(Format No. 3)

SUMMARY OF DOCTORAL THESIS

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Title: Novel Non-NadB Type L-Aspartate Dehydrogenases from *Pseudomonas aeruginosa* PAO1 and *Ralstonia eutropha* JMP134: Molecular Characterization, Application, and Physiological Function

(***Pseudomonas aeruginosa*** **PAO1と*Ralstonia eutropha* JMP134株由来新規非NadB型アスパラギン酸デヒドロゲナーゼの分子特性、応用と生理機能)

L-Aspartate dehydrogenase (L-AspDH, EC 1.4.1.21), a member of amino acid dehydrogenase family, catalyzes the reversible oxidative deamination of L-Asp to oxaloacetate (OAA) with the concomitant reduction of $NAD(P)^+$ to NAD(P)H. It is extremely rare and so far has only been reported in the thermophilic bacterium *Thermotoga maritima* (TmaAspDH) and the archaea species *Archaeoglobus fulgidus* (AfuAspDH). The crystal structures of these 2 enzymes have been well determined to be complexed with NAD^+ alone in TmaAspDH and with NAD^+ and citrate (a substrate analog) in AfuAspDH. Although both of them have been reported with strong thermal properties, the catalytic activity was very low at room temperature, which limits their applicability at mesophilic temperatures. At the genomic level, both AfuAspDH and TmaAspDH genes form an operon with the NAD biosynthesis genes *nadA* and *nadC*. Their products are therefore proposed to play roles as NadB type enzymes, previously known as LAO. To date, no mesophilic non-NadB type AspDH has been reported. Taking this into account, our interest in novel mesophilic AspDHs led us to screen for them in mesophilic microorganisms; many mesophilic AspDHs were found by performing a BLAST (tblastn program) search of DNA databases translated into protein using AfuAspDH as the seed. In this thesis, two mesophilic non-NadB type AspDHs, identified from bacteria *Pseudomonas aeruginosa* PAO1 (PaeAspDH) and *Ralstonia eutropha* JMP134 (ReuAspDH), were characterized in detail.

Biochemical characterization revealed that the enzymatic properties of ReuAspDH and PaeAspDH were extremely similar. The homogeneously purified PaeAspDH and ReuAspDH were dimeric proteins with molecular mass of about 28 kDa exhibiting very high specific activity for L-aspartate (L-Asp) of 127 and 137 U/mg, respectively, which are much higher than that of the thermophilic AfuAspDH at 50°C (4.6 U/mg). Both enzymes were capable of utilizing NAD and NADP as coenzyme. PaeAspDH and ReuAspDH showed optimal reaction temperatures of 48°C and the T_m values of 48 and 49°C, respectively, for 20 min that was improved to approximately 60°C by the addition of 0.4 M NaCl or 30% glycerol. The apparent K_m values of PaeAspDH for OAA, NADH and ammonia were 2.12, 0.045 and 10.1 mM, respectively; comparable results were observed with NADPH. The K_m values of PaeAspDH for L-Asp were 4.87 and 4.74 mM with NAD and NADP as coenzyme, respectively; same K_m value of 0.47 mM for NAD^+ and $NADP^+$ was also observed. In contrast, the apparent K_m values of ReuAspDH for L-Asp and OAA were 12- and 3-folds higher when NAD^+(H) were used as coenzyme than those when $NADP^+$(H) were used as coenzyme. Noting that the K_m value of ReuAspDH for $NADP^+$ was

approximately 8-fold higher than that of NAD^+, it is likely that NAD^+ is preferentially used as the cofactor for the oxidation of Asp *in vivo*. In addition, the apparent K_m value of PaeAspDH and ReuAspDH for NH_4^+ were much lower than that of AfuAspDH (167 mM), which suggests that these two mesophilic AspDHs recognizes NH_4^+ in a different manner than the thermophilic NadB type AspDHs.

L-Asp, an essential amino acid, is the main ingredient of the artificial sweetener and also serves as the precursor for biosynthesis of threonine, methionine, lysine, and NAD. In this study, PaeAspDH was applied for L-Asp production in three coupled enzymes systems. The L-Asp production system B consisting of PaeAspDH, *Bacillus subtilis* malate dehydrogenase (BsMDH) and *Escherichia coli* fumarase (EcFum) in the same cells, achieved a high level of L-Asp production (625 mM) from fumarate in fed-batch process with a molar conversion yield of 89.4%. Furthermore, the fermentative production system C (PaeAspDH and BsMDH) released 33 mM of L-Asp after 50 h by using succinate as carbon source. Interestingly, the addition of NAD^+ in enzymatic production system was not necessary at all, which reduces the production cost of L-Asp. In fermentation system, addition of over 0.8% of Triton X-100 could improve L-Asp production. These novel production systems illustrate the potential for developing new processes for production of important amino acids and industrial compounds from cheaper substrates.

Bioinformatic analysis revealed that ReuAspDH and PaeAspDH genes individually formed a gene cluster with their 12 neighboring genes in *R. eutropha* JMP134 and *P. aeroginosa* PAO1 genomes, respectively, which do not encode the enzymes for NAD biosynthesis. This gene cluster is also conserved in the genomes of 45 bacteria in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Release 57.0, January 1, 2011). Most of these bacteria, including representative PHA-producing bacteria, *R. eutropha* H16 and *P. aeruginosa* PAO1, possess *phaC*, which is absolutely necessary for poly-3-hydroxyalkanoate (PHA) biosynthesis. The existence of *nadB* genes in these 2 bacteria eliminates the possible involvement of the L-AspDH gene in NAD biosynthesis. These mesophilic L-AspDHs, considered as non-NadB type enzymes, might play the same physiological role described in the above bioinformatics analysis. Unlike the NadB type enzyme, ReuAspDH may have evolved to catalyze the first step in the catabolism of L-Asp. To gain insight into non-NadB type L-AspDH, Q-PCR was performed to determine the expression levels of 37 genes involved in the ReuAspDH gene cluster, TCA cycle, gluconeogenesis, PHA and NAD biosynthesis, and other pathways. GC-MS analysis was performed to detect PHA in wild-type *R. eutropha* strain JMP134 and ΔB3576 mutant grown in the presence of L-Asp or fructose. Q-PCR analysis indicated that the genes involved in AspDH gene cluster, PHA biosynthesis, and TCA cycle were considerably induced by L-Asp in wild-type cells. In contrast, the expression of aspartase (AspA) and aspartate aminotransferase (AspC) genes was substantially induced in AspDH gene knockout mutant (ΔB3576) but not in wild type. These results suggested that AspA and AspC substituted for AspDH to assimilate L-Asp in the ΔB3576 mutant. GC-MS analyses revealed that wild-type strain could synthesize poly-3-hydroxybutyrate (PHB) from fructose or L-Asp, whereas the ΔB3576 mutant could not synthesize any type of PHA from L-Asp. The AspDH gene cluster products might be involved in the biosynthesis of PHA precursor, thus revealing that the AspDH is a non-NadB type enzyme, which is entirely different from previously reported NadB type enzyme involved in NAD biosynthesis.