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Identification, characterization and structure analysis of a type I ribosome-inactivating protein from *Sapium sebiferum* (Euphorbiaceae)

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ABSTRACT

Ribosome-inactivating proteins (RIPs) are N-glycosidases (EC3.2.2.22) that universally inactivate the ribosome, thereby inhibiting protein biosynthesis. In this study, a novel type I RIPs named SEBIN was identified in *Sapium sebiferum*. Nuclear acid depurine experiment showed that SEBIN had rRNA N-Glycosidase activity. Further experiment indicated that SEBIN significantly inhibited *Caenorhabditis elegans* development as well as resulted in worm cell apoptosis. This is the first report to evaluate RIPs toxicity using *C. elegans*. We proposed that SEBIN may impaire *C. elegans* reproduction in a DNA-damage manner besides traditional protein synthesis inhibition approach. The predicted 3D structure was modeled using threading and *ab initio* modeling, and the r-RNA binding residue of SEBIN was identified through the protein-ligand docking approach. It showed the amino acid residues, Glu195, Asn81, Ala82, Tyr83, Glu164, Ser163, Ile159 and Arg167, played critical roles in catalytic process. Our results provided the theoretical foundation of structure–function relationships between enzymatic properties, toxicity and structural characterization of SEBIN.

1. Introduction

Ribosome-inactivating proteins (RIPs) are a unique family of proteins isolated from many plants and are abundant in angiosperms [1]. Historically, RIPs has been reported to exhibit significant cytotoxicity in several cell models [2]. The widely accepted mechanism suggests that RIPs possess characteristics N-glycosidase activity, which removes a single adenine from rRNA (A4324 from the 28S rRNA in the 60S subunit of ribosomes), thereby inactivating the ribosome and inhibiting polypeptide chain elongation [3]. For plants, RIPs have been linked to biological defense owing to their antiviral, antifungal and insecticidal properties [4].

Based on existing knowledge regarding their biological activities, RIPs from plants have potentially useful applications in agriculture and medicine [5], [6]. In medicine, recent work has been focused on the ability of RIPs against Human immunodeficiency virus (HIV) [7], [8]. Trichosanthin (*Trichosanthes kirilowii*), PAP (*Phytolacca Americana*) have been reported to inhibit HIV-1 replication *in vitro* [9], curcin (*Jatropha Curcas*) [10]. In agriculture, resistance to insects was obtained in maize by enhancing the expression of an endogenous RIP [11], [12]. In addition, the expression of RIPs was improved in plants when subjected to various abiotic stresses: heat and osmotic stress [13], salinity [14] [15] [16], mechanical injury [17], [18].

Sapium sebiferum which belongs to Euphorbiaceae family is a tropical and subtropical deciduous plant. It is indigenous to China with more than 1400 years' cultivation history [19]. The main cultivated area includes the Yangtze River basin and southern provinces. Japan and India are also distributed [20]. *S. sebiferum* is also traditional energy species in history because of outstanding advantages of biological and energy oil output as well as landscaping, it has currently become a new interest for researchers. Research shows that the seeds of the plant are not only a source of biodiesel, but also contain several toxic metabolites of pharmaceutical importance, for example diterpenoids called phorbol esters

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[20], less known is the toxic protein from the seeds. Like most Euphorbiaceae family plants, *Jatropha Curcas* seeds contain a ribosome-inactivating protein (RIP), curcin [21], [22]. Recently, some studies showed curcin exhibited various pharmacological and biological activities such as anti-tumor activity [23], pesticidal activity [24], and antifugal activity [25]. So far, many important RIPs were found in Euphorbiaceae family plants, for instance, ricin (*Ricinus communis*), curcin (*Jatropha Curcas*), gelonin (*Gelonium multiflorum*), mapalmin (*Manihot palmate*) and crotins (*Croton tiglium*) [26]. **Whether *S. sebiferum* (Euphorbiaceae) seeds contain RIP? Did the toxicity relate to the RIP in S. sebiferum, and does it have new activity and potential applications?** Not much information about the RIP from *S. sebiferum*, and the toxic properties are currently available in literature. In this report, we describe the identification of a novel Type I RIP in the immaturate seeds of *S. sebiferum*, and the study of their preliminary chemical and biological properties. The possible active sites between SEBIN and the adenine of r-RNA were also predicted. It would form the basis for designing future applications of SEBIN in pharmacology, and can also produce non-toxic varieties of *S. sebiferum* in agriculture, which would make the seed-cake suitable as animal feed.

2. Materials and methods

2.1. Cloning and sequence analysis of the SEBIN from S. sebiferum

Total RNA was extracted from young fruit using the E.Z.N.A.™ Plant RNA kit according to manufacturer's directions. To obtain the full length cDNA sequence of *SEBIN*, rapid amplification of cDNA ends (RACE)-PCR was performed using a SMART™ RACE cDNA Amplification Kit (Clontech Co., Palo Alto, CA) according to the manufacturer's instructions. For multiple sequence alignment of SEBIN amino acid sequences, 39 RIPs were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/protein/) including the type I RIPs and the A chain of type II RIPs. Multiple sequence alignments were performed using the ClustalW tool included in the MEGA5.0 [27]. Multiple sequence alignments were graphically represented by sequence logos [28] created with WebLogo 3 (http://weblogo.threeplusone.com/) [29].

2.2. rRNA N-Glycosidase activity assay

The assay was carried out basically determined according to Begam et al. with some modifications [30]. The *SEBIN* cDNA was amplified by PCR to subcloned into pGEX-4T-1. The recombinant plasmids were transferred into *E.coli* Rossetta cells for the overexpression of SEBIN protein. Total RNA (2 μg) extracted from leaves of tune tree was treated with different concentration of SEBIN (2 μg, 4 μg, 10 μg, 20 μg) and incubated at 30 °C for 30 min and divided in half, respectively. One part was treated with 1M freshly prepared aniline acetate (pH 4.5), whereas the other part was untreated. RNA incubated in the absence of RIPs served as a negative control.

2.3. In vivo toxicity of SEBIN in Caenorhabditis elegans

The developmental manifestation was investigated by comparing the length of the Bristol N2 worms, and the evaluation of reproductive toxicity from SEBIN was implemented by counting the number of germ cell apoptosis. The Bristol N2, *hus-1* (*op241*) and *clk-2* (*mn159*) strains were cultivated with one of the following SEBIN treatments: 0.1 μM or 1 μM respectively. Apoptotic germ cells were measured by AO vital staining as reported previously. Briefly, synchronized worms at L4 stage were exposed to graded doses of SEBIN protein for 24 h, and then transferred into a Costar 24-well plate staining with 500 ml of 25 mg ml^{-1} AO and OP50. After incubation at 20 °C for 60 min, the worms were transferred onto bacterial lawns for recovering. The worms immobilized by levamisole and the fluorescent staining were observed under an Olympus 1 × 71 microscope (Olympus Co., Japan). The number of germ cell corpse per gonad arm was measured. Buffer B (500 mM Tris-HCl, 10 mM Reduced Glutathione, pH8.0) was set as blank control. All treatments were repeated three times. Statistical differences between various concentrations of different strains were tested using analysis of variance (ANOVA) followed by Tukey's multiple comparison test ($p < 0.05$).

2.4. 3D structure modeling and active site prediction

The adenine binding site of SEBIN was predicted by superposition of SEBIN with the ricin-adenine complex (PDB id: 2P8N) by MatchMaker extension of UCSF Chimera v.1.9 [31]. To further identify adenine interacting residues, docking experiments were performed by using the Auto-Dock 4.2 [32]. The structure of SEBIN and ligand (adenine) were prepared by the manual of Auto-dock 4.2. AutoGrid 4.2 Program, supplied with AutoDock 4.2, was applied to generate grid maps for the ligands. The grid box was set at 60 Å × 60 Å × 60 Å in x-, y- and z-axis, so the residue of adenine binding site of SEBIN predicted from the above may be involved in docking. Lamarckian genetic search algorithm was selected to find suitable binding positions for a ligand on SEBIN.

3. Results and discussion

3.1. Cloning, sequence alignment and phylogenetic analysis of SEBIN

The RIPs homologue in *S. sebiferum*, *SEBIN*, was isolated by RT-PCR. Analysis of 1265 bp segments revealed that the gene included a 309 bp 5′ flanking region, a 789 bp ORF encoding a putative protein of 262 amino acids and a 167 bp 3′ flanking region. Its molecular weight was 29.77 kDa and isoelectric point was 6.66. The putative polypeptide showed 52% similarity with *Camellia sinensis* and 49% similarity with *Jatropha curcas*.

With the aim of understanding the conserved amino acid residues of the SEBIN, multiple sequence alignments were performed using Clustal W. Fig. 1 (left) shows a sequence logo representing the alignment of 40 RIP sequences **including type I RIPs and the A chain of type II RIPs**. 15 amino acids residues were present in at least 80% of the sequences: Y66, F69, R74, Y130, Y134, L171, L194, G195, L213, E252, A253, R255, F284, W296 and S300 (Fig. 1 left). In order to ascertain the function of these highly conserved amino acids, we located them all in the structure of SEBIN. Among these residues, twelve highly conserved amino acids appeared to be also conserved in SEBIN (Y25, F28, R33, Y83, Y122, L133, L154, E164, A165, R167, W198 and S202) (Fig. 1 right). Compared with Ebulin l A-chain structure (accession code 1HWP), the function of five amino acids is well-known: E164 and R167 are directly involved in the mechanism of acid-catalyzed depurination, Y83, Y122, and W198 are involved in the binding to the target adenine. The role of Ser 202 needs to be clarified for its adjacency to the catalytic Trp198 (296 in the sequence logo). We hypothesized that the amino acid could play an important role in stabilizing the conformation of W198 side chain, which was consistent with the report about S211 and W207 in the type 1 RIP PD-L4 [33]. Further analysis showed that SEBIN has the highest similarity with A chains of type II RIPs than any other type I RIPs. Peumans et al. suggested that, based on the comparison of RIPs sequences from 300 plants, an ancestor of modern seed plants developed the RIP domain at least 300 million years ago [34]. This ancestral RIP domain which gave rise to a direct lineage of type I RIPs (**i.e., primary type I RIPs**) still present today in

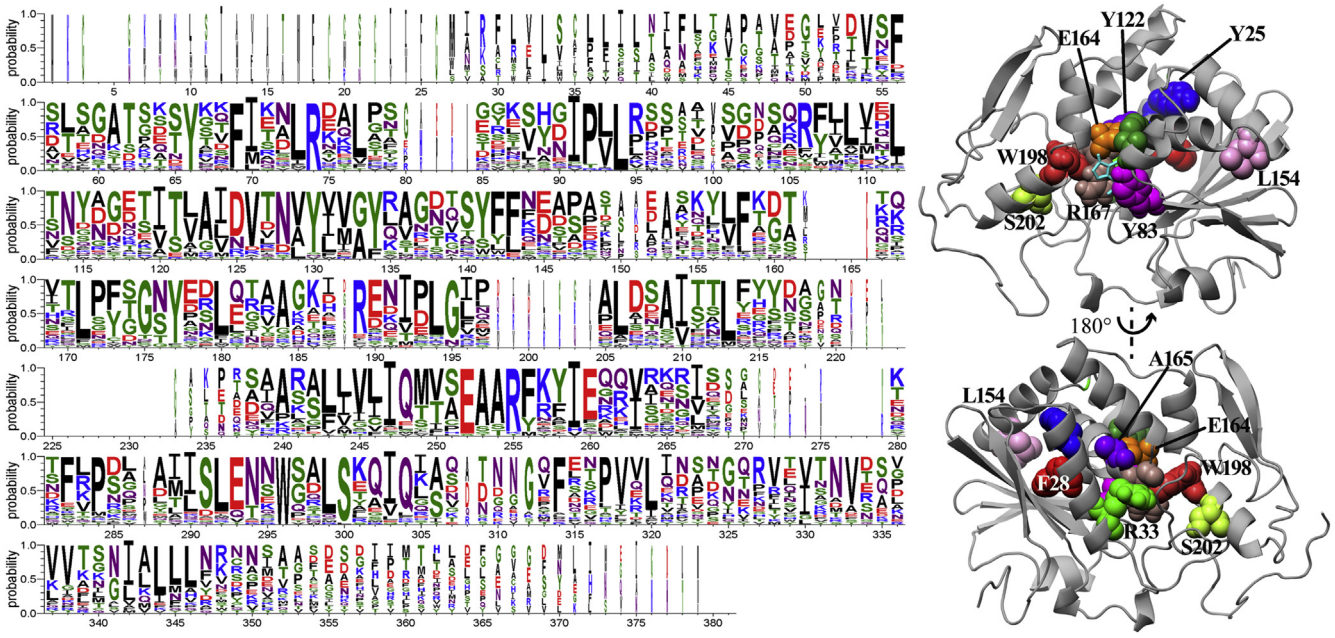

Fig. 1. Amino acid sequence conservation in the type I RIPs and type II RIP A-chains. Left: sequence logo representation of the alignment of 40 RIP sequences. Letter height is proportional to the frequency of that amino acid at that position in RIPs respect to all the amino acids; letter width is proportional to the frequency of that amino acid but including gaps. It was created as indicated in "Methods" section. Right: 3D structure modeling of SEBIN and the conserved amino acids labeled by PyMOL.

many monocots and at least one dicot. At a later stage, a plant succeeded in fusing the RIP domain to a duplicated lectin-B domain acquired from a bacterium, which formed the ancestral type II RIP. In contrast, the ancestral type II RIP gave rise to all modern type II RIPs and different lines of **"secondary" type I RIPs** as well as ricin-B type lectins by domain deletion. In this paper, the alignment results with 40 protein sequences indicated that SEBIN was more closely related to the A-chains of type II than type 1 RIPs, suggesting that SEBIN might derive from type II RIPs by deletion of the nucleotide sequence coding for the B chain. Therefore, **SEBIN should be classified into "secondary" type I RIP**.

3.2. rRNA N-Glycosidase activity of SEBIN

Fig. 2 showed that SEBIN, similar to the other type-I RIPs, displayed N-glycosidase activity by producing the endo-fragment when RIP-depurinated rRNA was treated with aniline. However, no small nucleotide fragment release was observed after the incubation of SEBIN without aniline. The enzymatic activity of SEBIN showed a same tendency with the SEBIN concentration. The release of endo-fragment suggested that SEBIN possessed rRNA N-glycosidase activity, which could lead to the inactivation of ribosome.

3.3. In vivo toxicity in C. elegans

Owing to cellular simplicity, genetic manipulability and evolutionarily conserved biology, *Caenorhabditis elegans* has been proved to be an excellent model organism for studying fundamental toxicity assessments and further mechanistic studies in the fields of environmental and biomedical toxicology [35], [36]. To evaluate the toxicity of SEBIN at individual level, we investigated the developmental appearance by comparing the length of *C. elegans* and reproductive toxicity of SEBIN by counting the number of cell corpses in the germline of nematodes. At the concentration of 1 μM,

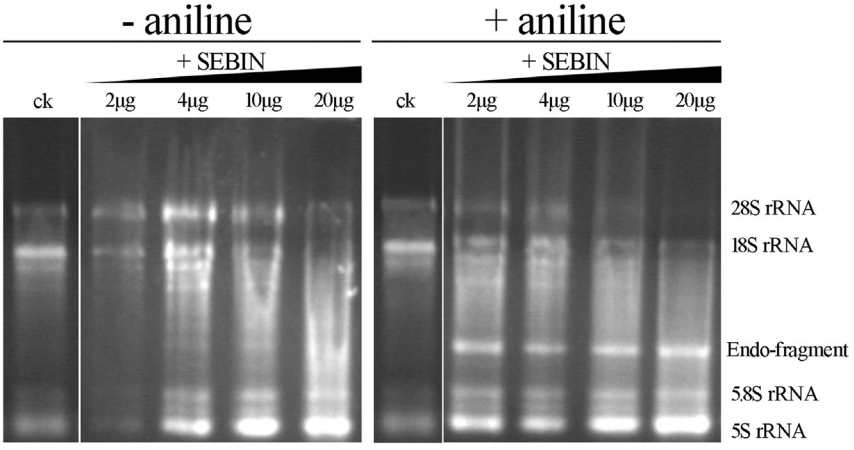

Fig. 2. rRNA N-glycosidase activity of SEBIN. The rRNA was extracted and treated (+) or untreated (−) with aniline hydrolysis and analyzed by 2% agarose gel electrophoresis. The amount of rRNA endo-fragment resulting from aniline-catalyzed hydrolysis increased with the concentration of SEBIN improving.

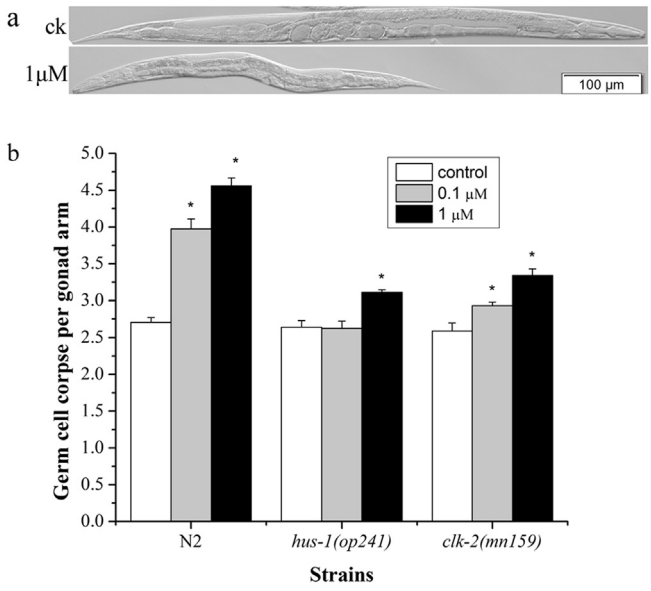

Fig. 3. SEBIN exposure induced *C. elegans* germline apoptosis. The Bristol N2 (wild-type), *hus-1(op241)* and *clk-2(mn159)* strains were cultivated with one of the following treatments: 0.0 μM, 0.1 μM or 1.0 μM SEBIN, respectively. a, Representative pictures of growth inhibiting in *C. elegans* by SEBIN. Scale bar = 100 μm b, Exposure of *C. elegans* to graded doses of SEBIN caused cell apoptosis. All values are mean ± SE, expressed as germ cell corpses per gonad arm. For each dose, about 50 worms were quantified. *p < 0.05 represents statistical difference compared to untreated control.

the body size of *C. elegans* was only about half of the blank control. It suggested that SEBIN exposure significantly inhibited *C. elegans* larval development (Fig. 3a).

To determine whether SEBIN exposure could causes germ cell apoptosis, synchronized young adult wild type N2 worms were exposed to SEBIN. As shown in Fig. 3b, following treatment with 0.1 μM and 1.0 μM of SEBIN, germ cell death exhibited significant increase compared with that of the control populations in a dose-dependent manner (in all cases, P < 0.05). The number of germ cell corpses per gonad arm was about 4.5 as exposed to SEBIN at 1.0 μM, corresponding to the toxicity of Diesel Particulate Extract (DPE) at 400 μg ml^{-1} or UVA more than 5.0 J cm^{-2} [37].

The mechanism of apoptosis in the *C. elegans* germline was assessed with the mutant strains *hus-1* and *clk-2*. Hus1 is one of the important checkpoint genes that functions as the sensor of DNA damage [38]. The *C. elegans hus-1* encodes a conserved checkpoint protein that is required for germline apoptosis triggered by DNA-damage agents, and *clk-2* acts in parallel to *hus-1* [39] [40], [41]. Under graded doses of SEBIN exposure, germ cell apoptosis were almost abolished in alleles of *hus-1* and *clk-2*, in contrast to N2 (Fig. 3b). The results clearly revealed that the loss-of-function of either *hus-1 or clk-2* could partly prevent germline from apoptosis under SEBIN exposure. Taken together, these phenotypes indicated that SEBIN might impaire *C. elegans* reproduction through DNA-damage. In previous studies, researchers focused on the effects of RIPs on protein synthesis, while our study showed that RIPs not only inhibited protein synthesis, but also impaired DNA to induce apoptosis.

Historically, RIPs has been reported to exhibit significant cytotoxicity in several cell models [2], [39]. The widely accepted mechanism suggests that RIPs possess characteristics N-glycosidase activity that inactivates the ribosomes, inhibiting cell protein synthesis irreversibly [42]. Here, we evaluated the toxicity of SEBIN by investigating the apoptosis of *C. elegans* germline [43], [44]. It showed that exposure of *C. elegans* to SEBIN caused significantly elevated level of embryonic lethality and dose-dependent germline apoptosis. Furthermore, this apoptotic response was blocked in loss-of-function mutants of *hus-1* (*op241*) and *clk-2* (*mn159*). We speculated that SEBIN exposure results in an increased number of meiotic DNA double-strand breaks in germ cells and impaired maintenance of genomic integrity, subsequently leading to activated DNA damage checkpoint. The method to evaluate *in vivo* activity of RIPs using *C. elegans* would provide a new way in understanding the molecular mechanisms of RIPs toxicity involved in multiple biological pathways.

3.4. 3D structure modeling and active site prediction of SEBIN

The structure of ricin A-chain complex with adenine (2P8N) is resolved at 1.94 Å [3].The overall RMSD deviation of 1.270 Å

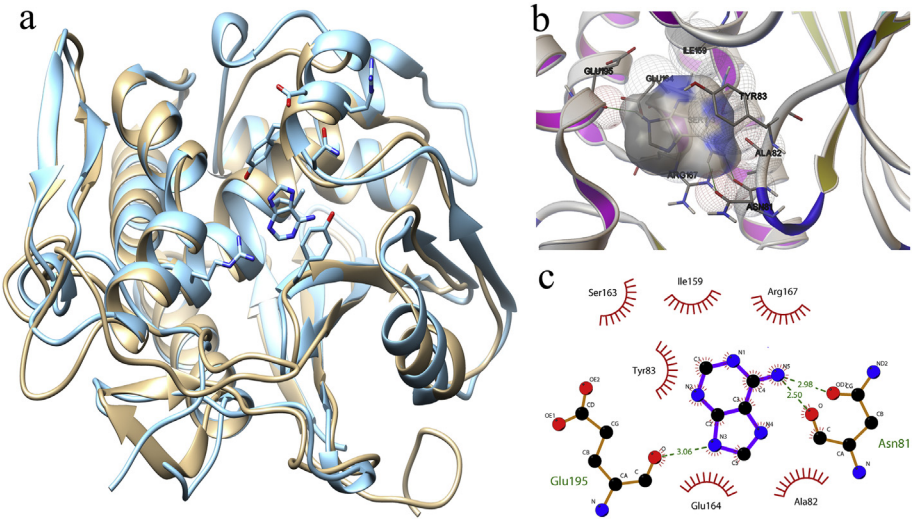

Fig. 4. Superposition of main chains of ricin (cyan) and SEBIN (brown), and adenine binding site residues of SEBIN. a. Superposition of main chains of ricin (cyan) and SEBIN (brown). b. Adenine binding site residues of SEBIN. Adenine is shown in gray shade and the hydrogen bond interactions are depicted by green lines. c. A schematic diagram of the distributions of hydrophobicity and hydrogen bonds for SEBIN-adenine complex, generated by the program LIGPLOT. The red arcs with radiating spokes represents the amino acids showing hydrophobic interaction with adenine and the green dotted lines represent hydrogen bond interactions. Carbon, nitrogen and oxygen atoms are shown in black, blue and red, respectively. Hydrogen bond interactions are depicted by green dotted lines. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

between ricin and SEBIN model indicated the SEBIN structural model was consistent with experimental coordinates of ricin and avaliable for further study (Fig. 4a).

In the present work, docking analysis on SEBIN structure was performed by Autodock 4.2 (Fig. 4b, c). The result indicated that residues Glu195, Asn81, Ala82, Tyr83, Glu164, Ser163, Ile159 and Arg167 were in close contact with adenine, and the binding site residues involved in hydrogen bonding were Glu195 and Asn81. Among the eight residues, six were exactly same as those in the active site of ricin (Asn122, Ala79, Tyr80, Ser176, Ile172, Arg180) [45]. It has been previously suggested that ricin has a very high specificity to adenine as it depurinates adenosine from roughly 7000 nucleotides in eukaryotic ribosomes [46]. Chan et al. found that ricin A chain (RTA) induces apoptosis of Hela cells with an IC50 for cell viability of 12×10^{-3} μg ml^{-1} [47]. Our previous study showed that SEBIN was about 10^4- fold less toxic than RTA. The difference of residues involved in adenine binding between ricin and SEBIN might be the main determinant in toxicity. Our preliminary findings thus warrant molecular mechanisms of the SEBIN mediated potential toxicity of *S. sebiferum* seeds.

In summary, our study shows a novel RIP was isolated from *S. sebiferum* and the relationship among enzymatic properties, toxicity and structure of SEBIN was investigated systematically. It provides new clues in understanding the molecular mechanisms of RIPs toxicity besides traditional protein synthesis inhibition manner. It also provided a new way to enrich the theoretical model of molecular mechanisms of RIPs toxicity. Moreover, understanding the characterization and possible active sites of SEBIN can facilitate the development and utilization of SEBIN in *S. sebiferum*.

Conflict of interest

The authors declare that they have no competing interests.

Acknowledgments

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