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Research Article

Simple method and strategy for biologically active biosynthetic recombinant human insulin protein production in *Escherichia coli*

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ABSTRACT

Simple and time-consuming strategy have placed to produce biologically active biosynthetic recombinant human Insulin in *E.coli* for pharmaceutical use. Starting with simple designing, modeling of the only functional gene of A & B polypeptide chain, joined with only 5 residues that studied well to replace *c-peptide* in function of folding with no effect on the quaternary structure of the protein. Gene optimization have been made using online software (OPTIMIZER) for converting rare codon to abundant codon used by *E.coli* without changing amino acid sequence of the protein. simple strategy of assembly step based on synthesis of two long forward & reverse oligonucleotides covering the full length of designed gene which annealed and amplified using only single PCR program to produce the gene, after that Insulin gene have been incorporated in pTriEX-4 bacterial vector, followed by Chemical transformation in Origami 2(DE3) pLacI competent *E.coli* that support disulfide bond formation,. screening using PCR, restriction enzyme and automated sequencing takes place for confirming the correct in frame gene, expression takes place by flask fermentation using IPTG, then successive purification step using Ni-NTA agarose beads that capture poly (His* tag) to obtain Human Insulin protein purified which indicated and confirmed by SDS-PAGE, western blot and spectrophotometric techniques. Bioactivity Assay performed in diabetic induced mice using streptozotocin (STZ) for increasing blood glucose level, showing lowering of blood glucose level after IM injection by produced and purified insulin, indicate well Biological activity and Biodistribution of the produced Insulin

Key words: *E.coli, Streptozotocin, SDS-PAGE*

INTRODUCTION

Diabetes mellitus, describes a metabolic disorder characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both[1],Most cases of diabetes mellitus fall into three broad categories: a) Insulin-dependent diabetes mellitus (IDDM) also known as type I diabetes, characterized by loss of the insulin-producing beta cells of the islets of Langerhans in the pancreas by a T-Cell mediated Autoimmune attack, leading to insulin deficiency, causes approximately 5 % of diabetes mellitus cases, b) Non–insulin dependent diabetes mellitus (NIDDM) also known as type II diabetes is the most common form of diabetes accounts for about 90% to 95% of all diagnosed cases of diabetes, It characterized by disorders of insulin action and secretion, c) Gestational diabetes mellitus (GDM) is a form of glucose intolerance diagnosed duringpregnancy[2]. Diabetes has been estimated to be the third-largest cause of death in developing and industrialized countries. Human Insulin hormone Secreted from beta cells in the islets of Langerhans in the pancreas, and passes directly into the bloodstream, where regulating the process of carbohydrate metabolism, where Insulin consider the first responsible for the process of adjusting the level of blood glucose, also have many other physiologic function like protein and amino acid metabolism[3,4]. Insulin is a 51-polypeptide hormone consisting of the A chain with 21 a.a. and the B chain with 30 a.a ,and has a molecular weight of 5.8 kDa, linked by two disulfide bridges (Cys7A–Cys7B/Cys20A–Cys19B). Secreted by pancreatic cells. Insulin is biosynthetically derived from the single-chain, 86-residue precursor, named Pro-Insulin with molecular weigh about 9.5 kDa, After

three disulfide bridges have been formed within pro-Insulin, it is converted by proteolytic excision of theinternal 31-residue C-chain, or C-peptide, and 4 residues removed altogether, forming the two-chained (the B chain and A chain) active hormone that remain connected by disulfide bonds, The development of recombinant DNA technology allowed for rapid, high-level production of insulin, and this technology has been widely used to treat diabetes[5]. Recently, in the international diabetes federation survey for ten countries which have diabetes patients worldwide, Egypt occupied the ninth position of 10with 8 million patients; this will grow to be 10 million by 2025. The Arab Emirates Union, Saudi Arabia, Bahrain, Kuwait, Oman States occupied the second, third, fourth, fifth, and sixth positions worldwide, respectively**.** The world population is growing at a rate of 1–2%, the annual reported increase the incidence of insulin-dependent diabetes can be as high as 5–6%.Theincreasing demand for insulin per patient can be 0.5–1gram per year[2]. Insulin discovered in 1921 by Fredrick Banting and Best[6],and was purified and crystallized by five years later[7]. The elucidation of the primary structure was described by Dorothy Crowfoot-Hodgkin[8]determined the spatial conformation of insulin in 1969s[5].The initial approach taken by the scientists at Genentech entailed inserting the nucleotide sequence coding for the human insulin A and B chains into two different *E. coli* cells (K12). It followed by severe all arge companies in the production recombinant version of this hormone at industrial level, such as Novo Nordisk and Eli Lilly[9]. Since this product has economic importance, make us think about starting to produce this protein on lab scale have our own construct, preparing to optimize for large industrial scale to cover the local demand of insulin for diabetic patients, also to cover the surrounding area.

MATERIALS AND METHODS

Modelling and structure prediction of full-length physiologically functional Human Insulin protein.

Addition of five residues as a connection between the two A & Bpolypeptide chains instead of *c-peptide*, according to previous research by Chang in 1998s [9], Also addition of eight Histidine (8*His tag) residuesupstreaming of to the sequence. Protein modelling, designing and structure prediction were achieved using SWISS-MODEL workspace (a web-based environment for protein structure homology modelling) [10,11,12]to compare with both structure of native human Insulin and pro-Insulin that expressed in human body.

Gene design, optimization and synthesis of Human Insulin gene.

Gene optimization of human insulin gene to was performed by online software called OPTIMIZER[13].Optimization performed for replacing rare human codon by abundant one to suit the translation machinery of the *E.coli*While maintaining the same amino acid sequence of Human Insulin gene after translation. With considering the G/C content not to be more than 50 %. Alsoaddition of *EcoRI* sequence (GAATTC) to upstream sequence and *Bgl II* down streaming (AGATCT), for easy sticky end ligation in the selected expression vector *pTriEx-4*(Novagen, USA). Two long oligonucleotides *MIPF & MIPR* (241 mer) covering the whole sequence of the Human Insulin Gene, including the five amino acids joining region between the two peptide chains, and the eight histidine tag (8* His-tag) sequence in the beginning of the sequence, also two short oligonucleotides *GPIF*(5´ TCGGAATTCCATC ATCACCATC3´)& *GPIR*(5´TCCTTTTGCGGCCG CTTATC 3´) have been synthesized complement with the start of the two forward and reverse long sequence, which will be used in the assembly step for building and amplification of the whole sequence of human insulin gene , all Oligo's have been synthesized in (Sigma Oligos, Germany).

Human Insulin gene assembly and amplification using PCR technique.

Fifteen PCR(Polymerase Chain Reaction) reaction have been made with different five annealing temperature (55 - 58 - 62 - 65 - 68 °C), to study the best annealing temperature for the reaction. Required for Assembly & amplification of double stranded DNA fragment of the desired Insulin gene using Single PCR program, which performed in Perkin- Elmer GeneAmp 9700 Thermal Cycler, where*MIPF & MIPR* used as a DNA template to build the gene, with aid of *pfu* DNA polymerase for correct proof reading gene building.

Human Insulin gene transformation, cloning and screening

Double digestion of both synthetic human Insulin gene and pTriEx-4 (5238 bp) Bacterial expression vector via *EcoRI*&*BglII* restriction endonucleases, purified then applied for sticky end ligation overnight in the presence of T4 DNA ligase.Ligation reaction immediately undergoes chemical transformation into *Origami 2(DE3) pLacI*Competent*E.coli*, then plated onto LB agar plate containing 100 mg/L Ampicillin as a selective medium. Only 8 colonies chosen for screening using both PCR technique and the double digestion by the same two restriction endonuclease *Eco RI*&*Bgl II*, to confirm right gene insertion.

Automated sequencing step for the positive results

Five samples were sentto (Beckman Coulter Genomics, UK) for sequencing, to identify the correct in-frame clone. According to the recommended requirements of sample preparation for the sequence by the Beckman Coulter Genomics, the samples placed in 1.5 ml microfuge tube with total concentration of (1.5 µg) in concentration of (100 ng/µl). Two forward and reverse primers were synthesized for automated sequencing, to identify a DNA piece about ~599 bp including our Insert gene of interest, with the sequence of (*S*Tag 18mer Primer* GAACGCCAGCACATGGAC) and (*TriExDOWN*TCGATCTCAGTGGTATTTGTG).

Human Insulin protein expression, production and purification

Chosen *E.coli* strain according to sequencing results that carry the pTriEx-4-HIG and shows correct target gene Insertion, has used to continue for target protein expression and production. Undergoes flask fermentation in LB/Ampicillin medium overnight at 37°C then 1M IPTG added for induction of proteinexpression for 5 hours. After induction cells collected by centrifugation at 4000 rpm under cooling, then resuspended in 0.1M PBS, sonication takes place then cell lysate centrifuged under cooling at 10000 rpm. Supernatant collected and checked for protein expression by SDS-PAGE for both induced and non-induced sample. Ni-NTA Agarose beadsused for purification of histidine containing recombinant insulin, then purified protein undergoes Chemical Cleavage of Protein at Methionyl-X Peptide Bonds using cyanogen bromide to obtain pure functional insulin protein Then purified again using Ni-NTA agarose beads. Identification of purified recombinant human insulin checked by western lot technique then quantifying using (IMPLEN Nano – drop) spectrophotometer. Chemical Cleavage of Protein at Methionyl-X Peptide Bonds using cyanogen bromide to obtain pure functional insulin protein (14). CNBr hydrolyzes peptide bonds at the C-terminus of methionine (Met) residues and leave the target protein alone. In this step collecting the washing material not elute, because it have protein of interest, while the other part is poly histidine with the remain portion of un-desirable sequence will be captured using Ni-NTA agarose beads. Then concentrate using lyophilized technique to obtain pure Insulin protein.

Bioactivity assay of Insulin

Male Swiss albino mice weighing 22-28 gm were housed 5 per cage into 3 group, in room temperature and artificial light from 6.30 am till 19.00 pm, and with controlling of humidity. The animal had free access to food and water. For group No.2 & 3 Diabetes was induced by an IP injection of 200 mg/kg streptozotocin (Sigma Aldrich, Germany) dissolved in 1 % citrate buffer at 4.5 pH, and administrated 5 days before the experiment day[15,16,17], Where group No.1 leave as a control. Blood glucose level measured to all groups at the beginning of the experiment using Glucometer (Roche, Germany), and then measured again after treating with streptozotocin for diabetes induction. Only group No.2 treated with 20 unit of Insulin (Human recombinant insulin which produced by us), and then blood glucose level had measured again after 2, 4, 6 hours.Data collected and exposed to analysis to know the efficiency of recombinant human insulin in lowering blood glucose level in mice.

RESULTS AND DISCUSSION

Designing and Modeling of Biologically active human Insulin

The proposed sequence design of the full length physiologically functional Human Insulin protein after addition of five amino acid as a connection between the two chains A & B, and Also addition of 8 Histidine amino acids up streaming to the sequence will not affect the structure and permit easy way for protein capture when purification with x-His tag bead. The resulted model showing structure similarity in the main domains of the predicted modified protein comparing with both native and pro-insulin model, (Fig.1). Which means that new sequence will not affect the external functional Quaternary structure of the desired protein, and indicating that the modified structure will be the same with native one in configurational structure and binding efficiency to its receptor.

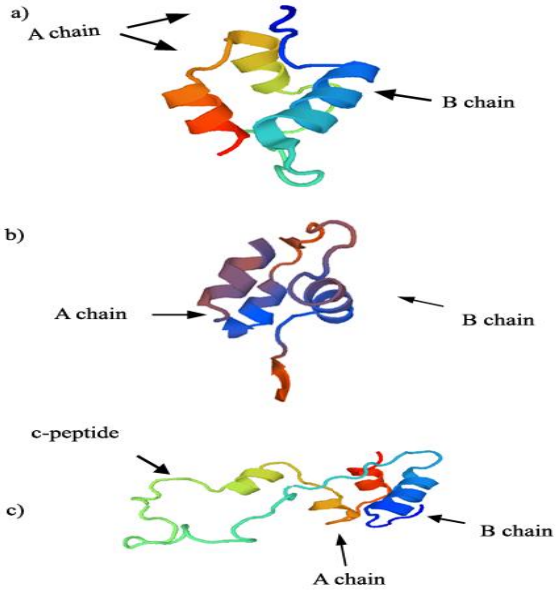

Fig.1: Modeling of predicted structure of. a) Modified Human Insulin, b) native Human Insulin, c) Human pro-Insulin. Where all models show structure similarity in the same active domain site without any distortion

Codon optimization and Gene design.

Computational codon optimization using OPTIMIZER: a web server for online optimizing the codon usage of DNA sequences, that convert the amino acid sequence of the desired modified Human Insulin polypeptide into Nucleotides sequence codons that are abundant use by *E.coli* to obtain the most suitable Human Insulin gene sequence of nucleotide for 60 amino acids residues of the target protein , with addition of start codon ATG of the methionine amino acid at the beginning of the sequence, and sequence of eight Histidine residue (8*His tag), also the connection sequence for *Eco RI &Bgl II* for easy sticky end ligation in the selected expression vector pTriEx-4, also addition of suitable two strong successive stop codon TGATAA down-streaming to the sequence (Fig.2).

Assembly and amplification results of the desired gene

Since it's the first time to do such PCR to this new sequence gene, so that no previous literature for the

annealing temperature T_m of the reaction where give the most and the best amplification result, Also we don't know if it possible to make the assembly and amplification using single DNA strand or must doing that step with the two long Oligo's

Fig.2: Diagram showing the components of the modeled insulin gene.

So fifteen PCR reaction have been made with different five annealing temperature (55 - 58 - 62 - 65 - 68 °C), to study the best annealing temperature for the reaction. For each individual annealing temperature three PCR reaction had made, all three reactions used two short primers with *MIPF*, *MIPR* and *MIPF/MIPR* respectively. False bands and bad results observed when using each of oligonucleotide singularly as a DNA template, even making reaction in five different annealing temperature, while when using the two long oligonucleotides, a very good results observed by presence of distinct DNA bands at the desired MW at about 241 bpsee (fig.3), These good results had given with all different Five annealing temperature. Fantastic good results obtained when using the two oligonucleotides MIPF/MIPR in the same reaction, all five reaction give the same good results although difference in annealing temperature (55 - 58 - 62 - 65 – 68) °C, this shown obviously in (fig.4),where all bands are sharp at about 241 bp comparing with the DNA ladder, this consider very good beginning step towards building a correct gene for recombination to plasmid.

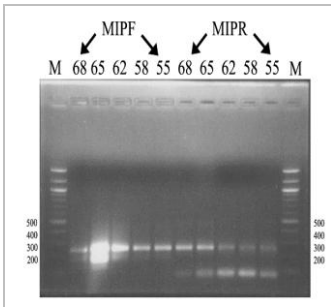

Fig.3: 1.5% Agarose gel electrophoresis showing results of assembly and amplification step using only one of each long oligonucleotides (MIPF or MIPR) as a template with different annealing temperature (68, 65, 62, 58, 55),, M is the DNA marker 100 bp range.

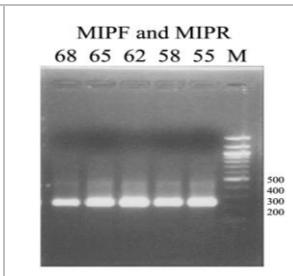

Fig.4: 1.5% Agarose gel electrophoresis showing results of assembly and amplification step using both long oligonucleotides (MIPF and MIPR) as a template with different annealing temperature (68, 65, 62, 58, 55), M is the DNA marker 100 bp range

Human Insulin Gene transformation and screening results

Transparent White colonies appears on LB plates that used in the plating of the transformation reaction for E.coli transfected by pTriEx-4-HIG vector (~5475 bp) (fig.5),with Large Number of colonies > 500/plate Which means good transformation and cloning in number and efficiency due to strong promotor used and well-chosen expression Bacterial host system. Eight colonies selected for screening , sub-cultured separately, and undergoes miniprep for plasmid isolation, then allowed for test of having the correct insert or not via three ways; 1) Plasmid DNA Digestion using Restriction Enzymes , 2) Target gene Human Insulin Gene detection using PCR technique. 3) Automated sequencing technique. Digestion of Isolated pTriEx-4-HIG plasmid from transformed *E.coli* using the *EcoRI&BglII*, resulting in two mathematically predictable DNA fragments (fig.6) the upper is for the digested pTriEx-4 (~ 5200 bp) plasmid while the lower is for the Inserted gene (~ 225 bp). Which indicate correct Gene Insertion. The second method by Performing PCR (polymerase chain reaction) for the 8 sample from the purified pTriEx-4-HIGvectors carrying Insulin gene using outermost forward and backward short primers *GPIF& GPIR*, it gives a distinct right DNA band at about 241 bp showed in (fig.7) These results assured and indicate that gene insertion were in the correct frame site. Automated sequencing that made for five of the eight selected colonies (data not shown) is an important step to assure the success in placing the desired gene in the frame without shifting, alteration in nucleotide sequence or mutations that may occurred during replication. Sequencing results showing that three of five samples sent to test in (Beckman Coulter Genomics, UK) have the correct in frame Insulin Gene.

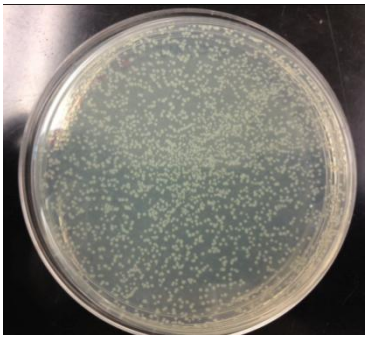

Fig.5: White transparent colonies with large numbers appear in the LB/Amp plates, as a result of transformation and cloning of pTriEx-4-HIG in Origami 2(DE3)pLacI Chemically Competent *E.coli*

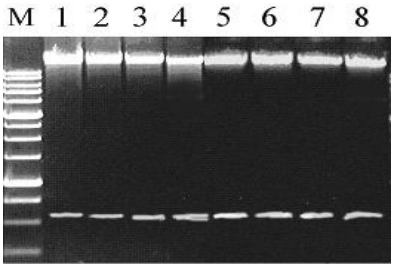

Fig.6: 1.5 % Agarose gel showing results of pTriEx-4-HIG digestion with *Eco RI &Bgl II*, giving Two Fragments the upper for the plasmid while the lower for the inserted gene.

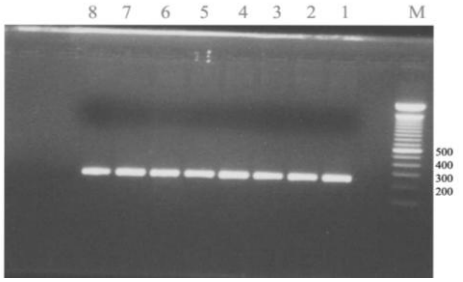

Fig.7: 1.5 % Agarose gel showing results of pTriEx-4-HIG PCR with *GPIF& GPIR* primers, giving one distinct DNA at 241 bp.

Human Insulin protein expression, purification and detection

Flask fermentation have been takes place for one of the succeeded clones in sequencing. Then bacterial cells after induction for 5 hours by IPTG subjected to analyze to test the protein profile content of the cells, SDS-PAGE performed for non-induced, induced and sample after purification via Ni-NTA agarose beads, which will survive only the His-Tag protein and getting rid of all other bacterial proteins. Results come satisfied to us because large expected protein band have been observed in the same position that calculated theoretically before at about 10.8 kDa (fig.8). Expected band at 6.92 kDa which consider the pure Insulin protein which modelled by us, obtained after cleavage with CNBr (fig.9). The reaction is highly specific, with few side reactions and a typical yield of 90–100%. It is also relatively simple and adaptable to large or small scale. Because methionine is one of the least abundant amino acids. (Table.1) represent the sequence of expressed protein before and after cleavage with CNBr. Cleavage reactions undergoes 2nd purification step by Ni-NTA agarose beads again, this time not for capturing the target protein, but to leave target protein goes down with washing where the undesired portion contacting his-tag which attached to beads. Washing solution containing target Human Insulin protein collected and lyophilized and protein quantification had been takes place using UV spectrophotometer at wavelength 280 nm for direct quantification of Protein, Protein Sample concentration was about 10 mg/ml, of pure expressed recombinant Human Insulin.

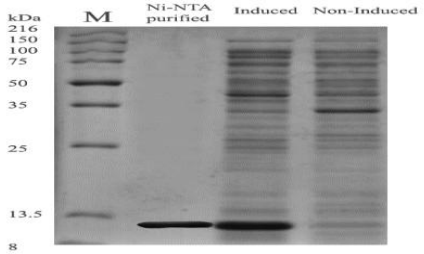

Fig.8: 15 % SDS-PAGE showing appearance of our target protein band at about 10.8 kDa, which become dense in after induction with IPTG and the single band in the same position of our target protein after Ni-NTA agarose beads for His-Tag proteins purification, while M in the left lane for the protein marker.

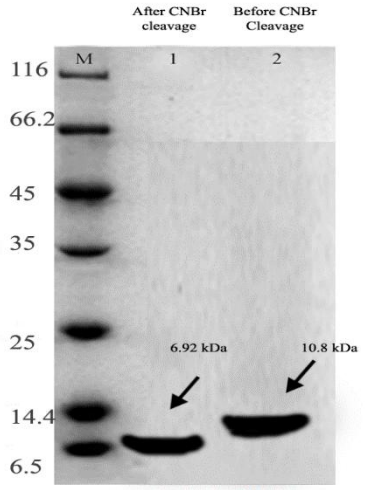

Fig.9:15 % SDS-PAGE showing purified recombinant Human insulin Protein before and after cleavage with CNBr, to remove all non-insulin part from the expressed protein.

Table.1: Represent the sequence of expressed protein before and after cleavage with CNBr.

Before CNBr cleavage Mw ~ 10.8 kDa	MTTTRVRASPQRYLSSWIRSEF**HHHHHHHH**MFVNQHLCGSHLVEALYLVCGERGFFYTPKTRRYPGDVKRGIVEQCCTSICSLYQLENYCN
After CNBr cleavage Mw ~ 6.92 kDa	FVNQHLCGSHLVEALYLVCGERGFFYTPKTRRYPGDVKRGIVEQCCTSICSLYQLENYCN

Bioactivity Assay

Blood glucose level measurement for all three groups in mice at the beginning of the experiment was (96 ± 4.5 mg/dl), where after treatment of group No.2&3 with injection with streptozotocin to induce diabetes in mice the blood glucose level increased to (325 ± 15 mg/dl). Treating group No.2 with our product of recombinant Human insulin that produced in *E.coli* lowering blood glucose level to (150 ± 12 mg/dl) after 2 hour from injection with insulin**,** which indicate positive effect of our insulin in treatment and lowering blood glucose level in diabetic mice (table.2).

Table .2: Represents value of Blood glucose levels during experiment step.

	Before experiment	**After injection with STZ**	**2 hour After treating with Insulin**	**4 hour After treating with Insulin**	**6 hour After treating with Insulin**
Group 1* (n=5)	92± 3				
Group 2 (n=5)	98± 2	340± 15	152± 8	175± 5	199± 10
Group 3 (n=5)	97± 1	315± 12	295± 16	312± 12	322± 14

****Group No.1 control., *Group No.2 treated with Insulin after Diabetes induction., * Group No.3 left without treatment., *n= number of mice per group.***

CONCLUSION

Increased world demand for the human insulin protein, requiring highly & efficient productive systems; *E.coli* based production of the human insulin protein offering several advantages such as relatively high correct cloned vector number per bacterium and high yield of the secreted recombinant proteins in addition to low cost production process, Goeddel[18] in 1979s and its working team following the precursor protein approach used for somatostatin by Itakura in 1977s[19] ,in Synthesis genes for human Insulin A and B chains, which cloned separately in plasmid pBR322. The cloned synthetic genes then fused to *Escherichia coliβ*-galactosidase gene to provide efficient transcription and translation and a stable precursor protein. The Insulin peptides cleaved from *β*-galactosidase, purified. Complete purification of the A chain and partial purification of the B chain were achieved. These products mixed, reduced, and re-oxidized. The presence of Insulin detected by radioimmunoassay. Although it was the first trial to clone and express the coding sequence of chain A and chain B of the functional human insulin protein, but a lot of disadvantages of this methods summarized in many point; a) Depending upon chemical ligation in rejoining separate parts of A and B chain which do give correct ligated yield no exceeding the 50 %, b) Separate purification process of peptide sequence for both chain A and B which increasing the cost of the manufacturing process, c) oxidizing step that made to rejoin the chain A and chain B via forming the intra-molecular disulphide bond between each of them, that will lower the yield of the final rejoined product than the starting material also affect the functionality of produced protein, d) using radioimmunoassay to detect the chain A and chain B inserted sequence of DNA and also the expressed protein, because PCR technique were not developed yet which could facilitate the detection process. Goeddel's trial followed by many trials through the next three decades to produce insulin, mainly depending upon recombinant technique but all tries to improve once by changing the host to higher like yeast, insect cells or mammalian cells, and another changing strategy of cloning by cloning whole proinsulin gene, then digest the c-peptide after purification. Our research aims to facilitate the recombinant protein production strategy especially small protein like human insulin, by designing whole functional protein accompanied with codon optimization using advanced bioinformatical software, building the gene requires only two long oligonucleotides which overlapped together in two successive specific PCR program producing the gene of interest, then insertion in pTriEX-4 bacterial vector followed by chemical transformation in Origami 2(DE3) pLacI chemical competent *E.coli* that support disulfide bond formation, screening using PCR, restriction enzyme and automated sequencing takes place for confirming the correct in frame gene. One of positive colonies suffering flask fermentation for protein production under IPTG stress, produced insulin detected by SDS-PAGE, western blot and bioactivity tested in diabetic induced mice, which show lowering in blood glucose level after IM injection by produced and purified insulin, indicate well Biological activity and Biodistribution of the produced Insulin. Now we have our own construct of human insulin protein that can be used for treating diabetic patients where they need insulin for treatment, this achieved by simple methods can be used to produce other proteins with the same strategy with easily and with low cost method.

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