

Cloning and Bacterial Expression of Karasurins, Ribosome-Inactivating Proteins (RIPs) from *Trichosanthes Kirilowii* var. *Japonica*

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Abstract

A genomic clone encoding a karasurin precursor was isolated from root tubers of *Trichosanthes kirilowii* var. *japonica*. The sequence analysis indicates that karasurin-A and karasurin-C exhibiting abortifacient and ribosome-inhibiting activities are produced from a common karasurin precursor by proteolytically cleaving at a different site. Northern hybridization analysis shows that the karasurin gene was expressed only in root tubers. An efficient system to overproduce the recombinant karasurin-A as fused protein with the maltose-binding protein was established.

Keywords: bacterial expression, cloning, karasurin, ribosome-inactivating protein, *Trichosanthes kirilowii* var. *japonica*.

Karasurins are very basic proteins accumulating in root tubers of *Trichosanthes kirilowii* var. *japonica* (Cucurbitaceae). Earlier we purified three karasurin isoforms (karasurin-A, karasurin-B and karasurin-C) and determined their complete amino acid sequences (Toyokawa *et al.*, 1991a; Kondo *et al.*, 1996). We also showed that karasurins exhibited various pharmacological activities including abortifacient (Toyokawa *et al.*, 1991b), cytotoxic (Toyokawa *et al.*, 1991c) and immunosuppressive (Terawaki *et al.*, 1997) activities. Recently we have proven that these activities are mainly due to their RNA N-glycosidase activity and that karasurins belong to type I ribosome-inactivating proteins distributing widely in Cucurbitaceae plants (data not presented). For further characterization of pharmacological activities sufficient and stable supply of the protein is desirable. Furthermore, engineering of karasurins may lead to novel plant proteins with improved pharmacological activities. Towards these ends we cloned a gene encoding karasurins from root tubers of *T. kirilowii* var. *japonica* and established a heterologous expression system enabling us to produce large amount of karasurins as fused proteins with maltose-binding protein (MalE protein) which is described in the present paper.

Because amino acid sequences of karasurins are highly homologous to the α -trichosanthin sequence

(Toyokawa *et al.*, 1991), a PCR primer set (KRN1F=5'-CATTGTAGAGAAATGATGAG-3' and KRN1R=ACGTAGCTTGAAGTTACT-3') covering both the whole coding region and 5' non-coding region of α -trichosanthin gene (Chow *et al.*, 1990). Total DNA was extracted from the root tubers according to the method described by Rogers and Bendich (1994) and used for subsequent PCR as template. The amplified fragment of about 1.2 kb was cloned to pGEM-T vector (Promega). Several clones were randomly selected and their complete nucleotide sequences were determined. The nucleotide and deduced amino acid sequences were described in a previous communication (Mizukami *et al.*, 1997) and appeared in the DDBJ, EMBL and GenBank databases under an accession number AB000666. The nucleotide sequence was 1189 bp and contained an open reading frame of 870 bp encoding 289 amino acids. The deduced amino acid sequence is consistent with that of native karasurin-A and karasurin-C except for a putative N-terminal signal peptide of 21 amino acids and 19 extra amino acids at C-terminus, indicating that karasurin-A and karasurin-C are the products of the same gene, but are proteolytically cleaved at a different site of the precursor protein. Although we sequenced several clones, we could not find a clone whose deduced amino acid sequence is consistent with the

karasurin-B sequence.

To analyze the karasurin gene transcript in various plant organs, total RNAs were prepared from leaves, roots and root tubers of *T. kirilowii* var. *japonica* using TRIZOL reagent (GIBCO/BRL), electrophoretically separated on 1.2% agarose-formaldehyde gel and transferred to a Hybond N+ membrane (Amersham Pharmacia). The coding region of the karasurin gene was labeled with digoxigenin using a PCR DIG Probe Synthesis Kit (Roche Biochemicals) and used as a hybridization probe. As shown in Fig. 1A, a strong signal corresponding to the karasurin mRNA was observed in the root tubers, but not present either in the roots or in the leaves. This indicates that expression of the karasurin gene was linked to root tuber formation.

Total DNA from the root tubers was digested with *Hind*III or *Eco*RI, either of which has no restriction site within the karasurin gene. The digested DNAs were separated by electrophoresis on 1% agarose gel, blotted to a Hybond N+ membrane and hybridized with the same probe used for the northern hybridization analysis. The membrane was finally washed in 0.1 X SSC, 0.1% SDS at 67°C for 15 min. There are four (two strong and two weak) bands between 10 kb and 2 kb in either digest (Fig. 1B).

This reveals that there are at least four copies of karasurin genes, and the two weak signals may arise from genes coding for karasurin-B and/or some other minor karasurin-related proteins.

The karasurin gene segment corresponding to mature karasurin-A was amplified by PCR and cloned into an expression cassette vector pMAL-c2. The pMAL-c2 vector was obtained from RIKEN DNA Bank, Japan. To facilitate cloning into the vector pMAL-c2, a restriction enzyme site of *Eco*RI was incorporated into a forward primer: 5'-GCGCGAATTCGATGTTAGCTTCCGTTTATC-AGG-3', while a *Hind*III site and a stop codon were flanked in a reverse primer: 5'-GCGCAA-GCTTCTATGCCATATTCTTTCTATTTAGCAG-C-3'. The resulting construct pMKRN-A was used to transform *E. coli* JM105 cells. A plasmid clone whose sequence was confirmed to be identical with the ORF sequence of karasurin-A by sequencing was selected and used for expression experiments.

The *E. coli* cells harboring pMKRN-A were grown at 37°C in LB medium until the absorbance at 600 nm reached about 1.0 and protein expression was induced by addition of IPTG to a final concentration of 1 mM. The cells were further incubated for 3 h and collected by centrifugation. One portion

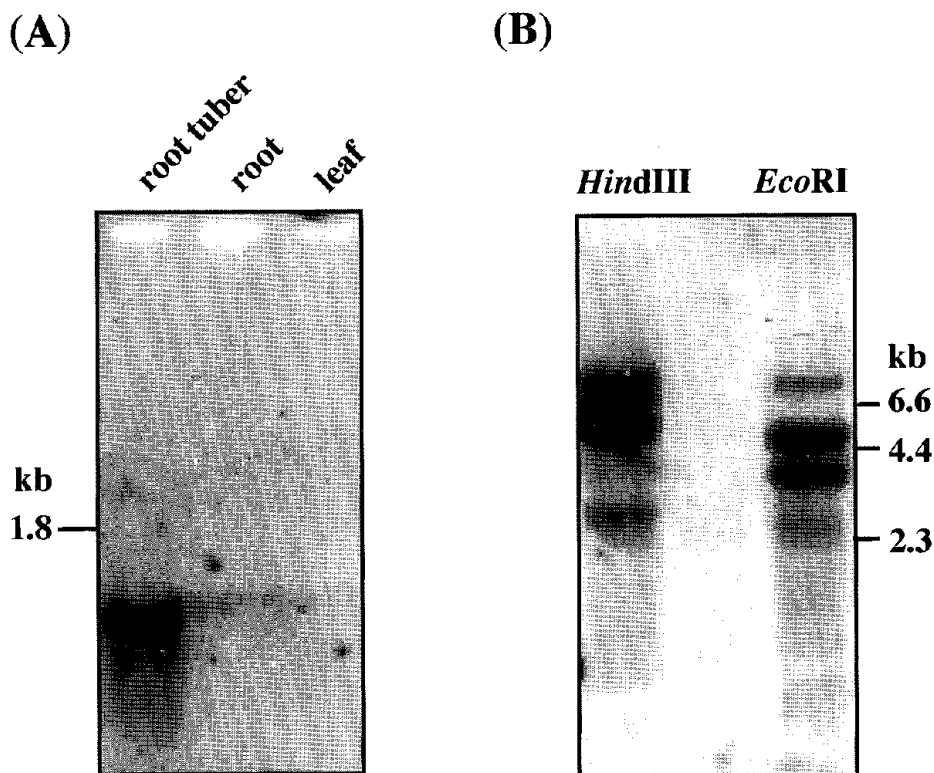


Fig. 1 Northern (A) and southern (B) hybridization analyses of karasurin gene. (A) Total RNAs (10 μ g each) from root tubers, roots and stems of *T. kirilowii* var. *japonica* were separated on 1.2% agarose-formaldehyde gel, transferred to a nylon membrane and hybridized with digoxigenin-labeled karasurin gene. (B) Genomic DNA (10 μ g each) was digested with *Hind*III or *Eco*RI, and probed with the digoxigenin-labeled karasurin gene.

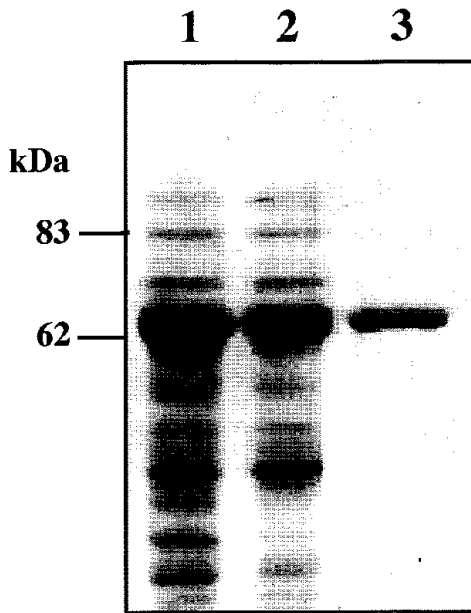


Fig. 2 Production of recombinant malE-karasurin-A fused protein in IPTG-treated *E. coli* harboring an expression vector pMKRN-A. SDS-PAGE analysis of total cell protein (lane 1), soluble protein (lane 2) and affinity-purified protein by Agarose resin column (lane 3).

of the bacterial pellet was boiled in 1 X sample buffer (50 mM tris-HCl, pH 6.8, 5 mM 2-mercaptoethanol, 2% SDS, 0.1% bromophenol blue and 10% glycerol) for 2 min to obtain a total cell protein fraction which was subjected to SDS-PAGE. The rest of the cell pellet was suspended in extraction buffer (50 mM tris-HCl, pH 7.6, 200 mM NaCl, and 5 mM 2-mercaptoethanol) containing 10 $\mu\text{g ml}^{-1}$ lysozyme, incubated at 30°C for 15 min and sonicated. After centrifugation at 12000g for 15 min, the supernatant (soluble protein fraction) was subjected to SDS-PAGE. Upon induction by IPTG, karasurin-A was expressed as a major protein product in the total cell protein (**Fig. 2, lane 1**). The molecular weight of the expressed protein was estimated to be about 70 kDa, 42 kDa larger than that of karasurin-A (28 kDa) due to the presence of fused maltose-binding protein (malE protein). The amount of the recombinant protein was roughly more than 30% of the total protein. The recombinant protein was mostly recovered in the soluble fraction of the bacteria (**Fig. 2, lane 2**). The soluble fraction was applied onto Amylose resin (New England Biolabs) column (bed volume, 1.0 ml). After the column was washed with column buffer (20 mM tris-HCl, pH 7.4, 200 mM NaCl and 1 mM EDTA) the protein was eluted with 20 mM maltose in the column buffer and the eluate was subjected to SDS-PAGE (**Fig. 2, lane 3**). The recombinant

protein was purified to near homogeneity by one-round of the affinity chromatography. The purified protein was successfully cleaved by factor Xa protease to yield malE protein and karasurin-A (data not shown). The yield of malE-fused protein was estimated to be approximately 0.2–0.5 mg/100 ml culture. Thus we established an efficient bacterial production system of the recombinant karasurin-A using the pMAL-c2 cassette vector and the cloned karasurin gene. This enables us to obtain large amounts of the protein for detailed pharmacological investigation and also to engineer the proteins for improving the activities.

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