The present study attempted to delineate the structure-activity relationship for D-hydantoinase from *Bacillus* sp. AR9 (bar9Hyd). D-hydantoinase is an industrial enzyme used for the commercial production of optically pure D- amino acids. It had been reported earlier that substituted hydantoins are precursors for synthesis of natural as well as unnatural amino acids. The relative ease and cost-effective way of synthesizing the 5-substituted hydantoins led many commercial organizations to make amino acid derivatives on large scales. However, the hydrolysis reaction gives racemic mixtures of both D and L-amino acids. Hydantoinase has been used to resolve the selectivity of the substrate for hydrolysis and to achieve the synthesis of the required isomer (Altenbuchner *et al.*, 2001). Hydantoinases have also been used for the synthesis of antibiotics (D-*p*-hydroxyphenylglycine), pesticides (D-valine), sweeteners (D-alanine) and peptide hormones through the production of non-natural amino acid intermediates (Ogawa and Shimizu, 1999). Many groups have attempted a directed evolution strategy to enhance the activity, stereospecificity and thermostability of hydantoinases (May *et al.*, 2000).

Hydantoinases belong to the urease family of enzymes and contain a catalytic TIM-barrel domain where the active site is present on the C-terminal end of the β-strands. Like all the other urease family enzymes (dihydroorotase (DHO), urease (URS), triphosphoesterase (TPE)), hydantoinases are dependent on two metal ions for their activity. Therefore, all the metal-binding amino acid residues in these enzymes are conserved. The hydantoinases contain a second domain formed from the N-terminal and C-terminal amino acid residues of the polypeptide chain. This domain is rich in β-strands and is believed to be involved in multimer formation. The TIM-barrel domain is an eightfold repetition of a β/α-motif wrapped in a circular fashion to make a central β-barrel. Each β-strand (B1 to B8) is connected to a α-helix (H1 to H8) through a front loop (FL1 to FL8), and each helix is connected to a strand by a back loop (BL1 to BL7). The active site in TIM-barrel enzymes is always situated either at the C-terminal ends of the β-strands or on the front loops (FLs). In the hydantoinases, the conserved metal-binding residues are present on the C-terminal ends of β-strands and on the FLs. So far, five structures of hydantoinases have been solved including the structure solution of bar9Hyd by Kishan and his co-workers (Kishan *et al.*, 2005). It had been observed that mercuric compounds and *N*-bromosuccinimde (NBS) completely abrogated the activity

of the enzyme. The active site of bar9Hyd is formed from amino acids His58, His60, Lys150, His183, His239 and Asp315. Surprisingly, Lys150, which was carboxylated in all the other urease family enzymes, has no modification in this hydantoinase. Whereas in all the other binuclear metal hydrolases, the Lys residue equivalent to Lys150 participates in the formation of coordination bonds with metal ions through carbamate modification (Kishan *et al.*, 2005).

By keeping all these facts into view, the present study was initiated to answer some interesting questions in context. As we know that mercury ions do have a preferential affinity to bind with the thiol groups (cysteine residues), and moreover these thiol groups can be alkylated by iodoacetate treatment to the proteins. Alkylation of bar9Hyd was carried out using iodoacetamide and iodoacetic acid. Iodoacetamide treatment led to a remarkable difference of 9% loss in activity, whereas iodoacetic acid treatment did not show any significant change. Then, mercury ions treatment to native and alkylated enzymes showed complete precipitation of proteins. Native-PAGE, SDS-PAGE, fluorescence and circular dichroism studies did not show much difference among the native and alkylated samples. Also the crystallization set-up of alkylated enzyme in a condition that yielded bullet shape crystals for native hydantoinase, did not produce any crystals. This indicated that some significant changes had happened in the enzyme, which inhibited the nucleus formation of crystal. Crystallization set-ups for mercury treated hydantoinase could not be attempted due to precipitation problem. Also initial crystallization trials for alkylated hydantoinase did not show any favourable results. Ultimately, mercury-soaking of bullet shape crystals was carried out with respect to variable time periods. It was found that only 14 hours of mercury soaking was feasible for diffraction data collection, because soaking crystals for more than 14 hours did not diffract well. Structure solution and refinement studies of this experimental data produced a mercury-bound structure (bar9Hydhg) at 2.8 Å resolution. At comparison level, four of the five cysteines were amalgamated and the only exception was Cys264. Additionally, there were some significant deviations near to the active site area, and the loop region close to Cys93 residue. So these structural changes might be the initiation of structural collapse of hydantoinase and thereby imparting diminished activity, possibly due to metal toxicity.

N-bromosuccinimide (NBS) also inhibited the activity of bar9Hyd completely. There are three considerations to accommodate this observation – first, the structure of NBS resembles hydantoin ring, so probably it may act as an inhibitor to the hydantoinase; second, it modifies the indole ring of tryptophans 108 / 287 (nearby the active site) to oxindole ultimately leading to the complete loss of activity, third, both of these phenomena may occur side by side resulting in complete loss of hydantoinase activity. It is noticeable that NBS prefers tryptophans to react with at acidic pH. So various optimization trials such as effect of acidic pH on enzyme activity, NBS treatment to enzyme at acidic pH, optimization of NBS concentration treatment to enzyme etc. were carried out. Our experimental results showed that 1mM concentration of freshly prepared NBS solution, D-hydantoinase at pH 6.0 and a total reaction time of about a minute is optimal to diminish the activity of enzyme completely. Activity assay, UV-absorption and fluorescence studies also showed that NBS reaction with bar9Hyd was very fast and was over within one minute. Fluorescence studies also demonstrated that NBS quenched tryptophans in majority. Native-PAGE profile convinced us that there was a large mobility shift, possibly due to important change in the net charge of hydantoinase molecule. CD studies revealed that NBS treatment disturbed the α-helical interactions of the enzyme. D-hydantoinase was also treated with the structural analogue of NBS, *N*-hydroxysuccinimide (NHS). This experiment proved that there was no significant difference in the activity of native and NHS treated hydantoinase, vaguely hinting towards the fact that NBS did not bind or block the active site of enzyme. Crystallization screens for NBS treated bar9Hyd did not produce any crystals. Alternatively, bullet shape crystals of bar9Hyd were tried for NBS soaking with respect to various time-periods. After various trials 15 hours soaked crystal diffracted, but produced relatively a discontinous electron density map. Importantly, there was no appropriate electron density for *N*- bromosuccinimde either bound to the hydantoinase or bound in the active site of the enzyme, which convinced us comprehensively that NBS did not bind to the molecule. Thus, NBS diminished the activity of hydantoinase by only modifying the tryptophans 287 or 108 near to the active site, also very well supported by biochemical studies.

Recombinant hydantoinase bar9Hyd (Nat-H) was expressed, purified and treated with 1 mM concentration of cobalt and nickel chlorides. Contrary to the reported results

for wild type bar9Hyd, Nat-H got complete precipitation after metals treatment. Consequently, hydantoinase was over-expressed in the absence of any metal ions otherwise, which were Mn ions as per standard procedure of expression for native hydantoinase. This hydantoinase was termed as no metal (NM-H) hydantoinase. SDS- and Native- PAGE profiles of NM-H were similar to that of Nat-H respectively, whereas relative activity of NM-H was found to be about 7% of Nat-H. Interestingly, Mn^{+2} ions treatment to purified NM-H led to the activity similar to that of Nat-H. Like *in vivo* $MnCl_2$ supplementation, various concentrations of $CoCl_2$ and $NiCl_2$ were also tried during the growth of culture. However this experiment was not successful because these ions proved to be toxic for the host strain *E. coli* BL21 (DE3). *In vitro* carboxylation of lysine residues of Nat-H and NM-H was carried out. First of all, Nat-H and NM-H were dialyzed into carbonate buffer, pH 9.6 and then treated with 50 mM $NaHCO_3$ at 25 °C. Further, these samples were treated with various metals ions such as Mn, Co, Ni and Zn followed by kinetic activity assay for each of the protein sample. Zn ions treatment led to the precipitation of enzymes, and Co and Ni ions treatment showed light precipitation, whereas Mn ions treatment showed no precipitation. Kinetic activity assay demonstrated that carboxylated hydantoinases have higher activities in comparison to that of native hydantoinase. As an interesting inference, it was found that metals treatment to carboxylated-NM-H showed higher activities, while the same metals treatment to NM-H had shown very less activity, in comparison to that of Nat-H. This experiment gave a hint in a vague manner that carboxylation of lysine residues, particularly Lys150, lead to proper coordination of metals into the active site, thus enhanced activity was observed. Native-PAGE was run for the native and carboxylated samples. It showed that carboxylated hydantoinases had higher mobility than that of uncarboxylated enzyme indicating carboxylation had happened and had imparted charge on to the molecule leading to higher mobility.

CD melting spectra at 215 nm showed that carboxylated hydantoinases were more stable than that of their native counterparts. Crystallization screens for carboxylated hydantoinase did not produce any favourable results. However, a new crystal form (spear-head shape) of native hydantoinase was obtained, whose diffraction statistics were good up to 3.2 Å. It was determined that crystal was of different symmetry i.e. orthorhombic C and that the space group was *C*222. On the other hand, structural comparisons of various hydantoinases could not argue why active site lysine 150 was not

carboxylated in case of bar9Hyd, whereas the corresponding lysine was carboxylated in all the other binuclear metal hydrolases. Even the nearest structural and sequence-wise neighbour of bar9Hyd, bsHyd had carbamated lysine. This unmodified lysine was not an artifact of the over-expression, for many enzymes of this family including bsHyd had been reported to be over-expressed in *E. Coli,* which contain carboxylated lysine residue at the active site. Conclusively this question still remained unsolved, but as per observations from carboxylation trials, it is certain that carboxylated lysine is required for higher activities in hydantoinases.