Presence of Protein Polymorphism in Karasurin, an Abortifacient and Anti-tumor Protein, Identified with Physicochemical Properties

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Karasurin is an abortifacient plant protein isolated from fresh root tubers of *Trichosanthes kirilowii* MAXIMOWICZ var. *japonicum* KITAMURA (Cucurbitaceae). This study describes the presence of protein polymorphism in karasurin (karasurin-A and karasurin-B) separated by ion-exchange chromatography. Two components showed no differences in the molecular weight (ca. 28000), the amino acid composition, the neutral sugar content and part of the amino acid sequence. A unique difference was observed in their isoelectric points (10.1 for karasurin-A and 10.2 for karasurin-B). However, modifications of glycosylation or phosphorylation were not found. Biological assays for inducing mid-term abortion in pregnant mice and inhibition of the growth of BeWo cells gave no significant differences between them. The presence of protein polymorphism in karasurin, whose biological significance is not yet understood, is a first finding among several abortifacient proteins.

Keywords plant protein; karasurin; *Trichosanthes kirilowii* var. *japonicum*; abortifacient protein; anti-tumor protein; ion-exchange chromatography; isoelectric focusing; amino acid sequence

Karasurin, which was isolated from fresh root tubers of *Trichosanthes kirilowii* MAXIMOWICZ var. *japonicum* KITAMURA (Cucurbitaceae), was a highly basic protein with pI 10.1, and showed potent activity in inducing mid-term abortion in pregnant mice.¹⁾ We have previously determined the complete amino acid sequence.²⁾

In Cucurbitaceae, many abortifacient proteins are now known and show similar physicochemical properties to karasurin.^{1–9)} They are present as a single molecular form. It is interesting that this protein family has several biological roles such as anti-tumor, ribosome-inactivation and anti-human immunodeficiency virus (HIV) (the virus causing acquired immune deficiency syndrome (AIDS)).^{10–14)}

We report herein the finding of an electrophoretic variant of karasurin, which was effective in inducing mid-term abortion in mice and showed strong inhibition of the growth of BeWo cells (human choriocarcinoma cell line) *in vitro* for the first time.

Materials and Methods

Purification Karasurin-A whose primary structure²⁾ was previously determined and karasurin-B (newly found in this study) were isolated from the acetone precipitate obtained from root tubers of *T. kirilowii* MAX. var. *japonicum* KITAM. (collected in the botanical garden of Nagoya City University) as previously described.¹⁾

Acetone powder (100 mg), fraction P1,¹⁾ was separated by ion-exchange chromatography on Toyopearlpack SP 650S (2.2 × 20 cm; Tosoh) with a linear gradient of 0–0.1 M NaCl in 20 mM potassium phosphate buffer (pH 6.5) at a flow rate of 4 ml/min. The eighth and tenth peaks, karasurin-A and -B, respectively, were collected, dialyzed against distilled water, and lyophilized.

Biological Assays The assay of mid-term abortifacient activity was performed by the administration of karasurin-A or -B intraperitoneally on day 12 post coitum (PC). Mice were autopsied on day 14 PC. The total number of implantation sites in uterine was recorded and their sizes measured. The numbers of live fetuses, dead fetuses whose hearts had stopped pulsating, and resorbing fetuses were recorded.¹⁾

The assay of the inhibition of cell growth was performed essentially according to the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) method¹⁵⁾ using a human choriocarcinoma cell line, BeWo (kindly supplied by Japanese Cancer Research Resources Bank). About 2 × 10³ cells in 0.1 ml of culture medium (RPMI 1640 supplemented with 10% fetal calf serum) were inoculated into 96-well culture plates. After 24 h of incubation in a humidified 5% CO₂ atmosphere at 37 °C, karasurin-A or -B in culture medium was added to each well at various concentra-

tions. Cells were incubated for 48 h. The inhibition of cell growth with karasurin-A or -B was assayed by measurement of dehydrogenase activity using MTT (Sigma). After incubation with MTT (50 µg in 10 µl phosphate-buffered saline per well) for 6 h, the resulting products were dissolved by adding 100 µl/well of 10% sodium dodecyl sulfate (SDS) for 24 h at 37 °C. The optical density (OD) measured the difference between 550 and 630 nm using an automatic plate reader (Corona Electric). The results were computed for each well against the incubate without karasurin as a control.

Physicochemical Analyses The molecular weight was determined by SDS-polyacrylamide gel electrophoresis¹⁶⁾ with 15% gel using the molecular weight standards (MW 14400–97400; Bio-Rad).

Deglycosylation was performed for sample (50 µg) with glycopeptidase F (2 munits; Takara) in 0.16 M sodium phosphate buffer, pH 8.6, at 37 °C for 16 h. Dephosphorylation was performed for sample (50 µg) with bacterial alkaline phosphatase (250 units; Bethesda Research Laboratories) in 0.01 M Tris-HCl, pH 8.0, at 37 °C for 30 min. Isoelectric focusing was performed with PhastSystem and PhastGel IEF 3–9 (Pharmacia) using the pI-Marker kit (pI 4.1–10.6; Oriental Yeast).

The neutral sugar content was analyzed by the phenol-sulfuric acid method,¹⁷⁾ with glucose as a standard. The amino acid composition was determined as previously described.¹⁾ The amino acid sequence of the N-terminal region was determined by automated Edman degradation using an Applied Biosystems model 477A automated gas phase sequencer.

Results and Discussion

Separation by ion-exchange chromatography on Toyopearlpack SP 650S (Fig. 1) revealed that karasurin-B was

Fig. 1. Ion-Exchange Chromatography on Toyopearlpack SP 650S of Fraction P1

TABLE I. Mid-Term Abortifacient Activities of Karasurin-A and -B

	Dose (mg/kg)	No. of mice	No. of aborted mice ^{a)} per No. of treated mice
Control	0.2 ml saline	5	0/5 (0%)
Karasurin-A	2.5	5	5/5 (100%)
Karasurin-B	2.5	6	6/6 (100%)

a) Mice were considered to be aborted when the dead fetuses were more than 50% of the total number of implantation sites on day 14 PC.

Fig. 2. Effects of Karasurin-A and -B on Growth of BeWo Cells

□, control (C); ▨, karasurin-A; ▩, karasurin-B. Each bar and vertical line represent the mean and S.E. of 6–8 experiments in each experimental group. a) $p < 0.05$, b) $p < 0.01$, c) $p < 0.001$ vs. control.

Fig. 3. SDS-Polyacrylamide Gel Electrophoreses of Karasurin-A and -B

Samples were: lane 1, marker proteins (molecular sizes are indicated at the left); lane 2, karasurin-A; lane 3, karasurin-B. The gel was stained with Coomassie brilliant blue R-250.

eluted at the tenth peak, whereas karasurin-A was present at the eighth peak.¹⁾ Karasurin-B was adsorbed on a column more strongly than karasurin-A. Karasurin-A and -B comprised 60% and 10% of the protein loaded, respectively.

The biological activity of *in vivo* mid-term abortifacient activity in mice was assayed for karasurin-A and -B. The results (Table I) show that a single intraperitoneal injection of a dose of 2.5 mg/kg of karasurin-A or -B on day 12 PC induced abortion in all pregnant mice.

The effects of karasurin-A and -B on the growth of BeWo cells are assayed (Fig. 2). Both proteins significantly inhibited the cell growth above 0.1 $\mu\text{g/ml}$. The biological activities of both proteins were dose dependent and almost the same.

Fig. 4. Isoelectric Focusing Profiles of Karasurin-A and -B

Samples were: lane 1, karasurin-A; lane 2, karasurin-B; lane 3, karasurin-A plus -B. The positions of pI markers are indicated at the left. The gel was stained with PhastGel Blue R.

TABLE II. Amino Acid Compositions (mol%) and Neutral Sugar Contents (mol/mol protein) of Karasurin-A and -B

	Karasurin-A	Karasurin-B
Asn/Asp	11.2	11.1
Thr	6.6	6.6
Ser	8.9	8.8
Gln/Glu	8.5	8.6
Pro	3.2	2.8
Gly	4.7	5.0
Ala	11.1	11.5
Cys	0.0	0.0
Val	6.1	6.1
Met	0.8	1.2
Ile	7.3	7.2
Leu	10.7	10.6
Tyr	5.9	5.8
Phe	3.8	3.8
Lys	4.8	4.8
His	0.3	0.3
Arg	5.7	5.7
Trp	0.4	0.4
Neutral sugar	<0.60	<0.63

TABLE III. The N-Terminal Sequences of Karasurin-A and -B

Karasurin-A	1	10	20	30
Karasurin-B	1	10	20	30

DVSFRLSGATSSSYGVFISNLRKALPYERKLYDIPLL
DVSFRLSGATSSSYGVFISNLRKALPYERKLYDIPLL

SDS-polyacrylamide gel electrophoresis of karasurin-A and -B showed an identical mobility with the molecular weight estimated to be 28000 (Fig. 3).

Since karasurin-B is more basic than karasurin-A on ion-exchange chromatography, non-equilibrium isoelectric focusing was performed by running the proteins from cathode to anode. Karasurin-B gave a single band and was shown to be more basic (pI 10.2) than karasurin-A (pI 10.1) (Fig. 4). To search for a modification of the karasurins, treatment of glycosidase or phosphatase was performed. The isoelectric focusing profiles of karasurin-A and -B did not change (data not shown).

Analyses of the amino acid composition of karasurin-A and -B showed no significant differences between them. The neutral sugar contents of two proteins were less than 0.63 mol/mol protein (Table II).

Table III shows the amino acid sequences of the N-terminal 37 residues of karasurin-A and -B and the sequences were identical.

Karasurin-B, which was newly isolated in this study, was obtained at about one-sixth of karasurin-A.¹⁾ Karasurin-B showed a strong abortion effect in pregnant mice and strong anti-tumor activity in BeWo cells, the same as karasurin-A. Moreover, physicochemical properties in the molecular weight, the neutral sugar content, the amino acid composition and the amino acid sequence of the N-terminal 37 residues showed no differences between karasurin-A and -B. The only difference between them is basicity as found in ion-exchange chromatography and isoelectric focusing. These facts suggested that electrophoretic variants are present in karasurin. However, the isoelectric focusing profiles of glycosidase- or phosphatase- treated karasurin-A and -B indicate the absence of glycosylation or phosphorylation. Furthermore, microheterogeneity of the amino acid sequence of karasurin is not convinced by the partial analyses of the peptides of karasurin-B (unpublished data). An alternative explanation of the polymorphism is a difference in the contents of Asn and Gln/Asp and Glu between Karasurin-A and -B.

Recently, it is becoming known that some abortifacient and anti-tumor proteins, a family of karasurin, obtained from the Cucurbitaceae subspecies have additional biological activities such as ribosome-inactivation¹¹⁻¹³⁾ and anti-HIV.¹⁰⁾ It will be interesting to know how the polymorphic karasurin molecules perform various biological activities in the future.

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