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Expression of Barley Endopeptidase B in *Trichoderma reesei*

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The gene for barley endopeptidase B (EPB) has been expressed in the filamentous fungus *Trichoderma reesei* from the *cbh1* promoter. The EPB signal sequence allowed secretion of over 90% of the recombinant protein. Yields reached about 500 mg of immunoreactive protein per liter and exceeded values for any other protein derived from a higher eukaryotic organism produced in *T. reesei*.

Trichoderma reesei is exploited in industry as a producer of hydrolytic enzymes (4). In high-cellulase-producing mutants, the major *T. reesei* cellulase, cellobiohydrolase I (CBHI), is secreted into the culture medium in the tens of grams per liter range under cellulase-inducing conditions (3). *T. reesei* has successfully been used as a host for heterologous fungal proteins (7, 13, 14, 18). Recently, *T. reesei* has also been shown to be able to produce immunologically active antibody derivatives of murine origin (17). The production level of 150 mg of CBHI-Fab fusion antibody per liter in a fermentor cultivation corresponds to 60 mg of Fab fragment per liter (17).

The starchy endosperm of barley grain is surrounded by a living aleurone layer which secretes hydrolytic enzymes to the endosperm, allowing mobilization of the reserve materials from their storage place to the growing seedling during germination. The cysteine proteinases endopeptidases A (EPA [37 kDa]) and B (EPB [30 kDa]) are secreted from isolated aleurone layers when they are incubated with gibberellic acid (10, 12) and appear to play an important role in breaking down the water-insoluble storage proteins during germination. EPB is reported to be a nonglycosylated protein and to exist as two closely related isozymes (12). Efficient production of these papain-type proteinases in an alternative host would pave the way for their use in various applications requiring proteolysis, such as in the food processing industry.

We have employed two different host strains, *T. reesei* Rut-C30 (ATCC 56765) (15) and ALKO2221, for the expression of EPB. *T. reesei* ALKO2221 is a low-protease mutant derived from strain VTT-D-79125 (2) by UV mutagenesis and by screening of the mutagenized conidia on minimal plates (19) containing 1% skim milk and 1% glycerol (12a). The host strains were transformed with the 8.9-kb EPB expression cassette of vector pALK584 (16) as described by Karhunen et al. (8). In pALK584, the 1.15-kb *Pvu*I fragment from a barley *epb* cDNA clone, pHVEP4, harboring the secretion signals and coding sequences for pro- and mature forms of the protein (11), was ligated to *Xho*I-linearized plasmid pALK493 (20a). The 2.2-kb *Stu*I-*Sac*II *cbh1* promoter fragment from *T. reesei* VTT-D-80133 (22) and the 1.7-kb *Bam*HI-*Eco*RI *cbh1* 3′ flanking region from *T. reesei* ALKO2466 (6) were used to target the integration of the transforming DNA into the *cbh1* locus. The 0.74-kb *Ava*II fragment, starting 113 bp before the stop codon of the *cbh1* gene, ensures termination of EPB transcription. The 3.1-kb *Stu*I-*Bam*HI fragment containing the selectable marker gene, *amdS*, was from plasmid p3SR2 (9).

In order to screen for production of the recombinant EPB, approximately 200 purified recombinant clones were cultivated in 250-ml conical flasks for 7 days at 30°C in 50 ml of cellulase-inducing medium (21). Proteins secreted into the cultivation medium were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. To detect strains containing the replacement of the *cbh1* locus with the EPB expression cassette, Western blot analysis was carried out with the CBHI-specific monoclonal antibody CI89 or CI261 (1) and the Protoblot AP System (Promega, Madison, Wis.) according to the instructions of the manufacturer. For detection of the recombinant EPB protein, Western blot analysis with polyclonal EPB antiserum was performed (11). Approximately 40 clones produced detectable amounts of immunoreactive recombinant EPB protein. Four clones, including one Rut-C30-based clone and three clones from ALKO2221, were chosen for further studies. The Rut-C30-based transformant ALKO3713 exhibited a $CBHI^+$ phenotype, indicating that the gene construct had not integrated into the *cbh1* locus. Among the ALKO2221 transformants, the *cbh1* locus had been replaced in two cases (Table 1).

Organization of the *cbh1* locus in the transformed strains was analyzed by Southern blotting. *T. reesei* chromosomal DNA was isolated according to the method described by Raeder and Broda (20). DNA probes were labelled with digoxigenin according to the recommendations of the manufacturer (Boehringer, Mannheim, Germany). Figure 1A shows a hybridization blot of chromosomal DNAs digested with *Xba*I and hybridized with the *cbh1* cDNA coding region. In *T. reesei* Rut-C30 and ALKO2221, the genomic *cbh1* gene with its promoter and terminator sequences is located in a 9-kb fragment. The transformed EPB expression cassette did not contain the CBHI coding region corresponding to the probe. No signal in transformants ALKO3708 and ALKO3709 was obtained, indicating that the *cbh1* locus was replaced with the cassette. The strains in which the *cbh1* locus had remained intact displayed the 9-kb fragment (ALKO3711 and ALKO3713).

To determine the numbers of copies of the intact EPB expression cassette, DNAs were digested with *Xho*I and hybridized with the 1.5-kb *Xba*I-*Sac*II fragment of pALK584, which is specific for the *cbh1* promoter (Fig. 1B). In the recipient strains Rut-C30 and ALKO2221, the *cbh1* region was released as a 9-kb DNA fragment. Because *Xho*I does not cut the

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TABLE 1. Production of barley EPB in *T. reesei*

Strain	Transformation host	Presence (+) or absence (−) of CBHI	Maximum cysteine proteinase activity (U/liter)	Amt of excreted EPB protein (mg/liter)
ALKO2221		+	0	0
ALKO3708	ALKO2221	−	1,700	500
ALKO3709	ALKO2221	−	1,500	50
ALKO3711	ALKO2221	+	3,400	100
Rut-C30		+	0	0
ALKO3713	Rut-C30	+	11,000	50

expression vector, the size of the *cbh1* locus was expected to increase by 1.9 kb (to 11 kb) in a single-copy transformant and by an additional 8.9 kb (to 20 kb) in a two-copy transformant. Three or more copies in the *cbh1* locus would give a band larger than 29 kb. Strain ALKO3709 carried an intact copy of the expression cassette integrated in the *cbh1* locus (Fig. 1B). The CBHI-negative strain ALKO3708 gave two hybridizing *Xho*I fragments (sizes of approximately 11 and 20 kb). The 11-kb fragment may represent a single copy of the expression cassette integrated into the *cbh1* locus, and the larger fragment originates from a copy elsewhere in the genome. In the CBHI-positive transformant ALKO3711, which also exhibited an intact 9-kb *Xba*I fragment when it was probed for the *cbh1* coding region (Fig. 1A), the size of the *Xho*I fragment was changed (Fig. 1B), indicating rearrangements in the *cbh1* locus. The result may be explained by the integration of the cassette (or parts of it) into the promoter region of the *cbh1* gene 5′ to the *Xba*I site of the promoter. The Rut-C30 transformant ALKO3713 had one copy at an unknown locus.

EPB-producing transformants and the recipient strains Rut-C30 and ALKO2221 were grown for 5 days in a 1-liter laboratory fermentor (Braun Biostat M; B. Braun Melsungen AG, Melsungen, Germany) in a cellulase-inducing medium (21) by standard procedures. Production of the recombinant EPB was analyzed by Western blotting with the EPB antiserum (11). The amount of EPB was estimated from 1-μl fermentor samples by comparison to 50 ng of the purified barley EPB protein. To determine the time dependency of EPB production, 10-μl samples, which were taken at different time points of fermentor cultivations (0 to 5 days), were analyzed by Western blotting. Cysteine proteinase activities from the fermentor samples were measured according to the method described by Koehler and Ho (10), except that the incubation time was extended to 5 h at 40°C. The true cysteine proteinase activity was verified with the specific inhibitor E-64 (Sigma Chemical Co., St. Louis, Mo.) (10). The A_{330} was calculated by subtracting the values obtained from incubation mixtures containing the enzyme sample and 10 μM inhibitor from those carried out without the inhibitor.

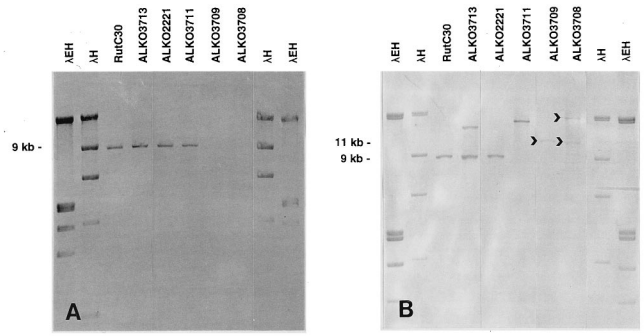

FIG. 1. Southern blot analyses of *Trichoderma* strains transformed with the EPB expression cassette from pALK584 and of the nontransformed host strains Rut-C30 and ALKO2221. (A) Genomic DNA digested with *Xba*I and hybridized with a digoxigenin-labelled *cbh1* cDNA probe; (B) genomic DNA digested with *Xho*I and hybridized with a digoxigenin-labelled *cbh1* promoter probe. Arrows, faintly hybridizing fragments. Molecular weight markers lambda *Hin*dIII (λH) and lambda *Eco*RI-*Hin*dIII (λEH) were used. Hybridizing fragment sizes are indicated on the left.

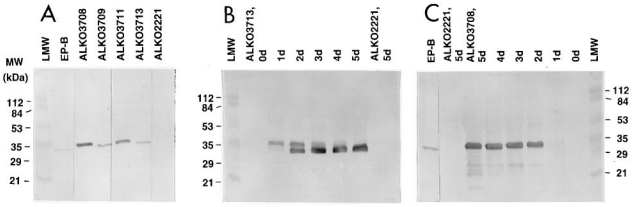

FIG. 2. Production of barley EPB protein by *T. reesei*. Western blotting of the culture media of strains transformed with the EPB expression cassette and of the nontransformed host strain ALKO2221 cultivated in a cellulase-inducing medium for 5 days was carried out with polyclonal EPB antiserum. (A) The amounts of EPB secreted by different *T. reesei* transformants estimated from fermentor samples by comparison to purified barley EPB protein (50 ng); (B) time course of EPB production by the recombinant strain ALKO3713 analyzed from fermentor samples taken at different time points of cultivation (0 to 5 days) (the 5-day sample of the nontransformed strain ALKO2221 served as a control); (C) time course of EPB production by recombinant strain ALKO3708 analyzed as described for panel B. Purified barley EPB protein (50 ng) was loaded for electrophoresis. The positions of the prestained molecular weight markers (LMW) are indicated. d, days.

The highest level of recombinant EPB protein, which was approximately 500 mg/liter, was produced by the *T. reesei* ALKO2221-derived transformant ALKO3708 (Fig. 2A). This exceeds the values reported for any other higher eukaryotic protein expressed in *T. reesei* or for initial transformants of any other filamentous fungus. In ALKO3708, the maximum enzyme activity produced in the fermentor was 1,700 U/liter (Table 1). The production levels of recombinant EPB protein correlated in general with the copy numbers of expression cassettes integrated in the fungal genome. In ALKO3708, the large amount of protein may be increased by the integration of the expression cassette at an unidentified, effective location in the fungal genome in addition to one copy at the *cbh1* locus. In the transformant ALKO3709, one copy of an intact expression cassette in the *cbh1* locus yielded approximately 50 mg of secreted EPB per liter (Fig. 2A). The Rut-C30-based transformant ALKO3713 produced approximately 50 mg of EPB per liter (Fig. 2A), and the highest enzyme activity measured was 11,000 U/liter (Table 1).

In the barley EPB, the mature sequence is preceded by a signal peptide and a propeptide, which is cleaved off outside the cell to activate the enzyme. Western blot analysis (Fig. 2A) revealed that in all *T. reesei* transformants, the recombinant EPB was slightly larger than the authentic purified barley enzyme. In strain ALKO3708, only one band of immunoreactive EPB was detected during the cultivation period (Fig. 2C), whereas in ALKO3713, two immunoreactive protein bands with different sizes were seen. At day 1, a band that was approximately 2 to 3,000 kDa larger than the dominating form was present. On day 2, this band appeared together with the main recombinant protein but disappeared gradually by day 5 (Fig. 2B). The larger protein may have represented a precursor form of EPB subjected to processing, since both forms were visible on the second day of cultivation. The processing was not mediated by acidic conditions when it was tested by addition of acetic acid to concentrations as high as 90 mM (data not

shown), which may imply the presence of a processing activity in the transformant. Interestingly, an enzyme that has not yet been identified which is capable of correct processing of CBHI-Fab fusion protein in *T. reesei* Rut-C30-based transformants has been reported by Nyyssönen et al. (17). Evidence exists that the barley EPB is glycosylated in *T. reesei*, which may interfere with the processing of the recombinant enzyme into an active form (16).

A protein somewhat larger than the authentic form was also detected in extracts of *Aspergillus oryzae* transformants expressing recombinant thaumatin which, however, was of the correct size when it was analyzed from the culture medium (5). As our study also found, the plant signal sequence was functional in the fungal host; however, the amount of secreted thaumatin in *A. oryzae* was considerably smaller, 3.2 mg/liter, compared to 500 mg of EPB per liter in *T. reesei*. In both studies, maximum accumulation of the extracellular immunoreactive protein was observed after 2 days. The measurements of intracellular protein from ALKO3708 and ALKO3713 mycelia grown for 2 days revealed that less than 10% of the immunoreactive EPB was retained inside the cells. On the fifth day, practically all of the EPB protein was found in the medium (not shown).

The specific activities of the recombinant EPB as calculated from Table 1 varied considerably (from 3.4 to 220 U/mg of immunoreactive protein) among transformants. They were less than the specific activity (approximately 1,000 U/mg of protein) reported for the purified barley EPB (12). The variation between transformants may partly be explained by the use of an enzyme activity assay operating at very low enzyme concentrations and protein amounts deduced from the Western blots. Differential processing of the enzyme in *T. reesei*, as seen in Fig. 2B and C, exhibiting the secreted forms of recombinant EPB from two host strains could also create variation. The exact nature of the processing could be verified by purification of the recombinant EPB produced in the transformants, followed by N-terminal sequencing of the proteins. Further experiments are needed to clarify why ALKO3713 from Rut-C30 produces more active EPB.

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