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Overexpression of the Catalytic Subunit of Protein Phosphatase Type 2A in *Escherichia coli*

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ABSTRACT

In this study we have established a system for expression of the catalytic subunit of protein phosphatase type 2A (PP2A) of bovine adrenal medulla in *E. coli*. Expression of PP2A in pQE31 vector, based on use of the phage T5 promoter and two *lac* operator sequences, produced the enzyme as an insoluble aggregate which constituted up to 30-35% of total cellular protein. Although enzyme activity was not detected, the protein had an Mr of 36 kDa, comparable to that of authentic phosphatase 2A isolated from human red blood cells. Both the recombinant protein and the authentic phosphatase 2A exhibited cross-reactivity with specific antibody against a synthetic peptide corresponding to PP2A amino acid residues 296-309. The insoluble protein, fused with a 6x His affinity tag was directly applied to a Ni-NTA column for purification by affinity chromatography. Approximately 90% homogeneity was obtained by a single purification and the enzyme was essentially homogeneous after further passage through a gel filtration column. These procedures yielded about 15-20 mg purified PP2A/liter culture, which will facilitate further renaturation experiments and future studies on structure-function relationships of the enzyme.

Key words : Protein phosphatase type 2A (PP2A), pQE31.

INTRODUCTION

Protein phosphatase 2A (PP2A) is one of four major classes of protein (serine/threonine) phosphatases which catalyze the dephosphorylation of a number of intracellular phosphoproteins, thereby reversing the actions of protein kinases[1]. PP2A is set apart from type 1 phosphatase by its specificity for the α subunit of phosphorylase kinase and its insensitivity towards inhibitors 1 and 2. It is distinguished biochemically from other type 2 phosphatases (protein phosphatases 2B and 2C) on the basis of divalent cationic independence[2,3]. The catalytic subunit of PP2A [termed C subunit] isolated from mammalian tissues has an Mr 36kDa which is complexed with a 65kDa regulatory subunit [termed A subunit] to construct a core dimer. The core dimer is associated with a third, variable subunit of 54, 55, 72, 74 or 130 kDa [termed B subunit] which may confer the distinct

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characteristics on the holoenzyme[4,5,6].

In fact, recent interest in serine/threonine protein phosphatases may be explained by the awareness that these enzymes are not simply passive "off-switches" of protein kinases, and that they may play a number of important roles in cell growth and regulation. Specific inhibition of PP2A by okadaic acid, a tumor promoter, has been investigated by several research groups, who have hypothesized that PP2A may be a tumor suppressor[7,8]. The increasing expression of PP2A in rat liver tumors has partially supported this hypothesis[9,10]. In addition, the importance of the PP2A in cell growth control is indicated by the selective association of PP2A with tumor antigens of polyomavirus and simian virus[11,12,13,14,15,16,16,17,18]. The enzyme activity is subverted by the binding of T antigens, and thereby may permit uncontrolled cell growth and viral replication. Moreover, the crucial insight into the function of PP2A *in vivo* has been obtained from the experiments on yeast, which have showed that the inactivation of two C subunit genes (PPH1 and PPH2) results in cell growth inhibition while inactivation of all three C subunit encoding genes (PPH1, PPH2, and PPH3) is lethal. By contrast, inactivation of A subunit causes the cell morphological and transcriptional defects[19] The catalytic subunit of PP2A was also noted to be phosphorylated and inactivated by an autophosphorylation-activated protein kinase, which could contribute to the significant increase in the phosphorylatin of cellular proteins in response to insulin and other mitogens [20,21]. These reports imply that PP2A may play a crucial role in the cell growth control as well as in the signal transduction pathways. Furthermore, our previous study also revealed that PP2A may play a positive role in regulating the NGF- directed neurite outgrouwth in PC12 cells [22].

The recent evidence that cellular functions of PP2A extend beyond cell growth control to other signal regulatory functions, particularly in response to extracellular stimuli, has initiated a greater impetus for understanding its structure/function relationships. However, the limited amounts of enzyme that can be obtained from natural sources have limited further biochemical and crystallographic studies. In this study we report the successful expression of the cDNA encoding the catalytic subunit of PP2Aα from bovine adrenal medulla[23] in *E. coli*. Because most of the enzyme was being aggregated in the insoluble protein, we also describe here our attempts to refold the recombinant PP2Aα to its active form.

EXPERIMENTAL PROCEDURES

I . *Materials*

Protein phosphatase 2Aα cDNA clone was isolated from bovine adrenal medulla as previously described[23]. The pQE31 vectors, Ni-NTA resin and *E. coli*[pREP4] were purchased from QIAGEN Inc. (CA, USA). pMAL-p and pMAL -c were from New England Biolabs, (NJ, USA). ρ-nitrophenol phosphate was obtained from Sigma, (FD, USA). Guanidine hydrochloride was from BDH Limited, (Poole, England). *Bam* HI and *Pst* I were purchased from New England Biolab, (NJ, USA) and Takara Biochemicals, (Kyoto, Japan), respectively. Primers were synthesized by TIB Molbiol, (Berlin, Germany). Purified protein phosphatase 2A holoenzyme from human red blood cells and PP2A peptide antibody were purchased from Upstate Biotechnology Inc. (NY.USA). Prestained SDS-PAGE standards and HPLC gel filtration column (Bio-Sil SEC-250, 600 X7.5mm) were purchased from Bio-Rad, (CA,USA).

II. *Methods*

(I). *Insertion of the PP2Aα cDNA in pQE31*

General cloning procedures used in this study were as described by Sambrook *et al.*[24]. The PP2Aα cDNA[23] was used as the template sequence for inroduction of a *Bam* HI site at two bases downstream from the initiating methionine

codon by PCR amplification. The sense primer 5′→3′

TGGCATC**ATG**GATCCGAAGGTGTTC

(nucleotides -7～18) was used for the 5′ end of the coding region (underlined residues show the *Bam* HI site and the initiating codon ATG is shown in **bold**). The antisense primer 5′→3′

GTTTACATCTTATCTGCAGTCTCTC

was used; which is corresponding to PP2Aα cDNA 3′ noncoding region nucleotides 1209-1233 comprising a *Pst* I site (underlined residues). PCR (94°C, 1min; 51°C, 1min; 72°C, 4 min) was carried out for 30 cycles in a total volume of 50 μl in 50mM KCl, 10mM Tris-Cl (pH 8.3), 1.5mM $MgCl_2$, 0.01% gelatin, 200μM of each dNTP, 1.5 unit Taq DNA polymerase, 30 ng PP2Aα cDNA template, 200ng 5′ sense and 3′ antisense primers. The PCR product was assured as a single band of 1.2 kb. This was digested with *Bam* HI and *Pst* I and inserted into pQE31 vector which had been previously digested with the same restriction enzymes and purified by agarose gel electrophoresis (Fig.1).

(II). *Expression of Protein Phosphatase 2A from pQE31*

Single colony from *E. coli* M15[pREP4] cells harboring pQE31-2A (pQE31 vector containing PP2Aα cDNA) was taken from cells plated on LB/carbenicillin/kanamycin agar plates and used to inoculate 5ml LB/carbenicillin (100μg/ml)/kanamycin (25μg/ml) media and grown overnight at 37°C. These were used to inoculate 1-liter super medium (25g bacto-tryptone, 15g bactoyeast extract and 5g NaCl per liter) and grown at 37°C until the absorbance at 600nm reached 0.3. IPTG was then added to a final concentration of 0.3 mM, and the culture maintained overnight at 28°C or 37°C.

(III). *Purification Recombinant PP2A*

The cells from two 1-liter cultures were ha-

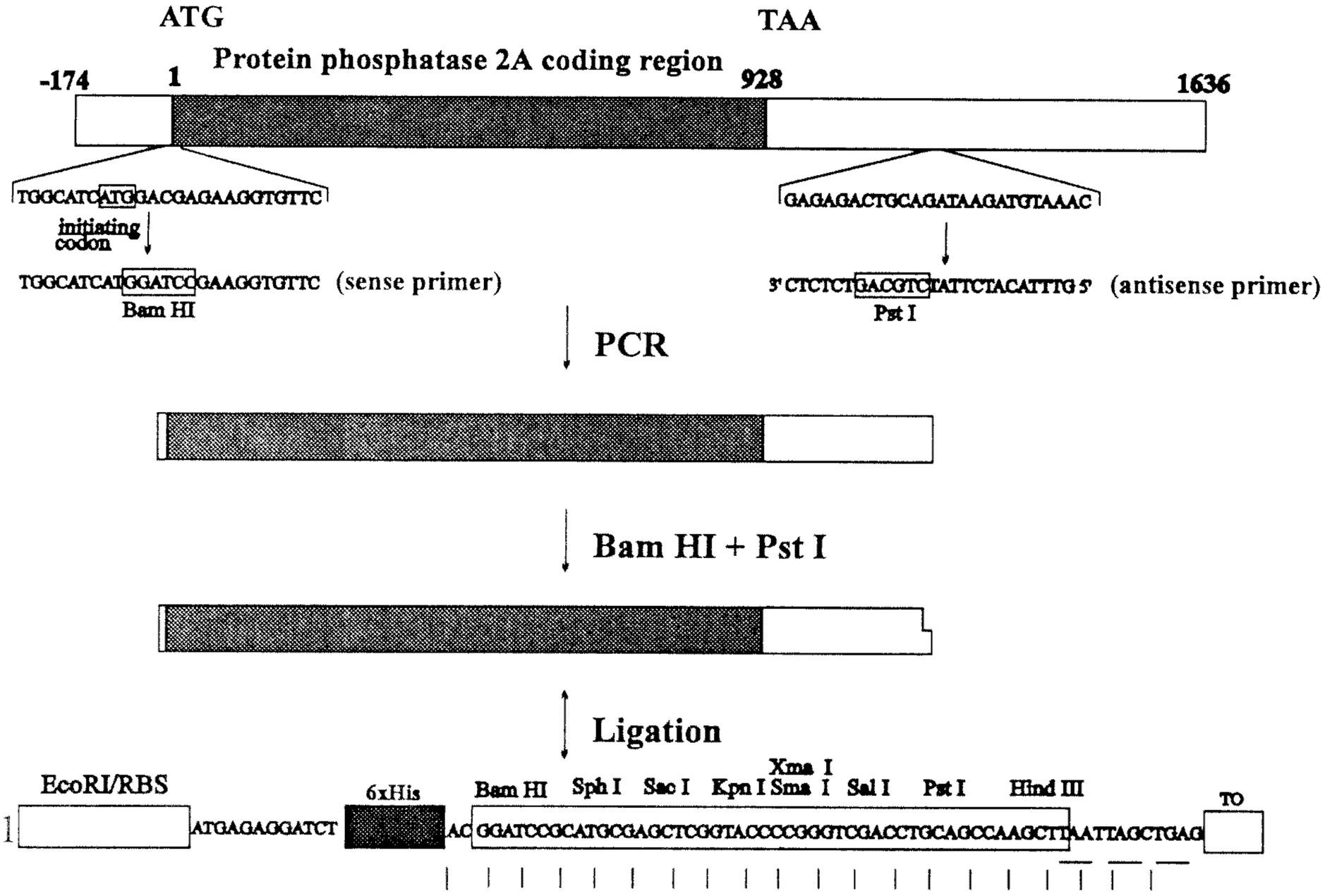

Figure 1. Cloning strategy of the expression of 6xHis-tagged protein phosphatase 2A in pQE31 vector. PCR conditions are described under experimental procedures.

rvested by centrifugation (4000xg at 4°C for 25 minutes) and resuspended in 0.1 volume (200ml) of 50mM Tris.Cl, 500mM NaCl, 10% glycerol, pH7.4. The cell pellet was disrupted intermittently by Bead-Beater (Biospec products, OK, USA) at 1 min intervals (4°C) and the lysate centrifuged (20,000xg) at 4°C for 20 minutes. Subsequently, the supernatant was assayed for PP2A activity and the pellet resuspended in Buffer A (6M Guanidine HCl, 0.1 M Na-phosphate, 0.01M Tris/HCl, pH8.0) at 5ml per gram wet weight and stirred for 1 hr at room temperature. The suspension was centrifuged (10,000xg) for 15 minutes at 4°C and the supernatant applied to Ni-NTA column for purification.

(IV). *Ni-NTA Affinity Chromatograpy*

The procedures were performed at room temperature. The pooled supernatant was added to 8 ml of a 50% slurry of Ni-NTA resin which was previously equilibrated in Buffer A. This was stirred at room temperature for 45 minutes and then loaded into a 1.6 cm diameter column. The column was washed with 10X column volume of Buffer A and 5X volume of Buffer B (8 M Urea, 0.1 M Na-phosphate, 0.01M Tris/HCl, pH8.0) until the A_{280} of the flow-through was < 0.01. Next, the column was washed with Buffer C (8 M urea, 0.1 M Na-phosphate, 0.01 M Tris. Cl, pH6.3) until the A_{280} was <0.01. The enzyme was then eluted with 20ml Buffer D (8M urea, 0.1M Na-phosphate, 0.01M Tris. HCl, pH 4.5). Fractions of 3ml were collected and analyzed by SDS-PAGE.

(V). *HPLC Gel Filtration Chromatography*

Fractions containing PP2A protein were pooled and dialyzed against HPLC column buffer containing 23.5mM Tris base, 0.1mM EGTA, 2mM $MnCl_2$, 2mM dithiothreitol, 800 mM NaCl, pH7.4 and centrifuged (20,000xg) for 20 minutes. An appropriate volume (20μ1, ~ 100μg protein) of supernatant was loaded onto an HPLC gel filtration column (600x7.5mm, Bio -Sil SEC-250, Bio-Rad, USA) equilibrated with column buffer. The fraction size was 0.8ml; flow rate 0.8ml/min, and the wavelength of detector was set at 280nm.

(VI). *Assay of PP2A Activity*

The preliminary assay of the recombinant enzyme activity was performed as described by Silberman *et al.* (1984)[25]. Basically, the reaction was carried out in 100μl of solution containing 50 mM Tris-HCl pH7.5, 0.1 mM EGTA, 0.1% (v/v) 2-mercaptoethanol at 30°C. Enzyme dilutions were made in 50mM Tris-HCl pH7.5, 0.1 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, 1mg /ml bovine serum albumin (BSA). One unit defined as the amount of enzyme that hydrolyzes 1 nmol of ρ-nitrophenol phosphate (50mM) per minute at 30°C.

(VII). *SDS-Gel Electrophoresis*

SDS-PAGE was performed as described by Laemmli (1970)[26] in 10% acrylamide. Gels were stained with Coomassie blue.

(VIII). *Protein Determinations*

Protein determinations were performed as described by Bradford (1976)[27] using bovine serum albumin as a standard or by comparing laser densitometric scans of gel tracks of unknown samples with known amounts of bovine serum albumin.

(IX). *Western Blotting*

Western blotting was performed following the procedures outlined in Immuno-blot assay kit (Bio-Rad). In brief, proteins in SDS-PAGE gel were transferred to nitrocellulose membrane and then submerged in 5% BSA at 4°C, overnight. PP2A was identified using antibody against a synthetic peptide corresponding to PP2A amino acid sequence 296-309 followed by goat anti-rabbit IgG(H+L) conjugated alkaline phosphatase. The primay and secondary antibodies were diluted at 1:2000 and 1:3000 dilutions, respectively.

RESULTS

I. *Expression of Bovine Adrenal Medulla PP2A as an Insoluble Protein*

The insertion of the coding sequence for bovine adrenal medulla PP2A into pQE31 vector led to the protein as insoluble aggregates found in inclusion bodies clearly visible by light micro-

scopy. The same results were also observed for the expression of cathepsin D in *E. coli*[28] and protein phosphatase 1 in insect cells[29]. Examination of the *E. coli* pQE31-2A transformant lysates failed to reveal any protein phosphatase activity. The insoluble pellet obtained by centrifugaiton was resuspended in SDS-PAGE sample buffer and subjected to electrophoresis. The result showed that the major components in the particulate were 36 kDa and 28 kDa proteins. Neither protein was found in the *E. coli* host and the *E. coli* transformed with the control plsamid pQE31. The 36 kDa protein was comparable to that of authentic phosphatase 2A isolated from human red blood cells. This indicates that the 36 kDa protein was encoded from the inserted PP2A coding region and the 28 kDa protein may be a proteolytic product of 36 kDa recombinant protein (Fig.2).

II. *Antibodies to Synthetic PP2A Peptide Cross-react with Recombinant PP2A Protein*

Western blot analysis showed that both rocombinant PP2A protein and authentic phosphatase 2A exhibited a cross-reactivity with antibody against a synthetic peptide corresponding to PP2A amino acid residues 296-309. In addition, the 28 kDa protein, a putative proteolytic product of the major recombinant PP2A protein, also exhibited a positive immunoreactivity with the peptide antibody. This 28 kDa polypeptide was therefore believed to be a carboxyl terminal containing fragment of the recombinant PP2A. An endogenous cross-reactive protein (Mr 49.5 kDa) was also detected in the insoluble pellet (Fig. 3). Since the peptide sequence used as an antigen for raising antibodies uniquely corresponds to PP2A carboxyl terminal sequence and thus far no phosphatase with an Mr 49.5 kDa has been reported. We suggest that 49.5 kDa protein band may be a nonspecific signal caused by overloading of secondary antibody.

III. *Insolubility of Recombinant PP2A is Independent of Rate of Synthesis and Degree of Accumulation*

We attempted to obtain soluble PP2A from

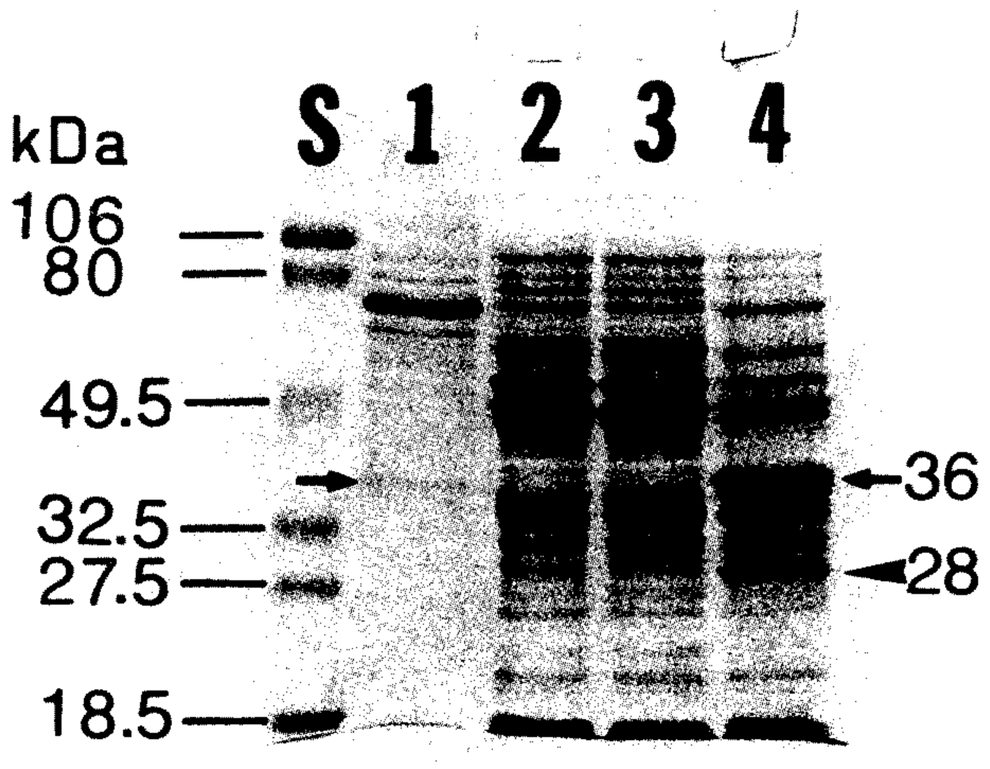

Figure 2. *E. coli* harboring pQE31-2A expresses the protein having an Mr 36 kDa which is comparable to human PP2A. The *E. coli* colony harboring plasmid pQE31-2A and a colony transformed with the control plasmid pQE31 were selected and transferred to 5ml of LB/carbenicillin/kanamycin media and grown at 37°C, overnight. Subsequently, 1.25ml of saturated cultures were inoculated into 8.75 ml of prewarmed LB/carbenicillin/kanamycin media and grew for one hour. The A_{600} of the cultures were measured to ensure that uniform cell densities were achieved. Next, the expression of PP2A was then induced by adding IPTG to a final concentration of 0.3mM, and the cultures were grown for an additional 3 hours. Cell pellets were harvested by centrifugation and resuspended in 1 ml of 50mM Tris. Cl, 500mM NaCl, 10% glycerol, pH7.4. Cell suspension was disrupted by Bead-Beater and the lysate was then divided into supernatant and pellet fractions by centrifugation. Aliquots of pellet were mixed with 100 μl 2X SDS/PAGE sample buffer and heated at 100°C for 10 min. Subsequently, the mixtures were subjected 10% SDS/PAGE. Lane S, protein molecular standards; lane 1, purified PP2A from human red blood cells (The major protein, apparent Mr 80 kDa, is a serum albumin added as an enzyme stabilizer.); lanes 2~4, crude extracts of *E. coli* host, *E. coli* transformed control vector pQE31, and *E. coli* harboring pQE31-2A, respectively.

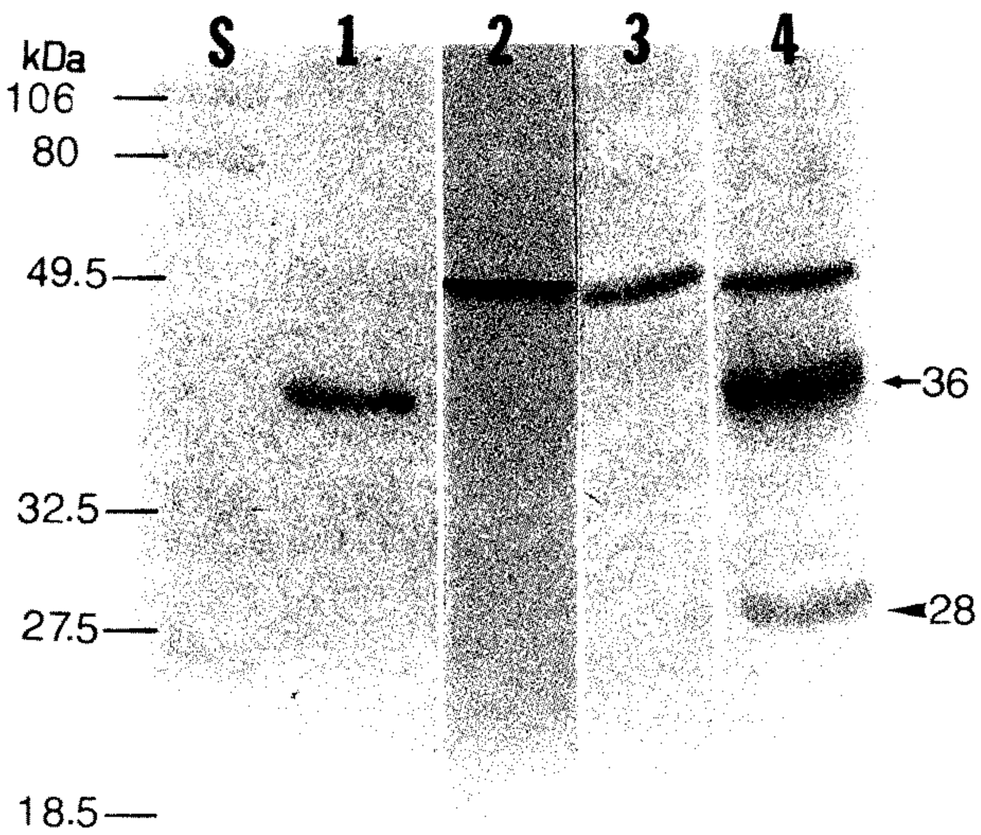

Figure 3. Both the *E. coli* recombinant protein and the human PP2A exhibit a cross-reactivity with specific antibody against a synthetic peptide corresponding to PP2A. Duplicate samples in Fig. 2 were transferred to nitrocellulose membrane and then subjectd to Western blot analysis as described in experimental procedures. The primay and secondary antibodies were at 1:2000 and 1:3000 dilutions, respectively. Lane S, protein molecular standards; lane 1, purified PP2A from human red blood cells; lanes 2~4, crude extracts of *E. coli* host, *E. coli* transformed control vector pQE31, and *E. coli* harboring pQE31-2A, respectively.

cells that were grown at lower temperatures and the expression period kept to a minimum after induction. These conditions slow the rate of protein synthesis and allow control over the accumulation.

Cells harboring pQE31-2A were grown in LB/carbenicillin/kanamycin at 37°C to A_{600}=0.3, a density at which no synthesis of PP2A was detectable. Cells were then shifted to 28°C and induced with IPTG. Cultures were analyzed at 0, 1, 5, and 24 hrs intervals. Proteins from pellet and supernatant fractions were separated on SDS/PAGE. Duplicate samples were used for Western blot analysis to determine if soluble PP 2A could be detected in the supernatant fractions after protein became visible in Coomassie blue stained gels. Our results showed that PP2A was present in an insoluble fraction under conditions which lower its rate of synthesis (Fig.4 A), and that no significant amount of soluble PP2A was detectable by Western blot analysis after the recombinant protein becomes a major cell product(Fig.4B). Similar results were obtained when cells were grown in super medium (25g bacto-tryptone, 15g bacto-yeast extract and 5g NaCl per liter), 2X YT medium (16g bacto-tryptone, 10g bacto-yeast extract and 5g NaCl per liter), and Psi-broth medium (LB medium + 4mM $MgSO_4$, 10mM KCl per liter) at 28 and 20°C in the presence of inducer. Different *E. coli* host strains *E. coli* JM109 and *E. coli* SG13009 [pREP4] used for the production of recombinant PP2A in the same culture conditions, were similar to what has been observed for *E. coli* M15 [pREP4] (data not shown). We conclude that insolubility of recombinant PP2A is not a function of the rate of protein synthesis, and is not correlated with protein accumulation, temperature-dependent factors, nor related to either *E. coli* host strains or culture media.

These results differ from other reports which have suggested that insolubility of recombinant proteins in *E. coli* may be a function of overexpression or a high rate of synthesis[30,31,32,33] and that solubility of recombinant proteins is favored by growth at lower temperatures[34].

IV. *Purification and Renaturation of the Recombinant PP2A Protein*

Due to its insolubility, the recombinant PP2A protein was purified by a process of denaturation and renaturation. A 2 liter culture was grown and induced as described earlier. Cell pellet was resuspended and solubilized in 6M guanidine HCl. Subsequent to centrifugation, the supernatant was collected and subjected to Ni-NTA affinity column. After a serial washing with buffer containing either guanidine HCl or urea, the final elution with buffer D (8M urea, 0.1M Na-phosphate, 0.01M Tris, pH4.5) was collected and analyzed with SDS/ PAGE. Densitometric scanning of the gel revealed that it

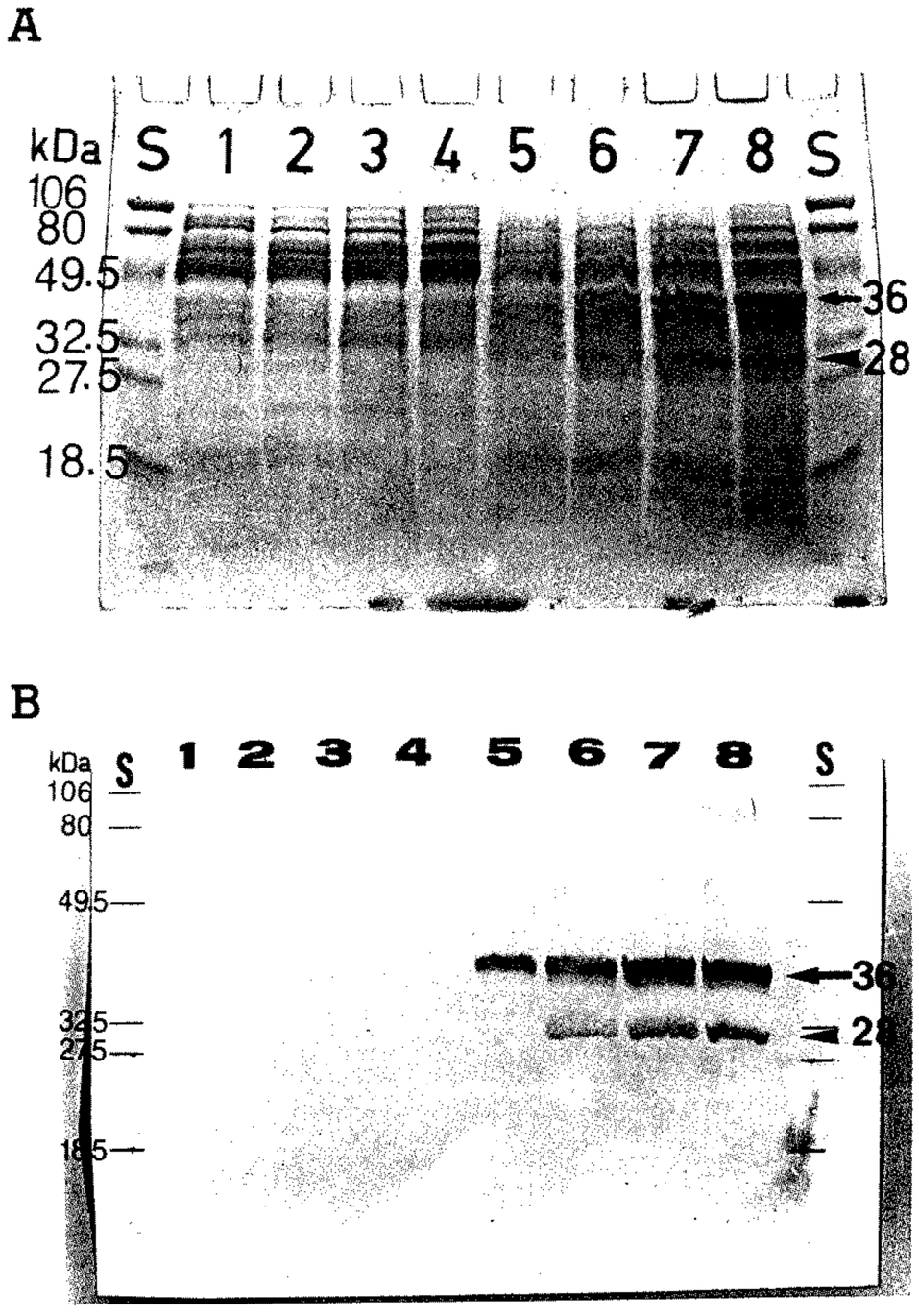

Figure 4. Insolubility of recombinant PP2A is not favored by low temperature and is independent of extent of accumulation. (A). *E. coli* cells harboring pQE31-2A were grown at 37°C (see experimental procedures). After IPTG induction, the cells were incubated at 28°C. Cells were harvested at intervals by centrifugation and resuspended in lytic buffer (10 mM Na_2HPO_4, 30 mM NaCl, 0.25% Tween 20, 10 mM β-mercaptoethanol, 10 mM EDTA, 10 mM EGTA). This suspension was disrupted by Bead-Beater and the pellet and supernatant separated by centrifugation (20,000xg) for 20 minutes. Lane S, Protein molecular weight standards; lanes 1～4, supernatant fractions of cell extract harvested at 0, 1, 5, and 24 hours after IPTG induction, respectively; lanes 5～8, pellet fractions of cell extract harvested at 0, 1, 5, and 24 hours after IPTG induction, respectively. (B). Duplicate sample were transferred to nitrocellulose and assayed by Western blotting for the presence of PP2A as described in experimental procedures. The primary and secondary antibodies were used at 1:2000 and 1:10000 dilutons, respectively. Immunoreactive material was detected only in the pellet fractions.

accounted for over 90% homogeneity of the recombinant protein obtained by one step purification and the enzyme was essentially homogenous after further passage through an HPLC gel filtration column (Fig.5). The yield in each case produced approximately 15～20 mg PP2A per liter culture. The purified enzyme was examined for phosphatase activity by numerous refolding conditions, including those used for reactivation PP1[29]. However, none of the refolding protocols generated active enzyme.

DISCUSSION

In this study we employed vector pQE31 using T5 promoter for PP2A expression. Our results show that overexpression in the vector pQE31 results in the accumulation of enzyme protein as an insoluble aggregate. Densitometric analysis reveals that the protein expressed from pQE31 accounts for 35% of the total cellular protein. A yield of 15～20 mg of purified protein/liter of culture was obtained in the *E. coli* expression system. This amount is higher than that reported by Zhang *et al.*[35] who used vector pTACTAC (with *trp-lac* promoter) and Berndt *et al.*[29] who used pAcYM1 (a baculovirus vector and polyhedrin promoter) to express the catalytic subunit of PP1 and is two folds higher than the expression of rabbit muscle phosphorylase b in pTACTAC[36].

Some laboratories have noted that the difficulty of expressing complex mammalian allosteric enzymes may be overcome by using weaker rather than a stronger promoter for expression and by lowering the culture temperature as well as modulation of the promoter activity[35, 36, 37, 38]. Based on these observations, Zhang *et al.*[35] subsequently reported that utilizing the vector pTACTAC at a lowered growth rate (28～30°C) produced soluble active PP1. Prior to pQE31 used in this study, bacterial vectors pMAL-p and pMAL-c were tested using the *trp-lac* fusion promoter to express PP2A. Basically, the structure of vectors pMAL-p and pMAL-c are similar; the only difference between these

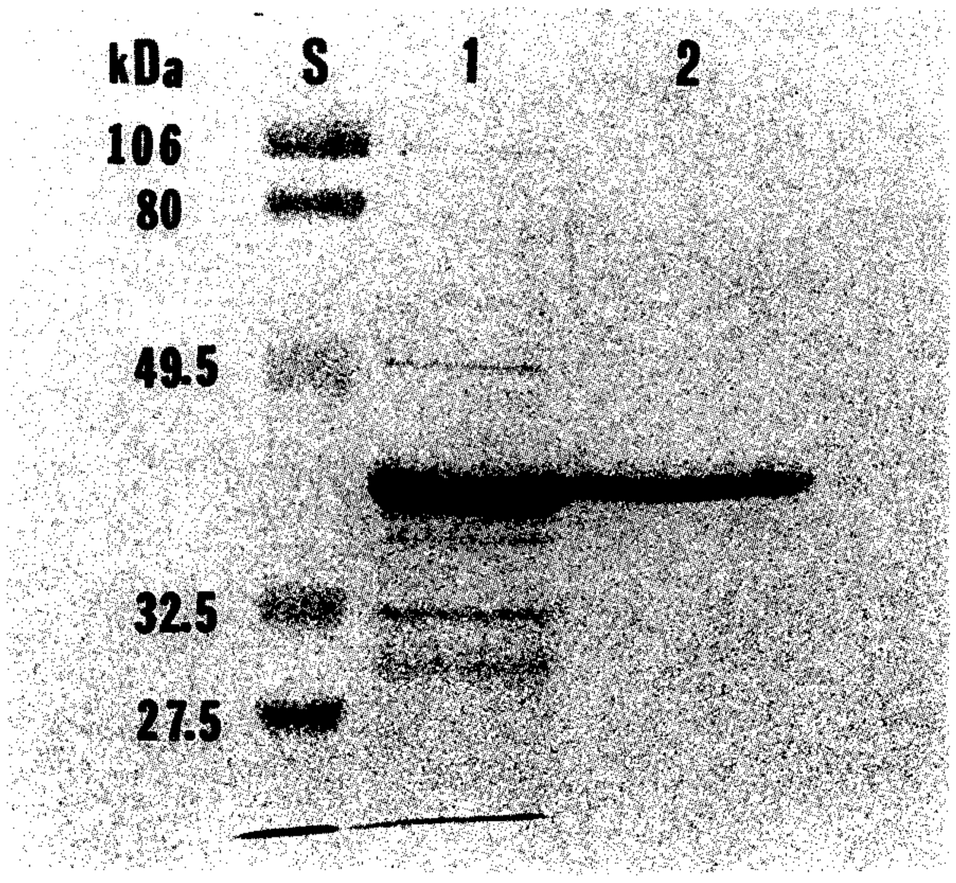

Figure 5. Homogenous recombinant PP2A can be obtained by passage through a Ni-NTA affinity and an HPLC gel filtration column. Lane S, protein molecular standards; lane 1, PP2A eluted from Ni-NTA affinity column; lane 2, homologous PP2A eluted from a Ni-NTA column and an HPLC gel filtration column.

two vectors lies in the signal sequence of the *mal*E gene on pMAL-p which is intact and potentially allows fusion proteins to be exported to the periplasm. However, our initial attempts to express PP2A at any appreciable level in *E. coli* were unsuccessful regardless of the varibles which were tested and included culture medium, *E. coli* host, culture temperature and metal ion. The expressed protein from both pMAL-p and pMAL-c was completely insoluble and could only be detected with antibodies (date not shown). The present results are similar to those of Tamura *et al.* who reported the expression of PP2C in *E. coli*[39]; and may be attributed to the structural difference between the catalytic subunit of PP1 and PP2A. If so, the *trp-lac* fusion promoter may not facilitate in the expression of PP2A as under the same conditions for PP1. Protein phosphatase type 2A is a heterotrimeric enzyme, thus the catalytic subunit may possess significant surface regions of hydrophobic nature that are involved in protein-protein interaction. This may explain why the catalytic subunit is present as an insoluble aggregation rather than folding to a relatively unstable tertiary structure during the process of bacterial expression in the absence of its other subunits. In fact, the free catalytic subunit of PP2A rarely occurs in mammalian cells. In addition, in our experience bovine adrenal medulla PP2A raises its specific activity when the concentrate enzyme is greater dilution. These lines of evidence seem to suggest a role of the regulatory subunits that contributes to enzyme specificity and the solubility of the catalytic subunit of PP2A. Thus, the expression of the active enzyme may be controlled by the availability of those regulatory subunits which bind to the catalytic subunit of PP2A. One possibility to test this attractive hypothesis would be to co-express the catalytic subunit with the A subunit of PP2A.

The significance of this current work in which we have expressed the protein in *E. coli* in mg amounts is that it provides an optimal means to obtain a pure preparation of a single isoform in lieu of isolation from tissue sources which is likely to result in a heterogenous yield. The fusion protein containing one 6xHis affinity tag, which binds to the Ni-NTA resin, facilitates the purification protocol. Moreover, the binding of the 6xHis/Ni-NTA does not require a functional proteinaceous structure and thus unaffected by strong denaturants such as guanidine hydrochloride or urea. Since 6xHis tag protein is itself of samll size, non-immunogenic and uncharged at physiological pH, the purified 6xHis fusion protein may be used directly to elicit an antigenic response in an immunized animal. Vector pQE31 employed in this study has been proved to be a rapid and efficient system to express enzyme[40], transcription factors[41] and antigens[42].

Berndt *et al.*[29] has shown that PP1 can be renaturated by rapid dilution out of 6M guanidine hydrochloride with buffer containing Mn^{2+} and 0.8M NaCl. However, we found preliminary refolding conditions via smaller dilution steps during dialysis against the buffer containing 50 mM Tris/Cl pH7.0, 50mM dithiothreitol, 1mM

$MnCl_2$, and 600mM NaCl reactivates about 1% of the total purified PP2A. The study of optimum conditions of refolding and characteristics of the reactivated enzyme is still in progress. Nevertheless, the active enzyme obtained in mg amount in our present work will be of significant use for the further study of PP2A since less than 1 mg of protein can be isolated by conventional preparations from about 100 bovine adrenal medulla glands[23].

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REFERENCES

1. Cohen, P. 1989. The Structure and Regulation of Protein Phosphatases. Annu. Rev. Biochem. 58:453-508.
2. Shenolikar, S. and Nairn, A.C. 1991. Protein Phosphatases: Recent Progress. Adv. Second Messenger Phosphoprotein Res. 23:1-121
3. Mackintosh, C. 1993. Assay and Purification of Protein (Serine/Threonine) Phosphatases. In "Protein Phosphorylation-a Practical Approach". Chapter 9. pp. 197-230. Hardie, D. G. (Ed). Oxford University Press Inc., NY. U.S.A.
4. Mayer-Jaekel, R.E. and Hemmings. B.A. 1994. Protein Phosphatase 2A-a 'ménage à trois'. Trends in Cell Biol. 4:287-291.
5. Walter, G. and Mumby, M. 1993. Protein Serine/Threonine Phosphatases and Cell Transformation. Biochimica et Biophysica Acta. 1155:207-226.
6. Mumby, M. and Walter, G. 1993. Protein Serine/Threonine Phosphatases: Structure, Regulation, and Function in Cell Growth. The American Physiol. Soc. 70:673-698.
7. Bialojan, C. and Takai, A. 1988. Inhibitory Effect of a Marine-sponge Toxin, Okadaic aicd, on Protein Phosphatases. Biochem. J. 256:283-290.
8. Haystead, T.A.J., Sim, A.T.R., Carling, D., Honnor, R.C., Tsukitani, Y., Cohen, P. and Hardie, D.G. 1989. Effects of the Tumor Promoter Okadaic Acid on Intracellular Protein Phosphorylation and Metabolism. Nature 337:78-81.
9. Kitagawa, Y.,Tahira, T., Ikeda, I., Kikuchi, K., Tsuiki, S., Sugimura, T. and Nagao, M. 1988. Molecular Cloning of cDNA for the Catalytic Subunit of Rat Liver Type 2A Protein Phosphatase and Detection of High Levels of Expression of the Gene in Normal and Cancer Cells. Biochim. Biophys. Acta. 951:123-129.
10. Kitagawa, Y., Sakai, R., Tahira, T., Tsuda, H., Ito, N., Sugimura, T. and Nagro, M. 1988. Molecular Cloning of Rat Phosphoprotein Phosphatase 2Aβ cDNA and Increases Expressions of Phosphatase 2Aα and 2Aβ in Rat Liver Tumors. Biochem. Biophys. Res. Commun. 157:821-827.
11. Virshup, D.M., Kauffman, M.G. and Kelly, T.J. 1989. Activation of SV40 DNA Replication *in vitro* by Cellular Protein Phosphatase 2A. EMBO J. 8:3891-3898.
12. Pallas D.C., Shahrik, L.K., Martin, B.L., Jaspers, S., Miller, T.B., Brautigan, D.L. and Roberts, T.M. 1990. Polyoma Small and Middle T Antigens and SV40 Small t Antigen Form Stable Complexes with Protein Phosphatase 2A. Cell 60:167-176.
13. Walter, G., Ruediger, R., Slaughter, C. and Mumby, M. 1990. Association of Protein Phosphatase 2A with Polyoma Virus Medium Tumor Antigen. Proc. Natl. Acad. Sci. U.S.A. 87:2521-2525.
14. Mumby, M. and Walter, G. 1991. Protein Phosphatases and DNA Tumor Viruses: Transformation through the Back Door? Cell Regul. 2:589-598.

15. Scheidtmann, K.H., Mumby, M.C., Rundell, K. and Walter, G. 1991. Dephosphorylation of Simian Virus 40 Large-T Atigen and p53 Protein by Protein Phosphatase 2A: Inhibition by Small-t Antigen. Mol. Cell. Biol. 11:1996-2003.
16. Yang, S., Lickteig, R.L., Ester, R., Rundell, K., Walter, G. and Mumby, M.C. 1991. Control of Protein Phosphatase 2A by Simian Virus 40 Small-t Antigen. Mol. Cell. Biol. 11:1988-1995.
17. Ruediger, R., Roeckel, D., Fait, J., Bergovist, A., Magnusson, G. and Walter, G. 1992. Identification of Binding Sites on the Regulatory A Subunit of Protein Phosphatase 2A for the Catalytic C Subunit and for Tumor Antigens of Simian Virus 40 and Polyomavirus. Mol. Cell. Biol. 12:4872-4882.
18. Cayla, X., Ballmer-Hoffer, K., Merlevede, W. and Goris, J. 1993. Phosphatase 2A Associated with Polyomavirus Small-t or Middle-T Antigen is an Okadaic Acid-sensitive Tyrosyl Phosphatase. Eur. J. Biochem. 214: 281-286.
19. Van Zyl, W., Huang, W., Sneddon, A.A., Stark, M., Camier, S., Wernwer, M., Marck, C., Sentenac, A. and Broach, J.R. 1992. Inactivation of the Protein Phosphatase 2A Regulatory Subunit A Results in Morphological and Transcriptional Defects in *Saccharomyces cerevisiae*. Mol. Cell. Biol. 12: 4946-4959.
20. Guo, H., Reddy, S.A. and Damuni, Z. 1993. Purification and Characterization of an Autophosphorylation-activated Protein Serine Threonine That Phosphorylates and Inactivates Protein Phosphatase 2A. J. Biol. Chem. 268:11193-11198.
21. Guo, H. and Damuni Z. 1993. Autophosphorylation-activated Protein Kinase Phosphorylates and Inactivates Protein Phosphatase 2A. Proc. Natl. Acad. Sci. U.S.A. 90:2500-2504.
22. Chiou, J.Y. and Westhead, E.W. 1992. Okadaic Acid, a Protein Phosphatase Inhibitor, Inhibits Nerve Growth Factor-directed Neurite Outgrowth in PC12 cells. J. Neurochem. 59:1963-1966.
23. Chiou, J.Y. 1992. Purification and Molecular Cloning of Protein Phosphatases of Bovine Adrenal Medulla: An Assessment of Their Physiological Role in PC12 Cells. Ph.D. Dissertation in Univ. of Massachusetts.
24. Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. Molecular Cloning: A Laboratory manual, 2nd ED., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
25. Silberman, S.R., Speth, M., Nemani, R., Ganapathi, M.K., Dombradi, V., Paris, H. and Lee, E.Y.C. 1984 Isolation and Characterization of Rabbit Skeletal Muscle Protein Phosphatases C-I and C-II. J. Biol. Chem. 259: 2913-2922.
26. Laemmli, U.K. 1970. Cleavage of Structural Proteins During the Assembly of the Head of Bacteriophage T4. Nature 227:680-685.
27. Bradford, M.M. 1976. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-dye Binding. Anal. Biochem. 72:248-254.
28. Conner, G.E. and Udey, J.A. 1990. Expression and Refolding of Recombinant Human Fibroblast Procathepsin D. DNA and Cell Biol. 9:1-9.
29. Berndt, N. and Cohen, P.T.W. 1990. Renaturation of Protein Phosphatase 1 Expressed at High Levels in Insect Cells Using a Baculovirus Vector. Eur. J. Biochem. 190:291-297.
30. Prouty, W.F., Karnovsky M.J. and Goldberg. A.L. 1975. Degradation of Abnormal Proteins in *Escherich*ia *coli*. Bio/technology 2:800-804.
31. Cheng, Y.-S., Kwoh, D.Y., Soltvedt, B.C. and Zipser, D. 1981. Stabilization of a Degradable Protein by Its Overexpression in *Escherichia coli*. Gene 14:121-130.
32. Williams, D.C., Van Frank, R.M.,Muth, W. L. and Burnett, J.P. 1982. Cytoplasmic Inclusion Bodies in *Escherichia coli* Producing Biosynthetic Human Insulin Proteins. Science

215:687-689.
33. Marston, F.A.O., Lowe, P.A., Doel, M.T., Schoemaker, J.M., White, S. and Angal, S. 1984. Purification of Calf Prochymosin (Prorennin) Synthesized in *Escherichia coli*. Bio/technology 2:800-804.
34. Schein, C.H. and Noteborn, M.H.M. 1988. Formation of Soluble Recombinant Proteins in *Escherichia coli* is Favored by Lower Growth Temperatures. Bio/technology 6:291-294.
35. Zhang, Z., Bai, G., Deans-Zirattu, S., Browner, M.F. and Lee, E.Y.C. 1992. Expression of the Catalytic Subunit of Phosphorylase Phosphatase (Protein Phosphatase-1) in *Escherichia coli*. J. Biol. Chem. 267, 1484-1490.
36. Browner, M.F. Rasor, P., Tugendreich, S. and Fletterick, R.J. 1991. Temperature-sensitive Production of Rabbit Muscle Glycogen Phosphorylase in *Escherichia coli*. Protein Eng. 4:351-357.
37. Takagi, H., Morinaga, Y., Tsuchiya, M., Ikemura, H. and Inouye, M. 1988. Mutant Subtilisin E with Enhanced Protease Activity Obtained by Site-directed Mutagenesis. Biotechnology 6:948-950.
38. Schein, C.H. and Noteborn, M.H.M. 1988. Formation of Soluble Recombinant Proteins in *Escherichia coli* is Favored by Lower Growth Temperatures. Bio/technology. 6:291-294.
39. Tamura, S., Yasui, A. and Tsuiki, S. 1989. Expression of Rat Protein Phosphatase 2C (IA) in *Escherichia coli*. Biochem. Biophys. Res. Commun. 163, 131-136.
40. Dobeli, H., Trecziak, A., Gillessen, D., Matile, H., Srivastava, I.K., Perrin, L.H., Jakob, P.E. and Certa, U. 1990. Expression, Purification , Biochemical Characterization and Inhibition of Recombinant Plasmodium Falciparum Aldolase. Mol. and Biochem. Parasitol. 41:259-268.
41. Janknecht, R., de Martynoff, G., Lou, J., Hipskind, R.A., Nordheim, A. and Stunnenberg, H.G. 1991. Rapid and Efficient Purification of Native Histidine Tagged Protein Expressed by Recombinant Vaccinia Virus. Proc. Natl. Acad. Sci. U.S.A. 88:8972-8976.
42. Stuber, D., Matile, H. and Garotta, G. 1990. System for High-level Production in *Escherichia coli* and Rapid Purification of Recombinant Proteins: Application to Epitope Mapping, Preparation of Antibodies, and Structure-function Analysis. In "Immunological Methods" (I. Lefkovits and B. Pernis, eds.) Vol. IV, 121-152.

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2A型蛋白去磷酸化酵素催化體在大腸桿菌中的高度表達

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摘　要

本研究的目的係利用分子選殖方式，發展一套能在大腸桿菌中高度表達牛腎上腺髓質2A型蛋白去磷酸化酵素催化體的系統。大量的酵素經分離純化後，將可供進一步研究該2A型酵素的結構及其活性基位和其催化體與調節體之相互關係。2A型酵素催化體已成功的在大腸桿菌中獲得表達，所使用的質體pQE31含有大腸桿菌噬菌體T5驅動子(T5 promoter)和兩段*lac* operator作爲2A型酵素的表達和調控，另含一段合成6個histidine標的胜肽基因以便利所表達的2A型酵素之純化和分離。所表達出來之酵素約佔菌體蛋白的30～35%，但大都爲水不溶性蛋白且不具酵素活性。SDS-PAGE分析顯示，其分子量爲36kDa，與自人的紅血球分離到之2A型酵素相同。且此基因重組蛋白和紅血球分離出來的2A型酵素一樣，能與合成2A型酵素胜肽(相對於2A型酵素催化體氨基酸順序296-309)之特異抗體作用。此不溶性蛋白可溶解於6M guanidine hydrochloride，並直接利用Ni-NTA resin來純化，單一的吸附層析純化可達90%以上的純化率，且完全的純化可再藉由通過凝膠過濾層析來達成。大約每公升的培養基可獲得15～20mg純化之2A型酵素。目前，酵素回性條件尚在探討中，若能有效的使蛋白再摺疊而活化該酵素，則本表達系統提供一個大量生產且快速純化2A型酵素的方法，將有助於探討該酵素的結構和其生理的功能。

關鍵詞：2A型蛋白去磷酸化酵素，pQE31。