

[12] One Bead–One Compound Combinatorial Peptide Library: Different Types of Screening

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Introduction

The application of combinatorial technologies has become a significant trend in pharmaceutical research. The combinatorial discovery process consists of two parts: the generation of collections of molecular diversity, known as libraries, by systematic connection of different building blocks, and screening of such libraries with target molecules to identify novel compounds that interact with them.^{1–3} Thus, the combinatorial approach requires not only the design and construction of libraries of chemical diversity, but also the development of novel screening and assay methodologies for library evolution. Using a split synthesis method, peptide bead libraries can be synthesized such that each bead contains only one peptide species: the “one bead–one peptide concept.”^{4–6} The lead discovery approach based on such libraries is known as the “Selectide process.”^{3–6} This chapter describes three different protocols that have been successfully applied for the screening of such “one bead–one peptide” libraries: (i) to identify substrates for two protein kinases, (ii) to find specific peptide sequences that bind small organic molecules, and (iii) to optimize the biological potency of a known human thrombin inhibitor. According to the characteristics and objectives of each system, we used different approaches including *in situ* autoradiography, direct binding of organic dye, and enzyme-linked binding assay.

¹ E. M. Gordon, R. W. Barrett, W. J. Dower, S. P. A. Fodor, and M. A. Gallop, *J. Med. Chem.* **37**(9), 1251 (1994).

² E. M. Gordon, R. W. Barrett, W. J. Dower, S. P. A. Fodor, and M. A. Gallop, *J. Med. Chem.* **37**(10), 1385 (1994).

³ M. C. Desai, R. N. Zuckermann, and W. H. Moos, *Drug Dev. Res.* **33**, 174 (1994).

⁴ M. Lebl, V. Krchnák, N. F. Sepetov, B. Seligmann, P. Strop, S. Felder, and K. S. Lam, *Biopolymers (Pept. Sci.)* **37**, 177 (1995).

⁵ K. S. Lam, S. E. Salmon, E. M. Hersh, V. J. Hruby, W. M. Kazmierski, and R. J. Knapp, *Nature (London)* **354**, 82 (1991).

⁶ K. S. Lam and M. Lebl, *Methods: A Companion to Methods Enzymol.* **6**, 372 (1994).

Synthesis of Random Peptide Libraries

Synthesis of random peptide libraries is accomplished by standard solid-phase peptide synthetic methods.⁶⁻⁹ Fluorenylmethyloxycarbonyl (Fmoc) protecting group utilizing chemistry⁹ is especially convenient because of its easy implementation and low demand for special instrumentation. The split synthesis method^{15,10,11} can be performed manually, or sophisticated instrumentation may be utilized.¹²⁻¹⁴ The most critical aspect for the success of library synthesis is the selection of an appropriate solid carrier. The resin has to be compatible not only with solid-phase peptide synthetic methods (i.e., stable to base, trifluoroacetic acid, and organic solvents), but also with the aqueous conditions used during the screening process. Additional important qualities of the carrier include its homogeneity (size, substitution), nonstickiness (both to the surfaces and to the other beads), mechanical stability (fracturing of beads may cause problems during screening and analysis), and physical appearance (color changes are much more pronounced on clear smooth beads than on the rough and irregularly shaped ones). The commercially available TentaGel-S (polystyrene grafted with polyoxyethylene, Rapp Polymere, Germany)¹⁵ or polydimethylacrylamide resin (Pepsyn Gel) first described by Sheppard's group¹⁶ has been found to be satisfactory.

Synthesis of Libraries for Bead-Binding Screening

The TentaGel-S resin (0.2–0.4 mEq/g, 120 μm) is first divided into several aliquots, and a fourfold excess of a different Fmoc amino acid is then added to each aliquot of resin. The coupling reaction is initiated

⁷ R. B. Merrifield, *J. Am. Chem. Soc.* **85**, 2149 (1963).

⁸ J. M. Steward and J. D. Young, "Solid Phase Peptide Synthesis." Pierce Chemical Co., Rockford, IL, 1984.

⁹ E. Atherton and R. C. Sheppard, "Solid Phase Peptide Synthesis." IRL Press, Oxford, 1989.

¹⁰ R. A. Houghten, C. Pinilla, S. E. Blondelle, J. R. Appel, C. T. Dooley, and J. H. Cuervo, *Nature (London)* **354**, 84 (1991).

¹¹ A. Furka, F. Sebestyén, M. Asgedom, and G. Dibó, *Int. J. Pept. Protein Res.* **37**, 487 (1991).

¹² H. H. Saneii, J. D. Shannon, R. M. Miceli, H. D. Fischer, and C. W. Smith, *Pept. Chem.* **31**, 117 (1993).

¹³ Z. Barták, J. Bolf, J. Kalousek, P. Mudra, M. Pavlík, V. Pokorný, M. Rinová, Z. Voburka, K. Zenisek, V. Krchnák, M. Lebl, S. E. Salmon, and K. S. Lam, *Methods: A Companion to Methods Enzymol.* **6**, 432 (1994).

¹⁴ R. N. Zuckermann, J. M. Kerr, M. A. Siani, and S. C. Banville, *Int. J. Pept. Protein Res.* **40**, 497 (1992).

¹⁵ W. Rapp, L. Zhang, R. Habich, and E. Bayer, in "Peptides 1988: Proceedings of the 20th European Peptide Symposium" (G. Jung and E. Bayer, eds.), p. 199. de Gruyter, Berlin, 1989.

¹⁶ R. Arshady, E. Atherton, D. L. J. Clive, and R. C. Sheppard, *J. Chem. Soc. Perkin Trans. 1*, 529 (1981).

with the addition of benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP), *N*-hydroxybenzotriazole (HOBT), and diisopropylethylamine (DIEA) with gentle mixing. Alternatively, diisopropylcarbodiimide (DIC) and HOBT can be used for coupling. In the latter case, the coupling reaction can be monitored by incorporating a trace of bromphenol blue into the reaction mixture.¹⁷ The completion of the reaction is confirmed by the ninhydrin test.¹⁸ The ninhydrin test is used here to ensure that even the slowest couplings are complete. Since every single bead contains a different peptide sequence, it is important to analyze relatively large samples of the resin and to inspect individual beads instead of an average solution signal. On rare occasions, double or even triple coupling is needed to ensure the completion of each coupling reaction. After each coupling cycle, all the resin aliquots are mixed and washed. The *N*^α-Fmoc group is deprotected and the resin is washed and divided into several aliquots for the next cycle of synthesis. The same process is repeated until the desired length of the library is achieved. The *N*^α-Fmoc group is deprotected by treatment with 20% piperidine in dimethylformamide (DMF) (v/v), and side chain protecting groups are removed by addition of a mixture of trifluoroacetic acid/phenol/water/thioanisole/ethanedithiol (82.5:5:5:5:2.5, v/w/v/v/v).¹⁹

Determination of Substrate Specificities of Protein Kinases

Posttranslational modification of proteins is common. There are approximately 200 derivatized amino acids known to be naturally present in proteins. Examples of some common posttranslational modifications are phosphorylation, acetylation, methylation, sulfation, hydroxylation, glycosylation, ubiquitination, and prenylation. The recognition site for some of these modifications is based on a relatively short stretch of amino acid primary sequence. Conventional methods for the determination of such recognition sites are time-consuming and labor-intensive. The Selectide process offers a rapid alternative approach for the elucidation of the primary structures of these recognition sites. We have successfully applied this method in the identification of substrate motifs for two protein serine/threonine kinases (cAMP-dependent protein kinase and a plant-derived serine protein kinase) and one protein tyrosine kinase (p60^{c-src}). The method involves the phosphorylation of the peptide-bead library with [γ -³²P]ATP

¹⁷ V. Krchnák, J. Vágner, P. Safár, and M. Lebl, *Collect. Czech. Chem. Commun.* **53**, 2542 (1988).

¹⁸ E. Kaiser, R. L. Colescott, C. D. Bossinger, and P. I. Cook, *Anal. Biochem.* **34**, 595 (1969).

¹⁹ D. S. King, C. G. Fields, and G. B. Fields, *Int. J. Pept. Protein Res.* **36**, 255 (1990).

and the protein kinase of interest. The library is washed and immobilized by agar on a glass plate. The ^{32}P -labeled beads are then identified by autoradiography.²⁰⁻²²

In principle, the same general method can also be applied to posttranslational modifications other than phosphorylations.

Phosphorylation of Peptide-Bead Library by cAMP-dependent Protein Kinase^{20,21}

The peptide-bead library is first washed extensively with double-distilled water followed by MES buffer [30 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), 10 mM MgCl_2 , 0.4 mg/ml bovine serum albumin (BSA), pH 6.8]. The phosphorylation reaction is conducted in MES buffer containing 1.8 $\mu\text{g}/\text{ml}$ of cAMP-dependent protein kinase (catalytic subunit from bovine heart, Sigma Chemical Co., St. Louis, MO) and 0.1 μM [γ - ^{32}P]ATP (specific activity 25 Ci/mmol, ICN Biomedicals, Irvine, CA). Usually, 500,000 to 2 million beads are screened in each experiment. The final mixture is incubated at room temperature for 1 hr with gentle mixing. The ^{32}P -labeled beads are then washed thoroughly with a buffer containing 0.68 M NaCl, 10 mM KCl, 40 mM Na_2HPO_4 , 7 mM KH_2PO_4 , and 0.05% Tween 20 (v/v), pH 7.2. Most of the nonspecific binding of [γ - ^{32}P]ATP is removed by this high ionic strength buffer. Background is greatly reduced by heating the bead library to 100° in 1 M HCl for 5 min. This is most likely due to the complete hydrolysis of [γ - ^{32}P]ATP under these conditions. For protein tyrosine kinase screens, the bead library is sometimes heated to 58° in 1 M NaOH for 1 hr. This completely dephosphorylates phosphoserine and phosphothreonine residues, while leaving most phosphotyrosines intact. This step is particularly important if the protein tyrosine kinase preparation is contaminated with undesirable serine/threonine protein kinase activities.

Detection and Isolation of ^{32}P -Labeled Beads

After thorough washing, the ^{32}P -labeled bead library is suspended in 5–20 ml (500,000 to 2 million beads) of agarose solution (1.5%, w/v) (Sea Kem LE agarose, FMC BioProducts, Rockland, ME) at 70–75°. The suspension is then carefully poured onto a clean glass plate (16 × 18 cm) and air-dried at room temperature overnight. Each glass plate can hold approximately 5 ml of the bead suspension. In order to facilitate alignment of

²⁰ J. Wu, Q. N. Ma, and K. S. Lam, *Biochemistry* **33**, 14825 (1994).

²¹ K. S. Lam and J. Wu, *Methods: A Companion to Methods Enzymol.* **6**, 401 (1994).

²² K. S. Lam, J. Wu, and Q. Lou, *Int. J. Pept. Protein Res.* **45**, 587 (1995).

the immobilized beads with the autoradiogram, Glogas II autoradiogram markers (Stratagene, La Jolla, CA) are taped to the corners of each glass plate. The glass plates with the dried agarose-embedded beads are then exposed to an X-ray film (X-OMAT LS, Kodak, Rochester, NY) for 20–30 hr at room temperature, and the film is then developed. The area of embedded beads corresponding to the dark spots on the autoradiogram is carefully excised with a razor blade. The excised beads/agar is then added to 5 ml of hot agarose solution (1.5%, w/v) at 75°. The resuspended beads are then plated, dried, and exposed to X-ray film as described before. Because the beads are now greatly diluted, single beads corresponding to dark spots on the autoradiogram can easily be located. Under a dissecting microscope, 30 μ l double-distilled water is added to swell the agar surrounding the 32 P-labeled bead. The bead is then dislodged with a 27-gauge needle attached to a syringe, removed with a micropipette, washed several times with double-distilled water, transferred onto a glass-fiber filter, and inserted into a protein sequencer (Model 477A, Applied Biosystems, Foster City, CA) for structure determination. Approximately 20–80 pmol amino acid/cycle should be recovered.

Screening for Peptides that Bind Small Organic Molecules

The Selectide process has been proven to be an invaluable tool for the identification of binding ligands to various macromolecular targets. The same method has been applied for the identification of ligands for small organic molecules such as organic dye.^{23,24} Organic dye was chosen as the model system because the probe is intrinsically colored, which greatly simplifies the screening process. In principle, the same general method can be applied to any molecules with intrinsic fluorescent property or that are radioactive. Fluorescent microscopy, fluorescence-activated cell sorter, or autoradiography can be used to identify the positive beads.

Identification of Linear Peptides that Interact with Indigo Carmine

The peptide–bead library is first washed thoroughly with double-distilled water followed by phosphate-buffered saline (PBS) with 0.1% Tween 20 (v/v). Indigo carmine is then added to the bead library to a final concentration of 10 μ M in 2 \times PBS with 0.1% Tween 20 (v/v) and poured into several petri dishes (10 \times 2 cm). After 1 to 2 hr, some beads will turn deep turquoise in color. There is no need to remove the dye from the incubating

²³ K. S. Lam, Z. G. Zhao, S. Wade, V. Krchnák, and M. Lebl, *Drug Dev. Res.* **33**, 157 (1994).

²⁴ H. Wennemers and W. C. Still, *Tetrahedron Lett.* **35**, 6413 (1994).

mixture as the background color is extremely light. The colored beads are then isolated under a dissecting microscope with a micropipette, washed thoroughly with double-distilled water, and microsequenced as described earlier.

High concentrations of salt (0.26 M NaCl) and nonionic detergents (Tween 20) are included in the incubation buffer to minimize the nonspecific ionic and hydrophobic interactions, respectively. Because the resin is fully compatible with organic solvent, the screening can also be performed under organic solvent conditions.

Selecting High-Affinity Protease Inhibitors

The process of blood coagulation involves a series of complex steps terminating in the formation of a fibrin clot.²⁵⁻²⁸ With each step in the coagulation sequence, the system is amplified to produce increasing numbers of activated coagulation molecules (serine proteases), resulting in the generation of thrombin, the enzyme that converts fibrinogen to fibrin.²⁶ Normally, *in vivo* coagulation is modulated by several efficient protective mechanisms, one of which is the neutralization of activated coagulation factors by naturally occurring protease inhibitors and anticoagulants.^{29,30} Screening combinatorial peptide libraries with the activated coagulation factors can effectively and rapidly select high-affinity inhibitors of these factors, generating and optimizing lead compounds for treating coagulation disorders. As an example, we have successfully used an enzyme-linked binding assay to improve the potency of the known peptide inhibitor (D-Phe-Pro-Arg-Pro-Gly) of thrombin about 1600-fold (based on our kinetic chromogenic assay). The screening protocol described here involves binding of streptavidin-alkaline phosphatase (SAP) conjugate complexed with biotinylated thrombin to the peptide library, and identification of the thrombin-binding beads by alkaline phosphatase cleavage of 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt (BCIP) and precipitation of its blue insoluble product 5,5'-dibromo-4,4'-dichloro indigo on the surface of these beads. In order to obtain a high-affinity specific inhibitor of thrombin, we developed consecutive steps of staining, which include varying concen-

²⁵ E. W. Davie and K. Fujikawa, *Annu. Rev. Biochem.* **44**, 799 (1975).

²⁶ E. W. Davie, K. Fujikawa, K. Kurachi, and W. Kisiel, *Adv. Enzymol.* **48**, 277 (1979).

²⁷ K. Fujikawa, M. H. Coan, M. E. Legaz, and E. W. Davie, *Biochemistry* **13**, 5290 (1974).

²⁸ R. D. Rosenberg, *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **36**, 10 (1977).

²⁹ P. C. Harpel, R. W. Colman, J. Hirsh, V. J. Marder, and E. W. Salzman, "Basic Principles and Clinical Practice." Lippincott, Philadelphia, 1987.

³⁰ W. Kisiel, W. M. Canfield, L. H. Ericsson, and E. W. Davie, *Biochemistry* **16**, 5824 (1977).

trations of thrombin and using known contact site thrombin inhibitor hirudin as competitor during the screening process.

In addition to the thrombin inhibitors, we have also been able to identify high-affinity inhibitors of other coagulation serine proteases (not described here) using similar methods.

Biotinylation of Human α -Thrombin

Approximately 5 mg of human α -thrombin (Enzyme Research Labs, South Bend, IN) is dialyzed exhaustively against 100 mM NaHCO₃ buffer (pH 9.0) at 4°. Right before starting biotinylation, about 1 mg of immunopure NHS-LC-biotin (Pierce, Rockford, IL) is dissolved in double-distilled water to a final concentration of 10 mM. The NHS-LC-biotin solution is then mixed with the dialyzed thrombin at a molar ratio of 1.5 to 1 and is incubated at room temperature for 1 hr with gentle shaking. The reaction mixture is then loaded onto a preequilibrated Sephadex G-25 (Sigma Chemical Co., St. Louis, MO) gel-filtration column and eluted with pH 6.5 citrate buffer [50 mM citric acid, 200 mM NaCl, 0.05% polyethylene glycol (PEG) 8000 (w/v)]. To determine the distribution of thrombin in the eluant, 10 μ l from each collected fraction is mixed with either 100 μ l BCA (protein assay kit, Pierce) working reagent or 100 μ l of 150 μ M S2366 (thrombin substrate, Chromogenix, Molndal, Sweden) and absorbances are obtained at 650 or 405 nm, respectively. The fractions with the highest protein content and thrombin activity are combined and dialyzed against elution buffer overnight. This biotinylated thrombin stock is then aliquoted and stored at -80°.

Primary Staining of Library Beads

Approximately 3 g (9 million) of library beads (D-Phe-Pro-Arg-Pro-XXXXX-linker-TentaGel, X stands for randomization of 19 L-amino acids, excluding cysteine) is used for screening. Beads are transferred into 50-ml chromatography columns and are washed extensively with double-distilled water followed by HSB-B-T [50 mM HEPES, 800 mM NaCl, 0.05% PEG 8000 (w/v), 0.1 mg/ml BSA, 0.25% Tween 20 (v/v), 0.02% NaN₃ (w/v), pH 7.4]. Beads are then washed three times with QHSBB-T (HSBBT diluted 1:4) and are incubated with QHSBB-TG [QHSBB-T with 0.05% gelatin (w/v)] for 1 hr on a Nutator (Model 1105, Clay Adams, Division of Becton Dickinson, Parsippany, NJ). This blocking step minimizes the nonspecific binding of proteins to the surface of the beads. After blocking, the beads are washed once with QHSBB-TG and incubated with 120 nM biotinylated thrombin for 2 hr at 4° on a Nutator. The incubation is conducted at low temperature so that possible cleavage of on-bead substrate sequences by

thrombin is minimized. The beads are then washed three times with QHSBB-TG to remove the excess and nonspecifically bound biotinylated thrombin. The next step is to incubate the washed beads with 8.6 nM SAP (Pierce) in QHSBB-TG at 4° for 1 hr with the same gentle mixing. To remove the excess SAP, the beads are washed three times with HSBB-T followed by three times with staining buffer (250 mM Tris-base, 250 mM NaCl, 6 mM MgCl₂, pH 8.5). Beads are mixed quickly with 0.165 mg/ml BCIP (Pierce) in the just-described staining buffer and aliquoted into large (100 × 15 mm) polystyrene petri dishes to monitor the color deposition on the beads. One petri dish can hold about 20 ml of liquid and a single layer of beads is distributed in each petri dish to facilitate picking of stained beads. Under ideal conditions, the stained beads (shown as blue/turquoise color) should be clearly visible within 1 hr. This color development step is generally stopped within 2 to 3 hr by removing the BCIP-containing solution and washing the beads three times with 0.01% HCl (w/v). It is important that the time of color development is recorded and that each subsequent staining follows the same time to get comparable results. Evenly stained beads are picked up with the aid of a dissecting microscope and a micropipette and are stored in 0.01% HCl at 4° before the next staining.

Confirmation of Staining

The primary stained beads are sonicated in a 1.7-ml microcentrifuge tube with 8 M guanidine hydrochloride (Amresco, Solon, Ohio) twice for 15 min each time to remove the bound proteins. This is the stripping step. Destaining is done by vortexing the beads at low speed in dimethyl formamide (Baxter Scientific, McGaw Park, IL), spinning in a microcentrifuge, and removing the supernatant. This process is repeated until the beads are colorless. The washing and blocking steps are carried out as before. While the beads are being blocked, the binding-signaling complex is prepared by incubating 200 nM biotinylated thrombin with 200 nM SAP and 600 nM biotin at room temperature for 1 hr. Upon completion of blocking, the mixture is diluted 1:20 in QHSBB-TG and used for incubation with the beads for 2 hr at 4°. The beads are then washed and stained in BCIP as described earlier. The blue and nonblue beads are separated by picking out the one kind with fewer number of beads with a micropipette. The blue beads are the ones that have been "confirmed" of binding to the thrombin/alkaline phosphatase complex and are stored in 0.01% HCl at 4° before the next step.

Competition with Hirudin

In order to determine that the sequences on the selected blue beads are specific binders to thrombin molecule, we use recombinant hirudin

(American Diagnostica Inc., Greenwich, CT) as the competitor for further selection. Hirudin is a polypeptide of about 66 amino acids originally derived from the salivary gland of the medicinal leech (*Hirudo medicinalis*). It has been used as an anticoagulant and interacts tightly with α -thrombin to form a remarkably stable noncovalent complex with a dissociation constant at about $2 \times 10^{-14} M$.^{31,32} Several studies suggest that hirudin interacts not only with the catalytic site of thrombin, but also with a distant fibrinogen-binding site on the α -thrombin molecule.³²⁻³⁴ To perform the competition staining, the blue beads from the last step are stripped, decolorized, and blocked as described earlier. After blocking, 50 nM hirudin is included in the biotinylated thrombin/SAP-binding complex solution to incubate with the beads. At the end of BCIP staining, the competed beads that show no staining are separated from the blue ones with a micropipette.

Selection of High-Affinity Thrombin-Binding Sequences

In order to select on-bead peptides with high affinities to thrombin, the 0.5 nM biotinylated thrombin/0.5 nM SAP/1.5 nM biotin complex is used to probe the beads competed by hirudin (colorless ones) according to the binding/staining protocol described. A total of four beads are stained at this concentration of thrombin and are picked up, washed several times with 0.01% HCl, and microsequenced as described earlier. Two complete and two partial sequences are obtained.

Chromogenic Assay of Thrombin Inhibition by Selected Sequences

The two complete on-bead sequences are resynthesized as free peptides. The peptides are dissolved in dimethyl sulfoxide, serially diluted in chromogenic assay buffer [50 mM Tris, 200 mM NaCl, 0.05% PEG 8000 (w/v), 10 mM CaCl₂, 0.02% NaN₃ (w/v), pH 7.8], and assayed in a half-area microtiter plate (Costar, Cambridge, MA) against 0.5 nM thrombin and 400 μM chromogenic substrate S2366 at a 405-nm wavelength on a microtiter plate reader (Ceres UV900 Hidi, Bio-tek Instruments Inc., Winooski, VT). The IC₅₀ values are derived from the kinetic plots and K_i values are calculated according to the K_m and concentration of S2366. One of the selected peptides, D-Phe-Pro-Arg-Pro-Phe-Gly-Tyr-Arg-Val- β Ala, showed an apparent K_i of 25 nM to thrombin. Thus, roughly 1600-fold improvement over the original D-Phe-Pro-Arg-Pro-Gly peptide ($K_i = 40 \mu M$) has been achieved through our enzyme-linked screening.

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³² S. R. Stone, P. J. Braun, and J. Hofsteenge, *Biochemistry* **26**, 4617 (1987).

³³ P. J. Braun, S. Dennis, J. Hofsteenge, and S. R. Stone, *Biochemistry* **27**, 6517 (1990).

³⁴ J. Y. Chang, P. K. Ngai, H. Rink, S. Dennis, and J. M. Schlaeppi, *FEBS Lett.* **261**, 287 (1990).