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An Aspartic Protease Analogue: Intermolecular Catalysis of Peptide Hydrolysis by Carboxyl Groups

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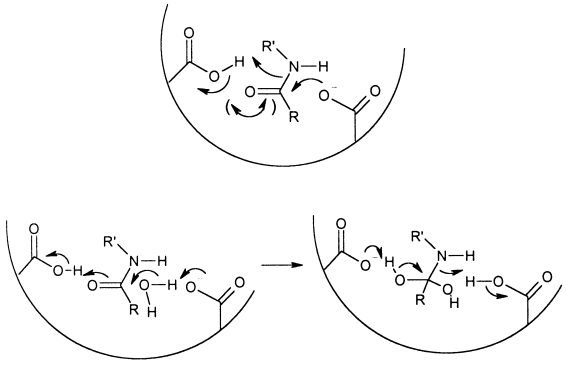
Abstract—Two aspartic carboxyl groups act as key catalytic groups in the active site of an aspartic protease. We synthesized an aspartic protease analogue by positioning three salicylate residues in close proximity on a cross-linked polystyrene. The immobile artificial protease effectively hydrolyzed albumin into many small fragments by the catalytic action of carboxyl groups contained in the active site. The artificial protease manifested optimum activity at pH 3 just as aspartic proteases.

An aspartic protease such as pepsin, penicillopepsin, renin, or HIV protease exploits two aspartic carboxyl groups as key catalytic groups in hydrolyzing peptide substrates. To explain that aspartic proteases are most active at pH 1.9–4.0, it is generally accepted that one of the carboxyl groups is in the acid form and the other is in the ionized form at the optimum pH. In the mechanism 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] Intramolecular catalysis of peptide hydrolysis by carboxyl groups tethered to the peptide substrates has been intensively investigated.[4,5] To obtain a catalyst, however, the catalytic part should be separated from the substrate.

Previously, we synthesized an organic artificial protease whose active site contained carboxyl groups.[6,7] 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 branched poly(ethylenimine) (PEI) to obtain ($FeSal_3$)-PEI. 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 a 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 in contrast to aspartic proteases. Thus, it is likely 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 mimic an aspartic protease, we have built the trisalicylate sites on the backbones of other synthetic polymers. An effective artificial aspartic protease has been obtained in the present study by cross-linking $FeBAS_3$ with poly(aminomethylstyrene-co-divinylbenzene) (PAD). PAD is a cross-linked polystyrene in which the styryl residue contains aminomethyl group. Thus, PAD produces insoluble catalysts which are often more useful



Scheme 1. Mechanisms proposed for aspartic proteases.

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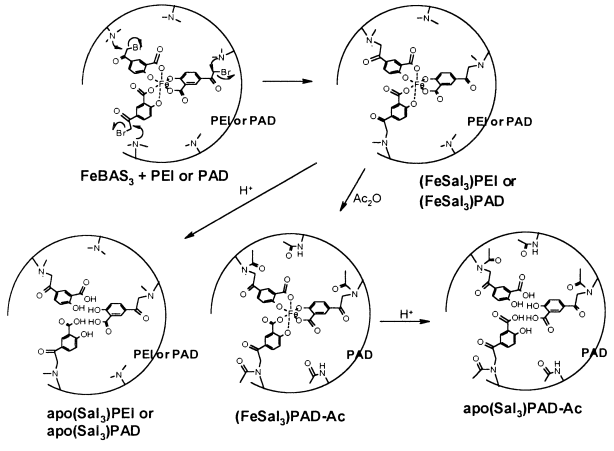
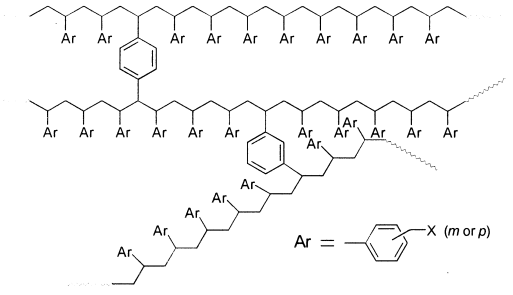
than soluble catalysts in practical applications. Here, we report an insoluble artificial protease that reproduces major catalytic features of aspartic proteases.

By shaking poly(chlroromethylstyrene-co-divinylbenzene) (PCD)[8] with 2.4 equiv NaN_3 in DMF for 3 days at 65 °C, the chloro groups of PCD were substituted with azido groups, which were subsequently reduced[9,10] by shaking with 2.1 equiv $SnCl_2$ and a catalytic amount of $AlCl_3$ in DMF at 65 °C for 3 days leading to the formation of PAD. IR spectra indicated complete disappearance of C–Cl peak (1264 cm^{-1}) upon treatment with NaN_3 and azido peak (2092 cm^{-1}) upon reduction. Elemental analysis (C/N ratio) indicated that the content of amino groups in PAD is 85 residue mol%. It is possible that some of the chloro groups of PCD had been replaced by water during suspension polymerization[11] in water.

$FeBAS_3$ was generated and cross-linked with PAD according to the general procedure reported previously[6] leading to $(FeSal_3)$PAD, which was subsequently acetylated in dimethyl sulfoxide with 10 equiv acetic anhydride and 10 equiv triethylamine to prepare $(FeSal_3)$PAD-Ac (Scheme 2). Complete acetylation of amino groups was confirmed by Kaiser's test[12] for primary amines using ninhydrin. Fe(III) ion was removed from $(FeSal_3)$PAD or $(FeSal_3)$PAD-Ac by shaking the resin in 1 N HCl at 25 °C for 24 h four times and was quantified by ICP analysis. The yield for attachment of $FeBAS_3$ to PAD was almost quantitative, as estimated from the amount of Fe(III) released from $(FeSal_3)$PAD. Based on the amount of Fe(III) ion, the content of $Fe(Sal)_3$ was quantified as 1.0 residue mol%. Acetamido groups of the acetylated PAD were not hydrolyzed by the treatment with HCl as checked by the Kaiser's test.





PCD: X = Cl
PAD: X = NH_2

Scheme 2. Synthetic routes to apo(Sal_3)PEI and apo(Sal_3)PAD-Ac.

Formation constant (K_f) of the Fe(III) complex in $(FeSal_3)$PAD was measured by using EDTA as the competing chelating reagent according to the literature method.[8,13] The log K_f value for $(FeSal_3)$PAD was estimated as 24.08 ± 0.06 at 25 °C and pH 7.50 whereas Fe(III) binding by PAD was negligible. The log K_f for $(FeSal_3)$PAD is considerably larger than that (log $K_f = 17.70$ at pH 7.50)[7] for $(FeSal_3)$PEI. This is attributable to lower polarity of the microenvironment on polystyrene compared with PEI, which facilitates complexation between the anionic ligand and the cationic metal ion.

$(Sal)^{ran}$PAD-Ac, in which salicylates are attached randomly, was prepared by using excess Fe(III) (3 equiv of BAS) when BAS was attached to PAD. Assembly of two or three BAS moieties by Fe(III) was suppressed by adding excess Fe(III). Content of salicylate residue in $(Sal)^{ran}$PAD-Ac was 3.0 residue mol% as measured by the amount of Fe(III) released on treatment with HCl.

Activity of apo(Sal_3)PAD-Ac was tested in the hydrolysis of bovine serum albumin (M_r 66,000).[14] The PAD derivatives were swollen in the buffer solution at room temperature for 12 h prior to kinetic measurements. When the buffer solution containing albumin was stirred with the resin, disappearance of albumin was observed by SDS-PAGE[15] gel electrophoresis (Fig. 1).[16] Pseudo-first-order rate constant (k_0) for cleavage of albumin was calculated from the intensity of the electrophoretic bands as described previously.[7,8] The value of k_0 increased considerably as the stirring speed was raised up to 1000 rpm and reached the plateau value at 1000–1200 rpm as checked with the hydrolysis of albumin ($S_0 = 1.5 \times 10^{-6}$ M) catalyzed by apo(Sal_3)PAD-Ac ($C_0 = 0.5$ mM: initially added concentration of the catalyst defined as the concentration of Sal_3 moiety attainable when the resin is assumed to be dissolved) at pH 3.00 and 50 °C. Kinetic data were, therefore, collected at the stirring speed of 1200 rpm. To check the effect of ionic strength on the catalytic activity, kinetic data were collected with 0.05, 0.1, or 0.2 M phosphoric acid at pH 3.00. Since the k_0 value was not affected considerably by the changes in buffer concentration, kinetic data were collected with 0.05 M buffer.

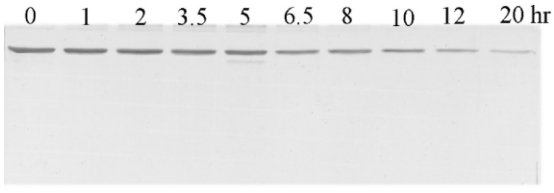


Figure 1. Results of SDS-PAGE gel electrophoresis performed on albumin ($S_0 = 1.5 \times 10^{-6}$ M) incubated with apo(Sal_3)PAD-Ac ($C_0 = 0.12$ mM) at pH 3.00 and 50 °C.

The electrophoresis indicated that no intermediate proteins accumulated in amounts detectable by the electrophoresis during the cleavage of albumin by the resin. The MALDI-TOF-MS spectrum of a product solution obtained by cleavage of albumin with apo(Sal_3)PAD-Ac revealed that albumin was cleaved into fragments smaller than 2 kDa. HPLC analysis of a product solution with and without treatment with phenyl isothiocyanate indicated that amino groups of the product peptides were modified by phenyl isothiocyanate, confirming[7] the hydrolytic nature of the protein cleavage by the resin (Fig. 2).

The pH profile of k_0 for the hydrolysis of albumin by apo(Sal_3)PAD-Ac is illustrated in Figure 3. At pH 3.00, k_0 was proportional to C_0 when measured up to $C_0 = 0.5$ mM. The kinetic data obtained with the hydrolysis of albumin by (Sal)^{ran}PAD-Ac are also included in Figure 3.

For catalytic reactions proceeding through complex formation between the substrate and the artificial active sites built on synthetic polymers, kinetic data are analyzed in terms of the Michaelis–Menten scheme.[7,8,17,18] Under the conditions of $C_0 \approx [C] \gg [CS]$, pseudo-first-order kinetic behavior is predicted with k_0 being equal to $k_{cat}C_0/(C_0 + K_m)$. When $K_m \gg C_0$, k_0 is proportional to C_0 and k_0/C_0 corresponds to k_{cat}/K_m. The pH profile of Figure 3, therefore, represents pH effects on k_{cat}/K_m. Artificial protease apo(Sal_3)PAD-Ac manifests optimum activity at pH 3. This agrees with the optimum pH for aspartic proteases.

Participation of two or more salicylate residues[19] in the catalytic action of apo(Sal_3)PAD-Ac is evident from the very low catalytic activity of (Sal)^{ran}PAD-Ac (Fig. 3) in which the salicylate residues are mostly separated from one another.[20] The active site of apo(Sal_3)PAD-Ac contains carboxyl and phenol groups. At pH 3, phenol may act as a general acid but its activity should be much lower than that of carboxyl group. Moreover, optimum activity would be manifested at pH 6–8 if the peptide hydrolysis is achieved by collaboration between a carboxylate ion and a phenol group. Thus, the proteolytic activity of apo(Sal_3)PAD-Ac at pH 3 is attributable to cooperation of two or more carboxyl groups by a mechanism analogous to the mechanisms of Scheme 1.

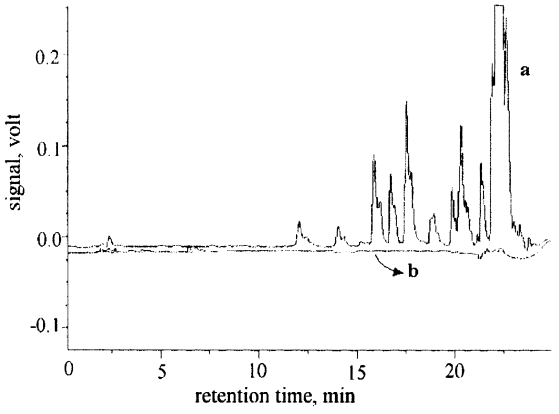


Figure 2. Results of HPLC analysis for product solution of Figure 1 after treatment with phenyl isothiocyanate (a). Line b represents the signal observed without treatment with phenyl isothiocyanate. Typical conditions employed by the Waters PicoTag System were used for the elution.

An effective analogue of aspartic proteases is synthesized in the present study. Important catalytic features of aspartic proteases reproduced by the synthetic analogue are: facile hydrolytic cleavage of a protein substrate, catalytic cooperation by multiple carboxyl groups positioned in proximity, and optimum activity at pH 3.

Half-life for spontaneous hydrolysis of peptides at pH 7 and 25 °C has been measured as 500–1000 years.[21–23] Recently, several types of artificial enzymes with peptidase-like activities have been reported. The highest catalytic activity (up to 10^9-fold acceleration) reported so far for amide hydrolysis with artificial peptidases has been achieved with artificial metallopeptidases built with coordinatively polymerized synthetic bilayer membranes[24] or Cu(II) complex of cyclen[8] attached to a PCD derivative. These synthetic metallopeptidases hydrolyzed proteins with half-lives of 1–10 min at 4 °C and pH 5–7.

An example of organic artificial proteinase is the catalytic antibody elicited by a joint hybridoma and combinatorial antibody library approach.[25] This is the catalytic antibody with the highest peptidase activity reported to date, with k_{cat} of 0.18 h^{-1} at pH 9, the optimum pH, and 25 °C. For apo($Sal)_3$PAD-Ac, k_{cat} is much greater than 0.17 h^{-1} at pH 3, the optimum pH, and 50 °C.[26] This indicates that the immobile artificial aspartic protease prepared in the present study has reasonably high catalytic activity.

Recently, we have designed another organic artificial protease on the backbone of polystyrene.[27] This protease achieved effective protein hydrolysis by collaboration of

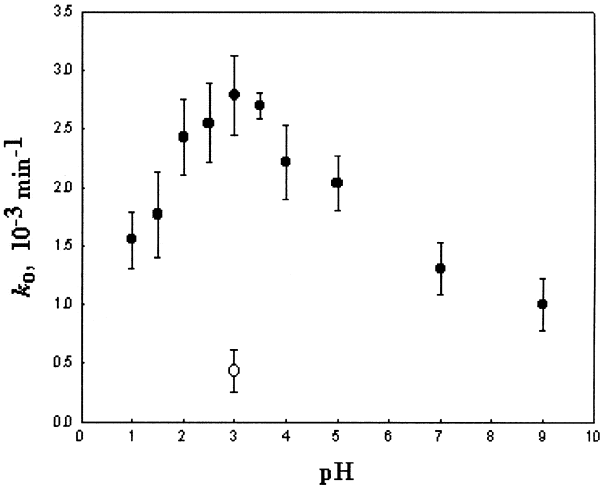


Figure 3. pH profile of k_0 for hydrolysis of albumin ($S_0 = 1.5 \times 10^{-6}$ M) catalyzed by apo(Sal_3)PAD-Ac (●) ($C_0 = 0.5$ mM) or by (Sal)^{ran} PAD-Ac (○) ($C_0 = 0.5$ mM; defined as one third of the concentration of salicylate residue attainable when the resin is assumed to be dissolved) at 50 °C. Buffers (0.05 M) used for the kinetic measurements were HCl (pH 1–2), phosphoric acid (pH 3), acetic acid (pH 4–5), 4-morpholineethanesulfonic acid (pH 6), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7–8), and boric acid (pH 9).

imidazoles attached to the backbone. In the present study, facile protein hydrolysis is achieved by collaboration of carboxyl groups attached to the backbone. It would be possible to synthesize artificial proteases with catalytic rates comparable to natural enzymes by positioning several organic functional groups in close proximity.

Acknowledgements

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26. For Michaelis–Menten kinetics, k_{cat} represents the maximal rate constant achievable in the presence of excess catalyst ($C_0 \gg K_m$). In the present study, $C_0 \ll K_m$ and, thus, $k_{cat} \gg k_0$. The value of k_{cat} for the hydrolysis of albumin catalyzed by apo(Sal_3)PAD-Ac is, therefore, $\gg 0.17\ h^{-1}$ at pH 3.00 and 50 °C.
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