Monitoring the kinetics of glycoprotein synthesis and secretion in the filamentous fungus *Trichoderma reesei*: cellobiohydrolase I (CBHI) as a model protein

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The authors have developed methodology to study the kinetics of protein synthesis and secretion in filamentous fungi. Production of cellobiohydrolase I (CBHI) by *Trichoderma reesei* was studied by metabolic labelling of the proteins *in vivo* with [³⁵S]methionine or [¹⁴C]mannose, and subsequent analysis of the labelled proteins using two-dimensional gel electrophoresis. Analysis of the different pI forms of the nascent proteins allowed monitoring of the maturation of CBHI during the transport along the biosynthetic pathway. The maturation of the pI pattern of CBHI as well as secretion into culture medium was prevented by treatment with the reducing agent DTT. The pI forms of CBHI detectable in the presence of DTT corresponded to the early endoplasmic reticulum forms of the protein. Removal of *N*-glycans by enzymic treatment (endoglycosidase H or peptide-*N*-glycosidase F), or chemical removal of both *N*- and *O*-glycans, changed the pI pattern of CBHI, showing that glycan structures are involved in formation of the different pI forms of the protein. By quantifying the labelled proteins during a time course, parameters describing protein synthesis and secretion were deduced. The mean synthesis time for CBHI under the conditions used was 4 min and the minimum secretion time was 11 min. The methodology developed in this study provides tools to reveal the rate-limiting factors in protein production and to obtain information on the intracellular events involved in the secretion process.

Keywords: glycosylation, protein transport, two-dimensional gel electrophoresis, metabolic labelling

INTRODUCTION

The filamentous fungus *Trichoderma reesei* has been widely used for industrial enzyme production, especially its cellulolytic system, which is well characterized and exploited in industry (reviewed by Nevalainen & Penttilä, 1995; Penttilä *et al.*, 1991; Teeri *et al.*, 1992). The fungus is able to produce extracellular enzymes at very high levels. Under inducing conditions, production

of nearly 40 g extracellular protein l⁻¹ has been reported (Durand *et al.*, 1988). Cellobiohydrolase I (CBHI) forms about 60 % of the total amount of secreted protein. Due to the exceptionally high capacity of protein production, the fungus is also a potent host organism for production of heterologous proteins. However, the production of heterologous proteins in *Trichoderma* as well as in other industrially important filamentous fungi, e.g. *Aspergillus* species, is usually much less efficient compared to the vast production of endogenous proteins (reviewed by Keränen & Penttilä, 1995; Penttilä, 1998; Gouka *et al.*, 1997; Archer & Peberdy, 1997). This is especially the case with proteins from phylogenetically distant species, e.g. of mammalian origin.

The strong promoter of the *cbh1* gene has been

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Abbreviations: CBHI, cellobiohydrolase I; 2D, two-dimensional; Endo H, endoglycosidase H; ER, endoplasmic reticulum; PNGase F, peptide-*N*-glycosidase F.

commonly used for heterologous expression in *Trichoderma* (for a review see Penttilä, 1998). The abundantly expressed CBHI is encoded by a single copy gene, the regulation of which is well characterized (Ilmén *et al.*, 1996, 1997; Ilmén, 1997). The properties of CBHI are assumed to be beneficial in the different steps of protein production, and CBHI has therefore been successfully used as a fusion partner to alleviate the rate-limiting steps in production of heterologous proteins (Nyyssönen & Keränen, 1995; Penttilä, 1998).

The molecular mechanisms of protein secretion in the yeast *Saccharomyces cerevisiae* and in mammalian cells are known in great detail and the general features of the secretory pathway seem to be well-conserved in evolution. However, knowledge of the secretory pathway in filamentous fungi is rather limited despite their high capacity to produce secreted proteins and interesting multicellular filamentous morphology. Recent progress in the dissection of the fungal pathway has been reviewed by Archer & Peberdy (1997). With the aid of information obtained from other organisms, several genes encoding factors involved in these processes have now been cloned and characterized from filamentous fungi, e.g. those encoding a component of the signal recognition particle (Thompson *et al.*, 1995), foldases, such as peptide disulfide isomerase (Kajino *et al.*, 1994; Hjort, 1995; Lee *et al.*, 1996; Malpricht *et al.*, 1996; Ngiam *et al.*, 1997; Saloheimo *et al.*, 1999) and BiP (van Gemeren *et al.*, 1997; Hijarrubia *et al.*, 1997), as well as components from the vesicle transport machinery, such as Sar1 (Veldhuisen *et al.*, 1997). The actual site of protein secretion in the hypha has been located to the hyphal tips (Wösten *et al.*, 1991). However, in some cases the secretion has been reported to occur from subapical compartments (Keizer-Gunnink *et al.*, 1996; Nykänen *et al.*, 1997). It is even possible that different proteins are secreted through different sites on the hypha (Nykänen *et al.*, 1997).

The first events in *N*-glycosylation of the proteins and the trimming of the glycans in the endoplasmic reticulum (ER) occur in a similar fashion in yeast and animal cells (reviewed by Goochee *et al.*, 1991). The glycan structures are then modified further in the Golgi complex, in animal cells to produce the complex type glycans, and in yeast to produce the highly mannosylated large glycan structures. The O-glycans of *S. cerevisiae* proteins consist mainly of mannose units. The first two residues are attached to the protein in the ER, and the O-linked glycan chain is further extended in the Golgi complex (reviewed by Herscovics & Orlean, 1993). In animal cells, the O-glycosylation occurs in the Golgi complex, and the composition of the glycans is more complex. In filamentous fungi, the glycans present on the proteins seem to differ from those in both *S. cerevisiae* and higher eukaryotes. In *Trichoderma*, the glycosylation of proteins has mainly been studied by analysing the glycan structures of CBHI. CBHI has four potential *N*-glycosylation sites, three of which have been reported to carry glycans in strain Rut-C30. Typically, the *N*-glycans are high-mannose type structures of limited size, in which

the mammalian type core glycan is extended with mannose and/or glucose units (Salovuori *et al.*, 1987; Maras *et al.*, 1997a, b). In addition, some of the *N*-glycans have been found to carry atypical structures like terminal mannose α -1,6-phosphodiester (Maras *et al.*, 1997a, b). Different strains of *T. reesei* seem to display variation in the *N*-glycan structures synthesized; e.g. in some strains the *N*-glycans are reported to be composed of only a single *N*-acetylglucosamine (Harrison *et al.*, 1998; Klarskov *et al.*, 1997). The O-glycans of *T. reesei* CBHI are mainly formed of 1–4 mannose units, resembling the ones found in *S. cerevisiae* (Fägerstam *et al.*, 1984; Salovuori *et al.*, 1987; Harrison *et al.*, 1998).

In this study, we have developed methodology for the metabolic labelling and subsequent analysis of the maturation and secretion of proteins in *Trichoderma*. The techniques enable the kinetic analysis of protein synthesis and transport at a controlled physiological state of the fungus, and will be of great importance in determining the rate-limiting steps of these processes.

METHODS

Strains and cultivation conditions. *Trichoderma reesei* strain Rut-C30 (Montenecourt & Eveleigh, 1979) was cultivated in a minimal medium containing: $(\text{NH}_4)_2\text{SO}_4$, 7.6 g l⁻¹; KH_2PO_4 , 5.0 g l⁻¹; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g l⁻¹; $\text{CaCl}_2 \cdot \text{H}_2\text{O}$, 0.2 g l⁻¹; CoCl_2 , 3.7 mg l⁻¹; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 5 mg l⁻¹; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 1.4 mg l⁻¹; $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$, 1.6 mg l⁻¹; and lactose, 8 or 20 g l⁻¹. The inoculum for the cultivations was prepared by inoculating 2×10^7 spores (stored at -80 °C in 20% glycerol) into 200 ml growth medium (lactose 20 g l⁻¹), growing for 4 d in conical flasks at 30 °C with shaking at 200 r.p.m., and finally transferring to the bioreactor (1.8 l laboratory fermenter, Chemap) to a final volume of 1.5 l growth medium. The cultivations were carried out at 30 °C, with aeration of 1.3 vols air per vol. liquid min⁻¹ and stirring with impeller tip speed 2 m s⁻¹. The pH was kept at 4.8 ± 0.2 by the addition of 10% (w/v) KOH, and occasionally Struktol SB 2023 (Schill & Seilacher) was added to prevent foaming. When performing the carbon-limited chemostat cultivations, the feed (minimal medium containing 8 g lactose l⁻¹) was started at the time when the lactose in the batch phase was nearly consumed. A dilution rate of 0.07 h⁻¹ was used in the study.

Steady state of the chemostat cultivation was confirmed by analysing the fungal dry weight and the lactose, phosphate and ammonium concentrations in the culture broth. Dry weight was measured by filtering and drying mycelium samples at 105 °C to constant weight (24 h). Residual lactose and glucose in the cultivations was measured using either HPLC or an enzymic test kit (Boehringer Mannheim 176303) for lactose and the GOD-Perid method (Boehringer Mannheim 124036) for glucose. The amount of phosphate was measured as described in Basset *et al.* (1987). An enzymic test kit (Boehringer Mannheim 1112732) was used for measuring ammonium concentration.

Metabolic labelling of the proteins. The metabolic labelling experiments were carried out in 25 ml aliquots transferred gently from the bioreactor into shake flasks (temperature 30 °C, stirring 210 r.p.m.). The labelling was started immediately after the transfer. When labelling cells from chemostat cultivations, fresh medium was added into the shake flasks in order to avoid changes in the concentrations of

the nutrients. Fresh medium was added at intervals of 15 s during the labelling; the amount of medium added per culture volume was calculated to be the same that would be added in the chemostat during the time period (15 s). Either 5.5 or 12.5 μCi [^{35}S]methionine (Amersham SJ 1015, *in vivo* cell labelling grade, 1000 Ci mmol^{-1} , 10 μCi μl^{-1}) was added ($\text{mg dry wt biomass}^{-1}$ (1 Ci = 3.7×10^{10} Bq)). In some cases, [^{14}C]mannose (Amersham CFB26, 200–300 mCi mmol^{-1} , 200 μCi ml^{-1}) was used for the labelling of the glycan structures of the proteins [$3.5 \mu\text{Ci}$ ($\text{mg biomass dry wt}^{-1}$)]. Samples of 1 ml were collected during a time course. The mycelium in the samples was separated from the culture medium by filtering through Millipore HVLP02500 filters, which were washed with 10 ml double-distilled water and frozen immediately in liquid nitrogen. For the analysis of the labelled proteins the cells were resuspended in 20 mM *N*-ethylmaleimide, 10 mM NaN_3 ($3\text{--}7 \text{ mg biomass dry wt ml}^{-1}$), and disrupted by sonication ($8 \times 8 \text{ s}$ with an MSE 150 W sonicator, 18 micron amplitude, 30 s cooling on ice between the sonication cycles).

Analysis of the labelled proteins. Two-dimensional (2D) gel electrophoresis was carried out using the Immobiline DryStrip system (Pharmacia), and sample preparation was performed essentially according to the manufacturer's instructions. The mycelium samples were prepared by resuspending the lyophilized cell lysate first into 2D lysis buffer [9 M urea; 2%, v/v, Triton X-100; 286 mM β -mercaptoethanol; 2%, v/v, Pharmalyte 3–10; 560 $\mu\text{g biomass dry weight}$ ($25 \mu\text{l buffer}^{-1}$)], adding 3 vols 2D sample buffer (the lysis buffer containing 0.5%, v/v, Triton X-100), and incubating the samples at room temperature for 1 h. Insoluble material was removed by centrifugation. Equal amounts of total protein from the different time points were loaded in the gels (as determined by the Bio-Rad Protein Assay kit). The amount of protein loaded was confirmed to be in a range giving a linear response between the CBHI signal and the sample volume. Typically, 20 μg intracellular protein or 3 μg extracellular protein was loaded.

The total proteins in the mycelium lysates or culture supernatants were precipitated in 20% (w/v) TCA and 0.1% (w/v) BSA overnight on ice. The precipitate was heated at 95 °C for 10 min in 5% (w/v) TCA to release labelled tRNA (Braakman *et al.*, 1991), washed with 5% (w/v) TCA, and the radioactivity in the precipitate was measured by scintillation counting.

CBHI was immunoprecipitated from cellular extracts or culture supernatants using mAbs against CBHI. The epitopes of the antibodies have been mapped to amino acids 426–447 located in the C-terminal part of the core domain of the protein (α -CBHI 258, 261 or 271; Aho *et al.*, 1991). The immunoprecipitations were carried out in 800 μl 280 mM NaCl, 28 mM Tris/HCl (pH 7.5), 5.6 mM EDTA, 1.1% (v/v) Nonidet P-40. The cell lysates or samples of the culture supernatants were first incubated in the precipitation buffer for 30 min on ice. The insoluble material was removed by centrifugation for 5 min at 14000 r.p.m. at 4 °C. Protein G Sepharose 4 Fast Flow (Pharmacia) (50 μl) was added to the supernatants, and the samples were incubated with the beads with gentle mixing at 4 °C for 1 h, after which the beads were removed by centrifugation. The mAb against CBHI (7–8 μg α -CBHI 258, 261 or 271; Aho *et al.*, 1991) and 20 μl fresh Protein G Sepharose were added to the supernatants, followed by overnight incubation at 4 °C with gentle mixing. The immunoprecipitates were then washed twice with 1 ml of the precipitation buffer containing 0.1% (v/v) Nonidet P-40, and

once with 1 ml double-distilled water, and resuspended in an appropriate buffer for enzymic treatment (as described below) or into 2D gel sample buffer.

For digestion with endoglycosidase H (Endo H expressed in *E. coli*; Boehringer Mannheim), the immunocomplexes bound to Protein G Sepharose beads were resuspended in 50 μl 0.125 M sodium citrate (pH 5.3) and 0.2% (w/v) SDS and heated at 95 °C for 5 min. Half of the suspension was used for the digestion with 1 mU Endo H for 18 h at 37 °C, and the other half was used as a control sample. Peptide-*N*-glycosidase F (PNGase F expressed in *E. coli*; Oxford GlycoSystems) digestion of the immunoprecipitates was carried out in 20 mM sodium phosphate (pH 7.5), 50 mM EDTA, 0.5% (w/v) SDS and 5% (v/v) β -mercaptoethanol. Before the digestion the immunocomplexes were denatured by heating for 5 min at 95 °C, and 1% (v/v) Triton X-100 was added to the samples. The digestion with PNGase F was carried out for 18 h at 37 °C (10 U PNGase F was used per 30 μl reaction mixture).

Removal of O- and N-glycans by anhydrous trifluoromethane-sulfonic acid was performed using the Glycofree Deglycosylation kit (Oxford GlycoSystems). Before the deglycosylation treatment the samples of mycelium and culture supernatant were either dialysed against 0.1% (v/v) trifluoroacetic acid and lyophilized, or alternatively extracted with phenol and precipitated with acetone. The phenol extraction was carried out by adding an equal volume of phenol into the samples, vortexing for 30 s, centrifuging at 15000 g for 5 min, and collecting the organic phases. The extraction was carried out twice, and the phenol phases from the extractions were combined. Proteins were precipitated from the organic phases by adding 4 vols cold (-20 °C) acetone, incubating the samples on ice for 10 min, and centrifuging at 15000 g for 10 min. The pellets were washed with cold acetone, kept on ice for 10 min, centrifuged for 10 min, and finally dried. The deglycosylation of the proteins was confirmed using the GlycoTrack Carbohydrate Detection kit (Oxford GlycoSystems).

RESULTS

Labelling of the cells with [^{35}S]methionine or [^{14}C]mannose *in vivo*

To be able to carry out *in vivo* labelling of proteins in a controlled physiological state, the *T. reesei* Rut-C30 strain was grown in a carbon-limited chemostat. A chemically defined minimal medium with lactose as a carbon source was used in the cultivations. To obtain maximal labelling of the proteins, organic nitrogen sources and free methionine were excluded from the medium.

For metabolic labelling, aliquots of the fungal cultivations were gently withdrawn from the bioreactor into shake flasks. The conditions during the labelling experiment were designed to mimic the conditions in the fermenter as closely as possible. Any disturbances, such as filtering or centrifuging the fungal mycelium, which have been noticed to disturb, for example, label uptake, were omitted. The labelled amino acid precursor [^{35}S]methionine was added to the aliquots without changing the culture medium or its composition and without a chase with unlabelled compounds in order to avoid changes in the physiological state of the fungus. The

Fig. 1. Uptake of [^{35}S]methionine and incorporation of the labelled precursor into proteins. Metabolic labelling of the proteins was carried out as described in Methods. The total radioactivity [$\text{Bq (mg dry wt biomass)}^{-1}$] in the cellular extract (■) and in the corresponding samples of culture supernatant (○) at different time points after addition of [^{35}S]methionine was determined by scintillation counting. The label incorporated into proteins was analysed by TCA precipitation of the samples (□, TCA-insoluble radioactivity in the cell lysate; ○, in the culture supernatant).

labelled newly synthesized proteins were analysed during a time course from the cell extract and the culture medium. Labelling with [^{14}C]mannose was carried out to detect glycoproteins.

Uptake of the labelled methionine occurred very efficiently. About 95% of the label was found in the cellular extracts after the first minute of labelling in repeated experiments (Fig. 1). Because of the efficient uptake of [^{35}S]methionine, the amount of the label used was adjusted according to the biomass present in the experiments. Incorporation of [^{35}S]methionine into the proteins started immediately after addition of the precursor, as detected by TCA precipitation of the mycelial samples. During the first 10 min of labelling, the TCA-insoluble radioactivity increased rapidly, representing a minor portion of the total cellular label pool. Towards the end of the labelling period the incorporation continued with a declining rate. After 30 min of labelling, the TCA-insoluble pool represented 60% of the total cellular radioactivity. Therefore, free intracellular label was available and there did not seem to be limitations in incorporation of [^{35}S]methionine into the proteins in the early stages of labelling.

Synthesis and secretion of CBHI

The appearance of labelled CBHI in the intracellular and extracellular samples was analysed by 2D gel electrophoresis. The mobility of CBHI in the gel system was confirmed by Western blotting and by immunoprecipitation using mAbs against the protein. The parameters for the mean synthesis time and the minimum secretion time for the molecule were determined

Fig. 2. Kinetics of CBHI synthesis and secretion. Extracellular (●) and intracellular (▲) CBHI labelled with [^{35}S]methionine was quantified from 2D gels at different time points after addition of [^{35}S]methionine to the cultivation. The Molecular Dynamics Phosphorimager and the ImageQuant software were used for the quantification. The amount of labelled CBHI is shown as arbitrary phosphorimager units ($\text{mg dry wt biomass}^{-1}$).

by plotting the quantity of labelled CBHI in the cellular extract and in the culture medium during a time course (Fig. 2). The linear parts of the curves were extrapolated to the abscissa. Half of the synthesis time was determined as the intercept of the CBHI synthesis curve and the abscissa (Fig. 2; Braakman *et al.*, 1991; Horwitz *et al.*, 1969; Loftfield & Eigner, 1958). The lag time for incorporation of the labelled amino acid precursor into proteins was considered to be negligible (Fig. 1) and was thus omitted when determining the synthesis time of CBHI. The minimum time for secretion of the full-length molecules was determined as the difference between the intercepts of the curves representing the synthesis and production of CBHI into the culture medium (Fig. 2). Under the conditions used, at the exponential growth phase in batch fermentations or at the highest practical dilution rate, $D = 0.07 \text{ h}^{-1}$, in the chemostat cultivations, the mean synthesis time obtained for CBHI was 4 min and the minimum secretion time for the synthesized CBHI molecules was 11 min. The determined mean synthesis time of CBHI corresponded to translation of 128 aa min^{-1} . Similar results were obtained in several repeated experiments (data not shown).

The slopes determined for the linear parts of the CBHI synthesis and secretion curves (Fig. 2) represent the synthesis and secretion rates of the protein, respectively. To obtain the specific rates, the values were normalized for the mycelium biomass. It is evident that the slopes are not identical, and the secretion rate is somewhat lower than the synthesis rate. The balance between the synthesis, folding and intracellular transport processes on the one hand, and degradation of the synthesized protein on the other, determines the secretion rate. A parameter that describes the efficiency of the secretion process can be obtained as the ratio of the secretion and

Fig. 3. 2D gel electrophoretic analysis of CBHI synthesized by *T. reesei* Rut-C30. (a) Major extracellular proteins produced by the fungus labelled with [35 S]methionine for 30 min. The spots representing CBHI are boxed. The positions of molecular mass and pI markers are indicated on the left and on the top, respectively. (b) [35 S]Methionine-labelled CBHI immunoprecipitated from mycelial lysates at different time points (time after addition of [35 S]methionine is indicated above each sample). The mobility of the major early form of CBHI is indicated by an arrowhead.

synthesis rates. Under the conditions used in this study, the specific secretion rate of CBHI was about half of the specific synthesis rate of the molecule.

Analysis of the CBHI maturation process

The use of 2D gel electrophoresis enables the detailed analysis of the different molecular forms of CBHI. The major extracellular proteins (labelled with [35 S]-methionine) secreted by *T. reesei* Rut-C30 are shown in Fig. 3(a). The spots representing CBHI, as identified using monoclonal CBHI antibodies, are indicated by a box. By silver staining it is possible to identify at least eight different pI forms of CBHI, located in the pI range of 3.5–4.3, in the culture medium. At the beginning of the labelling time course there is only one major nascent pI form of CBHI detectable in the cellular extract (Fig. 3b). The full-length molecule is already detectable within 30 s of labelling, which was the first time point analysed. The additional pI forms positioned on the acidic side of the first one appear later in the intracellular maturation process of CBHI. These pI forms can first be detected after 7 min of labelling and their relative abundance increases thereafter (Fig. 3b). The analysis did not reveal significant amounts of partially synthesized CBHI polypeptides. Their proportion is probably too low to be detected in the cell extracts analysed directly in 2D gels, and the shorter forms are not precipitated with the CBHI antibodies, whose epitope is located in the C-terminal part of CBHI.

Glycosylation of the CBHI forms observed in the 2D gel pattern was monitored by labelling the cells with [14 C]mannose. When the labelled CBHI molecules were visualized in 2D gels, a pI pattern practically identical to the [35 S]methionine-labelled one was observed, indicating that the pI forms detected are modified with N- and/or O-glycan structures. The comparison of pI patterns of extracellular proteins labelled with either [35 S]methionine or [14 C]mannose is shown in Fig. 4. When the [14 C]mannose-labelled CBHI was quantified during a labelling time course, a delay of 2 min in the synthesis kinetics was observed compared to the [35 S]-methionine labelling (data not shown). The delay is probably due to a lag in incorporation of the mannoses into the glycan precursors, or perhaps in the protein glycosylation process itself. The minimum secretion time measured for the mannose-labelled CBHI was the same as that measured for the [35 S]methionine-labelled protein (11 min).

The impact of glycosylation on the formation of the different pI forms of CBHI was studied further by deglycosylation treatment of the labelled CBHI molecules. Removal of the N-glycan structures by Endo H was found to reduce the apparent molecular mass of CBHI (70–78 kDa) by approximately 10 kDa (Fig. 5). Furthermore, a slight shift of the CBHI pattern to the basic direction was observed, and the proportion of the most acidic forms of CBHI was reduced in the spot pattern. All the intracellular and extracellular forms of CBHI detected were sensitive to the Endo H treatment,

Fig. 4. Comparison of [^{35}S]methionine- and [^{14}C]mannose-labelled extracellular proteins resolved in 2D gels. Culture supernatants of *T. reesei*, metabolically labelled with either [^{35}S]methionine or [^{14}C]mannose for 40 min, were resolved in 2D gels. The proteins labelled with [^{35}S]methionine are shown in the leftmost panel, the proteins labelled with [^{14}C]mannose in the middle, and a mixture of the two specimens is shown in the rightmost panel. CBHI is indicated by a box.

Fig. 5. Treatment of CBHI with Endo H: analysis by 2D gel electrophoresis. *T. reesei* cultures were labelled with [^{35}S]methionine for 30 min, and CBHI immunoprecipitated from the cell lysate (C; upper panel) or the culture medium (M; lower panel) was treated with Endo H as described in Methods. –Endo H, Untreated control samples; +Endo H, Endo H-treated samples; –Endo H/+Endo H, mixtures of the two.

consistent with the *N*-glycans on the protein being of the high-mannose type (Dunphy *et al.*, 1985; Lodish *et al.*, 1983; Yeo *et al.*, 1985). Treatment of the CBHI

immunoprecipitates with PNGase F yielded results highly similar to those obtained with Endo H (data not shown).

Fig. 6. Chemical deglycosylation of CBHI: analysis by 2D gel electrophoresis. *T. reesei* cultures were labelled with [^{35}S]methionine for 30 min, and total proteins from the cell lysate or the culture medium (M; bottom panels) were deglycosylated using the Oxford GlycoSystems GlycoFree kit and analysed using 2D gel electrophoresis. Mobility of CBHI in cell lysate samples is shown in the panels at the top (C) and in the culture medium samples in the bottom panels (M). -Deglyc, Untreated control samples; +Deglyc, deglycosylated samples; -Deglyc/+Deglyc, mixtures of the two. The mobility of deglycosylated (+) and glycosylated (-) CBHI is indicated by a vertical line on the right side of each panel. (The additional spots below CBHI in the non-treated samples correspond to other glycoproteins in the sample.)

To analyse the effect of O-glycans on the pI pattern of CBHI, total protein samples from cell extracts and culture supernatants were subjected to chemical removal of both the N- and O-glycans followed by 2D gel analysis. The deglycosylated CBHI appeared in the 2D gels as a broad spot with increased pI, and the apparent molecular mass of the protein was reduced by about 15 kDa (Fig. 6). The results, together with those of the Endo H treatment, suggest that the glycosylation of CBHI at least partially explains the pI heterogeneity of the protein.

Treatment of live cells with the reducing agent DTT is reported to inhibit the intrachain disulphide bond formation during the folding of proteins in the ER in mammalian (Alberini *et al.*, 1990; Braakman *et al.*, 1992) and yeast (Jämsä *et al.*, 1994) cells, leading to a secretion block and ER accumulation of specific proteins. Therefore, the [^{35}S]methionine-labelling experiments were also carried out in the presence of DTT in order to study its effect on the protein secretion by *T. reesei*, and specifically, on the maturation of CBHI. As judged from the TCA-insoluble radioactivity, 10 mM DTT in the labelling medium efficiently blocked secretion of extracellular proteins into the culture medium, even though the total protein synthesis was not

affected by the reducing agent during the 40 min labelling period used (data not shown). Secretion of CBHI into the culture medium was totally inhibited in the presence of 10 mM DTT (Fig. 7a). When the pI pattern of the intracellular CBHI labelled in the presence of DTT was studied by 2D gel electrophoresis, only the very first pI forms of CBHI could be detected, even at the later stages of the labelling time course (Fig. 7b).

DISCUSSION

In this study, we have developed methodology to analyse the kinetics of protein synthesis and secretion in the filamentous fungus *T. reesei*. Production of the major cellulase CBHI by the strain Rut-C30 was used as a model system. In order to avoid changes of the culture medium disturbing the controlled physiological state of the fungus, we devised for this purpose metabolic labelling techniques in which a chase with unlabelled precursors was not used. Analysis of the different pI forms of CBHI using 2D gel electrophoresis allowed us to monitor the maturation of the enzyme during its transport along the biosynthetic pathway. During the first minutes of [^{35}S]methionine labelling, only one major form of labelled CBHI was detected. Additional intracellular forms of CBHI appeared later during the

Fig. 7. Effect of DTT on the maturation and secretion of CBHI. A [35 S]methionine-labelling time course experiment was carried out in the absence or presence of 10 mM DTT added to the medium 10 min before the labelled precursor. (a) Quantification of labelled CBHI in the culture supernatants in the absence of DTT (◆) and in the presence of 10 mM DTT (■). The quantification of CBHI from 2D gels was carried out using the Molecular Dynamics Phosphorimager and the ImageQuant software. The units of CBHI are arbitrary phosphorimager units. (b) 2D gel pattern of CBHI in mycelial specimens after 30 min labelling. -DTT, untreated specimen; 10 mM DTT, DTT-treated one.

labelling experiments, giving rise to a spot pattern resembling the one found in the extracellular samples. If the normal folding of the protein and formation of disulphide bonds, which occur in the ER, were prevented by adding the reducing agent DTT to the medium, the appearance of the more acidic pI forms and the secretion of CBHI into the culture medium were inhibited. This indicates that the exit of CBHI from the ER was blocked in the presence of DTT. Thus the pI forms detected at the beginning of the labelling probably represent the early ER forms of CBHI, the more acidic forms representing modifications occurring later along the secretory pathway.

The addition of the N-linked core glycan structures on proteins occurs while the polypeptide is being translocated through the ER membrane in higher eukaryotes and yeast, and in *S. cerevisiae* also the initial steps of O-glycosylation are known to take place in the ER (reviewed by Herscovics & Orlean, 1993). The earliest forms of CBHI detected already have a clearly higher apparent molecular mass than the partially (Endo H or PNGase F treatments) or fully deglycosylated molecules. Further, the deglycosylation treatments indicated that the more acidic pI forms of CBHI are largely due to

heterogeneous glycan structures generated in post-ER intracellular compartments. The N-glycan structures of CBHI produced by the strain Rut-C30 have been reported to contain phosphate groups (Maras *et al.*, 1997a, b), which may affect the pI of the protein. It is, however, noteworthy that the removal of both O- and N-glycans had a much more pronounced effect on the pI forms than treatments that merely affect the N-glycans. The O-glycans of CBHI have been reported to mainly consist of the neutral carbohydrate mannose (Salovuori *et al.*, 1987), but the present work indicates that these glycans may also contain acidic structures that significantly lower the pI of the protein. Sulfation of the linker glycopeptide region has been reported in CBHI produced by the *T. reesei* strain ALKO2788 (Harrison *et al.*, 1998). Even the chemically deglycosylated protein appeared in the 2D gels as a spot covering a fairly broad pI range. This indicates that CBHI may also carry some other type of post-translational modification that affects the mobility of the protein in the IEF. Alternatively, the deglycosylation treatment may have slightly affected the integrity of the polypeptide itself.

By quantifying the CBHI labelled metabolically during a time course, parameters describing the different steps in protein production were deduced. A mean synthesis time of 4 min was measured for CBHI, corresponding to a translation rate of 2.1 amino acids s^{-1} . The newly synthesized CBHI was secreted into culture medium with a minimum time of 11 min, the secretion rate being significantly lower than the synthesis rate (about 50% under the conditions used in the study). The rates of translation and intracellular transport of proteins have been addressed most thoroughly in mammalian cell systems. There, faster translation rates have been measured, e. g. 4.5 aa s^{-1} for influenza virus haemagglutinin in CHO cells (Braakman *et al.*, 1991) and 6 aa s^{-1} for apolipoprotein B-100 in Hep G2 cells (Boström *et al.*, 1986). On the other hand, the minimum times of secretion for several mammalian proteins have been reported to be somewhat longer than that observed here, 20–30 min (Boström *et al.*, 1986; Fries *et al.*, 1984; Matlin & Simons, 1983). However, the intracellular retention half times of different mammalian glycoproteins vary to a great extent, from about 30 min to several hours (Boström *et al.*, 1986; Fries *et al.*, 1984; Lodish *et al.*, 1983; Yeo *et al.*, 1985). In addition, the protein folding, maturation and transport processes in mammalian cells are dependent on the cell type and expression system used, as well as on external parameters such as temperature and stress conditions (Braakman *et al.*, 1991). The variability in secretion rates can mainly be attributed to differences in retention of the proteins in the ER and their transport to the medial Golgi level (Fries *et al.*, 1984; Lodish *et al.*, 1983; Yeo *et al.*, 1985).

In lower eukaryotes, the kinetics of protein transport has mainly been studied in the yeast *S. cerevisiae*. Secretion of invertase (Novick *et al.*, 1981) and Hsp150 (Jämsä *et al.*, 1994) has been shown to occur with somewhat faster kinetics compared to protein transport

in *Trichoderma*, the transport time of invertase being 5 min and the half time of secretion of Hsp150 2 min. In other systems, there are relatively scarce kinetic data on protein secretion and intracellular events in protein transport that would be comparable to the present study in terms of methodology. Metabolic labelling of proteins has been applied in the study of chymoelastase Pr1 biosynthesis in the filamentous fungus *Metarhizium anisopliae* (St Leger *et al.*, 1991): the time from uptake of labelled amino acid precursor to extracellular release of the enzyme was 6.5–7.2 min. This is markedly faster than the corresponding time observed for CBHI in *Trichoderma* (13 min). Thus it seems that the rate of protein secretion by *Trichoderma* is faster compared to mammalian cells but somewhat slower compared to *Saccharomyces*, but this does not necessarily apply to all filamentous fungi.

The kinetic analysis of *Trichoderma* protein synthesis and secretion described here will provide tools essential for future studies on protein production in the fungus. The efficiency of protein synthesis and transport is likely to be affected by both external conditions and the physiological state of the organism. The methodology developed in this study allows analysis of these events under different cultivation conditions. The techniques can be applied to analyse the rate-limiting events in the transport process, and to compare the kinetics of production of endogenous fungal proteins and heterologous products.

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