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U.S. Dept. of Energy: "Cellulase - A Key Enzyme for Fermentation
Feedstocks

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Final report and summary, Proposed Experimentation and Revised Budget

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Report	Page 1
Publication/Presentations/Theses	Page 4
Final Report	Page 6

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Report

Biomass can be fermented to a range of useful products. For efficiency a well characterized and effective cellulase is necessary to gain fermentable sugars from cellulotics (forest by-products and agricultural wastes and municipal refuse). Microbial cellulase systems are comprised of multiple components namely, endoglucanase (EG), cellobiohydrolase (CBH), and beta-glucosidase, which act synergistically. However, their mechanism of interaction in the hydrolysis of crystalline cellulose is poorly understood.

We have been clarifying the cellulase system especially that of *Microbispora bispora* through isolation of the individual components. As there is no direct assay for the cellobiohydrolase component, we have made monoclonal antibodies to them, and are using them to characterize the cellobiohydrolases of *Trichoderma reesei* and *M. bispora*. With *T. reesei*, rapid purification has been achieved through MAb affinity chromatography and also a highly sensitive MAb assay for CBH in crude cellulase broths has been developed. For *M. bispora*, the monoclonal antibodies towards CBH have been prepared and are being used for selection of clones from a *M. bispora* gene library. We have focussed on the *M. bispora* cellulase.

The cellulase components of *M. bispora* have been clarified. There are thermally stable endoglucanases, an exo-glucanase and two beta-glucosidases (Bgl) of the latter, Bgl B is noteworthy being resistant to end-product inhibition. Bgl A is noteworthy in that it attacks small oligomers and is a glucan-glucanohydrolase.

Sequence Analysis and Characterization of Endoglucanases from *Microbispora bispora*

We have previously constructed a gene library of *Microbispora bispora* in *Escherichia coli*. The library was screened via *in situ* zymograms for the detection of endoglucanase activity. Twelve colonies exhibited endoglucanase activity. These colonies yielded five unrelated clones (as shown by restriction mapping of the inserts). However only two clones exhibit detectable endoglucanase activity by reducing sugar assay. A major endoglucanase gene, MBICEL1, from clone CC3622 is encoded on a fragment of 4.28 kb. This fragment was represented seven times in the gene bank. Another endoglucanase- MBICEL2, from clone CC6704 is encoded on an fragment of 7.0 kb. The MBICEL1 fragment produces two major activity bands of 58 kd and 47 kd and a minor activity band of 38 kd (in zymograms). The MBICEL2 fragment produces a single activity band of 50 kd. When the enzymes produced by these cloned fragments and *M. bispora* culture both enzymes are analyzed electrophoretically on denaturing or nondenaturing PAGE, the endoglucanases from both preparations comigrate.

The insert from plasmid pCC3622 was subcloned into pUC119 (Vieira and Messing) in both orientations to create plasmids pCCMY116 and pCCMY117. These plasmids were used to construct unidirectional nested deletion sets from each end of the insert. The nucleotide sequence of the gene was then determined by the Sanger dideoxy method.

From a combination of nucleotide sequence with 5' and 3' deletion analysis we have determined the that coding region of the MBICEL1 gene is. 1374 nucleotides and encodes a protein with a predicted molecular weight of 60,162 daltons. This size is consistent with the size of the active enzyme as analyzed on SDS-PAGE (58 kd). The coding region is 70.1% G or C and thus the codon usage is severely restricted. The occurrence of G or C in the third position of codons is 98%. Twenty-seven codons are not utilized and several are seen one to three times.

The MBICEL1 gene is expressed in *E. coli* by the activity of it's native promoter. A putative Pribnow box is positioned some 300 bp from the AUG. Removal of these sequences results in a dramatic drop in activity much like a promoter down mutation. Alternatively the native Pribnow box may be closer and not well recognized by *E. coli* RNA polymerase and that the sequence 300bp upstream is fortuitously recognized. Therefore we cannot absolutely assign the position of the native promoter. The gene has a Shine-Dalgarno sequence 8 bp upstream from the AUG. At the 3' end of the gene a strong stem-loop structure can form 20 bp from the termination codon, reminiscent of a rho independent transcription termination signal.

The protein sequence deduced from the MBICEL1 gene shows regions of primary amino acid sequence and conformational homology with the endoglucanases of other microorganisms. The enzyme has a positively charged NH₂ ter-minus followed by a hydrophobic leader sequence of 34aa. proline rich sequence (which could form an alpha-helix) at aa325 to aa375 is similar to such possible helices in other endoglucanases. We refer to this amino acid sequence as the Ser-Pro box. This proline rich sequence in MBICEL1 shows strongest homology to the Thr-Pro box in the *Cellulomonas fimi* endo- and exoglucanases which could also form alpha-helices. The Ser-Pro box in MBICEL1 occurs in approximately the same position in the enzyme as seen in the *C. fimi* exoglucanase (approximately 100 amino acids from the COOH terminal end of the enzyme). The sequence contains a stretch of 33 amino acids with 14 proline, 11 serine and 3 threonine residues. This stretch includes three heptapeptides: SPSPTPS, TPSPSPS, SPSPSPS. The first two peptides are colinear and separated from the third by QSDPG.

The MBICEL1 endoglucanase appears to be cleaved in *E. coli* yielding three active bands (in zymograms) of 58 kd, 47 kd and 38 kd. The -two larger bands seem equally active and the third is a minor band. These three bands correspond to bands present in extracellular cellulase from *M. bispora* culture fluid, the implication being that *E. coli* may be capable of processing this protein in a manner similar to *M. bispora*.

We have conducted preliminary truncation studies on the enzyme. Approximately 100 COOH-terminal amino acids can be removed without loss of activity. When these amino acids are removed the enzyme produced is 47kd. This enzyme is also cleaved in *E. coli* as the minor 38kd active band can be seen in zymograms. The elimination of more than 100 amino acids from the COOH terminus results in removal of the heptapeptides discussed above and concomitant loss of enzyme activity.

There are three putative active sites (pac) in the MBICEL1 endoglucanase. Two, pac1 and pac2 show homology to the active site of 774 lysozyme and one (pac3) shows homology to the active site of Hen Egg White lysozyme. The pac2 site also shows limited homology to the proposed. active site of *T. reesei* CBRI. The pac2 site shows the Asp-Glu-9aa-Asp seen in the T4 lysozyme active site as well as other matches of identical and conservatively replaced amino acids for a stretch of 19aa. As active site

arrangements are elucidated more will be understood about the optimum spacing needed between acidic amino acids for cleavage of different β -linked polysaccharides by acid catalysis. For now the MBICEL1 active site position and conformation is an Open investigation.

Cloning of Beta-Glucosidase from *Microbispora bispora*.

Fourteen colonies exhibiting beta-glucosidase activity yielded six independent clones. These six clones fall into two groups based on homologous regions seen by restriction fragment mapping. The relative specificity of the enzymes produced by these two types of fragments differ dramatically. The Type I fragment produces an enzyme which exhibits a strong preference for methylumbelliferyl- β -D-glucose while the Type II fragment exhibits a broader specificity including MU- β -D-glucose, MU- β -D-cellobiose, MU- β -D-N-acetylglucosamine, and MU- β -D-xylose. The β -glucosidases are cloned on relatively large fragments. At present these genes are being subcloned for sequencing.

Cloning of Cellobiohydrolase from *Microbispora bispora*

We have not yet been able to detect colonies exhibiting cellobiohydrolase (CBH) activity using MU- β -D-cellobiose. At present we are screening the gene bank with the newly developed monoclonal antibodies to the CBHI from *M. bispora*. At present we have detected presumptive clones in this manner. Further analysis will be required to confirm whether these clones contain the CBH genes of *M. bispora*.

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Eveleigh, D.E. 1987. This Rotten World: Mushrooms, History and Music. Sigma Xi Lecture, Waksman Institute, Rutgers University, November, New Brunswick, NJ.

Riske, F.J., D.E. Eveleigh and J.D. Macmillan. 1987. Development of a Monoclonal Antibody Assay for Cellobiohydrolase from *Trichoderma reesei*. Theobald Smith Society, New Jersey.

Kahrs, S.K., M.D. Yablonsky, Z.P. Shalita and D.E. Eveleigh. 1987. Characterization of Exo- and Endoglucanases of *Microbispora bispora* by Physiological and Immunological Techniques. Theobald Smith Soc. New Jersey.

Eveleigh, D.E. "Cellulose to Ethanol Problems and Successes", University of Graz, Austria. Österreichische Gesellschaft für Biotechnologie and Österreichische Biochemische Gesellschaft at the Institute for Biochemistry. April 19.

Eveleigh, D.E. "Magnificent Microbes - Biotechnology to Biomass". International Atomic Energy Agency, Vienna, France. April 29.

Eveleigh, D.E. "Development of Improved Rural Methane Production from Biomass Utilizing Nuclear Techniques". Third FAO/IAEA Research Coordination Meeting, Tiubliana, Yugoslavia. May 10-14.

Eveleigh, D.E. "Cellulose to Ethanol - Problems and Successes". Slovenian Microbiological Society, Boris Kidric. Institute, Ljubljana, Yugoslavia. May 12.

Eveleigh, D.E. "Cellulose to Ethanol - Problems and Successes". Central Food Research institute and Technical University, Budapest, Hungary. June 1.

Eveleigh, D.E. "Cellulose to Ethanol - Problems and Successes". Technical University, Lodz, Poland. June 4-5.

Eveleigh, D.E. "Cellulose to Ethanol - Problems and Successes". Slovak Academy of Sciences, Bratislava, Czechoslovakia. June 15.

THESES:

F. Riske, Ph.D. Thesis 1988, Production and Use of Monoclonal Antibodies to Cellobiohydrolase I from *Trichoderma reesei*.

S. Kahrs, M.S. Thesis 1988, Purification and Characterization of the Cellulase Enzymes of *Microbispora bispora* through Physiological and Immunological techniques.

Final Report

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Cellulase - A Key Enzyme for Fermentation Feedstocks D.E. Eveleigh, J.D. Macmillan, Dept. of
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Biomass can be fermented to a range of useful products. For efficiency a well characterized and effective cellulase is necessary to gain fermentable sugars from cellulosics (forest by-products and agricultural wastes and municipal refuse). Two cellulases have been further characterized, the classic from *Trichoderma reesei* and a thermally stable complex from the actinomycete *Microbispora bispora*. Thus there is no direct assay for one of the major cellulase components, cellobiohydrolase (CBH). Immunologic (monoclonal antibody - MAb) approaches have been used for the cellobiohydrolases of each system. With *T. reesei* rapid purification has been achieved through MAb affinity chromatography and also a highly sensitive MAb assay for CBH in crude cellulase broths has been developed. For *M. bispora*, the monoclonal antibodies towards CBH have been prepared and are being used for selection of clones from a *M. bispora* gene library. Characterization of the other *M. bispora* cellulase components via rDNA protocols has indicated five endoglucanases in comparison to four found by classical biochemical characterization. One gene (MBI.CEL1) has been sequence and shows certain homologies with other cellulases from *Cellulomonas* and *Trichoderma*. Both immunologic and cloning approaches have been used successfully to yield novel insights into these cellulase complexes.