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Enhanced Hydrolysis of Soluble Cellulosic Substrates by a Metallocellulase with Veratryl Alcohol-Oxidase Activity†

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ABSTRACT

A cellulase enzyme fraction was separated from *Trichoderma reesei* Pulpzyme HA™, and its characteristics suggested that it was mainly composed of cellobiohydrolase II (CBH II). The covalent attachment of pentaammineruthenium(III) to this enzyme resulted in threefold and fourfold enhancements of its hydrolytic activity on carboxymethyl cellulose (CMC) and barley β-glucan, respectively, as well as endowing it with veratryl alcohol-oxidase activity. Enhancement of hydrolysis was not affected by addition of tartrate or hydrogen peroxide to the reaction mixture. Both native and pentaammineruthenium-modified enzymes had negligible activity on cellobiose and *p*-nitrophenyl β-cellobioside (PNPC).

Index Entries: Cellulase; metallocellulase; cellobiohydrolase II: *Trichoderma reesei;* ruthenium; modification.

INTRODUCTION

We have been investigating the effects of the chemical modification of cellulases and xylanases, by the attachment of pentaammineruthenium (III), on their catalytic properties. The rationale for such a modification of

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xylanases is that it could confer oxidoreductase activity on them and possibly increase their utility in biobleaching of wood pulp (*1–3*). We intended to modify the xylanases produced by *T. reesei*. Two xylanases have been reported to be produced by this fungus: XYN I, with a pI of 5.5 and a mol wt of 19 kDa, and XYN II, with a pI of 9.0 and a mol wt of 21 kDa. XYN II is reported to posses 10-fold higher hydrolytic activity on beechwood xylan than XYN I (*4,5*). In our previous work, pentaammineruthenium(III)-modified cellobiohydrolase I (CBH I) from *T. reesei* has been shown to possess veratryl alcohol-oxidase activity and enhanced cellulase activity compared to the native enzyme (*6,7*).

During the present study, a protein was isolated from a commercial xylanase (*T. reesei* Pulpzyme HA™), and appeared to be largely composed of cellobiohydrolase II (CBH II), the second most abundant cellulase component produced by *T. reesei* (*8*). Comparison of the properties of Pulpzyme HA and Celluclast™, a cellulase mixture produced by *T. reesei*, on polyacrylamide gel electrophoresis, and in assays of cellulolytic activity, revealed that Pulpzyme HA contains the full complement of *Trichoderma* cellulases. It does contain an increased amount of the two xylanases as compared to Celluclast, but they still only comprise about 5–10% of total protein in the crude fungal filtrate sold as Pulpzyme HA.

Attachment of pentaammineruthenium(III) to this CBH II preparation endowed it with enhanced activity toward soluble carboxymethyl cellulose (CMC) and barley β-glucan as well as conferring veratryl alcohol-oxidase activity on it. The details of this study are now described and represent the first report of the enhancement of the specific activity of a cellulase enzyme toward soluble cellulosic substrates by chemical modification.

MATERIALS AND METHODS

Carboxymethyl cellulose (medium viscosity, 0.65-0.85 substitution), hexokinase glucose assay reagent, and barley β-glucan were obtained from Sigma Chemical Co., St. Louis, MO. Veratryl alcohol was obtained from Aldrich Chemical Co., Milwaukee, WI. Celluclast and Pulpzyme HA were generous gifts from Novo Nordisk Bioindustrials, Inc., Danbury, CT. The PhastSystem™ and DEAE Sephadex were purchased from Pharmacia LKB Biotechnology, Piscataway, NJ.

Separation and Purification of CBH II

Purification of a cellulase protein with molecular weight, pI, and hydrolytic activity corresponding to that reported for CBH II (*9–14*) was carried out by ion-exchange chromatography. Pulpzyme HA™, a culture filtrate from *T. reesei*, was dialyzed overnight against 5 m*M* ammonium carbonate buffer, pH 7.8, at 4°C. The dialyzed solution (470 mg protein; 25 mL) was then applied to a 2.5 × 8 cm column of DEAE Sephadex equilibrated in the same buffer, and 20 mL fractions were eluted. Pool 1,

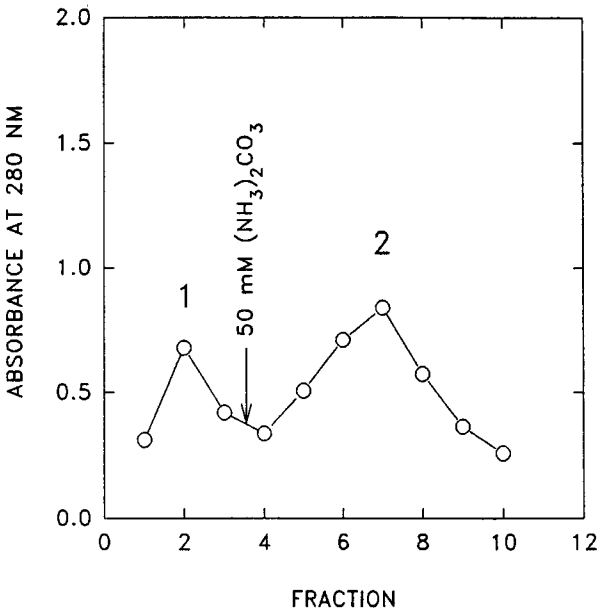

Fig. 1. DEAE chromatography of Pulpzyme HA. Pulpzyme HA (700 mg) was applied to a 50-mL column (2.5 × 8 cm) of DEAE Sephadex in 5 m*M* ammonium carbonate, pH 7,8, as described in the text. Pool 1 was eluted with 5 m*M* buffer and contained xylanase II and β-glucosidase; pool 2 eluted with 50 m*M* ammonium carbonate, pH 7.8, and contained predominantly CBH II.

fractions 1–4 (21 mg protein), that did not bind to the column, appears to contain xylanase II (*1*) and β-glucosidase (Fig. 1). Pool 2, fractions 5–10, containing primarily CBH II (76 mg protein), was eluted by application of 50 m*M* ammonium carbonate, pH 7.8 (Fig. 1). The proteins that remain bound to the DEAE Sephadex, largely CBH I and endoglucanases, were eluted with 1*M* sodium chloride.

Attachment of Pentaammineruthenium to Cellulase

Pentaammineruthenium(II) was attached to the CBH II preparation as described previously (*6*). A 10-fold excess of aquopentaammineruthenium(II) was incubated with 30 mg of CBH II pool for 2 h at 23°C in 50 m*M* HEPES buffer, pH 7. After attachment of pentaammineruthenium, the reaction was quenched by addition of 50 m*M* sodium acetate buffer, pH 5.0, and excess reagent was removed by filtration in an Amicon filtration cell. The attached pentaammineruthenium was allowed to air-oxidize to the 3+ state.

Enzyme Assays

Native CBH II and pentaammineruthenium(III)-CBH II were dialyzed against distilled water at 4°C to remove the buffer used in preparation before assays were carried out to study the effects of pH on the hydrolytic

activity of the proteins. Hydrolysis of cellulosic substrates was carried out at 45°C. Hydrolysis of carboxymethyl cellulose (CMC, 1%) and barley β-glucan (0.5%) was carried out in either 25 m*M* sodium acetate or sodium phosphate buffer at pH 5.0, unless otherwise indicated; sodium phosphate was used for pH 6.0. Cellobiase activity was assayed with 10 m*M* cellobiose in 50 m*M* sodium acetate, pH 5.0. Production of reducing sugar or glucose was followed with the dinitrosalicylic acid reagent (*15*) or the hexokinase assay reagent (Sigma), respectively. Assays of PNPCase activity were carried out with 10 m*M* PNPC at pH 5.0 in 50 m*M* sodium acetate buffer. Production of PNP was determined by adding 100-μL aliquots of reaction mixtures to 1 mL of 1*M* sodium carbonate and measuring the absorbance of the solution at 402 nm. The concentration of CBH II in all the assays was 0.040 mg/mL.

Veratryl alcohol-oxidase assays were carried out in the dark at 45°C, with vigorous stirring, in a reaction mixture containing 0.10 mg/mL pentaammineruthenium(III)-CBH II, 3.0 m*M* veratryl alcohol, and 1.8 m*M* hydrogen peroxide in 50 m*M* sodium acetate, pH 4.3. Production of veratraldehyde was monitored by measuring the increase in absorbance at 310 nm. After subtraction of the absorbance of control solutions containing hydrogen peroxide and veratryl alcohol, and pentaammineruthenium(III)-CBH II and veratryl alcohol, the yield of veratraldehyde was calculated from its molar extinction coefficient at 310 nm, $9300M^{-1}$/cm (*16*).

Analytical Procedures

The Pharmacia PhastSystem was used to perform polyacrylamide gel electrophoresis. For isoelectric focusing, Pharmacia precast gels with a pH gradient of pH 3–10 were used. For SDS-PAGE, 10–15% gradient precast Pharmacia gels were used. All samples run on SDS gels were denatured by incubation with 2% SDS and 5% β-mercaptoethanol at 90°C for 4 min. Papain digests were carried out as previously described (*17,18*), using a ratio of 250:1 (w/w) cellulase to papain.

RESULTS AND DISCUSSION

Identification of the Cellulase as CBH II

The cellobiohydrolase II (CBH II; EC 3.2.1.91) component of *T. reesei* cellulase catalyzes the hydrolysis of crystalline cellulose to cellobiose primarily (*6,7*). The protein consists of an N-terminal cellulose-binding domain, a glycosylated linker region, and a C-terminal catalytic domain. The active site of CBH II is a closed tunnel 2 nm in length, with catalytic residues at D175 and D221. CBH II contains four histidines, which are located in the catalytic domain at positions 266, 331, 340, and 414 of the amino acid sequence (*9–14*).

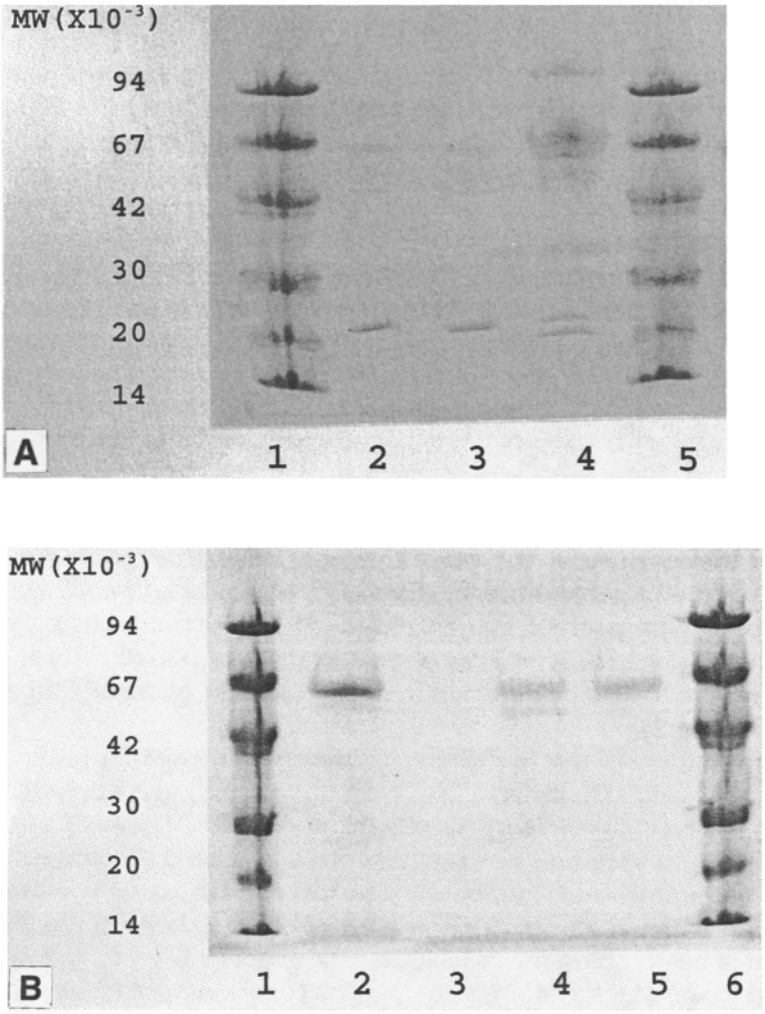

Fig. 2. (**A**) SDS gels of Pulpzyme HA fractions. Lanes 1 and 5, low-mol-wt markers; lanes 2 and 3, Pulpzyme DEAE pool 1; lane 4, crude, unfractionated Pulpzyme HA. (**B**) Comparison of DEAE pool 2 from Pulpzyme HA with CBH II from Celluclast. Lanes 1 and 6, low-mol-wt markers; lane 2, CBH II isolated from Celluclast by ion-exchange chromatography and chromatofocusing; lane 4, pentaammineruthenium(III)-modified DEAE pool 2; lane 5, Pulpzyme HA DEAE pool 2.

SDS gel electrophoresis of crude, unfractionated Pulpzyme HA revealed the presence of large amounts of both CBH I and II, as well as endoglucanases and β-glucosidase (Fig. 2A, lane 4). Pool 1 from DEAE Sephadex chromatography of Pulpzyme HA appears to contain XYN II, the 21-kDa xylanase of *T. reesei* (Fig. 2A, lanes 2 and 3; [4]). SDS electrophoresis of native pool 2 protein from DEAE Sephadex chromatography (Fig. 2B, lanes 4 and 5) showed that the molecular weight of the major

component corresponded to that of a preparation of CBH II purified from Celluclast with a mol wt of 56 kDa (Fig. 2B, lane 2). The *T. reesei* xylanases are both small (19 and 21 kDa), and so are readily distinguishable from CBH II on SDS gels. On SDS gel electrophoresis, only the 56-kDa band corresponding to CBH II appears to be the main component of pool 2 (Fig. 2B, lane 5) and pentaammineruthenium(III)-modified pool 2 (Fig. 2B, lane 4).

Isoelectric focusing electrophoresis showed that pool 1 contained mainly the pI 9.0 xylanase, XYN II (Fig. 3A, lane 3) (*4,5*). The isoelectric focusing gel pattern of pool 2 appeared to contain a major component with a pI of 5.9, a lesser component with a pI of 5.5, and minor components with pI of 5.2 and 4.6 (Fig. 3A lane 5). The major components in Pool 2, therefore, appear to be CBH II (pI 5.9) and a xylanase, XYN I (pI 5.5).

Cleavage with papain has been demonstrated to remove the cellulose-binding domain and linker region from the catalytic core of CBH II (*9,14*). The core protein has a mol wt of 45 kDa and a pI of 4.4 (*9,14*). To confirm the identity of the pool 2 protein, papain digests were carried out. Digestion of pentaammineruthenium(III)-modified pool 2 with papain produced a core protein that migrated on isoelectric focusing gels with the same pI (Fig. 3B, lane 1) as papain digests of native CBH II controls. This band could also be detected in papain digests of crude Pulpzyme HA (Fig. 3B, lane 2).

Examination of the hydrolytic activity of the pool 2 protein revealed that it possessed activity on CMC, barley β-glucan, and birchwood xylan, but barely detectable activity on cellobiose or PNPC (Tables 1 and 2). CBH II cloned and expressed in yeast has been reported to possess xylanase activity on birchwood xylan, but its activity on xylan is one-hundredth of its activity on barley β-glucan (*5*). Since the xylanase activity of the pool 2 preparation is nearly the same as its glucanase activity (Table 1), this would correspond to the identification of the pool as consisting of CBH II with a small amount of highly active XYN I not detectible on SDS gel electrophoresis.

Chemical Modification

Attachment of pentaammineruthenium(III) to DEAE pool 2 from Pulpzyme HA was verified by the appearance of a voltage peak at +95 mV (vs NHE) on square-wave voltammetry, and by the appearance of an absorbance peak at 360–370 nm in the UV-visible spectrum of the protein that is not present in the spectrum of the native protein (data not shown). The voltage and absorbance peaks correspond to those reported for pentaammineruthenium(III) groups attached to a protein through a histidine imidazole (*19*). At the present time, we have not yet determined the number of pentaammineruthenium groups attached to CBH II by the modification.

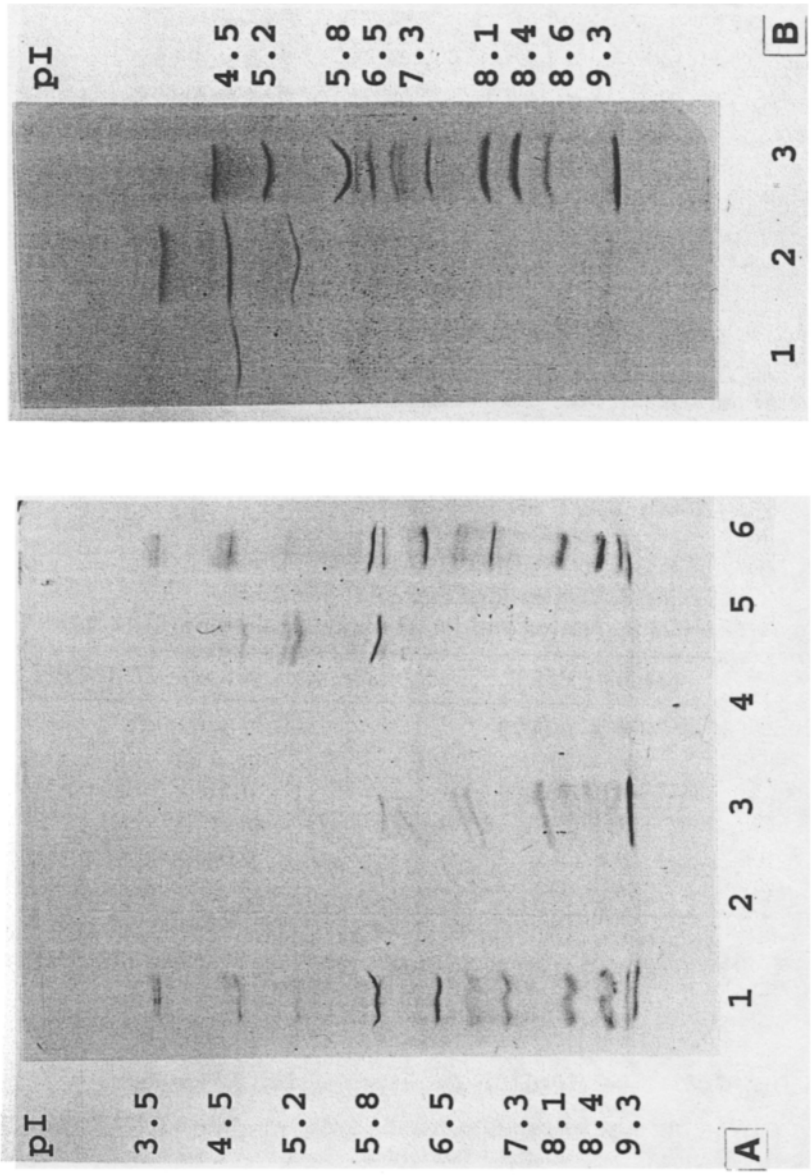

Fig. 3. (**A**) Isoelectric focusing gel of pulpzyme HA fractions. Lanes 1 and 6, pI 3–10 markers; lane 3, pool 1 of DEAE chromatography; lane 5, pool 2 of DEAE chromatography. (**B**) Isoelectric focusing gel of papain digests. Lane 1, papain digest of pentaammineruthenium(III) DEAE pool 2 Pulpzyme HA, lane 2, papain digest of crude, unfractionated Pulpzyme HA, lane 3, pI 3–10 markers.

Table 1
Specific Activities of Native and Pentaammineruthenium(III)-Modified CBH II[a]

Substrate	Native CBH II	Pentaammineruthenium(III)-CBH II
Veratryl alcohol	ND	0.0240
Carboxymethyl cellulose	0.916 ± 0.404 (n = 4)	2.50 ± 0.14 (n = 5)
Barley β-glucan	1.43 ± 0.40 (n = 3)	5.56 ± 0.84 (n = 3)
Birchwood xylan	1.16 ± 0.24 (n = 2)	1.38 ± 0.04 (n = 2)
Cellobiose	0.0268 (n = 1)	0.0257 (n = 1)
p-Nitrophenyl β-cellobioside	0.00170 (n = 1)	0.00594 (n = 1)

[a]Activities are in $\mu mol/min^{-1}$ mg^{-1} for production of veratraldehyde from veratryl alcohol; production of reducing sugar from carboxymethyl cellulose, barley β-glucan, and birchwood xylan; production of glucose from cellobiose; and production of p-nitrophenol from p-nitrophenyl β-cellobioside. ND = not detected; n = number of trials; values are the mean of the values from n trials ± SD.

Table 2
Specific Activities for Glucose Production
from Various Substrates by Native and Pentaammineruthenium(III)-CBH II[a]

Substrate	Native CBH II	Pentaammineruthenium(III)-CBH II
Carboxymethyl cellulose	0.0288 ± 0.0079 (n = 3)	0.120 ± 0.0042 (n = 2)
Barley β-glucan	0.0473 (n = 1)	0.1106 (n = 1)
Cellobiose	0.0268 (n = 1)	0.0257 (n = 1)

[a]Specific activities are given in $\mu mol/min^{-1}$ mg^{-1} for glucose determined with the Sigma hexokinase assay reagent. n = number of trials; values are the mean of the values from n trials ± SD.

Effect of Chemical Modification on Hydrolytic Activity

Native control and pentaammineruthenium(III)-modified DEAE pool 2 from Pulpzyme HA was assayed for hydrolytic activity with various cellulosic substrates (Tables 1 and 2). Both modified CBH II and native controls had essentially no activity on the chromophoric substrate p-nitrophenyl β-cellobioside; if an endoglucanase had been present in the preparations, significant PNPCase activity corresponding to the activity seen on barley β-glucan and CMC would be evident. To determine if β-glucosidase was present in the preparations, hydrolysis of cellobiose was assayed and

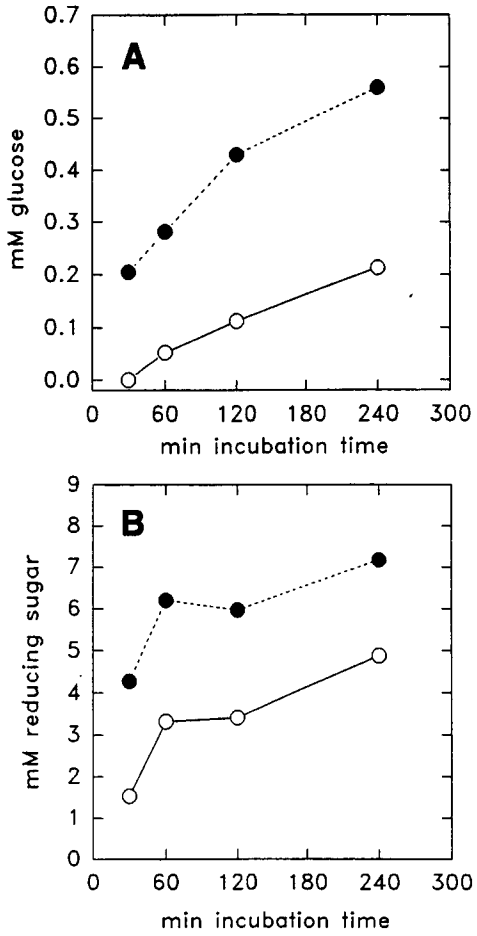

Fig. 4. Hydrolysis of CMC by native and pentaammineruthenium(III)-modified DEAE pool 2. Reactions were carried out in 25 m*M* sodium phosphate, pH 5.0, as described in Materials and Methods. (**A**) Production of glucose determined with the hexokinase assay reagent. (**B**) Production of reducing sugar determined with dinitrosalicylic acid. ○ Native DEAE pool 2; ● pentaammineruthenium(III)-modified DEAE pool 2.

found to be negligible (125-fold less than that of a β-glucosidase [Novozym 188™] control). The initial rates of production of reducing sugar from the soluble cellulosic substrates CMC (Sigma medium viscosity, substitution 0.65–0.85) and barley β-glucan were increased by three- and fourfold, respectively, for the pentaammineruthenium(III)-modified enzyme as compared to native controls (Table 1). Production of glucose from CMC and barley β-glucan, although much lower in concentration than that of reducing sugar, also appeared to be enhanced by fourfold and over two-fold, respectively (Table 2). The time-course of hydrolysis of CMC and barley β-glucan to reducing sugar or glucose is shown in Figs. 4 and 5.

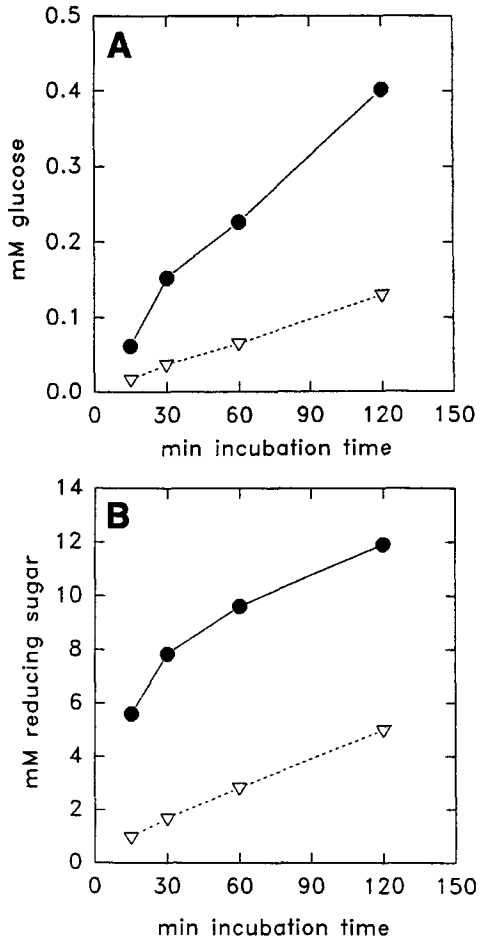

Fig. 5. Hydrolysis of barley β-glucan by native and pentaammineruthenium(III)-modified DEAE pool 2. Reactions were carried out in 25 m*M* sodium acetate, pH 5.0, as described in Materials and Methods. (**A**) Production of glucose determined with the hexokinase assay reagent. (**B**) Production of reducing sugar determined with dinitrosalicylic acid. ∇ Native DEAE Pool 2; ● pentaammineruthenium(III)-modified DEAE pool 2.

The effects of pH on the hydrolysis of carboxymethyl cellulose and barley β-glucan were compared for native and pentaammineruthenium(III)-CBH II (Figs. 6 and 7). No changes in pH dependence owing to the attachment of pentaammineruthenium(III) were apparent in the case of barley β-glucan. The optimal pH range for the hydrolysis of carboxymethyl cellulose appeared to be broadened in the case of the pentaammineruthenium(III)-modified enzyme. This may be because of the interaction of positively charged pentaammineruthenium(III)-(histidine) groups on the modified CBH II with the negatively charged carboxymethyl groups of the carboxymethyl cellulose. Trivalent cations, such as ferric ions, are known to form precipitates with and increase viscosity of

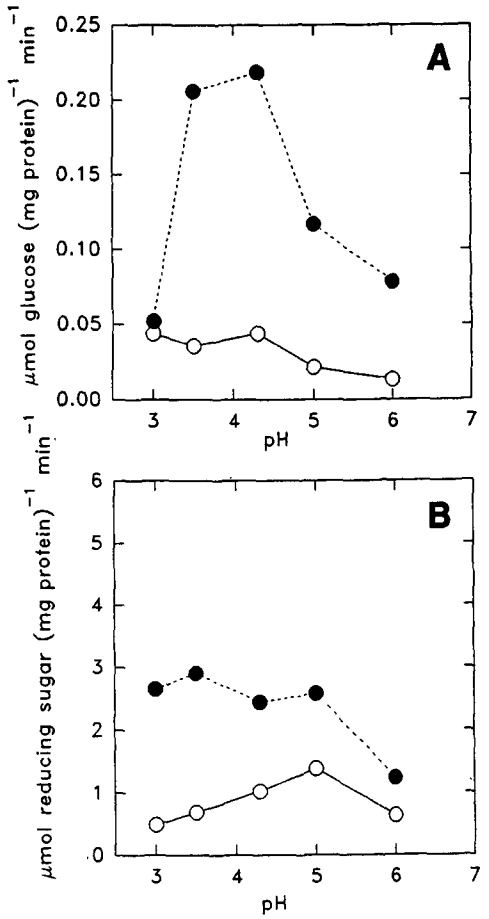

Fig. 6. The effect of pH on hydrolysis of CMC by native and pentaammineruthenium(III)-modified DEAE pool 2 from Pulpzyme HA. Assays were carried out as described in Materials and Methods. (**A**) Glucose production at 60 min. (**B**) Reducing sugar production at 60 min. ○ Native DEAE pool 2; ● pentaammineruthenium(III)-modified pool 2.

carboxymethyl cellulose solutions (Aqualon Company, Wilmington, DE, 1988); ruthenium, with a chemistry resembling that of iron, may cause a similar effect, which may influence the adsorption of modified CBH II to the substrate.

Veratryl Alcohol-Oxidase Activity

In our previous work (*6,7*), we found that attachment of pentaammineruthenium(III) to CBH I conferred veratryl alcohol oxidase on that protein. The pentaammineruthenium(III)-modified CBH II also proved to be able to oxidize veratryl alcohol, with an SA of 0.0240 μmol/mg/min (Fig. 8).

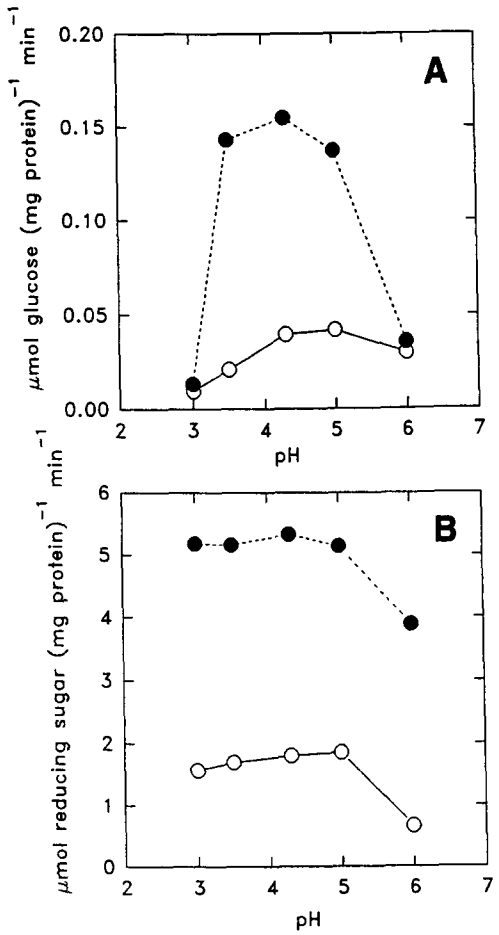

Fig. 7. The effect of pH on hydrolysis of Barley β-glucan by native and pentaammineruthenium(III)-modified DEAE pool 2 from Pulpzyme HA. Assays were carried out as described in Materials and Methods. (**A**) Glucose production at 60 min. (**B**) Reducing sugar production at 60 min. ○ Native DEAE pool 2; ● pentaammineruthenium(III)-modified pool 2.

Mechanism of Enhanced Hydrolytic Activity

In order to investigate the mechanism responsible for the enhancement of hydrolytic activity toward the soluble cellulosic substrates, CMC and barley β-glucan, we examined the effects of various redox reagents on hydrolysis catalyzed by native and pentaammineruthenium(III)-modified DEAE pool 2. Tartrate has been demonstrated to inhibit the veratryl alcohol-oxidase activity of pentaammineruthenium(III)-modified CBH I (7). In order to determine if the enhanced hydrolysis observed for modified CBH II was also inhibited by tartrate, native and pentaammineruthenium(III)-modified CBH II were incubated with CMC or barley β-glucan in the presence of 1.0 m*M* tartrate. For these two substrates, no

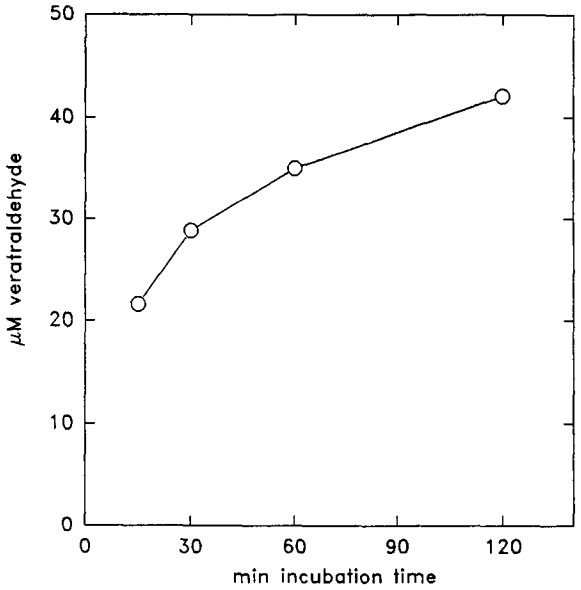

Fig. 8. Veratryl alcohol-oxidase activity of pentaammineruthenium(III)-modified DEAE pool 2 from Pulpzyme HA. The assay was carried out at 45°C as described in Materials and Methods.

inhibition of hydrolytic activity in the presence of tartrate was observed for either native or modified CBH II. This result implies that the mechanism that endows pentaammineruthenium(III)-CBH II with enhanced hydrolytic activity toward cellulosic substrates is not related to its veratryl alcohol-oxidase activity.

We also examined the effects of the addition of hydrogen peroxide to the modified enzyme during hydrolysis of CMC. If enhanced activity were the result of an oxidative reaction similar to the oxidation of veratryl alcohol, the addition of hydrogen peroxide would be expected to increase the hydrolysis rate. However, no difference between activity on CMC with and without hydrogen peroxide (3.0 m*M*) was observed for the pentaammineruthenium(III)-modified pool 2 enzyme.

Addition of free hexaammineruthenium(III) chloride or aquopentaammineruthenium(III) to reactions containing native CBH II and CMC or barley β-glucan did not result in any increase in glucose or reducing sugar production. Additionally, even after dialysis against distilled water, the pentaammineruthenium(III)-CBH II preparation maintained enhanced activity on barley β-glucan and CMC. It is therefore unlikely that the enhanced activity observed with the modified enzyme was the result of residual free pentaammineruthenium(III) from the modification procedure. It appears that interaction between amino acid residues of the protein and a pentaammineruthenium(III) group that is covalently attached to a histidine is necessary for an increase in hydrolysis to result.

Evidence to suggest that the hydrolysis of an insoluble crystalline cellulosic substrate is enhanced by pentaammineruthenium(III)-modified CBH II has, so far, not been obtained. However, in a preliminary experiment, addition of 3.0 m*M* hydrogen peroxide to a reaction mixture containing modified CBH II and newsprint increased hydrolysis of the cellulose component to reducing sugars twofold, but did not afffect the activity of the native enzyme (data not shown). The action of the pentaammineruthenium(III)-modified enzyme on crystalline cellulose and lignocellulosic substrates needs to be investigated further to determine the effects of various redox reagents on the activity of the enzymes. It remains to be established whether participation of the pentaammineruthenium(III)-histidine in catalysis is occurring, or an alteration in the structure of the protein caused by the attachment of the pentaammineruthenium(III) group is responsible for this increase in hydrolysis of soluble cellulosic substrates.

CONCLUSIONS

We have purified a protein from *T. reesei* Pulpzyme HA that appears to be CBH II. Attachment of pentaammineruthenium(III) to this protein has been shown to confer on it both veratryl alcohol-oxidase activity and increased hydrolytic activity toward CMC and barley β-glucan. Our initial attempts to determine the mechanism of the enhancement of hydrolytic activity indicate that it is not the result of a redox reaction with a potential similar to the veratryl alcohol oxidation, since the hydrolysis of the cellulosic substrates is not inhibited by tartrate or increased by hydrogen peroxide. However, the data obtained so far cannot exclude the possibility of participation in the hydrolysis reaction by a molecular species with a redox potential lower than that of tartrate.

In order to investigate this effect further, we plan to attach pentaammineruthenium groups to CBH II core protein prepared from Celluclast by proteolytic digest and purified to homogeneity by methods published previously (*18*). The effects of additional redox reagents, such as ascorbic acid and dithiothreitol, that have been previously used with other pentaammineruthenium-modified proteins (*19*) on the activities of the ruthenium-modified proteins will also be investigated.

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