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Cinnamomin: separation, crystallization and preliminary X-ray diffraction study

T. Wang[1], **Y.-S. Zou**[1], **D.-W. Zhu**[2], **A. Azzi**[2], **W.-Y. Liu**[1], and **S.-X. Lin**[1,2]

[1] Structural Biology Platform, Institute of Biochemistry and Cell Biology (SIBCB), Shanghai Institutes for Biological Sciences (SIBS), Chinese Academy of Sciences, Shanghai, China
[2] Laboratory of Oncology and Molecular Endocrinology, CHUL Medical Center (CHUQ) and Laval University, Québec, Canada

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Summary. Cinnamomin from *Cinnamonum camphora* seeds, a type II ribosome-inactivating protein that interferes with protein biosynthesis in mammalian cells, can induce the apoptosis of carcinoma cells and be used as an insecticide. A rapid and improved method has been developed for the extraction and purification of cinnamomin from camphora seed. Purification of cinnamomin is achieved with two successive steps of hydrophobic interaction chromatography carried out on a fast protein liquid chromatography (FPLC) system. Crystals suitable for X-ray diffraction analysis were obtained by vapor diffusion method. A complete data set at 2.8 Å resolution has been collected. Data indexation and refinement indicate that the crystal is orthorhombic with space group $P2_12_12_1$ and unit cell dimensions $a = 52.39$ Å, $b = 126.33$ Å, $c = 161.45$ Å. There are two molecules per asymmetric unit. Initial phasing by molecular replacement method yielded a solution, which will contribute to the structure determination. A molecular model will further the understanding of the mechanism of cinnamomin function. The latter will be combined with bio-informatics to facilitate the medical and other applications of cinnamomin.

Keywords: Cinnamomin – Crystallization – FPLC – Hydrophobic interaction chromatography – Ribosome-inactivating protein – X-ray crystal structure

Introduction

Ribosome-inactivating proteins (RIPs) are a group of ribotoxins, that have been extensively studied. RIPs inhibit mammalian protein biosynthesis by irreversible inactivation of the 60S ribosomal subunit (Barbieri et al., 1993). Of particular interest is their ability to selectively kill tumor cells in preference to normal cells. Thus, an interesting new approach is to use RIPs as immunotoxins for cancer therapy. Based on their primary amino acid sequences, RIPs were divided into three categories. Type I RIPs consist of a single peptide chain with a molecular mass of between 11 and 30 kDa, while type II RIPs are composed of two polypeptide chains (A- and B-chains) linked by a disulfide bond. Both the type I RIPs and the A-chain of type II RIP contain a single RNA N-glycosidase domain. The latter can remove an adenine base from the highly conserved sarcin/ricin domain in the largest RNA of mammalian ribosome. This abolishes ribosomes' ability to bind elongation factors thereby inhibiting protein biosynthesis (Endo et al., 1987; Zhang and Liu, 1992). The B-chain of type II RIP is a galactose-specific lectin that is responsible for the recognition of D-galactose of galactose-terminated receptors on the cell surface, and facilitates the internalization of the toxic A-chain. Additionally, a type III RIP has been identified and this protein is composed of a type I RIP-like N-terminal domain covalently linked to a C-terminal domain of unknown function (Reinbothe et al., 1994).

Cinnamomin, a toxic protein purified from the seeds of the camphor tree, belongs to the type II RIPs. Its physico-chemical, biochemical and enzymatic properties have been documented (Ling et al., 1995; Li et al., 1997; Pu et al., 1998; He and Liu, 2004). It has a molecular mass of 61 kDa and consists of two chains (A- and B-chains) held together by an S–S bond (Xu et al., 2000). The two chains differ in many aspects. The B-chain is responsible for the binding to specific receptors on cell membranes whereas the A-chain displays specific activity as an RNA N-glycosidase. The protein has a total of 535 residues with 271 in A-chain and 264 in B-chain (Yang et al., 2002).

As the first step towards understanding the detailed structure-function relationship, we report the purification, crystallization and preliminary crystallographic study of natural cinnamomin.

Materials and methods

Plant material

Cinnamomum camphora seeds were purchased from the Shanghai Botanic Garden. Sepharose 6B (Li et al., 1997) and Phenyl-Sepharose were supplied by Pharmacia. The AT (acid treated)-Sepharose 6B was obtained by treatment of Sepharose 6B with 0.2 N HCl at 50 °C for 2 h.

Extraction and purification of cinnamomin from seeds

A new purification method for cinnamomin has been developed by modifying an earlier reported protocol (Li et al., 1997; Pu et al., 1998). One hundred grams of the wet seeds were homogenized in one liter of the extraction solution (5% Acetic Acid), and stirred at 4 °C overnight. The homogenate was centrifuged (15,000 *g*, 4 °C) for 30 min. The supernatant was filtered through eight sheets of gauze to remove the wax. The protein in the supernatant was precipitated by adding solid ammonium sulfate to 80% saturation. The pellet was collected by centrifugation (24,000 *g*, 4 °C) for 40 min, and then dissolved in a minimal volume of phosphate buffer (50 mM, pH 7.2). Phenylmethylsulfonylfluoride (PMSF) was added to the solution to a final concentration of 1 mM, and then the solution was dialyzed against a phosphate buffer (50 mM, pH 8.0, NaCl 0.5 M) three times for 8 h each time. The insoluble materials were removed by a brief centrifugation. The supernatant was applied onto an AT-Sepharose 6B column (3 × 20 cm) pre-equilibrated with buffer A (50 mM sodium phosphate, pH 7.2, 0.5 M NaCl). The column was then washed with approximately one column volume of buffer A. The target protein was eluted by 0.1 M lactose in buffer A and collected. The protein was precipitated by adding solid ammonium sulfate to 80% saturation again. The pellet was collected by centrifugation (24,000 *g*, 4 °C) for 40 min, and then dissolved in buffer B (Tris–HCl 50 mM, pH 8.0, 1 M ammonium sulfate). The protein solution was applied to a phenyl-Sepharose column which was pre-equilibrated with buffer B. The column was washed with approximately three column volumes of buffer A, then the protein was eluted with a linear gradient of ammonium sulfate (from 1 to 0 M) in Tris–HCl buffer (50 mM, pH 8.0). The protein fractions containing the least contaminants were identified by SDS-PAGE and collected. The sample was re-precipitated and loaded onto a second phenyl-Sepharose column. The homogeneous protein fraction was finally collected and concentrated by Centricon YM-30 (Millipore) to a concentration of 50 mg/ml.

Crystallization and data collection

Cinnamomin crystals were obtained by vapor-diffusion (Ducruix and Giege, 1999) in hanging drops at 298 K. The initial crystallization screening was performed with Crystal Screen kits I (sparse-matrix screening kit; Jancarik and Kim, 1991) and II from Hampton Research, USA. The crystallization was initiated by mixing 0.5 µl of protein solution with 0.5 µl of each precipitation solution. Needle-shaped crystals gradually appeared under one of the conditions after four days at room temperature.

Diffraction data were collected from crystals flash frozen at 100 K on an in-house Rigaku R-AXIS IV++ image plate detector and CuK α radiation (wavelength of 1.5418 Å) focused with a confocal mirror. The crystals were briefly soaked in cryo-protectant solution containing 20% glycerol (w/v) added to the reservoir solution.

Results and discussion

The purification of cinnamomin has been reported (Li et al., 1997; Pu et al., 1998), however, the protein obtained by the previous method did not yield crystals at that homogeneity level. We have modified the extraction solutions (see methods) and accelerated the procedure using fast protein liquid chromatography. The purification has been shortened from a week to only three days. Two succeeding hydrophobic interaction columns were used in order to remove cinphorin, a major contaminant which is regarded as the enzymatically degraded product of cinnamomin (unpublished).

Because cinnamomin belongs to type II RIPs, its B-chain can bind to D-galactose. Base on this fact, cinnamomin should bind to the D-galactose which the Sepharose 6B would provide after being treated with 0.2 N HCl at 50 °C for 2 h (Li et al., 1997).

Compared to the previous reports, modification of extraction solutions not only effectively avoided the protein degradation, but also eliminated several other contaminants.

Cinphorin can also be extracted from the *Cinnamonum camphora* seeds. As cinphorin shares similar biochemical characteristics to cinnamomin, many attempts have failed to separate the two proteins. Cinphorin is probably an enzymatically degraded product of cinnamomin (unpublished). However, after several attempts we found that a hydrophobic interaction chromatography (HIC) step can successfully remove the contaminants. Homogeneous cinnamomin can be obtained after two rounds of HIC as demonstrated by the observation of a single band on SDS-PAGE (Figs. 1, 2). After the first round of HIC was carried out, the protein fractions (43–78 ml), which contained tiny cinphorin contamination as identified by SDS-PAGE, were collected (Fig. 1). The protein was then precipitated by adding solid ammonium sulfate and bringing the solution to 80% saturation. The precipitate was collected by centrifugation for 40 min, dissolved in a minimal volume of buffer B which contained 1 M ammonium sulfate at pH 8.0. Then the dissolved protein solution was loaded onto a second phenyl-Sepharose column. The protein fractions were identified again and the fractions from 35 to 45 ml were collected and concentrated to 50 mg/ml (Fig. 2). About 20 mg homogenous cinnamomin was obtained from 100 g *Cinnamonum camphora* seeds.

From the first hydrophobic interaction chromatography, cinphorin, the major contaminant, was eluted as the main peak, while cinnamomin appeared in the initial peak shoulder, as revealed by SDS-PAGE. From the second hydrophobic chromatography, cinnamomin was eluted as the

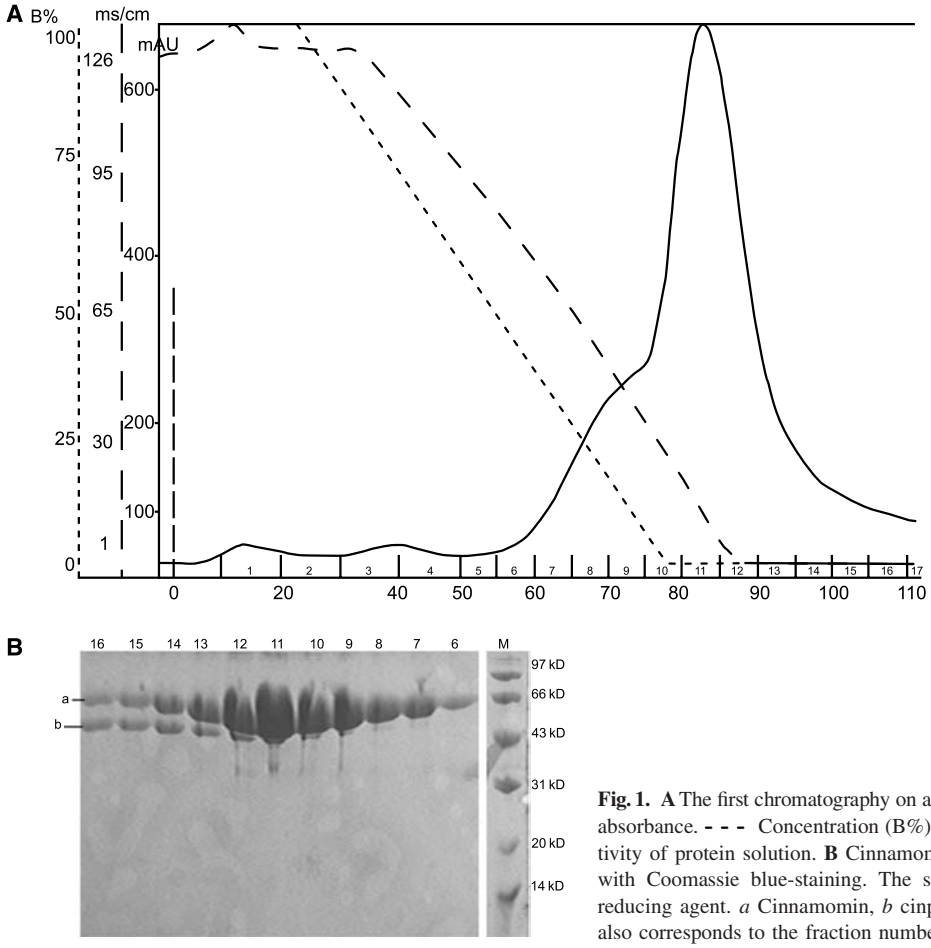

Fig. 1. **A** The first chromatography on a Phenyl-Sepharose column. —— UV280 absorbance. - - - Concentration (B%) of ammonium sulfate. — — Conductivity of protein solution. **B** Cinnamomin was identified by SDS-PAGE (12%) with Coomassie blue-staining. The sample buffer is in the absence of the reducing agent. *a* Cinnamomin, *b* cinphorin. The lane number on SDS-PAGE also corresponds to the fraction number. *M* Molecular marker

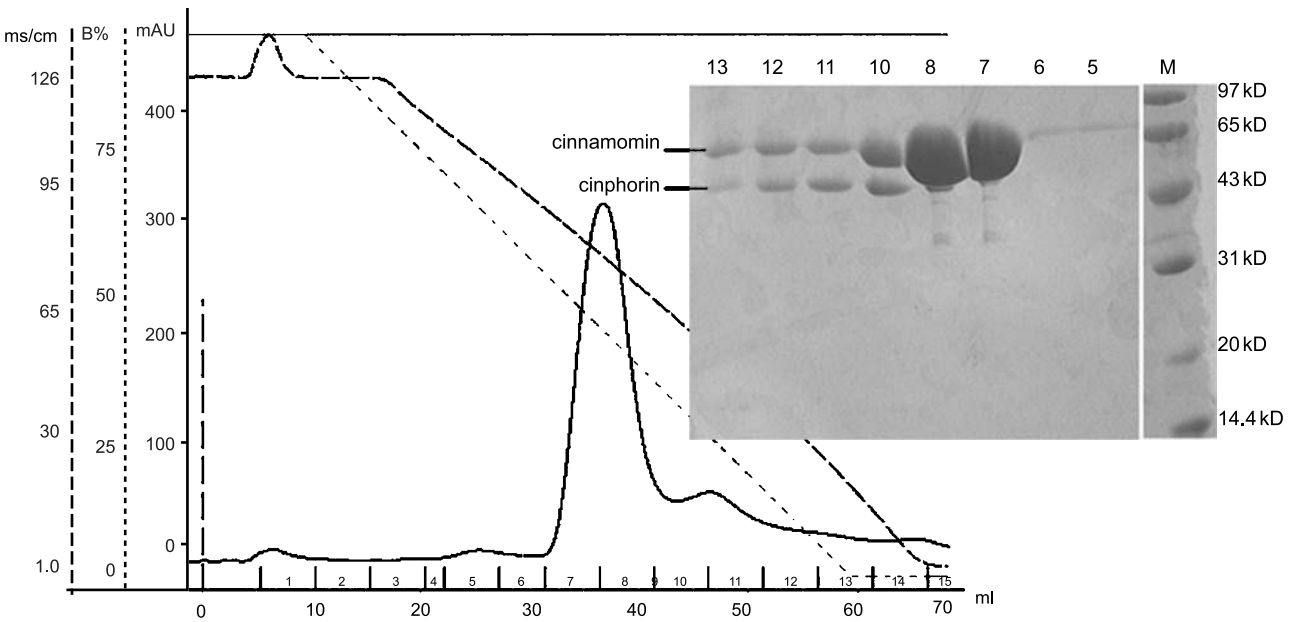

Fig. 2. The second chromatography by Phenyl-Sepharose column. —— UV280 line. - - - Percentage of ammonium sulfate concentration in buffer B (B%). — — Conductivity of protein solution. Cinnamomin was identified by a 12% SDS-PAGE with Coomassie blue-staining. The sample buffer is in the absence of the reducing agent. The lane number on SDS-PAGE also corresponds to the fraction number. *M* Molecular marker

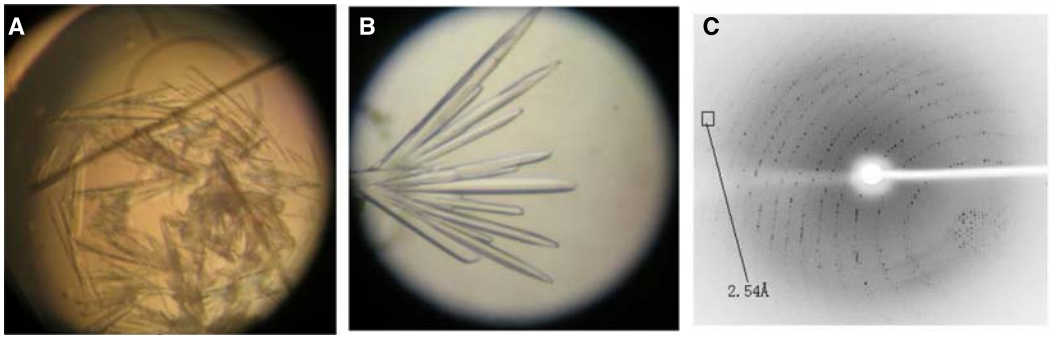

Fig. 3. Cinnamomin crystals. **A** The first crystals that were obtained. **B** Crystals after optimization. **C** A representative oscillation image taken with 1° oscillation and collected on Raxis IV detector. The diffraction limit of the crystal is 2.54 Å

main peak with good symmetry while the contaminating cinphorin appeared as a small shoulder after the main peak, thus resulting in homogeneous cinnamomin protein (Figs. 1 and 2).

Needle-shaped crystals of cinnamomin were grown by vapor-diffusion (Ducruix and Giege, 1999). These crystals were identified as cinnamomin crystals by SDS-PAGE. The washed crystals were dissolved and resulted in a protein band of the same size as that of the purified cinnamomin by SDS-PAGE. The initial crystallization condition was 0.2 M $CaCl_2$, 0.1 M sodium cacodylate pH 6.5 and 18% (w/v) PEG 8000. The crystallization conditions were optimized by varying the salt concentration from 0.1 to 0.2 M by 0.02 M increments. The PEG concentration was increased from 6% (w/v) to 24% (w/v) by 2% (w/v) increments and the pH was increased from 5.6 to 7.0 by 0.2 units. These conditions were tested at the following temperatures: 4, 10, 24 and 28 °C. The best crystal was obtained with 0.15 M $CaCl_2$, 10% (w/v) PEG8000, and 0.1 M sodium cacodylate at pH 6.5 and 24 °C (Fig. 3).

The diffraction data were processed and scaled with d*TREK (Pflugrath, 1999) and the results are shown in Table 1. The crystals were indexed to space group $P2_12_12_1$ with unit cell dimensions of $a = 52.39$ Å, $b = 126.33$ Å, $c = 161.45$ Å. From the Matthews coefficient the solvent fraction was estimated to 47%. This led us to assume that the asymmetric unit contains two molecules. Molecular replacement was successfully performed using Molrep (Tahirov et al., 1995; Vagin and Teplyakov, 2000) using the ricin structure as the starting model (Mlsna et al., 1993; Weston et al., 1994; Mishra et al., 2005). The building and refinement of the cinnamomin model are presently underway.

Table 1. Data processing statistics

	Cinamomin
Space group	$P2_12_12_1$
Unit cell (Å)	$a = 52.39$, $b = 126.33$, $c = 161.45$
Resolution range (Å)	20–2.78
Resolution shell range (Å)	2.88–2.78
Total reflections	121538
Unique reflections	27068
Mosaicity (°)	0.7
Multiplicity	4.5
Completeness (%)	98.1
Rmerge (%)[b]	12.7
Mean (I)/σ (I)	5.3

Although cinnamomin and ricin share a 65% amino-acid sequence identity (Xie et al., 2001), the cytotoxicity of cinnamomin is more than 130 times less than that of ricin because of B-chain differences (Wang et al., 2006). After the structure of cinnamomin will be resolved, extensive comparison of existing structures of different ribosome-inactivating proteins with cinnamomin will be carried out, to better understand the structural variation of B-chain and its binding to cell membrane among type II ribosome-inactivating proteins. As the association of the A- and B-chains may be relevant for toxicity and appears to be mediated by hydrophobic forces, further investigations on the conservation of polar and hydrophobic interactions occurring at chain interfaces will clarify the structure-function responsible for the toxicity. Based on the crystallographic structure, we may design improved RIP potential for cancer treatment with the help of bioinformatics (Chou, 2004, 2005) and as well as other bioinformatical tools (Lubec et al., 2005; Chou and Cai, 2006; Chou and Shen, 2006).

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Authors' address: Sheng-Xiang Lin, Visiting professor, SIBCB, SIBS, Shanghai, China,
Fax: +862154921084, E-mail: structuralbiology@sibs.ac.cn