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ATTACHMENT OF PENTAAMMINERUTHENIUM(III) TO *TRICHODERMA REESEI* CELLOBIOHYDROLASE I INCREASES ITS CATALYTIC ACTIVITY

Barbara R. Evans*, Ruth Margalit+, and Jonathan Woodward*‡

*Chemical Technology Division, Oak Ridge National Laboratory
Oak Ridge, TN 37831-6194

+Jet Propulsion Laboratory, Pasadena, CA 91109

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Pentaammineruthenium(III) was covalently attached to cellobiohydrolase I (CBH I, EC 3.2.1.91), the major component of *Trichoderma reesei* cellulase, resulting in 0.7 mol ruthenium/mol CBH I and an electrode potential of +95 mV. Fractionation of modified CBH I by chromatofocusing resulted in the separation of fractions with a 1.4- to 3.2-fold increase in specific activity toward p-nitrophenylcellobioside, depending on the assay conditions, over that of native enzyme. The extent of the hydrolysis of insoluble cellulosic substrates (Avicel and newsprint) to glucose by modified CBH I was also greater than that observed by the native enzyme.

Cellulose is a rapidly renewable source of sugar that can be fermented into fuels and chemicals that are currently obtained from fossil fuels (1,2). The enzyme cellulase from *T. reesei* contains cellobiohydrolases, endoglucanases, and β-glucosidases that act synergistically to hydrolyze insoluble crystalline cellulose to glucose (3).

Cellulases, in general, possess low specific activity toward insoluble cellulosic substrates (4). There are several reasons for this, including the crystallinity of cellulose and, in the case of lignocellulosic materials such as wood, the presence of lignin which impedes the ability of cellulase to act on the glycosidic bonds in cellulose.

In nature, organisms such as the white rot fungus *Phanerochaete chrysosporium* degrade lignin in lignocellulose by means of flavin or protoheme containing oxidoreductases via aryl cation, or other intermediate, radical formation (5-11). We wondered whether the enzymatic hydrolysis of cellulose or lignocellulose could be enhanced by conferring upon cellulase, oxidoreductase activity, through the attachment of pentaammineruthenium(III). Such a cellulase, we hypothesized, might oxidize and degrade lignin and lead to subsequent

‡To whom correspondence should be addressed. Fax: 615-574-6843.

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enhanced hydrolysis of the exposed glycosidic bonds in cellulose. The rationale for such a modification was based on the findings of Stanbury et al., who showed that a series of ruthenium(II) amines catalyzed the reduction of O_2 to H_2O_2 and that during this reaction, superoxide (O_2^-) and perhydroxyl (HO_2^-) radical intermediates were formed (12,13). Pentaammineruthenium(III) has been successfully attached to sperm whale myoglobin, cytochrome C, and azurin (by attachment of the nitrogen atom of accessible histidine residues) modifying their redox reactions without affecting their conformation (14-16).

Cellobiohydrolase I (CBH I; EC 3.2.1.91), the major component of crude *T. reesei* cellulase, was used in this study. It comprises 60% of total cellulase protein and is an exoglucanase (molecular weight 66,000 g/mol; comprising 497 amino acids; pI 3.6-4.2) primarily catalyzing the hydrolysis of crystalline cellulose (at the chain ends) to cellobiose, a dimer of glucose. The tertiary structure of CBH I has been shown to be tadpole-shaped with a catalytic head or core domain linked through a glycosylated hinge region to a C-terminal tail region known as the cellulose-binding domain. Digestion of CBH I with papain cleaves the hinge and tail region from the catalytic domain (17). There are a total of 5 histidines/mol CBH I, 4 of them residing in the catalytic domain at positions 11, 42, 206, and 228, and one residing in the cellulose-binding domain at position 465 (18). The present paper describes the enhancement of the catalytic activity of CBH I by the attachment of pentaammineruthenium(III).

MATERIALS AND METHODS

Purification and Chemical Modification of CBH I

A crude *T. reesei* cellulase preparation from Novo Nordisk Bioindustrials was subjected to batch ion exchange chromatography using DEAE-Sepharose equilibrated at pH 5.0 with 50 mM sodium acetate buffer. The fraction that bound to the gel containing CBH I was eluted with 1 M sodium chloride and re-equilibrated in 25 mM methyl piperazine buffer, pH 5.4, by gel filtration. CBH I was purified from this fraction by chromatofocusing on an FPLC employing a Mono P column (Pharmacia LKB) as described previously (19). Analytical isoelectrophoresis showed the expected subforms of purified CBH I as described by Kubicek (20). SDS PAGE indicated CBH I to be 99% homogeneous.

The modification was carried out as follows. Pentaammineruthenium(II), $Ru(NH_3)_5H_2O^{2+}$, was freshly prepared by Zn/Hg amalgam reduction of $Ru(NH_3)_5Cl^{2+}$ as described previously (21). A 50-fold molar excess of this reagent was reacted with CBH I (1.5 mg/mL) in 100 mM HEPES buffer, pH 7.0, under argon for 2 h after which the reaction was quenched by dilution with 50 mM sodium acetate buffer, pH 5.0. The excess Ru reagent was separated at 4°C using Amicon stirred filtration cells containing YM-3 or YM-10 membranes by washing with the pH 5.0 buffer. The product was oxidized to $Ru(NH_3)_5H_2O^{2+}$ by overnight incubation with K[CoEDTA] (a generous gift from D. Wuttke) at 4°C, pH 5.0, followed by filtration to remove excess oxidant. The amount of ruthenium in modified CBH I was determined by inductively coupled platinum atomic absorption spectroscopy carried out by the Analytical Chemistry Division of Oak Ridge National Laboratory. Solutions of pentaammineruthenium chloride were used as standards.

Measurement of CBH I Activity and Cellulose Hydrolysis

CBH I catalyzes the hydrolysis of the aglycon bond in p-nitrophenylcellobioside (PNPC) releasing p-nitrophenol (PNP). The concentration of PNP was determined by its absorption of light (402 nm) in 1 M sodium carbonate, pH 11.0. The PNPCase activity of the native and modified fractions of CBH I was assayed in a 1.0-mL volume containing 10 mM PNPC in 50 mM sodium acetate buffer, at pH 4.3 or 5.0, and 40 μg protein at 23°C, 42°C, and 55°C. At intervals of time, 0.1 mL was removed and its PNP concentration determined. The formation of PNP was linear for at least 4 h.

The hydrolysis of microcrystalline cellulose or newspaper (unprinted) was carried out in a 1.0-mL stirred volume of 50 mM sodium acetate buffer, pH 4.3, at 42°C containing 1.0 mg cellulose (Avicel PH-105 from FMC Corporation, Philadelphia, Pennsylvania) or newspaper, 40 μg native or modified CBH I, 15.7 μg ß-glucosidase and 0.01% sodium azide. The ß-glucosidase preparation was obtained from Novo Nordisk Bioindustrials, Inc., Danbury, Connecticut (Novozyme 188), and diluted 100 times with buffer prior to the addition of an aliquot containing 15.7 μg protein to the reaction mixture. Glucose concentration was determined using the hexokinase assay reagent (Sigma).

RESULTS AND DISCUSSION

The ruthenium content in modified CBH I was determined to be 0.7 mol/mol; other evidence obtained for establishing the attachment of pentaammineruthenium(III) to CBH I included spectroscopic and electrochemical measurements. Thus, a UV/visible spectrum of modified CBH I revealed a characteristic shoulder around 300 nm, reflecting the absorption of the ruthenium complex (22). Square-wave voltametry of modified CBH I at pH 5.0 revealed a current peak at +95 mV (vs NHE), which is in agreement with the E°, +90 mV for the model complex $Ru(NH_3)_5Cl_3$. Since native CBH I had no redox activity, the current peak in the voltammogram was due to the pentaammineruthenium(III) coordinated to CBH I via the nitrogen atom of a histidine.

Analytical isoelectric focusing during the purification of CBH I (Fig. 1a) indicated the usually observed subforms of purified CBH I (pI 3.6-3.8). An expected increase in the pI values of these subforms was seen after modification as shown in Fig. 1b due to the presence of positively charged ruthenium cations on CBH I. Modified CBH I was subjected to chromatofocusing (see materials and methods) and eluted as a broad band of protein with decreasing pI values (Fig. 2). This was done in order to separate unmodified from modified CBH I. Fractions containing protein (termed A through F) were pooled and assayed for their ability to hydrolyze PNPC. The specific activity of the modified CBH I fractions A, B, and C was found to be enhanced between 1.4 and 3.2 over that of all of the subforms of native CBH I which possessed similar activity (Table 1). The greatest enhancement was observed when the assay was conducted at pH 4.3 and 55°C, which were determined to be the optimum pH and temperature, respectively, for both native and modified CBH I acting on PNPC. Fractions D-E possessed similar or lower specific activity compared to the native enzyme.

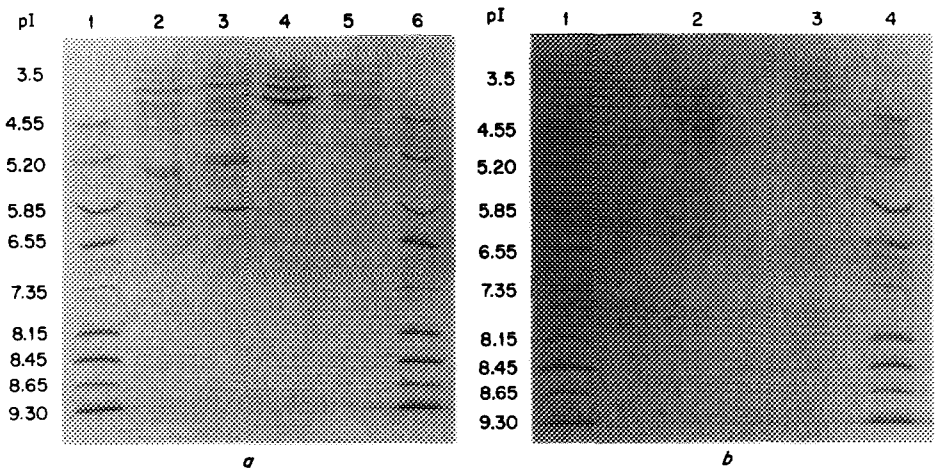

Fig. 1. Purification and modification of *T. reesei* CBH I monitored by analytical isoelectrofocusing. Details are referenced (19). (a) Lanes 1 and 6, pI markers; Lane 2, crude cellulase; Lane 3, fraction of cellulase not bound to DEAE-Sepharose; Lane 4, fraction bound to DEAE-Sepharose; Lane 5, purified CBH I after chromatofocusing. (b) Lanes 1 and 4 pI markers; Lane 2, pentaammineruthenium(III)-modified CBH I before chromatofocusing; Lane 3, native CBH I.

We also observed an enhancement of the hydrolysis of microcrystalline cellulose (Avicel) to glucose by modified CBH I in the presence of β-glucosidase over that by native CBH (Fig. 3a). Although the rates of cellulose hydrolysis by both enzymes were initially

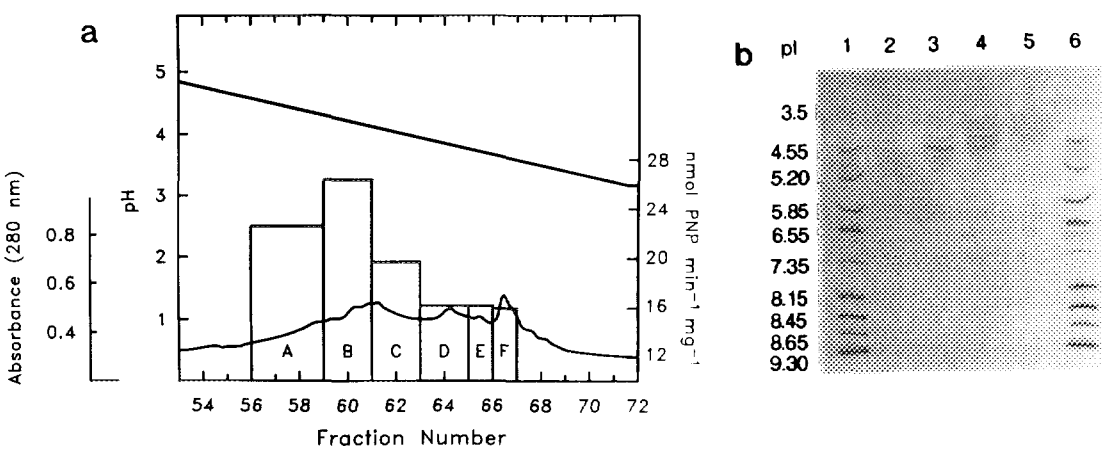

Fig. 2. (a) Chromatofocusing of pentaammineruthenium(III)-modified CBH I. Fractions A through F were pooled as indicated and analyzed for their protein concentration and enzyme activity (PNPCase). The native enzyme eluted between fractions 64 and 68 as a sharper peak (19). (b) Analytical isoelectric focusing of Fractions A through D. The pIs of the modified CBH I subforms in Fractions E through F were similar to those of native CBH I.

Table 1. Catalytic activity of native and modified CBH I toward PNPC

		Modified CBH I fractions		
	Native CBH I	A	B	C
pH 5.0				
23°C	16.4	22.7	26.3	19.6
42°C	45.0	67.0	90.0	67.0
55°C	81.9	110.6	139.4	122.3
pH 4.3				
55°C	88.2	223.1	285.8	193.1

Values are m units mg^{-1} protein.

similar, after 5 d the percentage of hydrolysis of Avicel by modified CBH I was approximately 90% compared to 60% for the native enzyme. Purified CBH I can degrade crystalline cellulose preparations, such as Avicel, to glucose providing β-glucosidase is present in the reaction mixture to catalyze the hydrolysis of cellobiose to glucose. The extent of hydrolysis of the cellulose component of newspaper (representing approximately 50% of total weight) was also greater by modified CBH I compared to that by the native

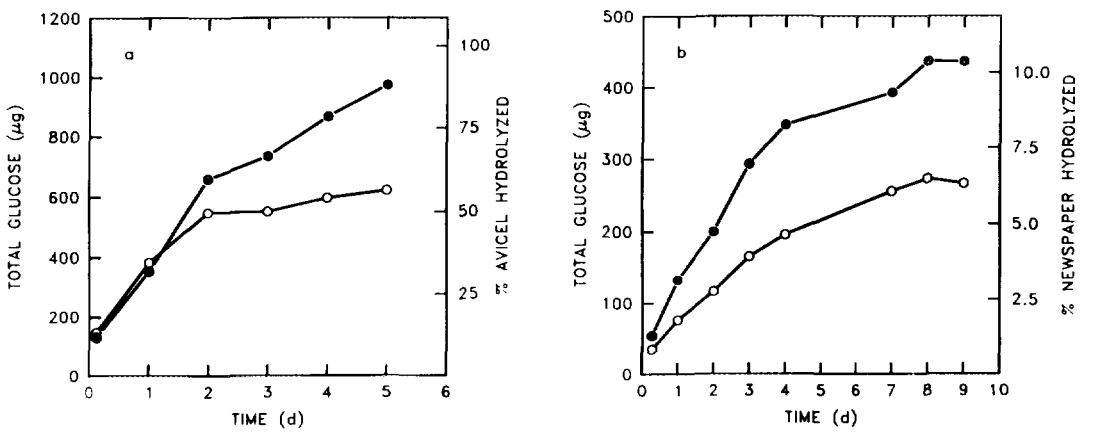

Fig. 3. Comparison of the hydrolysis of pure microcrystalline cellulose and newspaper by native and pentaammineruthenium(III)-modified CBH I. a: Microcrystalline cellulose (Avicel). The values for glucose concentration are the average of four hydrolyses by native CBH I (○-○) and the average of three hydrolyses by modified CBH I (●-●). The three hydrolyses by modified CBH I were done with Fractions A, B, and C. b: Newspaper. Hydrolysis by native CBH I (○-○) and modified CBH I, Fraction B (●-●).

enzyme (Fig. 3b). In this case about 20% of the cellulose in newspaper was converted to glucose by modified CBH I compared to only 6.5% by native CBH I.

The mechanism for the enhanced activity of modified CBH I toward PNPC, microcrystalline cellulose, and newsprint is not yet understood. The thermal stability of CBH I was determined to be unaffected by the modification.

The possibility that the mechanism for the enhanced activity of modified CBH I toward microcrystalline cellulose and newspaper involves its endowment with oxidoreductase activity with the generation of H_2O_2 (via free radical formation) resulting from the reaction of O_2 with ruthenium(II), as described by Stanbury et al. (12,13), also exists, and we have preliminary evidence indicating modified CBH I possesses low lignin peroxidase-like activity based upon veratryl alcohol oxidation (not shown). At the present time we do not know whether the enhanced activity of modified CBH I is correlated with its possible oxidoreductase activity. While this may explain the enhanced activity toward Avicel and newspaper, the explanation for enhanced PNPC hydrolysis is less obvious. If this were the mechanism, the pentaammineruthenium(III) group attached to CBH I would first be reduced to ruthenium(II); how this may be achieved without an exogenously added reductant is yet to be determined, but in the case of the enzyme cellobiose oxidase, reducing end groups of cellulose, cellobiose, and glucose are substrates of this enzyme (23). Although ruthenium(II) amines possess characteristic spectra (9), ruthenium(II) in modified CBH I is difficult to detect because of its low molar absorptivity (30 M^{-1} cm^{-1}, λ max 400 nm) (24).

If free radicals are generated by modified CBH I, they could aid in the disruption of the crystalline structure of cellulose (7). The finding that the enhancement of microcrystalline cellulose hydrolysis by modified CBH I was observed 2 days after the reaction commenced could be explained by the fact that the amorphous part of Avicel cellulose, approximately 50% of the total (25), is equally susceptible to hydrolysis by native and modified CBH I. The enhancement would be observed when modified CBH I was acting mainly on the crystalline portion of cellulose in Avicel. On the contrary, the enhancement of the hydrolysis of cellulose in newspaper was observed from the start of the reaction. In this case, exposure of cellulose by the action of free radicals on lignin could explain this difference, although this has yet to be established.

Although we have yet to identify the site of modification, analytical isoelectric focusing of the catalytic domain of modified CBH I revealed its pI to be higher than that of the catalytic domain of native CBH I (see reference 19). This suggests that pentaammineruthenium(III) is attached to a histidine in the catalytic domain of CBH I. Peptide mapping of modified CBH I to determine which histidine residue is modified is in progress.

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