Iranian Journal of Pharmaceutical Sciences
Autumn 2011: 7(4): 247-254
ijps.sums.ac.ir

Original Article

Extraction, Cloning and Expression of RTB, as a Vaccine Adjuvant/Carrier, in *E. coli* and Production of Mouse Polyclonal Antibody (Anti-B chain Abs)

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Abstract

Ricin, the toxic lectin extracted from the castor bean plant (Ricinus communis), consists of an A chain (RTA) and a B chain (RTB). Anti-A chain Abs and anti-B chain Abs can neutralize toxins *in vivo* and *in vitro* via blocking the binding of the toxin to the cell. Also, RTB protein is able to serve as an antigen deliver to the mucosal immune system and act as an immunoadjuant. Here, the genomic DNA was extracted from the fresh leave of the castor plant. The *RTB* gene was amplified by PCR. The prokaryotic expression vector pET-28a (+)- RTB was constructed, and used to transform *E. coli* Rosetta(DE3). The expression of recombinant protein induced by IPTG was examined by SDS-PAGE. Western blot were used to determine immunoreactivity of RTB-His by a rabbit monoclonal antibodies against His-tag. The SDS-PAGE profile exhibited the constructed prokaryotic expression efficiently produced RTB at the 1 mmol/L of IPTG. Addition of glycine and Triton X-100 enhanced native extracellular protein excreted into the culture medium. Anti-RTB polyclonal serum was generated by repeated immunization of mice with recombinant RTB protein. Finally, the antigenicity of recombinant RTB was identified by Western blot and indirect ELISA. A relative high titer of anti-RTB antibody was detected after the fourth injection. Western blot analysis was carried out with the polyclonal antibody revealed almost a 32-kDa band which corresponds to RTB protein. In conclusion, we herein report the expression of fully biologically active RTB as a plant lectin by a new strategy. This recombinant Ricin protein could be a promising drug for cancer therapy, vaccine as an immune response enhancement and even viral infected cells.

Keywords: Cloning; *E. coli* Rosetta (DE3); Expression; Lectin; Polyclonal antibody; Ricin toxin B.
Received: April 15, 2011; *Accepted:* July 15, 2011.

1. Introduction

Ricin is a cytotoxic lectin from Ricinus communis seeds which was the first plant Ribosome Inhibitor Protein (RIP) to be cloned [1]. In 1985 both cDNA and genomic clones encoding preproricin were isolated as a cDNA clone encoding for preproricin [2]. The DNA sequence of the genomic ricin clone showed

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that the gene did not contain introns. Ricin is a heterodimeric toxin of the AB form. The A chain is a 32 kDa enzyme which attacks the 60 S subunit of eukaryotic ribosomes and inactivates them. The B chain (RTB), a glycoprotein which is linked to the A chain by a disulfide bond whose molecular mass is also around 32 kDa [2, 3]. The B chain functions to assist the A chain in escaping from the endocytotic vesicle, thereby killing the intoxicated cell. Also RTB in fusion form with vaccine candidates may function as a mucosal vaccine adjuvant/carrier [4]. Disulfide bonds for galactose-binding activity, and also N-glycosylation give stability on the molecule. With these characteristics, RTB can be expressed in *E. coli*, provided some appropriate conditions, that are applied to direct the product to the periplasmic space. The RTB produced in *E. coli* is initially biologically active, but due to the lack of glycosylation, it is relatively unstable [5]. The absence of glycosylation appears to be less crucial for stability, if individual ricin toxin subdomains are expressed independently [6]. Today, scientists have found several therapeutic applications of ricin and its subdomains. Delivery to the target cell is achieved by linking the toxin to an antibody or growth factor to create conjugates called immunotoxins, which show great promise in the design of specific antitumor and other therapeutic agents [2]. At present, the *in vivo* application of whole ricin immunotoxins is not feasible since the ricin B chain galactose-binding capacity overrides the cell-type targeting specificity conferred by the antibody [6].

Both anti-A chain and anti-B chain Abs can neutralize toxins *in vivo* and *in vitro*. B chain Abs blocks binding of the toxin to the cell because one mechanism by which Abs can protect is by blocking the entry of toxins into cells [7]. It shows that producing a polyclonal antibody can protects animals against this toxin [8]. On the other hand, expression of RTB as protein-based adjuvant capable to facilitate antigen delivery to mucosal immune-responsive cells and to function as a mucosal adjuvant is critical for the development of mucosal subunit vaccines [9]. A prerequisite for this case is the ability to express biologically active, recombinant ricin B chain. Previous work has shown that this can be achieved in eukaryotic hosts if the recombinant B chain is directed into the endomembrane system using homologous or heterologous eukaryotic signal sequences

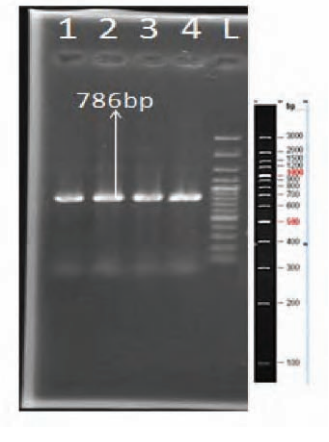

Figure 1. The target fragment of RTB gene amplified from *Castor bean*. Lane 1,2,3,4: The target amplification of RTB gene. Lane L: 100 bp DNA size marker. (#SM0623 purchased from Fermentas Co.).

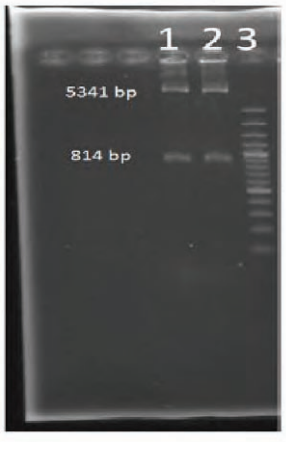

Figure 2. Agarose gel electrophoresis analysis of recombinant pET-28a(+)-*RTB*. Lane1, 2: Double digests of recombinant pET-28a(+)-*RTB* with *sal*I and *Hind*III. Lane3: 100bp DNA size marker (#SM0623 purchased from fermentas co.). Notice: The addition length of gene is because of restriction enzyme sites in primers.

[10]. In this study, we show that biologically active recombinant ricin B chain not only can be produced in *E. coli* using a bacterial signal sequence which targets the product to the periplasmic space, [10] but also with chemical treatments using Triton X-100 and glycine, we can produce soluble protein [11]. Finally through this method, we provided anti-Ricin chain B polyclonal antibody that will help us in our next studies.

2. Materials and methods

2.1. Enzymes, vectors and bacterial strains

Pfu DNA polymerase (2.5 U/µl, Fermentas, Lithuania), Enzymes NotI, *Hind*III and *Sal*I (Fermentas, Lithuania), IPTG (Vivantis, Malesia), Vector pET-28a (+) (Novagen USA), Vector pGEM-T (Promega, USA), 96-well ELISA plates (Greiner-GmbH, Kremsmunster, Austria), *Ricinus communis* seeds was prepared from Tehran University. The genomic DNA of plant was Extracted and used as the template in PCR experiment. *E. coli* DH5α and *E. coli* Rosetta (DE3) were used for cloning and expression experiments, respectively. Plasmid pGEM-T Vector and pET-28a (+) were used as cloning and expression vectors, respectively.

2.2. Amplification of RTB gene

Genomic DNA was extracted from fresh leave by a routine CTAB NaCl method [14]. The DNA fragment coding for RTB gene, with accession number X52908, was amplified using 2 primers. These primers which were designed by Oligo software were RTBF (5'GTC GAC GGA GGT TGT ATG GAT CCT GAG CCC ATA GAT3') as a forward primer with an endonuclease site of SalI and RTBR (5′AAG CTT ATTA AAA TAA TGG TAA CCA TAT TTG3') as reverse primer with an endonuclease site of *Hind*III and a strong stop codon (ATTA). Synthesis of those primers was performed by Sinaclon Bio-tech Company. Amplification was made in a total volume of 50 µl of reaction mixture containing 1µl of genomic DNA(0.5 µg/µl), 5 µl of 10X *Pfu* buffer with $MgSo_4$, 5 µl dNTP Mix(2 mM each), 2 µl of each primer (10 pmol) and 0.25 µl of *Pfu*DNA polymerase (2.5 unit/µl). By adding double-distilled water, the reaction mixture reached the Final volume 50 µl. A total of 32 cycles was performed with the first denaturation at 95 °C for 5 min., then 30 cycles at 95 °C for 30 second, 57 °C for 30 second and 72 °C for 30 second, and the final extension at 72 °C for 5 min. The amplified products were analyzed by electrophoresis with a 1% agarose gel followed by

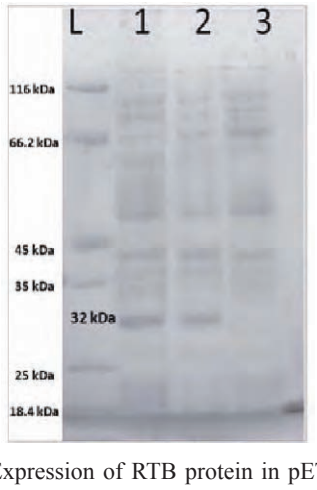

Figure 3. Expression of RTB protein in pET-28a(+)-*RTB*-Rosetta(DE3). Lane 1: around 32 kDa protein resulted from periplasm yield. Lane 2: around 32 kDa protein resulted from culture supernatant. Lane 3: Negative control (Non-induced recombinant bacterial cells). Lane L: Protein size marker (cat No:#sm0671 purchased from fermentas co.).

Figure 4. Western blotting analysis of expressed pET-28a-*RTB* products, using an anti-Histidine antibody. Lane Control (C): Non-induced recombinant bacterial cells. Lane Test (T): Induced recombinant purified protein by nickel column. Lane Ladder (L): Protein size marker (cat No:#SM0671 purchased from fermentas co.).

ethidium bromide staining and UV-transilluminator visualization [19].

2.3. Cloning, subcloning and sequencing

The PCR products of RTB were trailed with dATP, ligated with pGEM-T vector and transformed into *E. coli* DH5α. Ampicillin selection and some control tests ensured the presence of the recombinant plasmid. In order to subcloning, Fragments of *Sal*I and *Hind*III-digested RTB were inserted into the *SalI/Hind*III site of expression vector pET-28a (+), through enzyme digestion and ligation reactions. Then the recombinant plasmid pET-28a (+)-*RTB* was confirmed by PCR and restriction enzyme digestion. Recombinant pET-28a (+)-*RTB* was amplified in competent *E. coli* DH5α and then extracted by alkaline lysis method. The resulting plasmid pET-28a (+)-*RTB* was transformed into competent final host *E. coli* Rosetta (DE3). Kanamycin resistance was used for early selection and chloramphenicol was used for Rosetta (DE3) for next selection [12]. A recombinant plasmid was prepared and identified by restriction enzymes. In order to sequence the inserted fragment, 20 µl of purified recombinant plasmid was sequenced by Nasle Omid Biotech Company [19].

2.4. Expression and identification of the fusion protein

The RTB expression system pET-28a (+)-RTB Rosetta(DE3) was cultured in LB medium at 37 °C. After the OD of bacteria reached to 0.6, it was initiated by isopropylthio-β-D-galactoside (IPTG) at final concentration of 1 mmol/L. The culture medium was proportional to concentrations of glycine and Triton X-100 and then precipitated and incubated for 5 h. The bacteria were collected by centrifugation and the cell pellet was broken by B Buffer (NaH2PO4:13.8 g, Tris.HCl:1.2 g, urea 480.5 g and adding DDW to 1 liter then adjust pH in 8 value). The molecular weight of RTB fusion protein was identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Immunobloting analysis of immuno-reactivity of RTB fusion protein was determined by Western blot. For Western blotting, proteins were transferred to nitrocellulose membrane and incubated with the rabbit antiserum against Histidine and HRP labeling sheep anti-rabbit IgG as the first and second antibodies, respectively [19].

2.5. Anti-RTB antibody production

Prior to a course of immunizations, a 2-3 ml of test bleed was taken from the mouse to

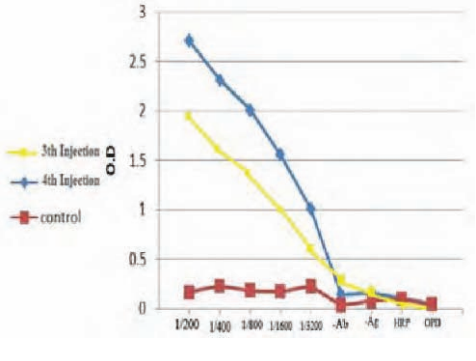

Figure 5. The titre of anti-RTB antibody after the third and fourth injections.

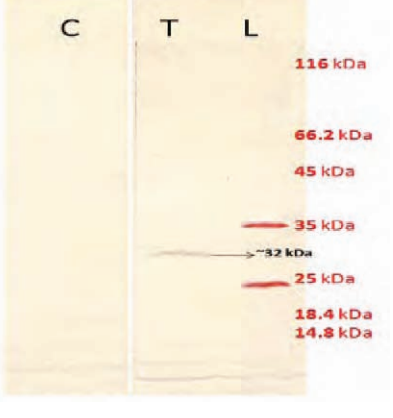

Figure 6. Western blotting analysis of expressed pET-28a-*RTB* products, using anti-RTB polyclonal serum. Lane Control (C): Non-induced recombinant bacterial cells. Lane Test (T): Induced recombinant cell extract. Lane Ladder (L): Protein size marker (cat No:#SM0671 purchased from fermentas co.).

provide a source of pre-immune antiserum. The RTB protein containing 500 µg was mixed with the Freund's adjuvant according to the manufacturer's instructions to achieve a final volume of 0.5 ml/injection. This mixture was injected intraperitoneally into the mouse. A total of four injections of RTB protein in Freund's adjuvant were performed at days 0, 14, 28 and 56 before final bleeding procedure was taken at day 90. Blood samples were collected of each anesthetized mouse. After centrifugation at 5,000 × g for 10 min. at 4 °C, supernatant fluids were collected and stored at -20 °C [19].

2.6. ELISA and Western blot analysis

Finally, the antigenicity of recombinant RTB was identified by Western blot and indirect ELISA. Individual mouse serum was examined for IgG antibodies against RTB by enzyme-linked immunosorbent assay (ELISA). Each sample well was coated with 0.1 ml of this suspension diluted 100-fold in 0.1 M carbonate buffer (pH 9.6). The 96-well ELISA plates were blocked with PBS containing 0.05% Tween 20 and 1% bovine serum albumin for 2 h at 37 °C. After washing with PBS, an anti-isotype secondary antibody (Sigma) was added and plates were incubated for 2 h at 37 °C. Serum samples were incubated with alkaline phosphatase-conjugated goat anti-mouse IgG antibodies. Plates were washed with PBS, developed with a solution of *p*-nitrophenyl phosphate (1 mg/ml) in 1 M diethanolamine buffer (pH 9.8), and read in an ELISA reader (Titertek Multiscan) at 405 nm. Titers were expressed as the last dilution that gave an optical density at 405 nm of 0.1. Under these experimental conditions, pre-immune samples always gave an optical density at 405 nm of 0.1 from the first dilution [19].

For Western blotting, proteins were transferred to nitrocellulose membrane and incubated with the produced mouse antiserum against recombinant RTB protein as the first antibody and HRP labeling sheep anti-rabbit IgG as second antibodies, respectively.

3. Results

3.1. Construction of recombinant pET-28a (+)-RTB

The PCR products that were amplified from genomic DNA of *Castor bean* are shown in Figure 1. About 786-bp of expected fragment amplified by PCR, contained a RTB gene. The PCR product was cloned into pGEM-T vector at first step. The recombinant pGEM-T-RTB vector digested with *Sal*I and *Not*I enzymes and RTB fragments ligated into the corresponding sites of pET-28a (+). The recombinant plasmids pET-28a (+)-RTB were digested by *Sal*I *and Hind*III and analyzed on agarose gel electrophoresis is shown in Figure 2.

3.2. Expression of recombinant fusion protein

The recombinant pET-28a (+)-RTB was transformed into *E. coli* Rosetta (DE3) strains and the fusion protein was expressed. The 1 mmol/L of IPTG was able to efficiently induce expression of RTB fusion protein and also addition of glycine and Triton X-100 enhanced native extracellular protein excreted into the culture medium. The yield of protein was obtained in the periplasm and culture supernatant with a predicted molecular mass of 32 kDa (lanes 1 and 2 in Figure 3). A culture of recombinant *E. coli* Rosetta (DE3) that wasn't induced by IPTG used as a negative control (lane 3 in Figure 3), as compared with those bacteria containing empty vector (data not shown). Concentration of recombinant protein after purification was measured by Nano Drop apparatus trough Bradford assay. This concentration was about 4.2 mg/ ml which equals to 32% of the total protein.

3.3. Western blot analysis using a specific antibody against Histidine tag

Expression of RTB was investigated by

Western blotting, as an antibody against the Histidine tag was employed. Expression of recombinant protein was then confirmed by appearing a proper band control is shown in Figure 4.

3.4 ELISA and Western blot analysis by Serum antibody

Anti-RTB polyclonal serum was generated by repeated immunization of mice with recombinant RTB protein (14). Titration of antiserum was performed by ELISA assay with purified RTB-protein coated onto microplates. A relative high titer of anti-RTB antibody was detected after the fourth injection (Figure 5).

In order to confirm the specificity of the purified antibodies, Western blot analysis carried out with the polyclonal antibody revealed almost a 32-kDa band which corresponds to RTB protein (Figure 6).

4. Discussion

Previous studies have shown when the expressed product is directed into the *E. coli* periplasmic space, however, the recombinant protein often folds into the native conformation and forms the correct disulphide bonds. Lynne M. Roberts and his colleagues have shown that secretion into the periplasmic space is accompanied by OmpA signal peptide cleavage resulting in a biologically active recombinant protein [13]. It is now clear that besides the signal peptide, other techniques in expression of proteins are involved in membrane transport [14].

In this study, we described expression and purification of recombinant RTB protein in two strains of *E. coli*, such as we have followed a new strategy for the improved translocation of biologically active recombinant ricin B chain to the periplasm in *E. coli*. The correctly folded protein in periplasm was obtained by the addition of glycine and Triton X-100 to the culture medium. Triton X-100 is only appropriate for small protein [15, 18]. By this way, we got over the problem of numerous cysteines in the peptide sequence of RTB that may cause the conformation of incorrect disulfide bands [17]. Also, to overcome the abundant rare codons of this eukaryotic gene, we used Rosetta (DE3) strain of *E. coli*.

The results of ELISA and western blotting confirmed that the anti-RTB polyclonal antibody could detect rRTB protein. We herein reported the successful expression of ricin toxin chain B as a plant lectin of Castor bean and the identification of the anti-RTB polyclonal antibody. As RTB is a bi-functional molecule not only as an immuneadjuvant, but also as a gene delivery system for mucosal vaccine candidates. Furthermore, the preparation of the antibody will be very important in our next studying for the immunization animals against ricin toxin.

Acknowledgements

The authors are grateful to Prof. Lynne M. Roberts for her valuable papers and suggestions which made me accomplish my thesis work.

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