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Trypsin-catalyzed peptide synthesis with *m*-guanidinophenyl and *m*-(guanidinomethyl)phenyl esters as acyl donor component

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Summary. Two series of inverse substrates, *m*-guanidinophenyl and *m*-(guanidinomethyl)phenyl esters derived from *N*-(*tert*-butyloxycarbonyl)-amino acid, were prepared as an acyl donor component for trypsin-catalyzed peptide synthesis. The kinetic behavior of these esters toward tryptic hydrolysis was analyzed. They were found to couple with an acyl acceptor such as L-alanine *p*-nitroanilide to produce dipeptide in the presence of trypsin. *Streptomyces griseus* trypsin was a more efficient catalyst than the bovine trypsin. Within the enzymatic peptide coupling methods, this approach was shown to be advantageous, since the resulting peptides are resistant to the enzymatic hydrolysis.

Keywords: Amino acid esters – Inverse substrate – Kinetics of tryptic hydrolysis – Protease catalysis – Peptide synthesis

Abbreviations: Boc: *tert*-butyloxycarbonyl; Aib: α-aminoisobutyric acid; DMSO: dimethylsulfoxide; Tris: tris(hydroxymethyl)aminomethane; MOPS: 3-morpholino-1-propanesulfonate; G: guanidinophenyl; GM: (guanidinomethyl)phenyl; *p*NA: *p*-nitroanilide.

Introduction

Peptide synthesis by protease-catalyzed reverse reaction has been extensively studied with a variety of amino acids and peptide derivatives as coupling compounds (Nakatsuka et al., 1987; Schellenberger et al., 1991; Tsuzuki et al., 1980; Wong, 1989). It is known that enzymatic peptide synthesis is more advantageous than chemical synthesis in many respects; it is highly stereoselective and racemization-free, and requires minimal side-chain protection (Nakatsuka et al., 1987; Schellenberger et al., 1991; Tsuzuki et al., 1980; Wong, 1989). A most serious drawback of the enzymatic method, however, is the restrictive substrate specificity. Thus, the application of proteases for peptide synthesis has been limited due to the specificity of the enzymes.

In a previous paper, we reported that the *p*-amidinophenyl and *p*-guanidinophenyl esters behave as specific substrates for trypsin and trypsin-like enzymes (Nakano et al., 1980; Nozawa et al., 1980). In these esters the site-specific groups (charged amidinium and guanidinium) for the enzyme are included in the leaving-group portion instead of being in the acyl moiety. Such a substrate is termed an "inverse substrate" (Tanizawa et al., 1977). Inverse substrates allow the specific introduction of an acyl group carrying a non-specific residue into the trypsin active site without recourse to a cationic acyl moiety, which is a characteristic of conventional substrates. These acyl trypsin intermediates are expected to play a key role in trypsin-catalyzed peptide synthesis. In this respect, we have been studying the trypsin-catalyzed peptide synthesis employing substrates of a new type "inverse substrate" such as *p*-amidinophenyl (Itoh et al., 1996), *p*-guanidinophenyl (Sekizaki et al., 1996b), and *p*-(guanidinomethyl)phenyl esters (Sekizaki et al., 1997, 1998) as an acyl donor component. Previous work revealed that the *m*-guanidinophenyl ester was still qualified as the specific "inverse substrates" for trypsin (Nakano et al., 1980; Nozawa et al., 1980). In the present work, we investigated trypsin-catalyzed peptide synthesis using *m*-guanidinophenyl and *m*-(guanidinomethyl)phenyl esters instead of *para*-esters. Comparison was made between two trypsins of different origin (bovine and *Streptomyces griseus* (SG) trypsin) as the catalyst for the peptide synthesis.

Materials and methods

Materials

Bovine pancreas trypsin (EC 3.4.21.4) was purchased from Worthington Biochemical Corp. (twice crystallized, lot TRL). *Streptomyces griseus* trypsin was prepared following the reported procedures (Yokosawa et al., 1976; Yoshida et al., 1971).

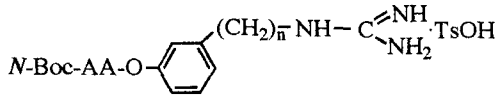
Synthesis of inverse substrates in Fig. 1

N-(*tert*-Butyloxycarbonyl)amino acid *m*-guanidinophenyl esters *p*-toluenesulfonate (1a–d) were synthesized as reported (Sekizaki et al., 1996). Pure compounds 1a–d were obtained as a colorless amorphous solid. 1a: $[\alpha]_D^{25}$ −25.0° (*c* = 1.0, MeOH). FAB-MS m/z: 323 $(M + H)^+$. 1b: $[\alpha]_D^{25}$ +26.0° (*c* = 1.0, MeOH). FAB-MS m/z: 323 $(M + H)^+$. 1c: FAB-MS m/z: 323 $(M + H)^+$. 1d: FAB-MS m/z: 337 $(M + H)^+$.

N-(*tert*-Butyloxycarbonyl)amino acid *m*-(guanidinomethyl)phenyl esters *p*-toluenesulfonate were synthesized following the procedure described for the *para*-isomers (Sekizaki et al., 1997, 1998). 2a: Colorless needles. mp 131–133°C (EtOH-AcOEt-hexane). $[\alpha]_D^{25}$ −30.4° (*c* = 1.0, MeOH). *Anal.* Calcd. for $C_{23}H_{32}N_4O_7S$: C, 54.32; H, 6.34; N, 11.02; S, 6.30. Found: C, 54.22; H, 6.32; N, 10.89; S, 6.32. 2b: Colorless fine needles. mp 130 ~ 132°C (AcOEt-hexane). $[\alpha]_D^{25}$ +27.4° (*c* = 1.0, MeOH). *Anal.* Calcd. for $C_{23}H_{32}N_4O_7S$: C, 54.32; H, 6.34; N, 11.02; S, 6.30. Found: C, 54.20; H, 6.46; N, 10.77; S, 6.24. 2c: Colorless amorphous solid. FAB-MS m/z: 337 $(M + H)^+$. 2d: Colorless needles. mp 185–187°C (EtOH-AcOEt-hexane). *Anal.* Calcd. for $C_{24}H_{33}N_4O_7S$: C, 55.16; H, 6.56; N, 10.72; S, 6.30. Found: C, 54.20; H, 6.46; N, 10.77; S, 6.24.

Kinetic measurements

Enzyme concentration was determined by active site titration using *p*-nitrophenyl *p'*-guanidinobenzoate (Chase and Shaw, 1967). The kinetic parameters, K_s, k_2 and k_3 for



AA	n=0	n=1
a : L-Ala	**1a**	**2a**
b : D-Ala	**1b**	**2b**
c : β-Ala	**1c**	**2c**
d : Aib	**1d**	**2d**

Fig. 1. Structure of acyl donors

bovine-catalyzed hydrolysis, were determined by means of the thionine displacement method using a stopped-flow technique (Brandt et al., 1967; Nozawa et al., 1980). The reaction was carried out in 0.05 M Tris-HCl buffer, pH 8.0, containing 0.02 M $CaCl_2$ at 25°C. In these experiments, the concentrations were: enzyme, $3.31 \times 10^{-6} \sim 7.83 \times 10^{-6}$ M; substrate, $2.40 \times 10^{-5} \sim 1.04 \times 10^{-3}$ M; thionine; 2.50×10^{-5} M. The kinetic paremters, K_m and k_{cat} for bovine-catalyzed hydrolysis, were determined by using pH-stat following the reported procedure (Tanizawa et al., 1977). The reaction was carried out in 0.1 M KCl, pH 8.0, containing 0.02 M $CaCl_2$ at 25°C. In these experiments the enzyme concentration was $1.95 \times 10^{-8} \sim 1.50 \times 10^{-6}$ M, and the substrate concentration was $1.76 \times 10^{-5} \sim 1.07 \times 10^{-4}$ M.

Trypsin-catalyzed peptide coupling reaction

A solution of 50 μl of acyl donor (10 mM solution of 1a–d or 2a–d in DMSO), 50 μl of acyl acceptor (200 mM solution of L-Ala-*p*NA in DMSO), 240 μl of 50 mM MOPS buffer (containing 20 mM of $CaCl_2$, pH 8.0) and 150 μl of DMSO were mixed. To this mixture, 10 μl of trypsin solution (1 mM solution in 1 mM HCl) was added and incubated at 25°C. The progress of the coupling reaction was monitored by HPLC under the following conditions: Wakosil 5C18–200 (column i.d. 4.0 × 250 mm), isocratic elution at 1 ml/min, 0.1% trifluoroacetic acid/acetonitrile. An aliquot of the reaction mixture was injected and the eluate was monitored at 310 nm (*p*-nitroanilide moiety). The retention times of elution peaks were correlated to those of authentic samples which were chemically synthesized (Bieth and Weremath, 1970; Kawai et al., 1982; Okada et al., 1982; Sekizaki et al., 1996a, 1996b, 1997). Coupling yields were calculated on the basis of the peak intensity.

Results and discussion

Kinetic parameters for trypsin-catalyzed hydrolysis

The kinetics of tryptic hydrolysis of the N^{α}-Boc-L-alanine *m*-(guanidinomethyl)phenyl ester (2a) was analyzed as a representative example of the series of compounds. Kinetic parameters were determined as described in the preceding paper (Sekizaki et al., 1996a), and they were compared with those of our previous work (Sekizaki et al., 1997; Nozawa et al., 1980) (Table 1). Binding of the *meta*-derivatives is diverse. Affinity of 2a for trypsin is less than that of the *para*-isomer, though the result is opposite in the comparison

Table 1. Comparison of kinetic parameters for trypsin-catalyzed hydrolysis of *p*- and *m*-guanidinophenyl acetate, and *p*- and *m*-(guanidinomethyl)phenyl ester

Substrate	K_s (K_m) (M)	k_2 (s^{-1})	k_3 (s^{-1})	k_{cat} (s^{-1})	$k_2/K_s(k_{cat}/K_m)$ ($s^{-1}\cdot M^{-1}$)
p-Ac-OG[a]	8.84×10^{-4}	8.16×10	1.25×10^{-2}	1.25×10^{-2}	9.23×10^{4}
m-Ac-OG[a]	8.83×10^{-5}	3.03×10^{-3}[c]	1.25×10^{-2}[c]	2.44×10^{-3}	3.06×10^{2}
p-N^{α}-Boc-L-Ala-OGM[b]	(4.31×10^{-4})	nd	nd	7.60×10^{-1}	(1.76×10^{3})
m-N^{α}-Boc-L-Ala-OGM (2a)	(1.75×10^{-3})	nd	nd	1.85×10^{-1}	(1.06×10^{2})

[a]See reference (Nakano et al., 1980; Nozawa et al., 1980). [b]See references (Sekizaki et al., 1997, 1998). [c]Theoretically deduced.

of spatially small guanidinophenyl acetates. In general, both *meta*-isomers have moderate binding affinity for trypsin with K_s (K_m) values in the range of 10^{-3}–10^{-5}M nearly comparable to that of the *para*-isomers. Hydrolysis of inverse substrate has been shown to proceed by consecutive acylation and deacylation processes (Tanizawa et al., 1977). If the acylation is much faster than the subsequent deacylation process, each rate constant (k_2 and k_3) was determined directly from the spectrometric trace of the presteady-state and steady-state parts of the hydrolysis, respectively. *p*-Guanidinophenyl acetate is a substrate of this case; k_2 and k_3 were determined in this manner (Nozawa et al., 1980). The behavior of *m*-guanidinophenyl acetate was different from that of the *para*-derivative. The spectrometric trace of the reaction did not show the typical biphasic pattern which was shown for the *para*-derivative. This is due to the kinetic characteristic of *m*-guanidinophenyl acetate, in which k_2 is not excessively larger than k_3. In Table 1 the experimentally determined, apparent overall k_{cat} is listed. It was recognized that the rate of the deacylation process (hydrolysis of the acyl enzyme intermediate) should not be dependent on the leaving phenol portion of the ester substrates if the identical acyl group is designed. Thus the k_3 value for *m*-AcOG was determined theoretically to be $1.25 \times 10^{-2}s^{-1}$. Taking this value into the equation (1) (Ryan et al., 1977), k_2 was calculated to be $3.03 \times 10^{-3}s^{-1}$ as listed in Table 1. On the whole, *m*-guanidinophenylesters are qualified as expected as

$$1/k_{cat} = 1/k_2 + 1/k_3 \qquad (1)$$

a good substrate for trypsin with their moderate binding affinity and acylation rate.

Trypsin-catalyzed hydrolysis of *p*- and *m*-N^{α}-Boc-L-Ala-OMG (2a) was rapid compared to that of *p*- and *m*-AcOG. This is shown from the k_{cat} in Table 1. The specificity indices (k_2/K_s or k_{cat}/K_m) are listed in Table 1 which were presented for the evaluation of the substrates (Brot et al., 1969). Guanidinophenyl esters are rather more specific than (guanidinomethyl)phenyl esters as judged by this index. It was also noted that *p*-derivatives are generally more specific than *m*-derivatives. No presteady-state hydrolysis (initial burst of liberation of (guanidinomethyl)phenol)

Table 2. Reaction yield of trypsin-catalyzed peptide coupling with *m*-guanidinophenyl ester and *m*-(guanidinomethyl)phenyl ester as acyl donor[a]

Entry No.	Trypsin	Acyl donor (No.)	Reaction time (h)	Product (No.)	Yield[b] (%)
1	Bovine	N^{α}-Boc-L-Ala-OG (1a)	12	N^{α}-Boc-L-Ala-L-Ala-*p*NA (3a)	58
2	SG	N^{α}-Boc-L-Ala-OG (1a)	12	N^{α}-Boc-L-Ala-L-Ala-*p*NA (3a)	60
3	Bovine	N^{α}-Boc-L-Ala-OGM (2a)	12	N^{α}-Boc-L-Ala-L-Ala-*p*NA (3a)	67
4	SG	N^{α}-Boc-L-Ala-OGM (2a)	3	N^{α}-Boc-L-Ala-L-Ala-*p*NA (3a)	80
5	Bovine	N^{α}-Boc-D-Ala-OG (1b)	24	N^{α}-Boc-D-Ala-L-Ala-*p*NA (3b)	20
6	SG	N^{α}-Boc-D-Ala-OG (1b)	24	N^{α}-Boc-D-Ala-L-Ala-*p*NA (3b)	48
7	Bovine	N^{α}-Boc-D-Ala-OGM (2b)	24	N^{α}-Boc-D-Ala-L-Ala-*p*NA (3b)	36
8	SG	N^{α}-Boc-D-Ala-OGM (2b)	20	N^{α}-Boc-D-Ala-L-Ala-*p*NA (3b)	75
9	Bovine	N^{β}-Boc-β-Ala-OG (1c)	24	N^{β}-Boc-β-Ala-L-Ala-*p*NA (3c)	32
10	SG	N^{β}-Boc-β-Ala-OG (1c)	48	N^{β}-Boc-β-Ala-L-Ala-*p*NA (3c)	31
11	Bovine	N^{β}-Boc-β-Ala-OGM (2c)	24	N^{β}-Boc-β-Ala-L-Ala-*p*NA (3c)	40
12	SG	N^{β}-Boc-β-Ala-OGM (2c)	10	N^{β}-Boc-β-Ala-L-Ala-*p*NA (3c)	54
13	Bovine	N^{α}-Boc-Aib-OG (1d)	48	N^{α}-Boc-Aib-L-Ala-*p*NA (3d)	13
14	SG	N^{α}-Boc-Aib-OG (1d)	48	N^{α}-Boc-Aib-L-Ala-*p*NA (3d)	18
15	Bovine	N^{α}-Boc-Aib-OGM (2d)	48	N^{α}-Boc-Aib-L-Ala-*p*NA (3d)	5
16	SG	N^{α}-Boc-Aib-OGM (2d)	48	N^{α}-Boc-Aib-L-Ala-*p*NA (3d)	28

[a] Conditions: acyl donor, 1 mM; acyl acceptor, 20 mM; trypsin, 10 μM; 50% DMSO-MOPS (50 mM, pH 8.0, containing 10 mM $CaCl_2$); 25°C.
[b] The values represent the mean of two runs (each value is within 2% variation).

prior to the steady-state hydrolysis was observed in the case of (guanidinomethyl)phenyl esters. This means that acylation and deacylation rates are close and, accordingly, their rate constants are undeterminable. Thus the overall catalytic rate constant and K_m were the parameters determined, and they are listed in Table 1. As a conclusion, *m*-(guanidinomethyl)phenyl esters are expected to be eligible for peptide synthesis in view of their moderate binding and acylation rate as well as *p*-(guanidinomethyl)phenyl esters.

Trypsin-catalyzed peptide coupling reaction

The reaction yields of the trypsin-catalyzed peptide coupling are summarized in Table 2. Each product completely coincides with the respective, authentic peptide sample. Coupling reactions of *N*-Boc-AA-OG (1) and *N*-Boc-AA-OGM (2) with L-Ala-*p*NA were compared in both catalysts, bovine and SG trypsin. Coupling reaction of the *m*-(guanidinomethyl)phenyl ester (2a–d) is much more efficient than that of the *m*-guanidinophenyl ester (1a–d).

In our previous work, it was summarized that peptide coupling by use of *para*-substrates proceeded rather rapidly (Sekizaki et al., 1996, 1997). The highest coupling yield (~95%) was realized with bovine trypsin rather than with SG trypsin.

In the present case, for both substrates 1a and 2a, bovine and SG trypsin behaves as moderately effective catalysts of the peptides synthesis (Entry 1–4). The best yield (80%) was obtained by the combination of 2a with SG trypsin. In the case of D-alanine, the coupling yields strongly depend on the substrate and catalyst (Entry 5–8). The combination of 2b with SG trypsin gave the coupling product in satisfactory yield (Entry 8). The coupling reaction of inverse substrates containing Aib is not favorable and the reaction is very slow (Entry 13–16). The reverse tendency of entry 15 compared with entry 13 was seen similarly to the previous observation (Sekizaki et al., 1997). The peptide (3c) containing β-amino acid was also obtained in moderate yield by the combination of *m*-(guanidinomethyl)phenyl ester derivative (2c) with SG trypsin (Entry 12). These results indicated that the *m*-(guanidinomethyl)-phenyl esters (2) behave as specific substrates for SG trypsin by means of specific binding and efficient acylation, whereas the *m*-guanidinophenyl esters (1) do not sufficiently meet the sterical requirement of the active site of either SG or bovine trypsin.

The coupling yields did not drop even after prolonged reaction period. This result indicated that enzymatic secondary hydrolysis of the resulting peptides is negligible. The combination of *m*-(guanidinomethyl)phenyl esters (2) with SG trypsin is proposed as a useful method for the preparation of a wide variety of peptides.

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References

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

Brandt KG, Himoe A, Hess GP (1967) Investigations of the chymotrypsin-catalyzed hydrolysis of specific substrates. J Biol Chem 242: 3973–3982

Brot FE, Bender ML (1969) Use of the specificity constant of α-chymotrypsin. J Am Chem Soc 91: 7187–7191

Chase T, Jr., Shaw E (1967) *p*-Nitrophenyl *p'*-guanidinobenzoate-HCl: a new active site titrant for trypsin. Biochem Biophys Res Commun 29: 508–514

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

Kawai M, Nagai U (1982) Upon DNP-aromatic rule. Effect of chromophore exchange on the CD spectra of *N*-DNP-arylalkylamines. Bull Chem Soc Jpn 55: 1327–1328

Nakano M, Tanizawa K, Nozawa M, Kanaoka Y (1980) Efficient tryptic hydrolysis of aryl esters with a cationic center in the leaving group. Further characterization of "inverse substrates". Chem Pharm Bull 28: 2212–2216

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

Nozawa M, Tanizawa K, Kanaoka Y (1980) "Inverse substrates" for trypsin-like enzymes. J Pharmacobio-Dyn 3: 213–219

Okada Y, Tsuda Y, Hirata A, Nagamatsu Y, Okamoto U (1982) Synthesis of chromogenic substrates specific for human spleen fibrinolytic protenase (SFP) and human leukocyte elastase (LE). Chem Pharm Bull 30: 4060–4068

Ryan TJ, Fenton JW, II, Chang T-l, Feinman RD (1977) Specificity of thrombin: evidence for selectivity in acylation rather than binding for *p*-nitrophenyl α-amino-*p*-toluate. Biochemistry 15: 1337–1341

Schellenberger V, Jakubke H-D (1991) Protease-catalyzed kinetically controlled peptide synthesis. Angew Chem Int Ed Engl 30: 1437–1449

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 esters as acyl donors. Chem Pharm Bull 44: 1585–1587

Sekizaki H, Itoh K, Toyota E, Tanizawa K (1997) Enzymatic coupling of α, α-dialkyl amino acids using inverse substrates as acyl donors. Tetrahedron Lett 38: 1777–1780

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

Tanizawa K, Kasaba Y, Kanaoka Y (1977) "Inverse substrates" for trypsin. Efficient enzymatic hydrolysis of certain esters with a cationic center in the leaving group. J Am Chem Soc 99: 4485–4488

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

Yokosawa H, Hanba Y, Ishii S (1976) Affinity chromatography of trypsin and related enzymes III. Purification of *Streptomyces griseus* trypsin using an affinity adsorbent containing a tryptic digest of protamine as a ligand. J Biochem 79: 757–763

Yoshida N, Sasaki A, Inoue H (1971) An anionic trypsin-like enzyme from *Streptomyces eythreus*. FEBS Lett 15: 129–132

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