Bull. Fac. Agr., Saga Univ. No. 82 : 83～90 (1997)

Study on the Expression of Pokeweed Antiviral Protein in *Escherichia coli* as a Fusion with Maltose-binding Protein

Eijiro HONJO, Keiichi WATANABE and Takuji TSUKAMOTO
(Laboratory of Life Chemistry)
Received September 5, 1997

Summary

A genomic clone encoding pokeweed antiviral protein (PAP) and its C-terminal extrapeptide was obtained from the DNA of leaves by a polymerase chain reaction using two specific primers based on the known cDNA sequence of PAP. The nucleotide sequence of the obtained clone was identical to the cDNA sequence except for a single base substitution of C for T, resulting in a change of Val113 to Ala. The DNA was inserted into expression vector pMAL-p2, and PAP was expressed in *E. coli* and exported to the periplasm as a soluble fusion with maltose-binding protein (MBP). The recombinant PAP (rPAP) was cleaved from MBP by treatment with factor Xa and subsequently purified with a yield of 4 mg/liter of culture. The analysis by SDS-polyacrylamide gel electrophoresis suggests that the C-terminal extrapeptide of rPAP was partially digested by some proteases in the process of transport or in the periplasm. The rPAP was 60-fold more effective than the MBP-PAP fusion in depurinating rat liver ribosomes, and as active as the native PAP purified from pokeweed leaves.

Keywords: Pokeweed antiviral protein; Ribosome inactivating protein; Expression; Fusion protein.

Introduction

Many plant species synthesize the polypeptides called ribosome inactivating proteins (RIPs) that inhibit protein synthesis by cleaving a single *N*-glycosidic bond of 28S rRNA (A4324 in rat ribosomes)[1]. Type I RIPs have a single polypeptide chain, while type II RIPs consist of two polypeptides, an active A chain and a B chain which is a galactose-binding lectin[2].

Pokeweed antiviral protein (PAP), isolated from several organs of *Phytolacca americana*, belongs to the type I RIP family. PAP, PAP-II and PAP-S are forms of pokeweed antiviral protein that appear in spring leaves, summer leaves and seeds, respectively[3-5]. PAPs efficiently inactivate mammalian, plant and *E. coli* ribosomes[6,7] in contrast to the

Correspondence to K. Watanabe, Department of Applied Biological Sciences, Saga University, Saga 840, Japan. Fax: +81-952-28-8774, E-mail: watakei@cc.saga-u.ac.jp
Abbreviations: RIP, ribosome inactivating protein; PAP, pokeweed antiviral protein; MBP, maltose binding protein, PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; FPLC, fast protein liquid chromatography.

RIPs like ricin A-chain that show a slight activity on plant ribosomes and no activity on prokaryotic ribosomes[8,9]. PAP inhibits infection and replication of plant and animal viruses[10,11]. The most likely mechanism of antiviral action is inactivation of ribosomes by PAP that enters the cytoplasm of the infected cells[12-14]. PAP has recently been of considerable interest due to its therapeutic application as chimeric toxins[11,15], targeted to a particular cell type such as cancer cells[16,17].

Some RIPs have been successfully expressed in *E. coli* to give a high yield[18,19], whereas others are difficult to express in *E. coli* due to the severe inhibition of growth of the host by the recombinant product[20,21]. To overcome this problem, PAP has been expressed with a signal peptide[22,23] and exported to the periplasm of *E. coli*. The growth of *E. coli* was, however, still arrested by the expression with a signal peptide, and rPAP was in some case expressed as an inclusion body. In the present report, we described the expression of recombinant PAP in *E. coli* as a soluble fusion protein with maltose-binding protein (MBP). The rPAP cleaved from MBP was as active as native PAP on rat liver polysomes.

Materials and Methods

Materials

Restriction endonucleases and T4 DNA ligase were purchased from Nippon Gene Co., Ltd. (Osaka, Japan). Ex Taq DNA polymerase was from Takara shuzo (Kyoto, Japan). The expression vector pMAL-p2, amylose resin and restriction protease factor Xa were from New England BioLabs, Inc. Mono-S column was from Pharmacia LKB. Rat liver polysomes were prepared by the established method[24]. Protein concentrations were measured by the method of Lowry *et al.*[25] using bovine serum albumin as a standard, and ribosome concentrations were measured by assuming 17 pmol/A_{260} unit[26].

Preparation of genomic DNA

Genomic DNA was extracted from spring leaves of *Phytolacca americana*. Harvested leaves were frozen with liquid nitrogen and ground to a fine powder in a mortar and pestle. A solution of 200 μl containing 0.5 M Tris-HCl, pH 8.0, 0.25 M EDTA and 5% SDS was added to 1 g of leaf powder, and the mixture was extracted with phenol saturated with 45 mM sodium citrate and 0.45 M NaCl. The isolated aqueous phase was extracted with phenol/chloroform (1:1), and then with chloroform/isoamyl alcohol (24:1) three times. The genomic DNA in the aqueous phase was ethanol-precipitated with 0.3 M ammonium acetate, washed with 70% ethanol, and dissolved in water.

Amplification

Two oligonucleotides (*PapN* and *PapC*) for polymerase chain reaction (PCR) were designed according to the cDNA sequence[30] of PAP. The primer *PapN* (5'-ACTGGATCC-GTGAATACAATCTACAATGTTG-3') encodes N-terminal amino acid sequence and has *Bam*H I site for subcloning. *PapC* (5'-TTTAAGCTTAGAATCCTTCAAATAGATCAC-CAAG) encodes the C-terminal sequence of PAP retaining the extrapeptide with stop

codon, and has *Hin*d III site. The reaction mixture contained 510 ng of genomic DNA, 2.5 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 100 pmol of *PapN* and *PapC*, 200μM of each dNTP, and 1.25 units of Ex Taq polymerase in a total volume of 100 μl. The reaction was performed for 35 cycles, and 1 μl of the mixture was analyzed by 1.5% agarose gel electrophoresis.

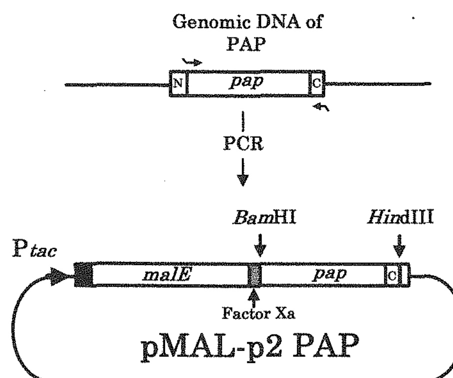

Fig. 1. Schematic diagram of expression vector for PAP. Small arrows indicate the PCR primers, and N and C denote the regions encoding N-terminal signal peptide and C-terminal extrapeptide, respectively. MalE signal sequence is shown as a black box.

Subcloning and sequencing of PAP gene

The amplified DNA (approx. 900 bp) was purified by 1.5% agarose gel electrophoresis, digested with *Bam*H I and *Hin*d III, and subcloned into the pBluescript II SK- vector. The nucleotide sequence was determined using a Dye Terminator Cycle Sequencing kit (Perkin Elmer).

Cloning of PAP gene into an expression vector

Genomic clone encoding PAP, inserted in pBluescript, was excised by digestion with *Bam*H I and *Hin*d III and ligated into pMAL-p2 (Fig. 1). The recombinant plasmids were used to transform competent XL1-Blue cells. The plasmid pMAL-p2 is designed to create fusions between a cloned gene and the *E. coli malE* gene which encodes MBP, resulting in the expression of an MBP fusion protein in the periplasm[27].

Expression and purification of rPAP

The *E. coli* strain XL1-Blue, transformed by pMAL-p2 with a PAP gene insert (pMAL-p2/PAP), was grown at 37°C in LB medium containing ampicillin (100 μg/ml) and tetracycline (12 μg/ml) to an OD of 1.5 at 600 nm. After the addition of 0.35 mM IPTG for inducing the expression of MBP-PAP fusion protein, the culture was further incubated at 25 or 37°C.

Cells were harvested by centrifugation, resuspended in 30 mM Tris-HCl (pH 8.0) containing 20% sucrose and 1 mM EDTA, and incubated for 30 min at room temperature with shaking. Cells were collected by centrifugation, resuspended in ice-cold 5 mM $MgSO_4$ and incubated for 30 min on ice with shaking. After separation of cells by centrifugation, 1 M Tris-HCl (pH 7.4) and 50 mM phenylmethanesulfonyl fluoride were added to the supernatant to a final concentrations of 20 mM and 0.25 mM, respectively. The supernatant was applied on an amylose resin column (1.5 x 4.7 cm) equilibrated with 20 mM Tris-HCl (pH 7.4) containing 1 mM EDTA. Proteins were eluted from the column with the above buffer containing 0.2 M NaCl and 10 mM maltose. The obtained MBP-PAP fusion protein was cleaved with factor X a (1:100 by weight) at 20°C for 60 h. After dialysis against 10 mM Na phosphate (pH 6.5), the sample was applied on a Mono-S

column equilibrated with the above buffer. Proteins were eluted with a linear gradient of 0-0.15 M NaCl in the buffer, and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE)[28]. The fractions containing PAP were pooled and stored as a 50% glycerol solution at -20°C.

Measurement of adenine released from ribosomes by PAP

The amount of adenine released from ribosomes was measured as described previously[29]. Rat liver ribosomes (8 pmol) were reacted with the increasing amounts of PAP at 37°C for 10 min, in 40 μl of reaction mixture containing 20 mM Tris-HCl buffer, pH 7.5, 100 mM NH_4Cl, 5 mM magnesium acetate, 1 mM dithiothreitol, and 0.05 mg/ml BSA. Adenine released from ribosomes by PAPs was recovered as an ethanol-soluble fraction and converted into the fluorescing derivative, 1,N^6-ethenoadenine, by the reaction with chloroacetaldehyde. The resulting ethenoadenine was analyzed by reverse-phase HPLC. The amount of adenine released from ribosomes was calculated from the heights of known amounts of adenine added to control ribosomes.

Results and Discussion

Sequencing of a genomic clone for PAP

A single DNA fragment (approx. 900 bp) was amplified from the genomic DNA of *Phytolacca americana*, by PCR using the primers based on the known cDNA sequence of PAP[30] (Fig. 2). The amplified DNA was subcloned into pBluescript, and then sequenced. It encoded a mature protein of 262 amino acids with a C-terminal extrapeptide of 29 amino acids and was identical to the sequence of cDNA reported previously[30], except for a base substitution of C for T, resulting in a change of Val 113 to Ala.

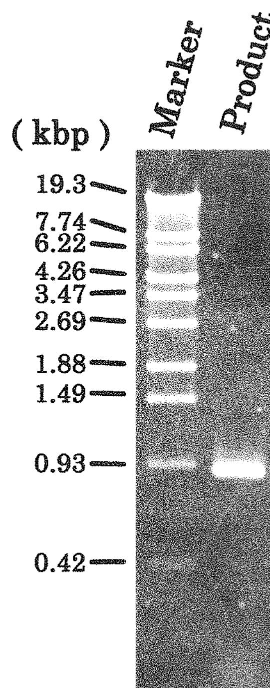

Fig. 2. Product amplified by polymerase chain reaction. PCR product was analyzed by electrophoresis in 1.5% agarose gel. The λDNA digested with *Sty* I was used as size marker.

Expression of rPAP in E. coli as a fusion with MBP

The effect of the MBP-PAP fusion protein expressed in *E. coli* on the host cell growth was examined both at 25°C and at 37°C. The bacterial transformant containing pMAL-p2/PAP was grown at 37°C to an OD_{600} of 0.5, and then the expression of MBP-PAP fusion was induced by addition of 0.35 mM IPTG. As shown in Fig. 3A, the expression at 37°C

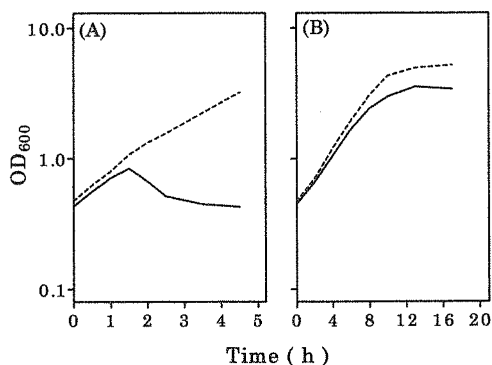

Fig. 3. Growth curves of XL1 Blue [pMAL-p2/PAP] at 25 and 37°C. Cells were grown in LB medium with 0.35 mM IPTG (—) or without IPTG (---) at 37°C (A) and 25°C (B). After various times, the OD was measured at 600 nm.

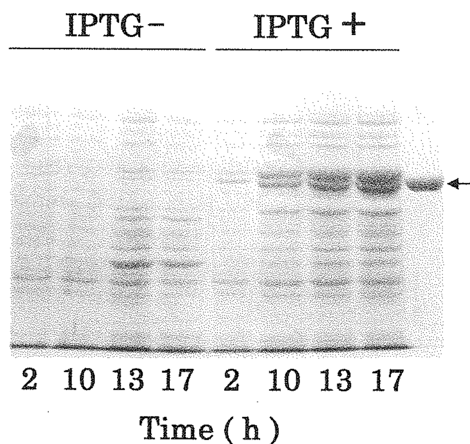

Fig. 4. SDS-PAGE of total proteins in *E. coli* transformant grown at 25°C. Expression of MBP-PAP fusion in *E. coli* was induced with 0.35 mM IPTG at 25°C for indicated times. Cells were collected by centrifugation, and cellular proteins were analyzed on 10% SDS-PAGE. The arrow indicates MBP-PAP fusion protein.

caused the growth-inhibition and lysis of the host cells, giving a maximum OD_{600} of 0.85 at 1.5 h of incubation. In contrast, the expression at 25°C did not arrest the growth of host, resulting in an OD_{600} of 2.8 at 13 h of incubation (Fig. 3B). The analysis of total cellular proteins on SDS-PAGE (Fig. 4) showed the high expression of fusion protein at 25°C. From these results it is found that the lower temperature is an important factor for expression of a higher amount of MBP-PAP fusion protein.

Purification and analysis of rPAP

After 12 h of incubation with IPTG at 25°C, the bacterial cells were harvested. The periplasmic fraction containing fusion protein (67 mg/liter of culture) was prepared by cold osmotic shock, and applied on an amylose resin column. The fractions (11 mg protein/liter of culture), eluted with 10 mM maltose, were digested with protease factor Xa to separate PAP from MBP. The pMAL-p2 vector contains the sequence encoding the recognition site of the specific protease factor Xa[31], located just 5' to the polylinker insertion site. Fig. 5 shows the elution profile of the digested fusion protein on a Mono-S column. Analysis of each fraction by SDS-PAGE revealed that the break through fraction contained maltose-binding protein (45 kDa), and the first and second eluted peaks consisted of the fusion protein (76 kDa) and rPAP (31 kDa), respectively (Fig. 5, inset). The final yield of rPAP was 4 mg/liter.

The native PAP has two intramolecular disulfide bonds[32], in contrast to ricin A-chain containing no intramolecular disulfide bond. The mobility of rPAP, as well as native PAP, on SDS-PAGE under non-reduced condition was larger than that under reduced condition, while ricin A-chain showed the same mobility under the both conditions (Fig. 6). This result indicates the formation of intramolecular disulfide bond in rPAP. The molecular

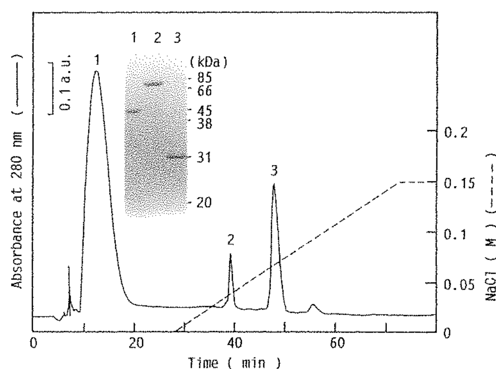

Fig. 5. Chromatogram of MBP-PAP fusion protein cleaved by factor Xa on a Mono-S column. The MBP-PAP fusion protein was digested with factor Xa, dialyzed against 10 mM Na phosphate (pH 6.5), and then applied on a Mono-S column equilibrated with the same buffer. The adsorbed proteins were eluted with the indicated linear gradient of NaCl in the same buffer. Inset, the SDS-polyacrylamide gel electrophoretic analysis of peaks 1-3.

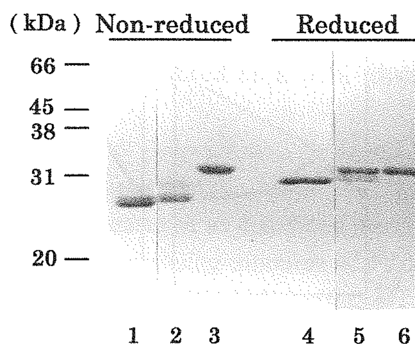

Fig. 6. SDS-PAGE of native and recombinant PAP. Purified proteins were treated with 1% SDS in the presence or absence of 5% 2-mercaptoethanol at 100°C for 3 min, and then analyzed on 12.5% SDS-PAGE. Lane 1 and 4, native PAP from leaves of pokeweed; lane 2 and 5, recombinant PAP; lane 3 and 6, ricin A-chain from the seeds of *Ricinus communis*.

mass of recombinant PAP is expected to be 33 kDa from its amino acid sequence. This value is 3 kDa larger than the native mature PAP because the genomic clone encodes a mature protein of 262 amino acids and a C-terminal extrapeptide of 29 amino acids. rPAP was detected, however, as a main band of 31 kDa protein and two minor bands of slightly smaller proteins on SDS-PAGE. The apparent molecular mass of the lowest minor protein was 30 kDa identical to mature PAP. This result suggests the degradation of C-terminal extrapeptide by some proteases in the process of secretion or in the periplasm. This is supported by the fact that the digestion of rPAPs with proteinase K produced a single protein of 30 kDa (Data not shown).

The RNA *N*-glycosidase activity of rPAP and MBP-PAP fusion protein was assayed by measurement of adenine released from rat liver polysomes. The concentration of rPAP required for 50% of the maximum release of adenine was 0.53 nM similar to that of PAP from pokeweed leaves (Table 1), while that of MBP-PAP fusion protein was 34 nM, 64-fold larger than rPAP. This weaker activity of fusion protein may allow the host cell to produce a higher amount of rPAP.

The present results demonstrate that MBP-fusion system is one of the effective methods for expression and purification of a large amount of PAP. The mutational analysis together with kinetics and X-ray crystallography may be useful to elucidate the exact mechanism of ribosome recognition by PAP. We are currently preparing several

Table 1. RNA *N*-glycosidase activities of native and recombinant PAP

PAPs	Amount of PAP giving 50% of maximum release of adenine (nM)
Native PAP	0.50
Recombinant PAP	0.53
MBP-PAP	34.0

mutant PAPs by using this expression system.

References

1. Endo, Y., Mitsui, K., Motizuki, M. and Tsurugi, K. (1987) *J. Biol. Chem.* **262**, 5908-5912.
2. Barbieri, L., Battelli, M. G. and Stirpe, F. (1993) *Biochim. Biophys. Acta* **1154**, 237-282.
3. Irvin, J. D. (1976) *Arch. Biochem. Biophys.* **169**, 522-528.
4. Irvin, J. D., Kelly, T. and Robertus, J. D. (1980) *Arch. Biochem. Biophys.* **200**, 418-425.
5. Barbieri, L., Aron, G. M., Irvin, J. D. and Stirpe, F. (1982) *Biochem. J.* **203**, 55-59.
6. Taylor, B. E. and Irvin, J. D. (1990) *FEBS Lett.* **273**, 144-146.
7. Hartley, M. R., Legname, G., Osborn, R., Chen, Z. and Lord, J. M. (1991) *FEBS Lett.* **290**, 65-68.
8. Harley, S. M. and Beevers, H. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 5935-5938.
9. Stirpe, F. and Hughes, R. C. (1989) *Biochem. J.* **262**, 1001-1002.
10. Chen, Z. C., White, F. R., Antoniw, J. F. and Lin, Q. (1991) *Plant. Pathol.* **40**, 612-620.
11. Zarling, J. M., Moran, P. A., Haffar, O., Sias, J., Richman, D. D., Spina, C. A., Myers, D. E., Kuebeldeck, V., Ledbetter, J. A. and Uckun, F. M. (1990) *Nature* **347**, 92-95.
12. Watanabe, K., Kawasaki, T., Sako, N. and Funatsu, G. (1997) *Biosci. Biotech. Biochem.* **61**, 994-997.
13. Ready, M. P., Brown, D. T. and Robertus, J. D. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 5053-5056.
14. Chen, Z. C., Antoniw, J. F. and White, R. F. (1993) *Physiol. Mol. Plant Pathol.* **42**, 249-258.
15. Uckun, F. M., Mnivel, C., Arthur, D., Cheistrom, L. M., Finnegan, D., Tuel-Ahlgren, L., Irvin, J. D., Myers, D. E. and Gunther, R. (1992) *Blood* **79**, 2201-2214.
16. Grossbard, M. L., Freedman, A. S., Ritz, J., Coral, F., Goldmacher, V. S., Eliseo, L., Spector, N., Dear, K., Lambert, J. M., Blattler, W. A., Taylor, J. A. and Nadler, L. M. (1992) *Blood* **79**, 576-585.
17. Yefenof, E., Abbsud, G., Etszteyn, S. and Vitetta, E. S. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 728-732
18. O'Hara, M., Roberts, L. M., Thorpe, P. E., Watoson, G. J., Prior, B. and Lord, J. M. (1987) *FEBS Lett.* **216**, 73-78.
19. Piatak, M., Lane, J. A., Laird, W., Bjorn, M. J., Wang, A. and Williams, M. (1988) *J. Biol. Chem.* **263**, 4837-4843.
20. Habuka, N., Murakami, Y., Noma, M., Kudo, T. and Horikoshi, K. (1989) *J. Biol. Chem.* **264**, 6629-6637.
21. Habuka, N., Akiyama, K., Tsuge, H., Miyano, M., Matsumoto, T. and Noma, M. (1990) *J. Biol. Chem.* **265**, 10988-10992.
22. Chen, Z. C., Antoniw, J. F., Lin, Q. and White, R. F. (1993) *Physiol. Mol. Plant Pathol.* **42**, 237-247.
23. Radom, J., Schlick, J. L., Poyet, J. L. and Hoeveler, A. (1996) *Plant Sci.* **120**, 19-28.
24. Staehelin, T. and Falvey, A. K. (1971) *Methods Enzymol.* **20**, 433-446.
25. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **38**, 65-70.
26. Baliga, B. S. and Munro, H. N. (1972) *Biochim. Biophys. Acta* **277**, 368-383.
27. Guan, C., li, P., Riggs, P. D. and Inouye, H. (1987) *Gene* **67**, 21-30.
28. Laemmli, U. K. (1970) *Nature* **227**, 680-685.
29. Watanabe, K., Dansako, H., Asada, N., Sakai, M. and Funatsu, G. (1994) *Biosci. Biotech. Biochem.* **58**, 716-721.
30. Lin, Q., Chen, Z. C., Antoniw, J. F. and White, R. F. (1991) *Plant Mol. Biol.* **17**, 609-614.
31. Nagia, K. and Thogersen, H. C. (1984) *Nature* **309**, 810-812.
32. Monzingo, A. F., Collins, E. J., Ernst, S. R., Irvin, J. D. and Robertus, J. D. (1993) *J. Mol. Biol.* **233**, 705-715.

アメリカヤマゴボウ抗ウイルスタンパク質のマルトース結合タンパク質との融合体としての大腸菌での発現に関する研究

本庄栄二郎・渡辺　啓一・塚本　卓治
(生命化学研究室)
平成9年9月5日　受理

摘　　　要

アメリカヤマゴボウ抗ウイルスタンパク質（PAP）及びそのC末端プロ領域をコードするゲノムDNAを，すでに報告されているPAPのcDNA配列をもとに作成したプライマーを用い，PCR法によりアメリカヤマゴホウの葉DNAからクローニングした．得られたクローンの塩基配列をPAPのcDNA配列と比較したところ1カ所だけ塩基が置換しており，PAPの113番目のバリンがアラニンへ変異していた．このDNAを発現ベクターpMAL-p2に組み込むことで，PAPをマルトース結合タンパク質（MBP）との融合体として大腸菌に発現させた．得られた融合タンパク質をプロテアーゼ（Factor Xa）処理することによりPAPをMBPと切り離し，精製を行った．PAPは大腸菌培養液1リットルより約4㎎得られた．SDSポリアクリルアミドゲル電気泳動による分析の結果，得られたPAPのC末端プロペプチドは輸送過程もしくはペリプラズム中でプロテアーゼの作用により部分的に切断されていると推察された．PAPのラット肝リボソームに対する脱プリン活性はMBPとの融合体の64倍であった．また，この活性はアメリカヤマゴボウ葉より精製されたPAPと同じあった．