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Interrelationship of Xylanase Induction and Cellulase Induction of *Trichoderma longibrachiatum*

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Xylose oligomers rapidly induced xylanase activity of *Trichoderma longibrachiatum*, whereas induction was delayed in the presence of glucose. Cellobiose, cellopentaose, and xylobiose did not induce detectable levels of cellulase activity. However, mixtures of xylobiose with cellobiose or cellopentaose rapidly induced cellulase activity. In addition, mixtures of xylobiose with cellopentaose or cellobiose induced xylanase activity more effectively than xylobiose alone. Both xylanase and cellulase activity were detected after a lag period in the presence of lactose.

Xylanases and cellulases are produced by a variety of fungi and bacteria (1, 6, 8, 28). These enzymes hydrolyze glycosidic bonds in cellulose and xylan, the two most abundant polysaccharides in nature. Both enzymes have potential application in the bioconversion of lignocellulose to useful end products (17, 25, 39). In addition, certain xylanases could be utilized for the selective removal of xylan from fiber and other plant materials (27, 30).

High levels of specific xylanase activity are secreted by noncellulolytic yeasts (4, 14–16). However, high yields of enzyme are dependent upon xylan in the culture medium; this fact may preclude enzyme generation on an industrial scale. In contrast, a variety of cellulolytic fungi, particularly *Trichoderma* spp., generate high levels of both xylanase and cellulase when cultured on heterogeneous wood pulp (37). The specificities in induction and activity of these enzymes have not been well characterized and may vary with different organisms (11, 33, 36, 37; J. C. Royer, Ph.D. thesis, State University of New York, Syracuse, 1988). Cellobiose has been proposed as the natural inducer of cellulase activity (13, 19). Xylobiose and xylan were shown to induce specific xylanases of *Trichoderma reesei*, while sophorose induced enzymes active on both xylan and cellulose (11).

For xylanase production to be studied, fungi are generally cultured on xylan. However, some filamentous fungi generate higher levels of xylanase when cultured on wood pulp than when cultured on pure xylan (31, 33). Conversely, cellulase generation by *T. reesei* QM 9414 was reduced when xylan was removed from wood pulp (11). Mutations affecting cellulase activity have often affected xylanase activity as well (22, 24). Together, these data suggest that the regulation of xylanase activity and the regulation of cellulase activity may be closely linked.

Trichoderma longibrachiatum Rifai generated high levels of xylanase and relatively low levels of cellulase when cultivated on lignocellulosic substrates (29, 31). Growth on xylan resulted in reduced levels of xylanase and very low levels of cellulase, while growth on lactose resulted in low levels of both xylanase and cellulase. Culture on a mixture of lactose (0.8%) and xylan (0.2%) resulted in significantly higher levels of both xylanase and cellulase than did culture on either substrate alone. Since the two major xylanases of *T. longibrachiatum* lack cellulase activity (Royer, Ph.D. thesis), the data suggested an interaction between the induction of these two enzyme systems. The purpose of the present study was to examine the specificity and interrelationship of xylanase induction and cellulase induction of *T. longibrachiatum*.

MATERIALS AND METHODS

Organism and culture. *T. longibrachiatum* was obtained from C. J. K. Wang at the State University of New York College of Environmental Science and Forestry, Syracuse, N.Y. The fungus was maintained on potato dextrose agar (Difco Laboratories, Detroit, Mich.). Spore formation was enhanced by culture on lactose (1.0%) plus Vogel salts (21).

Carbohydrate sources. Xylan from oat spelts was obtained from Koch Light (Haverhill, England); xylan from *Populus tremuloides* was isolated by the procedure of Timell (35). Carboxymethyl cellulose (type C-4888, lot no. 58C-0156) was purchased from Sigma Chemical Co., St. Louis, Mo. Cellopentaose was obtained from K. Grohman (Solar Energy Research Inst., Golden, Colo.); xylose oligomers used as standards were provided by T. E. Timell at the State University of New York College of Environmental Science and Forestry.

Additional xylobiose and xylotriose were generated by a modification of the method of Timell (35). Xylan from *Populus tremuloides* (6 g) was mixed with 100 ml of distilled water and 20 mg of purified xylanase A of *T. longibrachiatum* (Royer, Ph.D. thesis). The mixture was dispensed into dialysis bags (Spectrapore 132650, 23 mm), which were transferred to a 4-liter beaker containing 3.5 liters of distilled water. The mixture was stirred and maintained at 38 to 40°C on a hot plate-stirrer. The resulting oligomer-rich solution surrounding the dialysis bags was changed at 1, 2, and 4 days and concentrated by flash evaporation. Oligomers containing glucuronic acid residues were removed from concentrated oligomer solutions by sequential passage through AG 1X8 (carbonate form; Bio-Rad Laboratories, Richmond, Calif.) and Dowex 50W columns. The nonadsorbed neutral sugar fractions were concentrated by rotary evaporation after each column run and were resolved by chromatography through a column (1.5 by 100 cm) of BioGel P-2 (−400 mesh; Bio-Rad). The flow rate was maintained at 10 ml/h, with deionized water as eluant, and 1-ml fractions were collected. Oligomers were identified by high-performance liquid chromatography by using an HPX 42A column (Bio-Rad) maintained at 85°C. Water was used as eluant at a flow rate of 0.5 ml/min. Peaks were detected on the basis of the A_{192} and identified

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by comparison to standards. Fractions containing pure xylobiose or xylotriose were combined and concentrated before use in induction experiments.

Enzyme assays. The buffer used in all enzyme assays was sodium citrate-HCl (0.05 M, pH 4.8). Xylanase assays were modified carboxymethyl cellulase assays, described previously (18). Xylan substrate was either a 1% (wt/vol) suspension of xylan in sodium citrate-HCl or the soluble fraction remaining after centrifugation of a 1% suspension at 5,000 × *g* for 20 min. Reaction mixtures contained 0.50 ml of substrate and 0.50 ml of enzyme diluted in buffer to produce between 0.1 and 0.2 mg of reducing sugar in a 30-min assay at 50°C. Reducing sugars were detected by the dinitrosalicylic acid procedure (20), with xylose as the standard. Activity was expressed as micromoles of reducing sugar (as xylose) liberated per minute per milliliter of undiluted enzyme (units per milliliter).

Carboxymethyl cellulase assays were identical to xylanase assays, except that carboxymethyl cellulose was used as the substrate and activity was expressed as micromoles of reducing sugar (as glucose) liberated per minute per milliliter of undiluted enzyme.

Enzyme induction experiments. Fifty-milliliter portions of Vogel salts plus glucose (1.0%) were inoculated with spores and incubated on a rotary shaker (150 rpm) at 28°C. The resulting mycelium was ground in an Omnimixer (Ivan Sorvall, Inc., Norwalk, Conn.) for two 10-s bursts at high speed and used as inoculum (10% [vol/vol]) for a larger culture containing the same medium. After 20 h, the culture was washed with sterile distilled water and suspended in one-half the original culture volume of 2× Vogel salts. Aliquots of mycelium were next transferred to flasks containing an equal volume of carbon source (at twice the desired final concentration) in distilled water. Incubation was resumed, and replicate samples (1.5 ml each) were periodically removed, placed on ice, and centrifuged (12,000 × *g*, 10 min, 4°C). Supernatants were frozen before use in enzyme assays. Results are the mean values of the two replicates.

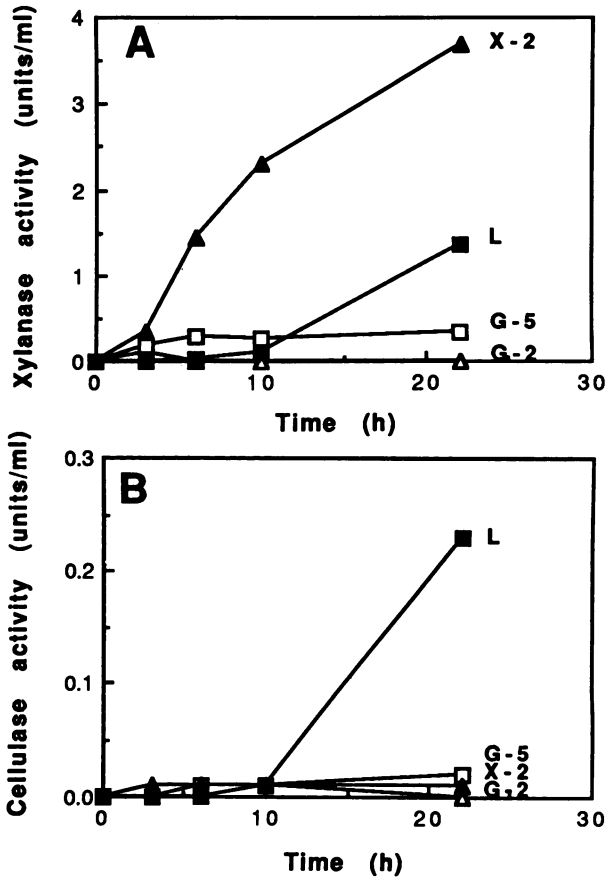

FIG. 1. Induction of xylanase activity (A) and cellulase activity (B) by various oligosaccharides. Washed, glucose-grown mycelium was suspended in medium containing 0.05% (wt/vol) oligosaccharide. Assay substrates were soluble oat spelt xylan (A) and carboxymethyl cellulose (B). Abbreviations: X-2, xylobiose; G-2, cellobiose; G-5, cellopentaose; and L, lactose.

RESULTS

Induction of xylanase activity by various oligosaccharides is shown in Fig. 1. Xylobiose rapidly induced xylanase activity. Lower levels were induced by lactose after a several-hour lag period. Neither cellobiose nor cellopentaose (nor xylose or methyl xyloside [data not shown]) induced xylanase activity. Of the oligosaccharides examined, only lactose induced significant levels of cellulase activity (Fig. 1B). The time course of cellulase generation on lactose mirrored that of xylanase generation.

Xylanase induction by various concentrations of xylobiose and xylotriose is shown in Fig. 2. Large increases in the concentration of either oligomer had relatively small effects on enzyme induction. Differences in xylanase induction by xylobiose and xylotriose were not notable at the highest oligosaccharide concentration tested. At an oligosaccharide concentration of 0.5% (wt/vol), induction by xylobiose was more rapid. However, induction by xylotriose continued for a longer period and resulted in the generation of significantly more enzyme. The presence of glucose delayed induction by several hours (Fig. 2A).

Xylanase induction by various combinations of oligomers is shown in Fig. 3A. Combination of either cellobiose or cellopentaose with xylobiose resulted in an enhancement of induction relative to induction by xylobiose alone. The effect was most notable at 3 h, when the combined-oligosaccharide treatments induced over sevenfold-higher activity than was induced by the treatment with xylobiose alone (Fig. 1A). The highest levels of xylanase were induced by the combination of lactose plus xylobiose, despite a lag period of several hours. After 22 h, activity was more than fourfold higher than the sum of the activities induced by each of these substrates individually.

Despite the fact that neither cellobiose, cellopentaose, nor xylobiose alone induced cellulase activity, combination of xylobiose with either glucose oligomer rapidly induced low levels of cellulase activity (Fig. 3B). Similar final yields of cellulase activity were obtained in the lactose (Fig. 1B) and the lactose-plus-cellopentaose treatments. However, formation was much more rapid in the treatment involving combined oligosaccharides. The highest levels of cellulase activity were generated in the lactose-plus-xylobiose treatment.

When crude enzyme preparations induced by the individual carbon sources were mixed and assayed, the increases in activity were only additive. Also, none of the oligosaccharides affected enzyme activity when included in assays at the initial concentrations described in Fig. 1.

DISCUSSION

Xylobiose rapidly induced specific xylanase activity of *T. longibrachiatum* and also that of *T. reesei* (11). However,

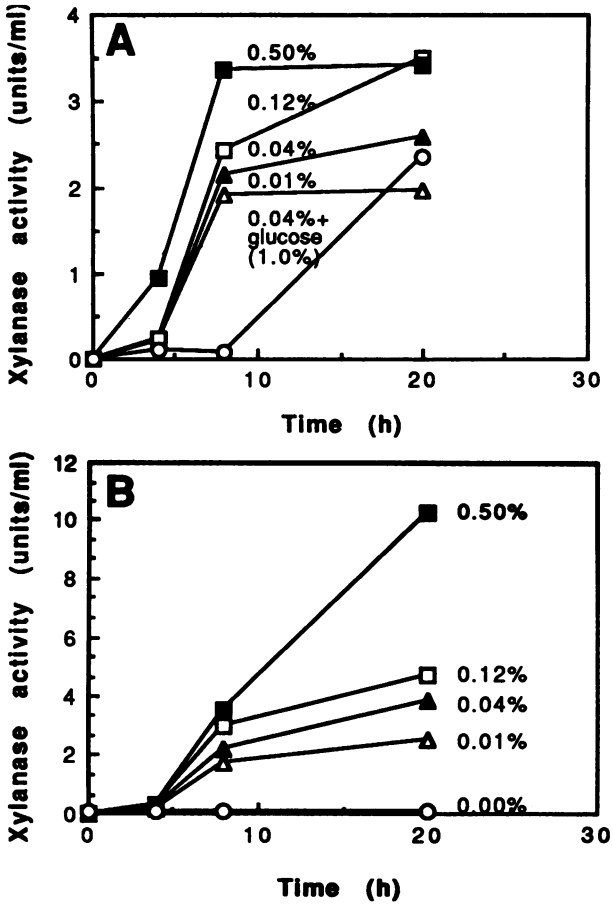

FIG. 2. Induction of xylanase activity by various concentrations of xylobiose (A) and xylotriose (B). Washed, glucose-grown mycelium was suspended in medium containing the indicated concentration (percent [weight/volume]) of oligomer. The assay substrate was aspen xylan (1% suspension).

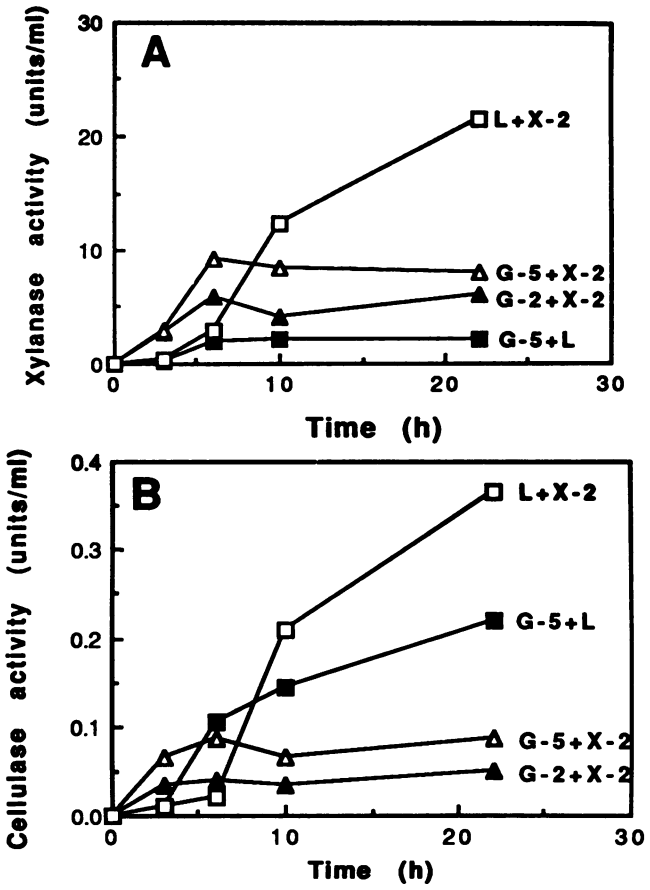

FIG. 3. Induction of xylanase activity (A) and cellulase activity (B) by combinations of xylobiose, cellobiose, cellopentaose, and lactose. Washed, glucose-grown mycelium was suspended in medium containing one of the oligosaccharides at a concentration of 0.05% (wt/vol). The assay substrates were soluble oat spelt xylan (A) and carboxymethyl cellulose (B). Abbreviations: X-2, xylobiose; G-2, cellobiose; G-5, cellopentaose; and L, lactose.

higher concentrations of xylobiose had little effect on the amount of enzyme produced by *T. longibrachiatum* in the present study. Similar results were obtained with *Cryptococcus albidus* (5), and it was concluded that the effect was due to catabolite repression by degradation products at high xylobiose concentrations. However, a saturation of some step in the inducing mechanism is also a possibility. Either possibility could explain the relatively low level of xylanase activity obtained with growth on pure xylan relative to growth on wood pulp (31, 33). A slight lag and prolongation of the period of induction at high xylotriose concentrations (Fig. 2B) suggests that the actual inducer of xylanase (xylobiose?) was formed gradually from xylotriose. A longer induction period or a lack of repression may have been responsible for the higher activity induced by xylotriose than by xylobiose at lower molar concentrations.

Neither xylose or methyl xyloside, which induced xylanase activity of yeast (5, 15, 38), induced xylanase activity of *T. longibrachiatum*. Delay of xylobiose induction in the presence of glucose suggests that the inducer is not metabolized in the presence of a repressing carbon source.

While cellobiose has been proposed as an inducer of cellulase activity, it failed to induce *Trichoderma* cellulase activity in this and several other studies (12, 26, 34). Variability in results could be due to genetic variability of the fungi or to differences in the purity of the inducing substrate. Cellodextrins also failed to induce cellulase in replacement cultures of *Trichoderma viride* (26).

Lactose induced both xylanase and cellulase activity of *T. longibrachiatum* after a lag time of several hours. A slow rate of induction of *T. reesei* cellulase activity by lactose has also been observed (34). Lactose has been referred to as an inducer of cellulase (9) and as a nonrepressive carbon source (5). The difference between the rates of enzyme formation in the presence of xylobiose and lactose suggests that different mechanisms may be involved. Alternatively, lactose may be slowly modified to a true inducer of xylanase activity.

Concurrent formation of cellulase and xylanase with lactose suggested that nonspecific enzymes may be responsible for both xylanase and cellulase activity. The two most active xylanases of *T. longibrachiatum* grown on wood pulp are highly specific (Royer, Ph.D. thesis). However, growth on lactose could result in synthesis of a different set of nonspecific enzymes. The question could be resolved through the use of cellulase and xylanase zymograms (2, 32).

The results of our induction studies, presented here, substantiate previous evidence for an interaction between xylanase induction and cellulase induction. Synergistic effects were not observed when enzyme preparations induced by the individual oligomers were mixed together and as-

sayed. This suggested that the interaction was on enzyme induction rather than formation. It is unlikely that the enhancement of enzyme induction with mixtures of inducers was solely due to the increase in carbon content. In previous work (31), culture on 1.0% cellobiose failed to result in measurable cellulase formation, and in the present study (Fig. 2) a 50-fold increase in xylobiose concentration resulted in a less than twofold increase in xylanase induction. In addition, a higher level of xylanase was induced by a mixture of 0.05% lactose and 0.05% xylobiose (Fig. 3A) than was generated after 1 week of culture on 1.0% lactose (31).

Enhancement of xylobiose induction of xylanase by lactose, cellobiose, or cellopentaose could result from increased biomass formation or a prolongation of the period of induction due to decreased utilization of the inducer. Nanda et al. (23) proposed that sorbose enhanced cellobiose induction of cellulase by *T. reesei* by reducing the rate of uptake of the inducer. However, the rapid rate of induction in the xylobiose-plus-glucose-oligomer treatments suggests a different mechanism.

Induction of cellulase by combinations of xylobiose with cellulose oligomers is difficult to interpret since none of these substrates was an effective inducer alone. It has been proposed that the actual inducer of cellulase in *Trichoderma* spp. is not cellobiose but an enzymatically modified product of cellulose or cellobiose such as sophorose (7, 10, 26). It was assumed that β-glucosidases or cellulases were responsible for such modifications. However, many xylanases, including an enzyme from *T. longibrachiatum* (Royer, Ph.D. thesis), exhibit transglycosidase activity, and a specific xylanase of *C. albidus* was capable of transferring a cellobiose unit to a xylosyl acceptor (3). Therefore, xylobiose-induced xylanases (or cellulases that are either poorly expressed or inactive individually on cellulose) may be capable of modifying cellulose oligomers (or lactose) to form inducers of cellulase activity. Alternatively, xylobiose may induce cellulase only during growth on nonrepressive carbon sources, such as lactose or cellulose oligomers.

The results of the present study suggest that both similarities and differences, as well as an interaction, exist between the induction of xylanase activity and cellulase activity of *T. longibrachiatum*. Both enzyme activities are generated on lactose. However, induction of specific xylanase activity by xylose oligomers and lack of cellulase induction by cellobiose and cellopentaose point to major differences in the regulation of these two enzyme systems. Cellulase activity of *T. longibrachiatum* has not been observed in the absence of xylanase activity, suggesting that induction of cellulase activity may be linked with induction of xylanase activity. Such a relationship is not surprising when one considers the intimate association of hemicellulose and cellulose in plant biomass.

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