Organic Artificial Proteinase with Active Site Comprising Three Salicylate Residues

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Abstract: The first organic artificial proteinase based on synthetic materials is obtained by building active sites on the backbone of poly(ethylenimine) (PEI). The active site comprising three salicylate residues is prepared by preassemblage of three molecules of a salicylate derivative with Fe(III) ion followed by cross-linkage of the salicylates with PEI and demetalation of the resulting polymer. Proteinase activity of the artificial enzyme is demonstrated with hydrolytic cleavage of γ-globulin (Gbn). Both the heavy (50 kDa) and the light (25 kDa) chains of Gbn are effectively cleaved into peptides smaller than 5 kDa. The optimum activity of the artificial proteinase is manifested at pH 5−7. The half-life for cleavage of the two chains of Gbn by the artificial proteinase is ∼1 h at pH 7 and 50 °C when the concentration of the artificial active site is 0.4−1 mM. The activity (at pH 7) of the artificial proteinase prepared with PEI is comparable to that (at pH 9) of a catalytic antibody elicited by a joint hybridoma and combinatorial antibody library approach and to that (at pH < 3) achieved by the intramolecular carboxyl group in the hydrolysis of *N*-methyl maleamic acid. Thus, the approach reported here is useful for designing active sites of artificial enzymes.

Introduction

Design of biomimetic catalysts is intensively investigated in various branches of chemistry and biology. In the area of molecular recognition,[1] for example, catalysis by selective recognition of transition states is one of the major targets. Several host molecules such as cyclodextrin derivatives have been designed as biomimetic catalysts. By utilizing versatile catalytic repertories of metal ions,[2,3] catalysts for various reactions have been devised through use of mononuclear or multinuclear metal centers.[4−7] Catalytic antibodies are examples of artificial enzymes based on macromolecules of biological origins.[8−11] Artificial enzymes are also designed by using synthetic macromolecules.[12−16]

For reproduction of characteristics of enzymatic action such as formation of complexes with substrates, large degrees of rate acceleration, and high selectivity, it is highly desirable to build active sites. Just as polypeptides form the backbone of the active sites of natural enzymes comprising several catalytic elements, macromolecules have been used as the skeleton of artificial enzymes. For example, catalytic antibodies use immunoglobulins as the skeleton, whereas synthetic macromolecules are used as skeletons in the design of synzymes[12] (synthetic polymers with enzyme-like activities).

Detailed mechanisms of enzymatic actions were difficult to understand in the early stages of enzymology; similarly information on structure and mechanism of artificial enzymes built on skeletons of natural or synthetic macromolecules is not easy to obtain. At present, in the area of artificial enzymes such as catalytic antibodies or synzymes, major efforts are being made to develop new strategies for designing the active site. Whether the strategy is successful is judged by the activity of the artificial enzymes produced, although the structure of the active site may not be well-characterized, and the mechanism of catalysis may not be understood on the molecular level.

In attempts to design active sites of artificial enzymes, various methods have been developed to position functional groups in proximity on the macromolecular backbone. With catalytic antibodies, transition-state analogues are used as haptens to induce several catalytic groups in the artificial active site built on immunoglobulins.[8−11] In molecular imprinting, functional groups preassembled with a template mimicking the transition state are attached to monomers that are to be subjected to copolymerization.[16] In addition, several types of specific binding sites have been attached to synthetic polymers, and methods have been developed to attach catalytic groups and binding sites in proximity to one another.[17−20] Although detailed information on the structure of the resulting active sites is seldom obtained, several strategies have been tested to improve the activity of artificial enzymes.

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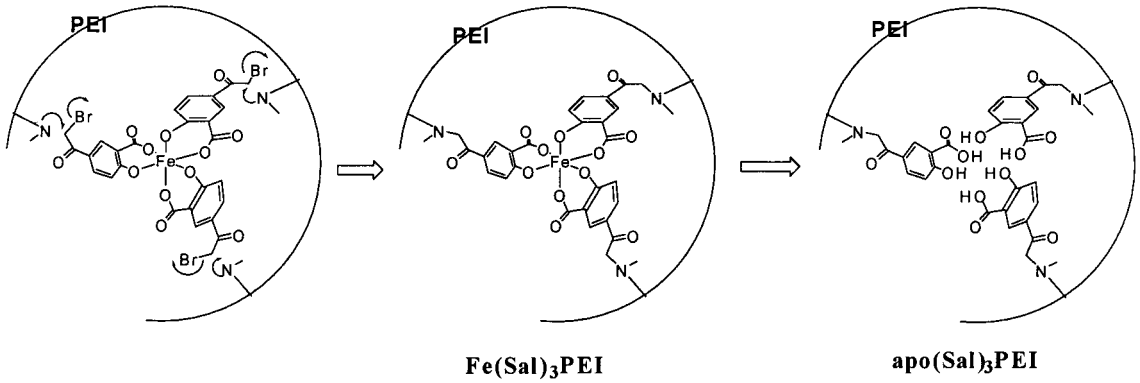
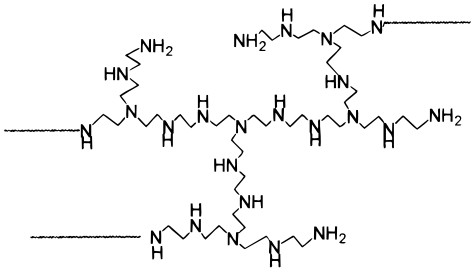
Scheme 1



For a novel methodology to place several organic functional groups in proximity on a synthetic macromolecular backbone, we developed the method of preassemblage of organic molecules with a template, followed by cross-linkage with a branched polymer.[20−23] For example, when three molecules of 4-bromoacetylsalicylate were preassembled with Fe(III) ion and then cross-linked with branched poly(ethylenimine) (PEI),[24] three



PEI

salicylate residues were positioned in proximity on the resulting polymer, $Fe(Sal)_3PEI$, as illustrated in Scheme 1.[22] Upon removal of the Fe(III) ion, $Fe(Sal)_3PEI$ was converted into apo-$(Sal)_3PEI$. The three salicylates remained in proximity even after the template Fe(III) ion was removed. This was demonstrated by the effective molarity of 1000 M for a salicylate residue of $apo(Sal)_3PEI$ toward Fe(III) ion complexed to another salicylate residue.[22]

$Apo(Sal)_3PEI$ contains sites comprising three salicylate moieties [$(Sal)_3$]. Each $(Sal)_3$ site contains three carboxyl groups and three phenol groups. The carboxyl group is the functional group of aspartate or glutamate residues of enzymes, whereas phenol is the functional group of tyrosine residues. In addition, the PEI backbone contains amino groups, the functional group of lysine. The original geometry of the complex formed between three molecules of 4-bromoacetylsalicylate and an Fe(III) ion is effectively conserved in $apo(Sal)_3PEI$. Each molecule of the salicylate is, however, connected to PEI by a single attachment, and so the salicylate residues in each $(Sal)_3$ unit have some conformational flexibility. If the salicylate residues can occupy the appropriate positions to stabilize transition states of certain chemical reactions, this would constitute effective biomimetic catalysts. In attempts to search for catalytic activities of $apo(Sal)_3PEI$, we tested its activity on protein hydrolysis, in view of the vast amount of information available for structure and mechanism of proteinases. In this paper, we report the activity of $apo(Sal)_3PEI$ for hydrolysis of γ-globulin (Gbn).

Experimental Section

Preparation of PEI Derivatives. Preparation and characterization of $Fe(Sal)_3PEI$, $apo(Sal)_3PEI$, and $(Sal)_{ran}PEI$ were reported previously.[22] As indicated in Scheme 1, $Fe(Sal)_3PEI$ was prepared by preassemblage of three molecules of 4-bromoacetylsalicylate with Fe(III) ion in dimethyl sulfoxide followed by cross-linkage of the salicylates with PEI. Removal of Fe(III) ion from $Fe(Sal)_3PEI$ by dialysis against 1 M HCl produced $apo(Sal)_3PEI$. By random (ran) alkylation of PEI with 4-bromoacetylsalicylate, $(Sal)_{ran}PEI$ was obtained. The PEI derivatives were purified by repetitive dialysis.

Measurements. Distilled, deionized water was used in preparing the PEI derivatives and in the kinetic studies. The degree of cleavage of bovine serum γ-globulin (Gbn) (purchased from Sigma; used without further purification) in the presence of the PEI derivatives was measured by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). Densities of the electrophoretic bands were analyzed with software purchased from Jandel Corporation. Rate data for cleavage of Gbn were collected at 50 ± 0.1 °C and various pH values. Buffers (0.05 M) used for the kinetic measurements were citric acid (pH 3.0−3.5), sodium acetate (pH 4−5), 4-morpholineethanesulfonic acid (pH 6), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7−8), boric acid (pH 9), glycine (pH 10), and sodium phosphate (pH 12). pH measurements were carried out with a Dongwoo Medical DP-880 pH/Ion meter. HPLC analysis of N-labeled peptide mixtures, amino acid analysis, and N-terminal sequencing of peptides by Edman degradation were performed by the Korea Basic Science Research Institute, using a Waters PicoTag System and a Perkin-Elmer Procise 491 model. Matrix-assisted laser desorption/ionization mass spectrometric (MALDI-MS) measurements were carried out with a Voyager PerSeptive linear model. Inductively coupled plasma absorption emission spectroscopic (ICP-AES) measurements were performed with a Shimadzu ICPS-1000IV model. NMR spectra were taken with a Bruker DPX 300-MHz model.

Results

$Fe(Sal)_3PEI$ and $apo(Sal)_3PEI$ were prepared and characterized according to the procedure reported previously.[22] The content of $Fe(Sal)_3$ units in $Fe(Sal)_3PEI$ was 0.57 residue mol %. ICP-AES analysis of $apo(Sal)_3PEI$ indicated that Fe(III) was almost completely (>99%) removed. The content of salicylate residue in $(Sal)_{ran}PEI$ was 1.9 residue mol %, as checked by NMR spectrometry.

As illustrated in Figure 1, SDS−PAGE[25,26] revealed facile cleavage of both the heavy (50 kDa) and the light (25 kDa)

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(24) PEI (M_w 60 000) contains ethylamine as the repeating unit. Among ~1400 amino groups, ~25%, 50%, and 25% are primary, secondary, and tertiary amines, respectively. The tertiary amines represent the branching points, and PEI is highly branched and soluble in water.

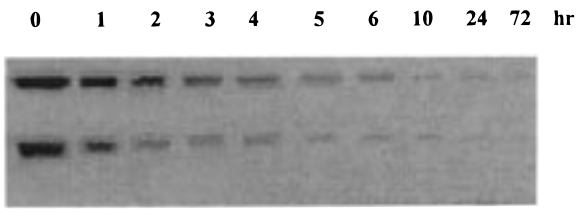

Figure 1. Electrophoretic analysis of Gbn (5.35×10^{-6} M) incubated with apo(Sal)$_3$PEI (4.95×10^{-4} M) at pH 6.00 and 50 °C. The numbers indicate the period of incubation. The upper and the lower bands are the heavy and the light chains of Gbn, respectively.

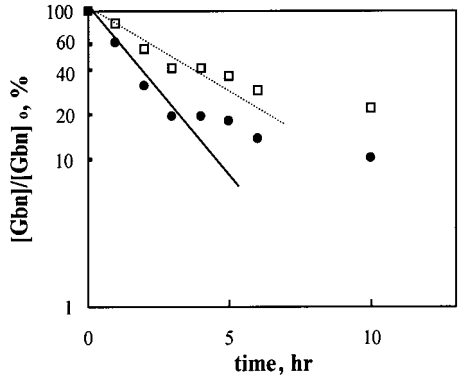

Figure 2. Plot of log [Gbn]/[Gbn]$_o$ against time for the data illustrated in Figure 1. The relative concentrations of the heavy (□) and the light (●) chains of Gbn were measured by analyzing the densities of the electrophoretic bands.

chains of bovine serum Gbn during incubation with apo-(Sal)$_3$PEI. The rate of protein cleavage was measured by monitoring the decrease in the density of the bands corresponding to the heavy and light chains. As illustrated in Figure 2, pseudo-first-order kinetic behavior was observed for up to 50−80% of the reactions. The initial pseudo-first-order rate constant ($k_{in} = v_o/S_o$) was estimated from the initial linear portion of the logarithmic plot as exemplified by Figure 2.

The dependence on C_o (the initially added concentration of catalyst) of k_{in} for cleavage of both chains of Gbn was examined under the conditions $C_o \gg S_o$ (the initially added concentration of substrate). The results obtained at pH 6.00 are illustrated in Figure 3. For apo(Sal)$_3$PEI, the concentration of the (Sal)$_3$ unit was used as the concentration of the catalyst. Proportionality between k_{in} and C_o was observed for both the heavy and the light chains of Gbn. The proportionality constant (k_2) was estimated from the plot of k_{in} against C_o at various pHs. The pH dependence of k_2 is illustrated in Figure 4 for the two chains. The pH dependence was analyzed with Scheme 2.[27] Here, CH_2, CH, and C represent the catalyst in various ionization states, with CH being the catalytically active species. The values for k_2^{lim}, pK_1, and pK_2 estimated from the nonlinear regression of the pH profiles are summarized in Table 1.

MALDI-MS measurement was performed with a product solution obtained after the cleavage of Gbn (5.35×10^{-6} M) in the presence of apo(Sal)$_3$PEI (4.95×10^{-4} M) at pH 6.00 was complete. Results of MALDI-MS indicated that the products obtained were smaller than 5 kDa.

After the cleavage of Gbn (5.35×10^{-6} M) in the presence of apo(Sal)$_3$PEI (4.95×10^{-4} M) at pH 6.00 was complete, the solution was subjected to ultrafiltration with a centrifugal concentrator (cutoff MW 10 000). Since apo(Sal)$_3$PEI does not penetrate the membrane, peptides smaller than 10 kDa are expected to be separated from apo(Sal)$_3$PEI unless they are tightly bound to apo(Sal)$_3$PEI or adsorbed to the membrane. The mixture of peptide products separated by ultrafiltration was treated with phenyl isothiocyanate to label the primary amino group of each peptide product. Phenyl isothiocyanate is the

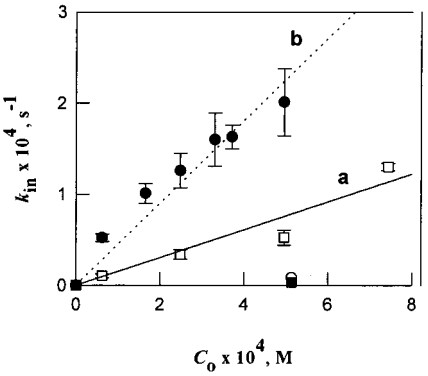

Figure 3. Plot of k_{in} against C_o for the hydrolysis of the heavy (□; line a) and the light (●; line b) chains of Gbn (5.35×10^{-6} M) catalyzed by apo(Sal)$_3$PEI at pH 6.00 and 50 °C. Also included are the rate data for cleavage of the heavy (■) and the light (○) chains of Gbn in the presence of (Sal)$_{ran}$PEI at pH 6.00 and 50 °C. For the data points of (Sal)$_{ran}$PEI, the total concentration of the salicylate residues on the polymer was divided by 3 because of the definition of the C_o for apo(Sal)$_3$PEI.

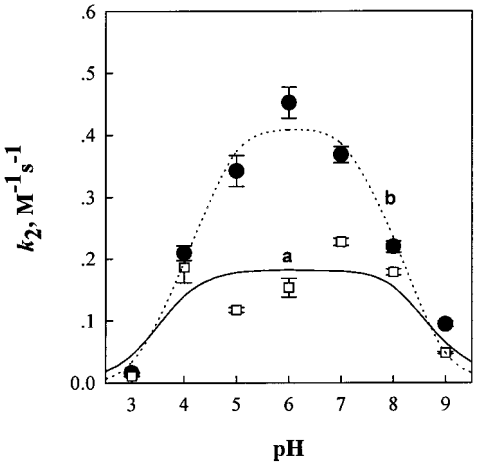

Figure 4. pH dependence of k_2 for the hydrolysis of the heavy (□; curve a) and the light (●; curve b) chains of Gbn catalyzed by apo-(Sal)$_3$PEI at 50 °C. The curves are obtained by fitting the data in terms of Scheme 2.

Scheme 2

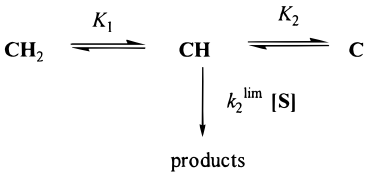

Table 1. Parameter Values Estimated from Analysis of pH Profiles of k_2 for Cleavage of the Two Chains of Gbn with Apo(Sal)$_3$PEI at 50 °C

	k_2^{lim}, M^{-1} s^{-1}	pK_1	pK_2
light chain	0.43 ± 0.03	4.06 ± 0.17	8.11 ± 0.17
heavy chain	0.18 ± 0.03	3.49 ± 0.53	8.74 ± 0.52

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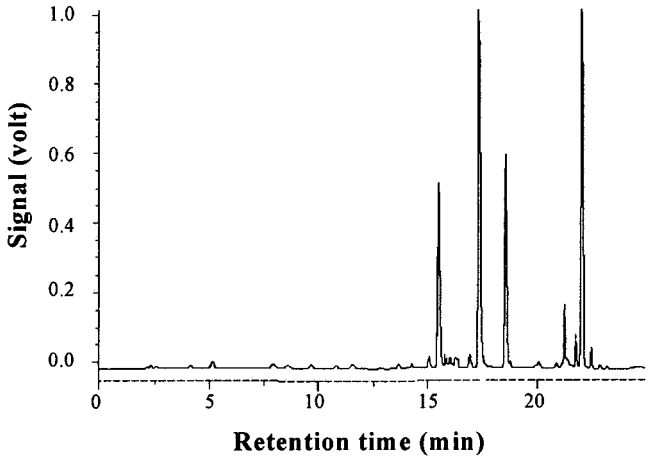

Figure 5. HPLC chromatogram of peptide products separated by ultrafiltration after cleavage of Gbn (7.53×10^{-6} M) by apo$(Sal)_3$PEI (4.95×10^{-4} M) at pH 6.00 and 50 °C. The primary amino groups of the peptide products were labeled with phenyl isothiocyanate before the HPLC analysis. Typical conditions for operating the Waters PicoTag System were used for the elution.

Table 2. Effect of Initially Added Fe(III) Ion and CDTA on Rates for Cleavage of Two Chains of Gbn by Apo$(Sal)_3$PEI[a]

	k_{in}, 10^{-5} s^{-1}	
additive	for heavy chain	for light chain
3.89×10^{-5} M Fe(III)	6.6 ± 0.8	6.9 ± 0.5
7.77×10^{-5} M Fe(III)	6.0 ± 0.8	6.5 ± 0.7
1.25×10^{-3} M CDTA	6.9 ± 0.2	8.2 ± 0.7
none	7.5 ± 0.9	8.0 ± 0.3

[a] $C_o = 3.71 \times 10^{-4}$ M, $S_o = 5.35 \times 10^{-6}$ M, pH 6.00, 50 °C.

reagent used in Edman degradation to convert the primary amino groups of peptides to phenylthiocarbamoyl derivatives. The HPLC chromatogram of the *N*-labeled peptide products is illustrated in Figure 5. In a separate experiment, the product solution separated by ultrafiltration was completely hydrolyzed with acid and the contents of the amino acids were measured. The amino acid analysis indicated that about 60% of the amino acids originally included in Gbn were collected by ultrafiltration. *N*-terminal sequencing of the mixture of peptides obtained as the product of the Gbn cleavage indicated that 10−15% of the amino acids initially contained in Gbn became *N*-terminal amino acid residues of the peptide products. Thus, the average number of amino acids for each peptide product appears to be 7−10.

Rates for hydrolysis of both the heavy and the light chains of Gbn by apo$(Sal)_3$PEI were measured in the presence of initially added Fe(III) ion or *trans*-1,2-cyclohexylenedinitrilotetraacetic acid (CDTA), and the results are summarized in Table 2. When the amount of Fe(III) ion added was ca. 10 or 20 mol % of the Fe(III) binding sites of apo$(Sal)_3$PEI, the rate was slowed by ca. 10 or 20%, respectively. It is known that CDTA removed about 80% of Fe(III) ions of Fe$(Sal)_3$PEI under the experimental conditions.[22] Addition of CDTA, however, did not affect the rate of the apo$(Sal)_3$PEI-catalyzed cleavage of Gbn beyond the error limit.

Rates for cleavage of the two chains of Gbn were also measured with $(Sal)_{ran}$PEI instead of apo$(Sal)_3$PEI, and the results are indicated in Figure 3.

Discussion

Analysis of the reaction products by MALDI-MS revealed that the peptide products obtained from the cleavage of the heavy (50 kDa) and the light (25 kDa) chains are smaller than 5 kDa. More than half of the peptide products were separated by ultrafiltration and subjected to HPLC analysis after their primary amino groups were labeled. A large number of products were obtained (Figure 5). That the peptides are labeled with phenyl isothiocyanate and undergo Edman degradation indicates that the N-terminal amino groups of the peptide products are generated by the cleavage of Gbn by apo$(Sal)_3$PEI. The results also reveal that the cleavage of Gbn in the presence of apo$(Sal)_3$PEI is hydrolytic. If Gbn were cleaved through transacylation by the amino groups of PEI backbone instead of hydrolysis, a major portion of the peptides obtained after multiple cleavage of Gbn would have been covalently attached to the polymer instead of separated from the polymer by the ultrafiltration.

The half-life for amide bonds under the conditions of spontaneous hydrolysis at pH 7 and 25 °C has been estimated as ∼1000 years.[28,29] The half-life of both the heavy and the light chains of Gbn is ∼1 h at 50 °C in the presence of 0.5−1 mM apo$(Sal)_3$PEI at pH 7. This corresponds to acceleration of the hydrolysis of amide bonds by $\sim 10^6$-fold.

Electrophoresis of the reaction mixtures indicated that only the parent proteins of the heavy and the light chains were detected during the cleavage reaction. Thus, any other proteins large enough for detection by the electrophoretic method did not accumulate appreciably during the reaction. Under the conditions of electrophoresis, proteins smaller than several kilodaltons are not detected. Even proteins with greater sizes are not detected at low concentrations. Initial cleavage of the heavy and the light chains of Gbn by apo$(Sal)_3$PEI should produce intermediate proteins of >25 kDa and 12 kDa, respectively. Thus, cleavage of intermediate proteins into peptides of <5 kDa is much faster than the cleavage of the parent proteins, the degree of acceleration being considerably greater than 10^6-fold.

As the reaction proceeded, the plot of log $[Gbn]/[Gbn]_o$ against time (Figure 2) deviated considerably from pseudo-first-order behavior. This is attributable to complexation to apo$(Sal)_3$PEI of products obtained by cleavage of Gbn into several small pieces and the consequent inhibition of the proteolytic activity of apo$(Sal)_3$PEI. The inhibition was supported by the result of a separate kinetic study for the cleavage of Gbn in the presence of preformed products.

When the salcylate residues were attached randomly to PEI, the proteolytic activity was substantially lower. This demonstrates that the proteolytic activity of apo$(Sal)_3$PEI originates from the salicylate groups positioned close together.

Adding a small amount of Fe(III) to apo$(Sal)_3$PEI to convert a fraction of $(Sal)_3$ sites into the Fe(III) complexes reduced the proteolytic activity of apo$(Sal)_3$PEI. The reduction in activity was approximately proportional to the $(Sal)_3$ sites occupied by Fe(III) ion. When CDTA was added to apo$(Sal)_3$PEI to extract Fe(III) ion that might have contaminated apo$(Sal)_3$PEI, the proteolytic activity of apo$(Sal)_3$PEI was not affected appreciably. These results demonstrate that the proteolytic activity of apo$(Sal)_3$PEI is not due to any Fe(III) ion that might have not been completely removed during preparation of apo$(Sal)_3$PEI. The proteolytic activity of apo$(Sal)_3$PEI originates from the active site that comprises three salicylates. Thus, the active site of apo$(Sal)_3$PEI is equipped with three carboxyl groups and three phenolic hydroxyl groups as well as amino groups of the PEI backbone.

The kinetic data observed for the reactions catalyzed by PEI derivatives conform to the Michaelis−Menten scheme.[14,17−19]

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Under the conditions of $C_o \gg S_o$, the Michaelis−Menten scheme leads to the expression of eq 1 for the pseudo-first-order rate constant (k_o). When complexation of the substrate to the catalyst is weak ($C_o \ll K_m$), eq 1 predicts proportionality between k_o and C_o, the proportionality constant being equal to k_{cat}/K_m. Thus, k_2 illustrated in Figure 4 corresponds to k_{cat}/K_m. The linear dependence of k_{in} on C_o indicates that K_m is much greater than the greatest C_o used ($\sim 10^{-3}$ M at pH 6.00; Figure 3) and that k_{cat} is much greater than the largest k_{in} observed (1×10^{-4} to 2×10^{-4} s^{-1}; Figure 3).

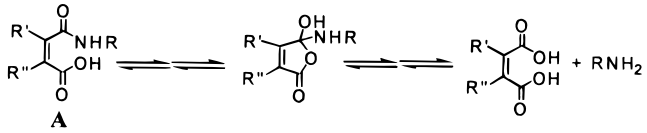
$$k_o = k_{cat}C_o/(K_m + C_o) \quad (1)$$

The pH profiles of k_{cat}/K_m for enzymatic reactions provide information on the catalytic functional groups of the enzyme uncomplexed with the substrate as well as those of uncomplexed substrate.[27] Similarly, the pH profiles illustrated in Figure 4 reflect ionization of the functional groups of apo(Sal)$_3$PEI and Gbn. The pH profiles for the heavy and the light chains of Gbn have similar shapes, except that the range of optimum pH is wider for the heavy chain (pH 4−8) than for the light chain (pH 5−7). The p*K* values estimated by analysis of the pH profiles according to the method[27] used in enzymology are summarized in Table 1. The basic limbs of the pH profiles are characterized by pK_2 of 8−9, suggesting that the phenol groups or ammonium groups (or both) at the active site have to be in acidic form to activate the catalyst. The acidic limbs of pH profiles are characterized by pK_1 = 3−4, suggesting that the carboxyl group is ionized in the active form of apo(Sal)$_3$PEI.[30] The deactivation seen above pH 9 and below pH 3 may result from protonation or deprotonation of functional groups of apo(Sal)$_3$PEI that participate in conversion of the complexed substrate. The deactivation could also be caused by suppression in complexation between the catalyst and the substrate, caused by changes in the overall charges on both the catalyst and the substrate. The small but definitive differences in the pH profile between the heavy and the light chains of Gbn are attributable to the different functional groups of apo(Sal)$_3$PEI involved in the catalytic steps and to the groups affecting complexation between apo(Sal)$_3$PEI and the substrates.

Many proteinases such as serine proteinases or aspartic proteinases utilize only the functional groups of amino acid residues to catalyze hydrolysis of proteins. An aspartic proteinase (also called carboxyl proteinase or acid proteinase) such as pepsin, penicilopepsin, renin, or HIV-1 protease contains two aspartate residues in the active site.[31−37] In aspartic proteinases, organic functional groups other than carboxyl group would also play catalytic roles. Thus the synthetic artificial proteinase of the present study may be related to aspartic proteinases.

The catalyst studied in the present investigation, apo(Sal)$_3$PEI, is the first organic artificial proteinase constructed from synthetic materials. A few artificial proteinases containing transition metal ions such as Pd(II), Cu(II), Fe(III), or Co(III) have been reported.[38−40] Except for the artificial metalloproteinase based on coordinatively polymerized bilayer membranes,[40] proteinase activity of apo(Sal)$_3$PEI is much better than that of the other artificial metalloproteinases.

Scheme 3



Organic artificial proteinases based on biotic materials have been obtained with catalytic antibodies.[41,42] The monoclonal catalytic antibody prepared with a phosphinate hapten exhibited optimum activity at pH 9.5. The k_{cat} measured with an amide substrate at pH 9 and 37 °C was 1.65×10^{-7} s^{-1}.[41] Thus, the half-life is 49 days when the substrate is fully complexed to the active site of the catalytic antibody. A much more improved antibody catalyst for amide hydrolysis has been elicited very recently as a result of a joint hybridoma and combinatorial antibody library approach.[42] The k_{cat} measured with a primary amide substrate at pH 9 and 25 °C was 5×10^{-5} s^{-1}. This corresponds to a half-life of 4 h when the substrate is fully complexed to the active site. Compare these half-lives for the amide hydrolysis catalyzed by the antibodies with that by apo(Sal)$_3$PEI (~1 h at pH 7 and 50 °C when the substrate is only partially complexed to the active site).

Effectiveness of apo(Sal)$_3$PEI in proteolysis can be compared with that of amide hydrolysis by intramolecular catalysis. The most efficient intramolecular catalysis by organic groups in hydrolysis of unactivated amide has been achieved with maleamic acid derivatives. Maleamic acid derivatives are hydrolyzed through nucleophilic catalysis by the intramolecular carboxyl group (Scheme 3).[43,44] The half-life for hydrolysis of *N*-methyl maleamic acid (with R′ = R″ = H and R = CH_3) at 39 °C and optimum pH (<3) is 3 h.[43] This is similar to that (~1 h at 50 °C) for hydrolysis of Gbn with apo(Sal)$_3$PEI (C_o = 0.4−1 mM) at neutral pH's. The catalytic group is tethered to the substrate to increase the effective molarity in the maleamic acid, whereas the catalytic action proceeds as an intermolecular process in the hydrolysis of Gbn by apo(Sal)$_3$PEI.

Amide hydrolysis is accelerated at acidic or alkaline pH's through acid or base catalysis.[45] Many artificial metalloproteinases as well as the catalytic antibodies and maleamic acid mentioned above show optimum activity at acidic or basic pH's. On the other hand, apo(Sal)$_3$PEI exhibits optimum activity at neutral pH, as many proteinases do. The effectiveness of apo(Sal)$_3$PEI should originate from multiple catalytic factors incorporated into the active sites comprising three salcylates. Although the detailed mechanism for the proteinase action is not delineated at present, collaboration among the multiple catalytic elements is most effective at neutral pH.

(30) Below pH 4, a substantial proportion of the amino groups of PEI backbone is protonated (Suh, J.; Paik, H.-J.; Hwang, B. K. *Bioorg. Chem.* **1994**, *22*, 318). The resulting cationic environment would suppress protonation of carboxylate anions.

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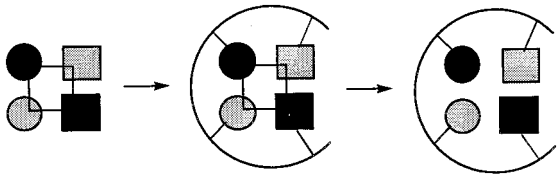
Scheme 4



In conclusion, we obtained an effective artificial proteinase by the strategy adopted in the present study. As illustrated in Scheme 4, catalytic elements are first preassembled with a template and then cross-linked with a branched polymer. Subsequent removal of the template generates active sites of the artificial enzyme. This strategy can be further elaborated to prepare more-effective artificial enzymes. Adding catalytic elements to the skeleton, fine-tuning the geometry of the active site by changing the template, and assembling nonidentical catalytic groups around a template are among the improvements to be made for this strategy.

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