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Basic Proteins Produced by Hairy Root Cultures of *Trichosanthes kirilowii* var. *japonica*

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Abstract

Hairy root cultures of *Trichosanthes kirilowii* var. *japonica* (Cucurbitaceae) were established by direct inoculation of *Agrobacterium rhizogenes* ATCC15834 on sterile seedlings. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and western blot analysis of intracellular proteins showed that karasurins, ribosome-inactivating proteins (RIPs) in root tubers of *T. kirilowii* var. *japonica*, were not accumulated in the hairy roots. A major basic protein produced by the hairy roots was tentatively identified as a class III chitinase based on N-terminal amino acid sequence homology comparisons. In spite of the apparent lack of karasurins, the basic protein fraction from the hairy roots exhibited ribosome-inactivating activity in the rabbit reticulocyte system, suggesting the presence of RIPs in the hairy roots.

1. Introduction

Trichosanthin and α-trichosanthin are ribosome-inactivating proteins (RIPs) isolated from root tubers of *Trichosanthes kirilowii* Maxim., a Cucurbitaceae plant distributed in China. Among all the RIPs, trichosanthin and α-trichosanthin have been attracting special interest due to their possible utility as anti-HIV agents [1]. We earlier purified three basic proteins (karasurin-A, karasurin-B and karasurin-C) from root tubers of a variety of *T. kirilowii* distributing in Japan, *T. kirilowii* Maxim. var. *japonica* Kitamura [2]. Molecular weight and pI of karasurins are about 27 kDa and 10, respectively. The amino acid sequences of karasurins are highly homologous to the sequences of trichosanthin and α-trichosanthin [3]. We have also shown that karasurins are potent RIPs [3] and that karasurin-A exhibits both abortifacient and cytotoxic activities [4].

Transformation of susceptible plant cells with *Agrobacterium rhizogenes* yields hairy roots which are characterized by a high degree of root branching and many meristematic root tips. Hairy roots have been recognized to be a good source of plant metabolites because they exhibit high overall growth rates comparable to cultured plant cells and are much more genetically stable than cultured plant cells. However, there have been few reports on protein production by hairy root cultures.

In order to attain an efficient production of karasurins, we established hairy root cultures of *T. kirilowii* var. *japonica* by inoculating sterile seedlings with *Agrobacterium rhizogenes*. Here we describe the characterization of basic proteins produced by the resulting hairy roots.

2. Materials and Methods

2. 1 Hairy root cultures

Hairy roots of *Trichosanthes kirilowii* var. *japonica* were obtained by direct inoculation of *Agrobacterium rhizogenes* ATCC15834 on sterile seedlings. Hairy roots induced at the inoculation site were excised and transferred onto Murashige and Skoog (MS) agar medium [5] containing 2% sucrose and 0.3 mg/m*l* Claforan (Hoechst, Japan). They were then subcultured on the same medium for several times at 2-week intervals to eliminate the bacteria. The axenic hairy roots were routinely subcultured using the same medium without antibiotics at 25°C in the dark. For large scale culture, hairy roots (about 500 mg) were inoculated into 150 m*l* MS liquid medium in a 500 m*l* Erlenmeyer flask and cultured on a rotary shaker at 100 rpm at 25°C in the dark. To confirm transformation, agropine and mannopine were detected by paper electrophoresis [6], and the presence of *rol* genes in the genomic DNA was detected by PCR [7].

2. 2 Isolation of basic proteins from hairy roots

Typically, about 50 g fresh hairy roots was homogenized in 150 m*l* water. The homogenate was centrifuged at 9,000 × g for 20 min., and the supernatant was dialyzed overnight against water and lyophilized (Fraction HR-P).

A 30 mg aliquot of the extract thus obtained was dissolved in 3 m*l* 20 mM sodium phosphate buffer (pH 7.0) and applied onto a cation-exchange cartridge

column (Accell Plus CM, Waters, U.S.A.). The protein was eluted with 15 m*l* 20 mM sodium phosphate buffer, pH 7.0, (Fraction HR-PA) and then with 15 m*l* 250 mM sodium phosphate buffer, pH 7.0, (Fraction HR-PB). Both fractions were lyophilized after dialysis against water. Protein content was determined according to the method of Bradford [8] using bovine serum albumin as a standard.

2. 3 Purification and sequencing of a major basic protein

A 0.15 mg aliquot of the HR-PB fraction was dissolved in water and applied to gel filtration HPLC using TSK gel G3000PWXL column. The protein was eluted with acetonitrile-water-trifluoroacetic acid (45: 55: 0.1) at a flow rate of 0.3 m*l*/min. The elution was monitored at 215 nm and the elute corresponding to a major peak was collected. N-terminal amino acid sequence of the purified protein was determined by automated Edman degradation using gas phase sequencer (model 477A, Applied Biosystems, U.S.A.).

2. 4 Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing (IEF-PAGE) were performed using PhastSystem (Pharmacia, Sweden) according to a protocol supplied by a manufacturer. The gels were silver-stained for detection of proteins. Marker proteins for molecular weight and pI were obtained from Bio-Rad (U.S.A.) and Oriental Yeast Co. (Japan), respectively.

2. 5 Western blot analysis

SDS-PAGE was performed with 15% polyacrylamide gels using a Mini-PROTEAN II electrophoresis cell (Bio-Rad). The separated proteins were electroblotted to two pieces of nitrocellulose membrane (Hybond C Extra, Amersham, U.S.A.) with an electrotransfer cell (Mini-Trans, Bio-Rad). One of the two membranes was stained with Amido Black 10B and the other membrane was used for immunodetection using a rabbit polyclonal anti-karasurin antibody and a goat anti-rabbit IgG-peroxidase conjugate (Cappel, U.S.A.) as a secondary antibody. The rabbit anti-karasurin antibody was a generous gift of Prof. K. Wakabayashi, Gunma University. Karasurins were detected by peroxidase-catalyzed color reaction using 4-chloro-1-naphthol as a chromogenic substrate.

2. 6 Assay for ribosome-inactivating activity

The effects of various protein fraction on *in vitro* translation in a rabbit reticulocyte lysate system (Promega, U.S.A.) were estimated according to a protocol supplied by a manufacturer.

3. Results

Hairy root cultures of *Trichosanthes kirilowii* var. *japonica* were established by inoculation with *Agrobacterium rhizogenes* ATCC15834. Integration of the T-DNA region of Ri-plasmids into the plant genome was confirmed by both opine assay on paper electrophoresis and PCR-based detection of *rol* genes (data not presented).

The basic protein fraction (Fraction HR-PB) eluted

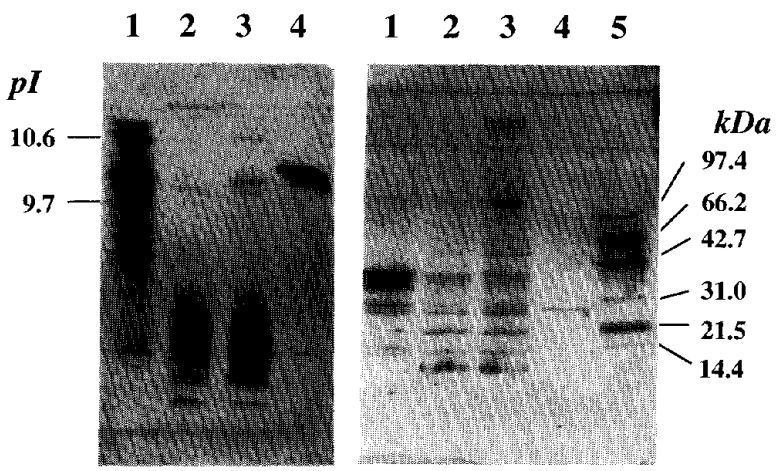

Fig. 1 Silver-stained IEF-PAGE gel (left) and SDS-PAGE gel (right) of the proteins from *T. kirilowii* var. *japonica* hairy roots.
IEF-PAGE gel was loaded with 20 μg of each protein fraction or with 0.2 μg karasurin A. SDS-PAGE gel was loaded with 10 μg of each protein fraction or with 0.1 μg karasurin-A. Lane 1, the basic protein (HR-PB) fraction; lane 2, the acidic and neutral protein (HR-PA) fraction; lane 3, crude protein (HR-P) fraction; lane 4, karasurin-A; lane 5, molecular weight markers (sizes are indicated in the figure). Molecular weight and pI of karasurin-A are 27.1 kDa and 10.1, respectively.

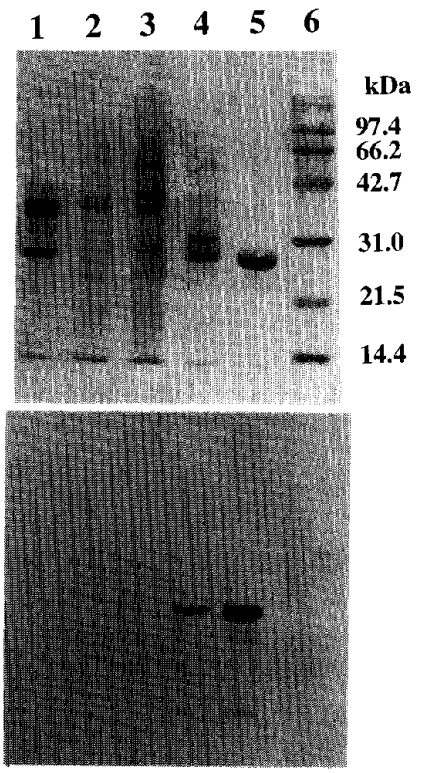

Fig. 2 Western blot of the proteins from *T. kirilowii* var. *japonica* hairy roots.
Above: Amido black 10 B staining of proteins resolved by SDS-PAGE. Lane 1, the basic protein fraction (HR-PB fraction, 20 μg protein) from the hairy roots; lane 2, the acidic and neutral protein fraction (HR-PA, 20 μg protein) from the hairy roots; lane 3, the crude protein fraction (HR-P, 20 μg protein) from the hairy roots; lane 4, the crude protein fraction from root tubers of *T. kirilowii* var. *japonica* (10 μg protein); lane 5, karasurin-A (1.0 μg); lane 6, molecular weight markers (sizes are indicated in the figure). Below: immunodetection using a karasurin-A antiserum. Two-fold more protein was loaded than loaded on the Amido Black 10B-stained gel.

from a CM-cellulose cartridge column was analyzed by SDS-PAGE and IEF-PAGE. As shown in **Fig. 1**, several bands were detected at the pI typical of those of karasurins on the IEF-PAGE. However, no bands of molecular weight of 27 kDa corresponding to the molecular weight of karasurins were found on the SDS-PAGE gel, indicating that karasurins might not be produced in the hairy roots. This was confirmed by western blot analysis (**Fig. 2**), which revealed that there were no proteins immunoreactive to the rabbit anti-karasurin antiserum.

The basic protein fraction was fractionated by gel-filtration HPLC. The main peak (HR-PB1) was collected and the N-terminal amino acids were sequenced. SDS-PAGE of HR-PB1 showed the presence of a major band at molecular weight of about 33 kDa with few faint bands (data not presented). Sequence analysis of HR-PB1 yielded a single N-terminal sequence.

	1														16
HR-PB-1	A	G	I	A	I	Y	W	G	Q	N	G	N	E	G	S	L
[*N-terminal amino acid sequences of class III chitinases*]															
Cucumber	A	G	I	A	I	Y	W	G	Q	N	G	N	E	G	S	L
Chickpea	A	G	I	A	V	Y	W	G	Q	N	G	N	E	G	S	L
Arabidopsis	G	G	I	A	I	Y	W	G	Q	N	G	N	E	G	N	L
Adzuki bean	A	G	G	I	S	V	Y	W	G	Q	N	G	N	E	G	S	L

Fig. 3 Multiple alignment of the partial N-terminal amino acid sequences of the major protein in HR-PB 1 purified from *T. kirilowii* var. *japonica* hairy root cultures and class III chitinases from various sources.
The amino acid sequences of the class III chitinases were retrieved from the Swiss Prot database.

A computer search of the SWISS PROT database showed that the N-terminal sequence of the major protein in HR-PB1 is highly homologous to the sequences of class III chitinases from other species (**Fig. 3**), suggesting its identity as a class III chitinase.

Finally, effects of the protein fractions from the hairy roots on cell-free protein synthesis were examined. When the basic protein fraction (HR-PB) was added to a rabbit reticulocyte system at a concentration of 30 ng protein/m*l*, strong inhibition of protein translation, comparable to 1 ng/m*l* karasurin-A in terms of percent inhibition, was observed (**Fig. 4a**). Inhibition by the acidic and neutral protein fraction (HR-PA) at the same concentration is about 50% of the inhibition caused by HR-PB. Ribosome-inactivating activity of HR-PB fraction was dose-dependent with an IC_{50} of about 5 ng/m*l*, compared to an IC_{50} for karasurin-A of 0.1 ng/m*l* (**Fig. 4b**).

4. Discussion

Ribosome-inactivating proteins (RIPs) from plants catalytically damage eucaryotic ribosomes, making them unable to perform the elongation step of protein synthesis [9]. Type 1 RIPs are single-chain proteins whereas type 2 RIPs consist of two polypeptide chains and possess a galactose-specific binding domain to cell surfaces. Type 1 RIPs are more common and have been identified and purified from more than 30 plants [10]. Interest in type 1 RIPs has been growing due to their widespread physiological activities as abortifacient agents [11] and immunotoxins [12]. The antiviral activity of RIPs has also focused attention on their potential use as anti-HIV agents [13].

There have been few reports on the production of RIPs by plant tissue or cell cultures. Low levels of trichosanthin was reported to accumulate in transformed hairy root cultures of *Trichosanthes kirilowii* var. *japonica* [14]. Trichosanthin was also identified in cell extracts of the transformed callus tissues resulting

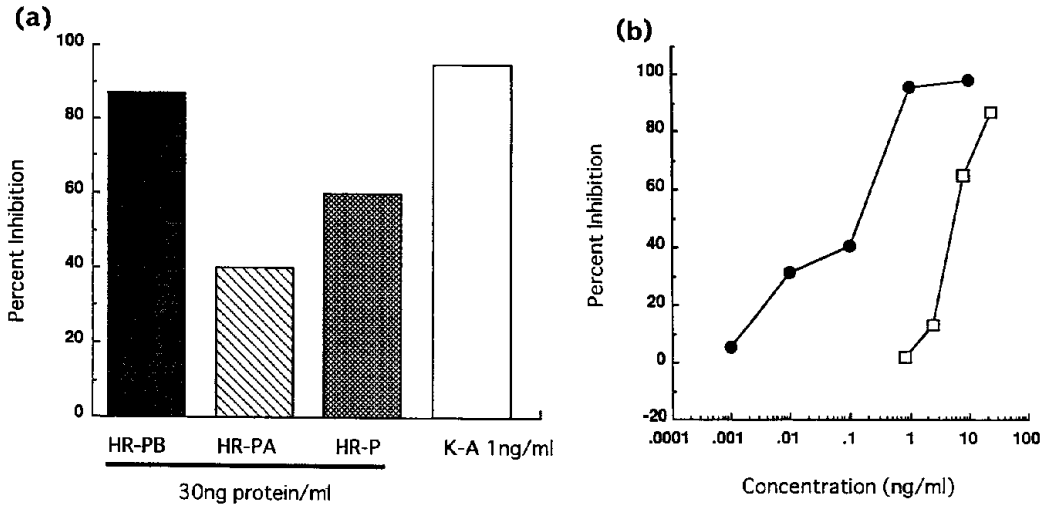

Fig. 4 Inhibition of protein synthesis in a rabbit reticulocyte lysate by the proteins from *T. kirilowii* var. *japonica* hairy root cultures.
(a) The amount of [U-^{3}H] leucine incorporation into protein precipitated by trichloroacetic acid (TCA) in the presence of 30 ng/m*l* protein fraction or 1 ng/m*l* karasurin-A (K-A) was measured.
(b) The amount of [U-^{3}H] leucine incorporation into protein precipitated by TCA in the presence of varying concentrations of the basic protein fraction (HR-PB; open squares) or karasurin-A (closed circles) was measured.

from infection by *Agrobacterium rhizogenes* but not in the untransformed callus of *T. kirilowii* [15]. Contrary to these reports, the present investigation revealed that the hairy root cultures were unable to produce karasurins in immunologically detectable amounts although several basic protein bands were detected by IEF-PAGE. The major protein in the basic protein fraction was tentatively identified as a class III chitinase based on the N-terminal amino acid sequence. This is consistent with the report by Savary and Flores [14], who identified two major extracellular basic proteins and one intracellular basic protein produced by *T. kirilowii* var. *japonica* hairy roots as class III chitinases. However, the N-terminal sequence of HR-PB 1 was very similar to but not identical with the sequence of any of these proteins.

The present result, together with the report describing very low level production of trichosanthin in the hairy roots [14] and transformed callus tissues [15], suggests that production of karasurin or trichosanthin may be associated with induction of secondary growth of roots. Chemical treatments, including addition of sucrose or jasmonates, have been shown to induce accumulation of sporamine in the leaf-petiole region of sweet potatoes [16] and expression of vegetative storage protein genes in soybean [17]. It would be interesting to investigate the effects of these chemicals on karasurin production in the hairy root cultures of *T. kirilowii* var. *japonica*. In fact, methyl jasmonate has been reported to induce RIP production in barley [18].

In spite of the apparent lack of karasurins, some ribosome-inactivating activity was detected in the crude protein from the hairy root cultures, and this RIP activity was concentrated in the basic protein fraction. Since it is unlikely that the class III chitinase has RIP activity, a minor protein having a potent ribosome-inactivating activity might be present in the basic fraction. This possibility seems to be justified by the fact that an RIP (PAP-C) purified from cell cultures of pokeweed exhibited a molecular weight and pI similar to an RIP from pokeweed (PAP) but the amino acid composition and chromatographic behavior of PAP-C are quite different from those of PAP [19].

It might therefore be possible to obtain a new RIP from the hairy root cultures of *T. kirilowii* var. *japonica*, but this requires further investigation.

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