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Protein Expression and Purification 40 (2005) 142–151
Protein Expression & Purification
www.elsevier.com/locate/yprep

Bacterial expression and enzymatic activity analysis of ME1, a ribosome-inactivating protein from *Mirabilis expansa*

Ramarao Vepachedu[a], Sang-Wook Park[b], Neelam Sharma[c], Jorge M. Vivanco[d,e,*]

[a] *Department of Medicine, National Jewish Medical and Research Center, 1400 Jackson Street, Denver, CO 80206, USA*
[b] *Boyce Thompson Institute for Plant Research, Ithaca, NY 14853, USA*
[c] *Department of Biochemistry and Molecular Biology, Colorado State University, Fort Collins, CO 80523, USA*
[d] *Department of Horticulture and Landscape Architecture, Colorado State University, Fort Collins, CO 80523-1173, USA*
[e] *Cell and Molecular Biology Program, Colorado State University, Fort Collins, CO 80523-1173, USA*

Received 15 September 2004, and in revised form 30 November 2004
Available online 5 January 2005

Abstract

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. However, study of the activity of type I RIPs has been hampered since their expression in *Escherichia coli* has typically been toxic to the model system. *Mirabilis expansa*, an Andean root crop, produces a type I RIP called ME1 in large quantities in its storage roots. In this study, the cDNA sequence of ME1 was used to successfully express the recombinant ME1 protein in *E. coli*. The production of recombinant ME1 in *E. coli* was confirmed by Western blot analysis using anti-ME1 antibodies. The studies with fluorescence-labeled ME1 showed that ME1 can enter bacteria and be distributed in the cytoplasm uniformly, indicating its ability to access the protein synthesis machinery of the bacteria. The recombinant enzyme was active and depurinated yeast ribosomes. However, both native and recombinant ME1 proteins failed to depurinate the *E. coli* ribosomes, explaining the non-toxicity of recombinant ME1 to *E. coli*. Structural modeling of ME1 showed that it has folding patterns similar to other RIPs, indicating that ME1 and PAP, which share a similar folding pattern, can show different substrate specificity towards *E. coli* ribosomes. The results presented here are very significant, as few reports are available in the area of bacterial interaction with type I RIPs.

Keywords: Ribosome-inactivating protein; ME1; Depurination; *E. coli*; Yeast ribosomes; Naked rRNA

Ribosome-inactivating proteins (RIPs) are a family of cytotoxic enzymes widely distributed in the plant Kingdom [1]. 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 [2,3]. RIPs show depurination activity against eukaryotic and prokaryotic ribosomal RNA (rRNA) in the presence and absence of ribosomal proteins [4]. Depurination of the S/R loop prevents binding of the elongation factor 2 to the ribosome, and results in protein synthesis inhibition [5]. Apart from the depurination of rRNA, RIPs exhibit the ability to depurinate DNA, poly(A), and viral RNA [6–8]. Studies on substrate specificity indicate that some RIPs can also interact with and depurinate mRNA [9], leading to a recent study demonstrating that depurination by ME1 does not require the cap structure for recognition [10].

RIPs have been characterized from various plant sources and are classified as type I, II, and III proteins. Type I RIPs consist of a single polypeptide chain of

* Corresponding author.
E-mail address: j.vivanco@colostate.edu (J.M. Vivanco).

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doi:10.1016/j.pep.2004.12.005

approximately 30 kDa. In contrast, type II RIPs contain a catalytically active A-chain and a carbohydrate-binding B-chain. Both chains are derived from a single precursor by the excision of a linker sequence between the A and B domains and are held together by a disulfide bridge between the C-terminus of the A-domain and the N-terminus of the B domain. Type III RIPs have a single chain but are distinctly different from both type I and II RIPs in their sequence.

RIPs hold broad interest because of their potential use as plant defense factors against viruses and fungi. Transgenic plants expressing RIPs show enhanced resistance to disease-causing pathogens: the barley RIP expressed in tobacco (*Nicotiana tabacum*) protected plants from the soil-borne pathogen *Rhizoctonia solani* [11,12], and maize RIP expressed in tobacco plants conferred resistance against the corn earworm, *Helicoverpa zea* (Boddie) [13]. Additionally, many RIPs are used as immunotoxins by linking them to antibodies specific to target cells [14,15]. For instance, the anti-CD19 and anti-CD22 immunotoxins made of murine IgG(1) monoclonal antibodies (Mabs) conjugated to a deglycosylated ricin A-chain (dgRTA) were effective in killing the B-lineage of non-Hodgkin's lymphoma (NHL) [16]. Understanding the mechanism of biological activity of RIPs greatly enhances our ability to use them as defense proteins against pathogens. Production of RIPs as biologically active recombinant proteins enhances our ability to use them as immunotoxins.

The expression of RIPs in bacteria as recombinant proteins is complicated because of their cellular toxicity. The expression of type I RIPs in *Escherichia coli* has typically been toxic to the model system because of their ability to depurinate prokaryotic ribosomes. Studies with dianthin and Phytolacca antiviral protein, PAP, both type I RIPs, indicate that these RIPs cleave a bond between the A-2660 and the ribose of the 23S rRNA of *E. coli*, a position equivalent to the A-4324 of eukaryotic 28S rRNA [17]. PAP mutants, which do not inhibit *E. coli* growth but inhibit eukaryotic protein synthesis, have been constructed and expressed in *E. coli* [18]; however, PAP mutants, despite their non-toxicity to *E. coli*, are apparently able to retain their ability to inactivate prokaryotic ribosomes [19]. *Mirabilis* antiviral protein (MAP) from *Mirabilis jalapa* has been expressed with its own signal sequence in *E. coli*, but the synthesis resulted in low yields and inhibition of bacterial growth [20]. Our recent work with ME1, an RIP from *Mirabilis expansa*, has shown that ME1 has close sequence similarity with MAP [10]. Previous studies of RIP activity on bacterial ribosomes have shown that type I RIPs show varying degrees of toxicity against bacterial ribosomes [21,22]. Interestingly, ME1 has both anti-fungal and anti-bacterial activities [23] that make this RIP an interesting target for expression studies aimed at understanding the biological activities of the RIPs. The toxicity of ME1 depends on its localization and the ability of the ME1 to depurinate the ribosomes of the host cells. Expression in bacteria allows us to understand how prokaryotes respond to a synthesis of these plant toxins. In the present study, we describe how ME1 was successfully expressed in *E. coli*, and discuss the enzymatic activity of this recombinant protein and its structural similarities to other RIPs.

Materials and methods

Plant material

Seeds of *M. expansa* (CIP Accession 208001, ARB 5395) were obtained from Dr. M.K.V. Zant, Southern Illinois University, USA, and the native ME1 protein was purified from the storage roots of *M. expansa* [23].

Construction of the recombinant expression system

ME1 was cloned into the pET28 vector at the *Nde*I and *Bam*HI sites for expression in *E. coli* pLysS. The open reading frame of ME1 has 317 amino acids and was obtained from a *M. expansa* storage root cDNA library using polymerase chain reaction (PCR). The enzyme sites *Nde*I and *Bam*HI were generated using the following 5′ and 3′ primers: CATATGGAAACTATGAGGTTGC TCTTCC and GGATCCTTAAGAAGATGCAACTA CAACACTA. The PCR product containing the open reading frame was cloned into a TA cloning vector, pGEM-T Easy (Promega, Madison, WI). The TA clone plasmid was isolated by mini-prep using a plasmid isolation kit (Bio-Rad, Hercules, CA) and digested with *Nde*I and *Bam*HI. The fragment with the gene sequence was separated on a 1% agarose gel and ligated into a pET28b expression vector (Invitrogen, Carlsbad, CA) digested with the same enzymes. The resulting vector, ME-pET 28b with an N-terminal His_6-tag, was sequenced to confirm the sequence of ME1 and proper ligation to the vector. The ME-pET 28b was transformed into pLysS (Invitrogen) bacterial cells for expression.

Expression of recombinant protein

The ME1 protein was expressed in *E. coli* pLysS strain by induction with IPTG (isopropyl-β-D-thiogalactopyranoside). The *E. coli* BL21(DE3) pLysS strain containing the ME1 expression plasmid was grown at 37 °C with constant shaking in Luria–Bertani (LB) medium supplemented with 50 μg/ml kanamycin and chloramphenicol, until the culture reached an absorbance of 0.5 at 620 nm. ME1 protein synthesis was induced by the addition of 1 mM IPTG to the medium and the culture was grown for 3 h at 200 rpm under constant shaking. To study the toxicity of the induced ME1 against the host