Amino Acids (2008) 34: 149–153
DOI 10.1007/s00726-006-0489-5
Printed in The Netherlands

Amino Acids

Application of several types of substrates to ficin-catalyzed peptide synthesis

H. Sekizaki[1], **E. Toyota**[1], **T. Fuchise**[1], **S. Zhou**[2], **Y. Noguchi**[1], and **K. Horita**[1]

[1] Faculty of Pharmaceutical Sciences, Health Sciences University of Hokkaido, Hokkaido, Japan
[2] Pharmic Department, Medical College, Qingdao University, Qingdao, China

Received July 10, 2006
Accepted December 13, 2006
Published online July 6, 2007;

Summary. The capability of ficin, a cystine protease, to form peptide bonds was investigated using several types of *N*-Boc-amino acid phenyl and naphthyl esters as acyl donor components. Enzyme-catalyzed peptide synthesis was carried out under optimized reaction conditions of pH, acyl acceptor concentration and selection of the best yield organic solvent. It used a condensation of *N*-Boc-Ala-O*p*Gu and Ala-*p*NA as a model reaction. The products were obtained in 72–96% yield using 10 different substrates, within a few minutes of reaction time.

Keywords: *N*-Boc-Amino acid – Amidinophenyl esters – Guanidinophenyl esters – (Guanidinomethyl)phenyl ester – Guanidinonaphthyl ester – Enzymatic peptide synthesis – Ficin

Abbreviations: Am, Amidinophenyl; Gu, guanidinophenyl; GM, (guanidinomethyl)phenyl; GN, guanidinonaphthyl; Ph, phenyl; Boc, *tert*-butyloxycarbonyl; DMSO, dimethyl sulfoxide; DMF, *N*,*N*-dimethylformamide; *p*-TsOH, *p*-toluensulfonic acid; Z, benzyloxycarbonyl; GTA, 3,3-dimethylglutaric acid (G), tris(hydroxymethyl)aminomethane (T), 2-amino-2-methyl-1,3-propanediol (A); CS, chum salmon; MOPS, 3-molpholinopropanesulfonic acid

Introduction

A large number of biologically active peptides has been recently isolated from bacterial, fungal, plant and animal sources and characterized in some detail. In particular, short sequence peptides play an important role in the sensory appreciation of food toward four basic taste sensations (sweet, bitter, sour and salty) (Nishimura and Kato, 1988). Synthetic chemistry has witnessed remarkable progress with the development of novel biologically active peptides. Enzymatic peptide synthesis has emerged as a powerful approach to the preparation of short sequences. Especially, peptide synthesis using protease-catalyzed reverse reaction has been extensively studied with a variety of amino acids and peptide derivatives as coupling components (Tsuzuki et al., 1980; Nakatsuka et al., 1987; Wong, 1989; Schellenberger and Jakubke, 1991; Gill et al., 1996; Bordusa, 2002; Kumar and Bhalla, 2005). It has been reported that the protease-catalyzed peptide synthesis is superior compared to the chemical coupling method due to the requirement of less side chain protection. The major drawback of the enzymatic method, however, is the respective substrate specificity. Among several enzymes already known, the cystein protease ficin has proven to be a versatile low-cost biocatalyst. Previously, some useful methods of ficin-catalyzed peptide synthesis using *N*-protected amino acids alkyl esters as an acyl donor component were reported. However, in these cases, long reaction time is required (Monter et al., 1991). On the other hand, immobilized ficin on starch was applied to peptide synthesis in organic solvent (Tai et al., 1995), and they showed good example for preparation of (Boc-L-Cys)$_2$-L-TyrOMe. Moreover, Hänsler et al. reported that with kinetically-controlled peptide synthesis in frozen aqueous solutions using Bz-Arg-OEt as acyl donor component it was possible to obtain satisfactory results (Hänsler et al., 1995). This report prompted us to use substrates containing a guanidino group for ficin-catalyzed peptide synthesis.

In the present work, we investigated ficin-catalyzed peptide synthesis using *N*-protected amino acid aromatic esters, which contained amidinium and guanidinium groups, as acyl donor components. We found that ficin-catalyzed peptide synthesis offers good yields of the condensation products and increased reaction rate.

We also found neither a charged amidinium nor a guanidinium group was required for ficin-catalyzed peptide synthesis.

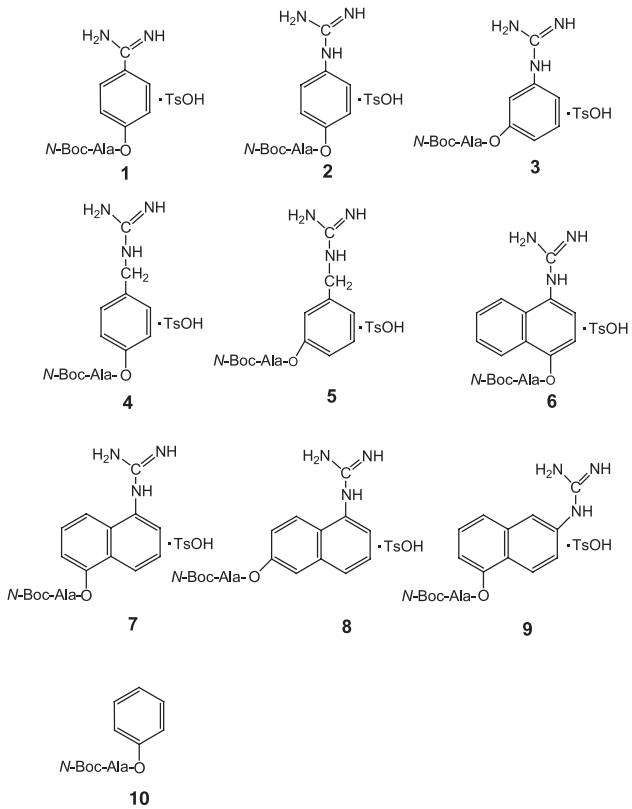

Fig. 1. Structure of substrates

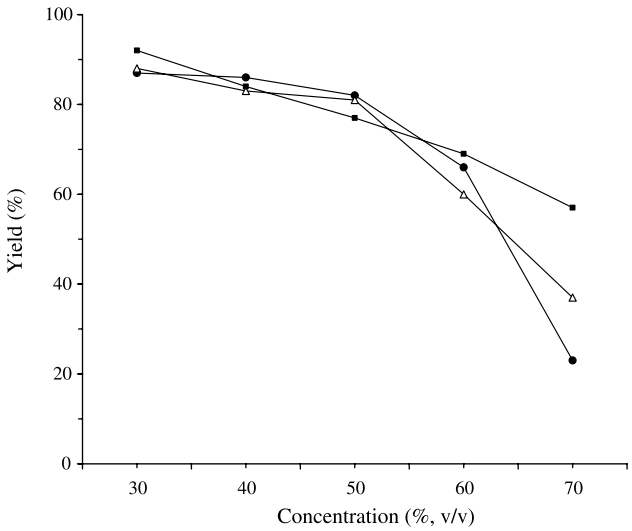

Fig. 2. Effect of organic solvent on ficin-catalyzed condensation of *N*-Boc-Ala-O*p*Gu (**2**) with Ala-*p*NA. Reaction was carried out in 50 mM GTA buffer (pH 9.0) containing DMSO (•), DMF (△), and acetonitrile (▪) at 37 °C. Product yield was analyzed after a reaction period of 5 min in which the coupling was completed. *N*-Boc-Ala-O*p*Gu (**2**), 1 mM; Ala-*p*NA, 35 mM; ficin 0.1 U

Materials and methods

Materials

All substrates were prepared according to our previous paper (Itoh et al., 1996; Sekizaki et al., 1996a, b, 1998, 1999a, b) as shown in Fig. 1. Ficin (EC 3.4.22.3, 0.11 U/mg), extracted from fig tree, was purchased from Sigma-Aldrich Co./Japan. HPLC grade DMSO, DMF, and acetonitrile from Kanto Chemical Co. Inc., were used. L-Ala-*p*NA was purchased from

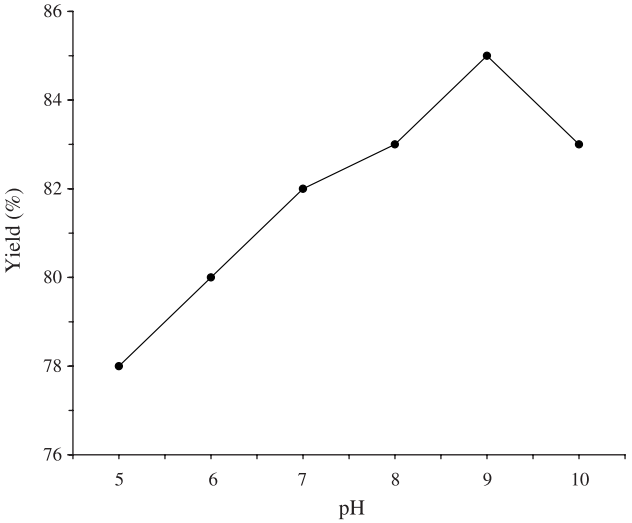

Fig. 3. pH Dependency of ficin-catalyzed condensation of *N*-Boc-Ala-O*p*Gu (**2**) with Ala-*p*NA. Reaction was carried out in 50 mM GTA buffer (pH 5.0–9.0) containing 40% DMSO at 37 °C. Product yield was analyzed after a reaction period of 5 min in which the coupling was completed. *N*-Boc-Ala-O*p*Gu (**2**), 1 mM; Ala-*p*NA, 35 mM; ficin 0.1 U

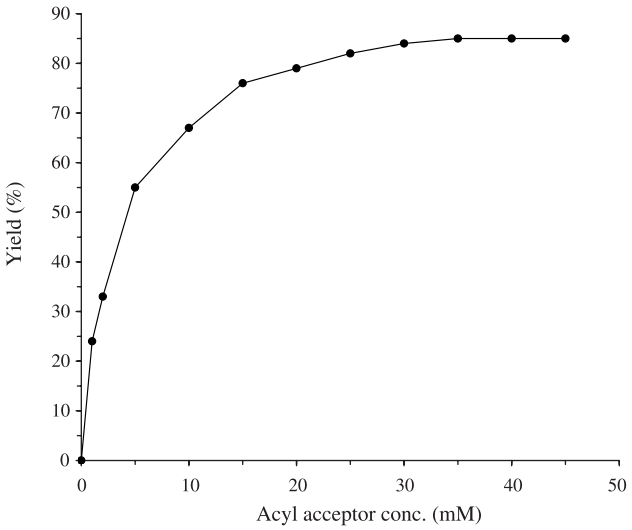

Fig. 4. Effect of acyl acceptor concentration on ficin-catalyzed condensation of *N*-Boc-Ala-O*p*Gu (**2**) with Ala-*p*NA. Reaction was carried out in 50 mM GTA buffer containing 40% DMSO at 37 °C. Product yield was analyzed after a reaction period of 5 min in which the coupling was completed. *N*-Boc-Ala-O*p*Gu (**2**), 1 mM; Ala-*p*NA, 0–45 mM; ficin 0.1 U

Peptide Institute Inc. 3,3-Dimethylglutaric acid, 2-amino-2-methyl-1,3-propanediol, and Tris(hydroxymethyl)aminomethane were obtained from Tokyo Chemical Industry Co. Ltd. and ICN Biomedicals Inc., respectively.

Ficin-catalyzed peptide synthesis

A solution of 10 µl of acyl donor (10 mM solution of substrates in DMSO), 10 µl of acyl acceptor (350 mM solution of L-Ala-*p*NA in DMSO), 50 µl of 50 mM GTA buffer (pH 9.0) and 20 µl of DMSO were mixed. To this mixture, 10 µl of ficin solution (10 U solution in 1 ml of 50 mM GTA buffer) was added and incubated at 37 °C. The progress of the coupling reaction was monitored with HPLC under the following conditions: Shim-pack CLC-ODS (M) (column i.d. 4.6 × 250 mm), isocratic elution at 1 ml/min, 0.1% aqueous trifluoroacetic acid/acetonitrile. An aliquot of the reaction mixture was injected and the eluate was monitored at 310 nm wave length at which it demonstrates chromophore properties due to the *p*-nitroanilide moiety. Peak identification was made by correlating the retention time with that of authentic samples which were chemically synthesized (Bieth and Weremath, 1970). Observed peak areas were used for the estimation of sample concentration.

Results and discussion

Ficin-catalyzed coupling reaction of *N-tert*-butyloxycarbonyl-L-alanine *p*-guanidinophenyl ester (*N*-Boc-L-Ala-O*p*Gu) (**2**) and L-Ala-*p*NA to give *N*-Boc-L-Ala-L-Ala-*p*NA was examined using co-solvents separately: DMSO,

Table 1. Yields of ficin[a], bovine trypsin[b], CS trypsin[c] and thrombin[d]-catalyzed peptide synthesis with amino acid ester as acyl donor

Entry no.	Trypsin	Acyl donor (no.)	Reaction time min (h)[e]	Product	Yield (%)[f]	Reference
1	Ficin	*N*-Boc-Ala-OAm (**1**)	2	*N*-Boc-Ala-Ala-*p*NA	83	This work
2	Ficin	*N*-Boc-Ala-O*p*Gu (**2**)	2	*N*-Boc-Ala-Ala-*p*NA	85	This work
3	Ficin	*N*-Boc-Ala-O*m*Gu (**3**)	3	*N*-Boc-Ala-Ala-*p*NA	72	This work
4	Ficin	*N*-Boc-Ala-O*p*GM (**4**)	2	*N*-Boc-Ala-Ala-*p*NA	94	This work
5	Ficin	*N*-Boc-Ala-O*m*GM (**5**)	5	*N*-Boc-Ala-Ala-*p*NA	77	This work
6	Ficin	*N*-Boc-Ala-O(1-4)GN (**6**)	5	*N*-Boc-Ala-Ala-*p*NA	82	This work
7	Ficin	*N*-Boc-Ala-O(1-5)GN (**7**)	5	*N*-Boc-Ala-Ala-*p*NA	82	This work
8	Ficin	*N*-Boc-Ala-O(1-6)GN (**8**)	5	*N*-Boc-Ala-Ala-*p*NA	73	This work
9	Ficin	*N*-Boc-Ala-O(2-5)GN (**9**)	5	*N*-Boc-Ala-Ala-*p*NA	96	This work
10	Ficin	*N*-Boc-Ala-Ph (**10**)	5	*N*-Boc-Ala-Ala-*p*NA	88	This work
11	Bovine trypsin	*N*-Boc-Ala-OAm (**1**)	10	*N*-Boc-Ala-Ala-*p*NA	77	Itoh et al. (1996)
12	Bovine trypsin	*N*-Boc-Ala-O*p*Gu (**2**)	10	*N*-Boc-Ala-Ala-*p*NA	64	Sekizaki et al. (1996b)
13	Bovine trypsin	*N*-Boc-Ala-O*m*Gu (**3**)	(12)	*N*-Boc-Ala-Ala-*p*NA	58	Sekizaki et al. (1999a)
14	Bovine trypsin	*N*-Boc-Ala-O*p*GM (**4**)	(5)	*N*-Boc-Ala-Ala-*p*NA	95	Sekizaki et al. (1998)
15	Bovine trypsin	*N*-Boc-Ala-O*m*GM (**5**)	(12)	*N*-Boc-Ala-Ala-*p*NA	67	Sekizaki et al. (1999a)
16	Bovine trypsin	*N*-Boc-Ala-O(1-4)GN (**6**)	(1)	*N*-Boc-Ala-Ala-*p*NA	79	Sekizaki et al. (1999b)
17	Bovine trypsin	*N*-Boc-Ala-O(1-5)GN (**7**)	(2)	*N*-Boc-Ala-Ala-*p*NA	72	Sekizaki et al. (1999b)
18	Bovine trypsin	*N*-Boc-Ala-O(1-6)GN (**8**)	(12)	*N*-Boc-Ala-Ala-*p*NA	75	Sekizaki et al. (1999b)
19	Bovine trypsin	*N*-Boc-Ala-O(2-5)GN (**9**)	(12)	*N*-Boc-Ala-Ala-*p*NA	88	Sekizaki et al. (1999b)
20	CS trypsin	*N*-Boc-Ala-OAm (**1**)	10	*N*-Boc-Ala-Ala-*p*NA	72	Sekizaki et al. (2001)
21	CS trypsin	*N*-Boc-Ala-O*p*Gu (**2**)	10	*N*-Boc-Ala-Ala-*p*NA	52	Sekizaki et al. (2001)
22	CS trypsin	*N*-Boc-Ala-O*m*Gu (**3**)	(1)	*N*-Boc-Ala-Ala-*p*NA	41	Sekizaki et al. (2001)
23	CS trypsin	*N*-Boc-Ala-O*p*GM (**4**)	(24)	*N*-Boc-Ala-Ala-*p*NA	67	Sekizaki et al. (2001)
24	CS trypsin	*N*-Boc-Ala-O*m*GM (**5**)	(6)	*N*-Boc-Ala-Ala-*p*NA	61	Sekizaki et al. (2001)
25	CS trypsin	*N*-Boc-Ala-O(1-4)GN (**6**)	30	*N*-Boc-Ala-Ala-*p*NA	72	Sekizaki et al. (2001)
26	CS trypsin	*N*-Boc-Ala-O(1-5)GN (**7**)	(1)	*N*-Boc-Ala-Ala-*p*NA	64	Sekizaki et al. (2001)
27	CS trypsin	*N*-Boc-Ala-O(1-6)GN (**8**)	(15)	*N*-Boc-Ala-Ala-*p*NA	67	Sekizaki et al. (2001)
28	CS trypsin	*N*-Boc-Ala-O(2-5)GN (**9**)	(12)	*N*-Boc-Ala-Ala-*p*NA	67	Sekizaki et al. (2001)
29	Thrombin	*N*-Boc-Ala-OAm (**1**)	30	*N*-Boc-Ala-Ala-*p*NA	73	Sekizaki et al. (1999c)
30	Thrombin	*N*-Boc-Ala-O*p*Gu (**2**)	(1)	*N*-Boc-Ala-Ala-*p*NA	63	Sekizaki et al. (1999c)
31	Thrombin	*N*-Boc-Ala-O*m*Gu (**3**)	(3)	*N*-Boc-Ala-Ala-*p*NA	51	Sekizaki et al. (1999c)
32	Thrombin	*N*-Boc-Ala-O*p*GM (**4**)	30	*N*-Boc-Ala-Ala-*p*NA	76	Sekizaki et al. (1999c)
33	Thrombin	*N*-Boc-Ala-O*m*GM (**5**)	(5)	*N*-Boc-Ala-Ala-*p*NA	55	Sekizaki et al. (1999c)
34	Thrombin	*N*-Boc-Ala-O(1-4)GN (**6**)	45	*N*-Boc-Ala-Ala-*p*NA	69	Sekizaki et al. (1999c)
35	Thrombin	*N*-Boc-Ala-O(1-5)GN (**7**)	(1.5)	*N*-Boc-Ala-Ala-*p*NA	63	Sekizaki et al. (1999c)
36	Thrombin	*N*-Boc-Ala-O(1-6)GN (**8**)	(1)	*N*-Boc-Ala-Ala-*p*NA	53	Sekizaki et al. (1999c)
37	Thrombin	*N*-Boc-Ala-O(2-5)GN (**9**)	(3)	*N*-Boc-Ala-Ala-*p*NA	71	Sekizaki et al. (1999c)

[a] Conditions: Acyl donor, 1 mM; acyl acceptor (Ala-*p*NA), 35 mM; ficin, 0.1 U; 40% DMSO-GTA (50 mM, pH 9.0); 37 °C. [b] Conditions: Acyl donor, 1 mM; acyl acceptor (Ala-*p*NA), 20 mM; bovine trypsin, 10 µM; 50% DMSO-MOPS (50 mM, pH 8.0, containing 20 mM $CaCl_2$); 25 °C. [c] Conditions: Acyl donor, 1 mM; acyl acceptor (Ala-*p*NA), 20 mM; chum salmon trypsin, 10 µM; 50% DMSO-MOPS (50 mM, pH 8.0, containing 20 mM $CaCl_2$); 25 °C. [d] Conditions: Acyl donor, 1 mM; acyl acceptor (Ala-*p*NA), 20 mM; thrombin, 5 µM; 50% DMSO-GTA (50 mM, pH 8.0, containing 20 mM $CaCl_2$); 25 °C. [e] The values in parentheses are reaction time (h). [f] The values represent the mean of two runs (each value in within 2% variation)

DMF and acetonitrile. The reaction was evaluated by changing the conditions such as the pH of the medium and the concentration of the acyl acceptor (L-Ala-*p*NA).

Effects of DMSO, DMF and acetonitrile concentration on coupling yields are shown in Fig. 2. The coupling product was obtained in high yield with acetonitrile rather than with DMSO and DMF. The effect of DMF concentration was similar to that of DMSO, but the coupling yields were slightly lower. Coupling yields higher than 50% were observed at the DMSO concentration range of 30–50%, and the best yield (85%) was obtained at 40% DMSO. The acetonitrile demonstrated a slight difference in comparison with DMSO or DMF and its best yield (92%) was obtained at a concentration of 30%. At a low concentration, acetonitrile demonstrates a decrease in the solubility of the substrates such as GN derivatives. The DMSO was used, therefore, to organic solvent in this study. Although high concentration of an organic solvent prevents the hydrolysis of the acyl enzyme, a decrease in the enzymatic activity is visible due to the denaturation of its enzyme (West and Wong, 1986). Consequently, the coupling yield was diminished at a concentration of organic solvents above 60%.

The effect of the buffer component that regulates the pH of the medium was also analyzed. DMSO was mixed with 50 mM solution of GTA buffer at several pHs. The pH values shown in Fig. 3 are those of the buffer before addition of the organic solvent. Reaction yields were determined for 40% DMSO solutions, with different pH of the buffer solution. The pH dependency on the yield at the reaction time of 5 min was determined as shown in Fig. 3. The best yield was obtained at pH 9 even though the optimum pH value for ficin-catalyzed hydrolysis is considered to be around 7 (Hammond and Gutfreund, 1959).

Its dependency can be explained to be due to the saturation of the enzymes binding site that couples with the acyl acceptor. The reaction yield reached the maximum of 85% at a concentration around 35 mM of the acyl acceptor (Fig. 4).

As described in this method, standard conditions was selected for the ficin-catalyzed peptide coupling reaction as follows: acyl donor, 1 mM; acyl acceptor, 35 mM; ficin, 0.1 U; solvent, 40% DMSO-GTA (50 mM, pH 9.0); reaction temperature, 37 °C.

The products of ficin-catalyzed coupling reaction were compared with those of bovine trypsin (Itoh et al., 1996; Sekizaki et al., 1998, 1999c), CS trypsin (Sekizaki et al., 2001) and thrombin-catalyzed coupling reaction (Sekizaki et al., 1999c) as previously reported. The results are summarized in Table 1.

A comparative study of cystein protease and serine protease as the catalyst of peptide coupling led us to the conclusion that all substrates (**1**–**9**) behave as a good acyl donor for both cystein and serine protease-catalyzed reaction. The coupling yields by cystein protease (Entry 1–9) are somewhat more significant compared with those of the serine protease (Entry 11–37). The coupling time of cystein protease is considerably faster than that of the serine protease. Moreover, ficin-catalyzed condensation of *N*-Boc-alanine phenylester (**10**) with Ala-*p*NA resulted in the formation of *N*-Boc-Ala-Ala-*p*NA in good yield (Entry 10). This result showed that the charged amidinium and guanidinium groups (site-specific groups for trypsin) were not required as acyl donor components. This result was important, because generally the synthesis of these compounds was not so simplicity (Fujioka et al., 1980; Sekizaki et al., 1996a, b, 1999a, b).

Acknowledgment

This work was supported in part by a grant from the Japan Private School Promotion Foundation.

References

Bieth J, Weremath C (1970) The action of elastase on *p*-nitroanilide substrates. Biochem Biophys Res Commun 53: 383–390

Bordusa F (2002) Protease in organic synthesis. Chem Rev 102: 4817–4867

Bordusa F, Ullmann D, Elsner C, Jakubke H-D (1997) Angew Chem Int Ed Engl 36: 2473–2475

Fujioka Y, Tanizawa K, Nakayama H, Kanaoka Y (1980) Inverse substrates IX. Amidinophenyl esters from amino acids and peptides: synthesis and properties as trypsin substrates. Chem Pharm Bull 28: 1899–1905

Gill I, Fandino RL, Jobra X, Vulfson EN (1996) Biologically active peptides and enzymatic approaches to their production. Enzyme Microb Technol 18: 162–183

Hammond BR, Gutfreund H (1959) The mechanism of ficin-catalyzed reaction. Biochem J 72: 349–357

Hänsler M, Ullmann G, Jakubke H-D (1995) The application of papain, ficin and clostripain in kinetically controlled peptide synthesis in frozen aqueous solutions. J Peptide Sci 1: 283–287

Itoh K, Sekizaki H, Toyota E, Fijiwara N, Tanizawa K (1996) Application of inverse substrates to trypsin-catalyzed peptide synthesis. Bioorg Chem 24: 59–68

Kumar D, Bhalla TC (2005) Microbial proteases in peptide synthesis: approaches and application. Appl Microbiol Biotechnol 68: 726–736

Monter B, Herzog B, Stehle P, Fürst P (1991) Kinetically controlled synthesis of dipeptides using ficin as biocatalyst. Biotechnol Appl Biochem 14: 183–191

Nakatsuka T, Sasaki T, Kaiser ET (1987) Peptide segment coupling catalyzed by the semisynthetic enzyme thiolsubtilisin. J Am Chem Soc 109: 3808–3810

Nishimura T, Kato H (1988) Taste of free amino acids and peptides. Food Rev Int 4: 175–194

Schellenberger V, Jakubke H-D (1991) Protease-catalyzed kinetically controlled peptide synthesis. Angew Chem Int Ed Engl 30: 1434–1449

Sekizaki H, Itoh K, Toyota E, Tanizawa K (1996a) Synthesis and tryptic hydrolysis of *p*-guanidinophenyl esters derived from amino acids and peptides. Chem Pharm Bull 44: 1577–1579

Sekizaki H, Itoh K, Toyota E, Tanizawa K (1996b) Trypsin-catalyzed peptide synthesis with various *p*-guanidinophenyl ester as acyl donors. Chem Pharm Bull 44: 1585–1587

Sekizaki H, Itoh K, Toyota E, Tanizawa K (1998) Enzymatic peptide synthesis with *p*-guanidinophenyl and *p*-(guanidinomethyl)phenyl esters as acyl donors. Chem Pharm Bull 46: 846–849

Sekizaki H, Itoh K, Toyota E, Tanizawa K (1999a) Trypsin-catalyzed peptide synthesis with *m*-guanidinophenyl and *m*-(guanidinomethyl)-phenyl esters as acyl donor component. Amino Acids 17: 285–291

Sekizaki H, Itoh K, Toyota E, Tanizawa K (1999b) The structural requirements for an inverse substrate for enzymatic peptide synthesis: position isomers of guanidinonaphthyl esters as the acyl donor component. Chem Pharm Bull 47: 104–110

Sekizaki H, Itoh K, Toyota E, Tanizawa K (1999c) Application of various inverse substrates to thrombin-catalyzed peptide synthesis. Chem Pharm Bull 47: 444–447

Sekizaki H, Murakami M, Itoh K, Toyota E, Tanizawa K (2000) Chum salmon trypsin-catalyzed peptide synthesis with inverse substrates as acyl donor components at low temperature. J Mol Catal B Enzym 11: 23–28

Sekizaki H, Itoh K, Toyota E, Tanizawa K (2001) Chum salmon trypsin-catalyzed preferential formation of peptides containing D-amino acid. Amino Acids 21: 175–184

Tai D-F, Huang H-Y, Huang C-C (1995) Immobilized ficin catalyzed synthesis of peptides in organic solvent. Bioorg Med Chem Lett 5: 1475–1478

Tsuzuki H, Oka T, Morihara K (1980) Coupling between Cbz-Arg-OH and Leu-X catalyzed by trypsin and papain. J Biochem 88: 669–675

Wong C-H (1989) Enzymatic catalysts in organic synthesis. Science 244: 1145–1152

Authors' address: Prof. Haruo Sekizaki, Faculty of Pharmaceutical Sciences, Health Sciences University of Hokkaido, Ishikari-Tobetsu, Hokkaido 061-0293, Japan,
Fax: +81-133 23 3791, E-mail: sekizaki@hoku-iryo-u.ac.jp