

**Cloning and Characterization of Cellobiose
Dehydrogenase from *Phanerochaete chrysosporium***

Bin Li

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The dissertation "Cloning and Characterization of Cellobiose Dehydrogenase from *Phanerochaete chrysosporium*" by Bin Li has been examined and approved by the following Examination Committee:

V. Renganathan, Advisor
Associate Professor

James M. Cregg
Professor

Michael H. Gold
Professor

Srinivasa R. Nagalla
Assistant Professor
Oregon Health Sciences University

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ABSTRACT

Cloning and Characterization of Cellobiose Dehydrogenase from *Phanerochaete chrysosporium*

Bin Li

Supervising Professor: V. Renganathan

Cellobiose dehydrogenase (CDH) is an extracellular hemoflavoenzyme produced by cellulose-degrading cultures of the wood-degrading basidiomycete *Phanerochaete chrysosporium*. CDH contains one flavin adenine dinucleotide and one heme *b* per molecule, and it oxidizes cellobiose to cellobionolactone.

A 2.4-kb cDNA fragment encoding CDH was isolated by screening an expression library of *P. chrysosporium* OGC101 with a CDH-specific polyclonal antibody. The cDNA encoded a 755 amino acid protein with a predicted mass of 80,115. Sequence analysis suggested that the heme domain is located at the N-terminus and that the flavin domain is at the C-terminus. The flavin domain exhibited high sequence similarity with several FAD-dependent enzymes. Nevertheless, little sequence similarity was found with heme-dependent enzymes or hemoflavoenzymes. Northern blot analysis suggested that cellulose regulates CDH expression at the transcriptional level.

CDH is encoded by two alleles of a single gene, *cdh-1* and *cdh-2*. The nucleotide sequences of *cdh-1* (3627 bp) and *cdh-2* (3623 bp) exhibited 97% similarity. Both alleles had 14 exons, and the introns were located at exactly the same positions. The translation products of these alleles had identical amino acid sequences. Restriction fragment length polymorphism analyses of homokaryotic derivatives indicated segregation of the CDH alleles.

Homologous expression of CDH in *P. chrysosporium* was achieved by placing *cdh-1* under the control of a glucose-6-phosphate dehydrogenase promoter. Recombinant CDH (rCDH) activity was found in the extracellular medium, and rCDH protein constituted approximately 40% of the total extracellular proteins. rCDH was purified to homogeneity. Molecular weight and spectral and kinetic characteristics of pure rCDH were similar to those of wild-type CDH.

The heme iron in CDH is hexacoordinated; Met65 and His114 have been proposed to function as the fifth and sixth heme iron ligands. Site-directed mutagenesis of Met65 to an Ala produced only a flavoprotein ($M_r = 67,000$ Da). The mutant protein was competent in oxidizing cellobiose. It had absorbance only at 380 nm and 450 nm, indicating the presence of only an FAD. It is probable that the Met65Ala mutant protein is unable to bind heme, and, in the absence of heme, the heme domain is further processed by proteases leading to the production of a 67-kDa flavoprotein.

CHAPTER 1

INTRODUCTION

1.1 STRUCTURE OF CELLULOSE

Cellulose is a linear homopolymer of β -1,4-linked glucose residues. The degree of polymerization varies between 10^2 and 10^4 residues. In the cellulose chain, each pyranose ring rotates by 180° relative to the neighboring pyranose ring. As a result, the actual structural repeating unit in the cellulose chain is cellobiose (Fig. 1.1).

In nature, cellulose does not occur as a single chain, but exists in the form of microfibrils which are its fundamental structural units. In the microfibrils, the extended cellulose chains are oriented in parallel, forming a flat ribbon-like structure which is strengthened by numerous intra- and intermolecular hydrogen bonds (Fig. 1.1). This leads to a highly ordered crystalline arrangement of cellulose chains. The crystalline domains are interspersed by more disordered amorphous regions. Microfibril size can vary from the elementary fibril, which contains approximately 36 chains, to the large microfibrils of the alga *Valonia macrophysa*, which contains more than 1200 chains [Sugiyama et al., 1985].

The majority of cellulose exists as a component of plant cell walls. In the secondary plant cell wall, the microfibrils are embedded in a matrix of hemicellulose and lignin. Hemicellulose is a heterogenous carbohydrate polymer. The polymers can be either linear or branched. The building blocks include D-xylose, D-mannose, L-arabinose, and D-galactose. Xylans and glucomannans are the two major polymeric components of hemicellulose. In the matrix, hemicelluloses are associated with lignin through ester or ether linkages [Scalbert et al., 1985]. Lignin is a highly branched aromatic heteropolymer. It is formed by the oxidative polymerization of coumaryl,

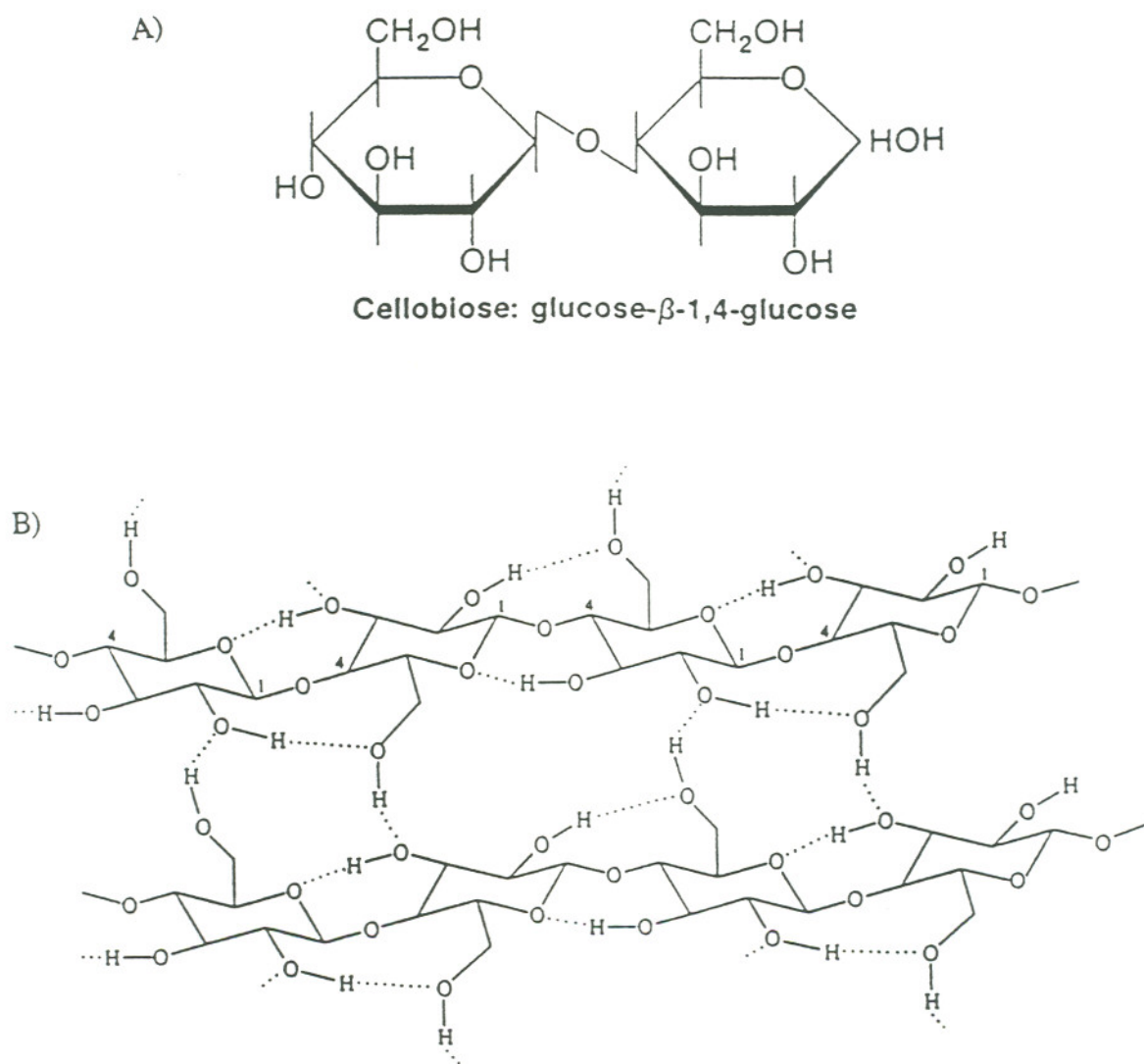


Fig. 1.1 (A) Structure of cellobiose. (B) The hydrogen bond network of cellulose.

coniferyl, and sinapyl alcohols. These building blocks are linked by covalent bonds, such as ether and carbon-carbon bonds [Freudenberg, 1968; Sarkanen & Ludwig, 1971] (Fig. 1.2).

1.2 APPLICATIONS OF CELLULOSE BIODEGRADATION

Interest in cellulose biodegradation was stimulated by the oil crisis in the late 1970s. Cellulosic biomass, such as agricultural and forest residues which are renewable, can be converted by cellulases to glucose. Fermentation of glucose into alcohol-based fuels could provide a partial substitution for fossil fuels. However, the biological conversion of cellulose is not economically feasible at present, because of the costs of substrate pre-treatment and enzyme production [Saddler, 1993]. Further study of the cellulose hydrolysis process by cellulolytic enzymes is needed to remove the economic barriers of lignocellulose bioconversion.

Many applications of cellulases involve limited hydrolysis of cellulose. Addition of cellulases to animal feed enhances its utilization and digestibility. Partial hydrolysis of the plant cell wall liberates starch and protein masked by the cell structure in cereals and increases the utilization of metabolizable energy and protein [Jorgensen & Cowan, 1989; Linko et al., 1989; Vanbelle & Bertin, 1989]. In some low-grade animal feeds, the soluble non-starch polysaccharides cause high viscosity in the small intestine of monogastric animals and impair digestion. Cellulases can partially degrade these non-starch polysaccharides, lowering viscosity in the intestine and improving feed utilization.

Cellulases have been used successfully in textile processing. Cellulases added to laundry powder remove the microfibrils that become partly detached from the main cotton fibers after several washing cycles. This restores the softness and color brightness of cotton fabrics and assists dirt removal. In the finishing process of blue jeans known as "bio-stoning," cellulases loosen and remove excess indigo dye from denim fabric and give the jeans a pre-faded appearance [Beguin & Aubert, 1994].

The paper industry is under consumer and environmental pressure to use more wastepaper in its products. Before wastepaper can be recycled, the ink has to be removed. Cellulases can also be used to facilitate the de-inking of recycled paper.

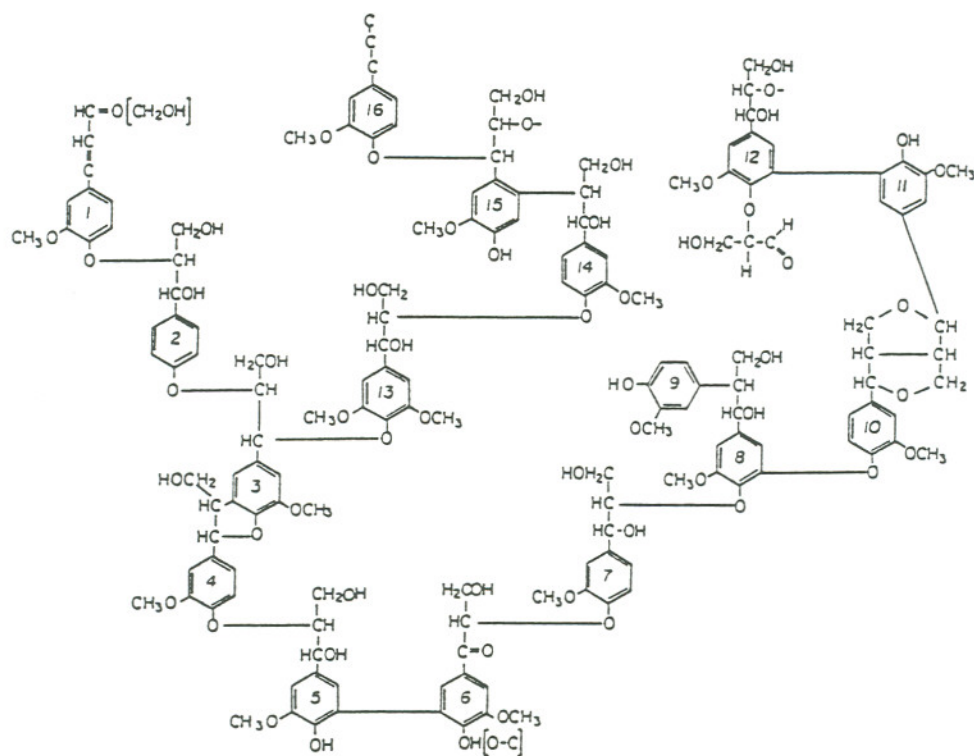


Fig. 1.2 A schematic structure of softwood lignin [Adler, 1977].

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1.3 FUNGAL CELLULOSE DEGRADATION

1.3.1 Wood-Rot Fungi

Fungi which degrade wood components can be categorized into three classes: white-rot fungi, brown-rot fungi and soft-rot fungi. White-rot fungi can degrade all components of wood [Eriksson et al., 1990]. They attack cellulose, hemicellulose, and lignin almost simultaneously. After lignin is degraded and removed, the wood becomes pale. White-rot fungi are mainly basidiomycetes [Kirk, 1971].

Brown-rot fungi can degrade cellulose and hemicellulose, but cannot degrade lignin [Kirk, 1975; Ander & Eriksson, 1978]. When the carbohydrates are removed, only the lignin remains; the wood becomes dark brown in color and fragile. Brown-rot fungi are mainly basidiomycetes.

Soft-rot fungi attack highly moist wood. Like brown-rot fungi, they can degrade cellulose and hemicellulose, but cannot degrade lignin. Soft-rot fungi are mainly ascomycetes [Corbett, 1965; Ander & Eriksson, 1978].

1.3.2 The Fungal Cellulase System

Since cellulose is insoluble, cellulose-degrading fungi secrete a system of soluble enzymes into the extracellular medium to convert cellulose to soluble products that can be transported into the cells. These enzymes include endoglucanases, cellobiohydrolases, and β -glucosidases. Endoglucanases hydrolyze cellulose in the amorphous regions. They are active against the soluble carboxymethyl cellulose but are inactive against crystalline cellulose [Eriksson & Wood, 1985].

Cellobiohydrolases splice cellobiose units from the ends of cellulose chains.

β -Glucosidases hydrolyze cellobiose to glucose and complete the cellulose degradation process.

Cellulase systems from some microorganisms are capable of hydrolyzing both amorphous and crystalline cellulose; these systems are known as complete cellulase systems. Some cellulase systems can only degrade amorphous cellulose; they are called low value cellulase systems [Klyosov, 1990]. The low value system does not have cellobiohydrolase [Klyosov, 1990].

The first model of cellulose degradation was proposed by Reese et al. [1950]. They hypothesized that the cellulase system is composed of two components, C_1 and C_x . C_1 is a nonhydrolytic chain-separating enzyme which was proposed to break hydrogen bonds in crystalline cellulose and enable separation of cellulose chains. Crystalline cellulose was first attacked by C_1 . Then, the separated cellulose chains (the reactive cellulose) were hydrolyzed by the C_x component and by β -glucosidase [Mandels & Reese, 1964]. The complete cellulase system which can hydrolyze crystalline cellulose contained both C_1 and C_x enzymes. The low-value cellulase system, which can only hydrolyze amorphous or modified cellulose, does not have the C_1 component. However, the C_1 enzyme as a "hydrogen bondase" was not identified.

In 1969, Eriksson proposed that the C_1 enzyme is an exoglucanase and revised the model for crystalline cellulose degradation [Eriksson, 1969]. The C_x enzyme (carboxymethyl cellulase, endoglucanase) first attacks crystalline cellulose at random over the cellulose chain, exposing both reducing and nonreducing ends. The C_1 enzyme (cellobiohydrolase, exoglucanase) then cleaves off cellobiose units from the nonreducing end. This model explains the synergism between these two enzymes. This enzyme mechanism of crystalline cellulose degradation has been identified in many fungi, such as *Phanerochaete chrysosporium* [Eriksson & Pettersson, 1972], *Trichoderma koningii* [Halliwell et al., 1972; Wood & McCrae, 1972], *Trichoderma reesei* [Berghem & Pettersson, 1973; Emert et al., 1974], *Fusarium solani* [Wood & McCrae, 1977], and *Sclerotium rolfsii* [Patil & Sadana, 1984].

The synergism among the components of the fungal cellulase system (endoglucanase, cellobiohydrolase, and β -glucosidase) can be explained by the current model of cellulose degradation (Fig. 1.3). Endoglucanases cleave the β -1,4-glucosidic bond in the amorphous regions of cellulose and provide cellulose chain ends for cellobiohydrolases to attack. Cellobiohydrolase then attacks the nonreducing ends and releases cellobiose units. Cellobiose is an inhibitor of endoglucanases and cellobiohydrolases [Wood & McCrae, 1978]. β -Glucosidases reduce the inhibition by hydrolyzing cellobiose to glucose [Eriksson, 1978; Wood & McCrae, 1978].

The cellulase system of the soft-rot fungus *T. reesei* is the best understood system. Two cellobiohydrolases with molecular masses of 65,000 and 58,000 Da,

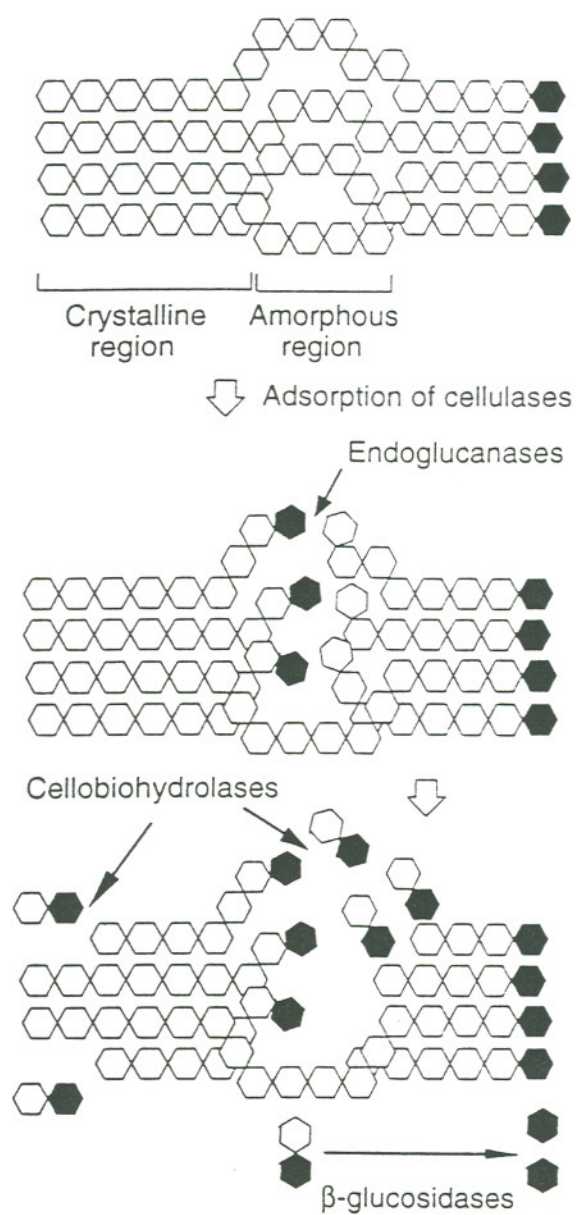


Fig. 1.3 Mechanism of cellulose degradation by cellulases. Glucose residues are indicated by hexagons; reducing ends are shown in solid hexagons [Beguín & Aubert, 1994].

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respectively, six endoglucanases with molecular masses ranging from 20,000 to 67,000 Da, and one β -glucosidase with a molecular mass of 73,000 Da have been isolated from *T. reesei* [Enari & Niku-Paavola, 1987].

1.3.3 Enzyme Families

All cellulases can be classified based on the amino acid sequence similarities of their catalytic domains. Originally, hydrophobic cluster analysis was used for cellulase and xylanase classification, and 21 cellulases and xylanases were classified into six families [Henrissat et al., 1989]. The primary sequences of more than 400 glycosyl hydrolases have been determined, and they are grouped into more than 50 families based on sequence similarity [Henrissat, 1991; Henrissat & Bairoch, 1993; Warren, 1996]. Fungal endoglucanases are distributed into five families: families 5, 6, 7, 12, and 45 [Henrissat, 1991; Henrissat & Bairoch, 1993; Warren, 1996]. Fungal cellobiohydrolases are distributed in families 6 and 7 [Henrissat, 1991; Henrissat & Bairoch, 1993; Warren, 1996]. Fungal β -glucosidases are all in family 3 [Henrissat, 1991; Henrissat & Bairoch, 1993].

1.3.4 X-ray Structures

The X-ray crystallographic structures of two fungal endoglucanases, the endoglucanase EGV of *Humicola insolens* [Davies et al., 1993] and the endoglucanase Eg1 of *Aspergillus aculeatus* [Okada, 1991], have been reported. EGV from *H. insolens* contains a six-stranded β -barrel core, connected by loops, and three α -helices. The active site is shaped like a long groove that runs the entire length of the molecule [Davies et al., 1993]. Eg1 from *A. aculeatus* comprises three antiparallel β -sheets and one α -helix. The active site is formed by a deep cleft between two of the β -sheets [Okada, 1991]. The fact that the active sites of endoglucanases are shaped like an open cleft fits their mode of action. An endoglucanase should be able to act anywhere along a cellulose chain. Thus, an open active site structure should enable the endoglucanase to bind to cellulose anywhere along a cellulose chain.

The X-ray crystallographic structures of two fungal cellobiohydrolases, *T. reesei* cellobiohydrolase II (CBHII) [Rouvinen et al., 1990] and *T. reesei*

cellobiohydrolase I (CBHI) [Divne et al., 1994], have been reported. The crystal structure of the *T. reesei* CBHII catalytic domain was the first cellulase structure to be solved. It has an α/β barrel structure which contains five α -helices and seven β -strands [Rouvinen et al., 1990]. Two loops on the C-terminal end of the barrel form a tunnel-shaped structure which holds the active site. The three-dimensional structure of the catalytic domain of *T. reesei* CBHI is a β -sandwich sheet which contains four short α -helices and 15 β -strands [Divne et al., 1994]. The active site is also shaped like a tunnel. The tunnel-shaped active site fits the exo reaction mode of cellobiohydrolases. The active site tunnel in *T. reesei* CBHII contains at least four subsites (A–D) and can accommodate four glucose residues; the cleavage site is between subsites B and C [Rouvinen et al., 1990]. The cellulose chain is threaded through the tunnel from the ends, and cellobiose units are cleaved.

1.3.5 Reaction Mechanisms

The cellulases catalyze the hydrolysis of the β -1,4-glucosidic linkage through an acid-base mechanism [Sinnott, 1990; Beguin & Aubert, 1994]. With respect to the configuration of the anomeric carbon, the stereochemistry of the reaction can involve either retention or inversion. There are generally two acidic amino acid residues, aspartic acid, or glutamic acid involved in the catalytic mechanism (Fig. 1.4). Of these two residues, one is protonated and the other is deprotonated. In the retaining mechanism, the protonated acidic residue donates one proton to the oxygen of the glycosidic bond. This causes its breakage. The charge developed in the process is stabilized by the deprotonated residue. Subsequent attack by water occurs from above the plane of the molecule, leading to the introduction of a β -hydroxyl group. In the inverting mechanism, the deprotonated carboxylate generates a hydroxide ion by removing a proton from a water molecule. The hydroxide ion attacks the glycosidic bond from below the plane, leading to the introduction of an α -hydroxyl group.

It has been shown that representatives of a given glycosyl hydrolase family have the same stereoselectivity: all are either inverting or retaining enzymes, which suggests that family members share a common catalytic mechanism [Gebler et al., 1992; Schou et al., 1993]. Thus, for the fungal endoglucanases, which are distributed

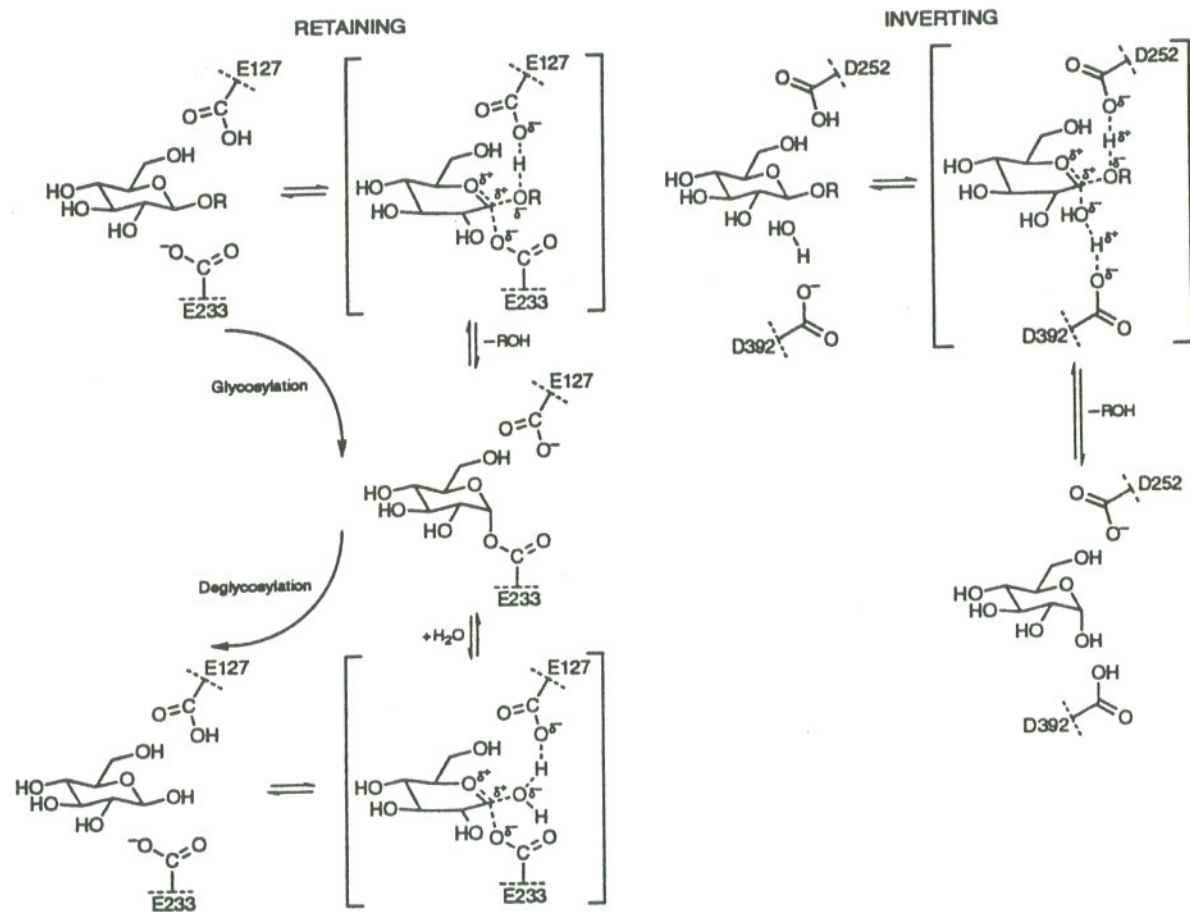


Fig. 1.4 Reaction mechanisms of retaining and inverting β -1,4-glycanases [Tomme et al., 1995b].

in families 5, 6, 7, 12, and 45, those in families 5, 7, and 12 are retaining enzymes, while those in families 6 and 45 are inverting enzymes [Gebler et al., 1992; Henrissat & Bairoch, 1993; Schou et al., 1993; Warren, 1996]. Fungal cellobiohydrolases are distributed in families 6 and 7. Those in family 6 are inverting enzymes; whereas, those in family 7 are retaining enzymes [Gebler et al., 1992; Henrissat & Bairoch, 1993; Schou et al., 1993; Warren, 1996]. Fungal β -glucosidases are all in family 3 and apparently are all retaining enzymes [Henrissat & Bairoch, 1993; Warren, 1996].

1.3.6 Cellulose-binding Domains (CBD)

Most of the cellulases are modular proteins with one catalytic domain and one cellulose-binding domain. Two domains are usually joined together by a linker region which is rich in proline and hydroxyl amino acid residues [Gilkes et al., 1991]. The domain structure of cellulases has been identified by studies of proteolytic products. Since the linker regions of many cellulases are very sensitive to proteases like papain, limited proteolysis will release the individual domains as discrete functional polypeptides which can be purified and analyzed [Van Tilbeurgh et al., 1986; Gilkes et al., 1988; Tomme et al., 1988]. Fungal CBDs are either at the C-terminus or at the N-terminus of the enzyme.

There are more than 120 CBDs with known sequences, and they are classified into ten families (I–X) [Tomme et al., 1995a]. All fungal CBDs are grouped into family I. They have highly conserved protein sequences and contain 32–36 amino acid residues. The three-dimensional structure of *T. reesei* CBHI CBD has been solved by NMR spectroscopy [Kraulis et al., 1989] (Fig. 1.5). The structure contains three antiparallel β -strands with an overall shape like a wedge. It has two flat faces, one being hydrophobic and the other hydrophilic. There are three highly conserved tyrosine residues on the hydrophobic face which are implicated in the interaction with cellulose [Kraulis et al., 1989; Reinikainen et al., 1992]. Four strictly conserved cysteine residues form two disulfide bonds which hold the three antiparallel β -strands together. The CBDs from *T. reesei* cellulases bind to both amorphous and crystalline cellulose [Tomme et al., 1988; Reinikainen et al., 1992; Srisodsuk et al., 1993].

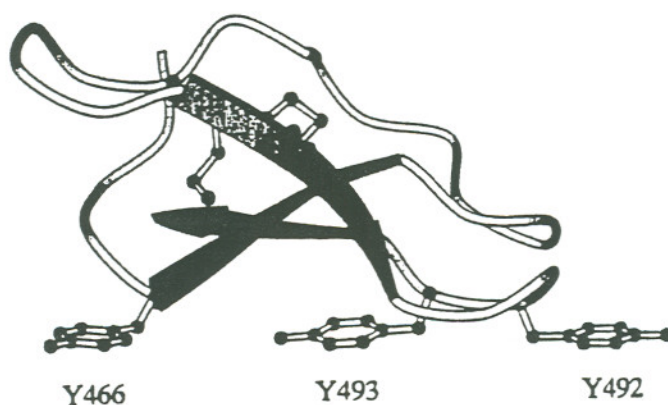


Fig. 1.5 Ribbon diagram of the solution structure of the family I CBD from *T. reesei* CBHI [Tomme et al., 1995a].

[Reprinted with permission of the American Chemical Society; originally published in: Tomme, P., Warren, A. J., Miller, R. C., Jr., Kilburn, D. G., and Gilkes, N. R. (1995) Cellulose-binding domains: classification and properties. In *Enzymatic Degradation of Insoluble Carbohydrates* (Saddler, J. N. and Penner, M. H., eds.), ACS Symp. Ser. 618, American Chemical Society, NY, pp. 142-163.]

The CBD can enhance cellulase activity against crystalline cellulose by attaching the cellulase catalytic domains to the cellulose surface and increasing their local concentration on the substrate surface [Knowles et al, 1987]. Removal of the CBD from cellulolytic enzymes by proteolysis often reduces their activity toward insoluble cellulose substrates [van Tilbeurgh et al., 1986; Tomme et al., 1988]. CBD can also enhance crystalline cellulose hydrolysis by disrupting the crystalline structure of the substrate [Knowles et al, 1987]. The wedge-like fungal CBD has been proposed to peel off cellulose chains from the surface of cellulose [Knowles et al., 1988].

1.3.7 Molecular Cloning of Cellulase Genes

More than 150 cellulase genes have been cloned and sequenced [Henrissat, 1991; Henrissat & Bairoch, 1993; Tomme et al., 1995a]. Most of the cellulase systems are composed of individual cellulase genes which are scattered throughout the genomes [Tomme et al., 1995a]. In other cellulase systems, some cellulase genes are located close to each other [Tomme et al., 1995a]. *T. reesei* is one of the most efficient and most studied cellulose-degrading organisms. It produces a complete cellulase system which includes endoglucanases, cellobiohydrolases and β -glucosidases acting in synergy. The number of enzymes in each class is not clear, but the genes of at least two cellobiohydrolases (CBHI and CBHII), four endoglucanases (EGI, EGII, EGIII, and EGIV), and one β -glucosidase (BGL) have been cloned and characterized [Shoemaker et al., 1983; Teeri et al., 1983; Penttila et al., 1986; Chen et al., 1987; Teeri et al., 1987; van Arsdell et al., 1987; Saloheimo et al., 1988, 1994]. In the genome of *T. reesei*, *cbh1*, *cbh2*, *egl1*, and *bgl* gene are present as single copies. There are six chromosomes in total in the *T. reesei* genome. The *cbh1*, *cbh2*, and *egl3* genes are on chromosome II, and *egl1* is on chromosome VI [Kubicek et al., 1993].

1.3.8 Regulation of Cellulase Synthesis

The regulation of cellulase biosynthesis involves two types of mechanisms. First, the presence of easily metabolized carbon sources, such as glucose, represses

cellulase biosynthesis in all cellulase systems [Bisaria & Mishra, 1989]. Second, cellulase synthesis is induced when cellulose is used as the carbon source in many cellulolytic microorganisms. A current popular hypothesis for cellulase induction proposes that fungi produce low levels of cellulases constitutively. The cellulases hydrolyze cellulose to soluble products (cellobiose, cellobionolactone, sophorose) which are transported into the cell and induce cellulase synthesis [Beguin & Aubert, 1994]. Many observations support this hypothesis. In *T. reesei*, the occurrence of cellulase system induction by cellulose coincides with the presence of basal level cellulases. Constitutive endoglucanases at a basal level have been detected in conidia, but not in mycelia. Also, cellulose has been shown to induce the synthesis of cellulase only in conidia, but not in mycelia; however, cellooligosaccharides and sophorose induce the synthesis of cellulase in both conidia and mycelia [Kubicek, 1987; Kubicek et al., 1988]. These observations demonstrate that constitutive cellulases present at a basal level are essential for cellulase system induction by cellulose. The *T. reesei* CBHI mRNA is induced by cellulose as indicated by northern blot analysis; but when antibodies against major cellulases in the system are added to the culture medium prior to the addition of cellulose, CBHI mRNA could not be detected [El-Gogary et al., 1989]. Cellobiohydrolases CBHI, CBHII, and endoglucanases EGI, EGII are the major cellulases in *T. reesei*; CBHII is shown to be the major conidial-bound cellulase in *T. reesei* [Messner et al., 1991]; insertional inactivation of CBHII or EGII abolishes the expression of other cellulase genes [Seiboth et al., 1992].

Identification of the cellulase synthesis inducer and the process by which the soluble inducer is formed from cellulose are not well understood. Sophorose (β -1,2-glucopyranosyl-D-glucose) appears to be an inducer since it induces cellulase synthesis in *Trichoderma* sp. [Mandels et al., 1962; Sternberg & Mendels, 1979]. A constitutive, membrane-bound β -glucosidase is involved in cellulase synthesis in *T. reesei* [Kubicek, 1987], and it can catalyze the formation of sophorose by transglycosylation [Umile & Kubicek, 1986]. However, sophorose may not be the only cellulase synthesis inducer since it can only induce two out of the five endoglucanases in *T. reesei* [Messner et al., 1988].

1.4 THE CELLULOLYTIC SYSTEM OF *P. CHRYSOSPORIUM*

Phanerochaete chrysosporium is the best studied white-rot basidiomycete. It was first isolated from pine pulp wood chips and was given the tentative name *Chrysosporium lignorum*. Later, it was renamed *Sporotrichum pulverulentum*. Now *P. chrysosporium* is the widely accepted name for this basidiomycete [Eriksson et al., 1990]. The cellulase system of *P. chrysosporium* is a complete system which includes endoglucanase, cellobiohydrolase, and β -glucosidase; these enzymes act synergistically to efficiently degrade crystalline cellulose.

1.4.1 Endoglucanases

Eriksson and Pettersson [1975a] first purified five endoglucanases from cellulose-degrading cultures of *P. chrysosporium*. The endoglucanases had molecular masses varying from 28,300 to 37,500 Da. All endoglucanases degraded carboxymethyl cellulose. With cotton linters or Avicel as substrates, strong synergism was observed between different endoglucanases and the cellobiohydrolase purified from the same cellulose-degrading cultures of *P. chrysosporium*. The degradation of crystalline cellulose by a mixture of endoglucanase and cellobiohydrolase was 40–392% more than the numerical sum of the endoglucanase activity and the cellobiohydrolase activity [Streamers et al., 1975].

Although endoglucanases have been isolated from *P. chrysosporium*, the corresponding endoglucanase genes have not been cloned. It has been hypothesized that CBHI-like proteins might exhibit endoglucanase-like activity [Sims et al., 1994].

1.4.2 Cellobiohydrolases

Eriksson and Pettersson [1975b] first purified one cellobiohydrolase from cellulose-degrading cultures of *P. chrysosporium*. The enzyme had molecular mass of 48,600 Da, and a pI of 4.3. It was not active against carboxymethyl cellulose.

Uzcategui et al. [1991] isolated three cellobiohydrolases (CBHI, CBH62, and CBH50) from *P. chrysosporium* cultures. CBHI had a molecular mass of 60,000 Da

and a pI of 3.82. CBH62 had a molecular mass of 62,000 Da and a pI of 4.85. CBH50 had a molecular mass of 50,000 Da and a pI of 4.87. CBHI is the major form of cellobiohydrolase and constitutes 12% of the total extracellular protein [Uzcategui et al. 1991].

Like *T. reesei*, *P. chrysosporium* appears to have two types of cellobiohydrolases, CBHI and CBHII. There is no sequence similarity between these two cellobiohydrolases; CBHI belongs to glycosyl hydrolase family 7, while CBHII belongs to glycosyl hydrolase family 6 [Henrissat & Bairoch, 1993]. Partial protein sequences showed that CBHI and CBH62 of *P. chrysosporium* were similar to CBHI from *T. reesei*, and CBH50 was similar to CBHII from *T. reesei* [Uzcategui et al. 1991].

In *P. chrysosporium*, at least six genes which have high sequence homology to the *T. reesei cbh1* gene have been identified [Covert et al., 1992a]. The most highly transcribed cellobiohydrolase gene is *cbh1-4*, which encodes the major *P. chrysosporium* cellobiohydrolase isozyme, CBHI [Wymelenberg et al., 1993]. The gene *cbh2* encodes CBHII from *P. chrysosporium* [Tempelaars et al., 1994]. Unlike the *cbh1* gene of *P. chrysosporium*, *cbh2* appears to be the only gene coding for CBHII [Tempelaars et al., 1994].

1.4.3 β -Glucosidases

P. chrysosporium produces three different types of β -glucosidases—extracellular, intracellular and cell-wall-bound—depending on the carbon source [Deshpande et al., 1978; Smith & Gold, 1979]. Deshpande et al. [1978] reported that cellulose induces intracellular and cell-wall-bound enzymes, and they purified five isozymes of extracellular β -glucosidases from cellulose-degrading cultures of *P. chrysosporium*. Molecular masses of these glucosidases ranged from 165,000 to 182,000 Da. Smith and Gold [1979] partially purified an extracellular β -glucosidase from *P. chrysosporium* OGC101 and characterized it as a monomer with a molecular mass of 90,000 Da. Lyman et al. [1995] purified and characterized a cellulose-binding extracellular β -glucosidase (CBGL) with a molecular mass of 114,000 Da

from cellulose-supplemented cultures of *P. chrysosporium* OGC101. When CBGL was treated with papain, its molecular weight decreased to 95,000 Da and it lost the ability to bind to cellulose; however, its catalytic activity was unchanged. This suggested that CBGL is organized into two domains—a cellulose-binding domain (CBD) and a catalytic domain [Lyman et al., 1995]. The glucosidase isolated previously by Smith and Gold [1979] from this strain might be the non-cellulose-binding form. The kinetic properties of the cellulose-binding and nonbinding forms were similar, indicating that CBD is not involved in catalysis.

1.4.4 Cellobiose-oxidizing Enzymes

In addition to cellulases, *P. chrysosporium* produces two cellobiose-oxidizing enzymes: cellobiose dehydrogenase and cellobiose:quinone oxidoreductase [Westermarck & Eriksson, 1974; Ayers et al., 1978; Bao et al., 1993]. These two enzymes oxidize cellobiose to cellobionolactone in the presence of electron acceptors such as cytochrome *c*, dichlorophenol-indophenol, quinones, Fe(III) complexes, Mn(III) complexes, and O₂ [Ayers et al., 1978; Morpeth, 1985; Henriksson et al., 1991; Kremer & Wood, 1992; Samejima & Eriksson, 1992; Bao et al., 1993].

1.5 CELLOBIOSE DEHYDROGENASE FROM *P. CHRYSOSPORIUM*

1.5.1 Discovery, Purification, and Distribution of CDH

Cellobiose dehydrogenase (CDH) was discovered by Eriksson and coworkers in 1974 [Westermarck & Eriksson, 1974]. They showed that cellulose degradation by the cell-free extracellular medium of *P. chrysosporium* in the presence of oxygen is significantly faster than in the absence of oxygen. Since the known cellulases do not require oxygen for their activity, it was rationalized that an oxidative enzyme which requires oxygen for its activity must be involved in the cellulose degradation process of *P. chrysosporium*. Later, this oxidative enzyme was characterized to be cellobiose oxidase, an extracellular hemoflavoenzyme [Ayers et al., 1978]. It had one b-type heme and one flavin as cofactors. It oxidized cellobiose, and other cellodextrins to their corresponding aldonic acids with molecular oxygen as the electron acceptor

[Ayers et al., 1978]. Subsequently, in addition to molecular oxygen, cellobiose oxidase was demonstrated to accept cytochrome *c*, quinones, 2,6-dichlorophenol-indophenol (DCPIP), Fe(III) and Mn(III) complexes as electron acceptors [Ayers et al., 1978; Morpeth, 1985; Wilson et al., 1990; Kremer & Wood, 1992a,b; Samejima & Eriksson, 1992; Bao et al., 1993]. Cytochrome *c* was identified as the most preferred electron acceptor and oxygen as the least preferred electron acceptor [Samejima & Eriksson, 1992; Bao et al., 1993]. In contrast, oxidases such as glucose oxidase prefer to transfer electrons to oxygen, but not cytochrome *c* or quinones. On the other hand, most dehydrogenases prefer transferring electrons to acceptors such as quinone, DCPIP, and cytochrome *c*, but not to oxygen. Because cellobiose oxidase preferred cytochrome *c* as the electron acceptor, it was renamed cellobiose dehydrogenase by Bao et al. [1993].

Since its discovery, several laboratories have reported the purification of CDH from *P. chrysosporium* [Ayers et al., 1978; Morpeth, 1985; Henriksson et al., 1991; Bao et al., 1993]. The purification procedure developed by Bao et al. [1993] produced CDH with high yield and specific activity. CDH was purified from cellulose-grown culture media of *P. chrysosporium* to homogeneity through five steps including ammonium sulfate precipitation, DEAE-Sephadex chromatography, Phenyl-Sepharose chromatography, Sephacryl S-200 chromatography, and FPLC separation using Mono-Q column [Bao et al., 1993]. Purified CDH had a specific activity of $10.3 \mu\text{mol min}^{-1} \text{mg}^{-1}$ for cytochrome *c* reduction [Bao et al., 1993]. CDH is a monomer and its molecular weight was 90,000 Da as determined by SDS-PAGE; it is a glycoprotein with 9.4% neutral carbohydrate; it has one heme b and one flavin adenine dinucleotide (FAD) as cofactors [Bao et al., 1993]. Cellobiose was the preferred electron donor and cytochrome *c* was the preferred electron acceptor as determined by kinetic studies [Bao et al., 1993]. CDH was a stable enzyme within pH 3-10 and below 50°C. Inactivation at low pH and at higher temperature is caused by the release of FAD from the enzyme; however, the heme remained bound to the protein under these conditions [Bao et al., 1993].

Besides *P. chrysosporium*, CDH is produced by many other cellulolytic fungi, such as *Sporotrichum thermophile* [Canevascini et al., 1991], *Coniophora puteana* [Schmidhalter & Canevascini, 1993], *Humicola insolens* [Schou et al., 1998], *Schizophyllum commune* [Fang et al., 1998], *Trametes versicolor* [Roy et al., 1996], and *Sclerotium rolfsii* [Sadana & Patil, 1985; Baminger et al., 1998]. Characteristics of CDH from these fungi are listed in Table 1.1.

Cellobiose-oxidizing enzymes have been reported in many other fungi, such as *Monilia* sp. [Dekker, 1980] and *Fomes annosus* [Huttermann & Noelle, 1982]. These enzymes have not been well characterized.

1.5.2 Spectroscopic Studies

The UV-visible absorption spectra of oxidized and reduced CDH are shown in Fig. 1.6. Oxidized CDH has three absorption peaks at 420, 529, and 570 nm. In the spectrum of reduced CDH produced by the addition of cellobiose or dithionite, the peaks shift to 428, 534, and 564 nm [Ayers et al., 1978; Morpeth, 1985; Bao et al., 1993]. These characteristics are very similar to those of flavocytochrome b_2 , a well-characterized hemoflavoenzyme [Tabor & Kellogg, 1970; Desbois et al., 1989]. The heme iron of CDH appears to be hexacoordinated since azide or cyanide does not bind to ferric CDH [Bao et al., 1993]. Spectroscopic studies, including optical absorption spectroscopy, nuclear magnetic resonance (NMR) spectroscopy, electron paramagnetic resonance (EPR) spectroscopy, and magnetic circular dichroism (MCD) spectroscopy, indicate that a histidine and a methionine act as the fifth and sixth ligands of the heme iron in CDH [Cox et al., 1992]. Cohen et al. [1997] reported the resonance Raman spectra of the oxidized, reduced, and deflavo forms of CDH as well as the individual flavin and heme domains of the enzyme obtained by peptide proteolysis. Proteolytic cleavage of the CDH domains had very little spectroscopic effect on the vibrational modes of the heme and FAD cofactors. Excitation of the oxidized CDH holoenzyme at 413 or 442 nm caused photoreduction of the heme. However, excitation of the deflavo form of the enzyme or excitation of the heme domain alone at the same wavelength did not cause photoreduction, indicating that photoinitiated electron transfer requires the FAD cofactor. These observations

Table 1.1 Characteristics of CDHs.

Source	Fungal Classification	Molecular Weight (kD)	Subunits	pH Optimum	K _m for Cellobiose (μM)	References
<i>Phanerochaete chrysosporium</i>	White-rot	90	Monomer	4.5	25	Bao <i>et al.</i> , 1993
<i>Sporotrichum thermophile</i>	Soft-rot	95 , 192	Monomer Dimer	5.0	3.5	Canevascini <i>et al.</i> , 1991; This study
<i>Trametes versicolor</i>	White-rot	97	Monomer	4.5	—	Roy <i>et al.</i> , 1996
<i>Sclerotium rolfsii</i>	Soft-rot	102	Monomer	4.5	—	Baminger <i>et al.</i> , 1998
<i>Coniophora puteana</i>	Brown-rot	192	Dimer	—	84	Schimidalter & Canevascini, 1993
<i>Humicola insolens</i>	Soft-rot	92	Monomer	6 - 8.5	11	Schou <i>et al.</i> , 1998
<i>Schizophyllum commune</i>	White-rot	102	Monomer	4.5	30	Fang <i>et al.</i> , 1998

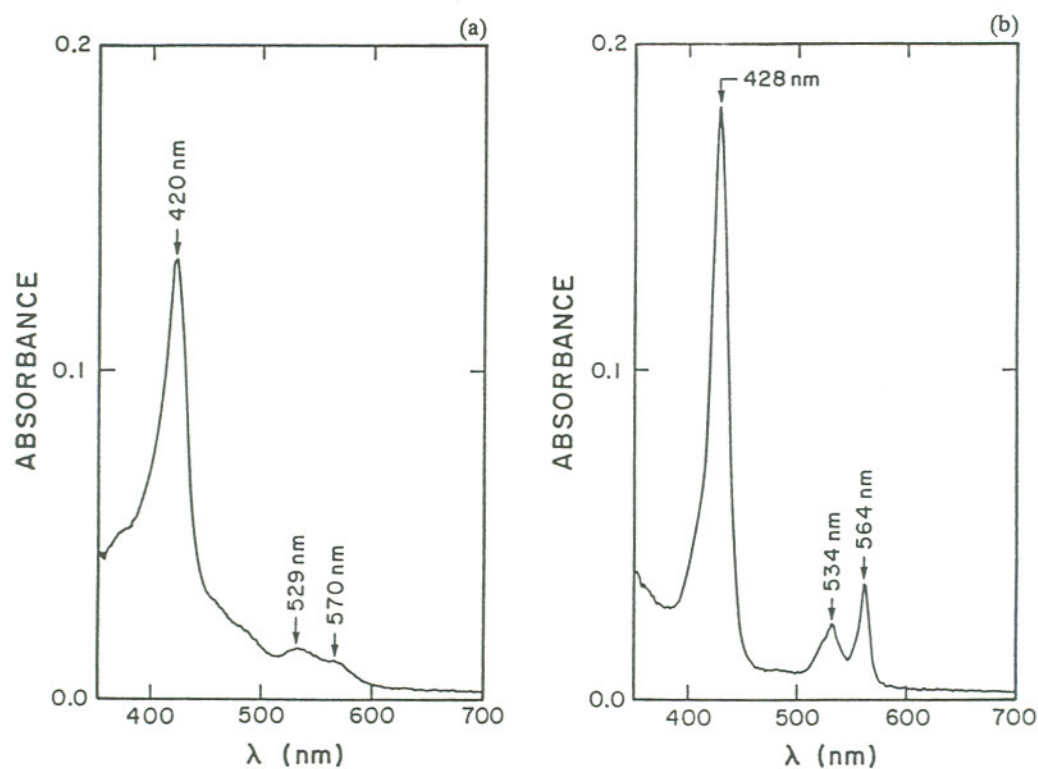


Fig. 1.6 UV-visible spectra of oxidized (a) and reduced (b) CDH. Reduced CDH was generated by the addition of cellobiose (50 μ M) to the oxidized form [Bao et al., 1993].

[Reprinted with permission of Academic Press; originally published in: Bao, W., Usha, S. N., and Renganathan, V. (1993) Purification and characterization of cellobiose dehydrogenase, a novel hemoflavoenzyme from the white-rot fungus *Phanerochaete chrysosporium*. *Arch. Biochem. Biophys.* 300, 705–713.]

suggested that the electrons obtained from the oxidation of cellobiose are transferred from the FAD to the heme. This mechanism is similar to what has been proposed for flavocytochrome *b*₂ [Desbois et al., 1989].

1.5.3 Domain Structure

CDH is organized into two domains, one domain contains the heme and the other contains the FAD [Henriksson et al., 1991; Wood & Wood, 1992; Habu et al., 1993]. Henriksson et al. [1991] reported that CDH can be cleaved into two fragments by papain. One fragment had a molecular mass of 35,000 Da and contained the heme cofactor; the other fragment had a molecular mass of 55,000 Da and contained the FAD cofactor. The flavin fragment oxidized cellobiose in the presence of 2,6-dichlorophenol-indophenol or 3,5-di-*t*-butyl-*O*-benzoquinone; however, cytochrome *c* did not serve as an electron acceptor for this fragment. Habu et al. [1993] demonstrated that the flavin domain of CDH can be cleaved by endogenous proteases from cellulolytic cultures of *P. chrysosporium* only when CDH is bound to cellulose. In addition to CDH, *P. chrysosporium* produces another cellobiose-oxidizing enzyme, cellobiose:quinone oxidoreductase (CBQase) [Westermarck & Eriksson, 1975]. CBQase is a flavoenzyme that oxidizes cellobiose and transfers electrons to quinones or 2,6-dichlorophenol-indophenol. CBQase appears to be a proteolytic degradation product of CDH. Wood and Wood [1992] showed that limited digestion of CDH and CBQase with staphylococcal V8 protease or cyanogen bromide produced many identically sized fragments as seen by SDS-PAGE. Also, culturing conditions which favored protease production decreased the yield of CDH and increased the yield of CBQase [Habu et al., 1993; Bao et al., 1994]. All these findings supported the proposal that CBQase is a degradation product of CDH [Henriksson et al., 1991; Wood & Wood, 1992; Habu et al., 1993]. CDH binds to microcrystalline cellulose, indicating that it might have a cellulose-binding domain [Renganathan et al., 1990]. Many bacterial and fungal cellulases are modular proteins with a catalytic domain and a non-catalytic cellulose-binding domain. The two domains are usually connected by a linker region that is rich in proline and hydroxy amino acid residues [Gilkes et al., 1991; Beguin & Aubert,

1994]. In fungal cellulases, the cellulose-binding domain is either at the C-terminus or at the N-terminus of the enzyme. Henriksson et al. [1991] demonstrated that the cellulose-binding site is located on the flavin domain.

1.5.4 Reaction Mechanism

CDH oxidizes cellobiose, cellotriose, cellotetraose, cellopentaose, and lactose in the presence of electron acceptors such as cytochrome *c*, 2,6-dichlorophenol-indophenol, quinones, ferricyanide, Mn(III)-malonate complex, and ferric iron and its complexes. However, cellobiose is the preferred substrate and cytochrome *c* is the preferred electron acceptor [Ayers et al., 1978; Morpeth, 1985; Henriksson et al., 1991; Kremer & Wood, 1992a; Samejima & Eriksson, 1992; Bao et al., 1993]. The physiological electron acceptor for CDH has not been identified.

The proposed catalytic cycle of CDH is shown in Fig. 1.7. Cellobiose is oxidized to cellobionolactone, and the electrons are first transferred to the FAD of CDH. Then, the reduced form of FAD transfers electrons to the heme iron, one electron at a time. The reduced heme in turn transfers the electrons to cytochrome *c*. The stopped-flow spectrophotometric analysis by Jones and Wilson [1988] indicated that, at pH 6.0, the reduction of flavin is a fast reaction, whereas the reduction of the heme is a slow reaction. Reduction of heme was observed to increase with CDH concentration. This suggested that the heme center in CDH is reduced preferentially by flavins in different molecules rather than by the flavin in the same molecule. This was in contrast to flavocytochrome *b*₂, in which the electron transfer from flavin to heme is intramolecular [Walker & Tollin, 1992]. Samejima et al. [1992] studied the reduction of 2,6-dichlorophenol-indophenol and cytochrome *c* by CDH at two pH levels. At pH 4.2, both compounds were effective electron acceptors. At pH 5.9, only 2,6-dichlorophenol-indophenol was active. They also studied the reduction of the FAD and heme of CDH by cellobiose at different pH using stopped-flow spectrophotometry. At pH 4.2, both FAD and heme were reduced with a high rate constant. At pH 5.9, only FAD was reduced. They concluded that the reduction of cytochrome *c* by CDH is dependent on heme, which functions at lower pH [Samejima et al., 1992].

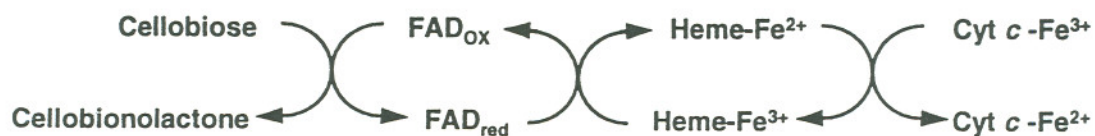


Fig. 1.7 Catalytic cycle of CDH [Renganathan & Bao, 1994].

[Reprinted with permission of the American Chemical Society; originally published in: Renganathan, V. and Bao, W. (1994) Cellobiose dehydrogenase, a hemoflavoenzyme from *Phanerochaete chrysosporium*. In *Enzymatic Conversion of Biomass for Fuels Production* (Himmel, M. E., Baker, J. O., and Overend, R. P., eds.), American Chemical Society, Washington, DC, pp. 179–187.]

Oxygen acts as a poor electron acceptor for CDH and is reduced to H_2O_2 [Wilson et al., 1990; Samejima et al., 1992; Bao et al., 1993]. The ratio of cellobiose oxidized to H_2O_2 produced has been determined to be approximately 1:1 [Bao et al., 1993]. Wilson et al. [1990] suggested that oxygen only reacts with the heme of CDH based on pre-steady-state kinetic studies. Kremer and Wood [1992a,b] reported that CDH produced hydroxyl radicals through Fenton reaction. The Fenton's reagents, H_2O_2 and Fe^{2+} , were produced by the reduction of O_2 and Fe(III) complex by CDH [Kremer & Wood, 1992a,b]. Subramaniam and Renganathan [1998] studied the production of H_2O_2 and hydroxyl radicals in the CDH reaction with oxygen and concluded that the stoichiometry of cellobiose oxidized and oxygen consumed was 1:1; the relationship between cellobiose oxidation and H_2O_2 formation was non-stoichiometric; a substantial portion of H_2O_2 appeared to be reduced to hydroxyl radicals depending on the reaction condition [Subramaniam, 1998]. Hydroxyl radical generation was inhibited in the presence of iron chelators such as EDTA and ferrozine. Surprisingly, chelators also inhibited oxygen uptake suggesting involvement of a non-heme iron as the site for oxygen reaction [Subramaniam, 1998].

1.5.5 Role of CDH in Lignocellulose Degradation

It has been proposed that CDH is involved in lignin degradation [Ander et al., 1990; Samejima & Eriksson, 1992]. *P. chrysosporium* secretes two important peroxidases—lignin peroxidase (LiP) and manganese peroxidase (MnP)—to degrade lignin [Kirk & Farrell, 1987; Gold et al., 1989]. Ander et al. [1990] demonstrated that the presence of CBQase and cellobiose increased lignin depolymerization by LiP. In the presence of cellobiose, CDH inhibits a variety of reactions catalyzed by LiP, MnP, and horseradish peroxidase (HRP) [Samejima & Eriksson, 1992; Bao et al., 1993]. Samejima and Eriksson [1992] demonstrated that CDH can reduce phenoxy radicals which are produced by phenol oxidases (LiP, MnP, and laccase) during lignin degradation. Phenoxy radicals can condense with themselves and with lignin [Wariishi et al., 1991]. The reduction of phenoxy radicals by CDH was hypothesized to prevent the repolymerization of phenoxy radicals and enhance lignin degradation [Ander et al., 1990; Samejima and Eriksson, 1992].

In addition to phenols, LiP also oxidizes nonphenolic compounds, such as 3,4-dimethoxybenzyl alcohol and methoxybenzenes, through the corresponding aromatic cation radical intermediate [Kirk & Farrell, 1987; Gold et al., 1989]. CDH can reduce these cation radical intermediates and inhibit LiP oxidation of nonphenolic compounds [Samejima & Eriksson, 1992].

In the LiP catalytic cycle, H_2O_2 oxidizes native LiP by two electrons to produce compound I. Suitable peroxidase substrates such as veratryl alcohol then reduce compound I by one electron to generate compound II. A further one-electron reduction of compound II by the substrate regenerates the native LiP [Renganathan & Gold, 1986; Kirk & Farrell, 1987; Gold et al., 1989]. In the presence of cellobiose, CDH reduces H_2O_2 -oxidized forms of LiP, MnP, and HRP [Ander et al., 1993; Renganathan & Bao, 1994]. The reversion rates for MnP compound II and LiP compound II back to native enzymes are increased significantly in the presence of CDH and cellobiose [Ander et al., 1993]. In the presence of a very low concentration of veratryl alcohol, the reduced CDH protected LiP from H_2O_2 -dependent inactivation [Wariishi & Gold, 1990; Cai & Tien, 1992; Renganathan & Bao, 1994].

CDH binds to microcrystalline cellulose, suggesting the presence of a cellulose-binding domain like those of cellulases [Renganathan et al., 1990]. CDH also can oxidize cellulose [Kremer & Wood, 1992a]. These findings suggest CDH is involved in cellulose degradation. Bao and Renganathan [1992] demonstrated that CDH from *P. chrysosporium* enhanced crystalline cellulose degradation by cellulases from *Trichoderma* sp. Addition of $10 \mu\text{g ml}^{-1}$ CDH to the cellulose hydrolysis reaction by *T. viride* cellulase increased both reducing sugar (glucose and cellobiose) production and cellulose weight loss. At a higher CDH level ($60 \mu\text{l}^{-1}$), reducing sugar production and cellulose weight loss were decreased due to inactivation of cellulase by H_2O_2 produced by the CDH reaction; addition of catalase to the hydrolysis mixture increased both reducing sugar production and cellulose weight loss [Bao & Renganathan, 1992].

There are two possible mechanisms for the CDH-dependent enhancement of cellulose hydrolysis. Cellobiose is an inhibitor of cellulase activity [Wood &

McCrae, 1978]; thus, by oxidizing cellobiose, CDH can reduce this inhibition and accelerate cellulose hydrolysis [Renganathan & Bao, 1994]. Alternatively, hydroxyl radicals produced by CDH might depolymerize cellulose and thus enhance cellulose hydrolysis [Kremer & Wood, 1992a,b; Henriksson et al., 1994; Mansfield et al., 1997].

Hydroxyl radicals are probably not involved in cellulose hydrolysis enhancement, because addition of catalase, which decomposes H_2O_2 , or addition of iron chelators, which inhibit hydroxyl radical production, did not affect CDH-dependent cellulose hydrolysis enhancement [Bao & Renganathan, 1992].

Igarashi et al. [1998] investigated the interaction of CDH with CBHI from *P. chrysosporium* and demonstrated that CDH enhanced CBHI activity by relieving competitive inhibition of cellobiose through its oxidation to cellobionolactone. The activity of CBHI for hydrolysis of the model substrate, *p*-nitrophenyl- β -D-cellobioside, was enhanced in the presence of the CDH/ferricyanide redox system. In the absence of the CDH/ferricyanide redox system, the K_m for hydrolysis of *p*-nitrophenyl- β -D-cellobioside was 384 μ M, whereas in the presence of the redox system, the K_m was much lower, 142 μ M. There was no significant difference between the k_{cat} values. Addition of cellobiose inhibited CBHI activity with a K_i value of 65 μ M. However, the inhibition did not occur if cellobiose was incubated with CDH before addition of CBHI. Their finding supports the hypothesis that CDH enhances cellulose hydrolysis by reducing the inhibition to cellulases caused by cellobiose.

1.6 RESEARCH SUMMARY

In order to further understand CDH structure, we initiated cloning of CDH cDNA. We isolated a 2.4-kb cDNA encoding CDH by screening an expression library of *P. chrysosporium* OGC101 with a CDH-specific polyclonal antibody. The cDNA encodes a 755 amino acid protein with a predicted mass of 80,115 Da. Sequence analysis suggests that the heme domain is located at the N-terminus and that the flavin domain is at the C-terminus. The flavin domain shows a $\beta 1\alpha A\beta 2$ motif for

FAD binding and has high sequence similarity with several FAD-dependent enzymes. Little sequence similarity is found with heme-dependent enzymes or hemoflavoenzymes. CDH binds to cellulose similarly to cellulases [Renganathan et al., 1990]. However, little sequence similarity is observed with the conserved cellulose binding sequences of cellulases. This suggests that CDH might possess a specific sequence for cellulose binding which is different from that of cellulases. Northern blot analysis of total RNA from cellulose-, glucose-, and cellobiose-grown *P. chrysosporium* indicated that CDH mRNA is produced only in cellulose-grown cells. This suggests that CDH expression is regulated at the transcriptional level by either cellulose or by one of its degradation products. Southern blot analysis suggests the presence of only a single gene for CDH in *P. chrysosporium* OGC101 [Li et al., 1996; see also Chapter 2].

We also isolated genomic clones of CDH by screening a λ EMBL3 genomic library of *P. chrysosporium* OGC101 [Godfrey et al., 1990] with a CDH cDNA probe. We cloned and sequenced both alleles of the *cdh* gene, *cdh-1* (3627 bp) and *cdh-2* (3623 bp). The nucleotide sequences of *cdh-1* and *cdh-2* exhibit 97% homology. A total of eighty-six different nucleotides are observed between *cdh-1* and *cdh-2*. Both alleles have 14 exons, and the introns are located at exactly the same position. The translation products of these alleles have identical amino acid sequences. Restriction fragment length polymorphism analyses of homokaryotic derivatives show segregation of *cdh-1* and *cdh-2*, indicating that they are two alleles of the same gene [Li et al., 1997; also see Chapter 3].

To further understand structure-function relationships of CDH by site-directed mutagenesis, an efficient expression system for recombinant CDH protein was required. Gold and coworkers achieved homologous expression of manganese peroxidase (MnP) from *P. chrysosporium* by placing *mnp* under the control of the glyceraldehyde-3-phosphate dehydrogenase (*gpd*) promoter [Mayfield et al., 1994]. By using the same homologous expression system, we expressed recombinant CDH (rCDH) at a high level in glucose cultures by placing *cdh1* under the control of the *gpd* promoter. We purified rCDH by ammonium sulfate precipitation, Sephacryl S-

200 chromatography, and FPLC using a Mono-Q 5/5 column. Molecular weight and spectral and kinetic characteristics of pure rCDH were similar to those of wild-type CDH. We constructed a mutant pM65A in which the putative heme iron ligand, methionine 65, is mutated to an alanine. The purified mutant protein is a flavoprotein with a molecular mass of 67,000 Da and is capable of transferring electrons to DCPIP, but not to cytochrome *c* [Chapter 4].

In Chapter 5, we report the cDNA and genomic sequences of a novel cellulose-binding extracellular β -glucosidase (CBGL) from cellulose-degrading cultures of *P. chrysosporium* OGC101. A 2.4-kb cDNA was isolated by screening an expression library of *P. chrysosporium* with a CBGL-specific polyclonal antibody. The cDNA encoded an 802-amino acid protein with a predicted mass of 83,439 Da. The N-terminal sequence of the mature protein showed high sequence similarity with the cellulose-binding domain (CBD) sequence of cellobiohydrolase II from *P. chrysosporium* indicating the presence of a CBD at the N-terminus. The CBD is linked to the C-terminal catalytic domain via a hydroxyamino acid-enriched linker sequence. The catalytic domain showed high sequence similarity with family 3 glucosidases from *T. reesei*, *A. aculeatus*, and *Pichia capsulata*. A genomic clone of *cbgl*, *cbgl-2* (4.5 kb), was obtained by screening a λ EMBL3 genomic library of *P. chrysosporium* [Godfrey et al., 1990] with *cbgl* cDNA. Comparison of cDNA and genomic (*cbgl-2*) sequences suggested that CBGL is encoded by 30 exons. Exon sequences of *cbgl-2* exhibited 98% homology to the *cbgl* cDNA sequence. A total of 50 bp in the exon sequences of *cbgl-2* did not match the cDNA sequence. However, the amino acid sequences differed only at four locations. Southern analysis of *P. chrysosporium* DNA suggested that *cbgl* is probably encoded by two alleles (*cbgl-1* and *cbgl-2*) of a single gene and that the cDNA sequence was presumably derived from *cbgl-1*. Northern blot analysis of total mRNA from cellulose-, glucose-, and cellobiose-grown *P. chrysosporium* indicated that CBGL mRNA is produced only in cellulose-grown cells. This suggested that CBGL expression is regulated at the transcriptional level by cellulose or one of its degradation products.

CHAPTER 2

CLONING OF A cDNA ENCODING CELLOBIOSE DEHYDROGENASE, A HEMOFLAVOENZYME FROM *PHANEROCHAETE CHRYSOSPORIUM**

2.1 INTRODUCTION

Cellulolytic fungi such as *Phanerochaete chrysosporium*, *Sporotrichum thermophile*, and *Coniophora puteana* produce extracellular cellobiose dehydrogenases (CDH). CDH is a novel hemoflavoenzyme containing one heme *b* and one FAD per molecule [Ayers et al., 1978; Canevascini et al., 1991; Bao et al., 1993; Schmidhalter & Canevascini, 1993]. CDH oxidizes the reducing ends of cellobiose, celooligosaccharides, and even cellulose in the presence of electron acceptors such as cytochrome *c*, quinones, and Fe(III) and Mn(III) complexes [Ayers et al., 1978; Henriksson et al., 1991; Kremer & Wood, 1992; Samejima & Eriksson, 1992; Bao et al., 1993]. Steady-state kinetic studies suggest that cellobiose is the preferred substrate and that cytochrome *c* is the preferred electron acceptor [Bao et al., 1993]. In the absence of suitable electron acceptors, oxygen functions as an electron acceptor and is reduced to H₂O₂ [Bao et al., 1993]. The heme and flavin cofactors of CDH are bound to separate domains [Henriksson et al., 1991; Habu et al., 1993]. Extracellular protease(s) from cellulose-degrading cultures of *P. chrysosporium* and papain hydrolyze CDH into a flavopeptide and a heme peptide [Henriksson et al., 1991; Habu et al., 1993]. The flavopeptide is catalytically active and oxidizes

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Li, B., Nagalla, S. R., and Renganathan, V. (1996) Cloning of a cDNA encoding cellobiose dehydrogenase, a hemoflavoenzyme from *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **62**, 1329–1335.

cellobiose in the presence of all electron acceptors for CDH except cytochrome *c* [Henriksson et al., 1991; Habu et al., 1993]. Cellulose-degrading cultures of *P. chrysosporium*, in addition to CDH, produce another cellobiose-oxidizing flavoenzyme, cellobiose:quinone oxidoreductase (CBQase) [Westermarck & Eriksson, 1975; Bao et al., 1994]. However, recent proteolysis experiments suggest that CBQase is possibly the flavopeptide formed from extracellular proteolytic degradation of CDH [Henriksson et al., 1991; Habu et al., 1993]. The heme iron of CDH is ferric and hexacoordinate [Ayers et al., 1978; Bao et al., 1993]. Cox et al. have suggested that CDH has a histidine and a methionine as the fifth and sixth coordinations to the heme iron [Cox et al., 1992].

CDH appears to be part of the cellulolytic system of *P. chrysosporium*, because CDH is produced only when cellulose is provided as the carbon source [Bao et al., 1994] and its substrate cellobiose is formed from exocellobiohydrolase hydrolysis of cellulose. Bao and Renganathan [1992] demonstrated that CDH enhances microcrystalline cellulose hydrolysis by *Trichoderma viride* cellulase. Cellobiose is an inhibitor of cellulase; CDH could be reducing this inhibition by oxidizing cellobiose to cellobionolactone [Bao & Renganathan, 1992]. CDH has also been suggested to play a role in lignin degradation by *P. chrysosporium* [Ander, 1994]. Depolymerization of lignin by lignin and manganese peroxidases generates reactive phenoxy radicals which tend to condense with themselves and with the lignin substrate. Reduction of such phenoxy radicals by CDH and CBQase has been proposed to prevent these polymerization reactions and thus increase the rate of depolymerization [Ander, 1994].

Among the cellulolytic enzymes produced by *P. chrysosporium*, only the cellobiohydrolase gene has been cloned and sequenced [Covert et al., 1992a; Covert et al., 1992b; Sims et al., 1994]. Cellobiohydrolase appears to be encoded by a family of genes [Covert et al., 1992a; Covert et al., 1992b; Sims et al., 1994]. Though endoglucanase has been purified from *P. chrysosporium*, a specific gene for that enzyme has not been isolated and sequenced [Sims et al., 1994]. Sims et al. have suggested that a CBH I-like protein might be exhibiting endoglucanase activity in *P.*

chrysosporium [Sims et al., 1994]. The *P. chrysosporium* cellulolytic system is unique in that all of its enzymes—cellobiohydrolase, endoglucanase, β -glucosidase, and CDH—bind to cellulose [Renganathan et al., 1990; Lymar et al., 1995]. We recently demonstrated that the extracellular β -glucosidase from *P. chrysosporium* binds to cellulose and that it might be organized into two domains: a cellulose-binding domain and a catalytic domain [Lymar et al., 1995]. Herein we report the molecular cloning and characterization of a cDNA encoding CDH from *P. chrysosporium*. Recently, Raices et al. [1995] reported a cDNA sequence for a CDH from *P. chrysosporium* strain K3. Whereas the two sequences are homologous, differences are observed in the cDNA and the deduced amino acid sequences.

2.2 MATERIALS AND METHODS

2.2.1 Organisms

P. chrysosporium OGC101 (a derivative of BKM-F-1767) was obtained from Dr. Michael H. Gold of the Oregon Graduate Institute [Alic et al., 1987]. *Escherichia coli* strains XL1-Blue, XL1-Blue MRF', and SOLR were obtained from Stratagene, La Jolla, CA.

2.2.2 Enzymes and Nucleotides

λ ZAP-cDNA synthesis and Picoblue immunoscreening kits and Gigapack II Gold packaging extract were purchased from Stratagene, La Jolla, CA. [α -Thio- 35 S]ATP was obtained from DuPont NEN Research Products, Boston, MA. Oligonucleotides were prepared by Oligos Etc., Wilsonville, OR. The plasmid isolation kit was obtained from Qiagen Inc., Chatsworth, CA. Papain was purchased from Boehringer Mannheim Inc.

2.2.3 CDH Polyclonal Antibody

A polyclonal antibody against CDH was raised in rabbits at the Pocono Rabbit Farm and Laboratory (Canadensis, PA). The CDH antiserum was used in

immunoscreening without further purification. A 2,000-fold-diluted antiserum was able to detect 1 ng of CDH.

2.2.4 N-Terminus Protein Sequence Analyses

The N-terminus sequences of CDH and its heme and flavin domains were analyzed. CDH was purified from cellulose-degrading cultures of *P. chrysosporium* as described [Bao et al., 1993; Bao et al., 1994]. The flavin and heme domains were prepared by incubating homogeneous CDH (3 mg) with papain (75 μ g) in 0.1 M phosphate buffer (pH 7, 1 ml) containing EDTA (2 mM) and dithiothreitol (2 mM) for 3 h at room temperature as described previously [Henriksson et al., 1991]. The reaction products were purified on a Sephacryl S-200 column (58 \times 2.8 cm) equilibrated with 50 mM phosphate (pH 6). CDH elution was monitored by cytochrome *c* assay [Bao et al., 1993]. Elution of the flavin and heme domains was followed by their absorbance at 420 and 450 nm, respectively. Heme and flavin domains were further purified by fast protein liquid chromatography (FPLC) using a Mono-Q column (Pharmacia Fine Chemicals, Piscataway, NJ). FPLC separations were performed in 10 mM Tris-HCl (pH 8), and proteins were eluted with a 1 M NaCl gradient. Fractions containing heme and flavin domains were separated and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The N-terminus sequence was analyzed using the Edman degradation method. These analyses were performed at the Center for Gene Research and Biotechnology, Oregon State University, Corvallis, OR.

2.2.5 cDNA Library Construction and Screening

P. chrysosporium was grown in a succinate medium containing 1% cotton linters as the carbon source [Bao et al., 1994]. On the eleventh day, cells were harvested by filtration and homogenized using a Polytron homogenizer in sodium citrate buffer (25 mM, pH 7) containing guanidinium isothiocyanate (50%) and lauryl sarcosine (0.5%). The total RNA was isolated from the cell extract by CsCl centrifugation [Chirgwin et al., 1979]. Poly(A) RNA was separated from the total RNA with an Oligo(dT) cellulose column [Aviv & Leder, 1972]. A cDNA library

was prepared from the poly(A) RNA by the method of Gubler and Hoffman [1983], with a commercial λ ZAPII cDNA synthesis kit (Stratagene, La Jolla, CA). The lambda library was packaged (Gigapack II Gold packaging extract, Stratagene, La Jolla, CA) and plated on *E. coli* XL1-Blue MRF'. The plaques were screened with anti-CDH antibody and a secondary antibody labeled with alkaline phosphatase.

2.2.6 cDNA Sequencing

The pBluescript SK(-) plasmid containing a putative CDH cDNA insert was rescued by *in vivo* excision using a helper phage. The plasmid was purified using a commercial plasmid isolation kit (Qiagen Inc., Chatsworth, CA) [Birnboim & Doly, 1979]. The cDNA was sequenced by the dideoxy method utilizing the "primer walking" strategy [Sanger et al., 1977; Strauss et al., 1986]. All DNA sequencing was performed with a TAQuence Version 2.0 sequencing kit (United States Biochemicals, Cleveland, OH). Initial sequencing was performed with vector primers flanking the cDNA. The internal sequences were obtained using cDNA-specific 17-mer oligonucleotides. Chain extension products were labeled with [α -³⁵S]ATP. 7-Deaza-dGTP was substituted for dGTP to avoid compression artifacts in G+C rich regions of cDNA. Sequence analysis was performed using DNASTAR (Madison, WI). PCR was used for determining the 5'-end sequence of CDH cDNA. The cDNA library was amplified using the vector-flanking T3 primer as the 5' primer and a 3' primer (ACAGGGTCGGTGATACCAGTG) designed from a CDH cDNA sequence [Nagalla et al., 1994]. PCR conditions were 35 cycles of 92°C for 1 min, 55°C for 1 min, and 72°C for 5 min, using 2.5 U *Taq* polymerase (Promega, Madison, WI). The PCR product was subcloned into the PGEM-T vector (Promega) and sequenced as described [Nagalla et al., 1994].

2.2.7 Northern Blot Analysis

Total RNA was isolated from 11-days-old mycelia of *P. chrysosporium* cultured with 1% cotton linters, cellobiose, or glucose as the carbon source. RNA was electrophoresed in 1.5% agarose gel containing 2.2 M formaldehyde, transferred

to Magnagraph nylon membranes (Microseparations Inc.) and probed with CDH cDNA at 42°C as described [Brown, 1994b].

2.2.8 Southern Blot Analysis

DNA from *P. chrysosporium* strains OGC101 and K3 was restriction digested and electrophoresed using a 0.7% agarose gel. The DNA was transferred to Magnagraph nylon membranes and hybridized to a ³²P-labeled CDH cDNA fragment (192–591 nucleotides) [Brown, 1994a]. For this purpose, a PCR product of CDH cDNA from 192–591 nucleotides was prepared, and this DNA fragment was used as a template for random-primed synthesis of a ³²P-labeled probe.

2.2.9 Nucleotide Sequence Accession Number

The *P. chrysosporium* OGC101 CDH cDNA sequence data reported here have been deposited in GenBank under accession number U46081.

2.3 RESULTS AND DISCUSSION

CDH is a novel hemoflavoenzyme containing one FAD and one heme *b* per molecule [Ayers et al., 1978; Bao et al., 1993]. Other enzymes that are characterized as hemoflavoenzymes include flavocytochrome *b*₂ or lactate dehydrogenase from *Saccharomyces cerevisiae* [Xia et al., 1987], flavocytochrome *c* or fumarate reductase from *Shewanella putrifaciens* [Pealing et al., 1992], spermidine dehydrogenase from *Serratia marcescens* [Tabor & Kellogg, 1970], mandelate dehydrogenase from *Rhodotorula graminis* [Yasin & Fewson, 1993], rubredoxin oxidase from *Desulfovibrio gigas* [Chen et al., 1993], fatty acid monooxygenase from *Bacillus megaterium* [Narhi & Fulco, 1986], and the nitric oxide synthetases from murine macrophages, rat cerebellum, and bovine aortic endothelial cells [Marletta, 1993]. Except for CDH, all the other hemoflavoenzymes are intracellular [Tabor & Kellogg, 1970; Narhi & Fulco, 1986; Xia et al., 1987; Pealing et al., 1992; Chen et al., 1993; Marletta, 1993; Yasin & Fewson, 1993]. Among these enzymes, flavocytochrome *b*₂,

fumarate reductase, fatty acid monooxygenase, and nitric oxide synthetase have been cloned and sequenced [Guiard, 1985; Ruettinger et al., 1989; Bredt et al., 1991; Pealing et al., 1992; Marletta, 1993].

2.3.1 CDH cDNA Sequence

Twenty-six positive clones of CDH were isolated by immunoscreening the λ cDNA expression library. A clone with the largest insert (2.5 kb) was sequenced and analyzed (Fig. 2.1). The sequence at the 5' end was obtained by PCR amplification of the library using a 3'-specific primer and a T3 primer. Sequence analysis revealed an open reading frame (ORF) consisting of 2319 bp encoding 773 amino acids. The ORF is flanked by 17 bp of 5'-noncoding sequence and 88 bp of 3'-noncoding sequence excluding the poly(A) tail. The G+C content of the coding region, 5'- and 3'-noncoding regions are 59.2%, 58.8%, and 38.7%, respectively. The G+C content of the coding and the 5'-noncoding regions are similar to that of *P. chrysosporium* genomic DNA (59%); however, the G+C content of the 3'-noncoding region is lower than that of genomic DNA [Raeder & Broda, 1984]. The order of preference for the third codon in a codon family is C > G > T > A. Highly expressed or constitutive genes of filamentous fungi prefer codons ending in C and avoid codons ending in A [Ballance, 1986]. A similar codon bias has been observed for lignin and manganese peroxidases produced by *P. chrysosporium* [de Boer et al., 1987; Ritch & Gold, 1992].

2.3.2 Authenticity of the CDH cDNA Clone

To verify that this cDNA codes for CDH, the protein sequence of CDH was compared with the cDNA sequence. The N-terminus of CDH is blocked; consequently an N-terminal sequence of CDH protein could not be obtained for comparison with the cDNA sequence. The holoenzyme was hydrolyzed into two peptide fragments, one containing the heme and the other containing the flavin cofactor [Henriksson et al., 1991]. The individual peptides were purified and their N-termini were sequenced. The heme domain did not provide any sequence suggesting that its N-terminus is blocked. This finding also suggested that this domain is located

1	ct	tcggtcgagt	tcacg	atg	cta	ggt	cga	tcg	tta	ctt	gcg	ctt	ctg	cct	ttt	gta				
				M	L	G	R	S	L	L	A	L	L	P	F	V	-6			
	ggc	ctc	gcg	ttc	tcg	cag	agt	gcc	tca	cag	ttt	acc	gac	cct	acc	aca	gga	ttc	cag	
	G	L	A	F	S	Q	S	A	S	Q	F	T	D	P	T	T	G	F	Q	14
	ttc	act	ggt	atc	acc	gac	cct	ggt	cat	gac	gtg	acc	tac	ggc	ttc	gtt	ttc	ccc	cct	
	F	T	G	I	T	D	P	V	H	D	V	T	Y	G	F	V	F	P	P	33
	ctg	gcc	acc	tcc	gga	gcg	caa	tcc	act	gag	ttc	atc	gga	gag	gtt	gtt	gcc	ccc	atc	
	L	A	T	S	G	A	Q	S	T	E	F	I	G	E	V	V	A	P	I	52
	gca	tca	aaa	tgg	att	ggt	att	gcc	ctc	ggt	ggc	gcc	atg	aac	aac	gac	ctg	cta	ctt	
	A	S	K	W	I	G	I	A	L	G	G	A	M	N	N	D	L	L	L	71
	gtg	gct	tgg	gcc	aac	ggc	aac	caa	att	gtt	tcc	tcc	act	cgc	tgg	gct	act	ggc	tat	
	V	A	W	A	N	G	N	Q	I	V	S	S	T	R	W	A	T	G	Y	90
	gta	cag	cgc	act	gca	tat	acg	gga	act	gcc	act	ttg	aca	aca	ctc	cct	gag	aca	acc	
	V	Q	P	T	A	Y	T	G	T	A	T	L	T	T	L	P	E	T	T	109
	atc	aac	tcc	acg	cac	tgg	aag	tgg	gtc	ttc	agg	tgt	cag	ggc	tgc	act	gag	tgg	aac	
	I	N	T	H	W	K	W	V	F	R	C	Q	G	C	T	E	W	N		128
	aat	ggc	ggc	gga	atc	gac	gtc	act	agc	cag	ggc	gtt	ctg	gcg	tgg	gca	ttc	tcc	aac	
	N	G	G	G	I	D	V	T	S	Q	G	V	L	A	W	A	F	S	N	147
	gtc	gcc	gtc	gac	gac	ccc	tcc	gac	ccg	cag	agt	acc	ttc	agc	gag	cac	acc	gac	ttc	
	V	A	V	D	P	S	D	P	Q	S	T	F	S	E	H	T	D	F		166
	ggc	ttc	ttc	gga	att	gac	tac	tcg	acc	gcg	cac	agc	gcc	aac	tac	cag	aac	tac	ctt	
	G	F	F	G	I	D	Y	S	T	A	H	S	A	N	Y	Q	N	Y	L	185
	aat	ggc	gac	tcc	ggc	aac	cct	acg	acc	acg	agc	acc	aag	ccc	aca	agc	acg	agc	agc	
	N	G	D	S	G	N	P	T	T	T	S	T	K	P	T	S	T	S	S	204
	tca	gtc	acg	act	gga	ccc	act	gtt	tct	gct	aca	cct	tac	gat	tac	atc	atc	gtc	ggc	
	S	V	T	T	G	P	T	V	S	A	T	P	Y	D	Y	I	I	V	G	223
	gct	ggc	cct	ggc	ggt	atc	att	gca	gct	gat	cgt	ctg	tcg	gag	gct	ggc	aag	aag	gtt	
	A	G	P	G	G	I	I	A	A	D	R	L	S	E	A	G	K	K	V	242
	ctc	ctt	ctc	gag	cgc	ggt	ggc	cct	agc	acc	aag	cag	acc	ggt	gga	acg	tat	gtc	gct	
	L	L	L	E	R	G	G	P	S	T	K	Q	T	G	G	T	Y	V	A	261
	cca	tgg	gct	act	agc	agt	ggt	cta	acg	aag	ttc	gat	att	ccc	gga	ctg	ttc	gag	tcc	
	F	W	A	T	S	S	G	L	T	K	F	D	I	P	G	L	F	E	S	280
	ttg	ttc	act	gat	tcc	aac	ccc	ttc	tgg	tgg	tgc	aaa	gac	atc	aca	gtc	ttc	gct	ggc	
	L	F	T	D	S	N	P	F	W	W	C	K	D	I	T	V	F	A	G	299
	tgc	ctg	gtc	ggc	ggc	ggt	act	tcg	gtc	aac	gga	gct	ctc	tac	tgg	tac	cct	aac	gac	
	C	L	V	G	G	G	T	S	V	N	G	A	L	Y	W	Y	P	N	D	318
	ggc	gac	ttc	tcc	tcg	agc	gtt	ggc	tgg	cca	agc	agc	tgg	acc	aac	cac	gcc	ccg	tac	
	G	D	F	S	S	S	V	G	W	P	S	S	W	T	N	H	A	P	Y	337
	acg	agc	aag	ctt	tcg	tct	cgt	ctc	ccc	agt	acg	gac	cac	cct	tcg	act	gat	ggc	cag	
	T	S	K	L	S	S	R	L	P	S	T	D	H	P	S	T	D	G	Q	356
	cgc	tac	ctt	gag	caa	tca	ttc	aac	gtc	gtg	tct	caa	ctt	ctc	aaa	ggc	caa	ggc	tac	
	R	Y	L	E	Q	S	F	N	V	V	S	Q	L	L	K	G	Q	G	Y	375
	aac	cag	gcc	acc	atc	aac	gac	aac	ccc	aac	tac	aag	gac	cac	gtc	ttc	ggc	tac	agc	
	N	Q	A	T	I	N	D	N	P	N	Y	K	D	H	V	F	G	Y	S	394
	gca	ttc	gat	ttc	ctt	aac	ggc	aag	cgt	gct	ggc	cca	gtc	gcc	acc	tac	ctc	cag	acg	
	A	F	D	F	L	N	G	K	R	A	G	P	V	A	T	Y	L	Q	T	413
	gca	ttg	gct	cgc	ccc	aac	ttc	act	ttc	aag	acc	aac	gtc	atg	gtc	tcg	aac	ggt	gtc	
	A	L	A	R	P	N	F	T	F	K	T	N	V	M	V	S	N	V	V	432
	cgc	aac	ggc	tcg	cag	atc	ctc	ggt	gtc	cag	acg	aac	gac	ccg	acg	ctc	ggc	ccc	aac	
	R	N	G	S	Q	I	L	G	V	Q	T	N	D	P	T	L	G	P	N	451
	ggt	ttc	atc	ccc	gtg	acc	ccg	aag	ggg	cgt	gtc	atc	ctc	tct	gct	ggc	gca	ttt	ggc	
	G	F	I	P	V	T	P	K	G	R	V	I	L	S	A	G	A	F	G	470
	act	tcg	cgc	att	ctc	ttc	caa	agc	ggt	att	ggc	ccc	acg	gat	atg	att	cag	act	ggt	
	T	S	R	I	L	F	Q	S	G	I	G	P	T	D	M	I	Q	T	V	489
	cag	agc	aac	ccg	acc	gcc	gcc	gcc	gcg	ctc	ccg	ccg	cag	aac	cag	tgg	atc	aac	ctc	
	Q	S	N	P	T	A	A	A	A	L	P	P	Q	N	Q	W	I	N	L	508
	cca	gtc	ggc	atg	aac	gca	cag	gac	aac	ccc	tcg	atc	aac	ctg	gtc	ttc	acc	cac	ccc	
	P	V	G	M	N	A	Q	D	N	P	S	I	N	L	V	F	T	H	P	527
	agc	atc	gat	gcc	tat	gag	aac	tgg	gct	gac	gtc	tgg	agc	aac	ccg	cgc	ccg	gct	gac	
	S	I	D	A	Y	E	N	W	A	D	V	W	S	N	P	R	P	A	D	546
	gct	gca	cag	tac	ctc	gcg	aac	cag	tcc	ggc	gtc	ttc	gca	ggc	gct	tct	ccc	aaa	ctc	
	A	A	Q	Y	L	A	N	Q	S	G	V	F	A	G	A	S	P	K	L	565
	aac	ctt	tgg	cgc	gca	tac	tct	ggc	tcg	gat	ggc	ttt	acc	cgt	tat	gcc	cag	ggg	acg	
	N	F	W	R	A	Y	S	G	S	D	G	F	T	R	Y	A	Q	G	T	584
	gtg	cgc	ccg	ggc	gca	gcc	tcc	gtg	aac	tcc	tcg	ctg	ccg	tac	aac	gcg	agc	cag	atc	
	V	R	P	G	A	A	S	V	M	S	S	L	P	Y	N	A	S	Q	I	603
	ttc	acg	atc	acc	gtg	tac	ctc	tct	acg	ggc	atc	cag	tcg	cgt	ggg	cgc	atc	ggc	atc	
	F	T	I	T	V	Y	L	S	T	G	I	Q	S	R	G	R	I	G	I	622
	gat	gca	gcg	ctc	cgc	ggt	acg	gtg	ctc	aca	ccg	ccg	tgg	ctc	gtg	aat	ccg	gtc	gac	
	D	A	A	L	R	G	T	V	L	T	P	P	W	L	V	N	P	V	D	641
	aag	acc	gtg	ctc	ctg	cag	gcg	ctg	cac	gac	gtc	gtc	tcg	aac	ata	ggg	tcg	att	ccc	
	K	T	V	L	L	Q	A	L	H	D	V	V	S	N	I	G	S	I	P	660
	ggc	ctg	acg	atg	atc	acg	ccc	gac	gtc	acg	cag	aca	ctc	gag	gag	tac	gtc	gat	gcg	
	G	L	T	M	I	T	P	D	V	T	Q	T	L	E	E	Y	V	D	A	679
	tac	gac	ccc	gcg	acg	atg	aac	tcg	aac	cac	tgg	gtc	tcg	tcc	acg	acg	atc	ggc	tca	
	Y	D	P	A	T	M	N	S	N	H	W	V	S	S	T	T	I	G	S	698
	tct	ccc	cag	agc	gcg	gta	gtc	gat	tcg	aac	gtc	aag	gtc	ttt	ggc	acg	aac	aac	ctg	
	S	P	Q	S	A	V	V	D	S	N	V	K	V	F	G	T	N	N	L	717
	ttt	atc	gtc	gac	gca	ggc	atc	att	ccc	cac	ctg	ccc	acg	ggc	aac	ccc	cag	ggc	acg	
	F	I	V	D	A	G	I	I	P	H	L	P	T	G	N	P	Q	G	T	736
	ctc	atg	tct	gcc	gag	cag	gcg	gcc	gcg	agc	atc	ctc	gcg	ctt	gcg	gga	ggc	cct		
	L	M	S	A	A	E	Q	A	A	A	K	I	L	A	L	A	G	G	P	755
	tgagcg	aaattctttta	tattactgtc	ctcggggctg	tagacgtaaa	aacggatgtt	atactatcga													
	tgga	aaaaaaa	tacgtttgtc	ccatcaaaaa	aaaaaaaaaa	aaa	2445													

Fig. 2.1 Nucleotide and deduced amino acid sequence of CDH from *P. chrysosporium*. Amino acids are numbered on the right and nucleotides on the left. The potential signal peptide sequence is overlined. The predicted heme domain is boxed. Amino-terminus sequence of the flavin domain obtained from protein sequencing is indicated by a dotted underline. Potential N-glycosylation sites are circled. Amino acid sequence which does not correspond to that of Raices et al. [1995] is indicated in bold type.

at the N-terminus of the holoenzyme. A sequence of 28 amino acids at the N-terminus of the flavin domain was obtained. This sequence, TGPXVXAXPYDYIIVGAGPGGIIAADRL, matched residues 208–235 predicted by the cDNA except at positions 211(T), 213(S), and 215(T) (Fig. 2.1). Since the unidentified residues are hydroxyamino acids, they might be glycosylated and thus could have escaped detection. Expression cloning with a CDH antibody, and the match between the cDNA and protein sequences, suggested that this cDNA encodes CDH.

2.3.3 CDH Structure

CDH is a secreted enzyme and, therefore, it is likely to possess a signal peptide (SP) sequence. Since the N-terminal amino acid sequence of mature CDH was not determined, the SP cleavage site and the size of the signal peptide could not be deduced. However, the empirical approach of von Heijne [1985, 1986] for SP identification suggested that the first 18 amino acids of the cDNA translation product constituted the SP sequence, and that the most probable cleavage site was located between Ser and Gln. The latter was presumably the N-terminal amino acid and is numbered 1 in Fig. 2.1. This is in agreement with Raices et al. [1995]. Thus, the mature protein of CDH appears to consist of 755 amino acids with a calculated molecular mass of 80,115 Da. According to Raices et al. [1995], the mature protein of CDH consists of 752 amino acids with a calculated molecular mass of 80,313 Da. The CDH molecular weight as determined by SDS-PAGE is 90,000 Da [Ayers et al., 1978; Bao et al., 1993]. CDH is a glycoprotein [Bao et al., 1993] and the difference in molecular weights could be attributable to carbohydrate. The cDNA sequence revealed six potential N-glycosylation sites conforming to the general rule Asn-X-Thr/Ser in which X is not a proline [Kornfeld & Kornfeld, 1985]. In addition, numerous potential O-glycosylation sites were present.

In the yeast flavocytochrome b_2 , the heme domain is at the N-terminus and the flavin domain is at the C-terminus. The two domains are joined by a linker region [Guiard, 1985; Xia et al., 1987]. A similar organization is found in CDH. Amino acids 1–192 appear to form the heme domain; and residues 193–207, which are

enriched with hydroxyamino acids, appear to form the linker region, and residues 208–755 appear to form the flavin domain. A methionine and a histidine have been suggested as the fifth and sixth ligands of the heme iron [Cox et al., 1992]. The predicted presence of four histidines and at least one methionine in the heme domain sequence further supports this suggestion.

2.3.4 Comparison with FAD-dependent Enzymes

FAD-dependent enzymes possess a conserved $\beta 1$ - α A- $\beta 2$ motif for binding the ADP substructure of FAD [Wierenga et al., 1986]. This motif is usually located at the N-terminus of the protein. In this motif, comprising about 30 amino acids, there are three conserved glycine residues with the sequence Gly-X-Gly-X-X-Gly, where X is any residue [Wierenga et al., 1986]. In addition, there are six hydrophobic residues, which form a hydrophobic core between the helix and the β -strand, and one conserved Asp [Wierenga et al., 1986]. In CDH, this FAD-binding fingerprint is located at the N-terminus of the flavin domain between residues 218 and 246. The flavin domain of CDH exhibited sequence similarity at the N-terminus with other FAD-dependent enzymes such as glucose oxidase from *Aspergillus niger* [Frederick et al., 1990], methanol oxidase from *Hansenula polymorpha* [Ledeboer et al., 1985], and alcohol dehydrogenase from *Pseudomonas oleovorans* [van Bielen et al., 1992] (Fig. 2.2). The C-terminus of CDH also exhibits extensive sequence similarity with these enzymes. The overall sequence similarity between the flavin domain of CDH and these FAD-dependent enzymes is approximately 50%.

2.3.5 Comparison with Hemoflavoenzymes

Hemoflavoenzymes are organized into two domains—an N-terminal heme-binding domain, and a C-terminal flavin-binding domain [Narhi & Fulco, 1986; Xia et al., 1987; Pealing et al., 1992; Marletta, 1993; Yasin & Fewson, 1993]. In the case of flavocytochrome b_2 and mandelate dehydrogenase, the FMN-binding domain oxidizes the organic substrate by two electrons and, subsequently, transfers these electrons to the heme domain one electron at a time [Xia et al., 1987; Yasin & Fewson, 1993]. The heme domain in turn transfers the electrons to ferricytochrome

A. Amino - terminus

CDH 218	D	Y	I	I	V	G	G	P	G	I	I	A	D	R	L	S	E	-	-	A	G	K	-	K	V	L	L	E			
GOD 21	D	Y	I	I	A	G	G	L	T	G	L	T	T	A	A	R	L	T	E	-	-	N	P	N	I	S	V	L	V	E	
ADH 3	D	Y	I	I	V	G	A	G	S	A	G	C	V	L	A	N	R	L	S	A	-	-	D	P	S	K	R	V	C	L	E
MO 8	D	I	I	V	V	G	G	S	T	G	C	C	I	A	G	R	L	A	N	L	D	D	Q	N	L	T	V	A	L	I	E

B. Carboxy - terminus

CDH 704	V	V	D	S	N	V	K	V	F	G	T	N	N	L	F	I	V	D	A	G	I	I	P
GOD 532	V	V	D	N	A	A	R	V	Y	G	V	Q	G	L	R	V	I	D	G	S	I	P	P
ADH 484	V	V	D	P	C	L	K	I	R	G	L	A	N	I	R	V	V	D	A	S	I	M	P
MO 590	V	L	D	A	R	L	N	V	Y	G	V	Q	N	L	K	V	A	D	L	S	V	C	P

Fig. 2.2 Comparison of CDH flavin domain with FAD-dependent enzymes. (A) Amino-terminus region of CDH (218–264) is compared with glucose oxidase (GOD) (21–50) from *A. niger* [Frederick et al., 1990], alcohol dehydrogenase (ADH) (4–33) from *P. oleovorans* [van Bielen et al., 1992], and methanol oxidase (MO) (8–39) from *H. anomala* [Ledeboer et al., 1985]. (B) Carboxy-terminus region of CDH (704–726) is compared with GOD (532–555), ADH (484–506), and MO (590–612). N-Terminus residues involved in FAD binding are indicated in bold type. Identical amino acids are enclosed in solid boxes.

c. The heme domain of the dehydrogenase class is a hexacoordinated cytochrome b_5 or cytochrome c [Xia et al., 1987; Pealing et al., 1992; Yasin & Fewson, 1993], whereas the corresponding domain for monooxygenases such as fatty acid monooxygenase and nitric oxide synthetases is a cytochrome P-450 [Narhi & Fulco, 1986; Marletta, 1993]. Although many biochemical properties of CDH are similar to those of flavocytochrome b_2 [Ayers et al., 1978; Bao et al., 1993], CDH exhibits very little sequence similarity with flavocytochrome b_2 .

2.3.6 Cellulose Binding by CDH

Earlier, we demonstrated that CDH binds to microcrystalline cellulose and enhances the hydrolysis of this cellulose by *Trichoderma* cellulases [Bao & Renganathan, 1992; Renganathan et al., 1990]. Recent experiments suggest that CDH can also bind to cotton linters and filter paper and enhance the hydrolysis of these celluloses by *Trichoderma* cellulases (Subramaniam, Bao, and Renganathan, unpublished results). The flavin domain of CDH binds to crystalline cellulose, whereas the heme domain does not bind to cellulose, suggesting that the cellulose-binding characteristic of CDH resides in the flavin domain [Henriksson et al., 1991; Habu et al., 1993].

Cellulose-binding bacterial and fungal cellulases are organized into two domains, a cellulose-binding domain (CBD) and a catalytic domain [Gilkes et al., 1991]. These two domains are connected usually by a linker region consisting of proline and threonine repeats. In fungal cellulases such as those of *Trichoderma reesei* and *P. chrysosporium*, the CBD consists of a conserved sequence of 33 amino acids [Gilkes et al., 1991]. In bacterial cellulase, CBD is larger and consists of approximately 100 amino acids [Gilkes et al., 1991]. Proteolytic hydrolysis experiments have not indicated a separate CBD in CDH [Henriksson et al., 1991; Habu et al., 1993]. Also, comparison of the CDH amino acid sequence with those of fungal and bacterial cellulases did not reveal any obvious sequence similarity. Thus, the amino acid sequence which enables CDH to bind to cellulose remains unknown.

P. chrysosporium produces CDH abundantly only when cellulose is provided as the sole carbon source [Bao et al., 1994]. To obtain evidence that CDH expression is regulated at the transcriptional level by cellulose, total RNA was isolated from 11-days-old cellulose, cellobiose, or glucose cultures and analyzed by Northern blotting (Fig. 2.3). A band corresponding to 2.5 kb was observed only with the RNA isolated from cellulose-grown cells. Also, the size of this RNA was very similar to the size of the cDNA insert. These preliminary findings suggest that either cellulose or one of its degradation products is controlling the expression of CDH at the transcriptional level.

A Southern blot of DNA from *P. chrysosporium* strains OGC101 and K3 is shown in Fig. 2.4. Strain OGC101 was used in this study and strain K3 was used by Raices et al. [Raices et al., 1995]. DNA was digested with *Hind*III and *Sac*I. Southern blot analysis for OGC101 DNA showed that only one restriction fragment (2.1 kb for *Hind*III, 8.0 kb for *Sac*I) from each digest hybridized to the probe which suggests that CDH is probably encoded by a single gene in this strain (Fig. 2.4). However, two fragments (2.1 and 4.0 kb for *Hind*III, 8.0 and 9.4 kb for *Sac*I) from strain K3 hybridized to the probe, suggesting the presence of a potentially distinct allele for CDH in strain K3 (Fig. 2.4).

The findings of this study with regard to the heme, flavin, and cellulose-binding domain structures of CDH are similar to those of Raices et al. [1995]. The two cDNA sequences are 99.6% homologous. However, variations are observed in the amino acid sequences deduced from the corresponding cDNA sequences. In the heme domain at residues 176 and 177, our cDNA predicts an Ala and a His and the corresponding cDNA sequence is GCG and CAC. In the Raices et al. [1995] report, a single Asp176 with a cDNA sequence GAC is predicted in the place of Ala176 and His177. In the flavin domain at position 460 predicted by our cDNA, a Gly is present which is absent in the other sequence. Protein sequence similarity was not observed between residues 587–621 of the CDH sequence of Raices et al. [1995] and 589–624 of our CDH. However, the nucleotide sequence in that region showed 97% homology (Fig. 2.5). Two base pairs, G1836 and C1837, found at the 5'-end of our cDNA sequence are absent in the other sequence, possibly resulting in a reading



Fig. 2.3 Northern analysis of *P. chrysosporium* RNA. Total RNA was isolated from 11-days-old mycelia obtained from 1% cellulose (lane 1), glucose (lane 2), or cellobiose (lane 3). The RNA was fractionated by electrophoresis in 1% agarose containing 2.2 M formaldehyde and transferred to a Magnagraph nylon membrane [Brown, 1994b]. The blot was probed with ^{32}P -labeled CDH cDNA. Bars to the left indicate the positions of 18S and 28S rRNA.

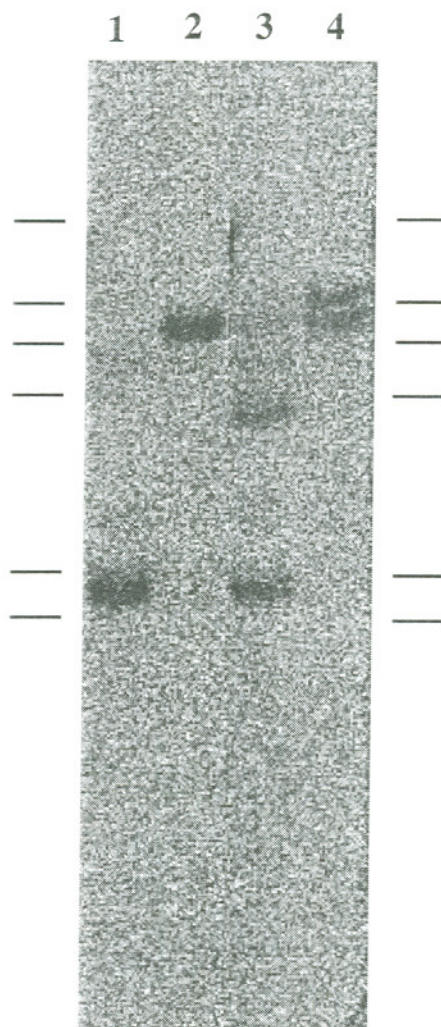


Fig. 2.4 Southern analysis of genomic DNA from *P. chrysosporium* strains OGC101 and K3. Genomic DNA, isolated by standard procedures, was digested with restriction enzymes *Hind*III (lanes 1,3) and *Sac*I (lanes 2,4). It was electrophoresed in 0.7% agarose and transferred to a Magnagraph nylon membrane. The blot was probed with a ^{32}P -labeled fragment (nucleotides 192–591) of CDH cDNA. Bars indicate the positions of molecular weight standards, from top to bottom: 23, 9.4, 6.6, 4.4, 2.3, 2.0 kilobases.

1830	CCG	GGC	GCA	GCC	TCC	GTG	AAC	TCC	TCG	CTG	CCG	TAC	AAC	GCG	AGC
587	P	G	A	A	S	V	N	S	S	L	P	Y	N	A	S
1807	CCG	GGC	--A	GCC	TCC	GTG	AAC	TCC	TCG	CTG	CCG	TAC	AAC	GCG	AGC
585	P	G		S	L	R	E	L	L	A	A	V	Q	R	E
	CAG	ATC	TTC	ACG	ATC	ACC	GTG	TAC	CTC	TCT	ACG	GGC	ATC	CAG	TCG
	Q	I	F	T	I	T	V	Y	L	S	T	G	I	Q	S
	CAG	ATC	TTC	ACG	ATC	ACC	GTG	TAC	CTC	TCT	ACG	GGC	ATC	CAG	TCG
	D	L	H	D	H	R	V	P	L	Y	G	H	P	V	A
	CGT	GGG	CGC	ATC	GGC	ATC	GAT	GCA	GCG	CTC	1949				
	R	G	R	I	G	I	D	A	A	L	626				
	CGT	GGG	CGC	ATC	GGC	ATC	GAT	-CA	GCG	CTC	1923				
	W	A	H	R	H	R	S		A	L	623				

Fig. 2.5 Comparison of nucleotide and deduced amino acid sequences from 1830–1949 of this study (top) and 1807–1923 of Raices et al. [1995] (bottom). Nucleotides that were absent in the Raices et al. [1995] sequence are indicated in bold type.

frame that has been shifted by 2 bp. The reading frame-shifted sequence apparently returns to the original reading frame by the loss of a G1941 of the Raices et al. [1995] cDNA sequence (Fig. 2.5). The net result is that the Raices et al. [1995] sequence lacks 3 bp or one amino acid in this region. A portion of the sequence in question in the flavin domain is partially conserved in FAD-dependent enzymes (Fig. 2.6). Only our sequence contains the full conserved sequence (Fig. 2.6). The amino acid composition calculated from our CDH cDNA sequence correlates with the amino acid composition of the CDH protein reported by Raices et al. [1995]. A significant deviation is found in the number of His and Arg. The earlier study reported 15 His and 21 Arg, whereas protein amino acid analysis indicated 11 His and 17 Arg, and our cDNA sequence predicts 11 His and 18 Arg.

It is possible that the two groups have sequenced different alleles of the same gene and the observed variations reflect the allelic sequence differences. This is supported by Southern analysis (Fig. 2.4) which suggested that, whereas the CDH from strain OGC101 is encoded by one gene, the CDH from strain K3 might be encoded by different alleles. Differences could also be due to sequencing error(s) by one or both groups, in which case further studies will be necessary to identify the correct sequence. However, genomic sequence of CDH from strain OGC101 obtained by our laboratory [Li and Renganathan, unpublished results] matched that of the CDH cDNA sequence reported here.

CDH 615	Q	S	R	G	R	I	G	I	D
ADH 390	K	S	R	G	R	I	G	L	K
CDH 392	P	S	R	G	H	V	R	I	K
GDH 457	R	S	R	G	F	I	G	L	R
GOD 431	F	T	R	G	Y	V	H	I	L
CDH 612	P	V	A	W	A	H	R	H	R

Fig. 2.6 Comparison of dissimilar protein sequences of CDH with FAD-dependent enzymes. The CDH sequence at the top is from this study. The CDH sequence at the bottom was reported by Raices et al. [1995]. ADH, alcohol dehydrogenase from *P. oleovorans* [van Bielen et al., 1992]; CHD, choline dehydrogenase from *Escherichia coli* [Raices et al., 1995]; GDH, glucose dehydrogenase from *Drosophila melanogaster* [Raices et al., 1995]; GOD, glucose oxidase from *A. niger* [Frederick et al., 1990]. Identical amino acids are enclosed in boxes.

CHAPTER 3
CELLOBIOSE DEHYDROGENASE FROM
PHANEROCHAETE CHRYSOSPORIUM IS ENCODED BY TWO ALLELES*

3.1 INTRODUCTION

The extracellular cellulolytic systems of fungi usually consist of an endoglucanase, an exocellobiohydrolase, and a β -glucosidase [Strauss et al., 1986]. Cellulose-degrading fungi, including *Phanerochaete chrysosporium* [Ayers et al., 1978; Bao et al., 1993], *Sporotrichum thermophile* [Canevascini et al., 1991], *Trametes versicolor* [Roy & Archibald, 1994], and *Coniophora puteana* [Schmidhalter & Canevascini, 1993], also produce extracellular cellobiose dehydrogenase (CDH), in addition to cellulases. CDH oxidizes cellobiose to cellobionolactone in the presence of electron acceptors such as cytochrome *c*, quinones, and ferric iron [Bao et al., 1993]. CDH from *P. chrysosporium* can enhance crystalline cellulose hydrolysis by *Trichoderma veride* and *Trichoderma reesei* cellulases [Bao & Renganathan, 1992]. This finding suggests that a probable physiological function of CDH in *P. chrysosporium* is to augment cellulose hydrolysis by cellulase [Bao & Renganathan, 1992].

All CDHs are hemoflavoenzymes and contain one heme *b* and one flavin per molecule [Ayers et al., 1978; Canevascini et al., 1991; Bao et al., 1993; Schmidhalter & Canevascini, 1993]. The cofactors are noncovalently bound to

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Li, B., Nagalla, S. R., and Renganathan, V. (1997) Cellobiose dehydrogenase from *Phanerochaete chrysosporium* is encoded by two allelic variants. *Appl. Environ. Microbiol.* **63**, 796-799.

specific domains of the protein [Canevascini et al., 1991; Henriksson et al., 1991]. Analysis of the cDNA sequence for *P. chrysosporium* CDH suggested that the heme domain is located at the N terminus and the flavin domain is located at the C terminus. The two domains are linked by a hydroxyamino acid-rich linker peptide [Raices et al., 1995; Li et al., 1996]. The amino acid sequence of the flavin-binding domain shows similarity to a number of FAD-dependent dehydrogenases [Raices et al., 1995; Li et al., 1996]. Northern blot analysis revealed that in *P. chrysosporium* CDH expression is transcriptionally regulated by cellulose [Li et al., 1996]. Similar to cellulases, CDH binds strongly to cellulose [Renganathan et al., 1990; Henriksson et al., 1991]. However, the sequence which enables CDH to bind to cellulose is not known [Raices et al., 1995; Li et al., 1996]. Earlier, based on Southern blot analysis, we suggested that there is potentially only one gene for CDH in *P. chrysosporium* OGC101 [Li et al., 1996]. Here, the nucleotide sequence of two *cdh* genes (*cdh-1* and *cdh-2*) from *P. chrysosporium* OGC101 is reported. Furthermore restriction fragment length polymorphism analysis of homokaryotic derivatives of *P. chrysosporium* suggested that these genes segregate and proved that they are two alleles of the same gene.

3.2 MATERIALS AND METHODS

3.2.1 Isolation of *cdh-1*

A λ EMBL3 genomic library of *P. chrysosporium* OGC101 was screened at high stringency (50°C, $4.8 \times$ SSC [$1 \times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 48% formamide) with two probes constructed from the 3' and 5' ends of the *cdh* cDNA [Brown, 1994]. The 5'-end probe was a PCR product of CDH cDNA (nucleotides 192–591) and the 3'-end probe was a *Xho*I digestion fragment of the *cdh* cDNA (nucleotides 2008–2445) [Li et al., 1996]. Based on the restriction mapping of the genomic clones, a 4-kb fragment (*Bam*HI-*Sac*I) containing the entire coding region was subcloned into pBluescript SK (Stratagene) and sequenced by the primer walking method [Strauss et al., 1986]. Sequencing was performed using an automatic

sequencer (Model 377, Applied Biosystems) and an Amplitaq FS kit. Both strands of the genomic clone were sequenced.

3.2.2 Isolation of *cdh-2*

The *cdh* cDNA clone, which was sequenced previously [Li et al., 1996], was probably derived from *cdh-2*. Comparison of the exon sequences of *cdh-1* and the cDNA sequence suggested that *Nde*I restriction analysis could be used for differentiating *cdh-1* and *cdh-2*. Several CDH clones were amplified by PCR (nucleotides 708–1968), digested with *Nde*I, and fractionated on an agarose gel. Clones, whose PCR product was not restricted by *Nde*I, were identified as *cdh-2*. Two overlapping fragments, one from a *Sal*I digest and another from a *Sac*I-*Bam*HI digest of a *cdh-2* clone, were subcloned into pBluescript(SK) and sequenced by the primer walking method [Strauss et al., 1986].

3.2.3 Isolation and Analysis of Homokaryons

Single homokaryotic basidiospores were isolated as described elsewhere [Alic et al., 1987]. DNA from homokaryotic cultures was isolated by standard procedures and restriction digested with *Bam*HI, size fractionated in 0.8% agarose gel, blotted onto a Magnagraph nylon transfer membrane (Micron Separations, Westboro, MA), and probed with a ³²P-labeled *Bam*HI fragment (1.6 kb) of *cdh-2*. The *Bam*HI fragment was obtained by digesting a *Sac*I-*Bam*HI fragment of a *cdh-2* clone with *Bam*HI.

3.2.4 Nucleotide Sequences Accession Numbers

cdh-1 and *cdh-2* gene sequences reported here have been deposited in GenBank under accession numbers U50409 and U65888, respectively.

3.3 RESULTS AND DISCUSSION

3.3.1 The *cdh-1* Sequence

Eleven CDH clones were isolated by screening a λ EMBL3 genomic library of *P. chrysosporium* OGC101 and one of the clones, *cdh-1*, was sequenced. *cdh-1* consists of 3627 bp, including 474 bp of 5' non-coding sequence and 153 bp of 3' non-coding sequence (Fig. 3.1). The 5' upstream region contains a potential TATAA box (TTTAAAAA) 59 bp upstream from the translation start codon. The TATAA box is flanked by GC-rich sequences. A CCAAT box sequence, GGCCAATCT, is found 139 bp upstream from the translation start codon. Comparison of the genomic and cDNA sequences of CDH indicated the presence of 13 introns varying in size from 49 to 59 bp (Figs. 3.1 and 3.2). All of the intron splice junctions conform to the GT—AG rule (Fig. 3.1) [Breathnach et al., 1978]. CDH is encoded by 14 exons. Of these, exons 2 through 5 code for the heme domain. The fifth exon also codes for the linker region. Exons 6 through 14 code for the flavin domain (Fig. 3.2).

The exon sequences of *cdh-1* exhibit 98% homology to the *cdh* cDNA sequence [Li et al., 1996]. A total of 47 bp in the exon sequences of *cdh-1* does not match the previously determined cDNA sequence [Li et al., 1996]. In spite of these differences, the amino acid sequence predicted from the translation of the exon sequences of *cdh-1* was identical to the amino acid sequence predicted from the cDNA sequence. Since Southern blot analysis of *P. chrysosporium* OGC101 genomic DNA previously indicated the presence of only one gene for CDH [Li et al., 1996], we inferred that CDH in *P. chrysosporium* was probably encoded by two alleles and that *cdh-1* is one of the two alleles. The *cdh* cDNA sequence determined previously was presumably derived from the other CDH allele, *cdh-2*.

3.3.2 The *cdh-2* Sequence

To isolate the second gene, *cdh-2*, a 1260 bp PCR fragment (nucleotides 708–1968) was synthesized from each of the CDH genomic clones and then digested with *Nde*I. Restriction site analysis suggested that only the PCR product from *cdh-1*

TTTTGGTCGGGTTTACGTCGGGCAACGCCGCACACCGCTCGCCCTCGGTGGAAGATAGCTGGCAACATCTGCACGGGCTGGCACCCCATGCTGCTCACGCGCACCGTGAGGTATTCTGG 120
 GCTTCCTACGATTTTACCTCGGTGTTATCAACAACATCTGTCGCTTTCTTACCTCATCAGACCGGTGATCAGAGCGGGTACACGTATCCGACGTGTCTCCGCCGCTCCGTCAGCG 240
 AGCTACATTGCGCGCCCCGCCGCCAATAACATGTGCCACGACGTCCCTCTATAGCGGATCTTTTCTGCGTACCTCACGAGGACTGTGCGCAATCTCTGAACATGGATTGTGTTT 360
 TCTCAGCTGCCGGCATATCTTCGCCCCTCGGAAAGAAAAATAGAGCAAGCCCGCTTTTAAAAACCAGAGAGAGGGCAGCAACAGCAGGCCAGGTGCACCTCCGGTCGAGTTCACGATGCTA 480
 GGTGCGATCGTTACTTGCGCTTCTGCCTTTTGTAGGCCTCGgtacgcggtttttgacgtcctcacagaaggttgcgaactgatgcaagcgcgcgccagCGTTCTCGCAGAGTGCCCTCAC 600
 G R S L L A L L P F V G L A F S Q S A S 22
 AGTTTACCGACCTTACCACAGGATTCCAGTTCACTGGTATTACCGACCTGTTCATGACGTGACCTACGGCTTCGTTTTCCCCCTCTGGCCACCTCCGAGCGCAATCCACTGAGTTCA 720
 Q F T D P T T G F Q F T G I T D P V H D V T Y G F V F P P L A T S G A Q S T E F 62
 TCGGAGAGGTGTGCCCCCATCGCATCAAAATGGATTGGCATTTGCCCTCGGTGGCGCCATGAACAACGACCTGCTACTTGTGGCTTGGGCCAACGGCAACCAATTTGTTTCTCCACTC 840
 I G E V V A P I A S K W I G I A L G G A M N N D L L L V A W A N G N Q I V S S T 102
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 R W A T G Y V Q P T M L 2
 tgtgttacagTGCATATACGGGAACCTGCACTTTGACAACACTCCCCGAGACAACCATCAACTCCACGCACTGGAAGTGGGTCTTCAGGTGCCAAGGCTGCACTGgtaagcggttccttc 1080
 A Y T G T A T L T T L P E T T I N S T H W K W V F R C Q G C T 143
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 E W N N G G G I D V T S Q G V L A W A F S N V A V D D P 171
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 S D P Q S T F S E H T D F G F F G I D Y S T A H S A N Y Q N Y L N G D S G N P T 211
 ACCACGAGCAACCAAGCCCAAGCAGCAGCTCAGTCACGACTGGGCCCACTGTTTCTgtgctgttggcaactctctgggtagggcatctagttgacttgcgtgatcatagGCTACACCT 1440
 T T S T K P T S T S S V T T G P T V S A T P 234
 TACGATTACATCATCGTCGGTGTCTGGTCTTGGCGGTATCATTGCAGCTGATCGTCTGTCGGAGGCTGGCAAGAAGTTCTCCTTCTCGAGCGCGGTGGCCCTAGCACCAAGCAGACCGGT 1560
 Y D Y I I V G A G P G G I I A A D R L S E A G K K V L L L E R G G P S T K Q T G 274
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 E S L F T D S N P F W W C K D I T V F A G C 318
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 F L N G K R A G P V A T Y L Q T A L A R P N F T F K T N V M 445


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      C                                     G
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      gt      t
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      G G       C
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A Q G T V R P G A A S V N S S L P Y N A S Q I F T I T V Y L S T G I Q 633
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I G S I P G L T M I T P D V T Q T L E E Y V D A Y D P A T M N S N H W V S S T T 713
      A T       A       C       -       g       t
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I G S S S P Q S A V V D S N V K V F G T N N L 735
      C       G       G
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F I V D A G I I P H L P T G N P Q G T L M S A A E Q A A A K I L A L A G G P * 773
      T
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      G
GCCGTCGCGCGACCACTGCGCGAGCTC

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Fig. 3.1 The nucleotide and amino acid sequences of *cdh-1* and *cdh-2*. The continuous sequence is the *cdh-1* nucleotide sequence. The *cdh-2* nucleotide sequence is the same as that of *cdh-1* except at the positions indicated in the upper line by boldface letters. The intron sequences are indicated by lowercase letters. The putative CCAAT and TATAA boxes are underlined.

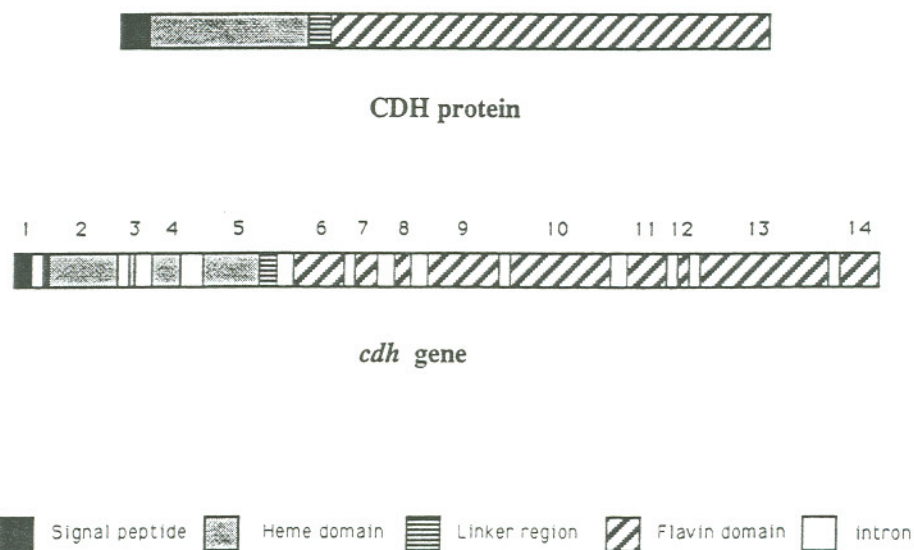


Fig. 3.2 Schematic representation of the protein and gene structures of CDH.

would be cut by *NdeI* to yield 858- and 402-bp fragments, whereas the PCR product from *cdh-2* would not be restricted (Fig. 3.3). Of the nine clones examined, PCR products from five clones were not restricted, suggesting that these were the *cdh-2* clones. Two overlapping fragments from one of the *cdh-2* clones, obtained by *SalI* and *SacI*-*BamHI* digestion, were subcloned and sequenced.

The exon sequences of *cdh-2* are 100% homologous to our *cdh* cDNA sequence (Fig. 3.1) [Li et al., 1996]. Intron locations in *cdh-2* were the same as those in *cdh-1* (Fig. 3.1 and 3.2). The *cdh-2* sequence is 97% homologous to that of *cdh-1*, with changes in both intron and exon sequences. Amino acid sequences of the protein products expressed by *cdh-1* and *cdh-2* are identical since the exon changes are all conservative and usually found in the third base of a codon. Earlier we isolated several cDNA clones for *cdh*, and a partial nucleotide sequence of one of the clones matched that of the *cdh-1* sequence (data not shown). This suggests that both alleles are expressed during cellulose degradation, although the relative levels of expression are unknown.

3.3.3 *cdh-1* and *cdh-2* Allelism

P. chrysosporium is a heterokaryon with two or more genetically distinct nuclei [Alic et al., 1987, Gold & Alic, 1993]. The genomic library, from which the CDH clones were isolated, is derived from such a heterokaryon. However, the basidiospores are homokaryons and contain two identical nuclei [Alic et al., 1987, Gold & Alic, 1993]. If *cdh-1* and *cdh-2* are truly allelic, then they should segregate among the homokaryons. Segregation of allelic variants of lignin peroxidase and glyoxal oxidase genes from *P. chrysosporium* has been reported [Gaskell et al., 1991; Kersten et al., 1995]. DNAs from homokaryotic and heterokaryotic cultures were restricted with *BamHI*, size-fractionated on an agarose gel, and probed with a 1.6 kb *BamHI* fragment of *cdh-2*. *cdh-1* has one *BamHI* site in the 5'-noncoding region (Fig. 3.3). *cdh-2* has two *BamHI* cleavage sites, one in the 5'-noncoding region and another within the gene sequence (Fig. 3.3). Based on this rationale, in the Southern analysis, the probe was anticipated to hybridize to a smaller fragment in a

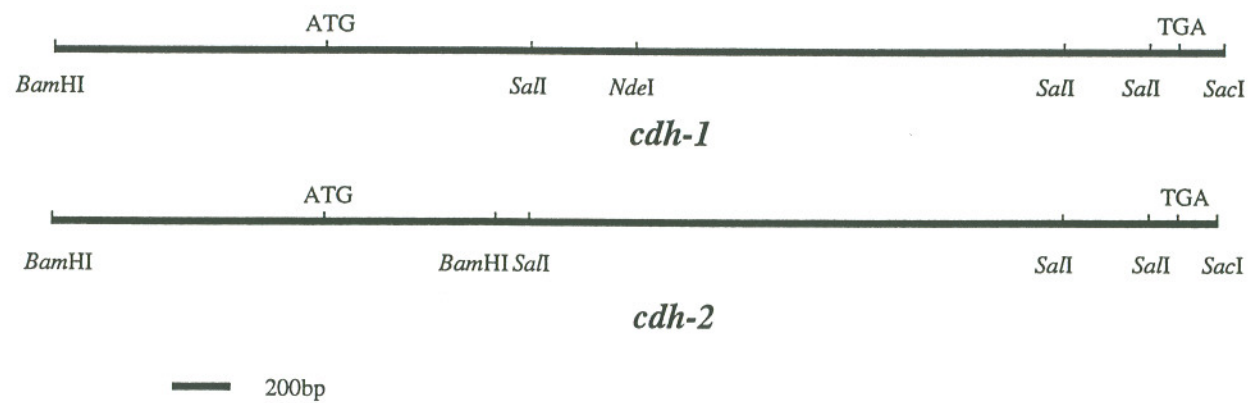


Fig. 3.3 Restriction maps of *cdh-1* and *cdh-2*. Only selected restriction sites are shown.

homokaryon containing *cdh-2*, to a larger fragment in a homokaryon containing *cdh-1*, and to two fragments in a heterokaryon. As expected, the probe hybridized to two bands from a heterokaryon and only one band from the homokaryons (Fig. 3.4). Among the eight homokaryons examined, five contained *cdh-2* and three contained *cdh-1* (data not shown). Thus, segregation of *cdh-1* and *cdh-2* in homokaryons confirmed their allelic relationship.

The molecular genetics of the cellulolytic system of *P. chrysosporium* is not well studied. Among the cellulolytic components of *P. chrysosporium*, only cellobiohydrolase and CDH have been cloned and sequenced [Covert et al., 1992a; Covert et al., 1992b; Raices et al., 1995; Li et al., 1996]. Cellobiohydrolase is encoded by multiple genes [Covert et al., 1992a; Covert et al., 1992b; Sims et al., 1994], whereas the present study shows that CDH is likely encoded by a single gene. Although endoglucanase has been purified from *P. chrysosporium*, a specific gene for that enzyme has not been isolated. Sims et al. [1994] have proposed that a cellobiohydrolase I-like protein has endoglucanase activity in *P. chrysosporium*. The extracellular β -glucosidase of *P. chrysosporium* OGC101 is novel in that it is the only glucosidase that is known to bind to cellulose by using a cellulose-binding domain [Lymar et al., 1995].

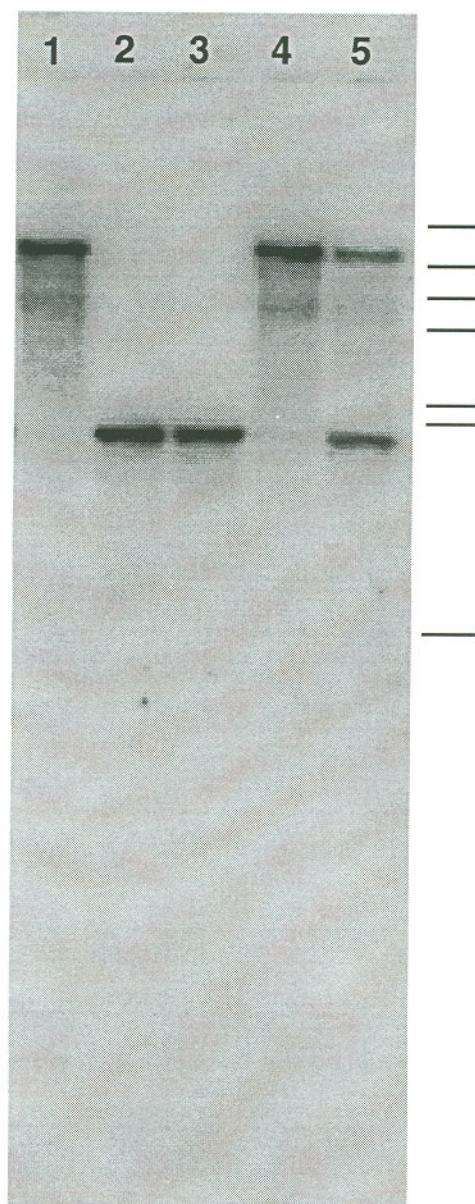


Fig. 3.4 Segregation of CDH alleles into homokaryons. Homokaryons or single-spore cultures were prepared as described elsewhere [Alic et al., 1987]. DNA from four separate single-spore cultures (lanes 1–4) and one parental heterokaryon culture of *P. chrysosporium* OGC101 were restricted with *Bam*HI, size-fractionated on an agarose gel, and probed with a 1.6-kb *Bam*HI fragment of *cdh-2*. Bars indicate the positions of molecular size standards (from top to bottom) 23, 9.4, 6.6, 4.4, 2.3, 2.0, and 0.5 kb.

CHAPTER 4

SITE-DIRECTED MUTAGENESIS OF Met65, A POTENTIAL HEME IRON LIGAND LEADS TO CLEAVAGE OF THE HEME DOMAIN IN CELLOBIOSE DEHYDROGENASE

4.1 INTRODUCTION

Cellobiose dehydrogenase (CDH) from *P. chrysosporium* contains 755 amino acids and has a predicted molecular weight of 80,115 [Raices et al., 1995; Li et al., 1996]. We have predicted that amino acid residues 1–192 could form the heme domain, residues 208–755 the flavin domain, and residues 193–207 the linker region which connects the heme and flavin domains [Li et al., 1996]. CDHs from *Trametes versicolor* and *Sporotrichum thermophile* have also been cloned [Dumonceaux et al., 1998; Subramaniam, 1998]. CDH from *T. versicolor* consists of 747 amino acids and is organized into two domains: an N-terminus heme domain and a C-terminus flavin domain [Dumonceaux et al., 1998]. CDH from *S. thermophile* contains 807 amino acids and is organized into three domains: an N-terminus heme domain, a middle flavin domain, and a C-terminus cellulose-binding domain [Subramaniam, 1998]. The three CDHs have more than 38% identity and more than 55% similarity [Subramaniam, 1998]. The heme domains of CDHs do not show significant similarity to any known heme protein. Nevertheless, the flavin domain shows strong similarity to the glucose-methanol-choline (GMC) oxidoreductase family which includes flavin-adenine-dinucleotide-dependent alcohol dehydrogenase, choline dehydrogenase, and glucose dehydrogenase [Li et al., 1996]. Northern blot analysis suggested that *P. chrysosporium cdh* is expressed only in cellulose-supplemented cultures [Li et al., 1996]. CDH from *P. chrysosporium* is encoded by two alleles (*cdh-1* and *cdh-2*) of a single gene [Li et al., 1997]. The alleles show 97% sequence homology. The two

sequences differ at 86 locations. Both alleles have 14 exons, and the introns are located at the same position. The translation products of the two alleles are identical, because the changes are found only in the third base of a codon [Li et al., 1997]. Sequence analysis of several CDH cDNA clones suggested that both alleles are expressed [Li et al., 1996; Li et al., 1997].

Spectroscopic studies first suggested that a methionine and a histidine form the fifth and the sixth heme iron ligands [Cox et al., 1992]. Comparison of the amino acid sequences of CDHs from *P. chrysosporium*, *T. versicolor*, and *S. thermophile* suggest that Met65 and His114 in *P. chrysosporium* CDH, which are located within conserved sequences of the heme domains, are the most probable fifth and sixth heme iron ligands [Raices et al., 1995; Li et al., 1996; Dumonceaux et al., 1998; Subramaniam, 1998]. CDH binds to cellulose strongly but lacks the cellulose-binding domain usually found in cellulases [Renganathan et al., 1990; Henriksson et al., 1991]. Recently, Henriksson et al. [1997] hypothesized that a region containing aromatic residues in the flavin domain is responsible for cellulose binding. This region is conserved in the CDHs from *P. chrysosporium* and *T. versicolor* but not in *S. thermophile*. However, the CDH from *S. thermophile* possesses a cellulose-binding domain [Subramaniam, 1998]. Confirmation of these structural predictions requires an efficient expression system. Gold and coworkers achieved expression of manganese peroxidase (MnP) from *P. chrysosporium* [Mayfield et al., 1994]. MnP, a peroxidase involved in lignin degradation, is expressed only under nutrient nitrogen-limiting conditions and in the presence of Mn(II) [Gold & Alic, 1993]. By placing *mnp* under the control of the glyceraldehyde-3-phosphate dehydrogenase promoter, Mayfield et al. [1994] accomplished expression of MnP in high carbon and high nitrogen cultures. Several mutants of MnP have been expressed successfully using this system [Kusters-van Someren et al., 1995; Kishi et al., 1996, 1997]. Under these culture conditions, wild-type MnP expression was undetectable. Here, high-level expression of rCDH using the *P. chrysosporium* expression system is reported. Using this system, a Met65Ala was expressed. Met65 is a potential ligand to heme iron.

4.2 MATERIALS AND METHODS

4.2.1 Organisms

Growth and maintenance of *P. chrysosporium* wild-type strain OGC101, an auxotrophic strain OGC107-1 (Ade1), were described previously [Alic et al., 1990]. *Escherichia coli* XL1-Blue, used in subcloning, was obtained from Stratagene (La Jolla, CA).

4.2.2 Construction of the Expression Vector pAGC1

cdh-1 was amplified using a polymerase chain reaction (PCR). Primers were designed to create a unique *Sph*I site at the 5' end and a unique *Eco*RI site at the 3' end of the PCR product. The 3.15-kb *Sph*I-*Eco*RI fragment contained the entire CDH coding region and 150 bp from the 3' non-coding region. In a one-step three-way ligation reaction, the *cdh-1* PCR fragment was ligated to a 1.16-kb *Xba*I-*Nsp*I fragment of the *gpd* promoter containing the ATG translation codon at the 3' end and pOGI18 [Mayfield et al., 1994] digested with *Eco*RI and *Xba*I to give pAGC1 (Fig. 4.1).

4.2.3 Construction of Mutant Plasmid pM65A

Site-directed mutagenesis was performed by using two PCR reactions. The plasmid pAGC1 was used as the template in the PCR reactions. Four oligonucleotides were used as primers. Primers Fmet and Rmet were complementary to the minus and plus strands of *cdh1* over nucleotide positions 771-790, which contained a *Kas*I site [Li et al, 1997]. Primer Fpuc was a 21-mer, corresponding to the pAGC1 sequence 367-387 bp upstream of the *Xba*I site (Fig. 4.2). Primer Rpuc was a 21-mer, corresponding to the pAGC1 sequence 216-236 bp downstream of the *Eco*RI site (Fig. 4.2). Primers Fmet and Rmet contained the codon and anticodon for Ala, GCG replacing ATG, which encoded Met65. The PCR product Fpuc-Rmet was digested with *Xba*I-*Kas*I, and Fmet-Rpuc was digested with *Kas*I-*Eco*RI. These two digestion products were used in a three-way ligation with pOGI18 digested with *Eco*RI and *Xba*I to give pM65A.

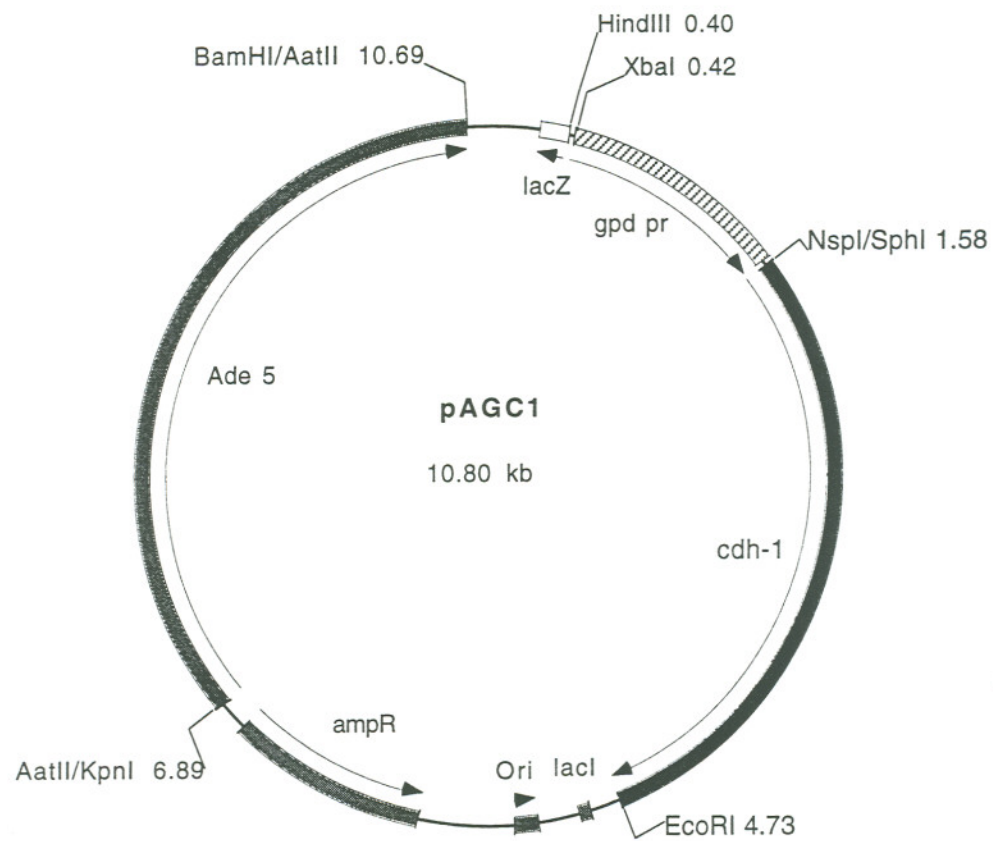


Fig. 4.1 Construction of CDH expression vector.

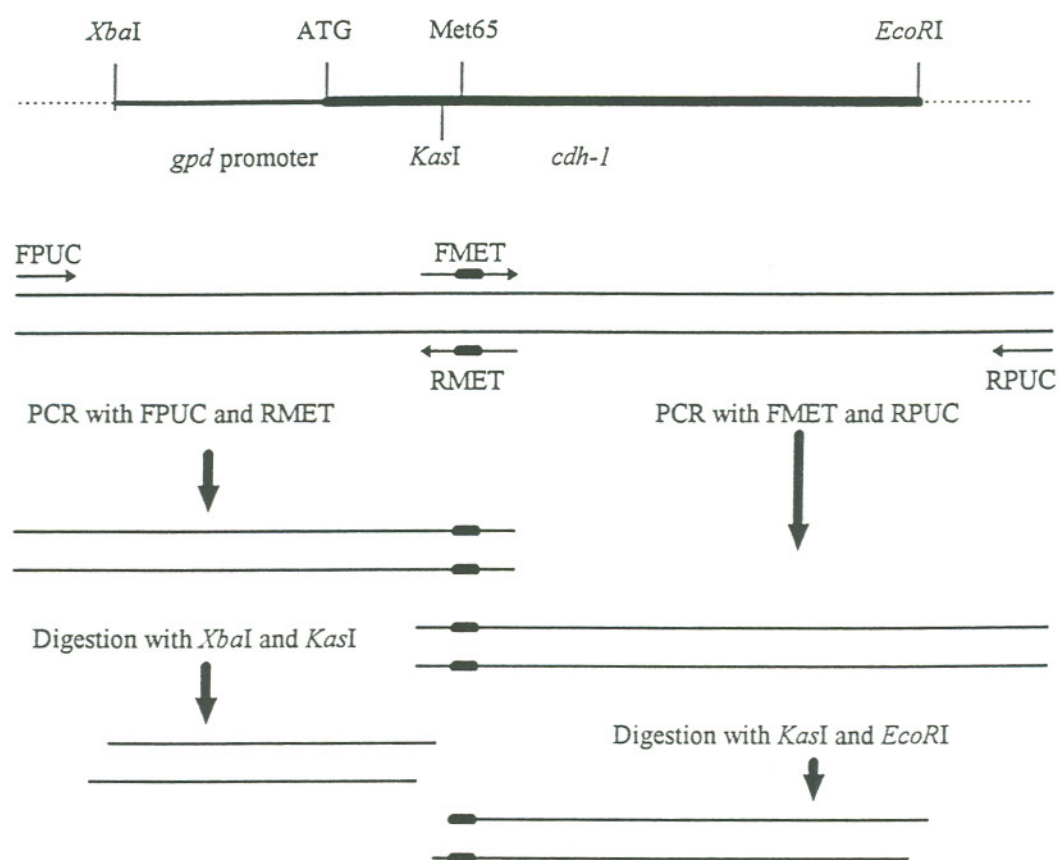


Fig. 4.2 Site-directed mutagenesis strategy of Met65Ala mutant.

4.2.4 *P. chrysosporium* Transformation

Protocols for the preparation and transformation of protoplasts of *P. chrysosporium* auxotrophic strain OGC107-1 (Ade1) were as described [Alic et al., 1989, 1990, 1991]. The adenine auxotroph was induced to fruit on one-sixth-strength modified Vogel medium [Smith & Gold, 1979] supplemented with 4.5% acid-treated cellulose and 0.01% adenine. The spores were washed off from the lids of fruiting plates with modified Vogel medium supplemented with 3% malt extract and 0.15% yeast extract, pH 4.8. Spores were swollen at 36°C for 4 h with shaking (150 rpm). The swollen spores were protoplasted by Novozyme 234 (10 mg/ml) and Cellulase CP (10 mg/ml) treatment. Protoplasts were transformed with 5 µg of *EcoRI*-linearized pOGI18, pAGC1, or pM65A, as described [Alic et al., 1989, 1990, 1991]. Transformed protoplasts were grown on minimal medium containing asparagine, glucose, and salts [Alic et al., 1989, 1990, 1991].

4.2.5 Screening for rCDH and the M65A Mutant Protein Expression

pAGC1 or pM65A transformants were inoculated into 250-ml Erlenmeyer flasks containing 20 ml of a medium as described by Kirk et al. [1978] supplemented with 2% glucose, 12 mM ammonium tartrate, 0.2% tryptone, and 20 mM sodium-2,2-dimethyl succinate. The pH of the medium was adjusted to 6.0. The cultures were incubated without shaking at 37°C. Periodically, 200 µl of extracellular medium was monitored for CDH activity using a cytochrome *c* assay and a DCPIP assay [Bao et al, 1993]. The cytochrome *c* assay mixture contained cellobiose (100 µM) and cytochrome *c* (12.5 µM) in 1.0 ml of 20 µM succinate buffer pH 4.5. The DCPIP assay mixture contained cellobiose (100 µM) and DCPIP (25 µM) in 1.0 ml of 20 µM succinate buffer pH 4.5.

4.2.6 Production of rCDH and the M65A Mutant Protein

Production of rCDH and mutant protein in stationary cultures: The pAGC1 or pM65A transformants were grown from conidial inocula at 37°C in 20-ml stationary cultures in 250-ml Erlenmeyer flasks. The medium was the same as described in Section 4.2.5.

Production of rCDH in shake cultures: The medium composition of the shake cultures was the same as for the stationary cultures as described above. The mycelial mats from four 2-day-old stationary cultures were blended and added to a 2-liter Erlenmeyer flask containing 1 liter of culture medium. The cultures were incubated at 28°C with shaking (150 rpm). CDH activity was monitored as described in Section 4.2.5.

4.2.7 Purification of rCDH and the M65A Mutant Protein

Extracellular medium from 7-day-old stationary cultures was filtered on a Buchner funnel and treated with protease inhibitors EDTA (5 mM) and phenylmethylsulfonyl fluoride (0.5 mM). The filtrate was concentrated and dialyzed against 50 mM phosphate (pH 7). The proteins were precipitated with ammonium sulfate (410 g/liter), and the precipitation was allowed to proceed overnight at 4°C. The resulting precipitate was separated by centrifugation and dialyzed against 50 mM potassium phosphate (pH 6) and concentrated using an ultrafiltration unit. The concentrate was then applied to a Sephacryl S-200 column (65 × 2.5 cm) equilibrated with 50 mM potassium phosphate (pH 6). Fractions containing CDH activity, as determined by cytochrome *c* or DCPIP assay, were pooled, dialyzed against 10 mM Tris-HCl (pH 8), concentrated, and purified further 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 rCDH or the M65A mutant protein was eluted with 1 M NaCl gradient.

4.2.8 Spectroscopic and Kinetic Methods

The UV-visible absorption spectra were obtained using a Shimadzu Model-260 spectrophotometer. The kinetics of cellobiose oxidation were determined by monitoring ferrocycytochrome *c* formation at 550 nm. The assays contained cytochrome *c* (12.5 μM) and varying levels of cellobiose (8–50 μM) in 20 mM succinate (pH 4.5). The kinetics of cytochrome *c* reduction were determined in a similar fashion except in those assays cellobiose concentration was 100 μM and cytochrome *c* levels ranged from 4.2 to 12.5 μM.

4.2.9 FAD Estimation

Purified M65A mutant protein was denatured by trichloroacetic acid treatment. In a microcentrifuge tube, 500 μg of the mutant protein in 800- μl volume was mixed with 200 μl of 50% trichloroacetic acid. The denatured protein was removed by centrifugation and the supernatant was estimated for flavin by measuring the absorbance at 450 nm. The amount of flavin released was calculated from a standard curve constructed with purified rCDH.

4.2.10 Chemicals

Cellobiose, ammonium sulfate, DCPIP, and cytochrome *c* were obtained from Sigma Chemical Company (St. Louis, MO). Sephacryl S-200 was purchased from Pharmacia LKB Biotechnology (Alameda, CA). Molecular biology reagents were obtained from New England Biolabs (Beverly, MA).

4.3 RESULTS AND DISCUSSION

Several interesting aspects relating to the structure and function of CDH, such as cellulose-binding sequence, heme iron ligations, and residues involved in substrate binding, could be addressed if an expression system was available. Homologous expression of *cdh* in *P. chrysosporium* was considered because of the recent successes in the expression of rMnP and several mutant rMnPs by Gold and coworkers [Mayfield et al., 1994; Kusters-van Someren et al., 1995; Kishi et al., 1996, 1997]. In this expression system, MnP was expressed by placing *mnp* under the control of a *P. chrysosporium gpd* promoter [Mayfield et al., 1994]. This enabled the production of MnP under nitrogen-sufficient culture conditions. Wild-type MnP expression was controlled by Mn(II) concentration in the medium, whereas rMnP expression was independent of Mn(II). Similar to MnP, CDH is also an inducible enzyme, the inducer being cellulose [Bao et al., 1994; Li et al., 1996]. No CDH is produced in glucose- or cellobiose-added cultures [Bao et al., 1994]. Thus, when *cdh* is placed under the control of a *gpd* promoter, rCDH protein is expected to be produced in glucose-supplemented cultures, and wild-type CDH expression is not anticipated.

Also, by including the native signal peptide sequence, secretion of CDH was expected. CDH has two cofactors, a heme and a flavin [Ayers et al., 1978; Canevascini et al., 1991; Bao et al., 1993; Schmidhalter & Canevascini, 1993; Roy et al., 1997]. Proper insertion of stoichiometric amounts of these cofactors is necessary to obtain a highly active CDH preparation. This was not expected to be a problem, because MnP, a heme enzyme, could be expressed in the active form [Mayfield et al., 1994].

4.3.1 Homologous Expression of rCDH

Transformation of *P. chrysosporium* Ade1 with the *cdh* expression vector, pAGC1, produced 19 Ade⁺ transformants. Production of rCDH by the transformants was determined by culturing them individually in stationary cultures supplemented with glucose. Sixteen of the transformants exhibited CDH activity. The activity levels varied significantly. No CDH activity was detected under the same culture conditions with the Ade1 auxotroph, three Ade⁺ transformants obtained with pOGI18 [Mayfield et al., 1994], nor the wild-type OGC101 parent strain (Fig. 4.3).

Of the 16 pAGC1 transformants, one which synthesized high levels of CDH was investigated further. Stationary cultures produced more rCDH than the shake cultures (Fig. 4.4). rCDH concentration in the medium increased with time and reached maximum levels on day 7. rCDH production was dependent on pH, method of cultivation, and culture volume. The optimum culture pH for expression was 6.0. Stationary cultures produced more CDH than shake cultures. Smaller stationary cultures (20 ml in a 250-ml flask) produced 500 U/liter of rCDH, whereas, larger stationary cultures (250 ml in a 2800-ml flask) produced 350 U/liter. In contrast, wild-type CDH production in shake cultures supplemented with cellulose was 140 U/liter. Based on CDH activity, rCDH constituted approximately 40% of the total extracellular protein. Also, *cdh1* expression was much higher than *mnp* expression [Mayfield et al., 1994], possibly because CDH is more stable than MnP.

Extracellular medium from 30 7-day-old 20-ml stationary cultures was collected for rCDH purification. rCDH was purified by a simple three-step procedure involving ammonium sulfate precipitation, a Sephacryl S-200 column, and FPLC

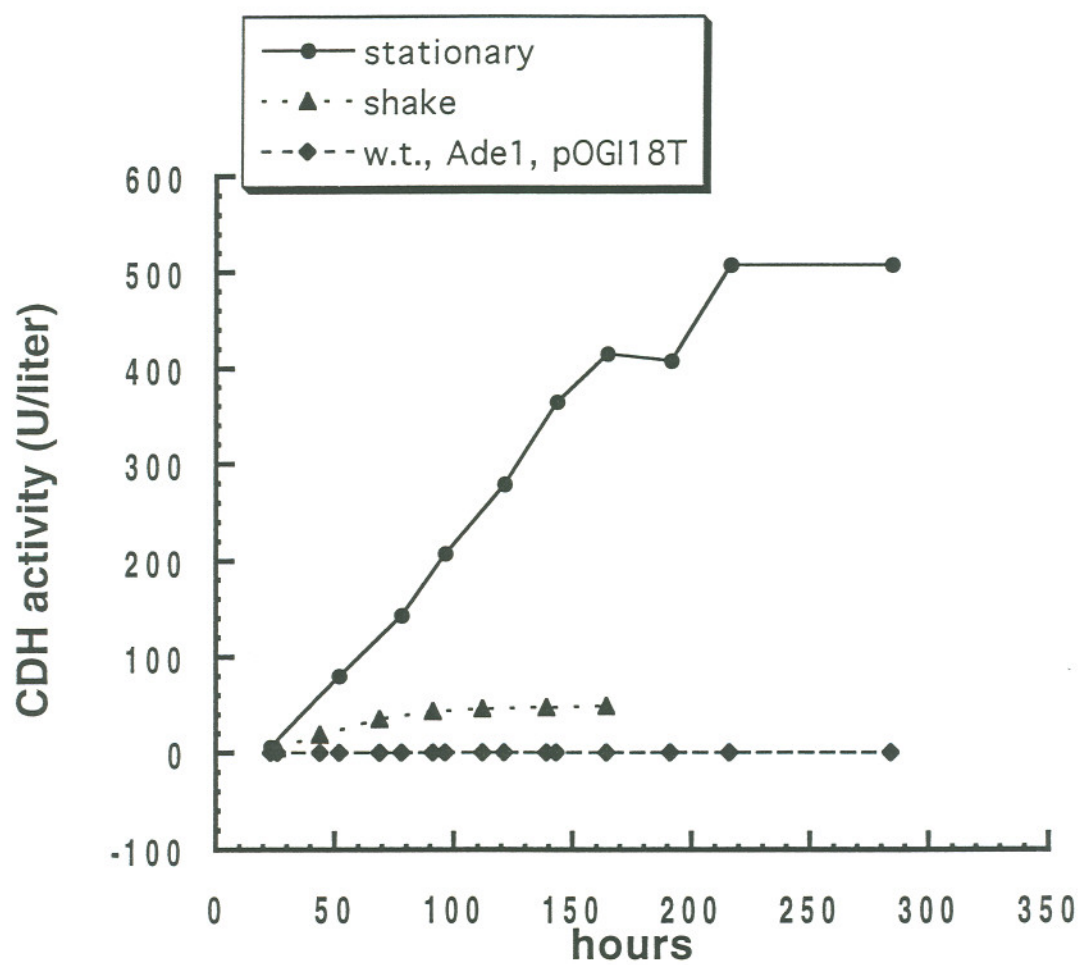


Fig. 4.3 Production of rCDH. CDH activity was monitored using cytochrome *c* in 20 mM succinate (pH 4.5).

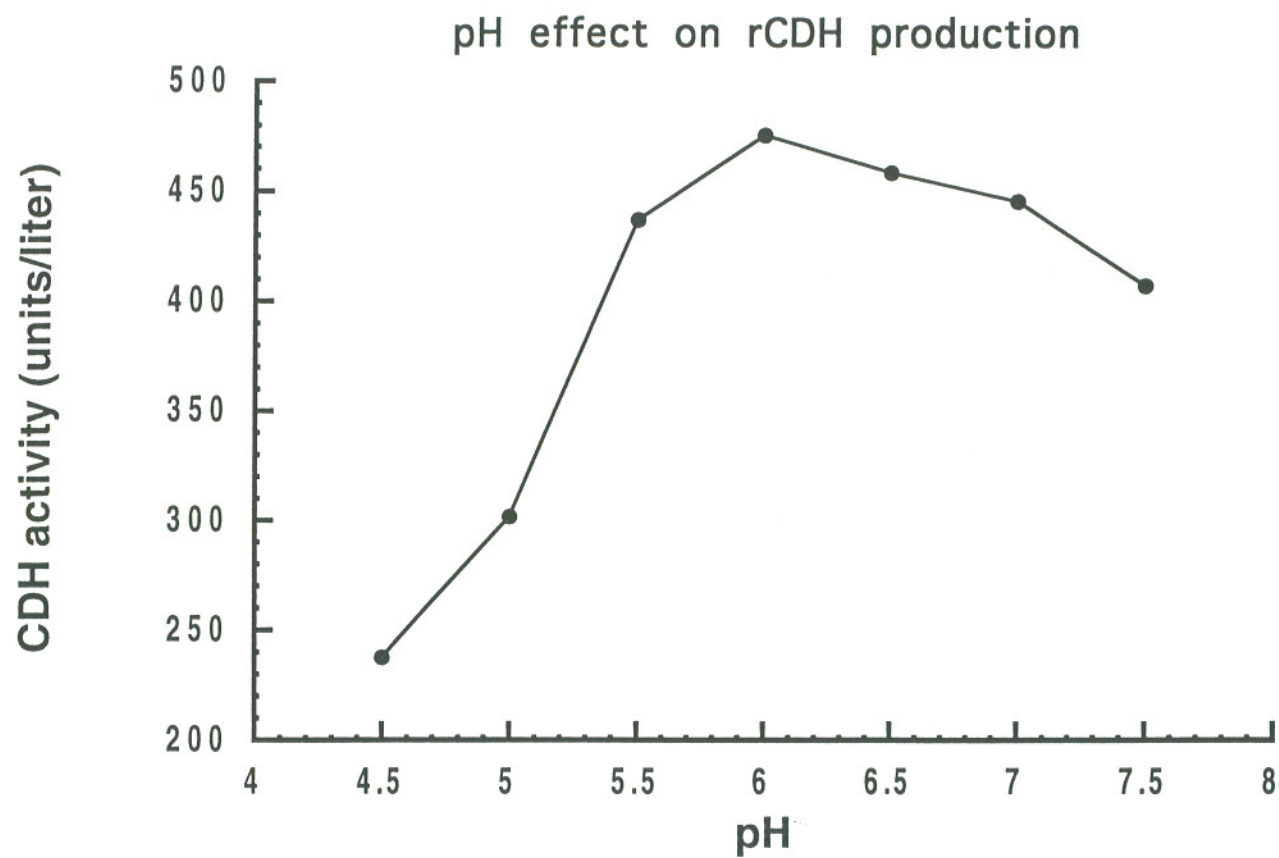


Fig. 4.4 Effect of initial medium pH on rCDH production. Culture conditions were as described in Section 4.2, except that the initial pH of the culture medium was adjusted from 4.5 to 7.5. CDH activity was assayed using the cytochrome *c* assay.

using a Mono-Q HR 5/5 column (Table 4.1). A mere four-fold purification yielded a homogeneous enzyme. The molecular weight of rCDH was the same as that of wild-type CDH (Fig. 4.5). rCDH-specific activity was 8 U/mg, which was slightly less than that of wild-type CDH. The native UV-visible spectrum showed absorptions at 420, 529, and 570 nm. On reduction with cellobiose, absorptions shifted to 428, 534, and 564 nm. These spectral characteristics were identical to those of wild-type CDH, suggesting similar heme environments for both CDHs. Like wild-type CDH, rCDH bound strongly to cellulose [Renganathan et al., 1990]. rCDH's K_m and k_{cat} values for cellobiose oxidation were 29 mM and 19 s⁻¹, respectively. K_m and k_{cat} values for cytochrome *c* were 0.7 mM and 12 s⁻¹ respectively. These kinetic values were comparable to those of wild-type CDH from *P. chrysosporium* [Bao et al., 1993].

4.3.2 Expression of the CDH Met65Ala Mutant

The heme iron of a heme protein can be either pentacoordinate or hexacoordinate. Hexacoordinated heme iron is generally found in dehydrogenases such as flavocytochrome *b₂* and proteins involved in electron transfer such as cytochrome *c* and cytochrome *b₅*. A distinguishing feature of hexacoordinated heme iron is that it does not bind azide or cyanide. Pentacoordinated heme iron is found in myoglobin, hemoglobin, peroxidase, catalase, and cytochrome P450. The ferric form of these proteins binds azide and cyanide, which changes their UV-visible spectral characteristics. The heme iron of CDH is hexacoordinate, and Met65 and His114 appear to serve as the fifth and sixth ligands to the heme iron [Cox et al., 1992; Subramaniam, 1998]. To provide evidence of this ligation, a Met65Ala mutant was created. If Met65 is indeed a ligand to the heme iron, then two outcomes are possible. In the absence of Met65, the heme domain might not be able to bind the heme, and the mutant protein would lack the heme absorption characteristic of wild-type CDH. If the mutant binds the heme, then the heme iron would be pentacoordinate, and the ferric form would be capable of binding azide and cyanide. Such binding could be monitored spectroscopically. As for the enzyme activity, Met65Ala was expected to be catalytically active, because it is the flavin domain

Table 4.1 Purification of Recombinant Cellobiose Dehydrogenase

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Fold purification
Medium concentrate	142.5	285	2.0	100	1.0
(NH ₄) ₂ SO ₄ precipitate	72.8	251	3.4	88.1	1.7
Sephacryl S-200	30.2	208	6.5	73.0	3.3
Mono-Q (FPLC)	11.4	91	8.0	31.9	4.0

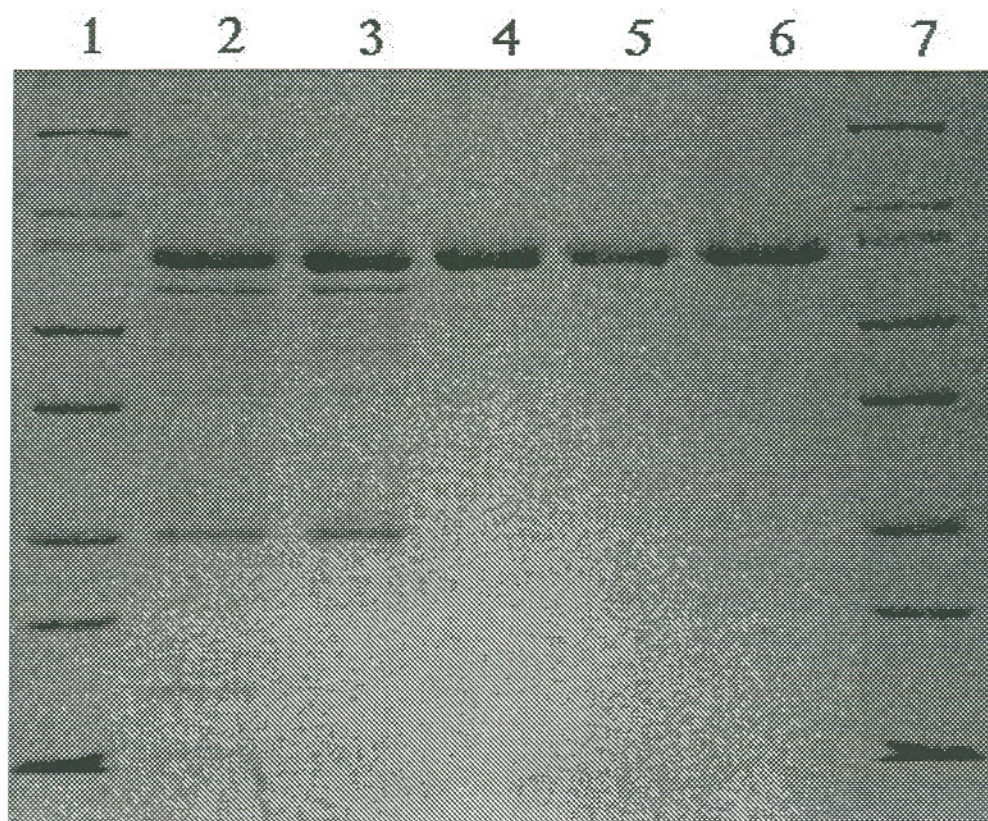


Fig. 4.5 SDS-PAGE of purified recombinant CDH. *Lanes 1 and 7*, molecular weight standards (from top to bottom: myosin, 200,000 Da; β -galactosidase, 116,300 Da; phosphorylase b, 97,400 Da; bovine serum albumin, 66,300 Da; glutamate dehydrogenase, 55,400 Da; lactate dehydrogenase, 36,500 Da; and carbonic anhydrase, 31,000 Da). *Lane 2*, extracellular proteins. *Lane 3*, ammonium sulfate precipitate. *Lane 4*, Sephacryl S-200 column fractions. *Lane 5*, FPLC fractions. *Lane 6*, wild-type CDH.

which catalyzes cellobiose oxidation. However, electron transfer to cytochrome *c* was not expected, because it needs a hexacoordinated heme iron.

The extracellular medium from twenty pM65A transformants was examined for CDH activity using cytochrome *c* or DCPIP as the electron acceptor. Sixteen transformants exhibited activity with DCPIP but none of the transformants exhibited activity with cytochrome *c*. CDH-M65A was purified from a transformant which showed the highest levels of DCPIP activity. Homogeneous CDH-M65A exhibited a relative molecular mass of 67,000 Da (Fig. 4.6). It oxidized cellobiose in the presence of DCPIP but not in the presence of cytochrome *c*. Specific activity for cellobiose oxidation was 12 U/mg, which was comparable to that of wild-type CDH. Its UV-visible absorption spectrum had absorbances at 380 and 450 nm which was indicative of the presence of only a flavin (Fig. 4.7). The ratio of flavin to CDH-M65A protein was estimated to be approximately 1.3:1. These findings strongly suggest that Met65 is liganded to the heme iron.

As for the formation of the truncated flavoprotein, the possibility of truncation of the *cdh* gene in the transforming plasmid was ruled out, because restriction digestion of pAGC1 and pM65A with *Hind*III showed the same restriction endonuclease fragments (data not shown), and the presence of M65A mutation was confirmed by sequencing. In the absence of heme, the heme domain might not be able to fold correctly which might target it for proteolytic processing leading to the heme domain cleavage. Flavocytochrome *b₂* is also hexacoordinate and His43 and His66 serve as heme iron ligands [Xia & Mathews, 1990]. When His43 was replaced with a Met, heme binding was significantly affected and only 5% of the heme sites were occupied [Miles et al., 1993]. The mutant protein had a green color. Lactate oxidation by the mutant enzyme was normal with ferricyanide as the electron acceptor but was reduced 750-fold with cytochrome *c* [Miles et al., 1993].

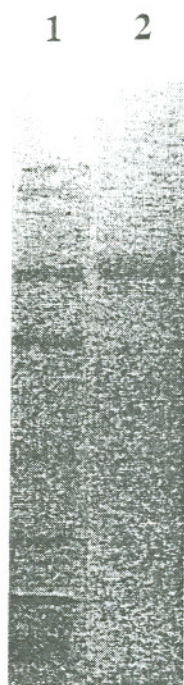


Fig. 4.6 SDS-PAGE of purified Met65Ala mutant protein. *Lane 1*, molecular weight standards (from top to bottom: myosin, 200,000 Da; β -galactosidase, 116,300 Da; phosphorylase b, 97,400 Da; bovine serum albumin, 66,300 Da; glutamate dehydrogenase, 55,400 Da; lactate dehydrogenase, 36,500 Da; and carbonic anhydrase, 31,000 Da). *Lane 2*, purified Met65Ala mutant protein.

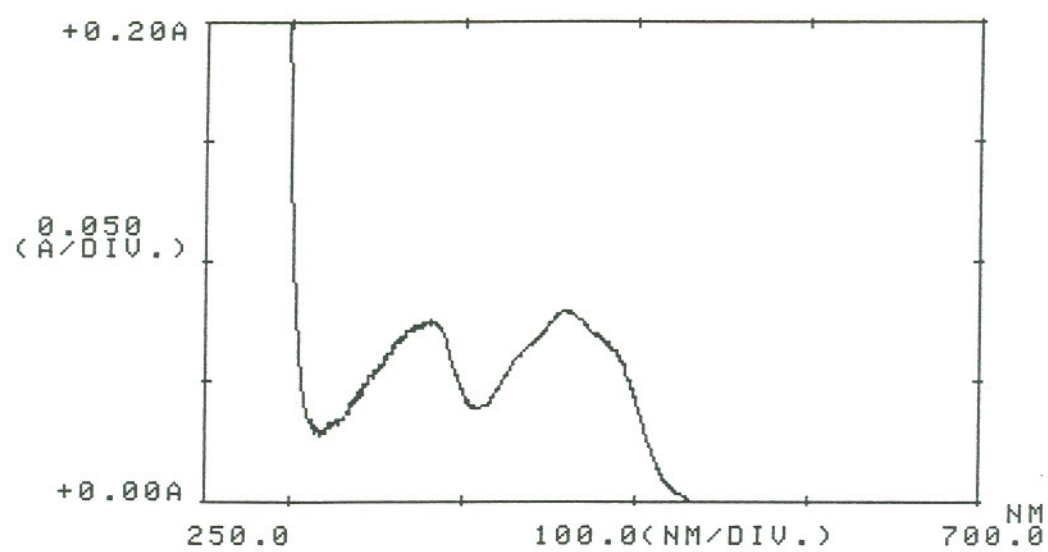


Fig. 4.7 UV-visible spectrum of M65A mutant protein.

CHAPTER 5

CLONING AND CHARACTERIZATION OF A NOVEL CELLULOSE-BINDING β -GLUCOSIDASE FROM *PHANEROCHAETE CHRYSOSPORIUM**

5.1 INTRODUCTION

The extracellular fungal cellulolytic system usually consists of at least one endoglucanase, one exocellobiohydrolase, and one β -glucosidase [Coughlan & Ljungdahl, 1988]. Synergistic action of endoglucanase and exocellobiohydrolase leads to the hydrolysis of cellulose to cellobiose, which is hydrolyzed to glucose by extracellular β -glucosidase. Intracellular β -glucosidases might also catalyze the hydrolysis of cellobiose. Thus, the role of β -glucosidases in cellulose hydrolysis is to complete the hydrolysis process initiated by cellulases. In addition, two other roles have been assigned for β -glucosidases in cellulose degradation. Cellobiose is an inhibitor of cellulases, and β -glucosidase could reduce this inhibition by hydrolyzing cellobiose and thereby enhancing cellulose hydrolysis [Stenberg et al., 1977]. For example, a cellulase with low levels of β -glucosidase hydrolyzes cellulose slowly and, in such cases, addition of glucosidase enhances cellulose hydrolysis. Sophorose is an endogenous inducer of cellulases produced by *Trichoderma reesei*, and it is suggested to be generated via a transglycosylation reaction of a β -glucosidase [Kubicek, 1987].

Cellulose-degrading cultures of *P. chrysosporium* produce three exocellobiohydrolases, five endoglucanases, one cellobiose dehydrogenase, and one β -

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Li, B. and Renganathan, V. (1998) Gene cloning and characterization of a novel cellulose-binding β -glucosidase from *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **64**, 2478-2754.

glucosidase in the extracellular medium [Eriksson & Pettersson, 1975a; Eriksson & Pettersson, 1975b; Ayers et al., 1978; Uzcategui et al., 1991; Bao et al., 1993; Bao et al., 1994]. A unique feature of this system is that all of the cellulolytic components bind to cellulose [Legler et al., 1979; Kraulis et al., 1989]. A family of six *cbh*-like genes has been cloned and characterized from *P. chrysosporium* [Covert et al., 1992a; Covert et al., 1992b]. *cbh1-4* has been identified as the most expressed *cbh* gene which produces the most dominant enzyme CBHI [Covert et al., 1992b]. A specific gene for endoglucanase has not been found. Sims et al. [1994] proposed that differential splicing of *cbh*-like genes might produce an enzyme with both exocellobiohydrolase and endoglucanase activities. CDH is an extracellular hemoflavoenzyme, and it oxidizes cellobiose produced from cellulose hydrolysis to cellobionolactone [Ayers et al., 1978; Bao et al., 1993]. Recently, CDH has also been cloned and sequenced [Raices et al., 1995; Li et al., 1996; Li et al., 1997]. CDH is apparently coded by a single gene [Li et al., 1997].

P. chrysosporium apparently produces three different β -glucosidases—extracellular, intracellular, and cell-wall-bound—depending on the carbon source [Deshpande et al., 1978; Smith & Gold, 1979]. Deshpande et al. [1978] reported that cellulose induced intracellular and cell-wall-bound enzymes and further purified five isozymes of extracellular β -glucosidases from cellulose-degrading cultures of *P. chrysosporium*. Molecular weights of these glucosidases ranged from 165,000 to 182,000 Da. Smith and Gold [1979] partially purified an extracellular β -glucosidase and characterized it as a monomer with a molecular weight of 90,000 Da. Recently, we purified and characterized a cellulose-binding extracellular β -glucosidase (CBGL) from *P. chrysosporium* [Lyman et al., 1995]. This glucosidase has a molecular mass of 114,000 Da and is produced only in cellulose-supplemented cultures. When CBGL was treated with papain, its molecular weight decreased to 95,000 Da. It lost the ability to bind to cellulose, but its catalytic activity was unchanged. This suggested that CBGL is organized into two domains—a cellulose-binding domain (CBD) and a catalytic domain [Lyman et al., 1995]. The kinetic properties of cellulose-binding and non-binding forms were similar, indicating that CBD was not involved in catalysis. The cellulose-binding form was more thermostable compared to the non-binding form.

Only one other β -glucosidase, 1,4- β -D-glucan glucohydrolase, produced by the thermophilic actinomycete *Microbispora bispora* apparently can bind to cellulose [Goyal & Eveleigh, 1996]. This glucosidase has an 83-amino acid C-terminal stretch which exhibits sequence similarity with the cellulose-binding sequence of *Clostridium stercorarium* [Goyal & Eveleigh, 1996]. Here, cloning and characterization of a cDNA clone and a genomic clone encoding CBGL is reported. Sequence analysis suggested the presence of a conserved cellulose-binding sequence which confirmed our prediction that this β -glucosidase consists of a catalytic domain and a CBD.

5.2 MATERIALS AND METHODS

5.2.1 Organisms

P. chrysosporium OGC101 (a derivative of BKM-F-1767) was obtained from Michael H. Gold of the Oregon Graduate Institute [Alic et al., 1987]. *Escherichia coli* XL1-Blue MRF' and SOLR were obtained from Stratagene (La Jolla, CA).

5.2.2 Nucleotides

Oligonucleotides were prepared by the Oregon Regional Primate Research Center (Beaverton, OR). The plasmid isolation kit was obtained from Qiagen, Inc. (Chatsworth, CA).

5.2.3 β -Glucosidase Polyclonal Antibody

A polyclonal antibody against homogeneous CBGL was raised in rabbits at the Pocono Rabbit Farm and Laboratory (Canadensis, PA). The antibody was used in immunoscreening without further purification. A 2000-fold-diluted antiserum was able to detect 1 ng of CBGL.

5.2.4 Isolation of a cDNA Clone of *cbgl*

The cDNA λ ZAP-expression library, prepared as described [Li et al., 1996], was screened with anti-CBGL antibody and a secondary antibody labeled with alkaline phosphatase. The pBluescript SK(-) plasmid containing a putative β -glucosidase

cDNA insert was rescued by *in vivo* excision with a helper phage. The plasmid was purified with a commercial plasmid isolation kit (Qiagen, Inc.). The cDNA was sequenced by the dideoxy method with the primer walking strategy [Sanger et al., 1977; Strauss et al., 1986].

5.2.5 Isolation of a Genomic Clone of *cbgl*

A λ EMBL3 genomic library of *P. chrysosporium* OGC101 was screened at high stringency [$4.8\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 48% formamide, 50°C] with a 550-bp *ApaI* fragment from the 3'-end of the *cbgl* cDNA clone. Based on the restriction mapping of the genomic clones, four overlapping restriction fragments (3.6 kb *SacI*, 1.7 kb *SacI*, 4.5 kb *SalI*, and 1.2 kb *SalI*) covering the entire region of *cbgl* were subcloned into pBluescript SK (Stratagene) and were sequenced by the primer walking method. Sequencing was performed at the Oregon Regional Primate Research Center.

5.2.6 Isolation of Full-length cDNA Clone of *cbgl*

The cDNA library of *P. chrysosporium* was probed with a restriction fragment (66–324 bp) obtained by digesting *cbgl*-2 with *MscI* and *NdeI*. Hybridization was performed at high stringency ($4.8\times$ SSC, 48% formamide, 50°C). Positive clones were purified by further screening. The pBluescript II SK plasmid containing the putative *cbgl* cDNA insert was rescued by *in vivo* excision with a helper phage. The plasmid was purified with a QIAGEN Plasmid Midi kit (Qiagen, Inc.).

5.2.7 Isolation and Analysis of Homokaryons

Single homokaryotic basidiospores were isolated as described [Alic et al., 1987; Gold & Alic, 1993]. DNAs from homokaryotic cultures were isolated by standard procedures and restriction digested with *SalI*, size fractionated in a 0.7% agarose gel, blotted onto a Magnagraph nylon transfer membrane (Micron Separations, Westboro, MA), and probed with a 32 P-labeled 1.4-kb *SacI* fragment of *cbgl* (nucleotides 1446–2866).

5.2.8 Northern (RNA) Blot Analysis

Total RNA was isolated from 11-days-old mycelia of *P. chrysosporium* cultured with 1% cotton linters, cellobiose, or glucose as the carbon source. RNA was electrophoresed in 1.5% agarose gel containing 2.2 M formaldehyde, transferred to Magnagraph nylon membranes (Micron Separations), and probed with cDNA for CBGL at 42°C as described previously [Brown, 1994b].

5.2.9 Southern Blot Analysis of *cbgl*

DNA from *P. chrysosporium* was restriction digested and electrophoresed with a 0.7% agarose gel. The DNA was transferred to Magnagraph nylon membranes and hybridized to a ³²P-labeled 1.4-kb *SalI* cDNA fragment of *cbgl* [Brown, 1994a].

5.2.10 Phylogenetic Analysis

All β -glucosidase sequences were retrieved from the NCBI database. Phylogenetic analysis was accomplished using CLUSTAL W and PHYLIP version 3.5c.

5.2.11 Nucleotide Accession Number

The *P. chrysosporium* OGC101 CBGL cDNA and gene sequence data reported here have been deposited in GenBank under accession numbers AF036873 and AF036872.

5.3 RESULTS AND DISCUSSION

Seventy-two positive clones of *cbgl* were isolated by immunoscreening of the *P. chrysosporium* λ cDNA expression library with CBGL-specific polyclonal antibodies. Genomic clones were isolated by screening a λ EMBL3 genomic library of *P. chrysosporium* with a 500-bp *ApaI* fragment from the 3' region of the cDNA sequence. This screening yielded ~50 positive genomic clones. Restriction fragment analyses of five clones which hybridized strongly to the probe indicated that they were

very similar, and one of them was subcloned and sequenced. A full-length cDNA clone was isolated by screening the cDNA library with a *MscI-NdeI* fragment from one genomic clone *cbgl-2*.

5.3.1 *cbgl* cDNA Sequence

Sequence analysis of the cDNA clone (2.4 kb) revealed an open reading frame consisting of 2469 bp encoding 823 amino acids, including a potential 21 amino acid N-terminal signal peptide sequence (Fig. 5.1). The N-terminal sequence of mature CBGL could not be determined, because its N-terminus is blocked. Prediction of the signal peptide cleavage site suggested Gln22 as the N-terminal amino acid [Nielsen et al., 1997]. The mature CBGL apparently consists of 802 amino acids with calculated molecular mass of 83,439 Da. The CBGL molecular weight as determined by SDS-PAGE was 114,000 Da. CBGL is a glycoprotein, and the difference in molecular weight could be attributable to carbohydrate. The cDNA sequence revealed six potential N-glycosylation sites conforming to the general rule Asn-X-Thr/Ser in which X is not a proline (Fig. 5.1) [Bause, 1983]. In addition, numerous potential O-glycosylation sites exist.

5.3.2 The Cellulose-binding Domain

Fungal and bacterial cellulases are organized into two distinct domains—a cellulose-binding domain (CBD) and a catalytic domain. A short linker peptide sequence connects the two domains. CBDs tend to be present at either the C- or N-terminus of a protein [Gilkes et al., 1991]. CBD sequences have been classified into ten families [Tomme et al., 1995]. Family 1 apparently contain only CBDs from fungal enzymes. Fungal CBDs consist of a conserved 30–36 amino acid sequence. Conserved residues in this sequence include four cysteines, two glutamines, and four aromatic residues. Analysis of the amino acid sequence predicted by the *cbgl* cDNA suggested that the amino acid sequence from 22–57 had a high sequence similarity with the conserved cellulose-binding sequence of CBHII from *P. chrysosporium* and other fungal CBD sequences (Fig. 5.2). This supported our hypothesis that CBGL would possess a CBD similar to that of cellulases.

TCGAAGTTGCCGTGTTTGTCCGCGCCTTATCCGCTCGACGCGGCTGCTGTTGGGCAACGACGTGGCCAAGACCATTTCGATCTACATATCGACTTCTTCAAACATGTTTCGAGAAGTAT 120
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M G L T L V V L L H L A L G L L T G V 19
AAGCTCAGTCCGCGCTGTATCAGCAATGTGGCGGTATCGGATGgttagctggcttgcctcctcgctccggagtgatgctcatatgttccctcgcatgcagGACTGGTGCAACTACTTG 360
Q A Q S G L Y Q Q C G G I G W T G A T T C 40
CGTCAGCGGGCGGACCTGACAGTGTGAACCCATgtacgtcgccacacagtcacagcagccgagctcatgacacgtagACTACTCGCAATGTCTCCCTGGCGCCGCCACCACG 480
V S G A T C T V L N P Y Y S Q C L P G A A T T 63
AGTGTTCGTCTAGTCATTTCGTCAAGTAGCAGCGTCAGCAGCCACTCCAGCAGTGCAAGCAGCAGCAGCATAAGTTCTACTAGCACCAGCCCCCGGCGCCCTCCAGACCGTGGCGAAC 600
S V S S S H S S S S S V S S H S S S A S S S S I S S T S T S P P A P S Q T V A N 103
G T T C
GTGTCTCCGAATGGGCGCGCTTACGTCAAGgttgaacgacgtcttcagatgtatatgttgatgtcactgaaacacgcgcagGCACAAGCTGTGTAGCAAAGCTATCCGGTGACTGA 720
V S P E W A A A Y V K A Q A A V A K L S V T D 126
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M V N L A T G V Q W Q K G P C V G N T P A I S S 150
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T S V F P P E I N V A A T W N R T L M R Q 192
CGCGAGCTGCTATGGAGCGGAATTAAAGGGGAAAGCGTGCACGTCGCTCTCGGTCCCATGATGAACCTCATGCGGTGTCGCCCGCGCTGGCCGAAACTGGGAAGGgtgagtttctttg 1200
R G A A M G A E F K G K G V H V A L G P M M N L M R V P A A G R N W E G 228
gagtggttgacacgcttgcgcgcgagcacttaatttctcgtcttggattgtagTGGCGGTGGTATCCTTTCCTCTGCGCAAGTGGCGTTTCGAGACCATCACTGGCATTTCAGTCCT 1320
G G G D P F L S G E V A F E T I T G I Q S 249
G (S)
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S G A Q A C A K H F I N N E Q E H F R D S S S 272
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E H E L Y G H P F L R S V Q A N V A S V M C S 302
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Y N Q 305
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D M A T R I L A A W Y L L G Q D Q N F P A V N F N S W N S G Q G 418
C
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Q H V N V S G N H A S L I R T I G A A S Q I 440
C G G
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L L K N V N S A L P L K K P K T I G I I G N G A G S N P N G P N A 473
(A) (G)


```

      T
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      T C
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      A C
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A R A S Q D G T T V S S S L S D T D L T G A A N T A T G K D V A M V F I T A D S 546
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      V W S G L P
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      G N A V T D V L F G A V N P G
      A
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I V P I P Y N E G L F I D Y R H F D Q
      A G I
      A
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D P W
      L H D S V V T V S F T L T N N G T V
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S I T N *
      A CG
GAGGTACACTGACTGACTTTACATGGAGGCAGTTTGGCCATATAAGCTAGCTTCCCCTTGCGTGCGAACAGGTAACACGACGGGTAAAAATGTTTCTGCAGCCCAACCATGTACAAA 4438
ACTACAGATTAGGGGGATCACGTCACTGCGAAGAGTCATGTTGCTAGAAGTGAATATGCGGGTAGTACAATGATATATCCAGACTCGGGGAGTTACGTCGCCAGTGACGCTTTTGA 4555

```

Fig. 5.1 Nucleotide and deduced amino acid sequence of CBGL from *P. chrysosporium*. Genomic and amino acid sequences were derived from *cbgl-2*. Amino acid sequence deduced from the cDNA sequence of *cbgl-1* was same as that of *cbgl-2* except at some positions. These positions are indicated below the amino acid sequence and are enclosed in parentheses. The exon sequence of *cbgl-1* is the same as that of *cbgl-2* except that at some positions. These positions are indicated above the nucleotide sequence. Nucleotides and amino acids are numbered on the right. The potential signal peptide sequence is overlined. The potential cellulose binding domain is boxed. Potential N-glycosylation sites are indicated in bold letters.

```

cbg1      22 QSGLYQQCGGIGWTGATTCVSGATCTVLNPYYSQCL 57
pcCBHII   21 QASEWGQCGGIGWTGPTTCVSGTTCTVLNPYYSQCL 56
pcCBHI    481 TVPQWGQCGGIGYTGSTTCASPYTCHVLNPYYSQCY 516
trCBHI    478 TQSHYGQCGGIGYSGPTVCASGTTTCQVLNPYYSQCL 513
trEGIII   22 QQTVWGQCGGIGWSGPTNCAPGSACSTLNPYYAQCI 57
          ***** . * * * . * ***** . **

```

Fig. 5.2 Comparison of cellulose-binding domain sequences of cellulose-binding glucosidase (CBGL), cellobiohydrolase I (pcCBHI), and cellobiohydrolase II (pcCBHII) from *P. chrysosporium* and cellobiohydrolase I (trCBHI) and endoglucanase III (trEGIII) from *Trichoderma reesei*.

5.3.3 The Linker Region

Unlike the CBD sequences, the linker sequences which connect the CBDs to the respective catalytic domains are not conserved. They vary greatly in length and sequence. They are enriched with either hydroxyamino acids or proline and hydroxyamino acids. The linker sequence of CBGL is approximately 43 amino acids long and enriched with serines.

5.3.4 The Catalytic Domain

Amino acids 101 to 823 at the C-terminus appear to form the catalytic domain. Henrissat and coworkers have classified glycosidases into 57 families based on amino acid sequence similarities [Henrissat & Bairoch, 1993]. β -Glucosidases belong to either family 1 or 3. Family 1 β -glucosidases are small ($M_r \cong 50,000$ – $60,000$), intracellular, and are generally found in bacteria and plants. They are identified by conserved motifs such as ENG and QIEGA [Rojas & Romeu, 1996]. Family 3 β -glucosidases are larger ($M_r \cong 85,000$ – $120,000$), extracellular or intracellular, and are produced by bacteria, yeast, and fungi. Family 3 β -glucosidases are identified by conserved motifs such as VMSDW and GLDM (Fig. 5.3). According to Henrissat's classification [Henrissat & Bairoch, 1993], CBGL would be categorized under family 3 along with the extracellular β -glucosidases from *T. reesei*, *Aspergillus aculeatus*, *Saccharomycopsis fibuligera*, and *Pichia capsulata* [Kohchi & Toh-e, 1985; Machida et al., 1988; Barnett et al., 1991; Henrissat & Bairoch, 1993; Janbon et al., 1995]. A phylogenetic diagram was developed by pair-wise comparison of family 3 β -glucosidase amino acid sequences (Fig. 5.4). This analysis clusters family 3 glucosidases into three groups. One group consists of fungal and yeast glucosidases. CBGL from *P. chrysosporium* is a member of this group. Also, this analysis showed that CBGL is closely related to the *T. reesei* glucosidase. The other two groups consist mostly of bacterial glucosidases.

5.3.5 Catalytic Residues

Glu and Asp have been found in the active sites of numerous glycosidases including β -glucosidase, cellulase, amylase, and lysozyme (Table 5.1).

Saf1 1 MLMIVQLLVFALGLAVAVPIQNYTQSPSQ-RDE-SSQWVSPHYYPPTQGG--RLQDVWQEAAYARAKAIVGQMTIVEKVN
Saf2 1 MLLILELLVLIIGLGVALPVQTHNLTDNQGFDEESSQWISPHYYPTPQGG--RLQGVWQDAYTKAKALVSQMTIVEKVN
Phc 1 -----VAN---VSPWAAAAYVKAQAAYAKLSVTDNVN
Trr 1 -----MRYRTAAALALATGPFARADS-----HSTSGASAEAVVPP---AGTPWGTAYDKAKAALAKLNLQDKVGI
Asa 1 -----MKLSWLEAAALTAASVVSAD-----LAFSPFPYFSPWAN---GQGEWAEAYQRAVAIVSQMTLDEKVN
Sel 1 -----MVSSLFNIAALAGAVIALSHED-----QSKHFTTIPFTFPDST---GEG-WKAAFEKAADAVSRLNLTQKVAL
Pic 1 ----MKSTIIILSVLAAATAKNISKAEMENLEHWWSYGRSDPVYPSPEIS---GLGDWQFAYQRAREIVALMTNEEKTNL
Pia 1 ----MLLPLYGLASFLVLSQAALVNTSAPQASNDPFPNHSPSYFPTPQGGRI-NDGKWQAAYFRARELVDQMSTAEKVN

Saf1 77 TTCTGWQLDPCVGN TGSVPRFG-IPNLCLQDGELGVRFADFVTGYPSGLATGATFNKDLFLQRGQALGHEFNSKGVHIAL
Saf2 79 TTCTGWQLGPCVGN TGSVPRFG-IPNLCLQDGELGVRLTDFSTGYPSGMATGATFNKDLFLQRGQALGHEFNSKGVHIAL
Phc 31 ATGVQWQKGPCVGNTPAISSIPGFTGLCLQDSPVGVRYADGTSVFPEINVAATWNRTLMRQRGAAMGAEEKGKGVHVAL
Trr 63 VSGVGVWNGGPCVGN TSPASKIS-YPSLCLQDGELGVRYSTGSTAFTPGVQAASTWDVNLIRERGGQFEGEVKASGIHVIL
Asa 63 TTCTGWELEKCVGQTGGVPRLN-IGGMCLQDSPGLGRDSDYNSAFPAQVNVAAATWDKNLAYLRGQAMGQEFSDKIDVQL
Sel 66 TTCTTAGLS-CNGNIAPLPEIN-FSGLCLADGFSVRIADLATVFPAGLTAAATWDRQLIYERARALGSEERKGSQVHL
Pic 74 TFGSSGDTG-CSGMISDVDPVD-FPGLCLQDAGNGVRGTDMMNAYASGLHVGA SWNRQLAYDRAVYMGAEFRHKGVNVLL
Pia 76 TTGVGSASGPCSGN TGSVPRLN-ISSICVQDGELSVRAADLTDFVPCGMAASSSFNKQLIYDRAVAIGSEFKGKGADAIL

Saf1 156 GPAVGPLGVKARGGRNFEAFGSDPYLQGTAAAAATIKGLQENNVMA CVKHFIGNEQEKYRQPDD-----INPATNQTKEA
Saf2 158 GPAVGPLGVKARGGRNFEAFGSDPYLQGTAAAAATIKGLQENNVMA CVKHFIGNEQDIYRQPSNS---KVDPEYDPATKES
Phc 111 GPMMN-LMRVPAAGRNWEGGGGDPFLSGEVAFETISGIIQSSGAQACAKHFINNNEQEHFR-----DS
Trr 142 GPVAGPLGKTPQGRNWEFGFVDPYLTGIA MGQTINGIQSVGVQATAKHVILNEQELNR-----ET
Asa 142 GPAAGPLGRSPDGRNWEFGFSPDPALTVLFAETIKGIQDAGVVAATAKHVILNEQBHFQVAE-----AAGYGFNISDT
Sel 144 GPASGALGRHPLGGRNWEFSFDPYLSGVAMDFSIRGIIQEMGVQANRKHFIGNEQETQR--SN-----TFTDDGTEIQA
Pic 152 GPVVGPIGRVATGGRNWEFGFTNDPYLAGALVYETTKGIIQEN-VIACTKHFIGNEQETNR-----NPSG--TYNQS
Pia 155 GPVYGPMGVKAAGRGWEGHGDPPYLEGVIAYLQTIGIQSQGVVSTAKHLIGNEQBHFRAKKDKHAGKIDPGMFNTSSS

Saf1 231 ISANIPDRAMHLYLWPFADSVRAGVGSVMCSYNRVNNTYACENS SYMMNHLKKEELGFQGFVVS DWGAQLSGVYS AISGL
Saf2 235 ISANIPDRAMHLYLWPFADSVRAGVGSVMCSYNRVNNTYACENS SYMINHLKKEELGFQGFVVS DWAAQMSGAYS AISGL
Phc 171 SSSNVDDRTEHELYLWPFELRSVQANVASVMCSYNQINGTFS CENEKTL SGLKGEYGFQGYVMS DWWATHSGAPAVNAGL
Trr 203 ISSNFDDRTLHELYTWPFADAVQANVASVMCSYNKVNTTWACEDQYTLQTVLKDQLGFPGYVMTDWDQAHTTVQSANSGL
Asa 216 ISSNVDDKTIHEMYLWPFADAVRAGVGAIMCSYNQINNSYGCQNSYTLNKLKAEELGFQGFVMSDWGAHHS GVGSALAGL
Sel 216 ISSNIDDRTMHELYLWPFANAVRSGVASVMCSYNRLNQTYACENS SKLMNGILKGEELGFQGYVVS DWYATHSGVESV NAGL
Pic 219 VSANIDDKTMHELYLWPFQDSVRAGLGSIMGSYNRVNNSYACKNSKVLNGLKSELGFQGFVVS DWGGQHTGIASANAGL
Pia 235 LSSEIDDRAMHEIYLWPF AEAVRGVSSIMCSYNKLNLSHACQNSYLLNLYLKEELGFQGFVMTDWDGALYSGIDAANAGL

Saf1 311 DMSMPGEVYGGWNTG---TSFWGQNLTKAVYNETVPIERLDDMATRILAALYATNSFPTED---HLPNFSSWTTKEYGNK
Saf2 315 DMSMPGELLGGWNTG---KSYWGQNLTKAVYNETVPIERLDDMATRILAALYATNSFPTKD---RLPNFSSFTTKEYGNE
Phc 251 DMTMPGDETLSSGTT---YFGQNLVN AVNSGQVSQARVKDMATRILA AWYLLGQDQNF---PAVNFSWNSGQ---
Trr 283 DMSMPG-TDFNGNNR---LWGPALTNAVNSNQVPTSRVDDMVTRILA AWYLTGQDQ-----AGYPSFNISR---
Asa 296 DMSMPGDITFDSATS---FWGTNLTIAYLVNGTVPQWRVDDMAVRIMAA YKVGDRDLY---QPPNFSSWTRDEYGFK
Sel 296 DMTMPGPLDSPSTALRPPSYLGGNLTEAVLVNGTIPEARVDDMARILMPYFFLGQD TDFPTVDPSTGFVFARTYNYPDE
Pic 299 DMAMPS-----ST-----YWEGLIEAVKNGTVDQSRLLDMATRILA AWYKYARLDD-----PGFGMPVSLAED--
Pia 315 DMDMPCEA-----Q-----YFGGNLTAVLVNGTLPQDRLLDMATRILSALIYSVHNHP-----DGPNYNAQTFLTEGHE

Saf1 385 -YADNTTEIVKVNYNVDPNSNDYFTEDTALKVAEESI VLLKNEENTLPTSPERAKRLLLSGIAAG---PDPICYQ-----C
 Saf2 389 FFVDKTSPPVVKVNHVDPNSND-FTEDTALKVAEESI VLLKNEKNTLPTSPNVRKLLLSGIAAG---PDPKGYE-----C
 Phc 319 -----QHVNVSNG--HASLRTIGAASQVLLKNANGALPLK--KPKTIGIIGNGAG---SNPNGPN----AF
 Trr 345 -----NVQGN--HKTNVRAIARDGIVLLKNDANILPLK--KPAStAVVGSAAI---IGNHARNSP--SC
 Asa 367 YFYPQEGPYEKVNHFVNVRN--HSEVIRKLGADSTVLLKNNN-ALPLTG--KERKVAILEGDA---SNSYGAN----GC
 Sel 376 YLTGGGLDPYNPPPPARDVRGN--HSDIVRKVAAAGTVLLKNVNNVLPK--EPKSVGIFGNGAA---DVTEGLTFTGDDS
 Pic 358 -----HELVDARDP-AAASTIFQGAVEGHVLLKNNEN-ALPLK--KPKYISLFGYDGVSTDVNTVGGGFSFFSF
 Pia 379 YFKQQEGDIVVLNKHVDVRS- INRAVALRSAGEV VLLKNEHETLPLGREKVRKISILGQAAG---DDSKGTS-----C

Saf1 456 EDQSCNTGALFQGWGSGSVGSPKYQVTFEEISYLARKNKMQFDYIRESYDLAQVTK--VASDAHLSIVVVSAAASGECYI
 Saf2 460 SDQSCVDGALFEGWGSQSVGYPKYQVTFEEISANARKNKMQFDYIRESFDLTQVST--VASDAHMSIVVVSASVSGECYL
 Phc 375 SDRAGDVCVLALGWGSGTANFP-YLVAPVDAITARASQDGTTVSSSLSDTLTGAAN--TATGKDVAMVFITADSGECYL
 Trr 400 NDKGCDGALGMLGWGSGAVNYP-YFVAPYDAINTRASSQGTQVTLSDNTDNTSSGAS---AARGKDVAVFITADSGECYI
 Asa 436 SDRGCDNGTLAMAWGSGTAFFP-YLVTPAQIAEVLKHKGSVYAITDNWALSQVET--LAKQASVSLVFNSDAGECYI
 Sel 449 GPWGADICALSVGGGSGAGRHT-HLVSPLAARKRRTESVGGRVQYLLSNSRIVNDDFTSIYPTPEVCLVFLKTWARECTD
 Pic 422 DVKAIENKTLISGGGSG-TNTPSYVDAPFNAFVAKARE-----NTFLSWDFTSAEP-VANPASDADIDFINAAASECYD
 Pia 450 SLRGCSSGAGTGYGSGAGTFS-YFVTPADGTGARAQKEKISYEFIGDSWNQAAAMD--SALYADAAIEVANSVAGBEIG

Saf1 534 TVDGNQGDNRKNLTLWNNGDKLIETVAENCANTVVVVVTSQGQINFEGFADHPNVTAIIVWAGPLGDRSGTAIANILFGKANP
 Saf2 538 IVDGNRGDKNNVTLWHNSDNLKAVAENCANTVVVVITSQGQVDVESFADHPNVTAIIVWAGPLGDRSGTAIANILFGNANP
 Phc 452 TVEGNAGDRNDLQAWHGGDALVQQVASHNKNITIVVINSVGPINMEAWVNHPNVTAIIVWSGLPGQEAGNAVTDVLFCAVNP
 Trr 476 TVEGNAGDRNNLDPWHNGNALVQAVAGANSNVIVVVHVSVAIILEQILALPQVKAUVWAGLPSQESGNALVDVLWGDVSP
 Asa 513 SVDGNEGDRNNLTLWKNGDNLKAAANNCNNTIVVIHVSVPVLVDEWYDHPNVTAILWAGLPGQESGNSLADVLVGRVNP
 Sel 528 RLS-YEND-----WN-STAVVNNVARRCPTITIVVTHSGC-INTMPWADNANVTAILAAHYPGQENGNSIMDILYGDVNP
 Pic 495 RPN--LAD-----KY-SDKLVEAVASQCSNTIVVIHNAIRLVNDNWIENHTVGVILAHLPQDGTGTSLEVLVYGNQSP
 Pia 527 DVDGNYGDLNNLTLWHNAVPLIKNISSINNTIVIVTSGQQIDLEPFIDNEHTVAVIYSSYLQDQFGTVLAKVLFQDENP

Saf1 614 SGHLPFTIAKT---DDYIPIETYSPPSSG-EPED--NHLVENDLLVDYRYFEKNIEPRYAFGYGLSYNEYEVSNKAVSA
 Saf2 618 SGHLPFTVAKS---NDDYIPIVTYNPPNG-EPED--NTLAHEDLLVDYRYFEKNIEPRYAFGYGLSYNEYKVSNAKUSA
 Phc 532 GGLPFTIGKS---ISDYSAQIITTGSGI-VP-----IPYNEGLFIDYRHFQAGIAPRFEFGFGLSYTTFDYSNLVITG
 Trr 556 SKLVYTTIAKS---PNDYNTRIVSGGS-----DSFSEGLFIDYKHFDDANITPRYFEGYGLSYTKFNYSRLSVLS
 Asa 593 GAKSPFTWCKTREAYGDYLVRELNNNGA-PQ----DDFSEGVFIDYRGFDKRNETPIYEFEGHGLSYTTFNYSGLHIQV
 Sel 599 SGRLPYTIIPKL---ATDYDFPVVNITNEA-QDPYVWQADTEGLLDYRHFDAARNITPLYEFGYGLSYTTFEIEGVANLV
 Pic 566 SGRLPYTVAKK---ASDYGGLLWPTPEGDLDLFYFPQSNTEGVYIDYKYFIQKNITPRYFEGYGLSYTTFDYSELEVDA
 Pia 607 SGRLPFTIAKD---VNDYIPVIEKVDVP--DP----VDKFTESIYVDYRYFDKYNKPVRYEFGYGLSYSNFSLSDIEIQT

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Saf1 688 AKKVDEELPEPATYLSEFSYQNAKDSKNPDAFAPADLNRVNEYLYPYLDSNVTLKDGNYEYPDGYSTEQRTPNQPGGG
Saf2 692 AKKVDEELPQPKLYLAEYSYNKTEEINNPEDAFFPSNARRIQEFLLPYLDSNVTLKDGNYEYPDGYSTEQRTPPIQPGGG
Phc 603 STAG-----G-TRQPPGPGSS
Trr 623 TAKS-----GPATGAVVPGGP
Asa 667 LNASSNAQVATETGAAPTFTGQVG---NASDYVYPEGLTRISKFIYPWLNSTDLKASS---GDPYVGVDTAEHVPEGA
Sel 675 AKSAKLS-----AFFASTDISHPGGN
Pic 643 ITNQS-----YLPPDCTIEEGGA
Pia 678 LQPFSENAEPAANYSETYQYKQ-----SNMDPSEYTVPEGF

Saf1 768 LGG-----NDALWEVAYNSTDKFVPQGNSTDKFVPOLYLKHPEDGK-FETP-IQLRGFEKV---ELSPGE
Saf2 772 LGG-----NDALWEVAYKVEVDVQNLGNSTDKFVPOLYLKHPEDGK-FETP-VQLRGFEKV---ELSPGE
Phc 618 LDP-----WLHDSVTVSFTLTNNGTVDGTEVPOLYLSPPASAK--SAP-QNLKGFDSV---FLPAGA
Trr 639 SD-----LFQNVATVTVDIANSQVGTGAQVLYITYPSSAP--RTPPKQLRGFAKL---NLTPGQ
Asa 738 TDGSPQPVLPAGGGSGGNPRLYDELIRVSVTVKNTGRVAGDAVPOLYVS--LGGP--NEPKVVLRFKFDRL---TLKPSE
Sel 696 PD-----LWEEVSVTAAVKNTGSVSGSQVQVLYISLPADGIPENSPMQVLRGFVKV---DLQPGQ
Pic 661 KS-----LWDIVATVKFTVTNTGDVAAAEVPLVVGIP-NGP---P-KVLRGFDDKK---LTHPGQ
Pia 714 KE-----LANYTYPYIHDASSIKANSSYDYPEGYSTEQLDGP-KSLAAGGLGNHTCGMLVTL SLLK

Saf1 828 KKTVDLRLRLRRDLSVWDTRQSWIVESGTYEALIGVAVNDIKTSVLFTI--
Saf2 832 KKTVEFELLRRDLSVWDTRQSWIVESGTYEALIGVAVNDIKTSVLFTI--
Phc 675 STTVSFELSRYSFSVWDVVSQSWQIPAGVTGISVCASSRDRLKGSITN--
Trr 695 SGTATFNIRRRDLSYWDTASQKWVVPSSGSGISVCASSRDRLTSTLSVA-
Asa 810 ETVWTTTLTRRDLSNWDVAAQDWVITSYPKKVHVSGSSRQLPLHAALPKVQ
Sel 754 SKSVEFSIMRRDLSFWNTAQDWELPNGQIEFRVCFSSRDIKSIVSRSL-
Pic 713 SEEFVVELTRRDLSWDVVAQNWGLQAGTYQFYVGRSVFDVPLTSALVFTN
Pia 775 SQIKVLMVLVGLHLNCLMDIQIMMNSQHLQCNYVDLKRCFWIKIILKLFLLN

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Fig. 5.3 Comparison of the catalytic domain sequence of CBGL with the β -glucosidases from *Trichoderma reesei* (Trr), *Aspergillus aculeatus* (Asa), *Saccharomycopsis fibuligera* (Saf1 and Saf2), *Pichia anomala* (Pia), *Pichia capsulata* (Pic) and *Septoria lycopersici* (Sel).

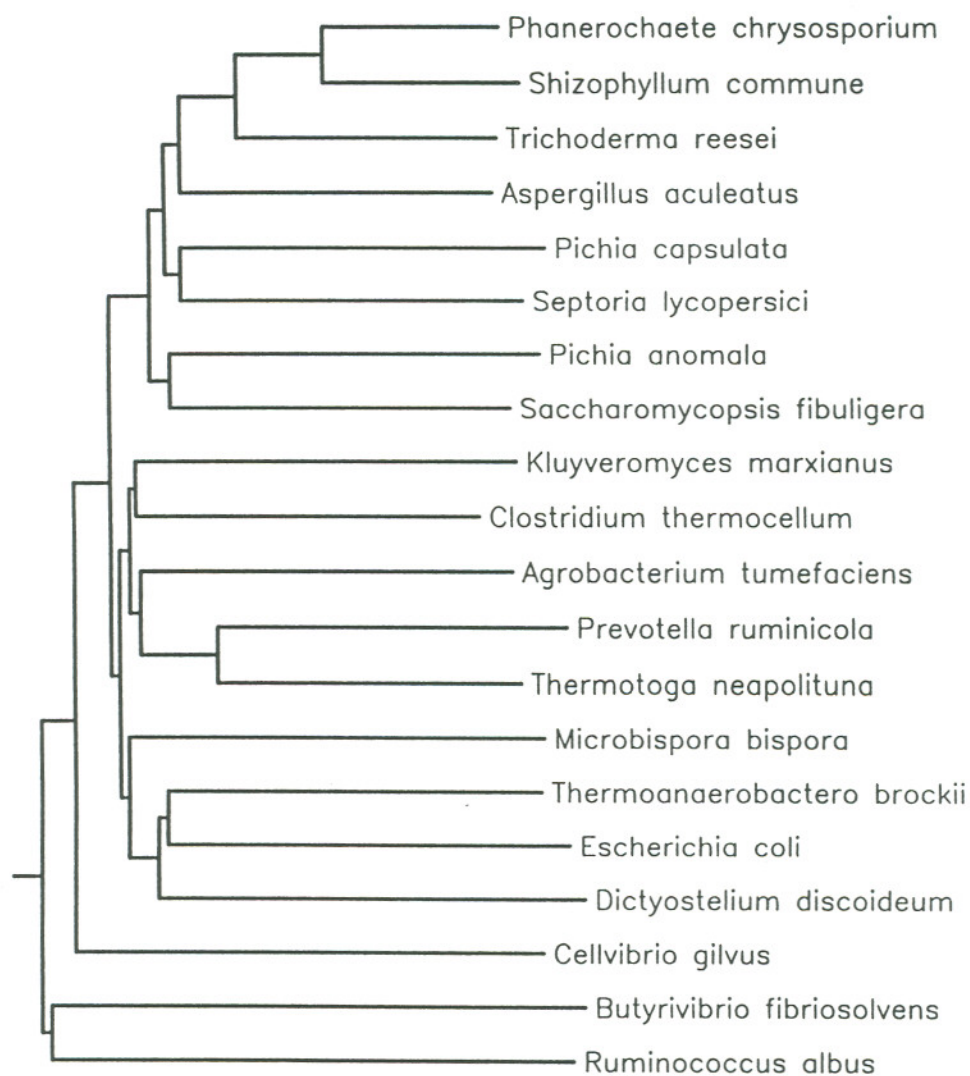


Fig. 5.4 Phylogenetic analysis of CBGL.

Table 5.1 Catalytic Residues of Glycosyl Hydrolases

Enzyme	Source	Catalytic residues	Reference
Lysozyme	hen egg white	Glu35, Glu52	Chipman & Sharon, 1969
Taka-amylase	<i>Aspergillus oryzae</i>	Glu230, Asp297	Matsuura et al., 1984
α -Amylase	procine pancreas	Asp197, Asp300	Buisson et al., 1987
CBHII	<i>Trichoderma reesei</i>	Asp175, Asp221	Rouvinen et al., 1990
Xylanase A	<i>Streptomyces lividans</i>	Glu128, Glu236	Derewenda et al., 1994
Endocellulase	<i>Thermomonospora fusca</i>	Asp117, Asp265	Stenberg et al., 1977
β -Glucosidase	<i>Agrobacterium fecalis</i>	Glu170, Glu358	Vaheri et al., 1979

The catalytic mechanism of lysozyme serves as a model for these glycosidases. The lysozyme active site has two catalytically important acidic residues, Glu35 and Asp52 [Chipman & Sharon, 1969]. Glu35 is protonated, whereas Asp52 is deprotonated. In the lysozyme reaction, Glu35 functions as a general acid and protonates the glycosidic linkage. This results in glycosidic bond cleavage and generation of a carbonium ion which is stabilized by the deprotonated Asp52. In the next step, the deprotonated Glu35 functions as a general base and generates a hydroxide ion by accepting a proton from H_2O . The hydroxide ion neutralizes the charge on the carbonium ion and thus completes the hydrolysis reaction. Our analysis of the catalytic domain sequences of eight family 3 fungal and yeast glycosidases indicated conservation of 13 acidic amino acid residues (Fig. 5.3). Sequences surrounding seven conserved acidic residues are preserved (Fig. 5.3). The three-dimensional structure of any family 3 glycosidase has not been solved yet. Consequently, the active site structure of these enzymes remains unknown. Legler et al. [1979], using an active site affinity label, conduritol epoxide, identified an aspartate residue at the active site which was part of an VMSDW motif. This motif has been found in several family 3 glycosidases. Twelve amino acids downstream from the VMSDW motif is another conserved motif, GLDM. In *P. chrysosporium* β -glucosidase, the VMSDW motif is located between residues 239 and 243 and the GLDM motif between residues 256 and 259. In analogy with lysozyme in which the catalytic residues are separated by only 17 residues, Barnett et al. [1991] suggested that the Asp in the GLDM motif as the possible second catalytic amino acid residue. Raynal et al. [1987] again in analogy with lysozyme, proposed that a conserved Glu located approximately nine amino acids upstream from the Asp of the VMSDW motif might be the second acidic amino acid residue. Table 5.1 shows the specific Asp and Glu residues present at the active site of a number of glycosidases. Unlike lysozyme, where the Asp are separated by only 17 residues, the catalytic residues are separated by much longer sequences in all the other glycosidases. So, the two putative acidic amino acid residues in family 3 glucosidases need not be in proximity. Potentially, any two of the conserved acidic amino acid residues could be involved in catalysis.

5.3.6 *cbgl* Gene Expression

P. chrysosporium produced CBGL abundantly only when cellulose is provided as the sole carbon source [Bao et al., 1994; Lymar et al., 1995]. To obtain further evidence that CBGL synthesis is regulated by cellulose, total RNA was isolated from 11-day-old cellulose, cellobiose, or glucose cultures and analyzed by Northern blotting (Fig. 5.5). A band corresponding to 2.4 kb was observed only with the RNA isolated from cellulose-grown cells. Also, the size of this RNA was similar to the size of the cloned *cbgl* cDNA. These preliminary findings suggest that either cellulose or one of its degradation products controls the expression of *cbgl* at the transcriptional level.

5.3.7 Gene Sequence of *cbgl-2*

cbgl-2 consists of 4555 bp, including 182 bp in the 5'-flanking region and 339 bp in the 3'-flanking region (Fig. 5.1). The 5'-upstream region contains a potential TATAA box (TATAAGT) 64 bp upstream from the translation start codon. Comparison of the genomic and cDNA sequences of CBGL indicated the presence of 29 introns varying in size from 47 to 68 bp. All of the intron splice junctions conformed to the GT-AG rule. Exon 1 codes for the signal peptide and a portion of the CBD (Fig. 5.6). Exon 2 codes only for the CBD. Exon 3 codes for the rest of the CBD, the linker peptide and a small part of the catalytic domain. Exons 4-30 code for the catalytic domain. Interestingly, exons 10 and 13 code for only one and two amino acids, respectively (Fig. 5.6). In contrast to *cbgl*, *T. reesei bglu1* has only two introns [Barnett et al., 1991]. The number of introns found in *cbgl* is the highest among the known *P. chrysosporium* genes.

cbgl-2 exon sequences exhibited 98% homology to the *cbgl* cDNA sequence. A total of 50 bp in the sequences (in the exon regions) did not match the cDNA sequence; however, the predicted amino acid sequences differed at only four positions. Restriction analysis of *P. chrysosporium* DNA indicated that, except for *HindIII* restriction, only one fragment from all the other restriction digestions hybridized to a 1.4-kb *SalI* fragment of *cbgl-2* (Fig. 5.7). This suggested that *cbgl* is

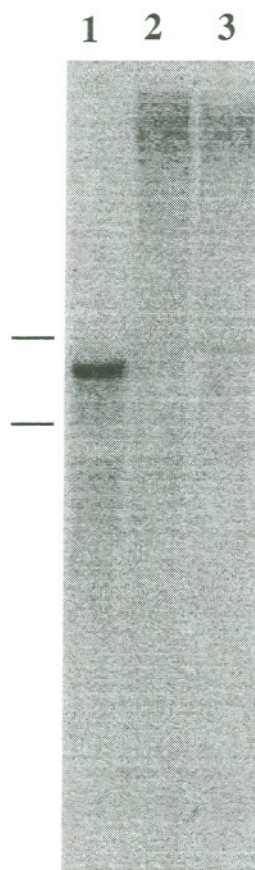


Fig. 5.5 Northern blot analysis of *P. chrysosporium* RNA. Total RNA was isolated from 11-days-old mycelia obtained from 1% cellulose (lane 1), glucose (lane 2), and cellobiose (lane 3). The RNA was fractionated by electrophoresis in 1% agarose containing 2.2 M formaldehyde and transferred to a Magnagraph nylon membrane [Brown, 1994b]. The blot was probed with ^{32}P -labeled CBGL cDNA. Bars to the left indicate the positions of 18S and 28S rRNA.

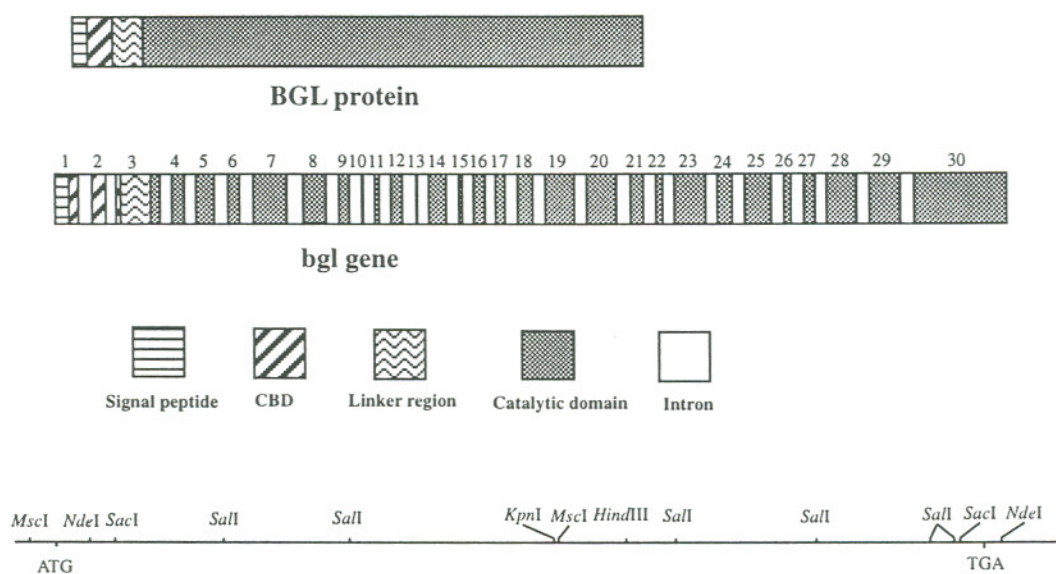


Fig. 5.6 Schematic representation of the protein and gene structures of CBGL and the restriction map of *cbgl-2*.

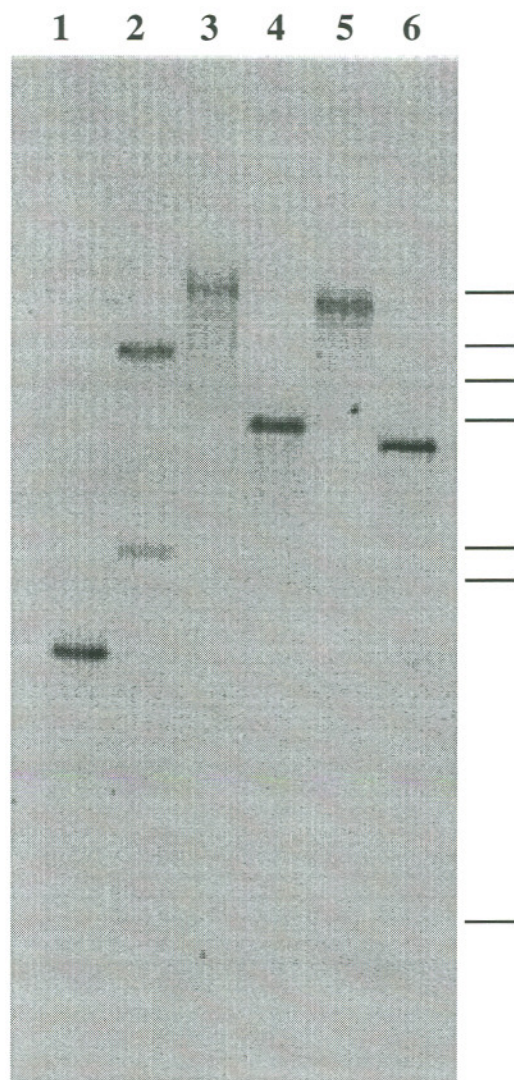


Fig. 5.7 Southern analysis of genomic DNA from *P. chrysosporium*. Genomic DNA, isolated by standard procedures, was digested with restriction enzymes *SalI* (lane 1), *HindIII* (lane 2), *BamHI* (lane 3), *NdeI* (lane 4), *EcoRI* (lane 5), and *SacI* (lane 6). DNA was electrophoresed in 0.7% agarose and transferred to a Maganagraph nylon membrane. The blot was probed with a ^{32}P -labeled 1.4-kb *SalI* fragment of *cbgl-2*. Bars indicate the position of molecular size standards (from top to bottom) 23.1, 9.4, 6.6, 4.4, 2.3, 2.0 and 0.6 kb.

probably encoded by two alleles (*cbgl-1* and *cbgl-2*) of a single gene. The cDNA clone was presumably derived from *cbgl-1*.

5.3.8 *cbgl-1* and *cbgl-2* Allelism

P. chrysosporium is a heterokaryon with two or more genetically distinct nuclei [Alic et al., 1987]. The genomic library from which the *cbgl* clones were isolated was derived from such a heterokaryon. However, mycelia derived from the basidiospores are homokaryons and contain two identical nuclei. If *cbgl-1* and *cbgl-2* are truly allelic, they should segregate and all basidiospores should contain only one or the other *cbgl* allele but not both. Segregation of allelic variants of lignin peroxidase, glyoxal oxidase, and cellobiose dehydrogenase from *P. chrysosporium* is known [Gaskell et al., 1991; Kersten et al., 1995; Li et al., 1997]. A comparison of the cDNA sequences of *cbgl-1* and the exon sequences of *cbgl-2* suggested that *cbgl-2* has at least one extra *SalI* site at nt 2866 (Fig. 5.6). This difference was utilized in distinguishing between two alleles. DNAs from homokaryotic and heterokaryotic cultures were restricted with *SalI* and probed with a 1.4-kb *SalI* fragment (1446–2866 bp). The probe was expected to hybridize to only a 2-kb fragment from *cbgl-1*, a 1.4-kb fragment from *cbgl-2*, and to two fragments (1.4- and 2-kb) from a wild-type heterokaryon. Southern analysis showed that only one fragment (1.4- or 2-kb) was present in homokaryon DNA, and two fragments from heterokaryon DNA (Fig. 5.8). These findings support the proposal that *cbgl-1* and *cbgl-2* are alleles.

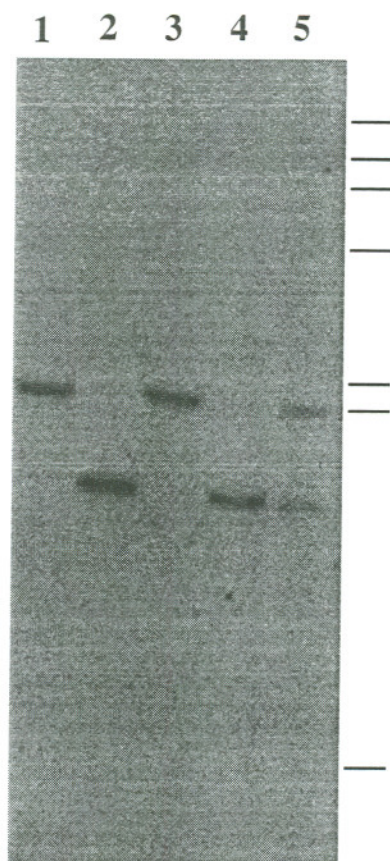


Fig. 5.8 Segregation of CBGL alleles into homokaryons. DNA from four separate single spore cultures (lanes 1-4) and one parenteral heterokaryon culture of *P. chrysosporium* OGC101 were restricted with *SalI*, size fractionated on an agarose gel, and probed with a 1.4-kb *SalI* of *cbgl-2*. Bars indicate the positions of molecular size standards (from top to bottom) of 23.1, 9.4, 6.6, 4.4, 2.3, 2.0 and 0.6 kb.

Chapter 6

FINAL COMMENTS

Using the homologous expression system in *Phanerochaete chrysosporium* developed by Gold and coworkers [Mayfield et al., 1994], we achieved a high-level expression of CDH. With this expression system, several interesting aspects relating to the structure and function of CDH, such as cellulose-binding sequence, heme iron ligation, active site residues involved in catalysis, substrate binding site, and the role of the linker region could be addressed.

6.1 CELLULOSE-BINDING DOMAIN OF CDH

CDH binds to microcrystalline cellulose, indicating that it might have a cellulose-binding domain like cellulases [Renganathan et al., 1990]. Many bacterial and fungal cellulases are modular proteins with a catalytic domain and a non-catalytic cellulose-binding domain. The two domains are usually connected by a linker region which is rich in proline and hydroxy amino acid residues [Gilkes et al., 1991; Beguin & Aubert, 1994]. In fungal cellulases, the cellulose-binding domain consists of about 30 amino acid residues and is highly conserved. It is located either at the C-terminus or the N-terminus. It has been proposed that the conserved aromatic amino acids present in the cellulose-binding domain sequence are involved in cellulose binding [Beguin & Aubert, 1994].

Henriksson et al. [1991] demonstrated that the cellulose-binding site in CDH from *P. chrysosporium* is located in the flavin domain. However, analysis of its primary sequence deduced from a cDNA clone did not identify a conserved cellulose-binding domain sequence in CDH [Raices et al., 1995; Li et al., 1996]. In order to identify the potential cellulose-binding sequence, Henriksson et al. [1997] partially

hydrolyzed CDH using a protease and then treated it with cellulose. It was assumed the peptides that bound to cellulose were involved in cellulose binding. Based on the cellulose-binding studies and amino acid sequence comparison of CDH and the glucose-methanol-choline (GMC) oxidoreductase family of enzymes, it was hypothesized that a region located internally in the flavin domain sequence of CDH is responsible for cellulose binding. This region corresponds to residues 252–297 in CDH from *P. chrysosporium* [Li et al., 1996] and contains nine aromatic residues, three of which are tryptophan residues. In fungal cellulose-binding domains, the stacking interaction between the aromatic ring in residues, such as tyrosine and tryptophan, and the glucose ring in cellulose is proposed to be responsible for cellulose binding [Beguin & Aubert, 1994]. Mutation of each of these aromatic amino acid residues and analysis of the mutant protein for cellulose binding would help in the identification of the cellulose-binding motif in CDH.

6.2 HEME IRON LIGAND

Spectroscopic studies—including optical absorption spectroscopy, nuclear magnetic resonance (NMR) spectroscopy, electron paramagnetic resonance (EPR) spectroscopy, and magnetic circular dichroism (MCD) spectroscopy—indicate that a histidine and a methionine act as the fifth and sixth ligands of the heme iron in CDH from *P. chrysosporium* [Cox et al., 1992]. There is only one methionine residue (Met65) in the heme domain of CDH from *P. chrysosporium* [Raices et al., 1995; Li et al., 1996]. Mutation of this methionine to an alanine produced a truncated flavoprotein. This suggested but did not prove that Met65 is a ligand to the heme iron. It is possible that the heme domain is susceptible to proteolytic degradation when the heme is not bound. A few more experiments need to be done. We should first confirm that the entire mutated gene has been integrated into the *P. chrysosporium* genome. A Southern blot analysis of the genomic DNA of the mutant strain with different regions of the transforming plasmid as probes should confirm the integrity of the mutated gene. Once this is confirmed, we should examine the size of the transcription product of the mutant *cdh* gene. A northern blot analysis of RNA

from the mutant strain grown in glucose medium with *cdh* cDNA as a probe should show the size of the mutated *cdh* gene transcript. Finally, we should check the size of the mutant protein at an early stage of culturing. We harvested the culture at day 7 for mutant protein purification, but the CDH activity was observed since day 2. Western blot analysis of extracellular proteins from day 2 to day 7 cultures with CDH antibodies might show the proteolysis of mutant CDH.

Four histidine residues are present in the heme domain of CDH from *P. chrysosporium* [Raices et al., 1995; Li et al., 1996]. Comparison of protein sequences of CDHs from *P. chrysosporium*, *T. versicolor*, and *S. thermophile* indicate that His 114 from *P. chrysosporium* is well conserved and is likely the second heme iron ligand [Dumonceaux et al., 1998; Subramaniam, 1998]. Creation of the H114A mutant would confirm this.

6.3 ACTIVE SITE RESIDUES

In the glucose-methanol-choline (GMC) oxidoreductase family, the crystal structures of two enzymes, cholesterol oxidase from *Brevibacterium sterolicum* and glucose oxidase from *Aspergillus niger*, have been determined [Hecht et al., 1993; Li et al., 1993]. Although there is only limited sequence similarity between these two enzymes, they share significant structural homology. Superimposition of these two structures indicates that a conserved histidine residue might be the active site residue which receives a proton from the substrate during the oxidase reaction [Li et al., 1993]. Comparison of the primary sequences of all GMC oxidoreductases and the CDHs indicates that this histidine is conserved in all these proteins [Subramaniam, 1998]. In CDH from *P. chrysosporium*, this histidine corresponds to His689 (Fig. 6.1). Site-directed mutagenesis of this histidine would help identify the active site residue in CDH.

CDH	MNSN H WVSSTTIGSSPQS-AVVDSNVKVFGTNNLFIVDAGIIPHLPTGNP	733
CHD	ETAF H PCGTCKMG--YDEMSVVDGEGRVHGLEGLRVVDASIMPQIITGNL	516
ADH	ETIY H PVGTCTRMG--KDPASVVDPCCLKIRGLANIRVVDASIMPHLVAGNT	512
GDH	GPEN H QAGSCKMGPSHDPMAVVNHELRVHGIRGLRVMDTSIMPKVSSGNT	589
GOD	RPNY H GVGTCSMMP-KEMGGVVDNAARVYGVQGLRVIDGSIPPTQMSSHV	582
	* ** . . . * . . . * . . * . . .	

Fig. 6.1 Conservation of potential catalytic residue in CDH and glucose-methanol-choline oxidoreductases. The conserved histidine is in bold. CHD, choline dehydrogenase; ADH, alcohol dehydrogenase; GDH, glucose dehydrogenase; GOD, glucose oxidase.

6.4 Substrate Binding Site

The amino acid sequences of three CDHs have been determined by molecular cloning. They are CDH from *P. chrysosporium*, CDH from *S. thermophile*, and CDH from *T. versicolor*. The flavin domain of these three CDHs shows high sequence similarity to the GMC oxidoreductase family enzymes, including cholesterol oxidase from *B. sterolicum* and glucose oxidase from *A. niger*. However, multiple sequence alignment of flavin domains of these three CDHs and the GMC oxidoreductase family enzymes identified a region which is highly conserved among CDHs but is not conserved in the GMC oxidoreductase family [Subramaniam, 1998]. In CDH from *P. chrysosporium*, this region corresponds to amino acid residues 342–358, and the corresponding sequence is SSRLPSTDHPSTDGQRY. A possible function of this region could be substrate binding. Thus, site-directed mutagenesis study of this conserved region might lead to the identification of a putative substrate binding site of CDH.

6.5 The Role of Linker Region in CDH

CDH has a similar domain organization to that of flavocytochrome b_2 or lactate dehydrogenase from *Saccharomyces cerevisiae*. Flavocytochrome b_2 is a hemoflavoenzyme which oxidizes lactate to pyruvate and transfers electrons to cytochrome c [Xia et al., 1987]. In flavocytochrome b_2 , the heme domain is at the N-terminus and the flavin domain is at the C-terminus. The two domains are connected by a hinge sequence that contains proline, glycine, and various charged residues [Guiard, 1985; Xia et al., 1987]. The most likely role of this hinge region has been proposed to provide domain mobility and allow movement of the heme domain with respect to the flavin domain [White et al., 1993]. To investigate the importance of the structural integrity of the hinge region for efficient intraprotein electron transfer, Sharp et al. [1994, 1996] made several mutant enzymes in which three, six, and nine amino acids, respectively, were deleted from the hinge region. Steady-state and stopped-flow kinetic studies indicated that all three hinge-deletion

enzymes remained competent lactate dehydrogenases, but the electron transfer from the flavin domain to the heme domain was significantly decreased. They concluded that complete structural integrity within the hinge region is essential for efficient intramolecular electron transfer from the flavin to the heme.

In CDH, a region (amino acid residues 193–207) between the N-terminal heme domain and the C-terminal flavin domain is rich in hydroxy amino acids and could form the linker region [Li et al., 1996]. This hinge region sequence shows no similarity to the flavocytochrome b_2 sequence. Like the hinge region in flavocytochrome b_2 from *S. cerevisiae*, this linker region in CDH might be important for intraprotein electron transfer. Similar deletion mutants in the linker region as those for flavocytochrome b_2 can be constructed for CDH. Steady-state and stopped-flow kinetic studies of the mutant proteins will show the electron transfer rate in different steps of the CDH catalytic cycle. The reduction of the flavin and the intraprotein electron transfer from FAD to heme can be monitored by stopped-flow kinetic analysis. Electron transfer from cellobiose to the FAD of the deletion mutant proteins would probably remain the same as wild-type CDH. If the linker region is important for intraprotein electron transfer, the rate of heme reduction would decrease in the mutant proteins. The reduction of cytochrome c can be studied by steady-state kinetic analysis, and the rate would probably be equal to the rate of heme reduction. The results of these experiments would provide information about the role of the linker region in CDH.

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BIOGRAPHICAL SKETCH

Bin Li was born in Changchun, China, on November 20, 1966. He received his Bachelor of Science degree from Jilin University in 1990. He worked at the Changchun Institute of Biological Products as a research assistant for three years. In September, 1993, he began his graduate study at the Oregon Graduate Institute of Science and Technology where he completed the requirements for the degree of Doctor of Philosophy in Biochemistry and Molecular Biology.