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Analogues of Aspartic Proteases Synthesized by Densely Covering Silica Gel with Carboxyl Groups

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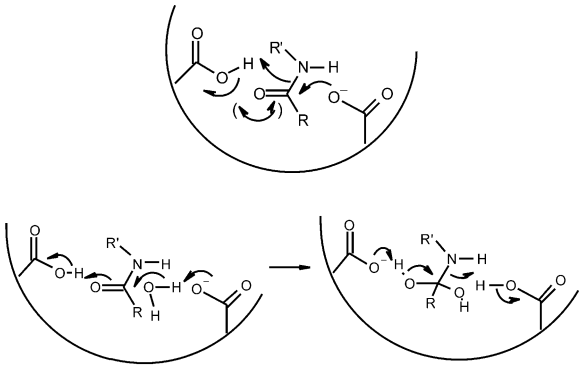
Abstract—Aspartic protease analogues synthesized by covering the surface of silica gel with carboxyl groups effectively hydrolyzed hemoglobin and γ-globulin. It is proposed that the carboxyl group is involved in both complexation of the protein substrate and the catalytic cleavage of the peptide bonds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

Aspartic proteases such as pepsin, renin, penicillopepsin, and HIV protease hydrolyze proteins by using two aspartic groups as the key catalytic groups. In the mechanisms widely proposed for the aspartic proteases, one carboxyl group acts as a general acid and the ionized form of the other carboxyl group as a nucleophile or a general base (Scheme 1).[1–3]

Attempts have been made to synthesize catalytic systems for amide hydrolysis by using carboxyl groups as catalytic groups. Some successful results were obtained with carboxyl groups tethered to amide bonds.[4,5] The intramolecular catalytic systems are mimicries of enzyme–substrate complexes rather than enzymes.

The first synthetic organic artificial protease was designed by creating artificial active sites comprising three proximal salicylates on the backbone of branched polyethylenimine (PEI). As summarized in Scheme 2, three molecules of 5-bromoacetylsalicylate (BAS) were complexed to Fe(III) ion, and the resulting complex ($FeBAS_3$) was cross-linked with PEI.[6] The polymer [apo(Sal_3)PEI] obtained after removal of Fe(III) ion manifested high reactivity in the hydrolytic cleavage of γ-globulin.[7] Since PEI is water-soluble, apo(Sal_3)PEI is an homogeneous artificial protease. The active site of apo(Sal_3)PEI contained three carboxyl and three phenol groups in addition to the amino groups of PEI backbone. Catalyst apo(Sal_3)PEI manifested optimum activity at pH 6. Thus, it is possible that peptide hydrolysis by apo(Sal_3)PEI involves catalytic participation of phenol and/or amino groups as well as carboxyl groups.

In search of artificial proteases that hydrolyze proteins by using carboxyl groups as the sole catalytic groups, we have built the trisalicylate (Sal_3) sites on the backbones of other synthetic polymers. An effective artificial aspartic protease was obtained by cross-linking $FeBAS_3$ with poly(aminomethylstyrene-co-divinylbenzene) (PAD).[8] PAD is a cross-linked polystyrene in which the styryl residue contains aminomethyl group. The immobile artificial protease effectively degraded albumin by the catalytic action of salicylic acids contained in the active



Scheme 1. Mechanisms proposed for aspartic proteases.

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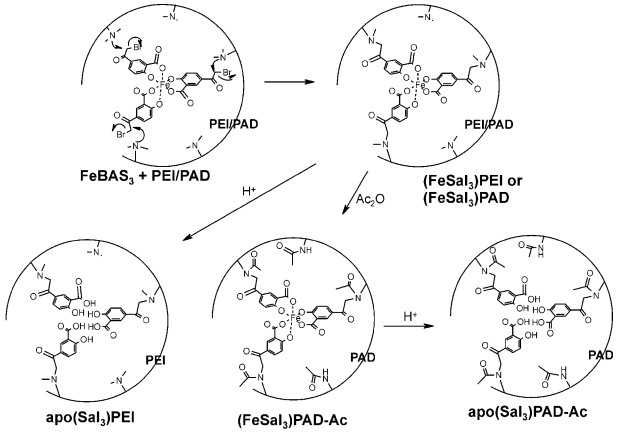
site. Artificial protease apo(Sal_3)PAD-Ac manifested optimum activity at pH 3. The proteolytic activity of apo(Sal_3)PAD-Ac at pH 3 was attributed to cooperation of two or more carboxyl groups by a mechanism analogous to the mechanisms of Scheme 1.

In view of effective proteolytic activity of the three carboxyl groups contained in the active site of apo(Sal_3)-PAD-Ac, we attempted to synthesize aspartic protease analogues by simply covering the surface of a branched polymer with carboxyl groups. If the polymer surface is densely covered with carboxyl groups, some proximal carboxyl pairs may have geometry suitable for degradation of the complexed protein. In addition, some carboxylate anions may act as binding sites for protein substrates by forming ion pairs with the ammonium or guanidinium ions of the proteins.

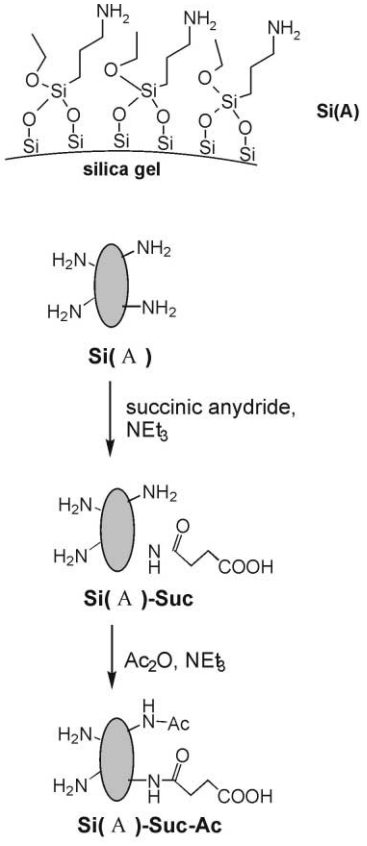
The most obvious candidate for the aspartic protease analogue based on insoluble resin covered with carboxyl group was Amberlite weakly acidic cation exchanger. In the Amberlite resin, the matrix is polystyrene and the active group is carboxyl group. When the Amberlite resin (purchased from Sigma) was tested for its proteolytic activity by using bovine serum albumin or bovine serum γ-globulin as the substrate, the proteins were adsorbed[9] strongly onto the resin. Apparently, multiple interaction between the carboxylate anions of the resin and the ammonium and/or guanidinium ions of the proteins led to the strong adsorption.

Adsorption of protein onto the solid support would depend on the structure and the microenvironment of the solid surface. It would be possible, therefore, to suppress the unproductive adsorption by changing the solid support. Subsequently, we tested silica gel as the solid support. 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] 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 3 predominantly under anhydrous conditions.[11,12] Elemental analysis indicated that the content of amino groups in Si(A) synthesized in the present study was 1.3 mmol/g.

The amino groups of Si(A) were succinylated and acetylated to obtain Si(A)-Suc and Si(A)-Suc-Ac as illustrated in Scheme 3. Si(A)-Suc was prepared by shaking (shaking speed at 200 rpm) Si(A) (40 g), succinic anhydride (25 g, 250 mmol), and triethylamine (68 mL, 490 mmol) in 250 mL acetonitrile at 25 °C for 40 h, followed by thorough washing with water, methanol, and methylene chloride. The amount of succinyl group incorporated to Si(A)-Suc was estimated as 34 mol% relative to the amino groups of Si(A) on the basis of elemental analysis. Si(A)-Suc (10 g) was acetylated by shaking with acetic anhydride (13 mL, 93 mmol) and triethylamine (9.0 mL, 93 mmol) in 100 mL methylene chloride at 25 °C and at the shaking speed of 200 rpm for 24 h, followed by thorough washing with water, methanol, and methylene chloride. The content of acetyl group in Si(A)-Suc-Ac was 23 mol% relative to the amino groups of Si(A) as estimated by elemental analysis. It is expected that essentially all of the amino groups of Si(A) accessible to succinic anhydride are succinylated in the preparation of Si(A)-Suc. Similarly, most of the amino groups of Si(A)-Suc sterically allowed to react with acetic anhydride should be acetylated in Si(A)-Suc-Ac. Thus, only the amino groups sterically protected from the acylating reagents would remain unaltered in Si(A)-Suc and Si(A)-Suc-Ac.



Scheme 2. Synthesis of apo(Sal_3)PEI and apo(Sal_3)PAD-Ac.



Scheme 3. Synthesis of Si(A)-Suc and Si(A)-Suc-Ac.

Chicken egg ovalbumin (M_r 44 kDa), bovine serum albumin (M_r 66 kDa), bovine serum γ-globulin (M_r 150 kDa), and bovine hemoglobin (M_r 62 kDa) were tested as the 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.[7,8,10] Intensities of electrophoretic bands were estimated with an AlphaImager™ IS-1220 model. 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 at 50 °C. The two chains of γ-globulin 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 hemoglobin have similar molecular weights (15 and 16 kDa), and, therefore, disappearance of the combined band of the two subunits was followed for hemoglobin. Possibility that disappearance of the parent protein was due to adsorption onto the silica 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.

Pseudo-first-order kinetic behavior was observed for degradation of protein substrates in the presence of the artificial proteases. 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 by using a heated shaking incubator (a Vor-Temp 56 model). Kinetic data were not collected under acidic conditions (pH ≤ 3) due to instability of silica gel under acidic conditions as well as possible intramolecular catalysis[4,5] of hydrolysis of succinyl monoamides of the catalysts.

The k_o values measured for degradation of γ-globulin by the catalysts at several pHs are summarized in Table 1 and Figure 1. The k_o values measured at the optimum pHs correspond to half-lives ($=0.69/k_o$) of 6.6 and 6.2 h for the degradation of the heavy and the light chains, respectively, of γ-globulin by Si(A)-Suc and 6.1 and 2.2 h for the degradation of the heavy and the light chains, respectively, of γ-globulin by Si(A)-Suc-Ac. When pH was raised to 8, the catalytic activity diminished greatly.

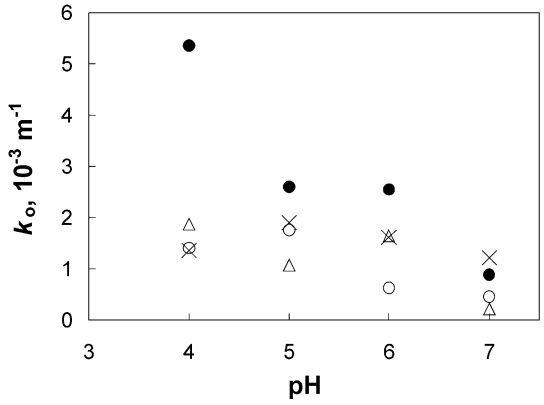


Figure 1. The pH dependence of k_o for the degradation of the heavy (○) and light (△) chains of γ-globulin by Si(A)-Suc or that of the heavy (X) and light (●) chains of γ-globulin by Si(A)-Suc-Ac.

When ovalbumin or albumin was used as the substrate, the kinetic measurement was hampered at pH ≤ 4 or 5 due to protein adsorption.[13] Optimum activity was manifested at pH 7 with half-lives of 33 and 43 h for the degradation of ovalbumin by Si(A)-Suc and Si(A)-Suc-Ac, respectively, or 17 and 44 h for the degradation of albumin by Si(A)-Suc and Si(A)-Suc-Ac, respectively, under the conditions indicated above.

When hemoglobin was used as the substrate, the kinetic measurement was hampered even at pH = 7 due to protein adsorption.[13] Optimum activity was manifested at pH 8 with half-lives of 0.50 and 1.1 h for the degradation of hemoglobin by Si(A)-Suc and Si(A)-Suc-Ac, respectively, under the conditions indicated above.[14]

Kinetic data for the protein degradation cannot be obtained when either the protein substrate or the hydrolysates are adsorbed onto the catalyst. It is difficult to differentiate whether the protein substrate is adsorbed without degradation or the protein is hydrolyzed before the hydrolysates are adsorbed. Protein adsorption was most serious for hemoglobin and/or its hydrolysates whereas γ-globulin and its hydrolysates were not significantly adsorbed at the pH range examined. The adsorption of the proteins onto Si(A)-Suc and Si(A)-Suc-Ac may involve multiple interaction between the carboxylate anions of the silica gel and the ammonium and/or guanidinium cations of the protein. The degree of protein adsorption would depend on the amount of ammonium and/or guanidinium cations as well as carboxyl anions on the protein surface and the shape of the protein.[15]

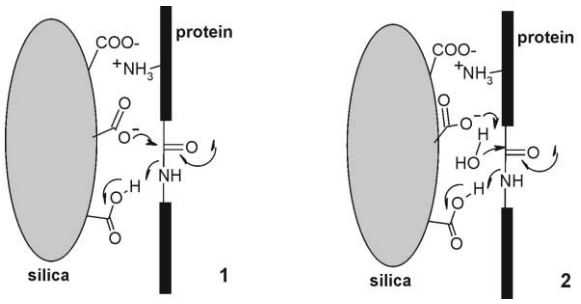
Although the pK_a values carboxyl groups (4–5 in water) may be altered somewhat on attachment to silica gel, the pH profiles of Figure 1 suggest that optimum activity is manifested by the silica derivatives at pH 3–5 by analogy with pepsin, a typical aspartic protease. Thus, the pH profile illustrated in Figure 1 is consistent with catalytic participation of both carboxyl group and carboxylate anion of the catalyst. Since the carboxylate ion of the catalyst can be exploited in binding of the substrate protein, the catalytic action of Si(A)-Suc and

Table 1. Pseudo-first-order rate constants (10^{-3} m^{-1})[a] for degradation of two chains of γ-globulin by silica derivatives measured at 50 °C[b]

pH	Si(A)-Suc		Si(A)-Suc-Ac	
	Heavy chain	Light chain	Heavy chain	Light chain
4.0	1.4	1.9	1.4	5.4
5.0	1.7	1.1	1.9	2.6
6.0	0.6	1.6	1.6	2.5
7.0	0.4	0.2	1.1	0.9

[a]Relative standard deviation: 10–30%.
[b]See the text for conditions of kinetic measurements.

Si(A)-Suc-Ac can be attributed to the mechanism of **1** or **2** by analogy with the mechanism of aspartic proteases. Here, the carboxylate ion of the silica may also interact with the guanidinium cation of the protein.[16]



Although protein adsorption was serious for hemoglobin and/or its hydrolysates, the fastest rate for protein degradation by Si(A)-Suc or Si(A)-Suc-Ac was achieved with hemoglobin. Half-life of 30 min at pH 8 and 50 °C measured for degradation of hemoglobin by Si(A)-Suc may be compared with the fastest rate observed for apo(Sal_3)PEI (half-life of 60 min at pH 6 and 50 °C in degradation of the light chain of γ-globulin) or apo(Sal_3)PAD-Ac (half-life of 4 h at pH 3 and 50 °C in degradation of albumin). An example of organic artificial protease produced by a living body is the catalytic antibody. The catalytic antibody elicited by a joint hybridoma and combinatorial antibody library approach[17] is the one with the highest peptidase activity reported to date. The fastest rate achieved by the catalytic antibody in the hydrolysis of an amide corresponded to the half-life of 3.8 h at 25 °C.

The organic proteases [apo(Sal_3)PEI and apo(Sal_3)-PAD-Ac] having artificial active sites comprising three salicylates manifested very narrow substrate specificity. They effectively hydrolyzed only either albumin or γ-globulin when both of the proteins were tested as substrates. The substrate specificity is not improved considerably with Si(A)-Suc or Si(A)-Suc-Ac. The silica-based aspartic protease analogues are prepared by random modification of the solid support whereas the Sal_3-containing active sites were deliberately constructed. Yet, the catalytic activity of the silica-based catalysts is comparable to or better than the $(Sal)_3$-containing artificial proteases.

Acknowledgements

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9. After the electrophoretic band for the protein disappeared, the amino acid content[10] of the buffer solution was measured.
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13. At pH 4–7, a significant portion of carboxyl groups of Si(A)-Suc and Si(A)-Suc-Ac would be present as carboxylate anions, and amino groups of the proteins are to be mostly protonated. Thus, protein adsorption would be significant at weakly acid pHs, although the degree of adsorption depends on the nature of proteins.
14. In the product solution separated from the resin after electrophoretic bands of hemoglobin disappeared completely, more than 50–80% of amino acids of the protein were recovered.[9,10] This indicates that the rate data mostly represent the protein degradation.
15. The adsorption of the proteins to Si(A)-Suc or Si(A)-Suc-Ac is not correlated with pI (isoelectric point) values (4.5 for ovalbumin, 4.7 for albumin, 6.8 for hemoglobin, and 7.2 for γ-globulin).
16. As mentioned above, some amino groups of Si(A) are inaccessible to succinic anhydride or acetic anhydride most likely due to steric strain. Interaction of such amino groups of silica gel with bulky molecules such as protein substrates or hydrolysates would be insignificant.
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