Peptide Synthesis Mediated by Thiolsubtilisin Using Peptide Thioester as Building Block

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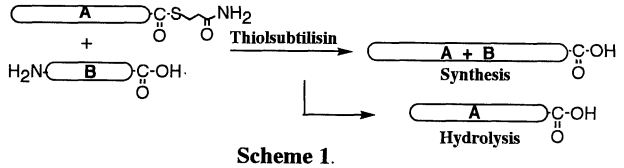
Peptides were efficiently synthesized by the catalysis of chemically engineered protease, thiolsubtilisin, with peptide thioesters as substrate building blocks. The ligation method was successfully applied to the synthesis of a partial sequence of DNA-binding *c*-Myb protein.

Considerable effort has been devoted toward the peptide synthesis mediated by proteases[1,2] as a counterpart to the chemical method such as solid-phase synthesis (SPS). There are several problems to be solved including secondary hydrolysis of products by proteases.[2] The critical side reaction is usually accompanied with peptide condensations using amidase activities of proteases. Use of enzyme with only the esterase activity would be free from the side reaction. On the basis of this principle, Kaiser *et al.* presented the approach using thiolsubtilisin, in which the active site Ser221 was chemically converted to Cys, for the synthesis of oligopeptides.[3] The amidase activity of thiolsubtilisin is diminished to about $1/10^5$, while its esterase activity is reduced to about $1/10^3$. Therefore, the modified enzyme can efficiently hydrolyze peptide active esters. They used peptide *p*-chlorophenyl esters as substrates for the peptide synthesis. However, it is not easy to achieve the synthetic application to large peptides due to difficulty in the synthesis of peptide active esters without side chain protections.

On the other hand, Hojo and Aimoto reported an efficient synthetic method for proteins composed of more than 100 amino acids using peptide thioester as building blocks.[4] Large peptide thioesters can be easily synthesized by SPS. However, the method was only applicable to Gly-Xaa bond formation, because of poor coupling efficiency and possible racemization. Therefore, combination of the rapid synthesis of peptide thioesters and the catalytic condensation with thiolsubtilisin would afford a useful method for the synthesis of longer peptides and proteins. In the present study, we studied applicability of the thiolsubtilisin-catalyzed peptide synthesis with peptide thioesters as substrate building blocks (Scheme 1).



Scheme 1.

At first, we carried out the catalytic reaction of thiolsubtilisin using *t*-butyloxycarbonyl (Boc)-amino acid thioesters (-S-CH_2CH_2-$CONH_2$: MPA-NH_2)[4] and a dipeptide amine to examine whether the thioester was appropriate for the enzyme-catalyzed coupling. Amino acid and peptide thioesters were synthesized by the solution method. Thiolsubtilisin was prepared by the chemical modification of subtilisin Carlsberg[3] and purified with a thiopropyl-Sepharose 6B column.[5] The enzyme reactions were performed with an ester component (20 mM; 1 M=1 mol dm^{-3}) and an amine component (40 mM) in 0.1 M phosphate buffer (pH 8.0)/dimethylformamide (DMF) (1/1, v/v) in the presence of thiolsubtilisin (10 μM) at 25 °C, unless otherwise indicated. The reaction was followed by HPLC and yields were calculated with HPLC integral values. Products were purified with HPLC and identified by FAB-MS and amino acid analysis. When amino acid thioesters, Boc-Xaa-MPA-NH_2 (Xaa: Gly, Ala, Val, Leu, Phe), and H-Ala-Phe-NH_2 were used as substrates, the coupling reactions proceeded with the reaction rates in the following order, Leu>Phe>Ala>>Val, Gly (Table 1). In the cases of Leu and Phe, the reactions reached to an equilibrium after 3 days to give the tripeptide products (Boc-Xaa-Ala-Phe-NH_2) in good yield (>60%). In contrast to the other amino acids, the Boc-Gly-Ala-Phe-NH_2 (35%) was obtained without the enzyme. Thiolsubtilisin seems to keep the substrate specificity at the S1 subsite of native subtilisin,[6] even though the thioesters are used as a substrate. It is noteworthy that Boc-Leu-OH and the peptide amine were not reacted with the enzyme.

Table 1. Results of the reaction with amino acid thioesters

Boc-Xaa-MPA-NH_2	Yield /%	$k_2 \times 10^{2a}$ /$M^{-1}s^{-1}$
Phe	67	60
Leu	63	100
Val	7	1.2
Ala	53	34
Gly	47 (35)[b]	3.4

[a] Apparent second-order rate constant.
[b] Yield without enzyme.

When the peptide thioester, Boc-Ala-Ala-Pro-Phe-MPA-NH_2, was used, the reaction reached to an equilibrium after 7 h and the reaction rate increased about 10-times as compared with amino acid substrates (Figure 1A). The hexapeptide Boc-Ala-Ala-Pro-Phe-Ala-Phe-NH_2 was obtained in 66% yield and the secondary hydrolysis of the product was not observed. Because the tetrapeptide Ala-Ala-Pro-Phe is known as a good substrate for native subtilisin, thiolsubtilisin retains the substrate specificity at the subsite region. The tetrapeptide Boc-Ala-Ala-Pro-Phe-OH, which was the hydrolysate of the peptide thioester, was obtained in 20% yield. The conversion in the catalytic reaction of thiolsubtilisin using the peptide thioester was high and the aminolysis reaction of the acylenzyme intermediate proceeded more effectively than the hydrolysis.

Furthermore, the insertion of Phe to the peptide thioester substrate, *i.e.*, peptide-MPA-Phe-NH_2, accelerated the catalytic reaction by 4 times and the product hexapeptide was obtained in

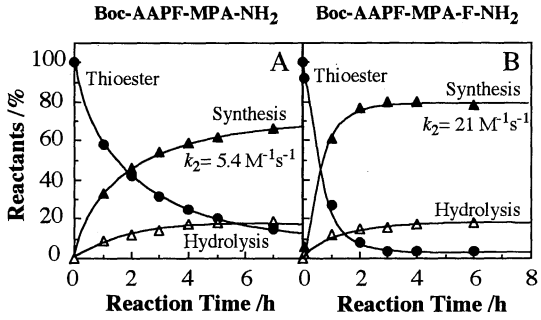


Figure 1. Time course of the reaction with peptide thioesters.

80% yield after 2 h (Figure 1B). Thiolsubtilisin prefers hydrophobic amino acids at the P2' position as like as native subtilisin. The same finding was obtained in the coupling reaction of *O*-ester depsipeptides with a S221C/P225A mutant of subtilisin.[7] The hydrolysate was still in 18% yield. Substitution of Gly, Ala, Val, and Leu for Phe at the P1 position changed the reaction rates as following (k_2/M^{-1}s^{-1}); Ala(35)>Leu(30)> Phe(21)>Gly(11)>Val(9.0), though the yields of the products were not so varied (75-85%). The different propensity at the P1 position from the amino acid ester substrates indicated the broad subsite specificity of thiolsubtilisin to the peptide substrates. This character would be convenient to synthesis of peptides with various sequences. The peptides were not obtained without the enzyme.

The optimum content of water was examined in this reaction ranging from 20% to 80% water in DMF, the best result was obtained at 50% content (Figure 2). The product was successfully obtained in high yield under the conditions more than 50% water content. However, the reaction could not sufficiently proceed in the solvent containing water less than 30%. DMF and methysulfoxide (DMSO) were favorable organic solvents among the solvents examined (acetonitrile, ethanol, and dioxane). These solvents are appropriate for the synthesis of longer peptides. It is worth to note that the peptide could not be obtained using subtilisin Carlsberg as a catalyst due to the rapid secondary hydrolysis of the product.

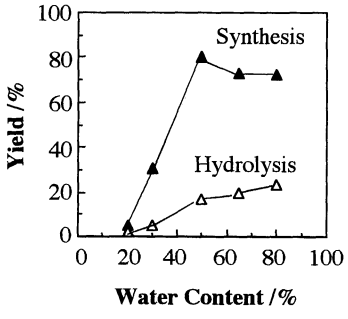


Figure 2. Effect of water content on the reaction in DMF.

On the basis of the successful reactions with model peptides, we attempted to synthesize a natural peptide corresponding to a partial sequence of the DNA-binding *c*-Myb protein,[8] *c*-Myb(29-48), by the combined method of the thiolsubtilisin-catalyzed reaction with the thioester SPS. The ester component, *i*Noc-Leu-Leu-Pro-Lys-Ser-Gly-Lys-Arg-His-Leu-MPA-Phe-NH$_2$, was synthesized by the SPS method on *p*-methylbenzhydryl amine resin with Boc amino acids (*i*Noc: *iso*-nicotinyloxycarbonyl[4]). The amine component, H-Gly-Lys-Thr-Arg-Trp-Thr-Arg-Glu-Glu-Asp-OH, was prepared on *p*-alkoxybenzyl alcohol resin with fluorenylmethyloxycarbonyl amino acids. These two components were subjected to the catalytic reactions under various conditions. The good result (70% product on HPLC and the hydrolysate 22%) was obtained in 50% aqueous DMSO solution (pH 8.0) with the ester component (1.0 mM) and the amine component (2.0 mM) in the presence of thiolsubtilisin (20 μM) at 3 h.[9]

Significant roles of proteins containing non-natural amino acids, selectively labeled ones, and phosphorylated and glycosylated proteins have been successively elucidated. To study detailed mechanism of such proteins and to find their application to biotechnological means, chemical method for peptide and protein synthesis would be inevitable tool to produce such large peptides and proteins. The ligation approach with thiolsubtilisin and the peptide thioesters has great advantages to the chemical methods such as solid-phase synthesis, for example, greater stereo- and regio-selectivity, mild reactions with reduced need for protecting groups. Although studies on the basis of the same principle were reported during this work,[10,11] combination with the thioester method established by the synthesis of various proteins[4] will be more practical for the synthesis of peptides and proteins with special modifications described above. Application to the synthesis of conjugated peptides and proteins is under progress.

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References and Notes

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9 FAB-MS: the ester component, *m/z* 1517 $(M+H)^+$; the amine component, *m/z* 1277 $(M+H)^+$, *c*-Myb(29-48) *m/z* 2542 $(M+H)^+$. Amino acid analysis of *c*-Myb(29-48), Asp 0.97 (1), Thr 1.76 (2), Ser 0.93 (1), Glu 1.92 (2), Pro 1.11 (1), Gly 2.00 (2), Leu 3.11 (3), His 1.05 (1), Lys 3.00 (3), Arg 2.87 (3). In the reaction, other compounds were only the hydrolysate of the ester component and the resulted thiol.
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