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

Wen-Jun HE and Wang-Yi LIU[1]

State Key Laboratory of Molecular Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 320 Yue-Yang Road, Shanghai 200031, People's Republic of China

Cinnamomin is a type II ribosome-inactivating protein and its A-chain exhibits RNA N-glycosidase activity to remove an adenine in the conserved sarcin/ricin loop of the largest RNA in ribosome, arresting protein synthesis at the elongation step. In this report, deadenylation of both rRNA and supercoiled DNA by native and recombinant cinnamomin A-chain expressed in *Escherichia coli* was demonstrated. However, the mutants of cinnamomin A-chain devoid of N-terminal 52 or/and C-terminal 51 amino acid residues lost both the activity of RNA N-glycosidase and the ability to release adenines from supercoiled DNA. Additionally, supercoiled DNA could not be cleaved into nicked and linear forms by these mutants. These results indicate that both N- and C-terminal regions are essential for the cinnamomin A-chain to deadenylate rRNA and supercoiled DNA. It was suggested that phosphodiester bonds in the extensively deadenylated region of supercoiled DNA would become fragile and liable to be broken spontaneously owing to the existence of tension in the supercoiled DNA.

Key words: cinnamomin A-chain, deadenylation, deletion mutation, ribosome-inactivating protein, supercoiled DNA.

INTRODUCTION

Plant RIPs (ribosome-inactivating proteins) are a group of toxic proteins that inhibit protein synthesis irreversibly by removing an adenine from the highly conserved sarcin/ricin domain of the largest RNA in the eukaryotic ribosome [1]. Plant RIPs have been divided into three types [2]. Among them, type II RIP is composed of two polypeptide chains (A- and B-chains) joined together by a disulphide bond, and its A-chain displays activity of RNA N-glycosidase (rRNA N-glycosidase, EC 3.2.2.22).

Cinnamomin is a type II RIP purified in our laboratory from mature seeds of the camphor tree [3]. Its primary structure and gene structure have been determined [4,5]. The A-chain of this toxic protein is an RNA N-glycosidase composed of 271 amino acid residues. It was shown that invariant residues in the active site of known RIPs are located mainly in central region of the A-chain [6–8]. Moreover, based on modelling of the three-dimensional structure of the cinnamomin A-chain, the functional roles of five amino acid residues in its active site have been investigated by site-directed mutagenesis. Single mutation of the five conserved amino acid residues led to loss of its RNA N-glycosidase activity to differing extents [4]. However, the roles of N- and C-terminal regions of cinnamomin A-chain in its RNA N-glycosidase activity remain unknown.

Besides its RNA N-glycosidase activity to rRNA, the cinnamomin A-chain has been found to cleave supercoiled double-stranded DNA into nicked and linear forms *in vitro* [9]. Similar activity was also observed in other RIPs [10–14].

Whether this enzymic activity is a common feature of RIPs is still a matter of dispute [2,15]. Moreover, it was reported that RIPs could release adenines extensively from single-stranded DNA [16], so they have been classified as polynucleotide:adenosine glycosidases [17,18].

Here, the functional roles of N- and C-terminal regions of cinnamomin A-chain in its enzymic activities have been investigated by deletion mutagenesis. Interestingly, the N-terminal 52 and C-terminal 51 amino acid residues of cinnamomin A-chain were found to be essential for its deadenylation activity to both rRNA and supercoiled DNA. In addition, by comparing the activities of cinnamomin A-chain and its deletion mutants, it was confirmed that the cleavage of supercoiled DNA to nicked and linear forms is due to deadenylation by the cinnamomin A-chain instead of possible nuclease contamination. The mechanism of spontaneous cleavage of supercoiled DNA at the apurinic site produced by deadenylation is discussed briefly.

MATERIALS AND METHODS

Materials

Oligodeoxyribonucleotide primers for producing cDNA of deletion mutants by PCR were designed and synthesized by Shanghai Genecore Technology Company. Restriction endonucleases (*Nde*I and *Hin*dIII), T4 DNA ligase and *Taq* DNA polymerase were products of Promega. IPTG (isopropyl β-D-thiogalactoside) and X-gal were purchased from Sangon. DEAE–Sepharose and ECL® (enhanced chemiluminescence) reagent were obtained from Amersham Biosciences. Chloroacetaldehyde was purchased from Sigma. Cinnamomin A-chain was purified according to the method of Pu et al. [19].

Bacterial strains and vector

Escherichia coli B strain BL21 (DE3), F^- *ompT hsdS*$_B$ ($r_B^-m_B^-$) *pLysS Cm*^{r} *dcm gal* λ (DE3) lysogen was purchased from Promega. *E. coli* K12 strain TG1, *supE hsd5 thi* Δ (lac-proAB)/F$'$ [*traD36*, *proAB*+, *lac I*^{q} *lacD*Δ*M15*] was a gift of Dr Lixin Zhu (Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China). The cloning and expression vector pMFT7 (*Amp*^{r}) was kindly provided by Dr En-Duo Wang (also at the Institute of Biochemistry and Cell Biology).

Abbreviations used: RIP, ribosome-inactivating protein; ECL®, enhanced chemiluminescence; IPTG, isopropyl β-D-thiogalactoside.

[1] To whom correspondence should be addressed (e-mail wyliu@sibs.ac.cn).

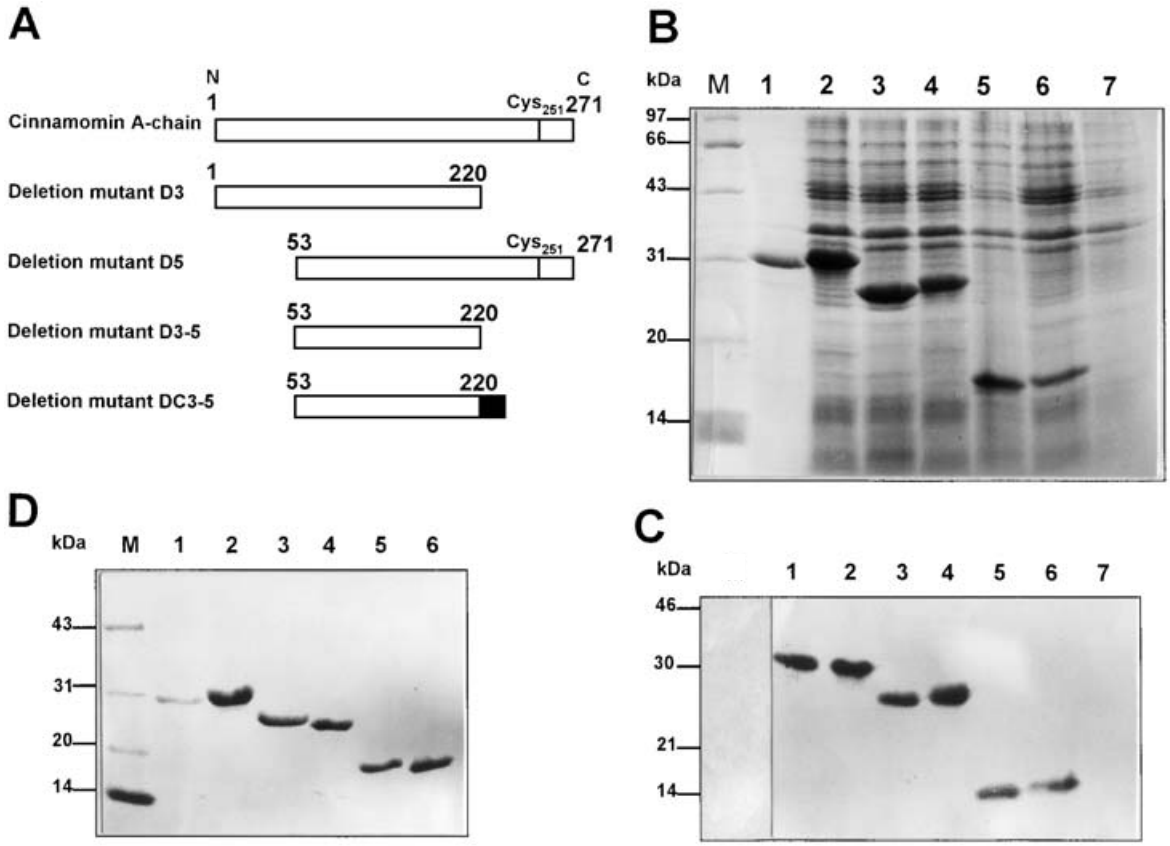

Figure 1 Recombinant expression of cinnamomin A-chain and its four deletion mutants in *E. coli*

(**A**) Schematic illustration of recombinant expression of cinnamomin A-chain and its four deletion mutants in *E. coli*. ■ represents ^{250}Ile–Cys–Arg^{252} added to the C-terminus of mutant DC3-5. (**B**) Expressed proteins on SDS/PAGE (15 % gel), stained with Coomassie Blue. Lane M, protein molecular-mass markers. (**C**) Western blot pattern. In both (**B**) and (**C**): lane 1, native cinnamomin A-chain; lanes 2–6, proteins in the lysate from *E. coli* BL21 (DE3) harbouring the modified pMFT7 expressing cinnamomin A-chain and its deletion mutants D3, D5, D3-5 and DC3-5 respectively; lane 7, proteins in lysates of *E. coli* BL21 (DE3) only harbouring pMFT7. (**D**) Identification of purity of recombinant cinnamomin A-chain and its deletion mutants purified from *E. coli*. The purified recombinant cinnamomin A-chain and its four deletion mutants were separated by SDS/PAGE (15 % gel) followed by Coomassie Blue staining. Lane M, as above; lane 1, 0.1 μg of native cinnamomin A-chain; lane 2, 1.0 μg of recombinant cinnamomin A-chain; lanes 3–6, 1.0 μg of deletion mutants D5, D3, D3-5 and DC3-5 respectively.

Construction of deletion mutants of cinnamomin A-chain

Plasmid pMFT7 containing the coding region of cinnamomin A-chain was used as a template for mutagenesis and DNA amplification by PCR. The upstream and downsteam primers to produce mutants D3, D5, D3-5 and DC3-5 were: D3 upstream, 5′-ATTCCAT**ATG**TACCAGACCGTGACC-3′, D3 downstream, 5′-GGCAAGCTT**CTATTA**CCCTTGGTTGGATTGCTG-3′; D5 upstream, 5′-ATTCCAT**ATG**GTG GAACTCTCAAATTGG-3′, D5 downstream, 5′-GGCAAGCTT**CTATTA**ATTGTCTGCATCTGTT-3′; D3-5 upstream, 5′-ATTCCAT**ATG**GTGGAACTCTCAAATTGG-3′, D3-5 downstream, 5′-GGCAAGCTT**CTATTA**CC CTTGGTTGGATTGCTG-3′; DC3-5 upstream, 5′-ATTCCAT**ATG**GTGGAACTCTCAAATTGG-3′, DC3-5 downstream, 5′-GGCAAGCTT**CTATTA**TCTGCAGATCCCTTGGTTGGATTGCTC-3′. In the downstream primers, a *Hin*dIII restriction site was introduced followed by two stop codons after the coding sequence. The upstream primer had an *Nde*I restriction site followed by an initiator ATG codon that annealed downstream to the T7 promoter in the expression vector. These stop and initiator codons are indicated in bold. As shown in Figure 1(A), in the deletion mutants D5 and D3, the N-terminal 52 and C-terminal 51 amino acid residues were deleted. In mutants D3-5 and DC3-5, both N-terminal 52 and C-terminal 51 amino acid residues were deleted. Additionally, at the C-terminal end of mutant DC3-5, three additional amino acid residues (Ile-Cys-Arg) were added.

Cycling protocols for amplification by PCR were as follows: denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min and extension at 72 °C for 1 min, for 30 cycles, followed by a final extension cycle at 72 °C for 10 min. The amplified fragment was isolated by gel electrophoresis and digested with *Nde*I and *Hin*dIII. Then these cDNA mutant fragments were cloned into the expression vector pMFT7. Initial transformation was done in the TG1 host strain and clones containing the insert were selected. Clones containing the desired mutations were transformed into the *E. coli* BL21 (DE3) host strain for expression.

Expression of cinnamomin A-chain and its deletion mutants in *E. coli*

E. coli BL21 (DE3) cells transformed with the appropriate plasmid were inoculated in Luria–Bertani medium containing 100 μg/ml ampicillin to a D_{600} of 0.1. The culture was grown with shaking (300 rev./min) at 37 °C until the D_{600} value reached 0.7–0.8. Expression was induced by adding IPTG to a final concentration of 1 mM, and the culture was shaken at 30 °C for 4–5 h. Then the cells were harvested by centrifugation (5000 ***g***, 4 °C) for 10 min.

Solubilization and purification of the expressed proteins from inclusion bodies

Wet *E. coli* cells (1 g) were suspended in 3 ml of buffer A (50 mM Tris/HCl, pH 8.0, 1 mM EDTA, 100 mM NaCl, 1 mM PMSF and 0.35 mg/ml lysozyme) and incubated on ice for 30 min. Then the lysate was treated with 4 mg of sodium deoxycholate and 20 μg of DNase I at 37 °C for 1 h. The inclusion bodies were sedimented by centrifugation (12000 ***g***, 4 °C) for 15 min. The pellet was washed twice with buffer A containing 1 % Triton X-100 and 10 mM EDTA, followed by centrifugation (20000 ***g***, 4 °C) for 30 min, and then solubilized in buffer B (10 mM Tris/HCl, pH 8.0, 1 mM EDTA, 1 mM 2-mercaptoethanol and 8 M urea). The insoluble materials were removed by centrifugation (20000 ***g***, 4 °C) for 30 min. The supernatant was diluted rapidly with 10 vol. of buffer C (10 mM Tris/HCl, pH 8.0, 1 mM EDTA and 1 mM 2-mercaptoethanol) and kept at 4 °C for 20–30 min. The expressed proteins that were released from the inclusion bodies were precipitated and recovered by centrifugation (12000 ***g***, 4 °C) for 10 min. Then the precipitated proteins were solubilized in buffer B at a concentration of 10 mg/ml. The solution was mixed with an equal volume of glycerol and dialysed extensively against 10 vol. of mixture of glycerol and buffer C (3:1, v/v) at 4 °C. The expressed protein in 75 % glycerol solution was rapidly diluted with 10 vol. of buffer C containing 0.1 M NaCl for renaturation. The diluted solutions were placed in an ice-bath for 10–20 min and then centrifuged (12000 ***g***, 4 °C) for 5 min to remove the precipitate. The supernatant containing the renatured protein was applied to a DEAE–Sepharose column pre-equilibrated with buffer C containing 0.1 M NaCl, and the expressed protein was eluted with a gradient of NaCl (0.1–0.5 M). Fractions that contained the recombinant cinnamomin A-chain or its deletion mutants were identified by SDS/PAGE and pooled. Protein concentration was determined by the method of Bradford [20].

Western blot analysis of the expressed cinnamomin A-chain and its deletion mutants

Preparation of antisera against intact cinnamomin A-chain, purification of polyclonal antibodies and Western blot analysis of the expressed proteins were carried out using standard procedures [21]. Blots were visualized with the ECL® detection system.

Protein synthesis in the cell-free system

Rabbit reticulocyte lysate was prepared as described in [22]. The assay for activity to inhibit protein synthesis in the lysate system was carried out according to the method of Xie et al. [4].

Assay for RNA N-glycosidase activity

Rat liver ribosomes were isolated by the method of Spedding [23] and the concentration of 80 S ribosomes was calculated from the molar absorption coefficient at 260 nm [23]. The assay for the RNA N-glycosdiase activity of the recombinant cinnamomin A-chain and its mutants was carried according to the method of Endo et al. [24]. rRNAs were separated by 3.5 % denaturing PAGE containing 8 M urea. RNA bands were stained with Methylene Blue.

Qualitative and quantitative determination of the bases released from supercoiled DNA by cinnamomin A-chain

Plasmid pMFT7 DNA (2 μg) was incubated with 50 ng of native or recombinant cinnamomin A-chain or its deletion mutants in 50 μl of buffer D (50 mM Tris/HCl, pH 7.5, 1 mM dithiothreitol, 50 mM KCl and 10 mM $MgCl_2$) at 37 °C for 4 h. After plasmid DNA was removed by precipitation with 2 vol. of ethanol, the ethanol supernatant was collected and evaporated by spinning. The sample was dissolved in 3 μl of acetic acid solution (20 mM), then applied to a polyamide thin-layer plate and developed in solution (ethyl acetate/acetic acid/methanol/water, 20:2:1:1, by vol.) [25]. The positions of the bases were located under UV light (254 nm). Adenine was quantified by the method of chloroacetaldehyde fluorescence labelling as described by Zamboni et al. [26].

Determination of cleavage of supercoiled DNA [12]

The plasmid pMFT7 DNA (0.5 μg) was incubated with various concentrations of cinnamomin A-chain or its deletion mutants in 20 μl of buffer E (50 mM Tris/HCl, pH 7.5, 50 mM KCl and 10 mM $MgCl_2$) at 37 °C for 1 h. The linear form of pMFT7 DNA was prepared by incubation of the supercoiled form with *Hin*dIII, phenol/choroform extraction and ethanol precipitation. The DNA samples were analysed by 1 % agarose gel electrophoresis and visualized by ethidium bromide staining.

Aniline reaction [14]

After supercoiled pMFT7 DNA (0.5 μg) was treated with native cinnamomin A-chain or recombinant cinnamomin A-chain, the resultant DNA sample was treated with the freshly prepared solution (aniline/acetic acid/water, 2:2:7, by vol.), then kept in an ice bath for 10 min, precipitated with ethanol and analysed by 1 % agarose gel electrophoresis and visualized by ethidium bromide staining.

Deadenylation and cleavage of supercoiled DNA by chemicals

The supercoiled pMFT7 DNA (0.5 μg) was incubated with 0.5 M NaOH and 1 mM EDTA at 90 °C for 5 min or with 1 % diethyl pyrocarbonate at 90 °C for 10 min. After precipitation by absolute ethanol, the deadenylated DNA was treated with 1.0 M piperidine at 90 °C for 10 min or 1.0 M freshly prepared acidic aniline (pH 4.5) at 60 °C for 20 min according to the methods described in [27,28]. The DNA sample was frozen, lyophilized and analysed by 1 % agarose gel electrophoresis with ethidium bromide staining.

RESULTS

High expression and renaturation of recombinant cinnamomin A-chain and its four deletion mutants

Cinnamomin is a type II RIP and its A-chain is an RNA N-glycosidase composed of 271 amino acid residues. Based on the simulated three-dimensional modelling of cinnamomin A-chain, five conserved amino acid residues (Tyr-75, Tyr-115, Glu-167, Arg-170 and Trp-201) were supposed to constitute the active site and to play crucial roles in substrate binding and catalysis [4]. In addition, it was reported that certain amino acid residues outside the active-site cleft of the ricin A-chain were involved in the enzymic activity [29]. Here, in order to study the functional roles of N- and C-terminal regions of cinnamomin A-chain and whether the central region that contained the active site was sufficient to function as an RNA N-glycosidase, four deletion mutants (D3, D5, D3-5 and DC3-5) were constructed (Figure 1A) and highly expressed in *E. coli* cells (Figure 1B). These proteins were further confirmed by Western-blot analysis (Figure 1C). To obtain highly purified and renatured proteins, the inclusion bodies

were first separated from cellular organelles and other soluble proteins by super-centrifugation, then the expressed proteins were obtained by two steps of solublization with 8 M urea of the purified inclusion bodies and rapid-dilution renaturation. In this way, the purity of the recombinant cinnamomin A-chain and the deletion mutants was 90%. The expressed proteins were further purified by DEAE–Sepharose column chromatography and shown to be homogenous by SDS/PAGE (Figure 1D).

Involvement of N- and C-terminal regions in enzymic activities of cinnamomin A-chain

RNA N-glycosidase activity

The ability of the recombinant cinnamomin A-chain and its four deletion mutants to inhibit protein synthesis *in vitro* was assayed and is shown in Figure 2(A). The IC_{50} of the recombinant A-chain was approx. 100 nM (3 μg/ml) and that of the native A-chain was 50 nM (1.5 μg/ml), whereas the four deletion mutants showed only weak inhibitory activity. The RNA N-glycosidase activities of these expressed proteins to rat ribosome were also determined. It was known that the rRNA deadenylated by RNA N-glycosidase could release a diagnostic RNA fragment (R-fragment) after acidic aniline treatment. Here, 10 ng of recombinant A-chain could produce the specific R-fragment similar to that produced by 10 ng of native A-chain. However, 100 ng of each of the four deletion mutants did not show such an activity (Figure 2B). The four deletion mutants devoid of N- or/and C-terminal regions had no RNA N-glycosidase activity, indicating that these deleted regions were essential for deadenylation activity of cinnamomin A-chain to rRNA, and the central region containing the five conserved amino acid residues in the active site alone could not function as an RNA N-glycosidase.

Cleavage of supercoiled double-stranded circular DNA

Cinnamomin A-chain was reported to cleave the supercoiled double-stranded DNA into nicked and linear forms [9]. However, it was argued later that this enzymic activity was caused by the nuclease contamination in the preparation of RIPs [2,15]. In order to exclude the possibility of nuclease contamination, the experiments were carried out as follows: supercoiled pMFT7 DNA was incubated with the recombinant cinnamomin A-chain at increasing concentration. The supercoiled pMFT7 DNA was gradually converted into nicked and linear forms (Figure 3A), similar to the action of native cinnamomin A-chain (Figure 3B). However, none of the four mutants could cleave the supercoiled DNA (Figure 3C). Moreover, neither recombinant cinnamomin A-chain nor deletion mutants could degrade the linear DNA into small fragments (Figure 3D). These results strongly excluded the possibility that cleavage of supercoiled DNA was due to nuclease contamination. All the above results indicated that cleavage of supercoiled double-stranded DNA was an intrinsic property of cinnamomin A-chain. The N- and C-terminal regions that were required for deadenylation of rRNA were also necessary for deadenylation of the supercoiled double-stranded circular DNA.

Multiple-site deadenylation of supercoiled double-stranded circular DNA by cinnamomin A-chain

It was reported that some RIPs, such as pokeweed antiviral protein and gelonin, could deadenylate single-stranded DNA [16]. Here, the supercoiled double-stranded DNA was used as a substrate to test whether cinnamomin A-chain could also deadenylate this kind

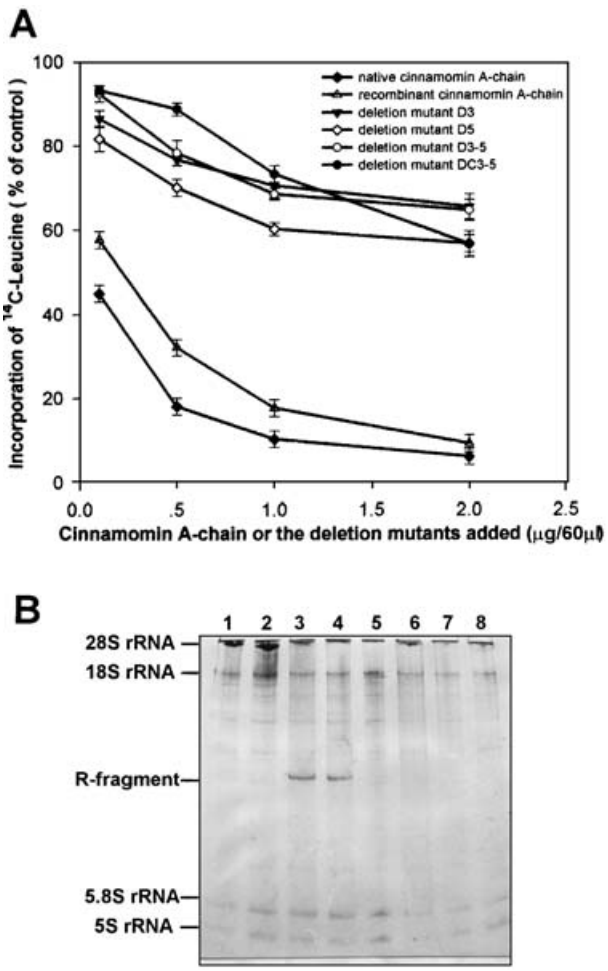

Figure 2 Assay for activity of native cinnamomin A-chain, recombinant cinnamomin A-chain and deletion mutants towards protein synthesis and rRNA

(**A**) Inhibition of protein synthesis by native cinnamomin A-chain, recombinant cinnamomin A-chain and deletion mutants in rabbit reticulocyte lysate. The protein synthesis system contained the indicated amounts of native cinnamomin A-chain or the renatured expression products in a final volume of 60 μl. After incubation, acid-insoluble radioactivity was measured as described in the Materials and methods section. The first concentration of various proteins examined is 0.1 μg/60 μl. In contrast to the negative control (no inhibitors, 100%), low concentrations of the deletion mutants (D3, D5, D3-5 and DC3-5) showed a weak inhibition of protein synthesis; therefore the initial incorporation of [^{14}C]leucine in the deletion mutants is below 100%. (**B**) Identification of R-fragment for assaying RNA N-glycosidase of recombinant cinnamomin A-chain and deletion mutants. Rat ribosome was incubated with native cinnamomin A-chain or various expression products, rRNAs were extracted and treated with acidic aniline as described in the Materials and methods section. Identification was carried out on 8 M urea-denatured PAGE (3.5% gel). Lane 1, ribosome without RIP treatment; lane 2, ribosome treated by native cinnamomin A-chain without acidic aniline treatment; lanes 3 and 4, ribosome treated by native cinnamomin A-chain and recombinant cinnamomin A-chain respectively, then with acidic aniline; lanes 5–8, ribosome treated with one of the four deletion mutants D5, D3, D3-5 and DC3-5, respectively, followed by acidic aniline. RNA bands were stained with Methylene Blue.

of DNA. As shown in Figure 4(A), among four bases only adenine could be released from supercoiled pMFT7 DNA after treatment with native or recombinant cinnamomin A-chain. Meanwhile, the deletion mutants could not deadenylate supercoiled DNA. Moreover, quantitative fluorescence determination of the adenines released from supercoiled DNA showed that cinnamomin A-chain could remove adenine residues at multiple sites on one DNA molecule, since the ratio of released adenines to DNA exceeded 50:1 (Table 1). This conclusion was also confirmed

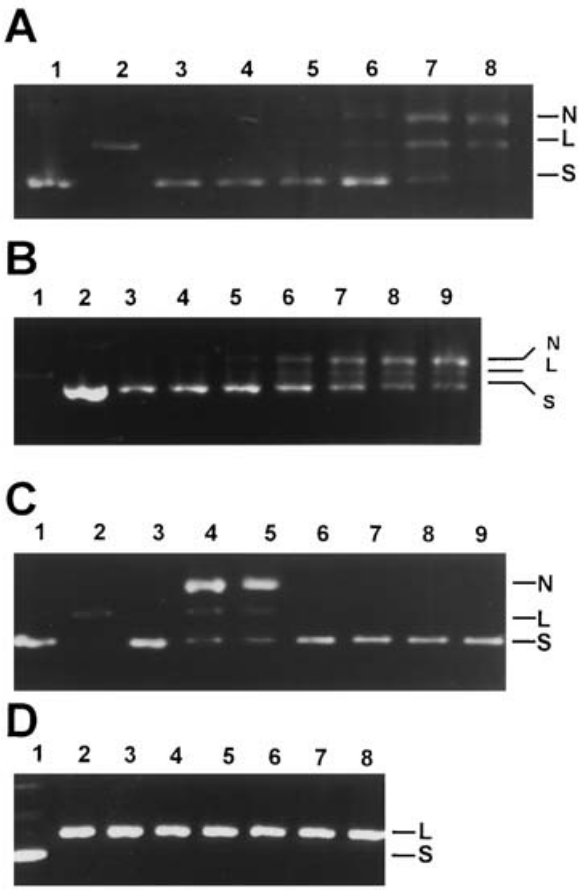

Figure 3 Deadenylation of supercoiled DNA by native cinnamomin A-chain and various expressed proteins

(**A**) Lane 1, supercoiled pMFT7 DNA; lane 2; linear form of pMFT7 DNA; lanes 3–8, 0.5 μg of supercoiled pMFT7 DNA incubated with 0, 0.05, 0.1, 0.5, 1.0 and 2.0 μg of recombinant cinnamomin A-chain respectively. (**B**) Lane 1, linear form of pMFT7 DNA; lane 2, supercoiled pMFT7 DNA; lanes 3–9, 0.5 μg of supercoiled pMFT7 DNA incubated with 0, 0.05, 0.1, 0.2, 0.4, 0.6 and 0.8 μg of native cinnamomin A-chain respectively. (**C**) Lane 1, supercoiled pMFT7 DNA; lane 2, linear pMFT7 DNA; lane 3, 0.5 μg of supercoiled pMFT7 DNA incubated without any protein; lanes 4 and 5, 0.5 μg of supercoiled pMFT7 DNA incubated with 1.0 μg of native or recombinant cinnamomin A-chain; lanes 6–9, 0.5 μg of supercoiled pMFT7 DNA incubated with 10.0 μg of deletion mutants D5, D3, D3-5 and DC3-5 respectively. (**D**) Effect of native cinnamomin A-chain, recombinant cinnamomin A-chain and deletion mutants on linear pMFT7 DNA. Lane 1, supercoiled pMFT7 DNA; lane 2, 0.5 μg of linear pMFT7 DNA incubated without protein; lanes 3–8, 0.5 μg of linear pMFT7 DNA incubated sequentially with 10.0 μg of native cinnamomin A-chain, recombinant cinnamomin A-chain or four deletion mutants D5, D3, D3-5 and DC3-5 respectively. N, nicked circular DNA; L, linear DNA; S, supercoiled DNA.

by the fact that only the nicked and linear forms of DNA, but not supercoiled DNA, could be degraded into small fragments after acidic aniline treatment, as shown in Figure 4(B). Acidic aniline treatment in the absence of cinnamomin A-chain did not show any significant degradation of the supercoiled DNA. When the supercoiled DNA was incubated with the deletion mutants, no obvious change could be observed after acidic aniline treatment (results not shown). The nicked and linear forms of DNA that were produced from the supercoiled DNA by cinnamomin A-chain were hydrolysed into small fragments which ran out of the gel after acidic aniline treatment, suggesting that cinnamomin A-chain deadenylated DNA at multiple sites that were susceptible to acidic aniline cleavage. The deletion mutants could not deadenylate both rRNA and supercoiled DNA. Supporting our above assumption, the supercoiled DNA deadenylated by chemicals could be broken

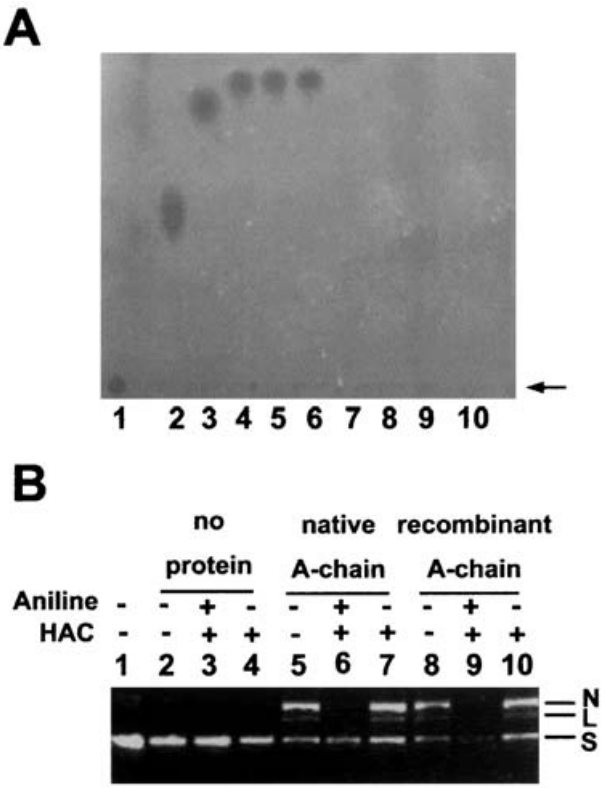

Figure 4 Multiple-site deadenylation of supercoiled DNA by cinnamomin A-chain

(**A**) Polyamide TLC for identifying bases released from supercoiled pMFT7 DNA by native cinnamomin A-chain, recombinant cinnamomin A-chain and deletion mutants. The supercoiled DNA was incubated with native or recombinant cinnamomin A-chain as well as deletion mutants. The released bases were recovered and identified as described in the Materials and methods section. Lanes 1–4, four bases: guanine (staying at origin point), cytosine, thymine and adenine respectively; lanes 5 and 6, adenines released from supercoiled pMFT7 DNA treated with native and recombinant cinnamomin A-chain respectively; lanes 7–10, the supernatants from supercoiled pMFT7 DNA treated with four deletion mutants D5, D3, D3-5 and DC3-5 respectively. The arrow indicates the origin points of the various samples examined. (**B**) Susceptibility of the deadenylated pMFT7 DNA to acetic aniline cleavage. The supercoiled DNA (0.5 μg) was incubated with native or recombinant cinnamomin A-chain, and then treated with acidic aniline as described in the Materials and methods section. HAC, acetic acid; other abbreviations are as defined for Figure 3.

Table 1 Adenine released from supercoiled pMFT7 DNA treated with cinnamomin A-chain

The amount of adenine released was measured as described in the Materials and methods. Pure adenine was used for generating the standard curve. In each treatment, 1 pmol of pMFT7 was used.

Treatment	Adenine released (pmol)
pMFT7 + reaction buffer only	0
pMFT7 + native cinnamomin A-chain	71 ± 4
pMFT7 + recombinant cinnamomin A-chain	58 ± 8

into nicked and linear forms that could also be degraded into small fragments by piperidine or aniline (Figure 5).

DISCUSSION

The results of this study show that both N-terminal 52 and C-terminal 51 amino acid residues of the cinnamomin A-chain

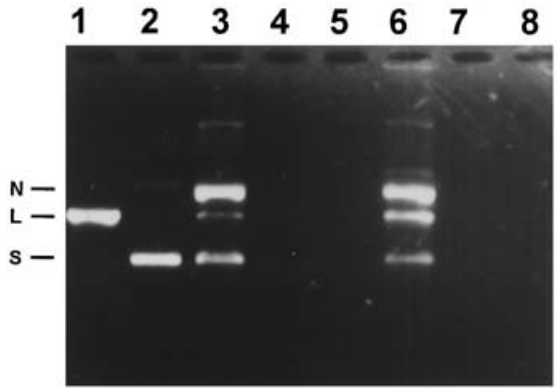

Figure 5 Deadenylation and cleavage of supercoiled pMFT7 DNA by treatment with chemicals

The supercoiled DNA was deadenylated by alkali or diethyl pyrocarbonate, and then cleaved at an apurinic site by piperidine or acidic aniline as described in the Materials and methods section. Lane 1, linear form of pMFT7 DNA; lane 2, supercoiled pMFT7 DNA; lane 3, supercoiled pMFT7 DNA treated with alkali; lanes 4 and 5, supercoiled pMFT7 DNA treated with alkali and cleaved by piperidine or acidic aniline respectively; lane 6, supercoiled pMFT7 DNA treated with diethyl pyrocarbonate; lanes 7 and 8, supercoiled pMFT7 DNA treated with diethyl pyrocarbonate and then cleaved by piperidine or acidic aniline respectively. Abbreviations are as defined for Figure 3.

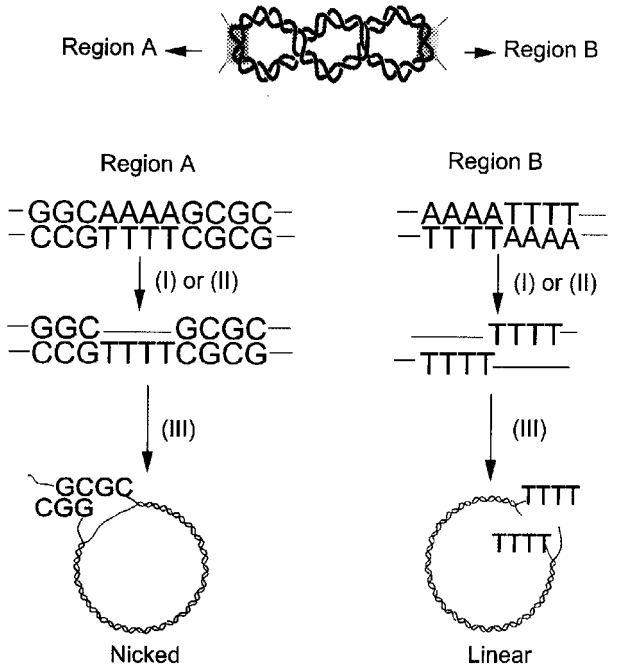

Figure 6 Proposed pathway for production of nicked and linear forms from supercoiled double-stranded DNA deadenylated by cinnamomin A-chain by chemical means

There are many deadenylation sites that are randomly located in the AT-rich regions of one supercoiled DNA molecule. The sequences of regions A and B indicate two possible cases. (I) Deadenylation by cinnamomin A-chain; (II) deadenylation by treatment with chemicals; (III) spontaneous cleavage at any one apurinic site by tension in the supercoiled DNA molecule.

are essential for its activity to deadenylate rRNA and supercoiled DNA. Also, several lines of evidences confirmed that cleavage of supercoiled DNA into nicked and linear forms was caused by deadenylation with cinnamomin A-chain instead of contaminating nuclease.

Native cinnamomin A-chain is a glycopeptide with 0.3 % sugar [3]. The recombinant A-chain expressed in *E. coli* devoid of glycan chain still retained the ability to deadenylate rRNA and supercoiled DNA, demonstrating that the sugar chains in the native A-chain did not play an important role in deadenylation activity.

Our previous results showed that the five conserved amino acid residues (Tyr-75, Tyr-115, Glu-167, Arg-170 and Trp-201) in cinnamomin A-chain constituted the active site and played crucial roles in substrate binding and catalysis [4]. Here, deletion of about 50 amino acid residues at N- or/and C-terminal regions could result in obvious losses of deadenylation activity of cinnamomin A-chain to rRNA and supercoiled DNA, indicating that the amino acid residues in the two terminal regions far from the active site cleft are also essential for alignment of the key residues for catalysis and interaction with substrate. According to simulated three-dimensional structure of cinnamomin A-chain, its N-terminal region is an α-helix structure that is composed of around 50 amino acid residues. This α-helix structure is juxtaposed closely with the central region in which a cluster of α-helixes forms a globular module where the key amino acid residues form the active site. On the other hand, the C-terminal region consisted of a random coil composed of about 50 amino acid residues [4]. Deletion of the N-terminal α-helix or the C-terminal random coil would perturb the spatial structure of the central region and correct the conformation of the active-site cleft, leading to inactivation of the cinnamomin A-chain. Consistent with this result, ricin A-chain could not tolerate the deletion of its C-terminal 20 amino acid residues, although it was reported that 222 (83 %) of the 267 amino acid residues in ricin A-chain could be deleted without losing its ability to catalyse hydrolysis of a single N–C glycosidic bond in 7000 nucleotides [30].

The substrate specificity of RIPs and their activity on supercoiled DNA is still a matter of debate. The activity of RIPs to cleave and linearize superoiled DNA *in vitro* was argued as a result of nuclease contamination [2,15]. In our study, the recombinant cinnamomin A-chain and its four deletion mutants were prepared and purified under the same conditions. Like natural cinnamomin A-chain, the recombinant A-chain could specifically remove adenines from rRNA and supercoiled DNA. Furthermore, the deadenylated supercoiled DNA was converted into nicked and linear forms. In contrast, the deletion mutants devoid of the N-terminal 52 and/or C-terminal 51 amino acid residues lost these enzymic characteristics, as mentioned above, demonstrating that cleavage of supercoiled DNA was caused by deadenylation with the cinnamomin A-chain. In addition, the finding that the linearized DNA could not be degraded into small fragments by cinnamomin A-chain, even in large amounts, excluded possible nuclease contamination.

Regarding molecular mechanism of cleavage of supercoiled DNA to nicked and linear forms by deadenylation with the cinnamomin A-chain without aniline treatment, it is proposed to be due to the spontaneous breakage of phosphodiester bonds after removing adenines, since the DNA molecule in the supercoiled state is inherently less stable than uncoiled DNA. The stress present within supercoiled DNA sometimes leads to regions rich in AT base pairs coming apart [31], and thus being accessible to the action of the 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. The cleavage at one apurinic site in one strand of the deadenylated supercoiled DNA would produce the nicked form. In another case, the linear form could emerge when cleavage occurred at adjacent apurinic sites in AT-rich regions of both stands in the deadenylated supercoiled DNA, converting supercoiled DNA into the unstrained, energetically more favourable relaxed state (Figure 6). Moreover, it was

reported in the literature that cleavage at apurinic sites in DNA molecule could be catalysed by amino groups of chemicals, e.g. Tris [32]. In our reaction system, existence of Tris would accelerate cleavage of phosphodiester bonds at apurinic sites and produce nicked and linear DNA. In conclusion, cleavage of the supercoiled DNA into the nicked and linear form occurs spontaneously after adenine residues are removed from DNA molecules by the enzyme; thus the cleavage is not due to the direct action of RNA N-glycosidase, but rather is a consequence of its action.

The lower activity of the recombinant cinnamomin A-chain to inhibit protein sythesis and to deadenylate supercoiled DNA could be explained by the purification process. During purification through the many steps of column chromatography the A-chain is easily denatured in comparison with that in a mixture of A- and B-chains or the intact cinnamomin molecule [33].

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