Expression and Secretion of Barley Cysteine Endopeptidase B and Celllobiohydrolase I in *Trichoderma reesei*

MARKO NYKÄNEN,1,4 RITVA SAARELAINEN,2 MARJATTA RAUDASKOSKI,3 K. M. HELENA NEVALAINEN,4 AND ANITA MIKKONEN†

Department of Biological and Environmental Science, University of Jyväskylä, 40351 Jyväskylä,1 Primalco Ltd. Biotec, Valta-akseli, FIN-05200 Rajamäki,2 and Department of Biosciences, 00014 University of Helsinki,3 Finland, and School of Biological Sciences, Macquarie University, Sydney, New South Wales 2109, Australia4

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Localization of expression and secretion of a heterologous barley cysteine endopeptidase (EPB) and the homologous main celllobiohydrolase I (CBHI) in a *Trichoderma reesei* transformant expressing both proteins were studied. The transformant was grown on solid medium with Avicel cellulose and lactose to induce the cbh1 promoter for the synthesis of the native CBHI and the recombinant barley protein linked to a cbh1 expression cassette. Differences in localization of expression between the two proteins were clearly indicated by in situ hybridization, indirect immunofluorescence, and immunoelectron microscopy. In young hyphae, native-size recombinant epb mRNA was localized to apical compartments. In older cultures, it was also seen in subapical compartments but not in hyphae from the colony center. The recombinant EPB had a higher molecular weight than the native barley protein, probably due to glycosylation and differential processing in the fungal host. As was found with its transcripts, recombinant EPB was localized in apical and subapical compartments of hyphae. The cbh1 mRNA and CBHI were both localized to all hyphae of a colony, which suggests that the endogenous CBHI was also secreted from these. In immunoelectron microscopy, the endoplasmic reticulum and spherical vesicles assumed to contribute to secretion were labeled by both CBHI and EPB antibodies while only CBHI was localized in elongated vesicles close to the plasma membrane and in hyphal walls. The results indicate that in addition to young apical cells, more mature hyphae in a colony may secrete proteins.

The ability to secrete hydrolytic enzymes into the external medium is a characteristic of filamentous fungi which makes them important as scavengers of plant material in nature and as producers of enzymes in industry. One of the best-known secretors of extracellular proteins is the cellulolytic mesophile fungus *Trichoderma reesei* (reviewed by Nevalainen et al. [25]). Mutant strains can secrete up to 40 g of protein per liter into the growth medium, about 60% of which consists of celllobiohydrolase I (CBHI), produced by one gene (5, 7). After cloning of the gene encoding CBHI, its strong inducible promoter was used for the expression of both homologous and heterologous gene products in the eukaryotic *T. reesei* system (reviewed by Nevalainen and Penttilä [23]).

At the molecular level, very little is known about the secretory pathway, posttranslational modifications, and release of extracellular enzymes from the hyphae into the growth medium in filamentous fungi. Also, the yields of heterologous gene products from *T. reesei* and other fungi have so far been quite low when compared to those of homologous proteins. Since transcription has not been considered to be the main restricting factor in the production of heterologous proteins in filamentous fungi (17), it is probable that they are lost while secreted. Thus, it would contribute to the basic understanding of fungal cell biology and help improve production strains if the sorting, targeting, and excretion of proteins from the hyphae of *T. reesei* were better understood, as is the case for animal cells, yeasts and bacteria (16, 30, 32, 39).

The main secretory route suggested for proteins in filamentous fungi is through the growing hyphal apex (40). Rapid apical growth of hyphae requires an efficient system for exocytosis of cell wall precursors. The proteins destined for secretion are thought to follow the bulk flow and traverse the growing apex (38). Immuno electron microscopic localization of the homologous endoglucanases in *T. reesei* (35) showed intensively labeled hyphal tips and some label in the hyphal walls. More recent ultrastructural studies with an alkaline xylanase produced by a high-cellulase-secreting mutant of *T. reesei*, Rut-C30, indicated that two major subcellular structures, the endoplasmic reticulum (ER) and secretory vesicles/vacuoles, were involved in secretion (14). No Golgi-like structures were identified in the study.

To increase our understanding of protein processing and excretion of heterologous proteins in fungi, a cDNA of a barley (*Hordeum vulgare*) cysteine endopeptidase B (EPB) has been introduced into *T. reesei* (35). In barley, the secretion of EPB is fast and is apparently a constitutive process in a tissue surrounding a biosynthetically inactive storage compartment of the grain. The secretion occurs through the Golgi complex and inside small vesicles without accumulation (20). The function of the EPB is to hydrolyze storage proteins to amino nitrogen for further use in the seedling. This is a process comparable to the secretion of cellulolytic enzymes of *T. reesei* into its surroundings to hydrolyze polymeric cellulose to oligosaccharides and glucose for growth. Thus, the EPB is a good model for investigating the transcription, translation, and secretion of a foreign protein in *T. reesei*. In the present work, we localize and compare the expression and secretion of EPB and the endogenous CBHI in a recombinant strain, *T. reesei* ALKO3713, which produces both proteins on solid cultivation media.

† Present address: University of Oulu, Research and Development Centre of Kajaani, FIN-87100 Kajaani, Finland.

* Corresponding author. Mailing address: Department of Biological and Environmental Science, University of Jyväskylä, P.O. Box 35, FIN-40351 Jyväskylä, Finland. Phone: 358-14-602297. Fax: 358-14-602221. E-mail: nymaju@tukki.jyu.fi.

4 Present address: University of Oulu, Research and Development Centre of Kajaani, FIN-87100 Kajaani, Finland.
immunolabeling of EPB. Monoclonal antibody against CBHI, CI-261 (1.6 mg/serum (0.34 mg/ml) was separated by protein G-Sepharose (MAbTrap G; Pharmacia LKB Biotechnology, Uppsala, Sweden) and used for immunoblotting and immunolabeling of EPB. Monoclonal antibody against CBHI, CI-261 (1.6 mg/ml) was used as the primary antibody for CBHI immunostaining. The monoclonal antibody for CBHI at room temperature. Antibodies against EPB and CBHI were diluted to 25 and 3.2 mg/ml, respectively. To control the specificity of the EPB immunoblotting, the EPB antibody was preincubated for 2 h with growth medium containing the recombinant EPB before immunostaining.

In situ hybridization. The quick-frozen, freeze-substituted, and hyphal wall preparations were then described by Raudaskoski et al. (31). After these treatments, hyphae were rinsed for 5 min with phosphate-buffered saline (PBS), at pH 5.5 and then at pH 7.3. The specimens were placed onto aminocarboxylasinate-coated slides (9), which were laid on a thermal block at 40°C. To enhance the penetration of the probe, the hyphae were treated with protease K (10 μg/ml; Boehringer, Mannheim, Germany) for 10 min at 37°C and washed briefly with distilled water. Prehybridization and hybridization were then described by Marttila et al. (19) with the following modifications. Prehybridization was carried out for 1 h at 40°C in the hybridization buffer (50% formamide, 10% dextran sulfate, 0.3 M NaCl, 1× Tri-EDETA [TE], 1× Denhardt’s solution, 10 mM diithiothreitol) containing 20 mg of tRNA per ml from RNase-free Escherichia coli. For in situ hybridization, the cDNAs described above were 3′-S-DATP labeled by the random-primer kit as for Northern blot analysis, giving a specific activity of 1 × 10⁷ to 5 × 10⁷ cpm/μg. The cDNA of Rubisco was used as a control probe. T. reesei strains that did not synthesize cbh1 or epb mRNAs were treated with the cbh1 or epb probes as negative controls. The final probe concentration in the hybridization buffer was 0.5 ng/μl. Hybridization was carried out for 16 h at 42°C, and posthybridization washes were done at 60°C, twice with 2× SSC for 30 min and once with 0.5× SSC for 1 h. The slides were then rapidly dehydrated in ethanol, air dried, and laid on Kodak X-Omat X-ray film for 3 days for preexamination. The slides were then processed as described by Raudaskoski et al. (20). The IgG fraction of EPB antiserum was diluted 1:1 with distilled water, and exposed for 1 to 3 weeks at 4°C. They were developed in Kodak D19 developer for 3 min and fixed in Kodak Unifix for 5 min.

In situ specimens were also treated with N-acetylgalactosamine-specific rhodamine-conjugated wheat germ agglutinin (WGA; Sigma) to visualize fungal cell walls and septa. The developed slides were washed with PBS (pH 7.3), and rhodamine-WGA (25 mg/ml) in PBS containing 100 μM each CaCl2, MgCl2, and MnCl2, was added to the specimens. The slides were incubated for 2 h at 37°C and rinsed with PBS at pH 7.3 and then at pH 8.5. The specimens were then mounted into glycerol-PBS buffer (1.2) containing 1 μg of 4′,6-diamidino-2-phenylindole (DAPI) per ml to visualize the nuclei.

Indirect immunofluorescence microscopy. For protein localization by indirect immunofluorescence microscopy, samples from the T. reesei ALKO3713 strain and host Rut-C30 colonies were quick-frozen and freeze-substituted, and the cell walls were treated as described by Raudaskoski et al. (31). For detection of CBHI and EPB, cells were treated with the primary antibody with the dilution buffer were used as controls. Treatments with the rhodamine-WGA lectin were then carried out and the samples were rinsed as described above.

Immunoelectron microscopy. Mycelia from the ALKO3713 transformant and Rut-C30 strain were fixed for 2 h at room temperature in a mixture of 4% (wt/vol) paraformaldehyde and 1% (vol/vol) glutaraldehyde in PBS (pH 7.3) and then rinsed thoroughly with PBS. The fixed samples were incubated at 4°C overnight with rotation in 20% (wt/vol) polyvinylpyrrolidone (M, 10,000; Sigma, St. Louis, Mo.) containing 1.84 M sucrose and then frozen in liquid nitrogen as described by Tokuyasu (36). Ultrathin sections were cut at −90°C with the Reichert Ultratome FC4 ultramicrotome equipped with a cryostat. Sections were then placed on nickel grids coated with Formvar (Monsanto) and carbon. After immunolabeling, the specimens were first incubated in PBS blocking solution containing 10% (wt/vol) fetal calf serum (Sigma) and 0.12% (wt/vol) glycine for 30 min and then incubated in the IgG fraction for EPB or the monoclonal antibody for CBHI at room temperature. Antibodies against EPB and CBHI were diluted to 25 and 10 μg/ml in the blocking solution. After a 2-h incubation, the grids were washed in PBS-glycine for 25 min, and...
those treated with the polyclonal EPB antibody were incubated for 60 min with protein A conjugated to 10-nm-diameter gold particles (ICN Immunochemicals, Irvine, Calif.). Protein A-gold was prepared by the method of Slot and Geuze (34) and diluted to 1:125 with blocking solution. For CBHI localization, the grids were incubated for 60 min with the primary antibody. The grids were examined with a JEOL 1200EX transmission electron microscope at an acceleration voltage of 60 kV.

RESULTS

In this work, we have studied the simultaneous expression and secretion of a heterologous EPB and homologous cellulase CBHI enzyme in the T. reesei transformant strain producing both proteins. Preliminary tests with transformants showed that features of the transformant ALKO3713 made the strain best suitable for our purposes of the expression studies in T. reesei. Fungal cultivations were performed on solid media to facilitate the localization of transcription and enzyme secretion.

Expression of CBHI and EPB in T. reesei. Northern hybridization of the total RNA isolated from the T. reesei transformant strain ALKO3713 with epb cDNA showed that the transformant produced epb-specific transcripts of the same size as epb mRNA found in barley aleurone layers (Fig. 2A). A signal for the natural-size cbh1 mRNA, about 2.2 kb, was also obtained (Fig. 2B), because integration of the ebp construct had not occurred in the endogenous cbh1 locus (33). The host strain, T. reesei Rut-C30, did not produce any epb mRNA (Fig. 2A, lanes 4 and 5), and no signal for cbh1 mRNA was detected in the barley aleuronal RNA (Fig. 2B, lane 6). The presence of lactose in the growth medium significantly increased the synthesis of the epb and cbh1 transcripts in T. reesei (Fig. 2A and B; compare lanes 1, 2, and 3), as expected (13, 22).

In the immunoblots of extracts from barley aleurone layers, the mature EPB was seen as a 30-kDa band and its propeptide was seen as a 42.5-kDa band (Fig. 3A). When the EPB antibody was used in Western blotting to detect the EPB produced by the T. reesei transformant ALKO3713, a range of secreted polypeptides with molecular masses between 32 and 36 kDa were obtained on days 4 and 5 (Fig. 3A). From days 1 to 4, intracellular T. reesei extracts showed considerably larger forms, ranging from about 50 to 70 kDa. The specificity of the antibody used was indicated by the absence of signal when the host strain extract was treated with the antibody (Fig. 3A) or when the EPB antibody was preincubated with growth medium containing the recombinant EPB (data not shown). Approximately the same amount of the 67-kDa CBHI protein was produced by both the transformant strain ALKO3713 and its host strain, Rut-C30 (Fig. 3B). Interestingly, the production of CBHI in the transformant started 1 day earlier than in the host strain. The absence of lactose in the growth medium decreased the amounts of EPB and CBHI produced in T. reesei (Fig. 3).

In situ localization of epb and cbh1 transcripts in fungal hyphae. In the hyphae of the transformant ALKO3713 grown for 1 to 5 days on Avicel-lactose plates, strong signals were obtained when the transcripts of epb and cbh1 were localized by in situ hybridization (Fig. 4 and 5, respectively). When hybridization was carried out with the cDNA probe of Rubisco for the transformant strain ALKO3713 or with the epb probe for the host strain Rut-C30, only low background labeling occurred (Fig. 4A and B).

In 1- to 5-day-old samples, the epb transcripts were located only in the apical and subapical cells of hyphae at the edge of the colony. After growth for 16 h, dispersed epb transcripts
The signal for the \textit{cbh1} mRNA was also detected in the subapical compartments of the mycelia of 1-day-old samples. The signal was clear also in the mycelium at the colony edges. In the samples from 1-day-old colonies, the signal for \textit{cbh1} was seen only at the apex of the hyphae (Fig. 5B). In 3- to 5-day-old samples, the \textit{cbh1} signal was strong in the entire mycelium including the old hyphal cells (Fig. 5D, F, and G). Similar signals for \textit{cbh1} transcripts were also detected in the host strain Rut-C30 (data not shown). In all 13 colonies of the transformant ALKO3713 and the host strain Rut-C30 inspected, over 95% of young and old hyphal cells throughout each colony had the characteristic signal for \textit{cbh1} mRNA.

**Localization of EPB and CBHI proteins in hyphae.** Labeling of the EPB protein in the \textit{T. reesei} transformant ALKO3713 showed that the recombinant cysteine proteinase was located in the hyphae at the colony edges. In the samples from 1-day-old colonies, the signal for EPB was seen only at the apex of the hyphae (Fig. 6B). In 3-day-old samples, the label was seen as bright spots in the apical cells and their branches (Fig. 6E), as well as in the adjacent subapical cells (data not shown). The bright spots, visualized with fluorescein, were interpreted to represent vesicle-like structures containing EPB protein. The hyphal structure was well preserved after the immunolabeling procedure. The hyphae accommodated several nuclei, and a certain distance was always observed between the tip and the nucleus closest to it (Fig. 6D and G). Similar hyphal structures were also visualized in the host strain Rut-C30 but without a signal for EPB after the immunolabeling process (Fig. 6F to H). Over 95% of 200 randomly chosen apical and subapical cells gave the positive label for EPB in each of 12 colonies of the transformant ALKO3713. The signal was totally absent in the cells of the central area of each analyzed colony.

In the transformant strain ALKO3713, which produced both CBHI and EPB, a signal for CBHI was visible throughout the mycelium of 1-day-old samples. The signal was clear also in the apical cells (Fig. 6I). After 2 days, stronger labeling was seen than did \textit{epb} transcripts. Similarly to the \textit{epb} signal, these were absent from the extreme apex of hyphae (Fig. 4C and 5B). In 3- to 5-day-old samples, the \textit{cbh1} signal was strong in the entire mycelium including the old hyphal cells (Fig. 5D, F, and G). Similar signals for \textit{cbh1} transcripts were also detected in the host strain Rut-C30 (data not shown). In all 13 colonies of the transformant ALKO3713 and the host strain Rut-C30 inspected, over 95% of young and old hyphal cells throughout each colony had the characteristic signal for \textit{cbh1} mRNA.
throughout the mycelium both at hyphal tips and in areas behind them. The signal was also seen in association with hyphal walls but not with the septa (Fig. 6I and J). Nuclei occurred as dark areas surrounded by the labeled CBHI, as shown in an example from the older parts of the mycelium (Fig. 6I and K). In the host strain Rut-C30, a strong signal for CBHI was frequently obtained close to the hyphal wall (Fig. 6L) and labeling for CBHI was detected in the entire mycelium including apical cells (Fig. 6M). Each of 10 inspected colonies of the transformant ALKO3713 and the host strain Rut-C30 had the characteristic label for CBHI in over 99% of all the young and old hyphal cells throughout the colony.

Subcellular localization of EPB and CBHI. A more detailed localization of EPB and CBHI was carried out by immunoelectron microscopy investigation of cryosections from the transformant strain T. reesei ALKO3713 (Fig. 7 and 8). A common ultrastructural feature of the host strain Rut-C30 and the recombinant strain ALKO3713 was the large amount of ER. This has also been reported in a previous ultrastructural study of the host strain Rut-C30 (7).

In the immunoelectron microscopic investigation, the recombinant EPB was localized in the ER and small vesicles, which had perhaps been budded from the ER. All hyphae in which the label was found had thin hyphal walls, which suggested that they were from the young parts of the mycelial colony (Fig. 7A to D). Vesicles with the label were also detected in the proximity of the plasma membrane. Characteristic of EPB immunolabeling was the occurrence of the label in conspicuous aggregates next to the ER. No membrane surrounding the aggregation or individual immunogold particles could be distinguished even at high magnification (Fig. 7D). EPB was also localized in the area close to flat, tubular endomembrane cisternae that seemed to be associated with the plasma membrane and to open to the cell wall (Fig. 7E). The function of these structures is unknown, but they resemble the membrane compartments shown to be responsible for Golgi functions in wild-type yeast cells (29). In control sections treated with buffer instead of the EPB antibodies, only sporadic labeling was observed (Fig. 7F).

In the ALKO3713 strain, CBHI was associated with the ER throughout the cross sections of young (Fig. 8A, E, and F) and old (Fig. 8B to D) hyphae, where the age was deduced from the thickness of the cell wall. CBHI label was also detected in the hyphal wall but not in the septa (Fig. 8B to D), which was consistent with the indirect immunofluorescence microscopic results. In the hyphae, the label for CBHI was often present inside elongated vesicles adjacent to the septum and cell wall (Fig. 8E and F). In addition, small spherical vesicles which resembled those detected by the EPB antibody were labeled. Electron-dense putative protein bodies surrounded by the membrane did not give any positive signal for CBHI (Fig. 8D).

DISCUSSION

In this study, we have focused on visualization of the expression and structures involved in the secretion of the main endogenous CBHI enzyme and a heterologous barley (H. vulgare) EPB in T. reesei. This was possible in the selected transformant ALKO3713, since the integration of the expression cassette had not disrupted the cbh1 locus. Thus, the strain produced both CBHI and EPB, of which about 50 mg/liter was found in the culture medium, when grown in shake flask cultures (33). In strain ALKO3713, the level of recombinant epb transcripts was only slightly lower than that of endogenous cbh1 transcripts. This supports the idea that transcription may not be the main restricting event in the production of heterologous proteins in filamentous fungi (17).

The cDNA sequence of the EPB used for expression encodes a preproenzyme of 42.5 kDa. During processing, the 2.5-kDa signal sequence and a propeptide of 12.3 kDa are cleaved off to give the mature form of 25.7 kDa. On SDS-polyacrylamide gel electrophoresis, the mature enzyme migrates with a size of about 30 kDa (12). In Western blots presenting EPB produced by T. reesei ALKO3713, the EPB antibody recognized intracellular high-molecular-weight polypeptides and multiple forms of secreted recombinant EPB. The 42.5-kDa preproform of the 30-kDa mature enzyme was not seen in the transformant T. reesei, and the secreted form was slightly larger than the mature barley enzyme. However, the secreted EPB from T. reesei is enzymatically active (33), which indicates that the fungus is capable of processing at least part of the enzyme into an active form. An explanation for the large EPB polypeptides could be the glycosylation of the barley enzyme in the fungal host. Our preliminary in vitro deglycosylation studies suggest that the recombinant protein, unlike in H. vulgare, is indeed N glycosylated (25a).

In situ hybridization experiments indicated that in the transformant ALKO3713, the epb mRNA was located mainly in the young hyphae while the cbh1 mRNA also appeared in the old compartments of the colony. Occurrence of the transcripts in apical compartments which are involved in the growth of the
FIG. 7. Intracellular localization of EPB in T. reesei transformant grown on Avicel-lactose medium for 2 days. Ultrathin cryosections were treated with the EPB antibody followed by protein A-conjugated 10-nm-diameter gold particles. Arrows point to membrane-coated vesicles (B and E). The open arrow indicates an EPB aggregate adjacent to the ER (C). The arrowhead marks the nuclear envelope (F). cw, cell wall; er, endoplasmic reticulum; gly, glycogen granules; m, mitochondrion; pm, plasma membrane; ec, endomembraneous cisterna; n, nucleus; pb, protein body. Bars, 0.2 μm (A to D and F) and 0.1 μm (E).
FIG. 8. Intracellular localization of CBHI in the T. reesei transformant grown on Avicel-lactose medium for 2 days. Ultrathin cryosections were treated with monoclonal CBHI antibody followed by anti-mouse IgG-conjugated 10-nm-diameter gold particles. Arrowheads indicate the label for CBHI (B and C). Arrows point to various labeled vesicles (D to F). cw, cell wall; er, endoplasmic reticulum; gly, glycogen granules; m, mitochondrion; pb, protein body; pm, plasma membrane; s, septum. Bar, 0.2 μm.
hyphae suggests that nuclei which are closely coupled to the progression of the cell cycle (4) are also involved in the production of transcripts of secreted proteins. The epb mRNA observed in subapical compartments and that of cbh1 in the hyphae from the center of the colony could represent stable transcripts originating at the time these compartments belonged to the actively growing parts of the colony. If this is the case, the absence of epb mRNA from the old hyphae could indicate that it is less stable than the endogenous cbh1 mRNA.

In the T. reesei transformant studied, EPB and CBHII were generally localized in the same hyphal compartments as their transcripts, except that both proteins were also found at the very apex of the hyphae, where transcripts were not detected. This means that transport of the proteins in the hyphae must take place from the site of synthesis, for example, to the tip. In yeast and animal cells, transport through the secretory pathway occurs in transport vesicles (30). In the immunogold-labeled cryosections of hyphae from ALKO3713, EPB and CBHII were localized in the ER and close to it in vesicles, which could represent transport vesicles leaving the ER. Only a few structures comparable to Golgi compartments in yeast (29) have been identified. The lack of data about the morphology of the Golgi compartments in T. reesei made it difficult to deduce the part of the secretion pathway targeting to the plasma membrane, although vesicles containing EPB and CBHII were distinguished close to it.

Electron-dense elongated vesicles were found to be only CBHII labeled in both strains studied. These elongated vesicles could originate from Golgi bodies (8). The tubular elongated vesicles could form a part of the secretion mechanism for CBHII and represent an ultrastructural counterpart to structures with strong fluorescence seen close to the plasma membrane in the indirect immunofluorescence microscopic studies. In the labeled cryosections, CBHII was also observed inside the hyphal wall but not in the septa. These results, together with the occurrence of the cbh1 transcripts and the CBHII protein in all hyphae independent of age, suggest that each hyphal compartment throughout the T. reesei mycelium has the ability to secrete the CBHII protein. This may partially explain the gram-level yields of CBHII protein excreted in the cultivation medium and suggests a complementary mechanism for fungal protein secretion in addition to the bulk flow associated with polarized apical growth of hyphae (38). An alternative protein secretion pathway in T. reesei hyphae could, for example, function with a translocator resembling the P glycoprotein on the plasma membrane as suggested for Aspergillus (3).

Unlike the endogenous CBHII, immunolocalization results for the heterologous EPB suggest that the main secretion of EPB takes place at the hyphal apex. The EPB label was detected only in the youngest part of the colony, and no label was seen next to the plasma membrane along the lateral walls of hyphae by indirect immunofluorescence microscopy or in the hyphal walls in labeled cryosections. In the immunoelectron microscopic investigation, aggregates of EPB-labeled granules were found adjacent to the ER. Whether these EPB aggregates are ultrastructural equivalents of the large proteins in the intracellular fraction revealed by Western blotting, perhaps representing poorly secretable/unsecretable EPB, remains to be clarified.

In summary, this is the first report on a microscopic study of simultaneous expression and secretion of a native protein and a foreign protein in a filamentous fungus. We were able to show that the translational sites of EPB and CBHII correspond to their transcriptional locations, which are different for the recombinant EPB and the native CBHII. The cbh1 gene is expressed and protein is secreted throughout the mycelium, whereas expression of the recombinant EPB occurs only in the apical and subapical cells. Thus, CBHII could contain a signal(s) promoting effective secretion from the fungal cell. We plan to explore this further with the aid of different fusion proteins. Comparison of the levels of transcripts for the immunoreactive EPB and the endogenous CBHII showed that the efficiency of translation of the recombinant mRNA was reasonably high and thus will not explain the low yields of the secreted recombinant EPB. The most likely reasons for this include degradation, incomplete processing, and/or activation of the barley protein produced in the fungus. In future studies, we will focus on the processing of EPB in Trichoderma hosts.

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