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Synthesis, cloning and expression of a novel pre-miniproinsulin analogue gene in *Escherichia coli*

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A R T I C L E   I N F O

A B S T R A C T

In the present study, a novel pre-miniproinsulin analogue was designed to have a short 9 residue sequence replacing the 35 residue C-chain, one lysine and one arginine added to the C-terminus of the B-chain in combination with glycine and arginine substitution at A21 and B29, respectively, and a 16-residue fusion partner comprising the pentapeptide sequence (PSDKP) of the N-terminus of human tumor necrosis factor-α (TNF-α), 6 histidine residues for Ni^{2+} chelated affinity purification and a pentapeptide ending with methionine for ease of chemical cleavage fused at the N-terminus. Homology modeling of the designed protein against miniproinsulin (protein databank file 1 efeA) as a template showed that the distance between the α-carbons of the C-terminus of the B-chain and the N-terminus of the A-chain did not change; the root-mean-square deviation of the backbone atoms between the structures of modeled miniproinsulin and miniproinsulin template was 0.000 Å. DNA sequencing of the synthesized gene showed 100% identity with theoretical sequence. The gene was constructed taking into account the codon preference of *Escherichia coli* (CAI value 0.99) in order to increase the expression rate of the DNA in the host strain. The designed gene was synthesized using DNA synthesis technology and then cloned into the expression plasmid pET-24a(+) and propagated in *E. coli* strain JM109. Gene expression was successful in two *E. coli* strains: namely JM109(DE3) and BL21(DE3)pLysS. SDS–PAGE analysis was carried out to check protein size and to check and optimize expression. Rapid screening and purification of the resulting protein was carried out by Ni–NTA technology. The identity of the expressed protein was verified by immunological detection method of western blot using polyclonal rabbit antibody against insulin.

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Introduction

With the advent of recombinant DNA technology, a large number of recombinant proteins have been produced in different host organisms. However, this field is still of a very limited application in Egypt. Egyptian industrial organizations interested in biological products still depend on imported final biotechnology products or imported recombinant raw materials for local formulation. In best cases, few depend on imported

biotechnology processes with the turnkey approach for technology transfer.

Recombinant insulin was the first biopharmaceutical agent approved for human use in 1982 [1–3] and its analogues were among the first engineered biopharmaceutical products [4–6]. It represents the essential treatment of all Type I and a considerable number of Type II diabetes patients. Diabetes mellitus is a significant public health problem globally and in Egypt. In a recent study it is estimated that 346 million people worldwide have diabetes [7,8]. In Egypt 6.2% of males and 8.2% of females are diabetics with an average prevalence of 7.2% in both sexes [8]. On the economic side, insulin annual consumption in Egypt was estimated to be 33 million US dollars for retail market in 2009 [9] and consequently 60–65 million dollars total consumption.

Human insulin is a small protein molecule made up of two separate polypeptide chains (A and B, consisting of 21 and 30 amino acids, respectively) linked together by two inter-chain disulfide bridges and one intra-chain A disulfide bridge. Substitutions occur at many positions of either chain without affecting the bioactivity [10–12]. Although the amino acid sequence of insulin varies among species, certain positions and regions of the molecule are highly conserved including positions of the 3 disulfide bonds, amino and carboxyl regions of the A chain and hydrophobic residues in the carboxyl terminal region of the B chain [10].

In the pancreas, these chains are first synthesized as a single-chain precursor called pre-proinsulin, which is sequentially processed into proinsulin and finally into insulin. The pre-proinsulin is made up of the A and B chains linked by a middle peptide chain (C) made of 35 amino acids flanked by two pairs of basic amino acids, Arg Arg and Lys-Arg and preceded by a 24 residue N-signal peptide [10–12]. The signal peptide is first eliminated leading to the formation of proinsulin which undergoes proteolytic cleavage to release C peptide and mature insulin.

Using recombinant DNA technology, it is possible to produce human insulin biosynthetically and to modify the insulin molecule for potential therapeutic and physiological advantages by altering its absorption characteristics to achieve near-normal glucose levels. Recombinant DNA technology has provided rapid-acting and long-acting insulin analogues for the treatment of diabetes mellitus, with an efficacy and safety that have improved the treatment for this disease. Long acting insulin analogues, created via recombinant DNA technology, are modified versions of human insulin that primarily alter the duration of absorption of the molecule from site of injection. In recombinant insulin glargine, two arginine molecules are added at position B30 and asparagine is replaced by glycine at position A21. In insulin detemir the amino acid threonine in position B30 is removed and lysine at B29 is acylated with a myristic fatty acid residue. In insulin degludec, a basal insulin analogue with a potential of a thrice-weekly dosing schedule currently in Phase III development, human insulin amino acid sequence is retained but there is a deletion of threonine at B30 and addition of a 16-carbon fatty diacid attached to lysine at B29 via a glutamic acid spacer [13].

However, concerns have been raised in connection with the structural modification of the insulin molecule. Increased binding affinities to the insulin like growth factor 1 (IGF1) receptor with potential for increased mitogenic action compared to human insulin has been reported [14–16]. Addition of positively charged basic arginine residues at position B31–32 of the C-terminus of the B chain increases the affinity for the IGF1 receptor [16]. This may potentially constitute a major health problem, particularly with the long term dosages required for diabetes mellitus patients. Development of new insulin analogues devoid of the enhanced IGF-1 binding affinity would represent an attractive alternative. The increased IGF-1 affinity could be attenuated through the addition of a single arginine to the C terminus of the B chain [15,16] or substitution of one or both arginine residues with less positively charged basic amino acids.

Recombinant human mini-proinsulin is a novel insulin precursor with a shortened C-peptide chain which could be easily converted by enzymatic cleavage into human insulin [10]. Recently, a number of mini proinsulins with various mini C-peptides were produced to increase the folding efficiency of the insulin precursor and the production yield of insulin [10,17,18].

In the present study, a gene coding for a novel pre-miniproinsulin analogue with a potential industrial application for recombinant human insulin production was synthesized using DNA technology, cloned in a suitable expression vector and expressed in a selected *Escherichia coli* strain. SDS–PAGE analysis was used to detect the expressed protein and to evaluate the efficiency of the used expression system.

Material and methods

The expression vector pET-24a(+) was obtained from Novagen and the pMA-PMPA plasmid (a pMA plasmid harboring Pre-miniproAbollien gene) from Geneart, Germany. Restriction enzymes, T4 DNA ligase, kits for DNA gel extraction and purification and kits for plasmid extraction and purification were purchased from Promega, USA. Protein purification Ni–NTA Spin Kit was purchased from Qiagen, Germany. *E. coli* host strains JM109 (Genotype: endA1, recA1, gyrA96, thi, hsdR17 (rk$^-$, mk$^+$), relA1, supE44, Δ(lac-proAB), [F′ traD36, proAB, laqI^{q}ZΔM15]), JM109(DE3) (Genotype: endA1, recA1, gyrA96, thi, hsdR17 (rk$^-$, mk$^+$), relA1, supE44, λ$^-$, Δ(lac-proAB), [F′, traD36, proAB, lacI^{q}ZΔM15], λDE3) and BL21(DE3)pLysS (Genotype: F$^-$, ompT, hsdSB (rB$^-$, mB$^-$), dcm, gal, λ(DE3), pLysS, Cmr) were obtained from Qiagen, Germany. Designed gene was synthesized and optimized in Geneart, Germany. Forward and reverse T7 promoter primer and T7 terminator primer were synthesized by Operon Company, France.

Design of pre-miniproinsulin analogue

A new basal pre-miniproinsulin analogue was designed by making three modifications in the structure of a human miniproinsulin template (protein databank file 1efeA): inclusion of a pre-area consisting of a fusion partner (N-terminal pentapeptide sequence (PSDKP) of human tumor necrosis factor-α, TNF-α), a spacer (six histidine residues) and five peptide sequence ending by methionine (Ser-Ser-Gly-Ser-Met); replacement of the 35-residue C-peptide with 9 residues (Lys-Arg-Tyr-Pro-Gly-Asp-Val-Lys-Arg) as mini turn forming sequence; and change in the insulin chains by the addition of one lysine at B31 and one arginine at B32 of the C-terminus of the B-chain in combination replacement of asparagine at A21 with glycine and of lysine at B29 by arginine. The final

designed three areas were named Pre-miniproAbollien and the resulting analogue was named Abollien.

Homology modeling of the designed miniproAbollien using miniproinsulin (protein databank file 1efeA) as a template was done by DeepView/Swiss-Pdb Viewer. Target-template alignment and model construction were carried out. After optimization the distance between the α-carbons of the C-terminal of the B-chain and the N-terminal of the A-chain was measured. Model quality assessment was done by calculating the root-mean-square deviation of the backbone atoms (240 atoms) between the structures of modeled miniproAbollien and miniproinsulin, search residues making clash generally or with backbone and search for threading energy high values.

A gene coding for Pre-miniproAbollien (231 bp not including restriction sites) was optimized for expression in *E. coli* using GeneOptimizer® at Geneart to select the proper codons to be used during translation of amino acid sequence to codon sequence. It was then synthesized by DNA synthesis technology flanked by two restriction sites NdeI and XhoI at 5' end and 3' end respectively. The fragment was cloned into pMA-plasmid (ampR) to form the construct pMA-PMPA. The final construct was verified by DNA sequencing then alignment against original DNA sequence using multiple-alignment algorithm in Megallign (DNASTAR, Window version 3.12e). The construct was then lyophilized.

Gene sub-cloning

Sub-cloning strategy depended on directional cloning to decrease background of self-ligated vector and prevent inverted orientation insert [19]. *E. coli* strain JM109 was chosen for sub-cloning to stabilize insert and prevent restriction of construct and protect it to be able to transform wild type r_k^+ *E. coli* strains. Reconstituted pMA plasmid harboring Pre-miniproAbollien gene (pMA-PMPA) was transformed into *E. coli* JM109 competent cells according to the method of Chung et al. [20]. The cells were then spread over SOB media plates containing 1% glucose and 100 µg/ml ampicillin and incubated overnight at 37 °C. Transformed cells were then used to propagate pMA-PMPA plasmid. Propagated pMA-PMPA plasmid was extracted and purified from the transformed cells using plasmid extraction kit, and a sample of the purified propagated pMA-PMPA plasmid was also loaded to 1% agarose gel to check concentration against a pMA-PMPA standard solution. Double restriction digestion of pMA-PMPA was carried out using restriction enzymes NdeI and XhoI and the insert was then separated by agarose gel electrophoresis. The insert gel slices were cut and the insert was purified using DNA gel extraction and purification kit. The purified insert was then loaded onto 2% agarose gel to determine its concentration.

For linear vector preparation, reconstituted pET-24a(+) plasmid was transformed into *E. coli* JM109 competent cells according to the method described by Chung et al. [20]. The cells were then spread over the surface of SOB media plates containing 1% glucose and 30 µg/ml kanamycin and incubated overnight at 37 °C. Transformed cells were then used to propagate pET-24a(+) plasmid. Propagated pET-24a(+) plasmid vector was extracted and purified from the transformed cells using plasmid extraction kit, and a sample of the purified propagated pET-24a(+) plasmid vector was loaded to 0.8% agarose gel to check its concentration against pET-24a(+) standard solution. Double restriction digestion of pET-24a(+) plasmid vector was done using restriction enzymes NdeI and XhoI in order to get a to linear vector. The linear vector was then separated by agarose gel electrophoresis. The linear vector gel slices were cut and purified and sample of the purified linear vector was loaded to 0.8% agarose gel to determine its concentration.

The expression construct (pPMPA) was prepared by ligation of purified Pre-miniproAbollien insert and purified linear pET-24a(+) plasmid vector using T4 DNA ligase. The ligation reaction was used to transform *E. coli* JM109 competent cells and the cells were then spread over SOB media plate containing 1% glucose and 30 µg/ml kanamycin and incubated overnight at 37 °C. Transformed cells were used to propagate expression construct (pPMPA).

The propagated expression construct was extracted and purified from *E. coli* JM109 transformed cells by plasmid extraction and purification kit. The purified expression construct was checked by performing PCR reaction to amplify the fragment comprising the site of cloning of Pre-miniproAbollien using T7 promoter primer and T7 terminator primer then run on agarose gel to check its base pair number (theoretically to be 423 bp while that of plain pET-24a(+) is 255 bp).

The purified expression construct pPMPA was used to transform two bacterial expression strains *E. coli* JM109(DE3) and *E. coli* BL21(DE3)pLysS. Control experiments were done for each expression strain. *E. coli* JM109(DE3) competent cells transformed with pPMPA were spread over a SOB media plate containing 1% glucose and 30 µg/ml kanamycin; sufficient number of separated colonies was obtained after incubation overnight at 37 °C. Similarly, *E. coli* BL21(DE3)pLysS competent cells transformed with pPMPA were spread over a SOB media plate containing 1% glucose, 30 µg/ml kanamycin and 34 µg/ml chloramphenicol. It showed growth of sufficient number of separated colonies after incubation overnight at 37 °C.

The expression construct was extracted from each strain and purified by plasmid extraction and purification kit for verification. Each purified construct was checked by performing PCR reaction to amplify the fragment comprising the site of cloning of Pre-miniproAbollien then run on agarose gel to check its base pair number (which is different from plain pET-24 a(+)) against DNA ladder. After the gel was examined using Fotodyne UV transilluminator (Foto/UV 15) bands were excised and the expression construct from each strain was extracted and purified by the gel extraction kit and then sent for sequencing and the two sequences were aligned to theoretical sequence.

Expression of recombinant Pre-miniproAbollien analogue

A loopful from a glycerol stock of *E. coli* JM109(DE3) expression strain transformed with pPMPA was streaked on SOB media plates containing 1% glucose and 30 µg/ml kanamycin under Laminar Air Flow (LAF) cabinet and a loopful from a glycerol stock of *E. coli* BL21(DE3)pLysS expression strain transformed with pPMPA was streaked on SOB media plates containing 1% glucose and 30 µg/ml kanamycin and 34 µg/ml chloramphenicol. The plates were incubated inverted at 37 °C overnight. One of the transformed *E. coli* JM109(DE3)

grown colonies was used to inoculate 10 ml LB broth containing 1% glucose and 30 µg/ml kanamycin and *E. coli* BL21(DE3)pLysS transformed cells was inoculated into 10 ml LB broth containing 1% glucose and 30 µg/ml kanamycin and 34 µg/ml chloramphenicol and incubated overnight at 37 °C with shaking at 225 rpm. Two ml of each of the overnight cultures was centrifuged at $1000g$ for 5 min and the cells were then resuspended in new media with same overnight media compositions and immediately used to inoculate 50 ml of LB broth containing 1% glucose and 30 µg/ml kanamycin in case of *E. coli* JM109 (DE3) and in 50 ml LB broth containing 1% glucose and 30 µg/ml kanamycin and 34 µg/ml chloramphenicol in case of *E. coli* BL21(DE3)pLysS. The inoculated broth was incubated at 37 °C with shaking at 300 rpm until the optical density of the broth (measured at 600 nm) was between 0.6 and 0.9. Induction was then done by adding 500 µl of sterile 100 mM IPTG to each culture. A negative control for each strain with pET 24a(+) transformed cells were treated exactly the same for each strain. One ml sample was taken from each expression strain just before induction and at 1, 2, 4, 6 and 8 h after induction respectively.

Samples from *E. coli* expression cultures were loaded onto One-dimensional gel electrophoresis under denaturing conditions to check the whole cell lysates including the expressed protein. The expressed protein was lower than 10 kDa (average molecular weight 8.4 kDa). Gel preparation and running were done using Nonurea peptide separations with tris buffers method comprising the traditional Laemmli buffer system with slight modification [21]. Gel preparation, staining and de-staining were done according to Coligan [22].

For rapid screening and purification, expression of Pre-miniproAbollien in *E. coli* JM109(DE3) was done according to previously described methods except that 4 h after the OD_{600} had reached 0.8, 40 ml of the culture was harvested by centrifugation. The resulting pellet was freezed by liquid nitrogen and stored at −20 °C. The cells (pellet) were thawed for 15 min and resuspended in 4 ml Buffer (8 M urea; 0.1 M NaH$_2$PO$_4$; 0.01 M Tris·Cl; pH 8.0) and incubated with shaking for 1 h at room temperature. Cells lysate was centrifuged at 10,000g for 20–30 min at room temperature. The supernatant (cleared cell lysate) was collected to be purified and a sample of it was saved for SDS–PAGE analysis. Purification of Pre-miniproAbollien from the cleared cell lysate was done using Ni–NTA Spin Kit according to the manufacturer's instructions [23]. Purification depended on the interaction of six histidine amino acids in the pre area of Pre-miniproAbollien and immobilized nickel ions held to the NTA resin in the kit columns.

Immunological detection of Pre-miniproAbollien by western blot using polyclonal rabbit antibody against insulin was carried out to check the identity of the expressed protein. New two parallel SDS–PAGE's were done as previously described. Each gel was loaded with 10 µl and 20 µl of purified Pre-miniproAbollien using prestained Pierce blue protein molecular weight as protein marker. After the end of the gel running one gel was stained and the second gel was transferred to nitrocellulose membrane according to the method described by Coligan [22]; the membrane was then cut into two strips, one strip was stained with Coomassie blue and the other was stored at −20 °C until used in immunoblotting. Antibodies against human insulin (prepared in a healthy male New Zealand rabbit according to Hu et al. [24]) were diluted (1:50) in PBS–0.3% Tween 80 solution containing 5% nonfat dry milk and then

the membrane strip containing Pre-miniproAbollien were immersed in it and incubated for one hour on the shaker platform. The strips were then washed 4 times (1 min for each washing) with hot PBS/Tween 80. Goat anti-rabbit IgG labeled with peroxidase conjugate was used as 1/500 in 1× PBS–0.3% Tween to cover the strip for 1 h at room temperature. This was followed by washing 3 times with PBS/Tween 80 and twice with 1× PBS. The substrate [(0.5 ml from 5% DAB) in (50 ml PBS + 5 µl 30% H$_2$O$_2$)] was used to cover the strip with shaking until the color was developed. The reaction was stopped by washing the membrane strip with distilled H$_2$O [25].

Results

Design of Pre-miniproAbollien

The homology modeling of miniproAbollien using protein databank file 1efeA referring to miniproinsulin as a template done by DeepView/Swiss-Pdb Viewer (Fig. 1) showed that the distance between the α-carbons of the C-terminal of the B-chain and the N-terminal of the A-chain was the same (11.01 Å) and the root-mean-square deviation of the backbone atoms (240 atoms) between the structures of modeled miniproAbollien and miniproinsulin was 0.000 Å. Threading energy did not show high values on the changed residues and there was no significant structural constraints on the remaining parts of the miniproinsulin. There were no residues making clash generally or with backbone. All previous homology modeling checks indicated that the small β turn and the changes in the chains do not affect the three dimensional structure of miniproinsulin.

Sequencing of the synthesized *Pre-miniproAbollien* gene showed 100% identity with theoretical sequence. The codon adaptation index (CAI) value was 0.99 which means that codons used in the optimized gene was near 100% of best choice of codon preference in *E. coli*.

### Cloning and expression of Pre-miniproAbollien

Expression construct was prepared by ligation and then transformed into *E. coli* JM109 competent cells. The purified construct was checked by amplifying the fragment comprising the site of cloning of Pre-miniproAbollien using PCR and then loaded into 2% gel. As shown in Figs. 2a and 2b, strong band on 423 bp was observed on the construct lane; sample from multiple cloning site in plain pET-24a(+) showed strong band on 255 bp (as expected) and negative control showed no bands.

Purified construct was used to transform two *E. coli* expression strains *E. coli* JM109(DE3) and BL21(DE3)pLysS harboring a chromosomal copy of the bacteriophage T7 RNA polymerase. Construct was retested after transformation into expression strains in the same way as tested after transformation into *E. coli* JM109 and results are shown in Figs. 2a and 2b. Sequencing of the amplified fragment showed 100% identity with the theoretical sequence.

Expression cultures were sampled just before induction and at 1, 2, 4, 6 and 8 h after induction with the synthetic lactose analogue isopropyl-B-D-thiogalactopyranoside (IPTG) and those samples were loaded to denaturing SDS discontinuous gel. Stained gel showed distinct bands of intensity increasing with time between 7 and 15 kDa (expected molecular weight of Pre-miniproAbollien was 8.4 kDa) as shown in Figs. 3a
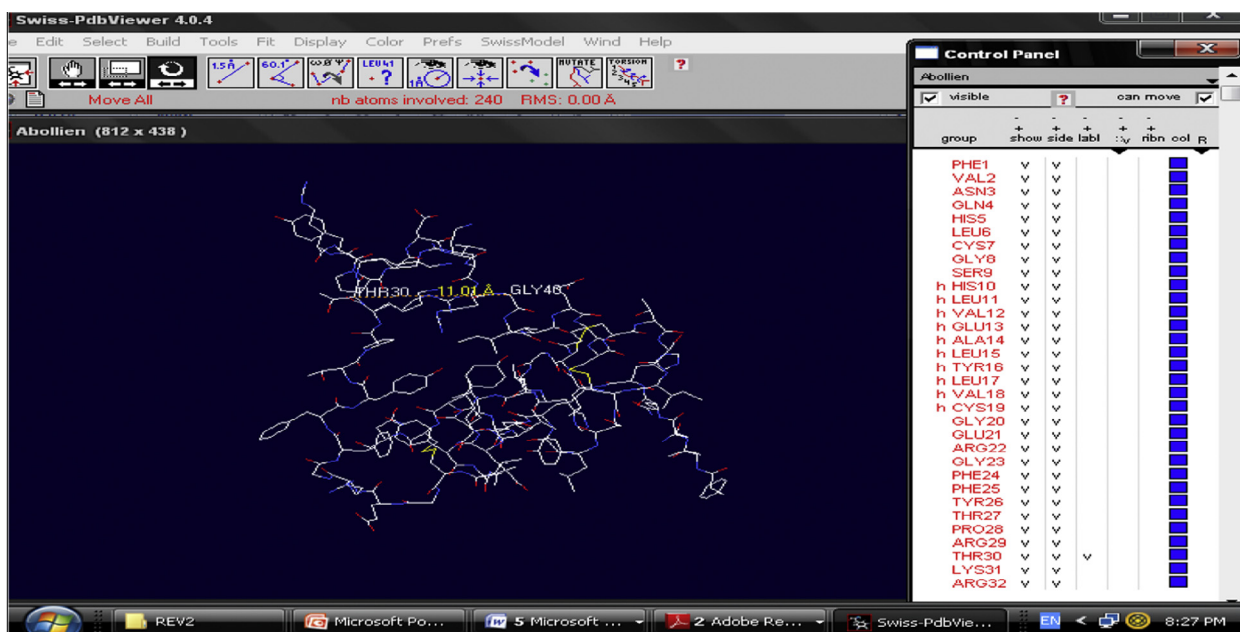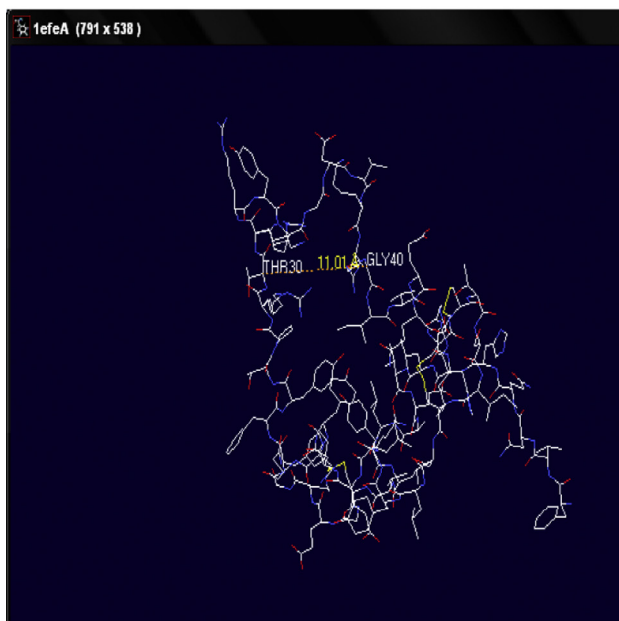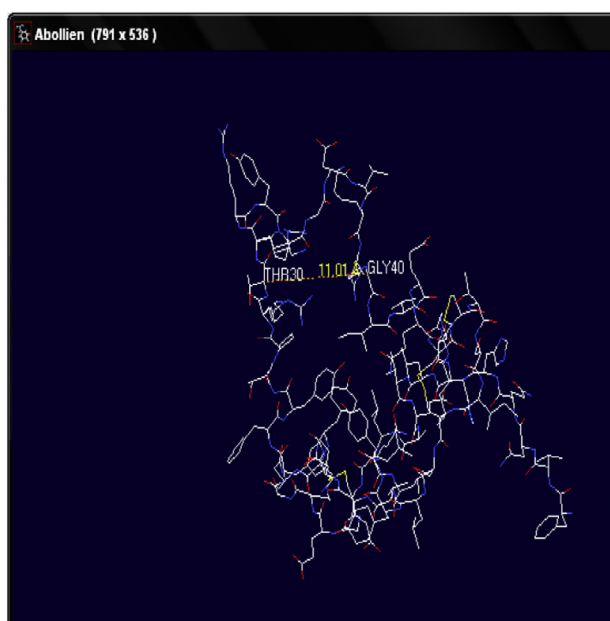
Fig. 1 Homology modeling of miniproAbollien against miniproinsulin using DeepView/Swiss-Pdb Viewer. (a) Modeled miniproAbollien (named Abollien in program) showing 0.000 Å root-mean-square deviation of the backbone atoms. (b) Miniproinsulin (named 1efeA in program) showing 11.01 Å distance between B30 Thr and A1 Gly. (c) miniproAbollien (named Abollien in program) showing 11.01 Å distance between B30 Thr and A1 Gly.

and 3b. Intensity of band observed on the gel for *E. coli* JM109(DE3) was higher than *E. coli* BL21(DE3)pLysS.

Rapid screening and purification of Pre-miniproAbollien

As shown in Fig. 4, flow of cleared total cell lysate through Ni–NTA column followed by washing and elution resulted in a distinct band of Pre-miniproAbollien at between 7 and 15 kDa (nearer to 7 kDa). Flow through and washing showed

cell proteins without any band at same size of Pre-miniproAbollien. First elution showed very high intensity band at the same size of Pre-miniproAbollien and second elution showed from one-third to half intensity of elution 1.

Immunological detection of Pre-miniproAbollien

After immunoblotting with polyclonal rabbit antibody against human insulin, the nonstained bands of the expressed protein
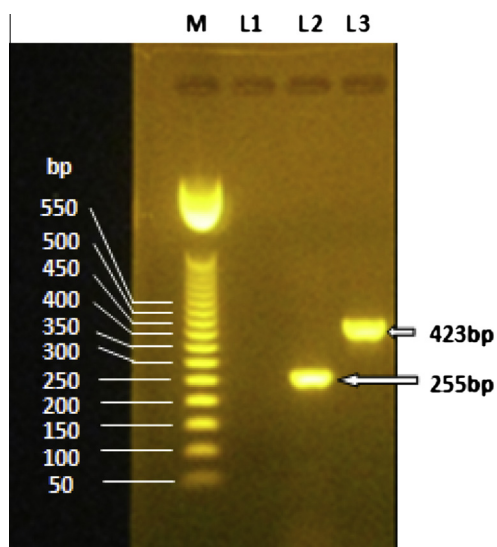
Fig. 2a PCR analysis of expression construct extracted from *E. coli* JM109. M: DNA 50 bp step ladder. L1: negative control reaction. L2: PCR product of pET-24a(+) (positive control). L3: PCR product of expression construct extracted from *E. coli* JM109.
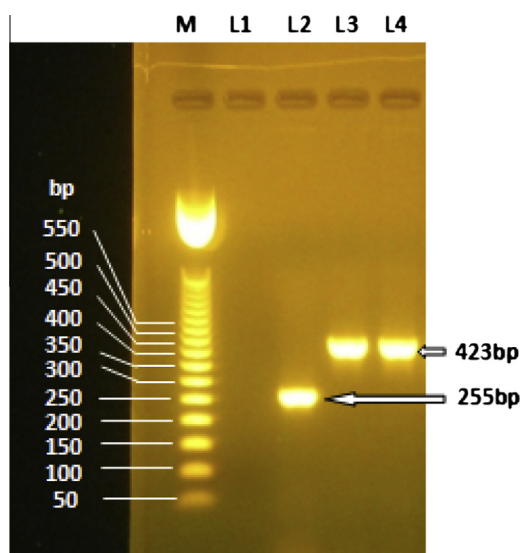

Fig. 2b PCR analysis of expression constructs extracted from *E. coli* strains JM109(DE3) and BL21(DE3)pLysS. M: DNA 50 bp step ladder. L1: negative control reaction. L2: PCR product of pET-24a(+) (positive control). L3: PCR product of expression construct extracted from *E. coli* JM109(DE3). L4: PCR product of expression construct extracted from *E. coli* BL21(DE3)pLysS.

showed clear blots on the nitrocellulose membrane with intensity increasing with concentration (Fig. 5, right). They were of the same size of stained bands on the lane loaded with purified Pre-miniproAbollien (Fig. 5, left).

Discussion

The present study describes the design and genetic expression of new pre-miniproinsulin analogue (*Pre-miniproAbollien*) with
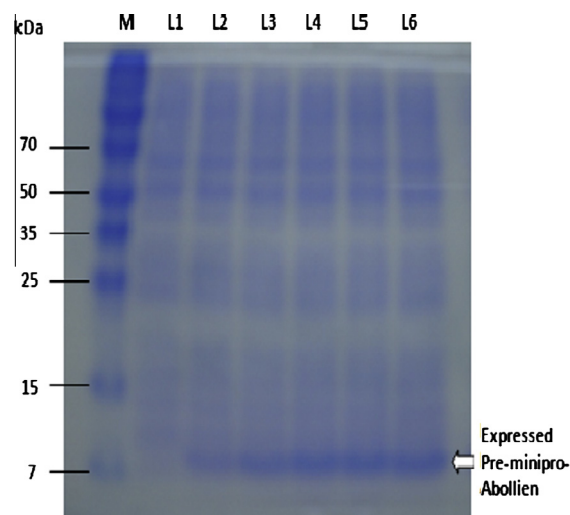

Fig. 3a SDS–PAGE of the expressed Pre-miniproAbollien in *E. coli* strain JM109(DE3). M: molecular weight marker. L1: noninduced control sample. L2–6: samples taken at 1, 2, 4, 6 and 8 h after induction respectively.
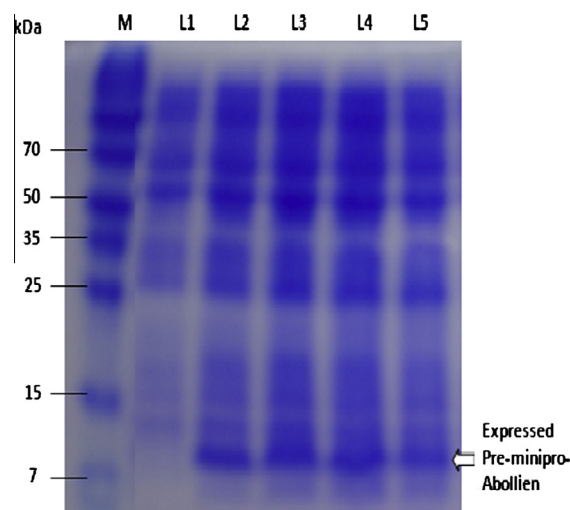

Fig. 3b SDS–PAGE of the expressed Pre-miniproAbollien in *E. coli* strain BL21(DE3)pLysS. M: Molecular weight marker. L1: noninduced control sample. L2–5: samples taken at 2, 4, 6 and 8 h after induction respectively.

potential for use as a precursor for the production of recombinant insulin. In the design of Pre-miniproAbollien, a 16-residue pre area comprising the pentapeptide sequence (PSDKP) of N-terminus of human TNF-α as a fusion area, a six histidine residues spacer and a five peptide sequence ending by methionine residue was used for the following reasons. The N-terminal (PSDKP) sequence of human TNF-α is characterized by its high-level expression and the characteristic β-sheet structure of TNF-α has the property of protecting the protein from degradation during expression [10,26]; it also increases the relative proportion of miniproAbollien to the TNF-α moiety in the fusion protein. In addition, the yield of *E. coli* JM109(DE3) and *E. coli* BL21(DE3)pLysS expression cultures proved that the N-terminal pentapeptide sequence (PSDKP) of human TNF-
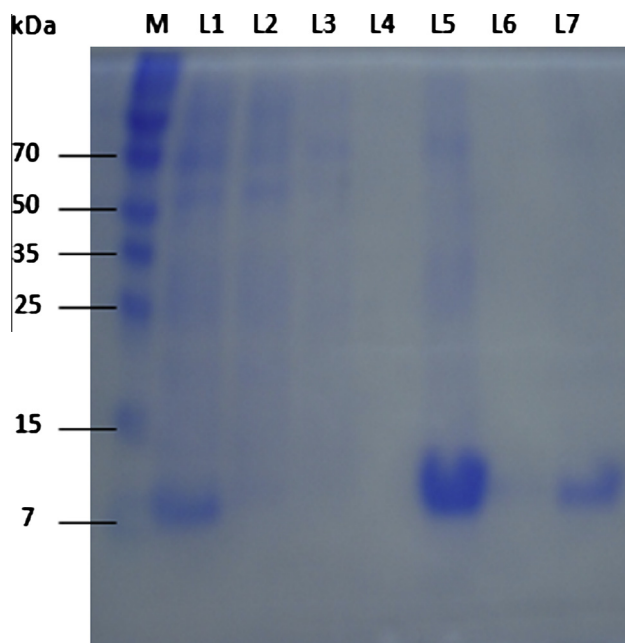
Fig. 4 SDS–PAGE of purification stages of pre-minipro-Abollien. M: molecular weight marker. L1: total cell lysate. L2: flow through Ni–NTA column. L3: first wash. L4: second wash. L5: first elution. L6: third elution. (L7): second elution.

α protected the Pre-miniproAbollien from degradation as there was no degradable protein in the gel even after 8 h of fermentation as shown in Figs. 3a and 3b. The use of only 6 histidine residues instead of 10 histidine residues used in other study [10] further decreases the relative proportion of pre section in the produced protein without affecting Ni^{2+} chelated-affinity purification. The six histidine residues were sufficient as the Pre-miniproAbollien binds to Ni^{2+} in the Ni–NTA Spin Kit and the elution contained a very high concentration of Pre-miniproAbollien relevant to the starting cleared total cell lysate (Fig. 4). To ease the chemical cleavage, a pentapeptide

ending by methionine residue was inserted between the PSDKP sequence and the target protein as reported in a previous study [10].

Recently, a number of miniproinsulins with various mini C-peptides were produced to increase the folding efficiency of the insulin precursor and the production yield of insulin [10,17,18].

Substitution of the 35-amino acid C-chain in human proinsulin with a shorter 9 residues as mini turn forming sequence in the designed Pre-miniproAbollien was adopted for a number of reasons: to increase the proportion of Abollien relative to connecting chain comprising the β-turn and help turn formation in protein folding [10,27]. It also aids enzyme processing required to release the native Abollien as most dibasic processing sites were predicted to occur in a β-turn adjacent to a region of helix or β-sheet [28].

The strategy followed in designing of the insulin analogue glargine was specifically directed toward a soluble formulation at low pH but of reduced solubility relative to native insulin at physiologic pH by the addition of two arginines to the C-terminus of the B-chain. This change in combination with a glycine substitution at A21 provides insulin with extended duration of action [29]. In this study we added one lysine and one arginine to the C-terminus of the B-chain in combination with glycine substitution at A21 and arginine substitution at B29. This change has the potential of attenuating the IGF-1 receptor binding affinity where concerns have been raised in connection with the in vitro biochemical profile of insulin glargine specifically increased IGF-1 receptor affinity and potential for increased mitogenic potency [14–16].

The homology modeling of miniproAbollien against miniproinsulin as a template (protein databank file 1efeA) done by DeepView/Swiss-Pdb Viewer (Fig. 1) showed that the distance between the α-carbons of the C-terminal of the B-chain and the N-terminal of the A-chain was not altered (11.01 Å) and the root-mean-square deviation of the backbone atoms (240 atoms) between the structures of modeled minipro-Abollien and miniproinsulin was 0.000 Å. Threading energy was low on the changed residues. There were no residues making clash generally or with backbone. It can be concluded that
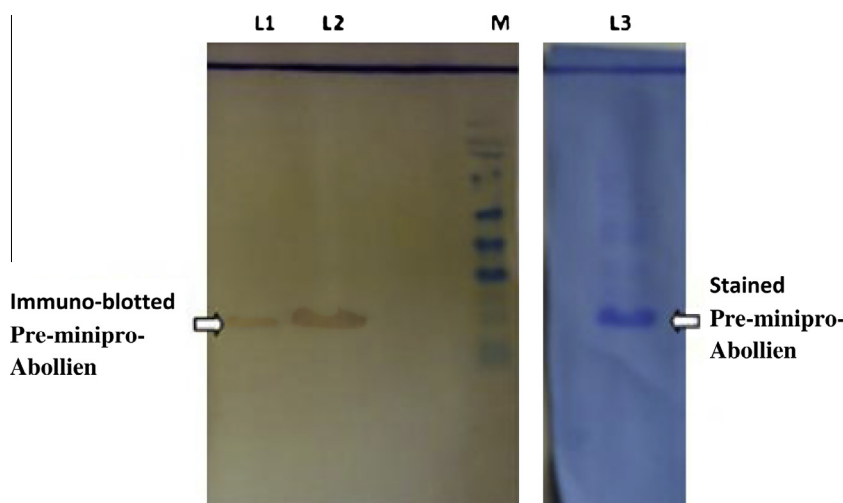

Fig. 5 Nonstained (left) and stained (right) Western blots of purified Pre-minipro-Abollien expressed in *E. coli* strain JM109(DE3). M: prestained Pierce blue protein molecular weight as protein marker. L1: 10 μl of purified Pre-minipro-Abollien expressed in JM109 DE3. (L2): 20 μl of purified Pre-minipro-Abollien expressed in JM109(DE3). L3: 10 μl of purified Pre-minipro-Abollien expressed in JM109(DE3).

all homology modeling checks indicated that the small β turn and the changes in the chains did not affect the three dimensional structure of miniproinsulin.

The expression system was composed of three basic elements: a gene coding for the target protein, an expression vector to harbor the gene and a suitable host organism. The gene was constructed with due consideration to the codon preference of *E. coli* in order to allow high and stable expression rates of the *Pre-miniproAbollien* gene in the host strain. The designed gene was optimized then synthesized by DNA synthesis technology. It was then sub-cloned to *E. coli* strain JM109 then expressed in two *E. coli* strains JM109(DE3) and BL21(DE3)-pLysS. Synthetic gene as an approach was chosen to bypass hard mRNA isolation, go directly to exons without introns, easy optimization and finally and most importantly to modify sequence to the chosen analogue sequence. The success of expression and 20% yield as appeared in SDS–PAGE (Figs. 3a and 3b) showed that optimization was successful. Success of sub-cloning strategy appears in the high yield of pure nonnicked construct that yielded one sharp band representing neither truncated nor elongated cloned gene upon amplification by PCR and gel run (Figs. 2a and 2b).

Expression strategy depended on transformation of construct in two *E. coli* strains JM109(DE3) and BL21(DE3)-pLysS then expression under control of T7 lac promoter in construct and maintaining catabolite repression condition by glucose supplement in the media to aid the stability of eukaryotic gene sequences and prevent the toxic effect reported for insulin and specially miniproinsulin [30,31]. *E. coli* JM109(DE3) and *E. coli* BL21(DE3)pLysS were chosen because of their properties of having r_k^- and r_B^- respectively to protect the construct. *E. coli* JM109(DE3) also contains *lac*I^q to inhibit transcription from lac promotor and to prevent leaks of expressed protein before induction and decrease the toxicity level of Pre-miniproAbollien after induction. *E. coli* BL21(DE3)pLysS was chosen to do the same job but with expression of T7 lysosymes that bind to T7 RNA polymerase. Glucose fed in media prevents auto induction by cAMP [32]. Expression strategy showed good expression levels as shown in Figs. 3a and 3b that bypass the apparent toxicity of Pre-miniproAbollien noticed on the great suppression of cell growth after induction compared to negative control of cultures of the same strains transformed with plain pET-24a(+). SDS–PAGE showed distinct bands without attached bands with lower or higher size. This proved that there were no truncated proteins due to incomplete translation or proteolysis of protein. It proved also that there were not any proteins with higher size as a result of inefficient stop of transcription or polymerization of the targeted protein. This conclusion was supported by one band in the elution of purified Pre-miniproAbollien and no bands in the Ni–NTA column flow through or washing in SDS–PAGE (Fig. 4).

The identity of the protein amino acids were screened for the six histidine residues in the pre area of Pre-miniproAbollien as the Pre-miniproAbollien binds to Ni^{2+} in the Ni–NTA Spin Kit and the elutions contained a very high concentration of Pre-miniproAbollien relevant to the starting cleared total cell lysate as illustrated in Fig. 4. Study using the western blot on the structural and immunological similarity showed specific and quantity dependent interaction between antibodies against human insulin and Pre-minipro-Abollien (Fig. 5).

Conclusions

A novel pre-miniproinsulin gene was synthesized, cloned and expressed in *E. coli*. A distinct nontruncated protein having the same immunological interaction of pure insulin and can be purified with Ni–NTA technology was obtained.

Conflict of interest

The authors have declared no conflict of interest.

Compliance with Ethics Requirements

This article does not contain any studies with human or animal subjects.

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