Identification and Characterization of Insect Cellulolytic Systems for Plant Biomass Degradation

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Project Location: University of Tennessee

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1. Planned Activities:

Our proposed activities for this time period included:

A) Characterization of cellulolytic activity in emerald ash borer (EAB) larvae and in samples from coleopteran larvae from New Zealand using activity assays and zymograms.

B) Cloning of an endocellulase from *Tribolium castaneum* and expression in yeast cultures to provide proof-of-concept for our cDNA library cellulase screening approach.

2. Actual Accomplishments:

A) We have started a collaboration with colleagues at AgResearch New Zealand received samples of digestive fluids collected from larvae of two coleopteran species autochthonous to New Zealand that are known for feeding on lignocellulosic materials. Samples tested included midgut and hindgut of New Zealand grass grub (*Costelytra zealandica*) and red-headed cockchafer (*Adoryphorus couloni*) larvae. We performed cellulase quantitative assays using the DNSA methodology and carboxymethyl cellulose (CMC), microcrystalline cellulose (MCC), unprocessed switchgrass, and filter paper as substrates. The samples arrived to us after one week since their shipment date. With no refrigeration left in the package, it is possible that some of the cellulases were degraded during shipment. Thus, data from these assays has to be taken cautiously. Furthermore, the midgut sample from *C. zealandica* was lost due to an error during data acquisition, limiting the assays with this species to the hindgut sample.

The highest average activity for each insect sample was observed when using CMC as substrate, which is common to previously tested insect samples (see Oppert et al, 2010). This abundance of activity against CMC suggests that endoglucanase activity is predominant in these samples. The midgut of *A. couloni* had the highest overall activity (0.401 U/mg), which is relatively high when compared to activity in other insects (Oppert et al., 2010). Lower levels of activity were detected when using filter paper as substrate. When compared to the cellulase standard (cellulase from *Aspergillus niger*), the midgut of *A. couloni* showed a remarkably similar level of activity (0.274 U/mg for the cellulase standard and 0.2565 U/mg for *A. couloni*). This is a relevant level of activity, especially when considering that the insect samples are a complex mixture of enzymes and the control cellulase a purified sample. These data also suggest that cellulase activity in this
insect is mostly localized to the midgut and not the hindgut, which may suggest the presence of insect-derived cellulases. The lowest average activity was observed when the samples where assayed with MCC or switchgrass, with none of the samples producing even 10% of the glucose that the cellulase standard did. This low activity against crystalline forms of cellulose is also observed in other insect samples (see Oppert et al, 2010), and may suggest lack of efficient exoglucanases in these samples. However, it is also possible that these enzymes are less thermostable and more prone to degradation during shipping and experimentation.

The in-gel zymogram assay allowed us to detect cellulase protein bands in the insect samples. For the C. zealandica samples we detected two main areas of cellulase activity of the same molecular size (approx. 30-kDa and 40-kDa) as the commercial cellulase mixture. In contrast, only the smaller activity band was observed in samples from A. couloni.

These data demonstrate the existence of relatively high cellulase activity in C. zealandica and A. couloni. Further testing with additional materials would be necessary to characterize these activities and to test their potential use in degradation of biofuel feedstocks.

We also tested using the DNSA assays digestive fluids from larvae of the Asian longhorn beetle (Anoplophora glabripennis), a devastating invasive species that tunnels in hardwood trees. Our collaborator at USDA-APHIS (Dr. Damon J. Crook) has a colony of these insects and sent us fluids from larvae fed an artificial diet or on wood. Due to limited sample amounts, our activity assays were done using CMC as substrate. We observed that activity in wood-fed larvae was not measurable, probably due to wood-derived contaminants that inhibited the DNSA assay. Activity against CMC was approximately 0.2 U/mg, which is similar to alternative insect species (Oppert et al., 2010).

B) We previously cloned an endoglucanase from Tribolium castaneum that we named TcEG1. This cellulase displayed activity in alkaline buffer (Ph 9) when expressed in cultured insect cells. We were interested in developing an expression cDNA midgut library to identify and directly obtain sequence data for our most active insect species. To test the possibility of using yeast as our heterologous expression system, we cloned and expressed TcEG1 in yeast, and further characterize its activity. The advantage of this yeast system is that transformed yeast can be selected using media containing CMC as the only carbon source, so that only clones expressing active cellulases would be able to survive. Our intention is to use this system then would be used to screen expression cDNA libraries. Transformation of TcEG1 into yeast resulted in expression and secretion of TcEG1. Activity assays demonstrated that the expressed enzyme is fully active and retains its unique activity at high pH.

3. **Explanation of Variance:**

The only setback we had was that the samples from New Zealand arrived thawed and probably partially degraded. We are planning to develop a more efficient method to obtain these samples for further testing. Our data with TcEG1 expression in yeast suggests that this system may be used to select for clones expressing active cellulases.
The advantage of this genomic strategy (cDNA expression library) is that only active cellulases are detected, limiting identification of cellulases with low activity.

4. **Plans for Next Quarter:**
   We plan the following activities to attain the project goal:
   - Obtain additional samples from New Zealand species to test their activity. Together with our collaborators we are submitting a proposal to the New Zealand government for funding to visit our collaborators and perform sampling and activity assays with native species. In our preliminary assays these species display high cellulytic activity compared to other screened species.
   - Further characterization of the TcEG1 cellulase expressed in yeast. We plan to obtain information on its stability in ionic liquids used for pretreatment of lignocellulosic biomass, as well as data on its specificity and the products it generates during cellulose digestion.

5. **Budget:**
   a. Funds Expended to Date (End of Reporting Period): $189,695.54
   b. Remaining Balance of Funds: $25,299.46

6. **Patents**

   Patent application submitted through UTRF on 17th September, 2008: Application number 61097754, Title: Cellulases from insects, Inventors: Jurat-Fuentes, JL, Oppert, C. Klingeman, B., Willis, J., and Oppert, B.

   Invention disclosure: “Novel cellulases from Lepismatidae and applications for biofuel production” UTRF file No. PD10064.

7. **Publications / Presentations:**

   Oppert, C., Klingeman, W., Willis, J. D., Oppert, B., and J. L. Jurat-Fuentes (2010). “Prospecting for cellulytic activity in insect digestive fluids”. Comp. Biochem. Physiol., 155B(2):145-154. In this publication we describe data from our cellulase screening of more than 100 different insect species as our basis to select a short list of species with high activity to target for cloning of cellulases.

   Willis, J. D., Oppert, B., Oppert, C., Klingeman, W. E., and J. L. Jurat-Fuentes (2010) “Cloning, expression, and characterization of a GHF9 cellulase from *Tribolium castaneum* (Coleoptera: Tenebrionidae)” Insect Physiol., submitted, in review. This publication reports the identification, cloning, expression, and initial characterization of a cellulase from *T. castaneum*. This cellulase has unique activity under alkaline pH.

the insects displaying high cellulase activity from our screening. The paper presents the quantitative estimation of activity, purification of enzymes, and zymograms.


Invited speaker: “Prospecting insects for cellulases to optimize biofuel production” J. L. Jurat-Fuentes. Invited seminar at the Biofuels Institute, Jiangsu University, Zhanjiang (China), June 2010.

Poster presentation: Derek Shirley, Cris Oppert, Bethany Miracle, Todd Reynolds, William Klingeman, and Juan Luis Jurat-Fuentes “Functional characterization of an endoglucanase from Tribolium castaneum in Saccharomyces cerevisiae” Presented at the 58th Annual Meeting of the Entomological Society of America (San Diego, CA), December 2010. This presentation won the ESA President’s Award.