Biochimica et Biophysica Acta 1808 (2011) 1476–1482

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Mode of action of parasporin-4, a cytocidal protein from *Bacillus thuringiensis*

Shiro Okumura [a,*], Hiroyuki Saitoh [a], Tomoyuki Ishikawa [a], Kuniyo Inouye [b], Eiichi Mizuki [a]

[a] Fukuoka Industrial Technology Centre, 1465-5 Aikawa, Kurume, Fukuoka 839-0861, Japan
[b] Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan

ARTICLE INFO

Article history:
Received 15 June 2010
Received in revised form 2 November 2010
Accepted 3 November 2010
Available online 10 November 2010

Keywords:
Bacillus thuringiensis
β-pore-forming toxin
Cytotoxic activity
Parasporin

ABSTRACT

Parasporin-4 (PS4) is a cytotoxic protein produced by *Bacillus thuringiensis* strain A1470. It exhibits specific cytotoxicity against human cancer cell lines, CACO-2, Sawano, and MOLT-4 cells, in particular. When cells were administrated with PS4, cell swelling and nuclear shrinkage were induced, and, the ballooned cells burst within 24 h. PSI-BLAST search showed that the protein shared homology not only with *B. thuringiensis* Cry toxins but also with aerolysin-type β-pore-forming toxins. Circular dichroism measurements suggested that PS4 was a β-sheet-rich protein. PS4 aggregated into oligomers on the plasma membrane of PS4-susceptible CACO-2 cells, but not on that of PS4-resistant HeLa cells. Leakage of lactate dehydrogenase and influx of extracellular FITC-dextrans were observed only in susceptible cells. The activation of effectors caspase 3 and/or 7 was not observed in PS4-treated CACO-2 cells. It was shown that cytotoxicity of the PS4 against CACO-2 cells was exhibited when treated by cyclodextrin which induces cholesterol depletion. These results suggest that PS4 is a unique β-pore-forming toxin with a cholesterol-independent activity.

1. Introduction

Bacillus thuringiensis strain A1470 produces multiple cytotoxic proteins with similar molecular masses [1]. Parasporin-4 [Cry45Aa as designated by the *B. thuringiensis* δ-endotoxin nomenclature committee (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/); hereafter abbreviated as PS4] is one of the cytotoxic proteins produced by A1470. It exhibits strong cytotoxicity against several human tumor cell lines when activated by protease treatment, although it does not exhibit insecticidal or hemolytic activities [2]. Pro-parasporin-4 (proPS4) is comprised of 275 amino acid residues with a molecular mass of 31 kDa, and is processed to the active 27 kDa form by pepsin cleavage of the C-terminus at Glu-252 [3]. The activated toxin exhibits high cytotoxic activity against CACO-2, Sawano, MOLT-4, TCS, and HL60 cells. It does not, however, exhibit this activity against 4 normal tissue cells such as human primary hepatocyte cells, UtSMC, MRC-5, and normal T cells [4].

B. thuringiensis is a Gram-positive endospore-forming bacterium that produces large crystalline parasporal inclusions in sporulating cells. This bacterium is well known for the insecticidal protein produced in the parasporal inclusion. The protein is thought to bind to the surface of the apical brush border membrane of epithelial columnar cells, where it forms pores and eventually kills susceptible insects [5–7]. However, non-insecticidal *B. thuringiensis* strains are more widely distributed than insecticidal ones [8]. Mizuki et al. reported that some non-insecticidal *B. thuringiensis* strains produced human cancer cell-killing proteins, which were named parasporins [9]. At present, 6 parasporins have been reported by their primary rank grouping (http://parasporin.fitc.pref.fukuoka.jp/), each possessing a unique cell-killing spectrum [10]. Parasporin-1 activates apoptotic signaling in sensitive cells because of the increased Ca^{2+} level [11], and parasporin-2 acts as a cytolysin that permeabilizes the plasma membrane with target cell specificity [12]. It seems that each parasporin has its own mechanism for inducing cell death.

A position-specific iterated (PSI)-BLAST search [13] of PS4 indicated that it shares homology with pore-forming toxins. In this study, we examined the typical activities of pore-forming toxins to elucidate the mechanism of PS4.

2. Materials and methods

2.1. Purification of PS4

Inclusion bodies from *Escherichia coli* BL21 (DE3) cells transformed with a plasmid containing the proPS4 gene were purified as described previously [3] and were solubilized in 10 mM HCl at 37 °C for 1 h. The solution was clarified by centrifugation at 5600×*g* for 5 min, and proPS4 solution in the supernatant was processed with 200 μg/ml pepsin (pepsin 1:10,000, lot PTL1716, purchased from Wako Pure Chemicals, Osaka, Japan) for 90 min at 37 °C. The proteolytic reaction

Abbreviations: β-PFT, β-pore-forming toxin; BSA, bovine serum albumin; BSA-PBS, PBS containing 1% BSA, 0.05% sodium azide; CD, circular dichroism; CDC, cholesterol-dependent cytolysin; LDH, lactate dehydrogenase; MTT, 3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide; PSI-BLAST, position-specific iterated-BLAST; proPS4, pro-parasporin-4; PS4, parasporin-4; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

* Corresponding author. Tel.: +81 942 30 6644; fax: +81 942 30 7244.
E-mail address: sokumura@fitc.pref.fukuoka.jp (S. Okumura).

0005-2736/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2010.11.003

was stopped by 20 μg/ml pepstatin A (Sigma Aldrich, St. Louis, MO, USA; lot 21K8632). The solution was clarified by centrifugation at 15,000×*g* at 4 °C for 10 min. The supernatant was subjected to a Resource S cation-exchange column (6.4 mm inner diameter×30 mm length; GE Healthcare, Buckinghamshire, England) pre-equilibrated with 20 mM glycine–HCl buffer, pH 3.0 at a flow-rate of 1.0 ml/min. Unwanted proteins were eluted by several washes with the same buffer containing 3 M NaCl. The running buffer was changed to 5 mM CAPS-NaOH (pH 12.0), and the eluted solution containing activated PS4 was collected. The solution was applied to a SuperdexG-75 XK 16/70 gel-chromatography column (16 mm inner diameter×70 mm length; GE Healthcare) pre-equilibrated with the same CAPS buffer. Purified PS4 samples were collected and stored at −80 °C until use. Protein concentration was measured by BCA protein assay [14] with bovine serum albumin (BSA) as the standard. An average of 20 mg purified PS4 was obtained from a liter of medium.

2.2. Circular dichroism spectra

Circular dichroism (CD) spectra of native and heated PS4 were measured on a model J-720W (JASCO, Tokyo, Japan) at room temperature. Molar ellipticity data were converted to mean residue molar ellipticity. A value of 109.0 was taken as the mean residue weight of PS4. Estimation of the α-helix, β-structure, and unordered structure content was performed according to the method reported by Andrade et al. [15] and Merelo et al. [16].

2.3. Mammalian cell lines

CACO-2, HeLa, MOLT-4, and K562 cells were obtained from the Riken Bioresource Center (Tsukuba, Japan) and were maintained under the conditions recommended by the supplier. CACO-2 was cultured in MEM (Nissui Pharma., Tokyo, Japan) containing 20% fetal bovine serum. HeLa cells were cultured in MEM containing 10% fetal bovine serum. MOLT-4 and K562 were cultured in RPMI1640 medium (Nissui Pharma.) containing 10% fetal bovine serum and kanamycin (30 μg/ml). Because previous studies have indicated that PS4 is most cytotoxic against CACO-2 cells, we focused on this cell type and used PS4-resistant HeLa cells as a negative control. CACO-2 cannot be dispersed by trypsin or EDTA, and thus cannot be analyzed by flow cytometry. We therefore also used nonadherent MOLT-4 and K562 cells, which are PS4-susceptible and -resistant, respectively.

2.4. Leakage of lactate dehydrogenase

Leakage of lactate dehydrogenase (LDH) from CACO-2, MOLT-4, and HeLa cells treated with PS4 was measured. A 90 μl aliquot of cell suspension, containing 2×10^4 cells, was applied to each of a 96-well plate (Costar 3595 purchased from Corning, Corning, NY, USA) and incubated at 37 °C overnight. The cell concentration was determined by direct counting in an improved Neubauer counting chamber (Sunlead Glass, Saitama, Japan). The cells were then treated with PS4 (0.1–1.0 μg/ml) and streptolysin O (3 U/ml), and were incubated at 37 °C for 10–360 min. The activity of LDH leaked from the cells was measured by a CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega, Madison, WI, USA). Absorbance at 492 nm was measured on a Multiskan Bichromatic microplate reader (Labsystems Oy., Helsinki, Finland).

2.5. Influx of extracellular FITC-dextrans

MOLT-4 and K562 cells were cultured and FITC-conjugated dextrans with various molecular weights (Life Technologies, Carlsbad, CA, USA) were added to the culture medium at a final concentration of 50 μM FITC. PS4 was added, and the cells were incubated at 37 °C for 60 min. The cells were fixed with 10% formalin-PBS for 10 min and washed with PBS containing 1% BSA, 0.05% sodium azide (BSA-PBS). Fluorescence intensity was determined with an Epics XL flow cytometer (Beckman Coulter, Brea, CA, USA). Streptolysin O (Sigma Aldrich) was used as a positive control. The activity of streptolysin O is expressed in units; one unit of toxin is defined by the supplier as that which causes 50% lysis of 50 μl of a 2% human red blood cell suspension in PBS at 37 °C in 30 min.

2.6. Flow cytometric analysis of PS4 binding

HeLa, MOLT-4, and K562 cells were used for PS4 binding analysis by flow cytometry. HeLa cells were recovered from their culture dishes with EDTA (5 mM in PBS). Each cell sample was washed with BSA–PBS, followed by fixation with 10% formalin–PBS for 10 min and washed with BSA–PBS thrice. The cells were treated with PS4 at a final concentration of 10 μg/ml in BSA–PBS, incubated for 1 h at room temperature, and thrice washed with BSA–PBS. Then they were treated with a rabbit antiserum raised against PS4 for 1 h and washed thrice, followed by treatment with commercially supplied Alexa488-conjugated anti-rabbit immunoglobulins (Promega, Madison, WI, USA) for 1 h. The treated cells were analyzed with an Epics XL flow cytometer.

2.7. Formation of PS4 multimer

CACO-2, MOLT-4 and HeLa cells were prepared in a 96-well plate (Costar 3595). PS4 was added to the cells and was incubated for 3–60 min. Then, 50 μl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (47 mM Tris–HCl, pH 6.8, 70 mM sodium dodecyl sulfate, 10% glycerol, 5% 2-mercaptoethanol, 0.0125% bromophenol blue) was added to each well and the plate was agitated. The supernatant was recovered from each well and heated at 95 °C for 5 min. A 10-μl aliquot of each sample was analyzed by SDS-PAGE [17] with a polyacrylamide gradient gel (3–10%; purchased from Wako Pure Chemicals). Immunoblotting was performed with a rabbit antiserum raised against PS4 and commercially supplied HRP-conjugated anti-rabbit immunoglobulins (Dako, Glostrup, Denmark). Positive bands were detected with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Waltham, MA, USA).

2.8. Caspase-3/7 activation by PS4

A 90 μl aliquot of cell suspension containing 4×10^4 MOLT-4 cells was applied to each of the 96-well plate. PS4 (0.6 or 3 μg/ml final concentration), actinomycin D (1 μM), camptothecin (1 μM), or TritonX-100 (0.1%) was added to the cells. A chemiluminescent substrate, Caspase GLO (Promega) was added and was incubated at room temperature for 30 min. Actinomycin D [18] and camptothecin [19], which are apoptosis positive controls, were purchased from Wako Pure Chemicals.

2.9. Effect of cholesterol depletion on PS4 cytotoxicity

CACO-2 cells were cultured in medium containing 4 μM lovastatin and 0.25 mM mevalonate in a 96-well plate (Costar 3595) for 48 h to minimize *de novo* synthesis of cholesterol. Residual cholesterol was extracted by treatment with 10 mM methyl-β-cyclodextrin in MEM for 80 min [20]. The cells were washed twice with medium and were incubated with PS4 or streptolysin O for 20 h before being assayed for cytotoxicity. The cytotoxic activities of the toxins were measured by the MTT [3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide] method [21,22] using the CellTiter96 Aqueous One Solution Reagent (Promega). Ten μl of MTT reagent was added to each well, followed by further incubation at 37 °C for 4 h. Finally, the absorbance at 492 nm was measured on a microplate reader with a control