

Persistence of pierisin-1 activities in the adult cabbage white butterfly, *Pieris rapae*, during storage after killing

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Abstract: Crude extracts from larvae, pupae and adults of cabbage white butterflies, *Pieris rapae* and *Pieris brassicae*, and green-veined butterfly, *Pieris napi*, have an ability to induce apoptosis in the human cancer cell lines. As apoptosis inducing protein, pierisin-1 and -2 have been isolated from pupae of *P. rapae* and *P. brassicae*, respectively, and shown to exhibit DNA ADP-ribosylating activity. Although the highest activity was detected in the late phase of larvae and early phase of pupae, certain activity was found in adult butterflies. In order to investigate distribution of substances having pierisin-like activities in butterflies, many species need to be analyzed. However, fresh samples of larvae and pupae are hard to obtain, especially if samples are of scarce species or from overseas. The usage of adult butterflies is practical to examine the distribution of pierisin-like activity in many species. In this study, we examined the cytotoxicity of crude extracts from adults of *P. rapae* against HeLa cells and DNA ADP-ribosylation ability during storage for 1, 2 and 8 weeks at room temperature after killing adult butterflies after eclosion. Body weights decreased to 18% for 8 weeks through dehydration. Cytotoxicity of samples from butterfly kept for 1, 2 and 8 weeks decreased to 47, 39 and 22%, respectively, of the control value. DNA ADP-ribosylating activity of the samples also decreased to 30, 27 and 23%. Similar reduction was observed on western blot analysis with anti-pierisin-1 antibody. Fortunately, these results suggest that cytotoxic and DNA ADP-ribosylating activity persists to some extent in the body after killing, at least for 8 weeks. Thus, butterfly adult samples kept for two months at room temperature can still be useful for examination of the presence of substance having pierisin-like activity.

Keywords: pierisin, cytotoxicity, DNA ADP-ribosylation, *Pieris rapae*

Introduction

Pierisin-1 was initially identified as a cytotoxic protein from pupae of the cabbage white butterfly, *Pieris rapae*, against various mammalian cell lines with measured IC₅₀ values ranging from 0.043 to 270 ng/ml.^{1)–3)} Pierisin-1 is a 98 kDa protein comprising 850 amino acids, which shares sequence similarity with ADP-ribosyltransferases such as cholera and pertussis toxins in its N-terminal region, and

with the lectin domain of the ricin superfamily in its C-terminal region.^{4)–6)} Pierisin-1 binds to receptors, such as globotriaosylceramide and globotetraosylceramide,⁷⁾ on target cell surfaces by interaction of its C-terminal region. The entire protein is then internalized into the cell and cleaved into the N- and C-terminal fragments with intracellular protease. Finally, unlike other ADP-ribosyltransferases, which act on proteins as target substrates,⁸⁾ N-terminal fragment catalyzes the transfer of the ADP-ribose moiety of NAD to N² amino groups of guanine residues in DNA to induce cytotoxicity.⁹⁾

In early stage of studies, we examined cytotoxicity of extracts from adults of 18 species of butterfly using the TMK-1 human gastric cancer cell line. Positive results were obtained with extracts from *P. rapae*, *Pieris brassicae* and *Pieris napi* among the genus *Pieris*.¹⁾ However, no cytotoxicity was ob-

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served in the extracts from other examined butterflies, *Eurema hecabe*, *Colias erate* and *Hebomoia glaucippe*, among family Pieridae and species belonging to other families. Moreover, the active protein was purified from pupae of *P. brassicae* and was named pierisin-2, and this protein showed an amino acid sequence 91% identical to that of pierisin-1 isolated from pupae of *P. rapae*.¹⁰⁾ Pierisin-2 also targets DNA, and the structure of the DNA adduct produced by pierisin-2 is the same as that produced with pierisin-1.¹¹⁾

Pierisin-1 is mainly distributed in fat bodies during the final larval instar, and is highly expressed in fifth instar larvae and early pupae, although certain activities are found in adult butterflies. The levels of pierisin-1 per total protein in crude extract vary with developmental stage: 1st–3rd larval stage, 0.005–0.05%; 4th larval stage, 0.2%; 5th larval to early pupal stage, 0.5%; late pupal stage, 0.2%; adult stage, 0.025%.¹²⁾ It has been suspected that pierisin-1 may play important roles such as induction of apoptosis to remove larval cells fated to die in the pupation of *P. rapae* for metamorphosis. Another possibility is that the strong cytotoxicity of pierisin-1 may be effective as a protective agent against microbes and/or parasitoids. In any case, it would be expected that pierisin-1-like proteins might be present in various butterflies, not only in the genus *Pieris* and related species.

Butterflies belonging to the family Pieridae are nearly 2,000 species in the world.¹³⁾ We need to examine many species to generate an accurate picture of the distribution of substances showing pierisin-like activities in butterflies. Adult butterfly specimens are, in general, more easily obtained than larvae and pupae, so the usage of them is practical. However, it is practically impossible to obtain live butterflies that are rare species or only available in foreign countries. An important question is therefore whether pierisin-like activity in the adult butterfly persists to some extent in the body after killing them. Thus, to determine how pierisin-1 activity decreases after killing, we examined the cytotoxicity of crude extracts from adults of *P. rapae* against HeLa cells, along with their ability to form ADP-ribosylated DNA adducts after storage of adults butterflies for 1, 2 and 8 weeks at room temperature after killing. Immunoblot analysis with anti-pierisin-1 antibody was also included.

Materials and methods

Insects. Adult female butterflies (*P. rapae*) were caught in Tochigi prefecture and kept under live conditions in a net cage on 10% honey solution at room temperature. Eggs oviposited on fresh cabbage leaves were removed and reared on fresh natural diet until the third instar larvae at $21 \pm 1^\circ\text{C}$ of 16 h light: 8 h dark. The fourth and fifth instar larvae were reared on an artificial diet in 3.5 cm diameter petri dish individually. The artificial diet was prepared from 5 g Insecta F-II (Nosan corp., Kanagawa, Japan) and 7 g kale powder (Mykale[®]) (Endo-aojiru co., Osaka, Japan) in 75 ml water, being mixed and boiled for a few minutes. The diet was introduced into the 3.5 cm diameter petri dish.

Preparation of crude extracts. Adult butterflies (male and female), which lived for several days after new adult eclosion in our laboratory, were chosen at random and killed by firm pressure over the thorax, and kept at room temperature for the set periods of time. For the determination, three individuals were separately examined. Abdomens of the adults were weighed and homogenized in nine volumes of 50 mM Tris-HCl (pH 7.5) and 1 mM dithiothreitol (DTT) with a disposable microtube pestle. The homogenates were then centrifuged for 10 min at $10,000 \times g$ to remove debris and protein concentrations of each supernatant were measured by the Bradford assay with a Bio-Rad Protein Assay kit I (Bio-Rad Laboratories, CA, USA).

Cytotoxicity and DNA ADP-ribosylating activity in crude extracts. Cytotoxicity of crude extract samples against HeLa cells was examined by measuring cell viability using WST-1 [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt; Dojindo Laboratory, Kumamoto, Japan] as described previously.^{2), 3)} DNA ADP-ribosylating activity was measured as previously described¹⁴⁾ with slight modifications. Briefly, 5 μg of calf thymus DNA, 18.5 kBq of β -[adenylate-³²P]NAD, and 10 μM β -NAD were incubated with 0.1–10 μg of protein of crude extract from butterflies in 50 μl of reaction solution (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 50 mM NaCl, 1 mM DTT, 25 $\mu\text{g}/\text{ml}$ trypsin) for 30 min at 37°C . Then, 1 μg of proteinase K was added to the reaction mixtures, followed by incubation for an additional 30 min at 37°C . DNA was recovered by phenol extraction, chloroform ex-

traction and ethanol precipitation with ammonium acetate salt. Recovered DNA was dissolved in 9 μ l of 20 mM Bis-Tris-HCl (pH 6.5) containing 5 mM CaCl_2 , and 6.3 μ l aliquots of the DNA mixture were incubated with 2 units of micrococcal nuclease, and 0.02 unit of phosphodiesterase II in a volume of 7 μ l at 37 °C for 12 h. DNA mixtures were digested to deoxyribonucleoside 3'-phosphates, and 3 μ l aliquots were spotted onto TLC sheets. To detect background noise, remaining 2.7 μ l aliquots of DNA mixture without nuclease treatment were spotted onto a TLC sheet. After development using 6 M acetic acid, 0.1 M LiCl, and 3 M urea, these were exposed to Fuji Imaging Plates (Fujifilm, Tokyo, Japan) and DNA adducts were detected with a Bio-Image Analyzer (Fujifilm). Levels of adduct formation were calculated by densitometric analysis of DNA adducts using Scion Image software (Scion, USA), values being normalized to that for 1 pg pierisin-1 in each test. The mean value for pierisin-1 was 2.16 ± 0.79 per picogram of protein.

Immunoblot analysis. Crude extract samples were separated by SDS-polyacrylamide gel electrophoresis and blotted on PVDF transfer membranes (Millipore, MA, USA). Anti-pierisin-1 rabbit polyclonal antibodies were prepared as described previously.¹⁵⁾ ECLTM Anti-rabbit IgG, Horseradish Peroxidase linked F(ab')₂ fragments (from donkey) (GE Healthcare, UK) and ECLTM western blotting detection reagents (GE Healthcare) were employed to visualize protein-antibody complexes.

Results and discussion

Body weights of butterflies kept for 1, 2 and 8 weeks decreased to 32%, 24% and 18%, respectively, of the value for fresh adults. When crude extracts from these samples were examined for cytotoxicity against HeLa cells by WST-1 assay, 50% cell viability was noted with 636 ng/ml from fresh adults (indicated as time period 0 week in Table 1 and Fig. 1). Cytotoxicity of samples from butterflies kept for 1, 2 and 8 weeks at room temperature decreased to 47%, 39% and 22%, respectively (Table 1). Applicable protein concentration on this WST-1 assay is 10-300 μ g/ml, and the detection limit of 50% cell viability appears to be around 400 μ g/ml.

When calf thymus DNA was incubated with crude extracts from the samples and β -[adenylate-³²P]NAD, ADP-ribosylated DNA adducts were detected on TLC sheets in all cases (data not shown),

Table 1. Change of cytotoxicity and DNA ADP-ribosylating activity of crude extracts from adults of *P. rapae* on storage at room temperature.

Period (Week)	n	50% cell viability ^{a)}	ADP-ribosylating activity ^{c)} (pg/ μ g)
0	3	$0.6 \pm 0.2^{\text{d}}$ (100) ^{b)}	$13.5 \pm 5.4^{\text{d}}$
1	3	1.5 ± 0.7 (47)	4.0 ± 1.9
2	3	2.1 ± 1.2 (39)	3.7 ± 1.3
8	3	3.9 ± 2.5 (22)	3.2 ± 0.7

^{a)} Protein concentration (μ g/ml) of crude extracts at the point of 50% cell viability. ^{b)} The inverse values for 50% cell viability were calculated to show the percentage of the value at time point zero. ^{c)} The values for the DNA ADP-ribosylating activity with each sample were converted into picogram of pierisin-1 per microgram of protein. These data were obtained from three independent samples. ^{d)} Mean \pm SD.

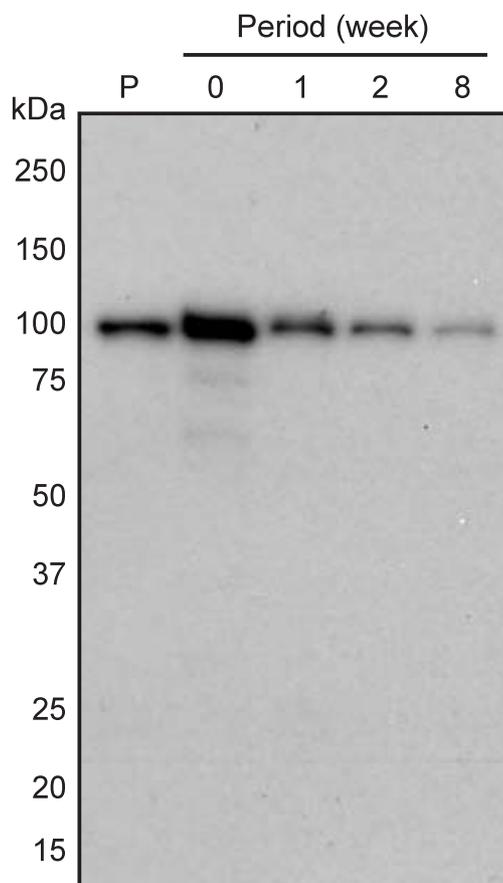


Fig. 1. Decrease of pierisin-1 in crude extracts from butterflies stored for the indicated periods at room temperature. Five microgram protein aliquots of crude extracts were subjected to SDS-PAGE and immunoblot analysis. Lane P; 2.5 ng purified pierisin-1.

and the values for extracts from adults stored for 1, 2 and 8 weeks decreased to 30%, 27% and 23%, respectively, of the value for fresh samples (Table 1).

Under these conditions, the detection limit of ADP-ribosylating activities was calculated to be about 0.2 pg pierisin-1 equivalent per μg of protein.

To quantify pierisin-1 protein in these samples, western blot analysis using anti-pierisin-1 antibody was performed. The level of full-length pierisin-1, 98 kDa band, in the samples from butterflies kept for 1, 2 and 8 weeks decreased to 49%, 29% and 15%, respectively (Fig. 1). Thus, amounts of pierisin-1 protein in extracts from adults stored at room temperature showed a similar tendency for decreasing as found for both biological activities.

In this study, we could demonstrate that around 20% of the pierisin-1 cytotoxic and DNA ADP-ribosylating activity persists in the bodies of adult *P. rapae* kept for 8 weeks at room temperature after killing. These results suggested that samples of adult butterflies, collected and stored in wax paper envelope after killing, at least up to two months, might still be useful for examination of the presence of pierisin-like protein. It was reported that the DNA ADP-ribosylating activity of purified pierisin-1 in solution at pH 9.0 decreases to around half for 42 h at 37°C.¹⁶⁾ In the bodies of butterflies after killing, it is assumed that degradation of pierisin-1 might be suppressed by coexistence with other substances. We are now examining cytotoxicity and DNA ADP-ribosylating activity of crude extracts from various Pieridae butterflies obtained from many other countries. These researches on distribution of pierisin or pierisin-like proteins will contribute to understanding of their biological significance.

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