Ethanol Perturbs Glycosylation and Inhibits Hypersecretion in
Trichoderma reesei

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The effects of ethanol and phenylethanol on the growth of and glycoprotein secretion by Trichoderma reesei were studied. Low levels (1.5%, vol/vol) of ethanol perturbed the glycosylation process as shown by alterations in the isoelectric profile of the secreted proteins and a reduction in the rate of incorporation of mannose into oligosaccharides. In addition to these effects on posttranslational modification, ethanol drastically lowered the protein secretion level of a hypersecretary strain.

The effects of short-chain alcohols, and particularly ethanol, on microbial physiology in both procaryotic and eucaryotic systems have been studied. A comprehensive review (3) and a very recent update (2) have presented strong arguments that much of the overall effect of ethanol on microorganisms is due to the physicochemical interaction of the alcohol with cell membranes.

It has become clear in recent years that protein transport and secretion processes in eucaryotic microorganisms occur via a membrane-mediated pathway similar to that found in animal cells (9). Secretory and membrane-bound proteins are cotranslationally transported into the lumen of the rough endoplasmic reticulum and initially (core) glycosylated there. The proteins are transported to the Golgi complex, where oligosaccharide modifications take place. The mature glycoproteins are then transported to their final destinations. Both the pre- and post-Golgi routings occur by the use of vesicle transport systems.

We are studying the synthesis, processing, and transport of glycoproteins by the cellulolytic filamentous fungus Trichoderma reesei. Since short-chain alcohols have been shown (3) convincingly to act as membrane perturbers in microorganisms, we explored and report here the effects of ethanol and phenylethanol on glycoprotein synthesis and secretion by T. reesei.

The protocols for growing T. reesei QM6a (wild type) and RL-P37 (a hypersecretory multistep derivative of QM6a), as well as the methods for measuring secreted cellulase activities (7) and analyzing the secreted protein mixtures by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (5) and isoelectric focusing gel electrophoresis, have been described previously (8).

We measured the effects of ethanol and phenylethanol on the mycelial growth rate of T. reesei and found that this fungus is fairly sensitive to these short-chain alcohols (Fig. 1). At concentrations at or above 2% (vol/vol) ethanol or 0.2% (vol/vol) phenylethanol, mycelial growth was inhibited significantly. T. reesei is thus much more sensitive to short-chain alcohols than are ethanol-producing eucaryotic microorganisms such as Saccharomyces cerevisiae, some of which are able to grow in media containing greater than 10% ethanol (3). For concentrations of ethanol less than those preventing growth of T. reesei, we measured the levels of protein secreted by mycelial cultures and the specific activities of the various enzymes that compose the secreted cellulase system. For wild-type strain QM6a, secretion levels (normalized to cell growth) remained fairly constant. However, for the hypersecretory strain RL-P37, we found that in the presence of low levels of ethanol, protein secretion was inhibited much more strongly than was mycelial growth. The data presented in Table 1 show that in the presence of ethanol at 1% in the growth medium, T. reesei RL-P37 secretes only about one-quarter as much protein, normalized to growth, as it does in the absence of ethanol. The reduced level of total secreted protein was also reflected clearly in a corresponding reduction in the levels of cellulase activities (7) detectable in the culture fluid, again normalized to mycelial dry weight (data not shown).

Because membrane-related effects of ethanol can be similar to the effects of growth at elevated temperatures (3, 6, 10), we investigated the effects of growth temperature on protein secretion by T. reesei. Following the same protocols as for the alcohol experiments, we consistently found that strain RL-P37 secreted only about one-half as much protein (normalized to dry weight) when it was grown at 37°C than when it was grown at 25°C, although the mycelial growth rates at these two temperatures were roughly equal. In general, the effects of ethanol and phenylethanol on protein secretion in T. reesei were similar to those obtained at the elevated temperatures (463-465).

<table>
<thead>
<tr>
<th>Strain</th>
<th>ethanol (%, vol/vol)</th>
<th>growth (mg [dry wt]/ml)</th>
<th>protein secreted (mg/μl)</th>
<th>secretion/growth (mg of protein/mg of [dry wt])</th>
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</thead>
<tbody>
<tr>
<td>QM6a</td>
<td>0</td>
<td>3.1</td>
<td>71</td>
<td>22</td>
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<tr>
<td></td>
<td>1</td>
<td>2.9</td>
<td>79</td>
<td>26</td>
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<tr>
<td></td>
<td>2</td>
<td>1.9</td>
<td>58</td>
<td>30</td>
</tr>
<tr>
<td>RL-P37</td>
<td>0</td>
<td>3.9</td>
<td>395</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>3.7</td>
<td>98</td>
<td>26</td>
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<tr>
<td></td>
<td>2</td>
<td>1.3</td>
<td>20</td>
<td>15</td>
</tr>
</tbody>
</table>

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TABLE 1. Effect of alcohol on growth and protein secretion by T. reesei

* Cultures were incubated as described in the legend to Fig. 1. Ethanol was added at 24 h. Cultures were harvested at 96 h, and dry weights and total secreted protein were measured as described previously (8).
corresponding studies on phenylethanol, we detected a slight preferential reduction in protein secretion (data not shown), but the effect was much less significant than that for ethanol or elevated temperature.

Polyacrylamide gel isoelectric focusing analyses were carried out on proteins secreted by *T. reesei* grown in the absence or presence of ethanol (Fig. 2). Growth in 1.5% ethanol resulted in two major alterations in the isoelectric focusing profiles from both strains. First, there were clear changes in the relative abundances of the various isoelectric forms of endoglucanases that band in the pH 4.5 to 5.0 region. Second, the normal multiple banding of the acidic cellobiohydrolase I (near pH 4) was reduced, and the forms of this enzyme that were secreted in the presence of ethanol had slightly altered isoelectric points. These changes in the isoelectric heterogeneity of the two most abundant enzyme types secreted by *T. reesei* suggest that one or more aspects of postranslational modification are altered by the presence of ethanol.

All of the proteins secreted by *T. reesei* were stained by the Schiff reagent for carbohydrate, but we repeatedly observed a decreased intensity of staining for carbohydrate (compared with duplicate gels stained with Coomassie blue for protein) in the protein bands from the *T. reesei* cultures grown in ethanol, suggesting that under these growth conditions, the proteins secreted by the fungus may be underglycosylated. To study this further, we determined the effect of ethanol on the incorporation of $[^3H]$mannose and $[^35S]$methionine into acid-precipitable material in viable protoplasts of *T. reesei*. Under conditions for which the incorporation of methionine (into protein) was unaffected, the rate of incorporation of labeled mannose (into glycoprotein or proteoglycan) was reduced approximately twofold in both strains QM6a and RL-P37 (Fig. 3). These data indicate that ethanol...
(at 1.5%) affects either the specific incorporation rate of mannose or the intracellular mannose pool. Taken together, the separate studies showing reduced staining for carbohydrate, the lowered incorporation rate of mannose, and the alterations in the electrophoretic heterogeneities of the major secreted enzymes strongly suggest that glycosylation was perturbed during growth in the presence of low levels of ethanol.

The effects of ethanol on secretion of glycoproteins by T. reesei are distinct from those caused by the drug tunicamycin (8), which is a specific inhibitor of the cotranslational addition of core glycans to asparagine residues (N-linked glycosylation) on the nascent polypeptide in the lumen of the rough endoplasmic reticulum. For example, ethanol caused no significant changes in the apparent molecular weights of the secreted glycoproteins, as measured by mobilities in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown). Thus, it is likely that ethanol does not inhibit the addition of N-linked core glycans in the rough endoplasmic reticulum, but rather that the alcohol perturbs the addition of short O-linked glycans or the modification of the N-glycans in the Golgi complex. The recent characterization of the N and O glycan structures of cellobiohydrolase I from T. reesei (I. Salovuori, M. Makarow, H. Rauvala, J. Knowles, and L. Kaariainen, BioTechnology, in press) provides a basis for future detailed oligosaccharide analyses of glycoproteins produced under interesting abnormal conditions.

In summary, we report here that the presence of ethanol alters posttranslational modification, including glycosylation, in T. reesei and dramatically lowers the protein secretion level of a hypersecretory strain of this filamentous fungus. We do not know the basis for the specific secretion-reducing effect of ethanol on the hypersecretory strain, but a possible explanation is that “normal” secretion, including that necessary for mycelial wall growth, and “hypersecretion” are to some extent distinct pathways, involving biochemically or spatially distinguishable secretory vesicles. If this is the case, a preferential inhibitory effect of ethanol on the formation or function of the hypersecretion vesicles might be the basis of the effect reported here. Another possibility is that ethanol causes a reduction in the abundance of endoplasmic reticulum, perhaps analogous to the heat-shock-induced destruction of endoplasmic reticulum recently reported to occur in the secretion-specialized aleurone layers in barley seeds (1).

The results reported here for the filamentous fungus T. reesei suggest that short-chain alcohols and other amphiphiles may be useful tools as membrane-perturbing agents in filamentous fungi for studies on the synthesis, processing, and targeting of both native and foreign glycoproteins.

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