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# Purification and NMR studies on Phosphatase domain of UBLCP1

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***Abstract*** : UBLCP1 is composed of Ubiquitin Like domain and RNA Polymerase II Phosphatase I domain. Phosphatase domain (25.9KDa) has been cloned into the *E.coli* using pET32a vector with TEV protease cleavage site and successfully purified as a monomer using affinity chromatography and histidine tag was cleaved with TEV protease for structural studies. Our results indicated that the Phosphatase domain showed well-defined folded structure based on data from one-dimensional and two-dimensional NMR spectroscopy. Data form circular dichroism also suggested that Phosphatase domain consisted of both α -helix and β -sheet. This information will be used for detailed structural study of UBLCP1.
**Keywords** : RNA Polymerase II, CTD-Phosphatase, Circular dichroism, NMR spectroscopy, Cloning, Purification

## INTRODUCTION

UBLCP1 consists of Ubiqitin Like domain(UBL) and Phosphatase domain, which are structurally independent each other [1]. RNA polymerase II(RNAP II) is an enzyme discovered in eucaryotic cells and catalyzes the transcription of DNA and its synthesis [2-5]. RNAP II consists of two domain, folded structural domain and mobile C-terminal repeat domain (CTD) [6]. Structural domain involves in mRNA biogenesis whereas the mobile CTD regulates RNA polymerase II activity [7]. CTD(C-terminal domain) is a target of enzymes such as kinases and Phosphatase. The C-terminal domain(CTD) is considered as

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transcription factors, which has an important regulatory mechanism whereas CTD can either be dephosphrylated or hyperphosphorylated in the elongation stage of transcription [8]. In this report, we present cloning, purification and characterization of Phosphatase domain. We also performed the preliminary structural studies using NMR spectroscopy and circular dichroism [9-10].

## MATERIALS AND METHODS

### *Cloning and expression of Phosphatase domain.*

The Phosphatase domain was obtained from Hela cell. The Phosphatase was cloned into the expression vector, pET32a. cDNA was produced using primers containing BamHI/EcoRI for Phosphatase and tabacco etch virus (TEV) protease (ENLYFQG) was added at sense primer to cleave tag from fusion protein. Sense primer was 5' -cgc gga tcc gaa aac ctg tat ttt cag ggc ccc agg gaa ggg aaa aag-3' and anti primer was 5' -cgc gga tcc gaa aac ctg tat ttt cag ggc gat att gaa gat gaa gta-3' . After cloning, we confirmed the DNA sequence at COSMO Co. Plasmid containing Phosphatase was over-expressed in Escherichia coli strain BL21 (DE3) and colonies with Phosphatase domain were selected LB (Luria-Bertani)plate with ampicillin (0.1 mg/mL). To verity the protein cells were sonicated in lysis buffer(25mM NaPi, 300mM NaCl, 5mM β-mercaptoethnol, pH7.3), protease inhibition cocktail (Roche) was added and protein expression verified using SDS-PAGE(sodium dodecyl sulfate polyacrylamide gel electrophoresis).

### *Isotope($^{15}N$) labeling of Phosphatase domain.*

For hetero-nuclear NMR experiments, we prepared isotope labeled form of Phosphatase domain. Cells were cultured in M9 minimal medium containing $^{15}N$ labeled $NH_4Cl$($^{15}N$, 99 %, Cambridge Isotope Laboratories, Inc.) at 37℃/220rpm and protein expression was induced by 0.1mM IPTG (isopropyl-beta-D-thiogalactopyranoside) when the cell density reached $OD_{600}$ of 0.6, then the cells were cultured at 25℃ for 20hr, $OD_{600}$>1.5.

***Purification of Phosphatase domain.***

The cells containing fusion Phosphatase domain proteins (TRX-His6-TEV-target protein), were sonicated in lysis buffer(25mM NaPi, 300mM NaCl, 5mM β-mercaptoethanol, pH7.3). Protease inhibition cocktail (Roche) 50 $\mu\ell$ was added to inhibit protease that could degrade the target protein and centrifused to obtain supernatant. We purified to obtain fusion protein using Ni-NTA open column(affinity chromatography), and removed imidazole by dialysis. To the acquire target protein, we performed SDS-PAGE. The SDS-PAGE shown to other band except target protein. For removing the impurity we performed SP column and ion exchange chromatography. We finally confirmed the molecular weight of the protein using the size exclusion chromatography, HiLoad$^{TM}$ 16/60 Superdex$^{TM}$ 75. Finally, the target protein was to concentrated ~0.5mM for NMR or CD experiments.

***NMR spectroscopy.***

Target protein(Phosphatase domain) were dissolved in buffer(10mM HEPES, 150mM NaCl, 2mM DTT, 0.01% $NaN_3$ in 90% $H_2O$, 10% $D_2O$) for NMR experiments. The protein concentration was about 0.5mM. To defined the stabled buffer condition, we executed $^{1}H$-$^{15}N$ 2D-HSQC experiment[11] in 3 differnet pH conditions (pH, 6.0, 6.5, 8.5) using Bruker DRX 500MHz equipped with Cryoprobe$^{TM}$.

***CD experiment.***

The concentration of Phosphatase domain(target protein) was 0.3mg/ml in 20mM HEPES, 20mM NaCl and pH6.5. CD spectra were recorded with a JASCO model J-810 spectropolarimeter(JASCO, Tokyo, Japan) with 1mm path-length cell at room temperature.

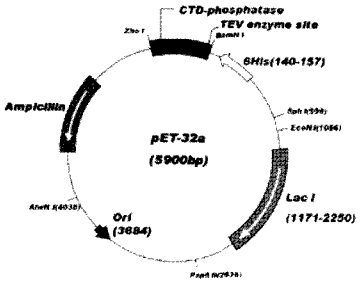
## RESULTS AND DISCUSSION

***Domain construct of Phosphatase domain.***

The sequence alignment of Phosphatase domain was Tcoffee program and secondary prediction was calculated using PSI-PRED program. We designed the construct of

Phosphatase from 100 to 318 amino acid. Phosphatase (100-318) was cloned into the *E.coli* over-expression vector, pET32a, and was expressed as TRX-$His_6$-TEV protease sequence from pET32a (Fig. 1A). We executed sequence alignment for Phosphatase domain from HUMAN, RAT, MOUSE (Fig. 1B). As a result, these constructs showed high homology. Secondary structure prediction suggests that Phosphatase consisted of 10 α-helix and β-sheet each other using PSI-PRED (Fig. 1B).

**A.**




**B**

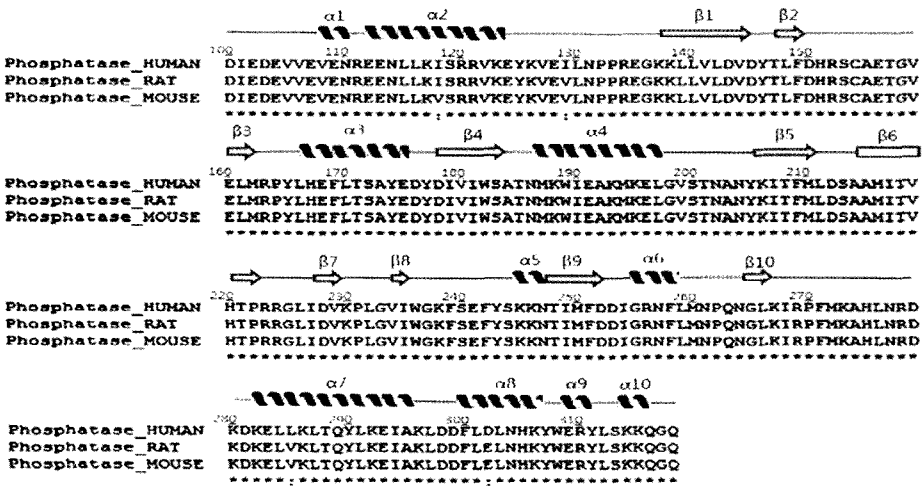



Fig. 1. Vector map of Phosphatase domain and sequence alignment. We designed the construct of Phosphatase domain sequence from 100 to 318. The vector map expressed in *E.coli* and added TEV cleavage site was present (A). Amino acid sequence and sequence alignment for Phosphatase

domain from Phosphatase_HUMAN, Phosphatase_RAT, Phosphatase_MOUSE and secondary structure for Phosphatase domain of Phosphatase_HUMAN; It was consist of 10 α-helix and β-sheet separately.

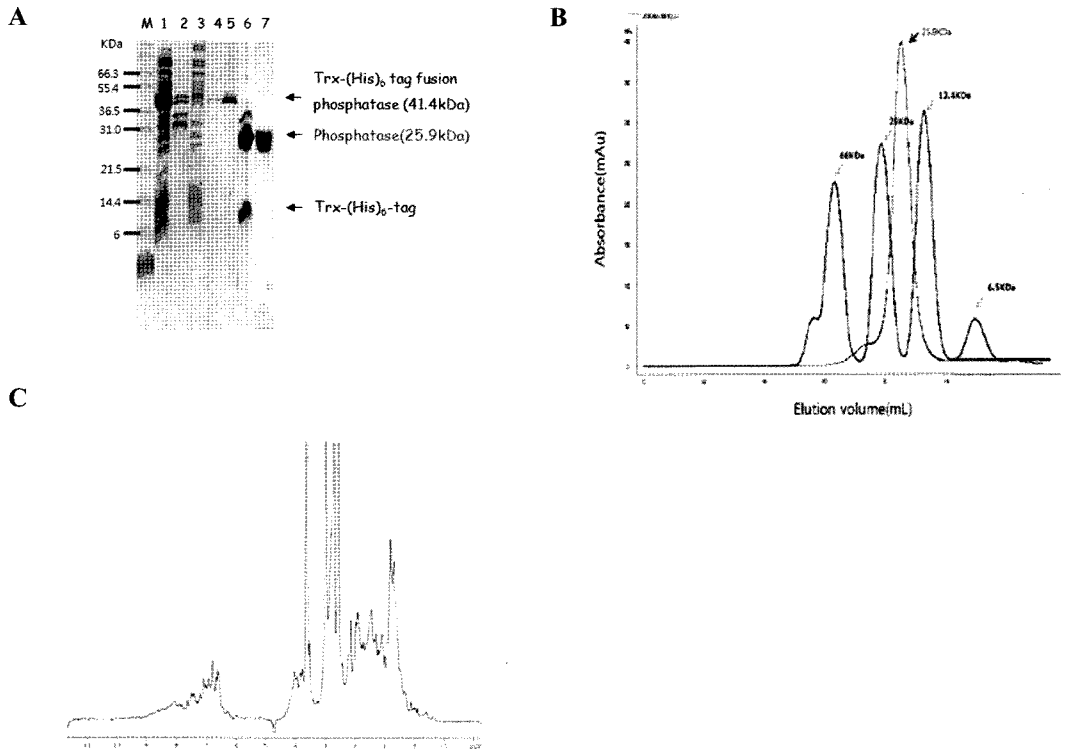



Fig. 2. Purification, gel filtration, and $^{1}H$ NMR spectroscopy of Phosphatase domain. (A) SDS-PAGE of purified Phosphatase domain. Column of 1-5 shows 1$^{st}$ Ni-NTA column work. (M: size marker, 1: supernatant, 2: precipitated form, 3: flow through, 4: 40mM wash, 5: 500mM elution). Column of 5 is TRX-$His_6$ tagged fusion Phosphatase. Column of 6 shows result after TEV cleavage. Column of 7 was the result after SP column. (B) confirming the size of Phosphatase domain using gel filtration. $^{1}H$ NMR spectrum was recoded in buffer, 10mM HEPES, 150mM NaCl, 2mM DTT, 0.01% $NaN_3$, pH 6.5, 25℃

***Purification of Phosphatase domain.***

Cells cloned with Phosphatase domain was expressed in LB(Luria-Bertani) medium. At $OD_{600}$ was 0.6, 0.1mM IPTG was added and cells were havasted after 20hr. Induction profile was confirmed using SDS-PAGE(data not shown). Phosphatase domain cells were sonicated to obtain the target protein and fusion protein (TRX- $His_6$-TEV-target protein) was purified using Ni-NTA affinity chromatography (Fig. 2A-5). To acquire target protein(Phosphatase), TEV cleavage experiment(0.5X, 6hr, 25℃)(Fig. 2A-6) was done. the TRX-$His_6$-TEV tag was removed using Ni-NTA affinity column. Nevertheless, the impurity was not removed between 36.5 and 31.0 KDa(Fig. 2A-6). We further executed other type column work for the removing the impurity using SP ion exclusion chromatography and successfully removed the impurities (Fig. 2A).

***Size exclusion chromatography of Phosphatase domain.***

To determine the molecular weight of Phosphatase domain, we performed the gel filtration experiment (Fig. 3B) using HiLoad$^{TM}$ 16/60 Superdex$^{TM}$ 75 and confirmed that peak was shown from 80 to 100ml. When prepare with size marker, there was located from 29 to 12.4 KDa (Fig. 2B). As a this experiment, confirmed the size of Phosphatase domain.

***Structural studies of Phosphatase domain.***

In other define the secondary structure of Phosphatase domain, we performed NMR spectroscopy and CD spectropolarimetry. As a result of $^{1}H$ NMR spectrum, all of proton resonances of Phosphatase domain existed structural stably(Fig. 2C). We tested which condition was stabled, so we confirmed the buffer condition using $^{1}H$-$^{15}N$ HSQC spectrum (Fig. 3A, B, C). At pH 6.0, little peaks were seen (Fig. 3A) and at pH 8.5, peaks were lump together(Fig. 3B) and at pH 6.5, There was regularly in peaks(Fig. 3C). As a result, it is more stable at buffer condition of pH 6.5. We also executed other experiment to define secondary structure, Circular Dichroism(CD) spectropolarmerty. Fig. 4A shows the CD spectra of Phosphatase in 20mM HEPES, 20mM NaCl buffer(pH 6.5). It shows an α-helical propensity, which have strong minimum around 210 and 220. The spectrum in aqueous solution have maximum around 210nm and a strong minimum around 220nm(Fig. 4A). As

a result of circular dichroism and homology modeling (Fig. 4B), we propose that Phosphatase domain consists of about 70% α-helix.

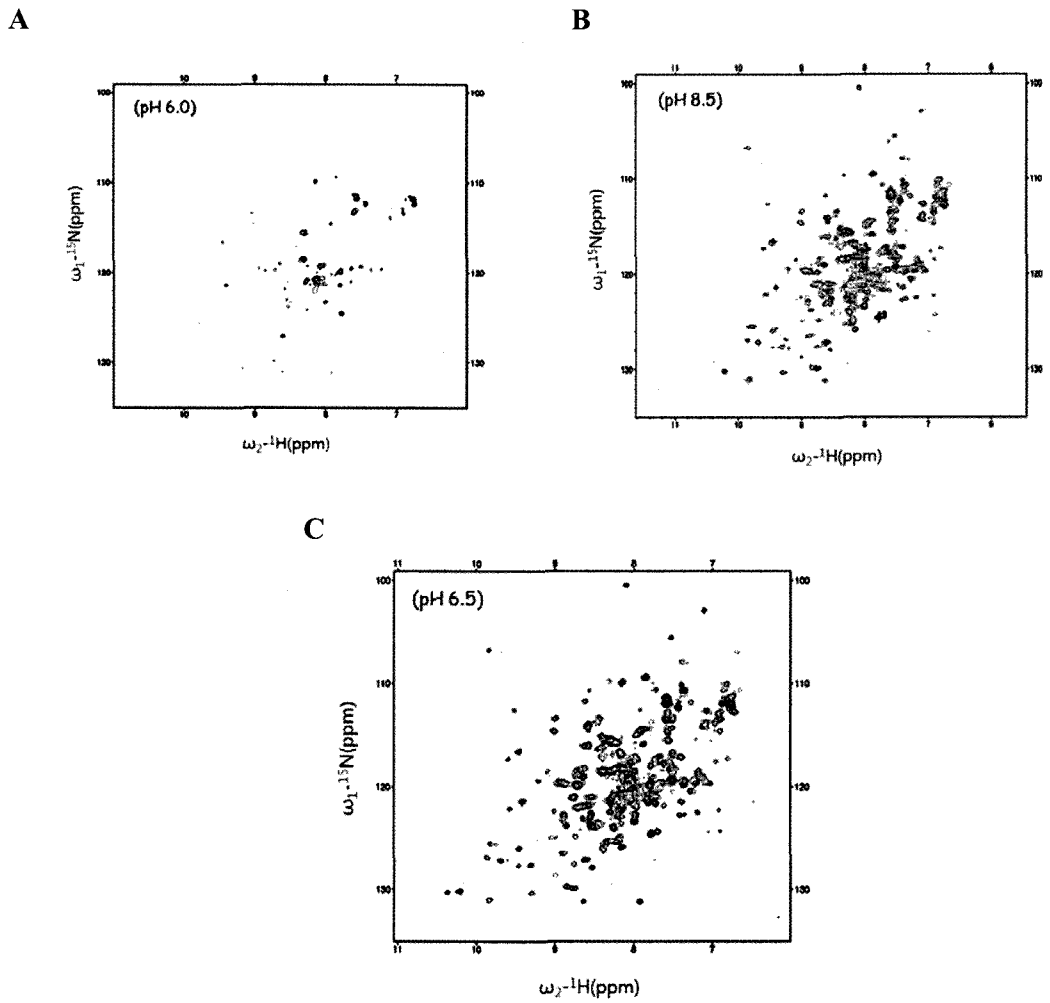



Fig. 3. $^1$H-$^{15}$N 2D-HSQC spectrum of Phosphatase domain. The spectrum were recorded on a 0.3mM solution in 10mM HEPES, 150mM NaCl, 2mM DTT, 0.01% $NaN_3$, 25℃. $^1$H-$^{15}$N HSQC spectrum performed in Bruker DRX 500MHz spectrometer equipped with Cryoprobe™ for three conditions, (A) pH6.0 (B) pH8.5 and (C) pH6.5, respectively.

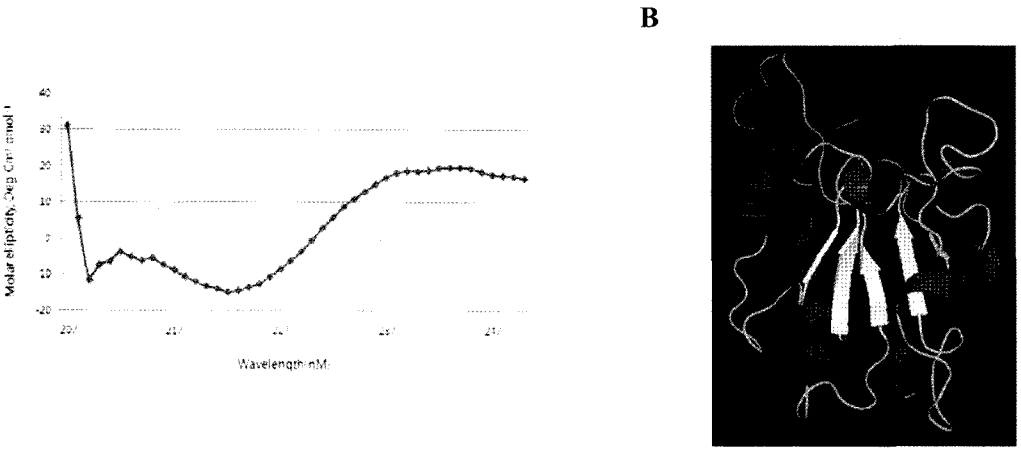



Fig. 4. CD spectropolarimetry experiment and homology modeling of Phosphatase domain. It recoded in buffer, 20mM HEPES, 20mM NaCl, pH 6.5(A). Homology modeling was calculated using SWISS-MODEL, ExPASyRed. It was formed that red is α-helix, yellow is β-sheet, green is loops(B).

## CONCLUSION

Human Phosphatase domain has been successfully cloned into the E.coli expression vector, with TEV cleavage protease site and it was purified as a monomer using affinity, ion exchange and gel filtration chromatography. Data from two-dimensional $^{1}H$-$^{15}N$ HSQC and circular dichroism provided an optimum condition for structural study. The purified protein is stable at pH 6.0, and it consists of about 70% of α-helical structure.

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