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A Thermostable Chymotrypsin Derivative Soluble in Toluene

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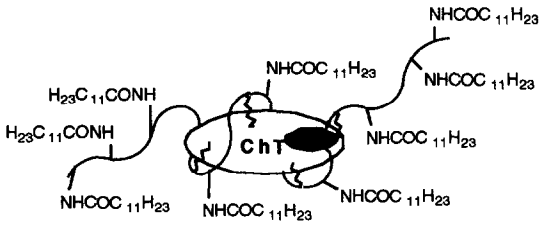
Abstract: α-Chymotrypsin (ChT) was attached to a lauroylated derivative of poly(allylamine). The resulting ChT derivative, which was soluble in toluene, manifested greater resistivity to thermoinactivation compared with the native ChT and a shift in the substrate specificity upon changing solvents.

Utility of an enzyme in biotechnology and organic synthesis may be improved by enhancement of the thermostability of the enzyme, solubilization of the enzyme in organic solvents, or changes in substrate specificity. Various methods have been developed for such improvements. For example, thermostability of enzymes has been raised through cross-linkage of the enzymes.[1] In one of our previous studies, thermostability and resistivity against denaturing conditions of α-chymotrypsin (ChT) were enhanced by conjugation of the enzyme to derivatives of poly(allylamine) (PAA: M.W. 100 000) or poly(ethylenimine).[1a,1b] The cross-linkage of ChT with the water-soluble polyamines appears to result in a multiple attachment of the enzyme to the polymers and suppression of unfolding of the tertiary structure of the enzyme. Solubilization of enzymes in organic solvents has been achieved by attaching poyl(ethylene glycol) moieties to the enzymes.[2] Changes in substrate specificity have been observed when enzyme crystals are suspended in different organic solvents.[3] In the present study, we have accomplished improvement of all of the aforementioned three properties of ChT by attaching the enzyme to a lauroylated PAA.

Cross-linkage of ChT with Lauroyl$_{0.27}$PAA (PAA containing 27 residue molar % lauroyl groups)[4] and purification of the resulting Lauroyl$_{0.27}$PAA-ChT were carried out according to the method reported previously.[1a] As indicated by the schematic structure of Lauroyl$_{0.27}$PAA-ChT, structural features such as a multiple attachment to PAA and creation of hydrophobic microenvironments are added to ChT.



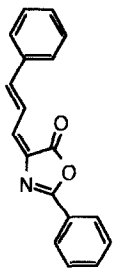
Lauroyl$_{0.27}$PAA-ChT

At 25 °C and pH 7.6 in water, k_{cat} of (3.6 ± 0.2) X 10^{-2} s^{-1} and k_{cat}/K_m of 570 + 50 $M^{-1}s^{-1}$ were obtained for the hydrolysis of *N*-benzoyl-L-tyrosine-*p*-nitroanilide (BTNA) catalyzed by Lauroyl$_{0.27}$PAA-ChT. When compared with those[1a] measured with the native ChT, k_{cat} and k_{cat}/K_m are about 4 and 6 times, respectively, smaller.

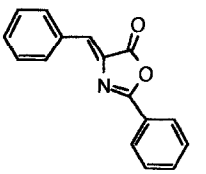
Rate of the thermoinactivation of Lauroyl$_{0.27}$PAA-ChT (E_o = 6.4 X 10^{-7} M) in water was measured at 50°C using BTNA as the substrate according to the method described previously[1a] The half-life for thermoinactivation of Lauroyl$_{0.27}$PAA-ChT was 20 ± 2 min whereas those[1a] of the native ChT and ChT attached to PAA (PAA-ChT) were 3.0 ± 0.4 min and 150 ± 20 min, respectively Thermostability of Lauroyl$_{0.27}$PAA-ChT is considerably greater than that of ChT although the degree of stabilization is smaller than that of PAA-ChT. Apparently, the multiple attachment of ChT to Lauroyl$_{0.27}$PAA was not as extensive as that to PAA probably due to blockage of 27 % of the amino groups of PAA with lauroyl group

When an aqueous solution of Lauroyl$_{0.27}$PAA-ChT was adjusted to pH 7.6 and lyophilized, powders of Lauroyl$_{0.27}$PAA-ChT were obtained, which were soluble in toluene The thermostability of Lauroyl$_{0.27}$PAA-ChT (E_o = 1.2 X 10^{-7} M) in toluene was measured at 50 °C by using *N*-acetyl-L-phenylalanine *p*-nitrophenyl ester (APNE)[5] (S_o = 5.1 X 10^{-4} M) as the substrate and methanol (MeOH) (2.4 % (v/v)) as the nucleophile for transesterification. The half-life for thermoinactivation of Lauroyl$_{0.27}$PAA-ChT was 650 + 80 min at 50°C in toluene, being 33 times greater than that in water.

Different substrate specificity of Lauroyl$_{0.27}$PAA-ChT in toluene and water may be exemplified by deacylation of 2-phenyl-(*E*)-[4-(*E*)-cinnamylidene]-oxazolin-5-one (PCO)[6] and 2-phenyl-(*Z*)-4-benzylidene-oxazolin-5-one[7] (PBO). In water, the rate-determining step is the breakdown of acyl-ChT intermediates for the ChT-catalyzed hydrolysis of both PCO and PBO [6,7] Values of the rate constant (k_{br}) for the breakdown step measured under the conditions of $E_o >> S_o$ and those of k_{cat}/K_m measured under the conditions of $E_o << S_o$ for PCO and PBO in water are summarized in Table 1 The values of k_{cat}/K_m for the deacylation of PCO and PBO catalyzed by Lauroyl$_{0.27}$PAA-ChT in toluene are also summarized in Table 1.



PCO



PBO

In water, PBO is hydrolyzed by ChT considerably faster than PCO In toluene, however, the reactivity toward Lauroyl$_{0.27}$PAA-ChT of PBO is appreciably smaller than that of PCO. More interesting to note is that both k_{cat} and k_{cat}/K_m for PCO increase as the content of MeOH is raised whereas those for PBO is little affected by the content of MeOH. In toluene, MeOH is the nucleophile for the attack at the acyl-ChT intermediate and

Table 1 Values of Kinetic Parameters for the Deacylation of PCO and PBO Catalyzed by Lauroyl$_{0.2}$-PAA-ChT at 25 °C

solvent	PCO	PBO
toluene with 2.4 % (v/v) MeOH[a]	$k_{cat} = (1.4 \pm 0.1) \times 10^{-2}$ s^{-1} $k_{cat}/K_m = 13 \pm 1$ M^{-1}s^{-1}	$k_{cat} = (5.9 \pm 0.8) \times 10^{-3}$ s^{-1} $k_{cat}/K_m = 5.8 \pm 0.3$ M^{-1}s^{-1}
toluene with 3.6 % (v/v) MeOH[a]	$k_{cat} = (3.3 \pm 0.3) \times 10^{-2}$ s^{-1} $k_{cat}/K_m = 32 \pm 1$ M^{-1}s^{-1}	$k_{cat} = (6.5 \pm 0.5) \times 10^{-3}$ s^{-1} $k_{cat}/K_m = 5.9 \pm 0.1$ M^{-1}s^{-1}
toluene with 4.7 % (v/v) MeOH[a]	$k_{cat} = (5.0 \pm 0.3) \times 10^{-2}$ s^{-1} $k_{cat}/K_m = 49 \pm 1$ M^{-1}s^{-1}	$k_{cat} = (7.7 \pm 0.2) \times 10^{-3}$ s^{-1} $k_{cat}/K_m = 7.0 \pm 0.2$ M^{-1}s^{-1}
water[b,c]	$k_{cat}/K_m = (1.7 \pm 0.1) \times 10^{3}$ M^{-1}s^{-1}	$k_{cat}/K_m = (1.3 \pm 0.1) \times 10^{4}$ M^{-1}s^{-1}

[a] $E_o = 2.4 \times 10^{-7}$ M and $S_o = (0.03 - 3) \times 10^{-3}$ M.

[b] pH 6.5 with 0.02 M *N*-(2-hydroxyethyl)-1-piperazineethanesulfonate, 0.5 M NaCl. $E_o = 1.6 \times 10^{-7}$ M and $S_o = (1.7 - 8) \times 10^{-6}$ M. S_o was not raised above 0.8×10^{-5} M due to the limited solubility of the substrate

[c] When measured under the conditions of $E_o >> S_o$, the following values of k_{hr} were obtained: 0.023 s^{-1} at pH 5.0, 0.058 s^{-1} at pH 5.5, and 0.20 s^{-1} at pH 6.0 for PCO and 0.079 s^{-1} at pH 4.5 and 0.19 s^{-1} at pH 5.0 for PBO

deacylation of the substrates results in methanolysis instead of hydrolysis. The different effects of the MeOH content on kinetic parameters for PCO and PBO are best accounted for by assuming that the rate-determining step is the breakdown of the acyl-ChT intermediate for PCO and the formation of the acyl-ChT intermediate for PBO. The rate constant (k_{form}) for the formation step of acyl-ChT for PCO is to be much larger than the observed value of k_{cat}. The k_{form} for PBO is, therefore, markedly smaller than that for PCO.

The rate-determining step is the breakdown of acyl-ChT for both PCO and PBO in water, whereas it is the breakdown of acyl-ChT for PCO and the formation of acyl-ChT for PBO in toluene. Toward Lauroyl$_{0.2}$-PAA-ChT, PBO is more reactive than PCO in water, but less reactive than PCO in toluene. These kinetic features of PBO manifested toward Lauroyl$_{0.2}$-PAA-ChT are attributable to the small k_{form} of PBO in toluene.

When the nucleophile that attacks the acyl-ChT intermediate is changed from water to methanol, a bulkier substrate may suffer from greater steric disadvantages and, therefore, the breakdown of the intermediate would be slower for the bulkier substrate (PCO). The major kinetic feature observed is, however, the slower

formation of the acyl-ChT intermediate for the smaller substrate (PBO). At present, the molecular basis of the small k_{form} for the deacylation of PBO in toluene catalyzed by Lauroyl$_{0.27}$PAA-ChT is unknown. The different behavior of PBO and PCO, however, suggests that the active site of the enzyme has different conformations when dissolved in water and in toluene.

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References and Notes

1 (a) Suh, J , Hwang, B. K. *Bioorg. Chem.* **1992**, *20*, 223. (b) Suh, J.; Cho, Y ; Kwag, G. *Bioorg. Chem.* **1992**, *20*, 236. (c) Wang, P ; Hill, T. G , Wartchow, C A.; Huston, M. E., Oehler, L. M., Bradley Smith, M.; Bednarski, M. D.; Callstrom, M. R *J. Am. Chem. Soc.* **1992**, *114*, 378. (d) St. Clair, N. L ; Navia, M. A. *J. Am. Chem. Soc.* **1992**, *114*, 7314.

2 (a) Takahashi, K ; Ajima, A., Yoshimoto, T , Inada, Y *Biochem. Biophys. Res. Comm.* **1984**, *121*, 261 (b) Matsushima, A.; Okada, M.; Inada, Y *FEBS Lett* **1984**, *178*, 275 (c) Yoshimoto, T., Ritani, A.; Ohwada, K , Takahashi, K., Kodera, Y.; Matsushima, A ; Saito, Y., Inada, T. *Biochem. Biophys. Res. Comm.* **1987**, *148*, 876. (d) Inada, Y.; Matsushima, A.; Takahashi, K.; Saito, Y *Biocatalysis* **1990**, *3*, 317.

3 (a) Zaks, A.; Klibanov, A M. *J. Am. Chem. Soc.* **1986**, *108*, 2767 (b) Sakurai, T., Margolin, A. L ; Russel, A. J., Klibanov, A M. *J. Am. Chem. Soc.* **1988**, *110*, 7236. (c) Rubio, E , Fernandez-Mayorales, A ; Klibanov, A. M. *J. Am. Chem. Soc.* **1991**, *113*, 695. (d) Parida, S., Dordick, J. S. *J. Am. Chem. Soc.* **1991**, *113*, 2253. (e) Fitzpatrick, P. A., Klibanov, A M. *J. Am. Chem. Soc.* **1991**, *113*, 3166 (f) Tawaki, S , Klibanov, A M. *J. Am. Chem. Soc.* **1992**, *114*, 1882. (g) Terradas, F , Teston-Henry, M., Fitzpatrick, P A.; Klibanov, A. M *J. Am. Chem. Soc.* **1993**, *114*, 390. (h) Wescott, C. R., Klibanov, A. M *J. Am. Chem. Soc.* **1993**, *113*, 1629.

4. Lauroyl$_{0.27}$PAA was prepared by neutralization of the HCl salt of PAA (10 residue mmol) to pH 8.2 with NaOH in 100 ml water followed by reaction of the resulting PAA with *N*-lauroyloxysuccinimide (11 mmol) in the presence of additionally added 100 ml acetone at 4 °C for 3 days and was purified by removal of the remaining *N*-lauroyloxysuccinimide through extraction with methylene chloride followed by dialysis.

5. In toluene at 25 °C, k_{cat} = 1.1 ± 0.1 s^{-1} and k_{cat}/K_m = 2200 ± 140 M^{-1}s^{-1} were obtained for the deacylation of APNE (S_o = 0.5-5 1 X 10^{-4} M) catalyzed by Lauroyl$_{0.27}$PAA-ChT in the presence of 2.4 % (v/v) MeOH For the ChT-catalyzed hydrolysis of APNE at 25 °C and pH 7.0, k_{cat} of 77 s^{-1} and k_{cat}/K_m of 3.2 X 10^6 M^{-1}s^{-1} have been observed in water containing 3.2 % (v/v) acetonitrile and k_{cat} of 73 s^{-1} and k_{cat}/K_m of 6.3 X 10^5 M^{-1}s^{-1} in water containing 15 % (v/v) acetonitrile (Zerner, B , Bond, R. P M ; Bender, M. L. *J. Am. Chem. Soc.* **1964**, *86*, 3674) In these solvents, water is the nucleophile for the attack at the acyl-ChT intermediate and its concentration is 45-55 M. In the present study in toluene, however, MeOH is the nucleophile and its concentration is only 0.6 M

6. Park, H. S ; Lee, S.; Suh, J. *J. Biochem. Biophys. Meth.* **1993**, *27*, 261

7. Hamilton, S E ; Zerner, B. *J. Am. Chem. Soc.* **1981**, *103*, 1827.

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