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Initial Proteome Analysis of Caffeine-Induced Proteins in *Aspergillus tamarii* Using Two-Dimensional Fluorescence Difference Gel Electrophoresis

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Abstract Caffeine is toxic to most microorganisms. However, some filamentous fungi, such as *Aspergillus tamarii*, are able to metabolize this alkaloid when fed caffeine as the sole nitrogen source. The aim of the present work was to identify intracellular *A. tamarii* proteins, regulated by caffeine, using fluorescence difference two-dimensional gel electrophoresis. Specific proteins from two culture media of *A. tamarii* grown either on ammonium sulfate or caffeine as the sole nitrogen source were analysed by mass spectrometry. Thirteen out of a total of 85 differentially expressed spots were identified after database search. Identified up-regulated proteins include phosphoglycerate kinase, malate dehydrogenase, dyp-type peroxidase family protein, heat shock protein, Cu, Zn superoxidase dismutase and xanthine dehydrogenase. Some of the proteins identified in this study are involved in the caffeine degradation pathway as well as in stress response, suggesting that stress proteins could be involved in caffeine metabolism in filamentous fungi.

Keywords Two-dimensional electrophoresis · Difference gel electrophoresis · Proteomics · Filamentous fungi · Caffeine degradation · Mass spectrometry

This paper is dedicated to the memory of Christopher Augur, esteemed colleague and friend.

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Introduction

Coffee is a major commodity for Latin American countries, which produce over 60% of the world's coffee. However, the by-products generated in the process result in severe environmental pollution. The major solid product is coffee pulp, generated after removal of the seed from the coffee berry. Coffee pulp is comparable to corn in total protein, and superior to it in calcium and phosphorus content. It should be possible, therefore, to use coffee pulp as a component of mixed livestock feed [2]. However, presence of caffeine in coffee pulp is known to be a limiting factor in the use of the pulp as animal feed [1, 2]. There is, therefore, a need for new technologies that can detoxify coffee pulp and, at the same time, improve the nutritional quality of the final product.

Microbial decaffeination shows tremendous potential. Attempts have been made to degrade caffeine present in coffee pulp and husk by solid-state fermentation using filamentous fungi species such as *Aspergillus*, *Penicillium*, *Rhizopus*, *Phanerochaete*, *Trichoderma* and *Fusarium* [3, 8, 28, 29, 33]. Variations in mineral nitrogen in the media culture have been shown to affect caffeine degradation by *Aspergillus* and *Penicillium* species [4, 14, 28].

The filamentous fungi species most studied and best characterized both physiologically and biochemically for caffeine degradation are *Penicillium* and *Aspergillus* [4, 11–14, 18]. The results from these studies show that in *Aspergillus* V12A25, the first three fungal enzymes of the caffeine metabolism are intracellular and cytoplasmatic [4, 11, 12]. To our knowledge, only one of the fungal enzymes from *Aspergillus tamarii* (theophylline demethylase) involved in caffeine degradation has been partially characterized [12].

Attempts made to purify caffeine demethylases to homogeneity have been unsuccessful, mainly due to their instability once extracted from the cell. Furthermore, it has been shown that the fungal enzymatic system involved in caffeine degradation is inducible by caffeine [4]. Previous studies suggest that caffeine metabolism is regulated by ammonium. In the presence of a simple nitrogen source (ammonium sulphate), caffeine degradation is inhibited. However, degradation of caffeine will resume once the simple nitrogen source had been consumed [11]. Therefore, analysing the *A. tamarii* proteome should reveal the identity of specific proteins regulated by caffeine, considering that from those studied fungi, *A. tamarri* is the most suited to caffeine degradation [14].

Proteomics has relied heavily on two-dimensional (2D) gel electrophoresis, since this is a long established technology. In 2D gel electrophoresis, large numbers of proteins present in biological extracts are separated according to their isoelectric point (p*I*) and molecular weight (*Mr*) [23]. Proteomic analysis using 2D gel electrophoresis is often criticized in part due to the time-consuming process of image analysis that is necessary to determine differential protein expression. This process can be laborious due to variations between gels.

Difference gel electrophoresis (DIGE) is a technique that allows co-separation and visualization of multiple samples on a 2D gel. DIGE technology has been applied successfully to identify differentially expressed proteins in bacteria [36, 39], yeast [17], mouse tissues [19, 30, 32, 34], cancer cells in human tissue [40], the visual cortex of cats [37, 38] and more recently in filamentous fungi.

In the present study, a proteome analysis was performed on *A. tamarii* proteins regulated by caffeine. To do so, intracellular proteins from caffeine-treated and ammonium-treated fungi were separated by fluorescence difference two-dimensional gel electrophoresis (2D-DIGE). In addition, differentially expressed proteins were analysed by mass spectrometry (MS), followed by database searching for identification.

Materials and Methods

Microorganism and Culture Media The *A. tamarii* V12A25 strain was obtained from the IRD-UAM (Institut de Recherche pour le Développement, France—Universidad Autónoma Metropolitana, Mexico) collection [28] and was selected because of its ability to grow in the presence of caffeine [13]. The strain was maintained on coffee infusion agar medium (CIAM) at 4 °C, as described by Denis [4]. CIAM medium was prepared as follows (g/L): a coffee infusion was prepared with ground coffee [33], and sucrose (2.0), KH_2PO_4 (1.3), $Na_2HPO_4{\cdot}7H_2O$ (0.19), $MgSO_4$ (0.15) and $CaCl_2$ (0.2) were dissolved in the coffee infusion. The pH was adjusted to 5.6 and the volume was brought to 1 L. Agar (20 g) was added and the medium was sterilized at 121 °C for 20 min [4, 11]. For inoculation, spores of 6-day-old cultures grown on CIAM were harvested with 0.1% (*v*/*v*) Tween 20 solution and 1×10^6 spores/mL were used to inoculate Fernbach culture flasks. Each flask contained 990 mL of defined synthetic medium as described elsewhere, containing caffeine (1 mM), and/or $(NH_4)_2SO_4$ (1 mM) as the nitrogen source [11]. The pH was adjusted to 4.0 with H_2SO_4. After sterilization at 121 °C for 15 min, 10 mL of filter sterilized trace element solution was added [4]. After 48 h of fermentation, a pulse of nitrogen source was added by sterile injection of either caffeine (15 mM) or ammonium sulphate (15 mM), to each flask. As described in Gutierrez-Sanchez et al. [11], sampling for protein extraction and caffeine-demethylase assay was carried out at/for 48 h when enzyme activity was maximal. Mycelium was filtered through Whatman filter paper No. 41. Mycelium was washed three times with physiological solution and dried at 60 °C to constant weight.

Caffeine-Demethylase Assay Caffeine-demethylase activity was performed in 1.5 mL Eppendorf vials at 30 °C. The reaction solution (1 mL) contained 50 mM phosphate buffer (pH 7.4), 0.2 mM caffeine and 1 mM DTT. The reaction was initiated by adding 560 μL of Crude Extract (CE), followed by vortexing. The reaction was stopped after 90 min with 25 μL of Trichloroacetic acid (TCA, 50%, *w*/*v*) and centrifuged at 12,000 rpm for 10 min at 4 °C. The supernatant was analysed by HPLC. One unit of activity was defined as the amount of enzyme required to transform 1 nmol of caffeine per min. Data are reported as the average of three separate experiments.

Caffeine Analysis Caffeine analysis was performed on a HPLC Waters Millennium (Milford, MA, USA) system with a 626 pump, a 717 plus auto sampler with 100 μL injection loop, a 600 s controller and a 996 PDA detector. Methylxanthine was separated on a Prodigy column (C18, 5 μm, 250×4.6 mm) from Phenomenex (Torrace, CA, USA) at room temperature with a flow rate of 1.5 mL/min by a gradient elution system. The mobile phase used was made up of 1.75 mM KH_2PO_4, acetonitrile and tetrahydrofuran (98:1:1; *v*/*v*/*v*) for pump A, and acetonitrile and tetrahydrofuran (99:1; *v*/*v*) for pump B. Samples were diluted in deionized water in order to obtain a concentration within the range of the standard curve.

Protein Extraction For 2-D DIGE analysis, mycelium was collected at the late exponential phase (t=48 h after induction). Protein extraction was performed at 4 °C. Mycelium was filtered through a Büchner funnel with a 20-μm mesh net and washed three times with 500 mL saline solution (0.9% *w*/*v* NaCl). Cells free of culture broth were then rinsed twice with 300 mL of 50 mM phosphate buffer, pH 7.7 (buffer A), and twice more with buffer A containing 0.1 mM dithiothreitol (DTT). Lysis of the resulting biomass (50 g wet weight) was performed with a 40-mL glass homogenizer (Tenbrock, Fisher Scientific, Pittsburgh, PA, USA) containing 15 mL of lysis buffer (buffer A and 10 mM DTT) and protease

inhibitor cocktail (Sigma, Cat. No. P8215, Saint Louis, Missouri, USA) as recommended by the manufacturer. The mixture was centrifuged at 17,600×*g* for 40 min at 4 °C, and the supernatant was filtered through a 45-μm filter (Millipore, USA) and dialyzed immediately against H_2O overnight. The sample was further treated with 20% TCA (*v*/*v*) on ice for 30 min in order to precipitate proteins. The precipitate was collected by ultracentrifugation at 17,500×*g* for 10 min at 4 °C. The precipitated proteins were washed with acetone to remove traces of TCA. Acetone was then removed by vacuum centrifugation. Precipitated proteins were resuspended in sample solubilization buffer (SSB; 30 mM Tris pH 8, 4% 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane sulfonate [CHAPS], 8 M urea) and then homogenized by passing through a 25-gauge needle 6 times. Insoluble material was removed by ultracentrifugation at 17,500×*g* for 5 min at 4 °C. The protein concentration of *A. tamarii* lysate was determined using the Bio-Rad Bradford protein assay dissolving bovine serum albumin in SSB for the standard curve and applying the manufacturer's recommended protocol (Bio-Rad, Hercules, CA, USA). Protein samples were dissolved in SSB to give stock solutions with a final concentration of 100 mg/mL, which were used to prepare experimental samples.

Protein Labelling with CyDye A. tamarii lysates were labelled using the *N*-hydroxy succinimidyl ester-derivatives of the cyanine dyes Cy3 and Cy5 developed for 2D-DIGE technology (Amersham Biosciences, Inc.) following the manufacturer's recommended protocols. Fifty microgrammes of each extract were minimally labelled with 200 pmol of either Cy3 or Cy5 (Amersham Biosciences, San Francisco, CA, USA) at 4 °C for 30 min in the dark. The labelling reaction was then quenched with 10 nmol lysine for 10 min on ice. Lysates from two separate time course experiments were run in parallel. Ammonium sulphate and caffeine lysates were labelled with Cy3 and Cy5 in both combinations.

2D-DIGE Separation and Image Analysis Prior to isoelectric focusing (IEF), Cy3-labelled and Cy5-labelled samples were mixed and added to 350 μL of rehydration sample buffer containing CHAPS 4%, urea 8 M, 0.2% (*v*/*v*) Bio-Lytes 3–10, 10 mM DTT and 1% bromophenol blue. IEF was performed using a PROTEAN IEF cell system from Bio-Rad. Immobilized linear pH gradient (IPG) strips (17 cm; pH 3–10) from Bio-Rad (Hercules, CA, USA) were rehydrated with Cy-labelled samples under passive conditions according to the manufacturer's guidelines. Strips were focused using focusing trays for a total of 50 kV h^{-1} at 20 °C. Briefly, the second dimension was carried out as follows: prior to running the second dimension, IPG strips were equilibrated with 10 mL equilibration buffer 1 from Bio-Rad (No 163–2107, Hercules, CA, USA) on a rocking table for 10 min, followed by 10 mL equilibration buffer 2 from Bio-Rad (No 163–2108, Hercules, CA, USA) for another 10 min. These strips were then loaded and run on 12% SDS polyacrylamide gels using an Ettan DALT 12 apparatus (Amersham Pharmacia Biotech Inc.) in conjunction with low fluorescence glass plates (18× 16 cm) and 1 mm thick × 1 cm wide spacers. Gels were run at 25 mA/gel for 10 min and then increased to 50 mA/gel until the bromophenol blue dye (Sigma) front ran off the bottom of the gel. Gels were fixed in 30% ethanol and 7.5% acetic acid overnight at room temperature. The Cy3-labelled images were collected at an excitation wavelength of 540 nm, whereas the Cy5-labelled gel images were collected at an excitation wavelength of 620 nm using the Typhoon 9400 imager (Amersham Biosciences, USA), converted to 16-bit TIF files and analysed using DeCyder software V4.0 (Amersham Biosciences, Inc.).

Protein Identification by Mass Spectrometry Identification of proteins by MS was performed as follows. After 2D gel electrophoresis and gel image analysis by DeCyder software, gels were stained with Sypro Ruby (Molecular Probes, Cat. No. S12000, Eugene,

OR, USA), destained in 10% methanol and 6% acetic acid for 30 min at room temperature, imaged and matched to the Cy images using DeCyder software. A list of spots to pick was created based on the above criteria. From the spots of interest, 2 mm gel plugs were picked, washed, digested with trypsin and the resulting peptides were extracted and spotted using the Spot Handling Workstation (Amersham Biosciences, Inc, USA.). Briefly, plugs were washed twice with 50 mM ammonium bicarbonate in 50% methanol for 20 min at room temperature. Plugs were washed with 75% acetonitrile for 20 min at room temperature and dried at 40 °C for 10 min. The plugs were then incubated with 140 ng of sequencing-grade trypsin (Promega) at 37 °C for 1 h. Peptides were extracted twice with 50% acetonitrile in 0.1% trifluoroacetic acid (TFA) for 20 min at room temperature. Approximately 25% of the resulting peptide mixture was spotted onto the MALDI sample target plate with a saturated solution of α-cyano-4-hydroxy-cinnamic acid (Sigma, Cat. No. 70990). MS and MS/MS data were acquired on a 4700 Proteomics Analyzer (Applied Biosystems, CA, USA) using standard acquisition methods. MS spectra were internally calibrated using two trypsin autolysis peaks (1,045.5 and 2,211.1 *m*/*z*). MS/MS spectra were calibrated using the instrument default processing method.

Peak lists were submitted to NCBInr (http://www.ncbi.nlm.nih.gov/BLAST/) and SwissProt (http://www.expasy.org/sprot/) using Mascot (MatrixScience, London, UK; http://www.matrixscience.com; Fungi Taxonomy used).

Capillary LC/MS/MS The tryptic digested samples of 2D gel protein spots were introduced into the Quadrupole time-of-flight (Q-TOF) 2 (Micromass, Manchester, UK) mass spectrometer using a Waters CapLC (Waters Corporation, MA, USA). The original CapLC solvent delivery configuration was modified by bypassing the UV cell to minimize the dead volume between the mixer and the nanocapillary column. The tryptic peptides were acidified with 0.1% formic acid and loaded onto a 75 μm×15 cm C18 column (LC Packings) at a flow rate of 180 nL/min. The mobile phases used for gradient elution consisted of water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). The linear gradient conditions used to elute the peptides were 10% B to 70% B over 70 min and a flow rate of 180 nL/min. The Q-TOF 2 was operated in a data-dependent acquisition mode. The survey MS spectra were acquired from 450 to 1,700 Da and the switch criteria for MS to MS/MS mode was an ion count of 5. The Q-TOF was set to ignore singly charged ions and acquire MS/MS data for up to three co-eluting peptides. Collision energy varied depending on the peptide mass and charge state.

The MS/MS spectra obtained from the gel spots were processed into a peak list with Masslynx software and searched against databases (NCBInr, dbEST; http://www.ncbi.nlm.nih.gov) to identify proteins using the MASCOT search engine (MatrixScience, London, UK; http://www.matrixscience.com; Fungi Taxonomy used). Database searches were performed with the following parameters: peptides were derived from enzymatic cleavage with trypsin and one missed cleavage was allowed; no modifications were allowed and peptide Mw tolerance was 200 ppm. Proteins were considered identified if their candidate peptides matched with probability-based Mowse scores that exceeded the predetermined threshold ($p<0.05$).

Results

A. tamarii is a filamentous fungus isolated from coffee-growing areas of Mexico [27]. The strain is able to grow in the presence of caffeine. When *A. tamarii* is grown on a synthetic medium with caffeine as the sole nitrogen source, a long adaptation period or lag phase is observed prior to efficient caffeine degradation [11].

We were interested in studying protein expression during caffeine degradation by *A. tamarii*. Of particular interest was the time point at which caffeine degrading enzymes were present. Therefore, we used 2D-DIGE to study *A. tamarii* intracellular proteins induced or repressed by caffeine as the sole nitrogen source. In the present study, a two-stage fermentation process was used [11]. During the first stage, *A. tamarii* was grown in the presence of a low concentration of simple nitrogen source (ammonium sulphate) and caffeine to reduce the lag phase and enhance caffeine degradation within the second stage. The second stage was the induction stage, adding either a pulse of caffeine or ammonium sulphate. We assayed enzyme activity for caffeine demethylase in both caffeine-treated and non-caffeine-treated intracellular extracts, and observed that caffeine demethylase activity was present only in caffeine-treated extracts (Fig. 1).

Changes in Protein Expression Comparison of protein expression patterns between ammonium sulphate-treated (Cy3) and caffeine-treated (Cy5) *A. tamarii* intracellular proteins was analysed using DeCyder software V4.0 (Amersham Bioscience, Inc.) setting a threshold of 1.5. The two samples were analysed simultaneously in two gels by testing the reproducibility of Cy dye labelling. The same sample (ammonium sulphate-treated protein extract or caffeine-treated protein extract) was "forward" labelled (ammonium sulphate-treated labelled with Cy3 and caffeine-treated with Cy5), and the mixed labelled samples were run on the same gels. We were able to detect the same differences in the expression of particular proteins from gel to gel (data not shown). These differences were also detectable when the sample–dye combinations were "reversed" labelled (data not shown). Taken together, these results are in agreement with the conclusions drawn from the more rigorous validation of the technique carried out by Tonge et al. [34] and show that this technique is both sensitive and reproducible.

The spot detection by the Decyder software resulted in the detection of 532 spots in both treatments, and this gel was set as the master gel for this analysis. After matching the protein patterns obtained from the gels, spots were filtered according to their average ratio and significance (*t* test). At first, 85 spots were selected by setting filter parameters for an averaged ratio to greater than 1.5 or smaller than −1.5, and for *p* values smaller than 0.05. According to their average ratio, 48 (9%) spots demonstrated up-regulation upon ammonium

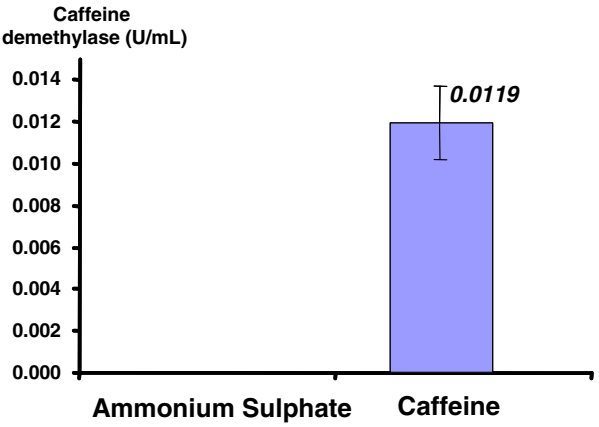


Fig. 1 Caffeine demethylase activity in both caffeine-treated and non-caffeine-treated intracellular extracts of *Aspergillus tamarii* at 48 h of culture

treatment and 37 (7%) up-regulation upon caffeine treatment. Values such as spot volumes (expressed with background subtraction) and spot ratios (indicating the change in spot volume between two images) were calculated. Figure 2 shows examples of the image analysis after Decyder software simulation.

Once the Cy3 and Cy5 images were obtained, the same gel was stained with Sypro Ruby, and the 85 filtered spots showing differences in the software analysis were assigned to corresponding spots on Sypro Ruby stained gels (Fig. 3) for further analysis by MS. The protein spots were spread out over a range of p*I* between 4.8 and 6.4. Most of the differently expressed proteins exhibited molecular masses smaller that 75 kDa and were detected at p*I* 4.5–6.8 (Fig. 3).

Identification of Down-Regulated or Up-Regulated Proteins The spot numbers in Fig. 3 indicate the proteins analysed by MS analysis using TOF/TOF or Q-TOF-MSMS followed by database searching and are listed in Table 1. Nine proteins were identified with confidence (Table 1); among these, one was down-regulated (pectate lyase A) with caffeine treatment and six were up-regulated (phospoglycerate kinase, two malate dehydrogenase, dyp-type peroxidase family protein, hypothetical protein, Cu,Zn superoxide dismutase SOD1, heat shock protein 30 and a probable xanthine dehydrogenase). When the number of matching peptides or sequence coverage was low, the information was not sufficient for protein identification. For those samples, de novo sequencing was conducted using PEAKS Studio V4.2 software, and three putative peptide sequences were obtained (two peptides for spot 9 and one peptide for spot 12). PEAKS performs de novo sequencing directly from the MS/MS data and therefore does not rely on a protein database; it computes the best possible sequence among all possible amino acid combinations and outputs positional confidence scores, which reliably determine which sequence or amino acids are correct [21]. Peptide sequences identified with PEAKS underwent a Blastp search in an attempt to identify these proteins.

Discussion

In this study, we present the first attempt to identify intracellular proteins in *A. tamarii* regulated by caffeine using 2D-DIGE and MS. The proteins produced in the presence of

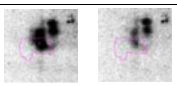
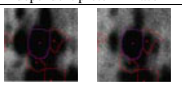
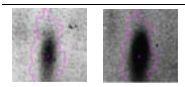
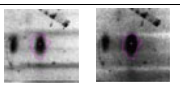
	Proteins resulting from ammonium sulfate treatment		**Proteins resulting from caffeine treatment**	
	Down-regulated		**Up-regulated**	
Spot identification	**Spot 1** pectate lyase A	**Spot 8** Putative cyclic AMP-dependent protein kinase	**Spot 13** Unknown	**Spot 7** Cu, Zn SOD
Zoom on gel				
3D view				
Fold change ratio	-2.10	-2.47	2.26	1.92

Fig. 2 Comparison of protein expression between ammonium sulphate and caffeine treatment using Decyder sofware, setting a threshold of 1.5. Example of proteins resulting from ammonium sulphate treatment (*left panel*) and caffeine treatment (*right panel*) are shown. The *upper panel* shows the images of the protein spots in the 2D gel. 3D simulation view of protein spots is shown in *lower panel* and their calculated spot value and volume ratio

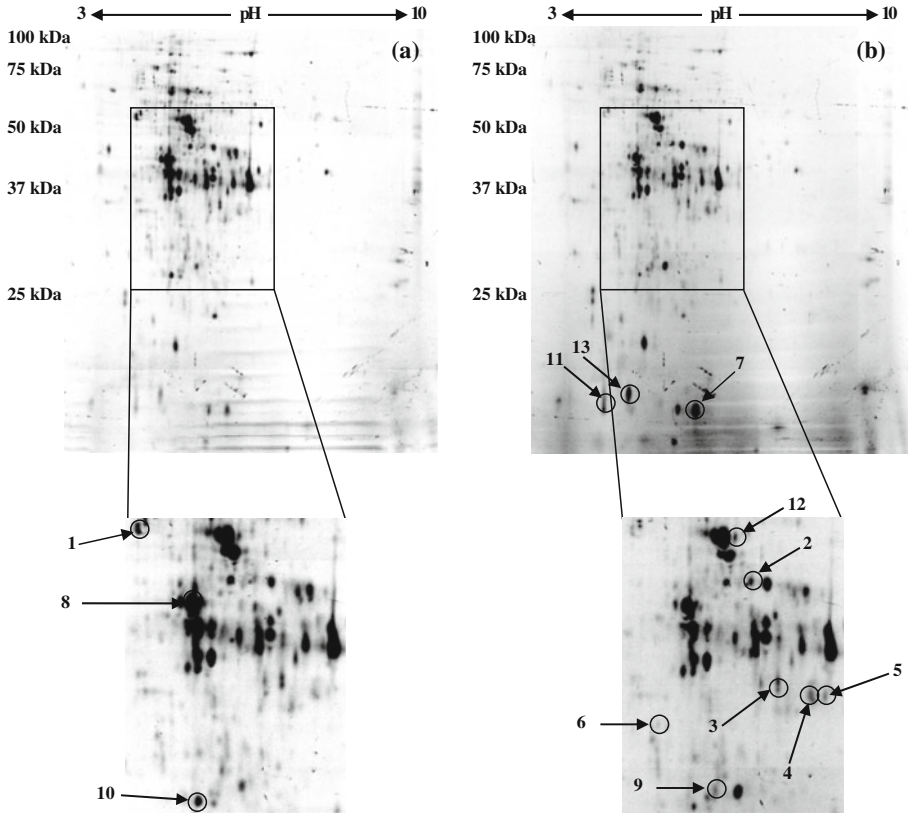


Fig. 3 2D-DIGE gel images from *A. tamarii* intracellular proteins. **a** Cy3 image of ammonium sulfate-treated proteins. **b** Cy5 image of caffeine-treated proteins

ammonium sulphate or caffeine as a nitrogen source were extracted in order to identify down-regulated or up-regulated proteins under these conditions.

Unfortunately, the genome of *A. tamarii* has not as yet been sequenced. In the NCBI protein database, only 41 protein sequences from *A. tamarii* are present and none of these are enzymes involved in nitrogen metabolism, and no fungal proteins involved on caffeine metabolism have been purified. This is a limiting factor in the search for functions to the differentially expressed proteins studied. When a genome is not complete or has not yet been sequenced, de novo sequencing must be carried out in order to obtain sequences or partial sequences. For the deduction of protein sequences from MS/MS data, we used software called PEAKS. These new sequences obtained present homology in the databases searched to a heat shock protein 30 and to a probable xanthine dehydrogenase (Table 1).

Down-Regulated Proteins One protein was identified as regulated after caffeine treatment. After database searching, spot one showed homology to a pectate lyase. The enzyme is produced by phytopathogenic fungi to penetrate carbohydrate barriers associated with host plant cell walls. A previous analysis of an inducible pectate lyase gene from *Fusarium solani* showed that when the fungus was grown in a simple carbon source, the transcription of the lyase gene was not detected nor was lyase activity found [9]. This suggests a similarity

Table 1 Identification of intracellular proteins spots from *A. tamarii* regulated by caffeine with a significant 1.5-fold higher or lower protein amount in caffeine-treatment samples

Spot[a]	Abundance	Volume Ratio	Putative function	Species	Gene Id	Peptide sequence	Score	% prot. covered	Identification method
1	Decrease	−2.1	Pectate lyase A	*Aspergillus niger*	GI:145256261	DGAQLLVESNQFVDSK	34	4	LC-MSMS
1	Decrease	−2.1	Sterigmatocystin biosynthesis lipase/esterase StcI	*Aspergillus nidulans*	GI:67901658	SFFDCYGAPPDDPR	18	5	
2	Increase	1.52	Phosphoglycerate kinase	*A. oryzae*	GI:1172456	ATGGQIILLENLR	76	18	MALDIMS
						GLTALGDVYINDAFGTAHR			
						ALESPQRPFLAILGGAK			
						VSDKIQLIDNLLPK			
						IGNSLFDEAGSK			
3	Increase	1.91	Malate dehydrogenase	*A. flavus*	GI:220700638	DDLFNTNASIVR	44	3	LC-MSMS
4	Increase	2.16	Dyp-type peroxidase family protein	*A. flavus*	GI:220700186	GVVEPTFVDSPLYK	66	4	LC-MSMS
5	Increase	1.61	Malate dehydrogenase	*Penicillum.marneffei*	GI:212537107	ILPVGQVNAYEEK	36	20	LC-MSMS
						DDLFNTNASIVR	45		
						GVVEPTFVDSPLYK	42		
						VSELALYDIR	53		
						GSEIVLIPAGVPR	36		
6	Increase	2.26	Hypothetical protein	*A. oryzae*	GI:169772123	IGYTVGVEPAPK	73	6	LC-MSMS
7	Increase	1.92	Cu, Zn superoxide dismutase SOD1	*A. terreus*	GI:11540026	LIGAESVLGR	46	23	MALDIMS
						SEHPESKK			
						HVGDLGNFK			
						TDAEGNAVGSK			
						EHGAPEDENR			
						TLVIHAGTDDLGR			
9	Increase	2.12	Heat shock protein 30	*Aspergillus oryzae*	GI:38564191	SVTAGDFSPLFR	81%	10	LC-MSMS
						LSLVVPK			

Table 1 (continued)

Spot[a]	Abundance	Volume Ratio	Putative function	Species	Gene Id	Peptide sequence	Score	% prot. covered	Identification method
12	Increase	1.61	Probable xanthine dehydrogenase protein	Sinorhizobium medicae	GI:67901658	VDGLTLAAGVGHR	46%	3	LC-MSMS

Identification was done by LC/MS/MS and MS/MS/MS. The percentage of protein covered and corresponding Mascot score are listed below. Volume ratio shows the fold difference between the ammonium sulphate treatment and caffeine treatment. Putative peptide sequence derived after the de novo sequencing of differentially expressed proteins in *A. tamarii*. The percentage of protein covered and corresponding Mascot score are listed below

GI number of NCBInr database

[a] Spot numbers correspond to gels in Fig. 4

between the *A. tamarii* pectate lyase found in this study and the *F. solani* enzyme. In *Colletotrichum gloeosporioides*, pectate lyase secretion was not observed at pH 4.0. However, pectate lyase PL secretion was observed only when the pH was 6.0 or more and nitrogen, either inorganic or organic, was present [6].

Up-Regulated Proteins Among the up-regulated proteins, one protein with function in glycolysis (phosphoglycerate kinase, PGK) was identified. The synthesis of some glycolytic enzymes and respiratory proteins has been found to increase during heat shock. In yeast, PGK was identified as one of a very small number of proteins that was made as efficiently as Hsps after a heat shock [26]. It has also been reported that caffeine elevated ATP levels and the energy charge of *Saccharomyces cerevisiae* [35]. The over expression of this glycolytic enzyme may help cells continue to generate sufficient ATP for essential metabolic processes that could be related with caffeine degradation.

Malate dehydrogenase (MDH) was another protein found to be up-regulated by caffeine. In the cytosol, NADH efficiently reduces oxaloacetate to malate via cytosolic MDH. This MDH is transported into mitochondria by the α-ketoglutarate/malate antiporter. Inside the mitochondria, MDH catalyzes the oxidation of the hydroxyl group of malate into ketone to generate oxaloacetate and complete the cycle around the citric acid cycle. This enzyme is a key in the TCA cycle and could be overexpressed in order to supply NADH to the electron-transport chain or to provide substrate for biosynthesis reactions. In addition, it has been shown that caffeine significantly increased the levels of MDH in rat myotubes [20].

The expression of some stress-related proteins was found to be increased during caffeine-induced growth. One of these proteins, Spot number 9, presented significant homology to a Hsp30. Hsps, also called *stress proteins*, are a group of proteins present in all cells of any organism. Proteins, such as Hsps, Cu, Zn and SODs, are induced when a cell undergoes any type of environmental stress like heat, cold and oxygen deprivation [27]. These enzymes are, therefore, important in protecting the cell against oxidative stress. Two of the identified proteins (Hsps and SOD), which are involved in protein synthesis/folding, have been reported to be induced when cells undergo any type of environmental stress such as heat, cold and oxygen deprivation [7, 15, 16, 24, 25]. In addition, it has been shown that caffeine ingestion for 22–30 days increased the activity of SOD in mice [22].

In filamentous fungi, caffeine is converted into xanthine by caffeine, theophylline and 3-methylxanthine demethylase [13, 18, 31]. Xanthine dehydrogenase converts xanthine into uric acid [18]. All of these reactions generate hydroxy radicals (H_2O_2). Similar radicals are also generated when allantoic acid is degraded to uric acid (uric acid oxidase), generating highly toxic metabolites of molecular oxygen. SOD transforms superoxide to hydrogen peroxide and dioxygen, and the conversion of superoxide to peroxide minimizes production of hydroxy radicals, the most potent of the oxygen free radicals [5]. It is interesting to note that some of the enzymes identified in this study correspond to the xanthine degradation pathway (Cu,Zn, SOD and xanthine dehydrogenase) and stress response (Hsp30). Despite the size of the current database, enzymes in filamentous fungi corresponding to the first steps of caffeine demethylation have not yet been found, although this does not mean they cannot be present under conditions carried out in this work. Since caffeine demethylase from *A. tamarii* or any other species of *Aspergillus* or fungi has not been sequenced, it makes their identification by mass spectrometry more difficult. Moreover, caffeine is produced by plants to protect against predators and is considered a growth inhibitor for bacteria and fungi. Spots 7 and 9 could, therefore, be stress proteins responding to caffeine. Further work is required to study the regulation pattern of these proteins after caffeine treatment.

Initial studies related to the purification of caffeine demethylase showed that it has a p*I* around 4.9 and 6.1 and a molecular weight around 30,000 Da [10]. When comparing with the up-regulated proteins present in 2D gels (Fig. 3), spots 4 and 5 seem to be the best candidates. However, further characterization studies will need to be performed to corroborate this hypothesis.

From these results we may conclude that besides PGK and MDH, stress proteins might play a vital role in caffeine degradation. Taking into account these data, the hypothesis that Hsp30, SOD and a probable xanthine dehydrogenase are involved in caffeine metabolism in filamentous fungi could be raised.

This work indicates that despite the lack of sequence knowledge of the *A. tamarii* genome, a proteome analysis can provide information on responses induced during filamentous fungi growth in different nitrogen sources. There is no doubt that as the gene and protein databases grow the ability of the search programs that use MS data will also grow proportionally. Therefore, the data collected in this study could be re-analysed in the future when the *A. tamarii* genome is more complete. It is clear that this first-pass screen has raised more biological questions, but it also opens up new avenues to explore the regulation by caffeine of specific fungal proteins. Present work is focused on the purification of caffeine demethylases in order to better characterize the caffeine response in *A. tamarii*.

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