Int. J. Peptide Protein Res. 47, 1996, 91–97
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INTERNATIONAL JOURNAL OF
PEPTIDE & PROTEIN RESEARCH
ISSN 0367-8377

Automated multiple peptide synthesis: Improvements in obtaining quality peptides

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Received 9 May, revised 20 June, accepted for publication 20 August 1995

Production of multiple overlapping peptides is a key step in the identification of T-cell epitopes. A large number of peptides can be produced by using ABIMED's automated multiple peptide synthesizer. We report here considerable improvement in the software and chemistry of peptide synthesis by introducing a resin mixing step during coupling, when using this synthesizer. A comparison of two solvent systems for synthesis was performed. Six test peptides were synthesized by standard and modified methods. The purity of peptides, assessed by HPLC and mass spectrometry, showed a substantial improvement when automated resin mixing and mixed solvent system were used. These improvements enable us to produce 48 peptides within a week each of sufficient purity to be used for rapid screening of T-cell epitopes. © Munksgaard 1996.

Key words: coupling solvent; multiple peptide synthesis; resin mixing; T-cell epitopes

Solid-phase peptide synthesis (SPPS), invented by Merrifield (1), involves three distinct steps: (1) chain assembly on the solid phase; (2) cleavage of peptide from the solid support along with deprotection of side chains of amino acid residues; (3) purification and characterization of peptides. In the preparation of a large number of peptides for T cell epitope screening, the purification step is time consuming and not practical. Ideally, for the production of multiple peptides, the cleaved crude peptides should be sufficiently pure that they can be directly used in T cell epitope screening. Several automated and semi-automated multiple peptide synthesizers (MPS) are available commercially (2–6) which can be used to prepare multiple peptides. However, to achieve the goal of producing pure peptides of more than 12 amino acids, several modifications have to be made such as the use of superior coupling agents, multiple couplings of N^{α}-blocked amino acids or transfer to a better solvent system. Sometimes one or all these modifications has to be introduced in order to get optimum peptide purity.

We were interested in the preparation of 14–20-mer overlapping peptides of antigenic proteins invoked in the autoimmune diseases, viz. multiple sclerosis (MS), myasthenia gravis (MG), rheumatoid arthritis (RA) and insulin-dependent diabetes mellitus (IDDM). We employed an ABIMED-Gilson AMS 422 MPS for this purpose. However, the peptides synthesized by PyBOP coupling chemistry in dimethylformamide (DMF) using standard protocols provided by ABIMED did not produce the desired level of purity. There were several problems in using the standard protocol. First, with no mixing of resin during coupling of amino acids to the growing chain on the resin, the polystyrene resin used as solid support settled at the bottom of the column, thus decreasing the efficiency of aminoacylation, as well as Fmoc deprotection. Second, although DMF is considered to be a good solvent for peptide synthesis, it is not an ideal solvent for the preparation of longer peptides in which growing peptide chains tend to form a β-sheet structure (7). Efforts to eliminate β-sheet structures by the addition of more polar solvents such as trifluoroethanol (TFE) and hexafluoroisopropanol have been successful (8, 9). A problem that is usually ignored is that in cases when aggregation occurs due to apolar side-chain protecting groups, increased solvent polarity does not help to disrupt the aggregation and a nonpolar

AA, amino acid; Ac, acetyl; Ac-DR4DW4β57-76, acetyl-human major histocompatibility complex DR4DW4 β-chain sequence 57–76; BOP, benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphonate; CDI, carbonyldiimidazole; DCM, dichloromethane; DIEA, diisopropylethylamine; DMF, dimethylformamide; HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate; HOAt, 1-hydroxy-7-azabenzotriazole; HOBt, 1-hydroxybenzotriazole; MBP, myelin basic protein; NMM, *N*-methylmorpholine; PyBOP, benzotriazole-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate; GP, guinea pig.

TABLE 1

Sequences of peptides synthesized on AMS422 multiple peptide synthesizer

Peptide no.	Peptide name	Sequence
1	Ac-MBP83-102Y83	Ac-YDENPVVHFFKNIVTPRTPP-NH_2
2	Ac-DR4DW4b57-76	Ac-DAEYWNSQKDLLEQRAAVD-NH_2
3	GP-MBP69-88	H-GSLPQKSQRSQDENPVVHF-NH_2
4	Ac-MBP1-14	Ac-ASQKRPSQRHGSKY-NH_2
5	RatMBP87-99	H-VHFFKNIVTPRTP-NH_2
6	Collagen Type II 274-288	H-GIAGFKGEQGPKGEP-NH_2

solvent may be necessary. These problems require improvements in both robotics and chemistry of coupling to suppress deletion or addition-peptide formation and obtain high-purity peptides. To resolve these technical hurdles, six test peptide fragments from different antigenic proteins associated with autoimmune disease were prepared by standard, and several modifications of standard, coupling procedures. The purity of these peptides were compared.

EXPERIMENTAL PROCEDURES

Peptide synthesis

Six peptides with sequences (Table 1) were synthesized on a 0.25 mmol scale using an ABI 431A peptide synthesizer by FastMoc chemistry (10). HBTU/HOBt activation of N^{α}-protected amino acids was employed for coupling. The side-chain protecting groups used in all the syntheses are given in Table 2. Rink amide-MBHA (Novabiochem, San Diego; substitution: 0.55 mmol/g) resin was used for all syntheses. The peptides were cleaved using trifluoroacetic acid containing 4-methoxybenzenethiol and 4-(methylmercapto)phenol as scavengers. The crude peptides were precipitated in pentane:acetone (4:1). They were purified by preparative reversed-phase high-performance liquid chromatography (RH-HPLC), using 0–70% aqueous acetonitrile containing 0.1% TFA gradient. The purity of each final product was assessed by analytical RP-HPLC, and the peptides were characterized by fast atom bombardment mass spectrometry (FAB-MS). These peptides were used as standards for coelution studies for the peptides obtained with the multiple peptide synthesizer.

TABLE 2

Side-chain protecting groups used in the peptide synthesis

Amino acid	Side-chain protecting group
S, T, Y, D and E	*tert*-Butyl (t-Bu)
C, N, Q and H	Trityl (Trt)
R	2,2,5,7,8-Pentamethylchloraman-6-sulfonyl (Pmc)
K and W	Butyloxycarbonyl (Boc)

Peptide synthesis on a multiple peptide synthesizer

Our ABIMED-Gilson AMS 422 multiple peptide synthesizer was obtained from Gilson. The synthesizer consists of a Gilson auto-sampler which is capable of *X-Y-Z* movements, a 48-column reactor module and amino acid and activating reagent reservoirs. While the reagents and solvents were added to each column by a micro-injector sequentially, the washing of resin in all reaction columns was performed simultaneously.

Multiple peptide synthesis

Method A: multiple peptide synthesis in DMF by standard protocol without resin mixing. The six peptides (Table 1) were synthesized on MPS-AMS 422 using PyBOP chemistry following a standard protocol suggested by ABIMED (Fig. 1a). Rink amide-MBHA resin (0.025 mmol of active sites per reaction column) was used for synthesis. The coupling reactions were performed in DMF. Deprotection of Fmoc groups was carried out in 20% piperidine in DMF for 6.5 min at the start of the synthesis and gradually increased to 19.5 min when amino acid chain length on the resin was increased to 20. A double deprotection strategy was used at each step to ensure complete deprotection of Fmoc groups before the next amino acid coupling. The coupling of amino acids was achieved by using a molar ratio of active sites on the coupling resin to Fmoc-amino acid as 1:6. The amino acids were double-coupled to ensure the completion of reaction.

Method B: multiple peptide synthesis by mixing the resin in DMF. The six peptides were prepared on MPS-AMS-422 by the protocol given in Fig. 1. The software on the computer was modified to accommodate resin mixing after the coupling solutions were delivered to each reaction column. The resin mixing was performed for 35 s by gently bubbling nitrogen through the reaction col-

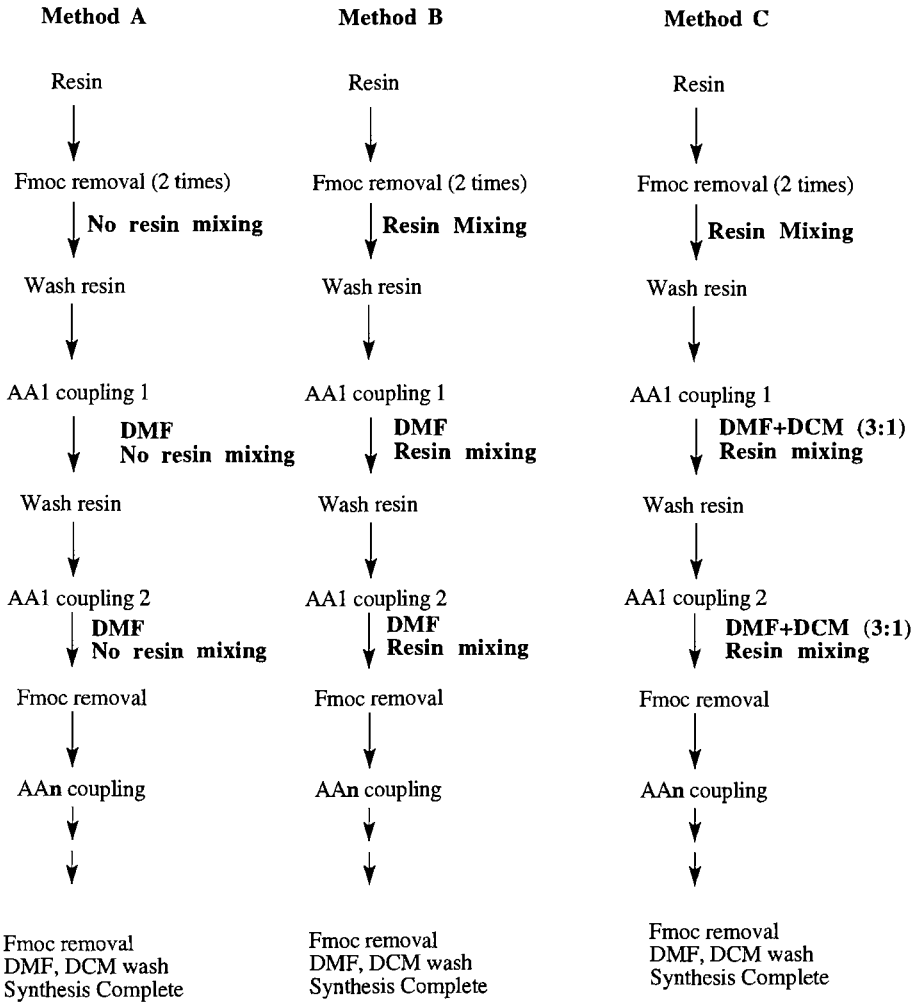

FIGURE 1

The flow chart of three methods used for the synthesis of peptides on AMS 422 multiple peptide synthesizer. Deprotection was carried out for 6.5 min for the first amino acid and it was linearly increased at each cycle to achieve a deporotection time of 19.5 min at cycle 20. A similar strategy was used for coupling time with basic coupling time of 30 min for the first cycle, increasing to 90 min the for 20th cycle.

umn. Each delivery of coupling solution and deprotection solution was repeated three times for each column before delivering the next amino acid to another reaction column.

Method C: multiple peptide synthesis by improved protocol (resin mixing and DMF-DCM mixed solvent). The six peptides were also prepared on MPS AMS 422 by the new protocol as given in Fig. 1. The method was similar to Method B described above except one minute after Fmoc-amino acid, PyBOP, HOBt and NMM were delivered to the reaction column, DCM was delivered to it. The ratio of DCM:DMF (v:v) in the reaction mixture was 1:3. Resin mixing was performed similarly to Method B after the DCM was delivered.

Cleavage and isolation of crude peptides synthesized on multiple peptide synthesizer by standard or improved protocol

After the final Fmoc deprotection, the peptide resins were washed with DCM and dried in the reaction columns by applying vacuum on the synthesizer. Columns were removed from the synthesizer and capped at one end using syringe caps (Gilson part #3980025). TFA (1.5 mL), containing 0.07 g of 4-(methylmercapto)-phenol, and 0.1 mL of 4-methoxybenzenethiol, was added to each column followed by mixing at room temperature (r.t.) for 2 h. Upon completion of cleavage, the caps were replaced by PTFE minifilters (Nalgene part #199-2045). The reaction mixture was filtered and the filtrate was collected into 100 mL of pentane:acetone (4:1). The peptides were allowed to precipitate for 2 h

at room temperature and isolated by centrifugation. They were washed three times with pentane:acetone and twice with pentane. The crude peptides were dried in vacuum for 2 h then subjected to analytical RP-HPLC and mass spectrometry. HPLC coelution studies of these peptides were performed using purified reference standards.

RESULTS AND DISCUSSION

Six model peptides were designed to optimize multiple peptide synthesis techniques. By using resin mixing and two different solvent systems, peptides ranging from 14–20 amino acids can be produced in high purity. These peptides were chosen based on the fact that they contain difficult sequences. The problems in the syn-

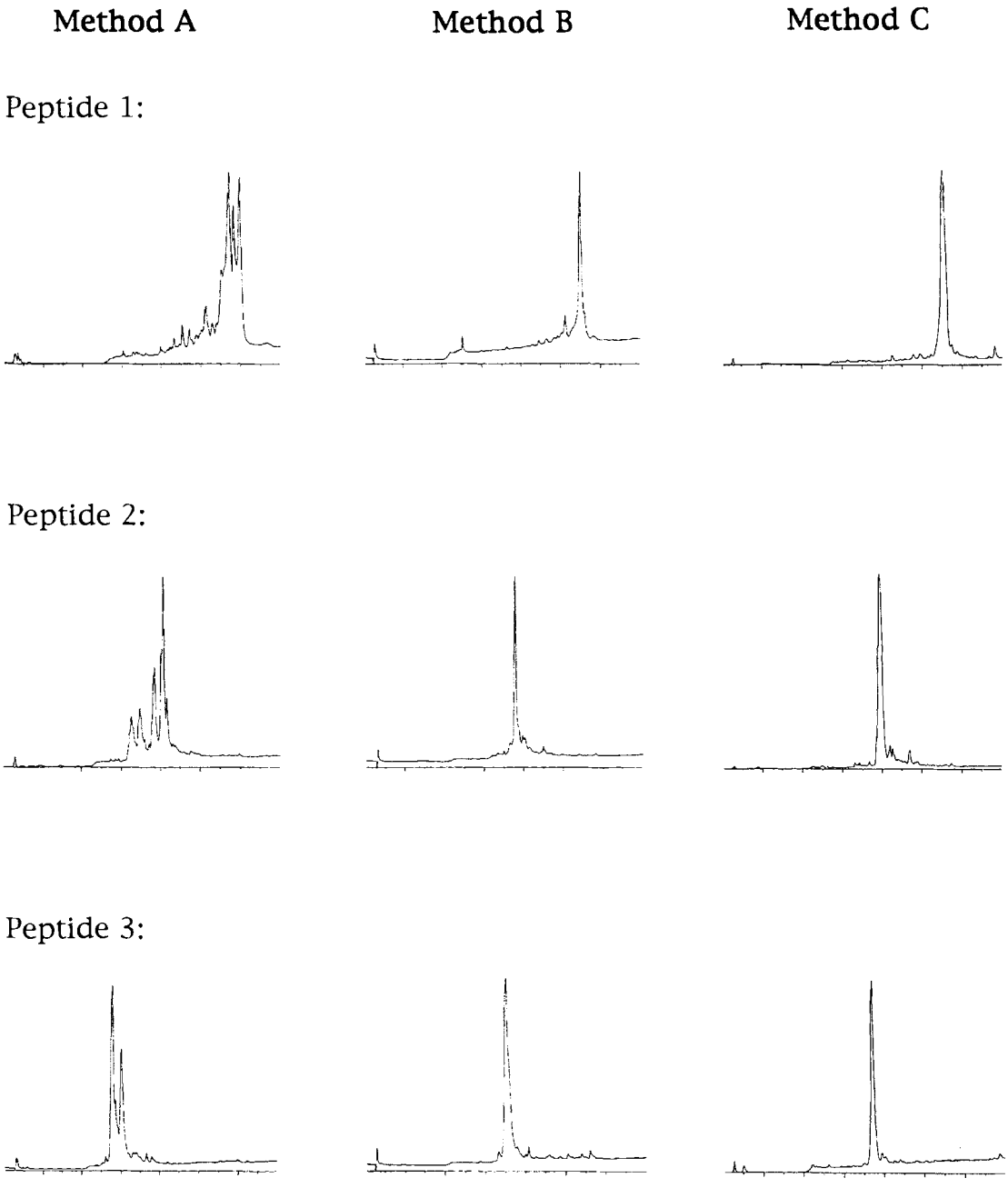

Fig. 2a.

thesis of these T-cell epitopes were well documented in our laboratory. The three protocols which were used in synthesis of the six peptides on MPS are given in Fig. 1. Reversed-phase HPLC traces of peptides prepared by these protocols are given in Figs. 2a and 2b.

The HPLC results of crude peptides prepared by these three methods indicated that the purity of peptides increased substantially by using a resin mixing step. The standard protocols (Method A) gave crude peptides that contained deletion and/or addition compounds as indicated by multiple peaks on HPLC. Coelution of the crude peptides with purified peptides (data not shown) suggested that peptides from Methods B and C coeluted with the purified peptides. On the other

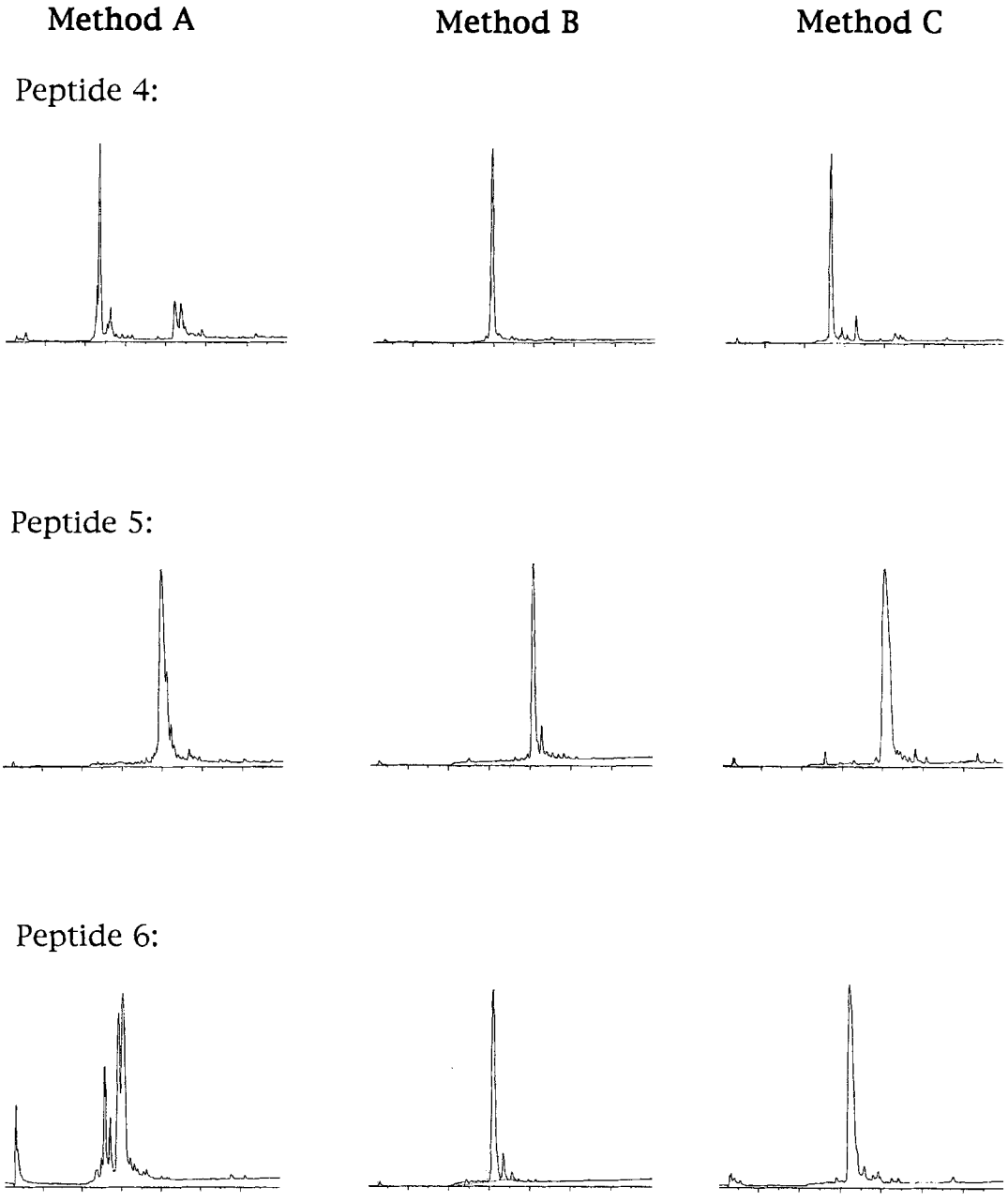

Fig. 2b.

FIGURE 2

Reversed-phase HPLC of peptides prepared by multiple peptide synthesizer by different protocols. Column 1 shows peptides prepared by Method A where there was no mixing of resin. Column 2 shows peptides prepared by Method B in which mixing of the resin during coupling in DMF and deprotection was introduced. Column 3 shows peptides prepared by Method C in which mixing of the resin during coupling in mixed solvents.

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