Cloning of a Microbispora bispora cellobiohydrolase gene in Escherichia coli

P. Hu, S.K. Kahrs, T. Chase, Jr. and D.E. Eveleigh

Department of Biochemistry and Microbiology, Cook College, Rutgers University, New Brunswick, NJ, USA

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SUMMARY

Cellobiohydrolase II was purified from a Microbispora bispora culture filtrate and a monoclonal antibody to it was prepared. Screening a M. bispora genomic library in Escherichia coli with this antibody yielded three equivalent clones. Subcloning resulted in greater expression, and activity could be monitored using 4-methylumbelliferylcellobioside. Southern analysis provided evidence that there is a single gene coding for CBH II. The original 22-kb fragment was reduced to 4 kb and subcloned into pUC118/119 resulting in a doubling of expression CBH II. The gene was expressed via its own promoter. The optimal pH (6.5) and the optimal temperature (60 °C) of the cloned enzyme are similar to that of the native CBH II.

INTRODUCTION

Cellulose, the β-1,4-polymer of glucose, is the major component of biomass, and is central to the ecological cycle. It also forms a major component of solid waste from agriculture and the food, pulp and paper industries, and is a potential renewable source of fuels and chemicals. Cellulose would be best utilized through fermentation, but first it must be converted to sugars. Cellulase, which converts cellulose to glucose, plays the central role in transforming biomass, and has attracted major attention with regard to biotechnology and deterioration [1,5,11,13-15,30].

We have been studying the cellulase of a thermophilic actinomycete Microbispora bispora [23]. This bacterium grows at 60 °C. It produces a thermally stable, extracellular cellulase system which converts crystalline cellulose to glucose in good yields, and which is resistant to end-product (glucose) inhibition [29]. The cellulase system is comprised of four endo-glucanases, two cellobiohydrolases, CBH I (70 kDa) and CBH II (93 kDa) [33], and also two β-glucosidases. The endo- and exoglucanases act synergistically. A M. bispora genomic library was originally constructed in Escherichia coli using pBR322 [33]. We now report the isolation of a M. bispora cellobiohydrolase gene from this genomic library by selection with a monoclonal antibody specific against CBH II.

MATERIALS AND METHODS

Materials. Restriction endonucleases were purchased from Boehringer Mannheim (Indianapolis, IN) or Stratagene (La Jolla, CA), and restriction digestion were performed as recommended by the manufacturer. Basic procedures for cloning followedAusubel et al. [2] and Sambrook et al. [25].

The reagents were obtained from the following sources: cellulose (Avicel PH105), FMC Corp., Wilmington, DE; cell culture medium NCTC 135 and penicillin/streptomycin, Gibco, Grand Island, NY; fetal calf serum and polyethylene glycol 1500, M.A. Bioproducts, Walkersville, MD; goat anti-mouse peroxidase conjugate, Kirkegaard and Perry Labs. Inc., Gaithersburg, MD; female BALB/c mice, 6–8 weeks old, Charles River Breeders, Wilmington, MA; and a mouse myeloma cell line P3-X63-Ag8.8653, the Institute for Medical Research, Camden, NJ. Other chemicals were from Aldrich Chemicals, Milwaukee, WI, Fisher Scientific Co., Springfield, NJ, Pharmacia, Piscataway, NJ, or Sigma Chemical Co., St. Louis, MO.

Enzyme production and purification. The basal salt medium of Hägerdal et al. [12] + 1% (w/v) Avicel (500 ml in a 2.8 l baffled flask), was inoculated with 10 ml M. bispora NRRL 15568 (dense mycelial homogenate grown in the
same medium). The culture was shaken (250 rpm) at 55 °C for 5 days. For preparation of the extracellular enzyme, the mycelia were removed by centrifugation, the supernatant broth filtered through Whatman GF/A glass fiber paper, and then (NH₄)₂SO₄ was added to 80% saturation. Precipitated protein from 500 ml culture was redissolved in 30-40 ml 10 mM Tris-HCl, pH 7.5, and desalted by gel filtration on BioGel P-2. The protein (67 mg) was applied to a DEAE-Sepharose CL-6B column (2.5 x 30 cm), which was then eluted with a linear gradient, 0–0.6 M NaCl in the same buffer at room temperature. The last two peaks eluted gave single bands as visualized by silver staining in SDS-PAGE [19]. These proteins showed activity towards acid-swollen cellulose, but did not cleave carboxymethylcellulose (Fig. 1). They were designated CBH I and II, and comprised approximately 28% and 21%, respectively, of the total protein in the crude broth [31].

Injection and fusion. Mice were injected intraperitoneally on days 0 and 14 with 150 μl (30 μg) purified CBH II + 150 μl Freund’s complete adjuvant. On days 42, 43 and 44 they were injected with 100 μg CBH II in 300 ml phosphate-buffered saline (PBS), the first intravenously, the last two intraperitoneally. On day 46, spleen lymphocytes were collected from the immunized mouse and fused in ratio 1:10 with mouse myeloma cells, grown in exponential phase for 7–10 days in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum, using polyethylene glycol 1500 as fusigen [27]. Fused cells, plus unfused spleen cells as feeders, were plated in 24-well tissue culture plates and incubated at 37 °C under 8% CO₂. Hybridoma cells were maintained in DMEM supplemented with 20% fetal calf serum, 1% penicillin/streptomycin and 2 mM L-glutamine. Beginning the day after the fusion, spent medium was replaced with H²xAT-DMEM (hypoxanthine, 2 x aminopterin, thymidine in DMEM) to select for hybrid cells.

Detection of antibodies. An enzyme-linked immunosorbent assay (ELISA) based on that outlined by Voller et al. [28] was used. Incubations were at room temperature unless otherwise indicated. Purified CBH II (50 ng) was added to wells of a 96-well polystyrene flat-bottomed plate and incubated for 2 h. The wells were washed with PBS, filled with 2% bovine serum albumin (BSA), covered and stored overnight at 4 °C. The BSA was then removed and the plate washed with PBS containing 0.05% Tween 20. Dilutions of serum (collected on days 18, 42 and 46) in PBS-Tween, or undiluted spent hybridoma medium, were added to wells and incubated for 1 h at room temperature. After washing the wells with PBS, a 1:400 dilution of peroxidase-labelled rabbit anti-mouse IgG was added to each well (50 μl) and incubated for 1 h. After a final wash, peroxidase was added to each well (50 μl) and the plate incubated 20 min in the dark. Absorbance at 410 nm was then read in an ELISA reader (Biotek, Burlington, VT). Control wells contained monoclonal antibodies (MAb) to other proteins, or no conjugate, or no sensitizing antigen; cultures were considered positive for monoclonal antibodies if absorbance was 3-times that of controls.

The same procedure, substituting CBH I for CBH II as first addition to the plate, was used to test cross-reactivity of the MAb to CBH II against CBH I.

Cloning, subtyping and propagation. Cultures producing antibody to CBH II were cloned twice by limiting dilution [9], using dilutions containing 3.0, 1.0 and 0.5 cells/well on spleen cells from an unimmunized mouse as a feeder layer, and 0.2 ml of each dilution were plated in each of 30 wells of a 96-well round-bottomed plate. Single clones were selected and their immunoglobulin class and sub-