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Minireview

Cinnamomin—a Versatile Type II Ribosome-inactivating Protein

Hong XU and Wang-Yi LIU*

State Key Laboratory of Molecular Biology, the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, the Chinese Academy of Sciences, 320 Yueyang Road, Shanghai 200031, China

Abstract Ribosome-inactivating proteins (RIPs) are a group of toxic proteins that can specifically act on the universally conserved sarcin/ricin domain (S/R domain) of the largest RNA in ribosome and thus irreversibly inactivate ribosome for protein synthesis. Cinnamomin is a multifunctional type II RIP isolated in our laboratory from the mature seeds of the camphor tree. This protein has been extensively studied with regard to its purification, characteristics, structure and function, genetic expression, enzymatic mechanism, physiological role in seed cell and toxicity to cancer cells and insect larvae. The research results of cinnamomin obtained in our laboratory are summarized in this review. Understanding of cinnamomin and the relative new proteins will help expand our knowledge of RIPs and may accelerate theoretical study and the development of their potential applications.

Key words cinnamomin; cinphorin; ribosome-inactivating protein

Ribosome-inactivating proteins (RIPs) are a group of ribotoxins widely distributed in the plant kingdom as well as in certain fungi, algae and bacteria. RIPs have been thoroughly reviewed in references [1–6]. These proteins act as RNA *N*-glycosidase (rRNA *N*-glycosidase, EC 3.2.2.22) to specifically remove an adenine from the universally conserved sarcin/ricin domain (S/R domain) of the largest RNA in ribosome [7–9] and to render it incapable of carrying out protein synthesis (Fig. 1).

Based on the structures of both proteins and their corresponding genes, RIPs are classified into three types as shown in Fig. 2. In general, type I RIPs are single-chain proteins with RNA *N*-glycosidase activity. Besides the typical type I RIPs, a few type I RIPs that are built up of two smaller polypeptide chains held together by noncovalent interaction have been identified [10,11]. Many type II RIPs are highly toxic heterodimeric proteins consisting of an A-chain with RNA *N*-glycosidase activity and a galactose-specific lectin B-chain. The A- and B-chain are linked together by a disulfide bond. The lectin B-chain can bind to sugars on the surface of eukaryotic cells and mediate retrograde transport of the A-chain to cytosol, where the A-chain inactivates ribosome and thus kills the cell. Type III RIPs are reserved for the proteins that are structurally and evolutionarily related to a 60-kD jasmonate-induced protein (JIP60) with RIP activity from barley [12–14]. The JIP60 consists of an N-terminal domain resembling type I RIPs tandemly arrayed to a function-unknown C-terminal domain that has no sequence similarity with any other plant protein.

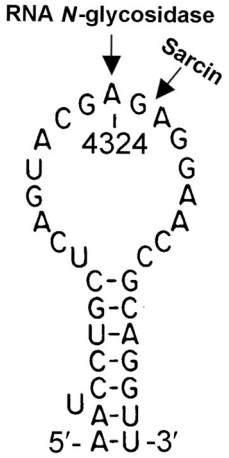

Fig. 1 The cleavage-site of RIP on the universally conserved S/R domain in rat ribosome

RNA *N*-glycosidase hydrolyses the N-C glycosidic bond of adenosine at 4324. Sarcin hydrolyses the phosphodiester bond between guanosine 4325 and adenosine 4326.

The most well-studied member of type II RIP is ricin

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*Corresponding author: Tel, 86-21-54921238; Fax, 86-21-54921011; E-mail, wyliu@sibs.ac.cn

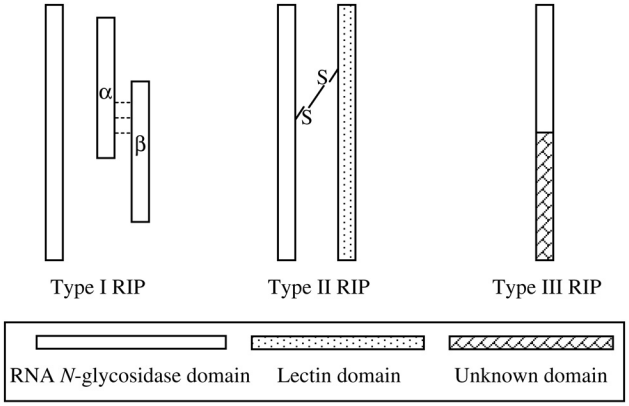

Fig. 2 Schematic structure of three different types of RIPs

The dot lines between the α and β chains in type I RIP indicate that the two chains interact with each other non-covalently.

[15]. Other type II RIPs, for example, abrin [16], viscumin [17], ebulin 1 [18], and nigrin b [19], have similar structure and function. Ricin, abrin, viscumin and other such toxins have been used in an unpurified form by traditional medicines since ancient time and are still in use today in herbal medicine. In particular, extract from mistletoe that contains viscumin is used extensively by alternative medicine for treating terminal cancer [20].

Cinnamomin is a novel type II RIP isolated in our laboratory from the mature seeds of the camphor tree (*Cinnamomum camphora*) [21,22]. It has been extensively investigated and major results are summarized in this review article.

Purification of Cinnamomin and Its A- and B-Chain

Cinnamomin was purified from the mature seeds of the camphor tree that belongs to lauraceae family. At the beginning, several steps of chromatography were taken to purify cinnamomin [21]. Subsequently, Li *et al.* [23] developed an improved method for large-scale preparation of cinnamomin in a single step of acid-treated Sepharose CL-4B affinity column chromatography. 620 mg of dried cinnamomin was obtained from 500 g of wet seeds, while only 10.6 mg of dried cinnamomin was obtained by previous method.

The A- and B-chain of cinnamomin were also separated and purified to homogeneity [24]. The strong hydrophobic interaction between the A- and B-chain of cinnamomin made it difficult to separate them under usual conditions after the disulfide bond was broken. A convenient method for purification of the A- and B-chain of cinnamomin in a large scale was developed. Urea was chosen to weaken the non-covalent interaction between the A- and B-chain. In the presence of 4 M urea, the A- and B-chain of the reduced cinnamomin were separated effectively by DEAE-cellulose chromatography. The purified A-chain still exhibited RNA *N*-glycosidase activity, while B-chain lost its lectin activity after removing urea. By refolding in the presence of lactose, the B-chain was renatured and active B-chain with lectin activity could be further purified by acid-treated Sepharose 4B affinity column chromatography. From 80 mg of cinnamomin, 10 mg of A-chain (25%) and 38 mg of the B-chain (95%) were obtained. In addition, Wang and Liu developed another method to purify A- and B-chain [25].

It should be noted that a small amount of free A- and B-chain of cinnamomin were found to be present in mature seeds of *C. camphora* apart from intact cinnamomin [26]. The percentage of free A- and B-chain was 2.6%–2.8% of the total cinnamomin in the seed extract. Of these free A- and B-chain, approximate 80% already existed in the seed cell, and only about 20% were produced during purification operation. It was proposed that free A- and B-chain were derived from enzymatic reduction of the interchain disulfide bond of cinnamomin in the seed cell. Additionally, it was discovered that camphorin, a type I RIP previously reported to be contaminated with a superoxide dismutase in the seed extract of *C. camphora*, actually was the A-chain of cinnamomin [27].

The Characteristics of Cinnamomin and Its A- and B-Chain

The molecular mass of cinnamomin was around 61 kD, and it displayed three isoforms. The A- and B-chain derived from three isoforms exhibited similar mobility shown by SDS-PAGE. The isoelectric points of two isoforms were at pH 8.10 and 7.82 respectively [21].

Cinnamomin was a glycoprotein. Its A- and B-chain contained 0.3% and 3.9% sugar respectively [28]. After sugar chains of cinnamomin were oxidized with periodate and then fluorescence-labeled with fluorescein-5-thiosemicarbazide (FTSC), the RNA *N*-glycosidase activity of its A-chain and the lectin activity of its B-chain decreased three-fold [29]. However, the physiological function of the sugar chains was still unclear. Three major glycopeptides had been isolated and purified from cinnamomin B-chain by gel filtration chromatography, anion-exchange chromatography and HPLC. Their primary structures were

determined by two-dimensional nuclear magnetic resonance (NMR) [28].

As shown in Fig. 3, ten cysteine residues were titrated with 5,5′-dithiobis(2-nitrobenzoate) (DTNB) in the reduced cinnamomin in denatured condition, one in A-chain and other nine in B-chain [29]. This result was completely confirmed by primary structure deduced from its cDNA and gene structure [30,31]. In cinnamomin, all the cysteine residues formed interchain and intrachain disulfide bonds, which accounted for its high level of structural stability [32]. Cys^{251} in the random coil of A-chain formed the interchain di-sulfide bond with the Cys^{4} of B-chain. Meanwhile, the B-chain was stabilized by four intrachain disulfide bridges: Cys^{20}-Cys^{39}, Cys^{63}-Cys^{80}, Cys^{151}-Cys^{164} and Cys^{194}-Cys^{209} [30,31]. Based on the simulated three-dimensional structure of cinnamomin A-chain using ricin A-chain as a template, it was determined that the N-terminal region of A-chain was composed of an α-helix structure of around 50 amino-acid residues. This α-helix was juxtaposed closely to the central region where a cluster of α-helixes formed the globular module. The central region contained key amino-acid residues and the active site cleft. The C-ter-minal region of cinnamomin A-chain consisted of a random coil of about 50 amino-acid residues [30].

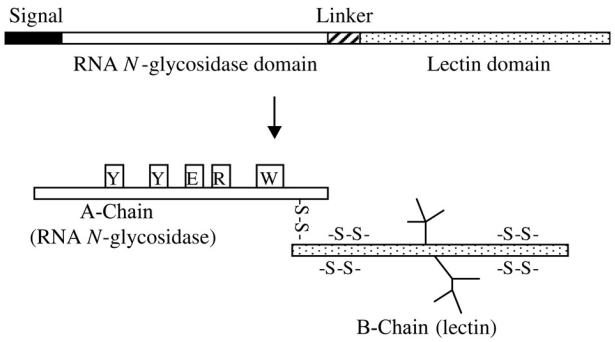

Fig. 3 Schematic structure of cinnamomin

The precursor polypeptide chain is posttranslationally processed to its mature form by removing the signal peptide and the 14 amino-acid linker. The letters in the frames indicate the amino-acid residues involved in the active site of cinnamomin A-chain (E, glutamate 167; R, arginine 170; W, tryptophan 201; Y, tyrosine 75 and tyrosine 115). The branches in the B-chain indicate the sugar chains.

When being stored at 4 °C or heated, pure cinnamomin A-chain was easy to denature compared to that in the mixture of A- and B-chain or the intact cinnamomin molecule. When heated at 45 °C for 20 min, the A-chain generated a partially unfolded intermediate and lost its tertiary structure, thus resulting in the inactivation of its RNA *N*-glycosidase, though it retained most of its secondary structure. This partially unfolded intermediate was sensitive to protease, exhibiting the properties of a molten globule. Changes in conformation and activity were irreversible upon cooling. The partially unfolded intermediate could fully restore its RNA *N*-glycosidase activity in the presence of cinnamomin B-chain. The phenomenon of the cinnamomin B-chain mediating the refolding of partially unfolded A-chain probably played an important role in the intracellular transport of the cytotoxic protein, *i.e.*, keeping the structural stability of A-chain and refolding partially unfolded A-chain that occasionally appeared in the process of intracellular transport, to avoid the proteolysis that occurred in most denatured proteins in the cell [25].

The Genes Encoding Cinnamomin

There were three genes encoding cinnamomin with sequence identity > 98.0% [31]. Three genes without intron were all functional. Three cinnamomin precursors that were inferred from the cDNA sequence of three *cinnamomin* genes exhibited relatively high sequence homology with other type II RIPs. It was proposed that three cinnamomin genes might encode three isoforms. One of the genes encoded a large precursor of cinnamomin consisting of 581 amino-acid residues deduced from the nucleotide sequence. This precursor protein comprised a signal peptide of 32 residues, A-chain of 271 residues containing only one cysteine residue, a linker peptide of 14 residues, and B-chain of 264 residues containing nine cysteines. The lengths of precursors encoded by the other two genes were both 580 residues—only one residue shorter in B-chain [31].

Enzymatic Activity of Cinnamomin A-Chain

Site-specific RNA *N*-glycosidase activity toward the ribosome

Cinnamomin A-chain exhibited specific RNA *N*-glycosidase activity toward rat ribosome [21], like A-chain of other type II RIPs. It modified the largest RNA among the four species of RNAs in ribosome. After being treated with acid aniline, a diagnostic R-fragment was released from the modified 28S rRNA. The cleavage site by the cinnamomin A-chain was the adenosine at position 4324 embedded in the highly conserved S/R domain in rat 28S

rRNA. Cinnamomin A-chain could not cleave its autologous ribosome isolated from the leaves of *C. camphora* [33]. Similar phenomena were reported in other RIPs.

A prominent cleft was proposed to be the active site of ricin A-chain, and most of the amino-acid residues invariant in RIPs were clustered at the bottom of the putative cleft [34,35]. Based on modeling of the three-dimensional structure of the cinnamomin A-chain, five invariant amino-acid residues (Tyr^{75}, Tyr^{115}, Glu^{167}, Arg^{170} and Trp^{201}) were proposed to be in its active site. The role of these amino-acid residues was investigated by site-directed mutation and chemical modification. It was found that a single mutation of the five amino-acid residues led to 8- to 50-fold loss in enzymatic activity. Such results suggest that these residues are crucial for RNA *N*-glycosidase activity of A-chain. The strong electric charge introduced at the position of the single cysteine residue in the A-chain seemed to play a role in the enzyme/substrate binding [30]. Results of chemical modification indicated that tyrosine residues are involved in the recognition and binding of adenine in ribosomal RNA [36].

In addition, mutants of the cinnamomin A-chain devoid of N-terminal 52 and/or C-terminal 51 amino-acid residues lost RNA *N*-glycosidase activity [37]. These results demonstrate that both N- and C-terminal regions are essential for the activity of cinnamomin A-chain to deadenylate ribosomal RNA.

DNA-cleaving activity of cinnamomin A-chain

Cinnamomin A-chain could deadenylate DNA molecules at multiple sites. Furthermore, our work has revealed that the native and the recombinant cinnamomin A-chain could cleave the supercoiled double-stranded DNA instead of the linear double-stranded DNA, excluding the possibility of nuclease contamination in RIP preparation [38,39]. Regarding the molecular mechanism of cleavage of supercoiled DNA to nicked and linear forms by deadenylation with cinnamomin A-chain without aniline treatment, it is proposed to be due to the spontaneous breakage of phosphodiester bonds after the removal of adenines, since DNA molecules in supercoiled states are inherently less stable than uncoiled DNA [37]. The stress present within supercoiled DNA sometimes causes regions rich in AT base pairs to come apart, and to thereby become more accessible to the action of cinnamomin A-chain. The phosphodiester bonds in extensively deadenylated regions of supercoiled DNA would become fragile and liable to be broken due to the existence of tension in supercoiled DNA. The cleavage at one apurinic site in one strand of the deadenylated supercoiled DNA would produce the nicked form. In another case, the linear form could emerge when cleavage occurred at adjacent apurinic sites in AT rich regions of both strands in the deadenylated supercoiled DNA, converting supercoiled DNA to the unstrained, energetically more favorable relaxed state. In conclusion, cleavage of the supercoiled DNA into the nicked and linear forms occurs spontaneously after adenine residues are removed from DNA molecules by enzyme, and thus the cleavage is not a direct action of RNA *N*-glycosidase, but a consequence of its action [37].

In addition, the mutants of cinnamomin A-chain devoid of N-terminal 52 or/and C-terminal 51 amino-acid residues lost both RNA N-glycosidase activity and the activity of cleaving the supercoiled double-stranded DNA. This indicates that two activities of cinnamomin A-chain probably use the same activity site [37].

Other enzymatic activities of cinnamomin A-chain

The *N*-glycosidase activity of cinnamomin A-chain has been characterized by ^{1}H-NMR. Cinnamomin A-chain cleaves the *N*-glycosidic bond and releases an adenine base from AMP, ADP, dAMP and adenosine. However, it cannot act on GMP, CMP and UMP, indicating a base preference of the hydrolysis of the N-C glycosidic bond of adenosine [40].

The Activity of Cinnamomin B-Chain

Cinnamomin B-chain was a lectin and displayed hemoagglutination activity [29]. Thus the hemoagglutination activity of cinnamomin attributed to the lectin properties of the B-chain. It was found that galactose and lactose could potently inhibit the hemoagglutination activity of cinnamomin. Other sugars (*D*-glucose, *D*-mannose, *N*-acetyl-*D*-galactoamine, and the sialic sugar, 6′-sialyllactose) tested were not inhibitory even at concentration of 100 mM. These results indicated that cinnamomin B-chain was a galactose-binding lectin.

Biological Activity of Cinnamomin

Toxicity of cinnamomin to carcinoma cells

Cinnamomin displayed inhibitory effects on cultured carcinoma cells. The IC_{50} of cinnamomin to the human hepatocarcinoma cell-line 7721 and the melanoma cell-line M21 were 18.8 nmol and 11.7 nmol respectively. Furthermore, cinnamomin exhibited a remarkable inhibi-

tory effect on growth of solid melanoma in the skin of nude mouse. Moreover, an R-fragment could be isolated from ribosomes of cinnamomin-treated carcinoma cells after incubation with acidic aniline, indicating that the cytotoxicity of cinnamomin to carcinoma cells might result from the modification of ribosomal RNA [41].

In addition, the cytotoxicity of intact cinnamomin and the *in vitro* RNA *N*-glycosidase activity of cinnamomin A-chain have been studied and compared with that of ricin. Cinnamomin A-chain exhibited RNA *N*-glycosidase activity in inhibiting *in vitro* protein synthesis similar to that of ricin A-chain, whereas the cytotoxicity of intact cinnamomin was obviously lower than intact ricin to BA/F3β cells. In order to clarify that it was the B-chains of the two RIPs that bore the difference in cytotoxicity, two hybrid RIPs were reconstructed from the purified A- and B-chain of cinnamomin and ricin by the disulfide exchange reaction. It was found that hybrid RIP reconstructed from cinnamomin A-chain and ricin B-chain was more toxic than the native cinnamomin, and equivalent to the native ricin to BA/F3β cells. However, the cytotoxicity of the hybrid RIP reconstructed from the ricin A-chain and cinnamomin B-chain was lower than ricin and equivalent to the native cinnamomin to BA/F3β cells. Furthermore, the bound amounts of two purified B-chains on the cell surface were determined by the method of direct cellular enzyme-linked immunosorbent assay (ELISA). Scatchard analysis of binding two B-chains to cell indicated that the two RIPs exhibited obviously different affinities although they shared similar binding sites.

Toxicity of cinnamomin to insects

Cinnamomin demonstrated toxicity not only to carcinoma cells, but also to insects larvae [42]. The LC_{50} of cinnamomin to bollworm larvae was 1839 ppm and the LC_{50} to mosquito larvae was 168 ppm fed on a diet containing cinnamomin. The gut extract of bollworm larvae could apparently hydrolyze cinnamomin. Inhibition of protein synthesis by cinnamomin was tested in an *in vitro* translation system of bollworm larvae, and its IC_{50} was determined to be 14 nM. Bollworm larvae ribosome treated with cinnamomin produced a specific RNA fragment (R-fragment) characterized by urea-denatured PAGE. In addition, experimental data suggested that hidden breaks exist in the largest ribosomal RNA of bollworm larvae [42].

The toxicity of cinnamomin and ricin to silkworm larvae were also tested and compared. They exhibited different toxicities to silkworm (*Bombyx mori*) larvae by oral feeding bioassay. The LC_{50} of ricin to the silkworm larvae at third instar was much lower than that of cinnamomin. When the isolated 80S ribosome from silkworm pupae was treated separately with the reduced cinnamomin or the reduced ricin, a specific RNA fragment (R-fragment) was produced as characterized by 8 M urea-denatured PAGE. The purified A-chains of both cinnamomin and ricin showed approximately identical RNA *N*-glycosidase activity to silkworm ribosome. Thus it was proposed that the difference of their toxicity to silkworm larvae was not due to their A-chains, but the properties of their B-chains. It was also found that the liquid extracted from the midgut of silkworm larvae could apparently hydrolyze these two proteins to the same extent.

The interaction of cinnamomin with model membrane

Cinnamomin was found to be able to induce the release of calcein loaded in lecithin small unilamellar vesicles and the fusion or aggregation of the lecithin liposomes. Such induction could be greatly increased by an acidic environment (pH 5.0), a condition similar to that in endocytic vesicles. Lowering the pH value from 7.5 to 5.0 evoked conformational changes of cinnamomin and unmasked its hydrophobic areas, including the exposure of 1-anilino-8-naphthalenesulfonate (1,8-ANS) binding sites of the molecule. Some tryptophan residues with affinity to acrylamide were demonstrated to participate in the lipid-protein interaction. The pH-dependent fusogenicity of type II RIP probably suggested its *in vivo* function as a fusogen to exert its cytotoxicity [43].

The interaction of cinnamomin and its A-chain with model membrane was further investigated and compared with that of ricin. It was revealed that ricin, cinnamomin and their A-chains could pH-dependently aggregate liposome and induce the release of calcein from liposome. Ricin was more effective in interacting with model membrane than cinnamomin. Ricin A-chain (RTA) interacted with model membrane more violently than cinnamomin A-chain (CTA). The difference in the interaction of cinnamomin and ricin or their A-chains with model membrane likely indicates the different levels of cytotoxicity between cinnamomin and ricin.

Additionally, cinnamomin was reconstituted into membranes of planar lipid bilayer and giant liposome. The channel-forming activity of cinnamomin was found and cation permeability of the channel was characterized by patch clamp. In an asymmetric solution system, bath 150/pipette 100 mM KCl, the unit conductance was (140 ± 7) pS and the reversal potential was (10.4 ± 0.6)

mV, very close to the theoretical value of the K^+ electrode. The results offered an interpretation for internalization of RIP and cytotoxicity difference between type I and type II RIPs [44].

The Physiological Function of Cinnamomin in the Seed Cell

There were arguments about physiological function that RIPs played in plant cell. It was reported that many RIPs acted as a defense mechanism in plant cell or terminated protein synthesis under appropriate physiological conditions and thus regulated metabolism. In addition, some RIPs accumulated in a large amount in plant vegetable organs like cotyledon, bark and root. Some researchers proposed that RIP might play as a storage protein in these tissues. However, there had been no conclusive evidence to support the proposal role of RIP to date. Cinnamomin could not cleave its autologous ribosome, so it could not inactivate its own ribosome *in vitro* and it was proposed that cinnamomin was not toxic to itself [33]. It is very interesting that cinnamomin was found to be a storage protein in seed cell. Northern and Western blotting revealed that cinnamomin was only expressed in cotyledons [31,45,46]. It accumulated in large amounts simultaneously with other proteins at the post-stages of seed development. Cinnamomin degraded rapidly during the early stages of seed germination. Endopeptidase was proven to play an important role in degradation of cinnamomin. Western blotting of total proteins from the protein body with antibodies against cinnamomin demonstrated that it only existed in this specific cellular organelle as a storage protein. The properties of cinnamomin similar to other seed storage proteins of dicotyledons were comparable. It was concluded that cinnamomin was a special storage protein in the seed of *C. camphora* [46].

Cinphorin: a Miniature Type II RIP Coexists with Cinnamomin in the Seed of *C. camphora*

A small protein named cinphorin of about 46 kD in the mature seeds of the camphor tree was eluted together with cinnamomin by lactose from the acid-treated Sepharose 4B column. Like cinnamomin, cinphorin consists of two chains (A- and B-chain) connected by the disulfide bond since it could be reduced by 2-mercaptoethanol to produce a miniature A-chain and a normal B-chain. The miniature A-chain exhibited RNA *N*-glycosidase activity and strong inhibitory activity to protein synthesis *in vitro* in almost the same manner as the normal A-chain did. Preliminary analytical results indicated that the sequence of N-terminal 10 amino-acid residues of cinphorin A-chain was identical with that of cinnamomin A-chain and that both B-chains were completely compatible [47]. The structural homology of these two type II RIPs was further confirmed by Western blotting using the antibody against cinnamomin. Does mRNA of cinphorin exist in seed cell? It was unusual that Northern blotting showed that mRNA of cinnamomin existed as only one normal form in the cotyledons of *Cinnamomum camphora* seeds. Based on these facts, it was proposed that the miniature A-chain was composed of all essential amino-acid residues related to the RNA *N*-glycosidase activity of the cinnamomin A-chain and that it was probably a cleaving product of cinnamomin A-chain or its mRNA. If this is the case, cinphorin would be interesting as a good model to study cleaving process of protein or mRNA [31,46].

Other Type II RIPs Exist in Genus *Cinnamomum*

Besides cinnamomin, porrectin and bodinierin were identified and isolated from the mature seeds of other two species of genus *Cinnamomum* (*porrectum* and *bodinieri*) [47–49]. These three large proteins had similar structure and biological activities, which provided phylogenetic evidence for three species [48]. Interestingly, miniature type II RIPs similar to cinphorin also existed in the mature seeds of these two species of *Cinnamomum*.

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Correction

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An Engineered PrP^{sc}-like Molecule from the Chimera of Mammalian Prion Protein and Yeast Ure2p Prion-inducing Domain

Shao-Man YIN, Man-Sun SY [1], and Po TIEN*

Page 131: Legend of Fig. 4 was incorrect. The correct legend is shown following:

Amyloid fibril forms (A) and amorphous forms (B) with equal protein quantities (10 μg) were subjected to protease K (1 μg/ml) digestion for variant time, then reaction was stopped by boiling and the digested products were analyzed by SDS-PAGE. Lane M shows the molecular weight markers (from bottom to top) of 14.4, 20, 31, 43, 66.2, 97 kD. The protease K digestion time (min) is indicated above each gel. M, molecular marker; 1–9, PK digestion for 0, 2, 4, 6, 10, 15, 20, 30 and 40 min respectively.