岡大医短紀要, 9 : 105～111, 1998
Bull Sch Health Sci, Okayama Univ
(Original)

Affinity purification of phosphacan core protein expressed in *Escherichia coli* as histidine-tagged fusion protein

Sekiko ITO, Motoi OKAMOTO and Shuji MORI

Abstract

Specific regions of core protein of phosphacan, one of the chondroitin sulfate proteoglycans, were expressed as fusion proteins with histidine-tag (His-tag) in *Escherichia coli (E.coli)* and were affinity purified using nickel-nitrilotriacetic acid (Ni-NTA) matrix. cDNA fragments encoding amino acid residues 343-446 (P3) and 1-340 (P4) of phosphacan core protein were amplified by polymerase chain reaction from E18 rat brain mRNA as template. The amplified products were subcloned into pQE30 vector and were introduced into *E.coli* strain M15 [pREP4] for the expression. The His-tagged fusion proteins were expressed by cultivating the transformants at 37°C for 5h in the presence of 1mM IPTG. His-tagged P3 fusion protein (His-P3) was expressed as soluble form, and was purified using Ni-NTA matrix. His-tagged P4 fusion protein (His-P4) which was sequestered into insoluble inclusion bodies was treated with 8.0M urea to solubilize, and then was purified under denaturing conditions.

Key words : phosphacan, core protein, His-tagged proteins, recombinant protein

Introduction

Recombinant proteins have been widely used for structural and functional analysis of trace amount of native proteins. Bacterial expression system, which can overproduce foreign proteins, is simple and easy cDNA expression system. In general, foreign protein is expressed as fusion protein with specific affinity tag at its N-terminal or C-terminal, because of its convenience on purification.

Histidine-tag (His-tag) is the well known affinity tag consisting of six histidine residues, it have been reported to be capable of binding to specific affinity matrix, nickelnitrilotriacetic acid (Ni-NTA) matrix. Two of the six ligand binding sites of coordination sphere of the nickel ion interact with His-tag, leaving four sites bind to NTA[1]. The binding between His-tag and Ni-NTA matrix is too tight, which allows His-tagged protein to be affinity purified efficiently even under denaturing condition. In addition, because of the lack of its steric hindrance, His-tag is thought to have slight or no influence on the conformation and biological activity of expressed protein, as compared with other tags with the greatly higher molecular weight such as glutathione S-transferase (GST) and maltose binding protein (MBP).

Chondroitin sulfate proteoglycans (CSPGs) have various biological functions in cell prolif-

Faculty of Health Sciences, Okayama University Medical School

eration, migration, differentiation and extension of processes[2-9]. They also play important roles in survival or death of neuronal cells, viral infection, and degenerative disorders of central nervous system[10-14]. CSPGs are structurally complex molecules having polysaccharide side chains attached to core proteins.

In previous studies, we reported that soluble CSPGs purified from neonatal rat brain could protect glutamate-induced acute and delayed cell death of rat primary cultured cortical and hippocampal neurons, and that the protective action depended on core proteins rather than glycosaminoglycans[10,11].

In addition, we found that the peptide derived from phosphacan, which was one of CSPGs known as extracellular variant of receptor-like protein-tyrosine phosphatase[15,16], might have the protective action against delayed neuronal cell death induced by glutamate (unpublished data). However, we could not neglect the contributions of other regions. In order to specify the specific region essential for protective action, some recombinant proteins from phosphacan were tried to be expressed in *E.coli* as fusion protein with GST in previous study[17]. While GST-fused proteins were expressed, they were sequestered into inclusion bodies, hence could not be purified.

In this study, two specific regions (P3 and P4) from phosphacan were chosen as candidates for protective action, then were expressed and were purified as His-tagged fusion proteins (His-P3 and P4). P3 was the region corresponding to the residue 343-446 of phosphacan, which was expected to be responsible for protective action. P4 was the N-terminal side region of P3, which corresponded to the residue 1-340.

Materials and Methods

1. PCR and cloning

Using the Fast Track mRNA Isolation Kit (Invitrogen), mRNA was extracted from brain of 18-day-old SD rat fetuses (Kurea), and mRNA was reverse-transcribed to obtain the cDNA as described previously[17]. Then, two DNA fragments encoding P3 and P4 (312bp for P3, 1125bp for P4) were amplified by PCR with cDNA as the template, respectively. Amplification reactions were performed with RNA PCR Kit (Takara) according to the manufacture's protocol. For amplification, two primer sets (OKA-3S and OKA-3AS for P3; OKA-4S and OKA-4AS for P4) were used (Fig. 1). The amplified DNA were cloned into pCR II vector using TA Cloning Kit (Invitrogen), and were subcloned into pGEX4T-1 vector (Amersham Pharmacia Biotech). Then, *E.coli* strain BL21 was transformed with pGEX4T-1 encoding P3 or P4 according to the method of Hanahan[18], the obtained transformants were used as follows.

2. Plasmid construction

The pGEX4T-1 plasmid encoding P3 or P4 was purified using boiling method[19]. After digestion with restriction enzymes (BamH I and Sma I), two DNA fragments were separated by agarose gel electrophoresis. The fragments were ligated to pQE30 expression vector (QIAGEN) for preparing the proteins fused with His-tag. Transformation of *E.coli* strain M15 [pREP4] with ligated pQE30 vectors was done in the same manner as described above.

3. Expression of His-tagged fusion proteins

For expression of His-tagged fusion proteins, a colony of transformants was picked up from LB plate containing ampicillin at 100μg/ml and kanamycin at 25μg/ml. The colony was inoculated in 50ml of LB medium (with antibiotics) and was grown overnight at 37°C with vigorous shaking (small scale culture). Then, all of the small scale culture was added to

OKA—3S (sense)
343 349
5'— GGATCCATTGAGAAGTTTGCGGTTCTG —3'

OKA—3AS (antisense)
417 410
5'— GAATTCGAGGTCAAGTTCAGCATCCTC —3'

OKA—4S (sense)
1 6
5'— GGATCCATGCGAATCCTGCAGAGC —3'

OKA—4AS (antisense)
344 340
5'— GAATTCGTCATAAACAACCCGAGGCCT —3'

P3 : OKA-3S—OKA-3AS 312bp
P4 : OKA-4S—OKA-4AS 1125bp

Fig. 1 Sequences of four synthetic oligonucleotide primers for PCR. These primers for PCR were designed to amplify cDNA corresponding to the specific regions of phosphacan core protein. The recognition sequences (underlined) for restriction enzymes (BamH I, Sma I) were attached to 5'-terminal of each primers.

450ml of prewarmed media (with antibiotics) and was grown for 2h at 37°C with vigorous shaking (large scale culture). To induce the expression of fusion proteins, IPTG was added at a final concentration of 1mM. The incubation was continued to grow at 37°C for 5h after the addition of IPTG. The culture medium was centrifuged at 4000×g at 4°C for 15min, and cell pellet was resuspended in 50ml of 10mM Tris-HCl (TB, pH7.5) containing 0.3M NaCl. The lysate was obtained by sonicating cell suspension twice for 1min on ice. After centrifugation at 10,000×g for 15min at 4°C, resultant supernatant was mixed with Ni-NTA affinity matrix.

In the purification of His-tagged P4 fusion protein (His-P4), insoluble fraction after the sonication was resuspended in 8ml of 10mM TB (pH8.0) containing 8.0M urea and 0.1M NaH_2PO_4, and was shaken at room temperature for 10h. After centrifugation at 10,000×g for 15min at 4°C, the supernatant was recovered and was applied to Ni-NTA affinity purification step.

4. Affinity purification of fusion proteins

The supernatant containing His-tagged P3 fusion protein (His-P3) (50ml) or His-P4 (8ml) was added to 3ml of Ni-NTA matrix, respectively. After mixing for 12h at 4°C, the mixture was poured into a small column. Then, column for His-P3 was washed with 200ml of 10mM TB(pH7.5) containing 0.3M NaCl and 0.1M imidazole. For His-P4, column was washed with 200ml of 10mM TB(pH7.5) containing 0.3M NaCl. His-P3 and His-P4 were eluted with 20ml of 10mM TB (pH7.5) containing 0.3M NaCl and 0.5M imidazole. For sequencing N-terminal amino acids, the eluted proteins were electrophoresed on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli[20]. Separated proteins were Western-blotted to PVDF membrane at 45V for 3h at 4°C in 10mM CAPS buffer (pH11.0) containing 10% meth-

anol.

Automated N-terminal amino acids analysis was performed on a Perkin-Elmer model 491 protein sequencer. Standard Edman chemistry was used and the amino acid phenylthiohydantoins were analyzed. Results were analyzed using the Perkin-Elmer data analysis software. The amino acid sequence obtained was compared with sequences of proteins contained in the sequence data base SWISS-PROT[21].

Results and Discussion

In preliminary study, we tried to express and to affinity purify P3 and P4 regions fused with GST. However, GST-fused proteins were sequestered into insoluble inclusion bodies. The expression as insoluble aggregates did not permit the purification of those. It has well known that eukaryotic proteins are frequently produced in *E.coli* as insoluble aggregates. The intermolecular association of hydrophobic domains during folding has been believed to play a role in the formation of inclusion bodies. For proteins with cysteine residues, improper formation of disulphide bonds in the reducing environment of the *E.coli* cytoplasm may also contribute to incorrect folding and formation of inclusion bodies. Also, affinity tags with the high molecular weight such as GST and MBP might affect the conformation of expressed proteins by way of its steric hindrance. If so, lowing the IPTG concentration and temperature for cultivation, which lead to the decrease in expression, might allow to recover fused proteins as soluble form. Some attempts were done to improve the yield of soluble GST-fused proteins, however, the proteins were found in insoluble fraction in all cases tried. Treatment with some detergents, such as N-lauroryl-sarcosine, Triton X-100 and SDS, allowed to solubilize GST-fused proteins from insoluble aggregates, but solubilized fused proteins could no longer be affinity purified because of the lack of their binding activities to specific affinity matrix (Glutathione-Sepharose 4B).

In this paper, we tried to express and to affinity purify His-P3 and P4 proteins. After IPTG-induced expression, His-P3 could be recovered in soluble fraction, since His-P4 was found to form insoluble aggregates. Insoluble His-P4 was solubilized efficiently with 8.0M urea, and resultant solubilized fraction was used for purification. After loading onto Ni-NTA matrix, both His-tagged proteins were bind to the matrix. Then those could be eluted as a single peak by increasing the concentrations of imidazole stepwizely (Fig. 2a and Fig. 3a), because imidazole has structural homology to the side chain of histidine. Eluates containing His-P3 or His-P4 were pooled and analyzed by SDS-PAGE. Electrophoretic patterns of the proteins were shown in Fig. 2b and Fig. 3b. Analysis of N-terminal amino acid sequences of purified His-P3 and His-P4 revealed that those were identical with deduced amino acid sequences of corresponding region of phosphacan core protein. In both cases, trace amounts of contaminants were observed. At present, the reason for this phenomenon remains to be elucidated. While some speculations might be proposed that non-neighboring histidines were accommodated the binding to the Ni^{2+} by use of denaturant during purification steps.

In conclusion, His-P3 and P4 could be expressed and purified. As described, phosphacan has been reported to have several biological activities[2-9]. His-P3 and P4 may be provided as the useful tools for studying biological implications of phosphacan. Some studies using His-tagged proteins are currently under way along this line.

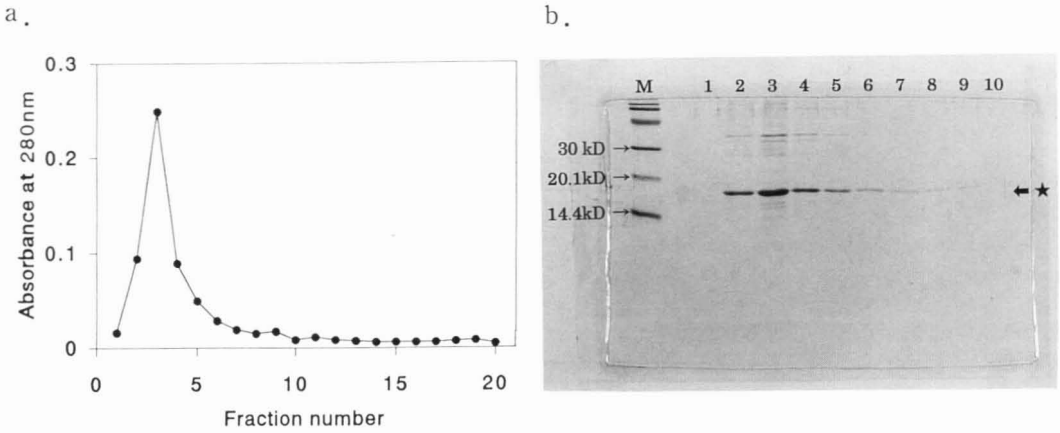

Fig. 2 Purification of His-P3.

Fifty milliliters of the supernatant containing His-P3 expressed in *E.coli* were added to 3ml of Ni-NTA matrix.

a : After washing with 200ml of 10mM TB (pH7.5) containing 0.3M NaCl and 0.1M imidazole, His-P3 was eluted with 20ml of 10mM TB (pH7.5) containing 0.3M NaCl and 0.5M imidazole. Absorbance of fractionated His-P3 was measured at 280nm.

b : Aliquots of each eluate were separated onto 20.0% SDS-PAGE, and then were stained with Coomassie Brilliant Blue R-250. His-P3 was indicated with star (★). The positions of molecular size markers (kDa) were shown on the left.

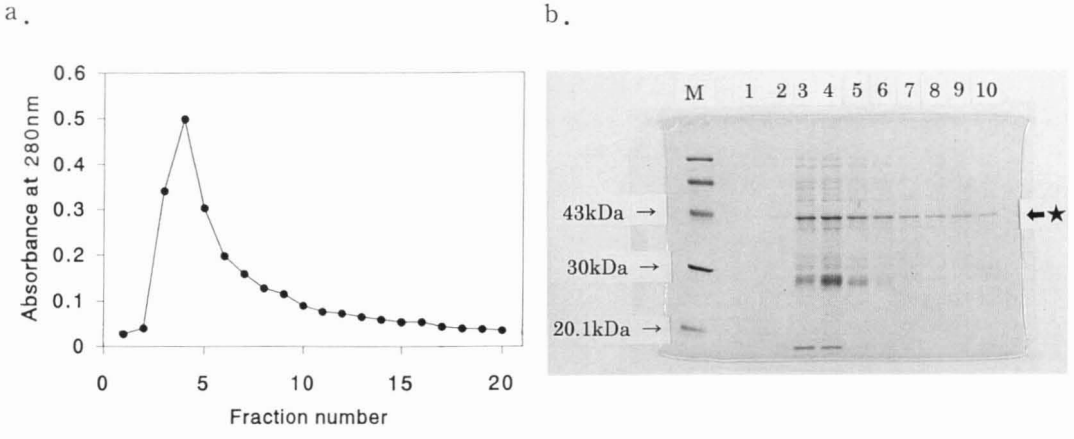

Fig. 3 Purification of His-P4.

Eight milliliters of the supernatant containing His-P4 expressed in *E.coli* were added to 3ml of Ni-NTA matrix.

a : After washing with 200ml of 10mM TB (pH7.5) containing 0.3M NaCl, His-P4 was eluted with 20ml of 10mM TB (pH7.5) containing 0.3M NaCl and 0.5M imidazole. Absorbance of fractionated His-P3 was measured at 280nm.

b : Aliquots of each eluate were separated onto 12.5% SDS-PAGE, and then were stained with Coomassie Brilliant Blue R-250. His-P4 was indicated with star (★). The positions of molecular size markers (kDa) were shown on the left.

References

1) Hochuli E, Dobeli H and Schacher A : New metal chelate adsorment selective for proteins and peptides containing neighboring histidine residues. J Chromato 411 : 177-184, 1987.
2) 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.
3) Iijima N, Oohira A, Mori T, Kitabatake K and Kohsaka S : Core protein of chondroitin sulfate proteoglycan promotes neurite outgrowth from cultured neocortical neurons. J Neurochem 56 : 706-708, 1991.
4) Kato-Semba R, Matsuda M, Kato K and Oohira A : Chondroitin sulfate proteoglycan 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.
5) 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.
6) Oohira A, Matsui F and Kato-Semba R : Inhibitory effects of brain chondroitin sulfate proteoglycans on neurite outgrowth from PC12 cells. J Neurosci 11 : 822-827, 1991.
7) 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.
8) Snow DM, Watanabe M, Letourneau PC and Silver J : A chondroitin sulfate proteoglycan may influence the direction of retinal ganglion cell outgrowth. Development 113 : 1473-1485, 1991.
9) 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.
10) 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.
11) 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.
12) 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. Neurosci Lett 172 : 51-54, 1994.
13) 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.
14) 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.
15) Maurel P, Rauch U, Flad M, Margolis RK and Margolis RU : Phosphacan, 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.
16) 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.
17) Ito S and Okamoto M : Expression of phosphacan, a chondroitin sulfate proteoglycan, core protein in Escherichia coli as a fusion protein with glutathione S-transferase. Bull Sch Health Sci, Okayama Univ 6 : 63-72, 1995.
18) Hanahan Đ : Studies on transformation of Escherichia coli with plasmids. J Mol Biol, 166 : 557-580, 1983.
19) Holmes DS and Qiogley M : A rapid boiling method for the preparation of bacterial plasmids. Anal Biochem 114 : 193, 1981.
20) Laemmli UK : Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227 : 680-685, 1970.
21) Bairoch A and Boeckmann B : The SWISS-PROT protein sequence data bank. Nucleic Acids Research 19 suppl : 2247-2249, 1991.

岡大医短紀要，9：105～111，1998

（原　著）

ヒスチジンタグを持つホスファカンコア蛋白の大腸菌での発現と精製

伊藤昔子　　岡本　基　　森　秀治

要　　約

コンドロイチン硫酸プロテオグリカンの一つであるホスファカンのコア蛋白の特定領域を，ヒスチジンタグ(His-tag)を持つ融合蛋白として大腸菌内で発現させニッケル-ニトリロ3酢酸(Ni-NTA)アフィニティ担体を用いて精製した。

ホスファカンコア蛋白のアミノ酸残基343-446(P3)及び1-340(P4)に相当するcDNA断片を，胎性18日目のラット脳由来のmRNAを鋳型としたPCRによって増幅した。増幅された断片は発現ベクターpQE30に組み込まれ，これで大腸菌（M15[pREP4]）を形質転換した。

His-tag融合蛋白の発現は形質転換株を1mM IPTG存在下で37℃，5時間培養することによって行われた。

His-tagged P3融合蛋白は可溶性蛋白質として発現し，Ni-NTA担体を用いて精製された。His-tagged P4融合蛋白は不溶性の封入体を形成したが，8M尿素によって可溶化され，変性条件下で同様に精製された。

キーワード：ホスファカン，コア蛋白，His-tagged proteins，融合蛋白

岡山大学医学部保健学科検査技術科学専攻