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Affinity Chromatography Based on a Combinatorial Strategy for rErythropoietin Purification

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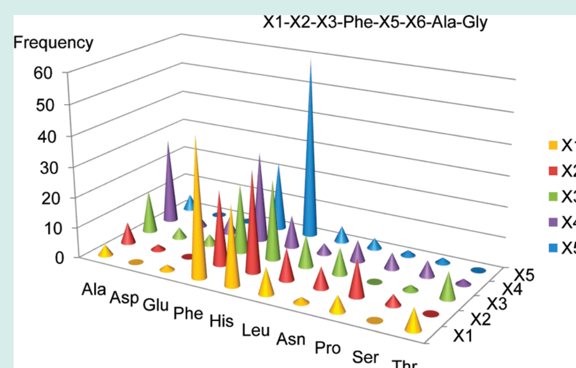
ABSTRACT: Small peptides containing fewer than 10 amino acids are promising ligand candidates with which to build affinity chromatographic systems for industrial protein purification. The application of combinatorial peptide synthesis strategies greatly facilitates the discovery of suitable ligands for any given protein of interest. Here we sought to identify peptide ligands with affinity for recombinant human erythropoietin (rhEPO), which is used for the treatment of anemia. A combinatorial library containing the octapeptides X-X-X-Phe-X-X-Ala-Gly, where X = Ala, Asp, Glu, Phe, His, Leu, Asn, Pro, Ser, or Thr, was synthesized on HMBA-ChemMatrix resin by the divide-couple-recombine method. For the library screening, rhEPO was coupled to either Texas Red or biotin. Fluorescent beads or beads showing a positive reaction with streptavidin-peroxidase were isolated. After cleavage, peptides were sequenced by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Fifty-seven beads showed a positive reaction. Peptides showing more consensuses were synthesized, and their affinity to rhEPO was assessed using a plasma resonance biosensor. Dissociation constant values in the range of 1−18 μM were obtained. The best two peptides were immobilized on Sepharose, and the resultant chromatographic matrixes showed affinity for rhEPO with dissociation constant values between 1.8 and 2.7 μM. Chinese hamster ovary (CHO) cell culture supernatant was spiked with rhEPO, and the artificial mixture was loaded on Peptide-Sepharose columns. The rhEPO was recovered in the elution fraction with a yield of 90% and a purity of 95% and 97% for P1-Sepharose and P2-Sepharose, respectively.



KEYWORDS: affinity chromatography, rhEPO, one-bead-one-peptide combinatorial libraries, surface-plasmon-resonance, ChemMatrix resin, solid-phase peptide synthesis

INTRODUCTION

Erythropoietin (EPO), a glycoprotein hormone produced in mammalian kidney and liver, regulates the proliferation and differentiation of erythroid progenitor cells and maintains physiological levels of circulating red blood cells. Recombinant human erythropoietin (rhEPO) is used for the therapeutics of anemia associated with chronic renal disease, AZT-induced anemia of AIDS, the treatment of cancer patients on chemotherapy, and for surgical patients to prevent the need for a red blood cell transfusion.[1] The sugar moiety, which accounts for about 40% of the molecular weight of EPO, is essential for its full biological activity. The Chinese hamster ovary (CHO) cell line is one of the most widely used mammalian cells for rhEPO expression. This is because the N-linked carbohydrate structures of the glycoprotein it synthesizes show the characteristic features of the glycans naturally occurring in human proteins.[2]

Access to biopharmaceuticals is limited by price. Therefore, reduction of the biopharmaceutical manufacturing costs has become a critical challenge for the industry. A major expense in the production of a recombinant protein is that of the separation and purification of the product from the culture medium in which it is produced. These procedures may account for as much as 60−80% of the total manufacturing costs. Such expense is due to the stringent quality criteria that protein products intended for human use should meet.[3] Typically, purification processes consist of multiple separation steps. Consequently, there is a need not only to improve individual purification steps but also to combine these steps into integrated unit operations to increase the overall efficiency of the process train. Affinity chromatography

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(AC) combines the steps of coarse and fine purification by introducing a specific interaction between the protein of interest and the respective chromatographic ligand and is ideally suited for the purification of therapeutic proteins.[4]

Monoclonal antibodies (mAbs),[5] triazinic dyes,[6] and metals[7] are ligands used in AC for EPO purification.[8−14] Antibodies display high affinity and selectivity but are expensive and can lose activity or leach into products as a result of the harsh elution and cleaning conditions used in industrial purification processes.[3] Cibacron Blue and Cu(II)-iminodiacetic acid were also used for EPO purification because of their low cost, availability, simple immobilization reaction, and resistance to biological and chemical degradation.[8−12] However, both ligands also adsorbed bovine serum albumin (BSA) with high affinity,[15,16] which is the main contaminant when expressing EPO in CHO cells because of the use of fetal bovine serum.

Small peptide ligands containing fewer than 10 amino acids are much more physically and chemically stable than antibody ligands and are more resistant to proteolytic cleavage.[17−19] They can be readily synthesized by standard chemistry in bulk amounts at a lower cost under good manufacturing practices (GMPs).[20] Also, peptides can be easily modified by chemical methods to facilitate product elution under mild conditions. Furthermore, peptides allow site-directed immobilization and high ligand density, and the matrixes are more robust during elution and regeneration than protein-based affinity matrixes such as monoclonal antibodies. Moreover, in the case of leakage into the product, peptides show lower toxicity and generate lower immune responses as compared with proteins, dyes, and transition metal ion ligands.[17,20] Even when leakage does occur, small peptide molecules can be easily removed from the macromolecular product by diafiltration or size exclusion chromatography.

The combinatorial methods allow the selection of ligands from large peptide libraries constructed randomly by synthetic techniques. The divide-couple-recombine (DCR), also known as the split-and-mix method[21−23] assures a theoretically even representation of the library members and a "one-bead-one-compound" distribution. To screen these combinatorial libraries, tens of thousands to millions of compound beads are first mixed with the probe molecule. The beads that interact with it will be identified and then isolated for compound structure determination.[24]

In previous studies, we developed a rapid and inexpensive strategy using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) for the identification of synthetic peptides from positive beads. The peptide synthesis was carried out using fluorenylmethoxycarbonyl (Fmoc)/*tert*-butyl (*t*Bu) chemistry and the linker 4-hydroxymethylbenzoic acid (HMBA) immobilized on ChemMatrix resin.[25−28] This resin is compatible with both organic solvents, used for the peptide synthesis, and aqueous ones, which are useful for the screening step, and therefore highly suitable for the whole process.[29]

The aim of this study was to apply that strategy for the identification of peptides with affinity for rhEPO and to attach these peptides to agarose to prepare chromatographic matrixes for rhEPO purification by AC.

■ RESULTS AND DISCUSSION

Peptide Library Design. In previous studies, we had synthesized a "one-bead-one-peptide library" composed of 130,321 tetrapeptides with the combination of all the natural amino acids except Cys, which was omitted to prevent disulfide bridge formation. By the screening of that library we found high affinity tetrapeptide ligands for the purification of an antigranulocyte macrophage-colony stimulating factor monoclonal antibody[30] and soybean peroxidase;[31] however, the peptides found for rhEPO were of low affinity.[32] To find peptides with higher affinity and suitable for acting as chromatographic ligands, we prepared an octapeptide library with five variable positions. Ten different amino acids were used at each of five variable positions in the sequence, resulting in a library of 10^5 = 100,000 peptide sequences. Considering that for statistical reasons the number of beads must exceed the number of peptides by a factor of at least 10 and that 1 g of resin contains 10^6 beads, only 1 g of resin was required to synthesize the library. Although the library was much smaller than the "complete" library in which all the 20 proteinogenic amino acids would have been used at each position, the total number of sequences in the one-bead-one-peptide library was of a size that could easily be manipulated.[33] The 10 building blocks of the variable positions and the Phe in the fix position were selected on the basis of results previously obtained by our group with the tetrapeptide library.[32] Ala-Gly residues were incorporated as a spacer arm at the C termini of the library.

Peptide Library Screening and Analysis by MALDI-TOF MS. The screening was performed by two methods. When using rhEPO-Texas Red, fluorescent beads were separated using the Complex Object Parametric Analyzer and Sorter (COPAS) BIOBEAD 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. A manual inspection of the fluorescent beads sorted by the COPAS was performed to separate positive beads caused by peptide-rhEPO interaction and false-positive beads produced by peptide-fluorescent dye interaction. As we had previously demonstrated, false-positive beads presented bright homogeneous fluorescence while positive ones had a characteristic halo appearance (with the highest fluorescence intensity at the bead surface and the lowest in the core).[27] Only positive beads were isolated for MALDI-TOF MS analysis. When using rhEPO-Biotin, beads were detected with streptavidin-peroxidase conjugate (SA-POD) and 4-Cl-naphtol, and positive beads turned violet. Motifs that bind streptavidin (His-Pro-Gln and His-Pro-Met) previously reported by Lam et al.[34] were excluded from the library, thus precluding the selection of false-positive beads. All violet beads were isolated manually. Fifty-seven beads showed a positive reaction. Peptides from the isolated beads were cleaved from the resin using NH_4OH and identified by MALDI-TOF MS analysis. Table 1 shows the positive sequences and Figure 1 the position frequency for all the amino acids assayed. In position X1 and X2, His and Phe occurred at high frequency. This was also the case in position X3 but Ala, Leu, Asn, and Thr also appeared with reasonable frequency. At position X5 Ala and Phe were found to occur at high frequency while at position X6 the predominant amino acid was His. The peptide Phe-His-His-Phe-Ala-His-Ala-Gly was selected for further experimentation since it appeared with the highest frequency in both screening methods, which was in accordance with the frequency of amino acids obtained in each position. Phe-His-Asn-Phe-Ala-His-Ala-Gly, although appearing only twice, was also selected because of its high similarity with the most frequent peptide.

Interaction Study by Surface Plasmon Resonance (SPR) in the Biacore T-100 System. The affinity interaction of Ac-Phe-His-His-Phe-Ala-His-Ala-Gly-NH_2 and Ac-Phe-His-Asn-Phe-Ala-His-Ala-Gly-NH_2 with rhEPO was studied. The non-specific

peptide ligand Ac-Ser-Gly-Ser-Gly-Ser-Gly-Ser-Gly-NH_2 was also studied as negative control. The Biacore system exploits the phenomenon of SPR to monitor the interaction between molecules in real time without labeling. It involves the attachment of one potentially interacting partner (ligand) to the surface of a sensor chip, and then the pass of the sample containing the other partner (analyte) over the surface. During sample injection, the binding of molecules to the sensor surface generates a response proportional to the bound mass. The response, measured in resonance units (RU), increases as a function of time as analyte binds to the surface, until it reaches the steady state value. Equilibrium constants are calculated by measuring the concentration of free interactants and complex at the steady state. The concentration of complex can be measured as the steady state response. The concentration of free analyte is the bulk analyte concentration (since the analyte is constantly replenished during sample injection). The concentration of free ligand on the surface can be calculated from the concentration of complex if the total surface binding capacity is known.[35]

In the present study, rhEPO was attached to the sensor surface. Attachment of a protein to the sensor surface may be covalent or through high affinity binding to another molecule, which is in turn covalently attached to the surface (capture molecule). The rhEPO produced by CHO cells was characterized previously.[36,37] It is composed of 11 isoforms with isoelectric points between 3.6 and 5.9. Although amine coupling chemistry is widely applicable for attaching biomolecules directly to a carboxymethylated sensor chip, levels of immobilized rhEPO were low because of the electrostatic repulsion between the rhEPO and the negative charge of the sensor chip. A preconcentration necessary for efficient binding would have not been possible and isoforms with a higher isoelectric point would have coupled with higher efficiency than those with lower isoelectric point.[38] Hence, biotinylated rhEPO was immobilized on a commercial sensor chip with streptavidin preimmobilized on the surface, assuring a homogeneous coupling of all the rhEPO isoforms. The immobilization level reached was 695 RU (active channel). To compensate for the matrix refractive index effect and non-specific binding of the analyte, a reference was prepared with biocytin (reference channel). Peptides Ac-Phe-His-His-Phe-Ala-His-Ala-Gly-NH_2, Ac-Phe-His-Asn-Phe-Ala-His-Ala-Gly-NH_2, and Ac-Ser-Gly-Ser-Gly-Ser-Gly-Ser-Gly-NH_2 were injected at different concentrations, and their binding to the sensor chips was assessed. For analysis, a double reference was applied: the response obtained at the reference channel and the response obtained in the active channel at zero analyte concentration was subtracted from the response obtained at the active channel at each analyte concentration. Figure 2 shows the maximal response in RU versus different concentrations of each peptide. Dissociation constants (K_d) of 1.5 ± 0.2 μM and 18 ± 7 μM were obtained for Ac-FHHFAHAG-NH_2 and Ac-FHNFAHAG-NH_2,

Table 1. Amino Acid Sequences of Peptides in Beads That Interacted with rhEPO[a]

AAAFAHAG (1)	**FHHFAHAG** (5)	HFFFAHAG (1)	LLFFFFAG (1)
ANTFDHAG (1)	FHLFAFAG (2)	HFFFHAAG (1)	LLNFAFAG (1)
EHEFFFAG (1)	**FHNFAHAG** (2)	HFHFFSAG(1)	LNHFHHAG(1)
FAAFFLAG (1)	FHTFEHAG (1)	HFHFPLAG (2)	LPFFLPAG (1)
FAFFSHAG (1)	FHTFFHAG (1)	HFSFSHAG (1)	LPLFEHAG (1)
FAHSHAG (1)	FNHFAHAG (1)	HHHFHHAG (1)	NNDFNHAG (1)
FDDFFAAG (1)	FPAFPHAG(1)	HHLFDHAG (1)	PHAFFHAG (1)
FFEFFFAG (1)	FPFFNFAG (1)	HLAFAHAG (1)	PHHFHNAG(1)
FFFFFHAG (1)	FPHFHHAG (1)	HLFFEFAG (1)	PHNFFHHAG (1)
FFLFNFAG (1)	FPTFAFAG (1)	HLFFFFAG (1)	TFFFFAAG (1)
FFLFNFAG (1)	FSTFTFAG (1)	HLHFANAG (1)	TFFFFHAG (1)
FHFFLHAG (1)	HFAFFHAG (1)	HSNFHHAG (1)	THAFFHAG(1)
			TPFFFHAG (1)

[a] The number in parentheses denotes the number of times that these sequences were detected.

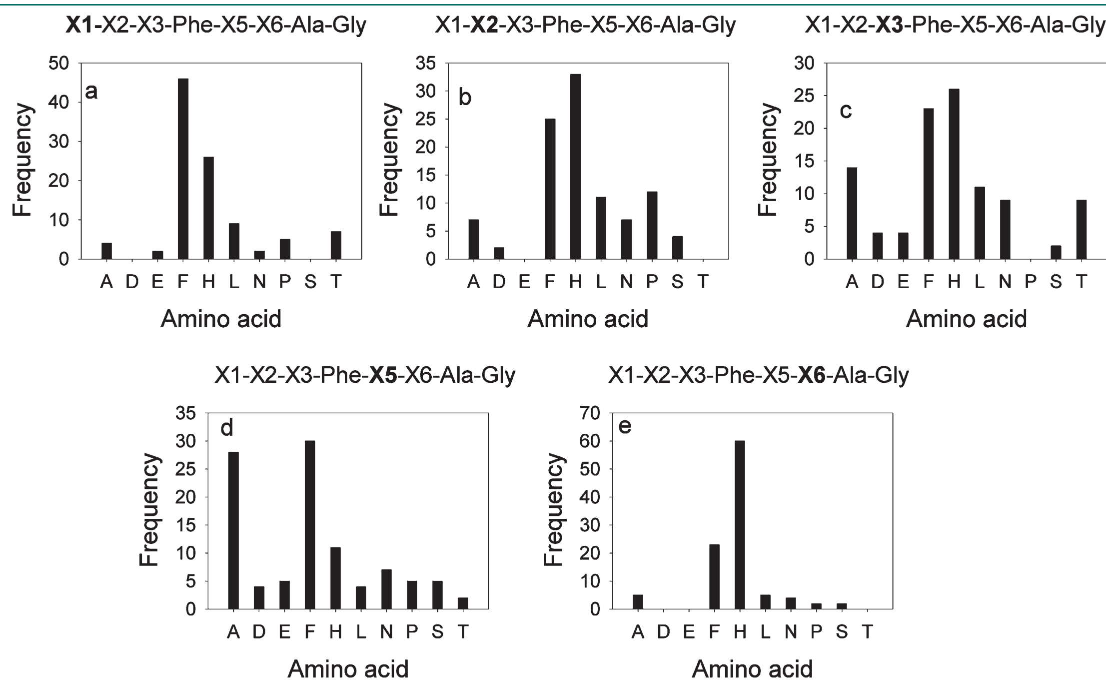

Figure 1. Analysis of the frequency data for amino acids found in positions (a) X1, (b) X2, (c) X3, (d), X5, and (e) X6 of the peptides identified by screening of the peptide library.

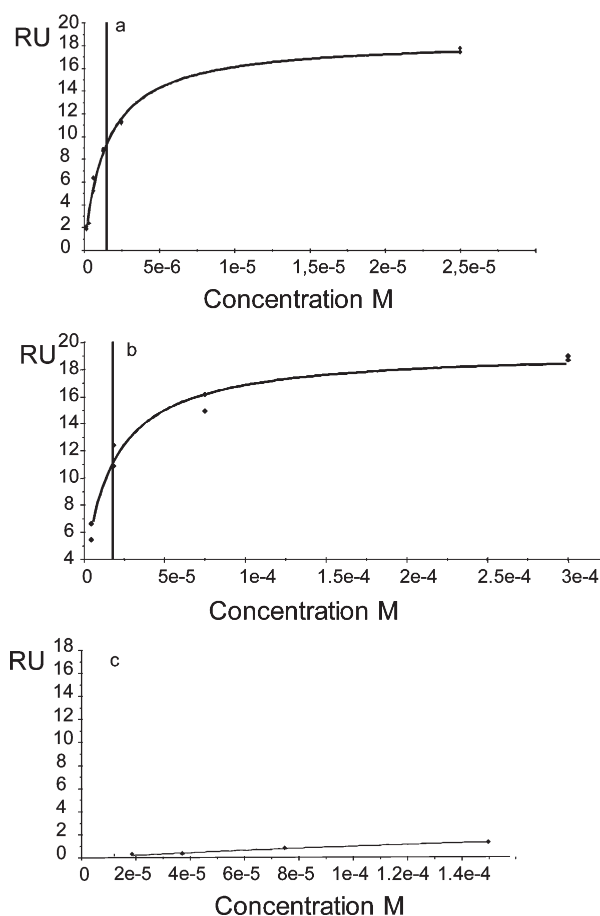

Figure 2. Binding of peptides Ac-FHHFAHAG-NH_2 (a), Ac-FHNFAHAG-NH_2 (b), and Ac-SGSGSGSG-NH_2 (c) to rhEPO. Running buffer was 10 mM HEPES, pH 7.4, 150 mM NaCl. From the affinity plots, K_d's of 1.5 ± 0.2 μM and 18 ± 7 μM were obtained for peptides Ac-FHHFAHAG-NH_2 (a) and Ac-FHNFAHAG-NH_2 (b), respectively.

respectively. The negative control peptide, Ac-SGSGSGSG-NH_2, showed no specific interaction with rhEPO. The reported K_d value was unreliable because the response against concentration plot did not flatten out sufficiently.

Synthesis of Peptide Affinity Adsorbents. Peptide ligands Ac-Phe-His-His-Phe-Ala-His-Ala-Lys-NH_2 (P1) or Ac-Phe-His-Asn-Phe-Ala-His-Ala-Lys-NH_2 (P2) were synthesized by solid-phase chemistry on a Rink-amide-polystyrene resin using the Fmoc strategy and analyzed by HPLC, Electrospray Ionization Mass Spectrometry (ESI MS), and MALDI MS. The purity of the ligands was over 96%. The main peak corresponded to the m/z of the peptides synthesized. These peptides were then immobilized on Sepharose. 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 Sepharose support as that in the library bead. The concentration of immobilized peptides was measured indirectly, from the uncoupled amount of peptide ligand present in the gel washings obtained after the immobilization reaction. A ligand density of 17 μmol per mL of matrix was achieved.

Influence of pH on rhEPO and Bovine Serum Albumin (BSA) Adsorption to Peptide-Sepharose Matrixes. The main contaminant when expressing rhEPO in CHO cell culture is the BSA from fetal bovine serum. Samples of BSA and rhEPO were loaded on chromatography columns with P1-Sepharose, P2-Sepharose, and ethanolamine (EA)-Sepharose at different pHs between 4.0 and 8.25. Neither the BSA nor the rhEPO were adsorbed by EA-Sepharose, thus evidencing the absence of interaction between the proteins and the unreacted groups on the matrix. Figure 3 shows the yield of the elution peak (as adsorption percentage) of BSA and rhEPO at these pHs. RhEPO was fully adsorbed by the peptide chromatographic matrix at all the pHs assayed. In contrast, BSA elution yield decreased as the pH was lowered. Given this observation, we selected a pH 4.0 for rhEPO adsorption, where 100% of rhEPO is adsorbed and 100% BSA passed through.

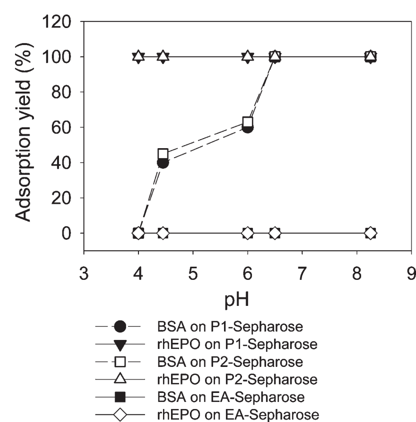

Figure 3. Influence of pH on rhEPO and BSA adsorption to peptide-Sepharose and ethanolamine (EA)-Sepharose. Samples of rhEPO and BSA in 20 mM sodium phosphate buffers with pHs from 4.00 to 8.25 were loaded on columns (0.5 × 5 cm) filled with EA-Sepharose, P1-Sepharose, or P2-Sepharose. The columns were washed with the equilibrating buffer at a flow rate of 0.25 mL/min until the absorbance at 280 nm reached its initial value. Thereafter, the elution of the bound protein was accomplished with 100 mM sodium acetate buffer, pH 3.0, 0.25 M NaCl at the same flow rate.

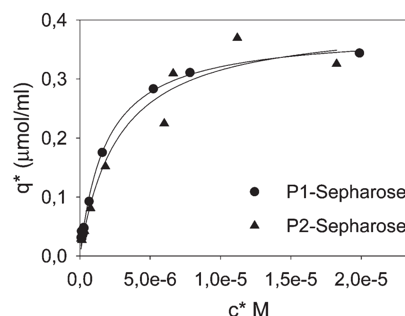

Figure 4. Equilibrium adsorption isotherm for binding of rhEPO to P1-Sepharose and P2-Sepharose. Chromatographic matrix aliquots, 30 μL, were put into tubes containing 1 mL of pure rhEPO solution at different concentrations in 20 mM sodium phosphate buffer, pH 4.0. The suspension was gently shaken overnight at 24 °C. Protein concentration was measured with Bradford reagent. K_d's of 1.8 ± 0.1 μM and 2.7 ± 0.7 μM for P1-Sepharose and P2-Sepharose, respectively, and qms of 0.38 ± 0.01 and 0.40 ± 0.03 μmol rhEPO mL^{-1} for P1-Sepharose and P2-Sepharose, respectively, were obtained.

Adsorption Isotherms at pH 4. Figure 4 shows the adsorption isotherms of rhEPO on P1-Sepharose and P2-Sepharose,

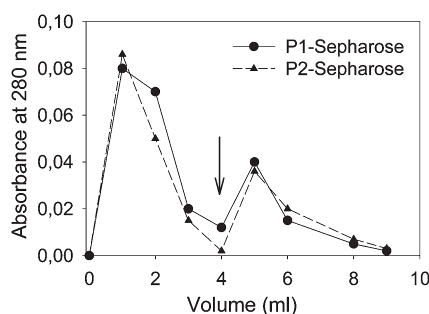

Figure 5. CHO cell culture supernatant was spiked with rhEPO. Sample conditioning was performed by loading the sample on a PD-10 desalting column containing Sephadex G-25 equilibrated with 20 mM sodium phosphate buffer, pH 4.0. One milliliter of the artificial mixture was loaded on columns (0.5 × 5 cm) filled with P1-Sepharose or P2-Sepharose. 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. Thereafter, elution of the bound protein was accomplished with 100 mM sodium acetate buffer, pH 3.0, 0.25 M NaCl at the same flow rate. The arrow indicates the buffer change.

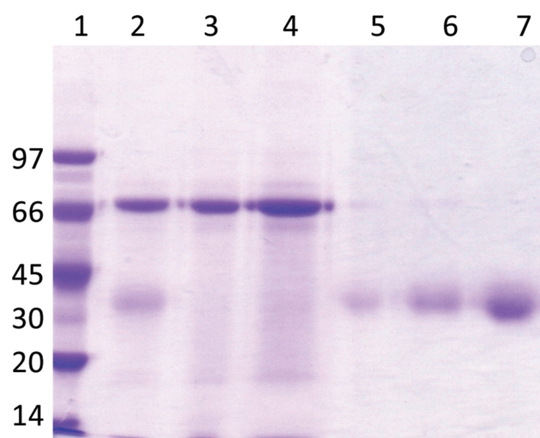

Figure 6. SDS-PAGE of CHO cell culture-rhEPO mixture fractionation: Lane 1, molecular weight markers; lane 2, CHO cell culture spiked with rhEPO, lane 3, P1-Sepharose pass-through fraction; lane 4, P2-Sepharose pass-through fraction; lane 5, P1-Sepharose-eluted fraction; lane 6, P2-Sepharose-eluted fraction; lane 7, rhEPO standard.

developed with pure rhEPO in 20 mM sodium phosphate buffer, pH 4.0. The isotherms show a good fit of experimental data to a Langmuir-type isotherm and allows calculation of a maximum capacity (qm) of 0.38 ± 0.01 and 0.40 ± 0.03 μmol rhEPO/mL matrix for P1-Sepharose and P2-Sepharose, respectively, and a K_d of 1.85 ± 0.14 μM and 2.72 ± 0.70 μM for P1-Sepharose and P2-Sepharose, respectively. The qms obtained were comparable to commercial matrixes.[39] The moderate K_d value is appropriate for AC as rhEPO can be adsorbed at low concentrations from culture supernatants and then eluted under mild conditions without impairing its biological activity. The apparent binding constant ($1/K_d$) of the immobilized peptides was estimated at 5.2×10^5 L/mol and 3.7×10^5 L/mol for P1 and P2, respectively. They were just in the range of desired affinity interaction applicable to affinity chromatography separation of proteins ($10^5 - 10^6$ L/mol).[40]

Peptide-Affinity Chromatography of CHO Cell Cultures Spiked with rhEPO. A CHO cell culture supernatant was spiked with rhEPO, and the artificial mixture was conditioned and then loaded on P1 and P2 Sepharose columns (Figure 5). According to the enzyme-linked immunosorbent assay (ELISA) analysis, the total mass of rhEPO in the pass-through and the elution fraction were 2.9 and 44.0 μg respectively for P1-Sepharose fractionation and 1.8 and 43.5 μg in the pass-through and elution fraction respectively for P2-Sepharose fractionation. The yield was 90% in both cases. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis shows the high effectiveness of the peptide-chromatography for rhEPO purification. According to the densitometric quantification, the purity was 95 and 97% for P1-Sepharose and P2-Sepharose respectively (Figure 6).

CONCLUSION

The strategy previously designed by our group[25−28] was useful for finding appropriate peptide ligands for rhEPO. The peptide-affinity matrixes with immobilized Ac-Phe-His-His-Phe-Ala-His-Ala-Lys-NH_2 and Ac-Phe-His-Asn-Phe-Ala-His-Ala-Lys-NH_2 proved to be suitable for the future development of a rhEPO purification method from crude CHO cultures. The moderate dissociation constants of the two ligands allowed, at the same time, adsorbing the rhEPO at a low concentration, such as that usually found in CHO cell culture supernatants, and quantitative rhEPO elution under mild conditions. The low cost of the peptide ligands makes them suitable for the industrial scale-up of the chromatographic process.

EXPERIMENTAL PROCEDURES

Materials. HMBA-ChemMatrix resin was kindly donated by Matrix Innovation Inc. (Montreal, Québec, Canada). Fmoc-amino acids, 1-hydroxybenzotriazole (HOBt), and 1-[bis(dimethylamino)methylene]-1*H*-benzotriazolium hexafluorophosphate 3-oxide (TBTU) were from Peptides International Inc. (Louisville, KY, U.S.A.). 4-(*N*,*N*-dimethylamino)pyridine (DMAP), α-cyano-4-hydroxycinnamic acid (CHCA), BSA (Fraction V), and biocytin were from Sigma-Aldrich (St. Louis, MO, U.S.A.). 1,3-Diisopropylcarbodiimide (DIPCDI), *N*,*N*-diisopropylethylamine (DIPEA), and triisopropyl silane (TIS) were from Fluka Chemie AG (Buchs, Switzerland). *N*-hydroxysuccinimide (NHS)-Biotin was from Pierce Protein Research products (Thermo Fisher Scientific Inc., Rockford, IL, U.S.A.). NHS-Texas Red was from Invitrogen (Carlsbad, CA, U.S.A.). SA-POD was from Roche (Basel, Switzerland). NHS-Sepharose, PD-10 desalting columns, Biacore carboxymethylated, and streptavidin commercial chips, HBSN buffer (10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 7.4, 150 mM NaCl), HBSEP buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% surfactant P20) were from GE Healthcare (Waukesha, WI, U.S.A.). rhEPO and CHO cell culture supernatant was from Zelltek S.A. (Santa Fe, Argentina). All other reagents were AR grade.

Previous Washings of HMBA-ChemMatrix Resin. HMBA-ChemMatrix resin was washed before use as follows: 1 N HCl (5 × 1 min), H_2O (5 × 1 min), CH_3OH (5 × 1 min), CH_2Cl_2 (5 × 1 min), *N*,*N*-dimethylformamide (DMF) (5 × 1 min).

Combinatorial Peptide Library Synthesis. 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. A "one-bead-one-peptide" library of 100,000 octapeptides X1-X2-X3-Phe-X5-X6-Ala-Gly, where X = Ala, Asp, Glu, Phe, His, Leu, Asn, Pro, Ser, or Thr (variable positions), was synthesized using the divide-couple-recombine (DCR) method as per Lam et al.[21] The peptide library was synthesized using the

Fmoc strategy with a 4-fold excess of the amino acids at each coupling step. HMBA-ChemMatrix resin (100−200 mesh and 0.64 mmol/g substitution) was used. The C-termini Gly was incorporated with DIPCDI in the presence of DMAP in DMF as described by Mellor et al.[41] The remaining protected amino acids were incorporated with DIPCDI/HOBt. Randomization was carried out in the variable positions (X). Fmoc removal was achieved with piperidine/DMF (1/4). 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.

rhEPO Labeling. NHS-activated biotin and NHS-activated Texas Red were coupled to rhEPO following the manufacturer's protocol.[42,43]

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 × 1 min), DMF (5 × 1 min), DMF/H_2O (7:3, 5:5, 3:7) (5 × 1 min each one) and H_2O (5 × 1 min). Subsequently, they were blocked with 10% skim milk, 2% BSA in phosphate buffered saline (PBS), pH 6.8. The beads were then washed 5 × 1 min with 0.05% Tween 20 in PBS (PBS-Tween) and incubated with the target protein coupled with Texas Red or biotin in PBS-Tween for 1 h. The beads were then thoroughly washed with PBS-Tween (5 × 1 min). When using rhEPO-biotin, beads were incubated with 1 U/mL SA-POD in PBS-Tween for 1 h. The beads were thoroughly washed with PBS-Tween (5 × 1 min) and PBS (5 × 1 min) and revealed with a mixture of 1 mL solution A (3 mg 4-Cl-naphtol in 1 mL CH_3OH) and 4 mL solution B (4 mL PBS containing 20 μL of 30 vol H_2O_2). After 5 min, positive beads turned in violet. Violet-colored beads were then isolated manually with needles. When using rhEPO-Texas Red, fluorescent beads were detected using a stereoscopic microscope Leica MZ FLIII (Leica Microsystems GmbH, Wetzlar, Germany) and isolated manually with needles, or using the COPAS BIO-BEAD flow sorting equipment (Union Biometrica).[44,45] In the latter, the beads were suspended in a COPAS GP Sheath reagent and poured into the sample cup at a density of about 50 beads per mL. Gating and sorting regions were defined for sorting beads on COPAS on the basis of their time-of-flight (TOF) to sort uniform sized beads and red fluorescence intensity (RED). All sorted beads were transferred into a Petri dish and examined under a fluorescence microscope.

Bead Washing after Screening. Positive beads were isolated and washed with H_2O (5 × 1 μL). They were then treated with 10 μL of acetic acid (AcOH)/acetonitrile (MeCN)/H_2O (3:4:3) and washed sequentially with MeCN (5 × 1 μL), CH_2Cl_2 (5 × 1 μL), and air-dried.[28]

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 20 μL of AcOH/MeCN/H_2O (3:4:3) overnight.[28]

MALDI-TOF MS Analysis of the Eluted Peptides. MALDI-TOF MS were recorded in a 4700 Proteomics Analyzer instrument (Applied Biosystems, Foster City, CA, U.S.A.). The analysis was performed following Martínez-Ceron et al.[28] A 1 μL portion of eluted peptide from a single bead was loaded onto the sample plate, air-dried at room temperature, and then 1 μL 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-Ser-Gly-Ser-Gly-Ser-Gly-Ser-Gly-NH_2, Ac-Phe-His-His-Phe-Ala-His-Ala-Gly-NH_2, Ac-Phe-His-His-Phe-Ala-His-Ala-Lys-NH_2, Ac-Phe-His-Asn-Phe-Ala-His-Ala-Gly-NH_2, and Ac-Phe-His-Asn-Phe-Ala-His-Ala-Lys-NH_2 were synthesized by the Fmoc chemistry on Rink-amide resin, as described by Chan and White.[46] 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). 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 were removed 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 SunFire reverse-phase C18 column (4.6 × 100 mm, 3.5 μm) and a linear gradient from 100% 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 SunFire reverse-phase C18 column (2.1 × 100 mm, 3.5 μm) and a linear gradient from 100% 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, Foster City, CA, U.S.A.). A 1-μL aliquot of peptide solution was loaded onto the sample plate, air-dried at room temperature, and then 1 μL 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.

Interaction Study with the SPR Biacore T-100 System. Biotin-rhEPO (50 μg/mL in 100 mM HEPES, pH 7.4, 150 mM NaCl) was immobilized at a flow rate of 5 μL/min during 24 s on a Biacore streptavidin sensor chip in the active channel following the manufacturer's instructions. To compensate for matrix, refractive index effects, and non-specific binding of the analyte, a reference was prepared with biocytin 10 μM immobilized at a flow rate of 10 μL/min during 24 s on a Biacore streptavidin sensor chip in the reference channel. Peptides Ac-Phe-His-His-Phe-Ala-His-Ala-Gly-NH_2, Ac-Phe-His-Asn-Phe-Ala-His-Ala-Gly-NH_2, and Gly-Ser-Gly-Ser-Gly-Ser-Gly-NH_2 were injected at different concentrations in 10 mM HEPES, pH 7.4, 150 mM NaCl at a flow rate of 30 μL/min at 20 °C. The sensor chips were regenerated with NaOH 50 mM. Double reference was applied and assessed by fitting to a one-to-one Langmuir binding model provided by the BiaEvaluation 2.0 software. The non-specific peptide ligand Ac-Ser-Gly-Ser-Gly-Ser-Gly-Ser-Gly-NH_2 was studied as a negative control.

Preparation of the Affinity Chromatographic Matrix. Affinity matrixes were prepared as described by Hermanson et al.[39] A 3-fold excess of Ac-Phe-His-His-Phe-Ala-His-Ala-Lys-NH_2 (P1) or Ac-Phe-His-Asn-Phe-Ala-His-Ala-Lys-NH_2 (P2) dissolved in 1 mL of dimethyl sulfoxide (DMSO) was added to 1 mL of NHS-Sepharose 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. After incubation at room temperature for 2 h, the P1-Sepharose and P2-Sepharose matrixes were washed with DMSO. Any remaining unreacted group was blocked by addition of a slight excess of EA at the end of the reaction. A reference matrix (EA-Sepharose) was prepared by blocking all the NHS groups with EA.

Monitoring Protein Coupling. The peptide concentration of the solution prior to the immobilization step and that of the soluble (unbound) peptide remaining in the coupling and wash solutions was monitored by HPLC. The difference of the total peptide mass before and after coupling equaled the total peptide mass immobilized. HPLC analysis was performed in a Shimadzu LC-20AT System with a μBondapack reverse-phase C18 column (4.6 × 150 mm, 5 μm) and a linear gradient from 100% 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. A calibration curve was built with peptide solutions of known concentration.

Peptide-Affinity Chromatography of rhEPO and BSA. Pure samples of rhEPO and BSA in equilibrating buffer (20 mM sodium phosphate with a preselected pH, from 4.00 to 8.25) were loaded on columns (0.5 × 5 cm) filled with P1-Sepharose, P2-Sepharose, or EA-Sepharose. The column was washed with equilibrating buffer at a flow rate of 0.25 mL/min until the absorbance at 280 nm reached its initial value. Thereafter, the elution of the bound protein was accomplished with 100 mM sodium acetate buffer, pH 3.0, 0.25 M NaCl at the same flow rate.

rhEPO Adsorption Isotherm Determination. Chromatographic matrix aliquots, 30 μL, were put into tubes containing 1 mL of pure rhEPO solution at different concentrations in 20 mM sodium phosphate buffer, pH 4.0. The suspension was gently shaken overnight at 24 °C to enable the system to reach its equilibrium. Protein concentration was determined with Bradford reagent. The equilibrium concentration of rhEPO bound to the matrix was calculated as the total amount of rhEPO present at the beginning of the experiment less the amount still in the soluble phase at equilibrium. qm and K_d for each matrix were calculated using a one-to-one Langmuir binding model as described by Chase[47] using the SigmaPlot 2001 regression program (2001 SPSS Inc.).

Peptide-Affinity Chromatography of CHO Cell Culture Spiked with rhEPO. A 2.400 mL portion of CHO cell culture supernatant was spiked with 0.100 mL of rhEPO 1,7 mg/mL. Sample conditioning was performed by loading all the sample (2.5 mL) on PD-10 desalting columns containing Sephadex G-25 equilibrated with 20 mM sodium phosphate buffer, pH 4.0, and eluted with 3.5 mL of the same buffer. One milliliter of the artificial mixture was loaded into columns (0.5 × 5 cm) filled with P1-Sepharose or P2-Sepharose. 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. Thereafter, elution of the bound protein was accomplished with 100 mM sodium acetate buffer, pH 3.0, 0.25 M NaCl at the same flow rate.

Enzyme-Linked Immunosorbent Assay (ELISA). RhEPO concentration was measured by indirect ELISA according to Amadeo et al.[36]

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE was performed as per Laemmli[48] and stained with Coomassie Blue following the standard procedure. Densitometric quantification of gels was performed with a Bio-Rad GS-800 densitometer and an Image Quant TL equipment.

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ABBREVIATIONS

AC, affinity chromatography; AcOH, acetic acid; BSA, bovine serum albumin; CHCA, α-cyano-4-hydroxycinnamic acid; CHO, Chinese hamster ovary; COPAS, Complex Object Parametric Analyzer and Sorter; DIPCDI, 1,3-diisopropylcarbodiimide; DIPEA, *N,N*-diisopropylethylamine; DCR, divide-couple-recombine; DMAP, 4-(*N,N*-dimethylamino)pyridine; DMF, *N,N*-dimethylformamide; DMSO, dimethylsulfoxide; EA, ethanolamine; ESI MS, electrospray ionization mass spectrometry; EPO, erythropoietin; Fmoc, fluorenylmethoxycarbonyl; GMPs, good manufacturing practices; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HMBA, 4-hydroxymethylbenzoic acid; HOBt, 1-hydroxybenzotriazole; K_d, dissociation constant; mAb, monoclonal antibody; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MeCN, acetonitrile; NHS, *N*-hydroxysuccinimide; P1, Ac-Phe-His-His-Phe-Ala-His-Ala-Lys-NH_2; P2, Ac-Phe-His-Asn-Phe-Ala-His-Ala-Lys-NH_2; PBS, phosphate-buffered saline; qm, maximum capacity; rhEPO, recombinant human erythropoietin; RU, resonance units; SA-POD, streptavidin-peroxidase conjugate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SPR, Surface Plasmon Resonance; TBTU, 1-[bis(dimethylamino)methylene]-1*H*-benzotriazolium hexafluorophosphate 3-oxide; *t*Bu, *tert*-butyl; TFA, trifluoroacetic acid; TIS, triisopropyl silane; and TOF, time-of-flight

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