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IDENTIFICATION OF ANTIBODY MIMOTOPES CONTAINING NON-NATURAL AMINO ACIDS BY RECOMBINANT AND SYNTHETIC PEPTIDE LIBRARY AFFINITY SELECTION METHODS

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ABSTRACT: A 512-component synthetic hexapeptide library containing non-natural amino acids was generated based on a mimotope obtained from screening a diverse bacteriophage library with an anti-gp120 monoclonal antibody. Four peptides were identified by affinity selection with ≤ 2 μM IC_{50}s, three of which contained non-natural amino acids.

The discovery of novel ligands in drug screening programs has been greatly facilitated by the advent of biological and chemical methodologies for generating and screening complex peptide libraries (1-8). The use of recombinant peptide library technologies to generate a lead compound, followed by synthetic methodologies directed towards optimization (e.g., the use of non-natural amino acids), is perhaps an ideal route to obtaining peptides of desirable binding and stability properties. We report here the use of a *synthetic* hexapeptide library containing non-natural amino acids to investigate an antibody mimotope (9) originally discovered by screening a diverse bacteriophage library.

Hexamer mimotopes of an anti-gp120 monoclonal antibody were obtained by performing two rounds of affinity selection with a diverse (1×10^8 clones) bacteriophage library (10). DNA sequencing of 120 randomly chosen clones revealed two consensus sequences: ESTRPM and PCCRAF. These sequence motifs differ greatly from the original decapeptide epitope: RAFHTTGRII. The binding properties of these two sequences were verified by their chemical synthesis as free hexapeptides, followed by ELISA evaluation. The IC_{50} values were 630 nM for both the ESTRPM and PCCRAF (oxidized form) peptides. The ESTRPM sequence was chosen for affinity optimization studies, as opposed to PCCRAF, because of complications due to disulfide exchange in a mixture of cysteine-containing peptides.

The critical binding residues of the ESTRPM peptide were determined by systematically replacing each amino acid in the parent sequence with alanine. Competition ELISA assays of these six analogues indicated a ≥ 10-fold loss in affinity for four peptides: EATRPM, ESARPM, ESTAPM and ESTRPA (Table 1). The glutamic acid and proline positions were the least affected by alanine substitution, showing a ≤ 3-fold loss in affinity.

TABLE 1: CRITICAL RESIDUE DETERMINATION OF THE *ESTRPM* MIMOTOPE

Peptide	IC_{50} (μM)[b]	Loss of affinity
ESTRPM[a]	0.63	--
ASTRPM	1.80	3x
E**A**TRPM	6.30	10x
ES**A**RPM	6.30	10x
EST**A**PM	10.0	16x
ESTR**A**M	0.63	--
ESTRP**A**	10.0	16x

[a]Sequence of the original mimotope obtained from the affinity selection of a bacteriophage hexapeptide library.
[b]Determined by competition ELISA on the synthetic N-acetylated and C-amidated free peptides.

Synthetic peptide pools were then generated by conservative substitution of the four critical residues of the ESTRPM peptide (Figure 1). These replacements, which included some non-natural amino acids (11), were simultaneously made at each of these four positions ($EX_1X_2X_3PX_4$) to investigate other possible *combinations* of residues that bind. The X_1 and X_2 positions were each substituted with four small neutral hydrophilic residues, X_3 was substituted with four positively charged residues and X_4 was substituted with eight hydrophobic residues. Synthesis was performed using a resin-splitting algorithm (6-8, 12) on an automated peptide mixture synthesizer (13) which generates mixtures of *equimolar* composition. A library of 512 peptides was synthesized by holding the N-terminal X_1 and X_2 positions constant, thereby generating 16 pools of 32 peptides each. IC_{50} values of these free peptide mixtures were determined by a solution-phase competition ELISA. The IC_{50} values are reported as the concentration of *each* component in order to normalize for the pool's diversity (32 components) and thus represents an upper limit for the affinity of any *one* component. ELISA assays of the 16 peptide pools indicated that only 1 pool had an $IC_{50} \leq 1$ μM (Figure 2). The $EX_1X_2X_3PX_4$ pool where $X_1 = S$ and $X_2 = T$ had the greatest overall

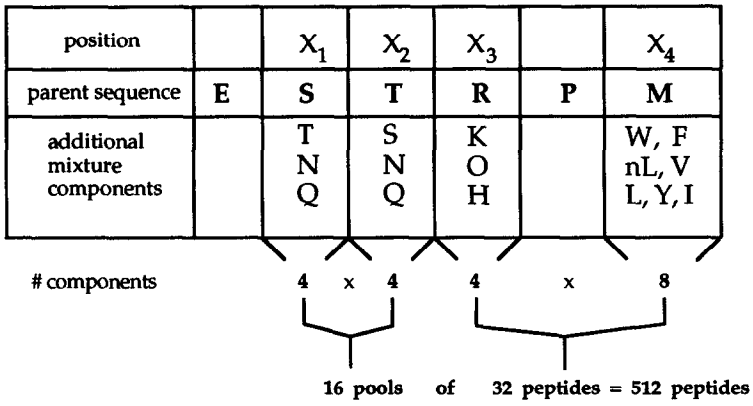

Figure 1. A library of 512 synthetic peptides was generated as 16 pools of 32 peptides by simultaneously substituting each critical residue (X_{1-4}) of the ESTRPM parent sequence with a variety of chemically similar amino acids.

affinity with an IC_{50} value of 400 nM, and contains the parent ESTRPM sequence (IC_{50} = 630 nM). The IC_{50} values are reported as the concentration of *each* component in the 32-fold degenerate mixture; therefore, the affinity of any one component could range from 400 nM to 13 μM (32 x 400 nM). The second best $EX_1X_2X_3PX_4$ pool where X_1 = N and X_2 = T had an affinity of 1.5 μM; therefore, the affinity of any one component in this pool could range from 1.5 μM to 48 μM.

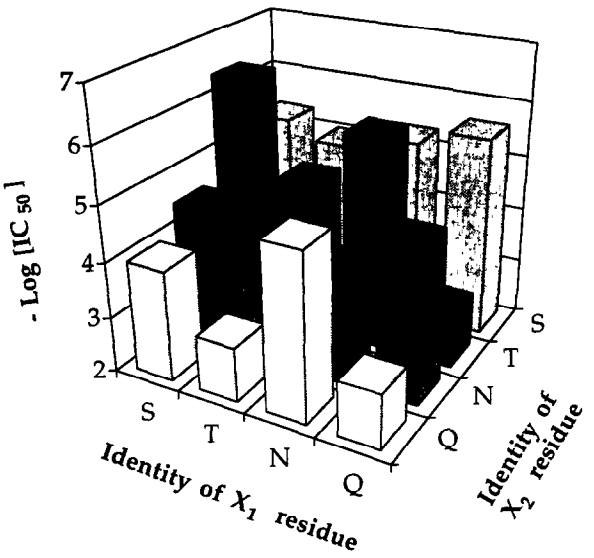

Figure 2. Competitive inhibition of the antibody-gp120 interaction by the 16 $EX_1X_2X_3PX_4$ pools. Each pool contains 32 peptides and the IC_{50}'s reported indicate the concentration of *each* peptide component. Each pool has a different combination of residues at X_1 and X_2, and the same combination of residues at X_3 and X_4. The most inhibitory pool was X_1 = S, X_2 = T.

The identification of the highest affinity peptide(s) in the ESTX$_3$PX$_4$ pool was then determined by affinity selection (8, 14) with a monoclonal antibody. This technique involved incubating the pool with a 10-fold molar excess of *each* peptide over the antibody to ensure competition between ligands. The high-affinity peptide/antibody complex was then separated from the excess peptide pool by gel filtration, and the bound peptide(s) isolated by HPLC. Analytical reversed-phase HPLC (15) of the ESTX$_3$PX$_4$ pool before and after affinity selection is shown in Figure 3. The affinity selected pool was substantially enriched in only four peptide components. These were identified as ESTRPM, ESTRPnL,

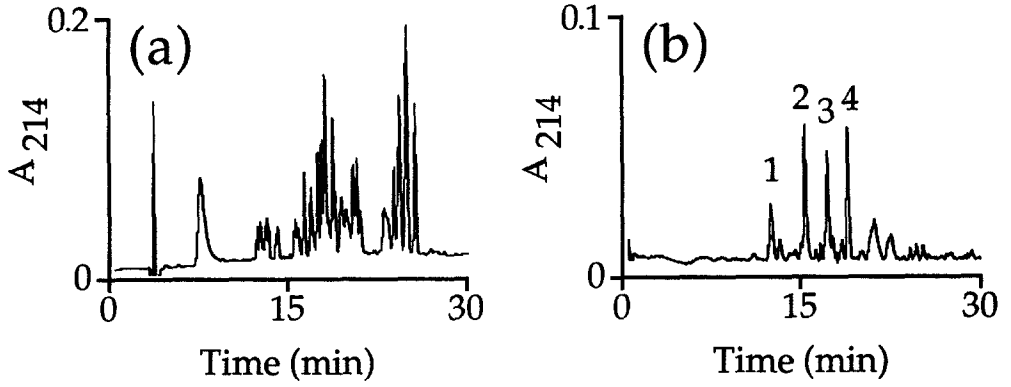

Figure 3. Affinity selection experiment of the highest affinity pool: ESTX$_3$PX$_4$. Analytical reversed-phase HPLC analysis of (a) the 32 component peptide mixture, and (b) the same mixture after affinity selection with an anti-gp120 monoclonal antibody.

ESTOPM and ESTOPnL by mass spectrometry and amino acid analysis. The binding properties of these analogues were verified by their chemical synthesis as free peptides, followed by competition ELISA evaluation (Table 2). The parent sequence ESTRPM had the greatest affinity (630 nM), however, three new analogues were discovered that contained non-natural amino acids, one of which had submicromolar affinity: ESTRPnL. Although conservative amino acids were chosen for substitution at each residue, the antibody only selected a small subset of all possible combinations. Only two of the possible eight hydrophobic residues at the C-terminal position were accepted: Met and nLeu. Similarly, only ornithine was accepted in place of arginine at the positively charged X$_3$ position.

TABLE 2: AFFINITIES OF PEPTIDES IDENTIFIED BY AFFINITY SELECTION FROM THE ESTX3PX4 POOL

Peak #	X_3	X_4	IC_{50} (μM)
1	O	M	1.5
2	R	M	0.63
3	O	nL	2.0
4	R	nL	0.80

In summary, we investigated 512 mimotope analogues by a quantitative solution-phase screening/selection method. Only two peptides were found to bind with submicromolar affinities, the original ESTRPM mimotope and an analogue containing a non-natural amino acid: ESTRPnL. The fact that the original mimotope was the highest affinity analogue suggests that the bacteriophage-peptide affinity selection experiments were successful in selecting the best peptide of the ESTRPM motif. The screening of a recombinant bacteriophage library to discover novel ligands, followed by optimization with a synthetic peptide library containing non-natural amino acids, therefore, represents a powerful tool for the discovery of non-natural protein ligands.

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14. The anti-gp120 monoclonal antibody (1.0 nmol, IgG, Chiron #26-8-F8-E3) was incubated with a 32-component mixture at a 10-fold molar excess of each peptide (10 nmol) in 200 mM NaCl, 10 mM phosphate, pH 7.5 (100 μL) for 1 hour at room temperature. The mixture was then fractionated by gel filtration on a Sephadex G-25 Fast Desalting column (Pharmacia, 1 x 10 cm) in 150 mM NaCl, 10 mM phosphate buffer (pH 7.5) at a 3.0 mL/min flow rate.
15. HPLC characterization of peptides was performed on a Rainin HPX system controller with a C18 reversed-phase HPLC column (Vydac, 25 cm x 4.6 mm) and a gradient elution (solvent A: H_20/0.1% TFA and solvent B: CH_3CN/0.1% TFA; 0%-37.5% B in 30 min).