FEBS Letters 347 (1994) 257–260

FEBS 14168

# Luffin-S — a small novel ribosome-inactivating protein from *Luffa cylindrica*

## Characterization and mechanism studies

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Received 27 December 1993

**Abstract**

We purified and characterized a novel RIP (ribosome inactivating protein), Luffin-S from the seeds of *Luffa cylindrica*. Different from Luffin-A and B, which are RNA *N*-glycosidases with molecular weights of 27 and 28 kDa, respectively, Luffin-S has an M.W. of only approx. 10 kDa, much smaller than any other RIPs so far investigated. Its abundant resources, toxicity similar to TCS in a cell-free protein synthesis system and unique mechanism as phosphodiesterase, like α-sarcin, promisingly make it a potential toxic moiety of immunotoxin.

*Key words:* Ribosome inactivating protein; Luffin; RNA *N*-glycosidase; Phosphodiesterase; Immunotoxin

## 1. Introduction

Widely distributed in the plant kingdom, ribosome inactivating proteins (RIPs) are a class of toxic proteins which can catalytically inactivate ribosomes by specifically cleaving high-M.W. rRNA. Plant RIPs are grouped into two major types based on their structure and function. The type I RIPs (e.g. TCS) have a single chain with M.W. ranging from 25 to 30 kDa. The type II RIPs (e.g. ricin and abrin) with M.W. around 60 kDa are usually composed of two chains (A and B) connected by a disulfide bond. When entering the cytoplasm with the help of the B (binding) chain, which binds to the galactose-containing receptors on the cell surface, the A chain can inactivate the 60S ribosome subunits [1], as its type I counterparts do.

It has been demonstrated that all plant RIPs catalytically cleave the *N*-glycosidic bond of a specific adenine nucleoside residue, $A_{4324}$, in rat liver 28S rRNA and are thereby designated as RNA *N*-glycosidases [2,3]. Fungal toxin α-sarcin can inactivate the 60S subunits in a distinctive manner. It cleaves the single phosphodiester bond between $G_{4325}$ and $A_{4326}$ in rat liver 28S rRNA and releases a fragment of 393 nt (α-fragment) [4], showing ribonucleolytic activity. So far, there is no report on plant RIPs acting in this way.

While isolating Luffin-A and B from *Luffa cylindrica*, we discovered another toxic protein and named it 'Luffin-S', where 'S' means 'small molecule', for its M.W. is only approx. 10 kDa. Mechanism studies of Luffin-S indicated that it is a novel phosphodiesterase, acting in a way similar to α-sarcin.

## 2. Materials and methods

### 2.1. Materials

Seeds of *Luffa cylindrica* were bought from Shanghai Seeds Company. Sephacryl S-100 was the product of Phamacia Fine Chemicals (Uppsala, Sweden). CM-52 was purchased from Whatman Ltd. (Springfield, UK). L-[U-$^{14}$C]Leucine (319 mCi/mmol) was from Amersham (Buckinghamshire, UK). Other reagents were of analytical grade.

### 2.2. Purification of Luffin-S

Smashed seeds of *Luffa cylindrica* were defatted with ice-cold ether, and extracted with 20 mM PB, pH 7.0, containing 0.2 M NaCl. The crude extract was then precipitated with 55–85% saturation of ammonium sulfate at 0°C. The precipitate was dissolved and dialyzed against 5 mM PB, pH 7.6, and then applied to a CM-52 ion exchange column (2.5 × 15 cm) previously equilibrated with the same buffer, followed by elution with a linear 0–0.5 M NaCl gradient in 5 mM PB, pH 7.6. Fractions containing proteins showing toxicity in cell-free system and a M.W. of approx. 10 kDa were pooled and applied to a Sephacryl S-100 column (1.6 × 110 cm), equilibrated and washed with 0.1 M PBS, pH 8.0.

### 2.3. Molecular weight estimation and carbohydrate detection

The M.W. of Luffin-S was estimated by SDS-PAGE [5]. The presence of carbohydrate was detected using the periodic acid-schiff (PAS) method [6] after SDS-PAGE, using ricin and TCS as positive and negative controls.

### 2.4. Amino acid composition of Luffin-S

Luffin-S was hydrolyzed in 6 N HCl at 110°C for 24 h and analyzed on a Beckman System 6300 amnio acid analyzer.

### 2.5. Inhibition of protein synthesis in cell-free system

Rabbit reticulocyte lysate was prepared as described [7]. 14 μl containing increasing amounts of toxic proteins (in 10 mM Tris-HCl, pH 7.7) and 16 μl L-[U-$^{14}$C]leucine solution(3 μCi/ml) were added to 30 μl crude rabbit reticulocyte lysate. Protein synthesis was terminated after incubation at 37°C for 1 h with 0.1 N NaOH, 0.3% $H_2O_2$. Proteins were

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*SSDI* 0014-5793(94)00554-9

precipitated onto Whatman GF/C filters with 50% trichloroacetic acid (TCA). After washing with 5% TCA and ethanol, the radioactivity of L-[U-$^{14}$C]leucine incorporated into protein was measured by standard scintillation counting techniques.

*2.6. Cleavage of ribosomal RNA by toxic proteins*

Rat liver ribosomes were prepared by the method of Wettstein [8]. The ribosomes were stored at −70°C, 1.0 $A_{260}$/Eppendorf, in medium A (50 mM Tris-HCl, pH 7.7, 25 mM KCl, 5 mM $MgCl_2$, 0.3 M sucrose). Naked rRNA was extracted from ribosomes with 1.0% SDS (in 50 mM Tris-HCl, pH 7.5) and phenol. Modification of ribosomes and naked rRNA was carried out according to the method of Endo and Wool [9]. Ribosomes or naked rRNA were incubated with toxins for 15 min at 37°C in a final volume of 50 μl reaction buffer (50 mM Tris-HCl pH 7.0, 5 mM EDTA). The reaction was terminated by the addition of 5 μl 1.0% SDS. For analysis of the rRNA fragments, total rRNA was prepared by phenol extraction followed by ethanol precipitation and electrophoresed on a 1.8% agarose gel.

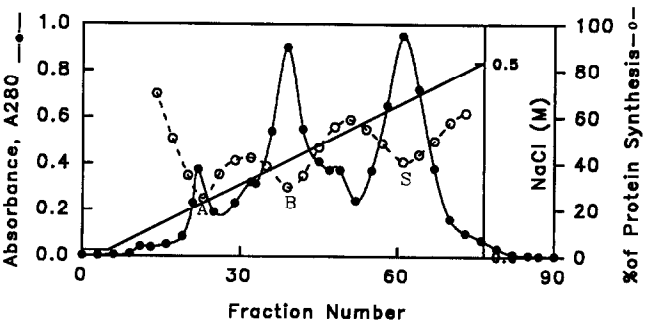


Fig. 1. Purification of Luffin-S by ion-exchange chromatography. A,B,S indicate the fractions showing inhibitory activities in a cell-free protein synthesis system, from which Luffin-A,B,S can be further purified, respectively. Samples were adjusted to final concentrations of $6.0 \times 10^{-10}$ M, activities were determined as described in section 2, and cpm 27115 was taken as 100% protein synthesis.

## 3. Results and discussion

### *3.1. Characterization of Luffin-S*

The M.W. of single chain RIPs from higher plants usually range from 25 to 30 kDa. Traditionally, the strategy for isolating these proteins is applying gel filtration before using ion-exchange chromatography. Our modified procedures enabled us to discover a toxic protein beyond that range but with strong inhibitory activity in a cell-free protein synthesis system (Fig. 1.). Its M.W. is only around 10 kDa as determined by 12.5% SDS-PAGE (Fig. 2.). We named it Luffin-S, in which 'S' means 'small molecule'. The positive result of PAS staining (not shown) indicated that Luffin-S is a glycoprotein.

Amino acid composition (Table 1) indicates that Luffin-S contains fairly high quantities of acidic amino acids (Glx) as well as basic amino acids (Arg), which is quite different from Luffin A (or B), α-sarcin and RNase A, suggesting that Luffin-S is not likely to be an active fragment degraded from these intact toxins.

In the cell-free protein synthesis system from rabbit reticulocyte lysate, Luffin-S displayed an inhibitory activity comparable with TCS and α-sarcin[10]. Their $IC_{50}$ values are $3.4 \times 10^{-10}$ M, $2.9 \times 10^{-10}$ M and $8.8 \times 10^{-10}$ M, respectively (Fig. 3). In the 55–85% $(NH_4)_2SO_4$ precipitate, Luffin-S makes up about 50% of the content. The abundant resources, easy preparation and potent

Table 1
Amino acid composition of Luffin-S, Luffin A, α-sarcin and RNase A

| Amino acid | Luffin-S | Luffin A | α-sarcin | RNase A |
|---|---|---|---|---|
| Asx | 6.00 (6) | 26.34 (26) (23)[a] | 23.90 (24) (22)[b] | 17.35 (17) |
| Thr | 0.00 (0) | 22.91 (23) (23) | 11.24 (11) (11) | 10.68 (11) |
| Ser | 2.78 (3) | 27.96 (28) (30) | 9.56 (10) (9) | 15.93 (16) |
| Glx | 29.94 (30) | 22.20 (22) (20) | 10.05 (10) (9) | 14.20 (14) |
| Pro | 3.79 (4) | 8.72 (9) (6) | 16.17 (16) (13) | 5.56 (6) |
| Gly | 4.93 (5) | 16.33 (16) (13) | 13.45 (13) (13) | 3.53 (4) |
| Ala | 6.00 (6) | 22.72 (23) (22) | 4.99 (5) (5) | 13.11 (13) |
| Cys | 4.21 (4) | 0.00 (0) (0) | 1.73 (2) (4) | 4.27 (4) |
| Val | 1.33 (1) | 15.57 (16) (17) | 3.28 (3) (3) | 8.41 (8) |
| Met | 2.41 (2) | 1.61 (2) (2) | 0.00 (0) (0) | 3.61 (4) |
| Ile | 1.61 (2) | 15.62 (16) (18) | 2.70 (3) (4) | 1.95 (2) |
| Leu | 5.31 (5) | 22.95 (23) (26) | 9.13 (9) (9) | 2.12 (2) |
| Tyr | 0.00 (0) | 9.57 (10) (13) | 5.28 (5) (8) | 4.31 (4) |
| Phe | 1.46 (1) | 9.00 (9) (10) | 5.42 (5) (6) | 2.89 (3) |
| His | 1.03 (1) | 1.57 (2) (1) | 7.73 (8) (8) | 4.25 (4) |
| Lys | 0.00 (0) | 17.22 (17) (16) | 22.42 (22) (20) | 12.14 (12) |
| Arg | 19.20 (19) | 6.72 (7) (7) | 3.96 (4) (4) | 3.69 (4) |
| Trp | N.D. | N.D. (1) | N.D. (2) | N.D. |
| Total | 89 | 249 (248) | 150 (150) | 128 |
| M.W. | 10745 | 26729 (27021) | 16655 (16980) | 13998 |

[a] Calculated from published sequence of Luffin-A. (Isiam, M.R. et al. (1990) Agric. Biol. Chem. 54, 2967–2978.)
[b] Calculated from published sequence of α-sarcin. (Oka, T. et al. (1990) Nucleic Acids Res. 18, 1897)

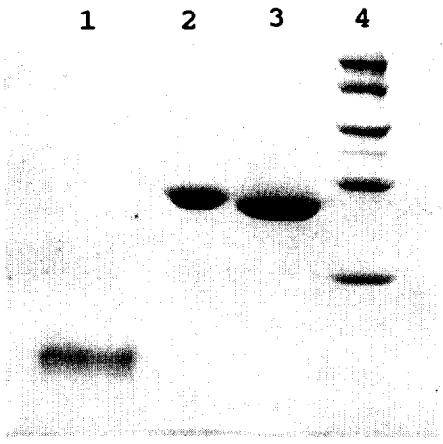


Fig. 2. Molecular weight of purified Luffin-S was estimated on 12.5% SDS-PAGE. Lane 1, Luffin-S (10 kDa); lane 2, Luffin B (28 kDa); lane 3, Luffin A (27 kDa); Lane 4, standard markers (94, 67, 43, 30, 17.5 kDa).

toxicity suggest that Luffin-S may be used as toxic moiety in immunotoxin. But as it is much smaller than the common RIPs, whether the substitution will reduce the immunoresponses against the toxic moiety and even facilitate the penetration of immunotoxin into tumors, it is worthwhile to be further investigated.

### 3.2. *Ribonucleolytic activity of Luffin-S*

Compared with all reported plant RIPs (RNA *N*-glycosidases) [2,3], Luffin-S showed a different mechanism for inactivating ribosomes. The common RIPs cannot generate cleaved rRNA fragments without aniline treatment after incubation with ribosomes. But just like α-sarcin (phosphodiesterase), Luffin-S did cleave the large rRNAs and generated directly a major band nearly at the same position of the α-fragment on a 1.8% agarose gel (Fig. 4a). We named it the S-fragment. 0.5 μg/ml α-sarcin was usually used in the experiment and only this single cleaved α-fragment was observed even when the concentration of α-sarcin was increased to 50 μg/ml [11]. But this was not the case as to Luffin-S. Though 2.0 μg/ml of Luffin-S was sufficient, we used a higher concentration of 40μg/ml, two orders of magnitude higher than that of α-sarcin in the experiment, to observe the generation and characteristics of the specific S-fragment, as well as the degradation of high M.W. rRNAs.

Luffin-S is heat-labile (100°C for 5 min, not shown), but Subtilisin-resistant even at 2 μg Luffin-S/1 unit Subtilisin, 37°C for 15 min (Fig. 4a, lane 4). We are confident to draw the conclusion that the activity of Luffin-S is not due to any contamination of the common RNase. This is not only true for their difference in M.W., but also because RNase cannot be so easily heat-inactivated, and just a little of it will cause complete degradation of the ribosomal RNA without any observable specific band. In 25 mM Tris-HCl buffer, pH 7.5, addition of 5 mM $MgCl_2$ totally inhibited the ribonucleolytic activity of Luffin-S (result not shown). But RNase activity is not decreased even at a much higher concentration of $Mg^{2+}$.

When naked rRNA was used as substrate, α-sarcin cleaved the rRNAs at multiple sites, generating discrete fragments (Fig. 4b, lane 2), whereas Luffin-S degraded all large rRNAs. However, the Subtilisin-treated Luffin-S showed some specificity and the S-fragment could be still observed (Fig. 4b, lane 4). This suggested that Luffin-S was 'gently inactivated' by the protease. The reduction of Luffin-S' ribonucleolytic activity may render its specificity enhancement. Since only rRNA was concerned in the reaction, these results implied that the structure of rRNA itself, but not ribosomal proteins, may account for this specificity. It was demonstrated that the cleavage site of α-sarcin is embedded in a purine-rich single-stranded segment of 17 nucleotides that is nearly universal [9]. This is one of the most strongly conserved regions of rRNA and is crucial for the function since ribosomes survive treatment with ribonucleases despite many nicks other than this region [12]. The cleaved S-fragment may also contain this region and the highly conserved structure must somehow resist treatment with Luffin-S. The on-going sequencing of the S-fragment will reveal the exact cleavage site on rat liver 28S rRNA.

Recently, Trichosanthin was reported to be a potent inhibitor of HIV-1 replication [13]. Other than a plant RIP, TCS is also a DNase which can cleave the supercoiled double-stranded DNA into nicked circular and linear DNA [14]. In our other preliminary experiments, Luffin-S also shows potent DNase-like activity. Further research is now under way to explore the possibility of its use as an inhibitor against HIV-1 replication.

Actually, we have observed several other Luffin-S-like small M.W. RIPs in the plants of the *Cucurbita* family. They are all potential toxic moieties of immunotoxin. So far, there is no report on plant RIPs acting as the unique α-sarcin. This may be the discovery of a new family of

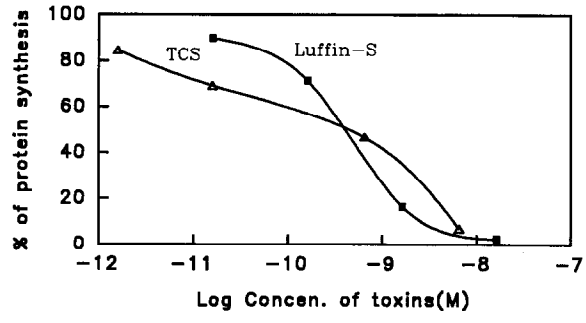


Fig. 3. Inhibitory activities of Luffin-S (■) and TCS (△) in a cell-free protein synthesis system from rabbit reticulocyte lysate. Increasing amounts of sample were added as described. The radioactivity of L-[U-$^{14}$C]leucine incorporated into protein was measured and cpm 14898 was taken as 100% protein synthesis.

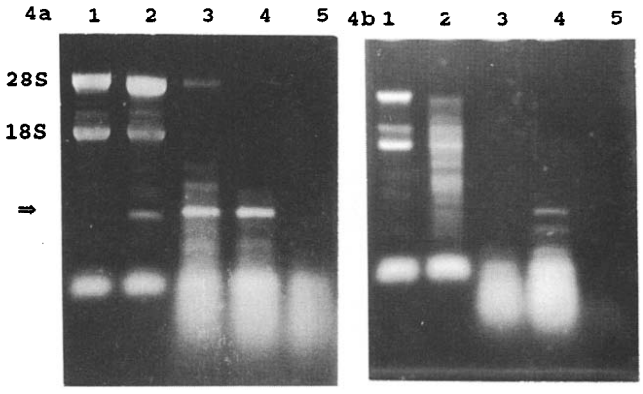


Fig. 4. The effect of purified Luffin-S on ribosome (a) and naked rRNA substrates (b). Total rRNA was extracted from reaction mixtures and analyzed on a 1.8% agarose gel. Samples were: lane 1, only substrates; lane 2, α-sarcin (0.5 μg/ml); lane 3, Luffin-S (40 μg/ml); lane 4, subtilisin-treated Luffin-S (40 μg/ml); lane 5, RNase (1 unit). The arrow shows the α- and S-fragment. The disappearance of the trace amount of 28S rRNA in Fig. 4a, lane 4 was due to the continued presence of the enzyme in the reaction mixture which caused the proteolysis of the ribosomal proteins, facilitating Luffin-S to exert its activity.

plant RIPs with specific phosphodiesterase activity for the first time.

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