

Artificial Metalloproteases with Broad Substrate Selectivity Constructed on Polystyrene

Eun Hwa Ko and Junghun Suh*

Department of Chemistry, Seoul National University, Seoul 151-747, Korea

Received August 31, 2004

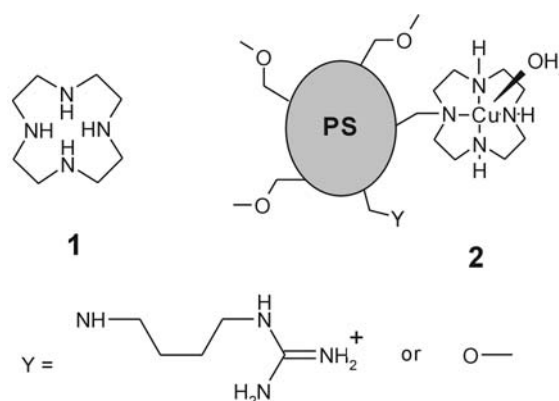
Although the proteolytic activity of the Cu(II) complex of cyclen (Cyc) is greatly enhanced upon attachment to a cross-linked polystyrene (PS), the Cu(II)Cyc-containing PS derivatives reported previously hydrolyzed only a very limited number of proteins. The PS-based artificial metalloproteases can overcome thermal, mechanical, and chemical instabilities of natural proteases, but the narrow substrate selectivity of the artificial metalloproteases limits their industrial application. In the present study, artificial metalloproteases exhibiting broad substrate selectivity were synthesized by attaching Cu(II)Cyc to a PS derivative using linkers with various structures in an attempt to facilitate the interaction of various protein substrates with the PS surface. The new artificial metalloproteases hydrolyzed all of the four protein substrates (albumin, myoglobin, γ globulin, and lysozyme) examined, manifesting k_{cat}/K_m values of 28-1500 $\text{h}^{-1}\text{M}^{-1}$ at 50 °C. The improvement in substrate selectivity is attributed to steric and/or polar interaction between the bound protein and the PS surface as well as the hydrophobicity of the microenvironment of the catalytic centers.

Key Words : Artificial protease, Metalloprotease, Substrate selectivity, Cu(II)-cyclen, Polystyrene

Introduction

Designing artificial proteases¹ is an important subject in the area of bio-related chemistry in view of the importance of proteins in modern biology as well as the high stability^{2,3} of peptide bonds. Hydrolysis of protein mixtures into smaller fragments is carried out in various industries.^{4,5} Proteolysis can be also applied to utilization of agricultural, seafood, and meat byproducts and to improvement of nutritional and functional properties of proteins.^{4,5} Moreover, proteolysis produces peptides with various kinds of bioactivity.⁶⁻¹² Industrial processes for proteolysis have been performed so far with biotic catalysts. Synthetic catalysts hydrolyzing a variety of proteins can lead to alternative processes for proteolysis. To design artificial proteases applicable to protein industries, it is desirable to synthesize immobile catalysts that hydrolyze a variety of proteins at near neutral pH's.

Metal ions can play various catalytic roles in peptide hydrolysis acting as Lewis acids.¹³ When various metal complexes were tethered to peptide substrates, highly effective intramolecular catalysis of peptide hydrolysis was achieved.^{14,15} As intermolecular catalysts for peptide hydrolysis,¹⁶⁻¹⁸ however, metal complexes frequently suffered from low reactivity under physiological conditions. Remarkable improvement of the proteolytic activity of the Cu(II) complex of cyclen (Cyc: **1**) has been achieved when Cu(II)Cyc was attached to a cross-linked polystyrene (PS).¹⁹ Thus, very high proteolytic activity of **2** was observed when bovine serum γ globulin was used as the substrate. Despite the high proteolytic activity toward γ globulin, **2** manifested narrow substrate selectivity, having negligible activity toward bovine serum albumin.



Although the artificial metalloproteases based on PS can overcome limitations of natural proteases such as thermal, mechanical, and chemical instability, the narrow substrate selectivity of the artificial metalloproteases would limit their industrial applications. In order to use in various types of protein industries, the artificial protease should manifest broad substrate selectivity, hydrolyzing almost every protein substrate that the catalyst encounters. In the present study, linkers with various structures were inserted between the PS support and Cu(II)Cyc moiety in an attempt to facilitate the interaction of various protein substrates with the polymer surface.

Experimental Section

Synthesis of the PS-based artificial metalloproteases. Poly[(aminomethyl)_{0.17}styrene-co-divinylbenzene] (PAD), a derivative of PS with 17% of styrene residues aminomethylated (1.5 mmol NH₂ per gram polymer) and with 2% cross-linkage, was purchased from Fluka and employed as

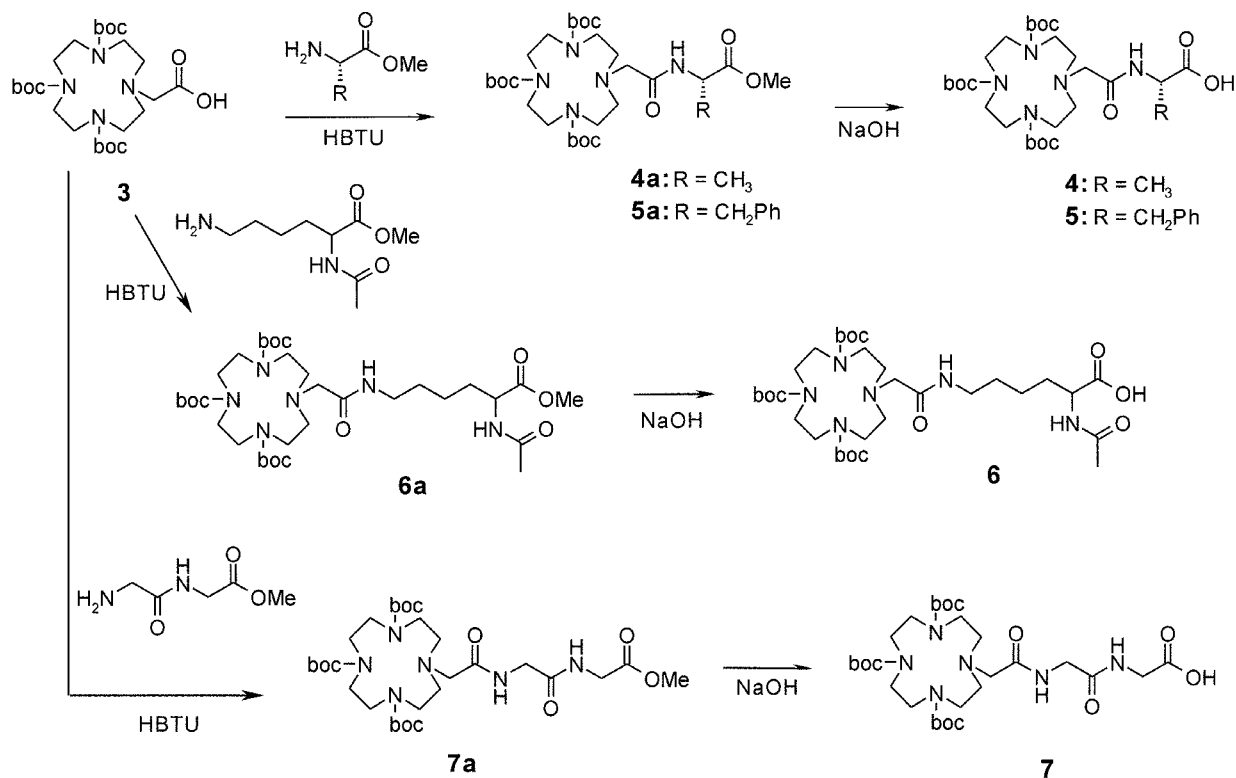
*Corresponding Author. e-mail: jhsuh@snu.ac.kr

the solid support of the artificial metalloproteases.

Catalytic module **3** was prepared as reported in the literature²⁰ and **4-7** were prepared according to the synthetic paths summarized in Scheme 1. For *N*-{[4,7,10-tris(*t*-butoxycarbonyl)-1,4,7,10-tetraazacyclododecan-1-yl]acetyl}-L-alanine (**4**): ¹H NMR (300 MHz, CDCl₃) δ 4.55 (q, 1H), 3.45-3.65 (br, 8H), 3.30-3.45 (m, 4H), 3.20 (s, 2H), 2.60-2.80 (br, 4H), 1.35-1.50 (br, 30H); MS (MALDI-TOF) *m/z* 602.95 (M+H)⁺, calcd for C₂₈H₅₂N₅O₉ 602.75. For *N*-{[4,7,10-tris(*t*-butoxycarbonyl)-1,4,7,10-tetraazacyclododecan-1-yl]acetyl}-L-phenylalanine (**5**): ¹H NMR (300 MHz, CDCl₃): δ 7.20-7.30 (br, 5H), 4.83 (q, 1H), 3.00-3.60 (br m, 16H), 2.97-2.60 (br, 4H) 1.43 (br, 27H); MS (MALDI-TOF) *m/z* 678.99 (M+H)⁺, calcd for C₃₄H₅₆N₅O₉ 678.85. For *N*-α-acetyl-*N*-ε-[4,7,10-tris(*t*-butoxycarbonyl)-1,4,7,10-tetraazacyclododecan-1-yl]acetyl}-L-lysine (**6**): ¹H NMR (300 MHz, CDCl₃) δ 4.52 (s, 1H), 3.10-3.65 (br, 16H), 2.40-2.55 (br, 4H), 1.91 (s, 3H), 1.65-1.80 (br s, 4H), 1.44 (m, 27H), 1.20-1.35 (br s, 2H); MS (MALDI-TOF) *m/z* 701.03 (M+H)⁺, calcd for C₃₃H₆₀N₆O₁₀ 701.88. For *N*-{[4,7,10-tris(*t*-butoxycarbonyl)-1,4,7,10-tetraazacyclododecan-1-yl]acetyl}-glycylglycine (**7**): ¹H NMR (300 MHz, CDCl₃): δ 4.02 (d, 2H), 3.90 (d, 2H), 3.55 (br, 8H), 3.40 (br, 4H), 3.23 (s, 2H), 2.81 (s, 4H), 1.43 (m, 27H); MS (MALDI-TOF) *m/z* 645.99 (M+H)⁺, calcd for C₂₉H₅₂N₆O₁₀ 645.77.

PAD was converted to **A** by the synthetic route summarized in Scheme 2. The solution (5 mL) of **3** (350 mg) mixed with *O*-benzotriazol-1-yl-*N,N,N',N'*-tetramethyluraniu-hexafluoro phosphate (HBTU) (250 mg), diisopropylethylamine (DIEA) (0.3 mL), *N*-hydroxybenzotriazole (HOBT)

(90 mg) in *N,N*-dimethylformamide (DMF) was added to the suspension of PAD (3 g) in 30 mL of DMF. The suspension was degassed for 30 minutes and shaken at 45 rpm and room temperature for 1 day. The product polymer (**Ai**) was collected by filtration, washed with DMF (30 mL × 5), dichloromethane (DCM) (30 mL × 5), MeOH (30 mL × 5), and dried *in vacuo*. The amino groups of **Ai** were acetylated by shaking the polymer (3 g) with acetic anhydride (Ac₂O) (0.5 mL) and DIEA (1 mL) dissolved in 30 mL DMF at room temperature for 1 day. The product polymer (**Aii**) was collected by filtration, washed with DMF (30 mL × 5), MeOH (30 mL × 5), and dried *in vacuo*. Kaiser test²¹ indicated that the yield of the acetylation step was greater than 99%. **Aii** (3 g) was shaken in the mixture of 6 mL trifluoroacetic acid (TFA) and 24 mL DCM at 45 rpm and room temperature for 1 h. The product polymer (**Aiii**) was collected by filtration, washed with DCM (30 mL × 5), MeOH (30 mL × 5), and dried *in vacuo*. **Aiii** (2.5 g) was suspended in 0.23 M CuCl₂·2H₂O solution in DMF (25 mL), and the resulting mixture was shaken at 45 rpm and room temperature for 1 day. The product polymer (**A**) was collected by filtration, washed with DMF (30 mL × 5), water (30 mL × 5), and MeOH (30 mL × 5). Then **A** was suspended again in a pH 5 buffer and the mixture was shaken at 45 rpm and room temperature for 1 day. **A** was collected by filtration, washed with water (30 mL × 5) and MeOH (30 mL × 5) and dried *in vacuo*. PS-based artificial metalloproteases **B-E** were synthesized by the same procedure by using **4-7**



Scheme 1. Synthetic routes to various carboxylic acid derivatives containing Cyc moieties.

instead of **3**. Contents of the Cu(II)Cyc moieties of A-E were determined by measuring the amount of Cu(II) ion bound to the PS. The catalyst (20 mg) was suspended in 1 N HNO₃ solution (10 mL), shaken at the speed of 100 rpm for a day at room temperature, separated by filtration, and washed with 1 N HNO₃ (5 mL × 2). This process was repeated twice. The concentrations of Cu(II) ion in the filtrates were determined by Inductively Coupled Plasma-Atomic Emission Spectroscopy (ICP-AES). The contents of the Cu(II)Cyc moieties of the artificial metalloproteases thus determined are 1.4% (relative to the content of styrene moieties) for A, 1.4% for B, 1.5% for C, 1.2% for D, and 1.4% for E.

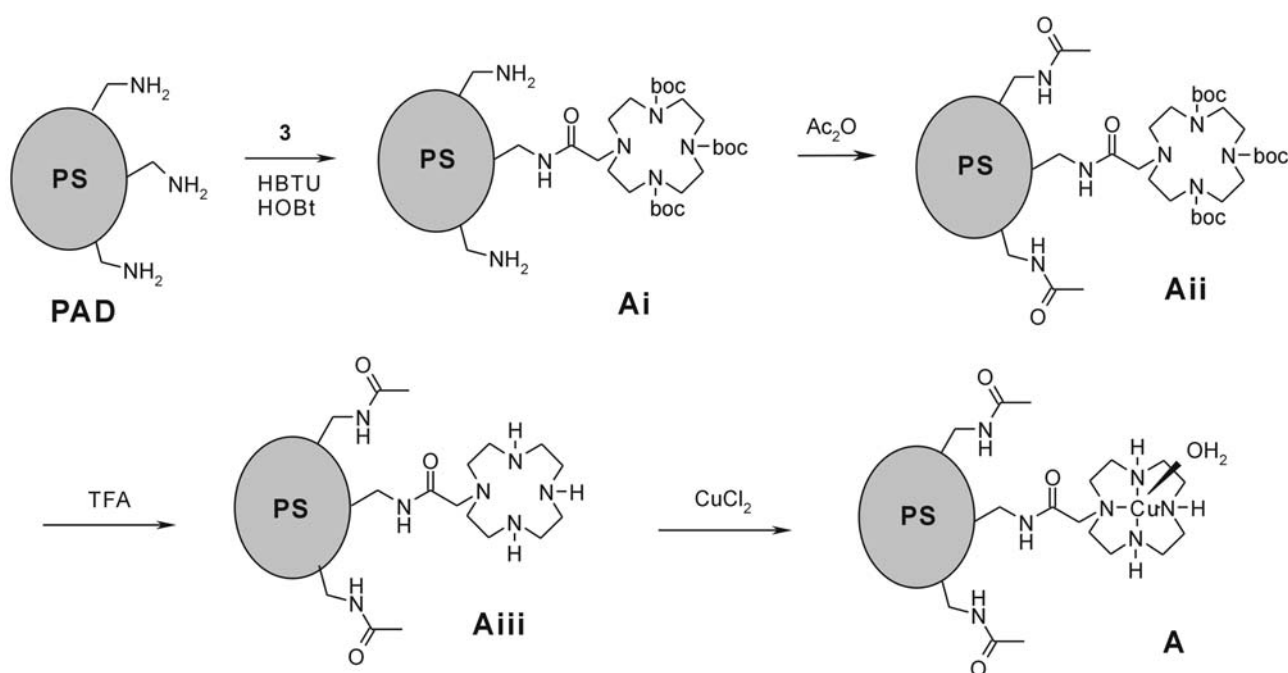
Measurements. In kinetic measurements, the shaking speed and temperature were controlled with a VORTEMP manufactured by Labnet. pH measurements were carried out with a Dongwoo Medical DP-880 pH/Ion meter. The degree of cleavage of proteins was measured by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)²² with a Mighty Small II SE 250 model. Densities of the electrophoretic bands were analyzed with a AlphaImagerTM 2200 model and a AlphaEaseTM model. Distilled and deionized water was used for preparation of buffer solutions. Buffers (0.05 M) used for the kinetic measurements were sodium acetate (pH 5.0), 4-morpholineethanesulfonic acid (pH 6.0, 6.5), *N*-2-hydroxyethylpiperazine-*N'*-ethansulfonate (pH 7.0, 7.5, 8.0), *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonate (pH 8.5), boric acid (pH 9.0, 9.5, 10.0) and 3-(cyclohexylamino)-1-propanolsulfonate (pH 10.5). All of the buffer solutions were filtered with a 0.45 μm Millipore microfilter and autoclaved before use in the kinetic measurements. Protein substrates (bovine serum albumin, horse skeletal muscle myoglobin, chicken egg white lysozyme, and bovine serum γ-globulin) were purchased

from Sigma and used without further purification. The PS-based catalyst (20 mg) was suspended in the buffer solution (0.90 mL) and swollen for 1 h prior to kinetic measurement. A portion (0.10 mL) of the substrate stock solution was added to the heterogeneous mixture, and the resulting mixture was shaken at the speed of 1000 rpm and at 50 °C. The mixture was centrifuged at various intervals and the upper layers (20 μL) of the mixture were collected and subjected to analysis by SDS-PAGE.

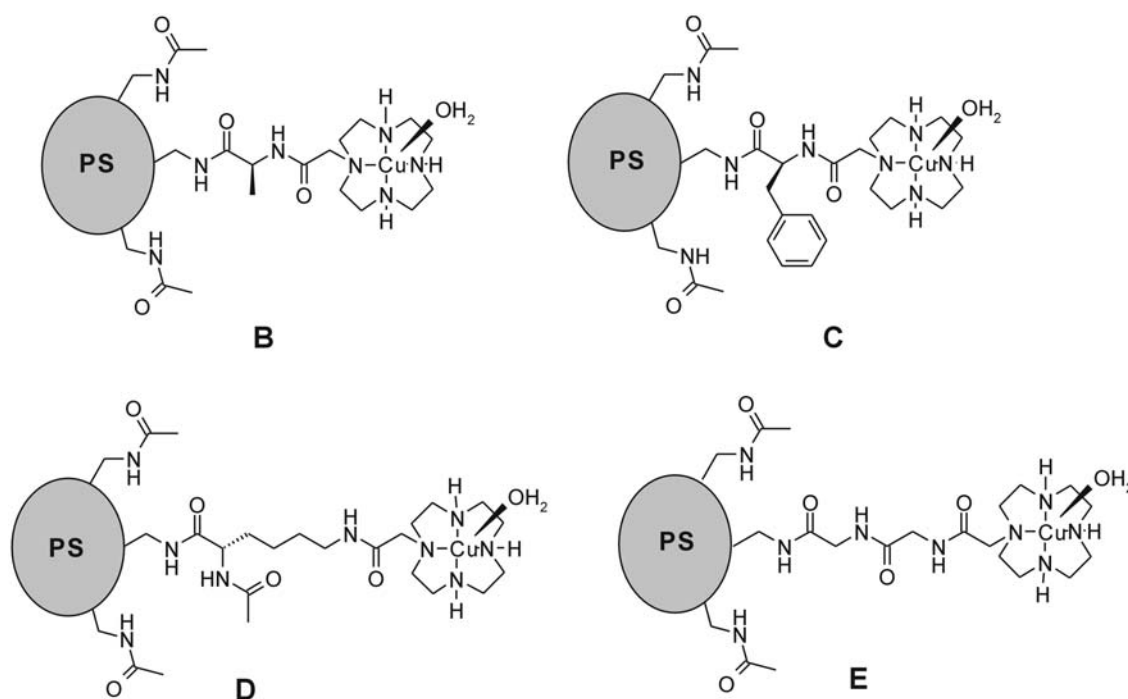
Results

Various linkers were inserted between the phenyl group of the styrene residue of PS and Cu(II)Cyc to alter the microenvironments of the catalytic Cu(II)Cyc moieties. For this purpose, several carboxylic acids containing a protected form of Cyc were synthesized according to the synthetic routes summarized in Scheme 1. PAD, a PS derivative with 17% of styrene residues aminomethylated and with 2% cross-linkage, was employed as the solid support of the artificial proteases. The catalyst was synthesized by coupling of a Cyc-containing carboxylic acid (**3-7**) with PAD, acetylation of the excess amino groups of PAD, deprotection of the Cyc moiety, and insertion of Cu(II) ion as summarized in Scheme 2. The structures of the PS-based artificial metalloproteases prepared in this study are indicated by A-E (Schemes 2 and 3). Contents of Cu(II)Cyc moieties in A-E were 1.2-1.5% (relative to the content of styrene moieties).

Proteolytic activity of A-E was tested by using albumin (M.W. 66 kDa), myoglobin (M.W. 17 kDa), γ-globulin (M.W. 150 kDa), and lysozyme (M.W. 14 kDa) as the substrates. While the buffer solution containing each substrate



Scheme 2. Synthetic route to catalyst A.



Scheme 3. Structures of catalysts B-E synthesized by the route summarized in Scheme 2.

was shaken in the presence of the PS derivative, rates for cleavage of the protein were measured by following disappearance of the parent band in the gel electrophoresis (SDS-PAGE). Typical results of electrophoresis are illustrated in Figure 1. That the disappearance of the electrophoretic band of each protein was not due to the adsorption onto the insoluble support was confirmed by measuring the total amino acid content (70-100% yield) of the product solution separated from the insoluble catalyst by filtration.²³ The rate

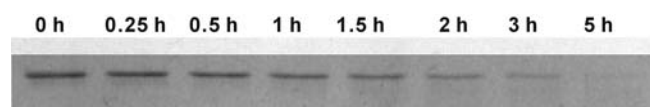


Figure 1. Results of SDS-PAGE performed on albumin ($S_0 = 3.01 \times 10^{-5}$ M) incubated with B ($C_0 = 1.27$ mM) at pH 9.0 and 50 °C.

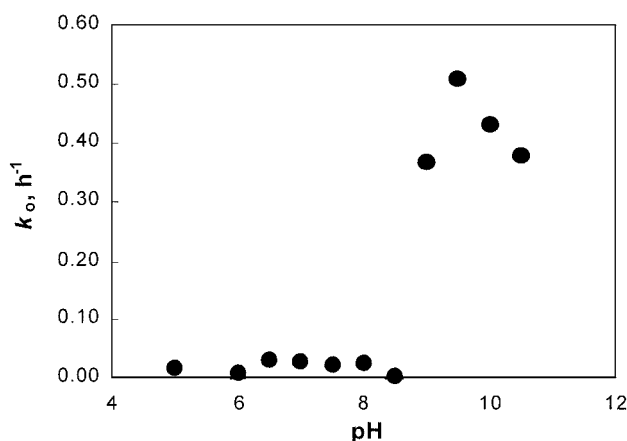


Figure 2. Values of k_0 measured for cleavage of albumin by B at 50 °C and various pH's ($S_0 = 3.01 \times 10^{-5}$ M, $C_0 = 1.27$ mM).

of protein cleavage was measured by monitoring the decrease in the intensity of the electrophoretic bands corresponding to the protein substrates.^{19,20} The values of pseudo-first-order kinetic constant (k_0) were measured at the shaking speed of 1000 rpm since k_0 reached the plateau value at the shaking speed exceeding 800 rpm. For γ globulin, which contains two types of chains, the kinetic data were collected for the heavy chain although the light chain was also cleaved.

The k_0 values for degradation of each protein substrate by A-E were measured at several pH's (pH 5.0-10.5) and 50 °C by fixing S_0 (the initially added concentration of the substrate) and C_0 (the initially added concentration of the catalyst: calculated by assuming that the catalyst is dissolved in the buffer solution) for each catalyst, as exemplified by

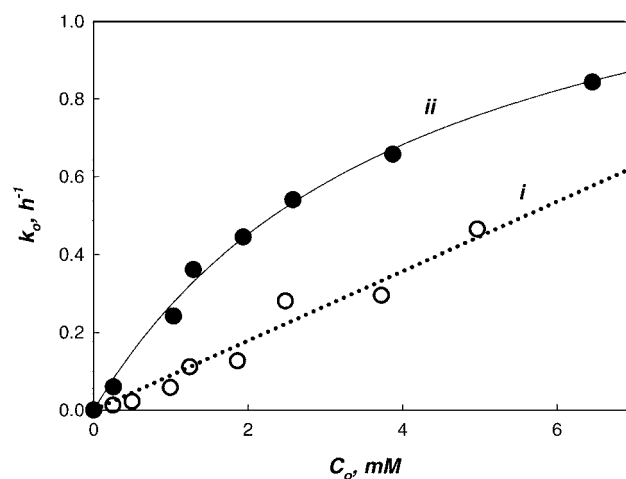


Figure 3. Dependence of k_0 on C_0 for cleavage of lysozyme by A (○ : i) at pH 9.5 and by E (● : ii) at pH 10.0.

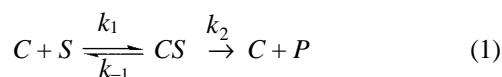
Table 1. Values of kinetic parameters for cleavage of protein substrates by catalysts A-E^{a,b}

		albumin	myoglobin	γ -globulin	lysozyme
A	k_{cat}	—	—	1.1	—
	K_{m}	—	—	4.9	—
	$k_{\text{cat}}/K_{\text{m}}$	28 (9.5)	50 (10.0)	220 (10.0)	89 (9.5)
B	k_{cat}	1.2	4.9	—	1.6
	K_{m}	2.6	3.3	—	3.0
	$k_{\text{cat}}/K_{\text{m}}$	450 (10.0)	1500 (9.0)	570 (9.5)	520 (9.0)
C	k_{cat}	1.0	—	5.4	1.4
	K_{m}	1.3	—	5.6	2.1
	$k_{\text{cat}}/K_{\text{m}}$	770 (10.0)	190 (9.5)	960 (10.0)	630 (9.0)
D	k_{cat}	2.0	—	—	2.2
	K_{m}	5.0	—	—	4.9
	$k_{\text{cat}}/K_{\text{m}}$	410 (10.0)	200 (9.5)	530 (10.0)	440 (9.0)
E	k_{cat}	3.2	1.1	2.7	1.4
	K_{m}	7.5	2.6	3.5	4.2
	$k_{\text{cat}}/K_{\text{m}}$	420 (10.0)	410 (9.5)	770 (9.5)	330 (10.0)

^aMeasured at 50 °C and the optimum pH in the presence of 0.05 M buffer. Units are h⁻¹ for k_{cat} , mM for K_{m} , and h⁻¹M⁻¹ for $k_{\text{cat}}/K_{\text{m}}$.
^bOptimum pH's are indicated in parentheses.

the results illustrated in Figure 2.

At the optimum pH thus selected, the dependence of k_0 on C_0 was measured under the conditions of $C_0 \gg S_0$. Either a saturation curve or a linear line was observed for the plot of k_0 against C_0 as illustrated in Figure 3. The kinetic data were analyzed in terms of Michaelis-Menten scheme (eq. 1).²⁰ Under the conditions of $C_0 \approx [C]$, k_0 is expressed as eq. (2), predicting saturation kinetic behavior for the plot of k_0 against C_0 . When $K_{\text{m}} \gg C_0$, however, k_0 is proportional to C_0 (eq. 3), with the proportionality constant being equal to $k_{\text{cat}}/K_{\text{m}}$. For the reactions manifesting saturation kinetic behavior, the values of k_{cat} , K_{m} , and $k_{\text{cat}}/K_{\text{m}}$ were estimated by analyzing the dependence of k_0 on C_0 with a nonlinear regression program. For the reactions manifesting linear kinetic behavior, the values of $k_{\text{cat}}/K_{\text{m}}$ were taken as the proportionality constant. The values of kinetic parameters thus obtained are summarized in Table 1. For the reactions with no values given for k_{cat} and K_{m} in Table 1, the linear kinetic behavior indicates that K_{m} is greater than 10 mM.

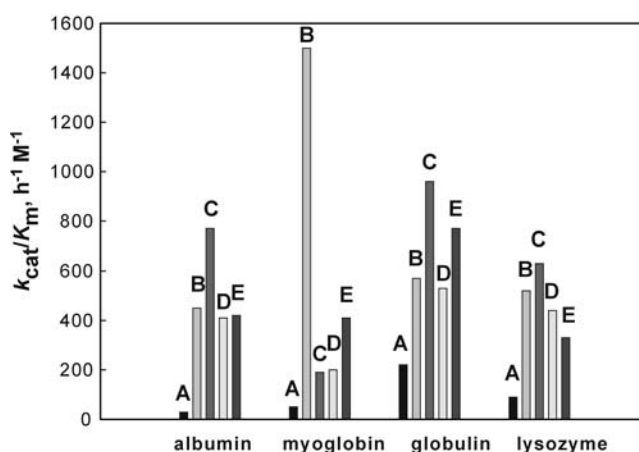


$$k_0 = k_{\text{cat}}C_0/(K_{\text{m}} + C_0)$$

$$\text{where } k_{\text{cat}} = k_2 \text{ and } K_{\text{m}} = (k_{-1} + k_2)/k_1 \quad (2)$$

$$k_0 = k_{\text{cat}}C_0/K_{\text{m}} \text{ when } K_{\text{m}} \gg C_0 \quad (3)$$

The values of $k_{\text{cat}}/K_{\text{m}}$ are estimated for all of the proteolytic reactions examined. Among the kinetic parameters of the Michaelis-Menten scheme, $k_{\text{cat}}/K_{\text{m}}$ stands for the reactivity between the catalyst and the substrate and is regarded as the

**Figure 4.** Values of $k_{\text{cat}}/K_{\text{m}}$ for cleavage of protein substrates by catalysts A-E summarized in Table 1.

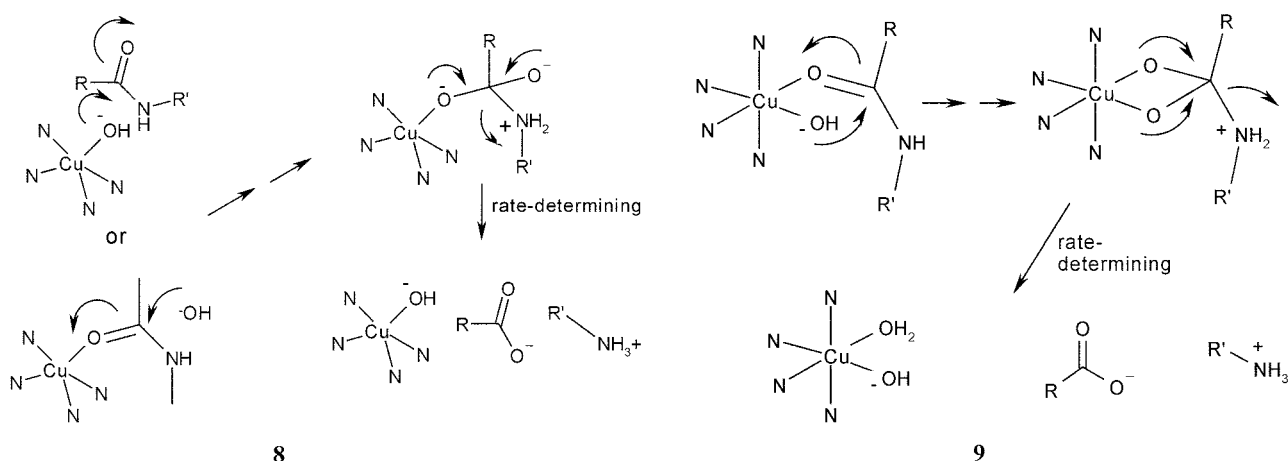
typical parameter to represent enzyme activity. The values of $k_{\text{cat}}/K_{\text{m}}$ measured for the proteolytic action of A-E at the respective optimum pH's are compared in Figure 4. The relative reactivity of artificial metalloproteases represented by $k_{\text{cat}}/K_{\text{m}}$ reflects both the ability of the catalysts to form productive complexes with substrates and the reactivity of the catalyst-substrate complexes.

Discussion

It is well established that the cleavage of proteins by Cu(II)Cyc attached to PS occurs by hydrolysis of the polypeptide backbones.^{19,20} In view of the catalytic roles¹³ of metal ions acting as Lewis acid catalysts in peptide hydrolysis, possible mechanisms for the Cu(II)Cyc-catalyzed proteolysis are indicated as **8** and **9**. Here, the Cu(II) center can activate the carbonyl group of the scissile peptide bond by binding at the carbonyl oxygen. Cu(II)-bound hydroxide ion can act as a potent nucleophile. Those catalytic roles can take place cooperatively as indicated by **9**.

The Cu(II) complex of Cyc itself has negligible proteolytic activity. The proteolytic activity of Cu(II)Cyc is remarkably activated, however, when it is attached to a PS derivative.¹⁹ A possible explanation for the effect of PS is that dimerization of the Cu(II) complex through formation of hydroxo or oxo bridge is prevented when the metal center is fixed on a polymer surface. Upon dimerization, the metal center would not be able to interact with peptide bonds of the substrate proteins. Deactivation of Cu(II)Cyc by dimer formation is not likely, however, since no evidence has been reported for dimerization of related complexes (Zn(II)Cyc and Co(III)Cyc)^{24,25} although their solution behavior has been thoroughly investigated.

Instead, the effects of the PS support can be related to the unique microenvironments created on the surface of the PS which can enhance the intrinsic reactivity of the metal center. Rates of many organic reactions are sensitive to the nature of the reaction media. In both **8** and **9**, the transition state of the rate-determining step has considerably lower



charge density than the corresponding ground state, which can lead to remarkable acceleration²⁶ simply by lowering polarity of the medium. The microenvironment of the Cu(II)Cyc moiety attached to PS would be affected by the styrene phenyl groups of the PS backbone, organic pendants attached to the polymer, and water molecules from the solvent. It has been suggested that the nonpolar microenvironment is important for the catalytic activity of the PS-bound metal complexes of Cyc in the cleavage of not only proteins but also DNAs.¹

In order for the PS-based Cu(II)Cyc to cleave a protein, it is prerequisite that the protein molecule is complexed to the PS surface. Some factors would affect the ability of the protein to bind to the polymer. First, steric strain would be imposed by the PS surface when a peptide bond of the protein interacts with the Cu(II)Cyc center. Second, the structure of the polymer surface would affect the polar and the nonpolar interactions between the protein and the catalyst.

As illustrated in Figure 4, A-E hydrolyze all of the protein substrates examined in the present study, exhibiting wide substrate selectivity. This stands in contrast to **2**, which manifested narrow substrate selectivity as mentioned above. Among A-E, A has the lowest proteolytic activity.

The difference in the reactivity of the catalysts may be related to the different microenvironments of the catalytic center. For **2**, most of the styrene residues of the PS support contained chloromethyl groups which were mostly converted to ether groups. On the other hand, only 17% of the styrene residues of the PS support of A-E contained aminomethyl groups which were mostly converted to acetamide groups. Thus, the microdomains on the PS surface are considerably different for **2** and A-E. The polar or the hydrophobic interaction between the functional groups located on the protein surface and those on the PS surface would play important roles in complexation of the protein substrate to the PS surface.

The Cu(II)Cyc catalytic center of **2** is attached to the styrene residue of PS by using the methylene group as the linker. Thus, A-E have longer linkers than **2**. When the protein substrate interacts with the Cu(II)Cyc center, the

protein can suffer from steric strain with the PS surface when a shorter linker is used. Length of the spacer would affect not only the steric strain but also the interaction between the functional groups located on the protein surface and those on the PS backbone. In addition, the polarity of microenvironment and, consequently, the proteolytic reactivity of the Cu(II)Cyc center would be affected by the length and the structure of the linker. The narrow substrate selectivity of **2** in marked contrast with A-E, therefore, can be related to its short linker.

Rate data for catalytic reactions that involve formation of the catalyst-substrate complexes can be analyzed by the Michaelis-Menten scheme. As demonstrated by many enzymatic reactions, Michaelis-Menten parameters k_{cat} , K_m , and k_{cat}/K_m are composed of several rate constants and mechanistic information derived from those kinetic parameters is generally very limited. Among the parameters, k_{cat}/K_m best stands for the reactivity of the catalyst. The data summarized in Figure 4 indicate that A has the lowest proteolytic activity among A-E for all of the protein substrates examined in the present study. For albumin, γ -globulin, and lysozyme, C manifests the highest proteolytic activity among the PS-based catalysts. For myoglobin, B has uniquely high proteolytic activity. The lowest activity manifested by A may be related to the shortest linker connecting the Cu(II)Cyc moiety to PS. Although the linker is shorter for C than for D or E, better proteolytic activity is observed with C for albumin, γ -globulin, and lysozyme. This implicates that the lengthening the linker beyond an optimum size does not improve the activity. The high catalytic activity of C may be also attributed to the phenyl side chain attached to the linker which may keep the microenvironment more hydrophobic. The much higher activity of B compared with C for the cleavage of myoglobin suggests that the phenyl ring of C may imposed additional steric strain in the complex formed with myoglobin.

Although longer linkers with more complex structures have not been tested, the results of the present study indicate that the substrate selectivity and the proteolytic activity of the artificial metalloproteases based on the Cu(II)Cyc-containing PS can be controlled by variation in the structure

of the linker connecting Cu(II)Cyc to PS and the functional groups covering the PS surface.

Conclusions

In the previous study,¹⁹ the first artificial metalloproteases operating under physiological conditions were synthesized by enhancing the proteolytic activity of Cu(II)Cyc through attachment to PS. Both the PS backbone and the Cu(II)Cyc moiety are responsible for the proteolytic activity of the Cu(II)Cyc-containing PS derivatives. The proteolytic activity is further enhanced by changing the structure of the linkers connecting Cu(II)Cyc and PS in this work. If the artificial metalloproteases are further improved to exhibit amino acid selectivity with regard to cleavage sites, they may be able to replace natural proteases in various types of industrial applications.

Acknowledgments. This work was supported by Korea Research Foundation Grant (KRF 2001-015-DS0029).

References

1. Suh, J. *Acc. Chem. Res.* **2003**, *36*, 562.
2. Radzicka, A.; Wolfenden, R. *J. Am. Chem. Soc.* **1996**, *118*, 6105.
3. Smith, R. S.; Hansen, D. E. *J. Am. Chem. Soc.* **1998**, *120*, 8910.
4. Ward, O. P. In *Comprehensive Biotechnology*; Moo-Young, M., Ed.; Pergamon: Oxford, 1985; Vol. 3, pp 789-818.
5. Tramper, J. In *Applied Biocatalysis*; Cabral, J. M. S., Best, D., Boross, L., Tramper, J., Eds.; Harwood Academic Publishers: Chur, 1994; pp 1-46.
6. Brantl, V.; Pfeiffer, A.; Herz, A.; Henschen, A.; Louttspeich, F. *Peptides* **1982**, *3*, 793.
7. Meisel, H. *FEBS Lett.* **1986**, *196*, 223.
8. Saito, H.; Miyakawa, H.; Tamura, Y.; Shimamura, S.; Tomita, M. *J. Dairy Sci.* **1991**, *74*, 3724.
9. Fiat, A.-M.; Migliore-Samour, D.; Jolles, P.; Drouet, L.; Sollier, C. B. D.; Caen, J. *J. Dairy Sci.* **1993**, *76*, 301.
10. Kayser, H.; Meisel, H. *FEBS Lett.* **1996**, *383*, 18.
11. Matar, C.; Nadathur, S. S.; Bakalinski, A. T.; Goulet, J. *J. Dairy Sci.* **1997**, *80*, 1965.
12. Nakagomi, K.; Yamada, R.; Ebisu, H.; Sadakane, Y.; Akizawa, T.; Tanimura, T. *FEBS Lett.* **2000**, *467*, 235.
13. Suh, J. *Acc. Chem. Res.* **1992**, *25*, 273.
14. Sutton, P. A.; Buckingham, D. A. *Acc. Chem. Res.* **1987**, *20*, 357.
15. Rana, T. M.; Meares, C. F. *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 10578.
16. Chin, J.; Jubian, V.; Mrejen, K. *J. Chem. Soc., Chem. Commun.* **1990**, 1326.
17. Zhu, L.; Qin, L.; Parac, T. N.; Kostic, N. M. *J. Am. Chem. Soc.* **1994**, *116*, 5218.
18. Hegg, E. L.; Burstyn, J. N. *J. Am. Chem. Soc.* **1995**, *117*, 7015.
19. Jang, B.-B.; Lee, K. P.; Min, D. H.; Suh, J. *J. Am. Chem. Soc.* **1998**, *120*, 12008.
20. Yoo, C. E.; Chae, P.; Kim, J. E.; Jeong, E. J.; Suh, J. *J. Am. Chem. Soc.* **2003**, *125*, 14580.
21. Sarin, V. K.; Kent, S. B. H.; Tam, J. P.; Merrifield, R. B. *Anal. Biochem.* **1981**, *117*, 147.
22. Hames, B. D. In *Gel Electrophoresis of Proteins*; Hames, B. D.; Rickwood, D., Eds.; IRL Press: New York, 1990; Chap. 1.
23. Suh, J.; Oh, S. *J. Org. Chem.* **2000**, *65*, 7534.
24. Koike, T.; Takamura, M.; Kimura, E. *J. Am. Chem. Soc.* **1994**, *116*, 8443.
25. Clarkson, A. J.; Buckingham, D. A.; Rogers, A. J.; Blackman, A. G.; Clark, C. R. *Inorg. Chem.* **2000**, *39*, 4769.
26. Carroll, F. A. *Perspectives on Structure and Mechanism in Organic Chemistry*; Brooks/Cole: Pacific Grove, 1998; p 329.