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A RECOMBINANT SYSTEM FOR PLO1 EXPRESSION: AN AMENABLE TOOL TO STUDY *SCHIZOSACCHAROMYCES POMBE* POLO-LIKE KINASE *IN VITRO*

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ABSTRACT

Eukaryotic cell cycle events are highly regulated by cyclin dependent kinases (CDKs) and the Polo like kinases (PLKs). Polo like kinases belongs to a large subfamily of serine-threonine kinases conserved both structurally and functionally from yeast to humans. plo1, homologue of budding yeast Cdc5, is solitary Plk in *Schizosaccharomyces pombe*. plo1 is the key regulator of fission yeast mitosis as well as meiosis. However, the biochemical functions of plo1 in cell division are poorly understood in fission yeast. So, the present *in-vitro* study was undertaken to clone *plo1* gene in pGEX4T2 vector and screen the different *E. coli* expression strains for soluble GST-plo1p expression. While BL21 (DE3) and BL21 codon-plus expressed the GST-plo1p maximally in insoluble fraction, soluble expression of GST-plo1p was observed in BL21DE3 (C43), BL21DE3 (C41), pLys, BL21 star, BL21DE3 (RIL) and rosetta. The recombinant plo1 protein of 103 kDa was successfully over expressed as GST-plo1 in different *E. coli* strains. Recombinant GST-plo1p expression was confirmed by Western analysis using anti- GST antibodies. From Western blotting, highest soluble expression of GST-plo1p was found in BL21DE3 (C43) expression strain of *E. coli*. The recombinant plo1 will provide an amenable tool to explore the biochemical functions of plo1 in vitro, following purification of GST-plo1 protein.

Key words: Cell cycle, *Schizosaccharomyces pombe*, Polo like kinase, plo1, recombinant plo1, BL21DE3 (C43), GST

INTRODUCTION

Schizosaccharomyces pombe, also known as 'fission yeast', is a member of yeast species, and a unicellular eukaryote [1]. Like other eukaryotic cells, the fission yeast cell cycle can be divided into four discrete phases: G_1 (gap 1), S (synthetic), G_2 (gap2), and M (mitotic) [2]. G_1 and S phases are shortest (each of ~10% of total), but G_2 phase (~70% of total) is the longest phase of *S. pombe* cell cycle, during which most of the growth occurs [3]. Most of the genes and cell cycle events of fission yeast are very similar to the metazoans, e.g., *Caenorhabditis elegans, Drosophila melanogaster* and *Homo sapiens*. Several such functions of fission yeast are highly divergent or absent in budding yeast (*Saccharomyces cerevisiae*) [4] e.g., chromosome structure and metabolism, relatively large chromosomes, large repetitive centromeres, heterochromatic histone methylation, chromo - domain heterochromatin proteins, siRNA-regulated heterochromatin and TRF-family telomere binding proteins), RNAi pathway, the signalosome pathway and spliceosome components [5].

Polo like kinases (PLKs) are the member of a large subfamily of serine-threonine kinases, conserved from yeast to mammals [6, 7]. PLKs share a highly conserved catalytic protein kinase domain and non-catalytic polo-box domain [8]. The fission yeast *S. pombe* contains a PLK named plo1 [7, 9]. *plo1*gene, encoding polo like kinase is the first kinase which is to be identified during fission yeast mitotic progression [10]. The role of plo1 protein kinase is critical during fission yeast cell cycle because, its loss or overexpression leads to defective cell cycle. Deletion of *plo*1 leads todefect in the formation and establishment of bipolar spindle and failure in septa formation or misplacement of septa [11], and formation of cytokinetic actomyosin ring early in mitosis [12]. plo1 also regulates the timing of septation [13], and transcription of several genes [14]. The association of plo1 at spindle pole body diminishes after APC activation but still persists till the end of mitosis [15]. This indicates that, unlike mammalian PLKs and budding yeast PLK, plo1 is not degraded towards the end of mitosis [13]. Although the functions of plo1 have been studied *in vivo*, there are no reports on biochemical characterization of plo1 as a kinase. Therefore, the present study was undertaken to establish an easy tool to study plo1 *in vitro*. Amongst most pronounced yeast model systems, fission yeast is the most evolutionary closest member to humans. Most of the cancer related proteins are present in fission yeast whose mutated products are found in human's cancer related proteins [4], and

plo1 is one of those proteins. So, studies on fission yeast plo1 can be used to understand the mammalian/human counterpart, Plk1, and its spectrum for anticancer targets can be expanded.

MATERIAL AND METHODS

Chemicals and strains

All chemicals were of analytical grade, and were procured from Himedia Labs, Mumbai. Restriction enzymes and T4 DNA ligase were purchased from Fermentas Inc., USA. *Schizosaccharomyces pombe* standard strain ABP20 (MTCC 3240) was procured from IMTECH, Chandigarh, India. *S. pombe* yeast strain was cultured on YEPD supplemented with 0.8 % adenine (0.5 % in 0.05 N HCl) agar/broth and grown at 30 °C. Standard laboratory strains of *E. coli* were used for the study and cultured in nutrient broth (NB) and grown at 37 °C.

DNA manipulation

Genomic DNA of *S. pombe* and pGEX4T2 plasmid DNA was isolated by glass bead method and alkaline lysis method, respectively [16]. The quality of genomic DNA and pGEX4T2 plasmid preparation was analyzed by agarose gel electrophoresis.

PCR amplification and cloning of *plo1* gene in pGEX4T2 vector

Amplification of *plo1* gene from *S. pombe* genomic DNA was carried out using forward 25-mer (5'-AATTGGATCCATGGCGAGTGTTGCA-3'), bearing *BamH1* restriction site (underlined) and reverse 25-mer (5'-AGCCCTCGAGTTAACTCACTTC CAT-3') bearing *XhoI* restriction site (underlined) using Taq polymerase and Pfu polymerase and cloned into pGEX4T2 vector at the *BamH1/XhoI* restriction sites. Cloning of *S. pombe plo1* ORF into pGEX4T2 vector containing a T7 promoter allows T7 RNA polymerase dependent expression of plo1 along with a protease-cleavable N-terminal GST tag. The resulting recombinant plasmid was transformed in *E. coli* DH5α. 100 ng of ligation mixture was transformed into *E. coli* strain DH5α competent cells by heat shock method [16] at 42 °C/ 2 minutes followed by addition of 1 ml nutrient broth and incubated at 37 °C/ 1 h. Transformants were selected on NB plates containing 100 µg/ml ampicillin. Error free plo1 DNA sequence was ensured by restriction analysis and DNA sequencing. Then pGEX4T2/*plo1* was transformed into various expression host strains of *E. coli.*

Screening for soluble expression of recombinant GST-plo1 protein in different *E. coli* host strains

Over expression of *S. pombe* plo1 was monitored by inoculating a fresh colony of the following expression strain bearing pGEX4T2/*plo1* plasmid DNA into 5 ml

HiVeg LB broth (Himedia Labs) supplemented with 100 µg/ml ampicillin as described below: BL21 DE3, BL21 codon plus, BL21 DE3 (C43), BL21 DE3 (C41), Rosetta, BL21DE3(RIL), BL21 star, and pLys. The cultures were grown at 37 °C for overnight. One percent of overnight grown primary culture was used to inoculate 5 ml of HiVeg LB medium supplemented withampicillin (100 µg/ml) and grown at 37 °C. At an A_{600} of ~ 0.5, an aliquot was removed as the uninduced control, and the remaining culture was induced by the addition of IPTG to a final concentration of 0.5 and 1 mM, and incubation was continued for an additional 3 hours at 37°C or 15 h at 18°C . The cells were harvested by centrifugation (6000 rpm for 10 min), and the pellets were resuspended in buffer A (50 mM Tris–Cl, pH 8, 50 mM NaCl, 2 mM β-mercaptoethanol, and 1X protease inhibitors). The cells were disrupted by sonication at 52 % duty cycle pulse mode on ice for 2 min. The cell suspension was centrifuged at 12,000 rpm for 20 min at 4°C. Aliquots from the pellet (resuspended in buffer A) and supernatant fractions were each mixed with 6X SDS-PAGE loading buffer, and incubated at 95°C for 10 minutes. The samples were loaded (20 µg of total protein per lane) on a 10% SDS–polyacrylamide gel. The gels were stained with coommassie brilliant blue (CBBR-250) and inspected visually for protein expression. To assess relative plo1 protein abundance in the pellet and supernatant fractions, 20µg of total protein was resolved by SDS–PAGE and transferred to the nitrocellulose membrane (Millipore, Immobilon P 0.45µm) by electroblotting in transfer buffer for 1 h 30 minutes/ room temperature. Blot was incubated at 4°C for 3h in blocking solution containing 3% BSA. Membranes were then incubated with anti-GST antibodies (1: 5000, Abcam) overnight at 4°C. lmmunoreactive proteins were detected by using anti-rabbit IgG conjugated to horseradish peroxidase (1: 10000, Abcam). After several washes, blots were processed for enhanced chemiluminescence (ECL) detection.

RESULTS

***S. pombe* genomic DNA and pGEX4T2 plasmid DNA isolation**

To clone *plo1* gene in pGEX4T2 vector, *S. pombe* genomic DNA and pGEX4T2 plasmid DNA were isolated as described in material and methods [16]. The quality of the DNA preparations DNA was analysed by agarose gel electrophoresis, which revealed the presence of intact genomic (Fig. 1A) and plasmid DNA (Fig. 1B).

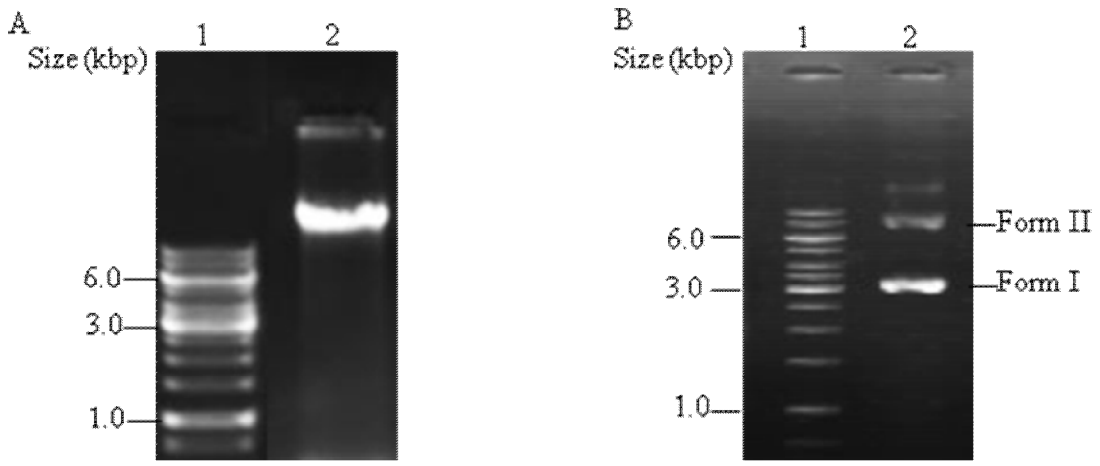

Figure 1: Analysis of *S. pombe* genomic DNA and pGEX4T2 plasmid isolation. A: *S. pombe* genomic DNA analysis. lane 1, 1 kb DNA marker; lane 2, *S. pombe* genomic DNA. B: pGEX4T2 plasmid DNA analysis. Lane 1, 1 kb DNA marker; lane 2, pGEX4T2 plasmid DNA showing form I (supercoiled), form II (nicked). The size of marker bands is indicated in kbp.

PCR amplification of *plo1* gene

To achieve *plo1* gene amplification, *S. pombe* genomic DNA was taken as template for PCR using *plo1* gene specific primers flanked by BamHI (forward) and XhoI (reverse) as described in material and methods. The PCR product was analysed by agarose gel electrophoresis. A band of~ 2.0 kb was observed on agarose gel, which is the expected size for *plo1* gene amplicon (Fig. 2).

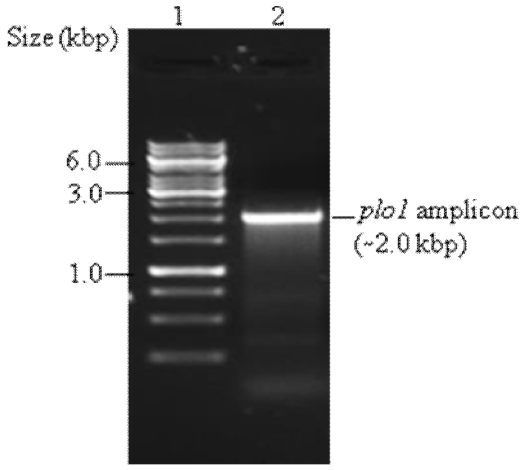

Figure 2: PCR amplification of *plo1*: The reaction product of PCR using plo1 primers was analysed by 1 % agarose gel followed by gel documentation. Lane 1, 1 kb DNA marker; lane 2: PCR product showing amplification of *plo1* gene (~ 2.0 kb).

Cloning of *plo1* gene in pGEX4T2 vector

Cloning of *Schizosaccharomyces pombe plo1* ORF into pGEX4T2 vector was accomplished by restriction digestion of *plo1* and pGEX4T2 vector with a combination BamHI and XhoI restriction enzymes. Double digested pGEX4T2 vector and *plo1* were ligated at 4 °C/ 16 h. The resulting recombinant plasmid

(pGEX4T2/*plo1*) was transformed in *E. coli* DH5α as described in material and methods. Further error free *plo1* DNA sequence of~ 2.0 kb was confirmed by DNA sequencing. To confirm the presence of *plo1* gene, pGEX4T2/*plo1* clone was double digested with cloning enzymes BamHI and XhoI (Fig. 3). The release of 2.0 kb (*plo1*) and 4.9 kb (pGEX4T2) bands, confirmed the presence of *plo1* insert in pGEX4T2 vector (Fig. 3).

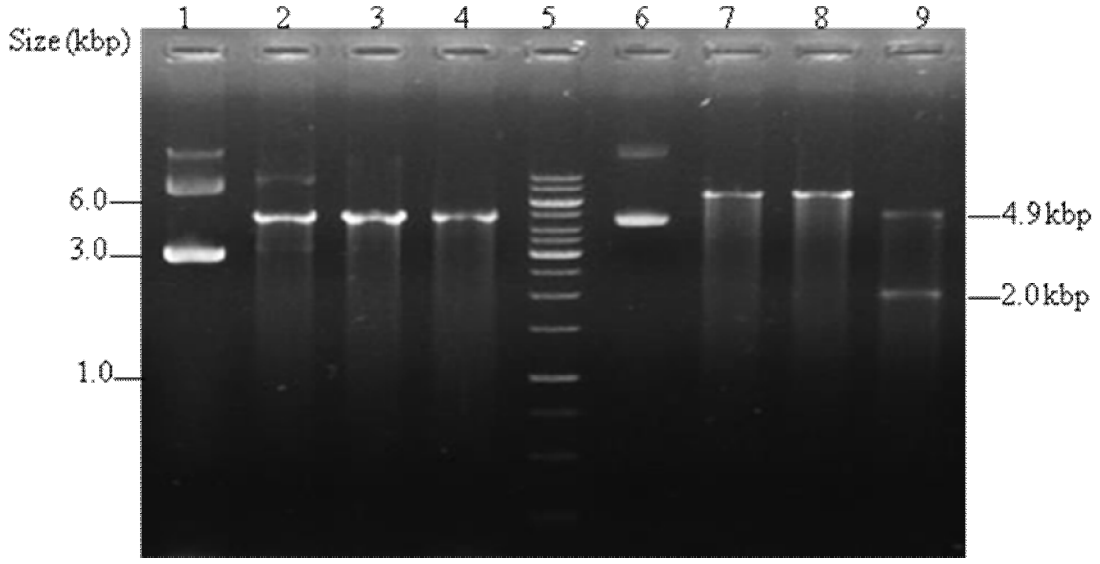

Figure 3: Restriction mapping of pGEX4T2/*plo1* construct. lane 1, Undigested pGEX4T2 vector; lane 2, pGEX4T2 vector digested with *BamH1*; lane 3, pGEX4T2 vector digested with *Xho1*; lane 4, double digested pGEX4T2 vector with *BamHI* and *XhoI*; lane 5, 1 kb DNA marker; lane 6, Undigested pGEX4T2/*plo1* construct; lane 7-9, pGEX4T2/*plo1* digested with *BamHI* , *XhoI* and *BamHI* - *XhoI* respectively.

Recombinant expression of GST-plo1 protein in *E. coli*

To express the GST-plo1, pGEX4T2/plo1 plasmid was transformed into two *E. coli* expression hosts, BL21 (DE3) and BL21 codon-plus. Expression of GST-plo1 was induced by addition of IPTG as described in material and methods. As shown by Fig. 4, inducible expression of protein of~ 103 kDa come to the size of GST-plo1 was observed in the induced fraction of both the strains (Fig. 4, lane 2 and 4). However, GST-plo1 expression in both BL21 (DE3) and BL21 codon-plus was found in insoluble fraction was found in the insoluble fraction at 37°C as well as at 18°C (data not shown). Nevertheless, those results indicate that plo1 has been cloned in frame with GST-tag in pGEX4T2, and inducible expression of GST-plo1 is also confirmed.

Soluble GST-plo1 expression is highest in BL21DE3/C43

To achieve the soluble expression of GST-plo1, pGEX4T2/plo1 was transformed into different *E. coli* host strains including BL21DE3 (C43), BL21DE3 (C41), pLys, BL21DE3 (RIL), Rosetta and BL21 star. Expression of GST-plo1 was induced by

addition of 1 mM IPTG and incubation at 37°C for 3 hours. Although a similar pattern of induction was observed in all the expression strains, of significant amount of GST-plo1 protein was obtained in soluble fraction. However leaky expression of GST-plo1 was also observed in the uninduced fraction of some host strains including, and pLys, rosetta, BL21 star, BL21DE3 (C41) (Fig. 5, lane 6, 10, 13, 17 respectively). Induction at early or mid-log phase gave the best results. Among the different expression strains studied, the expression of GST-plo1 was highest in BL21DE3 (C43) and BL21DE3 (C41), as compared to other host strains (Fig. 5). To confirm the expression of GST-plo1 protein, the soluble fractions were subjected to Western blotting GST-tag specific antibody (Figure 6). Therefore, BL21DE3 (C43) strain showed maximal soluble GST-plo1 expression followed by BL21DE3 (C41) and BL21DE3 (RIL). BL21DE3 (C41), Rosetta, BL21 star and pLys host strains showed leaky GST-plo1 expression (expression in uninduced supernatant fraction) as confirmed by western blot (Fig. 6).

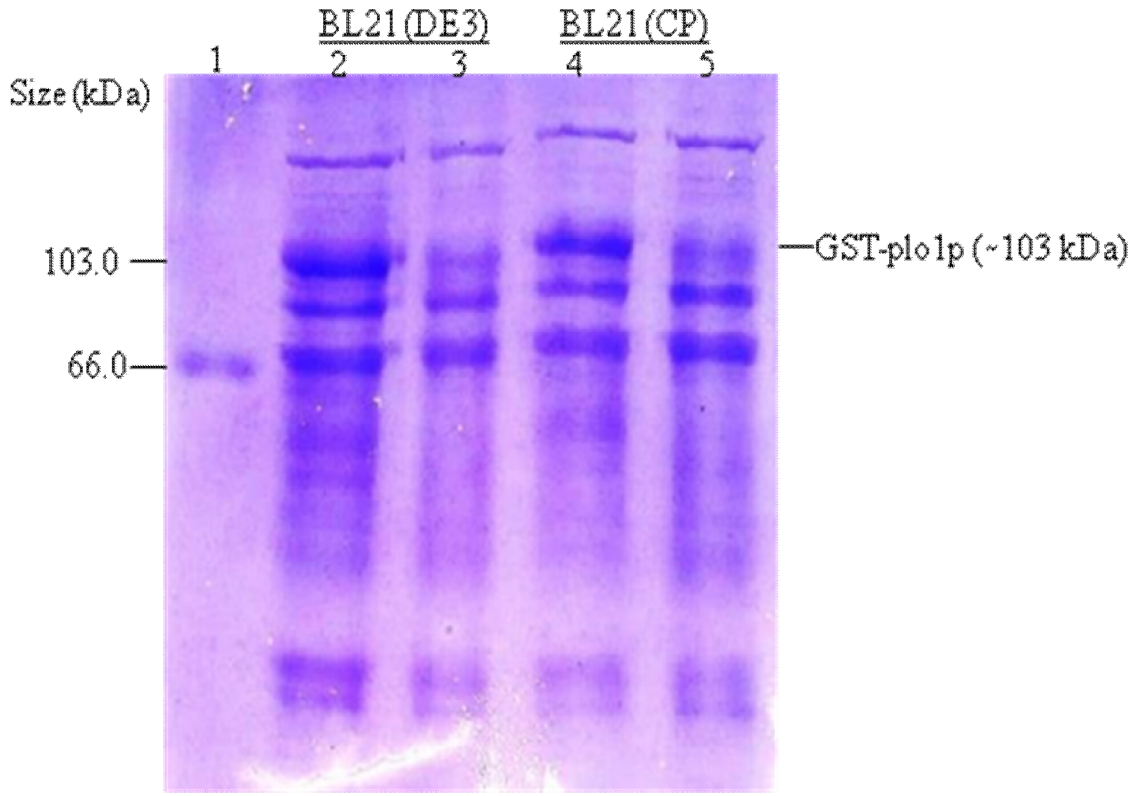

Figure 4: Recombinant expression of GST-plo1 protein in BL21 (DE3) and BL21 (codon- plus) strains of *E. coli*. Expression of GST-plo1 was induced by addition of 1 mM IPTG and protein extracts were analysed by 10 % SDS-PAGE. Lane 1,~66 kDa size marker; lane 2 and 3, induced and uninduced supernatant in BL21 (DE3) strain; lane 4 and 5, induced and uninduced supernatant in BL21 codon-plus strain

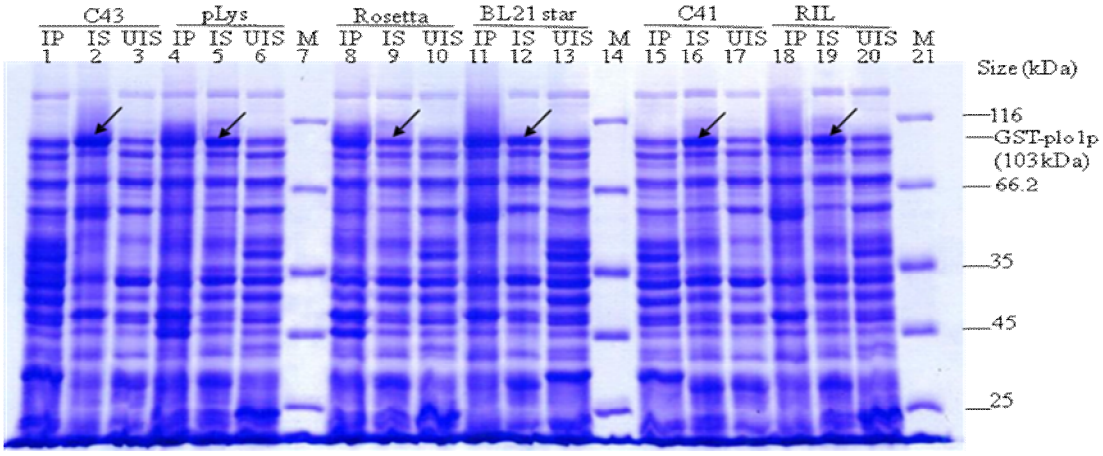

Figure 5: Expression of GST-plo1 protein in different *E. coli* host strains (37 ^{0}C with 1 mM IPTG). To get soluble GST-plo1p expression, induction was carried in the indicated strains of *E. coli.* Protein extracts were prepared from cell free lysates of expressed GST-plo1 and separated by 10 % SDS-PAGE followed by staining with CBBR-250. The protein fractions from the expression host strains are indicated. Lane 1-3 : BL21DE3 ; lane 4-6 : pLys ; lane 8- 10 : rosetta ; lane 11-13 : BL21 star ; lane 15-17 : BL21DE3 (C41) ; lane 18-20 : BL21DE3 (RIL). IS – induced supernatant; IP – induced pellet; UIS – uninduced supernatant; M – size marker (kDa). The position of induced expression of GST-plo1 is indicated by arrow heads.

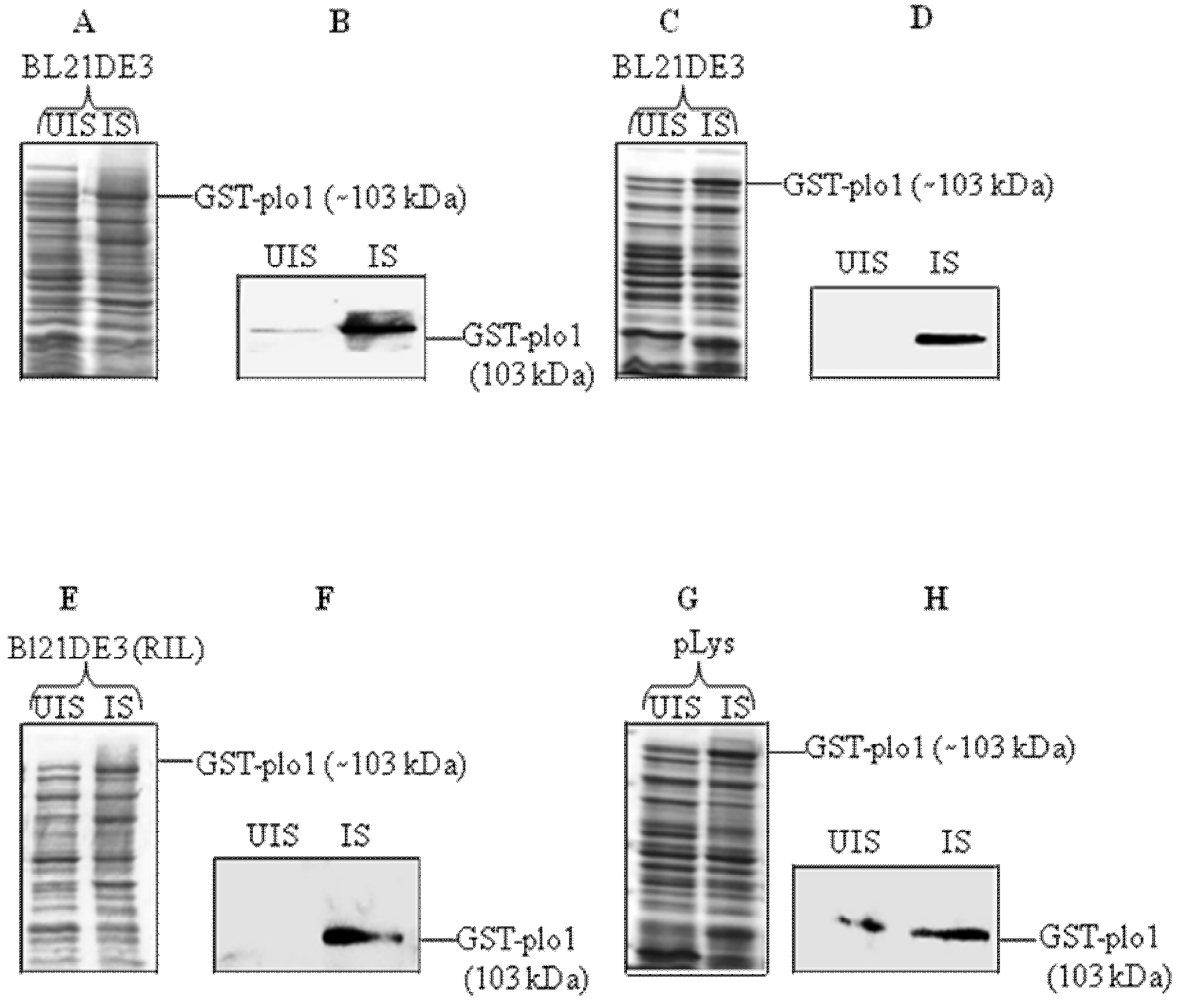

Figure 6: Western analysis of GST-plo1p expression in different *E. coli* expression strains with anti-GST antibody: The soluble protein extracts were prepared from uninduced and induced samples of the indicated expression strains harbouring pGEX4T2/plo1. The proteins were resolved by 10 % SDS-PAGE and subjected to staining with CBBR-250 [A, C, E & G] and western blotting with GST antibodies [B, D, F & H). UIS – uninduced supernatant; IS – induced supernatant

DISCUSSION

PLKs play a vital role in governing cell cycle in most eukaryotes studied, yet the kinome of PLKs is largely unexplored except for few of its substrates [18]. Adding to this, PLKs have acquired species-specific functions in many eukaryotes [7]. The present study was initiated to generate an *in vitro* system for exploration of plo1, the solitary PLK in the fission yeast, *S. pombe*. Although the role of plo1 during cell cycle have been largely characterized, but there are no reports on the biochemical studies on plo1. In this study, we have cloned, over expressed *S. pombe* plo1 protein in different *E. coli* host strains. Previous studies have reported the cloning, expression of functionally active PLK from the budding yeast, *Saccharomyces cerevisiae* (Cdc5) and *Candida albicans* (CaCdc5) [19, 20]. Similar to the present study, *S. cerevisiae* Cdc5 and CaCdc5 were cloned in pGEX4T2, GST-tagging vector. Expression of recombinant plo1 from *E. coli*host strains was complicated by the insolubility of the expressed protein at varying temperature (6 °C – 37 °C) and IPTG concentrations (0.1 mM – 1 mM). Generally, difficulties are encountered by researchers during overexpression of recombinant proteins in heterologous host strains. Difference in codon usage could be one of the possible reasons for insoluble expression. BL21 (DE3) and BL21 codon-plus strains were used to screen for high-level expression of plo1 protein in soluble fraction. However, none of the conditions of induction including varying temperature and IPTG concentrations resulted in soluble expression of GST-plo1.

BL21 (DE3) and BL21codon-plus host strains could not get the plo1 protein in soluble fraction. So, various BL21DE3 derivative strains like, BL21DE3 (C43), BL21DE3 (C41), BL21DE3 (RIL), pLys and BL21 star and rosetta were screened for soluble GST-plo1 protein expression [Fig. 5] Interestingly, soluble expression of GST-plo1 was obtained in all the expression strains at 37 °C with 1 mM IPTG [Fig. 5]. These results indicate that the choice of host strain plays a vital role in obtaining the soluble expression of the recombinant protein.

Maximal soluble GST-plo1 protein expression was found in BL21DE3 (C43) followed by BL21DE3 (C41) and BL21DE3 (RIL). These results are in contrast to those of budding yeast PLKs Cdc5 and CaCdc5. Both these PLKs exhibited soluble expression in BL21 codon – plus at low temperature (18 °C) [19, 20]. The BL21DE3 (C41) and BL21DE3 (C43) host strains are effective in overexpressing toxic proteins from different organisms, including viruses, bacteria, yeasts, plants, insects, and

mammals. BL21 (DE3) derivative strains have Lambda DE3 lysogen, which expresses T7 RNA polymerase from the lacUV5 promoter by IPTG induction [17].

Leaky GST-plo1 protein expression was observed in various host strains like BL21DE3 (C41), pLys and BL21 star and rosetta which could be due to possible leaky expression of T7 RNA polymerase in these host strains. BL21DE3 (C43) host strain has not been explored for recombinant expression of yeast proteins. Thus present study forms the first study for recombinant expression of a fission yeast protein in BL21DE3 (C43).

CONCLUSION

This study provides an example to express the GST-tagged yeast toxic proteins in BL21DE3 (C43) system. Beside this, recombinant expression of plo1 protein will provide the key for the characterization of enzymatic features of plo1 as a kinase, pull down of substrates of plo1 from fission yeast, mutational analysis for structure-function analysis, and decipher the consensus sequences for PLK phosphorylation. Substrate identification of plo1 is still a mystery to be solved. Hence, purification of the GST-plo1 recombinant protein and its functional characterization could be used for many downstream applications

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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