Indian Journal of Chemistry
Vol. 46B, July 2007, pp. 1143-1147

Simple, economical and flexible apparatus for solid phase peptide synthesis

Kota Satyanarayana*, K V R C Rajesh Kumar & Ch Venkanna

Natco Research Center, Natco Pharma Ltd, B-13, Sanatnagar, Hyderabad 500 080, India

E-mail: dr_ksn@natcopharma.co.in

Received 30 October 2006; accepted (revised) 6 March 2007

Solid phase peptide synthesis basically involves repetitive deblocking and coupling reactions. In order to make these repetitive operations fast and convenient, the deblocking and coupling reactions are carried out in specially designed apparatus, which are called solid phase peptide synthesis apparatus. These apparatus are expensive, hence unaffordable for many laboratories. In addition, these apparatus lack the operational flexibility for varying reaction parameters such as reaction time, temperature, pressure, sonication, microwave irradiation, *etc*. In order to make peptide synthesis convenient and flexible with respect to reaction conditions and affordable for many laboratories, a two-vessel approach has been proposed. In this approach, coupling reaction is carried out in a round bottom flask with overhead stirrer, and the filtration (work-up) is carried out in a specially designed filtration-vessel. Using the two-vessel approach, D-Met-enkephalins (pentapeptides, Tyr-Gly-Gly-Phe-D-Met) and some of its derivatives involving lengthy reactions have been synthesized on Merrifield resin. This approach is very convenient for studying variations in reaction conditions and is good for synthesizing short peptides (<10 amino acids). By this approach 1-100 g resin can be handled in a laboratory without any additional cost. This approach can be extrapolated to solid phase syntheses as well.

Keywords: Peptide synthesis, solid phase, manual solid phase synthesis, two-vessel solid phase peptide synthesis, enkephalins

Solid phase peptide synthesis technique consists of two operations, namely coupling reaction and removal of reagents and by-products by filtration process. Since the invention of solid phase peptide synthesis technique by Merrifield in 1963, all efforts were directed towards combining these two operations in a single vessel to make repetitive processes convenient and speedy in both manual and automated solid phase peptide synthesis[1]. Doubtless, that these efforts helped in synthesizing complicated long peptide/proteins but at the expense of cost escalation. These efforts made vessels complicated, fragile, and unaffordable for many research groups. The major constraint of one-vessel approach has been the lack of operational flexibility for variability of reaction parameters such as reaction time, temperature, pressure, ultrasonication, microwave irradiation, photo-irradiation, *etc*. Another disadvantage of one-vessel approach is that it is not the way a chemist usually works, hence creates the mental blocks. In this communication, a two-vessel approach for solid phase peptide synthesis is proposed and describes the design, fabrication and working of a simple, convenient, scalable, filtration-vessel involving no additional cost. Using the two-vessel approach, the synthesis of enkephalins modified at fifth position is described. Enkephalins are pentapeptides (Tyr-Gly-Gly-Phe-Met), and are produced endogenously to relieve pain[2]. The suitability and flexibility of two-vessel approach is further demonstrated by synthesizing enkephalin derivatives involving lengthy reactions. This communication describes the complete solid phase peptide synthesis approach beginning from attachment of amino acid to Merrifield resin, synthesis of peptide and ending with cleavage of peptide and peptide derivatives.

Results and Discussion

In the conventional solid phase peptide synthesis apparatus deblocking and coupling reactions and related work-up to remove excess reagents and by-products from the solid support are carried out in one vessel. It is a glass cylinder or a round bottom flask fitted with medium porosity sintered disc at bottom. The contents of the vessel are stirred by rocking movements by a mechanical agitator, or an overhead stirrer, or a magnetic stirrer, or by nitrogen bubbling. The disadvantages of these conventional apparatus are:

(i) These apparatus are operated at RT only and do not have provision for heating, cooling and varying the reaction conditions. Hence the study of reaction parameters such as temperature, microwave irradiation, photo-irradiation, pressure, ultrasonication, time, *etc.*, which are the essential requirements in a chemical reaction, are not possible in these apparatus.

(ii) Apparatus are fragile, complicated, and unaffordable for many laboratories.

(iii) Scalability is difficult, inconvenient and not linear.

Keeping the above disadvantages in mind, the technique of carrying out solid phase peptide synthesis in two vessels was developed. In this new technique, deblocking and coupling reactions are carried out in conventional reaction vessels such as round bottom flask with overhead or magnetic stirrer. The work-up operations to remove the excess reagents and by-products from the solid support are carried out in a filtration vessel described in this paper. The present paper describes the design, fabrication and working of filtration vessel and synthesis of Met-enkephalins using this apparatus.

Boc-D-Met-OH was attached to Merrifield resin by following Gisin procedure[3]. Cesium salt of Boc-D-Met-OH and Merrifield resin in DMF were stirred at 50°C for 12 hr in the reaction-vessel and filtered in the filtration-vessel to remove excess reagents and by-products. The yield was 99.8% and D-methionine concentration was 0.99 mmole/g of resin. Boc-D-Met-OH attached to Merrifield resin was deblocked with trifluoroacetic acid and the resin was then transferred to the filtration-vessel for washings. Deblocked resin, Boc-Phe-OH, 1-hydroxybenzotriazole and DMF were added into the reaction-vessel. Dicyclohexylcarbodiimide was added to the mass and stirred for 5 hr at RT. After the completion of coupling as indicated by Kaiser test, the reagents and by-products were filtered in the filtration-vessel, washed with DMF and methanol alternately for six times and finally with methanol three times. Similarly, other amino acids in the following order Boc-Gly-OH, Boc-Gly-OH, Boc-Tyr-OH were attached. Coupling yield was 93%. Boc protected pentapeptides was deblocked by trifluoro-acetic acid to give Tyr-Gly-Gly-Phe-D-Met-Merrifield resin. This was treated with HBr-acetic acetic acid (33%) to give peptide acid in 68.6% yield with 66.0% HPLC purity. Further, Tyr-Gly-Gly-Phe-D-Met-Merrifield resin was treated with ammonia for 48 hr to yield D-Met-enkephalin amide in 58.0% yield with 38.0% HPLC purity. Similarly, other derivatives such as monomethylamine, isopropylamine, hydrazine hydrate were prepared in 57.0, 73.0, 47.7% yield with 63.9, 56.2, 66.4% HPLC purity respectively (**Table I**). The crude peptides were purified to >90%, and characterized by amino acid analysis and mass spectrometry. New compounds monomethylamine **3**, propylamine **4** and hydrazide **5** derivative were further characterized by ^{1}H NMR. All these peptides were tested for analgesic activity.

The technique of carrying out solid phase peptide synthesis by the two-vessel approach using filtration vessel described in this paper has the following advantages. The two-vessel approach is simple, flexible, convenient for modifying reaction conditions, and is similar to regular laboratory approach. The filtration vessel described in the paper can be fabricated in a glassblowing department of an institution in-house, hence is highly convenient. The present approach involves no additional cost and most suitable for preparing small peptides (≅5-10 amino acids). Since both coupling reaction and filtration are carried out under mild conditions, the possibility of resin breakage is less as compared to mechanical mixing as done in the case of one-vessel approach. 1-100 g resin can be handled easily in a laboratory by this method. However, it is difficult to prepare long peptides due to handling losses. The approach can be extrapolated to other solid phase syntheses. Thus, by using manual two-vessel approach D-met-enkephalin and its derivatives involving long reaction times were conveniently prepared by two-vessel approach.

Experimental Section

Merrifield resin was purchased from Bharavi Laboratories, Bangalore, India. Boc protected amino acids were synthesized according to reported procedures[4]. Analytical HPLC was performed on Waters Alliance 2996 instrument. Preparative chromatography was performed on AKTA purifier, GE Health Care, Biosciences machine using Simpack ODS column, Shimadzu. EI-MS was carried out on API 2000, SCIEX, Perkin-Elmer instrument. Amino acid analysis was carried by Waters Pico tag method. ^{1}H NMR spectra were recorded on Varian Innova 500 MHz instrument.

Design, Fabrication and Working of Filtration-vessel

Filtration-vessel is a glass cylinder (6.5 cm diameter × 15 cm length) fitted with fritted disc (G2 porosity) and the bottom is tapered like a Buchner

Table I — Physiochemical properties of D-Met-enkephalins synthesized by two-vessel approach Tyr-Gly-Gly-Phe-D-Met-X

Compd	X	Mol. formula[a]	Mol. wt.	MS	time (hr)	HPLC[b]	Yield (%)	^{1}H NMR (500 MHz, DMSO-d_6) δ, ppm
1	OH	$C_{27}H_{35}N_5O_7S$	573.70	574.5	4	66.0	68.6	-
2	NH_2	$C_{27}H_{36}N_6O_6S$	572.16	573.1	48	34.5	58.0	-
3	$NHCH_3$	$C_{28}H_{38}N_6O_6S$	586.19	587.2	72	63.9	57.0	6.68-7.43(9H,m, Ar-H, Phe and Tyr), 4.93 (1H, t, Phe-CH_2), 4.53 (1H, t, Met-CH), 4.09 (4H, s, Gly-CH_2), 3.90 (1H, t, Tyr-CH), 3.19-3.55 (m, 4H, CH_2, Phe and Tyr), 3.05 (3H, s, N-CH_3), .50-2.60 (2H, m, Met-S-CH_2), 2.14 (3H, s, Met-S-CH_3), 2.05-2.10 (2H, m, Met-CH_2-CH_2).
4	NHC_3H_7	$C_{30}H_{42}N_6O_6S$	614.24	615.1	48	56.1	73.0	6.65-7.40(9H,m, Ar-H, Phe and Tyr), 4.92 (1H, t, Phe-CH_2), 4.53 (1H, t, Met-CH), 4.09 (4H, s, Gly-CH_2), 3.90 (1H, t, Tyr-CH), 3.19-3.65 (m, 4H, CH_2, Phe and Tyr), 2.60 (2H, t, Met-S-CH_2), 2.14 (3H, s, Met-S-CH_3), 2.06-2.10 (2H, m, Met-CH_2-CH_2), 1.50-1.60 (2H, m, NH-CH_2-C*H*$_2$), 0.9 (3H, t, NH-CH_2-CH_2-C*H*$_3$).
5	$NHNH_2$	$C_{27}H_{37}N_7O_6S$	587.18	588.1	48	66.4	44.7	6.70-7.43(9H,m, Ar-H, Phe and Tyr), 4.93 (1H, t, Phe-CH_2), 4.53 (1H, t, Met-CH), 4.22 (NH-N*H*$_2$), 4.03 (4H, s, Gly-CH_2), 3.96 (1H, t, Tyr-CH), 3.19-3.55 (m, 4H, CH_2, Phe and Tyr), 2.50-2.72 (2H, m, Met-S-CH_2), 2.14 (3H, s, Met-S-CH_3), 2.05-2.10 (2H, m, Met-CH_2-CH_2).

[a]Amino acid analysis was within ±10% of expected value[b]. Analytical HPLC was performed on C18 column (Develosil) using a gradient of 0.1% TFA in water and 0.1% TFA in acetonitrile at a flow rate of 1 mL/min with linear gradient 0-100% in 30 min.

filtration funnel with a 24/29 ground glass cone. Just above the cone a side tube of about 2 cm length is provided for vacuum connection and the upper end of the cylinder is tapered with 34/35 sockets. This vessel is fabricated using commercially available (Borosil, India) 200 mL capacity Buchner funnel with fritted disc and vacuum cone (G2, disc diameter 6.5 cm, height 7.7 cm, cone 24/29) having side tube (design similar to Aldrich Cat. no. Z547565-1EA). To it 8 cm length glass tube of similar dimension is attached, then 34/35 socket is attached (**Figure 1**).

The reaction-vessel is a 3-necked round bottom flask, having a glass shaft with Teflon blade and overhead stirrer provided with a speed controlling regulator. Reactions such as attachment of amino acid to resin, elongation of peptide chain and deprotection are conducted in the reaction vessel.

Filtration apparatus is fitted to 250 mL capacity conical flask with 24/29 ground glass joints and having a side tube for applying vacuum as shown in **Figure 2**. After the completion of reaction, the mass is transferred into the filtration-vessel, and vacuum is applied to drain out the solvent. Then, fresh solvent is added and nitrogen gas is bubbled from the side tube of

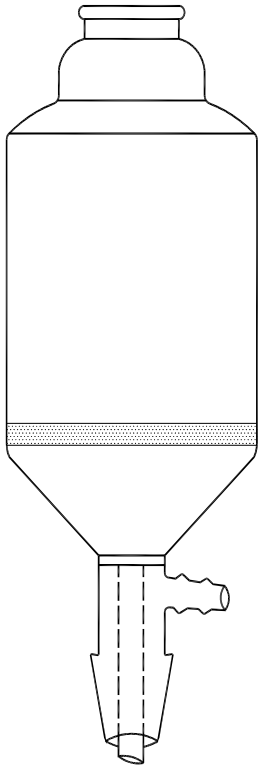

Figure 1 — Filtration apparatus

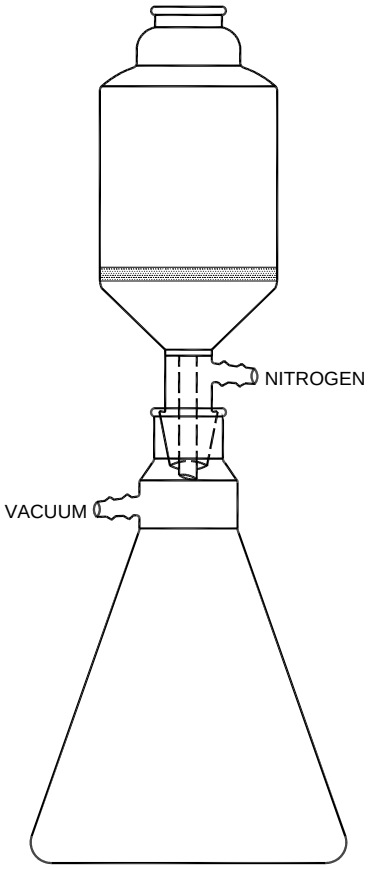

Figure 2 — Assemble of filtration apparatus during operation

the conical flask for about 3-5 min for mixing. Resin attached with peptide is again taken into the reaction-vessel and deprotection is initiated. After complete deprotection, the reaction mass is transferred to the filtration-vessel and the washing process is repeated.

Synthesis of Boc-D-Met-Merrifield resin

To a solution of Boc-D-Methionine (5 g, 20 mmole) in dry DMF (20 mL), cesium carbonate (3.25 g, 0.01 mole) in methanol was added till a clear solution was obtained. Solvent was removed and the dry powder was added into the reaction-vessel containing Merrifield resin (10 g, 0.1 mmole/g, 100-200 mesh), DMF (100 mL) and stirred at 60 rpm for 10 hr at 40-50°C. Reaction mass was transferred into the filtration-vessel and washed with DMF–methanol (20 mL each) alternatively 6 times and finally with methanol 3 times (20 mL). Yield 12.67 g (99.8%), substitution 0.99 mmole.

Synthesis of Tyr-Gly-Gly-Phe-D-Met-Merrifield resin

Boc-D-Met-Merrifield resin (12 g) and trifluoro-acetic acid were mixed and stirred in the reaction-vessel at 60 rpm for 45 min (completion of deprotection was checked by Kaiser test[5]). Reaction mass was transferred to the filtration-vessel and washings were done in the following order: acetic acid (30 mL, 3 min), DMF (20 mL, 4 min), triethylamine (5 mL in 20 mL DMF, 4 min) and finally with methanol (40 mL, 4 min). The above deblocked D-Met-Merrifield resin and Boc-L-Phe-OH (10.6 g 40 mmole), 1-hydroxy-benzotriazole (6.2 g, 40 mmole) and DMF (50 mL) were added into the reaction-vessel, cooled to 0-5°C, then dicyclohexylcarbodiimide (8 g, 40 mmole) was added and stirred for 5 hr at RT. After the completion of reaction, washings were carried out in the filtration-vessels in the following order, DMF (30 mL, 5 min) and methanol (20 mL, 3 min) alternatively for six times, and finally with methanol (20 mL, 2 min) three times. Similarly, other amino acids were attached in the following order: Boc-Gly-OH, Boc-Gly-OH, Boc-Tyr-OH. Yield 14.75 g (93%). Tyr, 1.0; Gly, 1.95; Phe, 1.0; Met, 0.98.

Synthesis of H-Tyr-Gly-Gly-Phe-D-Met-OH

H-Tyr-Gly-Gly-Phe-D-Met-Merrifield resin (2 g), 10% anisole (1 mL), *m*-cresol (1 mL) and HBr-acetic acid (33%, 10 mL) were added into the reaction-vessel and stirred at 100 rpm for 3 hr. The reaction mass was transferred to the filtration-vessel, washed twice with acetic acid (10 mL each). The filtrate was concentrated to a syrupy mass, which was washed with chilled solvent ether (20 mL × 4) and then dissolved in aqueous acetic acid (4 *N*) and lyophilized to obtain the brown coloured crude product. Yield 500 mg, (68.6%), 573(M^+), HPLC purity 66.0%.

The crude peptide was purified to >90% by preparative HPLC using 1% TFA in acetonitrile and water linear gradient system.

Synthesis of H-Tyr-Gly-Gly-Phe-D-Met-X

H-Tyr-Gly-Gly-Phe-D-Met-Merrifield resin (2 g), DMF (5 mL) were taken into a reaction-vessel cooled to 0-5°C then ammonia gas was passed for 48 hr (monitored by Kaiser test). The reaction mixture was filtered, washed with methanol (10 mL × 2), and the filtrate concentrated to a syrupy mass. This was dissolved in acetic acid (4 *N*) and lyophilized to yield a brown coloured mass. Similarly, other derivatives were prepared using monomethylamine, *n*-propyl-amine and hydrazine hydrate. Crude peptides were further purified to >90% as above.

Acknowledgements

The authors sincerely thank the glass blowing section, Natco Research Center for helping with the fabrication of filtration-vessel and Dr. A K S Bhujanga Rao for encouragement and helpful discussions.

References

1 a) Merrifield R B, *J Am Chem Soc*, 85, **1963**, 2149; b) Merrifield R B, *Science*, 150, **1965**, 178; c) Khosla M C, Leese R A, Maloy A T, Smeby R R & Bumpus F M, *J Med Chem*, 15, **1972**, 792; d) Stewart J M & Young J D, in *Solid Phase Peptide Synthesis*, 2nd edition, (Pierce Chemical Company, Rockford) **1984**; e) Atherton E & Sheppard R C, in *Solid Phase Peptide Synthesis*, edited by D Rickwood and B D Hames Series, (IRL Press, Oxford) **1989**, 137; f) Brown R A, Herkens P H H, Ottenheijm H C J & Rees D C, *Synlett*, 1**996**, 817; g) Chang C C & White P D, in *Fmoc Solid Phase Peptide Synthesis*, edited by C C Chang and P D White, (Oxford University Press, Oxford) **2000**, 41; h) Kiyoshi N, Shizue Y, Chikako T & Jun W, *The Jap Pep Soc*, **2002**, 61; i) Glassware vendors to name a few are Aldrich, Activotec, Wilmad LabGlass, Ace Glass Incorporation, Kimble Kotes, Advanced ChemTech, CS Bio, *etc*.

2 Gustein H B, Akil H & Goodman G, in *Pharmacological Basis of Therapeutics*, edited by J G Hardman and L E Limbird (McGraw-Hill, New York) **2001**, 10th Edn, 569.

3 Gisin B F, *Helv Chim Acta*, 56, **1973**, 1476.

4 Keller O, Keller W E, van Look G & Wersin G, *Org Syn Coll Vol* 7, **1990**, 70.

5 Mergler M & Dureix J P, in *The Bachem Practice of SPPS* (Bachem, Switzerland) **2000**, p 43.