Chapter 4

Introduction

Analysis of the genes encoded by the apicoplast genome of *Plasmodium falciparum* and *Toxoplasma gondii*, has only offered us a small glimpse of what is going inside the apicoplast. The apicoplast genome contains genes for Ribosomal RNA predicted to fold into proper ribosomal subunits, ORF's predicted to encode many ribosomal proteins, translational components such as elongation factor – Tu (EF – Tu) and a full set of tRNA's. Persistence of these and other genes provide strong evidence for a translation system. Moreover, ribosome like particles of bacterial size are visible in the apicoplast, and polysomes containing plastid rRNA and mRNA can be partially purified from erythrocytic stages of the parasite. Several drugs that block the prokaryotic translational system are found to be parasiticidal. Thus, it becomes vital to understand the protein products translated in the apicoplast. (Ralph *et. al*., 2001)

Elongation Factor Tu (EF – Tu)

The translation elongation factor, EF-Tu, the product of *tufA* gene, is one of the best conserved proteins encoded by the apicoplast DNA circle. The protein translation and presence has been shown successfully in *P. falciparum* (Chaubey *et. al*., 2005). The EF-Tu has been shown to be localized within the apicoplast. It is maximally synthesized in the trophozoite (middle to late) or early schizont stages. Also, the protein translation is sensitive to the inhibitors (thiostrepton) of prokaryotic translation. There are as yet no reports on the characteristics or the functional nature of this protein in *P. vivax*. Various other available reports detail this protein from prokaryotic organisms and among apicomplexans it has been used mainly for phylogenetic studies related with apicoplast genes.

ORF470/ SufB

The *ORF470* gene in the Apicoplast genome was reported as a homologue of a red algae protein ycf24 and recently has been understood as a close relative of the *suf*

operon in bacteria that regulates the primary assembly of [Fe – S] cluster. This cluster plays a primary role in Iron and Sulphur mobilization during various enzymatic pathways related with redox and non – redox catalysis. Most of the genes of the *suf* operon (*suf*A, C, D, E and S) have been found to be present in the *P. falciparum* genome database. The missing part of this family from the genome is *sufB* gene which is present in the plastid genome. (Ellis *et. al*., 2001)

There are very few studies detailing the *sufB* gene and protein in *Plasmodium* species. Except for some hypothetical explanations to the function or existence of this gene or protein, there are as yet no experimental data directly related to the protein.

Results and Discussions

In order to understand the function of the *P. vivax* EF – Tu protein and to characterize the differences from that of *P. falciparum* protein, protein expression trials of amplified *EF – Tu* gene product and B – cell epitope scanning studies with the *P.vivax* EF – Tu and SufB proteins were performed.

Protein Expression of EF – Tu

The amplified *tufA* gene product was first cloned in pRSET A expression vector at PvuII site by blunt end cloning method (Sambrook *et. al.*, 1989), as there were no restriction sites present at the start or end of the gene. But the gene was found to be out of reading frame, thus it was digested and religated into the pET21c expression vector system at Eco RI / Sac I site (Figure 4.1).

The obtained product was transformed into *E. coli* BL21 (DE3) and *E. coli* BL21 (DE3) pLysS cells. Protein expression trials were carried out by growing a primary culture followed by a secondary culture, induction (IPTG concentration varying from 0.5 mM, 1.0 mM, 1.5 mM, 3.0 mM to 5.0 mM) at 2 – 2 ½ hours and sampling at various time intervals from ½ hour to 8 hours. The samples were treated with lysis buffer and checked on SDS – PAGE.

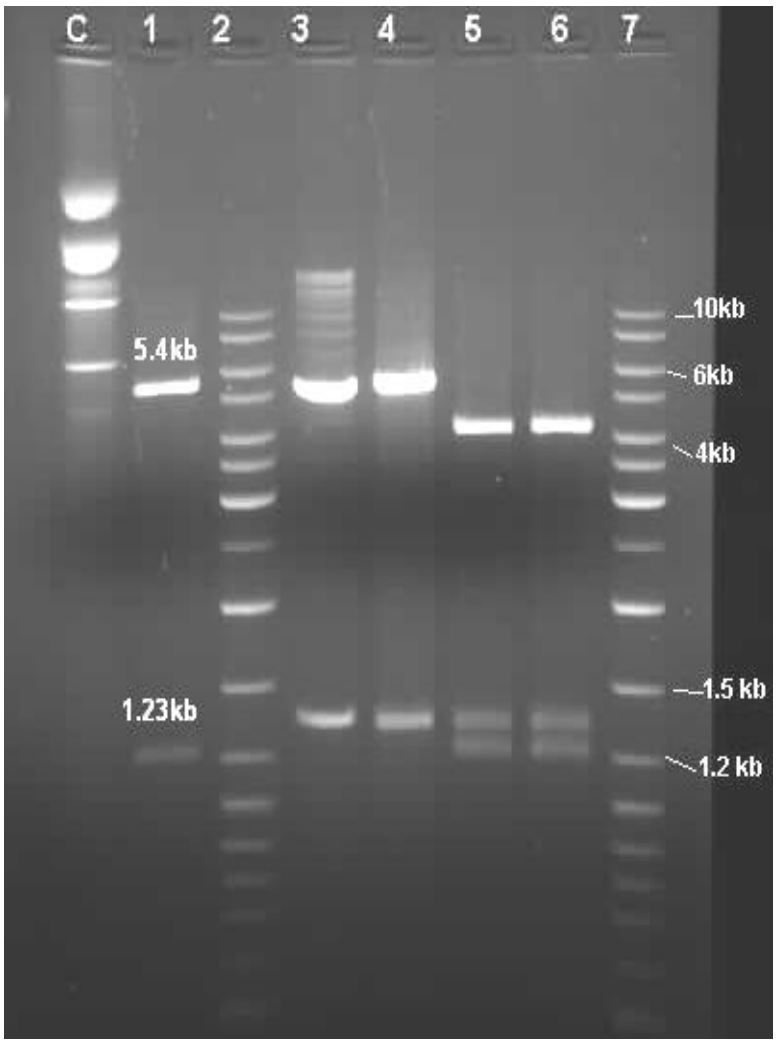

Figure 4.1: Restriction digestion profile of pET 21c (5.4 kb) – *tufA* (1.23kb) clone.
Lanes C = Clone as plasmid preparation;
1 = Res. Dig. with Sac1/ Eco R1;
2, 7 = 1kb DNA Ladder Mix (SM#0331, MBI Fermentas);
3 = Res. Dig. with Bgl2;
4 = Res. Dig. with Pst1;
5 = Res. Dig. with Pst1/ BamH1;
6 = Res. Dig. with Pst1/ EcoR1.

The *P. vivax* EF – Tu protein is approximately 45KDa in size and was expected at approximately 47KDa due to the N terminal T7 His – Tag. After repeated trials of varying the IPTG concentration or the time duration of sampling, a faint band could be obtained at the desired ~47KDa position (Fig. 4.2 a & b) but its appearance was transient. Trials were also carried out by changing the expression host without any success. A feeble expression would appear rarely in *E. coli* BL21 (DE3) host while no expression could be seen in other hosts.

The expression profile of EF-Tu protein raised doubts over the correct and 'in-frame' orientation of the *tufA* gene. To verify this, the pET21c – *tufA* clone was sequenced using T7 promoter primers. The obtained sequences verified that the gene was present 'in – frame' in the vector and should express.

Figure 4.2 a: Gel 1

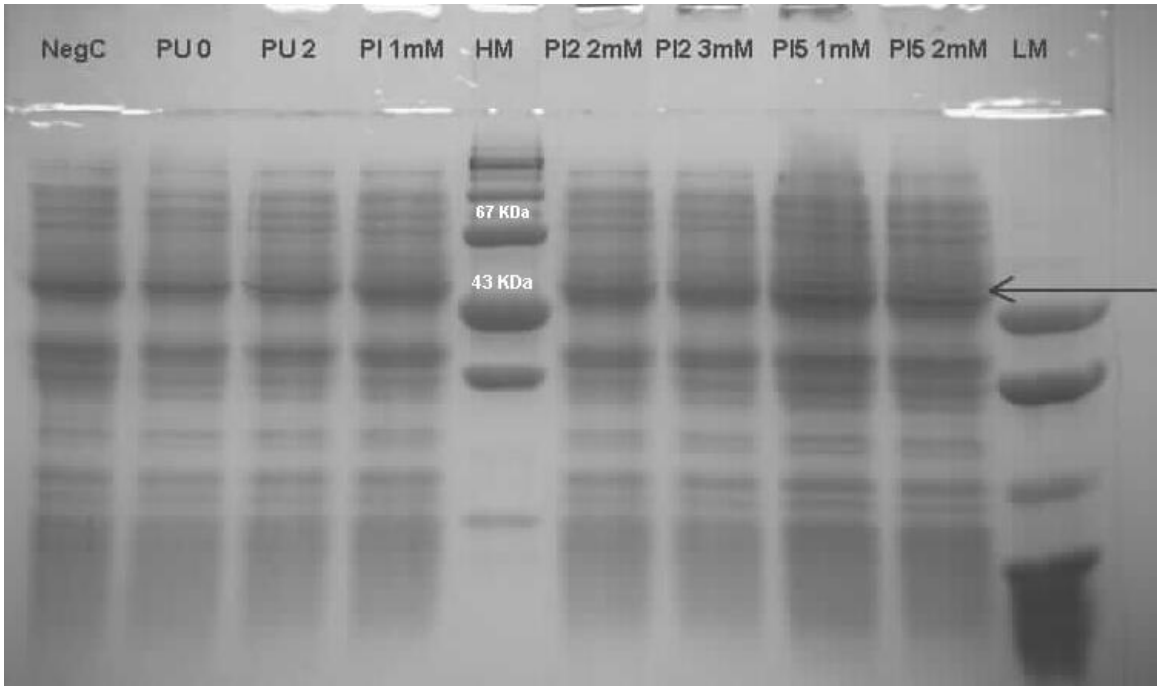

Figure 4.2 b: Gel 2

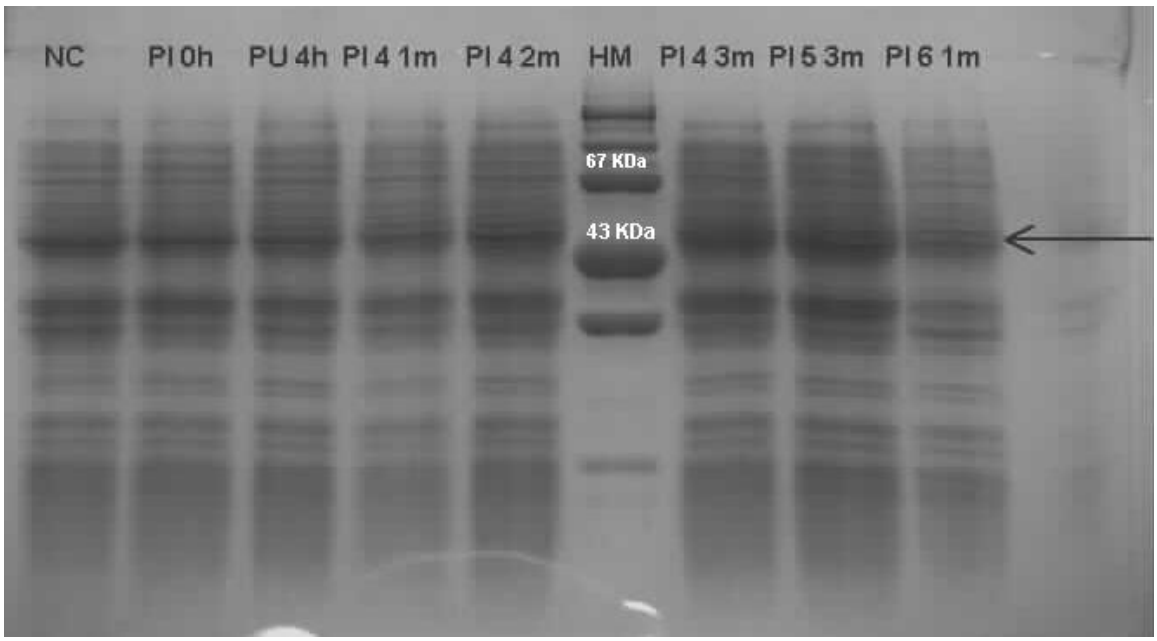

Figure 4.2: Protein sample profile of EF-Tu on SDS – PAGE. NegC/ NC= Negative control, pET21c vector alone; PU= Uninduced samples; PI= Induced samples at different time intervals and different IPTG concentrations. Arrow marks the position of expected ~47KDa band.

The other reason for the feeble expression of protein was considered as codon bias problem. The codons present in the eukaryotic genes (especially the A/T rich genes) may not always be translated by bacterial host tRNAs. This problem is usually seen during eukaryotic protein expressions in the bacterial hosts due to absence of some tRNAs for which codons are present in the gene. To solve this problem, RIG plasmid (Baca and Hol, 2000) was used which carries genes for 4 tRNAs (AGA, AUA, GGA and AGG) that are absent in *E. coli* hosts. The RIG plasmid was co – transformed with the clone into the expression host and expression trials were carried out as before but without any success.

RNA isolation and RT – PCR

Further to study whether gene is actually getting transcribed, mRNA isolation (Ausubel *et. al*., 1999) was carried out from the clone cultures and negative control pET21c culture. Similar to protein expression trials, the primary cultures were grown overnight followed by the secondary culture at 12^{th} hour. The secondary cultures were induced (IPTG 1.0 mM) after 2 hours. Induced samples were collected at different time intervals, pelleted and total RNA was isolated. This was followed by cDNA synthesis using Sensiscript Reverse Transcriptase kit (QIAGEN). For this an RNA mix consisting of total RNA as template, Oligo dT primers and water was incubated at 65^0C for 5 minutes. To this a reaction mix consisting of RT buffer, dNTP mix, Reverse Transcriptase enzyme and water was added and the whole mix was incubated at 37^0C for an hour. The product from this reaction was amplified using normal *tufA* gene amplification PCR.

Blazing bands were obtained at slightly above to 1200bp marker position (Figure 4.3) in uninduced as well as all induced samples. The negative controls (RT – PCR using vector culture RNA and simple PCR for *tufA* gene using clone culture RNA) did not show any amplification. This result could mean that the EF – Tu protein transcription is taking place but the translation is either not at all happening or the translated protein is highly unstable which is getting denatured very fast. The probability of later reasoning may be more because we were able to see faint protein band in the SDS gel occasionally. Since, the expression for EF – Tu was very feeble and transient, so the

protein isolation could not be accomplished. Thus, to study the immunogenic aspects of the protein, B – cell epitope regions were identified in the protein sequence.

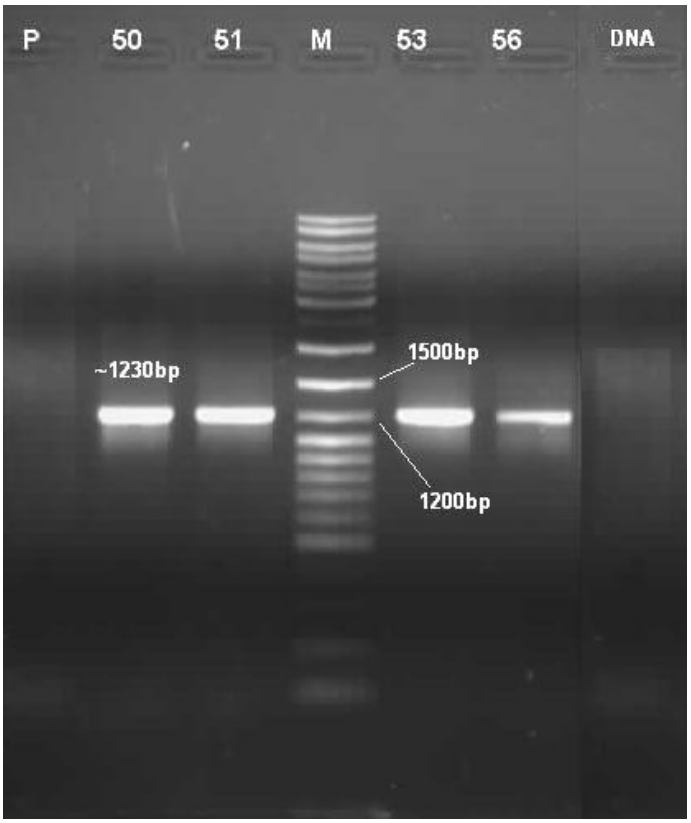

Figure 4.3: *tufA* cDNA synthesized from the mRNA of pET21c – *tufA* clone. P = pET21c culture; 50 – 56=Clone (No. 5) culture samples at 0 hr (before induction with IPTG), 1 hr, 3 hrs and 6 hrs after induction; DNA = normal *tufA* gene amplification from clone RNA.

B – cell Epitope Scanning

The available protein sequences were studied to identify the presence of B – cell Epitope regions using Bcepred software freely available by IMTECH, Chandigarh (http://www. imtech.res.in/raghava/bcepred/). Using the physio-chemical properties of amino acids, such as hydrophilicity, flexibility/mobility, accessibility, polarity, exposed surface and turns, the program assists in locating putative B – cell epitope regions for immunological studies. Quantification of these properties is determined by assigning a value to each of the 20 natural amino acids. Users can select any physico-chemical property or combination of two or more properties for epitope prediction. The server presents the results in graphical and tabular frame. In case of graphical

frame, this server plots the residue properties along protein backbone, which assist the users in rapid visualization of B-cell epitope on protein. The peak of the amino acid residue segment above the threshold value (default is 2.38) is considered as predicted B-cell epitope. The tabular output gives the normalized score of the selected properties with the corresponding amino acid residue of a protein along with the maximum, minimum and average values of the combined methods selected. The server is able to predict epitopes with 58.7% accuracy.

Based upon these parameters, the obtained EF – Tu and SufB sequences were submitted to the software and following images (Figures 4.4 A – E; 4.5 A – C) were obtained along with tabular data from which peptides (Table 4.1) were selected. Four peptides (AKD19 – AKD22) were designed from *P. vivax* TufA protein sequence, three peptides (AKD25 – AKD27) from *P. vivax* SufB protein sequence, and one peptide (AKD24) from *P. falciparum* TufA protein. Among the four *P. vivax* TufA peptides two were overlapping peptides.

Table 4.1: List of peptides designed using Bcepred server.

Peptide ID.	Peptide Sequence	Peptide position
TufA		
AKD19	LSKKYNYSDIDSAPEEKIRG	40 – 60
AKD20	KYNFNLNNIHILAGSA	160 – 177
AKD21	PNKLKVYKSFIAETYILT	308 – 326
AKD22	YILTKEEGGRHKPFNIGYK	323 – 350
AKD24	IIQKNKDYELIKSNIWIQ	181 – 199
ORF470/ SufB		
AKD25	YNLNYKYQYKNKINLYLIR	10 – 29
AKD26	NLSPYFKTNSSDFAQFG	195 – 213
AKD27	PYIKNYNNTSYVKQEAFV	395 – 410

Figure 4.4: Peptides from *Plasmodium vivax* EF – Tu (TufA) protein

a) Peptide 1: AKD19

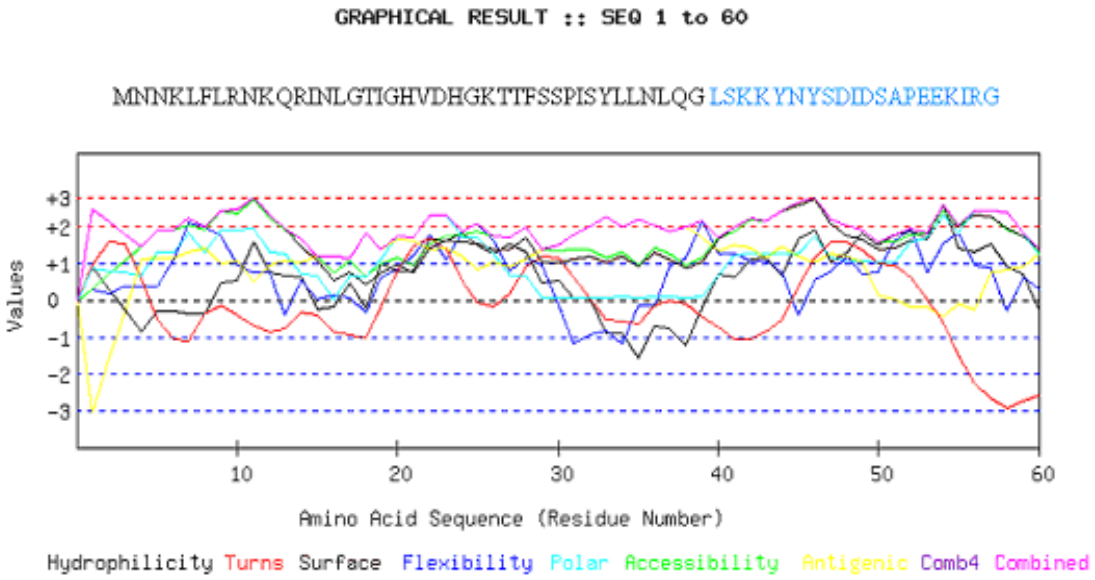

Figure 4.4 A: A scan of *P. vivax* TufA peptide AKD19

b) Peptide 2: AKD20

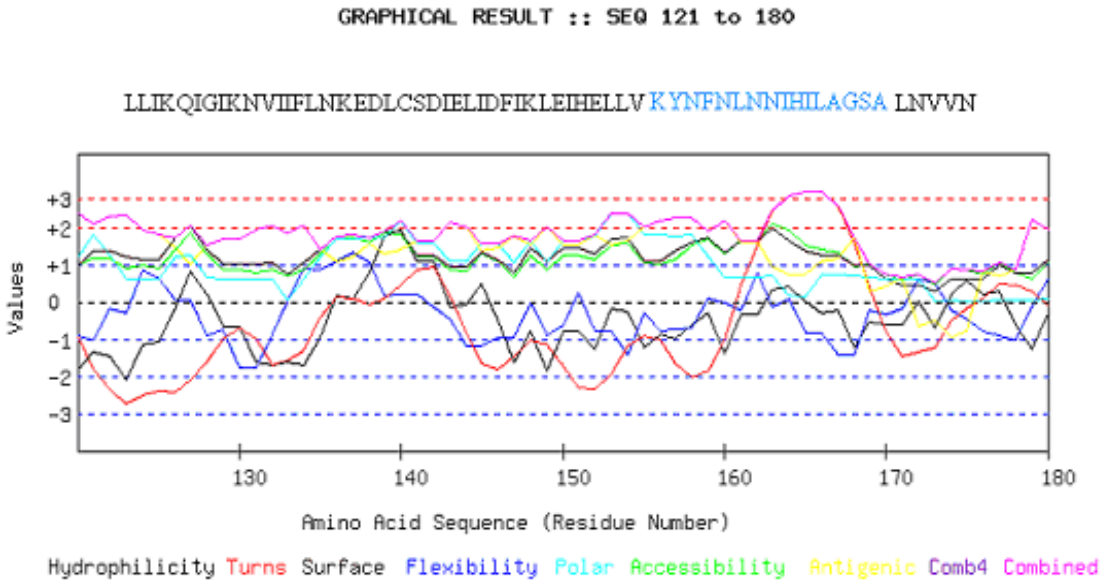

Figure 4.4 B: A scan of *P. vivax* TufA peptide AKD20

c) Peptide 3: AKD21

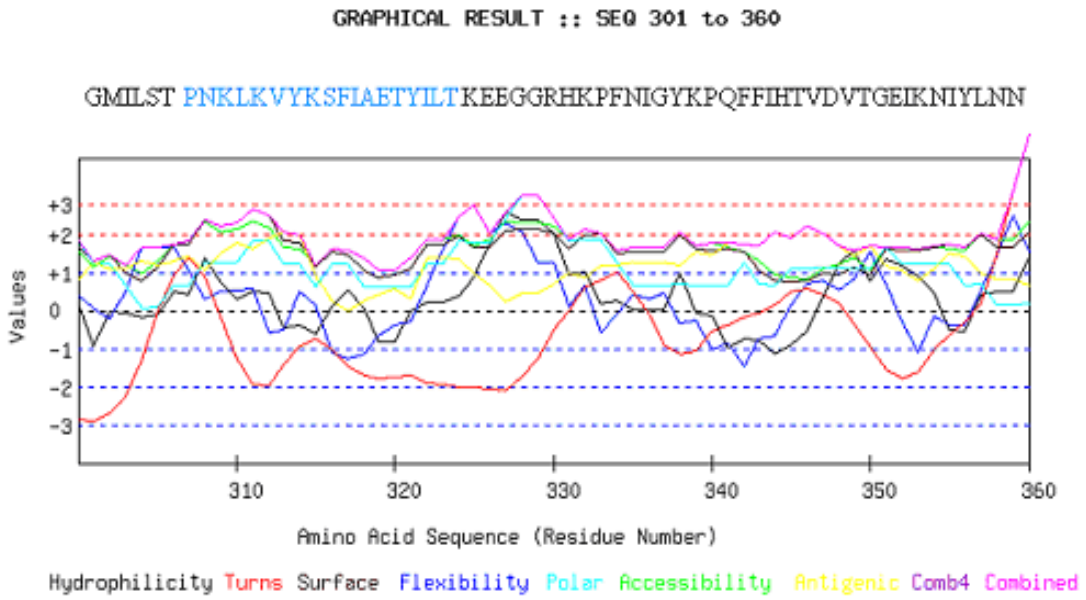

Figure 4.4 C: A scan of *P. vivax* TufA peptide AKD21

d) Peptide 4: AKD22

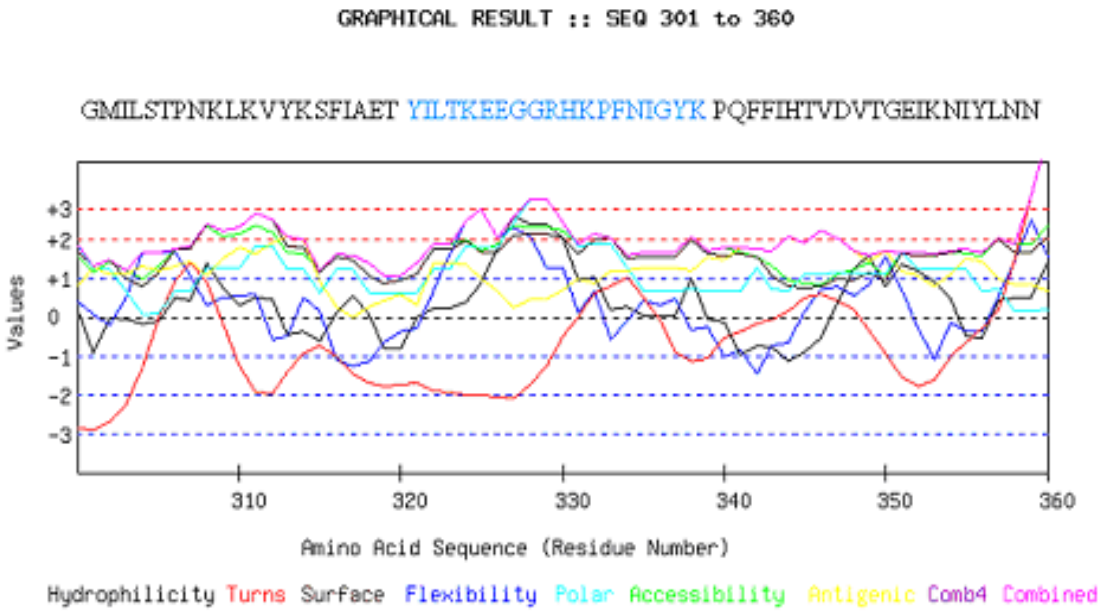

Figure 4.4 D: A scan of *P .vivax* TufA peptide AKD22

e) Peptide 5: AKD24 (*P. falciparum* TufA)

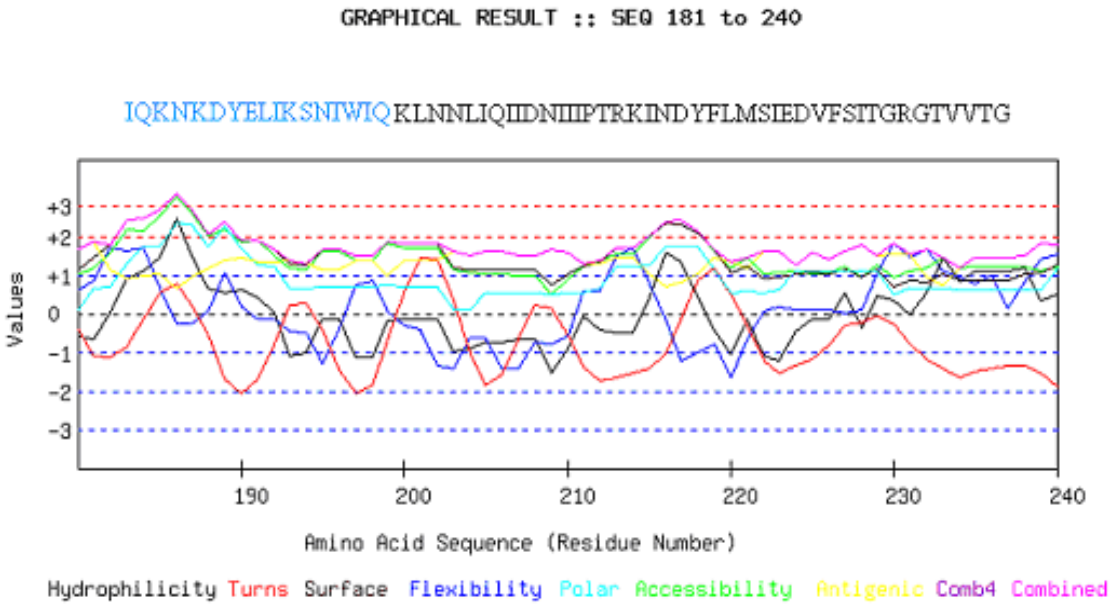

Figure 4.4 E: A scan of *P. falciparum* TufA peptide AKD24

Figure 4.5: Peptides from *P. vivax* SufB protein

a) Peptide 1: AKD25

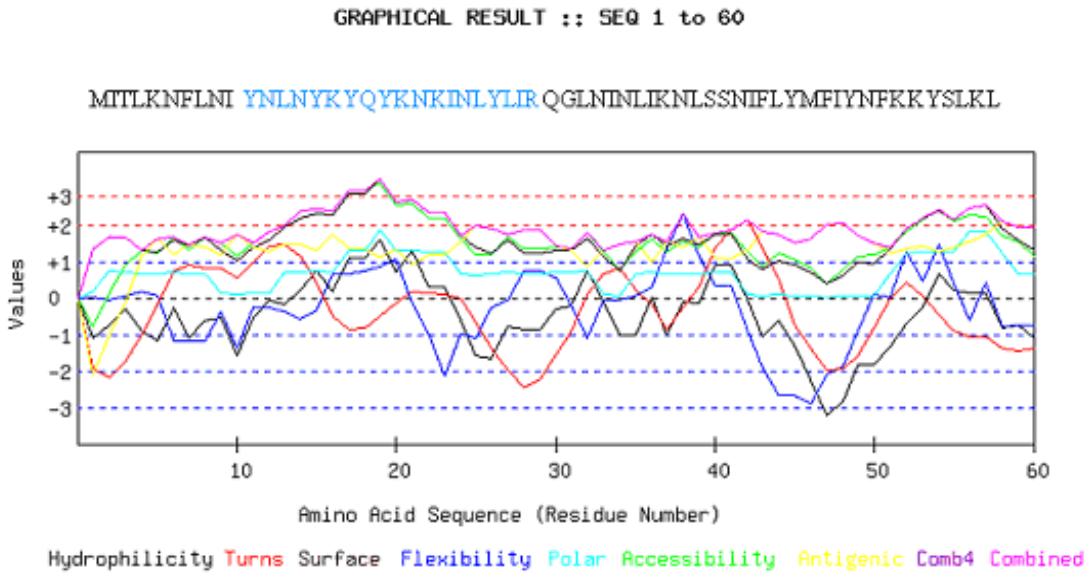

Figure 4.5 A: A scan of *Plasmodium vivax* SufB peptide AKD25

b) Peptide 2: AKD26

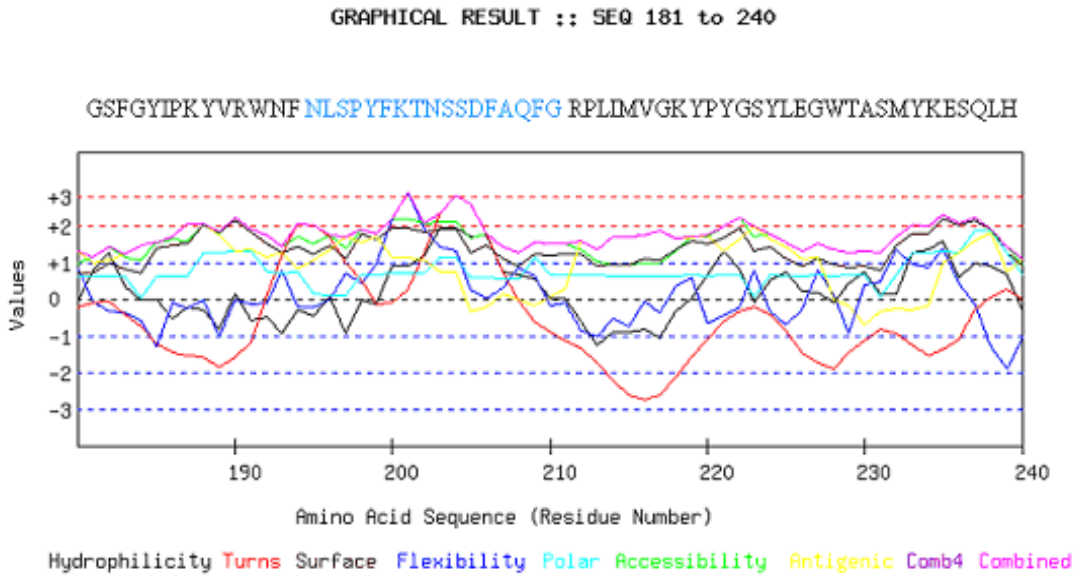

Figure 4.5 B: A scan of *P. vivax* SufB peptide AKD26

c) Peptide 3: AKD27

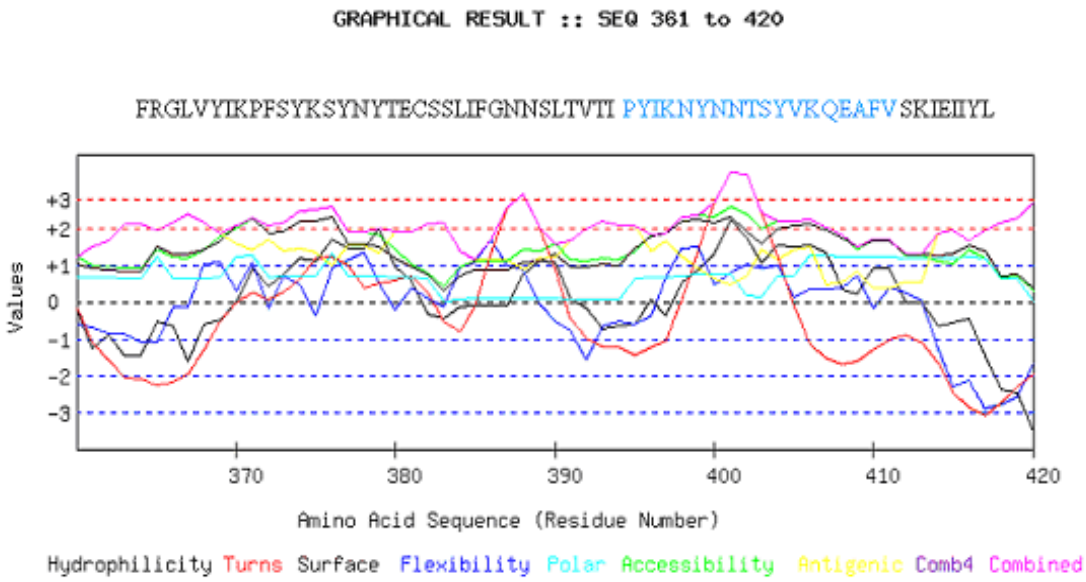

Figure 4.5 C: A scan of *P. vivax* SufB peptide AKD27