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Discriminatory protein binding by a library of 96 new affinity resins: A novel dye-affinity chromatography tool-kit

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ABSTRACT

Initial acceptance of Cibacron Blue 3G-A® based matrices has made dye-ligand affinity chromatography an attractive proposition. This prompted the synthesis and search for new dye structures. A systematic library of 96 affinity resins was generated using novel analogs of Cibacron Blue 3G-A® and also by varying spacer lengths for immobilization. The library was tested in a batch binding and elution mode using seven different proteins – four Aspergillus enzymes namely, NADP-glutamate dehydrogenase, laccase, glutamine synthetase and arginase, bovine pancreatic trypsin and the two serum proteins human serum albumin and immunoglobulin G. Unique binding patterns were observed for each of them indicating that the library displayed discriminatory interactions. The significance of spacer length in the interaction with proteins was discernable. Trypsin interacted best with affinity resins that had no spacer. It was possible to resolve IgG and HSA from a mixture using a combination of resins. There was a good spread of HSA binding capacity in the 96 affinity resins. While some showed better HSA binding capacity than the commercial CB3GA-based matrix, a few with lower capacity were also observed. Subsequent to an initial screen, one affinity resin (CR-017) could be used to enrich *Aspergillus terreus* NADP-GDH from crude cell extracts. The efficacy of this dye-affinity resin was rationalized by characterizing NADP-GDH inhibition kinetics with the corresponding free dye ligand. In the sum, the library provides a set of dye-ligand affinity matrices with a potential for use in high throughput screening for protein purification.

1. Introduction

The serendipitous discovery of the binding of Cibacron Blue 3G-A® (CB3GA) to enzymes gave birth to the concept of developing textile dyes as ligands for affinity chromatography [1]. Textile dyes belonging to the monochlorotriazine class of compounds gained importance when biochemists observed a reversible binding between phosphofructokinase and Blue Dextran® – commonly applied macromolecular marker for measuring void volume in gel filtration chromatography [2]. Cibacron Blue F3G-A is a mixture of *meta* and *para* substituted isomers of the textile dye while CB3GA represents pure *ortho* component [1]. Subsequently, the reactive dye CB3GA has been found useful for the purification of several proteins and enzymes. Fractionation of plasma proteins was performed on Cibacron Blue F3G-A immobilized cross-linked agarose beads. SDS-PAGE was subsequently used to monitor 27 plasma proteins which could be eluted out from this column by a pH gradient [3]. Albumin has been purified successfully on large scale by using CB3GA immobilized on agarose [4]. Trypsin-like serine proteases can be purified on CB3GA-agarose and interaction of CB3GA with the serine protease active site is also reported [5].

The applications of CB3GA as a ligand in dye-affinity chromatography have been reviewed periodically [1,6,7]. Interactions of this dye with various proteins and enzymes are also reported. Several theories attempt to explain the binding of CB3GA to proteins or enzymes. An early idea, referred to as the 'dinucleotide-fold hypothesis', suggested that the dye mimics nicotinamide adenine dinucleotide (NAD^+) moiety and hence interacts with proteins and enzymes that possess a dinucleotide-fold. In such cases, the bound proteins can be eluted by specific nucleotides [8]. However, a range of proteins like albumin, aldolases, hemoglobin and cytochrome C also bind to CB3GA [1]. Such nonspecific binding could be ionic, hydrophobic or a combination thereof and can be disrupted by

Abbreviations: CB3GA, Cibacron Blue 3G-A®; CR, CibaFix® Resin; HSA, human serum albumin; IgG, immunoglobulin G; GS, glutamine synthetase; NADP-GDH, NADP-glutamate dehydrogenase.

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Table 1
Various commercially available 96-well kits for protein purification.

Product	Source	Application
MultiTrap™ 96-well filter plates	GE Healthcare, Little Chalfont, UK	GST-tag, His-tag and immunoaffinity
Mimetic ligand 96-well screening kit	ProMetic BioSciences Inc, Maryland, USA	Chromatography screen for ProMetic dye adsorbents
High throughput resin screening tools	ATOLL GmbH, Weingarten, Germany	Any commercial resins individually packed in 96 MiniColumns
HisLink™ 96 protein purification system	Promega, Madison, USA	Purification of expressed proteins from bacterial culture
TALON HT 96-well plate	Clontech, Mountain View (California), USA	Purification of His-tagged proteins
Vivapure(TM) 8 to 96-well cobalt-chelate kit	Sartorius AG, Göttingen, Germany	Membrane-based purification of His-tagged proteins
RoboPop purification kit	EMD Chemicals, Inc., Gibbstown, UK (Novagen)	Purification of GST/His-tagged fusion proteins
Superflow 96 BioRobot	QIAGEN, Valencia (California), USA	Purification of His-tagged proteins

chaotropic salts. The bound proteins could be eluted by changing the ionic strength or pH of the medium. While a theoretical basis for such protein binding is yet to be fully understood, their specificity may be determined mainly by hydrophobic interactions and the stability by electrostatic forces [9].

Following the initial success of CB3GA a number of commercially available affinity resins like Blue Sepharose from GE Healthcare, Uppsala, Sweden and Blue Affigel from Bio-Rad Laboratories, Hercules, USA make use of CB3GA as their dye-affinity ligand. Subsequently triazine dyes (other than CB3GA) were also used in the purification of proteins and some of these products are patented [10–12]. Triazine dye ligand-based adsorbents like MAbsorbent® A1P and A2P are also available as alternative to Protein A columns for the purification of IgGs [13]. The utility of these affinity resins on an industrial scale has lagged because of concerns of selectivity and toxicity. Issues of toxicity were addressed and tolerance levels have been defined for CB3GA [14,15].

Several directed and empirical attempts were made in the past to generate novel triazine-based dye-affinity resins [16–18]. The issues of selectivity were addressed by preparing synthetic ligands, synthesizing tailor made ligands based on molecular modeling and using other combinatorial approaches. Triazine group containing structures are also amenable to high throughput screening by microarray-based techniques [19]. Similarly, methods for simultaneous screening large number of dye-ligands for protein purification are described in the literature [20–24]. Different platforms in the 96-well format are commercially available (Table 1). There is further scope for achieving efficient purification processes through the synthesis of novel dye-ligand structures and their presentation for large through put screening. A number of dyes analogous to CB3GA – a well-studied pseudo affinity ligand – were synthesized and 96 different affinity resins based on these CB3GA analogs were prepared [25]. Here we describe the characterization of these novel affinity resins by defining their discriminatory binding to different enzymes and proteins. The potential utility of this affinity resin library as an affinity chromatography tool-kit for developing a down stream protein purification strategy is discussed.

2. Materials and methods

2.1. Chemicals and reagents

Human serum albumin (HSA) was obtained from Sanquin, Amsterdam, The Netherlands (Product name: Cealb) and pure immunoglobulin G (IgG) fraction was from VHB Life Sciences, Maharashtra, India (Product name: Iviglob*EX). The IgG fraction is a clinical, commercially available product from a pharmacy. It is available in solution form at 50 mg protein per ml. The solution (of pH 4.0) contains total human immunoglobulin G fraction (≥95% purity, polyclonal) and is made iso-osmotic with 10% maltose. Chromatography matrices namely, Sephadex G-25, Blue Sepharose and DEAE-Sephacel were obtained from GE Healthcare, Singapore. Water purified from Millipore-Milli-Q® Ultrapure Water Purification Systems from Millipore, Massachusetts, USA was used throughout this study. Nα-benzoyl-DL-arginine-4-nitroanilide hydrochloride, 2,2'-azino-bis(3-ethylbenzthiazoline) sulfonic acid (ABTS) and NADPH were purchased from Sigma–Aldrich Co., St. Louis, Missouri, USA. All other chemicals were of analytical grade and were either obtained from Sigma or from the local suppliers. All the Cibacron dyes used in this study were synthesized in-house and were from Ciba Research (India) Pvt. Ltd., Mumbai, India.

2.2. Synthesis of 96 new affinity resins

Five different anthraquinone moieties were coupled to cyanuric chloride during the synthesis of a range of reactive dyes analogous to CB3GA. Variations were introduced in the 'A region' (Fig. 1) by varying substitutions on the aromatic ring or by replacing the aromatic ring by an aliphatic functionality. The different dichlorotriazine intermediates thus formed were treated in the next step with different amines, aromatic or aliphatic in order to vary 'B region'. Thirty-three different dye analogs of CB3GA (numbered CD-1 to CD-33) were synthesized in purities greater than 90% and were subsequently used to prepare different affinity resins. These dyes were immobilized on 6% cross-linked agarose beads, either directly or through spacer arms of varying lengths (from 7–13 atom spacers). Details on the preparation of these dyes and the affinity resin library may be found in a patent [25]. All the 96 affinity resins were characterized in terms of – (a) ligand density i.e. the amount of dye immobilized (per g of packed/drained resin) and (b) stability of each resin towards storage, elution and cleaning conditions.

2.3. Enzyme sources, extraction and buffers used

Aspergillus niger (NCIM 565) and *Aspergillus terreus* (NCIM 656) were obtained from National Collection of Industrial Microorganisms at NCL, Pune, India. Laccase, arginase, and glutamine synthetase were prepared from *A. niger* (NCIM 565) grown on minimal medium (without nitrogen source, [26]) supplemented with ammonium nitrate (28 mM), arginine (14 mM) or glutamate (50 mM), respectively. NADP-glutamate dehydrogenase (NADP-

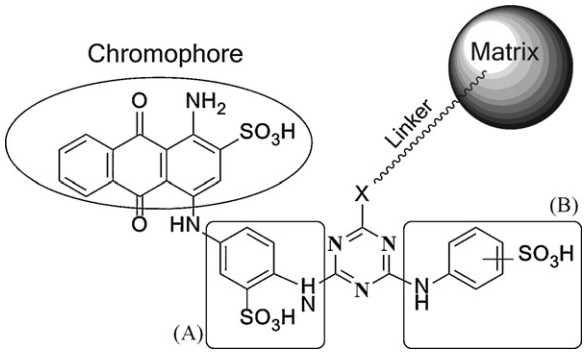

Fig. 1. Structure of Cibacron Blue 3G-A® and its linkage to the matrix. The reactive dye is immobilized through a reaction at the group X. Molecular diversity was achieved by incorporating various substitutions/changes in regions A and B of the structure.

Table 2
Buffers used to extract various *Aspergillus* enzymes.

Enzyme	Extraction buffer
Laccase	20 mM phosphate buffer at pH 7.0
NADP-GDH	100 mM phosphate, 1 mM PMSF, 1 mM EDTA and 4 mM 2-Mercaptoethanol at pH 7.5 [15]
Arginase	200 mM imidazole HCl, 1 mM PMSF, 12 mM $MnSO_4$, and 2 mM 2-Mercaptoethanol at pH 7.5 [17]
Glutamine synthetase	20 mM imidazole, 0.1 mM EDTA, 5% glycerol, 2 mM 2-Mercaptoethanol at pH 7.8 [15]

GDH) was obtained from *A. terreus* grown on minimal medium with sodium nitrate (50 mM) as sole nitrogen source. Enzyme extraction from the mycelia was essentially according to Choudhury et al. [26] but with different extraction buffers as mentioned in Table 2.

For each enzyme, the protein precipitating between 30% and 80% saturation of ammonium sulfate was collected and stored at −20 °C until further use. These enzyme samples were re-dissolved in appropriate purification buffer and desalted on Sephadex G-25 before loading on to the affinity resin. For laccase and glutamine synthetase, the purification buffer and the extraction buffer were the same. However, NADP-GDH and arginase purification was conducted in phosphate (20 mM potassium phosphate, 1 mM EDTA and 4 mM 2-mercaptoethanol) and imidazole (25 mM imidazole-HCl, 1.2 mM $MnSO_4$, 2 mM 2-mercaptoethanol) buffers at pH 7.5, respectively. Trypsin (Sisco Research Laboratories; Product code 204844) binding was monitored using Tris–HCl buffer (25 mM Tris, 0.5 M $CaCl_2$ and 0.5 M $MgCl_2$ at pH 7.4) [5]. HSA and IgG binding/separation was followed in Tris–HCl (50 mM) buffer, at pH 8.0 [27]. Respective buffers used in individual examples for binding and elution are also summarized in Table 3.

2.4. Enzyme and protein estimations

Arginase was assayed by estimating the urea formed in a standard assay, using Arachibald method with certain modification [28]. Gutamine synthetase was monitored through γ-glutamylhydroxamate formed by its Mn-dependent γ-glutamyl transferase activity [29]. NADP-GDH activity was estimated by NADPH disappearance in its reductive amination reaction [30]. Laccase was assayed in a standard 1 ml reaction by the continuous oxidation of ABTS at 436 nm [31]. Trypsin was monitored using DL-BAPA as substrate according to Erlanger et al. [32]. One unit of activity was defined as the amount that enzyme required to produce 1 μmol of the product per min in the standard assay. However, the unit for trypsin was defined as 1 nmol of p-nitroaniline formed per minute.

The protein samples containing HSA and IgG were directly monitored through their absorbance at 280 nm. Corresponding standard curve in each case was used to determine the amount of protein in the samples. In case of enzyme samples, protein concentration was estimated [33] using bovine serum albumin as a reference.

2.5. Electrophoretic procedures

Electrophoresis was performed in a Hoefer SE250 system according to manufacturer's guidelines. Proteins were separated in 1 mm thick acrylamide gels. Native PAGE [34] (in 7.5% gels) was run at pH 8.8 without 2-mercaptoethanol while the SDS-PAGE [35] (in 10% gels) was performed at pH 8.8 under reducing conditions (protein samples treated with 5% 2-mercaptoethanol). The protein bands on gels were visualized by staining with Coomassie Blue R-250. The gels were stained with freshly prepared Coomassie Blue R-250 for 30 min followed by destaining in a mixture of methanol:water:acetic acid (v/v, 3:6:1). The destaining was done till the bands were prominent and the background was clear.

2.6. Screening of affinity resins

Batch binding and elution was used to assess the interaction of various proteins with the affinity resins. Affinity resins (0.3 ml drained medium) were taken in the 1.5 ml Eppendorf tubes and equilibrated with purification buffer. Various enzyme/protein samples were applied on to these matrices and incubated on ice for 30 min with occasional mixing (at least twice). The unbound fraction was collected as supernatant after centrifugation at 10,000 × *g* for 5 min. These affinity resins were washed twice with the purification buffer. Typically, elution of the bound proteins was attempted in two batches – first with a low salt containing buffer followed by high salt buffer. In all these steps the affinity resins were incubated for about 10 min (on ice) to achieve proper equilibration. After each operation supernatant was collected by centrifugation at 10,000 × *g* for 5 min. Amounts of various enzyme/protein samples loaded and volumes of buffer used in washing and elution steps are listed in the Table 3.

For mass balance calculations, the enzyme activity (in appropriate units) recovered after the respective binding and elution protocol was computed as below.

$$\text{Enzyme activity recovered}(\%) = \frac{\text{enzyme units in (elution 1 + elution 2 + elution 3)}}{\text{enzyme units loaded}} \times 100$$

Table 3
Binding and elution protocols used to evaluate affinity resins.

Sample	Load	Wash 1	Wash 2	Elution 1	Elution 2	Elution 3
Laccase[a]	0.5 U in 0.5 ml	0.5 ml	0.5 ml	0.5 ml (with 0.3 M KCl)	0.5 ml (with 0.3 M KCl)	0.5 ml (with 0.6 M KCl)
Arginase[b]	0.5 U in 0.5 ml	0.5 ml	0.5 ml	0.5 ml (with 0.3 M KCl)	0.5 ml (with 0.3 M KCl)	0.5 ml (with 0.6 M KCl)
Glutamine synthetase[c]	0.5 U in 0.5 ml	0.5 ml	0.5 ml	0.5 ml (with 0.3 M KCl)	0.5 ml (with 0.3 M KCl)	0.5 ml (with 0.6 M KCl)
NADP-GDH[d]	0.1 U in 0.5 ml	0.5 ml	0.5 ml	0.5 ml (with 0.3 M KCl)	0.5 ml (with 0.3 M KCl)	0.5 ml (with 0.6 M KCl)
Trypsin[e]	10 U	0.5 ml	0.5 ml	0.5 ml (with 0.3 M KCl)	0.5 ml (with 0.3 M KCl)	0.5 ml (with 0.6 M KCl)
IgG[f]	0.5 mg in 1.0 ml	1.0 ml	1.0 ml	1.0 ml (with 1.5 M KCl)	1.0 ml (with 1.5 M KCl)	1.0 ml (with 2.5 M KCl)
HSA[g]	1.0 mg in 1.0 ml	1.0 ml	1.0 ml	1.0 ml (with 1.5 M KCl)	1.0 ml (with 1.5 M KCl)	1.0 ml (with 2.5 M KCl)

Protein recovered from three elution steps together was considered to calculate percent recovery (Section 2.6).
The following buffers were used in the binding experiments; respective buffers with KCl (molarity as in the table above) were used for elution.

[a] Laccase: 20 mM potassium phosphate buffer at pH 7.0.
[b] Arginase: 25 mM imidazole-HCl, 1.2 mM $MnSO_4$, 2 mM 2-mercaptoethanol, at pH 7.5.
[c] Glutamine synthetase: 20 mM imidazole, 0.1 mM EDTA, 5% glycerol, 2 mM 2-mercaptoethanol at pH 7.0.
[d] NAPD-GDH: 20 mM potassium phosphate, 1 mM EDTA and 4 mM 2-mercaptoethanol, at pH 7.5.
[e] Trypsin: Tris–HCl buffer (25 mM Tris, 0.5 M $CaCl_2$ and 0.5 M $MgCl_2$) at pH 7.4.
[f,g] IgG and HSA: Tris–HCl (50 mM) buffer, at pH 8.0.

With HSA and IgG however, percentage recovery was calculated based on the amount of protein loaded and recovered.

2.7. HSA binding capacity of affinity resins

The protocol used to assess the capacity of affinity resins to bind HSA was essentially same as mentioned for screening these resins in Section 2.6 (Table 3). Batch binding and elution was scaled up using 2 ml of each affinity resin in 15 ml conical bottom plastic centrifuge tubes (Tarsons Products Pvt. Ltd., Kolkata, India). Accordingly, increased volume (5 ml) of buffer was used for each wash and elution step. The protein binding was followed by monitoring the A_{280}, as mentioned before. The maximal binding capacity of each affinity resin was evaluated by loading 10 mg, 50 mg or 200 mg of HSA, in three separate experiments. Blue Sepharose served as control dye-affinity resin in these experiments; CR-034 from the library of affinity resins corresponds to (and is comparable with) commercially available CB3GA-based matrix.

The data presented in these binding studies showed a statistical variation of about 5%. However with binding experiments involving lower amount of affinity resins (0.3 ml drained medium, see Section 2.6) the variation was up to 10%.

2.8. Dye inhibition of NADP-GDH activity

The standard reductive amination reaction of NADP-GDH was followed to monitor dye inhibition. Different fixed concentrations of the dye inhibitor were used while performing NADPH saturation. Aqueous stock solutions of the three dyes namely, Cibacron Red LS/B, Cibacron Blue 3G-A® and CD-11 were prepared for this inhibition study.

The data were fitted using nonlinear regression analysis software (SigmaPlot 9.1) to obtain the kinetic constants. In order to determine the nature of dye inhibition, the inhibition data were fitted to various forms of the equation (competitive, noncompetitive, partially noncompetitive and mixed) and best fit was chosen. Enzyme kinetic data presented are typical of at least three independent experiments. An R^2 value of 0.98–0.99 was always achieved in these cases.

2.9. Scale-up studies on NADP-GDH and arginase purification

Few affinity resins were selected to attempt purification of *A. terreus* NADP-GDH and *A. niger* arginase by column chromatography. However, representative data for *A. terreus* NADP-GDH alone are shown. Subsequent to protein binding, the columns (18 ml bed volume; liquid chromatography columns from Sigma–Aldrich; product code: C4169) were developed with a linear (0–0.3 M) KCl gradient. Flow rate of 10 ml/h was maintained during the loading step while 18 ml/h was maintained during washing and elution. Peristaltic pump P1 (GE Healthcare, product code 18-1110-91) was used in negative pressure mode to maintain the flow rate. Gradient Mixer GM-1 (GE Healthcare, product code 19-0495-01) was used to maintain the KCl gradient (40 ml total). Regeneration of the dye-ligand affinity columns were performed by washing them with 3 M NaCl and then with deionized water. For long term storage, these affinity resins were maintained in 20% ethanol at 4 °C.

3. Results and discussion

3.1. Synthesis of novel dye structures and new affinity resins

Cibacron Blue 3G-A® (Fig. 1) is the most often used textile dye for dye-ligand affinity chromatography. Consequently much effort has gone in to define the nature of its interaction with different proteins [36]. Its structure was compared with that of pyridine dinucleotide cofactors namely, NAD and NADP [37]. The anthraquinone moiety of CB3GA was assumed to occupy the hydrophobic pockets on the surface of the protein whereas its sulfonate groups may be responsible for ionic interactions. Subsequent literature has shown that CB3GA interaction with various proteins (that it was used to purify) is more complex and is not a straight forward affinity ligand. It is often therefore considered as a pseudo-affinity ligand. Few structural variants of trizine-based ligands as well as different spacer lengths are described to generate novel affinity resins [16–18]. However there have been no systematic efforts to generate structural variants of CB3GA, and to establish a structure-activity relationship. In this context, a library of CB3GA structural variants was generated [25]. While the skeletal structure comprising of the triazine nucleus and the anthraquinone were retained, structural variations were introduced in regions 'A' and 'B' of the molecule (Fig. 1). The third substitution 'X' on the triazine nucleus serves as a point of linkage to the polymer matrix used (e.g. agarose). This rational, in combination with the possible use of different spacer lengths, led to the development of more than 30 novel dye structures and over a hundred new affinity resins [25]. Ninety six most promising affinity resins were selected from this library for detailed analysis and with a view to subsequently present them in a 96-well format for high throughput screening.

3.2. Binding discrimination by the affinity resins

The 96 affinity resins were screened for their ability to discriminate and bind various enzymes and proteins. Enzymes from different representative classes were chosen for this study. These included - NADP-GDH (EC 1.4.1.4, a dehydrogenase), laccase (EC 1.10.3.2, an oxidase), arginase (EC 3.5.3.1, an ureohydrolase), trypsin (EC 3.4.21.4, a mammalian protease) and glutamine synthetase (EC 6.3.1.2, a synthetase). In addition, two serum proteins – HSA and IgG – were also included in this analysis. In a representative binding protocol (see Section 2.6 and Table 3) the ability of these 96 affinity resins to interact with these proteins was evaluated. The results are presented as percent enzyme activity (or protein amount) recovered in the eluate for each specific elution protocol. Unique binding patterns were observed in each example (Fig. 2; for detailed data see Supplementary data). For instance, NADP-GDH did not bind to CR-002 (position A2), bound poorly to CR-016 (position B4), and showed intermediate binding to CR-057 (position E9) and strong binding to CR-017 (position B5). Trypsin did not bind to CR-075 (position G3); glutamine synthetase showed intermediate binding while laccase and arginase bound strongly to this matrix. On the whole, the binding patterns observed for the model enzymes/proteins were unique (Fig. 2) and the library of affinity resins exhibited sufficient selectivity as anticipated. While these studies were done using one set of conditions for binding and elution (by KCl in the elution buffer) there is ample scope to vary these conditions and achieve desired resolution and improve recovery. In principle, one could vary pH (data available but not shown) and buffer species or include ligands (like substrate, inhibitor etc) during binding. It may also be possible to employ a kinetic locking-on strategy during bio-affinity purifications [38].

Importance of spacer in protein binding is evident from earlier reports [39,40]. The significance of spacer length in proper binding to the proteins was also evident from the data presented. Laccase did not bind to the affinity resin when the dye-ligand (CD-25; Supplementary data) was directly immobilized on to agarose (Fig. 3); spacer was a requirement for its binding. Binding to the same ligand was improved upon introducing a spacer between the ligand and the matrix. NADP-GDH showed a similar trend (with a different dye-ligand however, namely CD-11), where it exhibited strongest binding to the affinity resin with a 13-atom spacer. An

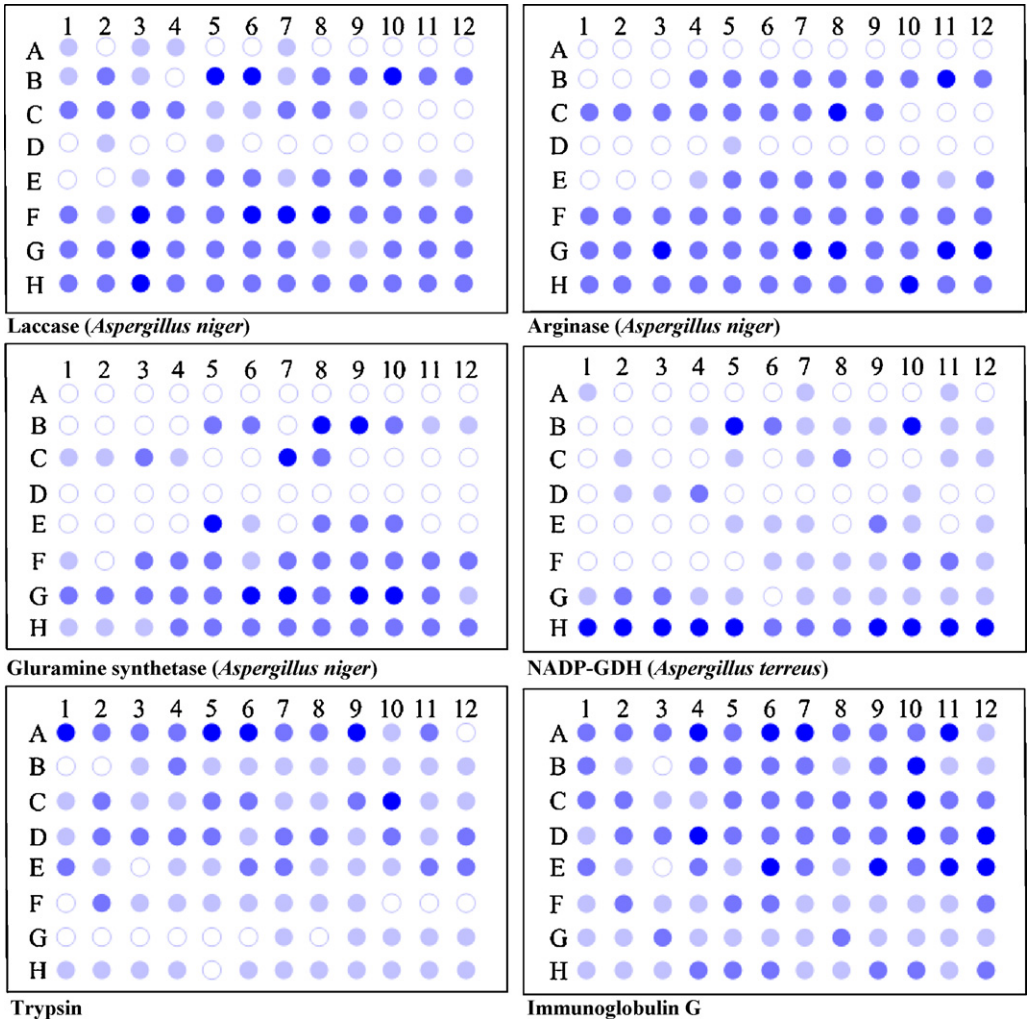

Fig. 2. Discriminatory binding and relative efficacy of affinity resins. The 96 CRs were screened for their ability to bind and elute (Table 3) six different model proteins. Protein/enzyme recovered in the eluate (relative to amount loaded as 100%) ranged from 0% to 100%. The affinity resins were grouped into four categories according to percent recovered as – none (<10%; ○), poor (10–33%; ●), intermediate (34–66%; ●) and high (>66%; ●). Affinity resins showing more than 80% recovery are – CR-053 and CR-082 (for glutamine synthetase); CR-086, CR-087, CR-088, CR-089, CR-093 and CR-095 (for NADP-GDH); CR-032, CR-063, CR-064, CR-068, CR-076, CR-077 and CR-078 (for HSA); CR-004 and CR-007 (for IgG).

11-atom spacer was optimal for arginase binding. Trypsin was a unique example with best binding to the affinity resin that had no spacer; the binding in fact, was reduced when the spacer was introduced (Fig. 3). Interaction of glutamine synthetase was similar to that of NADP-GDH and arginase, and this was facilitated by an 11-atom spacer. Clearly, the range of affinity resins not only provides a selectivity space through a library of novel dye structures but also through a range of spacer lengths for each dye immobilized.

Affinity chromatography has become the mainstay of industrial purification of pharmaceutical protein products [41]. Commercial CB3GA-based resins are available that are mechanically stable to high flow rates and sanitization protocols. They are being used in several commercial processes such as the production of albumin from Cohn fraction I, Recombulin® (yeast derived recombinant human albumin) and Thyrogen® (thyroid stimulating hormone) [42]. There have been very few applications of the dye-ligand chromatography in the purification of antibodies including monoclonal antibodies. Bruck et al. [43] first described the use of CB3GA in purifying mouse IgG class of proteins. Interestingly, this matrix was not a simple dye-matrix but a Cibacron Blue F3 GA–DEAE combination adsorbent. A further improvement of this method was to use the matrix in an FPLC mode [44]. Also, IgM monoclonal antibodies from murine ascitic fluid were successfully purified in a two-step procedure using agarose immobilized Cibacron Blue F3 GA [45]. A scalable CB3GA-based method was designed primarily to eliminate impurity in antibody preparation post Protein-A chromatography [42]. Here monoclonal antibodies interacted weakly with the resin and were unbound whereas impurities (like contaminating fraction of albumin!) are either bound to the resin or occur at the distal end of the flow through. It was therefore of interest to evaluate the 96 affinity resins for their HSA and IgG binding ability. Both HSA and IgG showed distinct binding patterns with these resins. Some of the affinity resins (like CR-004, CR-007, CR-048 and CR-059) bound IgG better than the others (Fig. 2). Similarly there were affinity resins to which HSA bound better than to the commercial Blue Sepharose. For instance, under identical conditions of chromatography, CR-078 was better at resolving HSA and IgG (from a mixture of these two proteins in 10:3 ratio as reported in Ref. [46]) than the commercial Blue Sepharose (Fig. 4A). Although they bound HSA to varying degrees, not even one of the 96 affinity resins failed to bind HSA (see Section 3.3 below). Despite this, it was of interest to design a strategy for enriching and resolving IgG component from HSA.

On the basis of their binding properties, a combination of CR-078 (good HSA binding but with poor IgG binding) and CR-052

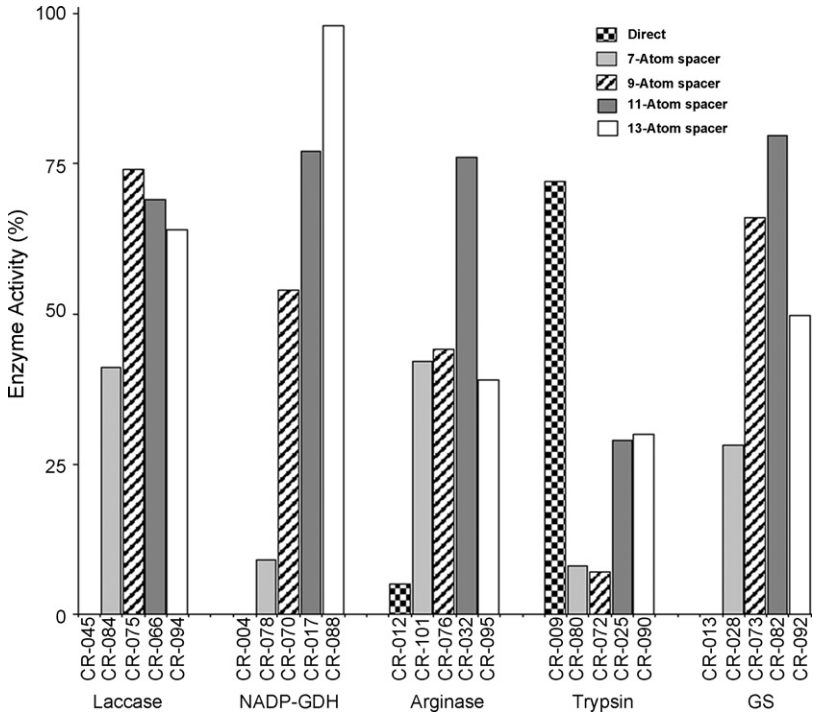

Fig. 3. Effect of spacer on binding of different enzyme/protein with affinity resins. Representative binding data for different enzymes, each interacting with one unique dye structure is shown. The five dyes, namely, CD-11 (for NADP-GDH), CD-14 (for trypsin), CD-19 (for GS), CD-25 (for laccase) and CD-29 (for arginase) were attached to agarose either directly or through a spacer (of 7-13-atoms). CR-101 (for arginase) is not part of the 96 affinity resins list (Appendix) but represents CD-29 immobilized with a 7-atom spacer.

(good IgG binding but with poor HSA binding) was used for resolving IgG from HSA. CR-078 served as an excellent negative step for IgG while it bound and eluted HSA very well. In a subsequent step one could use CR-052 a positive step for IgG recovery (Fig. 4B). Further refinements of such a strategy, employing other affinity resin combinations and elution conditions, to purify IgG from plasma fractions are feasible. Only a few dye-affinity adsorbents are reported for fractionation of IgGs. A large number of dyes were screened for the separation of human IgG_2 from the other IgG subclasses [24]. IgG fraction consists of many isoforms and few of them display differential binding to well known affinity resins like Protein A Sepharose [24]. Such a differential interaction by different IgG isoforms may also occur with the dye ligands. It is difficult to explore these features since only total IgG fraction (polyclonal IgG) was used in the present study (Fig. 2). Nevertheless, the range of affinity resins presented here open up interesting possibilities for purification and fractionation of antibodies.

3.3. Binding capacity for HSA

Limited amount of HSA was loaded in a typical screening protocol (see Table 3; Section 2.6). The 96 affinity resins exhibited differential ability to bind HSA in this study (Fig. 5A). Binding capacity of these resins defines yet another important parameter for chromatography. Protein binding capacity of the 96 affinity resins was evaluated using HSA as the standard. Maximal HSA binding (in mg per ml of the drained medium) was determined and compared with that of commercial CB3GA based affinity resin (Fig. 5B). There was a good spread of binding capacity in the 96 affinity resins. While many of them showed better HSA binding capacity than Blue Sepharose, some with lower capacity were also observed. Poor binder like CR-013 and CR-014 (Fig. 5B) are potentially interesting in further development of affinity resins, suitable for HSA elimination. Large scale purification of few proteins has been established using CB3GA-based dye-affinity resins [47]. Reusability and reproducibility of CB3GA-based matrices for the purification of human

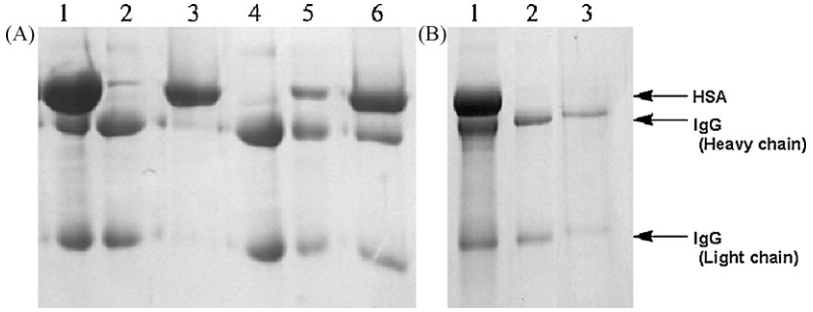

Fig. 4. Combination of affinity resins used to resolve HSA and IgG. A solution of HSA and IgG mixture (10:3, mg/mg) was chromatographed on 1.0 ml of different resins. SDS-PAGE of various fractions is shown. Panel A: Lane 1 – mixture of HSA and IgG (sample load), Lane 2 – unbound fraction from CR-078, Lane 3 – eluted fraction from CR-078, Lane 4 – unbound fraction from Blue Sepharose, Lane 5 – eluted fraction from Blue Sepharose. Panel B: Sequential use of CR-078 and CR-052 to resolve IgG from HSA. Lane 1 – mixture of HSA and IgG (sample load), Lane 2 – unbound fraction from CR-078, Lane 3 – unbound fraction from the first column (CR-078) loaded on to CR-052 and eluted.

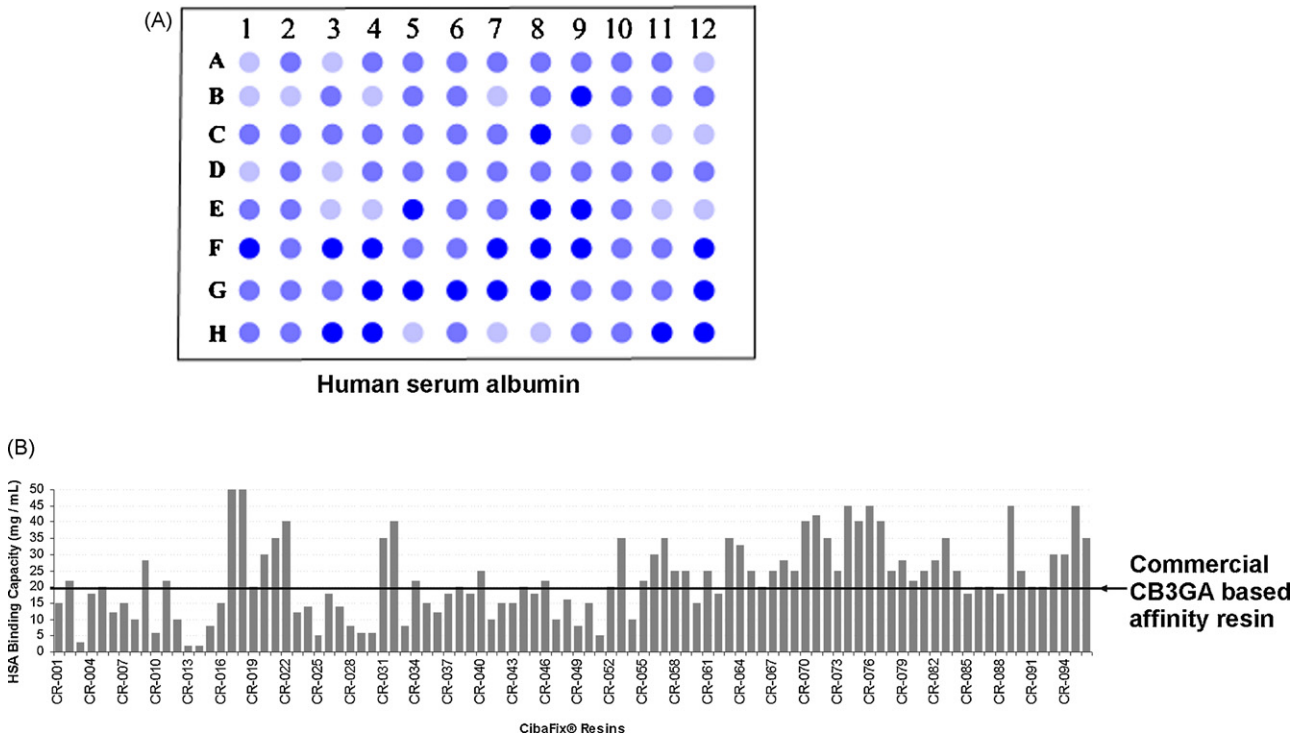

Fig. 5. Interaction of HSA with the 96 affinity resins. Panel A: Discriminatory binding and relative efficacy of affinity resins to bind and elute (Table 3) HSA was assessed. The affinity resins were grouped into four categories as mentioned (see legend to Fig. 2). Panel B: Binding capacity of the 96 affinity resins was evaluated by loading up to 200 mg of HSA per 2 ml of each resin. In this library of affinity resins, CR-034 corresponds to (and is comparable with) commercially available CB3GA-based matrix. The data was also compared with Blue Sepharose whose capacity is shown by the horizontal line (at 20 mg/ml).

albumin (final purity remaining constant at 98–100%) is reported [4,48]. The dye-affinity resins described here thus offer a range of possibilities at least in the selective enrichment and/or elimination of albumin.

3.4. Scale-up studies with laccase, arginase and NADP-GDH

Subsequent to the screen (Fig. 2), some of the affinity resins were chosen for scale up experiments in a column mode. Attempts were made to bind and elute laccase (from *A. niger*), arginase (from *A. niger*) and NADP-GDH (from *A. terreus*). Three affinity resins (CR-023, CR-081 and CR-079) have shown promise in arginase purification while CR-022 was useful in laccase purification (not shown). Similarly, CR-017, CR-018, CR-022 and CR-057 were promising candidates for *A. terreus* NADP-GDH purification.

CR-017 was chosen to purify NADP-GDH in a column mode. Substantial enrichment of the enzyme (from crude *A. terreus* protein extracts) was possible in a single step of binding followed by elution with KCl gradient (Fig. 6). Interestingly, this protein could not be purified using a dye-affinity method reported for the *A. niger* enzyme [30]. Specific interaction with affinity (and pseudo affinity) ligands may be captured through enzyme inhibition kinetics. Therefore possible interaction of NADP-GDH with the corresponding free dyes was carried out. Cibacron Red LS/B (effective for the *A. niger* NADP-GDH purification) and CD-11 (corresponding to CR-017) were tested for their ability to inhibit *A. terreus* NADP-GDH while CB3GA served as a control. These three dyes (although are reactive dyes) were reversible inhibitors of NADP-GDH. Since CB3GA and related dyes are reported to interact with the enzyme dinucleotide fold [6], the inhibitions were set up with $NADP^+$ as the varied substrate. From the inhibition data it is obvious that CD-11 interacts noncompetitively while Cibacron Red LS/B and CB3GA are competitive inhibitors of this enzyme (Fig. 7). This unique feature may be responsible for the efficacy of CR-017 in *A. terreus* NADP-GDH enrichment. Apart from the expected role of ligand immobilization (direct or indirect), two factors critical

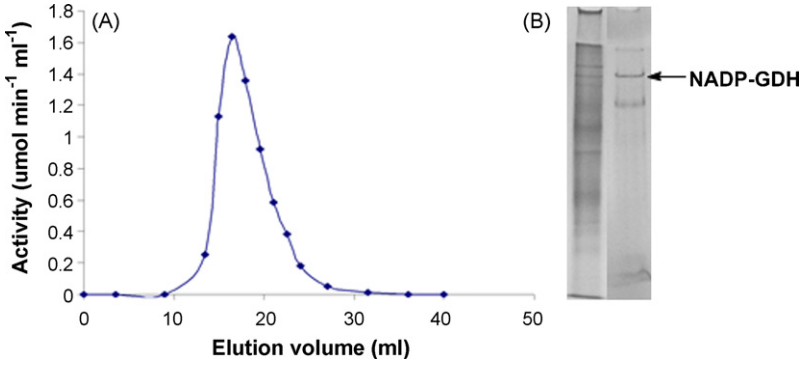

Fig. 6. Enrichment of NADP-GDH from *A. terreus*. Panel A: Elution profile of NADP-GDH from a CR-017 column. Panel B: Native PAGE of the enriched *A. terreus* enzyme. Lane 1 – Crude mycelial extract and Lane 2 – Peak protein fraction eluting from CR-017. The two lanes are from separate gel runs.

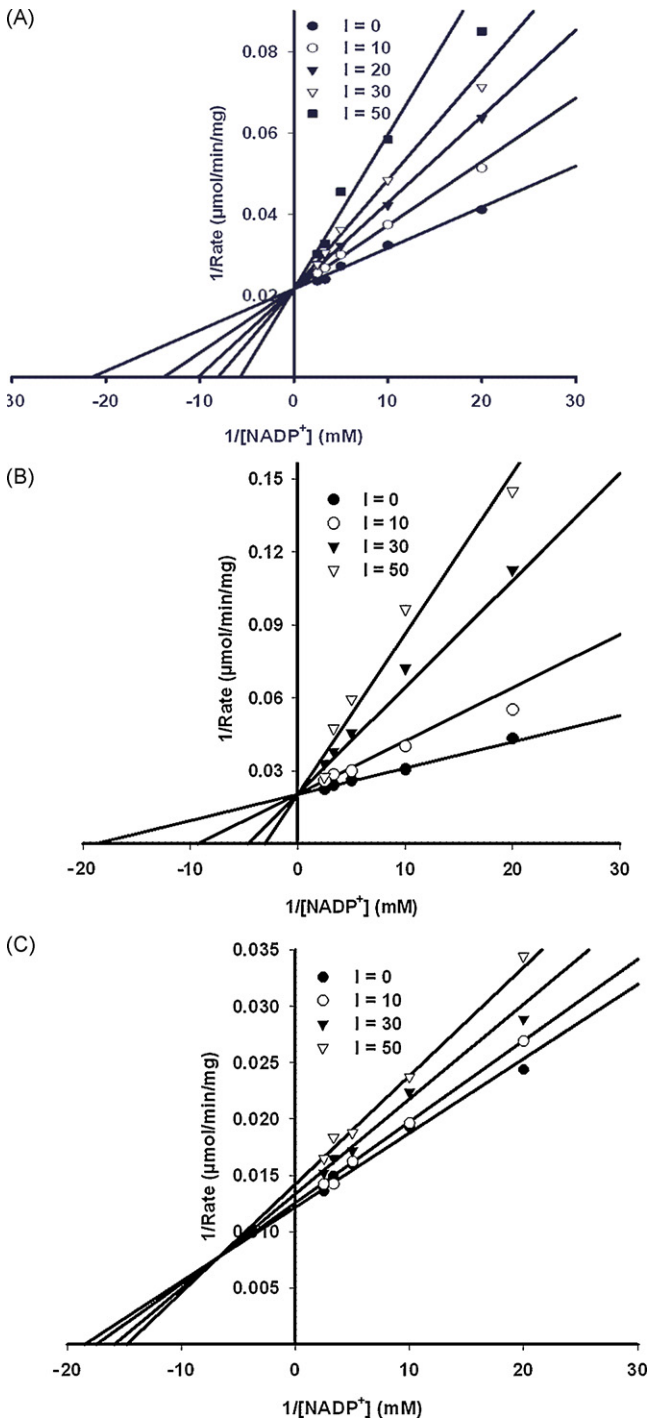

Fig. 7. Interaction of *A. terreus* NADP-GDH with reactive dye ligands. The $NADP^+$ saturation was performed at different fixed concentrations (in μM; shown in inset) of each dye. The inhibition data for Cibacron Red LS/B (A), Cibacron Blue 3G-A® (B) and CD-11 (corresponding to CR-017; C) is shown. Cibacron Red LS/B and Cibacron Blue were competitive inhibitors (with K_Is of 18.1 ± 0.5 μM and 9.8 ± 1.6, respectively) while CD-11 was non-competitive (K_I of 108.7 ± 1.5 μM).

in determining the effectiveness of the dye as an affinity ligand are – (a) the nature of inhibition and (b) the strength of interaction (reflected in the K_I values). Therefore, dyes interacting in a non-competitive manner (Cibacron Red LS/B for *A. niger* enzyme and CD-11 for *A. terreus* enzyme) performed better as affinity ligands. For instance, complete purification of *A. terreus* NADP-GDH was recently achieved by including an enrichment step on CR-017 column [49].

4. Conclusions

Availability of CB3GA and other dye-based matrices has made dye-ligand pseudo affinity chromatography an attractive proposition. A systematic library of 96 affinity resins, using different dye structures and varying spacer lengths for immobilization, was generated and tested. These dye-affinity matrices displayed excellent discrimination for binding and elution with seven model proteins which also includes human serum albumin. Interesting binding trends for hormones, antibodies and viral proteins have also been observed (proprietary data, not shown). While we have tested a limited set for demonstration, variations in chromatographic conditions such as pH, ionic strength and presence of ligands are feasible. As demonstrated for *A. terreus* NADP-GDH, after initial screening the best affinity resin can be further adopted for purification and scale up. These affinity resins when presented in a 96-well format (Table 1) would facilitate high throughput screening and selection. Finally, the structural information of these dyes could be correlated with their ability to bind with various enzyme/protein classes thereby defining the unique structural features that define such binding. Such structure-activity correlation analysis is in progress and is anticipated to help develop bio-mimetic dye-ligand variants.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2009.08.045.

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