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Cloning and expression of Human glutamic acid decarboxylase (*GAD 65*) gene in *Escherichia coli*

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Abstract

Diabetes is a chronic autoimmune disease characterized by the inability of body to produce or respond to insulin a hormone required by body to burn glucose for energy. Type I *Diabetes mellitus,* also known as Insulin Dependent *Diabetes mellitus* is a most frequent chronic disease of childhood, afflicts 0.2-0.3% of human individuals due to auto immune destruction of insulin secreting pancreatic β cells. GAD65 is the major auto antigen in Insulin Dependent Diabetes Mellitus (IIDM). Thus, this project is aimed at expression of GAD65 in *E. coli*. *GAD65* gene was cloned into pET-28a bacterial expression vector and expression was studied in BL21 DE3 cells. Different parameters of induction like isopropyl-β-D-thiogalactopyranoside (IPTG), temperature, time interval were standardized. The recombinant clones induced with 2 µM of IPTG at 30°C for 4 h at flask level produced the protein upto 537µg/ml. Furthermore, the specificity of the purified recombinant protein was confirmed by western blot analysis using monoclonal antibodies. This work establishes a strategy in *E. coli* for the expression of GAD65 with optimized parameters.

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Introduction

Diabetes mellitus is a disease or disorder that is gaining importance and increasing at the alarming rate, which is also called as hyperglycemia where the blood glucose level is higher than the normal. According to World Health Organization, there are mainly two principle types of diabetes; Type 1 diabetes (juvenile or insulin dependent diabetes) and type 2 diabetes (insulin independent diabetes).Type 1diabetes an autoimmune form of diabetic disorder where the insulin producing β cells of the pancreatic islets are destroyed. It is commonly seen in children and the worldwide numbers of prevalent cases of T1D in children (<15 years) have increased and the estimates indicate that there are almost 500,000 children aged under 15 years with T1D worldwide (Patterson *et al.,* 2014). In this type of diabetes, the autoimmune response occurs against self-antigens which include insulin, intracellular membrane proteins such as GAD, IA2, ZnT8 transporter protein (La Torre and Lernmark, 2010; Wenzlau *et al*., 2009). There is currently no cure for T1D and the only available treatment is insulin therapy. Insulin therapy can lead to hypoglycemia if the doses are not taken properly without monitoring blood glucose level and patients requires two or more injections of insulin daily which is painful. (Alvarez *et al.,* 2013).In this regard, GAD65 a major auto antigen have much potential as an important marker for the prediction, diagnosis and in the prevention of T1D in humans. Autoantibodies to GAD65 are observed months to years before the clinical onset of diabetes and are present in the sera of 70–80% of patients with T1D and this anti-GAD 65 antibodies are now serving as an important marker for the prediction and diagnosis of type 1 diabetes (Jayakrishnan *et al.,* 2011; Oak *et al.,* 2011; Wang *et al.,* 2012).

Glutamic acid decarboxylase is an enzyme which is involved in the production of Gamma amino butyric acid (GABA) by the decarboxylation of glutamate is an inhibitory neurotransmitter in neurons and pancreatic beta cells. This GAD exists in two major protein isoforms. One isoform has a molecular size of 65kDa and is termed GAD 65, while the second one, of 67kDa size, is termed GAD67 (Towns and Pietropaolo, 2011). The GAD65 enzyme isolation in large quantities from the human pancreatic (beta cells) tissue is unrealistic so the expression of GAD 65 enzyme as a recombinant protein in heterologous system is mandatory. The large scale production of GAD 65 enzyme currently involves the use of baculovirus infected Sf9 insect cells and methylo-trophic yeast (Mauch *et al.,* 1993; Moody *et al.,* 1995). However, these expression system are technically, economically demanding and highly vulnerable to contamination.

Number of heterologous biopharmaceutical proteins expressed in *E. coli* which are at commercial level are innumerable when compared to all other hosts system this is because of reasons like, it is inexpensive, offers rapid culture times and the ability to achieve high biomass and high protein yields (Assenberg *et al.,* 2013). *E. coli* expression system remain to be the first choice for laboratory investigations, scaling up activities at commercial level and are useful benchmark for comparison among various expression platforms. The sufficient quantity of soluble and functional recombinant GAD 65 protein is required to carry out any molecular and immunological studies. Poor expression of protein because of cellular toxicity and formation of inclusion bodies are the major hindrance in recombinant protein expression. Therefore, this study was carried out for the optimization of different parameters to enhance GAD 65 protein expression and purification in *E. coli*.

Materials and methods

Bacterial strains, plasmids and growth conditions

The *E. coli* strains DH5α and BL21 (DE3) were used for the cloning and expression experiments. The expression vector pET-28a used in this study was purchased from Novogen, USA. The pTNT vector carrying GAD 65 gene was provided by Ake Lernmark, Professor, Lund University Sweden. The *E. coli* was grown in Luria Bertini Broth (LB) at 37°C with shaking 200rpm or on LB plates solidified with 1.5 w/v agar.

As per the requirement LB broth and agar plates were supplemented with kanamycin in a final concentration of 50 mg/L. The GAD 65 gene was cloned and maintained in pTNT cloning vector. Bacterial expression vector pET-28a was selected to express the gene encoding for GAD 65 protein in *E. coli*. It contains T7 promoter driven expression of recombinant proteins with the addition of a 19 amino acid N-terminal fusion tag containing a 6X His tag followed by a thrombin protease cleavage site. Two stop codons are included in the vector at the C-terminal cloning site with kanamycin resistance (*nptII*) gene as selection marker and poly linker sequence with multiple cloning sites (Fig. 1.). This vector replicates in *E. coli* through it sp BR322 origin of replication. Plasmid DNA was isolated and purified using QIA prep Miniprep Kit (Qiagen, USA) according to the manufacturer protocol.

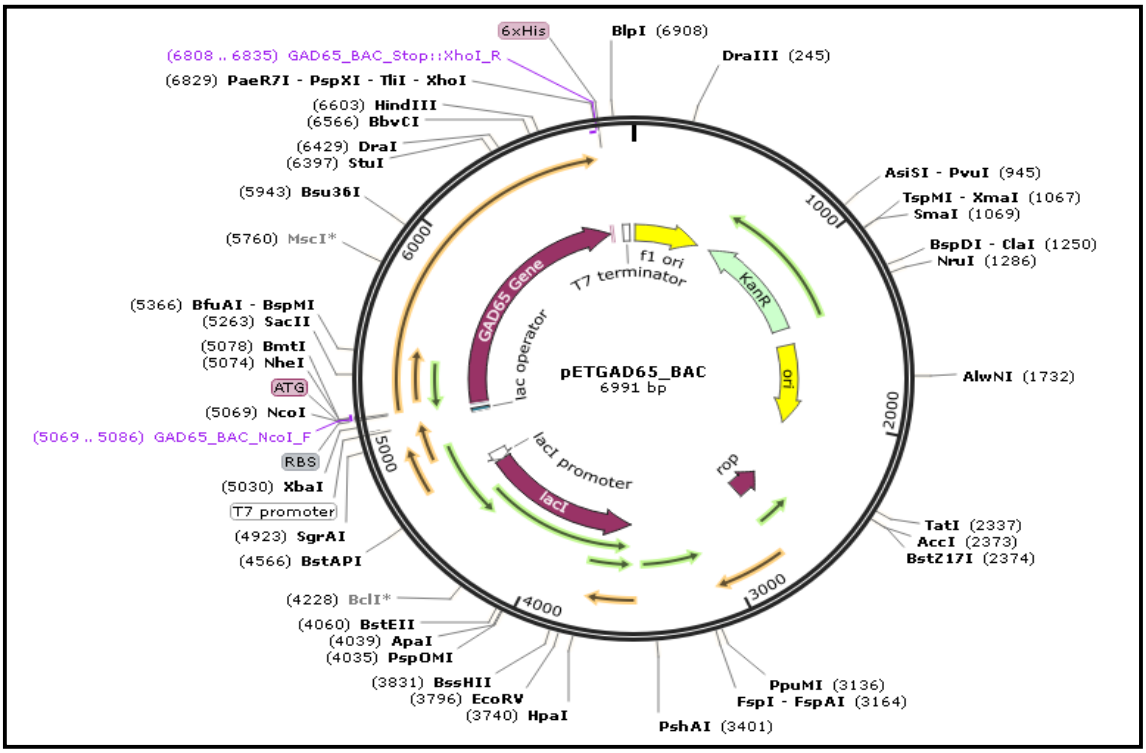

Fig. 1. Vector map of recombinant pET-28a_GAD 65.

Primer designing for the cloning of GAD65 gene

Oligonucleotides primers were designed for PCR amplification of *GAD65* gene. To facilitate the cloning of GAD 65 gene into the expression vector pET-28a, *Nco*I restriction site was inserted in the forward primer (5'-atatcatg CCATGGCTAGCCCAGGCT-3') and *Xho*I site in the reverse primer sequence (5'- ccg CTCGAGTAAATCTTGTCCAAGGCG-3') due to the absence of the two sites in the GAD 65 gene.

Cloning of GAD65 gene into bacterial expression vector pET-28a

The GAD 65 gene in the pTNT vector was used as a template and was amplified with *Pfu* poly meraseusing a pair of gene specific primers *GAD65 XhoI* forward primer and GAD65 *NcoI* reverse primer in 50 µL reaction mixture.

The programme is as follows; Initial denaturation at 98°C for 3 minutes followed by 30 cycles of final denaturation at 98°C for 10 sec, annealing at 60°C for 30 sec and extension at 68°C for 1 min with a final extension for 7 min at 68°C. The PCR product was resolved on 1 per cent agarose gel and the 1.75 Kb GAD 65 gene was eluted from the agarose using Qiagen gel elution kit. The purified PCR product (GAD 65 gene) was restriction digested in 50µL reaction mixture (2µg DNA, *Xho*I and *NcoI*).

The bacterial expression vector pET-28a was restriction digested with *XhoI* and *NcoI*. The pET-28avector carries an N-terminal 6X His tag. The *GAD65* gene was cloned to the upstream of N-terminal coding portion of pET-28a vector (Fig. 1.).

The digested PCR product was ligated into digested pET-28a vector in 1:3 ratio using T_4 DNA ligase enzyme. The ligated mixture was transformed into *E. coli* DH5α competent cells by electroporation as described by Sam brook and Russel (2001). Transform ants were screened using 50 mg/l kanamycin selection.

To confirm the recombinant clones, the colony PCR was done using a pair of gene specific primers. Further these clones were also confirmed by restriction digestion with *NcoI* and *XhoI* and confirmed on 0.8% agarose gel.

GAD 65 protein expression

The *E. coli* BL 21 competent cells were transformed with confirmed recombinant clone as described by Sam brook and Russel (2001) and screened using 50 mg/Lkanamycin selection. The single colony was cultured by inoculating it in 10 mL LB broth containing kanamycin (50 mg/L) and incubated at 37°C overnight. From the overnight grown culture one mL was inoculated into 100 mL LB broth to make 1:100 dilution and incubated at 37°C until the OD reached 0.4-0.6. At this point, different parameters were used to optimize GAD 65 protein expression in *E. coli* i.e., different IPTG concentration (0.1, 0.3, 0.5, 0.7, 1, 1.5, 2.0, 2.5, 3.0 3.5 and 4.0 µM), temperature (25°C and 30°C) and time interval (4, 8, 12, 16 and 18 h). The cells were harvested by centrifugation at 10000 rpm for 15 min at 4°C.

GAD 65 protein extraction and purification

The harvested cells were suspended in lysis buffer [(1X PBS,), 7µL PMSF and 1mg/m Llysozyme] and incubated on ice for 15 min. Cells were lysed by sonication at 200-300 W for 15 min with alternative 10 sec sonication and 10 sec rest on ice. The cells were sonicated and pelleted out at 10000 rpm for 10 min at 4°C. The supernatant was collected and subjected for purification using Ni-NTA agarose column. The column of Ni-NTA agarose was prepared, equilibrated and activated with 1X PBS. The column was loaded with protein sample and flow throw was collected.

The column was washed with 1X PBS to remove unbound foreign or host proteins. Then the GAD 65 protein was eluted from the column using different concentration of imidazole (50, 150,250 mM). The concentration of purified GAD 65 protein isolated from *E. coli* BL 21 cell was measured by using Bradford protein assay (Bradford, 1976).

SDS PAGE and Western blot analysis

The purified GAD 65 protein was analyzed by SDS-PAGE as per the standard procedure of Lamelli (1970). The protein samples were prepared by denaturing the proteins by boiling for 15 min with SDS loading dye. The protein samples were run on 12 per cent acrylamide gel along with standard protein marker. Further the expression of the GAD 65 protein was confirmed by western blot as per the standard protocol of Tow bin *et al.,* 1979. The protein bands were transferred from SDS-PAGE gel to activated PVDF membrane by electro blotting. The unspecific protein binding sites were blocked with blocking buffer containing skimmed milk and incubated at 37°C for 1 h. Then the membrane was washed with PBST and incubated for 1 h at 37°C with GAD65 monoclonal antibody. The binding of the antibody was visualized using goat antibody antimouse Ig G conjugate with peroxidase.

Results and discussion

Generation of recombinant clones of pET-28a_GAD65

The *E. coli* strain DH5αwas transformed with the ligation mixture and the recombinants were grown on kanamycin selection media. The colony PCR resulted in the amplification of *GAD65* gene, amplicon size ~1.75Kb. (Fig. 2.). Further, restriction digestion of recombinant clones with *XhoI* and *NcoI* restriction enzymes resulted in linearization of pET-28a vector (~5.23 kb) and the release of the GAD 65 gene (~1.75Kb). This confirms the proper integration of the gene into the expression vector. Thereafter, clones are designated as recombinant clones (Fig. 3.).

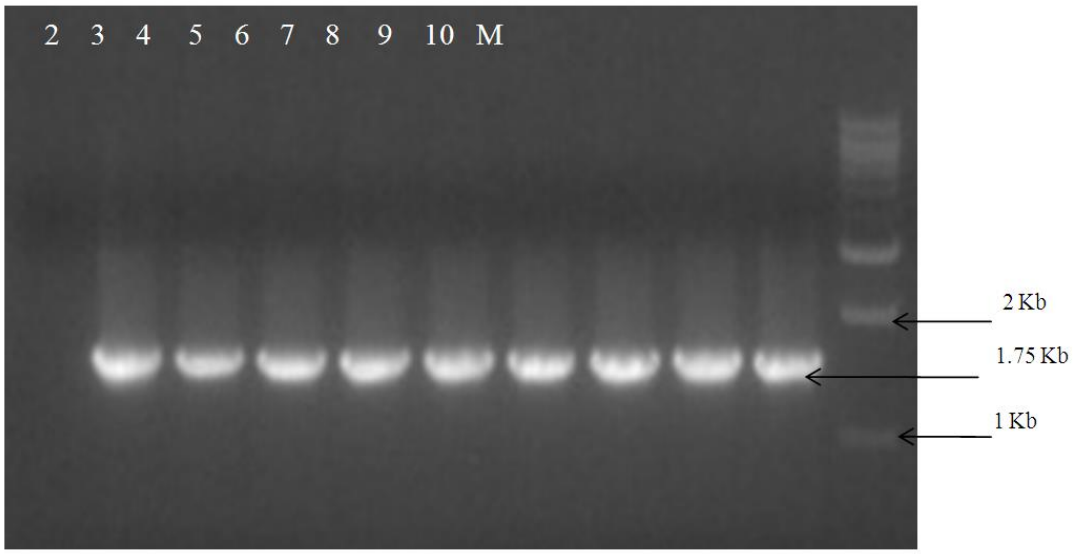

Fig. 2. Colony PCR of transformed colonies of pET-28 a_GAD 65 gene. M: 1 Kb DNA ladder; lane 1: Negative control, lane 2: Positive control; lane 3-10: PCR amplicon of GAD 65 gene.

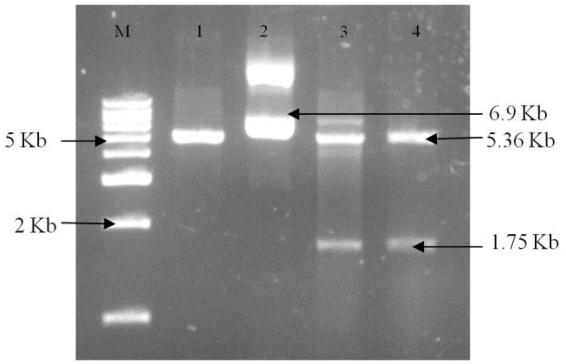

Fig. 3. Confirmation of recombinant pET-28a_GAD 65 gene construct with *Xho*I and *NcoI* restriction digestion. M: 1Kb DNA ladder; Lane 1: undigested pET-28a vector; lane 2: undigested recombinant pET-28a *GAD65* gene construct; lane 3-4: Restriction digestedre combinant pET-28a *GAD65* construct.

Effect of IPTG concentration on gene expression

Different parameters like IPTG concentration, induction temperature and length of induction influences both the solubility and yield of the protein. For the expression vector pET-28a_GAD 65, the foreign protein expression was triggered by adding IPTG into the culture medium. The Fig. 4. showed overall growth pattern of recombinant clones on different concentrations of IPTG ranging from 0.1, 0.3, 0.5, 0.7, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 µM. Although the maximum cell growth was observed at 0.3 to 0.5µ M IPTG.

The optimum growth and maximum expression i.e. 530µg/mL of protein was seen at 2.0 µM IPTG at 30°C after 4 h of induction (Fig. 4.). The decrease in protein expression after 2 µM IPTG concentration is may be due to the fact that further increase in IPTG concentration acts as inhibitory for the bacterial cells. Lower IPTG concentration, lower temperature with longer induction time leads to the slower expression rate of the protein which allows better folding of the protein but reduces yield. The higher IPTG concentration with shorter induction time at low temperature may allow higher properly folded protein expression which leads to increased yields of protein (Tolia and Joshua- Tor, 2006). As the GAD 65 gene was cloned into pET-28a expression vector, it has *lacI* promoter which codes for repressor protein and a T7 promoter which is specific to only T7 *RNA polymerase*. The control of pET expression system is accomplished through the *lac* promoter and operator. Addition of IPTG displaces the repressor from the *lac* operator and induces the expression of T7 RNA polymerase which in turn transcribes the target gene cloned into the pET-28a bacterial expression vector system. By adjusting the concentration of IPTG, expression can be regulated from very low level expression up to the robust, fully induced expression levels commonly associated with pET vectors (German and Eduardo, 2014).

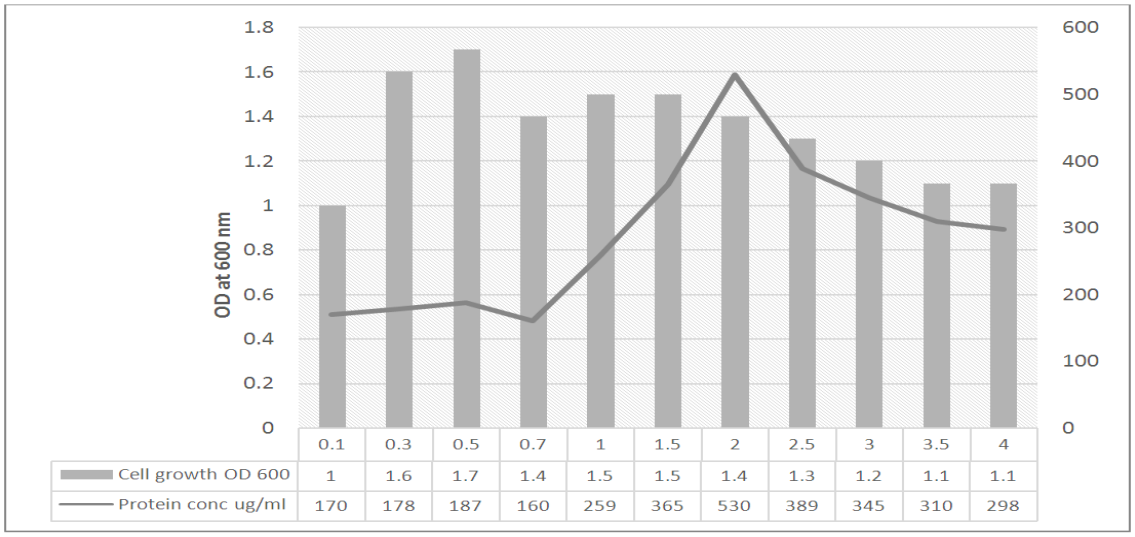

Fig. 4. Effect of IPTG concentrations on bacterial cell growth and GAD 65 protein expression.

Effect of different temperature and time interval on gene expression

The optimum temperature for *E. coli* growth is 37°C but induction for recombinant protein expression are carried out at low temperature to retain the solubility (folding) of the expressed protein. The recombinant clones showed maximum growth at 37°C as compared with the other temperatures. Although the cells grew well at 37°C but the protein expression was not maximum at this temperature. At 25°C with 4, 8, 12, 16, 18 h time interval the protein expression was 259, 391, 498, 250 and 245µg/mL respectively while at 30°C with same length of induction period, the GAD 65 protein expression was 537, 436, 319, 298 and 256 µg/mL respectively. Hence, *E. coli* cells induced at 30°C for 4 h and 25°C for 12 h at 2 µM IPTG concentration showed the highest GAD65 protein expression of 537 µg/mL and 498 µg/mL respectively (Fig. 5.). This may be due to slower expression rate at 25°C when compared to 30°C. The temperature is known to influence the expression of recombinant proteins in *E. coli*. When the temperature decreases, induction time increases because of reducing culture temperature usually leads to slower growth of bacteria and slower rate of protein production (Gong *et al.,* 2009). The bacterial growth at 37°C causes some proteins to accumulate as inclusion bodies, while incubation at 30°C leads to soluble, active protein (Vera *et al.,* 2007).

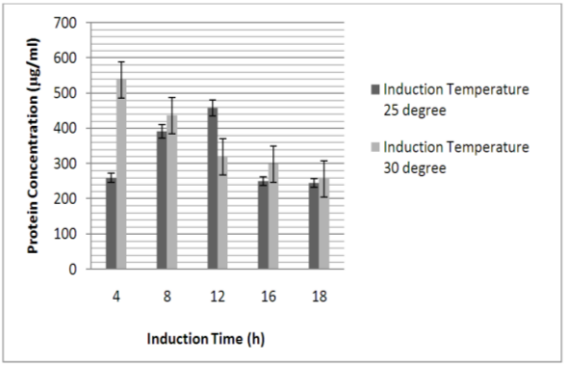

Fig. 5. Effect of different induction temperatures and time intervals on GAD 65 protein expression. The error bars indicate the standard deviation.

Expression and Purification of GAD 65 protein

After the optimization of the different parameters, the histidine tag fused GAD 65 protein expressed in *E. coli* BL 21 cell was extracted and purified by metal affinity chromatography. The purification of protein is essential for any biochemical analysis, therapeutics or structural studies. The GAD 65 protein samples were passed through Ni-NTA agarose column and the optimum elution of GAD65 protein was obtained when 250 mM imidazole was used. Similar result was reported by Papouchado *et al.,* (1997) where they expressed GAD 65 protein as a fusion protein with thioredoxin which helped in the purification of GAD 65 protein. The purified GAD 65 protein expressed in *E. coli* was confirmed by SDS-PAGE where 65 kDa GAD 65 protein band was observed (Fig. 6A.).

Similarly, Rohmah *et al.,* (2013) reported 65 kDa GAD 65 protein band on SDS-PAGE. This may be due to the correct expression and proper folding of GAD65 protein in *E coli*. Further it was confirmed by western blotting using monoclonal antibody. The presence of single 65 kDA protein band on the PVDF membrane confirmed the expression of GAD 65 protein (Fig.6B.).

This is in line with Papouchado *et al.,* (1997) who expressed GAD 65 protein in *E. coli*. The expression and subsequent purification of recombinant proteins are widely employed in biochemical and molecular studies.

A powerful purification method involves the use of peptide affinity tags, which are fused to the protein of interest and used to expedite protein purification *via* affinity chromatography (Bornhorst and Falke, 2000). Hence, immobilized metal-affinity chromatography (IMAC) can be used to purify recombinant proteins containing a short affinity tag consisting of polyhistidine residues, theoredoxin residues etc., and the Ni NTA passed protein could keep its function.

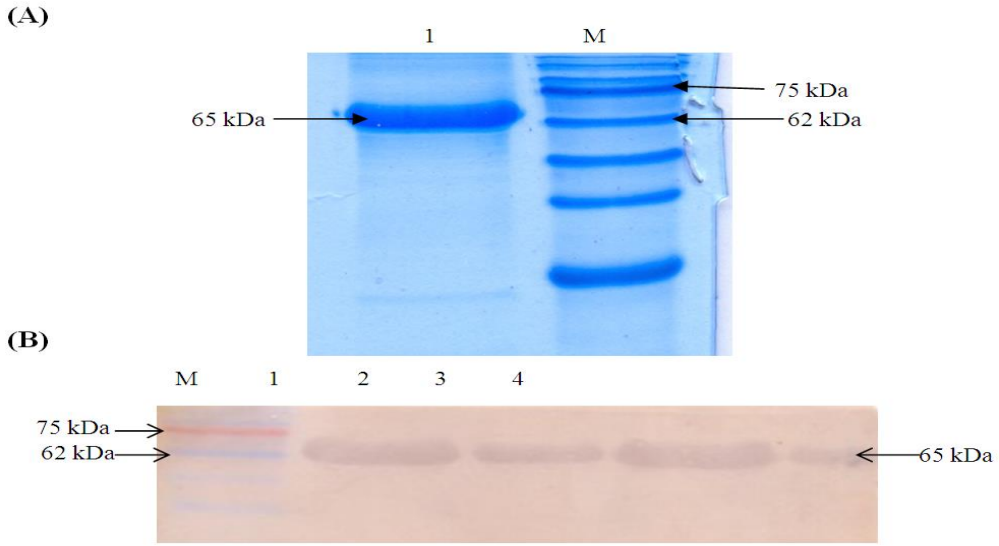

Fig. 6. SDS-PAGE and western blot analysis of recombinant GAD 65 protein expressed in *E. coli*. (A) Lane M: Protein ladder; lane 1: Purified protein sample (B) Western blot analysis. Lane M: Protein ladder; lane 1-4: GAD 65 protein samples expressed in *E. coli*.

In conclusion, the different parameters were standardized for the expression of GAD 65 protein in *E. coli*. The protein produced can be aimed at developing a diagnostic kit for the prediction of insulin-dependent diabetes mellitus (Type-1).

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