Cloning and Expression of Recombinant Human Insulin Gene in

*Pichia pastoris*

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**ABSTRACT**

The main goal of the current study was cloning and expression of the human insulin gene in *Pichia pastoris* expression system, using genetic engineering techniques and its treatment application. Total RNA was purified from fresh normal human pancreatic tissue. RNA of good quality was chosen to obtain a first single strand cDNA. Human preproinsulin gene was amplified from cDNA strand, by using two sets of specific primers contain EcoRI and NotI restriction sites. The amplified preproinsulin gene fragment was double digested with EcoRI and NotI restriction enzymes, then inserted into *pPIC9K* expression vector. The new *pPIC9K-hpi* constructive expression vector was transformed by the heat-shock method into the *E. coli* DH5α competent cells. *pPic9k – hpi*, which was propagated in the positive transformant *E. coli* cells, was isolated from cells and then linearised by restriction enzyme *SalI*, then transformed into *Pichia pastoris* GS115 using electroporation method. Genomic DNA of His+ transformants cell was extracted and used as a template for PCR analysis. The results showed, that the *pPic9k – hpi* was successfully integrated into the *P. pastoris* genome, for selected *His*+ transformers clones on the anticipated band at 330 bp, which is corresponded to the theoretical molecular size of the human insulin gene. To follow the insulin expression in transformans, Tricine–SDS gel electrophoresis and Western blot analysis were conducted. The results showed a successful expression of recombinant protein was detected by the presence of a single major band with about (5.8 KDa) on the gel. These bands correspond well with the size of human insulin with the theoretical molecular weight (5.8 KDa).

**Keywords:** Recombinant Human insulin, *Pichia Pastoris*, cloning, Tricine SDS- page.

**INTRODUCTION**

The use of modern recombinant DNA technology permits the synthesis and purification of human proteins in heterologous cell systems, which are potentially useful for many medical applications. Many proteins such as human insulin cannot be obtained from their natural sources in suitable quantities, because of their low abundance or difficulty of purification by conventional methods from human tissue samples, organs or cell lines. Recombinant DNA technology enabled the production of large quantities of protein drugs as cost-efficient sources; these proteins perform the same function as naturally occurring proteins in body. Proteins started being used as pharmaceuticals in the 1920s, with insulin extracted from pig pancreas. In the early 1980s, human insulin was prepared in recombinant bacteria and this protein, which proved of better quality than the conventional insulin, recombinant human insulin is almost exact to natural human insulin, and has completely replaced animal insulin because animal insulin is a slower rate of absorption into the bloodstream. It causes Immunological complications, allergy has been particularly common, with local symptoms still occurring in ~5% of patients, it is less effective, and it is expensive to produce. The human insulin gene (*INS*) is located on the short arm of chromosome (11p15.5), which is the homologous to chromosomes 1 and 7 in mice and rats respectively. This gene contains three expressed sequences (exons) separated by two intervening sequences (introns) or IVS. This gene encodes a 1430-nucleotide insulin messenger RNA precursor, which contains two intervening sequences of 179 and 786 nucleotides that are excised from the precursor to generate the insulin messenger RNA molecule. The insulin messenger RNA directs the synthesis of the insulin precursor protein, pre-proinsulin. This manuscript is concerned with the chromosome localization of the human insulin gene. The three exons code for the signal peptide, the B-chain, C-peptide, and the A-chain of the insulin molecule. Human insulin is naturally occurring polypeptide hormone, with a molecular weight of about 5,800 KD, it is secreted by the beta cells of the islets of Langerhans from the pancreas in response to increased levels of glucose in the blood, as well as to the parasympathetic nervous system and other stimuli. Mature insulin consists of 51 amino acid residues organized in two polypeptide chains A and B chains interconnected via two disulphide bridges chain, A chain consists of 21 residues with an extra disulphide bond between A6 and A11, B chain consists of 30 amino acids. Insulin structure is highly conserved among vertebrates, which are showed by its high degree of

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homology. Porcine insulin has a single amino acid variation from the human variety (alanine substituting threonine in position B30), while bovine insulin has three amino acid variations) alanine instead of threonine in positions A8 and B30 and valine instead of leucine in position A10°.

Figure 1: Agarose gel electrophoresis for total RNA isolated from pancreatic tissue was fractionated on 1% agarose. lane 1,2,3,4 and 5 the bands of 28S and 18S rRNA.

Figure 2: Amplification of human preproinsulin gene by RT-PCR. The products were fractionated on 1.5 % agarose gel electrophoresis visualized under U.V light after staining with Ethidum bromide. Lane M: DNA marker 2000 bp. molecular weight standard (2000, 1000, 750, 500, 250, and 100) Lane 2-5: preproinsulin gene.

Figure 3: Agarose gel electrophoresis of constructive expression vector pPIC9K-hpi fractionated on 1% agarose gel electrophoresi Lane M: DNA marker 15000 bp. molecular weight standard (15000, 10000, 7500, 5000, 2500, 1000, 250). Lanes 2,3,4,5,6 7 and 8: the size of pPIC9K P. pastoris expression vector which containing the PCRproduct amplified preproinsulin gene.
Figure 3: Agarose gel electrophoresis of Linearised hpi- pPic9k Plasmid with SalI. DNA was fractionated on 1% agarose gel electrophoresis Lane M: DNA marker 15000 bp, molecular weight standard (15000, 10000, 7500, 5000, 2500, 1000, 250). Lanes 2, 3, 4 and 5: band of Linearised pPIC9K.

Figure 3.12: Agarose gel electrophoresis of DNA isolated from His+ transformants grown on higher concentrations of geneticin G418 antibiotic, DNA was fractionated on 1.5% agarose gel electrophoresis visualized under U.V light after staining with Ethidum bromide. Lane M: DNA marker 2000 bp molecular weight standard (2000, 1000, 750, 500, 250, 100) Lane 2-5: His+ selected transformants.

Figure 3.13: Tricine–SDS–PAGE analysis of the supernatant from fermentation of recombinant P. pastoris. Samples were collected at 24 h intervals after methanol induction, analysed by 12% Tricine-SDS-PAGE reducing gel and subsequently stained with Coomassie brilliant blue R-250. Lane 1 = Uninduced Pichia pastoris GS115 Lane M = Standard molecular weight protein marker (200, 116.5, 97.2, 66.4, 29.0, 20.1, 14.3, and 6.5 KD). Lane 2 = Induced recombinant Pichia pastoris GS115 (24 hrs) Lane 3 = Induced recombinant Pichia pastoris GS115 (48 hrs) Lane 4 = Induced recombinant Pichia pastoris GS115 (72 hrs) Lane 5 = Uninduced recombinant Pichia pastoris GS115 Lane 6 = Uninduced recombinant Pichia pastoris GS115 Lane 7 = Uninduced recombinant Pichia pastoris GS115.
Isolation of Total Cellular RNA from Human pancreatic tissue

Normal human pancreatic tissue of a brain patient was provided from the Cancer center for organ recovery and education resource (C.O.R.E). Fudan medical university, the organ was obtained from deceased healthy multi-organ donor with the ages of 38 who consented to research use. The sample was immediately frozen in liquid nitrogen and then kept at -80°C, total RNA was extracted by trizol method according to Sambrook & Russell 2011, using total RNA Qiagene RNeasy extraction mini kit according to manufacturer’s protocol. Concentration and purity of eluted RNA were determined by measuring their absorbance at 260 (A260) and 280 (A280) using DU-640 nucleic acid and protein analyzer (Backmen,USA). Total pancreatic RNA integrity was evaluated by agarose gel electrophoresis, the quality of the isolated total RNA was assayed by visualization of discrete 28S, 18S and 5S rRNAs. Through 0.8% agarose gel, according to the instructions.

PCR amplification of human preproinsulin gene

The amplification of the human preproinsulin gene was carried out by using the first-strand cDNA as a template using non - proofreading thermostable DNA polymerase, Nova Taq. DNA polymerase, and two sets of specific primers were used the forward primer was 5’TAC GAATTC ATG GCC CTT GGG ATG CGC CTG -3’ and the reverse primer was 5’ -CAT GCGGCC GTT GCA GTA GTT CTC CAG CTG -3’. Primers contain EcoRI and NotI restriction sites on the 5’ends for amplification of human preproinsulin fragment. Construction of human preproinsulin into expression vector

The modified pPIC9K expression vector used for ligation of hpi gene was provided from (invitrogene). The vector employed for this study containing the a-factor secretion signal directed release of the recombinant protein into the culture supernatant media, and has an N-terminal flag-tag for immunodetection and a stretch of ten consecutive histidine residues as an affinity handle. The vector and the PCR amplified gene double restricted with EcoRI and NotI enzymes. Both the restricted products were cleaned up using (Axygen) gel clean up kit. The product obtained after cleanup is checked on 1 % agarose gel for sizes, and ligation reactions was carried out by the hpi into the EcoRI and NotI sites of pPIC9K to produce the expression plasmid, in which the hip gene inserted in front of under methanol-inducible alcohol oxidase (AOX1) promoter and is under its control. The gene insertions at the EcoRI site of pPIC9K were used to generate an in-frame protein fusion of the inserted DNA with the Saccharomyces cerevisiae α-mating factor signal sequence to enable the secretion of recombinant protein.

Transformation of competent cells with pPic9k

In the present work, the strain of E. coli DH5α competent cells were transformed by the heat-shock method as described by11. Expression vector, pPic9k- hpi which was propagated in the positive transformant E. coli, was isolated from cells and linearised using the restriction enzyme Sall prior to the transformation process to increase integration through recombination events. Pichia pastoris GS115 was used for the transformation procedure with recombinant plasmid by the electroporation method as described by12. This strain
has a mutation in histidinol dehydrogenase (his4) that prevents it from synthesizing histidine
Screening and expression of Recombinant Protein in \textit{P. pastoris} strains
The genomic DNA was extracted from His+ transformants and used as template for PCR analysis to
confirm whether or not the \textit{P. pastoris} transformants contain \textit{pPic9k- hpi} plasmid expression vector according to Linder 1996.

The reaction cycle was carried out under the following conditions, started at 94°C for 5 min followed by 30
cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 1 min and a
final extension at 72°C for 5 min. PCR products were purified by using PCR purification Kit, (Axygen, china).
Successful PCR amplification was confirmed by electrophoresis on a 1.0% agarose gel
Recombinant protein expression was assessed by performing small scale expression studies using a single
colony able to grow on MD plates without histidine supplement. Tricine - Sodium dodecyl sulfate
polyacrylamide gel electrophoresis was employed to follow the insulin expression in cultures, as described by
Schagger and von15, after that Western blot analysis was performed according to the procedure described by
Bjerrum16.

RESULTS AND DISCUSSION
Extraction of total RNA from human pancreatic tissue
Extraction of total RNA pancreatic cells was prepared by
homogenization of normal human pancreatic tissue of a
brain death healthy multiorgan donor patient. These cells
represent the specific cells type which expresses a
characteristic set of gene (that is preproinsulin genes) and
mRNA preparation from a particular tissue usually
contains some specific sequence of higher abundance.
Total RNA was extracted directly from the cells using
trizol method which recommended by Sambrook &
Russel, (2001), and purification of RNA was done using
phenol /chlorormethane. Concentration and purity of
RNA was estimated as 4.91 µg/µl and 1.87 respectively,
depending on starting material (copies of DNA)which
increased proportionally with initial number.
Appearance of this band was expected as long as the
recombinant plasmid, it was successfully integrate in the
genome through heterologous recombination between
sequences of vectors and homologous sequences in
\textit{AOX1} locus of \textit{P. pastoris} GS115 and the sequence was
amplified by using 5′\textit{AOX1} and 3′\textit{AOX1} primers which
anneal to the 5′ and 3′ \textit{AOX1} sequence in Mut+ clones,
where endogenous \textit{AOX1} is present along with inserted
recombinant DNA, These primers amplify \textit{AOX1}
promoter region of the \textit{P. pastoris} genome which is
considered a strong inducible promoter that induced the
gene inserted within the 5′-\textit{AOX1}and 3′-\textit{AOX1}primming
sites by the use of methanol along with the insert18.

Construction of human preproinsulin into expression
vector
To generate a cloning vector that contains a desired
insert, genes to be cloned and backbone vector were
digested with the same restriction endonucleases.
Therefore the PCR product was double digested with
\textit{EcoRI} and \textit{Not 1} sites which cuts in the primers part as
long as it contains the restriction sites and no internal
sites present within the sequence of the gene. The gene
was inserted in the correct direction, following the
multiple cloning sites for unidirectional insertion which
are in front of and under the control of methanol-
ducible alcohol oxidase (\textit{AOX1}) promoter, and in the
correct reading frame. Result shown in Figure (3)
indicated the presence of a sharp band of about 9630 bp,
which represent the size of pPic9K.

Transformation of \textit{Pichia pastoris} \textit{pPic9k- \textit{hpi}} expression vector, which propagated in the
positive \textit{E. coli} transformants was isolated and linearised
using \textit{SalI} prior to the transformation process to increase
integration into the \textit{P. pastoris} genome through
recombination events and according to the strategy of
insertion in an expression cassette. Results shown in
Figure (3) indicate the presence of band of about 9630 pb
as compared to a 1 kb DNA ladder. Linearisation of \textit{hpi -}
\textit{pPic9k} facilitated integration of the plasmid with the cell
chromosome and prevents disruption of gene of interest
during integration. Moreover the number of clones we get
would be higher with linearized plasmid17.

PCR analysis of \textit{Pichia Pastoris integrants}
Genomic DNA was isolated from His+ transformants and
used as template for PCR reaction in order to confirm
integrants of \textit{hpi} gene using homologous recombination
events. Results shown in Figure (3.12) indicate the
presence of intense band of 330 bp which is referred to
\textit{hpi} gene multicopies. PCR amplify DNA in a manner
depending on starting material (copies of DNA)which
increased proportionally with initial number.

Recombinant Protein expression in \textit{P. pastoris} strains
In the present study, tricine- sodium dodecyl sulfate
polyacrylamide gel electrophoresis was employed to
follow the insulin expression in cultures. Samples from
small scale methanol inducible culture were subjected to
12 % Tricine-SDS- page analysis. Results shown in
Figure (3.13) indicate the successful expression of
recombinant protein as indicated by the presence of a
single major band with about 5.800 KDa this band.
correspond well with the size of human insulin with the theoretical molecular weight of 5,800 kDa as compared with the result of the control negative P. pastoris competent cells free of pPIC9K-hpi plasmid. The Tricine-SDS–PAGE revealed the successful production and secretion of the recombinant human insulin into the culture supernatants under the induction of methanol which increased gradually with time as compared to plasmid free samples lane .6, 7, 8, in fig (3.13). In conclusion the high level of expression can be attributed to two factors; first, human insulin gene was successfully integration downstream of methanol-induced alcohol oxidase promoter into the genome of P. pastoris and under its control. Most P. pastoris expression systems use the methanol-induced alcohol oxidase (AOX1) promoter for the expression of the heterologous gene upon induction by methanol. Second, target gene was cloned in frame and downstream of the α-factor signal sequence in order to allow secretion of the recombinant protein into the surrounding medium to facilitate harvesting and purification\[19\]. Moreover, result of tricine-SDS–PAGE were coincided with the similar results of expression patterns of western blot analysis that shown in fig. (3. 14) which confirms the presence of the expressed recombinant protein according to the size of protein which was secreted in the medium and developed on the SDS page gel. Hence western blot analysis could be taken as an evidence for successfully expression of the recombinant protein.

Results were shown in figure (3.14) indicate the presence of band of approximately 5,800 kDa which mimic the calculated band of human insulin –protein (5,800 kDa). It can be concluded, that recombinant protein had been successfully expressed and subjected to the efficient proteolytic cleavage in P. pastoris of fusion proteins based on the alpha-factor prepro-leader in the P. pastoris cells and secreted in the medium and developed on the SDS page gel, as well as detection on western blot membrane\[30\].

REFERENCES


