Amino Acids (2001) 21: 175–184

Amino
Acids

Chum salmon trypsin-catalyzed preferential formation of peptides containing D-amino acid

H. Sekizaki, K. Itoh, E. Toyota, and **K. Tanizawa**

Faculty of Pharmaceutical Sciences, Health Sciences University of Hokkaido, Ishikari-Tobetsu, Hokkaido, Japan

Accepted December 2, 2000

Summary. Chum salmon trypsin-catalyzed peptide synthesis has been studied by using nine series of "inverse substrates," i.e., *p*-amidinophenyl, *p*- and *m*-guanidinophenyl, *p*- and *m*-(guanidinomethyl)phenyl, and four position isomers of guanidinonaphthyl esters derived from N^{α}-(*tert*-butyloxycarbonyl)amino acid as acyl donor components. They were found to couple with an acyl acceptor such as L-alanine *p*-nitroanilide to produce dipeptide in the presence of trypsin. All substrates tested in this study undergo less enantioselective coupling reaction, and the coupling product was the favorably obtained D-series rather than L-series (in the present case; N^{α}-Boc-D-Ala and N^{α}-Boc-L-Ala). The optimum condition for the coupling reaction was studied by changing the organic solvent, buffer solution, pH, and acyl acceptor concentration. It was found that the enzymatic hydrolysis of the resulting product was negligible.

Keywords: Amino acids – Inverse substrate – Chum salmon trypsin – Enzymatic peptide synthesis – D-Amino acid – Amidinophenyl ester – Guanidinophenyl ester

Abbreviations: Boc: *tert*-butyloxycarbonyl; DMSO: dimethylsulfoxide; DMF: *N*,*N*-dimethylformamide; Tris: tris(hydroxymethyl)aminomethane; MOPS: 3-morpholino-1-propanesulfonate; GTA: mixture of 3,3-dimethylglutaric acid (G), Tris (T), and 2-amino-2-methyl-1,3-propanediol (A); HEPES: *N*-2-hydroxyethylpiperazine-*N*′-ethanesulfonic acid; Tricine: *N*-tris(hydroxyethyl)methylglycine; Am; amidinophenyl; *p*Gu: *p*-guanidinophenyl; *m*Gu: *m*-guanidinophenyl; *p*GM: *p*-(guanidinomethyl) phenyl; *m*GM: *m*-(guanidinomethyl)phenyl; GN: guanidinonaphthyl; *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

components (Nakatsuka et al., 1987; Schellenberger et al., 1991; Tsuzuki et al., 1980; Wong, 1989). The protease-catalyzed 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 possible synthesis of a number of biologically important peptides containing D-amino acid or other unusual amino acid, because the enzymatic method is subject to restrictive substrate specificity and stereoselectivity.

In a previous paper, we reported that the *p*-amidinophenyl and *p*-guanidinophenyl esters behave as specific substrates for trypsin (Itoh et al., 1996; Sekizaki et al., 1996a). 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 substrate is termed an "inverse substrate" by us (Tanizawa et al., 1977) and "substrate mimetics" by Bordusa et al. (1997). 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. We previously reported bovine trypsin-catalyzed peptide synthesis by use of N^{α}-Boc-amino acid *p*-amidinophenyl (N^{α}-Boc-AA-OAm) (AA = amino acid) (Itoh et al., 1996), *p*-guanidinophenyl (N^{α}-Boc-AA-O*p*Gu) (Sekizaki et al., 1996b), *m*-guanidinophenyl (N^{α}-Boc-AA-O*m*Gu) (Sekizaki et al., 1999a), *p*-(guanidinomethyl)phenyl (N^{α}-Boc-AA-O*p*GM) (Sekizaki et al., 1997, 1998), *m*-(guanidinomethyl) phenyl (N^{α}-Boc-AA-O*m*GM) (Sekizaki et al., 1999a), and guanidinonaphthyl esters (N^{α}-Boc-AA-OGN) (Sekizaki et al., 1999b) as acyl donors.

Many studies on the characterization of trypsins from cold-adapted species have been reported (Vecchi and Coppes, 1996). These trypsins display substantially higher catalytic efficiency than their mammalian counterparts (Asgeirsson et al., 1989; Asgeirsson and Bjarnason, 1991, 1993; Kristjansson and Nielsen, 1992; Gildberg and Overbo, 1990; Osnes et al., 1985; Simpson and Haard, 1984). Thus, trypsin from chum salmon (*Onchorhynchus keta*) is expected to be a highly efficient catalyst for enzymatic peptide synthesis. Therefore, it was assumed that chum salmon trypsin-catalyzed coupling reaction by use of an inverse substrate as the acyl donor component is the most promising procedure for peptide synthesis. In fact, we recently reported the chum salmon trypsin-catalyzed reaction using N^{α}-Boc-AA-OAm at low temperature was applicable to the synthesis of peptides which are sensitive to spontaneous and enzymatic hydrolyses (Sekizaki et al., 2000b). Studies on the requirements for chum salmon trypsin-catalyzed reaction by use of various inverse substrates (Fig. 1) were investigated.

Materials and methods

Materials

All inverse substrates were prepared according to our previous papers (Itoh et al., 1995, 1996; Sekizaki et al., 1996a, 1997, 1998, 1999a, 1999b). Chum salmon trypsin was prepared

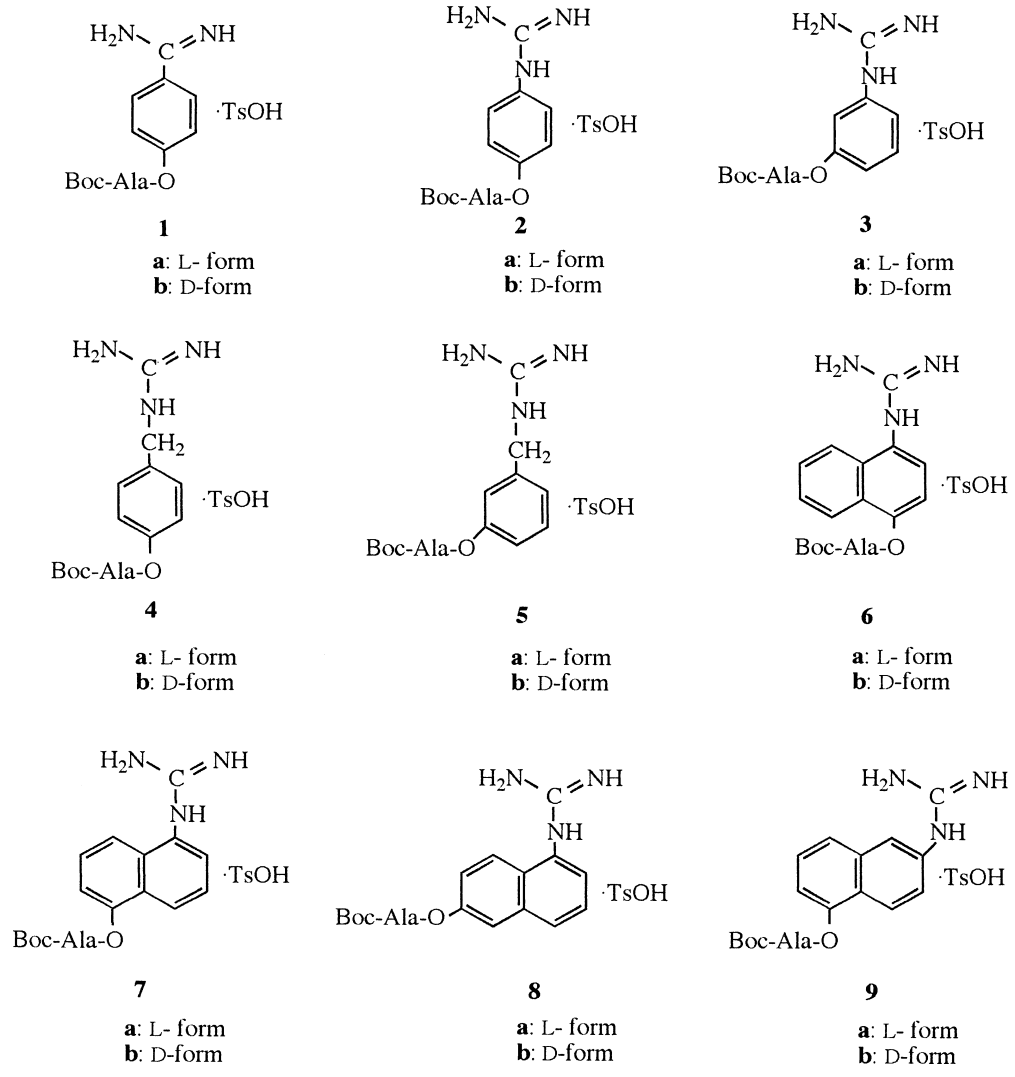

Fig. 1. Structure of inverse substrates

following our reported procedures (Sekizaki et al., 2000a). HPLC grade DMSO, DMF, and acetonitrile from Kanto Chemical Co., Inc. were used. L-Ala-*p*NA and glycylglycine were purchased from Peptide Institute, Inc. MOPS and HEPES were from Wako Pure Chemical Industries. 3,3-Dimethylglutaric acid and 2-amino-2-methyl-1,3-propanediol, and Tris were obtained from Tokyo Chemical Industry Co., Ltd. and ICN Biomedicals, Inc., respectively. Tricine and *p*-methylumbelliferyl *p'*-guanidinobenzoate were purchased from Merck & Co., Inc.

Enzymatic peptide coupling reaction

Peptide coupling reaction was carried out at 25°C in 50% DMSO-HEPES buffer (pH 8.0, containing 20 mM $CaCl_2$). Concentrations of acyl donors (**1–9**), acyl acceptor (L-Ala-*p*NA), and enzyme were 1 mM, 20 mM, and 10 μM, respectively. Concentration of chum salmon trypsin was determined by active site titration as 64% using *p*-methylumbelliferyl

p'-guanidinobenzoate according to the literature (Coleman et al., 1976). 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% aqueous trifluoroacetic acid/acetonitrile. An aliquot of the reaction mixture was injected and the eluate was monitored at 310 nm (chromophore 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; Kawai and Nagai, 1982; Okada et al., 1982). Observed peak areas were used for the estimation of sample concentration.

Results and discussion

Chum salmon trypsin-catalyzed coupling reaction of N^{α}-*tert*-butyloxycarbonyl-L-alanine *p*-guanidinophenyl ester (N^{α}-Boc-L-Ala-O*p*Gu) (**2a**) and L-Ala-*p*NA to give N^{α}-Boc-L-Ala-L-Ala-*p*NA was examined in DMSO, DMF, or acetonitrile as a co-solvent. The reaction was also evaluated under the condition where the pH of the medium was changed and the concentration of the acyl acceptor (L-Ala-*p*NA) was changed. The coupling product was obtained in high yield with DMSO, but unsatisfactorily with other organic solvents.

Effects of DMSO, DMF, and acetonitrile concentration on coupling yields are shown in Fig. 2. Coupling yields higher than 50% were observed at DMSO concentration in the range of 50–60%, and the best yield (52%) was at around

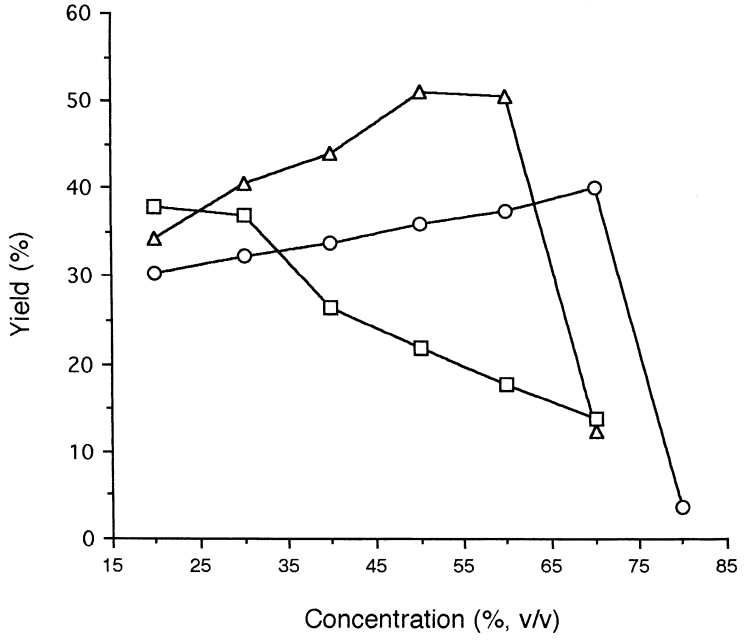

Fig. 2. Effect of organic solvent on chum salmon trypsin-catalyzed condensation of N^{α}-Boc-L-Ala-O*p*Gu with L-Ala-*p*NA. Reaction was carried out in 50 mM HEPES buffer (pH 8.0) containing DMSO (△), DMF (○), and acetonitrile (□) at 25°C. Product yield was analyzed after a reaction period of 1.5 hours in which the coupling was completed. N^{α}-Boc-L-Ala-O*p*Gu, 1 mM; L-Ala-*p*NA, 20 mM; chum salmon trypsin, 10 μM

50% DMSO. The effect of DMF concentration was similar to that of DMSO, but the coupling yields were much lower than those in DMSO. However, the extreme decrease of coupling yield, which occurred in 70% DMSO and 80% DMF, respectively. On the other hand, acetonitrile showed different behavior from DMSO and DMF; the maximum yield (38%) was observed at lower concentration of the organic solvent (20%), and the coupling yields decreased progressively with the increase of the acetonitrile. Although high concentration of an organic solvent prevents the hydrolysis of the acyl enzyme, it will decrease the enzymatic activity due to the denaturation of chum salmon trypsin as well as bovine trypsin (West and Wong, 1986). Consequently, the coupling yield was presumed to be decreased at the concentration of organic solvents higher than 60% DMSO and 70% DMF, respectively.

The effects of pH and the variety of the reaction medium on the coupling yields were analyzed. DMSO was mixed with a variety of buffers (50 mM, containing 20 mM $CaCl_2$) at various pH. The pH values given in Fig. 3 are those of the buffer itself before mixing with an organic cosolvent. The effect of pH on the coupling yield was determined (Fig. 3). The coupling yield was not simply dependent on the pH, and was greatly affected by the kind of buffer solution. The best yield was obtained at pH 8 in HEPES even though the optimum pH for chum salmon trypsin-catalyzed hydrolysis is around 10 (Sekizaki et al., 2000a).

The effect of acyl acceptor concentration on the coupling yields in 50% aqueous DMSO is shown in Fig. 4. The dependency can be explained to be due to the saturation of the enzyme binding site with the acyl acceptor. The reaction yield reached maximum (52%) at the concentration around 20 mM of acyl acceptor.

Consequently, standard conditions for chum salmon trypsin-catalyzed peptide coupling reaction were selected as described in the methods. The

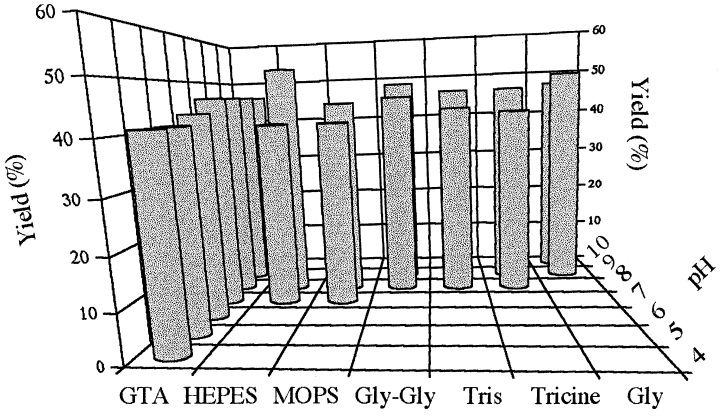

Fig. 3. Effects of pH and variety of reaction medium on chum salmon trypsin-catalyzed condensation. Reactions were carried out in 50 mM GTA, HEPES, MOPS, Glycylglycine, Tris, Tricine, and Glycine buffer containing 50% DMSO at 25°C. Product yield was analyzed after a reaction period of 1.5 hours in which the coupling was completed. N^{α}-Boc-L-Ala-O*p*Gu, 1 mM; L-Ala-*p*NA, 20 mM; chum salmon trypsin, 10 μM

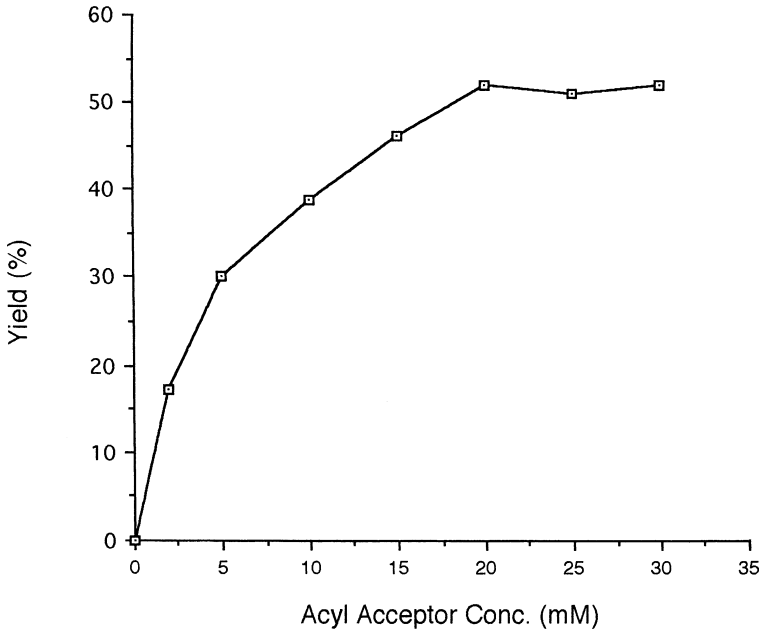

Fig. 4. Effect of acyl acceptor concentration on chum salmon trypsin-catalyzed condensation. Reactions were carried out in 50 mM HEPES buffer (pH 8.0) containing 50% DMSO at 25°C. Product yield was analyzed after a reaction period of 1.5 hours in which the coupling was completed. N^{α}-Boc-L-Ala-O*p*Gu, 1 mM; chum salmon trypsin, 10 μM; L-Ala-*p*NA, 1–30 mM

results of chum salmon trypsin-catalyzed coupling reaction were compared with those of bovine trypsin-catalyzed coupling reaction previously reported (Itoh et al., 1996; Sekizaki et al., 1996b, 1998, 1999a, 1999b), and they are summarized in Tables 1 and 2. In general, toward all inverse substrates derived from N^{α}-Boc-D-Ala (**1b–9b**), chum salmon trypsin behaved as an effective catalyst for the synthesis of the peptides (Entry 19–27 in Table 2). As shown in Table 1, chum salmon trypsin can be also utilized for the synthesis of peptides containing L-amino acid (Entry 1–9 in Table 1).

Comparative study of chum salmon trypsin and bovine trypsin as the catalyst of peptide coupling led to the conclusion that L-series substrates (**1a–9a**) behave as a good acyl donor for both chum salmon and bovine trypsin-catalyzed reaction, and the coupling yields by bovine trypsin are somewhat better than those by chum salmon trypsin. The D-series substrates (**1b–9b**) behave as a good acyl donor for both enzymes as well. Coupling yields by chum salmon trypsin, however, are better than those by bovine trypsin in this case.

In any event, all inverse substrates afforded the coupling product in good yield regardless of their structures. In the enzymatic peptide synthesis, secondary hydrolysis of the resulting peptide is unavoidable to some extent. This serious problem can be largely overcome by use of inverse substrates since the resulting peptide is much less specific to the enzyme than the substrate – "preferential coupling to hydrolysis".

Table 1. Yield of chum salmon[a] and bovine[b] trypsin-catalyzed peptide synthesis with L-amino acid ester as acyl donor

Entry No.	Trypsin	Acyl donor (No.)	Reaction time (h)	Product	Yield (%)[c]	Reference
1	Chum Salmon	N^{α}-Boc-L-Ala-OAm (**1a**)	0.2	N^{α}-Boc-L-Ala-L-Ala-*p*NA	72	This work
2	Chum Salmon	N^{α}-Boc-L-Ala-O*p*Gu (**2a**)	0.2	N^{α}-Boc-L-Ala-L-Ala-*p*NA	52	This work
3	Chum Salmon	N^{α}-Boc-L-Ala-O*p*GM (**3a**)	1	N^{α}-Boc-L-Ala-L-Ala-*p*NA	67	This work
4	Chum Salmon	N^{α}-Boc-L-Ala-O*m*Gu (**4a**)	24	N^{α}-Boc-L-Ala-L-Ala-*p*NA	41	This work
5	Chum Salmon	N^{α}-Boc-L-Ala-O*m*GM (**5a**)	6	N^{α}-Boc-L-Ala-L-Ala-*p*NA	61	This work
6	Chum Salmon	N^{α}-Boc-L-Ala-O(1–4)GN (**6a**)	0.5	N^{α}-Boc-L-Ala-L-Ala-*p*NA	72	This work
7	Chum Salmon	N^{α}-Boc-L-Ala-O(1–5)GN (**7a**)	1	N^{α}-Boc-L-Ala-L-Ala-*p*NA	64	This work
8	Chum Salmon	N^{α}-Boc-L-Ala-O(1–6)GN (**8a**)	15	N^{α}-Boc-L-Ala-L-Ala-*p*NA	67	This work
9	Chum Salmon	N^{α}-Boc-L-Ala-O(2–5)GN (**9a**)	12	N^{α}-Boc-L-Ala-L-Ala-*p*NA	67	This work
10	Bovine	N^{α}-Boc-L-Ala-OAm (**1a**)	0.2	N^{α}-Boc-L-Ala-L-Ala-*p*NA	77	Itoh et al., 1996
11	Bovine	N^{α}-Boc-L-Ala-O*p*Gu (**2a**)	0.2	N^{α}-Boc-L-Ala-L-Ala-*p*NA	64	Sekizaki et al., 1996b
12	Bovine	N^{α}-Boc-L-Ala-O*p*GM (**3a**)	5	N^{α}-Boc-L-Ala-L-Ala-*p*NA	95	Sekizaki et al., 1998
13	Bovine	N^{α}-Boc-L-Ala-O*m*Gu (**4a**)	12	N^{α}-Boc-L-Ala-L-Ala-*p*NA	58	Sekizaki et al., 1999a
14	Bovine	N^{α}-Boc-L-Ala-O*m*GM (**5a**)	12	N^{α}-Boc-L-Ala-L-Ala-*p*NA	67	Sekizaki et al., 1999a
15	Bovine	N^{α}-Boc-L-Ala-O(1–4)GN (**6a**)	1	N^{α}-Boc-L-Ala-L-Ala-*p*NA	79	Sekizaki et al., 1999b
16	Bovine	N^{α}-Boc-L-Ala-O(1–5)GN (**7a**)	2	N^{α}-Boc-L-Ala-L-Ala-*p*NA	72	Sekizaki et al., 1999b
17	Bovine	N^{α}-Boc-L-Ala-O(1–6)GN (**8a**)	12	N^{α}-Boc-L-Ala-L-Ala-*p*NA	75	Sekizaki et al., 1999b
18	Bovine	N^{α}-Boc-L-Ala-O(2–5)GN (**9a**)	12	N^{α}-Boc-L-Ala-L-Ala-*p*NA	88	Sekizaki et al., 1999b

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

Table 2. Yield of chum salmon[a] and bovine[b] trypsin-catalyzed peptide synthesis with D-amino acid ester as acyl donor

Entry No.	Trypsin	Acyl donor (No.)	Reaction time (h)	Product	Yield (%)[c]	Reference
19	Chum Salmon	N^{α}-Boc-D-Ala-OAm (**1b**)	0.3	N^{α}-Boc-D-Ala-L-Ala-*p*NA	74	This work
20	Chum Salmon	N^{α}-Boc-D-Ala-O*p*Gu (**2b**)	0.6	N^{α}-Boc-D-Ala-L-Ala-*p*NA	74	This work
21	Chum Salmon	N^{α}-Boc-D-Ala-O*p*GM (**3b**)	6	N^{α}-Boc-D-Ala-L-Ala-*p*NA	82	This work
22	Chum Salmon	N^{α}-Boc-D-Ala-O*m*Gu (**4b**)	36	N^{α}-Boc-D-Ala-L-Ala-*p*NA	45	This work
23	Chum Salmon	N^{α}-Boc-D-Ala-O*m*GM (**5b**)	36	N^{α}-Boc-D-Ala-L-Ala-*p*NA	74	This work
24	Chum Salmon	N^{α}-Boc-D-Ala-O(1–4)GN (**6b**)	1	N^{α}-Boc-D-Ala-L-Ala-*p*NA	87	This work
25	Chum Salmon	N^{α}-Boc-D-Ala-O(1–5)GN (**7b**)	5	N^{α}-Boc-D-Ala-L-Ala-*p*NA	80	This work
26	Chum Salmon	N^{α}-Boc-D-Ala-O(1–6)GN (**8b**)	72	N^{α}-Boc-D-Ala-L-Ala-*p*NA	51	This work
27	Chum Salmon	N^{α}-Boc-D-Ala-O(2–5)GN (**9b**)	48	N^{α}-Boc-D-Ala-L-Ala-*p*NA	67	This work
28	Bovine	N^{α}-Boc-D-Ala-OAm (**1b**)	1	N^{α}-Boc-D-Ala-L-Ala-*p*NA	65	Itoh et al., 1996
29	Bovine	N^{α}-Boc-D-Ala-O*p*Gu (**2b**)	0.3	N^{α}-Boc-D-Ala-L-Ala-*p*NA	74	Sekizaki et al., 1996b
30	Bovine	N^{α}-Boc-D-Ala-O*p*GM (**3b**)	24	N^{α}-Boc-D-Ala-L-Ala-*p*NA	63	Sekizaki et al., 1998
31	Bovine	N^{α}-Boc-D-Ala-O*m*Gu (**4b**)	24	N^{α}-Boc-D-Ala-L-Ala-*p*NA	20	Sekizaki et al., 1999a
32	Bovine	N^{α}-Boc-D-Ala-O*m*GM (**5b**)	24	N^{α}-Boc-D-Ala-L-Ala-*p*NA	36	Sekizaki et al., 1999a
33	Bovine	N^{α}-Boc-D-Ala-O(1–4)GN (**6b**)	1	N^{α}-Boc-D-Ala-L-Ala-*p*NA	75	Sekizaki et al., 1999b
34	Bovine	N^{α}-Boc-D-Ala-O(1–5)GN (**7b**)	6	N^{α}-Boc-D-Ala-L-Ala-*p*NA	74	Sekizaki et al., 1999b
35	Bovine	N^{α}-Boc-D-Ala-O(1–6)GN (**8b**)	24	N^{α}-Boc-D-Ala-L-Ala-*p*NA	46	Sekizaki et al., 1996b
36	Bovine	N^{α}-Boc-D-Ala-O(2–5)GN (**9b**)	24	N^{α}-Boc-D-Ala-L-Ala-*p*NA	39	Sekizaki et al., 1996b

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

It appears indeed that secondary hydrolysis of the coupling product was negligible in our enzymatic procedure, since a separate experiment incubating the coupling product for 72 h resulted in no detectable change.

Acknowledgment

This work was supported in part by a Grant-in-Aid for Scientific Research (c) (No.10671994) from Japan Society for the Promotion of Science and for High Technology Research Program from the Ministry of Education, Science, Sports and Culture of Japan, the Akiyama Foundation, and by a grant from the Japan Private School Promotion Foundation.

References

Asgeirsson B, Bjarnason JB (1991) Structural and kinetic properties of chymotrypsin from Atlantic cod (*Gadus morhua*). Comparison with bovine chymotrypsin. Comp Biochem Physiol 99B: 327–335

Asgeirsson B, Bjarnason JB (1993) Properties of elastase from Atlantic cod, a cold-adapted proteinase. Biochim Biophys Acta 1164: 91–100

Asgeirsson B, Fox JW, Bjarnason JB (1989) Purification and characterization of trypsin from the poikilotherm *Gadus morhua*. Eur J Biochem 180: 85–94

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

Bordusa F, Ullmann D, Elsner C, Jakubke H-D (1997) Substrate mimetic mediated peptide synthesis: an irreversible ligation strategy that is independent of substrate specificity. Angew Chem Int Ed Engl 36: 2473–2475

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

Coleman PL, Latham HG, Shaw EN Jr (1976) In: Colowick SP, Kaplan NO (eds) Methods in enzymology, vol. 45. Academic Press, New York, pp 12–26

Gildberg A, Overbo K (1990) Purification and characterization of pancreatic elastase from Atlantic cod (*Gadus morhua*). Comp Biochem Physiol 97B: 775–782

Itoh K, Sekizaki H, Toyota E, Tanizawa K (1995) Synthesis and properties of N^{α}-(tert-butyloxycarbonyl)peptide *p*-guanidinophenyl esters as trypsin substrates. Chem Pharm Bull 43: 2082–2087

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

Kristjansson MM, Nielsen HH (1992) Purification and characterization of two chymotrypsin-like proteases from the pyloric caeca of rainbow trout (*Oncorhynchus mykiss*). Comp Biochem Physiol 101B: 247–253

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

Osnes K Kr, Mohr V (1985) On the purification and characterization of three anionic serine type peptide hydrolases from Antarctic krill. *Euphausia superba*. Comp Biochem Physiol 82B: 607–619

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

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, Murakami M, Toyota E, Tanizawa K (2000a) Anionic trypsin from chum salmon: activity with *p*-amidinophenyl ester and comparison with bovine and *Streptomyces griseuse* trypsin. Comp Biochem Physiol 127B: 337–346

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

Simpson BK, Haard NF (1984) Purification and characterization of trypsin from the Greenland cod (*Gadus ogac*), 1. Kinetic and thermodynamic characteristics. Can J Biochem Cell Biol 62: 894–900

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

Vecchi SD, Coppers Z (1996) Marine fish digestive protease – Relevance to food industry and the south-west Atlantic region – A review. J Food Biochem 20: 193–214

West JB, Wong C-H (1986) Enzyme-catalyzed irreversible formation of peptides containing D-amino acid. J Org Chem 51: 2728–2735

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

Authors' address: Prof. Kazutaka Tanizawa, Faculty of Pharmaceutical Sciences, Health Sciences University of Hokkaido, Ishikari-Tobetsu, Hokkaido 061-0293, Japan

Received August 10, 2000