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Novel Therapeutic Property of Recombinant Human GAPDH Peptide

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The development of new antimicrobial agents from natural resources by genetic engineering methods has become a trend in biotech research today. There are enormous numbers of peptide and proteins present in plants, animals, and microbial system that has the specific ability in inducing innate immunity as well as controlling the growth of infectious agents. In the present study the partial Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene of size 584 bp (94-677) from the full coding region of human system was amplified, cloned and sequenced. The sequence analysis of the gene exhibits the psuedogene nature with five stop codons at positions 328, 331, 502, 553, 557. Based on the cutting site of the restriction enzyme HindIII exclusively at 198 position a fragment of 104 bp (94-198) was selected and expressed in prokaryotic system. The recombinant GAPDH peptide was purified. The bactericidal property of the recombinant GAPDH peptide alone and its synergistic effect with the antibiotic ampicillin were confirmed by the Antibacterial sensitivity test (AST). The presence of positive charged aminoacids lysine and the hydrophobic aminoacids isoleucine, valine and tyrosine in the recombinant GAPDH peptide strongly supports the mode of action of the peptide in suppressing the bacterial growth.

Key words: Glyceraldehyde-3-phosphate dehydrogenase, Antibacterial sensitivity test, Recombinant GAPDH peptide, Positively charged residues, *Psuedogene.*

Mammalian system especially humans have a plethora of enzymes for maintaining a biochemical balance in the total metabolism. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12) is one of the major glycolytic enzymes in human systems involved in glucose metabolism[1]. Mammalian GAPDH genes, including human, have a complex genetic organization[2] and the gene expression studies reveal that human GAPDH has only one functional gene located on chromosome 12[3]. Based on the observation of multiple stop codons in the coding region of human GAPDH sequence, it exhibits more than 150 *psuedogene* with only one functional gene[4]. The role of mammalian GAPDH in various biological signaling pathways over the last couple of decades has increased interest in this classical glycolytic enzyme[1,5,6]. Traditionally, GAPDH was used as a model, or control, in protein and gene structural and catalytic mechanism related studies because of its high degree of gene and protein sequence conservation across species[1,5,6]. Recently, the Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), has become a subject of interest as more and more studies reveal a surfeit of diverse GAPDH functions, extending beyond traditional aerobic metabolism of glucose. As a result of multiple isoforms and cellular locales, GAPDH was able to

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come in contact with a variety of small molecules, proteins, membranes which play important roles in normal and pathologic cell functions. However, a variety of recent studies are now suggesting that GAPDH is a multifunctional protein[1,7]. With respect to the genetic and functional diversity of GAPDH, the present study was undertaken with an objective of cloning, sequencing and the prokaryotic expression of GAPDH gene. The study also focused on the functional characterization of the recombinant GAPDH peptide with reference to its therapeutic property.

MATERIALS AND METHODS

Isolation of RNA from human blood

Human blood samples were collected from healthy person and RNA was isolated by GTC method[8]. The purity of RNA was checked by formaldehyde gel electrophoresis.

cDNA synthesis

4 µg of RNA was used for cDNA synthesis using 35 units of AMVRT (Vivantis, Malaysia), 30 units of RNase Inhibitor and 100 ng of PolyT primer (Fermentas, Canada).

Designing of primers

Gene specific primers were designed using the software Vector NTI (Invitrogen, USA) for 584 bp partial GAPDH gene. All the parameters essential for maintaining the optimum conditions of good quality primer was maintained.

PCR assay of human GAPDH gene

cDNA encoding 584 bp of the partial human GAPDH gene was amplified by PCR using forward (5tGCCATCAATGACCCCTTCAT3t) and reverse primers (5tCCGTTCAGCTCAGGGGAT GAC3t). PCR reaction was carried out in 25 µl containing 100 ng of cDNA, 0.5 µl of dNTPs (0.2mM), 20 pmol each of forward and reverse primers and 1 unit of Taq DNA polymerase (Fermentas, Canada) by following the conditions: Initial denaturation of 3 min at 95ºC, 35 cycles of 30 seconds at 94ºC, 45 sec at 60ºC and 1 min at 72ºC, followed by final extension of 72ºC for 10 minutes. The PCR product was separated on 1.2% agarose gel and the amplified product was purified using Gel elution kit (Genei, Bangalore).

Cloning and Sequencing

The PCR product was cloned into a TA cloning vector, pTZ57R/T (Fermentas, Germany) using T4 DNA ligase (5 U/µl) and positive clones were identified by blue white screening following the procedure of Krishna et al[8]. The clone was confirmed by restriction enzyme digestion (HindIII). The cloned GAPDH gene was subjected to automated sequencing (Xcelris, Ahmadabad).

Sequence analysis of GAPDH gene

The primary sequence analysis was done for understanding the physicochemical properties of the sequence. Secondary structure prediction was done for structural analysis, the open reading frame (ORF) and the translated DNA was checked for detecting the functionality of the gene. The sequence was submitted to NCBI.

Prokaryotic expression and Purification of recombinant human GAPDH peptide

Considering the nature of the nucleotide sequence with the presence of stop codon within the sequence, a functional fragment of size 104 bp from the 5' end of 584 bp was subcloned to prokaryotic vector pET32b (Novagen, USA) for the production of recombinant peptide. The pTZ57R/T-GAPDH and pET32b plasmids were digested using restriction enzymes HindIII and BamH1. The gene of interest 104 bp and the linearized pET32b were eluted out. The eluted product was subcloned into pET32b expression vector and was then transformed into an expression host (*E.coli*). Partial GAPDH gene of size 104 bp in the pET32b-GAPDH plasmid was confirmed by PCR. The pET32b-GAPDH cloned was transformed with *E.coli* BL21De3pLysS expression host. Induction of recombinant peptide was done using IPTG (1 mM) at 37ºC. As a control pET32b empty vector transformed with *E.coli* BL21De3pLysS was also subjected to induction. An induced culture of pET32b and pET32b-GAPDH was drawn at different time intervals (0 hr, 1 hr, 2 hr & 3 hr). Collected fractions were subjected to SDS-PAGE analysis at a concentration of 12% for profiling it. The recombinant peptide was purified by Affinity chromatography using Ni^{2+} coated IMAC Hypercel column (PALL)[8].

Antibacterial Sensitivity Analysis of Recombinant GAPDH Peptide

Antibacterial sensitivity analysis of recombinant peptide was done using purified recombinant peptide. *E.coli 0111* an enteropathogenic intestinal bacteria (donated by Michael Donnenberg University of Maryland, US)

was used to investigate the antibacterial activity of recombinant peptide. The antibacterial sensitivity analysis was done in two steps. (i) the inoculum was allowed to grow in the LB culture in the presence of recombinant peptide alone at a concentration of 500 µg. (ii) In the second phase of the experiment the culture was grown in the medium containing recombinant GAPDH peptide (500 µg) and 20 µg of ampicillin (MIC 50 µg /ml). As the control of the experiment the antibiotic ampicillin (20 µg) along with the culture was used as positive control and the culture alone without ampicillin or recombinant GAPDH peptide was used as the negative control.

RESULTS AND DISCUSSION

Amplified and Sequenced Partial Human GAPDH Gene

Total RNA isolated from the blood sample shows intact bands of 18S and 28S indicating the purity of RNA (Figure 1). Partial GAPDH gene of 584 bp was amplified from cDNA (Figure 2). The amplicon was cloned and sequenced. The sequence analysis revealed five stop codons at positions 328, 331, 502, 553, 577 indicates the non functional nature of the gene (Figure 3). Based on the cutting site of HindIII exclusively at 198 position (A |A G C T T) a fragment of 104 size (94-198) was

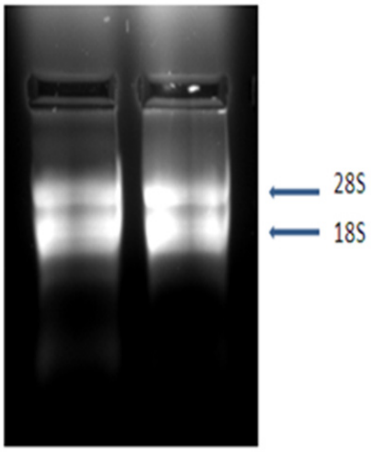

Fig. 1. RNA isolated from blood

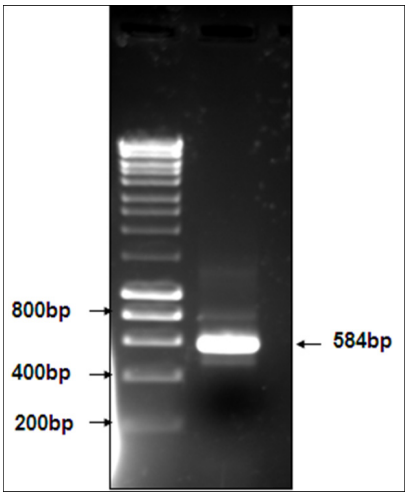

Fig. 2. PCR amplified partial GAPDH gene (584 bp)

GCC ATC AAT GAC CCC TTC ATT GAC CTC AAC TAC
ATG GTC TGT GTG TTC CAG TAT GAT TTT GTC TGT
GGC AAA TTC CAT GGC ACT GCC AAG GCT GAG AAT
GGG A|AG CTT GTC ATC AAC GAA AAT CCT ATC ACC
ATC TTC CAG GAG CGA GAT CCC TCC AAA ATC AGA
TGG GGC AAT GCT GGT GCT GAG TAT ATC ATG GAA
TCC ACC GGT GTC CTC ACC ACC ATG GAG AAG GCT
GGG ACT CAC TTG CAG CGG GGA GCC AAA AGG
GTC ATC ATC TCT GCC CCT TCT GCT GAC ACC CCC
ATG TCT GTG ATG GGC ATG AAG CAT GAG AAG TAA
TGA CAA CAG CCT CAA GAT CAT CAG CAT GCC TCC
TGT ATC ACC AAC TGC TTA GTG CCT CTG GCC AAG
GTT ATC CAT GAC AAC TCC GGT ATC GTG GAA GGA
CTC ATG AAT GAC CAC AGT CCA TGC CAT CAC TGC
CAC CCA GAA GAC TGT GGA TGG CCC CTC TGG
GAA ACT GTG GTG TGA CAG CCA CGG CGC TCT CCA
GAA CAT CAT CCC TGC CTC TAC TGG TGC TGC TAA
GGT TGT GGG CAA GGT CAT CCC TGA GCT GAA

Fig. 3. Partial GAPDH gene showing five stop codons (red), the cutting site for HindIII enzyme (blue) and the 104 bp portion used for protein expression (green)

selected for expression (Figure 3).The presence of the positively charged lysine residue and the hydrophobic residue valine, isoleucine and tyrosine in the random units of alpha helix region supports the functional efficacy of the peptide. The sequence was registered as accession number: JN038570 in NCBI.

Recombinant GAPDH Peptide in Prokaryotic System:

The GAPDH clone was subcloned to prokaryotic expression vector pET32b. Recombinant plasmid was transformed with *E.coli* BL21De3pLysS cells for protein expression. The positive colonies were used to raise starter cultures for expression studies. Cultures for expression were inoculated using cell pellet from the starter culture. Induction was given as the cultures crossed an OD600 value of 0.4 by adding IPTG at a final concentration of 1 mM. Samples were collected at intervals of 1 h starting from 0 h to 3 h. The efficacy of gene expression at time intervals was analyzed by SDS-PAGE (Figure 4). A fusion protein band of size 24 kDa was observed representing the recombinant GAPDH protein along with the fusion partner His tag protein .Since the major portion of the recombinant GAPDH protein was found in the soluble phase of the bacterial culture, the fraction was centrifuged and the supernatant was collected as protein source. The recombinant protein was purified using IMAC Hypercel His tag purification

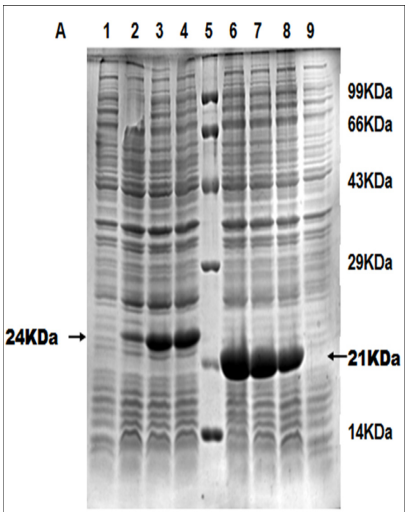

Fig. 4. Expression profile of GAPDH

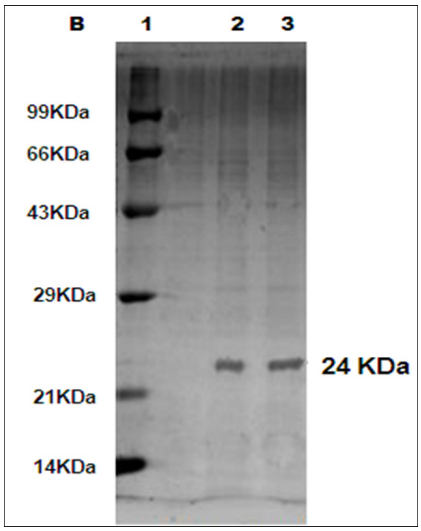

Fig. 5. Purified recombinant GAPDH

affinity column. Figure 5 demonstrates the purified recombinant protein with a clear band of expected molecular weight of 24 kDa. The results clearly demonstrate the successful expression and purification of the recombinant GAPDH protein.

Antibacterial Activity of Recombinant GAPDH Peptide

The recombinant GAPDH was subjected to antibacterial assay using *E.coli 0111* strain for determining the functionality of the expressed peptide. The efficacy of the interference of the recombinant peptide in inhibiting *E.coli* growth was checked by two means (i) Presence of recombinant GAPDH peptide as such in the bacterial culture. (ii) Addition of recombinant peptide with antibiotic ampicillin for determining the synergistic effect. For both the experiments ampicillin at a concentration of 20 µg/ml (MIC 50 µg/ml) was used as control. The culture was incubated at 37°C for a period of 6 hr and the growth was checked at the interval of every 30 minutes. The bacterial growth curve was drawn and the inhibitory rate was determined from the colony forming unit (CFU) per ml of each culture. The comparison of growth inhibition was made with the standard curve of bacterial culture without any inhibition.

Effect of ampicillin in bacterial growth

Effect of ampicillin inhibiting the growth of the enteropathogenic strain *E.coli* 0111 was determined as standard. In the control an active proliferation of bacterial cells was observed from 0.5 to 4 hrs, but in the culture in the presence of

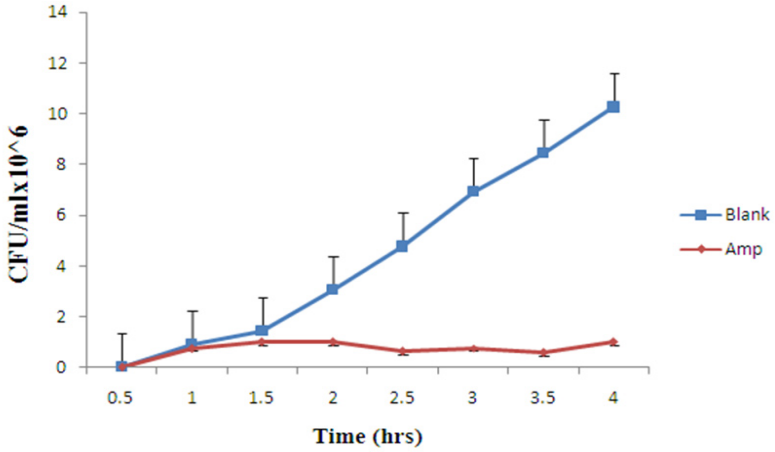

Fig. 6. Effect of ampicillin in bacterial growth. The experiment was repeated ten times and the data was presented as mean ± standard deviation (SD) of mean values of ten measurements

ampicillin has shown a sound level of inhibition of bacterial growth from 0.5 to 4 hrs at consistent rate indicating the effect of ampicillin in depleting the bacterial cell proliferation (Figure 6).

Effect of recombinant GAPDH peptide in bacterial growth:

In order to detect the functional property of the rGAPDH peptide bacterial growth was measured in presence of recombinant peptide and the CFU values were determined. It could be seen from Figure 7, that the recombinant peptide of GAPDH exhibits an effective suppression which is almost similar to that of ampicillin. It has been noticed that the aminoacid residues in the protein sequence especially the positively charged lysine residue and the hydrophobic Isoleucine, valine, tyrosine are responsible for digesting the cell wall membrane electrostaticaly for suppressing the bacterial growth[9]. The structural prediction analysis indicates the position of positively charged aminoacids and hydrophobic aminoacids residue were observed in the random units of alpha helix and beta sheet region. The bactericidal property of recombinant GAPDH peptide in suppressing the growth of *E coli,* the gram negative bacteria can be interpreted that the positively charged aminoacid residues of the peptides can interact with the negatively charged acid phosphate group of lipopolysacchrides of the membrane of gram negative bacteria to form blisters in the bacterial system[10]. This electrostatic signaling will trigger the disintegration of cell membrane leading to inactivation of cell growth (rGAPDH-LPS interaction). Subsequent to the disintegration of

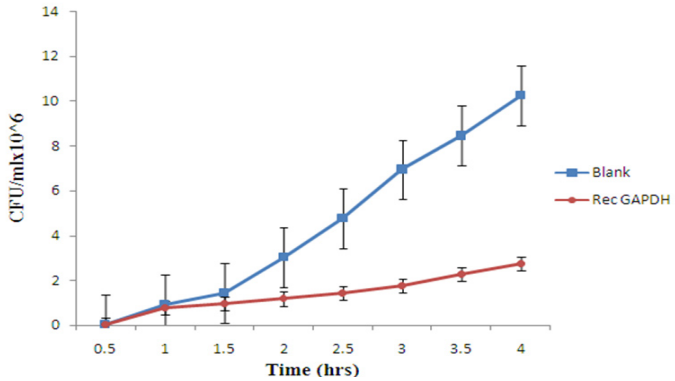

Fig. 7. Effect of recombinant GAPDH peptide in bacterial growth. The experiment was repeated ten times and the data was presented as mean ± standard deviation (SD) of mean values of ten measurements

the bacterial outer membrane of *Ecoli*, the positive charge of the peptides reacts with the anionic molecule of lipoteichoic acid of the cell wall resulting in the reduction of the negative charge of the cell wall which inturn trigger the digestion of cell wall.

Synergistic effect of recombinant GAPDH peptide with ampicillin:

Based on the interference of recombinant GAPDH peptide in suppressing the bacterial growth compared to antibiotic ampicillin, the experiment was repeated with the medium containing recombinant peptide and ampicillin in the ratio of 25 : 1 (500 µg:20 µg). Remarkable inhibition of bacterial growth was observed in the combination of recombinant peptide and ampicillin. Even though the concentration of ampicillin is negligible compared to peptide concentration a consistent level of suppression was noticed in the following repeated trials. The addition of ampicillin conventionally inactivates the cell wall synthesis in the culture which would enhance the activity of recombinant peptide more effectively on the membrane. In other words the 20 µg of ampicillin (MIC 50 µg /ml) is adequate for increasing the mode of action of recombinant GAPDH peptide as catalyst. This interference of recombinant peptide GAPDH in presence of ampicillin strongly indicates a synergistic effect of the peptide and the ampicillin (Figure 8).

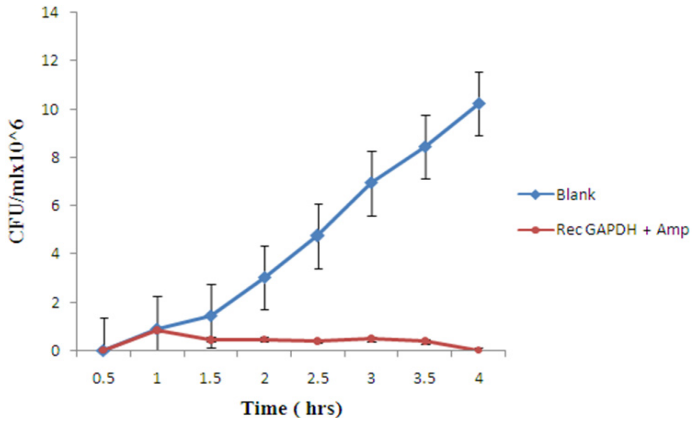

Fig. 8. Synergistic effect of recombinant GAPDH peptide with ampicillin. The experiment was repeated ten times and the data was presented as mean ± standard deviation (SD) of mean values of ten measurements

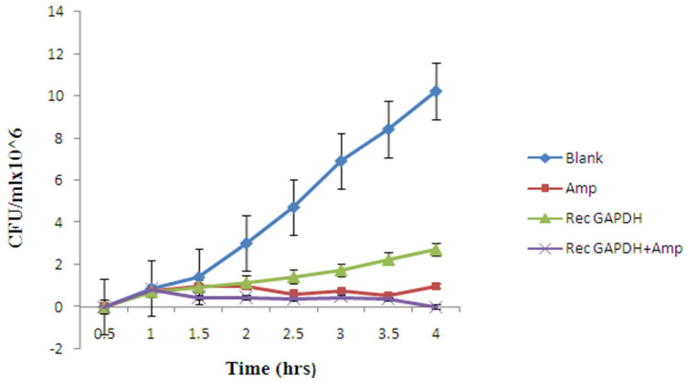

Fig. 9. Antibacterial sensitivity analysis of GAPDH peptide. The experiment was repeated ten times and the data was presented as mean ± standard deviation (SD) of mean values of ten measurements

Figure 9 demonstrates the antibacterial activity of recombinant GAPDH in cultures with different preparations of GAPDH at same concentration. Thus the antimicrobial sensitivity test data using recombinant peptide has conclusively proved its effect of inhibiting the growth of bacteria. The synergistic effect of recombinant peptide and ampicillin in suppressing the bacterial growth more

effectively than ampicillin alone provide sufficient insight to establish the bactericidal properties of recombinant GAPDH peptide. So the data of the study provides a novel outlook in the development of a new antimicrobial agent with more efficacy than the antibiotic.

CONCLUSION

Exploitation of therapeutic property of Antimicrobial peptides has become a trend of clinical research today. Since peptide has a unique mechanism of suppressing the growth of microbes by directly destructing the cell membranes unlike antibiotics, the production of recombinant peptide from new source can induce a novel change in the field of drug industry. To the best of our knowledge this is the first time report of showing the antibacterial property of human recombinant GAPDH peptide expressed in prokaryotic system. The bactericidal effect of recombinant human GAPDH peptide was ascertained from the study.

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