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Development of Mammalian Serum Albumin Affinity Purification Media by Peptide Phage Display

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Several phage isolates that bind specifically to human serum albumin (HSA) were isolated from disulfide-constrained cyclic peptide phage-display libraries. The majority of corresponding synthetic peptides bind with micromolar affinity to HSA in low salt at pH 6.2, as determined by fluorescence anisotropy. One of the highest affinity peptides, DX-236, also bound well to several mammalian serum albumins (SA). Immobilized DX-236 quantitatively captures HSA from human serum; mild conditions (100 mM Tris, pH 9.1) allow release of HSA. The DX-236 affinity column bound HSA from human serum with a greater specificity than does Cibacron Blue agarose beads. In addition to its likely utility in HSA and other mammalian SA purifications, this peptide media may be useful in the proteomics and medical research markets for selective removal of mammalian albumin from serum prior to mass spectrometric and other analyses.

Introduction

Human serum albumin (HSA) is the most abundant protein in serum and serves as a metabolite/drug transporter and an osmotic regulator. It is used clinically in protein replacement therapy and as a plasma expander after blood loss resulting from surgery, burns, trauma, and/or shock. Because patients often receive large quantities of HSA in a single treatment, commercial HSA preparations require a higher degree of purity than other protein therapeutics. Even under these restrictions, commercial albumin preparations (colloid solutions) contain many impurities (*1*, *2*) that may cause adverse side effects in patients (*3*).

Because commercial HSA is purified from serum from human donors, possible human pathogens must be removed by laborious and costly processes. Moreover, current HSA affinity purification, e.g., those relying on Cibacron Blue Sepharose beads (*4*−*7*) and/or conventional chromatography (ion exchange, hydrophobic interaction), do not yield highly pure and correctly folded HSA, making more effective purification schemes desirable (*1*, *2*).

We sought a peptide ligand that could be immobilized on a chromatographic support for an improved affinity purification of HSA. Peptide phage display (PPD) was chosen to identify such a ligand because it has the most desirable properties of available peptide selection technologies (*8*, *9*). First, PPD allows one to rapidly screen several billion peptide sequences against a protein target (∼20 days). Second, binders can be selected iteratively. Third, because all manipulations are carried out in vitro, conditions for peptide binding and release can be specified during the selection process. Finally, the libraries are produced using standard molecular biology techniques without the need for peptide syntheses.

For this study, cyclic peptides constrained via an intramolecular disulfide bond were displayed on the N-terminus of the gene III protein of M13 bacteriophage. Disulfide-constrained loop peptides offer advantages over linear peptides by providing amino acids having some structure for binding to the target (*10*, *11*).

Several disulfide-constrained cyclic peptide libraries with various sizes of loops were utilized to find peptides that bind to HSA. HSA was either immobilized directly on polystyrene plates or was bound to streptavidin beads via biotin. After positive phage isolates were identified by ELISA, their DNA was sequenced and corresponding peptides were synthesized. High affinity peptides were identified using fluorescence anisotropy and then immobilized on amine reactive Sepharose beads for testing HSA capture and purification.

Materials and Methods

Chemicals, Supplies, and Equipment. Human serum (type AB) was purchased from Sigma Chemical Company (St. Louis, MO). Affinity purified monomeric HSA was purchased from ICN (Costa Mesa, CA). All other mammalian albumins were purchased as Fraction V purified material from Sigma Chemical Company. *N*-Hydroxysuccinimide (NHS) activated Sepharose beads and Cibacron Blue Sepharose cartridges (1 mL) were purchased from Amersham-Pharmacia Biotech (Uppsala, Sweden). All empty chromatographic columns were purchased from Omnifit (Rockville Centre, NY). All other chemicals were of the highest grade available. Sera-Mag Streptavidin Magnetic Beads (Level 1 and 5) were purchased from Seradyn (Indianapolis, IN). NHS-LC-LC-Biotin was purchased from Pierce Chemical Company (Rockford, IL) for the biotinylation of HSA. A 2-compo-

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nent tetramethyl benzidine (TMB) system was purchased from Kirkegaard and Perry (Gaithersburg, MD).

Plates were washed with a Bio-Tek 404 plate washer (Winooski, VT). ELISA signals were read with a Bio-Tek plate reader (Winooski, VT). Agitation of 96-well plates was on a LabQuake shaker (Labindustries, Berkeley, CA).

Construction of Phage Display Libraries. All of the cyclic peptide libraries were constructed in a derivative of M13mp18, MANP, having the following modifications: *bla* (Amp^R) gene, modified junction between signal of *iii* and coding region for mature III, and removal of LacZ complementation system. MANP comprises 8164 bases. The *bla* gene was from pGEM3Zf1(+), was bound by *Bam*HI-*Hin*dIII sites at the 5′ end and *Hin*dIII-*Sal*I sites at the 3′ end, replaced bases 6001 through 6432 of M13mp18, and was in the same orientation as the phage genes. The junction between the III signal sequence and mature III was modified as shown in Table 1 with an *Nco*I restriction site embedded in the last three codons of the signal sequence. After the III signal, MANP contained the sequence of BPTI with restriction sites for the enzymes *Sty*I, *Xho*I, *Pfl*MI, *Apa*I, *Bsp*120I, *Eco*O109I, *Psp*OMI, *Bss*HII, *Stu*I, *Bst*Z17I, *Blp*I, *Eag*I, and *Sph*I, which were all unique within MANP. Following BPTI, a unique *Pst*I site and a Factor Xa cleavage site were inserted in a short linker region before mature III.

The DNA encoding the library was synthesized with constant DNA on either side so that the DNA could be PCR amplified using TAQ DNA polymerase (Perkin-Elmer), cleaved with *Nco*I and *Pst*I, and ligated to similarly cleaved vector. The variegated parts were synthesized with TRIM technology (*12*), which incorporates trinucleotides and allows mixtures of any set of amino acid types in any desired proportions. The flanking and varied DNA sequence positions of each library are depicted in Table 1. XL1-Blue MFR′ *E. coli* cells were transformed with the ligated DNA. All of the libraries were constructed in same manner.

HSA Selection and ELISA Protocols for Passively Immobilized HSA Screening. Three phage libraries (TN6-6, TN10-9, and TN12-1, Table 1) were selected against caprylate-bound HSA in Immulon 2HB 96-well plates (DYNEX Technologies, Inc.). For each library, two wells were coated with caprylate-bound HSA (10 μg/mL, 100 μL/well) in PBS (137 mM NaCl, 2.7 mM KCl, 1.5 mM KPO_4 (monobasic), and 3.5 mM KPO_4 (dibasic), pH 7.4) at 4 °C overnight. Excess HSA was then removed, and these wells were blocked with PBS, 0.1% caprylate, and 0.1% Tween-20 (PBSCT) for 2 h at room temperature; wells were washed in PBSCT six times. Next, each phage library was diluted in PBSCT to 10^9 pfu/μL. To each HSA-coated well, 100 μL/well of a given diluted phage library was added (10^{11} total phage). Phage were incubated at room temperature for 2 h and then washed with PBSCT six times. Using 100 μL/well of CBS (50 mM sodium citrate, 150 mM NaCl, pH 2) at pH 2.0 for 5 min, bound phage were eluted and then neutralized with 2 M Tris, pH 8, at 250 μL/well. All elutions were pooled for a 1.5 mL total volume.

The pooled 1st round elution was mixed with aliquots of XL1-Blue MRF′ *E. coli* cells that had been chilled on ice after having been grown to mid-logarithmic phase. After approximately 15 min at room temperature, the phage/cell mixture was spread onto a bioassay dish (243 mm × 243 mm × 18 mm, Nalge Nunc) containing 250 mL of NZCYM agar with 50 μg/mL of ampicillin. The plate was incubated overnight at 37 °C. Amplified phage were harvested from the bioassay dish.

A second round of selection was performed as in the first round except phage input was decreased to approximately 3×10^{10} pfu. The 2nd round elution was plated on a large square plate (243 mm × 243 mm × 18 mm Nunc bioassay plates containing NZCYM agar and 100 μg/mL ampicillin) at a density of about 10^4 colonies/plate and grown overnight at 37 °C. Colonies were picked (BioRobotics BioPick, Cambridge, U.K.) into 96-well flat-bottom plates (Greiner Labortechnik, Germany) containing 100 μL/well of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.5). From these plates, ten 96-well overnight culture plates for ELISA analysis were prepared.

For analysis of caprylate-HSA as a target, Immulon 2HB plates were prepared by the addition of 340 μL/well of caprylate-HSA at 5.6 μg/mL; plates were incubated overnight at 4 °C. Coated plates were washed with PBSCT six times (300 μL/well). To account for plate binders, empty plates were also washed with PBSCT and used as negative controls. An equal volume of each phage clone isolate was added to a plate well containing 70 μL of PBSCT in both the target plate and the control plate. Plates were incubated for 1 h at room temperature. The plates were washed seven times with PBSCT (300 μL/well). To each well, a 1:10,000 dilution of Pharmacia HRP-αM13 conjugate in PBSCT was added at 100 μL/well. Plates were incubated for 1 h. After incubation, plates were washed six times with PBSCT (300 μL/well). Following the wash, a 1:1 mixture of the 2-component TMB substrate mixture (Kirkegaard and Perry) at 100 μL/well was added; plates were read at 620 nm after 30 min of incubation.

HSA Selection and ELISA Protocols for Soluble Capture Screening. TN6-6 and TN12-1 phage libraries were screened separately against caprylate-biotinylated-HSA in solution. For this procedure, Level 1 Sera-Mag streptavidin magnetic beads (Seradyn) were washed five times in PBSCT. To remove bead binders, 3 to 4×10^{11} plaque-forming units (pfu) from a library in 100 μL of PBSCT were introduced to an aliquot of PBSCT washed beads. The beads were agitated in a microfuge tube for 10 min, beads were pelleted at 14,000 × *g*, and the supernatant containing phage was transferred to a fresh tube with another aliquot of PBSCT washed beads. A total of 5 × 10 min exposures of phage to aliquots of beads were used.

One hundred microliters of the processed phage solution was made 1 μM in caprylate-biotinylated-HSA by the addition of 2 μL of a 50 μM stock solution of biotinylated HSA. After 1 h, the mixture was added to an aliquot of Level 5 Sera-Mag streptavidin magnetic beads, which had previously been washed 5 times with PBSCT. The tube was placed on a Labquake shaker for 5 min to allow capture of free and phage bound caprylate-biotinylated-HSA phage complexes onto the beads. Beads were washed as rapidly as possible with 5 × 1 mL PBSCT + 0.1 mM biotin using a magnetic stand (Promega, Madison, WI) to separate the beads from the PBSCT + 0.1 mM biotin which was discarded. Phage still bound to beads were eluted with 2 × 250 μL aliquots of CBS, pH 2, 15 min for each elution. The eluates were neutralized with 100 μL of 2 M Tris, pH 8. Eluted phage were amplified and harvested as above.

A second, third and fourth round of selection were performed as in the first round except phage input was decreased to approximately 3×10^{10} pfu.

For analysis of biotinylated caprylate-HSA as a target, Immulon 2HB plates were prepared by the addition of 340 μL/well of streptavidin solution (5 μg/mL, dissolved in 0.1 M $NaHCO_3$, pH 8.5) and incubated overnight at

Table 1. (Top) Signal::BPTI::Mature Junction in MANP Gene *iii*; (Bottom) Constant and Varied Peptide Sequence in Phage Libraries

```
          ----- III signal sequence ---------------------------->
           M   K   K   L   L   F   A   I   P   L   V   V   P   F
1574 caac GTG aaa aaa tta tta ttc gca att cct tta gtt gtt cct ttc

     III signal---->| cleavage site
      Y   S   M   A
1620  tat tCC ATG Gcc
          NcoI

     BPTI ------------------------------------------->
      R   P   D   F   C   L   E   P   P   Y   T   G   P   C
1632  cgc cca gat ttc tgt CTC GAG cca CCA TAC ACT GGG CCC Tgc
                         XhoI     PflMI
                         AvaI                    Bsp120I
                                                 EcoO109I

      K   A   R   I   I   R
1674  aaa GCG CGC atc atc cgc
          BssHII

      Y   F   Y   N   A   K   A   G   L   C
1692  tat ttc tac aat gct aaa gcA GGC CTg tgc
                               StuI

      Q   T   F   V   Y   G   G   C   R   A   K   R
1722  Cag acc ttt GTA TAC Ggt ggt tgc cgt GCT AAG Cgt
                  Bst1107I                EspI

      N   N   F   K   S   A   E   D
1758  aac aac ttt aaa tCG GCC Gaa gat
                       EagI

      C   M   R   T   C   G   S   A   D   P
1782  tGC ATG Cgt acc tgc ggt tCT GCA Gat cct
       SphI                    PstI

      S   Y   I   E   G   R   I   V   G   S   A
1812  tca tac att gaa ggt cgt att gtc ggt agc gcc
          Factor Xa site

     Mature III: Domain 1 ------------->
      A   E   T   V   E   S   C   L   A
1845  gct gaa act gtt gaa agt tgt tta gca ....
```

Library Designation	Left Flank	Insert	Right Flank
TN6-6	AEGTGS	X1X2X2 CX2X2X2X2C X2X2X1 X1 = A D FGH L NPQRS VWY X2 = A DEFGHIKLMNPQRSTVWY	APGPTDS
TN10-9	AEGTGS	X1X1X2 CX3X3X3X3X3X3X3C X2X1X1 X1 = D F H L NP RS WY X2 = A D FGH L NPQRS VWY X3 = A DEFGHIKLMNPQRSTVWY	APGPTDS
TN12-1	AEGTGD	X1X1X2 CX2X2X2X2X2X2X2X2X2C X2X1X1 X1 = A D FGH L NP RS WY X2 = A DEFGHIKLMNPQRSTVWY	DPGPTDN

4 °C. Coated plates were washed with PBSCT six times (300 μL/well). To half of the streptavidin plates, 100 μL of 5 μg/mL biotinylated HSA target was added to each well and was incubated at room temperature for 2 h. Blank streptavidin plates are a negative control to detect streptavidin binders. An equal volume of each phage isolate (50 μL) was added to a well containing 50 μL PBSCT in both the target and control plates and incubated for 1 h at room temperature. The plates were washed seven times with PBSCT (300 μL/well). To each well, 100 μL of a 1:5,000 dilution of Pharmacia HRP-αM13 conjugate in PBSCT was added. Plates were incubated for 1 h. After incubation, plates were washed six times with PBSCT (300 μL/well). Then a 1:1 mixture of the 2-component TMB substrate mixture at 100 μL/well was added; plates were read at 620 nm after 30 min.

Peptide Synthesis and Fluorescein Labeling. Peptides corresponding to positive phage isolates were synthesized on solid phase using 9-fluorenylmethoxycarbonyl protocols and purified by reverse phase chroma-

tography. Peptide masses were confirmed by electrospray mass spectrometry, and peptides were quantified by absorbance at 280 nm. For synthesis, the phage-derived sequence that was not varied (**bold**) surrounding each selected sequence was retained and a Gly-Gly-Gly-Lys-NH_2 or (6-aminohexanoic acid)-Lys-NH_2 linker was added to the C-terminus of each peptide, yielding peptides with sequences such as Ac-**AEGTGS**(3)**C**(4−10)**C**(3)**D(or A)-PE***GGG*K-NH_2, where the numbers in parentheses are the numbers of varied residues in the libraries and the underline indicates a disulfide-closed loop. In some cases, the *GGG* spacer was replaced by 6-aminohexanoic acid (X). For peptides with selected lysine residues, these were protected with 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methybutyl (ivDde) (*13*). ivDde allows selective coupling to the C-terminal lysine, is not removed during peptide cleavage, and can be removed after coupling with 2% hydrazine in DMF or 0.5 M hydroxylamine, pH 8, in water.

Fluorescence Anisotropy Measurements. Fluorescence anisotropy measurements were performed in 384-well microplates in a volume of 10 μL in binding buffer using a Tecan Polarion fluorescence polarization plate reader. The concentration of fluorescein labeled peptide was held constant (20 nM), and the concentration of HSA was varied. For the PBS (pH 7.4) binding conditions, the same selection buffer recipe (±0.1% caprylate) was used. For the pH 6.2 binding conditions, a 3 mM phosphate, 0.01% Tween-20 buffer (±0.14 M NaCl) was used. For pH 9.1 binding conditions, a 3 mM sodium bicarbonate buffer, 0.01% Tween-20 buffer (±0.14 M NaCl) was used. Binding mixtures were equilibrated for 10 min in the microplate at 30 °C before measurement. The observed change in anisotropy was fit to the equation below via nonlinear regression to obtain the apparent K_D. Equation 1 assumes that the synthetic peptide and HSA form a reversible complex in solution with 1:1 stoichiometry:

$$r_{obs} = r_{free} + (r_{bound} - r_{free}) \times \frac{(K_D + HSA + P) - \sqrt{(K_D + HSA + P)^2 - 4 \cdot HSA \cdot P}}{2 \cdot P} \quad (1)$$

where r_{obs} is the observed anisotropy, r_{free} is the anisotropy of the free peptide, r_{bound} is the anisotropy of the bound peptide, K_D is the apparent dissociation constant, HSA is the total HSA concentration, and P is the total fluorescein-labeled peptide concentration.

Peptide Immobilization on NHS-Sepharose. For producing immobilized peptide test columns, 5 μmol of each peptide was dissolved in DMSO in a minimal volume and then added to 1 mL of NHS-Sepharose beads, which had been washed once with DMSO. The immobilization reaction was initiated by addition of diisopropylethylamine to 2% v/v. After 4 h of slow mixing on a shaker table at room temperature, the reaction was quenched by the addition of an equal volume of 0.5 M hydroxylamine, pH 8, in water. For those peptides with ivDde-protected internal lysines, the hydroxylamine quench treatment also removed the ivDde group. To ensure complete removal of ivDde, the reaction was continued overnight at room temperature. Once quenched and deprotected, the immobilized peptide media were washed at least three times with water to remove solvent and unbound peptide. Noncovalently bound peptide was eluted from the resin by washing at least three times in 30 mM phosphoric acid, pH 2. After washing, the resin was resuspended in water as a 50% v/v mixture. A 50-μL aliquot of beads suspension was examined to determine the ligand density on the resin by quantitative amino acid analysis. Finally, the resin slurry was packed into 0.35 mL Omnifit glass columns (3 mm × 50 mm, diameter × length) for analytical testing.

For larger preparative columns, the amounts of peptide and Sepharose beads were scaled up proportionally, and the final batches of peptide-coupled resin were packed into larger 10 mL Omnifit columns (10 mm diameter).

HSA Column Testing. For analytical affinity column testing, albumin was dissolved at 1 mg/mL concentration in 3 mM phosphate, pH 6.2, 0.01% Tween-20 (EB1). One milliliter of albumin solution was passed through each column (0.35 mL) previously equilibrated in EB1. The columns were washed with EB1 and then eluted with 100 mM Tris, pH 9.1 (flowrate, 0.5 mL/min = 430 cm/h for all steps). A Biorad BioLogic System was used throughout with absorbance monitoring at 280 nm.

To test the binding properties of the immobilized DX-236 peptide, a preparative DX-236-Sepharose affinity column (10 mL) was made. Human serum was dialyzed against 3 mM phosphate, pH 6.2, 20 mM NaCl, 0.01% Tween-20 (EB2). One hundred microliters of dialyzed serum was injected on the preparative DX-236-Sepharose column, which was previously equilibrated with EB2. The column was washed with EB2, followed by a gradient between 20 and 44 mM NaCl, and finally the HSA was eluted with 100 mM Tris, pH 9.1. For all steps, the flowrates were 5 mL/min.

For Cibacron Blue Sepharose column testing, human serum was dialyzed into PBS, pH 7, 0.01% Tween-20 (equilibration buffer). One hundred microliters of dialyzed serum was injected on a 1-mL Cibacron Blue Sepharose cartridge (Amersham Pharmacia Biotech), which was previously equilibrated with equilibration buffer. The column was washed with the same equilibration buffer and then HSA was eluted with PBS, 1 M NaCl, pH 7. For all steps, the flowrates were 1 mL/min.

For all of the above analyses, the amount of albumin in each fraction was determined using C4 reverse phase chromatography and an albumin standard curve. The bound HSA (mg) per column volume (mL) was determined by dividing the amount of eluted HSA by the column volume (0.35 mL). All reverse phase analyses were done using the 214 nm absorbance signal.

Results and Discussion

In this study, several peptides that bind HSA with high affinity were identified using PPD to develop robust HSA affinity purification media. The disulfide constrained cyclic peptide format was chosen over linear peptides or antibodies, since HSA is known to bind small metabolites having rigid structures (*14*−*17*). We immobilized HSA for selection, either directly on polystyrene plates, or indirectly by immobilizing biotinylated HSA on streptavidin beads or plates. Following 2−4 rounds of selection, single phage isolates were grown in bacterial colonies, picked from plates, and assayed for HSA binding by ELISA. The ELISA positive isolates were sequenced, and corresponding peptides were synthesized for affinity determination by fluorescence anisotropy. Peptides that bound well to HSA were immobilized on Sepharose beads and tested for HSA binding.

Three disulfide-constrained cyclic peptide phage display libraries (TN6-6, TN10-9, and TN12-1, Figure 1D) were pooled in approximately equal amounts. The pooled phage libraries were used in selections against HSA

Table 2. Direct Fluorescence Anisotropy Data[a]

		K_D (μM)				
	pH	**6.2**	6.2	7.4	**7.4**	9.1
	NaCl (±0.14 M)	**-**	**+**	**+**	**+**	**+**
DX- Sequence	**Caprylate (±0.1%)**	**-**	**-**	**-**	**+**	**-**
232 Ac-AEGTGSVAWCTIFLCLDVAPEGGG**K**-NH_2		**0.22**	0.5	0.18	**1.05**	N.A.
295 Ac-AEGTGSFKICDQWFCLMPAPE-X-**K**-NH_2		**1.8**	>100	86	**210**	N.A.
296 Ac-AEGTGSHVGCNNALCMQYAPE-X-**K**-NH_2		**17**	>100	76	**>200**	N.A.
297 Ac-AEGTGSWKVCDHFFCLSPAPE-X-**K**-NH_2		**18**	>200	>200	**>200**	N.A.
298 Ac-AEGTGSNHGCWHFSCIWDAPE-X-**K**-NH_2		**1.9**	>200	127	**73**	>200
238 Ac-AEGTGSFRNCEPWMLRFGCNPRAPEGGG**K**-NH_2		**4.8**	61	79	**110**	N.A.
234 Ac-AEGTGDADFCEGKDMIDWVYCRLYDPEGGG**K**-NH_2		**2.5**	85	109	**118**	N.A.
236 Ac-AEGTGDFWFCDRIAWYPQHLCEFLDPEGGG**K**-NH_2		**1.9**	8.7	35	**26.8**	99
313 Ac-AEGTGDDWDCVTRWANRDQQCWGPDPE-X-**K**-NH_2		**9.5**	80	>200	**121**	90
315 Ac-AEGTGDDWDCVTRWANRDQQCWALDPE-X-**K**-NH_2		**13**	>200	113	**>100**	N.A.
317 Ac-AEGTGDDWDCVTDWANRHQHCWALDPE-X-**K**-NH_2		**6.7**	>200	74	**45**	N.A.
319 Ac-AEGTGDDWQCVKDWANRRRGCMADDPE-X-**K**-NH_2		**17**	>200	>200	**26**	N.A.
321 Ac-AEGTGDRNMCKFSWIRSPAFCARADPE-X-**K**-NH_2		**0.9**	9	84	**75**	N.A.
- Fluorescein		**30**	>200	>200	**>200**	N.A.
CytC **X**-GAQGHTVEK-NH_2		**335**	N.A.	N.A.	**N.A.**	N.A.

[a] **232**, **236**, **321** indicates peptides used for affinity columns; **K**, **X** indicates site fluorescein labeling.

immobilized directly on polystyrene microtiter plates. For each round of selection, phage were incubated with HSA in PBS, 0.1% sodium caprylate, 0.1% Tween-20, pH 7.4. To obtain a more homogeneous population of structurally stabilized HSA molecules, sodium caprylate was included. Caprylate is known to stabilize HSA against temperature-induced denaturation and proteolytic digestion (most likely by promoting a tightening of the C-terminal domain), and sodium caprylate promotes release of HSA-bound metabolites from serum-purified albumin (*18*−*22*).

After two rounds of selection, phage peptide selection on directly immobilized HSA yielded several phage isolates (232, 234, 236, and 238) that showed positive HSA binding by ELISA (Figure 1A). Phage isolate 232 showed the highest ELISA signal. DNA sequencing identified phage isolates 232, 234, 236, and 238 as members of the TN6, TN12, TN12, and TN10 libraries, respectively (Figure 1D, Table 1, and Table 2). The phage isolates all possessed unique DNA sequences, which is not unusual for isolates taken from the second round of selection.

Proteins that change conformation as a function of their environment (pH, salt, temperature), such as HSA, often change conformation on binding plastic. Numerous studies have shown that albumin shows losses in α helix and gains in β sheet and random coil upon hydrophobic surface adsorption (*23, 24*). In selections against surface adsorbed HSA, the isolated phage displayed peptides may bind specifically to the plastic-bound conformer over the soluble form (*25*). To circumvent this potential selection problem, we also employed a second selection scheme using biotinylated HSA (bioHSA) and magnetic streptavidin beads. In this selection, bioHSA was incubated with the phage library in solution for 1 h; then, HSA-binding phage were captured by the addition of magnetic streptavidin beads for 15 min. As in the previous selection, all incubations were done in PBS, 0.1% sodium caprylate, and 0.1% Tween-20. Unlike the first selection, the libraries were not pooled and TN6-6 and TN12-1 were selected separately. Selection from these libraries yielded several positive isolates from each library that were identified by ELISA (Figure 1B,C and Table 2). Phage isolates 298 (from library TN6) and 321 (from library TN12) showed the highest ELISA signal.

DNA from all ELISA-positive phage isolates was sequenced, and corresponding peptides were synthesized. A small amount of constant phage sequence surrounding each selected sequence region was retained in the synthesized peptides because these residues could affect binding. To determine HSA binding affinity, each peptide was labeled with fluorescein on a C-terminal lysine side

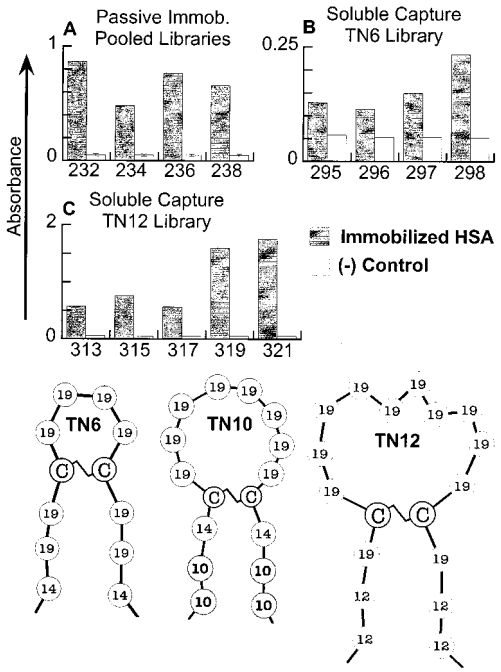


Figure 1. Phage ELISA data. (A) Directly immobilized HSA selection/pooled libraries. (B) Soluble bioHSA selection/TN6 library. (C) Soluble bioHSA selection/TN12 library. Individual phage isolates were assayed for HSA binding using ELISA as described in Materials and Methods. (D) Formats of the constrained loop peptides displayed on phage in libraries TN6, TN10, and TN12. The numbers in each circle represent the possible number of amino acids in each position.

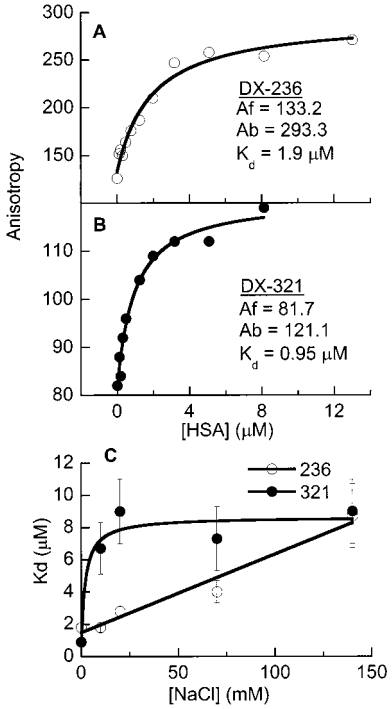


Figure 2. Fluorescence anisotropy. (A) Direct binding data for DX-236 at pH 6.2, no NaCl. (B) Direct binding data for DX-321 at pH 6.2, no NaCl. (C) Salt titration for DX-236 and DX-321 at pH 6.2. Fluorescence anisotropy measurements conducted as described in Materials and Methods.

chain. Using fluorescence anisotropy, the affinity of each peptide was determined in PBS (pH 7.4), 0.01% Tween-20, with or without 0.1% sodium caprylate. Most of the peptide affinities for HSA in the screening buffer, in the presence or absence of caprylate, were lower than expected based on our previous experience screening phage peptide libraries against proteins. Historically, the majority of phage display-derived peptides bind their selected targets with dissociation constants (K_D) less than 5 μM. As shown in Table 2, only peptide DX-232 had a K_D below 5 μM under the tested conditions. Additionally, most of the peptides did not show a dramatic dependence on caprylate for binding in PBS.

On the basis of the modest dissociation constants derived above, we proceeded to evaluate the HSA binding of each peptide under a variety of pH, salt concentration (±0−140 mM NaCl), and caprylate (±0.1%) conditions. We discovered that many of the peptides bound better at a lower pH (3 mM phosphate, pH 6.2) and in the absence of added salt (Table 2). Since HSA bound fluorescein in the no salt, pH 6.2 buffer with a K_D of 30 μM, we have taken this as the upper limit for specific HSA binding by these peptides (Table 2). In keeping with our observation of pH-dependent peptide binding to HSA, others have observed that HSA undergoes dramatic pH-dependent structural changes (*26*). HSA transitions through a series of 5 different conformational states from pH 2 to pH 11 (*17*). The predominant form found in serum is the N form, which is present in pH range of 4.3−8. Even at constant pH, HSA is also extremely flexible and rapidly changing in shape. This flexibility most likely allows albumin to bind such a wide array of molecules of different shape, charge, and hydrophobicity (*27*).

Although selection was conducted in PBSCT, it is evident from Table 2 that, at pH 6.2, all 13 peptides examined had lower affinity for HSA in the presence of 140 mM NaCl than they did at low concentrations of added salt. At pH 6.2 in the no salt buffer, DX-232, DX-236, and DX-321 were the only peptides that had K_D values of less than 2 μM. Moreover, in contrast to the other peptides studied, these peptides were relatively resistant to the addition of 140 mM NaCl, i.e., K_D(140 mM NaCl) < 10 × K_D(no salt) (Table 2, Figure 2A, B). The K_D of peptide DX295, for example, increased more than 10-fold from 1.8 to >100 μM. More rigorous analysis revealed that the K_D of DX-321 increased sharply from 0.9 to 9 μM upon the addition of 10 mM NaCl (Figure 2C). In contrast, the K_D of DX-236 increased linearly from 1.9 to 8.7 μM upon titrating up to 140 mM NaCl (Figure 2C). Since chloride is known to bind HSA and compete with some Sudlow site II ligands (*28, 29*), e.g., tryptophan, these results suggest that both peptides may be binding in this site on HSA. The gradual salt dependence of peptide DX-236, however, suggests that some salt could be added during HSA purification to reduce nonspecific binding of other proteins to the chromatography matrix.

Truncation variants of peptide DX-236 were examined to determine the contribution of different portions of the peptide to HSA binding affinity (no salt, pH 6.2 buffer). This fluorescence anisotropy analysis (Table 3) demonstrated that the surrounding C and N terminal residues do influence HSA binding by the ligand. Truncation of either the C- or N-terminus resulted in an approximately 4-fold increase in the K_D. In keeping with this result, truncation of both C- and N-termini resulted in an additive 8-fold increase in the K_D. Therefore, although the flanking residues make a positive contribution to HSA binding, the 10 selected residues of the core sequence contribute most to the binding energy. When truncation is extended to include both cysteine residues, the residual linear core sequence of peptide DX-236 showed low affinity binding (K_D, 125 μM), consistent with

Table 3. (Top) DX-236 Truncation Peptides;[a] (Bottom) DX-236 Alanine Mutant Peptides[b]

Sequence	K_D (μM)
Ac-AEGTGDFWFCDRIAWYPQHLCEFLDPEGGGK-NH_2	1.9
Ac-FWFCDRIAWYPQHLCEFLDPEGGGK-NH_2	8.9
Ac-CDRIAWYPQHLCEFLDPEGGGK-NH_2	8.7
Ac-AEGTGDFWFCDRIAWYPQHLCEFLGGGK-NH_2	9.9
Ac-AEGTGDFWFCDRIAWYPQHLCGGGK-NH_2	8.9
Ac-CDRIAWYPQHLCGGGK-NH_2	16.0
Ac-DRIAWYPQHLGGGK-NH_2	125

		K_D (μM)		
DX-	Sequence	pH 6.2	PBS	Binding
236	Ac-AEGTGDFWFCDRIAWYPQHLCEFLDPEGGGK-NH_2	1.7	35.0	
737	Ac-AEGTGDFWFCDRIAWYPQHLCEFL**A**PEGGGK-NH_2	1.7	10.5	++
739	Ac-AEGTGDFWFCDRIAWYPQHLCEF**A**DPEGGGK-NH_2	2.1	47.0	-
741	Ac-AEGTGDFWFCDRIAWYPQHLCE**A**LDPEGGGK-NH_2	1.7	44.0	-
743	Ac-AEGTGDFWFCDRIAWYPQHLC**A**FLDPEGGGK-NH_2	1.7	6.6	++
745	Ac-AEGTGDFWFCDRIAWYPQH**A**CEFLDPEGGGK-NH_2	2.0	34.5	
747	Ac-AEGTGDFWFCDRIAWYPQ**A**LCEFLDPEGGGK-NH_2	1.7	4.2	++
749	Ac-AEGTGDFWFCDRIAWYP**A**HLCEFLDPEGGGK-NH_2	2.2	22.5	+
751	Ac-AEGTGDFWFCDRIAWY**A**QHLCEFLDPEGGGK-NH_2	1.3	43.0	
753	Ac-AEGTGDFWFCDRIAW**A**PQHLCEFLDPEGGGK-NH_2	1.4	26.0	+
755	Ac-AEGTGDFWFCDRIA**A**YPQHLCEFLDPEGGGK-NH_2	1.4	44.0	-
757	Ac-AEGTGDFWFCDR**A**AWYPQHLCEFLDPEGGGK-NH_2	1.1	4.5	++
759	Ac-AEGTGDFWFCD**A**IAWYPQHLCEFLDPEGGGK-NH_2	1.5	17.5	++
761	Ac-AEGTGDFWFC**A**RIAWYPQHLCEFLDPEGGGK-NH_2	1.6	5.0	++
763	Ac-AEGTGDFW**A**CDRIAWYPQHLCEFLDPEGGGK-NH_2	1.1	43.0	
765	Ac-AEGTGDF**A**FCDRIAWYPQHLCEFLDPEGGGK-NH_2	2.3	36.5	
767	Ac-AEGTGD**A**WFCDRIAWYPQHLCEFLDPEGGGK-NH_2	5.1	26.5	
769	Ac-AEGTG**A**FWFCDRIAWYPQHLCEFLDPEGGGK-NH_2	5.1	27.0	

[a] Binding Affinities determined in pH 6.2, low salt buffer. [b] Binding: −, 25% lower affinity, +, 25% higher affinity, ++, 50% higher affinity in PBS.

the idea that a disulfide-constrained structure is involved in the higher affinity HSA binding behavior of the core sequence.

To more precisely map the interaction between DX-236 and HSA, a series of alanine mutants were synthesized and tested for binding in both the no salt, pH 6.2 and PBS (pH 7.4) buffers (Table 3). Historically, if a single alanine mutant peptide shows a lower affinity for the target, then the mutated residue is deemed important to the peptide-target binding interaction. In the no salt, pH 6.2 buffer, only DX-767 (F7A) and DX-769 (D6A) bound with lower affinity to HSA relative to the DX-236. Since these residues are on the N-terminal flank of the peptide and not inside the selected constrained loop, we speculated that no one residue was particularly crucial to the binding event under the no salt, pH 6.2 buffer condition.

Unlike the binding affinities determined in no salt, pH 6.2 buffer, the set of alanine mutant peptides bound HSA with a wide range of affinities in the PBS buffer (pH 7.4).

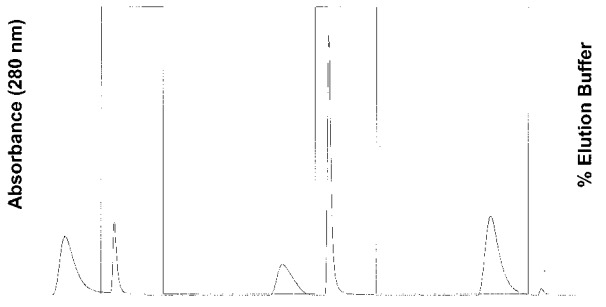
DX-755 (W15A), DX-741 (F23A), and DX-739 (L24A) bound HSA with at least 25% lower affinity than DX-236 (1.25 times the K_D of DX-236). In PBS, these hydrophobic residues may play an important role in HSA binding. Unexpectedly, many of the alanine mutant peptides bound HSA with higher affinities than DX-236. DX-761 (D11A), DX-759 (R12A), DX-757 (I13A), DX-747 (H19A), DX-743 (E22A), and DX-737 (D25A) bound HSA with at least 50% higher affinity than DX-236 (0.5 times the K_D of DX-236). Since all of these residues, except I13, are acidic or basic residues, these particular residues may be mediating a repulsive interaction between HSA and the peptide in PBS. Alternatively, the addition of alanine (a small, hydrophobic residue) at key positions in the peptide may add additional flexibility or hydrophobicity that aids in peptide binding.

Peptides DX-232, DX-236, and DX-321 had K_D values of <2 μM in no salt buffer at pH 6.2 and, of those we examined, were the only peptides having K_D values of <10 μM in the presence of 140 mM NaCl at pH 6.2. At ligand densities between 1 and 5 μmol immobilized peptide per milliliter of affinity media, we and others (*30*, *31*) have observed that peptides must bind their target protein with solution K_D values of <10 μM to bind with sufficient capacity in immobilized form. Therefore, only these three peptides were pursued for affinity chromatography development. Each peptide was immobilized on NHS-Sepharose beads using the procedure outlined in Materials and Methods. In an effort to preserve the original HSA binding structure, the peptides were immobilized on the same C-terminal lysine used for fluorescein labeling. The ligand densities for DX-321-, DX-236-, and DX-232-Sepharose bead columns were 3.2, 0.8, and 2.4 μmol/mL, respectively. Each column was tested for the ability to bind 1 mg of HSA in the optimal binding buffer, 3 mM sodium phosphate, 0.1% Tween-20, pH 6.2 (EB1).

Unexpectedly, each immobilized peptide column performed distinctly in the initial HSA binding tests (Figure 3). Although in solution DX-232 bound HSA with the highest affinity, the DX-232-Sepharose column performance was the worst of the three, binding no detectable HSA. In contrast, the DX-236-Sepharose column was the best performer and quantitatively bound the entire 1 mg injection (0.15 μmol). At higher HSA loads in the no salt, pH 6.2 buffer, the DX-236-Sepharose column (0.35 mL) bound upward of 4 mg of HSA, which corresponds to a dynamic capacity of greater than 11 mg/mL. With the addition of 20 mM NaCl to this same buffer, the 100% breakthrough capacity did not decrease and remained constant at approximately 10 mg/mL (data not shown). Ligand utilization on this resin is high: greater than 19% (0.8 μmol/mL resin can capture >0.15 μmol HSA/mL resin). The DX-321-Sepharose column was an intermediate performer and captured about 40% of the total material in this test.

Since some of the peptides showed a sharp increase in K_D as the pH was increased to 9.1 with 140 mM NaCl (Table 2), we speculated that a 100 mM Tris, pH 9.1 buffer would elute HSA effectively from the peptide-Sepharose columns. As expected, the Tris elution buffer eluted all of the bound HSA from both DX-236- and DX-321-Sepharose columns.

To test the binding specificity of DX-236 and DX-321 for HSA over other albumins, we determined their dissociation constants against a panel of mammalian albumins both in 3 mM sodium phosphate, pH 6.2, and in PBS (Table 4). In the 3 mM phosphate, pH 6.2 buffer,



Peptide Column	Fraction	µg HSA	% Initial	HSA (mg) Volume (ml)
DX-321	FT	554	55.4	
	Elution	370	37.0	1.1 mg/ml
DX-236	FT	0	0	
	Elution	947	94.7	2.7 mg/ml

Figure 3. HSA Binding Profiles of DX-321 (3.2 mmol/mL), DX-236 (0.8 mmol/mL), and DX-232 (2.4 mmol/mL) Sepharose columns in 3 mM phosphate, pH 6.2, 0.01% Tween-20. One milligram of HSA was injected onto each column (0.35 mL) previously equilibrated in 3 mM phosphate, pH 6.2, 0.01% Tween-20 (EB1). The columns were washed with EB1 and then eluted with 100 mM Tris, pH 9.1 (flowrate, 1 mL/min). The table contains reverse phase analytical results from the DX-321 and DX-236 runs. The percent of initial HSA loaded in each fraction is shown (% initial, μg HSA in fraction/1000 μg). The bound HSA (mg) per column volume (mL) was determined by dividing the amount of eluted HSA by the column volume (0.35 mL). HSA showed no binding to a control hydrolyzed NHS-sepharose column (data not shown).

Table 4. Species Specificity Data

			DX-236		DX-321	
species	pI	% identity	pH 6.2, 0 M NaCl	PBS, 0.14 M NaCl	pH 6.2, 0 M NaCl	PBS, 0.14 M NaCl
human	5.67	100	1.9	35	**0.9**	84
rhesus	5.67	93.2	1.1	23	38	82
bovine	5.60	75.6	1.1	13.3	**21**	>200
goat	NA	NA	1.6	23	**95**	83
pig	5.75	75.0	0.5	12	21	>200
rabbit	5.65	75.0	0.5	18	32	>200
rat	5.80	73.2	1.6	25	23	117
mouse	5.53	72.0	5.5	32	>**200**	>200
chicken, egg	5.19		>200	>200	>200	>200

fluorescein-labeled DX-236 bound albumin from each species tested with $K_D < 2$ μM, with the exception of murine serum albumin (MSA, $K_D = 5$ μM). In PBS, binding of DX-236 to albumin from all species was weakened between 5-fold (mouse) and 36-fold (rabbit) relative to the low salt, pH 6.2 buffer. Unlike DX-236, Cibacron Blue dye binds albumins from different species with a wide range of dissociation constants (*32*). Fortunately, for HSA processing work, Cibacron Blue binds human serum albumin with the highest affinity ($K_D \approx$ 16 μM against immobilized Cibacron Blue sepharose, Tris/HCl buffer, pH 8.6) relative to other mammalian albumins (bovine, 196 μM; rat, 80 μM; rabbit, 150 μM; sheep, 150 μM; goat, 150 μM; horse, 51 μM) (*32*).

Fluoresceinated DX-321 bound each mammalian albumin with a substantially higher K_D compared to HSA in the no salt, pH 6.2 buffer. In particular, MSA bound DX-321 with a K_D greater than 200 μM compared to HSA, which bound DX-321 with a submicromolar K_D (Table 4). Similar to Cibacron Blue (*32*), all the other nonhuman albumins also bound weakly to DX-321 and had K_D

Table 5. Mammalian Serum Albumin Testing[a] with DX-236 and DX-321

albumin	initial protein load	DX-236 FT	DX-236 elution	DX-236 HSA/vol[b]	DX-321 FT	DX-321 elution	DX-321 HSA/vol[b]
bovine	1 mg	0	0.72	2.04	0.86	0.15	0.42
goat	1 mg	0	0.79	2.26	0.93	0.11	0.32
mouse	0.5 mg	0.05	0.59	1.68	0.49	0.13	0.36

[a] One milligram of albumin was injected onto each column (0.35 mL) previously equilibrated in 3 mM phosphate, pH 6.2, 0.01% Tween-20. The columns were washed with equilibration buffer and then eluted with 100 mM Tris, pH 9.1 (flowrate, 1 mL/min). Table below contains reverse phase analytical results from the DX-321 and DX-236 runs. [b] In mg per mL.

values at least 10 times greater than that for HSA. In PBS, however, the differences in DX-321 binding affinities to HSA and the other albumins were less pronounced than those found at pH 6.2. As a negative control, we tested each peptide (DX-236 and DX-321) for binding to chicken ovalbumin in both buffers, since chicken ovalbumin is not homologous to HSA in amino acid sequence. Neither peptide showed any significant binding to ovalbumin (Table 4).

From the above binding analysis, we predicted that immobilized DX-236-Sepharose beads could be used to purify mammalian albumins, whereas DX-321-Sepharose beads may show differential binding to other albumins, in the pH 6.2 buffer. To evaluate this prediction, we tested the DX-236- and DX-321-Sepharose columns used previously (Figure 3) for capture of bovine serum albumin (BSA), goat serum albumin (GSA), and murine serum albumin (MSA) from pH 6.2, no salt buffer (EB1). As expected from the results in Table 4, DX-236-Sepharose bound all three albumins extremely well (Table 5). DX-236-Sepharose media may prove useful as a pan-albumin binder for the affinity purification of nearly any mammalian albumin from serum. This behavior contrasts with that of Cibacron Blue, which binds with higher affinity to human and baboon albumin over other mammalian albumins (*4*). DX-236 coupled media, however, could not be used to purify HSA of nonhuman serum albumins in transgenic mammalian expression systems, e.g., HSA expressed in murine milk (*33*). However, it could effectively purify HSA from a recombinant system devoid of mammalian albumin, e.g., yeast (*34, 35*), tobacco (*36*), chicken eggs, or potato (*36*). In addition to albumin purification applications, DX-236-Sepharose could also be used to deplete albumin from serum samples prior to mass spectrometric or other analyses.

In contrast to DX-236 media, DX-321-Sepharose beads bound the nonhuman albumins weakly, if at all, according to the same hierarchy as the relative solution peptide affinities for the ligand: BSA > GSA > MSA (Table 4). Of the three nonhuman albumins, BSA may have been captured with slightly higher efficiency than GSA, whereas MSA may not have been captured at all. The albumin binding properties of DX-321 column complement the properties of the DX-236 column and may prove useful in the partial purification of HSA away from other nonhuman serum albumins in recombinant expression systems.

To test the potential utility of the DX-236 peptide in HSA purification, we examined the ability of a DX-236-Sepharose column (10 mL, 0.3 μmol ligand/mL resin) to capture and elute HSA from serum. Since we planned to test the binding of large serum samples (greater than 0.1 mL), we employed a larger DX-236 column to ensure complete capture. Both the column and serum sample

Table 6. DX-236 HSA Purification from Human Serum

fraction	μg of HSA	% initial
initial	4805	100
FT	565	12
wash/gradient	88	1.8
elution	4003	83
total	4656	96.8

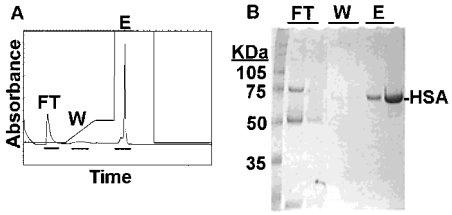


Figure 4. Purification of HSA from human serum with gel analysis. (A) Chromatogram of HSA purification using the DX-236 Sepharose. Whole human serum was dialyzed against 3 mM NaPi, 20 mM NaCl, pH 6.2 (EB2), and 100 μL (~5 mg HSA) was injected onto a preparative DX-236 column (10 mL, 0.3 μmol/mL) previously equilibrated in EB2. A salt gradient between 20 and 44 mM was applied. HSA was eluted with 100 mM Tris, pH 9.1. (B) SDS−PAGE analysis of DX-236 column fractions (shown by underlined areas). Flowthrough (FT), wash gradient (W), and elution (E) fractions were collected for analysis (depicted by underlines) and then analyzed by SDS−PAGE (boiled under reducing conditions). The quantity of HSA in each fraction was determined using a reverse phase assay (Table 6).

were exchanged into 3 mM phosphate, 20 mM NaCl, 0.1% Tween-20, pH 6.2 (EB2). Salt was added to the EB1 binding buffer to minimize nonspecific protein binding to the column. The 10-mL column quantitatively captured the HSA in a 0.1-mL serum injection (~5 mg HSA total). Further, the vast majority of HSA applied to the column (93%) was recovered by means of a 100 mM Tris, pH 9.1 wash (Table 6). The purity of the eluted HSA was greater than 99% as determined by both sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE; Figures 4B, 5B) and reverse phase chromatography (Figure 5C, dashed line). The DX236-column-purified material was compared to HSA purified from serum using Cibacron Blue (CB) Sepharose (Figure 5A) and was found to be of slightly higher purity (Figure 5B, C, solid line). In Figure 5B, C, the arrows indicate impurities in the Cibacron Blue elution fraction.

In addition to Cibacron Blue Sepharose, other immobilized peptide media have been used to purify HSA. For example, Pignali et al. (*37*) reported the HSA purification properties of an immobilized peptide derived from cytochrome *c* (**G**AQ**G**HTVEK), which contains 2 Cys-to-Gly mutations (bolded **G** residues) of the peptide originally characterized by Adams et al. (*38*). This linear peptide (CytC) was synthesized directly on POROS-amine resin (Applied Biosystems) via a single C-terminal 6-aminohexanoic acid linker. The final peptide loading of the media was 93 μmol/g resin, which is significantly higher than the peptide density of the DX-236-Sepharose media (ca. 0.3−0.8 μmol/mL) described above. It appears from SDS−PAGE analysis (*26*) that the high ligand density CytC-column specifically captured and eluted HSA from human serum. Strikingly, the CytC-column bound HSA best in low salt, pH 6 buffer (20 mM MES), a condition very similar to the optimized conditions for DX-236 as determined above. Pignali et al. (*37*) have compared the dynamic binding capacity of the CytC-column (~19 mg/mL) to an anti-HSA antibody column (~2 mg/mL). Remarkably, the capacity of the CytC-column was no more than 2-fold greater than that of the

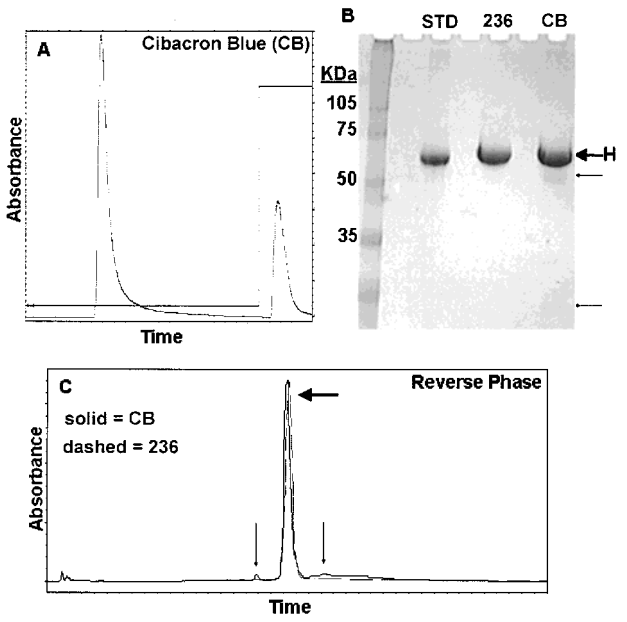


Figure 5. Comparison of HSA purification using DX-236 vs Cibacron Blue Sepharose. (A) Chromatogram of HSA purification from human serum using Cibacron Blue Sepharose. Whole human serum was dialyzed against PBS, pH 7, and 100 μL (~5 mg HSA) was injected onto a 1 mL Cibacron Blue Sepharose column previously equilibrated in the same buffer used for dialysis. The column was washed extensively with PBS and then the HSA was eluted with PBS, 1.0 M NaCl. The Cibacron Blue Sepharose elution fraction was compared to the DX-236 elution fraction using SDS−PAGE [(B) STD, HSA standard (2.5 μg); 236, DX-236 elution; CB, Cibacron Blue elution] and reverse phase chromatography (C). For the same quantity of total protein, the Cibacron Blue elution fraction had lower purity than the corresponding DX-236 elution fraction (arrows, protein contaminants). In both A and C, absorbance was monitored at 280 nm.

DX-236-Sepharose affinity column (greater than 11 mg/mL), although the CytC media carried ~100-fold more peptide.

Since Pignali et al. (*37*) did not report the affinity of the CytC peptide for HSA, we synthesized a fluorescein labeled peptide based on the CytC sequence. The labeled CytC peptide bound weakly to HSA in solution with a K_D of approximately 335 μM, which is approximately 100 fold greater than the DX-236 K_D in the same buffer (Table 2). This weak affinity elucidated the high ligand density most likely required to effectively bind HSA from human serum. As a result of the much tighter HSA binding affinity, the DX-236-Sepharose HSA affinity purification media required much less peptide to achieve a comparable level of performance.

In conclusion, we have isolated two new peptides that bind HSA with low micromolar affinity. At pH 9.1 and 140 mM NaCl, however, the affinity drops sufficiently to allow efficient elution of HSA. DX-236 coupled beads, in particular, captured HSA very effectively from human serum and gave higher purity material than Cibacron Blue Sepharose. Serendipitously, we discovered two peptides with complementary albumin binding properties: DX-236 bound tightly to albumin from a variety of species, whereas DX-321 shows a strong preference for albumin from humans. The binding properties of these peptides highlight the power of phage display for isolating molecules that bind a specific protein target with the desired specificity. Additionally, this study underscores the power and utility of phage display in designing highly specific affinity purification media. Finally, peptides generated from studies such as this promise to find fruitful application in basic, clinical and pharmaceutical research and development processes.

Notation

HSA	human serum albumin
BSA	bovine serum albumin
GSA	goat serum albumin
MSA	murine serum albumin
MS	mass spectrometry
TN	thio-nut
NHS	*N*-hydroxysuccinimide ester
FT	flowthrough
DMSO	dimethyl sulfoxide
TMB	tetramethyl benzidine
SDS−PAGE	sodium dodecyl sulfate−polyacrylamide gel electrophoresis

Amino acid single letter codes

A	Ala
C	Cys
D	Asp
E	Glu
F	Phe
G	Gly
H	His
I	Ile
K	Lys
L	Leu
M	Met
N	Asn
P	Pro
Q	Gln
R	Arg
S	Ser
T	Thr
V	Val
W	Trp
Y	Tyr
X	6-aminohexanoic acid

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References and Notes

(1) Darcel, C. L.; Kaldy, M. S. Further evidence for the heterogeneity of serum albumin. *Comp. Biochem. Physiol., B* **1986**, *85*, 15−22.

(2) Van Liedekerke, B. M.; Nelis, H. J.; Kint, J. A.; Vanneste, F. W.; De Leenheer, A. P. Quality control of albumin solutions by size-exclusion high-performance liquid chromatography, isoelectric focusing, and two-dimensional immunoelectrophoresis. *J. Pharm. Sci.* **1991**, *80*, 11−16.

(3) Bunn, F.; Lefebvre, C.; Li Wan Po, A.; Li, L.; Roberts, I.; Schierhout, G. Human albumin solution for resuscitation and volume expansion in critically ill patients. The Albumin Reviewers. *Cochrane. Database Syst. Rev.* **2000**, *2*.

(4) Mahany, T.; Khirabadi, B. S.; Gersten, D. M.; Kurian, P.; Ledley, R. S.; Ramwell, P. W. Studies on the affinity chromatography of serum albumins from human and animal plasmas. *Comp. Biochem. Physiol., B* **1981**, *68*, 319−323.

(5) Mohammad, J.; Zeerak, A.; Hjerten, S. Dye-ligand affinity chromatography on continuous beds. *Biomed. Chromatogr.* **1995**, *9*, 80−84.

(6) Travis, J.; Pannell, R. Selective removal of albumin from plasma by affinity chromatography. *Clin. Chim. Acta* **1973**, *49*, 49−52.
(7) Travis, J.; Bowen, J.; Tewksbury, D.; Johnson, D.; Pannell, R. Isolation of albumin from whole human plasma and fractionation of albumin-depleted plasma. *Biochem. J.* **1976**, *157*, 301−306.
(8) Roberts, B. L.; Markland, W.; Ladner, R. C. Affinity maturation of proteins displayed on surface of M13 bacteriophage as major coat protein fusions. *Methods Enzymol.* **1996**, *267*, 68−82.
(9) Ladner, R. C. Polypeptides from phage display. A superior source of in vivo imaging agents. *Q. J. Nucl. Med.* **1999**, *43*, 119−124.
(10) Ladner, R. C. Constrained peptides as binding entities. *Trends Biotechnol.* **1995**, *13*, 426−430.
(11) McLafferty, M. A.; Kent, R. B.; Ladner, R. C.; Markland, W. M13 bacteriophage displaying disulfide-constrained microproteins. *Gene* **1993**, *128*, 29−36.
(12) Virnekas B, G. L.; Pluckthun, A.; Schneider, K. C., Wellnhofer, G., Moroney, S. E. Trinucleotide phosphoramidites: ideal reagents for the synthesis of mixed oligonucleotides for random mutagenesis. *Nucleic Acids Res.* **1994**, *22*, 5600−5607.
(13) Chan, W. C. An appraisal of new variants of dde amine protecting group for solid-phase synthesis. *Tetrahedron Lett.* **1998**, *39*, 1603−1606.
(14) Hage, D. S.; Austin, J. High-performance affinity chromatography and immobilized serum albumin as probes for drug- and hormone-protein binding. *J. Chromatogr., B* **2000**, *739*, 39−54.
(15) Kragh-Hansen, U. Structure and ligand binding properties of human serum albumin. *Dan. Med. Bull.* **1990**, *37*, 57−84.
(16) Davies, N. M. Clinical pharmacokinetics of ibuprofen. The first 30 years. *Clin. Pharmacokinet.* **1998**, *34*, 101−154.
(17) Peters, T. *All About Albumin: Biochemistry, Genetics, and Medical Applications*; Academic Press: San Diego, CA, 1996; pp 54−62.
(18) Cheruvallath, V. K.; Riley, C. M.; Narayanan, S. R.; Lindenbaum, S.; Perrin, J. H. The effect of octanoic acid on the binding of the enantiomers of ibuprofen and naproxen to human serum albumin: a chromatographic implication. *Pharm. Res.* **1996**, *13*, 173−178.
(19) Kragh-Hansen, U. Octanoate binding to the indole- and benzodiazepine-binding region of human serum albumin. *Biochem. J.* **1991**, *273*, 641−644.
(20) Arakawa, T.; Kita, Y. Stabilizing effects of caprylate and acetyltryptophanate on heat-induced aggregation of bovine serum albumin. *Biochim. Biophys. Acta* **2000**, *1479*, 32−36.
(21) Ross, P. D.; Finlayson, J. S.; Shrake, A. Thermal stability of human albumin measured by differential scanning calorimetry. II. Effects of isomers of *N*-acetyltryptophanate and tryptophanate, pH, reheating, and dimerization. *Vox Sang* **1984**, *47*, 19−27.
(22) Shrake, A.; Finlayson, J. S.; Ross, P. D. Thermal stability of human albumin measured by differential scanning calorimetry. I. Effects of caprylate and *N*-acetyltryptophanate. *Vox Sang.* **1984**, *47*, 7−18.
(23) Wu, H.; Fan, Y.; Sheng, J.; Sui, S. F. Induction of changes in the secondary structure of globular proteins by a hydrophobic surface. *Eur. Biophys. J.* **1993**, *22*, 201−205.
(24) Lenk, T. J.; Ratner, B. D.; Gendreau, R. M.; Chittur, K. K. IR spectral changes of bovine serum albumin upon surface adsorption. *J. Biomed. Mater. Res.* **1989**, *23*, 549−569.
(25) Norde, W.; Giacomelli, C. E. BSA structural changes during homomolecular exchange between the adsorbed and the dissolved states. *J. Biotechnol.* **2000**, *79*, 259−268.
(26) Luik, A. I.; Naboka Yu, N.; Mogilevich, S. E.; Hushcha, T. O.; Mischenko, N. I. Study of human serum albumin structure by dynamic light scattering: two types of reactions under different pH and interaction with physiologically active compounds [letter]. *Spectrochim. Acta, Part A* **1998**, *54*, 1503−1507.
(27) Weber, G. Energetics of ligand binding to proteins. *Adv. Protein Chem.* **1975**, *29*, 1−83.
(28) Halle, B.; Lindman, B. Chloride ion binding to human plasma albumin from chlorine-35 quadrapole relaxation. *Biochemistry* **1978**, *17*, 3774−3781.
(29) Longsworth, L. G.; Jacobsen, C. F. An electrophoretic study of the binding of salt ions by β-lactoglobulin and bovine serum albumin. *J. Phys. Colloid Chem.* **1949**, *53*, 126−135.
(30) Huang, P. Y.; Carbonell, R. G. Affinity purification of proteins using ligands derived from peptide libraries. *Biotechnol. Bioeng.* **1995**, *47*, 288−297.
(31) Huang, P. Y.; Baumbach, G. A.; Dadd, C. A.; Buettner, J. A.; Masecar, B. L.; Hentsch, M.; Hammond, D. J.; Carbonell, R. G. Affinity purification of von Willebrand factor using ligands derived from peptide libraries. *Bioorg. Med. Chem.* **1996**, *4*, 699−708.
(32) Metcalf, E. C.; Crow, B.; Dean, P. D. The effect of ligand presaturation on the interaction of serum albumins with an immobilized Cibacron Blue 3G-A studied by affinity gel electrophoresis. *Biochem. J.* **1981**, *199*, 465−472.
(33) Shani, M.; Barash, I.; Nathan, M.; Ricca, G.; Searfoss, G. H.; Dekel, I.; Faerman, A.; Givol, D.; Hurwitz, D. R. Expression of human serum albumin in the milk of transgenic mice. *Transgenic Res.* **1992**, *1*, 195−208.
(34) Kobayashi, K.; Nakamura, N.; Sumi, A.; Ohmura, T.; Yokoyama, K. The development of recombinant human serum albumin. *Ther. Apher.* **1998**, *2*, 257−262.
(35) Okabayashi, K.; Nakagawa, Y.; Hayasuke, N.; Ohi, H.; Miura, M.; Ishida, Y.; Shimizu, M.; Murakami, K.; Hirabayashi, K.; Minamino, H. et al. Secretory expression of the human serum albumin gene in the yeast, *Saccharomyces cerevisiae*. *J. Biochem. (Tokyo)* **1991**, *110*, 103−110.
(36) Sijmons, P. C.; Dekker, B. M.; Schrammeijer, B.; Verwoerd, T. C.; van den Elzen, P. J.; Hoekema, A. Production of correctly processed human serum albumin in transgenic plants. *Biotechnology* **1990**, *8*, 217−221.
(37) Pingali, A.; McGuinness, B.; Keshishian, H.; Jing, F. W.; Varady, L.; Regnier, F. Peptides as affinity surfaces for protein purification. *J. Mol. Recognit.* **1996**, *9*, 426−432.
(38) Adams, P. A.; Goold, R. D.; Thumser, A. A. Heme-peptide/protein interactions: the binding of heme octa and undecapeptides, and microperoxidase-8 and −11, to human serum albumin. *J. Inorg. Biochem.* **1989**, *37*, 91−103.

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