COMBINATORIAL PEPTIDE LIBRARY PROTOCOLS

METHODS IN MOLECULAR BIOLOGY™

Combinatorial Peptide Library Protocols

Edited by

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Preface

During the course of evolution, an imbalance was created between the rate of vertebrate genetic adaptation and that of the lower forms of living organisms, such as bacteria and viruses. This imbalance has given the latter the advantage of generating, relatively quickly, molecules with unexpected structures and features that carry a threat to vertebrates. To compensate for their weakness, vertebrates have accelerated their own evolutionary processes, not at the level of whole organism, but in specialized cells containing the genes that code for antibody molecules or for T-cell receptors. That is, when an immediate requirement for molecules capable of specific interactions arose, nature has preferred to speed up the mode of Darwinian evolution in preference to any other approach (such as the use of X-ray diffraction studies and computergraphic analysis).

Recently, Darwinian rules have been adapted for test tube research, and the concept of selecting molecules having particular characteristics from random pools has been realized in the form of various chemical and biological combinatorial libraries. While working with these libraries, we noticed the interesting fact that when combinatorial libraries of oligopeptides were allowed to interact with different selector proteins, only the actual binding sites of these proteins showed binding properties, whereas the rest of the protein surface seemed "inert." This seemingly common feature of proteins—having no extra potential binding sites—was probably selected during evolution in order to minimize nonspecific interactions with the surrounding milieu. From a practical point of view, it allows the user of combinatorial libraries to "fish out" the actual ligands.

Combinatorial Peptide Library Protocols endeavors to cover most of the basic techniques needed for the construction and use of peptide libraries. The initial chapters describe various methods for the chemical synthesis of peptide libraries and their use in the screening of various acceptor proteins for cognate ligands, as well as in the screening of enzymes for substrates and inhibitors. The intermediate chapters deal with biological libraries that are displayed over the coat protein of filamentous phage or on the surface of bacteria. The final chapters are devoted to some possible practical applications of combinatorial library technology.

I would like to thank and express my gratitude to the contributing authors, to Ephraim Katchalski-Katzir, Judith Heldman, Dina Zafriri, and the Rashi Foundation, who helped me through the process that gave birth to this volume. I dedicate this book to my parents, and to Orly, Yuval, Roni, and Itai.

Shmuel Cabilly

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Contents

1

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

Kit S. Lam and Michal Lebl

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 in 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

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positive peptide-beads are then physically isolated for microsequencing with an automatic protein sequencer. In this chapter, detailed methods for the synthesis of a random "one-bead one-compound" combinatorial peptide library will be described. Chapters 2 and 10 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*-hydroxybenzotriazole (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.