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Research Article

Production of functional rat liver PSP protein in *Escherichia coli*

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Abstract. An efficient *Escherichia coli* expression system for the production of a perchloric acid-soluble protein (PSP) has been constructed. Complementary DNA encoding PSP was inserted into an inducible bacterial expression vector pGEX-4T-1. After the plasmid introduced into *E. coli* was expressed by isopropyl 1-thio-β-D-galaetopyranoside (IPTG), the recombinant product was purified by glutathione-Sepharose 4B affinity chromatography. The purified product showed the expected NH_2-terminal sequence, but the translation inhibitory activity of this product was 10 times lower compared with that of authentic PSP isolated from rat liver.

Key words. Perchloric acid-soluble protein; recombinant PSP; *Escherichia coli*; translation.

In a previous paper, we reported isolation and characterization of a novel perchloric acid-soluble protein (PSP) in the cytosolic fraction of rat liver [1]. The PSP is a homodimer, each subunit consisting of 136 amino acid residues with a molecular mass of 14,149 Da. It inhibits cell-free protein synthesis in the lysate of rabbit reticulocyte in a different manner than RNase A. Incubation of the purified PSP with the lysate leads to disaggregation of polysomes, suggesting that PSP exerts its inhibitory effects at the initiation stage of the cell-free protein synthesis [1]. Examination by immunoblotting, Northern blotting and immunohistochemistry revealed that PSP is present in the kidney in addition to the liver [1].

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Recently, a 14-kDa protein which inhibits translation was characterized from human monocyte [2] and mouse liver [3]. The 14-kDa proteins and their messenger RNA (mRNAs) were reported to be preferentially expressed in the liver and kidney as in the case of PSP. Furthermore, the sequences of complementary DNA (cDNA) encoding the 14-kDa protein and PSP are highly similar to those of cDNA encoding a new hypothetical family (YER057c/YJGF) of small proteins with presently unknown function [2]. Thus the sequences of PSP-like proteins are highly conserved in prokaryotes, cyanobacteria, fungi and eukaryotes. The high degree of evolutionary conservation of these proteins suggests that these proteins play an important role in cellular regulation.

To understand the physiological significance of the PSP protein, we have expressed the PSP cDNA clone in

bacterial cells. The protein purified from the over-producing strain was active in inhibiting protein synthesis.

Materials and methods

Materials. Restriction endonuclease and nucleic acid-modifying enzymes were purchased from Takara Biochemicals (Kyoto, Japan). The other reagents were purchased from BioRad (Richmond, USA). The expression vector pGEX-4T-1 was purchased from Amersham Pharmacia Biotech, USA.

General methods. Plasmid DNA was prepared by the alkaline method and purified by Qiagen pack-100 (Qiagen Inc., USA). Restriction enzyme digestion and ligations were carried out essentially as recommended by the supplier. The nucleotide sequence of the insert was determined by the dideoxy chain termination procedure of Sanger et al. [4] employing Sequenase with 7-deazadeoxy GTP (dGTP) (United States Biochemicals, USA). Amino acid sequencing of the recombinant protein was carried out as described previously [1].

Construction of the *E. coli* expression vector. The outline of the construction of pGEX-PSP is shown in figure 1. A plasmid pPSP was constructed by inserting PSP cDNA [1] into pUC19. The coding sequence of PSP from plasmid pPSP was amplified by polymerase chain reaction (PCR). Two oligonucleotide primers containing Eco RI sites were designed for the PCR; primer 1 (25 mer) 5′-3′ TCGACGAATTCTCGTCAATAATCAG and primer 2 (25 mer) 5′-3′ TCGACGAATTCAGCATGGCTCCTTA. A PCR fragment containing the PSP coding sequence was excised by subsequent digestion with Eco RI. pGEX-PSP was constructed by ligating this sequence with Eco RI-digested pGEX-4T-1. The resulting construct, pGEX-PSP, encoded a fusion protein of the glutathione-S-transferase (GST) and PSP.

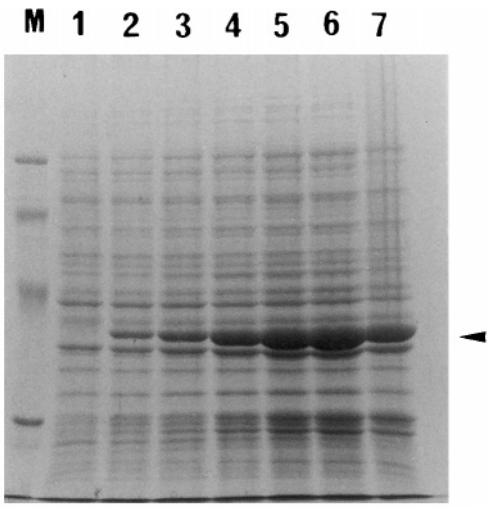

Figure 2. Time course of PSP production after induction by the addition of IPTG. Bacteria were precultured in L-broth, and expression was initiated by the addition of 1 mM IPTG. Each extract, containing 30 μg of protein, was separated on 15% SDS-PAGE, and the protein bands were visualized by Coomassie brilliant blue. Lane 1, start of incubation; lanes 2–7, 0.5, 1, 2, 3, 4, 5 h after induction, respectively. The arrowhead indicates the position of expressed recombinant PSP-GST fusion protein. Molecular weight markers (M) are phosphorylase b, 94,000; bovine serum albumin, 67,000; ovalbumin, 43,000; carbonic anhydrase, 30,000 (from top to bottom).

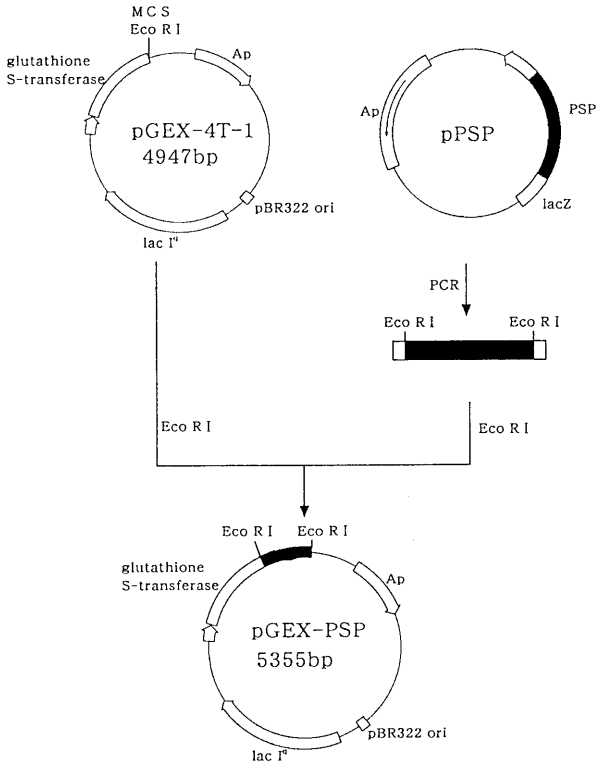

Figure 1. Construction of expression vector, pGEX-PSP.

Expression of PSP in *E. coli* and purification of recombinant PSP. Recombinant plasmid pGEX-PSP was propagated in *E. coli* strain BL21. *E. coli* transformants grew in L-broth containing 50 μg/ml of ampicillin at 30 °C. When the absorbance at 550 nm of the culture reached 0.8, the expression was initiated by addition of 1 mM IPTG. Production of PSP was achieved by continuous aerobic incubation at 37 °C.

Cells (from 2 l of culture) were harvested by centrifugation at 10,000*g* for 10 min, and the pellet was suspended in 10 volumes of water and disrupted by sonication. Purification of PSP from the extract was carried out by glutathione Sepharose 4B. The lysate was applied on an

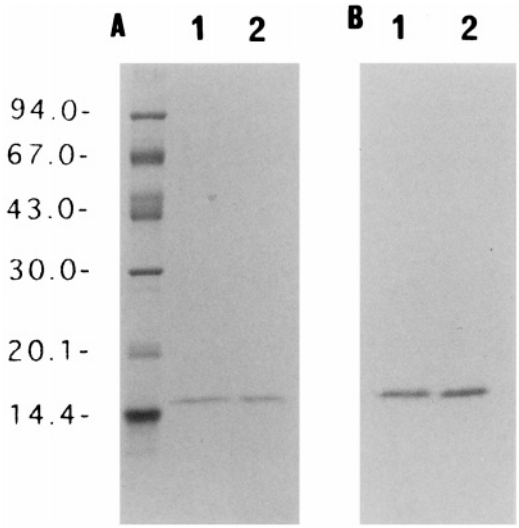

Figure 3. SDS-PAGE analysis of purified PSP. Authentic (lane 1) and purified recombinant (lane 2) PSP proteins were separated, and stained (A) or immunoblotted (B) as described in the text. Molecular weight markers are the same as in figure 2 except for trypsin inhibitor, 20,100 and α-lactoalbumin, 14,400 (from top to bottom).

affinity column of glutathione Sepharose 4B equilibrated with phosphate-buffered saline (PBS). After washing the column with 10 volumes of PBS, the GST-PSP fusion proteins were eluted with 10 mM glutathione in 50 mM Tris-HCl buffer (pH 8.0). To separate the recombinant protein from the GST moiety, the site-specific proteolysis was accompanied by incubation with thrombin in buffer (2.5 mM $CaCl_2$, 50 mM Tris-HCl pH 7.5, 150 mM NaCl) for 8 h at 37 °C followed by dialyzing against PBS. To remove the GST, the dialysate was again applied on the glutathione Sepharose 4B. The recombinant PSP was present in a flow-through fraction. It was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and found to be homogeneous.

Identification of PSP. Proteins in the samples were separated on 15% polyacrylamide gels containing 0.1% sodium dodecylsulfate (SDS), according to Laemmli [5]. Gels were stained with Coomassie brilliant blue R-250, or proteins were transferred to nitrocellulose membrane. Immunodetection was carried out by incubating the membrane first with rabbit polyclonal antibody raised against PSP protein from rat liver [1] and then with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) (Cappel Products, USA) [1].

Functional analysis of the recombinant PSP. Effects of the recombinant PSP on cell-free protein synthesis were examined using a rabbit reticulocyte lysate assay system (Amersham Pharmacia Biotech) and a tobacco mosaic virus mRNA (Boehringer Mannheim GmbH, Germany) as described previously [1].

Results

Expression of recombinant PSP. Figure 2 shows the time course of PSP production in the *E. coli* transformant BL21/pGEX-PSP. The extracts obtained by brief sonication of the cell were analyzed by SDS-PAGE. The intensity of a band gradually increased during 4 h after the induction by IPTG and slightly decreased at 5 h. Immunostaining experiments further showed that the increase in band intensity was due to expression of recombinant PSP (data not shown). Using enzyme-linked immunosorbent assay (ELISA), the amount of recombinant PSP produced in the cells 4 h after the induction was estimated to be 1.0 mg/l of culture.

Characterization of recombinant PSP. PSP was extracted and purified as described in 'Materials and methods'. An analysis of the purified protein by SDS-PAGE revealed a single band, having the same mobility as authentic PSP (fig. 3A), which was immunostained

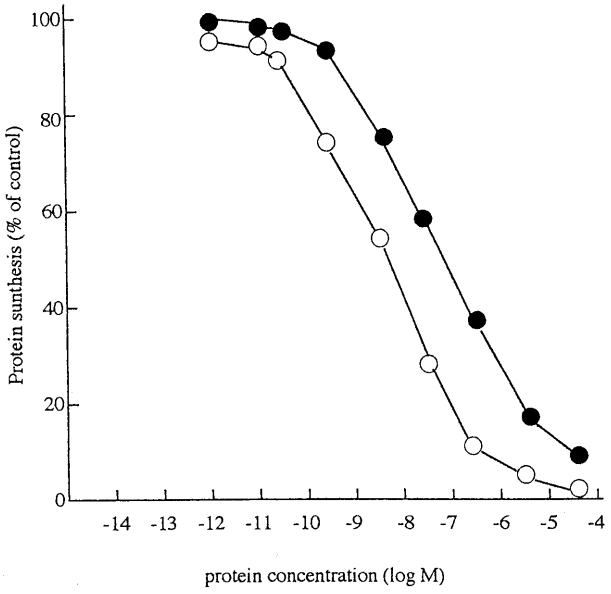

Figure 4. Inhibition of cell-free protein synthesis by recombinant PSP. Protein synthesis by a rabbit reticulocyte lysate mixture was measured in the presence of different concentrations of authentic PSP (○) and recombinant PSP (●), described under 'Materials and methods'. Each point represents the mean value of [^{14}C]leucine incorporation into protein determined in five separate experiments and expressed as a percentage of the incorporated leucine (30,000 cpm) in the absence of PSP.

by anti-PSP antibody (fig. 3B). The first eight amino acid residues from the NH_2-terminal of the purified protein were found to be Ser-Ser-Ile-Ile-Arg-Lys-Val-Ile. This result indicates that the cleavage of a peptide bond in the fusion protein by thrombin between GST and the designed PSP took place at the expected site in the bacterial cells.

Translational inhibition of recombinant PSP. The ability of recombinant PSP to inhibit cell-free protein synthesis was examined by rabbit reticulocyte lysate. As shown in figure 4, both authentic and recombinant PSP proteins inhibited protein synthesis, but the IC_{50} of recombinant protein was 10 times higher compared with that of authentic PSP (IC_{50}, 10 nM).

Discussion

In our previous study, we found a PSP in rat liver and completely elucidated its primary structure by amino acid sequencing and nucleotide sequencing. Interestingly, PSP was shown to inhibit cell-free protein synthesis in a rabbit reticulocyte lysate system. Recently, Schmiedknecht et al. [2] isolated and characterized a 14.5-kDa trichloroacetic acid-soluble translational inhibitor protein (p14.5) from human monocytes which shows a remarkable similarity to PSP. Interestingly, these authors demonstrated that the p14.5 mRNA is weakly expressed in freshly isolated monocyte but significantly upregulated when the monocytes are subjected to differentiation to macrophages. Furthermore, the expression of K-PSP, that is PSP from rat kidney, increased from fetal day 17 to postnatal week 4, and it remained almost the same until the postnatal week 7 [6]. On the other hand, the expression of K-PSP in renal tumor cells was downregulated as compared with intact tissue. Thus, the above-mentioned results suggest that expression of PSP might be differentiation-dependent. At present, investigations into the involvement of PSP proteins in the regulation of cell differentiation is being actively pursued.

We have now developed an efficient *E. coli* system for the production of functional PSP and a convenient procedure for its purification. Using a rabbit reticulocyte lysate system, we demonstrated that rat recombinant PSP inhibits protein synthesis. Authentic purified PSP was shown to inhibit in vitro protein synthesis at an IC_{50} of 10 nM, whereas recombinant PSP inhibits at a 10 times higher concentration of IC_{50}. This difference is likely to be due to loss of activity when recombinant PSP is expressed in a prokaryotic system. We believe that our system will provide a useful tool for further study of the mechanism of action of PSP.

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