Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 5, No. 14, pp. 1475-1478, 1995

0960-894X/95 $9.50+0.00

0960-894X(95)00240-5

Immobilized Ficin Catalyzed Synthesis of Peptides in Organic Solvent

Dar-Fu Tai*, Hui-Yao Huang and Ching-Chong Huang

Biochemical Engineering Center, Da-Yeh Institute of Technology, Chang Hwa, Taiwan, ROC.

Abstract: Immobilized ficin catalyzed the synthesis of peptides between *N*-protected amino acids and amino acid esters in ethyl acetate. Under similar conditions *N,N'*-diBoc-L-cystine was furnished the monopeptidation by controlling the duration of the reaction. The influence of pH, reaction time and amount of triethylamine on mono-peptidation was also studied.

In recent years enzymatic methodology has been widely used for the synthesis of peptide bonds.[1] Enzymatic formation of peptide bonds offers an attractive alternate method to their chemical synthesis. Ficin a sulfhydryl protease has previously been shown to possess the ability for the hydrolysis of esters[2,3] and the formation of peptides.[4] Recently, we have reported the papain catalyzed synthesis of unsymmetric cystine peptides.[5] However, the ficin catalyzed synthesis of peptides in organic solvents has not been reported. In this paper we disclose the results of our investigations on the immobilized ficin catalyzed synthesis of peptides in organic solvents. A variety of *N*-protected amino acids were incubated with L-tyrosine methyl ester in the presence of ficin immobilized on starch[6] and obtained the corresponding dipeptides in reasonable yields (Eq. 1 and Table 1).

$$\begin{matrix} \text{P-NH-CHR-CO}_2\text{H} + \text{Et}_3\text{N} \\ + \text{HCl·NH}_2\text{CHR'COOR"} \end{matrix} \xrightarrow[\text{EtOAc}]{\text{Ficin}} \begin{matrix} \text{P-NH-CHR-CONHCHR'COOR"} \\ + \text{Et}_3\text{NHCl} + \text{H}_2\text{O} \end{matrix} \qquad \text{(Eq. 1)}$$

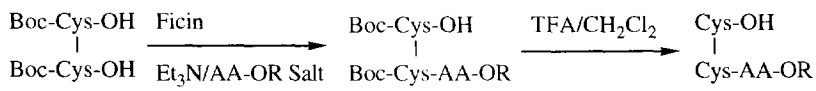
In a typical experiment, ficin (1 g) was immobilized by mixing with starch (2 g) and a solution of 3M, pH 5.5, citrate-potassium buffer (1 mL) in a vial and shaking in a incubator at 30°C for 30 min. The salt of amino acid ester (2 mmole), triethylamine (0.6 mL), ethyl acetate (20 mL) and *N*-protected amino acid (2 mmole) were added to a reaction vessel, followed by the above immobilized ficin. The reaction mixture was incubated at 37°C for 24 hours. The residues were filtered and the filtrates were concentrated and purified by column chromatography (silica gel, *n*-hexane:ethyl acetate = 1:1 to ethyl acetate). All new compounds were characterized by IR, polarimeter and ^{1}H NMR (200 MHz). The hydrolysis of the newly formed peptide bonds was not observed under these reaction conditions. These results led us to successfully couple Cbz-L-Asp with L-Phe-OMe to form the dipeptide, the precursor of aspartame (Table 1, entry 13). The rates of formation of tripeptide or tetrapeptide were slower (Table 1, entries 15 to 17). Interestingly, transacylation was occurred when Cbz-Gly-Gly was used as a substrate. The yields of Cbz-Gly-L-Phe-OMe and Cbz-Gly-Gly-L-Phe-OMe amounted to 47 and 4%, respectively.

Table 1: Immobilized ficin catalyzed formation of dipeptides.

No.	Substrate	Nucleophile	Time	Yield(%)	Product
1	Cbz-L-Ala	L-Tyr-OMe	24hr	45.3	Cbz-L-Ala-L-TyrOMe
2	Cbz-L-Asn		24hr	46.5	Cbz-L-Asn-L-TyrOMe
3	Cbz-L-Asp		24hr	82.2	Cbz-L-Asp-L-TyrOMe
4	Cbz-L-Glu		6hr	15	Cbz-L-Glu-L-TyrOMe
5	Cbz-Gly		24hr	61.4	Cbz-Gly-L-TyrOMe
6	Cbz-L-Pro		24hr	0	
7	Cbz-L-Met		24hr	40	Cbz-L-Met-L-TyrOMe
8	Cbz-L-Ser		24hr	82.9	Cbz-L-Ser-L-TyrOMe
9	Cbz-L-Thr		24hr	20	Cbz-L-Thr-L-TyrOMe
10	Cbz-L-Val		24hr	3	Cbz-L-Val-L-TyrOMe
11	Cbz-L-Leu		24hr	30	Cbz-L-Leu-L-TyrOMe
12	Cbz-L-Phe		24hr	97.9	Cbz-L-Phe-L-TyrOMe
13	Cbz-L-Asp	L-Phe-OMe	24hr	84.8	Cbz-L-Asp-L-PheOMe
14	Cbz-L-Glu	L-Asn-O^{t}Bu	24hr	26.7	Cbz-L-Glu-L-AsnO^{t}Bu
15	Cbz-L-Glu	L-Phe-L-LeuNH2	48hr	10.2	Cbz-L-Glu-L-Phe-L-LeuNH2
16	Cbz-Gly-Gly	L-Phe-OMe	48hr	46.9	Cbz-Gly-L-PheOMe +
				3.9	Cbz-Gly-Gly-L-PheOMe
17	Cbz-Gly-Gly	L-Phe-L-LeuNH2	48hr	55.6	Cbz-Gly-L-Phe-L-LeuNH2 +
				21.6	Cbz-Gly-Gly-L-Phe-L-LeuNH2

The selective monomodification of a multifunctional molecule is a challenging task especially for a symmetric one. Cystine-containing peptides are critical for controlling the structure and biological activity of protein.[7] In this respect, the synthesis of glutathione analogous[8] and cystine containing cyclic peptides[9] or proteins[10] were promoted. However, chemical synthesis of the disulfide bond requires a special environment and it is extremely difficult to join several disulfide bonds properly.[11,12] The yields are usually low due to their random choice of partners[13,14] as well as the protection, deprotection and oxidation of the thiol groups. Separation and purification are often required.

Scheme I



To overcome these problems, preconnected disulfide bond obviously is a better approach. The disulfide bond can be easily destroyed or oxidized during subsequent peptide coupling and deprotecting steps. Therefore, the two cysteines need to be differentiated. Chemical synthesis by DCC method[15] generally gives lower monoamide/diamide ratios than the enzymatic method. Upon coupling an equal molar of *N,N'*-diBoc-L-cystine and L-tyrosine methyl ester with DCC for 2 hours resulted in a monoamide/diamide ratio of 5 to 3. Enzymatic coupling of peptide bonds usually occurs in very mild conditions, without racemization and with high selectivity. For the differentiation of two cysteines, enzymatic monopeptidation is a promising alternative. Accordingly, we have studied the monopeptidation of *N,N'*-diBoc-L-cystine with L-tyrosine methyl ester by using immobilized ficin as biocatalyst (Scheme I) and obtained the unsymmetric cystine monopeptide and dipeptide in the ratio of 11:1 (Table 2, entry 1).

Previously, a lower ratio of monoamide/diamide (3.5:1) was obtained by using papain as the catalyst.[5] As shown in Table 2, unsymmetric cystine peptides were obtained by monocoupling of diBoc-L-cystine with different nucleophiles. Most of the non-tyrosine nucleophiles can undergo only mono coupling, the diamides were not detected. The only exception was tyrosine derivatives. Unlike other amino acid esters, L-tyrosine esters have a lower monoamide/diamide ratio.

Table 2: Monopeptidation of diBoc-L-cystine with various nucleophiles.

No	Substrate	Nucleophile	Time	Yield(%)	Products
1	(Boc-L-Cys)2	L-Tyr-OMe	24hr	93.4	(Boc-L-Cys)2-L-TyrOMe + (Boc-L-Cys-L-TyrOMe)2
2		L-Tyr-OBn	24hr	94.2	(Boc-L-Cys)2-L-TyrOBn + (Boc-L-Cys-L-TyrOBn)2
3		L-Phe-OBn	24hr	59.8	(Boc-L-Cys)2-L-Phe-OBn
4		L-Pro-OBn	24hr	0	
5		L-Phe-OMe	24hr	57.8	(Boc-L-Cys)2-L-Phe-OMe
6		Gly-OMe	24hr	10.5	(Boc-L-Cys)2-Gly-OMe
7		$Gly\text{-}NH_2$	24hr	0	
8		$L\text{-}Leu\text{-}NH_2$	24hr	56.3	$(Boc\text{-}L\text{-}Cys)_2\text{-}L\text{-}Leu\text{-}NH_2$

$(L\text{-Cysteine})_2$-L-tyrosine is an important part of the oxytocin-an oxytocic.[16] Therefore, the enzymatic coupling of tyrosine methyl ester with diBoc-L-cystine was studied. We checked the influences of pH, reaction time and the amount of triethylamine on the yield of the coupling. Best results were obtained around pH 5.0 to 6.5 and the yields were decreased with the increase in pH to 8.5 (Table 3).

Table 3: The effect of pH and reaction time on the yield and ratio of monoamide to diamide.

Reaction Time \ pH		4.5	5.0	5.5	6.0	6.5	7.5	8.5
2hr	Monoamide / Diamide	11.3	9.9	14.2	12.2	15.3	12.5	11.5
	Yield	53.2	69.4	67.9	61.0	69.7	44.5	62.9
4hr	Monoamide / Diamide	10.9	8.7	11.5	11.2	13.0	10.2	10.3
	Yield	79.7	84.9	86.8	69.6	84.6	53.4	64.5
6hr	Monoamide / Diamide	10.5	8.5	10.4	10.2	12.2	9.6	10.5
	Yield	80.8	87.4	88.3	76.0	87.6	56.7	63.3
24hr	Monoamide / Diamide	10.2	6.5	8.8	9.7	10.1	8.4	9.4
	Yield	87.3	92.5	93.4	89.9	89.2	84.4	76.3

As the duration of the reaction increased, the monopeptidation also increased. Cystine derivatives slowly decomposed on longer incubations at 37°C. As shown in Table 3, a decent yield was obtained when the reaction had been operated for 6 hours. The ratio of monoamide to diamide was decreased, when the yield of the peptide was increased. The monoamidation can be controlled kinetically by using higher stoichiometric ratio of *N,N'*-diBoc-L-cystine. At the stoichiometric ratio of 1:1 (*N,N'*-diBoc-L-cystine to L-tyrosine methyl ester), the best time to stop the reaction is 24 hours.

We have also observed that the amount of triethylamine has a propound influence on the yield and the ratio of monoamide to diamide (Table 4). These results show that mono coupling can be achieved by controlling the amount of triethylamine. At the stoichiometric ratio of 1:2 (L-tyrosine methyl ester to triethylamine), the yield is significantly better. As the yield of the peptide was increased, the ratio of monoamide to diamide decreased. By using this enzymatic method, we have differentiated the two carboxylic groups in the cystine amino acid. Free cystine peptides[17] were obtained after removing the *t*-butoxyl group by trifluoroacetic acid (Scheme I). Hydrolysis of benzyl ester or methyl ester by lipase or esterase can generate free acid. Esterification or peptidation of the free carboxylic group in the cystine monopeptide by enzymatic or chemical methods will produce a number of unsymmetric cystine derivatives for the synthesis of cystine-containing peptides.

Table 4: The influence of triethylamine on the yield and ratio of monoamide to diamide.

Tyr-OMe·HCl (mmole)	$(Et)_3N$ (mmole)	Yield (%)	Monoamide/Diamide
2	2	44.4	24.3
2	4	88.3	10.4
2	6	38.9	15.6
2	8	21.7	31.8

In summary, we have enzymatically synthesized dipeptides and disulfide-bridged peptides by using ficin as a catalyst in organic solvent. Application of these ficin catalyzed reactions may be useful in the synthesis of a number of biologically active peptides.

References

1. Wong, C. -H.; Wang, K. -T. *Experientia* **1991**, 47, 1123-1129.
2. Kortt, A.; Hinds, J. A.; Zerner, B. *Biochemistry* **1972**, 13, 2029-2037.
3. Carotti, A.; Casini, G.; Hansch, C. *Biotech. App. Chem.* **1991**, 14. 183-191.
4. Monter, B.; Herzog, B.; Stehle, P.; Furst, P. *J. Med. Chem.* **1984**, 27. 1427-1431.
5. Tai, D. -F.; Chen, C. -E.; Huang, C. -C.; Huang, H. -Y. *Biotechnol. Lett.* **1993**, 15, 961-964.
6. Ficin and starch were purchased from sigma.
7. Richardson, J. S. *Adv. Prot. Chem.*. **1981**, 34, 167-339.
8. Adang, A. E. P.; Duindam, A. J. G.; Brussee, J.; Mulder, G. J.; Gen, A. V. D. *Biochem. J.* **1988**, 255, 715-720.
9. Milburn, P. J.; Meinwald, Y. C.; Takahashi, S.; Ooi, T.; Scheraga, H. A. *Int. J. Pept. Prot. Res.* **1988**, 31, 311-321.
10. Yajima, H.; Fujii, N.; Funakoshi, S.; Watanabe, T.; Murayama, E.; Otaka, A. *Tetrahedron* **1988**, 44, 805-819.
11. Shih, H. *J. Org. Chem.* **1993**, 58, 3003-3008.
12. Ruiz-Gayo, M.; Albericio, F.; Royo, M.; Pedroso, E.; Giralt, E. *Tetrahedron Lett.* **1988**, 29, 3845-3848.
13. Houk, J.; Whitesides, G. M. *J. Am. Chem. Soc.* **1987**, 109, 6825-6836.
14. Ponsati, B.; Ruiz-Gayo, M.; Giralt, E.; Albericio, F.; Andreu, D. *J. Am. Chem. Soc.* **1990**, 112, 5345- 5347.
15. Sheehan, J. C.; Yang, D. -D. H. *J. Am. Chem. Soc.* **1957**, 79, 1158- 1164.
16. Boissonnas, R.A.; Guttmann. St.; Jaquenoud, P.-A.; Waller, J.-P. *Helv. Chem. Acta.* **1955**, 38, 1491-1501.
17. Upson, D.A.; Hruby, V.J. *J. Org. Chem.* **1976**, 41, 1353-1358.

(*Received in Japan* 11 *March* 1995; *accepted* 29 *May* 1995)