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

I PURIFICATION*

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

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

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SUMMARY

1 A cellulase from *Polyporus schweinitzii* Fr has been purified by a 4-stage process including $(NH_4)_2SO_4$ precipitations, gel filtration and ion exchange gel chromatography, a 1550-fold increase in the specific activity was obtained

2 So that appropriate tests could be used to follow the purification, the mode of action of the cellulase was first determined by analyzing the products after an heterogeneous degradation of a regenerated cellulose with culture filtrate, the results showed that the activity was due almost exclusively to an endocellulase (β-1,4-glucan 4-glucanohydrolase, EC 3 2 1 4) A viscosimetric test using CM-cellulose therefore was adopted

3 The culture filtrate was tested for the presence of other enzymes, particularly other glycanases, and the separation of these from the cellulase was determined after successive stages of the purification

4 In the final product only one of the original glycan hydrolases detected, namely mannanase, remained

5 The purified product has been used as a starting material for characterizing the cellulase to be reported in a subsequent paper

INTRODUCTION

The components of wood cell walls are subject to degradation by the extracellular enzymes of numerous invading organisms, particularly fungi We are examining some of these enzymes as part of a study on the processes involved in the biodeterioration of wood and on the further characterization of the different types of fungal decay recognized in wood pathology (see refs 1–3)

Our initial attention is focused on the cellulolytic enzymes and, in particular, on

* Dedicated to Professor Dr E Husemann on the occasion of her 60th birthday

** Present address Institut fur Makromolekulare Chemie der Universitat Freiburg, 78 Freiburg, Stefan-Meier-Strasse 31, Germany

the number of components involved (C_1, C_x, *cf* ref 4), their mode of action (random or endwise hydrolysis of the cellulose molecules) and their molecular weights (hence size and diffusibility) A knowledge of such properties of the cellulolytic enzymes of fungi producing the different types of wood decay would be very useful, for example, in comparing the so-called brown rot fungi with the soft rot fungi, both of these fungi initially attack the carbohydrate components of the cell wall although the respective micromorphological changes produced are quite different (see ref 3) As discussed by these authors, the different behaviors of these groups of fungi may be connected not with their cellulolytic systems but with their pre-cellulolytic systems There is, however, very little detailed knowledge of the cellulases of wood-destroying fungi (refs 5–7) and even less concerning pre-cellulolytic systems

In the present paper the purification of a cellulase from *Polyporus schweinitzii* Fr is described, the properties of this enzyme will be reported in a subsequent paper *P schweinitzii* utilizes principally carbohydrate fractions of cell walls and occurs mainly as a parasite at the bottom of young conifers, causing the so-called cubical brown rot, it can continue to grow in felled stems as long as the moisture content remains high

MATERIALS AND METHODS

Organism

P schweinitzii Fr , Strain No VII a, CBS, Baarn

Culture medium

1 l of culture medium contained 1 0 g asparagine, 2 0 g NH_4NO_3, 2 0 g KH_2PO_4, 0 5 g $MgSO_4\ 7H_2O$, 0 1 g $CaCl_2\ 2H_2O$, 1 0 mg $FeCl_3\ 6H_2O$, 0 5 mg $ZnSO_4 \cdot 7H_2O$, 0 25 mg $MnSO_4 \cdot H_2O$, 0.05 mg $CuSO_4 \cdot 5H_2O$, 2.0 mg thiamine dichloride, 5 0 g yeast extract (Merck), and 25 g glucose The initial pH was adjusted to 5 0 The medium was sterilized by heating in steam for 20 min on three consecutive days.

Culture conditions

As pre-cultures, 50 ml of nutrient medium in 300-ml conical flasks were inoculated from the stock cultures After a 3-week incubation at 27° without shaking, the main cultures were inoculated with mycelium from the pre-cultures They were then incubated for 3 weeks at 27° without shaking

Culture filtrate

The cultures were filtered through filter paper without suction The filtrate (approx 1 l per 25 cultures) was centrifuged for 30 min at 16 000 × *g* to ensure the removal of cellular matter and was finally adjusted to pH 4 0

Gel chromatography, ion exchange chromatography

The Sephadex gels used were obtained from Pharmacia (Uppsala) and were prepared in accordance with the manufacturer's instructions (details of individual experiments are given in the legends to the figures)

Electrophoresis

An electrophoresis apparatus from Bender and Hobein (Munich) was used The polyacrylamide gel was prepared from a 25 25 1 (by vol) mixture of the following solutions I, 47 5 g acrylamide *plus* 2 5 g *N,N'*-methylene diacrylamide in 500 ml water, II, 1 ml *N,N,N',N'*-tetramethylenediamine diluted to 500 ml with water, III, 10% aqueous ammonium peroxidisulfate 51 ml of the mixture were polymerized

in a glass trough (internal dimensions of 18 cm × 8 cm × 0 4 cm) fitted with aluminium foil to exclude air The gel was then equilibrated overnight with the buffer to be used (*cf* ref 8) Fixation and staining was achieved with trichloroacetic acid and Coomassie blue (Serva, Heidelberg), and the staining intensity was determined by absorption at 583 nm with a Vitatron densitometer

Enzyme assays

Cellulase (β-1,4-glucan 4-glucanohydrolase, EC 3 2 1 4) was determined viscosimetrically at 20° using a 0 05% solution of CM-cellulose in a 0 05 M acetate buffer (pH 4 0) as described by HUSEMANN AND WERNER[9] and by AFTING[10] CM-cellulose (Tylose C 10 000 p) was supplied by Kalle (Wiesbaden-Biebrich), it had a 0 65–0.75 degree of substitution, a weight average molecular weight of 200 000 and was purified by reprecipitation from water with acetone The SCHULZ–BLASCHKE[11] constant (K), required for calculating the intrinsic viscosity $[\eta]$ according to Formula 1, was found to be 0.530 for this CM-cellulose in 0 05 M acetate buffer (pH 4 0).

$$[\eta] = \frac{\eta_{sp}/c}{1 + k\eta_{sp}} \qquad (1)$$

One cellulase unit[12] was taken as the amount of enzyme required to hydrolyze 1 μequiv of glucosidic bonds per min at 20° in a 0 05% solution of CM-cellulose (degree of substitution 0 65–0 75) in 0 05 M acetate buffer (pH 4 0)

β-Glucosidase (EC 3 2 1 21) was detected by testing for the production of glucose in a 0.2% solution of cellobiose in a 0 05 M acetate buffer (pH 5 5) using a coupled hexokinase and glucose-6-phosphate dehydrogenase system[13,14] (Boehringer, Mannheim).

Xylanase (EC 3 2 1 8) was detected by observing the increase in the reducing power resulting in a 0 2% solution of a wheat straw xylan of P_w* 100 in a 0 05 M acetate buffer (pH 4 0), using dinitrosalicylic acid[15].

Mannanase was detected viscosimetrically with a 2% solution of mannan (from Tubera Salep, P_w = 1200) in a 0.05 M acetate buffer (pH 4.2), according to the method of KEILICH[16]

Dextranase (EC 3 2 1 11) was detected by measuring the increase in the reducing power in a 0 2% solution of Dextran (P_w = 800, Pharmacia, Uppsala) in a 0 05 M acetate buffer (pH 5 0), using dinitrosalicylic acid[15]

β-Amylase (EC 3 2 1 2) was determined as described by BERNFELD[17].

Glucoamylase (EC 3 2 1 3) was detected by observing the amount of glucose released in a 0 1% solution of synthetic amylose[18–20] (P_n** = 1200) in 0 01 M phosphate buffer (pH 6 0), using a coupled hexokinase and glucose-6-phosphate dehydrogenase system[13,14]

α-Amylase (EC 3 2 1 1) was detected viscosimetrically[21] with a 0 1% solution of synthetic amylose[18–20] (P_n = 1200) in a 0 05 M sodium citrate buffer (pH 5 5) at 20°. α-Glucosidase (EC 3 2 1 20) was detected by measuring the amount of glucose released from a 0 1% maltose solution in a 0 05 M phosphate buffer (pH 7 0), using a coupled hexokinase and glucose-6-phosphate dehydrogenase system[13,14]

Protein was determined by the spectrophotometric method measuring $\varepsilon_{260}/\varepsilon_{280}$ (ref. 22)

* P_w = weight average degree of polymerization
** P_n = number average degree of polymerization

A regenerated cellulose was prepared by the slow addition of a solution of cotton linters in cuoxam to an aqueous solution of Rochelle salt[23,24] under N_2 with stirring

The weight average degree of polymerization (P_w) of the cellulose samples was determined from measurements of the intrinsic viscosity of cellulose solutions in cuoxam[25]

Soluble sugars were determined using the anthrone method[26]

Paper chromatography of water soluble mono- and oligosaccharides

The solutions were desalted with Amberlite IR 120 and IR 45 ion exchange resins and then were concentrated under vacuum One dimensional chromatography was carried out on Schleicher and Schull paper No 2043b Mgl with a mixture of *n*-butanol–pyridine–water (6 4 3, by vol) as the solvent carrier, running time 48 h at 20° The chromatogram was developed by spraying with (a) saturated solution of $AgNO_3$ in acetone and (b) ethanolic 10% KOH followed by clearing in 10% $Na_2S_2O_3$ (10 min), washing and drying

RESULTS AND COMMENTS

It is well known that wood-destroying fungi secrete, in addition to cellulases, other enzymes capable of degrading various other polysaccharides It is relevant to investigate these enzymes not only for the general understanding of the action of such fungi but also because they are known to be similar in many respects to cellulases, consequently they may be the source of considerable difficulties in trying to obtain a pure cellulase from culture filtrates In the present work they have been investigated only qualitatively and primarily as an aid in following the cellulase purification It was also necessary to determine the mode of cellulose hydrolysis, *i e* , whether primarily by a random action (endocellulase), by endwise action (exocellulase) or by a combination of both It is therefore convenient to divide the results into two sections as follows

Preliminary tests

Presence of other polyglycan hydrolases

Qualitative tests showed that the crude *P schweinitzii* culture filtrate contains enzymes which split the homopolysaccharides cellulose, amylose, mannan and xylan, dextran was not degraded For the hydrolysis of α-1,4-glucosidic bonds in polymers (as in amylose, amylopectin or glycogen) three different enzymes were detected, namely α-amylase, β-amylase and glucoamylase In addition disaccharide-splitting enzymes such as α-glucosidase and β-glucosidase were found to be present The activity of the culture filtrate on heteropolysaccharides was not investigated

Mode of action of the cellulase

Apart from being of general interest, this must be established so that an appropriate test can be used during the purification To determine whether *P schweinitzii* produces an endocellulase or an exocellulase or both, preliminary experiments must be made with cellulose as the substrate (cellulose derivatives are substrates only for endocellulases[21,24,27]) The regenerated cellulose used by HUSEMANN AND LOETTERLE[24] and LOETTERLE[27] is suitable for such tests, as it may be hydrolyzed by exo- as well as by endocellulases independently of the presence of the so-called C_1 factor[4]

Our test involves the incubation of a regenerated cotton cellulose (P_w = 3900) with the culture filtrate after removal of sugars using gel filtration with Sephadex G-25

TABLE I

DEGRADATION OF A REGENERATED CELLULOSE WITH CULTURE FILTRATE FROM *P schweinitzii* Fr

Expt	*Substrate*	*Buffer*	*Enzyme*	*Temp*	*Time (h)*	*P_w* of remaining cellulose*	*Cellulose weight loss (%)*	*Sugars* in solution*
1	250 mg cellulose, P_w about 3900	25 ml 0 05 M acetate buffer (pH 4 0)	10 ml culture filtrate**	35°	24	About 1050	1	<1 mg glucose <1 mg cellobiose
2	250 mg cellulose, P_w about 3900	25 ml 0 05 M acetate buffer (pH 4 0)	10 ml culture filtrate**	35°	72	About 380	2	<1 mg glucose <1 mg cellobiose
3	250 mg cellulose, P_w about 3900	25 ml 0 05 M acetate buffer (pH 4 0)	None (control)	35°	72	About 3500	0	None

* See MATERIALS AND METHODS

** Lower molecular weight substances such as mono- and oligosaccharides were first removed from the culture filtrate on a Sephadex G-25 column

The weight loss, fall in P_w of the cellulose and sugars produced are determined (the latter by paper chromatography and quantitatively with anthrone) From combining the results obtained, it is generally possible to conclude whether degradation by an exo- or an endocellulase has occurred The conditions and the results of these experiments are shown in Table I

It is seen that after 72 h, the P_w of the cellulose has fallen to about 1/10th of its original value This result, in combination with the negligible weight loss, suggests that an endocellulase (β-1,4-glucan 4-glucanohydrolase, EC 3 2 1 4) is certainly present As only traces of glucose and cellobiose were detected after incubation, the presence of an exocellulase may be excluded Furthermore, the appearance of these traces of glucose and cellobiose may be due to the action of the detected β-glucosidase on water-soluble β-glucosidic bonded oligomers (degree of polymerization 3–7) produced as final products of the endocellulase action

Having established that an endocellulase is present in the culture filtrate, the purification was carried out using a test for cellulase activity with CM-cellulose as the substrate

Purification of the cellulase

Starting material

The culture filtrate contained 2 05 units of cellulase per ml and had a protein content of 11 35 mg/ml, giving a specific activity of 0 180 unit/mg protein

First $(NH_4)_2SO_4$ precipitation

Preliminary experiments with small aliquots of the culture filtrate showed that a satisfactory fractionation was not possible, even after concentrating the filtrate with coarse Sephadex G-25 to a solution with approximately double the protein content However, it was found that an almost quantitative precipitation of the cellulase was obtained using the procedure described below, this offered the advantage of a large decrease in volume and an approx 30-fold increase in the specific activity Therefore this step was included in the purification The ultraviolet spectrum of the product after this step was a typical protein curve with $\varepsilon_{280}/\varepsilon_{260} = 1\ 2$

All operations were carried out at 3–5° About 1 l of the culture filtrate was brought to an $(NH_4)_2SO_4$ saturation of $s = 0\ 92$ by the addition, with slow stirring, o-

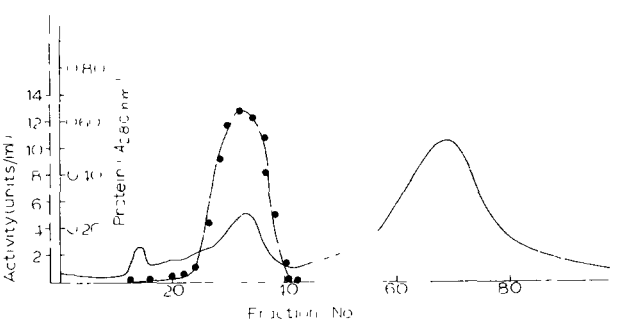

Fig 1 Gel filtration of cellulase on Sephadex G-100 10 ml of the enzyme solution containing approx 500 mg protein were layered on a column (40 mm × 400 mm) which was equilibrated at 10° with a 0 05 M acetate buffer (pH 5 0) , 8-ml fractions were collected at a flow rate of 20 ml/h, ———, $A_{280\ nm}$, ●— —●, cellulase activity in units/ml

finely milled $(NH_4)_2SO_4$ When all the salt had dissolved, the pH was adjusted to 4 0 After standing for 18 h, the solution was centrifuged for 40 min at 16 000 × *g*, and the precipitate was washed with 60 ml of an $(NH_4)_2SO_4$ solution (*s* = 0 90, pH 4 0) The precipitate was then separated by centrifugation at 48 000 × *g* for 20 min and finally was dissolved in about 10 ml of 0 05 M acetate buffer (pH 4 0), a small amount of insoluble matter was removed by further centrifugation.

Gel filtration on Sephadex G-100

The protein solution obtained from the $(NH_4)_2SO_4$ precipitation was fractionated on a Sephadex G-100 column A typical elution pattern is shown in Fig 1, cellulase activity was present only in the second protein peak

This step generally results in about a 7-fold increase in the specific activity over the $(NH_4)_2SO_4$ precipitation product However, activity tests for different glycan hydrolases showed that the cellulase at this stage is still accompanied by enzymes hydrolyzing mannan, xylan, amylose and maltose, only the β-glucosidase present in the culture filtrate is no longer present, suggesting that the proteins responsible for the various activities may be of very similar molecular weight

Separation of the product into various components is, however, achieved using gel electrophoresis on polyacrylamide gel (pH 7 2 and 5 0) Satisfactory staining of cellulases could not be achieved using Amido Black 10B, but good results were obtained using trichloroacetic acid and Coomassie blue as described by CHRAMBACH *et al* [28] At both pH 7 2 and 5 0, several components are separated (Figs 2 and 3), by elution and activity testing it was determined that cellulase was present only in component No 2 in both Figs 2 and 3 This behavior of the cellulase suggests that a separation might

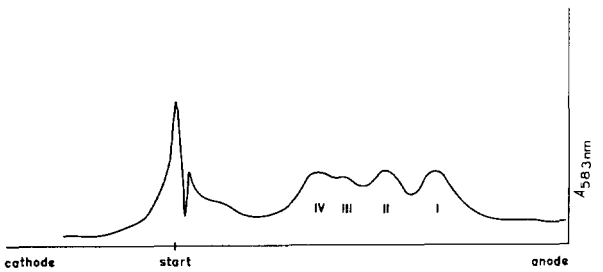

Fig 2 Polyacrylamide-gel electrophoresis of protein solution after the Sephadex G-100 step, in 0 05 M triethanolamine–HCl buffer pH 7 2 at 15°, 10 mA, about 250 V, running time, 5 0 h The curve represents absorption at 583 nm after fixing and staining with trichloroacetic acid and Coomassie blue

also be possible using ion exchange chromatography on DEAE-Sephadex It is interesting that at both pH 7 2 and 5 0, the *P schweinitzii* cellulase migrates towards the anode, other authors, *e g* WHITAKER *et al* [29], have reported that cellulases from other microorganisms migrate towards the cathode

Ion exchange chromatography on DEAE-Sephadex A-25

The fractions from the Sephadex G-100 step containing cellulase activity were pooled and further fractionated on DEAE-Sephadex A-25 A typical elution pattern is shown in Fig 4 The increase in the specific activity is generally about 2 5-fold with respect to the gel filtration step

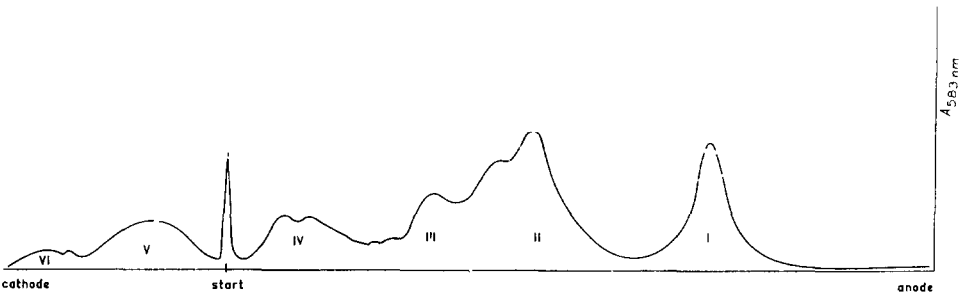

Fig 3 Polyacrylamide-gel electrophoresis of protein solution after the Sephadex G-100 filtration step, in 0 05 M acetate buffer (pH 5 0) at 15°, 10 mA, about 250 V, running time, 4 5 h

Second $(NH_4)_2SO_4$ precipitation

A second $(NH_4)_2SO_4$ precipitation was made in order to obtain a more concentrated final product This step also gave a further 2 8-fold increase in the specific activity From the DEAE-Sephadex the fractions containing the cellulase activity were pooled, the pH was adjusted to 4 5 and the protein was fractionated at 3–5° with finely ground $(NH_4)_2SO_4$ (inactivation occurs if the precipitation is carried out at pH 7 2), the cellulase was recovered between $s = 0\,60$ and $s = 0\,80$ The precipitate was collected after centrifugation at 16 000 × *g* for 40 min, was washed with an $(NH_4)_2SO_4$ solution ($s = 0.80$) and then was dissolved in about 5 0 ml of acetate buffer (pH 4 0) A small amount of insoluble matter was removed by further centrifugation

A summary of the purification procedure is given in Table II The specific activity of the purified cellulase (280 units/mg protein) is about 1550-fold higher than that of the crude filtrate The removal of nucleic acids present in the culture filtrate (presumably due to autolysis, *cf* ref 30) is indicated by the increasing $\varepsilon_{280}/\varepsilon_{260}$ values The final product may be assumed to be virtually free of nucleic acid Tests for

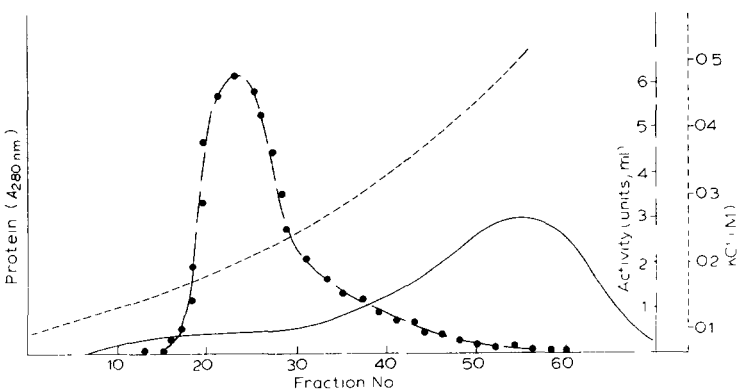

Fig 4 Chromatography of cellulase on DEAE-Sephadex A-25 The solution from the Sephadex G-100 stage (about 130 ml) was layered on the column (10 mm × 100 mm) which was equilibrated at 10° with 0 01 M triethanolamine–HCl buffer (pH 7 2) containing 0 1 M KCl, after washing the column with the same buffer until no protein could be detected in the eluate, the protein was eluted with a salt gradient of 0 1–0 5 M KCl in 0 01 M triethanolamine–HCl buffer (pH 7 2) The flow rate was 10 ml/h and 5-ml fractions were collected ———, $A_{280\,nm}$, ●—●, cellulase activity in units/ml, — — —, KCl (M)

TABLE II

PURIFICATION OF CELLULASE FROM *P schweinitzii* Fr

	Volume (ml)	*Protein (mg/ml)*	$\varepsilon_{280}/\varepsilon_{560}$ *ratio*	*Cellulase activity (total units)*	*Specific activity (units/mg)*	*Total yield (%)*	*Total purification factor*
Culture filtrate	1000	11 35	0 900	2050	0 180	100	—
$(NH_4)_2SO_4$ precipitation (pH 4 0, s = 0 92)	12	27 10	1 20	1850	5 80	90	32
Sephadex G-100 (pH 5 0)	130	0 335	1 45	1760	40 5	86	224
DEAE-Sephadex A-25 (pH 7 2, 0 14–0 16 M KCl)	70	0 495	1 60	1440	100	70 5	555
$(NH_4)_2SO_4$ precipitation (pH 4 5, s = 0 6–0 8)	4 8	0 86	1 75	1150	280	56	1550

the different glycan hydrolases detected in the culture filtrate show that the final cellulase product is contaminated by only mannanase, a mannan splitting endohydrolase The product is free of carbohydrate as shown by the anthrone test[26] and is stable for at least several months

DISCUSSION

The production of cellulase in a culture medium containing glucose as the sole carbon source is noteworthy Cellulase is generally found to be an adaptive or inducible enzyme, although other workers (*e g* , LYR[31]) have reported its production by wood-destroying fungi on media containing no cellulose However, the effects of other substances, such as malt and yeast extracts often included in nutrient media, are not clear (see refs 32–34)

The cellulase content of the culture filtrates used in our work was 1 5–2 0 units/ml with a specific activity of 0 1–0 18 units/mg protein It is difficult to compare this with the amounts of cellulase produced by other cellulolytic microorganisms because of the wide range of tests and activity units in use (see ref 35) Our results clearly show, however, that only a small amount of the protein in *P schweinitzii* culture filtrates is cellulase It is accompanied by numerous other proteins especially other glycanases, including xylanase, mannanase, α-amylase, β-amylase and glucoamylase

By degradation of a regenerated cellulose it was shown that the cellulase activity is almost exclusively due to a random acting, *i e* , endocellulase It is not clear how the traces of glucose and cellobiose found in these experiments arise The action of a small amount of exocellulase on either the cellulose itself or on oligomers produced by the action of the endocellulase could be responsible (*cf* ref 36) Alternatively the sugars may be produced by the hydrolysis of soluble oligomers by the β-glucosidase known to be present

Using the purification procedure described above, it was possible to obtain a

1550-fold increase in the specific activity (specific activity of the final product 280 units/mg protein) Numerous other polysaccharide-splitting enzymes were removed, ion exchange chromatography being particularly effective in this respect Persistence of the mannanase indicates that it is so similar to the cellulase in its molecular weight, electrophoretic behavior, pH and temperature stabilities that the two enzymes are not easily separated using the usual techniques More sensitive methods, such as substrate precipitation or preparative electrophoresis, might be more successful

The final cellulase product is, however, free of other glucan hydrolases and may therefore be used for the characterization of the cellulase This will be reported and discussed in a subsequent paper

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