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ARTICLE

Antidermatophytic effect of *Bacillus mojavensis* SZMC 22228 and its secreted chymotrypsin-like protease

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ABSTRACT The aim of the present study was to investigate the antifungal effect of *Bacillus mojavensis* SZMC 22228 against different dermatophytes and to isolate the antidermatophytic compound from the bacterial ferment broth. *B. mojavensis* SZMC 22228 and its cell-free ferment broth effectively inhibited the growth of clinical reference strain of *Microsporum canis*, *Microsporum gypseum*, *Trichophyton mentagrophytes*, *Trichophyton rubrum* and *Trichophyton tonsurans* in agar diffusion test *in vitro*. An antidermatophytic, ~25 kDa protein (*B. mojavensis* SZMC 22228 antidermatophyitc protein, BMAP) was purified from the antifungally active, cell-free ferment broth using size exclusion and ion-exchange chromatography. BMAP showed antifungal effect against all of the investigated dermatophytes both in agar diffusion and broth microdilution susceptibility tests. *M. gypseum* proved to be the most susceptible dermatophyte to BMAP (MIC=40 µg/ml), all the other investigated fungi were less susceptible (MIC=80 µg/ml). The enzymatic activity of this protein was investigated in microtiter plate assay using hydrolase specific chromogenic substrates. BMAP showed high proteolytic activity towards N-Succ-Ala-Ala-Pro-Phe-*p*NA, and proved to be a chymotrypsin-like protease. These results suggest that the antidermatohytic activity of *B. mojavensis* SZMC 22228 correlates with its chymotrypsin-like protease production. After further investigations, the purified BMAP could be a promising base of a novel antidermatophytic strategy. **Acta Biol Szeged 58(2):157-162 (2014)**

KEY WORDS

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The worldwide distributed dermatophytes are a group of keratinophilic fungi. They are highly adapted to digest keratinous debris thus these fungi are capable of invading the superficial layer of the skin, hair, and nails of humans and other vertebrates (Bond 2010; Leite Jr et al. 2014). Approximately 40% of the human population is affected in worldwide (Leite Jr et al. 2014), but the spectrum of isolated dermatophytes is diverse in time and geography (Havlickova et al. 2008; Seebacher et al. 2008). They rarely cause life-threatening infection in healthy individuals, but they may have a debilitating effect on the patients and affect their quality of life (Rotta et al. 2012). However, it is a well-known fact that the incidence of superficial and fatal systemic dermatophyte infections has been increasing continuously during recent years as a consequence of the rise in the number of immunocompromised patients (Rotta et al. 2012). The choice of the appropriate therapy in the cases of these patients is not simple because of the possible drug interactions and severe side effects (Dias et al. 2103). The more frequent occurrence of antimycotic resistant dermatophyte strains further sharpens this problem (Jones 1990; Mukherjee et al. 2003; Martinez-Rossi et al. 2008). Therefore, there is a substantial demand for new safely applicable antidermatophytic compounds without side effects.

Biologically active substances secreted by the members of *Bacillus* genus are promising compounds of drug industry for development of novel antifungal agents (Tserkovniak et al. 2009). The plant endophyte *Bacillus mojavensis* is a relatively new member of the *Bacillu*s genus (Nair et al. 2002; Bacon and Hinton 2011). This species is closely related to *Bacillus subtilis* complex, but it can be distinguishable based on the fatty acid composition, DNA sequence, and the resistance to genetic transformation (Bacon and Hinton 2011). Several studies reported that *B. mojavensis* actively secretes different compounds with remarkable or potential antifungal activity, *e.g.* lipopeptides such as surfactin, iturin, fengycin; proteases, cellulases (Youcef-Ali et al. 2014). Due to this feature *B. mojavensis* is also known as an effective biocontrol agent against plant and post-harvest pathogenic filamentous fungi (Bacon and Hinton 2011).

This paper describes the antidermatophytic activity of a *B. mojavensis* strain isolated from mumijo, a traditional Mongolian medicine (Galgóczy et al. 2011); furthermore, the

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isolation and identification of an extracellular chymotrypsin-like protease (*B. mojavensis* SZMC 22228 antidermatophyitc protein, BMAP) which plays a role in it.

Materials and Methods

Strains and media

The following clinical reference dermatophyte strains were used in the present study: *Microsporum canis* (American Type Culture Collection, USA; ATCC 36299), *Microsporum gypseum* (ATCC 24102), *Trichophyton mentagrophytes* (ATCC 9533), *Trichophyton rubrum* (ATCC 28188) and *Trichophyton tonsurans* (ATCC 28942). All these isolates were maintained on potato dextrose agar (PDA, Sigma-Aldrich, St Louis, MO, USA) slants at 4 °C. For inoculum preparation they were subcultured onto oatmeal agar (10 g/l oatmeal, 1.5 g/l KH_2PO_4, 1 g/l $NaNO_3$, 1 g/l $MgSO_4$, 20 g/l agar) to induce conidium production (Jessup et al. 2000; Ghannoum et al. 2006). Antifungal activity tests were performed in PDA plates. *B. mojavensis* SZMC 22228 was maintained on LB (10 g/l tryptone, 2 g/l yeast extract, 10 g/l NaCl, 20 g/l agar, pH 7.0) slant and was grown in low salt LB broth (LSLB: 10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl, pH 7.0) for investigation of antifungal activity of the cell-free ferment broth and for purification of the BMAP.

Isolation and molecular identification of *B. mojavensis* SZMC 22228

B. mojavensis was isolated from a Mongolian mumijo preparation (Galgóczy et al. 2011) and indentified for partial sequencing of the *gyrA* gene according to Reva et al. (2004). After sequencing (performed at LGC Genomics GmbH, Berlin, Germany) of the amplified partial *gyrA* gene, the result was compared with similar sequences available in the National Center for Biotechnology Information (NCBI) database using Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990).

Purification of *B. mojavensis* SZMC 22228 antidermatophytic protein (BMAP)

BMAP was isolated from the ferment broth of *B. mojavensis* SZMC 22228, which was cultivated in 250-ml Erlenmeyer flask containing 100 ml LSLB inoculated with 10^5 bacteria, and incubated at 30 °C for 48 h under continuous shaking (160 rpm). After bacterial cell harvest (30 min, 10.000×*g*, 4 °C), the supernatant was filtered (MILLEX-HP, pore size: 0.45 µm, Millipore, Billerica, MA, USA) then the extracellular protein fraction was precipitated with 80% of ammonium sulfate at 4 °C for 24 h. Precipitated proteins were collected by centrifugation (30 min, 10.000×*g*, 4 °C) and resuspended in 10 ml of 20 mM Tris buffer (pH 7.2). This protein solution was dialyzed (Snake Skin dialysis tubing, 3.5K MWCO, Thermo Scientific, Logan, UT, USA) against 20 mM Tris buffer (pH 7.2) in 1:100 volume ratio based on the instruction of manufacturer at 4 °C for 48 h with buffer changing after 24 h.

The different molecular weight protein fractions of the ten-fold concentrated sample were separated with size exclusion chromatography. The column (20×250 mm, Sephadex G-50, GE Healthcare, Uppsala, Sweden) was equilibrated with 20 mM Tris buffer (pH 7.2). Protein fractions were eluted (flow rate: 2 ml/min) with the above mentioned buffer. Protein fractions which showed high antidermatophytic activity in agar diffusion test (data not shown) were pooled, concentrated to six-fold, and then this solution was separated with centrifugal ultrafiltration (Corning® Spin-X® UF 20 ml Centrifugal Concentrator, 30,000 MWCO Membrane, Corning, Tewksbury, MA, USA). The <30 kDa fraction (showed antidermatophytic effect in agar diffusion test, data not shown) was dialyzed against 20 mM Tris buffer (pH 7.2), then purified with ion-exchange chromatography on a CM Sepharose Fast Flow (Sigma-Aldrich, St Louis, MO, USA) column (13×60 mm, equilibrated with 20 mM Tris buffer (pH 7.2) at a flow rate of 1 ml/min. The proteins were eluted with a NaCl gradient (0.1-1.0 M) prepared in the equilibrating buffer. The protein fractions were dialyzed against 20 mM Tris buffer (pH 7.2); finally, they were sterilized by filtration (MILLEX-HP, pore size: 0.45 µm, Millipore, Billerica, MA, USA) and their antidermatophytic activity was investigated in agar diffusion test. Protein content of the different fractions was checked in SDS-PAGE (NuPAGE Novex 4-12% Bis-Tris Gel, 1.0 mm, 10 well, Invitrogen-Life Technologies, Eugene, OR, USA) visualized with Coomassie Brilliant Blue R-250 and silver staining. Purification experiments were repeated three times.

Antidermatophytic activity assays

Fungal inocula were prepared as described previously (Galgóczy et al. 2008). An agar diffusion technique was used to estimate the antagonism ability of *B. mojavensis* SZMC 22228 and the size of growth inhibition caused by the cell-free bacterial ferment broth (filtered through MILLEX-HP, pore size: 0.45 µm) and the purified BMAP. Solid culture medium (PDA) was overlaid with 1 ml of potato dextrose broth (PDB, Sigma-Aldrich, St Louis, MO, USA; pH 7.0) that were inoculated with 10^6 microconidia/ml and was dried. For the investigation of antagonism ability, 10 µl 10^4 bacteria/ml suspended in LB was dropped on the surface of this plate and was dried. For investigation of the antifungal activity of *B. mojavensis* SZMC 22228 cell-free ferment broth and purified BMAP, an agar diffusion technique was used: 100 µl of sterile bacterial ferment broth or BMAP (160 µg/ml) diluted in 20 mM Tris buffer (pH 7.2) were filled into the wells of inoculated PDA plates. Sterile LSLB or Tris buffer (pH 7.2) was used as negative control. Diameters of the inhibition zones were measured after incubation at 30 °C for 72 h.

Table 1. Antidermatophytic activity of the *Bacillus mojavensis* SZMC 22228, its cell-free ferment broth and the 160 µg/ml purified ~25 kDa antidermatophytic protein (BMAP) in agar diffusion test on PDA after incubation at 30 °C for 72 h.

Species	Diameter of inhibition zone (mm)		
	B. mojavensis SZMC 22228	Cell-free ferment broth	160 µg/ml BMAP
Microsporum canis ATCC 36299	58.0±10.5	14.3±2.1	15.3±2.9
Microsporum gypseum ATCC 24102	48.0±2.9	18.3±2.1	23.7±3.1
Trichophyton mentagrophytes ATCC 9533	53.3±14.5	12.0±3.0	14.7±1.5
Trichophyton rubrum ATCC 28188	32.7±11.6	15.0±2.6	17.30±0.6
Trichophyton tonsurans ATCC 28942	56.7±12.6	16.3±2.1	18.3±2.3

Standard deviations (±SD) derived from three replicates (N=3).

Minimal inhibitory concentrations (MIC) of BMAP against the investigated dermatophytes were determined in broth microdilution susceptibility test using flat-bottom 96-well microtiter plate. One hundred microliter of BMAP diluted in PDB was mixed with 100 µl of microconidial suspension (10^5 microconidia/ml) prepared also in PDB. The final concentrations of BMAP were 160, 80, 40, 20, 10, 5, 2.5 and 1.25 µg/ml. The plates were incubated at 30 °C for 72 h, and the optical densities were then measured at 620 nm with microtiter plate reader (ASYS Jupiter HD-ASYS Hitech, Salzburg, Austria). Two hundred microliter fresh PDB was used as background for the spectrophotometric calibration. To determine MICs, optical densities of the untreated control cultures (100 µl of microconidial suspension mixed with 100 µl PDB) were referred to 100% of growth, in each case. MIC was defined as the lowest BMAP concentration at which growth was not detected on the basis of the OD_{620} values as compared with the untreated control. Antifungal activity experiments were repeated three times.

Enzymatic activity assays

The enzymatic activity of BMAP was determined in 96-well microtiter plate by the use of N-bz-Phe-Val-Arg-*p*NA, N-α-bz-L-Arg-*p*NA, N-acetyl-L-Leu-*p*NA, N-Succ-Ala-Ala-Pro-Phe-*p*NA, *p*-nitrophenyl-N-acetyl-β-D-glucosaminide, *p*-nitrophenyl-N,N'-diacetyl-β-D-chitobioside, *p*-nitrophenyl-β-D-N,N′,N′′-triacetylchitotriose, *p*-nitrophenyl-β-D-cellobioside, and *p*-nitrophenyl-β-glucopyranoside. All substrates were purchased from Sigma-Aldrich (St Louis, MO, USA). Twenty microliter of 1 mg/ml substrate was given to 80 µl of 40 µg/ml BMAP (diluted in 20 mM Tris buffer; pH 7.2) and incubated for 3 h at 30 °C. The reaction was stopped by addition of 25 µl 10% (w/v) sodium carbonate and the *p*-nitrophenol or *p*-nitroaniline release was measured at 405 nm using an ASYS Jupiter HD microplate reader. Twenty microliter of 1 mg/ml substrate mixed with 20 mM Tris buffer (pH 7.2) was used as background for the spectrophotometric calibration. Enzymatic activity assays were repeated three times.

Results

Isolation and identification of *B. mojavensis* SZMC 22228

The BLAST analysis of the amplified partial *gyrA* sequence revealed 99% similarity to the partial *gyrA* sequence of *B. mojavensis* NRRL BD-600 strain (accession number: EU138644). Based on this result, the *Bacillus* strain isolated from Mongolian mumijo preparation was identified as *B. mojavensis*. This *B. mojavensis* strain was deposited in the Szeged Microbiological Collection (SZMC, http://www2.sci.u-szeged.hu/microbiology/collection.htm) under the SZMC 22228 number, and the partial *gyrA* gene sequence in the European Nucleotide Archive under the HG964636 identifier.

Antifungal activity of *B. mojavensis* SZMC 22228 and its ferment broth

The isolated *B. mojavensis* SZMC 22228 effectively inhibited the growth of *M. canis*, *M. gypseum*, *T. mentagrophytes*, *T. rubrum* and *T. tonsurans* in different rates (Table 1). The cell-free ferment broth of *B. mojavensis* SZMC 22228 also showed antidermatophytic activity on these isolates in the applied agar diffusion test (Table 1). In these tests *M. canis* (58.0±10.5 mm inhibition zone caused by *B. mojavensis* SZMC 22228) and *M. gypseum* (18.3±2.1 mm inhibition zone caused by the cell-free ferment broth of *B. mojavensis* SZMC 22228) were the most susceptible dermatophytes (Table 1).

Purification of *B. mojavensis* SZMC 22228 antidermatophytic protein (BMAP) and its antifungal activity

Gel electrophoresis revealed the presence of a protein with a molecular mass of approx. 25 kDa in the purified fraction of the ferment broth which had antidermatophytic activity (BMAP, Figure 1). After purification the final yield of BMAP from three independent productions was 3.64±0.53 mg. One hundred sixty microgram per milliliter BMAP was able to inhibit the growth of *M. canis*, *M. gypseum*, *T. mentagrophytes*, *T. rubrum* and *T. tonsurans* causing inhibition halos with different diameters in agar diffusion tests (Table 1). *M. gypseum* (inhibition zone: 23.7±3.1 mm) was the most susceptible isolate (Table 1). Based on these results we suppose that the isolated ~25 kDa molecular weight BMAP plays a role in the antidermatophytic effect. The purified BMAP maintained

its antifungal activity against all investigated dermatophytes in broth microdilution susceptibility test, and also the *M. gypseum* proved to be the most susceptible fungus (MIC = 40 µg/ml). All the other dermatophytes were less susceptible (MIC = 80 µg/ml).

Enzymatic activity assays

BMAP did not show enzymatic activity towards N-acetyl-L-Leu-*p*NA, *p*-nitrophenyl-N-acetyl-β-D-glucosaminide, *p*-nitrophenyl-N,N'-diacetyl-β-D-chitobioside, *p*-nitrophenyl β-D-N,N′,N′′-triacetylchitotriose, *p*-nitrophenyl-β-D-cellobioside, *p*-nitrophenyl-β-glucopyranoside, *p*-nitrophenyl-N-acetyl-β-D-glucosaminide; but hydrolysed the N-α-bz-L-Arg-*p*NA, N-bz-Phe-Val-Arg-*p*NA, N-Succ-Ala-Ala-Pro-Phe-*p*NA proteolytic substrates. The most effective proteolytic activity was exerted on N-Succ-Ala-Ala-Pro-Phe-*p*NA (Figure 2). Based on these results and the amount of the released *p*-nitroaniline from digested proteolytic substrates (Figure 2), BMAP is considered as a chymotrypsin-like protease.

Discussion

Several *B. mojavensis* strains are considered as biocontrol agents because they can enhance plant growth and protect the plant against its fungal pathogens due to the produced surfactin, iturin, fengycin and various enzymes. Although, the factors which are responsible for growth enhancement are unknown but the antifungal mechanism of the biocontrol strains is well-characterized especially on *Fusarium verticillioides* (Bacon and Hinton 2011). Growth inhibition ability of *B. mojavensis* on other plant pathogenic (*e.g. Ambrosiella macrospora*, *Botrytis cinerea*, *Fusarium oxysporum*, *Fusarium moniliforme*, *Macrophomina phaseolina*, *Mucor rammanianus*, *Alternaria solani*, *Phytophthora meadii*, *Pythium aphanidermatum* and *Rhizoctonia solani*), and (opportunistic) human pathogenic (such as *Aspergillus flavus*, *Aspergillus niger*, *Candida utilis*, *Candida albicans* and *Rhizopus* spp.) fungi was demonstrated by previous studies (Nair et al. 2002; Maachiaet al. 2011; Youcef-Ali et al. 2014). In our work we observed that *B. mojavenis* SZMC 22228 can inhibit effectively the growth of different dermatophytes. Such kind antifungal activity of *B. mojavenis* has not been reported in the literature yet; however, data are available about the antidermatophytic effect of other *Bacillus* species. Previously, it was proven that *Bacillus amyloliquefaciens* HNA3 exerts growth inhibition effect on *T. mentagrophytes* (Nastro et al. 2011) and different *B. subtilis* isolates on *Trichophyton* and *Microsporum* spp. (Kumar et al. 2005, 2009; Lukmanova et al. 2008). Kumar et al. (2009) observed that a peptide belonging to the group of antifungal antibiotic peptides is responsible for this effect. Compared to this finding, a ~25 kDa extracellular chymotrypsin-like protease (BMAP) plays a role in the antidermatophyotic activity of *B. mojavensis* SZMC 22228.

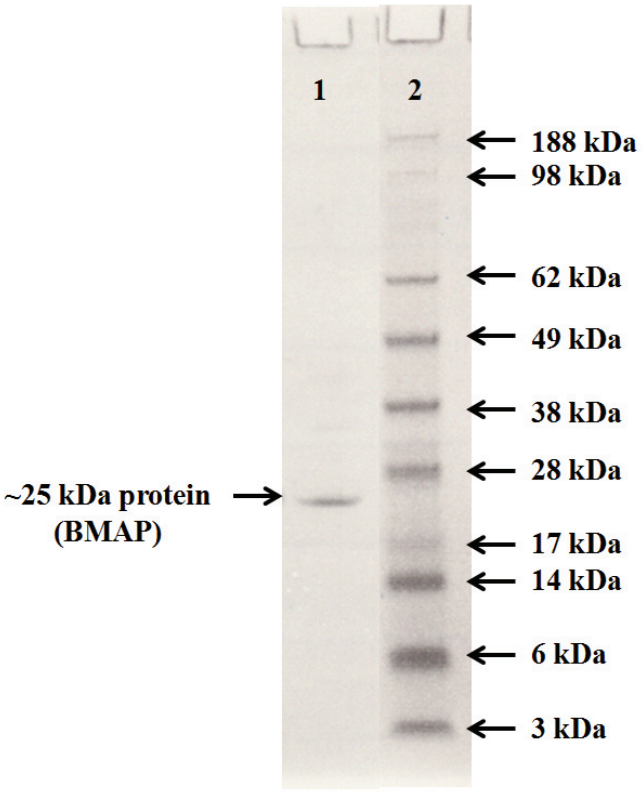

Figure 1. Bis-Tris SDS PAGE (4-12%) of the antidermatophytically active purified ~25 kDa protein (BMAP) from the ferment broth of *Bacillus mojavensis* SZMC 22228. Lane 1, purified ~25 kDa BMAP; lane 2, molecular weight marker (SeeBlue Plus2 Pre-Stained Standard, Invitrogen-Life Technologies, Eugene, OR, USA).

Numerous species of *Bacillus* have been involved in industrial protease production and *B. mojavensis* is also considered as a great source of different (alkaline) proteases (Schallmey et al. 2004). Three different alkaline proteases from *B. mojavensis* A21 strain have been isolated, which show differences in their molecular weight (15-29 kDa) and enzymatic features, such as specific activity, pH and thermal optimum/stability, and calcium-dependence (Haddar et al. 2009a, 2009b). Proteases showing same substrate specificity as BMAP were isolated from the ferment broth of *B. mojavensis*-related *B. subtilis* strains. A 30 and 43-46 kDa molecular weight fibrinolytic enzymes with high activity to N-Succ-Ala-Ala-Pro-Phe-*p*NA chromogenic substrate were purified from the ferment broth of *B. subtilis* DC33 and *Bacillus* sp. nov. SK006 (Wang et al. 2006; Hua et al. 2008). The antifungal activity of these proteases has not been investigated yet, but few reports demonstrated a correlation between the biocontrol function of *Bacillus* strains and their extracellular protease production (Luo et al. 2013). Information about the

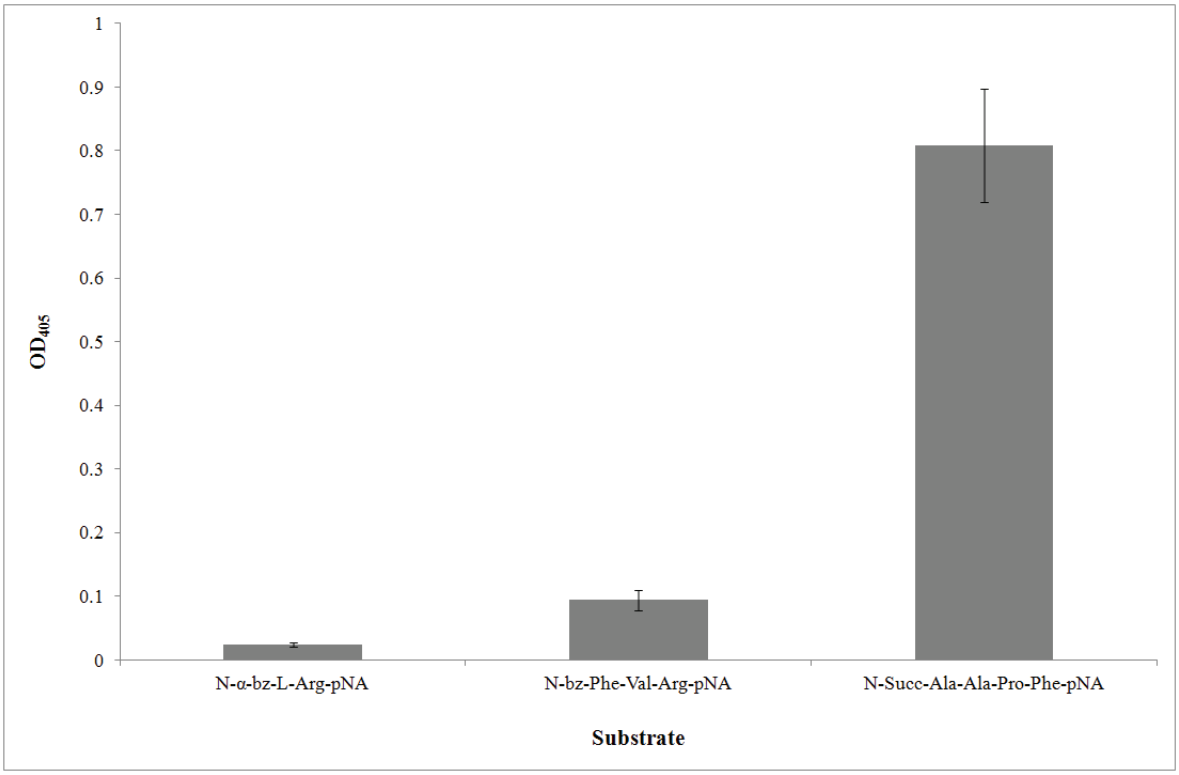

Figure 2. Substrate activity of the of the purified ~25 kDa antidermatophytic protein (BMAP) at 30 °C after 3 h. Standard deviations (±SD) derived from three replicates (N=3).

antifungal activity of extracellular proteases is available in the case of *B. mojavensis*-related *B. subtilis*. Its culture filtrate which had protease activity inhibited the growth of aflatoxigenic Aspergilli (Thakaew and Niamsup 2013). Basurto-Cadena et al. (2012) found connection between the presence of proteases and antagonistic activity of *B. subtilis* against plant pathogenic fungi such as *F. verticillioides* and *R. solani*. In a more detailed study, it was observed that the 41.38 kDa alkaline protease P6 from *B. subtilis* N7 effectively inhibits the spore germination and mycelial growth of *F. oxysporum* f.sp. *cucumerium* (Luo et al. 2013). Until the present study, antidermatophytic effect of bacterial proteases has not been described in the literature.

Our results indicate that the isolated ~25 kDa BMAP could be a promising agent in the treatment of dermatophytosis, however, further *in vitro* and *in vivo* investigations and characterization experiments (such as MALDI-TOF analysis, N-terminal sequencing, enzyme kinetics, pH and thermal optimum/stability, ion-dependence, hematolytic and toxic effect, etc.) are needed.

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