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Journal of Molecular Catalysis B: Enzymatic 11 (2000) 23–28

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

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Chum salmon trypsin-catalyzed peptide synthesis with inverse substrates as acyl donor components at low temperature

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Received 16 May 2000; received in revised form 16 June 2000; accepted 16 June 2000

Abstract

Chum salmon trypsin-catalyzed peptide synthesis has been studied by using *p*-amidinophenyl esters of N^{α}-(*tert*-butyloxycarbonyl)amino acid as the acyl donor components at 0°C. The reaction conditions were optimized for an organic solvent, pH, and concentration of the acyl acceptor. The method was shown to be successful as a general method for the synthesis of the peptide, and also useful for the preparation of peptides containing D-amino acids. The enzymatic hydrolysis of the resulting products was negligible. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Chum salmon trypsin; Enzymatic peptide synthesis; Inverse substrate; N^{α}-(*tert*-Butyloxycarbonyl)amino acid *p*-amidinophenyl ester; Oligopeptide

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1. Introduction

Peptide synthesis by protease-catalyzed reverse reaction has been extensively studied with a variety of model oligopeptides [1–4]. 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 [1–4]. The most serious drawback of the enzymatic method, however, is the restrictive substrate specificity. Thus, the application of proteases to peptide synthesis has been limited by the specificity of the enzymes.

Previously, we reported that inverse substrates such as *p*-amidino- [5], *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. Jakubke and co-workers [9–11] and we [12–16] demonstrated successful application of inverse substrates to trypsin-catalyzed coupling.

Many studies on the characterization of trypsins from cold-adapted species have been reported [17]. These trypsins display substantially higher catalytic efficency than their mammalian counterparts [18–24]. Thus, trypsin from chum salmon (*Onchorhynchus keta*) is expected to be a highly efficient catalyst for

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PII: S1381-1177(00)00192-2

enzymatic peptide synthesis. Recently, we reported the isolation and properties of trypsin from chum salmon [25]. We are interested in the application of chum salmon trypsin to synthesis at low temperature.

The purpose of the present study is to elucidate the requirements for chum salmon trypsin-catalyzed peptide coupling reaction of *p*-amidinophenyl esters with a view of utilizing the reaction as a preparative method for peptide synthesis at low temperature.

2. Experimental

2.1. Analysis

HPLC analysis was performed by using reversed-phasecolumn (Shim-pack, CLC-ODS(M), 4.6 mm × 250 mm)on a Shimadzu LC-6A pump system equipped with a Shimadzu SPD-6AV UV-VIS spectrophotometric detector. UV analysis was carried out with a Hitachi U-2000 spectrophotometer.

2.2. Materials

Chum salmon trypsin was prepared following our reported procedures [25]. Dimethylsulfoxide (DMSO), dimethylformamide (DMF), and acetonitrile of HPLC grade from Kanto Chemical Co., Inc. were used. L-Alanine-*p*-nitroanilide (L-Ala-*p*NA) was purchased from Peptide Institute, Inc. 3,3-Dimethylglutaric acid and 2-amino-2-methyl-1,3-propanediol, and tris(hydroxymethyl)aminomethane were obtained from Tokyo Chemical Industry Co., Ltd. and ICN Biomedicals, Inc., respectively. *p*-Methylumbelliferyl *p*′-guanidinobenzoate was purchased from Merck and Co., Inc. Other chemicals used for the analytical study and synthetic work were obtained from Kanto Chemical Co., Inc. Inverse substrates (**1–10**) were prepared, following our reported procedures [12,26].

2.3. Enzymatic peptide coupling reaction

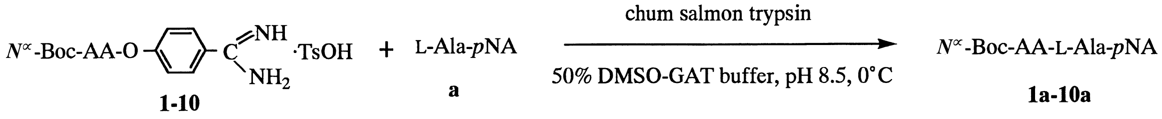
Peptide coupling reaction was carried out at 0°C in 50% DMSO solution, which was mixed with 50 mM solution of 3,3-dimethylglutaric acid (G), tris(hydroxymethyl)aminomethane (T), and 2-amino-2-methyl-1,3-propanediol (A) (GTA buffer) (pH 8.5, containing 20 mM $CaCl_2$). Concentrations of acyl donors (**1–10**), acyl acceptor (L-Ala-*p*NA), and enzyme were 1 mM, 20 mM, and 5 μM, respectively. Concentration of chum salmon trypsin was determined by active site titration as 64%, using *p*-methylumbelliferyl *p*′-guanidinobenzoate according to the literature [27]. The progress of the peptide coupling reaction was monitored by HPLC under the following conditions: isocratic elution at 1 ml min^{-1}, 0.1% trifluoroacetic acid/acetonitrile. An aliquot of the reaction mixture was injected and the eluate was monitored at 310 nm (chromophore due to *p*-nitroanilide moiety). Peak identification was made by correlating the retention time with that of authentic samples which were chemically synthesized [28–30]. Observed peak areas were used for the estimation of sample concentration.

3. Results and discussion

Hydrolysis of reactant components is a serious problem incurred in the enzyme-catalyzed synthetic method. We previously investigated the occurrence of nonenzymatic reactions. At 50% solvent content N^{α}-(*tert*-butyloxycarbonyl)-L-alanine *p*-amidinophenyl ester (N^{α}-Boc-L-Ala-OAm) (**2**) underwent some spontaneous hydrolysis with a half-life of about 15 h at 25°C. In the case that L-alanine *p*-nitroanilide (L-Ala-*p*NA) (**a**) was present, the coupling product (**2a**) was detected in 3% yield after 5 min [12].

Therefore, we expected to solve this problem by use of cold-adapted trypsin at low temperature. The chum salmon trypsin-catalyzed peptide coupling reaction has been studied by using synthetic inverse substrates (**1–10**) as the acyl donors. The coupling reaction was carried out by incubating an acyl donor with an acyl acceptor (L-Ala-*p*NA) (a) and chum salmon trypsin in a mixture of GTA buffer (50 mM, containing 20 mM $CaCl_2$, pH 8.5) and an organic solvent at 0°C (Scheme 1). The progress of the coupling reaction was monitored by HPLC.

Chum salmon trypsin-catalyzed coupling reaction of N^{α}-Boc-L-Leu-OAm (**3**) and L-Ala-*p*NA (**a**) to



1: AA = Gly; **2**: AA = L-Ala; **3**: AA = L-Leu; **4**: AA = L-Phe; **5**: AA = L-Pro;
6: AA = D-Ala; **7**: AA = D-Leu; **8**: AA = D-Phe; **9**: AA = D-Pro; **10**: AA = β-Ala

Scheme 1.

give N^{α}-Boc-L-Leu-L-Ala-pNA (**3a**) was examined in DMSO, DMF, or acetonitrile as a co-solvent. The reaction was also evaluated under various conditions where the pH of the medium and the concentration of the acyl acceptor, L-Ala-pNA (**a**), were changed.

Effects of DMSO, DMF, and acetonitrile concentration on coupling yields are shown in Fig. 1. Coupling yields higher than 80% were observed at DMSO concentration in the range of 30–60%, and the best yield (86%) was around 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, was not observed in 70% DMF. On the other hand, acetonitrile showed different behavior from DMSO and DMF, the maximum yield (80%) was observed at lower concentration of the organic solvent (30%), and the coupling yields decreased progresively 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 [31]. Consequently, the coupling yield was presumed to be decreased at the concentration of organic solvents higher than 60%, but chum salmon trypsin seemed still stable even in 70% DMF.

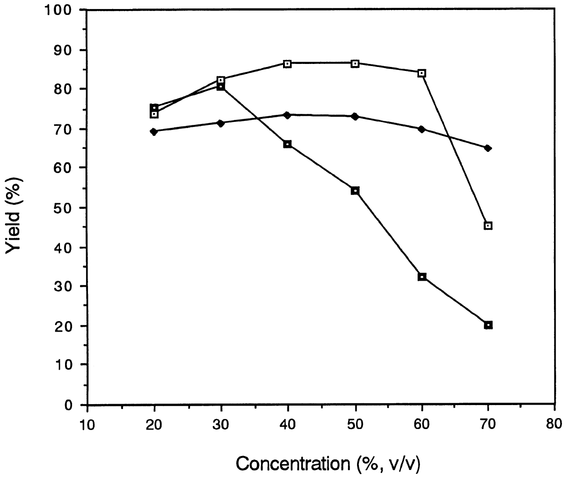


Fig. 1. Effect of organic solvent on chum salmon trypsin-catalyzed condensation of N^{α}-Boc-L-Leu-OAm (**3**) with L-Ala-pNA (**a**). Reaction was carried out in 50 mM GTA buffer (pH 8.5) containing DMSO (⊡), DMF (◆), and acetonitrile (■) at 0°C. Product yield was analyzed after a reaction period of 1.5 h in which the coupling was completed. N^{α}-Boc-L-Leu-OAm (**3**), 1 mM; L-Ala-pNA (**a**), 20 mM; chum salmon trypsin, 5 μM.

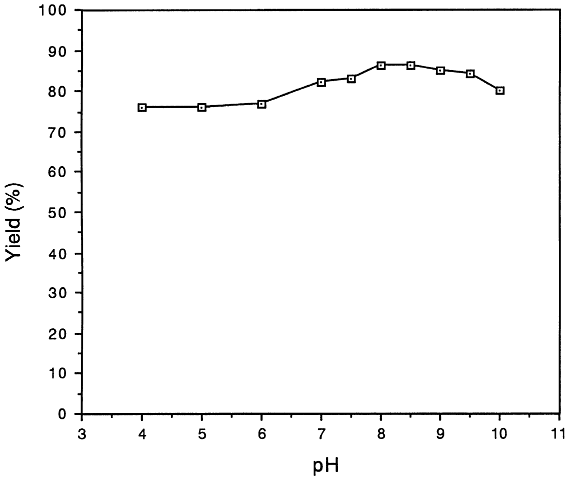


Fig. 2. pH dependency of chum salmon trypsin-catalyzed condensation. Reactions were carried out in 50 mM GTA buffer containing 50% DMSO at 0°C. Product yield was analyzed after a reaction period of 1.5 h in which the coupling was completed. N^{α}-Boc-L-Leu-OAm (**3**), 1 mM; L-Ala-pNA (**a**), 20 mM; chum salmon trypsin, 5 μM.

The effect of pH of the reaction medium on the coupling yields was analyzed. DMSO was mixed with GTA buffer (50 mM, containing 20 mM $CaCl_2$) at various pH. The pH values given in Fig. 2 are those of the buffer itself before mixing with an organic co-solvent. The pH-dependency of the coupling yield was determined (Fig. 2). The coupling yield was essentially unaffected by pH change in the reaction media after the analysis with the reaction period of 90 min. The best yield (86%) was obtained at around pH 8.0–8.5 even though the optimum pH for chum salmon trypsin-catalyzed hydrolysis is around 10 [25].

The effect of acyl acceptor concentration on the coupling yields in 50% aqueous DMSO is shown in Fig. 3. The dependency can be explained to be due to the saturation of the enzyme binding site with the acyl acceptor. The reaction yield reached to the maximum (86%) at concentrations 15–20 mM of the acyl acceptor.

Consequently, the standard conditions for chum salmon trypsin-catalyzed peptide coupling reaction were selected as described in the experimental. The time courses of the coupling of N^{α}-Boc-L-Leu-OAm (**3**) and N^{α}-Boc-D-Leu-OAm (**7**) with L-Ala-*p*NA (a) are shown in Fig. 4. Fig. 4 showed that the D-acyl donor is a versatile substrate for the enzymatic synthesis, as is also the L-acyl donor, albeit the reaction is slow.

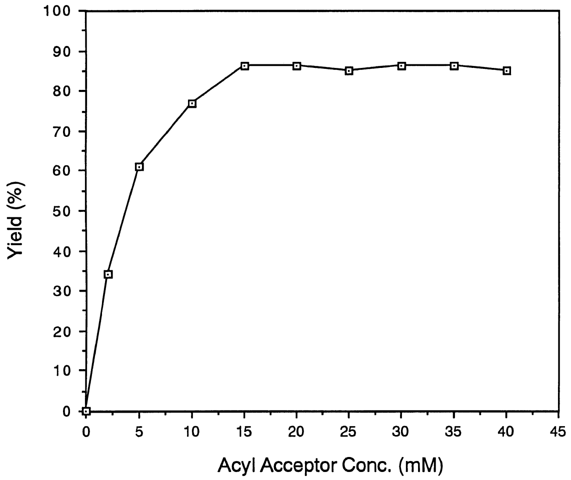


Fig. 3. Effect of acyl acceptor concentration on chum salmon trypsin-catalyzed condensation. Reactions were carried out in 50 mM GTA buffer (pH 8.5) containing 50% DMSO at 0°C. Product yield was analyzed after a reaction period of 1.5 h in which the coupling was completed. N^{α}-Boc-L-Leu-OAm (**3**), 1 mM; chum salmon trypsin, 5 μM; L-Ala-*p*NA (**a**), 2–40 mM.

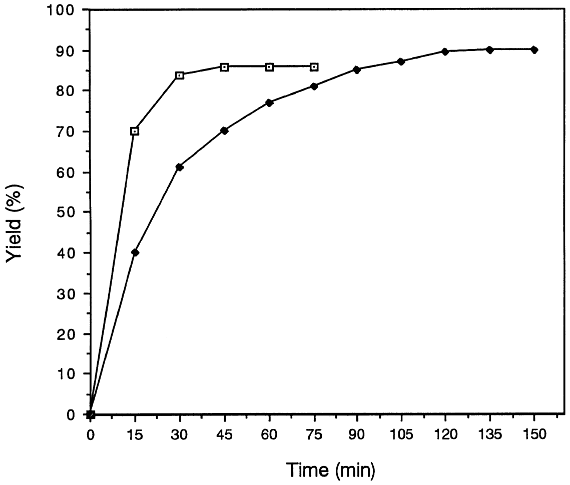


Fig. 4. Time course for the chum salmon trypsin-catalyzed synthesis of N^{α}-Boc-L-Leu-L-Ala-*p*NA (**3a**) (□) and N^{α}-Boc-D-Leu-L-Ala-*p*NA (**7a**) (◆). Conditions: acyl donor, 1 mM; acyl acceptor (L-Ala-*p*NA)(a), 20 mM; chum salmon trypsin, 5 μM; 50% DMSO-GTA (50 mM, pH 10, containing 20 mM $CaCl_2$); 0°C.

The results of chum salmon trypsin-catalyzed coupling reaction were compared with those of trypsin-catalyzed coupling reaction previously reported [12], and they are summarized in Table 1. In general, toward all inverse substrates (**1–10**), chum salmon trypsin behaved as a moderately effective catalyst for the synthesis of the peptides (Entry 1–10 in Table 1). As shown in Table 1, chum salmon trypsin can be also utilized for the synthesis of peptides containing D-amino acid and β-amino acid though the reaction requires long reaction time (Entry 6–10 in Table 1).

In conclusion, the utility of chum salmon trypsin can be proposed as a catalyst for the synthesis of peptide by use of inverse substrates, *p*-amidinophenyl esters. The method, which is operative at low temperature, is advantageous since the spontaneous hydrolysis of the acyl donor is retarded; i.e. the half-life for the spontaneous hydrolysis of **2** in 50% aqueous DMSO at 0°C was determined to be 113 h. It must be emphasized that the coupling yields in the present method were not decreased by a longer period of incubation. This result suggested that secondary hy-

Table 1
Yield of chum salmon trypsin-catalyzed peptide synthesis[a]

Entry no.	Acyl donor	Reaction		Product (no.)	Yield	
		time (min)	[ ][b]		(%)	[ ][c]
1	N^{α}-Boc-Gly-OAm (**1**)	15	[5][d]	N^{α}-Boc-Gly-L-Ala-*p*Na (**1a**)	56	[77][d]
2	N^{α}-Boc-L-Ala-OAm (**2**)	30	[5][d]	N^{α}-Boc-L-Ala-L-Ala-*p*Na (**2a**)	82	[77][d]
3	N^{α}-Boc-L-Leu-Oam (**3**)	45	[5][d]	N^{α}-Boc-L-Leu-L-Ala-*p*Na (3**a**)	86	[85][d]
4	N^{α}-Boc-L-Phe-Oam (**4**)	30	[5][d]	N^{α}-Boc-L-Phe-L-Ala-*p*Na (**4a**)	85	[82][d]
5	N^{α}-Boc-L-Pro-Oam (**5**)	12[e]	[120][d]	N^{α}-Boc-L-Pro-L-Ala-*p*Na (**5a**)	47	[80][d]
6	N^{α}-Boc-D-Ala-Oam (**6**)	60	[60][d]	N^{α}-Boc-D-Ala-L-Ala-*p*Na (**6a**)	77	[65][d]
7	N^{α}-Boc-D-Leu-Oam (**3**)	120	[120][d]	N^{α}-Boc-D-Leu-L-Ala-*p*Na (**7a**)	90	[68][d]
8	N^{α}-Boc-D-Phe-Oam (**4**)	60	[120][d]	N^{α}-Boc-D-Phe-L-Ala-*p*Na (**8a**)	59	[64][d]
9	N^{α}-Boc-D-Pro-Oam (**5**)	48[e]	[12 h][d]	N^{α}-Boc-D-Pro-L-Ala-*p*Na (**9a**)	70	[76][d]
10	N^{α}-Boc-β-Ala-Oam (**6**)	90	[120][d]	N^{α}-Boc-β-Ala-L-Ala-*p*Na (**10a**)	4	[8][d]

[a] Conditions: acyl donor, 1 mM; acyl acceptor (L-Ala *p*NA), 20 mM; chum salmon trypsin, 5 μM; 50% DMSO-GTA (50 mM, pH 8.5, containing 20 mM $CaCl_2$); 0°C; concentrations of enzymes (chum salmon and bovine trypsins) were used, those determined by active site titration method [27].

[b] The values in brackets are reaction times (min) of bovine trypsin-catalyzed peptide synthesis.

[c] The values in brackets are yields (%) of bovine trypsin-catalyzed peptide synthesis.

[d] See reference [12].

[e] Rection time is in hours.

drolysis of the resulting products by enzyme is negligible.

Acknowledgements

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.

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