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SUMMARY OF DOCTORAL THESIS

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Title: Molecular studies on trimethylaminobutanol dehydrogenase and trimethylaminobutyraldehyde dehydrogenase produced by *Pseudomonas* sp. 13CM

（*Pseudomonas* sp. 13CMのトリメチルアミノブタノール脱水素酵素及びトリメチルアミノブチルアルデヒド脱水素酵素の分子的研究）

The present study was aimed at a molecular investigation of trimethylaminobutanol dehydrogenase (TMA-Butanol-DH) and trimethylaminobutyraldehyde dehydrogenase (TMABaldehyde-DH) produced by *Pseudomonas* sp. 13CM. Genes encoding these enzymes have been cloned and sequenced. The recombinant enzymes were over-expressed in *Escherichia coli* and properties of recombinant TMABaldehyde-DH were investigated. Newly discovered TMABaldehyde-DH (TMABaldehyde-DH II) is also discussed. Apart from the characterization of both types of TMABaldehyde-DHs, several critical residues of TMABaldehyde-DHs are investigated.

The introductory part defines the background of this study and set the aim. The review of literature section provides the selective reviews relevant to this work.

In Chapter 1, genes encoding TMABaldehyde-DH and TMA-Butanol-DH produced by *Pseudomonas* sp. 13CM were identified. Oligonucleotidic probes were designed corresponding to the N-terminal amino acid sequences of the both enzymes and corresponding to the conserved sequences in alcohol and aldehyde dehydrogenases. DNA fragment containing core regions were obtained by PCR. To get the unknown part of the gene, inverse-PCR primers were synthesized using the known region in the genomic DNA. The sequences achieved from the inverse-PCR approach, connecting the flanking region and core region led the construct of full sequences of the genes encoding TMABaldehyde-DH and TMA-Butanol-DH. The primary structures of TMABaldehyde-DH and TMA-Butanol-DH showed high identities with, respectively, putative aldehyde dehydrogenase (94.0%) and iron containing alcohol dehydrogenase (87.0%) of *Pseudomonas putida* GB-1. The section also covers the over-expression of these enzymes in *E. coli* and properties of recombinant TMABaldehyde-DH. The enzyme had a molecular mass about 160 kDa by gel filtration, and was trimer with identical 50 kDa subunits. Optimum pH and temperature were found to, respectively, pH 9.5 and 50 ºC. The kinetic analysis showed the K_m values for 4-*N*-trimethylaminobutyraldehyde

(TMABaldehyde), 4-dimethylaminobutyraldehyde (DMABaldehyde), and NAD^+ respectively, 0.12 mM, 0.07 mM, and 0.15 mM. Characterization of purified recombinant TMABaldehyde-DH, confirmed that the enzyme had essentially the same properties as TMABaldehyde-DH purified from *Pseudomonas* sp. 13CM. Molecular cloning with *Pseudomonas* sp. 13CM as a gene donor led to the production of a large quantity of the enzyme in a recombinant strain.

In Chapter 2, the gene encoding TMABaldehyde-DH from *Pseudomonas* sp. 13CM was isolated by shotgun cloning and expressed in *E. coli* DH5α. The structural gene encoding TMABaldehyde-DH inserted into an expression vector pET24b (+) and transformed to *E. coli* BL21 (DE3). Over-expressed enzyme was purified to apperant homogeneity and biochemically characterized. The molecular mass of the enzyme was estimated at about 150 kDa using gel filtration. The enzyme was found to be a trimer with identical 52 kDa subunits by SDS−PAGE, and about 51 kDa by MALDI-TOF MS analysis. The isoelectric point was found to be 4.5. Optimum pH and temperature were found, respectively, pH 9.5 and 40 °C. The K_m values for TMABaldehyde, DMABaldehyde, and NAD^+ were 0.31 mM, 0.62 mM, and 1.16 mM, respectively. It was observed that the molecular and catalytic properties clearly differed from those of TMABaldehyde-DH I, which was discovered initially in *Pseudomonas* sp. 13CM and renamed the obtained second enzyme "TMABaldehyde-DH II". Discovered TMABaldehyde-DH can now be obtained in large quantity necessary for further biochemical characterization and possible applications.

The study in Chapter 3 was attempted to investigate the several critical residues located in the portion of the substrate binding pocket of TMABaldehyde-DHs (TMABaldehyde-DH I and TMABaldehyde-DH II) of *Pseudomonas* sp. 13CM. Based on the homology model and alignment, clear difference in residues between TMABaldehyde-DH I and II were found around the entrance to the substrate channel (the residues Asp457, Glu458, and Leu459 in TMABaldehyde-DH I; and corresponding positioned residues Gln437, Ala438, and Val 439 in TMABladehyde-DH II). Site-specific mutagenesis was conducted with the replacement of selected residues of one enzyme with that of the corresponding positioned residues of another, and *vice versa*. Concerning the catalytic properties towards substrates, all of the mutants exhibited a substantial decrease in activity. Additionally, TMABaldehyde-DH I mutants displayed a consistent increase of K_m both for substrate and cofactor. In contrast, mutant A438E and V439L of TMABaldehyde-DH II exhibited kinetic properties with a significant improvement in the affinity. Because Q437D mutant of TMABaldehyde-DH II exhibited extremely low level of activity, the Gln residue and its corresponding residue of *Pseudomonas* sp. 13CM TMABaldehyde-DHs are important for binding and formation of successive products. The reported data represent a valuable resource to understand the relationship between structure and function of TMABaldehyde-DHs.