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Characterization and cytotoxic activity of apoptosis-inducing pierisin-5 protein from white cabbage butterfly

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ABSTRACT

In this study, caspase-dependent apoptosis-inducing pierisin-5 gene was identified and characterized from cabbage white butterfly, *Pieris canidia*. A thousand-fold increase in expression of pierisin-5 gene was observed from second to third instar larvae, gradually decreasing before pupation. Pierisin-5 was purified from the fifth-instar larvae and was found to exhibit cytotoxicity against HeLa and HepG2 human cancer cell lines. Pierisin-5 showed growth inhibition and several morphological changes such as cell shrinkage, chromatin condensation and apoptotic body formation with programmed cell death in HeLa and HepG2 cells. Moreover, DNA fragmentation was observed after gel electrophoresis analysis. Caspase substrate assay showed further cleavage of Ac-DEVD-pNA, suggesting the activation of Caspase-3. Flow cytometry analysis revealed the cell cycle arrest at G1 phase and increased the percentage of apoptotic cells in cancer cell lines treated with pierisin-5. These findings suggest that pierisin-5 could significantly induce apoptosis in cancer cell lines and is mediated by activation of caspase-3 in the mitochondrial pathway. Phylogenetic analysis using pierisin proteins from Pierid butterflies, ADP-ribosylating toxins from bacteria, human, rat, and mouse indicated the possibility of horizontal transfer of pierisin genes from bacteria to butterflies. The single copy of pierisin gene unlike other insect toxin genes also supports lateral transfer.

1. Introduction

Some specific subtribes of Pierinae from Pieridae family (Pierina, Aporiina, and Appiadina) have proteins that exhibit cytotoxicity and DNA ADP-ribosylating activity. Pierisin-1 is a cytotoxic protein identified from the pupae of cabbage white butterfly, *Pieris rapae*, against human gastric TMK-1 and cervical HeLa cancer cell lines [1]. Pierisin-1, a 98-kDa DNA-ADP ribosylating protein, transfers the ADP-ribose moiety from NAD to the N2 position of guanine base of DNA [2]. The N-terminus of pierisin-1 shows partial sequence similarity with ADP-ribosylating toxins such as cholera, pertussis, diphtheria and mosquitocidal toxin (MTX), whereas the C-terminal region has sequence similarity with Botulinum toxin [3]. Pierisin-1 has a unique enzymatic feature, catalyzing ADP-ribosylation of guanine residues of DNA but not amino acid residues of proteins. Pierisin-1 induces apoptosis by mitochondrial pathway involving Bcl2 and caspases [4]. The amount of pierisin-1 protein increased around 100 folds from the first-instar to fifth-instar larvae and then immediately decreased by over 90% during the pupal stage [5]. In other aspects, the potent cytotoxicity of pierisin-1 might be efficient as a protective agent against microbes and parasitoids. However, the real biological significance of pierisin-1 in Pieris butterflies is yet to be fully elucidated [6].

To date, pierisin-1, -1b, -2, -3 and -4, have been identified. All of them have been observed to have cytotoxicity against human cancer cell lines. Pierisin-1b was also found in *P. rapae* and shares 91.2% sequence similarity with pierisin-1 [7]. The other cabbage butterfly, *Pieris brassicae*, also contains an apoptosis-inducing protein named pierisin-2. cDNA of pierisin-2 encodes 850 amino acids. The amino acid sequence deduced from the cDNA shows that pierisin-2 is 91% similar to pierisin-1. Pierisin-2 exhibits cytotoxicity similar to pierisin-1 [8]. Pierisin-3 from *Pieris melete* (gray-veined white) and pierisin-4 from *Aporia cratagi* (black-veined white) have been characterized which encodes 850 and 858 amino acids, respectively [9].

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In this study, we report the cDNA cloning of apoptosis-inducing protein named pierisin-5 from the cabbage white butterfly, *Pieris canidia*, which belongs to subtribes Pierina and these extracts had been shown to have pierisin-like activity [10]. The deduced amino acid sequence of the cDNA indicated that pierisin-5 have a close sequence similarity to pierisin-1, -1b, -2, -3 and -4, respectively. The purified proteins exhibited cytotoxicity and apoptotic activity in HeLa and HepG2 cells were similar to pierisin-3. Pierisin-5 induces apoptosis via mitochondrial pathway by activation of caspase-3. The evolutionary conservation of amino acid sequence of ADP-ribosylating proteins was compared with pierisin-5 by phylogenetic analysis, and the results are discussed.

2. Materials and methods

2.1. Butterfly collection and rearing

The adult butterfly of *P. canidia* was collected from the mustard fields in and around Sihphir village, Aizawl district (23.8208N, 92.7357°E), Mizoram, and Northeast India. Species identification was carried out based on the morphological information [11–13]. Five pairs of butterflies consisting of equal sex ratio were released in the fresh potted shoots of natural host plants (*Brassica juncea*) with 20% of honey solution for egg-laying in the laboratory and covered with the thin net for mating. Butterflies were maintained in the rhythm of day and night condition (10 l: 14 D h) at 25 ± 2° C. Cabbage leaves were supplied to each Petri dish as food, and the leaves were renewed at 12 h interval and inspected periodically. Wet cotton was used to keep them fresh. The identification and characterization of each instar of larvae were done by expert entomologists based on the size of head width and body length using micrometric grid incorporated in a binocular microscope (1 division = 0.035 mm). The adult females were regularly observed at the eight-hour interval for their oviposition. After mating the female butterflies laid eggs in clusters on the ventral side of the cabbage leaves. Each instar larvae, pupae and adult butterfly tissues were also stored in *RNAlater* solution (Life Technologies, USA) at −80 °C for RNA isolation and real time PCR studies.

2.2. Nucleic acid isolation

Genomic DNA was extracted from the whole larvae of *P.canidia* by SDS method [14]. Total RNA was isolated from various larval stages, pre-pupa and pupal stages using RNeasy Plus Mini kit (Qiagen, Netherlands). The quality and quantity of total RNA was analyzed using denaturing agarose gel and Nanodrop (Implen NanoPhotometer), respectively.

2.3. PCR amplification of pierisin genes

First strand cDNA synthesis was carried out using RevertAid first strand cDNA synthesis kit (Thermo Scientific, USA). For the amplification of conserved regions, primers were designed by the Primer3 tool using pierisin gene sequence available in the NCBI database. These primers were used to obtain overlapping PCR products using either cDNA or genomic DNA as a template (Supplementary Table 1). PCR was performed in 20 μl master mix reaction containing 0.5 μM of each primer, two μl of 10x PCR buffer, 1.5 mM of Mgcl2, and 1 unit of Pfu DNA polymerase and 0.5 mM of each of the dNTPs. PCR was performed for 35 cycles with an initial denaturing at 98 °C for 10 s, followed by annealing at 60 °C for 20 s and extension at 68 °C for 30 s and a final extension of 68 °C for 7 min.

2.4. Rapid Amplification of cDNA ends of pierisin genes

RACE experiments were performed to obtain the cDNA ends of *P. canidia*. Gene-specific primers were designed using the newly obtained cDNA sequences corresponding to individual species (Supplementary Table 1). The 5′ and 3′ ends of the transcript were obtained using GeneRacer RACE ready cDNA kit (Invitrogen, USA) according to the manufacture's protocol.

2.5. Cloning and sequencing of PCR product

The amplified PCR fragments from each reaction were subjected to electrophoresis on 1% agarose gels and purified using the QIAquick Gel Purification Kit (Qiagen, Netherlands). These products were cloned into a pJET1.2/blunt cloning vector (Thermo Scientific, USA), verified by restriction digestion analysis and sequenced by ABI PRISM 310 Genetic Analyzer in both the directions (Genotypic, Bangalore, India). PCR products were reproduced by Pfu polymerase, cloned and sequenced three times and confirmed for the sequence identity before submission.

Full-length cDNA of pierisin genes were amplified using the primers obtained from 5′ and 3′ RACE sequences (Supplementary Table 1). These products were cloned and sequenced by Primer walking method. The sequences were submitted to GenBank (NCBI) database.

2.6. Developmental stage-specific expression using real-time qRT- PCR analysis

The quantitative expression of pierisin gene in different developmental stages such as first instar larvae (5–6 days), second instar (9–11 days), third instar (12–15 days), fourth instar (15–19 days), fifth instar (19–24 days), pre-pupae (20–26 days), and pupae (26–34 days) of *P. canidia* was tested.

First-strand cDNA synthesis was performed using Maxima first strand cDNA synthesis kit (Qiagen, Netherlands) using a mixture of oligo (dT) primer and random hexamer. The reverse transcription was performed in a total reaction volume of 20 μl containing 2 μg of total RNA from each sample, 1X reaction buffer containing 0.4 mM of each primer, 2.5 mM Mgcl2, 0.2 mM dNTPs, 1X PCR enhancer, and 0.04 unit/ml of Maxima reverse transcriptase. The reverse-transcribed cDNA from various developmental stages of *P. canidia* larvae (1st–5th instar), pre-pupae and pupae were amplified using the SYBR® Premix Ex Taq containing Tli RNaseH Plus (Takara, Japan) with the primers (10 μM each) from the intron-exon spanning region of pierisin-5 (forward- GACCGCCAACCTTACATGAC; reverse-AATACGGTTGGTCTCCATCG) The real-time PCR was carried out in a StepOnePlus Lifecycler (Life Technologies, USA) as follows: 95 °C for 5 min, 40 cycles of 5 s at 95 °C, and 30 s at 60 °C, followed by a melting step at 60 °C, temperature increasing to 95 °C by 1 °C every 5 s. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward-CCATCGACAAGGCATCTGCTCAC and reverse-GCAGTTGGTTGTGCATGAGG), which has been reported to be relatively stable in the expression of different developmental stages in insects, was chosen as an endogenous control gene to normalize the pierisin expression [15]. Relative expression was studied by comparative Ct method with two μl of the cDNA from each sample. The cDNA of first instar larvae served as the calibrator for gene expression studies. A total of three qPCR replicates were performed for each sample. PCR amplification efficiencies and threshold cycle (Ct) values were calculated by the real-time StepOne program version 2.2 (Life Technologies, USA), and the relative expression of pierisin gene at different developmental stages was estimated using the Pfaffl method [16].