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Expression of a pokeweed antiviral protein in *Escherichia coli* and its characterization

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Two expression vectors were constructed to produce a putative mature α-pokeweed antiviral protein (α-PAP) in *Escherichia coli* with its NH_2- and COOH-terminal extrapeptides excised. One was for its intracellular expression with a methionine at its NH_2-terminal. The other was for its secretion using an ompA signal peptide. The former product was purified from the total soluble proteins of the transformant with a yield of 1.74 mg/liter and the latter had a yield of 5.55 mg/liter. Both products exhibited RNA *N*-glycosidase activity on wheat ribosomes and inhibitory activity to protein synthesis in a rabbit reticulocyte system.

α-Pokeweed antiviral protein; Expression; RNA *N*-glycosidase activity

1. INTRODUCTION

Many, and possibly all, plants contain ribosome-inactivating proteins (RIPs) [1], e.g. ricin from *Ricinus communis* [2], α-trichosanthin from *Trichosanthes kirilowii* Maxim. [3], and *Mirabilis* antiviral protein (MAP) from *Mirabilis jalapa* L. [4]. It has been indicated that RIPs cleave the *N*-glycosidic bond at adenine^{4324} of rat liver 28S rRNA with their RNA *N*-glycosidase activity to inactivate the ribosomes, resulting in the inhibition of protein synthesis [5,6]. Pokeweed antiviral protein (PAP) is an RIP isolated from several organs of *Phytolacca americana* [7–9]. It inhibits not only infection by plant viruses [8,10,11], but also the replication of mammalian viruses including Type 1 human immunodeficiency virus [10,12–15]. Thus, PAP has attracted the interest of many researchers for its possible use as a therapeutic agent.

We have previously reported the nucleotide sequence of the genomic gene for α-PAP and its expression in *Escherichia coli* [16]. The gene product depurinated wheat ribosomes, indicating that it is a functional protein exhibiting RNA *N*-glycosidase activity [16]. However, the protein was obtained from the total soluble proteins of the transformant with a very low yield of 30 μg/liter. A comparison of its amino acid sequence to that of native PAP from the seeds suggested that it could exert its RNA *N*-glycosidase activity without putative NH_2- and COOH-terminal extrapeptides [16]. Therefore, two expression vectors were constructed to produce a putative mature α-PAP in *E. coli*. Here we report the substantial expression of the protein with undiminished activity.

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2. MATERIALS AND METHODS

2.1. *Materials*

Restriction endonucleases and DNA modification enzymes were purchased from Takara Shuzo (Kyoto, Japan) and Bethesda Research Laboratory (Tokyo, Japan). CM-Sepharose, Blue-Sepharose and Mono-S columns were from Pharmacia LKB (Tokyo, Japan). Wheat germ extract, rabbit reticulocyte lysate and L-[^{35}S]methionine were from Amersham (Tokyo, Japan).

2.2. *Modification of an α-PAP genomic gene by PCR*

PCR primers were synthesized using a 392 DNA/RNA Synthesizer (Applied Biosystems Inc.). PCR was carried out in the conventional way using a GeneAmp PCR Reagent Kit and a DNA Thermal Cycler (Takara Shuzo). Template DNA constitues 10 ng of the genomic gene in EMBL3 [16]. The nucleotide sequences of PCR products were determined using a ^{T7}Sequencing Kit (Pharmacia) after cloning in pBluescript (Stratagene). The expression vector pSHompA was a derivative of pSH7 [17] with the P_L promoter and *c*I857 repressor replaced by *tac* and *lac*I^Q, respectively [16].

2.3. *Expression and purification of a recombinant mature α-PAP*

The *E. coli* strain MM294 [17] was transformed using each expression vector. An overnight culture of each transformant (50 ml) was incubated in 1 liter of LBroth at 37°C for 3 h. After the addition of IPTG to a final concentration of 0.1 mM, culture was continued for 3 h. Cells were harvested by centrifugation and then ruptured. After removal of the insoluble precipitate by 30% saturation with $(NH_4)_2SO_4$, the protein was purified through CM-Sepharose, Blue-Sepharose and Mono-S column chromatography as described previously [4,17]. The NH_2-terminal peptide sequence of each protein was determined with a 477A Protein Sequencer (Applied Biosystems Inc.) using automatic Edman degradation [17]. The protein in the culture

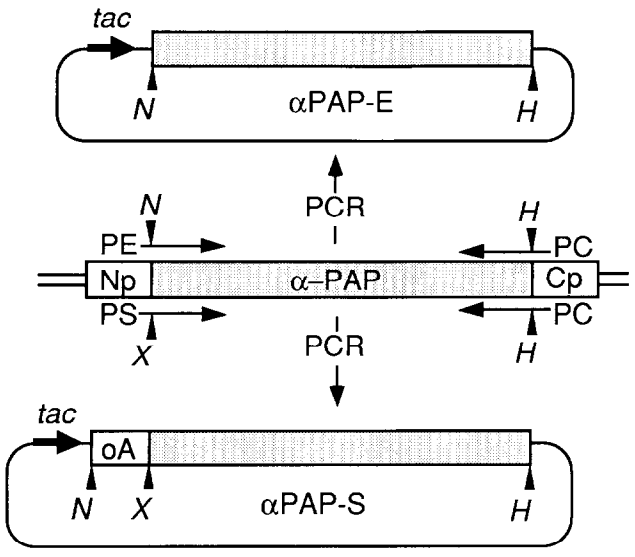

Fig. 1. Schematic diagrams of expression vectors for αPAP-E and αPAP-S. Thin arrows indicate the directions of the PCR primers PE, PS and PC. Np and Cp denote the putative NH_2- and COOH-terminal extrapeptides of α-PAP [16], respectively. *tac* and oA indicate *tac* promoter and ompA signal peptide, respectively. *N*, *Nde*I; *X*, *Xba*I; and *H*, *Hin*dIII.

medium was precipitated with 90% $(NH_4)_2SO_4$ and purified through the same column chromatography procedures.

2.4. *RNA N-glycosidase activity of a recombinant mature α-PAP and its inhibitory activity to in vitro protein synthesis*

The RNA *N*-glycosidase activity of the protein was assayed using wheat germ extract. Removal of the specific adenine from the ribosomes was analyzed by a primer extension procedure using the primer 5′-TTCGCGCCACTGGCTTT-3′, which is complementary to the region 44–60 bases downstream from the adenine attacked by RIPs in wheat 25S rRNA [18] as described previously [16,19].

The inhibitory activity of the protein to protein synthesis was measured using a rabbit reticulocyte system as described previously [17]. Cucumber mosaic virus RNA or Brome mosaic virus RNA was used as template mRNA. MAP was the positive control [17].

3. RESULTS

The PCR primers PE, PS and PC were synthesized to modify the α-PAP genomic gene. Primer PE (5′-CCCCATATGATAAATACAATCACCTTCGAT-3′) was designed to replace the NH_2-extrapeptide with a methionine and create an *Nde*I site. Primer PS (5′-GGGCATATGACTCTAGAATAAATACAA-TCACCTTCGAT-3′) was designed to excise the extrapeptide and create an *Xba*I site. Primer PC (5′-GGGGAAGCTTCATTAAGTTGCCTGGCA-GGTCCC-3′) was designed to replace the COOH-extrapeptide with two stop codons and create an *Hin*dIII site (Fig. 1). The PCR product from PE and PC (αPAP-E gene) was digested with *Nde*I and *Hin*dIII, and then inserted in the corresponding sites of pSHompA for the intracellular expression of the putative mature α-PAP. The PCR product from PS and PC was digested with *Hin*dIII and *Xba*I, and then inserted in the corresponding sites of pSHompA to create a chimeric gene combining an ompA signal peptide and the putative mature α-PAP (αPAP-S gene) with a linker peptide (APTLE) between them for the secretion of the protein (Fig. 1).

Each expression vector was introduced into the *E. coli* strain MM294 [17, 20]. The fresh weights of the transformants expressing αPAP-E and αPAP-S were 2.71 g/liter and 2.19 g/liter, respectively, when cultured without IPTG, and 1.75 g/liter and 1.32 g/liter, respectively, when cultured with IPTG for the expression of the genes. αPAP-E and αPAP-S were extracted from the total soluble proteins of whole transformants. αPAP-S was also extracted from the culture medium. The proteins purified through CM-Sepharose and Blue-Sepharose column chromatography were analyzed on 15% SDS-PAGE (Fig. 2). Large amounts of αPAP-E and αPAP-S were recovered from whole transformants (lanes 1 and 2), while only a trace level of αPAP-S was found in the culture medium (lane 3). The proteins from the whole transformants were further purified to homogeneity by Mono-S column chromatography. The final yields of αPAP-E and αPAP-S were 1.74 mg/liter and 5.55 mg/liter, respectively. The NH_2-terminal peptide sequence of αPAP-E was 'MINTITFDVGNATIN-KYATFMKSIHNQA', which is identical to that of the putative mature α-PAP except for the first methionine

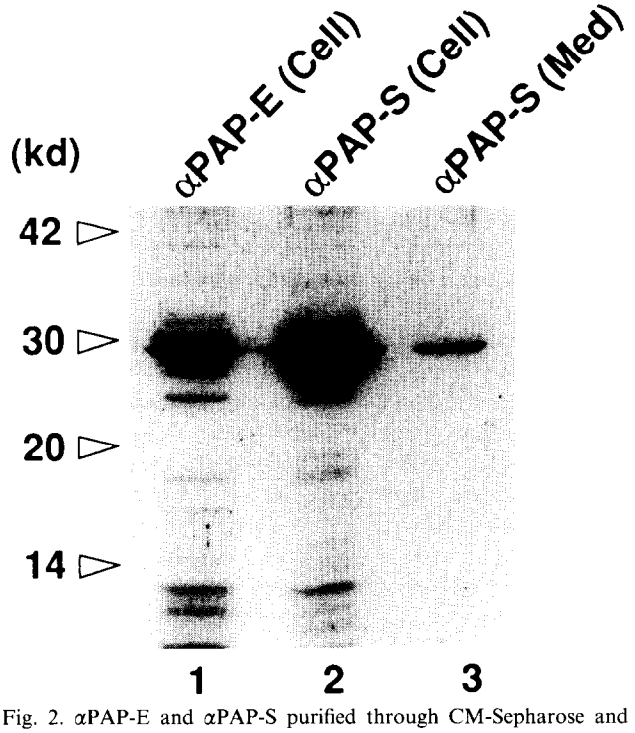

Fig. 2. αPAP-E and αPAP-S purified through CM-Sepharose and Blue-Sepharose column chromatography. 4 μl of each sample (5 ml) was analyzed on 15% SDS-PAGE. Lane 1: αPAP-E from total soluble proteins of the transformant. Lane 2: αPAP-S from total soluble proteins of the transformant. Lane 3: αPAP-S from a culture medium of the transformant. An arrow indicates the position of αPAP-E and αPAP-S.

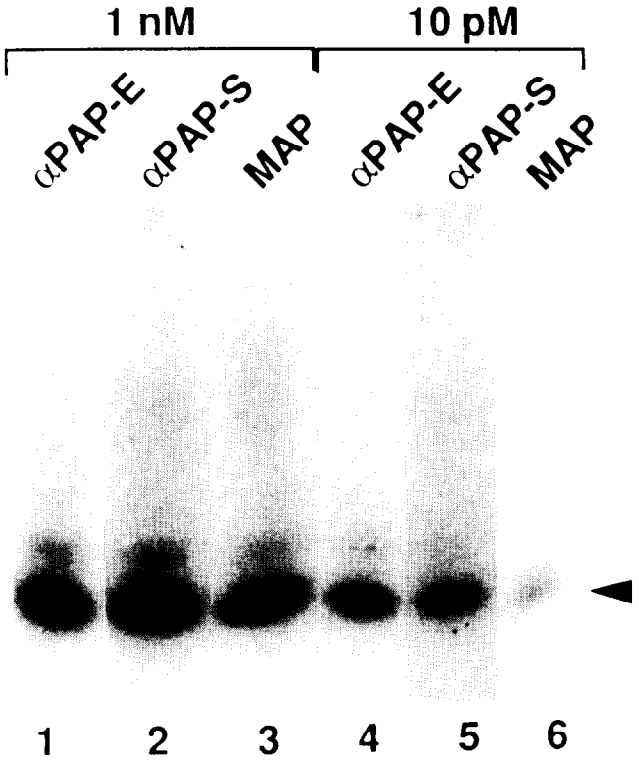

Fig. 3. RNA *N*-glycosidase activity of αPAP-E and αPAP-S on wheat ribosomes. An arrowhead indicates the extended primer terminated after the addition of 43 nucleotides. MAP denotes *Mirabilis* antiviral protein as a positive control [17].

introduced by the PCR. The NH_2-terminal peptide sequence of αPAP-S was 'APTLEINTITFDVGNA TINKYATFMKSI-HN'. It indicates that the ompA signal peptide was cleaved from αPAP-S and only a linker peptide (APTLE) was retained in the protein at its NH_2-terminal.

The RNA *N*-glycosidase activity of αPAP-E and αPAP-S was assayed by the primer extension procedure using wheat germ extract [16,19]. The target adenine was specifically removed from the ribosomes by both proteins (Fig. 3) as reported previously [16]. The radioactivity of the bands from ribosomes treated with either αPAP-E or αPAP-S at a concentration of 10 pM (lanes 4 and 5) was far stronger than that treated with MAP at the same concentration (lane 6). These data indicate that the proteins have the RNA *N*-glycosidase activity specific to RIPs and that their activity is stronger than that of MAP. The inhibitory activity of both proteins to in vitro protein synthesis was measured using a rabbit reticulocyte system (Fig. 4). In this experiment, the median inhibitory concentrations of L-[^{35}S]methionine incorporation of αPAP-E and αPAP-S were 0.007 and 0.005 nM, respectively, whereas that of MAP was 0.09 nM. These results indicate that both recombinant proteins have an inhibitory activity at least 12 times as high as MAP.

4. DISCUSSION

We have previously reported the trace level expression (30 mg/liter) of α-PAP with the putative extrapeptides [16]. The present study shows that a substantial amount of the putative mature α-PAP without extrapeptides can be produced by *E. coli* while maintaining its activity. We speculate that the putative NH_2-terminal extrapeptide, which contains many methionine and valine residues [16], might make efficient expression difficult and/or the putative COOH-terminal extrapeptide might make the protein susceptible to endogenous proteases in *E. coli*.

The inhibitory activity of native PAPs from the leaves and seeds to *in vitro* protein synthesis was reported to be about 8 and 54 times higher than that of MAP, respectively [1,17]. Since αPAP-E and αPAP-S were at least 12 times as active as MAP on rabbit ribosomes, both recombinant proteins were concluded to have an inhibitory activity comparable with that of the native PAPs.

The transformant expressing αPAP-S was too sticky and weak to fractionate periplasmic proteins by the osmotic shock method [21]. αPAP-S was mainly recovered from the soluble proteins of whole transformant, and only a slight amount was obtained from the culture medium. The NH_2-terminal sequence of αPAP-S revealed that the ompA signal peptide was cleaved from the chimeric protein, probably during secretion. Therefore, αPAP-S seems to have been secreted into the periplasmic space of the transformant.

It has been reported that *E. coli* 23S rRNA is slightly depurinated by native PAPs [22]. αPAP-S secreted into the periplasmic space could not depurinate ribosomes of the transformant, in contrast to αPAP-E expressed intracellularly. This might be the reason why about 3 times more αPAP-S was produced in *E. coli* than αPAP-

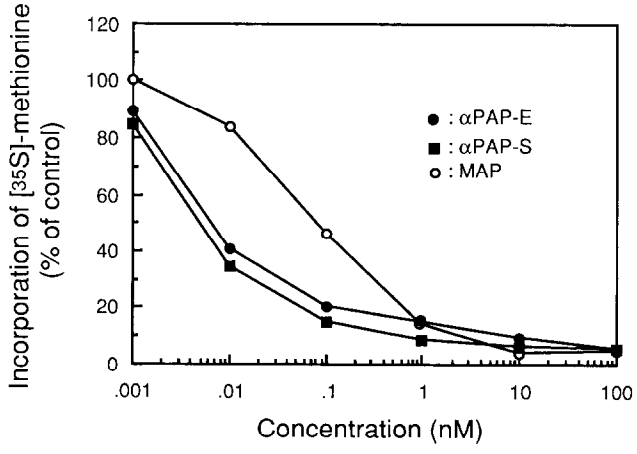

Fig. 4. Inhibitory activity of αPAP-E and αPAP-S on in vitro protein synthesis in a rabbit reticulocyte system. Closed circles and boxes indicate αPAP-E and αPAP-S, respectively. Open circles indicate MAP as a positive control [17]. The incorporation of L-[^{35}S]methionine is presented relative to that with (100%) and without (0%) template mRNA.

E in spite of the lower cell yield of the αPAP-S transformant than of the αPAP-E transformant.

αPAP-S and αPAP-E were produced in substantial amounts by *E. coli* as functionally active forms in the present experiment, even though it has been reported that some researchers encountered difficulty in expressing PAP in *E. coli* [22]. Therefore the putative mature α-PAP seems to be a suitable form for obtaining a pokeweed antiviral protein by genetic engineering for practical use such as a therapeutic agent.

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