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SUMMARY OF DOCTORAL THESIS

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Title: Mutational Studies on Catalytic Domain of D-stereospecific Amidohydrolase from *Streptomyces* sp. 82F2.

（放線菌由来D体特異的アミド加水分解酵素の触媒領域における変異解析）

Recently D-Stereospecific amidohydrolase (DAH) obtained from *Streptomyces* sp. 82F2 isolated from soil samples. The enzyme belongs to S12 family serine peptidase and categorized as D-Stereospecific peptidase. Comparison of the DAH crystal structure with the structures of other members of the S12 peptidase family showed that the substrate specificity of DAH arises from its active site structure. DAH exhibited high aminolysis reaction in accordance with hydrolysis. DAH recognizes D-amino acyl ester derivatives as substrates and catalyzes hydrolysis and aminolysis to yield D-amino acids and D-amino acyl peptides or amide derivatives, respectively. In the aminolysis, DAH preferentially utilizes D-amino acyl derivatives as acyl donor and L-amino acyl derivatives as acyl acceptor, the enzyme is of particular interest for synthesizing dipeptides with a DL-configuration.

Crystal structure of DAH that bind with 1,8-diaminooctane was resolved at resolution 1.49Å((PDB: 3WWX), the structural analysis has revealed that DAH possesses large cavity leads to catalytic center S86 which positioned at the center of the large cavity, close to the catalytic center of Ser86, lys89 and Tyr191 there is a small pocket at the bottom. Because the pocket is close to the catalytic center and is thought to interact with substrates during the catalytic reaction. Structural compersion of S12 family members and DAH revealed the overall structures of S12 enzyme family members are similar, although there are significant differences in terms of the shapes and sizes of the cavities and active site pockets among them. In DAH the enzyme has a large cavity and active site pocket. This large cavity in DAH allows the peptide substrate to enter, and the large space in the active site pocket accommodates the large side chain of the acyl donor substrate. DAH pocket composes of a number of hydrophobic residues these residues seem to involved in acyl donor and acyl acceptor during the catalytic reaction. the active site pocket plays a functional role in substrate recognition, and the factors related to the hydrolysis, preference of acyl acceptor in the aminolysis reaction and stereoselectivity of the substrate are still unclear. Therefore, the present study was conducted to elucidate the function of the pocket in the catalytic activity of DAH. we investigated the role of residues constituting the active site pocket via mutational analysis(we used site-directed mutagenesis), these investigations have illustrated the strong relationship between the pocket structure and catalytic activity such as acyl-enzyme intermediate formation, hydrolysis reaction, and aminolysis. overall results provide useful information insight into the mechanism of substrate recognition aiming to develop a convenient biocatalyst for peptide synthesis.

Chapter one outlines the aims of this dissertation, provides a literature review of the relative studies on serine protease and their structure and mechanism of the reaction, and also provides a brief background on the origin of DAH, crystal structure, mechanism of catalytic reaction and function of biologically active dipeptide synthesis by aminolysis reaction of DAH.

In Chapter two, we analyzed the function of the eight residues that form the DAH active site pocket and seem to be involved in substrate recognition, (Tyr144, Thr145, Phe150, Val154, Phe155, Ile266, Ile338, and His339), in terms of substrate recognition and aminolysis by mutational analysis (site-directed mutagenesis). The formation of the acyl-enzyme

intermediate and catalysis of aminolysis by DAH were changed by substitutions of selected residues with Ala. In particular, I338A DAH exhibited a significant increase in the condensation product of Ac-D-Phe methyl ester and 1,8-diaminooctane (Ac-D-Phe-1,8-diaminooctane) compared with the wild-type DAH. A similar effect was observed by the mutation of Ile338 to Gly and Ser. The pocket shapes and local flexibility of the mutants I338G, I338A, and I338S are thought to resemble each other. The data indicated that IIe I338 is an important residue for substrate recognition among the pocket residues. Thus, changes in the shape and local flexibility of the pocket of DAH by mutation presumably alter substrate recognition for aminolysis.

In chapter three, this study addresses the effects of modifying the active site pocket of DAH on the recognition of stereoselectivity and hydrophobicity of substrates and on the formation of enzyme-acyl intermediate. Structural comparison of DAH with substrate-bound D-amino acid, amidase revealed that three residues located in the active site pocket of DAH (Thr145, Ala267, and Gly271) might be involved in interactions with D-phenylalanine substrate. We substituted Ala267 and Gly271, which are located at the bottom of the hydrophobic pocket of DAH, with Phe and observed changes in the stereoselectivity and specific activity toward the free and acetylated forms of D/L-Phe-methyl esters. In contrast, the mutation of Thr145, which likely supplies a negative charge for recognition of the amino group of the substrate, hardly affected the stereoselectivity of the enzyme. Substrate binding by DAH was disrupted by the mutation of Ala267 to Val or Trp and kinetic analysis showed that the hydrophobicity of the bottom of the active site pocket (Ala267 and Gly271) is important for both stereoselectivity and recognizing hydrophobic substrates.

In Chapter four, this study aims to assess the effect of the space filling of the active site pocket of D-stereospecific amidohydrolase (DAH) on catalytic activity in order to enhance the aminolysis activity of the enzyme. Two mutants A267F and G271F DAHs were designed to fill the space of active site pocket of DAH. Then we investigated the effect of space modification on the acyl-enzyme intermediates formation, hydrolysis, and aminolysis catalysis. Methanol release from acetylated D-Phe methyl ester, which represents the acyl-enzyme intermediate formation, was observed to be higher in A267F by three-fold than that of WT. In addition, A267F DAH exhibited a significant increase in condensation production by aminolysis reaction when L-Trp was used as acyl acceptor substrate. In contrast to A267F DAH, no enhancement rather decrease in activity was observed by the mutation of Gly271 to Phe. A similar effect of mutation was observed in the efficiency of the synthetic activity of chitinases inhibitor cyclic dipeptide, cyclo(D-Pro-L-Arg), the lead compound for the development of antifungal reagents and insecticides. A276F DAH showed high aminolysis activity and improved the reaction rate for cyclo(D-Pro-L-Arg) production. This mutant might be considered as a high-performance biocatalyst for the synthesis of the biologically active dipeptides.

Overall, this study provides insight into the mechanism and structural factors affecting substrate recognition and stereoselectivity of DAH, the DAH binding pocket plays a potent role in recognition for both the hydrophobic and stereoselective substrates. The results of this study also show the relationship between the pocket size/shape, electrostatic environment, local flexibility of active site and aminolysis reaction. the overall results that obtained from the present study will contribute to the understanding of the substrate specificity and the structural basis of the catalytic functions of DAH and family S12 serine peptidase.