Protein-synthesis inhibitory protein from seeds of *Luffa cylindria roem*

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A protein (designated as luffin) with an app. M_r of 26000, which inhibits protein synthesis in rabbit reticulocyte lysate, was purified to homogeneity from the seeds of *Luffa cylindria roem* by extraction with 20 mM Na phosphate buffer (pH 7.2) containing 0.2 M NaCl, ammonium sulfate fractionation, and chromatography on Sephacryl S-200 and CM-Sephadex C-25. Luffin exhibited 10-times as strong inhibitory activity against protein synthesis in rabbit reticulocyte lysate (IC_{50}, 0.42 ng/ml) as that of ricin A-chain, but it showed only a weak cytotoxicity against murine leukemia L1210 cells, an activity of $1/10^6$ to $1/10^5$ that of ricin.

Luffa cylindria roem *Luffin* *Protein synthesis* *Inhibition of protein synthesis* *Rabbit reticulocyte lysate* *Cytotoxicity*

1. INTRODUCTION

Several plant proteins having inhibitory activity against eukaryotic protein synthesis have been reported. These proteins can be classified into two groups: proteins composed of two functionally different subunits like ricin [1] and abrin [2] and those having no subunits like gelonin [3], momordin [4], and PAP [5,6]. In the former proteins, one subunit, the B-chain, binds to the cell surface and helps to transfer into the cell the other subunit, the A-chain, which, once internalized, inhibits protein synthesis. The former proteins exhibit cytotoxicity; the latter proteins are not significantly cytotoxic because they are unable to bind to the cells.

In this study we have isolated, from the seeds of *Luffa cylindria roem*, a protein of the second type having an extremely potent protein-synthesis inhibitory activity in rabbit reticulocyte lysate and designated as luffin.

Abbreviations: Buffer I, 20 mM Na phosphate buffer (pH 7.2) containing 0.2 M NaCl; FBS, fetal bovine serum; 2-ME, 2-mercaptoethanol; PAP, *Phytolacca americana* antiviral protein; PBS, 10 mM phosphate buffer (pH 7.2) containing 0.14 M NaCl; SDS–PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis

2. MATERIALS AND METHODS

2.1. *Tumor cell lines*

Murine leukemia L1210 and sarcoma-180 cell lines were provided by Dr T. Kataoka, Cancer Chemotherapy Center, Japanese Foundation for Cancer Research (Tokyo) and Dr D. Mizuno, Teikyo University (Kanagawa), respectively. L1210 cells were maintained by intraperitoneal passage in DBA/2 mice, and sarcoma-180 cells in ICR mice. For cytotoxicity tests, 7 days after inoculation, ascites tumor cells were collected in PBS and washed 3-times with the culture medium before use.

2.2. *Antisera against ricin A-chain and momordin*

Guinea-pig antiserum against ricin A-chain was prepared as in [7]. For the preparation of antiserum against momordin [4], rabbits were given one intramuscular and one subcutaneous injection of momordin in Freund's complete adjuvant 12

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days apart. Twenty days after the second injection, the rabbits were bled, and the resulting sera inactivated at 56°C for 30 min.

2.3. *Extraction and purification of luffin*

Step 1: Extraction Decorticated seeds (15 g) of *Luffa cylindria roem* (Tomizawa Seedlings Store, Chiba) were ground for 2 min in cold ethyl ether (500 ml) and filtered. The material on the filter was further washed with cold ethyl ether (2 × 500 ml). Extraction of the remaining powder with 10 vol. buffer I at 4°C overnight, followed by centrifugation, gave a crude luffin preparation (supernatant).

Step 2: Ammonium sulfate fractionation Ammonium sulfate was added to the crude extract to 40% saturation and centrifuged at 15000 rev./min for 15 min. The supernatant was separated and saturated with ammonium sulfate. The precipitate was dissolved in 6 ml buffer I and dialyzed against the same buffer.

Step 3: Sephacryl S-200 gel-filtration The solution from step 2 (50 mg protein) was subjected to gel filtration on a Sephacryl S-200 column (3.0 × 134 cm) with buffer I and, based on cell-free protein synthesis assay using rabbit reticulocyte lysate and assay on SDS–PAGE, the fractions containing partially purified ruffin were pooled for further purification.

Step 4: CM-Sephadex C-25 chromatography The pooled fractions in step 3, after concentration by ultrafiltration on a PM-10 filter membrane, were dialyzed against 5 mM Na-phosphate buffer (pH 6.0) and applied to a CM-Sephadex C-25 column (1.0 × 6.6 cm) equilibrated with the same buffer. The column was eluted successively with phosphate buffer containing 0.05, 0.3, and 1 M KCl, respectively, to give luffin in the fractions eluted with 0.3 M KCl, as determined by cell-free protein synthesis assay and SDS–PAGE.

2.4. *SDS–PAGE*

SDS–PAGE was carried out essentially as in [8] on 6 or 8% polyacrylamide gels in 40 mM Tris acetate buffer (pH 7.4) containing 0.1% SDS.

2.5. *Cell-free protein synthesis assay*

Using rabbit reticulocyte lysate prepared by the method in [9], the cell-free protein synthesis assay was performed as described in [10].

3. RESULTS AND DISCUSSION

As summarized in table 1, a protein having a protein-synthesis inhibitory activity in rabbit reticulocyte lysate was extracted and purified from the seeds of *Luffa cylindria roem* and designated as luffin. The protein exhibited a 90-fold higher spec. act. as compared to that of the crude extract prepared with buffer I. During purification, a large increase in activity was gained by Sephacryl S-200 chromatography, which might be due to the removal of inhibitor(s) present in the material.

Fig.1A shows the results of SDS–PAGE of the respective materials obtained at each purification step. The material obtained after CM-Sephadex C-25 chromatography gave a single band at the position of an M_r of 26000 on SDS–PAGE after

Table 1

Purification of luffin from decorticated seeds of *Luffa cylindria roem*

Step	Protein (mg)	Activity (U × 10^{-9})	Spec. act. (U/mg) × 10^{-6}	Yield (%)
Crude extract	618.9	1.63	0.264	100
$(NH_4)_2SO_4$ fraction (40–100% saturation)	161.9	0.61	0.377	37
Sephacryl S-200	26.8	2.55	9.50	156
CM-Sephadex C-25	7.6	1.80	23.7	110

One unit (U) denotes the amount of protein necessary for 50% inhibition of protein synthesis in rabbit reticulocyte lysate. Luffin was purified from 15 g decorticated seeds of *Luffa cylindria roem*

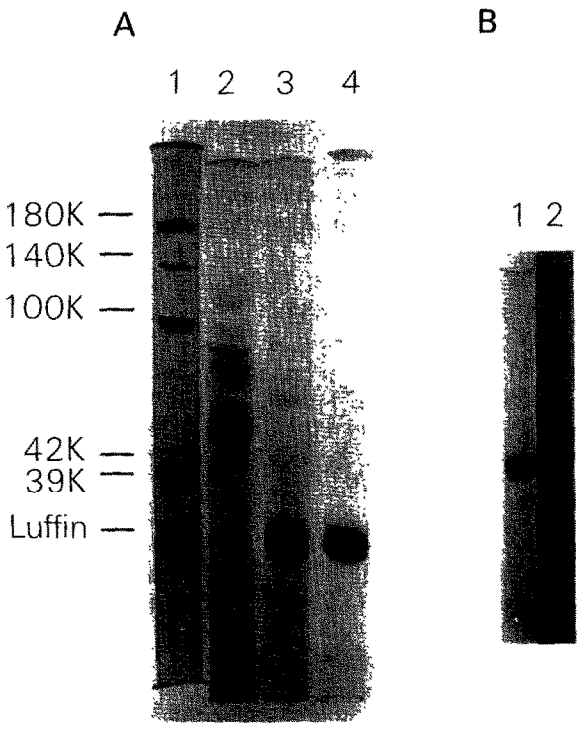


Fig.1. SDS–PAGE analysis. (A) Analysis of proteins in various steps of purification. SDS–PAGE was carried out on 6% gels: (1) luffin (CM-Sephadex C-25 fraction) plus SDS–PAGE marker I [5 subunits of RNA polymerase B (Seikagaku Kogyo, Tokyo)]; (2) 40–100% saturated ammonium sulfate fraction; (3) Sephacryl S-200 fraction; (4) CM-Sephadex C-25 fraction; (B) Analysis of 2-ME- and urea-treated luffin. Luffin was treated with 200 mM 2-ME and 8 M urea for 1 h at 37°C, and the mixture analyzed on SDS–PAGE on 8% gels: (1) untreated luffin; (2) luffin treated with 200 mM 2-ME and 8 M urea.

staining with Coomassie Brilliant Blue R-250 for proteins. The protein (luffin), even when analyzed on SDS–PAGE after treatment with 200 mM 2-ME and 8 M urea, gave a single band (fig.1B). The M_r of 26000, as estimated by SDS–PAGE, was in accord with the elution position of the protein chromatographed on a Sephacryl S-200 column calibrated for M_r determination with goat IgG (150000), ricin (66000), PAP (27000), and cytochrome *c* (12400). These results indicate that luffin is composed neither of subunits linked by disulfide bond(s) nor of those held together by noncovalent bonds, but is a single polypeptide chain of an M_r 26000.

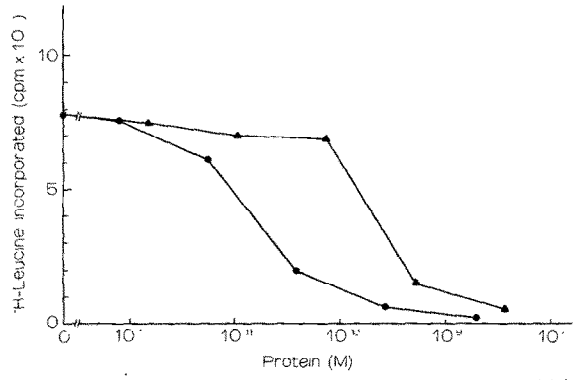


Fig.2. Inhibition of protein synthesis in rabbit reticulocyte lysate by luffin. Rabbit reticulocyte lysate was incubated with serially diluted test materials for 10 min at 37°C in an assay mixture containing L-[4,5-^{3}H]leucine, and the amount of L-[4,5-^{3}H]leucine incorporated was determined: (●—●) luffin; (▲—▲) ricin A-chain.

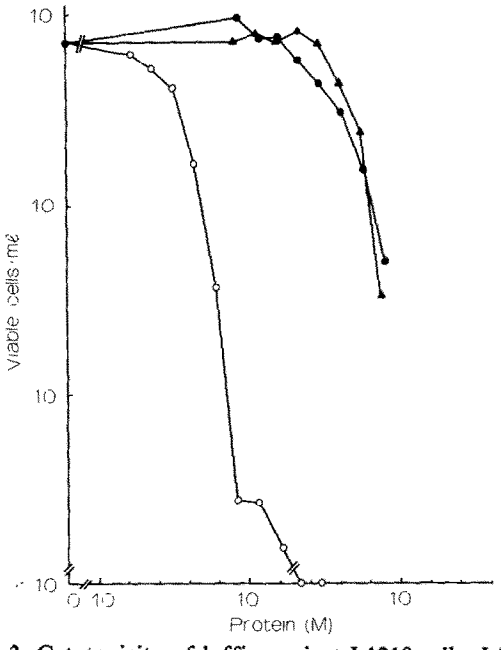


Fig.3. Cytotoxicity of luffin against L1210 cells. L1210 cells were cultured in medium RPMI 1640 (Nissui Seiyaku, Tokyo) (2×10^4 cells/ml) containing 10% heat-inactivated FBS (Grand Island Biological, NY), 20 μM 2-ME, kanamycin sulfate (0.1 mg/ml) and a serially diluted test sample in a 96-well microtest plate (Nunclon no.167008, A/C Nunc, Roskilde) in a humidified atmosphere of 5% CO_2 in air at 37°C for 48 h, and the number of viable cells was determined by counting undyed cells after addition of 1/10 volume of 3% trypan blue in PBS. Cells were cultured in duplicates. The means of the numbers of viable cells are given here: (●—●) luffin; (○—○) ricin; (▲—▲) ricin A-chain.

Luffin exhibited a strong activity of inhibiting protein synthesis in rabbit reticulocyte lysates (IC_{50}, 0.42 ng/ml). This activity is about 10-times as strong as that of the ricin A-chain (fig.2). However, the protein was only weakly cytotoxic to cultured murine leukemia L1210 cells, an activity of $1/10^6$ to $1/10^5$ that of ricin (fig.3). Similarly, no significant cytotoxicity was observed against murine sarcoma-180 cells (not shown).

To investigate whether luffin and ricin A-chain or momordin have such similar structural features which may be regarded as common antigenic determinants, the reactions of anti-ricin A-chain antiserum and of anti-momordin antiserum with luffin were examined by the Ouchterlony method. Neither anti-ricin A-chain antiserum nor anti-momordin antiserum showed a precipitin line with luffin. In addition, there were no cross reactivities in anti-ricin A-chain and anti-momordin antisera between ricin A-chain and momordin. These results indicate that there are no immunologically detectable common structures in ricin A-chain, momordin and luffin.

Investigations on the mechanism of action of luffin in inhibiting protein synthesis will be done in the future.

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