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Characteristics of Chymotrypsin Modified with Water-soluble Acylating Reagents and Its Peptide Synthesis Ability in Aqueous Organic Media†

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Several kinds of modified chymotrypsin were prepared with water-soluble acylating reagents, and their characteristics after hydrolyzing with unmodified chymotrypsin in aqueous-*N*,*N*′-dimethylformamide (DMF) media were compared. It was found that chymotrypsin (Csin), of which a 20% amino group was modified with a benzyloxycarbonyl group (Z(20)Csin), had more favorable characteristics than unmodified chymotrypsin with regard to hydrolytic activity in an aqueous DMF media. We also investigated the Z(20)Csin-catalyzed peptide synthesis in two different solution systems. In the one-layer system containing water and DMF, Z(20)Csin catalyzed the peptide bond formation in a higher yield than that by unmodifide chymotrypsin and enabled a synthetic reaction in even an 80% (v/v) DMF media, in which the hydrolytic reaction could not be carried out. Z(20)Csin catalyzed the condensation between some *N*-acyl amino acids or peptide derivatives and amino acids in 90% ethylacetate, 90% hexane or 50% benzene. This latter method employs a two-layer system, and the modified enzyme may be able to reduce the number of synthetic steps when preparing acyl peptides.

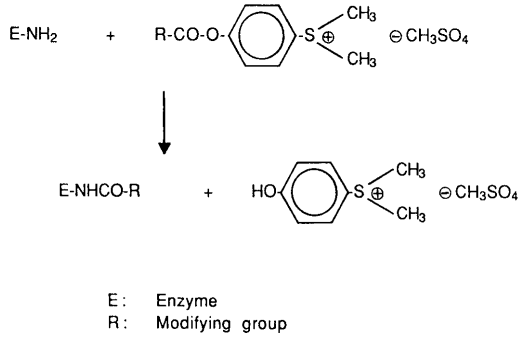
Fundamental research into enzymes has so far been developed to apply them in laboratories and industries. Research on remodeling enzymes has been undertaken to create new or additional functions by the recent recombinant DNA technique or by available chemical modification methods.

Enzyme-catalyzed peptide bond formation is one of these additional enzyme functions. It is known that proteases catalyze not only the hydrolysis of peptide bonds but also the formation of peptide bonds.[1] In comparison with chemical methods, enzymatic peptide bond formation proceeds stereospecifically without side reactions and requires no side protection. Hence, enzymatic peptide syntheses are often employed because of these advantages. A more effective process for enzymatic peptide synthesis uses organic solvents in a reaction system to shift the equilibrium in favor of synthesis over hydrolysis, resulting in a higher yield.[2,3] However, inactivation of the enzyme occurs depending on the concentration of organic solvents and decreases the yield. It seems reasonable to prepare more stable enzymes in organic solvents to overcome this problem.

Inada and co-workers[4] have prepared some amphipathic enzymes conjugating polyethylene glycol, which could be dissolved in organic solvents, and the polyethylene glycol-conjugated chymotrypsin (Csin) effectively catalyzed peptide bond formation in organic solvents.

Recently, we have developed a method to introduce the benzyloxycarbonyl (Z) group to amino groups in Csin by using the water-soluble acylating reagent, [*p*-(benzyloxycarbonyl)phenyl]-dimethylsulfonium methylsulfate (Z-DSP), as shown in Fig. 1. We[5,6] found that modified Csins were able to catalyze hydrolysis and peptide bond formation in *N*,*N*′-dimethylformamide (DMF)-water mixed solutions. This chemical modification method using a water-soluble acylating reagent enables chemical modification to be performed under mild conditions with less damage to the enzyme. Moreover, the desired modifying group can be easily introduced to the enzyme.

In this report, Csin was modified with several water-soluble acylating reagents, and its hydrolyzing ability and characteristics in a water-DMF mixed solution were evaluated against the unmodified material. We also investigated modified Csin-catalyzed peptide synthesis in two different systems. The synthesis of Z-Tyr-Gly-NH_2 was catalyzed by modified or unmodified Csin in a one-layer system containing water and DMF. Furthermore, the condensation between some acyl amino acids or peptide derivatives and amino acids was carried out in 90% ethylacetate (EtOAc), 90% hexane or 50% benzene as a two-layer system.



E: Enzyme
R: Modifying group

Fig. 1. Preparation of a Modified Enzyme.

† Remodeled Enzyme. Part I.

Materials and Methods

Materials. Csin was purchased from Boehringer Mannheim Yamanouchi. All amino acid derivatives and peptides were synthesized by conventional methods. Methyl-2,3-di-*O*-(phenylalanyl)-α-D-glucopyranoside (2,3-di-*O*-(Phe)-GlcOMe) was synthesized by Okai and co-workers.[7] The synthetic procedures for the water-soluble acylating reagents have been described elsewhere.[8]

Preparation of modified Csins. Csin (14 mg) was dissolved in 5 ml of 0.1 M $Na_2B_4O_7$-HCl (pH 7.9), and the intended amount of each water-soluble acylating reagent was added. The reaction mixture was allowed to stand at 10°C for 16 hr. To remove the precipitates, the reaction mixture was centrifuged at 20,000 × *g* for 10 min, before dialyzing the supernatant against deionized water for 72 hr with a dialysis membrane (Spectra/Por 1, 8,000 molecular weight cut off, Spectrum Medical Industries). After the dialysis, the supernatant was lyophilized, and the lyophilized powder was used for subsequent enzymatic reactions. The abbreviations of modified Csins are listed in Table I.

Determination of the rate of modification. The rates of modication were determined on the basis of the amount of the free amino group, which was measured by using sodium 2,4,6-trinitrobenzenesulfonate (TNBS).[9] Modified Csin (1 mg), or unmodified Csin for the control, was dissolved in 1 ml of water. The solution was mixed with 6 ml of a 0.15 M borate buffer (pH 9.2) and incubated at 50°C for 5 min. Then, 1 ml of a 0.2% (v/v) TNBS solution and 1 ml of 0.03 M Na_2SO_3 were successively added, before incubating at 50°C for 1 hr. After cooling the reaction mixture to room temperature, the absorbance was determined at 425 nm. The rate of modification was then calculated as

$$\text{Rate of modification } (\%) = (1 - A/B) \times 100 .$$

where A is A_{425} of the modified Csin and B is A_{425} of unmodified Csin.

Hydrolysis of 2,3-di-O-(Phe)-GlcOMe. A 0.4 mg (0.016 μmol) quantity of each modified Csin, or unmodified Csin for the control, and 5.6 mg (12 μmol) of 2,3-di-*O*-(Phe)-GlcOMe were dissolved in 1 ml of DMF containing a 0.1 M Tris–HCl buffer (pH 8.0), and the reaction mixture was incubatd at 40°C for 30 min. The reaction yields were determined on the basis of the amount of free Phe by reversephase high-performance liquid chromatography (HPLC) (column, Finepak SIL C_{18}, 4.6 × 250 mm, Japan Spectroscopic, eluent: 0.2 M sodium citrate (pH 2.5); detection, *o*-phthalaldehyde).

Peptide synthesis in a one-layer system. A 2 mg (0.08 μmol) quantity of each modified Csin, or unmodified Csin for the control, 0.315 mg (0.1 mmol) of Z-Tyr-OH and 1.1 mg (10 mmol) of H-Gly-NH_2·HCl were dissolved in 1 ml of DMF containing a 0.1 M Tris–HCl buffer (pH 6.7), and the reaction mixture was incubated at 20°C for 24 hr with gentle stirring. A quantitative determination of each resulting peptide was carried out by HPLC (column, Finepak SIL C_{18}, 4.6 × 250 mm; eluent, 50% acetonitrile, detection, UV (278 nm)).

Peptide synthesis in a two-layer system. A 1 ml quantity of a two-layer solution system containing 2 mg (0.08 μmol) of modified Csin, or unmodified Csin for the control, 1 μmol of an acyl amino acid or a peptide derivative, and 50 μmol of a amino acid was incubated at 37°C for 24 hr with stirring. The resulting peptides and their approximate yields were detected by thin-layer chromatography (Kieselgel G nach Stahl (type 60), Merk) with 1-butanol–acetic acid–pyridine–water (4 : 1 : 1 : 2, v/v).

Table I. Abbrevations for Modified Chymotrypsins

Modifying group	Rate of modification (%)	Abbreviation
Benzyloxycarbonyl	5	Z(5)Csin
	20	Z(20)Csin
	31	Z(31)Csin
	50	Z(50)Csin
t-Butyloxycarbonyl	29	Boc(29)Csin
	35	Boc(35)Csin
9-Fluorenyl-methyloxycarbonyl	22	Fmoc(22)Csin
Lauroyl	21	Lau(21)Csin

Results and Discussion

Modified Csin catalyzed-hydrolysis in aqueous-DMF media

Figure 2 shows the influence of the concentration of DMF on the hydrolysis of 2,3-di-*O*-(Phe)-GlcOMe catalyzed by modified Csin, compared with that of unmodified. 2,3-di-*O*-(Phe)-GlcOMe has previously been found to be a suitable substrate for Csin.[7] The hydrolytic activity of Boc(29)Csin, Boc(35)Csin, Fmoc(22)Csin and unmodified Csin was reduced in the same manner, depending on the concentration of DMF. In 0 to 10% (v/v) DMF containing a medium, the hydrolytic activity of Fmoc(22) and Lau(21)Csin was certainly lower than that of other modified Csins, but Lau(21)Csin possessing the most hydrophobic modifying groups in this series still maintained its enzymatic activity in a medium even containing 30% (v/v) DMF. One possible reason for this is that the modification contributed to stabilizing the enzume in aqueous-DMF media, and prevented denaturation of the enzyme caused by DMF. We know that such chemical modification derived from a stabilizing effect on enzymes has been occasionally observed.[10] The hydrolytic activity of modified Csins with the Z group, except for Z(50)Csin, was higher than that of the unmodified materials in 30 to 50% (v/v) DMF containing a medium, whereas in 0 to 10% (v/v) DMF containing a medium, all modified Csins were less active, and their hydrolytic activity decreased in correlation with

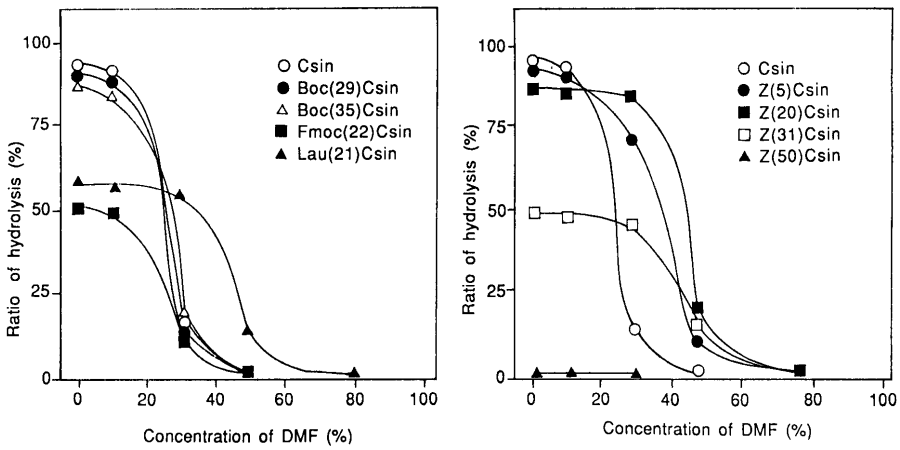


Fig. 2. Hydrolysis of 2,3-di-*O*-(Phe)-GlcOMe Catalyzed by Modified or Unmodified Csin in Aqueous-DMF Media.

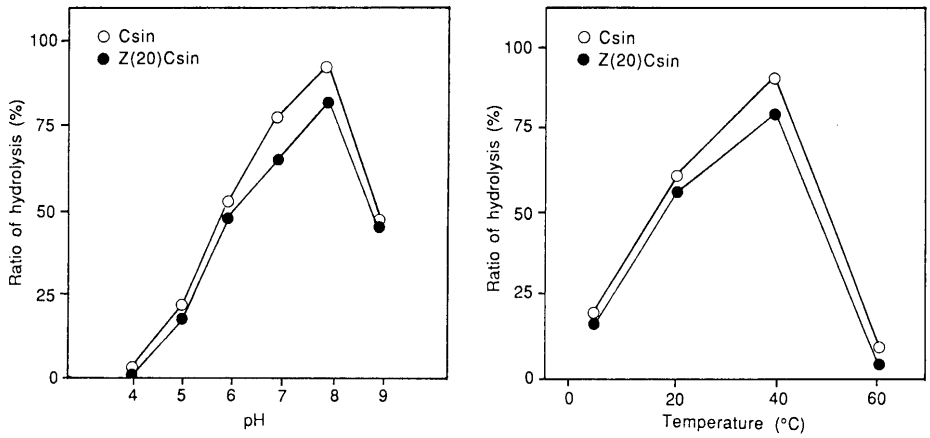


Fig. 3. Effect of pH and Temperature on the Hydrolysis of 2,3-di-*O*-(Phe)-GlcOMe Catalyzed by Csin or Z(20)Csin in Aqueous Media.

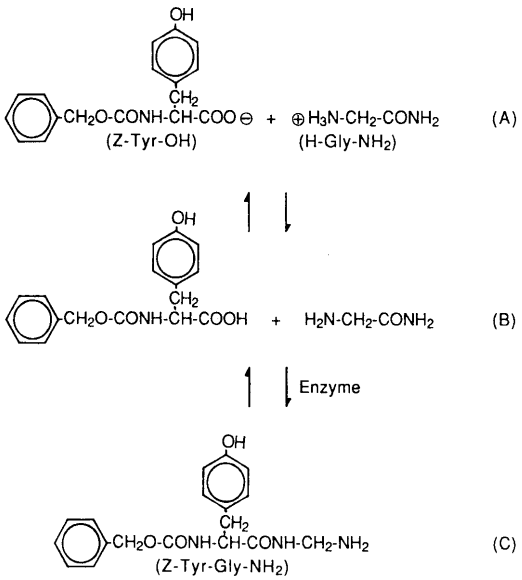


Fig. 4. Enzymatic Synthesis of Z-Tyr-Gly-NH_2.

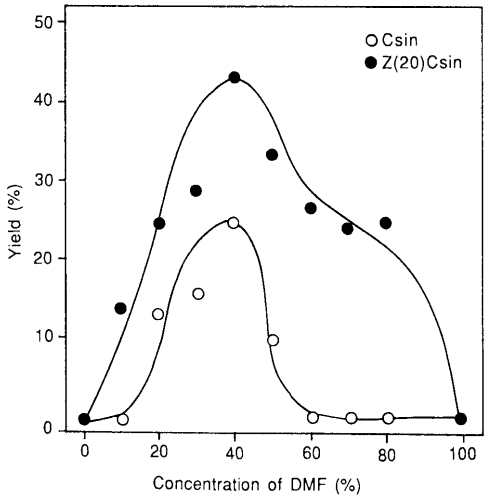


Fig. 5. Synthesis of Z-Tyr-Gly-NH_2 Catalyzed by Csin or Z(20)Csin in Aqueous-DMF Media.

the ratio of modification. In spite of possessing a different modifying group, Lau(21)Csin and Z(31)Csin behaved similarly with the hydrolytic reaction in an aqueous-DMF medium. Z(20)Csin possessed the most favorable characteristics in this series in regard to its hydrolytic activity in a medium containing 30% (v/v) DMF. Additionally, it seemed that no significant alteration in pH- or temperature-stability derived from the modification occurred to Z(20)Csin (Fig. 3).

Peptide synthesis catalyzed by Z(20)Csin in a one-layer system

According to previous studies, the reaction system must contain an appropriate amount of an organic solvent to synthesize Z-Tyr-Gly-NH_2.[3] The reason for this that is generally accepted is that adding an organic solvent causes the equilibrium of amino acid derivatives to move from an ionized state (Fig. 4A) to a non-ionized state (Fig. 4B); therefore, the equilibrium of the reaction would shift toward synthesis. However, the concentration of organic solvents is naturally limited for the reaction system, because excess organic solvents would cause denaturation of the enzymes. Modified enzymes with sufficient activity in organic solvents containing a medium can be used to overcome this limitation. Employing Z(20)Csin actually resulted in a higher yield, in comparison with unmodified Csin, and also extended the suitable concentration rang of DMF for peptide synthesis (Fig. 5). Z(20)Csin was allowed to catalyze the synthetic reaction in 80% (v/v) DMF containing a medium, in which hydrolysis could not be carried out. This finding shows that the loss of hydrolytic activity by an organic solvent is not necessarily linked with denaturation of the enzyme.

Peptide synthesis catalyzed by Z(20)Csin in a two-layer system

Endoprotease-catalyzed peptide synthesis has been restricted to the use of amino acid derivatives (amides, hydrazides and esters) as the amino components, while the possibility of applying free amino acids has been generally rejected. Recently, however Stehle *et al.*[11] have described a papain-catalyzed synthesis of a dipeptide, using a large amount of free amino acids as nucleophiles. We have also previously reported[12] that the synthesis of sweet peptide Ac-Phe-Lys-OH could not be catalyzed by Csin modified with the Z group in aqueous-DMF media, but by employing a two-layer system, the reaction could be performed. The established solvent system to obtain more favorable results in the synthesis of Ac-Phe-Lys-OH was 90% EtOAc, 90% hexane and 50% benzene. The condensation reaction

Table II. Synthetic Reactions Catalyzed by Z(20)Csin in a Two-layer System[a]

Substrate		Solvent system[b]		
		90% EtOAc	90% Hexane	50% Benzene
Ac-Phe	Lys	+++	++	++
	Gly	++	++	++
	Pro	++	++	++
	Asp	++	++	++
	Leu	+	−	−
	Phe	−	−	−
Boc-Phe	Lys	++	++	++
	Gly	−	−	−
	Phe	−	−	−
Z-Gly-Phe-OEt	Lys	−	+	+
	Gly	−	+	+
	Phe	−	+	+
Z-Tyr	Lys	+	+	n.d[c].

[a] Resulting yields: +++, >10%; ++, 5—10%; +, 0—5%; −, 0%.
[b] The aqueous-layer was a 0.15 M phosphate buffer (pH 6.7).
[c] Not determined.

applying other free amino acids (Gly, Pro, and Asp) successively gave corresponding acyl dipeptides. It is still not clear why these endoproteases acted like carboxypeptidases on peptide bond formation.

As shown in Table II, condensation between acyl amino acids or peptide derivatives and amino acids was attempted by using Z(20)Csin. In spite of excess amino acids as nucleophiles, in most cases the yield was less than 10% and no by-product was detected. One possible reason for these unsatisfactory yields is that the three-solvent system was originally stablished for the synthesis of Ac-Phe-Lys-OH. Consequently, suitable conditions must be individually selected for each reaction. In practice, Lys acted as the most favorable nucleophile in all reaction systems. Recently, Aso[13] has synthesized sweet peptide Ac-Phe-Lys-OH from Ac-Phe-OEt as a donor and lysine esters as acceptor nucleophiles by a Csin-catalyzed reaction in a 75% yield, but in order to obtain the final product, a saponification step was required. Our synthetic method in a two-layer system adopted modified Csin as a catalyst, successfully reducing the number of synthetic steps. We intend to examine the establishment of more efficient synthetic procedures. The peptide bond between two Phe residues, except for the synthesis of Z-Gly-Phe-Phe-OH, could not be formed in these experiments. According to the mechanism for Csin-catalyzed peptide synthesis, the formation of an acyl enzyme as an intermediate between an acyl amino acid and Csin may be essential, and acyl Phe derivatives are especially appropriate as acylating groups to form the acyl enzyme because an aromatic group of Phe fits the S_1 site of Csin.[14] In these reaction systems, excess Phe might have acted as a competitive inhibitor for the formation of acyl enzymes, which would have made it difficult to form the peptide bond between two Phe residues. Additionally, slightly soluble amino acids or peptide derivatives such as Z-Gly-Phe-OEt can be applied by using a two layer system.

In order to establish more effective procedures for modified enzyme-catalyzed synthetic reactions, many important factors exist; for example, the modifying group, rate of modification, solvent system, and concentration of substrates. We are considering preparing modified enzymes possessing profitable characteristics and designing appropriate procedures for practical peptide synthesis, in which enzymatic synthesis can be applied to advantage.

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