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Original Article

Research on ribosome-inactivating proteins from angiospermae to gymnospermae and cryptogamia

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Abstract: Ribosome-inactivating Proteins (RIPs) are a group of cytotoxin proteins that usually contain a RNA N-glycosidase domain, which irreversibly inactivates ribosome, thus inhibiting protein synthesis. During the past 14 years (1990-2004), the studies conducted in our laboratory had been focusing on the structure and enzymatic mechanism of several PIPs. Herein, we briefly described a summary of the studies conducted mainly in our laboratory on RIPs from angiospermae to gymnospermae and cryptogamia as follows. (1) Cinnamomin is a novel type II RIP isolated from mature seeds of camphor tree. Like ricin, it specifically removes the adenine at A4324 in rat liver 28S rRNA. We systematically studied this low-toxic RIP in term of its enzymatic mechanism, the primary and crystal structure and the nucleotide sequence of its gene, the genetic expression, and its physiological role in the seed cell and the toxicity to human cancer cells and insect larvae. The cleavage of supercoiled double-stranded DNA was its intrinsic property of cinnamomin A-chain, its N- and C-terminal regions were found to be required for deadenylation of rRNA and also necessary for deadenylation of supercoiled double-stranded circular DNA. These results strongly excluded the possibility that cleavage of supercoiled DNA was due to nuclease contamination. (2) Trichosanthin, an abortifacient protein, was purified from the Chinese medicinal herb, Tian-hua-fen, obtained from root tubers of Chinese trichosanthes plant. We proved that trichosanthin was a RNA N-glycosidase, inactivating eukaryotic ribosome by hydrolyzing the N-C glycosidic bond of the adenose at site 4324 in rat 28S rRNA, and inhibited protein synthesis *in vitro*. (3) A unique *Biota orientalis* RNase (RNase Bo) was extracted from the mature seeds of the cypress cypress tree (*Oriental arborvita*), which was gymnospermae plant. It cleaved only a specific phosphodiester bond between C4453 and A4454 of 28S RNA in rat ribosomes, producing a small RNA-fragment (S-fragment), thus inhibiting protein synthesis and belonging to RNase-like RIP, similar to α-sarcin, a special RIP. (4) Lamjapin, the first RIP purified from kelp, the marine cryptogamia algal plant, was shown to be the first single-chained RNA N-glycosidase from marine plant to date. It hydrolyzed rat ribosomal 28S RNA to produce meanly a rather smaller RNA, shorter than the diagnostic R-fragment under the restricted condition. The significance of existence of type I RIP in the lower marine algal plant was briefly discussed.

Keywords: Ribosome-inactivating proteins (RIPs), RNA N-glycosidase, ribosome, cinnamomin, trichosanthin, RNase Bo, lamjapin angiospermae, gymnospermae, cryptogamia, sarcin/ricin domain, R-fragment, protein synthesis, S-fragment, aldehyde, cleave double-stranded supercoil DNA, cinphorin

Introduction

Ribosome-inactivating proteins (RIPs) are a group of cytotoxin proteins that usually contain a RNA N-glycosidase domain, which irreversibly inactivate ribosome, thus inhibiting protein synthesis. Most RIPs are widely distributed in the higher plants. The RNA N-glycosidase can specifically remove an adenine from an adenosine (A4324) in the highly conserved sarcin/ricin domain loop of 28S RNA in rat ribosome. Based on the diversity of chemical structure, plant RIPs have been classified into three types (**Figure 1A**).

Type I RIPs are composed of a single, intact polypeptide chain with a molecular mass around 11-30 kDa. In some cases, it is proteolytically processed into two shorter polypeptides which are held together by non-covalent interaction. Type II RIP is consisted of two polypeptides (A-and B-chain) linked by a disulfide bond that can be reduced into two separate A- and B-chains. Both type I RIP and the A-chain of

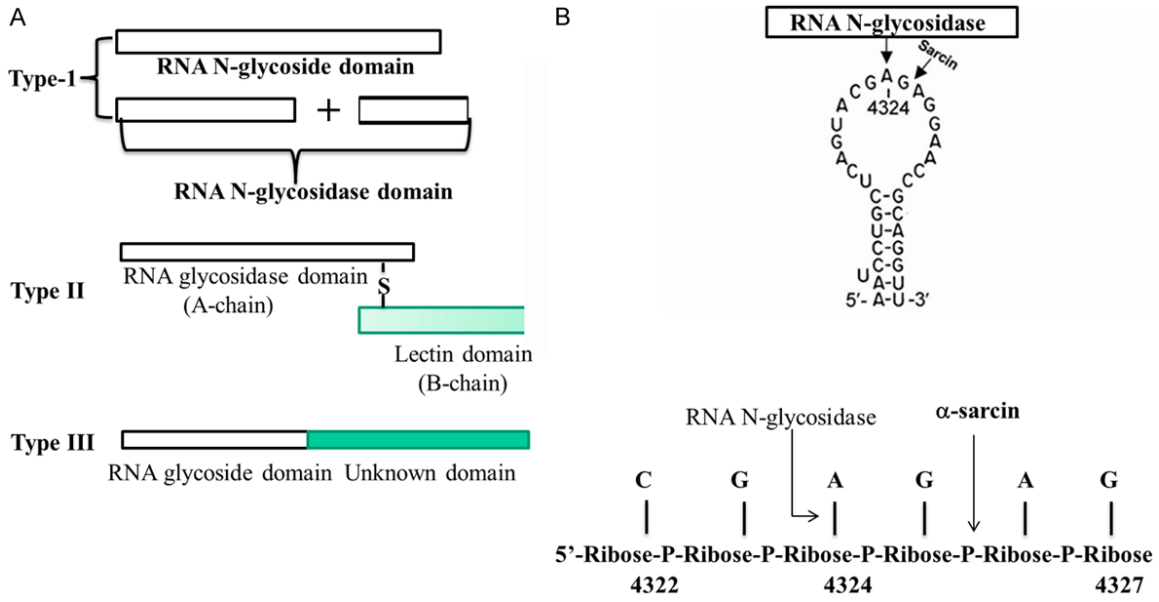

Figure 1. Schematic illustration of the structures of three type RIPs and sarcin/ricin domain (Adapted partially from reference [3]). (A) Three types of RNA N-glycosidase; and (B) The cleavage-sites of RIPs in the conserved sarcin/ricin domain of rat 28S ribosomal RNA.

type II RIP are consisted exclusively of a single RNA N-glycosidase domain. The B-chain of type II is a galactose-specific lectin responsible for the recognition of D-galactose on galactose-terminated receptors present on the cell surface, thus facilitating the internalization of the toxic A-chain into cytoplasm. Additionally, a special type III RIP has also been identified. Type III is composed of a type I-like N-terminal RNA N-glycosidase domain covalently linked to a C-terminal domain with unknown function. Most of RIPs possess RNA N-glycosidase activity. There is another different kind of RIP, whose enzymatic activity is quite distinct from that of RNA N-glycosidase. It is a unique ribonuclease-like RIP possessed only by α-sarcin hitherto investigated. Alpha-sarcin is capable of selectively hydrolyzing a single phosphodiester bond between G4325 and A4326 in rat 28S rRNA, where nearby the cleaving position (A4324) of ricin A-chain in rat largest rRNA. Therefore, this RNA domain (loop) is named sarcin/ricin domain (**Figure 1B**). This domain is responsible for the interaction between the elongation factors and ribosome during the process of protein synthesis.

RIPs have drawn increasing attentions because of their antiviral activity and the potential use as a toxin moiety in immunotoxins used for the treatment of severe human diseases, such as cancer and AIDS. RIPs also have promising applications in biotechnology of crop plants with the aim of increasing resistance to insects, fungal and viral pathogens. In addition, RIPs are also a powerful tool to probe the topographic structures of ribosomal RNAs and elucidate the molecular mechanism of protein synthesis. Many review articles on the RIPs had been published in the past years. Several of them were listed as references in [1-4].

Cinnamomin, a multifunctional type II RIP with low cytotoxicity

Identification, purification of cinnamomin and its RNA N-glycosidase activity

Our studies had been concentrating on the RIPs. Especially, we identified a novel type II RIP, cinnamomin, from the matured seeds of camphor tree *(Cinnamomum camphora).* Cinnamomin is a glycoprotein with a molecular mass around 61 kDa. Three isoforms of cinnamomin have been identified in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (see below for three genes of cinnamomin). Similar to typical type II RIP, such as ricin, cinnamomin is composed of two chains: A-chain and B-chain. The A-chain has the specific RNA N-glycosidase activity, which specifically removes the adenine at A4324 of rat 28S rRNA,

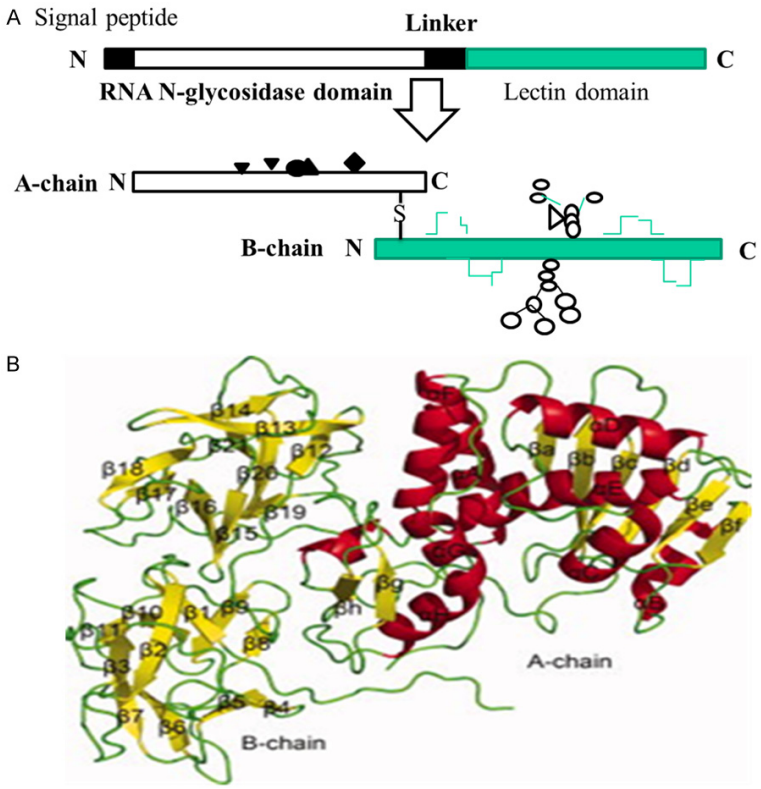

Figure 2. The primary and tertiary structure of cinnamomin (Adapted from references [3] and [12]). A. The precursor of the primary structure of cinnamomin. The precursor polypeptide chain is cleaved proteolytically to remove the signal peptide and the 14 amino-acid linker. The structure of glycan chains of the B-chain and the positions of disulfide bridges as well as the conserved amino-acid residues in the active site of the A-chain are indicated. (○) Mannose; (◇) GlcNAc; (Δ) Xylulose; (▼) Tyr75 and Tyr115; (●) Glu167; (▲) Arg170; and (◆) Trp201. B. The crystal structure of cinnamomin. Cartoon representation of A-chain and B-chain with its two homologous domains vertically aligned. [Color figure could be viewed in the online issue, which is available at www.interscience.wiley.com].

while the B-chain is a lectin, which specifically binds to galactose. Cinnamomin potently inhibits the *in vitro* protein synthesis in the rabbit reticulocyte lysate system or a reconstituted protein synthesis system composed of rat ribosome (IC_{50}, ~9.7 nM). As compared with the intact cinnamomin, the A-chain of cinnamomin displays stronger inhibitory activity on the *in vitro* protein synthesis (IC_{50}, 1 nM).

In addition to intact cinnamomin, there are small amounts of free A- and B-chain of cinnamomin in the mature seed cells of camphor tree. It should be stressed that we later found that the so called camphorin, an assumed type I RIP in the seeds of camphor tree, was the co-purified with cinnamomin A-chain and the detected RIP activity of the camphorin is actually due to the contamination of the cinnamomin A-chain.

In order to systematically study cinnamomin, we developed a method for purification of cinnamomin in a large scale. Initially, we purified cinnamomin from the seed of *C. camphora* using size-exclusive chromatography and ionic interaction chromatography. Because of the low capacity of size-exclusive chromatography, only small amount of pure cinnamomin could be obtained. Later, we found that similar to ricin (A type II RIP), cinnamomin was a galactose-binding lectin able to bind to Sepharose-4B. However, cinnamomin had the affinity to galactose lower than that of ricin and cannot strongly bind to Sepharose-4B. To purify cinnamomin, we made the two modifications similar to the affinity chromatography for ricin as follows. First, we used an acid-treated (AT) Sepharose-4B, which somehow had an enhanced binding ability to cinnamomin as compared to the untreated Sepharose-4B. Second, we used smaller volume of washing solution to remove the unwanted proteins. Using the modified method, we were able to obtain about 620 mg of cinnamomin from 500 g of wet seeds of *C. camphora* [5, 6].

It should be noted that about 20% of the free B-chain of cinnamomin was presented in this cinnamomin preparation. Because there were about 20% of the free B-chain of cinnamomin in the seeds, both cinnamomin and its free B-chain were able to bind to AT-Sepharose and then eluted by lactose [7].

The primary and crystal structure of cinnamomin

The amino-acid sequence of cinnamomin was analyzed by cDNA cloning of cinnamomin (A- and B-chains) via the RT-PCR method. The

Table 1. Effect of modification of cysteine on the enzymatic activity of cinnamomin A-chain

Method	Structure	Inhibition of protein synthesis (%)
Control (native cinnam omin A-chain)	R-CH_2-SH	100
Site-direct mutagenesis		
Cysteine→Alanine	R-CH_2-H	95
Cysteine→Serine	R-CH_2-OH	98
Cysteine→Arginine	R-CH_2-CH_2-CH_2-NH-C(NH_2^+)-NH_2	150
Cysteine→Lysine	R-CH_2-CH_2-CH_2-CH_2-NH_3^+	86
Chemical modification		
With iodoacetic acid	R-CH_2-S-CH_2-COO^-	85
With Na_2SO_3 and $Na_2S_4O_6$	R-CH_2-S-SO_3^{2-}	150

R represents the cinnamomin A-chain minus the side chain of cysteine. The letters in bold stand for the sulfhydryl group of cysteine and its modified structure.

result showed that it was composed of total of 549 amino-acid residues, including 271 residues in the A-chain, 14-residues in linker, and 264 residues in the B-chain (**Figure 2A**). Several lines of evidence proved the accuracy of the deduced primary structure of cinnamomin [8]. First, as we previously reported, a total of ten cysteine residues were confined in disulfide bond form in the native cinnamomin molecule by 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) titration method, one cysteine in the A-chain and the other nine cysteines in the B-chain. All the cysteines existed in the disulfide form in cinnamomin molecule, which probably was accounted for its high structural stability [9]. In addition, it was known that the fourth position of amino-acid in N-terminal region of B-chain in many RIPs, including B-chain of cinnamomin, ricin, abrin and porrectin, was occupied by cysteine. While in the cinnamomin A-chain, cysteine 251 was the sole cysteine only in the amino-acid residues. Therefore, we suggested that the inter-chain disulfide bridge between A- and B-chains was constructed by cysteine 251 in A-chain and cysteine 4 in B-chain [9, 10]. These data were identical to that of the deduced sequence of cinnamomin.

Second, in our other work, the primary structure of two glycopeptides of the native cinnamomin B-chain was determined by the nuclear magnetic resonance (NMR) method, indicating that their structure was as follows: (1) Gly-Asn*-Asn-Thr and (2) Ala-Asn*-Gly-Thr (*indicates the amino-acid residue to be linked to the glycan chain). These two peptides existed indeed in the deduced primary structure of B-chain [11]. Third, the amino-acid compositions of native cinnamomin were consistent with that of the deduced amino-acid sequence of cinnamomin. All of these results made it convincing that the cDNA we obtained really encoded the cinnamomin molecule (**Figure 2A**).

In order to understand the relationship of structural/functional relationship and of the structural differences between various RIPs, we and coworkers deciphered firstly the crystal structure of the native cinnamomin III, one of cinnamomin isomers expressed as three different isomers as mentioned above (**Figure 2B**). The structural information could be compared with the indicated enzyme activity and the substrate specificity, benefitting the understanding of the molecular mechanism of these kinds of proteins with potential medical and agricultural applications [12].

Effect of modification of cysteine in cinnamomin A-chain on the enzyme activity

It has been known that the five conserved amino-acid residues of cinnamomin A-chain play an essential role in its RNA N-glycosidase activity. We wanted to know if any other amino acid residues also played a role in the enzymatic activity. In cinnamomin A-chain, there was only one cysteine residue (Cys251) that was located on the surface of the molecule and formed the disulfide bond with Cys4 in the B-chain of cinnamomin. After reduction of the inter-chain disulfide bond, cinnamomin A-chain became the active enzyme.

In order to study the effect of this Cys251 on the enzymatic activity of cinnamomin A-chain, both methods of chemical modification and site-directed mutagenesis were employed. The results [**Table 1**] indicated that if when Cys251 was replaced by Ser or Ala similar in structure with Cys251, the enzymatic activity did was not changed at all. When Cys251 was modified with iodoacetic acid or substituted by lysine, there was only a slight decrease in enzymatic activity. These results suggested that the sulfhydryl group of Cys251 was not essential to the enzymatic activity. However, when the Cys251 was replaced by arginine with a much stronger positive charge or modified with Na_2SO_3 and $Na_2S_4O_6$ to introduce much stronger negative charge, the enzymatic activity was increased by 0.5 fold in both cases, as compared to that of the wild-type A-chain, indicating that the extremely acidic or basic environment at position of Cys251 was propitious to enzymatic activity [8, 9].

The role of key amino-acids on enzyme activity of expressed cinnamomin A-chain and its mutants

The ability of the re-natured recombinant A-chain and its mutants to inhibit protein synthesis was systematically studied, showing that the IC_{50} of the recombinant A-chain was 1.2 nM, while that of the native A-chain was 1.0 nM. In addition, the RNA N-glycosidase activity of the recombinant A-chain was also determined. Here, 0.5 ng of recombinant A-chain could clearly produce R-fragment from ribosome after treatment with acidic aniline, similar to that produced by the native A-chain. About 80% of RNA N-glycosidase activity of the recombinant A-chain could be recovered by about 80% as compared with that of the native A-chain, suggesting that it still meets the requirements of structure and function study of the cinnamomin A-chain.

Usually, the five conserved amino-acid residues, including Tyr75, Tyr115, Glu167, Arg170, and Trp 201 of the cinnamomin A-chain, are probably involved in hydrolysis of N-glycosidic bonds of the adenosine 4324 in 28S rRNA. Thus, our initial mutagenesis work on cinnamomin A-chain was focused on these five conserved residues. Five site-specific mutants were constructed by PCR-directed mutagenesis, which were named as Y75F, Y115F, E167D, R170K, and W201F, respectively. In each mutant, a conserved amino-acid residue was substituted by another one with a similar structure. In our experiments, the *in vitro* transcription and translation methods were employed to determine the RNA N-glycosidase activity.

Experimental results revealed that the enzymatic activities of all mutants were decreased to different extents. Mutant E167D led to an almost 50-fold loss in activity, and R170K resulted in a 45-fold decrease in activity. As for the activities of the other three mutants, there was a 20-fold loss in Y75F and an eight-fold loss in both Y115F and W201F. Based on these results, we concluded that these conserved amino-acid residues, especially Glu 167 and Arg170, were crucial to maintain the RNA N-glycosidase activity of cinnamomin A-chain [8].

Furthermore, in addition to five conserved amino-acid residues mentioned above, we had also studied again two tyrosines involved in activity of RNA N-glycosidase of cinnamomin A-chain by the way of chemical modification. It was observed that modification of two tyrosine residues by N-acetylimidazole (N-AI) caused almost complete loss activity of the A-chain. Adenine base seems appears to partially protect two tyrosine residues from modification by N-AI. Thus, it was proposed that two conserved tyrosine residues were clearly involved in activity of cinnamomin A-chain and they were crucial in recognition and binding of ribosomal RNA [13].

Structural studies of the glycopeptides of cinnamomin B-chain by nuclear magnetic resonance

Most RIPs are glycoproteins and their sugar contents are around 1-7%. The B-chain of type II RIPs is a galactose-specific lectin. Usually, the methods to determine the primary structure of sugar chain are inconvenient and time-consuming. Due to the development of the technology of nuclear magnetic resonance (NMR), the determination of the primary structure of a new sugar chain by NMR has become convenient.

Cinnamomin B-chain is a glycoprotein with the sugar content of 3.9%. Frequently, the glycosylation of proteins have a dramatic influence on protein structure, impacting either overall

structure or local conformation. In some cases, the glycan chain could stabilize the structure of the fully folded protein by making specific contacts with the protein. Although the physiological function of the sugar chains in cell is unclear yet, it was worthy to understanding their structural role in some important proteins. For example, the sugar chains of cinnamomin had been oxidized with periodate and then labeled with a fluorescent probe. In this way, both the RNA N-glycosidase activity of its A-chain and the lectin activity of its B-chain were decreased by three folds after fluorescence-labeling [14].

To further purify cinnamomin B-chain, we carried out three steps of purification, including gel filtration chromatography, anion-exchange chromatography and HPLC. We cooperated with co-workers and obtained three major glycopeptides from cinnamomin B-chain and finally the primary structures of these glycopeptides (GP1, GP2 and GP3) were determined by NMR. Of two peptide sequences, GP1 and GP2 had been found in the complete amino-acid sequence of cinnamomin B-chain (**Figure 2A**). GP3 glycopeptide chain did not existed in the B-chain [11].

Three genes encoding three cinnamomin isomers and their expression patterns

As mentioned above, cinnamomin had three molecule isomers. We attempted to clone the full-length cDNA and then to determine the genomic sequences of cinnamomin isomers. Its full-length cDNA of cinnamomin was obtained by 5' rapid amplification of cDNA ends (5'-RACE). Subsequently, the polymerase chain reaction (PCR) amplification of its genomic DNA was performed. Unexpectedly, three closely homologous genes were acquired, including the one that matched the previously published cDNA were acquired. Because three genes had shared very high sequence identity (>98%) with similar size (2024, 2020, and 2010 bp), they appeared as a single band in gel electrophoresis and could only be distinguished by sequencing. The analytical results indicated that the longest sequence (cinnamomin I, 2024 bp) was correspondent to the cDNA cloned by Xie [8], whereas the other two sequences (cinnamomin II and III) were the new genes. In the previous work, we found there were three isomers of natural cinnamomin in the mature seeds of camphor tree. Three isomers had identical molecular mass of around 61 kDa and could be separated into three bands on native PAGE gel [5]. The N-terminal amino-acid sequences of A- and B-chains of three isomers were identical. We had cloned three functional genes of cinnamomin. Their molecular masses and the N-terminal amino-acid sequences of A- and B-chains were inferred to be consistent with those of the published isomer of cinnamomin A- and B-chains. In addition, three genes are expressed specifically only in seeds, but not in other tissues of camphor tree. All these facts together suggest that three different isomers of natural cinnamomin were appeared not to be the products of mRNA splicing or post-translational modification, but to be the expressive products of three slightly different genes separately inside cells. The amino-acid sequences of cinnamomin isomers were highly homologous. Ten cysteine residues that formed five disulfide bonds, one at inter-chain and four at intra-chain, were located at the correspondent positions in three isomers of cinnamomin compared to other type II RIPs. The five conserved amino-acid residues that formed the conserved active-site of RNA N-glycosidase of A-chain and two carbohydrate binding sites of B-chain are highly reserved in all three isomers of cinnamomin, except that His575 in the second carbohydrate binding site of B-chain of cinnamomin I replaced Gln570 in ricin B-chain. This substitution introduced a positive charge in the second carbohydrate binding site of cinnamomin I [15].

Cleavage of supercoiled double-stranded DNA by RNA N-glycosidase

Besides the RNA N-glycosidase activity of RIPs on ribosomal 28S RNA, a special enzymatic activity to cleave supercoiled double-stranded DNA producing nicked circular and linear forms of DNA by trichosanthin was the first case. Whether this enzymatic activity was a common characteristic of RIPs is still in dispute. The clarification of this question will be helpful for understanding the intrinsic effectiveness of RIPs in eukaryotic cell *in vivo*. We used several RIPs, such as α-sarcin, ricin A-chain, cinnamoin A-chain, luffin, and heat-inactivated ricin A-chain, to examine this enzymatic activity on supercoiled DNAs (e.g. pGEM-4z, pBR322, and pSP70). Our experimental results showed that when the supercoiled DNA pGEM-4z was incubated with ricin A-chain in a titration test, a significant change in the conformation of the

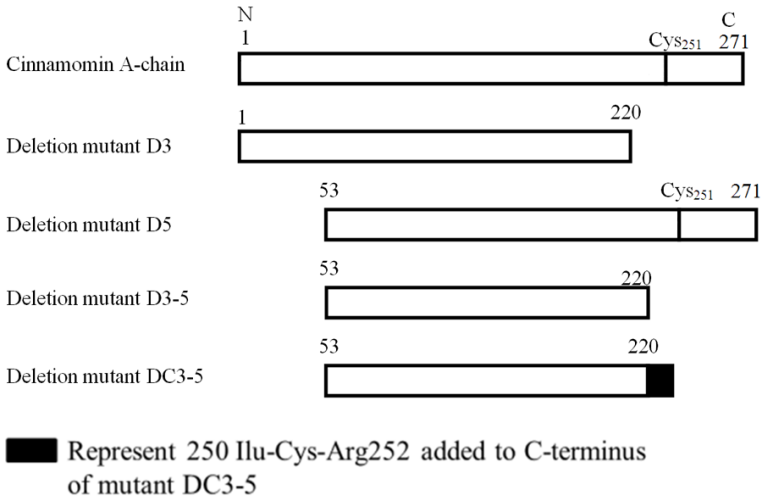

Figure 3. Schematic illustration of recombinant cinnamomin A-chain and its four mutants.

supercoiled DNA was detected. The supercoiled DNA was firstly cleaved into nicked circular DNA, which migrated significantly slower than the supercoiled DNA through agarose gel electrophoresis [16]. As the concentration of ricin A-chain was increased up to 3 μg, the linear form of DNA emerged, which migrated faster than the nicked circular form but slower than the supercoiled DNA. Similar results were also observed, when the other supercoiled DNA and RIPs were employed in the same assay system. Therefore, we concluded that the enzymatic activity on supercoiled double-stranded DNA was a common characteristic pertaining to RIPs. Our hypothesis is that there must be a topographic prerequisite for supercoiled DNA to interact with RIPs since no cleaving activity of RIPs was detected when the linear double-stranded r-DNA or the limited enzymatic linearized-pGEM-4z DNA was used as the substrates [17].

Both N- and C-terminal regions are essential for cinnamomin A-chain to deadenylate ribosomal RNA and supercoiled double-stranded DNA

Working on this project, we are cheerful that we have successfully obtained many satisfactory results. Especially, we reported an unvarnished account of the deadenylation of the supercoiled DNA by RNA N-glycosidase.

As mentioned above, cinnamomin A-chain was is composed of 271 amino-acid residues. Five conserved amino-acid residues are located in its central region, which are and supposed to constitute the active-site. In addition, it was known that certain amino-acid resides outside the active-site cleft were also involved in enzymatic activity. In order to study the functional roles of the amino-acids in both N- and C-terminal regions of cinnamomin A-chain and to clarify whether the central region was sufficient to function as a RNA N-glycosidase as well as the cleaving activity to the supercoil DNA, the recombinant intact cinnamomin A-chain and its four deletion mutants (D3, D5, D3-5, and DC3-5) were designed (**Figure 3**) and highly expressed in *E.coli.* These recombinant proteins were further purified, then re-natured and shown to be homogenous by SDS/PAGE.

The recombinant A-chain expressed the full activity of RNA N-glycosidase as the native cinnamomin A-chain did, while the four deletion mutants showed only very weak activity. The results to test the RNA N-glycosidase activity of the recombinant A-chain and four deletion mutants indicated that 10 μg of recombinant A-chain was sufficient to produce the diagnostic R-fragment similar to that produced by 10 μg of native A-chain. However, 100 μg of each four deletion mutants did not show such high activity, respectively.

These four deletion mutants devoid of N- or/and C-terminal regions could not inhibit protein synthesis *in vitro*, and did not have RNA N-glycosidase activity, indicating that these deleted regions were essential for inhibiting protein synthesis and deadenylation activity of cinnamomin A-chain, but also that the central region containing the five conserved amino-acid residues in the active site alone could not function as an active RNA N-glycosidase.

We had found that cinnamomin A-chain could cleave the supercoiled double-stranded DNA into nicked and then linear forms of DNA. However, it was argued later that this enzymatic activity might be caused by the nuclease contamination in the RIP preparations. In order to exclude the possibility of nuclease contamination, we designed and performed the experi-

Table 2. Adenine was released from supercoiled pMFT7 DNA treated by cinnamomin A-chain

pMFT7 DNA (pmol)	Treatment	Adenine released (pmol)
1	Reaction buffer only	0
1	Native cinnamomin A-chain	71±4
1	Recombinant cinnamomin A-chain	58±8

The amount of adenine released was measured. Pure adenine was used for generating the standard curve. In each experiment, 1 pmol of pMFT7 was used.

ments as follows: the supercoiled pMFT7 DNA was incubated with the recombinant cinnamomin A-chain and its deletion counterparts at increasing concentration, respectively. Similar to the result obtained from native cinnamomin A-chain, the supercoiled pMFT7 DNA was gradually converted into nicked and then linear forms of DNA in the presence of recombinant full-length cinnamomin A-chain. However, none of the four deletion mutants could cleave the supercoiled DNA. Moreover, neither recombinant cinnamomin A-chain nor the deletion mutants could degrade the linear DNA into small fragments. These results strongly excluded the possibility that cleavage of supercoiled DNA was due to nuclease contamination.

All of the above results indicate that cleavage of supercoiled double-stranded DNA is an intrinsic property of cinnamomin A-chain, the N- and C-terminal regions required for deadenylation of rRNA are also necessary for deadenylation of supercoiled double-stranded circular DNA [16-18].

Proposed mechanism of multi-sites deadenylation of supercoiled DNA by cinnamomin A-chain

It was shown that among four bases only adenine could be released from pMFT7 DNA after treatment by native or recombinant cinnamomin A-chain, while the four deletion mutants could not deadenylate this supercoiled DNA. Moreover, quantitative fluorescence determination of adenines released from supercoiled DNA showed that cinnamomin A-chain could remove adenine residues at multiple-sites in one molecule of supercoiled DNA, since the ratio of released adenines to DNA molecule exceeded 50:1 (**Table 2**). This point was also confirmed by the fact that only the nicked and linear forms of DNA but not the supercoiled DNA could be degraded into small fragments after acidic aniline treatment. In the absence of cinnamomin A-chain, acidic aniline treatment did not show any significant degradation of supercoiled DNA. When the supercoiled DNA was incubated with the deletion mutants, no obvious change could be observed after acidic-aniline treatment. The nicked and linear forms of DNA derived from the supercoiled DNA after the incubation with cinnamomin A-chain were further hydrolyzed into small fragments by the following acidic-aniline treatment and run out of the gel, suggesting that cinnamomin A-chain deadenylated DNA at multiple sites that were susceptible to acidic-aniline cleavage. The deletion mutants could not deadenylate both 28S rRNA and supercoiled DNA, supporting our assumptions mentioned above.

In summary, the above results demonstrated that both the 52 amino acid residues in N-terminal and 51 amino-acid residues in C-terminal of cinnamomin A-chain were essential for its activity to deadenylate rRNA and supercoiled DNA. Also, several lines of evidence clearly confirmed that cleavage of supercoiled DNA into nicked and then linear forms of DNA was definitely caused by deadenylation with cinnamomin A-chain instead of contaminating nuclease as described elsewhere.

Native cinnamomin A-chain was a glycopeptide chain containing tiny amount (0.3%) of sugar. The recombinant cinnamomin A-chain devoid of glycan chain still retained the ability to deadehylate rRNA and supercoiled DNA, indicating that sugar chain in native cinnamomin A-chain did not play a crucial role in its deadenylation activity. Our results mentioned above showed that the five conserved amino-acid residues in cinnamon A-chain constituted the active site and played crucial roles in substrate binding and catalysis. Here, deletion of 50 amino-acid residues at N- or C-terminal regions could result in obvious losses of deadenylation activity of cinnamomin A-chain to ribosomal RNA and supercoiled DNA, demonstrating that the amino-acid residues in two terminal regions far from the active-site cleft are also essential for alignment of the key residues to interact with and catalyze the substrate.

Regarding the molecular mechanism of cleaving supercoiled DNA into nicked and linear

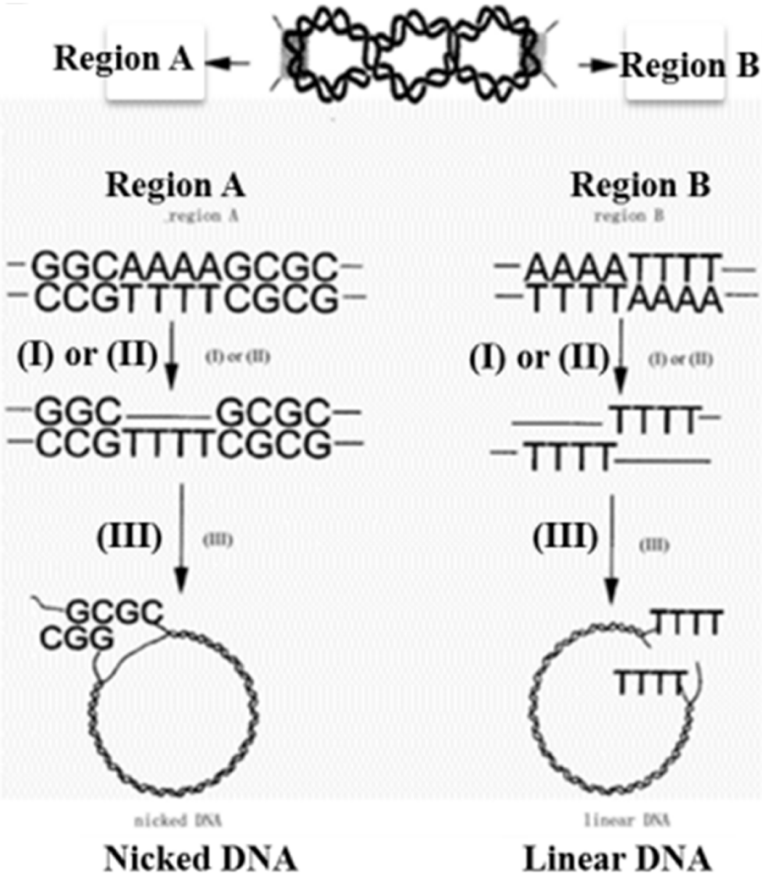

Figure 4. Proposed cleavage path of supercoiled DNA to produce the nicked and linear forms of DNA by cinnamomin A-chain or chemicals. There are many deadenylation sites that are randomly located in the AT-rich regions of one supercoiled DNA molecule. The partial sequences of regions A and B indicate two possible cases: (I) deadenylation by cinnamomin A-chain; (II) deadenylation by chemicals; and (III) Spontaneous cleavage at any one deadenylated site by tension in the supercoiled DNA molecule.

forms of DNA by deadenylation with cinnamomin A-chain without aniline-treatment, it is proposed that the spontaneous breakage of phosphodiester bonds would occur after an adenine being removed since the DNA molecule in the supercoiled state was inherently less stable than uncoiled DNA. The stress presented within supercoiled DNA sometimes leaded to regions rich in AT base pairs coming apart, and thus being accessible to the action of cinnamomin A-chain. The phosphodiester bonds in extensively deadenylated regions of supercoiled DNA would become fragile and liable to breakage owing to the existence of tension in supercoiled DNA.

In one case, cleavage at least one deadenylated site in one strand of the deadenylated supercoiled DNA would produce the nicked form of DNA. In another case, the linear form could emerge when cleavage occurred at adjacent deadenylated sites in AT-rich regions of both strands in the deadenylated supercoiled DNA, converting supercoiled DNA into unstrained, energetically more favorable relaxed state (**Figure 4**). Moreover, it was reported that the cleavage at deadenylated sites in DNA molecule could be catalyzed by amino group of chemicals, e.g. Tris. In our assay system, existence of tris would accelerate the cleavage of phosphodiester bonds at deadenylated sites and produced nicked and linear forms of DNA.

In conclusion, the cleavage of supercoiled DNA into nicked and linear forms of DNA occurs spontaneously after adenine residues were removed from DNA molecule by cinnamomin A-chain. Thus the cleavage was not due to the direct action on phosphodiester bond, but rather a result of deadenylation catalyzed by the RNA N-glycosidase [18].

There was another example of supercoiled DNA to be cleaved by an enzymatic catalysis. We unexpectedly observed that a eukaryotic cambialistic superoxide dismutase (SOD) extracted from the mature seeds of camphor tree, could also induce the cleavage of supercoiled double-stranded DNA into nicked and linear DNA form *in vitro*, but it could not cleave the linear DNA or RNA, besides its well-known role of being protected cells against oxidative stress [19]. The result demonstrated there was no DNase or RNase presented in the cambialistic SOD sample. Furthermore, the SOD could linearize circular pGEM-4Z DNA that was relaxed by topoisomerase I. These results indicated that the DNA-cleaving activity of SOD requires also constrainedly topological substrates. The supercoiled DNA-cleaving activity of cambialistic SOD could be also inactivated by SOD inhibitors,

showing that the dismutation activity was crucial to the supercoiled DNA cleavage [20].

Lower cytotoxcity of cinnamomin to carcinoma cells was due to its B-chain

The weak cytotoxicity of cinnamomin was early observed and investigated before molecular mechanism of cinnamomin action was known. Generally, higher toxicity of RIPs to carcinoma cells was particularly interesting because it could shed a light on the use of RIPs as anticancer agents. When the human hepatocarcinoma cell-line 7721 was treated with cinnamomin, the inhibitory effect on the cell growth was observed clearly after a time lag of 30 min. The treated cells first swelled and then burst leading to death after 10 h. After 20 h, most of cells were killed, leaving cell debris suspended in medium. Similar effects occurred in the melanoma cell-line M21, which was slightly more sensitive to cinnamomin than the human hepatocarcinoma cell-line 7721 was. Our preliminary result also showed that cinnamomin could damage the solid melanoma cells induced in the skin of nude mice. After the daily injection of cinnamomin into tumor cells for 5 days, the size of the melanoma was decreased. Meanwhile, no side-effects were noticed. The nude mice appeared healthy [21].

Based on the biochemistry viewpoint, both cinnamomin and ricin belong to the type II RIP that was a heterodimeric ribotoxin. They contain a RNA N-glycosidase chain (A-chain) and a lectin chain (B-chain). After treatment of ribosome by cinnamomin A-chain, the protein synthesis consequently was stopped. The B-chain expressed a carbohydrate-preferential lectin activity with two binding sites that contribute to *in vivo* toxicity. By binding the carbohydrate-containing receptors on cell surface, the B-chain triggers endocytosis of the A-chains.

Ribosome was located in cytoplasm, the entrance of enzyme, *i.e.*, RNA N-glycosidase must be efficacious in cytoplasm to inactivate the ribosome. Whatever and whether the RIPs could fluently enter cells is a key point in exerting its cytotoxicity. It was well-known that the amino-acid residues of two carbohydrate-binding sites of the B-chain of type II RIPs were conserved, demonstrating that the B-chain is crucial in type II RIP cytotoxicity. Cinnamomin and ricin shared 65% amino-acid sequence identity. In comparison of their A-chain, they have comparable RNA N-glycosidase activities. However, as the cytotoxicity was considered, cinnamomin was less toxic to cells, meaning that it exhibited a low cytotoxicity to the human hepatocarcinoma cell-line 7721 than that to the melanoma cell-line M21. The reason of lower cytotoxicity of cinnamomin in comparison with ricin and other type II RIPs still remained to be a mystery. In order to understand this point, the toxicity of cinnamomin to BA/F3β cells could be tested as a sample to compare with that of ricin. The result indicated that cinnamomin was an efficacious toxin to BA/F3β cells although its cytotoxicity was lower than that of ricin. After 22 h treatment, the viability of BA/F3β cells decreased with the increased concentration of cinnamomin and the IC_{50} (concentration for 50% cells killed) was 1.1×10^{-11} M for cinnamomin. Comparatively, ricin was more efficacious in killing BA/F3β cells, and the IC_{50} was 1×10^{-13} M. Also, protein synthesis in BA/F3β cells was inhibited after being treated by cinnamomin or ricin. The IC_{50} of cinnamomin to BA/F3β cells was 1×10^{-11} and that of ricin to BA/F3β cells was 8×10^{-14} M. The effect on protein synthesis was consistent with the effect on cell viability. The death of cells was due to the inhibition of protein synthesis by RIPs *in viv*o. The cytotoxicity of cinnamomin was 137.5 times less toxic than that of ricin.

Whether the different cytotoxicities of the two toxins were due to the different RNA N-glycosidase activities of their A-chains? The results showed already that cinnamomin A-chain and ricin A- chain had almost equivalent activity. The IC_{50} of cinnamomin A-chain was 8×10^{-10} M, and that of ricin was 4×10^{-10} M. This slight difference in RNA N-glycosidase activity of two A-chains could not explain the distinct cytotoxicity of two native proteins. Reversely, these results suggested that the B-chains of two RIPs might play a more important role in the difference in cytotoxicity to BA/F3β cells. In the case of cinnamomin to BA/F3β cell, it was reasonable to suppose that the lower toxicity was due to the lower affinity of cinnamomin B-chain to the cell surface [22].

In order to examine whether the above supposition is reasonable, two mixed hybrid RIPs (CTA-RTB and RTA-CTB) were reconstructed separately from A- and B-chain of cinnamomin and

ricin using the disulfide exchange reaction. The freshly purified ricin B-chain and cinnamomin A-chain were mixed to form a disulfide-linked mixed hybrid via thiol-disulfide interchange. Approximately 60% of separated chains were converted into mixed hybrid (about 60 kDa). A purified and expected hybrid CTA-RTB was obtained and another mixed hybrid RTA-CTB was also prepared and identified by the same method as in preparing CTA-RTB.

In this way, two distinct mixed hybrids were obtained. Their purities were subjected to SDS-PAGE analysis. If the difference of two native RIPs in cytotoxicity to BA/F3β cells was caused by the different affinity of their B-chains to the lectin binding sites on the cell surface, it would be expected that the hybrid (CTA-RTB) will display the toxicity similar to native ricin, while the hybrid (RTA-CTB) will exhibit an equivalent cytotoxicity as compared to that of the native cinnamomin.

The experimental results of the inhibition of protein synthesis in the BA/F3β cells showed that the inhibition of the hybrid (RTA-CTB) was similar to that of cinnamomin, while that of the hybrid (CTA-RTB) was the same as that of ricin. These results confirmed that ricin B-chain was more efficient than cinnamomin B-chain in exerting cytotoxicity to BA/F3β cells. Though cinnamomin was roughly similar to ricin in their chemical structures, and their A-chains expressed almost equivalent RNA N-glycosidase activity, but the two RIPs exhibited distinct cytotoxicity to BA/F3β cells. By comparative studies on the cytotoxicity of the two native RIPs with that of their hybrids, we know that two B-chains were responsible for the different cytotoxicity of the native RIPs. Despite the two B-chains shared similar numbers of binding sites on cell surface, the affinity was highly different, that was consistent with the difference in cytotoxicity of two native RIPs [22].

On the other hand, the physical properties of cinnamomin A-chain were also importance in its enzymatic activity. The pure cinnamomin A-chain was unstable as compared to that in the mixture of A- and B-chain or in intact cinnamomin molecule either stored at 4°C or being heated. When being heated at 45°C for 20 min, the A-chain generated partially unfolded intermediate and lost its tertiary structure as monitored by circular dichroism (CD) and tryptophan fluorescence, thus resulting in the inactivation of its RNA N-glycosidase activity albeit it retained most of its secondary structure. This partially unfolded intermediate was sensitive to protease, exhibiting property of a molten globule. The 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 that the cinnamomin B-chain mediated 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 destiny of proteolysis that would occur in most denatured proteins in cells [23].

It has been known that cinnamomin is less cytotoxic to cell as compared with ricin. In order to further clarify the mechanism of their difference in cytotoxicity, the interaction of cinnamomin and its A-chain with model membrane was investigated and compared with that of ricin and its A-chain. It was revealed that cinnamomin was less effective than ricin was in interaction with model membrane. Cinnamomin A-chain interacted much less violently with model membrane than ricin A-chain did. The differences in the interaction of cinnamomin, ricin or their A-chains with model membrane might at least in part indicate the different cytotoxicity between cinnamomin and ricin [24].

Cinnamomin was a storage protein in the seed of the camphor tree

Many studies on RIPs have been performed on various aspects. However, the physiological function that RIPs played in the plant cells and where RIPs are produced are still unclear. Many RIPs were presumed to be involved in the defensive role in plant cells, and stopped protein synthesis under appropriate physiological conditions, thus were related to metabolic regulation. In addition, some RIPs accumulated in non-productive tissues, such as cotyledon, bark and root. Thus, it was proposed that RIPs might play a role as a storage protein in these tissues. To date, there had been no conclusive evidence to support the storage role of RIPs. The roles of cinnamomin played in the seed cell

of camphor tree still remained to be studied. In a preliminary study in our laboratory, we found that the synthesis of cinnamomin in seeds during a period of autumn was gradually increased up to November [25].

In order to study in the detailed function of cinnamomin in plant cells and where cinnamomin was produced, many aspects of study were carried out as follows:

Tissue-specific expression of cinnamomin: Total RNAs and proteins were extracted from various tissues including roots, stems, leaves of seedlings, leaves of adults, fruits and cotyledons of the mature seeds of camphor tree. The expression of cinnamomin in these tissues was examined by Northern blots, showing that mRNA of cinnamomin was expressed exclusively in the cotyledons, a storage organ of the plant. Western blotting of the total proteins from these tissues using antibodies against cinnamomin indicated that it could only be detected in the cotyledons. These results demonstrated that cinnamomin was expressed specifically in the cotyledons.

Synthesis of cinnamomin during seed development: To study the course of accumulation of cinnamomin during the development of the seeds, proteins extracted from cotyledons at different developmental stages were analyzed by SDS/PAGE and Western blotting. Little protein was synthesized in the early developmental stages and no cinnamomin was detected at these stages. Cinnamomin first appeared at 60 DAF in the cotyledon and subsequently accumulated over the following 30 days. Such a pattern of synthesis and accumulation of cinnamomin in the later stages of seed development was very similar to that of other seed storage proteins of other species.

Degradation of cinnamomin during seed germination: The change in cinnamomin content during germination of the seeds was monitored for a period of 42 days after imbibition (DAI). By 42 DAI, when plantlets developed three to four leaves and their total dry weight was equal to or larger than that of the initial seeds, total proteins were extracted from the seeds at different stages of germination and analyzed by both SDS-PAGE and Western blotting. The results revealed that the amounts of cinnamomin was not changed during the first stage (7 DAI), but it was degraded rapidly during the subsequent stages (7-12 DAI), and became undetectable at 21 DAI. The rapid degradation of cinnamomin in the early stages of seed germination was similar to that of seed storage proteins of other species.

Effect of endopeptidase on cinnamomin degradation: Our results showed that endopeptidase played an important role in the degradation of storage proteins in dicotyledonous seeds. Changes in the activities of the endopeptidase as well as the two exopeptidases, carboxypeptidase, i.e., carboxypeptidase and aminopeptidase, were measured during the germination of camphor tree seeds. The activity of exopeptidase was very low throughout the germination period. However, endopepidase activity increased dramatically on 7-14 DAI, and was maintained at a high level thereafter. This increment of endopeptidase activity coincided with the rapid breakdown of cinnamomin (7-28 DAI). Furthermore, the endopeptidase of the seeds was found to be a serine protease, since it was sensitive to PMSF but not to N-ethylmaleimide.

The effect of endopeptidase on the degradation of cinnamomin was studied further by using the protease inhibitor PMSF. The extracts from camphor seeds at different stages of germination were treated with PMSF and then auto-digested. The cinnamomin content in extracts treated with PMSF was much higher than those not treated with PMSF, demonstrating that the inhibition of endopeptidase by PMSF could prevent the degradation of cinnamomin. All of these results suggested that endopeptidase played an important role in the degradation of cinnamomin during seed germination.

Localization of cinnamomin in the protein body: Most plant storage proteins were located in the protein body of the cell. This highly specialized cellular organelle could be purified by sucrose-density-gradient centrifugation, and appearing as a sharp band at the interface of 40% and 80% sucrose. More than 80% of total cotyledon proteins were found in the band (protein-body fractions). Western blotting showed that cinnamomin also existed in these fractions. Whereas no cinnamomin was detected in the other components over 40% sucrose, here other cytoplasmic organelles were concentrated. This showed that cinnamomin was localized specifically in protein bodies, consistent with the pro-

Table 3. Partial restoration of protein synthesis activity of ribosome inactivated by cinnamomin A-chain

A			B		C	
Addition		Activity of control (%)	Addition	Activity of control (%)	Addition	Activity of control (%)
None (control)		100	None (control)	100	None (control)	100
Cinnamonin		29.5	Cinnamonin	29.5	Cinnamonin	29.5
Alanine	L-	45.4	L-amino-butyric acid	47.3	Sodium borohydride	64.2
	D-	44.7	D-3.4-Dichlorophenylalanine	39.2	L-Tryptophan	63.5
Glutamic acid	L-	49.4	L-3, 4-Dichlorophenylalanine	35.7	L-Histidine	57.5
	D-	50.0	L-2-Aminohexanoic acid	38.1	L-Lysine	58.5
Phenylalanine	L-	42.3	N-Acetyl-tryptophan	23.8	L-Glutamic acid	48.3
	D-	45.0	N-Acetyl-Tyr-Val-Gly	24.6	α-Sarcin	26.3
Threonine	L-	50.6	N-Boc-glycine	23.9	Sodium borohydride	28.7
	D-	49.2	N-Boc-glycine	28.3	L-Tryptophan	28.9
Valine	L-	53.3	N-HCO-proline	27.9	L-Histidine	24.5
	D-	49.5	Spermidine	40.7	L-Lysine	29.4
			Spermine	43.2	L-Glutamic acid	30.1

Partial restoration of the protein synthesis activity of cinnamomin-inactivated ribosome. Assay method: rat 80S ribosomes were incubated with cinnamomin followed by the treatment with different chemicals in A, B, C. The modified ribosomes were recovered by centrifugation and then assayed for polyphenylalanine synthesis. The protein synthesis activity of the native ribosome was 100%. The experimental data were the average value of triplicates. A. Five L-, D-amino-acids or their enantiamers; B. amino acids derivatives or two polyamines; C. $NaBH_4$ or L-amino acids to react with cinnamomin-treated ribosome; To compare with cinnamomin-inactivated ribosome, here $NaBH_4$ instead of cinnamomin in part to inactivate ribosome.

posed role as a storage protein. The results described above indicated that expression of cinnamomin was limited only in the cotyledon of C. camphora seeds. It synthesized cinnamomin in large amounts during the latter stages of seed development, and its rapid degradation was in the early and intermediate stage of seed germination, suggesting that cinnamomin should function as a seed storage protein. Cinnamomin was post-translationally processed. These processes included all of proteolytical cleavage, the formation of intra- and interchain disulfide bonds as well as the attachments of glycosyl chains with mannose N-linked to asparagine residues. After processing, the mature cinnamomin is transported to the protein body, the specific cellular organelle for storage of proteins. Herein, we provided lines of evidence that cinnamomin was one of the most prevalent storage protein in C. camphora seeds, accounting for about 11% of total proteins. This content was higher than those of many other storage proteins. Most importantly, cinnamomin is very rich in amide-containing amino-acids, including Asn (12.6%) and Gln (9.4%), and also contains relatively higher contents of sulfur-containing amino-acids, including Cys (1.9%) and Met (2.6%). All of these properties suggest that cinnamomin functions as a storage protein, which provides the sources of carbon, nitrogen and sulfur during seedling growth and development [26].

The involvement of the active aldehyde group generated by RNA N-glycosidase in partial restoration of the inactivated ribosomes

After the adenine was removed by RNA N-glycosidase, an active aldehyde group emerged at the ribose C'1 of ribose 4324 in rat ribosomal 28S RNA, and thus, protein synthesis was stopped. We had paid serious attention to this newly emergent aldehyde group that is involved in inactivation of ribosomes during the process of protein synthesis.

It was found earlier in my laboratory that sodium borohydride and L-amino-acids could partially restore the translational activity of the inactivated ribosome that was already damaged by RNA N-glycosidase [27, 28]. We wanted to find out whether some of D-amino-acids and other compounds bearing the amino groups would have the same capacity to restore the translational activity of the inactivated ribosome. The results showed that the inactivated ribosome had no selective preference for L- or D-amino-acids to restore the activity [29]. To investigate further, we used some amino-acid derivatives with free- or blocked amino-group of amino-acids, and two polyamines (spermi-

dine or spermine), to block the aldehyde group. The results indicated that the amino-acid derivatives with free amino group or polyamines could also partially restore protein synthesis activity of the inactivated ribosome, while those with blocked amino group could not [**Table 3**]. These facts indicated that the inactivation of ribosome was related, at least in part, to the emergence of the aldehyde group in the sarcin/ricin domain of 28S RNA in ribosome.

The reason explaining the partial restoration of the inactivated ribosome would be an important point to further understanding of the mechanism of the translational process in protein synthesis. From the viewpoint of the enzymatic mechanism of RNA N-glycosidase and sarcin, both enzymes inactivate ribosome in quite different ways. In the case of RNA N-glycosidase, the inactivated ribosome by the enzyme lost an adenine base, and, at the same time, produced an active aldehyde group at C'1 of ribose 4324 in rat 28S rRNA. Here, losing an adenine from 28S rRNA was a little trifling as comparable with such a big ribosome. The tertiary structure of ribosome at least of sarcin/ricin domain would be unchangeable under the condition. In general, it would be conceivable that losing an adenine base could not effect on the activity of ribosome. But, the emergence of an active aldehyde would cause harm to ribosome.

When the aldehyde was treated with then sodium borohydride or the amino-acids, the inactivated ribosomes obtained again rather higher restoration in protein synthesis. Since the former reduced the aldehyde into hydroxyl group, while the amino group of the latter reacted with aldehyde to produce Schiff's base. In both cases, the aldehyde group would be eliminated and the activity of the inactivated ribosomes could be partially restored. We proposed that within the cells, the active aldehyde emerged in the sarcin/ricin domain could occasionally react with the amino group of the elongation factors, which have already carried tRNA, entrancing the sarcin/ricin domain to form a Schiff's base with aldehyde group. Thereby, they occupy steadily this space, thus, preventing the entrance of the next elongation factors. In this way, protein synthesis would completely be stopped.

However, α-sarcin worked in a different way comparing with that of RNA N-glycosidase. Like a special ribonuclease, α-sarcin hydrolyzed only one phosphodiester bond in the sarcin/ricin domain of 28S rRNA, neither sodium borohydride nor compounds carrying the amino group could react with any groups producing by α-sarcin anyhow. Furthermore, owing to the cleavage of a phosphdiester bond in 28S rRNA, α-sarcin might loosen the topographical structure of sarcin/ricin domain in ribosome, and the activity of the inactivated ribosome could certainly not be restored by the processes mentioned above, resulting in protein synthesis entirely stopped (**Table 3**).

In order to strengthen the viewpoint that the emergence of aldehyde in inactivated ribosome was crucial to the activity of protein synthesis, we used three RNA N-glycosidases (trichosanthin, cinnamomin A-chain and ricin A-chain) to perform more experiments. All the results demonstrated that aldehyde group in 28S rRNA of the inactivated ribosome remarkably inhibited eEF1A-dependent Phe-tRNA binding activity. While the aldehyde group was reduced first into the hydroxyl group with sodium borohydride, then the binding activity of eEF1A-dependent Phe-tRNA to the inactivated ribosome could partially be restored. The restoration of activity was 64.7% (trichosamthin), 52.6% (cinnamomin A-chain), and 62.2% (ricin A-chain), respectively.

Blockage of aldehyde group with valine or threonine also resulted in significant restoration of the eEF1A-dependent Phe-tRNA binding activity. In another case, the binding activity to eEF1A-dependent GTPase was decreased obviously, owing to the emergence of aldehyde groups generated by RNA N-glycosidase. In addition, after the aldehyde group was reduced to a hydroxyl group by sodium borohydride, the activity of eEF1A-dependent GTPase was only partially restored. The result showed that restoration also occurred remarkably when the aldehyde group was blocked with valine or threonine [30, 31].

Based on the aldehyde group could react with $NaBH_4$ or amino-acids producing hydroxyl or schiff's base, a radioassy method for RNA N-glycosidase with tritium-labeled sodium borohydride or amino-acids was developed. The radioactivity incorporated into the modified 28S ribosomal RNA allowed a rapid and sensitive determination and quantification of RNA N-glycosidase activity [32].

Table 4. Species distinction of three type II RIPs from the seeds of *Cinnamomum* genus

Genus		*Cinnamomum*		
Species		*Camphora*	*Porrectum*	*Bodinieri*
Plant tissue		Kernel		
RIP name		Cinnamomin	Porrectin	Bodinierin
Yield (mg/500 g)		620	112	40
Molecular mass (kDa)	A-chain	31	30.5	31
	B-chain	34	33.5	34
Content of neutral sugar (total)		2.2%	2.5%	2.0%
IC_{50} (nM) in cell-free system		1.1	1.0	1.2
Carbohydrate-binding activity*		12	38	15

*The carbohydrate-binding activity is defined as the lowest concentration (nM) required to agglutinate rabbit erythrocyte.

Three type II RIPs from seeds of three species of the same genus Cinnamomum

Cinnamomum genus belongs to family *lauraceae*, and has three species: *camphora, porrectum* and *bodinieri.* In addition to cinnamomin, we identified two new type II RIPs, porrectin and odinierin, from other two species of *Cinnamomum* genus: *C. porrectum and C. bodinieri,* respectively. Similar to *C. camphora,* both *C. porrectum and C. bodinieri* are well-known for their resistance to virus infection and insect diseases. Taking an approach similar to the purification of cinnamomin, *i.e.*, the affinity chromatography on AT-sepharose 4B, we purified porrectin and odinierin, from the seeds of *C. porrectum and C. bodiniei*. The chemical and biological properties of three RIPs are listed in **Table 4**. Although their contents in the mature seeds are significantly lower than that of cinnamomin, both porrectin and odinierin displayed the properties similar to those of cinnamomin in many aspects, including molecular mass, molecular structure, inhibition of the protein synthesis, inactivation of rat ribosome, and so on. Therefore, we proposed that cinnamomin-like type II RIPs were the hallmark of genus *cinnamomum* [33, 34].

Species divergence of activity in various RNA N-glycosidase to cleave the different sorts of ribosomes

Rat or rabbit ribosome is usually used as the bettersubstratetoassaytheactivityofRNAN-glycosidase. Many RIPs showed very high RNA N-glycosidase activity on animal ribosome, especially rat ribosome, but expressed obviously changeable activity on different plant ribosomes. It has been reported that certain RIPs could not inactivate their own (autologous) ribosomes, while some type I RIPs could deadenylate well plant ribosomes, including their own ribosomes without species specificity.

Our results showed that a tiny amount (3 nM) of cinnamomin A-chain was sufficient to produce the R-fragment from rat 28S ribosomal RNA after acidic aniline treatment. However, when *C. camphora* ribosome was used as substrate, even a large amounts (3 μM) of cinnamomin A-chain were added, only little amount of R-fragment appeared after acidic aniline treatment through the SDS-PAGE analysis. This result demonstrated clearly that cinnamomin A-chain was unable to inactivate its own ribosome, *i.e.*, *C. camphora* ribosome.

It had been reported that some soluble factors, such as ATP, tRNAs, from animals and other unknown factors could promote the action of RNA N-glycosidase on plant ribosome. On the other hand, lack of such factors was proposed to account for the lower efficiency of certain RIPs on plant ribosome.

We wanted to examine if these factors could specifically promote the activity of cinnamomin A-chain. The result indicated that rat extra-ribosomal factors (S-100) were unable to promote RNA N-glycosidase activity of cinnamomin A-chain on its own ribosome. In order to find if other kinds of RIPs could cleave *C. camphora* ribosome, five RIPs, including two A-chains of type II (cinnamomin and ricin) and three type I (trichosanthin, saporin-S6, and gelonin), were used in rather high concentrations. The experimental results indicated that only one, *i.e.,* saporin-S6, could obviously produce the diagnostic R-fragment from *C. camphora* ribosome after acidic aniline treatment.

It was known that the extra-ribosomal factors (S-100) could also promote gelonin activity in cleaving animal ribosome. However, the activity of gelonin on *C. camphora* ribosome could not be promoted by rat extra-ribosomal factors (S-100), indicating again extra-ribosomal fac-

tors did not account for the insensitivity of *C. camphora* ribosome itself to many RIPs.

Furthermore, it should be noted that the sizes of the R-fragment released from *C. camphora* ribosome by saporin-S6 was a little smaller than the R-fragment from rat ribosome. A comparison of saporin-S6 acting on both rat and *C. camphora* ribosome was carefully carried out. The results showed that saporin-S6 was extremely efficient in deadenylation of the rat ribosomal RNA, while approximately 1000 times greater amounts of saporin-S6 was needed to inactivate *C. camphora* ribosome under the same condition.

C. camphora ribosome was not only insensitive to its autologous RIP, *i.e.*, cinnamomin A-chain, but also insensitive to other RIPs such as ricin A-chain, trichosanthin, and gelonin. However, these five RIPs tested were all extremely efficient in catalyzing the deadenylation of rat ribosome. Whereas the type I were also suggested to efficiently deadenylate plant ribosomal RNA without species specificity. This conflicting result indicated that the structure of sarcin/ricin domain in *C. camphora* ribosome seems to be different obviously from that in rat and other plant ribosomes. Now we would reach to conclude that the structural difference in ribosomal RNA between *C. camphora* and rat ribosomes had been clearly expressed in our work, *i.e.,* the R-fragment released from *C. camphora* ribosome was shorter than that from rat ribosome. In addition, despite saporin-S6 could deadenylate *C. camphora* ribosome, the efficiency of the enzyme was 1000 times less than it acts on rat ribosome [35].

Cinphorin, a novel type II RIP containig a active miniature A-chain that expressed RNA N-glycosidase activity

Fortunately, we had obtained an interesting result that in the seed extract of camphor tree a small protein was co-bound with cinnamomin on the AT-sepharose 4B column and eluted together with cinnamomin by lactose from the column. This tiny protein was named as cinphorin (46 kDa), much smaller than that of cinnamomin (64 kDa). It could be separated well from cinnamomin by SDS-PAGE (12%). Cinphorin contained also two polypeptide chains (A- and B-chain). The A-chain exhibited N-glycosidase activity and strongly inhibited protein synthesis *in vitro* with the IC_{50} 1.2 nM, which is comparable to that of cinnamomin A-chain (1.0 nM). Two chains of cinphorin were connected by a disulfide bond since it could be reduced by 2-mercaptoethanol to produce a miniature A-chain and a normal B-chain. Further results showed that the miniature A-chain contained the entirely identical N-terminal 10 amino-acid sequence compared with that of normal cinnamomin A-chain. However, the N-terminal 10 amino-acid sequence and the molecular mass of cinphorin B-chain were the same as compared with normal cinnamomin B-chain. This fact indicated that the B-chain of cinphorin was entirely identical to that of cinnamomin B-chain.

Further study showed that cinphorin A-chain was also linked to its B-chain by a disulfide bond. It could be deduced that in the C-terminus of cinphorin A-chain should had a cysteine residue which linked to the cysteine 4 residue in its B-chain. Cinphorin A-chain contained N- and perhaps C-terminus of the same sequence of amino-acids, compared with normal cinnamomin A-chain. However, the molecular mass of cinphorin A-chain was small (16 kDa), only half of that of normal cinnamomin A-chain (31 kDa). Now, the problem was how the cinphorin A-chain was produced *in vivo*, and how such a small cinphorin A-chain accounted for its RNA N-glycosidase activity? These questions were quite intriguing [36].

Preliminary records of application of cinnamomin in practice

To investigate the potential application of cinnamomin as a pesticide, we examined the toxicity of cinnamomin to the larvae of both cotton bollworm (*Helicoverpa armigera*) and mosquito *(Culex pipines pallens*) by feeding the insects on the diet containing cinnamomin. We found that bollworm larvae were more resistant to cinnamomin than were mosquito larvae (LC_{50} of 1839 ppm to the former and 168 ppm to the latter). One apparent reason for the relatively low toxicity of cinnamomin to bollworm was that cinnamomin could be effectively digested by the gut extract of bollworm larvae. Although protein synthesis could be stopped by inactivating bollworm ribosome with cinnamomin and producing the R-fragment, carefully quantitative analysis showed that the susceptibility of bollworm ribosome was about 10-100 times less than that of rat ribosomes to cinnamomin.

These results suggested that cinnamomin was less toxic to ribosome of bollworm than to rat, showing that at least the structures of sarcin/ricin domain of ribosomes between bollworm and rat were different [37].

Cinnamomin and ricin exhibited also a different toxicity to domestic silkworm *(Bombyxmori)* larvae by oral feeding bioassay. The IC_{50} of ricin to the silkworm larvae at third instar was much lower than that of cinnamomin. When the isolated 80S ribosome from domestic silkworm pupae was treated separately with the reduced cinnamomin or the reduced ricin, a specific RNA (R-fragment) was produced as characterized by 8 M urea-denatured polyacrylamide gel (3.5%) electrophoresis. The purified A-chains of both cinnamomin and ricin showed only slight different RNA N-glycosidase activity to the domestic silkworm pupae ribosome. It was proposed that the difference of their toxicity to domestic silkworm larvae was not related to their A-chains, but due to again the structural differences of their B-chains [22, 38].

Trichosanthin, a versatile abortifacient protein from a climbing plant, is RNA N-glycosidase

Trichosanthin expressed RNA N-glycosidase activity

Trichosanthin was an abortifacient protein isolated from a climbing plant, the Chinese medicinal herb, Tian-hua-fen, which was obtained from the root tubers of the Chinese medicinal plant trichosanthes tree *(Trichosanthes kirilowii* Maxim). For a long time, Tian-hua-fen had been using to induce midterm abortion in China. The active ingredient had been purified and shown to be a basic protein with a single-chain of MW 27,000 Da and was named trichosanthin that belonged to the family of type I RIP, inhibiting protein synthesis in the cell-free system. It was found that trichosanthin could selectively kill choriocarcinoma cells *in vitro* and had potent inhibitory activity against human immunodeficiency virus (HIV) *in vitro*. These medical effects of trichosanthin might be ascribed to its enzymatic action on ribosomal RNA. In my laboratory, its enzymatic mechanism on ribosomal RNA had been clarified as a RNA N-glycosidase.

We used ricin A-chain as a reference in investigating the molecular mechanism of trichosanthin action. Ribosome after treatment with trichosanthin and acidic-aniline could release a R-fragment (450 nucleotides) indistinguishable in size from the diagnostic R-fragment released by ricin A-chain and aniline treatment on gel, demonstrating trichosanthin really hydrolyzed the the ribosomal RNA that produced a R-fragment. These results showed that trichosanthin expressed RNA N-glycosidase activity as ricin A-chain did.

In order to determine the cleaving site of trichosanthin on rat 28S rRNA, the 5'-terminal nucleotide sequence of the R-fragment released by trichosanthin and aniline treatment was determined. To compare with the complete sequence of rat 28S rRNA, the 5'-terrninal nucleotide sequence of the R-fragment were determined by an enzymatic method, thus A4324 in the conserved sarcin/ricin domain of 28S rRNA was deduced to be the cleaving site of trichosanthin. The results demonstrated that quantitative recovery of adenine and no other bases were released in the solution of trichosanthin and aniline treated ribosome, providing evidence that trichosanthin cleaved the N-C glycosidic bond of A4324 of 28S RNA in ribosome. It should be noted that the cleaving fashion of trichosanthin on ribosomal RNA could have two different ways.

The N-C glycosidic bond of the adenosine residue in 28S rRNA might be enzymatically cleaved by either phosphorolysis or hydrolysis. In the presence of inorganic [P32]-phosphate, if it was phosphorolytic mechanism, the [^{32}P]-phosphate should be incorporated into ribose residue of A4324 to form ribose C'1-phosphate. The result showed that almost no [P32]-phosphate incorporated into 28S rRNA after cleavage with trichosanthin. In fact, it was demonstrated that trichosanthin cleaves the N-C glycosidic bond by a non-phosphorolysis. In short, trichosanthin inactivates rat ribosomes by cleaving the N-C glycosidic bond of A4324 in 28S rRNA in a hydrolytic fashion.

RNA N-glycosidase cleaved the N-C glycosidic bond of adenosine 4324 in the hydrolytic fashion. Thus it should generate a hemiacetal radical at ribose C'1-ribose 4324 position in 28S rRNA. In fact, chemically dynamic equilibrium exists between the configuration of hemiacetal and aldehyde group. Furthermore, the aldehyde group could be either reduced to hydroxyl group by [H^3]-$NaBH_4$ or converted to Schiff's base

by nucleophilic addition with [H^3]-alanine. The reactions could break the equilibrium, converting most hemiacetal group into aldehyde group. If tritium was introduced into the final products, the modified 28S rRNA was radioactive. These results demonstrate that hemiacetal radical indeed existed in 28S RNA of trichosanthin-treated ribosome, indicating that trichosanthin inactivates ribosome by cleaving the N-C glycosidic bond of A4324, leaving a hemiacetal radical at C'1 of ribose 4324 in 28S rRNA with a hydrolytic fashion [39].

Low-density lipoprotein receptor-related protein 1 was an essential receptor for trichosanthin in 2-choriocarcinoma cell lines

Trichosanthin was highly toxic to trophoblasts and choriocarcinoma cells. However, the mechanism for this selectivity had not been completely elucidated. There was increasing evidence that the selective cytotoxicity of trichosanthin was associated with its RNA N-glycosidase activity. As a RNA N-glycosidase, trichosanthin had to translocate across the plasma membrane and traveled intracellularly to reach its molecular target (ribosome) within the cytoplasm of mammalian cells, thereby indicating that trichosantin might have a selective transport mechanism for entering cells that were sensitive to it.

Low-density lipoprotein receptor-related protein 1 (LRP1) was a type-1 transmembrane receptor, which functioned in the endocytosis of over 40 structurally and functionally distinct ligands. Previous studies reported that saporin, ricin A-chain and trichosanthin could directly bind to LRP1, however, it was not clear whether LRP1 was an essential receptor for trichosanthin endocytosis.

Our experimental results demonstrated that trichosanthin is capable of binding and internalizing into 2 choriocarcinnoma cell lines. In contrast, trichosanthin displays no obvious binding and endocytosis into Hella cell line. Furthermore, silencing LRP1 gene in JAR and BeWo cell lines blocked trichosanthin binding. Trichosanthin could also interact with LRP1. The results of our studies established that LRP1 was a major receptor for phagocytosis of trichosanthin in JAR and beWo cell lines and might be the molecular basis of trichosanthin abortifacient and anti-2-choriocarcinoma activity [40].

A novel ribosome-inactivating Protein with special ribonuclease activity that specifically cleaves only a single phosphodiester bond in rat 28S ribosomal RNA and inactivates ribosome

It was found that the crude extract from mature seeds of cypress (*Biota orientalis*), a gymnosperm tree, inhibited protein synthesis in rabbit reticulocyte lysate system. During incubation of rat ribosome with the crude extract under restricted condition (25 mM Mg^{2+}), a specific RNA fragment appeared. This RNA was smaller than the usual diagnostic RNA fragment (R-fragment) produced by RNA N-glycosidase. The smaller RNA fragment was designated S-fragment and it could be released from rat ribosome without acidic-aniline treatment. Thus, it was presumed that a special ribonuclease existed in the crude extract of mature seeds of *Biota orientalis* tree.

Isolation and purification of Biota oriental RNase (RNase Bo)

To isolate this special ribonuclease, the crude extract of mature seeds of *Biota orientalis* tree was subjected to several steps of different column chromatography. The partially purified ribonuclease was characterized by negative-staining in gel with a molecular mass of approximate 13 kDa and named *Biota oriental* RNase (RNase Bo). RNase Bo retained its specificity to release S-fragment from rat ribosome after many steps of purification even heating at 100°C for 10 min under acidic condition.

A small RNA fragment (S-fragment) was released from rat 28S rRNA treated with RNase Bo

Several lines of evidence strongly indicated that S-fragment was derived from the 3'-terminus of rat 28S rRNA. First, the 3'-terminal nucleotide of S-fragment was determined as U by two-dimensional thin-layer chromatography, which was consistent with the 3'-terminal nucleotide U of rat 28S rRNA, while that of 18S rRNA was A. Thus, it was very likely that S-fragment was derived from the 3'-end of 28S rRNA. Second, the partial sequence of guanosine and adenosine at 3'- and 5'-terminal regions of S-fragment were determined by enzymatic methods. Compared with the known sequence at 3'-terminal region of rat 28S rRNA, it was found

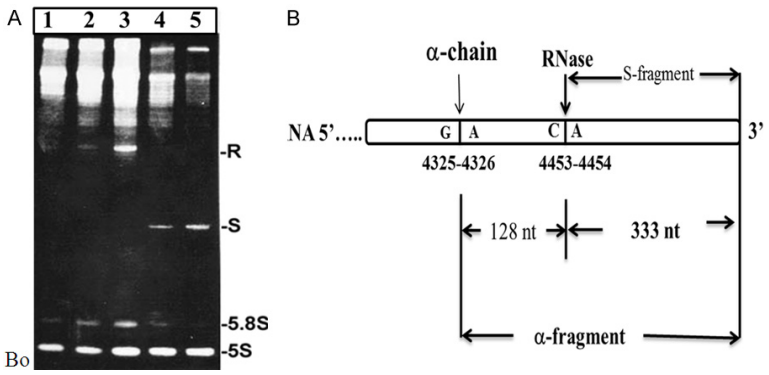

Figure 5. Special cleavage-site of RNase Bo on rat 28S rRNA. (NA represents rat 28S rRNA, RNase means RNase Bo). A. S-fragment produced by RNase Bo on rat ribosomal 28S RNA in the presence of Mg^{2+} (25 mM) by gel electrophoretic analysis. Lane 1, ribosome treated with DEPC water; lane 2, ribosome treated with ricin A-chain; lane 3, ribosome treated with ricin A-chain and acidic aniline, the R-fragment was produced; lanes 4 and 5, ribosome treated with RNase Bo without aniline treatment; R, representing the diagnostic RNA fragment that was released from rat rRNA treated with ricin A-chain and acidic aniline; S, refers as to the small RNA fragment produced from rat rRNA by RNase Bo without aniline treatment; 5.8S, representing 5.8S RNA; 5S, meaining 5S RNA; B. Schematic summary of the cleavage-site of RNase Bo in rat 28S ribosomal RNA compared with that of α-sarcin.

that the partial sequence of guanosine and adenosine of S-fragment were the same as the counterpart region of rat 28S rRNA. Third, using primer-extension to analyze the 3'-terminal sequence of S-fragment, the 3'-terminal 37-nucleotide sequence of S-fragment obtained in this way was the same as that of the corresponding sequence of rat 28S rRNA. Furthermore, the complete cDNA of the full-length of S-fragment was obtained by RT-PCR. The sequencing result of the cDNA directly confirmed that S-fragment was derived from the 3'-terminal region of rat 28S rRNA cleaved by RNase Bo (**Figure 5A**, **5B**).

The cleavage-site by RNase Bo in rat 28S rRNA was the single phosphodiester bond between C4453-A4454

All above data definitely indicated that the phosphodiester bond cleaved by RNase Bo was located between C4453 and A4454 of rat 28S rRNA. Accordingly, it was deduced that the S-fragment was totally composed of 333 ribonucleotides (nt). The distance between the cleavage-sites by RNase Bo and α-sarcin was 128 nucleotides (**Figure 5B**). The region around C4453 and A4454 was named RNase Bo region. In addition, the 5'-terminal nucleotide of S-fragment could be directly labeled by [^{32}-P]-ATP and T4 polynucleotide kinase without pretreatment with monophosphatase, demonstrating the 5'-terminal nucleotide of S-fragment bore a hydroxyl group. The 5'-terminal nucleotide of S-fragment was determined as A by two-dimensional thin-layer chromatography. We concluded that RNase Bo specifically cleaved a single phosphodiester bond of 28S RNA in rat ribosome under restricted condition (25 mM Mg^{2+}). It was worthy to study whether the unique specificity of RNase Bo is attributed only to the intrinsic property of the enzyme itself. When rat ribosome was used as substrate, RNase Bo cleaved only a specific site in 28S rRNA and released S-fragment under restricted conditions. However, if naked rat 28S rRNA was used as substrate, RNase Bo caused extensive digestion of the RNA.

The specific cleavage-site targeted by RNase Bo was a conserved region in several higher mammalian 28S ribosomal RNA

Comparing the region of cleavage-site of RNase Bo in rat 28S rRNA with the corresponding regions of 28S rRNA of the other species, it was found that this region was highly conserved among higher mammalian species, such as human, rat, and mouse. When ribosomes of rat, mouse, yeast, rice or *E.coli* was incubated with RNase Bo, S-fragment was released only from rat and mouse ribosomes, while no S-fragment was released from ribosomes of other species. These results indicated that RNase Bo selectively acted on the conserved region of mammalian ribosomal RNAs under restricted condition (25 mM Mg^{2+}). All the above facts demonstrated that the specificity of RNase Bo in cleaving 28S rRNA depended on both the intrinsic property of the enzyme itself (RNase Bo) and the structural variability of ribosomes. RNase Bo had no detectable dexoyribonuclease (DNase) activity.

Inhibition of protein synthesis was due to the direct cleavage of ribosomal RNA by RNase Bo

The inhibition of protein synthesis by RNase Bo might be due to other reasons, such as cleavage of tRNA and mRNA presented on the surface of ribosomes. To exclude this possibility, rat ribosomes were pre-incubated with RNase

Bo and the pretreated ribosomes were separated from the enzyme by high-speed centrifugation. The activity of synthesizing polypeptide of pretreated ribosome was obviously decreased as the concentration of RNase Bo that was used to treat ribosome was increased. The inhibition of polypeptide synthesis was a direct result of specific cleavage on rat ribosomal 28S RNA by RNase Bo, since S-fragment was released also from pretreated ribosome.

The effect of RNase Bo on EF-1α-dependent aminoacyl-tRNA binding to ribosome

The mechanism of inhibiting polypeptide synthesis was further investigated. The specific cleavage of rat 28S rRNA by RNase Bo affected directly EF-1α-dependent binding of aminoacyl-tRNA to ribosome, while it had no effect on non-enzymatic binding of aminoacyl-tRNA to ribosome. In addition, the damage to 28S rRNA caused by RNase Bo did not significantly interfere with the formation of EF-2/GDP/ribosome complex, while ricin dramatically inhibited this complex formation. These results indicated that RNase Bo inhibited polypeptide synthesis at least partially by affecting EF-1α-dependent binding of aminoacyl-tRNA to ribosome.

As for the fact that RNase Bo specifically cleaved 28S RNA of rat ribosome under restricted conditions (25 mM Mg^{2+}), one may argue that such high concentration of Mg^{2+} was much higher than that in cell. But it had been reported that high concentration of cation, such as Mg^{2+}, could create folding or stabilizing condition, permitting a greater degree of internalization of the rRNA. At such high concentration of Mg^{2+} (25 mM), it was possible that only the RNase Bo region was available for the enzymatic cleavage by RNase Bo. Although the biological role of RNase Bo in the cell was unknown, we could take the advantage of its specificity to cleave rRNA under restricted conditions and used it as a tool to study the structure and function of ribosome. Similarly, in the case of α-sarcin, the production of α-fragment was also performed under non-physiological conditions, *i.e.,* restricted condition (5 mM EDTA). In the absence of EDTA, no α-fragment was released by α-sarcin. The specificity of RNase Bo to cleave the largest RNA in ribosome was similar to that of α-sarcin, which also inhibited protein synthesis by causing a single cleavage of phosphodiester bond of the largest rRNA. As the isolated ribosome was used as substrate, both α-sarcin and RNase Bo could cleave only one of thousands of phosphodiester bonds at different sites in 28S rRNA. When the naked rRNA was used as substrate instead of natural ribosome, α-sarcin also caused extensive cleavage as RNase Bo did.

The specificity of RNase Bo to ribosome was also similar to that of another unique ribonuclease colicin E3, which inactivated *E.coli* ribosome by cleaving a single phosphodiester bond between A1493 and A1494 in 16S rRNA, releasing a small 48-nucleotide fragment from its 3'-end. The RNase Bo only specifically attacked ribosomes of certain mammalian species while it had no effect on ribosomes of several other species under restricted conditions (25 mM Mg^{2+}). Similarly, it was known that the activity of RIPs to ribosome from different species also showed quite differences. In our laboratory, we observed that *C. camphora* ribosome was considerably insensitive, while rat liver ribosome was extremely sensitive to cinnamomin A-chain [35]. Thus we proposed that the specificity of RNase Bo to ribosomes of different species was dependent on RNA sequence and/or the conformational structure of ribosomal RNA/protein complex [41].

To our knowledge, RNase Bo is the first ribonuclease isolated from gymnosperm. It had a unique activity to certain ribosomes under restricted conditions. Up to now, its physiological function in the seed cell is still not known and whether RNase Bo played an important role in the maturation of *oriental arborvitae* seeds remaining to be studied.

Lamjapin, the first ribosome-inactivating protein from kelp, the marine cryptogamic algal plant

Extraction and purification of lamjapin

Many Ribosome-inactivating proteins, including type I, II, and III RNA N-glycosidase, have been isolated and characterized mostly from terrestrial flowering plant (Angiosperm), while no RIP had been isolated from the lower marine plants (e.g. cryptogamic alga) so far. In our laboratory, the first RIP was discovered from cryptogamic plant kelp and was shown to be a single-chain RNA N-glycosidase and named as lamjapin.

Work on extraction of protein from brown alga was tedious, because of its richness in phenotic compounds, pigments and polyanionic cell wall consisting of alginates. The difficulty in extraction of active lamjapin was that usual methods of homogenization could not be used in the presence of above inhibitory substances. An alternatively desired method that the lyophilized kelp was powdering in liquid nitrogen and then extracted gently at low temperature in the alkaline solution in the presence of ascorbic acid had been chosen. These conditions could efficiently decrease interaction of proteins with phenolic compounds and alginates etc., and hence preserved the enzymatic activity of lamjapin.

After four steps of column chromatography, the pure lamjapin was obtained. Approximately 30 μg of pure lamjapin could be obtained from 30 g of dry kelp powder. The low yield of lamjapin obtained was due to its low abundance in the total proteins and also to the low efficiency of extraction as described. Lamjapin obtained from the last step with Mono Q column migrated as a single band on both 12% SDS/PAGE and isoelectric focusing gel electrophoresis, indicating that it was a homogeneous protein. Furthermore, it appeared as a single band by SDS/PAGE in the presence or absence of thiothreitol, showing that it was a type I RIP composed of a single peptide chain without an intra-disulfide bond. Lamjapin had an apparent molecular mass of about 36 kDa, which was a slightly larger than the average molecular mass (about 30 kDa) of other known single-chain RIPs from the higher plants. Like other type I RIPs, Lamjapin was a basic protein with a pI of 8.4, as determined by isoelectrophoresis.

Inhibition of protein synthesis and RNA N-glycosidase activity of lamjapin

Lamjapin inhibited protein synthesis in the cell-free system of rabbit reticulocyte. The protein synthesis was decreased gradually with increment of lamjapin in the reaction mixture. The IC_{50} (the concentration of RIP causing 50% inhibition of translation) of lamjapin was about 0.69 nM, a very low value for inhibition of protein synthesis and still in the range of the IC_{50} (0.03-4 nM) of type I RIP. The IC90 (the concentration of RIP causing 90% inhibition of translation) of lamjapin in the cell-free system of rabbit reticulocyte was about 5.56 nM. Furthermore, the enzymatic activity of lamjapin to ribosome was compared with that of cinnamomin A-chain. At a molar ratio of lamjapin/ribosome was 1/48, *i.e.* only 20 ng of lamjapin was sufficient to cleave rat liver ribosomal 28S RNA, producing the R-fragment after acidic-aniline treatment. The R-fragment did not appear when the ribosome was incubated with only lamjapin without acidic-aniline treatment. Therefore, it was concluded that the R-fragment was not caused by the RNase contamination in the purified sample, showing that lamjapin expressed RNA N-glycosidase activity, like other RIPs from the higher plants.

Multiple cleaving sites of lamjapin on 28S RNA in rat ribosome

Besides the predominant R-fragment produced by lamjapin from rat ribosomal 28S RNA, three additional RNA fragments larger than the R-fragment were also found when the treated ribosomal RNAs were subjected to 8 M urea-denatured polyacrylamide gel (4.5%) electrophoresis for a longer period of time (3 h). The R-fragment and three larger fragments did not appear when the ribosome was incubated with only lamjapin without acidic-aniline treatment. These data demonstrated conclusively that the emergence of R-fragment and the larger RNA fragments by lamjapin action was not an artifact caused by the nuclease contaminant. It was also shown that ratio of three fragments a, b, c and R-fragment was constant (0.2:0.3:0.1:10), independent on the amount of lamjapin employed. This indicated that lamjapin specially cleave the three sites of ribosomal 28S RNA, but the sensitivity of these sites to lamjapin was much lower than that of R-fragment size (A4324) in the sarcin/ricin domain of ribosome.

In order to confirm the multiple cleaving sites of deadenylation of lamjapin on the rat ribosomal RNA, the adenine base released from ribosomal RNA by lamjapin was quantitatively analyzed by the chloroacetaldehyde method. A time course study demonstrated that the release of adenine by lamjapin continued at a linear rate for at least 40 min when lamjapin and ribosome were presented at 1:1 molar ratio. The analytical result revealed that lamjapin could release more than one mole of adenine from each mole of ribosomes in 10 min and even reach to the level of 12 moles of adenines in 40

min. Therefore, we reached the conclusion that lamjapin acted at multiple cleaving sites, but mainly on A4324 site in ribosomal RNAs.

The synthetic oligoribonucleotide (35-mer) that mimics the sarcin/ricin domain (SRD RNA) of ribosomal 28S RNA was an useful substrate for studying the mechanism of action of RIP and analysis of the chemical structure of RNA recognized by RIP (**Figure 1B**). Cinnamomin A-chain deadenylated A20 of SRD RNA, the site corresponds to position A4324 of rat ribosomal 28S RNA, producing two fragments (20-mer and 15-mer). The SRD RNA was treated with lamjapin and acidic aniline also released the two fragments with the same size as that obtained by cinnamomin A-chain, whereas no fragment was released when the SRD RNA was treated only with lamjapin. Furthermore, the activity of lamjapin on the SRD RNA exhibited clearly the base specificity as showed by the fact that both the transitional and transversional mutants (A20 to G20, C20 or U20) were insensitive to lamjapin and no any fragment appeared from these mutants treated with lampapin and acidic-aniline. These results showed that only A20 but no other adenines of the mutants could be released by lamjapin from SRD RNA domain. The activity of lamjapin to the SRD RNA was absolutely dependent on the preservation of adenine at a proper site (A20). The base- and site-specificity of RNA N-glycosidase activity of lamjapin was the same as that of other RIPs from terrestrial higher plants, such as cinnammomin A-chain. However, the study on the distribution of RIP in lower plants is still scare.

Most of plant species examined belong to the class of the flowering plants, *i.e.*, Angiospermae. Up to today, no RIPs have yet been isolated from the class of gymnospermae and cryptogamia, the lowest species in the plant kingdom. The existence of lamjapin in marine kelp showed that RIP indeed exists outside of the flowering plants. Our group screened three marine algae and three freshwater algae in cryptogamai and the RNA N-glycosidase was only found in marine kelp (*L. japonic*a A). It was very likely that the distribution of RIP was sporadic rather than ubiquitous in the plant kingdom as proposed by Van Damme [2]. The existence of lamjapin in *L. japonica* A demonstrated that such sporadic distribution of RIP in plant kingdom ranged widely from the lowest plants to the higher plants [42].

Nonspecific deadenylation on sarcin/ricin domain of 28S rRNA catalyzed by gelonin under acidic conditions

It has been known that RIPs, including types I, II and III, specifically cleave the N-C glycosidic bond at A4324 in rat 28S ribosomal RNA, releasing an adenine and thus inhibiting protein synthesis under usual condition. Gelonin is a single-chain RIP (type I) that does the same thing on rat ribosome as the other type I do. Here, one of instance was reported that the specificity in substrate recognition was challenged by the fact that gelonin could remove adenine from other oligoribonucleotide substrates that differentiate from usual sarcin/ricin domain. However, the mechanism underlying the site specificity of gelonin to deadenylate various substrates is still unknown. Hereby, in our laboratory, the effect of pH values upon site specificity of the deadenylation activity of gelonin was examined using the synthetic oligoribonucleotide (mimic SRD RNA) that mimicked the ribosomal srcin/ricin domain. Interestedly, gelonin gradually acquired the ability to nonspecifically remove adenine that was not at position A20 of SRD RNA when pH values were changed from neutral to acidic conditions. Another two SRD RNA mutants, either with the conserved adenosine deleted or with the tetraloop converted one, showed very similar cleavage style to that of the wild-type SRD RNA, meaning that the mutant SRD RNA were seldom cleaved by gelonin under weakly acidic values. The facts underscored the key role of pH value in site specificity of recognition by gelonon. Furthermore, the RNA N-glycosidase activity of gelonin was also enhanced with decreasing the pH values. It should be noted that no obvious change was observed in the molecular conformation of gelonin at various pH values. Taking together, our data implied that the protonation of adenosines in SRD RNA was potentially an important factor responsible for the non-specific deadenylation by gelonin. The physiological significance of the non-specific deadenylation on sarcin/ricin domain RNA catalyzed by gelonin under acidic conditions is still remained to be clarified. But the acidic conditions may offer a powerful tool to decipher the topological structure of RNA [43].

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