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"Paralogs", sorbent families for protein separations

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ABSTRACT

Novel peptide-based (paralog) sorbents are evaluated with respect to performance, reproducibility and reusability in a 96-well test plate screening format, and to utility in protein separations. The results demonstrate that this approach to constructing sorbents provides a new and generally applicable set of tools for separating proteins.

INTRODUCTION

Liquid chromatography produces physical separations by exploiting differences in affinity of dissolved analytes for a stationary phase [1]. In conventional single-mode chromatography, such as ion-exchange or hydrophobic interaction chromatography, the differential affinities of analytes is relatively small.

Higher differential affinities can be achieved by using specific antibodies or other affinity sorbents. High affinity can be a drawback for chromatography, however, because it often renders impossible the recovery of bound analytes under mild conditions which would preserve bioactivity [2] and because it eliminates moderately related variants that may be of interest. Further, the use of strong elution conditions, effectively creating a binary on/off mechanism, eliminates the opportunity to identify microheterogeneities by the traditional chromatographic process in which numerous partitioning events take place between the stationary and mobile phases.

An antibody's high specificity for its target protein relative to accompanying contaminants is attributable to (i) the molecular scale mixture of separation modes provided by the polypeptide recognition site (paratope), and (ii) the rigidity of the binding environment due to the framework structure of immunoglobulins [3]. This rigidity contributes to high binding affinities and probably accounts for difficulties in eluting bound proteins in active form. Even in the case of ion exchangers, rigid spatial localization of the binding moieties can irreversibly distort the structure of the bound protein [4].

Molecular scale multi-mode sorbents for protein separation can be created by coupling short peptides to a solid support [5,6]. Systematic diversification of sorbent characteristics can be achieved by manipulating multiple properties of the ligands described by parameters drawn from the protein structure literature [7].

The use of short peptide-based ligands as paratope analogues, or "paralogs", overcomes in principle drawbacks inherent in high-affinity sorbents while retaining the desirable feature of providing specificities that are distinct from those available in single-mode sorbents. Unlike most previous mixed-

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mode sorbents (see ref. 8, where random co-polymers of amino acids as a chromatographic matrix are described. Polysorb MP-3 (Interaction Chemicals) is a mixed-mode polymeric sorbent containing both C_{18} and sulfonic acid moieties. Several varieties of Cibacron-Blue attached to DEAE- or CM-agarose are available, *e.g.* from Bio-Rad.) which combine modes at a level equivalent to bulk mixing of single mode sorbents, paralogs combine modes at the molecular size scale where binding actually occurs. Chemically synthesized paralogs may also contain non-peptide constituents which, in principle, allow the creation of specificities not possible with antibodies.

Previous assessment of paralog sorbents has been by examination of the patterns of proteins the sorbents bind, using a yeast extract as the source material and sodium dodecyl sulfate (SDS) gel electrophoresis as the analytical tool [7,9]. The qualitative conclusions from these experiments were that a variety of proteins can bind to a particular sorbent, that the same protein (or more accurately, SDS gel band) can bind to a variety of sorbents, and that the pattern of relative binding strengths to the different sorbents is characteristic of each protein, just as is true for single-mode sorbents such as DEAE-cellulose.

For the present study, a set of paralog sorbents, selected to mimic existing sorbents with varying degrees of fidelity [9], were studied in greater detail and used to establish general operating guidelines for handling these novel reagents.

EXPERIMENTAL

Reagents

DEAE-Cellulose (DEAE), CM-cellulose (CM), yeast extract, phenylmethylsulfonyl fluoride (PMSF) and all single proteins were purchased from Sigma (St. Louis, MO, USA). Affi-Gel 10 was purchased from Bio-Rad (Richmond, CA, USA). Paralog sorbents were synthesized at Terrapin Technologies (South San Francisco, CA, USA). Paralog sorbents each consist of a single paralog peptide coupled to N-hydroxy-succinimide activated agarose (Affi-Gel 10) at a particular loading density, generally *ca.* 4 μmol/ml sorbent settled bed volume (SBV) unless otherwise indicated. The coupling of paralogs to the agarose support was performed, with minor modifications, according to the manufacturer's recommendation. After completion of the coupling reaction, the remaining non-attached paralog was quantitated by HPLC. The difference between the initial peptide amount in the reaction mixture and the amount present post-coupling, after washing the beads, was considered to be covalently attached to the agarose sorbent. All peptides were synthesized by Multiple Peptide Systems (La Jolla, CA, USA), Advanced Chemtech (Louisville, KY, USA) or Coast Scientific Products (La Jolla, CA, USA).

A slurry of each sorbent was placed in replicate wells of a membrane-bottomed (flow-through) 96-well test plate (Silent Monitor, Pall Biosupport Corporation, Glen Cove, NY, USA) effectively creating miniature "columns" of 150 μl SBV. The wells of such sorbent plates were filled with storage buffer and the plates sealed and refrigerated until further use.

Collection plates (Falcon) were pretreated with Tween-20 to block protein adsorption to the plastic surfaces. The protein contents in the flow-through and retained-eluted fractions were determined using the Bio-Rad protein assay, adapted to the 96-well plate format; absorbances were read in a Vmax Plate Reader using SOFTmax software for curve fitting (Molecular Devices, Menlo Park, CA, USA). Homologous protein was used to generate a standard curve for each protein.

Paralog structures

The properties of the side chains of the various paralogs are summarized in Table I. An N-terminal amino acid spacer is present in all cases, the free amino group of which is used for coupling to the sorbent. With the exception of P3 and P7, this residue is α-aminoisobutyric acid (aib); for P3 and P7, it is 2-aminobutyric acid (2ab). In all cases, the C-terminus is capped with an amide group. Hydrophobic amino acids used (phenylalanine, alanine, valine) are D-isomers. Positive (ornithine), negative (aspartate) and neutral hydrophilic residues (serine) have the shortest length side chains readily available. Intra-chain cyclization is via a disulfide bond between two cysteine residues.

Using the standard three letter codes for the amino acids, along with the abbreviations noted above, the structures of the paralogs used in this study are

TABLE I

PARALOG STRUCTURES

Short peptides attached to a solid support allow proteins to bind to the sorbent through a controlled mixture of binding modes. Side chain properties (positive or negative, hydrophilic or hydrophobic) for sorbents that mimic conventional ion exchangers to varying degrees are illustrated. Internal cyclization via disulfide bonds are also indicated.

Paralog sorbent	Charge structure	Paralog sorbent	Charge structure
P1	+++++	P6	-----
P2	++OO++	P7	Δ----Δ
P3	Δ++++Δ	P8	Δ-++-Δ
P4	ΔO++OΔ	P9	H--OO+
P5	-H+++O	P10	HHOHH

as follows: P1 = aib–Orn–Orn–Orn–Orn–Orn; P2 = aib–Orn–Orn–Ser–Ser–Orn–Orn; P3 = 2ab–Cys–Orn–Orn–Orn–Orn–Cys; P4 = aib–Cys–Ser–Orn–Orn–Ser–Cys; P5 = aib–Asp–Ala–Orn–Orn–Orn–Ser; P6 = aib–Asp–Asp–Asp–Asp–Asp; P7 = 2ab–Cys–Asp–Asp–Asp–Asp–Cys; P8 = aib–Cys–Asp–Orn–Orn–Asp–Cys; P9 = aib–Phe–Asp–Asp–Ser–Ser–Orn; P10 = aib–Tyr–Ala–Gly–Ala–Tyr.

Buffers

The standard buffer used for preparing the sorbent slurries and sample loading was 10 m*M* Tris–HCl, pH 7.5, 1 m*M* EDTA (TE). The standard elution buffer was 10 m*M* Tris–HCl, pH 7.5, 1 m*M* EDTA, 1000 m*M* NaCl (TEN-1000). In fractionation experiments, we used TE- and NaCl-based elution buffers where the NaCl concentrations (m*M*) are indicated by the number following TEN-. For long-term storage of the sorbent plates, 2% glycerol and 0.01% sodium azide in TE was used. All reagents and steps were at room temperature.

Chromatography in the 96-well test plate format

The storage buffer was removed from the sorbent plate by centrifugation into an empty 96-well plate. The columns were washed with 200 μl TE three times. Centrifugation steps to drain the plates were performed on a Beckman TJ-6 centrifuge (Beckman Instruments, Fullerton CA, USA), equipped with 96-well test plate carriers, at 750 *g* (*ca.* 2000 rpm) for 2 min. The columns were then equilibrated in TE by the addition of 200 μl TE and incubation for 15 min. Excess buffer was again removed by centrifugation.

For binding profile experiments, 50 μl of a purified protein solution (1 mg/ml in TE buffer) were loaded onto each column in the sorbent plate. The plate was incubated at room temperature for 15 min to allow adsorption of the sample to the chromatographic sorbents, after which 50 μl of TE was added to each well and the buffer and unbound sample collected by centrifugation into a 96-well plate. An additional 50 μl TE were added to each well and removed by centrifugation into the same collection plate. This microplate then contained *ca.* 150 μl of unbound or flow-through (FT) protein fraction.

Proteins bound to the chromatographic sorbents were typically eluted by the addition of 75-μl TEN-1000 to each column and equilibration for 15 min at room temperature. The eluted proteins were collected into a second pretreated microplate by centrifugation. A second 75-μl TEN-1000 equilibration and elution step was performed, collecting into the same plate. These samples are the TEN-1000 retained-eluted (RE) fraction. The plates were washed with 6 *M* urea and re-equilibrated in TE prior to loading the next protein samples.

Affinity binding constant measurement

Equal volumes (200 μl) of a bovine serum albumin (BSA) solution series at varying concentrations (2.5, 5.0, 10.0, 20.0 and 50.0 mg/ml) were loaded onto 80-μl SBV "columns" of DEAE and 200-μl SBV "columns" of paralog sorbents P3 and P4 at a ligand density of *ca.* 14 μmol/ml SBV. After incubation for 15 min, the sorbents were washed with TE buffer to remove the non-adsorbed BSA. The adsorbed BSA was then eluted with TEN-1000 buffer and the protein concentration of the fraction determined. Adsorption isotherms were constructed and affinity binding constants calculated by Scatchard analysis [10].

Yeast extract prefractionation

Yeast extract, a whole cell acetone lysate, was dissolved in TE (180 mg solid in 2 ml buffer) with 10 μl of freshly prepared 50 m*M* PMSF added as a

protease inhibitor. After centrifugation at 10 000 *g* for 10 min, the sample solution was loaded on a 5-ml SBV DEAE-cellulose column and washed with 80 ml loading buffer. The adsorbed proteins were eluted with approximately 100 ml TEN-200 buffer, dialyzed against distilled water and lyophilized. This protein mixture was then used as starting material for further fractionation experiments comparing the performance of DEAE-cellulose and selected paralog sorbents, again using the microplate format for sequential step gradient elutions. For these experiments, all fractions were collected, dialyzed, lyophilized and then analyzed by SDS-polyacrylamide gel (10%) electrophoresis (PAGE) [11] using silver staining [12] as the visualization technique.

RESULTS

As detailed in the Experimental section, we developed a protocol that allows rapid evaluation of the adsorption characteristics of a large number of sorbents in parallel experiments. The key to this technique is use of a flow-through 96-well test plate, a device orginally developed for immunochemistry assays on large numbers of parallel samples and thus well adapted for efficient processing using tools such as multi-channel pipettors and 96-well plate readers. We selected BSA for our standard protein because of its widespread occurrence in the biotechnology industry, as a contaminant in fermentation products and assays, and as a stabilizer in drug formulations.

Diversification of sorbent characteristics was created by starting with a homopolymer of an amino acid and then substituting amino acids with other properties for particular positions [9]. Variations in ligand structure were tested which involve the absolute number and the spacing of substitutions as well as the extent to which the substitution differs in properties from the dominant monomer. A third parameter which was varied was stabilization of the backbone conformation by an intramolecular cross-link.

The paralogs examined in this paper were chosen to deviate to varying extents from traditional single-mode exchangers (Table I). Paralog sorbents P1, P2, P3, P4 and P5 represent new anion-exchange-like sorbents, designed to deviate from DEAE-cellulose to varying extents. Paralog sorbents P6 and P7 are designed to be cation-exchanger variants that deviate from CM-cellulose. P8 and P9 are mixed property sorbents and P10 is a hydrophobic paralog sorbent. Complete structures are provided in the Experimental section above. Ethanolamine-blocked Affi-Gel 10 (AFG) served as a "negative control".

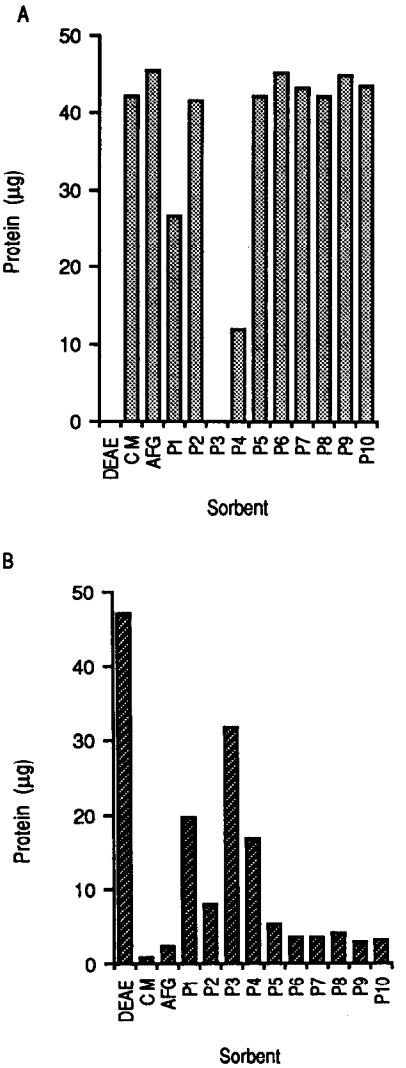

Fig. 1. Binding profile of BSA on a sorbent plate. (A) amount of BSA in the flow-through fraction (FT); (B) retained and eluted (RE) fraction. The sorbent plate contains DEAE- and CM-cellulose, and Affi-Gel control (AFG), and ten different sorbents prepared by coupling paralogs to Affi-Gel 10.

Protein binding profiles

The test protocol was designed to provide basic information on the adsorption, elution and recovery profile of single proteins. The two step protocol provided the amount of material which does not bind to the paralog sorbents and the amount of material which can be eluted from the columns under such conditions that maximum recovery is expected. The protein concentration in the FT and the RE fractions allow calculation of the mass balance of the procedure. Similarly, activity balance and/or composition analysis can be performed on the fractions.

The characteristic binding profile of BSA to the paralog sorbents is shown in Fig. 1. For comparison, the binding profile includes the adsorption of BSA to DEAE-cellulose, CM-cellulose and ethanolamine-blocked Affi-Gel (AFG). The amounts of BSA in the FT and the RE fractions for each sorbent are shown in Fig. 1A and B, respectively. Under these experimental conditions, DEAE-cellulose and paralog sorbents P1, P3, and P4 bind a significant amount of protein while CM-cellulose and the control AFG do not bind BSA.

Reproducibility of the protein binding profile protocol

Since the sorbent plate is not a standard chromatography format, we evaluated the plate-to-plate reproducibility. A 50-μg amount of BSA was loaded into each well and eluted according to the standard protocol. Using three different sorbent plates, we calculated the amount of protein in the FT and the RE fractions (Table II). The average relative standard deviation for the FT and RE fractions with significant protein content was *ca.* 15%. The mass balance averaged to 88% of starting material for all sorbents.

Reusability of the sorbent plates

Since short peptides are not subject to denaturation, it should be possible to regenerate the sorbents by stripping all bound proteins with a strong denaturant. To test this expectation, the sorbents from the previous experiment were treated with 6 *M* urea, re-equilibrated in TE, and tested again for their ability to bind BSA. A total of five binding/elution/regeneration cycles were performed on three different sorbent plates. Typical results, from one plate, are diagrammed in Fig. 2 in a three-dimensional

TABLE II

PLATE-TO-PLATE REPRODUCIBILITY

The BSA screening profile against the paralog sorbents illustrated in Table I provides the micrograms of total protein recovered in the flow-through and retained fractions, out of 50 μg applied. All sorbents have high % protein recovery except P3 and P4, the sorbents which have the highest affinity for BSA. The results for three plates were averaged for each determination.

Sorbent	Flow-through			Retained-eluted			Total protein		
	Average	S.D.	Recovery (%)	Average	S.D.	Recovery (%)	Average	S.D.	Recovery (%)
DEAE	0.0	0.0	0	47.3	4.4	95	47.3	4.4	95
CM	42.2	9.2	84	0.8	0.9	2	43.0	8.5	86
AFG	45.6	2.8	91	2.2	1.9	4	47.8	4.7	96
P1	26.7	2.6	53	19.7	1.6	39	46.4	3.8	93
P2	41.8	2.5	84	8.0	0.8	16	49.7	1.7	99
P3	0.0	0.0	0	31.7	1.7	63	31.7	1.7	63
P4	11.9	6.5	24	16.8	3.5	34	28.7	8.3	57
P5	42.3	2.6	85	5.0	1.3	10	47.3	2.6	95
P6	45.2	1.5	90	3.5	1.8	7	48.7	1.6	97
P7	43.3	3.5	87	3.5	3.0	7	46.8	6.4	94
P8	42.3	3.2	85	4.1	3.2	8	46.4	6.3	93
P9	45.2	3.3	90	3.0	1.3	6	48.2	4.5	96
P10	43.8	8.0	88	3.1	0.7	6	46.9	7.3	94

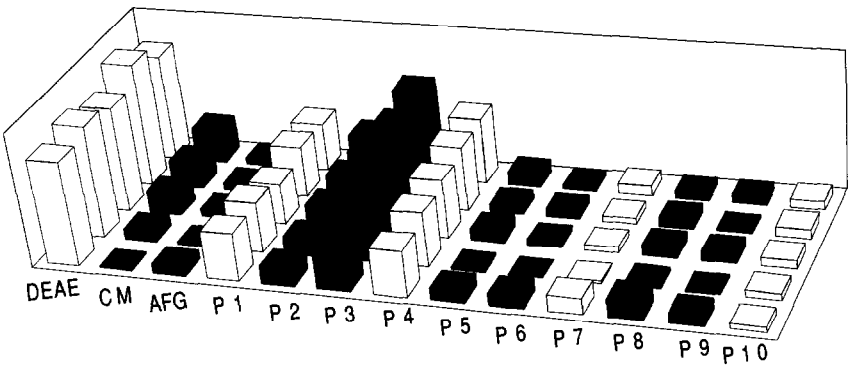

Fig. 2. Reusability of the sorbent plates. BSA retained and eluted profiles on a sorbent plate, determined as in Fig. 1, are conserved through five cycles of screening and sorbent regeneration.

bar-chart which displays the BSA binding profile on a panel of sorbents across five successive cycles.

Protease resistance of the paralog sorbents

A concern regarding re-use of paralog sorbents is the inherent protease sensitivity of peptide ligands. The paralog sorbents examined here have both N and C termini blocked; they also incorporate several non-standard amino acids. To test the efficacy of these features in hindering proteolysis, the BSA binding capacity of fresh paralog sorbents was compared to that of sorbents incubated with 1 mg/ml trypsin solution for 30 min. The acitivity of the trypsin solution was confirmed in parallel experiments by observing release into solution of dye from azocoll, an insoluble dye–protein conjugate [13]. After removing the trypsin, washing the sorbents with 6 *M* urea and re-equilibrating the sorbent plate in TE buffer, we repeated the BSA binding experiment. The amount of BSA bound was comparable to that shown in Table II indicating that at least those paralog sorbents which bind BSA are resistant to proteolysis. During a different set of experiments, we saw that trypsin treatment of plates used many times with a variety of proteins helped to restore the BSA binding characteristics, presumably by degrading irreversibly bound protein (data not shown).

Binding profiles of a panel of single proteins

After establishing the reproducibility of operations with BSA, we used the standard protocol to determine the binding profile of several other commercially available purified single proteins. The binding experiments were performed at least three times for each protein. Periodically, BSA was run on the plates as a quality assurance marker. The BSA profiles were similar to Table II providing additional evidence for reusability of the plates. Fig. 3 displays the binding profile of several proteins on DEAE-cellulose, CM-cellulose, blocked Affi-Gel, and the paralog sorbents. For this figure, the results are presented as a transformed bar chart in which the height of the bars have been transferred into gray scale values. We established five levels on the gray scale, which correspond to <5, 5–15, 15–25, 25–35 and >35 μg adsorbed protein out of the 50 μg applied. Fig. 3 thus allows data for all three parameters to be easily visualized: 13 sorbents $\times$ 10 proteins $\times$ 5 qualitative adsorption values.

Measurement of the affinity binding constant

The binding affinity of BSA for two paralog sorbents was determined in TE buffer using standard Scatchard analysis [10]. The results (Fig. 4) indicate that the sorbents provide binding strengths comparable to traditional ion-exchange resins, *ca.* 10^4 M^{-1}, a range that also characterizes low to moderate affinity antibodies [14]. The affinity of protein–paralog interactions is thus in the range which is typically used for chromatographic resolution of similar proteins by repeated differential partitioning, a process not generally possible in the on/off step elution mode of traditional affinity chromatography using high-affinity ligands.

At a ligand density of 14 μmol/ml of paralog sor-

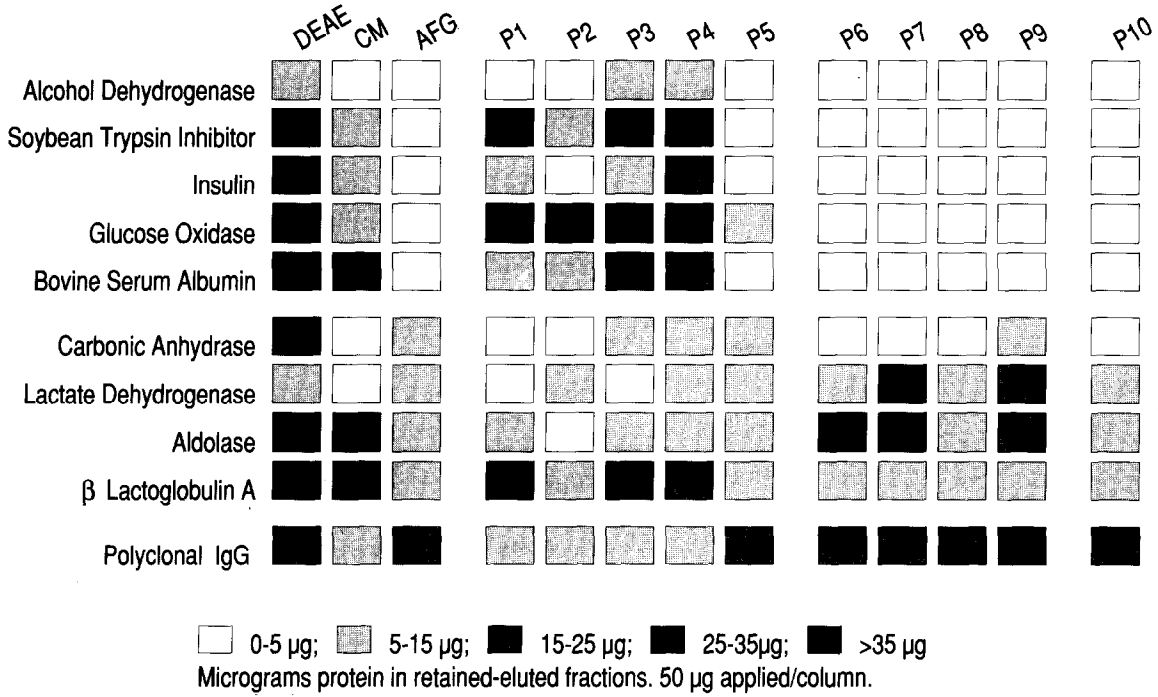

Fig. 3. Comparative binding profiles of single proteins. The squares represent the amount of protein retained on the sorbents: the darker the square, the more protein retained and eluted from the sorbent; in all cases, 50 μg of protein was applied.

bent, the capacity of P3 for BSA is about 13% the capacity of a comparable amount of DEAE-cellulose, in reasonable accord with the fact that the number of positive charges is about 9% that of DEAE-cellulose. Independently prepared batches of paralog sorbents, with constant amount of paralog put into the coupling reaction, yielded sorbents with equal ligand densities to within the accuracy of the determination. For these experiments, the amount of ligand conjugated to the solid support was estimated from the difference between ligand added to the conjugation reaction and ligand recovered free in solution following the reaction. As a functional test, two independently prepared batches of P4 were used to generate BSA binding profiles, with the results matching to within the precision of the determination. A second pair of sorbents using paralog P4 was also prepared with half the amount of ligand put into the coupling reaction resulting in approximately half the amount of ligand attached to the solid phase; the maximal binding for BSA to these sorbents was reduced appoximately by half compared to the higher ligand density sorbent.

Application of paralog sorbents to sequential fractionation of a complex protein mixture

Differential binding profiles of proteins is expected to aid in protein purification. To further examine the utility of the novel sorbents in a model protein fractionation system, a complex mixture of yeast proteins was first fractionated on DEAE-cellulose using a steep NaCl gradient. The protein fractions were eluted with 0, 20, 50, 80, 100 and 140 m*M* NaCl in TE buffer. The 80 m*M* NaCl fraction was then dialyzed against TE. Following a commonly practiced protein purification strategy, that fraction was then re-fractionated on DEAE using a shallower NaCl gradient (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 m*M* NaCl) to provide higher resolution. In parallel, other aliquots of the same fraction were chromatographed on paralog sorbents P3 and P4, anion-exchange variants. The use of a flow-through microplate, following the protocol described above for single protein profile determinations, helped to insure that these parallel processing steps were conducted under identical conditions. Three wells of each sorbent were used, and fractions pooled, to

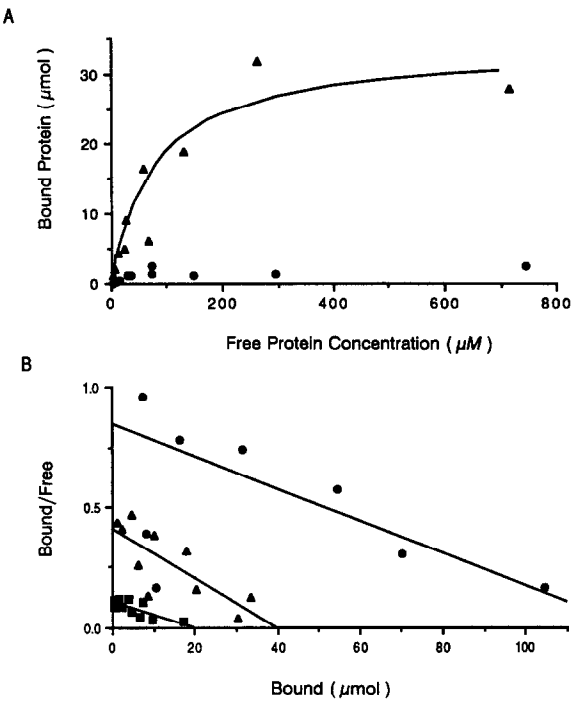

Fig. 4. Adsorption isotherm of BSA on DEAE-cellulose, P3 and P4. (A) specific BSA binding, in μmol, on sorbent P3 (▲) and a blank Affi-Gel control (●). (B) Scatchard analysis of these data along with comparable data for P4 (■) and DEAE (●) (▲ = P3). The paralog ligand density on the sorbents was 14 μmol/ml SBV. The calculated affinity constants for BSA binding are: $1 \cdot 10^4\ M^{-1}$ (P3), $5 \cdot 10^3\ M^{-1}$ (P4), $7 \cdot 10^3\ M^{-1}$ (DEAE). The maximal binding in the DEAE case is 2.5 × larger than the graph shows because a 40% smaller settled bed volume of DEAE sorbent was used than in the paralog cases.

provide enough capacity for the sequential fractionation experiments.

The SDS-PAGE analysis of the resulting fractions are provided in Fig. 5. A constant proportion of each fraction was loaded onto the gel, thus resulting in certain lanes being overloaded with regard to optimal staining for visualization of individual bands but allowing clear visualization of the differences in overall binding between the various fractions. The differences in selectivity between DEAE and the paralog sorbents are clearly illustrated in Fig. 5B. The ionic strength necessary for the elution of the protein mixtures is lower on the paralog sorbents than on DEAE-cellulose. The composition differences of the coresponding fractions (*e.g.* same ionic strength) collected from P3 and P4 are also distinct.

To further compare the utility of the differing selectivities provided by the family of paralog sorbents, we selected the 50 m*M* NaCl fraction from the secondary separation on P3 and, following dialysis against TE, subjected it to a tertiary fractionation on sorbent P4. Similarly, the 50 m*M* NaCl fraction from P4 was applied to P3. The proteins in these tertiary fractionation steps on both paralog sorbents were eluted with a salt gradient containing 30, 40, 50 and 80 m*M* NaCl steps. The SDS-PAGE analysis of these fractions are displayed in Fig. 5C. It is evident that the composition of the analogous fractions are significantly different. These observations indicate that consecutive purification steps on different paralog sorbents can provide favorable selectivity for a variety of proteins.

DISCUSSION

The availability of sorbents that deviate to varying extents in binding properties from conventional ion-exchangers is expected to be of general utility in protein purification due simply to the differences themselves, quite apart from the absolute specificity that the sorbents offer, because the differences allow sequential separation of a variety of contaminants. The data provided here show, for both individual purified proteins and for the many different proteins in a yeast extract, that one protein's binding affinity to each member of a panel of paralog sorbents is largely uncorrelated with the binding affinity of other proteins for each member of the same panel. By contrast, conventional sorbents of the same "type", such as anion exchangers of strong, medium, and weak varieties, show a high degree of correlation in binding pattern to the panel of one protein compared to other proteins, with essentially all analytes binding more tightly to the strong form at a given pH.

Because paralogs differ in both character of binding motifs and their spatial distribution, individual proteins were expected to show less uniformity in their binding profiles across a panel of paralog sorbents than against a panel of sorbents differing in only one parameter. The experiments reported here document the validity of this hypothesis and thereby establish the generalized applicability of the pa-

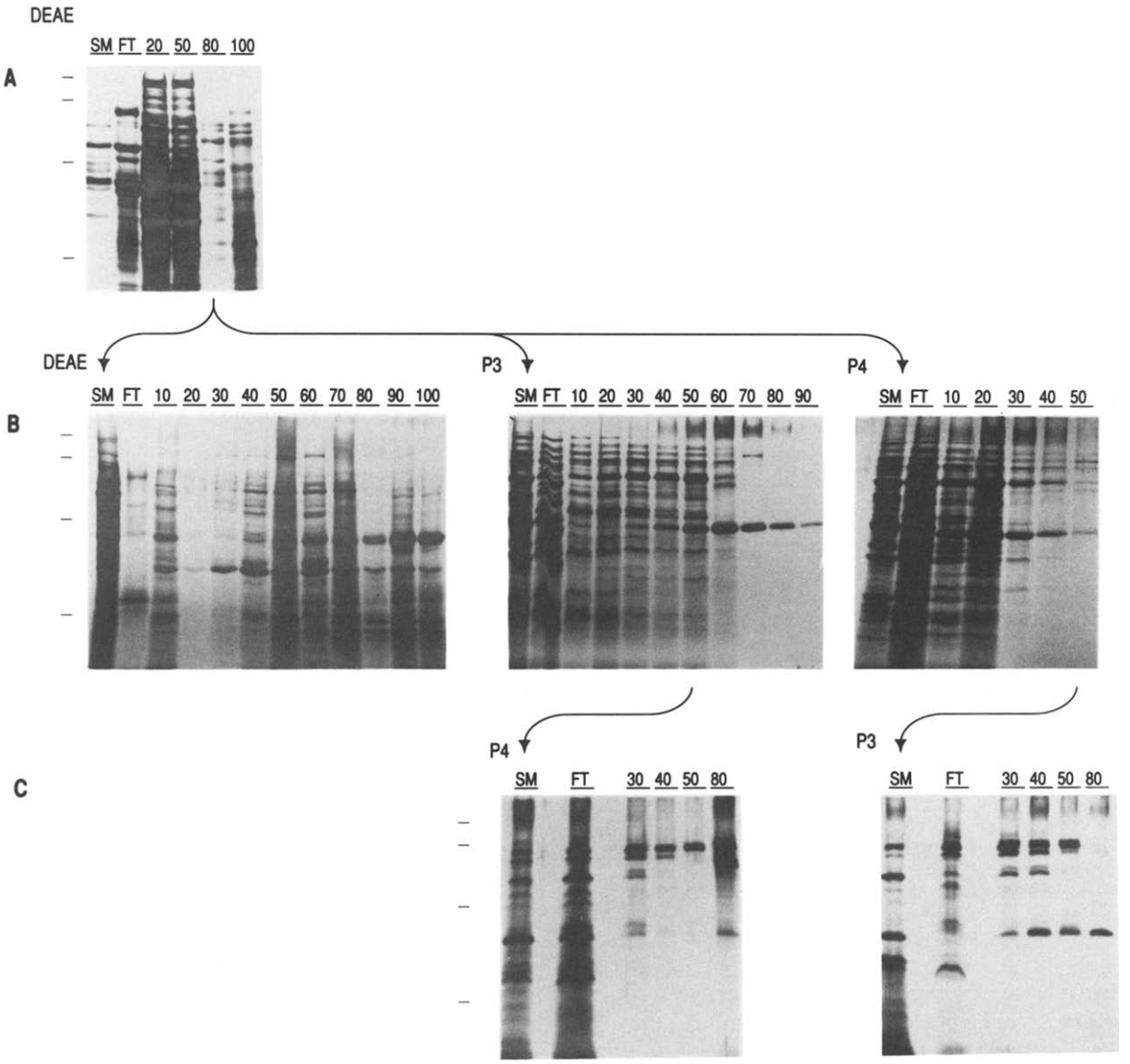

Fig. 5. Fractionation of a yeast cell lysate by sequential purification. First lanes are the starting material (SM), second lanes are the flow-through fractions (FT) and the numbers over the remaining lanes indicate the NaCl concentration (m*M*) of the elution buffer. SDS-PAGE (10%) and silver staining was used to visualize the fractionated protein patterns. Equal-sized aliquots of each fraction were loaded on the gel, resulting in some overloading of lanes for fractions with very high protein contents. Molecular mass markers (Bio-Rad, pre-stained) are M_r 80 000, 49 500, 32 500 and 18 500. (A) Primary fractionation of yeast proteins on DEAE-cellulose sorbent. (B) Secondary fractionation of yeast proteins on DEAE-cellulose, P3 and P4; (C) Tertiary fractionation of yeast proteins on sequentially run paralog sorbents. The 50 m*M* eluate on P3 from (B) was loaded onto P4; likewise, the 50 m*M* eluate on P4 from (B) was loaded onto P3.

ralog concept. By providing molecular scale combinations of known single-mode separations, analogous to that furnished by antibody binding sites (paratopes), paralogs allow a wide range of separation characteristics useful for distinguishing proteins. The set studied here mimics conventional ion

exchangers to varying degrees. Additional sets can in principle also be produced, centered on other structural motifs. Such reagents offer a number of advantages in purification optimization. First, the selectivity differences between family members is a valuable parameter by itself as in traditional affinity chromatography. Second, the moderate affinity allows gradient elution, providing additional utility analogous to traditional uni-modal chromatography. Further, since the ionic strength necessary for the elution is low, properly selected sorbents can be applied in sequence without prior desalting of the sample. These desirable operating charactersistics of the paralog sorbents should reduce the losses in yield and activity generally associated with other sequential purification schemes.

The novel properties of the anion exchanger paralogs are more evident in the experiments reported here than for the cation exchangers in part because many of the test proteins also bind to the blocked Affi-Gel, most likely due to free carboxylic acid residues which remain exposed on the final sorbents. These excess carboxylic acid groups are attributable to the manufacturing procedure of Affi-Gel 10.

While providing some of the selectivity characteristics of antibodies, paralogs can be treated with strongly denaturing buffers without the loss of activity encountered with antibodies, and can be used without the introduction into the purified protein of macromolecular contaminants resulting from degradation and leaching of the antibody off the support. Theoretically, other regeneration conditions compatible with peptide and agarose chemistry may also be used, such as guanidine hydrochloride, sodium thiocyanate, 0.2 *M* propionic acid, methanol, or a variety of detergents. We note, however, that the efficiency of regeneration and the stability of these unique sorbents might be different when more complex protein mixtures are used than those tested here. Therefore, the reuseability should be directly established for such samples.

The standard screening protocol described here used only one arbitrarily selected buffer, with pH and ionic strength conditions chosen to correspond to a frequently applied protein purification condition. We anticipate that other conditions would provide additional diversity in the binding profiles. The sorbent plate format allows not only rapid evaluation of numerous sorbents under identical mobile phase conditions, but also evaluation of a single sorbent under a variety of mobile phase conditions. The effects of ionic strength, counter-ion type, organic modifiers, and pH on the adsorption of a single protein or of a protein mixture can thus be conveniently measured.

CONCLUSIONS

A wide range of protein purification and characterization applications should benefit from the development of families of diverse sorbents with properties intermediate between existing sorbents [15]. The sorbent design strategy illustrated here provides one route to building such an array. Another example of the same concept is the recently introduced panel of sorbents derivatized with individually synthesized textile dyes, a panel that provides some additional selectivity over the previous sorbents derivatized with the mixture of dyes known as Cibacron Blue [16]. The utility of any such panel of sorbents is augmented when combined with a convenient format for their rapid evaluation, such as the flow-through microplate used here. More broadly, interactions between short peptides and macromolecules have become an area of increasing interest due to work in immunology [3], drug design [17], and through intriguing observations of particular peptides binding to particular proteins [6,18]. The results reported here establish the immediate feasibility of studying such peptide-macromolecule interactions by use of simple chromatographic procedures that provide highly sensitive indications of differential affinity.

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