Biochem. J. (1983) **215**, 677–683
Printed in Great Britain

Cellobiohydrolase from *Trichoderma reesei*

Martti NUMMI,* Marja-Leena NIKU-PAAVOLA,* Arja LAPPALAINEN,* Tor-Magnus ENARI* and Veijo RAUNIO†
**Technical Research Centre of Finland, Biotechnical Laboratory, Tietotie 2, SF-02150 Espoo 15, Finland, and †National Public Health Institute, Helsinki, Finland*

(*Received 20 May 1983/Accepted 22 August 1983*)

A 1,4-β-D-glucan cellobiohydrolase (EC 3.2.1.91) was purified from the culture liquid of *Trichoderma reesei* by using biospecific sorption on amorphous cellulose and immunoaffinity chromatography. A single protein band in polyacrylamide-gel electrophoresis and one arc in immunoelectrophoresis corresponded to the enzyme activity. The M_r was 65000. The pI was 4.2–3.6. The purified enzyme contained about 10% hexose. The enzyme differs from previously described cellobiohydrolases in being more effective in the hydrolysis of cellulose.

Fungi capable of degrading cellulosic materials secrete into their culture medium a set of cellulolytic enzymes: endoglucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91) and β-glucosidases (EC 3.2.1.21). All these enzymes are regarded as necessary in the hydrolysis of native cellulose.

Several attempts to purify cellulolytic enzymes have been published. All these procedures reveal the existence of a number of 'isoenzymes' for each type of enzyme (Enari, 1983). In our previous studies it was found that two enzymes produced by *Trichoderma reesei* were specifically bound to amorphous cellulose (Nummi *et al.*, 1981*b*). The aim of the present study was to purify the most abundant of these enzymes (anodic in immunoelectrophoresis) and study its properties.

The purification of cellulolytic enzymes described in the present paper is based on the formation of an enzyme–substrate complex, i.e. sorption of enzymes on amorphous cellulose and their subsequent release during the enzymic hydrolysis of the substrate. Furthermore, immunological specificity is applied to the identification of a single protein throughout the procedure. This technique has been proposed (Håkansson *et al.*, 1979), but not previously used systematically, in the purification of cellulolytic enzymes. The combination of these methods has facilitated the elimination of non-cellulolytic proteins from the final preparation. With other techniques the impurities are difficult to exclude from preparations of cellulolytic enzymes because of the close similarity in physicochemical properties of many extracellular *Trichoderma* proteins and the lack of specific activity-assay methods for cellulases.

Abbreviation used: D.S., degree of substitution.

Materials and methods

Enzyme preparation

Cellulolytic enzymes from the culture liquid of *Trichoderma reesei* VTT-D-80133 were sorbed on amorphous cellulose (Whatman CF11, ball-milled; Whatman, Maidstone, Kent, U.K.) and purified from other extracellular proteins as described previously (Nummi *et al.*, 1981*b*).

Activity determinations

Determinations were performed in 50mM-sodium citrate buffer, pH5.0. Cellulolytic activity was measured nephelometrically (Nummi *et al.*, 1981*a*) with amorphous cellulose (Nummi *et al.*, 1980) as substrate. The incubation temperature was 40°C and time 10min. The same method was used for measuring activity towards insoluble birch xylan. The unit of activity is defined as the decrease in nephelometric value per minute.

Endoglucanase activity was measured with hydroxyethyl-cellulose (D.S.0.9; Fluka, Buchs, Switzerland) as substrate and determination of the reducing groups liberated during a 10min hydrolysis at 50°C (Enari *et al.*, 1981). Activity towards barley β-glucan (Biocon, Eardiston, U.K.) and starch (soluble; Merck, Darmstadt, Germany) was measured by the same method. Endoglucanase activity was also measured with CM-cellulose (D.S.0.88, type 7H3SXF; Hercules, Wilmington, DE, U.S.A.) as substrate in a viscosimetric method

(Almin *et al.*, 1967). The incubation time was 15 min and the temperature 25°C.

β-Glucosidase activity was measured with *p*-nitrophenyl β-glucoside (Merck) and cellobiose (Fluka) as substrates and determination of the products liberated, nitrophenol and glucose respectively (Enari *et al.*, 1981). The incubation time was 10 min and the temperature 50°C. Endoglucanase and β-glucosidase activities are expressed as katals.

Hydrolysis studies

Hydrolysis of native cotton (Suomen Vanutehdas, Jokela, Finland) was studied with mixtures containing 20 mg of cotton and 200 μg of enzyme protein in 5 ml of 50 mM-citrate buffer, pH 5.0. The soluble sugars released into the medium during 20 days at 50°C were determined by the anthrone/H_2SO_4 method (Whistler & Wolfrom, 1962).

Hydrolysis of cellohexaose was measured by incubating 400 μg of substrate with 1 μg of enzyme protein in 1 ml of 50 mM-citrate buffer, pH 5.0, for 10 min. The hydrolysis products were analysed by liquid chromatography (Optilab 931 HSRI apparatus equipped with Multiref 902C; Tecator, Vällingsby, Sweden) on an Aminex HPX-87C column in water at 65°C. The hydrolysis products from amorphous cellulose were analysed by the same method.

Carbohydrate determination

The amount of carbohydrate of the purified cellobiohydrolase was determined by the anthrone/H_2SO_4 method (Whistler & Wolfrom, 1962).

Electrofocusing chromatography

The isoelectric point was determined by using electrofocusing chromatography. PGE 94 ion-exchanger (Pharmacia, Uppsala, Sweden) (20 ml) stabilized in 25 mM-histidine/HCl buffer, pH 6.5, was used to start the focusing. The sample (20–200 mg) was eluted with diluted (1:10) PB 76 buffer (Pharmacia) with a gradient from pH 5.0 to 3.5. The fractions were collected and activities for cellulolytic enzymes were assayed.

Preparation of cellodextrins

Ball-milled amorphous cellulose (Nummi *et al.*, 1980) was hydrolysed with 75% (v/v) formic acid at 100°C for 4 h. The soluble hydrolysis products were deionized by evaporation and by use of a mixed-bed ion-exchanger. The cellodextrins were fractionated in 500 mg batches in two successive chromatographic runs on Bio-Gel P2 (Bio-Rad Laboratories, Richmond, CA, U.S.A.) columns (500 ml and 200 ml respectively) at 60°C, with deionized water as eluent. The purity of the cellodextrins collected was checked by paper chromatography, and their identity was confirmed (Storvick *et al.*, 1963). Cellohexaose was used as substrate in hydrolysis experiments.

Preparation of cellobiohydrolase antiserum

Cellulolytic enzymes were purified by biospecific sorption (Nummi *et al.*, 1981*b*) and thereafter separated from each other by using zone electrophoresis on agarose gel in 25 mM-sodium barbital buffer, pH 8.2. The anodic component was extracted from the crushed gel with 50 mM-citrate buffer, pH 5.0, and used as antigen.

During 3 months' immunization 0.5 mg of antigen was injected once a month into a sheep and the serum was collected. The γ-globulin fraction containing the antibodies was purified from the serum by using ion-exchange chromatography (Livingston, 1974). The fraction that formed immunoprecipitate only with cellobiohydrolase was used for preparation of immunoabsorbent for immunoaffinity chromatography (Livingston, 1974) and in quantitative immunological determination of enzyme protein (Mancini *et al.*, 1965).

Immunoaffinity chromatography

Immunoabsorbent consisted of 15 ml of CNBr-activated Sepharose 4B gel (Pharmacia) to which 200 mg of purified specific cellobiohydrolase antibody was bound (Livingston, 1974). Concentrated (Amicon PM 10 filter) cellulolytic enzymes from the biospecific sorption step were adsorbed on the gel at pH 8.2 in 50 mM-barbital buffer containing 0.1 M-NaCl.

Non-specifically bound proteins were eluted with 0.1 M-sodium acetate buffer, pH 4.6, containing 0.5 M-NaCl. Cellobiohydrolase was released at pH 5.0 with 4 M-$MgCl_2$, dialysed against 50 mM-citrate buffer, pH 5.0, and concentrated by ultrafiltration. Protein concentration was measured by the Lowry method (Lowry *et al.*, 1951), with bovine serum albumin (Sigma Chemical Co., St. Louis, MO, U.S.A.) as standard.

Quantitative immunological analysis

To measure the amount of cellobiohydrolase protein at different purification steps a quantitative immunological method (Mancini *et al.*, 1965) was adapted for *T. reesei* proteins. The specific antiserum against cellobiohydrolase was mixed (1:50, v/v) with 1% agarose gel in 50 mM-sodium phosphate buffer, pH 6.8, containing 0.5 M-NaCl. The purified cellobiohydrolase was used as standard in 1 μl samples containing 0.1–0.5 μg of protein.

Combined polyacrylamide-gel electrophoresis–immunodiffusion

Electrophoresis was performed on 7.5% polyacrylamide gel slabs at pH 7 in Tris/barbital buffer

(Maurer, 1968) and at pH 8.5 with 10% gel in Tris/HCl buffer (Laemmli, 1970).

The separation on polyacrylamide gel was analysed by immunodiffusion against the specific cellobiohydrolase antiserum and an antiserum prepared against all *T. reesei* proteins (Nummi *et al.*, 1980). The polyacrylamide-gel strips containing the proteins separated by electrophoresis were embedded in agarose in 25 mM-sodium barbital buffer, pH 8.2. The antisera were applied in wells cut alongside the polyacrylamide gel. The diffusion and washing steps were identical with those after conventional immunoelectrophoresis. The activity was demonstrated with amorphous-cellulose-substrate overlay plates (Nummi *et al.*, 1980). The proteins were stained with Coomassie Brilliant Blue R-250 (Serva, Heidelberg, Germany) (Diezel *et al.*, 1972).

Electrophoresis in denaturating conditions

Electrophoresis was run on 10% polyacrylamide gel containing 0.1% sodium dodecyl sulphate (Laemmli, 1970). The calibration mixture of large-molecular-mass proteins (Pharmacia) and the pure proteins (Sigma) *Escherichia coli* β-galactosidase, phosphorylase *b*, transferrin and bovine serum albumin were used as references.

End-product inhibition

End-product inhibition was studied by using the nephelometric assay with additions of pure glucose and cellobiose. The inhibitor concentrations used were 0.1, 1.0, 10 and 50 mM.

Results

The amounts of cellobiohydrolase protein at different purification steps, determined by the quantitative immunological method, are presented in Table 1. In the biospecific sorption a great excess of protein is used. This eliminates non-specific adsorption of proteins other than cellobiohydrolase, but leads, of course, to a low yield. The values given in Table 1 correspond to the amount of sample that can be purified by one immunoaffinity run. The 15 ml affinity column binds only 0.5 mg of cellobiohydrolase. However, of the total extracellular protein in the culture liquid, as much as 60% is cellobiohydrolase, as estimated by the precipitation test. Thus the purification attained is only 1.7-fold, although the preparation is extremely pure.

The cellulolytic activities at different purification steps are shown in Table 2. The specific activity of cellobiohydrolase decreases through the purification procedure. This decrease is typical for purified

Table 1. *Purification of cellobiohydrolase protein*

For full experimental details see the text. Total protein was determined by the method of Lowry *et al.* (1951). Cellobiohydrolase protein was determined by a quantitative immunological method (Mancini *et al.*, 1965).

Purification step	Total protein (mg)	Cellobiohydrolase protein (mg)	Protein yield (%)	Cellobiohydrolase protein/total protein	Purification (fold)
Culture liquid	100	60	100	0.6	1
Biospecific sorption on cellulose	1	0.8	1.3	0.8	1.3
Immunoaffinity chromatography	0.5	0.5	0.8	1.0	1.7

Table 2. *Cellulolytic activities during the purification procedure*

For full experimental details see the text.

Substrate	Product analysed	Purification step of cellulolytic enzymes ...	Activity: Culture liquid	Activity: Biospecific sorption	Activity: Immunoaffinity
Amorphous cellulose (0.3 mg/ml)	Decrease in particle size		28*	28*	8*
	Reducing sugars		15†	14†	2.5†
	Cellobiose		14†	8†	2.4†
	Glucose		1.7†	4†	0†
Hydroxyethyl-cellulose (10 mg/ml)	Reducing sugars		145†	22†	0†
Cellobiose (8.8 mM)	Glucose		6†	2†	0.3†
p-Nitrophenyl β-glucoside (1 mM)	Nitrophenol		12†	0†	0†

* Activity units: Δ (nephelometric value)/min per mg of protein.

† Activity units: nkat/mg of protein.

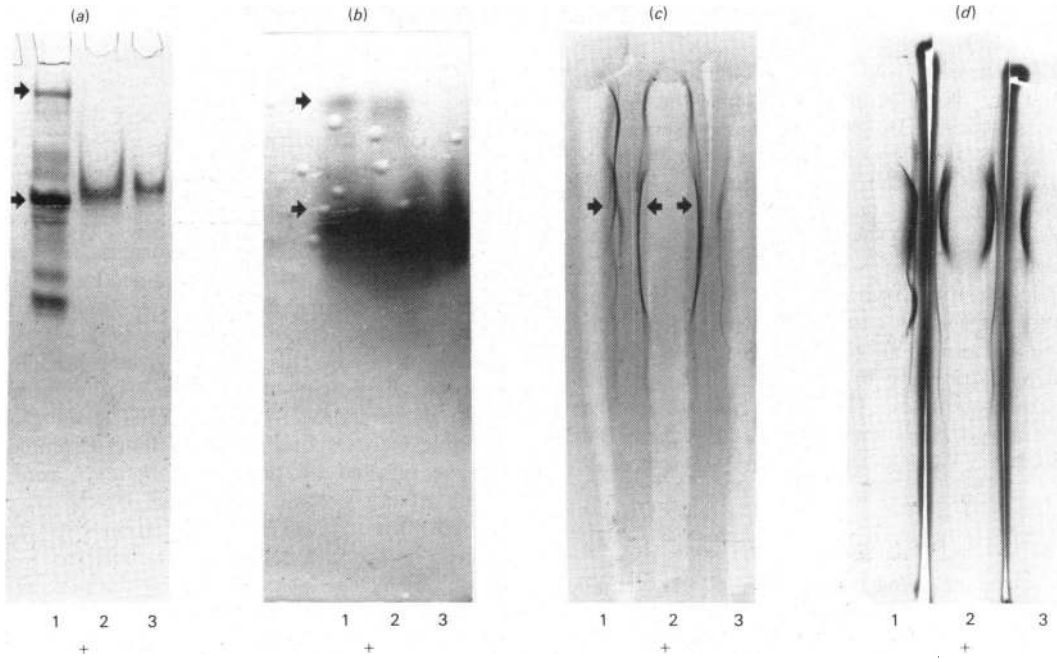

Fig. 1. *Combined polyacrylamide-gel electrophoresis–immunodiffusion from the purification steps of cellobiohydrolase*
Electrophoresis was performed at pH 7.0 in 7.5% polyacrylamide gel, and immunodiffusion at pH 8.2 in agarose gel (*a*) Total protein staining of polyacrylamide gel; (*b*) cellulose-degrading activity on the polyacrylamide gel; (*c*) polyacrylamide embedded in agarose, immunodiffusion against antiserum containing antibodies towards all *T. reesei* proteins, protein staining; (*d*) as (*c*), but with antiserum specific towards cellobiohydrolase. Samples: 1, culture liquid, 50 μg for protein, 4 μg for activity determination; 2, preparation from biospecific-sorption step, 20 μg for protein, 8 μg for activity determination; 3, pure cellobiohydrolase, 20 μg for protein, 12 μg for activity determination. Arrows in (*a*) and (*b*) show the cellulolytically active proteins, and in (*c*) the two immunoarcs corresponding to one protein band in (*a*).

cellulolytic enzymes and has been explained as evidence for synergistic action of cellulolytic enzymes (Wood & McCrae, 1978). The purified cellobiohydrolase did not show any endoglucanase or β-glucosidase activity, and cellobiose was hydrolysed very slightly.

Polyacrylamide-gel electrophoresis (Figs. 1*a* and 1*b*) shows that the culture liquid (sample 1) contains two electrophoretically different cellulolytically active proteins (arrows). These are both sorbed on amorphous cellulose (Figs. 1*b* and 1*c*, sample 2). The specific antiserum used in immunoabsorption (Fig. 1*d*) reacts with only one of them. Thus it is likely that the two cellulolytic enzymes are antigenically different proteins (Nummi *et al.*, 1980, 1981*b*). Figs. 1(*a*) and 1(*c*) demonstrate the great sensitivity of immunoprecipitation in the detection of these proteins. Comparison of samples 1 and 2 (arrows) shows that one protein band in polyacrylamide-gel electrophoresis gives two arcs in immunodiffusion. This means that the single band in fact contains two antigenically different proteins. Thus impure cellulolytic fractions are obtained if purification is based on ionic properties or molecular size only.

The analysis with the specific cellobiohydrolase antiserum (Fig. 1*d*) shows that in culture liquid (sample 1) there is a group of proteins reacting with this antiserum. After the biospecific sorption step two arcs are seen, and after immunoabsorption only one arc is obtained.

The lability of cellobiohydrolase was investigated by incubating the purified enzyme at pH 8.5 at 50°C for 20 h. Such conditions are used, for example, to assay cellulase activity on the gel after electrophoresis. After this treatment, the homogeneous cellobiohydrolase was found to disintegrate into two proteins with different electrophoretic mobilities (Fig. 2). The cellobiohydrolase activity after this treatment was only 35% of that of the untreated cellobiohydrolase.

The pI value of the purified cellobiohydrolase as measured by electrofocusing chromatography was 4.2–3.6. The enzyme is a glycoprotein containing about 10% hexoses, as determined by the anthrone reaction.

The purified cellobiohydrolase shows in sodium dodecyl sulphate/polyacrylamide-gel electrophoresis a single polypeptide band corresponding to M_r approx. 65000.

The hydrolytic properties of preparations from different purification steps were compared in short-term hydrolysis with the use of the same amount of protein for each sample (Tables 2 and 3). The pure cellobiohydrolase hydrolyses amorphous cellulose, producing cellobiose as the only hydrolysis product. It does not attack substituted celluloses, cellohexaose, *p*-nitrophenyl β-glucoside, insoluble xylan, barley β-glucan or starch. Cellobiose is hydrolysed only slightly. The cellobiose-hydrolysing activity corresponds to 5% of that in the culture liquid.

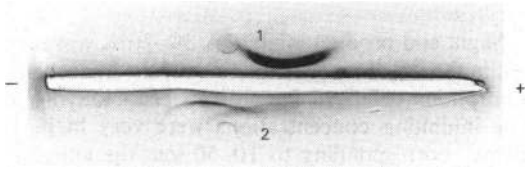

Fig. 2. *Modification of pure cellobiohydrolase at pH 8.5 at 50°C*
Immunoelectrophoresis was performed at pH 8.2 on agarose gel, with antiserum specific for cellobiohydrolase. Samples: 1, 4 μg of pure cellobiohydrolase; 2, 4 μg of modified pure cellobiohydrolase. Protein staining was used.

As shown in Table 2, in short-term (10 min) hydrolysis the purified cellobiohydrolase did not hydrolyse amorphous cellulose as fast as did the preparation after the biospecific sorption step. However, when hydrolysis was extended to longer times or greater cellobiohydrolase amounts were used, the amorphous substrate was completely hydrolysed by the purified cellobiohydrolase alone (Table 4).

The same results were obtained with ball-milled spruce wood (Table 4). The hydrolysis of cotton is indicated by formation of short insoluble fibres and soluble sugars. This hydrolysis is very slow. Only after a hydrolysis time of 10 days did the culture-liquid enzymes and enzymes from the biospecific-sorption step split cotton completely into short fibres. The same result was accomplished in 20 days with the purified cellobiohydrolase. At this point the amount of soluble sugars produced by the purified

Table 3. *Hydrolysis of polysaccharides by cellulolytic enzymes*
For full experimental details see the text.

Substrate	Product analysed	Purification step of cellulolytic enzymes ...	Activity: Culture liquid	Activity: Biospecific sorption	Activity: Immuno-affinity
Soluble starch (15 mg/ml)	Reducing sugars		0.3*	0*	0*
Barley β-glucan (1 mg/ml)	Reducing sugars		680*	280*	0*
Cellohexaose (0.4 mg/ml)	Cellobiose		0*	10*	0*
	Glucose		0*	0*	0*
Soluble CM-cellulose (2.6 mg/ml)	Glucosidic linkage broken		6*	0.9*	0.01*
Insoluble xylan (0.4 mg/ml)	Decrease in particle size		8†	0†	0†

* Activity units: nkat/mg of protein.
† Activity units: Δ (nephelometric value)/min per mg of protein.

Table 4. *Hydrolysis of natural cellulosic materials by cellulolytic enzymes*
For full experimental details see the text.

Substrate	Amounts of enzyme/substrate (mg/mg)	Analysis method	Hydrolysis time (days)	Purification step of cellulolytic enzyme ...	Extent of hydrolysis (%): Culture liquid	Extent of hydrolysis (%): Biospecific sorption	Extent of hydrolysis (%): Immuno-affinity
Amorphous cellulose	0.1/3	Nephelometric	1		82	82	60
			2		83	83	82
Ball-milled spruce wood	0.025/1	Nephelometric	1		41	38	24
			3		44	41	30
Natural cotton	0.2/20	Soluble sugars	1		6	8	3
			20		37	33	16

cellobiohydrolase was still lower than in the other incubation mixtures. However, it is likely that the hydrolysis by the purified enzyme would continue if the hydrolysis were further prolonged.

It has been reported that glucose and cellobiose serve as end-product inhibitors for cellulolytic enzymes of *T. reesei* (Wood & McCrae, 1978; Ladisch *et al.*, 1981). This could not be confirmed with the pure cellobiohydrolase. Inhibition from 10 to 25% was found in the initial hydrolysis velocity of amorphous cellulose when 10–50mM-glucose or -cellobiose was added to the reaction mixture.

Discussion

The purified cellobiohydrolase resembles in its protein properties (glycoprotein nature, pI and M_r) the cellobiohydrolases of *T. reesei* described previously (Berghem *et al.*, 1975; Gum & Brown, 1977). In fact, cellobiohydrolase I (Berghem *et al.*, 1975) (kindly supplied by Dr. G. Pettersson) was found to be immunologically identical with our cellobiohydrolase. The hydrolytic properties of cellobiohydrolase purified in the present work differ, however, from those of cellobiohydrolases described previously (Berghem *et al.*, 1975; Gum & Brown, 1977; Wood & McCrae, 1978; Ladisch *et al.*, 1981). Hydrolysis of amorphous cellulose and ball-milled spruce wood was observed as rapid decrease in the turbidity of the substrate suspension and as liberation of cellobiose. Furthermore, the hydrolysis of native cotton produced short insoluble fibres and minor amounts of cellobiose. Fibre-forming activity is normally attributed to endoglucanases (Halliwell & Riaz, 1970). An endoglucanase of *T. reesei* has been claimed to be responsible for the main hydrolysis of insoluble cellulose (Klyosov *et al.*, 1980). The cellobiohydrolase purified does not correspond to this endoglucanase, since it does not decrease the viscosity of CM-cellulose and does not produce reducing groups from hydroxyethylcellulose.

According to hydrolysis experiments with other substrates, aryl β-glucoside, cellobiose, cellohexaose, barley β-glucan, insoluble xylan and starch, the purified cellobiohydrolase attacks only unsubstituted insoluble polyglucans containing 1,4-β-linkages. It has been previously reported that purified cellulolytic enzymes of *T. reesei* are unable, alone, to hydrolyse native crystalline cotton (Wood & McCrae, 1978). The purified cellobiohydrolase discussed in the present paper clearly attacks native cotton.

Immunological analysis showed that the cellobiohydrolase is sensitive to extreme pH and high temperatures. Depending on the purification procedures used, the original cellobiohydrolase protein can become modified in different ways and partly inactivated. In the purification procedure described in the present paper the cellobiohydrolase seems to be less inactivated than in the preparations obtained with other techniques. The lability of cellobiohydrolase protein indicates that at least some of the isoenzyme forms described with cellobiohydrolase activity may be due to conversion of the native protein. The low rate of hydrolysis by purified cellobiohydrolase could be an indication of a requirement for synergistic action between several enzymes, but also could be an indication of partial inactivation of the original cellobiohydrolase during purification.

Slight end-product inhibition, 10–20%, was observed in the initial reaction velocity of the hydrolysis of amorphous cellulose by purified cellobiohydrolase. The inhibiting concentrations were very high, 10–50mM, corresponding to 10–50-fold the amount of cellulose in the reaction mixture. Thus the end-product inhibition observed in hydrolysis with culture liquids (Wood & McCrae, 1978; Ladisch *et al.*, 1981) seems not to be caused by inhibition of cellobiohydrolase.

T. reesei also produces another enzyme that is sorbed by amorphous cellulose. Addition of this enzyme to the pure cellobiohydrolase does accelerate the hydrolysis of cellulose, but it is not necessary for complete hydrolysis.

The pure cellobiohydrolase isolated does not show any activity towards the substituted celluloses generally used as substrates in the measurement of the activity of cellulolytic enzymes. There are several endoglucanases capable of hydrolysing substituted celluloses produced by the VTT-mutant strain of *T. reesei*, but they do not hydrolyse amorphous cellulose (M. Nummi, M.-L. Niku-Paavola, A. Lappalainen, T.-M. Enari & V. Raunio, unpublished work).

Thanks are due to Mrs. Raija Arnkil for the preparation and analysis of cellodextrins. T.-M. E. is a Research Professor of the Academy of Finland. A. L. is a Research Assistant of the Academy of Finland.

References

Almin, K. E., Eriksson, K.-E. & Jansson, C. (1967) *Biochim. Biophys. Acta* **139**, 248–253

Berghem, L. E. R., Pettersson, L. G. & Axiö-Fredriksson, U.-B. (1975) *Eur. J. Biochem.* **53**, 55–62

Diezel, W., Kopperschläger, G. & Hofmann, E. (1972) *Anal. Biochem.* **48**, 617–620

Enari, T.-M. (1983) in *Microbial Enzymes and Biotechnology* (Fogarty, W. M., ed.), pp. 183–223, Applied Science Publishers, London

Enari, T.-M., Niku-Paavola, M.-L. & Nummi, M. (1981) in *Proc. Bioconversion Biochem. Eng. Symp.* (Ghose, T. K., ed.), vol. 1, pp. 87–95, Indian Institute of Technology, New Delhi

Gum, E. K., Jr. & Brown, R. D., Jr. (1977) *Biochim. Biophys. Acta* **492**, 225–231
Håkansson, U., Fägerstam, L. G. & Pettersson, L. G. (1979) *Biochem. J.* **179**, 141–149
Halliwell, G. & Riaz, M. (1970) *Biochem. J.* **116**, 35–42
Klyosov, A. A., Sinitsyn, A. P. & Rabinowitch, M. L. (1980) *Enzyme Eng.* **5**, 153–165
Ladisch, M. R., Hong, Y., Voloch, M. & Tsao, G. T. (1981) *Basic Life Sci.* **18**, 55–83
Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
Livingston, D. M. (1974) *Methods Enzymol.* **34**, 723–731
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
Mancini, G., Carbonara, A. O. & Heremans, J. F. (1965) *Immunochemistry* **2**, 235–254
Maurer, H. R. (1968) *Disk-Elektrophorese*, W. de Gruyter, Berlin
Nummi, M., Niku-Paavola, M.-L., Enari, T.-M. & Raunio, V. (1980) *FEBS Lett.* **113**, 164–166
Nummi, M., Fox, P. C., Niku-Paavola, M.-L. & Enari, T.-M. (1981*a*) *Anal. Biochem.* **116**, 133–136
Nummi, M., Niku-Paavola, M.-L., Enari, T.-M. & Raunio, V. (1981*b*) *Anal. Biochem.* **116**, 137–141
Storvick, W. O., Cole, F. E. & King, K. W. (1963) *Biochemistry* **2**, 1106–1110
Whistler, R. L. & Wolfrom, M. L. (1962) *Methods Carbohydr. Chem.* **1**, 390
Wood, T. M. & McCrae, S. I. (1978) *Biochem. J.* **171**, 61–72