Two-stage screening of combinatorial peptide libraries. Application to bovine serum albumin ligand selection

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RESUMEN. Los péptidos cortos son excelentes ligandos para cromatografía de afinidad. El método dividir-acoplar-recombinar permite obtener bibliotecas peptídicas con todas las combinaciones de los aminoácidos en la forma de "una bolilla-un péptido". En este trabajo, se diseñó un método de doble *screening* de bibliotecas para seleccionar ligandos peptídicos. El primer *screening* de la biblioteca se realizó con la proteína blanco (seroalbúmina bovina, BSA) marcada con Texas Red. Las bolillas fluorescentes se aislaron en forma automática utilizando el equipo *Complex Object Parametric Analyzer and Sorter* (COPAS). Las bolillas aisladas se lavaron y sometieron a un segundo *screening* realizado con BSA marcada con biotina y con estreptavidina-peroxidasa y las bolillas se revelaron con 3,3'-diaminobencidina. Los péptidos presentes en las bolillas aisladas se secuenciaron por espectrometría de masas. Se sintetizaron los péptidos obtenidos con mayor frecuencia y se acoplaron a agarosa. Las matrices adsorbieron la BSA y no adsorbieron el Texas-Red ni la estreptavidina-peroxidasa. La BSA es el principal contaminante en los sobrenadantes de cultivo cuando se produce rhEPO en células CHO. Con la intención de utilizar estas matrices para la remoción de la BSA de dichos sobrenadantes, se ensayó además, la adsorción de rhEPO pura a las matrices, la cual no fue retenida en ninguno de los casos.

ABSTRACT. Short peptides are excellent ligands for affinity chromatrography. Divide-couple-recombine method allows obtaining a peptide library with all possible combinations of the amino acids in the form of "one bead-one peptide". It was designed a two-stage library screening method for peptide ligands selection in this work. The library screening was performed with the target protein (bovine seroalbumin, BSA) coupled to Texas Red. Fluorescent beads were automatically

isolated using the Complex Object Parametric Analyzer and Sorter (COPAS) equipment. Isolated beads were washed. The second screening was performed with BSA coupled to biotin and with streptavidin-peroxidase and revealed with 3,3'-diaminobenzidine. Peptides from isolated beads were sequenced using a mass spectrometry analyzer. Those peptides appearing with more frequency were synthesized and immobilized on agarose. All peptides adsorbed BSA but not Texas-Red nor streptavidin-peroxidase. As BSA from fetal bovine serum is the main contaminant, when expressing rhEPO in CHO cell culture, it was studied rhEPO adsorption on these matrices for their future application in BSA removal from cell cultures. When a pure sample of rhEPO was loaded on peptide-agarose columns, all rhEPO passed through.

INTRODUCTION

Small peptides consisting of a few amino acids represent promising affinity ligand candidates for industrial separations. Peptide ligands are much more physically and chemically stable than antibody ligands and very resistant against proteolytic cleavage. They can be readily synthesized in bulk quantities by standard chemistry at good manufacturing practices with the advantage of more than 30 years of experience in peptide synthesis. Also, peptides may be easily modified by existing chemical methods to facilitate product elution under mild conditions. Furthermore, site-directed immobilization is possible, high ligand densities can be achieved and the matrices are more robust during elution and regeneration as compared to protein-based affinity matrices. Even if any leakage occurs, small peptide molecules can be easily removed from a macromolecular product. [1-4]

The application of combinatorial peptide synthesis strategies greathy for any given protein interest facilitates the discovery of suitable ligands. Also known as the split-and-mix method, the divide-couple-recombine (DCR), is the most advantageous among the solid-phase strategies available for peptide library preparation.4 This procedure assures a theoretically even representation of the library members and a "one-bead-one-peptide" distribution.[5, 6]

To screen these combinatorial peptide libraries, tens of thousands to millions of compound beads are first mixed with a target molecule. A reporter group such as biotin or a fluorescent dye is conjugated to those targets that cannot be detected directly. When using biotin as reporter, the identification is accomplished by incubating the library with streptavidine-peroxidase (SA-POD) and then with different chromogen-peroxidase substrates such as 3,3'-diaminobenzidine or 4-Cl-naphtol. Color beads are isolated manually with needles using a stereoscopic microscope. When using targets labeled with fluorescent dyes, fluorescent beads are detected using a fluorescence stereoscopic microscope and isolated manually with needles or using the Complex Object Parametric Analyzer and Sorter (COPAS) BIOBEAD flow sorting equipment (Union Biometrica). COPAS has the capacity to analyze and sort large objects (120-300 μm) on the basis of the physical characteristics of size, density, and fluorescence signals.[7, 8]

Afterwards, peptides contained on each isolated bead are sequenced.

Non-ionic detergents and blocking agents such as gelatin or powdered milk are used during the screening to avoid no specific interactions. However false positives are commonly selected.[9, 10] For instance, beads with His-Pro-Gln and His-Pro-Met motifs with high affinity for streptavidin (SA), are selected when using SA-POD.11 Thus the library can be designed excluding those motifs that bind SA to preclude the selection of false-positive beads. However, beads that interact directly with SA-POD may be still identified with these libraries. The library may be first incubated with SA-POD in order to discard, manually, false positive beads. The drawback of this strategy is that it is time-consuming and laborious and lots of beads may be damaged or lost.

When screening the library with the target protein coupled with fluorescent dyes, peptides with high content of hydrophobic amino acids interact with the dye due to their hydrophobicity.12 We have previously reported that false positive beads showed bright homogeneous fluorescence while positive beads displayed a heterogeneous fluorescence, exhibiting a halo appearance with high

fluorescence intensity on the bead surface and low in the core.[13] A manual inspection of the fluorescent beads sorted by the COPAS allows separating positive beads from false positive ones; the difference between them is not so obvious, depending on the target and the fluorescent dye used.[14] Therefore, a lot of false positive beads are usually isolated and their peptides sequenced.
The aim of this work was to design a two-stage one-bead-one-peptide library screening where the positive beads from the first screening were subject to a different screening process to avoid false positives beads selection. Bovine serum albumin (BSA) was used as the probe protein and the strategy was employed to search BSA affinity ligands.

MATERIALS AND METHODS

Materials

Hydroxymethylbenzamide-ChemMatrix (HMBA-CM) resin was donated by Matrix Innovation Inc. (Montreal, Quebec, Canada). Fmoc-amino acids, 1-hydroxybenzotriazole (HOBt), *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium tetrafluoroborate *N*-oxide (TBTU) were from Peptides International Inc. (Louisville, KY, USA). 4-(*N*,*N*-dimethylamino)pyridine (DMAP), α-cyano-4-hydroxycinnamic acid (CHCA), BSA (Fraction V), 3,3'-diaminobenzidine (DAB), Texas red, BSA-Texas red were from Sigma-Aldrich (St. Louis, MO, USA). 1,3 -Diisopropylcarbodiimide (DIPCDI),*N*,*N*-diisopropylethylamine (DIPEA), triisopropylsilane (TIS) were from Fluka Chemie AG (Buchs, Switzerland). N-hydroxysuccinimide (NHS)-Biotin, NHS-activated agarose were from Pierce Protein Research products (Thermo Fisher Scientific Inc., Rockford, IL, USA). SA-POD was from Roche (Basel, Switzerland). PD-10 desalting columns were from GE Healthcare (Waukesha, WI, USA). GP Sheath Reagent was from Union Biometrica (Somerville, MA, USA). All other reagents were AR grade.

Experimental part

Combinatorial Peptide Libraries Synthesis

HMBA-CM was washed before use as follows: 1 mol/L HCl (5 × 1 min), H_2O (5 × 1 min), CH_3OH (5 × 1min), CH_2Cl_2 (5 × 1min), *N*,*N*-dimethylformamide (DMF) (5 × 1 min).
Manual solid-phase peptide elongation and other solid-phase manipulations were carried out in polypropylene syringes fitted with a polyethylene porous disk. Solvents and soluble reagents were removed by suction. “One-bead-one-peptide” libraries were synthesized using the divide-couple-recombine (DCR) method as per Lam et al.[6]”Library A had 161051 nonapeptides Ac-His-X_2-X_3-X_4-X_5-X_6-Gly-Ala-Gly-NH_2, where X = Ala, Glu, Phe, Gly, His, Leu, Asn, Pro, Arg, Ser or Thr (variable positions). Library B had 65536 nonapeptides H-X_1-X_2-Glu-X_4-X_5-Arg-Gly-Ala-Gly-NH_2, where X = Ala, Asp, Glu, Phe, His, Ile, Lys, Leu, Asn, Pro, Gln, Arg, Ser, Thr, Val or Tyr (variable positions). Peptide libraries were synthesized using the Fluorenylmethoxycarbonyl (Fmoc) strategy with a 3-fold excess of the amino acids at each coupling step. HMBA-CM resin (100-200mesh and 0.64 mmol/g substitution) was used. The C-termini Gly was incorporated with DIPCDI in the presence of DMAP in DMF.16 The remaining protected amino acids were incorporated with DIPCDI/HOBt. Randomization was carried out at the variable positions (X). Fmoc removal was achieved with piperidine/DMF (1/4). HMBA-CM resin was stirred during coupling steps using an orbital shaker (Viking SRL). Washings between deprotection, coupling, and subsequent deprotection steps were accomplished with DMF and CH_2Cl_2. Following elongation completion, the side-chain protecting groups were removed from the peptide-linker-resin by treatment with a mixture of trifluoroacetic acid (TFA)/TIS/H_2O (95 : 2 . 5 : 2 . 5) for 2 h, leaving the unprotected peptide anchored to the resin.

BSA labeling
NHS-activated biotin was coupled to BSA following the manufacturer's protocol.[17]

Library screening
This procedure was carried out at room temperature in syringes, each fitted with a polyethylene porous disk. The peptide beads were first soaked in CH_2Cl_2 (5 × 1min), DMF (5 × 1min), DMF/H_2O(7 : 3, 5 : 5, 3 : 7) (5 × 1min each one) and H_2O (5 × 1min). Subsequently, they were blocked with 10 % skim milk in phosphate buffered saline (PBS), pH 6.8. The beads were then washed 5 × 1 min with 0.1% Tween 20 in PBS (PBS-Tween) and incubated with BSA-Texas red (Sigma-Aldrich, BSA-TR) at a concentration of 3 μM in PBS-Tween for 1 h. The beads were then thoroughly washed with PBS-Tween (5 × 1 min). Fluorescent beads were isolated using the COPAS BIO-BEAD flow sorting equipment (Union Biometrica).7 They were suspended in COPAS GP Sheath Reagent and poured into the sample cup at a density of about 50 beads/mL. Gating and sorting regions were defined for sorting beads on the basis of their time-of-flight (TOF) to sort uniform sized beads and red fluorescence intensity (RED). Selected beads were washed with acetic acid (AcOH)/acetonitrile (MeCN)/H_2O (3 : 4 : 3) (2 × 15 min) to eliminate the adsorbed protein and then soaked subsequently in MeCN (5 × 1min), CH_2Cl_2 (5 × 1min), DMF (5 × 1min), DMF/H_2O(7 : 3, 5 : 5, 3 : 7) (5 × 1min each one) and H_2O (5 × 1min). After that, they were blocked with 10% skim milk in PBS, pH 6.8, and then washed 5 × 1 min with PBS-Tween and incubated with biotinylated-BSA 3 μmol/L in PBS-Tween for 1 h. The beads were then washed with PBS-Tween (5×1 min) and incubated with SA-POD (1 U/mL) in PBS-Tween. Again, they were washed with PBS-Tween (5 × 1 min) and beads were revealed with a mixture of 200 μL DAB (10 mg tablet in 15 mL of Tris-buffered saline, pH 7.6) with 12 μL of fresh 30 % hydrogen peroxide. After 5 min, positive beads turned brown. The beads were thoroughly washed with PBS-Tween (5 × 1 min) and PBS (5 × 1 min). Brown beads were isolated manually with needles using a stereoscopic microscope Leica MZ FLIII (Leica Microsystems GmbH, Wetzlar, Germany).Positive beads were isolated and washed with H_2O (5 × 1μL). Then, they were treated with 10 μL of AcOH/MeCN/H_2O (3 : 4 : 3) and washed sequentially with MeCN (5 × 1μL), CH_2Cl_2 (5 × 1μL), and air-dried.18

Peptide Cleavage and Elution from the Bead
Peptides were cleaved from the beads using ammonia vapor. Single peptide-beads were placed into separate micro tubes, which were placed in a drying chamber together with a flask containing NH_4OH 30 %. The drying chamber was clamped shut and left to stand overnight at room temperature. Released peptides were eluted from each bead with 20μL of AcOH/MeCN/H_2O (3 : 4 : 3) overnight.18

MALDI-TOF MS Analysis of the Eluted Peptides
Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) were recorded in a 4700 Proteomics Analyzer instrument (Applied Biosystems). The analysis was performed following Martínez-Ceron *et al*., 2010.18 One microlitre of eluted peptide from a single bead was loaded onto the sample plate, air-dried at room temperature, and then 1μL of CHCA 4 mg/mL in MeCN/H_2O (1:1) with 0.1 % TFA was added on the sample dry layer (successive-dry-layers deposit method). Mass spectra were acquired in the MS reflector positive-ion mode. Tandem mass spectra were obtained using the MS/MS positive acquisition method.

Peptide Synthesis
Peptides: Ac-His-His-His-His-Thr-Asn-Gly-Ala-Lys-NH_2 (Peptide 1) and Ac-Thr-His-Glu-His-Arg-Arg-Gly-Ala-Lys-NH_2 (Peptide 2) were synthesized by the Fmoc chemistry on Rink-amide resin, as described by Chan and White, 2000.19 Manual solid-phase peptide elongation and other

solid-phase manipulations were carried out in polypropylene syringes fitted with a polyethylene porous disk. Solvents and soluble reagents were removed by suction. Peptides were synthesized using the Fmoc strategy with 3-fold excess of the amino acids at each coupling step incorporated with DIPEA/TBTU. Fmoc removal was performed with piperidine/DMF (1:4). Rink-amide resin was stirring during coupling steps using an orbital shaker (Viking SRL). Washings between deprotection, coupling, and subsequent deprotection steps were carried out with DMF and CH_2Cl_2. After completion of elongation, the peptide cleavage and side-chain protecting groups removal was performed by treatment with a mixture of TFA/TIS/H_2O (95 : 2 . 5 : 2 . 5) for 2 h. Peptides were isolated by precipitation with cold diethyl ether and then dissolved in MeCN/H_2O (1 : 1) and lyophilized.

Peptide Analysis

Peptides were analyzed by HPLC, ESI MS, and MALDI-TOF MS. HPLC analysis was carried out in a Waters Alliance 2695 instrument with a Waters Sun Fire reverse-phase C18 column (4.6 - 100 mm, 3.5 μm) and a linear gradient from100% A to 80 % B in 15 min, where A=H_2O (0.045 % TFA) and B=MeCN (0.036 % TFA), at a flow rate of 1.0 mL/min. ESI MS analysis was performed in a Waters Micromass ZQ with a Waters Sun Fire reverse-phase C18 column (2.1 - 100 mm, 3.5 μm) and a linear gradient from100% A to 80% B in 15 min, where A = H_2O(0.1 % formic acid) and B = MeCN (0.07 % formic acid), at a flow rate of 1.0 mL/min. MALDI-TOF MS was recorded in a 4700 Proteomics Analyzer instrument (Applied Biosystems). One microlitre aliquot of peptide solution was loaded onto the sample plate, air-dried at room temperature, and then 1μL of CHCA 4 mg/mL in MeCN/H_2O (1:1) with 0.1% TFA was added on the sample dry layer (successive-dry-layers deposit method). Mass spectra were acquired in the MS reflector positive-ion mode. Tandem mass spectra were obtained using the MS/MS positive acquisition method.

Preparation of the Affinity Chromatographic Matrix

Affinity matrices were prepared as described by Hermanson *et al*., 1992.20 A 3-fold excess of Peptide 1 or Peptide 2 dissolved in 1 mL of dimethyl sulfoxide (DMSO) was added to 1 mL of NHS-agarose provided with a spacer arm with a ligand density of 16 - 23 μmol NHS/mL drained medium. Anhydrous triethylamine was added to a level that was equimolar to the amount of peptide charged. The gel/peptide slurry was stirred for 4 h at room temperature using a orbital shaker (Vicking SRL). After incubation at room temperature for 2 h, the Peptide 1-agarose and Peptide 2-agarose matrices were washed with DMSO. Any remaining group was blocked by addition of a slight excess of ethanolamine (EA) at the end of the reaction. A reference matrix (EA-agarose) was prepared by blocking all the NHS groups with EA.

Proteins and Texas-Red adsorption on peptide-agarose matrix

Pure samples of BSA, Texas-Red, SA-POD or recombinant human erythropoietin (rhEPO) in equilibrating buffer were loaded on columns (0.5 × 5 cm) filled with Peptide 1-agarose, Peptide 2-agarose or EA-agarose. The columns were washed with equilibrating buffer at a flow rate of 0.25 mL/min until the absorbance at 280 nm reached its initial value. When the equilibrating buffers assayed were 25 mol/L sodium phosphate, pH 7.0, or phosphate buffered saline (PBS), pH 7.0, the elution was performed with 100 mol/L sodium acetate buffer, pH 3.0, 0.25 M NaCl, while when the equilibrating buffers assayed were 20 mol/L sodium citrate, 10, 100, 150 or 200 mM NaCl, pH 3.0, the elution was accomplished with 20 mol/L sodium phosphate, pH 8.5, at the same flow rate.

BSA adsorption isotherm determination

Chromatographic matrix aliquots, 30 µL, were put into tubes containing 1 mL of pure BSA solution at different concentrations in 20 mol/L sodium citrate, 150 mol/L NaCl buffer (Peptide 1-agarose) or 20 mM sodium citrate, 200 mol/L NaCl buffer (Peptide 2-agarose).The suspensions were gently shaken overnight at 24º C to enable the system to reach its equilibrium. Protein concentration was determined with Bradford reagent.21 The equilibrium concentration of BSA bound to the matrix (q*) was calculated as the total amount of BSA present at the beginning of the experiment less the amount still in the soluble phase at equilibrium (c*). One-to-one Langmuir binding model was used to calculate qm and Kd as described by Chase, 1984,[22] using the SigmaPlot 2010 regression program (SPSS Inc.).

RESULTS AND DISCUSSION

Peptide library screening and analysis by MALDI-TOF MS

In the first step of the screening strategy, BSA-TR was used and fluorescent beads were separated using the Complex Object Parametric Analyzer and Sorter (COPAS) flow sorting equipment, which has the capacity to analyze and sort large objects (120 - 300 µm) at a high rate (up to 50 objects per second) on the basis of the physical characteristics of size, density, and fluorescence signals. The isolated beads were subjected to a second screening with BSA-biotin. Beads were reveled with SA-POD and DAB, and positive beads turned brown. Whole brown beads were isolated manually. Fig. 1 summarizes the two-stage one-bead-one-peptide libraries screening strategy developed.

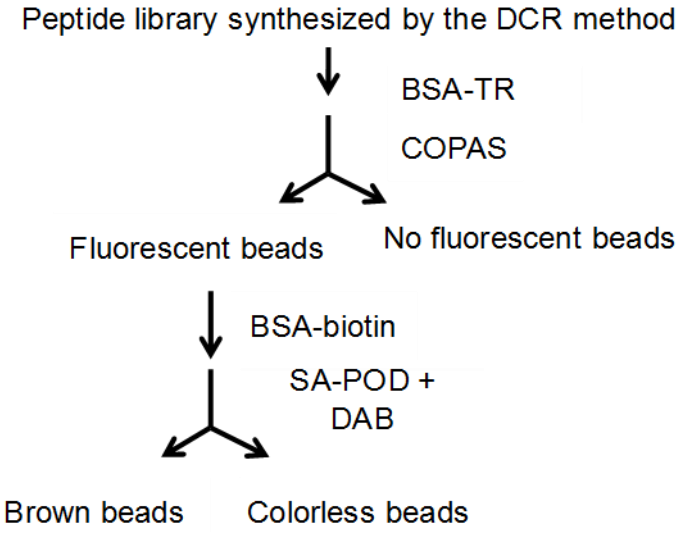

Fig 1. *Two-stage peptide libraries screening strategy.*

Fifteen beads showed a positive reaction for library A and forty-five beads for library B. Peptides from the isolated beads were cleaved from the resin using NH_4OH and identified by MALDI-TOF MS analysis. Table 1 and 2 shows positive sequences obtained after screening with libraries A and B respectively. Peptide Ac-His-His-His-His-Thr-Asn-Gly-Ala-Gly-NH_2 from library A and H-Thr-His-Glu-Arg-Arg-Gly-Ala-Gly-NH_2 from library B were selected for further experimentation since they appeared with the highest frequency.

Synthesis of peptide affinity adsorbents

Peptide ligands Ac-His-His-His-His-Thr-Asn-Gly-Ala-Lys-NH_2 (Peptide 1) or Ac-Thr-His-Glu-Arg-Arg-Gly-Ala-Lys-NH_2 (Peptide 2) were synthesized by solid phase chemistry. The purity of the ligands was over 96 % according to the HPLC analysis, and the m/z obtained by mass spectrometry analysis corresponded to the molecular weight of the peptides synthesized. These

peptides were then immobilized on agarose. The peptide amide was synthesized to prevent peptide polymerization during coupling. The N-terminus was acetylated and the Gly residue at the C-terminal was replaced by a Lys residue to allow the peptide to be coupled only through the side-chain amine group of Lys, thus assuring the same peptide orientation in the agarose support as that in the library bead.

Table 1. Sequences of the peptide-beads selected after screening library A with BSA

Ac-HEHNPPGAG (1)	Ac-HHHFHFGAG (1)	Ac-HLFHPFGAG (1)
Ac-HFLAFAGAG (1)	Ac-HHHGFHGAG (1)	Ac-HLHGHTGAG (1)
Ac-HHAPRHGAG (1)	Ac-HHHHTNGAG (2)	Ac-HSTTPFGAG (1)
Ac-HHARANGAG (1)	Ac-HHRAHPGAG (1)	Ac-HTFNHHGAG (1)
Ac-HHEHTNGAG (1)	Ac-HLFFRAGAG (1)	

Library A: Ac-His-X_2-X_3-X_4-X_5-X_6-Gly-Ala-Gly-NH_2; where X = Ala, Glu, Phe, Gly, His, Leu, Asn, Pro, Arg, Ser or Thr (variable positions). The number in parentheses denotes the number of times that these sequences were detected.

Proteins and Texas-Red adsorption to peptide-agarose matrix

BSA, Texas-Red and SA-POD adsorption on each column was evaluated. Both matrices adsorbed BSA but did not adsorbed Texas-Red or SA-POD while no one of the compounds assayed were adsorbed in EA-agarose, thus evidencing the absence of interaction between the proteins and the unreacted groups on the matrix.

BSA adsorption in each column (Peptide 1-agarose or Peptide 2-agarose) was assessed under different buffer conditions. The pH decrease and the increase in ion strength improve BSA adsorption. The best result for Peptide 1-agarose matrix was achieved when employing 20 mM sodium citrate, 150 mol/L NaCl, pH 3.0 buffer as the adsorption buffer and for Peptide 2-agarose when using 20 mol/L sodium citrate, 200 mol/L NaCl, pH 3.0, buffer. These buffers were chosen for further analysis.

As the main contaminant when expressing rhEPO in CHO cell culture is the BSA from fetal bovine serum,23 we studied rhEPO adsorption on these matrices for their future application in BSA removal from cell cultures. When a pure sample of rhEPO (0.40 mg/mL) was loaded on both columns with different equilibrating buffers, 100% of rhEPO passed through.

Table 2. Sequences of the peptide-beads selected after screening library B with BSA.

ANERFRGAG (1)	PPENYRGAG (1)	RHEQHRGAG (1)
ERERPRGAG (1)	PPEYTRGAG (1)	RLEFERGAG (1)
FQEYTRGAG (1)	QAEAVRGAG (1)	RQEFQRGAG (1)
HFETVRGAG (1)	QAEPPRGAG (1)	RREHTRGAG (1)
HHELNRGAG (1)	QAERLRGAG (1)	RRESNRGAG (1)
HHEPQRGAG (1)	QHEFQRGAG (1)	RSEERRGAG (1)
HHETSRGA (1)	QPEQHRGAG (1)	RSETQEGAG (1)
HLESLRGAG (1)	QQEQYRGAG (1)	RTETHRGAG (1)
HREQARGAG (1)	QRELERGAG (1)	RTEVHRGAG (1)
HREYRRGAG (1)	QTEDFRGAG (1)	TFEHHRGAG (1)
HYENRRGAG (1)	QVENARGAG (1)	THEHRRGAG (2)
LPEQHRGAG (1)	QYEYVRGAG (1)	TPERRRGAG (1)
LQEQVRGAG (1)	RAERNRGAG (1)	VTEFQRGAG (1)
NHEVERGAG (1)	RDEHQRGAG (1)	
NLEQQRGAG (1)	RHEFQRGAG (1)	

Library B: H-X_1-X_2-Glu-X_4-X_5-Arg-Gly-Ala-Gly-NH_2;where X = Ala, Asp, Glu, Phe, His, Ile, Lys, Leu, Asn, Pro, Gln, Arg, Ser, Thr, Val or Tyr (variable positions). The number in parentheses denotes the number of times that these sequences were detected. Lys is indistinguishable from Gln in MALDI-TOF-MS sequencing. To simplify the table we added only one of the possibilities

Adsorption Isotherms

Figure 2 shows the adsorption isotherms of BSA on Peptide 1-agarose and Peptide 2-agarose, developed with pure BSA in 20 mM sodium citrate, 150 mM NaCl, pH 3.0, for Peptide 1-agarose and 20 mM sodium citrate, 200 mM NaCl, pH 3.0, for Peptide 2-agarose. The isotherms showed a good fit of experimental data to a Langmuir-type isotherm and allowed calculation of a maximum capacity (qm) of 0.043 ± 0.0025 and 0.040 ± 0.0021 μmol BSA/mL matrix for Peptide 1-agarose and Peptide 2-agarose, respectively, and a dissociation constant (Kd) of 3.4 ± 0.6 μmol/L

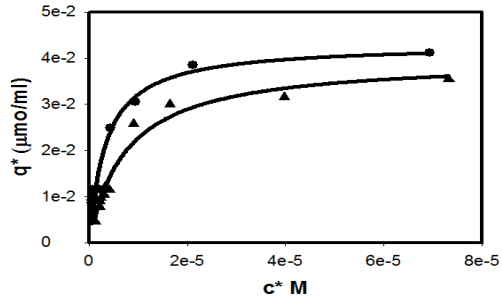

Fig 2. *Equilibrium adsorption isotherm for binding of BSA to Peptide 1-agarose (▲) and Peptide 2-agarose (●). Chromatographic matrix aliquots, 30 μL, were put into tubes containing 1 mL of pure BSA solution at different concentrations in 20 mol/L sodium citrate, 150 mol/L NaCl, pH 3.0, for Peptide 1-agarose and in 20 mol/L sodium citrate, 200 mol/L NaCl, pH 3.0, for Peptide 2-agarose. The suspension was gently shaken overnight at 24 ºC. Protein concentration in the supernatants was measured with Bradford reagent. Kds of 3.4 ± 0.6 μmol/L and 7.55 ± 1 μmol/L for Peptide 1-agarose and Peptide 2-agarose respectively, and qms of 0.043 ± 0.0025 and 0.040 ± 0.0021 μmol BSA/mL matrix for Peptide 1-agarose and Peptide 2-agarose, respectively were obtained.*

and 7.55 ± 1 μmol/L for Peptide 1-agarose and Peptide 2-agarose respectively. The qms obtained were comparable to commercial matrices.20 The moderate Kd value is appropriate for affinity chromatography as BSA can be adsorbed at low concentrations from culture supernatants and then eluted under mild conditions. The apparent binding constant (1/Kd) of the immobilized peptides was estimated at 1.09×10^5 L/mol and 2.73×10^5 L/mol for Peptide 1 and Peptide 2, respectively. They were just in the range of desired affinity interaction applicable to affinity chromatography separation of proteins (10^5 - 10^6 L/mol).24

CONCLUSION

The two stage screening here designed allowed the selection of peptides with specific affinity for the protein of interest reducing the selection of false positive beads in a simpler way than previous protocols.9

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