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Discovery of Alpha-1-Proteinase Inhibitor Binding Peptides from the Screening of a Solid Phase Combinatorial Peptide Library

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ABSTRACT

Alpha-1-proteinase inhibitor (A1PI, also known as alpha-1-antitrypsin), a blood plasma protein, was radiolabeled and screened against a portion of a hexameric, solid-phase combinatorial peptide library. The screening of 2% of a library containing 34 million peptides yielded 19 sequences. All 19 sequences bound A1PI when immobilized to a chromatographic support, but with varying avidities. Several of these sequences proved capable of purifying A1PI from aqueous mixtures with human serum albumin and from Effluent II+III, a process intermediate in the Cohn plasma fractionation process. A single chromatography step was shown to provide high yields with significant purification and no measurable loss in activity.

Key Words. Protein purification; Alpha-1-proteinase inhibitor; Combinatorial peptide library; Affinity chromatography

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INTRODUCTION

The production of proteins by traditional and recombinant methods has experienced tremendous growth in recent years. The efficient and economic recovery and purification of these high value products is of critical importance. The low concentration of the target molecule as well as the presence of several proteins with similar properties in a complex feed can make this task difficult. Most current large-scale processes, like ion exchange chromatography and precipitation, are limited by their selectivity. A single operation with increased selectivity, such as affinity chromatography, could potentially replace several steps while increasing product purity and yield. While affinity chromatography is commonly employed in laboratories, it is not as widespread industrially due to the cost, stability, and safety concerns typically associated with affinity ligands like antibodies (1).

The ability of small peptides (3–8 amino acids) to bind very specifically to a desired protein is well established in the literature. Peptides can provide higher specificity than metal and dye ligands without the toxicity of dye ligands should leakage occur. In comparison to antibodies, small peptides can be produced more inexpensively and by synthetic means; they are less likely to generate an immune response and are more stable (1). Peptides typically bind proteins with a lower avidity than antibodies, resulting in milder elution conditions for chromatographic separation (2). However, small peptides normally exhibit lower specificities than antibodies. Accordingly, peptide affinity resins may be best suited for capture and concentration of target molecules in a crude feed stream, rather than as a polishing step requiring very high purity.

The main challenge for peptide affinity chromatography lies in the identification of a peptide with sufficient specificity and avidity for separation. The design of an amino acid sequence that will prove complementary to a portion of the surface of a desired protein, even when the crystal structure of the protein is known, has proved difficult (3, 4). The use of combinatorial peptide libraries makes it possible to screen thousands to millions of potential compounds at once, obviating the need for detailed knowledge of the target molecule's structure. Baumbach and Hammond successfully demonstrated the concept of immobilizing a library-derived peptide ligand for the purification of streptavidin (1). The technique has since been demonstrated for a variety of proteins including s-protein (2), von Willebrand Factor (5), fibrinogen (6–8), Factor IX (9), Factor VIII (10), and albumin (8). These ligands have been discovered from both phage and chemical libraries.

Two major types of peptide libraries have been employed, phage display and synthetic libraries. Phage display libraries were introduced earlier this decade and have found use in a wide array of applications (11–14). These libraries can be propagated through multiple rounds of screening, allowing for different selection criteria through the process. In contrast to synthetic li-

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braries, phage libraries can be endlessly regenerated, and the peptides themselves can be longer and more complex, including cyclic structures. They do, however, suffer from natural selective pressures that sometimes prevent achieving full diversity and are limited to only naturally occurring amino acids while solid phase libraries allow for the introduction of non-L-amino acid structures (15).

Solid phase synthetic libraries have a distinct advantage for purification applications as the peptide is already immobilized as a ligand on a chromatographic support. Earlier studies have found that peptides from a solution phase screening will sometimes not bind the target protein when immobilized to resin (10). The reuse of solid phase libraries can be limited. In addition, their size (i.e., number of sequences) is physically limited by the solid support. Therefore, cost considerations sometimes prohibit screening the full library. A smaller library would reduce the size and cost, but would require a reduction in diversity by limiting either the peptide length or the number of amino acid building blocks. Without exposure to the entire library, a true consensus sequence may fail to emerge. However, prior screenings of a portion of a solid phase library have successfully identified ligands for fibrinogen (6, 7) and Factor IX (9).

Alpha-1-proteinase inhibitor (A1PI, also known as alpha-1-antitrypsin) is a blood plasma glycoprotein belonging to a family of enzymes known as serine protease inhibitors or Serpins (16). A1PI consists of a single chain of 394 amino acids with three carbohydrate side chains (total mass of 53,000 daltons) and no disulfide bonds (17). A1PI protects lung tissue by inhibiting neutrophil elastase (18) and is approved as a therapeutic for people with a genetic deficiency (19). A1PI has a pI of 4.8 and is present in plasma at 1.3–2 g/L (20). The native (enzymatically active) form has a lower stability than most globular proteins (18). The protein is irreversibly inactivated below its pI of 4.7 (21, 22) and at elevated temperature (50°C and above) (23).

The purification of A1PI from blood plasma is complicated primarily due to human serum albumin (hSA) which has very similar physical properties including pI and molecular weight (21, 24). In small-scale separations, several affinity and pseudoaffinity methods have been explored including immobilized Concanavalin A (25), an immobilized anti-A1PI monoclonal antibody (26), thiol-exchange chromatography (27), and triazine dye-affinity resins used to remove hSA and other proteins from the mixture (28).

Large-scale A1PI purification from blood plasma has been demonstrated from IV-1 paste and Effluent II+III (Eff. II+III), two intermediates in the Cohn ethanol fractionation process (29). Redissolved IV-1 paste is an aqueous stream (pH 7–8, 20°C) while Eff. II+III, which is upstream of IV-1 paste, contains 20% ethanol and must be processed at 4°C or below. A1PI constitutes only 3% of the total protein in Eff. II+III. Eff. II+III contains 87% of the total A1PI in plasma, compared to only 49% in the redissolved IV-1 paste (30).

Purification has been achieved from these streams using either DEAE chromatography alone (24) or DEAE chromatography in combination with polyethylene glycol (PEG) precipitation (31–34), cation-exchange chromatography (30, 35), or gel filtration (36).

In this work a solid phase peptide library of hexamers was screened to find peptide affinity ligands for A1PI purification. The peptides were identified using a radiological screening technique (7). The screening identified 19 peptides that bound A1PI. These peptides exhibited varying avidities and purification potential. We have demonstrated the ability of selected peptides to purify A1PI from sample mixtures of albumin and from the Cohn fraction Eff. II+III.

EXPERIMENTAL

Materials

The Bayer Corporation donated purified human A1PI (30, 35) and human serum albumin (the Bayer product Plasbumin). The Eff. II+III used was a manufacturing intermediate from the Bayer Corporation (Clayton, NC). Casein (Blocker Casein in phosphate buffered saline) and guanidine hydrochloride (sequanal grade) were purchased from Pierce (Rockford, IL). Fmoc amino acids were obtained from Novobiochem (La Jolla, CA). ^{14}C-labeled formaldehyde came from Dupont-NEN (Boston, MA), and Low Melt Agarose (preparative grade) was from Biorad (Hercules, CA). All other chemicals were reagent grade or better.

Synthesis of Combinatorial Peptide Library

A linear, hexameric combinatorial peptide library was synthesized on Toyopearl AF-Chelate-650 (Tosohaas, Montgomeryville, PA). The resin was chosen for its ability to withstand peptide synthesis conditions and its mechanical rigidity for HPLC and large-scale column chromatography (9). The methacrylate-based resin has a particle size of 100–300 μm with a 1000 Å average pore diameter. The particle size used was higher than the standard material sold (40–90 μm diameter) and was obtained specially from the manufacturer. The resin is substituted with iminodiacetic acid and is marketed for metal chelate affinity chromatography. The carboxyl groups were aminated using 4,7,10-trioxa-1,13-tridecanediamine (TOTDA, Aldrich, St. Louis, MO). The TOTDA and amino acid couplings were performed using standard Fmoc chemistry with benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP, Novabiochem) as described by Buettner et al. (9).

The "one bead, one structure" library was constructed using the divide, couple, recombine (DCR) technique (37). The library was synthesized using 18 of

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the 20 natural L-amino acids (excepting cysteine and methionine) following the procedure of Buettner et al. using a Gilson AMS 422 (9). The DCR procedure was repeated six times to make a linear, hexapeptide library that theoretically contained 34×10^6 sequences. The density of peptide on the resin in the library ranged from 0.2–0.5 mmol/g resin.

Synthesis of Individual Peptides

Single peptide sequences were synthesized directly onto the Toyopearl AF-Chelate-650M Chelate resin (40–90 μm particle size, 1000 Å pore diameter) using the Gilson AMS 42. The peptide library was fully substituted with peptide to ensure sufficient peptide for sequencing. The individual peptides were synthesized to a density of 0.1 mmol peptide/g, a level closer to the expected range for a production scale resin. To control the peptide density, the Chelate resin was modified with TOTDA and then a mixture of 1:10 of Fmoc-L-Alanine to tBoc-L-Alanine as described by Buettner et al. (6). The subsequent peptide synthesis was performed maintaining a 5–10-fold excess of amino acid derivative to available Fmoc-L-Alanine residues. Peptides prepared by direct synthesis were found to be 77% pure (9).

The peptide composition was confirmed and the degree of substitution of the resin calculated by quantitative amino acid analysis performed at Commonwealth Biotechnologies, Inc., Richmond, VA. Each peptide resin was subjected to propionic acid/HCl hydrolysis for 2 hours at 155°C followed by analysis on a Hewlett-Packard Amino Quant Chemistry system (38). Unless otherwise stated, all peptide resins had a peptide density of approximately 0.1 mmol peptide/g resin.

Radiolabeling of A1PI

A1PI was labeled by reductive methylation utilizing sodium cyanoborohydride and ^{14}C-formaldehyde as described by Jentoft and Dearborn (39). A1PI was reconstituted in water and subjected to buffer exchange with water by ultrafiltration using a Centricon-10 (MWCO 10,000 Da, Amicon) to remove any amine-containing salts. The sodium cyanoborohydride was recrystallized prior to use due to possible hydrate formation during storage (39). The formaldehyde was added at a 5-fold molar excess over the total number of amines present on A1PI (35 total, 34 lysines + N-terminus). Sodium cyanoborohydride was added at 1.25× the amount of formaldehyde. The reagents were added to a 0.1 M sodium phosphate, pH 7 buffer along with the protein. The reaction was performed at 25°C for 4 hours. Following the reaction, the labeled protein was separated from reagents using an EconoPac 10DG Desalting Column (Biorad, Hercules, CA) equilibrated with 0.1 M sodium phosphate, pH 7. The radioactivity of samples was determined using

a 1500 Tri-Carb Liquid Scintillation Analyzer from Packard (Meridian, CT) and CytoScint ES scintillation liquid from ICN (Costa Mesa, CA).

Library Screening

A radiological detection approach similar to the approach used by Mondorf et al. (7) was utilized to identify peptide sequences from the library. Two 200 mg aliquots of library beads (dry weight) were placed in separate 3 mL reaction vessels (Gilson, Middleton, WI) with polyethylene frits. The library was washed four times with 2 mL of 20% methanol in water and then four times with 2 mL of equilibration buffer, 10 mM Hepes, 0.1 M NaCl, 0.1 v/v Tween 20, pH 7. To minimize nonspecific interactions between the library and ^{14}C-A1PI, the library beads were first incubated with 1 mL of a 0.5 w/v casein solution in equilibration buffer for 2 hours on a rotating plate. 20 μg of ^{14}C-A1PI was added directly to each of the bead/blocking solution slurries to a final concentration of 0.5 μM and rotated for 1.5 hours at 25°C.

Following incubation, the beads were washed in the reaction columns with 30 mL of equilibration buffer. The beads were then aliquoted equally into four containers. A solution of 2% low-melting agarose solution at 35°C was added to each vessel, and the slurry poured onto an 8″ × 10″ sheet of Gelbond (FMC, Rockland, ME) to form a monolayer of beads. The agarose gel was air-dried overnight and exposed to a photographic film (Hyperfilm-βmax, Amersham Life Science, Arlington Heights, IL) at −70°C for 3–7 days. Positive signals on the film were confirmed by reexposure of the gel to a new film. Beads resulting in confirmed signals were identified by careful alignment of the film and gel (via punched holes), and isolated with a scalpel under a microscope. The beads were placed in a glass vial with water and heated to 70°C to remove remaining agarose prior to sequencing. Library beads were sequenced at the Protein Technologies Laboratories, Texas A&M University, TX, by Edman degradation using a Hewlett-Packard G1005A (40).

Binding Confirmation (secondary screening)

Individual peptide sequences were synthesized directly onto Toyopearl AF-650M Chelate resin that had been modified with TOTDA followed by an alanine residue (A-TOTDA-Toyopearl) to yield a final substitution density of 0.1 mmol/g resin. The resin was packed into 0.4 × 5 cm columns and tested in a chromatographic format on a microbore HPLC with a built-in UV detector (Ultrafast Microprotein Analyzer, Michrom BioResources, Auburn, CA). Samples were injected via a Gilson 231XL auto-injector (Middleton, WI) with a 1-mL loop. A Gilson FC 204 fraction collector at the HPLC outlet collected individual eluates. The samples for injection and collected fractions were kept at 4°C by thermostatted racks connected to a Lauda 6M recirculating bath. For runs at 4°C, a 200 μL stainless steel loop was placed in the flow path imme-

diately prior to the column with both the loop and the column immersed in the water bath. The apparatus was automated to run several injections and collect the data utilizing an EZChrom Chromatography Data System (Scientific Software, Inc., San Ramon, CA).

Tests were made by injecting 1 mL of several test solutions: 5 mg/mL hSA in buffer, 1 mg/mL A1PI in buffer, 1 mg A1PI + 5 mg hSA in buffer, or Effl. II+III. The protein samples were in equilibration buffer (10 mM Hepes, 0.1 M NaCl, 0.1 v/v Tween 20, pH 7) unless otherwise stated. The column was equilibrated with equilibration buffer (as above). Equilibration buffer was used to load the injection with sufficient time for unbound material to flow through. The column was then washed with step elutions of 1 M NaCl and 3 M NaCl in equilibration buffer. The column was reexposed to equilibration buffer with 0.1 M NaCl. Any remaining protein was eluted with elution buffer (2% by volume glacial acetic acid in water) and then reequilibrated for the next run. Fractions collected from the 2% acetic acid eluates were neutralized with 2 M Tris, pH 10.5, to bring the pH to neutrality.

Some runs used 4–6 M guanidine hydrochloride (GdnHCl) in water in lieu of 2% acetic acid for elution and recovery and A1PI activity. The GdnHCl eluate was immediately diluted 20-fold in a buffer containing 200 mM Tris, 10 mM EDTA, pH 8, to dilute the GdnHCl and recover the A1PI activity. The diluted sample was incubated for 1 hour at room temperature and concentrated using an Amicon Centriplus centrifugal ultrafiltration unit (MWCO 10 or 30 kDa).

The chromatography method was run in one of two modes. When injecting single protein samples, a constant flow rate of 0.52 mL/min (250 cm/h) was used. For protein mixtures, samples were loaded at an initial flow rate of 0.87 mL/min (413 cm/h) for 1 minute, at which point the flow was reduced to 0.02 mL/min for 10 minutes to allow sufficient residence time for binding. The flow was returned to 0.87 mL/min for the remainder of the run.

Analytical Methods

Protein concentrations were determined by laser immunonephelometry. Immunoreactive A1PI and other proteins were quantified using a laser nephelometer immunoprecipitation assay on either a Behring Nephelometer, model BNA, with assay kits purchased from Behringwerke AG (Marburg, DEU), or on a Beckman Array 360CE, with assay kits purchased from Beckman Instruments (Brea, CA). The machines were used according to the manufacturer's recommendations. Controls showed that A1PI eluted with 2% acetic acid would not retain full biological activity, but could still be quantified by immunonephelometry.

A1PI activity was determined by inhibition of porcine pancreatic elastase (Calbiochem) using a chromogenic substrate [succinyl-(alanine) 3-*p*-ni-

troanilide] (Sigma, St. Louis, MO, USA) at 405 nm (41). The assay was performed manually or using a BioMek workstation. The microtiter plates were read with a Molecular Devices microtiter plate reader with a 405-nm filter and SoftMax software. The increase in adsorption was monitored continuously. Comparisons of the linear changes of absorbance with time in the presence and absence of sample were made. The amount of inhibitor was calculated based on a calibration with known standards of A1PI and based on the 1:1 stoichiometric relationship between A1PI and elastase (16). Activity was expressed as milligrams of A1PI per milliliter of sample.

RESULTS AND DISCUSSION

Radiolabeling of A1PI

Protein labeling with sodium cyanoborohydride and formaldehyde converts free amino groups in proteins to dimethyl derivatives at neutral pH and mild reaction conditions. The reaction is very selective to label only the free *N*-terminal amine and lysyl residues. The change in size and structure of the labeled protein is minimal due to the small size of the methyl group and retention of the positive charge (39).

The labeling resulted in a radioactive yield of 0.4 mCi/mol A1PI. The percent of amines methylated was calculated assuming 35 possible labeling sites [i.e., N-terminus and 34 lysine residues (17)] as:

$$\%\ \text{Methylated} = \frac{\text{mCi}}{\text{mmol A1PI}} \times \frac{\text{mmol } ^{14}\text{C}}{15.8\ \text{mCi}} \div \left(\frac{35\ \text{mmol NH}_2}{\text{mmol A1PI}} \times \frac{2\ \text{mmol } ^{14}\text{C}}{\text{mmol NH}_2} \right)$$

The reaction methylated 37% of the total residues available. Busby et al. (42) reported no loss of inhibitory activity or change in molecular size for methylation levels over 50% (>18 of the 35 residues) for A1PI. Labeling experiments in our lab also showed no loss in inhibitory activity and no detectable shift using SDS-PAGE electrophoresis (results not shown).

Library Screening Results

The screening procedure was designed to identify peptide sequences that bound A1PI. Our original target feedstream was redissolved in IV-1 paste, an aqueous stream (pH 7–8) processed at 20°C (30, 43) which serves as the current source of production (31–33). Therefore, the screening was performed in an aqueous system at room temperature. Equilibrium adsorption studies between proteins and peptide resins found equilibrium was typically established

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in 15–30 minutes (P. Huang, unpublished data). Therefore, an incubation time of $1\frac{1}{2}$ hours was used to ensure equilibrium. Casein was used as the blocker to minimize nonspecific interactions between A1PI and the library. Other screening work in our lab showed casein a more effective blocking agent in reducing background detection levels than bovine serum albumin (7).

After 3 days exposure at −70°C, the films were developed and provided very low background levels. Reexposure of the gels for an additional 7 days at −70°C resulted in the confirmation of 19 signals. While Mondorf et al. (7) demonstrated that image analysis of the film could correlate image intensity with radioactivity level, no attempt was made due to significant heterogeneity in bead size diameters.

Only 2% of the total library was screened, representing approximately 680,000 sequences out of a total of 34 million. Based on the (31 m^2/g) surface area of the resin (5) and assuming a monolayer of protein has a surface density of 2 mg/m^2 (44, 45), the amount of A1PI added could saturate 0.2% of the bead surface area in the screening. The 19 signal-producing beads represent 0.003% of the beads in the screening. Due to the close proximity of some of the beads in the agarose, 21 beads were excised and sequenced. The resulting sequences are presented in Table 1. All sequences are listed from the N to the C terminus, with the C-terminal residue bound to the resin. The table provides the sequences using both the standard three and one letter amino acid abbreviations.

No true consensus sequence or motif emerged for all of the sequences, which is not unexpected given that not all of the library was screened. However, sequences 1–19 in Table 1 exhibited a large number of similarities. They all contained at least one aromatic (Tyr, Phe, Trp) and one positively charged residue (Arg, His, Lys), while the majority contained two or more of each. A majority of the sequences also contained either Ile or Leu residues. Thus, all the peptides were positively charged due to the positively charged residues and the free N-terminal amino group. The peptides all had some hydrophobic character imparted due to the hydrocarbon side chains of Ile, Leu, and Val and the aromatic residues. For sequences 1–19 in Table 1, 90% of the amino acid residues came from nine possible amino acids. There were a few repeated motifs and many similarities among the sequences. Sequences 1 and 2 (VIWLVR and IIWLYK) differed only slightly at position 1 (two aliphatic residues), by the substitution of a hydrophobic valine (V) for aromatic tyrosine (Y) at position 5, and different positively charged residues at position 6 (R and K). Sequences 18 and 19, IKRYYN and IKRYYL, respectively, differed only in their C-terminal residues. By contrast, sequences 20 and 21 did not share the same characteristics as the previous 19. Neither contained a positively charge residue, and sequence 21 (NQIQVL) had no aromatic residues.

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TABLE 1
Sequences Deduced from the Screening at ^{14}C-A1PI Against a Peptide Library[a]

ID	Sequence						Shorthand
1	Val	Ile	Trp	Leu	Val	Arg	VIWLVR
2	Ile	Ile	Trp	Leu	Tyr	Lys	IIWLYK
3	Arg	Tyr	Arg	Ile	Phe	Ile	RYRIFI
4	Arg	Ala	Phe	Trp	Tyr	Ile	RAFWYI
5	Arg	Phe	Ile	Tyr	Tyr	Thr	RFIYYT
6	Tyr	Lys	Phe	Arg	Phe	Trp	YKFRFW
7	Leu	Ile	Val	His	Arg	Trp	LIVHRW
8	Pro	Tyr	Trp	Ile	Val	Arg	PYWIVR
9	Trp	Lys	Leu	Trp	Arg	Trp	WKLWRW
10	Ala	Arg	Trp	Tyr	Ile	His	ARWYIH
11	Gln	Tyr	His	Phe	Trp	Tyr	QYHFWY
12	Trp	Ser	Ser	Lys	Arg	Tyr	WSSKRY
13	Trp	Ile	Lys	Trp	Thr	Lys	WIKWTK
14	Arg	Arg	Lys	Tyr	Leu	Trp	RRKYLW
15	Arg	Leu	Trp	Arg	Tyr	Gly	RLWRYG
16	Asn	Trp	Lys	Arg	Val	Arg	NWKRVR
17	Ile	Trp	Arg	Lys	Tyr	Ser	IWRKYS
18	Ile	Lys	Arg	Tyr	Tyr	Asn	IKRYYN
19	Ile	Lys	Arg	Tyr	Tyr	Leu	IKRYYL
20	Asn	Phe	Pro	Leu	Ile	Ala	NFPLIA
21	Asn	Gln	Ile	Gln	Val	Leu	NQIQVL

[a] 400 mg of a hexameric, solid phase peptide library was incubated in a solution containing 5 mg/mL casein and 0.5 μM A1PI at 25°C for 1.5 hours. The buffer solution contained 10 mM Hepes, 0.1 M NaCl, 0.1% Tween 20, pH 7. Following screening, the beads were suspended as a monolayer in agarose and exposed to autoradiography film. 19 signals were detected on the autoradiography film. 21 beads were isolated and sequenced by Edman degradation. Sequences are listed from N to C terminal positions.

A1PI Binding to Peptide Resins

Pure A1PI was injected onto the peptide columns at a constant flow rate with step elutions of 1 M NaCl, 3 M NaCl, and 2% acetic acid. Tables 2 and 3 summarize the elution behavior for all 21 sequences for the injection of pure A1PI at 20 and 4°C, respectively. The "Percent A1PI eluted by fraction" represents the amount eluted in the individual flow-through, 1 M NaCl wash, and 2% acetic acid fractions relative to the total A1PI eluted. The percentages are based on the mass balance by the integration of the peak areas at 280 nm or by immunonephelometry. A comparison of these two quantitative methods yielded nearly identical results. The mass balances for the runs closed to within experimental error (15%) for a control injection.

TABLE 2
A1PI Binding at 20°C[a]

Sequence ID	Peptide sequence	Percent A1PI eluted by fraction		
		Flow-through	1 M NaCl	2% Acetic acid
1	VIWLVR	4	16	80
2	IIWLYK	28	17	55
3	RYRIFI	0	35	65
4	RAFWYI	0	72	28
5	RFIYYT	0	64	36
6	YKFRFW	0	75	25
7	LIVHRW	0	64	36
8	PYWIVR	0	55	45
9	WKLWRW	0	67	33
10	ARWYIH	0	84	16
11	QYHFWY	0	81	19
12	WSSKRY	0	97	3
13	WIKWTK	7	91	2
14	RRKYLW	0	82	18
15	RLWRYG	0	88	12
16	NWKRVR	0	93	7
17	IWRKYS	0	81	19
18	IKRYYN	0	95	5
19	IKRYYL	0	92	8
20	NFPLIA	94	0	6
21	NQIQVL	100	0	0
	Amino	89	11	0

[a] 1 mg of pure A1PI was injected at a constant flow rate of 0.53 mL/min (250 cm/h) in a buffer of 10 mM Hepes, 0.1 NaCl, 0.1% Tween 20, pH 7 at 20°C. The column was washed with step elutions of 1 M NaCl in the loading buffer and 2% acetic acid in water. The "Percent A1PI eluted by fraction" columns represent the amount eluted in each fraction relative to the total A1PI eluted from the column. The mass balance was calculated by the integration of the absorbance at 280 nm or immunonephelometry. The mass balance for all runs closed to within 15% of the control injection.

At 20°C, sequences 1–19 in Table 1 bound A1PI, with all but one of those capturing more than 90% of the injected A1PI. At 4°C, the same 19 sequences bound greater than 95% of the A1PI. It is reasonable to conclude that the sequences 1–19 that showed the sequence similarities mentioned previously and that the bound A1PI were those which produced signals on the autoradiography film. Sequences 20 and 21 did not share the same sequence characteristics as the other 19 and did not bind A1PI. Therefore, sequences 20 and 21 were likely the beads excised in addition to the others.

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TABLE 3
A1PI Binding at 4°C[a]

Sequence ID	Peptide sequence	Percent A1PI eluted by fraction		
		Flow-through	1 M NaCl	2% Acetic acid
1	VIWLVR	5	46	49
2	IIWLYK	0	52	48
3	RYRIFI	0	40	60
4	RAFWYI	0	55	45
5	RFIYYT	0	57	43
6	YKFRFW	0	64	36
7	LIVHRW	0	74	26
8	PYWIVR	0	74	26
9	WKLWRW	0	80	20
10	ARWYIH	0	55	45
11	QYHFWY	0	74	26
12	WSSKRY	0	91	9
13	WIKWTK	0	100	0
14	RRKYLW	0	92	8
15	RLWRYG	0	88	12
16	NWKRVR	0	95	5
17	IWRKYS	0	94	6
18	IKRYYN	0	100	0
19	IKRYYL	0	97	3
20	NQIQVL	89	0	11
21	NFPLIA	99	1	0
	Amino	97	3	0

[a] These results are based on the injection of 1 mg A1PI onto the peptide columns performed identically to the experiments in Table 2 except at 4°C.

Therefore, the radiological screening method itself provided no false positives. The two extra sequences resulted not from false signals in screening but from human error in bead isolation. The extra sequences could have been eliminated by the separation of the beads and reexposure to autoradiography film to identify the true signal-producing bead. The success of the technique can be attributed to the blocking of nonspecific binding and the direct detection method using radiolabeled protein in lieu of introducing other detection species like antibodies.

As seen in Table 2, when A1PI bound to the peptide columns, it exhibited two possible modes of elution. The A1PI could be eluted either wholly or partly in 1 M NaCl, with the remainder eluted in 2% acetic acid. Two representative chromatograms are presented in Fig. 1. The sequence IKRYYN (sequence ID 18) showed complete A1PI capture with total elution in the 1 M NaCl wash. The sequence VIWLVR (sequence ID 1) showed capture with

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some elution in 1 M NaCl, but the majority of A1PI eluted in 2% acetic acid. Approximately half of the 19 sequences in Tables 2 and 3 eluted more than 25% of the bound A1PI in the acetic acid wash. By applying a 1 M NaCl gradient, it was found that the protein that eluted in the 1 M NaCl step gradient could be eluted between 0.2 to 0.4 M NaCl, which is consistent with the levels required to disrupt electrostatic interactions.

The resin Toyopearl AF-Amino-650 M is the same base resin as the Toyopearl Chelate except with the amine functionality. Amino resin serves as a control with the same base resin and charge characteristics. The results for the injection of A1PI only onto the Amino resin are also presented in Tables 2 and 3. The Amino resin captured only 3 and 11% of the A1PI loaded at 4 and 20°C, respectively, illustrating that the base resin and charge alone do not account for binding.

The mixed elution of A1PI by both NaCl and acid may have several causes. The peptide sequences may bind to different portions of A1PI with different affinities. Also, the peptides were prepared by direct synthesis onto the resin and, therefore, were only 77% pure with the impurities consisting of truncated and deleted sequences (9). The desired and altered sequences could exhibit

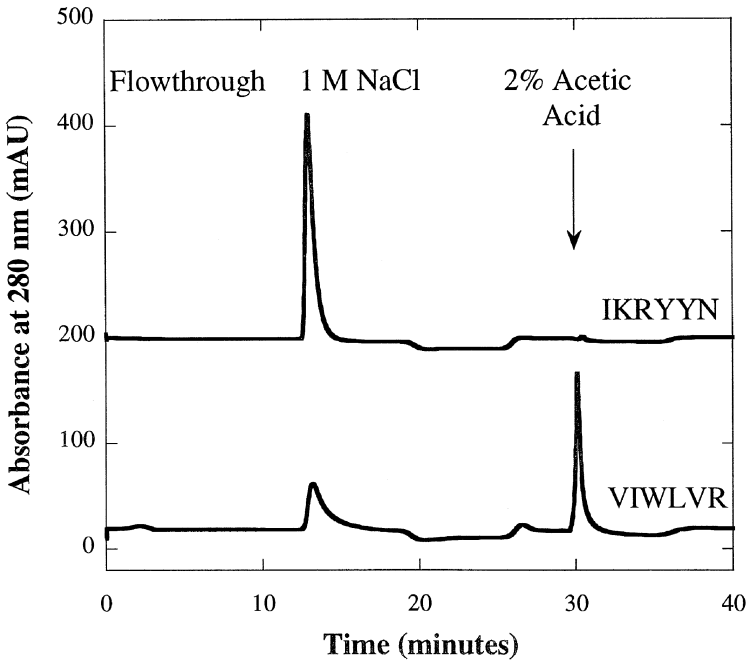

FIG. 1 A1PI elution profile. A280 traces for the injection of 1 mg A1PI onto 2 peptide resin columns. The runs were conducted at a constant flow rate of 0.52 mL/min (250 cm/h) at 4°C. The A1PI was dissolved and loaded in 10 mM Hepes, 0.1 M NaCl, 0.1% Tween 20, pH 7. After sufficient time for flow-through, the column was subjected to step elutions with 1 M and 3 M NaCl in the equilibration buffer, and then 2% acetic acid in water.

different interactions. Additionally, the distribution of the peptides on the resin surface may not be uniform. Nonuniform spacing of peptides could lead to populations of sites with the avidity mediated by factors including surface orientation, steric hindrance, or multipoint interactions.

Some peptides showed a difference in the elution of A1PI at 4 and 20°C. While the capture was largely unaffected, certain sequences experienced a shift in the relative amounts of A1PI eluted in the 1 M NaCl and 2% acetic fractions. The sequence VIWLVR showed more elution in the acid eluate at 20°C compared to 4°C (80 and 49%, respectively).

hSA Binding to Peptide Resins

hSA serves as the primary contaminant in A1PI purification from blood plasma. The ability of the peptides to bind hSA was tested by the injection of 5 mg hSA and using the same chromatography method as the A1PI tests. Sequences 1–19, which bound A1PI, also bound hSA. The bound hSA eluted exclusively in 1 M NaCl for most sequences, while a few eluted the hSA primarily in 1 M NaCl with the remainder in the acid eluate. Sample chromatograms for the separate injections of hSA and A1PI on the column VIWLVR at 4°C are shown in Fig. 2. The figure shows that while not all of the hSA bound, the bound hSA eluted in 1 M NaCl, as did some A1PI. The other

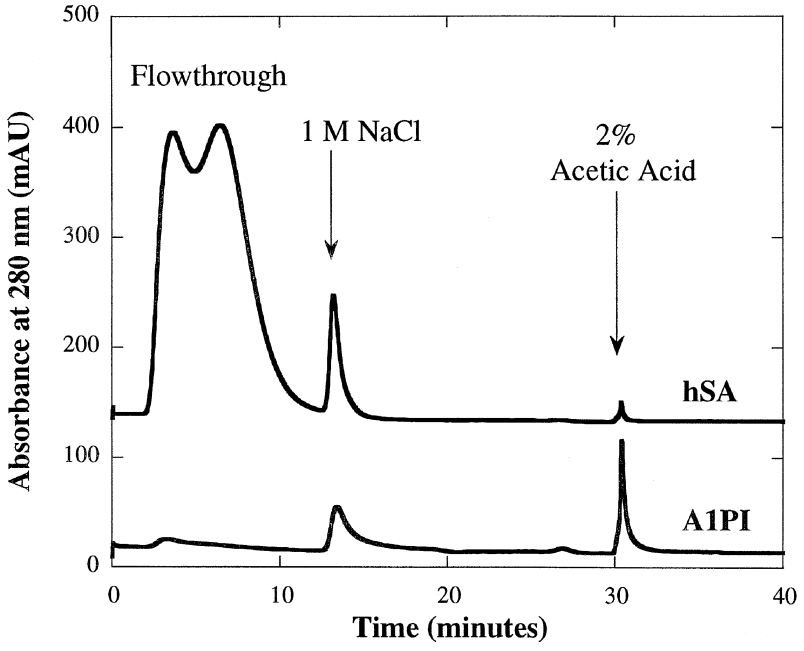

FIG. 2 Albumin and A1PI elution profiles for the peptide VIWLVR. The two traces are for the separate injections of 5 mg hSA and 1 mg A1PI in equilibration buffer at 4°C. The chromatography method used was the same as for Fig. 1.

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peptides all showed similar behavior with many capturing up to 100% of the hSA loaded.

Purification of A1PI/hSA Mixtures

All 19 peptide resins captured both hSA and A1PI. When tested with mixtures of hSA and A1PI, those peptides that eluted A1PI in the 1 M NaCl wash exclusively coeluted hSA and A1PI with no purification. Attempts to separate the A1PI and hSA eluted in the 1 M NaCl wash by the use of a gradient were unsuccessful. As a result, sequences that eluted A1PI only in the 1 M NaCl wash were unable to purify A1PI from hSA.

The chromatograms for the separate injections of hSA and A1PI in Fig. 2 for the peptide VIWLVR showed that while the peptide resin captured both hSA and A1PI, the acid eluate should be primarily A1PI. Therefore, sequences like VIWLVR that eluted A1PI in the acid eluate should purify A1PI from hSA. The purification of A1PI in the acid eluate can be seen in Fig. 3 where a series of mixtures containing 5 mg hSA with 0, 0.2, 0.5, and 0.9 mg A1PI were

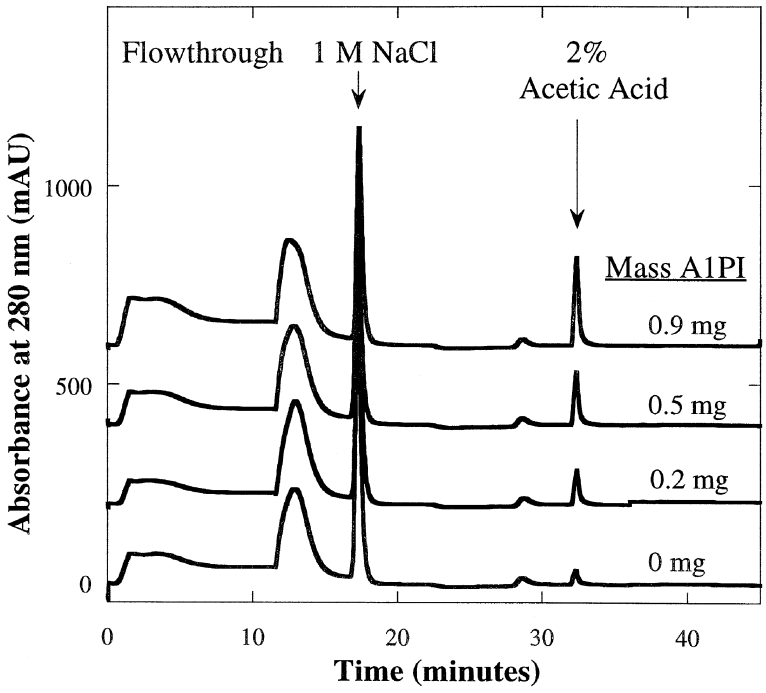

FIG. 3 Purification of A1PI from hSA at 20°C. The chromatograms represent the injection of 5 mg hSA with increasing quantities of A1PI on the peptide column VIWLVR. The samples were injected at a flow rate of 0.87 mL/min (413 cm/h) for 1 minute, followed by the reduction of the flow rate to 0.01 mL/min for 10 minutes. The flow rate was returned to 0.87 mL/min for the remainder of the run. The column was washed with step elutions of 1 M and 3 M NaCl in equilibration buffer, and then 2% acetic acid in water. The increase in the acid peak height was confirmed as A1PI by immunonephelometry.

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injected onto the column VIWLVR at 20°C. The bottom chromatogram for the hSA only injection shows significant hSA in the flow-through and 1 M NaCl fractions, but little in the acid eluate. As the mass of A1PI in the injections increased, the peaks for the acid eluates increased. The protein in the acid eluate was confirmed as A1PI by immunonephelometry. Table 4 presents the mass balance for these injections. The acid eluates did not contain detectable levels of hSA by immunonephelometry. With the given detection limit of nephelometry, the A1PI in the acid eluate for the highest injection is at least 90% pure.

Several peptide columns were assayed with a mixture of 5 mg hSA + 1 mg A1PI at 20 and 4°C. The mass balances for selected columns are presented in Tables 5 and 6. The purity and yield are defined as follows:

$$\text{Yield} = \frac{\text{A1PI}_{\text{Acid}}}{\text{A1PI}_{\text{Total}}} \times 100\%$$

$$\text{Purity} = \frac{\text{A1PI}_{\text{Acid}}}{\text{A1PI}_{\text{Acid}} + \text{hSA}_{\text{Acid}}} \times 100\%$$

As seen in Tables 5 and 6, the tested sequences captured from a third to all of the hSA loaded on the column with the bound hSA eluting entirely in 1 M NaCl for most of the sequences shown. Purified A1PI was eluted in the acid fraction with yields ranging from 27% for RAFWYI (sequence ID 4) to 75% for the sequence VIWLVR (sequence ID 1) with purities in excess of 90%.

TABLE 4
Purification of an A1PI/hSA Mixture Using the Peptide VIWLVR[a]

A1PI Injected (mg)	Protein	Protein mass by fraction (mg)			
		Flow	1 M NaCl	Acid	Total
0.2	A1PI	*	*	0.18	0.18
	hSA	1.1	3.4	*	4.5
0.5	A1PI	0.03	0.04	0.48	0.55
	hSA	1.1	3.1	*	4.2
0.9	A1PI	0.11	0.12	0.70	0.93
	hSA	1.6	2.8	*	4.4

[a] The mass balances for the columns are based on the injection of approximately 5 mg hSA with increasing amounts of A1PI at 20°C in a buffer of 10 mM Hepes, 0.1 M NaCl, 0.1% Tween 20, pH 7. These runs correspond to the chromatograms shown in Fig. 3. The masses are based on immunonephelometry. No protein was detected in the 3 M NaCl eluate fraction. All runs shown gave mass balances that closed within error for nephelometry (±15%) when compared to a control run on the date of the experiment. "*" denotes a value below the detection limit.

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TABLE 5
A1PI Purification at 20°C[a]

			Protein mass by fraction (mg)				% A1PI in acid	
ID	Sequence	Protein	Flow	1 M NaCl	3 M NaCl	Acid	Yield	Purity
1	VIWLVR	A1PI	0.11	0.12	*	0.70	75	100
		hSA	1.57	2.80	*	*		
3	RYRIFI	A1PI	0.22	0.21	*	0.50	53	94
		hSA	0.78	4.17	*	0.03		
4	RAFWYI	A1PI	0.09	0.14	*	0.54	70	94
		hSA	0.31	5.15	*	0.04		
5	RFIYYT	A1PI	0.29	0.11	*	0.44	53	93
		hSA	3.07	1.68	*	0.03		
13	WIKWTK	A1PI	0.02	0.80	*	*	0	—
		hSA	0.13	3.70	*	*		
	Amino	A1PI	0.81	0.05	*	*	0	—
		hSA	2.66	0.71	*	*		

[a] Mass balance for columns based on the injection of 5 mg hSA + mg A1PI at 20°C. The chromatography method in the same as used for Table 4. The mass balance values are from a single, representative run. "*" denoted a value below the detection limit.

TABLE 6
A1PI Purification at 4°C[a]

			Protein mass by fraction (mg)				% A1PI in acid	
ID	Sequence	Protein	Flow	1 M NaCl	3 M NaCl	Acid	Yield	Purity
1	VIWLVR	A1PI	0.12	0.22	*	0.68	67	100
		hSA	1.02	3.94	*	*		
3	RYRIFI	A1PI	*	0.27	*	0.71	72	100
		hSA	*	4.69	0.10	*		
4	RAFWYI	A1PI	0.31	0.34	*	0.24	27	100
		hSA	0.84	3.69	*	*		
5	RFIYYT	A1PI	0.03	0.27	0.04	0.64	66	78
		hSA	0.11	4.98	*	0.18		

[a] Mass balance for columns based on the injection of 5 mg hSA + 1 mg A1PI at 4°C. The chromatography method is the same as used for Table 4. The mass balance values are from a single, representative run. "*" denotes values below the detection limit.

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A comparison between sequences VIWLVR and WIKWTK (sequences 1 and 13, respectively, in Table 5) shows the range of behavior exhibited by the peptide resins. VIWLVR yielded 75% of the A1PI in the acid eluate with no detectable hSA. WIKWTK showed more than 95% capture of both A1PI and hSA but eluted both in 1 M NaCl with no purification. The results for the injection of the hSA/A1PI mixture onto the Amino resin at 20°C (Table 5) showed no purification of A1PI from hSA. Those sequences with the highest percentages of A1PI eluted in acid for the injection of A1PI alone (Tables 2 and 3) showed the highest yields of purified A1PI for the A1PI/hSA mixtures.

Sequence Optimization

The peptide resin VIWLVR (sequence ID 1) proved one of the best peptide resins for A1PI purification due to its high A1PI yield with no detectable hSA. Like other peptides, VIWLVR eluted A1PI in both the 1 M NaCl and acid washes. The elution of A1PI in NaCl decreased the yield of purified A1PI in the acid eluate. Because only a fraction of the library was screened, an attempt was made to rationally alter the sequence to improve its performance. The selective mutation of a lead sequence is a routine tool in structure/activity studies. The library screening identified A1PI binding peptides, but mutation may reveal closely related sequences better suited for purification. A single amino acid substitution of a phage-derived peptide for the purification of von Willebrand Factor led to increased yield and purity (5).

It was surmised that the aromatic tryptophan (W) residue at position 3 was important for binding. Tryptophan is subject to degradation, which could reduce the column's useful life. In an attempt to improve the yield and column life, the tryptophan was replaced with the other two aromatic residues, tyrosine and phenylalanine, to create the peptide resins VIYLVR and VIFLVR, respectively.

The replacement of the tryptophan residue with phenylalanine to create VIFLVR led to a shift in the elution of A1PI. When tested with pure A1PI, both sequences captured more than 90% of the protein, but VIFLVR eluted more than 90% of the A1PI in the acid eluate at both 4 and 20°C. In contrast, the VIWLVR peptide resin eluted 49 and 80% of the A1PI in the acid eluate at 4 and 20°C, respectively. The chromatograms for the injection of pure A1PI on both columns at 4°C are shown in Fig. 4.

When tested with an hSA/A1PI mixture, VIFLVR provided superior yields over VIWLVR. The mass balances for the peptide resins VIFLVR and VIWLVR at 4 and 20°C are shown in Table 7. The yield of A1PI in the acid eluate increased from 67 to 83% at 4°C and from 75 to 90% at 20°C when using the VIFLVR resin. However, the increased yield for VIFLVR was accompanied by a decrease in the eluate purity. While no hSA was detected in the VI-

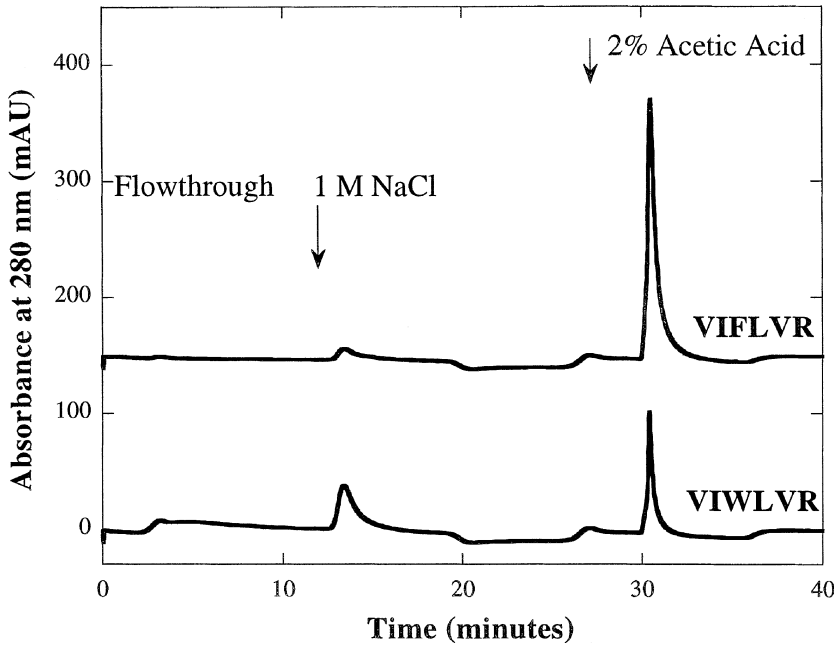

FIG. 4 A1PI adsorption by a mutated sequence. The A280 traces are for the injection of 1 mg A1PI at 4°C. The chromatography method was the same as used in Fig. 1.

WLVR eluate, hSA constituted 10–15% of the VIFLVR eluate. The substitution of tryptophan to phenylalanine improved the yield at the expense of purity. The VIYLVR peptide resin's performance fell in between that of VIWLVR and VIFLVR. The VIYLVR peptide achieved the same yields as the

TABLE 7
A1PI Purification with a Mutated Sequence[a]

Peptide sequence	Protein	Temperature (°C)	Protein mass by fraction (mg)			% A1PI in acid	
			Flow	1 M NaCl	Acid	Yield	Purity
VIWLVR	A1PI	4	0.12	0.22	0.68	67	100
	hSA		1.02	3.94	*		
VIFLVR	A1PI	4	0.09	0.05	0.68	83	89
	hSA		1.64	2.95	0.09		
VIWLVR	A1PI	20	0.11	0.12	0.70	75	100
	hSA		1.57	2.80	*		
VIFLVR	A1PI	20	0.06	0.01	0.59	90	84
	hSA		2.01	1.31	0.12		

[a] Mass balance for columns based on the injection of 5 mg hSA + 1 mg A1PI at 20 and 4°C. The chromatography method is the same as used for Table 4. "*" denotes values below the detection limit.

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VIWLVR column in purification experiments, but with the same reduction in purity as witnessed for VIFLVR.

Purification of A1PI from Eff. II+III

Several of the peptide resins were tested with Eff. II+III, a process intermediate in the Cohn plasma fractionation process. The columns were assayed with 1 mL of neat material using the same chromatography method as for the hSA/A1PI mixtures. To prevent protein denaturation and precipitation in the 20% ethanol feed, all experiments were conducted at 0°C.

The mass balance for each run was calculated by determining the concentration of each of the eight major proteins (A1PI, hSA, Hpt, AAG, Apo A-1, IgA, IgG, Trf) by immunonephelometry. Table 8 summarizes the yield and purity of A1PI in the acid eluate for a variety of sequences. The purity was calculated as the mass of A1PI in the acid eluate divided by the sum of the masses of all proteins in the acid eluate based on nephelometry.

Several of the peptides showed A1PI yields of 70–80% from Eff. II+III with purities ranging from 42 to 77%, representing a 13 to 26-fold purification. The mutated sequence VIFLVR showed an average yield of 79% with a purity slightly above 50%, a 17-fold purification. VIFLVR provided a significantly higher yield than the sequence VIWLVR (79 vs 43%), but with the

TABLE 8
Yield and Purities Achieved from Effluent II+III[a]

Sequence ID	Peptide sequence	% A1PI in acid	
		Yield	Purity
	VIFLVR	79	52
1	VIWLVR	43	52
2	IIWLYK	79	42
3	RYRIFI	69	46
4	RAFWYI	80	69
5	RFIYYT	78	62
11	QYHFWY	75	77
15	RLWRYG	14	19

[a] The table presents the percent A1PI eluted in the acid eluate and the percentage of A1PI in the acid eluate as part of the total based on the injection of 1 mL Eff. II+III onto the column at 0°C. The chromatography method was the same as used in Fig. 3. The mass balance for all protein components is from immunonephelometry. The results for VIFLVR are the average from multiple runs.

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TABLE 9
Composition of Purified Eluate from Effluent II+III[a]

	Mass in acid eluate (mg)	
Protein	VIFLVR	RAFWYI
A1PI	0.57	0.87
hSA	0.06	*
A1-AG	*	*
Apo A-1	0.29	0.29
IgA	0.07	0.1
IgG	*	*
Hpt	*	*
Trf	*	*

[a] The table presents the masses of proteins in the acid eluates for the peptides VIFLVR and RAFWYI for the injection of 1 mL Eff. II+III onto the columns at 0°C. The mass balance is the result of a single run. The two runs were done with different lots of Eff. II+III, which explains the differences in the mass of A1PI. "*" denotes values below the detection limit by nephelometry.

same level of purification. As in tests with aqueous feeds, Amino resin without peptide showed no purification from Eff. II+III (data not shown). Several other sequences including RYRIFI, RAFWYI, RFIYYT, and QYHFWY (sequence IDs 3, 4, 5, and 11, respectively) also showed yields from 69 to 80%, but provided higher purity eluates ranging from 46 to 77%, a maximum 26-fold purification. The acid eluate compositions for the peptide resins VIFLVR and RAFWYI are presented in Table 9. The purification from Eff. II+III by these peptide columns exceeds the reported yields in the literature (40–75%) and matches or surpasses the reported purification factors obtained by DEAE column chromatography (24, 34, 36).

All of the peptide resins in Table 8 allowed 90–100% of the α_1-acid glycoprotein, IgG, haptoglobin, and transferring to flow through unbound. The peptides bound 10–50% of the hSA loaded depending upon the sequence with the vast majority eluted in 1 M NaCl. IgA was typically found in all three fractions. The peptides typically bound 60–80% of the IgA with some elution in 1 M NaCl and about 40–60% of the total IgA loaded eluting in the acid fraction. The major contaminant present in the acid eluate was apolipoprotein A-I. This protein has a molecular weight of 28,000 Da and a pI of approximately 5.5. It is the major apolipoprotein in high-density lipoproteins (46). Apo A-1 was completely bound and coeluted with A1PI in the acid eluate for all of the peptide resins in Table 8.

The peptide resins with the highest A1PI yields from Eff. II+III exhibited some differences in their capture and elution of other proteins in the feed. The peptides VIFLVR and RAFWYI are both approximately 80% of the A1PI, but VIFLVR gave a lower purity of 52 vs 69% for RAFWYI. The acid eluates for VIFLVR and RAFWYI both contained A1PI, Apo A-1, and IgA. VIFLVR had a lower purity because of the presence of hSA in its acid eluate, while the eluate for RAFWYI contained no detectable hSA.

The binding and elution of hSA highlighted another difference among the various peptide resins. VIFLVR allowed 90+% of the hSA in Eff. II+III to flow through unbound, while the sequence RAFWYI (and others with a positively charged residue near the N-terminus, sequence IDs 3–5, 11) captured up to 50% of the hSA loaded. The hSA captured by all of these peptides eluted in 1 M NaCl, except for the small amount in the acid eluate for VIFLVR.

The capture of hSA by the peptide columns has two important implications on their potential use. First, the binding of hSA and other proteins could ultimately limit the capacity for A1PI and reduce the column throughput due to competition for binding sites. Additionally, in the case of plasma protein production, the impact on downstream processes must be considered. An ideal capture step would selectively capture A1PI, leaving the remaining stream relatively unchanged for further processing. The significant capture of a downstream product like hSA in Eff. II+III could necessitate other process changes.

A number of peptides found from the library screening using an aqueous solution at 20°C purified A1PI from the 20% ethanol, 0°C, Eff. II+III. The A1PI yields for the same peptide were the same and sometimes higher from Eff. II+III than from the aqueous hSA/A1PI mixtures, despite the higher total protein concentration and number of proteins in Eff. II+III. However, the presence of other proteins in Eff. II+III led to lower purities from Eff. II+III.

Elution and Reactivation of A1PI

For all of the results presented, the peptide resins were subjected to the same chromatography cycle with step elutions of 1 M NaCl and 2% acetic acid. The acid eluate was used to elute bound protein by using a pH below the pI of both hSA and A1PI. When subjected to a pH around or below its pI of 4.7, A1PI is irreversibly inactivated (21, 22). Literature data suggested A1PI activity was recoverable when exposed to very low pH (i.e., 2–3) followed by titration with basic media and incubation (47). Attempts to recover activity with our samples in this manner proved unsuccessful.

We found that elution was possible with 4–6 M guanidine hydrochloride (GdnHCl) in water in lieu of 2% acetic acid. Bound A1PI was eluted from the

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peptide column VIFLVR with GdnHCl from both the aqueous and Eff. II+III feedstreams. The yields and purities from the peptide columns were identical whether using 2% acetic acid or GdnHCl as the eluate. Exposure to GdnHCl can inactivate A1PI by the formation of dimers and higher molecular weight polymers (48). Therefore, the GdnHCl eluate was immediately diluted 20-fold in a buffer containing 200 mM Tris, 10 mM EDTA, pH 8, based on a previous study in the literature (49). After incubation for 1 hour at room temperature, we were able to fully recover the A1PI activity from both aqueous and ethanol containing feedstreams.

CONCLUSIONS

The radiological screening of 2% of a linear, hexapeptide library identified 19 sequences with similar characteristics, all of which bound A1PI. The screening procedure did not generate any false positives, proving the method an effective and efficient technique for ligand identification.

The peptide sequences bound not only A1PI but also a significant amount of hSA. hSA was not used in the library screening as it was not shown to be an effective blocking agent. Future screening for A1PI or other targets should include the main contaminants during the target-library incubation to improve the ligand selectivity. A shortcoming of the direct detection system lies in its inability to distinguish between beads with only the radiolabeled target adsorbed and those with both target and contaminant bound, although the signal strength during detection may allow for differentiation of such beads.

Several peptides provided 60+% yields with 90+% purity in a single step from aqueous mixtures at 4 and 20°C. Using Eff. II+III, the peptides VIFLVR and RAFWYI demonstrated A1PI yields and purification factors equal to or better than reported ion-exchange chromatography (24, 34, 36). The peptides in this study performed best in the aqueous conditions in which the library was screened. The purification of A1PI from the more complex, ethanol-containing Eff. II+III was fortuitous given the large differences between the two feeds. Screening under conditions closer to the Eff. II+III solution would likely lead to even better performing peptides for purification from this feed.

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