J|A|C|S
ARTICLES
Published on Web 06/14/2005

Artificial Metalloprotease with Active Site Comprising Aldehyde Group and Cu(II)Cyclen Complex

Sang Ho Yoo, Byoung June Lee, Hyunsook Kim, and Junghun Suh*

Contribution from the Department of Chemistry, Seoul National University, Seoul 151-747, Korea

Received April 6, 2005; E-mail: jhsuh@snu.ac.kr

Abstract: To design artificial proteases that cleave peptide backbones of a wide range of proteins at selected sites, artificial active sites comprising the Cu(II) complex of cyclen (Cu(II)Cyc) and aldehyde group were synthesized on a cross-linked polystyrene. The aldehyde group was employed as the binding site in view of its ability of reversible formation of imine bonds with ϵ-amino groups of Lys residues exposed on the surface of proteins and Cu(II)Cyc as the catalytic group for peptide hydrolysis. The two polymeric artificial metalloproteases synthesized in the present study cleaved all of the protein substrates examined (myoglobin, γ-globulin, bovine serum albumin, human serum albumin, lysozyme, and ovalbumin), manifesting saturation kinetic behavior. At 50 °C and pH 9.0 or 9.5, K_m was (1.3−22) $\times$ 10^{-4} M, comparable to those of natural proteases, and k_{cat} was (6.0−25) $\times$ 10^{-4} s^{-1}, corresponding to half-lives of 4.6−19 min. Intermediacy of the imine complexes formed between the aldehyde group of the catalyst and the ϵ-amino groups of Lys residues of the substrates was confirmed by the trapping experiment with $NaB(OAc)_3H$. MALDI-TOF MS of the proteolytic reaction mixtures revealed formation of various cleavage products. Structures of some of the cleavage products were determined by using carboxypeptidase A and trypsin. Among various cleavage sites thus identified, Gln(91)−Ser(92) and Ala(94)−Thr(95) were the major initial cleavage sites in the degradation of myoglobin by the two catalysts. The selective cleavage of Gln(91)−Ser(92) and Ala(94)−Thr(95) was attributed to general acid assistance in peptide cleavage by Tyr(146) located in proximity to the two peptide bonds. Broad substrate selectivity, high cleavage-site selectivity, and high proteolytic rate are achieved, therefore, by positioning the aldehyde group in proximity to Cu(II)Cyc attached to a cross-linked polystyrene.

Introduction

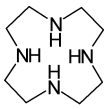
Designing enzyme-like catalysts for hydrolysis of peptide bonds of proteins is challenging in view of the high stability[1,2] of peptide bonds as well as importance of proteins and peptides in modern biology and bio-related chemistry. Intramolecular catalysis of hydrolysis of unactivated peptides by organic functional groups[3] or metal ions[4] as well as by the cooperative action[5] of a metal ion and an organic functional group has been intensively investigated since the 1970s. Intermolecular catalysts with peptidase activity, however, have been synthesized only in recent years. Now, several intermolecular catalysts have been reported for hydrolysis of peptide bonds by using either metal complexes[4−14] or organic functionalities[15−19] as the catalytic groups. When organic functional groups are exploited as the catalytic centers, cooperation of two or more catalytic groups is needed. On the other hand, a single metal complex can act as the catalytic center by playing various catalytic roles.[20]

Metal complexes capable of site-selective cleavage of proteins or peptides have been designed in attempts to obtain novel biochemical reagents or more advanced biomimetic catalysts. Early studies in this area employed metal complexes in combination with oxidoreductive additives to achieve either hydrolytic[21] or oxidative[22−24] cleavage of the target protein. Site-

(1) Radzicka, A.; Wolfenden, R. *J. Am. Chem. Soc.* **1996**, *118*, 6105−6109.
(2) Bryant, R. A. R.; Hansen, D. A. *J. Am. Chem. Soc.* **1998**, *120*, 8910−8913.
(3) Kirby, A. J. *Adv. Phys. Org. Chem.* **1980**, *17*, 183−278.
(4) Sutton, P. A.; Buckingham, D. A. *Acc. Chem. Res.* **1987**, *20*, 357−364.
(5) Suh, J.; Park, T. H.; Hwang, B. K. *J. Am. Chem. Soc.* **1992**, *114*, 5141−5146.
(6) Chin, J. *Acc. Chem. Res.* **1991**, *24*, 145−152.
(7) Suh, J. *Acc. Chem. Res.* **2003**, *36*, 562−570.
(8) Chin, J.; Jubian, V.; Mrejen, K. *J. Chem. Soc., Chem. Commun.* **1990**, 1326−1328.
(9) Zhu, L.; Qin, L.; Parac, T, N.; Kostic, N. M. *J. Am. Chem. Soc.* **1994**, *116*, 5218−5224.
(10) Hegg, E. L.; Burstyn, J. N. *J. Am. Chem. Soc.* **1995**, *117*, 7015−7016.
(11) Jang, B.-B.; Lee, K.-P.; Min, D.-H.; Suh, J. *J. Am. Chem. Soc.* **1998**, *120*, 12008−12016.
(12) Kaminskaia, N. V.; Johnson, T. W.; Kostic, N. M. *J. Am. Chem. Soc.* **1999**, *121*, 8663−8664.
(13) Saha, M. K.; Bernal, I. *J. Chem. Soc., Chem. Commun.* **2003**, 612−613.
(14) Kasai, M.; Ravi, R. G.; Shealy, S. J.; Grant, K. B. *Inorg. Chem.* **2004**, *43*, 6130−6132.
(15) Suh, J.; Hah, S. S. *J. Am. Chem. Soc.* **1998**, *120*, 10088−10093.
(16) Suh, J.; Oh, S. *J. Org. Chem.* **2000**, *65*, 7534−7540.
(17) Oh, S.; Chang, W.; Suh, J. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 1469−1472.
(18) Kim, H.; Paik, H.; Kim, M.-s.; Chung, Y.-S.; Suh, J. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 2557−2560.
(19) Kim, H.; Kim, M.-S.; Paik, H.; Chung, Y.-S.; Hong, I. S.; Suh, J. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 3247−3250.
(20) Suh, J. *Acc. Chem. Res.* **1992**, *25*, 273−279.
(21) Rana, T. M.; Meares, C. F. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 10578−10582
(22) Shepartz, A.; Cuenoud, B. *J. Am. Chem. Soc.* **1990**, *112*, 3247−3249.
(23) Hoyer, D.; Cho, H.; Schultz, P. G. *J. Am. Chem. Soc.* **1990**, *112*, 3249−3250.
(24) Gallagher, J.; Zelenko, O.; Walts, A. D.; Sigman, D. S. *Biochemistry* **1998**, *37*, 2096−2104.

selective hydrolytic cleavage[25−28] of peptides or proteins without the oxidoreductive additives requires highly effective catalytic centers for hydrolysis of peptide bonds. Several kinds of metal complexes have been examined for their ability to hydrolyze peptide bonds,[6−14] but most of them had low catalytic rates unless they were tethered to peptide substrates especially under physiological conditions.

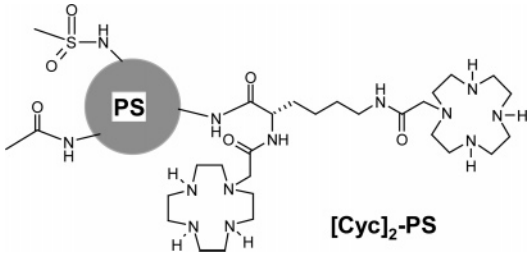
It was observed that the protein-hydrolyzing activity[11] of Cu(II) complex of cyclen (Cu(II)Cyc) and DNA-hydrolyzing activity[29,30] of Co(III) complex of cyclen were enhanced remarkably upon attachment to cross-linked polystyrene. Subsequently, it was attempted to design artificial enzymes for peptide hydrolysis possessing selectivity for cleavage sites by using the Cu(II) complexes of cyclen and related ligands as the catalytic centers and cross-linked polystyrene as the solid support. To introduce a substrate-recognizing site in proximity to the catalytic center, an artificial peptidase was prepared by attaching the guanidinium group close to the Cu(II) complex of a tetraaza ligand attached to polystyrene.[25] In another study, an active site comprising three proximal Cu(II) centers was prepared by transfer of a trinuclear metal center confined in a bowl-shaped molecule to polystyrene.[26] Those polystyrene derivatives selectively hydrolyzed carboxyl-containing amides by cleaving the peptide bonds adjacent to the carboxyl groups. The cleavage-site selectivity was attributed to recognition of the carboxylate anion of the substrate by the carboxylate-binding group of the artificial active site.



cyclen

The first artificial protease with selectivity for cleavage site in the hydrolysis of a protein instead of simple amides was obtained in a later study:[27] the active site was designed by attachment of a molecular entity containing two cyclens to a polystyrene derivative to obtain a polymer indicated by $[Cyc]_2$−PS, followed by addition of Cu(II) ion to the cyclen moieties to obtain $[Cu(II)Cyc]_2$−PS. In the hydrolytic degradation of myoglobin by $[Cu(II)Cyc]_2$−PS, two pairs of intermediate proteins accumulated. Peptide linkages of Gln(91)−Ser(92) and Ala(94)−Thr(95) were the initial cleavage sites leading to the formation of the protein fragments. On the basis of a molecular modeling study, the site selectivity was attributed to anchorage of one Cu(II)Cyc unit of the catalytic module to a heme carboxylate of myoglobin. Although high site selectivity for the initial cleavage of a protein substrate was achieved with the artificial metalloprotease, the catalyst failed to cleave other common proteins such as γ-globulin or albumin.

One of the next challenges in the area of synthetic artificial proteases is to design catalysts that hydrolyze a wide range of protein substrates (broad substrate selectivity) by cleaving the substrate at selected positions on the polypeptide backbone (high site selectivity). To possess both the broad substrate selectivity and the high site selectivity as digestive proteases or proteasomes, the artificial proteases should be able to form complexes with a variety of protein substrates effectively and to cleave peptide bonds at selected positions in the resulting complexes.

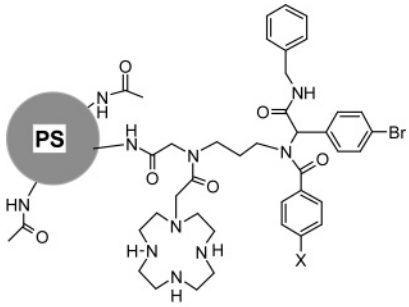


$[Cyc]_2$-PS

For this purpose, the aldehyde group is employed in the present study as the binding site of the artificial proteases because the aldehyde group can form imine bonds with the ϵ-amino groups of Lys residues exposed on the surface of proteins. Since the imine bonds are readily hydrolyzed, the artificial protease equipped with an active site containing the aldehyde group may be able to form complexes with a variety of proteins reversibly. If a catalytic group with high proteolytic activity is positioned in proximity to the aldehyde group, the catalytic group may cleave a peptide bond in vicinity to the Lys residue of the substrate complexed to the artificial protease. In the present study, polymeric artificial metalloproteases with active sites comprising Cu(II)Cyc and the aldehyde group were synthesized. The cooperation between the aldehyde group and the metal center in the action of the artificial proteases in cleavage of various proteins is described in this article.

Results

Synthesis of Artificial Metalloproteases. The cyclen−aldehyde conjugates built on the backbone of polystyrene in the present study are indicated by A−PS and B−PS. As the control catalysts lacking the aldehyde groups, A^{cont}−PS and B^{cont}−PS were also prepared.

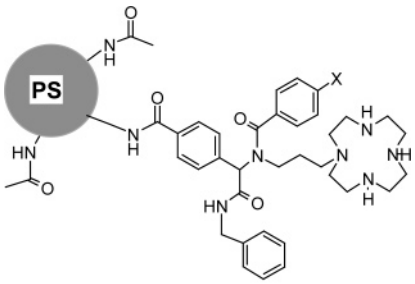


A-PS (X = CHO)

A^{cont}-PS (X = H)

The synthetic routes for preparation of A−PS, A^{cont}−PS, B−PS, and B^{cont}−PS are summarized in Schemes 1 and 2. The precursors (A5, $A5^{cont}$, B1, or $B1^{cont}$) of catalytic modules were synthesized by the Ugi condensation reaction. In the Ugi reaction, one-pot condensation of a carboxylic acid, an aldehyde, an amine, and an isocyanide produces an *N*-acyl amino acid amide.[31] As the polymeric support of the artificial proteases, a

(25) Suh, J.; Moon, S.-J. *Inorg. Chem.* **2001**, *40*, 4890−4895.
(26) Moon, S.-J.; Jeon, J. W.; Kim, H.; Suh, M. P.; Suh, J. *J. Am. Chem. Soc.* **2000**, *122*, 7742−7749.
(27) Yoo, C. E.; Chae, P. S.; Kim, J. E.; Jeong, E. J.; Suh, J. *J. Am. Chem. Soc.* **2003**, *125*, 14580−14589.
(28) Milovic, N. M.; Badjic, J. D.; Kostic, N. M. *J. Am. Chem. Soc.* **2004**, *126*, 696−697.
(29) Jeung, C. S.; Kim, C. H.; Min, K.; Suh, S. W.; Suh, J. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 2401−2404.
(30) Jeung, C.-S.; Song, J. B.; Kim, Y.-H.; Suh, J. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 3061−3064.

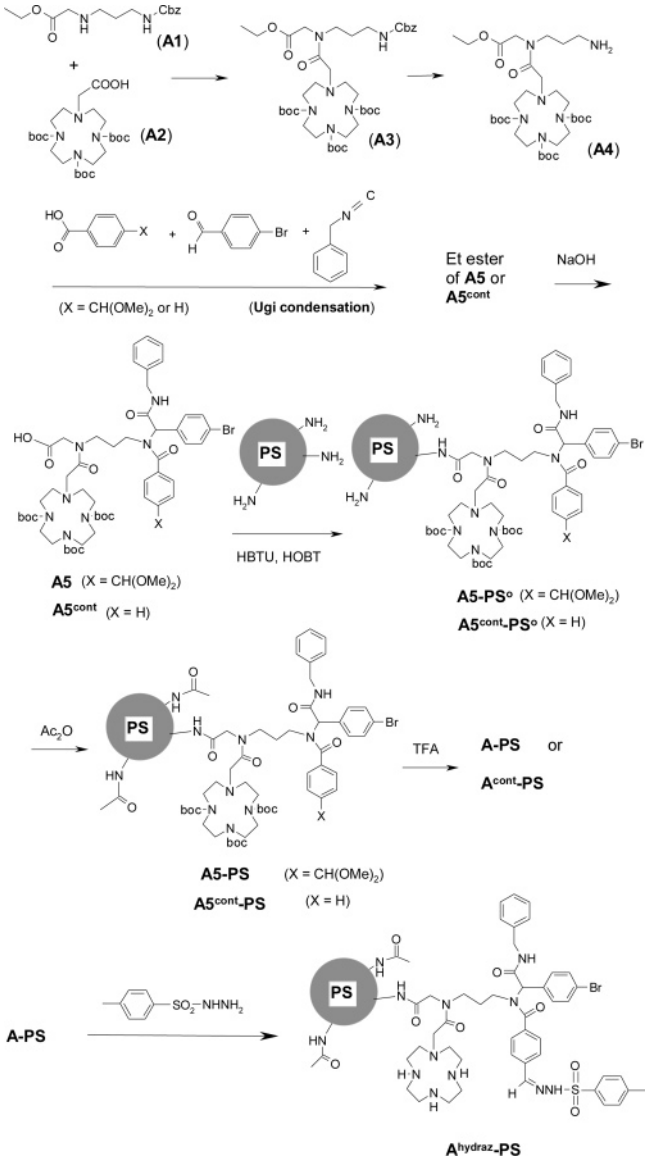


B-PS (X = CHO)

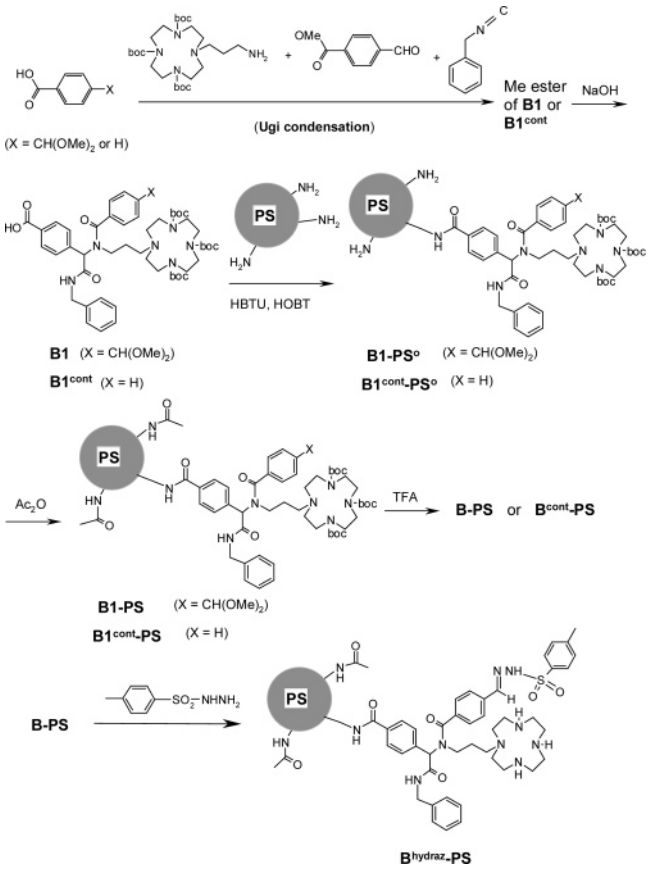
B^{cont}-PS (X = H)

cross-linked polystyrene derivative containing aminomethyl groups attached to a part of the phenyl rings was used. The content of aminomethyl group in the polystyrene derivative was 1.5 mmol/g: 17% of the phenyl rings of polystyrene contained aminomethyl group. After A5, A5^{cont}, B1, or B1^{cont} was attached to the polystyrene derivative, the primary amines on the polymer were acetylated, and the protecting groups on the catalytic modules were removed to obtain A−PS, A^{cont}−PS, B−PS, and

Scheme 1. Synthetic Route to A−PS and Derivatives



Scheme 2. Synthetic Route to B−PS and Derivatives



B^{cont}−PS. By the reaction with *p*-toluenesulfonylhydrazide, the aldehyde groups of A−PS and B−PS were converted to the corresponding hydrazones leading to the formation of A^{hydraz}−PS and B^{hydraz}−PS. By treatment of the cyclen-containing polymers with $CuCl_2$, Cu(II) ion was complexed to the cyclen moieties to form Cu(II)A−PS, Cu(II)A^{cont}−PS, Cu(II)A^{hydraz}−PS, Cu(II)B−PS, Cu(II)B^{cont}−PS, and Cu(II)B^{hydraz}−PS.

The contents of Cu(II) ion in Cu(II)A−PS, Cu(II)A^{cont}−PS, Cu(II)A^{hydraz}−PS, Cu(II)B−PS, Cu(II)B^{cont}−PS, and Cu(II)-B^{hydraz}−PS were measured by inductively coupled plasma absorption−emission spectroscopy (ICP-AES) after Cu(II) ion was released from the polystyrene derivatives by multiple treatment with 1 N HNO_3. Based on the amounts of the Cu(II) ion, the contents of the catalytic modules were calculated as 0.49 mol % (relative to styrene moieties) for Cu(II)A−PS, 0.59 mol % for Cu(II)A^{cont}−PS, 0.47 mol % for Cu(II)A^{hydraz}−PS, 1.3 mol % for Cu(II)B−PS, 0.90 mol % for Cu(II)B^{cont}−PS, and 1.3 mol % for Cu(II)B^{hydraz}−PS.

Electron probe microanalysis (EPMA) estimated the molar ratio of Cu(II), S, and Br on the surface of Cu(II)A^{hydraz}−PS as 1.00:0.81:0.88 and that of Cu(II) and S on the surface of Cu-(II)B^{hydraz}−PS as 1.00:0.87, respectively. Considering the possible nonspecific adsorption of the Cu(II) ion, incomplete formation of the hydrazones, and/or the experimental error involved in EPMA measurement, these data are consistent with the structure of the catalytic modules introduced to Cu(II)A−PS and Cu(II)B−PS.

Kinetic Measurement. Proteolytic activity of Cu(II)A−PS, Cu(II)A^{cont}−PS, Cu(II)B−PS, and Cu(II)B^{cont}−PS was exam-

(31) Domling, A.; Ugi, I. *Angew. Chem., Int. Ed.* **2000**, *39*, 3168−3210.

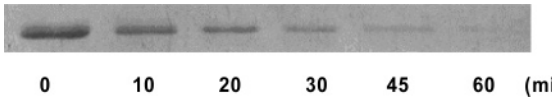


Figure 1. Results of SDS-PAGE performed on myoglobin (4.9 μM) incubated with Cu(II)A−PS (C_o = 0.45 mM) at pH 9.5 and 50 °C.

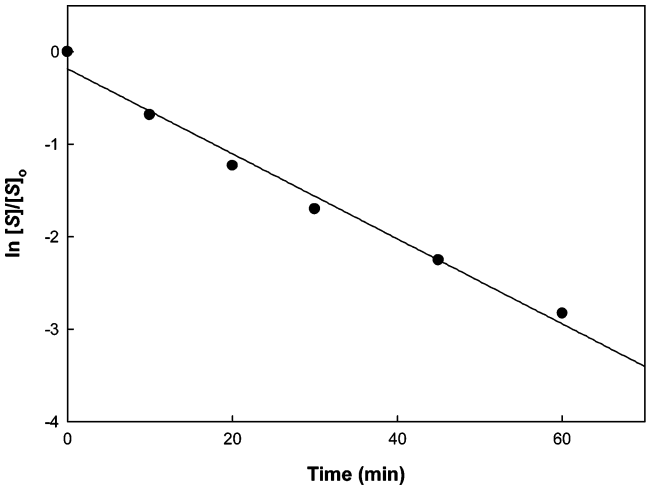


Figure 2. Plot of ln[S]/[S]$_o$ against time for the electrophoretic bands presented in Figure 1. The relative concentration of substrate was measured by analyzing the density of the electrophoretic bands. The straight line corresponds to k_o of 7.7 × 10^{-4} s^{-1}.

ined by using horse heart myoglobin, bovine serum γ-globulin, bovine serum albumin, human serum albumin, chicken egg white lysozyme, and chicken egg ovalbumin as the substrates. Myoglobin is oxidized to metmyoglobin in the presence of oxygen. The myoglobin purchased from a commercial source and used in the present study was also in the met form, as checked by its visible spectrum.[32]

While the buffer solution containing a protein substrate (1.4−7.0 μM) was shaken with the resin, disappearance of the protein was observed by sodium dodecyl sulfate polyacrylamide gel electrophoresis[33,34] (SDS-PAGE). Typical results of SDS-PAGE performed on the protein substrates cleaved by the polystyrene-based catalysts are illustrated in Figure 1. That the disappearance of the electrophoretic bands of the protein substrates was not due to the adsorption onto the resin was confirmed by measuring the total amino acid contents of the product solution separated from the resin according to the method described previously.[16]

The rate of protein cleavage at 50 °C was measured by monitoring the decrease in the intensity of the electrophoretic bands corresponding to the substrate protein.[11,15−19,27] The pseudo-first-order kinetic constant (k_o) was estimated from the logarithmic plot such as that illustrated in Figure 2. Rate data were collected by varying the speed of shaking the reaction mixture containing the polystyrene-based catalyst and the protein substrate, and it was found that k_o reached a plateau value at the shaking speed of 1200 rpm. Thus, kinetic data were collected at this shaking speed. The k_o values stand for cleavage of the substrate protein molecule itself and do not provide information on further fragmentation of the initial cleavage products.

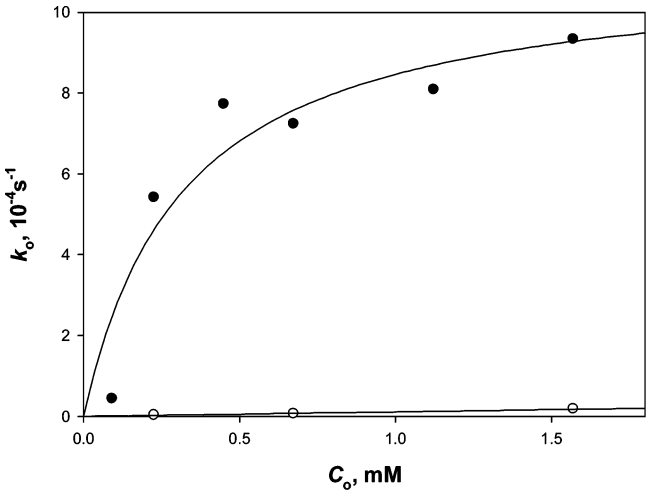


Figure 3. Plot of k_o against C_o for the hydrolysis of myoglobin catalyzed by Cu(II)A−PS (●) and Cu(II)A^{cont}−PS (○) at pH 9.5 and 50 °C.

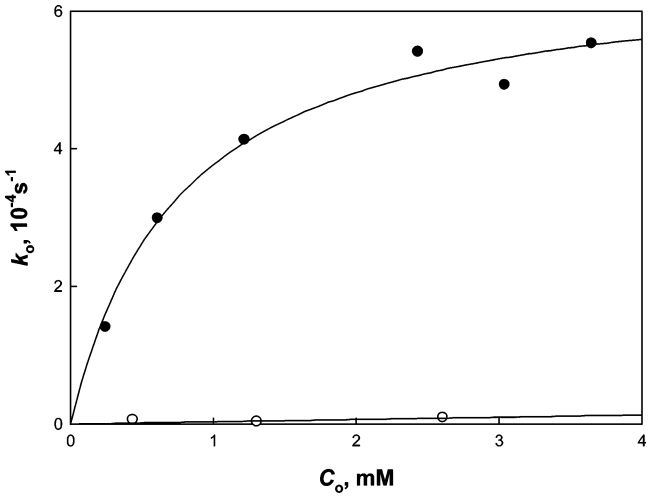


Figure 4. Plot of k_o against C_o for the hydrolysis of myoglobin catalyzed by Cu(II)B−PS (●) and Cu(II)B^{cont}−PS (○) at pH 9.5 and 50 °C.

γ-Globulin has two subunits with distinctly different molecular weights (25 and 50 kDa). Since the density of the electrophoretic band of the light chain was weak, the kinetic data were collected only for the degradation of the heavy chain.

Rate data were collected by varying the amount of the catalyst (C_o). Here, C_o is expressed as the concentration of the catalytic module obtainable when the resin is assumed to be dissolved. The k_o values were measured for each substrate in the presence of each polystyrene derivative at various pH's and at fixed C_o and S_o concentrations under the conditions of $C_o \gg S_o$ to identify the optimum pH. At the optimum pH thus selected, the k_o values were measured at various C_o concentrations under the conditions of $C_o \gg S_o$. As shown by the dependence of k_o on C_o (Figures 3, 4, and S1−S10 of Supporting Information), saturation kinetic behavior was observed for the cleavage of all of the protein substrates by Cu(II)A−PS and Cu(II)B−PS. Under the same conditions, cleavage of the protein substrates by Cu(II)A^{cont}−PS or Cu(II)B^{cont}−PS was much slower than that by Cu(II)A−PS and Cu(II)B−PS.

Kinetic data for the cleavage of the protein substrates by Cu(II)A−PS or Cu(II)B−PS can be analyzed in terms of Michaelis−Menten scheme (eq 1) as was done with the reactions catalyzed by other polystyrene-based artificial proteases.[11,27]

(32) Bodwell, C. E.; McClain P. E. In *The Science of Meat and Meat Products*, 2nd ed.; Price, J. F., Schweigert, B. S., Ed.; W. H. Freeman: San Francisco, CA, 1971; p 97.
(33) Lameli, U. K. *Nature* **1970**, *227*, 680−685.
(34) Hames, B. D. In *Gel Electrophoresis of Proteins*; Hames, B. D., Rickwood, D., Eds.; IRL Press: New York, 1990; Chapter 1.

Table 1. Values of Kinetic Parameters[a] for the Cleavage of Various Protein Substrates by Cu(II)A−PS and Cu(II)B−PS at 50 °C and at the Optimum pH

substrate	catalyst	pH	k_{cat} (10^{-4} s^{-1})	K_m (10^{-3} M^{-1})	k_{cat}/K_m (s^{-1} M^{-1})[b]
myoglobin[c]	Cu(II)A−PS	9.5	11	0.32	3.5 (0.005)
myoglobin	Cu(II)B−PS	9.5	6.7	0.77	8.7 (0.003)
γ-globulin[d]	Cu(II)A−PS	9.5	22	1.3	1.7 (0.008)
γ-globulin	Cu(II)B−PS	9.5	7.8	0.57	1.4 (0.005)
bovine serum albumin[d]	Cu(II)A−PS	9.5	8.0	0.92	0.87 (0.005)
bovine serum albumin	Cu(II)B−PS	9.5	8.7	1.2	0.73 (0.005)
human serum albumin	Cu(II)A−PS	9.5	18	0.79	2.3 (0.008)
human serum albumin	Cu(II)B−PS	9.0	6.7	1.1	0.61 (0.006)
lysozyme	Cu(II)A−PS	9.5	6.2	0.13	4.8 (0.002)
lysozyme	Cu(II)B−PS	9.5	6.0	1.3	0.46 (0.002)
ovalbumin	Cu(II)A−PS	9.5	25	1.2	2.1 (0.003)
ovalbumin	Cu(II)B−PS	9.5	15	2.2	0.68 (0.002)

[a] Parameters k_{cat} and K_m become k_{cat}^{app} and K_m^{app}, respectively, when the scheme of eq 5 is used instead of eq 1. [b] Values in parentheses are k_{cat}/K_m measured with the respective control polymer (Cu(II)A^{cont}−PS or Cu(II)B^{cont}−PS) under the same conditions. Accuracy of these values is low. [c] For [Cu(II)Cyc]$_2$−PS,[27] $k_{cat} = 0.95 \times 10^{-4}$ s^{-1}, $K_m = 2.0 \times 10^{-3}$ M, and $k_{cat}/K_m = 0.047$ s^{-1} M^{-1} at pH 9.0 and 50 °C. [d] No catalysis in protein degradation was observed with [Cu(II)Cyc]$_2$−PS.[27]

Under the conditions of $C_o \gg [CS]$, pseudo-first-order kinetic behavior is expected with k_o being derived as eq 2. Kinetic data illustrated in Figures 3, 4, and S1−S10 were analyzed according to eq 2 with a nonlinear regression program. The values of k_{cat}, K_m, and k_{cat}/K_m for the cleavage of the protein substrates by Cu(II)A−PS or Cu(II)B−PS are summarized in Table 1. For Cu(II)A^{cont}−PS or Cu(II)B^{cont}−PS, k_o was proportional to C_o, and the proportionality constant can be taken as k_{cat}/K_m ($K_m \gg C_o$, eq 2). The values of k_{cat}/K_m thus estimated for the action of Cu(II)A^{cont}−PS or Cu(II)B^{cont}−PS are also summarized in Table 1.

$$C + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} CS \xrightarrow{k_2} C + P \quad (1)$$

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

$$k_{cat} = k_2 \quad (3)$$

$$K_m = (k_{-1} + k_2)/k_1 \quad (4)$$

After Cu(II)A−PS or Cu(II)B−PS was incubated with bovine serum albumin at various pH's until the protein was almost completely degraded, the polymer was recovered and used again to cleave bovine serum albumin. The activity of the recovered polymer was almost identical with that of the fresh polymer. The active sites of the artificial proteases, therefore, are not appreciably damaged during incubation with the proteins under the conditions adopted for collection of the kinetic data.

Identification of Cleavage Sites. During incubation of a protein substrate with a polystyrene-based catalyst, aliquots of the buffer solution were taken to measure the matrix-assisted laser desorption/ionization time-of-flight mass spectrum (MALDI-TOF MS). An example is illustrated in Figure 5, with MALDI-TOF MS taken at various intervals for the degradation of myoglobin in the presence of Cu(II)A−PS. Disappearance of myoglobin, formation and decay of some intermediate proteins, and accumulation of product proteins are shown in the MALDI-TOF MS of Figure 5. Each new MALDI-TOF MS peak represents a cleavage product obtained by the action of the catalysts.

Cleavage sites for protein degradation can be identified by determining the structures of the cleavage products corresponding to the peaks in the MALDI-TOF MS. The content of each

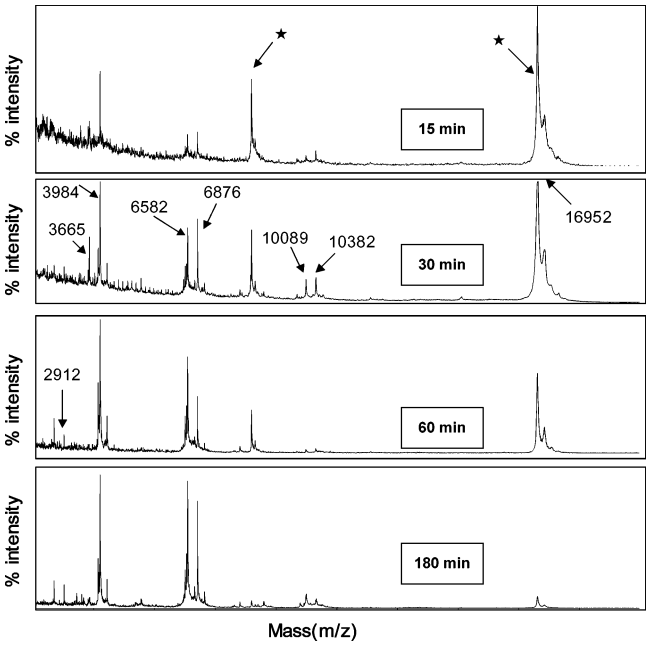


Figure 5. MALDI-TOF MS obtained during incubation of myoglobin (S_o = 4.9 μM) with Cu(II)A−PS (C_o = 0.45 mM) at pH 9.5 and 50 °C. The peaks labeled with asterisks (m/z = 16 952 and 8476) are those of myoglobin. The peaks with m/z of 10 382, 10 089, 6876, 6582, 3984, 3663, and 2912 are identified as Gly(1)−Ala(94), Gly(1)−Gln(91), Ser(92)−Gly(153), Thr(95)−Gly(153), Ser(117)−Gly(153), Gly(1)−Phe(33), and Ser(92)−His(116), respectively.

reaction mixture isolated at various time intervals was partially separated by HPLC to obtain fractions containing fewer numbers of cleavage products. Each cleavage product contained in the fraction was subjected to structural analysis by treatment with carboxypeptidase A or trypsin followed by MALDI-TOF MS measurement. Carboxypeptidase A releases *C*-terminal amino acid residues of a protein in a sequential manner, whereas trypsin cleaves peptide bonds on the carboxyl side of Lys or Arg.

The sizes of new protein fragments obtained after treatment of a cleavage product with carboxypeptidase A provide information for amino acid sequence at the *C*-terminus of the cleavage product as described previously.[27,35] When the *C*-terminal amino acid of a cleavage product is Gly, Asp, Glu,

(35) Chae, P. S.; Kim, M.-s.; Jeung, C.-S.; Lee, S. D.; Park, H.; Lee, S.; Suh, J. *J. Am. Chem. Soc.* **2005**, *127*, 2396−2397.

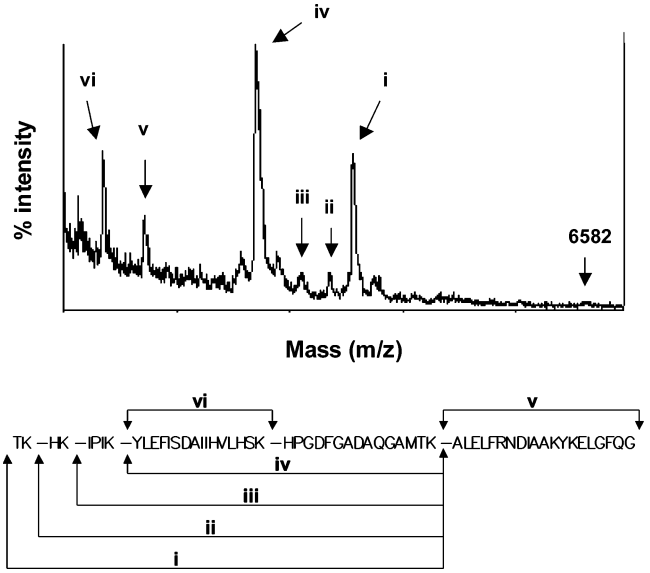


Figure 6. MALDI-TOF MS of the protein fragment with *m*/*z* of 6582 (obtained as indicated in Figure 5) after treatment with trypsin (1.0 μM) at pH 8.0 and 25 °C for 20 min. The *m*/*z* values of i, ii, iii, iv, v, and vi are 4316, 4086, 3821, 3370, 2284, and 1885, respectively. The diagram shows how the structures of the fragments were assigned.

Pro, or Arg, the cleavage product resists the action of carboxypeptidase A. Then, treatment of the cleavage product with trypsin can provide a different kind of information on the structure of the cleavage product, as exemplified by the MALDI-TOF MS illustrated in Figure 6.

The same analysis was performed for the other protein substrates, but structures were positively identified for fewer cleavage products compared with myoglobin degradation. This was because MALDI-TOF MS peaks of the cleavage products obtained from the other proteins were weaker and broader than those from myoglobin. The structures of the cleavage products and the corresponding cleavage sites identified for the proteolytic actions of Cu(II)A−PSand Cu(II)B−PS are summarized in Table 2.

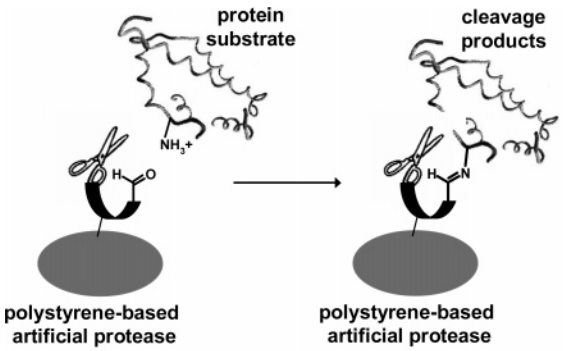
Trapping of Imine Intermediate. Since the aldehyde groups of the artificial proteases can form imine bonds with amino groups of the protein substrates, attempts were made to trap the imine intermediates with $NaB(OAc)_3H$, which is known to reduce imines much faster than aldehydes. Since reductive amination with $NaB(OAc)_3H$ is usually carried out under weakly acidic conditions[36] and Cu(II)A−PS and Cu(II)B−PS are deactivated under acidic conditions, the trapping experiments were carried out at pH 6.0. When myoglobin (S_o = 4.9 μM) was degraded by Cu(II)A−PS (C_o = 0.45 mM) at pH 6.0 and 50 °C, k_o was 6.8×10^{-6} s^{-1}. After disappearance of myoglobin was complete, 88% of the total amino acid residues initially introduced by myoglobin were recovered in the buffer solution. When 5 mM $NaB(OAc)_3H$ was initially introduced to the reaction mixture, k_o was 1.3×10^{-4} s^{-1} and only 15% of the total amino acid residues were recovered in the buffer solution. When the same reaction was carried out first without $NaB(OAc)_3H$ to complete the degradation of myoglobin, $NaB(OAc)_3H$ was added to the degradation mixture, and the mixture was incubated for 3 days; 72% of the total amino acid residues were recovered in the buffer solution.

Discussion

It is well-established that the Cu(II) complex of cyclen catalyzes cleavage of peptide bonds by hydrolysis: formation of amino and carboxyl groups upon cleavage of a peptide bond was confirmed, and catalytic turnover in peptide hydrolysis was observed.[27,37,38]

As demonstrated by the kinetic data summarized in Figures 3, 4, and S1−S10 as well as in Table 1, Cu(II)A−PS and Cu(II)B−PS effectively degraded all of the six protein substrates examined in the present study. The values of k_{cat}/K_m for myoglobin degradation by Cu(II)A−PS and Cu(II)B−PS are 20−80 times greater than that by $[Cu(II)Cyc]_2$−PS reported[27] previously, as summarized in Table 1. Catalysts Cu(II)A−PS and Cu(II)B−PS are even more effective for γ-globulin and bovine serum albumin, since these proteins were not cleaved by $[Cu(II)Cyc]_2$−PS.[27] On the other hand, $Cu(II)A^{cont}$−PS and $Cu(II)B^{cont}$−PS lacking the aldehyde groups in the active sites exhibited negligible proteolytic activity. By positioning the aldehyde group in proximity to Cu(II)Cyc attached to a cross-linked polystyrene, both broad substrate selectivity and high proteolytic rate are achieved.

The broad substrate selectivity and high proteolytic rate can be attributed to the imine formation between the aldehyde group of the artificial protease and the amino groups of the protein substrate as schematically indicated by **1**. Essentially all globular proteins possess Lys residues which provide ammonium cations on the protein surfaces. Imine formation between the Lys-amino group of the protein substrate and the aldehyde group of the polystyrene-based artificial protease would anchor the protein on polystyrene. The broad substrate selectivity manifested by Cu(II)A−PS and Cu(II)B−PS is attributable to the anchorage of the protein substrates through imine bonds. Upon formation of the covalent complex, cleavage of the polypeptide backbone of the bound protein substrate could occur more readily since the reaction between the catalytic Cu(II) center and the scissile peptide bond becomes entropically more favorable.



1

The effect of imine formation can be analyzed quantitatively in terms of a modified Michaelis−Menten scheme of eq 5. Here, *CS* stands for the noncovalent intermediate formed between the

(36) Abdel-Magid, A. F.; Carson, K. G.; Harris, B. D.; Maryanoff, C. A.; Shah, R. D. *J. Org. Chem.* **1996**, *61*, 3849−3862.

(37) Jeon, J. W.; Son, S. J.; Yoo, C. E.; Hong, I. S.; Song, J. B.; Suh, J. *Org. Lett.* **2002**, *4*, 4155−4158.

(38) Jeon, J. W.; Son, S. J.; Yoo, C. E.; Hong, I. S.; Suh, J. *Bioorg. Med. Chem.* **2003**, *11*, 2901−2910.

Table 2. Cleavage Products and the Corresponding Cleavage Sites Identified for the Proteolytic Action of Cu(II)A−PS and Cu(II)B−PS

substrate	cleavage product	cleavage site
myoglobin[a]	Gly(1)−Phe(33)[b,c]	Phe(33)−Thr(34)
	Gly(1)−Gln(91)[b,c]	Gln(91)−Ser(92)
	Gly(1)−Ala(94)[b,c]	Ala(94)−Thr(95)
	Ser(92)−His(116)[b]	Gln(91)−Ser(92), His(116)−Ser(117)
	Ser(92)−Gly(153)[b,c]	Gln(91)−Ser(92)
	Thr(95)−Gly(153)[b,c]	Ala(94)−Thr(95)
	Ser(117)−Gly(153)[b]	His(116)−Ser(117)
bovine serum albumin[a]	Asp(1)−Gln(33)[b]	Gln(33)−Cys(34)
	Ile(141)−Trp(213)[b,c]	Gln(140)−Ile(141), Trp(213)−Ser(214)
	Gly(206)−Val(230)[b]	Phe(205)−Gly(206), Val(230)−Thr(231)
human serum albumin[a]	Asp(1)−Ile(25)[b]	Ile(25)−Ala(26)
	Asp(1)−Gln(33)[b]	Gln(33)−Cys(34)
	Met(298)−Met(329)[c]	Glu(297)−Met(298), Met(329)−Phe(330)
lysozyme[a]	Ser(91)−Arg(114)[b,c]	Ala(90)−Ser(91), Arg(114)−Cys(115)
	Gly(104)−Arg(125)[b,c]	Asn(103)−Gly(104), Arg(125)−Gly(126)
	Gly(104)−Leu(129)[b]	Asn(103)−Gly(104)

[a] The numbers of amino acid residues contained in the protein molecules are 153 for myoglobin, 583 for bovine serum albumin, 585 for human serum albumin, and 129 for lysozyme. [b] Identified for the cleavage by Cu(II)A−PS. [c] Identified for the cleavage by Cu(II)B−PS.

substrate and the catalyst, *CS′* the imine formed between the substrate and the catalyst, and CP_1 the imine formed between the cleavage product and the catalyst. By applying steady-state approximation to [*CS*] and [*CS′*], rate parameters are derived as eqs 6−8 under the condition of $C_o \gg S_o$ for the disappearance of *S*. The same saturation kinetic behavior (eqs 2 and 6) is predicted for the dependence of k_o on C_o by both eqs 1 and 5.

$$C + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} CS \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} CS' \xrightarrow{k_3} CP_1 + P_2 \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} C + P_1 + P_2 \quad (5)$$

$$k_o = k_{cat}^{app} C_o/(K_m^{app} + C_o) \quad (6)$$

$$k_{cat}^{app} = k_2 k_3/(k_2 + k_{-2} + k_3) \leq k_3 \quad (7)$$

$$K_m^{app} = (k_2 k_3 + k_{-1} k_3 + k_{-1} k_{-2})/k_1(k_2 + k_{-2} + k_3) \quad (8)$$

$$k_{cat}^{app} = k_3 \,(\text{when } k_3 \ll k_{-2} \ll k_2) \quad (9)$$

$$K_m^{app} = k_{-1} k_{-2}/k_1 k_2 = (k_{-2}/k_2) K_d \ll K_d \quad (\text{when } k_3 \ll k_{-2} \ll k_2 \ll k_{-1}) \quad (10)$$

When the kinetic scheme is modified to include more intermediates, the expressions of k_{cat} (or k_{cat}^{app}) and K_m (or K_m^{app}) become more complex. Under special circumstances, the physical meaning of k_{cat}^{app} and K_m^{app} represented by eqs 7 and 8 is clearer. It is likely that the rate-determining step is the peptide-cleaving step ($k_3 \ll k_{-2}$). In view of the broad substrate selectivity and high catalytic rates observed with Cu(II)A−PS or Cu(II)B−PS, it is reasonable to assume that [*CS′*] is much higher than [*CS*] ($k_{-2} \ll k_2$). One may assume that the *CS* complex is in fast equilibration with *C* and *S* ($k_2 \ll k_{-1}$ and, thus, $K_m = K_d = k_{-1}/k_1$). Under these conditions, k_{cat}^{app} and K_m^{app} can be approximated as eqs 9 and 10.

The values of k_{cat}^{app} are $(6.0-25) \times 10^{-4}$ s^{-1}, corresponding to half-lives of 4.6−19 min. Since k_3 stands for the rate constant for peptide cleavage in *CS′* to form CP_1, k_{cat}^{app} represents effectiveness of the catalytic center in peptide cleavage. Under the identical conditions, the half-life for the spontaneous hydrolysis of peptide bonds is 10−30 years.[1,2] The values of K_m^{app} are $(1.3-22) \times 10^{-4}$ M, being comparable to the K_m values of proteolytic enzymes.[39] The low values of K_m^{app} (= $(k_{-2}/k_2)K_d$; eq 10) can be attributed to the high stability ($k_{-2}/k_2 \ll 1$) of the imine complex (*CS′*) compared with that of the noncovalent complex (*CS*).

The intermediacy of the imine complex is further supported by the trapping experiment using $NaB(OAc)_3H$. The rate for the disappearance of myoglobin in the presence of Cu(II)A−PS was raised by 19 times upon addition of $NaB(OAc)_3H$ under the conditions described in the Results section. The enhanced rate for disappearance of the protein substrate is consistent with trapping of the imine intermediate with the borohydride reagent. A major portion of the amino acid residues originally contained in the protein substrate was recovered after completion of the proteolytic reaction in the absence of $NaB(OAc)_3H$. When $NaB(OAc)_3H$ was added after completion of the proteolysis, most of the amino acid residues were still recovered. On the other hand, addition of $NaB(OAc)_3H$ in the initial stage of the proteolysis by Cu(II)A−PS resulted in immobilization of most of the amino residues on the polystyrene surface. This again supports trapping of imine intermediate by $NaB(OAc)_3H$.

The structures of the seven cleavage products and the positions of four cleavage sites were identified for the action of Cu(II)A−PS on myoglobin. The four cleavage sites are indicated on the X-ray crystallographic structure[40] of horse heart metmyoglobin in Figure 7. Among the seven cleavage products, five were also identified in the action of Cu(II)B−PS. It is certain that Gln(91)−Ser(92) and Ala(94)−Thr(95) are among the initial cleavage sites since the two protein fragments (Gly(1)−Gln(91) and Ser(92)−Gly(153); Gly(1)−Ala(94) and Thr(95)−Gly(153)) obtained by the cleavage at each of the two sites were positively identified. Figure 5 does not reveal any other large fragment arising from another possible initial cleavage site.

Myoglobin contains 19 Lys residues which are marked with K in Figure 7. With the whole structure of myoglobin including the side chains of Lys residues being frozen, molecular mechanics calculations were carried out by using molecular modeling programs (HyperChem and Sculpt) to identify the potential anchorage sites leading to the cleavage at Gln(91)−Ser(92) or Ala(94)−Thr(95). With the aldehyde groups of the

(39) Polgár, L. *Mechanisms of Protease Action*; CRC Press: Boca Raton, FL, 1989.
(40) Evans, S. V.; Brayer, G. D. *J. Mol. Biol.* **1990**, *213*, 885−897.

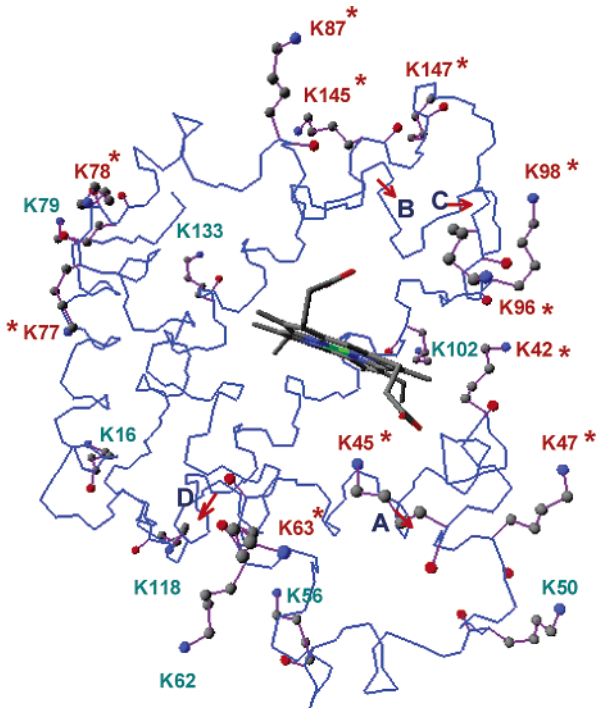


Figure 7. Backbone of the X-ray crystallographic structure of metmyoglobin. In the center is the heme group. Red arrows indicate the cleavage sites: A for Phe(33)−Thr(34), B for Gln(91)−Ser(92), C for Ala(94)−Thr(95), and D for His(116)−Ser(117). Lys residues are marked with K followed by numerals.

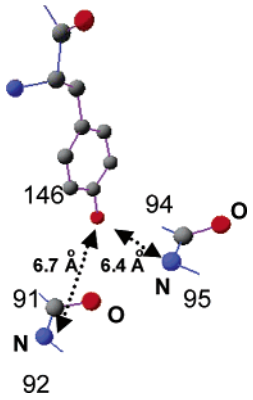
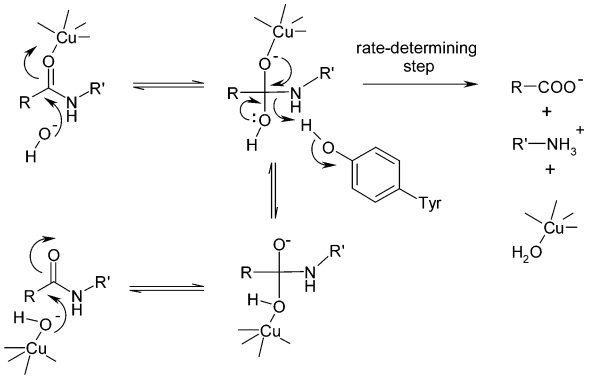


Figure 8. Locations of the scissile peptide bonds of Gln(91)−Ser(92) and Ala(94)−Thr(95) as well as the phenol group of Tyr(146) in the X-ray crystallographic structure of myoglobin.

Scheme 3. Possible Action of Tyr-146 as a General Acid Catalyst



catalyst linked to the amino group of a Lys residue, whether the attack of the Cu(II)Cyc moiety of the catalyst at the peptide group of Gln(91)−Ser(92) or Ala(94)−Thr(95) is feasible was examined. The modeling programs indicated that the anchorage at one of the 11 Lys resides marked in red and with * in Figure 7 did not involve appreciable steric strain for the attack of the Cu(II)Cyc at either Gln(91)−Ser(92) or Ala(94)−Thr(95). This was mainly due to the flexibility of the artificial active site comprising Cu(II)Cyc and the aldehyde group. If the flexibility of the protein, especially the long side chains of the Lys residues, is taken into consideration, it is more difficult to identify the anchorage site.

It is not possible to identify the Lys residue of myoglobin that is anchored on the aldehyde group of the catalytic module prior to the initial peptide cleavage. The catalytic center, however, selectively attacks peptide bonds of Gln(91)−Ser(92) or Ala(94)−Thr(95) despite the flexibility of the artificial active site comprising Cu(II)Cyc and the aldehyde group. Interestingly, Gln(91)−Ser(92) and Ala(94)−Thr(95) were also the initial cleavage sites in the degradation of myoglobin by $[Cu(II)Cyc]_2$−PS investigated in the previous study.[27] As noted above, the site selectivity in the action of $[Cu(II)Cyc]_2$−PS was attributed to anchorage of one of the two Cu(II)Cyc units of the active site by the heme carboxylate of myoglobin followed by the attack by the other Cu(II)Cyc unit at the scissile peptide bond. In the action of $[Cu(II)Cyc]_2$−PS, why Gln(91)−Ser(92) and Ala(94)−Thr(95) were selectively cleaved among several peptide groups having access to the catalytic center was unanswered.

The selective cleavage of Gln(91)−Ser(92) and Ala(94)−Thr(95) in the action of Cu(II)A−PS, Cu(II)B−PS, and $[Cu(II)Cyc]_2$−PS despite the structural differences in the catalytic modules implicates that some structural features may assist the peptide cleavage at Gln(91)−Ser(92) and Ala(94)−Thr(95). Examination of the crystallographic structure of myoglobin suggested that the phenol group of Tyr(146) may promote the cleavage of both Gln(91)−Ser(92) and Ala(94)−Thr(95) by acting as an extra catalytic group. As illustrated in Figure 8, the distance between the phenol oxygen of Tyr(146) and the amide nitrogen atom of Ser(92) or Thr(95) is 6.7 or 6.4 Å, respectively, in the crystallographic structure. Little steric strain is imposed, however, when the distance between the phenol oxygen atom and either of the two amide nitrogen atoms is reduced to 3 Å.

As summarized in Scheme 3, the Cu(II) center may act as a Lewis acid catalyst to polarize the carbonyl group of the scissile peptide bond and facilitate attack by hydroxide ion at the carbonyl carbon. Alternatively, the Cu(II)-bound hydroxide ion may act as the nucleophile. In either case, the rate-determining step would be the expulsion of amine moiety from the tetrahedral intermediate, which requires protonation of the amine nitrogen by the specific acid or a general acid.[20] The phenol of Tyr(146) may act as the general acid catalyst to accelerate the rate-determining amine expulsion. At pH 9.0−9.5, the optimum pH for the polystyrene-based artificial proteases, the phenol of Tyr is the strongest acid among the organic functionalities present in the protein molecule.

The initial cleavage of myoglobin at Gln(91)−Ser(92) or Ala(94)−Thr(95) involves anchorage of the substrate onto the polystyrene-based artificial protease by reversible formation of the imine complexes as well as cleavage of peptide bonds by the Cu(II)Cyc moiety of the artificial protease with general acid assistance from the Tyr phenol group provided by the substrate.

The optimum pH of 9.0−9.5 reflects the overall efficiency of complexation of the protein by the catalyst, the formation of imine intermediates, and the catalytic action of Cu(II)Cyc combined with hydroxide ion and the Tyr phenol group.

Figure 5 indicates that Gly(1)−Gln(91) and Gly(1)−Ala(94) are degraded rapidly, whereas Ser(92)−Gly(153) and Thr(95)−Gly(153) are more stable during incubation with Cu(II)A−PS. Those protein fragments contain Lys residues which can interact with the aldehyde group of Cu(II)A−PS. The faster degradation of Gly(1)−Gln(91) and Gly(1)−Ala(94) suggests the presence of extra catalytic groups provided by the protein fragments, although no information is available for the three-dimensional structure of Gly(1)−Gln(91) and Gly(1)−Ala(94).

When myoglobin or its degradation products are reductively linked to polystyrene by treatment with $NaB(OAc)_3H$, the amount of total amino acids released to the buffer solution is considerably reduced, indicating suppression of cleavage of the polypeptide backbones. The aldehyde group of the polystyrene-based protease forms imine complexes with various Lys amino groups of the protein substrate reversibly. In the absence of $NaB(OAc)_3H$, various forms of the imine complexes would reversibly transform into one another until the productive forms undergo protein cleavage. In the presence of $NaB(OAc)_3H$, however, reduction of the unproductive forms of the imine complexes can immobilize the proteins blocking the protein fragmentation.

In vivo, digestive proteases and proteasomes mostly hydrolyze unfolded or precleaved proteins. For example, pepsin cleaves proteins unfolded in the acidic medium of stomach producing protein fragments, which are cleaved further by other proteases in the intestine. Proteasomes, the main enzymes of the nonlysosomal pathway of protein degradation in cells of higher organisms, cleave cellular proteins unfolded with ATP.[41] In the industrial application of artificial proteases, however, it is desirable to hydrolyze a variety of proteins without addition of denaturing agents. Hydrophilic residues such as ammonium or carboxylate ions are exposed on the surface of undenatured globular proteins.[42] To synthesize artificial proteases recognizing undenatured protein molecules, we chose to design recognition sites targeting the ammonium groups exposed on the surface of protein substrates. As we expected, artificial proteases Cu(II)A−PS and Cu(II)B−PS cleaved all of the protein substrates tested in the present study by using both the aldehyde and the Cu(II)Cyc moiety in the active site. Furthermore, the artificial proteases manifested high cleavage site selectivity.

The immobile artificial proteases can overcome thermal, chemical, and mechanical instabilities of natural proteases. Broad substrate selectivity, high proteolytic rate, and high cleavage-site selectivity are the three major objectives in designing artificial proteases applicable to protein industry. By introducing an aldehyde group in proximity to the Cu(II)Cyc attached to polystyrene, remarkable improvement has been achieved in those three major goals. By incorporating more catalytic elements to the artificial active site with a better-defined structure, the artificial proteases would become more effective and achieve higher reaction rates and high amino acid specificity.

Experimental Section

Preparation of Catalysts. The derivative of poly(styrene-*co*-divinylbenzene) with 17% of styryl residues aminomethylated (1.5 mmol NH_2 per gram resin) and with 2% cross-linkage was purchased from Fluka. A solution of a precursor (A5, $A5^{cont}$, B1, or $B1^{cont}$) of the catalytic module or the control module (0.73 mmol) mixed with 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (280 mg; 0.73 mmol), di-*i*-propylethylamine (DIEA) (0.40 mL; 2.3 mmol), and *N*-hydroxybenzotriazole (HOBT) (89 mg; 0.66 mmol) in 5 mL of *N*,*N*-dimethylformamide (DMF) was added to the suspension of the polystyrene (4.0 g) in 30 mL of DMF. The suspension was degassed for 30 min and shaken at 45 rpm and room temperature for 1 day. The product resin (A5−PS^o, $A5^{cont}$−PS^o, B1−PS^o, or $B1^{cont}$−PS^o) was collected by filtration, washed with CH_2Cl_2 (30 mL × 5) and CH_3OH (30 mL × 5), and dried in vacuo. The amino groups of the resulting resin were acetylated by shaking the resin (4.0 g) with acetic anhydride (5.7 mL) and DIEA (10 mL) dissolved in 30 mL of DMF at room temperature for 1 day. The product resin was collected by filtration, washed with DMF (30 mL × 5) and CH_3OH (30 mL × 5), and dried in vacuo to obtain the acetylated derivative (A5−PS, $A5^{cont}$−PS, B1−PS, or $B1^{cont}$−PS). Kaiser test[43] indicated that the remaining amino groups were quantitatively acetylated. The acetylated resin (4.0 g) was shaken in the mixture of 6 mL of trifluoroacetic acid (TFA) and 30 mL of CH_2Cl_2 at 45 rpm and room temperature for 1 h. The product resin was collected by filtration and washed with CH_2Cl_2 (30 mL × 5). Then the resin was shaken in 6 mL of DIEA and 24 mL of CH_2Cl_2 at 45 rpm and room temperature for 1 h. The product resin was collected by filtration, washed with CH_2Cl_2 (30 mL × 5) and CH_3OH (30 mL × 5), and dried in vacuo to obtain A−PS, A^{cont}−PS, B−PS, or B^{cont}−PS. To the suspension of A−PS or B−PS (0.10 g) in CH_3OH (1.5 mL) were added acetic acid (15 μL) and *p*-toluenesulfonhydrazide (2.6 mg, 0.014 mmol). The resulting mixture was shaken at 45 rpm and room temperature for 5 h. The resin was filtered, washed with CH_3OH (10 mL × 5) and CH_2Cl_2 (30 mL × 5), and dried in vacuo to obtain A^{hydraz}−PS or B^{hydraz}−PS. To a 0.23 M $CuCl_2 \cdot 2H_2O$ solution in DMF (25 mL), the cyclen-containing polystyrene derivative (4.0 g) was suspended, and the resulting mixture was shaken at 45 rpm and room temperature for 1 day and washed with DMF (30 mL × 5) and CH_3OH (30 mL × 5) to obtain the Cu(II) complex of the cyclen-containing polystyrene derivative (Cu(II)A−PS, Cu(II)A^{cont}−PS, Cu(II)A^{hydraz}−PS, Cu(II)B−PS, Cu(II)B^{cont}−PS, or Cu(II)B^{hydraz}−PS).

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 SDS-PAGE with a Mighty Small II SE 250 model. Densities of the electrophoretic bands were analyzed with a AlphaImager 2200 model and a AlphaEase model. MALDI-TOF MS analysis was performed with a Voyger-DE STR Biospectrometry Workstation model. NMR spectra were recorded with a Bruker DPX 300 MHz model, and the UV−vis spectra with a Beckman DU 68 UV−vis spectrometer. ICP-AES measurement was carried out with a Shimadzu ICPS-1000IV model. EPMA was performed with a CAMECA SX-57 model. Distilled and deionized water was used for preparation of buffer solutions. Buffers (0.05 M) used in this study were acetate (pH 5.0), 2-(*N*-morpholine)-ethanesulfonate (pH 6.0), *N*-2-hydroxyethylpiperazine-*N*′-ethansulfonate (pH 7.0−8.0), *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonate (pH 8.5), and boric acid (pH 9.0−10.5). Kinetic measurements and trapping experiments were carried out in the presence of 0.3 M NaCl.

(41) Kisselev, A. F.; Goldberg, A. L. *Chem. Biol.* **2001**, *8*, 739−758.
(42) It has been recently reported[28] that an artificial peptidase based on a Pd(II)−cyclodextrin conjugate recognized the phenyl residue of an oligopeptide and hydrolyzed the adjacent peptide bond of the oligopeptide. This catalyst would not cleave the peptide backbones of globular proteins if the phenyl residues are not exposed on the surface of the protein substrates.
(43) Sarin, V. K.; Kent, S. B. H.; Tam, J. P.; Merrifield, R. B. *Anal. Biochem.* **1981**, *117*, 147−157.

All buffer solutions were filtered with 0.45 μm Millipore microfilter and autoclaved before use in the kinetic measurements. Horse heart myoglobin, bovine serum γ-globulin, bovine serum albumin, human serum albumin, chicken egg white lysozyme, chicken egg ovalbumin, bovine carboxypeptidase A, and bovine trypsin were purchased from Sigma and used without further purification. The resins were suspended in a buffer solution and swollen for 1 h prior to kinetic measurement.

Acknowledgment. This work was supported by Korea Research Foundation Grant (KRF 2004-041-C00220). This article is dedicated to the late Prof. Irving M. Klotz, who pioneered the area of synthetic macromolecules with enzyme-like activities.

Supporting Information Available: Experimental procedures for synthesis of A5, A5^{cont}, B1, and B1^{cont} and plots of k_o against C_o (Figures S1−S10) for hydrolysis of γ-globulin, bovine serum albumin, human serum albumin, lysozyme, and ovalbumin by the polystyrene-based catalysts. This material is available free of charge via the Internet at http://pubs.acs.org.

JA052191H