

Extracellular proteome of *Aspergillus terreus* grown on different carbon sources

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Abstract Extracellular proteins of filamentous fungi are important for biomedical and biotechnological applications. *Aspergillus terreus* not only comprises an important class of organisms that have significant commercial relevance to the biotechnology industry, but also is an emerging fungal pathogen. However, no information is available on the extracellular proteome of *A. terreus*. Thus, we analyzed the extracellular proteomes of *A. terreus* under different culture conditions using sucrose, glucose, or starch as a main carbon source. A total of 82 protein spots including 39 unique proteins was successfully identified by 2-DE and nano-LC-MS/MS. Of these, 12 proteins were detected in the presence of at least two different carbon sources, whereas 16 proteins were unique to sucrose-, 3 to glucose-, and 8 to starch-

grown *A. terreus*. Most of the proteins with known functions are hydrolytic enzymes, such as hydrolases, glycosylases and proteases, some of which include potential allergens. Both oryzin and a predicted protein (ATEG_07481) were the most abundant in all three media. Particularly, oryzin was highly secreted in high concentration sucrose medium. These proteomic data will be useful for studying protein secretion in further detail, and finding fusion partners for the extracellular production of homologous or heterologous proteins in *A. terreus*.

Keywords *Aspergillus terreus* · Extracellular proteins · Fungus · Secretome

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Introduction

Filamentous fungi are important organisms with significant commercial relevance in industrial biotechnology. The fungi are often used to produce a wide variety of products ranging from human therapeutics (e.g., antibacterial and antifungal agents) to chemicals (e.g., commercial enzymes and organic acids), which together represent billions of dollars in annual sales (Carlile et al. 2001; Nevalainen et al. 2005). For example, the cholesterol-lowering statins that represent a market of almost US\$15 billion per year in the USA (Manzoni and Rollini 2002; Maggon 2005) could be naturally produced by species of filamentous fungi. Lovastatin is mainly produced by *Aspergillus terreus* strains (Manzoni and Rollini 2002). Also, *A. terreus* is the frequently used commercial producer of itaconic acid that has been categorized as one of the “top 12” building block molecules from sugars in advanced biorefineries and used worldwide in the fields of polymer chemistry, pharmacy, and agriculture (Okabe et al. 2009).

On the other hand, fungi are notorious pathogens in both humans (Segal and Walsh 2006; Cutler et al. 2007) and plants (Maor and Shirasu 2005). Invasive fungal infections caused by *Aspergillus* species have become a major cause of morbidity and mortality among immunocompromised patients worldwide (Ellis 1999; Remington et al. 2005). In case of the absence of effective therapy, mortality rates are high, between 30 and 90%. In particular, *A. fumigatus*, which is the most common *Aspergillus* species associated with invasive aspergillosis, causes approximately 4% of all hospital-based deaths in Europe (Brookman and Denning 2000). Although *A. fumigatus* is most frequently isolated from clinical specimens, other species including *A. flavus*, *A. niger*, and *A. terreus* have emerged as significant causes of opportunistic infection at hospitals. In particular, there have been increasing reports of pneumonia and disseminated infections due to *A. terreus* strains derived from environmental and clinical isolates (Tokimatsu et al. 2007; Castón et al. 2007). The fact that *A. terreus* is becoming more frequent in relation to other *Aspergillus* species (Baddley et al. 2003) causes concern because of its resistance to amphotericin B, which is the main antibiotic used for the treatment of fungal infections (Walsh et al. 2003).

The increasing number of fungal genomes that have been sequenced highlights the importance of studying fungi (Kim et al. 2007b). The addition of proteome analysis to these studies facilitates better understanding of the various altered protein expression patterns that occur in filamentous fungi under industrial culture conditions and vastly improve our knowledge of pathogenesis, antibiotic resistance, and the biology of fungi. Several proteomic studies of intracellular proteins from *Aspergillus* species, such as *A. nidulans* (Melin et al. 2002; Kim et al. 2007a), *A. flavus* (Medina et al. 2004), *A. fumigatus* (Carberry et al. 2006; Asif et al. 2006), and *A. oryzae* (Oda et al. 2006), have been reported. Some of the studies, known as subcellular proteomics or subproteomics, have also focused on a particular subset of protein complement such as membrane proteins (Medina et al. 2004; Asif et al. 2006) or secreted proteins (Oda et al. 2006; Tsang et al. 2009). Secretome-related studies are particularly relevant in understanding fungi, because many fungi secrete a large number of proteins to accommodate their saprotrophic lifestyle. Secreted proteins are of particular interest in the studies of pathogens (Remington et al. 2005) and finding fusion partners for extracellular production of biotechnologically relevant heterologous proteins in filamentous fungi (Nevalainen et al. 2005). Although a few of secretomic studies on *Aspergillus* species, such as *A. flavus* (Medina et al. 2004, 2005) or *A. oryzae* (Oda et al. 2006) have been previously reported, none involved in-depth proteomic analysis using both

two-dimensional gel electrophoresis (2-DE) and advanced mass spectrometry (MS) techniques. Recently, a large fraction of the *A. niger* secretome was identified by a combination of direct MS analysis without separation on 2-D gel and by prediction by computational methods (Tsang et al. 2009). Since that method did not provide any information on protein quantitation, the relative amounts of each protein secreted under different growth conditions could unfortunately not be determined. To date, no research has described any part of the extracellular proteome of *A. terreus*.

Taking advantage from the recently annotated *A. terreus* genome, we used 2-DE and nano-liquid chromatography (LC)-MS/MS to identify the proteins secreted from *A. terreus* grown at under three different culture conditions utilizing sucrose, glucose, and starch, which are the most commonly used carbon sources in industrial biotechnology. The extracellular proteomes of *A. terreus* produced in the presence of high concentrations of sucrose and glucose were also determined. In addition, computational approaches were used to further confirm the subcellular localizations of the identified proteins.

Materials and methods

Strain, culture conditions, and analytical procedures

A. terreus NRRL 1960 (ATCC10020) was maintained on a spore-forming solid medium (pH 5.0) containing 20 g/L glucose (Samyang Genex, Incheon, Korea), 35 g/L corn steep liquor, 20 g/L NaCl, and 20 g/L agar (Dwiarti et al. 2002). Spores (1×10^8) were transferred into Czapek-Dox solution (pH 7.3 ± 0.2) (Atlas and Parks 1993) containing 30 g/L sucrose (Junsei Chemical Co., Ltd, Tokyo, Japan), 3.0 g/L NaNO₃, 1.0 g/L K₂HPO₄, 0.5 g/L MgSO₄·7H₂O, 0.5 g/L KCl, and 0.01 g/L FeSO₄·7H₂O and cultures incubated at 30°C and 170 rpm for 48 h (Dwiarti et al. 2002; Thom and Raper 1945). Then, 10% of the seed was transferred into 500-mL Erlenmeyer flasks containing 50 mL of Czapek-Dox solution that was supplemented with 30 g/L of one of three carbon sources: sucrose, glucose, or starch (soluble starch, Yakuri Pure Chemicals Co. Ltd. Osaka, Japan). All of the media had the same common composition except for the carbon source. To examine the effect of a higher concentration of sugars on secretion, media containing 100 g/L of either sucrose or glucose, which are widely used in industrial production processes (Okabe et al. 2009), were also tested. The effects of high starch concentration were not studied since cultivation in a medium containing 100 g/L of starch resulted in a highly viscous solution, making it difficult to obtain extracellular proteins from the culture supernatant. The cultivation was

performed at 30°C for 84 h (3.5 days) on a rotary shaker at 170 rpm. Cell growth of *A. terreus* grown under the five different conditions was measured by dry cell weight (DCW, g/L) at the sampling point. To determine DCW, the cell suspension was centrifuged in pre-weighed tubes for 10 min at 3,500×g, followed by drying at 85°C until there was no further change in total weight. For all cultures, the pH was measured after growth to ensure that the values were maintained without a significant change in the pH of the initial inoculated medium. Total nucleic acids were measured fluorometrically using the fluorescent dye bis-Benzimide (Sigma Chemical Co., St. Louis, MO, USA) (Nandakumar et al. 2006; Xia et al. 2008). The concentrations of glucose and acetic acid were measured by high-performance liquid chromatography (HPLC) using the ProStar 210 system (Varian, Palo Alto, CA, USA) equipped with a MetaCarb 87H column (300 mm × 7.8 mm, Varian) and ProStar 320 UV/visible-light (Varian) and RI (Shodex RI-71; Tokyo, Japan) detectors. The column was eluted isocratically with 0.01 M H₂SO₄ at 60°C at a flow rate of 0.6 mL/min. The residual starch and sucrose in the broth was estimated after acid hydrolysis (Pirt and Whelan 1951) by HPLC analysis using the conversion factors of (0.9 × glucose) and (0.95 × sum of glucose and fructose), respectively. Flask cultures were carried out in triplicate and checked for the consistency as to the profiles of cell growth and metabolite formation.

Preparation of extracellular proteins

The extracellular proteins were prepared as previously described by Xia et al. (2008). Briefly, cells were removed by centrifugation at 3,500×g for 20 min at 4°C. The clear supernatant was collected, passed through a membrane (pore size of 0.2 µm; Sartorius, Goettingen, Germany), mixed with an equal volume of cold 20% (w/v) TCA (trichloroacetic acid; Sigma, St. Louis, MO, USA) in acetone, and incubated overnight at −20°C. The precipitate was collected by centrifugation at 13,000×g for 1 h at 4°C. The precipitated protein pellet was then washed with cold acetone to remove traces of TCA. The washed protein pellet was air dried for 1 h at room temperature and resolubilized in a 2-DE sample buffer composed of 8 M urea, 2 M thiourea, 4% (w/v) CHAPS, 20 mM DTT, and 1% (v/v) protease inhibitor cocktail (Complete Mini EDTA-free; Roche Diagnostics GmbH, Mannheim, Germany). Insoluble materials were removed by centrifugation at 13,000×g for 15 min. The supernatant was collected, and the total extracellular protein concentration was measured by the Bradford method (Bradford 1976). The resulting solution was stored at −80°C until 2-DE analysis.

Preparation of whole cellular proteins

Whole cell extracts were obtained as reported previously by Kniemeyer et al. (2006) with some modifications. Briefly, frozen biomass was ground to a fine powder in liquid nitrogen using a cooled mortar. About 50 mg of the powder was directly dissolved in the 2-DE sample buffer and incubated for 30 min at room temperature. After centrifugation at 16,000×g for 15 min at 20°C, the supernatant was collected. Proteins were precipitated with TCA/acetone and quantified as described in the preparation of extracellular proteins.

Two-dimensional gel electrophoresis (2-DE) and gel analysis

The 2-DE experiments were carried out with the IPGphor IEF system (GE Healthcare; Chalfont St. Giles, Bucks, UK) and Protean II xi Cell (Bio-Rad, Hercules, CA, USA) as described previously (Han et al. 2005). The samples of extracellular proteins (100 µg) or whole cell extracts (100 µg) were diluted with the 2-DE sample buffer containing 1% (v/v) IPG buffer (GE Healthcare; pH 3–10 NL and pH 4–7). IbpB purified from recombinant *E. coli* was added at a concentration of 5% (w/w) to inhibit the proteolysis of the samples during isoelectric focusing (Han et al. 2005) and was used as a standard marker for gel-to-gel comparison and spot quantitation. Protein samples were carefully loaded on the IPG strips (GE Healthcare; 18-cm, pH 3–10 NL and pH 4–7), followed by rehydration of the loaded IPG strips for 12 h. and the isoelectric focusing was performed at 20°C for 15 min at 250 V, followed by 3,500 V until a total of 60 kV h was reached. The strips were first equilibrated for 15 min in equilibration buffer I, which was composed of 6 M urea, 0.375 M Tris-HCl (pH 8.8), 20% (w/v) glycerol, 2% (w/v) SDS, and 130 mM DTT, then incubated for 15 min in equilibration buffer II, which was composed of 6 M urea, 0.375 M Tris-HCl (pH 8.8), 20% (w/v) glycerol, 2% (w/v) SDS, and 135 mM iodoacetamide, and lastly placed on 12% (w/v) SDS-PAGE gels as described by Laemmli (1970). Protein spots were visualized using a silver staining kit (GE Healthcare), and the stained gels were scanned by a UMAX PowerLook 2100XL Scanner (UMAX Technologies Inc., TX, USA).

PDQuest 2-D Analysis Software (Bio-Rad) was used to automate the process of finding protein spots within the image and to quantify the density of the spots in the percentage of volume basis (i.e., values were calculated by the integration of spot optical intensity over the spot area). After features resulting from non-protein sources were filtered out and protein spots were normalized, pairwise image comparisons were performed. All 2-DE experiments

were carried out in triplicate, and the mean and the standard deviation of each spot were calculated.

Nano-LC-MS/MS analysis

Samples for the MS/MS analysis were prepared as described previously (Han et al. 2001) with some modifications. Briefly, protein spots were excised and destained by incubation in 30 mM potassium ferricyanide and 65 mM sodium thiosulfate for 10 min. Gel pieces were washed in Milli-Q water until they were colorless and transparent and then vacuum-dried. These pieces were proteolyzed with 0.02 µg/µL of modified trypsin (Promega, Madison, WI, USA) in 40 mM ammonium bicarbonate overnight at 37°C. Tryptic peptides (10 µL aliquots) were analyzed by a nano-LC/MS system consisting of an Ultimate HPLC system (LC Packings, Amsterdam, Netherlands) and a quadrupole-time-of-flight (Q-TOF) MS (Micromass, Manchester, UK) equipped with a nano-ESI source, as described previously (Lee et al. 2006). The acquired MS/MS spectra were processed using MassLynx v 3.5 (Waters, Milford, MA, USA). The MASCOT search server (version 1.8; <http://www.matrixscience.com/>) was used for the identification of protein spots by querying the sequence of the tryptic peptide fragments. The database taken from the Broad Institute at MIT and Harvard (http://www.broad.mit.edu/annotation/genome/aspergillus_terreus/GenomesIndex.html) was used for the identification of target proteins. Both the UniProt Knowledgebase (Swiss-Prot and TrEMBL; <http://kr.expasy.org/>) and NCBI (<http://www.ncbi.nlm.nih.gov/>) reference databases were used to help determine protein functions.

Computational analysis

The subcellular localization of proteins was predicted by using the WoLF PSORT program (<http://wolfsort.org/>) (Horton et al. 2006). The probability of the presence of a signal peptide was predicted for those proteins found in the secretome using SignalP v.3.0 (<http://www.cbs.dtu.dk/services/SignalP/>) (Bendtsen et al. 2004). Putative function of proteins annotated as hypothetical or predictive proteins

from *A. terreus* database were identified by the BLASTP search (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) against non-redundant protein sequences (nr) in the NCBI database.

Results

The concentration of secreted proteins of *A. terreus* under different carbon sources

Although many secreted proteins in fungi are of special interest in the study of pathogens (Rementeria et al. 2005) or to the biotechnology industry (Nevalainen et al. 2005; Okabe et al. 2009), the proteins secreted from *A. terreus* have not been thoroughly studied. Thus, this study involved the extracellular proteome profiling of *A. terreus* grown in the presence of one of three different carbon sources: sucrose, glucose and starch. At the selected sampling point, the amount of proteins secreted from *A. terreus* was similar among the culture media supplemented with 30 g/L of different carbon sources (Table 1). In contrast, *A. terreus* cells grown under high concentrations of sucrose or glucose (100 g/L) secreted considerably lower amounts of protein than cells grown under moderate concentrations of the carbon source of interest (30 g/L). This result indicates that protein excretion of *A. terreus* is highly suppressed in the presence of a high concentration of a carbon source.

As shown in Table 1, the accumulation of acetic acid produced by cell metabolism was negligible at the sampling points. In addition, the culture pH, which was maintained within ~0.15 unit of the starting pH values, did not change during the culturing period. These results confirm that, with the exception of the varying carbon source, the experimental culture conditions were kept constant in this study.

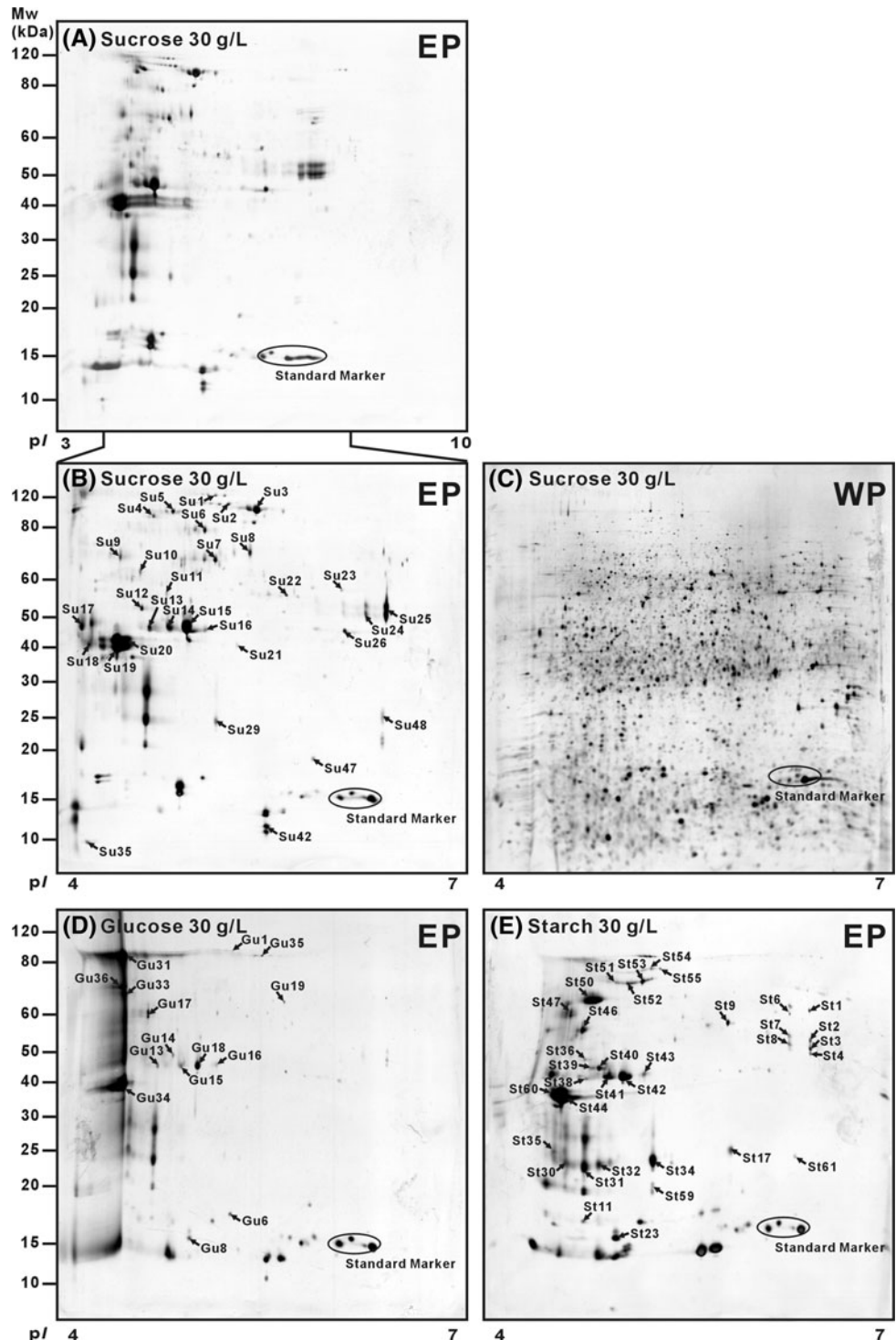
The extracellular proteome maps of *A. terreus* under different carbon sources

The representative 2-DE maps of extracellular proteins of *A. terreus* at each carbon source are shown in Figs. 1 and 2.

Table 1 The secreted protein amounts of *A. terreus* under various culture conditions

Culture condition (g/L)	Dry cell weight (DCW; g/L)	Extracellular protein/culture supernatant (mg/L)	Extracellular protein/DCW (mg/g)	Concentration of residual carbon source (g/L)	Concentration of produced acetic acid (g/L)	pH at starting point	pH at sampling point
Sucrose 30	14.7 ± 0.4876	11.23 ± 0.0771	0.7638	3.02	0.10	7.04	7.13
Sucrose 100	29.1 ± 1.249	9.146 ± 0.0321	0.3143	58.7	0.06	7.04	7.10
Glucose 30	13.1 ± 2.081	11.15 ± 0.0177	0.8513	4.01	0.23	7.05	7.10
Glucose 100	28.6 ± 1.987	10.56 ± 0.0963	0.3692	62.1	0.07	7.05	7.07
Starch 30	12.2 ± 0.2598	10.97 ± 0.0642	0.8993	17.2	0.08	7.04	7.28

Fig. 1 The representative 2-DE maps of extracellular proteins (EP) of *A. terreus* under three different culture conditions: (a, b) 30 g/L sucrose; (d) 30 g/L glucose; and (e) 30 g/L starch. For comparison, the whole cellular proteins (WP) extracted from *A. terreus* under a sucrose medium is also shown (c). Protein samples were precipitated from the supernatants at the given conditions. The precipitated proteins were separated on IPG strips of pH 3–10 NL (a) or pH 4–7 (b, c, d, and e), followed by 12% (w/v) SDS–PAGE, and stained with silver. The positions of identified proteins and the IbpB used as a standard marker are indicated. More information on these proteins can be found in Table 2

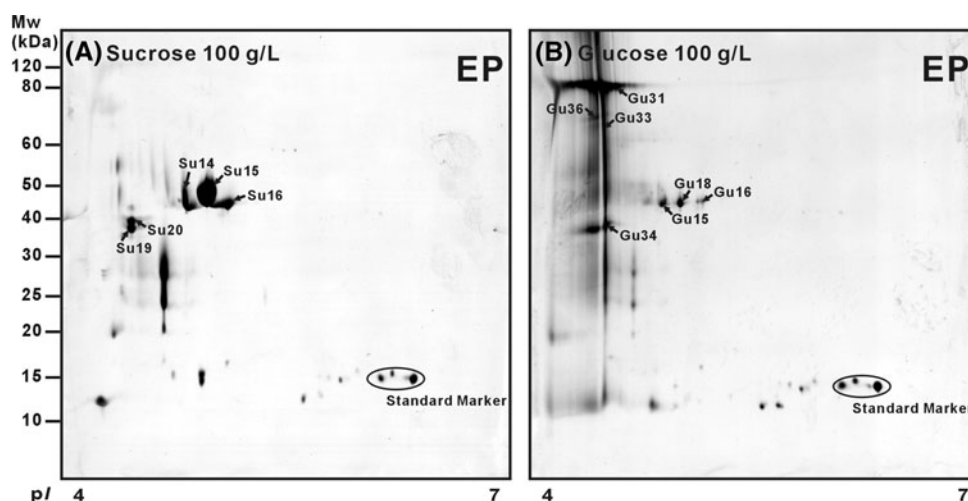


The initial separation of proteins secreted from *A. terreus* grown on sucrose as the carbon source was carried out on the broader pH range (pH 3–10) IPG gels (Fig. 1a). Most proteins were detected at a pH range between 4 and 7, being particularly clustered at acidic pHs. To increase the resolution of the 2-D gels, samples were further analyzed on the medium pH range (pH 4–7) IPG gels (Fig. 1b). As expected,

there was better separation on the medium pH range IPG gels. Given that result, the medium pH range IPG gels (Fig. 1c and d) were used to analyze the proteins secreted from *A. terreus* cultured on either glucose or starch.

An important issue in this analysis is the control of the contamination of cellular proteins in the extracellular proteome. To examine whether any of the identified

Fig. 2 The representative 2-DE maps of extracellular proteins of *A. terreus* at high concentrations of the carbon source: (a) 100 g/L sucrose; and (b) 100 g/L glucose. The positions of identified proteins and the IbpB used as a standard marker are indicated. More information on these proteins can be found in Table 2



extracellular proteins were actually released as a result of cell lysis, the culture supernatant was analyzed as described previously (Nandakumar et al. 2006; Xia et al. 2008). Nucleic acids were not detected in the culture supernatant under the period of proteome sampling. In addition, the 2-D gels of extracellular proteins were significantly different from the gel of *A. terreus* cell lysates under the same culture condition (Fig. 1b and c), indicating that the extracellular protein accumulation was not a result of simple cell lysis.

In order to identify the proteins secreted by *A. terreus* cultured with different carbon sources, 2-DE analysis followed by MS/MS was performed. The initial analysis of each 2-D gel revealed a total of 148 protein spots, which included 48 for sucrose, 38 for glucose, and 62 for starch. Of these, 82 protein spots, including 31 for sucrose, 15 for glucose, and 36 for starch, were successfully identified, and these spots corresponded to a total of 39 non-redundant proteins (Table 2). The positions of the protein spots on the gels are shown in Figs. 1 and 2. Among the 39 non-redundant proteins, the numbers of proteins identified from culture supernatants of *A. terreus* cultivated on different carbon sources are summarized in Fig. 3a. In the cultures grown on starch, the 36 protein spots were represented by only 19 proteins, demonstrating that 47% of the identified proteins existed in multiple isoforms. Twelve proteins were co-detected in the presence of at least two different carbon sources. Five proteins, which include beta-glucosidase 1 (ATEG_03047), oryzin (ATEG_03900), a conserved hypothetical protein (ATEG_07817), and two predicted proteins (ATEG_04955 and ATEG_07481), were secreted on all three carbon sources. Of these, the most abundant extracellular proteins were oryzin and the predicted protein ATEG_07481. Twenty-two unique proteins, representing over half of the secreted proteins, were classified as hypothetical or predicted proteins. The largest number of

different proteins (16 proteins) was secreted by cultures grown on sucrose (Table 2). The secreted proteins were further classified into three subgroups: hydrolases (thioether and trialkylsulfonium hydrolases, glycosylases, and proteases); proteins with miscellaneous functions; and hypothetical or predicted proteins. A major function of the annotated proteins was hydrolysis for the utilization or degradation of a variety of substrates (Table 2).

As shown in Table 1, the amounts of proteins secreted from *A. terreus* cells grown under high concentrations of sucrose or glucose (100 g/L) were approximately twofold lower than those grown under moderate concentrations of sucrose or glucose (30 g/L). Regardless of the concentration, the overall extracellular proteomic pattern was similar for a particular carbon source (Figs. 1 and 2) even though the relative levels of some proteins might differ to some extent. For example, arabinan endo-1,5- α -L-arabinosidase A (Su24 and 25) and the predicted protein (ATEG_07481; Su19 and Su20) were significantly repressed at high sucrose concentrations. In contrast, oryzin (Su14, Su15 and Su16), which is a member of the subtilisin family, was highly secreted at high sucrose concentrations. Therefore, both the concentration and type of the carbon source highly influences the pattern of secreted proteins.

Prediction of subcellular localization of proteins identified in the culture medium

In order to further confirm the subcellular localization of identified proteins, we predicted their subcellular localization by computational methods (Table 2). Of the 39 non-redundant proteins, 69% (27 proteins) were predicted to be extracellular proteins by the program WoLF PSORT (Fig. 3b). Six proteins were predicted to have a cytoplasmic origin, and six proteins were predicted to originate from other compartments, including the nuclear and

Table 2 Identification of the secreted proteins of *A. terreus* under three different carbon sources

Accession number ^a	Protein description ^a	Score ^b	Theoretical ^b Mw	pI ^b	Sequence coverage ^b (%)	Predicted location ^c	Signal peptide ^d	Sucrose ^e	Glucose ^e	Starch ^e
Hydrolases										
Acting on ether bonds-thioether and trialkylsulfonium hydrolases										
ATEG_00163	Adenosylhomocysteinease	62	47420	6.02	6	Cyto	No			St6 (0.69 ± 0.12)
Glycosylases										
ATEG_03688	Arabinan endo-1,5-alpha-L-arabinosidase A precursor	228	35368	6.1	22	Extr	Yes	Su24 (14.77 ± 1.22), Su25 (41.77 ± 4.23)		St2 (2.52 ± 0.47), St3 (3.60 ± 0.45), St4 (1.06 ± 0.14), St7 (1.76 ± 0.20), St8 (2.19 ± 0.30) St56 (5.94 ± 0.85)
ATEG_03047	Beta-glucosidase 1 precursor	642	93298	5.08	25	Extr	Yes	Su1 (1.96 ± 0.13)	Gu1 (0.71 ± 0.12)	
ATEG_00809	Endo-1,4-beta-xylanase A precursor	167	30869	4.77	25	Extr	Yes	Su18 (4.50 ± 0.44)		
ATEG_04660	Endochitinase 1 precursor	228	44492	5.84	26	Extr	No	Su23 (1.40 ± 0.16)		
ATEG_00658	Mannosyl-oligosaccharide alpha-1,2-mannosidase precursor	402	56254	5.15	26	Extr	Yes	Su7 (7.79 ± 0.29)		
Act on peptide bonds—proteases										
ATEG_04941	24 kDa Metalloproteinase precursor	63	37038	5.3	5	Extr	Yes			St61 (0.71 ± 0.27)
ATEG_06546	Cerevisin precursor	273	52409	5.58	18	Extr	Yes	Su26 (6.12 ± 0.13)		
ATEG_09875	Dipeptidyl-peptidase 5 precursor	405	79577	5.19	20	Extr	Yes	Su2 (2.62 ± 0.05)		
ATEG_03900	Oryzin precursor	113	42654	5.37	18	Extr	Yes	Su14 (14.95 ± 1.65; 86.67 ± 3.93*), Su15 (69.59 ± 0.75; 433.4 ± 19.63*), Su16 (6.78 ± 0.47; 90.11 ± 7.83*), Su17 (17.11 ± 4.51)	Gu15 (4.37 ± 0.51; 13.37 ± 0.97*), Gu16 (1.73 ± 0.05; 6.89 ± 1.36*), Gu18 (16.55 ± 1.02; 15.62 ± 0.19*)	St36 (1.52 ± 0.13), St41 (23.28 ± 5.14), St4 2 (63.09 ± 5.98), St43 (4.10 ± 0.56)

Table 2 continued

Accession number ^a	Protein description ^a	Score ^b	Theoretical ^b Mw pI	Sequence coverage ^b (%)	Predicted location ^c	Signal peptide ^d	Sucrose ^e	Glucose ^e	Starch ^f
ATEG_04676	Vacuolar protease A precursor	521	43084 4.78	37	Extr	Yes	Su12 (5.28 ± 0.23)		
Proteins with miscellaneous functions									
ATEG_01242	Allergen Asp f 15 precursor	46	14483 8.73	7	Extr	Yes	Gu8 (4.35 ± 0.31)	St23 (12.67 ± 1.41)	
ATEG_07477	Catalase B precursor	842	77850 5.38	33	Extr	Yes	Su3 (37.18 ± 2.22)	Gu35 (1.52 ± 0.24)	
ATEG_04573	Dolichol-phosphate mannosyltransferase	179	54397 5.20	13	Cyto	No			St9 (4.54 ± 0.64)
ATEG_02902	Enolase	144	47318 5.3	13	Cyto	No		Gu19 (0.89 ± 0.13)	
ATEG_08149	Glycolipid-anchored surface protein 5 precursor	462	47761 5.22	39	Extr	Yes	Su11 (2.17 ± 0.02)		
ATEG_00551	Spermidine synthase	144	33183 5.33	28	Nucl; Cyto_nucl	No	Su21 (0.80 ± 0.20)		
Hypothetical or predicted proteins (putative function) ^f									
ATEG_01031	Conserved hypothetical protein (hydrolase, ref. <i>A. flavus</i> (XP_002376361), identities = 76%)	284	71533 5.13	23	Extr	Yes	Su5 (5.46 ± 0.05)		
ATEG_01375	Conserved hypothetical protein	39	38968 6.20	5	Extr	Yes			St1 (1.65 ± 0.28)
ATEG_05549	Conserved hypothetical protein (GPI-anchored cell wall organization protein Ecm33, ref. <i>A. clavatus</i> (XP_001271571), identities = 66%)	176	41149 4.57	18	Extr	Yes	Gu33 (27.40 ± 1.03), Gu36 (30.68 ± 1.56; 10.41 ± 1.03*)		
ATEG_06125	Conserved hypothetical protein (amidase family protein, ref. <i>A. fumigatus</i> (EDP55345), identities = 75%)	236	64842 5.09	18	Extr	Yes			St54 (0.96 ± 0.15), St55 (0.57 ± 0.10) St38 (3.17 ± 0.76)
ATEG_07817	Conserved hypothetical protein (endo-arabinase, ref. <i>A. flavus</i> (XP_002379171), identities = 81%)	334	35094 4.92	49	Extr	Yes	Su13 (7.46 ± 0.90)	Gu13 (2.55 ± 0.41)	
ATEG_08870	Conserved hypothetical protein (cyanovirin-N family protein, ref. <i>Pyrenophora tritici-repentis</i> (XP_001931533), identities = 54%)	62	11568 4.32	36	Cyto_nucl	No	Su35 (3.19 ± 0.21)		
ATEG_09844	Conserved hypothetical protein (cellulase, ref. <i>Neosartorya fischeri</i> (XP_001260430), identities = 77%) (endo-beta-1,6-glucanase, ref. <i>A. fumigatus</i> (EDP54465), identities = 76%)	430	45785 5.19	35	Extr	Yes	Su8 (9.69 ± 0.35)		
ATEG_10323	Conserved hypothetical protein (C6 transcription factor, ref. <i>A. flavus</i> (XP_002375447), identities = 78%)	211	98619 6.94	6	Plas	Yes	Su42 (8.37 ± 1.55)		

Table 2 continued

Accession number ^a	Protein description ^a	Score ^b	Theoretical ^b Mw	pI	Sequence coverage ^b (%)	Predicted location ^c	Signal peptide ^d	Sucrose ^e	Glucose ^e	Starch ^e
ATEG_09393	Hypothetical protein similar to adenosine kinase (adenosine kinase, ref. <i>A. flavus</i> (XP_002383011), identities = 91%)	264	38271	5.57	30	Cyto	No	Su22 (3.03 ± 0.30)		
ATEG_05177	Hypothetical protein similar to alpha-glucosidase (alpha-glucosidase, ref. <i>A. fumigatus</i> (XP_752991), identities = 74%)	210	98380	5.19	7	Extr	Yes			St50 (51.15 ± 0.87)
ATEG_00336	Hypothetical protein similar to beta-1,3-glucanotransferase (1,3-beta-glucanotransferase Bgt1, ref. <i>N. fischeri</i> (XP_001264620), identities = 71%)	344	33398	5.02	33	Extr	Yes		Gu14 (1.76 ± 0.13)	St39 (4.04 ± 0.54), St40 (6.73 ± 1.14)
ATEG_02590	Hypothetical protein similar to cysteine rich protein (cysteine-rich secreted protein, ref. <i>A. flavus</i> (XP_002374495), identities = 62%)	79	37162	5.78	15	Extr	Yes	Su48 (4.90 ± 0.08)		
ATEG_08718	Hypothetical protein similar to glutaminase A (glutaminase GtaA, ref. <i>A. flavus</i> (XP_002383995), identities = 68%)	410	76654	4.66	18	Extr	Yes	Su4 (2.61 ± 0.12)		
ATEG_07222	Hypothetical protein similar to peroxisomal membrane protein (allergen Asp F3, ref. <i>A. fumigatus</i> (XP_747849), identities = 91%)	52	18440	5.35	16	Cyto	No		Gu6 (2.62 ± 0.20)	
ATEG_05185	Hypothetical protein similar to rAsp f 9 (extracellular cell wall glucanase Crf1/allergen Asp F9, ref. <i>N. fischeri</i> (XP_001264143), identities = 74%)	40	40700	4.41	4	Extr	Yes			St35 (9.79 ± 1.20)
ATEG_02515	Hypothetical protein similar to taka-amylase (alpha-amylase 1, ref. <i>Gibberella moniliformis</i> (AAZ73168), identities = 69%)	230	51249	4.51	25	Extr	Yes	Su9 (5.47 ± 0.51)		St47 (6.07 ± 0.47)
ATEG_00338	Predicted protein (ABC transporter related protein, ref. <i>Mesorhizobium opportunistum</i> (ZP_05808120), identities = 27%)	30	19582	6.04	6	Mito	No	Su47 (2.65 ± 0.26)		
ATEG_04955	Predicted protein (aspartyl protease, ref. <i>Talaromyces stipitatus</i> (XP_002487453), identities = 40%)	106	37694	4.52	11	Cyto	No	Su10 (3.79 ± 0.14)	Gu17 (7.69 ± 0.57)	St46 (12.79 ± 1.68)

Table 2 continued

Accession number ^a	Protein description ^a	Score ^b	Theoretical ^b Mw	pI	Sequence coverage ^b (%)	Predicted location ^c	Signal peptide ^d	Sucrose ^e	Glucose ^e	Starch ^e
ATEG_05691	Predicted protein (peptidase S8 and S53 subtilisin kexin sedolisin, ref. <i>Actinosynnema mirum</i> (YP_003101524), identities = 41%)	311	58639	5.49	23	Mito	No	Su6 (4.20 ± 0.47)		St51 (1.77 ± 0.02), St52 (4.97 ± 0.03), St53 (4.09 ± 0.46)
ATEG_07481	Predicted protein (F0F1-type ATP synthase, beta subunit, ref. <i>Burkholderia cenocepacia</i> (ZP_04941086), identities = 30%)	82	24649	4.6	10	Extr	Yes	Su19 (70.99 ± 4.40; 17.82 ± 2.37*), Su20 (70.30 ± 2.20; 16.07 ± 2.54*)	Gu31 (196.39 ± 24.57; 259.57 ± 23.14*), Gu34 (195.61 ± 25.67; 23.90 ± 1.08*)	St44 (84.57 ± 3.02), St60 (66.42 ± 8.18)
ATEG_07648	Predicted protein (conserved membrane protein, ref. <i>A. flavus</i> (XP_002374182), identities = 25%)	43	14528	9.86	5	Nucl	No			St11 (2.71 ± 0.37)
ATEG_09250	Predicted protein (IgE-binding protein, ref. <i>A. clavatus</i> (XP_001270190), identities = 42%)	72	20475	6.54	23	Extr	Yes	Su29 (4.62 ± 1.41)		St17 (6.24 ± 2.00), St30 (17.57 ± 1.90), St31 (34.23 ± 0.24), St32 (17.19 ± 3.28), St34 (37.43 ± 4.81), St59 (4.05 ± 0.64)

^a The accession number and description of identified proteins are from the database of Broad Institute (http://www.broad.mit.edu/annotation/genome/aspergillus_terreus/GenomesIndex.html)

^b The score, calculated *pI* and *MW*, and sequence coverage (%) are from MASCOT search results of Matrix science (<http://www.matrixscience.com/>) based on the genome information. MASCOT scores >26 indicate identity or extensive homology (*p* < 0.05)

^c The subcellular localization of proteins was predicted by using WoLF PSORT (<http://wolfpsort.org/>) (Horton et al. 2006). The predicted subcellular localization for the protein is listed: *cyto* cytoplasmic, *nucl* nuclear, and *plasma* plasma membrane-bound or integral membrane protein

^d The probability of signal peptide was predicted for the proteins found in the secreted proteomes using SignalP v.3.0 (<http://www.cbs.dtu.dk/services/SignalP/>) (Bendtsen et al. 2004)

Quantity values were calculated by integrating spot intensity and area of each spot on 2-D gels and then were normalized as total quantities of each gel (mean value $\times 1,000 \pm \text{SD}$)

Putative function of proteins annotated as hypothetical or putative proteins from *A. terreus* database was further examined using the BLASTP search (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The possible function information of top protein in matched against non-redundant protein sequences (nr) in the NCBI database was indicated in parenthesis (protein function, reference organism containing NCBI locus number, percentage of identities)

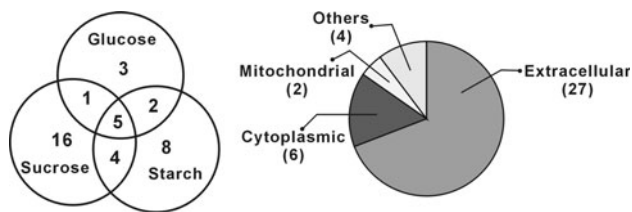


Fig. 3 Analyses of the 39 non-redundant proteins secreted by *A. terreus*. (a) The number of proteins secreted under the three different culture conditions was compared. (b) As predicted by WoLF PSORT (<http://wolfpsort.org/>), the subcellular localization of the 39 proteins predicted could be classified into 4 groups: cytoplasmic; extracellular; mitochondrial; and other, including nuclear and plasma membrane-bound

mitochondrial inner membranes. This result served as additional evidence that the identified extracellular proteins were not substantially contaminated by cellular protein components.

Since many known secreted proteins are secretory proteins in *Aspergillus* species, these proteins were also examined for the possession of signal peptides, using the Signal P software program (Bendtsen et al. 2004), with a probability score setting of higher than 0.98 to minimize false positives. Among the 39 non-redundant proteins identified, N-terminal signal peptide sequences, which function to target proteins for secretion from the cytoplasm, were present in 27 proteins (~69%) (Table 2). Thus, all of the proteins that were predicted to have an extracellular location by the WoLF PSORT program contained an N-terminal signal peptide, most of which would direct their respective proteins to the secretory protein translocation (Sec) pathway.

Discussion

An attraction of filamentous fungi is their natural ability to secrete large amounts of proteins (mainly hydrolytic enzymes) into the growth medium. However, very few reports of using 2-DE on extracellular proteins of *Aspergillus* are available. Medina et al. (2004) identified 15 extracellular proteins from *A. flavus* using 2-DE and MALDI-TOF MS that were specifically induced when the flavonoid rutin (quercetin 3-O-glycoside) was the only source of carbon. Using LC-MS/MS, they also identified a total of 51 uniquely secreted proteins from *A. flavus* grown in media containing rutin, glucose, or potato dextrose, with 18 of these found to be in the rutin degradation pathway (Medina et al. 2005). Oda et al. (2006) reported the secretome of *A. oryzae* and identified 29 extracellular proteins that were produced when the fungi were grown in either liquid or solid-state culture, with taka-amylase A (TAA), glucoamylase (GLAA), and aspergillopepsin A (PEPA)

comprising the majority of secreted proteins, as determined by 2-D gel analysis.

Most of the proteins secreted by *A. terreus* that were identified in this study using combination of 2-DE and nano-LCMS/MS techniques, have not been documented previously (Table 2). *A. terreus* cultivated on a sucrose medium clearly secretes a greater number of different proteins than the cells cultivated on a glucose medium, with 16 unique proteins versus 3, respectively (Fig. 3a). Seventeen of the thirty-six total secreted from *A. terreus* cultivated on a starch medium were found to be present as two or more isoforms; specifically, arabinan endo-1,5- α -L-arabinosidase A (ATEG_03688) and one of the predicted proteins (ATEG_09250) had five isoforms each, and oryzin (ATEG_03900) had four isoforms. These results showed that different kinds of proteins and their isoforms secreted by *A. terreus*, along with their relative levels, are affected by different carbon sources.

The extracellular proteins specifically produced by *A. terreus* in the presence of one of three carbon sources were mainly hydrolases (Table 2), such as thioether and trialkyl-sulfonium hydrolases including adenosylhomocysteinase, glycosylases including arabinan endo-1,5- α -L-arabinosidase A, beta-glucosidase 1, endo-1,4-beta-xylanase A, endochitinase 1, and mannosyl-oligosaccharide α -1,2-mannosidase, and proteases including 24-kDa metalloproteinase, cerevisin, dipeptidyl-peptidase 5, and oryzin.

Some of extracellular proteins are related to allergens which can be grouped into several categories: proteases; glycosidases; oxidative stress response proteins; and enzymes involved in gluconeogenesis or the pentose phosphate shunt (Denning et al. 2006). For example of *A. fumigatus* (Rementeria et al. 2005), Asp f5/Mep has a metalloprotease activity, Asp f10/Pep appears to be an aspartic protease, Asp f11 or cyclophilin has a dipeptidylprolyl-isomerase activity, Asp f13/Alp2 possesses alkaline serine protease activity, and Asp f18 is a vacuolar serine protease. In addition, an enolase has been reported to be similar to the allergen Asp f22 of *A. fumigatus* (Lai et al. 2002). Thus, several extracellular proteins identified in *A. terreus* might represent potential allergens under infective conditions that can serve as candidates for vaccination against this fungal organism. These may include 24-kDa metalloproteinase (Rhodes et al. 1990; Rementeria et al. 2005), cerevisin (Nebes and Jones 1991), oryzin (Chou et al. 1999), dipeptidyl-peptidase (Monod et al. 2002), vacuolar protease A (Monod et al. 2002), allergen Asp f 15, catalase B (Blum et al. 2008) and enolase (Lai et al. 2002). A 24-kDa metalloproteinase (ATEG_04941) identified in this study, however, differs from those metalloproteases, since it has a smaller molecular mass. Instead, it might represent a homolog of an elastinolytic proteinase of *A. flavus* (Rhodes et al. 1990). Catalase B was only secreted

by *A. terreus* in the presence of moderate concentrations of sucrose (spot no. Su3) or glucose (spot no. Gu35). Blum et al. (2008) showed that *A. terreus* produced significantly more catalases than *A. fumigatus* and that the high-level production of catalases by *A. terreus* contributes to its resistance to amphotericin B. Thus, the secreted catalase may serve as one of several types of reactive-oxidant scavengers that play roles in amphotericin B resistance in *A. terreus*.

Interestingly, oryzin (ATEG_03900; *Aspergillus* alkaline proteinase) is a predominant alkaline endopeptidase secreted by *A. terreus* on all carbon sources. This is similar to what has been reported for *A. oryzae* cultured under solid-state conditions (Oda et al. 2006). In addition, oryzin appeared at more than three different positions on the 2-D gels (Table 2, Fig. 1). High concentrations of sucrose resulted in higher excretion levels of this enzyme. Our result is consistent with a previous study that reported that an alkaline serine protease (IJIRA 6.2) was one of the major extracellular proteases produced by *A. terreus* (Chakrabarti et al. 2000). Recently, a recombinant alkaline protease derived from *A. oryzae* was shown to be efficiently produced in *Pichia pastoris* with a maximum yield of 513 mg/L, which indicates its potential for wide use in a variety of industrial applications (Guo and Ma 2008).

In industrial biotechnology, especially with regard to the expression of heterologous proteins, products of interest are synthesized by fusing the corresponding gene to the 3' end of a gene, encoding a well-secreted endogenous protein, such as glucoamylase (encoded by *glaA*) in filamentous fungi (Gouka et al. 1997). The gene fusion method has resulted in higher levels of excretory production of various proteins, including bovine prochymosin (Ward et al. 1990), porcine pancreatic phospholipase A2 (Roberts et al. 1992), human interleukin-6 (Broekhuijsen et al. 1993), and human lactoferrin (Ward et al. 1995). However, even when proteins are finally secreted efficiently, a major problem for heterologous proteins is their degradation by extracellular proteases. In the current study, we have identified efficiently secreted endogenous proteins that may be used as fusion partners to heterologous gene products. In addition, we have identified several proteases that may interfere with the extracellular protein yields. Genes encoding extracellular proteases have been inactivated to successfully improve the yields of heterologous gene products produced in fungal hosts (Broekhuijsen et al. 1993; van den Hombergh et al. 1997).

More than half of these secreted proteins (22 of 39 non-redundant proteins) were classified as hypothetical or predicted proteins. This finding may be due to the scarce annotation of the completely sequenced *A. terreus* genome, with only approximately 7.5% of the open reading frames having assigned functions. The function of these proteins

can be predicted by using the BLASTP search (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) (Table 2). The majority of proteins identified in this study were hydrolytic in nature, such as alpha-amylase, alpha-glucosidase, amidase, aspartyl protease, cellulase, endo-arabinase, glutaminase, hydrolase, and peptidase. Two potential allergens Asp F3 and F9 were also identified. Despite the lack of proteins with known functions, our data confirms that hypothetical proteins predicted from the genomic sequence are expressed, thereby identifying additional extracellular proteins or enzymes.

Finally, although the coupling of the 2-D gel analysis with the nano-LC-MS/MS technique identified many of new extracellular proteins, the limitation that results from the lower successful identification rate of protein spots than typically found in typical biological samples (e.g., bacterial proteins) still remains. This limitation is caused by the high number of glycosylated proteins in fungi, resulting in a poor recovery of tryptic peptide sequences that are used for protein identification. In the future, an advanced proteomic approach that allows a much greater depth of analysis is required.

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References

- Asif AR, Oellerich M, Armstrong VW, Riemenschneider B, Monod M, Reichard U (2006) Proteome of conidial surface associated proteins of *Aspergillus fumigatus* reflecting potential vaccine candidates and allergens. *J Proteome Res* 5(4):954–962
- Atlas RM, Parks LC (1993) Handbook of Microbiological Media. CRC Press, Boca Raton
- Baddley JW, Pappas PG, Smith AC, Moser SA (2003) Epidemiology of *Aspergillus terreus* at a university hospital. *J Clin Microbiol* 41(12):5525–5529
- Bendtsen JD, Nielsen H, von Heijine G, Brunak S (2004) Improved prediction of signal peptides: SignalP 3.0. *J Mol Biol* 340(4):783–795
- Blum G, Perkhof S, Haas H, Schrettl M, Würzner R, Dierich MP, Lass-Flörl C (2008) Potential basis for amphotericin B resistance in *Aspergillus terreus*. *Antimicrob Agents Chemother* 52(4):1553–1555
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254
- Broekhuijsen MP, Mattern IE, Contreras R, Kinghorn JR, van den Hondel CA (1993) Secretion of heterologous proteins by *Aspergillus niger*: production of active human interleukin-6 in a protease-deficient mutant by KEX2-like processing of a glucoamylase-hIL6 fusion protein. *J Biotechnol* 31(2):135–145

- Brookman JL, Denning DW (2000) Molecular genetics in *Aspergillus fumigatus*. *Curr Opin Microbiol* 3(5):468–474
- Carberry S, Neville CM, Kavanagh KA, Doyle S (2006) Analysis of major intracellular proteins of *Aspergillus fumigatus* by MALDI mass spectrometry: identification and characterisation of an elongation factor 1B protein with glutathione transferase activity. *Biochem Biophys Res Commun* 341(4):1096–1104
- Carlile MJ, Watkinson SC, Gooday GW (2001) *The Fungi*, 2nd edn. Academic Press, San Diego
- Castón JJ, Linares MJ, Gallego C, Rivero A, Font P, Solís F, Casal M, Torre-Cisneros J (2007) Risk factors for pulmonary *Aspergillus terreus* infection in patients with positive culture for filamentous fungi. *Chest* 131(1):230–236
- Chakrabarti SK, Matsumura N, Ranu RS (2000) Purification and characterization of an extracellular alkaline serine protease from *Aspergillus terreus* (IJIRA 6.2). *Curr Microbiol* 40(4):239–244
- Chou H, Lin WL, Tam MF, Wang SR, Han SH, Shen HD (1999) Alkaline serine proteinase is a major allergen of *Aspergillus flavus*, a prevalent airborne *Aspergillus* species in the Taipei area. *Int Arch Allergy Immunol* 119(4):282–290
- Cutler JE, Deepe GS Jr, Klein BS (2007) Advances in combating fungal diseases: vaccines on the threshold. *Nat Rev Microbiol* 5(1):13–28
- Denning DW, O'Driscoll BR, Hogaboam CM, Bowyer P, Niven RM (2006) The link between fungi and severe asthma: a summary of the evidence. *Eur Respir J* 27(3):615–626
- Dwiarti L, Yamane K, Yamatani H, Kahar P, Okabe M (2002) Purification and characterization of cis-aconitic acid decarboxylase from *Aspergillus terreus* TN484-M1. *J Biosci Bioeng* 94(1):29–33
- Ellis M (1999) Therapy of *Aspergillus fumigatus*-related diseases. *Contrib Microbiol* 2:105–129
- Gouka RJ, Punt PJ, van den Hondel CA (1997) Efficient production of secreted proteins by *Aspergillus*: progress, limitations and prospects. *Appl Microbiol Biotechnol* 47(1):1–11
- Guo JP, Ma Y (2008) High-level expression, purification and characterization of recombinant *Aspergillus oryzae* alkaline protease in *Pichia pastoris*. *Protein Expr Purif* 58(2):301–308
- Han MJ, Yoon SS, Lee SY (2001) Proteome analysis of metabolically engineered *Escherichia coli* producing Poly(3-hydroxybutyrate). *J Bacteriol* 183(1):301–308
- Han MJ, Lee JW, Lee SY (2005) Enhanced proteome profiling by inhibiting proteolysis with small heat shock proteins. *J Proteome Res* 4(6):2429–2434
- Horton P, Park KJ, Obayashi T, Nakai K (2006) Protein Subcellular Localization Prediction with WoLF PSORT, Proceedings of the 4th annual Asia Pacific bioinformatics conference APBC06. Taipei, Taiwan, pp 39–48
- Kim Y, Nandakumar MP, Marten MR (2007a) Proteome map of *Aspergillus nidulans* during osmoadaptation. *Fungal Genet Biol* 44(9):886–895
- Kim Y, Nandakumar MP, Marten MR (2007b) Proteomics of filamentous fungi. *Trends Biotechnol* 25(9):395–400
- Kniemeyer O, Lessing F, Scheibner O, Hertweck C, Brakhage AA (2006) Optimisation of a 2-D gel electrophoresis protocol for the human-pathogenic fungus *Aspergillus fumigatus*. *Curr Genet* 49(3):178–189
- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227(5259):680–685
- Lai HY, Tam MF, Tang RB, Chou H, Chang CY, Tsai JJ, Shen HD (2002) cDNA cloning and immunological characterization of a newly identified enolase allergen from *Penicillium citrinum* and *Aspergillus fumigatus*. *Int Arch Allergy Immunol* 127(3):181–190
- Lee JW, Lee SY, Song H, Yoo JS (2006) The proteome of *Mannheimia succiniciproducens*, a capnophilic rumen bacterium. *Proteomics* 6(12):3550–3566
- Maggon K (2005) Best-selling human medicines 2002–2004. *Drug Discov Today* 10(11):739–742
- Manzoni M, Rollini M (2002) Biosynthesis and biotechnological production of statins by filamentous fungi and application of these cholesterol-lowering drugs. *Appl Microbiol Biotechnol* 58(5):555–564
- Maor R, Shirasu K (2005) The arms race continues: battle strategies between plants and fungal pathogens. *Curr Opin Microbiol* 8(4):399–404
- Medina ML, Kiernan UA, Francisco WA (2004) Proteomic analysis of rutin-induced secreted proteins from *Aspergillus flavus*. *Fungal Genet Biol* 41(3):327–335
- Medina ML, Haynes PA, Brei L, Francisco WA (2005) Analysis of secreted proteins from *Aspergillus flavus*. *Proteomics* 5(12):3153–3161
- Melin P, Schnurer J, Wagner EG (2002) Proteome analysis of *Aspergillus nidulans* reveals proteins associated with the response to the antibiotic concanamycin A, produced by *Streptomyces* species. *Mol Genet Genomics* 267(6):695–702
- Monod M, Capoccia S, Léchenne B, Zaugg C, Holdom M, Jousson O (2002) Secreted proteases from pathogenic fungi. *Int J Med Microbiol* 292(5–6):405–419
- Nandakumar MP, Cheung A, Marten MR (2006) Proteomic analysis of extracellular proteins from *Escherichia coli* W3110. *J Proteome Res* 5(5):1155–1161
- Nebes VL, Jones EW (1991) Activation of the proteinase B precursor of the yeast *Saccharomyces cerevisiae* by autocatalysis and by an internal sequence. *J Biol Chem* 266(34):22851–22857
- Nevalainen KM, Te'o VS, Bergquist PL (2005) Heterologous protein expression in filamentous fungi. *Trends Biotechnol* 23(9):468–474
- Oda K, Kakizono D, Yamada O, Iefuji H, Akita O, Iwashita K (2006) Proteomic analysis of extracellular proteins from *Aspergillus oryzae* grown under submerged and solid-state culture conditions. *Appl Environ Microbiol* 72(5):3448–3457
- Okabe M, Lies D, Kanamasa S, Park EY (2009) Biotechnological production of itaconic acid and its biosynthesis in *Aspergillus terreus*. *Appl Microbiol Biotechnol* 84(4):597–606
- Pirt SJ, Whelan WJ (1951) The determination of starch by acid hydrolysis. *J Sci Food Agric* 2:224–228
- Rementeria A, López-Molina N, Ludwig A, Vivanco AB, Bikandi J, Pontón J, Garaizar J (2005) Genes and molecules involved in *Aspergillus fumigatus* virulence. *Rev Iberoam Micol* 22(1):1–23
- Rhodes JC, Amlung TW, Miller MS (1990) Isolation and characterization of an elastinolytic proteinase from *Aspergillus flavus*. *Infect Immun* 58(8):2529–2534
- Roberts IN, Jeenes DJ, MacKenzie DA, Wilkinson AP, Sumner IG, Archer DB (1992) Heterologous gene expression in *Aspergillus niger*: a glucoamylase-porcine pancreatic phospholipase A2 fusion protein is secreted and processed to yield mature enzyme. *Gene* 122(1):155–161
- Segal BH, Walsh TJ (2006) Current approaches to diagnosis and treatment of invasive aspergillosis. *Am J Respir Crit Care Med* 173(7):707–717
- Thom C, Raper KB (1945) *A manual of the Aspergilli*. Williams & Wilkins, Baltimore
- Tokimatsu I, Kushima H, Iwata A, Hashinaga K, Umeki K, Ohama M, Kohno K, Ishii H, Kishi K, Ogata M, Hiramatsu K, Saikawa T, Kadota J (2007) Invasive pulmonary aspergillosis with hematological malignancy caused by *Aspergillus terreus* and in vitro susceptibility of *A. terreus* isolate to micafungin. *Intern Med* 46(11):775–779

- Tsang A, Butler G, Powlowski J, Panisko EA, Baker SE (2009) Analytical and computational approaches to define the *Aspergillus niger* secretome. Fungal Genet Biol 46:S153–S160
- van den Hombergh JP, van de Vondervoort PJ, Fraissinet-Tachet L, Visser J (1997) *Aspergillus* as a host for heterologous protein production: the problem of proteases. Trends Biotechnol 15(7):256–263
- Walsh TJ, Petraitis V, Petraitiene R, Field-Ridley A, Sutton D, Ghannoum M, Sein T, Schaufele R, Peter J, Bacher J, Casler H, Armstrong D, Espinel-Ingroff A, Rinaldi MG, Lyman CA (2003) Experimental pulmonary aspergillosis due to *Aspergillus terreus*: pathogenesis and treatment of an emerging fungal pathogen resistant to amphotericin B. J Infect Dis 188(2):305–319
- Ward M, Wilson LJ, Kodama KH, Rey MW, Berka RM (1990) Improved production of chymosin in *Aspergillus* by expression as a glucoamylase-chymosin fusion. Biotechnology (NY) 8(5):435–440
- Ward PP, Piddington CS, Cunningham GA, Zhou X, Wyatt RD, Conneely OM (1995) A system for production of commercial quantities of human lactoferrin: a broad spectrum natural antibiotic. Biotechnology (NY) 13(5):498–503
- Xia XX, Han MJ, Lee SY, Yoo JS (2008) Comparison of the extracellular proteomes of *Escherichia coli* B and K-12 strains during high cell density cultivation. Proteomics 8(10):2089–2103