JOURNAL OF BACTERIOLOGY, July 1984, p. 294–299
0021-9193/84/070294-06$02.00/0

Vol. 159, No. 1

Extracellular Proteins Secreted by the Basidiomycete *Schizophyllum commune* in Response to Carbon Source†

GORDON E. WILLICK,[1,2]* ROLF MOROSOLI,[1]‡ VERN L. SELIGY,[2] MAKOTO YAGUCHI,[2] AND MICHEL DESROCHERS[3]

Molecular Research Group, Ontario Paper Company,[1] and Molecular Genetics Section,[2] Division of Biological Sciences, National Research Council, Ottawa, Canada K1A 0R6; and Pulp and Paper Research Institute of Canada, Pointe Claire, Québec, Canada H9R 3J9[3]*

Received 25 July 1983/Accepted 2 April 1984

The secretion of 1,4-β-D-glucanases by the basidiomycete *Schizophyllum commune* in response to cellulose or cellobiose has been studied. The proteins were labeled with ^{35}S, and the secretion of enzymes was measured by β-glucosidase and carboxymethyl cellulase activities and by immunoprecipitation with specific antibodies. The antigen proteins used were a β-glucosidase (M_r, 93,000), an avicelase (avicelase II; M_r, 64,000), and a carboxymethyl cellulase (carboxymethyl cellulase I; M_r 41,000). The β-glucosidase was initially secreted as an M_r 110,000 form, which was followed later by lower-molecular-weight (88,000 to 93,000) forms. The avicelase II, which accounted for about 50% of the secreted labeled protein, had an M_r of 64,000. Secretion of the related avicelase I (M_r 61,000) followed later. The carboxymethyl cellulase I was secreted in two molecular weight forms, M_r 44,000 and 41,000. The evidence is consistent with the idea that three genes account for the secreted glucanase activities. Other species result from different glycosylation or proteolytic cleavage processing, which may occur during or after secretion. The β-glucosidase secretion appears to be regulated differently than that of avicelase II or carboxymethyl cellulase I; the latter two were regulated coordinately under the conditions used in this work. No common immune determinants between the three antigens were observed.

In recent years considerable interest has been shown in the fungal cellulases. The fungal cellulases are capable of efficiently degrading cellulose, the world's most abundant glucose polymer, to glucose, thus providing a substrate for further microbial conversion to ethanol or other important chemicals. The cellulases from *Phanerochaete chrysosporium*, *Trichoderma reesei*, *Trichoderma koningii*, and, more recently *Schizophyllum commune* have been particularly well studied. The attack on cellulose has been shown to involve the cooperative action of three types of 1,4-β-D-glucanases (4, 5, 18), including endo-1,4-β-D-glucanases (EC 3.2.1.4), exo-1,4-β-D-glucanases (EC 3.2.1.91) (which release either glucose or cellobiose), and 1,4-β-D-glucosidases (EC 3.2.1.21) (which may also attack oligosaccharides higher than cellobiose [2]).

We are interested in isolating the genes coding for these cellulases and understanding the control of their expression and secretion. As part of this study, a number of extracellular proteins of *S. commune* have been isolated, and some have been partially sequenced. These include two β-glucosidases (βG-I and βG-II), two avicelases (Av-I and Av-II), two carboxymethyl cellulases (CMC-I and CMC-II), and two proteins with as yet unknown enzymatic activities (PX-I and PX-II).

In general, multiple cellulases have been identified in fungal culture filtrates (5). It is not known for certain whether this multiplicity arises from multiple genes, post-transcriptional processing, or a combination of both. The latter can include extracellular protease attack, and this has been shown to produce a multiplicity of cellulases in *P. chrysosporium* (5).

Gong et al. (7) showed that the chromatographic profile of the secreted proteins of *T. reesei* grew progressively more complicated as the culture aged, and they spectulated that this might be due to extracellular processing. In general, however, studies have been done on cultures with maximum cellulase activity; this may lead to obscuring the initial secretion profile. Indeed, it has been shown that extracellular proteolytic processing increases the activity of cellulases in *P. chrysosporium* (6). We are interested in the basic response at the gene level when *S. commune* is presented with different carbon sources. We have approached this problem by labeling the proteins of this organism at the time it is presented with cellobiose or cellulose in a minimal medium. Using antibody to several purified proteins, we have been able to identify the specific proteins secreted at early stages. We here demonstrate that the secretion profile is indeed relatively simple under these conditions.

MATERIALS AND METHODS

Growth and labeling of *S. commune* extracellular proteins. *S. commune* Fries strain 13 Delmar (ATCC 38548) was used throughout this work. A 1% inoculum from a 3-day growth of mycelium in malt extract (1%) was added to 100 ml of a defined medium containing the following (grams per 100 ml): KH_2PO_4 (0.1), K_2HPO_4 (0.1), $MgSO_4 \cdot 7H_2O$ (0.05), asparagine (0.2), metal ion mixture (12) (0.1 ml), and cellulose (Solka Floc, Brown Co.), cellobiose, or glucose (0.1) as the carbon source (1). Growth proceeded for 7 days at 30°C in a polypropylene flask containing a 2-cm glass marble with shaking at 200 rpm. The mycelium was harvested by centrifugation at 6,000 × *g* for 15 min, and fresh medium (60 ml) was added with the carbon source as indicated. For ^{35}S labeling, 80 to 120 μCi of $H_2{}^{35}SO_4$ (New England Nuclear Corp.) per ml was added, and the $MgSO_4$ was replaced by $MgCl_2$.

Purification of extracellular proteins. *S. commune* was grown in a medium optimized for cellulase production (3),

* Corresponding author.
† National Research Council of Canada publication no. 23297.
‡ Present address: Institute Armand Frappier, Montréal, Québec.

TABLE 1. Physical characteristics of extracellular proteins purified from *S. commune*

Protein	Mol wt[a]	pI[b]
βG-I (3)	97,000	3.2, 3.1[c]
βG-II (3)	92,000	
Av-II	64,000	3.0
Av-I	61,000	3.15
Px-II[d]	42,000	ND[e]
CMC-Ia	44,000	2.95
CMC-Ib	41,000	2.95
CMC-II	38,000	2.90

[a] Molecular weights were estimated with sodium dodecyl sulfate-gel electrophoresis (11) and standard proteins.
[b] Isoelectric points were estimated by using a pH 2.5 to 8.0 gradient as described in the text.
[c] Molecular weight correlation not known.
[d] Protein purified, but enzymatic activity, if any, as yet unidentified.
[e] ND, Not determined.

and the culture filtrate was fractionally precipitated by the addition of cold ethanol (−18°C) (14a). The fraction containing cellulolytic activity was dissolved in 0.2 M pyridine acetate buffer (pH 5.0) and freeze dried. The precipitate was redissolved in the above buffer and applied to a column of DEAE–Bio-Gel A (2.5 by 100 cm) (Bio-Rad Laboratories) as previously described (3). Proteins were eluted with a 4-liter linear gradient of pyridine acetate (pH 5.0; 0.2 to 0.6 M). Xylanase activity was in the void volume, and proteins CMC-I, PX-II, CMC-II, βGlu-I, Av-I, β-Glu-II, and Av-II were eluted in order. Av-I and Av-II were passed through a Bio-Gel P300 column (Bio-Rad) (1.5 by 90 cm) equilibrated with 0.1 M pyridine acetate (pH 5.0) to remove β-glucosidase activity.

Antibody production. Antibodies were induced in New Zealand white rabbits by an initial intramuscular injection of 50 to 100 μg of antigen in complete Freund adjuvant, followed by multiple intradermal injections of 25 μg in incomplete Freund adjuvant at 3 weeks and every additional 2 weeks until the desired response was obtained. The antiserum was precipitated once with 40% $(NH_4)_2SO_4$ at 20°C and passed through a DEAE-cellulose (Bio-Rad) column equilibrated with 25 mM sodium phosphate (pH 7.3). The unbound material (immunoglobulin G [IgG]) was concentrated five times by $(NH_4)_2SO_4$ precipitation, dialyzed against 10 mM sodium phosphate–0.14 M NaCl (pH 7.2), aliquoted, and stored at −70°C.

Immunoprecipitation of ^{35}S-labeled cellulases. Cellulases were precipitated from the extracellular medium (50 to 200 μl) by the addition of 1 to 5 μl of antibody. After 2 h at 4°C, the antibody was precipitated by the addition of 10 μl of protein A-agarose (Sigma Chemical Co.) or protein A-Sepharose (Pharmacia Fine Chemicals, Inc.), and allowed to stand at 20°C for 30 min with periodic agitation. The precipitate was washed three times with TEN-40 (50 mM Tris-hydrochloride, 5 mM EDTA, 150 mM NaCl, 0.05% Nonidet P-40, pH 7.4) suspended in Laemmli buffer (11) containing 6 M urea, and boiled for 5 min before use in gel electrophoresis experiments.

Gel electrophoresis. Gels were run in the discontinuous system of Laemmli (11) with 12.5% acrylamide in the separation gel. Gels were treated with En^{3}Hance (New England Nuclear) according to the manufacturers' instructions. Autoradiography was carried out at −70°C with preflashed Kodak X-Omat-R film. Standards for molecular weight calibration were either prestained high-molecular-weight standards (Bethesda Research Laboratories) or radiolabeled high-molecular-weight standards (Amersham Corp.). Isoelectric focusing was carried out in 0.5-mm gels containing 4.85% acrylamide, 13% glycerol, and 6% Pharmalyte (Pharmacia) (pH range 2.5 to 5 and 5 to 8 mixed 1.4:1 [vol/vol]) at 12°C and a potential of 1,500 V for 1.5 h and 2,000 V for 0.5 h.

Enzyme assays. Carboxymethyl cellulase activity was measured by mixing a 0.5-ml sample of the mycelium-free medium with 0.5 ml of 1% carboxymethyl cellulose (Sigma; high viscosity) in 0.2 M sodium acetate (pH 5.0) and incubating for 10 min at 30°C. Reducing sugar was estimated by adding 1 ml of dinitrosalicylic acid reagent, boiling for 15 min, diluting 1/10 with Rochelle salt, and reading the absorbance at 575 nm (13). β-Glucosidase activity was measured by following the release of *p*-nitrophenol at 420 nm from *p*-nitrophenyl-β-D-glucoside (Sigma) (14). Cellobiase activity was measured by release of glucose. Glucose was measured with a commercial assay kit (Sigma; no. 510). Enzyme activities are expressed as international units (micromoles of glucose or reducing sugar produced per minute).

RESULTS

Antibodies to cellulases of *S. commune*. Several cellulolytic enzymes have been purified from the filtrate of relatively mature cultures of *S. commune*. These are listed in Table 1, along with their estimated molecular weights and isoelectric points. Preparations of these enzymes were used to generate specific antibodies (Fig. 1). It is immediately clear that the labeled protein initially secreted in response to cellulose is not necessarily exactly the same in molecular weight as the antigen that was used originally. The β-glucosidase sample used as antigen had a molecular weight of 93,000, whereas that initially secreted has a molecular weight of 110,000 (Fig. 1A). The Av-II used as antigen was devoid of Av-I (Fig. 1B), but the immunoprecipitated sample shows a small amount of faster-migrating material, with a molecular weight equivalent to that of Av-I. The CMC-I used as antigen had a molecular weight of 41,000 (Fig. 1C) and showed a single band on

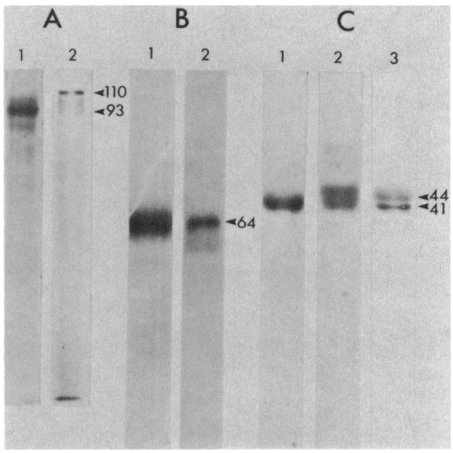

FIG. 1. Specificity of cellulase antibodies. Antibodies were raised to β-glucosidase, Av-II, and CMC-I and used to precipitate samples of the ^{35}S-labeled proteins secreted in response to cellulose. Antigen samples were on the same gel. The gel was stained with Coomassie blue. (A) Lanes: 1, β-glucosidase, 2, β-glucosidase immunoprecipitate. (B) Lanes: 1, Av-II; 2, Av-II immunoprecipitate. (C) Lanes: 1, CMC-I used as antigen (single tube from purification column); 2, total pooled CMC-I; 3, CMC-I immunoprecipitate.

isoelectric focusing (data not shown). The resulting immunoprecipitate showed two major bands, with indication of even greater molecular weight microheterogeneity. We concluded that CMC-I is secreted predominantly in two molecular weight forms, here labeled CMC-Ia and CMC-Ib (Table 1, Fig. 1C). No evidence of any accumulation of CMC-II was found, although this species was present in the cultures used for enzyme purification. CMC-I and CMC-II are related by 16 additional amino acids at the NH_2 terminus in CMC-I (14a). There is no evidence for microheterogeneity of the sequence of the CMC-I preparations shown in Fig. 3C (lanes 1 and 2) (M. Yaguchi, unpublished results). Other evidence, including [^{3}H]mannose incorporation and concanavalin A binding (G. Willick, unpublished results) indicate that the highest-molecular-weight forms of these three enzymes are glycosylated. In summary, the evidence suggests that the multiplicity is a result of differential glycosylation or NH_2-terminal proteolytic cleavages. However, we do not know whether this occurs because of changes in the processing during secretion or is a result of extracellular processing by enzymes in the culture filtrate.

Secretion of cellulolytic activity in response to cellulose. The secretion of glucanase activities in response to cellulose was measured by using *p*-nitrophenyl-β-D-glucoside and carboxymethyl cellulose as substrates (Fig. 2). The *p*-nitrophenyl-β-D-glucosidase activity is due entirely to the β-glucosidase (Fig. 1A, Table 1). The purified β-glucosidase had a cellobiase activity approximately one-half of that on *p*-nitrophenyl-β-D-glucoside. The activity on higher oligosaccharides has not yet been explored, but in analogy to other fungal β-glucosidases such an activity is likely (2). The carboxymethyl cellulase activity is accounted for almost entirely by the CMC-I species. This enzyme has been shown to hydrolyze soluble oligosaccharides, but does not release glucose (14a).

The release of glucanase activities was immediate when the organism has previously been growing on cellulose, but there was a lag when the previous substrate was either glucose or cellobiose. Such a lag suggests that some type of induction phenomenon was involved. This might include the synthesis of secretory organelles and does correlate with the appearance of mRNA transcripts coding for the glucanases (16).

Identification of secreted proteins in response to cellulose. The secreted proteins were labeled by the addition of $H_2{}^{35}SO_4$ at the time of transfer into the cellulose medium. The antibodies described above were used to precipitate labeled components (Fig. 3). This type of detection allows the identification of some proteins before detectable enzyme activity has been secreted. Thus, CMC-I and Av-II can be discerned in the earliest samples. By comparison, the components present in the total secreted labeled protein could be identified (Fig. 4). It is clear that a very large portion of the secreted label has been accounted for. The autoradiograms were scanned, and the proportions of secreted proteins estimated from the peak areas. These calculations indicated that 85% of the protein has been accounted for, with Av-II being almost 50% of the total.

It is clear from Fig. 3 that the earliest secreted species observed (1 to 1.25 days) are always the highest-molecular-weight variant, with lower-molecular-weight forms appearing later. As shown in Fig. 1, the antigen used corresponded to a lower-molecular-weight form for two of the enzymes (βGase and CMC-I). Possible explanations for the appearance of these altered forms were discussed above. It is very likely that the multiplicity of molecular weights arises from differential processing during or after secretion.

Secreted proteins in response to cellobiose. Cellobiose has been demonstrated to be an inducer of cellulase expression in *S. commune* (15), and it has been hypothesized that the release of cellobiose from the attack of a cellobiohydrolase on cellulose results in the expression of glucanases observed

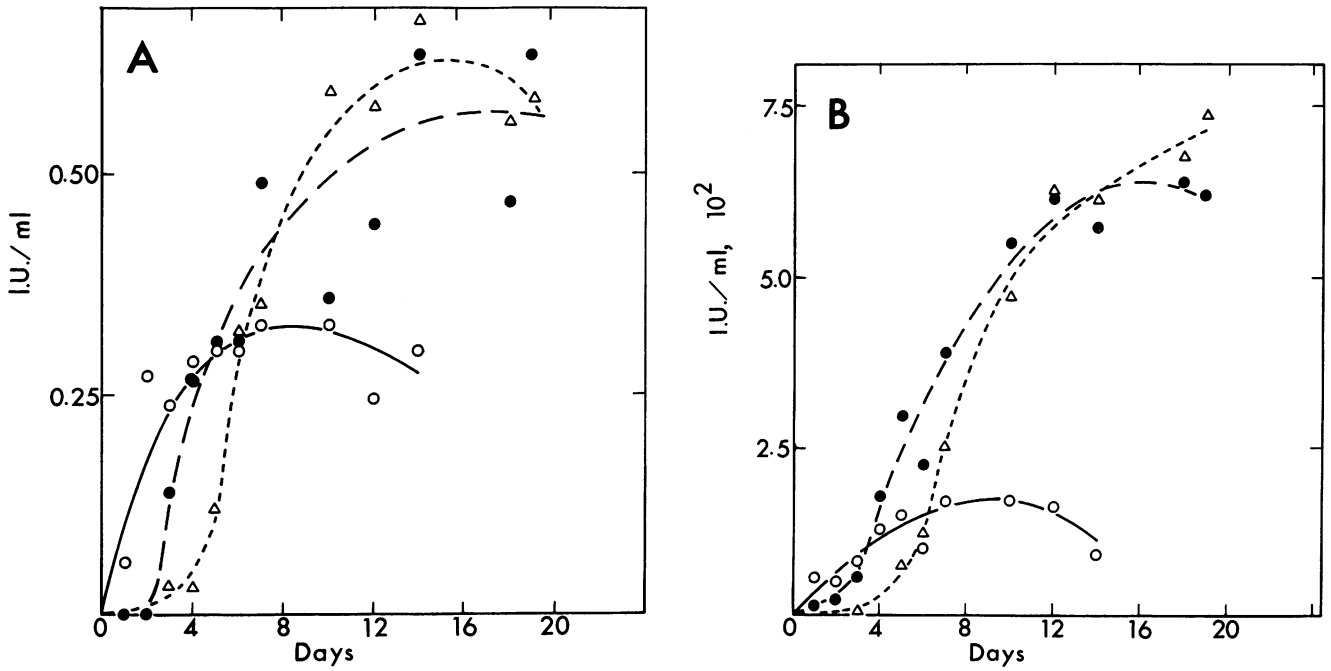

FIG. 2. Release of β-glucosidase (A) or carboxymethyl cellulase (B) activities into the culture filtrate after the addition of medium containing cellulose (0.1%) to mycelium. Cultures were initially grown on cellulose (○), cellobiose (●), or glucose (△).

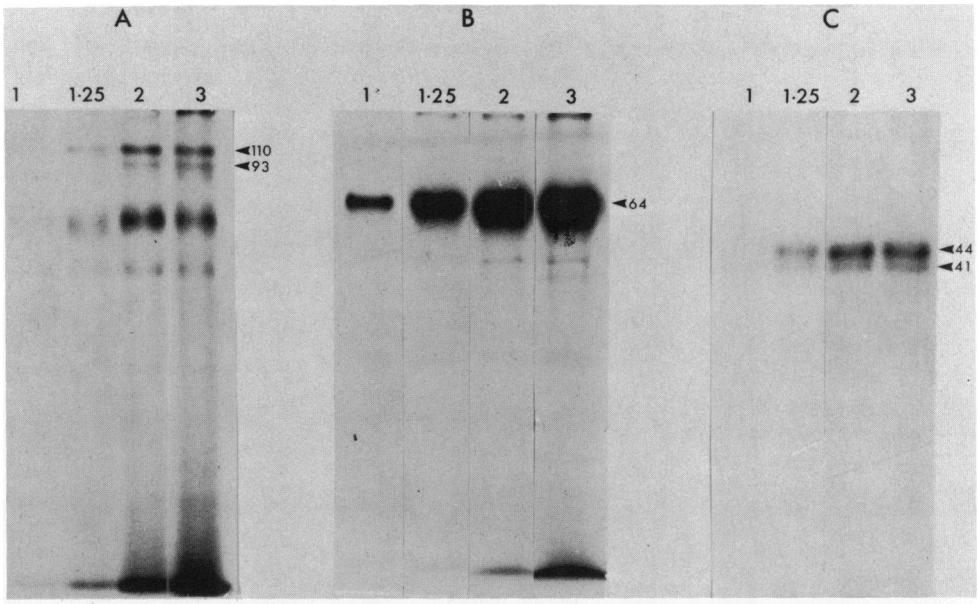

FIG. 3. Appearance of glucanases after the addition of cellulose medium containing $H_2{}^{35}SO_4$. Mycelium was grown on 0.1% cellulose for 7 days, the mycelium was removed by centrifugation, and fresh medium containing cellulose (0.1%) and $H_2{}^{35}SO_4$ (100 μCi/ml) was added. Samples were immunoprecipitated with the indicated antibodies. Lanes are labeled in time (days) after transfer. Protein species are labeled with molecular weights in thousands. (A) Anti-β-glucosidase. The antigen used to prepare this antibody also contained AV-II. (B) Anti-Av-II. (C) anti-CMC-I.

when cellulose is the substrate. Are the cellulases expressed in a similar manner in response to cellobiose as in response to cellulose? If they were under common regulation, then they should show coordinate expression. The expression of enzyme activities is shown in Fig. 5, along with the anti-CMC-I and anti-Av-II precipitations. The data in Fig. 5 demonstrate that β-glucosidase activity is expressed long after carboxymethyl cellulase activity has ceased and been destroyed. Fig. 5 (inset) shows that CMC-I and Av-II are coordinately expressed under the conditions of this experiment. We conclude that the β-glucosidase is under separate regulation as compared with CMC-I and Av-II, and that the latter two enzymes may be under a common regulation.

At the time of cessation of carboxymethyl cellulase expression, another protein appears which we have identified as Px-II. Our available data are consistent with this protein being a protease. If so, it would explain the sudden destruction of CMC-I and Av-II that was observed.

DISCUSSION

This work supports the idea that *S. commune*, in the presence of cellulose, secretes the products of three 1,4-β-D-glucanase genes: a β-glucosidase, an endoglucanase (CMC-I), and an avicelase (Av-II). The antibody studies presented here suggest that other species are related to one or another member of this set. Given the known specificities of these enzymes, this is likely to be the minimum gene response necessary to bring about the total degradation of cellulose.

It has been observed that fungi produce many glucanases to degrade cellulose (6, 17). More recent studies with *T. reesei*, with early cultures (7) or sophorose induction (9), suggest that only three or four glucanases may be secreted.

A multiplicity of secreted products could arise from differences in glycosylation or proteolytic processing during or after secretion. Our preliminary evidence, from [^{3}H]mannose incorporation or concanavalin A binding, suggests

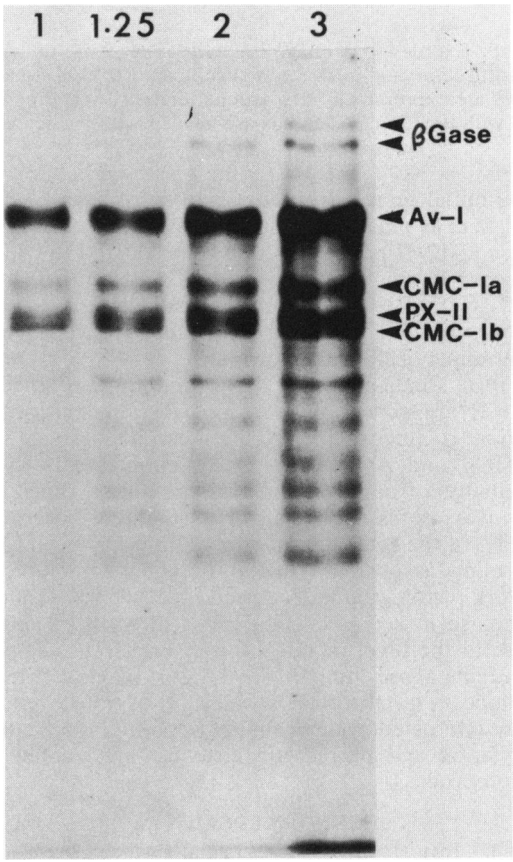

FIG. 4. Total secreted proteins. Lanes are labeled in time (days) after transfer into fresh cellulose (0.1%) medium containing $^{35}SO_4{}^{-2}$ (100 μCi/ml).

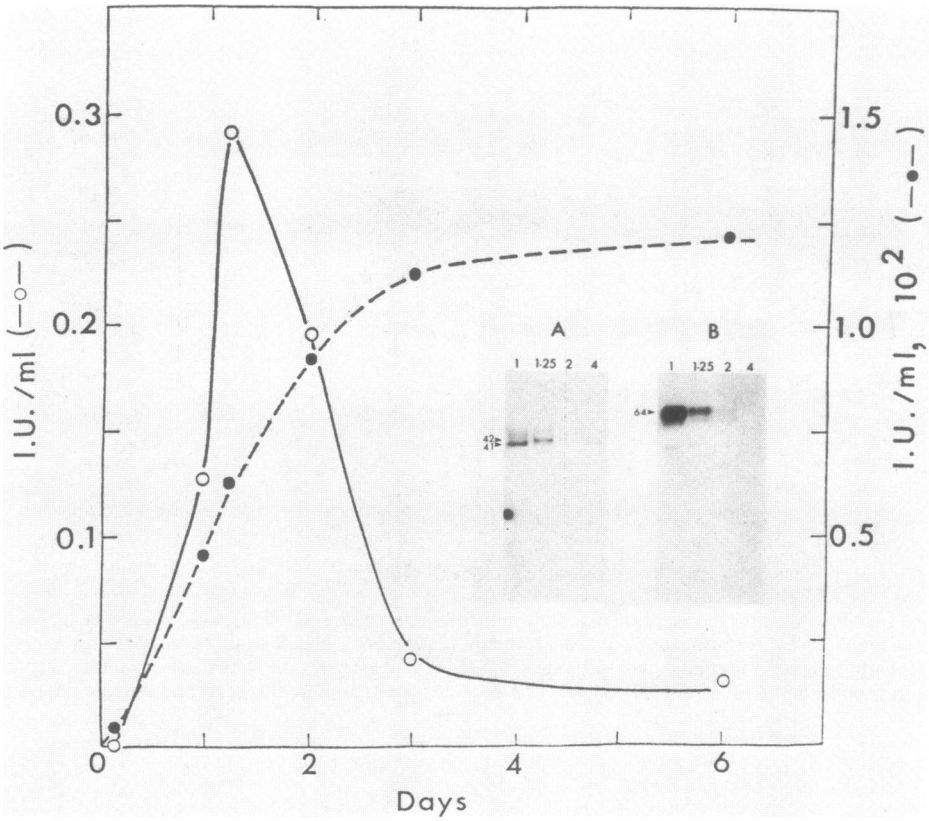

FIG. 5. Expression of enzyme activity in response to cellobiose. Mycelium was grown on cellobiose (0.1%) for 7 days and then removed by centrifugation, and fresh medium containing 0.1% cellobiose and $H_2{}^{35}SO_4$ was added. Carboxymethyl cellulase (○) and β-glucosidase (●) activities are expressed in international units. (Inset) Precipitation of cellobiose samples with anti-CMC-I (A) or anti-Av-II (B). Lanes are labeled with time in days. Bands are labeled with molecular weights in thousands.

that the initial secreted products are more heavily glycosylated. There is also evidence for proteolytic cleavage. The cellulase CMC-II has been found to be related to CMC-I by cleavage of 16 amino acids of CMC-I. This may be a fungal analogy to the late secretion of a penicillinase by *Bacillus licheniformis* which results from proteolytic cleavage at the NH_2 terminus of the early form (10).

Our results indicate that CMC-I (underglycosylated form) and Av-II are cosecreted and possibly under a common regulation. β-Glucosidase secretion can be dissociated from the CMC-I and Av-II (Fig. 5) secretion and is therefore differentially regulated. Unlike some other fungi (2), *S. commune* secretes almost all of its β-glucosidase activity. Only 10% of the total activity in a culture has been found to be mycelium associated (D. Rho, personal communication).

In work parallel to that reported here, we have found that the increase in secreted cellulase is directly related to an increase in the level of mRNA transcripts associated with the three glucanases (unpublished observations). Thus, regulation appears to take place at the level of transcription. We are now interested in determining how the modifications of glycosylation and proteolytic cleavage are related to the secretion process.

ACKNOWLEDGMENTS

We thank Lien Hoa Huang, Elisa de Miguel, and Fernand Rollin for their capable assistance with various aspects of this study.

This project was funded in part by the program for Industry Laboratory Projects (contract NRC IPO-163) of the National Research Council of Canada.

LITERATURE CITED

1. **Baradi, J., and L. Jurasek.** 1968. Effect of pH on mycelium, carboxymethylcellulase, and xylanase formation in submerged culture of *Schizophyllum commune*. Drevarski vyskum. 193–202.
2. **Coughlan, M. P., P. J. Considine, A. P. Moloney, and A. J. McHale.** 1982. The β-glucosidases of *Talaromyces emersonii*: properties and possible functions in cellulose digestion in vivo, p. 75–80. TAPPI Proceedings, Research and Development Conference, Asheville, N.C.
3. **Desrochers, M., L. Jurasek, and M. G. Paice.** 1981. High production of β-glucosidase in *Schizophylum commune*: isolation of the enzyme and effect of the culture filtrate on cellulose hydrolysis. Appl. Environ. Microbiol. **41**:222–228.
4. **Eriksson, K.-E.** 1978. Enzyme mechanisms involved in cellulose hydrolysis by the rot fungus *Sporotrichium pulverulentum*. Biotechol. Bioeng. **20**:317–332.
5. **Eriksson, K.-E.** 1981. Cellulases of fungi, p. 19–31. *In* A. Hollaender (ed.), Trends in the biology of fermentations. Plenum Publishing Corp., New York.
6. **Eriksson, K.-E., and B. Pettersson.** 1982. Purification and partial characterization of two acidic proteases from the white-rot fungus *Sporotrichum pulverulentum*. Eur. J. Biochem. **124**:635–642.
7. **Gong, C.-S., M. R. Ladisch, and G. T. Tsao.** 1979. Biosynthesis, purification, and mode of action of cellulases of *Trichoderma reesei*. Adv. Chem. **181**:261–287.
8. **Gong, C.-S., and G. T. Tsao.** 1979. Cellulase and biosynthesis regulation. Annu. Rep. Ferment. Proc. **3**:111–140.
9. **Gritzali, M., and R. D. Brown, Jr.** 1979. Relationships between purified extracellular enzymes from induced or cellulose-grown cells. Adv. Chem. **181**:237–260.

10. **Izui, K., J. B. K. Nielsen, M. P. Caulfield, and J. O. Lampen.** 1980. Large exopenicillinase, initial extracellular form detected in cultures of *Bacillus licheniformis*. Biochemistry **19**:1882–1886.
11. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
12. **Mandel, M., R. Andreotti, and C. Roche.** 1976. Measurement of saccharifying cellulase. Biotechol. Bioeng. Symp. **6**:21–33.
13. **Miller, G. L.** 1959. Use of dinitrosalycilic acid reagent for determination of reducing sugar. Anal. Chem. **31**:426–428.
14. **Nisizawa, T., H. Suzuki, and K. Nisizawa.** 1971. 'De novo' synthesis of cellulase induced by sophorose in *Trichoderma viride* cells. J. Biochem. **70**:387–393.
14a. **Paice, M. G., M. Desrochers, D. Rho, L. Jurasek, C. Roy, C. F. Rollin, E. De Miguel, and M. Yaguchi.** 1984. Two forms of endoglucanose from the basidiomycete *Schizophyllum commune* and their relationship to other β-1,4-glycoside hydrolases. Biotechnology **2**:535–539.
15. **Rho, D., M. Desrochers, L. Jurasek, H. Driguez, and J. Defaye.** 1982. Induction of cellulase in *Schizophyllum commune*: thiocellobiose as a new inducer. J. Bacteriol. **149**:47–53.
16. **Seligy, V. L., J. R. Barbier, K. D. Dimock, M. J. Dove, F. Moranelli, R. Morosoli, G. E. Willick, and M. Yaguchi.** 1983. Current status in the application of recombinant DNA technology in the construction of cellulolytic yeast strains, p. 167–185. *In* M. Korhola and E. VÂisänen (ed.), Gene expression in yeast. Proceedings of the Alko Yeast Symposium, Helsinki, 1983. Publisher, city.
17. **Shoemaker, S. P., and R. D. Brown, Jr.** 1978. Characterization of endo-1,4-β-D-glucanases purified from *Trichoderma viride*. Biochim. Biophys. Acta **523**:147–161.
18. **Wood, T. M.** 1982. Enzymic and other factors affecting the microbial generation of soluble sugars from cellulosic materials, p. 327–334. TAPPI Proceedings, Research and Development Conference, Asheville, N.C.