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Cloning of a *Microbispora bispora* cellobiohydrolase gene in *Escherichia coli*

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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.

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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 followed Ausubel 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 $(NH_4)_2SO_4$ 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 × 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]. SDS gels were also stained for glycoprotein using a modified periodic acid-Schiff method [9]. Non-denaturing gels were assayed for carboxymethylcellulase activity using Congo red [3]. Isoelectric focusing was carried out in 5% acrylamide gels, using Pharmalyte ampholyte, pH 4–6.5,

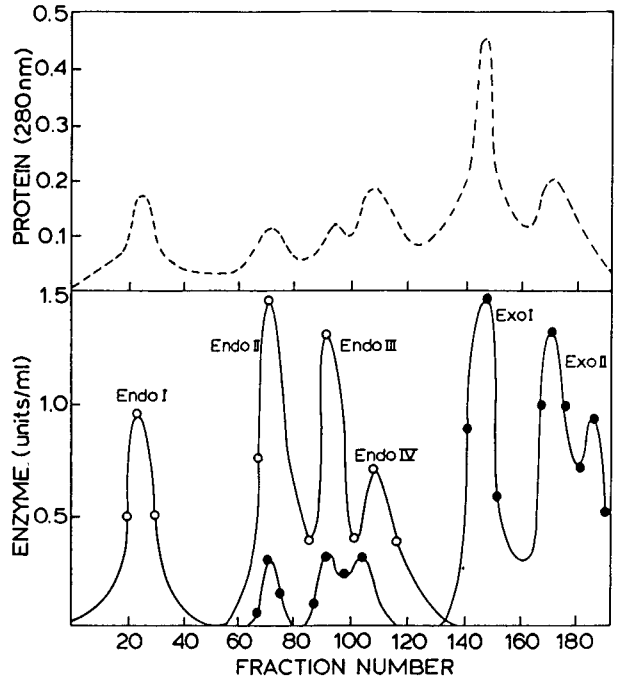

Fig. 1. Elution profile of *M. bispora* crude enzyme on DEAE-Sepharose CL-6B. Crude enzyme (67 mg protein) was applied to the column (92.5 × 30 cm) in 10 ml 0.01 M Tris-H Cl, pH 7.5. The column was eluted with 1.8 l of a linear salt gradient, 0–0.6 M NaCl in the same buffer, at a flow rate of 20 ml/h, collecting 6-ml fractions. ○, carboxymethylcellulase activity; ●, reducing sugar production from acid-swollen cellulose.

and gel pH was determined with a flat tip electrode (Ingold Electrodes, Inc., Wilmington, MA) after focusing.

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% CO_2. 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 × 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-

class were determined by ELISA with the mouse antibody sub typing kit (Boehringer Mannheim Biochemicals, Indianapolis, IN). MAb were produced in culture broth by growth of hybridomas in 75 cm^2 tissue culture flasks in DMEM + 15% fetal calf serum, using supernatant fluid for ELISA and immunoblot tests.

Immunoblotting. Crude *M. bispora* cellulase and purified CBH I and II were separated by SDS-PAGE and the proteins electrotransferred to nitrocellulose paper. Unbound sites were blocked with BSA, the paper washed and sealed in plastic bags with one of the following: (1) PBS (control), (2) MAb to CBH II, (3) antisera from mice immunized with CBH I, (4) antisera from mice immunized with CBH II. The papers were rocked overnight at 4 °C, washed and then incubated 2 h with peroxidase-conjugated anti-mouse IgG. After final washing, immune complexes were visualized with 4-chloro-1-naphthol + H_2O_2.

Screening of the genomic library. E. coli clones from the genomic library were grown on LB agar plates containing tetracycline (10 μg/ml) at 37 °C overnight, and the colonies transferred onto nitrocellulose filters. For lysis, the cells were exposed to chloroform for 20 min and then to lysozyme (40 μg/ml) and DNAse (1–10 μg/ml) in 50 mM Tris-HCl (pH 7.6) buffer, containing 150 mM NaCl, 5 mM $MgCl_2$ and 3% bovine serum albumin (BSA), for 10 h. The lysed cells on the membranes were incubated in TBS (150 mM NaCl, 10 mM Tris-HCl, pH 8.0) with 3% BSA and the CBH II monoclonal antibody (1:5 dilution of the cell culture supernatant) overnight. The excess antibody was removed by washing the membranes four times with TBS. The membranes were then incubated with anti-mouse IgG conjugated with alkaline phosphatase (Sigma) in TBS/1% BSA for 2 h. After the membranes were washed 6–7 times with TBS, CBH II positive clones were visualized using 5-bromo-4-chloro-3-indolyl-phosphate and Nitro blue tetrazolium.

Plasmids from CBH-positive clones were isolated by alkaline lysis and described in Sambrook et al. [25]. A restriction map was constructed following restriction endonuclease digestion and separation of the fragments on 0.7% agarose gels in 0.04 M Tris-acetate–1.0 mM EDTA buffer.

Southern analysis was done using a nonradiolabelled method according to the manufacturer's suggestions (ChemiProbe kit, FMC Bioproducts, Rockland, ME). An 8-kb *Pst*I fragment from the positive clone was used as the probe. The probe is chemically modified by the cytosines being sulfonated at carbon 6 by sodium bisulfite. This non-radiolabelled protocol is particular useful for Southern analysis of genes from high GC content genome, such as occurs in *M. bispora*. Hybridization was performed in 50% formamide (deionized), 1% SDS, 1 M NaCl, 5% dextran sulfate, 100 μg/ml freshly denatured salmon sperm DNA and 100 ng/ml freshly denatured probe, in a shaking water bath at 42 °C for 3 h. High stringency conditions were used in the post-hybridization washing: the membrane was sequentially washed in 2 × SSC (15 mM sodium citrate, 150 mM sodium chloride, pH 7.0), 0.1% SDS at room temperature for 15 min; 2 × SSC, 0.1% SDS at 68 °C for 15 min; 0.2 × SSC, 0.1% SDS at 68 °C, 5 × 10 min each. The chemically modified DNA was detected by a monoclonal antibody that specifically recognizes the sulfone groups on the modified DNA and an alkaline phosphate-anti IgG conjugate.

Enzyme assays. In the purification of CBH from the *M. bispora* culture filtrate, general cellulase activity was measured using acid-swollen cellulose. Diluted enzyme (0.25 ml) was added to 0.5 ml 1% acid-swollen cellulose and incubated for 30 min at 60 °C. Production of reducing sugars was measured by the Nelson-Somogyi method

Fig. 2. Plasmids from CBH positive clones digested with *Pst*I and separated on a 0.7% agarose gel. Lanes: (1) molecular size markers (λ/*Hin*dIII); (2) pBR322 cut with *Pst*I; (3,5,7) Plasmids from CBH positive clones cut with *Pst*I, (4,6,8) Plasmids from CBH positive clones (pRME55, pRME101, pRME104).

[21]. For study of CBH II expressed in *E. coli*, cells of the CBH-positive clone were grown overnight in LB broth (20 ml). The cells were harvested by centrifugation (10 min at 10000 × *g*), washed and resuspended in 12 mM citrate-50 mM Na phosphate-5 mM dithiothreitol, buffer, pH 6.5 (2 ml). Intracellular enzymes were obtained by breaking the cells by sonication (3–5 min at 40 W on ice), while periplasmic enzymes were obtained by osmotic shock [22].

For assay of cellobiohydrolase, 4-methylumbelliferyl-cellobioside (MUC) was used as the substrate. Prior to the cellobiohydrolase assay, enzyme samples were heated at 60 °C for 10 min, and the precipitated protein was removed by centrifugation before assaying. The substrate (2.0 mM in citrate-phosphate buffer, pH 6.5, 0.5 ml) were mixed with an equal volume of appropriately diluted enzyme (0.5 ml). Following incubation at 60 °C for 30 min, the reaction was terminated by addition of 0.15 M glycine-NaOH buffer (3.0 ml; final pH 10.3). The fluorescence of the aglycone product, 4-methylumbelliferone, was measured with excitation at 360 nm and emission at 448 nm (Turner fluorometer, Model 111, Turner Associates, Palo Alto, CA), and correlated with a standard curve. One enzyme unit is defined as the amount of enzyme that released 1 μmol 4-methylumbelliferone per min under the above conditions. δ-Gluconolactone (1.0 mM), an inhibitor of β-glucosidase [31], was used to prevent the hydro-

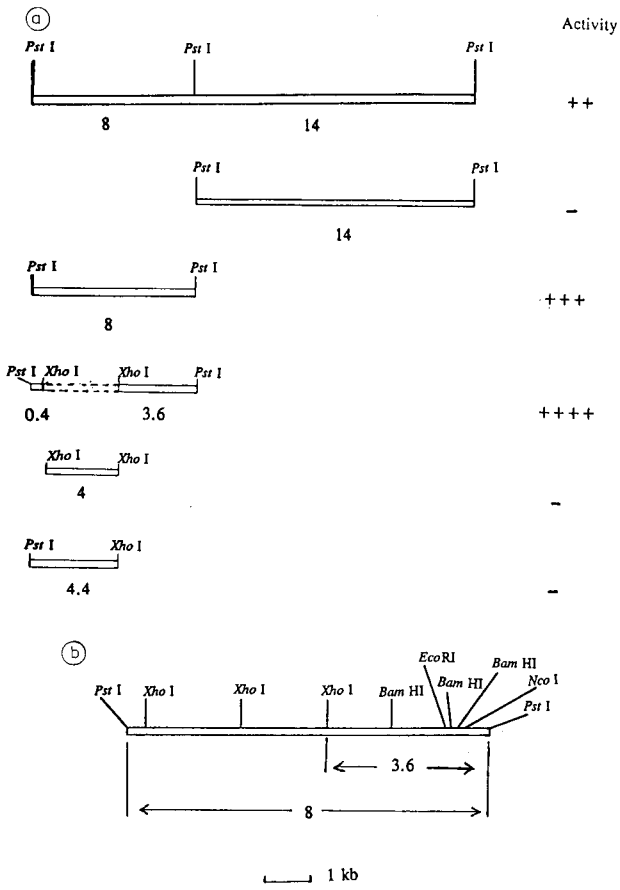

Fig. 3. (a) Location of the CBHII gene. The cloned fragment was digested with restriction endonuclease and fragments subcloned into pU119/118. Cellobiohydrolase activity of the resulting *E. coli* clones was measured. = = = = *Xho*I site was reclosed. (b) Restriction map of *M. bispora* cellobiohydrolase II gene (8-kb fragment). The fragment was digested with restriction endoglucanases with subsequent separation on a 0.7% agarose gel.

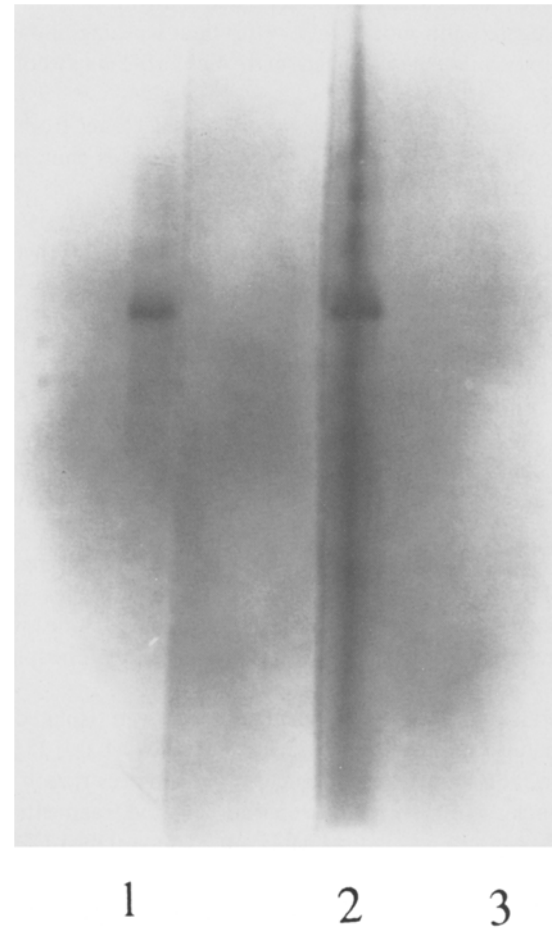

Fig. 4. Southern analysis of *M. bispora* genomic DNA using the 8-kb *Pst*I fragment as the probe. The 8-kb *Pst*I fragment coding for cellobiohydrolase activity was purified by electroelution and used as the probe. *M. bispora* genomic DNA and salmon sperm DNA were digested with *Pst*I and separated on 0.7% agarose gel. Hybridization was detected using the ChemiProbe system (FMC Bioproducts). Lanes: (1) 8-kb *Pst*I fragment (see Fig. 3); (2) *M. bispora* genomic DNA digested with *Pst*I; (3) Salmon sperm DNA digested with *Pst*I.

TABLE 1

M. bispora 4-methylumbelliferylcellobiohydrolase activity in *E. coli* clones

Clones	Intracellular[a] total (mU/ml)	Particulate[a] total (mU/ml)	Periplasmic[b] total (mU/ml)	Culture broth total (mU/ml)
pBR322-cbh	0.01	0.001	0	0
pUC119-cbh (with IPTG)	0.02	0.001	0	0
pUC119-cbh (No IPTG)	0.02	0.001	0	0
pUC118-cbh (with IPTG)	0.018	0.001	0	0
pUC118-cbh (No IPTG)	0.017	0.001	0	0

Alkaline phosphatase [18] was used as a positive control for periplasmic enzymes.

[a] Cells were grown and then washed with 12 mM citrate – 50 mM phosphate buffer (pH 6.5), and sonicated. The cell homogenates were centrifuged at $10\,000 \times g$ for 10 min. The supernatant was designated the intracellular fraction and the debris as the particulate fraction.

[b] The periplasmic fraction was prepared according to Neu and Heppel [22].

lysis of the substrate to glucose and 4-methylumbelliferylglucoside.

Protein concentration was measured using the Bradford dye binding assay [4], or during CBH purification by the Lowry method [17] or absorbance at 280 nm.

RESULTS

Cellobiohydrolase II purification

Purified CBH II gave a single band, molecular mass (M_r) 92.5 kDa, in SDS-PAGE, a single band in native gel electrophoresis without carboxymethylcellulase activity, and a single band in isoelectric focusing, pI = 4.0 [33]. In contrast, CBH I gave a single band by SDS-PAGE, M_r 74 kDa. It gave two bands in native gel electrophoresis, but both had similar activity on cellulose and differed in pI by only 0.1 unit (pIs 4.3, 4.2 [3]). Both CBHs stained as glycoproteins with periodic acid-Schiff reagent (data not shown).

Monoclonal antibody isolation

Hybridoma growth occurred in 92 of 95 wells seeded; 34 produced antibody to CBH II. Nineteen cell lines were propagated, of which six were successfully cloned. All six secreted MAb containing κ light chains and IgG_1 heavy chains. Lines 3A3H1 and 3A2G6 grew well, were strong producers of MAb to CBH II, and were used for further study. Neither MAb cross-reacted with CBH I in an ELISA or in immunoblots; in contrast, polyclonal antibodies (mouse antisera) to either CBH I or CBH II reacted with CBH I in immunoblots.

Isolation of a clone expressing cellobiohydrolase II

Screening 3840 clones with CBH II monoclonal antibody (3A3HI) yielded three clones (Fig. 2). They appeared to be identical, as the inserts of all three were 22 kb and their restriction endonuclease digestion patterns were the same.

The 22 kb insert was digested with *Pst*I, resulting in 8- and 14-kb fragments which were subcloned into pUC 118/119. The activity was located on the 8-kb fragment (Fig. 3a). Probing a Southern blot of *M. bispora* genomic DNA digested with *Pst*I with this cloned 8-kb *Pst*I fragment, yielded one specific band (Fig. 4), of which a more detailed restriction map was constructed (Fig. 3b). This 8-kb fragment was further digested with *Xho*I (Fig. 3) and all the fragments were subcloned into pUC118/119. The cellobiohydrolase activity was located in a 3.6-kb *Xho*I-*Pst*I fragment (Fig. 3a). For the convenience of making nested deletion sets for sequencing and for further subcloning into *Streptomyces*, a larger *Pst*I-*Pst*I 4-kb fragment (400-bp *Pst*I – *Xho*I fragment + 3.6-kb *Xho*I– *Pst*I) was maintained in the construct (Fig. 3a).

The antibody-positive clones were assayed for cellobiohydrolase activity using 4-methylumbelliferylcellobioside as the substrate. The enzyme is intracellular (Table 1), its specific activity in total cell homogenates being 0.0001 U/mg protein. Expression of enzymatic activity was unaffected by the orientation of the fragment or the presence of IPTG as an inducer (Table 1).

The optimal pH for the cloned enzyme was 6.5 (Fig. 5) with an optimal temperature of 60 °C (Fig. 6), both parameters being similar to that of the native enzyme.

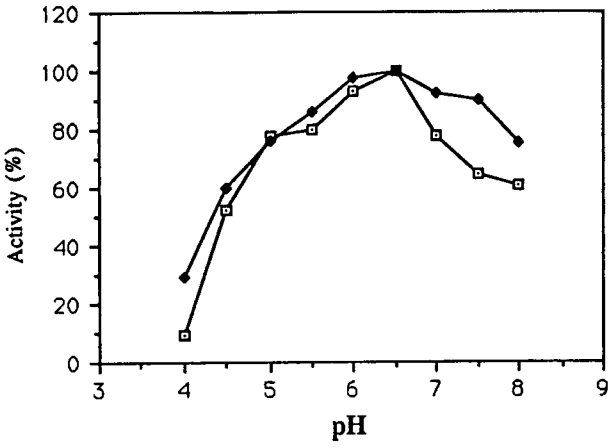

Fig. 5. The optimal reaction pH of *M. bispora* CBHII. The activities were determined at different pHs at 60 °C for 30 min. (—⊡—) *E. coli* (cloned CBHII); (—◆—) *M. Bispora* (native CBHII).

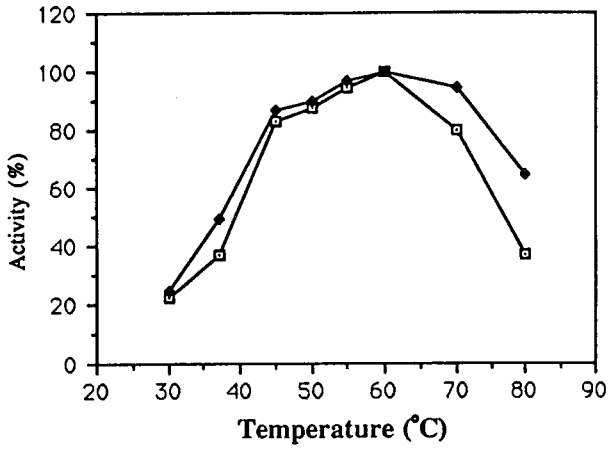

Fig. 6. The optimal reaction temperature of *M. bispora* CBHII. The activities were determined at different temperatures for 30 min. (—⊡—) *E. coli* (cloned CBHII); (—◆—) *M. bispora* (native CBHII).

δ-Gluconolactone, an inhibitor of β-glucosidase, also inhibits cellobiohydrolase activity of crude *M. bispora* culture supernatant but only at high concentration (Fig. 7).

DISCUSSION

M. bispora CBH II is an essential major cellulase component, comprising 21% of extracellular cellulase protein [33]. It was proposed to further characterize it and its gene through cloning. A genomic library was prepared, but the initial direct expression of CBH II in *E. coli* was very low. Indeed, in initial screening of the genomic library using 4-methylumbelliferylcellobioside to detect CBH II

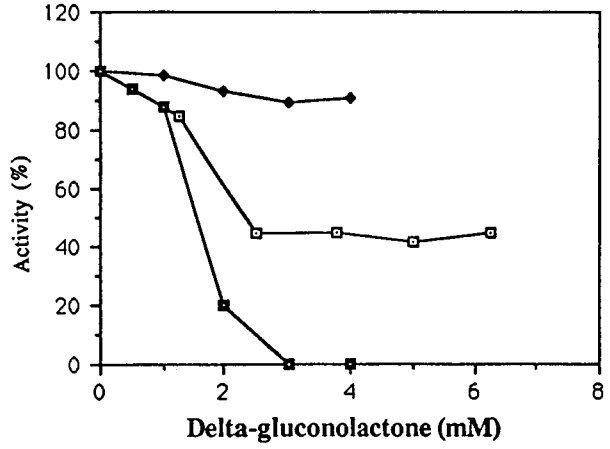

Fig. 7. Differential inhibition of δ-gluconolactone on *M. bispora* cellobiohydrolases. Cellobiohydrolase activity was measured in the presence of a range of concentrations of δ-gluconolactone, and comparison of its effect mode on CBHI and CBHII. (—⊡—) CBHI and II (culture broth); (—◆—) Purified CBHI; (—■—) Cloned CBHII.

activity no positive clones were found. Therefore, we decided to use the more sensitive antibody selection technique. As polyclonal antibodies to either CBH I or CBH II cross-reacted with the other, we therefore prepared monoclonal antibodies to CBH II. MAb from cell line 3A3HI proved useful and was used to rescreen the library. This yielded three apparently identical clones. The MAb clone also allows preparation of CBH II free of other cellulase components, a useful advance as the native CBH I and CBH II tend to co-purify [33]. The separation shown in Fig. 1 was not reliable. The CBH-specific antibody will be of further practical value to develop a cellobiohydrolase assay, for which no specific method exists. We have used this approach for the *Trichoderma reesei* CBH I [24].

Though positive, enzyme activity in *E. coli* was low (Table 1). However, low expression of actinomycete genes in *E. coli* is a general phenomenon, and cellulase expression in the milliunit range has been previously observed [20]. The low yield of *M. bispora* cellobiohydrolase in *E. coli* could be due to several factors. For instance, the GC content of *Microbispora bispora* is 70–72.3% [23], typical of actinomycetes but quite distant from that of *E. coli* (50%). The *E. coli* RNA polymerase may not recognize the high GC actinomycete promoter efficiently, resulting in insufficient message. A further reason could be that as a result of high GC content, *M. bispora* tends to use G or C at the third position of the codon, and *E. coli* cannot provide sufficient amount of these specific tRNA species, thus resulting in a reduced elongation rate or premature termination during the synthesis of the enzyme and/or increased

errors. The problem of promoter recognition can be solved by fusing the start codon directly to a strong *E. coli* promoter [6]. The low expression resulting from differences in codon usage can be improved by employing a more appropriate cloning host. For instance, *Streptomyces lividans* would be a good candidate. Its GC content is 70%, and it has been successfully used as a cloning host to obtain a high level expression of several polysaccharase genes [7,8,26].

There was no difference in activity with or without IPTG, indicating the cloned enzyme was expressed via its own promoter. Decreasing the size of the insert and the subcloning to a higher copy number plasmid vector (pUC118/119) resulted in better expression -- twice the yield of the original clone. However, the yield of CBH II in *E. coli* was still relatively low (Table 1). The properties of the cloned cellobiohydrolase were compared to those of the native enzyme. The cloned CBH II has the same optimal pH and temperature as the native enzyme (Figs. 5, 6).

Not all bacterial cellulase complexes contain cellobiohydrolases [32]. Thus cloning and characterization of CBH II from *M. bispora* can provide further insight into the importance of cellobiohydrolases and the evolution of the cellulases. For this purpose, sequencing of the gene is underway.

δ-Gluconolactone, an inhibitor of β-glucosidase [31], at 1 mM gave little inhibition of cellobiohydrolase activity (Fig. 7), and thus its use is appropriate to selectively inhibit the more sensitive β-glucosidase. At higher concentrations (3–4 mM), δ-gluconolactone inhibited 60% of the cellobiohydrolase activity in the *M. bispora* culture supernatant (containing CBH I and II) and completely inhibited cloned CBH II. In comparison, partially purified CBH I (from a DEAE-Sepharose column) showed little inhibition by δ-gluconolactone. Presumably the 40% activity that remains when culture broth containing CBH I and CBH II is treated with δ-gluconolactone is contributed by CBH I. Such selective inhibition by δ-gluconolactone could be useful in assays to distinguish between CBH I and CBH II.

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