Monatshefte für Chemie 123, 1015–1022 (1992)

Monatshefte für Chemie
Chemical Monthly

Model Studies on Protease-Catalysed Peptide Synthesis Using 9-Fluorenylmethoxycarbonyl Protected Amino Acid Derivatives

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Summary. *Fmoc*-Phe-O*Me*, *Fmoc*-Ala-O*Me* and *Fmoc*-Gly-OH were coupled with H-Leu-NH_2 under catalytic action of chymotrypsin, papain and thermolysin, respectively. The influence of different reaction media and several reaction parameters, such as reactants and enzyme concentrations as well as reaction time, on the peptide bond formation was investigated.

Keywords. Enzymatic peptide synthesis; *Fmoc*-protected amino acid derivatives; Chymotrypsin; Papain; Thermolysin.

Modelluntersuchungen zur Protease-katalysierten Peptidsynthese unter Verwendung 9-Fluorenylmethoxycarbonyl-geschützter Aminosäurederivate

Zusammenfassung. *Fmoc*-Phe-O*Me*, *Fmoc*-Ala-O*Me* und *Fmoc*-Gly-OH wurden unter katalytischer Wirkung von Chymotrypsin, Papain bzw. Thermolysin mit H-Leu-NH_2 gekuppelt und der Einfluß verschiedener Reaktionsmedien und -parameter, wie Reaktanden- und Enzymkonzentration sowie Reaktionszeit, auf die Peptidbindungsbildung untersucht.

Abbreviations: IUPAC-IUB rules for peptides are followed, see (1984) Eur. J. Biochem. **138**: 9. All amino acids except of Gly are of *L*-configuration. *DMF* = N,N-dimethylformamide, *DTE* = dithioerythritol, *EDTA* = ethylenediaminetetraacetic acid, disodium salt, *N/C* = ratio nucleophile component/carboxyl component, -O*Me* = methyl ester, *TLC* = thin layer chromatography.

Introduction

Whereas in solid phase peptide synthesis the 9-fluorenylmethoxycarbonyl-(*Fmoc*)-group is well established, only a few reports on the use of N^{α}-*Fmoc*-protected amino acid or peptide derivatives in enzyme-catalysed reactions can be found [1–4]. Although the markedly hydrophobic character of this group may involve some solubility problems of the protected compounds in mainly aqueous reaction systems, there is experimental evidence that homogeneous media are not a *conditio sine qua non* for good synthesis yields [5].

Two inherent advantages of the base labile *Fmoc* group might also be of interest when special target peptides are to be synthesized by a combination of chemical and enzymatic steps: (i) its usefulness in orthogonal combination with acid labile side chain blocking groups, and (ii) after deprotection, a peptide with an unprotonated amino group results in contrast with the amine salt formed in acidolysis.

In the following we want to report on protease-catalysed model reactions using *Fmoc*-Phe-O*Me*, *Fmoc*-Ala-O*Me* and *Fmoc*-Gly-OH as substrates for chymotrypsin, papain and thermolysin, respectively, and H-Leu-NH_2 as nucleophile. In order to obtain reasonable peptide yields, different reaction media, ranging from homogeneous aqueous-organic systems to suspensions in hydrophobic organic solvents, have been tried.

Results and Discussion

According to the substrate specificity of chymotrypsin we studied the coupling of *Fmoc*-Phe-O*Me* with H-Leu-NH_2 to *Fmoc*-Phe-Leu-NH_2 (**1**). For reactions in ho-

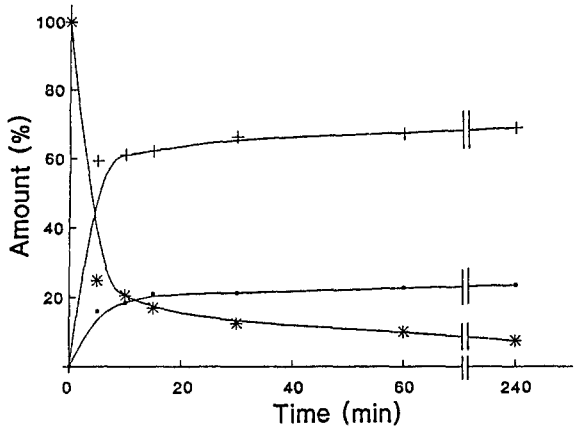

Fig. 1. Time course of the chymotrypsin-catalysed reaction of *Fmoc*-Phe-O*Me* (*) with H-Leu-NH_2 giving **1** (+) and *Fmoc*-Phe-OH (■). Reaction conditions: 0.2 *M* carbonate buffer (*pH* 9.2)/40% (*v*/*v*) *DMF*; 5 m*M* *Fmoc*-Phe-O*Me*; 100 m*M* H-Leu-NH_2; 50 μ*M* chymotrypsin

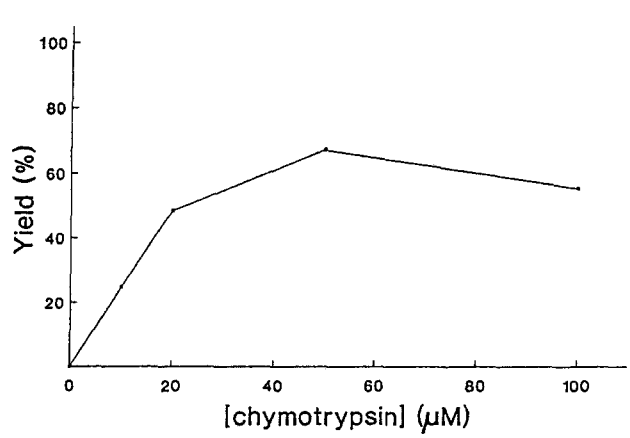

Fig. 2. Yield of **1** after 1 h reaction time in dependence on various chymotrypsin concentrations. For reaction conditions see legend to Fig. 1

mogeneous phase *DMF* proved to be the best cosolvent in addition to 0.2 *M* carbonate buffer (*pH* 9.2). As a compromise between reasonable chymotrypsin activity and sufficient ester concentration 40% (*v/v*) *DMF* were used giving 5 m*M* substrate solutions. Figure 1 shows the time course of the chymotrypsin-mediated formation of **1**. A twentyfold nucleophile concentration allowed to obtain **1** in a yield of about 70% after 30 minutes.

However, from an economical point of view it is highly desirable to reduce the excess of nucleophile and the amount of protease. Therefore we studied the formation of the dipeptide derivative **1** in dependence on chymotrypsin (Fig. 2) and nucleophile concentration (Fig. 3). As it can be seen from Figure 2, the peptide yield increases with the amount of catalyst. In order to obtain an at least 50% yield of **1**, a substrate/enzyme ratio of 250 : 1 was necessary. Figure 3 demonstrates that an increase of the peptide yield can also be achieved by augmenting the nucleophile content, which in any case has to be in considerable excess to the carboxyl component.

The application of aqueous-organic biphasic reaction media with ethyl acetate as organic phase in ratios of $\alpha = V_{org}/V_{aq} = 1$ and 50, respectively, did not prove to be more advantageous (Tab. 1). Presumably, the concentration of the very hydrophobic *Fmoc*-Phe-O*Me* in the buffer phase is too low for an effective coupling. A somewhat better result was obtained in buffer saturated ethyl acetate, although in

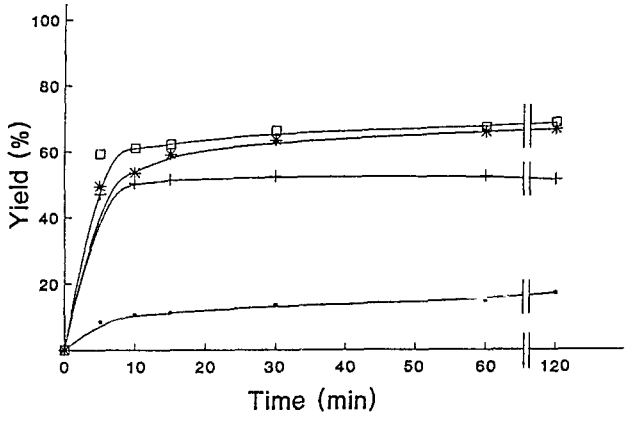

Fig. 3. Synthesis of **1** at various nucleophile concentrations. [Leu-NH_2] = 25 (■), 50 (+), 75 (*), 100 (□) m*M*. For other reaction conditions see legend to Fig. 1

Table 1. Chymotrypsin-catalysed synthesis of **1** in 0.2 *M* carbonate buffer (*pH* 9.2)/ethyl acetate systems ($\alpha = V_{org}/V_{aq}$)[a]

Solvent system	Total volume (ml)	Chymotrypsin (mg)	Yield of **1** (%)
$\alpha = 1$	1.00	5	26
$\alpha = 50$	1.02	0.2	34
Buffer saturated ethyl acetate	1.00	10	51

[a] Reactants concentration 10 m*M* *Fmoc*-Phe-O*Me*; 200 m*M* H-Leu-NH_2; time 24 h

this case the higher protease content should be considered. Substituting chloroform as organic solvent under the same reaction conditions was not advantageous.

In recent studies we were able to show that enzymic synthesis of peptide bonds can proceed effectively even when most of the reactants are undissolved [5–7]. Because of the rather restricted solubility of the *Fmoc* derivative, a reaction medium, in which the reactants are suspended, deserved our attention. Figure 4 shows the yields of **1** in hexane in dependence on reaction time at two different chymotrypsin concentrations, the only water source being the hydration water of sodium carbonate. At a *N*/*C*-ratio of only 4:1, but using a relatively large amount of chymotrypsin, nearly 90% of **1** could be produced. It is not advisable to use more soda (e.g. 0.5 mmol, as we experienced in a further experiment), because the releasing water tends to stick the suspended material to the wall of the reaction tube, preventing effective mixing.

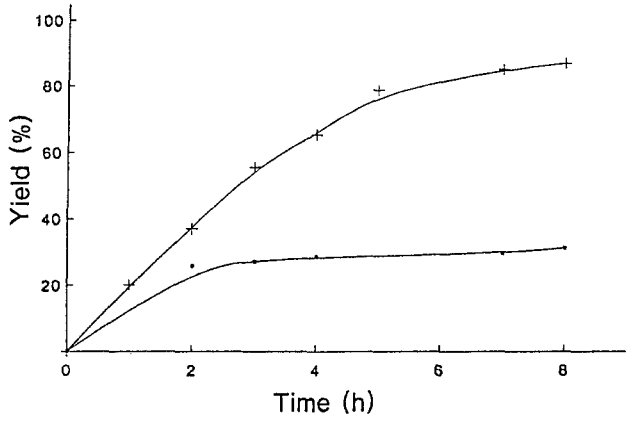

Fig. 4. Yield dependence of **1** on reaction time in a hexane/$Na_2CO_3 \cdot 10\,H_2O$ medium at two different chymotrypsin concentrations. Reaction conditions: 2 ml hexane; 0.1 mmol *Fmoc*-Phe-O*Me*; 0.4 mmol H-Leu-NH_2; 0.1 mmol $Na_2CO_3 \cdot 10\,H_2O$; [chymotrypsin] = 10 (■) and 20 (+) mg

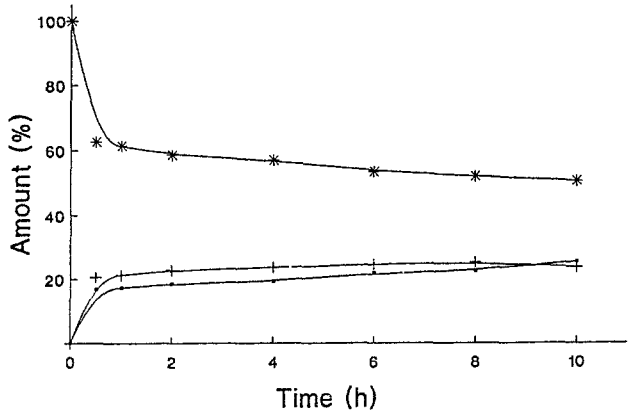

Fig. 5. Time course of the papain-catalysed reaction of *Fmoc*-Ala-O*Me* (*) with H-Leu-NH_2 giving **2** (+) and *Fmoc*-Ala-OH (■). Reaction conditions: 0.2 *M* Mc Ilvaine buffer (*pH* 7.8, containing 5 m*M* *DTE* and 1 m*M* *EDTA*)/40% (*v*/*v*) *DMF*; 5 m*M* *Fmoc*-Ala-O*Me*; 200 m*M* H-Leu-NH_2; 1 m*M* papain

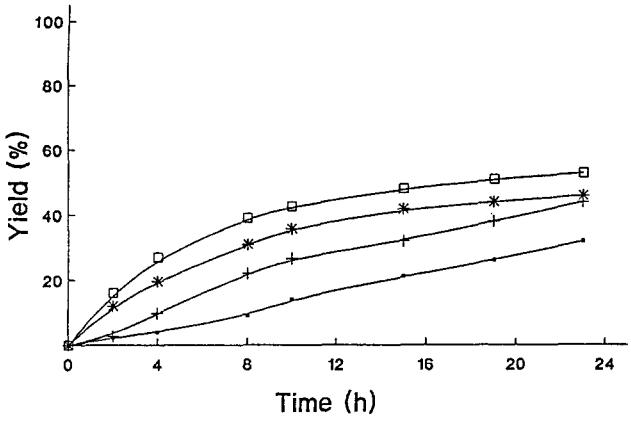

Fig. 6. Time course of the thermolysin-catalysed synthesis of **3** from *Fmoc*-Gly-OH and H-Leu-NH_2 at different enzyme concentrations. Reaction conditions: 0.2 *M* Tris-maleate buffer (*pH* 7.0)/40% (*v/v*) *DMF*; 10 m*M* *Fmoc*-Gly-OH; 200 m*M* H-Leu-NH_2; [thermolysin] = 0.1 (■), 0.25 (+), 0.5 (*), 1.0 (□) m*M*; temperature 37°C

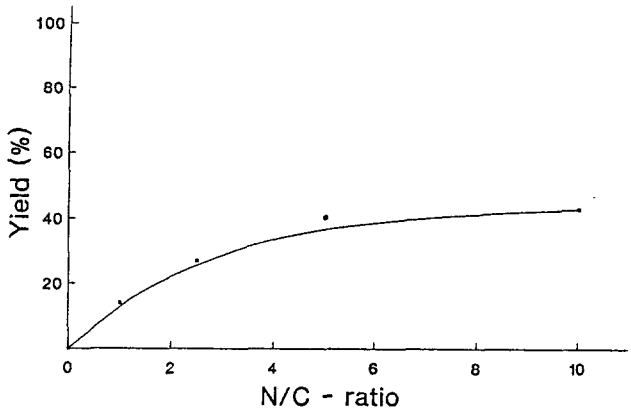

Fig. 7. Yield of **3** in dependence on the *N/C*-ratio. 10 m*M* *Fmoc*-Gly-OH; 10 − 100 m*M* H-Leu-NH_2; 1 m*M* thermolysin; for other reaction conditions see legend to Fig. 6

Table 2. Thermolysin-catalysed synthesis of **3** in 0.2 *M* Tris-maleate buffer (*pH* 7)/ethyl acetate or chloroform[a]

Solvent system	Total volume (ml)	Thermolysin (mg)	Yield of **3** (%) Organic solvent	
			Ethyl acetate	Chloroform
$\alpha = 1$	1.00	2.5	23	18
$\alpha = 50$	1.02	0.1	98	92
Buffer saturated organic solvent	1.00	5	98	92

[a] Reactants concentration 10 m*M* *Fmoc*-Gly-OH; 200 m*M* H-Leu-NH_2; temperature 37°C; time 24 h

For the coupling of *Fmoc*-Ala-O*Me* and H-Leu-NH_2 to *Fmoc*-Ala-Leu-NH_2 (**2**) the cysteine protease papain was chosen as biocatalyst. As a homogeneous aqueous-organic reaction medium we applied 0.2 *M* Mc Ilvaine buffer (*pH* 7.8) and 40% *DMF* as cosolvent. The concentrations of the ester and the amino component were 5 and 200 m*M*, respectively. Despite of this large *N*/*C*-ratio, **2** was only synthesized at papain concentrations > 0.2 m*M*, the maximum yield obtained with 1 m*M* papain being about 20% after 2 h (Fig. 5).

Under two-phase conditions using Mc Ilvaine buffer and ethyl acetate or chloroform no acceptable amounts of **2** resulted ($\leqslant 5\%$). The hydrolysis product *Fmoc*-Ala-OH was formed with 40 or 17% yield, respectively. We did not observe any influence of α on the synthesis of **2**, neither was the use of buffer saturated ethyl acetate successful.

Working with a suspension of reactants, papain and soda in hexane gave similar low yields up to 10% after 24 h. No better results were obtained varying concentrations of enzyme, reactants and the amount of added salt hydrate.

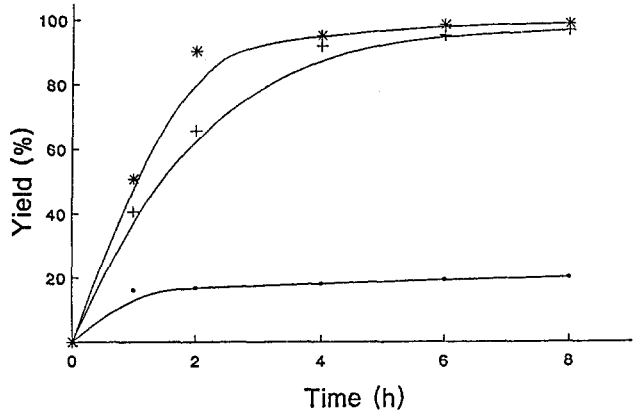

Fig. 8. Time course of the thermolysin-catalysed formation of **3** in different chloroform containing solvent systems. $\alpha = 1$ (■), $\alpha = 50$ (+), buffer saturated chloroform (*); for other reaction conditions see Table 2

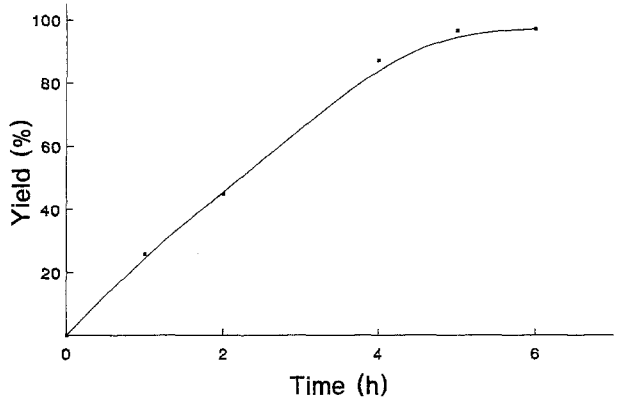

Fig. 9. Yield dependence of **3** on reaction time in a hexane/$Na_2SO_4 \cdot 10\,H_2O$ medium. Reaction conditions: 2 ml hexane; 0.1 mmol *Fmoc*-Gly-OH; 0.4 mmol H-Leu-NH_2; 0.1 mmol $Na_2SO_4 \cdot 10\,H_2O$; 10 mg thermolysin

As a thermolysin-catalysed model reaction we investigated the thermodynamically controlled synthesis of *Fmoc*-Gly-Leu-NH_2 (**3**) from *Fmoc*-Gly-OH and H-Leu-NH_2. In a homogeneous aqueous-organic reaction medium with 40% *DMF*, **3** was obtained after 23 h in a yield of 53% using the N-component in a twentyfold excess. Figure 6 shows the reaction progress at different thermolysin concentrations, and in Fig. 7 the dependence of the peptide yield on the *N*/*C*-ratio is given.

It is well documented in literature that thermolysin-catalysed reactions can proceed effectively in buffer saturated ethyl acetate or biphasic systems containing this organic solvent (see review [8]). When we applied these conditions to the synthesis of **3**, we got very good results in reaction media with high portions of ethyl acetate or chloroform (Tab. 2). In solvent mixtures with ethyl acetate at $\alpha > 50$ similar good yields were obtained even within a reaction time of 3 hours. The time course of the synthesis of **3** in chloroform containing media is represented in Fig. 8.

With regard to a more economic *N*/*C*-ratio the use of a hexane/ $Na_2SO_4 \cdot 10\,H_2O$ medium proved to be the best choice enabling coupling yields of more than 90%. The reaction progress under these conditions is plotted in Fig. 9.

Summing up, we were able to demonstrate for an example of model reactions under different media conditions that *Fmoc* protected amino acids or their derivatives can be coupled enzymatically with H-Leu-NH_2. Whereas we obtained good results in the chymotrypsin-catalysed synthesis of **1** and the thermolysin-catalysed synthesis of **3**, the reaction of *Fmoc*-Ala-O*Me* with H-Leu-NH_2 in the presence of papain gave only poor yields up to 25%. In order to obtain satisfying yields, relatively high *N*/*C*-ratios and enzyme concentrations had to be used in most cases.

Experimental Part

Bovine α-chymotrypsin (EC 3.4.21.1; 3× recrystallized and lyophilized, 44 U/mg) from Serva (Heidelberg), papain (EC 3.4.22.2; 3 000 USP-U/mg) obtained from Merck (Darmstadt) and thermolysin (EC 3.4.24.4; from Bacillus thermoproteolyticus, lyophilized) supplied by Boehringer (Mannheim) were used without further purification. H-Leu-NH_2 was purchased from Bachem (Bubendorf, Switzerland) and *Fmoc*-Gly-OH from Novabiochem (Läufelfingen, Switzerland). *Fmoc*-Phe-O*Me* (yield 80%, m.p. 119 – 123°C, $[\alpha]_D^{25} = -18.7$, $c = 1$ in *DMF*) and *Fmoc*-Ala-O*Me* (yield 61%, m.p. 110 – 113°C, $[\alpha]_D^{25} = -16.5$, $c = 1$ in *DMF*) were synthesized from the corresponding amino acid methyl ester according to a known procedure [9] and checked by *TLC* in $CHCl_3$/*Me*OH (9 : 1) and hexane/*Et*OH (1 : 3 and 3 : 1).

Enzymatic synthesis reactions were carried out in small sample tubes held on a reciprocal shaker at room temperature. The thermolysin-catalysed reactions proceeded in a thermostat at 37°C. For reactions with *DMF* as cosolvent the total volume was 0.4 ml. In biphasic systems the organic solvent had been saturated in advance with the corresponding buffer. Its volume was 0.5 ml for $\alpha = 1$ and 1 ml both for $\alpha = 50$ and reactions in buffer saturated solvent. All results were obtained in duplicate experiments. Substrates and products were analysed by HPLC using a LC-6A pump (Shimadzu), a 4 × 250 mm column packed with LiChrosorb RP 18 (7 µ), a UV detector LCD 2040 (Laboratorni Pristoje, ČSFR) at 280 nm and an integrator C-R6A (Shimadzu). As eluant a mixture of 10 m*M* ammonium acetate solution/acetonitril (35 : 65, *v*/*v*) was used. The peptide products were identified by comparison with authentic samples.

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Received November 28, 1991. Accepted February 3, 1992