Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 306 (2003) 39–45
BBRC
www.elsevier.com/locate/ybbrc

Refolding of partially thermo-unfolded cinnamomin A-chain mediated by B-chain

Bao-Zhong Wang and Wang-Yi Liu*

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, China

Received 17 April 2003

Abstract

The pure cinnamomin A-chain is unstable compared to that in the mixture of A- and B-chain or in intact cinnamomin molecule either being stored at 4 °C or being heated. When being heated at 45 °C for 20 min, the A-chain generates partially unfolded intermediate and loses its tertiary structure as monitored by circular dichroism (CD) and tryptophan fluorescence, thus resulting in the inactivity of its RNA *N*-glycosidase albeit it retains most of its secondary structures. This partially unfolded intermediate is sensitive to protease, exhibiting property of a molten globule. The changes in conformation and activity are irreversible upon cooling. The partially unfolded intermediate can fully restore its RNA *N*-glycosidase activity in the presence of cinnamomin B-chain. The phenomenon, that the cinnamomin B-chain mediates the refolding of partially unfolded A-chain, probably plays 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 occurs in most denatured proteins in cell.

Keywords: Cinnamomin; Circular dichroism; Refolding and unfolding; RNA *N*-glycosidase activity; Ribosome-inactivating protein; Tryptophan fluorescence

Plant ribosome-inactivating protein (RIP) is RNA *N*-glycosidase. On the basis of structural diversity, RIPs have been classified into three types [1,2]. Type II RIP is a heterodimer that consists of an A-chain and a B-chain linked together by a disulfide bond [3]. The A-chain is catalytic unit that exhibits RNA *N*-glycosidase activity by removing a specific adenine from an exposed loop (ricin/sarcin loop) of the largest RNA in ribosome [4]. After this modification, the phosphodiester bonds on either side of the depurination site become susceptible to acidic aniline hydrolysis, releasing a small fragment (R-fragment) from the 3′-end of the largest ribosomal RNA, which is diagnostic indicator of the RIP activity. The B-chain has lectin properties, binding to specific receptors on the surface of eukaryotic cells and mediating the active A-chain into the cytosol [5,6]. Before the A-chain is translocated into the cytosol, the disulfide bond between the two chains is reduced and dissociates in the endoplasmic reticulum (ER) [7].

Type II RIP has potential prospects in clinical and agricultural applications [8,9]. Since the substrate of the RNA *N*-glycosidase is located in ribosome inside the cell, the problem of how its A-chain enters into cells and transports to access to its substrate is especially important in application. Furthermore, the stability of A-chain during transport in cell must be considered. Recently, the mechanisms of receptor-mediated endocytosis and intracellular transport have been investigated in the case of ricin [10]. Plenty of experimental data suggest that the ricin B-chain plays a role in cytotoxicity beyond the initial binding of cell surface receptors [11]. By studying a series of ricin point mutants, it has been known that the ricin B-chain galactose binding activity is important not only in cell surface binding but also intracellularly for ricin cytotoxicity. The lectin binding sites interact with intracellular galactose-containing receptors and that this alters the

* Corresponding author. Fax: +86-21-54921011.
E-mail address: wyliu@sibs.ac.cn (W.-Y. Liu).

0006-291X/03/$ - see front matter © 2003 Elsevier Science (USA). All rights reserved.
doi:10.1016/S0006-291X(03)00915-X

subcellular routing of the toxin to facilitate toxicity [12]. It is supposed that the ricin B-chain protects the ricin A-chain from degradation during transport from the cell surface to the place where the A-chain is translocated into the cytosol [13].

Cinnamomin is a new type II RIP purified in our laboratory. It contains two chains (A- and B-chains). The A-chain is an RNA *N*-glycosidase and the B-chain is a lectin [14,15]. Although many aspects of cinnamomin are extensively investigated [14–19], the thermal stability of the A-chain remains to be studied. This paper investigates the thermo-unfolding of the cinnamomin A-chain and the restoration of RNA *N*-glycosidase activity of partially unfolded A-chain in the presence of cinnamomin B-chain. The phenomenon that B-chain can mediate the refolding of unfolded A-chain supposes that the B-chain can keep the stability of A-chain and refold the unfolded A-chain to avoid the destiny of proteolysis in intracellular transport.

Materials and methods

Materials. Cinnamomin was isolated from the mature seeds of camphor tree (*Cinnamomun camphora*) as described previously [15]. Sepharose 4B and CM-Sephadex C-50 were purchased from Pharmacia. AT-Sepharose 4B was prepared according to the method of Li et al. [15]. DEAE-cellulose and CM-cellulose were products of Whatman. All other chemicals were of analytic grade.

Preparation of cinnamomin A-chain. Cinnamomin (1 mg/ml) was reduced with 5% β-mercaptoethanol in buffer A (20 mM sodium phosphate, pH 8.0) at 37 °C for 3 h. Under this condition, most of cinnnamomin B-chain was denatured. After a brief centrifugation, the supernatant was dialyzed against buffer B (20 mM sodium phosphate, pH 8.0, 0.15 M NaCl, and 1 mM DTT) for three changes, then the resulting dialyzate was loaded onto an AT-Sepharose 4B column pre-equilibrated with buffer B. The pass-through fraction was collected and dialyzed against buffer C (20 mM sodium phosphate, pH 6.0, and 1 mM DTT) exhaustively. Then the dialyzate was loaded onto a CM-cellulose column pre-equilibrated with buffer C. After washing the column adequately with buffer C, the proteins were eluted with a linear gradient of NaCl (0–0.15 M) in buffer C and two peaks appeared. The pure A-chain in the second peak was collected. After dialysis against distilled water and lyophilization, the pure A-chain was stored at −20 °C.

Preparation of cinnamomin B-chain. Since cinnamomin B-chain was easy to be denatured, a different condition was employed in the breakage of disulfide bond of cinnamomin in the preparation of cinnamomin B-chain compared to that of A-chain. Cinnamomin (1 mg/ml) was reduced with 50 mM DTT in buffer D (10 mM Tris/HCl, pH 8.5, and 0.5 M galactose) at 25 °C overnight. Then, the mixture of A- and B-chain obtained was loaded onto a DEAE-cellulose column pre-equilibrated with buffer D. After washing the column with three column-volumes of buffer D, the B-chain was eluted with buffer E (10 mM Tris/HCl, pH 8.5, 0.5 M NaCl, 50 mM galactose, and 1 mM DTT). The eluate was dialyzed against buffer F (20 mM sodium phosphate, pH 6.0, and 1 mM DTT), and then loaded onto a CM-Sephadex C-50 column pre-equilibrated with buffer F. The B-chain was eluted with a linear gradient of NaCl (0–0.4 M) in buffer F. The fraction of the first peak contained the pure cinnamomin B-chain. The pure B-chain was condensed in a centrifugal concentrator and stored at 4 °C for not more than 4 days. Before being used in further experiment, the pure B-chain needed a brief centrifugation to remove the denatured protein.

Western blotting analysis. Intact cinnamomin, purified A- or B-chain was resolved by SDS–PAGE and transferred to PVDF film (Bio-Rad). Western blots were visualized with enhanced chemiluminescence (ECL) and exposed to Kodak X-Omat diagnostic film immediately.

Circular dichroism. Circular dichroism (CD) spectra were recorded on a Jasco J-715 spectropolarimeter attached to a Jasco PTE-348W Peltier heating system. An aqueous of d-10-camphor sulfonic acid solution (1 mg/ml) was used for calibration. The molar ellipticity was calculated for the mean residue molecular weight in cinnamomin A-chain. In far UV CD spectra measurements, 0.1 mg/ml of cinnamomin A-chain in buffer G (20 mM sodium phosphate buffer, pH 7.2) and 0.2 cm path length cuvette were used. The recorded range was from 250 to 190 nm. In near UV CD spectra measurements, 1 mg/ml of cinnamomin A-chain in the buffer G and 1 cm path length cuvette were employed. The recorded range was from 320 to 250 nm. All CD spectra were smoothed using the Jasco analysis software.

Tryptophan fluorescence. Emission spectra of cinnamomin A-chain (0.02 mg/ml) in buffer G were recorded in a Perkin–Elmer LS-50B spectrofluorimeter attached to a LKB Bromma thermostatic circulator. The path length was 1 cm and an excitation wavelength was 295 nm. Both excitation and emission bandwidths were set at 5 nm. In the presence of 1-anilinonaphthalene-8-sulfonate (ANS), the excitation wavelength was 380 nm.

Assay for RNA N-glycosidase and protein synthesis in cell-free system. The assay for activity of RNA *N*-glycosidase of intact cinnamomin, A- or B-chain, and treated A-chain was carried out according to the method as described previously [15]. Inhibition of protein synthesis in a rabbit reticulocyte lysate system by RIPs was assayed by incorporation of L-[^{3}H]leucine according to the method of Li et al. [15]. For determining the changes of IC_{50} in the time course, the samples in buffer G contained 1 mM PMFS and 0.02% NaN_3.

Protease digestion. Cinnamomin A-chain (0.02 mg/ml) in buffer G was heated at different temperatures as indicated for 20 min and cooled in ice-bath for 10 min. Then thermolysin was added (final concentration was 0.004 mg/ml containing 5 mM $CaCl_2$) to the cinnamomin A-chain solution and incubated at room temperature for 30 min. The reaction was stopped by adding 10 mM EDTA. The digested products were analyzed by SDS–PAGE.

Results and discussion

Instability of the purified cinnamomin A-chain

The A-chain is the catalytic unit of cinnamomin in inactivating ribosomes as described previously, but the A-chain in the purified state is unstable compared to that in the mixture of A- and B-chain or in intact cinnamomin. The data in Table 1 indicated that the IC_{50} of cinnamomin A-chain to protein synthesis in cell-free system increased more speedily than that of mixture of A- and B-chain or intact cinnamomin did in the time course at 4 °C. After 30 days, the IC_{50} of pure A-chain increased from 0.68 to 11.2 nM (15.5-folds). Comparatively, the IC_{50} of the mixture of A- and B-chain and intact cinnamomin increased from 1.26 to 4.96 nM (2.93-folds) and 48.2 to 68.0 nM (1.42-folds), respectively. The data in Table 2 showed that the purified cinnamomin A-chain was easy to denature under thermo-treatment also. When it was heated at 45 °C for 20 min, the A-chain lost most of its catalytic activity and the IC_{50} increased to 484 nM (713-folds). Under the

Table 1
IC_{50} of inhibiting protein synthesis by cinnamomin A-chain stored for various times

Time (day)	0	5	10	15	20	25	30
Cinnamomin A-chain (nM)	0.68	0.87	1.36	2.90	3.74	6.41	11.20
In mixture of cinnamomin A- and B-chain (nM)[a]	1.26	1.54	1.90	2.23	3.01	3.74	4.96
In intact cinnamomin (nM)	48.2	48.5	50.0	52.7	55.6	61.9	68.0

All samples were stored at 4 °C for different times as indicated. The data are the mean of three independent experiments.
[a] The values were calculated according to the concentration of cinnamomin A-chain.

Table 2
Effect of temperature on the IC_{50} of inhibiting protein synthesis by cinnamomin A-chain

Temperature (°C)	0	25	37	45	55	65	75
Cinnamomin A-chain (nM)	0.68	0.69	0.79	484	>5000	—	—
In mixture of cinnamomin A- and B-chain (nM)[a]	1.26	1.26	1.30	41.7	620	>5000	—
In intact cinnamomin (nM)	48.2	48.4	48.9	50.0	93.6	364	>2800

Samples were heated at different temperatures as indicated for 20 min. The data were the means of three independent experiments.
[a] The values were calculated according to the concentration of cinnamomin A-chain.

same condition, the IC_{50} of the mixture of A- and B-chain was 41.7 nM (33.6-folds) and that of the intact cinnamomin was 50.0 nM (1.04 folds). However, if the treatment was performed at 55 °C, the purified A-chain showed almost no inhibition activity to cell-free system, while the IC_{50} of the mixture of A- and B-chain was 620 nM (492-folds of control) and that of the intact cinnamomin was 93.6 nM (1.94-folds of control). These results demonstrated that the presence of cinnamomin B-chain could stabilize the A-chain either the B-chain was dissociated or in the intact molecule of cinnamomin.

Effect of temperature on the conformational changes of cinnamomin A-chain

In order to understand what happened in the above processes, the structural changes of cinnamomin A-chain at different temperatures were monitored by CD spectroscopy in both near and far ultraviolet regions. As shown in Fig. 1A, cinnamomin A-chain retained its tertiary structure at 0 and 37 °C. If the protein was heated at 45 °C for 20 min, a significant change in its tertiary structure took place as revealed by near UV CD spectra, but still retained most secondary structure compared to that of native protein (Fig. 1B). The secondary structure was disrupted at 55, 65, or 75 °C (Fig. 1B). Elaborate examination of Fig. 1 revealed that the tertiary structure of cinnamomin A-chain lost prior to its secondary structure and the thermal unfolding of cinnamomin A-chain was not a two-state process but went through partially unfolded intermediates, for example, the state formed by heating at 45 °C for 20 min. In this state, cinnamomin A-chain kept most of its secondary structures but lost its tertiary structure as revealed by CD spectra. If the thermo-treatments were performed at 55, 65, or 75 °C, cinnamomin A-chain was substantially unfolded since the ellipticity did not further decrease.

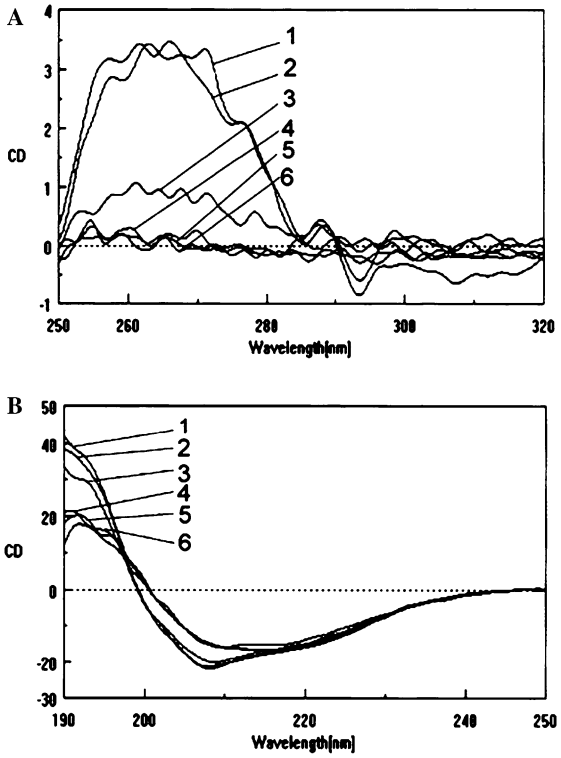

Fig. 1. Effect of temperature on UV CD spectra of cinnamomin A-chain: (A) Near UV CD spectra of cinnamomin A-chain. CD represented molar ellipticity ($\times 10^2$ deg cm^2 dmol^{-1}). (B) Far UV CD spectra of cinnamomin A-chain. CD was molar ellipticity ($\times 10^{-2}$ deg. cm^2 dmol^{-1}). Curves 1, 2, 3, 4, 5, and 6 indicated cinnamomin A-chain heated at 0, 37, 45, 55, 65, and 75 °C for 20 min, respectively.

Effect of temperature on tryptophan fluorescence of cinnamomin A-chain

In the thermal unfolding of cinnamomin A-chain, tryptophan residues and tryptophan-containing hydrophobic clusters were involved. This was demonstrated

by fluorescence emission spectra recorded at different temperatures. Fig. 2A revealed that thermal unfolding of the protein at 45 °C resulted in a large decrease in the quantum yield without any evident shift in emission maximum compared with that of native cinnamomin A-chain. It was known from the sequence data that cinnamomin A-chain contained three tryptophan residues located at 59, 144, and 201, respectively. In the three-dimensional models, all the three tryptophan residues were positioned in three α-helixes [18]. The microenvironments of indole chromophore of tryptophans were similar to that of native protein, since α-helix structures did not lose at 45 °C as shown in far UV CD spectra (Fig. 1B). Consistent with this point, partially unfolded cinnamomin A-chain formed at 45 °C showed little change in the position of emission maximum. If the temperature enhanced to 55, 65, or 75 °C, there was evident fluorescence quenching coupled with significant changes in the band position of emission maximum (about 10 nm). The red shift in emission maximum could be attributed to exposure of tryptophan residues to the solvent as a result of disruption of α-helical structures.

Fig. 2B indicated that thermal unfolding of cinnamomin A-chain was coupled with an increase in the fluorescence intensity when an extrinsic probe (ANS) was added. This probe could recognize hydrophobic clusters and be used to judge the formation of molten globular states in unfolding and refolding of protein [20,21]. The experimental results showed that when the cinnamomin A-chain was heated at 45 °C for 20 min, there was an increase in fluorescence intensity observed in the presence of ANS although the protein retained mainly secondary structure. This change was caused by the partial exposure of the hydrophobic clusters to solvent. The interaction of hydrophobic cluster was the crucial factor in supporting the tertiary structures and the integrity of catalysis. When the temperature was enhanced to 55, 65, or 75 °C, the interaction of hydrophobic cluster was disrupted completely. Thus, the ANS extrinsic fluorescence attained a plateau, and then did not increase any more as shown in Fig. 2B.

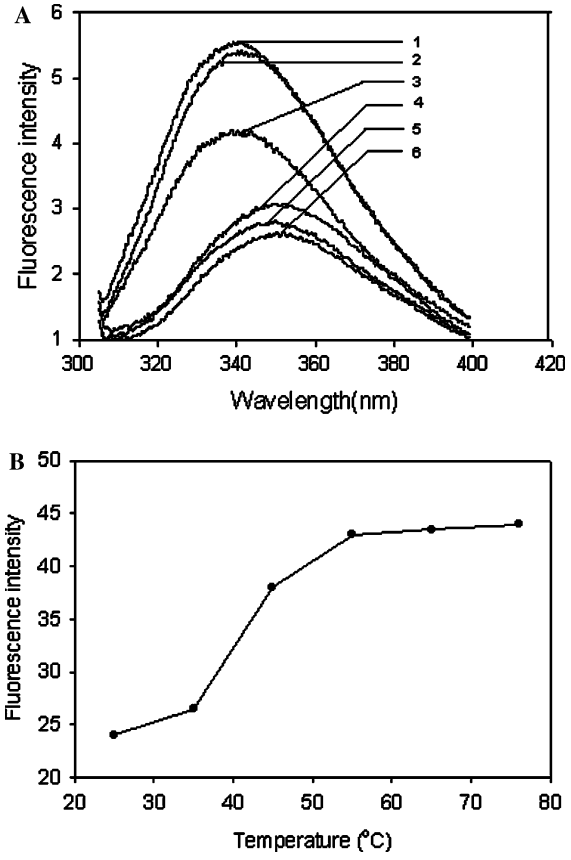

Fig. 2. Effect of temperature on fluorescence of cinnamomin A-chain tryptophan residues. (A) Fluorescence emission spectra of cinnamomin A-chain. Curves 1, 2, 3, 4, 5, and 6 represented cinnamomin A-chain samples (0.02 mg/ml in buffer G) that were heated at 0, 37, 45, 55, 65, and 75 °C for 20 min, respectively. (B) Changes of fluorescence intensity of cinnamomin A-chain. Cinnamomin A-chain was heated at different temperatures for 20 min, then a 10-fold molar excess of ANS was added and the concentration of protein was adjusted to 0.02 mg/ml with buffer G. Fluorescence intensity was recorded at 520 nm (excitation at 380 nm).

Existence of partially unfolded cinnamomin A-chain intermediate in molten globule

Fig. 3 showed the resistance of cinnamomin A-chain to protease digestion. The A-chain heated at 37 °C for 20 min was protease-insensitive as that of native protein. However, if the protein was treated at 45 °C for 20 min, it disrupted most of tertiary structures and was suscep-

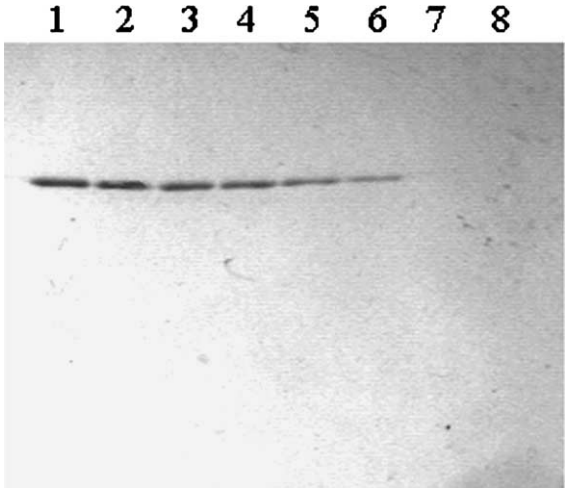

Fig. 3. Protease sensitivity of cinnamomin A-chain. Lane 1: native cinnamomin A-chain in the absence of thermolysin; lanes 2, 3, 4, 5, 6, 7, and 8: cinnamomin A-chain was heated at 0, 25, 37, 45, 55, 65, and 75 °C for 20 min, respectively, and then digested by thermolysin. For SDS–PAGE analysis, 1 μg of cinnamomin A-chain was loaded in each lane and the protein bands were visualized with Coomassi brilliant blue R250.

tible to protease digestion, whereas it kept most of secondary structures as shown in Fig. 1. Additionally, the cinnamomin A-chain unfolded non-cooperatively (not a two-state process) and bound ANS as described previously. Thus, the data indicated that the partially unfolded cinnamomin A-chain formed at 45 °C resembled a molten globular intermediate, because in this it possessed all features of typical molten globular states [21]. Since the experimental data could not exclude the presence of other intermediates, we supposed $(I)_n$ presented all possible intermediates. Therefore, the following model was proposed to explain the unfolding of cinnamomin A-chain by heating:

$$N \rightarrow (I)_n \rightarrow U$$

where N and U represented the native and completely unfolded cinnamomin A-chain, respectively.

Refolding of partially unfolded cinnamomin A-chain mediated by cinnamomin B-chain

As shown in Fig. 4, cinnamomin A-chain still retained its RNA *N*-glycosidase activity if the thermo-treatments performed at 37 even 42 °C. However, it lost enzymatic activity when being heated at 45 °C for 20 min though cinnamomin A-chain still retained most of the secondary structures. This change was irreversible, since cooling of the partially unfolded protein at room temperature for 1 h did not restore its tertiary structure and RNA *N*-glycosidase activity as shown in Figs. 5A and B, respectively. In addition, the fact that the intermediate formed in this condition completely lost its catalytic activity demonstrated that the substrate (rat ribosome)

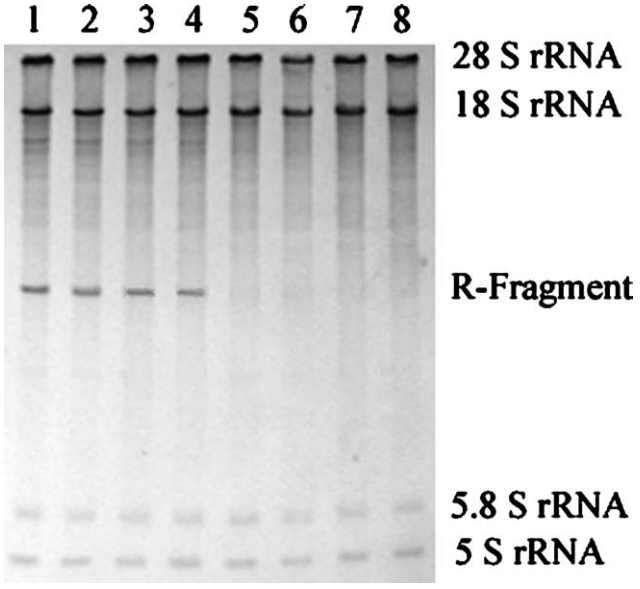

Fig. 4. RNA *N*-glycosidase activity. Lanes 1, 2, 3, 4, 5, 6, 7, and 8 represented cinnamomin A-chain that was heated at 0, 25, 37, 42, 45, 55, 65, and 75 °C for 20 min, respectively.

of cinnamomin A-chain could not induce the refolding of partially unfolded cinnamomin A-chain. It was reported that partially unfolded ricin A-chain by transient heating depurinated rat 28S rRNA and the result suggested that the ribosomes (substrates) themselves were responsible for the refolding of partially unfolded ricin A-chain [22]. In this point, cinnamomin A-chain was different from ricin A-chain although the two proteins were similar in structure and function as described previously [18]. Interestingly, when partially unfolded cinnamomin A-chain was cooled to 0 °C,then mixed with purified cinnamomin B-chain, which was dissolved in buffer G containing 2 mM DTT and followed by incubation at 25 °C for 1 h, cinnamomin A-chain restored its RNA *N*-glycosidase activity (Fig. 5B, lane 6). To exclude the possibility that the B-chain contained trace contamination of native A-chain or intact cinnamomin in preparation, the purified A- or B-chain was identified by Western blotting analysis using polyclonal antibody of intact cinnamomin that could arise immune conjunction with intact cinnamomin or separate A- or B-chain. Fig. 5C showed that the purified A- or B-chain had no intact cinnamomin contamination or cross-contamination with each other. In the same time, the activity assay showed no *N*-glycosidase activity with the pure B-chain (Fig. 5B, lane 5). These results suggested that the B-chain could mediate refolding of partially unfolded cinnamomin A-chain. Fig. 5B also revealed that the B-chain could not induce refolding of completely unfolded A-chain. This demonstrated that the intermediate containing significant secondary structure was necessary to the refolding of partially unfolded cinnamomin A-chain.

In order to know the interaction of the two subunits in the process that the B-chain mediated the refolding of partially unfolded cinnamomin A-chain, different concentrations of cinnamomin B-chain were tested. As shown in Fig. 6, IC_{50} of refolded cinnamomin A-chain decreased with the enhancive concentration of B-chain added. The decrease reached to a plateau when the molar ratio of added B-chain with partially unfolded A-chain approached 1:1. The data here demonstrated that the two parts were equally molar in the refolding of partially unfolded cinnamomin A-chain mediated by the B-chain.

According to the recent advance in uptake and intracellular transport of ricin, the toxin is transported via endosomes to the Golgi apparatus and by retrograde transport to endoplasmic reticulum (ER), then its active unit, A-chain, separates from B-chain and translocates to the cytosol to inactivate ribosomes [23]. However, many minutes keep unknown in this process. For example, now that the function of B-chain is to bind receptors on surface of cells to help the entrance of A-chain, why does it not separate from A-chain after the entrance until before the A-chain translocates to the cytosol from ER? Tonevitsky et al. [13] supposed that ricin B-chain protected ricin A-chain from degradation during transport from

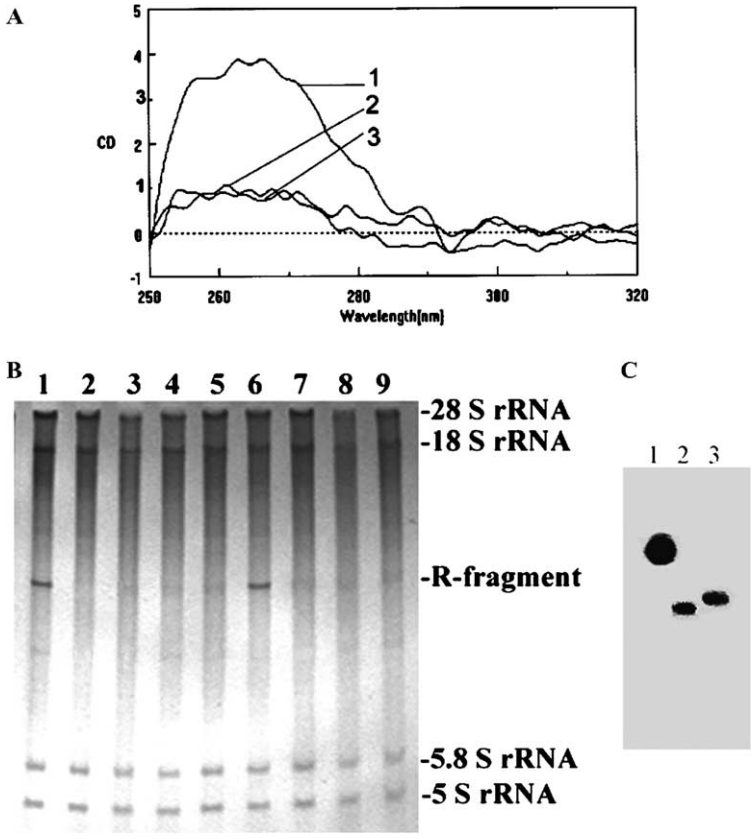

Fig. 5. Irreversibility of thermo-induced unfolding of cinnamomin A-chain and refolding of partially unfolded cinnamomin A-chain mediated by cinnamomin B-chain. (A) Near UV CD spectra. 1, The native cinnamomin A-chain; 2, cinnamomin A-chain was heated at 45 °C for 20 min; 3, cinnamomin A-chain was heated at 45 °C for 20 min following cooling upon room temperature (25 °C) for 1 h. CD was molar ellipticity ($\times 10^{-2}$ deg. cm^2 dmol^{-1}). (B) RNA *N*-glycosidase activity. Lane 1: native cinnamomin A-chain (positive control); lanes 2, 3, and 4, cinnamomin A-chain (0.2 mg/ml in buffer G) was, respectively, heated at 45, 55, and 65 °C for 20 min, then cooled to 25 °C for 1 h; lane 5: cinnamomin B-chain; lanes 6, 7, and 8: cinnamomin A-chain (0.2 mg/ml) was, respectively, heated at 45, 55, and 65 °C for 20 min, then cooled to 0 °C and cinnamomin B-chain in equal molar ratio with cinnamomin A-chain was added, subsequently incubated at 25 °C for 1 h. (C) Purity analysis of cinnamomin A- and B-chain by Western blotting. Lane 1: intact cinnamomin; lane 2: cinnamomin A-chain; and lane 3: cinnamomin B-chain.

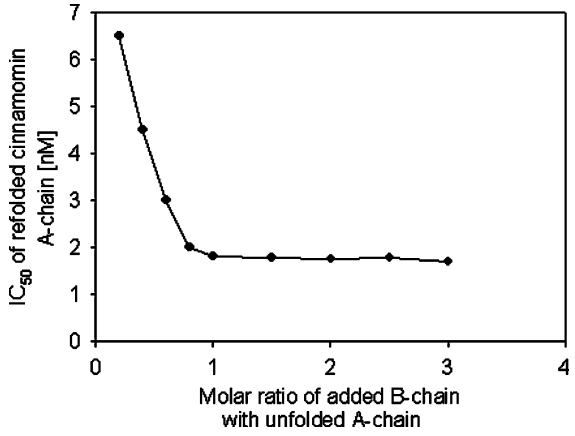

Fig. 6. Effect of cinnamomin B-chain on the refolding of partially unfolded cinnamomin A-chain cinnamomin A-chain (0.2 mg/ml) in buffer G was heated at 45 °C for 20 min, then cooled to 0 °C and native cinnamomin B-chain (1 mg/ml in buffer G) was added at different molar ratio (B-chain/unfolded A-chain) as indicated. The final concentration of partially unfolded A-chain was adjusted to 0.1 mg/ml using buffer G. The mixture was incubated at 25 °C for 1 h and the IC_{50} of inhibiting protein synthesis by the mixture was determined as described in Materials and methods.

cell surface to cytosol. Our data here confirmed that the B-chain could keep the structural stability of A-chain even help the refolding of partially unfolded A–chain. This hinted the other intracellular functions of B-chain in cytotoxicity of type II RIP.

Acknowledgments

The authors are grateful to Professor Hong-Yu Hu and Dr. Mong Zhao of our institute for their favorable advice in CD measurement and data analysis. We are also indebted to Dr. Ru-Qiang Liang of our institute for his help in fluorescence measurements and analysis. This work was supported by a grant of Natural Science Foundation of China and a grant of the Chinese Academy of Sciences.

References

[1] K. Nielsen, R.S. Boston, Ribosome-inactivating protein: a plant perspective, Annu. Rev. Plant Physiol. Pant Mol. Biol. 52 (2001) 785–816.

[2] E.J.M. Van Damme, Q. Hao, Y. Chen, A. Barre, E. Vandenbussche, S. Desmyster, P. Roue, W.J. Peumans, Ribosome-inactivating

proteins: a family of plant proteins that do more then inactivate ribosome, Crit. Rev. Plant Sci. 20 (2001) 395–465.
[3] Y. Endo, K. Tsurugi, RNA *N*-glycosidase activity of ricin A-chain: mechanism of action of the toxic lection ricin on eukaryotic ribosomes, J. Biol. Chem. 262 (1987) 8128–8130.
[4] S. Olsnes, A. Pihl, Treatment of abrin and ricin with mercaptoethanol opposite effects on their toxicity in mice and their ability to inhibit protein synthesis in a cell-free system, FEBS Lett. 28 (1972) 48–50.
[5] S. Olsnes, K. Sandvig, How protein toxins enter and kill cells, Cancer Treat. Res. 37 (1988) 39–73.
[6] B. Beaumelle, M. Alami, C.R. Hopkins, ATP-dependent translocation of ricin across the membrane of purified endosomes, J. Biol. Chem. 268 (1993) 23661–23669.
[7] I.I. Agapov, A.G. Tonevitsky, M.M. Moysenovich, N.V. Maluchenko, R. Weyhenmeyer, M.P. Kirpichnikov, Mistletoe lectin dissociates into catalytic and binding subunits before translocations across the membrane to the cytoplasm, FEBS Lett. 452 (1999) 211–214.
[8] S. Ramakrishnan, D. Fryxell, D. Mohanraj, M. Olson, B.Y. Li, Cytotoxic conjugates containing translational inhibitory proteins, Annu. Rev. Pharmacol. Toxicol. 32 (1992) 579–621.
[9] D.A. Vallera, Immunotoxins: will their clinical promise be fulfilled? Blood 83 (1994) 309–317.
[10] K. Sandvig, B. Deurs, Endocytosis and intracellular transport of ricin: recent discovery, FEBS Lett. 452 (1999) 67–70.
[11] V.G. Johnson, R.J. Youle, in: C.J. Steer, H.J. Hanover (Eds.), Intracellular Trafficking of Proteins, Cambridge University Press, Cambridge, UK, 1991, pp. 183–225.
[12] D.L. Newton, R. Wales, P.T. Richardson, S. Walbridge, S.K. Saxena, E.J. Ackerman, L.M. Roberts, J.M. Lord, R.J. Youle, Cell surface and intracellular functions for ricin galactose binding, J. Biol. Chem. 267 (1992) 11917–11922.
[13] A.G. Tonevitsky, A.Yu. Toptygin, A.T. Shamshiev, I.I. Agapov, E.V. Ershova, U. Pfuller, K. Pfuller, Comparison of properties of mistletoe lectin 1 A-chain and ricin B-chain conjugate with native toxins, FEBS Lett. 336 (1993) 100–102.
[14] J. Ling, W.Y. Liu, T.P. Wang, Simultaneous existence of two types of ribosome-inactivating proteins in the seeds of *Cinnamomum camphora*—characterization of the enzymatic activities of these cytotoxic proteins, Biochim. Biophys. Acta 1252 (1995) 15–22.
[15] X.D. Li, W.F. Chen, W.Y. Liu, G.H. Wang, Large-scale preparation of two new ribosome-inactivating proteins, cinnamomin and camphorin from the seeds of *Cinnamomum camphora*, Prot. Expr. Purif. 10 (1997) 27–31.
[16] J. Ling, W.Y. Liu, Cytotoxicity of two ribosome-inactivating proteins, cinnamomin, and camphorin, to carcinoma cells, Cell Biochem. Funct. 14 (1996) 157–161.
[17] W.Y. Liu, H.Y. Chen, W.F. Chen, X.D. Li, N. Ulbrich, W. Schroder, A proposed position of a disulfide bridge in the molecules of cinnamomin and porrectin—two new type II ribosome-inactivating proteins isolated from the seeds of camphor trees, Biochem. Mol. Boil. Int. 44 (1998) 1045–1049.
[18] L. Xie, B.Z. Wang, R.G. Hu, H.B. Ji, L. Zhang, W.Y. Liu, Structural and functional studies of cinnamomin, a new type II ribosome-inactivating protein isolated from the seeds of the camphor tree, Eur. J. Biochem. 268 (2001) 5723–5733.
[19] W.J. He, W.Y. Liu, Cinnamomin: a multifunctional type 2 ribosome-inactivating protein, Int. J. Biochem. & Cell Biol. 1409 (2002) 1–7.
[20] G. Weber, B.L. Young, J. Biol. Chem. 239 (1964) 1415–1420.
[21] O.B. Ptitsyn, Molten globule and protein folding, Adv. Protein Chem. 47 (1995) 83–229.
[22] R.H. Argent, A.M. Parrott, P.J. Day, L.M. Roberts, P.G. Stockley, J.M. Lord, S.E. Radford, Ribosome-mediated folding of partially unfolded ricin A-chain, J. Biol. Chem. 275 (2000) 9263–9269.
[23] K. Sandvig, B. Deurs, Endocytosis and intracellular transport of ricin: recent discoveries, FEBS Lett. 452 (1999) 67–70.