Bull Sch Health Sci, Okayama Univ 6 : 63~72, 1995
(Original article)

Expression of Phosphacan, a chondroitin sulfate proteoglycan, core protein in Esherichia coli as a fusion protein with glutathione S-transferase

Sekiko ITO and Motoi OKAMOTO

Abstract

Optimal conditions for expressing a specific region of core protein of phosphacan, a chondroitin sulfate proteoglycan known as receptor type protein tyrosine phosphatase, as fusion protein with glutathione S-transferase (GST) in E.coli were examined. DNA fragments inserted into the expression vector (pGEX-4T-1) were amplified by RT-PCR using mRNA purified from E18 rat brain as template. Primers attached with BamH I or EcoR I restriction site on 5' end were used to amplify first strand cDNA by PCR. Before ligation into the pGEX-4T-1 for GST fusion protein, PCR products were once cloned using T-A cloning system because they were not directly ligated into the pGEX-4T-1. E.coli strain BL21 was transformed by pGEX-4T-1 ligated with restriction DNA fragment cut out from pCR II plasmid vector of T-A cloning system. The growth of transformed BL21 was not different between the colony incubated at 37°C for 24-48 h and the colony stored at 4°C for 7-10 days after 24h incubation at 37°C. The desirable OD_{550} of culture medium for inducing the expression of fusion protein by isopropylthio-β-D-galactoside (IPTG) was from 0.6 to 1.0, because expression of native E.coli proteins per ml of culture medium was increased relatively when IPTG was added at OD_{550} more than 1.0. The expression of fusion protein reached plateau around 6h after the induction. Relative expression of native E.coli proteins per ml of culture medium increased thereafter. Therefore, it may be desirable to purify the fusion protein around 6h after the induction.

Key words : phosphacan, glutathione S-transferase, BL21, IPTG, fusion protein.

INTRODUCTION

There are increasing evidence that chondroitin sulfate proteoglycan (CSPGs), which constitute a family of extracellular matrix molecules, have various biological functions in cell proliferation, migration, differentiation, extension of processes[1-8]. They also play important roles in survival or death of neuronal cells, viral infection, and degenerative disorders of central nervous system[9-13]. CSPGs are structurally complex molecules having polysaccharide side chains attached to core proteins. Glycosaminoglycan side chains are indispensable for some biological functions[7,14-15], while core proteins play the leading role in other biological functions[2,5,16]. In conventional biochemical strategies, core proteins or glycosaminoglycans separated by enzymatic

School of Health Sciences, Okayama University

digestion of CSPGs with glycosidases or proteinase for discriminating functions of core proteins from those of glycosaminoglycans.

In previous studies, we reported that soluble CSPGs purified from neonatal rat brain protected both acute and delayed cell death induced by glutamate of primary cultured cortical and hippocampal neurons of the rat, and that the protective action depended on core proteins rather than glycosaminoglycans[9-10]. We found that a specific domain of the core protein of phosphacan[17-18], a brain soluble CSPGs, might have the leading role in protective action against delayed neuronal death induced by glutamate in experiments using fragments of core proteins purified by chondroitinase digestion and CNBr treatment (unpublished data). However, we could not rule out the role of polysaccharides, because phosphacan have keratan sulfate side chains and oligosaccharides beside the chondroitin sulfate side chains, and keratan sulfates and oligosaccharides are not removed from core protein after chondroitinase digestion. In this context, we planed to purify recombinant phosphacan core protein after expressing it as a fusion protein with glutathione S-transferase (GST) in Esherichia coli.

In this study, we examined the optimal conditions for ligation of DNA fragments into plasmid vector, transformation of E.coli, and expression of the fusion protein with GST. Primers for PCR were designed to amplify DNA correspond to the domain of phosphacan core protein which was identified to have protective action against delayed neuronal death induced by glutamate in our other experiments.

MATERIALS and METHODS

1. EXTRACTION OF mRNA FROM THE RAT BRAIN

mRNA was extracted from the brain of 18-day-old rat fetuses (Sprague-Dawley rat, Kurea, Osaka, Japan), using the Fast Track mRNA Isolation Kit (Invitrogen, USA). Brains were placed in Fast Track-lysis buffer, homogenized with a machine-driven Teflon pestle homogenizer for 15-30 seconds, and incubated at 45°C for 60 min. The NaCl concentration of the lysate was adjusted to 0.5M by adding 5M NaCl stock solution. Oligo (dT) (Invitrogen, USA) Cellulose were added to the lysate, incubated at room temperature for 60min, and spined at room temperature for 5min at 3200rpm. Following centrifugation, the Oligo (dT) Cellulose were washed three times with low salt wash buffer. Oligo (dT) Cellulose were suspended in low salt wash buffer, pipetted onto a spin-column, and spined at room temperature for 10sec at 5000rpm. The column was washed repeatedly with low salt wash buffer until the OD_{260} of the "flow-through" was $\leq$ 0.05. Elution buffer was added to the column, and spined for 10sec to collect the elution containing mRNA. mRNA were precipitated with 0.15 volume of 2M Sodium Acetate and 2.5 volume of 100% ethanol, and spined for 15min at 15000rpm. Following centrifugation and drying by vacuum, the mRNA pellet was resuspended in 10mM Tris-HCl (pH7.5).

2. REVERSE TRANSCRIPTION/PCR

Using the First-Strand cDNA Synthesis Kit (Pharmacia Biotech) and the PCR Master Kit (Boehringer Mannheim), 1.0μg of mRNA was reverse-transcribed for 60min at 37°C in a volume of 15μl. Oligo d(T)12-18 (Takara, KYOTO, Japan) was used as the primer for reverse transcription. Following incubation, All sens and antisense primers, and PCR components except the Taq polymerase were added and heated for 2min at 95°C. Primers used in PCR, combination of primers, and the size of PCR products are shown in Fig. 1. After addition of Taq

```
OKA-1S (sense)
            343                    349
  5 '-GGATCCATTGAGAAGTTTGCGGTTCTG-3'

OKA-1AS (antisense)
            417                    410
  5 '-GAATTCGAGGTCAAGTTCAGCATCCTC-3'

OKA-2S (sense)
            407                    413
  5 '-GGATCCGACATGCCTACTGAGGATGCT-3'

OKA-2AS (antisense)
            445                    439
  5 '-GAATTCACCGGGATTCAAGCCAGTGTC-3'

P1:OKA-1S - OKA-1AS  225bp
P2:OKA-2S - OKA-2AS  120bp
P3:OKA-1S - OKA-2AS  312bp
```

Fig. 1. Sequences of synthetic oligonucleotide primer for PCR. These primers for PCR were designed to amplify DNA correspond to the domain of phosphacan core protein which identified to have protective action against delayed neuronal death induced by glutamate. Primers are attached the recognition sequence for a restriction enzyme (BamH I, EcoR I) at 5' terminus.

polymerase, the template was placed in an heating/cooling block (Zymoreactor AB-1800, ATTO, Japan). The reaction was started by heat denaturation of the template (2min, 94°C). The template was amplified by 30 PCR cycles (94°C (1min), 55°C (1min), and 72°C (2min)) followed by a 7-min extension at 72°C after the final cycle. After purification by phenol/chloroform extraction followed by precipitation with ethanol, the PCR products were analyzed by agarose (3%-Nu Sieve 3 : 1 agarose, FMC, USA)-ethidium bromide (0.5mg/dl) gel electrophoresis.

3. SUBCLONING OF THE PCR PRODUCTS AND DNA SEQUENCING

Using the T-A Cloning Kit (Invitrogen), 500ng of PCR products were ligated into 25ng of pCR II vector with 3'T-overhangs[19]. The reaction medium was incubated at 16°C for overnight, transformed into One Shot cells (INVαF) by heat shock, and spreaded on labeled LB agar plates containing 100μg/ml of ampicillin, 20mg/dl of 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (WAKO, OSAKA, JAPAN) and 100mM of isopropylthio-β-D-galactoside (IPTG) (AMBION). The LB plates were incubated at 37°C for 24hr. Plasmid DNA were purified from One Shot cells (INVαF) by using boiling methods. At least 20 single white colonies were transferred into 2ml of ×2 YTA medium (Tryptone 16g, Yeast extract 10g and ampicillin 100mg/l), and incubated overnight at 37 with vigorous agitation. The culture medium was spined at 3000rpm for 10min at 4°C. Following centrifugation, the bacterial pellet was resuspended in 350μl of STET (8% sucrose, 0.5% Triton-X100, 50mM EDTA, 10mM Tris-HCl (pH8.0)). A prepared solution of lysozyme (50mg/ml) (Boehringer Mannheim) was added to the lysate, and placed in a boiling-water bath for 1min. The bacterial lysate was spined at 12000rpm for 15min. After centrifugation, the pellet of bacterial debris were removed from the microfuge tube with a sterile toothpick. The isopropanol and 3M sodium acetate was added into the supernatant, mixed by vortex, and placed the tube for 15min at −20°C. After precipitation for 15min, microfuge tube was spined at 4°C for 15min at 12000rpm. The pellet was rinsed with 70% ethanol, and evaporated in desiccator. The plasmid DNA was redissolved in 50μl of RNase (200μg/ml), incubated at room temperature for 1hr. A prepared solution of 20% Polyethylene Glycol 6000 was added to the lysate, placed at 4°C for 1hr, and spined at 12000rpm for 5min. After centrifugation, the pellet was rinsed with 70% ethanol, and evaporated in desiccator. Plasmid DNA sequencing was performed by using PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems). The plasmid DNA was amplified with ampli taq DNA polymerase and universal primers for

the sequencing of bacteriophage M13 recombinant clones (universal M13 forward (−20) primer and universal M13 reverse primer). Amplification was performed using 25 cycles of 96°C (1min), 50°C (0.5min), 60°C (4min). After amplification, the PCR products were extracted by phenol/chloroform, and precipitated with ethanol. The purified plasmid DNA was suspended in sample loading buffer. The samples were loading onto polyacrylamide gel containing 5.25% long ranger (FMC Bioproducts-Europe) and 8.3M urea for 16hr.

Beside the DNA sequencing (373 DNA sequence system, Applied Biosystems) pCR II plasmid was purified from INVαF, digested with BamH I and EcoR I, and separated by agarose gel electrophoresis to purify sticky-end PCR product. The resulting fragments were subcloned into pGEX (4T-1) by using E.coli strain BL21.

4. EXAMINATION OF OPTIMAL CONDITION FOR EXPRESSION OF THE FUSION PROTEIN

Purified PCR product was ligated into pGEX-4T-1 plasmid vector at 37°C for 30min or 16°C overnight by using Ligation Kit (Takara, KYOTO, JAPAN), and introduced by a heat shock of 42°C for 90sec to transform E.coli BL21. A single colony of E coli transformed with the pGEX (4T-1) recombinant were transferred into 2ml of ×2 YTA medium in test tubes, and incubated at 30°C or 37°C with vigorous agitation until the OD_{550} reached from 0.6 to 2.0. 100mM of IPTG was added to the bacterial medium at a final concentration of 0.1mM, and then the bacterial lysate was continued incuba-

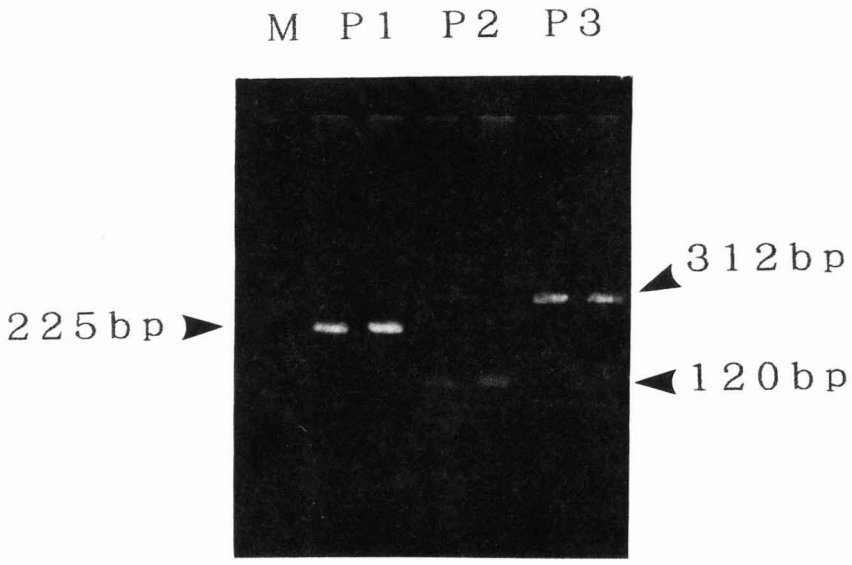

Fig. 2. Ethidium bromide-agarose gel electrophoresis of the PCR products. mRNA was extracted from the brain of 18-day-old rat fetuses. Oligo $d(T)_{12-18}$ was used as the primer for reverse transcription. The templates were amplified with Taq polymerase and synthesized primers with restriction enzyme recognition site, using 30 temperature-step cycles of 94°C (1min), 55°C (1min) and 72°C (2min) with a 2min denaturation at 94°C before the cycles and a 7min extension at 72°C after the final cycle. The reaction products were electrophoresed on 3% agarose (NuSieve 3 : 1) gel and stained with ethidium bromide.
M ; DNA biomarker low (Bioventures, INC) : 1.0, 0.7, 0.52, 0.5, 0.4, 0.3, 0.2, 0.1 and 0.05kb respectively. 1 ; OKA 1S-OKA 1AS : 225bp. 2 ; OKA 2S-OKA 2AS : 120bp. 3 ; OKA 1S-OKA 2AS : 312bp.

tion at 30°C or 37°C for another 24hr. During induction, 1.5ml aliquots of induced culture were sampled at 1, 2, 4, 6, 12, 18 and 24hours after the addition of IPTG. These samples were immediately spined at 10000rpm for 1min at room temperature. Each pellet was resuspended in 100μl of SDS gel-loading buffer, heated to 100°C for 5min and spined at 15000rpm for 1min at room temperature. Ten μl of each supernatant was loaded on SDS-polyacrylamide gel of 15% concentration. The gel was stained with Coomassie Brilliant Blue.

RESULTS

1. REVERSE TRANSCRIPTION/PCR

The PCR products from rat's brain mRNA were shown in Fig. 2. All three PCR products were electrophoresed at expected base-pair size of 225bp (P1), 120bp (P2) and 312bp (P3) respectively on agarose gel. At first, we used primers without restriction enzyme-recognition site for RT/PCR. The PCR products was digested with Klenow fragment, phosphorylated, and then a bluntended fragment of PCR products was ligated into pGEX (4T-2), but not successfully ligated into the vector. Therefore, we used primers having 5'restriction enzyme-recognition site to produced double-strand DNA having BamH I and EcoR I site at each end.

2. LIGATION AND TRANSFORMATION

The PCR products were not successfully ligated into pGEX plasmid (4T-1) even when they were digested with BamH I and EcoR I to have asymmetric sticky-end on each side. Then we used a T-A cloning kit, in which the PCR products with 3'A-overhangs could be ligated into pCR II vector with 3'T-overhangs. The

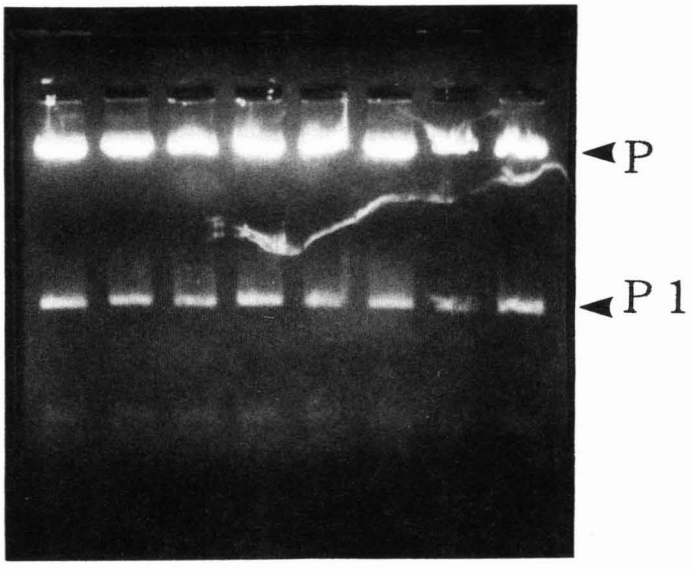

Fig. 3. Ethidium bromide-agarose gel electrophoresis of 225bp BamH I-EcoR I restriction fragment. Plasmid DNA was purified from One Shot cells (INVαF) by using boiling methods as described in materials and methods. Purified plasmid DNA was digested with BamH I and EcoR I, and separated by agarose gel electrophoresis. M ; DNA biomarker low : 1.0, 0.7, 0.52, 0.5, 0.4, 0.3, 0.2, 0.1 and 0.05kb. The fragments of 225bp (indicated by the arrow) were cut out from the gel, and purified as described in materials and methods to ligate into pGEX-4T-1.

PCR products were successfully ligated into pCR II by T-A cloning kit. We could confirm that PCR product P1 and P2 had correct nucleotide sequence but nucleotide sequence of P3 was incorrect by DNA sequencing. Then we used PCR product P1 to examine the optimal conditions for expression of fusion protein with GST. The pGEX (4T-1) ligated a 225bp BamH I-EcoR I restriction fragment (Fig. 3) was purified from One shot cell (INVαF). At first, we used E.coli strain JM109 for transformation, but this E.coli strain JM109 express little fusion protein. Then we changed the strain of E.coli to BL21, E.coli strain BL21 expressed the fusion protein.

3. OPTIMAL CONDITION FOR EXPRESSION OF FUSION PROTEIN

The colonies of BL21 were picked up from two different LB plate containing ampicillin.

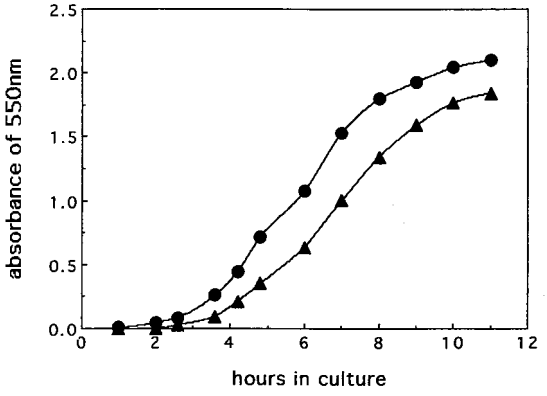

Fig. 4. Growth curve of transformed E.coli BL21 at 30°C. The colonies of BL21 were picked up from two different LB plate containing ampicillin and incubated at 30°C with vigorous agitation. The one was incubated at 37°C for 24 to 48hrs after the plating of E.coli. Another was stored at 4°C for 7 to 10 days after the incubation at 37°C for 24hrs. During incubation, OD_{550} of culture medium was measured at every 30 or 60min.
Closed circles: colonies incubated at 37°C for 24 to 48hrs. Closed triangles: colonies stored at 4°C for to 10 days after the incubation at 37°C for 24hrs.

The one was incubated at 37°C for 24 to 48hrs after the plating of E.coli. Another was stored at 4°C for 7 to 10 days after the incubation at 37°C for 24hrs. The growth curve of two colonies were very similar to each other (Fig. 4). We examined the effect of temperature on growth curve of transformed E.coli in the next experiment. As expected , E.coli grew rapidly at 37°C compared to growth temperature at 30° C. However, majority of GST fusion protein was held in insoluble fraction when E.coli was grown at 37°C. Therefore, transformed BL21 was incubated in a shaking water bath at 37°C in the next two experiments.

To examine the adequate OD_{550} to induces the expression of GST fusion protein, IPTG was added to the medium at OD_{550} from 0.6 to 1.6. Following the another 13-18hrs incubation at 30°C, the expression of fusion protein was analyzed by SDS-PAGE (Fig. 5). OD_{550} at the induction with IPTG did not have so much effect on the expression of fusion protein, but production of native proteins of E.coli was slightly increased when IPTG was added at OD_{550} more than 1.0. There was no difference between colonies incubated at 37°C and colonies stored at 4°C.

Finally, duration of incubation for the expression of fusion protein was examined. A colony was picked up from LB plate incubated at 37°C for 48hrs, and incubated in 20ml of 2× YTA medium at 30°C. IPTG was added to the medium at $OD_{550}=0.6$. One ml of the medium was sampled at 1, 2, 4, 6, 12, 18 and 24hours after the induction. Each sample was centrifuged at 10000rpm for 10sec, and pellet was stored at −20°C until the analysis. The expression of fusion protein was analyzed by SDS-PAGE. The expression of fusion protein reached the maximum level around 6h after the IPTG induction, and relative concentration of

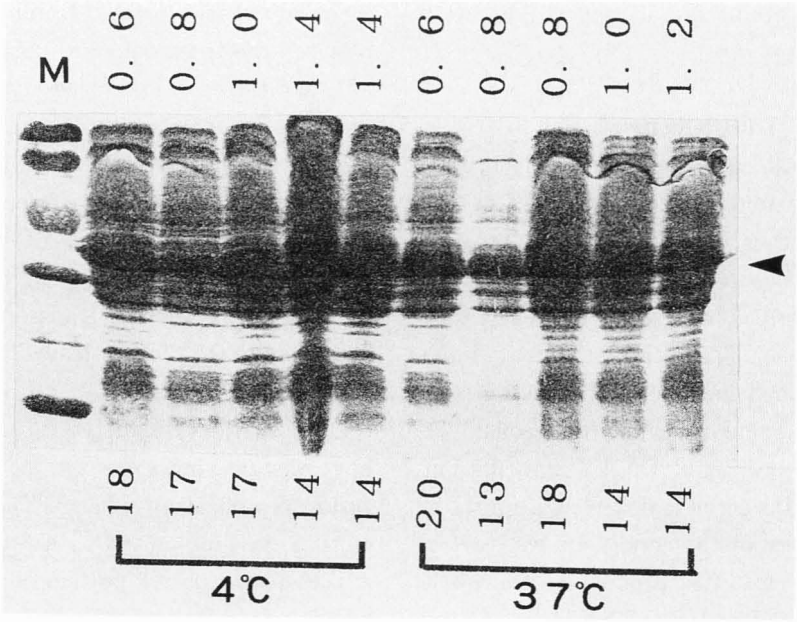

Fig. 5. Relation between the OD_{550} at the time of IPTG induction and the expression of fusion protein. The colonies of BL21 were picked up from two different LB plate as described in results and Fig. 4 and incubated in 2× YTA medium at 37°C with vigorous agitation until the OD_{550} reached from 0.6 to 1.4. When the OD_{550} reached from 0.6 to 1.4, IPTG was added to the bacterial medium at a final concentration of 0.1mM. One ml of medium was sampled at 13-18h after the IPTG induction, and the pellet of bacteria was boiled for 5min in SDS sample buffer. Ten μl of the aliquot was electrophoresed on SDS-polyacrylamide gel (4.5% stacking, 15% separating) to examine the expression of fusion protein. The numbers on the top of gel indicate OD_{550} when IPTG was added to the bacterial medium.

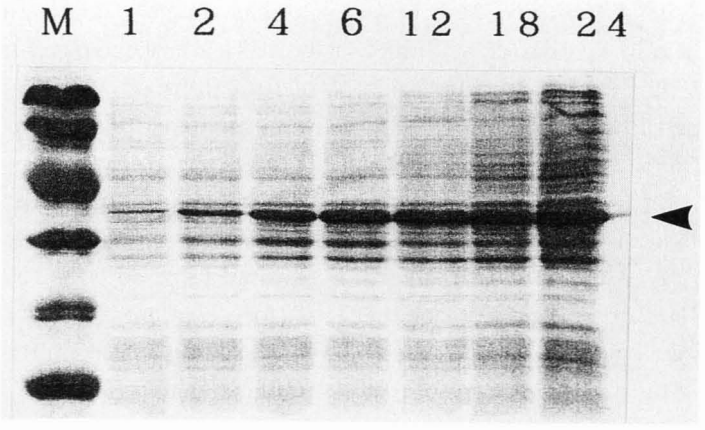

Fig. 6. Relation between the time after the addition of IPTG and the expression of fusion protein. A colony of BL21 was picked up from LB plate incubated at 37°C for 24 to 48hrs. The colony was incubated at 30°C until the OD_{550} reached 0.6, and then IPTG was added to the bacterial medium at a final concentration of 0.1mM. One ml aliquot of culture medium was sampled at 1, 2, 4, 6, 12, 18 and 24hours, after the addition of IPTG. The pellet was boiled for 5min in SDS sample buffer, and the aliquot of 10μl was electrophoresed on SDS-polyacrylamide gel (4.5% stacking, 15% separating). The expression of fusion protein (indicate by the arrow) reached the peak at 12h after the addition of IPTG.

native E.coli proteins was increased thereafter (Fig. 6).

DISCUSSION

The PCR products were not directly ligated into pGEX plasmid vector regardless of the constitution of their ends ; blunt or sticky, or of the protocol of ligation reaction ; ligated at 37°C for 30min or at 16°C overnight. However, they were successfully ligated into the expression vector after cloned once by T-A cloning system. The results indicate that the biochemical or biophysical properties of DNA fragments cut out from pCR II vector is different from those of PCR products, or the conditions we used in direct ligation of PCR products into pGEX vector were inadequate. The lattered was supported by the fact that PCR products having sticky ends were directly ligated by using another ligation kit (data not shown), although we are confirming that transformed E.coli have pGEX plasmid vector properly ligated with PCR products in our ongoing experiments. The ratio between insert DNA and plasmid is a important factor for ligation, and may be a reason of our failure in ligating directly into plasmid vector. But this is not probable because we examined all ratios from 10 : 1 to 1 : 1.

The expression of fusion protein was slow when E.coli JM109 was used as the host of expression vector. That is to say, it required longer time in JM109 than in BL21 to reach the peak of expression of the fusion protein. Although we did not fully examined the optimal conditions for the expression of fusion protein in JM109, this imply that the strain of host bacteria influence the expression of protein concerned, and that JM109 is not suitable for the expression of GST fusion protein or core protein of phosphacan.

Growth of transformed E.coli BL21 was same between the fresh colonies incubated 24-48h at 37°C and the colonies stored at 4°C for 7-10days after the incubation at 37°C (Fig. 4). This was the case when transformed BL21 was incubated at 37°C (data not shown). There were no differences in the expression of fusion protein between the two colonies (Fig. 5). The results indicate that colony of transformed E.coli can be stored at 4°C for at least 7-10 days on LB plate.

OD_{550} at the time of IPTG induction did not have distinct influence on the expression of fusion protein itself when IPTG was added to culture medium at OD_{550} 0.6-1.4. However, expression of native protein of the host BL21, which may influence the efficiency of purification of GST fusion protein, was increased when IPTG was added at OD_{550} over 1.0. Therefore, it may be desirable to induce the expression of fusion protein at OD_{550}.

The expression of fusion protein was considered to reach the maximum level around 6h after the induction with IPTG although we did not calculate the relative amount of expressed protein in the same mass of E.coli. The amount of fusion protein in per ml of culture medium was increased almost in parallel with the increase of OD_{550} thereafter. But expression of native E.coli protein in per ml culture medium was also increased when the culture was continued for more than 12h. It is desirable to exclude contamination of native E.coli protein for purifying fusion protein, because unknown proteins may affect the recovery of fusion protein. Therefore it may desirable to purify fusion protein around 6h after the induction.

Reference

1) Bicknese AR, Sheppard AM, O'Leary DDM and Pearlman AL.: Thalamocortical axons along a

chondroitin sulfate proteoglycan-enriched pathway coincident with the neocortical subplate and distinct from the efferent path. J Neurosci 14 : 3500-3510, 1994.

2) Iijima N, Oohira A, Mori T, Kitabatake K and Kohsaka S : Core protein of chondroitin sulfate proteoglycan promotes neuraite outgrowth from cultured neocortical neurons. J Neurochem 56 : 706-708, 1991.

3) Kato-Semba R, Matsuda M, Kato K and Oohira A : Chondroitin sulfate proteoglycans in the rat brain : candidates for axon barrier of sensory neurons and the possible modification by laminin of their actions. Eur J Neurosci 7 : 613-621, 1995.

4) Miller B, Sheppard AM, Bicknese AR and Pearlman AL : Chondroitin sulfate proteoglycans in the developing cerebral cortex : the distribution of neurocan distinguishes forming afferent and efferent axonal pathways. J Comp Neurol 355 : 615-628, 1995.

5) Oohira A Matsui F and Kato-Semba R : Inhibitory effects of brain chondroitinsulfate proteoglycans on neuraite outgrowth from PC12 cells. J Neurosci 11 : 822-827, 1991.

6) Snow DM, Lemmon V, Carrino DA , Caplan AI and Silver J : Sulfated proteoglycans in astroglial barriers inhibit neurite outgrowth in vitro. Exp Neurol 109 : 111-130, 1990.

7) Snow DM, Watanabe M, Letourneau PC and Silver J. : A chondroitin sulfate proteoglycan may influence the direction of retinal ganglion cell outgrowth. Develoment 113 : 1473-1485, 1991.

8) Watanabe E, Aono S, Matsui F, Yamada Y, Naruse I and Oohira A : Distribution of a brain-specific proteoglycan, neurocan, and the corresponding mRNA during the formation of barrels in the rat somatosensory cortex. Eur J Neurosci 7 : 547-554, 1995.

9) Okamoto M, Ichimura M and Mori S : Excitotoxic death of cultured cortical neurons : developmental changes and prophylactic effects of chondroitin sulfate proteoglycans. Neurosci 19 : 163-172, 1993.

10) Okamoto M, Mori S and Endo H : A protective action of chondroitin sulfate proteoglycans against neuronal cell death induced by glutamate. Brain Res 637 : 57-67, 1994.

11) Okamoto M, Mori S, Ichimura M and Endo H : Chondroitin sulfate proteoglycans protect cultured rat's cortical and hippocampal neurons from delayed death induced by excitatory amino acids. Nurosci Lett 172 : 51-54, 1994.

12) Pangalos MN, Shioi J and Robakis NK : Expression of chondroitin sulfate proteoglycans of amyloid precursor (appican) and amyloid precursor-like protein 2. J Neurochem 65 : 762-769, 1995.

13) Banfield BW, Leeuc Y, Esford L, Visalli RJ, Brandt CR and Tufaro F : Evidence for an interaction of herpes simplex virus with chondroitin sulfate proteoglycans during infection. Virology 208 : 531-539, 1995.

14) Muir D, Engvall E, Varon S and Manthorpe M. : Schwannoma cell-derived inhibitor of the neurite-promoting activity of laminin. J Cell Biol 109 : 2353-2362, 1989.

15) Snow DM, Steindler DA and Silver J. : Molecular and cellular characterization of the glial roof plate of the spinal cord and optic tectum : a possible role for a proteoglycan in the development of an axon barrier. Dev Biol 138 : 359-376, 1990.

16) Grumet M, Flaccus A and Margolis RU. : Functional characterization of chondroitin sulfate proteoglycans of brain : interactions with neurons and neuronal cell adhesion molecules. J Cell Biol 120 : 815-824, 1993.

17) Maurel P, Rauch U, Flad M, Margolis RK and Margolis RU. : CloniPhosphacan, a chondroitin sulfate proteoglycan of brain that interacts with neural cell-adhesion molecules, is an extracellular variant of receptor-type protein tyrosinephosphatase. Proc Natl Acad Sci. USA. 91 : 2512-2516, 1994.

18) Shitara K, Yamada H, Watanabe K, Shimonaka M and Yamaguchi Y. : Brain-specific receptor-type protein-tyrosine phospatase RPTPβ is a chondroitin sulfate proteoglycan in vivo. J Biol Chem. 269 : 20189-20193, 1994.

19) Mead DA, Pey NK, Herrnstadt C, Marcil RA and Smith LM. : A universal method for the direct cloning of PCR amplified nucleic acid. Bio/Technology 9 : 657-663, 1991.

大腸菌を用いたフォスファカン（コンドロイチン硫酸プロテオグリカン）の融合コア蛋白の発現条件の検討

伊藤昔子　岡本　基

要　　約

コンドロイチン硫酸プロテオグリカンの一種であるフォスファカンのコア蛋白の特異領域を，グルタチオン-S-トランスフェラーゼ（GST）との融合蛋白として大腸菌内で発現させ，その発現条件の検討を行った。胎生18日目のラット脳から抽出したmRNAを鋳型として，RT-PCRによって増幅したDNAフラグメントを発現ベクターに挿入した。PCRの為のプライマーは，BamH IとEcoR Iの酵素消化部位を5'末端に組み込んだものを用いた。GST融合蛋白を作製するために，PCR産物を一旦，T-Aクローニングシステムに組込み，その後プラスミド精製と制限酵素消化によって目的のフラグメントを切り出した後，pGEXベクターに再度組み込ませ，大腸菌（BL21）を形質転換した。形質転換した大腸菌（BL21）について，24～48時間培養後に，37℃で保存したものと，37℃で24時間培養後に4℃で7～10日間保存したものとの増殖曲線を比較したところ，両者に有意な差は認められなかった。融合蛋白の誘導に必要なイソプロピルチオ-β-D-ガラクトシド（IPTG）の添加の時期は，菌培養液の吸光度（550nm）が1.0以上の場合には融合蛋白に比べて他の大腸菌固有の蛋白の割合が相対的に増大してしまう為，また，吸光度が0.6以下では菌量が少ない為，吸光度が0.6～1.0を示す時期に添加する方がより望ましいことがわかった。IPTGによる誘導後，融合蛋白の発現は6時間でプラトーに達し，その後は大腸菌固有の内在蛋白量の割合が相対的に増加した。以上の結果より，融合蛋白の発現を誘導するための至適条件は，次のように決定された。1．IPTG誘導は，培養液の吸光度（550nm）が0.6～1.0の際に開始する。2．IPTGによる誘導時間は，6時間とする。

キーワード：フォスファカン，グルタチオン-S-トランスフェラーゼ，BL21，IPTG，融合蛋白

岡山大学医療技術短期大学部衛生技術学科