BIOORGANIC CHEMISTRY **25,** 307–319 (1997)
ARTICLE NO. BH971076

Methyltrypsin-Catalyzed Peptide Coupling: Comparison of Alkyl Ester and Guanidinophenyl Ester Derivatives as Acyl Donor Component[1]

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Received August 15, 1997

Methyltrypsin-catalyzed peptide synthesis has been studied by using conventional alkyl ester and *p*-guanidinophenyl ester derivatives of α-amino acid as the acyl donor component. They were found to be coupled with α-amino acid derivatives (acyl acceptor component) to produce dipeptide. The behavior of methyltrypsin toward both the substrates has been studied.

INTRODUCTION

It is well known that arginine- or lysine-containing peptides interact specifically with trypsin-like enzymes and hence possess a variety of pharmacological activities. However, chemical synthesis of these peptides is tedious. Synthesis of arginyl peptides, for example, requires the extensive protection of the guanidino side-chain. Enzymatic peptide synthesis is more advantageous than chemical synthesis in many respects since it is highly stereoselective and racemization-free and requires minimal side-chain protection (*1*, *2*). Enzymatic peptide synthesis, however, has two serious drawbacks. One of them is the potential loss of the product due to the hydrolysis of the resulting peptide bonds by the enzyme. Another serious defect is the discrimination of amino acid residues by the enzyme being employed in the enzymatic synthesis. In the case of trypsin-catalyzed coupling, for example, the acyl donor is limited to peptides containing a positively charged arginine or lysine residue at the carboxyl end position. As a solution to the former problem, application of chemically modified enzymes such as methylchymotrypsin (*3*, *4*) and methylsubtilisin (*5*) has been developed by C.-H. Wong *et al*. The latter problem could be solved by use of inverse substrates, as reported in our previous papers (*6–8*).

It has been demonstrated that methyltrypsin can be easily prepared from the inexpensive commercial bovine trypsin (*9–11*). Methyltrypsin is known to lose the amidase activity of the original enzyme whereas the esterase activity is retained (*3*, *4*). We wish now to report the application of methyltrypsin as a catalyst for peptide

[1] Dedicated to Dr. Yuichi Kanaoka (Editorial Board of this Journal, Emeritus Professor of Hokkaido University) on the occasion of his 70th birthday.

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0045-2068/97 $25.00

synthesis by using conventional substrates (Z-L-Lys-OR and Bz-L-Arg-OEt) and inverse substrates for trypsin (Boc-amino acid *p*-guanidinophenyl esters) (*12*, *13*) as the acyl donor component.

EXPERIMENTAL PROCEDURES

Melting points were determined on a Yanaco MP-500 melting point apparatus and were uncorrected. IR spectra were recorded on a JASCO VALOR-III FT-IR spectrometer. ^{1}H-NMR spectra were recorded on a JEOL JNM-FX-400 FT NMR spectrometer. Optical rotations were measured with a JASCO DIP-360 digital polarimeter. Kinetic parameters were determined with a Union Giken RA-401 stopped-flow spectrometer, a Hitachi U-2,000 UV spectrophotometer, and a Radiometer TTT-80 pH-stat. HPLC was performed on a Shimadzu LC-6A pump system equipped with a Shimadzu SPD-6AV UV–VIS spectrophotometric detector.

Materials

Bovine pancreatic trypsin (EC 3.4.21.4) was purchased from Worthington Biochemical Co. (twice recrystallized, lot TRL). It was further purified by the treatment with L-1-*p*-tosylamino-2-phenylethyl chloromethyl ketone (TPCK) and subsequently by affinity chromatography (*14*) using Benzamidine Sepharose 6B (Pharmacia). *m*-Aminobenzenesulfonic acid (metanilic acid) and cyanamide were purchased from Kanto Chemical Co., Inc. Trimethyloxonium tetrafluoroborate and TPCK were products of Aldrich Chemical Co., Inc. *p*-Nitrophenyl-*p'*-guanidinobenzoate hydrochloride (NPGB) was obtained from Merck Co., Inc. (*p*-Amidinophenyl)methanesulfonyl fluoride hydrochloride was purchased from Wako Pure Chemical Industries, Ltd. N^{α}-(benzyloxycarbonyl)-N^{ε}-(*tert*-butyloxycarbonyl)-L-lysine [Z-L-Lys(Boc)-OH] and N^{α}-(benzyloxycarbonyl)-L-lysine methyl ester hydrochloride (Z-L-Lys-OMe · HCl) were obtained from Calbiochem-Novabiochem International, Inc. L-Alanine *p*-nitroanilide (L-Ala-*p*NA), L-leucine amide (L-Leu-NH_2), and N^{α}-(benzoyl)-L-arginine ethyl ester hydrochloride (Bz-L-Arg-OEt · HCl) were supplied from Peptide Institute, Inc. N^{α}-(benzoyl)-L-phenylalanyl-L-valyl-L-arginine *p*-nitroanilide (Bz-L-Phe-L-Val-L-Arg-*p*NA) was purchased from Sigma Chemical Co. Inverse substrates [N^{α}-(*tert*-butyloxycarbonyl)amino acid *p*-guanidinophenyl ester *p*-toluenesulfonate (Boc-amino acid-OG*p* · TsOH)] were prepared following reported procedures (*12*, *13*).

Preparation of Methyltrypsin

Methylation of the enzyme was carried out by a modification of literature procedure (*9–11*). A solution of bovine trypsin (350 mg, 60% purity, determined from active site titration using NPGB (*15*)) in 100 ml of 50 mM Tris–HCl buffer (pH 7.4, containing 20 mM $CaCl_2$) was treated with 10 ml of 0.1 M solution of methyl *m*-guanidinobenzenesulfonate tetrafluoroborate in acetonitrile. The reaction mixture was incubated at 25°C for 3 h. During the incubation the pH was kept at 7.4 by occasional addition of 50 mM NaOH. After the pH of the reaction mixture was

lowered to 7.0 by the addition of 0.1 M HCl, (*p*-amidinophenyl)methanesulfonyl fluoride hydrochloride (5 mg) was added to the reaction mixture and incubation was continued for 10 min at 25°C. During the incubation the pH was kept at 7.0 by occasional addition of 50 mM NaOH. After the resulting precipitate was removed by centrifugation, the pH of the supernatant was adjusted to 3.0 by addition of 1 M HCl. The solution was dialyzed against 1 mM HCl (pH 3.0) at 4°C, and then pH and salt concentration of the dialysate was adjusted to those of eluate buffer. The solution was loaded on a column of Benzamidine Sepharose 6B (2.0 × 7.0 cm), which was equilibrated with 0.05 M sodium acetate buffer (pH 5.0, containing 0.02 M $CaCl_2$, and 0.1 M KCl). The column was washed with the same buffer until the effluent exhibited negligible absorbance at 280 nm. The bound methyltrypsin was eluted with 5 mM HCl (pH 2.3, containing 0.1 M KCl). The collected fractions were dialyzed against 1 mM HCl (pH 3.0). Methyltrypsin (90 mg) was obtained as a colorless powder after lyophilization.

The molar concentration of methyltrypsin was estimated spectrophotometrically from its absorbance at 280 nm assuming $E_{1\ cm} = 15.4$ and a molecular weight of 23,800 (*16*). The purity of the preparation was determined by NPGB titration (*15*). The amidase activity was determined using Bz-L-Phe-L-Val-L-Arg-*p*NA as a substrate according to the reported method (*17–19*).

Synthesis of Substrate

Synthesis of N^{α}-(tert-Butyloxycarbonyl)-L-tyrosine p-aminophenyl ester. A solution of Boc-L-tyrosine *p*-nitrophenyl ester (*20*) (443 mg, 1.1 mmol) in EtOH (30 ml) containing 10% Pd-C (10 mg) was vigorously stirred in an atmosphere of hydrogen at room temperature for 20 h. The catalyst was filtered off and the filtrate was evaporated to dryness *in vacuo*. The crude residue was subjected to the next reaction without purification.

Synthesis of N^{α}-(tert-butyloxycarbonyl)-L-tyrosine p-[N′,N″-bis(benzyloxycarbonyl)guanidino]phenyl ester (*Boc-L-Tyr-OGp*(Z_2)]. A solution of the crude aminophenyl ester (described above) and 1-[*N*,*N*′-bis(benzyloxycarbonyl)amidino]-pyrazole (*21*, *22*) (378 mg, 1.0 mmol) in absolute THF (0.5 ml) was stirred for 3 h at room temperature in an atmosphere of nitrogen. The reaction mixture was diluted with benzene-ethyl acetate (5:1) and passed through a short silica gel column (2.5 × 30 cm). The eluate was evaporated to dryness *in vacuo* and the solid residue was recrystallized from MeOH. Boc-L-Tyr-OG*p*(Z_2) (478 mg, 70%) was obtained as colorless solid. mp 131–132°C. $[\alpha]_D^{25}$ −6.9° (*c* = 1.1, CH_3CN). *Anal*. Calcd. for $C_{37}H_{38}N_4O_9 \cdot H_2O$: C, 63.42; H, 5.75; N, 8.00. Found: C, 63.52; H, 5.66; N, 7.99.

Synthesis of N^{α}-(tert-Butyloxycarbonyl)-L-tyrosine p-guanidinophenyl ester p-toluenesulfonate (*Boc-L-Tyr-OGp · TsOH*). A solution of Boc-L-Tyr-OGp(Z_2) (341 mg, 0.5 mmol) and TsOH · H_2O (95 mg, 0.5 mmol) in MeOH (30 ml) containing 10% Pd-C (5 mg) was vigorously stirred overnight in an atmosphere of hydrogen at room temperature. The catalyst was filtered off, and the filtrate was evaporated to dryness *in vacuo*. The residue was washed with dry diethyl ether. The procedure gave Boc-L-Tyr-OG*p* · TsOH (282 mg, 96%) as a colorless amorphous material. $[\alpha]_D^{25}$ + 1.6°(*c* = 1.0, MeOH). FAB-MS *m*/*z*: 415 $(M + H)^+$.

Synthesis of N^{α}-(tert-butyloxycarbonyl)-D-tyrosine p-guanidinophenyl ester p-toluenesulfonate (*Boc-D-Tyr-OGp · TsOH*). This series of compounds was obtained from Boc-D-tyrosine *p*-nitrophenyl ester by a procedure similar to that described for the L-tyrosine derivatives. Boc-D-Tyr-OG*p*(Z_2) was obtained in 62% yield as a colorless solid. mp 130–131°C. $[\alpha]_D^{25}$ + 6.4°(*c* = 1.1, CH_3CN). *Anal.* Calcd for $C_{37}H_{38}N_4O_9 \cdot H_2O$: C, 63.42; H, 5.75; N, 8.00. Found: C, 63.71; H, 5.77; N, 8.10. Boc-D-Tyr-OG*p* · TsOH was obtained in 94% yield as a colorless amorphous material. $[\alpha]_D$ − 1.6°(*c* = 1.0, MeOH). FAB-MS *m/z*: 415 $(M + H)^+$.

Synthesis of N^{α}-(benzyloxycarbonyl)-N^{ε}-(tert-butyloxycarbonyl)-L-lysine p-chlorophenyl ester (*Z-L-Lys*(*Boc*)-*OCp*). A solution of Z-L-Lys(Boc)-OH (1.141 g, 3.0 mmol) and *p*-chlorophenol (424 mg, 3.3 mmol) in a mixture of dimethylformamide (5 ml) and ethyl acetate (10 ml) was treated with dicyclohexylcarbodiimide (681 mg, 3.3 mmol) at 0°C. The reaction mixture was stirred for 1 h at 0°C and for 12 h at room temperature. The resulting precipitate of dicyclohexylurea was filtered off and the filtrate was concentrated to dryness *in vacuo*. Recrystallization from diethyl ether-hexane afforded a colorless needle (930 mg, 63%). mp 80–81°C. $[\alpha]_D^{25}$ − 6.7°(*c* = 1.0, $CHCl_3$). *Anal.* Calcd for $C_{25}H_{31}N_2O_6Cl$: C, 61.16; H, 6.36; N, 5.71; Cl, 7.22. Found: C, 61.16; H, 6.41; N, 5.69; Cl, 7.18.

Synthesis of N^{α}-(benzyloxycarbonyl)-L-lysine p-chlorophenyl ester (*Z-L-Lys-OCp*). Z-L-Lys(Boc)-OC*p* (490 mg, 1.0 mmol) was treated with 2 M HCl in dioxane (6 ml) for 2 h at room temperature. The resulting precipitate was filtrated and washed with dry diethyl ether. Recrystallization from diethyl ether-ethanol gave a colorless solid. mp 162–164°C, 385 mg (90% yield). $[\alpha]_D^{25}$ − 30.6°(*c* = 1.1, MeOH). *Anal.* Calcd for $C_{20}H_{23}N_2O_4Cl \cdot HCl$: C, 56.21; H, 5.66; N, 6.56; Cl, 16.48. Found: C, 55.70; H, 5.61; N, 6.48; Cl, 16.59.

Measurements of Enzyme Activity

The kinetic parameters, K_s, k_2, and k_3, for the hydrolysis of Bz-L-Arg-OEt, were determined by the thionine displacement method using stopped-flow techniques. The reaction was carried out in 50 mM Tris–HCl (pH 8.0, containing 20 mM $CaCl_2$) according to the reported method (*17–19*). The kinetic parameters, K_m and k_{cat}, for Boc-L-Phe-OG*p* · TsOH, were determined with a pH-stat device. The reaction was carried out in 0.1 M KCl (containing 20 mM $CaCl_2$), with 10 mM NaOH as the titrant.

Enzymatic Peptide Coupling Reactions

Peptide coupling reactions were carried out at 25°C in 50 mM 4-morpholinepropanesulfonate (MOPS) buffer, pH 8.0, containing dimethylsulfoxide (DMSO). The concentrations of acyl donor (conventional substrate), acyl acceptor (L-leucine amide, L- or D-alanine *p*-nitroanilide), and methyltrypsin were 1 mM, 100 mM (or 20 mM), and 10 μM, respectively. In the case of inverse substrate, the concentrations of acyl donor (inverse substrate), acyl acceptor (L-arginine amide, L-lysine amide, or L-alanine *p*-nitroanilide), and methyltrypsin were 1 mM, 40 mM, and 20 μM, respectively. The time course of the peptide coupling reaction was determined aliquots of the reaction mixture by HPLC. HPLC conditions were as follows: column

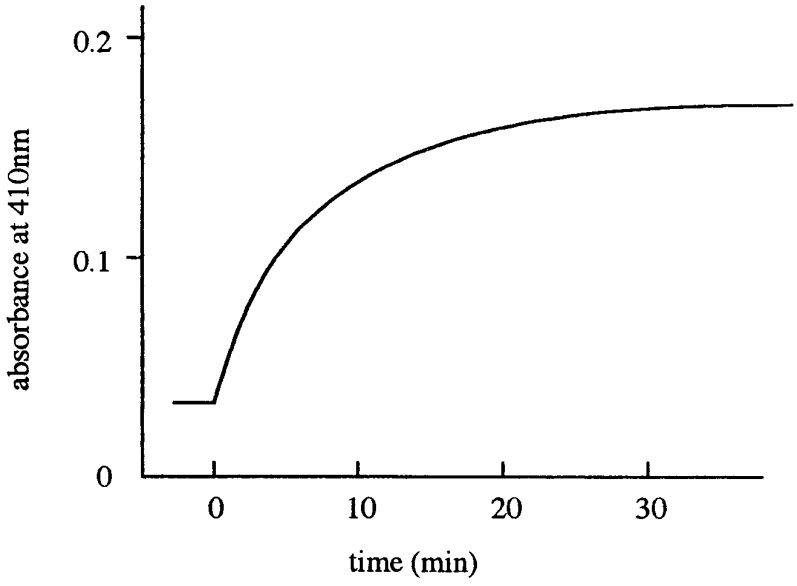

FIG. 1. Time course of methyltrypsin-catalyzed hydrolysis of NPGB. Reaction was carried out in 0.1 M Veronal buffer (pH 8.3, containing 20 mM $CaCl_2$) at 25°C. Concentration of methyltrypsin and NPGB were 8.45 and 98 μM, respectively.

TABLE 1
Comparison of Kinetic Parameters for Native Trypsin and Methyltrypsin-Catalyzed Hydrolysis of Conventional and Inverse Substrate

	$K_s(K_m)$ (M)	k_2 (s^{-1})	$k_3(k_{cat})$ (s^{-1})	k_2/K_s (k_{cat}/K_m) (s^{-1} M^{-1})	Reference
NPGB[a]					
Native trypsin	3.65×10^{-5}	2.69×10^{2}	4.10×10^{-5}	7.37×10^{6}	23
Methyltrypsin	1.84×10^{-4}	4.06×10^{-2}	Not determined	1.21×10^{2}	This work
Bz-L-Arg-OEt[b]					
Native trypsin	4.26×10^{-6}	1.10×10^{2}	2.04×10^{0}	2.58×10^{5}	This work
Methyltrypsin	1.69×10^{-3}	5.01×10^{-1}	4.02×10^{-3}	2.96×10^{2}	This work
Boc-L-Phe-OG*p*[c]					
Native trypsin	(2.25×10^{-5})	Not observed	(2.68×10)	(1.19×10^{6})	12
Methyltrypsin	(3.73×10^{-5})	Not observed	(1.13×10^{-3})	(3.03×10)	This work

[a] Reaction was carried out in 0.1 M Veronal (pH 8.3, containing 20 mM $CaCl_2$) at 25°C.

[b] Reaction was carried out in 50 mM Tris–HCl (pH 8.0, containing 20 mM $CaCl_2$) at 25°C and parameters were estimated by thionine displacement method using stopped-flow technique.

[c] Reaction was carried out in 0.1 M KCl (pH 8.0, containing 20 mM $CaCl_2$) at 25°C and parameters were estimated using pH-stat technique.

TABLE 2
Methyltrypsin-Catalyzed Peptide Coupling by Use of Conventional Substrate as Acyl Donor Component[a]

Entry no.	Acyl donor	Reaction time (h)	Product	Yield[b] (%)
1	Z-L-Lys-OMe	3	Z-L-Lys-L-Leu-NH_2	83 (53)
2	Z-L-Lys-OC*p*	0.5	Z-L-Lys-L-Leu-NH_2	28 (7)
3	Bz-L-Arg-OEt	4	Bz-L-Arg-L-Leu-NH_2	66 (35)
4	Z-L-Lys-OMe	24	Z-L-Lys-D-Leu-NH_2	Not detected
5	Z-L-Lys-OMe	3	Z-L-Lys-L-Ala-*p*NA	80 (48)
6	Z-L-Lys-OC*p*	0.5	Z-L-Lys-L-Ala-*p*NA	55 (24)
7	Bz-L-Arg-OEt	1	Bz-L-Arg-L-Ala-*p*NA	86 (76)

[a] Condition: acyl donor, 1 mM; actyl acceptor, 100 mM; methyltrypsin, 10 μM; 20% DMSO–MOPS (50 mM, pH 8.0, containing 10 mM $CaCl_2$); 25°C.
[b] The values in parentheses are yields by using 20 mM acyl acceptor.

(4.6 × 250 mm, Wakosil 5C18–200), isocratic elution at 1 ml/min, 0.1% trifluoroacetic acid/acetonitrile. Elution peaks were detected at 310 nm for the *p*-nitroanilide moiety, at 254 nm for the benzyloxycarbonyl moiety, and at 260 nm for the *p*-hydroxybenzyl moiety, respectively.

RESULTS AND DISCUSSION

Methyltrypsin

The purity of the commercial preparation of bovine pancreatic trypsin was verified by active site titration using NPGB according to the reported method (*15*), and the preparation used was determined to be 60% pure. Methyltrypsin was prepared using methyl *m*-guanidinobenzenesulfonate tetrafluoroborate as described in the experimental procedure. The pH was kept at 7.4 by occasional addition of NaOH. It was found that strict control of pH during the methylation step is crucial for the purity of the modified enzyme. The amidase activity of methyltrypsin was measured with Bz-L-Phe-L-Val-L-Arg-*p*NA and no appreciable activity was detected. The purity of methyltrypsin (95%) was determined by active site titration according to a method similar to that described for bovine trypsin using NPGB.

The time course of methyltrypsin-catalyzed hydrolysis of NPGB is shown in Fig. 1. After mixing enzyme and substrate, a very slow but clear acylation reaction was observed but no distinct steady-state turnover process. As shown in Table 1, the affinity of methyltrypsin toward NPGB is weaker than that of native trypsin (fivefold larger K_s value) (*23*). In contrast, the k_2 value for methyltrypsin is approximately four orders of magnitude smaller than that of native trypsin. The parameter, k_2/K_s (or k_{cat}/K_m), introduced by Brot and Bender (*24*) was used for the evalution of the specificity of the substrates. In terms of these k_2/K_s (or k_{cat}/K_m) values, the specificity of methyltrypsin is four orders of magnitude lower than those of native trypsin.