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Kinetically Controlled Peptide Synthesis Mediated by Papain Using the Carbamoylmethyl Ester as an Acyl Donor

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Abstract A series of dipeptides were synthesized generally in good yields with carbamoylmethyl (Cam) esters as acyl donors in the presence of a cysteine protease, papain, immobilized on Celite. Several segment condensations were also achieved generally in high yields without danger of racemization and formation of the secondary-hydrolysis product. Moreover, partial sequences of some bioactive peptides were prepared through segment condensations, and aimed-at peptides were obtained generally in high yields without the racemization of *C*-terminal residues of the carboxyl components. Thus, the superiority of the Cam ester in the kinetically controlled peptide synthesis was once again ascertained in couplings mediated by the *cysteine* protease as in those catalyzed by the serine proteases reported earlier.

Keywords Carbamoylmethyl ester · Cysteine protease · Kinetically controlled peptide synthesis · Papain · Segment condensation

Introduction

The specificities of enzymes, especially proteases, have been increasingly exploited for peptide-bond formation. As the consequence of intensive investigations during the last few decades, protease-catalyzed peptide synthesis is now becoming popular as an alternative or complement to chemical synthesis of biologically active peptides (Nuijens et al. 2012). This enzymatic methodology has a lot of advantages compared to the conventional chemical approaches: freedom from racemization, high regio- and stereoselectivity, and minimal side-chain protection. On the other hand, narrow substrate specificity and the secondary hydrolysis of the growing peptides are counted as major disadvantages. We previously demonstrated the superiority of the carbamoylmethyl (Cam) ester as an acyl donor in the kinetically controlled peptide-bond formation mediated by the mammalian serine protease α-chymotrypsin: the above-mentioned drawbacks were overcome by employing this particular ester (Miyazawa et al. 2001a, b, 2002a, b). The effectiveness of the Cam ester was ascertained also in peptide syntheses mediated by some microbial serine proteases (Miyazawa et al. 2002c, 2003, 2008).

In peptide synthesis mediated by a serine or cysteine protease, the kinetically controlled approach employing ester substrates has often been chosen, because compared with the thermodynamically controlled approach which represents the direct reversal of proteolysis it generally gives a higher yield of the targeted peptide in a shorter reaction time and it requires a lower concentration of the enzyme employed (Nuijens et al. 2012). Döring et al. (1981) first observed that papain-mediated peptide syntheses could proceed when esterified acyl group donors were employed. These syntheses were performed in a biphasic aqueous-organic system at pH 5.5. Mitin et al. (1984) reported later on peptide syntheses at pH values ranging from 8 to 9.5 where the peptidase activity of the protease was largely reduced.

In the present paper, using the Cam ester as the acyl donor and the immobilized papain (EC 3.4.22.2) in an organic solvent with a minimum amount of water, we have examined a series of dipeptide syntheses and several model segment condensations, and the synthesis of partial

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sequences of some bioactive peptides, in order to demonstrate the superiority of the Cam ester in the kinetically controlled peptide synthesis mediated by the *cysteine* protease (Wang et al. 2011; de Beer et al. 2011, 2012).

Materials and Methods

Materials

Papain (twice-crystallized, lyophilized powder from papaya latex) was purchased from Sigma Chemical Co. and had a specific activity of 2.8 U (mg solid)$^{-1}$ with *N*-benzoyl-L-Arg-OEt. α-Chymotrypsin (type II, from bovine pancreas) and *Bacillus licheniformis* protease (subtilisin Carlsberg) were also from Sigma. The Cam esters of *N*-Z-amino acids (Z = benzyloxycarbonyl) or *N*-protected peptides were prepared through the reaction of their Cs salts with 2-chloroacetamide in DMF at 60 °C overnight (Miyazawa et al. 2001b, 2002b). Amino acid amide hydrochlorides were purchased from Kokusan Chemical Works. Gly-L-Leu-L-Met-NH_2·HBr and L-Phe-Gly-NH_2·HBr were prepared from their corresponding *N*-Z-peptide amides through debenzyloxycabonylation with HBr in acetic acid. The authentic samples of *N*-protected di- and tripeptide amides were prepared by the conventional chemical methods. The racemization-free authentic samples of partial sequences of bioactive peptides were prepared by the EDC [1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide]–HOBt (1-hydroxybenzotriazole)–$CuCl_2$ method (Miyazawa et al. 1992), while a mixture of epimeric peptides was prepared through the same segment condensations by the EDC method which usually results in a large amount of racemization of the *C*-terminal residue. All organic solvents were distilled and dried over molecular sieves prior to use.

Preparation of Immobilized Proteases

The immobilization procedure was essentially the same as that adopted previously for α-chymotrypsin (Miyazawa et al. 2001a). Papain (150 mg) was dissolved in 15 ml of phosphate buffer (pH 8.0), and mixed up with cysteine hydrochloride (25 mg) and Celite No. 535 (1 g), and the mixture was lyophilized using an Eyela freeze-dryer FDU-830 for 24 h. *B. licheniformis* protease was immobilized in a similar way as shown in the footnote f of Table 3.

HPLC Analyses

The liquid chromatograph employed was a Shimadzu LC-10AS instrument equipped with a Shimadzu SPD-10A variable wavelength UV monitor and a Shimadzu C-R6A data processor. HPLC analyses were undertaken under the following conditions: column, YMC ODS (4.6 mm i.d. × 250 mm); mobile phase, 50–65 % MeOH aq. containing H_3PO_4 (0.01 M); flow rate, 1.0 ml min^{-1}; column temperature, 30 °C; detection, UV at 254 nm.

Papain-Catalyzed Peptide Synthesis

In a typical coupling experiment, a mixture of the Cam ester of an *N*-Z-amino acid or *N*-protected peptide (0.05 mmol), an amino acid amide hydrochloride or a peptide amide hydrobromide (0.2 mmol), triethylamine (28 µl, 0.2 mmol) and the immobilized enzyme (150 mg) was incubated with shaking in a solvent composed of 2 ml of acetonitrile and 42 µl of 0.1 M phosphate buffer (pH 8.0) at 30 °C. The yields of the desired peptide, its epimer which might occur as the result of the racemization of the *C*-terminal residue, the hydrolysis product of the donor ester and possible by-products were determined by HPLC analysis.

Results and Discussion

In the kinetically controlled peptide bond formation mediated by a serine or cysteine protease, the ester substrate acting as an acyl group donor first forms the enzyme–substrate (ES) complex, and then it affords the acyl-enzyme intermediate (Nuijens et al. 2012). This intermediate is then deacylated either by a nucleophilic amino component to yield the peptide product or by water to form the hydrolysis product of the donor ester. Accordingly, an anhydrous or nearly anhydrous organic solvent should be an ideal reaction medium if the enzyme of question is active enough in that environment, because any undesirable hydrolytic side reactions are, in principle, avoidable. Thus far, papain-catalyzed kinetically controlled peptide syntheses have been conducted mainly in aqueous-organic solvent mixtures employing conventional esters such as the methyl ester as the acyl donor. We intended to use the Cam ester as the acyl donor in an organic solvent with low water content to minimize the secondary hydrolysis of the growing peptide. The minimal amount of water necessary for guaranteeing reasonable activity varies with each enzyme, and the kind of buffer solution which contains the water might have some effect on the enzyme's activity. Moreover, we found previously that the coupling efficiency was largely dependent on the pH of the buffer solution from which the immobilized protease was prepared (Miyazawa et al. 2002c).

Based on the preliminary results obtained by employing model couplings, e.g., Z-L-Phe-OCam + L-Leu-NH_2 and Z-L-Ala-OCam + L-Ile-NH_2, we decided to conduct

Table 1 Papain-catalyzed couplings of Z-Xbb-OCam with Xcc-NH_2 in aqueous acetonitrile

Xbb	Xcc	Yield (%)	
		Peptide	Z-Xbb-OH
Gly	L-Leu	87.9	0.6
L-Ala	L-Leu	87.2	2.8
L-Leu	L-Leu	82.4	3.3
L-Ser	L-Leu	83.9	3.1
L-Met	L-Leu	85.1	7.7
L-Phe	Gly	67.4	19.3
L-Phe	L-Val	57.5	15.1
L-Phe	L-Leu	86.1	8.4
L-Phe	L-Ile	77.4	12.4
L-Phe	L-Ser	81.2	8.1
L-Phe	L-Met	75.7	13.6
L-Phe	L-Phe	87.5	8.6

Reactions were conducted using 0.05 mmol of Z-Xbb-OCam, 0.2 mmol of Xcc-NH_2·HCl, 0.2 mmol of TEA and 150 mg of the immobilized papain (prepared at pH 8.0) in 2 mL of acetonitrile and 2 % (v/v) of phosphate buffer (pH 8.0) at 30 °C for 24 h

papain-catalyzed couplings in acetonitrile containing 2 % (v/v) phosphate buffer (pH 8.0) at 30 °C in the presence of immobilized papain prepared at pH8.0. Thus, a series of Z-Xbb-OCam (Xbb = Gly, L-Ala, L-Leu, L-Ser, L-Met or L-Phe) were allowed to react with Xcc-NH_2 (Xcc = Gly, L-Val, L-Leu, L-Ile, L-Ser, L-Met or L-Phe). The yields of the targeted peptide (Z-Xbb-Xcc-NH_2) and the hydrolysis product of the donor ester (Z-Xaa) after 24 h of incubation, quantified by HPLC analysis, are compiled in Table 1. The coupling efficiencies were generally low compared with those obtained using α-chymotrypsin as a biocatalyst (Miyazawa et al. 2001b). First, the influence of the amino acid residue as the carboxyl component (Xbb) was examined when the amino acid residue as the amino component (Xcc) was fixed to L-Leu. The differences among the residues were rather small: the peptide yields were more than 80 % in all the cases. This is in accordance with the well-known fact that the S_1-site of papain can be occupied by a variety of amino acid residues (Kullmann 1987). The primary specificity of papain is for a bulky aliphatic or aromatic residue in the P_2-position and this requirement is met with the use of the *N*-Z-protecting group. The influence of the amino acid residue as the amino component (Xcc) was examined next when the amino acid residue as the carboxyl component (Xbb) was fixed to L-Phe. Since an amino acid nucleophile competes with water in the reaction with the acyl-enzyme intermediate, the coupling efficiency must be affected by the amino component employed. Leu, Phe and Ser proved to be rather good residues: the peptide yields were more than 80 % and the production of Z-Phe was comparatively small among the residues examined. On the other hand, Val was a poor amine nucleophile, probably because of its bulky side chain which might interfere with the nucleophilic attack toward the acyl-enzyme intermediate. Gly, Ile and Met occupied the intermediate position between the two groups of residues. Data on the specificity for the S_1'-site of papain have been less documented thus far. In the papain-catalyzed coupling (via the thermodynamically controlled approach) of Z-DL-Gla-OH (Gla = γ-carboxyglutamic acid) with L-Xcc-NHNHPh, Čeřovský and Jošt (1985) reported that the yields of the homochiral dipeptides Z-L-Gla-L-Xcc-NHNHPh were dependent on the chemical nature of Xcc, following the order: Leu > Phe > Met > Ala > Val > Asn. Thus, the two different types of approaches, i.e., kinetically-controlled or thermodynamically-controlled, showed almost the same tendency toward the preference for the S_1'-site of papain.

Table 2 Papain-catalyzed couplings of Z-Xaa-Xbb-OCam with L-Leu-NH_2 in aqueous acetonitrile

	Yield (%)			
	Xbb = L-Phe		Xbb = L-Ala	
Xaa	Peptide	Z-Xaa-L-Phe-OH	Peptide	Z-Xaa-L-Ala-OH
Gly	62.7	7.8	89.4	4.2
L-Ala	89.3	4.8	97.2	1.8
L-Phe	46.9	5.0	79.9	1.5

Reactions were conducted using 0.05 mmol of Z-Xaa-Xbb-OCam, 0.2 mmol of L-Leu-NH_2·HCl, 0.2 mmol of TEA and 150 mg of the immobilized papain (prepared at pH 8.0) in a solvent composed of the specified amount of acetonitrile and 2 % (v/v) of phosphate buffer (pH 8.0) at 30 °C for 24 h

We examined next some model segment condensations, Z-Xaa-L-Phe-OCam + L-Leu-NH_2 or Z-Xaa-L-Ala-OCam + L-Leu-NH_2 (Xaa = Gly, L-Ala, or L-Phe), under the same reaction conditions as above. The results obtained after 24 h of incubation are compiled in Table 2. The peptide yields were generally much higher and the amounts of the hydrolysis products of the donor esters were lower with Z-Xaa-L-Ala-OCam than with Z-Xaa-L-Phe-OCam. As stated above, the S_1-site of papain can be occupied by a variety of amino acid residues. However, in the couplings (via the thermodynamically controlled approach) of *N*-Z-protected amino acids or peptides, it was reported that hydrophilic or small hydrophobic amino acids were more appropriate as acyl group donors (Isowa et al. 1977). Our present result is in accordance with the reported specificity for the P_1-position. As far as the specificity for the P_2-position is concerned, the less hydrophobic Ala residue gave better peptide yield than the more hydrophobic Phe residue. This is not in accordance with what was observed by Isowa et al.: with some remarkable exceptions, improved peptide yields resulted if bulky hydrophobic amino acid residues occupied the P_2-position of the substrate. In the present segment

Table 3 Protease-catalyzed syntheses of substance P (7-11)-pentapeptide

Protease	2 + 3 Coupling[a]		4 + 1 Coupling[b]	
	Yield (%)		Yield (%)	
	Peptide	Boc-L-Phe-L-Phe-OH	Peptide	Boc-L-Phe-L-Phe-Gly-L-Leu-OH
Papain[c]	82.8	4.8	78.3	7.2
α-Chymotrypsin[d]	83.9	5.1	75.2	6.9
B. licheniformis protease[e]	84.1	1.7	73.5	9.9

Boc-L-Phe-L-Phe-Gly-L-Leu-L-Met-NH_2: mp 166–168 °C; $[a]_D^{25}$-23.8° (c 1.0, DMF)

[a] Reactions were conducted using 0.05 mmol of Boc-L-Phe-L-Phe-OCam, 0.2 mmol of Gly-L-Leu-L-Met-NH_2·HBr, and 0.2 mmol of TEA at 30 °C for 48 h

[b] Reactions were conducted using 0.05 mmol of Boc-L-Phe-L-Phe-Gly-L-Leu-OCam, 0.2 mmol of L-Met-NH_2·HCl, and 0.2 mmol of TEA at 30 °C for 48 h

[c] Using 150 mg of the immobilized papain (prepared at pH 8.0) in 2 mL of acetonitrile and 2 % (v/v) of phosphate buffer (pH 8.0)

[d] Using 150 mg of the immobilized α-chymotrypsin (prepared at pH 8.0) in 2 mL of acetonitrile and 2 % (v/v) of phosphate buffer (pH 8.0)

[e] Using 150 mg of the immobilized subtilisin Carlsberg (prepared at pH 10.7) in 2 mL of acetonitrile and 2 % (v/v) of carbonate buffer (pH 10.7)

condensations, the formation of the defective peptides and the racemization of the *C*-terminal residues were not detected on HPLC, as clarified by preparing the epimeric authentic samples chemically.

Furthermore, the synthesis of partial sequences of some bioactive peptides was examined through segment condensations employing Cam esters as acyl donors. These peptides are the partial sequences containing the minimum sequences which retain their bioactivities. For comparison, the same couplings were also conducted by using α-chymotripsin and *B. licheniformis* protease as biocatalysts. These proteases were also employed in immobilized form. In these couplings the quantification of the aimed-at peptide, its epimer which might occur as the result of the racemization of the *C*-terminal residue, and the hydrolysis product of the donor ester were done by HPLC analysis. Substance P is an undecapeptide functioning as a neurotransmitter and neuromodulator which belongs to the tachykinin neuropeptide family. The minimum sequence was found to be the *C*-terminal (8-11)-tetrapeptide, i.e., L-Phe-Gly-L-Leu-L-Met-NH_2 (Nakamura et al. 1999). We examined the synthesis of the (7-11)-pentapeptide sequence through both 2 + 3 (Boc-L-Phe-L-Phe-OCam + Gly-L-Leu-L-Met-NH_2) (Boc = *t*-butoxycarbonyl) and 4 + 1 (Boc-L-Phe-L-Phe-Gly-L-Leu-OCam + L-Met-NH_2) segment condensations (Table 3). In both the segment condensations the desired peptide was obtained in ca. 80 % yield after 48 h of incubation. Although the concomitant formation of a small amount of the hydrolysis product of the donor esters was inevitable, no epimeric peptides were detected on HPLC. Moreover, no significant formation of defective peptides was observed from the HPLC profile. The two serine proteases yielded almost similar results to those obtained using papain in both of these segment couplings. Eledoisin is an undecapeptide of mollusk origin and also belongs to the tachykinin family of neuropeptides. Its minimum sequence is also the *C*-terminal (8-11)-tetrapeptide, i.e., L-Ile-Gly-L-Leu-L-Met-NH_2 (Broccardo et al. 2003). We tried the preparation of the (6-11)-hexapeptide sequence through a 3 + 3 (Boc-L-Ala-L-Phe-L-Ile-OCam + Gly-L-Leu-L-Met-NH_2) segment condensation (Table 4). The desired peptide was obtained in ca. 90 % yield after 48 h of incubation. No indications of the formation of the epimeric peptide and defective peptides were detected on HPLC. In this segment coupling also the two serine proteases yielded almost similar results to those obtained using papain.

Table 4 Protease-catalyzed syntheses of eledoisin (6-11)-hexapeptide

Protease[a]	Yield (%)	
	Peptide	Boc-L-Ala-L-Phe-L-Ile-OH
Papain	90.3	4.9
α-Chymotrypsin	88.0	1.9
B. licheniformis protease	86.8	4.5

Boc-L-Ala-L-Phe-L-Ile-Gly-L-Leu-L-Met-NH_2: mp 257–258 °C; $[a]_D^{25}$-29.6° (c 1.0, DMF). Reactions were conducted using 0.05 mmol of Boc-L-Ala-L-Phe-L-Ile-OCam, 0.2 mmol of Gly-L-Leu-L-Met-NH_2·HBr, and 0.2 mmol of TEA at 30 °C for 48 h

[a] See footnotes d–f of Table 3

Dermorphin is a heptapeptide having potent analgesic activity isolated from the skin of a South American frog. The *N*-terminal (1-4)-tetrapeptide, i.e., L-Tyr-D-Ala-L-Phe-Gly, is the minimum sequence that retains dermorphin activity (Montecucchi 1981). The synthesis of the tetrapeptide sequence, which contains a D-amino acid residue, was examined through both 2 + 2 (Boc-L-Tyr-D-Ala-OCam + L-Phe-Gly-NH_2) and 3 + 1 (Boc-L-Tyr-D-Ala-L-Phe-OCam + Gly-NH_2) segment condensations (Table 5). As previously reported (Miyazawa et al. 2001b), the use of the Cam ester allowed even the coupling of a D-amino acid as the carboxyl component mediated by α-chymotrypsin. Therefore, we thought it worthwhile to examine the 2 + 2 segment condensation in which the *C*-terminal residue was a D-amino acid, considering also the fact that the specificity of papain for the P_1-position is rather broad. In fact, the aimed-at peptide was obtained in a moderate yield (57 %) after 48 h of incubation in this case. No racemization of the D-Ala residue at the P_1-position was detected. The 3 + 1 segment condensation

Table 5 Protease-catalyzed syntheses of dermorphin (1-4)-tetrapeptide

Protease[a]	2 + 2 Coupling[b]		3 + 1 Coupling[c]	
	Yield (%)		Yield (%)	
	Peptide	Boc-L-Tyr-D-Ala-OH	Peptide	Boc-L-Tyr-D-Ala-L-Phe-OH
Papain	60.2	7.2	57.2	5.8
α-Chymotrypsin	33.3	5.6	41.1	3.2
B. licheniformis protease	34.5	4.0	37.0	2.8

Boc-L-Tyr-D-Ala-L-Phe-Gly-NH_2: mp 130–130.5 °C; $[a]_D^{25}$ + 29.2° (c 1.0, DMF)

[a] See footnotes d–f of Table 3

[b] Reactions were conducted using 0.05 mmol of Boc-L-Tyr-D-Ala-OCam, 0.2 mmol of L-Phe-Gly-NH_2·HBr, and 0.2 mmol of TEA at 30 °C for 48 h

[c] Reactions were conducted using 0.05 mmol of Boc-L-Tyr-D-Ala-L-Phe-OCam, 0.2 mmol of Gly-NH_2·HCl, and 0.2 mmol of TEA at 30 °C for 48 h

also afforded the desired peptide in almost a similar yield as the 2 + 2 condensation. In this case also no racemization of the Phe residue at the P_1 position was detected. Both the segment condensations no indications of the formation of defective peptides were detected on HPLC. The two serine proteases also gave the desired peptide in lower yields in both the segment condensations. In particular, the yields were about half of that obtained by the catalysis of papain in the 3 + 1 segment condensation.

Conclusions

A series of dipeptides were synthesized generally in good yields by employing Cam esters as acyl donors in the presence of immobilized papain. In these couplings the differences among the amino acid residues as the carboxyl components were rather small and Leu and Phe were good amino acid residues as the amino components. Several segment condensations were achieved generally in high yields without danger of racemization and formation of the secondary-hydrolysis product. Partial sequences of some bioactive peptides were prepared through segment condensations, and aimed-at peptides were obtained generally in high yields without the racemization of *C*-terminal residues of the carboxyl components. In the synthesis of partial sequences of bioactive peptides through segment condensations, aimed-at peptides were obtained generally in high yields without the racemization of *C*-terminal residues of the carboxyl components. Thus, the present results indicate the usefulness of the Cam ester as the acyl donor in the *cysteine* protease-catalyzed kinetically controlled peptide synthesis.

References

Broccardo M, Erspamer V, Falconieri G, Improta G, Linari G, Melchiorri P, Grace RCR, Chandrashekar IR, Cowsik SM (2003) Solution structure of the tachykinin peptide eledoisin. Biophys J 84:655–664

Čeřovský V, Jošt K (1985) Enzymatically catalyzed synthesis of dipeptides of γ-carboxy-L-glutamic acid from benzyloxycarbonyl-γ-carboxy-DL-glutamic acid. Coll Czech Chem Commun 50:878–884

de Beer RJAC, Zarzycka B, Amatdjais-Groenen HIV, Jans SCB, Nuijens T, Quaedflieg PJL, van Delft FL, Nabuurs SB, Rutjes FPJT (2011) Papain-catalyzed peptide bond formation: enzyme-specific activation with guanidinophenyl esters. ChemBioChem 12:2201–2207

de Beer RJAC, Zarzycka B, Mariman M, Amatdjais-Groenen HIV, Mulders MJ, Quaedflieg PJL, van Delft FL, Nabuurs SB, Rutjes FPJT (2012) Papain-specific activating esters in aqueous dipeptide synthesis. ChemBioChem 13:1319–1326

Döring G, Kuhl P, Jakubke H-D (1981) Modelluntersuchungen zur papainkatalysierten Peptidsynthese im wäßrig-organischen Zweiphasensystem. Monatsh Chem 112:1165–1173

Isowa Y, Ohmori M, Ichikawa T, Kurita H, Sato M, Mori K (1977) The synthesis of peptides by means of proteolytic enzymes. Bull Chem Soc Jpn 50:2762–2765

Kullmann W (1987) Enzymatic peptide synthesis. CRC Press, Boca Raton, pp 50–52

Mitin YV, Zapevalova NP, Gorbunova EY (1984) Peptide synthesis catalyzed by papain at alkaline pH values. Int J Pept Protein Res 23:528–534

Miyazawa T, Otomatsu T, Fukui Y, Yamada T, Kuwata S (1992) Simultaneous use of 1-hydroxybenzotriazole and copper(II) chloride as additives for racemization-free and efficient peptide synthesis by the carbodiimide method. Int J Pept Protein Res 39:308–314

Miyazawa T, Nakajo S, Nishikawa M, Hamahara K, Imagawa K, Ensatsu E, Yanagihara R, Yamada T (2001a) α-Chymotrypsin-catalysed peptide synthesis via the kinetically controlled approach using activated esters as acyl donors in organic solvents with low water content: incorporation of non-protein amino acids into peptides. J Chem Soc, Perkin Trans 1:82–86

Miyazawa T, Tanaka K, Ensatsu E, Yanagihara R, Yamada T (2001b) Broadening of the substrate tolerance of α-chymotrypsin by using the carbamoylmethyl ester as an acyl donor in kinetically controlled peptide synthesis. J Chem Soc, Perkin Trans 1:87–93

Miyazawa T, Ensatsu E, Yabuuchi N, Yanagihara R, Yamada T (2002a) Superiority of the carbamoylmethyl ester as an acyl donor for the kinetically controlled amide-bond formation mediated by α-chymotrypsin. J Chem Soc, Perkin Trans 1:390–395

Miyazawa T, Ensatsu E, Hiramatsu M, Yanagihara R, Yamada T (2002b) α-Chymotrypsin-catalysed segment condensations via the kinetically controlled approach using carbamoylmethyl esters as acyl donors in organic media. J Chem Soc, Perkin Trans 1:396–401

Miyazawa T, Hiramatsu M, Murashima T, Yamada T (2002c) *Bacillus licheniformis* protease-catalyzed peptide synthesis via the kinetically controlled approach using the carbamoylmethyl ester as an acyl donor in anhydrous acetonitrile. Lett Pept Sci 9:173–177

Miyazawa T, Hiramatsu M, Murashima T, Yamada T (2003) Utilization of proteases from *Aspergillus* species for peptide synthesis via the kinetically controlled approach. Biocatal Biotrans 21:93–100

Miyazawa T, Shindo H, Murashima T, Yamada T (2008) Peptide synthesis using carbamoylmethyl esters as acyl donors mediated by *Bacillus amyloliquefaciens* protease. Protein Pept Lett 15:1050–1054

Montecucchi PC (1981) Pharmacological data on dermorphins, a new class of potent opioid peptides from amphibian skin. Br J Pharmacol 73:625–631

Nakamura M, Chikama T, Nishida T (1999) Synergistic effect with Phe-Gly-Leu-Met-NH_2 of the C-terminal of substance P and insulin-like growth factor-1 on epithelial wound healing of rabbit cornea. Br J Pharmacol 127:489–497

Nuijens T, Quaedflieg PJLM, Jakubke H-D (2012) Synthesis of peptides. In: Drauz K, Gröger H, May O (eds) Enzyme catalysis in organic synthesis, 3rd edn. Wiley-VCH, Weinheim, pp 692–738

Wang M, Qi W, Yu Q, Su R, He Z (2011) Kinetically controlled enzymatic synthesis of dipeptide precursor of L-alanyl–L-glutamine. Biotechnol Appl Biochem 58:449–455