Trypsin-Catalyzed Peptide Synthesis with Various *p*-Guanidinophenyl Esters as Acyl Donors[1)]

Haruo SEKIZAKI, Kunihiko ITOH, Eiko TOYOTA, and Kazutaka TANIZAWA*

Faculty of Pharmaceutical Sciences, Health Sciences University of Hokkaido, Ishikari-Tobetsu, Hokkaido 061-02, Japan. Received January 12, 1996; accepted March 8, 1996

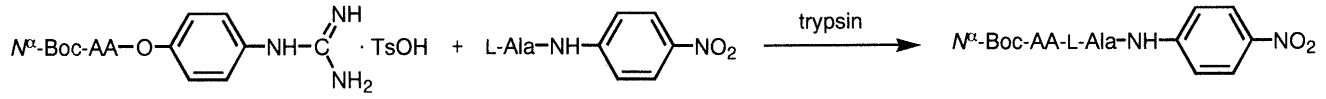
Trypsin-catalyzed peptide synthesis has been studied by using *p*-guanidinophenyl esters of N^{α}-(*tert*-butyloxycarbonyl)amino acid and peptide as acyl donor components. The reaction conditions were optimized for organic solvents, pH, and concentration of acceptor. The method was especially useful for the preparation of various peptides containing D-amino acids. The enzymatic hydrolysis of the resulting products was negligible.

Key words trypsin; inverse substrate; peptide synthesis; N^{α}-(*tert*-butyloxycarbonyl)amino acid *p*-guanidinophenyl ester; N^{α}-(*tert*-butyloxycarbonyl)peptide *p*-guanidinophenyl ester

Peptide synthesis by protease-catalyzed reverse reaction has been extensively studied with a variety of model oligopeptides.[2)] In the previous papers, we reported that the *p*-guanidinophenyl esters as inverse substrates were readily synthesized and these synthetic substrates are specifically hydrolyzed by trypsin and trypsin-like enzymes.[3-5)] It is supposed that the acyl moieties are transferred to the enzyme's catalytic residue in the form of acyl trypsin intermediates during the course of the hydrolysis. These intermediates are expected to be useful for trypsin-catalyzed peptide synthesis. Schellenberger *et al.* reported[6)] successful trypsin-catalyzed peptide synthesis by using N^{α}-Cbz-L-Ala and N^{α}-Cbz-L-Pro *p*-guanidinophenyl esters as acyl donors. However, the conditions of trypsin-catalyzed peptide synthesis was not described in detail. The purpose of the present study is to elucidate the requirements for trypsin-catalyzed peptide coupling reaction of inverse substrates with a view to utilizing the reaction as a preparative method for peptide synthesis.

Trypsin-Catalyzed Peptide Coupling Reaction The trypsin-catalyzed peptide coupling reaction has been studied by using synthetic inverse substrates (**1**—**15**) as acyl donors. The coupling reaction was carried out by incubating an acyl donor (1 mM) with an acyl acceptor (L-Ala-*p*NA) (**16**) (20 mM) and trypsin (10 μM) in a mixture of MOPS buffer (50 mM, pH 8.0, containing 20 mM $CaCl_2$) and DMSO (1 : 1) at 25 °C (Chart 1). The progress of the coupling reaction was monitored by HPLC. Elution peaks were correlated to those of authentic samples which were chemically synthesized according to the reported procedure.[7)] The yields of the coupling reaction are summarized in Table 1. In all cases, trypsin was moderately effective for the synthesis of the peptides. The effect of reaction conditions on the yield of N^{α}-Boc-L-Ala-L-Ala-*p*NA (IV) and N^{α}-Boc-D-Ala-L-Ala-*p*NA (V) was further investigated with regard to reaction media, pH, acyl acceptor concentration, and reaction time.

The effects of DMSO, DMF, and acetonitrile concentra-



1~15 **16** **I~XV**

Chart 1

Table 1. Yield of Trypsin-Catalyzed Peptide Synthesis[a)]

Acyl donor	Yield (%)	Product
N^{α}-Boc-Gly-OGp (**1**)	61	N^{α}-Boc-Gly-L-Ala-*p*NA (I)
N^{α}-Boc-Gly-Gly-OGp (**2**)	60	N^{α}-Boc-Gly-Gly-L-Ala-*p*NA (II)
N^{α}-Boc-Gly-Gly-Gly-OGp (**3**)	79	N^{α}-Boc-Gly-Gly-Gly-L-Ala-*p*NA (III)
N^{α}-Boc-L-Ala-OGp (**4**)	64	N^{α}-Boc-L-Ala-L-Ala-*p*NA (IV)
N^{α}-Boc-D-Ala-OGp (**5**)	74	N^{α}-Boc-D-Ala-L-Ala-*p*NA (V)
N^{α}-Boc-L-Leu-OGp (**6**)	82	N^{α}-Boc-L-Leu-L-Ala-*p*NA (VI)
N^{α}-Boc-D-Leu-OGp (**7**)	76	N^{α}-Boc-D-Leu-L-Ala-*p*NA (VII)
N^{α}-Boc-L-Phe-OGp (**8**)	82	N^{α}-Boc-L-Phe-L-Ala-*p*NA (VIII)
N^{α}-Boc-D-Phe-OGp (**9**)	78	N^{α}-Boc-D-Phe-L-Ala-*p*NA (IX)
N^{α}-Boc-L-Ala-Gly-OGp (**10**)	85	N^{α}-Boc-L-Ala-Gly-L-Ala-*p*NA (X)
N^{α}-Boc-D-Ala-Gly-OGp (**11**)	91	N^{α}-Boc-D-Ala-Gly-L-Ala-*p*NA (XI)
N^{α}-Boc-L-Ala-L-Ala-OGp (**12**)	80	N^{α}-Boc-L-Ala-L-Ala-L-Ala-*p*NA (XII)
N^{α}-Boc-D-Ala-D-Ala-OGp (**13**)	75	N^{α}-Boc-D-Ala-D-Ala-L-Ala-*p*NA (XIII)
N^{α}-Boc-L-Phe-D-Ala-OGp (**14**)	80	N^{α}-Boc-L-Phe-D-Ala-L-Ala-*p*NA (XIV)
N^{α}-Boc-L-Phe-L-Phe-OGp (**15**)	78	N^{α}-Boc-L-Phe-L-Phe-L-Ala-*p*NA (XV)

a) Conditions: acyl donor, 1 mM; acyl acceptor (L-Ala-*p*NA), 20 mM; trypsin, 10 μM; 50% DMSO–MOPS (50 mM, pH 8.0, containing 20 mM $CaCl_2$); 25 °C.

* To whom correspondence should be addressed.

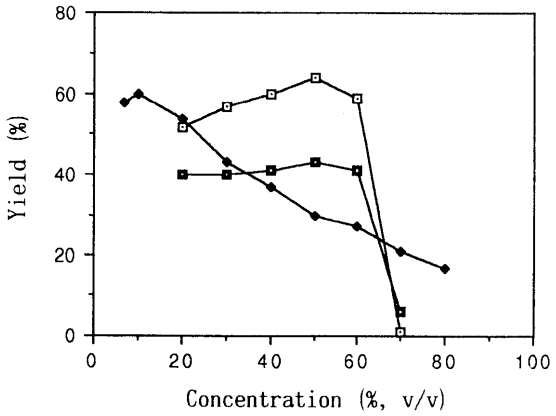


Fig. 1. The Effect of Reaction Solvent on the Trypsin-Catalyzed Synthesis of N^{α}-Boc-L-Ala-L-Ala-*p*NA (IV)

The reaction mixture contained 1 mM N^{α}-Boc-L-Ala-OGp (**4**), 20 mM L-Ala-*p*NA (**16**), and 10 μM trypsin in 50 mM MOPS (pH 8.0). Cosolvents are DMSO (⊡), DMF (■), and acetonitrile (◆).

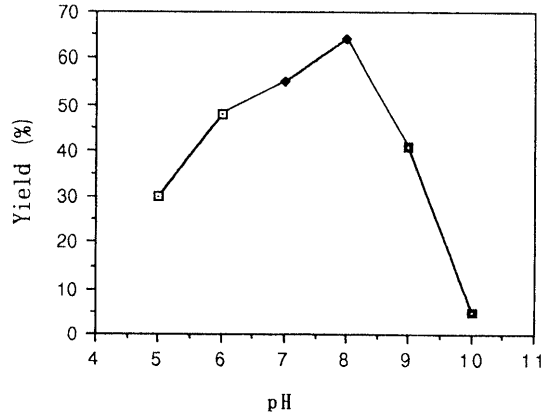


Fig. 2. pH Profile for the Synthesis of N^{α}-Boc-L-Ala-L-Ala-*p*NA (IV)

The concentrations of N^{α}-Boc-L-Ala-OGp (**4**), L-Ala-*p*NA (**16**), and trypsin were 1 mM, 20 mM, and 10 μM, respectively. The reaction was carried out in 50% DMSO solution. The pH values on the abscissa are those of MES (⊡), MOPS (◆), or carbonate (■) buffers used for the preparation of aqueous organic solvents.

tions on the coupling yields are shown in Fig. 1. Coupling yields higher than 50% were observed at the DMSO concentration range of 20—60%, and the highest yield (64%) was obtained at 50% DMSO. The effect of DMF concentration was similar to that of DMSO, but the coupling yields were lower than those in DMSO. On the other hand, acetonitrile showed different behavior; the highest yield (60%) was obtained at 10% concentration, and the peptide yields decreased at higher concentrations. Wong and West[8] reported that coupling yield generally increases with increasing dielectric constant of the reaction media and the two correlate quite well. The high yields were achieved at the dielectric constant range of 65—75 in 50—60% aqueous DMSO and 50—60% aqueous DMF, respectively. The dielectric constants of 10% and 20% aqueous acetonitrile are 73 and 69, respectively. The coupling was sufficient in this range of dielectric constant. Although a high concentration of organic solvent prevents the hydrolysis of the acyl enzyme, it decreases the enzymatic activity due to the denaturation of trypsin.[8] Consequently, the coupling yield was decreased at higher concentration of organic solvents than 60%. Less than 10% of organic solvents resulted in a turbid solution due to the insolubility of the acyl donor.

The effect of pH of the reaction medium on the coupling yields was analyzed. DMSO was mixed with MES (50 mM, containing 20 mM $CaCl_2$), MOPS, and carbonate buffers with various pH values, as shown in Fig. 2. The pH values given in Fig. 2 are those of the buffer itself before mixing with organic cosolvent. The pH-dependency of the coupling yield for the reaction period of 20 min was determined (Fig. 2). The yield was greatly decreased at higher pH than 9.

The effect of acyl acceptor concentration on the coupling yields in 50% aqueous DMSO is shown in Fig. 3. This result is similar to those in trypsin-catalyzed[9] and chymotrypsin-catalyzed[8,10] peptide synthesis using conventional substrates. The dependency can be interpreted as being due to the saturation of the enzyme binding site with the acyl acceptor. The reaction yield was improved to 64% when a twenty times higher concentration (20 mM) of acyl acceptor was used.

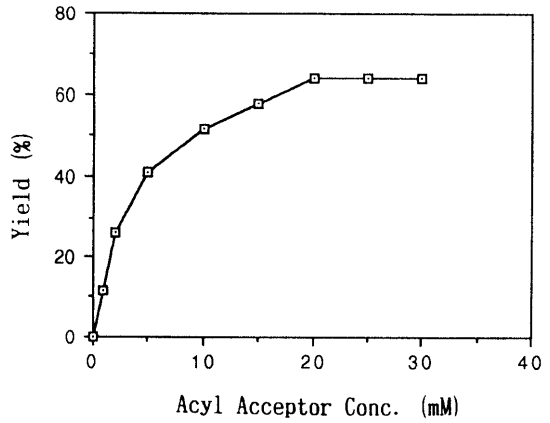


Fig. 3. The Effect of Acyl Acceptor Concentration on the Synthesis of N^{α}-Boc-L-Ala-L-Ala-*p*NA (IV)

Conditions are the same as those in Table 1.

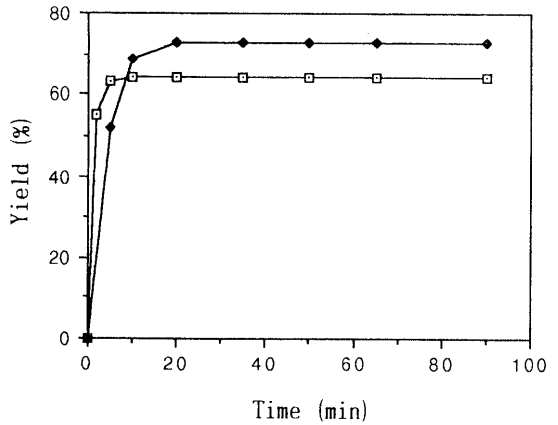


Fig. 4. Time Course for the Trypsin-Catalyzed Synthesis of N^{α}-Boc-L-Ala-L-Ala-*p*NA (IV) (⊡) and N^{α}-D-Ala-L-Ala-*p*NA (V) (◆)

Conditions are the same as those in Table 1.

The time courses of the coupling of N^{α}-Boc-L-Ala-OGp (**4**) and N^{α}-Boc-D-Ala-OGp (**5**) with L-Ala-*p*NA (**16**) are shown in Fig. 4. The D-acyl donor is an efficient substrate for the enzymatic coupling reaction, as well as the L-acyl donor. These results are summarized in Table 1. The coupling yields were not changed after a longer period of incubation. This result indicated that enzymatic hydrol-

ysis of the products is negligible. Schellenberger *et al.*[6] noted the suitability of inverse substrates for trypsin-catalyzed peptide synthesis, and they focused on the determination of "partition constant" of the substrates, an index of coupling efficiency over hydrolysis. We were interested in the development of inverse substrates as a new, practical tool for peptide synthesis.

Experimental

The melting points were measured on a Yanagimoto melting point apparatus. The optical rotations were measured with a JASCO DIP-360 digital polarimeter in a 5 cm cell. Bovine pancreas trypsin (EC 3.4.21.4) purchased from Worthington Biochemical Corp. (twice crystallized, lot TRL) was further purified by affinity chromatography using benzamidine-Sepharose 6B (Pharmacia).[11] The preparation of substrates (**1**—**15**) was reported previously.[2] L-Ala–*p*NA (**16**) and N^{α}-Boc-L-Ala-L-Ala-*p*NA (IV) were purchased from Peptide Research Foundation and Sigma Chemical Company, respectively.

Synthesis of Authentic Samples Authentic samples were chemically synthesized according to the reported procedure.[7] V: Colorless powder. mp 188—189 °C (AcOEt–hexane). $[\alpha]_D^{25}$ −93.8° (c=1.0, MeOH). *Anal.* Calcd for $C_{17}H_{24}N_4O_6$: C, 53.68; H,6.36; N, 14.73. Found: C, 53.85; H, 6.44; N, 14.56. VI: Colorless powder. mp 159—160 °C (AcOEt–hexane). $[\alpha]_D^{25}$ −76.0° (c=1.0, MeOH). *Anal.* Calcd for $C_{20}H_{30}N_4O_6$: C, 56.86; H, 7.16; N, 13.26. Found: C, 56.77; H, 7.16; N, 13.17. VII: Colorless needles. mp 204—205 °C (AcOEt). $[\alpha]_D^{25}$ −45.6° (c=1.0, MeOH). *Anal.* Calcd for $C_{23}H_{28}N_4O_6$: C, 60.52; H, 6.18; N, 12.27. Found: C, 60.47; H, 6.19; N, 12.29. IX: Colorless needles. mp 148—150 °C (AcOEt). $[\alpha]_D^{25}$ −89.4° (c=1.0, MeOH). *Anal.* Calcd for $C_{23}H_{28}N_4O_6$: C, 60.52; H, 6.18; N, 12.27. Found: C, 60.45; H, 6.20; N, 12.18.

Trypsin-Catalyzed Peptide Coupling Reaction Enzyme concentration was determined by active site titration with *p*-nitrophenyl *p*-guanidinobenzoate.[12] A mixture of 50 μl of acyl donor stock solution (10 mM DMSO solution of **1**—**15**), 50 μl of acyl acceptor stock solution (200mM DMSO solution of L-alanine *p*-nitroanilide (**16**)), 240 μl of 50 mM MOPS buffer (containing 20 mM of $CaCl_2$, pH 8), 150 μl of DMSO, and 10 μl of trypsin stock solution (1 mM solution in 1 mM HCl) was incubated at 25 °C. The progress of the coupling reaction was monitored by HPLC under the following conditions: column i.d. 4.0 × 250 mm Wakosil 5C 18-200, isocratic elution at 1 ml/min, 0.1% trifluoroacetic acid/acetonitrile. An aliquot of the reaction mixture was injected and peaks were detected at 310 nm (*p*-nitroanilide moiety).

Acknowledgment This work was supported in part by a Grant-in-Aid for Encouragement of Young Scientists (No. 07772105) from the Ministry of Education, Science, Sports and Culture of Japan.

References and Notes

1) The following abbreviations are used: Boc = *tert*-butyloxycarbonyl, Cbz = benzyloxycarbonyl, DMF = *N*,*N*-dimethylformamide, DMSO = dimethyl sulfoxide, MOPS = 3-morpholino-1-propanesulfonate, MES = 2-morpholino-1-ethanesulfonate, Gp = *p*-guanidinophenyl, *p*NA = *p*-nitroanilide.
2) For example: Tsuzuki H., Oka T., Morihara K., *J. Biochem.*, **88**, 669—675 (1980); Nakatuka T., Sakaki T., Kaiser E. T., *J. Am. Chem. Soc.*, **109**, 3808—3810 (1987); Wong C.-H., *Science*, **244**, 1145—1152 (1989); Schellenberger V., Jakubke H.-D., *Angew. Chem. Int. Ed. Engl.*, **30**, 1437—1449 (1991).
3) Itoh K., Sekizaki H., Toyota E., Tanizawa K., *Chem. Pharm. Bull.*, **43**, 2082—2087 (1995); Sekizaki H., Itoh K., Toyota E., Tanizawa K., *ibid.*, **44**, 1577—1579 (1996).
4) Tanizawa K., Kasaba Y., Kanaoka Y., *J. Am. Chem. Soc.*, **99**, 4485—4488 (1977).
5) Nozawa M., Tanizawa K., Kanaoka Y., *J. Pharmacobio-Dyn.*, **3**, 213—219 (1980).
6) Schellenberger V., Jakubke H.-D., Zapevalova N. P., Mitin Y. V., *Biotechnol. Bioeng.*, **38**, 104—108 (1991); Schellenberger V., Schellenberger U., Jakubke H.-D., Zapevalova N.P., Mitin Y.V., *ibid.*, **38**, 319—321 (1991).
7) Kawai M., Nagai U., *Bull. Chem. Soc. Jpn.*, **55**, 1327—1328 (1982); Okada Y., Tsuda Y., Hirata A., Nagamatsu Y., Okamoto U., *Chem. Pharm. Bull.*, **30**, 4060—4068 (1982); Bieth J., Weremath C. G., *Biochem. Biophys. Res. Commun.*, **53**, 383—390 (1970).
8) West J. B., Wong C.-H., *J. Org. Chem.*, **51**, 2728—2735 (1986).
9) Tsuzuki H., Oka T., Morihara K., *J. Biochem.*, **88**, 669—675 (1980).
10) Oka T., Morihara K., *J. Biochem.*, **84**, 1277—1283 (1978).
11) Hixson H. F., Jr., Nishikawa A. H., *Arch. Biochem. Biophys.*, **154**, 501—509 (1973).
12) Chase T., Shaw E., *Biochem. Biophys. Res. Commun.*, **29**, 508—514 (1967).