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Expression Purification and Immunodetection of a fusion protein Glutathione S Transferase from pGEX-3X vector in BL21

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Abstract: *Glutathione S Transferase(GST) is an enzyme of a multi gene family which is involved in reducing oxidative damage to cells and detoxification of Xenobiotic compounds and plays critical role in life processes. The entire work was completely qualitative and the objective of my work was to deal with the induction, extraction and purification of the GST fusion protein from pGEX 3X vector.In order to achieve high degree of transformed cells,the E.Coli BL21 host strain was made competent using 0.1M $CaCl_2$ and adding of pGEX 3X vector into host made it transformed.With the induction of GST protein by 0.1mM IPTG,the desired protein was purified through glutathione Cl agarose column and was detected by immunoblotting method with the use of anti GST HRP conjugate Ab which expressed the desired protein.*

Keywords: *Glutathione S Transferase, pGEX 3X, $CaCl_2$ and IPTG.*

I. Introduction

Glutathione S Transferase (GSTs)) is an enzyme with a molecular mass of 26KDa are widely distributed in nature from bacteria & yeasts to plants & animals.On the basis of their sequence identity the soluble mammalian enzymes have been categorized to eight families namely Alpha(α) ,Mu(μ), Pi(π), Sigma(σ), Theta(θ),Zeta(ζ),Omega(ω) and Kappa(κ).Four additional classes Beta(β),Delta(δ),Phi(ϕ),Tau(τ) are found in bacteria,insects and plants.GSTs catalyze the nucleophilic attack of glutathione(GSH) which is a tripeptide in which a cysteine- Glycine dipeptide is attached at the carboxyl carbon of the Glutamate side chain. Reduced glutathione with a free sulfhydryl group is required to combat oxidative stress and maintain the normal reduced state in the cell thus protects the cell from oxidative damage as well as in detoxification of Xenobiotic componds .Cells with a decreased level of glutathione are more susceptible to haemolytic anaemia and pathogenesis of many disease like cystic fibrosis, HIV, AIDS, cancer, heart attack, stroke and diabetes. More recently defined superfamily is composed of microsomal transferases designated MAPEG that is not principally involved in detoxification but rather involved in biosynthesis of leucotrienes and prostaglandins,endogenous lipid signalling molecules.

The entire work dealt with over expression of GST with suitable host cell BL21 which would otherwise be very difficult in normal condition to achieve this high degree of protein.In order to achieve high degree of purification of that protein just one step purification method is desired through affinity chromatography.The antibodies which get bound to desired protein confirm the presence of GST protein.

II. Materials And Methods

All the media used in this work were obtained from Himedia Laboratories Pvt. Ltd.(Mumbai,India).Pure distilled water was obtained with a Milli-Q system(Millipore,Japan). All the solutions and buffer used in this experiments were self prepared by using chemical reagents.

2.1 Plasmid DNA Isolation:

The Alkali lysis method is used to isolate pGEX-3X vector from *E.*Coli. 1.5ml of an overnight culture was transferred and centrifuged at 12000rpm.The composition of solutions used in this experiment were solution I 100µl , solution II 200µl, solution III 150µl, RNAse 1µl, 70% ethanol, 0.1X TE buffer 40µl, LB broth 10ml, ampicillin 20µl.The isolated plasmid was observed by electrophoresis on 0.8% agarose gel prepared with 0.24gm agarose in 600µl 1X TAE buffer and EtBr 5µl.The gel loading dye(5µl each)was added in 10µl of sample and the gel was visualized under the UV transilluminator.

2.2 Competent cell preparation and Transformation :

The *E.*Coli BL21 host strain was inoculated and incubated till the O.D at 600nm reaches 0.3 and arresting the growth by chilling on ice.The bacterial pellet was made competent by repeating treatment of 0.1M of $CaCl_2$. 5 aliquots of 125µl competent cell was made and 10µl of isolated pGEX 3X vector was added to 3 aliquots.The labelled vials (both CC & TC) were incubated on ice for 20min .The vials were exposed to heat shock adjusted to 42°C for 2min then immediately chilled on ice for 10min.Added 500µl of LB broth to each

vials before incubating at 37°C for 1hr then 100µl & 150µl of the TC was transferred to LB plate with 0.1mM ampicillin.

Similarly 100µl of CC was transferred to LB plate with and without ampicillin.The plates were incubated overnight at 37°C to observe the presence of transformed pGEX 3X vector.

2.3 GST induction with IPTG :

An isolated colony from transformed LB ampicillin plates were inoculated to 5ml of LB ampicillin broth and incubated overnight at 37°C with 130rpm. 2ml of overnight culture was reinoculated into 100ml of LB ampicillin broth.Incubated at 130rpm till the OD at 600nm reaches 0.5 then 5ml of culture was drawn and marked as before induction and 0.1mM of IPTG was added to remaining 95ml of culture to the final concentration of 0.1mM and marked as after induction.Both BI and AI samples were incubated for 3hrs at 30°C with 220rpm.

2.4 Cell lyses : Two methods can be used to lyse the incubated cultures.

Method I : 4ml of BI & AI were taken and centrifuged at 8000rpm for 10min .After discarding the supernatant the pellet was resuspended in 150µl of extraction buffer.25µl of the sample loading buffer was added to BI & AI cell suspensions and boiled to 70-100°C in a water bath for 20mins.The BI & AI samples were centrifuged at 6000-8000rpm for 10mins.transferred the supernatant and used them for SDS-PAGE.

Method II : The remaining AI suspension was centrifuged at 5000rpm for 10mins.the supernatant was discarded.The tubes were weighed with pellet and transferred to chilled mortar then again weighed without pellet and calculated pellet weight.2.5X Alumina(pellet wgt.×2.5) was added to pellet and grinded it to paste then added extraction buffer(2ml/100-200mg of pellet).the cell extract was centrifuged at 5000rpm for 50mins.Carefully transferred the supernatant without disturbing the pellet then added 0.5ml of Triton X 100 to 10ml of clarified lysate and again centrifuged at 14000rpm for 30mins and transferred the supernatant to sterile test tube and stored at 4^0C.

2.5 Purification using Affinity Chromatography with Glutathione Agarose column :

The Glutathione column was equilibriated with 10ml of equilibriation buffer.10ml of the cell lysate sample was allowed to flow into the gel bed and collected 1.5ml aliquots each.Again washed the column with equillibriation buffer(1X) in 2ml aliquots each till the OD reaches 0.05 at 280nm. The column was eluted with 10ml of elution buffer with reduced glutathione and collected 1ml fraction around 10numbers.Elution can be monitored by absorbance at 280nm(80% of the GST protein will come off in the first 3 eluted fractions).The eluted GST fusion protein can now be visualized through SDS-PAGE and confirmed by western blotting.

2.6 SDS-PAGE :

10% of resolving gel reaction mixture(5ml) was poured in between the glass plates.allowed the gel to polymerize then again 5% of stacking gel reaction mixture (2.5ml) was poured over the resolving gel.The comb was inserted immediately and allowed to polymerize.Then the plates were placed in electrophoresis unit with 1X tris glycine buffer(pH 8.3) and loaded 50µl of processed protein samples into wells.Electrophoresed the gel and dissected into 2 parts,one to commassie brilliant blue staining as in SDS and other half to western blotting.

2.7 Electro blotting and Immunodetection :

Assembled the cassette with gel and nitro cellulose membrane and inserted in the chamber with transfer buffer and started applying current at 50V after 5-8hrs at RT or 30V overnight at 4°C.

The membrane was blocked with 1X blocking buffer for 2hrs at room temperature.Incubated with anti GST-HRP conjugate Ab for 20mins(1:1000 dilution in 1X assay buffer,3.3µl of Ab conjugate + 10ml of 1X PBS).Washed with 1X wash buffer for 3-5mins thrice.Incubated with the 1X substrate solution(9.750 ml of distilled water + 250µl of substrate TMB/H_2O_2) in dark for 20mins and observed for protein detection. Blue colour indicated the presence of GST.

III. Results and Discussion

3.1 pGEX 3X bands on agarose gel :

All the work done and experiments were qualitative and standardized.The pGEX 3X plasmid DNA was isolated by alkali lysis method and was resolved on 0.8% agarose gel.Spinning down the cell separates the cell wall released from bacteria and floating around in the supernatant.Cells were resuspended in solution I where glucose helps in increasing osmotic pressure in and out of the cell,EDTA chelates divalent Mg^+ ion and Tris HCl maintains the pH of the solution.Net freshly prepared solution II was used which contains SDS and NaOH.SDS is an anionic detergent helps in rupturing the cell wall by breaking lipid membrane and NaOH

sharply increases the pH and denatures the chromosomal and plasmid DNA.Hence Highly alkaline solution gives rise to the name of this technique.Glacial acetic acid of ice cold solution III brings about a pH drop. Adding Potassium acetate to the SDS forms KDS which is insoluble.This will allow easy removal of SDS and allows circular DNA to renature and sheared cellular DNA remains denatured.RNase was added to remove RNA content.chilled absolute alcohol will precipitate the DNA and 70% ethanol will remove salt and 30% water will dissolve salt completely.Air dried plasmid was dissolved in TE buffer that maintains pH and prevent any DNase action.

The bands were observed on agarose gel.The supercoiled conformation of DNA is the most condensed form and traverses through the gel more easily than the other two.The linear conformation of DNA can be coiled but open nicked conformation is cut only on one strand so it is more difficult to be coiled and slow than the other two.The bands were seen in P1,P2,P3 and P4 as shown in Fig.1–

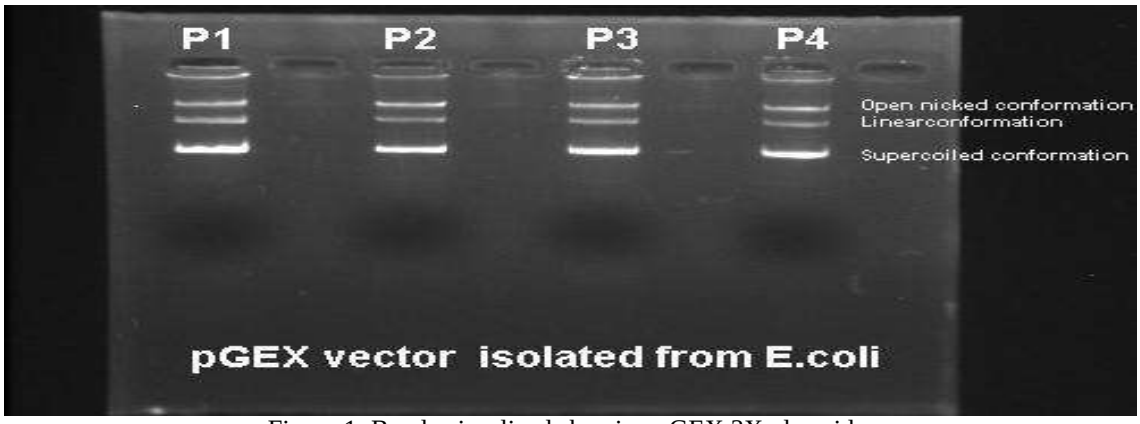

Figure 1: Bands visualized showing pGEX 3X plasmid.

3.2 Transformation :

Recombinant plasmid was transferred into a competent host cell and allowed to multiply with the host cell.Simple efficient transformation procedure with *E.*Coli BL 21 involves resuspending log phase cells with ice cold $CaCl_2$ and keeping them on ice.It affects the cell wall and may be responsible for binding DNA to the cell surface.The actual uptake of plasmid DNA by host cell was stimulated by brief heat shock, which leads transient opening of cell membrane.The control plate was prepared without any inoculums and antibiotic which shows that the plating was done under aseptic condition.As we can see no growth was seen in Fig. 2.In Fig. 3, competent cells(100µl) were plated on LB agar with ampicillin(200µl).The plate did not show any growth.It shows that competent cells does not have any ampicillin resistant gene.In Fig. 4,competent cells without ampicillin showed crowded growth after overnight incubation. In last Fig. 5 transformed cells with ampicillin showed growth as they have gained resistance to ampicillin.

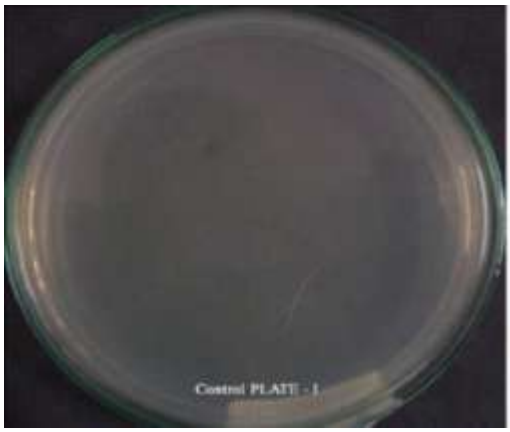

Figure 2: control plate

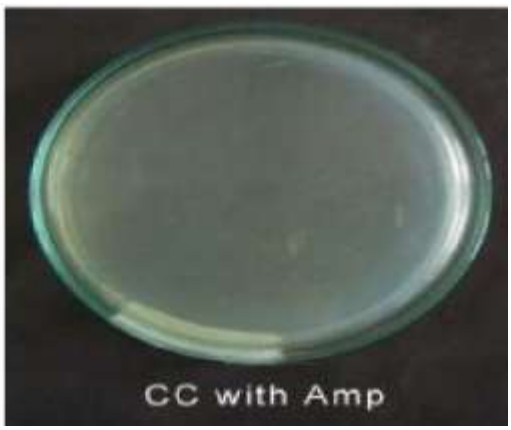

Figure 3: CC with amp

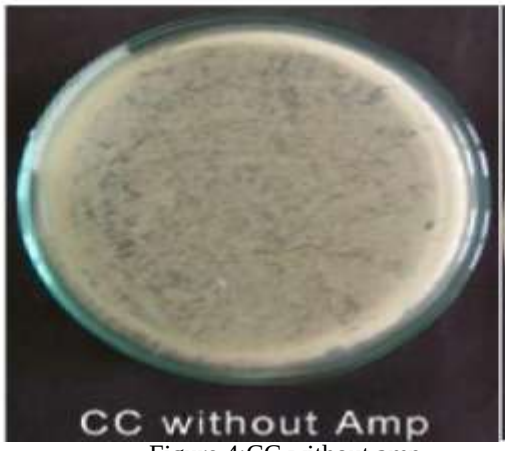

Figure 4:CC without amp

Figure 5: TC with amp

3.3 GST induction :

The after induction(AI) tubes were weighed with pellet and without pellet and calculated.2.5X alumina was added to net pellet weight as shown in TABLE-

TABLE 1: Calculation of GST Induction:

Tubes with pellet (W)	Tubes without pellet (W')	Net pellet weight × 2.5X alumina
W_1=12.98gm	W'_1= 12.71gm	0.27gm
W_2=13.20gm	W'_2= 13.02gm	0.18gm
W_3=13.22gm	W'_3= 12.88gm	0.34gm
W_4=12.70gm	W'_4= 12.33gm	0.37gm

3.4 SDS PAGE and Immunodetection :

IPTG induced GST was collected by cell lyses method (BI & AI) and analyzed by SDS.The purification of protein was done by affinity chromatography.The SDS was performed in two stages.First with marker ,BI,AI and cell lysate samples and second with eluted samples.Here eluted sample is shown in gel from E1 to E7.In Fig. 6, intensity of bands decreased along with wells except E1 as it contained some amount of equilibration buffer. In Fig. 7, Cell lysate showed GST but it had some amounts of other protein.E2 showed sharp band of purified GST compared with Std GST and was confirmed with immunodetection.

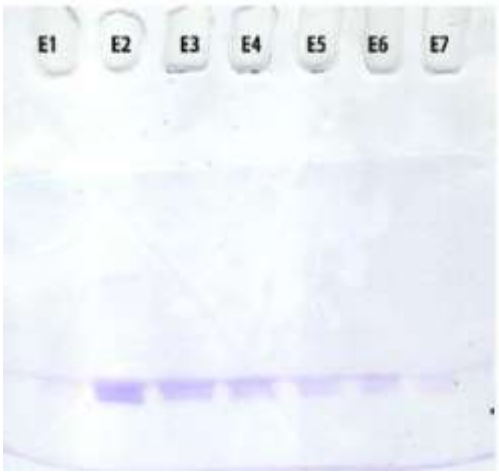

Figure 6:PAGE showing bands of GST sharp In E2.

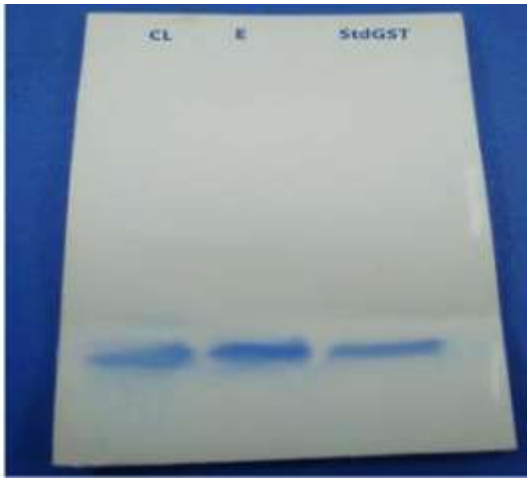

Figure 7:Membrane showing presence of GST protein compared with Std GST.

V. Conclusion

GSTs have been the subject of advance scientific research for about forty years.Recent studies and advances have been made in this topic to address the endogenous functions of GSTs within the cell.Besides their role in metabolism of foreign chemicals such as carcinogens and environmental pollutants as well as the detoxification of potentially harmful endogenously reactive compounds.It has been found to be worked in

intracellular transport of wide spectrum of hydrophobic compounds and modulation of signal transduction pathway. Collectively the catalytic actions of GST isoenzymes contribute to cellular detoxification and to autocrine and paracrine regulatory mechanisms.Besides its advantages the incidence of the most common form of glucose 6-phosphate dehydrogenase deficiency characterized by a tenfold reduction in enzymatic activity in red blood cells.Indeed G 6-P deficiency protects against *f*alciparum malaria because it is very much sensitive to ROS.An antimalarial drug Primaquine is believed to act by causing oxidative stress and causing serious medical problems.Sometimes in many people with normal cellular mechanism the reduced glutathione prevents the drug from being entering into the cell by modulating the channel most likely the MDR1.These people are very much susceptible to malaria.The whole work was to overexpress the protein so that it could be applicable .Recently GSTs have been found to work in signal transduction pathway which can directly target gene and can be helpful in the treatment of medical illness.

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