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CHEMO-ENZYMATIC SYNTHESIS OF FMOC-PEPTIDE FRAGMENTS

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Abstract: Optically pure Fmoc-peptide fragments have been prepared *via* dicyclohexylcarbodiimide coupling of N-protected amino acids with amino acid esters, followed by enzyme-catalyzed ester hydrolysis by alcalase with a high concentration of organic solvent in a high yield.

Peptide fragment condensation has been used to synthesize a large number of biologically active peptides[1]. Because the failure of coupling of the fragments in any of the synthetic steps will result in a significant difference in the physico-chemical property between the desired product and the missing-fragment peptide, the purification procedure is efficient and simple. Many methods have been reported for preparing of peptide fragments[2].

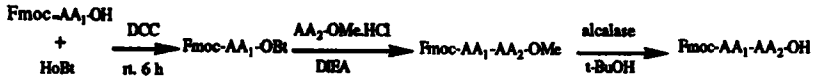
Fmoc-amino acid derivatives are widely used in solid and solution phase peptide synthesis[3]. The synthesis of Fmoc-peptide fragments has been little studied. This is due to the fact that i) Fmoc-peptide fragments can not be prepared *via* base-catalyze saponification from Fmoc-peptide ester, because the Fmoc-group is labile with base and will stripe during

the saponification[4], and ii) the side-chain protecting group of Fmoc-amino acid derivatives are labile with acids; therefore these Fmoc-peptide fragments cannot be obtained by means of acidolysis to remove the C-terminal protecting group either. Using enzyme-catalyze hydrolysis to remove the C-terminal protecting group in mild conditions is one of the best choices for preparing Fmoc-peptide fragments. Recently, we have found that the alkaline protease "alcalase" is stable and has high esterolytic activity in a high concentration of organic solvents such as ethanol, isopropanol, and t-butanol[5]. The Fmoc-peptide esters are insoluble in aqueous solution or a low concentration of organic co-solvent but soluble in a high concentration of organic co-solvent. This report describes a practical procedure for the preparation of Fmoc-peptide fragments by saponification of Fmoc-peptide esters in a high concentration of organic solvent catalysed by alcalase.

Results and discussion

Peptide synthesis was performed via the preformed-active ester method. As shown in scheme 1, coupling of an N-protected amino acid with 1-hydroxybenzotrizole (HoBt) in ethyl acetate, using dicyclohexylcarbodiimide as the coupling reagent, afforded the N-protected amino acid HoBt active ester. The N,N'-dicyclohexylurea formed as a by-product in such coupling was removed by filtration. The nucleophile was prepared by dissolving the amino acid methyl ester with one equivalent of diisopropylethylamine in dichloromethane. Peptide bond formation was achieved by reacting the nucleophile with the N-protected amino acid HoBt active ester, and the product was

Scheme 1



isolated simply by extraction with ethyl acetate. The results are shown in table 1 . The yields of each coupling were about 75-90%.

The stability of alcalase[5] in organic solvents was measured by the rate of inactivation of the enzyme using the basis of remaining activity method[6]. Figure 1 is the time course of alcalase inactivated under typical reaction conditions. The 100% activity was defined as the hydrolytic activity of an aliquot of the mixture towards benzoyl Arginine p-nitrophenol ester, 10 s after addition of the alcalase to the reaction mixture. In methanol solution, nearly half of the original activity was lost within 35 min. In t-butanol, ethanol and isopraponal, half of the original activity was maintained after about 24 hours. In pure water or buffer solution at the same reaction pH, the half live of the enzymtic activity can be maintained for more than 3 hours. In other solvents, such as acetone, dioxane, acetonitrile and chloroform, the enzyme is insoluble and precipitate to stick on the wall of the reaction flask.

Enzyme catalyzed saponification was carried out at 35°C using t-butanol as a solvent. In a preliminary test, alcalase (0.1 mL, 0.25 AU), Fmoc-Ala-Val-OMe (0.112 g. 0.25 mmol), in a mixture of t-butanol (20 mL) and phosphate buffer (2 mL, pH 8.2, 0.5M) were stirred at 35°C. Periodically, 50 µl aliquots were taken and analyzed on hplc. Figure 2

Table 1. Chemical synthesis of Fmoc-peptide esters and enzyme-catalyzed saponification.

substrate/product	mp: °C,	$[\alpha]_D$:[a]	yield(%):	amino acid analysis:
1. Fmoc-Ala-Val*-Ile-OMe	181-182	-11.12	90	Ala:Val:Ile
Fmoc-Ala-Val-Ile-OH	202-204	-7.14	85	1.01:0.97:0.97
2. Fmoc-Ala*-Val-OMe	157-159	-8.91	88	Ala:Val
Fmoc-Ala-Val-OH	184-186	-1.98	86	1.00:1.01
3. Fmoc-Phe*-Thr-OMe	98-100	+1.37	85	Phe:Thr
Fmoc-Phe-Thr-OH	158-160	-8.47	83	1.00:0.84
4. Fmoc-Asn*-Phe-OBZl	152-154	-3.72	81	Asp:Phe
Fmoc-Asn-Phe-OH	169-171	-2.64	90	1.03:1.00
5. Fmoc-Val*-Ile-OMe	162-164	-6.75	87	Val:Ile
Fmoc-Val-Ile-OH	156-158	-1.39	84	1.00:0.99
6. Fmoc-Ala*-Gly-OBzl	164-166	-9.45	83	Ala:Gly
Fmoc-Ala-Gly-OH	159-161	-18.34	91	1.00:0.98
7. Fmoc-Lys(Boc)-Thr-OMe	124-126	+0.12	89	Lys:Thr
Fmoc-Lys(Boc)-Thr-OH	122-124	+1.59	84	1.00:0.82
8. Boc-Met-Leu-*Phe-OMe	131-132	-31.40	89	Met:Leu:Phe
Boc-Met-Leu-Phe-OH	112-114	-28.50	80	0.97:1.00:1.00
9. Z-Met-Asp(OMe)-*Phe-OMe	150-152	-28.17	86	Met:Asp:Phe
Z-Met-Asp(OMe)-Phe-OH	169-171	-25.43	90	0.98:1.02:1.00

The * means in which the peptide bond was formed.
a. Optical rotation was measureds in methanol c=2.

shows the time course of the alcalase catalyzed hydrolysis of Fmoc-peptide esters. The initial rates of saponification were determined from time dependent plots of the increasing concentration of Fmoc-Ala-Val-OH or the decreasing concentration of Fmoc-Ala-Val-OMe. In a representive preparative scale reaction, alcalase (3 mL, 7.5 AU), Fmoc-Ala-Val-OMe (2.21 g, 5.0 mmol), phosphate buffer (10 mL, pH 8.2, 0.5M) and t-butanol (100 mL) were stirred at 35°C until all the Fmoc-Ala-Val-

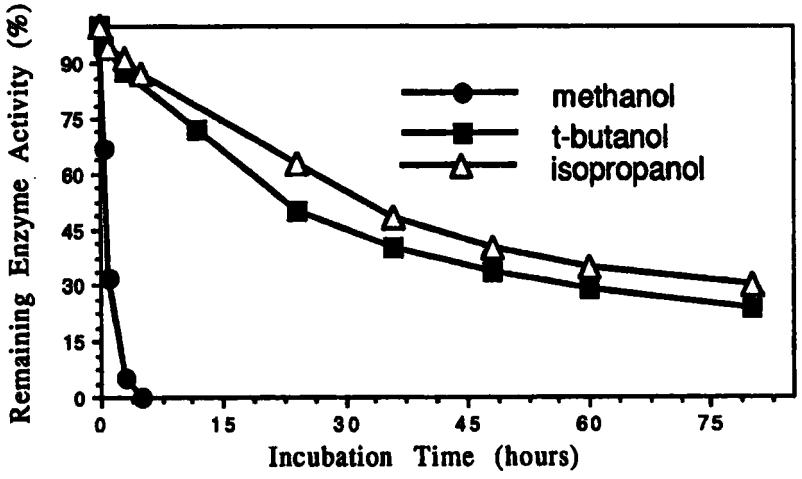


Figure 1. Stability of alcalase in various alcohols at 35oC

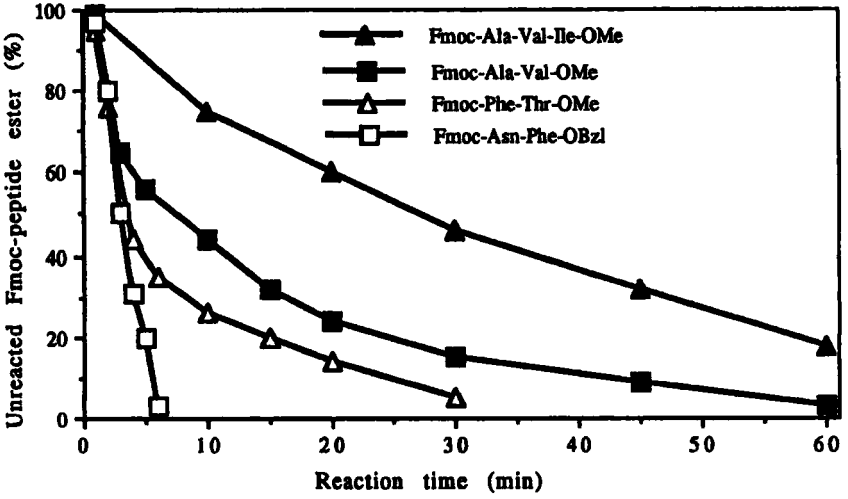


Figure 2. Time course of alcalase catalysed hydrolysis of Fmoc-peptide ester.

OMe had been consumed (hplc, about 50 min). The product was isolated by simple extraction with ethyl acetate and recrystallized with ethyl acetate/hexane (3:1) to give pure Fmoc-Ala-Val-OH (3.38 g, 80% yield). In a similar manner, all the synthesized Fmoc-peptide esters were hydrolyzed. The results are summarized in table 1.

Enzymatic hydrolysis of these peptides is probably the most simple method to prepare Fmoc-peptide fragments. The procedure illustrated here describes the practical application of alcalase in Fmoc-peptide fragment synthesis using a new approach. As shown in figure 2, no significant peptide bond hydrolysis was observed in the reactions. The alcalase, which has diastereo-selectivity at it's P_2-site, has been studied[7]. It can prevent the racemic by-product, which occurs during chemical method peptide bond formation, from hydrolyzing. This also makes the product more easily separated from the by-product. The procedure described here is not limited in saponification of Fmoc-peptide esters; peptide esters, with other commonly used protecting groups such as Cbz-, and Boc-, can be reacted and isolated in high yield. Chymotrypsin was used in hydrolyzing the α-carboxyl esters, but the rates were low[8]. Alkaline protease Bacillus subtilisin DY[9] and Thermitase[10] have been reported with esterase activity but may not suitable under these reaction conditions. In summary, this new enzymatic process has several advantages: 1) The high turnover rate and low cost of the enzyme make enzyme immobilization unnecessary. 2) The enzyme is stable at high concentrations of organic solvent, thus allowing operation at a high concentration of substrate. 3) The reaction is highly selective in ester hydrolysis[7], and the peptide bonds remain

intact. 4) Hydrolysis proceeds under mild conditions, so there is no danger of side reaction during saponication, and the product isolation is simple.

Experimental.

Alcalase was purchased from NOVO industrial as a brown liquid with a specific activity of 2.5 $AU.mL^{-1}$. It was used without further purification. The amino acids were purchased from Sigma, USA. Dicyclohexylcarbodiimide and hydroxybenzotriazole were purchased from E. Merck, Germany. The substrates were prepared by the established method and purified to meet homogeneous on tlc and hplc. The amino acid methyl esters were prepared in methanol with 2-equivalents of thionyl chloride. Optical rotation was measured on a Universal Polarimeter (Schmidt & Haensch, Germany). The hplc system consisted of two Waters model 6000 pumps, a Waters Model 450 uv detector, and an M-660 solvent programmer. A Suntex P.C. 303 Auto-pH Controller was used (Suntex Instruments Co., Taiwan). Tlc was performed on silica gel G pre-coated plates (E. Merck. Germany). Methanol, ethyl acetate, methylene chloride, acetonitrile, dioxane, ether, and acetone (hplc grade and reagent grade) were obtained from a local supplier, ALPS Chem. Co. (Taiwan).

Synthesis of Fmoc-peptide esters (Fmoc-Ala-Val-OMe as example.)

Dicyclohexylcarbodiimide (2.06g, 10 mmol) was added to a solution of Fmoc-Ala-OH (3.11g, 10 mmol) and HoBt (1.80g, 12 mmol) in ethyl acetate (30 mL) and DMF (5 mL). The mixture was stirred for 5-6 h at room temperature and filtered to remove the dicyclohexylurea. A solution of Val-OMe.HCl (1.92 g. 12 mmol) in dichloromethane (20 mL) and diisopropylethylamine (2.04 mL, 12 mmol) was added to the filtrate and reacted was continued for 3-4 h. The resulting mixture was diluted with ethyl acetate (150 mL), and this mixture was washed with 0.1 M HCl (4x20 mL), water (30 mL), 5% sodium bicarbonate (4x20 mL), water (4x 20mL) and then dried over anhydrous sodium sulfate. Evaporating organic solvent gave the crude product, which was recrystallized from ethyl acetate/n-hexane (1:3), yield 3.89 g (88%) mp: 157-159°C, $[\alpha]_D$:-8.91 (c 2.0 MeOH).

Saponification of Fmoc-peptide esters.(Fmoc-Ala-Val-OMe as example.)

Alcalase (4 mL, 10 AU) was added to a solution of Fmoc-peptide ester (4.42g, 10.mmol) dissolved in a mixture of t-butanol (200 mL) and phsophate buffer (20 mL, 0.5M, pH 8.2) at 35°C. The solution was stirred until the substrate completely disappeared (about 30 min, monitored by hplc). The reaction mixture was diluted with ethyl acetate (500 mL) and washed with 0.1 N HCl (6x30 mL), and water (3x 50 mL). The resulting solution was evaporated and ether was added to give Fmoc-Ala-Val-OH (3.68 g, 86% yield), mp: 184-186°C, $[\alpha]_D$: -1.98 (c 2, DMF).

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