Journal of Peptide Science, Vol. 2, 240–244 (1996)

Inclusion Volume Solid-phase Synthesis

JUTTA EICHLER[1], RICHARD A. HOUGHTEN[1] and MICHAL LEBL[2]

[1] Torrey Pines Institute for Molecular Studies, San Diego, California, USA
[2] Selectide Corporation, an Affiliate of Hoechst Marion Roussel, Tucson, Arizona, USA

Received 12 October 1995
Accepted 5 February 1996

Abstract: Solid-phase synthesis of peptides was carried out using only the volume of the solvent included in the swollen solid-phase resin beads (inclusion volume synthesis). This approach enables (i) the use of higher concentrations of activated amino acids, resulting in increased coupling rates, (ii) drastically decreased consumption of solvents, and (iii) the construction of multiple peptide synthesizers having virtually no reaction vessels.

Keywords: Keywords: solid-phase synthesis; peptide synthesis; multiple synthesis; inclusion volume synthesis

INTRODUCTION

The success and potential of solid-phase synthesis [1] relies upon the immobilization of the initial reactive component to an insoluble polymeric carrier, which enables all excess reactants to be removed by simple wash procedures. The excess of later incoming reactants is crucial in solid-phase synthesis in order to achieve high (i.e. > 99%) coupling yields, since, in contrast to solution synthesis, no purification of intermediates is involved in the solid-phase methods. The concentration of reactants, however, is more important for high reaction rates than is the molar excess. Therefore, it is rational to work with as little volume as possible with of as highly concentrated solutions as possible in order to achieve the highest coupling rates with as little activated incoming reagent as possible. This concept has been utilized for the peptide synthesis using cotton as the solid support [2]. The use of only the volumes equal to the inclusion volume of the cotton carrier is possible for all steps of the synthesis (i.e. coupling, wash and deprotection). An important prerequisite for successful inclusion volume synthesis (IVS) is an efficient means of liquid removal, since, if one solution is not removed from the solid support, the support cannot be soaked with the next incoming solution. It has been shown that DMF, a solvent commonly used for Fmoc-based peptide synthesis, can be efficiently (up to 96%) removed from the cotton carrier by centrifugation, thus enabling the construction of a new type of automated multiple peptide synthesizer [3, 4], in which the solid support seves not only as the solid support, but also as the reaction vessel itself. Another efficient means of solvent removal after the last wash prior to the deprotection or coupling is evaporation, which can be expedited by vacuum and/or elevated temperature. The inclusion volume concept of solid-phase peptide synthesis has also been partially utilized (i.e. for the coupling step) in another arrangement of peptide synthesis on cellulose-based carriers, termed 'spot synthesis' [5].

In the current study, we have used the inclusion volume method in combination with a traditional resin-type solid support, thus extending the applicability of this method to the most commonly used types of solid support. The results of the IVS are compared with the synthesis results of standard manual and automated solid-phase peptide synthesis.

MATERIALS AND METHODS

General

Fmoc-amino acids (Fmoc-AA) (side-chain protection: *t*Bu for Asp, Glu, Ser, Thr, Tyr; Trt for His, Asn; Boc for Lys; Pmc for Arg) were obtained from Bachem California (Torrance, CA) or from Advanced Chem-

Address for correspondence: Dr Jutta Eichler, Torrey Pines Institute of Molecular Studies, 3550 General Atomics Court, San Diego, CA 92121, USA.

CCC 1075-2617/96/040240-05

Tech (Louisville, KY). *N*-hydroxybenzotriazole (HOBt), spectroscopic grade dimethylformamide (DMF), dimethylacetamide (DMA), diisopropylethylamine (DIEA), trifluoroacetic acid (TFA) and piperidine were purchased from Aldrich (Milwaukee, WI), and Fmoc-2,4-dimethoxy-4′-(carboxymethyloxy)-benzhydrylamine from Bachem Bioscience, Inc. (Philadelphia, PA). 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) was obtained from Advanced ChemTech (Louisville, KY). TentaGel resin was from Rapp Polymere (Tübingen, Germany), diisopropylcarbodiimide (DIC) from Chem-Impex (Wood Dale, IL) and triisobutylsilane from Fluka (Ronkonkoma, NJ). All chemicals were used as received. Standard techniques of solid-phase synthesis were employed (for a review see [6].

Matrix-assisted laser desorption ionization was used for the generation of mass spectra on a MALDI instrument from Kratos Analytical (Ramsey, NJ). HPLC chromatograms were obtained using a System Gold instrument from Beckman (Fullerton, CA).

Manual 'Tea Bag' Synthesis

TentaGel resin (0.3 mmol/g, 90 μm) was contained in polypropylene mesh bags (100 mg resin per bag) [7]. The synthesis was carried out by vigorously shaking the resin-filled bags in the respective coupling, wash and deprotection solutions. The following protocol was used:

1. Coupling (0.1 M Fmoc-AA/DIC/HOBt, 1.5 ml/bag (60 min
2. Wash (DMF, 3 ml/bag) 3 × 1 min
3. Re-coupling (see 1)
4. Wash (DMF, 3 ml/bag) 5 × 1 min
5. Deprotection (20% piperidine/DMF, 3 ml/bag) 5 + 15 min
6. Wash (DMF, 3 ml/bag)3 × 1 min
7. Wash (DCM, 3 ml/bag) 2 × 1 min

Automated Inclusion Volume Synthesis

TentaGel resin was contained in polypropylene mesh bags (80 mg/bag), which were sealed to polypropylene frames used for the automated multiple peptide synthesizer COMPAS 242 (Spyder Instruments Inc., San Diego, CA). The frames were mounted on the perimeter of a centrifugal plate. Amino acid solutions were added by pneumatically operated spray pumps. All other reagent and wash solutions were added in independent lines by gear pumps. Reagent and wash solutions were removed from the resin by centrifugation. The following protocol was used:

1. Coupling (0.3 M Fmoc-AA/DIC/HOBt, 0.4 ml/bag) 60 min
2. Wash (DMF, 0.4 ml/bag) 3×
3. Re-coupling (see 1)
4. Wash (DMF, 0.4)ml/bag) 5×
5. Deprotection (20% piperidine/DMF, 0.4 ml/bag) 20 min
6. Wash (DMF, 0.4 ml/bag) 5×

Regular Automated Synthesis

Peptides were synthesized on TentaGel (100 mg/peptide) using the automated multiple peptide synthesizer ACT-396 (Advanced ChemTech, Louisville, KY). The following protocol was used:

1. Coupling (0.5 ml 0.5 M Fmoc-AA/HOBt in DMA, followed by 0.5 ml 0.5 M HBTU in DMF + 0.25 ml 2 M DIEA in DMF) 25 min
2. Wash (DMF) 1 ml
3. Re-coupling (see 1)
4. Wash (DMF) 1 ml
5. Wash (methanol) 1 ml
6. Wash (DMF) 2 × 1 ml
7. Deprotection (50% piperidine/DMF, 1.5 ml) 30 sec + 10 min
8. Wash (methanol) 1 ml
9. Wash (DMF) 7 × 1 ml

In all three syntheses, a TFA-cleavable linker (Fmoc-2,4-dimethoxy-4′-(carboxymethyloxy)-benzhydrylamine) [8] was coupled to the resin prior to assembling the peptide sequence.

Cleavage from the Resin

The peptide–resins from the manual 'tea bag' and inclusion volume syntheses were taken out of the polypropylene bags and placed into 'Quik-Snap' plastic tubes (5 ml, Isolab, Inc., Akron, OH) equipped with a sintered bottom disc. The peptides were cleaved as their C-terminal amides in 1.5 ml of a mixture of TFA/DCM/water/triisobutylsilane 70:20:5:5 for 3 h at room temperature. The tips of the tubes were snapped off, and the cleavage solutions added to centrifugation tubes containing 30 ml of cold *tert*-butylmethylether. The resins were washed with 2 ml of TFA, and the wash solution added to the *tert*-butylmethylether. The precipitated peptides were collected by centrifugation, washed

with 10 ml of cold *tert*-butylmethylether, dissolved in water and lyophilized.

For the peptide-resins from the ACT-396 synthesis, mixture K (TFA/phenol/water/1,2-ethanedithiol/thioanisole 82.5:5:2.5:5) [9] was used as the cleavage mixture. The crude peptides were characterized by mass spectrometry and analytical HPLC (Vydac C-18, 4 × 250 mm, 5 μm particle size, gradient of acetonitrile in 0.1% TFA in water, 0–60% in 60 min).

RESULTS AND DISCUSSION

Nine peptides, ranging in length from 7 to 13 amino acids (Table 1) were synthesized on polyoxyethylene-grafted polystyrene resin (TentaGel) [10] using three different methods. The first set was synthesized manually using the 'tea bag' method of multiple peptide synthesis [7]. In this synthesis, five equivalents of activated amino acids were used for the couplings at 0.1 M concentration. The second set of peptides was synthesized by the inclusion volume method using the automated multiple peptide synthesizer COMPAS 242 [3]. As for the first set, the resin was contained in polypropylene mesh bags, and five equivalents of activated amino acids were used for the couplings. The concentration of activated amino acids, however, was 0.3 M, so that the volume of coupling solutions was three times less than in the first set. The volumes of deprotection and wash solutions used for the second set were eight times lower than in the first set. Thus, the consumption of solvents was 7.5 times lower in the second set (inclusion volume method, 6.4 ml/peptide/cycle) than in the first set (standard method, 48 ml/peptide/cycle). The third set was synthesized using another automated multiple peptide synthesizer (ACT 396). The protocol that had been previously found to be optimal for this instrument was used for the third synthesis. The solvent consumption in this synthesis (18.5 ml/peptide/cycle) was almost three times higher than in the inclusion volume synthesis. It should be noted that the ACT 396 synthesizer works with 8.3 equivalents of activated amino acid for the couplings as compared to 5 equivalents used in the 'tea bag' and inclusion volume syntheses. The synthesis on the ACT 396 instrument was repeated after several months and the results of both syntheses were compared.

One could argue that in order to obtain truly comparable results, exactly identical conditions should have been used in all three synthetic arrangements. This, however, would mean that non-optimal conditions would have been used for at least two of the three methods. Lower concentration of Fmoc amino acids would lead to incomplete couplings in the inclusion volume synthesis, because the concentration inside the beads would be too low. Higher concentration in the tea bag method, on the other hand, would be a waste of amino acids and would not represent the real situation. The conditions used for the synthesis on the ACT 396 synthesizer had previously been optimized by trial and error during the synthesis of hundreds of peptides. These conditions include the use of HBTU as a coupling reagent, which provided consistently better results than DIC. Therefore, the experimental

Table 1 Purity of Crude Peptides[a] Synthesized Using Three Different Methods of Multiple Synthesis

Sequence	Manual tea bag' method	Automated inclusion volume method	Automated synthesizer
1 YAFGYPS-NH_2	87.3	75.0	91.0 (89.5)[b]
2 DPAFNSWG-NH_2	60.3	67.8	35.6 (44.2)
3 YGGFMRRV-NH_2	72.1	81.8	83.1 (76.8)
4 WAGGDASGE-NH_2	68.7	92.3	71.3 (73.2)
5 GNLWATGHFM-NH_2	41.2	74.8	62.4 (64.1)
6 ARPGYLAFPRM-NH_2	40.8	79.4	68.1 (66.8)
7 LEEEEEAYGWMDF-NH_2	31.0	58.3[c]	51.3[c] (49.6[c])
8 INLKALAALAKKIL-NH_2	14.1	44.5	30.6 (32.4)
9 YMFHLMD-NH_2	65.8	67.5	56.7 (58.2)
Average	53.5	71.3	61.1 (61.6)

[a] Determined by HPLC.
[b] Results of repeated synthesis in parentheses.
[c] Sulphoxide form.

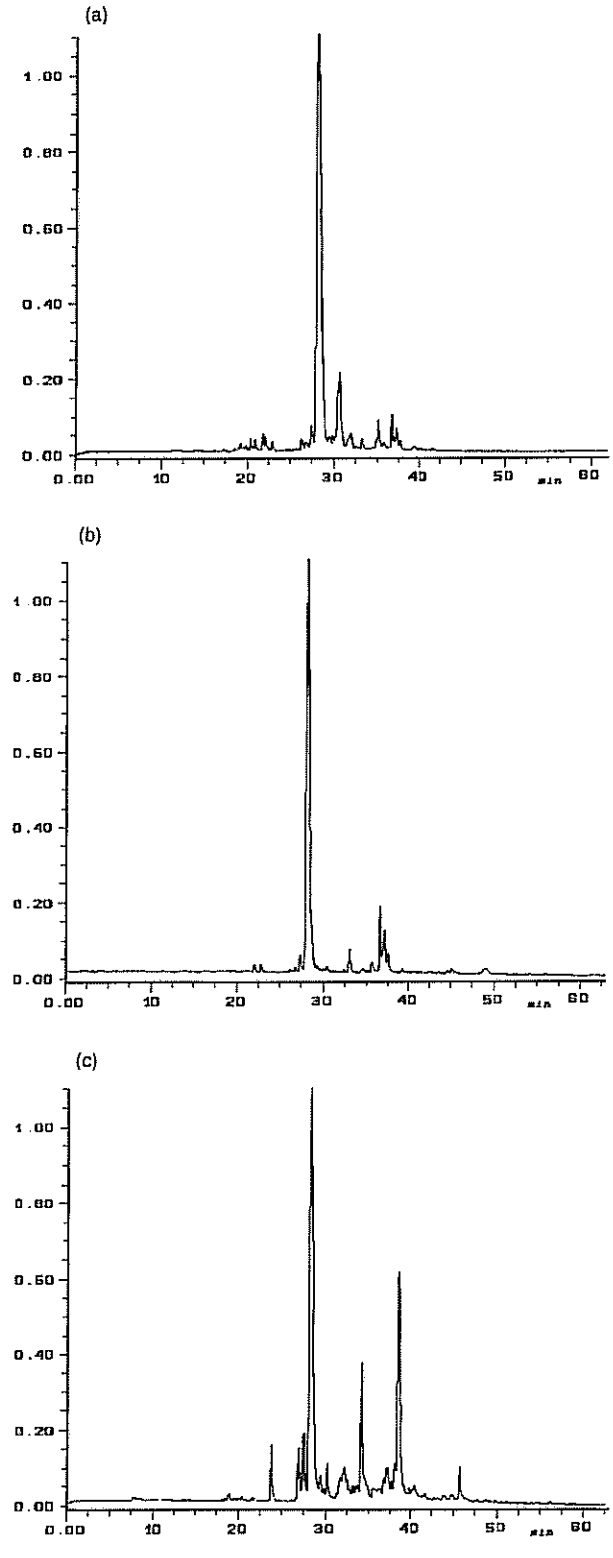

Figure 1 HPLC profiles of crude peptide 2 (see Table 1) synthesized by: (a)-'tea bag' method; (b) automated inclusion volume method; (c) Standard automated Synthesizer (ACT 396).

design for the comparative syntheses was based on the best conditions for each of the three methods, keeping constant only the resin and the sequences of the synthesized peptides.

The results of all syntheses with regard to purity of the crude peptides are listed in Table 1. On average, the purity of the peptides synthesized by IVS (71.3%) was higher than the peptides prepared by manual 'tea bag' synthesis (53.5%) or peptides prepared by automated multiple synthesis (61.1 or 61.6%). Seven out of the nine peptides had the highest purity when synthesized by the inclusion volume method, whereas only two peptides were the purest when synthesized by automated multiple synthesis. The HPLC profiles of the same peptide synthesized by the three different methods (Figure 1) show the correct peptide as the main peak; the patterns of impurities, however, are clearly different.

CONCLUSIONS

Inclusion volume synthesis (IVS), as illustrated for the synthesis of peptides, yielded peptides of equal or higher purity compared with other manual or automated solid-phase synthesis methods. The IVS consumes significantly (7.5 times) less solvent than manual 'tea bags' synthesis, and almost three times less than the synthesis using a standard multiple peptide synthesizer. This is noteworthy not only from the economic purity but also the environmental point of view. Furthermore, IVS makes possible the construction of mechanically simplified automated multiple peptide synthesizers with no separate reaction vessels, since the solid carriers themselves serve as the reaction vessels.

Acknowledgements

We thank Dasha Cabel, Adam W. Lucka and Edward Brehm for their excellent technical assistance.

REFERENCES

1. R. B. Merrified (1963). Solid phase peptide synthesis. I. The synthesis of a tetrapeptide. *J. Am. Chem. Soc. 85*, 2149–2154.
2. J. Eicher, M. Bienert, A Stierandová and M. Lebl (1991). Evaluation of cotton as a carrier for solid phase peptide synthesis. *Peptide Res. 4*, 296–307.
3. V. Pokorný, P. Mudra, J. Jehnička, K. Ženišek, M.

Pavlík, Z. Vobůrka, M. Rinnová, A. Stierandová, A. W. Lucka, J. Eichler, R. A. Houghten and M. Lebl in : *Innovation and Perspectives in Solid Phase Synthesis*, R. Epton, Ed., p. 643–648, Mayflower, Birmingham 1994.

4. M. Lebl, A. Stierandová, J. Eichler, M. Pátek, V. Pokorný, J. Jehnička, P. Mudra, K. Zenišek and J. Kalousek in: *Innovation and Perspectives in Solid Phase Peptide Synthesis*, R. Epton, Ed., p. 251–257, Intercept, Andover 1992.
5. R. Frank (1992). Spot synthesis: An easy technique for the positionally addressable, parallel chemical synthesis on a membrane support. *Tetrahedron 48*, 9217–9232.
6. G. A. Grant, Ed.: *Synthetic Peptides, A User's Guide*, Freeman, New York 1992.
7. R. A. Houghten (1985). General method for the rapid solid-phase synthesis of large numbers of peptides: Specificity of antigen–antibody interaction at the level of individual amino acids. *Proc. Natl. Acad. Sci. USA 82*, 5131–5135.
8. H. Rink (1987). Solid-phase synthesis of protected peptide fragments using a trialkoxy-diphenyl-methylester resin, *Tetrahedron Lett. 28*, 3787–3790.
9. D. S. King, C. G. Fields and G. B. Fields (1990). A Cleavage method which minimizes side reactions following Fmoc solid phase peptide synthesis. *Int. J. Peptide Protein Res. 36*, 255–266.
10. E. Bayer and W. Rapp in: *Poly(ethylene glycol) Chemistry: Biotechnical and Biomedical Applications*, J. M. Harris, Ed., p. 325–345, Plenum Press, New York 1992.