JOC Article

Fairly Marked Enantioselectivity for the Hydrolysis of Amino Acid Esters by Chemically Modified Enzymes[1]

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The hydrolysis (deacylation) of enantiomeric substrates by the chemically modified enzymes decanoyl-α-chymotrypsin and decanoyl-trypsin was studied. Reaction activity for decanoyl-α-chymotrypsin was lower than that for the native enzyme, although intriguingly the enantioselectivity was markedly enhanced as compared with the native enzyme. In particular, the apparently complete enantioselective catalysis was attained for the hydrolytic cleavage of *p*-nitrophenyl *N*-dodecanoyl-D(L)-phenylalaninates. The enhancement of enantioselectivity, however, was not observed for decanoyl-trypsin. These results suggest that the chemically modified α-chymotrypsin by addition of hydrophobic groups has promoted enantioselectivity for the hydrolysis of hydrophobic esters.

Introduction

Enzymes are attractive catalysts because of their exquisite chemo-, regio-, and stereospecificity and their impressive catalytic efficiencies. For example, α-chymotrypsin catalyzes the hydrolytic cleavage of peptide bonds at the carboxyl side of either Phe, Tyr, or Trp residues in proteins. Developing artificial enzymes with enzyme-like specificity is often carried out using biomimetic chemistry. Enzyme model studies have been the subject of continued interest in such areas as the development of stereoselective reaction sites for the hydrolysis of enantiomeric substrates and in aiding the understanding of the origins of stereoselectivity in the proteolytic enzyme. In particular, micelles are often used as an enzyme model, because the structure and properties provide a remarkably close analogy to those of globular proteins, including enzymes. The analogy between micelles and proteins may be extended for certain reactions since micelles exhibit a catalytic activity that has several characteristics of enzyme catalysis.

From this viewpoint, stereoselective cleavages of *N*-protected amino acid and peptide *p*-nitrophenyl esters in various surfactant aggregate systems have been used as models to probe the origins of proteolytic enzymes. In the course of the study on the stereoselective hydrolysis of amino acid esters in the coaggregate systems, Ueoka and others emphasized that the stereochemical control is attained by regulating temperature[2−10] and ionic strength[4,10−13] and by changing the composition of the coaggregates.[2,5,8−10,14] In particular, almost complete L-enantioselective catalysis[4,10−12,15] can be attributed to optimization in the conformation of the enzyme model in the coaggregate systems.[2,10,16] On the other hand, Okai and co-workers have developed a chemical modification method to introduce a modifying group to an amino moiety in α-chymotrypsin.[17]

In this study, we investigated the enantioselective hydrolysis of enantiomeric substrates (Z-D(L)-Phe-PNA, Z-D(L)-Phe-PNP, C_{12}-D(L)-Phe-PNP, and Z-D(L)-Lys-PNP·HCl) catalyzed by native enzymes (α-chymotrypsin (Csin); trypsin (Tsin)) and the chemically modified enzymes (decanoyl-α-chymotrypsin (Dec-Csin); decanoyltrypsin

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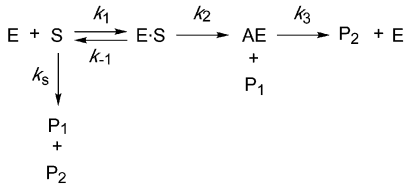
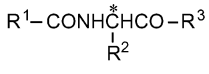
SCHEME 1



(Dec-Tsin)). The enantiomeric substrates employed in this study are listed in the following section.



Z-D(L)-Phe-PNA : $R^1=C_6H_5CH_2O$
$R^2=C_6H_5CH_2$
$R^3=p\text{-}NO_2C_6H_4NH$

Z-D(L)-Phe-PNP : $R^1=C_6H_5CH_2O$
$R^2=C_6H_5CH_2$
$R^3=p\text{-}NO_2C_6H_4O$

Z-D(L)-Lys-PNP·HCl : $R^1=C_6H_5CH_2O$
$R^2=ClH{\cdot}NH_2(CH_2)_4$
$R^3=p\text{-}NO_2C_6H_4O$

C_{12}-D(L)-Phe-PNP : $R^1=CH_3(CH_2)_{10}$
$R^2=C_6H_5CH_2$
$R^3=p\text{-}NO_2C_6H_4O$

Results and Discussion

Kinetics for Modified Enzyme on the Basis of the Michaelis−Menten Principle. It is well-known that native enzyme reactions obey Michaelis−Menten kinetics. We investigated catalytic effects of long-chain modified Dec-Csin and Dec-Tsin for the enantioselective hydrolysis (deacylation) of enantiomeric substrates on the basis of the Michaelis−Menten principle shown in Scheme 1. Here, E, S, E·S, AE, P, and *k* refer to enzyme, substrate, enzyme·substrate complex, acyl enzyme, product, and the rate constant for the reaction process shown in Scheme 1, respectively. Figures 1 and 2 show the concentration dependence of substrates on the initial velocity (v_{EO}) for the hydrolysis of Z-D(L)-amino acid esters catalyzed by the modified and unmodified enzymes.

Typical kinetic behavior based on the Michaelis−Menten principle was observed, that is, the enhancement of initial velocities was gradually saturated along with an increase in substrate concentration. The Lineweaver−Burk plots and Hofstee plots for the hydrolysis of Z-D-(L)-amino acid esters catalyzed by native and modified enzymes are shown in Figures 3 and 4, respectively. Both of these plots show good linear relationships. So, all of the enzyme reactions investigated in this study obeyed Michaelis−Menten kinetics.

Enantioselective Hydrolysis of Z-D(L)-Phe-PNA Catalyzed by Csin and Dec-Csin. With respect to the hydrolysis of Z-D(L)-Phe-PNA catalyzed by Csin and Dec-Csin, both of these enzymes accelerated the hydrolysis of L-substrate, while catalytic hydrolysis of D-substrate was not observed. The kinetic parameters V_{max}, K_m, and k_{cat} obtained on the basis of the Michaelis−Menten principle are shown in Table 1. K_m and k_{cat} (and V_{max}) values for the catalytic hydrolysis by Dec-Csin are smaller than those by Csin. This means that the binding affinity (reflected by $1/K_m$) for the formation of the Dec-Csin·L-substrate complex increased, but the reactivity

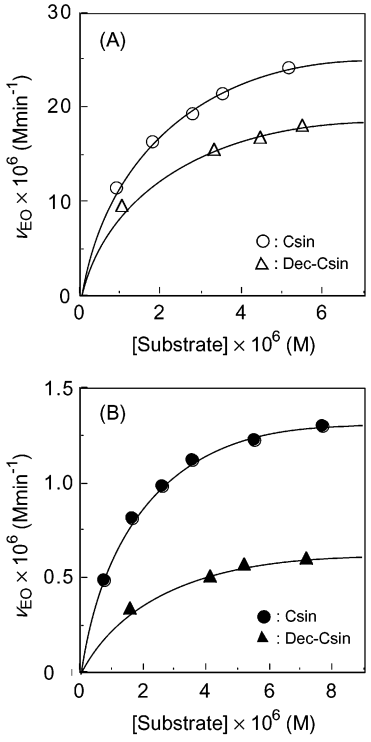


FIGURE 1. Michaelis−Menten plots for the hydrolysis of Z-L-Phe-PNP (A) and Z-D-Phe-PNP (B) catalyzed by Csin and Dec-Csin.

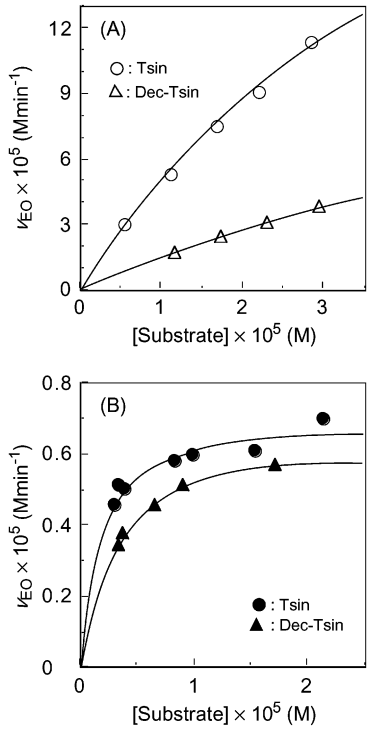


FIGURE 2. Michaelis−Menten plots for the hydrolysis of Z-L-Lys-PNP·HCl (A) and Z-D-Lys-PNP·HCl (B) catalyzed by Tsin and Dec-Tsin.

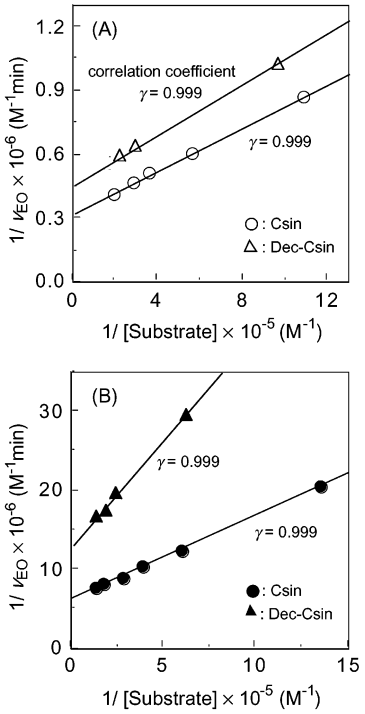


FIGURE 3. Lineweaver−Burk plots for the hydrolysis of Z-L-Phe-PNP (A) and Z-D-Phe-PNP (B) catalyzed by Csin and Dec-Csin.

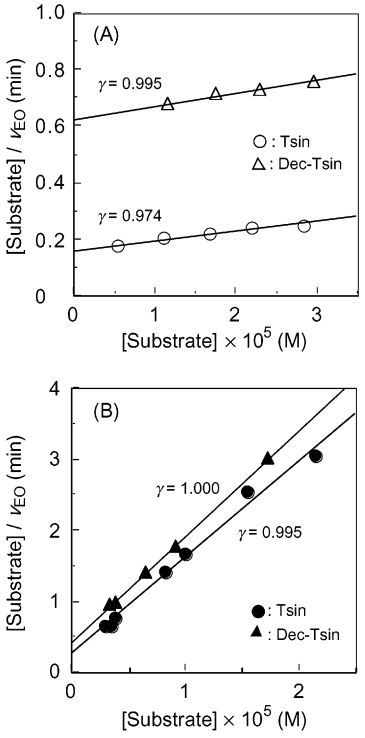


FIGURE 4. Hofstee plots for the hydrolysis of Z-L-Lys-PNP·HCl (A) and Z-D-Lys-PNP·HCl (B) catalyzed by Tsin and Dec-Tsin.

(reflected by k_{cat}) for the deacylation of substrate with Dec-Csin decreased as compared with those for Csin. As a result, the catalytic activity (reflected by k_{cat}/K_m) of Dec-Csin was slightly greater than that of Csin. This result seems to suggest that the long-chain modification of Csin induces a stronger affinity of Dec-Csin for the L-substrate through hydrophobic interactions, while the orientation of the L-substrate at the binding site in Dec-Csin should be less favorable for the acylation. The catalytic efficiency of the enzymes for the hydrolysis of D-substrate was not established.

Enantioselective Hydrolysis of Z-D(L)-Phe-PNP Catalyzed by Csin and Dec-Csin. We examined the catalytic efficiency of Csin and Dec-Csin for the hydrolysis of Z-D(L)-Phe-PNP, which are enantiomeric esters bearing the same Z-phenylalanine residue as for above Z-D(L)-Phe-PNA substrates. As shown in Table 2, significant L-selective catalysis was observed in the hydrolysis of Z-D(L)-Phe-PNP by Csin and Dec-Csin ($(k_{cat}^L/K_m^L)/(k_{cat}^D/K_m^D)$ = 210 and 460, respectively). The high enantioselectivities could be mainly attributed to the deacylation process rather than the binding process, since this was reflected by a fairly large k_{cat}^L/k_{cat}^D (Csin = 180, Dec-Csin = 270) and a small K_m^L/K_m^D value (Csin = 1.1, Dec-Csin = 1.7). It is intriguing that a similar trend was observed for the catalytic hydrolysis of L-substrates; the long-chain modification induces Csin to decrease in K_m and k_{cat} values as well as those for the hydrolysis of Z-D(L)-Phe-PNA. In addition, the binding affinity and reactivity of deacylation for D-substrates decreased for the hydrolysis mediated by Dec-Csin as compared with those by Csin. Thus, improved enantioselectivity for the L-substrate was observed for hydrolysis catalyzed by Dec-Csin. These observations indicate that the long alkyl chains in Dec-Csin must play an important role in governing the enantioselective catalysis.

Enantioselective Hydrolysis of Z-D(L)-Lys-PNP·HCl Catalyzed by Tsin and Dec-Tsin. Tsin is a serine protease that catalyzes the hydrolysis of peptide bonds at the carboxyl side of basic amino acid residues in proteins. The enantioselective hydrolysis of Z-D(L)-Lys-PNP·HCl catalyzed by both native Tsin and long-chain modified Dec-Tsin was studied, and the results are shown in Table 3. A large degree of L-enantioselectivity was observed in the deacylation process (k_{cat}^L/k_{cat}^D: Csin = 570, Dec-Csin = 880) catalyzed by Tsin and Dec-Tsin. Interestingly, however, D-enantioselectivity was observed in the binding process (K_m^D/K_m^L: Csin = 29, Dec-Csin = 50), so the L-selectivity in the catalytic hydrolysis was poor for both enzymes ($(k_{cat}^L/K_m^L)/(k_{cat}^D/K_m^D)$: Csin = 19, Dec-Csin = 17). As regards the effect of modification of Tsin on the enantiomeric catalysis, K_m values for the L- and D-substrates were larger than those for the native enzyme, especially the binding affinity for the L-substrate, which was markedly reduced. Although the k_{cat} value changed very little for the D-substrate and increased for the L-substrate, the overall catalytic activity and selectivity of Dec-Tsin were lower than those for Tsin. It can be concluded that long-chain modification of Tsin such as Dec-Tsin does not improve enantioselective catalysis.

Enantioselective Hydrolysis of C_{12}-D(L)-Phe-PNP Catalyzed by Csin and Dec-Csin. The enantioselective hydrolysis of long-chain substrates C_{12}-D(L)-Phe-PNP catalyzed by Csin and Dec-Csin was investigated. The kinetic results are summarized in Table 4. As compared with the catalytic efficiency of Csin for the hydrolysis of Z-D(L)-Phe-PNP (Table 2), K_m values of L- and D-

TABLE 1. Kinetic Parameters for α-Chymotrypsin (Csin) and Decanoyl-α-Chymotrypsin (Dec-Csin)[a]

enzyme	substrate	V_{max} (M/min)	K_m (M)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	L/D
Csin	Z-L-Phe-PNA[b]	5.61×10^{-5}	2.82×10^{-4}	0.233	8.56×10^{2}	∞
	Z-D-Phe-PNA		no catalysis			
Dec-Csin	Z-L-Phe-PNA[b]	1.62×10^{-5}	5.86×10^{-5}	0.068	1.16×10^{3}	∞
	Z-D-Phe-PNA		no catalysis			

[a] Conditions: 25 °C, pH 8.2, 0.05 M Tris-HCl buffer (0.05 M KCl), 6% (v/v) CH_3CN-H_2O. [b] [substrate] = $(0.47-2.71) \times 10^{-5}$ M, [enzyme] = 4×10^{-6} M.

TABLE 2. Kinetic Parameters for α-Chymotrypsin (Csin) and Decanoyl-α-Chymotrypsin (Dec-Csin)[a]

enzyme	substrate	V_{max} (M/min)	K_m (M)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	L/D
Csin	Z-L-Phe-PNP[b]	3.10×10^{-5}	1.56×10^{-6}	25.10	1.65×10^{7}	210
	Z-D-Phe-PNP[c]	1.64×10^{-6}	1.72×10^{-6}	0.137	7.94×10^{4}	
Dec-Csin	Z-L-Phe-PNP[b]	2.17×10^{-5}	1.28×10^{-6}	18.10	1.41×10^{7}	460
	Z-D-Phe-PNP[c]	7.87×10^{-7}	2.13×10^{-6}	0.066	3.08×10^{4}	

[a] Conditions: 25 °C, pH 8.2, 0.05 M Tris-HCl buffer (0.05 M KCl), 6% (v/v) CH_3CN-H_2O. [b] [substrate] = $(0.90-5.5) \times 10^{-6}$ M, [enzyme] = 2×10^{-8} M. [c] [substrate] = $(0.72-7.7) \times 10^{-6}$ M, [enzyme] = 2×10^{-7} M.

TABLE 3. Kinetic Parameters for Trypsin (Tsin) and Decanoyl-Trypsin (Dec-Tsin)[a]

enzyme	substrate	V_{max} (M/min)	K_m (M)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	L/D
Csin	Z-L-Lys-PNP·HCl[b]	3.32×10^{-4}	5.60×10^{-5}	69.20	1.24×10^{6}	19
	Z-D-Lys-PNP·HCl[c]	7.31×10^{-6}	1.90×10^{-6}	0.122	6.41×10^{4}	
Dec-Csin	Z-L-Lys-PNP·HCl[d]	2.36×10^{-4}	1.50×10^{-4}	98.30	6.56×10^{5}	17
	Z-D-Lys-PNP·HCl[c]	6.70×10^{-6}	2.98×10^{-6}	0.112	3.75×10^{4}	

[a] Conditions: 25 °C, pH 7.6, 0.05 M Tris-HCl buffer (0.05 M KCl), 6% (v/v) CH_3CN-H_2O. [b] [substrate] = $(0.55-2.83) \times 10^{-5}$ M, [enzyme] = 8×10^{-8} M. [c] [substrate] = $(0.30-2.14) \times 10^{-5}$ M, [enzyme] = 1×10^{-6} M. [d] [substrate] = $(1.16-2.95) \times 10^{-5}$ M, [enzyme] = 4×10^{-8} M.

TABLE 4. Kinetic Parameters for α-Chymotrypsin (Csin) and Decanoyl-α-Chymotrypsin (Dec-Csin)[a]

enzyme	substrate	V_{max} (M/min)	K_m (M)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	L/D
Csin	C_{12}-L-Phe-PNP[b]	4.93×10^{-5}	7.57×10^{-6}	41.10	5.43×10^{6}	3200
	C_{12}-D-Phe-PNP[c]	2.20×10^{-7}	1.09×10^{-5}	0.018	1.68×10^{3}	
Dec-Csin	C_{12}-L-Phe-PNP[b]	3.19×10^{-5}	1.01×10^{-5}	26.60	2.63×10^{6}	∞
	C_{12}-D-Phe-PNP[c]		no catalysis			

[a] Conditions: 25 °C, pH 8.2, 0.05 M Tris-HCl buffer (0.05 M KCl), 6% (v/v) CH_3CN-H_2O. [b] [substrate] = $(0.80-5.5) \times 10^{-6}$ M, [enzyme] = 2×10^{-8} M. [c] [substrate] = $(5.50-17.7) \times 10^{-6}$ M, [enzyme] = 2×10^{-7} M.

substrates increased, reflecting the decrease in the binding affinity. The k_{cat} value for the L-substrate increased, and that for the D-substrate decreased. The rate of reaction decreased markedly for L-enantioselective catalysis mediated by Csin. The long acyl chain of the substrate may interfere with binding to Csin. However, a favorable orientation of the L-substrate, but not the D-substrate, for the acylation at the catalytic site of the enzyme gives rise to the observed enantioselectivity $((k_{cat}^{L}/K_m^{L})/(k_{cat}^{D}/K_m^{D}) = 3200)$. Furthermore, in the catalytic hydrolysis of C_{12}-D(L)-Phe-PNP by Dec-Csin, exclusive cleavage of the L-substrate was observed. Binding of the substrate to Dec-Csin may be enhanced by hydrophobic interactions between the long alkyl chains in the modified enzyme. Steric effects may favor hydrolysis of the L-substrate over that of the D-substrate.

Conclusion

The most striking feature of this study was that the apparently complete enantioselective catalysis was attained for the hydrolytic cleavage of the long-chain amino acid esters (C_{12}-D(L)-Phe-PNP) by the long-chain modified α-chymotrypsin. Such a change in enantiomeric discrimination was not observed for decanoyl-trypsin. These results suggest that the hydrophobic groups introduced into α-chymotrypsin by chemical modification have markedly enhanced enantioselectivity for the hydrolysis of hydrophobic esters.

Experimental Section

Materials. *p*-Nitroanilide *N*-(Benzyloxycarbonyl)-D(L)-phenylalanine (Z-D(L)-Phe-PNA). The anilides of Z-amino acid (Z-D(L)-Phe-PNA) were synthesized by mixed anhydride coupling of *N*-(benzyloxycarbonyl)-D(or L)-phenylalaninate (Z-D(or L)-Phe-OH) and *p*-nitroaniline with trimethylacetyl chloride and TEA in anhydrous THF.[18] Satisfactory analytical data were obtained for Z-D(L)-Phe-PNA. Z-D-Phe-PNA: mp 159.5−161.5 °C; $[\alpha]_D^{20}$ −60.97° (*c* 1, $CHCl_3$). Anal. Calcd for $C_{23}H_{21}N_3O_5$: C, 65.86; H, 5.01; N, 10.01. Found: C, 65.51; H, 5.01; N, 10.02. Z-L-Phe-PNA: mp 157.0−159.0 °C; $[\alpha]_D^{20}$ +60.71 (*c* 1, $CHCl_3$). Anal. Found: C, 65.63; H, 5.08; N, 10.03.

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***p*-Nitrophenyl *N*-(Benzyloxycarbonyl)-D(L)-phenylalaninate (Z-D(L)-Phe-PNP).** The Z-amino acid esters (Z-D-(L)-Phe-PNP[2]) were prepared from Z-D(or L)-Phe-OH by the esterification of the COOH group with *p*-nitrophenol and DCC.[19] Satisfactory analytical data were obtained for Z-D(L)-Phe-PNP. Z-D-Phe-PNP: mp 121.5−123.5 °C. Anal. Calcd for $C_{23}H_{20}N_2O_6$: C, 65.70; H, 4.78; N, 6.66. Found: C, 65.88; H, 4.70; N, 6.67. Z-L-Phe-PNP: mp 119.0−120.0 °C. Anal. Found: C, 65.65; H, 4.73; N, 6.59.

***p*-Nitrophenyl N^{α}-(Benzyloxycarbonyl)-D(L)-lysinate hydrochloride (Z-D(L)-Lys-PNP·HCl).** The Z-amino acid esters (Z-D(L)-Lys-PNP·HCl) were synthesized from N^{α}-(benzyloxycarbonyl)-N^{ϵ}-(*tert*-butyloxycarbonyl)-D(L)-lysinate (Z-D-(or L)-Lys(Boc)-OH) according to a method similar to that for Z-D(L)-Phe-PNP, followed by decarbonylation of the Boc group with 4 N HCl/dioxane. Satisfactory analytical data were obtained for Z-D(L)-Lys-PNP·HCl. Z-D-Lys-PNP·HCl: mp 141.0−144.0 °C; $[\alpha]_D^{20}$ +34.20° (*c* 1, $CHCl_3$). Anal. Calcd for $C_{20}H_{24}N_3O_6$-Cl: C, 54.87; H, 5.53; N, 9.59. Found: C, 55.06; H, 5.50; N, 9.61. Z-L-Lys-PNP·HCl: mp 138.0−143.0 °C; $[\alpha]_D^{20}$ −34.28° (*c* 1, $CHCl_3$). Anal. Found: C, 54.64; H, 5.49; N, 9.52.

***p*-Nitrophenyl *N*-Dodecanoyl-D(L)-phenylalaninate (C_{12}-D(L)-Phe-PNP).** The long-chain amino acid esters (C_{12}-D(L)-Phe-PNP[2]) were prepared as described above, followed by hydrobromination of the NH_2 group[20] and then acylation of the NH_2·HBr group with dodecanoic anhydride.[19] Satisfactory analytical data were obtained for C_{12}-D(L)-Phe-PNP. C_{12}-D-Phe-PNP: mp 107.0−108.5 °C; $[\alpha]_D^{23}$ +10.8° (*c* 2, $CHCl_3$). Anal. Calcd for $C_{27}H_{36}N_2O_5$: C, 69.21; H, 7.74; N, 5.98. Found: C, 69.07; H, 7.67; N, 5.96. C_{12}-L-Phe-PNP: mp 106.5−108.0 °C, $[\alpha]_D^{23}$ −10.8° (*c* 2, $CHCl_3$). Anal. Found: C, 68.98; H, 7.77; N, 5.96.

Decanoyl-α-Chymotrypsin (Dec-Csin) and Decanoyl-Trypsin (Dec-Tsin). Csin and Tsin were purchased commercially. The chemically modified enzymes (Dec-Csin[1] and Dec-Tsin) were prepared from the native enzymes by the acylation of ϵ-NH_2 groups of lysine residue with water-soluble acylating reagent *p*-(decanoyl)phenyl-dimethylsulfonium methyl sulfate, which was synthesized by condensation of decanoic acid and *p*-hydroxyphenyl-dimethylsulfonium methyl sulfate with DCC.[17,21,22]

Determination of the Ratio of Modification. The ratio of modification for decanoyl enzymes was determined on the basis of the amount of the free amino group, which was measured by using sodium 2,4,6-trinitrobenzene sulfonate (TNBS).[17,23] Each native and modified enzyme was reacted with TNBS reagent, and then the absorbance was measured at 425 nm. The ratio of modification was calculated as follows,

$$\text{Ratio of modification (\%)} = (1 - A/B) \times 100 \qquad (1)$$

where *A* is absorbance of modified enzyme and *B* is absorbance of native enzyme. The ratios of modification for Dec-Csin and Dec-Tsin were determined to be 18.6 and 11.7%, respectively.

Kinetic Measurement. Rates of *p*-nitrophenol liberation from *p*-nitrophenyl esters or *p*-nitroaniline liberation from *p*-nitroanilides were monitored at 400 or 379 nm using UV/vis spectrophotometers. Each run was initiated by adding an acetonitrile solution (0.02 mL) of a substrate to the reaction medium of tris(hydroxymethyl)aminomethane (Tris) buffer (3.3 mL) containing native or modified enzymes. The reactions obeyed the conventional Michaelis−Menten equation on the basis of the steady-state method under an excess amount of substrate. Kinetic parameters such as maximum velocity (V_{max}) and Michaelis constant (K_m) were determined by the least-squares method from a Lineweaver−Burk plot between $1/v_{E0}$ (reciprocal value of initial velocity) and 1/[S] in eq 2 or a Hofstee plot between $[S]/v_{E0}$ and [S] in eq 3.

$$1/v_{E0} = (K_m/V_{max})/[S] + 1/V_{max} \qquad (2)$$

$$[S]/v_{E0} = K_m/V_{max} + [S]/V_{max} \qquad (3)$$

The reaction activity reflected in k_{cat} for the enzymatic hydrolysis (deacylation of substrates) was evaluated by eq 4,

$$k_2 = V_{max}/[E]_0 = k_{cat} \qquad (4)$$

where $[E]_0$ indicates the initial concentration of enzymes.

Supporting Information Available: Michaelis−Menten and Lineweaver−Burk plots for the hydrolysis of Z-L-Phe-PNA and C_{12}-D(L)-Phe-PNP catalyzed by Csin and Dec-Csin. This material is available free of charge via the Internet at http://pubs.acs.org.

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