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Antifungal properties and mode of action of psacotheasin, a novel knottin-type peptide derived from *Psacothea hilaris*

Bomi Hwang [a,1], Jae-Sam Hwang [b,1], Juneyoung Lee [a], Dong Gun Lee [a,*]

[a] School of Life Sciences and Biotechnology, College of Natural Sciences, Kyungpook National University, Daegu 702-701, Republic of Korea
[b] National Academy of Agricultural Science, Rural Development Administration, Suwon 441-100, Republic of Korea

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ABSTRACT

Psacotheasin is a 34-mer knottin-type peptide that is derived from *Psacothea hilaris* larvae. In this study, the antifungal activity and mechanism(s) by which psacotheasin affects human fungal pathogens were investigated. Psacotheasin shows remarkable antifungal properties without hemolytic activity against human erythrocytes. To understand the antifungal mechanism(s) of psacotheasin in *Candida albicans*, flow cytometric analysis with $DiBAC_4(3)$ and PI was conducted. The results showed that psacotheasin depolarized and perturbed the plasma membrane of the *C. albicans*. Three-dimensional (3D)-flow cytometric contour-plot analysis, accompanied by decreased forward scatter (FS), which indicates cell size, confirmed that psacotheasin exerted antifungal effects via membrane permeabilization. The membrane studies, using a single GUV and FITC-dextran (FD) loaded liposomes, indicate that psacotheasin acts as a pore-forming peptide in the model membrane of *C. albicans* and the radius of pores were presumed to be anywhere from 2.3 to 3.3 nm. Therefore, the current study suggests that the mechanism(s) of psacotheasin's antifungal properties function within the membrane.

1. Introduction

Every living organism, from prokaryotes to plants, insects, amphibians and mammals, including humans, is constantly confronted with attacks by various kinds of pathogens [1]. To defend themselves against infection by microbes, all organisms produce antimicrobial peptides (AMPs) as a major part of their immediately effective defenses. Many naturally-occurring AMPs have a wide spectrum of activity and high selectivity because of share the following features: cationicity, hydrophobicity, and amphipathcity [2]. Interestingly, they physically and swiftly interact with the cell plasma membrane, causing the increase of permeability and loss of membrane function, in contrast to conventional antibiotics which have specific molecular targets. Therefore, the development of resistance to AMPs is thought to be considerably reduced when compared with that of many current antibiotics [3].

Insects represent the largest class within the animal kingdom in terms of species number and have self-defense systems such as passive structural barriers, innate cellular and humoral immune reactions. As a powerful defense against harmful invading pathogens, insects transiently synthesize humoral factors like AMPs and secrete them into their hemolymph (blood) [4]. So far, more than 200 AMPs have been found in insects. Insect AMPs are typically cationic, and often consist of less than 100 amino acid residues. Although their structures are diverse, most of the peptides belong to a limited number of families [5].

Psacotheasin (CIAKGNGCQPSGVQGNCCSGHCHKEPGWVAGYCK-NH_2), a new knottin-type antimicrobial peptide, was isolated from the yellow-spotted long-horned beetle, *Psacothea hilaris*, and was found to be active against human pathogenic bacterial strains [6]. The primary structure of psacotheasin is characterized by a knottin-like cysteine motif [7]. Knottins are most frequently structured with 30 amino acids in length and have various biological functions. For example, many are toxic, enzyme inhibitory, antimicrobial, insecticidal, cytotoxic, or have anti-HIV properties or hormone-like activity. They typically share a unique knotted pattern characterized by three disulfide bonds. This is alternatively referred to the so-called cysteine knot [8].

In this study, the antifungal properties of psacotheasin against human pathogenic fungal strains were investigated. Additionally, the antifungal mechanism(s) of psacotheasin and its potential as a novel antifungal peptide were explored.

Abbreviations: MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide; $DiBAC_4(3)$, bis-(1,3-dibutylbarbituric acid) trimethine oxonol; PI, propidium iodide; ITO, indium tin oxide.

* Corresponding author. Address: School of Life Sciences and Biotechnology, College of Natural Sciences, Kyungpook National University, 1370 Sankyuk-Dong, Puk-Ku, Daegu 702-701, Republic of Korea. Fax: +82 53 955 5522.
E-mail address: dglee222@knu.ac.kr (D.G. Lee).

[1] These authors equally contributed to this work and should be considered co-first authors.

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2. Materials and methods

2.1. Peptide synthesis

Peptides were synthesized by the solid phase method using the Fmoc (9-fluorenyl-methoxycarbonyl) chemistry [9,10]. The crude peptide was repeatedly washed with diethyl ether and dissolved in 10 ml of 0.1 mM ammonium bicarbonate, 50 ml of H_2O and 50 ml of acetonitrile. The mixture was dried under a vacuum after salts were excluded, and purified using reversed-phase preparative HPLC on a Waters 15-μm Delta Pak C_{18} Column (19 × 30 cm). The purity of the peptide was checked by analytical reversed-phase HPLC on an Ultrasphere C_{18} column, (4.6 × 25 cm) (Beckman, Fullerton, CA, USA). The molecular weight of the synthetic peptide was determined using a matrix-assisted laser desorption ionization (MALDI) mass spectrometer [11].

2.2. Fungal strains and antifungal activity assay

Candida albicans (ATCC 90028) and *Candida parapsilosis* (ATCC 22019) were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). *Trichosporon beigelii* (KCTC 7707) and *Malassezia furfur* (KCTC 7744) were obtained from the Korean Collection for Type Cultures (KCTC) at the Korea Research Institute of Bioscience and Biotechnology (KRIBB) (Daejeon, Korea).

The fungal strains were cultured in YPD broth (Difco) with aeration at 28 °C, and the *M. furfur* was cultured at 32 °C in a modified YM broth (Difco) containing 1% olive oil. Fungal cells in the exponential phase (2×10^6/ml) were injected into 0.1-ml/wells of YPD or YM broth, and then dispensed in microtiter plates. Minimum inhibitory concentrations (MICs) were determined by a serial two-fold dilution of the test peptides, followed by a micro-dilution method [12] and MTT assay [13]. After 48 h of incubation, the minimal concentration of peptides required to prevent the growth of the microorganisms was determined, and was defined as the MIC. The growth was measured with a microtiter ELISA Reader (Molecular Devices Emax, CA, USA) by monitoring absorption at 580 nm. The MIC values were determined by three independent assays [14].

2.3. Hemolytic activity assay

The hemolytic activity of the peptides was evaluated by measuring the absorbance at 414 nm with an ELISA reader to determine the release of hemoglobin from a 4% suspension of human erythrocytes [15]. The hemolysis percentage was calculated using the following equation: hemolysis (%) = 100[(Abs_{414nm} in the peptide solution − Abs_{414nm} in saline)/(Abs_{414nm} in 0.1% Triton X-100 − Abs_{414nm} in saline)].

2.4. Change of the plasma membrane potential

For the analysis of the membrane integrity after peptide treatment, exponential phase *C. albicans* cells (2×10^4/ml) were harvested and resuspended in 1 ml YPD broth containing either psacotheasin or melittin at 5× the MIC. After incubation for 3 h, the cells were harvested by centrifugation and suspended in PBS, then treated with 50 μg $DiBAC_4(3)$ for 30 min at 4 °C in the dark [16]. Flow cytometric analysis was performed via a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

2.5. Flow cytometric analysis for membrane permeabilization and morphological change of C. albicans

To analyze the fungal membrane permeabilization and morphological changes of after peptide treatment, *C. albicans* cells (2×10^4/ml) were treated with the peptides at 5× the MIC and incubated for 3 h at 28 °C. After incubation, cells were harvested by centrifugation and suspended in PBS. Subsequently, the cells were treated with 9 μM PI and incubated for 5 min at room temperature. The cells were analyzed by a FACSCalibur flow cytometer [17–19].

2.6. Preparation and microscopic observation of a single GUV

Giant phospholipid unilamellar vesicles (GUVs) were prepared by the electroformation method as described by Angelova and Dimitrov [20,21]. A lipid solution of phosphatidylcholine (PC)/rhodamine-conjugated phosphatidylethanolamine (PE)/phosphatidylinositol (PI)/ergosterol (5:4:1:2, w/w/w/w) was prepared in chloroform at a concentration of 3.75 mg/ml. The lipid solutions were coated on ITO-coated glass for 5 min in the spin coater (Spin Coater, ACE-1020 Series), and then evaporated under a vacuum for 2 h. Next, a square frame was created from silicon to serve as a (2-mm thick) spacer between the lipid-coated glass and normal glass. The chamber was filled with 10 mM HEPES buffer (pH 7.2) through a hole in the silicon spacer. A 1.7-V pp (peak-to-peak) and 10-Hz sine wave was immediately applied to the ITO electrodes for 2 h at room temperature. GUVs from the ITO glass were then detached under the application of a 4-V pp and 10-Hz sine wave for 10 min. Ten microliters of GUV aliquots solution were place on an inverted fluorescence phase-contrast microscope (Leica, DFC 420C), and peptide solutions were added after the selection of a single GUV.

2.7. Preparation of dextran-loaded liposomes and leakage experiments

FITC-labeled dextrans (FD10 and 20; Sigma, USA) were used to evaluate the extent of the membrane damage induced by the peptides. To prepare FD-entrapped liposomes, PPB buffer (1 ml, 50 mM potassium phosphate, pH 7.4, with 0.1 mM EDTA) containing 2 mg/ml FD, was sonicated on ice for 30 min with 20 mg/ml lipid solution composed of PC/PE/PI/ergosterol (5:4:1:2, w/w/w/w) in chloroform. The chloroform was removed using a rotary vacuum evaporator at 25 °C, resulting first in the formation of a viscous gel and then, a liposome suspension. PPB buffer (2 ml) was added and the suspension was evaporated further for the removal of the chloroform. The liposome suspension was then centrifuged and washed twice at 13,000 rpm for 30 min to remove free FD. For the assay, the 5 μM peptides were treated in a suspension of FD-loaded liposomes (Peptide/Liposome = 0.1). The mixture solution (3 ml, final volume) was stirred for 10 min in the dark, centrifuged at 13,000 rpm for 20 min, and the supernatant was then recovered. The leakage of dextran-loaded liposomes was recorded by measuring the fluorescence intensity of FITC (λ_{ex} = 494 nm, λ_{em} = 520 nm), with a RF-3501PC spectrofluorophotometer (Shimadzu, Japan) [22]. The percentage of dye leakage was calculated by the following equation: dye leakage (%) = $100(F - F_0)/(F_t - F_0)$, where F represents the fluorescent intensity achieved by the peptide treatment, and F_0 and F_t represent the fluorescence intensities without the peptide treatment and with Triton X-100 treatment, respectively.

3. Results and discussion

3.1. Antifungal and hemolytic activities of psacotheasin

Psacotheasin is a 34-residue antimicrobial peptide that was isolated from the *P. hilaris*. It has been reported as a knottin-type peptide containing potent antibacterial activity against several human pathogenic bacterial strains [6], whereas its antifungal properties are not understood yet. Therefore, to examine the activity of psaco-

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Table 1
Antifungal activity of psacotheasin.

Fungal strains	MIC (μM)	
	Psacotheasin	Melittin
C. albicans ATCC 90028	12.5	3.125
C. parapsilosis ATCC 22019	6.25	3.125
T. beigelli KCTC 7707	6.25	1.56
M. furfur KCTC 7744	12.5	1.56

Table 2
Hemolytic activity of psacotheasin against human erythrocytes.

Peptide	% Hemolysis (μM)					
	100	50	25	12.5	6.25	3.13
Psacotheasin	0	0	0	0	0	0
Melittin	100	100	100	92	85	79

theasin, the antifungal susceptibility testing was conducted against human pathogens as listed in Table 1, specifically *C. albicans*, *C. parapsilosis*, *T. beigelli* and *M. furfur*. In this study, melittin (GIGAVLKVLTTGLPALISWIKRKRQQ-NH_2), the principal toxic component of the venom of the European honey bee (*Apis mellifera*) [23], was used as a reference peptide. Melittin is known to have powerful antimicrobial activity against many kinds of microorganisms [24,25]. It also binds to membrane surfaces in an interfacial orientation at low peptide-to-lipid ratios, but inserts into the membrane at higher concentrations. However, the major problems associated with the use of melittin as an antimicrobial agent are its relative toxicity and an ability to lyse eukaryotic cells, usually described as hemolytic activity [26].

The antifungal effect of psacotheasin was measured by MTT assay [13]. The results show that the fungal strains are highly susceptible to psacotheasin with MIC values in the range of 6.25–12.5 μM and that the antifungal activities of psacotheasin are less potent than that of melittin, which was determined to have MIC values of 1.56–3.13 μM (Table 1). We also examined the hemolytic activity of peptide against human erythrocytes as a measure of toxicity toward higher eukaryotic cells [3]. Contrary to the potent hemolytic activity of melittin, none of the tested concentrations of psacotheasin exhibit hemolytic activity toward human erythrocytes (Table 2). These results indicate that psacotheasin has a remarkable level of antifungal activity without cytotoxicity to human erythrocytes and therefore, has a potential use as a therapeutic agent for treating fungal diseases in humans.

3.2. Membrane-active mechanisms of psacotheasin

Most organisms produce AMPs as the first step of their innate defense system against invading microbial pathogens, and AMPs play an important role as major host defense effectors [27]. To kill pathogens rapidly, it is preferable to target the cell surface rather than the cell interior. There is much evidence indicating that AMPs act by permeabilizing the cell membranes of microorganisms [28,29]. Therefore, to initially assess whether psacotheasin can affect fungal plasma membrane function, its ability to dissipate the membrane potential of *C. albicans* was investigated. *C. albicans* is the most prevalent opportunistic fungal pathogen known to humankind and causes a wide variety of life-threatening systemic diseases such as oral thrush and disseminated candidiasis in immunocompromised patients [30]. Considering its medical importance, *C. albicans* was selected as a model organism for the experiments herein. Cell wall damage or cell death causes a membrane depolarization, which can be detected with membrane po-

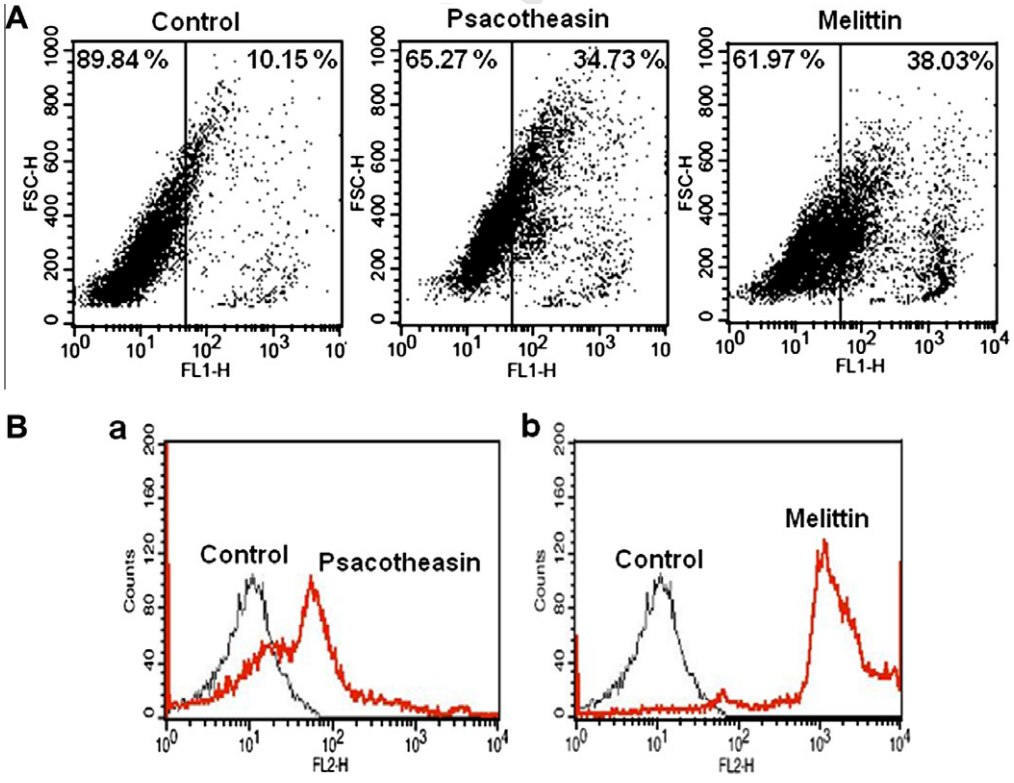

Fig. 1. Flow cytometric analysis of membrane depolarization and permeablization by $DiBAC_4(3)$ and PI staining in *C. albicans*. (A) The fluorescence intensities of stained $DiBAC_4(3)$ after treatment with the peptides for different cell populations. (B) Histograms show the fluorescence intensity of stained PI after peptide treatment of *C. albicans* cells: (a) psacotheasin-treated cells, (b) melittin-treated cells.

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tential sensitive dyes [31]. $DiBAC_4(3)$ is a green fluorescent anionic dye commonly used as an indicator of membrane potential. It only penetrates cells via a depolarized membrane, binds to hydrophobic intracellular components, and fluoresces [32]. Therefore, in the presence or absence of peptides, the disruption of membrane potential in cells was measured for the amounts of accumulated $DiBAC_4(3)$ fluorescence. As shown in Fig. 1A, cells treated with psacotheasin cause higher accumulations of $DiBAC_4(3)$ than peptide-untreated cells. The result indicates that psacotheasin damages the cell membrane and induces membrane depolarization.

To further examine the effects of psacotheasin on fungal membrane integrity, the influx of the membrane-impermeable fluorescent dye, PI, into cells was monitored by flow cytometry. PI only enters cells with damaged membranes, after which, binding to nucleic acids enhances the fluorescence of this probe by 20- to 30-fold [33]. The result shows that psacotheasin causes significant influx of PI in *C. albicans*. This indicates that psacotheasin affects fungal cells by injuring their membranes, and thus increasing membrane permeability (Fig. 1B).

The morphological changes induced by psacotheasin on the *C. albicans* were further investigated by flow cytometric analysis plotting the forward scatter (FS) and side scatter (SS) of treated and untreated cells. FS is an indicator of cell size and SS is an indicator of granularity [34]. As shown in Fig. 2, a homogeneous population of undamaged cells was dominant with the absence of any peptide. In the presence of psacotheasin, a cell population appeared in the 3D density plots with a smaller size (decreased FS), indicative of membrane damage [35]. The results indicate that psacotheasin and melittin cause the fungal cells to be permeable and damage the cell surfaces. Furthermore, the results could confirm the membrane-active mechanism of psacotheasin, which was suggested by the depolarization of the fungal membrane and assays regarding the PI influx.

3.3. Visualization of membrane-active mechanisms of psacotheasin

The morphological changes of a single GUV responding to a peptide offered additional information about the membrane-active mechanism of psacotheasin. GUVs with an average diameter of 10 to 100 μm were used for observation of the physical and biological properties of models imitating vesicle membranes, such as macroscopic and morphological changes [21,36]. The GUVs are easily formed from different lipid mixtures and observed under a fluorescent or confocal microscope, if the appropriate fluorescent probe is incorporated into the lipid phase during vesicle formation [37]. In this study, single yeast-sized GUVs of PC/PE/PI/ergosterol 5:4:1:2 (w/w/w/w) [38], which mimicked the plasma membranes of *C. albicans*, were created by employing the previously-described electroformation method [20,21]. Thereafter, the shape of a selected single vesicle, treated with the peptides, was continuously monitored for 5 min. The observed response of a single vesicle treated with psacotheasin was distinct from the response with the treatment of melittin (Fig. 3). When treated with melittin, not only did the rhodamine intensity decrease, but GUV membranes also burst and disintegrated. Although a single GUV treated with psacotheasin maintain the circular shape of a vesicle, the rhodamine intensity decreases. This result suggests that the major target site of psacotheasin is the fungal cell membrane, in line with previous observations, and that it might exert its antifungal activity by pore-forming action, not lytic action.

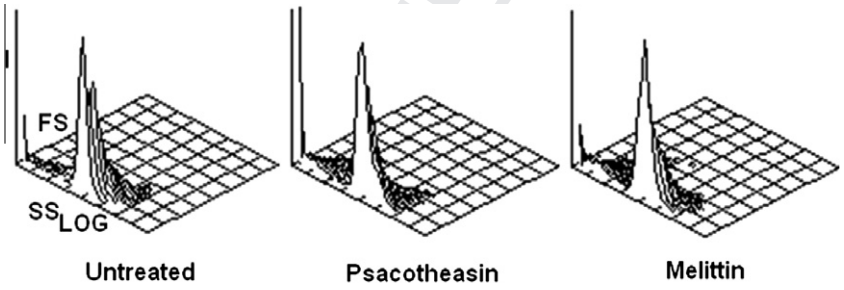

Fig. 2. Three-dimensional (3D) flow cytometric contour-plot analysis of *C. albicans* cells treated with peptides. FS (*y*-axis) and SS (90° scattering, SS_{LOG}, *x*-axis) is an indicator of cell size and granularity, respectively. The *z*-axis represents the cellular population intensity.

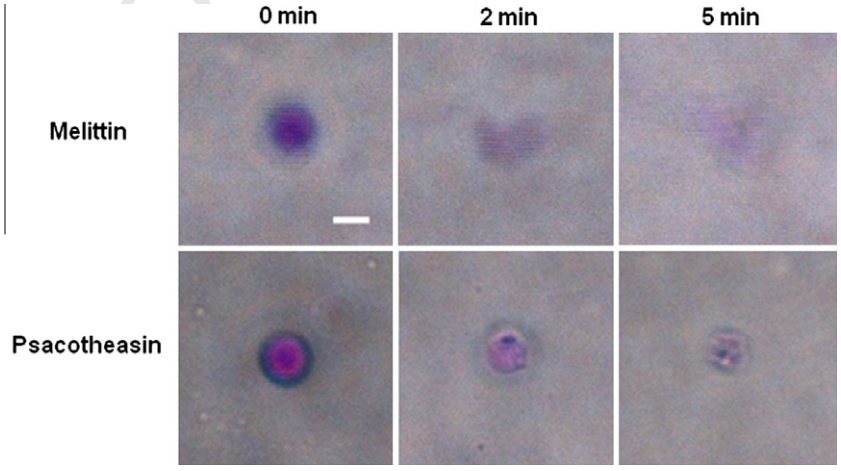

Fig. 3. The response of the single rhodamine-labeled GUV to the peptide treatment. The times above each image show the time after the peptide treatments. The bar represents 10 μm.

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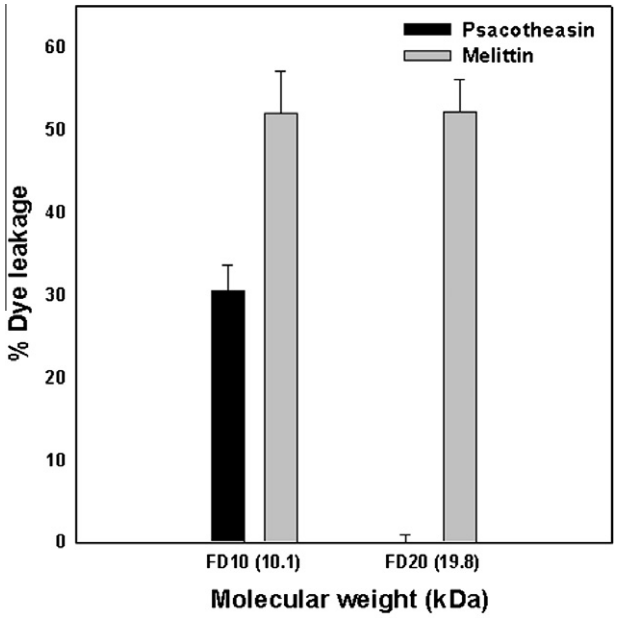

Fig. 4. FITC-dextrans (FD) release from *C. albicans* mimetic liposomes. *C. albicans* cell membrane mimetic liposomes composed of 5:4:1:2 (w/w/w/w) PC/PE/PI/ergosterol containing FD10 and FD20 were incubated with peptides for 10 min. Values are the mean of three independent measurements.

3.4. Size of the pore formed by psacotheasin in fungal model membranes

The leakage assay using fluorescent dyes of different molecular weights is used to determine the mechanism of pore formation in the membrane perturbation process [39]. To elucidate the extent of the psacotheasin-induced membrane damage, the release of entrapped FD of various sizes, i.e., FD10 (10.1 kDa, 2.3 nm radius) and FD20 (19.8 kDa, 3.3 nm radius) [40], was monitored. As shown in Fig. 4, on average, psacotheasin released 30.55% of the FD10 from the liposomes. Even though melittin induced a more potent release of all FDs, psacotheasin did not trigger the release of FD20 from the liposome at all. This indicates that psacotheasin could make pores in fungal model membranes and suggests that the pores have a radius between 2.3 nm and 3.3 nm. Therefore, this result also confirmed that psacotheasin contains a membrane-active mechanism.

In this study, the antifungal effects and mode of action(s) of psacotheasin were investigated. Psacotheasin exhibits potent antifungal effects against human pathogenic fungi without hemolytic activity. Although the exact mechanism(s) at the molecular level are not completely understood, this study suggests that psacotheasin likely exerts antifungal effects by disturbing fungal membranes. Furthermore, the membrane studies indicate that the mechanism of psacotheasin in *C. albicans* cells could be originated from the pore-forming action. Therefore, it can be concluded that an understanding of the antifungal effects and mode of action(s) of psacotheasin was gained from the work herein.

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