Extracellular Proteins Secreted by the Basidiomycete \textit{Schizophyllum commune} in Response to Carbon Source$^\dagger$

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The secretion of 1,4-\(\beta\)-d-glucanases by the basidiomycete \textit{Schizophyllum commune} in response to cellulose or cellobiose has been studied. The proteins were labeled with \(\text{\textsuperscript{35}}\text{S}\), and the secretion of enzymes was measured by \(\beta\)-glucosidase and carboxymethyl cellulase activities and by immunoprecipitation with specific antibodies. The antigen proteins used were a \(\beta\)-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 \(\beta\)-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 \(\beta\)-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 \textit{Phanerochaete chrysosporium}, \textit{Trichoderma reesei}, \textit{Trichoderma koningii}, and, more recently \textit{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-\(\beta\)-d-glucanases (4, 5, 18), including endo-1,4-\(\beta\)-d-glucanases (EC 3.2.1.4), exo-1,4-\(\beta\)-d-glucanases (EC 3.2.1.91) (which release either glucose or cellobiose), and 1,4-\(\beta\)-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 \textit{S. commune} have been isolated, and some have been partially sequenced. These include two \(\beta\)-glucosidases (\(\beta G-I\) and \(\beta G-II\)), 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, posttranscriptional 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 \textit{P. chrysosporium} (5).

Gong et al. (7) showed that the chromatographic profile of the secreted proteins of \textit{T. reesei} grew progressively more complicated as the culture aged, and they speculated that this might be due to extracellular processing. In general, however, studies have been done on cultures with maximum cellulose 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 \textit{P. chrysosporium} (6). We are interested in the basic response at the gene level when \textit{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.

\textbf{MATERIALS AND METHODS}

\textbf{Growth and labeling of \textit{S. commune} extracellular proteins.} \textit{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): \(\text{KH}_2\text{PO}_4\) (0.1), \(\text{K}_2\text{HPO}_4\) (0.1), \(\text{MgSO}_4 \cdot 7\text{H}_2\text{O}\) (0.05), asparagine (0.2), metal ion mixture (12) (0.1 ml), and cellulose (Sokla 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 \(\times\) \(g\) for 15 min, and fresh medium (60 ml) was added with the carbon source as indicated. For \(\text{\textsuperscript{35}}\text{S}\) labeling, 80 to 120 \(\mu\)Ci of \(\text{H}_2\text{\textsuperscript{35}}\text{SO}_4\) (New England Nuclear Corp.) per ml was added, and the \(\text{MgSO}_4\) was replaced by MgCl$_2$.

\textbf{Purification of extracellular proteins.} \textit{S. commune} was grown in a medium optimized for cellulase production (3),
TABLE 1. Physical characteristics of extracellular proteins purified from S. commune

<table>
<thead>
<tr>
<th>Protein</th>
<th>Mol wt*</th>
<th>pl†</th>
</tr>
</thead>
<tbody>
<tr>
<td>βG-I (3)</td>
<td>97,000</td>
<td>3.2, 3.1‡</td>
</tr>
<tr>
<td>βG-II (3)</td>
<td>92,000</td>
<td></td>
</tr>
<tr>
<td>Av-II</td>
<td>64,000</td>
<td>3.0</td>
</tr>
<tr>
<td>Av-I</td>
<td>61,000</td>
<td>3.15</td>
</tr>
<tr>
<td>Px-II ‡</td>
<td>42,000</td>
<td>ND§</td>
</tr>
<tr>
<td>CMC-Ia</td>
<td>44,000</td>
<td>2.95</td>
</tr>
<tr>
<td>CMC-Ib</td>
<td>41,000</td>
<td>2.95</td>
</tr>
<tr>
<td>CMC-Icd</td>
<td>38,000</td>
<td>2.90</td>
</tr>
</tbody>
</table>

* Molecular weights were estimated with sodium dodecyl sulfate-gel electrophoresis (11) and standard proteins.
† Isoelectric points were estimated by using a pH 2.5 to 8.0 gradient as described in the text.
‡ Molecular weight correlation not known.
§ ND. Not determined.

and the culture filtrate was fractionally precipitated by the addition of cold ethanol (−18°C) (14a). The fraction containing cellulytic 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, β-Glu-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% (NH₄)₂SO₄ 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 (NH₄)₂SO₄ precipitation, dialyzed against 10 mM sodium phosphate–0.14 M NaCl (pH 7.2), aliquoted, and stored at −70°C.

Immunoprecipitation of 35S-labeled celluloses. 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'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% Phama- lyte (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 dinitrosalicic 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 from p-nitropheryl-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 celluloses of S. commune. Several cellulytic 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...
isoelectric focusing (data not shown). The resulting immuno-
precipitate 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 NH2 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 [3H]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 NH2-
terminus proteolytic cleavages. However, we do not know
whether this occurs because of changes in the processing
during secretion or as a result of extracellular processing by
enzymes in the culture filtrate.

Secretion of cellulytic activity in response to cellulose. The
secretion of glucanase activities in response to cellulose was
measured by using p-nitrophenyl-β-D-glucoside and carbox-
ymethyl 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 cel-
lobiase activity approximately one-half of that on p-nitro-
phenyl-β-D-glucoside. The activity on higher oligosacchar-ides 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 mRNAs encoding the glucanases (16).

Identification of secreted proteins in response to cellulose.
The secreted proteins were labeled by the addition of
H235SO4 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 com-
ponents 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 appear-
ning 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 appear-
ance 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. communr (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 (△).]
when cellulose is the substrate. Are the cellulases expressed in a similar manner in response to cellulose 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 [3H]mannose incorporation or concanavalin A binding, suggests
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\textsubscript{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.

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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\textsubscript{2}SO\textsubscript{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.

LITERATURE CITED


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