Computational and Structural Biotechnology Journal 14 (2016) 49–57

COMPUTATIONAL AND STRUCTURAL BIOTECHNOLOGY JOURNAL

journal homepage: www.elsevier.com/locate/csbj

Prediction of anticancer peptides against MCF-7 breast cancer cells from the peptidomes of *Achatina fulica* mucus fractions

Teerasak E-kobon [a], Pennapa Thongararm [b], Sittiruk Roytrakul [c], Ladda Meesuk [d], Pramote Chumnanpuen [b,*]

[a] Department of Genetics, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand
[b] Department of Zoology, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand
[c] National Center for Genetic Engineering and Biotechnology, Thailand Science Park, Pathum Thani 12120, Thailand
[d] Faculty of Dentistry, Thammasat University, Pathum Thani 12120, Thailand

ARTICLE INFO

Article history:
Received 10 August 2015
Received in revised form 18 November 2015
Accepted 19 November 2015
Available online 7 December 2015

Keywords:
Peptidomics
Cytotoxic peptides
Achatina fulica
Breast cancer
Bioinformatics prediction
Snail mucus

ABSTRACT

Several reports have shown antimicrobial and anticancer activities of mucous glycoproteins extracted from the giant African snail *Achatina fulica*. Anticancer properties of the snail mucous peptides remain incompletely revealed. The aim of this study was to predict anticancer peptides from *A. fulica* mucus. Two of HPLC-separated mucous fractions (F2 and F5) showed in vitro cytotoxicity against the breast cancer cell line (MCF-7) and normal epithelium cell line (Vero). According to the mass spectrometric analysis, 404 and 424 peptides from the F2 and F5 fractions were identified. Our comprehensive bioinformatics workflow predicted 16 putative cationic and amphipathic anticancer peptides with diverse structures from these two peptidome data. These peptides would be promising molecules for new anti-breast cancer drug development.

1. Introduction

Breast cancer is one of the most common diseases in women globally [1]. Several factors make women at high risk of the breast cancer [2]. Early detection and the use of radiation therapy, surgery, and chemotherapeutic drugs including selective estrogen receptor modulators (SERMs) and aromatase inhibitors can reduce invasive breast cancer. However, the patients remain traumatized by the unfavorable side effects [3,4]. The search for target-specific and less side-effect cancer therapy is still undergoing.

Anticancer peptides have been proved to be effective small molecules (<50 amino acids) that can act specifically against cancerous cells by either membranolytic mechanism or disruption of mitochondria [5]. The net negative charge of the cancer membrane is an important factor for peptides' selectivity and toxicity [6], as compared to the typically zwitterionic property of non-cancerous eukaryotic membranes. Amphiphilicity levels and hydrophobic arc size allow penetration of these peptides through the cancerous cell membranes and lead to destabilization of the membrane integrity [7,8]. For example, pleurocidin-like peptides (NRCs) identified from fish could kill breast cancer cells and human mammary epithelial cells by causing membrane damage with subtle harm to human fibroblasts [9]. These cell-penetrating peptides could be used as cancer-specific drug delivery. For example, the non-specific cell-penetrating anticancer peptide buforin IIb was modified to enhance the cancer specificity with no effects on normal cells [10]. This cancer-specific peptide derivative was successfully used to deliver apoptosis-induced antibody into the cancer cells. Distinctively, a peptide SA12 could induce apoptosis on SKBr-3 breast cancer cells by the mitochondrial pathway [11]. Taken together, these physicochemical properties and experimentally validated information were used to develop bioinformatic programs for anticancer peptide prediction and design. AntiCP predicts anticancer peptides by using amino acid composition and binary profiles to develop support vector machine models (SVM) [12]. Another SVM-based program, ACPP, particularly screens for anticancer peptides that contain apoptotic domain [13]. In this regard, these prediction tools will assist high-throughput screening for anticancer peptides from complex peptidomes of an array of natural products.

Giant African snails (*Achatina fulica*) are invasive animals that seriously cause damages to agricultural and ornamental plants worldwide. Only one antimicrobial peptide, namely Mytimacin-AF, was identified from the mucus of *A. fulica* [14]. Mytimacin-AF (9.7 kDa) was a novel cysteine-rich peptide that could inhibit the growth of both fungi and bacteria with little hemolytic effect on human red blood cells. However, we hypothesized that the anticancer peptides from the mucus of *A. fulica* may not be completely revealed. Thus, this study aimed to predict putative anticancer peptides from the most effective HPLC-

* Corresponding author at: Department of Zoology, Faculty of Science, Kasetsart University, 50 Ngam Wong Wan Road, Chatuchak, Bangkok 10900, Thailand. Tel.: +66 812 854 672; fax: +66 257 955 28.
E-mail address: pramote.c@ku.ac.th (P. Chumnanpuen).

http://dx.doi.org/10.1016/j.csbj.2015.11.005

separated mucous fractions against the breast cancer cell line MCF-7 using mass spectrometric and bioinformatic analysis methods. Our results provide alternative high-throughput screening methods to identify potential anticancer peptides from nearly a thousand peptides within the snail mucus for further validation.

2. Experimental procedure

2.1. Cell culture

The breast cancer cell line MCF-7 and the kidney epithelial cell line Vero used in this study were kindly provided by the Department of Biochemistry, Faculty of Medicine, Chiangmai University, Thailand and the Genome Institute, National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand. The cells were cultured and passaged in Dulbecco's Modification of Eagle's Medium (DMEM, Gibco-RBL, Life Technologies, NY) supplemented with 10% Fetal Bovine Serum (FBS, Hyclone, Thermo Fisher Scientific Inc., USA), 1% Penicillin–Streptomycin (PAA, Laboratories GmbH, Austria) and 1% Amphotericin B (PAA, Laboratories GmbH, Austria). The cells were maintained at 37 °C in 95% relative humidified atmosphere containing 5% CO_2. Cell growth was measured under a light microscope and 80% confluence of the cells was used in all experiments.

2.2. Separation of A. fulica mucus by HPLC

The snail mucus samples were collected from adult *A. fulica* by intermittent irritation in an ultrasonicating bath at 30 °C sporadically. The crude mucous samples were separated by ZORBAX 300SB-4.6 × 150 mm C18 column, 5 μm, (Agilent, Palo Alto, CA) with Agilent® 1200 system using methanol–water (50:50) with 0.1% trifluoroacetic acid (adjusted from [15]) as mobile phase and the flow rate was 0.30 ml/min. Numbers of the HPLC peaks were used to determined numbers of the fractions. Six HPLC-separated mucous fractions were collected manually and named as F1, F2, F3, F4, F5 and F6 fractions. All HPLC fractions and the crude mucus were concentrated by freeze-drying at −100 °C and kept at −20 °C until use.

2.3. Determination of cytotoxicity of the mucous fractions by MTT assay

Cell viability count was performed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [16]. Cells were seeded at 2×10^4 cells per well (200 μl/well) in 96-well tissue culture plates and allowed cells to adhere for 24 h at 37 °C in the CO_2 incubator. The culture medium was then replaced with 200 μl/well of the fresh medium for the control group and 200 μl/well of the fresh medium containing the same concentration (1000 μg/ml) of the crude mucus or the six HPLC-separated fractions. After 72 h incubation, 50 μl/well of tetrazolium bromide salt solution (2 mg/ml of stock in phosphate buffered saline, PBS) was added into 150 μl of the cell suspension. Four hours before completion, the reaction mixture was carefully taken out and 200 μl/well of dimethyl sulfoxide or DMSO (Sigma, USA) was added to each well before the addition of 25 μl/well of Sorensen's glycine buffer (Research Organics, USA). The optical densities (OD) were measured at 570 nm using microplate reader (Tecan Sunrise, Switzerland). Finally, the highest effective anti-breast cancer fraction with the lowest percentage of cell viability was then selected for further analysis.

Cytotoxicity of the mucous fractions against the MCF-7 and Vero cells was compared by slightly modified the above-described method due to the limited quantity of the fractions. The cells were seeded at 4×10^3 cells per well in 96-well tissue culture plates and allowed cells to adhere for 24 h at 37 °C in the CO_2 incubator. The culture medium was then replaced with 100 μl/well of the fresh medium for the control group and 100 μl/well of the fresh medium containing three concentrations (1, 10 and 100 μg/ml) of the crude mucus, the F2, and F5 fractions. After 24 h incubation, 25 μl/well of tetrazolium bromide salt solution (5 mg/ml of stock in PBS) was added to the cell suspension. Four hours before completion, the reaction mixture was carefully taken out, and 100 μl/well of DMSO was added to each well. The optical densities were measured at 570 nm.

2.4. Statistical analysis of the MTT assay

The results were presented as mean ± sem (standard error of mean) or mean ± sd (standard deviation). The parameters were analyzed with one-way analysis of variance (One-way ANOVA) followed by Sidak's multiple comparisons test. Statistical analysis was conducted with Graphpad Prism version 6.0 for Windows (Graphpad software, San Diego, California, USA). Significant levels were considered at $p < 0.05$ and highly significant level at $p < 0.01$ comparing with control group.

2.5. Mass spectrometric analysis of the selected cytotoxic fractions

2.5.1. Sample fractionation

Individual selected fractions were fractionated based on their molecular size using Macrosep® 3 K, 10 K and 50 K Omega centrifugal devices (Pall Life Sciences, USA) into four sub-fractions: lower than 3 kDa, between 3 and 10 kDa, between 10 and 50 kDa, and larger than 50 kDa sub-fractions. These sub-fractions were mixed well with two volumes of cold acetone and incubated overnight at −20 °C. The mixture was centrifuged at 10,000 ×*g* for 15 min, and the supernatant was discarded. The pellet was freeze-dried and stored at −80 °C before use.

2.5.2. Determination of protein/peptide concentration by lowry method

The pellets were resuspended in 0.15% Sodium Deoxycholic acid (DOC) or 0.5% SDS and determined protein concentration by Lowry method [17]. The absorbance at 750 nm (OD_{750}) was measured, and the protein concentration was calculated using the standard curve, plotted between OD_{750} on Y-axis and BSA concentration (μg/ml) on X-axis.

2.5.3. In-solution digestion

Each protein sub-fractions were hydrolyzed by trypsin at an enzyme to the protein ratio of 1:50 at 37 °C for 24 h, except the lower than 3 kDa sub-fraction. The peptides were dried by vacuum centrifuge and kept at −80 °C for further mass spectrometric analysis.

2.5.4. HCTultra LC–MS analysis of the peptidomes

Peptide solutions were analyzed using an HCTultra PTM Discovery System (Bruker Daltonics Ltd., U.K.) coupled to an UltiMate 3000 LC System (Dionex Ltd., U.K.). Peptides were separated on a nanocolumn (PepSwift monolithic column 100 μm i.d. × 50 mm). Eluent A was 0.1% formic acid, and eluent B was 80% acetonitrile in water containing 0.1% formic acid. Peptide separation was achieved with a linear gradient from 10% to 70% B for 13 min at a flow rate of 300 nl/min, including a regeneration step at 90% B and an equilibration step at 10% B, one run took 20 min. Peptide fragment mass spectra were acquired in data-dependent AutoMS (2) mode with a scan range of 300–1500 *m/z*, 3 averages, and up to 5 precursor ions selected from the MS scan 50–3000 *m/z*.

2.5.5. Identification of peptide sequences

The MS/MS data from LC–MS were submitted to database search using the Mascot software (Matrix Science, London, U.K., [18]). The peptide sequence data was searched against the NCBI database for protein identification. Database interrogation was; taxonomy (other metazoans); enzyme (trypsin); variable modifications (carbamidomethyl, oxidation of methionine residues); mass values (monoisotopic); protein mass (unrestricted); peptide mass tolerance (1 Da); fragment mass tolerance (±0.4 Da), peptide charge state (1+, 2+ and 3+), max missed cleavages (1) and instrument = ESI-TRAP. Proteins considered as identified proteins had at least one peptides with an individual Mascot score corresponding to $p < 0.05$. The resultant peptides with