Pergamon

Bioorganic & Medicinal Chemistry Letters 12 (2002) 3247–3250

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Effective Artificial Proteases Synthesized by Covering Silica Gel with Aldehyde and Various Other Organic Groups

Hyunsook Kim,[a] Myoung-soon Kim,[a] Hyesun Paik,[a] Yeon-Sook Chung,[a] In Seok Hong[b] and Junghun Suh[a,*]

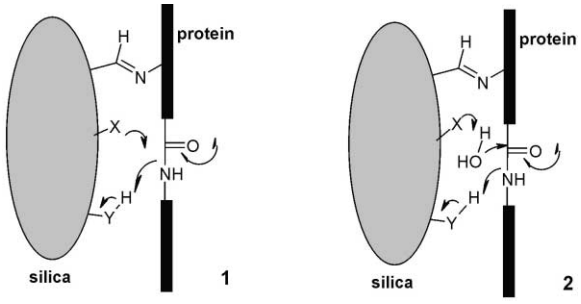
[a]*School of Chemistry and Center for Molecular Catalysis, Seoul National University, Seoul 151-747, Republic of Korea*
[b]*Artzyme Biotech Corporation, 403-1, Silim-2-Dong, Seoul 151-012, Republic of Korea*

Received 1 July 2002; accepted 30 August 2002

Abstract—Organic artificial proteases with broad substrate specificity were synthesized by covering the surface of silica gel with aldehyde and the functional groups present in amino acids. The artificial proteases hydrolyzed ovalbumin, albumin, hemoglobin, γ-globulin with half-lives as short as 50 min at 25 °C or 7 min at 50 °C.

Hydrolysis of protein mixtures into smaller fragments is carried out in various industries.[1,2] Proteolysis can be also applied to utilization of agricultural, seafood, and meat byproducts and to improvement of nutritional and functional properties of proteins.[1,2] Moreover, proteolysis produces peptides with various kinds of bioactivity.[3–10] 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 pHs.

Many proteases hydrolyze proteins by using only organic functional groups. The first synthetic organic catalyst that hydrolyzed unactivated peptide bonds was designed by preparing active sites comprising three proximal salicylates on polymeric backbones. This was achieved by cross-linkage of three salicylates preassembled by Fe(III) ion with branched polymeric amines.[11,12] Subsequently, we prepared the second type of organic synthetic peptidase by designing active sites comprising two or more proximal imidazoles on polystyrene backbone.[13] The third type of organic artificial protease was synthesized by using aldehyde groups attached to silica gel as the functional group for both the binding site and the catalytic site as illustrated by **1**/**2** [X = CH(OH)(O$^-$) and Y-H = CH(OH)$_2$].[14] In this mechanism, the imine bond is formed between the aldehyde group of the silica and the amino group of the protein.



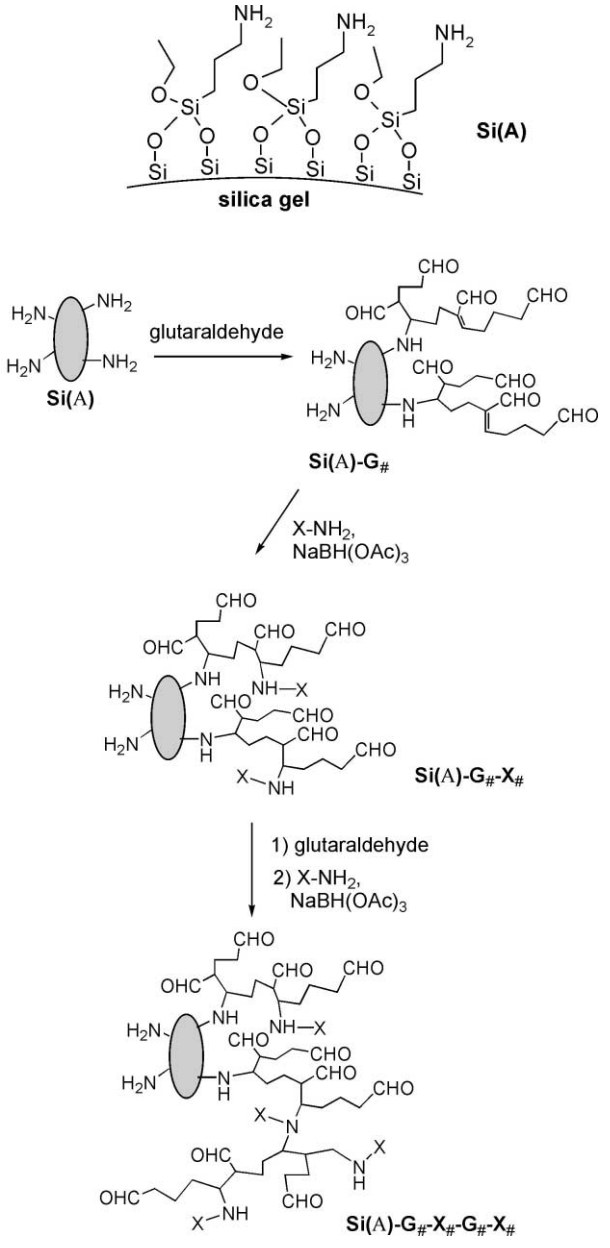
The artificial proteases constructed with salicylates or imidazoles manifested very narrow substrate specificity, hydrolyzing only either albumin (Alb) or γ-globulin (Gbn) when the two proteins were tested as substrates.[11–13] The aldehyde-containing catalyst hydrolyzed Alb and Gbn,[14] but it was not effective toward ovalbumin (Ovl) or hemoglobin (Hgb) due to strong protein adsorption. To design more effective immobile organic proteases by using the aldehyde-containing silica gel, it was necessary to broaden the substrate specificity and to raise the catalytic rate. To accomplish these objectives, we introduced various functional groups to the aldehyde-containing silica gel. In the present study, functional groups present in amino acids

*Corresponding author. Tel.: +82-2-886-2184; fax: +82-2-874-3704; e-mail: jhsuh@snu.ac.kr

0960-894X/02/$ - see front matter © 2002 Elsevier Science Ltd. All rights reserved.
PII: S0960-894X(02)00724-2

such as carboxyl, hydroxyl, phenolic, mercapto, and imidazolyl groups were attached to silica gel in combination with the aldehyde group according to the synthetic route summarized in Scheme 1.

Synthesis and characterization of Si(A), Si(A)-$G_{\#}$, and Si(A)-G_{180}-Trp_{32} was described previously.[14] In the nomenclature of the catalysts, G and X stand for glutaraldehyde and the amine, respectively, attached to Si(A), and # indicates the content of the pendant expressed in terms of mol% relative to the amino groups of Si(A). In the present study, Si(A)-$G_{\#}$-$X_{\#}$ was prepared from Si(A)-$G_{\#}$ by shaking Si(A)-$G_{\#}$ (20 g) with X-NH_2 (90–260 mmol) in the presence of $NaBH(OAc)_3$ (150–380 mmol) for 1–42 h. Here, the amine was added and shaken for 10–60 min before the powders of $NaBH(OAc)_3$ were added in small portions. The reaction was carried out in a buffer solution (0.05 M 4-morpholineethanesulfonate, pH 6) with or without an equal volume of methanol. Si(A)-$G_{\#}$-$His_{\#}$-$G_{\#}$-$His_{\#}$ was prepared by the reaction of Si(A)-$G_{\#}$-$His_{\#}$ (20 g) with glutaraldehyde followed by histidine by the method applied to Si(A)-$G_{\#}$ and Si(A)-$G_{\#}$-$X_{\#}$. Acetylation of Si(A)-$G_{\#}$-$His_{\#}$-$G_{\#}$-$His_{\#}$ was carried out by the acetylation procedure of the previous work.[14] Elemental analysis indicated that the content of amino groups in Si(A) was 1.3 mmol/g. The contents of glutaraldehyde and amines (XNH_2) incorporated to the silica-based catalysts were measured by elemental analysis. The silica-based catalysts prepared in the present study are indicated in Table 1.



Scheme 1. Synthetic routes to the silica-based artificial proteases.

As described previously,[14] it has been reported[15] that the silica-based amines undergo conjugate addition to the enal groups obtained by aldol condensation among glutaraldehyde molecules.[16] The previous study revealed that aldehyde groups of Si(A)-$G_{\#}$ are not reduced when the silica derivatives are treated with $NaBH(OAc)_3$ and that amines are added to Si(A)-G mainly by conjugate addition to the enal groups even in the presence of $NaBH(OAc)_3$.[14] In the synthesis of the catalyst to be used for protein degradation, however, amines were added to Si(A)-$G_{\#}$ in the presence of $NaBH(OAc)_3$ to maximize addition of the amines to the silica derivative in view of possible reductive amination of the aldehyde groups to a minor degree.

Chicken egg Ovl (M_r 44 kDa), bovine serum Alb (M_r 66 kDa), bovine serum Gbn (M_r 150 kDa), and bovine Hgb (M_r 62 kDa) were tested as substrate proteins. Rates for cleavage of the proteins by the silica-based catalysts were measured by following disappearance of parent bands in SDS-PAGE electrophoresis as described previously.[11–14] The two chains of Gbn have distinctly different molecular weights (25 and 50 kDa) and rates for their disappearance were separately measured. On the other hand, the two subunits of Hgb have similar molecular weights (15 and 16 kDa), and, therefore, disappearance of the combined bands of the two subunits was followed.

Possibility that disappearance of the parent protein was due to adsorption onto the silica was excluded by the method[13] described previously: a major portion of the amino acid residues of the protein substrate were recovered from the filtrate after the electrophoretic band of the substrate disappeared. Accumulation of electrophoretic bands of degradation products was also taken as evidence for the protein cleavage. For selected proteolysis products, HPLC analysis was carried out with and without treatment with phenyl isothiocyanate as described previously.[13] Phenyl isothiocyanate introduces an aromatic ring to primary amines of proteins and is used for N-terminal sequencing of proteins by Edman degradation. The results of HPLC analysis (Fig. 1) show the presence of a large number of peptides with N-terminal amino groups, providing evidence for hydrolysis of peptide bonds during the protein degradation.

Pseudo-first-order kinetic behavior was observed for degradation of protein substrates in the presence of the

Table 1. Values of k_0 (10^{-2} min^{-1}) for hydrolytic cleavage of various proteins by silica-based artificial proteases[a]

Catalyst[b]	Ovl	Alb	Hgb	Gbn
Si(A)	Ads	Ads	Ads	Ads/Ads
Si(A)-Ac	Ads	NR	Ads	Ads/Ads
Si(A)-G_{170}	Ads	Ads	Ads	Ads/Ads
Si(A)-G_{200}-Gly_{40}	2.4(6)	NR	Ads	0.33(7)/0.55(7)
Si(A)-G_{210}-Asp_{45}	0.17(6)	2.1(6)	Ads	0.14(6)/0.26(6)
Si(A)-G_{190}-Glu_{17}	0.16(4)	0.03(6)	2.2(7)	0.32(6)/0.24(6)
Si(A)-G_{200}-Bal_{40}	1.9(6)	NR	Ads	0.20(8)/0.19(7)
Si(A)-G_{200}-Abt_{39}	1.5(6)	0.12(5)	Ads	0.26(6)/0.30(6)
Si(A)-G_{180}-Ctm_{54}	Ads	0.52(6)	Ads	0.16(9)/0.46(6)
Si(A)-G_{180}-Etm_{42}	Ads	0.19(4)	Ads	0.18(9)/0.40(7)
Si(A)-G_{180}-Tym_{23}	Ads	0.22(6)	3.2(7)	0.06(9)/0.20(7)
Si(A)-G_{180}-Tpm_{32}	Ads	0.14(6)[c]	Ads	0.68(6)/0.52(6)[c]
Si(A)-G_{140}-Htm_{16}	10(5)	0.30(7)	3.5(8)	0.09(8)/0.08(8)
Si(A)-G_{250}-His_{22}-G_{16}-His_{10}	1.2(6)*	1.1(9)	1.1(7)*	1.3(9)*/0.96(8)*
Si(A)-G_{170}-His_{39}-G_{36}-His_3-Ac_{70}	0.76(6)*	0.81(7)	1.2(8)*	0.73(8)/0.52(8)

[a]Kinetic measurements were performed with 100 mg of catalyst added to 1.5 mL of 0.05 M buffer solution containing 0.15 mg of the protein substrate. Values of k_0 were measured at the optimum pH indicated in parentheses and at 50 °C except those marked with * which were measured at 25 °C. Relative standard deviations of k_0 were 10–25%. For Gbn, k_0 values for the heavy and the light chains are indicated in sequence with/put between them. When k_0 was smaller than 1×10^{-4} min^{-1}, the catalyst was considered to be inactive and marked as NR. When marked as Ads, rate measurements were hampered by strong adsorption of the substrate protein or hydrolysates.
[b]See Scheme 1 for nomenclature of catalysts. Amines used for XNH_2 are: glycine for Gly, L-aspartate for Asp, L-glutamate for Glu, β-alanine for Bal, γ-aminobutyrate for Abt, cysteamine for Ctm, ethanolamine for Etm, tyramine for Tym, tryptamine for Tpm, histamine for Htm, and L-histidine for His. Functional groups introduced by the amines are: carboxyl by Gly, Asp, Glu, Bal, and Abt; mercapto by Ctm; hydroxyl by Etm; phenolic by Tym; indolyl by Tpm; imidazolyl by Htm; imidazolyl and carboxyl by His.
[c]From ref 14.

artificial proteases under the conditions indicated in Table 1.[17] The pseudo-first-order rate constants (k_0) were little affected by the shaking speed as far as the shaking speed exceeded 600 rpm. The kinetic measurements were performed at the shaking speed of 1200 rpm. The k_0 values measured for the catalysts are summarized in Table 1. Kinetic measurements were carried out at several pHs and the values measured at the optimum pHs are listed in Table 1.

Some of the silica-based catalysts manifested broad substrate specificity as shown by the data of Table 1. The highest values of k_0 listed in Table 1 are equivalent to half-lives ($=0.69/k_0$) of 50 min at 25 °C and 7 min at 50 °C. Rates for the spontaneous hydrolysis of peptide bonds at pH 7 and 25 °C correspond to half-lives of about 500 years.[18,19] Then, disappearance of a protein substrate containing 300 amino acid residues due to the spontaneous hydrolysis of a peptide bond would proceed with a half-life of about 2 years. In this regard, the degree of acceleration achieved here is high.

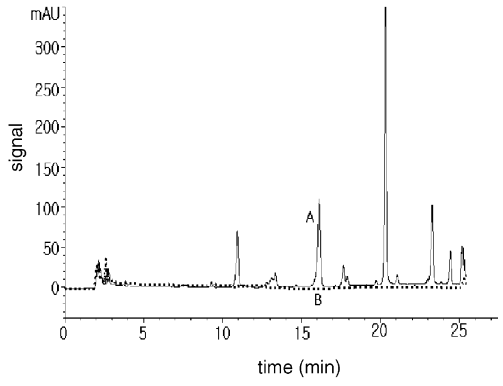


Figure 1. A typical result of HPLC analysis of protein hydrolysates. HPLC was performed after degradation of Alb with Si(A)-G_{250}-His_{22}-G_{16}-His_{10} for 5 h at pH 8 under the conditions of Table 1 with (solid line A) or without (dotted line B) subsequent treatment with phenyl isothiocyanate.

For the protein hydrolysis, complexation between the substrate and the catalytic sites of the catalyst must be facilitated. On the other hand, if the protein substrate and/or its hydrolysates are adsorbed too strongly onto the solid support, the solid is not useful as the catalyst. Si(A) is covered with amino groups and adsorbs the protein substrates strongly (Table 1). The strong adsorption may be attributed to multiple interaction with a protein molecule through ion pair formation between ammonium ions of Si(A) and carboxylate ions of the protein. Several enzymes have been immobilized onto Si(A)-G through formation of covalent bonds between the glutaraldehyde portions attached to silica and the amino groups of the enzyme.[15,20] Strong adsorption observed for some proteins onto Si(A)-G_{170} (Table 1) may be attributed to the same covalent linkage.

On attachment of amines with functional groups such as indolyl, carboxyl, imidazolyl, hydroxyl, phenolic, and mercapto groups to Si(A)-G, adsorption of the protein became much weaker. Immobilization of proteins to Si(A)-G was attributed to conjugate addition of the amino group of a protein to the enal group attached to silica.[15] Various pendants introduced to Si(A)-G in the present study apparently blocked the conjugate addition of the protein to the enal group. This may be because the amines used for introduction of the pendants had been already added to the enal groups.[14] It is also possible that the pendants located on the silica surface impose steric hindrance to the approach of the protein to the enal groups.[14]

The silica-based proteases contain various reactive organic functional groups. Imidazolyl, carboxyl, hydroxyl, phenolic, and mercapto groups act as nucleophiles, general acids, and/or general bases in the catalytic processes of enzymes as well as in simple organic reactions.[21] Diols obtained by hydration of aldehydes can play catalytic roles in transacylation reactions.[22] The surface of silica-based catalysts is covered densely with those potential catalytic groups.

Possible mechanisms for proteolysis by the silica-based catalysts synthesized in this work are proposed as **1**/**2**. Here, the protein substrate is complexed to the silica-based protease by forming the imine linkage. X acts as a nucleophile to attack at the carbonyl carbon in **1** or as a general base to assist attack of water molecule at the carbonyl carbon in **2**. In both **1** and **2**, Y-H acts as a general acid to protonate the leaving amine in the rate-determining expulsion of amine from the tetrahedral intermediate. X and Y-H may include aldehyde hydrate, carboxylic acid, alcohol, thiol, phenol, and imidazole as well as their ionized forms.

The indolyl group of Si(A)-G_{180}-Tpm_{32} has no specific catalytic roles.[14] When extra polar groups with potential catalytic roles are attached to Si(A)-$G_{\#}$, the silica-based catalysts showed a broad specificity hydrolyzing all of the four proteins tested. The proteolytic activity toward Alb or Gbn was not, however, improved considerably by some of the polar pendants. In this regard, whether the polar groups such as carboxyl, hydroxyl, mercapto, and phenolic groups play specific catalytic roles or simply modify the microenvironments to enhance the reactivity of aldehyde groups is not clear. On the other hand, much higher rate was observed by histamine- or histidine-containing derivatives. The high catalytic activity of the histamine- or histidine-containing catalysts suggests that imidazolyl and carboxyl groups of histamine and histidine play specific roles and collaborate effectively with each other as well as with the aldehyde group.

The silica-based artificial proteases are comparable to or better than the organic artificial proteases[11–14] prepared previously in terms of degree of rate acceleration. The silica-based artificial proteases, especially the histamine- or histidine-containing derivatives, are, however, considerably improved over the previous ones since they have broad substrate specificity and, thus, mimic natural enzymes better.

The silica-based catalysts are prepared by random attachment of a variety of organic functional groups to the solid support. Many different artificial proteases can be further synthesized according to the synthetic method developed here. Various other types of organic reactions can be exploited for functionalization of silica. Different silica-based artificial proteases may have different amino acid specificities producing a variety of different oligopeptides from the same protein.

Acknowledgements

This work was supported Artzyme Biotech Corporation. H.K., M.-s.K., H.P., and Y.-S.C. were recipients of BK 21 fellowship.

References and Notes

1. Ward, O. P. In *Comprehensive Biotechnology*; Moo-Young, M., Ed.; Pergamon: Oxford, 1985; Vol. 3, p 789.
2. Tramper, J. In *Applied Biocatalysis*; Cabral, J. M. S.; Best, D.; Boross, L.; Tramper, J., Eds.; Harwood Academic: Chur, 1994; p 1.
3. Brantl, V.; Pfeiffer, A.; Herz, A.; Henschen, A.; Louttspeich, F. *Peptides* **1982**, *3*, 793.
4. Meisel, H. *FEBS Lett*. **1986**, *196*, 223.
5. Saito, H.; Miyakawa, H.; Tamura, Y.; Shimamura, S.; Tomita, M. *J. Dairy Sci*. **1991**, *74*, 3724.
6. Fiat, A.-M.; Migliore-Samour, D.; Jolles, P.; Drouet, L.; Sollier, C. B. D.; Caen, J. *J. Dairy Sci*. **1993**, *76*, 301.
7. Kayser, H.; Meisel, H. *FEBS Lett*. **1996**, *383*, 18.
8. Matar, C.; Nadathur, S. S.; Bakalinski, A. T.; Goulet, J. *J. Dairy Sci*. **1997**, *80*, 1965.
9. Nakagomi, K.; Yamada, R.; Ebisu, H.; Sadakane, Y.; Akizawa, T.; Tanimura, T. *FEBS Lett*. **2000**, *467*, 235.
10. Nagaoka, S.; Futamura, Y.; Miwa, K.; Awano, T.; Yamauchi, K. *Biochem. Biophys. Res. Commun*. **2001**, 281.
11. Suh, J.; Hah, S. S. *J. Am. Chem. Soc*. **1998**, *120*, 10088.
12. Oh, S.; Chang, W.; Suh, J. *Bioorg. Med. Chem. Lett*. **2001**, *11*, 1469.
13. Suh, J.; Oh, S. *J. Org. Chem*. **2000**, *65*, 7534.
14. Kim, H.; Paik, H.; Kim, M.-s.; Chung, Y.-S.; Suh, J. *Bioorg. Med. Chem. Lett*. **2002**, *12*, 2557.
15. Singh, A. K.; Flounders, A. W.; Volponi, J. V.; Ashley, C. S.; Wally, K.; Schoeniger, J. S. *Biosens. Bioelectron*. **1999**, *14*, 703.
16. The imines formed by addition of the amine pendants to the aldehyde groups might be easily hydrolyzed. In this regard, **1** and **2** which include imine bonds represent transient intermediates.
17. For the kinetic Scheme applied to proteolysis by immobile catalysts involving complexation of the protein substrate to the solid catalysts, see: Jang, B.-B.; Lee, K. P.; Min, D. H.; Suh, J. *J. Am. Chem. Soc*. **1998**, *120*, 12008.
18. Radzicka, A.; Wolfenden, R. *J. Am. Chem. Soc*. **1996**, *118*, 6105.
19. Smith, R. S.; Hansen, D. E. *J. Am. Chem. Soc*. **1998**, *120*, 8910.
20. Burtreau, N.; Burton, S.; Crichton, R. R. *FEBS Lett*. **1989**, *258*, 185.
21. Silverman, R. B. In *The Organic Chemistry of Drug Design and Drug Action*; Academic: San Diego, 1992; p 103.
22. Bender, M. L.; Reinstein, J. A.; Silver, M. S.; Mikulak, R. *J. Am. Chem. Soc*. **1965**, *87*, 4545.