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Trypsin-Catalysed Synthesis of Oligopeptide Amides: Comparison of Catalytic Efficiency Among Trypsins of Different Origin (Bovine, *Streptomyces Griseus* and Chum Salmon)

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Abstract: A procedure has been developed for the synthesis of oligopeptide amide using inverse substrates as acyl donors with amino acid amide instead of *p*-nitroanilide as acyl acceptor and trypsins of different origin (bovine, *Streptomyces griseus* and chum salmon trypsins) as the catalyst. The effectiveness of this procedure was demonstrated by the synthesis of a pentapeptide, Boc-[Leu^5]-enkephalin amide, as a model compound. The method was the first enzymatic method shown to be successful at each successive coupling step for the synthesis of the oligopeptide. Bovine and chum salmon trypsins were superior to *Streptomyces griseus* trypsin as the catalyst. Copyright © 2002 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: bovine trypsin; *Streptomyces griseus* trypsin; chum salmon trypsin; inverse substrate; enzymatic peptide synthesis; *p*-guanidinophenyl ester

INTRODUCTION

Peptide synthesis by the protease-catalysed reverse reaction has been studied extensively with a variety of amino acids and peptide derivatives as coupling components [1–4]. The protease-catalysed peptide synthesis is superior to the chemical coupling method. The method requires less side-chain protection than the chemical coupling method. The method, however, has not been fully exploited for the possible synthesis of a number of biologically important peptides containing D-amino acids or other unusual amino acids, because the enzymatic method is subject to restriction by substrate specificity and stereoselectivity.

Previously, we reported that inverse substrates such as *p*-amidinophenyl esters [5] and *p*-guanidinophenyl esters [6–8] behave as specific substrates for trypsin and trypsin-like enzymes and allow the specific introduction of an acyl group carrying a non-specific residue into the enzyme active site. The characteristic features of inverse substrates suggested that they are useful for enzymatic peptide synthesis. Jabubke *et al.* [9–11] and we [12–15] demonstrated the successful application of inverse substrates for trypsin-catalysed coupling. Recently, Bordusa reported an enzymatic peptide coupling

Abbreviations: AA, amino acid; Boc, *tert*-butyloxycarbonyl; BOP, benzotriazol-1-yloxy-tris(dimethyl-amino)phosphonium hexafluorophosphate; BT, bovine trypsin; Bzl, benzyl; Cam, carboxamidomethyl; CS, chum salmon trypsin; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulphoxide; HOGp, *p*-guanidinophenol; HOGp(Z_2), *p*-[N',N''-bis(benzyloxycarbonyl)guanidino]phenol; MOPS, 3-morpholino-1-propanesulphonate; SG, *Streptomyces griseus* trypsin; *p*-TsOH, *p*-toluenesulphonic acid; Z, benzyloxycarbonyl.

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reaction using inverse substrates and a wide variety of proteases such as thrombin, clostripain, V8 protease, chymotrypsin and subtilisin [16].

A facile method was developed for the trypsin-catalysed synthesis of oligopeptide amide using inverse substrates as the acyl donor with amino acid amides as the acyl acceptor. The utility of this method was demonstrated for the synthesis of a pentapeptide, Boc-[Leu^5]-enkephalin amide (**1**), (Boc-L-Tyr-Gly-Gly-L-Phe-L-Leu-NH_2) [17] as a model compound.

Much attention has been paid to enkephalin and its analogues with regard to their biological activity, and a great deal of synthetic work has been carried out with respect to the enzymatic method. The synthesis of Boc-L-Tyr-Gly-Gly-L-Phe-L-Leu-NH_2 [18] was carried out by segment condensation of Boc-L-Tyr-Gly-Gly-L-Phe-OCam with L-Leu-NH_2 by immobilized α-chymotrypsin. In this case, the enzyme was used at only one stage owing to the substrate specificity of the enzyme. Therefore, a combination of several enzymes with different specificity was required for the full enzymatic procedure. For the synthesis of enkephalin analogues such as Z-L-Tyr-Gly-Gly-L-Phe-L-Leu-OEt [19], Z-L-Tyr-Gly-Gly-L-Phe-L-Leu-NHNHPh [20] and Boc or Z-L-Tyr-Gly-Gly-L-Phe-L-Leu-OEt [17], the combined use of α-chymotrypsin, thermolysin, papain and bromelain were employed.

In this paper, the procedure for the synthesis of oligopeptide amide using inverse substrates as the acyl donor with amino acid amide instead of *p*-nitroanilide is described. The procedure was applied for the synthesis of a pentapeptide, Boc-[Leu^5]-enkephalin amide (**1**). The procedure has shown that synthesis of oligopeptides was carried out using a single enzyme at different coupling stages. Also described is a comparison of catalytic efficiency among the trypsins of different origin (bovine, *Streptomyces griseus* and chum salmon), with a view to finding a preferable condition for the strategy.

MATERIALS AND METHODS

General Methods

The melting points were measured on a Yanaco MP-500 micro melting point apparatus and are 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 in a 5cm cell.

Materials

Bovine pancreas trypsin (EC 3.4.21.4) was purchased from Worthington Biochemical Corp. (twice crystallized, lot TRL). *Streptomyces griseus* trypsin was prepared according to the reported procedures [21,22]. Chum salmon trypsin was also prepared following the reported procedures [23]. Inverse substrates (**2**–**10**) were prepared following the reported procedures [7,8,24]. Acyl acceptors (**a**–**d**) were purchased from Calbiochem-Novabiochem International, Inc. The other peptide amide and authentic samples were prepared according to the reported procedure [25,26].

Synthesis of Inverse Substrates

***tert*-Butyloxycarbonyl-O-benzyl-L-tyrosylglycine *p*-(N′,N″-bis(benzyloxycarbonyl)guanidino)-phenyl ester (13).** A solution of [N',N''-bis(Z)guanidino]phenol (629 mg, 1.5 mmol), Boc-L-Tyr(Bzl)-Gly-OH [25] (643 mg, 1.5 mmol) and BOP reagent (664 mg, 1.5 mmol) in DMF (15 ml) was treated with Et_3N (415 μl, 1.5 mmol) at room temperature. The reaction mixture was stirred for 1 h, then the reaction mixture was evaporated *in vacuo* below 35 °C. The residue was diluted with AcOEt and purified by silica gel column chromatography. The pure Boc-L-Tyr(Bzl)-Gly-OGp(Z_2) was obtained (972 mg, 78%) as colourless fine needles (from AcOEt). mp 170°–174 °C; IR (KBr) cm^{-1}: 1770, 1729, 1678, 1667, 1645, 1625; ^{1}H-NMR (400 MHz, $CDCl_3$)δ: 1.40 (s, 9 H), 1.63 (br s, 2 H), 3.04 (d, 2 H, $J = 6.6$), 4.17–4.25 (m, 2 H), 4.31 (m, 1 H), 5.00 (s, 2 H), 5.14 (s, 2 H), 5.24 (s, 2 H), 6.48 (t, 1 H, $J = 7.0$), 6.90 (d, 2 H, $J = 9.0$), 7.05 (d, 2 H, $J = 9.0$), 7.13 (d, 2 H, $J = 9.0$), 7.26–7.49 (m, 15 H), 7.57 (d, 2 H, $J = 9.0$), 10.27 (s, 1 H), 11.88 (s, 1 H); for $C_{46}H_{47}N_5O_{10}$ (829.9) calculated: 66.57% C, 5.71% H, 8.44% N: found: 66.71% C, 5.87% H, 8.35% N.

***tert*-Butyloxycarbonyl-O-benzyl-L-tyrosylglycylglycine *p*-(N′,N″-bis-(benzyloxycarbonyl)guanidino)phenyl ester (15).** Compound (**15**) (946 mg, 71%) was obtained from Boc-L-Tyr(Bzl)-Gly-Gly-OH [27] (728 mg, 1 mmol) as colourless needles (from EtOH) by a procedure similar to that described for Boc-L-Tyr(Bzl)-Gly-OGp(Z_2) (**13**). mp 110°–112 °C; IR (KBr) cm^{-1}: 1764, 1740, 1648; ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 1.39 (s, 9 H), 1.72 (br

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s, 2 H), 2.91–3.08 (m, 2 H), 3.85–4.03 (m, 2 H), 4.19 (dd, 2 H, $J = 9.4$, 4.0), 5.01 (s, 2 H), 5.03 (t, 1 H, $J = 6.6$), 5.14 (s, 2 H), 5.23 (s, 2 H), 6.73 (t, 1 H, $J = 6.6$), 6.90 (d, 2 H, $J = 8.6$), 7.04 (d, 2 H, $J = 9.0$), 7.10 (d, 2 H, $J = 8.6$), 7.28–7.42 (m, 15 H), 7.54 (d, 2 H, $J = 9.0$), 10.25 (s, 1 H), 11.88 (s, 1 H); for $C_{48}H_{50}N_6O_{11}$ (886.9) calculated: 65.00% C, 5.68% H, 9.48% N: found: 65.19% C, 5.93% H, 9.41% N.

***tert*-Butyloxycarbonyl-*O*-benzyl-L-tyrosylglycylglycyl-L-phenylalanine *p*-(N′,N″-bis(benzyloxycarbonyl)guanidino)phenyl ester (17).** Compound (**17**) (1.015 g, 65%) was obtained from Boc-L-Tyr(Bzl)-Gly-Gly-L-Phe-OH [28] (1.55 g, 1.5 mmol) as colourless prisms (from benzene) by a procedure similar to that described for Boc-L-Tyr(Bzl)-Gly-OGp(Z_2) (**13**). mp 108°–110 °C; IR (KBr) cm^{-1}: 1750, 1700, 1652; ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 1.40 (s, 9 H), 1.66 (br s, 2 H), 2.93–3.08 (m, 3 H), 3.25 (d, 2 H, $J = 6.3$), 3.71–4.03 (m, 2 H), 4.24 (dd, 2 H, $J = 9.4$, 4.0), 4.99–5.06 (m, 4 H), 5.13 (s, 2 H), 5.23 (s, 2 H), 6.60 (t, 1 H, $J = 6.3$), 6.89–6.95 (m, 4 H), 7.06–7.25 (m, 4 H), 7.30–7.44 (m, 18 H), 7.53 (d, 2 H, $J = 9.0$), 10.25 (s, 1 H), 11.88 (s, 1 H); for $C_{57}H_{59}N_7O_{12}$(1034.1) calculated: 66.20% C, 5.75% H,9.48% N: found: 66.27% C, 6.04% H, 9.22% N.

***tert*-Butyloxycarbonyl-L-tyrosylglycine *p*-guanidinophenyl ester *p*-toluenesulphonate (8).** A suspension of Boc-L-Tyr(Bzl)-Gly-OGp(Z_2) (829 mg, 1 mmol) and *p*-TsOH·H_2O (190 mg, 1 mmol) in a mixture of EtOH (14 ml) and Et_2O (14 ml) containing 10% Pd-C (10 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 amorphous residue was washed with dry ether to give Boc-L-Tyr-Gly-OGp·TsOH (**8**) (636 mg, 99%). $[\alpha]_D + 1.4°$ (c = 1.0, MeOH); IR (KBr) cm^{-1}: 3328, 1771, 1635; FABMS m/z 472 $[M + H]^+$.

***tert*-Butyloxycarbonyl-L-tyrosylglycylglycine *p*-guanidinophenyl ester *p*-toluenesulphonate (9).** Compound (**9**) (625 mg, 89%) was obtained from Boc-L-Tyr(Bzl)-Gly-Gly-OGp(Z_2) (886 mg, 1 mmol) as a colourless amorphous solid by a procedure similar to that described for Boc-L-Tyr-Gly-OGp·TsOH (**8**). **9**: $[\alpha]_D + 9.4°$ (c = 1.0, MeOH); IR (KBr) cm^{-1}: 3321, 1772, 1671; FABMS m/z 529 $[M + H]^+$.

***tert*-Butyloxycarbonyl-L-tyrosylglycylglycyl-L-phenylalanine *p*-guanidinophenyl ester *p*-toluenesulphonate (10).** Compound (**10**) (418 mg, 99%) was obtained from Boc-L-Tyr(Bzl)-Gly-Gly-L-Phe-OGp(Z_2) (517 mg, 0.5 mmol) as a colourless amorphous solid by a procedure similar to that described for Boc-L-Tyr-Gly-OGp·TsOH (**8**). **10**: $[\alpha]_D + 1.1°$ (c = 1.1, MeOH); IR (KBr) cm^{-1}: 3329, 2964, 1765, 1667; FABMS m/z 676 $[M + H]^+$.

Enzymatic Peptide Coupling Reaction

Peptide coupling reaction was carried out at 25 °C in 50% DMSO-MOPS buffer (250 mM, pH 8.0, containing 20 mM $CaCl_2$). The concentrations of acyl donors (**2–10**), acyl acceptors (**a**-**h**) and enzyme were 25 mM, 250 mM, and 250 µM, respectively. The progress of the peptide coupling reaction was monitored by HPLC under the following conditions: column (4.6 × 250 mm, Wakosil 5C18-200), 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 215 nm (chromophore due to amide bond) and 270 nm (chromophore due to *p*-hydroxybenzyl moiety). Peak identification was made by correlating the retention time with that of authentic samples which were chemically synthesized. Observed peak areas were used for the estimation of sample concentration.

RESULTS AND DISCUSSION

The structures of inverse substrates (**2–10**) used in this study are shown in Scheme 1. These inverse

Boc-AA_1-O-C_6H_4-NH-C(=NH)NH_2 ·TsOH (**2-10**) + AA_2-NH_2 (**a-h**) —trypsin→ Boc-AA_1-AA_2-NH_2 (**2a-10d**)

2: AA_1 = L-Ala; **3**: AA_1 = D-Ala; **4**: AA_1 = L-Tyr; **5**: AA_1 = D-Tyr; **6**: AA_1 = L-Ala-L-Ala; **7**: AA_1 = D-Ala-D-Ala; **8**: AA_1 = L-Tyr-Gly;
9: AA_1 = L-Tyr-Gly-Gly; **10**: AA_1 = L-Tyr-Gly-Gly-L-Phe
a: AA_1 = L-Phe; **b**: AA_2 = D-Phe; **c**: AA_2 = L- Leu; **d**: AA_2 = D- Leu; **e**: AA_2 = L-Ala-L-Ala ; **f**: AA_2 = L-Phe-L-Leu ;
g: AA_2 = Gly-L-Phe-L-Leu; **h**: AA_2 = Gly-Gly-L-Phe-L-Leu

Scheme 1 Trypsin-catalysed oligopeptide synthesis using inverse substrates.

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substrates were prepared by condensation of the appropriate Boc-protected amino acid (Boc-peptide) and *p*-[*N*′,*N*″-bis(Z)guanidino]phenol (**11**), and subsequent deprotection by catalytic hydrogenation as shown in Scheme 2 [7,8,24].

The trypsin-catalysed coupling reaction of Boc-L-Ala-OGp (**2**) and L-Phe-NH$_2$ (**a**) to give Boc-L-Ala-L-Phe-NH$_2$ (**2a**) was examined under various conditions in which the concentrations of the acyl donor (**2**) and acyl acceptor (**a**) were varied. The results are shown in Table 1. The yield was increased with the increased concentration of either the acyl donor or the acyl acceptor. The coupling yield reached a stationary level when 25 mM and the higher acyl donor was used. Consequently, a standard procedure for the coupling reaction was fixed as follows: acyl donor (inverse substrate), 25 mM; acyl acceptor (amino acid amide), 250 mM; trypsin, 250 μM as described in Methods. 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 [25–26]. This optimum enzymatic coupling condition was applied to the synthesis of **2a** by SG and CS. As shown in Figure 1 (Entries 1–3), BT and CS exhibited almost same catalytic efficiency, but SG conducted less efficient catalysis. This tendency of SG was pronounced when the acyl acceptor was changed to amino acid amide from amino acid *p*-nitroanilide [14,29].

This optimum condition was also applied to the synthesis of peptides containing D-amino acid as shown in Table 2. Equivalent results were obtained when both enantiomers were compared as acyl donors, where the L-amino acid amide (**a**) was used as the acyl acceptor (Entries 1–6). These similar

Figure 1 Time course for the enzymatic synthesis of Boc-L-Ala-L-Phe-NH$_2$ (**2a**) catalysed by BT (□), CS (Δ) and SG (○).

Scheme 2 Synthesis of inverse substrates.

Table 1 Effect of Acyl Donor and Acyl Acceptor Concentration on the Synthesis of Boc-L-Ala-L-Phe-NH$_2$ **(2a)**

Acyl donor conc. (mM)	1	5	10	15	20	20	20	25	25	25	30	30	30	40	50
Acyl acceptor conc. (mM)	10	50	100	150	100	200	400	125	250	500	150	300	600	400	500
Yield (%)	13	38	54	65	51	73	76	56	77	78	67	77	79	71	64

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Table 2 Reaction Yields of Trypsin-Catalysed Peptide Coupling Reaction[a]

Entry No.	Acyl donor (No.)	Acyl acceptor (No.)	Enzyme	Reaction time (min)	Product (No.)	Yield (%)
1	Boc-L-Ala-OGp (**2**)	L-Phe-NH_2 (**a**)	BT	30	Boc-L-Ala-L-Phe-NH_2 (**2a**)	77
2	Boc-L-Ala-OGp (**2**)	L-Phe-NH_2 (**a**)	SG	30	Boc-L-Ala-L-Phe-NH_2 (**2a**)	45
3	Boc-L-Ala-OGp (**2**)	L-Phe-NH_2 (**a**)	CS	30	Boc-L-Ala-L-Phe-NH_2 (**2a**)	84
4	Boc-D-Ala-OGp (**3**)	L-Phe-NH_2 (**a**)	BT	30	Boc-D-Ala-L-Phe-NH_2 (**3a**)	82
5	Boc-D-Ala-OGp (**3**)	L-Phe-NH_2 (**a**)	SG	30	Boc-D-Ala-L-Phe-NH_2 (**3a**)	49
6	Boc-D-Ala-OGp (**3**)	L-Phe-NH_2 (**a**)	CS	30	Boc-D-Ala-L-Phe-NH_2 (**3a**)	85
7	Boc-L-Ala-OGp (**2**)	D-Phe-NH_2 (**b**)	BT	30	Boc-L-Ala-D-Phe-NH_2 (**2b**)	30
8	Boc-L-Ala-OGp (**2**)	D-Phe-NH_2 (**b**)	SG	30	Boc-L-Ala-D-Phe-NH_2 (**2b**)	11
9	Boc-L-Ala-OGp (**2**)	D-Phe-NH_2 (**b**)	CS	30	Boc-L-Ala-D-Phe-NH_2 (**2b**)	37
10	Boc-D-Ala-OGp (**3**)	D-Phe-NH_2 (**b**)	BT	30	Boc-D-Ala-D-Phe-NH_2 (**3b**)	17
11	Boc-D-Ala-OGp (**3**)	D-Phe-NH_2 (**b**)	SG	30	Boc-D-Ala-D-Phe-NH_2 (**3b**)	8
12	Boc-D-Ala-OGp (**3**)	D-Phe-NH_2 (**b**)	CS	30	Boc-D-Ala-D-Phe-NH_2 (**3b**)	18

[a] Conditions: acyl donor, 25 mM; acyl acceptor, 250 mM; trypsin, 250 μM; 50% DMSO-MOPS (250 mM, pH 8.0, containing 20 mM $CaCl_2$); 25 °C.

responses of the enzyme toward both enantiomers are probably due to the strong binding affinity that is common to inverse substrates. However, the coupling yields were decreased when the D-amino acid amide (**b**) was used as the acyl acceptor (Entries 7–12), probably because of the inherent nature of trypsins in that the L-configuration is advantageous for their P′1 interaction [30].

Table 3 shows that all three enzymes are capable of catalysing the $2+2$ fragment condensation. In these cases all three trypsins were effective catalysts for the formation of the tetrapeptides (**6e** and **7e**). The effect of the configuration of the acyl donor on the coupling yield was small in the case of $1+1$. SG trypsin, however, exhibited coupling efficiency comparable to that of CS and BT. It was assumed that the coupling yield was variable with the size of the fragment. Therefore, it was necessary to investigate the fragment condensation by changing the fragment in various ways. The investigation was carried out by way of the synthesis of Boc-[Leu^5]-enkephalin amide (**1**).

The BT-catalysed bond formation (1–2 bond) between the Boc-L-tyrosine *p*-guanidinophenyl ester (**4**) and tetrapeptide amide (**h**) was achieved in 53% yield (Entry 19). The diastereomer, Boc-D-Tyr^1-Gly^2-Gly^3-L-Phe^4-L-Leu^5-NH_2(**5h**), was prepared in 72% yield (Entry 22). In the $2+3$ fragment condensation, the coupling reaction of Boc-L-Tyr^1-Gly^2-OGp (**8**) with Gly^3-L-Phe^3-L-Leu^5-NH_2(**g**) was successful in giving the target product in 65% yield (Entry 25). In the $3+2$ fragment condensation, Boc-L-Tyr^1-Gly^2-Gly^3-OGp (**9**) was coupled with L-Phe^3-L-Leu^5-NH_2(**f**) to afford *N*-protected Leu-enkephalin amide in a

Table 3 Reaction Yields of Trypsin-Catalysed Peptide Coupling Reaction[a]

Entry No.	Acyl donor (No.)	Acyl acceptor (No.)	Enzyme	Reaction time (min)	Product (No.)	Yield (%)
13	Boc-L-Ala-L-Ala-OGp (**6**)	L-Ala-L-Ala-NH_2 (**e**)	BT	30	Boc-L-Ala-L-Ala-L-Ala-L-Ala-NH_2 (**6e**)	77
14	Boc-L-Ala-L-Ala-OGp (**6**)	L-Ala-L-Ala-NH_2 (**e**)	SG	30	Boc-L-Ala-L-Ala-L-Ala-L-Ala-NH_2 (**6e**)	90
15	Boc-L-Ala-L-Ala-OGp (**6**)	L-Ala-L-Ala-NH_2 (**e**)	CS	30	Boc-L-Ala-L-Ala-L-Ala-L-Ala-NH_2 (**6e**)	74
16	Boc-D-Ala-D-Ala-OGp (**7**)	L-Ala-L-Ala-NH_2 (**e**)	BT	30	Boc-D-Ala-D-Ala-L-Ala-L-Ala-NH_2 (**7e**)	75
17	Boc-D-Ala-D-Ala-OGp (**7**)	L-Ala-L-Ala-NH_2 (**e**)	SG	30	Boc-D-Ala-D-Ala-L-Ala-L-Ala-NH_2 (**7e**)	63
18	Boc-D-Ala-D-Ala-OGp (**7**)	L-Ala-L-Ala-NH_2 (**e**)	CS	30	Boc-D-Ala-D-Ala-L-Ala-L-Ala-NH_2 (**7e**)	70

[a] Conditions: acyl donor, 25 mM; acyl acceptor, 250 mM; trypsin, 250 μM; 50% DMSO-MOPS (250 mM, pH 8.0, containing 20 mM $CaCl_2$); 25 °C.

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Table 4 Reaction Yields of Trypsin-Catalysed Peptide Coupling Reaction[a]

Entry No.	Acyl donor (No.)	Acyl acceptor (No.)	Enzyme	Reaction time (min)	Product (No.)	Yield (%)
19	Boc-L-Tyr-OGp (**4**)	Gly-Gly-L-Phe-L-Leu-NH_2 (**h**)	BT	30	Boc-L-Try-Gly-Gly-L-Phe-L-Leu-NH_2 (**1**)	53
20	Boc-L-Tyr-OGp (**4**)	Gly-Gly-L-Phe-L-Leu-NH_2 (**h**)	SG	30	Boc-L-Try-Gly-Gly-L-Phe-L-Leu-NH_2 (**1**)	42
21	Boc-L-Tyr-OGp (**4**)	Gly-Gly-L-Phe-L-Leu-NH_2 (**h**)	CS	30	Boc-L-Try-Gly-Gly-L-Phe-L-Leu-NH_2 (**1**)	50
22	Boc-D-Tyr-OGp (**5**)	Gly-Gly-L-Phe-L-Leu-NH_2 (**h**)	BT	45	Boc-D-Try-Gly-Gly-L-Phe-L-Leu-NH_2 (**5h**)	72
23	Boc-D-Tyr-OGp (**5**)	Gly-Gly-L-Phe-L-Leu-NH_2 (**h**)	SG	45	Boc-D-Try-Gly-Gly-L-Phe-L-Leu-NH_2 (**5h**)	76
24	Boc-D-Tyr-OGp (**5**)	Gly-Gly-L-Phe-L-Leu-NH_2 (**h**)	CS	45	Boc-D-Try-Gly-Gly-L-Phe-L-Leu-NH_2 (**5h**)	56
25	Boc-L-Tyr-Gly-OGp (**8**)	Gly-L-Phe-L-Leu-NH_2 (**g**)	BT	45	Boc-L-Try-Gly-Gly-L-Phe-L-Leu-NH_2 (**1**)	65
26	Boc-L-Tyr-Gly-OGp (**8**)	Gly-L-Phe-L-Leu-NH_2 (**g**)	SG	45	Boc-L-Try-Gly-Gly-L-Phe-L-Leu-NH_2 (**1**)	21
27	Boc-L-Tyr-Gly-OGp (**8**)	Gly-L-Phe-L-Leu-NH_2 (**g**)	CS	45	Boc-L-Try-Gly-Gly-L-Phe-L-Leu-NH_2 (**1**)	66
28	Boc-L-Tyr-Gly-Gly-OGp (**9**)	L-Phe-L-Leu-NH_2 (**f**)	BT	30	Boc-L-Try-Gly-Gly-L-Phe-L-Leu-NH_2 (**1**)	51
29	Boc-L-Tyr-Gly-Gly-OGp (**9**)	L-Phe-L-Leu-NH_2 (**f**)	SG	30	Boc-L-Try-Gly-Gly-L-Phe-L-Leu-NH_2 (**1**)	15
30	Boc-L-Tyr-Gly-Gly-OGp (**9**)	L-Phe-L-Leu-NH_2 (**f**)	CS	30	Boc-L-Try-Gly-Gly-L-Phe-L-Leu-NH_2 (**1**)	42
31	Boc-L-Tyr-Gly-Gly-L-Phe-OGp (**10**)	L-Leu-NH_2 (**c**)	BT	30	Boc-L-Try-Gly-Gly-L-Phe-L-Leu-NH_2 (**1**)	79
32	Boc-L-Tyr-Gly-Gly-L-Phe-OGp (**10**)	L-Leu-NH_2 (**c**)	SG	30	Boc-L-Try-Gly-Gly-L-Phe-L-Leu-NH_2 (**1**)	76
33	Boc-L-Tyr-Gly-Gly-L-Phe-OGp (**10**)	L-Leu-NH_2 (**c**)	CS	30	Boc-L-Try-Gly-Gly-L-Phe-L-Leu-NH_2 (**1**)	85
34	Boc-L-Tyr-Gly-Gly-L-Phe-OGp (**10**)	D-Leu-NH_2 (**d**)	BT	45	Boc-L-Try-Gly-Gly-L-Phe-D-Leu-NH_2 (**10d**)	23
35	Boc-L-Tyr-Gly-Gly-L-Phe-OGp (**10**)	D-Leu-NH_2 (**d**)	SG	45	Boc-L-Try-Gly-Gly-L-Phe-D-Leu-NH_2 (**10d**)	13
36	Boc-L-Tyr-Gly-Gly-L-Phe-OGp (**10**)	D-Leu-NH_2 (**d**)	CS	45	Boc-L-Try-Gly-Gly-L-Phe-D-Leu-NH_2 (**10d**)	39

[a] Conditions: acyl donor, 25 mM; acyl acceptor, 250 mM; trypsin, 250 μM; 50% DMSO-MOPS (250 mM, pH 8.0, containing 20 mM $CaCl_2$); 25 °C.

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51% yield (Entry 28). Finally the 4 + 1 fragment condensation was attempted using L- (**c**) and D-Leu-NH_2 (**d**). The coupling reaction between Boc-L-Tyr-Gly-Gly-L-Phe-OGp (**10**) with L-Leu-NH_2 (**c**) and D-Leu-NH_2 (**d**) proceeded in 79% and 23% yields, respectively (Entries 31 and 34).

The results with BT-catalysed synthesis of enkephalin were compared with those with SG and CS-catalysed reactions as shown in Table 4. SG resulted in a low coupling yield, except in the case of inverse substrate derived from D-amino acid (Entry 23). CS-catalysed reactions were principally better than SG-catalysed reactions. It is noticeable that an exceedingly high yield was obtained with the CS for the synthesis of **10d** (Entry 36).

At the present stage, it is difficult to summarize the variety of experimental results. Therefore, the strategy for the peptide coupling, choice of enzyme and peptide fragment, is still unclear. In any event, BT, CS and SG used in this study are efficient catalysts for the inverse substrate mediated fragment condensation.

CONCLUSION

A procedure has been developed for the synthesis of oligopeptide amide using inverse substrates as acyl donors with amino acid amide as acyl acceptor and trypsins of different origin (bovine, *Streptomyces griseus* and chum salmon trypsins) as the catalyst. The effectiveness of this procedure was demonstrated by the synthesis of a pentapeptide, Boc-[Leu^5]-enkephalin amide, as a model compound. The method was the first example shown to be successful for the synthesis of the peptide using the same enzyme at each coupling step. It was shown that BT and CS were equally efficient for this strategy, and SG was less efficient. The method is useful for the preparation of enkephalin analogues containing D-amino acids which are resistant to hydrolysis by most of the serine proteases present in the living system. It is also proposed that the secondary hydrolysis of the coupling product can be disregarded in our synthetic procedure, since the coupling product was not decreased even after a 48 h reaction time.

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