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Isolation and characterization of a novel ribosome-inactivating protein, β-kirilowin, from the seeds of *Trichosanthes kirilowii*

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A novel ribosome-inactivating protein designated β-kirilowin was isolated from the seeds of *Trichosanthes kirilowii*. The proteins exhibited strong abortifacient activity in pregnant mice and produced a single band after SDS-polyacrylamide gel electrophoresis with an apparent mol. wt. of 27,500 daltons. It was active in inhibiting the cell-free translation system with an ID_{50} of about 1.8 ng/ml. No cross-reactivity with trichosanthin could be detected by immunodiffusion. Sequence comparison of the first 10 residues of β-kirilowin with ribosome-inactivating proteins isolated from *T. kirilowii* such as trichosanthin, trichokirin and karasurin indicated 70%, 60% and 70% identity, respectively.

Several biologically active proteins have been isolated from *Trichosanthes kirilowii* Maxim, the traditional medicinal plant Tian Hua Fen. The most intensively studied is trichosanthin (TCS), used as an abortifacient and for the treatment of trophoblastic tumors (1). TCS was later identified as a ribosome-inactivating protein (RIP)(2). Recently, TCS and some other RIPs were discovered to have anti-human immunodeficiency virus (HIV) activity, that is, they can specifically inhibit the infection and replication of HIV-infected cells (3). RIPs are proteins that can inhibit protein synthesis through their action on the ribosome (4-6). They possess RNA N-glycosidase activity that can cleave a specific glycosidic bond in the 28S rRNA (7).

Besides TCS, a related protein, *Trichosanthes* anti-HIV protein (TAP 29), has been isolated from the root tubers of *Trichosanthes kirilowii* (8). The N-terminal sequence of TAP 29 is highly homologous to TCS and they possess similar anti-HIV activities except that TAP 29 is less toxic to host cells. Another RIP, karasurin, with about 98% sequence homology to TCS, was also isolated from a related species of *Trichosanthes kirilowii* (9).

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Seeds were another major source of RIPs. Casellas et.al. (10) reported the isolation of a RIP from the seeds of *Trichosanthes kirilowii* designated as trichokirin. It is a basic glycoprotein with an apparent molecular weight of 27,000 daltons. The N-terminal sequence of trichokirin also exhibits a substantial degree of homology to TCS and TAP29. In a search for more RIPs in seeds of *Trichosanthes kirilowii,* we discovered a novel RIP, designated β-kirilowin. This report describes the isolation, the physiochemical property and biological activity of this novel RIP.

Materials and Methods

Seeds of *Trichosanthes kirilowii* were purchased form Ping Hu of Zhejiang, China. L-[4,5-^{3}H] leucine and methyl-^{3}H-thymidine were purchased from New England Nuclear. Chromatographic media and columns were obtained from Pharmacia, LKB. Antisera against trichosanthin and *T. kirilowii* seed acetone powder were obtained from rabbits immunized with the protein in complete and incomplete Freund's adjuvants according to a previously reported protocol (11).

Purification of β-kirilowin

Seeds of *Trichosanthes kirilowii* were decoated and soaked in normal saline (3ml/g) for approx. 2 hr. at 4°C. After homogenization in a Waring blender, the homogenate was stirred at 4°C overnight. The pH of the homogenate was adjusted to 4 with 1N HCl, and it was then centrifuged (1500 g, 10 min). The supernatant was filtered through 4 layers of cheesecloth. Ice-cold acetone (0.5V/V) was slowly added to the supernatant and the mixture was allowed to stand at 4°C for 2 hr. The precipitate (AP0.5) was collected by centrifugation (27,000 g, 20 min). The supernatant was precipitated again with an equal volume of ice-cold acetone. After standing for 2 hr at 4°C, the precipitate designated as crude acetone powder (AP2.0) was collected by centrifugation as described above. The acetone precipitates (both AP0.5 and AP2.0) were redissolved in distilled water and dialyzed against water. The dialyzate was lyophilized to give the acetone powder which was the starting material for further purification. The yields of AP0.5 and AP2.0 were usually around 2.8% and 0.5% by weight respectively.

CM-Sepharose CL-6B column chromatography

The crude acetone powder (AP2.0) was dissolved in sodium phosphate buffer (5 mM, pH 6.4). Insoluble material was pelleted by centrifugation. The supernatant was applied onto a column of CM-Sepharose CL-6B (1.5x28 cm) which had been equilibrated with the same buffer. After unadsorbed material has been eluted, a linear gradient of 0.05 to 0.4 M sodium chloride in 5 mM sodium phosphate buffer was applied. Protein peaks monitored by A280 were collected, dialyzed against distilled water and lyophilized. Fractions C7 and C7', which were eluted at around 270 mM sodium chloride, were found to possess the highest abortifacient activity in mice.

Gel filtration and FPLC on Mono-S column

Fraction C7' obtained from the CM-Sepharose CL-6B column, active in the assay for abortifacient activity, was further purified by gel filtration on Sephadex G-100 to produce two major peaks, G1 and G2. The active fraction G1 was purified by FPLC on a Mono-S column to give a single peak which was eluted at 94 mM NaCl. This purified protein was designated as β-kirilowin.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The fractions obtained during the purification process were monitored by SDS-PAGE according to the method of Laemmli (12). The apparent molecular weight of the active protein was determined by comparing its electrophoretic mobility with those of standard protein markers.

Automated Edman degradation and amino acid composition analysis

Protein sequence determination as well as amino acid composition analysis were performed at the Molecular Biology Resource Facility at University of Oklahoma Health Sciences Center in Oklahoma City, Oklahoma, USA. Sequence analysis of β-kirilowin was performed with an Applied Biosystems 470A gas-phase sequencer with on-line PTH identification using an Applied Biosystem 120 PTH analyzer. For amino acid composition analysis (13), samples were hydrolyzed in 6 N HCl in evacuated sealed tubes at 110°C for 20-24 hours. Hydrolyzed samples were vacuum-dried, dissolved in 0.01 N HCl, and filtered through a 0.45 micron nylon filter before analysis. Amino acid analysis was performed by cation exchange chromatography. Amino acid elution was accomplished by a two-buffer system. Samples equilibrated with 0.2 N sodium citrate, pH 3.28 were injected into the column. This buffer eluted the first nine amino acids. Next, 1.0 N NaCl (pH 7.4) was used to elute the remaining amino acids. Amino acids were detected by on-line post column reaction with ninhydrin (Trione, Pickering Laboratories, Inc.). Derivatized amino acids were quantitated by absorption at 570 nm, except for glutamic acid and proline, which were detected at 440 nm. This procedure was performed on a totally automated Beckman system Gold HPLC amino acid analyzer.

Inhibition of cell-free protein synthesis

Cell-free protein synthesis assay using a rabbit reticulocyte lysate was conducted by determining the incorporation of L-[4,5-^{3}H] leucine into trichloroacetic acid (TCA) precipitable proteins (14). The TCA precipitate collected by filtration onto a glass fiber disc (Whatman GF/C) was processed for liquid scintillation counting.

Assay of abortifacient activity (15)

Mature female ICR mice were caged with fertile male mice. The presence of copulation plug in the following morning was designated day 1 of pregnancy (PD1). Protein fractions were administered intraperitoneally on PD11. The animals were autopsied on PD14 to examine the condition of the fetuses. Mice were considered to be aborted when the number of dead fetuses was greater than 50% of the total implantation sites.

Immunodiffusion

Double immunodiffusion was performed as described previously (15) to test the immunological relationship between trichosanthin and β-kirilowin.

Results and Discussion

Isolation of β-kirilowin

The *T. kirilowii* seed acetone powder was fractionated by CM-Sepharose CL-6B column chromatography (Fig. 1). Gradient elution with NaCl revealed several major peaks. Abortifacient activity was found to reside in fractions C7 and C7' which were eluted at 0.26-0.28 M NaCl (Table 1). After further purification as described in

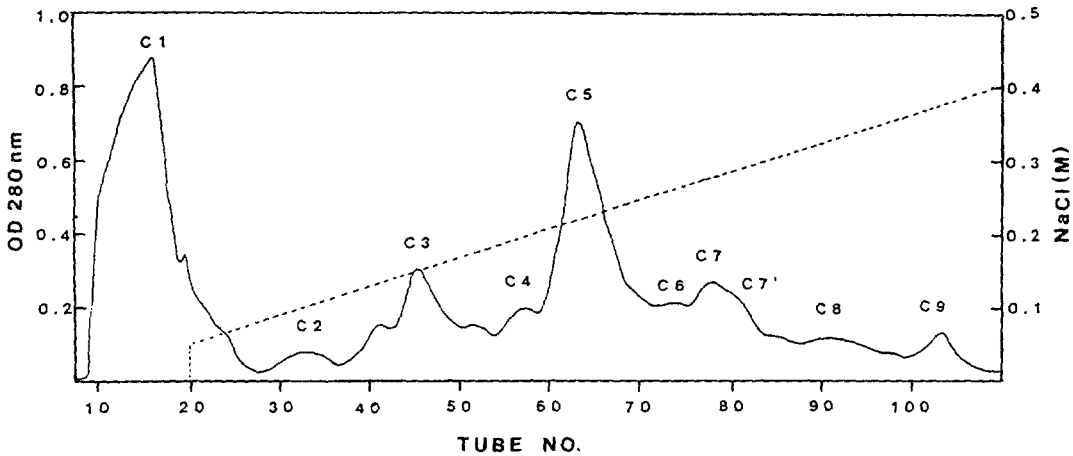

Fig.1. Elution profile of *Trichosanthes kirilowii* seed crude powder (A.P. 2.0) from a CM-Sepharose CL-6B column (1.5x28cm). Eluent for tubes 1-20: 5mM sodium phosphate buffer (pH 6.4). Eluent for tubes 20-110: linear gradient of 0.05 to 0.4M sodium chloride in 5 mM sodium phosphate buffer. Fraction size, 5ml.

the Materials and Methods section involving gel filtration and FPLC, a novel protein designated β-kirilowin was isolated from fraction C7' (Fig. 2). It displayed a single band in SDS-PAGE with an apparent molecular weight of 27,500 daltons (Fig. 2). The overall amino acid compositions of β-kirilowin and other RIPs from *T. kirilowii* were very similar (Table 2), especially with respect to the hydrophobic residues, val, ile and leu, and the aromatic residue phe. Major differences occurred in ser, arg and

Table 1. Abortifacient activity of fractions prepared from the seeds of *T. kirilowii*

Fractions	Sample dose (mg/25gm)	Abortifacient activity* (%)
Crude powder	1.0	80
Acetone powder	0.5	100
C7'	0.1	76.5
G1	0.1	100
β-kirilowin	0.05	80

*Abortifacient activity = no. of aborted mice** / no. of treated mice.

**Mice were considered to be aborted when the number of dead fetuses was > 50% of the total implantation sites.

Table 2. Amino acid compositions of β-kirilowin and other RIPs from *T. kirilowii*

Amino acids	β-kirilowin	trichokirin**	trichosanthin*	karasurin*
Asx	29	23	29	27
Thr	15	19	16	17
Ser	32	24	25	24
Glx	24	22	20	20
Pro	7	8	8	8
Gly	20	16	11	11
Ala	22	21	28	27/28
½Cys	---	2	---	---
Val	12	13	15	16
Met	3	3	4	3
Ile	18	16	19	19
Leu	23	24	25	25
Tyr	8	12	13	14
Phe	10	10	9	9
Lys	16	17	10	11
His	3	1	1	1
Arg	7	6	13	13
Trp	n.d.	n.d.	1	1
Total	**(249)**	**(237)**	**247**	**246/247**
Reference	this work	10	16	9

( ) = estimated value.
n.d.= not determined.
* composition was calculated from the sequence.
** composition was converted into integral numbers from the data revealed with mol/mol in the corresponding paper.

glx residues. β-kirilowin contained a high abundance of asx and glx residues and migrated towards the anode in an acidic polyacrylamide gel system (data not shown), indicating that it is a basic protein. Thus we expect that a considerable portion of the asx and glx residues to be derived from asn and gln. The N-terminal sequence of the first 10 residues of β-kirilowin in comparison with other RIPs such as TCS, karasurin and trichokirin is shown in Table 3. The sequence of β-kirilowin clearly shows an insertion between the first and second residues which is different from the other similar RIPs. Furthermore, there was no cross-reactivity between trichosanthin and antiserum against *Trichosanthes kirilowii* seed acetone powder indicating that

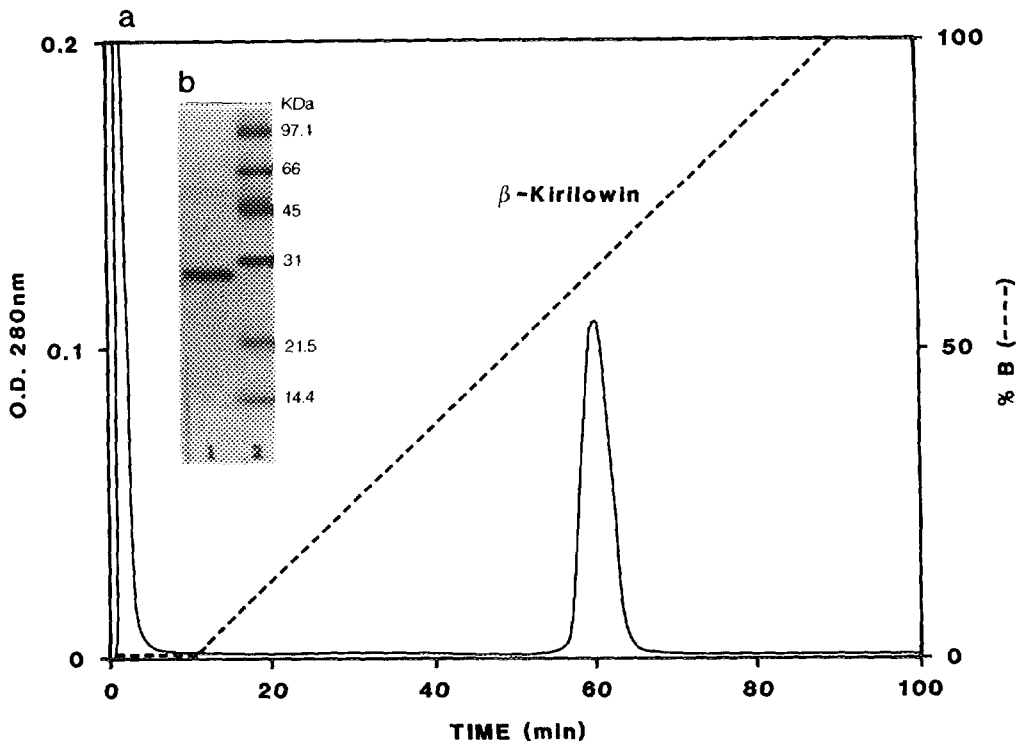

Fig.2a. Purification of β-kirilowin on a Mono-S column by FPLC. Solvent A: 2 mM sodium phosphate buffer pH 7.5, Solvent B: 150 mM sodium chloride in solvent A. Flow rate: 1ml/min.

Fig.2b. SDS-polyacrylamide gel electrophoresis of β-kirilowin. Lane 1, β-kirilowin; lane 2, molecular weight markers: 1) phosphorylase b, (97,400), 2) bovine serum albumin (66,200), 3) ovalbumin (45,000), 4) carbonic anhydrase (31,000), 5) soybean trypsin inhibitor (21,500) and 6) α-lactalbumin (14,400).

trichosanthin is immunologically distinct from β-kirilowin and that there is no or only a very low level of trichosanthin in the seeds (data not shown).

Biological activity of β-kirilowin

At a dose of 0.05 mg per 25 gm body weight, both purified β-kirilowin was able to induce 80% abortion in pregnant mice (Table 1). It exhibited similar activity

Table 3. Sequence comparison of ribosome inactivating proteins isolated from *T. kirilowii*

Proteins from *T. kirilowii*	**Tissue source**	**N-terminal sequence**										**ref.**
β-Kirilowin	seed	D	N	I	I	F	R	L	S	G	A	this work
Trichosanthin	root	D	--	V	S	F	R	L	S	G	A	16
Trichokirin	seed	D	--	V	S	F	R	L	S	G	G	10
Karasurin	root	D	--	V	S	F	R	L	S	G	A	9

as TCS in inhibiting cell-free protein synthesis in a rabbit reticulocyte lysate system with an ID_{50} in the range of 1.2-1.8 ng/ml (data not shown).

The isolation of a novel RIP from the seeds of *T. kirilowii* is reported herein. Though we were not able to locate the earlier reported trichokirin (10) from our study, we cannot preclude the possibility that it may be present in other fractions eluted from the CM-Sepharose column. Another protein with RIP activity, immunologically distinct from trichosanthin but with a molecular weight slightly larger than that of β-kirilowin, was detected in fraction C7 eluted from the CM-Sepharose column. The relationship of this protein, designated α-kirilowin, with β-kirilowin remains to be elucidated.

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