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Synthetic secapin bee venom peptide exerts an anti-microbial effect but not a cytotoxic or inflammatory response

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ABSTRACT

Our previous study demonstrated that the secapin peptide from the venom of the Asiatic honeybee (*Apis cerana*, AcSecapin-1) exhibits anti-fibrinolytic, anti-elastolytic, and anti-microbial activities. In the present study, we investigated the anti-microbial activity and cytotoxicity of a synthetic AcSecapin-1 peptide. Seven synthetic AcSecapin-1 peptides (AcSecapin-S1 to AcSecapin-S7) were synthesized based on the peptide sequence of AcSecapin-1. AcSecapin-S1, which consists of the 25-amino acid sequence identical to that of the mature AcSecapin-1 peptide, exhibited the highest anti-microbial activity against bacteria and fungi. This was followed by AcSecapin-S6, which was missing 10 N-terminal amino acids and 6 C-terminal amino acids from AcSecapin-S1. Furthermore, AcSecapin-S1 was not cytotoxic and did not activate macrophages. Taken together, our data demonstrated that a synthetic AcSecapin-1 peptide could exhibit anti-microbial activity without producing a cytotoxic or inflammatory response, suggesting that this synthetic AcSecapin-1 peptide can be used as an anti-microbial agent for biomedical applications.

Introduction

Bee venom is a complex mixture of biologically active compounds that have various biological, toxicological, and pharmacological activities (Winningham et al., 2004; Hoffman, 2006; Son et al., 2007; Chen and Lariviere, 2010; Choo et al., 2010, 2012; Qiu et al., 2011; Park et al., 2014; Danneels et al., 2015). Bee venom contains various peptide components. Secapin, a bee venom peptide, comprises 25 amino acid residues with a disulfide bond (Gauldie et al., 1976, 1978; Vlasak and Kreil, 1984). Secapin exerts no toxicity in mice but elicits signs of sedation, piloerection, and hypothermia at high injection doses (Gauldie et al., 1976). A previous study has suggested secapin as a potent neurotoxin (Taylor et al., 1984). Recent studies have reported that secapin possesses several biological effects such as hyperalgesic, edematogenic (Mourelle et al., 2014), and anti-bacterial properties (Hou et al., 2014).

Our most recent study demonstrated that the secapin peptide (AcSecapin-1) in venom from the Asiatic honeybee (*Apis cerana*) exhibits anti-fibrinolytic, anti-elastolytic, and anti-microbial activities (Lee et al., 2016b). Because honeybee secapin is not allergenic (Köhler et al., 2014), our results suggested that the secapin is a potent anti-fibrinolytic and anti-microbial agent. However, secapin peptides are inadequate for diverse applications owing to the difficulties in purifying secapin from bee venom and expressing recombinant secapin as a mature 25-amino acid peptide. To overcome these issues, creating a synthetic peptide is a possible approach to produce a high yield of secapin. Moreover, it is necessary to know the cytotoxicity and inflammatory response of honeybee secapin for biomedical applications.

In the present study, we examined the anti-microbial activity as well as the cytotoxic and inflammatory response of synthetic AcSecapin-1 peptides. We prepared seven synthetic AcSecapin-1 peptides based on the original peptide sequences and two cysteine residues of AcSecapin-1 (Lee et al., 2016b). We found that synthetic AcSecapin-1 peptides exhibit anti-microbial activity against bacteria and fungi, while the same synthetic AcSecapin-1 peptide does not exert cytotoxic or inflammatory characteristics.

Materials and methods

Peptide synthesis

Seven synthetic AcSecapin-1 peptides (AcSecapin-S1 through AcSecapin-S7) were synthesized by GL Biochem Ltd. (Shanghai, China): AcSecapin-S1 (the original 25-amino acid mature peptide of AcSecapin-1), AcSecapin-S2 (Cys20Ala in AcSecapin-S1), AcSecapin-S3 (Cys20Ser in AcSecapin-S1), AcSecapin-S4 (deletion of 7 N-terminal amino acids and 4 C-terminal amino acids from AcSecapin-S2), AcSecapin-S5 (deletion of 7 N-terminal amino acids and 4 C-terminal amino acids from AcSecapin-S3), AcSecapin-S6 (deletion of 10 N-terminal amino acids and 6 C-terminal amino acids from AcSecapin-S1), and

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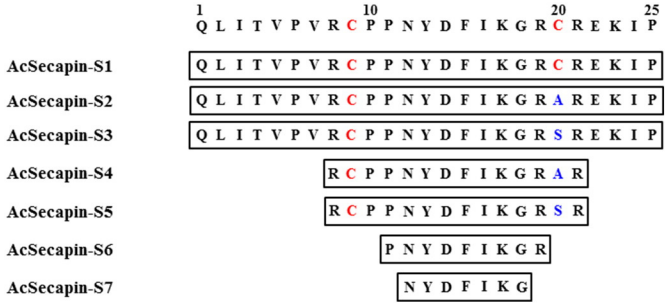

Fig. 1. The peptide sequences of the synthetic AcSecapin-1 peptides. The two cysteine residues are indicated.

AcSecapin-S7 (deletion of 11 N-terminal amino acids and 7 C-terminal amino acids from AcSecapin-S1) (Fig. 1).

Cytotoxicity and cell viability assays

To determine the cytotoxicity and viability of cells in response to AcSecapin-S1, the murine fibroblast cell line NIH 3T3 was used as previously described (Lee et al., 2016a). NIH 3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Gibco BRL, Gaithersburg, MD, USA) at 37 °C in an atmosphere containing 5% CO_2. Cells were cultured in 96-well plates (5×10^4 cells/well) for 24 h prior to the addition of either AcSecapin-S1 (0.1, 1, 10, 100, 250, or 500 μg per ml of medium) or 1% Triton X-100 (Sigma), as a positive control, to each well. After the cells were incubated for 24 h, the media were harvested by centrifugation at 1000*g* for 5 min and immediately used in the lactate dehydrogenase (LDH) assay using the LDH-Cytotoxicity Assay Kit II (BioVision, Milpitas, CA, USA). Next, the MTT [3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay was performed as previously described (Lee et al., 2016a). NIH 3T3 cells were cultured in 96-well plates (5×10^4 cells/well) with either AcSecapin-S1 (0.1, 1, 10, 100, 250, or 500 μg per ml of medium) or 1% Triton X-100 (Sigma), as a positive control, for 24 or 48 h, after which 50 μl of MTT reagent (BioVision) was added to each well. The media were discarded after 4 h of treatment, and the formazan crystals were dissolved in 150 μl of MTT solvent. The absorbance was measured at 590 nm using a microplate reader (Bio-Rad Model 3550, Bio-Rad, Hercules CA, USA). Each experiment was performed in triplicate.

Apoptosis assay

To determine the apoptotic response to AcSecapin-S1, NIH 3T3 cells were used as previously described (Lee et al., 2016a). Apoptosis levels were determined by measuring caspase-3 activity using the Caspase-3/CPP32 Colorimetric Assay Kit (BioVision). NIH 3T3 cells were cultured in 6-well plates (1×10^6 cells/well) for 24 h, and AcSecapin-S1 (10, 100, 250, or 500 μg per ml of medium) was added to each well. After the cells were treated for 24 h, they were harvested, washed with phosphate-buffered saline (PBS), resuspended in cold lysis buffer and placed on ice for 20 min. The lysed cells were centrifuged at 14,000*g* for 15 min, and the supernatants were mixed with a caspase substrate (Ac-DEVD-pNA) in a 96-well plate. The samples were incubated overnight at 37 °C, and the released *p*-nitroaniline levels were measured by reading the absorbance at 405 nm using a microplate reader (Bio-Rad Model 3550, Bio-Rad). Each experiment was performed in triplicate.

Macrophage stimulation and measurement of proinflammatory mediators and cytokine release

To determine the macrophage stimulatory response to AcSecapin-S1, the murine macrophage cell line J774 was used as previously described (Lee et al., 2016a). J774 cells were cultured in DMEM (Sigma) supplemented with 10% FBS (Gibco BRL) at 37 °C in an atmosphere containing 5% CO_2. Cells were cultured in 24-well plates (4×10^5 cells/well) for 24 h, and either AcSecapin-S1 (250 μg per ml of medium) or lipopolysaccharide (100 ng per ml of medium, LPS, Sigma), as a positive control, was added to each well. After 5 h of treatment, the cells were centrifuged at 600*g* for 10 min, and media were harvested to determine the release of proinflammatory mediators and cytokines by western blot analysis. The medium samples were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and detected via Western blot using an ECL (enhanced chemiluminescence) Western Blotting Detection System (Amersham Biosciences, Piscataway, NJ, USA). Mouse polyclonal antibodies targeting tumor necrosis factor (TNF)-α [diluted 1:1000 (v/v); Cell Signaling Technology, Beverly, MA, USA], cyclooxygenase (COX)-2 [diluted 1:1000 (v/v); Cell Signaling Technology], interleukin (IL)-1β [diluted 1:1000 (v/v); Abcam, Cambridge, United Kingdom], and IL-6 [diluted 1:1000 (v/v); Cell Signaling Technology] were used. The secondary antibody was an

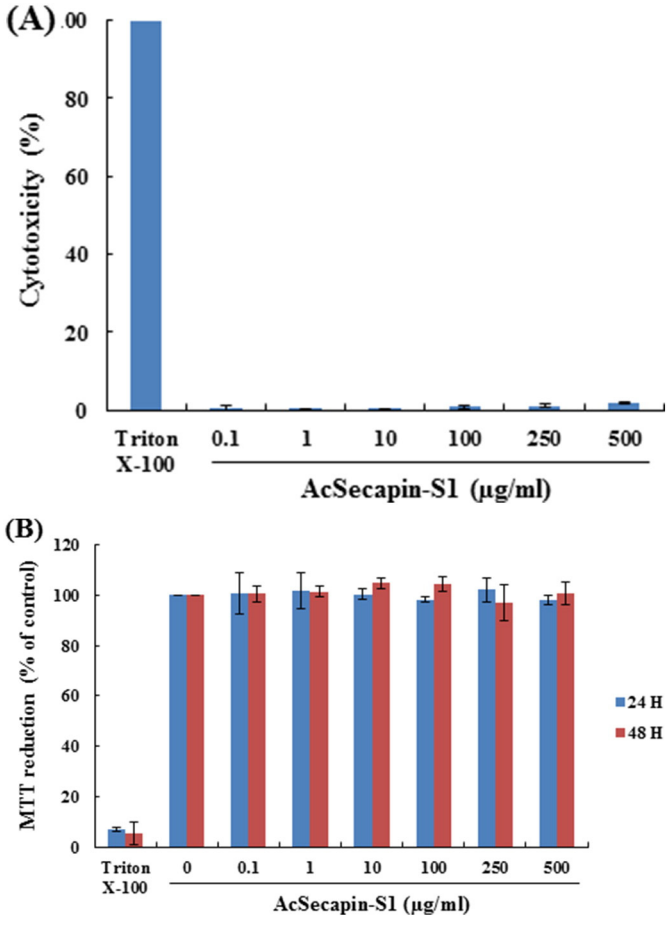

Fig. 2. Cytotoxicity and cell viability in response to AcSecapin-S1. (A) Cytotoxicity of AcSecapin-S1. A cytotoxicity assay was performed using NIH 3T3 cells with AcSecapin-S1 (0.1, 1, 10, 100, 250, or 500 μg per ml of medium) or Triton X-100 (positive control). Bars represent the mean ± SD ($n = 3$). (B) Cell viability after treatment with AcSecapin-S1. The MTT assay was performed using NIH 3T3 cells with AcSecapin-S1 (0.1, 1, 10, 100, 250, or 500 μg per ml of medium) or Triton X-100 (positive control). The percentage of MTT reduction was determined at 24 and 48 h after AcSecapin-S1 treatment. Bars represent the mean ± SD ($n = 3$).