Fungicidal activity of alpha-melanocyte stimulating hormone in specific solvent augmented at explicit pH against *Candida albicans*.

POONAM SINGH[1]

[1]*School of Environmental Sciences, Jawaharlal Nehru University, New Delhi, India-110067*

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ABSTRACT

Objective: Fungal infections are fourth most common cause of nosocomial bloodstream infections. *Candida* species contributes to majority of infections and among them, *Candida albicans* has emerged as most frequent opportunistic pathogen that causes systemic and mucosal infections. Its control is difficult in immunocompromised individuals because of emergence of multidrug-resistance as well as insufficiency in inhibitory potential of available drugs that pose challenge in controlling invasive mycosis. Additionally, antimycotic drugs exert multiple adverse effects and are occasionally dose limiting. In such circumstances, it has become crucial to find novel antifungal agent and its targets.

Methods: In this study, the antifungal potential of α-MSH and its mechanism of action was evaluated. *In vitro* fungicidal activity of α- MSH against *C. albicans* at range of concentrations, in different media, buffers, solvent (sterile water), at various pH and time points were examined. In order to understand its mode of action: membrane permeabilisation assay, electron microscopic and circular dichroism studies were performed.

Results: This study validate that α-MSH exhibits strongest activity in water as compared to any growth media and buffers. It also shows that it possesses strong and rapid antifungal activity at specific pH against *C. albicans* which was mediated via conformational change in water that leads to membrane permeabilisation and disruption of cell wall structure.

Conclusions: The study revealed that α-MSH activity is concentration, pH, solvent dependent and acquire characteristic conformation in water that is responsible for its candidacidal activity.

KEY WORDS: *Candida albicans*, Cell wall damage, Circular dichroism, Membrane permeabilisation, α-MSH

Introduction

Fungal infection is becoming serious medical problem because of difficulty of its control in immunocompromised patients and due to emergence of multidrug resistance against known conventional drug [1-4]. Recently, its incidences of infections have steadily increased and they have become the fourth most common cause of nosocomial bloodstream infection leading to death [5]. The major agents of fungal infections are *Candida* species, and among them, *C. albicans* accounts for about 50 to 60% of overall yeast isolates [6,7]. *C. albicans* is an opportunistic fungal pathogen, frequently found in the oral flora of normal individuals where it causes mucosal infectious diseases ranging from superficial to systemic [8]. Its infection is frequently associated with low-birth-weight infants, patients with human immunodeficiency virus, prolonged chemotherapy induced neutropenia, immuno-suppressant following transplantation or severe burns and growing use of implanted medical prosthetic devices and catheters [9,10]. These phenomena are aggravated by the rapid development of resistance against most of currently used antifungal drugs. Therefore, it is imperative to find novel antifungal agent and its targets. Eukaryotic organisms, from human to plants to insects all are well equipped with various proteins and peptides that constitute integral parts of innate immune system [11]. Host innate defense system are characterized by production of potent antimicrobial molecules that limit infection based on their capacity to selectively discriminate pathogens from self species [12]. These molecules are termed as 'Antimicrobial peptides (AMPs)'.

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Correspondence to: Dr. Poonam Singh
Email: poonam2018bio@gmail.com

One such AMP is α-MSH, it is cationic endogenous linear tridecapeptide with potent anti-inflammatory and anti-pyretic effects [13-15]. Its sequence is: Ac-Ser_1-Tyr_2-Ser_3-Met_4-Glu_5-His_6-Phe_7-Arg_8-Trp_9-Gly_{10}-Lys_{11}-Pro_{12}-Val_{13}-NH_2, and produced by post-translational processing of the larger precursor molecule proopiomelanocortin (POMC) [16,17].

α-MSH reduces production of proinflammatory mediators by host cells. It inhibits the activation of transcription factor NF-kB and thus regulates several proinflammatory cytokines, stimulates the production of anti-inflammatory IL-10 and reduces upregulation of intercellular adhesion molecule1 (ICAM-1) [18]. It is produced by many cell types including peripheral, central phagocytes and by keratinocytes [19]. It has been observed that α-MSH exhibits antimicrobial activity against widely spread organisms including, *C. albicans*, *Staphylococcus aureus* and *E. coli*. It has been reported that the active message sequence resides in the C terminal tripeptide α-MSH [(11-13) KPV] which has potent anti-inflammatory influences *in vivo* and *in vitro* that parallel those of the parent molecule. Further, it was revealed that the candidacidal effect of α-MSH may be mediated through induction of cyclic adenosine monophosphate (cAMP), a mediator of signalling pathway [20]. However its exact mechanism of action is not very clear. Although the anti-inflammatory properties of α-MSH are well documented, its antifungal mechanism is little understood. The aim of present study was to evaluate antifungal potential of α-MSH and also elucidate its mechanism of action against human pathogenic yeast *C. albicans*.

Materials and Methods

Yeast strain and its growth condition

C. albicans strain CAF 2,1 was grown in Yeast Extract-Peptone-Dextrose (YEPD) agar and were incubated at 30 °C. This strain was maintained in 15 % glycerol stock solution at -80 °C and freshly revived on YEPD agar plate before use and transferred into 10 ml of YEPD broth in a 50 ml falcon tube. After 16 h of incubation at 30 °C, 300 µl from this suspension was sub-cultured for 5 h in 100 ml of YEPD broth to obtain log phase culture. Before experiments, the cells were washed twice with Phosphate buffer saline (PBS). The cell turbidity was adjusted to OD_{600} = 0.1 in PBS that corresponds to 10^6 cfu/ml.

Antifungal activity of α-MSH

Antifungal assay of α-MSH and other conventional drugs such as Amphotericin B (AMB) and Fluconazole (FLC) was performed in RPMI-1640 media *in vitro* through broth microdilution procedure. The protocol was based on the recommendations of the Clinical and Laboratory Standards Institute (CLSI) method M27-A [21,22].

Candidacidal activity of α-MSH in media, buffers, water at various pH

Log phase cells were inoculated into sterile water, RPMI, YEPD, Sabouraud dextrose broth (SDB), Phosphate buffer (PB), PBS, Sodium citrate (SC) containing α-MSH (2 µM) and incubated for 60 min. at 30 ºC, 200 rpm. There after 100 µl aliquot was removed, diluted 10 times with water, plated on YEPD agar plates and incubated at 30 ºC for 24 h. After 24 h of incubation at 30 ºC, colony forming units (CFUs) were evaluated by counting. Same protocol was also followed for assessment of its activity in different pH of water, RPMI and SDB. All experiments were performed in triplicate [23].

Membrane permeabilisation assay

Propidium iodide (PI) is a membrane impermeable dye. It does not give any fluorescence but when it enters in membrane-compromised cells, its fluorescence is enhanced by 20-30 folds on binding with nucleic acid. For study of membrane permeabilization by α-MSH in different media, buffers and water, strain CAF 2,1 suspended in appropriate media and buffer along with 2 µM α-MSH for 60 min. at 200 rpm, 30 ºC. After incubation, PI (1.5 µM) was added to cell suspension for 20 min, harvested by centrifugation and suspended in water [24]. The cells were examined with Olympus Fluoview TM FV1000 confocal microscopy system (Olympus, Japan) equipped with a HeNe (G)/Ar laser with 100X 1.4 NA oil objective lens and 543 nm wavelength was used for PI. Untreated cells served as a control.

Antifungal activity of α-MSH in various concentration range

Initially, antifungal activity of α-MSH was assayed at lower concentration i.e. 2 µM and found that it has effective candidacidal activity. To examine whether antifungal activity of peptide was concentration dependent or not, a broad range of concentration from nM to µM were used to study killing kinetics in similar manner as described above in case of different media, buffer and water.

Circular dichroism (CD)

To study conformational change in water, media and buffer CD spectrophotometer of type Sx20 stopped flow spectrometer was used. Average of five scans represent spectrum (190-260 nm) by using Quartz cell of 1mm path length run with scanning speed of 0.5 s per point for 2 s response time and 0.1 nm band width at room temperature. Spectrum

of α-MSH was measured in media, water and buffers and 70 % 2,2,2-trifluoroethanol (TFE; mimic membrane environment) taken as a positive control for helical structure and blank spectrum of all without peptide counted as a base line. The acquired spectra were converted into mean residue ellipticity $[\theta]_{MR} = \theta_{obs} \times M_{RW})/(cxl)$, where θ_{obs} is the observed ellipticity corrected for the buffer at given wavelength (mdeg), M_{RW} is residue mol. Wt (mol. Wt/number of backbone amides), c is peptide concentration (mg/ml) and l is path length (mm) [25].

Electron microscopic studies

Scanning electron microscopy (SEM) was used to investigate mechanism of action of α-MSH with CAF 2,1 strain. Freshly revived cells in PBS was treated with α-MSH. The controls were run without peptide i.e. considered as untreated. After incubation at 37 ºC for 1 h, the cells were pelleted by centrifugation at 4226g for 8 min. followed by washing 3-4 times with 0.1 M PBS and then fixed with 2 % glutaraldehyde in PBS at 4 ºC for 1 hr, washed with PBS 3-4 times. After rinsing with PBS, cells were fixed in 1 % Osimum tetraoxide (OsO_4) for 1 h at 4 ºC, dehydrated with acetone and dropped on round glass cover slip with hexamethyldisilizane (HMDS, Sigma) for 20 min. and allowed to evaporate at room temperature for drying. Samples was sputtered with small amount of gold for avoiding the charge in the microscope. Microscopy was performed with Carl Zeiss EV040, (Germany) at 20 keV. Transmission electron microscopy (TEM) was performed to investigate ultra structure of *Candida* cells. Dehydration of samples was completed with graded acetone and cleared in toluene. At room temperature, the sample was infiltrated with Araldite CY212 and toluene mixture. Final filtration was done at 50 ºC in pure araldite and poured in Eppendoff tube. Ultramicrotome was used to cut semi-thin and ultra-thin sections (Ultramicrotome Lecia EM UC6). Copper grid of 3.05 mm diameter and 200 mesh was used to hold sections which was stained with 2 % Uranyl acetate (saturated with 50 % alcohol) followed with Lead citrate. Thereafter, the sections were placed for observation under TEM (Jeol-JEM 2100F, Japan) at 120 keV [23,24,26].

Statistical analysis

Data entry and analysis were performed using MS-Excel 2007. The data were expressed as mean ± SD and percentages.

Results

Antifungal activity of α-MSH

α-MSH sequence and its properties are reported (Table 1) where values of ΔPhe and GRAVY are calculated according to ProtParam, Expasy Server. ΔPhe (α, β didehydrophenylalanine) is a non-natural amino acid, inserted on hydrophobic face of any synthetic peptide to preserve its amphipathicity. Reported that the presence of more than one ΔPhe restrain the peptide in a 310 or α-helical conformation and increases its relative stability towards proteolytic degradation [27,28]. GRAVY (Grand Average of Hydropathy) is a simple method for displaying the hydropathic character of a protein. It is calculated as the sum of hydropathy values of all the amino acids, divided by the number of residues in the sequence [29].

Table 1. Sequence of α-MSH and its properties.

Peptide	Sequence[a]	Length	ΔPhe	Mw[b]	Charge[b]	pI[b]	GRAVY[b]	Extinction Coefficient ($M^{-1}cm^{-1}$)[b]
α-MSH	SYSMEHF RWGKPV	13	0	1623.8	+1	8.33	-0.923	6990

aOne letter amino acid code is shown to indicate peptide sequences
bCalculated values according to ProtParam at the Expasy Server (http://web.expasy.org/cgi-bin/protparam/protparam).

To assess the antifungal activity of α-MSH against CAF 2,1 strain along with AMB and FLC, minimum inhibitory concentration (MIC) assays were performed. The MIC_{100} is defined as the concentration which inhibits the growth of cells by 100 %. AMB and FLC showed MIC 0.032 µg/ml and 0.244 µg/ml respectively for CAF 2,1 while no detectable MIC value was found for α-MSH in RPMI-1640 media (Table 2).

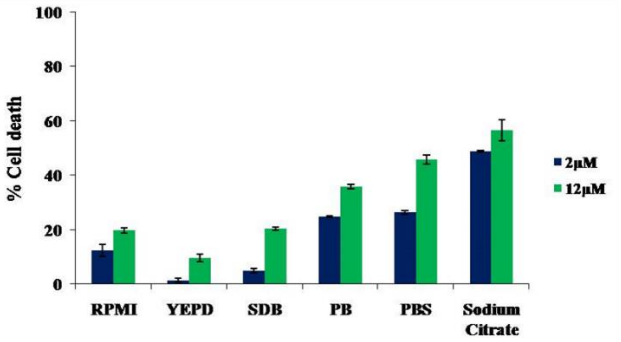
Table 2. MIC_{100} values (µg/ml).

Drugs / Peptide	Concentration (µg/ml)
Amphotericin B (AMB)	0.032
Fluconazole (FLC)	0.224
Alpha-Melanocyte Stimulating Hormone (α-MSH)	No Activity

Candidacidal activity of α-MSH in different media, buffers and water

Microdilution assay of α-MSH with RPMI was found to be negative, there was a need to search better media or buffer for α-MSH in which it could show better candidacidal activity. For this spread assay was performed to elucidate the activity of α-MSH at two different concentration (2 µM and 12 µM) in different media and buffers. It was seen that percentage growth inhibition (i.e. % cell death) at lower peptide concentration (2 µM): 12.4 % (RPMI), 1.4 % (YEPD), 4.9 % (SDB), 24.8 % (PB), 26.3 % (PBS), 48.8 % (SC); while at higher concentrations (12 µM): 19.7 % (RPMI), 9.6 % (YEPD), 20.4 % (SDB), 35.8 % (PB), 45.8 % (PBS), 56.6 % (SC) (Figure 1). The selected media and buffers were not able to cause 100 % cell death at both lower or higher concentration of α-MSH. Previous study suggested that AMPs which are effective at or below 3 µM are considered as active [30].

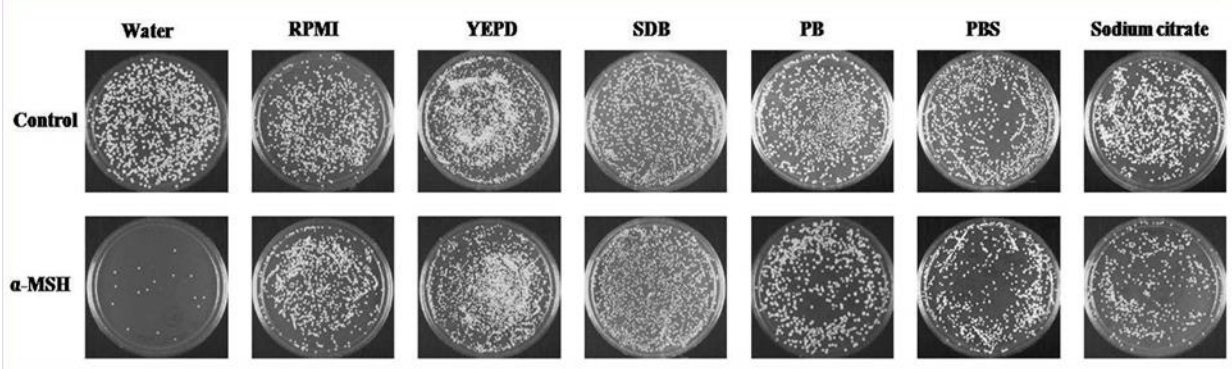
Figure 1. Activity of α-MSH in different media and buffers. Estimation of its activity at two different concentrations of α-MSH (2 µM and12 µM) in different media and buffers at 60 min.



Another study reported that α-MSH and its synthetic derivative exercise its antimicrobial activity against infectious agents that were suspended in physiologic solution or in water but in culture media no activity was observed [31]. In another study the well known peptide 'Defensin' also showed best activity in water [32].

These conclusions lead us to use sterile water as a solvent for activity of α-MSH at lower concentration. We performed candidacidal activity of α-MSH (2 µM) in sterile water along with selective media and buffer for comparative study by spread assay. After overnight incubation, it was observed that no CFUs was appeared in plate where cells were incubated with α-MSH in water as compared to selected media and buffers (**Figure 2**). These findings confirmed that water is best solvent for activity of α-MSH.

Figure 2. Comparison of α-MSH activity in different media, buffers and solvent (water). Antifungal activity of α-MSH in different media, buffers and water for 60 min. at 30 ºC. The results were obtained by counting the colony forming units after 24 h.



pH dependent activity of α-MSH

Since antifungal activity of α-MSH was sensitive to media composition, the effect of pH on its activity was assessed. Spread assay was performed in water, RPMI-1640 and PBS at 30 ºC for 24 h and observation were made by CFUs. It was observed that the fungicidal efficacy of α-MSH with variation in pH of water, RPMI-1640 and PBS within the range of 4- 8 (Figure 4). It is evident from % killing, the activity of α-MSH was best in water at pH 6.8 (> 90 % killing). The activity of peptide was very sensitive to media pH which fell sharply thereafter. None of the other media showed any improvement in % killing of *Candida* cells, however, PBS at pH 8 showed 50 % killing.

Concentration and time dependent activity of α-MSH

After confirmation of water as a best solvent for antifungal activity of α-MSH, a range of its concentrations (from nM to μM) were prepared for determination of MIC_{100} and CAF 2,1 cells were incubated with these concentrations at different time points from 0 to 120 min. Cells were exposed at different concentrations of peptide, grown after indicated time of incubation and observed ~ 90 % and 100 % cell death for 2 μM and 12 μM concentration, respectively (Figure 3A). It is evident that % killing increases with increasing concentration of peptide which was maximum after 120 min. of incubation. From these observations, 2 μM of α-MSH (MIC_{90}) was used as a MIC for further experiments. The dependence of its antifungal activity on time was further established by counting CFUs of CAF 2,1 cells before and after treatment with 2 μM of α-MSH which kills cells in 120 min. Interestingly, the well known potent antifungal FLC was unable to kill *C. albicans* cells completely at MIC 4.01 μM confirming its fungistatic activity (Figure 3B).

PI uptake assay

To investigate the mode of action of α-MSH on *Candida* cell in different media, buffer and water, PI uptake assay was performed to study membrane permeabilisation by peptide. Incubation of *Candida* cells with α-MSH resulted in PI uptake by the cells, confirmed by Confocal microscopic images and by measuring fluorescent intensity using Olympus software FV 10- ASW21. It was found that α-MSH causes membrane disruption in water by measuring red fluorescent signal of PI in membrane compromised cells in comparison to others (Figure 5A and B).

Figure 3. Candidacidal activity of α-MSH at various concentrations and time points. (A) Killing kinetics of CAF 2,1 by α-MSH at different concentrations (from nM to μM) at different time points from 0 to 120 min. (B) Activity of α-MSH and FLC as a function of time.

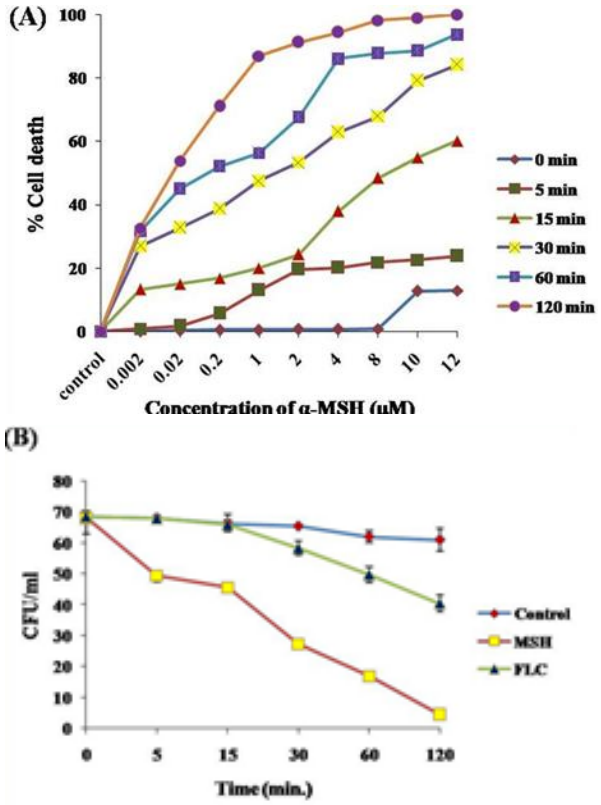
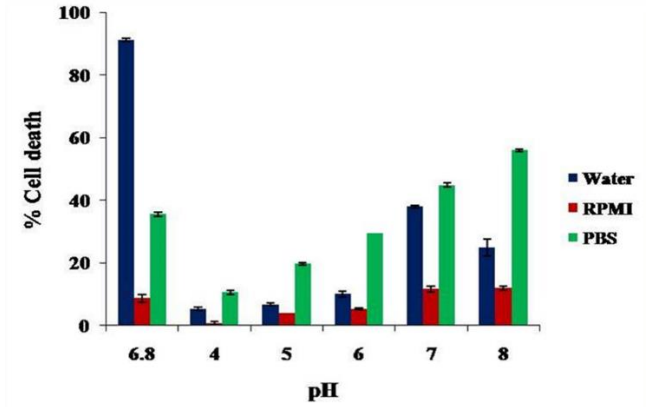


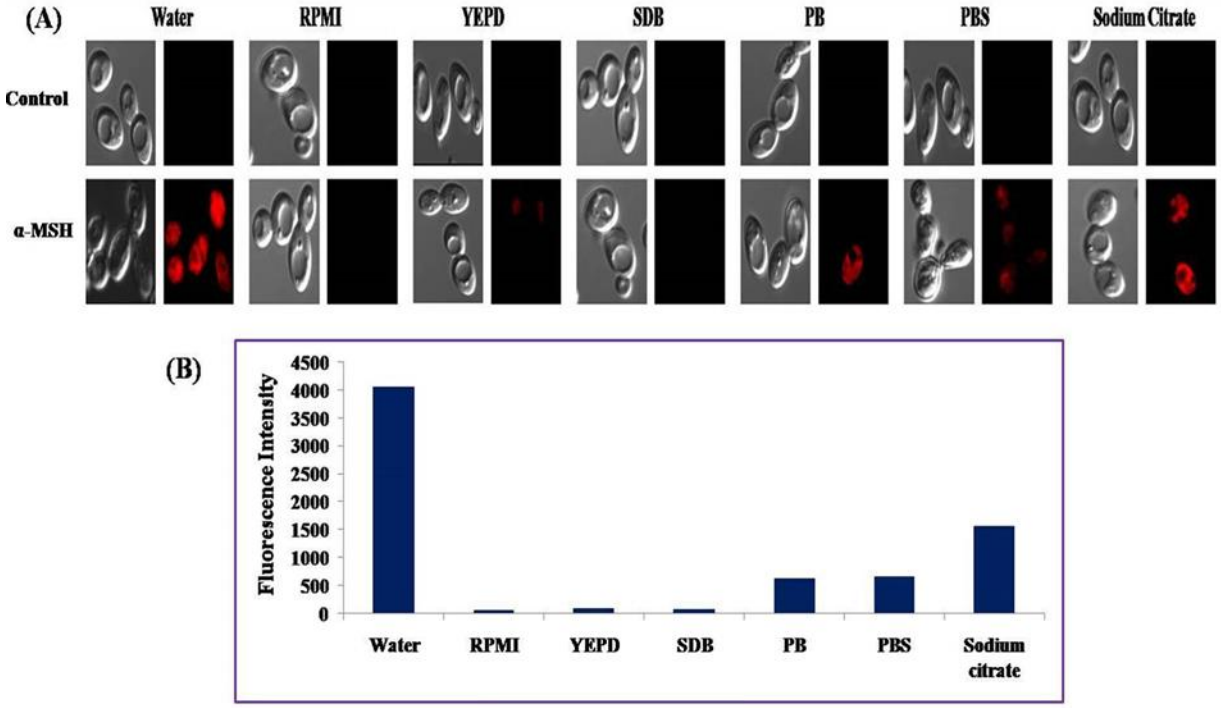
Figure 4. Fungicidal activity of α-MSH at different pH. Antifungal activity of α-MSH (2 μM) in different pH of water, RPMI and PBS for confirmation of its pH dependency at 60 min.



CD spectra analysis

The amino acid sequences of AMPs possess ability to adopt different conformation depending upon their solvent environment, inside a protein or upon the interaction between an enzyme and its substrate [33,34]. Most of the AMPs attain characteristic α-helical conformation that helpful in permeabilisation of the lipid bilayer of the cytoplasmic membrane which cause dissipation of transmembrane

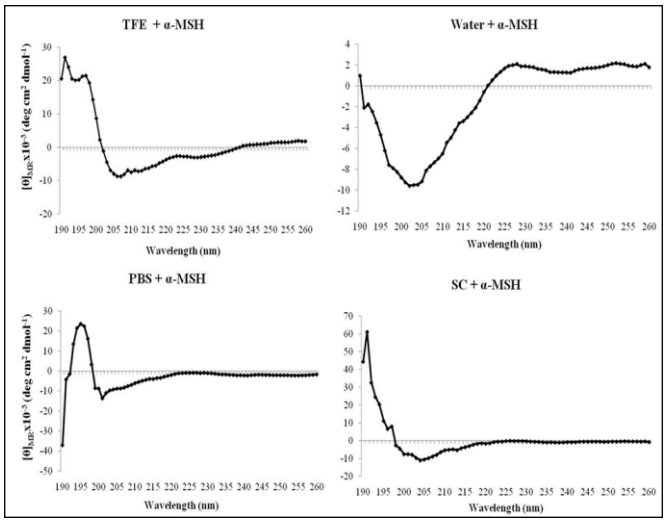
Figure 5. Membrane disruptive activity of α-MSH. (A) Confocal microscopic analysis in water as compared to other media and buffers. (B) Fluorescent intensity of PI uptake by membrane compromised *Candida* cells.



potential resulting cell death [35,36]. The cytotoxic function of other amphipathic AMPs depend on conformational switch regions with equal probability for different conformations. These AMPs having random coil initially that electrostatically associated with membrane surface followed with intercalation into lipid bilayer subsequently conformation change from random coil to an amphipathic α-helix ensuing ion-pairing of acidic phospholipids with positive residues of the peptide. Ultimately peptide reorient itself and inserted into the membrane perpendicularly to the monolayer surface to create pore in membrane [37].

To investigate conformational change in α-MSH by CD analysis in sterile water (solvent), different buffer (PBS and SC), media (RPMI-1640 and YEPD) and 70 % TFE. TFE is commonly considered as a helix-promoting solvent. TFE/H_2O mixtures are used widely to stabilize peptide helices [38]. This study revealed that change in structural conformation of α-MSH is responsible for candidacidal activity in water as compared to different media and buffers. It was found that in water α-MSH acquire random coil while in TFE it acquires α-helical conformation. Though in buffers α-MSH possess β-turn aggregation (Figure 6), whereas in media no peak was observed and background noise was high, it indicates that media components inhibit structural change in α-MSH.

Figure 6. CD spectrometry studies of α-MSH in water, buffers (PBS, SC) and TFE. Study was carried out on a Sx20 stopped flow spectrometer at scanning speed of 0.5 s per point for 2 s response time by using Quartz cell of 1mm path length over the wavelength range 190- 260 nm. Results are expressed in molar ellipticity (deg·cm^2·dmol^{-1}).

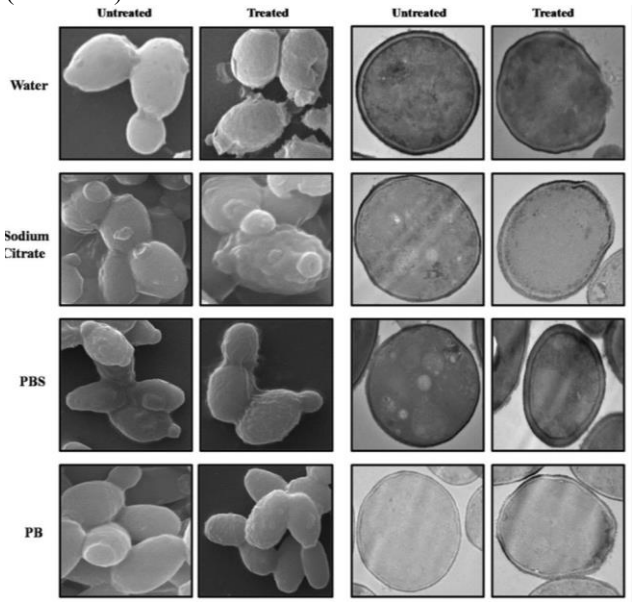


Electron microscopic studies

From CD and membrane permeabilisation observations it was substantiated that α-MSH causes membrane disruption by damaging the cell wall. To gain further insight into this *Candida* cells were treated with α-MSH (2 μM) for 60 min. at 30 ºC and examined by Electron microscopy (EM). SEM images show that integrity of membrane was grossly af-

fected as evident from the roughened cell surface as compared with smooth surface of untreated cells (Figure 7A). α-MSH induced breakage in cell wall ensuing release of cellular content that is clearly visible in the images of TEM (Figure 7B). EM micrographs confirmed that cell wall damage followed by cell membrane rupture resulting cell death.

Figure 7. Electron microscopic analysis shows cell wall damage by α-MSH. Electron micrographs of α-MSH treated *C. albicans* cell for 60 min. in water, SC, PBS and PB. (A) SEM images indicating cell wall damage by α-MSH (2 μM). (B) TEM images show significant cell wall damage and leakage of cellular contents. The control (untreated) is without α-MSH.



Discussion

α-MSH is cationic tridecapeptide with potent anti-inflammatory and anti-pyretic effects. However, its antifungal potential and molecular mechanisms are poorly understood. In this study antifungal activity of α-MSH was performed according to CLSI guidelines against *C. albicans* along with AMB and FLC. Both AMB and FLC showed expected MICs whereas no killing effect observed in case of α-MSH, this demonstrated that activity of α-MSH was sensitive to media (RPMI-1640) composition. Not only media composition even pH of the media has strong influence on its activity. By employing water, media and buffer (PBS) within pH range of 4 to 8, it was shown that the activity of peptide was very sensitive to pH of the media, as no improvement in percentage killing of *Candida* cells was observed while PBS at pH 8 showed 50 % killing. It was evident from the percentage killing that the activity of the peptide was best (> 90 % killing) in water at pH 6.8.

To determine MIC, a range of concentration (nM to μM) was used to perform killing assays. It was observed that the percentage killing increased with increase in concentration of peptide which was maximum after 120 min. of incubation. Several studies reported that majority of peptide utilizes membrane disruption as an important mode of action that are initiated by change in structural conformation. For understanding its potential mechanism we performed CD studies and it was confirmed that α-MSH prefer random coil conformation for performing antifungal activities which prevails in water as compared to buffer and media. Further it was supported by PI uptake assay which showed that peptide α-MSH was able to permeabilised the *Candida* cell membrane rapidly within 5 min. of treatment. It is evident by the time dependent increase of fluorescence signal of PI as visualized by confocal microscopy and by measuring its fluorescent intensity. These finding confirmed a direct relationship between conformational change and membrane permeabilisation viz. responsible for the leakage of cellular content leads to cell death. Electron microscopic images confirmed that α-MSH treatment result in permeabilisation of *Candida* cells following extensive cell wall damage that result in cell death.

This study revealed that α-MSH perform rapid and concentration dependent fungicidal activity at specific pH in water where it acquires random coil conformation. This characteristic change in conformation causes membrane perturbation in compromised cells, it causes leakage of cellular content that ultimately leads to cell death as confirmed by EM micrographs. Collectively the study suggests that α-MSH activity is concentration, pH, solvent dependent and particular conformation change in water responsible for its candidacidal activity.

Conflict of Interest

I declare that I have no conflict of interest.

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