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Investigation of the Cell–Wall Loosening Protein Expansin as a Possible Additive in the Enzymatic Saccharification of Lignocellulosic Biomass

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Introduction

Expansins are a class of proteins that enable and regulate the extension of plant cell walls, thereby, among other possible functions, permitting the growth of plant cells *(1–6).* Although the precise molecular mechanism of expansin action remains to be determined, considerable evidence points to their mediating the exchange of interpolymer interactions (between cellulose and matrix-polymers, or between cellulose microfibrils themselves) that allows microfibrils to "creep" past each other in response to mechanical stress applied by the expanding cell inside *(1,2).* Because expansins have not been found to have any detectable glycosidase activity *(7),* it seems likely that the interactions being broken and then reformed under expansin influence are noncovalent, although the involvement of some extremely specific glycosyl transferase activity cannot be ruled out *(1).* Although expansins do not hydrolyze cellulose, they have been reported to enhance the hydrolysis of microcystalline cellulose by low levels of a mixed *Trichoderma* cellulase preparation *(8).*

From the viewpoint of the biomass-to-ethanol program, an additional significant feature of expansins is that their cell–wall loosening action is activated by mildly acidic conditions (pH ≤ 5.0), whereas expansins are

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inactive at neutral pH *(1)*. With the current process pH for simultaneous saccharification and fermentation of biomass established near pH 5.0, expansins would appear to be of possible use as additives to facilitate enzymatic saccharification. The standard biomass substrate we are currently studying is dilute-acid-pretreated yellow poplar (PYP) sawdust. Pretreatment has removed most (but not all) of the hemicellulose from this substrate. Substantial lignin content remains (roughly one third of the substrate dry wt), and microscopic examination reveals that the substrate particles are structurally complex. Because steric hindrance is likely to be an important factor in the enzymatic depolymerization of the cellulose content of the biomass, an additive that could loosen the structure, and allow attack at new surfaces, could be quite helpful.

We therefore decided to investigate the use of an expansin as an additive in the enzymatic saccharification of biomass. Presented here are the results of a preliminary study on the effect of β-expansin-D on the saccharification of PYP by a *Trichoderma reesei* cellulase mixture produced by the National Renewable Energy Laboratory (NREL) and by a reconstituted mixture of purified cellulases.

Materials and Methods

Protein Production

T. reesei was grown at NREL on Solka-Floc; the filter paper activity of the culture filtrate was determined according to the recommendations of Ghose *(9)*. *T. reesei* CBHI was purified at NREL from a commercial mixture according to a procedure described previously *(10,11)*. The catalytic domain of *Acidothermus cellulolyticus* endoglucanase-1 (E1cd) was prepared at NREL by papain cleavage of the gene product expressed in *Streptomyces lividans*, as described previously *(12)*. Electrophoretically pure β-expansin (Zea m1, isoform D) was obtained by extraction of *Zea mays* pollen in 50 m*M* sodium acetate buffer (pH 4.5), followed by chromatography on carboxymethyl Sepharose (Pharmacia, Uppsala, Sweden) and a CM-high-performance liquid chromatography column. This protein preparation had no detectable cellulase or endoglucanase activity.

Cellulosic Substrates

PYP was produced at NREL by previously described procedures *(13,14)*. Microcrystalline cellulose (Sigmacell, Type 20) was purchased from Sigma (St. Louis, MO). Both substrates were pipeted from well-stirred slurries to generate sets of substrate aliquots having uniform dry weights and particle size distributions *(13)*.

Assay Procedure (Diafiltration Saccharification Assay)

Cellulase assays were carried out at 40°C in 20 m*M* acetate buffer, pH 5.0, using the diafiltration saccharification assay apparatus previously described *(13)*, except that Biomax-5 ultrafiltration membranes (5000 nomi-

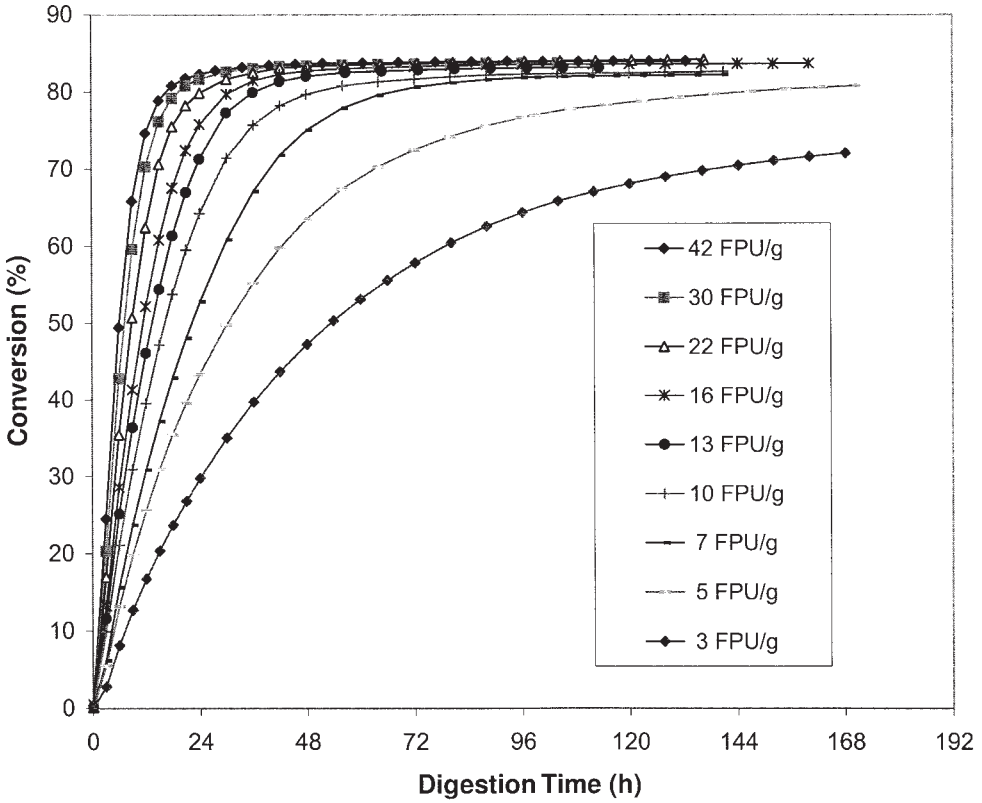

Fig. 1. Effect of enzyme loading on the saccharification of PYP by Solka-Floc-grown *T. reesei* cellulase at pH 5.0 and 40°C. **(Inset)** Numbers are the enzyme loadings in terms of filter paper units per gram of biomass cellulose.

nal molecular-weight-cut-off; Millipore, Burlington, MA) were used in place of the Amicon (Beverly, MA) PM-10 membranes (10,000 MWCO) used in the previous study. For all assays in this series, the buffer flux through each cell was maintained at 0.02 mL/min. Enzyme loadings were expressed in terms of measured filter paper units (FPU)/g of biomass cellulose for the *T. reesei* cellulase mixture, and in terms of the "protein equivalents of FPU" for the synthetic mixture of *A. cellulolyticus* E1cd and *T. reesei* CBHI. In the latter case, 1.67 mg of protein was considered to be equivalent to 1.0 FPU *(15).*

Results and Discussion

Figure 1 displays the results of a performance-vs-loading study of the digestion of PYP by a cellulase mixture produced at NREL by *T. reesei* growing on Solka-Floc. This loading study is presented here simply to illustrate the rationale behind our choice of the cellulase loading (5 FPU/g of biomass cellulose) used to obtain the remainder of the data given here. Figure 1 reveals that all cellulase loadings from the highest (42 FPU/g of

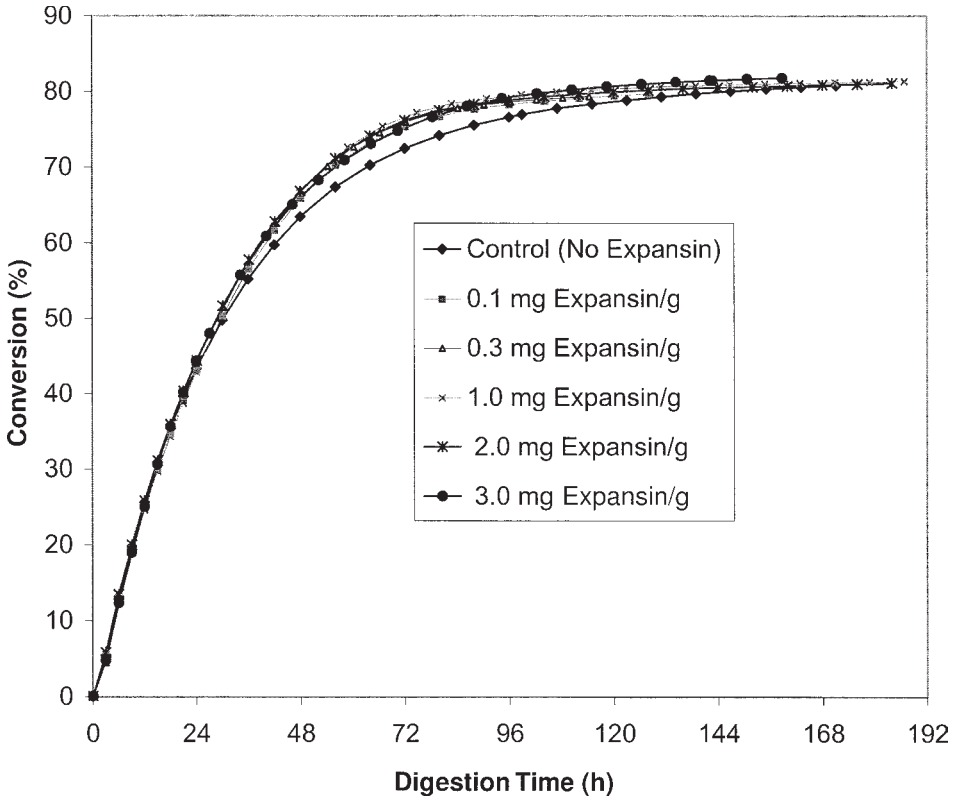

Fig. 2. Effect of expansin on saccharification of PYP by NREL Solka-Floc-grown *T. reesei* cellulase at pH 5.0 and 40°C, with a fixed cellulase loading of 5 FPU/g of biomass cellulose. **(Inset)** Numbers refer to the loading of expansin protein in milligrams per gram of biomass cellulose.

cellulose) down to 7 FPU/g of cellulose produced essentially the same soluble-sugar yields (approx 84%) by the end of the standard 120-h digestion period, although the rates at which this yield is approached do vary with enzyme loading. The two smaller loadings in this study (5 and 3 FPU/g) did not reach 84% conversion by the end of 120 h. Of the loadings investigated, the 5 FPU/g of cellulose loading was chosen as an "almost competent" loading that had more room for improvement in performance than did the higher loadings, but was effective enough as it stood to provide reasonable hope that an activity-enhancing additive might bring its performance into the acceptable range of 80%+ conversion by 120 h.

Figure 2 shows the performance of a 5 FPU/g of cellulose loading of the *T. reesei* cellulase mixture in saccharifying PYP in the presence of five levels of β-expansin, compared with a control run in which no expansin was added. At all levels of added expansin, the cell-wall elongation-promoting protein appears to enhance the rate of saccharification over the range of 55–80% conversion, but does not appear to increase significantly the ultimate (120 h and beyond) level of conversion. A striking additional

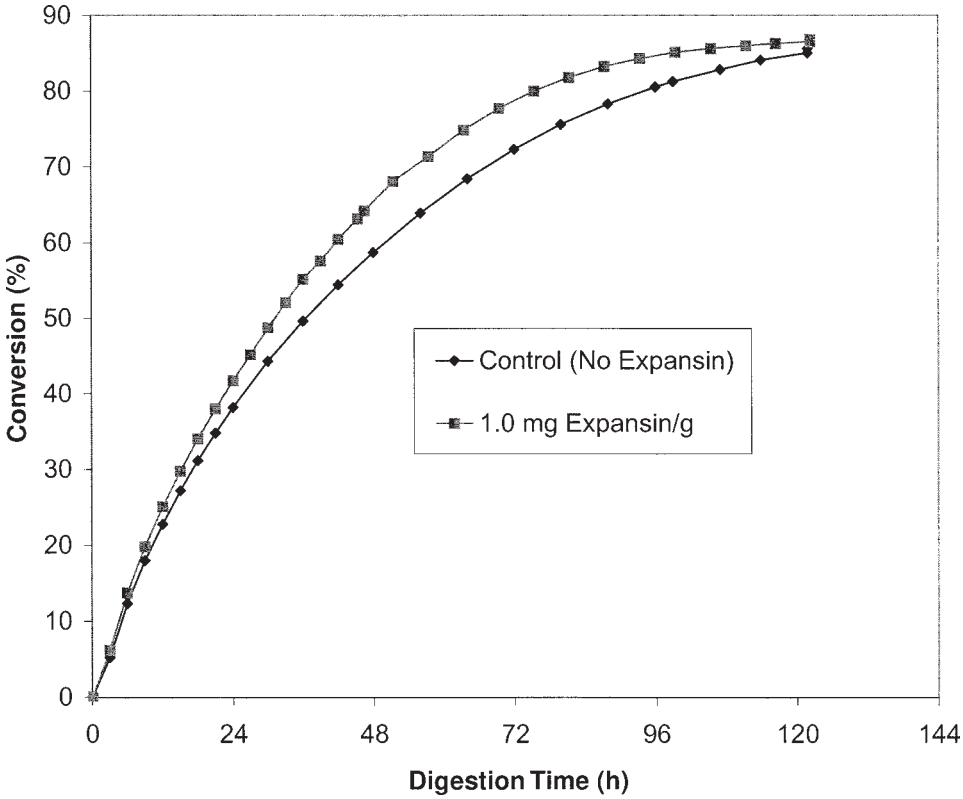

Fig. 3. Effect of expansin on saccharification of microcrystalline cellulose (Sigmacell-20) by NREL Solka-Floc-grown *T. reesei* cellulase at pH 5.0 and 40°C, with a fixed cellulase loading of 5.0 FPU/g of biomass cellulose.

observation is that the extent of the kinetic enhancement does not appear to depend significantly on the level of expansin added. For the range of expansin loadings used, at least, the expansin effect appears to be limited in magnitude, and to require only that a very small level of expansin be present.

Figure 3 shows a similar expansin effect for this cellulase system in the saccharification of a different substrate, microcrystalline cellulose (Sigmacell-20). As was observed for the lignin-containing PYP substrate, the kinetics of approach to the final conversion value appear to be enhanced, without a significant increase in the extent of saccharification obtained by 120 h.

The digestion data shown in Fig. 4 indicate that the expansin effect may be dependent on certain characteristics of the cellulase system to which expansin is added, and may not be seen with all cellulase systems. Figure 4 presents diafiltration saccharification assay digestion data for a reconstituted mixture of one purified endoglucanase (the catalytic domain of *A. cellulolyticus* E1, or E1cd) and one cellobiohydrolase (*T. reesei* CBHI), working against PYP as a substrate. Here the total cellulase protein loading (E1cd plus CBHI) is 8.35 mg protein/g of cellulose, which, given the

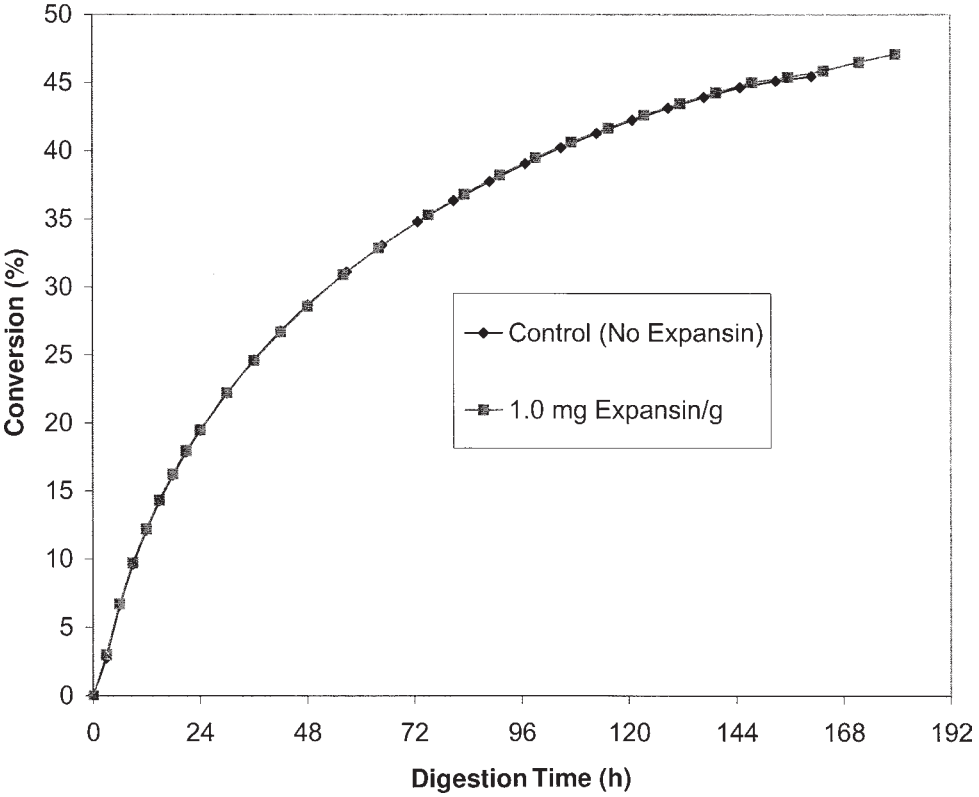

Fig. 4. Effect of expansin on the saccharification of PYP by a reconstituted cellulase mixture containing 5.3% *A. cellulolyticus* E1cd and 94.7% *T. reesei* CBHI on a molar basis, and used at a total loading of 8.35 mg/g of biomass cellulose (equivalent to the protein loading in a 5 FPU/g loading of typical *T. reesei* cellulase). Assays were run at pH 5.0 and 40°C, and contained *Aspergillus niger* β-glucosidase at 0.045 mg/mL to convert essentially all cellobiose produced to glucose.

assumption that 1.67 mg of protein in a typical *T. reesei* cellulase preparation provides 1.0 FPU of activity, is the protein equivalent of a loading of 5 FPU/g of cellulose if using *T. reesei* cellulase. In this case, the progress curves in the presence and absence of 1.0 mg of expansin/g of cellulose are identical. One possible explanation for this difference in the susceptibilities of the two enzyme systems to enhancement by expansin is that expansin may be synergizing with a component of the considerably more complex *T. reesei* system, which has no equivalent in the simple reconstituted mixture. One additional difference between the experiments of Fig. 4 and those of Fig. 2 is that the reconstituted "binary" enzyme mixture is acting only against the first 46% or so of the biomass cellulose, whereas the *T. reesei* system shows an expansin effect only during the saccharification of the (probably more recalcitrant) material attacked after 50% or more of the cellulose has been solubilized. The possible effect of this factor was addressed, to some extent, by an experiment in which a heavier loading of

the E1cd/CBHI mixture (25 mg/g of cellulose, equivalent to the protein loading in a 15 FPU/g of *T. reesei* loading) was used against PYP. Although this further experiment (data not shown) did push the solubilization of PYP cellulose beyond 60%, the curves obtained in the presence and absence of 1.0 mg of expansin/g of cellulose were as identical to each other as are the corresponding curves seen in Fig 4.

Conclusion

Although the expansin effect, as observed under the present conditions, appears to be somewhat limited in magnitude, the observation that extremely small additions of expansin (0.1 mg of expansin/g of cellulose added to approx 8.35 mg of *T. reesei* protein/g of cellulose; Fig. 2) suffice to produce a consistent effect, makes this protein worthy of additional study. In addition, the apparent selectivity of the effect for certain cellulase systems suggests that further studies may identify the specific portion of the cellulose-depolymerization process with which the expansin is interacting.

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