Remarkable Proteolytic Activity of Imidazoles Attached to Cross-Linked Polystyrene

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The insoluble resins synthesized by attaching imidazoles to poly(chloromethylstyrene-*co*-divinylbenzene) effectively hydrolyzed albumin with half-life as short as 20 min at pH 7 and 25 °C. Thus, peptide hydrolysis was accomplished with imidazole in an artificial system for the first time. The imidazole-based artificial proteinases manifested optimum activity at pH 7−8. The proteolytic activity of the imidazole-based artificial proteinases exceeded that of previously reported organic artificial proteinases including catalytic antibodies. High proteolytic activity was observed when imidazole was attached to the resin through the C-2 atom instead of the N atom. The catalytic activity was greatly reduced when the content of imidazole was lowered. This indicates catalytic cooperation of at least two proximal imidazole moieties attached to the resin. Possible mechanisms for the effective protein hydrolysis by the proximal imidazoles are presented.

Introduction

Functional groups provided by amino acids such as hydroxyl, phenolic, mercapto, carboxyl, imidazolyl, and amino groups are primary candidates for catalytic groups of artificial enzymes. In particular, imidazolyl and carboxyl groups ionize at near neutral pH and, thus, can play various catalytic roles such as nucleophiles, general acids, and general bases. Among enzymes, proteinases such as α-chymotrypsin, carboxypeptidase A, pepsin, and papain are the first that have been subjected to both intensive mechanistic studies and extensive model studies.[1−4] In designing proteinase-mimicking catalysts, imidazole has been often employed as the key functional group in view of its versatile catalytic roles. Various kinds of small molecules containing imidazole have been tested for catalytic activity in the hydrolysis of activated esters and anilides.[5−11] Sometimes *N*,*N*-dimethylpyridine, which has higher nucleophilicity than imidazole, was used as an imidazole analogue. Imidazole and *N*,*N*-(dimethylamino)pyridine attached to polymers have been also tested for proteinase-like activity.[12−17] To date, hydrolysis of unactivated peptides has not been achieved with synthetic catalysts exploiting imidazole or 4-(*N*,*N*-dimethylamino)pyridine.

Some enzymes are known to use either two imidazoles or two carboxyl groups as the key catalytic groups. In ribonuclease A, one imidazole acts as a general base and the protonated form of the other imidazole as a general acid.[18] In the mechanism widely proposed for aspartic proteases such as pepsin, penicillopepsin, rennin, and HIV protease, one carboxyl group acts as a general acid and the ionized form of the other carboxyl group as a general base or a nucleophile.[2,19] To mimic such enzymes, synthetic catalysts have been designed to induce cooperation between two identical catalytic groups. An example is the cyclodextrin derivative with two imidazoles attached to the rim, which has been designed as a mimic of ribonuclease A.[20] The half-lives for spontaneous hydrolysis of peptides[21−23] and RNAs[24] are 500−1000 yr and about 100 yr, respectively. If the imidazole pair is effective in hydrolysis of RNA, it might be also effective in hydrolysis of peptides just as the carboxyl pair of aspartic proteinases in view of similar stability of peptides and RNAs.

To design effective artificial proteinases employing imidazole as the sole catalytic group, the reactivity of imidazole incorporated in the catalyst should be improved remarkably. This may be achieved by locating two or more imidazoles in productive positions to induce effective cooperation among proximal imidazoles and by

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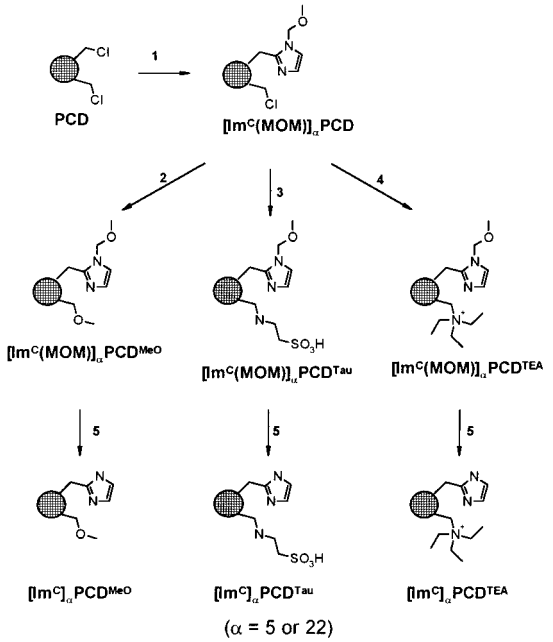
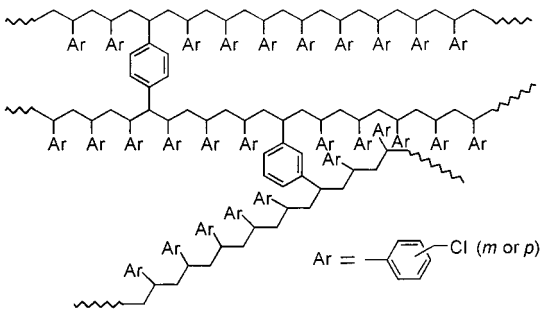
Scheme 1



tuning the microenvironment to optimize the medium effects. In the present study, we succeeded to prepare the first artificial proteinase based on imidazole. This artificial proteinase was synthesized through random attachment of imidazoles to poly(chloromethylstyrene-*co*-divinylbenzene) (PCD). PCD is a cross-linked polystyrene, where the styryl moiety is chloromethylated.[25] In this article, preparation of the imidazole-based immobile artificial proteinase is described, together with the kinetic data for the proteinase-like activity.



PCD

Results

PCD with 2% cross-linkage was prepared according to the literature procedure by suspension copolymerization of chloromethylstyrene and divinylbenzene.[25] Imidazole was attached to PCD via either the C-2 atom or the N atom as summarized in Schemes 1−3. By the route summarized in Scheme 1, the carbanion derived from 1-methoxymethylimidazole (MOM-imidazole) replaced the chloro groups of PCD through the C-2 atom. The chloro groups which remained unaffected by treatment with MOM-imidazole carbanion were subsequently treated with methoxide, taurine (2-aminoethanesulfonate), or triethylamine (TEA). To enhance the reactivity of halide in substitution with MOM-imidazole carbanion, chloro

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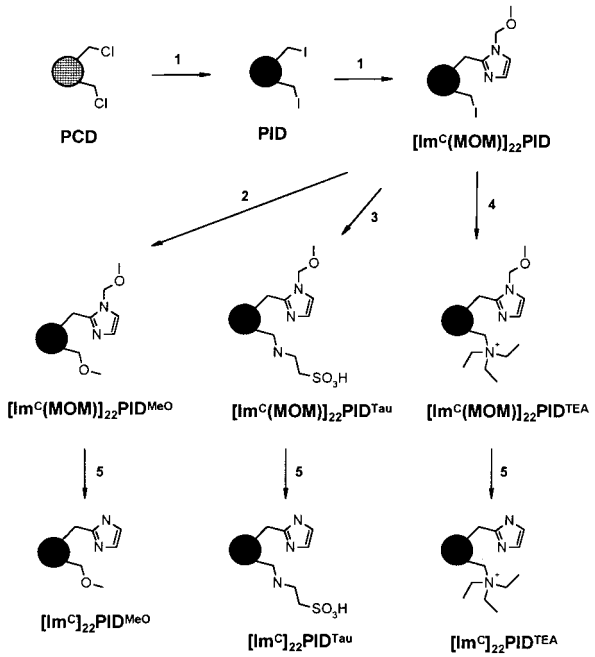
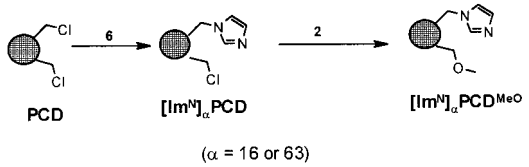
Scheme 2



Scheme 3



groups of PCD were treated with iodide ion to obtain poly(iodomethylstyrene-*co*-divinylbenzene) (PID). Electron probe microanalysis (EPMA) revealed that the amount of unsubstituted Cl on the surface of PID was negligible. PID was subsequently treated with MOM-imidazole carbanion followed by methoxide, taurine, or triethylamine (Scheme 2). By using imidazole itself, imidazole replaced the chloro groups of PCD through the N atom (Scheme 3). In the nomenclature of resins included in Schemes 1−3, Im^C and Im^N denote imidazoles attached to the polymer via the C-2 and the N atoms, respectively, superscripts (MeO, Tau, and TEA) stand for the extra functional groups attached to the resin, and subscripts (α or numbers) represent the contents (residue mol %) of imidazole in the resin.

For the PCD or PID derivatives, contents of imidazole, taurine, and TEA were estimated on the basis of results of elemental analysis. By modification of PCD with an excess amount (2.2 equiv compared with the chloromethylstyryl moiety of PCD) of MOM-imidazole carbanion and by the subsequent treatment with excess taurine $[Im^C(MOM)]_{22}PCD$ and $[Im^C(MOM)]_{22}PCD^{Tau}$ were obtained (Scheme 1). Based on the C/N ratio measured by elemental analysis for $[Im^C(MOM)]_{22}PCD$, the content of imidazole in the resin was calculated as 21.1 residue mol %. Based on C/N, C/S, and N/S ratios of $[Im^C(MOM)]_{22}PCD^{Tau}$, the contents of imidazole and taurine in the resin were calculated as 22.6 and 21.1 residue mol %, respectively. The content of imidazole in the PCD derivatives obtained from $[Im^C(MOM)]_{22}PCD$ was, therefore, assigned as 22 residue mol %. In addition, the content of taurine in $[Im^C(MOM)]_{22}PCD^{Tau}$ and $[Im^C]_{22}PCD^{Tau}$ was assigned as 21 residue mol %. From the C/N ratio

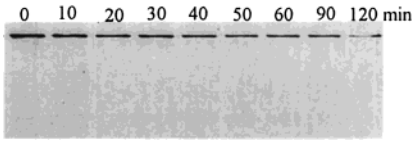


Figure 1. Results of SDS−PAGE gel electrophoresis performed on albumin ($S_o = 1.50 \times 10^{-6}$ M) incubated with $[Im^C]_{22}$-PCD^{MeO} ($C_o = 0.115$ M) at pH 6.00 and 25 °C.

measured by elemental analysis for $[Im^C(MOM)]_{22}$-PCD^{TEA}, the content of TEA moiety was calculated as 57 residue mol %.

To introduce a smaller amount of imidazole to PCD via the C-2 atom, the amount of MOM-imidazole carbanion used in the preparative step was reduced to 0.25 equiv of the chloromethylstyryl moiety of PCD. The content of imidazole in the resulting resin ($[Im^C(MOM)]_5$-PCD) was estimated as 5 residue mol % on the basis of elemental analysis.

Based on the results of elemental analysis, the content of imidazole in $[Im^C(MOM)]_{22}PID$ (Scheme 2) was estimated as 22 residue mol %. The contents of taurine and TEA in $[Im^C(MOM)]_{22}PID^{Tau}$ and $[Im^C(MOM)]_{22}PID^{TEA}$ were estimated as 22 and 70 residue mol %, respectively.

In $[Im^N]_{16}PCD$ (Scheme 3), the content of imidazole was estimated as 16 residue mol % on the basis of elemental analysis although 0.25 residue mol equiv of imidazole had been reacted with PCD. When 10 residue mol equiv of imidazole were used, the content of Im was raised to 63 residue mol %, leading to $[Im^N]_{63}PCD$.

For the resins treated with methoxide ion, elemental analysis does not estimate the content of methoxy group correctly, as the C/N ratio is not affected sensitively by the amount of methoxy group. Instead, the amount of Cl or I atoms remaining on the resin surface was examined by EPMA. EPMA performed on the derivatives of $[Im^C]$-PCD or $[Im^C]PID$ revealed that considerable amounts of Cl or I were left on the resin surface after treatment with methoxide, taurine, or TEA. When EPMA was performed with the resins incubated with bovine serum albumin in the pH 7 buffer for 2−3 h, however, the results showed that the amounts of Cl or I atoms on the resin surface were unaffected by incubation of the resins under the typical conditions of kinetic measurements.

Activity of the imidazole-containing resins was tested in the hydrolysis of bovine serum albumin (MW 66000). The PCD derivatives were swollen in the buffer solution at 25 °C for 10 h prior to kinetic measurement. When the buffer solution containing albumin was stirred with the resin, disappearance of albumin was observed by SDS−PAGE gel electrophoresis.[26] A typical result of electrophoresis performed on albumin cleaved by the resin is illustrated in Figure 1. In this study, the initially added concentration (C_o) of the catalyst is calculated as the concentration of imidazole moiety attainable when the resin is dissolved in the reaction mixture.

That the disappearance of the electrophoretic band of albumin is not due to the adsorption onto the resin was confirmed by measuring the total amino acid content of the product solution separated from the resin by filtration. Peptides present in the product solution were hydrolyzed with 13.5 M NaOH, and the amino acids thus generated were quantified with ninhydrin to estimate the total amino acid content.[27] The amino acid content of the product solution was then compared with that obtained with album untreated with the resin. For the kinetic run

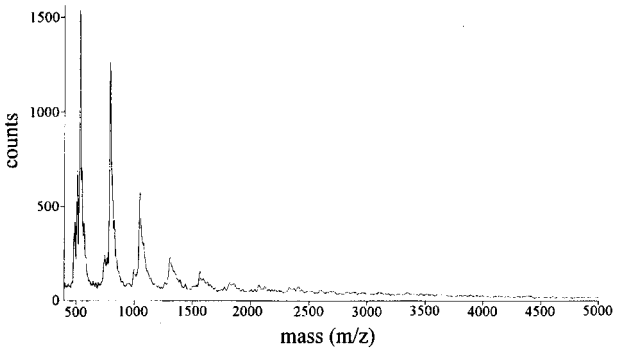


Figure 2. MALDI-TOF MS spectrum of product solution obtained after incubation of albumin ($S_o = 1.50 \times 10^{-6}$ M) with $[Im^C]_{22}PCD^{MeO}$ ($C_o = 0.115$ M) at pH 7.00 and 25 °C.

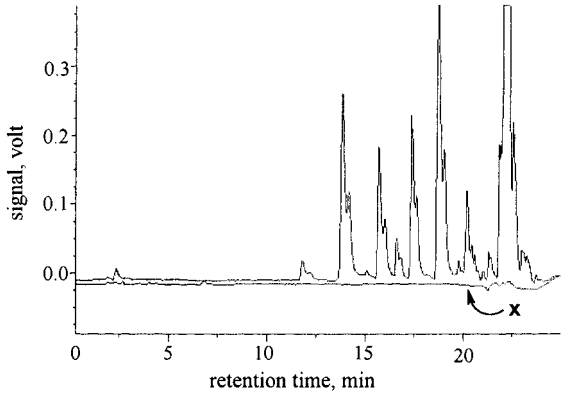


Figure 3. HPLC spectra of product solution of Figure 2 after treatment with phenyl isothiocyanate. Line **x** represents the signal observed without treatment with phenyl isothiocyanate. Typical conditions employed by the Waters PicoTag System were used for the elution.

indicated in Figure 1, about 70% of the amino acids were recovered in the filtrate after complete disappearance of albumin was confirmed by electrophoresis.

The electrophoresis indicated that no intermediate proteins accumulated in amounts detectable by the electrophoresis during the cleavage of albumin by the resin. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometric (MALDI-TOF MS) spectrum of a product solution obtained by cleavage of albumin by $[Im^C]_{22}PCD^{MeO}$ is illustrated in Figure 2, which reveals that albumin is cleaved into fragments smaller than 2 kDa. HPLC analysis of a product solution with and without treatment with phenyl isothiocyanate (Figure 3) shows that many fragments are obtained as the product and that the fragments are modified by phenyl isothiocyanate. Phenyl isothiocyanate is the reagent used in Edman degradation to convert the primary amino groups of peptides to phenylthiocarbamoyl derivatives. Modification of the fragments with phenyl isothiocyanate demonstrates that each fragment contains amino group, in agreement with the hydrolytic nature of the protein cleavage by the resin.

By measuring the density of the electrophoretic bands obtained at various time intervals during the hydrolysis of albumin by the resin, pseudo-first-order rate constants (k_o) were calculated as exemplified by Figure 4. Linear

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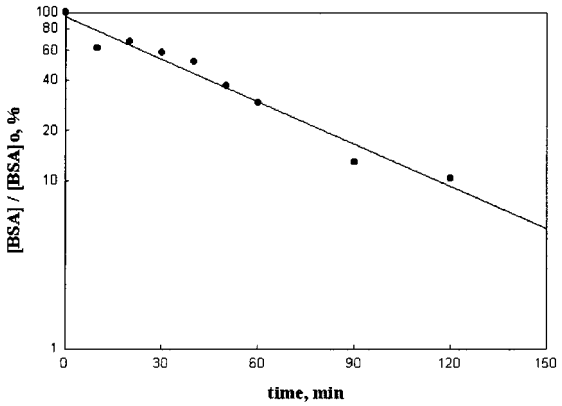


Figure 4. Logarithmic plot for the intensities of electrophoretic bands shown in Figure 1.

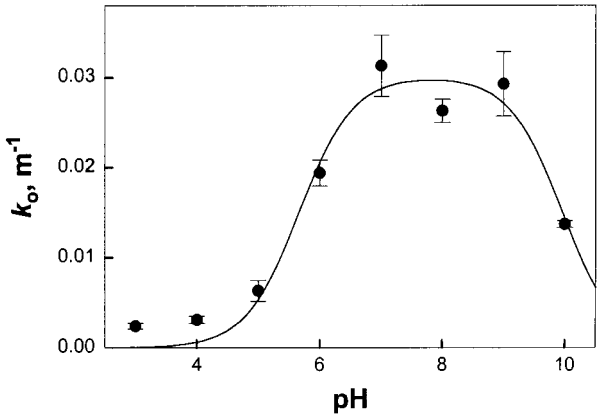


Figure 5. The pH profile of k_o for the hydrolysis of albumin ($S_o = 1.50 \times 10^{-6}$ M) by $[Im^C]_{22}PCD^{MeO}$ ($C_o = 0.115$ M) at 25 °C. The curve is obtained by analysis with Scheme 6.

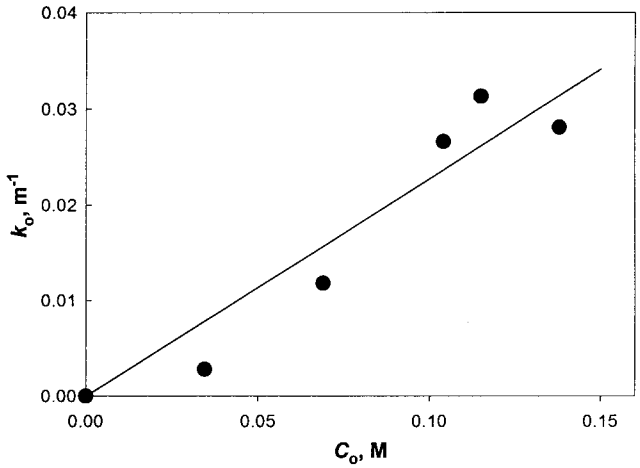


Figure 6. The dependence of k_o on C_o for the hydrolysis of albumin ($S_o = 1.50 \times 10^{-6}$ M) by $[Im^C]_{22}PCD^{MeO}$ at pH 7.00 and 25 °C.

relationship is observed for the data of Figure 4 up to 90% of the reaction, indicating the absence of appreciable inhibition by the product formed as many small fragments.

The pH profile of k_o for the hydrolysis of albumin by $[Im^C]_{22}PCD^{MeO}$ is illustrated in Figure 5. The dependence of k_o on C_o for the hydrolysis of albumin by $[Im^C]_{22}PCD^{MeO}$ at pH 7.00 is illustrated in Figure 6. For $[Im^C]_{22}PCD^{Tau}$, $[Im^C]_{22}PCD^{TEA}$, $[Im^C]_{22}PID^{MeO}$, $[Im^C]_{22}PID^{Tau}$, and $[Im^C]_{22}$-PID^{TEA}, pH 7 was the optimum pH in the hydrolysis of albumin. The values of k_o for the hydrolysis of albumin

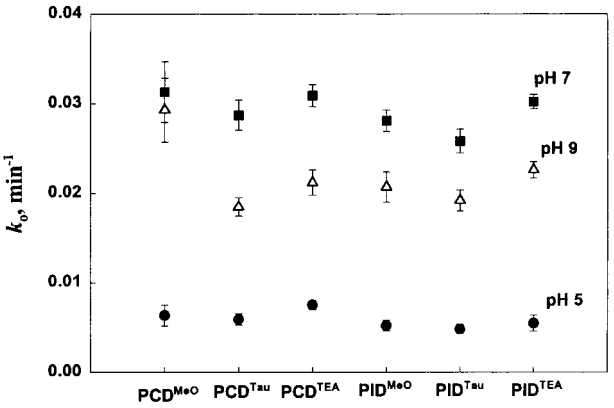


Figure 7. Effect of methoxide, taurine, and TEA attached to the surface of $[Im^C]_{22}PCD$ or $[Im^C]_{22}PID$ on k_o for the hydrolysis of albumin ($S_o = 1.50 \times 10^{-6}$ M, $C_o = 0.115$ M) at 25 °C.

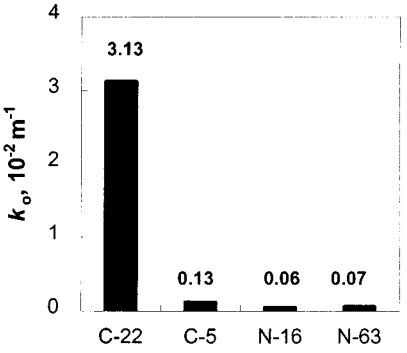


Figure 8. Comparison of k_o for the hydrolysis of albumin ($S_o = 1.50 \times 10^{-6}$ M) by $[Im^C]_{22}PCD^{MeO}$ (C-22), $[Im^C]_5PCD^{MeO}$ (C-5), $[Im^N]_{16}PCD^{MeO}$ (N-16), and $[Im^N]_{63}PCD^{MeO}$ (N-63) ($C_o = 0.115$ M) at pH 7.00 and 25 °C.

catalyzed by $[Im^C]_{22}PCD^{MeO}$, $[Im^C]_{22}PCD^{Tau}$, $[Im^C]_{22}$-PCD^{TEA}, $[Im^C]_{22}PID^{MeO}$, $[Im^C]_{22}PID^{Tau}$, and $[Im^C]_{22}PID^{TEA}$ at 25 °C were measured using the same C_o concentration (0.115 M) and are compared in Figure 7. The catalytic activities of $[Im^C]_5PCD^{MeO}$, $[Im^C]_{22}PCD^{MeO}$, $[Im^N]_{16}PCD^{MeO}$, and $[Im^N]_{63}PCD^{MeO}$ are compared in Figure 8 to examine the effects of imidazole content and the difference in activity between the *C*-alkylated and the *N*-alkylated imidazoles.

Discussion

Imidazole was attached to the cross-linked polystyrene resin via either the C-2 or the N atom by using either MOM-imidazole carbanion or imidazole, respectively. The chemical yield for attachment of MOM-imidazole carbanion to PCD was low: 22 residue mol % attached when 2.2 residue mol equiv of MOM-imidazole carbanion were used. To enhance the reactivity of the resin toward MOM-imidazole carbanion, PCD was converted to PID. The amount of MOM-imidazole attached to PID was almost the same as that to PCD. The maximum content of MOM-imidazole anion that can be attached to either PCD or PID prepared in the present study by the procedure employed here is, therefore, about 22 residue mol %.

Derivatives of $[Im^C]_{22}PCD$ or $[Im^C]_{22}PID$ are very effective in hydrolyzing albumin. The data points with the highest k_o values in Figures 5 and 6 correspond to the half-life of 20 min at pH 7 and 25 °C. This half-life is about 10^7-times shorter than that (500−1000 yr at pH 7 and 25 °C)[21−23] of spontaneous hydrolysis of amide bonds.

Albumin contains 583 amino acid residues, having higher probability of hydrolytic cleavage than short peptides. On the other hand, albumin is cleaved by the imidazole-containing resins into many small fragments without accumulation of intermediate proteins. The intermediate proteins are, therefore, cleaved much faster than albumin itself. If the hydrolysis of the intermediate proteins is considered together with the large number of cleavage sites, the degree of rate acceleration in peptide hydrolysis achieved by the imidazole-containing resins may be estimated as 10^6-10^7 fold.

For catalytic reactions proceeding through complex formation between the substrate and the artificial active sites built on synthetic polymers, kinetic data are analyzed in terms of the Michaelis−Menten scheme (eq 1).[25,28−30] Under the conditions of $C_o \approx [C] \gg [CS]$, pseudo-first-order kinetic behavior is predicted (eq 2). Since k_o is proportional to C_o for the reactions investigated in the present study (Figure 6), K_m is much greater than C_o. When $K_m \gg C_o$, k_o is proportional to C_o and k_o/C_o corresponds to k_{cat}/K_m. For Michaelis−Menten kinetics, k_{cat} represents the maximal rate constant achievable in the presence of excess catalyst ($C_o \gg K_m$). Thus, k_{cat} for the hydrolysis of albumin catalyzed by $[Im^C]_{22}PCD^{MeO}$ is $\gg 0.03\ m^{-1}$ at pH 7.00 and 25 °C (Figure 6). Kinetic data were not collected under the conditions of $S_o \gg C_o$ due to the slow rates. For immobile artificial enzymes used in practical applications, substrates may be passed through the column packed with the catalyst with the reaction taking place in the presence of excess catalyst.

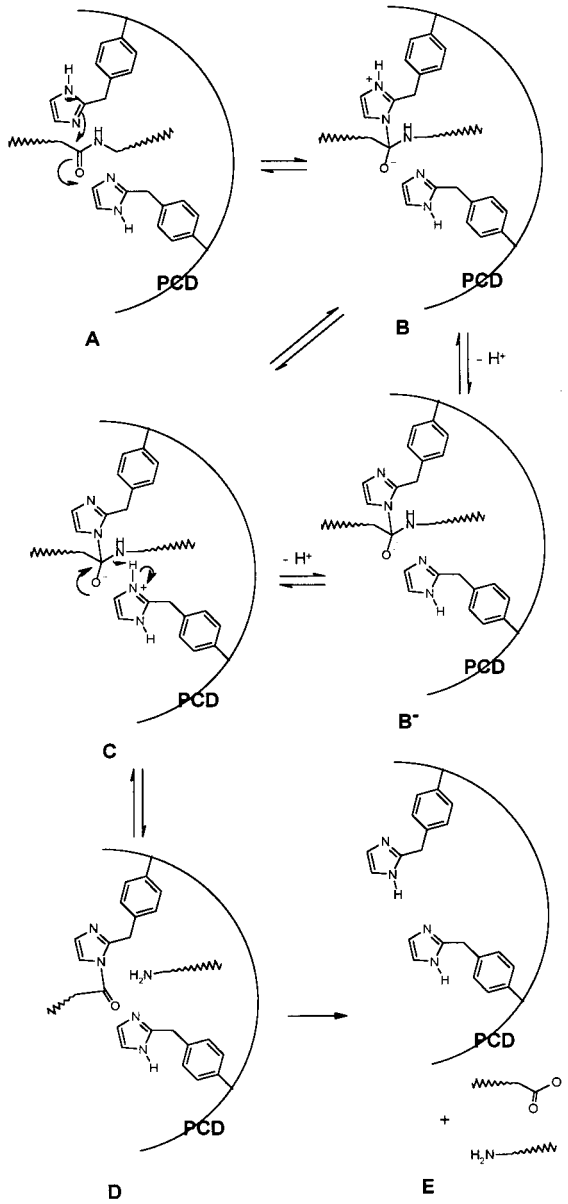
$$C + S \underset{K_m}{\rightleftharpoons} CS \xrightarrow{k_{cat}} C + P_i \qquad (1)$$

$$k_o = k_{cat}C_o/(C_o + K_m) \qquad (2)$$

The proteolytic activity of the imidazole-containing polystyrene per imidazole moiety is reduced markedly when the content of imidazole attached via the C-2 atom is lowered from 22 to 5 residue mol % (Figure 8). This indicates that the active site on $[Im^C]_{22}PCD^{MeO}$ contains two or more imidazole moieties. When the content of imidazole is increased from 5 to 22 residue mol %, the population of sites containing two proximal imidazoles would be increased by considerably more than 4.4 times. For 100 holes arrayed in 10 rows and 10 columns, for example, the probability that two adjacent holes are marked becomes 15-times greater when the number of holes randomly marked is increased from 5 to 22. The proteolytic activity of the imidazole-containing polystyrene is also reduced markedly when imidazole is attached via the N atom (Figure 8). This reveals that the imidazole moieties are much less active when attached to the resin via *N*-alkylation instead of *C*-alkylation.

A mechanism consistent with the catalytic action of two or more imidazoles with unalkylated N atoms is presented in Scheme 4. Here, the first imidazole makes nucleophilic attack at the carbonyl carbon of the scissile amide bond (**A**) to form a zwitterionic tetrahedral intermediate (**B**). Proton transfer within the intermediate generates **C**, which undergoes general acid-assisted expulsion of the amine (**D**). This mechanism presumes

Scheme 4



participation of two proximal imidazoles. Deprotonation of the imidazolium ion of **B** reduces the leaving ability of the imidazole moiety, suppressing the reverse formation of **A** from **B**. If the imidazole is attached to the resin via the N atom, deprotonation of the imidazolium ion of **B** is not possible.

An alternative mechanism is shown in Scheme 5. Here, the first imidazole acts as a general base to assist water attack at the carbonyl group. In this step, however, participation of another water molecule as a general base is presumed in order to rationalize the deactivation of imidazole upon *C*-alkylation. This double general base catalysis is analogous to the charge relay system proposed for chymotrypsin. Possibility of the double general base catalysis is not readily excluded for the present reaction which takes place in the gellike[31] microdomains of polystyrene surface.

The pH profile of Figure 5 represents pH effects on k_{cat}/K_m as k_o corresponds to k_{cat}/K_m when $C_o \ll K_m$. Nonlinear regression of the pH profile in terms of Scheme 6 by analogy with enzyme reactions[32] produced $pK_{a1} = 5.66 \pm 0.16$ and $pK_{a2} = 9.96 \pm 0.16$. The mechanisms of

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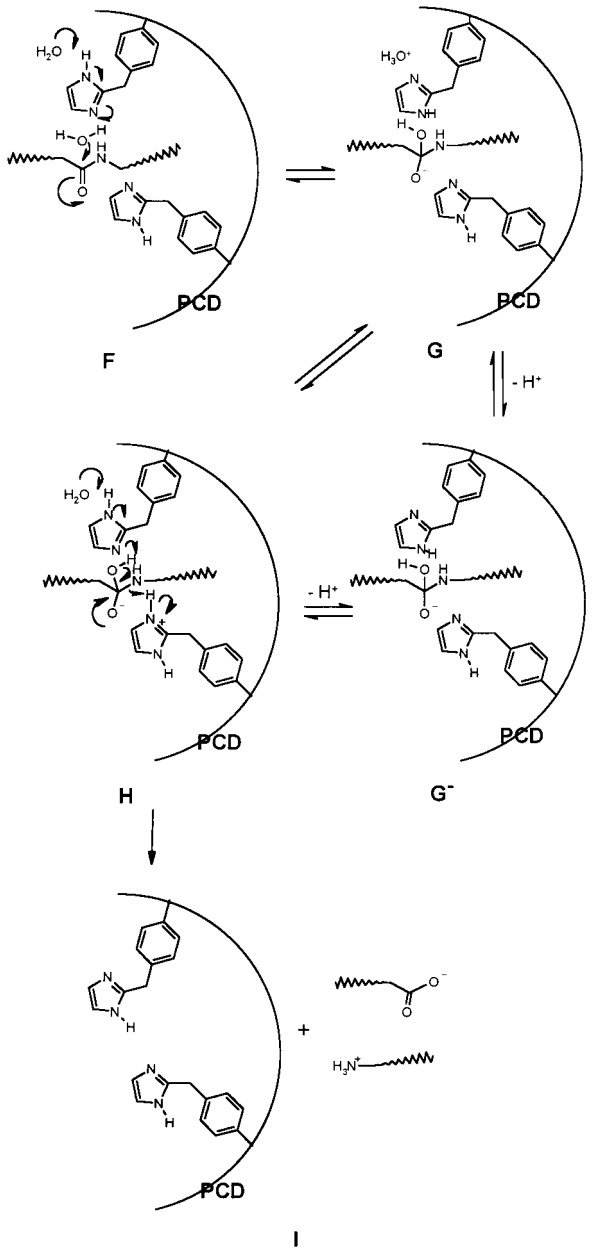
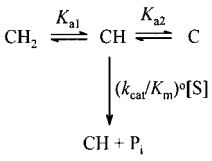
Scheme 5



Scheme 6



Schemes 4 and 5 require that both of the imidazole moieties be in the basic form for the catalytic activity, in agreement with the deactivation below pH 6. The acidic limb of the bell-shaped pH profile is, therefore, attributable to the ionization of the imidazole groups. Loss of catalytic activity in the alkaline region may be ascribed to the formation of unproductive intermediate B^- or G^-. Interpretation of the pH profile is, however, complicated by ionization of functional groups on albumin and the concomitant changes in reactivity.

Efforts to achieve high catalytic activity by cooperation of two proximal imidazoles have been made previously by using synthetic peptide made of 42 amino acid residues.[11,33] The synthetic peptide folded into a helix-loop-helix motif and dimerized to form a four-helix bundle. Two histidine residues attached to the surface of the folded polypeptide were able to catalyze hydrolysis of *p*-nitrophenyl acylates. Cooperation between the two imidazole moieties of histidine residues was quite effective and achieved second-order-rate constant that was more than 1000 times greater than that of 4-methylimidazole-catalyzed reactions. Detailed studies revealed that the two imidazoles acted as a nucleophile and a general acid.[11]

Peptide hydrolysis by the catalytic action of imidazole contained in a synthetic molecule is, however, unprecedented. The remarkable activity of imidazoles attached to the derivatives of $[Im^C]_{22}$PCD or $[Im^C]_{22}$PID should involve cooperation between proximal imidazoles. Some imidazole pairs on the resin surface may take highly productive positions, providing effective active sites. Additional imidazoles may be included in the active site, assisting complexation of the substrate or stabilization of transition states. It is also possible that the intrinsic reactivity of the catalytic groups is enhanced in the gellike microenvironment provided by the resin.

Previously, remarkable activation of the Cu(II) complex of cyclen in proteolytic action has been observed upon attachment of the complex to PCD, which was attributed in part to the medium effects of the microenvironments created on the resin.[25] In this regard, taurine and TEA as well as methoxide were attached to the resin surface for fine-tuning of the microenvironment. The results of Figure 7 reveal that creation of quaternary ammonium sites with TEA or zwitterionic microenvironment with taurine on the resin surface does not affect the catalytic rate significantly. Some of the chloro groups on the surface of PCD remained unsubstituted upon treatment with methoxide, taurine, or TEA during the preparation of the resins. Those chloro groups were, however, unaffected under the conditions of kinetic measurements for the albumin hydrolysis. Due to the lack of appreciable effects on catalytic rates by changes in the functional groups covering the resin surface, it was not attempted to reduce the amount of the unreacted chloro groups further. Lack of effects on catalytic activity by taurine and TEA may be taken to indicate that they do not affect the microenvironment in the immediate vicinity to the active site created by proximal imidazoles.

The $[Im^C]$PCD and the $[Im^C]$PID derivatives prepared in the present study are the first immobile artificial peptidases made from only organic materials. Recently, several types of artificial enzymes with peptidase-like activities have been reported. Small metal complexes such as Pd(II) complexes[34] of analogues of ethylenediamine or the Cu(II) complex[35] of [9]aneN_3 are the first fully synthetic catalysts reported to be capable of amide hydrolysis, although the degree of rate acceleration was moderate. The highest catalytic activity (up to 10^9-fold acceleration) reported so far for amide hydrolysis with artificial peptidases has been achieved with artificial metallopeptidases built with coordinatively polymerized

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synthetic bilayer membranes[36] or Cu(II) complex of cyclen[25] attached to a PCD derivative. These synthetic metallopeptidases hydrolyzed proteins with half-lives of 1−10 min at 4 °C and pH 5−7.

An example of purely organic artificial proteinase is the catalytic antibody elicited by a joint hybridoma and combinatorial antibody library approach.[37] This is the catalytic antibody with the highest peptidase activity reported to date, with k_{cat} of 0.0030 m^{-1} at pH 9, the optimum pH, and 25 °C. The only precedent of organic artificial proteinase prepared by totally synthetic method is the soluble catalyst obtained by building an active site comprising three proximal salicylate residues on the backbone of poly(ethylenimine).[28] The salicylate derivative achieved a half-life of 1 h at 50 °C and pH 7 in the hydrolysis of γ-globulin. The imidazole-containing polystyrenes of the present study are much more reactive than the salicylate-based catalyst. As shown by the k_{cat} value ($\gg$0.03 m^{-1} at pH 7 and 25 °C) for the [Im^C]PCD or [Im^C]PID derivatives and that (0.003 m^{-1} at pH 9 and 25 °C) for the aforementioned antibody, the imidazole-based polystyrene is the best organic artificial proteinase ever reported at least in terms of k_{cat}, being better than the best catalytic antibody.

High proteolytic activity is achieved in the present study by using imidazole as the sole catalytic group. Apparently, active sites comprising proximal imidazoles were prepared by random attachment of the MOM-imidazole carbanion. Introduction of additional catalytic groups near the active site comprising two or more imidazole moieties may improve the catalytic power further. Future studies in the design of imidazole-based artificial enzymes would involve development of methodologies other than random modification for construction of the active site comprising not only two or more imidazoles but also other catalytic groups on the backbone of the highly branched polymers.

Experimental Section

Preparation of PCD Derivatives. PCD. This polymer was obtained by suspension copolymerization of chloromethylstyrene and divinylbenzene as described previously.[25,38] The content of divinylbenzene was 2 mol % relative to chloromethylstyrene. Physical data of PCD such as specific area and mechanical strength were reported previously.[38]

[Im^C(MOM)]$_\alpha$PCD. Attachment of MOM-imidazole[39] to PCD to obtain [Im^C(MOM)]$_\alpha$PCD (α = 5 or 22) was carried out under nitrogen atmosphere with solvents degassed prior to use in synthesis. A solution of 1.6 M *n*-butyllithium in hexane (2.2 mL (3.5 mmol) when α = 5 and 8.8 mL (14 mmol) when α = 22) was added to a tetrahydrofuran (THF) solution (20 mL) of MOM-imidazole (0.39 g (3.5 mmol) when α = 5 and 1.6 g (14 mmol) when α = 22) at −60 °C, the mixture was stirred for 45 min, and then PCD (1.0 g; 6.4 residue mmol) was added to the mixture. The resulting mixture was stirred for 2 h at room temperature. When MOM-imidazole was attached to PCD, the resin became yellow. The yellow resin of [Im^C(MOM)]$_\alpha$PCD was collected by filtration and washed with 50 mL of THF, 50 mL of water, and 50 mL of acetone.

[Im^N]$_\alpha$PCD. PCD (1.0 g; 6.4 residue mmol) was added to a chloroform solution (20 mL) of imidazole (0.11 g (1.6 mmol) when α = 16 and 4.4 g (65 mmol) when α = 63), and the mixture was shaken for 48 h at 50 °C. The speed of shaking employed in the synthesis of various PCD derivatives in the present study was 60 rpm. The product resin was collected by filtration and washed with 50 mL of chloroform and 50 mL of acetone.

PID. A mixture of PCD (5.0 g; 32 residue mmol) and NaI (39 g; 260 mmol) in 100 mL acetone was refluxed for 24 h, and PID was collected by filtration and washed with 300 mL of acetone.

[Im^C(MOM)]$_{22}$PID. This resin was prepared from PID according to the procedure used for the preparation of [Im^C(MOM)]$_{22}$PID.

[Im^C(MOM)]$_\alpha$$PCD^{MeO}$, [$Im^N$]$_\alpha$$PCD^{MeO}$, and [$Im^C$(MOM)]$_{22}$-$PID^{MeO}$. The chloro groups of [$Im^C$(MOM)]$_\alpha$PCD, [$Im^N$]$_\alpha$PCD, or [$Im^C$(MOM)]$_{22}$PID were substituted with methoxide ion by shaking the resin (prepared from 1 g PCD) with sodium methoxide (0.86 g; 16 mmol) dissolved in 100 mL 2:1 (v/v) *N*,*N*-dimethylformamide (DMF)−methanol at 50 °C for 72 h. The product resin was collected by filtration and washed with 30 mL of DMF and 30 mL of methanol.

[Im^C(MOM)]$_{22}$$PCD^{Tau}$ and [Im^C(MOM)]$_{22}$$PID^{Tau}$. The chloro groups of [$Im^C$(MOM)]$_{22}$PCD or [$Im^C$(MOM)]$_{22}$PID were substituted with taurine by shaking the resin (prepared from 0.5 g PCD) with taurine (0.80 g; 6.4 mmol) and *N*,*N*-diisopropylethylamine (2.1 mL; 12 mmol) dissolved in 10 mL 1:1 (v/v) of DMF−acetonitrile at 50 °C for 48 h. The product resin was collected by filtration and washed with 50 mL of DMF, 50 mL of water, and 50 mL of acetone.

[Im^C(MOM)]$_{22}$$PCD^{TEA}$ and [Im^C(MOM)]$_{22}$$PID^{TEA}$. The chloro groups of [$Im^C$(MOM)]$_{22}$PCD or [$Im^C$(MOM)]$_{22}$PID were substituted with TEA by shaking the resin (prepared from 0.5 g of PCD) with TEA (4.2 g; 30 mmol) dissolved in 10 mL of dimethyl sulfoxide at 50 °C for 48 h. The product resin was collected by filtration and washed with 50 mL of DMF, 50 mL of water, and 50 mL of acetone.

[Im^C]$_5$$PCD^{MeO}$, [$Im^C$]$_{22}$$PCD^{MeO}$, [$Im^C$]$_{22}$$PCD^{Tau}$, [$Im^C$]$_{22}$-$PCD^{TEA}$, [$Im^C$]$_{22}$$PID^{MeO}$, [$Im^C$]$_{22}$$PID^{Tau}$, and [$Im^C$]$_{22}$$PID^{TEA}$. These resins were prepared from the respective [Im^C(MOM)] derivatives by refluxing the resin (1.0 g) in 50 mL of 2 N HCl for 3 h and washing the resin with 50 mL of water, 50 mL of ethanol, and 50 mL of acetone.

Measurements. Distilled and deionized water was used for preparation of buffer solutions. Buffers (0.05 M) used for the kinetic measurements were phosphoric acid (pH 3), acetic acid (pH 4−5), 4-morpholineethanesulfonic acid (pH 6), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7−8), and boric acid (pH 9−10). pH measurements were carried out with a Dongwoo Medical DP-880 pH/Ion meter. In kinetic measurements, the stirring speed was controlled with a tachometer, and temperature was controlled within ±0.1 °C with a circulator. The value of k_o increased considerably as the stirring speed was raised to 1000 rpm and reached the plateau value at 1000−1200 rpm as checked with the hydrolysis of albumin (S_o = 1.50 × 10^{-6} M) catalyzed by [Im^C]$_{22}$$PCD^{MeO}$ (C_o = 0.115 M) at pH 7.00 and 25 °C. Kinetic data were, therefore, collected at the stirring speed of 1200 rpm. To check the effect of ionic strength on the catalytic activity, kinetic data for the hydrolysis of albumin catalyzed by [Im^C]$_{22}$$PCD^{MeO}$ were collected with 0.05, 0.1, or 0.2 M Hepes. Since k_o value was not affected by the changes in buffer concentration, kinetic data were collected with 0.05 M buffer. HPLC analysis of *N*-labeled peptide mixtures was performed by Korea Basic Science Research Institute with a Waters PicoTag System. MALDI-TOF MS measurements were carried out with a Voyager PerSeptive linear model. EPMA analysis was performed with a CAMECA SX-57 model.

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Supporting Information Available: Experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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