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Preparation and Preliminary Application of Monoclonal Antibodies against Trichokirin-S1, a Small Ribosome-inactivating Peptide from the Seeds of *Trichosanthes kirilowii*

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Abstract Trichokirin-S1, a small ribosome-inactivating peptide recently purified from the seeds of *Trichosanthes kirilowii*, has potential clinical applications because of its small molecular mass. Two stable strains of hybridomas (1F11 and 2A5) that can secrete highly specific monoclonal antibodies (mAbs) against Trichokirin-S1 have been developed using the hybridoma technique. The isotypes of these two mAbs, 1F11 and 2A5, were determined to be IgG_{2a} and IgG_1, respectively. The affinity constants, which were measured by non-competitive ELISA, were found to be 2.3×10^8 M^{-1} and 2.8×10^8 M^{-1}, respectively. An immunoaffinity method using 2A5-coupled Sepharose 4B was successfully developed to purify Trichokirin-S1. These two antibodies have also been used to detect Trichokirin-S1 in Western blot.

Key words Trichokirin-S1; monoclonal antibody; Western blot; affinity chromatography

Plant ribosome-inactivating proteins (RIPs) are a group of plant proteins that inhibit protein synthesis because of their RNA N-glycosidase activity, which irreversibly inactivates eukaryotic ribosomes by removing a specific adenine from a highly conserved loop presented in 28S rRNA [1]. Generally, plant RIPs can be divided into two types according to their primary structure. Type I RIPs consist of single-chain proteins with a molecular mass of around 30 kDa. Type II RIPs are heterodimeric proteins with a molecular mass of around 60 kDa; they consist of a catalytically active A-chain, which is functionally equivalent to that in Type I RIPs, and a lectin-like chain linked by a disulfide bond.

Many researchers have reported that RIPs are involved in various biological activities, such as antivirus, antifungal, cell apoptosis, abortifacient, antitumor, immunosuppressive and HIV-1 integrase inhibitory activities [2–6]. As a result, RIPs have the potential to be widely applied in clinical therapy.

Recently, a new group of ribosome-inactivating peptides with a lower molecular mass of around 10 kDa has been identified, including γ-momorcharin [7], Charantin [8], S-trichokirin [9]. Because they are smaller molecules than conventional RIPs, their lower immunogenicity may have potential clinical applications.

Trichokirin-S1, a novel small ribosome-inactivating peptide derived from the seeds of *Trichosanthes kirilowii*, a cucurbitaceous plant, has been isolated and characterized in our laboratory [10].

In the present study, two hybridomas secreting monoclonal antibodies (mAbs) against Trichokirin-S1 were developed. The properties and preliminary applications of the mAbs were also studied.

Materials and Methods

Materials

Eight-week-old Balb/c female mice were obtained from Shanghai Laboratory Animals Centre, Chinese Academy of Sciences (Shanghai, China). The murine myeloma cell

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line X63-Ag8-653 was maintained in our laboratory. Polyethylene glycol (PEG, MW 3000–3700), complete Freund's adjuvant and incomplete Freund's adjuvant were from Sigma (St. Louis, MO, USA). The mouse antibody isotyping kit was from Zymed (CA, USA). The CNBr-activated Sepharose 4B and Protein A Sepharose CL-4B were both from Amersham Pharmacia (NJ, USA). Goat-anti-mouse IgG-alkaline phosphatase, nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were from Promega (Madison, USA). The Type I RIPs, Luffin A and Luffin B, were prepared as described by Gao *et al.* [11]. The small RIP Luffin S was prepared as described by Gao *et al.* [12]. The ribonuclease α-Sarcin, which is of fungal origin, was a generous gift from Prof. Wang-Yi LIU (IBCB, SIBS, CAS, Shanghai, China). The Type II RIP Ricin was prepared as described by Zhang *et al.* [13]. The single-chain RIP Trichosanthin (TCS) was supplied by Shanghai Jinshan Pharmaceutical Factory (Shanghai, China). The standard protein and polypeptide molecular weight marker was from Shanghai Yuyan Biotechnology Company Limited (Shanghai, China). Other reagents were of analytical grade.

Preparation of mAbs against Trichokirin-S1

The immunization of mice, cell fusion, hybridoma cell cloning and the large-scale production of mAbs were all performed according to "Antibodies: A Laboratory Manual" [14]. Briefly, eight-week-old female mice were immunized intraperitoneally with 50 μg of purified Trichokirin-S1 in 0.25 ml of PBS mixed with an equal volume of Freund's adjuvant per mouse at two-week intervals. In the first phase of immunization, the complete Freund's adjuvant was used and the incomplete Freund's adjuvant was used for the following immunization phase. The serum samples were collected one week after immunization and the antibody titers were measured by indirect enzyme-linked immunosorbent assay (ELISA) [15]. The titer was defined as the highest dilution fold of antiserum when the absorbance at 450 nm (A_{450}) reaches 0.1. The mouse with a titer of over 5×10^4 was boosted with 100 μg of Trichokirin-S1 in 0.5 ml of PBS (pH 7.2). On the fourth day after this boosting, the mouse was killed and splenocytes were harvested and washed with serum-free DMEM. The murine myeloma cells X63-Ag8-653 were previously cultured in DMEM containing 20% fetal calf serum (FCS; Hyclone). For fusion, the splenocytes and myeloma cells were mixed at a ratio of 5–10:1 and fused using the PEG method. The fused cells were resuspended in pre-warmed HAT (10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin, 1.6×10^{-5} M thymidine)-selective DMEM supplemented with 20% FCS, then dispensed into 96-well microtiter plates and cultured in a 5% CO_2 incubator at 37 °C. After being cultured for about 10 days, the media in wells with growing cell clones were collected for the anti-Trichokirin-S1 antibodies test using indirect ELISA. The positive clones were subcloned in 96-well culture plates by the limiting dilution method. After about three cycles of subcloning, the stable positive hybridoma cell lines were obtained.

For the large-scale preparation of anti-Trichokirin-S1 mAbs, the hybridoma cells were injected intraperitoneally into the pristane-primed Balb/c mice with approximately $1–5\times10^6$ cells per mouse. About 7 to 12 days later, the ascites were collected for the titer test and the antibodies were purified by ammonium sulfate precipitation and Protein A affinity chromatography.

Characterization of mAbs against Trichokirin-S1

The isotype of the mAbs was determined by using the mouse antibody isotyping kit according to the manufacturer's instructions.

The specificity of the mAbs was tested by indirect ELISA. The 96-well microtiter plates were coated with different antigens, including Luffin A, Luffin B, Luffin S, α-Sarcin, Ricin, TCS and Trichokirin-S1, at 5 μg/ml. After being blocked by 10% newborn calf serum (NCS) in PBS, 100 μl of cultured medium from different hybridoma was added to the wells. The plates were incubated at 37 °C for 1 h. After they were washed, the wells were incubated with goat-anti-mouse IgG-HRP at 37 °C for 30 min. Finally, the color was developed using 3,3′,5,5′-tetramethylbenzidine (TMB) and H_2O_2 as the substrate, and stopped by 1 M H_2SO_4. The absorbance at 450 nm was measured by an ELISA reader.

The antibody affinity constant was determined by non-competitive ELISA, as described by Beatty *et al.* [16]. The concentrations of pure mAbs were measured by the absorbance at 280 nm. For non-competitive ELISA, microtiter plates were coated with 5 μg/ml mixed protein solutions, which contained different concentrations of Trichokirin-S1 and complementary concentrations of bovine serum albumin (BSA). After being blocked by 10% NCS in PBS, mAbs against Trichokirin-S1 were added to the coated plates in a series of diluted concentrations and incubated at 37 °C for 1 h. The following steps were performed as described above. Figures showing the relationship between A_{450} and lg(M_{Ab}) were plotted, where M_{Ab} is the molar concentration of the mAb in the wells. When the value of A_{450} is equivalent to 50% of the largest absorbance (A-50), the affinity constant (K_{aff}) of mAbs

can be calculated according to **Equation (1)**:

$$K_{\text{aff}} = \frac{n-1}{2\times(n[\text{Ab}']_t - [\text{Ab}]_t)},\ n = \frac{[\text{Ag}]_t}{[\text{Ag}']_t} \quad (1)$$

in which, $[\text{Ag}]_t$ and $[\text{Ag}']_t$ are the concentrations of Trichokirin-S1 in two different coating solutions. $[\text{Ab}]_t$ and $[\text{Ab}']_t$ are the concentrations of the anti-Trichokirin-S1 monoclonal antibody at A-50 in the plates coated with two different concentrations of Trichokirin-S1.

Preliminary applications of mAbs against Trichokirin-S1

The mAb affinity gel was prepared using CNBr-activated Sepharose 4B according to the product manual. The resulting affinity gel was loaded on a column (1–1.5 ml) and equilibrated with PBS (pH 7.2). The 60%–100% saturated ammonium sulfate precipitation of the protein body extract from the seeds of *Trichosanthes kirilowii* was carried out as described by Li *et al.* [10]. The precipitation was dissolved in PBS (pH 7.2), dialyzed against the same buffer, and then applied onto the column. After being washed by 20-bed volumes of PBS and 10-bed volumes of 10 mM PB (pH 6.8), the absorbed protein was eluted by 0.1 M glycine-HCl (pH 2.0) containing 0.5 M NaCl. The eluted protein samples were neutralized by 2 M Tris-HCl (pH 8.0) and dialyzed against PBS. The purity and the N-glycosidase activity of purified Trichokirin-S1 were analyzed by 16.5% Tricine SDS-PAGE [17] and the method described by Endo and Tsurugi [1].

Trichokirin-S1 samples from the different purification steps were subjected to 16.5% Tricine SDS-PAGE, and transferred to a PVDF membrane (Millipore, USA) by a semi-dry transfer cell (Bio-Rad, USA). The membrane was blocked by 5% skimmed milk powder in 150 mM NaCl and 10 mM Tris-HCl (pH 7.5), and incubated with mAb ascites (1:2000). Then the membrane was incubated with goat-anti-mouse IgG labeled with alkaline phosphatase (1:10,000). The protein bands were visualized using the substrates NBT and BCIP.

Results

Preparation of mAbs against Trichokirin-S1

Two stable hybridoma cell lines secreting mAbs against Trichokirin-S1, which were named 1F11 and 2A5, were successfully established by the cell fusion method. By using the mouse antibody isotyping kit, the isotypes of 1F11 and 2A5 were determined as IgG_{2a} and IgG_1, respectively. The results of the antibody specificity analysis (**Table 1**) showed that both mAbs were highly specific in targeting Trichokirin-S1, except that 1F11 showed a weak reaction with Luffin S, a small RIP purified from the seeds of *Luffa cylindrica*.

Table 1 Specificity analysis of mAbs by indirect ELISA (A_{450})

Coated antigens	mAb	
	2A5	1F11
Trichokirin-S1	1.571	1.387
Luffin B	0.004	0.000
Luffin A	0.009	0.001
Ricin	0.031	0.005
α-Sarcin	0.001	0.000
TCS	0.019	0.006
Luffin S	0.029	0.367

For the large-scale preparation of mAbs, four pristane-primed Balb/c mice were injected intraperitoneally with 1F11 and 2A5 cells. About 3.5 ml of ascites was obtained on average from each mouse. The ascite titers measured by indirect ELISA were all higher than 1.0×10^6.

Then the 1F11 and 2A5 ascites were combined and purified by 40% saturated ammonium sulfate precipitation and Protein A affinity chromatography. The purified antibody samples showed a single staining band in the 7.5% SDS-PAGE analysis (data not shown), and were used in the following experiments.

According to **Fig. 1** and the formula described previously, the affinity constants of 1F11 and 2A5 were calculated as 2.3×10^8 M^{-1} and 2.8×10^8 M^{-1}, respectively.

Preliminary applications of mAbs against Trichokirin-S1

The affinity gel was prepared as described in "Materials and Methods", and about 5 mg of 2A5 antibody was conjugated on 1 ml of CNBr-activated Sepharose 4B. Under experimental conditions, about 0.3 mg of Trichokirin-S1 was purified by a 1 ml column of affinity gel from the crude Trichokirin-S1 sample of ammonium sulfate precipitation. From the Tricine SDS-PAGE [**Fig. 2(A)**], the immunoaffinity-purified Trichokirin-S1 showed a single band [**Fig. 2(A)**, lane 3] with an apparent molecular

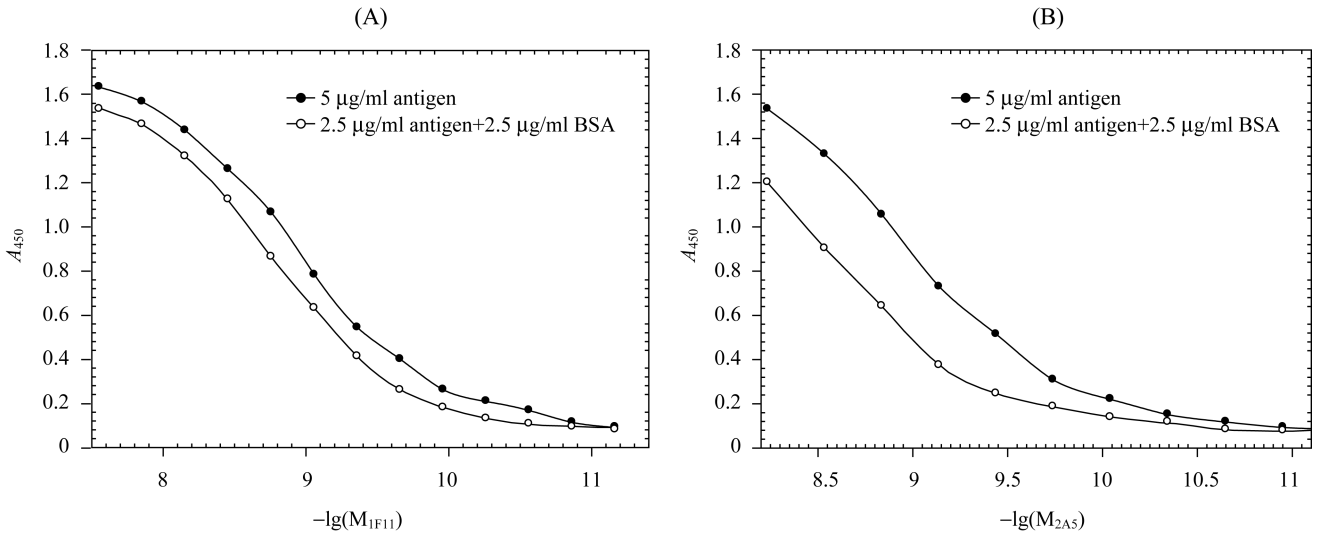

Fig. 1 Determination of the antibody affinity constant by non-competitive ELISA

(A) 1F11. (B) 2A5. M_{1F11} and M_{2A5} are the molar concentrations of mAbs 1F11 and 2A5 in each well, respectively. The non-competitive ELISA was performed and the affinity constant can be calculated as described in "Materials and Methods".

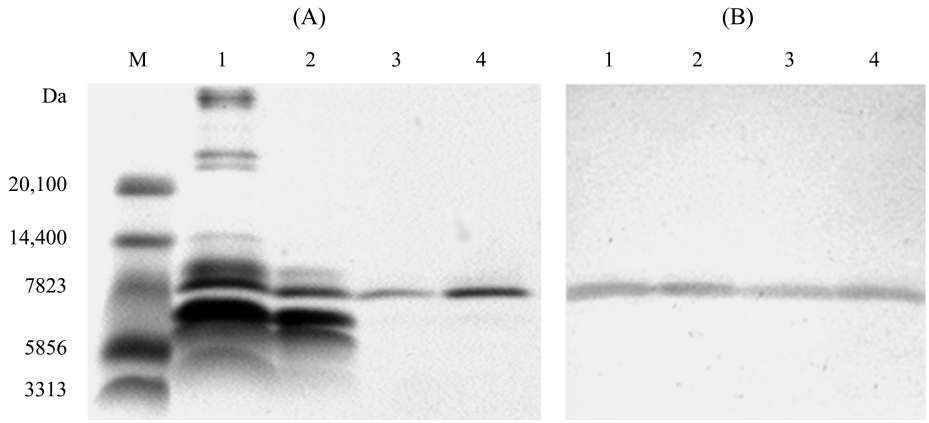

Fig. 2 16.5% Tricine SDS-PAGE and Western blot analysis of Trichokirin-S1

(A) Tricine SDS-PAGE. The gel was stained by Coomassie brilliant blue G-250. (B) Western blot. 1, protein extract of protein bodies from the seeds of *Trichosanthes kirilowii*; 2, 60%–100% saturated ammonium sulfate precipitation of crude protein extract; 3, immunoaffinity-purified Trichokirin-S1; 4, standard Trichokirin-S1; M, standard protein and polypeptide molecular weight marker.

mass of about 8 kDa, which is the same as that of standard Trichokirin-S1 [**Fig. 2(A)**, lane 4] purified by the conventional method [10]. Using immunoaffinity chromatography, about 8.6 mg of homogenous Trichokirin-S1 was obtained from 100 g of *Trichosanthes kirilowii* seeds. The yield was much higher than that of the conventional method, by which only about 2.6 mg of purified sample was obtained from the same amount of seeds [10].

The N-glycosidase activity of affinity-purified Trichokirin-S1 was analyzed by the method described by Endo and Tsurugi [1]. Plant RIPs can hydrolyze the N-C glycosidic bond of the adenosine residue A^{4324} of rat liver 28S RNA. When the hydrolyzed RNA is further treated with acidic aniline, the phospho-ribose bond is cleaved and a specific RNA fragment of about 400 bp (α-fragment)

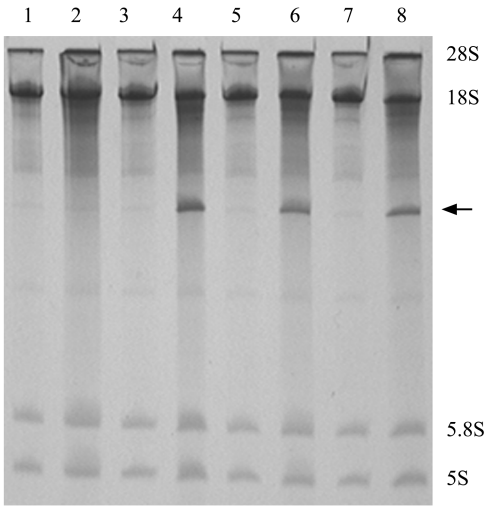

Fig. 3 rRNA N-glycosidase activity assay of Trichokirin-S1
Rat liver ribosomes were treated with TCS or Trichokirin-S1 and then with aniline, and the resulting rRNA fragments were analyzed by 3.5% UREA-PAGE. The arrow indicates the α-fragment released from the rRNA. 1, sample treated with PBS; 2, sample treated with PBS and aniline; 3, sample treated with 10 ng of TCS; 4, sample treated with 10 ng of TCS and aniline; 5, sample treated only with 10 ng of FPLC-purified Trichokirin-S1; 6, sample treated with 10 ng of FPLC-purified Trichokirin-S1 and aniline; 7, sample treated only with 10 ng of immunoaffinity-purified Trichokirin-S1; 8, sample treated with 10 ng of immunoaffinity-purified Trichokirin-S1 and aniline.

is released. **Fig. 3** shows the result of the analysis of N-glycosidase activity. As shown in lane 8, a tiny amount (10 ng) of affinity-purified Trichokirin-S1 was sufficient to yield a clear band of specific α-fragment, as did the positive control TCS (lane 4) and standard Trichokirin-S1 (lane 6).

The mAbs 1F11 and 2A5 were used for the Western blotting of Trichokirin-S1. As shown in **Fig. 2(B)**, the various Trichokirin-S1 preparations from the different purification steps all revealed only a single staining band with 1F11 or 2A5 (the figure only shows the result using 2A5) at the same position as the protein staining band of Trichokirin-S1. The results also indicated that these two mAbs have high specificity and can be used as Trichokirin-S1-detecting antibodies in future studies.

Discussion

Plant RIPs have multiple biological functions and their potential for various applications have been widely reported [2,3,5,18]. In recent years, a series of new ribosome-inactivating peptides with molecular mass ranging from 8 kDa to 12 kDa have been isolated from different plant organs. A novel ribosome-inactivating peptide, Trichokirin-S1, has recently been purified from the seeds of *Trichosanthes kirilowii* [10]. Trichokirin-S1 displays an ability to inhibit protein synthesis that is similar to that of TCS in the rabbit reticulocyte lysate protein synthesis system. TCS is a well-known single-chain RIP and it possesses anti-HIV and antitumor characteristics. It has been successfully used to construct immunotoxins (ITs) that selectively kill human melanoma and hepatoma cells *in vitro* [19, 20]. Trichokirin-S1 may have the potential as a new toxin moiety in the preparation of immunotoxins. Furthermore, it may also have the advantage of low immunogenicity because of its small molecule.

In the present study, we have established two hybridoma cell lines, 1F11 and 2A5, which secrete highly specific mAbs against Trichokirin-S1. The mAbs 1F11 and 2A5 were successfully used for the Western blot analysis of Trichokirin-S1 and may be used to investigate the intracellular location of Trichokirin-S1.

The preparation of Trichokirin-S1 using conventional methods is an arduous task because the seeds of *Trichosanthes kirilowii* have a low Trichokirin-S1 content. However, the mAb 2A5 has been successfully applied to the affinity purification of Trichokirin-S1. The yield is over 3 times of that obtained by the conventional method (8.6 mg versus 2.6 mg obtained from 100 g of seeds). The immunoaffinity-purified Trichokirin-S1 is homogeneous [**Fig. 2(A)**, lane 3], and has displayed strong rRNA glycosidase activity of the same level as that of standard Trichokirin-S1. The immunoaffinity purification method is efficient, easy to handle, economical, practical and time-saving.

The successful preparation of mAbs against Trichokirin-S1 will be of benefit to future basic and applied research on Trichokirin-S1.

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