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Application of a dual color detection scheme in the screening of a random combinatorial peptide library

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Abstract

Selectide Technology is a random synthetic combinatorial library method in which millions of random compounds are screened in parallel for their ability to bind to a tagged macromolecular target. The library consists of millions of beads and each individual bead expresses a unique chemical compound such as a peptide. In the standard enzyme-linked colorimetric detection scheme, the positive bead which turns color is isolated for microsequencing. In this paper, a dual color detection scheme using two sequential orthogonal probes is described. This dual color system enables one to rapidly differentiate false positive beads from true positive beads, resulting in a much more efficient use of the microsequencer.

Keywords: Combinatorial library; Selectide; Peptide; Screening; Substrate; (Color)

1. Introduction

Random combinatorial library methods have proven to be an invaluable tool in the identification of ligands for various macromolecular targets (Moos and Green, 1993; Lam, 1994). These methods not only facilitate the drug discovery process, but also provide extremely important information for the understanding of molecular recognition. In general there are three methods used to generate and screen huge peptide libraries (e.g., 10^6–10^8): (i) biologic peptide library systems such as filamentous phage (Scott and Smith, 1990; Cwirla et al., 1990; Devlin et al., 1990) and plasmid (Schatz, 1993), (ii) combinatorial peptide library with iterative process (Geysen et al., 1986; Houghten et al., 1991) or positional scanning (Dooley and Houghten, 1993), and (iii) combinatorial peptide libraries using the 'one bead-one peptide' concept, the Selectide process (Lam et al., 1991,1993a,1993b; Lam, 1994). The first method relies on a biologic system and therefore only L-amino acid ligands can be isolated. In

Abbreviations: BCIP, 5-bromo-4-chloro-3-indolyl phosphate; BOP, benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium-hexa-fluorophosphate; DIEA, diisopropylethylamine; DMF, dimethylformamide; Fmoc, 9-fluorenylmethoxy carbonyl; NBT, nitroblue tetrazolium; HOBt, *N*-hydroxybenzotriazole

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contrast, since synthetic chemistry is used in the latter two methods, unnatural amino acids (Lam et al., 1993b) or even non-amino acid chemical subunits can be incorporated into the libraries (Bunin and Ellman, 1992; Nikolaiev et al., 1993; Cho et al., 1993; Lebl et al., 1994; Chen et al., 1994).

In the Selectide process, with a 'split synthesis' method, the random combinatorial library is synthesized such that each individual bead expresses only one chemical entity although there are many copies ($>10^{13}$) of the same entity in and on the surface of each bead. In our standard screening method, the peptide library (10^6–10^8 beads) is then incubated with an acceptor-enzyme complex (e.g., acceptor-alkaline phosphatase conjugate). The acceptor molecule could be an antibody, biologic receptor, enzyme, adhesion molecule, or other macromolecular target. After thorough washing, the beads are then mixed with the substrate (e.g., BCIP plus NBT); the positive beads then turn purple and subsequently are isolated for microsequencing (Lam et al., 1991; Lam and Lebl, 1992). The screening of such a library usually takes less than a day. The rate limiting step, however, is the microsequencing step. With the standard microsequencer, about 3–4 heptapeptides can be sequenced every 24 h. Therefore, it is extremely important to eliminate the false positive beads prior to submitting to microsequencing. Besides using the general method of incorporating blocking agents (such as gelatin, BSA, or powdered milk), non-ionic detergents and high concentrations of salt (e.g., 0.26 M) into the incubation buffer in order to reduce non-specific staining, we have developed a dual color orthogonal screening method that enables us to identify and eliminate false positive staining beads prior to microsequencing.

2. Method

2.1. Preparation of random peptide library

The random peptide library was synthesized as described elsewhere (Lam et al., 1991; Lam and Lebl, 1992, 1994). Standard Fmoc chemistry (Atherton and Sheppard, 1988) and a 'split synthesis' method (Lam et al., 1991; Furka et al., 1991) were used in the preparation of the library. In brief, TentaGel S (Rapp Polymere, Tübingen, Germany) was divided into 19 aliquots. Four-fold excess of Fmoc-amino acids (all 18 eukaryotic L-amino acids except cysteine, plus glycine) were then added individually and separately to each aliquot of resin. Coupling was initiated by the addition of BOP, HOBt and DIEA. The coupling reaction was monitored by the ninhydrin test. Occasionally, double coupling was required. After completion of coupling, all the aliquots of beads were mixed, washed 5 × with DMF, $N\alpha$-Fmoc deprotected by 20% piperidine in DMF (v/v), washed 5 × with DMF, and the beads were divided into several aliquots again. The coupling cycle was then repeated until a peptide library of the desired length was completed. The side chain protecting groups were then removed by a mixture of trifluoroacetic acid-phenol-anisole-ethanedithiol (94:2:2:2, v/w/v/v). The pentapeptide library used in this experiment has the following structure: $XXXXX\beta\varepsilon$-TentaGel S (where X = all 18 eukaryotic L-amino acids except for cysteine, plus glycine $\beta = \beta$-alanine, and $\varepsilon = \varepsilon$-aminocaprioic acid). During the mixing and deprotection steps, the beads were thoroughly washed (10 ×) and mixed. Provided that the beads are distributed equally in each coupling step, the statistical distribution of an individual peptide is based on the Poisson Distribution and the number of possible peptides actually synthesized is determined and limited by the total number of beads present in the synthesis.

2.2. Screening of the library with a dual color detection scheme

The screening process was performed at room temperature. The library (approximately 500 000 beads) was first washed extensively with double distilled water, blocked with 0.05% gelatin for an hour, and washed with PBS-Tween (PBS with 0.1% Tween 20 (v/v) where PBS = 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, and 2.7 mM KCl, pH 7.2). The library was then mixed

with biotinylated anti-β-endorphin monoclonal antibody (4 ng/ml, clone 3E7, Boehringer Mannheim Biochemicals, Indianapolis, IN) in 2 × PBS with 0.05% Tween 20 (v/v) and 0.1% gelatin (w/v) for 2 h. The library was then washed with PBS-Tween and incubated with a 1:200000 dilution of streptavidin-alkaline phosphatase at 2 mg/ml (Pierce, Rockford, IL) for 1 h. The library was then washed thoroughly with PBS-Tween followed by two washes with 2 × PBS and one wash with TBS (2.5 mM Tris, 13.7 mM NaCl, and 0.27 mM KCl, pH 8.0). The beads were then placed in several 12 cm petri dishes with BCIP (substrate 1: 1.65 mg BCIP in 10 ml of 0.1 M Tris-HCl, 0.1 M NaCl with 2.34 mM $MgCl_2$, pH 9.0). After 1 h or so, approximately 100 light, and 65 medium to deep turquoise beads were visualized. Under a dissecting microscope, with the aid of a micropipette, the 65 darker beads were transferred one by one to a small petri dish. These positive beads were the result of either binding to the anti-β-endorphin, the streptavidin, or the alkaline phosphatase.

These 65 turquoise beads were then incubated with 6 M guanidine HCl, pH 1.0 for 15 min to strip the streptavidin-alkaline phosphatase or the antibody off the beads. These beads were washed and subjected to a second screening with biotinylated anti-β-endorphin monoclonal antibody as before for 2 h. These beads were then washed with PBS-Tween. Instead of using streptavidin-alkaline phosphatase conjugate, polyclonal goat anti-mouse Ig-alkaline phosphatase conjugate at 1/10000 dilution (Bio-Rad Laboratories, Richmond, CA) was used in this secondary step. After 1 h, the beads were washed and placed in the petri dish with a combination of BCIP and NBT (substrate 2: 0.66 mg NBT and 1.65 mg BCIP in 10 ml of 0.1 M Tris-HCl, 0.1 M NaCl, with 2.34 mM $MgCl_2$, pH 9.0). After 15–30 min, one-third of these beads turned purple while the rest remained turquoise in color. Eight of the deep turquoise beads and ten of the darkest purple beads were then treated with 6 M guanidine HCl, pH 1.0, washed with double distilled water and loaded individually into a protein sequencer (Model 477A, Applied Biosystems) for structure determination.

3. Results and discussion

Fig. 1 summarizes the dual color screening method described above. The result of the micro-sequencing is shown in Table 1. As expected, all the turquoise beads stained by the first but not by the second step have an HPQ motif, indicating they all bind to streptavidin. On the other hand, all the dark purple beads stained by both the first and the second step showed a YG-F sequence, the binding motif for anti-β-endorphin (Lam et al., 1991,1993a,1993b; Cwirla et al., 1990).

Our results can easily be explained by the schematic shown in Fig. 1. The beads that remain turquoise throughout the two staining steps bound to the secondary streptavidin-alkaline phosphatase complex in the first step and when BCIP was added, water insoluble indigo was formed and deposited on the surface of the beads, turning them turquoise in color. However, in the second step, these beads bound to neither the primary nor secondary reagents and therefore no new color deposit (purple) was formed. Upon

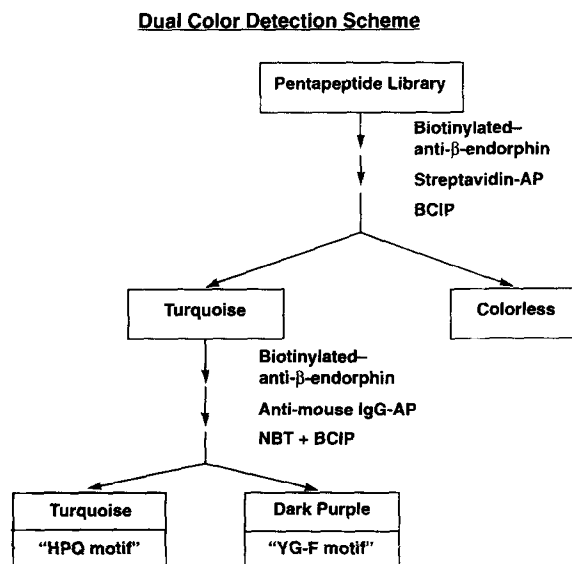

Fig. 1. Schematic summarizing the dual color detection method. After a two-step staining process, one can differentiate the streptavidin-binding beads ('HPQ motif') from the anti-β-endorphin binding beads ('YG-F motif'). AP = alkaline phosphatase, BCIP = 5-bromo-4-chloro-3-indolyl phosphate, NBT = nitroblue tetrazolium. (Please refer to the text for a detailed explanation.)

Table 1
Amino acid sequence of the peptides identified from the dual color screening system

Turquoise [a]	Purple [b]
WHPQV	YGAFF
WHPQG	YGWFN
IHPQF	YGQFV
WLHPQ	YGYFE
WEHPQ(2) [c]	YGVFI
WMHPQ	YGGFT
WAHPQ	YGVFE
	YGGFI
	YGWFQ

[a] The turquoise beads are streptavidin-specific with the HPQ motif.
[b] The purple beads are anti-β-endorphin-specific with the YG-F motif.
[c] WEHPQ was detected on two occasions.

microsequencing, all these beads showed HPQ, which is known to be a ligand motif for streptavidin (Lam et al., 1991; Lam and Lebl, 1992; Devlin et al., 1990). As for the beads that turned turquoise in the first step and dark purple in the second step, this can be explained by their binding to the primary biotinylated-anti-β-endorphin MoAb in both steps. In the presence of secondary streptavidin-alkaline phosphatase in the first step, indigo was formed and these beads turned turquoise. However in the second step, when anti-mouse IgG-alkaline phosphatase conjugate was used, this secondary reagent also bound to the anti-β-endorphin MoAb that had already bound these beads and therefore when BCIP plus NBT was used, indigo was formed which in turn reduced the NBT to an intensively dark purple formazon precipitate deposited on the surface of these beads. The combination of BCIP and NBT as a substrate is very sensitive and the color reaction can usually be completed in a much shorter time. As expected, microsequencing of all these dark purple beads showed YG-F, which is a known ligand motif for anti-β-endorphin (Cwirla et al., 1990; Lam et al., 1991, 1993a, b). Since alkaline phosphatase is present in both the first and second steps of screening, in principle, peptide beads that are specific for alkaline phosphatase should also turn purple. However, at the concentration of alkaline phosphatase used, we have never detected any alkaline phosphatase-specific peptides. It is conceivable that even if specific peptides for the enzyme active site of alkaline phosphatase are present in the library, the bound enzyme would be inactivated as the enzyme active sites are occupied by the bound peptide, resulting in no color change of the beads.

These results clearly illustrate that with a simple dual color detection scheme using two orthogonal secondary reagents and two color substrate systems (BCIP alone or BCIP + NBT), one can easily differentiate the false positive beads (in this case the HPQ) from the true positive beads (in this case the YG-F). This can save the valuable time of the protein microsequencer which is usually the rate-limiting step of the Selectide process.

The experimental scheme described in this paper is just one example. There are numerous different variations one can use. For example, the dual color staining in the presence or absence of a competing soluble ligand can allow one to differentiate specific binding to the ligand binding site of the macromolecular target from other non-specific binding. Besides using the BCIP and BCIP/NBT color systems described here, one can employ other color substrates such as 5-bromo-6-chloro-3-indolyl phosphate (magenta color) or 6-chloro-3-indoyl-phosphate (salmon color) (available commercially from Biosynth, Switzerland). Other tetrazolium salts, such as 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyl-2*H*-tetrazolium chloride (INT), may also be used, and when coupled with BCIP will turn reddish brown. In addition, one may even use a combination of different enzyme systems (e.g., alkaline phosphatase, horseradish peroxidase, glucose oxidase) and their corresponding multicolor substrates. Some of these substrate systems have already been applied to multicolor Western blotting or immunohistochemical staining. Similarly, if one decides to use a fluorescent probe to screen bead-libraries, one can easily apply fluorescent probes with a variety of different wavelengths.

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