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Enantioselective ester hydrolysis catalyzed by β-cyclodextrin conjugated with β-hairpin peptides

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This work is dedicated to the late professor Akihiko Ueno (deceased March 23, 2003)

Abstract—Designed cyclodextrin–peptide conjugates, which have one or two β-hairpin peptides, have been synthesized as catalysts for ester hydrolysis. One or two β-hairpin peptides were located at the primary hydroxyl group side of β-cyclodextrin so as to arrange two histidine residues that act as a general acid and a general base catalysts and provide the substrate recognition subsite. Kinetic studies revealed that the two-β-hairpin peptide was more effective than that of the one-β-hairpin peptide for substrate recognition.

Much effort has been devoted to demonstrate the construction methods of catalysts for the desirable reactions.[1] Enzymes generate their high catalytic activities and substrate selectivities by arranging multiple functional groups masterly in their catalytic centers. Then, a lot of entries as trials for artificial enzymes have been reported by chemical approaches.[2] One of the essential factors for constructing artificial enzymes is arranging the multiple functional groups at appropriate positions for a selective substrate binding and an effective catalytic reaction. In the design of artificial enzymes, conjugation of cyclodextrin (CD) and polypeptide could be a promising technique for providing binding sites and scaffolds. In the modified CD derivatives, CD acts as a substrate binding site because of its remarkable ability to bind a guest molecule.[3] In the de novo designed peptides, their well-defined secondary structures are useful as scaffolds on which multiple functional moieties are placed at their appropriate positions.[4] Previously, we reported that a cyclodextrin-peptide (CD-peptide) hybrid having a βCD, an imidazole and a carboxylate group on an α-helix peptide hydrolyzed ester substrates effectively with the cooperative work between the imidazole and the carboxylate groups.[5] In this case, however, the remarkable substrate selectivity was not observed because the simple substrate binding site of the βCD cavity might not be enough for selective substrate binding. The multi-points binding is important for selective binding of substrates. In addition to the binding site by a CD cavity, another substrate binding/recognition site is also necessary. Because it is difficult to construct substrate binding site using single α-helix peptide, three- or four-helix bundle structures are utilized for the construction of substrate binding site.[6]

Here, we selected an antiparallel β-sheet peptide as a scaffold to arrange functional groups for catalytic reaction and substrate binding. A β-sheet peptide can be designed in a smaller size so that the side chains are located vertically for its backbone. Although the principles behind β-sheet formation are less well understood, many trials to construct the stable β-sheet structure have been reported.[7] A β-hairpin structure is the smallest β-sheet structure unit in which two antiparallel β-strands are connected by a β-turn. The sequence of BH8 peptide (-ITVNGKTY-) is one of the β-hairpin peptides that afford a stable β-sheet structure in aqueous solution.[8] In this peptide, the side chains of Ile, Val and Tyr are arranged at the same side and form a hydrophobic face when this peptide takes β-hairpin conformation. Hence, two β-hairpin peptide chains were arranged at the primary hydroxyl group side of βCD so as to form a hydrophobic site as an additional recognition site. In this study, an ester hydrolysis was chosen as a model catalytic reaction. Two histidine

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residues were arranged so as to work as a general acid and a general base catalysts for the ester hydrolysis (Fig. 1). In β(AD)-AH, the β-hairpin peptide has one histidine residue at the C-terminal side and two peptide chains were linked to A and D positions of βCD, and totally two histidine residues were arranged around a βCD cavity. In order to confirm the effect of two-β-hairpin peptide, β-HH that has one-β-hairpin peptide was designed. In β-HH, two histidine residues were arranged at both N-terminal and C-terminal sides in a single chain (Fig. 1).

Both peptides were synthesized by stepwise elongation of Fmoc-amino acids on a Rink amide resin.[9] The side chains of histidine, asparagine and cystein were protected by trityl groups, and those of glutamic acid, threonine and tyrosine were protected by *tert*-butyl groups. The side chain of Lys was protected by *tert*-butyloxycarbonyl group. Synthesized peptides were cleaved from resin, and at this stage, all protecting groups were removed. Crude peptides were purified with reversed phase HPLC, and purified peptides were reacted with 6-mono(*N*-bromoacetylamino)-βCD or $6^A,6^D$-bis(*N*-bromoacetylamino)-βCD[10,11] in the Tris–HCl buffer solution (pH 8.5) to give the βCDs conjugated with peptides. Products were purified with reversed-phase HPLC and identified by MALDI-TOFMS (β(AD)-AH *m*/*z* 4360.9 $[(M+Na)^+]$, calcd 4361.0 and β-HH *m*/*z* 2803.2 $[(M+H)^+]$, calcd 2804.0).

Circular dichroism spectra of β(AD)-AH and β-HH are shown in Figure 2. In the circular dichroism studies, there was no pH dependence (from 5.5 to 8.0) and no concentration effect (from 5.0×10^{-6} M to 1.0×10^{-4} M) for both catalysts. β(AD)-AH had a negative maximum point at 216 nm, whereas β-HH showed a smaller peak around 216 nm. β(AD)-AH showed a similar spectrum pattern compared with BH8 in aqueous solution.[8a] In

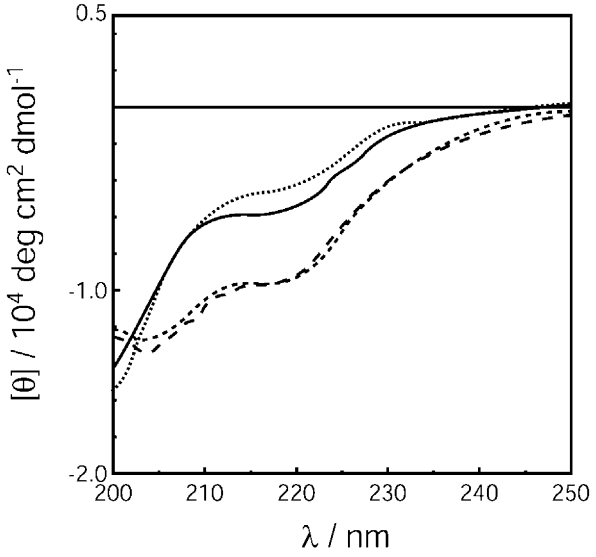

Figure 2. Circular dichroism spectra of β(AD)-AH and β-HH in 5.0×10^{-2} M, pH 6.5 phosphate buffer solution containing 0 or 30% TFE at 25 °C. β(AD)-AH (——) and β-HH (- - - - -) in 0 vol% TFE and β(AD)-AH (– – – –) and β-HH (— — — —) in 30 vol% TFE buffer solution (pH 6.5), respectively.

the presence of 30% 2,2,2-trifluoroethanol (TFE), both β-HH and β(AD)-AH showed almost the same spectra with a negative minimum point at 216 nm. In the NMR analyses of β(AD)-AH and β-HH in water, the long range NOE signals between Tyr10(CδH) and Ile3($C\delta H_3$), Tyr10(CεH) and Val5(CβH) were observed for only β(AD)-AH (data not shown). These results suggest that the peptide chains of β(AD)-AH interact with each other and stabilize their secondary structures but the structure of β-HH is more flexible. Since these peptides can form an amphiphilic structure as designed, the hydrophobic interaction between the two peptides might be effective.

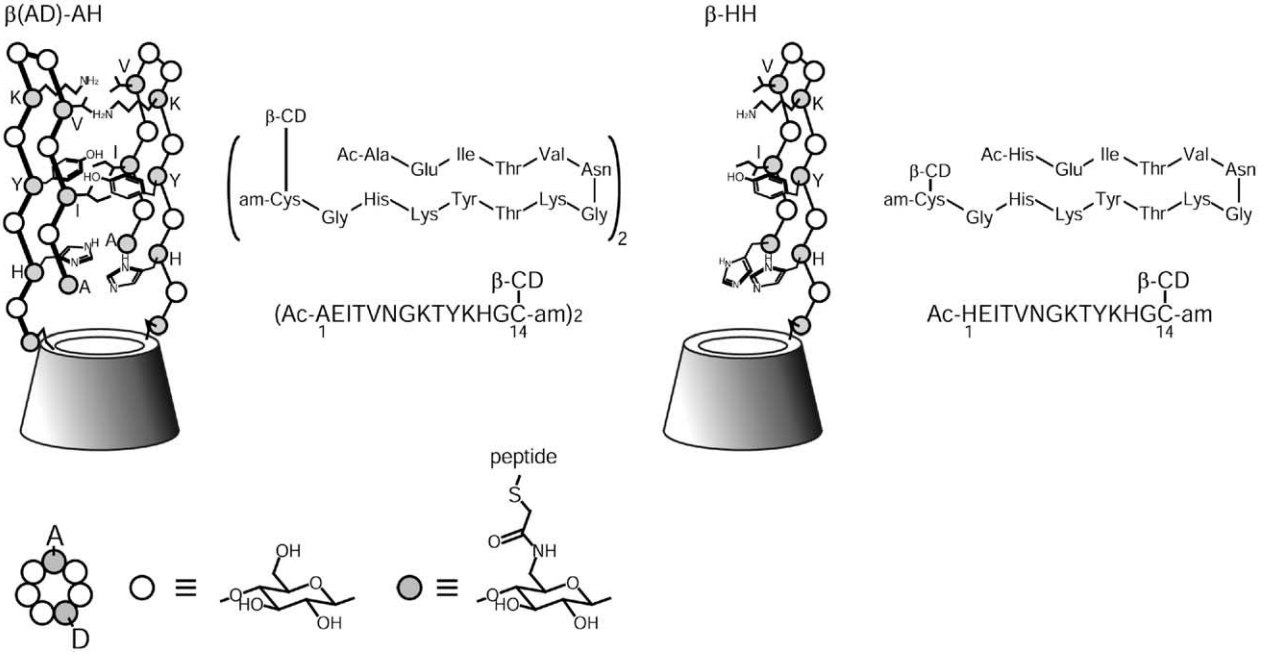

Figure. 1. Illustration of β(AD)-AH and β-HH and their amino acid sequences.

In the kinetic studies of ester hydrolysis, Boc-L-alanine *p*-nitrophenyl ester (L-AlaONp) and Boc-D-alanine *p*-nitrophenyl ester (D-AlaONp) were chosen as substrates. The hydrolysis was performed in various pHs of phosphate buffer solution and monitored as increasing UV–vis absorbance of the product, *p*-nitrophenolate (320 or 400 nm). In all measurements, the concentration of each catalyst was fixed at 2.0×10^{-5} M. The substrate concentration was varied from 1.0×10^{-4} to 5.0×10^{-4} M (L- and D-AlaONp). Excess conditions of the substrates were kept in each measurement. The pH dependence in initial rates of hydrolysis was measured under the conditions of the fixed substrate concentration of 3.0×10^{-4} M. Both catalysts had a maximum point around pH 6.5 in the hydrolysis of L-AlaONp and D-AlaONp. Since the secondary structure of the catalysts do not significantly depend on pH, the existence of a maximum point must arise from two imidazole groups. Two imidazole groups might exist as a protonated form and a free form, which could work as a general acid and a general base catalysts in this pH.[12] Then, kinetic analyses for the ester hydrolysis by β(AD)-AH and β-HH were performed at pH 6.5 and obtained kinetic parameters are summarized in Table 1. In the absence of any catalyst, the rate constants of the hydrolyses for D-AlaONp and L-AlaONp (k_{un}) were 1.86×10^{-5} s^{-1} and 1.89×10^{-5} s^{-1}, respectively. In the presence of catalysts, β(AD)-AH showed higher k_{cat} values than β-HH for both substrates. Because the secondary structure of β(AD)-AH appeared to be more stable than that of β-HH, two histidine residues of β(AD)-AH were fixed at their appropriate positions so as to hydrolyze the substrates efficiently, resulting that β(AD)-AH showed the higher k_{cat} values as compared with β-HH. However, β-HH also showed the maximum point for ester hydrolysis around pH 6.5, two histidine residues of β-hairpin peptide might be arranged near the βCD cavity and act cooperatively on the catalytic center. The K_m values of β(AD)-AH for L-AlaONp and D-AlaONp were 1.02×10^{-4} M and 7.66×10^{-4} M. β(AD)-AH showed 7.5 times superior K_m value for L-AlaONp. It is noted that there was no significant difference in K_m values between L-AlaONp and D-AlaONp by the reported bis-imidazole modified βCDs (for example, L-AlaONp: 8.0×10^{-4} M and D-AlaONp: 6.0×10^{-4} M).[12b] In addition, β(AD)-AH showed a superior K_m value to bis-imidazole modified βCDs for L-AlaONp, while β(AD)-AH showed almost the same value for D-AlaONp. These results suggest that the selectivity of β(AD)-AH for L-AlaONp is derived from the β-hairpin peptide scaffold. β(AD)-AH showed the slightly superior K_m value (1.02×10^{-4} M) to β-HH (1.63×10^{-4} M) for L-AlaONp. On the other hand, the K_m value of β(AD)-AH for D-AlaONp (7.66×10^{-4} M) was slightly inferior to that of β-HH (6.75×10^{-4} M). As a result, β(AD)-AH showed higher selectivity for L-AlaONp (7.5 fold) than β-HH (4.1 fold). This result suggests that the higher substrate selectivity of β(AD)-AH for L-AlaONp might be due to the hydrophobic site constructed with Ile and Tyr residues of two peptide chains. The hydrophobic site constructed with two β-hairpin peptides would serve favorably for the L-AlaONp binding. Thus, the two-β-hairpin peptide-βCD conjugate, β(AD)-AH, gained the higher catalytic activity for L-AlaONp than D-AlaONp (6.3 times higher k_{cat}/K_m value).

Table 1. Kinetic parameters for the hydrolysis of Boc-L-alanine *p*-nitrophenyl ester (L-AlaONp) and Boc-D-alanine *p*-nitrophenyl ester (D-AlaONp) catalyzed by β(AD)-AH and β-HH at 25 °C in pH 6.5 phosphate buffer solution (5.0×10^{-2} M)

Catalyst	Substrate	k_{cat} [10^{-4} s^{-1}]	k_m [10^{-4} M]	k_{cat}/K_m [M^{-1} s^1]
β(AD)-AH	L-AlaONp	12.1	1.02	11.9
	D-AlaONp	14.4	7.66	1.88
β-HH	L-AlaONp	8.89	1.63	5.45
	D-AlaONp	10.0	6.75	1.48

In this study, we have demonstrated that the β-hairpin peptide is useful as a new scaffold to arrange the functional groups and construct substrate binding subsite in the peptide-CD conjugates. The usage of two β-hairpin peptides is effective for the selective substrate recognition and catalysis. Although the reported peptide sequence is used as a model case in this study, design and selection of new β-hairpin scaffold suitable for catalysis will lead to the higher reaction and recognition abilities with smaller artificial molecules.

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