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Expression of a Fungal Cellobiohydrolase in Insect Cells

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The gene for *Trichoderma reesei* cellobiohydrolase I (CBHI) was expressed with a recombinant baculovirus and high levels of secreted protein were produced in *Spodoptera frugiperda* and *Trichoplusia ni* insect cells. Electrophoretic analysis indicated that the recombinant CBHI (rCBHI) was similar in apparent molecular weight to the native form and immunoblotting with anti-CBHI monoclonal antibodies confirmed its identity. The rCBHI was easily purified by affinity and hydrophobic interaction chromatography and demonstrated enzymatic activity on soluble substrate. © 1997 Academic Press

The filamentous fungus *Trichoderma reesei* secretes several cellulolytic enzymes that synergistically participate in the degradation of cellulose (1). One of these key enzymes is the 65 kDa glycoprotein cellobiohydrolase I (CBHI). CBHI is an exoglucanase that cleaves the cellulose chain from the reducing end to release single cellobiosyl units (2, 3). CBHI consists of a large *N*-glycosylated catalytic core domain and a smaller cellulose-binding domain (CBD) linked together by a heavily *O*-glycosylated peptide region (4). The three-dimensional structure for the catalytic core and cellulose-binding domains have been solved in high resolution (3, 5) spawning several studies into the structure-function relationship of CBHI, and thereby permitting the identification of specific residues involved in the catalysis and binding of cellulose (6, 7, 8, 9). However, most structure-function studies on the catalytic core have required the expression of CBHI in *T. reesei*. The use of the natural host to produce site-directed CBHI mutants ensures authentic protein production, but is hindered by a laborious and time-consuming cloning procedure as well as problems encountered in eliminating other contaminating synergistic cellulases, particularly the endoglucanases, during protein purification (10). Although efforts have been made to clone *T. reesei* CBHI in other hosts such as *Escherichia coli* (11, 12) and *Saccharomyces cerevisiae* (6, 13) poor solubility and overglycosylation, respectively, has made these recombinant forms of CBHI of limited use in structure-function studies.

The use of the baculovirus vector system for heterologous gene expression in insect cells has been widely and successfully implemented in the production of foreign proteins from viral, bacterial, fungal, plant, and mammalian sources. In general, recombinant proteins produced in insect cells are correctly processed, modified, and cellularly localized in a functionally active form (14, 15, 16). The advantages offered by the baculovirus expression system make it ideal for the production of *T. reesei* CBHI for use in structure-function studies. In this report, we describe the cloning and expression of *T. reesei* CBHI in the baculovirus system. CBHI was secreted from insect cells into the culture medium and purified to homogeneity in an enzymatically active form.

MATERIALS AND METHODS

Plasmid construction. To generate recombinant baculovirus containing the *cbh1* gene from *T. reesei*, a recombinant donor plasmid was first constructed. The plasmid pEM-F5 (17), which carries the CBHI coding region including the signal peptide sequence, was digested with *Sac*II, blunt-ended with T4 DNA polymerase treatment, and then digested with *Pst*I. The excised 2,190 bp cDNA fragment encoding the *cbh1* gene was then cloned into the *Stu*I-*Pst*I site of the donor plasmid pFASTBac1 (Gibco-BRL, Gaithersburg, MD, USA). The resulting *cbh1*-containing plasmid was designated as pBAC-CBHI (See figure 1 for an outline). All DNA manipulations were performed using established procedures (18).

Recombinant baculoviruses. The Bac-to-Bac expression system (Gibco-BRL), which employs transposon-directed integration of foreign genes into the baculovirus genome (19), was used to produce recombinant baculoviruses according to the manufacturer's instructions. In brief, the recombinant donor plasmid pBAC-CBHI carrying the cbh1 gene was used to transform *E. coli* DH10Bac by electroporation, and the cells plated onto Luria-Bertani agar supplemented with ampicillin, kanamycin, gentamycin, and isopropyl-1-thio-β-D-galactoside/Bluo-gal. Recombinant bacmid DNAs were isolated from large

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Abbreviations: MUG2, 4-methylumbelliferyl β-D-cellobioside; MOI, multiplicity of infection; PFU, plaque forming units.

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white colonies and then transfected into Sf21 cells using Cellfectin reagent (Gibco-BRL). At 72 h post-transfection, culture supernatants containing recombinant baculovirus were collected and then analyzed for the presence of secreted CBHI by immunoblotting with anti-CBHI monoclonal antibodies.

Cell culturing and infection. *Spodoptera frugiperda* (Sf21) and *Trichoplusia ni* (High Five) cell lines (20, 21)were routinely propagated as monolayer or suspension cultures at 27°C or room temperature with serum-free SF900-II culture medium using established procedures (14, 15). For small scale production of CBHI, 75-cm^2 tissue culture flasks containing insect cells at about 75% confluence were infected with recombinant baculovirus at a multiplicity of infection (MOI) of 10 and the culture supernatant recovered by low speed centrifugation at 72 h post-infection (p.i.).

Large scale expression. For CBHI production on a large scale, cells were infected in a 10 litre Biostat ECD bioreactor (B.Braun Biotech, Melsungen, Germany) as has been recently described in detail (22). Briefly, the cells were amplified in 2.8 litre Fernbach flasks on orbital shakers (130 rpm) to high density and transferred to the bioreactor. At a concentration of about 2×10^6 cells per ml of culture medium cells were infected with the recombinant virus at a MOI of 2-3. At 60 hours p.i. cells and media were separated as described previously (22).

Protein purification. The supernatant fraction from the 10 liter cultivation of rCBHI baculovirus infected High Five cells was filtered to separate the virus and then dialyzed against 50 mM sodium acetate (pH 5). The protein concentrate was loaded onto a thiocellobioside-based affinity column and the rCBHI purified as described previously (23). Hydrophobic interaction chromatography was performed using Phenyl Sepharose FF (Pharmacia) as described (24). All column fractions containing rCBHI were identified by dot-blotting analysis with anti-CBHI monoclonal antibody, pooled, concentrated, and dialyzed in 50 mM sodium acetate (pH 5).

Amino-terminal amino acid sequence determination. About 100 pmol of CBHI together with 5 and 20 pmol samples of beta lactoglobulin were separated by SDS-PAGE in a 12.5% acrylamide gel (25). The casted gel was aged for two days at +4C prior to electrophoresis to avoid acrylamide related modifications of proteins (26). After electrophoresis, the proteins were electroblotted onto a PVDF-membrane (ProBlot, Applied Biosystems, CA, USA) using the CAPS-buffer system (27). The protein bands were visualized by staining with Coomassie Brilliant blue and subjected to Edman-degradation in an Applied Biosystems 949A Procise protein sequencer.

Amino acid composition analysis. The CBHI protein samples were hydrolyzed for 24 h at 110°C in 6 N HCl containing 1 mg phenol/ml and the amino acid analyses performed on a LKB 4151 Alpha Plus amino acid analyzer by the Amino Acid Analysis Laboratory, Department of Biochemistry, Uppsala University.

Enzyme activity measurements. CBHI activity was measured using the soluble fluorogenic oligosaccharide derivative 4-methylumbelliferyl β-D-cellobioside (MUG2) essentially as previously described (28). Activity assays were performed at 50°C in 50 mM sodium acetate (pH 5) with 0.68 μM enzyme. An estimate of the enzyme concentration was calculated from UV absorbance at 280 nm using the molar extinction coefficient for CBHI of 73,000 M^{-1} cm^{-1}.

Gel electrophoresis and immunoblotting. Protein samples were analyzed by SDS-PAGE using 10% acrylamide gels according to the method of Laemmli (25) and then visualized by Coomassie brilliant blue staining as described (29). In western blotting, the proteins were transferred onto nitrocellulose membrane (30) and probed with the anti-CBHI monoclonal antibodies CI-261 and CI-89 (31). CBHI was recognized by commercial alkaline phosphatase labelled goat anti mouse IgG (Sigma) and stained with a Protoblot kit (Promega). For dot-blotting, sample aliquots of 2 μl were applied directly onto a nitrocellulose membrane and then treated as described above.

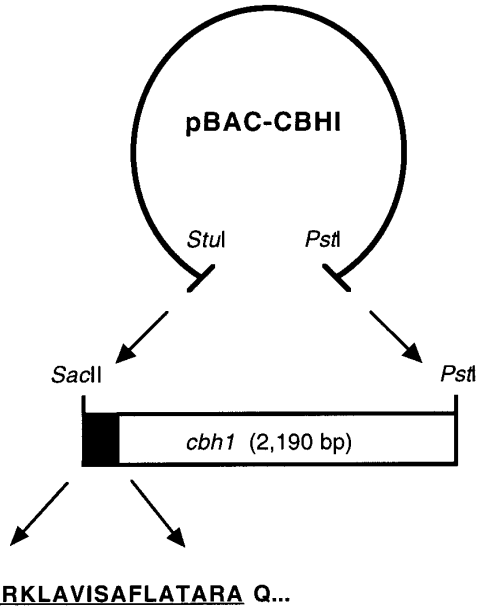

FIG. 1. Construct of the recombinant donor plasmid pBAC-CBHI. A 2,190 base pair cDNA fragment encoding the *T. reesei cbh1* gene including the signal peptide sequence was cloned into the multiple cloning site of the donor plasmid pFASTBac1 (Gibco-BRL) to generate the final plasmid pBAC-CBHI. (For details, see Methods and Materials .) Restriction sites and the amino acid sequence of the signal peptide (underlined) are given.

RESULTS AND DISCUSSION

Recombinant baculoviruses containing the signal peptide and coding sequence of the *T. reesei cbh1* gene were prepared with the Bac-to-Bac Baculovirus Expression System (Gibco-BRL). The recombinant donor plasmid designated pBAC-CBHI was first constructed by cloning the *cbh1* gene into the donor plasmid pFASTBac1 as outlined in figure 1 and then used to obtain recombinant bacmid DNA containing the *cbh1* gene for the transfection of the Sf21 insect cell line. The production of rCBHI baculoviruses from Sf21 cells was confirmed by immunoblotting analysis of supernatant fractions of the culture medium using the anti-CBHI monoclonal antibody CI-89. As shown in figure 2, an immunoreactive band (lane 1) comigrating with the 65 kDa band of native CBHI from *T. reesei* (lane 3) was detected. Mock and wild type virus infections failed to exhibit a similar immunoreactive banding pattern (results not shown). However, because the level of expression was relatively low with the Sf21 cells, the rCBHI baculovirus was used to infect the better secreting High Five cell line (lane 2). The expression levels of secreted rCBHI in the medium of infected High Five cells were electrophoretically estimated at 20 mg/l following three days incubation.

The purification of CBHI produced from a large-scale cultivation of High Five cells infected with recombinant

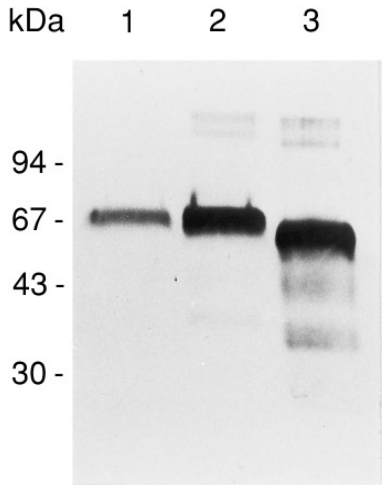

FIG. 2. Immunoblot analysis of rCBHI from insect cells. Supernatant fractions of culture medium from recombinant baculovirus infected Sf21 (lane 1) and High Five (lane 2) insect cells were loaded onto 10% SDS-gels, transferred onto nitrocellulose membrane, and treated with anti-CBHI monoclonal antibody CI-89. Native CBHI from *T. reesei* is shown in lane 3 and molecular weight markers are indicated.

baculovirus was carried out by thiocellobioside-based affinity (23) and hydrophobic interaction (24) chromatography, whereafter the protein samples were analyzed by SDS-PAGE and immunoblotting (Fig. 3). As shown by the Coomassie brilliant blue stained gels (Fig. 3A), rCBHI in the protein concentrate (lane 1) was virtually pure following the single step of affinity chromatography (lane 2). However, a faster migrating minor band revealed by the anti-CBHI core (CI-261) and anti-CBHI CBD (CI-89) monoclonal antibodies as proteolytically cleaved rCBHI core protein was also present (Figs. 3B and C; lane 2). An additional step of hydrophobic interaction chromatography was therefore employed to remove the core protein from the intact form of rCBHI. Coomassie brilliant blue staining of the SDS-gel and the corresponding immunoblots with CI-261 and CI-89 monoclonal antibodies (lane 3), demonstrate that only a single band corresponding to intact rCBHI is present following this chromatographic step. Although this purified rCBHI migrates as a single species on SDS-gels, its position on the gel is slightly higher than observed for native CBHI from *T. reesei* (lane 4). Recombinant CBHI produced from *S. cerevisiae* migrates much slower on SDS gels and this has been attributed to extensive overglycosylation (6). Since the difference in the migration pattern between the rCBHI from insect cells and the native form is only minor, overglycosylation is unlikely. However, an amino acid composition analysis of rCBHI from insect cells (results not shown) indicates the presence of four galactosamine and five glucosamine residues per protein molecule in contrast to only three glucosamine residues found in the native CBHI. Differences in the general patterns for glycosylation between insect cells and other eukaryotic species have been reported (32), and it has been shown that, e.g., a barley (1-3,1-4)-β-glucanase expressed in insect cells has a different glycan structure than the native plant enzyme (33). Since native CBHI contains both *N*- and *O*-linked glycosylation (34), differences in these glycan structures may well account for the observed difference in the apparent molecular weight.

To determine the signal sequence cleavage site of rCBHI, the protein from the culture supernatant of infected High Five cells was subjected to Edman degradation. However, no signals corresponding to the protein amount (about 100 pmol) on the PVDF-blot could be obtained from the rCBHI band even though the controls with considerably smaller protein amounts could easily be sequenced. It has previously been shown that the N-terminus of native CBHI contains a pyroglutamate residue (35) which makes the protein resistant to Edman-degradation. Lack of signals from the Edman-degradation of rCBHI suggests that it has been processed similar to the native protein, thus, containing a pyroglutamate residue at its N-terminus. All our efforts to enzymatically remove the cyclic N-terminal residue by pyroglutamyl aminopeptidase to facilitate sequencing proved unsuccessful. However, an amino acid composition determination (results not shown) revealed no apparent differences in amino acid content between rCBHI from insect cells and the mature form of native CBHI. Moreover, the N-terminal sequence of unprocessed rCBHI, which should lack a glutamyl residue at its N terminus, was never detected in the sequencing analysis. Therefore, it is likely that the signal peptide of rCBHI has been correctly recognized and cleaved by insect cells.

To investigate whether the purified rCBHI from insect cells is functionally similar to native CBHI, a comparison of the enzyme activity on the small, soluble substrate, MUG2 was performed. The k_{cat} values determined for rCBHI and native CBHI were 11.3 $\pm$ 0.3 min^{-1} and 14.6 $\pm$ 0.9 min^{-1}, respectively, indicating that rCBHI was slightly less active than the native form. A similar reduction in activity has been previously observed on insoluble but not on soluble substrates with the hyperglycosylated rCBHI produced in yeast (6). The reason for the slightly decreased activity obtained for the rCBHI from insect cells is not immediately obvious, although similar reductions in activity on some but not all substrates have been observed for a fungal lignin peroxidase (36)and a barley (1-3,1-4)-β-glucanase expressed in insect cells (33). In both these cases differences in glycosylation and/or processing of the recombinant enzymes were offered as a possible explanation. Clearly, further experiments including protein mass spectroscopy are needed to resolve the origin of the slightly decreased activity of the rCBHI.

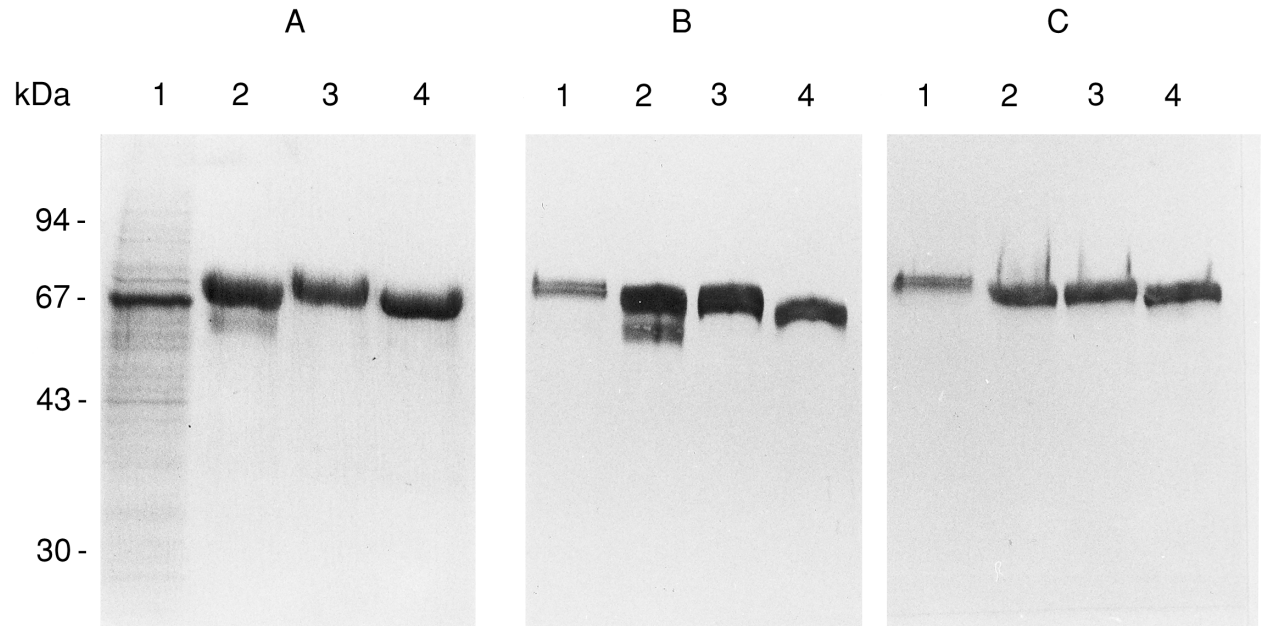

FIG. 3. Purification of rCBHI produced in High Five insect cells. The protein samples from the steps in the purification of rCBHI, the protein concentrate (lane 1), post-affinity chromatography (lane 2), and post-hydrophobic interaction chromatography (lane 3), were analyzed by SDS-PAGE, and stained with Coomassie blue (A) and immunoblotted with the anti-CBHI monoclonal antibodies CI-261 (B) and CI-89 (C). Native CBHI from *T. reesei* (lane 4) and molecular weight markers are included.

In conclusion, a recombinant baculovirus carrying the *T. reesei cbh1* gene has been constructed and used to obtain high level expression of enzymatically active and secreted rCBHI in insect cells. Although additional experiments are required to further characterize the rCBHI and possibly to optimize the expression system, the relative ease of purification and the absence of contaminating fungal cellulases make the recombinant baculovirus expression in insect cells a simple and convenient alternative expression system for CBHI. In particular, it will facilitate rapid screening of CBHI mutants which has been a significant impediment in protein engineering studies of this key cellulolytic enzyme.

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