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Biochemical Characteristics of a Killer Toxin Produced by *Ustilago maydis* Virus SH14 Isolated in Korea

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Toxin protein from *Ustilago maydis* virus SH14 isolated in Korea was purified using ethanol precipitation, cation exchange, gel filtration and anion exchange chromatography. The molecular weight of the purified protein was estimated to be 8.3 kDa by SDS-PAGE analysis. The N-terminal sequence of the protein is L-G-I-N-C(K)-R-G-S-S-Q-C(K)-G-L-S-G which is highly homologous with that of P4 toxin, but the amino acid composition and electrophoretic mobility in a native PAGE of the toxin protein were totally different from those of P4 toxin respectively. The SH14 toxin was shown to have immunological cross-reactivity about 50% with P4 toxin when examined by Western hybridization.

Key words: *Ustilago maydis*, dsRNA virus, toxin protein

Fungal cells of many species contain virus-like particles (9, 12). However, only two fungal species, *Saccharomyces cerevisiae* and *Ustilago maydis*, secrete killer toxins which kill the same or closely related species (3, 6). *U. maydis*, a basidiomycetous fungus which causes a corn smut, has a double stranded RNA virus that may or may not be encapsulated (16). Based on their sizes, three groups of the dsRNA segments are reported: heavy (H), medium (M), and light (L). H segment codes for capsid protein and RNA polymerase, and M segment codes for the killer toxin which kills other sensitive strains of the same species (8). There are three laboratory types of killer strains, designated P1, P4 and P6, and one universal sensitive strain P2 (7). Several biochemical characteristics of KP1, KP4 and KP6 toxins, each of which is associated with a different segmented virus subtype P1, P4 and P6, have been examined (5). KP4 toxin is a single polypeptide of 11.1 kDa processed from its prepropolypeptide whose molecular weight is 13.6 kDa (11). KP6 toxin consists of 2 polypeptides, α (a predicted 78 amino acids) and β (a predicted 81 amino acids), which are cleaved from a single preprotoxin of 219 amino acids (15).

We have isolated 25 corn smut fungal strains in Kangwon Province which contain virus particles or dsRNAs (4, 17), and their dsRNA band patterns and killing activities were examined. They usually showed typical P-type dsRNA band patterns and several strains had very strong killing activity. A toxin protein from the SH14 strain which showed the strongest killing activity was purified by ion exchange and gel filtration chromatography, and its biochemical characteristics were examined. Here we report the biochemical characteristics of the toxin protein such as its molecular weight, N-terminal sequences, and immunological cross-reactivity between anti-SH14 toxin antibody and SH1 (another Korean isolate), KP1 and KP4.

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Materials and Methods

Strains and culture conditions

All of the *U. maydis* strains used had been grown in *U. maydis* complete (UMC) medium (14) at 25°C and kept in 4°C. The SH14 strain for toxin production was grown in 30 ml of glucose minimal medium (1% glucose, 0.1% KH_2PO_4, 0.3% $(NH_4)_2SO_4$, 0.03% $MgSO_4.7H_2O$, 0.01% $CaCl_2$) at 25°C for 48 h, and then transferred to 3 L of glucose minimal medium in a 5 L fermentor. The cells were grown at 25°C, 150 rpm, with aeration (3 L/min) for 72 h.

Bioassay

A sensitive strain (SH10) which was virus-free and had no toxin production but was killed by other toxin producing strains was grown in UMC liquid medium for 2 days. Two hundred microliters

of 2 day culture (SH10) were mixed with UMC medium (0.8% agar), and then poured into a Petri dish. A filter paper disc (Whatman #1, 5 mm in diameter) on the UMC soft agar was soaked with 5 μl of specimen, and the inhibition zone was measured after incubation at 25 C for 36 h.

Purification of toxin protein

Three day culture supernatant was separated by centrifugation at 4,500×g for 10 min. The supernatant was cooled to 10°C and mixed with precooled ethanol (-20°C) to make 40% (v/v) concentration, and then the mixture was filtered through 5 sheets of cheese cloth. Precooled ethanol was added to the filtrates to make 80% (v/v) final concentration, and the precipitates were collected by centrifugation at 4,500×g for 20 min. The precipitates were washed twice with ethanol (95%), and 3 times with acetone. The protein suspension was filtered through the Whatman filter paper (No. 2) and the final precipitates were dried at room temperature overnight. The crude toxin protein was kept in-20 C freezer.

The dried protein (2 g) was dissolved in 1 ml of sodium phosphate buffer (20 mM, pH 6.0). This was loaded onto a CM-Sephadex C-50 column (2.5×40 cm), and the proteins were eluted with a NaCl gradient (0~0.5 M). The protein fractions which showed killing activity were collected, and concentrated by an ultrafiltration kit (M.W. cut-off, 5,000 Da).

The concentrated proteins were separated through a Sephadex G-75 column (2×80 cm; 20 mM Tris, pH 7.0). Fractions which showed good activity were collected and concentrated as above.

Toxin protein was further purified through the DEAE-Sepharose CL-6B column (2.5×25 cm; 20 mM Tris, pH 7.0). At this step, eluents were fractionated as soon as the protein suspension was loaded onto the resin. The purified toxin protein was divided into aliquots and kept in a freezer.

Electrophoresis of the toxin protein was carried out on a polyacrylamide gel for an alkaline protein (13). Determination of the molecular weight of the toxin protein was carried out by following a protocol for low molecular weight proteins (2).

Preparation of anti-toxin antibody

Purified native toxin (40 μg/150 μl in Tris, pH 7.0) with an equal volume of complete Freund's adjuvant was injected into a BALB/c mouse (8~10 weeks old) intraperitoneally. Two weeks later, the same dose of toxin with incomplete Freund's adjuvant was injected intraperitoneally. The third immunization was performed two weeks later as the second shot. Serum from the immunized mouse was used as the polyclonal anti-SH14 toxin antibody.

Biochemical characterization of the purified protein

Sequencing of the N-terminal amino acids of the toxin protein was carried out by the Edman degradation method with Sequetag column (Milligen 6600B). Amino acid composition of the toxin protein was determined with HPLC (Pico-Tag column, Waters). In order to compare the similarity and/or difference of the SH14 toxin with SH1, P1 and P4 toxins, toxin proteins in the culture supernatants of these strains (20 μg total protein) were separated by a low pH, non-denaturing PAGE (13). After the proteins were transferred to a nitrocellulose membrane, anti-SH14 toxin antibody and the alkaline phosphatase conjugated anti-mouse IgG (goat) antibody as a secondary antibody were applied. Toxin proteins were localized by NBT/BCIP color reaction.

Results and Discussion

U. maydis SH14 strain like other killer strains produced lots of viscous material in the culture supernatant when it was grown in the UMC medium. However the viscosity was extremely low when the fungus was cultivated in the glucose minimal medium. This helped in the purification of the toxin protein. When the crude toxin protein was separated through CM-Sephadex C-50 cation exchange chromatography, fractions in the concentrations of 0.15~0.35 M of NaCl showed killing activity (Fig. 1A). The killer toxin protein was further purified through Sephadex G-75 gel filtration chromatography (Fig. 1B) and DEAE-Sepharose CL-6B anion exchange chromatography (Fig. 1C). The toxin protein was purified 6.2-fold with a recovery of 8.7% (Table 1). When the purified toxin was analyzed by SDS-PAGE (Fig. 2A), it showed to consist of a single polypeptide and its molecular weight was 8.3 kDa (Fig. 2B). When its molecular weight was determined by gel filtration through a Sephadex G-50 column, its molecular weight was estimated at 6.5~7.5 kDa according to the column used (data not shown). When compared with other P-type killer toxins, the molecular weight of KP1 is 13.4 kDa (10), and that of KP4 is 11.1 kDa (11). KP6 consists of 2 polypeptides; the molecular weight of the α chain is 8.6 kDa and that of the β chain is 9.1 kDa (15).

The N-terminal sequence of the SH14 toxin protein was L-G-I-N-C(K)-R-G-S-S-Q-C(K)-G-L-S-G and

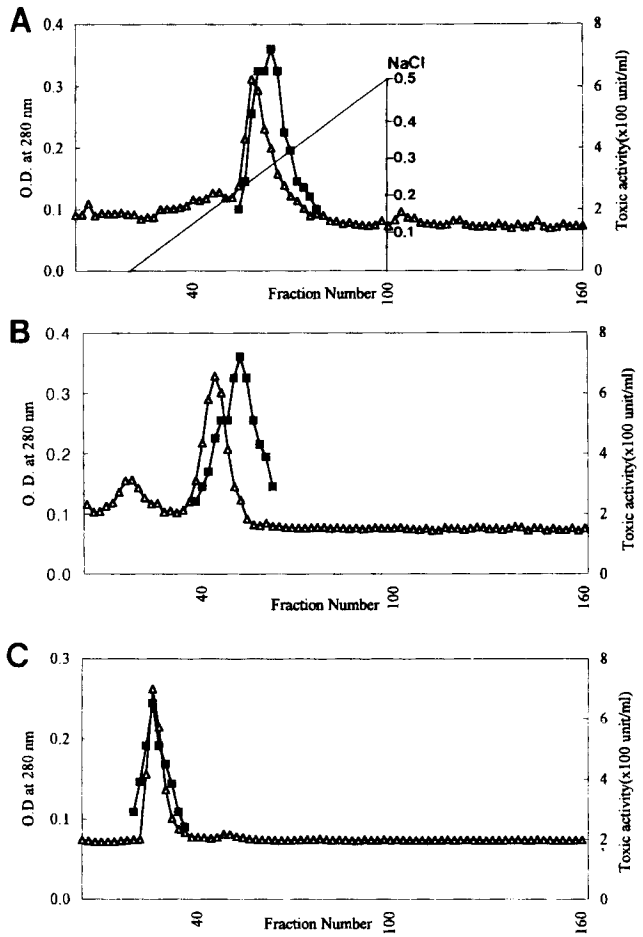

Fig. 1. Purification of killer toxin protein produced by *Ustilago maydis* SH14 virus. (A) CM Sephadex C-50 cation exchange chromatography. (B) Sephadex G-75 gel filtration chromatography. (C) DEAE Sepharose CL-6B anion exchange chromatography. Total protein (△-△). Toxin protein (■-■).

the total amino acid composition was shown in Table 2. The molar concentration of D/N and E/Q was 21.96%, that of basic amino acids (H, K, R) was 10.6%, that of hydrophobic amino acids (A, G, I, L, V) was 34.98%, and that of aromatic amino acids was 3. 82%. Even though the N-terminal sequence of the SH14 toxin protein was identical to that of KP4 if

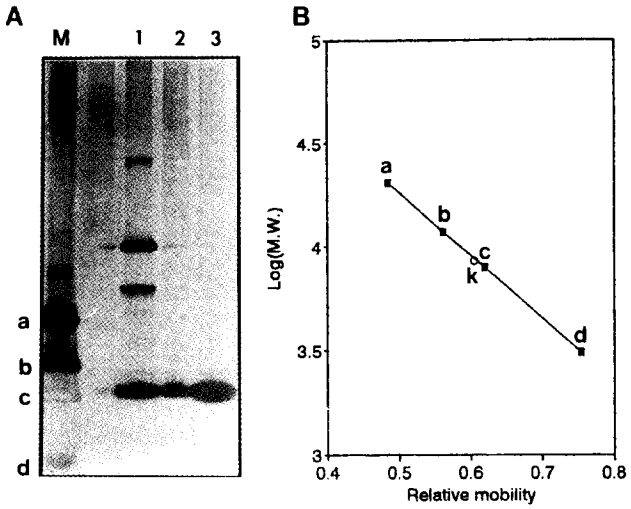

Fig. 2. Determination of the molecular weight of the toxin protein. (A) SDS-PAGE (10% stacking gel, 16% separating gel) analysis of toxin proteins. Lanes: M, molecular weight markers (a, trypsin inhibitor; 21.5 kDa. b, cytochrome, c; 12. 5 kDa, C, aprotinin; 6.5 kDa. D, insulin chain B; 3.4 kDa.); 1, toxin protein after CM Sephadex C-50; 2, toxin protein after Sephadex G-75; 3, toxin protein after DEAE Sepharose CL-6B. (B) Molecular weight determination of toxin protein. MW markers are same as the panel A, and k is the killer toxin.

the C(K) was C, the total amino acid composition was different (Table 2; 1). Most of all, SH14 toxin protein and KP4 showed different mobilities in a native PAGE analysis. Since the anti-SH14 toxin antibody was generated using the native protein, all of the toxin proteins were separated through the native PAGE, and then transferred to the nitrocellulose membrane. When the SH14 and KP4 toxins were analyzed by Western hybridization, these two toxin proteins showed different mobilities and different cross-reactivities (ca. 50% by scanning densitometry) against anti-SH14 toxin antibody (Fig. 3). It was thus concluded that SH14 toxin protein was different from KP4 even though their N-terminal sequence were identical. Since it is possible that the SH14 toxin might be a fragment of KP4, we plan to clone the toxin gene by the reverse transcription (RT) PCR using the N-terminal amino acid

Table 1. Purification of the *Ustilago maydis* SH14 killer toxin

	Total protein (mg)	Total unit[a] (U)	Specific activity (U/mg protein)	Yield (%)	Purification fold
Culture supernatant	320	541,700	1,692.8	100	1.0
Ethanol precipitation	88	518,400	5,890.9	95.7	3.5
CM-Sephadex C-50	20.7	126,720	6,121.7	23.4	3.6
Sephadex G-75	7.0	51,840	7,405.7	9.6	4.4
DEAE-Sepharose CL-6B	4.5	47,320	10,515.6	8.7	6.2

[a] 1 unit: Amount of toxin required to produce a growth inhibition zone of area 78.5 mm^2 when placed on a lawn of SH10 sensitive strain.

Table 2. Amino acid composition of *U. maydis* SH14 toxin protein

Amino acid	Mol%
Ala	5.86 (3.81)
Arg	5.87 (7.62)
Asp/Asn[a]	11.86 (14.29)
Cys	9.89 (9.52)
Glu/Gln[b]	10.10 (8.57)
Gly	12.02 (13.33)
His	1.95 (2.86)
Ile	3.70 (4.76)
Leu	8.05 (6.76)
Lys	2.81 (0.95)
Met	0.68 (0.95)
Phe	1.31 (0.00)
Pro	3.64 (1.90)
Ser	7.68 (9.52)
Thr	4.82 (4.76)
Trp	2.04 (0.00)
Tyr	0.47 (2.86)
Val	7.27 (7.62)

[a] Sum of aspartic acid and asparagine.
[b] Sum of glutamic acid and glutamine. Numbers in parentheses are mol % of KP4 toxin protein.

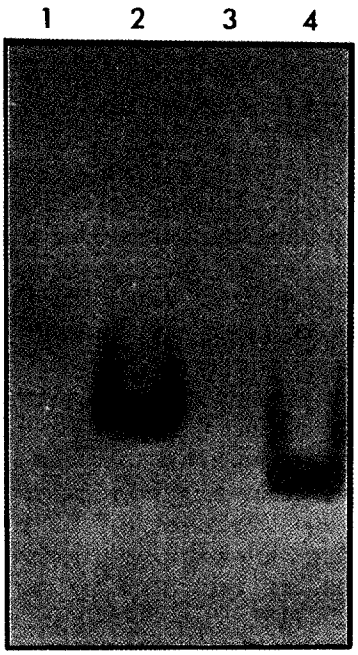

Fig. 3. Determination of cross-reactivity of toxin proteins with anti-SH14 toxin antibody by Western hybridization. Lanes: 1, SH1; 2, SH14; 3, P1; 4, P4.

sequence. Furthermore we are going to analyze the killing mechanism of the toxin protein.

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