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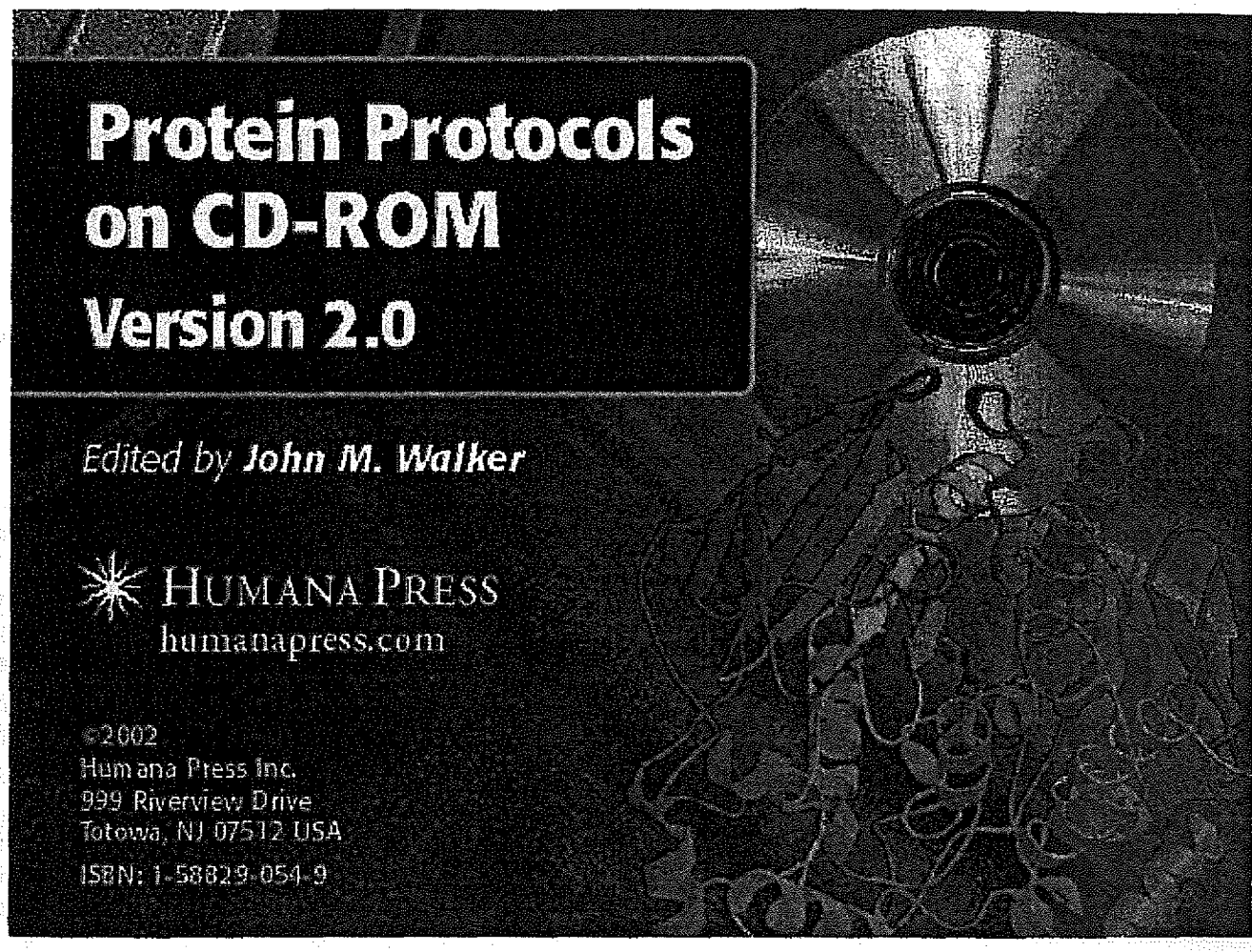

Section 23.4

Synthesis of a One-Bead One-Compound Combinatorial Peptide Library

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1. Introduction

The four general methods to generate and screen a huge combinatorial peptide library (>10^7 peptides) are: biological libraries such as filamentous phage ***(1)***, plasmid ***(2)***, or polysome ***(3)*** libraries; the "one-bead one-compound" synthetic combinatorial library method or the "Selectide process" ***(4-6)***; synthetic peptide library methods that require deconvolution, such as an iterative approach ***(7,8)***, positional scanning ***(9)***; orthogonal partition approach ***(10)***, or recurse deconvolution ***(11)***; and synthetic library using affinity column selection method ***(12,13)***.

There are advantages and disadvantages in each of these methods. In general, the main advantages of the biological library method are that large peptides can be displayed on a filamentous phage library, and that large protein folds can be incorporated into the library. However, the main disadvantage is that biological libraries, in general, are restricted to all L-amino acids. In contrast, the remaining three methods all use synthetic libraries; therefore, D-amino acids, unnatural amino acids, nonpeptide components, and small rigid scaffoldings can all be incorporated into these libraries.

The "one-bead one-compound" library is based on the concept ***(4,5)*** that when a solid-phase split synthesis method ***(4,8,14)*** is used, each solid-phase particle (bead) displays only one peptide entity although there are approx 10^{13} copies of the same peptide the same bead. The resulting peptide-bead library (e.g., 10^7 beads) is then screened in parallel using either "on-bead" binding assays ***(15)*** or "solution phase-releasable" assays ***(16)*** to identify peptide-beads with the desired biologic, biochemical, chemical, or physical properties. The positive peptide-beads are then physically isolated for microsequencing with an automatic protein sequencer. In this section, detailed methods for the synthesis of a random "one-bead one-compound" combinatorial peptide libra will be described. **Sections 23.5** and **23.13** give examples of two general screening methods for such libraries.

2. Materials

2.1. Chemicals

1. Tenta-Gel Resin S-NH_2 (90-100 μm) resin may be obtained from Rapp Polymere, Tubingen, Germany (*see* **Note 1**).
2. Fmoc amino acids with standard side chain-protecting groups, *N*-hydroxy-benzotriazole (HOBt), benzotriazolyl-oxy-trisdimethylamino-phosphonium hexafluorophosphate (BOP), diisopropylethylamine (DIEA), diisopropylcarbodiimide (DIC), piperidine, trifluoroacetic acid (TFA), ninhydrin, may be obtained from many different suppliers, such as Bachem (Torrance, CA), Bioscience (King of Prussia, PA), Advanced ChemTech (Louisville, KY),

Novabiochem (San Diego, CA), and Peptides International (Louisville, KY).

3. Technical grade solvents such as dimethylformamide (DMF) or dichloromethane (DCM) may be obtained from many different chemical suppliers. HPLC-grade DMF for the coupling may be obtained from Burdick and Jackson, Muskegon, MI. Ethanol, phenol, *p*-cresole, thioanisole, ethanedithiol, pyridine, and potassium cyanide may be obtained from many different chemical suppliers.
4. 0.1 g/mL Ninhydrin in ethanol.
5. 4 g/mL Phenol in ethanol.
6. 10 m*M* Potassium cyanide, stock solution.
7. 50% Piperidine in DMF.
8. Reagent K: TFA/*p*-cresole/water/thioanisole/ethanedithiol, 82.5:5:5:5:2.5. (v/v/v/v/v).
9. 10% DIEA in DMF.
10. Dimethylsulfoxide (DMSO)/Anisole/TFA, 10:5:85.

2.2. Apparatus

1. Polypropylene vials (5-10-mL) may be purchased from Baxter Scientific Products, McGaw Park, IL. Polyethylene disposable transfer pipets may be purchased from Elkay Products, Shrewsbury, MA.
2. Motorized rocking platform.
3. Randomization glass vessel (chromatography column 5-6 × 18 cm) fitted with a medium glass sintered frit connected to vacuum and nitrogen via a two-way valve from below. The three positions of the valve are "off," "vacuum," or "nitrogen."
4. Recirculating water aspirator or a solvent-resistant vacuum pump with cold trap.
5. Nitrogen tank.

3. Methods

3.1. Synthesis of a Linear Pentapeptide Library

As indicated earlier, a solid-phase split synthesis method ***(4,8,14)*** is used to generate a random peptide library. The composition and final structure of the peptide library depends on the number of amino acids (one or more) used in each coupling cycle and the number of coupling cycles used. The final peptide library may be linear or cyclic, or have specific secondary structures. For simplicity, the method for the synthesis of a linear pentapeptide library with all 19 eukaryotic amino acids except cysteine is given below:

1. Swell 10 g TentaGel Resin S-NH_2 beads (~ 0.25 mEq/g, *see* **Notes 1** and **2**) for at least 2 h in HPLC-grade DMF with gentle shaking in a siliconized flask.
2. Wash the beads twice with HPLC-grade DMF in the siliconized randomization vessel as follows: add 75 mL DMF from the top, gently bubble nitrogen from below through the sintered glass for 2 min, then remove the DMF by vacuum from below (*see* **Note 3**).
3. Transfer all the beads to a siliconized flask in HPLC-grade DMF. Then distribute the beads into 19 equal aliquots. A disposable polyethylene transfer pipet is extremely useful in the even distribution of the beads into each polypropylene vial (*see* **Note 4**).
4. Allow the beads to settle and remove most of the DMF above the settled bead surface from each polypropylene reaction vial.
5. Add threefold molar excess of each of the 19 Fmoc-protected amino acids (*see* **Note 5**) and threefold molar excess of HOBt to each reaction vial using a minimal volume of HPLC-grade DMF.

6. Add threefold molar excess each of BOP and DIEA to each reaction vial to initiate the coupling reaction.
7. Cap the reaction vials tightly and rock them gently for 1 h at room temperature.
8. To confirm the completion of coupling reaction, pipet a minute amount of resin from each reaction vial into small borosilicate glass tubes (6 × 50-mm) and perform ninhydrin test *(17)* as follows:

   Wash the minute quantity of resin in the small glass tubes (6 × 50-mm) sequentially with the following solvents: DMF, t-amyl alcohol (2-methylbutan-2-ol), acetic acid, t-amyl alcohol, DMF, and ether. Add to each tube one drop of each of the following three reagents, (ninhydrin in ethanol (0.1 g/mL), phenol in ethanol (4 g/mL), and potassium cyanide stock solution diluted 50 times with pyridine. Place the tubes in a heating block at 120°C for 2 min. Observe the color intensity of the beads under a microscope. To ensure complete coupling, every bead from the minute quantity of sample beads should be ninhydrin negative, i.e., straw yellow color.
9. If the coupling is incomplete (some beads remained purple or brown with ninhydrin test), remove the supernatant from those reaction vials and add fresh Fmoc-protected amino acids, BOP, DIEA, and HOBt into the reaction vial for additional coupling.
10. If the coupling is complete (beads remained straw yellow color with ninhydrin test) discard the supernatants of each reaction vial, and transfer and wash all the beads to the randomization vessel with technical grade DMF.
11. After all the 19 coupling reactions are completed, all the beads are transferred to the randomization vessel. Wash the beads (8 times, 2 min each) with technical grade DMF.
12. Add 75 mL 50% piperidine (in DMF) to the randomization vessel to remove the Fmoc protecting group. After 10 min, remove the piperidine and add 75 mL fresh 50% piperidine. After another 10 min, wash the beads 8 times with technical grade DMF and twice with HPLC-grade DMF.
13. Distribute the beads into each of the 19 reaction vials and carry out the next coupling reaction as described above.
14. After all the randomization steps are completed, remove the Fmoc protecting group with piperidine as described above.
15. After thorough washing with technical grade DMF (5X) followed by DCM (3X), add 10 mL of reagent K *(18)* to the randomization vessel for 3 h at room temperature.
16. Wash the deprotected resins thoroughly with DCM (3X), followed by technical grade DMF (5X), then once with 10% DIEA to neutralize the resin.
17. After thorough washing with technical grade DMF, store the bead library in HPLC-grade DMF at 4°C. Alternatively, the bead library can be washed thoroughly with water and stored in 0.1 *M* HCl or 0.1 *M* phosphate buffer with 0.05% sodium azide.

3.2. Synthesis of a Cyclic Peptide Library

The synthesis of a cyclic peptide library (disulfide bond formation) is essentially the same as that of the linear library except that Fmoc-Cys (Trt) is added at the carboxyl as well as amino terminus of the linear random peptide. After deprotection, add a mixture of DMSO/Anisole/TFA (*see* **Subheading 2.1., item 10**) into the resin; incubate overnight at room temperature. After thorough washing, store the library at 4°C as described above.

4. Notes

1. We have tested several commercially available resins for our library synthesis. The two satisfactory resins are TentaGel (polyethylene grafted polystyrene beads) and Pepsyn gel (polydimethylacrylamide beads). Overall, the TentaGel is preferable as it is nonsticky and mechanically more stable. However, unlike Pepsyn gel, the level of substitution of each TentaGel bead is far from uniform. With the advent of combinatorial chemistry, we anticipate newer resins entering the market in the near future.
2. TentaGel already has a long polyethylene linker and we do not routinely add additional linker for our library synthesis. In

contrast, a linker (preferably a hydrophilic linker) is necessary for the synthesis of a peptide library with polydimethylacrylamide beads. We have used Fmoc-β-alanine and/or Fmoc-aminocaproic acid as linkers in the past. However, aminocaproic acid is rather hydrophobic. A polyethyleneglycol-based amino acid (Shearwater, Polymers, Huntsville, AL) is probably preferable.

3. All glass vessels should be siliconized thoroughly prior to use. Besides using nitrogen bubbling through the randomization vessel to mix and wash the beads, we have also prepared libraries in hourglass reaction vessels (Peptides International, Louisville, KY), using rocking motion to mix the resins.
4. Each polypropylene reaction vial should be engraved with a letter corresponding to a specific amino acid to ensure no mix-up during the synthesis.
5. We often omit cysteines from the synthesis of linear peptide libraries to avoid the complication of intrachain and/or interchain crosslinking.

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