disk to remove the unhydrolyzed cellulose. The funnel containing the cellulose was dried in an oven at 60°C overnight, cooled for 1 h, and weighed to determine the cellulose weight loss.

4.2.4 Chemicals

All chemicals were reagent grade. Cytochrome *c*, microcrystalline cellulose (Sigmacell Type 50), and cellulases were purchased from Sigma Chemical Company,

St. Louis, MO. 2,3,4-Trihydroxybenzoic acid was obtained from Aldrich Chemical Company, Ronkonkoma, NY.

4.3 RESULTS

Low concentrations of CDH (5-10 μg/ml) enhanced glucose and cellobiose generation in *Trichoderma* cellulase catalyzed hydrolysis of microcrystalline cellulose (Fig. 4-1). When CDH (10 μg/ml) was added to *T. viride* cellulase, glucose production was increased by approximately 10%, whereas cellobiose yield was increased by 48% (Fig. 4-1A). Similarly, glucose and cellobiose production by *T. reesei* cellulase increased by approximately 10% and 20%, respectively, in the presence of low concentrations of CDH (Fig. 4-1B). However, at higher concentrations of CDH, reducing-sugar production by *T. viride* and *T. reesei* cellulases decreased (Fig. 4-1). This decrease was due to the CDH-catalyzed oxidation of cellobiose to cellobionolactone.

Cellobionolactone was estimated using an extensive modification of a previously reported colorimetric method [Jones, 1975]. This method is not specific for cellobionolactone in that other sugar lactones, such as gluconolactone, also give a positive response. The crude cellulase preparations used in these experiments possibly contained glucose oxidase as a contaminant resulting in the generation of glucanolactone as one of the cellulose degradation products. Therefore, to determine the amount of cellobionolactone formed with CDH, lactone produced in the absence of CDH was subtracted from the total lactone formed in the presence of CDH. In the *T. viride* cellulase reaction, cellobionolactone generation increased slowly between 10 and 40 μg of added CDH, and rapidly above the 40-μg level (Fig. 4-1A). In the case of *T. reesei*, cellobionolactone yield increased linearly as the CDH concentration in the reaction was increased (Fig. 4-1B).

The effect of CDH (10μg or 60 μg/ml) addition on cellulose weight loss was also determined (Table 4-1). In the presence of 10 μg/ml CDH, cellulose weight loss was increased by approximately 19%. Inclusion of 5 μg/ml catalase in this reaction further increased the weight loss to 27-28%. Catalase alone, in the absence of CDH,

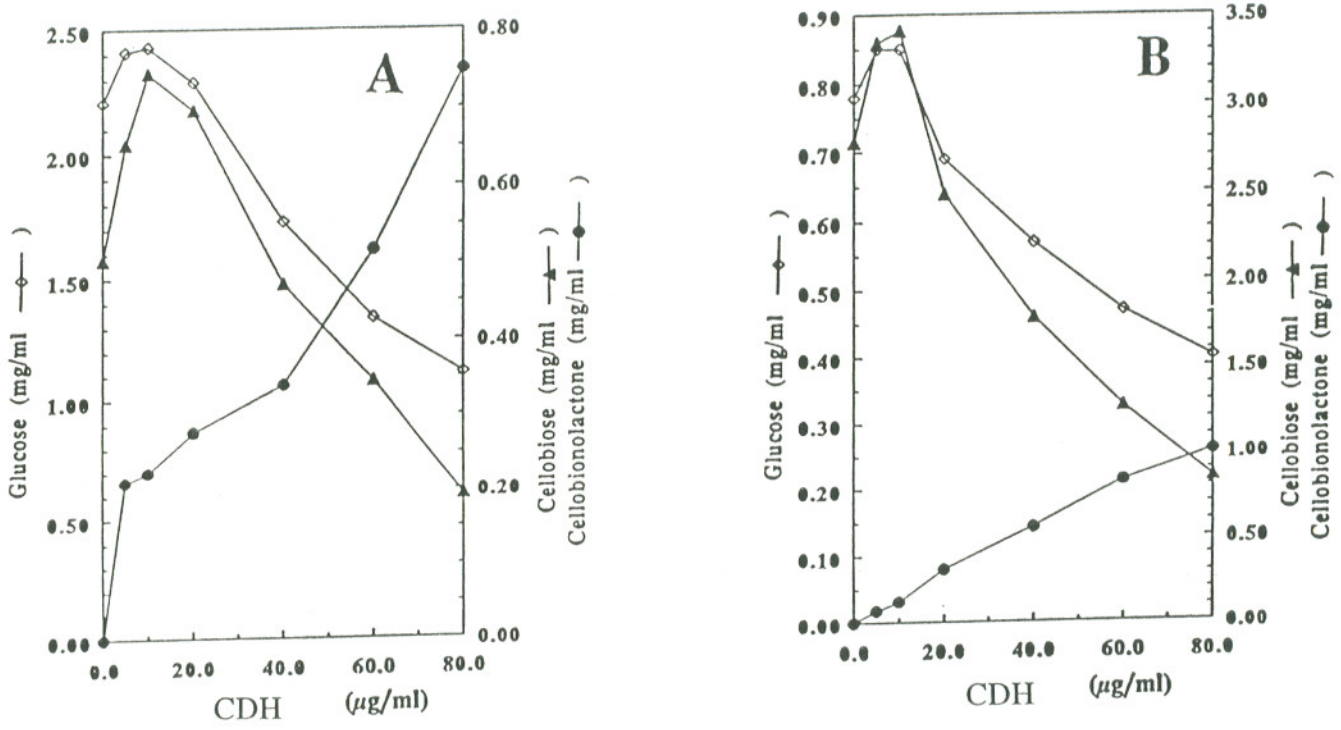

Fig. 4-1 *T. viride* (A) and *T. reesei* (B) cellulase-catalyzed conversion of microcrystalline cellulose to glucose (◇), cellobiose (▲) and cellobionolactone (●) in the presence of varying concentrations of CDH.

Table 4-1 Effect of CDH and/or catalase addition on microcrystalline cellulose weight loss[a]

Enzyme	Weight loss (mg)	Percentage weight loss relative to control[c]
Cellulase (control)[b]	91.5 ± 1.0	100% ±1.1%
Cellulase + CDH (10 μg/ml)	108.5 ± 0.6	118.6% ± 0.6%
Cellulase + CDH (60 μg/ml)	69.5 ± 0.1	76.0% ± 0.1%
Cellulase + CDH (10 μg/ml) + catalase (5μg/m)[d]	109.2 ± 2.3	119.3% ± 2.1%
Cellulase + CDH (60 μg/ml) + catalase (5μg/ml)[d]	86.7 ± 0.2	94.8% ± 0.2%

[a] Cellulase from *T. viride* (0.3 mg) was incubated with 600 mg microcrystalline cellulose in the presence of CDH and/or catalase in 50 mM acetate, pH 5.0, in a total volume of 30 ml. Weight loss was determined as described in Section 2.

[b] Cellulase is from *T. viride*.

[c] Cellulose weight loss observed in the absence of CDH and catalase was considered to be 100%.

[d] In a separate experiment, cellulose weight loss for the control in the presence of 5 μg/ml catalase was determined to be 97.8% ± 0.9%.

increased the weight loss by 3%. At the 60 μg/ml-CDH level, the weight loss was equal to that of the control cellulase incubations, and catalase further increased this weight loss to 21%.

4.4 DISCUSSION

Although Ayers et al. first discovered CDH in the cellulose-degrading cultures of *P. chrysosporium* in 1978 [Ayers et al., 1978], the role of CDH in cellulose degradation is still not well understood. One approach to determining the contribution of CDH to cellulose degradation is to study the effect of CDH addition on cellulose hydrolysis by cellulolytic systems such as those from *T. viride* and *T. reesei* which apparently do not contain CDH. Following such an approach, we have established that CDH enhances crystalline cellulose degradation by *Trichoderma* cellulases (Fig. 4-1, Table 4-1). At low concentrations (5-10 μg/ml), CDH increases both glucose and cellobiose production (Fig. 4-1). However, at higher levels, reducing-sugar generation decreases and cellobionolactone yield increases. Cellulose weight loss experiments suggest that, in the presence of 10 μg/ml CDH, cellulose weight loss is increased by 19% compared to the control cellulase reactions (Table 4-1). However at 60 μg/ml CDH, the cellulose weight loss is reduced about 24% compared to that of the control. CDH produces H_2O_2 during its catalytic cycle [Bao et al., 1993] and the oxy-radicals resulting from H_2O_2 may inactivate the cellulases. Therefore, the effect of catalase addition on cellulose weight loss was examined. Although, catalase did not have any effect on microcrystalline cellulose weight loss at 10 μg CDH, it significantly enhanced weight loss at 60 μg CDH.

In enzymatic cellulose hydrolysis, the synergistic actions of endocellulase and exocellobiohydrolase hydrolyze cellulose to cellobiose [Philips & Humphrey, 1983; Klyosov, 1990]. β-Glucosidase then hydrolyzes cellobiose to glucose. Cellobiose is the inhibitor of cellobiohydrolase [Enari & Niku-Paavola, 1987]. When a low concentration of CDH is included in a cellulase reaction, it oxidizes cellobiose to cellobionolactone which releases cellobiose inhibition and, thus, enhances cellulose degradation. At a higher concentration of CDH, both H_2O_2 and cellobionolactone are

produced much more compared to their production at a low level of CDH addition. H_2O_2 and the oxy-radicals inactivate cellulases. Cellobionolactone is a inhibitor of cellobiose dehydrogenase (Fig. 4-2). Thus, at a higher concentration of CDH, cellulose degradation by cellulases is reduced. The lignin-degrading system of *P. chrysosporium* contains two important peroxidases which require H_2O_2 as a substrate [Kirk & Farrell, 1987; Gold et al., 1989]. During lignocellulose-degradation, H_2O_2 produced via CDH may be consumed by lignin-degradation reactions, thus avoiding H_2O_2-dependent cellulase inactivation.

Hydrolysis of the crystalline structure of cellulose has been recognized to be rate-limiting in the saccharification of cellulose to glucose [Ohmine et al., 1983]. Findings presented in this report suggest that at low, perhaps physiologically relevant concentrations, CDH enhances cellulose degradation by cellulases and this may be the physiological function of CDH. Further experiments, aimed at understanding the interaction of CDH and *P. chrysosporium* cellulase in cellulose degradation and the mechanism of CDH-dependent enhancement of cellulose hydrolysis, are planned.

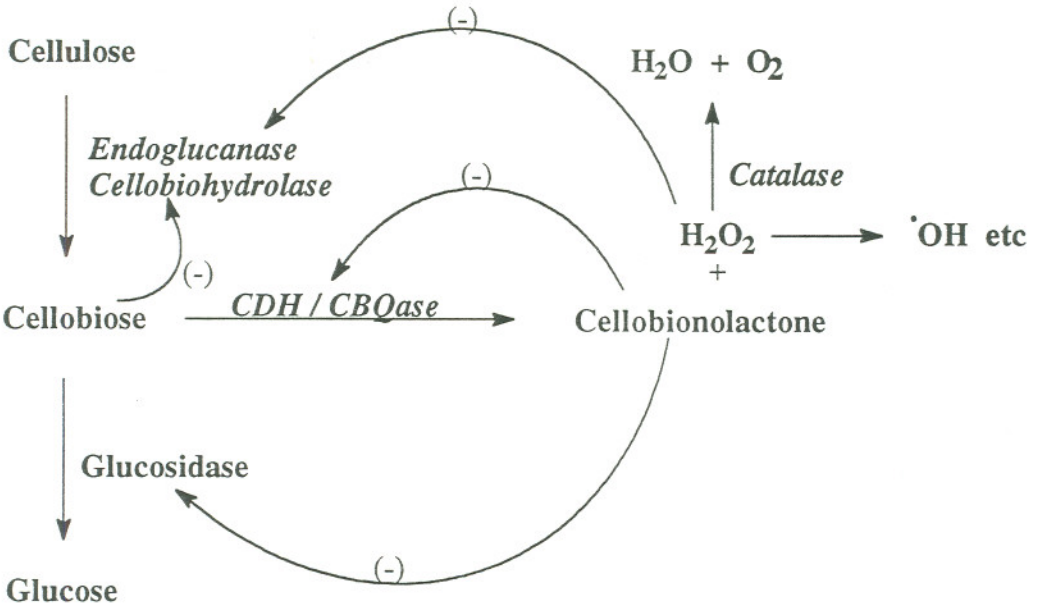

Fig. 4-2 CDH effects on cellulose degradation by cellulases.

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CHAPTER 5
EFFECT OF CELLOBIOSE DEHYDROGENASE ON DIFFERENT TYPES OF CELLULOSE DEGRADATION BY *TRICHODERMA VIRIDE* CELLULASES

5.1 INTRODUCTION

Although cellobiose dehydrogenase (CDH) was first isolated in 1978 [Ayers et al., 1978], the role of CDH in cellulose degradation by cellulase is not understood. One way to understand the role of CDH in cellulose degradation is to study the effect of CDH on cellulose hydrolysis by cellulases which do not contain CDH activity. We chose *Trichoderma* cellulases since they are well studied and apparently do not have CDH activity [Eriksson et al., 1990; Wood, 1989]. It was demonstrated that CDH enhances microcrystalline cellulose hydrolysis by cellulase from *Trichoderma* [Chapter 4; Bao & Renganathan, 1992]. Here, CDH was purified from a succinate medium with an initial pH of 4.5. Four types of cellulose—microcrystalline cellulose, cotton linter, filter paper, and acid-treated cellulose—were studied as hydrolysis substrates. The ability of CDH to bind to these celluloses was also examined. Our results suggest that CDH can significantly enhance microcrystalline cellulose hydrolysis by cellulase. It only slightly enhances cotton linter, filter paper and acid-treated cellulose hydrolysis. The possible relationship between the ability of CDH to bind to cellulose and its ability to enhance cellulose degradation is discussed.

5.2 MATERIALS AND METHODS

Most materials and methods, such as culture maintenance, CDH assay, glucose and cellobiose estimation, and weight loss determination, are the same as described in Chapter 4 except those mentioned below.

5.2.1 Cellobiose Dehydrogenase

Cellobiose dehydrogenase was produced in a succinate culture with an initial pH of 4.5 as described in Chapter 3. CDH was purified to homogeneity as previously reported [Bao et al., 1993; also Chapter 2] except that DEAE-Sephadex chromatography was omitted. CDH activity was assayed by cytochrome *c* methods as described in Chapter 2.

5.2.2 CDH Effect on Cellulose Degradation by *T. viride* Cellulase

The cellulose examined included microcrystalline cellulose (Sigmacell Type 50), cotton linter, filter paper (Whatman #1), and acid-treated microcrystalline cellulose. Preparation of acid-treated microcrystalline cellulose was described in Chapter 2. *T. viride* cellulase was dialyzed against a sterilized 50 mM acetate buffer. Cellulose (20 mg/ml) was incubated with cellulase (0.2 mg/ml) from *T. viride* in 3 ml sterilized 50 mM sodium acetate buffer, pH 5.0. The reaction mixtures were shaken (150 rpm) at 28°C for 18 h. Tetracycline (10 μg/ml) was added to the reaction to inhibit bacterial growth. To investigate the effect of CDH on cellulose degradation, 0.2-5 μg/ml of CDH was added to estimate the amount of glucose and cellobiose product. Triplicate samples were used in each experiment. At the end of the reaction, the samples were heated in boiling water for 2 minutes to stop the reaction, and then the amount of glucose and cellobiose in the supernatant was estimated [Bao & Renganathan, 1992].

To determine the cellulose weight loss, cellulose (600 mg) was incubated with cellulase (6 mg) and CDH (0.5 μg/ml) in 30 ml 50 mM acetate buffer, pH 5, as described in Chapter 4. After shaking for 18 h at 28°C, the reaction mixture was filtered through a pre-weighted Buchner funnel to separate undegraded cellulose. The

funnel containing cellulose was dried in an oven at 60°C, cooled for 1 h and weighed to determine the cellulose weight loss. Quadruplicate samples were used in each experiment.

5.2.3 CDH Binding to Cellulose

Cellulose (200 mg) was equilibrated with 50 mM acetate buffer, pH 5.0, and loaded in a column (0.5 cm diameter). CDH (0.38 units) was added to the column which was then washed with acetate buffer to elute unbound CDH. CDH activity was determined by cytochrome *c* assay.

5.2.4 Protein Estimation

Protein was estimated by the bicinchoninic acid method [Smith et al., 1985].

5.2.5 Chemicals

All chemicals were reagent grade. Cytochrome *c*, microcrystalline cellulose (Sigmacell Type 50) and cellulase were purchased from Sigma Chemical Company, St. Louis, MO. Cotton linter was purchased from Fluka Chemical Co., Ronkonkoma, NY.

5.3 RESULTS

In the presence of low concentrations of CDH_{succ} (0.2-1.0 μg/ml), cellulose degradation by *T. viride* cellulase yielded more glucose and cellobiose (Fig. 5-1). However, a higher concentration of CDH_{succ} decreased both glucose and cellobiose production (Fig. 5-1). When 0.5 μg/ml CDH_{succ} was added to *T. viride* cellulase, glucose generation was increased about 10% while cellobiose production was increased about 36% in microcrystalline cellulose hydrolysis. But when cotton linter, filter paper and acid-treated cellulose were the hydrolysis substrates, cellobiose yield was only enhanced by 14%, 1% and 8%, respectively, and the glucose yield remained almost the same compared to the control reaction. Similarly, microcrystalline cellulose weight loss was enhanced about 16% by the addition of CDH (0.5μg/ml).

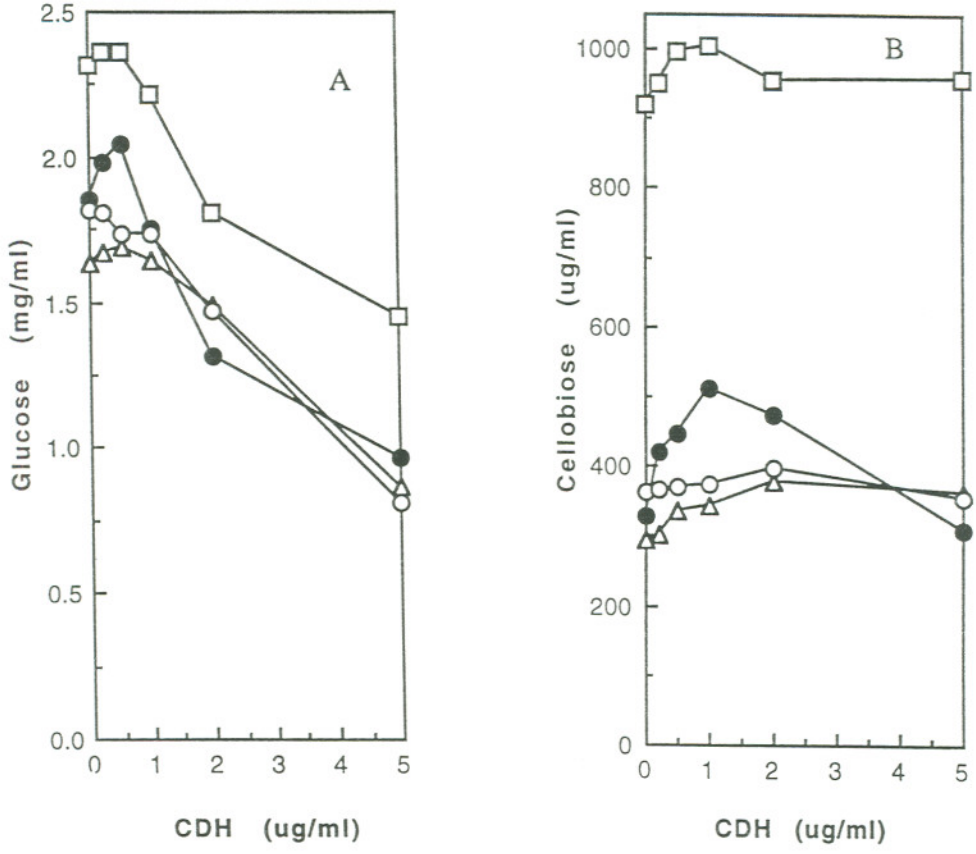

Fig. 5-1 CDH effects on glucose and cellobiose production by microcrystalline cellulose (●), cotton linter (▵), filter paper (o) and acid-treated cellulose (□) degradation by *T. viride* cellulases. (A) glucose production, (B) cellobiose production.

Only a small increase in weight loss (0.3-4.2%) was observed with cotton linter, filter paper or acid-treated cellulose (Table 5-1).

CDH binding to different types of cellulose was also studied. CDH bound very tightly to the microcrystalline cellulose column and no activity was detected in the elution buffer. CDH did not bind to acid-treated cellulose, and all CDH activity was eluted from the column with acetate buffer. About 45% and 36% of CDH activity was bound to cotton linter and filter paper, respectively.

5.4 DISCUSSION

Earlier we purified *P. chrysosporium* CDH from phosphate cultures with an initial pH of 7 [Bao et al., 1993; also Chapter 2]. Our later study on the production of CDH by cellulolytic cultures of *P. chrysosporium* showed that succinate cultures with an initial pH of 4.5 produced more CDH than other conditions we examined [Bao et al., 1994; Chapter 2]. In the present study, CDH was purified from succinate cultures. CDH from succinate cultures (CDH_{succ}) had the same molecular weight (90,000), pI (4.1) and specific activity by cytochrome *c* assay (10 units/mg) as CDH from phosphate cultures (CDH_{phos}). But CDH_{succ} had a higher carbohydrate content (17%) than that of CDH_{phos} (9.4%) (Table 5-2). CDH turnover with oxygen as the electron acceptor could be conveniently monitored by the change in absorbance at 564 nm due to the reduced CDH [Bao et al., 1993; Chapter 2]. Upon addition of cellobiose to CDH, the 564-nm absorbance increased and remained at that level for some time. The time at which the 564-nm absorbance starts to decrease is referred to as turnover time. When 50 μM cellobiose was added to 0.7 μM CDH, the aerobic turnover time of CDH_{succ} was <1 min, whereas the turnover time of CDH_{phos} was >10 min (Table 5-2). The reason for this difference in turnover time is not understood.

Different amounts of CDH from two culture conditions are needed to enhance cellulose degradation by cellulases. Earlier we reported that when 10 μg/ml CDH_{phos} was added to *T. viride* cellulases, microcrystalline cellulose weight loss increased 19%. Production of glucose and cellobiose increased 10% and 48%, respectively.

Table 5-1 Effect of CDH on different types of cellulose[a] degradation by *T. viride* cellulase

Reaction mixture	Weight loss (mg)	Percentage weight loss relative to control[f]	% CDH binding to cellulose
1A. MCC[b] + CLA(control)	99.2 ± 2.7	100% ± 2.7%	-
1B. MCC + CLA + CDH	114.9 ± 1.4	115.8% ± 1.2%	100
2A. CL + CLA(control)	100.5 ± 1.9	100% ± 1.9%	-
2B. CL + CLA + CDH	104.7 ± 0.9	104.2% ± 0.9%	45
3A. FP + CLA(control)	101.9 ± 1.2	100% ± 1.2%	-
3B. FP + CLA + CDH	102.2 ± 2.5	100.3% ± 2.4%	36
4A. ATC + CLA(control)	291.6 ± 3.1	100% ± 1.1%	-
4B. ATC + CLA + CDH	300.5 ± 2.8	103.3% ± 0.9%	0

[a] Cellulase from *Trichoderma viride* (6 mg) and CDH (15 μg) were incubated with 600 mg cellulose in 50 mM acetate, pH 5, in a total volume of 30 ml.

[b] Abbreviations used: MCC, microcrystalline cellulose; CL, cotton linter; FP, filter paper; ATC, acid-treated cellulose; CLA, cellulase from *Trichoderma viride*.

[c] CDH (0.38 U) was loaded on 200 mg cellulose and washed with 50 mM acetate buffer pH 5.0. CDH activity was determined by cytochrome *c* assay.

Table 5-2 Comparison of the properties of CDH_{phos} to CDH_{succ}

	CDH_{phos}[a]	CDH_{succ}
Molecular weight	90,000	90,000
Isoelectric focusing[b]	4.1	4.1
Specific activity	10.3	10
Turnover time (min)[c]	> 10	<< 1
% Carbohydrate content	9.4%	17%

[a] Data from Bao et al., 1993.

[b] Data from this work.

[c] CDH (0.7 μM) was mixed with cellobiose (50 μM) in 20 mM succinate buffer, pH 4.5. The time at which 564-nm absorbance started to decrease was treated as the turnover time.

When a higher concentration of CDH (60 μg/ml) was added to cellulases, microcrystalline cellulose weight loss was decreased 24% [Bao & Renganathan, 1992; Chapter 4]. Intriguingly, at 10 μg/ml concentration, CDH_{succ} decreased microcrystalline cellulose weight loss by 38% (Table 5-3). A similar effect on cotton linter, filter paper and acid-treated cellulose weight loss at 10 μg/ml CDH_{succ} was observed [data not shown]. In these reactions, O_2 serves as an electron acceptor for CDH, and O_2 itself is reduced to H_2O_2. Preliminary characterization of CDH_{succ} suggests that its turnover with O_2 as an electron acceptor is faster than CDH_{phos}. Thus CDH_{succ} produces higher levels of H_2O_2 and cellobionolactone. H_2O_2 inactivates cellulase, and cellobionolactone is an inhibitor of β-glucosidase and perhaps other cellulases.

Among the different cellulosic substrates used in this study, microcrystalline cellulose, cotton linter and filter paper are predominantly crystalline [Finch & Roberts, 1985], whereas the phosphoric acid-treated cellulose is amorphous [Walseth, 1952]. The degree of polymerization of crystalline substrates is 7,000 or more whereas the degree of polymerization of acid-treated cellulose is 150-200 [Finch & Roberts, 1985]. Only microcrystalline cellulose hydrolysis is significantly enhanced by CDH (Table 1-1). Interestingly, CDH binds strongly only to microcrystalline cellulose.

As for the mechanism of CDH-enhanced cellulose hydrolysis, by oxidizing cellobiose to cellobionolactone CDH may be relieving cellobiose inhibition on cellulase (Fig. 4-2; also chapter 4). Also, the cellulose binding characteristic of CDH locates it in the vicinity of the cellulases. Such an arrangement of cellulases and CDH could help maintain the microenvironment of cellulases devoid of cellobiose even at low concentrations of CDH (Fig. 5-2). It may be the reason why CDH enhancement of cellulose weight loss is apparently related to its ability to bind to cellulose.

Table 5-3 Comparison of the effect of CDH_{phos} and CDH_{succ} microcrystalline cellulose weight loss

	% weight loss	
Enzymes	CDH_{phos}	CDH_{succ}
Cellulase(control)[a]	100% ± 1.1%	100% ± 2.7%
Cellulase + CDH (0.5 μg/ml)	N.D.[b]	115.8% ± 1.2%
Cellulase + CDH (10 μg/ml)	118.6% ± 0.6%	62.6% ± 0.2%
Cellulase + CDH (60 μg/ml)	76% ± 0.1%	N.D.[b]

[a] Cellulase from *Trichoderma viride* (0.3 mg) was incubated with 600 mg microcrystalline cellulose in 50 mM acetate buffer, pH 5.0, in the presence of tetracycline (10 μg/ml). The total volume was 30 ml.

[b] Not determined.

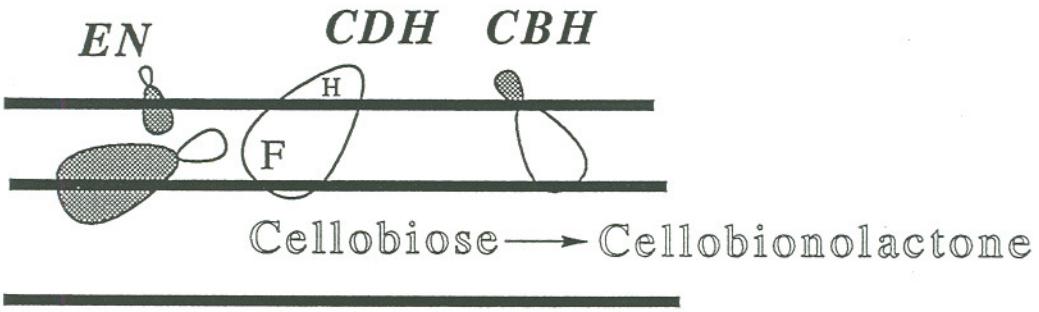

Fig. 5-2 Correlation between CDH affinity towards cellulose and its ability to enhance cellulose degradation by cellulases. EN, endoglucanase; CBH, cellobiohydrolase; CDH, cellobiose dehydrogenase; H, heme domain; F, flavin domain.

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CHAPTER 6
TRIIODIDE REDUCTION BY CELLOBIOSE:QUINONE OXIDOREDUCTASE OF *PHANEROCHAETE CHRYSOSPORIUM*

6.1 INTRODUCTION

White-rot basidiomycetous fungi are the only organisms known to be capable of degrading both lignin and cellulose to CO_2 and H_2O [Kirk & Farrell, 1987; Gold et al., 1989]. Cellulose-degrading cultures of the white-rot fungus *Phanerochaete chrysosporium* produce two extracellular oxidative enzymes, cellobiose:quinone oxidoreductase (CBQase) and cellobiose dehydrogenase, in addition to cellulases [Westermark & Eriksson, 1975; Ayers et al., 1978]. Both enzymes oxidize cellobiose to cellobionolactone. CBQase is a flavoenzyme and requires a quinone for activity [Westermark & Eriksson, 1975], whereas cellobiose dehydrogenase is a hemoflavoenzyme [Ayers et al., 1978]. CBQase reduces quinones to hydroquinones in the presence of cellobiose [Westermark & Eriksson, 1975]. Ligninolytic cultures of *P. chrysosporium* produce two extracellular peroxidases: lignin peroxidase (LiP) and manganese peroxidase (MnP) [Kirk & Farrell, 1987; Gold et al., 1989; Tien & Kirk, 1984; Gold et al., 1984; Glenn & Gold, 1985]. CBQase, in the presence of cellobiose, inhibits peroxidase-catalyzed decarboxylation of vanillic acid and LiP-catalyzed oxidation of 3,4-dimethoxybenzyl alcohol and polymerization of kraft lignin [Ander et al., 1990]. Ander et al. [1990] have proposed that CBQase inhibits these reactions through the reduction of phenoxy and aromatic cation radical intermediates. However, Odier et al. [1988] have suggested that CBQase does not reduce phenoxy radical intermediates. The mechanism of CBQase inhibition of peroxidase reactions requires further study.

Peroxidase reactions involve initial oxidation of the native enzyme by H_2O_2 to produce compound I which has two oxidizing equivalents more than the native enzyme [Dunford, 1982]. Donation of an electron from a substrate, such as a phenol, reduces compound I to II, and the addition of a second electron to compound II regenerates the native peroxidase [Dunford, 1982]. CBQase inhibition of peroxidase reactions requires cellobiose, and this suggests the involvement of reduced CBQase [Ander et al., 1990]. Electron transfer from the reduced CBQase to peroxidase compounds I and II would also inhibit peroxidase reactions because the peroxidase intermediates would not be available for substrate oxidation. Such a mechanism will inhibit all peroxidase reactions including the reactions that do not produce radical intermediates. To test this suggestion, we studied the effect of CBQase on the peroxidase catalyzed oxidation of iodide to I_3^-, which apparently involves only ionic rather than radical intermediates [Roman & Dunford, 1972]. CBQase, in the presence of cellobiose, inhibited iodide oxidation. However, this inhibition appears to be due to the two-electron reduction of I_3^- by CBQase and not to the reduction of peroxidase compounds I and II.

6.2 MATERIALS AND METHODS

6.2.1 CBQase Purification

P. chrysosporium strain OGC101 was grown with cotton linters (10 g/l) in agitated cultures as described previously [Renganathan et al., 1990]. CBQase was purified to homogeneity from the extracellular medium by a procedure involving ammonium sulfate precipitation, DEAE-Sephadex, Phenyl Sepharose, Sephacryl S-200 and Mono-Q (FPLC) chromatographic procedures (Bao and Renganathan, unpublished results). The specific activity of CBQase with dichlorophenol-indophenol (DCPIP) as an electron acceptor was 8 U/mg.

6.2.2 Peroxidases

LiP and MnP were purified from the extracellular medium of lignin-degrading cultures of *P. chrysosporium* as described previously [Gold et al., 1984; Glenn &

Gold, 1984; Wariishi and Gold, 1989; Wariishi & Gold, 1990]. HRP Type VI was purchased from Sigma Chemical Company, St. Louis, MO.

6.2.3 Enzyme Assays

CBQase was assayed with cellobiose (100 μM) and DCPIP (25 μM) at 515 nm (ϵ = 6.8 mM^{-1} cm^{-1}) in 20 Mm succinate buffer, pH 4.5 [Renganathan et al., 1990]. LiP activity was determined with 3,4-dimethoxybenzyl alcohol and H_2O_2 at 310 nm [Tien & Kirk, 1984; Gold et al., 1984]. MnP activity was assayed with 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and H_2O_2 at 415 nm in 20 mM lactate, pH 4.5 [Glenn & Gold, 1985].

6.2.4 Iodide Oxidation and Triiodide Reduction

Peroxidase-catalyzed oxidation of iodide and CBQase-catalyzed reduction of I_3^- were monitored by following the absorbance changes at 355 nm [Roman & Dunford, 1972]. Peroxidase assays contained iodide (1 mM), H_2O_2 (100 μM), and cellobiose (100 μM). CBQase assays contained cellobiose (100 μM), I_3^- (50 μM), and iodide (950 μM). All the reactions were performed in 20 mM succinate buffer, pH 4.5.

Triiodide solution was prepared by dissolving iodine in a potassium iodide solution with the aid of ultrasonication (30-120 sec).

Kinetics of CBQase reduction of I_3^- were performed at a fixed concentration of cellobiose (100 μM) and iodine (50 μM) and at varying concentrations of I_3^- solution (5 mM iodine in 2.5 M iodide solution). K_m and V_{max} were determined from double reciprocal plots of the substrate concentration (I_3^-) versus the initial velocity.

6.3 RESULTS AND DISCUSSION

CBQase is a flavin-dependent dehydrogenase present in the extracellular medium of cellulose-degrading cultures of the white-rot basidiomycete *P. chrysosporium* [Westermark & Eriksson, 1975]. CBQase oxidizes cellobiose to cellobionolactone in the presence of electron acceptors such as quinones and DCPIP [Westermark & Eriksson, 1975; Renganathan et al., 1990]. Peroxidases, such as

HRP, LiP, and MnP, oxidize iodide to I_3^- in the presence of H_2O_2 [Roman & Dunford, 1972; Renganathan et al., 1987; Valli and Gold, personal communication]. In this reaction, iodide reduces compound I by two electrons to produce the native peroxidase and hypoiodous acid or an iodonium ion ($HOI \rightleftharpoons OH^- + I$) [Roman & Dunford, 1972]. The latter complexes with two equivalents of iodide to yield I_3^-. CBQase, in the presence of cellobiose, inhibited the LiP-, MnP-, and HRP-catalyzed oxidation of iodide (Fig. 6-1). The initial velocity and the total I_3^- formed in the reaction decreased. CBQase or cellobiose alone did not cause any inhibition. The level of inhibition was dependent on both CBQase and cellobiose. CBQase, in the presence of cellobiose, also reduced chemically prepared I_3^-. The pH optimum for this reduction was 4.5, which is similar to that of the CBQase reaction with quinone or DCPIP as electron acceptors [Ayers et al., 1978; Renganathan et al., 1990]. The ratio of cellobiose oxidized to I_3^- reduced was 1:0.8. These findings suggest that CBQase inhibits peroxidase-catalyzed iodide oxidation by reducing I_3^- by two electrons ($I_3^- + 2e^- \rightarrow 3I^-$). The reducing equivalents are obtained by oxidizing cellobiose. The apparent K_m of I_3^- for this reaction was 120 μM, and the specific activity for I_3^- reduction was 57 μmol min^{-1} mg^{-1}.

I_3^- is an oxidant and the redox potential for two-electron reduction of I_3^- is 0.54 V [Skoog & West, 1982]. Two-electron reduction of I_3^- yields three iodide ions ($I_3^- + 2e^- \rightleftharpoons 3I^-$). The redox potentials of flavoenzymes range from -0.45 V to +0.15 V [Walsh, 1979]. Since I_3^- is at a higher potential compared to the flavin, electron transfer from the reduced flavin to I_3^- will be favored. Fig. 6-2 presents a possible mechanism for I_3^- reduction by CBQase. It involves substitution of one of the iodine atoms of I_3^- at the C(4a)-position of the reduced flavin and elimination of the other two iodine atoms as iodide ions. The C(4a)-iododihydroflavin intermediate is structurally similar to C(4a)-hydroxyflavin, proposed as an intermediate in the flavin-dependent monooxygenase reaction [Entsch et al., 1976]. C(4a)-Hydroxyflavin eliminates a molecule of water to regenerate the oxidized flavin. Similar elimination of HI from C(4a)-iododihydroflavin will yield the oxidized flavin (Fig. 6-2). Two other flavin-dependent enzymes, glucose oxidase and diaphorase (lipoyl dehydrogenase), were tested for their ability to reduce I_3^-. Glucose oxidase, in the

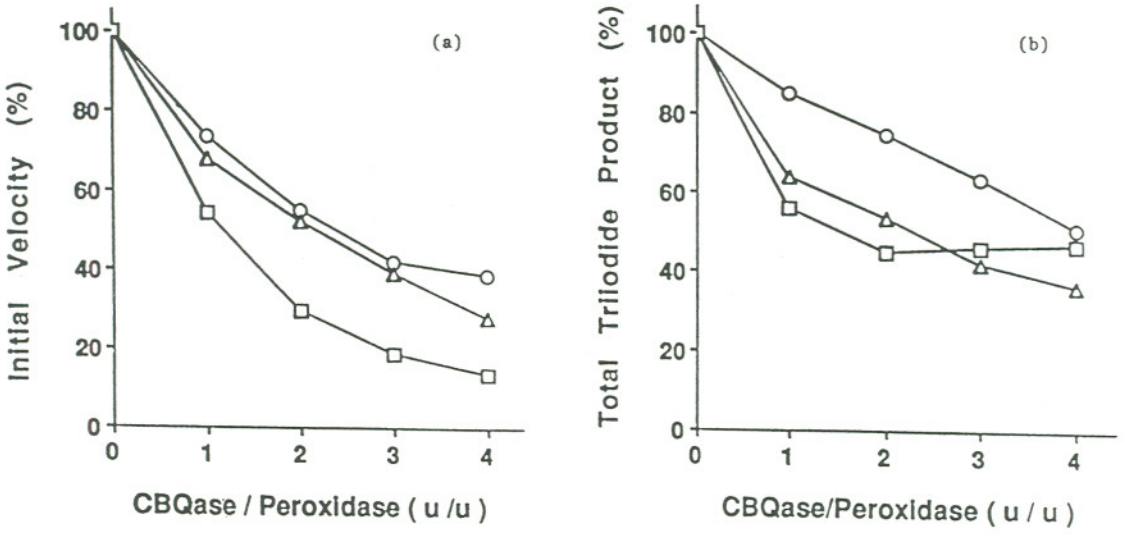

Fig. 6-1 CBQase inhibition of peroxidase-catalyzed oxidation of iodide to triiodide (I_3^-) in the presence of cellobiose: **(a)** Inhibition of initial velocity; **(b)** decrease in total I_3^- product formed. 100% initial velocity and 100% total triiodide product refer to the peroxidase activity observed in the absence of CBQase and cellobiose. Symbols: HRP (o), MnP (△), and LiP (□).

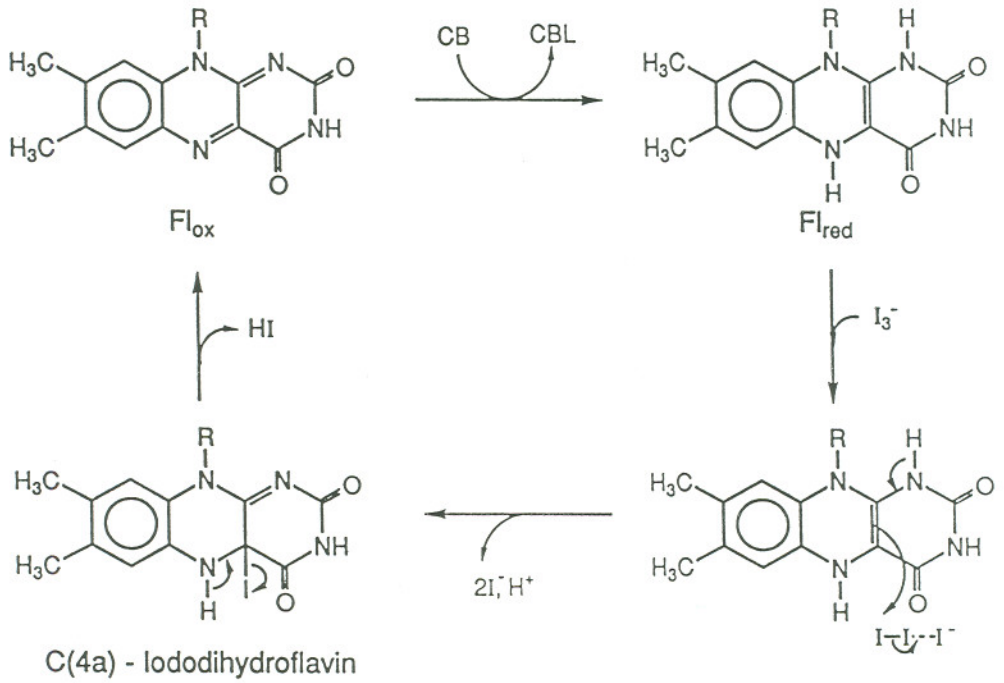

Fig. 6-2 A probable mechanism of triiodide (I_3^-) reduction by CBQase in the presence of cellobiose. Fl_{ox}, oxidized flavin; Fl_{red}, reduced flavin; CB, cellobiose; CBL, cellobionolactone.

presence of glucose, did not reduce I_3^- either under aerobic or anaerobic conditions. Since NAD(P)H reduced I_3^-, the ability of diaphorase to reduce I_3^- could not be determined.

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CHAPTER 7
FINAL COMMENTS

Among the hemoflavoenzymes, only flavocytochrome b_2 is well studied. Thus, a detailed study of cellobiose dehydrogenase will help in furthering our understanding of the structure, function and mechanism of hemoflavoenzymes. In addition, cellobiose dehydrogenase can tightly bind to crystalline cellulose which implies that, like cellulases, cellobiose dehydrogenase could be organized into two domains: a catalytic domain and a cellulose-binding domain [Renganathan et al., 1990]. Further study of cellobiose dehydrogenase could also help us understand the structure and function of cellulase domains.

7.1 HEME STRUCTURE OF CDH

We suggested that the heme iron of CDH is hexacoordinate since ferric CDH does not bind azide or cyanide and ferrous CDH does not bind CO. Preliminary resonance Raman (RR) spectroscopic study suggests that the CDH resonance Raman spectrum is very similar to that of flavocytochrome b_2 [Cohen, Bao, Renganathan and Loehr, unpublished results]. The heme iron of flavocytochrome b_2 contains two histidines as the fifth and sixth ligands [Xia et al., 1987]. Cox et al. [1992] suggested that CDH has a histidine and a methionine as the fifth and sixth coordinations to the heme iron based on NMR, EPR and near infra-red MCD spectroscopic studies. However, unambiguous assignment of the heme coordination structure could not be made from the RR spectrum of CDH. Other techniques, such as NMR and EXAFS, should be used to gather information about heme coordination.

7.2 CDH BINDING TO CELLULOSE

Endoglucanases (EG I and EG III) and cellobiohydrolases (CBH I and CBH II) from *T. reesei* comprise two functionally and structurally independent catalytic and cellulose-binding domains linked by a Pro/Thr/Ser rich region [Knowles et al., 1988; Béguin & Aubert, 1994] (Fig. 7-1). The amino acid sequences of cellulose-binding domains (CBD) are highly conserved (Block A). In CBH I and EG I, CBD is located at the C-terminal whereas, in CBH II and EG III, CBD is located at the N-terminal [Tomme et al., 1988; Knowles et al., 1988]. Cellulases produced by the cellulase-degrading bacterium *Cellulomonas fimi* (Cen A and Cex) are also similarly organized [Gilkes et al., 1988].

CDH binds strongly to crystalline cellulose. Its binding characteristics are similar to those of cellulases. But so far, there is no direct sequence evidence for the presence of a separate cellulose-binding domain in CDH. It is possible that CDH may lack a specific domain but has the conserved amino acid sequences required for cellulose binding. This information could be obtained through cDNA cloning of CDH and subsequent comparison of the amino acid sequence of CDH with that of cellulases.

Cellulases show a strong correlation between their ability to degrade crystalline cellulose and their affinity toward cellulose [Klyosov, 1990]. It has been proposed that CBDs could help peel off cellulose chains from the top layer of cellulose microfibers [Knowles et al., 1988]. Our studies indicate that CDH enhances the hydrolysis of microcrystalline cellulose to which it binds strongly. The CDH mechanism of cellulose hydrolysis enhancement could involve cellulose fiber splitting. This could be examined by electron microscopic studies.

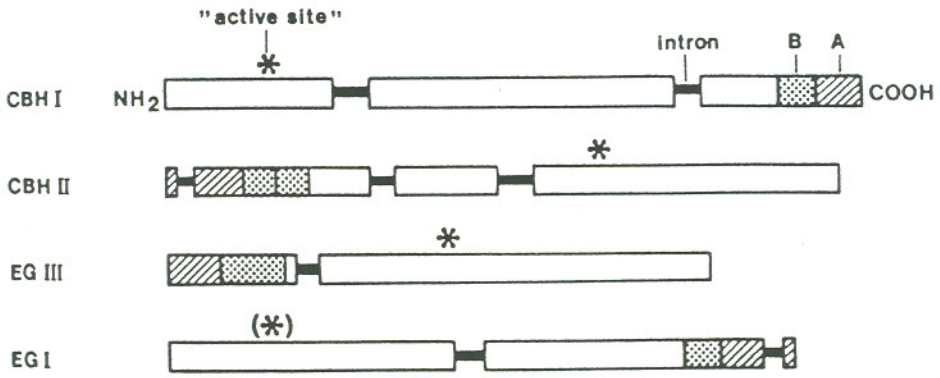

Fig. 7-1 Schematic structures of the four *Trichoderma* cellulase genes. Each enzyme consists of a catalytic domain (open box) and a binding domain (black A). The two domains are linked via a flexible hinge region (black B).

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VITA

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[1] Bao, W. and V. Renganathan (1991) Triiodide reduction by cellobiose: quinone oxidoreductase of *Phanerochaete chrysosporium*. *FEBS Lett.* **279**, 30-32.

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[8] Cohen, J.; W. Bao; V. Renganathan and T. Loehr (1994) A resonance Raman study on cellobiose dehydrogenase from *Phanerochaete chrysosporium* (to be submitted).

[9] Bao, W. and V. Renganathan (1994) Evidence for the electron transfer from cellobiose dehydrogenase to lignin peroxidase (to be submitted).

* The entire dissertation was written based on the above listed materials that have been published or will be published. Each chapter associated with the above publications is shown below [publication number]:

Chapter 2 [2], [3], [4], [5].

Chapter 3 [5], [6].

Chapter 4 [2], [3], [5].

Chapter 5 [6].

Chapter 6 [1].