Arch Microbiol (1990) 154:150–155

Archives of
Microbiology

Isolation of a β-glucosidase binding and activating polysaccharide from cell walls of *Trichoderma reesei*

Robert Messner, Karl Hagspiel, and Christian P. Kubicek

Institut für Biochemische Technologie und Mikrobiologie, Abteilung für Mikrobielle Biochemie, Getreidemarkt 9/1725, A-1060 Wien, Austria

Received December 7, 1989/Accepted February 10, 1990

Abstract. The extracellular β-glucosidase from the filamentous fungus *Trichoderma reesei* QM 9414 is mainly bound to the cell wall of the fungus and only partially released into the medium. Isolation of the cell walls and its hydrolysis by enzymatic treatment with *Aspergillus niger* cellulase released β-glucosidase, which appeared tightly associated with a cell wall polysaccharide. This polysaccharide was purified by gel filtration and ion exchange chromatography and was shown to consist of mannose, galactose, glucose, galacturonic acid and glucuronic acid. It was devoid of protein and phosphate. It reassociated both with extracellular β-glucosidase as well as β-glucosidase released from the fungus' cell wall. Addition of the polysaccharide to the β-glucosidase in vitro increased the enzyme's activity against 4-nitrophenyl-β-glucoside twofold. These findings suggest, that the isolated polysaccharide functions as an "anchor glycan" for the β-glucosidase in *Trichoderma reesei.*

Key words: *Trichoderma reesei* – β-Glucosidase – Polyheteroglycan – Cell wall – Enzyme activation – Reassociation

The filamentous fungus *Trichoderma reesei* is probably the most potent producer of cellulases currently known, with respect to the final amount of secreted protein (Mandels 1985) and its ability to break down cellulose synergistically (Coughlan and Ljungdahl 1988).

The bottleneck in the use of several commercial cellulase preparations for the production of glucose is the relatively low amount of β-glucosidase (Sternberg et al. 1977; Wood and Wiseman 1982). Attempts to improve the β-glucosidase content of *Trichoderma* cellulases have been made by the addition of β-glucosidase from other microorganisms (Enari et al. 1981; Sternberg 1976) or by isolation of mutants with increased rates of β-glucosidase formation (Kawamori et al. 1986a; Tagnu et al. 1981). The reason of this poor secretion of β-glucosidase into the culture fluid of *T. reesei* is that 50–100% of the β-glucosidase remains cell wall bound during cultivation (Kubicek 1981). The molecular basis of this "trapping" by the cell walls is not satisfactorily understood; release of β-glucosidase into the medium is enhanced in the presence of increased activities of β-1,3-glucanase, an enzyme responsible for cell wall turnover (Kubicek 1982, 1983a) or decreased cell wall β-1,3-glucan upon growth of *T. reesei* on sorbose (Kubicek 1983b; Bisaria et al. 1986; Nanda et al. 1986; Kawamori et al. 1985, 1986b). Whether or not a cell wall glucan is the actual place where β-glucosidase is bound, or whether its degradation facilitates subsequent extraction of the enzyme is not yet known.

We have recently described the release of β-glucosidase from *T. reesei* cell walls by an *Aspergillus niger* hydrolase mixture on a scale sufficient for characterization of the released enzyme (Messner and Kubicek 1990a). Here we describe the isolation of the β-glucosidase binding cell wall component, its association with and activation of β-glucosidase from *T. reesei.*

Materials and methods

Maintainance and growth of organism

Trichoderma reesei QM9414 (ATCC 26921) was maintained on malt agar slants. Submerged cultivations were performed as described earlier (Messner et al. 1988). Induction of β-glucosidase formation was carried out in a resting-cell system containing 0.7% (w/v) O-methyl-β-D-glucopyranoside according to Sternberg and Mandels (1982) using 22 h old mycelium pregrown on glucose.

Radiolabeling of mycelium

Specific labeling of cell wall components was performed according Messner and Kubicek (1990a). It must be noted, that for optimal recovery of ^{35}S-methionine labeled wall bound β-glucosidase, the

Offprint requests to: R. Messner

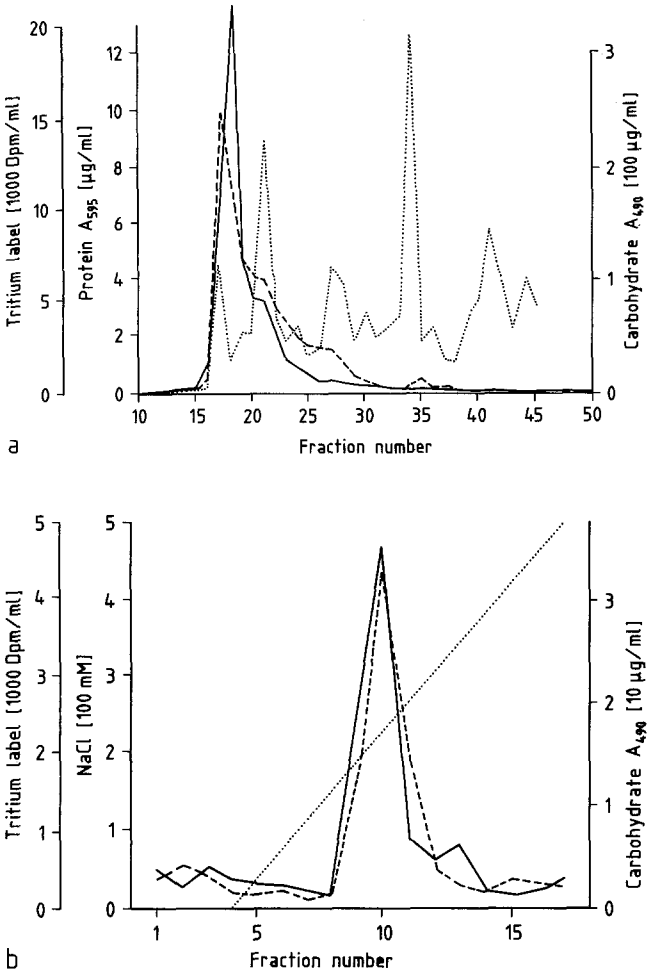


Fig. 1. A Gel filtration on Superose 12 of a digest by cellulase of *Aspergillus niger* of ^{3}H-glucose labeled cell wall of *Trichoderma reesei*. Conditions are described in the text. **B** Mono P anion exchange chromatography of the single carbohydrate peak from the gel filtration given in **A**. Conditions are described in the text

mycelium had to be at least 2 days old. For optimal silver staining or Western blotting of β-glucosidase, however, mycelium of 20–24 h age was used.

Cell wall fractionation

Chemical separation of purified cell walls was performed basically as described by Mahadevan and Tatum (1965), as modified by Messner and Kubicek 1990b).

Preparation of wall bound β-glucosidase

Purified cell walls were obtained from mycelia of different age (22–50 h) as described above and subjected to enzymatic hydrolysis. About 1 g cell walls (wet weight) was homogenously suspended in 2 volumes of sodium citrate buffer (50 mM, pH 5.0, 0.1 mg/ml final concentration of chloramphenicol from ethanolic stock solution) and digested by incubation with *Aspergillus niger* cellulase [Sigma Chemical Co., 1% (w/v), final concentration] for 46–52 h at 37° C. Suspensions were spun down in a bench top centrifuge (4° C, 15 min, 12000 *g*) and supernatants dialyzed exhaustively (3 times for 12 h at 4° C) against 25 mM imidazole buffer, pH 8.0.

The total volume of dialyzed digest was loaded on a Mono P anion axchanger (HR, size 0.5 × 20 cm), connected to an FPLC-apparatus (Pharmacia LKB Biotechnology), which had previously been equilibrated in 25 mM imidazole buffer, pH 8.0. A flow rate of 0.5 ml/min was used. Fractions of 1 ml were collected, lyophilized and stored at −20° C until analysis.

Preparations of β-glucosidase binding carbohydrate

Cell walls from 50 h old mycelium were purified and digested as described above. The supernatant obtained after centrifugation was membrane filtered (0.2 µm) instead of dialysis and subjected to gel filtration on Superose 12 (HR 16/50, V_t = 100 ml, Pharmacia), previously equilibrated with 50 mM imidazole buffer, pH 8.0 and operated by the FPLC-apparatus at a flow rate of 0.5 ml/min, 2 ml fractions were collected. The three fractions at 36% of V_t, which exhibited the highest carbohydrate content were diluted by 1 vol. of distilled water and subjected to Mono P anion exchange chromatography as described above. After 12 ml elution by the starting buffer, a linear salt gradient was applied (0–0.5 M NaCl in 32 ml). Fractions of 3 ml were collected and analyzed for carbohydrate. Positive fractions were lyophilized.

SDS-PAGE, Western blot and fluorography

SDS-PAGE/Western blotting and immunostaining was performed as described by Kubicek et al. (1987). Goat anti mouse IgG, coupled to alkaline phosphatase (Accurate Chemicals & Scientific Corp., USA) was used as second antibody. Silver staining of SDS-PAGE was done according Merril et al. (1981); radiolabeled proteins and carbohydrates were identified by fluorography described by Kubicek (1987).

Enzyme activity assays

Activity of β-glucosidase against 4-nitrophenyl-β-D-glucopyranoside (PNPG) was measured as described by Messner and Kubicek (1990b).

Chemical cell wall analysis

The concentration of protein was measured by the dye-binding-method (Bradford 1976). Total carbohydrate content was quantified b a miniaturized version of the phenol-sulfuric acid method (Dubois et al. 1966): 150 µl sample, 150 µl phenol in water (5%, w/v) and 900 µl H_2SO_4 (98%, w/v) were mixed in a 12 ml glass tube. After 15 min, the absorbance was read at 490 nm. Soluble starch was used for calibration. Hexuronic acid analysis was done according Chaplin (1986) and inorganic phosphate was measured after complete hydrolysis (parameters see below, TCL analysis) with the aid of the ammonium molybdate method (Ames 1966).

TLC analysis

Polysaccharides were desalted with the aid of PD 10 columns (0.8 × 5 cm, Pharmacia), using distilled water as a solvent. They were thereafter lyophilized and subsequently hydrolyzed in 0.1 N HCl at 110° C. Complete hydrolysis was achieved within 4 h. Thereafter samples were desiccated over solid KOH, dissolved in distilled water again and subjected to TLC on silica gel 60 (Merck). Plates were developed 2 times in acetone:n-butanol:water (5:4:1, v/v/v) for separation of monosaccharides (Hotta and Kurokawa 1968). For detection of total carbohydrate spots plates were treated with

a phenol-sulfuric acid spray reagent (Der Marderosian et al. 1968). For detection of reducing sugars, sugar acids and sugar alcohols the benzidine metaperiodate spray reagent was used (Badger et al. 1963).

GLC analysis of carbohydrate constituents

For recovery of neutral monosaccharides, the glycan – after anion exchange chromatography and concentration by lyophilization – was hydrolyzed in the presence of 200 mg/ml Dowex 50 cation exchanger (previously equilibrated in 1 N HCl and washed until neutral). Hydrolysis was allowed to proceed for 8 h at 110° C in Reacti-Vials (Pierce Chemical Co., Illinois). Thereafter, the suspensions were reacted with an equal amount of Dowex 1 anion exchanger (equilibrated in 1 N Na_2CO_3 and washed neutral) for 30 min at room temperature. The final supernatants were lyophilized and the sugars released were derivatized for GLC analysis; aliquots of maximal 100 µg were mixed with 200 µl reducing agent (12 mg/ml $NaBH_4$ in 1 M NH_4OH, freshly prepared) and incubated at 28° C for 2 h. The reaction was stopped by addition of 200 µl 4 N acetic acid, the samples lyophilized and the boric acid formed removed by the addition and subsequent evaporation of 1 ml methanol (repeated five times). The corresponding sugar alcohols were peracetylated in Reacti-Vials, containing 100 µl pyridine and 100 µl acetanhydride, for 30 min at 110° C. The acetylation reagent was removed in vacuo at 50° C and after resolving in chloroform the samples were filtrated through fine glass wool. The solvent was then again completely removed and the final residue taken up in 50 µl pyridine. All solvents used in this procedure had been from Merck (LiChrosolv HPLC purity grade).

The GLC used was a Model 437A from United Technology Packard equipped with a flame ionization detector and a Fused Silica Capillary Column CP-Sil 8 CB (25 m × 0.32 mm). Peak areas were integrated by an 3390A integrator (Hewlett Packard). Carrier gas was nitrogen 99.999%, flow rate 1.7 ml/min, detector temperature 200° C, injection block 230° C and temperature program was 160° C:2° C/min oven rise:190° C – 5 min hold. 1 µl of the sample was injected and splitted approximately 1:60.

For recovery of neutral and charged sugar monomers the polysaccharide was hydrolyzed as described above for TLC-analysis, and then subjected to derivatization for GLC after removal of hydrochloric acid.

Reassociation studies

Solutions of β-glucosidase as well as radiolabeled glycan were equilibrated in 20 mM sodium citrate buffer, pH 5.0 on PD-10 columns (Pharmacia) and lyophilized. They were then redissolved in an appropriate volume of water to give a final citrate concentration of 100 (± 10 mM) citrate. Individual aliquots of glycan were supplied with different amounts of β-glucosidase and diluted to a total volume of 100 µl. Reassociation was allowed to proceed at 30° C for 6 h. The whole sample was then subjected to SDS-PAGE and areas of interest cut out. Radioactive material was eluted from the gel slices by incubating each of them in 3 ml elution buffer [1 M glycine, 3% (w/v) SDS, pH 8.8 per Tris] at 100° C for 18 h. The eluted label was measured in 15 ml scintillation cocktail as recommended by the manufacturers of the liquid scintillation counter (1219 Rackbeta; LKB, Bromma, Sweden).

In vitro activation of β-glucosidase

Extracellular β-glucosidase was purified from 0.5 ml Celluclast (Novo Industri A/S, Denmark) after two times of desalting on PD 10 columns (Pharmacia) by anion exchange chromatography as described above. The "anchor glycan" was also prepared as given above and desalted on PD 10 columns (Pharmacia) against the buffer. Immediately before use the glycan was heated up to 100° C for 20 min in order to deactivate still attached, active traces of β-glucosidase.

The enzyme assay included in a total volume of 100 µl of 50 mM sodium citrate buffer, pH 5.0:3 µg β-glucosidase preparation; various amounts of the substrate PNPG; and freshly deactivated glycan. The enzyme reaction was stopped after 10 min of incubation at 50° C by addition of 100 µl 1 M sodium carbonate and diluted by 1.0 ml distilled water for photometric measurement at 405 nm.

Results

Comments on the procedure for isolation of the glycan

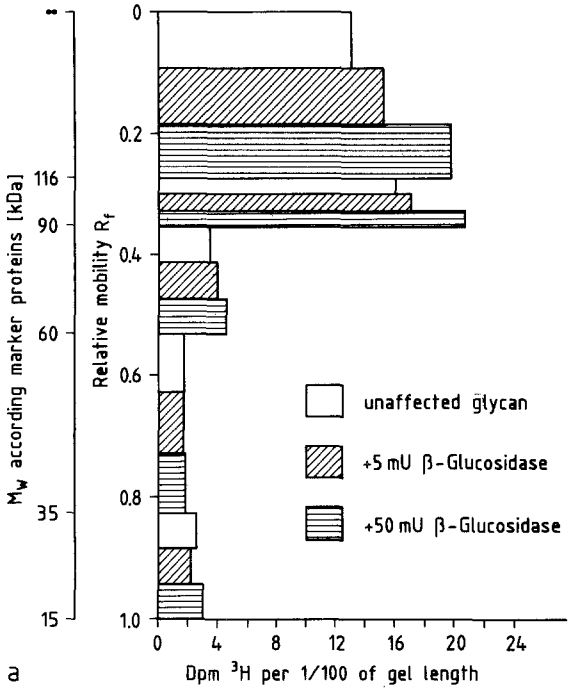
During our previous studies on the isolation of the cell wall bound β-glucosidase from *Trichoderma reesei*, several attempts to demonstrate the emzyme by electrophoretic methods failed. In the course of this work, it was realized that this was due to masking of the enzyme by a polysaccharide, which led to broad smears not reacting with antibodies (unpublished results). We have supposed therefore, that this polysaccharide may strongly bind to β-glucosidase. In the present study we have therefore purified it (see Materials and methods) and tested this possibility.

The elution profile of this glycan during Superose 12 (Fig. 1A) and Mono P (Fig. 1B) chromatography was shown to be identical, irrespective of the carbon source used to grow the mycelium for cell wall preparation: no differences with respect to area, position, geometry and number of peaks had been noticed, when the mycelium used had been grown on cellobiose, lactose and cellulose for 2 days, on lactose for 4 days or when it had been induced for β-glucosidase formation with β-methyl-glucoside for 14 h.

Some properties of the "anchor carbohydrate"

The carbohydrate, isolated as described above, yields a single, symmetrical peak in analytical gel filtration, but exhibits a slightly smaller size than the unpurified glycan, which still contains β-glucosidase and *Aspergillus niger* hydrolases (38% of V_t instead of 36%, Superose 12). Its monomere composition after hydrolysis was checked by chemical standart methods and assessed by both TLC and GLC analysis as follows (mol %): 45 (± 5) % mannose, 30 (± 3) % galactose, 11 (± 3) % glucose, 9 (± 4) % galacturonic acid and 5 (± 3) % glucuronic acid. The glycan exhibits high stability against several hydrolase systems, such as cellulase of *A. niger*, "lysing enzymes" (Sigma), "lyticase" (Sigma), laminarinase from *Arthrobacter sp.*, gamanase or promozym (Novo Industri A/S, Denmark). Only Novozym 234 was able to degrade the glycan, albeit at a very low rate. Its degradation was not inhibited by 600 µM δ-gluconolactone or 150 µM nojirimycin, which inhibits exo-β-glucanases.

The presence of low amounts of protein (1.4 ± 1.0%, w/w) was assessed whereas phosphate appears to be below the detection limit (< 0.3%, w/w).



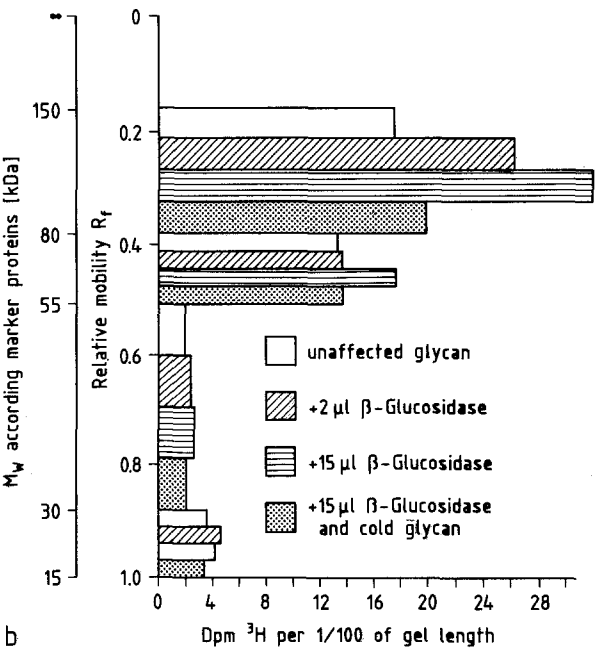


Fig. 2A, B. Reassociation of ^{3}H-glucose-labeled "anchor glycan" with (**A**) purified extracellular β-glucosidase (**A**) and wall released β-glucosidase (**B**) of *T. reesei*. The shift of label distribution, caused by the presence of β-glucosidase with respect to the relative mobility. Migration is given in % of total gel length × 100. Both β-glucosidase used for these experiments were purified by anion exchange chromatography as described in Material and methods

Reassociation of β-glucosidase and "anchor glycan"

In order to find out, whether binding between β-glucosidase and the "anchor glycan" might be reversible, β-glucosidase was mixed with glycan in vitro and the formation of a stable complex investigated by SDS-PAGE.

The pattern of migration of ^{3}H-labeled glycan in SDS-PAGE is clearly altered by the presence of either extracellular (Fig. 2A) or wall-released β-glucosidase (Fig. 2B). Migration of the pure glycan in SDS-PAGE is probably due to the negative charge of its uronic acid constituents. The simultaneous application of glycan and β-glucosidase retards the migration of the protein and accelerates that of the glycan, indicating their association. The specifity of this reassociation assay was ascertained by quenching the "shifting" effect by the addition of a 15-fold excess of unlabeled glycan (Fig. 2B). This quenching was not seen with various other polysaccharides (unpublished findings).

Activation of extracellular β-glucosidase by the "anchor glycan"

To answer the question, whether β-glucosidase binding to the glycan is due to a substrate-recognizing-mechanism, the effect of the addition of "anchor glycan" on the activity of β-glucosidase was investigated. It was found, that the enzymatic activity of extracellular β-glucosidase from *T. reesei* against PNPG is enhanced in the presence or purified glycan.

The activation (Table 1) adds up to 150% to the initial enzyme activity as estimated by double inverse plotting of relative enzyme velocity against glycan concentration. Most noteworthy, a similar activation is achieved by addition of yeast mannan, but not by other polysaccharides (unpublished results).

Table 1. Activation of β-glucosidase by the "anchor glycan". The activity is given in mU/ml at 50°C and pH 5.0

Substrate concentration [mM] PNPG	Glycan concentration [µg/ml]					
	0	36	109	364	909	1820
3	1363	1385	1490	1958	2171	2603
1.5	1186	1308	1444	1935	2198	2516
0.7	1097	1294	1354	1553	1653	1785

Discussion

Although the binding of fungal, extracellular enzymes to the cell wall is a frequent observation, the responsible cell wall polymer acting as receptors have not yet been identified. Data in favour of a β-glucan (Dickerson and Baker 1979; Chang and Trevithick 1972; Kubicek 1981), a "binding protein" (Yabuki and Fukui 1970) and a galactosamine containing polymer (Gratzner 1972) have been published. However, binding "in vitro" has so far not been demonstrated. The present data are the first report on an isolation of a cell wall polymer, which strongly binds β-glucosidase. Chemical analysis of the polymer suggests that it is a heteroglycan consisting of

mannose, glucose and galactose and which owes its negative charge to the presence of glucuronic and galacturonic acid. Since β-glucosidase is a positively charged protein (IP of 8.7), it is tempting to speculate that the negative charge of the heteroglycan could be responsible for its ability to bind the enzyme.

Extracellular polysaccharides from filamentous fungi often consist of neuronal sugars and amino sugars (Reissig and Glasgow 1971; Ruperez and Leal 1981; Gomez-Miranda and Leal 1981) but rarely contain uronic acids. The glycan isolated by us is quite similar to glycans from *Neurospora crassa* (Hiura et al. 1983; Nakajima et al. 1984a, b), wall fraction I of *N. crassa* (Cardemil and Pincheira 1979; Silva and Pincheira 1984) and *Penicillium erythromellis* (Ruperez and Leal 1987) and resembles closely the acidic heteropolysaccharide of *Aureobasidium pullulans* with respect to sugar composition (Brown and Lindberg 1967). In this paper, no attempts were made to assess the type of linkage of the *Trichoderma reesei* polymer; further methods, which enable the determination of the structure of the intact heteroglycan (i.e. NMR and MS) are required for determining the correct structure and linkage type of this carbohydrate.

Concerning the relation of the heteroglycan to the cell wall structure, it can be assumed that the glycan is a constitutive fragment of the cell wall, because different culture conditions do not alter its occurrence or properties. The amount of "anchor glycan" isolated from intact walls is 5 times the amount of that, which can be obtained by enzymatic hydrolysis of the cell wall, from which fraction I has already been removed. Hence it is obviously mainly a part of the alkali-soluble fraction I of cell walls. These findings are paralleled by the fact that β-glucosidase can also be detected immunologically in extracts of fraction I. Nevertheless, it must be borne in mind that a concomitant solubilization of wall fraction I and the heteroglycan does not necessarily mean that both polymers are associated with each other in the intact native cell wall.

A most interesting finding obtained was the ability of the heteroglycan to activate β-glucosidase. This shows that β-glucosidase does not bind to the polymer *via* an involvement of its active center, since in this case a competitive type of inhibition may have been anticipated. The very similar activation by yeast cell wall mannan in contrast to other soluble and insoluble polysaccharides suggests that the mannan residues in the heteroglycan may be responsible for this effect. Such an assumption does not necessarily contradict our hypothesis that the uronic acids are responsible for binding to β-glucosidase, since the carbohydrate chains may interact with more than one epitope on β-glucosidase. It is possible that by the binding of the glycan to the β-glucosidase, the polysaccharide alters the conformation of the enzyme in such a way as to render the active center better accessible to its substrates. Activation and stabilization of products by covalently bound carbohydrates has frequently been observed (Eylar 1965; Hayashida and Yoshioka 1979; Mizunaga et al. 1983). However, the fact that a protein can be activated by non-covalent association with a polysaccharide has not yet been reported. This observation may be of interest in the application of several industrial enzymes.

Acknowledgements. This work was supported by the Fonds zur Förderung Wissenschaftlicher Forschung (project P 6140). R.M. is thankful to the Bundesministerium für Wissenschaft und Forschung for a research grant.

References

Ames BN (1966) Assay of inorganic phosphate, total phosphate and phosphatases In: Colowick SP, Kaplan NO (eds) Methods in enzymology. Academic Press, New York London, pp 115–118

Badger GM, Donnelly JK, Spotawood TM (1963) Thin-layer chromatography using partially acetylated cellulose as adsorbent. J Chromatogr 10:97–98

Bisaria VS, Nanda M, Ghose TG (1986) Effect of L(−)sorbose on the release of β-glucosidase by *Trichoderma reesei* QM9414. J Gen Microbiol 132:973–978

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. Anal Biochem 72:248–254

Brown RG, Lindberg B (1967) Polysaccharides from cell walls of *Aureobasidium* (Pullularia) *pullulans*. II. Heteropolysaccharide. Acta Chem Scand 21:2383–2389

Cardemil L, Pincheira G (1979) Characterization of the carbohydrate component of fraction I in the *Neurospora crassa* cell wall. J Bacteriol 137:1067–1072

Chang LY, Trevithick JR (1972) Release of wall-bound invertase and trehalase in *Neurospora crassa* by hydrolytic enzymes. J Gen Microbiol 70:13–22

Chaplin MF (1986) Monosaccharides. In: Chaplin MF, Kennedy JF (eds) Carbohydrate analysis a practical approach. IRL Press, Eynsham, Oxford, pp 5–6

Coughlan MP, Ljungdahl LG (1988) Comparative biochemistry of fungal and bacterial cellulolytic enzyme systems In: Aubert JP, Beguin P, Millet J (eds) Biochemistry and genetics of cellulose degradation. Academic Press, San Diego, CA, pp 11–30

Der Marderosian AH, Pinkley HV, Dobbins MF (1968) Psychophotominetics and their abuse. Am J Pharm 140:83–96

Dickerson AG, Baker RCF (1979) The binding of enzymes to fungal β-glucans. J Gen Microbiol 112:67–75

Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1966) Colorimetric method for determination of sugars and related substances. Anal Chem 28:350–356

Enari TM, Niku-Paavola M-L, Harju L, Lappalainen A, Nummi M (1981) Purification of *Trichoderma reesei* and *Aspergillus niger* β-glucosidase. J Appl Biochem 3:157–163

Eylar EH (1965) On the biological role of glycoproteins. J Theor Biol 10:89–113

Gomez-Miranda B, Leal JA (1981) Extracellular and cell wall polysaccharides of *Aspergillus alliaceus*. Trans Br Mycol Soc 76:249–253

Gratzner HG (1972) cell wall alterations associated with the hyperproduction of extracellular enzymes in *Neurospora crassa*. J Bacteriol 111:443–446

Hayashida S, Yoshioka H (1979) The role of carbohydrate moiety on thermostability of cellulase from *Humicola insolens* YH-8. Agr Biol Chem 44:481–487

Hiura N, Nakajima T, Matsuda K (1983) Two cell wall β-D-glucans from *Neurospora crassa*. Agr Biol Chem 47:1317–1322

Hotta K, Kurokawa M (1968) Separation of fucose and actylhexosamines by two-dimensional thin-layer chromatography. Anal Biochem 26:272–274

Kawamori M, Morikawa Y, Takasawa S (1985) Inductive formation of cellulases by L-sorbose in *Trichoderma reesei*. Appl Microbiol Biotechnol 22:235–236

Kawamori M, Ado Y, Takasawa S (1986a) Preparation and application of *Trichoderma reesei* mutants with enhanced β-glucosidase. Agr Biol Chem 50:2477–2482

Kawamori M, Morikawa Y, Takasawa S (1986b) Induction and production of cellulases by L-sorbose in *Trichoderma reesei*. Appl Microbiol Biotechnol 24:449–453

Kubicek CP (1981) Release of carboxymethyl-cellulase and β-glucosidase from cell walls of *Trichoderma reesei*. Eur J Appl Biotechnol 13:226–231

Kubicek CP (1982) β-Glucosidase excretion by *Trichoderma pseudokoningii*, correlation with wall bound β-1,3-glucanase activities. Arch Microbiol 132:349–354

Kubicek CP (1983a) β-Glucosidase excretion in *Trichoderma* strains with different cell wall bound β-1,3-glucanase activities. Can J Microbiol 29:163–169

Kubicek CP (1983b) Influence of L-sorbose on the location of β-glucosidase in *Trichoderma pseudokoningii*. FEMS Microbiol Lett 20:285–288

Kubicek CP (1987) Involvement of a conidial endoglucanase and a plasma membrane bound β-glucosidase in the induction of endoglucanase synthesis by cellulose in *Trichoderma reesei*. J Gen Microbiol 133:1481–1487

Kubicek CP, Panda T, Schreferl-Kunar G, Gruber F, Messner R (1987) O-linked but not N-linked glycosylation is necessary for the secretion of endoglucanases I and II by *Trichoderma reesei*. Can J Biol 33:698–703

Mahadevan PR, Tatum EL (1965) Relationship of the major constituents of the *Neurospora crassa* cell wall to wild-typ and colonial morphology. J Bacteriol 90:1073–1081

Mandels M (1985) Applications of cellulases. Biochem Soc Trans 13:414–416

Merril CR, Goldman D, Sedman SA, Ebert MH (1981) Ultrasensitive stain for protein in polyacrylamid gel shows variation in cerebrospinal fluid proteins. Science 221:1437–1441

Messner R, Gruber F, Kubicek CP (1988) Differential regulation of synthesis of multiple forms of specific endoglucanases by *Trichoderma reesei* QM9414. J Bacteriol 170:3689–3693

Messner R, Kubicek CP (1990a) Synthesis of cell wall glucan, chitin and protein by regenerating protoplasts and mycelium of *Trichoderma reesei*. Can J Microbiol (in press)

Messner R, Kubicek CP (1990b) Evidence for a single, specific β-glucosidase in cell walls from *Trichoderma reesei* QM 9414. Enz Microb Technol (in press)

Mizunaga T, Oshida T, Takasaki A, Maruy Y (1983) Function of mannan chains of yeast repressible acid phosphatase on its enzymatic properties. Agr Biol Chem 48:1445–1450

Nakajima T, Yoshida M, Hiura N, Matsuda K (1984a) Structure of the cell wall proteogalactomannan from *Neurospora crassa*. I. Purification of the proteoheteroglycan and characterization of the alkali-labile oligosaccharides. J Biochem 96:1005–1011

Nakajima T, Yoshida M, Hiura N, Matsuda K (1984b) Structure of the cell wall proteogalactomannan from *Neurospora crassa*. II. Structural analysis of the polysaccharide part. J Biochem 96:1013–1020

Nanda M, Bisaria VS, Ghose TK (1986) Effect of L(−)sorbose on the release of β-glucosidase from *Trichoderma reesei* QM 9414. J Gen Microbiol 132:973–978

Reissig JL, Glasgow JE (1971) Mucopolysaccharide, which regulates growth in *Neurospora*. J Bacteriol 106:882–889

Ruperez P, Leal JA (1981) Extracellular galactosaminogalactan from *Aspergillus parasiticus*. Trans Br Mycol Soc 77:621–625

Ruperez P, Leal JA (1987) Mannoglucogalactane from the cell walls of *Penicillium erythromellis*: Isolation and partial characterization. Carbohydr Res 167:269–278

Silva M, Pincheira G (1984) Genetic control of a structural polymer of the *Neurospora crassa* cell wall. J Bacteriol 158:1104–1108

Sternberg D (1976) β-Glucosidase of *Trichoderma*: Its biosynthesis and role in saccharification of cellulose. Appl Environ Microbiol 31:648–654

Sternberg D, Mandels GR (1982) β-Glucosidase induction and repression in the cellulolytic fungus *Trichoderma reesei*. Exp Mycol 6:115–124

Sternberg D, Vijayakumar P, Reese ET (1977) β-Glucosidase: microbiol production and effect on enzymatic hydrolysis of cellulose. Can J Microbiol 23:139–147

Tagnu SK, Blanch HW, Charles RW (1981) Enhanced production of cellulase, hemicellulase and β-glucosidase by *Trichoderma reesei* (Rut C-30). Biotechnol Bioeng 23:1837–1849

White CA, Kennedy JF (1986) Oligosaccharides. In: Chaplin MF, Kennedy JF (eds) Carbohydrate analysis a practical approach. IRL Press, Eynsham, Oxford, pp 40–42

Woodwars J, Wiseman A (1982) Fungal and other β-glucosidases – their properties and applications. Enz Microb Technol 4:73–79

Yabuki M, Fukui S (1970) Presence of binding site for α-amylase and of masking protein for this site on mycelial cell wall of *Aspergillus oryczae*. J Bacteriol 104:138–144