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Cyclodextrin–Peptide Hybrid as a Hydrolytic Catalyst Having Multiple Functional Groups

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Abstract—A designed cyclodextrin–peptide hybrid, which has multiple functional groups on its α-helix peptide backbone, has been synthesized as a catalyst for ester hydrolysis. Kinetic study revealed that the carboxylate group plays a key role in this system.

Most enzymes have multiple functional groups in their catalytic center and those functional groups are placed on their appropriate positions. When those multiple functional groups work together, the enzyme can perform catalytic activity. For example, serine proteases, which are well known hydrolases, have hydroxyl (serine residue), imidazole (histidine residue), and carboxylate (aspartate residue) groups in their reaction center.[1] According to a previously proposed idea, Asp, His, and Ser form a stable triad as a strongly hydrogen-bonded and partially polarized system[1–3] that achieves efficient catalytic reaction. To construct a useful catalyst, precise placing of multiple functional groups is an essential requirement.

Among vast numbers of entries as trials for artificial enzymes, enzyme mimics based on cyclodextrin (CyD) or modified CyD have been studied extensively.[4] In these studies, CyD acts as a substrate binding site because of its remarkable property to include a guest molecule in its central cavity. On this basis, CyD derivatives having a benzoate conjugated imidazole[2,5] and tripeptide (serine-histidine-aspartate) attached CyD[6] were proposed as serine protease mimics. However, the effect and the role of those functional groups were ambiguous. On the other hand, recent development in solid phase peptide synthesis enables us to construct a molecule with well-defined secondary structure.[7] But, it is not easy to make a binding site for the substrate from only small peptides. Neither simple CyD derivatives nor small peptide fragments may deserve a superb artificial enzyme. The hybridization of CyD and peptide might overcome the difficulty, making a substrate binding site and placing the functional groups precisely. Under this circumstance, a CyD-peptide hybrid (EHβ19) containing imidazole (His10) and carboxylate (Glu6) was designed and synthesized.

In the design of EHβ19 (Fig. 1), for avoiding undesirable influence of the amino acid side chains, alanine that favors an α-helix structure[8] was chosen as the main component. In order to stabilize a helical structure of the peptide, two pairs of intramolecular salt bridges (Glu4-Arg8 and Glu12-Arg16)[9] were introduced into the opposite face of catalytic side. In place of aspartate in serine proteases, glutamate (Glu6) was arranged to maintain α-helix stability.[10] When the designed peptide forms an α-helix structure, all the functional groups, β-CyD, an imidazole (His10) and a carboxylate (Glu6) are supposed to be placed closely on the same site of the α-helix peptide to form a catalytic system by cooperation. In this system, β-CyD can act as a binding site for the substrate, and its primary or secondary hydroxyl groups may be expected to act like the hydroxyl group of serine. In order to examine whether the carboxylate works effectively or not, another CyD-peptide hybrid (Hβ19) which has an alanine (Ala6) in place of glutamate (Glu6) was prepared. Both α-helix peptides were synthesized by stepwise elongation of Fmoc-amino acid on a rink-amide resin. The side chains of His, Arg and Glu except for Glu14 have trityl (Trt), mesitylene-2-sulfonyl and benzyl groups, respectively, as side chain protection groups. Glu14 has *tert*-butyl (^{t}Bu) group for its side chain protection. Synthesized peptides were

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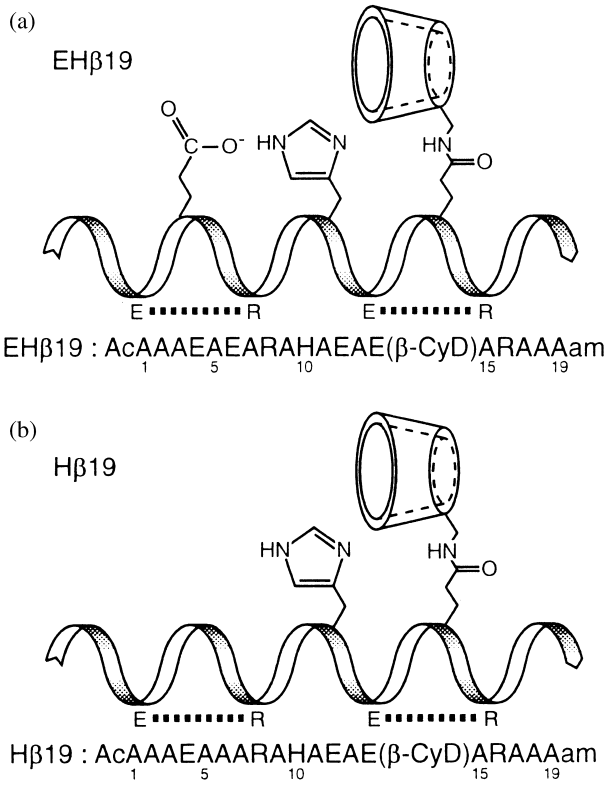

Figure 1. Illustration of EHβ19, Hβ19 and amino acid sequences.

cleaved from the resin by the treatment of trifluoroacetic acid, and the protection groups, ^{t}Bu of Glu14 and Trt of His10 were removed at this stage. 6-Mono-deoxy-6-amino-β-CyD was prepared as previously described[11] and selectively introduced into the side chain of Glu14 via an amide bond linkage. Finally, all protection groups were removed by trimethylsilyl trifluoromethanesulfonate.[12] Products were purified with reversed phase HPLC and identified by MALDI–TOFMS[13] and amino acid analysis.

The circular dichroism (CD) studies revealed that EHβ19 and Hβ19 showed a typical α-helical pattern with double negative maxima at 208 and 222 nm (Fig. 2) in the phosphate buffer solutions of various pHs.[14] The α-helix contents[15] of EHβ19 and Hβ19 were 73 and 67%, respectively. Both CyD–peptide hybrids have α-helix contents high enough to maintain proximity of the functional groups. The CyD-peptide hybrid with lower α-helix content (22%) results in lower catalytic activity, even if it has same functional groups.[16] *p*-Nitrophenyl acetate was chosen as a substrate. The hydrolysis was performed in various pHs of phosphate buffer solution and monitored as increasing UV absorbance of the product, *p*-nitrophenolate.[17] The concentration of each catalyst was fixed at 2.5×10^{-5} M,[18] and that of substrate was varied from 1.0 to 5.0×10^{-4} M. Excess conditions of the substrate were kept in each measurement. While both EHβ19 and Hβ19 did not have significant pH dependence on their α-helix contents, the catalytic activity of EHβ19 for the ester hydrolysis had a maximum point around pH 6.0. Hβ19 had no definite maximum point. Then, kinetic parameters of EHβ19 and

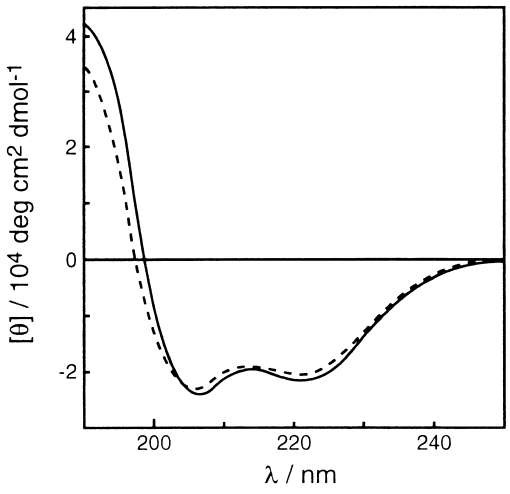

Figure 2. CD spectra of EHβ19(—) and Hβ19(- - -) in pH 6.0 phosphate buffer solution containing 1.25 vol% TFE (5.0×10^{-2} M).

Hβ19 were compared at pH 6.0 (Fig. 3) and summarized in Table 1. In the absence of any catalyst, the rate constant of the hydrolysis for *p*-nitrophenyl acetate (k_{un}) was 6.8×10^{-6} s^{-1}. The k_{cat}[19] values of EHβ19 and Hβ19 was 5.64×10^{-3} s^{-1} and 2.48×10^{-3} s^{-1}, respectively. Both catalysts accelerate the ester hydrolysis significantly comparing with the background. The Michaelis–Menten constants (K_m) for EHβ19 and Hβ19 were 9.87×10^{-4} M and 24.2×10^{-4} M, respectively. EHβ19 had 2.5 times higher advantage over Hβ19 in substrate binding. The apparent second order rate constant (k_{cat}/K_m) of EHβ19 was approximately 5 times higher than that of Hβ19 (Table 1). These results clearly suggest that the difference in the catalytic activity might derive from the existence of the carboxylate group of Glu6 in EHβ19. The values of transition state stability for the substrates (K_{TS})[20] were 0.12×10^{-5} M and

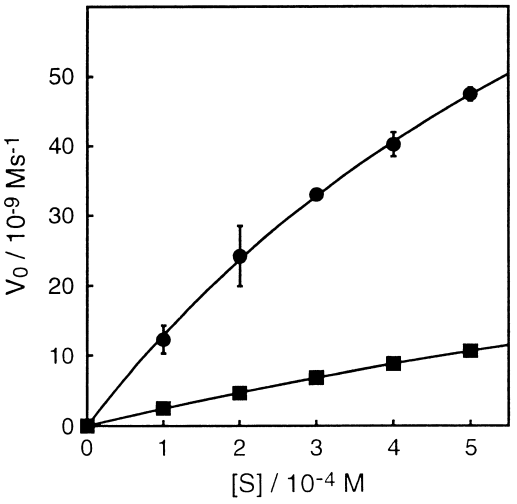

Figure 3. Hydrolysis of *p*-nitrophenyl acetate catalyzed by EHβ19(●) and Hβ19(■) in pH 6.0 phosphate buffer solution containing 1.25 vol% NMP. The concentration of EHβ19 and Hβ19 was 2.5×10^{-5} M. The concentration of the substrate was varied from 1.0×10^{-4} M to 5.0×10^{-4} M. The solid lines were obtained by least square curve fitting based on Michaelis–Menten equation.

Table 1. Kinetic parameters for the hydrolysis of *p*-nitrophenyl acetate catalyzed by EHβ19 or Hβ19 at 25 °C in pH 6.0 phosphate buffer solution containing 1.25 vol% NMP

	k_{cat} (10^{-3} s^{-1})	K_m (10^{-4} M)	k_{cat}/k_{un}	k_{cat}/K_m (M^{-1} s^{-1})	K_{TS} (10^{-5} M)
EHβ19	5.64	9.87	829	5.71	0.12
Hβ19	2.48	24.2	365	1.03	0.66

0.66×10^{-5} M for EHβ19 and Hβ19, respectively. EHβ19 shows also about 5 times superior value to Hβ19. This result suggests that EHβ19 could stabilize the transition state of the substrate more effectively than Hβ19. Namely, the existence of the carboxylate of Glu6 enhances the catalytic activity by stabilizing the transition state of the substrate. The k_{cat}/K_m and K_{TS} of the simple imidazole attached β-CyD (6Im-β-CyD) were 1.7 M^{-1} s^{-1} and 2.8×10^{-5} M, respectively.[21] Interestingly, the catalytic activity of EHβ19 is similar to that of 6Im-β-CyD, while both the CyD-peptide hybrids stabilize transition state of the substrate more effectively than 6Im-β-CyD. Especially, EHβ19 stabilizes the transition state of the substrate over 20 times than 6Im-β-CyD did. Those results suggest that placing of the functional groups at correct positions in the peptide backbone is favorable to stabilize the transition state of the substrate.

This is one of the successful examples of the artificial enzyme having appropriately placed multiple functional groups (an imidazole, a carboxylate, a binding site) on an α-helix peptide. Also this new approach of "cyclodextrin–peptide hybrid" may lead to novel possibility for the design and construction of the better artificial enzymes. The detailed study concerning the reaction mechanism and substrate selectivity is now under way.

Acknowledgements

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