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Full Length Research Paper

Expressing of rice ribosome inactivating protein as tool for treatment of cancer cells

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Ribosome-inactivating proteins (RIPs) are toxic proteins synthesized by many plants and some bacteria, that specifically depurinate the 28S RNA and thus interrupt protein translation. RIPs hold broad interest because of their potential use as plant defense factors against pathogens and cancer cells. The most promising way to exploit plant RIPs as weapons against cancer cells is either by designing molecules in which the toxic domains of RIPs are linked to selective tumor targeting domains or directly delivered as suicide genes for cancer gene therapy. In this research the sequence of rice RIP gene was synthesized in PUC57 plasmid. The gene was cloned in pPICZαA vector and expressed in *Pichia pastoris* KM71H (mut^S) strain. The expected protein which had an apparent molecular mass of 34 kDa was detected by SDS-PAGE analysis and confirmed by Western blot.

Key words: Ribosome-inactivating proteins, *Pichia pastoris,* N-glycosidases.

INTRODUCTION

Ribosome-inactivating proteins (RIPs) are a family of cytotoxic enzymes widely distributed in the plant Kingdom (Nielsen and Boston, 2001). RIPs are polynucleotide adenosine glycosidases that cleave the glycosidic bond of an adenosine base in an evolutionarily conserved sequence (GAGA) located in the α-sarcin/ricin (S/R) loop of eukaryotic ribosomes (Endo et al., 1988). RIPs show depurination activity against eukaryotic and prokaryotic ribosomal RNA (rRNA) in the presence and absence of ribosomal proteins (Endo et al., 1991). Depurination of the S/R loop prevents binding of the elongation factor 2 to the ribosome, and results in protein synthesis inhibition (Stirpe et al., 1992). Apart from the depurination of rRNA, RIPs exhibit the ability to depurinate DNA, poly(A), and viral RNA(Stirpe, 2004).RIPs of plants have been classified into three main types: type I is composed of a single polypeptide chain of approximately 30 KDa, Type II is a heterodimer consisting of an A chain, functionally equivalent to the Type I polypeptide (Olsnes and Pihl, 1973), linked to a B subunit, endowed with lectin-binding properties, (Lord et al., 1994) while Type III are synthesized as inactive precursors (ProRIPs) that require proteolytic processing events to form an active RIP (Peumans et al., 2001) and are not in use for therapeutic purposes.

Recent studies demonstrated that RIPs have anti-tumor and anti-viral properties (Lodge et al., 1993). For example, MAP30, a 30 kDa single-stranded RIP, was found to be able to inhibit HSV-2 and HSV-1 viral proliferation (Moon et al., 1997). Interestingly, MAP30 also showed robust and broad anti-cancer activities against multiple cancer cells such as lymphoid leukemia, lymphoma, choriocarcinoma, melanoma, breast cancer, (Hartley et al., 1991; Ready et al., 1986). RIPs are currently

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Abbreviations: SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis; **RIP:** Ribosome-inactivating proteins; **EGFR:** epidermal growth factor receptor; **IT:** Immunotoxin

skin tumor, prostatic cancer, squamous carcinoma, human bladder carcinomas, and Hodgkin's disease under study as therapeutic agents against cancer cells. The present study is a part of our continuing research to isolate active compounds from plants for anti-cancer activity. Rice ribosome-inactivating protein is not tested for anti-cancer activity. In first step we want to express its gene in a eukaryotic host and in the second step we want to test anti-cancer activity.

This paper describes the construction of a recombinant plasmid including pPICZαA vector and RIP gene for extracellular expression in *P. pastoris* strain KM71H.

MATERIALS AND METHODS

Strains, plasmids and material

E. coli TOP 10F' and *P. pastoris* KM71H (*arg4 aox1::ARG4*) strains (INVITROGEN) were used for plasmid con struction and expression, respectively. Zeocin and pPICZαA expression vector were purchased from INVITROGEN. Taq DNA polymerase, DNA ladders, T4 DNA ligase, restriction enzymes was supplied by FERMENTAS.

Plasmid extraction kit was from BIONEER and PCR purifying kit from ROCHE. Primers were synthesized by BIONEER. Low range protein molecular weight marker was from SIGMA. PUC57 plasmid containing synthetic RIP gene was ordered from GENSCRIPT. All other chemicals and media components were of analytical grade and obtained from MERCK.

Construction of the expression vector

The synthesized RIP gene that was synthesized in PUC57 plasmid was transformed to *E. coli*. After transformation *E. coli* cultured overnight at 37°C. The PUC57 plasmid was extracted and digested with *EcoRI* and *XbaI* enzymes which were designed for cloning in pPICZαA vector.

The RIP gene was 813 bp. After digestion, the gene was purified with purifying kit from 1% agarose gel. The gene designed with no ATG initiation codon and was in frame with α-factor of pPICZαA vector. This condition led to an open reading frame (ORF) starting from α-factor ATG to C-terminal *myc* epitope tag and C-terminal polyhistidine (6xHis) tag and finally to stop codon.

Cloning and transformation

The gel-purified gene was ligated with digested pPICZαA vector. After transforming into *E. coli* strain Top10, several recombinant plasmid designated as pPICZαA_RIP was selected on a low salt LB agar plate containing 25µg/ml zeocin. The insertion was checked by restriction analysis and sequencing. The enzymes for restriction analysis were *EcoRI* and *XbaI*. The primer for DNA sequencing primer was 5´*AOX1* primer. The sequence was 5´-GACTGGTTCCAATTGACAAGC-3´.

Due to the advantages of electroporation, such as high frequency of transformation (especially possibility of multicopy insertion), this method was employed to transform yeast cells by the constructed plasmid (pPICZαA -RIP).

For *P. pastoris* integration, 10 µg of recombinant plasmid was linearized with *Sac*I, and transformed into *P. pastoris* by electroporation. For electroporation, linearized recombinant plasmid was mixed with competent KM71H cells. The mixture was immediately transferred to a pre-chilled 0.2 cm electroporation cuvette and incubated on ice for 5 min. About 1 ml of ice-cold 1 M sorbitol was immediately added to the cuvette after electroporation on a Gene Pulser (Bio-Rad). The charging voltage, capacitance, and resistance were 1.5 kV, 25 µF, and 200 Ω, respectively. The transformants were selected at 28°C on the YPDS (1% (w/v) yeast extract,1 M sorbitol, 2% (w/v) peptone and 2% (w/v) D-glucose) agar plates containing 100 µg/ml zeocin for 2 days. The integration of the RIP gene into the genome of *P. pastoris* was confirmed by PCR using 5'AOX1 and 3'AOX1 primers. DNA extraction from *P. pastoris* for PCR was done following a standard protocol. The sequence of 3'*AOX1* primers was: 5´-GCAAATGGCATTCTGACATCC-3´. For screening of multicopy integration of rice RIP gene, clones were grown on 100 µg/ml zeocin YPDS medium and were transferred to 200 µg/ml then 500 µg/ml and finally to 1000 µg/ml zeocin YPDS medium. The clones grown on 1000 µg/ml zeocin YPDS medium were the multicopy integrants and selected for expression in KM71H.

Expression of RIP gene in *P. pastoris*

P. pastoris transformants were grown on 50 ml of fresh buffered minimal glycerol complex medium, BMGY (1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate (pH 6.0), 1.34% (w/v) YNB, 0.0004% (w/v) biotin, and 1% (v/v) glycerol) at 30°C (approximately 16-18 hours in 250 rpm) until an OD600 of 5 was reached. To induce rice RIP gene production in *P. pastoris*, the cell pellet was then harvested by centrifuging at 1500 to 3000 g for 5 min at room temperature and was resuspended in buffered minimal methanol medium, BMMY (1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate (pH 6.0), 1.34% (w/v) YNB, 0.0004% (w/v) biotin, and 0.5% methanol) using 1/5 volume of the original culture in a shaking incubator . Absolute methanol was added every 24 h to a final concentration of 0.5% (v/v) to maintain induction. The culture pellet was discarded after 2 days and supernatant stored at -80°C until needed for further assays.

Protein extraction and SDS-PAGE

Supernatant was stored at -80°C, thawed quickly on ice. Supernatant was precipitated with ammonium sulfate and pellet was dissolved in 50 mM Tris-HCL buffer pH: 7.2. The solution was dialyzed overnight at 4°C and was stored at -80°C. The expression of the recombinant RIP was analyzed by SDS-PAGE (15%). 16 µl of dialysed sample with 4 µl 5X SDS-PAGE Gel Loading buffer was mixed and boiled for 10 minutes and loaded per well. The bands were visualized by staining with silver nitrate.

Western blot analysis

After running total protein on 15% SDS-PAGE gel, Western blot analysis was performed by electroblotting of proteins from SDS-PAGE gel to Whatman nitrocellulose membranes. Tracking of the protein was achieved by employing monoclonal anti-His (C-term) antibody (INVITROGEN) as the primary and horseradish peroxidase-conjugated goat anti-mouse IgG (PROMEGA) as the secondary antibody. The bands were developed using DAB

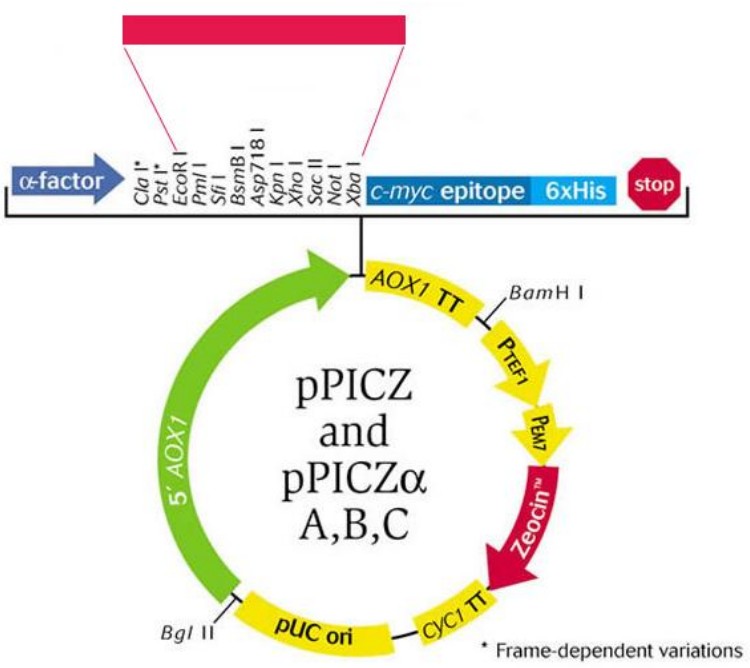

Figure 1. Schematic diagram of RIP gene and pPICZαA vector. RIP gene was cloned between EcoRI and XbaI sites and is in frame with α-factor, c-myc epitope and polyhistidine tag. The vector has a strong promoter (AOX1) and resistance gene for zeocin antibiotic.

chromogenic substrate (SIGMA).

RESULTS

For the construction of the pPICZαA-RIP recombinant plasmid, RIP gene from PUC57-hCT plasmid was subcloned into the pPICZαA vector using in frame with α-factor (Figure 1). After plasmid extraction from E. coli, restriction enzyme analysis was done for confirmation of cloning. PCR was done by 5´ AOX and 3´ AOX primers. PCR product was approximately 1335 bp. Since in start of the gene was an EcoRI site and end of gene a XbaI site, after digestion the cloned insert which was 813 bp exited from MCS of pPICZαA-RIP (Figure 2). The pPICZαA control vector had no insert while the pPICZαA-RIP produced one 813 bp fragment. No mutation was found in the nucleotide sequence of the inserted fragment after sequencing. The RIP gene sequence was inserted in frame with α-factor, the c-myc epitope and polyhistidine tag. The DNA sequence of the pPICZαA-RIP vector predicts that expected molecular weight of the recombinant product after successful removing of α-factor is 34 kDa.

To obtain the highest possible expression levels of RIP gene, transformants that contain multiple copies of the integrated vector was selected. Approximately 50 transformants of the KM71H strain were generated. 30 clones were isolated and screened by PCR with 5'AOX1 and 3'AOX1 primers. Some of the clones contained the expected 1335 bp DNA fragment, indicating that the RIP gene was integrated into the P. pastoris genome. These thirty clones also were screened on YPDS medium including 200, 500 and 1000 µg/ml zeocin. Moreover, the highest expression clone for scale-up experiment was screened from 20 randomly selected zeocin resistant transformants. The selected clone was cultured for induction of expression.

After 3 days, KM71H strain was harvested and protein extraction was performed. The recombinant RIP was produced extracellular in KM71H. The peptide was analyzed by SDS-PAGE and the band corresponding to the expected size (34 kDa) was visible on the gel. This protein band was not detected in the control KM71H strain (Figure 3). In western blotting also this band was detected (Figure 4).

DISCUSSION

As with many other types of difficult-to-cure cancers, a multi-disciplinary approach holds out hope for a

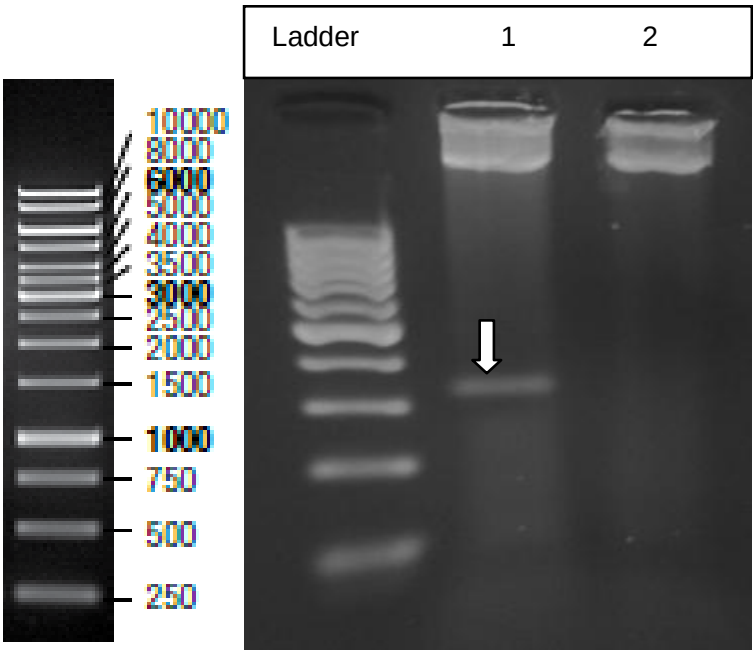

Figure 2. Restriction analysis of pPICZαA-RIP constructs using *EcoRI*/*XbaI* enzymes. DNA fragments were analyzed by 1% Agarose gel Electrophoresis. Ladder: DNA 1 Kb ladder, Lane 1: pPICZαA-RIP vector, the arrow shows 813bp fragment and Lane 2 is pPICZαA control vector.

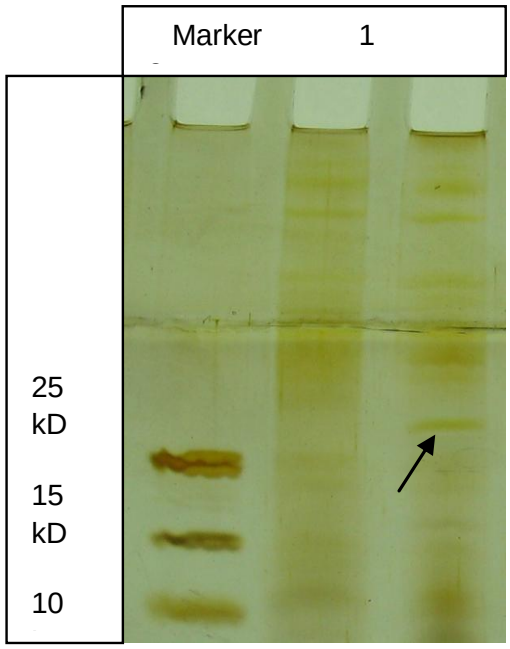

Figure 3. SDS-PAGE of secreted proteins of *P. pastoris*. Gel was 15% and stained with silver nitrate. Ladder is a Low molecular weight protein marker; Lane 1 is control and Lane 2 in induced samples. The arrow show expressed RIP gene in KM71H strain.

significant improvement in the therapeutic outcome.

Expanding the list of available weapons based on different modes of actions will also promote development of rational clinical protocols for effective and safe combinations. The choice of therapeutic transgenes and gene therapy strategies are rapidly evolving with the advances in identification of new genes and new targets, improvement of vectors and expression systems, and better understanding of molecular biology of cancer (Risberg et al., 2010).

For produce recombinant peptides and proteins, there is a need to have a set of different expression systems. Bacteria offer the advantage of high space-time yields and are favorable with respect to cultivation costs. However, as the major drawback, post-translational modification of peptides or proteins, needed for human applications, does not occur in bacteria. In the last decade, *P. pastoris* became one of the favorite expression systems for the production of various proteins of interest (Macauley-Patrick et al., 2005). Interestingly, it was found that RIP gene expression was not toxic to the yeast cells, although this RIP should be able to inactivate both prokaryotic and eukaryotic ribosome's(Hartley et al, 1991), thus suggesting that a rapid and/or very efficient secretion into the culture medium likely prevented RIPs from interacting with the yeast host ribosome

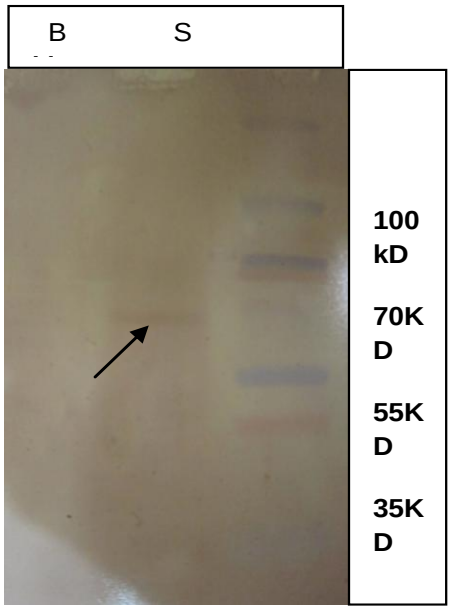

Figure 4. Western-blot analysis of rice RIP gene expressed in *P. pastoris*. Lane M, prestained molecular-weight marker. Lane B is control lane S is induced sample arrow shows RIP gene expressed in *P. pastoris*.

Rajamohan et al., 2000).

We have established an expression system based on recombinant DNA for extracellular expression of recombinant RIP in *P. pastoris*. To our knowledge, this is the first report describing expression of rice RIP gene in *P. pastoris* as methylotrophic yeast. In summary, we successfully expressed rice RIP gene in *P. pastoris*. The expressed RIP gene was detected by SDS-PAGE and Western blotting. This recombinant RIP can be made available on a scale which could meet the high demand of its use as a potential therapeutic agent for treating cancer cells. But in next step the product of gene should be purified and interacted with cancer cell lines as an immunotoxin.

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