An antifungal protein from *Escherichia coli*

V. Yadav,¹ R. Mandhan,² O. Pasha,¹ S. Pasha,¹ A. Katyal,³ A. K. Chhillar,⁴ J. Gupta,¹ R. Dabur⁵ and G. L. Sharma¹

Correspondence

G. L. Sharma

drglsharma@hotmail.com

¹Institute of Genomics and Integrative Biology, Mall Road, University Campus, Delhi, India

²Department of Biotechnology, Kurukshetra University, Kurukshetra, India

³Dr. B.R. Ambedkar Centre for Biomedical Research, Delhi, India

⁴Shriram Institute for Industrial Research, Delhi, India

⁵Regional Research Institute (Ay), Kothrud, Pune, India

A cytosolic protein was purified from *Escherichia coli* BL21 that demonstrated potent antifungal activity against pathogenic strains of *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus niger* and *Candida albicans*. The MIC of purified protein from *E. coli* BL21 (PPEBL21) against *Aspergillus* species and *C. albicans* was 1.95–3.98 and 15.62 $\mu\text{g ml}^{-1}$, respectively. *In vitro* toxicity tests demonstrated no cytotoxicity of PPEBL21 to human erythrocytes up to the tested concentrations of 1250 $\mu\text{g ml}^{-1}$. Amphotericin B was lethal to 100% of human erythrocytes at a concentration of 37.5 $\mu\text{g ml}^{-1}$. The N-terminal amino acid sequence of PPEBL21 was found to be DLAEVASR, which showed 75% sequence similarity with alcohol dehydrogenase of yeast. Mass fingerprinting by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry also substantiated these observations. The results suggested that *E. coli* BL21 might be an important bioresource of lead molecules for developing new peptide-based therapies for treating fungal infections.

Received 27 September 2006

Accepted 13 December 2006

INTRODUCTION

Mycotic infections are emerging as a major cause of morbidity and mortality in immunocompromised patients. Infections due to *Candida* species in immunocompromised patients are the most common; however, aspergilli and other pathogenic fungi are also emerging as a threat to public health (Chen *et al.*, 2001; Richardson & Warnock, 2003; Richardson, 2005). The mortality rate due to invasive aspergillosis has risen steadily with a 357% increase which caused significant morbidity and mortality during 1980–1997 (Rapp, 2004). Increased interest during the last 15 years in the area of development of new antifungal therapies has widened the options for preventing and treating fungal infections. However, amphotericin B has remained a preferred antifungal drug despite its severe renal and infusion-related toxicity (Oude Lashof & Kullberg, 2004). The nucleoside analogue class has only flucytosine, which targets the nucleus of fungal cells. Generally it is not given as monotherapy because of frequently developed resistance in pathogens. Ketconazole, fluconazole, itraconazole and voriconazole are important azoles often prescribed to treat fungal diseases but they all have limited efficacy (Gupta & Tomas, 2003). Fluconazole was demonstrated to be active largely

against *Candida* spp. and *Cryptococcus neoformans* whereas itraconazole is active against moulds (*Aspergillus*) and dimorphic fungi (*Histoplasma* and *Blastomyces*). Voriconazole is a relatively new molecule which has been found to be active against both yeasts and moulds. However, its strength under varied clinical settings has yet to be established (Herbrecht, 2004). Caspofungin belongs to the echinocandin class of antifungal drugs, which has been reported to be effective in the treatment of invasive candidiasis (Zaas & Alexander, 2005). It is also indicated as salvage therapy in cases of invasive aspergillosis. However, infusion-related phlebitis, tenderness, pain, itching, burning sensation and local discharge are commonly reported adverse effects in all patients treated with caspofungin (Sims-McCallum, 2003). Wegner *et al.* (2005) reported caspofungin to be antiproliferative and nephrotoxic. Most recent reports have demonstrated the refractoriness of aspergillosis and candidiasis to treatment with caspofungin (Ruchel *et al.*, 2006; Schelenz & Ross, 2006). Further, the emergence of resistance to most drugs in fungal pathogens has made the problem more complex and necessitated work to discover new lead molecules which eventually could be used to develop suitable antifungal formulations (Johnson *et al.*, 2004).

The exploration of natural bioresource for treating invasive fungal infections has been emphasized in recent years (Roessner & Scott, 1996; Dabur *et al.*, 2004, 2005). Micro-organisms have also been an important source of biologically active

Abbreviations: ADH, alcohol dehydrogenase; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight.

molecules (Demain, 1999). It was observed previously that the presence of an *Escherichia coli* strain in orally administered probiotic decreased the presence of pathogens, the number of infections and the need for antibiotics (Lodinová-Zádníková *et al.*, 2003). We recently observed the inhibitory effect of *E. coli* BL21 lysate on pathogenic aspergilli (Yadav *et al.*, 2005). However, what molecule in the lysate was endowed with antifungal activity is not yet known. Therefore, the present study was undertaken to identify and characterize the antifungal protein molecule(s) of *E. coli* having potential against fungi.

METHODS

Bacterial strain. *Escherichia coli* BL21 (MTCCB 1678) was procured from the Institute of Microbial Technology, Chandigarh (India). It was cultured in LB broth for 3 days at 37 °C in a shaking incubator. The cells were counted by the turbidity method and used for performing various experiments.

Fungal strains. Pathogenic strains of *Aspergillus fumigatus* [ITCC 4517 (IARI, Delhi), ITCC 1634 (IARI, Delhi), 190/96 (VPCI, Delhi)], *Aspergillus flavus* [ITCC 5192 (IARI, Delhi), ITCC 5076 (IARI, Delhi), 223/96 (VPCI, Delhi)], *Aspergillus niger* [ITCC 5405 (IARI, Delhi), 56/96 (VPCI, Delhi)] and *Candida albicans* [ITCC 4718 (IARI, Delhi)] were employed in the current study. All the *Aspergillus* and *Candida* strains were cultured in the laboratory on Sabouraud dextrose agar plates.

Preparation of lysate of *E. coli*. Lysate of *E. coli* BL21 was prepared as described previously (Yadav *et al.*, 2005). The 72 h culture of *E. coli* BL21 was centrifuged at 3300 *g* for 30 min using a Sorvall RC 5C centrifuge. The cells were sonicated using a sonicator (Misonix; Sonicator 3000) and sonicate was centrifuged at 16 350 *g* for 30 min. The supernatant was collected and used as lysate. The lysate was dialysed against distilled water at 4 °C for 24 h with several changes of water and lyophilized. The protein concentration of bacterial lysate was determined by the BCA method of Smith *et al.* (1985).

Purification of PPEBL21. An activity guided purification of the antifungal molecule from *E. coli* lysate was performed. The lysate prepared from *E. coli* BL21 was subjected to fractionation by ion exchange chromatography (Yadav *et al.*, 2005). The proteins recovered in an active ion exchange chromatographic fraction (F III) were fractionated by gel filtration chromatography. An amount of 3 mg F III proteins was applied onto a Sephadex G 100 column (1.5 × 75.0 cm) and eluted with Tris/HCl buffer at a flow rate of 0.5 ml min⁻¹ to obtain five subfractions (SF 1–SF 5). The active SF 3 was further examined for purity by HPLC (using a C8 column) and PAGE. The purified active protein of *E. coli* (PPEBL21) was subjected to detailed biochemical characterization.

Anti-*Aspergillus* activity. The activity of bacterial components against aspergilli was analysed by microbroth dilution, disc diffusion and spore germination inhibition assays (Rajesh & Sharma, 2002). Experiments were repeated at least three times to ascertain the activity of test samples.

Disc diffusion assay. This was performed in radiation-sterilized Petri plates of 10 cm diameter (Tarsons). Different concentrations in the range of 250–0.31 µg bacterial proteins were impregnated on the sterilized discs (5 mm in diameter) of Whatman filter paper no. 1. The discs were placed on the surface of the agar plates already inoculated with *A. fumigatus* spores. The plates were incubated at

37 °C and examined at 48 h for a zone of inhibition, if any, around the discs. The concentration that developed a zone of inhibition of at least 6 mm diameter was considered the MIC.

Microbroth dilution assay. The test was performed in 96-well culture plates (Nunc; Nunclon). Various concentrations of bacterial proteins in the range 125–0.97 µg ml⁻¹ were prepared in the wells by a twofold dilution method. The assay was performed as per the standard method described previously (Rajesh & Sharma, 2002).

Percentage spore germination inhibition assay. Different concentrations of the bacterial proteins in 90 µl culture medium were prepared in 96-well flat-bottom micro-culture plates (Nunc; Nunclon) by the double dilution method. The wells were prepared in triplicate for each concentration. Each well was then inoculated with 10 µl spore suspension containing 100 ± 5 spores. The plates were incubated at 37 °C for 16 h and then examined for spore germination with an inverted microscope (Nikon; Diphot). The number of germinated and non-germinated spores was counted to determine the percentage of growth inhibition (Rajesh & Sharma, 2002).

Anti-candidal activity. This was determined by a percentage growth inhibition assay (Iijima *et al.*, 1993) suitably modified in the laboratory. *C. albicans* was grown on Sabouraud dextrose agar plates at 37 °C. *C. albicans* cells in exponential phase were suspended in fresh medium at a density of 2 × 10⁶ cells ml⁻¹. The different wells of 96-well culture plates were inoculated with 100 µl cell suspension and treated with different concentrations of test preparations. The plates were incubated at 37 °C for 24 h and the OD₆₅₀ was measured to assess cell growth.

Cytotoxicity assay. The haemolytic assay was employed to determine the cytotoxic effect of PPEBL21 (Yadav *et al.*, 2005). Briefly, human erythrocytes in 2% (v/v) suspension were exposed to various concentrations of PPEBL21 ranging from 1250 to 1.4 µg ml⁻¹ at 37 °C for 1 h. The cells were pelleted at 3000 *g* for 10 min and the supernatant was collected to determine the A₄₅₀ using a spectrophotometer (UV Vis Spect Lambda Bio 20; Perkin Elmer). In negative control sets, only buffer was used for background lysis, whereas in positive controls, lysis buffer was used for completely lysing the erythrocytes. The percentage haemolysis was calculated and plotted against the concentration of PPEBL21 to determine the dose cytotoxic to human erythrocytes.

Elucidation of molecular mass. The molecular mass of PPEBL21 was determined by SDS-PAGE using the method of Laemmli & Favre (1973). The proteins were electrophoresed on 12.5% polyacrylamide gels and visualized by silver staining (Blum *et al.*, 1987). The gels were further analysed by Kodak 1D software to determine the precise molecular mass of PPEBL21.

N-terminal amino acid sequencing of PPEBL21. To analyse the N-terminal amino acid sequence of the subunits, purified PPEBL21 was subjected to SDS-PAGE under reducing conditions and then electroblotted onto a PVDF membrane. The protein band on the PVDF membrane was identified by Ponceau staining and cut out using a fine surgical blade. The membrane pieces containing PPEBL21 were rinsed with 100% methanol three times to eliminate the staining reagent. The protein band was analysed to determine the N-terminal amino acid sequence by the Edman degradation method on an automated protein sequencer (Perkin-Elmer Applied Biosystems). The resulting N-terminal amino acid sequences were compared with protein sequences in the SWISS-PROT database.

Peptide mass fingerprinting

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS. In-gel protein digests of PPEBL21 were prepared as

described by Gao *et al.* (1994). The silver-stained purified band from the SDS-PAGE gel was sliced out. After washing with distilled water, the gel slices were incubated for 20 min in wash solution (50 % acetonitrile in 50 mM NH_4HCO_3) to remove the staining dye. Gel pieces were treated with 150 μl 10 mM DTT in 100 mM NH_4HCO_3 for 10 min and incubated in 5 % acetonitrile for 1 h at 55 °C. The gel pieces were dehydrated in 100 μl 100 mM NH_4HCO_3 and 100 % acetonitrile for 10 and 20 min, respectively. For alkylation, 100 μl 50 mM iodoacetamide in 100 mM NH_4HCO_3 was added to the gel pieces and incubated in the dark at room temperature for 30 min. The gel pieces were washed with 100 μl 100 mM NH_4HCO_3 for 10 min and then dried in a vacuum centrifuge for 15 min. Subsequently, 20 μl trypsin solution (10 ng ml^{-1} in 50 mM NH_4HCO_3) was added to tubes having gel pieces and incubated at 37 °C for 16 h. Digested peptides were extracted with buffer (5 % trifluoroacetic acid in 60 % acetonitrile) and concentrated by Speed-Vac centrifugation for 3 h. The lyophilized peptides were resolubilized in a resuspension solution (1 % trifluoroacetic acid in 50 % acetonitrile) and then centrifuged for 2 min to obtain the supernatant. The peptide solution was mixed with matrix in a ratio of 1 : 1 and subjected to analysis by MALDI-TOF MS (Bruker TOF/TOF).

Mascot search. The peptide sequences along with their masses of PPEBL21 acquired from MALDI-TOF MS were analysed by the Mascot search of Matrix Science to find out the peptide matches present in the MSDB protein database.

Computational analysis. Multiple alignment of protein sequences was performed using CLUSTAL W software (Thompson *et al.*, 1994).

RESULTS

Purification of PPEBL21

The lysate of *E. coli* BL21 was initially fractionated by ion-exchange chromatography, which resulted in effective separation of an antifungal fraction, F III, at 0.3 M NaCl. Subsequent fractionation of F III by Sephadex G 100 column chromatography resulted in five subfractions, SF 1–SF 5. Evaluation of subfractions for antifungal potential showed that activity resided mainly in SF 3. SF 3, therefore, was subjected to PAGE under reducing and non-reducing conditions. The results showed a single protein band in both the gels on silver staining indicating the presence of only one protein in SF 3. The SF 3 was subjected to HPLC using a C8 column. The HPLC profile of SF 3 is shown in Fig. 1. A single peak of pure protein was obtained by HPLC at a retention time of 3.45 min. The protein molecule showed a single spot on 2D gel electrophoresis also (data not shown). The purified protein thus obtained from *E. coli* BL21 was called PPEBL21.

Antimycotic properties of PPEBL21

The activity of PPEBL21 against aspergilli was determined by using different assay systems. The inhibitory effect of PPEBL21 on *A. fumigatus* growth determined by the microbroth dilution assay is shown in Fig. 2. The MICs of PPEBL21 against filamentous fungi, i.e. *A. fumigatus* and *A. flavus*, were observed to be 1.95 $\mu\text{g ml}^{-1}$ by the microbroth dilution and percentage spore germination inhibition

Fig. 1. HPLC profile of PPEBL21.

assays. A slightly higher concentration of PPEBL21 (3.9 $\mu\text{g ml}^{-1}$) was required to completely inhibit the growth of *A. niger*. In the disc diffusion assay, PPEBL21 produced a clear zone of inhibition of 6.3 mm diameter at a concentration of 0.62 μg per disc against *A. fumigatus* (Fig. 3). There was no effect of the lower concentrations on the growth of *A. fumigatus* as the mycelia of the pathogen could grow even over to the discs impregnated with 0.31 μg

Fig. 2. Inhibitory effect of PPEBL21 on the growth of *Aspergillus* species analysed by a microbroth dilution assay. (a) Rows: 1, medium only; 2–8, normal growth of *A. fumigatus*. (b) Rows 1–8, wells treated with different concentrations of PPEBL21.

Fig. 3. Inhibition of growth of *Aspergillus* species by PPEBL21 analysed by a disc diffusion assay: (a) 2.50 μg Amp B; (b) 5.00 μg PPEBL21; (c) 2.50 μg PPEBL21; (d) 1.25 μg PPEBL21; (e) 0.62 μg PPEBL21; (f) 0.31 μg PPEBL21.

per disc or the solvent. PPEBL21 was also found to be active against yeast, i.e. *C. albicans*, the MIC being 15.62 $\mu\text{g ml}^{-1}$ determined by the percentage growth inhibition assay.

Molecular mass of PPEBL21

The SDS-PAGE gels having band of PPEBL21 along with appropriate molecular mass markers were analysed using Kodak 1D software. The precise molecular mass of PPEBL21 was found to be 39.3 kDa (Fig. 4).

N-terminal amino acid sequence

The first eight amino acids of PPEBL21 were found to be DLAEVASR. By comparing its N-terminal sequence with those available in databases, it was observed to be 75% identical with a previously described alcohol dehydrogenase (ADH) from yeast. The alignment of PPEBL21 with the ADH revealed the presence of some conserved aspartic acid and alanine residues.

MALDI analysis

The protein band of PPEBL21 was excised and subjected to in-gel digestion with trypsin. Proteolytic fragments were analysed by MALDI-TOF MS as well as nano liquid chromatography mass spectrometry (Bruker Ultraflex TOF/TOF) to identify potential peptide candidates. Fig. 5 shows the mass spectrum of trypsin-digested peptides of PPEBL21.

The Mascot algorithm was employed for protein identification by peptide mass fingerprinting. The MALDI-TOF and liquid chromatography mass spectra data revealed the potential match with ADH from *Bacillus* species, which

Fig. 4. SDS-PAGE analysis of PPEBL21. Lanes: 1, marker; 2, total protein lysate of *E. coli* BL21; 3, F III; 4, PPEBL21.

showed a significant MOWSE score of up to 94 by MALDI-TOF MS (Table 1). The peptide sequences of PPEBL21 obtained on tryptic digestion and which showed sequence homology with ADH of *Bacillus anthracis* are shown in Table 2. There were at least 14 peptides in PPEBL21 that were homologous to ADH of *B. anthracis*.

The matched peptide sequences of PPEBL21 were aligned based on their occurrence in *B. anthracis* ADH and subjected to an NCBI BLAST search. The results showed 45% homology with propanol-preferring ADH from *E. coli* (Fig. 6). Based on the sequence homology and peptide matching, PPEBL21 is proposed to be a propanol-preferring ADH.

Toxicity of PPEBL21

In vitro cell cytotoxicity of PPEBL21 was investigated using a haemolytic assay. The molecule exhibited no toxicity to human erythrocytes up to a concentration of 1250 $\mu\text{g ml}^{-1}$. The extended exposure of erythrocytes to 1250 $\mu\text{g PPEBL21 ml}^{-1}$ up to 24 h also did not show any toxicity to erythrocytes. Amphotericin B haemolysed all erythrocytes at a concentration of 37.5 $\mu\text{g ml}^{-1}$.

DISCUSSION

Members of the genus *Escherichia* are well-known antibiotic producers. It has been reported that a number of *E. coli* strains have a protective effect against antibiotic-resistant, colicin-sensitive and enterotoxigenic *E. coli*. It has been used as a probiotic for the treatment of recurrent infections of the urinary tract (Matricardi *et al.*, 2003). In the present work, a novel antifungal protein, PPEBL21, was

Fig. 5. Peptide mass spectrum of PPEBL21 by MALDI-TOF MS.

isolated from *E. coli* and investigated for antifungal potential. Experiments were conducted to characterize PPEBL21.

PPEBL21 showed potent antifungal activity against aspergilli and *Candida*. A concentration of $1.95 \mu\text{g ml}^{-1}$ PPEBL21 inhibited growth of *A. fumigatus* and *A. flavus* in microbroth dilution and percentage spore germination inhibition assays. In the disc diffusion assay, the MIC of PPEBL21 against *A. fumigatus* and *A. flavus* was found to be $0.62 \mu\text{g per disc}$. An antifungal compound purified by Lavermicocca *et al.* (2000) from *Lactobacillus plantarum* strain 21B was reported to have an inhibitory effect on growth of *Eurotium*, *Penicillium*, *Monillia*, *A. niger* and *A. flavus* at a concentration of 50 mg ml^{-1} . *Penicillium*

antifungal protein (PAF) isolated from *Penicillium* CL showed activity against 21 fungal isolates at a concentration of $50 \mu\text{g ml}^{-1}$ (Galgoczy *et al.*, 2005). The proteins isolated from *Pseudomonas* by Sorensen *et al.* (1996) were found to be much less effective than PPEBL21 against *A. fumigatus*; however, they demonstrated superior activity against *C. albicans*. Syringostatin A and syringotoxin B were the two main molecules obtained from *Pseudomonas*, which were reported to be lethal to *C. albicans* and *A. fumigatus* at MICs of 3.2 and $5.0 \mu\text{g ml}^{-1}$, respectively (Sorensen *et al.*, 1996). The strong antifungal activity of PPEBL21 against pathogenic fungi at a concentration of $1.95 \mu\text{g ml}^{-1}$ by the microbroth dilution and spore germination inhibition assays and $0.62 \mu\text{g per disc}$ by disc diffusion assay thus suggested that *E. coli* BL21 might be an important

Table 1. Analysis of PPEBL21 by MALDI-TOF MS

Identified protein (accession no.)	Molecular mass (Da)	MOWSE score	Tryptic matched peptides	Protein name
gil47527564	37 455	94	14	Alcohol dehydrogenase (Zn-containing)
gil49480019	37 468	81	13	Alcohol dehydrogenase (Zn-containing)
gil47565620	37 505	81	13	Alcohol dehydrogenase (Zn-containing)
gil30020354	37 521	81	13	Alcohol dehydrogenase (propanol-preferring)
gil42781360	37 715	59	10	Alcohol dehydrogenase (Zn-containing)

Table 2. Sequence of peptides matched with the alcohol dehydrogenase of *Bacillus anthracis* strain 'Ames Ancestor' after tryptic digestion of PPEBL21, analysed by the Mascot algorithm

Peptides are aligned by their residue numbers. Fixed modifications: carbamidomethyl (C); variable modifications: oxidation (M); sequence coverage: 45 %.

Start-end	Observed	Molecular mass (expected)	Molecular mass (calculated)	Delta	Miss	Sequence
82-93	1457.68	1456.67	1456.66	0.01	0	VSIAMWFQSCGR Oxidation (M)
94-101	1044.42	1043.41	1043.42	-0.01	0	CEYCVTGR
110-132	2419.08	2418.08	2418.08	-0.01	0	NAGYSVDGGMAEQCIVTADYAVK
133-154	2265.09	2264.09	2264.10	-0.01	0	VPEGLDPAQASSITCAGVTITYK
206-223	1842.89	1841.88	1841.88	-0.00	0	EVGADMTINPISQGPADK
224-242	1953.02	1952.02	1952.03	-0.01	0	IIQEEFGGAYAAVVTAVSK
243-253	1148.60	1147.60	1147.60	-0.00	0	VAFNSACDAVR
258-274	1823.01	1822.00	1822.00	0.00	0	VVAVGLPVETMDLNAPR
258-274	1839.00	1837.99	1838.00	-0.00	0	VVAVGLPVETMDLNAPR Oxidation (M)
291-304	1571.75	1570.74	1570.73	0.01	1	KDLEEFMFGEAGK
292-304	1443.65	1442.65	1442.65	0.01	0	DLEEFMFGEAGK
292-304	1459.64	1458.63	1458.63	-0.00	0	DLEEFMFGEAGK Oxidation (M)
317-328	1465.68	1464.67	1464.67	0.01	0	VQNVFEEMEQR
317-328	1481.68	1480.67	1480.66	0.01	0	VQNVFEEMEQR Oxidation (M)

bioresource of lead molecules for developing new anti-fungal therapies.

The results indicated that *A. fumigatus* and *A. flavus* were more susceptible than other species of fungi to the lethal effects of PPEBL21. Higher concentrations of PPEBL21, i.e. 3.9 and 15.62 $\mu\text{g ml}^{-1}$, were required to inhibit the growth of *A. niger* and *C. albicans*, respectively. The results thus indicated that the surface molecules in different species of pathogens may determine the susceptibility to treatment with PPEBL21. The difference in sensitivity of different *Aspergillus* species to treatment with proteins isolated from *Moringa oleifera* and *Indigofera oblongifolia* was observed by Dahot (1998, 1999) also.

In the present study, the results of toxicity experiments appeared to be of great interest. PPEBL21 was non-toxic up to a tested concentration of 1250 $\mu\text{g ml}^{-1}$ to human

erythrocytes. Amphotericin B lysed all erythrocytes at a concentration of 37.5 $\mu\text{g ml}^{-1}$. Hong *et al.* (1998) found that their novel antimicrobial peptides did not show haemolytic activity up to a concentration of 500 $\mu\text{g ml}^{-1}$. Leiter *et al.* (2005) reported PAF to be non-toxic up to 100 $\mu\text{g ml}^{-1}$ to human endothelial cells of the umbilical vein whereas Cammue *et al.* (1992) found their antifungal peptides to be non-toxic to human endothelial cells up to a concentration of 500 $\mu\text{g ml}^{-1}$. Woo *et al.* (2002) also found that antifungal SAP protein isolated from *Streptomyces* was non-toxic up to 250 $\mu\text{g ml}^{-1}$ to human dermal fibroblasts but was toxic at all higher doses. Sorensen *et al.* (1996) observed 100 % haemolysis by syringomycin at a concentration of 20 $\mu\text{g ml}^{-1}$ and found it to be more toxic than amphotericin B to erythrocytes. Thionins and defensins have been reported to be effective antifungal proteins against human pathogens but they exert non-specific

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>sp|P39451|ADHP_ECOLI Alcohol dehydrogenase, propanol-preferring
Length=336

Score = 172 bits (436), Expect = 3e-43
Identities = 111/246 (45%), Positives = 132/246 (53%), Gaps = 92/246 (37%)

Query 2 SIAUMFQSCGRCEYCVTG-----FNAGYSVDGGMAEQCIVTADYAVKVPFGLDPAQA 53
S+AU ++ CG CEYC +G +NAGYSVDGGMAE+CIV ADYAVKVP+GLD A A
Sbjct 61 SVAUFYEGCGHCEYCNSGNETLCRSVKFNAGYSVDGGMAEECIVVADYAVKVPDGLDSAAA 140

Query 54 SSITCAGVTITYK----- 65
SSITCAGVTITYK
Sbjct 141 SSITCAGVTITYKAVKLSKIRPGQWIAIYGLGLGNLALQYAKNVFNARVIAIDVNDQLK 200

Query 66 ---EVGADMTINPISQGPADKIIQEEFGGAYAAVVTAVSKVAFNSAVDAV---RVVAVG 118
E+GAD+ IN ++ A KI+OE+ GGA+AAVVTAV+E AFNSAVDAV RVVAVG
Sbjct 201 LATENGADLAINSHTEAA-KIVQKTCGAHAHVTAARAAFNASVDAVRAGGRVAVG 259

Query 119 LPVETNDLNIP-----RKDLEEFMFGEAGKV-----QNVFE 150
LP E+M L+IP R+DL EAF F AEGKV +F
Sbjct 260 LPPESSSLDIPRLVLDGIEVVGSLVGTQDLTEAFQFAAEGKVVPKVALRFLADINTIFT 319

Query 151 EMEQGR 156
EME+G+
Sbjct 320 EMEEGK 325
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Fig. 6. Sequence homology of trypsin-digested peptides of PPEBL21 aligned with propanol-preferring ADH of *E. coli* on reverse matching.

cytotoxicity against a wide range of normal and malignant targets, including cells resistant to TNF- α and NK-cytolytic factor. They appeared to kill mammalian target cells and micro-organisms by a common mechanism (Lehrer *et al.*, 1993). Antifungal peptides purified from *Bacillus cereus* were also reported to be highly toxic to human erythrocytes (Latoud *et al.*, 1986). The toxicity of most reported antifungal proteins to mammalian cells, therefore, has become a major limitation in using them as lead molecules for developing new antifungal drugs or preparing improved antifungal formulations. The extremely low toxicity of a potent antifungal protein, PPEBL21, identified in the current study thus emphasizes its usefulness in new antifungal therapeutics.

The N-terminal amino acid sequence of PPEBL21 (identified as ADH) is DLAEVASR. A computer-based search on N-terminal amino acid residues of PPEBL21 for homology with other reported antifungal protein sequences showed that the above sequence had no significant similarity with any antifungal proteins reported so far. Thus purified PPEBL21, which had 75 % sequence homology with ADH II and ADH III of *Saccharomyces cerevisiae* and ADH I of *Kluyveromyces marxianus*, might be a representative of a novel class of proteins having antifungal properties.

Mass fingerprinting also revealed the best matches with ADH from six different strains of *B. anthracis* and one strain each of *B. cereus* and *Bacillus thuringiensis*. These matched peptides were shown to be present in 45 % sequence coverage of ADH of *B. anthracis* and on reverse matching showed 45 % sequence similarity with the sequence of propanol-preferring ADH of *E. coli* available in the NCBI protein database. The alignment of PPEBL21 with these ADHs revealed the presence of conserved aspartic acid and alanine residues. These residues may be important for their structure-associated properties (Antoine *et al.*, 1999).

PPEBL21 appeared as a single band in PAGE under both reducing and non-reducing conditions. Analysis of SDS-PAGE gels by Kodak 1D software showed that PPEBL21 had a molecular mass of 39.3 kDa, which was very close to the molecular mass of ADH of *E. coli* (35.3 kDa), *Rhodococcus ruber* (38 kDa), *Clostridium beijerinckii* (38 kDa) and *Thermoanaerobacter ethanolicus* (38 kDa). Although the cloning and sequencing of the gene encoding PPEBL21 (which is under way) would prove the identity of the protein, the results convincingly predict the nature of PPEBL21. The molecular mass, MALDI-TOF MS and N-terminal amino acid sequence analysis suggested that PPEBL21 might be an ADH from a medium-chain dehydrogenase family which can inhibit growth of pathogenic fungi.

Conclusions

This is the first report showing that PPEBL21, a 39.3 kDa purified protein molecule isolated from *E. coli*, has strong antifungal potential against *Aspergillus* and *Candida* spp.

Compared to amphotericin B it had extremely low toxicity to human cells. Analysis of N-terminal amino acid sequences and mass fingerprinting of PPEBL21 suggested the protein to be ADH. Such preparations may be important leads for developing new antifungal therapies.

ACKNOWLEDGEMENTS

V. Y. wishes to thank the Council of Scientific and Industrial Research, India, for providing the necessary funding.

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