



Cloning, expression and purification of functionally active *Saccharomyces cerevisiae* Polo-like Kinase, Cdc5 in *E. coli*

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ABSTRACT

Polo-like Kinases (PLKs) belong to the serine/threonine family of protein kinases. They govern cell cycle progression in concert with CDKs and other cell cycle kinases in most eukaryotes studied. Polo-box domain, the signature motif of PLKs, functions to recruit the kinase to its substrates. However, the substrate specificity, and the complete repertoire of its substrates is not fully known, and their mode of action is still under investigation. The present study was undertaken to clone and express the budding yeast PLK, Cdc5 in *E. coli*. The recombinant Cdc5 was successfully expressed as a GST-Cdc5 fusion protein of ~ 107 kDa. GST-Cdc5 was purified to ~ 95% homogeneity. Interestingly, the recombinant GST-Cdc5 exhibited kinase activity *in vitro*. GST-Cdc5, but not GST-tag alone phosphorylated the generic substrate casein as well as showed autophosphorylation of the kinase itself. Thus, the recombinant GST-Cdc5 protein is functionally active *in vitro* and mimics its characteristics *in vivo*. The availability of the recombinant and active Cdc5 protein would facilitate structure-function investigations as well as the generation of appropriate truncated and site-directed mutant proteins, respectively for further insight into the cell cycle regulation mechanisms of PLKs.

1. INTRODUCTION

Eukaryotic cell cycle is mainly governed by Cyclin dependent kinases (CDK) and recently identified class of Polo-like Kinases (PLK). PLKs are central regulators of cell cycle, and control multiple mitotic events [1]. The conserved mitotic functions of PLK in all eukaryotes include entry into M phase, progression through mitosis, metaphase to anaphase transition, exit from mitosis and cytokinesis [1]. PLK belongs to Ser/Thr subfamily of protein kinases, characterized by kinase domain (KD) at the N-terminus and a conserved signature motif called polo-box domain (PBD) at C-terminus [2, 3].

The first identified member of PLK subfamily was polo in *Drosophila melanogaster*. Mutations in the *polo* gene result in abnormal mitotic and meiotic divisions, indicating PLK's vital role in cell cycle [1, 4]. After the discovery of polo, PLKs have been subsequently identified in other eukaryotes, except plants [1, 4]. Whereas mammals have multiple PLKs (Plk1, Plk2, Plk3, and Plk4) [2, 3], the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe* have only one PLK (Cdc5 in budding yeast [5] and Plo1 in fission yeast [6]). Both genes are essential, and loss of their functions leads to mitotic arrest [5, 6].

In budding yeast, Cdc5 was identified independently in early screens for genes required for cell cycle progression, which bears structural and functional homology with PLK subfamily members. Cdc5 regulates G2 to M phase transition [7], metaphase to anaphase transition during mitosis [8, 9], exit from mitosis and cytokinesis [9]. Cdc5 also plays an important role during meiosis in yeast [10]. Studies on PLKs in genetically amenable lower eukaryotes such as budding yeast can provide valuable insights into the functions of PLKs of higher eukaryotes.

Apart from the classical cell cycle functions, PLKs are implicated in unique post-mitotic functions acquired during evolution. PLKs function as an integral component of various mitotic regulatory circuits. PLKs studied in yeasts (Cdc5, and Plo1), fruit flies (Plo1), worms (Plc1), and mice (Plk1) also regulate the specialized cell division that gives rise to gametes, known as meiosis [10, 11]. In humans, deregulated PLKs are strongly implicated in tumorigenesis and therefore, represent an ideal protein kinase target for cancer drug development [12]. However, our understanding of its downstream targets for phosphorylation is still at infancy. Very few substrates of PLKs are known in yeast and humans [13]. Neither is the consensus of PLK substrates for phosphorylation completely understood. Therefore, the present study was undertaken to develop an amenable system for biochemical characterization of the yeast PLK, Cdc5. So far, there are no reports on the *in vitro* studies of Cdc5. *E. coli* was selected over budding yeast as host strain for recombinant expression of Cdc5, because of the drawback of proteolysis of over

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expressed proteins in yeast and the lowered yields in yeast system. The recombinant expression of Cdc5 will provide a versatile tool for characterization of enzymatic features of Cdc5 as a kinase, pull down of substrates of Cdc5 from yeast extracts, mutational analysis for deciphering structural- functional complementation, and analysis of consensus for PLK phosphorylation. Since PLKs exhibit a remarkable structural and functional homology, studies from yeast can be used to understand the mammalian counterpart, Plk1 and its spectrum for anticancer targets can be expanded.

2. MATERIALS AND METHODS

2.1 Chemicals and strains

All the fine chemicals were of analytical grade, and were procured from GE Biosciences and SIGMA. Restriction enzymes and T4 polynucleotide kinase were purchased from Fermentas. γ -P³²-ATP was procured from Bhabha Atomic Research Center, Mumbai and used at NIPGR, New Delhi. Standard laboratory strains of *E. coli* and *Saccharomyces cerevisiae* were used for the study.

2.2 Cloning of CDC5 gene from *Saccharomyces cerevisiae*

CDC5 gene was PCR amplified from *Saccharomyces cerevisiae* genomic DNA using forward 25-mer (5' ATATGGATCCATGTCGTTGGGTCCT3'), bearing BamHI restriction site (underlined) and reverse 25-mer (5' AGCGCTCGAGTTAATCTACGGTAAC3'), bearing XhoI restriction site (underlined) using Taq polymerase and Pfu polymerase and cloned into pGEX4T2 vector at the BamHI/XhoI restriction site. Cloning of *S. cerevisiae CDC5* ORF into this vector containing a T7 promoter allows T7 RNA polymerase dependent expression of *CDC5* along with a protease-cleavable GST tag. The resulting recombinant plasmid was transformed in *E. coli* DH5 α and error free *CDC5* DNA sequence was ensured by restriction analysis and sequencing.

2.3 Screening for maximal expression of soluble CDC5 protein

Overexpression of *S. cerevisiae CDC5* was monitored by inoculating a fresh colony of the indicated expression strain bearing pSc*CDC5* plasmid DNA into 5 ml HiVeg LB broth supplemented with antibiotics as described below: BL21 DE3 (ampicillin 100 μ g/ml); Rossetta, ampicillin (100 μ g/ml) and chloramphenicol (37 μ g/ml); BL21-CodonPlus, ampicillin (100 μ g/ml) were grown at 37°C for overnight. One percent of primary culture was used to inoculate 5 ml of HiVeg LB medium supplemented with the appropriate antibiotics and grown at 37°C. At an OD 600nm = 0.5, an aliquot was removed as the uninduced control, and the culture was induced with the addition of IPTG to a final concentration of 0.5, 0.3 and 0.1mM, and incubation was continued for an additional 16 h at 18°C and 4 hours at 37°C. The cells were pelleted by centrifugation (8000 rpm for 10 min), and the pellet were resuspended in buffer A (50 mM Tris-HCl, pH 8, 100 mM NaCl, 5 mM β -mercaptoethanol, and 1X protease inhibitors). The cells were disrupted by sonication (Model No.

UP200S, ultrasonic processor), set at 50% duty cycle pulse mode on ice for 2 min. The cell suspension was centrifuged at 12,000 rpm for 15 min at 4°C. Aliquots from the pellet (resuspended in buffer A) and supernatant fractions were each mixed with SDS-PAGE loading dye, and incubated at 90°C for 10 minutes. The samples were loaded (20 μ g per lane) on a 10% SDS-polyacrylamide gel.

The gels were stained with Coomassie blue and inspected visually for protein expression. To assess relative Cdc5 protein abundance in the pellet and supernatant fractions, 15 μ g protein was resolved by SDS-PAGE and transferred to the nitrocellulose membrane (Hybond ECL). Blot was kept at 4°C overnight in blocking solution containing 5% skim milk. Membranes were then incubated with anti-GST antibodies (Abcam) for 1 hour at room temperature. Immunoreactive proteins were detected by using anti-rabbit IgG conjugated to horseradish peroxidase (Abcam). After several washes, blots were processed for enhanced chemiluminescence (ECL) detection.

2.4 Purification of *S. cerevisiae Cdc5* protein

Following standardization of overexpression studies, *S. cerevisiae CDC5* was expressed in *E. coli* BL21 (Codon plus) because this host strain was capable of enhanced expression of protein, in the soluble form. The cell-free supernatant (from 100ml culture) was loaded onto a GST sepharose column, already pre-equilibrated with buffer A. The column was washed with 50 ml of buffer A containing Tris 50mM and NaCl 200mM and the bound protein was eluted with 10mM reduced glutathione. The eluted protein was dialyzed and the purity of the protein was evaluated on 10% SDS polyacrylamide gel. The molecular mass determined by SDS-PAGE was exactly the same as calculated of ~ 107 kDa. The fractions containing Cdc5 were pooled and dialyzed into storage buffer containing 10% glycerol. It was aliquoted and stored at -80°C for long term use. Approximately 0.5 mg Cdc5 protein was obtained from 1L of bacterial culture in LB broth.

2.5 Functional assay of Cdc5 kinase activity

The kinase assay was performed [14] with slight modifications. Dephosphorylated casein (sigma) was used as a substrate for the *in vitro* kinase assays. β - Glycerol phosphate and sodium ortho-vanadate (Na₃VO₄) served as phosphatase inhibitors. Kinase reactions were performed by incubating purified GST-Cdc5 kinase (2.5 μ g) in a 20 μ l reaction containing 50 mM Tris-Cl (pH 7.5), 10 mM MgCl₂, 5 mM MnCl₂, 1 mM dithiothreitol, 1 mM β -glycerol phosphate, 1 mM Na₃VO₄ supplemented with 0.25 mM ATP, 0.1 μ l of 10 mCi. μ l (γ -³²P) ATP (1 μ Ci; 3000Ci/mMol) and 5 μ g casein (Sigma).

The kinase reactions were then incubated for 30 min at 30°C. After the completion of the reaction, reaction was stopped by adding 4 μ l of 5X Laemmli buffer. Samples were loaded on 10% SDS-PAGE gel. After the run, the radioactive gel was wrapped in plastic (saran) wrap and exposed to the phosphor screen in a cassette for 12-16 hours. The screen was then scanned and developed using Typhoon ImageQ.

3. RESULTS AND DISCUSSION

3.1 Expression screening of *S. cerevisiae* Cdc5 in *E. coli*

Previously *CDC5* gene was cloned and expressed in pET28a but His₆-Cdc5 protein was not obtained in soluble fraction. Expression and purification of recombinant Cdc5 from *E. coli* host strains were complicated by the insolubility of the expressed protein at all temperature variations and IPTG concentrations. Generally difficulties are encountered by researchers while trying overexpression of recombinant proteins in heterologous host strains. Difference in codon usage could be one of the possible reasons behind this result. Consequently, different strains were used to screen for high-level expression of Cdc5 protein in soluble fraction. But none of the conditions resulted in soluble recombinant protein, so vector was changed and pGEX4T2 was selected. pGEX series of vector permits high levels of protein expression as compared to other vector systems [15]. The idea behind changing vector to pGEX4T2 was that the GST tag was itself big and highly soluble and when this tag was fused to gene, this construct was expressed successfully in soluble form. The three *E. coli* host strains used were BL21 (DE3) pLysS, BL21-Codon-Plus and Rossetta known for higher levels of expression as well as tighter control for expression of toxic proteins. The Rossetta (DE3) strain carries a chloramphenicol-resistant plasmid pRARE, that contributes tRNAs for codons rarely used in *E. coli*. BL21-Codon Plus is an engineered strain to optimize codon bias for eukaryotic proteins. It might be possible that the optimal codon bias in BL21-Codon Plus facilitates the proper folding and soluble expression of GST-Cdc5. *S. cerevisiae* *CDC5* gene was PCR amplified and cloned into expression vector pGEX4T2 in-frame with the GST -tag of this vector for expression in *E. coli*. Visual inspection of the gel shown in figure 1 revealed that GST-Cdc5

was found in the insoluble fraction at 37°C. Since recombinant protein overexpression in heterologous host strain is complicated by formation of inclusion bodies, but still protein expression in pellet or soluble form is not an all or none process. It depends on a variety of conditions like IPTG concentration used for induction, O.D. of the culture prior to induction, host strain used for expression, expression tag used for fusion with protein, temperature at which the induction is carried out and many other factors are involved. One has to play with these parameters to get overexpressed protein in soluble and active state. The strategy adopted here to improve protein solubility during heterologous expression was induction at low-temperature. Accordingly, cultures were grown and induced at 18°C for 15 hours. Although a similar pattern of induction was observed in different strains, expression at 18°C resulted in soluble GST-Cdc5 protein (Fig 2A). Overall, induction at early or mid log phase gave the best results. Among different strains used, the different strains can be ranked with BL21-CodonPlus performing the best, followed by Rosetta and BL21-DE3. Interestingly, the expression level of *CDC5* in BL21-Codon- Plus strain was not higher as compared to general host for protein expression BL21-DE3, suggesting that the codon context is not relevant to affect Cdc5 expression in *E. coli*. The data also show that significant amount of recombinant Cdc5 protein was also present in the pellet fraction in comparative more amounts. To ascertain the identity of native Cdc5 protein in the supernatant and pellet fractions from the overproducing strains, Western blotting was carried out using anti-GST antibodies. As shown in Fig. 2B, a protein band was detected in the IPTG-induced cell-free lysates, in both pellet and soluble fractions, with a size consistent with that determined for Cdc5-GST fusion protein (~ 107 kDa). Nevertheless, significant amount of Cdc5 protein was present in the soluble supernatant fraction.

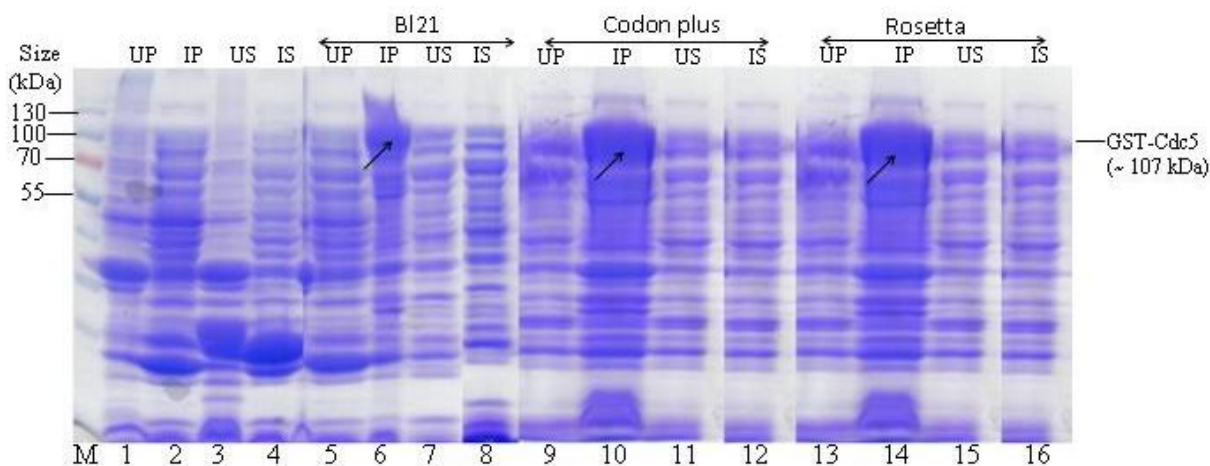


Fig. 1: Inducible expression of GST-Cdc5 protein from pGEX4T2/*CDC5* in different *E. coli* expression strains at 37°C. Protein extracts were prepared and separated by 10 % SDS-PAGE followed by Commassie staining. lanes 1 & 3, vector, uninduced pellet and supernatant respectively in BL21-DE3; lanes 2 & 4, vector, induced pellet & supernatant respectively at 0.1 mM IPTG concentration in BL21-DE3; lanes 5 & 7, pGEX4T2/*CDC5*, uninduced pellet and supernatant, respectively in BL21-DE3; lanes 6 & 8, pGEX4T2/*CDC5* induced pellet & supernatant, respectively at 0.1 mM IPTG concentration in BL21-DE3; lanes 9 & 11, pGEX4T2/*CDC5*, uninduced pellet and supernatant, respectively in Codon plus; lanes 10 & 12, pGEX4T2/*CDC5* induced pellet & supernatant, respectively at 0.1 mM IPTG concentration in Codon plus. lanes 13 & 15, pGEX4T2/*CDC5*, uninduced pellet and supernatant, respectively in Rossetta; lanes 14 & 16, pGEX4T2/*CDC5* induced pellet & supernatant, respectively at 0.1 mM IPTG concentration in Rossetta. UP, uninduced pellet; IP, induced pellet; US, uninduced supernatant; and IS, induced supernatant. Arrows show the position of the band corresponding to GST-Cdc5 protein.

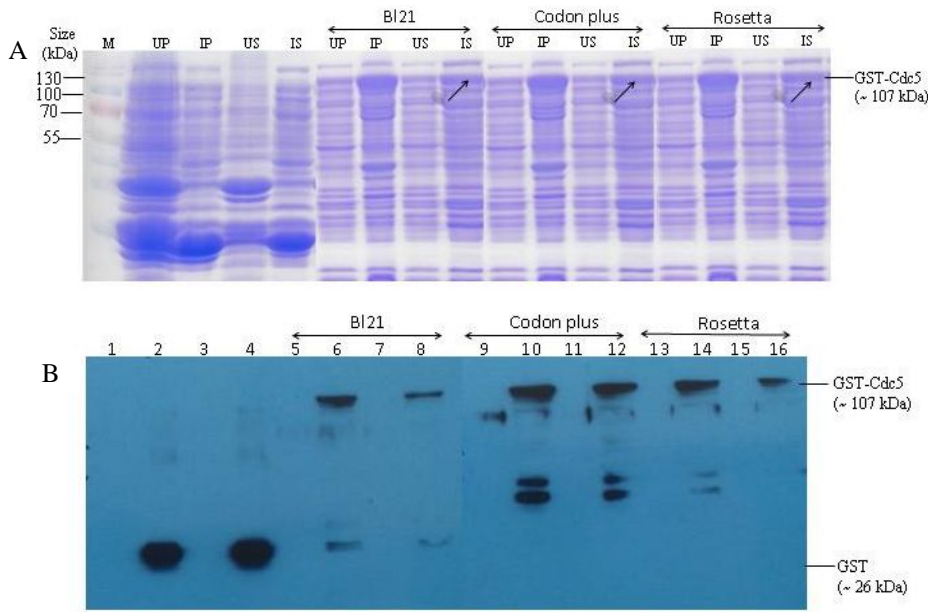


Fig. 2: Analysis of expressed GST-Cdc5 expression in different *E. coli* host strains at 18°C with 0.1 mM IPTG. GST-Cdc5 expression was analyzed in BL21-CP, BL21-DE3 and Rossetta strains of *E. coli*. Protein extracts were prepared from cell free lysates of expressed GST-Cdc5 and separated by 10 % SDS-PAGE followed by Commassie staining in panel A and Western blotting analysis in panel B. Panel A and B: Lanes 1 & 3, uninduced pellet and supernatant fractions of GST; lanes 2 & 4, induced pellet and supernatant fractions of GST; lanes 5 & 7, uninduced pellet and supernatant fractions of GST-Cdc5 in BL21-DE3 ; lanes 6 & 8, induced pellet and supernatant fraction of GST-Cdc5 in BL21-DE3; lanes 9 & 11, uninduced pellet and supernatant fractions of GST-Cdc5 in BL21-Codon-Plus ; lanes 10 & 12, induced pellet and supernatant fractions of GST-Cdc5 in Codon-Plus; lanes 13 & 15, uninduced pellet and supernatant fractions of GST-Cdc5 in Rossetta ; lanes 14 & 16, induced pellet and supernatant fraction of GST-Cdc5 in Rossetta. The size of protein bands is indicated in kDa.

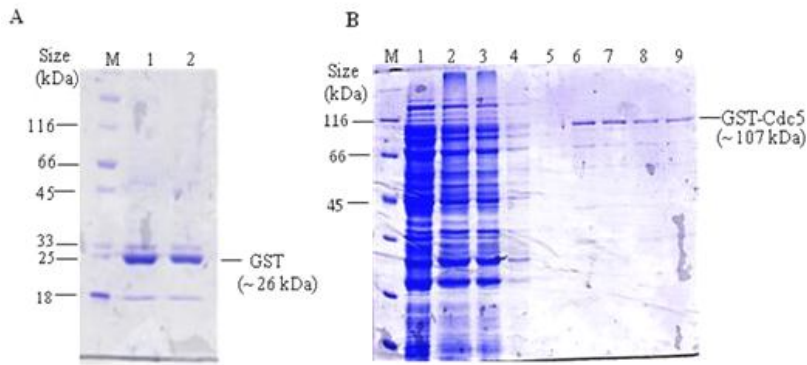


Fig. 3 Purification of recombinant GST-Cdc5 and GST from *E. coli*. SDS- PAGE analysis of purified GST (A) and GST-Cdc5 protein (B) along with other fractions. A. lanes 1 & 2, Eluates (E1 & E2) from reduced Glutathione sepharose showing presence of GST protein (~ 26kDa). B. Purification profile of GST-Cdc5 using reduced glutathione sepharose. Lane 1, Input- Induced lysate; lane 2, Flow through from glutathione sepharose column; lanes 3, 4, 5: Wash 1, 2 and 3 respectively; lanes 6, 7, 8 & 9: Eluate fractions of GST-Cdc5 protein from reduced glutathione sepharose column, E1, 2, 3 & 4, respectively. GST & GST-Cdc5 are indicated. M represents broad range protein marker. Size of marker band is indicated (kDa).

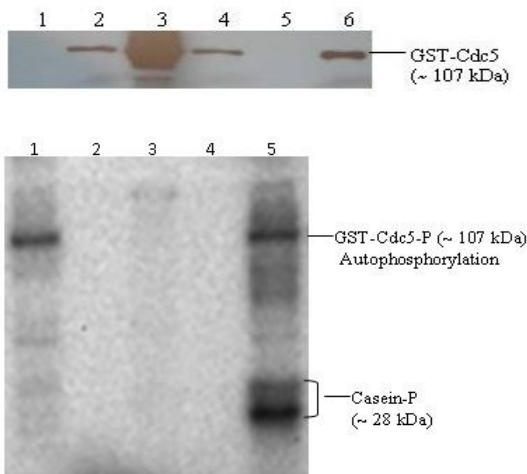


Fig. 4: Western blot analysis of purified GST-Cdc5 protein along with other fractions drawn during different stages of protein purification. The samples from different stages of purification shown in figure 3 were subjected to Western blotting with α -GST antibodies. The position of GST-Cdc5 protein is indicated. lane 1, Uninduced lysate; lane 2, Induced lysate (Input); lane 3, Flow through; lane 4, Wash 1; lane 5, Wash 2; lane 6, Purified GST-Cdc5 protein (2.5 μ g), from eluate E1 of reduced glutathione sepharose column.

Fig 5 Casein phosphorylation assay to assess GST-Cdc5 kinase activity. Kinase assays were performed as described in materials and methods. The reaction products were separated on 10 % SDS-PAGE gel followed by autoradiography of the gel. lane1: GST-Cdc5 kinase incubated in kinase assay buffer without casein; lane 2: Phosphorylation reaction without GST-Cdc5 kinase; lane 3: Casein phosphorylation assay without ATP; lane 4: GST protein incubated with casein in kinase assay as control; lane 5: GST-Cdc5 incubated with casein (substrate) in standard kinase assay reaction, confirming functional activity of purified GST-Cdc5 kinase.

3.2 Purification of GST-Cdc5 protein

We chose to express *S. cerevisiae CDC5* protein in *E. coli* BL21 codon plus because the level of expression of protein in the soluble was greatly enhanced from this host strain. The bacterial lysate containing protein of interest was loaded onto a GST sepharose column, which had been pre-equilibrated with

buffer A as described under Material and methods. The column was washed with Buffer A and eluted in Buffer B containing 10 mM reduced glutathione, and the bound protein was eluted. The eluted protein was dialyzed and protein was evaluated on 10% SDS polyacrylamide gel electrophoresis (Fig. 3) and confirmed with the Western of purified protein (Fig. 4). The molecular mass

determined by SDS–PAGE was in agreement with the calculated molecular mass of 107 kDa.

3.3 Functional validation of purified Cdc5 kinase

To assess the activity of recombinant GST-Cdc5, a kinase assay was performed using casein as a generic substrate as described in materials and methods. As shown in figure 5, GST-Cdc5 kinase showed intrinsic kinase activity and phosphorylated casein as evident in autoradiogram. Cdc5 kinase is a Ser/Thr kinase and showed autophosphorylation consistent with reported earlier for Cdc5 and Plk1 (15). Hence phosphorylated casein and autophosphorylated GST-Cdc5 band confirmed the presence of active kinase.

4. CONCLUSIONS

In this study, we have cloned, overexpressed and purified *S. cerevisiae* Cdc5 protein from *E. coli*. The temperature at which Cdc5 was expressed had a significant impact on its solubility. At low temperature induced protein is partitioned in pellet as well as soluble phase but the amount in soluble phase was enhanced substantially due to slow down of metabolic machinery of host strain. Even the tag used in this study affected the solubility of Cdc5. His tag could not get the protein in soluble fraction, but the GST tag did that.

The purified Cdc5 protein is highly soluble, biologically active and stable at -80°C. The availability of functionally active protein would facilitate the further optimization of kinase activity parameters and identification of its substrates during mitosis and meiosis. Biochemical characterization of Cdc5 kinase can be done that will shed light on its functional and structural aspects. Since the substrate specificity of Cdc5 is still in its infancy so purified kinase can be used for many downstream applications.

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