BIOORGANIC CHEMISTRY **24,** 59–68 (1996)
ARTICLE NO. 0007

Application of Inverse Substrates to Trypsin-Catalyzed Peptide Synthesis

KUNIHIKO ITOH, HARUO SEKIZAKI, EIKO TOYOTA, NORIHISA FUJIWARA, AND KAZUTAKA TANIZAWA[1]

Faculty of Pharmaceutical Sciences, Health Sciences University of Hokkaido, Ishikari-Tobetsu, Hokkaido 061-02, Japan

Received May 25, 1995

Trypsin-catalyzed peptide synthesis has been studied by using ''inverse substrate,'' i.e., *p*-amidinophenyl ester derived from α-amino acid derivative as an acyl donor component. Inverse substrate can afford acyl trypsin in a very specific manner, liberating the site-specific *p*-amidinophenyl moiety as the leaving group. Thus a variety of α-amino acid residues which are a part of *p*-amidinophenyl ester can be involved in the trypsin-catalyzed coupling reaction. The method has been shown to be successful as expected. In conclusion, the method was proposed as a new procedure which overcomes the disadvantage of enzymatic peptide synthesis.

INTRODUCTION

It is recognized that enzyme-catalyzed peptide synthesis is more advantageous than chemical synthesis in many respects (*1*, *2*). The enzymatic method is highly stereoselective, racemization-free, and requires minimal side-chain protection. The enzymatic method is at an advantage when discriminating enantiomers and amino acid side-chain residue and at a disadvantage as a general procedure of peptide synthesis. The reactants used as acyl coupling component are limited. Only the amino acid or peptide derivatives which meet the substrate specificity of the enzyme are applicable to the coupling reaction. This is because the enzymatic peptide coupling is considered to proceed via enzyme–substrate complex and subsequent acyl enzyme formation. In the case of trypsin-catalyzed reaction, for example, the acyl donor is limited to positively charged arginine or lysine residue at the carboxyl end position.

In our previous work (*3*), it has been shown that esters of *p*-amidinophenol are specifically hydrolyzed by trypsin and trypsin-like enzymes. In these esters the site-specific group for the enzyme, a charged amidinium group, is liberated during acylation to produce an acyl enzyme intermediate. Kinetic analyses showed that the binding affinity and catalytic efficiency at the acylation step of these esters are comparable to those for normal-type specific substrates, and so a new term, ''inverse substrates,'' was proposed for these esters. Thus the compounds provide a general method for efficient synthesis of acyl-enzymes without recourse to the structure of

[1] To whom correspondence should be addressed.

0045-2068/96 $18.00

the acyl component. The compounds are applicable to trypsin-catalyzed peptide synthesis for the same reason. The method is extremely useful since the acyl component is not restricted to cationic residues such as lysine and arginine.

Noting the characteristic feature of inverse substrate Schellenberger *et al.* (*4*) have tested the applicability of *p*-guanidinophenyl ester as tool for trypsin-catalyzed peptide synthesis. The paper prompted us to report the results on the general use of our inverse substrate, *p*-amidinophenyl esters, to tryptic peptide synthesis in detail.

EXPERIMENTAL PROCEDURES

Synthetic Chemistry

General. Dimethylformamide (DMF), ethyl acetate, and diethyl ether were obtained as the anhydrous solvents by usual manner. *N-tert*-butyloxycarbonylamino acid (Boc-amino acid) and *N,N′*-dicyclohexylcarbodiimide(DCC) were purchased from Peptide Institute, Inc. All other reagents were used without further purification from Aldrich Chemical Co., Inc., Wako Pure Chemical Industries, LTD., and Kanto Chemical Co., Inc. Melting points were determined on a Yanaco MP-500D apparatus and are uncorrected. Infrared spectra were recorded on a JASCO VALOR-III FT-IR spectrometer. NMR spectra were recorded on a JEOL JNM-FX400 FT NMR spectrometer.

General procedure for synthesis of inverse substrates. Inverse substrates, i.e., Boc-amino acid *p*-amidinophenyl esters, were prepared as follows according to our previous paper (*5*). DCC (454 mg, 2.2 mmol) was added to a solution of Boc-amino acid (2.0 mmol), *N*-benzyloxycarbonyl-*p*-amidinophenol (*5*) (540 mg, 2.0 mmol), and 4-dimethylaminopyridine (24 mg, 0.2 mmol) in 3 ml of DMF and 6 ml of ethyl acetate. The reaction mixture was kept at 0°C for 30 min and subsequently at room temperature overnight. The precipitated dicyclohexylurea was removed by filtration and the filtrate was evaporated *in vacuo.* The resulting *N*-benzyloxycarbonyl-*p*-amidinophenyl ester was recrystallized from benzene–hexane. Isolation yields of 64–80% resulted. Structure of the ester was confirmed by NMR spectra, ir spectra, and elemental analysis. Optical purity of each enantiomer was analyzed by optical rotation measurement. The ester (1.0 mmol) was dissolved in methanol (20 ml) containing *p*-toluenesulfonic acid monohydrate (190 mg, 1.0 mmol) and hydrogenated in the presence of 10% Pd-charcoal (10 mg). After the catalyst was removed, the filtrate was evaporated to dryness *in vacuo* and the residue was washed with dry ether. Recrystallization from ethanol–ether gave pure *p*-amidinophenyl ester tosylate.

p-Amidinophenyl esters prepared according to the general procedure are summarized in Table 1.

Enzyme Assay

Substrates and enzymes. Alanine *p*-nitroanilide (Ala-*p*NA) and leucine *p*-nitroanilide (Leu-*p*NA) were purchased from Peptide Institute, Inc. Glycine *p*-nitroanilide (Gly-*p*NA), D-alanine *p*-nitroanilide (D-Ala-*p*NA), and D-leucine *p*-nitroanilide (D-Leu-*p*NA) were prepared following the reported procedure (*6–8*).

TABLE 1
Physical Data of the Inverse Substrates (*N-tert*-Butyloxycarbonylamino Acid *p*-Amidinophenyl Ester Tosylate) Used for Trypsin-Catalyzed Coupling Reaction

Inverse substrate tosylate	mp(°C)	$[\alpha]_D^{20}$ (c = 1, methanol)	Formula	Analysis: calculated(found) C	H	N	S
Boc-GlyOAm	170–172		$C_{21}H_{27}N_3O_6S$ 4/3H_2O	52.65 (52.37	6.00 5.70	8.77 8.53	6.69 7.04)
Boc-AlaOAm	162–164	−41.8	$C_{22}H_{29}N_3O_7S$	55.10 (55.04	6.10 6.07	8.76 8.72	6.69 6.71)
Boc-D-AlaOAm	163–164	+41.2	$C_{22}H_{29}N_3O_7S$ 1/2H_2O	54.09 (54.00	6.19 5.94	8.60 8.48	6.56 6.69)
Boc-PheOAm	161–162	−7.2	$C_{28}H_{33}N_3O_7S$ 1/2H_2O	59.56 (59.56	6.07 5.80	7.44 7.46	5.68 5.96)
Boc-D-PheOAm	161–163	+7.0	$C_{28}H_{33}N_3O_7S$ 1/2H_2O	59.56 (59.68	6.07 5.93	7.44 7.49	5.68 5.99)
Boc-LeuOAm	153–154	−40.0	$C_{25}H_{35}N_3O_7S$ 2/3H_2O	56.27 (56.24	6.86 6.66	7.65 7.65	6.01 6.26)
Boc-D-LeuOAm	153–155	+39.2	$C_{25}H_{35}N_3O_7S$ 2/3H_2O	56.27 (56.37	6.86 6.63	7.65 7.71	6.01 6.43)

Bovine pancreatic trypsin (EC 3.4.21.4) purchased from Worthington Biochemical Corp. (twice recrystallized, lot TRL) was further purified by affinity chromatography (*9*), using Benzamidine Sepharose 6B obtained from Pharmacia. Enzyme concentration was determined from active site titration using *p*-nitrophenyl *p*-guanidinobenzoate (*10*).

Trypsin-catalyzed peptide coupling reaction. Peptide coupling reaction was carried out at 25°C in 50 mM 4-morpholinepropanesulfonic acid buffer (MOPS), pH 8.0, containing dimethylsulfoxide (DMSO). Concentrations of acyl donor (inverse substrate), acyl acceptor (amino acid *p*-nitroanilide), and trypsin were 1 mM, 20 mM, and 5μM, respectively. The progress of the coupling reaction was monitored by HPLC on a Hitachi Model L-6200 equipped with a Hitachi UV-VIS detector. The chromatograph was equipped with a Wakosil 5C18-200 column (4.0 × 250 mm). The mobile phase was acetonitrile-0.1% aqueous trifluoroacetic acid and the flow rate was 1.0 ml/min. Analysis was performed using isocratic system. An aliquot of the reaction mixture was injected and peaks were detected at 310 nm for *p*-nitroanilide moiety. Peak identification was made by correlation with authentic samples which were chemically synthesized (*11, 12*). Peak intensities were used to calculate relative concentration.

RESULTS

Trypsin-catalyzed coupling reaction of *N-tert*-butyloxycarbonylalanine *p*-amidinophenyl ester (Boc-Ala-OAm) and Ala-*p*NA to give Boc-Ala-Ala-*p*NA was determined in aqueous DMSO and aqueous DMF. The coupling reaction was instantaneous in the media less than 60% organic solvent and was completed within several minutes. During the reaction period the peak of Boc-Ala-OAm completely disappeared from the HPLC elution diagram. Only two peaks which were assigned as the coupling product and the hydrolysate newly emerged. Control experiments in the absence of trypsin showed that hydrolysis of Boc-Ala-OAm is so slow that no appreciable amount of the ester is consumed during the period that enzymatic reaction is complete. The rate constant for the spontaneous hydrolysis at pH 8.0 in 50% DMSO was determined to be 1.3×10^{-5} s^{-1} which is equal to 14.8 h of half-life time. In the presence of the acyl acceptor aminolysis takes place and affords coupling product even in the absence of the enzyme though the extent of the reaction is not large. It was found that the nonenzymatic aminolysis is sufficiently less than the enzymatic reaction; i.e., coupling yield of Boc-Ala-Ala-*p*NA in the absence of trypsin is 3% after 0.1 h. Thus the enzymatic hydrolysis of acyl donor (inverse substrate) is the only reaction competitive to the peptide synthesis, and the rest of the coupling is nearly equal to the part of hydrolysis.

Effects of DMSO and DMF concentration on coupling yields were shown in Fig. 1. Coupling yields higher than 65% were observed at the DMSO concentration range of 20–60%, and the best yield (85%) was obtained at 50% DMSO. Effect of DMF was nearly the same as that of DMSO but the coupling yield in aqueous DMF was less than that in aqueous DMSO. The diminished coupling yield at the low concentration of organic solvent could be due to the enzyme-catalyzed hydrolysis of acyl donor as mentioned previously. A higher concentration of organic solvent is not completely advantageous for the coupling, although it is expected to retard the hydrolysis. The high concentration of organic solvent results in the separation of the catalyst as well as the decrease of enzymatic activity.

The effect of pH of the buffer component in the medium on the coupling yields was analyzed. Reaction yields were determined for 50% DMSO solutions, changing pH of the buffer solution. The pH dependency of the yield at the reaction period of 10 min was determined as shown in Fig. 2. The pH dependency was also determined for 50% DMF in a similar manner. The pH dependency was not different for both cases though the reaction yield in 50% DMF was relatively low. The observed dependency was similar to that of trypsin-catalyzed hydrolysis of the specific ester substrates as reported previously in which the catalytic rate was increased at the higher pH and reached the limit at around pH 9 (*13*). Effect of cosolvent on the coupling yield was analyzed. Among six solvents so far tested, DMF and especially DMSO were found to be advantageous, as shown in Table 2. The analysis was made in MOPS buffer (pH 8.0) containing 50% organic solvent. For each case the coupling reaction was completed within 2 h. As shown in Table 2 the best result was obtained with DMSO after all. The observation was not different from that of enzyme-catalyzed coupling procedure by means of conventional substrates (*1*, *2*).

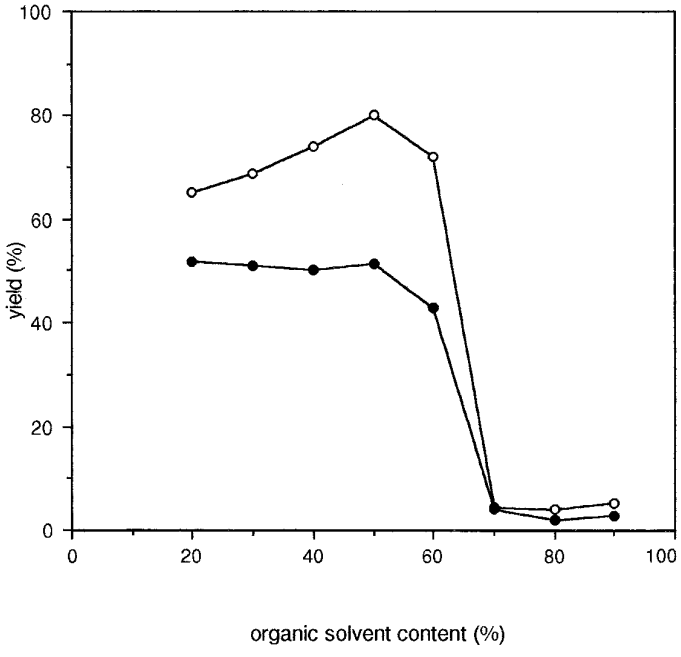

FIG. 1. Effect of organic solvent on trypsin-catalyzed condensation of *t*-butyloxycarbonylalanine *p*-amidinophenyl ester and alanine *p*-nitroanilide. Reaction was carried out in 50 mM MOPS buffer (pH 8.0) containing DMSO (○) or DMF (●) at 25°C. Product yield was analyzed after reaction period of 10 min in which the coupling was completed. Boc-Ala-OAm, 1 mM; Ala-*p*NA, 20 mM; trypsin, 5 μM.

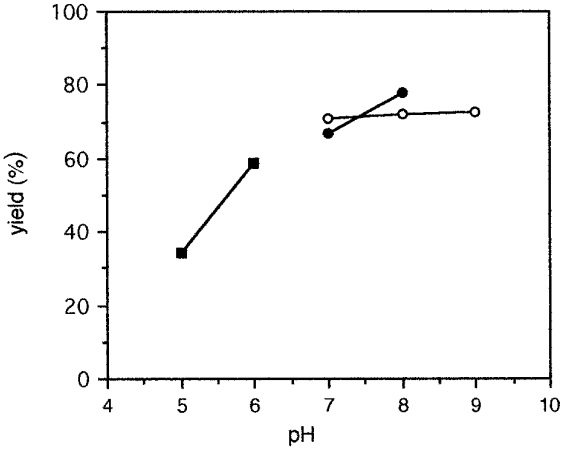

FIG. 2. pH dependency of trypsin-catalyzed condensation. Boc-Ala-OAm and Ala-*p*NA were reacted in 50 mM 4-morpholineethanesulfonic acid (MES) (■), 50 mM MOPS (●), and 50 mM Tris (○) buffers containing 50% DMSO at 25°C. Boc-Ala-OAm, 1 mM; Ala-*p*NA, 20 mM; trypsin, 5 μM. Coupling yield was determined after a reaction period of 10 min in which the coupling was completed.