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EXPRESSION OF GSH1GENE FOR THE PRODUCTION OF GLUTATHIONE IN *E.COLI*

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ABSTRACT

The wild Saccharomyces Cerevisiae consider as a good candidate for synthesizing high quantity of glutathione (red). GSH1 gene from Saccharomyces Cerevisiae was identified and cloned in E.coli expression vector for over expression of glutathione synthetase to leads to increased levels of GSH in cellular extracts and the product purified to confirm its expression. Primers were designed based on entire sequence of Saccharomyces cerevisiae and GC content of the genomic DNA. The PCR was carried out to amplify the template DNA with the primers. The amplified products were inserted in to 1-67&99-346 region of PUC18 used as a cloning vector for the higher expression of glutathione production. In view of comparative analysis of glutathione preparation and standarized the protocol used in various steps involved in the purification process, subsequently identify the product recovered from different samples used in the experiment. The cellular glutathione content extracted in a supernatant of the culture lysate was used for determined of glutathione. The efficiency of GSH production by recombinant E.coli has nearly 40 fold increased its production when compared to wild type strains. The prepared fractions were used for analysis of glutathione also the size of final purified products were checked by SDS PAGE and product should be concentrated by lyophilization process, it is recommended that application of glutathione on protecting freeze dried molecules in various field.

KEY WORDS

Cloning, E.coli , Glutathione, PCR, Saccharomyces Cerevisiae.

INTRODUCTION

Glutathione (γ – Glu-Cys-Gly) GSH is widely distributed in animal tissue, plants and microorganisms. The tripeptide glutathione is the thiol compound present in the highest concentration in cells of all organs. Glutathione has many physiological functions including its involvement in the defense against reactive oxygen species. Consequently, reactive oxygen species are continuously generated during oxidative metabolism will generated in high rates within the brain. Therefore, the detoxification of reactive oxygen species is an essential task within the brain and the involvement of the antioxidant glutathione in such processes is very important [1]. Glutathione is synthesized in vivo by the consecutive action of two enzymes. γ – Glu-Cys synthetase uses glutamate and cysteine as substrates forming the dipeptide γ – Glu-Cys is combined with glycine in a reaction catalyzed by glutathione synthetase to generate GSH. The intracellular level of glutathione is regulated by a feedback inhibition of γ – Glu-Cys synthetase by the end product GSH [2].

Glutathione is a very interesting biomolecule produced by the body and found in every cell, which occurs naturally in many foods and people who eat well probably have enough in their diets. Dietary glutathione occurs in highest amounts in fresh fruits, vegetables and found small amounts in grains [3] . The crucial role of the essential metabolite glutathione in cellular response to oxidative stress and metabolism has been documented [4]. GSH Synthesis proceeds via two ATP dependent steps; the first is the formation of the dipeptide γ – Glu-Cys from

glutamate and cysteine catalyzed by γ- glutamyl cysteine synthetase (GSH1). The GSH1gene encoding γ- glutamyl cysteine synthetase has been cloned from yeast by complementation of a GSH 1 mutation [5]. The final step in GSH synthesis is the ligation of glycine with L- γ – Glu-Cys catalyzed by GSH synthetase (GSH 2). Gene encoding GSH2 have been identified in several bacterial and eukaryotic species [6].

Glutathione is an antioxidant and detoxifying agent whose protective role has been demonstrated in numerous clinical studies. Glutathione is the strongest anticancer agent produced by the body system against illness and aging. Glutathione is a dietary supplement used as an antioxidant to help protect the body from many diseases and conditions. It is also used to treat infertility, cancer, cataract, accelerated aging, and disease of the immune system and detoxify various chemicals from the body. Biochemical studies have elucidated the enzymatic bases of the functions of GSH subsequently there is current interest in modulation of GSH levels during chemotherapy and radiotherapy of tumors including tumors that exhibit "multi drug" resistance. Method has been developed for increasing cellular levels of GSH thereby increased cellular levels protect cells against oxidative damage toxic compounds and radiation's. In the present, GSH1 gene from *Saccharomyces Cerevisiae* was identified and cloned in *E.coli* expression vector for over expression of glutathione synthetase to leads to increased levels of GSH in cellular extracts and the product purified to confirm its expression.

MATERIALS AND METHODS

Strains, Plasmids and Media:

The *Saccharomyces Cerevisiae* strains used in this study (MTCC No. 170) were grown in YPD medium (1%Yeast Extract, 2% Peptone, 2% D-glucose) and solidified by the addition of 2% agar under aerobic condition for 24 hours at 30^0C which was used to isolate GSH1 genes. TheGSH1 gene was isolated from yeast culture by PCR amplification of total yeast DNA with oligonucleotides specific for GSH1 sequence. Escherichia coli DH5α (Stratagene) was used for the cloning, sequencing and maintenance of various DNA fragments. The *E.coli* was grown in LB medium; the required antibiotics were added according to the reference recommendation [7].

CLONING CASSETTE:

Cloning is a basic fundamental process in bioengineering the selected interested gene to be inserted in to the cloning vector with the help of ligase enzymes, vector was constructed with a specific restriction sites. In the present work interested gene of GSH1 was digested with ECOR1&BamH1, and it was inserted in to the 1-67&99-346 region of the PUC18 used as a cloning vector. Now the recombinant plasmid was transformed in to E.coli, in which GSH1 gene was amplified represent the cloning cassette of GSH1gene.

PCR amplification of the GSH1 gene:

The GSH1 gene was isolated by PCR amplification of total yeast DNA with oligonucleotides specific for GSH1 sequences. The genomic DNA of *Saccharomyces Cerevisiae* (MTCC No. 170) has been prepared by CTAB – Proteinase k by the method of [7]. PCR amplification was carried out with the primers and the size of the amplicon was confirmed by 1.2% Agarose gel electrtophoresis. The size of the amplified product was confirmed on compared to λDNA Hind III digest used as a standard marker.

Analysis of Transformants:

Transformed colonies were isolated and grown in sterilized LB medium from this transformed DNA was prepared by CTAB proteinase K method. The size of the transformed DNA was identified on agarose gel when compared to λDNA Hind III digest standard marker.

Preparation of cell extract:

Recombinant *E.coli* extracts were grown in YEPD medium with antibiotics (ampicillin 200 mg/ml) at 37^0 C to an OD 600 of 0.8, after induction with 0.4mM IPTG/ the cultures were incubated at 37^0 C for hrs. After two cycles of centrifugation at 7000 rpm (10 min at 4^0C and re- suspended in 0.01 vol of 0.1 M Tris HCl buffer (pH 7. 5) cells were disrupted in a fresh press at 20000 p.s.i. subsequently cells debris was removed by 30 min centrifugation at 7000 rpm. Protein concentrations of the extracts were determined by the method of Lowry's [8].

Estimation of cellular Glutathione:

The wild *Saccharomyces Cerevisiae* strain, Recombinant *E.Coli* and wild *E.coli* were allowed to grown on the respected medium with a specific temperature for 48 hours.

Grown culture (10ml) was taken at every two hours intervals and centrifuged at 300 rpm under –4° C for 5minutes, this step was applied for the three selected experimental strains. The Cellular Glutathione content extracted in a supernatant of the culture lysate was used for determined of glutathione as per the method of [9]. Total GSH was determined by sedlak and Lindsay (1968) and the GSH1 encodes glutathione synthetase assay was performed according to protocols [10]. For the determination of GSH1 activity formation of the dipeptide γ- glutamyl cysteine was measured by using an amino acid analyzer and is expressed as nmoles of γ- glutamyl cysteine formed per minute per mg of protein.

Sephadex G-100 Column Chromatography:

The sample extracts were containing the glutathione activity subjected to column chromatography by using Sephadex G100. The column is fixed ¼ with buffer and the outlet is opened. Once the column is packed, a layer of the buffer should always be maintained on the gel surface [11]. About 1ml of extract was passed through Sephadex G100 column. Fraction (3ml) having high glutathione content alone was concentrated by lypholization and subjected for next step of purification.

Purification of Glutathione:

Glutathione-uniflow columns are pre packed with 1-ml Glutathione uniflow resin [12] 5x100mg of Glutathione (reduced). 10x Extraction/ Loading buffer compositions as follow: 1.4 mM NaCl, 100 mM Na_2HPO_4, 18 mM KH_2PO_4, pH 7.5. To prepare the extraction / loading buffer, dilute 4ml of 10 xextraction/ loading buffer with 36 ml of deionized water. If necessary warm the diluted buffer to room temperature to make it dissolved the precipitated salts (adjust the pH to 7.5). Elution Buffer: 50 mM Tris base, pH 8.0.Dissolve 100mg glutathione (reduced) in 10ml of the elution buffer and adjust the pH 8.0 if necessary, prepare fresh. Regeneration buffers: **Buffer 1:** 0.1M Tris-HCl (pH 8.5), 0.5M NaCl; **Buffer 2:** 0.1M Sodium acetate, 0.1M NaCl (pH 4.5**),Buffer 3:** 140mM NaCl, 10mM Na_2 HPO_4 (pH 7.5), 1.8mM KH_2PO_4.(Note: Solutions containing Glutathione must be kept at- 4°C or on ice at all times).

The batch/gravity flow protocol can be used with either glutathione. Super flow or uniflow Resins.However the purification protocol is only intended for use with the glutathione super flow resin. Experimental samples were transfer to the pre cooled mortar for homogenized the materials to release glutathione in the lysate.1ml of resin was used for every 100-500mg of cell lysate and added 2ml of precooled extraction buffer per 100-500mg of lysate mixture.(Centrifuge the homogenized mixture in the precooled centrifuge for 20 min at 10,000-12,000rpm. This step can pellet any insoluble material). Carefully transfer the supernatant to the clean, prechilled tube. Do not disturb the pellet. The supernatant was used as clarified sample.

Packing of Glutathione Resin into Disposable gravity columns:

Thoroughly resuspend the glutathione Resin to achieve a homogenous 50% suspension of resin in the storage solution, immediately transferred 2ml of resin suspension to a disposable gravity column. Ensure that the bottom of the column is plugged with a stopper and allowed the resin to settle in the column. Equilibration was done as follow: Remove the stopper and drain the storage from the column. Add 4ml of deionized water to the top of the column and allow it to drain Repeat (Step2) three times.(Do not distrub the resin) Equilibrate the column by adding 4ml of loading buffer, allow the buffer to drain. Repeat (step 4) three times and replaced the column's top and bottom stoppers, Place it on ice to prechill the resin. Selected column where internal diameter is at least 1cm, volumetric flow rate of 0.20ml/min can be used during loading and the recommend bed length of 3cm. Pack the column according to its manufacture's specifications. The linear flow rate is the volumetric flow rate, in ml/men, divided by the area of the cross section of the column. (r^2 Where r in the column radius in cm). The flow rate for washing and eluting can be increased significantly thus, reducing purification time and increasing yield. At a loading linear flow rate of 1cm/min, the capacity for glutathione protein (tripeptide) from selected experimental sample lysates was approximately 0.5 mg per ml of resin. The eluted fractions were used for

analysis of glutathione quantity by the method [9]. The purity of final isolated product should be checked by SDS PAGE and stored the product in lyophilized state.

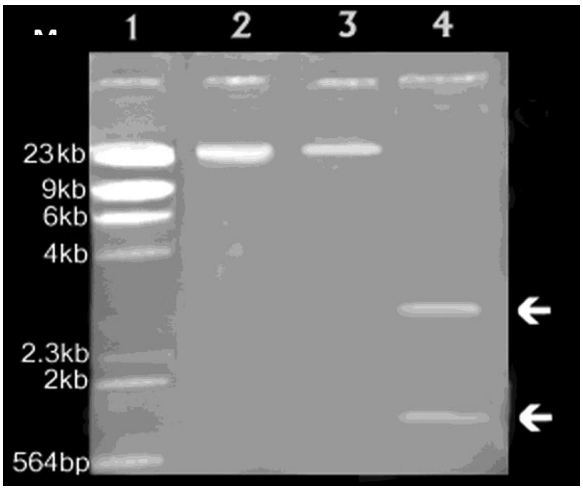
SDS PAGE ANALYSIS

Recombinant E,coli cell lysate purified product was analyzed for the presence of protein by SDS PAGE analysis [13]. The amount of total soluble protein content was estimated by Lowry's method and TSP was separated by 15% SDS PAGE. The separated protein bands were determined by electrophoretically and identified the size of the protein molecule compared with standard marker. The samples were mixed with sample buffer and were boiled for 5 minutes at 100^0C, centrifuged at 3000 rpm for 5 min. The sample volume containing 25-50-µg protein was loaded in the wells. Electrophoresis was carried out at a constant voltage of 100 V across the gel and the size of bands was identified on compared with standard commercial protein marker.

RESULTS AND DISCUSSION

In the present study, experimental samples were selected to recover glutathione from different sources such as recombinant *E.coli* which contain GSH1 gene encoding enzyme in GSH biosynthesis. In view of comparative analysis of glutathione preparation and standarized the protocol used in various steps involved in the purification process, subsequently identify the product recovered from different samples used in the experiment. The wild *Saccharomyces Cerevisiae* consider as a good candidate for synthesizing high quantity of glutathione (red). Genomic DNA of *Saccharomyces Cerevisiae* was prepared from the broth 10ml of culture and purity of the DNA contents were checked measuring the absorbance at 260 & 280nm. The size of the genomic DNA was confirmed by 1.2%Agarose Gel Electrophoresis (**Fig 1**). The size of the DNA was detected on gel with standard molecule of λDNA HindIII digest

Figure 1: ISOLATION, SEPARATION OF GSH1 GENE



LANE 1-λDNA Hind III digest
LANE 2-Genomic DNA of S.Cerevisiae
LANE 3-Genomic DNA of E.Coli
LANE 4-E.CoR1, BamH1 Digested GSH1
The genomic DNA of Saccharomyces cerevisiae was digested with ECOR1 and BamH1, size of fragments (GSH1) was detected on compared with standard markerλDNA Hind III digest.

Figure 2: TRANSFORMATION OF GSH1 IN TO *αDH5* (*E.COLI*)

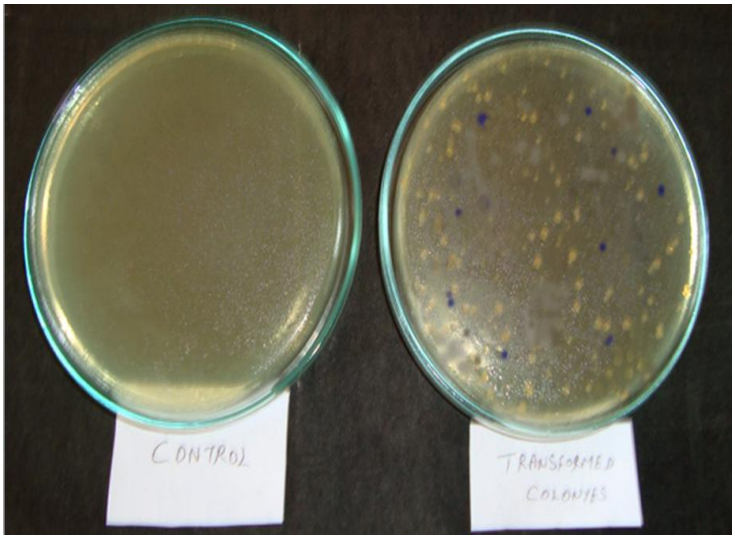


Figure 2: GSH1 gene was transformed through αDH5 used as a vector; the transformant was selected based on blue white selection techniques. White Colonies appeared on the plates were confirmed as transformant and the blue colonies observed as non transformant.

Construction of recombinant *E.coli* (GSH1 gene):

The cloning vector designed to contain GSH1 gene from *Saccharomyces cerevisiae* for the expression in E.coli. The N- terminal amino acids 1-67 and C terminal amino acids at 66-346 for the residue fusion of GSH1 which is digested with ECOR1 & BamH1 and blunt end with T4 DNA polymerase for the production of high quantity of GSH in E.coli. GSH1 gene was isolated from *Saccharomyces cerevisiae* and transformed into α DH5 (*E.coli* strain). Polymerase chain reaction amplification of GSH1 gene was carried out in 50µl volume and contained 100 ng of genomic yeast DNA, 0.5µM of each primer, 0.2mM of each dNTP (pharmacia), 1.5U Taq polymerase and 0.1 volume of 10x Taq buffer supplied with the polymerase. Amplification were performed in a thermal cycler programmed for one cycle of an initial denaturation step at 94^{o}C for 4 minutes annealing at 59^{o}C for 1 minute and an extension step for 1 minute at 72^{o}C at the 30th cycle included 6 minutes to ensure full extension of PCR products. Products were separated electrophoresis in 1% (W/V) agarose gels (**Fig 2**).

PRIMERS

- **5' – CGGGATCCATGAGAGACAGCAAGTTG-3'**

(BamH1 site is underlined)

- **5'GGGAATTCTTAGAGTCTTCCACCGGG-3'**

(EcoR1 site is underlined)

DNA of *Saccharomyces cerevisiae* digested with ECOR1&BamH1 were able to release a specific fragment contains 3000bp and 900bp size. Primers were designed based on entire sequence of *saccharomyces cerevisiae* and GC content of the genomic DNA. The PCR was carried out to amplify the template DNA with the primers. The amplified products were inserted in to 1-67&99-346 region of PUC18 used as a cloning vector for the higher expression of glutathione production.

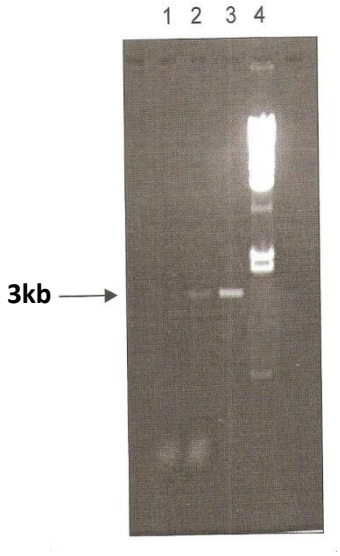
Determination of GSH1 gene in transformant:

Figure 3 shows transformed colonies (1-5 colonies were picked from agar plates) and resuspened in 100 µl of solution containing 10mM Tris-HCl and 1 mM EDTA (pH 8.0) for genomic DNA extraction. Mixture was heated at 95^{o}C for 5 min and removal of cell debris by a brief centrifugation, supernatant (1µl) containing genomic DNA was added to PCR mixture for amplification of 3000bp GSH1 gene using PCR system.

GSH is a tripeptide, low molecular mass compound present in various cells of all living systems mainly in eukaryotic organisms and aerobic gram negative organisms. The biosynthesis of GSH in the cells carried out by two ATP dependent enzymes such as GSH1 &

GSH2. The enzyme GSH1 involved in the first step of GSH synthesis to produce dipeptide, and which catalysed by GSH2 to produce glutathione as a tripeptide.GSH produced in two natures such as extracellular and intracellular also it was produced in two forms oxidized and reduced forms. Normally GSH was stored in reduced form due to its high antioxidant activity. The selected gene of GSH1 was cloned in to *E.coli* to produce high quantity of GSH and the production efficiency of wild type and recombinant *E.coli* was examined (**Fig 3**) when compare the production among these strains, recombinant *E.coli* has the tendency to produce nearly 40 fold increased quantity of GSH on compared to wild type was confirmed based on results on **Fig 4**. Glutathione is an essential metabolite protecting cells against oxidative stress and aging.

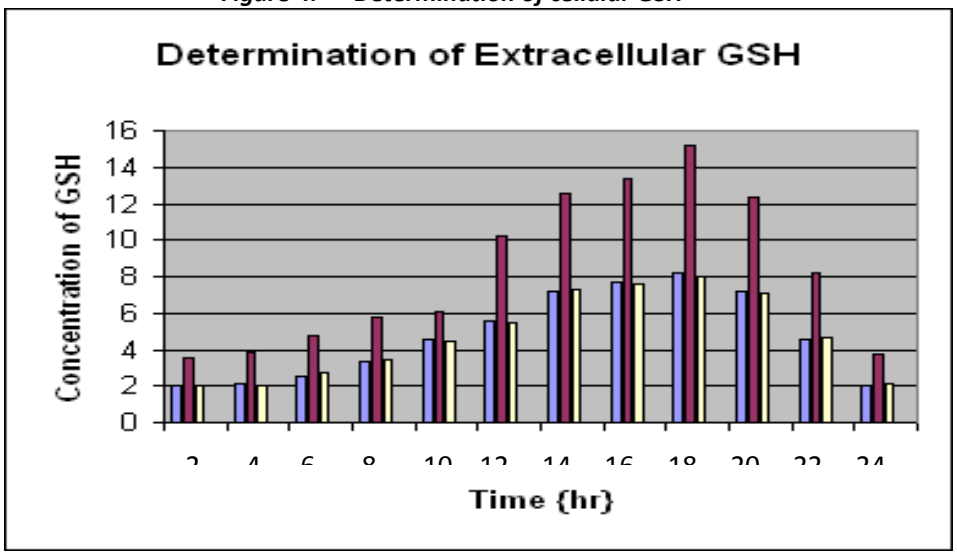
Figure 3:PCR AMPLIFICATION OF GSH1



Lane 1 -Control empty vector
Lane 2 -PCR product Without DNase
Lane 3 -PCR Product (DNase 1 RNase Free)
Lane 4 -λDNA Hind III digest

Gene of GSH1was obtained from Saccharomyces cerevisiae strain and amplified by using appropriate primers and the fragment size was confirmed on compared to standard marker λ DNA Hind III digest.

Figure 4: Determination of cellular GSH



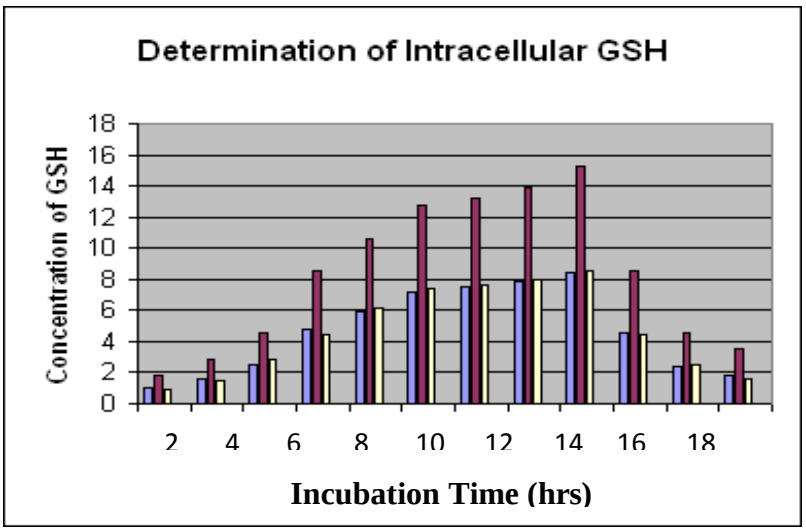


Figure 4 shows the quantity of intra and extra intracellular glutathione prepared from recombinant clone was determined by enzymatic recycling method. Results showed almost 40 fold increased in the production of glutathione (GSH content expressed as µmol /g wt weight) the constructed recombinant E.Coli strains which expressed increased yield of Glutathione were confirmed when compared to wild type strains.
Blue color indicates – Saccharomyces cerevisiae,
Red color represent – Recombinant E.Coli, and
Yellow color indicates – Wild E.Coli

Figure 5: Crude extracts of samples on SDS PAGE

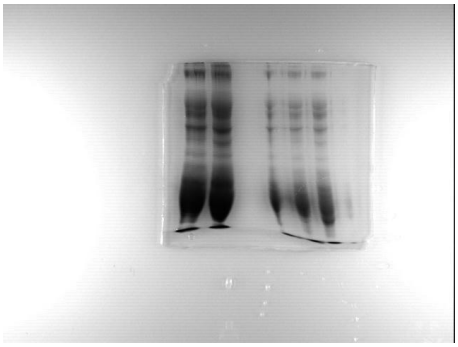


Figure 5: SDS-PAGE gel prepared from the specified fractions (Numbers above the Lanes). The arrow head indicates the 12 KDa protein band whose intensity corresponds to Glutathione activity in individual fraction. Lanes: 1 Crude extract; 2 soluble extract; 3 Gel filtration active fractions; 4 Glutathione uniflow resin column fraction; 5 Disposable gravity column fraction.

Figure 6: Confirmation of purified products on SDS PAGE

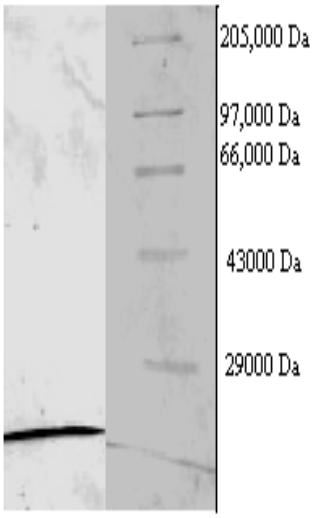


Figure 6 shows the sharp intense bands which indicate the purified molecule present in final eluted fractions could be considered as tripeptide (glutathione) its molecular size may be 12KDa on compared with standard protein marker.
Lane 1: Protein marker
Lane 2: Purified product from recombinant E.Coli.

Expression and Preparation of cell extract:

As stated in materials and methods, Protein was eluted at a flow rate of 14ml / hr. selected fractions were analyzed by SDS–PAGE. The fractions containing the highest Glutathione activity of each fraction was measured (**Fig 5**). The protein concentration also was measured using bovine serum albumin as the standard. Almost all analytical electrophoresis of proteins are carried out under conditions that ensure dissociation of the proteins into their peptide subunits and that minimize aggregation. The purified enzyme was resolved in SDS – PAGE by discontinuous buffer systems. SDS-PAGE analyses of total soluble protein (TSP) extracted from experimental samples an aliquot of 40 µl volume containing 50µg of protein were separated by 12% SDS PAGE and the molecular weight of the extracted product was detected on SDS PAGE gel. Figure 6 revealed showed only a thin band of tripeptide form with the expected molecular mass of near to associated glutathione (12 KDa) molecules. On comparison the size of the product comes close to 12 KDa on SDS-PAGE, only a single peak of tripeptide (has Glutathione activity) was detected at the final stage of purification and the silver stained gels of active fraction at this stage showed only a single band (fig 6). The intracellular concentration of ROS is tightly regulated by multiple defense mechanisms involving ROS scavenging enzymes and small antioxidant molecules [14]. Among these antioxidant systems acting as antioxidant or scavengers are glutathione and GSH, dependent enzymes, which are one of the protective mechanism vs oxidative damage, both in the circulation and in various tissues, including liver [15, 16]. GSH deficiency alone leads to the marked cellular degeneration observed; these effects are prevented by administration of GSH. Oral administration of GSH increases the GSH levels of colon mucous and protects these tissues against GSH might be of the therapeutic value in protecting the gastrointestinal epithelia against toxicity associated with inflammatory disease, oxidative damage, chemotherapy and radiation.

GSH used as a biomarker in the clinical field, involved in several pathologies related to oxidative stress.GSH is an essential metabolite in yeast, being required as a reluctant under normal growth conditions [17]. In addition, these strains are lacking GSH gene or altered in GSH redox state and superoxide anion as well as to the toxic products of lipid hydroperoxides [18] also found a role in the detoxification of endogenously

derived metabolites via a GSH –conjugate [19]. Administration of GSH, a relatively nontoxic way of supplying cysteine moieties as compared to giving cysteine may lead to cellular protectionb[20]. The available evidence indicates that protection always depends on the availability of intracellular GSH. Therefore, application of cellular GSH delivery agents such as GSH esters is advantageous. Administered GSH monoesters are more effective than GSH in protecting against the toxic effects of cisplatin [21]. It is of particular interest the treatment with GSH monoester leads to significant and apparently preferential increased GSH levels.

CONCLUSION

Glutathione is a protective agent in biological systems, the tripeptide, consisting of glutamine-cysteine glycine possesses a reduced thiol may readily reacts with potentially damaging oxidizing agents such as peroxides, that form in aqueous systems. The dipeptide appears to be as good as GSH in terms of its ability to serve as an Antioxidant in protection against hydrogen peroxide. Glu-Cys may act as antioxidant in addition it role as an intermediate in GSH biosynthesis. However molecule in cells extracts has purified these preparations are also sensitive to free radicals. Molecular oxygen dissolved in water can still from reactive oxygen species, through at a much lower rate than in metabolically active cells. As a result, molecules that are capable of donating an electron to a reactive oxygen species will become oxidized. In this way, glutathione molecules are purified for research and therapeutic purposes can slowly degrade over time. The use of protective reducing agent such as glutathione can mitigate oxidation and loss of biologically active molecules.

Glutathione was prepared its aliquots dispensed into glass vials. These aliquots were frozen 1 hrs at-40^{0}C capped with polypropylene caps. These samples were than stored at –70 ^{0}C. Freeze dried glutathione is rehydrated, shows virtually no loss of its reduced state as compared to freshly prepared glutathione. Based on this result, it is recommended that application of glutathione on protecting freeze dried biomolecules, high level of glutathione level will appear to protect against the danger of cancer, premature aging, heart disease, autoimmune disease and chronic illness. Further investigation on this work is scale up of the product and giving the instructions based on the production of glutathione (red) in industries by using the Recombinant *E.coli* which is produced by the above mentioned method. Glutathione produced by the recombinant *E.coli* may be used in the following fields, like pharmaceuticals, cosmetics, food industries, as a drug & biomarker in clinical field.

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