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Non-coded amino acids as acyl donor substrates for peptide bond formation catalyzed by thermoase in toluene

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Abstract

The peptide bond formation of *N*-protected non-coded amino acids having different structures as acyl donor substrates that is catalyzed by thermoase in organic media was investigated. In these reactions, *N*-protected L-α-non-coded amino acids, including L-Orn, L-Cit, α-aminobutyric acid (L-α-Abu) and phenylalanine homologues, were used as the acyl donors and phenylalanine derivatives were used as the acyl acceptors. This kind of enzymatic reactions cannot be carried out in an aqueous buffer due to the rigid specificity of proteases to coded amino acids in water. The results demonstrated that the substrate specificity of proteases could be broadened in organic solvents. In addition, the factors that influenced these protease-catalyzed reactions, including structures of the substrates, water content and the bases used, were systematically studied. Our work provided important evidence for broadening the application of protease in organic synthesis.

Keywords: Enzymatic reactions; Non-coded amino acids; Peptide synthesis; Proteases

1. Introduction

Many bioactive peptides contain non-coded amino acids [1,2]. And non-coded amino acids are effective building blocks for modifying bioactive peptides [3]. Moreover, non-coded amino acids are important for structure–activity relationship studies of peptides [4]. The synthesis of peptides containing non-coded amino acid residues is usually carried out by chemical methods. However, enzymatic peptide synthesis has became an important complement to chemical methods in recent years. The advantages of enzymatic synthesis of bioactive oligopeptides include mild reaction conditions, less requirement of side-chain protection, freedom from racemization, high regio- and stereo-selectivity [5–8]. However, the specificity of proteases limits their synthetic application; thus, non-coded amino acids are seldom accepted by proteases as substrates.

Therefore, the protease-catalyzed synthesis of peptides containing non-coded amino acid residues is a challenging task. This kind of enzymatic reactions cannot be carried out absolutely in an aqueous buffer due to the rigid specificity of proteases to coded amino acids in water. To overcome this limitation, the enzymatic reactions were performed in organic solvents because the substrate specificity of proteases could be broadened in organic solvents [9–19]. In our previous studies, estradiol derivatives [18] and amino alcohols with unprotected hydroxyl groups [19] were used as acyl acceptor substrates and a series of peptidomimetics were synthesized by proteases in organic solvents. These previous studies both related to about the utilization of proteases in the enzymatic reactions of non-amino acid compounds as acyl acceptor substrates. Generally, the specificity of proteases to the P_1 position (acyl donor) is more restricted than that to the P'_1 position (acyl acceptor) [20]. To our knowledge, there are only a few papers relating to the enzymatic formation of peptide bond of non-coded amino acids at P_1 position and subtilisin was used as catalyst in most cases [9–16]. Recently, we focused on the application of proteases to the synthesis of peptides of unnatural substrates as acyl donors in organic solvents. Dipeptide derivatives containing substitute phenylalanine residues at P_1 position were synthesized and the influence of different substituted groups on the enzymatic peptide synthesis was studied [14].

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In order to further extend the application of proteases, unnatural substrates containing non-coded amino acids were utilized as acyl donor substrates. In this paper, we report the successful synthesis of a series of dipeptide derivatives containing non-coded amino acid residues by thermoase (the crude powder mainly made up of thermolysin). Thermolysin was also used for the incorporation of non-coded amino acids into dipeptides at P_1 position by solid-to-solid conversion as that in Kula's report [10], while our research was carried out by solution method.

2. Experimental

2.1. Analysis of the samples

The melting points were determined by a Yanaco micro melting point apparatus (Yanagimoto Mfg. Co., Japan) and given without correction. Electrospray ionization–mass spectrometry (ESI–MS) was performed on a LCQ DECA XP^{plus} mass spectrometer (Thermo Finnigan, San Jose, CA, USA). Optical rotations were measured by a Perkin-Elmer (Wellesley, MA, USA) 341LC polarimeter.

2.2. Materials

Thermoase (specific activity 22 units/mg protein) was purchased from DAIWA KASEI K.K. Company. Toluene was analytical grade and was redistilled from Na/benzophenone. *N*-protected non-coded amino acids were purchased from BACHEM (Torrance, CA, USA). Phenylalanine derivatives were prepared by a standard technique [21]. The amino acid residues, which are not indicated configuration in this paper, are L-configuration. Standard abbreviations for amino acids and peptide derivatives are according to the suggestions of the IUPAC-IUB Commission on Biochemical Nomenclature (Eur. J. Biochem. 138 (1984), 9–37).

2.3. Typical experimental procedure

To a suspension of acyl donor substrate (0.3 mmol) and acyl acceptor substrate (0.6 mmol) in toluene (6 mL), water (6%) or $Na_2SO_4{\cdot}10H_2O$ (10%), *N*-ethylmorpholine or Et_3N (0.9 mmol) and thermoase (40 mg) were added. The reaction mixture was stirred at 37 °C for 3 days. At the end of the reaction, the mixture was poured into 100 mL of AcOEt ($CHCl_3$ was used in the synthesis of Fmoc-Cit-PheOMe). The organic layer was successively washed with 5% Na_2CO_3, saturated NaCl, 5% citric acid and saturated NaCl solution. The organic layer was dried over anhydrous Na_2SO_4 and filtered. The solvent of the filtrate was removed in vacuo and the obtained residue was purified by prepared thin layer chromatography ($CHCl_3$:MeOH = 20:1) and recrystallized from AcOEt/petroleum to afford pure product.

The following compounds were prepared using above procedure and the yields and MS of all compounds were listed in Tables 1 and 2.

Boc-Phe-Phe-OMe: mp 122–124 °C, $[\alpha]_D^{20}$ −12.2 (c 0.5, MeOH). Anal. calcd for $C_{24}H_{30}N_2O_5$ (%): C 67.59, H 7.09, N 6.57; found (%): C 67.49, H 7.15, N 6.40.
Boc-homoPhe-Phe-OMe: mp 116–118 °C, $[\alpha]_D^{20}$ −11.0 (c 1, MeOH). Anal. calcd for $C_{25}H_{32}N_2O_5$ (%): C 68.16, H 7.32, N 6.36; found (%): C 67.72, H 7.32, N 6.08.
Boc-Phg-Phe-OMe: mp 129–132 °C, $[\alpha]_D^{20}$ +40.7 (c 1, MeOH). Anal. calcd for $C_{23}H_{28}N_2O_5$ (%): C 66.97, H 6.84, N 6.79; found (%): C 66.67, H 6.86, N 6.61.

Table 1
Yields of dipeptide derivatives containing non-coded amino acid residue synthesized via Scheme 1

Entry	Product	Acyl donor	ESI–MS (*m/z*) $(M+Na)^+$	Yield (%)
1	Boc-Phe-Phe-OMe	Boc-Phe-OH	427[a]	63
2	Boc-homoPhe-Phe-OMe	Boc-homoPhe-OH	441[a]	49
3	Boc-Phg-Phe-OMe	Boc-Phg-OH	435	28
4	Boc-α-Abu-PheOMe	Boc-α-Abu-OH	387	84
5	Fmoc-Cit-PheOMe	Fmoc-Cit-OH	581	42
6	Boc-Orn(Z)-PheOMe	Boc-Orn(Z)-OH	550	70

[a] ESI–MS $(M+H)^+$.

Table 2
Yields of Boc-Orn(Z)-Phe-Y synthesized via Scheme 2

Entry	Product	Acyl acceptor	ESI–MS (*m/z*) $(M+H)^+$	Yield (%)
6	Boc-Orn(Z)-Phe-OMe	PheOMe	550[a]	70
7	Boc-Orn(Z)-Phe-OEt	PheOEt	542	25
8	Boc-Orn(Z)-Phe-OBzl	PheOBzl	604	6
9	Boc-Orn(Z)-Phe-OBu^t	$PheOBu^t$	–	0
10	Boc-Orn(Z)-Phe-NH_2	$PheNH_2$	513	83
11	Boc-Orn(Z)-Phe-N_2H_2Ph	$PheN_2H_2Ph$	604	78
12	Boc-Orn(Z)-Phe-$GlyNH_2$	$PheGlyNH_2$	570	85
13	Boc-Orn(Z)-Phe-$GlyN_2H_2Ph$	$PheGlyN_2H_2Ph$	661	86

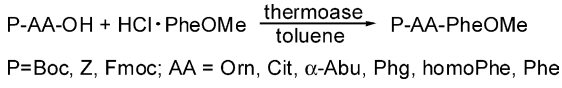
[a] ESI–MS $(M+Na)^+$.

Boc-α-Abu-PheOMe: mp 79–81 °C, $[\alpha]_D^{20}$ −22.9 (c 1.2, MeOH). Anal. calcd for $C_{19}H_{28}N_2O_5$ (%): C 62.62, H 7.74, N 7.69; found (%): C 62.55, H 7.78, N 7.53.
Fmoc-Cit-PheOMe: mp 166–169 °C, $[\alpha]_D^{20}$ −6.4 (c 0.5, DMF). Anal. calcd for $C_{31}H_{34}N_4O_6$ (%): C 66.65, H 6.13, N 10.03; found (%): C 66.42, H 6.23, N 9.76.
α-Boc-δ-Z-Orn-PheOMe: mp 116–118 °C, $[\alpha]_D^{20}$ −7.6 (c 1.1, MeOH). Anal. calcd for $C_{28}H_{37}N_3O_7$ (%): C 63.74, H 7.07, N 7.96; found (%): C 63.63, H 6.74, N 7.96.
α-Boc-δ-Z-Orn-Phe-OEt: mp 92–94 °C, $[\alpha]_D^{20}$ −10.0 (c 0.6, MeOH). Anal. calcd for $C_{29}H_{39}N_3O_7$ (%): C 64.31, H 7.26, N 7.76; found (%): C 64.25, H 7.13, N 7.74.
α-Boc-δ-Z-Orn-Phe-OBzl: mp 140–142 °C, $[\alpha]_D^{20}$ −16.8 (c 0.5, MeOH). Anal. calcd for $C_{34}H_{41}N_3O_7$ (%): C 67.64, H 6.85, N 6.96; found (%): C 67.21, H 6.74, N 6.82.
α-Boc-δ-Z-Orn-Phe-NH$_2$: mp 118–120 °C, $[\alpha]_D^{20}$ −21.0 (c 1, MeOH). Anal. calcd for $C_{27}H_{36}N_4O_6$ (%): C 63.26, H 7.08, N 10.93; found (%): C 63.50, H 7.15, N 10.68.
α-Boc-δ-Z-Orn-Phe-NHNHPh: mp 135–137 °C, $[\alpha]_D^{20}$ −34.5 (c 0.6, MeOH). Anal. calcd for $C_{33}H_{41}N_5O_6$ (%): C 65.65, H 6.85, N 11.60; found (%): C 65.39, H 6.85, N 11.58.
α-Boc-δ-Z-Orn-Phe-GlyNH$_2$: mp 120–123 °C, $[\alpha]_D^{20}$ −22.9 (c 0.8, MeOH). Anal. calcd for $C_{29}H_{39}N_5O_7$ (%): C 61.14, H 6.90, N 12.29; found (%): C 60.94, H 6.96, N 12.15.
α-Boc-δ-Z-Orn-Phe-GlyNHNHPh: mp 90–92 °C, $[\alpha]_D^{20}$ −4.0 (c 0.5, MeOH). Anal. calcd for $C_{35}H_{44}N_6O_7$ (%): C 63.62, H 6.71, N 12.72; found (%): C 63.37, H 6.84, N 12.43.

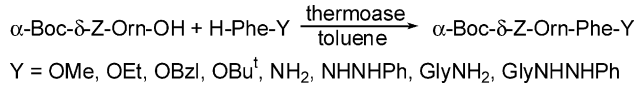
3. Results and discussion

N-protected non-coded amino acids with different structures, including basic amino acids L-Orn and L-Cit, neutral amino acid α-aminobutyric acid (L-α-Abu) and aromatic amino acids phenylglycine (L-Phg) and α-amino-γ-phenylbutyric acid (L-homoPhe), were utilized as acyl donor substrates for peptide bond formation catalyzed by thermoase in toluene (Scheme 1). A series of dipeptide derivatives containing non-coded amino acid residues were obtained (shown in Table 1). While no products were determined by TLC when these reactions were performed in an aqueous buffer. The results demonstrated that the substrate specificity of proteases could be broadened in organic solvents, so that unnatural substrates containing non-coded amino acids could be accepted by proteases as acyl donor substrates.

On the other hand, although there are many advantages in protease-catalyzed peptide synthesis, the rules and the mechanism of enzymatic peptide synthesis in organic solvents are not known clearly. Furthermore, the general approach for enzymatic peptide synthesis has not been achieved. The synthesis yields were influenced by many factors, including the structures of the substrates, water content, the bases used and so on [22–26]. To



Scheme 1. Synthesis of dipeptide derivatives containing non-coded amino acid residue at P_1 position by thermoase in toluene.



Scheme 2. Synthesis of Boc-Orn(Z)-Phe-Y catalyzed by thermoase in toluene.

seek the rules of enzymatic reactions in organic media, factors influencing these enzymatic reactions were systematically studied, too.

It is interesting to note that the yield of Boc-homoPhe-Phe-OMe (49%) was higher than that of Boc-Phg-Phe-OMe (28%) as shown in Table 1. This result was probably caused by the less steric hindrance of homoPhe. However, the yields of these two compounds were obviously lower than that of Boc-Phe-Phe-OMe with Boc-Phe-OH as acyl donor (63%), which indicated that such selectivity broadening of proteases in organic solvents was still limited.

In order to explore the effect of the different structures at C-terminus of acyl acceptors on the enzymatic reactions, Boc-Orn(Z)-OH was chosen as the acyl donor substrate and Phe derivatives with different structures at C-terminus were used as the acyl acceptor substrates (Scheme 2). The yields of these dipeptide derivatives Boc-Orn(Z)-Phe-Y (Y represents the groups at C-terminus of Phe) were displayed in Table 2. When the α-carboxyl groups of Phe were protected by ester bond (Table 2, entries 6–9), the yields were less than 70%. When Phe derivatives with amide bond at C-terminus were used as acyl acceptors (Table 2, entries 10–13), the yields were increased to 78–86%. There were two possible reasons; one was due to the poor solubility of these four products in toluene, the other was that the acyl acceptors with amide bond at C-terminus were easier to be recognized by thermolysin. Furthermore, the yields increased as the bulk of ester protecting groups of acyl acceptors decreased (Table 2, entries 6–9). The highest yield (70%) was obtained when PheOMe with the smallest ester at C-terminus was used as acyl acceptor. However, the product Boc-Orn(Z)-PheOBu^t could not be obtained because of the steric hindrance of tertiary butyl group. These results indicated that thermolysin was in favor of small blocking groups for α-carboxylic group when the α-carboxyl groups of Phe were protected by ester bond.

Next, we studied the effect of water content on the enzymatic reactions. In the synthesis of Boc-Orn(Z)-PheOMe, the water content in toluene was varied from 0 to 20% (Fig. 1). The results showed that the optimum water content was 4–10% and the yield was 53–58%. Higher yield (70%) was obtained when $Na_2SO_4{\cdot}10H_2O$ was used to supply essential water instead of free water. Furthermore, the yield of Boc-α-Abu-PheOMe in the presence of $Na_2SO_4{\cdot}10H_2O$ was 84%, which was higher than that using free water (71%). The reason was that the salt hydrates could take up or release water in organic solvents to keep a proper water activity (a_w) that is required for obtaining high activity of the enzymes and thus achieving high yields [26].

Finally, we studied the effect of bases on the enzymatic reactions (Scheme 3). *N*-Ethylmorpholine and Et_3N were both bases used for neutralizing hydrochloride salt of acyl acceptors and adjusting the pH value of the reaction media. In the synthesis of Boc-α-Abu-PheOMe and Boc-homoPhe-PheOMe catalyzed

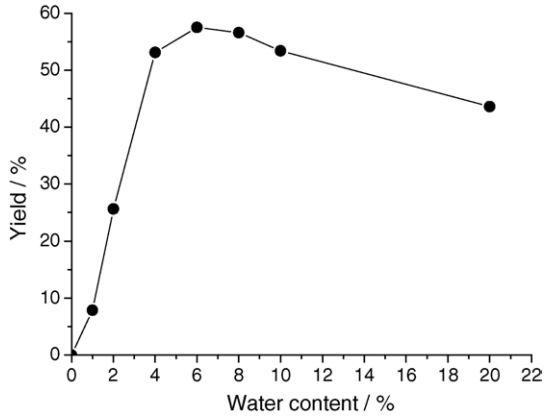
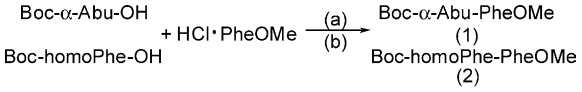


Fig. 1. Effect of water content on the synthesis of Boc-Orn(Z)-PheOMe catalyzed by thermoase in toluene.



(a) thermoase / toluene

	(1)	(2)
(b) *N*-ethylmorpholine	71%	49%
Et_3N	42%	35%

Scheme 3. Effect of bases on the enzymatic reactions.

by thermoase in toluene containing free water, the yields using *N*-ethylmorpholine were 71 and 49%, respectively, which were higher than those using Et_3N (42 and 35%, respectively). This suggested that *N*-ethylmorpholine was better than Et_3N in these enzymatic reactions.

4. Conclusions

Unnatural substrates containing non-coded amino acids were utilized as the acyl donor substrates for proteases in organic solvents, but this kind of enzymatic reactions could not be carried out in an aqueous buffer. The results demonstrated that the substrate specificity of proteases could be broadened in organic solvents. Factors influencing these protease-catalyzed reactions, including structures of substrates, water content and the bases used, were systematically studied. Some useful information was provided to broaden the application of proteases for peptide bond formation in organic synthesis.

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