

SH-Group Introduction to the *N*-terminal of Subtilisin and Preparation of Immobilized and Dimeric Enzymes

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An SH group was introduced at the *N*-terminal of subtilisin Carlsberg by reacting with 2-iminothiolane·HCl (Traut reagent) in weakly basic media. The obtained subtilisin-SH was coupled with maleimide connected to an amine polymer (poly(allylamine); PAA), both in soluble and insoluble forms, or connected to the *N*-terminal of another subtilisin molecule. The immobilized subtilisins and subtilisin dimer thus obtained showed higher specificity towards proteineous substrates, although they had lower activities towards a tetrapeptide substrate. Subtilisins immobilized (conjugated) to PAA were more stable against autolytic and thermal disactivation than the intact enzyme.

The chemical modification of proteins has a long history of research, and remains a powerful and versatile tool to introduce non-natural properties into proteins and enzymes,[1–3] even though molecular-biological or genetic technology for manipulating proteins has already attained numerous successful results.[4,5] The versatility of the chemical modification method is its major advantage; however, it also has some difficulties. One problem is the spatial or structural focusing of the modification site. We have used several physical or chemical perturbations to solve this problem and have attained some good results,[6–10] but they are not sufficient yet to pinpoint the modified site.

The introduction of a site-directed agent, such as a substrate analog or a specific inhibitor carrying a chemically active group is one way of solving this problem. However, this method is usually limited to the modification of an active site or substrate binding site. Such a change would result in a total loss in or severe damage to the enzyme activity. The combination of site-directed mutagenesis plus the chemical modification of introduced functional amino acid residues is another powerful method.[11–13]

Another possible method is to mask, chemically or physicochemically, the modifiable sites. As a very simple but relevant example, we utilized the pK_a difference of the α- and ε-amino groups; the former is usually more than one unit smaller than the latter. Thus, in weakly basic media, unprotonated α-amino groups are in 10-fold excess to the ε-amino groups, and we can almost selectively modify the former by carefully choosing the reaction conditions such as pH, reagent concentration, and reaction time.

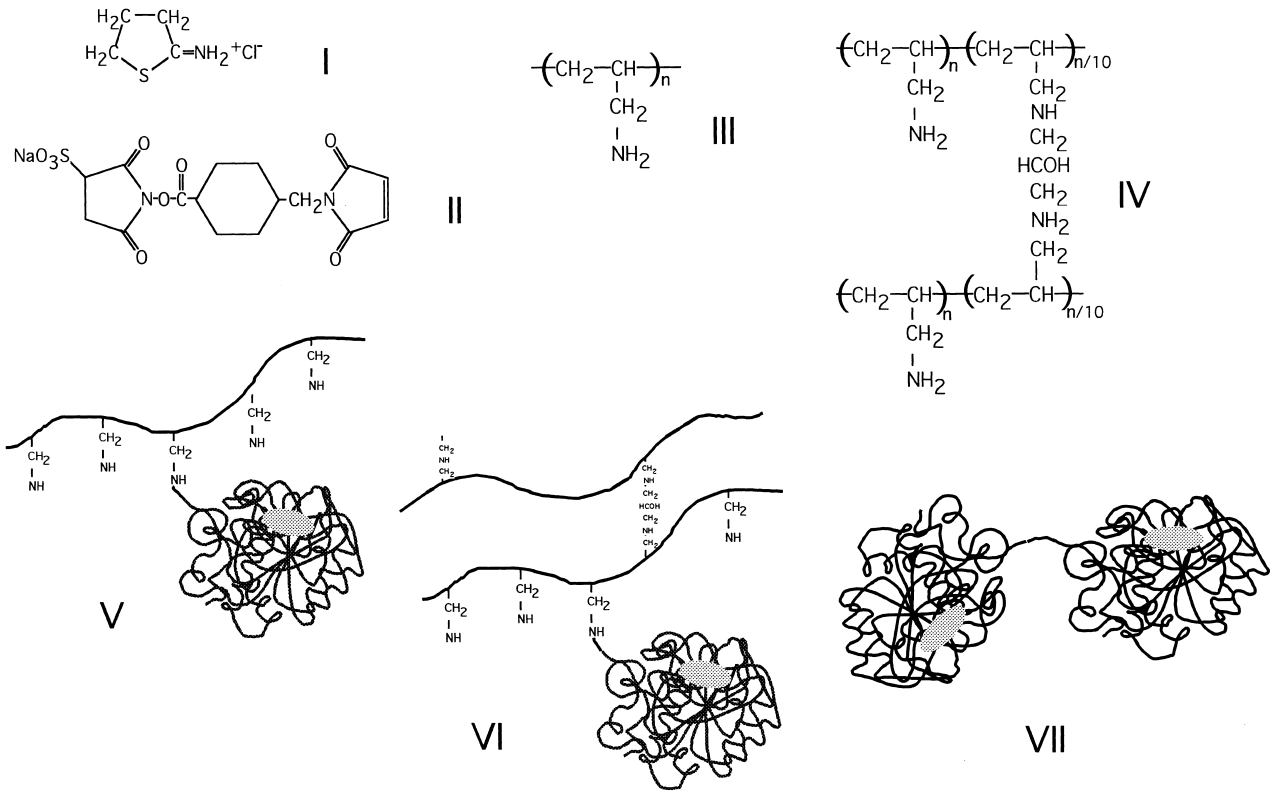
As for introducing a functional group, as a first trial we chose an SH group in combination with a non-SH protein. An SH-specific reagent can further couple this SH-protein with other polymers. Subtilisin (Carlsberg) was selected as the target protein. The characteristics of this microbial and SH-free enzyme have been well documented,[14,15] and it is one of the largest commercially consumed enzymes. The introduction of SH-group(s) into this enzyme has been studied by both chemical and genetic procedures.[16–18] Here, the *N*-terminal of subtilisin was modified by an amine-specific SH-reagent to obtain subtilisin-SH (Sub-SH).

An SH-specific reactive group (i.e. maleimide) can be introduced to the side chains of amine polymers or the α-amino group of another subtilisin molecule using an amine-specific/SH-specific hetero-bifunctional linker. As for the amine-specific SH-reagent and the amine-specific/SH-specific hetero-bifunctional linker, we used 2-iminothiolane (Traut reagent; **I**) and sodium sulfonatosuccinimido 4-(maleimidomethyl)cyclohexane-1-carboxylate (Sulfo-SMCC; **II**), respectively (Scheme 1). Poly(allylamine) (PAA; **III**) and its cross-linked microbeads (PAA-beads; **IV**) were used as amine polymers for chemical modification and chemical (covalent) immobilization of an enzyme on a soluble and insoluble support. Enzymes immobilized on insoluble[19] or soluble[20–25] supports have been studied extensively, but most studies used random modification and obtained randomly-oriented enzymes on (in) the support. The possible products are subtilisins immobilized on (conjugated with) soluble polymers (Sub-PAA; **V**), and immobilized on microbeads (Sub-PAA-beads; **VI**) through the *N*-terminal of the protein. When Sulfo-SMCC was reacted to the α-amino group of another subtilisin molecule and coupled with Sub-SH, a subtilisin dimer linked through the *N*-terminal of each protein (Sub-dimer; **VII**) should be obtained.

Thus, the modified subtilisins were investigated as for their catalytic activity, thermal and autolytic stability, and any changes in their substrate specificity.

Materials and Methods

Reagents. Subtilisin Carlsberg (Lot46H0522), bovine serum albumin (BSA) and bovine plasma hemoglobin (Hb) were obtained from Sigma (MO. USA). Skimmed milk and gelatin were from Difco (Detroit, MI, USA). 2-Iminothiolane·HCl (Traut reagent) and sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (a double agent™ cross-linker, Sulfo-SMCC) were purchased from Pierce (Rockford, I1, USA). Ellman reagent (5,5′-dithiobis(2-nitrobenzoic acid: DTNB), 2,4,6-trinitrobenzen



Scheme 1.

Table 1. Activity of Various Subtilisins towards Suc-AAPF-*p*NA at 25 °C

Enzyme	$10^{-6}\cdot k_{cat}/K_m/M^{-1}\cdot sec^{-1}$ at pH 8.0	Relative activity at pH 8.0	Relative activity at pH 10.0
Intact	1.5	1	1
Sub-SH	1.3	0.87	0.91
Sub-PAA	0.35	0.23	0.27
Sub-PAA-beads	0.041	0.03	0.07
Sub-Dimer	0.15	0.10	0.18

sulfonic acid (TNBS), *N*-benzoyl-L-tyrosine-*p*-nitroanilide (Bz-Tyr-*p*NA), and phenylmethanesulfonyl fluoride (PMSF) were from Wako Pure Chemicals (Osaka, Japan) or Sigma. *N*-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine *p*-nitroanilide (Suc-AAPF-*p*NA) was obtained from the Peptide Institute Inc. (Minoo, Japan).

Sephadex G-25:PD-10, Sephacryl S-200HR, and Sephadex G-75 were from Amersham Pharmacia Biotech Japan (Tokyo). Poly(allylamine) hydrochloric acid salt (PAA; Mw = 6×10^4) and its crosslinked form (poly(allylamine) beads; PAA-beads; 10 mol% crosslinked, 0.2–0.4 mmϕ) were from Nitto Boseki (Tokyo, Japan). All other chemicals were of reagent grade, and were obtained from Nacalai Tesque (Kyoto, Japan).

Preparation of Sub-SH. The concentration of subtilisin in solution was determined from its absorbance using a spectrophotometer (UV-2200; Shimadzu) with $\varepsilon_{280} = 23500\ M^{-1}\cdot cm^{-1}$ ($M \equiv mol/dm^3$). To a 0.5 mL aliquot of subtilisin solution at 2.6 mg/mL, 0.5 mL of 1.1 mg/mL Traut reagent (both in 8 mM phosphate buffer, pH 8.0) was added and the mixture reacted for 50 min at 25 °C. The reaction mixture was then desalinated through a Sephadex G-25:PD-10 column (eluant: 50 mM borate buffer, pH 7.5), and the concentration of thiolated and unthiolated enzyme in each fraction was detected. To the collected peak portions (total 2.5 mL), 0.01 g EDTA·2Na was added, and then nitrogen gas was bubbled through the mixture. A 0.1 mL aliquot of 2 mg/mL Ellman reagent was added. The absorbance change at 412 nm was measured to determine the modified ratio of amino residues, which was found to be 1.01/molecule. The lack of Edman degradation for the modified protein, in contrast to the Ala detection for the intact enzyme, indicated that only the *N*-terminal amino group was modified.

Preparation of PAA-Maleimide. A 0.5 mL aliquot of 50 mM borate buffer solution (pH 8.5) containing several mg's of PAA was mixed with 0.5 mL of Sulfo-SMCC solution (1.1 mg/250 mL in H_2O), and incubated at 5 °C for 15 min. The PAA-beads were preincubated in the borate buffer for swelling before being subjected to the reaction. The modified polymers were desalinated with Sephadex G-25:PD-10 (eluant: 50 mM borate buffer pH 7.5), and the free amino groups were quantified by monitoring the reaction with TNBS at 367 nm. For the PAA-beads, the concentration of Sulfo-SMCC in the supernatant after centrifugation was measured.

Conjugation and Immobilization. To a 0.5 mL aliquot of Sub-SH (6 μM), 0.5 mL of PAA- maleimide (50 μM) was added and the mixture was incubated at 5 °C for 15 min. The reaction mixture was fractionated by a Sephacryl S-200HR column (ϕ 17 mm × 450 mm), and the number of subtilisins conjugated to the polymer was estimated from the eluting time. The maleimidated PAA-beads were washed with borate buffer (50 mM, pH 7.5), desalinated by repeated washings and centrifugations, and a 3 mL dilute solution (1 μM) of Sub-SH was added. After 15 min at 5 °C, the suspension was centrifuged. The concentration of unreacted Sub-SH in the supernatant was measured by its UV absorbance, in order to assay the amount of immobilized enzyme. On average, PAA was found to conjugate one subtilisin per one polymer molecule. The PAA-beads immobilized about 0.26 subtilisin molecules per 100 NH_2-groups.

Dimerization of Subtilisin. In this case, maleimidated subtilisin was prepared by sulfo-SMCC in a similar manner to PAA, except for the temperature (25 °C) and reaction time (50 min). The modified ratio was also 1 per molecule. This maleimidated enzyme was then mixed with Sub-SH and the mixture was incubated for 90 min at 25 °C. After the reaction, the mixture was fractionated by a Sephadex G-75 column (ϕ 17 mm × 450 mm), and the portion corresponding to the dimeric subtilisin was obtained.

Activity Assay. Two types of substrates were used to assay the modified enzymes. One group contained the synthetic substrates: Suc-AAPF-*p*NA and Bz-Tyr-*p*NA. For these substrates, the *p*-nitroaniline liberated by the catalytic hydrolysis was continuously monitored by a spectrophotometer ($\varepsilon_{410} = 8900\ M^{-1}\cdot cm^{-1}$). The other group was composed of proteins (and mixtures): skimmed milk, gelatin, BSA, and hemoglobin. *De-novo* amino groups formed by the peptide hydrolysis were blocked with formaldehyde, and the corresponding carboxylate was measured by an auto-titrator (RTS 622/TTT60/ABU12; Radiometer, France) (formol titration).

Residual Activity. Various types of subtilisin solutions were incubated at pH 7.5 (50 mM borate buffer) and 4 °C or 45 °C. After the indicated time intervals, an aliquot was taken out, and the activity against Suc-AAPF-*p*NA was measured at 25 °C, pH 8.0 (0.1 M Tris-HCl).

DSC Measurement. DSC was measured by a high-sensitivity calorimeter (Nano-DSC II Model 6100: Calorimetry Sciences Co. Ltd. UT, USA). The protein concentration was 1–2 mg/mL, and the total sample volume was ca. 0.3 mL. The temperature increasing rate was usually 1 K/min.

Results and Discussion

Activity of Intact and Modified Enzymes towards Synthetic Substrates. The catalytic activity of the intact and modified subtilisins towards the synthetic peptide substrate, Suc-AAPF-*p*NA, was determined at 25 °C and pH 8.0. The results are compiled in Table 1. None of these enzymes showed any substantial hydrolytic activity towards Bz-Tyr-*p*NP, a chymotrypsin-specific substrate.

The introduction of an SH group at the *N*-terminal slightly lowered the activity. After conjugation to soluble PAA, the activity dropped to around one fourth of the intact enzyme, and conjugation to the PAA-beads reduced the activity further. This might be related to the accessibility of the substrate to the immobilized enzyme, the concentration of which had been calculated from the amount of unreacted Sub-SH in the supernatant. Subtilisin dimers conjugated through the *N*-terminal of each monomer showed one fifth to one tenth activity (in this case both enzymes in a dimer were counted in the concentration).

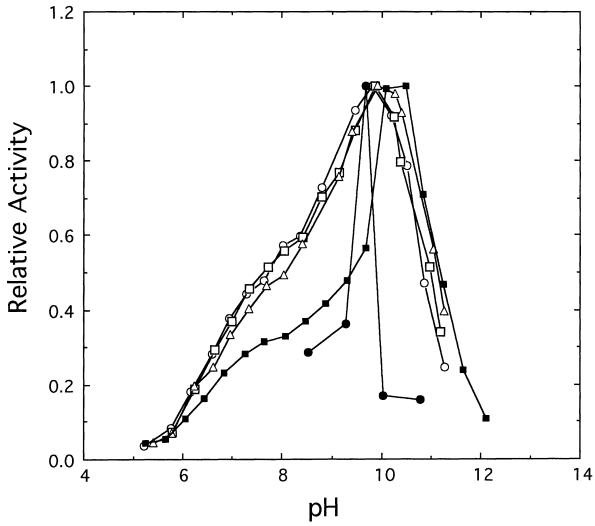


Fig. 1. pH-dependence of relative activity of intact and modified subtilisins. Intact (○), Sub-SH (□), Sub-PAA (△), Sub-PAA-beads (●), Sub-Dimer (■). Assayed in wide area buffer (Davies buffer) at 25 °C with Suc-AAPF-*p*NA. [E] = 0.0016 μM, except for $[E]_{Sub\text{-}Dimer}$ = 0.0049 μM. [S] = 0.01 mM.

Table 2. Proteolytic Activity of Intact and Modified Subtilisins

Substrate	Enzyme			
	Intact	Sub-SH	Sub-PAA	Sub-Dimer
Skim milk	0.51	0.18	0.37	0.28
	(1)	(0.35)	(0.73)	(0.55)
	<1>	<0.41>	<3.11>	<5.49>
Gelatin	0.31	0.39	0.26	0.17
	(1)	(1.26)	(0.84)	(0.55)
	<1>	<1.45>	<3.59>	<5.48>
BSA	0.35	0.79	0.33	0.11
	(1)	(2.26)	(0.94)	(0.31)
	<1>	<2.60>	<4.04>	<3.14>
Hemoglobin	0.42	0.50	0.048	0.025
	(1)	(1.19)	(0.11)	(0.06)
	<1>	<1.37>	<0.49>	<0.60>

( ): relative activity to the intact enzyme. < >: ratio of proteolytic activity (in μM/min) to the apparent second order rate constants listed in Table 1, relative to the intact enzyme.

The pH dependence of the activity is shown in Fig. 1, in the form of the relative value to the highest activity observed for each enzyme. There were no significant differences in the optimum pH (about 10), but the Sub-dimer showed a more asymmetric pH dependence, and the Sub-PAA-beads showed a very narrow activity range in pH.

Proteolytic Activity. The activity towards proteineous substrates is compiled in Table 2. It is notable that, compared with the activity towards the peptide substrate, these modified

subtilisins showed less compromised proteolytic activity. This becomes evident when the ratio of proteolytic activity (in μM/min) to the apparent second order rate constant (listed in Table 1) is compared for each enzyme (numbers in < >). Both Sub-PAA and Sub-dimer showed 3 to 5.5 times higher relative specificity towards proteineous substrates, except for hemoglobin, in which the rigid higher order structure of this protein seems to be unfavorable. This method of comparison is eqivalent to expressing the (active) enzyme concentration by its peptidase activity under standard conditions, rather than by its absorbance measurement; this is very common in kinetic studies of enzymes, in cases where there is no adequate active site titrant.[26] For the proteolysis of proteineous substrates with flexible or ambiguous higher structures, simultaneous attack by two or more proteases in proximity seems to be more efficient.[27] Trapping or holding of the same proteineous substrate in the vicinity of the enzyme, and a subsequent continuous or sliding attack could occur in the case of the immobilized enzyme.

Stability of Enzymes. Subtilisin is known to show autolytic degradation (disactivation) in solutions. The above-mentioned thiolation and dimerization reactions contained an incubation step at 25 °C (50 to 140 min). The effects of such a room temperature incubation on autolytic reactions were studied using DSC analysis. Autolysis-free incubation can be performed by the addition of a covalent inhibitor of serine protease, PMSF. When compared with incubations in the presence of PMSF, the intact subtilisin showed 6 and 8% smaller enthalpy of denaturation after PMSF-free incubations for 50 min and 140 min, respectively, at 25 °C. These values, however, are not sufficient to explain the lower activities found in the modified enzymes. There should be more unfavorable changes in the enzyme protein created by the various chemical modifications applied here.

The thermal and autolytic stability of the enzyme was improved by its conjugation to PAA. Table 3 shows the residual activity of subtilisins after 30 or 90 min incubations at 45 °C. The intact enzyme rapidly lost its activity. After 90 min, the activity had dropped to almost one fifth of the initial value. Although the initial activity of Sub-PAA was lower than the intact enzyme, this modified enzyme showed only a small disactivation during the incubation. Even after a 90 min incubation at 45 °C, it retained 90% of its initial activity. At this point, the net activity of Sub-PAA towards the peptide substrate became almost comparable to the control (intact enzyme incubated under the same conditions). It is notable that the presence of the PAA polymer, free from the enzyme, in the solution alone was able to increase the thermal stability of the intact subtilisin. In the presence of 17 μM PAA, the intact subtilisin showed 3 times higher activity than the control after a 90 min incubation at 45 °C.

Table 3. Residual Activity of Intact and Modified Subtilisins after Incubation at 45 °C

System	Residual activity*	
	After 30 min	After 90 min
Intact subtilisin	0.50	0.22
Sub-PAA	1.03	0.90
Subtilisin + PAA	0.94	0.66

*Relative to the activity before incubation for each system. The apparent second-order rate constant was measured with Suc-AAPF-*p*NA at 25 °C and pH 8.0 (0.1 M Tris-HCl). [E] = 0.013 μM, [S] = 0.01 mM.

The stability of Sub-PAA was also observed when the residual activity was studied after prolonged incubations at low temperature. Figure 2 shows the results for the incubations up to 9 days. Sub-PAA retained more than 90% of its initial activity, even after 8 days incubation at 4 °C. Intact subtilisin lost more than 60% of its initial activity after incubations for the same periods. The dimeric enzyme exhibited faster disactivation, and the immobilized one (Sub-PAA-beads) showed almost the same level of inactivation as the intact enzyme.

In our experiments, the amount of soluble enzyme was controlled by the absorbance-based concentrations. For enzymes of higher proteolytic activity, the autolytic inactivation should be faster. The results from the Sub-dimer could also be explained by this process. Although thermal and autolytic disactivation could not strictly be separated in the case of proteases such as subtilisin, the results at low temperature would typically represent the latter, whereas the incubations at higher temperatures would involve both processes. Immobilized proteases could show higher stability towards autolysis when the density of the enzymes in the matrix support is not very high, as in the present case, since there would be a low chance to encounter two enzyme molecules together. The multi-point conjugation explanation for the thermal stability of immobilized enzymes[28] is not applicable to the present case, since we limited the conjugation point for the present example.

The high stability of the immobilized enzymes as compared to the soluble polymer support, subtilisin-PAA, cannot be explained by the above mechanisms. There seems to be some

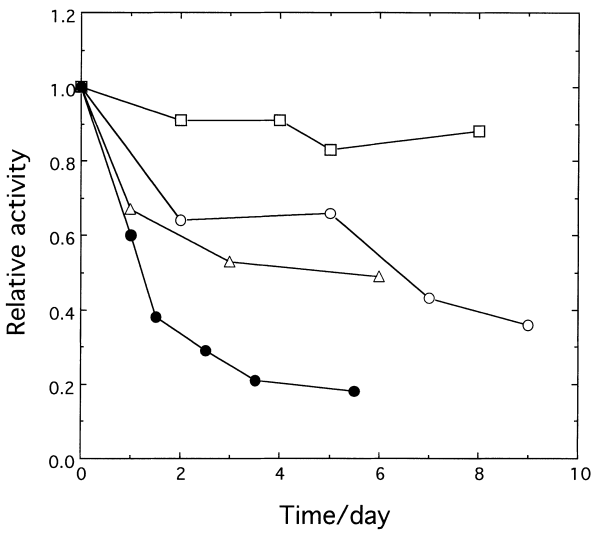


Fig. 2. Residual activity of subtilisins after incubation at 4 °C and pH 7.5 (50 mM borate buffer). Intact (○), Sub-PAA (□), Sub-PAA-beads (△), Sub-Dimer (●). Assayed in 0.1 M Tris-HCl (pH 8.0) at 25 °C with Suc-AAPF-*p*NA. $[E]_{Intact}$ = 0.003 μM, $[E]_{Sub\text{-}PAA}$ = 0.002 μM, $[E]_{Sub\text{-}PAA\text{-}beads}$ = 0.077 μM, $[E]_{Sub\text{-}Dimer}$ = 0.004 μM. [S] = 0.0125–0.1 mM.

positive and active participation from the soluble polymer support. The higher stability for the mixture of free PAA plus the enzyme would indicate that the direct or indirect interactions of the polymer, such as by ionic interactions, hydrogen-bonding or preferential hydration, would stabilize the protein structure against thermal perturbations. There have been several reports on the stabilizing effects of water-soluble polymers on proteins and enzymes.[29–33] The existence of a polymeric support in the vicinity of the enzyme could interfere with attack from another active subtilisin molecule. It would not interfere with attack against other proteineous substrates, thus increasing the relative specificity of the enzyme towards macromolecular substrates.

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