

Design and Test Improved Assays for Endoglucanases and Exoglucanases
Subcontract No. XDH-0-30009-03
under Prime Contract No. DE-AC36-99GO10337

Technical Status Report
Covers the period December 5, 2000 – February 5, 2001

Submitted by University of California, Davis

During this period, work was performed on tasks 2 and 4 of the Subcontract.

Task 2.

Apply the modified BCA method to characterizing cellulases selected by the NREL Research Monitor on insoluble high-molecular weight cellulose and lignocellulose. The substrates may include cotton cellulose or bacterial cellulose, and lignocellulosic feedstock(s) of interest to the NREL Ethanol Project. Differentiate between endo- and exo-mode of enzyme action based on the ratio between insoluble and soluble reducing end-groups formed during the enzymatic hydrolysis. (2 months, 05/01/00-07/01/00).

Task 4.

Develop the HPSEC-MALLS method for analysis of insoluble high-molecular weight cellulose using DMAc/LiCl solvent system. Substrates used for this task will include cotton cellulose or bacterial cellulose (4 months, 09/01/00 – 01/01/01).

Pertaining to **Task 2** of the UC Davis Statement of Work, two purified cellulases, CBHI from *Trichoderma reesei* and E1cd from *Acidothermus cellulolyticus*, are being characterized on insoluble high-molecular weight cellulose and lignocellulose using modified BCA method. The method allows differentiating between endo- and exo-mode of enzyme action based on the ratio between insoluble and soluble reducing end-groups determined in the same hydrolysis mixture.

The E1cd and CBHI cellulases were kindly provided by the U.S. DOE National Renewable Energy Laboratory (NREL). They were received on January 11, 2001. The following background information on the enzymes is available:

E1cd - E1 catalytic domain, 8 vials at a concentration of 279 µg/ml for a total of 2.23 mg, labeled S-rE1, TRB2602p033, 12/22/00.

CBHI - affinity purified (using p-Aminophenyl 1-thio-β-D-cellobioside (PAPC) affinity matrix, Piyachomkwan *et al.*, 1997), 5 vials at a concentration of 800 µg/ml for a total of 5 mg. Labeled TRB2602p028, OD – 1.2754, 11/30/00.

Both samples are in 20 mM acetate, 100 mM NaCl, pH 5.0 buffer.

The homogeneity of the enzymes is being confirmed by 7.5% SDS PAGE and IEF (PhastGel 4.0-6.5) with Coomassie-blue staining using Pharmacia PhastSystem. Protein content has been determined by the **Folin-Lowry method** (Lowry, 1951).

A number of other methods are being used to determine activity of cellulases on various substrates.

Viscometric activity is being determined as the initial rate of decrease in viscosity of 0.5% carboxymethylcellulose (CMC) at pH 5.0 and 40°C using a Schott Gerate automated capillary viscometer (Germany) with an Ubbelohde capillary tube (capillary I.D. 1.03 mm, constant $K=0.09277$, measuring range 20- 100 $\text{mm}^2 \text{sec}^{-1}$). Viscometric activity is also being determined by rotational viscometry using a Brookfield DV-III rheometer. Viscometric activity is expressed in relative units (cP/min/mg protein).

CMC-ase activity is being measured according to Vlasenko *et al.*, 1998, as the initial rate of reducing sugars (RS) formation during hydrolysis of 0.5% CMC at pH 5.0, 50°C, and expressed in International Units (IU). One IU corresponds to 1 μmol of β -1,4-glycosidic bonds of substrate hydrolyzed in one minute during the initial period of hydrolysis. Disodium 2,2'-bichinchoninate (BCA) method is used for RS determination in the CMC-ase activity assay (Doner & Irwin, 1992; Garcia *et al.*, 1993; Johnston *et al.*, 1998).

Filter Paper Activity (FPA) is being determined according to the standard procedure recommended by the Commission on Biotechnology, IUPAC (Ghose, 1987) and expressed in Filter Paper Units (FPU).

Cellulase activity is being measured on various insoluble cellulosic substrates (Avicel, Solka Flocc, cotton and bacterial cellulose) as the initial rate of RS formation determined by the BCA method. Cellulose (4 mg/ml) is hydrolyzed in 0.02 M Na-acetate buffer, pH 5.0, at 40°C under constant stirring (100 rpm) using from 10 to 1000 ng/ml of enzyme.

Pertaining to **Task 4** of the UC Davis Statement of Work, the HPSEC-MALLS method is being developed for analysis of cellulosic materials in DMAc/LiCl solvent system. The following steps have been completed to prepare the HPSEC-MALLS system for chromatographic analysis of cellulose:

- HPSEC-MALLS system has been moved to another laboratory and assembled under the fume hood. This was a necessity since DMAc is known to be an exceptional contact hazard that may be harmful if inhaled or absorbed through skin.
- A mixed bed Styragel HT 6E column has been purchased from Waters and equilibrated with DMAc/0.8% LiCl at 70°C. The column is designed for applications requiring high-temperature solvents and can be used in molecular weight range from 5000 to 10,000,000 D.
- The MALLS detector (DAWN DSP-F laser photometer, Wyatt technology Corp.) has been calibrated to enable ASTRA software to convert the detector's signals to Rayleigh ratios. The instrument was calibrated to the scattering from a pure HPLC-grade toluene, which has a high and accurately determined Rayleigh ratio. The solvent was injected into the flow cell using a syringe pump.

- The calibration constant for the RI detector (Model ERC-5712, Anspec Company) has been determined using several solutions of sodium chloride with accurately known concentrations. The solutions were injected into the sample cell of the RI detector using a syringe pump.
- The specific refractive index increments (dn/dc) have been determined for various cellulosic samples. The dn/dc value is the proportionality factor between the solute concentration and the refractive index of the solution.
- The DAWN-RI volume delay and the DAWN's normalization coefficients have been determined.

The first molecular-weight distributions of various cellulosic materials (cotton samples, bacterial cellulose, Avicel and Solka Flocc cellulose) have been obtained, and are now being analyzed using ASTRA software (Wyatt Technology Corp.). The results of this analysis will be included in the subsequent technical status reports.

References

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