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Site Directed Mutagenesis, Molecular Cloning and Expression of interleukin-17E to Generate Structural Variant with Enhanced Specific Activity Using Industrial Friendly Salt Inducible *Escherichia coli* GJ1158

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Abstract

The newly discovered Th2 pro-inflammatory cytokine, interleukin-17E belongs to the member of IL-17 family. In this study, bioactive recombinant mutated human IL–17E (rhIL–25) was synthesized using overlapping PCR strategy and amino acid mutations were carried out using site directed mutagenesis. Four cysteins at 78th, 83rd, 136th and 138th positions were involved in disulphide bond formation and were responsible for biological activity of mature protein. These four cysteins were replaced with serine using nucleotide substitution and the desired outcome was cloned into expression vector pRSET-A followed by expressed in a salt inducible Escherichia coli GJ1158. The transformants were selected by ampicillin resistance marker and also by DNA sequencing. SDS–PAGE analysis confirms 17.06 kDa purified protein against low molecular weight protein marker. Protein quantification was carried out using Lowry's method. Approximately 104 mg/L of recombinant IL-17E was produced at 37 ^{0}C. Biological activity of protein was determined by the release of IL–6 from PBMC cells using rhIL–17E. This is the first report on production of interleukin-17E structural variant with enhanced specific activity without compromising the biological activity.

Keywords: *Interleukin, PCR, disulphide bond, expression vector, SDS-PAGE, Lowry method*

1. Introduction

IL-17E is a distinct member of the IL-17 cytokine family and induces Th2 and Th9 cell responses and suppresses the Th1 and Th17 cell responses by inducing the expression of IL‑13 by dendritic cells (DCs) or by inhibiting IL‑23 production by macrophages [1]. In various organs, Interleukin (IL)-25 (also known as IL-17E) induces the expression of IL-4, IL-5 and IL-13 and promotes pathogenic T helper (Th)-2 cell responses. It is having structural homology to IL-17 family members [2, 3] and least structural homology with the prototypical IL-17 family member, IL-17A. In addition, allied studies demonstrated that other cell types like hematopoietic or non-hematopoietic compartments could produce IL-25 under certain circumstances, such as primary bone marrow-derived mast cells upon IgE cross-linking [4], eosinophils and basophiles upon stem cell factor

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stimulation [5, 6], mucosal and lung epithelial cells upon allergic response [7], alveolar macrophages after particle inhalation [8], NKT cells and cells of the gastrointestinal tract and uterus.

IL-25 production also happens in intestinal epithelial cells and brain capillary endothelial cells [9, 10]. Various investigations confirmed the ability of different cell types to produce IL-25, but on the same hand the mechanism to control the production of IL-25 production remains unclear. IL-25 share the same receptor with IL-17B and has higher binding affinity to IL-17RB compared to that of IL-17B [11, 12, 13].

In general, mature interleukin-17E contains ten cysteins at 7^{th}, 8^{th}, 42^{nd}, 78^{th}, 80^{th}, 83^{rd}, 113^{rd}, 124^{th}, 136^{th} and 138^{th} position. Among all, four cysteins at 78^{th}, 83^{rd}, 136^{th} and 138^{th} positions were involved in disulphide bond formation and were responsible for biological activity of the protein. In this study, 78^{th}, 83^{rd}, 136^{th} and 138^{th} positions were replaced with serine using site directed mutagenesis and upon expression will produce monomer form of protein. The protein thus produced is tested for its biological activity using PBMC cells.

2. Material and Methods

2.1. Microbial Strains and Plasmid

Maintenance host *E. coli* DH5α was procured from MTCC, Chandigarh and expression host *E.coli* GJ1158 was purchased from Genei, Bangalore. The expression vector pRSET-A and ampicillin were procured from Invitrogen. All custom oligos were procured from local vendor, Hyderabad.

2.2. Medium Composition

LBON (Tryptone – 10 g/L and Yeast extract – 5 g/L), TBON (Yeast extract – 24 g/L, Tryptone – 12 g/L, Glycerol – 0.4%, KH_2PO_4 – 2.31 g/L, and Na_2HPO_4 – 12.54 g/L) and M9ON media (K_2HPO_4 – 6 g/L, KH_2PO_4 – 3 g/L, NH_4Cl – 1 g/L, Yeast extract – 5 g/L, Dextrose – 5 g/L, 1M $MgSO_4$ – 2 mL, TMM – 1 mL $(Al_2 (SO_4)_3$. $7H_2O$ – 10 mg/L, $CuSO_4$. H_2O – 2 mg/L, H_3BO_4 – 1 mg/L, $MnCl_3$. $4H_2O$ – 20 mg/L, $NiCl_2$. $6H_2O$ – 1 mg/L, Na_2MoO_4. $2H_2O$ – 50 mg/L, $ZnSO_4$. $7H_2O$ – 50 mg/L, $FeSO_4$ – 50 mg/L) were used in the present study. All media ingredients were analytical grade and were procured from Merck India.

2.3. Gene Amplification using PCR

Wild type human IL-17E gene was isolated from human cDNA using forward primer 5’ ATGAGGGAGCGACCCAG3’ and reverse primer 5’ TTAGCCCATCACACGGGG3’. The PCR program was set to be initial denaturation at 95 °C for 5 min; 35 cycles of 95 °C for 1 min, 61 °C for 1 min, 72 °C for 1 min and final extension of 72 °C for 10 min. Codon optimized IL-25 sequence was synthesized using the following primers.

FP-01 (82 MER):
5’TACTCTCACTGGCCGTCTTGCTGCCCGTCTAAAGGTCAGGACACCTCTGAAG
AACTGCTGCGTTGGTCTACCGTTCCGGTTC3’
RP-01 (72 MER):
5’CGTCTTCAGACGCACGGCAAGATTCCGGGTGACGGTTCGGACGCGCCGGTT
CCAGCGGCGGAACCGGAACGG3’
FP-02 (92 MER):
5’CGTCTGAAGACGGTCCGCTGAACTCTCGTGCGATCTCTCCGTGGCGTTACGA
ACTGGACCGTGACCTGAACCGTCTGCCGCAGGACCTGTAC3’

RP-02 (86 MER):
5’AGAGTTACCACGCGGGTCCATGTGAGAACCGGTCTGCAGAGAAACGCAGTG
CGGGCACAGGCAACGCGCGTGGTACAGGTCCTG3’
FP-03 (98 MER):
5’CGTGGTAACTCTGAACTGCTGTACCACAACCAGACCGTTTTCTACCGTCGTC
CGTGCCACGGTGAAAAAGGTACCCACAAAGGTTACTGCCTGGAACG3’
RP-03 (70 MER):
5’CTAACCCATAACACGCGGACGAACGCAAACGCACGCCAGAGAAACACGGT
ACAGACGACGTTCCAGGCAG3’

2.4. Site Directed Mutagenesis

Amino acid cystein at position 78, 83, 136 and 138 was replaced with sulfur containing amino acid serine using site directed mutagenesis technique and over lapping PCR method. Hydroxylamine hydrochloride (Asn-Gly) cleavage site was incorporated at 5’ end of both forward and reverse primer. The PCR program was set to be initial denaturation at 95 °C for 3 min; 30 cycles of 95 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min and final extension of 72 °C for 15 min. amino acid replacement was carried out using the following primers.

FP-01 (91 MER):
5’CGCGGATCCAACGGCATGTACTCTCACTGGCCGTCTTGCTGCCCGTCTAAAG
GTCAGGACACCTCTGAAGAACTGCTGCGTTGGTCTACCGTTCCGGTTC 3’
RP-01 (72 MER):
5’CGTCTTCAGACGCACGGCAAGATTCCGGGTGACGGTTCGGACGCGCCGGTT
CCAGCGGCGGAACCGGAACGG 3’
FP-02 (92 MER):
5’CGTCTGAAGACGGTCCGCTGAACTCTCGTGCGATCTCTCCGTGGCGTTACGA
ACTGGACCGTGACCTGAACCGTCTGCCGCAGGACCTGTAC 3’
RP-02 (84 MER):
5’AGAGTTACCACGCGGGTCCATGTGAGAACCGGTCTGCAGAGAAACTGGGTG
CGGGCACAGTGGACGCGCGTGGTACAGGTCCTG3’
FP-03 (98 MER):
5’CGTGGTAACTCTGAACTGCTGTACCACAACCAGACCGTTTTCTACCGTCGTC
CGTGCCACGGTGAAAAAGGTACCCACAAAGGTTACTGCCTGGAACG3’
RP-03 (70 MER):
5’CGGGAATTC**CTA**GCCGTTACCCATAACACGCGGACGAACTGGAACTGGCGC
CAGAGAAACACGGTACAGACGACGTTCCAGGCAG 3’

2.5. Cloning of Structural Variant into Expression Plasmid pRSET-A

A 2.9 kb expression vector, pRSET-A was used for cloning the gene of interest. The double digested vector DNA (pRSET-A) and the gene of interest was ligated at a ratio of 1:4 using T4 DNA ligase for 20 min. The ligation was confirmed using ligation confirmation PCR using gene forward and vector specific primers [14]. After confirmation, the ligation mixture was transformed into the maintenance host *E. coli* DH5α competent cells. Control plasmid (pRSET-A) was used as positive control. Competent cells without DNA were used as negative control. The recombinant plasmid (pRSET-A – mutated IL-17E) was confirmed by using colony lysate PCR with gene specific primers, restriction digestion and DNA sequencing.

2.6. Heterologous Expression using *E.coli* GJ1158

The recombinant DNA was extracted from *E.coli* DH5α and transformed into salt inducible *E.coli* GJ1158. The recombinant expression host was grown in LBON and

TBON and supplemented with ampicillin (100 μg/ml) at 37°C. When the OD reaches to 0.8 to 1.0, appropriate amount of inducer (150 mM NaCl) was added and incubate the culture on shaker. After 4 hrs of induction, the induced culture was allowed for centrifugation at 13,800 rpm for 10 min. Pellet was collected and dissolved in PBS and equal concentration of sample solubulizing buffer (SSB) was also added. Later analyze the protein expression using 15% SDS–PAGE and concentration was determined by Lowry's method using BSA as a standard.

2.7. Purification of IL-17E structural variant

The expressed IL-17E structural variant containing an N–terminal 6X His tag was expressed and purified using Ni^{2+} resin affinity chromatography. Initially, 800 mg of harvested cells were obtained from freshly grown culture. After post induction, the culture was centrifuged at 13,800 rpm for 20 min at room temperature. Pellet was suspended in 12 mL of guanidinium lysis buffer containing 7 M guanidine HCl, 22 mM sodium phosphate, 510 mM NaCl with pH 7.8 at 4°C for 1 hr. The cell suspension was again centrifuged at 13,800 rpm for 20 min. 10 mL of cell lysate was purified on a column containing 1 mL resin equilibrated with denaturing binding buffer, incubated for 45 min at room temperature with gentle hand shaking for several times. The column was washed with 6 mL of denaturing wash buffer and twice with 6 mL of native wash buffer. The bounded peptide was eluted with 5 mL of elution buffer and analysed on 15% SDS–PAGE against standard protein marker.

After confirmation, IL-17E structural variant was incubated in hydroxylamine cleavage buffer (2 M hydroxylamine hydrochloride, 0.2 M Tris HCl, pH 9.0) for 3 hrs at 42 – 45 ^{0}C [15]. The reaction was terminated by cooling the samples to room temperature. After enzymatic cleavage the peptide samples was dialyzed twice with 10 mM Tri Hcl, pH 8.0 for 16 hrs at 4 ^{0}C using 16 kDa (initially) and 18 kDa (finally) molecular weight cut off membranes and later dialyzed sample was loaded on Q Sepharose column matrix equilibrated with the same buffer. The bound protein was eluted with a linear gradient of NaCl (0–1 M) in the same buffer. The elute containing purified IL-17E structural variant was dialyzed against 100 volumes of 20 mM sodium acetate buffer, pH 4.5 and passed through a SP-Sepharose column matrix. The bound protein was eluted using a linear gradient of NaCl (0–1 M) in the same buffer. The purity of the peptide was analyzed using 15 % SDS – PAGE.

2.8. Biological Activity of rhIL–25

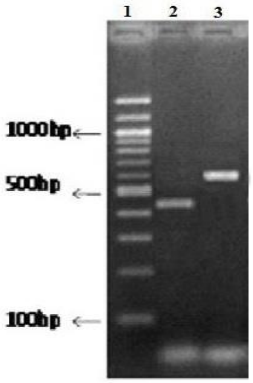
The biological activity of mutated rh IL–17E was determined by the amount of IL–6 released from IL–17E structural variant treated with PBMC (Peripheral Blood Mononuclear Cells). The activity was carried out using the human PBMC, by treating the cells with various concentrations of IL–25 structural variants to analyze the production of IL–6 levels. 10 mL of freshly citrated blood was centrifuged at 600xg for 20 min. Dilute the blood plasma in 30 mL in DPBS. 10 mL of ficoll hypaque solution was added to 30 mL blood plasma. Centrifuge the tubes 400 xg for 20 min at room temperature. Collect PBMC from inter phase of PBS/ficoll with a transfer pipette and transferred to new 50 mL tube and add 5 mL RBC lysis solution to pellet and incubate with mixing for every 5 min at room temepature. Dilute the cells to 10 mL with DPBS and centrifuge at 240xg for 8 min at room temperature. Resuspend the cells in 10 mL DPBS. Count the cells with the haemocytometer followed by cell viability. Resuspend the cells in 10 mL DPBS for final wash and centrifuge at 240xg, 8 min at room temperature. Transfer the cells to 1 mL cryovial and store in liquid N2 or use directly for analysis. 5 x 105 PBMC cells were plated into 96 well plates in 100 μL of culture medium. Recombinant human IL-25 purchased from R&D systems, USA used as standard. PBMC cells were treated with various concentrations of human IL-25 to generate the proper standard curve. PBMCs

were treated with ascending concentrations viz., 4, 8, 16, 32, 64, 125, 250, 500 and 1000 ng/mL of mutated rh IL-25 for 72 hours. Sample was added to the cells in 100 µl of culture medium. After 72 hrs of incubation, the cells with the medium were collected, boiled for 2 minutes at 90 0C and then centrifuged at 2000 rpm for 5 minutes. Then the lysate was used to analyze the IL–6 levels by ELISA method using the human IL-6 ELISA kit from Ray Biotech. The minimum detectable dose of IL–6 is < 3 pg/ml.

3. Results and Discussion

3.1. Amplification of Mutated rh17E

The full length 441 bp native mature and mutated rh17E gene was amplified using overlapping PCR. The results of the PCR methodologies were analyzed and confirmed the 441 bp amplified mutated rh17E gene product on 2% agarose gel by running against a 100 bp DNA ladder (Figure 1 & 2). Further this gene is sequenced (Figure-3) used as template to amplify the structural variant rh17E with a different set of primers in PCR and amplicon is confirmed on 1.2% agarose, followed by sequencing (Figure 4). The results are similar to our previous study [16].



(1) DNA Ladder;
(2) rhIL-17E structural variant;
(3) rhIL-17E with signal peptide

Figure 1. Amplification of Gene

MRERPRLGEDSSLISLFLQVVAFLAMVMGTHTYSHWPSCCPSKGQDTSEELLRWSTVPV
PPLEPARPNRHPESCRASEDGPLNSRAISPWRYELDRDLNRLPQDLYHARCLCPHCVSLQ
TGSHMDPRGNSELLYHNQTVFYRRPCHGEKGTHKGYCLERRLYRVSLACVCVRPRVMG

Figure 2. Full Length Sequence of rhIL-17E

YSHWPSCCPSKGQDTSEELLRWSTVPVPPLEPARPNRHPESCRASEDGPLNSRAISPWRY
ELDRDLNRLPQDLYHARCLCPHCVSLQTGSHMDPRGNSELLYHNQTVFYRRPCHGEKG
THKGYCLERRLYRVSLACVCVRPRVMG

Figure 3. Sequence of Mature rhIL-17E

YSHWPSCCPSKGQDTSEELLRWSTVPVPPLEPARPNRHPESCRASEDGPLNSRAISPWRY
ELDRDLNRLPQDLYHARSLCPHSVSLQTGSHMDPRGNSELLYHNQTVFYRRPCHGEKG
THKGYCLERRLYRVSLASVSVRPRVMG

Figure 4. Sequence of Structural Variant rhIL-17E with Serine Substitutes at 78, 83, 136 and 138 Amino Acid Sites

3.2. Generation of Recombinant DNA

The recombinant DNA was generated successfully by ligating the vector pRSET-A with passenger DNA, mutated rhIL-17E by T4 DNA ligase. The vector DNA and the insert (M*sak*) were ligated as per the protocol described as per Sambrook et al (1989) and the ligation reaction was confirmed by PCR with gene specific forward and vector specific reverse primers. The desired product size of structural variant consists of 441 bp DNA was identified against a 100 bp DNA ladder (Figure 5). The r-DNA was confirmed by PCR and transformed into *E.coli* DH5α by heat shock method.

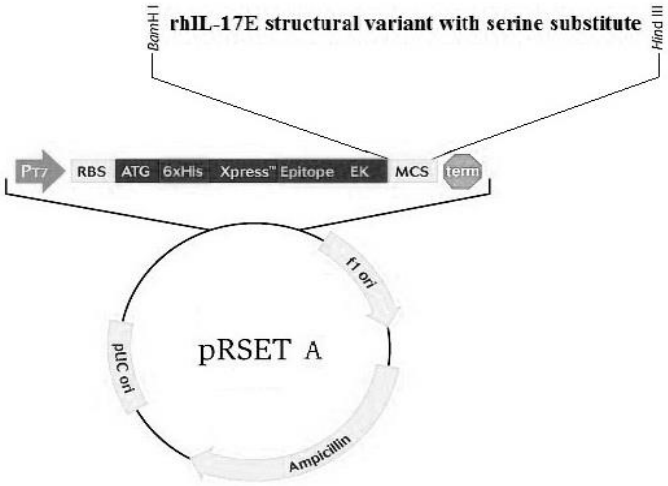
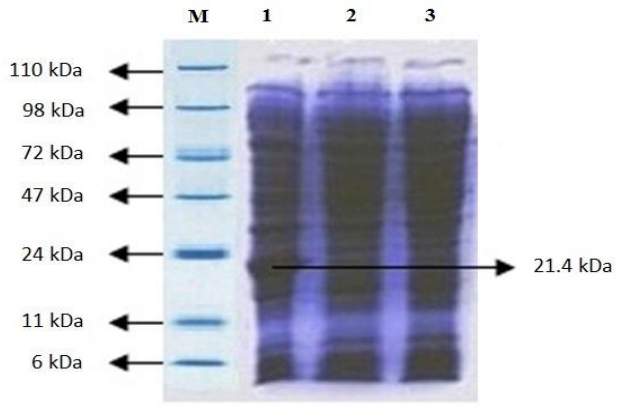


Figure 5. Structure of r-DNA Consisting of rh17E Structural Variant

3.3. Expression of rh17E structural variant

For the expression studies, salt inducible *Escherichia coli* GJ1158 was used. This expression system was chosen owing to its cost effective nature and simplicity. As mentioned earlier all expressions were analyzed by running on 12 % SDS-PAGE against standard protein marker and a very clear 21.4 kDa band was identified against a low molecular weight protein ladder (Figure 6). The observed results in the present investigation were coincided with the similar expression patterns in *Escherichia coli* [18].

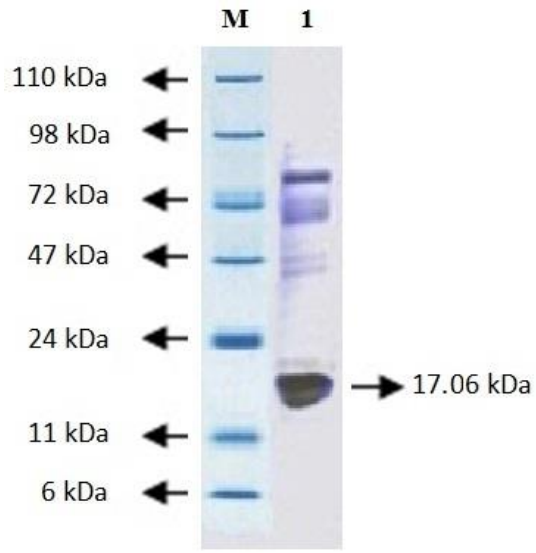


M = Marker protein
1 = Induced rh17E pRSET-A *Escherichia coli* GJ1158
2,3 = Uninduced rh17E pRSET-A *Escherichia coli* GJ1158

Figure 6. SDS PAGE Analysis of rh17E from pRSET-A Escherichia coli GJ1158

3.4. Purification of rhIL-17E structural variant

Purification of the rhIL-17E structural variant was carried out using 6X histidine column by loading the supernatant after sonication followed by enzymatic cleavage. SDS–PAGE analysis of the purified sample shows single band slightly at 17.06 kDa, which is consistent with the molecular weight of rhIL-17E structural variant and was also confirmed by western blot analysis (Figure – 7). Upon quantification, 103 mg/L of purified mutated rhIL-17E was achieved.



M = Marker protein
1 = Purified form of rhIL-17E structural variant

Figure 7. SDS PAGE Analysis of Purified rhIL-17E Structural Variant

3.5. Biological Activity Assay

Biological activity of recombinant human IL–17E structural variant was tested as per the standard protocol using human PBMC as described earlier [18]. IL–6 levels were estimated with the rhIL–17E structural variant treated cells were shown in the Figure 8. Correlation was observed between the concentration of rhIL–17E structural variant concentration and the secreted levels of IL–6.

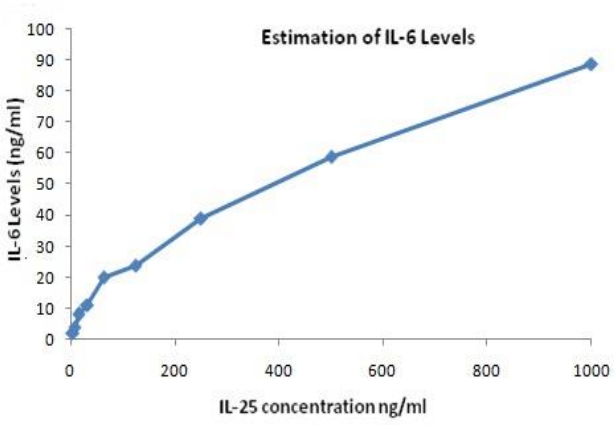


Figure 8. Biological Activity of the rhIL-17E Structural Variant

4. Conclusion

IL–17E plays a significant role in the pathogenesis of autoimmune diseases. Our previous study proved that 35% of protein was produced in soluble form after refolding and purification using prokaryotic host IPTG inducible *Escherichia coli* BL21(DE3). This production level was high when compared to production using *Pichia pastoris* strain X-33 [19]. But to avoid the disulphide bond formation here in this study we replaced cysteine with amino acid serine to produce rhIL-17E structural variant. This study will be useful to produce the rhIL–17E structural variant at large scale process. The biological activity of the structural variant was also not compromised. Hence it is useful to produce the rhIL-17E structural variant at large scale to satisfy the industrial needs.

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