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Silica-Based Artificial Protease Exploiting Aldehyde Groups as Catalytic Elements

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Abstract—An artificial protease synthesized by covering the surface of silica gel with aldehyde and indole groups effectively hydrolyzed albumin and γ-globulin. It is proposed that the aldehyde group is involved in both complexation of the protein substrate and the catalytic cleavage of the peptide bonds of the complexed proteins.

Designing artificial enzymes for hydrolysis of peptide bonds of proteins is challenging in view of the stability of peptide bonds, industrial and pharmaceutical importance of peptide hydrolysis, and interests in synthesis of biomimetic systems. Half-life for spontaneous hydrolysis of peptide bonds at pH 7 and 25 °C is about 500 years.[1,2]

Synthetic artificial enzymes are synthetic catalysts mimicking the catalytic principles and features of enzymes.[3,4] With synthetic artificial enzymes, it is possible to overcome limitations of natural enzymes such as instability to heat, incompatibility with organic solvents, inapplicability to abiotic reactions, and too narrow selectivity. To synthesize an effective enzyme-like catalyst, it is desirable to construct an artificial active site comprising both the binding site and the catalytic groups.

Many proteases such as serine proteases, aspartic proteases, and cysteine proteases hydrolyze proteins by using organic functional groups only.[5] Attempts have been made extensively to design organic catalytic systems for peptide hydrolysis. Some successful results were obtained with organic catalytic groups tethered to amide bonds.[6,7] The intramolecular catalytic systems are mimicries of enzyme-substrate complexes rather than enzymes. Intermolecular catalysis for hydrolysis of acyl derivates, however, was studied mostly with activated analogues such as nitrophenyl esters until a few years ago. To achieve effective intermolecular catalysis in peptide hydrolysis by artificial proteases exploiting organic functional groups exclusively, it is necessary to design artificial active sites that form complexes with the peptide substrates readily and hydrolyze the peptide bonds in the catalyst–substrate complexes through effective cooperation among the organic catalytic groups.

The first organic artificial protease has been synthesized by designing artificial active sites comprising three salicylates.[8] When such an active site was constructed on the backbone of cross-linked polystyrene, protein hydrolysis was achieved by collaboration among the carboxyl groups of the artificial protease, mimicking aspartic proteases.[9]

The second type of the organic artificial protease has been prepared by constructing artificial active sites comprising two or more imidazole groups on the backbone of cross-linked polystyrene.[10] Facile protein degradation was observed when imidazole was attached to polystyrene via C atom instead of N atom.

For the two types of organic artificial proteases reported to date, the artificial active site exploits functional groups present in the side chains of amino acids. It would be also possible to construct effective artificial active sites in which the catalytic groups are not related to amino acids. In the present study, an effective artificial protease is synthesized by using aldehyde group as the catalytic groups of the artificial active site.

Synthetic route to the aldehyde-containing artificial protease is summarized in Scheme 1. Silica gel was used as the solid support of the artificial protease.

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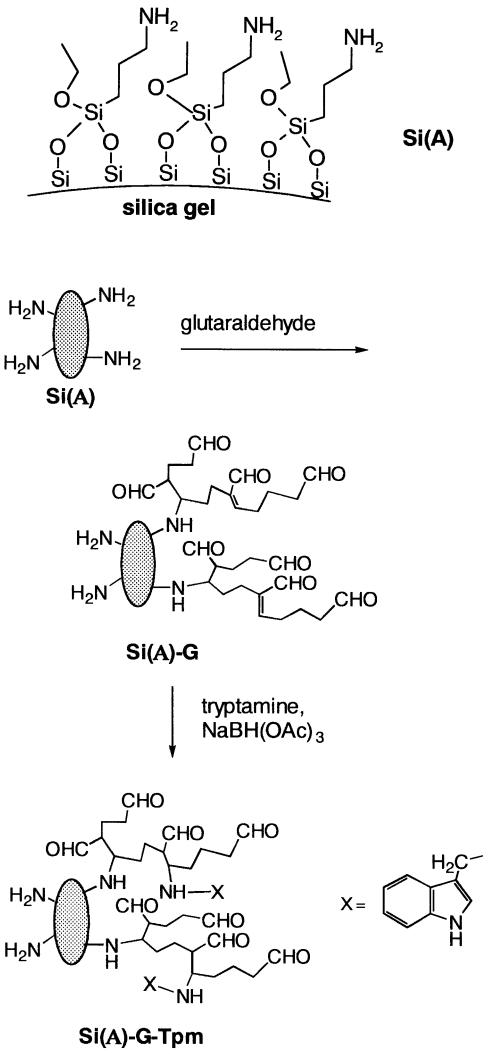


Scheme 1. Synthetic route to Si(A)-G and Si(A)-G-Tpm.

The silica surface was covered with primary amino groups by reacting silica gel (purchased from Merck) with γ-aminopropyltriethoxysilane in the absence of water by the procedure reported in the literature.[11,12] Elemental analysis indicated that the content of amino groups in Si(A) was 1.3 mmol/g. It is reported that the reaction of γ-aminopropyltriethoxysilane with the silanol hydroxyl groups located on the surface of silica gel forms the silica derivative with structure indicated as Si(A) in Scheme 1 predominantly under anhydrous conditions.[11,12]

Si(A)-G was prepared by shaking 80 g of Si(A) with 25% (v/v) aqueous solution of glutaraldehyde (1.1 L) for 6 h and by washing thoroughly with water and acetone. The shaking speed used for the modification of silica in the present study was 200 rpm. The amount of glutaraldehyde incorporated to Si(A)-G was estimated as 180 mol% relative to the amino groups of Si(A) on the basis of elemental analysis. It has been reported that the silica-based amines undergo conjugate addition to the enal groups obtained by aldol condensation among glutaraldehyde molecules.[13,14]

Si(A)-G-Tpm was prepared by shaking Si(A)-G (20 g) and tryptamine (14 g, 89 mmol) in a 1:1 (v/v) mixture (140 mL) of 0.05 M 4-morpholineethanesulfonate (Mes) buffer (pH 6) and methanol for 20 m at 25 °C followed by portionwise addition of $NaBH(OAc)_3$ (31 g, 150 mmol). After shaking the mixture for 16 h at 25 °C, the silica was separated by filtration and washed thoroughly with water, methanol, and methylene chloride. The amount of tryptamine incorporated to Si(A)-G-Tpm was estimated as 32 mol% relative to the amino groups of Si(A) on the basis of elemental analysis.

The content of aldehyde groups in the silica derivative was estimated by shaking the silica in methanol containing 0.5 M *p*-toluenesulfonylhydrazide and 2% (v/v) acetic acid at room temperature for 4 h to obtain the corresponding hydrazone from the reaction between the hydrazide and aldehyde groups. The amount of sulfur incorporated to the silica derivative was then estimated by elemental analysis.[15] When Si(A)-G was treated with $NaBH(OAc)_3$, a reducing agent selective for imines,[16] under the conditions of reductive amination experiments, the content of aldehyde group was not affected. This indicates that aldehyde groups are not reduced when the silica derivatives are treated with $NaBH(OAc)_3$.

Amines can be added to Si(A)-G by conjugate addition to the enal groups.[13] In the presence of $NaBH(OAc)_3$, amines may be also added to the aldehyde groups by reductive amination. After excess tryptamine was reacted with Si(A)-G in the presence or absence of $NaBH(OAc)_3$ under the conditions of the catalyst synthesis, the contents of tryptamine in the resulting silica derivatives were estimated by elemental analysis. The results revealed that the amount of tryptamine introduced to the silica derivatives was not increased appreciably by the addition of $NaBH(OAc)_3$. In the presence of $NaBH(OAc)_3$, therefore, the amines are added to Si(A)-G mostly by conjugate addition to the enal groups.[14] In the synthesis of the catalyst to be used for protein degradation, however, tryptamine was added to Si(A)-G in the presence of $NaBH(OAc)_3$ to maximize the amount of tryptamine incorporated to the catalysts.

Bovine serum albumin (BSA) (MW 66 kDa) and bovine serum γ-globulin (Gbn) (MW 150 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 illustrated in Figures 1 and 2. Intensities of electrophoretic bands were estimated with an Alpha-Imager™ IS-1220 model. Kinetic measurements were performed with 100 mg of catalyst added to 1.5 mL of 0.05 M buffer solution containing 9 mg of BSA or Gbn.[17] The two chains of Gbn have distinctly different molecular weights (25 and 50 kDa) and rates for their

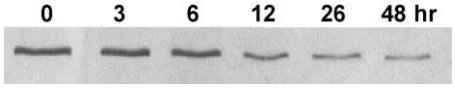


Figure 1. Results of SDS-PAGE obtained for BSA incubated with Si(A)-G-Tpm at 50 °C and pH 6.0.

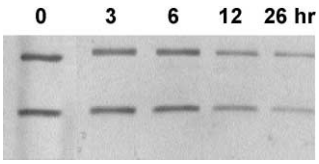


Figure 2. Results of SDS-PAGE obtained with the heavy (upper band) and light (lower band) chains of Gbn incubated with Si(A)-G-Tpm at 50 °C and pH 7.0.

disappearance were separately measured. Possibility that disappearance of the parent protein was due to adsorption onto Si(A)-G-Tpm was excluded by the method[10] 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. Gbn was not cleaved by incubation with 0.9 M HCHO in the absence of Si(A)-G-Tpm under the conditions of Figure 2. Effect of HCHO on BSA was not examined due to slow precipitation of the protein.

Pseudo-first-order kinetic behavior was observed for degradation of protein substrates in the presence of Si(A)-G-Tpm. 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 in a heated shaking incubator (a VorTemp 56 model). The k_0 values measured for fragmentation of protein substrates by Si(A)-G-Tpm at several pHs are summarized in Figure 3. The k_0 values measured at the optimum pHs correspond to half-lives ($=0.69/k_0$) of 240, 100, and 130 m for BSA, the heavy chain of Gbn, and the light chain of Gbn, respectively.[17]

For catalytic reactions proceeding through formation of catalyst–substrate complexes, the Michaelis–Menten scheme of enzymatic kinetics can be applied. For the Michaelis–Menten scheme, k_{cat} represents the highest k_0 value obtainable in the presence of excess enzyme. Thus, k_0 values illustrated in Figure 3 represent the lower limit of k_{cat} values.

When BSA or Gbn was stirred with Si(A) or Si(A)-G, the protein was adsorbed onto the silica, hampering

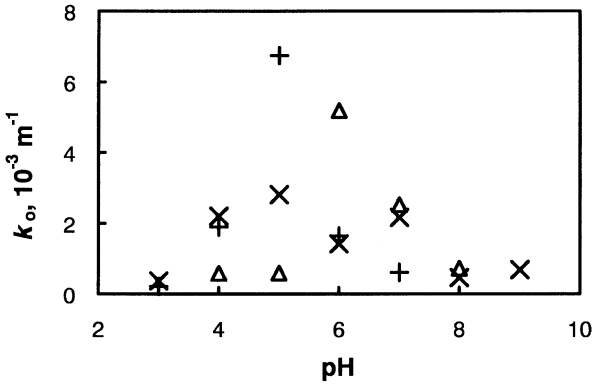


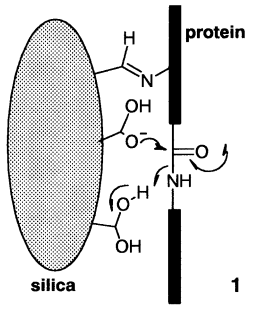
Figure 3. pH dependence of k_0 for the cleavage of BSA (×), the heavy chain of Gbn (+), and the light chain of Gbn (Δ) by Si(A)-G-Tpm at 50 °C. Buffers used were phosphate (pH 3), acetate (pH 4, 5), Mes (pH 6), 4-(2-hydroxyethyl)piperazineethanesulfonate (pH 7, 8), and borate (pH 9).

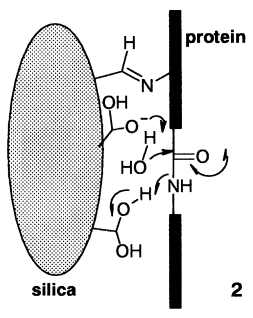
kinetic measurement for the protein degradation. Si(A) is covered with ammonium ions, which can interact with carboxylate anions of proteins. The strong adsorption of BSA or Gbn onto Si(A) may be attributed to multiple interaction between the protein molecule and Si(A) through ion pair formation between ammonium ions of Si(A) and carboxylate ions of the protein.

Enzymes can be immobilized onto Si(A)-G through formation of covalent bonds between the glutaraldehyde portions attached to silica and the amino groups of the enzyme.[13,18] Strong adsorption onto Si(A)-G observed for BSA and Gbn may be attributed to the same covalent linkage. On attachment of tryptamine to Si(A)-G, adsorption of the protein became negligible. 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.[13] Tryptamine 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 tryptamine was already added to the enal groups. It is also possible that indole groups located on the silica surface impose steric hindrance to the approach of the protein to the enal groups.

Hydrolysis of peptide bonds of BSA or Gbn by Si(A)-G-Tpm is remarkable, since aldehyde and amino groups are the only potential catalytic groups for Si(A)-G-Tpm. Indole group of tryptamine is an aromatic ring without specific catalytic roles. Since interaction of the amino groups of Si(A)-G-Tpm with protein substrates would suffer from steric strain, the aldehyde groups positioned on the silica surface are most likely responsible for the hydrolysis of the protein substrates.

The proteolytic action of Si(A)-G-Tpm may be attributed to the mechanism of **1** or **2**. Here, an aldehyde group anchors the protein substrate by imine formation. Diols obtained by hydration of aldehydes can play catalytic roles in transacylation reactions.[19] Two hydrated aldehydes are proposed to participate in cleavage of the peptide group: one makes nucleophilic attack at the carbonyl carbon or acts as a general base to assist addition of water molecule to the carbonyl group and the other acts as a general acid in the rate-determining expulsion of the amine moiety.[20] After hydrolysis of peptide bonds, the hydrolysates are released from the silica by hydrolysis of the imine linkage. The aldehyde groups, therefore, appear to serve as functional groups for both the binding part and the catalytic part of the artificial active site.





Effective proteolyisis by Si(A)-G-Tpm is attributable to the presence of artificial active sites comprising several proximal aldehyde groups. In addition, the microenvironment of the catalyst can exert favorable effects. In the mechanism of **1** or **2**, for example, the transition state may be stabilized by hydrogen bonding with hydrates of adjacent aldehyde groups. Such polar interactions would be facilitated in the hydrophobic microenvironment on the catalyst.

Results of the present study demonstrate that effective organic artificial proteases can be synthesized by using organic functional groups which are not exploited by amino acids. The catalytic effects of the aldehyde-containing artificial proteases may be improved by inducing collaboration of the aldehyde groups with other organic functional groups. Synthesis of derivatives of Si(A)-G containing various other functional groups is in progress.

Acknowledgements

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14. A significant amount (30–40%) of amino groups of Si(A) remained unaltered upon treatment of Si(A) with acetic anhydride in the presence of triethylamine, most likely due to steric protection. It is expected that a considerable portion of the amino groups would not be sterically allowed to react with glutaraldehyde. Yet, the content of glutaraldehyde introduced to Si(A)-G was 180 mol% relative to the amino group. This is consistent with addition of the amino groups to the glutaraldehyde oligomers. Although Scheme 1 indicates addition of the amino groups to the enal groups of the glutaraldehyde oligomers, it is possible that the amino group of Si(A) and the aldehyde group of glutaraldehyde oligomers form an imine linkage, which resists hydrolysis due to the hydrophobic microenvironment. The fact that tryptamine is attached to Si(A)-G mostly by conjugate addition at the enal groups even in the presence of $NaBH(OAc)_3$, however, supports the addition of amino groups of Si(A) to the enal groups of the glutaraldehyde oligomers.
15. Among the aldehydes attached to silica, only those exposed to attack by *p*-toluenesulfonylhydrazide would form the corresponding hydrazone. Nevertheless, the test provides information on the relative amounts of silica-based aldehydes readily reacting with the hydrazide.
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17. Although a large amount of silica catalysts were used, only a small fraction (probably less than 1%) of aldehyde groups located on the silica would be accessible to protein substrates or peptide intermediates. Each protein molecule is cleaved into many fragments. Thus, it is not easy to compare the amounts of catalytic sites and substrates available under the experimental conditions.
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20. The bell-shaped pH profiles of k_0 illustrated in Figure 3 may be related to the participation of ionized and neutral forms of aldehyde hydrates. The pH profile, however, reflects ionization of functional groups of the protein substrate as well as the catalyst.