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Enhancement of Durability of Chymotrypsin through Cross-Linking with Poly(allylamine)

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α-Chymotrypsin (ChT) is covalently linked to various derivatives of poly(allylamine) (PAA) by using a carbodiimide as a coupling reagent. The PAA derivatives contain cationic, anionic, or hydrophobic microdomains. The ChTs linked to the PAA derivatives exhibit considerable stability against denaturing agents such as 0.5 M sodium dodecylsulfate or 4 M guanidinium chloride. This suggests multiple intramolecular cross-linking of ChT by the linear PAA derivatives. Stabilization of ChT by cross-linking with PAA derivatives is also reflected in much greater resistance of the PAA-bound ChTs to thermoinactivation. The multiple attachment of ChT to PAA derivatives does not significantly damage the active site as indicated by the reactivity of the modified ChTs at optimum pHs.

INTRODUCTION

In view of the widespread industrial applications of enzymes and the recent advances in the exploitation of enzymes in organic synthesis (*1–6*), enzymatic properties such as reactivity, specificity, or compatibility with extreme reaction conditions are closely linked to the utility of enzymes. Various methods have been reported for the improvement of these properties. Immobilization of enzymes on insoluble supports is the most commonly used technique for enzyme stabilization (*7*). Chemical modification of enzymes can improve the utility of the enzymes(*8*). For example, attachment of a photosensitive functional group such as azobenzene residue to an enzyme resulted in the photoregulation of the enzyme activity (*9*). Recently, site-specific mutagenesis has been used to improve enzymatic properties such as thermal stability or stability in organic solvents (*10–13*). Semisynthetic enzymes with new catalytic properties have been obtained by fusing a catalytic domain to a binding domain (*14*) or by introducing an artificial catalytic unit into the active site of an enzyme (*15*). Entrapment or encapsulation is also used to modify enzyme structures (*16*). In addition, alteration of substrate specificity and the consequent enhancement of the versatility of the enzyme in organic synthesis have been achieved simply by changing reaction media without modifying enzyme structures (*5, 17*).

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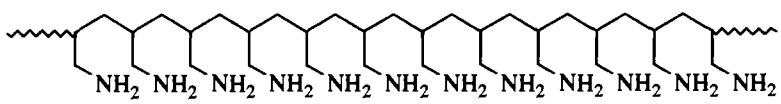
Durability of an enzyme may be improved by suppression of unfolding of the tertiary structure of the enzyme. One way to stabilize the tertiary structure is a multiple attachment of the enzyme to a matrix. Immobilization of enzymes to insoluble supports is often employed for this purpose. More effective cross-linking of an enzyme may be accomplished by using soluble polymer supports instead of solid supports because of the greater flexibility and conformational freedom of the soluble supports. Attachment of an enzyme to a soluble support produces a homogeneous catalyst. The majority of synthetic organic reactions utilize homogeneous catalysts, and soluble enzyme–polymer conjugates can be exploited in many practical applications. Furthermore, introduction of various functional groups to the soluble polymer support can create various microenvironments and additional catalytic features.

In the present investigation, improvement of enzyme properties through attachment to water-soluble polymers is tested by using various derivatives of poly(allylamine) (PAA). The molecular weight of PAA used in this study is about 100,000, which is greater than or comparable to that of many enzymes. PAA is soluble in water and in several common organic solvents. All of the nitrogen atoms in PAA are primary amines which can be readily modified by alkylation, acylation, and imine-formation, allowing incorporation of various structural elements. α-Chymotrypsin (ChT) is chosen in the present study as the enzyme linked to PAA derivatives containing anionic, cationic, and/or hydrophobic microenvironments.



PAA

EXPERIMENTAL PROCEDURES

Preparation of PAA Derivatives

(*1*) *$Ac_{0.6}$PAA*. The HCl salt of PAA (high molecular weight form) was purchased from Aldrich. The molecular weight of the neutral amine form of this polymer has been estimated as 9.9×10^4 (*18*). PAA · HCl (2.00 g; 21.4 residue mmol) was dissolved in 150 ml methanol by stirring with triethylamine (2.17 g; 21.4 mmol) at room temperature for 1 h. To the resultant solution, acetic anhydride (1.13 g; 12.8 mmol) was added under a nitrogen atmosphere over a period of 30 min. After completion of addition of acetic anhydride, a small amount of white solid precipitated, which was dissolved by adding 50 ml water 24 h later. The solution was dialyzed against 16 liters 1:1 (v/v) water–ethanol, ethanol, 0.1 M NaCl solution (twice), and water (twice). The ir spectrum of the white solid obtained after lyophilization of the purified sample indicated carbonyl stretching at 1640 cm^{-1}.

The content of acetyl group introduced to PAA was estimated as 60% of the monomer residue on the basis of ^{1}H NMR and elemental analysis.[2]

(*2*) *$Lau_{0.05}$PAA*. To the mixture of PAA · HCl (2.00 g; 21.4 residue mmol) and triethylamine (2.17 g; 21.4 mmol) obtained as indicated above, lauryl bromide (0.533 g; 2.14 mmol) was added under a nitrogen atmosphere. After stirring for 2 days at room temperature, water (50 ml) and acetic acid (1.1 ml) was added to the mixture to dissolve a small amount of white solid formed during the laurylation reaction. The resultant solution was purified by dialysis as indicated above for $Ac_{0.6}$PAA. The content of lauryl group introduced by PAA is estimated as 4.7 ~ 5.4% of the monomer residue on the basis of ^{1}H NMR and elemental analysis performed on the lyophilized sample.

(*3*) *Ni(II)*[PAA-BD]. To the solution of butanedione (0.522 g; 6.42 mmol) in methanol (50 ml) kept at 40°C, $NiCl_2 \cdot 6H_2O$ (8.83 g; 0.19 mol) dissolved in 50 ml water was added. To this solution, a solution of PAA · HCl (6.00 g; 64.2 residue mmol) and triethylamine (6.50 g; 64.2 mmol) in 500 ml methanol was added. The resulting mixture was refluxed for 3 days. The dark red solution of the product was purified by dialysis as indicated above for $Ac_{0.6}$PAA. The infrared spectrum of the dried sample showed a C=N peak at 1660 cm^{-1}. The content of the macrocyclic metal centers is estimated as 7.2% of the monomer residues on the basis of inductively coupled plasma (ICP) analysis[2] of Ni.

(*4*) *SucPAA*. To the solution of PAA · HCl (0.242 g; 2.59 residue mmol) dissolved in 0.2 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonate (Hepes) buffer (70 ml) at pH 7.7, succinic anhydride (1 g; 10 mmol) was added at room temperature over a period of 4 h in several portions. The pH of the reaction mixture was kept at 7.7 with 2 N NaOH. The product solution was purified by dialysis as indicated above for $Ac_{0.6}$PAA. The content of the succinyl group is estimated as 95 ~ 100% of the monomer residues on the basis of ^{1}H NMR and elemental analysis performed on the lyophilized sample.

Cross-Linking of ChT with PAA Derivatives

ChT was obtained from Sigma. To a solution of ChT (0.30 g, 1.2×10^{-5} mol) in 30 ml water, the hydrogen chloride salt of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (0.23 g, 1.2×10^{-3} mol) dissolved in 10 ml water was added and pH was maintained at 4.5 on a pH stat. The solution was stirred gently for 3 h at 10°C, and a solution of a PAA derivative (1.2×10^{-5} mol) made in 20 ml Hepes buffer (0.05 M, pH 8.2) was added to it and the pH was adjusted to 8.2 with 2 N NaOH. The resulting mixture was stirred gently for 40 h at 4°C. The volume of the reaction mixture was reduced to 30 ml by using an Amicon PM-30 membrane. Then, the mixture was purified by eluting on a Sephadex G-75 column at 4°C. Fractions manifesting large absorbance (>0.8 cm^{-1}) at 280 nm were subject to electrophoresis on a sodium dodecyl sulfate/12.5% polyacrylamide gel, which

[2] Elemental analysis was performed by Galbraith Laboratories, Tennessee, and ICP analysis by Korea Basic Science Center, Seoul.

identified the fractions that did not contain native ChT. These fractions were pooled and concentrated by using an Amicon PM-30 membrane. The isolated yield of the coupling reaction of ChT to the polymer derivatives was 20 ~ 70%. The concentration of the active site of ChT linked to a polymer derivative was determined by spectral titration with cinnamoyl imidazole according to the literature (*19*). The ChT derivatives prepared by this method are PAA-ChT (ChT linked to PAA), $Lau_{0.05}$PAA-ChT (ChT linked to $Lau_{0.05}$PAA), $Ac_{0.6}$PAA-ChT (ChT linked to $Ac_{0.6}$PAA), and Ni(II)[PAA-BD]-ChT (ChT linked to Ni(II)[PAA-BD]).

PAA-ChT and $Lau_{0.05}$PAA-ChT (2.0×10^{-5} M, 70 ml) were succinylated to produce SucPAA-ChT and $SucLau_{0.05}$PAA-ChT, respectively, by reaction with succinic anhydride (1 g, 10 mmol) at pH 7.7 (0.2 M Hepes or sodium phosphate buffer, pH was maintained at 7.7 during the succinylation by addition of 2 N NaOH) and 4°C followed by purification by dialysis.

Kinetic Measurements

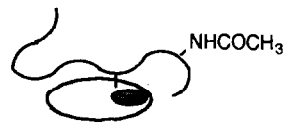
Rates of the hydrolysis of *N*-benzoyl-L-tyrosine-*p*-nitroanilde (BTNA) (purchased from Sigma and used after recrystallization from ethanol–water) were measured spectrophotometrically at 410 nm with a Beckman DU 64 UV/Vis spectrophotometer. Temperature was controlled to within 0.1°C with a Haake E12 circulator. In order to measure the resistance to sodium dodecyl sulfate (SDS), ChT or $Ac_{0.6}$PAA-ChT was incubated with 0.5% SDS at pH 7.5 (0.02 M Hepes) and 25°C. Residual activity of the enzyme was measured by mixing a 2-ml aliquot of the incubated sample with a 0.235 ml dimethyl sulfoxide (DMSO) solution of BTNA ($S_o = 2.0 \times 10^{-4}$ M) and following the absorbance increase accompanying the hydrolysis of BTNA for 1 ~ 2 min at 25°C. Stability of ChT, $Ac_{0.6}$PAA-ChT, and Ni(II)[PAA-BD]-ChT against 4 M guanidinium chloride at pH 7.44 and 25°C was measured by the same method. For measurements of thermoinactivation, various derivatives of ChT were assayed by measuring the absorbance increase during 2 ~ 5 min in the hydrolysis of BTNA ($S_o = 2.00 \times 10^{-4}$ M) at pH 7.5 (0.5 M NaCl, 0.02 M Hepes) in the presence of 10.6% (v/v) DMSO. Under the conditions employed for the measurement of residual activity during thermoinactivation, the absorbance increases did not deviate appreciably from straight lines.

For the kinetic measurements of k_{cat} or K_m values, the initially added concentration (S_o) of BTNA was $0.24 \sim 1.2 \times 10^{-4}$ M and that (E_o) of the ChT derivative was $0.8 \sim 1.2 \times 10^{-6}$ M. Buffer contained 0.5 M NaCl and 0.02 M chloroacetate (pH 3.5 ~ 4), acetate (pH 4.5 ~ 5), 4-morpholineethanesulfonate (pH 5.5 ~ 6.5), Hepes (pH 7 ~ 8), tris(hydroxymethyl)aminomethane (pH 8.5), borate (pH 9 ~ 9.5), or bicarbonate (pH 10 ~ 11) in addition to 1.2% (v/v) DMSO which was used as the solvent for the stock solutions of BTNA.

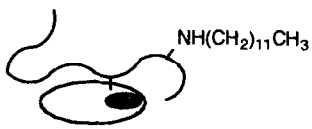
RESULTS

Characteristic structural features of the derivatives of ChT linked to various forms of PAA are schematically presented in Chart 1. Here, the large ellipses symbolize ChT and the shaded ellipses the active site. Many of the amino nitrogen

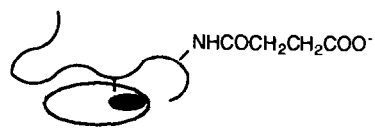
PAA-ChT



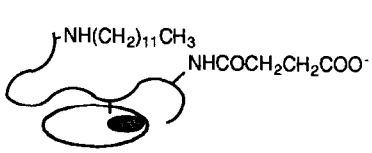
$Ac_{0.6}$PAA-ChT



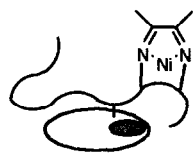
$Lau_{0.05}$PAA-ChT



SucPAA-ChT



Suc$Lau_{0.05}$PAA-ChT



Ni(II)[PAA-BD]-ChT

CHART 1

atoms of PAA are protonated at ambient pHs. The PAA backbones indicated in Chart 1 contain many monopositive cationic sites. Thus, the polyamines in PAA-ChT would provide ChT with cationic microenvironments. On $Lau_{0.05}$ PAA-ChT, about 5% of the nitrogen atoms on PAA contain lauryl groups. The polyamines in these ChT derivatives, therefore, would provide ChT with both cationic and hydrophobic microenvironments. Acetylation of 60% of the primary amines in PAA would lower the cationic character of the polymer considerably. In $Ac_{0.6}$PAA-ChT, the acetyl groups introduced to the backbone of PAA might interact with ChT by hydrogen bonding.

The contents of succinyl groups in SucPAA-ChT and Suc$Lau_{0.05}$PAA-ChT were not measured.[3] However, those in SucPAA-ChT might not be considerably different from those in SucPAA. Succinylation of PAA converts amino nitrogens into amide nitrogens, destroying the positive charges on the amino nitrogens. Thus, microenvironments of the succinylated polymers would be predominantly anionic.

[3] Succinylation of PAA-ChT or $Lau_{0.05}$PAA-ChT would succinylate ChT in addition to the polyamine backbone. Succinylation of ChT has been reported not to affect the thermal stability (*20*).

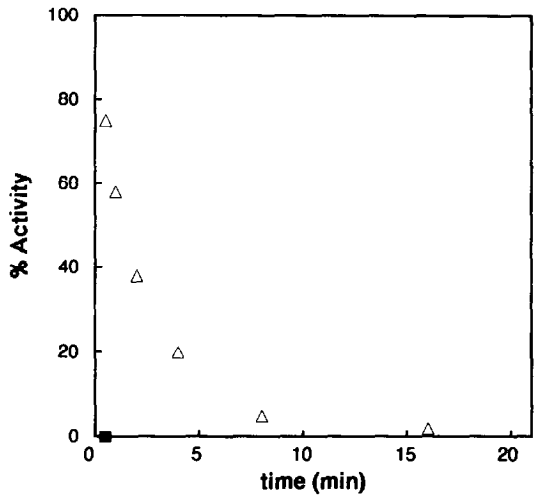


FIG. 1. Residual activity of ChT (■; E_0 = 1.0 × 10^{-6} M) and $Ac_{0.6}$PAA-ChT (Δ; E_0 = 2.1 × 10^{-6} M) during incubation at pH 7.5 and 25°C in the presence of 0.5% SDS.

In $SucLau_{0.05}$PAA-ChT, the lauryl group and the succinyl group would provide nonpolar and anionic microenvironments.

Preparation and kinetic studies on the macrocyclic metal complexes formed on poly(ethylenimine), another water-soluble polyamine, have been previously reported (*21*). Formation of macrocyclic complexes built on PAA such as Ni(II)[PAA-BD] is, however, reported for the first time in this article. The macrocyclic metal centers of Ni(II)[PAA-BD]-ChT would provide fixed dipositive cationic microdomains.

In order to check the stability of ChTs linked to the polyamines, $Ac_{0.6}$PAA-ChT and Ni(II)[PAA-BD]-ChT were chosen as typical examples and were incubated at 25°C in the presence of 0.5% SDS or 4 M guanidinium chloride.[4] Residual activity of the enzymes was checked at various time intervals by assay with BTNA in the presence of the denaturing agents as described under Experimental Procedures. The activity changes observed during the incubation of these ChT derivatives with the denaturing agents at 25°C are illustrated in Figs. 1 and 2.

In order to check the thermal stabiliy of the ChTs linked to the PAA derivatives, changes in the activity of various derivatives of ChT during incubation at 25 and 50°C were evaluated by assay with BTNA at 25°C. Typical semilogarithmic plots of the thermoinactivation process are illustrated in Fig. 3. Except for the initial portions corresponding to 10 ~ 20% activity decreases, the thermoinactivation did not deviate considerably from pseudo-first-order kinetics. The half-life estimated from the pseudo-first-order plots are summarized in Table 1.

[4] Measurement of stability of $Ac_{0.6}$PAA-ChT against 0.5% SDS was possible since $Ac_{0.6}$ PAA-ChT was soluble in the reaction media whereas most of other PAA-linked ChTs and the respective PAA derivatives precipitated in the presence of 0.5% SDS.

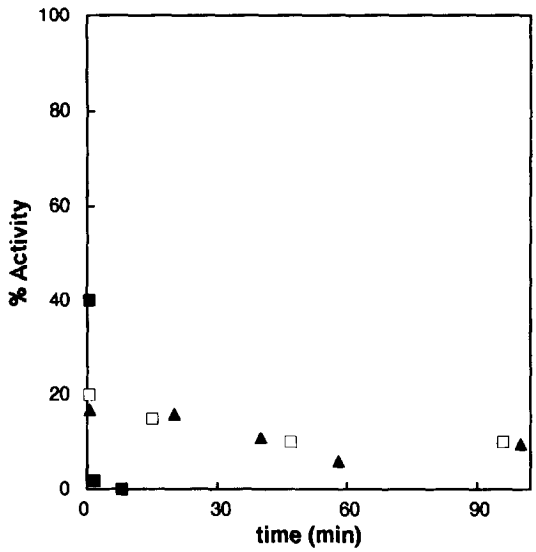


FIG. 2. Residual activity of ChT (■; $E_0 = 1.0 \times 10^{-6}$ M), $Ac_{0.6}$PAA-ChT (□; $E_0 = 2.1 \times 10^{-6}$ M), and Ni(II)[PAA-BD]-ChT (▲; $E_0 = 1.7 \times 10^{-6}$ M) during incubation at pH 7.44 and 25°C in the presence of 4 M guanidinium chloride.

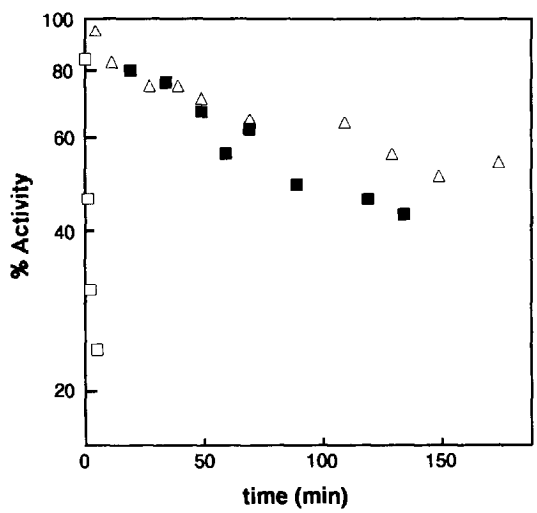


FIG. 3. Typical semilogarithmic plots of residual activity of various ChTs during thermoinactivation at 50°C. (□) ChT ($E_0 = 1.0 \times 10^{-5}$ M), (■) $Ac_{0.6}$PAA-ChT ($E_0 = 7{,}7 \times 10^{-6}$ M), and (△) PAA-ChT ($E_0 = 2.0 \times 10^{-6}$ M).

TABLE 1

Half-Lives of ChTs Linked to Various PAA Derivatives during Thermoinactivation at 25 and 50°C

Enzyme	$E_o(10^{-5}$ M)	Temperature	Half-life
ChT	1.0	25°C	300 ± 30 h
PAA-ChT	0.05	25°C	980 ± 20 h
$Ac_{0.6}$PAA-ChT	0.17	25°C	850 ± 70 h
$Lau_{0.05}$PAA-ChT	0.16	25°C	900 ± 170 h
Ni(II)[PAA-BD]-ChT	0.08	25°C	>1000 h[a]
ChT	1.0	50°C	3.0 ± 0.4 min
PAA-ChT	0.20	50°C	150 ± 20 min
$Ac_{0.6}$PAA-ChT	4.2	50°C	42 ± 2 min
$Ac_{0.6}$PAA-ChT	1.5	50°C	120 ± 20 min
$Ac_{0.6}$PAA-ChT	0.77	50°C	140 ± 20 min
$Ac_{0.6}$PAA-ChT	0.48	50°C	130 ± 10 min
$Ac_{0.6}$PAA-ChT	0.17	50°C	140 ± 10 min[b]
Ni(II)[PAA-BD]-ChT	0.20	50°C	120 ± 10 min
$Lau_{0.05}$PAA-ChT	0.16	50°C	110 ± 20 min

[a] Activity decreased by less than 20% in 500 h.

[b] Assay reaction was carried out at 50°C for this ChT derivative, whereas it was carried out at 25°C for the other derivatives.

The thermal stability at 50°C of $Ac_{0.6}$PAA-ChT was measured at several E_o concentrations ($0.17 \sim 4.2 \times 10^{-5}$ M) (Table 1). At $E_o \leq 1.5 \times 10^{-5}$ M, the thermal stability was independent of E_o. The thermal stability of $Ac_{0.6}$PAA-ChT at 50°C was also checked by performing the assay reaction at 50°C as well as 25°C. The same thermal stability is manifested by the assay methods employing the two different temperatures. This indicates that the deactivated form of the ChT derivative obtained by incubation at 50°C is not reactivated during the assay stage at 25°C.

Kinetics of the hydrolysis of BTNA catalyzed by the ChT derivatives were also measured under the conditions of $S_o \gg E_o$. The initial velocities (v_o) of the reactions were estimated by the statistical method described in the literature (*22*). Analysis of the v_o data according to the Lineweaver–Burk plot produced the values of k_{cat}, K_m, and k_{cat}/K_m. Typical pH profiles of k_{cat} and k_{cat}/K_m are illustrated in Figs. 4 and 5.

The pH profiles of k_{cat} and k_{cat}/K_m were analyzed according to Scheme 1 and Eqs. [1] and [2] (*23*) by a computer program based on the nonlinear regression method (*24*) reported in the literature. Values of pK_{E1}, pK_{E2}, pK_{ES1}, pK_{ES2}, k^o_{cat}, and $(k_{cat}/K_m)^o$ estimated by analyzing the pH profiles of k_{cat} and k_{cat}/K_m for the hydrolysis of BTNA by various derivatives of ChT are summarized in Table 2. The pH profiles were also measured for the hydrolysis of BTNA by native ChT in the presence of various derivatives of PAA. Values (Table 3) of pK_{E1}, pK_{E2}, pK_{ES1}, pK_{ES2}, k^o_{cat}, and $(k_{cat}/K_m)^o$ derived from these data indicate that the derivatives of PAA do not affect the kinetic behavior of ChT appreciably unless they are linked covalently to ChT.

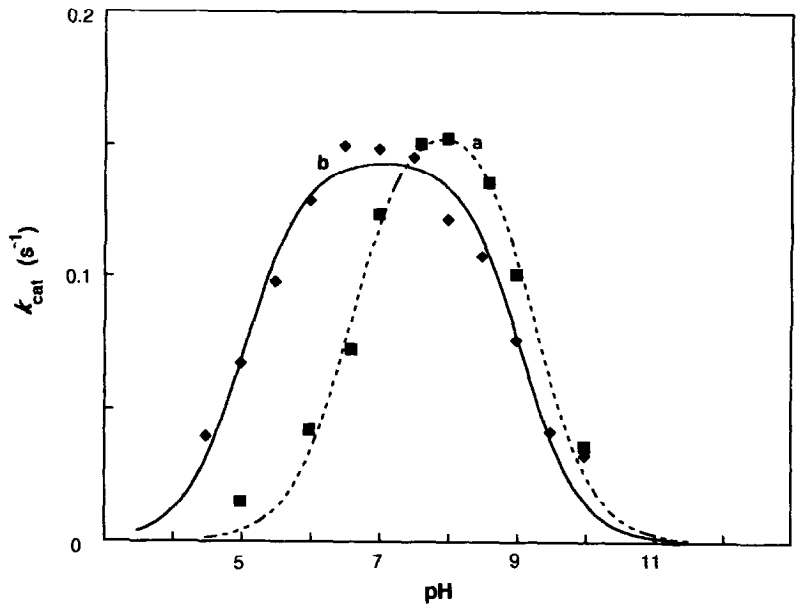


FIG. 4. pH profiles of k_{cat} for ChT [curve a (■)] and PAA-ChT [curve b (◆)] at 25°C.

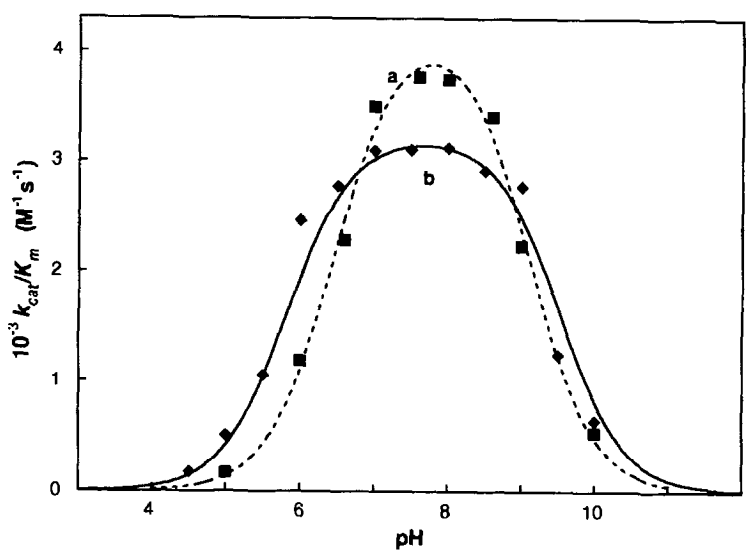
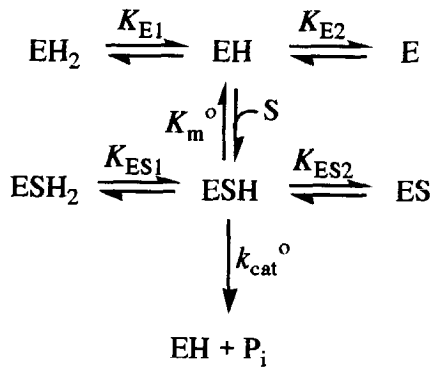


FIG. 5. pH profiles of k_{cat}/K_m for ChT [curve a (■)] and PAA-ChT [curve b (◆)] at 25°C.



SCHEME 1

$$k_{cat} = k^{o}_{cat}/(1 + [H^+]/K_{ES1} + K_{ES2}/[H^+]) \quad [1]$$

$$k_{cat}/K_m = (k_{cat}/K_m)^{o}/(1 + [H^+]/K_{E1} + K_{E2}/[H^+]) \quad [2]$$

DISCUSSION

ChT is linked to various derivatives of PAA by a coupling method using a water-soluble carbodiimide. Thus, the carboxyl groups of ChT are linked to the amino groups of the polyamines. It has been reported that 15 of 17 exposed carboxyl groups of ChT react with the water-soluble carbodiimide under the coupling conditions (*20, 25*). Since each molecule of PAA derivatives contains hundreds of primary amines, a ChT molecule can form several amide bonds with the polyamines. Once an amino group of a polyamine is linked to a carboxyl group of ChT, the subsequent attack by other amino groups of the same polyamine molecule at the other activated carboxyl groups of the ChT molecule is an intramolecular process and would proceed very readily. Therefore, attack by more than two polyamine molecules at the same ChT molecule is not very likely. As PAA has a

TABLE 2

Kinetic Parameters Estimated from Analysis of the pH Profiles of k_{cat} and k_{cat}/K_m for the Hydrolysis of BTNA by PAA-bound ChTs

Enzyme	pK_{E1}	pK_{E2}	pK_{ES1}	pK_{ES2}	$(k_{cat}/K_m)^{o}$(10^3 $s^{-1}M^{-1}$)	k^{o}_{cat}(10^{-1} s^{-1})
ChT	6.45 ± 0.07	9.11 ± 0.07	6.60 ± 0.09	9.26 ± 0.11	4.24 ± 0.15	1.67 ± 0.09
PAA-ChT	5.46 ± 0.10	9.45 ± 0.10	5.06 ± 0.09	9.06 ± 0.09	3.23 ± 0.13	1.46 ± 0.05
$Ac_{0.6}$PAA-ChT	5.82 ± 0.08	9.53 ± 0.08	5.98 ± 0.09	9.16 ± 0.09	3.45 ± 0.13	1.73 ± 0.08
$Lau_{0.1}$PAA-ChT	6.33 ± 0.18	8.40 ± 0.20	5.05 ± 0.28	8.44 ± 0.31	2.09 ± 0.27	0.26 ± 0.03
SucPAA-ChT	6.84 ± 0.11	9.60 ± 0.12	7.04 ± 0.22	9.23 ± 0.23	2.04 ± 0.12	0.85 ± 0.12
$SucLau_{0.1}$PAA-ChT	6.41 ± 0.10	9.76 ± 0.13	6.16 ± 0.23	9.48 ± 0.24	0.69 ± 0.04	0.74 ± 0.08
Ni(II)[PAA-BD]-ChT	6.32 ± 0.09	9.05 ± 0.10	5.66 ± 0.31	8.79 ± 0.25	2.32 ± 0.11	1.45 ± 0.20

TABLE 3

Kinetic Parameters Estimated from Analysis of the pH Profiles of k_{cat} and k_{cat}/K_m for the Hydrolysis of BTNA by ChT in the Presence of Equimolar Amounts of PAA Derivatives

Added polymer	pK_{E1}	pK_{E2}	pK_{ES1}	pK_{ES2}	$(k_{cat}/K_m)^o(10^3\ s^{-1}M^{-1})$	$k_{cat}^o(10^{-1}\ s^{-1})$
None	6.45 ± 0.07	9.11 ± 0.07	6.60 ± 0.09	9.26 ± 0.11	4.24 ± 0.15	1.67 ± 0.09
PAA	6.35 ± 0.07	9.10 ± 0.08	6.50 ± 0.12	9.19 ± 0.16	4.17 ± 0.15	1.75 ± 0.12
$Ac_{0.6}$PAA	6.41 ± 0.03	9.07 ± 0.04	6.52 ± 0.08	9.19 ± 0.10	4.12 ± 0.07	1.71 ± 0.08
$Lau_{0.1}$PAA	6.36 ± 0.05	9.08 ± 0.05	6.29 ± 0.08	9.52 ± 0.11	3.06 ± 0.15	1.28 ± 0.09
SucPAA	6.42 ± 0.09	9.65 ± 0.13	6.49 ± 0.17	9.40 ± 0.23	3.57 ± 0.16	1.48 ± 0.13
Ni(II)[PAA-BD]	6.33 ± 0.07	9.03 ± 0.08	6.34 ± 0.10	9.27 ± 0.12	3.94 ± 0.15	1.57 ± 0.08

greater molecular than ChT, it is possible that more than two ChT molecules are linked to the same molecule of PAA derivatives, resulting in the intermolecular cross-linking of the enzyme. As far as kinetic behavior is concerned, however, no anomalies that may be related to intermolecular cross-linking were observed for the ChTs linked to the PAA derivatives.

Stabilization of the tertiary structure of ChT upon attachment to the PAA derivatives is demonstrated by resistance to denaturing agents. In the presence of 0.5% SDS, native ChT is inactivated right after exposure to SDS, whereas $Ac_{0.6}$ PAA-ChT retains part of the activity over a period of several minutes. This suggests that the polypeptide chain is stabilized by cross-linking with the polyamine.

In the presence of 4M guanidinium chloride, the activity of native ChT, $Ac_{0.6}$ PAA-ChT, and Ni(II)[PAA-BD]-ChT decreases very rapidly. However, $Ac_{0.6}$PAA-ChT and Ni(II)]PAA-BD]-ChT keep 10 ~ 20% of the original activity even after 1 ~ 2 h, whereas the activity of native ChT is totally lost within a few minutes. It appears that attachment of ChT to $Ac_{0.6}$PAA or Ni(II)[PAA-BD] produces a mixture of ChT derivatives and that some of them are very stable against 4 M guanidinium chloride. At least 10 ~ 20% of $Ac_{0.6}$PAA-ChT and Ni(II)[PAA-BD]-ChT prepared in this study, therefore, strongly resist irreversible unfolding of tertiary structure by 4 M guanidinium chloride.

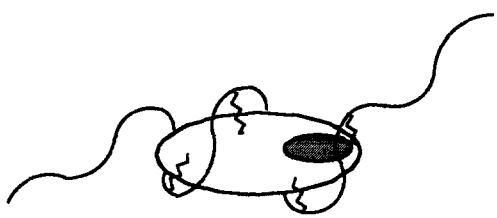
The thermal stability and the acid–base resistivity of an enzyme are more directly related to utility of the enzyme in practical applications, and most of the ChT derivatives prepared in this study were examined for thermoinactivation and pH-rate dependence. The thermal stability of ChT is enhanced considerably upon attachment to the PAA derivatives. Some of the polymer-bound ChTs retain significant activity after several hours at 50°C and after several weeks or months at 25°C. Dependence of the rates of thermoinactivation on E_o concentrations was examined with $Ac_{0.6}$PAA-ChT at 50°C. The results are consistent with the presence of both bimolecular and unimolecular paths. In the bimolecular path, ChT would be inactivated through autolysis. When E_o is sufficiently small, ChT is denatured in a unimolecular path. When $E_o \leq 1.5 \times 10^{-5}$ M, the rate of the thermoinactivation of $Ac_{0.6}$PAA-ChT at 50°C was independent of E_o, indicating that $Ac_{0.6}$PAA-ChT is inactivated by the unimolecular path. The rate for the thermoinactivation by the unimolecular path reflects the intrinsic stability of the polypeptide chain.

A previous thermoinactivation study (*20*) of native ChT at 50°C and pH 7

revealed the same rate (half-life, 2.8 min) for $E_0 = 1 \times 10^{-5}$ M and 0.07×10^{-5} M. A similar result is observed in this investigation with $E_0 = 1 \times 10^{-5}$ M (Table 1). The half-lives for the ChTs linked to the PAA derivatives are up to 50 times greater than that for native ChT. Thus, the intrinsic stability of the polypeptide chain of ChT is considerably enhanced upon attachment to the PAA derivatives. Autolysis of ChT might be effectively suppressed by attaching ChT to the PAA derivatives, since the PAA moieties would repel each other due to their large charge densities. The resistance to thermoinactivation revealed by ChTs linked to PAA derivatives is, however, mainly due to the enhanced intrinsic stability of the polypeptide chain instead of the suppression of autolysis.

Attempts have been made to stabilize enzymes by introducing artificial intramolecular cross-links into the enzymes. Many bifunctional compounds such as dialdehydes, diimido esters, diisocyanates, bisdiazonium salts, dicarboxylic acids, and diamines have been used for cross-linking of proteins (*26*). Thermostabilization of ChT by intramolecular cross-linking has been investigated by using diamines such as $H_2N(CH_2)_nNH_2$ (*20*). The thermostability of ChT modified with the diamines was the greatest when $H_2N(CH_2)_4NH_2$ was used as the cross-linking reagent, with the half-life of the thermoinactivation at 50°C being about 9 min (*20*). At 50°C, the half-life of the ChT cross-linked with $H_2N(CH_2)_4NH_2$ is only 3 times greater than that of native ChT, whereas those of the ChTs linked to the PAA derivatives are up to 50 times greater than that of native ChT.

The ChT derivatives examined in the present study manifest remarkable resistance to denaturing conditions, and much greater thermostabilization is achieved by cross-linking with PAA derivatives than with diamines. This may be attributed to the multiple attachment of the ChT to PAA derivatives and the consequent suppression of unfolding of the tertiary structure of ChT. This is schematically illustrated by A. The multiple attachment of ChT to the polyamines does not



A

significantly damage the active site, since $(k_{cat}/K_m)^o$ values of the polymer-bound ChTs is 50 ~ 80% of that of native ChT except for $SucLau_{0.05}PAA$-ChT.

In PAA-ChT, $Lau_{0.05}PAA$-ChT, and Ni(II)[PAA-BD]-ChT, the PAA derivatives would provide ChT with cationic microenvironments. On the other hand, SucPAA-ChT and $SucLau_{0.05}PAA$-ChT would create anionic microdomains. Although the pK values calculated from the pH profiles (Table 2) generally decrease upon

introduction of cationic microenvironments and increase by incorporation of anionic microdomains, anomalies are seen with pK_{E2} of PAA-ChT and pK_{ESI} of $SucLau_{0.05}$PAA-ChT. Since the pH profiles for the PAA-bound ChTs are shifted toward either lower or higher pHs compared with those for native ChT, the PAA-bound enzymes possess greater activity at acidic or basic pHs compared with native ChT.

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