Results and Discussion

Expression studies were conducted based on the results obtained from In-silico experiments to compare the wild type and mutant protein of novel variation c.596-597delinsTT identified in exon 7 of the present study resulting in the change of alanine to valine at 199 position (p.A199V). The differences in the activities of wild type and mutant proteins were studied by assay of IDO activity.

5.5 RECOMBINANT WILD TYPE AND MUTANT CLONES OF IDO GENE

Human IDO cDNA was cloned into pGEX-KG mammalian expression vector by restriction digestion of vector and IDO cDNA using xhoI and EcoRI enzymes followed by ligation of digested vector and IDO. The ligation product was transformed into Top10 competent cells and spread on agar plates and the plates were incubated at 37°C overnight. Colony PCR (Figure-5.22) was performed to identify IDO positive clone by amplifying the IDO gene of recombinant vector and also by restriction digestion (Figure-5.23). The pGEX-KG (WT) wild type recombinant plasmid was subjected to automated DNA sequencing to confirm the incorporation of the wild type sequence.

![Colony PCR](image1.png)

**Figure-5.22:** Colony PCR showing amplification of IDO CDNA in recombinant wild type (W) and mutant (M) plasmids. (L-1kb ladder)

![Restriction digestion](image2.png)

**Figure-5.23:** Restriction digestion of recombinant wild type (W) and mutant (M) plasmids showing release of IDO insert.

Mutant vector (p.A199V) was generated by using mutagenesis kit (Geneart® Site-Directed Mutagenesis kit, U.S). The pGEX-KG (WT) recombinant plasmid was used as template to introduce p.A199V mutation in IDO cDNA and also using complimentary mutagenic primers 5’GGGACACTTTGCTAAAGGTTCTGTTGGAAATAGCTTC3’. The pGEX-KG (MT) plasmid with mutation A199V was subjected to automated DNA sequencing to
confirm the incorporation of the mutant sequence (c.596-597delinsTT). The pGEX-KG (MT) recombinant plasmid was transformed in to Top10 cells as described above and confirmed by colony PCR (Figure-5.23) and restriction digestion (Figure-5.24).

5.6 OVER EXPRESSION AND PURIFICATION OF IDO WILD TYPE AND MUTANT PROTEINS

The pGEX-KG (WT) and pGEX-KG (MT) plasmids from Top10 cells were introduced into competent *Escherichia coli* strain BL21 as described under methods to check for over expression of recombinant proteins after inducing with IPTG (Figure-5.24). The wild type and mutant proteins were expressed as GST fusion proteins. The over expressed proteins were purified to homogeneity by a one-step procedure involving glutathione-agarose affinity chromatography. The homogeneity of purified mutant and wild-type proteins were assessed by SDS-PAGE. Figure- 5.25 depicts SDS-PAGE gel of the purified protein of wild-type and mutant (p.A199V) IDO proteins. The purified wild type and mutant proteins were used for further analysis.

![Figure-5.24](image)

**Figure-5.24:** SDS-PAGE showing expression of wild type and mutant IDO fusion proteins of 71kDa (IDO protein 45kDa + GST 26kDa). Lanes UW and IW represent uninduced and induced wild type proteins. Lanes UM and IM represent uninduced and induced mutant proteins. M represents molecular weight marker.

![Figure-5.25](image)

**Figure-5.25:** SDS-PAGE showing the purified protein of wild-type (W) and mutant (M) IDO proteins (71kDa) along with BSA standard (66kDa).
5.7 ASSAY OF IDO ACTIVITY

Concentration of the purified wild type and mutant proteins were estimated by a modified Bradford method using Bovine serum albumin (BSA) as standard (Bradford, 1976). Protein levels were observed to be reduced in mutants. Further, the differences in the kinetic properties of wild type and mutant IDO proteins were checked by the method of Takikawa, (2005). For this 125 ng of IDO wild type and mutant proteins were incubated with a range of tryptophan concentrations (0, 10, 20, 30, 40,50, 60,70,80,90,100 and 110 µM ) for 1 hr and formation of kynurenine was then measured at $\lambda_{\text{max}}$ 480 nm on Scinco S-3100 UV-visible spectrophotometer.

Using the reciprocal values of substrate concentration (1/[S]) and reaction velocity (1/V) Lineweaver-Burk graph was plotted to obtain a straight-line graph (Graph-6). From the graph, the Vmax and Michaelis-Menten constant (Km) were calculated through linear regression of the data. The difference in the Km and Vmax were compared between the wild type and mutant proteins. The Vmax for wild type and mutant proteins were 17.86 ±0.17 and 21.18±0.11 µmol/min/ng. The Km of wild type and mutants were 68.66±0.26 and 74.92±0.40µM respectively, which showed significance difference (p: 0.0001). It indicates reduced affinity between the enzyme and substrate for mutant IDO protein as compared to wild type.

The low affinity between mutant IDO enzyme and substrates may be causing reduction in the rate of UV filter synthesis in lens leading to the development of cataract. The low levels of UV filters in lens, makes the lens more vulnerable to the exposure of UV light resulting in cross linking of crystalline proteins which is considered as a major cause for the loss of lens transparency resulting in cataracts.