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Production of Tissue Plasminogen Activator (t-PA) in *Aspergillus niger*

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Abstract: A protease-deficient strain of *Aspergillus niger* has been used as a host for the production of human tissue plasminogen activator (t-PA). In defined medium, up to 0.07 mg t-PA (g biomass)$^{-1}$ was produced in batch and fed-batch cultures and production was increased two- to threefold in two-phase batch cultures in which additional glucose was provided as a single pulse at the end of the first batch growth phase. Production was increased [up to 1.9 mg t-PA (g biomass)$^{-1}$] by the addition of soy peptone to the defined medium. The rate of t-PA production in batch cultures supplemented with soy peptone (0.2 to 0.6 mg t-PA L^{-1} h^{-1}) was comparable to rates observed previously in high-producing mammalian or insect cell cultures. In glucose-limited chemostat culture supplemented with soy peptone, t-PA was produced at a rate of 0.7 mg t-PA L^{-1} h^{-1}. Expression of t-PA in *A. niger* resulted in increased expression of genes (*bipA*, *pdiA*, and *cypB*) involved in the unfolded protein response (UPR). However, when *cypB* was overexpressed in a t-PA-producing strain, t-PA production was not increased. The t-PA produced in *A. niger* was cleaved into two chains of similar molecular weight to two-chain human melanoma t-PA. The two chains appeared to be stable for at least 16 h in culture supernatant of the host strain. However, in general, <1% of the t-PA produced in *A. niger* was active, and active t-PA disappeared from the culture supernatant during the stationary phase of batch cultures, suggesting that the two-chain t-PA may have been incorrectly processed or that initial proteolytic cleavage occurred within the proteolytic domain of the protein. Total t-PA (detected by enzyme-linked immunoassay) also eventually disappeared from culture supernatants, confirming significant extracellular proteolytic activity, even though the host strain was protease-deficient. *Biotechnol Bioeng* **76**: 164–174, 2001.
Keywords: *Aspergillus niger;* tissue plasminogen activator (t-PA); cyclophilin B; unfolded protein response (UPR); protease-deficient

INTRODUCTION

As the commercial market for enzymes expanded during the second half of the 20th century, and as molecular techniques became available for the manipulation of filamentous fungi, *Aspergillus niger* became one of the few fungi developed for the production of not only native enzymes, but also of recombinant enzymes. As with the other *Aspergillus* spp. and *T. reesei*, *A. niger* is capable of producing and secreting high concentrations of protein (e.g., >20 g L^{-1} of glucoamylase) into the culture supernatant. This fact, combined with the cultivation technology available and the ability of fungi to glycosylate eukaryotic proteins, suggested that fungi such as *A. niger* would be suitable hosts for the production of high-value mammalian proteins, which are difficult to produce in bacteria and are produced only in small concentrations in animal cell culture (Devchand and Gwynne, 1991; Saunders et al., 1989). However, high concentrations of heterologous (nonfungal) products have generally not been obtained from any fungal host (Archer and Peberdy, 1997). Various reasons for the low yields of nonfungal products have been suggested, including incorrect folding or processing of the protein, upregulation of the unfolded protein response (UPR), and proteolytic degradation (Archer and Peberdy, 1997; Gouka et al., 1997). In addition, it is now recognized that, although filamentous fungi do not overglycosylate heterologous proteins, as *Saccharomyces cerevisiae* generally does, their glycosylation patterns differ

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significantly from those of mammalian proteins (Archer and Peberdy, 1997). Thus, to date, the use of filamentous fungi to produce therapeutic and other high-value mammalian products has been limited. Nonetheless, several mammalian proteins have been successfully produced in filamentous fungi and considerable understanding has been gained regarding the production bottlenecks that do occur (Gouka et al., 1997; Radzio and Kück, 1997).

Tissue plasminogen activator (t-PA) was one of the first mammalian proteins to be synthesized in a filamentous fungus, *A. nidulans* (Upshall et al., 1987). t-PA is a serine protease, which, when activated by fibrin, itself activates plasminogen, enabling the dissolution of blood clots (Pennica et al., 1983; Rijken et al., 1982). It is used therapeutically for the treatment of myocardial infarction, thrombosis, pulmonary embolism, and stroke. However, the serum level of t-PA is low and for clinical use it is necessary to obtain t-PA as a recombinant protein. Various hosts have been considered for the production of t-PA, including mammalian cell lines (Datar et al., 1993; Hippenmeyer and Highkin, 1993; Parekh et al., 1989), insect cells (Farrell et al., 1999), *S. cerevisiae* (Martegani et al., 1992), *A. nidulans* (Upshall et al., 1987), and *Escherichia coli* (Datar et al., 1993; Qiu et al., 1998). The main commercial source of t-PA (alteplase, Activase) is produced in Chinese hamster ovary (CHO) cells (Genentech: http://www.gene.com/products/activase/), although a nonglycosylated, truncated t-PA (reteplase, Retavase), which can be produced in *E. coli*, is also now available for therapeutic use (Centocor:http//www.centocor.com/retavase.htm). Both products cost >$2000 per dose (100 mg).

Using recombinant *A. nidulans*, Upshall et al. (1987) obtained 100 μg t-PA L^{-1} under the triosephosphate isomerase promoter and 1 mg t-PA L^{-1} under the alcohol dehydrogenase promoter, concentrations that are considerably lower than those now obtained from mammalian cell culture (10 to 64 mg t-PA L^{-1}, Datar et al. [1993]; see also Farrell et al. [1999]). *A. nidulans* was used for these experiments, although, unlike *A. niger*, it does not normally secrete large concentrations of protein. In addition, it has since been recognized that the concentrations of recombinant proteins secreted by filamentous fungi can be increased by the application of gene fusion strategies (Cullen et al., 1987; Gouka et al., 1997; Jeenes et al., 1993). We decided to use t-PA as a model protein for assessing the problems of producing a complex mammalian protein in *A. niger*. A protease-deficient host strain was used and the t-PA was produced as a fusion protein with the catalytic domain of the native glucoamylase protein. The gene encoding this fusion protein was expressed under control of either the constitutive promoter for glyceraldehyde-3-phosphate dehydrogenase (*gpdA*) or the glucoamylase (*glaA*) promoter. A dibasic processing site allowed cleavage of the t-PA from the glucoamylase and a $(His)_6$ tag was provided to facilitate purification.

MATERIALS AND METHODS

Strains and Plasmids

Aspergillus niger D15, the recipient strain for transformation, is a *pyrG*, protease-deficient (*prtT*), nonacidifying (*phmA*) mutant (Gordon et al., 2000). *A. niger* MGG018 is an olive-colored (*olvA*), *pyrG*, protease-deficient (*pepA;* Mattern et al., 1992), glucoamylase (*glaA*) deletion mutant.

Two t-PA expression vectors were constructed (Fig. 1). Both contained the coding region for mature (full-length) t-PA fused to the coding region of the *A. niger* glucoamylase gene (lacking the starch binding domain), linked with a nucleotide sequence encoding a dibasic processing site (N-V-I-S-K-R), followed by a sequence encoding a $(His)_6$ tag and the *trpC* terminator. The first vector contained the glyceraldehyde-3-phosphate dehydrogenase (p*gpdA*-Tpa) promoter, whereas the second contained the *A. niger* glucoamylase (p*glaA*-Tpa) promoter. The p*gpdA*-Tpa was generated from vector pJMU304 (provided by J. Uusitalo VTT Biotechnology and Food Research, Espoo, Finland), containing the *T. reesei cbh1* promoter, *cbh1* as a fusion gene, and the t-PA gene followed by a $(His)_6$ tag. From pJMU304, a 1.6-kb *Eco*RV-*Bcl*I fragment, carrying the t-PA gene, was ligated with a 0.8-kb *Bgl*II-*Xba*I fragment from pAN56-2 (Gordon et al., 2000) carrying the *trpC* terminator and cloned into the 7-kb *Xba*I-*Eco*RV fragment (the *gpdA* promoter and truncated *glaA* gene) from pAN56-4 (Broekhuijsen et al., 1993). For p*glaA*-Tpa, the t-PA gene was obtained by polymerase chain reaction from pJMU304 using the following primers (primer 1: 5′-GGC TCT AGA AAG AGA TCT TAC CAA GTG ATC; primer 2: 5′-GGC CCG GTC AAT GAT GAT GAT GAT GCG G), cloned into pCRbluntTOPO (Invitrogen), which was digested with *Xba*I and *Hpa*I to yield a 1.6-kb fragment. This fragment was cloned into the *Xba*I/*Hpa*I cloning site of pIGF (provided by Prof. D. Archer, University of Nottingham), containing the *glaA* promoter and truncated *glaA* gene (Archer et al., 1994). p*glaA*-Tpa was used to obtain a t-PA transformant in which the *cypB* gene (under control of the *gpdA* promoter) could be overexpressed.

Vector pBLUE-AmdSPyrG, containing both the *amdS* and *pyrG* selection markers, was used for cotransformation with the *gpdA* promoter t-PA expression vector. Vector pGW635, containing the *pyrG* gene (Goosen et al., 1987), was used for cotransformation with the *glaA* promoter t-PA expression vector.

The *cypB* vector (p*gpdA*-cypB; Fig. 1) expressed the *cypB* gene constitutively under control of the *gpdA* promoter and the *trpC* terminator (P. Derkx, unpublished results). Plasmid pAN8-1 (Punt and van den Hondel; 1992), containing the phleomycin resistance gene (*phleo*^{R}), was used for cotransformation with p*gpdA*-cypB.

Media

The defined medium of Vogel (1956) was used with the following modifications. Glucose (4 to 20 g L^{-1}), maltose

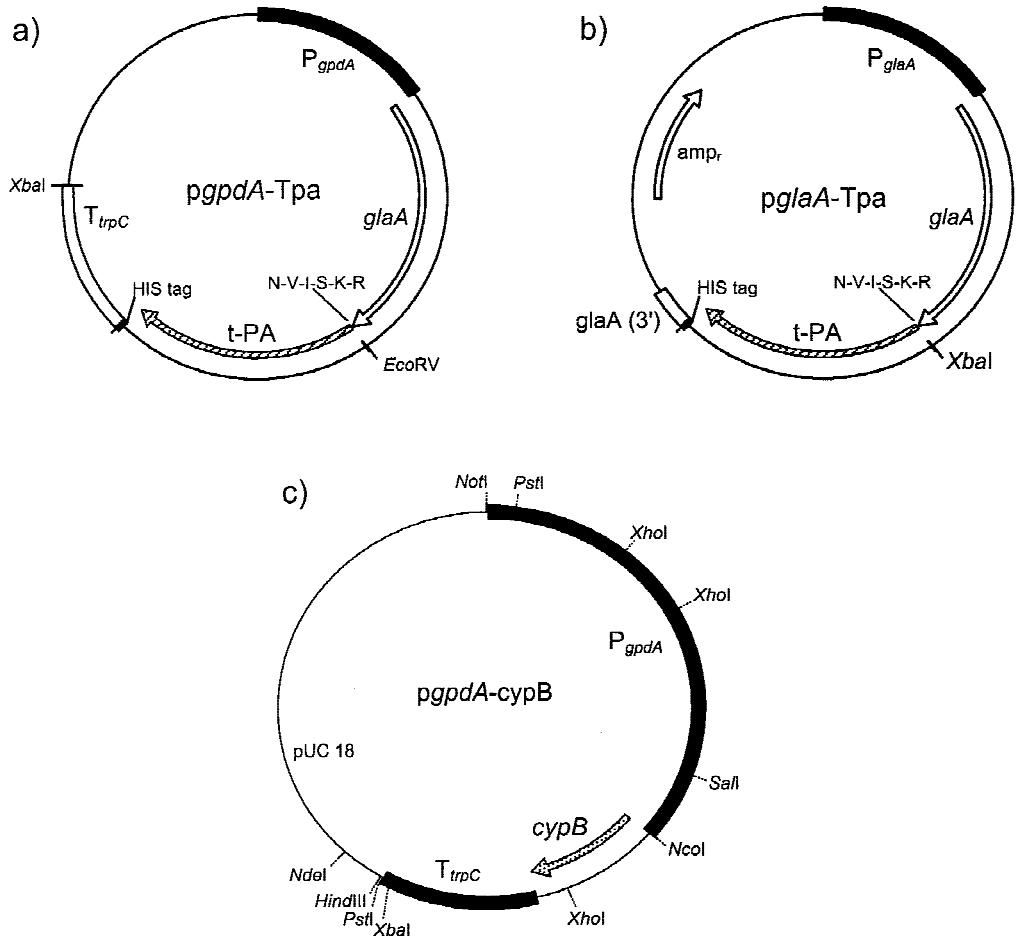

Figure 1. Schematic diagrams of the GLA-t-PA (a, b) and CYPB (c) vectors used for the transformation of *A. niger* D15. (a) Vector p*gpdA*-Tpa carrying the GLA-tPA fusion gene driven by the *gpdA* promoter (P_{gpdA}). (b) Vector p*glaA*-Tpa carrying the GLA-tPA fusion gene driven by the *glaA* promoter (P_{glaA}). (c) Vector p*gpdA*-cypB carrying the *cypB* gene driven by the *gpdA* promoter (P_{gpdA}). *glaA*, glucoamylase gene (catalytic domain); T_{trpC}, *trpC* terminator, N-V-I-S-K-R, region encoding dibasic processing site; amp_r, ampicillin resistance.

(15 g L^{-1}), or maltodextrin (10 or 100 g L^{-1}) was substituted for sucrose as the carbon source. $(NH_4)_2SO_4$ (1.75 to 15 g L^{-1}) was substituted for NH_4NO_3 as the nitrogen source. Some media were supplemented with soy peptone (4 to 15 g L^{-1}; Oxoid) or peptone (100 g L^{-1}). Some media were buffered to pH 6.0 with the addition of 50 m*M* 2-(*N*-morpholino)ethane-sulfonic acid (MES).

Alternatively, a medium (CAS-AM) containing (per liter) 10 to 50 g glucose, 0.6 g $NaNO_3$, 1.0 g casamino acids, 0.52 g KCl, 1.5 g KH_2PO_4, 0.24 g $MgSO_4$, 50 mg ethylenediamine tetraacetic acid (EDTA), 22 mg $ZnSO_4 \cdot 7H_2O$, 22 mg $FeSO_4 \cdot 7H_2O$, 22 mg $CuSO_4 \cdot 5H_2O$, 22 mg $Na_2MoO_4 \cdot 2H_2O$, 11 mg H_3BO_3, 5 mg $MnCl_2 \cdot 4H_2O$, and 1.7 mg $CoCl_2 \cdot 6H_2O$ was used. For some media, bovine serum albumin (10 g BSA L^{-1}) was substituted for casamino acids.

For induction of t-PA in the p*glaA*-Tpa transformant (D15-19, see later), cultures were grown in minimal medium (6 g $NaNO_3$ L^{-1}, 0.5 g KCl L^{-1}, 1.5 g KH_2PO_4 L^{-1}, 0.5 g $MgSO_4 \cdot 7H_2O$ L^{-1}, plus 1 mL trace metal solution L^{-1} [Vishniac and Santer, 1957]; pH 6.0) with fructose (10 g L^{-1}) as the carbon source and supplemented with 5 g yeast extract L^{-1} and 5 g casamino acids L^{-1}. Mycelium (1 g wet weight) was then transferred to the medium (as indicated earlier with the following exceptions) in which the fructose was replaced with either 20 g starch L^{-1} or 20 g maltodextrin L^{-1} and which did not contain casamino acids.

For selection of transformants, all media contained 1.2 *M* sorbitol as the osmotic stabilizer and were solidified with 1.5 g agar L^{-1}. p*glaA*-Tpa transformants were selected on minimal medium. Minimal medium containing either acetamide or acrylamide as the sole nitrogen sources was used for selection of p*gpdA*-Tpa transformants (Kelly and Hynes, 1985). Selection on acrylamide was expected to yield transformants with higher copy numbers of pBLUE-AmdSPyrG than selection on acetamide (Verdoes et al., 1993). For *cypB* transformants, minimal medium containing phleomycin (15 μg L^{-1}) was used.

Transformation of *A. niger* D15

D15 was cotransformed with pBLUE-AmdSPyrG and p*gpdA*-Tpa or with pGW635 and p*glaA*-Tpa), as described

by Punt and van den Hondel (1992). *AmdS* transformants (pBLUE-AmdSPyrG) were selected on acrylamide- or acetamide-containing medium. Those transformants showing the strongest growth on acrylamide medium were analyzed further. Transformants were screened for high-level expression of the t-PA gene by Northern analysis and for the ability to produce t-PA in shake-flask cultures in CAS-AM medium containing 10 m*M* uridine (to enable growth of the untransformed control), with either 5% (w/v) glucose or maltodextrin as carbon source. Cultures were grown for 40 to 44 h at 30°C.

PyrG transformants (pGW635) were selected on minimal medium and screened for integration of p*glaA*-Tpa by polymerase chain reaction (PCR) using primers (primer 1: 5′-TCC ATG TCC GAG CAA TAC GA, within the *glaA* coding region at −276 from the t-PA open reading frame; primer 2: 5′-TTC ACA GCA CTT CCC AGC AAA, at the start of the t-PA gene) that should give a 530-bp fragment. Transformants containing the plasmid were screened for high t-PA production on minimal medium supplemented with yeast extract and with starch as the carbon source in shake-flask cultures.

One transformant containing each vector was selected for further studies. Strain D15#25, containing p*gpdA*-Tpa, produced approximately 0.7 to 0.8 mg t-PA protein L^{-1} in the preliminary screen. Strain D15-19, containing p*glaA*-Tpa, produced approximately 0.1 mg t-PA protein L^{-1} in the preliminary screen.

Transformant D15-19 was cotransformed with plasmids p*gpdA*-cypB and pAN8-1 and transformants were selected on phleomycin-containing medium (Punt and van den Hondel, 1992). One transformant (D15-19::p*gpdA*-cypB) was selected that showed overexpression of the *cypB* gene by Northern analysis.

Culture Conditions

Small-scale batch cultures were grown in 250-mL flasks (25 to 50 mL medium per flask) at 20° to 30°C on a rotary shaker at 125 rpm (25-mL volumes) or 200 rpm (50-mL volumes). Flasks were inoculated with approximately 1.5×10^4 conidia mL^{-1} (final concentration) and incubated for up to 120 h.

All bioreactor cultures were inoculated with mycelial inoculum (10% working volume) grown in shake flasks. Inoculum for 10-L batch and fed-batch cultures was prepared by inoculating 1-L flasks (containing 250 mL of medium) with 25 mL from a 30-h small-scale batch culture followed by 48-h incubation at 28° to 30°C.

Batch bioreactor cultures were carried out in FT Applikon (2.3-L working volume) bioreactors or in a 15-L stirred-tank reactor with 10-L working volume (Giovanola). Chemostat cultures were carried out in the FT Applikon, and fed-batch cultures in the 15-L stirred tank. The FT Applikon was equipped with three six-bladed Rushton turbine impellers (48-mm diameter) and was agitated at 1000 rpm. The 15-L reactor was also equipped with three six-bladed Rushton turbine impellers (128-mm diameter) and agitated at speeds of up to 350 rpm (depending on dissolved oxygen concentrations). Cultures were aerated at rates between 0.33 and 1.5 L air (L culture)$^{-1}$ min^{-1}, with aeration rates adjusted manually to avoid oxygen limitation. pH was maintained constant by the automatic addition of NaOH (0.5 or 2 *M*) or either H_3PO_4 (10% v/v) or H_2SO_4 (10% v/v). Foaming was controlled by the addition of polypropylene-glycol (mixed molecular weight; Foamaster, Henkel Performance Chemicals) or Ucolub N115 (Fragol Industrieschmierstoffe, Mühlheim, Germany).

For fed-batch cultures, glucose solution (100 g glucose L^{-1} in fivefold concentrated salt and trace element solution of Vogel's defined medium) was fed at rates predicted by:

$$m_S(t) = F_S(t) \cdot cS_F = \frac{\mu_{set}}{Y_{X/S}} \cdot X(t_0) \cdot e^{\mu_{set}(t - t_0)} \qquad (1)$$

where $m_S(t)$ is the mass flow of the substrate (g h^{-1}), $F_S(t)$ the volumetric feeding rate (L h^{-1}) into the vessel at time t (h), cS_F is the carbon substrate concentration in the feeding solution (g L^{-1}), μ_{set} is the desired specific growth rate assuming pseudo-exponential growth (h^{-1}), X(g) the total biomass (i.e., biomass concentration multiplied by the working volume), and $Y_{X/S}$ the yield of biomass on carbon substrate (g g^{-1}). $Y_{X/S}$ was estimated to be around 0.79 (based on previous batch experiments). The glucose feed was started when the glucose (20 g glucose L^{-1}) present in the batch phase had been consumed. μ_{set} was initially 0.05 h^{-1} and was reduced to 0.03 h^{-1} after approximately 15 h. Ammonium and other salts were added as a single pulse at the start of glucose feeding, to ensure that N-limitation did not occur. The concentration of NH_{4^+} remained >0.5 g L^{-1} throughout the cultivation.

Two-stage batch cultivations were also carried out in which a single pulse of concentrated glucose (with ammonium and salts to avoid other limitations) was provided after the glucose from the initial batch phase (20 g glucose L^{-1}) had been consumed. A total of 40 g glucose L^{-1} was supplied in these experiments. Fed-batch and two-phase batch cultures were carried out at pH 5.5, 28°C, with the agitation rate (up to 350 rpm) and aeration adjusted to maintain pO_2 at >20%.

Chemostat cultures were carried out according to the methods of Wiebe and Trinci (1991) at a dilution rate of 0.07 h^{-1}, pH 6.0 ± 0.1, 25°C, 1000 rpm, and aeration of 0.6 to 0.8 L air (L culture)$^{-1}$ min^{-1}.

Biomass Determination

Biomass was measured as dry weight, either by harvesting biomass on predried, preweighed Whatman No. 1 filter papers and drying at 60°C, or by drying the biomass (separated from supernatant by centrifugation at 5000 *g* for 30 min) under vacuum at 40°C. For both methods, biomass was dried to a constant weight.

Enzyme Assays

Total t-PA was detected as antigen using sandwich enzyme-linked immunoassay (ELISA) assays (COALIZA t-PA from Haemochrom Diagnostica, Essen, Germany, or EIA kit from TNO). Microtiter plates were coated with anti-t-PA antibodies, after which sample (or standard t-PA) was added and incubated for 1 h at 37°C or overnight at 4°C, and then washed. Horseradish peroxidase (HP)-labeled monoclonal anti-t-PA antibody was added and the plates were incubated for 1 h at 37°C or up to 8 h at 4°C, then washed. Plates were incubated open for 30 min in the dark with substrate solution, after which the reaction was stopped by addition of sulfuric acid (1N). Absorbance was read at 450 nm.

t-PA activity was measured using D-Val-Leu-Lys-*p*-nitroanilide as a substrate with the BIA kit (TNO). As with the ELISA assay, sample t-PA was first bound to anti-t-PA antibody in microtiter plates in an overnight incubation (4°C). Substrate, plasminogen, and fibrinogen were added and the absorbance at 405 nm was read after 4-h incubation. t-PA activity was also measured using the Coaset t-PA kit (with D-Val-Leu-Lys-*p*-nitroanilide as substrate), following the manufacturer's instructions for assay conditions (Haemochrom Diagnostica). All assays were carried out in duplicate.

In vitro degradation of t-PA in culture supernatant was assessed by incubating human melanoma t-PA (2.5 ng) in culture supernatant (250 μL) of D15 or MGG018, which had been grown for 40 h in CAS-AM medium containing 10 m*M* uridine and 5 g yeast extract L^{-1}. Samples (50 μL) were removed after 0.5, 1, 2, and 16 h of incubation and t-PA was detected by Western analysis. For some samples, phenylmethylsulfonylfluoride (PMSF; 1 m*M*) or EDTA (2.5 m*M*) was added at the start of the incubation.

Protease activity was assayed using azocasein as a substrate, as described by Griffen et al. (1997), at pH 4 (Na-acetate buffer), 5 (Na-acetate buffer), 6 (Na-acetate buffer), and 7 (piperizine-*N*,*N*′-bis-2-ethane-sulfonic acid buffer). Protease assays were carried out in triplicate.

Protein Analysis

Partially purified t-PA was obtained either by metal chelate chromatography or by ion-exchange chromatography (IEC). The QIAexpress protein purification system (based on a nickel nitrilotriacetic acid [Ni-NTA] resin) was used according to the manufacturer's recommendations for metal chelate chromatography.

IEC was able to separate t-PA from glucoamylase (the only other major protein in the culture supernatant when cells were grown on Vogel's defined medium with glucose as carbon source). The culture supernatant was concentrated using Microcon filters to give 11.36 mg t-PA L^{-1}. Excess salts were removed by dialysis against 10 m*M* acetate buffer (pH 5.0 at 4°C for 24 h). An FPLC system (Fa. Pharmacia) with an ARS column ($h = 5.2$ cm, $d = 0.5$ cm, FF volume = 1.02 mL, with SP-Sepharose matrix) was used and absorbance monitored at 280 nm. Proteins were eluted (flow rate 1 mL min^{-1}) for 3 min in a 10 m*M* acetic acid buffer (pH 5), followed by a 15-min gradient from 0% to 100% of 1.5 *M* NaCl (with 10 m*M* acetic acid, 100% to 0%). t-PA was eluted at approximately 6 min.

Proteins were separated by sodium dodecylsulfate–polyacralymide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). Western analysis was carried out as described by Sambrook et al. (1989) using monoclonal t-PA antibody.

DNA and RNA Analyses

Fungal DNA was extracted from the mycelia as described by Kolar et al. (1988). RNA was extracted using RNAzol (Cinna-Biotecx) or RNeasy (Qiagen). As a probe for Northern hybridization of the t-PA transformants, a PCR-generated 1.6-kb t-PA fragment was used. The *cypB* probe (540 bp) was derived by *sma*I/*Hind*III digestion of the cDNA and the probe (650 bp) for 18S RNA was generated by PCR (forward primer: 5′-TCC AAG GAA GGC AGC AG-3′; reverse primer: 5′-AAT AGA AAC ACC GCC CG-3′). The *pdiA* probe (1005 bp) was derived by *Eco*RI digestion of the cDNA (Ngiam et al., 1997) and the *bipA* probe (667 bp) by *kpn*I digestion of the gene (van Gemeren et al., 1997). Probes were labeled with ^{32}P using a random primer labeling kit (Prime-It II Random Primer Labeling Kit, Stratagene) and purified with micro-biospin P30 columns (Biorad). Southern and Northern analyses were carried out essentially as described by Sambrook et al. (1989).

RESULTS

Selection of Host Strain for t-PA Production

Because previous experiments have shown that the choice of an appropriate protease-deficient host strain can be essential for successful protein production (see Gouka et al., 1997), two different protease-deficient host strains, D15 (*prtT*) and MGG018 (*pepA*), were used for in vitro t-PA degradation assays to assess their suitability for production of recombinant t-PA. t-PA standard (500 ng mL^{-1}) was incubated with culture supernatant of either strain, D15 or MGG018, for up to 16 h (Fig. 2a,b). Initially, both one- and two-chain t-PA could be identified in the D15 supernatant, whereas only two-chain t-PA was detectable after only 30-min incubation in MGG018 supernatant. t-PA was completely degraded within 1 h in MGG018 supernatant. One-chain t-PA was cleaved to form two-chain t-PA in D15 supernatant, but two-chain t-PA was still detectable after 16-h incubation at 30°C.

Cleavage of single-chain t-PA in D15 culture supernatant could be inhibited by addition of PMSF (1 m*M*) to the supernatant (Fig. 2c). EDTA (2.5 m*M*) did not inhibit the breakdown of single-chain t-PA in D15 culture supernatant (Fig. 2d). In contrast, neither PMSF nor EDTA significantly

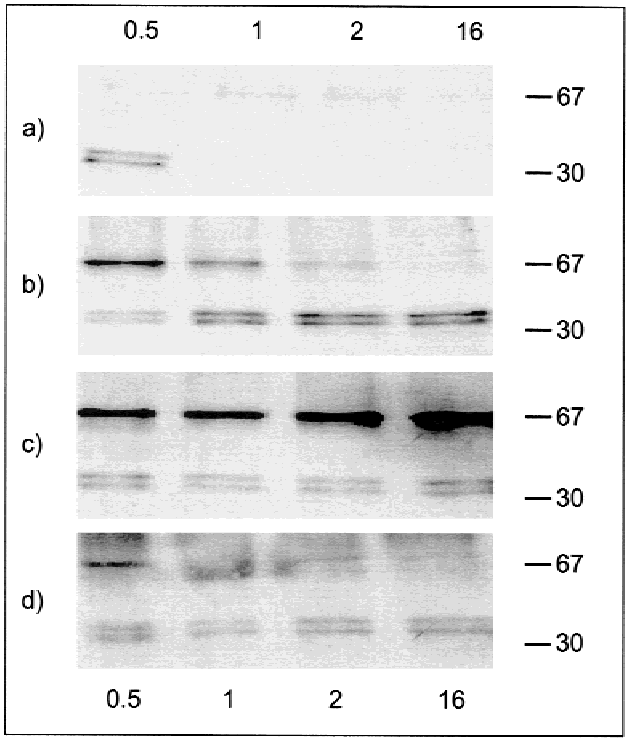

Figure 2. Western analysis of t-PA (500 ng mL^{-1}) incubated in the culture supernatant of: (a) *A. niger* MGG018; or (b), (c), and (d) *A. niger* D15, which had been grown for 40 h in CAS-AM-supplemented medium. Traces (a) and (b) are from untreated supernatant. D15 supernatant was also treated with (c) PMSF (1 m*M*) or (d) EDTA (2.5 m*M*). The lanes are labeled with the time interval (h) for which t-PA was incubated in the culture supernatants. Molecular weights (kDa) are given on the right side of each image.

inhibited degradation in the MGG018 supernatant (data not shown).

Based on these results, strain D15 was used for t-PA expression studies and two transformants, D15#25 (p*gpdA*-Tpa) and D15-19 (p*glaA*-Tpa), were isolated as described in Materials and Methods.

Production of t-PA in Batch Culture

D15#25 (*gpdA* promoter) produced 1.1 mg t-PA L^{-1} [69 μg t-PA (g biomass)$^{-1}$by ELISA] when grown for 38 h in Vogel's defined medium at 28°C and pH 5.5 ± 0.2. Less than 70 μg t-PA L^{-1} was detected in cultures grown on CAS-AM medium under the same conditions. When Vogel's medium was supplemented with soy peptone (5 g L^{-1}) concentrations of t-PA of 10.7 mg t-PA L^{-1} [1.2 to 1.9 mg t-PA (g biomass)$^{-1}$; ELISA] were observed after up to 55 h (25°C, pH 6.0 ± 0.1). Substituting bovine serum albumin (BSA; 1% w/v) for casamino acids in the CAS-AM medium, lowering the cultivation temperature from 30°C to 20° to 22°C, and prolonging cultivation from 40 to 94 h also increased the levels of total (ELISA) t-PA from 0.7 to 12 mg t-PA L^{-1} in shake-flask cultures. The incubation time required for maximum t-PA production in shake-flask cultures was more variable than in bioreactors, reflecting differences in inoculum and substrate concentrations.

In all cultures, most of the t-PA produced was not active. In Vogel's cultures supplemented with soy peptone (25°C, pH 6.0 ± 0.1), only 21.7 μg t-PA L^{-1} [4.3 μg t-PA (g biomass)$^{-1}$] was active; that is, only approximately 0.2% of the t-PA produced was active.

In batch cultures, t-PA was produced during the exponential growth phase (Fig. 3), with the maximum amount of active t-PA being observed around the time when the carbon source was exhausted from the medium. However, total t-PA (detected by ELISA) generally continued to increase for 10 to 20 h during deceleration or stationary phase (Fig. 4).

D15-19 (*glaA* promoter) produced 25 mg total (ELISA) t-PA L^{-1} in shake-flask cultures in Vogel's medium containing 100 g maltodextrin L^{-1} and 100 g peptone L^{-1} (pH 6.0, 25°C) and 280 μg active t-PA L^{-1}.

Effect of pH on t-PA Production in Batch Culture

D15#25 was also grown in Vogel's medium supplemented with soy peptone at pH 4.0 and 7.0 (25°C, bioreactor culture). Biomass production was similar at pH 4.0 and 6.0, but was significantly reduced at pH 7.0 (Fig. 4). Less t-PA was detected in culture supernatants of cultures grown at pH 4.0 (0.7 μg active t-PA L^{-1}, 2.9 mg total t-PA L^{-1}; ELISA) and pH 7.0 (14.6 μg active t-PA L^{-1}, 4.2 mg total t-PA L^{-1}; ELISA) than at pH 6.0 (21.7 μg active t-PA L^{-1}, 10.7 mg total t-PA l^{-1}; ELISA). However, specific t-PA production was similar at pH 6.0 [1.8 mg total t-PA (g biomass)$^{-1}$; ELISA] and pH 7.0 [2 mg total t-PA (g biomass)$^{-1}$; ELISA].

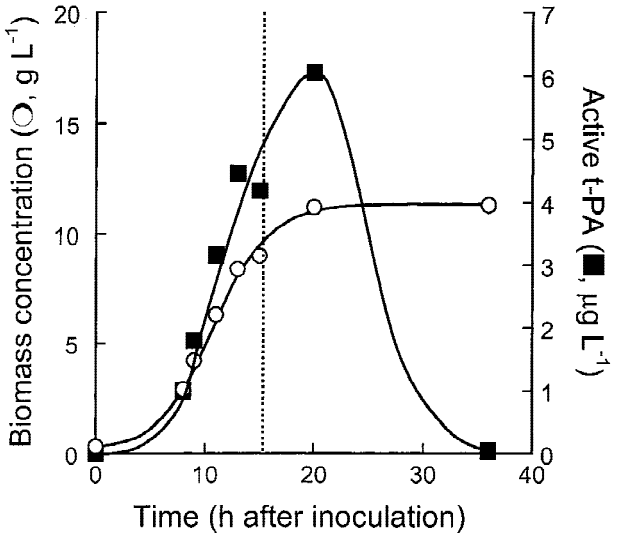

Figure 3. Production of active t-PA (■) and biomass (○) by *A. niger* D15#25 in batch culture on modified Vogel's medium (15 g maltose L^{-1}) supplemented with 15 g soy peptone L^{-1} and 15 g $(NH_4)_2SO_4$ L^{-1} [pH 6.0, 25°C, 1000 rpm, 0.6 to 0.8 L air (L culture)$^{-1}$ min^{-1}]. The dotted line indicates the end of exponential growth. Points are the means of two replicates.

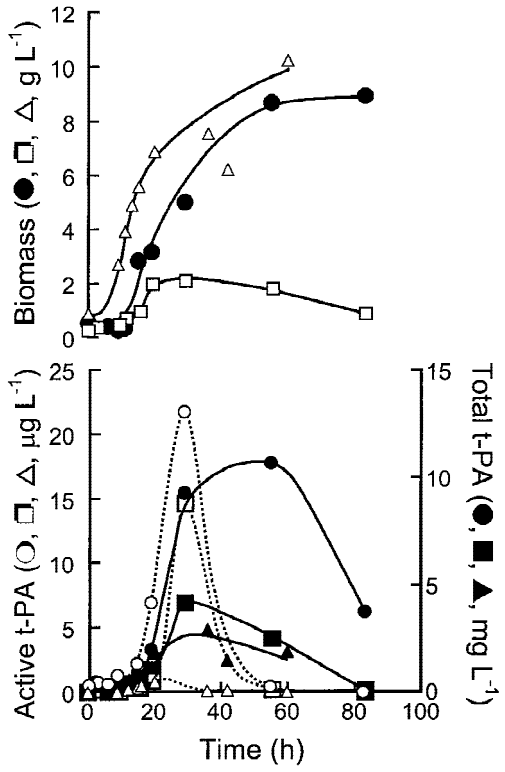

Figure 4. Production of t-PA (lower graph) and biomass (upper graph) by *A. niger* D15#25 in batch culture on modified Vogel's medium supplemented with 5 g soy peptone L^{-1} and 15 g $(NH_4)_2SO_4$ L^{-1} at pH 4 (▲, △), 6 (●, ○), and 7 (■, □). Cultures were maintained at 25°C, 1000 rpm, 0.6 to 0.8 L air (L culture)$^{-1}$ min^{-1}. Both total t-PA (■, ●, ▲, solid lines, determined by ELISA) and active t-PA (□, ○, △, dashed lines) are shown. Points are the means of two replicates.

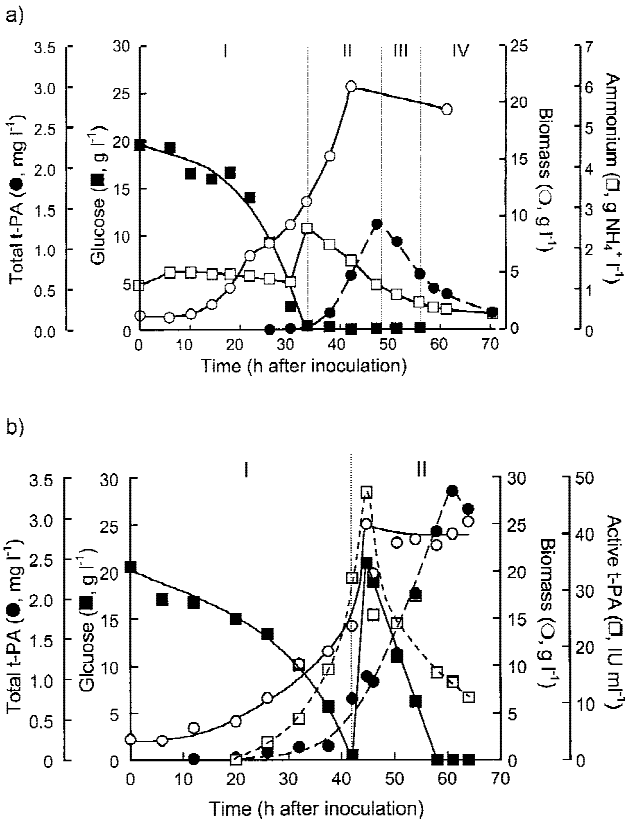

Figure 5. Production of t-PA in (a) fed-batch and (b) two-phase batch cultures of *A. niger* D15#25 growing in Vogel's defined medium (pH 5.5, 28°C). (a) The culture was fed (100 g glucose L^{-1}) at an exponential rate to sustain a specific growth rate of 0.05 h^{-1} (Phase II) after glucose had been consumed during the batch phase (I). During Phase III, the specific growth rate was reduced to 0.03 h^{-1}. The feed was discontinued in Phase IV. Ammonium was added as a single pulse at the end of the batch culture. The concentrations of total t-PA (●, determined by ELISA), glucose (■), biomass (○), and ammonium (□) are shown. (b) A single pulse of glucose (Phase II) was added after glucose had been consumed during the first batch phase (Phase I). Ammonium was also added as a single pulse at this time. The concentrations of total t-PA (●, determined by ELISA), active t-PA (□), glucose (■), and biomass (○) are shown.

Proteolytic Activity in D15#25 Culture Supernatant

Although the host strain, D15, was a protease-deficient strain, t-PA was degraded in culture supernatants (Figs. 3, 4, and 5). Assays of proteolytic activity against azocasein confirmed that protease concentrations in the culture supernatant were low. Maximum proteolytic activity at pH 6 in cultures grown at pH 6 did not exceed 1.7 U mL^{-1}. Maximal proteolytic activity at pH 4 and 7, in cultures grown at these pHs, did not exceed 0.8 and 0.6 U mL^{-1}, respectively. For each culture, there was generally more activity at pH 4 than at pH 5 or 6, and more activity at pH 7 than at pH 6. Preliminary experiments carried out in shake-flask cultures indicated that high concentrations of NH_4^+ in the culture medium resulted in increased concentrations of t-PA, suggesting that less degradation of the t-PA was occurring (data not shown).

Production of t-PA in Fed-Batch and Two-Phase Batch Cultures

Two high-biomass cultivations were carried out in Vogel's defined medium with D15#25: one with an exponential feed calculated to maintain a specific growth rate of 0.05 h^{-1} during the initial feeding phase, followed by another feeding phase with a specific growth rate of 0.03 h^{-1}; the second with a single pulse of concentrated glucose at the end of exponential growth in the batch culture. In the fed-batch culture, total t-PA continued to increase in the culture supernatant, to a maximum of 1.3 mg t-PA L^{-1} (ELISA) during the first feeding phase ($\mu = 0.05$ h^{-1}), but decreased during the second feeding phase ($\mu = 0.03$ h^{-1}; Fig. 5a). Biomass concentration also increased during the first feeding phase, reaching a maximum concentration of approximately 21 g biomass L^{-1}. Specific t-PA (ELISA) production was 62 µg (g biomass)$^{-1}$.

When glucose was provided as a single pulse (total glucose provided: 40 g glucose L^{-1}, with 20 g glucose L^{-1} in the initial batch phase), total (ELISA) t-PA increased to 3.3 mg t-PA L^{-1} at approximately 20 h after the addition of the glucose pulse (Fig. 5b), and then started to decrease. Approximately 25 g biomass L^{-1} was produced; specific t-PA

(ELISA) produced was approximately 140 μg (g biomass)$^{-1}$. As was observed in batch cultures, t-PA activities started to decline before the total t-PA concentration decreased, indicating deactivation of the enzyme prior to degradation. Maximum volumetric t-PA activity was observed at approximately 45 h, at the end of the first phase of batch growth. t-PA activity was measured in international units (IU) using the Coaset kit and the maximum specific t-PA activity obtained was 93 IU (μg t-PA)$^{-1}$ 37.5 h after inoculation, which corresponds to 16% of the specific activity reported for human melanoma t-PA [580 IU (μg t-PA)$^{-1}$]. After 37.5 h, the specific t-PA activity declined continuously until <1% of the total t-PA was active.

Production of t-PA in Chemostat Culture

D15#25 was grown in glucose-limited chemostat culture (D = 0.07 h^{-1}, pH 6.0, 25°C) supplemented with 4 g soy peptone L^{-1} (4 g glucose L^{-1}, pH 6.0, 25°C) for 140 h. During continuous medium flow, 10.1 mg total (ELISA) t-PA L^{-1} [approximately 2.7 mg t-PA (g biomass)$^{-1}$] was produced. Active t-PA was generally not detected.

Purification of t-PA

Purification of t-PA based on binding of the $(His)_6$ tag to Ni-NTA was not efficient. Maximum recovery of t-PA was approximately 14%. Separation by IEC was more efficient, with complete separation of glucoamylase and t-PA. SDS-PAGE indicated that the purified t-PA had been cleaved into two-chain t-PA, with molecular weights similar to those of human melanoma two-chain t-PA (Fig. 6).

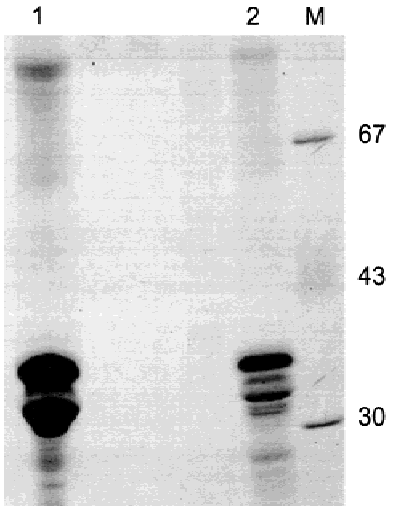

Figure 6. SDS-PAGE of human melanoma two-chain t-PA (lane 1) and of t-PA purified from *A. niger* D15#25 culture supernatant by IEC, as described in Materials and Methods (lane 2). The t-PA was purified from a batch culture grown on Vogel's defined medium with glucose as carbon source. Gels were stained with Coomassie Brilliant Blue R250. Lane M contains the molecular weight markers and the weights (kDa) are given on the right of the image.

Effect of *cypB* Overexpression on t-PA Production

Production of t-PA by strain D15-19 resulted in a 1.5- to 2-fold increase in expression of *cypB*, *pdiA*, and *bipA* (Fig. 7a). However, when *cypB* was overexpressed in D15-19::p*gpdA*-cypB (Fig. 7b) there was no increase in t-PA production compared with production of t-PA by D15-19 under the same conditions (Fig. 7c).

DISCUSSION

t-PA was produced in quantities of 12 to 25 mg L^{-1} (ELISA) by *A. niger* in a growth-associated manner, as would be expected for proteins produced under control of either the *glaA* (Swift et al., 1998) or the *gpdA* (Hanegraaf et al., 1991; Hellmuth et al., 1995) promoters. In some cultures (e.g., see Fig. 5), total t-PA continued to increase in the culture supernatant during stationary phase, which may indicate a bottleneck in the release of t-PA from the hyphae (cf. Pluschkell et al., 1996). There was no advantage in

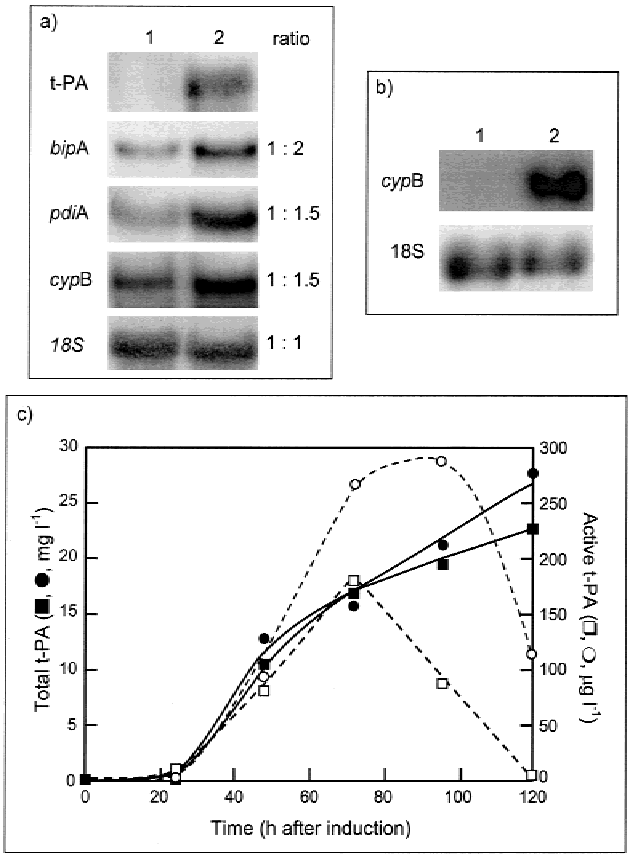

Figure 7. (a) Northern analysis of t-PA, *pdiA*, *bipA*, and *cypB* gene expression in *A. niger* D15-19 before (lane 1) and 6 h after induction with maltodextrin (lane 2) of t-PA production. (b) Northern analysis of *cyp*B gene expression in *A. niger* D15-19 (lane 1) and D15-19::p*gpdA*-cypB (lane 2). For (a) and (b), the concentration of 18S rRNA is shown as a control. (c) The production of total t-PA (■, ●, measured by ELISA) and active t-PA (□, ○, dashed lines) of *A. niger* D15-19 (●, ○) and d15-19::p*gpdA*-cypB (■, □) grown in shake-flask culture on modified Vogel's medium (100 g maltodextrin L^{-1}) supplemented with 100 g peptone L^{-1}.

producing the t-PA in a glucose-limited fed-batch culture with an exponential feed rate of 0.05 h^{-1} when compared with production in a batch culture; total (ELISA) t-PA (1.1 to 1.3 mg t-PA L^{-1}) and specific t-PA (ELISA) production [0.06 to 0.07 mg t-PA (g biomass)$^{-1}$] were the same in both systems. However, t-PA production could be increased two- to threefold by providing glucose for continued growth as a single pulse at the end of the initial batch culture (Fig. 5). Furthermore, t-PA production could also be increased by including soy peptone [1.2 to 1.9 mg t-PA (g biomass)$^{-1}$; ELISA] in the medium, but was reduced in the presence of casamino acids. Optimal t-PA production was observed at pH 5.5 to 6.0.

In *S. cerevisiae*, when the genes encoding foldases or ER chaperone binding proteins, such as protein disulfide isomerase (PDI) and BiP, are overexpressed, synthesis of heterologous proteins may be increased (Shusta et al., 1998). Both BiP and PDI may be upregulated in *A. niger* strains producing heterologous proteins; however, when BiP and PDI were overexpressed no increase in heterologous protein secretion was observed (Ngiam et al., 2000; Punt et al., 1998). The peptidyl prolyl *cis–trans* isomerases (PPIases) are another group of foldases, which includes the cyclophilins. The *A. nidulans* cyclophilin, CYPB, is targeted to the ER and appears to be involved in the heat-shock response (Joseph et al., 1999). As the *A. niger cypB* gene has also been cloned (P. Derkx, unpublished data), the effect of overexpressing *cypB* in a t-PA-producing strain was analyzed. Although production of t-PA induced *cypB*, *pdiA*, and *bipA* expression (Fig. 7a), overexpression of *cypB* did not result in increased levels of t-PA in the present study (Fig. 7b,c).

Several different production systems have been assessed for production of t-PA (Datar et al., 1993; Farrell et al., 1999; Martegani et al., 1992; Qiu et al., 1998). t-PA may be targeted to the periplasm in *E. coli* strains that overexpress cysteine oxidoreductases, thus eliminating the need for protein refolding (Qiu et al., 1998; Zhan et al., 1999), and *E. coli* (Datar et al., 1993; Qiu et al., 1998) and *S. cerevisiae* (Martegani et al., 1992) have each been used to produce 100 mg t-PA L^{-1} or more of nonsecreted protein. These systems both require recovery of the t-PA from the cell biomass, which adds to the cost and time required in harvesting the t-PA. Some t-PA may also be inactive. The rates of active t-PA production can be estimated as 10 to 100 μg active t-PA L^{-1} h^{-1} for *E. coli* (Qui et al., 1998) and up to 1500 μg active t-PA L^{-1} h^{-1} for *S. cerevisiae* (Martegani et al., 1992). Comparable concentrations of t-PA have also been attained from lepidopteran insect cells, from which up to 160 mg secreted t-PA L^{-1} could be obtained (Farrell et al., 1999). Like mammalian cells, insect cell cultures grow relatively slowly, so the rate of production was approximately 0.3 mg t-PA L^{-1} h^{-1}. Active t-PA was produced at a rate similar to that observed in *E. coli*, of approximately 40 to 100 μg active t-PA L^{-1} h^{-1}. CHO and other mammalian cells typically produce 10 to 60 mg t-PA L^{-1} or 0.1 to 0.3 mg t-PA L^{-1} h^{-1} (Choi et al., 1995; Datar et al., 1993). Although t-PA activity was not assayed in these reports, Lin et al. (1993) found consistently high specific activity for t-PA produced in CHO cells, suggesting that most, if not all, t-PA produced in CHO cells would be active (i.e., 100 to 300 μg active t-PA L^{-1} h^{-1}). In comparison, *A. niger* D15#25 produced 0.2 to 0.6 mg total (ELISA) t-PA L^{-1} h^{-1} in batch culture and 0.7 mg t-PA L^{-1} h^{-1} in chemostat culture. However, active t-PA was only produced at a rate of 1 to 3 μg active t-PA L^{-1} h^{-1}.

The results demonstrate that t-PA could be produced in *A. niger* and the simple partial purification process was designed based on ion-exchange chromatography. SDS-PAGE analysis of purified t-PA demonstrated that t-PA had been cleaved from the glucoamylase fusion protein and that most of the t-PA had been cleaved to yield two chains with molecular weights similar to those of two-chain human melanoma t-PA (30 to 35 kDa; Fig. 6). The cleavage of one-chain to two-chain t-PA was also observed when human melanoma t-PA was incubated in D15 culture supernatant (Fig. 2). Human melanoma t-PA was shown to be cleaved after the Arg^{275} residue to generate two-chain t-PA (Pennica et al., 1983), a site that would be subject to cleavage by serine proteases. Indeed, cleavage could be inhibited by the addition of the serine protease inhibitor PMSF to D15 culture supernatant, suggesting that the two-chain form was being processed correctly.

In spite of apparently correct processing of the t-PA to the two-chain form of the protein, most of the t-PA produced was not active or had a low specific activity compared with human melanoma t-PA. There are several possible reasons for this low activity. The protein produced in *A. niger* contained a $(His)_6$ tag to facilitate purification, but it is not known whether the presence of the $(His)_6$ tag may confer reduced activity to the protein. It is also possible that the t-PA was incorrectly glycosylated, even though the molecular weight of the two chains suggested that the level of glycoslyation was similar to that of human melanoma t-PA. Parekh et al. (1989) observed that the nature of the sugar residues and their linkages affected the activity of t-PA produced in Chinese hamster ovary (CHO) and mouse cell lines. In contrast, Datar et al. (1993) and Qiu et al. (1998) demonstrated that the absence of glycosylation on the t-PA produced in *E. coli* does not reduce the activity. Finally, the t-PA may have been incorrectly processed from one- to two-chain t-PA, even though the two fragments were of similar size to those of human melanoma t-PA. Lin et al. (1993) also observed a loss in t-PA activity, which could not be detected as degradation products in SDS-PAGE gels for t-PA produced in CHO cells.

Although D15#25 is a protease-deficient strain, there was evidence that t-PA was degraded by extracellular proteases during cultivation. Degradation could not be attributed to the presence of a specific protease, but data from the batch and fed-batch cultures suggest that a balance between synthesis and degradation was occurring, such that while the organism was growing and producing t-PA the concentration of t-PA increased in the supernatant even if proteases were present. However, when growth ceased or became

slower than 0.05 h^{-1} (fed-batch culture), the rate of t-PA degradation would exceed that of t-PA synthesis and the extracellular t-PA concentration would decrease rapidly. The presence of t-PA antigen in culture supernatants, which contained no t-PA activity, may indicate that initial proteolytic degradation occurred within the proteolytic domain of the protein.

Thus, although considerable progress has been made in the development of fungal strains in which complex mammalian proteins could be made, not all problems have been resolved. The $(His)_6$ tag did not enable easy product recovery and its removal might improve the yield of active t-PA from *A. niger* constructs. Problems related to glycosylation or proteolysis would be more difficult to address. van den Hombergh et al. (1994, 1995) noted that proteolytic activity in protease-deficient mutants of *A. niger* could be dependent on the protein source. Thus, although D15#25 had low proteolytic activity against casein, t-PA was still degraded. For production of t-PA in *A. niger*, a protease-deficient mutant with lower activity against t-PA would have to be found.

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