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CELLULASE (β-1,4-GLUCAN 4-GLUCANOHYDROLASE) FROM THE WOOD-DEGRADING FUNGUS *POLYPORUS SCHWEINITZII* FR

II CHARACTERIZATION

G KEILICH, P J BAILEY, E G AFTING AND W LIESE[a]

Institut fur Makromolekulare Chemie der Universitat Freiburg, Freiburg, and [a]Institut fur Holzbiologie und Holzschutz der Bundesforschungsanstalt fur Forst- und Holzwirtschaft, Reinbek (Germany)

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SUMMARY

1 Some properties of an endocellulase (β-1,4-glucan 4-glucanohydrolase, EC 3 2 1 4), previously isolated from culture filtrates of the wood-degrading fungus *Polyporus schweinitzii* Fr , have been investigated

2 The cellulase is stable between pH 2 6 and 7 5 Using a viscosimetric test with CM-cellulose as the substrate, an optimum activity was found at about pH 4 0, although only a very slight fall was observed at a lower pH With an increasing pH above 4 0, the activity decreased sharply, and the enzyme is almost inactive above pH 6 0

3 The cellulase has an optimum activity at about 60° and is almost completely stable up to this temperature, at higher temperatures it is rapidly denatured

4 The molecular weight was determined on a calibrated Sephadex G-100 column and was found to be 45 000 ± 4 500

5 Dewaxed cotton cellulose is practically unattacked by the cellulase both before and after purification and after the addition of a mycelium extract, whereas a regenerated cellulose was readily degraded, the amorphous regions are preferentially attacked

6 It was found that hydroxyethylcellulose was more easily degraded than a CM-cellulose with a similar degree of substitution and, in limited experiments with hydroxyethylcelluloses, that susceptibility to enzymatic attack is inversely proportional to the degree of substitution

7 The results are compared with those for cellulases from other sources and are discussed in relation to the behavior of *P schweinitzii in vivo*

INTRODUCTION

In a previous paper[1] the purification of a random acting (endo)cellulase (β-1,4-glucan 4-glucanohydrolase, EC 3 2 1 4) from culture filtrates of the wood-degrading

fungus *Polyporus schweinitzii* Fr was described Although the crude filtrate contains many other polysaccharide-splitting enzymes and much nucleic acid material, the final product of the purification is free of these except for a small amount of mannanase (*cf* ref 1)

The present paper concerns certain properties of this cellulase including its ability to degrade various celluloses and cellulose derivatives

MATERIALS AND METHODS

Organism, culture conditions

P schweinitzii Fr , strain number VIIa, CBS, Baarn was grown in a liquid culture medium as described by BAILEY *et al* [1]

Cellulase assay

A viscosimetric test using CM-cellulose in a 0 05 M, acetate buffer (pH 4 0) was used as described earlier[1]

Molecular weight determination using gel chromatography

The method employed is essentially that described by WHITAKER[2] Sephadex G-100 (Pharmacia, Uppsala) was equilibrated with 0 05 M imidazole–HCl buffer (pH 7 2) 0 1 M in KCl and was packed into a 1 cm × 50 cm column under a maximum pressure of a 10-cm water column The outer volume V_0 of the gel bed was determined in the usual way with Dextran Blue (Pharmacia) The elution volumes for the following proteins were determined using the spectrophotometric method for estimating protein[3] Cytochrome *c* (Boehringer, Mannheim), lysozyme (EC 3 2 1 17) (Boehringer, Mannheim), chymotrypsin (Boehringer, Mannheim), ovalbumin (Serva, Heidelberg) and bovine serum albumin (Serva, Heidelberg) In addition V_e for peroxidase (EC 1 11 1 7) (Boehringer, Mannheim) was determined using the enzyme assay[4] for the individual fractions A calibration curve of V_e/V_0 against log mol wt was prepared

Materials

A regenerated cellulose was prepared by the slow addition of a solution of cotton linters in cuoxam to an aqueous solution of Rochelle salt[5,6] under N_2 with stirring

A native cotton cellulose (Columbia) scoured in 0 5% NaOH under N_2 and extracted with benzene and acetone was donated by Temming, Gluckstadt

Hydroxyethylcellulose was prepared by the action of ethylene oxide on a solution of cotton linters in dimethyldibenzylammonium hydroxide (ref 7, E HUSEMANN AND E SIEFERT, unpublished results)

Index of crystallinity of cellulose (C_I)

The C_I values were determined as described by HERMANS AND WEIDINGER[8] and by KAST AND FLASCHNER[9] Dried cellulose samples were loaded with random orientation into the specimen window (1 cm × 1.5 cm) Radial traces of the X-ray diffraction patterns were obtained directly with a counter-tube goniometer These curves were evaluated by planimetering rather than by using diffraction peak heights (*cf.* ref 10). C_I is defined as $A_{CR}/(A_{CR} + A_{AM})$ where A_{CR} and A_{AM} are the integrated intensities due to the crystalline and amorphous contents, respectively.

The experimental curves were made using monochromatic Cu Kα radiation in a Philips X-ray diffractometer operating with a tube voltage of 35 kV/20 mA and a recording speed of 0 5° per min

RESULTS AND COMMENTS

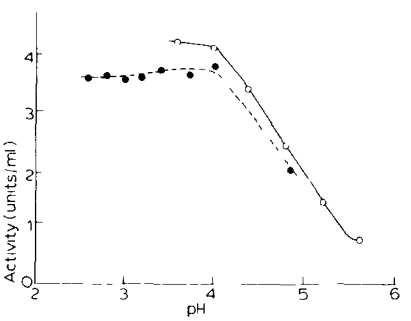
pH optimum and stability

The cellulase activity was determined at various pH's in the range 2 6–5 6 The standard test was used except that the substrate was dissolved in either an acetate or citrate buffer of the desired pH, and the enzyme preparation was suitably diluted with the same buffer As shown in Fig 1, no sharp pH optimum was found, but the activity is highest around pH 4 0 Discrepancies in the activity values in the region of overlap (Fig 1) are probably due to uninvestigated ionic effects Further measurements at lower pH's were not made, as it becomes increasingly difficult to exclude the effects of the acid hydrolysis of the substrate

The enzyme was found to be stable not only in the pH range tested but also at pH's up to 7 5 but above pH 6 0, the enzyme had practically no activity Other authors working with cellulases from different sources have found similar pH–activity relationships, although symmetrical and relatively sharp pH optima have also been reported (see for example refs 11–13) However, it is not possible to conclude that the pH dependence varies with the source of the enzyme, since the numerous different tests and substrates used prevent a strict comparison of the individual results

Temperature optimum and stability

P schweinitzii cellulase has optimum activity at about 60° (Fig 2), at higher temperatures inactivation predominates (Although the pH optimum is about 60°,



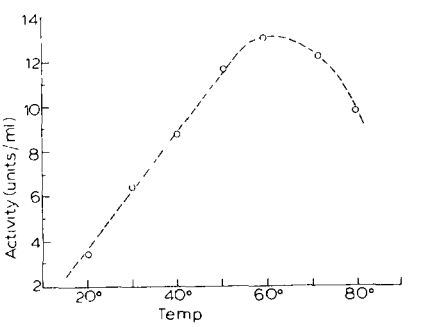


Fig 1 pH dependence of the cellulase activity at 20°, ●— — —●, in 0 05 M citrate buffer, ○— ○, in 0 05 M acetate buffer

Fig 2 Temperature dependence of the cellulase activity in 0 05 M acetate buffer (pH 4 0)

routine activity tests were carried out at 20° because of the inconvenience of making viscosimetric measurements at higher temperatures) To determine more precisely the effect of temperature, a diluted solution of the enzyme was incubated without the substrate for 10 min at various temperatures, was cooled in an ice–water bath and was tested in the usual way at 20° The results (Fig 3) indicate that the cellulase is completely stable up to about 60° but is very quickly denatured at higher temperatures This relatively high thermostability in both the presence and absence of the substrate appears to be characteristic of fungal cellulases[14]

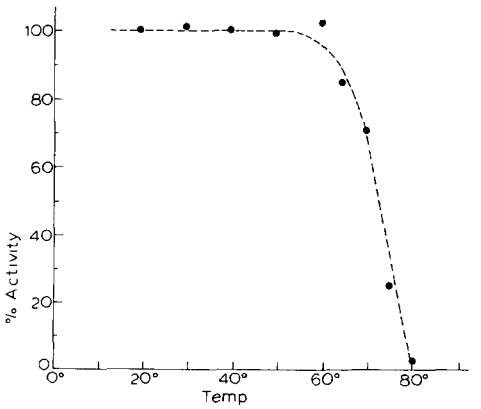


Fig 3 Heat stability of the cellulase The enzyme was maintained for 10 min at a given temperature in the absence of substrate After cooling the activity was tested in the usual way in 0 05 M acetate buffer (pH 4 0) at 20° These activities are shown in the figure, expressed as a percentage of the activity of unheated enzyme at 20°

Molecular weight

The molecular weight of the cellulase was determined from experiments using gel chromatography as described by WHITAKER[2] A calibration curve was prepared for a Sephadex G-100 column in 0 05 M imidazole–HCl buffer (pH 7 2) 0 1 M in NaCl using the following proteins of known molecular weight· cytochrome *c*, lysozyme, chymotrypsin, peroxidase, ovalbumin and bovine serum albumin Under the same experimental conditions, the *P schweinitzii* cellulase gave a value for V_e/V_0 of 1 54 which corresponds to a molecular weight of 45 000 ± 4500. Using the equation

$$\log M = 5\,914 - 0\,847\ (V_e/V_0)$$

proposed by DETERMANN[15] the results give a molecular weight of 43 000 The use of Sephadex (a cross-linked dextran) for determining the molecular weights of polyglycanases has been questioned because an interaction between the gel and enzyme may occur, leading to retardation and low estimations of molecular weight However, current work on the molecular weight determination of other polysaccharide-degrading enzymes using Bio-gel (polyacrylamide), as expected, shows that no interaction between such enzymes and this gel occurs, thus no retardation occurs, and the molecular weights determined in this way compare well with those found using other methods (*e g*, in the ultracentrifuge) Furthermore the results indicate that in the lower molecular weight range (< 50 000), the values derived from experiments with Sephadex columns are correct within the quoted error (G KEILICH AND K D FRANKEN, unpublished results)

Heterogeneous degradation of cellulose

Native and regenerated cellulose The ability of *P schweinitzii* cellulase to degrade a regenerated cellulose was demonstrated earlier[1] It is well known that native celluloses are less easily attacked by cell-free cellulase preparations than are regenerated celluloses or soluble cellulose derivatives (see ref 16) Therefore it was of interest to

TABLE I

DEGRADATION OF A DEWAXED NATIVE CELLULOSE (COLUMBIA COTTON) BY VARIOUS CELLULASE PREPARATIONS FROM *P schweinitzii*

In each case 250 mg of substrate were incubated at 35° for 72 h with an enzyme equivalent to 15 cellulase units diluted to a final volume of 35 ml with 0 05 M acetate buffer (pH 4 0) The mycelium extract was prepared by extraction with 0 05 M acetate buffer (pH 4 0) for 2 h with stirring, the amount of extract used corresponded to about the same amount of culture yielding 15 cellu lase units

Enzyme	*Cellulose weight loss (%)*	*P_w of cellulose after incubation (original P_w = 5800)*
Crude culture filtrate	<1	5600
Purified enzyme	<1	4300
Purified enzyme + mycelium extract	<1	5700
Control	<1	5700

investigate the ability of *P schweinitzii* cellulase to degrade a native cellulose Dewaxed cotton fibres were used, and we compared the ability of the crude culture filtrate, the culture filtrate *plus* mycelium extract and the purified enzyme to degrade it The experimental conditions and results are shown in Table I It is clear that little attack has taken place, *i e* , that no substance has been detected in the mycelium or in the culture filtrate which facilitates the degradation of native cellulose *in vitro* Using a regenerated cellulose and under similar experimental conditions, a decrease in P_w to about 1/10 of the original value was observed[1] The different susceptibilities of native and regenerated celluloses is clearly demonstrated

Effect of the crystallinity of the cellulose The inability of isolated cellulases to degrade native celluloses is usually associated with the higher crystallinity and the different crystalline form of native celluloses In order to learn if any similar effect occurs with a regenerated cellulose, we investigated the crystallinities of such a substrate before and after incubation with the *P schweinitzii* cellulase The crystal-

TABLE II

THE EFFECT OF THE ENZYMATIC DEGRADATION ON THE INDEX OF CRYSTALLINITY (C_I) OF A REGE NERATED CELLULOSE

C_I was determined by planimetry from the curves shown in Fig 4

Specimen	C_I	*Increase in C_I (%)*
Untreated	0 161	0
Incubated in buffer solution	0 159	0
Incubated in buffer *plus* enzyme	0 232	45

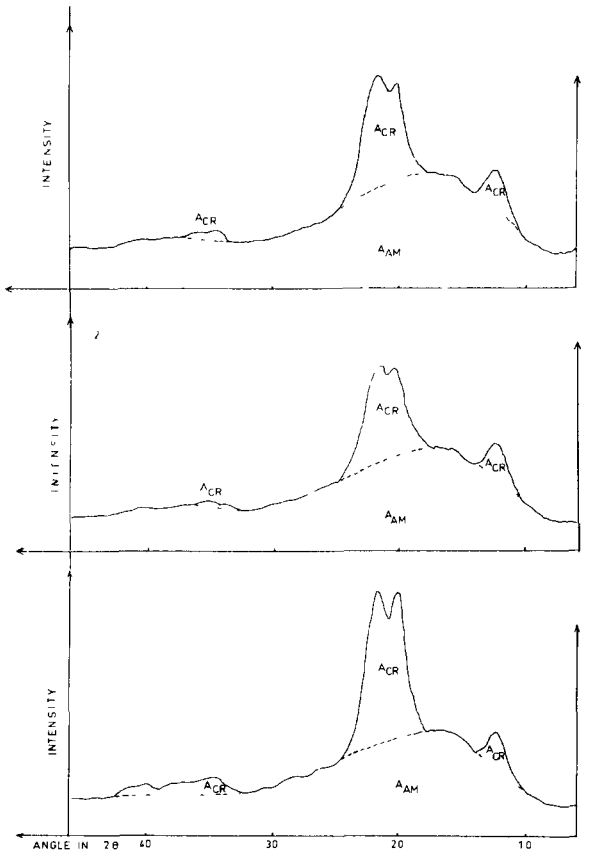


Fig 4 Index of crystallinity of regenerated cellulose (1) untreated, (2) incubated with buffer, (3) incubated with buffer and enzyme (under the same conditions as described for native cellulose in the legend to Table I) The curves represent radial traces of the X-ray diffraction patterns, obtained directly with a counter-tube goniometer A_{CR} and A_{AM} represent the integrated intensity of scattering arising from the crystalline and amorphous contents of the specimens, respectively

linities were compared on the basis of their index of crystallinity (C_0) values determined from X-ray diffraction experiments as described above Fig. 4 shows the experimental curves, and Table II shows the results after planimetric evaluation.

The increase in the index of crystallinity indicates that amorphous regions of the cellulose are preferentially attacked Although regenerated cellulose has a different crystalline lattice than has native cellulose[17], it is clear that such supramolecular structure still plays an important role in determining the susceptibility of cellulose to enzymatic degradation

Degradation of cellulose derivatives

In addition to the tests with CM-cellulose, we have also investigated the use of water-soluble hydroxyethylcelluloses having 0 3 and 0 6 as degrees of substitution as substrates The experimental conditions were the same as described for the test with

CM-cellulose[1]. The relative activities for these three substrates were approx. 1 4 8, respectively The effect of the degree of substitution on the susceptibility of cellulose derivatives to enzymatic hydrolysis has been investigated by a number of authors[18–23] Our results, which confirm that an increasing degree of substitution corresponds to a decreasing susceptibility, also substantiate the findings of WIRIK[19,20] that hydroxyethylcellulose is more easily degraded than is CM-cellulose of the same degree of substitution In order to discuss these effects further and to propose a mechanism for the splitting of such substrates, further kinetic experiments and analyses of the hydrolysis products are necessary In the absence of these, it is not possible to decide whether the proposed mechanisms of WIRIK[19,20] also apply to the action of *P schweinitzii* cellulase

DISCUSSION

In discussing cellulolytic enzyme systems three points are usually of most interest (1) whether one or more enzymes or other factors are necessary for the degradation of native cellulose, (2) the course of the hydrolysis (random or endwise) and (3) the number of hydrolytic components present.

REESE *et al* [16] proposed the following scheme

$$\text{Native cellulose} \xrightarrow{C_1} \text{linear polyanhydroglucose chains} \xrightarrow{C_x\text{'s}} \text{soluble small molecules}$$

There have been numerous reports providing evidence for a C_1 stage SELBY AND MAITLAND[24,25] and SELBY[26], working with *Trichoderma viride* and *Penicillum funiculosum*, claim to have completely separated a C_1 factor from the hydrolytic C_x enzymes BJOERNDAL AND ERIKSSON[13], however, report the isolation from the wood-degrading fungus *Stereum sanguinolentum* of a single enzyme capable of degrading native cellulose to cellobiose and glucose The role, and perhaps the universality, of a C_1 factor is still uncertain In the second step of the scheme of REESE *et al* [16], various enzymes may be involved The existence of endocellulases (random acting) is well known, but the occurrence of exocellulase (endwise acting) has still to be established in most cases.

Our tests with native cellulose showed that the *P schweinitzii* culture filtrate used contained no C_1 factor Furthermore we were unable to detect any in the mycelium It is not possible to account for this with any certainty as, a number of factors may be responsible, if C_1 is inductive it would not be produced in cultures with only glucose as the carbon source, and it may be unstable or inactive in cell-free preparations (although those isolated by SELBY and co-worker[25,26], particularly that from *P funiculosum*, appear to be relatively stable) In addition, it is possible that *P schweinitzii* does not produce a C_1 factor even *in vivo* There is certain evidence that native celluloses develop their high resistance to enzymatic degradation, and hence the need for a C_1 stage, after drying For example, it has been reported that cotton fibres which have never been dried and were obtained from the unopened boll are readily hydrolized by culture filtrates of various organisms (ref 27) If drying the cellulose does play an important role, then it is perhaps significant that *P schweinitzii* lives mainly as a parasite, *i e* , in undried wood, and may continue to live as a saprophyte as long as the wood remains wet, furthermore, it is virtually inactive in laboratory tests involving

TABLE III

MOLECULAR WEIGHT OF CELLULASES

The values given here are those mentioned in the literature, for more details regarding the degree of accuracy and the purity of the product used, see original literature Except where stated the enzyme is an endocellulase

Authors	*Source of enzymes*	*Molecular weight*	*Method*
PETTERSEN *et al* [28]	*Polyporus versicolor*	11 400	Ultracentrifuge
PETTERSEN AND PORATH[29]	*Polyporus versicolor*	51 000	Ultracentrifuge
DATTA, *et al* [30]	*Myrothecium verrucaria*	49 000	Ultracentrifuge
KING[31]	*Trichoderma viride*	42 000 61 000* 60 000**	Ultracentrifuge
IWASAKI *et al* [32]	*Trichoderma koningii*	50 000	Ultracentrifuge
LI *et al* [11]	*Trichoderma viride*	52 000 76 000*	Ultracentrifuge
SELBY AND MAITLAND[24]	*Myrothecium verracaria*	30 000 5 300*** 55 000**	Gel permeation chromatography
SELBY AND MAITLAND[25]	*Trichoderma viride*	12 000 48 000–62 000 61 000**	Gel permeation chromatography
BJORNDAL AND ERIKSSON[13]	*Stereum sanguinolentum*	21 200	Ultracentrifuge
PETTERSEN AND EAKER[33]	*Penicillum notatum*	35 000	Ultracentrifuge
OKADA *et al* [34]	*Trichoderma viride*	58 900 62 400 60 200	Ultracentrifuge

* Exocellulase
** C_1
*** Active against cotton

dried and re-wetted wood specimens, *viz P schweinitzii* is apparently unable to live on dried wood cell walls This behavior could be associated with an inability to effect a C_1 stage Unfortunately there are insufficient data on the cellulolytic enzyme systems of wood-destroying fungi and on the relationship between the physical form of celluloses and the C_1 stage, *etc* to allow further discussion of this idea It must be realized, of course, that other factors also determine the parasitic/saprophytic behavior of such fungi

Regarding the mode of action of *P schweinitzii* C_x cellulase, it was clearly shown in the first paper[1] that an endocellulase is present and that exocellulase is almost completely absent With respect to the number of C_x components present, it may be

mentioned that both during the purification and the molecular weight determination on Sephadex G-100, the cellulase activity was present in only one protein peak The electrophoretic separations also showed cellulase activity in only one component although it was apparently accompanied by two very similar proteins, probably a mannanase and a xylanase Therefore, we may conclude that *P schweinitzii* under the given culture conditions produces only one C_x type endocellulase of molecular weight 45 000 ± 4500

The molecular weights of a number of cellulases from other sources are listed in Table III It can be seen that the *P schweinitzii* cellulase compares with many of the other endocellulases investigated which have been reported as having molecular weights of 40 000–60 000 The only data on the size of such molecules are those of WHITAKER *et al* [35] who suggested a cigar-shaped molecule with dimensions of about 33 Å × 200 Å This represents a molecule much too large to diffuse freely through wood cell walls which, even when saturated with water, have practically a zero porosity[36] The mechanism by which fungi degrade cellulose embedded in wood cell walls remains unclear, particularly in those cases in which only the polysaccharide components are degraded The main question concerns the accessibility of the cellulose to the relatively large enzyme molecules This suggests that either much smaller cellulase components are also involved or that some modification of the cell wall occurs before the cellulose is attacked, *cf* the "pre-cellulolytic" stage[37] However, these are subjects requiring further investigation

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