Appl Microbiol Biotechnol (2010) 86:535–543
DOI 10.1007/s00253-009-2279-y

BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Factors affecting production and stability of the AcAFP antifungal peptide secreted by *Aspergillus clavatus*

Houda Skouri-Gargouri · Neila Jellouli-Chaker · Ali Gargouri

Received: 21 April 2009 / Revised: 24 September 2009 / Accepted: 25 September 2009 / Published online: 15 October 2009

Abstract We have previously reported the identification of a small, basic and cysteine-rich antifungal peptide (AcAFP) secreted by *Aspergillus clavatus* and shown its ability to prevent growth of various human- and plant-pathogenic filamentous fungi. In this study, we sought to determine the physiological/microbiological requirements to enhance the AcAFP production and the conditions influencing its stability. The maximum of AcAFP production was obtained when *A. clavatus* was grown on 2% glycerol as sole carbon source and 100 mM NaCl. The AcAFP expression was shown to be influenced by pH, being suppressed under acidic (pH 5) and strongly induced under alkaline conditions. The activity of the purified AcAFP was not affected by temperature; it loosed approximately 20% of its activity after 3 h at 100°C and was efficient through a large pH range (pH 5-12) with an optimum at pH 8. AcAFP activity decreased at high ionic strength and in the presence of 10 mM of divalent cations (Mn^{2+}, Fe^{2+} and Ca^{2+}).

Keywords *Aspergillus clavatus* · Antifungal activity · Salt effect · Thermostability · Carbon sources

Introduction

The risk of opportunistic infections is greatly increasing in immuno-compromised patients due to cancer chemotherapy, organ or bone marrow transplantation and in human immunodeficiency virus infections (Hoffman et al. 2000). Moreover, microorganisms became more and more resistant to drugs as far as chemical compounds are extensively adopted in current farming practices to protect crops against diseases. Due to the excessive use of the conventional chemical agents which led the development of resistant pathogen strains and the appearance of some side effects on users, new antifungal substances are in great demand for the combat of pathogenic fungi. The ultimate aim of new antifungal production strategies is to obtain novel drugs that combine high efficacies with a narrow susceptibility range, restricted toxicity and low cost of production.

During the last two decades, a wide range of antifungal peptides with different structural features have been isolated and characterised. They have been produced from different organisms, ranging from bacteria to humans, either constitutively or upon induction (Theis and Stahl 2004; Baker et al. 1997; Anderson and Yu 2005). In prokaryotes and lower eukaryotes, antifungal peptides might confer an ecological advantage for the producer organism in the competition for nutrients. In higher eukaryotes, these peptides are part of the innate immune system and are the first defence line against invading micro-organisms (Fritig et al. 1999; Lehrer and Ganz 1999; Raj and Dentino 2002). Among the large number of antifungal peptides, small peptides from imperfect filamentous fungi, endowed with potent antifungal activity, are more recently described (Marx 2004; Marx et al. 2008; Meyer 2008). The filamentous ascomycetes *Aspergillus giganteus*, *A. niger*, *Penicillium chrysogenum* and *P. nalgivense* secrete homologous antifungal peptides called AFP, AnAFP, PAF and NAF, respectively (Wnendt et al. 1994; Gun Lee et al. 1999; Marx et al. 1995; Geison 2000). Gene regulation and expression profile studies at different environmental conditions were limited to AFP and PAF peptides. Transcription of the *afp* gene was shown to be neither under the control of carbon catabolite repression nor regulated by nitrogen metabolite repression (Meyer et

H. Skouri-Gargouri · N. Jellouli-Chaker · A. Gargouri (✉)
Laboratoire de Génétique Moléculaire des Eucaryotes,
Centre de Biotechnologie de Sfax, BP «K» 3038, Sfax, Tunisia
e-mail: faouzi.gargouri@cbs.rnrt.tn

al. 2002). However, it is highly expressed under different stress conditions such as alkaline pH, salt stress, heat shock, carbon starvation and during co-cultivation with other fungi. In contrast, transcription is strongly inhibited under acidic pH and phosphate excess (Meyer and Stahl 2002; Meyer et al. 2002; Meyer and Stahl 2003; Meyer 2008). The transcription of *paf* seems to be under control of carbon catabolite repression and nitrogen metabolite repression (Marx et al. 1995), while it is not affected by heat shock or phosphate concentrations in the culture medium (Marx 2004). *afp* and *paf* genes reach their maximum expression level during the stationary growth phase. In addition, it seems that stress conditions have in general a positive impact on *afp* and *paf* expression (Meyer et al. 2002; Marx 2004; Marx et al. 2008; Meyer 2008).

We reported previously that the local strain named VR1 of the filamentous fungus *A. clavatus* abundantly secretes a thermostable, basic and amphitatic peptide named AcAFP, with a low molecular weight (5,773 Da) and a promising range of antifungal activity (Skouri-Gargouri and Gargouri 2008). This AcAFP peptide inhibited the growth of several filamentous fungi such as *A. niger* and *Fusarium oxysporum*. The last species is a phyto- pathogen responsible for wilt and cortical rot diseases of many economically important plant hosts (cereals, cotton and olive). The *F. oxysporum* f. sp. *albedinis* strain causes a vascular wilt of date palm known as Bayoudh disease that devastated a great number of palm plantations in North Africa and California (Fernandez et al. 1998). Therefore, due to its potent antifungal activity, the AcAFP exhibited a number of promising properties that make it a potentially appealing option for future application in biotechnological fields, particularly for the alleviation of current concerns pertaining to fungal infection and contamination.

The present study was then undertaken to gather information on the factors effecting the expression, activity and stability of AcAFP. Its production was assessed under various culture conditions, including the presence of different carbon sources, different salts, and different pH values. Several experimental conditions were carried out to determine the stability of AcAFP peptide towards heat treatment, pH variation, salt stress and metal ions.

Materials and methods

Microorganisms

A local fungal strain (named VR1) that we have previously isolated from a contaminated culture broth in our laboratory was identified by the Central Bureau voor Schimmelculture (CBS; the Netherlands) as *A. clavatus* and deposited in the collection of the "Centre de Biotechnologie de Sfax" as CTM 10.520 (Skouri-Gargouri and Gargouri 2008). It was used as the AcAFP producer strain while the *F. oxysporum* strain CTM:10.402 was used as the phytopathogenic target of the AcAFP activity.

Both strains were propagated on potato-dextrose-agar (PDA) plates and incubated at 30°C. For the preparation of the conidia of *F. oxysporum* and *A. clavatus*, the fungal cultures were transferred to Petri dishes that contained PDA and were allowed to grow at 30°C for 10 days; conidia were scraped, suspended in 1% tween solution and filtered through cotton. Spores were quantified, concentrated and stocked frozen in 20% glycerol at 10^9 spores ml^{-1}.

Culture media and conditions

During the investigations of culture conditions, growth was carried out in a basic medium containing 1% yeast extract, 0.5% pastone (pH 7) and gradually supplemented with various ingredients including several carbon sources (colloidal chitin, starch, glucose, sucrose and glycerol) and inorganic salts (sodium chloride and potassium chloride) at different concentrations. The resultant media (50 ml) were inoculated with spores of *A. clavatus* (10^6 spores ml^{-1}) in a 500 ml Erlenmeyer flask and then cultured for 120 h at 30°C on a rotary shaker (150 rpm). The culture supernatant was isolated through filtering by four layers of cheesecloth and then centrifuged for 20 min at 8,000 rpm.

Protein purification

The purification of AcAFP peptide from the culture supernatant of *A. clavatus* was performed as previously described (Skouri-Gargouri and Gargouri 2008). After 96 h of culture, thermo-labile and insoluble materials were removed by incubation of the supernatant at 70°C for 20 min followed by centrifugation at 6,000 rpm for 20 min. The pooled fraction was concentrated using centricon 3 kDa (Millipore) and finally purified by reverse-phase high-performance liquid chromatography (RP-HPLC; Agilent 1100 series; USA) on C18 column (μ-Bondpack C18: 3,000×4.6 mm; Waters USA) equilibrated with aqueous 0.05% trifluoroacetic acid (TFA). Elution was carried out using a linear gradient of 0–80% acetonitrile in aqueous 0.05% TFA at flow-rate of 0.5 ml min^{-1} (Skouri-Gargouri and Gargouri 2008).

Antifungal activity assay

The antifungal activity assay was conducted by an agar diffusion test. Two methods were compared: the use of antibiotic discs or wells made in agar. In both cases, the conidia of the target fungus *F. oxysporum* were spread out on PDA plates at a density of 10^6 spores ml^{-1} (30 μl of

spores stock per plate). Serially diluted-peptide solutions were deposited either on the antibiotic discs (0.65 mm) or in the wells made in the plates that contained the test fungus. The same peptide concentrations were used for both tests with several levels ranging from 3 μg to 100 μg ml^{-1}. Each sample volume was adjusted to 100 μl with sterilised Milli-Q water. The plates were incubated for 72 h at 30°C. Assays were performed in triplicate, and the inhibitory effect of the peptide was estimated by measuring the diameter of the inhibition zone of fungal growth minus the diameter size of the well or antibiotic disc (Ekengren and Hultmark 1999). The clear zone diameter around the antibiotic disc or around the well made in the agar plate was carefully measured with a Digimatic caliper (Mitutoyo series 500. Japan) to the nearest 0.1 mm.

Protein estimation and polyacrylamide gel electrophoresis

Protein concentration was determined using the method of Bradford (1976) with BSA as a standard. Analytical sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli and Favre (1973), the polyacrylamide concentration in the separating gel was 15% (*w*/*v*). Proteins were stained with Coomassie Brillant Blue R 250 (Sigma).

Effect of carbon source on the AcAFP expression

To study the effect of carbon sources on the expression of the antifungal peptide, the fungus was grown on a basic medium as described above. Several carbon sources were added separately to the defined medium, i.e. glucose, sucrose, colloidal chitin, starch and glycerol, at two different concentrations 1% and 2%. Antifungal activity, protein yield, pH variation and protein visualisation on the SDS-PAGE were determined for each set of conditions.

Effect of salt on the AcAFP production

Two different salts, NaCl and KCl, were added apart to the culture medium in a variety of concentrations (0.1, 0.5 and 1 M). Their effect on antifungal peptide production was investigated.

Effect of the pH on the AcAFP production

The optimal pH for AcAFP production was determined by measuring the antifungal activity in the crude culture supernatants obtained from various culture media according to their initial pH. The buffer systems used were equally set at 100 mM of sodium-acetate to get a pH value of 5; phosphate KH_2PO_4/K_2HPO_4 for a pH value of 7, and glycin-NaOH for a pH value of 9.

Effect of the pH on the AcAFP stability

The pH stability of AcAFP was evaluated by incubation of the purified peptide for 24 h at room temperature at pH ranging from 5 to 12 using different 100 mM buffers: sodium-citrate for pH 5; phosphate (KH_2PO_4/K_2HPO_4) for pH 6 and 7; Tris-HCl for pH 8, and glycin-NaOH for pH 9, 10, 11 and 12.

Effect of the temperature on the AcAFP stability

The AcAFP thermal stability was studied by heating the purified protein at 100°C for 1, 2 and 3 h in the same buffer: 10 mM Tris-HCl pH 8. The residual activities were then determined. AcAFP was used at a concentration of 100 μg ml^{-1}, standard deviation (±SD) of three independent experiments are given.

Effect of metal ions on the AcAFP stability

The effect of divalent cations on AcAFP stability was tested by measuring the growth inhibition of the target fungus. The ionic solutions used in this study were prepared in distilled water: $BaCl_2$, $CaCl_2$, $ZnCl_2$, $CoSO_4$, $CuSO_4$, $FeSO_4$, $MgSO_4$, $MnSO_4$, and used for the antifungal activity at 5 or 10 mM. For each ion, two tests were made on the test agar plate: one test for the purified AcAFP and the ion in front of a control containing only the ion.

Statistical analysis

Data were submitted to statistical analysis using the Statistical Package for the Social Sciences (SPSS) software version 11.0 for Windows (SPSS Inc., Chicago, USA). Classical descriptors such as mean and maximum and minimum values were determined. The correlation matrix was calculated for all variables. ANOVA was performed for detecting differences among and between all parameters.

Results

Evaluation of the antifungal activity

Antifungal activity, determined by inhibition zone assay, was assessed by two different methods in order to standardize the measurement of the AcAFP activity (Fig. 1a). The well agar diffusion method seemed to be more sensitive since it allowed the detection of low concentrations of the antifungal peptide that were not detectable by the antibiotic disc diffusion method. Moreover, the determined experimental value of the minimal inhibition concentration (MIC): 3.4 μg ml^{-1} was found to

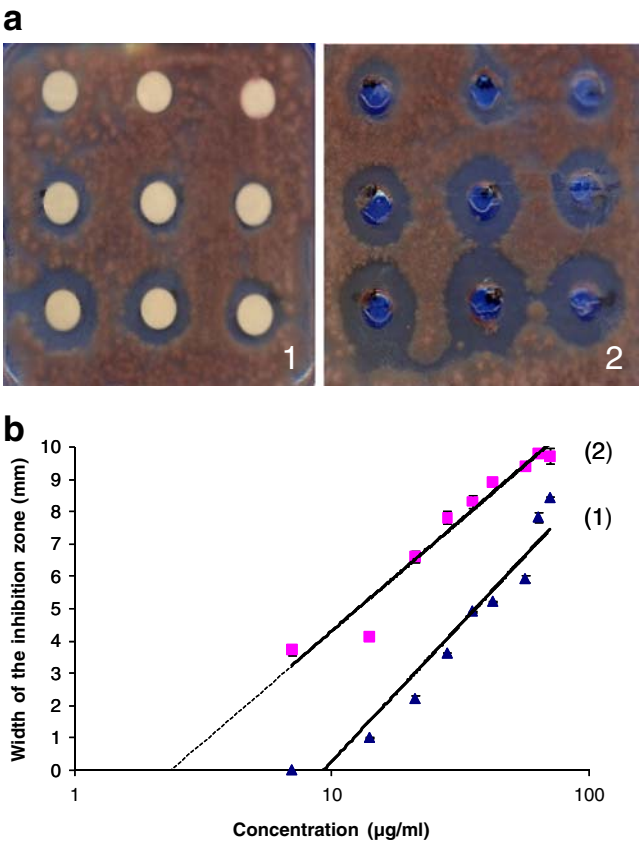

Fig. 1 Antifungal activity of AcAFP against *F. oxysporum*. **a** Antifungal activity assay using antibiotic discs (*1*) and wells in agar (*2*). AcAFP was used at a concentration of 3–100 µg ml^{-1}. **b** Plot of the antifungal activity against AcAFP concentration, method 1 (*filled triangle*) and method 2 (*filled diamond*). The width of the inhibition zone corresponds to the size (diameter) of the clear zone (millimetre) measured for each sample concentration minus the size of the central well/disc. Standard deviations (±SD) of three independent experiments are given

perfectly match with the "theoretical value" obtained by the projection of the plot of the second method on abscises axe (Fig. 1b). Interestingly, the diffusion test seems to be relevant to estimate the AcAFP peptide's antifungal activity since we observed a linear relationship between antifungal activity and the tested AcAFP concentrations. This proportionality is probably due to the AcAFP's small size which does not seem to disturb its diffusion properties into the agar.

Effect of carbon sources on the AcAFP production

Strain VR1 was cultured in basic media containing colloidal chitin, starch, glucose, sucrose or glycerol at 1% (*w*/*v*; *v*/*v*). A negative control medium without any additional carbon source was also used. Culture supernatants were used for the evaluation of the AcAFP production by measurement of the inhibition growth zones since the purification procedure (under different experimental culture conditions) led to the identification of only one single peptide presenting the described antifungal activity. By assessing the AcAFP production and total extracellular proteins, we found that the absence of added carbon source in the culture medium significantly decreased the concentration of the extra cellular proteins and the antifungal activity in the culture supernatants (Table 1). A slight increase of activity was observed when 1% of colloidal chitin was added, although the levels of total secreted proteins decreased (Table 1). A good growth with important release of extracellular proteins but low antifungal activity was observed on the media supplemented with sucrose (Table 1). Compared with colloidal chitin and sucrose, both starch and glucose had a significant positive effect on AcAFP production, which was enhanced by about four and five times, respectively. Interestingly, glycerol yielded the highest antifungal expression and activity, approximately two times higher than that obtained by glucose or starch (Table 1). The slight variation in biomass, quantified in each condition (Table 1), did not explain the variation in production of the antifungal activity. For instance, the biomass produced on sucrose is equal to that obtained on glycerol (9 g/l in both conditions) whereas the inhibition growth zone reached 0.49 mm (±0.07) on sucrose and 2.92 mm (±0.58) on glycerol. Thus, these results suggest firmly that the expression of the AcAFP is regulated by the carbon source administrated.

In a second step, the effect of the carbon source concentration on the AcAFP expression and production was investigated by raising the concentration of glycerol, glucose and sucrose up to 2% in culture media. Our data indicate that while antifungal activity was inhibited in the presence of sucrose, it was slightly increased with glucose and highly enhanced with glycerol (Fig. 2a). SDS-PAGE analysis confirmed the overproduction of AcAFP peptide in the presence of 2% glycerol (Fig. 2b).

Time course of AcAFP production

During the incubation process, the antagonistic activities against *F. oxysporum* were measured every 24 h for a total period of 120 h. When grown on glycerol, the antifungal activity was detected within 48 h of culture, reached a maximum at 96 h and dropped gradually afterwards (Fig. 3). However, when grown on glucose, the antifungal activity was detected after 72 h, showed a maximal activity after 120 h of culture (Fig. 3) and remained stable during the following 10 days of culture (data not shown). The maximal activity reached on glucose, nevertheless, remained lower than that reached on the glycerol medium.

Table 1 Effect of different C-sources (1% *w/v*) in basic media on the production of secreted proteins by *A. clavatus* VR1, pH variation and growth inhibition (gI)

Carbon source	Proteins (μg ml^{-1})	Biomass (g l^{-1})	gI (mm)[a]	pH of the culture
Absence	10.6±0.12	7.5	0.2±0.05	9
Colloidal chitin	8.25±0.14	6	0.43±0.04	6
Starch	21.7±0.21	7	1.13±0.09	7.4
Glucose	47.5±0.11	12	1.52±0.1	7.5
Sucrose	54.35±0.3	9	0.49±0.07	8
Glycerol	56.25±0.12	9	2.92±0.58	8

All cultures were incubated for 96 h and all antifungal assays were done with 200 μl of each set of culture conditions. Each value represents the means of three independent experiments

[a] The diameter of the inhibition growth zone

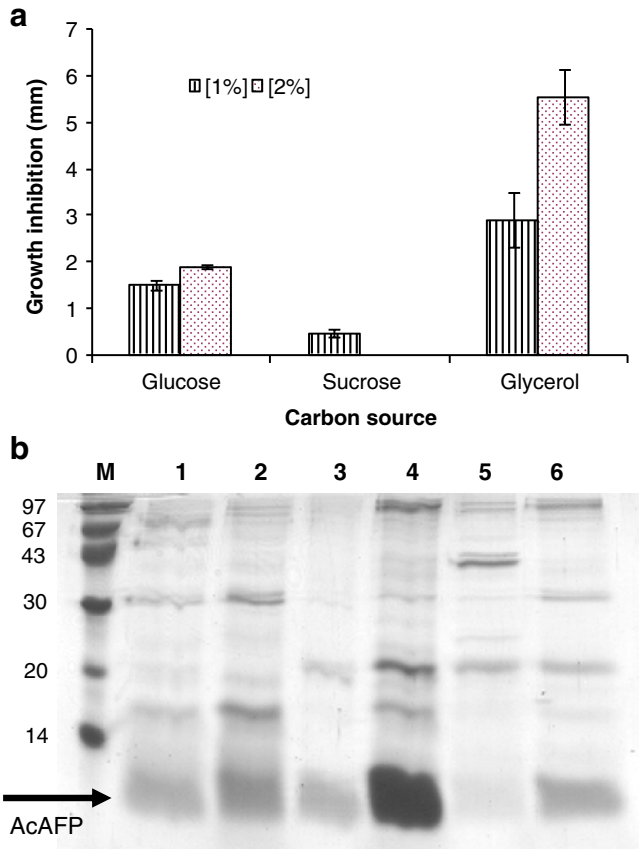

Fig. 2 **a** Effect of the carbon sources concentration on the antifungal activity production. The same volume (200 μl) from supernatant culture of each set of condition was used for antifungal activity assay. Each *bar* represents the means of three independent experiments (n= 3). **b** SDS-PAGE of proteins secreted by *A. clavatus* in cultures grown for 96 h. Each *lane* contains 50 μl of culture medium. *Lane 1*, glucose 1% (*w/v*); *lane 2*, glycerol 1% (*v/v*); *lane 3*, sucrose 1% (*w/v*); *lane 4*, glycerol 2%; *lane 5*, sucrose 2% and *lane 6*, glucose 2%. The band corresponding to the AcAFP peptide is indicated with an *arrow* according to the purification procedure (Skouri-Gargouri and Gargouri 2008)

Effect of salt on the AcAFP production

A variety of NaCl and KCl concentrations ranging from 100 mM to 1 M were used to evaluate the salt sensitivity of AcAFP. The optimal production of the AcAFP activity was obtained in the presence of 100 mM of salt (NaCl or KCl) on 2% glycerol (Fig. 4). On 2% glucose, the production is quite lower but, interestingly, an enhancement of about 2.5-fold of the AcAFP activity was observed in the presence of KCl at 500 mM compared with that obtained with 100 mM KCl. This result seems to be specific to KCl since we do not observe similar effect on the antifungal activity with NaCl. Finally, either in the presence of glucose or glycerol, the excess of salt in the culture medium to 1 M drastically increased the production of AcAFP (Fig. 4).

Effect of pH on the AcAFP production

Figure 5a shows that when the medium conditions (as above optimised) were adjusted at an initial pH of 5, the

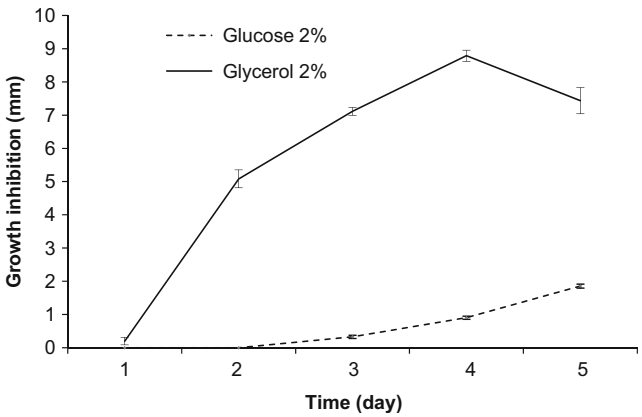

Fig. 3 Comparison of time course production of AcAFP on glycerol (**a**) and glucose (**b**) at 2%. A 200 μl of the crude supernatant culture of each set of condition was used for antifungal activity assay tested against *F. oxysporum* (n=3)

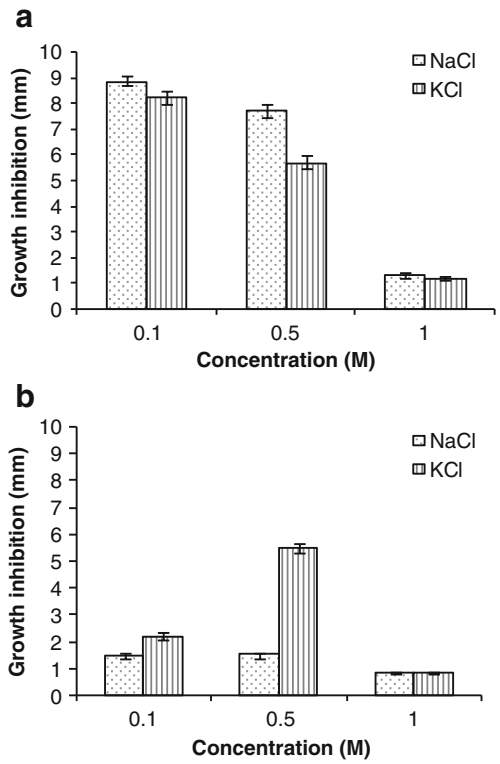

Fig. 4 Effect of salt on AcAFP production. **a** Glycerol 2%. **b** Glucose 2%. A 200 µl of the crude supernatant culture of each set of condition was used for antifungal activity assay tested against *F. oxysporum* (n=3)

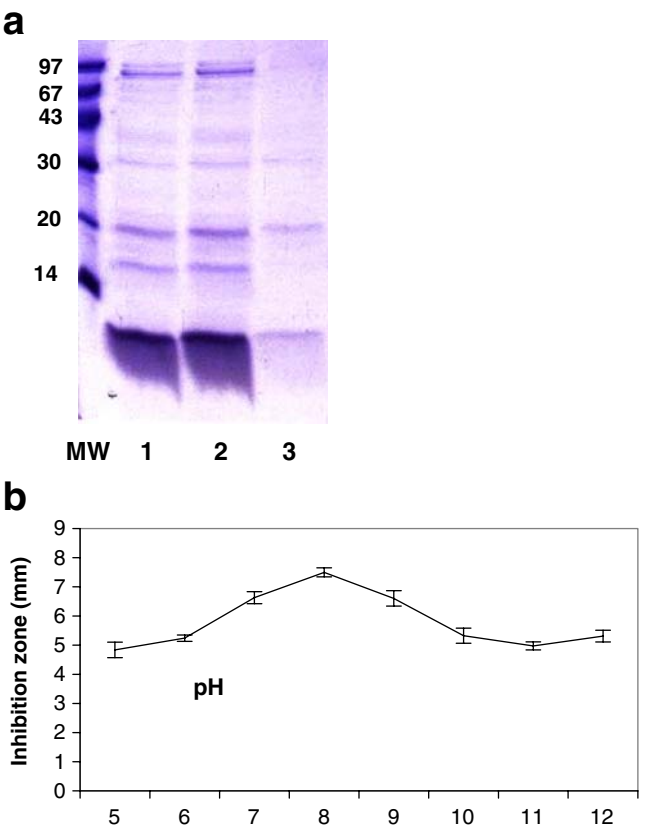

Fig. 5 **a** Effect of pH on AcAFP expression. *A. clavatus* was cultivated on 2% glycerol in the presence of different buffer systems. *Lane 1*, pH 9; *lane 2*, pH 7 and *lane 3*, pH 5. *MW* Molecular size marker. A 50 µl of crude supernatant for each set of condition were separated on a 15% SDS-PAGE. **b** Effect of pH on the stability of the purified AcAFP used at a concentration of 100 µg ml^{-1} (n=3)

antifungal activity was negligible. The same results were obtained for extracellular proteins and biomass, which suggested that the producer strain, *A. clavatus*, seems to be sensitive to acidic pH values. The secreted proteins and the antifungal activity were restored when the producer strain was grown in neutral (pH 7) or basic (pH9) conditions (Fig. 5a).

Effect of pH on the AcAFP stability

After incubation of the purified AcAFP for 24 h in the presence of different pH buffers, it remained active over a broad pH range with an optimum activity around pH 8 (Fig. 5b). It is worth noting that the AcAFP antifungal activity slightly decreased at extreme pH values.

Effect of temperature on the AcAFP stability

Since the thermostability of AcAFP has been studied during the purification procedure of the AcAFP protein (Skouri-Gargouri and Gargouri 2008), the effect of temperature on the activity of the antifungal peptide was determined by heating the purified peptide at 100°C for different time intervals ranging from 10 to 180 min. It was noted that AcAFP was quite stable at 100°C for 1 h (8.43 mm±0.38) and that its activity showed a slight alteration even after 2 h at 100°C (7.8 mm±0.28). However, antifungal activity decreased by approximately 20% after 3 h of heating (6.3 mm±0.09).

Effect of metal ions on AcAFP stability

Each metal ion was incubated for 24 h at room temperature with the purified AcAFP before testing their effect on AcAFP activity. The antifungal activity was substantially inhibited in the presence of divalent cations, particularly 10 mM of Mn^{2+} and Ca^{2+} and retained approximately 27% of its activity in both cases (Fig. 6). Moreover, the salt-sensitivity of AcAFP in the presence of monovalent ions was established by determining the antifungal activity against *F. oxysporum* at various NaCl and KCl concentrations that ranged from 0 to 500 mM. The antifungal activity was noted to be ionic strength dependent (decreasing with increasing ionic strength). While this activity was stable in the presence of 100 mM of KCl (7.2 mm±0.4), it was reduced in presence of 50 mM NaCl, the inhibition

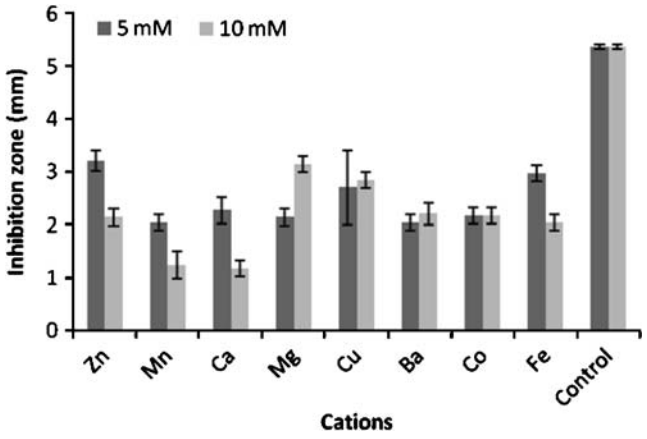

Fig. 6 Effect of some divalent cations on the stability of the purified AcAFP. AcAFP was used at a concentration of 100 μg ml^{-1}, standard deviations (± SD) of three independent experiments are given (n=3)

growth zone was instable as it completely disappeared after 3 days of incubation.

Discussion

This study aimed to further characterise the physiology of AcAFP peptide production by *A. clavatus*. Therefore, the nutritional requirements of this ascomycete were investigated with the objective of enhancing the antifungal peptide production and stability. AcAFP synthesis depends on the carbon source type present in the growth medium. Glycerol gave the best expression of antifungal activity level compared to glucose and sucrose.

Furthermore, the dramatic decrease of the secreted amount of AcAFP due to sucrose adjunction at 2% in the culture medium stands contradictory with the results reported by Marx et al. (1995) for the PAF antifungal peptide production from *P. chrysogenum*. It was repressed in the presence of 2% glucose and expressed in the presence of 2% sucrose or 2% starch. However, *A. giganteus* was reported to produce AFP either on 2% glucose or on 2% soluble starch (Wnendt et al. 1994; Meyer and Stahl 2002; Meyer et al. 2002; Theis et al. 2005) and *A. niger* was reported to produce AnAFP only on 2% glucose (Gun Lee et al. 1999). To our knowledge, no data were reported on the effect of glycerol on the known antifungal peptides expression and activity.

In the current study, while AcAFP activity was detected within 48 h in the presence of 2% glycerol, it was observed after 72 h of incubation on 2% glucose. This delay in the appearance of antifungal activity associated with the use of 2% glucose as sole carbon source is in congruence with a previously reported data on AFP expression by *A. giganteus* on 2% glucose (Meyer and Stahl 2002). In fact, Martinez-Ruiz et al. (1997) have shown that the protein AFP was barely detected after 24 h but was clearly expressed after 48 h and reached a maximum after 72 h, while the antifungal activity was detected only after 72 h of culture. To explain the delay between the AFP protein expression and the appearance of antifungal activity, the authors proposed that it could be attributed to the presence of an extracellular proteolysis leading to the AFP activation. Indeed, they remarked that a protein, which was slightly larger than AFP, appeared in the extracellular medium at 48–60 h and disappeared thereafter, as the culture proceeded (Martinez-Ruiz et al. 1997).

The AcAFP activity reached an optimum in the presence of 100 mM NaCl or KCl and glycerol, whereas on glucose, the highest yield was obtained specifically in the presence of 0.5 M KCl. It is worth noting in this respect that a previous study on AFP production reported that the addition of 1 M NaCl to the culture medium stimulated the activity by 2.2-folds. This difference could be related to the methodologies being employed. Indeed, The AFP mycelia were first continuously grown for 46 h in a salt-free medium subsequently washed and ultimately transferred to media supplemented with different concentrations of NaCl (Meyer and Stahl 2002).

Our data showed also an important decrease of AcAFP expression at acidic pH while it increased at pH 7 and 9, which was previously shown to occur with other antifungal peptides (Marx 2004; Meyer and Stahl 2002; Meyer et al. 2002). It has been already demonstrated that AFP expression is strongly inhibited in the presence of high external phosphate concentrations but induced as soon as phosphate becomes limiting. However, during our work, conscientious of the possible inhibition effect of the phosphate on the AFP expression, we compared between two buffer systems: Tris-HCl and KH_2PO_4/K_2HPO_4 (100 mM) to adjust the culture broth's pH values to 7. Despite the inefficacy of the Tris-HCl buffer to stabilise the pH value from the beginning to the end of the culture period (final pH 6.4), a comparable secretion was observed as for KH_2PO_4/K_2HPO_4. Moreover, the AcAFP secretion and the antifungal activity were approximately similar when the producer strain was grown on neutral conditions in the presence of phosphate or on basic tested conditions.

Furthermore, the AcAFP activity was reduced in the presence of 50 mM NaCl but it remained stable at the same concentration of KCl. Reduced AcAFP activity in the presence of KCl was shown from 0.1 M. This result is in agreement with previous reports which observed that while the presence of 0.1 M KCl or NaCl considerably reduced AFP activity (Theis et al. 2003), they had a limited effect on PAF potency (Kaiserer et al. 2003). In addition to being moderately resistant to salt, the AcAFP peptide was pH stable, with a maximum activity observed at pH 8.

The activity of the purified AcAFP was also shown to be affected by the presence of divalent cations, particularly Ca^{2+}, Mn^{2+} and Fe^{2+}. Concerning the PAF peptide, the antifungal activity was shown to be partially decreased in the presence of 20 mM Mg^{2+}. In plants, bacteria and insects, the cation-dependent neutralisation of the positively charged antimicrobial peptides was explained by the inhibition of their direct binding to negatively charged phospholipids present in the plasma membrane of target organisms (Marx et al. 2008; Thevissen et al. 1999; Osborn et al. 1995).

The thermostability of antifungal peptides is commonly attributed to their compact secondary structure (Martinez-Ruiz et al. 1997). Indeed, the AFP peptide does not display any decrease in its antifungal activity after 1 h of treatment at 80°C but looses approximately 90% of its antifungal activity when incubated for 15 min at 100°C (Theis et al. 2005). We have previously shown that AcAFP peptide retained its activity after incubation for 1 h at 100°C (Skouri-Gargouri and Gargouri 2008). Interestingly, in the current study we showed that, even after heating for 3 h at 100°C, the AcAFP peptide retained about 80% of its antifungal activity. To the best of our knowledge, the AcAFP peptide is the most thermostable amongst all fungal-produced antifungal peptides investigated so far.

In conclusion, the present study has demonstrated that the AcAFP peptide production is regulated by different environmental conditions, namely those pertaining to carbon source, ambient pH, and presence of different stress inducing agents. It has also demonstrated that the peptide under investigation is very robust; it can retain its activity over a large variety of conditions. These promising properties make the AcAFP peptide a potentially attractive candidate for further research and for future industrial applications.

Acknowledgment This work is dedicated to the memory of Professors Piotr Slonimski and Mohamed Marrakchi. Khmaies Benhaj and Radhia Gargouri are deeply thanked for reading the manuscript. This work was supported by the Tunisian "Ministry of Higher Education, Scientific Research and Technology."

References

Anderson RC, Yu PL (2005) Factors affecting the antimicrobial activity of ovine-derived cathelicidins against *E. coli* 0157:H4. Int J Antimicrob Agents 25:205–210

Baker B, Zambryski P, Staskawicz B, Dinesh-Kumar SP (1997) Signalling in plant-microbe interaction. Science 276:726–733

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

Ekengren S, Hultmark D (1999) *Drosophila* cecropin as an antifungal agent. Insect Biochem Mol Biol 29:965–972

Fernández D, Ouinten M, Tantaoui A, Geiger JP, Daboussi MJ, Langin T (1998) *Fot*1 insertions in the *Fusarium oxysporum* f. sp. *albedinis* genome provide diagnostic PCR targets for detection of the date palm pathogen. Appl Environ Microbiol 64:633–636

Fritig B, Heitz T, Legrand M (1999) Antimicrobial proteins in induced plant defense. Curr Opin Immunol 11:23–27

Geison R (2000) *P. nalgiovense* carries a gene which is homologous to the paf gene of *P. chrysogenum* which codes for an antifungal peptide. Int J Food Microbiol 62:95–101

Gun Lee D, Shin SY, Maeng CY, Jin ZZ, Kim KL, Ham KS (1999) Isolation and characterization of a novel antifungal peptide from *Aspergillus niger*. Biochem Biophys Res Commun 263:646–651

Hoffman HL, Ernest EJ, Klepser EE (2000) Novel triazole antifungal agents. Expert Opin Investig Drugs 9:593–605

Kaiserer L, Oberparleiter C, Weiler-Görz R, Burgstaller W, Leiter E, Marx F (2003) Characterization of the *Penicillium chrysogenum* antifungal protein PAF. Arch Microbiol 180:204–210

Laemmli UK, Favre M (1973) Maturation of the head of bacteriophage T4. I. DNA packaging events. J Mol Biol 80:573–599

Lehrer R, Ganz T (1999) Antimicrobial peptides in mammalian and insect host defence. Curr Opin Immunol 11:23–27

Martinez-Ruiz A, Martinez del Pozo A, Mancheno JM, Lacadena J, Onaderra M, Gavilanes JG (1997) Characterization of a natural larger form of the antifungal protein (AFP) from *Aspergillus giganteus*. Biochim Biophys Acta 1340:81–87

Marx F (2004) Small, basic antifungal proteins secreted from filamentous ascomycetes: comparative study regarding expression, structure, function and potential application. Appl Microbiol Biotechnol 65:133–142

Marx F, Haas H, Reindl M, Stöffler G, Lottspeich F, Redl B (1995) Cloning, structural organization and regulation of expression of the *Penicillium chrysogenum paf* gene encoding an abundantly secreted protein of *Aspergillus giganteus*. Gene 167:167–171

Marx F, Binder U, Leitner E, Pocsi I (2008) The *Penicillium chrysogenum* antifungal protein PAF, a promising tool for the development of new antifungal therapies and fungal cell biology studies. Cell Mol Life Sci 65:445–454

Meyer V (2008) A small protein that fights fungi: AFP as a new promising antifungal agent of biotechnological value. Appl Microbiol Biotechnol 78:17–28

Meyer V, Stahl U (2002) New insights in the regulation of the *afp* gene encoding the antifungal protein of *Aspergillus giganteus*. Curr Genet 45:36–42

Meyer V, Stahl U (2003) The influence of co-cultivation on expression of the antifungal protein in *Aspergillus giganteus*. J Basic Microbiol 43:68–74

Meyer V, Wedde M, Stahl U (2002) Transcriptional regulation of the antifungal protein in *Aspergillus giganteus*. Mol Genet Genomics 266:747–757

Osborn RW, De Samblanx GW, Thevissen K, Goderis I, Torrekens S, Van Leuven F, Attenborough S, Rees SB, Broekaert WF (1995) Isolation and characterisation of plants defensins from seeds of Asteraceae, Fabaceae, Hippocastanaceae and Saxifragaceae. FEBS Lett 368:257–262

Raj PA, Dentino AR (2002) Current status of defensins and their role in innate and adaptive immunity. FEMS Microbiol Lett 206:9–18

Skouri-Gargouri H, Gargouri A (2008) First isolation of a novel thermostable antifungal peptide secreted by *Aspergillus clavatus*. Peptides 29:1871–1877

Theis T, Stahl A (2004) Antifungal proteins: targets, mechanisms and prospective applications. Cell Mol Life Sci 61:437–455

Theis T, Wedde M, Meyer V, Stahl U (2003) The antifungal protein from *Aspergillus giganteus* causes membrane permeabilization. Antimicrob Agents Chemother 47:588–593

Theis T, Marx F, Salvenmoser W, Stahl U, Meyer V (2005) New insights into the target site and mode of action of the antifungal protein of *Aspergillus giganteus*. Res Microbiol 156:47–56

Thevissen K, Terras FRG, Broekaert WF (1999) Permeabilization of fungal membranes by plant defensins inhibits fungal growth. Appl Environ Microbiol 65:5451–5458

Wnendt S, Ulbrich N, Stahl U (1994) Molecular cloning sequence analysis and expression of the gene encoding an antifungal-protein from *Aspergillus giganteus*. Curr Genet 25:519–523