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Purification of Pierisin, an Inducer of Apoptosis in Human Gastric Carcinoma Cells, from Cabbage Butterfly, *Pieris rapae*

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A substance strongly cytotoxic to human carcinoma cell line TMK-1 has been found in pupae, larvae and adults of the cabbage butterfly, *Pieris rapae*, and named pierisin. Pierisin was purified from the pupae of *P. rapae* by ammonium sulfate precipitation followed by DEAE-cellulose, Phenyl-Sepharose and hydroxyapatite column chromatographies. The molecular weight of the purified pierisin, which was homogeneous on SDS-polyacrylamide gel, was analyzed by mass spectrometry and found to be 98 kDa. Pierisin showed a strong cytotoxic effect, with an IC_{50} of 0.75 ng/ml for human gastric carcinoma TMK-1 cells. The dying cells exhibited characteristic morphological features of apoptosis, such as cell shrinkage, chromatin condensation and nuclear fragmentation. Oligonucleosomal DNA fragmentation was also observed in DNA isolated from pierisin-treated cells. Moreover, similar characteristic changes showing apoptotic cell death were observed in TMK-1 cells treated with a crude extract of pupae of *P. rapae*. These results indicate that pierisin from the pupae of *P. rapae* induces apoptosis in human carcinoma cells.

Key words: Apoptosis — Human carcinoma cells — Butterfly — *Pieris rapae* — Pierisin

Self-defense systems in invertebrates differ from those in mammals. This suggests that invertebrates could be a good source of new bioactive substances. In fact, a number of invertebrate proteins, including lysozymes, defensins and prophenoloxidases, have been identified and characterized as having antibacterial activities.[1–4] In addition, extracts from several species of invertebrates, including mollusks,[5–8] have been reported to show anti-cancer activity. Cecropin, first isolated from the cecropia moth[9] and later shown to exist in many species of insects,[10] is cytotoxic to mammalian lymphoma and leukemia cells.[11] Galactosyl binding lectin, showing macrophage mediated anti-tumor activity *in vivo*, has been found in the flesh fly, *Sarcophaga peregrina*.[12, 13] The lectin was demonstrated to have an NF-κB binding motif in the 5′ upstream region of the gene.[14] These findings, in combination with the fact that insects are highly diverse, suggest that insects might contain novel anti-cancer substances, which could be useful for the study of molecular mechanisms of cell-killing.

Recently, we searched for cytotoxic activity against the human gastric carcinoma TMK-1 cell line in extracts of pupae and adults of 20 kinds of butterflies and moths, and found that three butterflies, *Pieris brassicae*, *Pieris napi* and *Pieris rapae*, contained cytotoxicity.[15] Among the three developmental stages, larvae, pupae and adults, of *P. rapae*, the pupae showed the strongest activity. The active principle in the pupae of *P. rapae* was heat-labile, precipitable with ammonium sulfate and inactivated by protease, suggesting that it is a protein. This cytotoxic principle was named pierisin.[15] Similarly, the cytotoxic factor present in the pupae of *P. brassicae* was suggested to be a protein.[16] Here we report the purification of pierisin from the pupae of *P. rapae* and its activity as an inducer of apoptosis in human gastric carcinoma cells.

MATERIALS AND METHODS

Purification of pierisin from the pupae of *P. rapae* Larvae of *P. rapae* were collected from a cabbage farm where insecticide had not been used, and grown on cabbages to pupate. The pupae, not later than 3 days after pupation, were frozen and stored at −80°C until extraction. The active principle in the pupae was purified by monitoring the cytotoxicity against the human gastric carcinoma cell line, TMK-1, as follows. Frozen pupae were thawed, and their body fluids were extracted by the method described previously, [15] utilizing nine volumes (v/w) of Dulbecco's phosphate-buffered saline (PBS) containing 1 m*M* dithiothreitol to suppress melanization of the pupal extract. The extracted solutions were centrifuged at 10,000*g* for 10 min and filtered through a Millex-GV filter (0.22 μm, Millipore Corp., Bedford, MA). The filtrate, termed the extract, was mixed with ammonium sulfate to give 35% saturation, allowed to stand for 1 h at 4°C and centrifuged. An aliquot of the precipitate, which is equivalent to 50 pupae, was dissolved in 20 ml of 20% (v/v) glycerol-20 m*M* Tris-HCl pH 7.5 (buffer A) and dialyzed against the same solution to remove ammonium sulfate. The dialyzed solution was applied to an anion-exchange DEAE-cellulose DE52 column (1.5×10 cm; Whatman, Fairfield, NJ), equilibrated with buffer A. Adsorbed proteins were eluted with a 100 ml linear gradient of 0–100 m*M* NaCl in buffer A at a flow rate of 20 ml/h. Fractions containing the cytotoxic activity (42–60 ml) were pooled, brought to 20% ammonium sulfate saturation and applied to a Phenyl-Sepharose CL-4B hydrophobic interaction column (0.7×7cm; Pharmacia, Uppsala, Sweden) equilibrated with 20% saturated ammonium sulfate in 20% glycerol-50 m*M* Tris-HCl pH 7.5 (buffer B) solution. Material was eluted with a 12.5 ml linear gradient of 10% to 0% saturated ammonium sulfate in buffer B, followed by 10 ml of buffer B without ammonium sulfate, at a flow rate of 5 ml/h. Fractions containing the cytotoxic activity (12–16 ml) were pooled, dialyzed with 20% glycerol-10 m*M* sodium phosphate pH 6.8 (buffer C) and applied to Bio-Gel HT hydroxyapatite column (1×5cm; Bio-Rad, Hercules, CA) equilibrated with buffer C. The column was developed with a 38 ml linear gradient of 10–200 m*M* sodium phosphate buffer pH 6.8 including 20% glycerol, at a flow rate of 5 ml/h. Active fractions (19–31 ml) were pooled and concentrated to 0.14 ml on a Centricon 50 (Amicon, Beverly, MA).

All the above procedures were performed on ice or at 4°C. Furthermore, native polyacrylamide gel electrophoresis was carried out to confirm that the purified protein is indeed the cytotoxic principle. A Voyager Linear matrix-assisted laser desorption ionization-time-of-flight mass spectrometer (MALDI-TOF/MS) (PerSeptive Biosystems, Framingham, MA) was used to estimate the molecular weight of the purified protein.

Cell line and culture conditions The human gastric carcinoma TMK-1 cells[17] were cultured with Eagle's MEM containing 10% fetal calf serum (Gibco BRL, Gaithersburg, MD). Exponentially growing cells were trypsinized, seeded at an appropriate density, and cultured for at least 1 day prior to use to allow adhesion and growth of the cells.

Cytotoxicity assays The trypsinized cells were suspended in the culture medium at a density of 1×10^5 cells/ml and 0.1 ml of the suspension was dispensed into each well of a 96-well dish. One day later, the medium was changed and samples for assays were added to the fresh culture medium of the cells. After 72 h incubation at 37°C in 5% CO_2 in air, the medium containing the sample was removed, then the cells were subjected to WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium; Dojindo Laboratories, Kumamoto) assay to measure their proliferation activity. Erythrosine B was used to stain and count the dead cells.

Morphological analysis Growing cells (ca. 5×10^5/3.5 cm dish) were incubated with the extract or pierisin in culture medium for 6 to 12 h, then trypsinized, harvested and fixed with 10% formalin in phosphate-buffered saline. The fixed cells were stained with 0.2 m*M* Hoechst 33342 (Sigma, St. Louis, MO) and subjected to fluorescence microscopic analysis. Phase-contrast micrographs of TMK-1 cells treated with samples were also taken.

DNA fragmentation analysis Cells incubated with the samples were trypsinized and collected. Cellular DNA was extracted with a solution containing 10 m*M* Tris-HCl pH 7.4, 10 m*M* EDTA and 0.5% Triton X-100. After treatments with RNase A (Sigma) and Proteinase K (Merck, Darmstadt, Germany), the DNA was precipitated with isopropanol and analyzed by agarose gel electrophoresis to detect the oligonucleosomal DNA ladder.

RESULTS

Table I summarizes the purification of pierisin from the pupae of P. *rapae*. The extract was prepared from fifty pupae of *P. rapae* and fractionated by means of ammonium sulfate precipitation. Most of the cytotoxic activity was detected in the precipitate obtained at 35% saturation of ammonium sulfate. The cytotoxic fraction was subsequently purified on DEAE-cellulose anion-exchange, Phenyl-Sepharose CL-4B hydrophobic interaction and Bio-Gel HT hydroxyapatite columns, to afford a cytotoxic

Table I. Purification of Pierisin from the Pupae of *P. rapae*

Step	Total protein (mg)	Recovery[a] (%)	Purification[a] (-fold)
Extract	1,060	100	1
Ammonium sulfate fractionation	91	79	9
DEAE-cellulose chromatography	5.3	60	120
Phenyl-Sepharose chromatography	2.2	41	200
Hydroxyapatite chromatography	1.4	31	240

a) Recovery and purification values were calculated from the cytotoxic activity towards TMK-1 cells, which was determined by use of the assay described in "Materials and Methods."

fraction that showed a single band on SDS-polyacrylamide gel electrophoresis, corresponding to a molecular weight of around 100 kDa (Fig. 1). In all three column chromatographies, the cytotoxic activity was observed only in fractions containing the 100 kDa protein, but not in other fractions. By these procedures, 1.4 mg of purified protein was obtained, and the cytotoxic activity was elevated 240-fold as compared to the extract. A fractionation experiment by native polyacrylamide gel electrophoresis showed that the cytotoxic activity of each fraction was in proportion to the band intensity of the 100kDa protein, indicating the active principle to be pierisin. Moreover, the molecular weight of pierisin was estimated to be 98kDa by MALDI-TOF/MS (data not shown).

The purified pierisin was subjected to a cell proliferation assay using TMK-1 cells to determine its cytotoxicity. As shown in Fig. 2, treatment of the cells with various doses of pierisin for 72 h resulted in dose-dependent cytotoxicity, with an IC_{50} value at 0.75 ng/ml. To determine the time required for cell death, the cells were treated at concentrations of 0.5, 5 or 50 ng/ml of pierisin, and their viability was examined at intervals using erythrosine. At any concentration, a major decrease of the viability of the cells was observed between 6 h and 12 h treatment (Fig. 3).

To clarify whether cell death induced by pierisin is apoptotic or necrotic, TMK-1 cells were incubated with pierisin at a dose of 5 ng/ml for 6, 9 and 12 h, and the morphology of the cells was analyzed by phase-contrast and fluorescent micrography. Cell death was hardly detected in the case of the 6 h treatment (Fig. 4B). After 9 and 12 h treatment, many detached, floating and fragmented cells were observed (Fig. 4, C and D). These

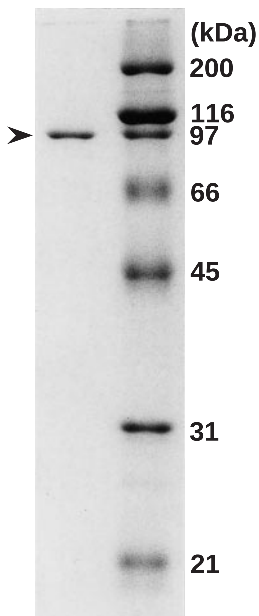

Fig. 1. SDS-polyacrylamide gel electrophoresis of pierisin purified from the pupae of *P. rapae*.

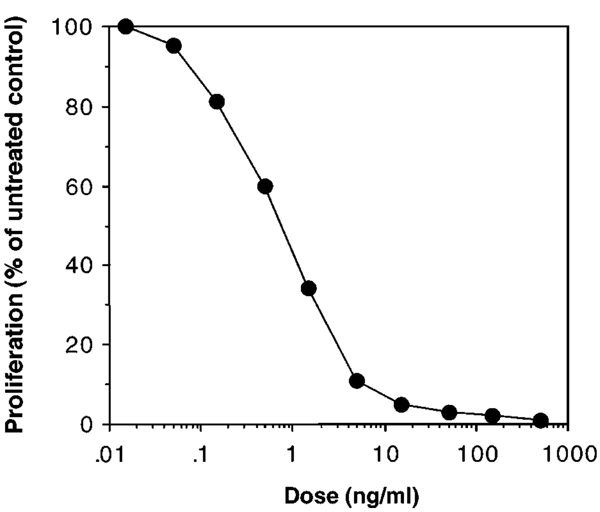

Fig. 2. Dose-dependent cytotoxic effects of purified pierisin on TMK-1 cells. Exponentially growing TMK-1 cells were incubated with different concentrations of pierisin for 72 h and subjected to WST-1 cell proliferation assays. The values shown at each point are the mean of three independent assays using three wells for each assay.

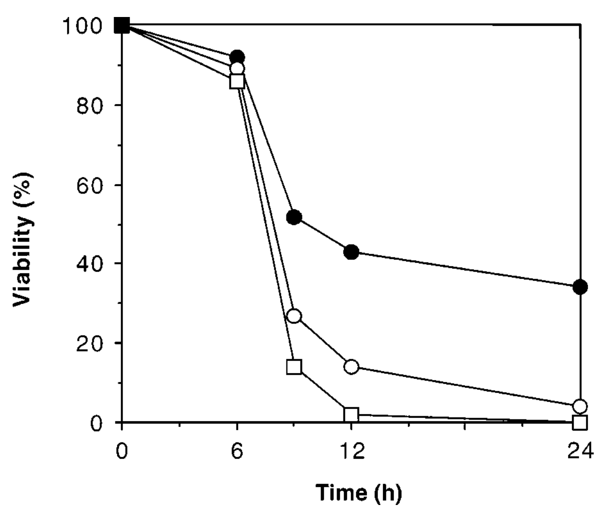

Fig. 3. Time course of cell death induced by pierisin. After treatment with pierisin for the indicated time, TMK-1 cells were stained with erythrosin B to determine their viability. The concentrations of pierisin were: ● 0.5 ng/ml, ○ 5 ng/ml, and □ 50 ng/ml.

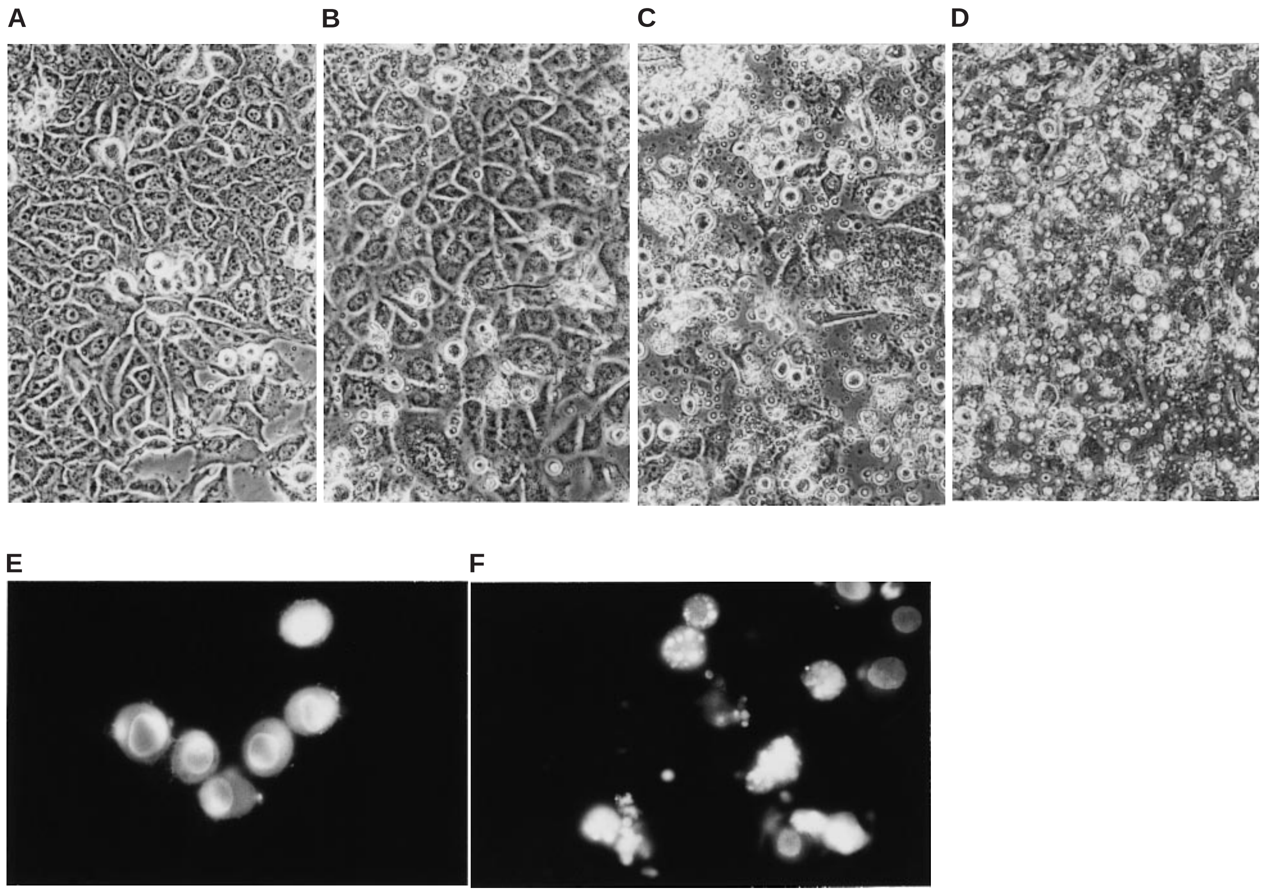

Fig. 4. Morphological analysis of TMK-1 cells undergoing pierisin-induced cell death. The cells were incubated with 5 ng/ml of pierisin for 6, 9 or 12 h. Phase-contrast micrographs of the cells are shown in A for no treatment, B for 6 h, C for 9 h and D for 12 h treatment. The incubated cells were harvested and their nuclei were stained with Hoechst 33342 for visualization by fluorescence microscopy. E, no treatment; F, 9 h treatment.

floating cells exhibited characteristic nuclear morphology of apoptosis such as chromatin condensation and nuclear fragmentation (Fig. 4F). Further evidence supporting apoptotic cell death was provided by DNA fragmentation analysis, as shown in Fig. 5. DNA was degraded to oligonucleosomal DNA fragments, showing apoptosis-specific laddering on agarose gel electrophoresis, when cells were treated with 5 ng/ml of pierisin for 9 h, whereas such laddering was not observed after 6 h treatment. A stronger DNA ladder was observed in the 12 h treated cells. It is concluded that pierisin induces apoptosis in human carcinoma cells.

To confirm that the presence of pierisin is responsible for the apoptosis-inducing activity in the crude extract from the pupae of *P. rapae*, the extract was incubated with TMK-1 cells at $1/10^5$ dilution for 9 h. This incubation also resulted in apoptotic cell death, as indicated by the occurrence of apoptotic bodies, chromatin condensation and oligonucleosomal DNA fragments in the cells.

DISCUSSION

In the present study, a cytotoxic principle, pierisin, was purified from the pupae of the cabbage butterfly, *P. rapae*, and its molecular weight was estimated to be 98 kDa. Chromatin condensation and nuclear fragmentation were observed when human gastric carcinoma TMK-1 cells were treated with purified pierisin. In addition, degradation of cellular DNA to oligonucleosomal fragments was observed in the pierisin-treated cells. These observations indicated that pierisin is an inducer of apoptosis to human carcinoma cells. The potency of pierisin was high: a concentration of 5 ng/ml was sufficient for effective induction of apoptosis. Based on the amount of pierisin in the

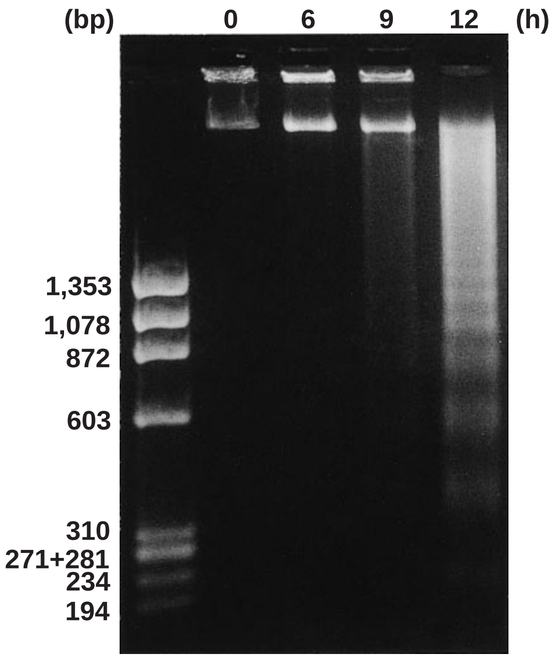

Fig. 5. DNA fragmentation in TMK-1 cells treated with pierisin. The cells were treated with 5 ng/ml of pierisin and the DNA from the cells was run on an agarose gel. Oligonucleosomal DNA fragmentation was observed in the cells treated for 9 and 12 h, but not 6 h.

extract of *P. rapae* and the effective dose of pierisin to induce apoptosis in TMK-1 cells, induction of apoptosis in the cells by the extract could be explained by the presence of pierisin in the extract of *P. rapae*. Pierisin-induced apoptosis may not be a fast pathway like the FasL/Fas pathway,[18–20] since the cells exhibited almost no morphological change after 6 h continuous treatment with pierisin. However, a further 6 h incubation with pierisin clearly induced apoptotic cell death in the TMK-1 cells. This characteristic cell death was also observed when the cells were incubated with pierisin for 1 h, followed by 11-h cultivation without pierisin. The doubling time of TMK-1 cells was around 24 h under the conditions in the present study. These observations suggest that pierisin might induce apoptosis at any cell cycle stage of the cells.

Although the physiological systems in insects are different from those in mammals, the mechanisms of apoptotic cell death are not totally different. The nematode contains several molecular analogues of apoptosis-related proteins existing in mammals, for example, CED-3, CED-4 and CED-9 in *Caenorhabditis elegans* and Caspase, Apaf-1 and Bcl-2 in humans, respectively.[21–23] Thus, it is reasonable that insects possess a protein inducing apoptosis of mammalian cells. Our study clearly demonstrated that the cabbage butterfly protein, pierisin, induced apoptosis of human carcinoma cells. However, it is currently unknown whether pierisin is an apoptosis-induction factor in insect cells. Further analysis on the cytotoxic effects of pierisin is ongoing, in order to clarify the molecular pathway of apoptosis by pierisin.

Sarcophaga lectin is reported to show cytotoxic activity against murine tumors.[12, 13] In addition, the lectin is known to be accumulated to the extent of more than 0.1% in the hemolymph at the pupal stage[24] and is involved in the promotion of the imaginal disk development in the flesh fly, *S. peregrina*.[25] The amount of pierisin in the pupae of *P. rapae* was about 0.4% of the total protein, and such a high concentration may imply some important role in the insect, as in the case of lectins. Our preliminary data indicated that the cytotoxic principle is accumulated in the pupal stage of *P. rapae*,[15] suggesting that pierisin may induce programmed cell death in larval cells to drive insect development. Understanding of apoptosis induction mechanisms in various kinds of cells, including normal cells, by pierisin will be very helpful to clarify the role(s) of this protein in the cabbage butterfly. cDNA cloning of the pierisin gene is under investigation in our laboratory.

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REFERENCES

1) Boman, H. G. and Hultmark, D. Cell-free immunity in insects. *Annu. Rev. Microbiol.*, **41**, 103–126 (1987).
2) Hultmark, D. Immune reactions in *Drosophila* and other insects: a model for innate immunity. *Trends Genet.*, **9**, 178–183 (1993).
3) Hoffmann, J. A. Innate immunity of insects. *Curr. Opin. Immunol.*, **7**, 4–10 (1995).
4) Beck, G. and Habicht, G. S. Immunity and the invertebrates. *Sci. Am.*, **275** (5), 42–46 (1996).
5) Kisugi, J., Kamiya, H. and Yamazaki, M. Purification and characterization of aplysianin E, an antitumor factor from sea hare eggs. *Cancer Res.*, **47**, 5649–5653 (1987).
6) Yamazaki, M., Kimura, K., Kisugi, J., Muramoto, K. and Kamiya, H. Isolation and characterization of a novel

cytolytic factor in purple fluid of the sea hare, *Aplysia kurodai*. *Cancer Res.*, **49**, 3834–3838 (1989).

7) Yamazaki, M., Tansho, S., Kisugi, J., Muramoto, K. and Kamiya, H. Purification and characterization of a cytolytic protein from purple fluid of the sea hare, *Dolabella auricularia*. *Chem. Pharm. Bull.*, **37**, 2179–2182 (1989).
8) Kisugi, J., Yamazaki, M., Ishii, Y., Tansho, S., Muramoto, K. and Kamiya, H. Purification of a novel cytolytic protein from albumen gland of the sea hare, *Dolabella auricularia*. *Chem. Pharm. Bull.*, **37**, 2773–2776 (1989).
9) Hultmark, D., Steiner, H., Rasmuson, T. and Boman, H. G. Insect immunity. Purification and properties of three inducible bactericidal proteins from hemolymph of immunized pupae of *Hyalophora cecropia*. *Eur. J. Biochem.*, **106**, 7–16 (1980).
10) Boman, H. G., Faye, I, Gudmundsson, G. H., Lee, J.-Y. and Lidholm, D.-A. Cell-free immunity in *Cecropia*. A model system for antibacterial proteins. *Eur. J. Biochem.*, **201**, 23–31 (1991).
11) Moore, A. J., Devine, D. A. and Bibby, M. C. Preliminary experimental anticancer activity of cecropins. *Pept. Res.*, **7**, 265–269 (1994).
12) Nakajima, H., Komano, H., Esumi-Kurisu, M., Abe, S., Yamazaki, M., Natori, S. and Mizuno, D. Induction of macrophage-mediated tumor lysis by an animal lectin, *Sarcophaga peregrina* aggulutinin. *Gann*, **73**, 627–632 (1982).
13) Itoh, A., Iizura, K. and Natori, S. Antitumor effect of *Sarcophaga* lectin on murine transplanted tumors. *Jpn. J. Cancer Res. (Gann)*, **76**, 1027–1033 (1985).
14) Kobayashi, A., Matsui, M., Kubo, T. and Natori, S. Purification and characterization of a 59-kilodalton protein that specifically binds to NF-κB-binding motifs of the defense protein genes of *Sarcophaga peregrina* (the flesh fly). *Mol. Cell. Biol.*, **13**, 4049–4056 (1993).
15) Koyama, K., Wakabayashi, K., Masutani, M., Koiwai, K., Watanabe, M., Yamazaki, S., Kono, T., Miki, K. and Sugimura, T. Presence in *Pieris rapae* of cytotoxic activity against human carcinoma cells. *Jpn. J. Cancer Res.*, **87**, 1259–1262 (1996).
16) Kono, T., Watanabe, M., Koyama, K., Sugimura, T. and Wakabayashi, K. Anti-cancer substance in *Pieris brassicae*. *Proc. Jpn. Acad.*, **73B**, 192–194 (1997).
17) Ochiai, A., Yasui, W. and Tahara, E. Growth-promoting effect of gastrin on human gastric carcinoma cell line TMK-1. *Jpn. J. Cancer Res. (Gann)*, **76**, 1064–1071 (1985).
18) Trauth, B. C., Klas, C., Peters, A. M. J., Matzku, S., Möller, P., Falk, W., Debatin, K.-M. and Krammer, P. H. Monoclonal antibody-mediated tumor regression by induction of apoptosis. *Science*, **245**, 301–305 (1989).
19) Itoh, N., Yonehara, S., Ishii, A., Yonehara, M., Mizushima, S., Sameshima, M., Hase, A., Seto, Y. and Nagata, S. The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. *Cell*, **66**, 233–243 (1991).
20) Suda, T., Takahashi, T., Golstein, P. and Nagata, S. Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. *Cell*, **75**, 1169–1178 (1993).
21) Yuan, J., Shaham, S., Ledoux, S., Ellis, H. M. and Horvitz, H. R. The C. elegans cell death gene *ced-3* encodes a protein similar to mammalian interleukin-1β converting enzyme. *Cell*, **75**, 641–652 (1993).
22) Hengartner, M. O. and Horvitz, H. R. C. elegans cell survival gene *ced-9* encodes a functional homolog of the mammalian proto-oncogene *bcl-2*. *Cell*, **76**, 665–676 (1994).
23) Zou, H., Henzel, W. J., Liu, X., Lutschg, A. and Wang, X. Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3. *Cell*, **90**, 405–413 (1997).
24) Komano, H., Mizuno, D. and Natori, S. Purification of lectin induced in the hemolymph of *Sarcophaga peregrina* larvae on injury. *J. Biol. Chem.*, **255**, 2919–2924 (1980).
25) Kawaguchi, N., Komano, H. and Natori, S. Involvement of *Sarcophaga* lectin in the development of imaginal discs of *Sarcophaga peregrina* in an autocrine manner. *Dev. Biol.*, **144**, 86–93 (1991).