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Original Article

Cloning and soluble expression of mature α-luffin from *Luffa cylindrica* and its antitumor activities *in vitro*

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Luffin-a, a single-chain Type I ribosome-inactivating protein, which is known to be the most toxic of the luffin family and apparently possesses antitumor activity, was isolated from *Luffa cylindrica* seeds. In the present study, mature α-luffin was cloned from *L. cylindrica* and it was found that mature α-luffin shared 96% amino acid similarity with luffin-a. The recombinant mature α-luffin was successfully expressed in a partly soluble form in *Escherichia coli* after optimization of expression conditions. The effects of the recombinant protein on bacterial growth and its *in vitro* protein synthesis inhibition activity were tested. Then, its antitumor activities against different human cancer cell lines were evaluated by CCK-8 assay and flow cytometry. The results indicated that the recombinant α-luffin was slightly toxic to *E. coli*. It could inhibit protein synthesis in the rabbit reticulocyte lysate system. At the same time, it inhibited the growth of the tumor cell lines in a dose- and time-dependent manner. Additionally, recombinant α-luffin was able to induce cell death by apoptosis. The cytotoxicity of α-luffin towards tumor cells makes it a potential antitumor agent.

Keywords luffin; expression; recombinant protein; antitumor activity

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Introduction

Ribosome-inactivating proteins (RIPs), mostly from plants, are a family of highly potent protein toxins that bring about inhibition of protein synthesis by directly interacting with and inactivating the ribosomes or by modification of factors involved in translation. This irreversible modification renders the ribosome unable to bind the elongation factors, leading to the interruption of protein translation. Besides, RIPs also possess a broad spectrum of activities encompassing antiproliferative, antitumor, antiviral, and antifungal activities. Thus, RIPs, especially single-chain Type I RIPs, have been extensively investigated in biological and biomedical fields, and are primary candidates for toxic moiety targeting drugs with potential application in cancer immunotherapy using immunotoxins [1,2].

There exist many inherent problems in the use of native toxins for cancer therapy, such as limited plant resources, complicated and multiple steps in the purification procedures. Therefore, generating the toxin by recombinant techniques may be of great advantage in terms of practical applications. Moreover, recombinant RIPs possess additional advantages for the construction of immunotoxins, either by chemical linkage or as recombinant fusion proteins with monoclonal antibodies or other suitable carriers, when compared with natural RIPs [3].

Luffin, a single-chain Type I RIP extracted from the seeds of the sponge gourd (*Luffa cylindrica*), is known to possess multiple biological activities, such as abortifacient, anti-tumor, and anti-HIV activities [4–7]. Within the luffin family, luffin-a has been the most extensively studied protein [8–10]. Moreover, luffin-a is known to be the most toxic among various types of luffin [11,12], so it was used to construct an immunotoxin with Ng76, a monoclonal antibody to human melanoma cells M(21) [13]. Homogeneous RIP generated using the recombinant DNA technology would be of great benefit for the therapeutic application of luffin-a. In this study, we cloned the α-luffin gene encoding mature α-luffin from *L. cylindrica* and also described the soluble expression in *Escherichia coli*. We further tested the toxicity of the recombinant protein against the host bacteria and investigated its protein synthesis inhibition activity in cell-free system and anti-tumor activities *in vitro*.

Materials and Methods

Plasmid construction

Total RNA was isolated from fresh immature seeds of *L. cylindrica* using Plant RNA Mini Kit (RNeasy Plant RNA Mini kit, Qiagen, Japan) following the manufacturer's instructions. The purity of the isolated RNA was checked spectrophotometrically. The isolated RNA was dissolved in nuclease-free water and stored at −70°C before further use for cDNA synthesis. The reverse transcription was carried out with 2 μg total RNA using AMV First-strand cDNA synthesis Kit (BBI) as per the manufacturer's protocol.

The cDNA sequence data of the signal sequence-deleted region of α-luffin available in the GenBank (GenBank: X62371.1, reported by J. Kataoka [14]) were used to design gene-specific primers for PCR amplification of the cDNA encoding the mature α-luffin. The forward primer (5′-CCATGGCAGATGTGAGGTTCAGTTTGTCAGGT-3′) and the reverse primer (5′-CTCGAGCGCAACATTT TGTTTGTA-3′) contain NcoI and XholI sites, respectively. The PCR product was firstly incubated at 94°C for 5 min, then incubated by a stepped program (94°C, 30 s; 59°C, 30 s; 72°C, 30 s) for 35 cycles, and by an extension at 72°C for 10 min. The PCR product was separated on 1% agarose gels and recovered by gel extraction, then cloned into PTA2 vector (Toyobo), and finally transformed into competent cells of *E. coli* strain DH5α. White colonies were checked by PCR, and the positive colonies were sequenced by Sangon Biotechnology Company (Shanghai, China). The constructed plasmid was named as PTA2-α-luffin.

The confirmed α-luffin sequence was subcloned into the NcoI and XholI sites of pET-28a(+) (Novagen) to construct the recombinant plamid pET-28a-α-luffin. The positive clones were selected and sequenced to ensure the in-frame insertion.

Expression and purification of recombinant proteins

The α-luffin protein was expressed in *E. coli* BL21(DE3)pLysS strain (Invitrogen) by induction with isopropyl-β-D-thiogalactopyranoside (IPTG). The *E. coli* pLysS strain transformed with expression plasmid was grown with constant shaking at 220 rpm in Luria-Bertani (LB) medium supplemented with 50 μg/ml kanamycin, until the OD_{600} of the culture reached 0.5. To identify the optimal conditions, expression was induced by adding IPTG to a final concentration of 1 mM followed by incubation with constant shaking at different temperatures (25°C, 30°C, and 37°C) and at different lengths of time (2, 4, and 6 h). To study the toxicity of the induced recombinant proteins against the host bacteria, the cultures were allowed to grow for 12 h at 37°C and 25°C with IPTG induction, respectively. Bacterial growth was monitored by absorbance at 600 nm. Bacteria cells were harvested by centrifugation at 1500 *g* for 10 min. The pellet was resuspended in 0.5 ml of ice-cold phosphate-buffered saline (PBS), and sonicated four times in ice-cold water using a Bioruptor (Cosmo Bio, Tokyo, Japan) at 200 W for 30 s each time, with a 120 s interval. The soluble and insoluble fractions were separated by centrifugation at 15,000 rpm for 15 min at 4°C. After expression, the recombinant was examined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The soluble fraction was purified using Ni-NTA agarose affinity chromatography according to the manual of QIA expressionist (Qiagen). Western blotting was conducted using RGS-His primary antibody and rabbit anti-mouse secondary antibody conjugated with alkaline phosphatase. The purified protein was dialyzed against TBS buffer (100 mmol L^{-1} Tris−HCl, 50 mmol L^{-1} NaCl, pH 8.0) at 4°C for 24 h. The protein concentration was determined by the BCA (bicinchoninic acid) method (Pierce).

Cell-free translation-inhibiting activity and anti-tumor activities of the recombinant protein *in vitro*

Protein synthesis in rabbit reticulocyte lysate was performed according to the Rabbit Reticulocyte Lysate System Technical Manual (Promega Co.) with slight modifications. Various concentrations of the recombinant protein (10 μl) were added to 40 μl of radioactive working rabbit reticulocyte lysate mixture containing 1 μl [^{3}H]leucine. The incubation proceeded at 37°C for 30 min before addition of 1 M NaOH and 2% H_2O_2. Further incubation for 10 min allowed decolorization and tRNA digestion. One volume of the reaction mixture was then added to 40% trichloroacetic acid with 2% casamino acids in a 96-well plate to precipitate radioactively labeled protein. The precipitate was collected on Whatman GF/A filter and [^{3}H]leucine incorporation was counted in an Beckman liquid scintillation counter.

The purified recombinant protein was tested by the Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) assay for cytotoxicity on JEG-3 (human placental choriocarcinoma cell line), HepG2 (human hepatoma cell line), and MCF-7 (human breast cancer cell line) cells, according to the recommendations of the supplier. Cells were inoculated to 96-well plates at the density of 5000 cells per well for 12 h and cultured in the medium with various concentrations (0.1, 0.5, 1, 2, 4, and 8 μg/ml, respectively) of recombinant protein for 24 h. Ten microliters of CCK-8 solutions was added to each well of the plates, and the plates were incubated for 4 h at 37°C. In addition, cells were incubated with 2 μg/ml recombinant protein at different periods of time (0, 6, 12, 24, and 36 h). Absorbance (*A*) was measured at 450 nm with a microplate reader. All experiments were performed in triplicate on three separate

Table 1 Comparison of the deduced amino acid sequence of mature α-luffin with luffin-a

α-Luffin	DVRFSLSGSS	STSYSKFIGD	LRKALPSNGT	VYNITLLLSS	ASGASRYTLM 50
Luffin-a	DVRFSLSGSS	STSYSKFIGD	LRKALPSNGT	VYNLTILLSS	ASGASRYTLM
				* *	
α-Luffin	TLSNYDGKAI	TVAVDVTNVY	IMGYLVNSTS	YFFNESDAKL	ASQYVFKGST 100
Luffin-a	TLSNYDGKAI	TVAVDVSQLY	IMGYLVNSTS	YFFNESDAKL	ASQYVFKGST
		***			
α-Luffin	IVTLPYSGNY	EKLQTAAGKI	REKIPLGFPA	LDSAITTLFH	YDSTAAAAAF 150
Luffin-a	IVTLPYSGNY	EKLQTAAGKI	REKIPLGFPA	LDSALTTIFH	YDSTAAAAAF
				* *	
α-Luffin	LVIIQTTAEA	SRFKYIEGQI	IERISKNQVP	SLATISLENE	WSALSKQIQL 200
Luffin-a	LVILQTTAEA	SRFKYIEGQI	IERISKNQVP	SLATISLENSL	WSALSKQIQL
	*			**	
α-Luffin	AQTNNGTFKT	PVVITDDKGQ	RVEITNVTSK	VVTKNIQLLL	NYKQNVA 247
Luffin-a	AQTNNGTFKT	PVVITDDKGQ	RVEITNVTSK	VVTKNIQLLL	NYKQNVA

*The different amino acid sites.

occasions. The cell inhibition ratio was calculated as follows: (1 − test group A/control group A) × 100%. The IC_{50} calculation software (LOGIT method) was used for 50% inhibiting concentration (IC_{50}) calculation.

Apoptosis was assessed using annexin V-FITC/propidium iodide (PI) staining according to the instructions of the manufacturer (Annexin V- FITC Apoptosis Detection Kit; BD Biosciences). In brief, exponentially growing JEG-3 cells were exposed to 2 and 4 μg/ml recombinant protein for 24 h, respectively. Then, the cells were resuspended in binding buffer, and incubated with Annexin V-FITC and PI at room temperature in the dark. Subsequently, analysis was performed in a FACSCan flow cytometer (Becton-Dickinson, USA) using the CellQuest software. Control cells were treated with the medium only. The data were obtained from three independent experiments with duplicates.

Statistical analysis

All data are expressed as mean ± standard deviation. Differences between groups were compared using analysis of variance (ANOVA) for repeated measures. Differences were considered significant when *P* value was less than 0.05.

Results

Cloning of mature α-luffin and sequence comparison

Total RNA was isolated from fresh immature seeds of *L. cylindrica* and the cDNAs were synthesized by reverse transcription and a 750 bp cDNA fragment was obtained. The deduced amino acids are shown in **Table 1**. There are 10 different amino acids between the mature α-luffin and luffin-a sequence submitted by Islam *et al*. [15], which shows 96% identity at amino acid level.

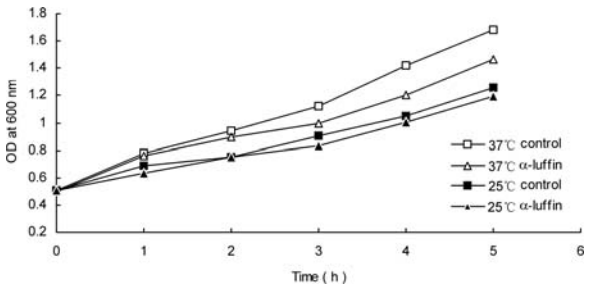

Figure 1 Effect of α-luffin on bacterial growth *E. coli* were grown in LB media with 1 mM IPTG induction when bacterial absorbance was 0.5 at 600. *E. coli* (pLysS) without plasmid at 37°C (open squares); *E. coli* (pLysS) with pET28a-α-luffin at 37°C (open triangles); *E. coli* (pLysS) without plasmid at 25°C (closed squares); *E. coli* (pLysS) with pET28a-α-luffin at 25°C (closed triangles).

Bacterial growth, expression and purification of the recombinant protein

α-luffin was labeled with an N-terminal His_6-tag and expressed in the *E. coli* pLysS strain by induction with IPTG. As seen in **Fig. 1**, bacterial expression of α-luffin was slightly toxic to *E. coli*, and the rate of bacterial growth was reduced as incubation time increased. Absorbance analysis suggested that prolonged exposure to recombinant α-luffin may have a negative effect on bacterial growth.

To evaluate the effect of temperature on the expression of α-luffin, we investigated the distribution of the recombinant protein expressed at different temperatures as analyzed by SDS-PAGE [**Fig. 2(A)**]. The results showed that the majority or almost all of the recombinant protein induced at 30°C or 37°C formed insoluble inclusion bodies. However,

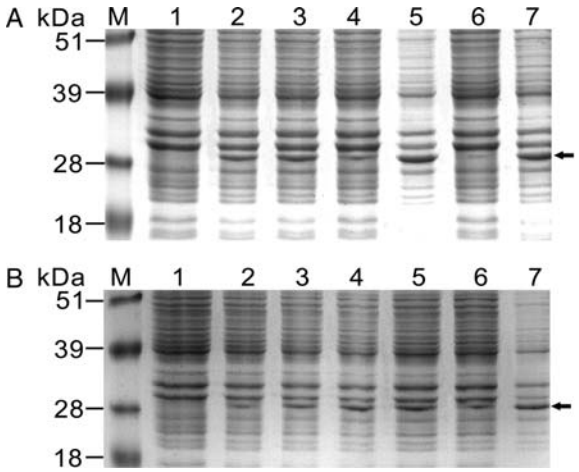

Figure 2 SDS-PAGE analysis of recombinant proteins expressed at different temperatures and at different times. (A) The distribution of recombinant proteins expressed at different temperatures: M, molecular standard marker; 1, control (*E. coli*/pET28 only at 25°C); 2, the supernatant at 25°C; 3, the pellet at 25°C; 4, the supernatant at 30°C; 5, the pellet at 30°C; 6, the supernatant at 37°C; and 7, the pellet at 37°C. (B) The distribution of recombinant proteins expressed for different time at 25°C: M, molecular weight markers; 1, control (*E. coli*/pET28 only); 2, the supernatant at 2 h; 3, the pellet at 2 h; 4, the supernatant at 4 h; 5, the pellet at 4 h; 6, the supernatant at 6 h; and 7, the pellet at 6 h.

the recombinant protein expressed at 25°C was distributed both in the pellet and in the supernatant. The recombinant mature α-luffin was produced in a partly soluble form.

To identify an optimal length of the incubation, the bacteria were induced at 25°C for 2, 4, and 6 h, respectively. The distribution of the recombinant proteins was analyzed on a 12% SDS-PAGE [**Fig. 2(B)**]. The results indicated that the maximum level of expression for α-luffin was attained in a soluble form if the length of the incubation was 4 h.

The size of the induced protein bands was about 28 kDa by SDS-PAGE, which was slightly larger than the cDNA-deduced amino acid sequence of the mature α-luffin (theoretical weight, 26.95 kDa). The difference was in part due to the presence of the extra N-terminal region containing six histidine amino acids. After purification (the final yield of soluble recombinant protein was about 0.45 mg/l bacterial cell culture), the recombinant protein exhibited a single band in 12% SDS-PAGE [**Fig. 3(A)**]. Western blotting analysis showed that the induced samples had a positive signal at a 28 kDa position, whereas there was no signal in the uninduced sample [**Fig. 3(B)**]. This result further confirmed that the induced *E. coil* had expressed the recombinant protein with a 6 × His-tag.

Cell-free translation-inhibiting activity, cytotoxic activities and apoptosis induction by recombinant α-luffin

The inhibitory effect of recombinant α-luffin on protein synthesis in the rabbit reticulocyte lysate translation system

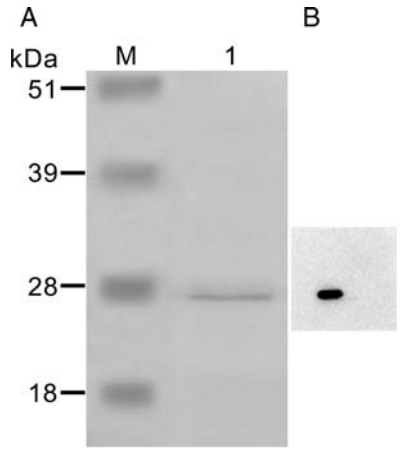

Figure 3 The soluble fraction of recombinant proteins purified by Ni-NTA agarose affinity chromatography (A) Protein molecular weight standard (M), and purified α-luffin (1). (B) Western blot of purified α-luffin.

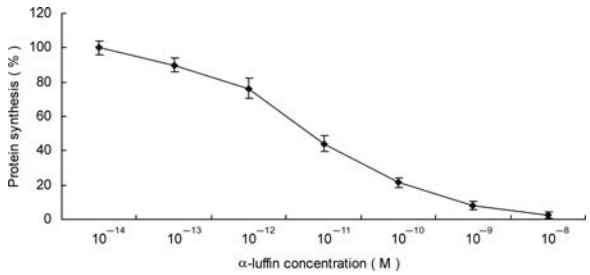

Figure 4 Inhibitory activity of α-luffin in a cell-free protein synthesis system from reticulocyte lysate (data represent means ± SD, $n = 3$).

is shown in **Fig. 4**. The IC_{50} value of recombinant α-luffin was determined as 3.41×10^{-11} M.

To test whether recombinant α-luffin affected the growth of tumor cells, viability assays were performed *in vitro*. When JEG-3, HepG2, and MCF-7 cells were incubated with α-luffin at concentrations ranging 0.1–8 μg/ml and at different time points post-treatment, the inhibitory effect on cell growth was measured by CCK-8 assay. The results showed that α-luffin inhibited the growth of JEG-3, HepG2, and MCF-7 cells in a dose-dependent manner at 24 h post-treatment with different concentrations (0.1, 0.5, 1, 2, 4, and 8 μg/ml) of α-luffin [**Fig. 5(A)**]. The inhibition of cell viability increased with the increase of α-luffin concentration. The IC_{50} values, determined after 24 h incubation, were 3.65 ± 0.66, 7.03 ± 1.03, and 10.42 ± 2.72 μg/ml for JEG-3, HepG2, and MCF-7 cells, respectively. The results are shown in **Fig. 6**. It seemed that the cytotoxicity of α-luffin to JEG-3 cells was stronger than to HepG2 cells, but there was no significant difference between the two groups ($P > 0.01$). Also, no statistically significant difference between HepG2 and MCF-7 group was found. The cytotoxicity of α-luffin to JEG-3 cells was significantly stronger, as compared with MCF-7 cells ($P < 0.01$). In general, JEG-3 cells showed higher sensitivity to

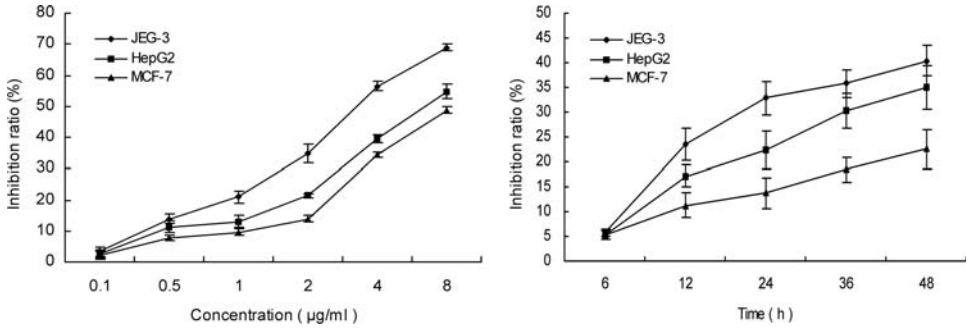

Figure 5 Cytotoxicity analysis of α-luffin to JEG-3, HepG2, and MCF-7 cells by CCK-8 method (A) The cells were treated with different concentrations of α-luffin for 24 h. (B) The cells were incubated with 2 μg/ml of α-luffin for the indicated periods. The results show means ± SD of triplicate experiments.

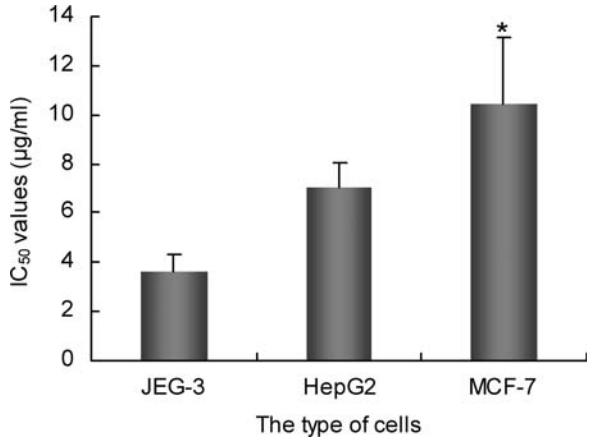

Figure 6 Comparison of the IC_{50} values for JEG-3, HepG2, and MCF-7 cells Horizontal axis and vertical axis indicate axis relative cell types and IC_{50} values, respectively (*$P < 0.01$, compared with JEG-3).

α-luffin. After treatment with 2 μg/ml α-luffin over different periods of time (6, 12, 24, 36, and 48 h), the results demonstrated that cell growth was inhibited in a time-dependent manner [**Fig. 5(B)**].

To elucidate the possibility that the inhibition of cell proliferation was associated with the induction of apoptosis after α-luffin treatment, further experiments were performed using JEG-3 cells to evaluate the effect of α-luffin on apoptosis using an annexin V/PI double-staining assay. As shown in **Fig. 7**, after treatment with 2 and 4 μg/ml α-luffin for 24 h, the percentage of early apoptotic cells increased from 3.39 ± 0.87% to 6.69 ± 1.59% and to 12.65 ± 2.23%, respectively. The percentages of late apoptotic and necrotic cells also increased from 4.39 ± 0.56% to 10.46 ± 1.33% and to 20.79 ± 1.33%, respectively. The use of α-luffin at 2 μg/ml significantly increased the number of cells in late apoptosis ($P < 0.01$), and at 4 μg/ml, α-luffin significantly increased the number of apoptotic cells during both early and late apoptosis ($P < 0.01$). These results suggested that α-luffin was able to induce cell death by apoptosis, especially at a higher concentration. It is supposed that apoptosis induced by α-luffin was involved in its antitumor effect.

Discussion

RIPs have been of considerable interest due to their multiple pharmacological properties and biological activities, such as their potential use as plant defense factors [16,17] and immunotoxins [18–20]. Type I RIPs, consisting of a single polypeptide chain, have great therapeutic importance in the construction of targeting drugs by coupling the toxin to cell-specific targeting molecules, such as antibodies, cytokines, and peptides to form an immunotoxin. Isolating and characterizing new RIPs or homogeneous recombinant RIPs will benefit the exploitation of novel agents that could play important roles in cancer treatment, immunotherapy, and treatment of viral diseases. Therefore, numerous studies have been carried out to search for novel and/or recombinant Type I RIPs with desirable properties.

In this study, the cDNA sequence encoding the mature α-luffin was cloned from the fresh seeds of *L. cylindrica*. It was found that the deduced amino acid sequence of the mature α-luffin identified was very similar to that of luffin-a. There were 10 different amino acids and significant homology between mature α-luffin and luffin-a, exhibiting 96% amino acids identity. The difference could be due to errors in protein sequencing as ricin [21], the heterogeneity of luffin-a expressed in variants of *L. cylindrica*, or to different forms of luffin-a in seeds.

The effect of α-luffin on bacterial growth was studied to understand its toxicity. Our results indicated that the bacterial expression of α-luffin was slightly toxic to *E. coli* when the incubation time was prolonged. In the past, many studies on the toxicity of RIPs against the host bacteria showed that the growth of *E. coli* harboring the RIP cDNA was retarded to varying degrees. This growth inhibition seemed to be due to the result of their partial depurination of bacterial ribosomes [22–26]. This depurination may result in protein synthesis inhibition in *E. coli*, as it has

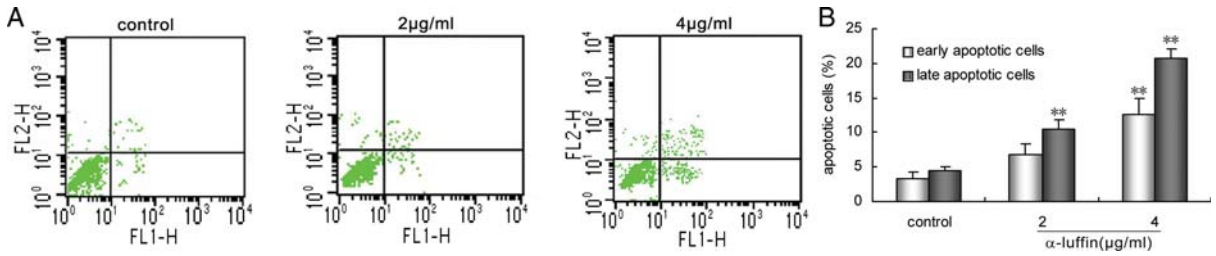

Figure 7 Apoptosis induction by α-luffin in JEG-3 cells JEG-3 cells were treated with 2 and 4 μg/ml α-luffin for 24 h, respectively. Apoptosis was quantified using Annexin V/PI staining followed by flow cytometric analysis. (A) The representative picture in each experimental group. (B) The percentage of apoptotic cells (*$P < 0.01$, compared with corresponding control).

been suggested that A-2660 interacts with the elongation factors EF-G and EF-Tu [23,27]. However, some other studies indicated that the RIP recombinant protein had no toxic effect on bacterial cells [28–30]. Our experimental results indicated that the expression of α-luffin was barely toxic to *E. coli* at 25°C. We proposed that a lowering of growth temperature brought about a reduction in growth rate of *E. coli*, which likely weakened the interaction with the host *E. coli* ribosomes.

The expression of RIPs in bacteria as recombinant proteins is complicated because of their cellular toxicity. Most of the expressed recombinant proteins were insoluble inclusion bodies [10,31–34]. The most critical problem in handling inclusion bodies is the solubilization step in which strong denaturing agents such as urea are used. Under these conditions, the protein has to be refolded subsequently to regain its native structure. Unfortunately, during such refolding, the protein might reaggregate or refold incorrectly. Eventually, this could lead to a very low recovery of the active product. In this study, different induction parameters have been modified to improve the expression levels of soluble proteins: growth temperature was lowered from 37°C and 30°C to 25°C and incubation time altered from 2 h and 4 h to 6 h. The results showed that a maximal yield of soluble proteins was achieved by induction with 1.0 mM IPTG for 4 h at 25°C. These data indicated that solubility of the protein increased as the induction temperature decreased. We suppose that conditions favoring a reduced growth rate could diminish recombinant protein synthesis to a reasonably low speed, facilitating the correct folding into a soluble and biologically active form. This is consistent with the observations of Balan *et al*. [35]. Temperature was the main factor that influenced the growth of *E. coli* and the expression of proteins. Moreover, the influence was not due to one factor alone, but the result of various factors interacting together [36].

In this study, the recombinant α-luffin displayed a strong inhibitory activity on protein synthesis in the cell-free rabbit reticulocyte system. To understand the cytotoxicity of recombinant α-luffin against tumor cells, we tested the inhibitory effect of α-luffin on JEG-3, HepG2, and MCF-7 cells growth. The results showed that recombinant α-luffin inhibited the proliferation of the three different types of cancer cell lines in a dose- and time-dependent manner. The data suggested that α-luffin had antitumor activities *in vitro*. Additionally, α-luffin showed various degrees of cytotoxicity against different cell types. The cytotoxicity of α-luffin to JEG-3 cells was significantly stronger than that to MCF-7 cells. Although Type I RIPs were devoid of the cell-binding B chain, and their capability of being uptaken into cells was very poor, they can be cytotoxic at high concentrations. Initially it was thought that Type I RIPs might enter cells via passive mechanisms such as fluid phase uptake [37]. However, different cell types showed a highly variable sensitivity toward each RIP. Those with normally high pinocytotic/phagocytic activity, such as macrophages, are more sensitive to Type I RIPs [38,39]. Further studies suggested a close correlation between the cytotoxicity and the intracellular routing of RIP, which may vary among different cell types depending on: (i) the expression of different types of binding molecules on the cell surface; (ii) the sorting of ligands leading to different compartments; and (iii) the availability of various pathways for the transport of the toxin to the cytosolic target [40]. Some studies had demonstrated that macrophages-, trophoblasts-, and choriocarcinoma-derived BeWo cell line, and the human neuroblastoma-derived NB100 cell line seem to be the most sensitive cell types to single-chain RIPs [22]. Our results also indicated that the different cell types varied in their sensitivities to α-luffin, and JEG-3 cells showed higher sensitivity to α-luffin. We supposed that the distinct inhibition of JEG-3 is possibly the result of more powerful pinocytosis of JEG-3 to α-luffin as trophoblastic cells and the ability to take up a wide variety of substances by different surface receptors, making α-luffin easier to enter into the cells and exert its cytotoxicity.

The cytotoxicity of RIPs could be commonly attributed to the inhibition of protein synthesis as a consequence of ribosomal damage. However, it was found that the morphology of cells treated with RIPs resembled that of cells undergoing death by apoptosis [41]. Many studies have

demonstrated that Type I RIPs are able to bring about cell death by inducing apoptosis [42–46]. Though many reports are available on toxin-induced apoptosis, the mechanisms involved are still unclear. In the present study, flow cytometry analysis of α-luffin-treated cells stained using annexin V-FITC/PI indicated that α-luffin significantly increased the number of apoptotic cells, especially at higher concentrations. We concluded that apoptosis induction by α-luffin was involved in its antitumor effects. However, the mechanism and pathways of apoptosis induction are still intriguing issues, which need further efforts to resolve. The present findings provide an experimental foundation for future researches.

In conclusion, this study described, for the first time, the cloning of mature α-luffin from *L. cylindrica* and its partly soluble expression in *E. coli.* The recombinant α-luffin could inhibit protein synthesis in rabbit reticulocyte lysate systems. Additionally, it was able to inhibit the growth of tumor cells and induce apoptosis. The cytotoxicity of α-luffin towards tumor cells makes it a potential antitumor agent, especially for developing RIP-based immunotoxin. This has provided us with much encouragement to continue studying α-luffin with respect to immunotoxins and anti-tumor medicine.

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References

1 Bolognesi A, Polito L, Tazzari PL, Lemoli RM, Lubelli C, Fogli M and Boon L, *et al*. *In vitro* anti-tumour activity of anti-CD80 and anti-CD86 immunotoxins containing type 1 ribosome-inactivating proteins. Br J Haematol 2000, 110: 351–361.

2 Qi L, Nett TM, Allen MC, Sha X, Harrison GS, Frederick BA and Crawford ED, *et al*. Binding and cytotoxicity of conjugated and recombinant fusion proteins targeted to the gonadotropin-releasing hormone receptor. Cancer Res 2004, 64: 2090–2095.

3 McGrath MS, Rosenblum MG, Philips MR and Scheinberg DA. Immunotoxin resistance in multidrug resistant cells. Cancer Res 2003, 63: 72–79.

4 Ng TB, Wong RN and Yeung HW. Two proteins with ribosome-inactivating, cytotoxic and abortifacient activities from seeds of *Luffa cylindrica* roem (Cucurbitaceae). Biochem Int 1992, 27: 197–207.

5 Ling J, Liu WY and Wang TP. Cleavage of supercoiled double-stranded DNA by several ribosome-inactivating proteins *in vitro*. FEBS Lett 1994, 345: 143–146.

6 Poma A, Miranda M and Spano L. Differential response of human melanoma and Ehrlich ascites cells *in vitro* to the ribosome-inactivating protein luffin. Melanoma Res 1998, 8: 465–467.

7 Au TK, Collins RA, Lam TL, Ng TB, Fong WP and Wan DC. The plant ribosome inactivating proteins luffin and saporin are potent inhibitors of HIV-1 integrase. FEBS Lett 2000, 471: 169–172.

8 Kishida K, Masuho Y and Hara T. Protein-synthesis inhibitory protein from seeds of *Luffa cylindria* roem. FEBS Letters 1983, 153: 209–212.

9 Kamenosono M, Nishida H and Funatsu G. Isolation and characterization of two luffins, protein-biosynthesis inhibitory proteins from the seeds of *Luffa cylindrica*. Agr Biol Chem 1988, 52: 1223–1227.

10 Xu XR, Zhan JB and Xia Z. Cloning and expression of luffin—a gene from the seeds of *Luffa cylindrical*. Zhejiang Da Xue Xue Bao Yi Xue Ban 2005, 34: 207–211, 216.

11 Stirpe F and Barbieri L. Ribosome-inactivating proteins up to date. FEBS Lett 1986, 195: 1–8.

12 Li F, Yang XX, Xia HC, Zeng R, Hu WG, Li Z and Zhang ZC. Purification and characterization of Luffin P1, a ribosome- inactivating peptide from the seeds of *Luffa cylindrica*. Peptides 2003, 24: 799–805.

13 Gao WD, Zhang RP, Cao HT, Ji RH and Zhang ZC. Construction of luffin A immunotoxin and its *in vitro* inhibition against human melanoma cell M21. Chin Sci Bull 1994, 39: 950–953.

14 Kataoka J, Habuka N, Miyano M, Masuta C and Koiwai A. Nucleotide sequence of cDNA encoding α-luffin, a ribosome-inactivating protein from *Luffa cylindrica*. Plant Mol Biol 1992, 18: 1199–1202.

15 Islam MR, Nishida H and Funatsu G. Complete amino acid sequence of luffin-a, a ribosome-inactivating protein from the seeds of *Luffa cylindrica*. Agric Biol Chem 1990, 54: 1343–1345.

16 Jach G, Gornhardt B, Mundy J, Logemann J, Pinsdorf E, Leah R and Schell J, *et al*. Enhanced quantitative resistance against fungal disease by combinatorial expression of different barley antifungal proteins in transgenic tobacco. Plant J 1995, 8: 97–109.

17 Dowd PF, Zuo WN, Gillikin JW, Johnson ET and Boston RS. Enhanced resistance to Helicoverpa zea in tobacco expressing an activated form of maize ribosome-inactivating protein. J Agric Food Chem 2003, 51: 3568–3574.

18 Barbieri L, Bolognesi A, Valbonesi P, Polito L, Olivieri F and Stirpe F. Polynucleotide: adenosine glycosidase activity of immunotoxins containing ribosome-inactivating proteins. J Drug Target 2000, 8: 281–288.

19 Herrera L, Yarbrough S, Ghetie V, Aquino DB and Vitetta ES. Treatment of SCID/human B cell precursor ALL with anti-CD19 and anti-CD22 immunotoxins. Leukemia 2003, 17: 334–338.

20 Smallshaw JE, Ghetie V, Rizo J, Fulmer JR, Trahan LL, Ghetie MA and Vitetta ES. Genetic engineering of an immunotoxin to eliminate pulmonary vascular leak in mice. Nat Biotechnol 2003, 21: 387–391.

21 Roberts LM, Lamb FI, Pappin DJ and Lord JM. The primary sequence of *Ricinus communis* agglutinin. Comparison with ricin. J Biol Chem 1985, 260: 15682–15686.

22 Barbieri L, Battelli MG and Stirpe F. Ribosome-inactivating proteins from plants. Biochim Biophys Acta 1993, 1154: 237–282.

23 Dore JM, Gras E, Depierre F and Wijdenes J. Mutations dissociating the inhibitory activity of the pokeweed antiviral protein on eukaryote translation and *Escherichia coli* growth. Nucleic Acids Res 1993, 21: 4200–4205.

24 Girbes T, Barbieri L, Ferreras M, Arias FJ, Rojo MA, Iglesias R and Alegre C, *et al*. Effects of ribosome-inactivating proteins on *Escherichia coli* and *Agrobacterium tumefaciens* translation systems. J Bacteriol 1993, 175: 6721–6724.

25 Kataoka J, Ago H, Habuka N, Furuno M, Masuta C, Miyano M and Koiwai A. Expression of a pokeweed antiviral protein in *Escherichia coli* and its characterization. FEBS Lett 1993, 320: 31–34.
26 Xu J, Kaloyanova D, Ivanov IG and AbouHaidar MG. The low expression level of pokeweed antiviral protein (PAP) gene in *Escherichia coli* by the inducible lac promoter is due to inefficient transcription and translation and not to the toxicity of the PAP. Arch Biochem Biophys 1998, 351: 82–88.
27 Moazed D, Robertson JM and Noller HF. Interaction of elongation factors EF-G and EF-Tu with a conserved loop in 23S RNA. Nature 1988, 334: 362–364.
28 Gholizadeh A, Kohnehrouz BB, Santha IM, Lodha ML and Kapoor HC. Cloning and expression of small cDNA fragment encoding strong antiviral peptide from *Celosia cristata* in *Escherichia coli*. Biochemistry (Mosc) 2005, 70: 1005–1010.
29 Vepachedu R, Park SW, Sharma N and Vivanco JM. Bacterial expression and enzymatic activity analysis of ME1, a ribosome-inactivating protein from *Mirabilis expansa*. Protein Expr Purif 2005, 40: 142–151.
30 Choudhary N, Kapoor HC and Lodha ML. Cloning and expression of antiviral/ribosome-inactivating protein from *Bougainvillea xbuttiana*. J Biosci 2008, 33: 91–101.
31 Rajamohan F, Engstrom CR, Denton TJ, Engen LA, Kourinov I and Uckun FM. High-level expression and purification of biologically active recombinant pokeweed antiviral protein. Protein Expr Purif 1999, 16: 359–368.
32 Kwon SY, An CS, Liu JR, Kwak SS, Lee HS, Kim JK and Paek KH. Molecular cloning of a cDNA encoding ribosome inactivating protein from *Amaranthus viridis* and its expression in *E. coli*. Mol Cells 2000, 10: 8–12.
33 Pittaluga E, Poma A, Tucci A and Spano L. Expression and characterisation in *E. coli* of mutant forms of saporin. J Biotechnol 2005, 117: 263–266.
34 Luo MJ, Liu WX, Yang XY, Xu Y, Yan F, Huang P and Chen F. Cloning, expression, and antitumor activity of recombinant protein of curcin. Russ J Plant Physiol 2007, 54: 202–206.
35 Balan A, de Souza CS, Moutran A, Ferreira RC, Franco CS, Ramos CH and de Souza Ferreira LC. Purification and in vitro characterization of the maltose-binding protein of the plant pathogen Xanthomonas citri. Protein Expr Purif 2005, 43: 103–110.
36 Strandberg L and Enfors SO. Batch and fed batch cultivations for the temperature induced production of a recombinant protein in *Escherichia coli*. Biotechnol Lett 1991, 13: 609–614.
37 Stirpe F, Barbieri L, Battelli MG, Soria M and Lappi DA. Ribosome-inactivating proteins from plants: present status and future prospects. Biotechnology 1992, 10: 405–412.
38 Skilleter DN, Paine AJ and Stirpe F. A comparison of the accumulation of ricin by hepatic parenchymal and non-parenchymal cells and its inhibition of protein synthesis. Biochim Biophys Acta 1981, 677: 495–500.
39 Simmons BM, Stahl PD and Russell JH. Mannose receptor-mediated uptake of ricin toxin and ricin A chain by macrophages. Multiple intracellular pathways for a chain translocation. J Biol Chem 1986, 261: 7912–7920.
40 Sandvig K, Grimmer S, Lauvrak SU, Torgersen ML, Skretting G, van Deurs B and Iversen TG. Pathways followed by ricin and Shiga toxin into cells. Histochem Cell Biol 2002, 117: 131–141.
41 Griffiths GD, Leek MD and Gee DJ. The toxic plant proteins ricin and abrin induce apoptotic changes in mammalian lymphoid tissues and intestine. J Pathol 1987, 151: 221–229.
42 Williams JM, Lea N, Lord JM, Roberts LM, Milford DV and Taylor CM. Comparison of ribosome-inactivating proteins in the induction of apoptosis. Toxicol Lett 1997, 91: 121–127.
43 Komatsu N, Oda T and Muramatsu T. Involvement of both caspase-like proteases and serine proteases in apoptotic cell death induced by ricin, modeccin, diphtheria toxin, and pseudomonas toxin. J Biochem 1998, 124: 1038–1044.
44 Luo MJ, Yang XY, Liu WX, Xu Y, Huang P, Yan F and Chen F. Expression, purification and anti-tumor activity of curcin. Acta Biochim Biophys Sin 2006, 38: 663–668.
45 Gan YH, Peng SQ and Liu HY. Molecular mechanism of apoptosis induced by ricin in HeLa cells. Acta Pharmacol Sin 2000, 21: 243–248.
46 Li M, Chen Y, Liu Z, Shen F, Bian X and Meng Y. Anti-tumor activity and immunological modification of ribosome-inactivating protein (RIP) from *Momordica charantia* by covalent attachment of polyethylene glycol. Acta Biochim Biophys Sin 2009, 41: 792–799.