Fused Silica as Solid Support for Artificial Protease Exploiting Aldehyde and Histidine as Catalytic Groups

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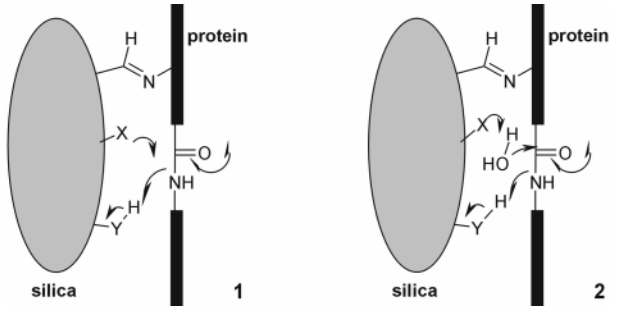
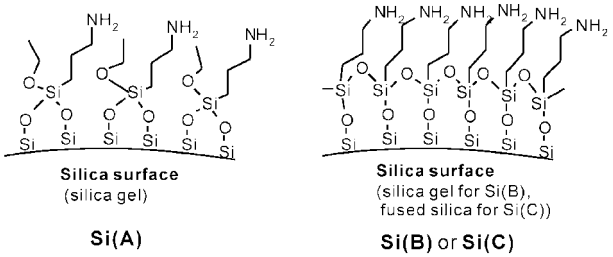
Received December 9, 2002

Key Words : Artificial enzyme, Fused silica, Proteolysis

Previously, we reported synthesis of effective artificial proteases by attaching aldehyde and other organic functional groups to silica gel.[1] As illustrated by **1**/**2**, the aldehyde group was proposed to act as a binding site by forming an imine with the amino group of the protein substrate. Polar groups X and Y-H can act as nucleophiles, general bases, or general acids. X and Y-H may include aldehyde hydrate, carboxylic acid, alcohol, thiol, phenol, and imidazole as well as their ionized forms. When the extra functional group attached to the aldehyde-containing silica gel was imidazole, the artificial protease hydrolyzed ovalbumin (Ovl), albumin (Alb), hemoglobin (Hgb), and γ-globulin (Gbn) with half-lives as short as 50 min at 25 °C or 7 min at 50 °C.

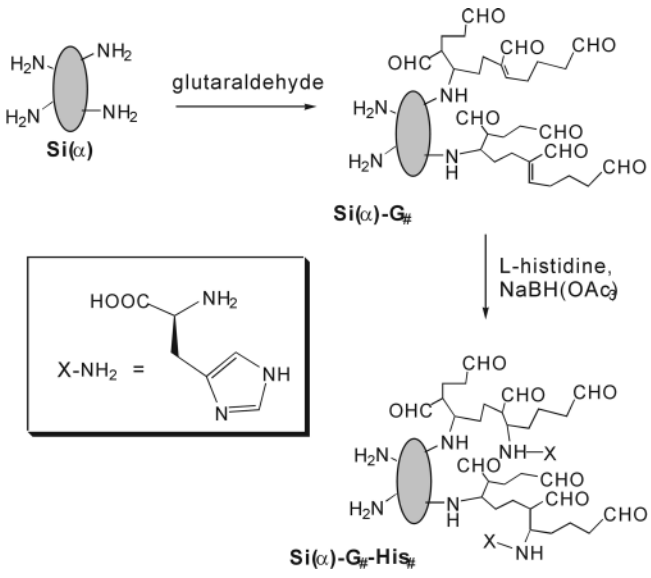


For the synthesis of the artificial proteases, the silica surface was covered with primary amino groups by reacting silica with γ-aminopropyltriethoxysilane (APS). It is reported that the reaction of APS with the silanol hydroxyl groups located on the surface of silica gel forms the silica derivative with structure indicated by Si(A) or Si(B) predominantly under anhydrous or hydrous conditions, respectively.[2,3] In the previous study, APS was attached to silica gel under anhydrous conditions to form Si(A).[1] The most effective catalyst obtained in the previous study contained both aldehyde groups and histidine moieties attached to the silica surface by the synthetic route illustrated in Scheme 1.[1] For example, catalyst Si(A)-G_{250}-His_{22}-G_{16}-His_{10} was synthesized by repeating the attachment of glutaraldehyde and histidine whereas catalyst Si(A)-G_{170}-His_{39}-G_{36}-His_3-Ac_{70} was prepared by acetylation of Si(A)-G_{170}-His_{39}-G_{36}-His_3. In the names of the catalysts prepared in the previous and the present studies, the subscript numbers (#) stand for the amount of glutaraldehyde (G) or histidine (His) in terms of mol % relative to the amino group of Si(A), Si(B), or Si(C).



Protein substrates have molecular weights usually exceeding 10 kDa. Most of the aldehyde groups or histidine moieties attached to silica gel would not be accessible to such large molecules. Only the functional groups positioned in open areas on the silica surface would act as catalytic groups in degradation of proteins and, possibly, even oligopeptides.

Fused silica is much cheaper (less than $1/kg) than silica gel. Unlike the functional groups attached to silica gel, most of the catalytic groups attached to fused silica would be accessible to large molecules. Thus, fused silica could be utilized as a more economical solid support for artificial proteases. In the present study, we attached glutaraldehyde and histidine to fused silica and compared the proteolytic activity of the resulting catalyst with those built on silica gel.



Scheme 1

Fused silica particles were treated with acid to release silanol hydroxyl groups on the surface.[4] The silica surface was subsequently covered with primary amino groups by reacting with APS in the presence of water to obtain Si(C). It was hoped that the content of amino groups can be raised by reacting silica with APS under hydrous conditions. Silica gel was also treated under hydrous conditions to obtain Si(B). The content of amino groups in Si(B) was estimated by elemental analysis. The contents of pendants attached to the surface of fused silica were too low to estimate by elemental analysis. The content of primary amino group in Si(C) was, therefore, estimated colorimetrically by Kaiser test using ninhydrin.[5] The contents of amines in Si(B) and Si(C) were 2.1 mmol/g and 0.17 μmol/g, respectively, whereas that in Si(A) prepared in the previous work was 1.3 mmol/g. Assuming that the population of silanol hydroxyl groups on silica surface is 4.55 residues/nm^2 as reported[6] in the literature and the fused silica particles are spherical with diameter of 0.1 mm and density of 2.21 g/mL, the amount of silanol hydroxyl group present on the surface of fused silica is estimated as 0.21 μmol/g. The surface of silica gel appears to be about 10^4-times greater than that of fused silica.

Attachment of glutaraldehyde and histidine to Si(B) and Si(C) was carried out according to Scheme 1 as reported previously.[1,7] Two new catalysts were prepared: Si(B)-G_{140}-His_{16} and Si(C)-G-His_{25}. The contents of glutaraldehyde and histidine in Si(B)-G_{140}-His_{16} were estimated by elemental analysis as reported previously.[1,7] Si(C)-G and Si(C)-G-His_{25} were treated with *m*-xylenediamine and $NaBH(OAc)_3$ in tetrahydrofuran for 12 h and the amount of primary amines introduced were estimated by Kaiser test. The content of His in Si(C)-G-His_{25} was calculated from the difference in the amount of the primary amines thus introduced, assuming that introduction of *m*-xylenediamine to Si(C)-G-His_{25} was blocked by the histidine residue.

Chicken egg Ovl (M.W. 44 kDa), bovine serum Alb (M.W. 66 kDa), bovine serum Gbn (M.W. 150 kDa), and bovine Hgb (M.W. 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.[7-12] The two chains of Gbn have distinctly different molecular weights (25 kDa 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 kDa and 16 kDa), and, therefore, disappearance of the combined band of the two subunits was followed. Examples of the electrophoretic results are illustrated in Figure 1.

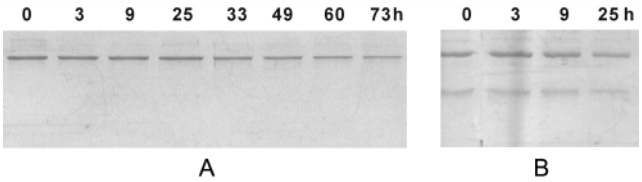


Figure 1. Results of SDS-PAGE obtained with Alb (a) or the heavy (upper band) and light (lower band) chains of Gbn (b) incubated with Si(C)-G-His_{25} at 50 °C and pH 6.0.

Pseudo-first-order kinetic behavior was observed for degradation of protein substrates in the presence of the artificial proteases under the conditions indicated in Table 1. The pseudo-first-order rate constants (k_o) 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 as described previously.[1,7] The kinetic data measured for degradation of protein substrates by the catalysts are summarized in Table 1.

The fused silica-based catalyst, Si(C)-G-His_{25}, is closely related to the silica gel-based catalyst, Si(B)-G_{140}-His_{16}, as both of them were obtained by treatment with APS under hydrous conditions and by introduction of histidine in one step. The catalytic activity of Si(C)-G-His_{25} toward the four protein substrates is about 7-50% of that of Si(B)-G_{140}-His_{16}. This difference stands in marked contrast with the about 10^4-fold difference in the content of amino, aldehyde, and histidyl groups of the two catalysts. Fused silica is much cheaper than silica gel. About 10^4-times greater amounts of APS, glutaraldehyde and histidine are needed for the synthesis of Si(B)-G_{140}-His_{16} compared with that of Si(C)-G-His_{25}. In spite of the large difference in the cost and materials needed to synthesize the catalysts, only minor difference is observed for their proteolytic activities.

Results of the present study indicate that fused silica can be used as an economical solid support for immobile catalysts using macromolecules as reactants or catalysts. Proteins are used as substrates in the present study, but many enzymes are linked to solid supports to obtain immobilized enzymes. Fused silica can be also exploited as an economical solid support for immobilized enzymes.

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

Catalyst[b]	Ovl	Alb	Hgb	Gbn
Si(A)-G_{250}-His_{22}-G_{16}-His_{10}[b]	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}[b]	0.76(6)*	0.81(7)	1.2(8)*	0.73(8)/0.52(8)
Si(B)-G_{140}-His_{16}	0.97(5)	1.3(7)	3.3(7)	1.2(7)/1.4(8)
Si(C)-G-His_{25}	0.51(6)	0.21(7)	0.22(8)	0.11(7)/0.13(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_o 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_o were 10-25%. For Gbn, k_o values for the heavy and the light chains are indicated in sequence with/put between them. Possibility that disappearance of the parent protein was due to adsorption onto the silica was excluded by the method[1] described previously. [b]Taken from ref. 1.

Experimental Section

Catalysts. Fused silica (particle size: 0.044-0.149 mm, density: 2.21 g/mL), purchased from Boram Chemical Co., was treated with HCl and H_2SO_4 according to the literature.[4] The acid-treated fused silica was treated with APS under hydrous conditions to obtain Si(C), according to the method reported in the literature.[2,3] Si(C)-G-His_{25} was obtained by shaking Si(C) (50 g) in a buffer (pH 7.5, 10 mM phosphate; 250 mL) containing 5%(v/v) glutaraldehyde for 2 h at the shaking speed of 200 rpm followed by shaking the resulting Si(C)-G (15 g) with L-His (12 mmol) and $NaBH(OAc)_3$ (25 μmol) dissolved in the Mes buffer (100 mL) for 2 d at 25 °C. Silica gel was treated with APS under hydrous conditions to obtain Si(B), which was modified to obtain Si(B)-G_{140}-His_{16} according to the procedure reported previously.[1]

Measurements. Ovl, Alb, Gbn, and Hgb were purchased from Sigma. For kinetic measurements, the reaction mixtures were shaken in a heated shaking incubator (a VorTemp 56 model). Pseudo-first-order rate constants were calculated from the intensities of electrophoretic bands of the protein substrate measured at various time intervals. Intensities of electrophoretic bands were estimated with an AlphaImager™ IS-1220 model.

Acknowledgment. This work was supported by Center for Molecular Catalysis and Artzyme Biotech Corporation. M.-s. K., H. K., H. P., and Y.-S. C. were recipients of BK21 fellowship.

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