Strategic recovery of recombinant human truncated Cyclin A and Cyclin D in *Escherichia coli* for characterization with putative inhibitors

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Introduction

Targeting cyclins enables us to interfere with cell cycle in order to inhibit the cancerous process which is the result of a non-prober regulation of this cell cycle control[1]. Through cell cycle, Cyclin dependent kinases (CDKs) as well as their activation partners, Cyclins, are regulators of progression and proliferation through activation of cell cycle checkpoints and inhibited by CKIs, thus they have been widely held as anti-cancer targets[2].

This work attempts the over-expression of recombinant truncated forms of human Cyclin A (His-tagged CCNA2) and Cyclin D (GST-tagged CCND1) seeking the high levels of yield and purity for crystallization and ligand characterization purposes.

Seeking to optimize binding affinity of CCNA2/inhibitor and CCND1/inhibitor complexes, in order to develop more active inhibitors against CDKs/Cyclins activity for cancer research.

Materials & Methods

Screening for Over-expression:

Different expression hosts tested BL21(DE3) and BL21(DE3)pLysS and were transformed with pET16b-cyclinA2 His-tagged and pET49-cyclinD1 GST-tagged. Expression of recombinant proteins induced by 0.1 to 1mM IPTG at several temperatures and time durations. Collected Washed with PBS. Lysis performed by sonication cycles with 1mg/mL Lysozyme, PMSF and Triton-X0.1%. Total, soluble and insoluble protein fractions were analyzed in 12% SDS-PAGE.

Denaturation means of Insoluble Aggragates:

A series of experimental protocols were implemented for purification of inclusion bodies. Sonication was performed with lysis Buffer 50 mM Tris-HCl, 2 mM EDTA, 300 mM NaCl, 1 mM PMSF Denaturations Buffers used: 8 M Urea, 50 mM Tris-Cl, 10 mM DTT and 6 M Guanidine-HCl, 0.1 M Tris, 80 mM GSH, 2 mM EDTA, for comparative purposes.

Transformation for Coexpression:

Transformation of E. coli BL21(DE3)-pET49CD1 with a chaperone plasmid. A group of E. coli's molecular chaperones (GroES, GroEL, DnaK, DnaJ, GrpE and Tf) and their combinations, co-expressed seeking soluble recombinant protein[6]. Inoculation into LB medium containing also chloramphenicol for plasmid selection and 0.5 mg/ml L-arabinose and/or 5 ng/ml tetracycline for induction of chaperone expression for 4h at °C.

Searching for suitable refolding buffer:

Protein refolding of CCNA2 and CCND1 were tested with 15 conditions of "QuickFoldTM Protein Refolding KIT"[3] for both denaturation buffers.

Expression of Molecular Chaperones:

At $O.D._{600}$ 0.5-0.6, IPTG was added to a final concertation up to 1mM for 5h at 37°C. Cells were collected by centrifugation, washed with PBS, weighted and stored at -20°C. Lysis Buffer was used with PBS, 0.5mM EDTA, 1mM DTT, 1mM PMSF, 1%Triton, 1mg/ml Lys.

Cooperation in folding process:

Coexpression of a target protein with these chaperone plasmids pG-KJE8, pGro7, pKJE7, pG-Tf2 and pTf16 increases recovery of expressed proteins in the soluble fraction.

Affinity column:

Protein purification of 6His-cyclin A2, performed with Chromatogram of Ni-IMAC purification column (Biorad) according to manufacturer's instruction.

Protein purification of GST-tagged CCND1 performed with Protino® Glutathione Agarose 4B column, according to manufacturer's instruction. Protein collected with elution buffer containing 50mM Tris-Base 10mM Glutathione, pH 8.0 according to manufacture's manual.

Results

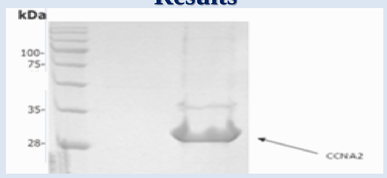

Figure 1. Optimum over-expression conditions of CCNA2 protein in BL21(DE3), 0.1mM IPTG, 20°C, 12h.

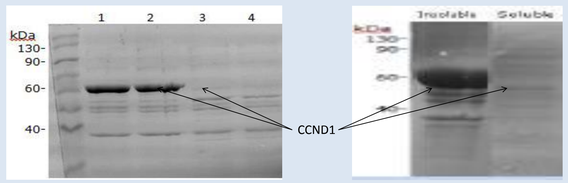

Figure 2. *Left:* Preliminary study indicates a significant over-expression in BL21(DE3) (lines 1-2) rather than BL21(DE3)pLysS (Lines 3-4) *Right:* Over-expression studies indicates lack of soluble CD1

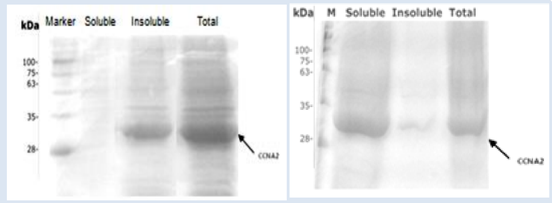

Figure 3. BL21 (DE3) with pET16b-CyclinA2, Over-expressed cyclin A2 fractions, 20°C, 1 mM IPTG, Overnight induction. *Left-Sonication*: Total protein samples after denaturation treatment with 8 M Urea. Fraction SDS analysis. *Right-Homogenization:* Total protein samples after denaturation treatment with 6 M Guanidine-HCl. Fraction SDS analysis

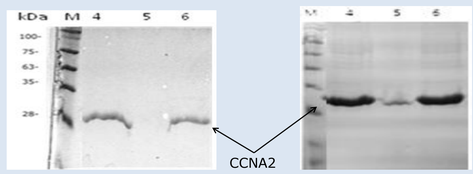

Figure 4. ***Left,*** Refolding strategies of unfolded CCNA2 with 15 buffer formulations. The 15 conditions were prepared by adding DTT, GSH and GSSH, according to protocol. ***Right,*** Refolded CCNA2 after TCA/Acetone precipitation .

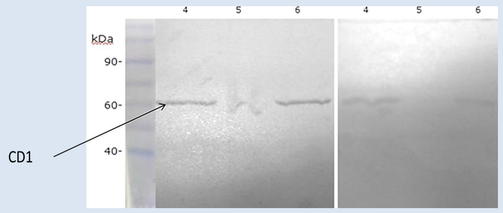

Figure 5. Refolding strategies of unfolded CCD1 with 15 buffer formulations. The 15 conditions prepared by adding DTT, GSH and GSSH according to provided protocol. *Left,* Treated with 8 M Urea *Right,* Treated with 6 M Guanidine-HCL. Protein concentration for both strategies adjusted to 1mg/ml before solutions were applied.

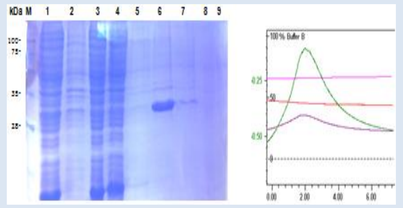

Figure 6. BL21(DE3) with 6xHis-CCNA2 /3h,25°C,0.5mM IPTG/(4.4mg/3.8g)>1.15mg/g (Electrophoresis of purification steps and chromatogram) **Lanes:** M: Pre stained Markers , 1:25°C 1mM +IPTG pellet, 2:Soluble fraction after sonication, 3:Flow through Ni-NTA, 4:Washing step, 5:Elution ,1, 6:Elution 2, 7:Elution 3

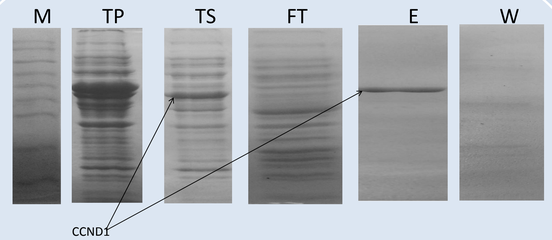

Figure 7. Incubated BL21(DE3) pET49-CCND1 with chaperone plasmid pTf16 for 4 hours with L-arabinose, followed by 5h with IPTG 1 M induction and co-expressed. Purification ofsoluble fraction of CCND1 according to Protino GST-tagged protein, manufacture's instruction. Co-expressed Pet49-CCND1 with Chaperone plasmid pTf16. From ***Left to Right***: Marker – Total proteins – Total soluble fraction – Flowthrough – Elution – Wash

Conclusions

BL21 (DE3) expression host is preferred for both recombinant proteins[4]. Homogenization increases the levels of regained protein from IB. Following denaturation of IB, Urea buffer is suggested for refolding. CCNA2 and CCND1 refolding was more efficient with GSH/GSSG rather than DTT, while stability of cyclins was achieved with elevated concentrations of $MgCl_2$.

In case of CCND1 co-expression with chaperone plasmid pTf16 increases substantially soluble protein.

Existing synthesized peptides, designed with REPLACE (REplacement with Partial Ligand Alternatives through Computational Enrichment). This structure-activity co-relation with non-fluorescent peptides as cyclin groove putative inhibitors (CGI) where tested[2].

References

1. B. D. Dynlacht, O. Flores, J. A. Lees, E Harlow, Genes Dev. 1994, 8, 1772–1786
2. *Andrews, M. J. I., Kontopidis, G., McInnes, et al, (2006), REPLACE: A Strategy for Iterative Design of Cyclin-Binding Groove Inhibitors. ChemBioChem, 7: 1909–1915*
3. QuickFold™ Protein Refolding Kit Application
4. Papaneophytou CP, Rinotas V, Douni E, Kontopidis GA (2013)A statistical approach for optimization of RANKL overexpression in Escherichia coli: purification and characterization of the protein, Protein Expression and Purification Volume 90, Issue 1, July 2013, Pages 9–19

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