Sonderdruck aus: **Material und Organismen** 17. Bd. 1982 Heft 2

Verlag Duncker & Humnblot, 1000 Berlin 41

Properties of a Carbohydrate-degrading Enzyme Complex from the Brown-rot Fungus *Poria plancenta*

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1. Introduction

A previous study reports the isolation of an enzyme complex from the brown-rot fungus, *Poria placenta* (Fr.) Cke., active on both polysaccharides and glycosides (T. L. HIGHLEY et al., 1981). The enzyme apparently consists of at least five subunits and may be a multifunctional enzyme (several autonomous functions on one polypeptide chain). Identification of properties of extracellular wall-degrading enzymes produced by decay fungi is important to provide information that can be utilized to stop their decaying activities in wood.

In this paper we describe the following properties of a carbohydrate-degrading enzyme complex isolated from *P. placenta*: (1) the effect of pH, temperature, metals, chelators, and sulfhydryl compounds on the cellulase (endo-1,4-ß-glucanase, C_x), xylanase, ß-glucosidase, and ß-xylosidase activities of the enzyme; (2) substrate specificity; (3) depolymerization reactions on cellulose and xylan; and (4) amino acid composition.

2. Materials[2] and Methods

Crude enzyme was prepared from sweetgum (*Liquidambar styraciflua* L.) decayed by *Poria placenta* in soil-block bottles as previously described (T. L.

[1] Maintained at Madison, Wis., in cooperation with the University of Wisconsin.

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HIGHLEY et al., 1981). The crude enzyme was purified for the characterization study by collodion filtration, thin layer isoelectricfocussing (TLIEF) and AcA 34 gel filtration described previously (T. L. HIGHLEY et al., 1981).

Polysaccharidase activities were assayed by increase in reducing groups (T. L. HIGHLEY, 1976). A unit of enzyme activity was defined as the amount needed to liberate reducing power equivalent to 1 μM of glucose per hour. Glycosidase activities were determined by either release of p-nitrophenol from sugar derivatives or by assaying for glucose with Glucostat reagent (Worthington) (T. L. HIGHLEY, 1976). A unit of enzyme activity was defined as the amount liberating 1 μM of p-nitrophenol or 1 μM of glucose per hour.

Substrates used were carboxymethylcellulose (Hercules), xylan (Aldrich), p-nitrophenol-ß-D-glucoside (Aldrich), and p-nitrophenol-ß-D-xyloside (Aldrich).

The effect of temperature on endoglucanase (C_x, CMCase), xylanase, ß-glucosidase, and ß-xylosidase was determined by measuring enzyme activity in 0.1 M, pH 5 citrate buffer at 23°, 50°, 60°, and 70°C. Temperature stability was determined by holding enzyme solution at the above temperatures for 1 hour, and then assaying for enzyme activity at pH 5 and 40° C.

The effect of pH on enzyme activity was measured at 40°C with the following buffers: pH 2 - 6, 0.05 M citrate; pH 7, 0.05 M phosphate; pH 8 and 9, 0.05 M tris-HCl. The effect of pH on stability was determined by incubating 0.5 ml of buffer with 0.5 ml of enzyme for 24 hours at 40°C and enzyme activity was determined at pH 5.0, 40° C.

A variety of metal ions, thiols, and chelators were tested for their effect on the various activities at a final concentration of 20 mM unless indicated differently: KBr, $CuSO_4$, $AgNO_3$, $MgCl_2$, $CaCl_2$, $BaCl_2$, $ZnCl_2$, $CdCl_2$, $HgCl_2$, $AlCl_3$, $SnCl_2$, p-chloromercuribenzenoate (10 mM), N-methyl malimide (10 mM) iodoacetate (10 mM), and ethylenediamine tetracetic acid. One-half ml of solution containing the test compound in 0.1 M acetate buffer pH 0.5 was mixed with 0.5 ml of enzyme. After 1 hour incubation at 40° C, enzyme activity was measured.

The ability of the purified enzyme to hydrolyze the following carbohydrate substrates was determined: a-mannan (yeast), holocellulose (sweetgum), Avicel, karaya gum, polygalacturonic acid, tragacanth gum, aspen sawdust, pectin, locust bean gum, guar gum, ballmilled aspen (*Populus grandidentata* Michx.; 8 hours in Sied Technik mill), filter paper, Walseth cotton, salicin, lactose, cellobiose, methyl-ß-D-xylopyranoside, p-nitrophenol-ß-D-mannoside, and p-nitrophenol-a-D-mannoside.

The products of hydrolysis of carboxymethylcellulose and xylan were determined by descending paper chromatography. Substrate in 0.05 M acetate buffer was incubated with the enzyme solution for 0, 4, 16, and 48 hours. After incubation, the mixture was boiled for 10 minutes, cooled, and spotted on a Whatman No. 1 chromatogram. Butanol-isopropanol-water (3 : 1 : 1) was used as a developing solvent. The separated sugars were detected by aniline spray reagent (I. SMITH, 1960).

The purified enzyme was hydrolyzed and the amino-acid profile determined by the Biophysics Laboratory. University of Wisconsin, Madison.

The constituent sugars of the enzyme were analyzed by paper chromatography of acid hydrolysates (W. E. MOORE and D. B. JOHNSON, 1967).

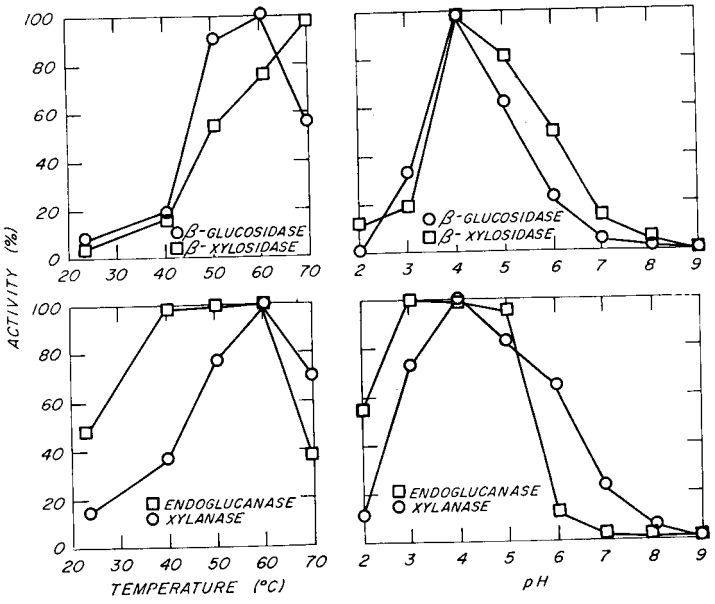

Fig. 1. Effect of temperature and pH on carbohydrolase activities of a purified enzyme complex from *Poria placenta.*

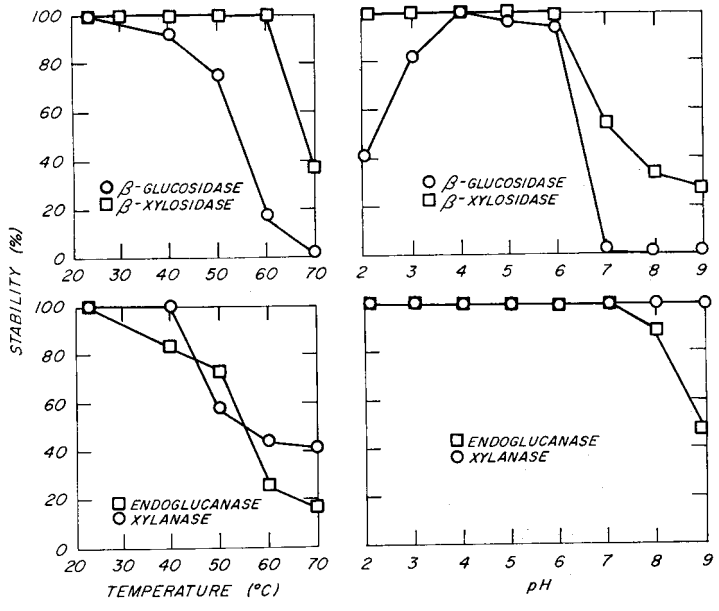

Fig. 2. Effect of temperature and pH on stability of activities of a purified enzyme complex from *Poria placenta.*

3. Results and Discussion

The relationships of pH and temperature to activity and stability were different for ß-glucosidase, ß-xylosidase, endoglucanase, and xylanase activities of the carbohydrate-degrading complex purified from *P. placenta* (Figs. 1 and 2). This follows what is reported for most multifunctional enzymes in that the domains or subunits on the polypeptide chain usually show differing pH optima and temperature sensitivity with respect to their functions (E. SCHMINCKE-OTT and H. BISSWANGER, 1980).

Very little temperature and pH data are available for extracellular carbohydrate-degrading enzymes from brown-rot fungi; but, like carbohydrolases from other fungi, they tend to be rather stable enzymes (G. KEILICH et al., 1970; T.L. HIGHLEY, 1975a, 1976; M. ISHIHARA et al., 1978; D. HERR et al., 1978a, 1978b). The carbohydrate-degrading activities in the purified complex isolated from *P. placenta* were also stable (Fig. 1). With the exception of ß-glucosidase, some activity was maintained after incubation at 70° C for 1 hour. ß-xylosidase was the most stable to high temperatures with no decrease in activity until 70° C. Endoglucanase and xylanase had high activities over a broad pH range.

Xylanase was stable for 24 hours at all pH's used (pH 2 - 9), while endoglucanase was most stable at pH 2 - 7. The glycosidases did not have such a wide pH spectrum. ß-xylosidase was stable from pH 2 - 6. Stability decreased considerably at pH 7. ß-glucosidase activity was the most sensitive to pH changes. Its stability decreased below pH 4 and was completely inactivated at pH 7.

ß-xylosidase had the highest temperature optimum, 70° C (highest temperature used), and endoglucanase the lowest temperature optimum, 40 - 60° C (Fig. 2). The temperature optima for carbohydrate-degrading enzymes from other brown-rot fungi are also relatively high. Xylanase from *Polyporus palustris* (Berk. et Curt.) Murr. had a temperature optimum of 76°C (M. ISHIHARA et al., 1978); ß-glucosidase from *Gloeophyllum trabeum* (Pers.), 75° C (D. HERR et al., 1978 a); endoglucanase from *G. trabeum*, 70° C (D. HERR et al., 1978 b); endoglucanase from *Phaeolus schweinitzii* (Fr.) Pat., 60° C (G. KEILICH et al., 1969); and endoglucanase from *Coniophora puteana* (Schum. ex Fr.) Karst., 50° C (G. KEILICH et al., 1970).

The pH optimum of endoglucanase was 3 - 4, while the pH optimum of the other enzyme activities was 4 (Fig.2). These pH optima are within the range of those reported for carbohydrolases from other

Table 1. Amino-acid composition of carbohydrate-degrading complex from *Poria placenta.*

Amino acid	Number of residues (closest integer)	Amino acid	Number of residues (closest integer)	Amino acid	Number of residues (closest integer)
Asp	160	Ala	97	Tyr	100
Thr	119	Val	75	Phe	68
Ser	120	Cys-Cys	12	NH_3	65
Glu	165	Met	17	Lys	24
Pro	70	Ile	49	His	30
Gly	94	Leu	105	Arg	49

brown-rot fungi (G. KEILICH et al., 1970; M. ISHIHARA et al., 1978; D. HERR et al., 1978 a, 1978 b). Endoglucanase and ß-glucosidase were the most sensitive to increased pH, activity dropping substantially at pH 6. Xylanase and ß-xylosidase activities were greatly reduced at pH 7. In the acid pH range, activity of the two glycosidases dropped considerably at pH 3. Little xylanase activity was detected at pH 2. Low pH had little effect on endoglucanase activity.

As in other studies (J.A. GASCOIGNE and M. M. GASCOIGNE, 1960; T. L. HIGHLEY, 1975b), the only metals with inhibiting activities were Hg and Ag. The chelator EDTA and the sulfhydryl inhibitors had no effect on the various activities. The sulfhydryl inhibitors would not be expected to inhibit activity because of the low amount of sulfur-containing amino acids found in the amino-acid profile of the enzyme complex (Table 1).

The initial products of xylan and carboxymethylcellulose hydrolysis by the enzyme complex were largely oligosaccharides with little free hexose or pentose. The early appearance of oligosaccharides is characteristic of random cleavage; random cleavage of polysaccharides is commonly reported (J.A. GASCOIGNE and M. M. GASCOIGNE, 1960; G. KEILICH et al., 1970; T. L. HIGHLEY, 1976).

The amino-acid composition of the enzyme complex shows a pattern similar to that reported for other carbohydrolases isolated from wood-decay fungi (K.-E. ERIKSSON and M. WINELL, 1968). There was a high content of aromatic and acidic amino acids and very few sulfur-con-

taining amino acids. The acidity of the enzyme complex was also verified previously by its low isoelectric point (1.8 - 2.0). Glucose was the only constituent sugar detected in significant quantities.

The enzyme complex was able to hydrolyze a number of carbohydrate substrates in addition to those previously reported (T. L. HIGHLEY et al., 1981). Of the polysaccharides assayed, the enzyme was active on locust bean gum, guar gum, and ballmilled aspen. It was unable to degrade microcrystalline cellulose (Avicel), holocellulose, polygalacturonic acid, pectin, tragacanth gum, karaya gum, aspen sawdust, filter paper, Walseth cotton and yeast mannan. The size of the enzyme (180,00 D) probably precludes degradation of ordered substrates such as Avicel and cotton. In addition to the glycoside activities already reported, the enzyme was active on salicin, p-nitrophenol-ß-D-mannoside, cellobiose, and lactose. It was inactive on a-D-mannoside and methyl-ß-D-xylopyranoside.

The ability of the enzyme complex to degrade a large number of different carbohydrate substrates differs from other reported carbohydrates. Because few purified carbohydrolases from brown-rot fungi have been studied, other brown-rot fungi should be looked at to determine if they also process carbohydrate-degrading complexes similar to *P. placenta*.

This study shows, however, that the properties of the carbohydrate-degrading complex isolated from *P. placenta,* with regard to pH, temperature, inhibitors, amino acid composition, and depolymerization of polysaccharides, are not greatly different from carbohydrate-degrading enzymes from other decay fungi. The great stability of the enzyme complex raises doubts about the feasibility of inactivating these enzymes as a means of controlling degradation in wood. It would probably be more profitable to aim at understanding the mechanics of enzyme formation and secretion, and utilize this information to develop control procedures.

4. Summary

The characteristics of an enzyme complex isolated from the brown-rot wood-decay fungus, *Poria placenta,* are described. The ability of the complex to degrade a large number of carbohydrate substrates differs from that of other reported carbohydrolases. However, its physical and chemical properties were similar to those of previously reported carbohydrolases from brown-rot fungi. Because of its great stability, inactivating this sort of enzyme as a means of controlling degradation may not be a feasible approach.

Zusammenfassung

Eigenschaften eines Kahlehydrat-abbauenden Enzym-Komplexes des Braunfäulepilzes *Poria placenta*

Die charakteristischen Eigenschaften eines aus dem holzabbauenden Braunfäulepilz *Poria placenta* isolierten Enzym-Komplexes werden beschrieben. Die Fähigkeit des Komplexes Zum Abbau einer großen Zahl von Kohlenhydrat-Substraten weicht von der anderer bekannter Carbohydrolasen ab. Die physikalischen und chemischen Eigenschaften ähnelten jedoch denen von Carbohydrolasen, die von Braunfäulepilzen bisher beschrieben waren. Wegen der großen Stabilität dieses Enzym-Typs scheint seine Inaktivierung kein gangbarer Weg für eine Verhinderung des Holzabbaus zu sein.

Résumé

Propriétés d'un complexe enzymatique dégradant les carbohydrates à partir d'un champignon de pourriture brune, le *Poria placenta*

On décrit les caractéristiques d'un complexe enzymatique isolé d'un champignon de pourriture brune, le *Poria placenta*. La capacité de ce complexe de dégrader un grand nombre de substrats de carbohydrates diffère de celle d'autres carbohydrolases rapportées. Cependant, ses propriétés physiques et chimiques étaient similaires à celles des carbohydrolases précédemment signalées à partir de champignons de pourriture brune. Par suite de sa grande stabilité, l'inactivation de cette sorte d'enzyme comme moyen de contrôle de la dégradation n'apparait pas être une approche réalisable.

Resumen

Propiedades de un eomplejo enzimático degradante de carbohidratos procedente del hongo de la pudrición parda, *Poria placenta*

Se procede a describir las propiedades de un complejo enzimático aislado a partir del hongo degradante de la madera y causante de la pudrición parda, *Poria placenta*. La capacidad del complejo para un gran número de substratos de carbohidrato difiere de la de otras carbohidrolasas conocidas. No obstante, sus propiedades fisicas y químicas fueron similares a las de otras carbohidrolasas procedentes de hongos de la pudrición parda descritas anteriormente. Dada su gran estabilidad, la inactivación de este tipo de enzima como medio para combatir la degradación no parece viable.

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