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Effects of tunicamycin on secretion and enzymatic activities of cellulase from *Trichoderma reesei*

Hannele Merivuori, Jeffrey A. Sands, and Bland S. Montenecourt

Department of Biology and Biotechnology Research Center
Lehigh University, Bethlehem, PA 18015, USA

Summary. The effects of tunicamycin, an inhibitor of N-asparagine linked glycosylation, on the synthesis, secretion, and activities of the cellulases produced by *Trichoderma reesei* wild type QM6a and hypersecreting mutant RL-P37 were studied. Neither the level of secreted cellulase nor the total amount of secreted protein was affected by the drug at a concentration (5 μg/ml) that slightly inhibited growth. SDS-polyacrylamide gel electrophoretic mobilities of proteins secreted during growth in tunicamycin were similar to those of proteins from control cultures that had their N-linked oligosaccharides removed by endoglycosidase H. Isoelectric focusing patterns of secreted proteins were also altered by growth in the presence of tunicamycin. All of the bands stained with Schiff's reagent, indicating that the secreted cellulases contained O-linked oligosaccharides in addition to N-linked sugars. Endoglucanase activity in culture broths from tunicamycin grown mycelia was more thermolabile and protease-sensitive than the same activity from control cultures. Thus, N-asparagine linked oligosaccharides do not appear to be necessary for *T. reesei* cellulase secretion or activity, but do seem to contribute to the stability of the enzymes. The role of O-linked oligosaccharides is being investigated.

Introduction

The cellulases produced by the filamentous fungus *Trichoderma reesei* consist of a complex mixture of enzymes that degrade cellulose into soluble oligosaccharides and glucose (Gong et al. 1978; Gritzali and Brown 1979). Enzymes involved in the cellulase complex are (a) endo-β-(1,4)-glucanases (EC 3.2.1.4), which cut randomly the β-(1,4)-linkages between glucose residues in cellulose, (b) (1,4)-β-D-glucan cellobiohydrolases (EC 3.2.1.91), which are exo-β-(1,4)-glucanases and cleave cellobiosyl units from the non-reducing ends of oligosaccharides and (c) (1,4)-β-glucosidases (EC 3.2.1.21), that cleave cellobiose into glucose. The precise mechanism of action of these enzymes is not clearly understood but it is generally accepted that endoglucanases and cellobiohydrolases act synergistically to convert crystalline cellulose into cellodextrins and cellobiose. These soluble cellulose derivatives are then converted to glucose by the action of β-glucosidases (Montenecourt 1983, a review).

The cellulases of *Trichoderma reesei* have undergone extensive study in view of their potential application in the conversion of renewable biomass to fermentable sugars. Many of the enzymes in the cellulase complex have been purified to homogeneity (Håkansson et al. 1979; Fägerstam and Pettersson 1979; Shoemaker et al. 1983). The N-terminal amino acid sequences of CBH I, CBH II and ENDO II were reported several years ago (Pettersson et al. 1981) and recently the entire amino acid sequence of CBH I has been elucidated (Fägerstam et al. 1984). Additionally, the gene for CBH I has been cloned into *Escherichia coli* (Shoemaker et al. 1983; Teeri et al. 1983) and the regulatory regions, intervening sequences and structural regions were sequenced (Shoemaker et al. 1983). Numerous reports have indicated that the cellulases of *Trichoderma* occur as multiple forms in the culture supernatant and the major enzymes have been shown to be glycoproteins (Fägerstam et al. 1984; Kubicek 1981; Sheir-Neiss and Montenecourt 1984; Shoemaker and Brown 1978).

Offprint requests to: B. S. Montenecourt

Although considerable headway has been made in understanding cellulases at the molecular level, little is known of the sequence of events involved in the active secretion of these extracellular glycoprotein enzymes. Recent studies (Fägerstam et al. 1984; Sheir-Neiss and Montenecourt 1984) have shown that all of the detectable cellulases secreted by the wild type QM6a, QM 9414 and the hypersecreting mutant RL-P37 are glycoproteins. Peptide analyses of CBH I from *T. reesei* mutant QM 9414 have suggested that this enzyme contains both N-asparagine linked and O-glycosidically linked carbohydrate (Fägerstam et al. 1984). Presumably, the endoglucanases and β-glucosidases contain similar carbohydrate linkages. We have initiated studies to determine the role of the carbohydrate in secretion and activity of *T. reesei* cellulases. We report here the use of tunicamycin, an antibiotic from *Streptomyces lysosuperificus,* which specifically inhibits the synthesis of dolichol-mediated protein glycosylation (Takatsuki et al. 1975; Tkacz and Lampen 1975), to study the role of N-linked glycosylation on the secretion and enzymatic activities of cellulases. In the presence of tunicamycin, the formation of N-glycosidically linked oligosaccharide chains is prevented but the biosynthesis of O-glycosidically linked oligosaccharides to serine and threonine residues proceeds normally. Thus, tunicamycin is a useful tool which allows an in vivo study of the effect of specific oligosaccharide chains on glycoprotein secretion and enzymatic activities.

Materials and methods

T. reesei strains and culture conditions. Wild type *Trichoderma reesei* QM6a and hypersecreting mutant RL-P37 (Sheir-Neiss and Montenecourt 1984) have been previously described. Stock cultures of QM6a were maintained on potato dextrose agar (Difco) and stock cultures of RL-P37 on Vogel's salts (Vogel 1956) containing 0.1% proteose peptone (Difco), 1% cellulose (Solka Floc, BW200, Brown Co., Berlin, NH) and 1.5% agar. Submerged fermentation studies were carried out in Vogel's medium containing 1% lactose as the carbon source. Cultures were inoculated with 10-day old spores at a final concentration of 2×10^5 spores/ml and incubated on a rotary shaker (250 rpm) at room temperature. After 24 h germination in lactose medium, varying concentrations of tunicamycin (Sigma, St. Louis, Mo.) were added to the culture and growth was continued for an additional 72 h. Samples were taken at 24 hour intervals and centrifuged (2,000 rpm) to remove the mycelia; the supernatant fluid was then analyzed for soluble protein and enzymatic activities. After 72 h of growth, mycelia were collected on glass fiber filters, washed three times with distilled water and used for dry weight determinations.

Cellulase assays. Supernatants were analyzed for protein concentration by the method of Lowry et al. (1957). Samples were precipitated with 0.15% deoxycholate and 72% TCA (Petterson 1983) prior to assay. Enzymatic activities were analyzed according to the methods of Mandels et al. (1976) in 0.05 M citrate buffer pH 4.8 at 50°C. Reducing sugars were quantified by the dinitrosalicyclic acid method (Miller 1969). β-glucosidase activity was measured using cellobiose (Sigma, St. Louis, Mo.) as substrate. Glucose was analyzed enzymatically by glucose oxidase/peroxidase (Sigma) assay. Supernatants from the final time point were concentrated approximately 50-fold by ultrafiltration using PM-10 membranes (Amicon, Danvers, Ma.).

In vitro cleavage by endoglycosidase H. To 100 µl of concentrated culture fluid, 100 µl 0.05 M Na-acetate buffer pH 5.3 were added, followed by 0.01 U of endoglycosidase H (EC 3.2.1.96, Miles Laboratories, Elkhart, In.). After incubation at 37°C for 72 h, the samples were boiled for 10 minutes to stop the reaction.

Electrophoresis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in 11% slab gels in an Aquabogue (Brookhaven, N. Y.) gel apparatus Model 100 according to Laemmli (1970). Apparent molecular weights were calculated according to Weber and Osborn (1969) using Sigma high molecular weight standards. Isoelectric focusing (IEF) was performed in 6% polyacrylamide slab gels containing 2% ampholytes, pH 4—6.5 (LKB), in Aquabogue gel apparatus Model 400. Equal amounts of protein were applied to each slot. IEF gels were run at 100 volts for 16 h followed by 300 volts for 6 h.

Both SDS-PAGE and isoelectric focusing gels were stained for protein with Coomassie blue R250 (Eastman Kodak Co., Rochester, N. Y.) and for carbohydrate with periodic acid/Schiff's reagent (Sigma). Cellulase enzyme activities in isoelectric focusing gels were localized employing agarose overlays containing either carboxymethyl cellulose or ball beaten Avicel as described previously (Sheir-Neiss and Montenecourt 1984).

Stability studies. Thermostability of endoglucanases in crude culture fluids was determined by preincubation at 60°C for up to 120 min. pH stability was measured over the pH range 3—9, using 0.05 M citrate buffer for pH range 3.0—5.5 and 0.05 M Na phosphate buffer for pH range 6.0—9.0.

Sensitivity to proteases. Crude culture fluids were incubated with trypsin (EC 3.4.21.4., Sigma), and proteinase K (Sigma protease Type XI). For trypsin digestion, 100 µl of culture fluid was added to 400 µl of 0.1 M Na-phosphate buffer pH 8.9. 50 µl of trypsin stock solution (1 mg/ml) was added and the mixture was incubated at 37°C for 2 h, at which time 40 µl of HCl was added to stop the reaction. In control samples 50 µl of distilled water was added in place of trypsin. Proteinase K digestions were carried out in 0.01 M Tris-HCl pH 7.8 as described for trypsin digestion by using 2 µl of proteinase K stock solution (20 mg/ml).

Results

Effect of tunicamycin on growth and secretion

The cellulases secreted by wildtype and hypersecretory mutants of *Trichoderma reesei* are glyco-

proteins, presumably having both O-linked and N-linked oligosaccharides. It was therefore of interest to determine the effect of tunicamycin, an inhibitor of N-linked glycosylation, on the secretion and activities of the *T. reesei* cellulase complex.

The effects of various concentrations (0—40 μg/ml) of tunicamycin on growth of wildtype *Trichoderma reesei* QM6a and hypersecreting mutant RL-P37 were determined (Fig. 1). Tunicamycin at 2.5 μg/ml had no detectable effect on growth of either strain, while at 5.0 μg/ml of the drug growth was inhibited slightly for both QM6a and RL-P37, as determined by final dry weights. Higher concentrations of tunicamycin significantly reduced growth; consequently, concentrations of 2.5 and 5.0 μg/ml were chosen for subsequent experiments. Under these conditions neither the level of secreted cellulase nor the total amount of secreted protein was affected following growth in the presence of tunicamycin (Table 1). Furthermore, no change in the final yield of endoglucanase, filter paper, or β-glucosidase activities could be demonstrated.

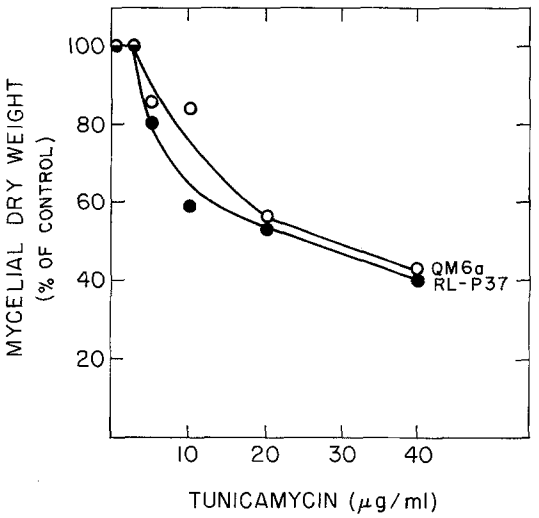

Fig. 1. The effect of tunicamycin on growth of *T. reesei* wild type QM6a and hypersecreting mutant RL-P37

Effect of tunicamycin on electrophoretic mobilities of extracellular enzymes

SDS-polyacrylamide gel electrophoretic analyses of proteins synthesized and secreted in the presence of tunicamycin showed that the effects of growth in the presence of the drug were very similar to those of in vitro cleavage by endoglycosi-

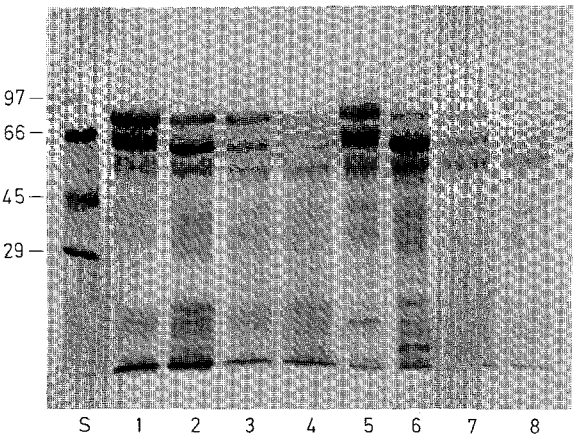

Fig. 2. SDS-PAGE analysis of proteins secreted by *T. reesei* QM6a and RL-P37. Cultures were grown in Vogel's-lactose medium in the presence (5 μg/ml) or absence of tunicamycin. *Lanes:* 1, QM6a control culture; 2, QM6a control, secreted proteins treated with endoglycosidase H; 3, QM6a tunicamycin-grown culture; 4, QM6a tunicamycin-grown culture, secreted proteins treated with endoglycosidase H; 5, RL-P37 control culture; 6, RL-P37 control culture, secreted proteins treated with endoglycosidase H; 7, RL-P37 tunicamycin-grown culture; 8, RL-P37 tunicamycin-grown culture, secreted proteins treated with endoglycosidase H. The numbers to the left of lane S indicate molecular weights ($\times 10^3$) of marker proteins

Table 1. Effect of growth in presence of tunicamycin on cellulase secretion by *T. reesei*

Strain	Tunicamycin μg/ml	Protein mg ($\times 10^{-2}$)/mg dry weight	Endoglucanase IU/mg dry weight	Filter paper IU ($\times 10^{-2}$)/mg dry weight	B-Glucosidase IU ($\times 10^{-2}$)/mg dry weight
QM6a	0	1.3	1.0	7.6	1.4
	2.5	1.3	1.0	7.9	1.3
	5.0	1.1	1.1	9.0	1.0
RL-P37	0	5.2	4.2	14.5	3.4
	2.5	8.0	3.1	13.5	2.8
	5.0	6.3	3.2	17.0	2.3

All data for 96 h of growth

dase H of secreted glycoproteins produced in the absence of tunicamycin (Fig. 2). The mobilities of some of the secreted proteins were increased while others did not show a noticeable change. In particular, the two prominent diffuse bands, containing cellobiohydrolase and endoglucanase as well as other proteins, corresponding to a molecular weight range of 50,000 to 80,000, were observed in repeated experiments to become sharper and have a slightly higher mobility in the tunicamycin and endo H samples than in the controls for both the wild type strain QM6a and the hypersecreting mutant RL-P37.

To further characterize the properties of the secreted cellulases following growth in the presence of tunicamycin, slab gel isoelectric focusing analyses were performed. The presence of tunicamycin in the growth medium had a clear effect on isoelectric focusing patterns of the cellulases secreted from both QM6a and RL-P37 (Fig. 3). As a result of the addition of tunicamycin, there were noticeable changes in the banding patterns for both strains. These changes included shifts in the position of some bands and a decrease in the number of multiple bands in the acidic CBH region.

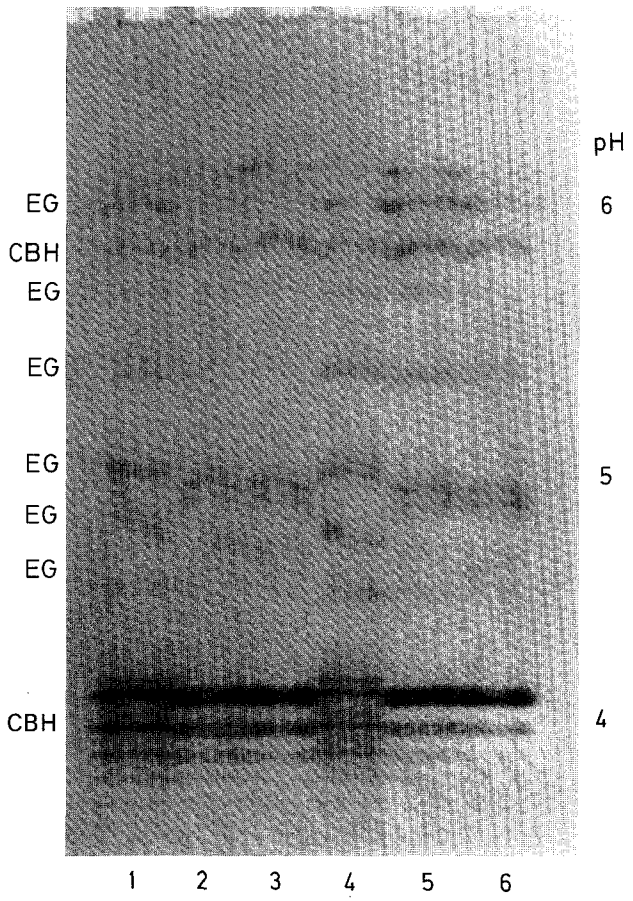

Fig. 3. Effect of growth in the presence of tunicamycin on isoelectric focusing of secreted cellulases from QM6a and RL-P37. The pH gradient in the gel was 4.0—6.5. *Lanes:* 1, QM6a control culture; 2, QM6a culture grown in the presence of tunicamycin at 2.5 μg/ml; 3, QM6a culture grown in the presence of tunicamycin at 5.0 μg/ml; 4, RL-P37 control culture; 5, RL-P37 culture grown in the presence of tunicamycin at 2.5 μg/ml; 6, RL-P37 culture grown in the presence of tunicamycin at 5.0 μg/ml. CBH, cellobiohydrolase; EG, endoglucanase

All the protein bands in isoelectric focusing and SDS-PAGE patterns stained with Schiff's reagent, confirming that all of the detectable proteins secreted by *T. reesei* were glycoproteins. Proteins secreted by cells growing in the presence of tunicamycin still were readily stained by Schiff's reagent, indicating that the attachment of some of the oligosaccharides to *T. reesei* cellulases is not inhibited by the drug. In the case of CBHI, it has been reported that the enzyme contains both N- and O-linked oligosaccharide (Fägerstam et al. 1984). Our results indicate that most, and perhaps all, of the secreted proteins of *T. reesei* contain significant amounts of O-linked (tunicamycin resistant) oligosaccharides.

Cellulase activities of the isoelectric focused proteins were qualitatively assayed by overlaying unfixed gels with an agarose gel containing Avicel 105 (cellulose) and incubating under conditions that allow clearing zones to develop due to cellulase activity in the bands in the IEF gel. We observed that the enzymes secreted from cells grown in the presence of tunicamycin were able to hydrolyze Avicel, but the activities of some of the bands seemed to be lower than for the controls (data not shown). The explanation for the lack of an effect of tunicamycin on enzyme activities in crude culture supernatants but the possible presence of an effect for some of the electrophoretically separated enzymes is unknown at this time.

Stability studies

Oligosaccharides are known to act as stabilizing factors for a variety of glycoproteins that have been biochemically characterized. For the *Trichoderma reesei* enzymes secreted by cells grown in the presence of tunicamycin, we observed a slight decrease in the thermostability of total endoglucanase activity in crude culture fluids for both QM6a and RL-P37 (Fig. 4). The decreased thermostability was very reproducible and evident in the initial slope of the 60° C inactivation curve. There were some minor changes in pH optima of

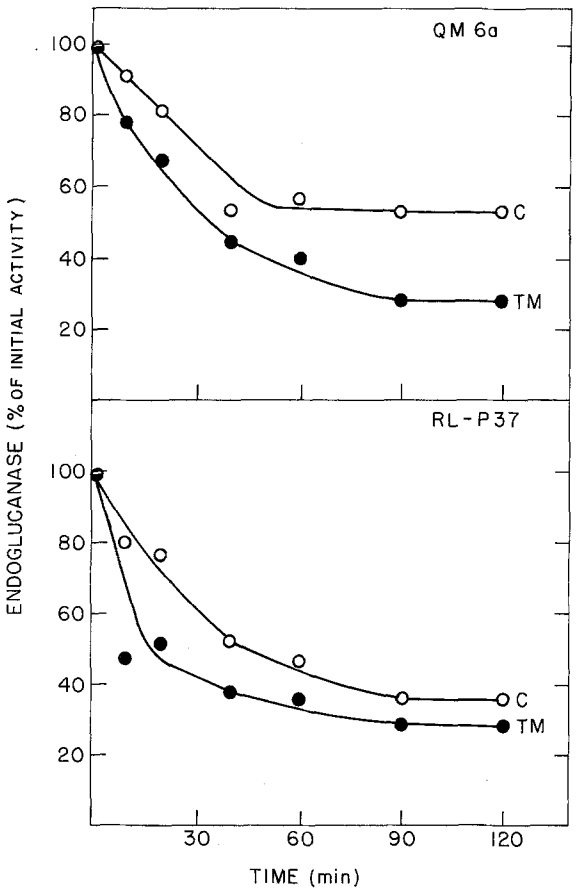

Fig. 4. Thermostabilities of crude endoglucanase activity from *T. reesei* cultures grown in the presence (5 μg/ml) (●) and absence (○) of tunicamycin. Preincubation was at 60° C for 0–120 min. The results presented are the average of three independent experiments

the enzymes produced in the presence of tunicamycin, but the overall shapes of the curves were not noticeably affected by the loss of the N-linked carbohydrate (data not shown). Endoglucanases in the crude culture fluid of QM6a and RL-P37 grown in the presence of tunicamycin showed somewhat enhanced sensitivity to trypsin and proteinase K compared to the endoglucanases of control cultures (Table 2).

Table 2. Effect of growth in presence to tunicamycin on sensitivity of crude secreted endoglucanase to proteases

Strain	Tunicamycin (μg/ml)	Endoglucanase activity (%) remaining after exposure to proteases	
		Trypsin	Proteinase K
QM6a	0	86	71
	2.5	70	69
	5.0	67	60
RL-P37	0	87	88
	2.5	70	69
	5.0	69	61

Discussion

The effect of tunicamycin on the synthesis and secretion of glycoproteins has been evaluated in many eukaryotic systems. The results obtained with animal cells, viruses and lower eukaryotes following treatment with tunicamycin have been diverse and have resulted in an inability to formulate generalizations concerning the role of N-linked oligosaccharides in enzyme activity and secretion. In this regard the most closely related system to *Trichoderma* which has been investigated to date is that of yeast, for which several enzymes have been studied. Invertase and acid phosphatase, which are highly glycosylated (50% carbohydrate), have been shown to be synthesized in an inactive form in the presence of tunicamycin (Kuo and Lampen 1974), presumably due to the fact that the unglycosylated forms of the enzymes are unable to form the proper tertiary configuration for the active enzyme. On the other hand, synthesis of active yeast alkaline phosphatase, which contains approximately 8% carbohydrate, is unaffected by tunicamycin. Although the enzyme was non-glycosylated, it was secreted normally and retained full activity (Onishi et al. 1979). An intermediate response was shown for the vacuolar enzyme carboxypeptidase Y (Hasilik and Tanner 1978). Tunicamycin was shown to depress enzyme synthesis and activity but did not abolish it completely and the enzyme formed in the presence of the antibiotic was shown to be unglycosylated. Tunicamycin was shown to have little or no effect upon exo-1,3-β-D-glucanase, a carbohydrase similar to cellulase (Sanchez et al. 1982). In the case of the β-1,3-glucanase, the activation energy and the K_m value of the glycosylated and non-glycosylated forms were shown to be identical and the investigators concluded that glycosylation was not necessary for either secretion or proper conformational folding of this enzyme. Thus, no clear cut role can be assigned for the carbohydrate moieties of yeast glycoproteins and their function may vary according to the ultimate location of the enzyme (vacuole or periplasmic) or the amount or location of the carbohydrate in the mature enzyme.

The results presented here suggest that the cellulases of *Trichoderma* conform more with the β-

1,3-glucanase and alkaline phosphatase of yeast than with yeast invertase and acid phosphatase. In addition, they provide physiological evidence in support of the biochemical studies of Fägerstam et al. (1984) that the polypeptides of the *T. reesei* cellulase complex contain both N- and O-linked carbohydrate, a situation reported for the invertase of *Fusarium* (Nishizawa and Maruyama 1981) and distinct from the yeast invertase which contains only N-glycosylation. N-linked glycosylation of proteins in *Trichoderma* appears to occur via a pathway similar to that described in yeast and animal cells which involves a lipid linked dolicholphosphate intermediate and can be blocked by tunicamycin. Blockage of N-glycosylation does not appear to affect O-glycosylation, the yield of the secreted proteins or the activity of the secreted cellulases, suggesting that N-asparagine linked carbohydrate is not necessary for proper targeting of the enzymes to their extracellular location or formation of an enzymatically active tertiary conformation. The underglycosylated forms of some of the extracellular proteins synthesized in the presence of tunicamycin have slighty increased electrophoretic mobilities in SDS polyacrylamide gels, as readily seen in the diffuse major bands containing cellobiohydrolase I and endoglucanases (Fig. 2). Growth in the presence of tunicamycin resulted in changes in the isoelectric focusing patterns of both the cellobiohydrolases and endoglucanases. In the acidic cellobiohydrolase I region at pH 4.0—4.5, the number of multiple bands capable of solubilizing Avicel but not reacting with carboxymethylcellulose decreased from seven to four or five and the intensity of the Coomassie stain was enhanced indicating an increase in protein content in comparison to equivalent bands in the control samples. Thus suggests that differences in glycosylation contribute to the heterogeneity of the acidic cellobiohydrolase I (Fig. 3).

Changes in the isoelectric focusing patterns of the endoglucanases in the region near pH 5 were also observed. The major band at pH 5 shifted toward the more acidic end of the gel and several additional bands appeared in the tunicamycin treated samples which were absent in the controls. A possible explanation for this observation is that the seemingly single protein band at pH 5.0 actually contains more than one protein. Blockage of N-glycosylation might result in exposure of different amino acid groups that would allow the underglycosylated forms to separate in a pH gradient. A second possible explanation is that the proteins represented in the major band at pH 5.0 consist of a complex held together by oligosaccharide chains and that the absence of some of the carbohydrate chains due to growth in the presence of tunicamycin results in easy dissociation of the complex. Such a complex of mixed cellulolytic activities has been postulated and identified from lyophilized culture filtrates of *T. reesei* QM 9414 (Sprey and Lambert 1983, 1984). Finally, if the N-linked carbohydrates function to protect the enzyme from endogenous or exogenous proteolytic degradation, inhibition of the carbohydrate addition in vivo might allow enhanced posttranslational proteolytic modification of the cellulases which would directly affect both SDS-PAGE and IEF protein separation patterns.

At levels which prevented N-linked glycosylation, tunicamycin did not cause a reduction in the overall levels of secreted protein or cellulase activities, but the underglycosylated endoglucanases were more thermolabile and more sensitive to proteases than the fully glycosylated forms, suggesting that the oligosaccharide chains enhance the structural stability of *T. reesei* cellulases. The conformation of underglycosylated forms may be slightly different from the fully glycosylated enzymes. Thus the proteins may maintain their activity under optimal conditions but be less stable at elevated temperatures and more accessible to proteases.

Similar effects of underglycosylation on enzyme stability have been reported for several other glycoprotein enzymes of lower eukaryotic origin. In the case of yeast exo-1,3β-glucanase, the underglycosylated form synthesized in the presence of tunicamycin was more sensitive to thermal and pH inactivation than the fully glycosylated enzyme (Sanchez et al. 1982). In an additional study, which dealt with the cellulases of the thermophilic fungus *Humicola isolensis*, partial removal in vitro of the carbohydrate moieties from cellobiohydrolase and endoglucanase resulted in decreased thermal and pH stabilities of the enzymes but did not significantly affect the specific activities (Hayashida and Yoshioka 1980).

The results presented in this paper thus indicate that the N-linked oligosaccharides do not play a crucial role in secretion and enzymatic activities of cellulases of *Trichoderma reesei* but do enhance thermal stability and protection against proteases. The results are also consistent with the suggestion that cellulases form complexes which may be held together at least in part by the oligosaccharide interactions. Much of the carbohydrate present in the fully glycosylated forms of the *T. reesei* cellulases appears to be of the O-

type, the formation of which is unaffected by the presence of tunicamycin. We are currently investigating the role of the O-linked carbohydrate in synthesis, secretion and stability of *Trichoderma* cellulases. A definitive understanding of the role of the carbohydrate in the synthesis and activity of *T. reesei* cellulases will aid in the development of new more efficient cellulolytic strains and increased hydrolysis efficiency.

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