

Automated multiple peptide synthesis: Improvements in obtaining quality peptides

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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-DR4DW4 β 57-76, acetyl-human major histocompatibility complex DR4DW4 β -chain sequence 57–76; BOP, benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate; 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-GIAGFKGEQGPKEP-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*²-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 (RP-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.

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-

TABLE 2
Side-chain protecting groups used in the peptide synthesis

Amino acid	Side-chain protecting group
S, T, Y, D and E	<i>tert</i> -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)

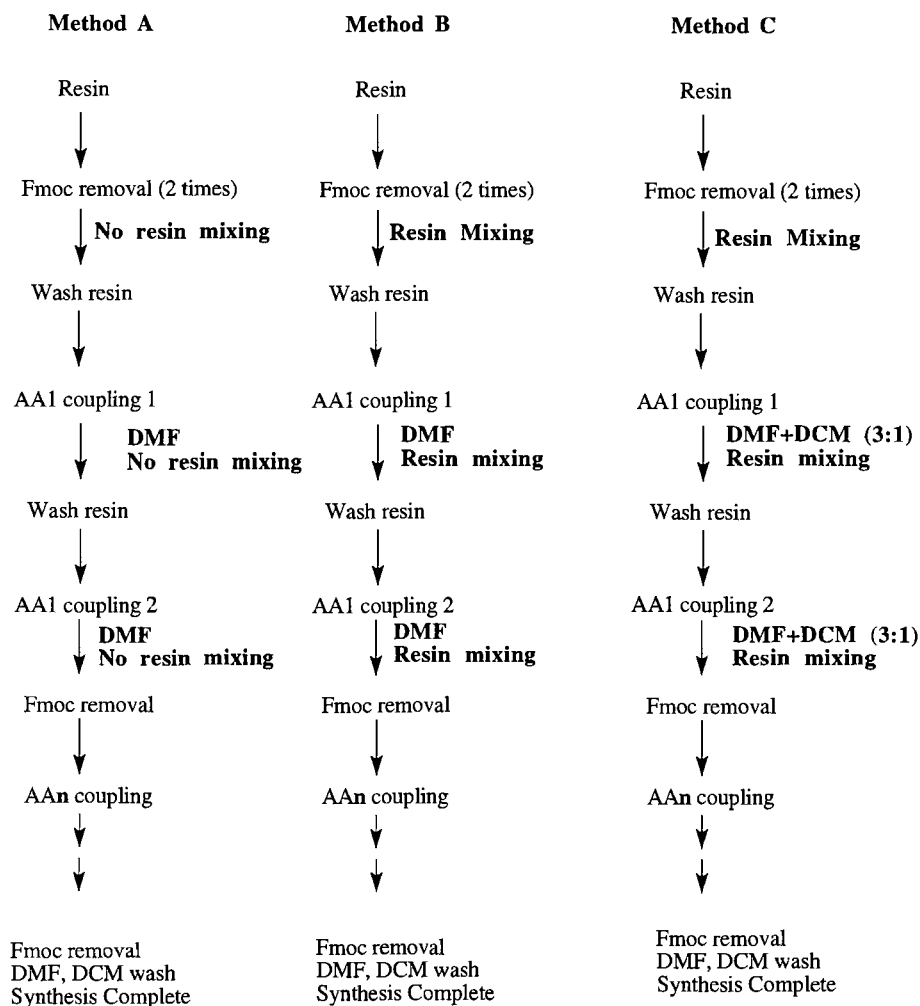


FIGURE 1

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 deprotection 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-

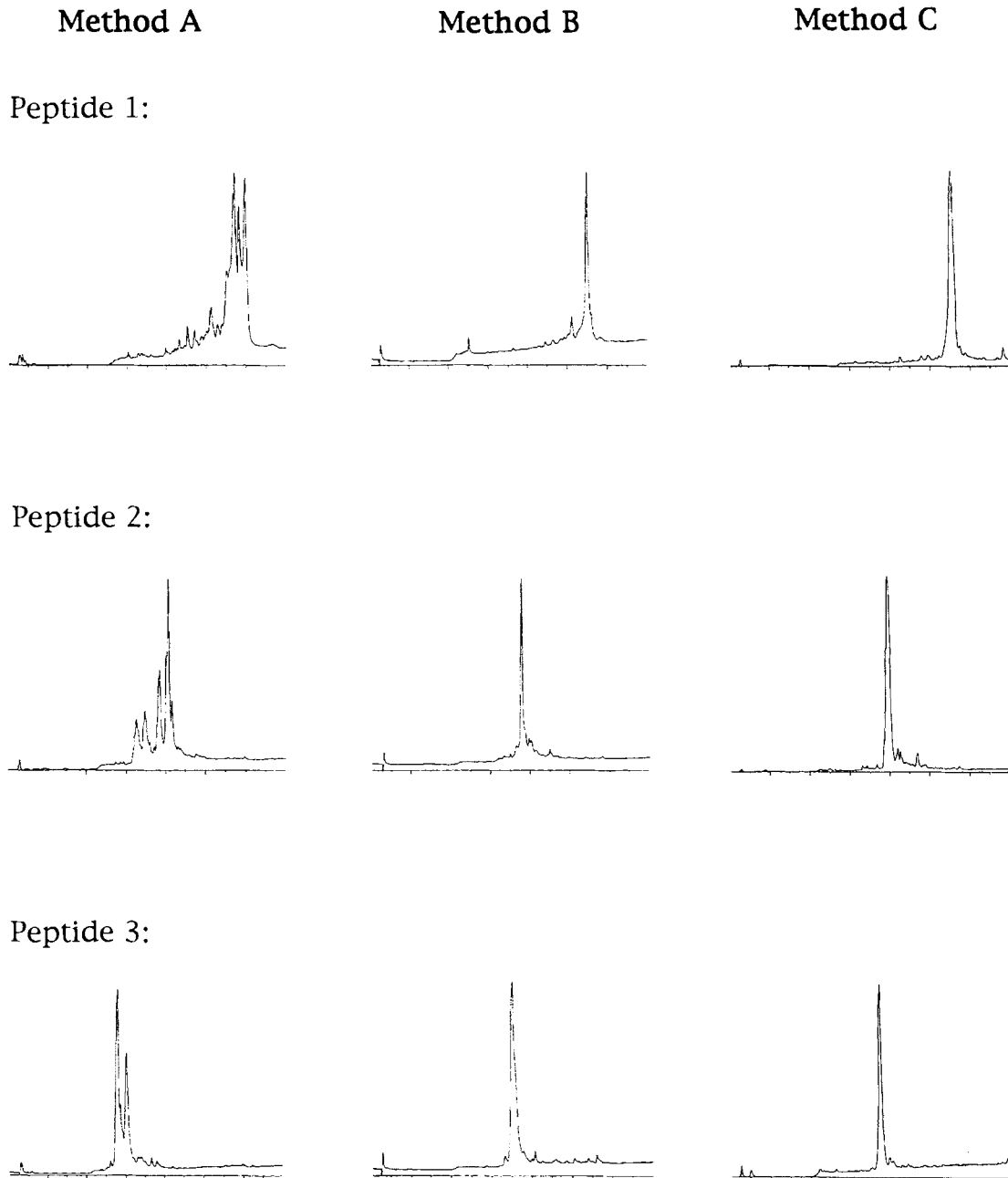


Fig. 2a.

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 pep-

tides 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. Co-elution 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

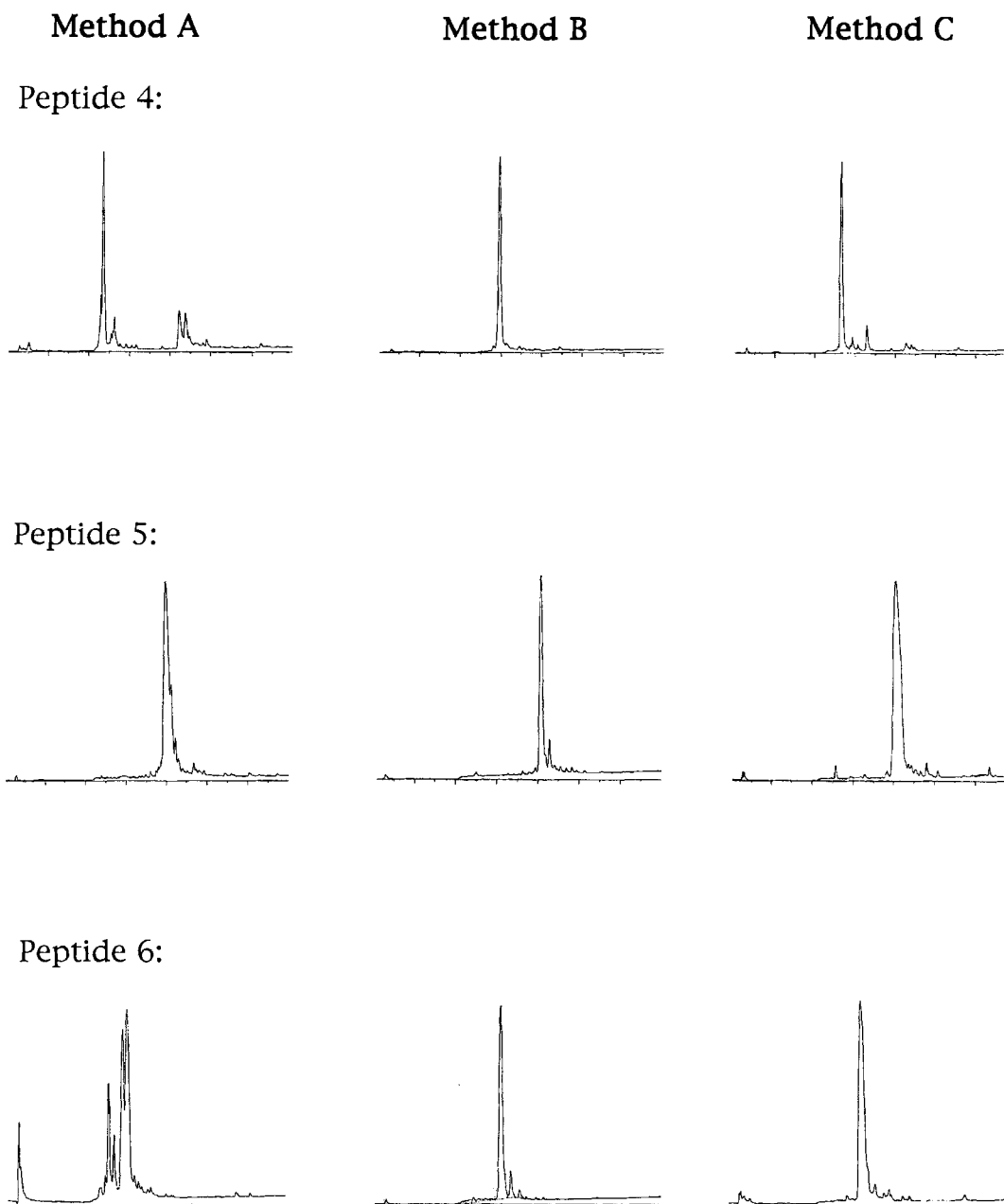


Fig. 2b.

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.

hand, some peptides prepared by Method A had very little or no desired product, indicating the incomplete assembly of the peptide chain.

The introduction of DCM in the coupling mixture has some effect on the purity of crude peptides. The effect was more prominent in cases of longer peptides, such as peptides 1–3. The amount of dichloromethane to be added was based on experiments in which the resin dispersion was evaluated at different ratios of DMF and DCM. The maximum dispersion was found when the ratio of DMF:DCM was 3:1. Peptide 1 contains four prolines. Unless care is taken to couple the prolines completely, synthesis of such peptides by Fmoc or Boc chemistry results in 20–30% of desproline products, whether the synthesis is automated or manual. Use of DCM seems to suppress the formation of these deletion structures.

Simultaneous, multiple peptide synthesis using SPSP is challenging in terms of successful peptide assembly, cleavage and simultaneous isolation of 48 or more peptides. The peptide assembly step involves the activation of protected amino acid using reagents such as carbodiimides, BOP, PyBOP, HBTU, CDI, and more recently by HOAt. These reagents render the carbonyl carbon of the carboxylic acid highly electrophilic and suitable for nucleophilic addition by the free amines on the resin. For successful peptide synthesis on solid phase the substitution reaction has to be complete, otherwise deletion peptides are formed. PyBOP is a fairly good coupling agent when used in the presence of a tertiary base such as NMM or DIEA and produces less toxic by-products as compared to BOP (11). In our synthesis, we routinely used a double coupling strategy with 5 molar excess of activated amino acids over the amines on the resin to force the completion of amide bond formation. In the synthesis of one peptide at a time, resins can be checked for completion of coupling reactions by ninhydrin or fluorescamine tests. Similarly, tests for complete Fmoc deprotection can be performed by subjecting small amounts of resin to additional deprotection and testing the supernatant for the presence of Fmoc by TLC or absorption at 290 nm. However, such testing is practically impossible in simultaneous synthesis of 48 or more peptides. Owing to the pseudodilution of reactive amines on the resin, activated amino acids are used in large excess over the nucleophiles available for reaction. The amino acid must be mixed thoroughly to increase the probability of amide bond formation. Therefore, changing the robotics to induce mixing of the resin and the activated amino acid solution resulted in higher quality of peptides with few deletion contaminants (Methods B and C).

In solid-phase peptide synthesis, sufficient solvation of the growing peptide chain containing side-chain-protected amino acids is also absolutely necessary to achieve an efficient coupling reaction. If a sub-optimal solvent or solvent system is used for coupling reactions, the growing peptide chain assumes intramolecular or

intermolecular β -sheet structure, thus making it difficult for the incoming amino acid to couple because of steric hindrance (12–14). Although this phenomenon is sequence-dependent, general solutions to this problem involve the use of more polar solvents and solvent additives such as HFIP to decrease the aggregation due to mostly β -sheet formation (9, 15). However, these solvents may not be sufficient, and in fact may increase the aggregation primarily due to apolar side-chain protecting groups such as *tert*-butyl, BOC and trityl, thus decreasing coupling efficiencies. It has been reported that the aggregation of side-chain protecting groups leads to a collapsed gel structure, limiting further coupling (16). Therefore, a delicate balancing must be achieved between using a solvent system efficient enough to disrupt β -sheet structures but does not interfere with side-chain protecting groups. The balance becomes more difficult to achieve when synthesizing several peptides simultaneously. For multiple peptide synthesis, the solvent system must be promiscuous and generally efficient for most of the sequences one commonly synthesizes. Several ways to achieve this balance such as the introduction of more polar side-chain protecting groups for individual amino acids or use of mixed solvent system have been discussed (17). The use of DCM to create mixed solvents has been suggested by Gausepohl *et al.* for the peptide synthesis using multiple peptide synthesizer (3). In our search for a promiscuous solvent system we further explored the use of mixed solvents for multiple peptide synthesis and found that a solvent system containing 3 parts DMF and 1 part DCM was optimal. In this method the amino acids are dissolved in DMF and delivered to the reaction vessel containing the growing peptide chain assembly on resin, and then the appropriate amount of dichloromethane is added to achieve the 3:1 ratio. This mixed solvent was able to yield pure peptides of 15 residues or longer, suggesting a general solution to β -sheet formation and solvation of apolar side-chain protecting groups.

In conclusion, the quality of crude peptides by mixing the resin during coupling increased 5–10-fold as compared to the quality of peptides produced by standard methods without resin mixing during coupling and deprotection. The introduction of a mixed solvent system containing DMF and DCM had marginal effect on the quality of shorter peptides, while it seemed to improve the purity of longer peptides.

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