Chapter 21

Synthesis and Application of Dye-Ligand Affinity Adsorbents

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Abstract

Dye-ligand affinity chromatography is a widely used technique in protein purification. The utility of the reactive dyes as affinity ligands results from their unique chemistry, which confers wide specificity towards a large number of proteins. They are commercially available, are inexpensive, and can easily be immobilized. Important factors that contribute to the successful operation of a dye-ligand chromatography include adsorbent properties, such as matrix type and ligand concentration, the buffer conditions used in the adsorption and elution stages, and contacting parameters like flow rate and column geometry. In general, with dye-ligand affinity chromatography, the specificity is provided by the adsorption and elution conditions employed in a particular purification, and these must often be worked out by trial and error. The present chapter provides protocols for the synthesis of dye-ligand affinity adsorbents as well as protocols for screening, selection, and optimization of a dye-ligand purification step. The purification of the glutathione transferases from *Phaseolus vulgaris* crude extract on Cibacron Blue 3GA-Sepharose is given as an example.

Key words Affinity chromatography, Cibacron Blue 3GA, Dye-ligand chromatography, Glutathione transferase, Triazine dyes

1 Introduction

Dye-ligand chromatography is affinity chromatography that utilizes immobilized textile dyes to purify proteins [1–3]. Dye-ligands can bind proteins either by specific interactions at the protein's binding site or by a range of nonspecific interactions. The interaction between the dye-ligand and proteins is achieved by complex combination of electrostatic, hydrophobic, hydrogen bonding interaction. Several dye-ligand affinity adsorbents have been used for the isolation of a variety of proteins including dehydrogenases, kinases, plasma proteins, and several others [3] owing to their ability to mimic the configuration of substrates, cofactors, or binding agents, thereby leading to high specificity. Some of these, such as Cibacron Blue 3GA or Procion blue, have been shown to display

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t1.1 **Table 1**
t1.2 **Advantages of dye-ligand affinity adsorbents**

t1.3 Advantages
t1.4 Low cost
t1.5 Readily available in bulk quantities
t1.6 t1.7 Chemical stable over a range of pH extremes enabling full recovery of activity after cleaning
t1.8 Easily coupled to matrixes via reactive groups
t1.9 Display high binding capacity for a wide range of proteins

high affinity for enzymes requiring adenylyl-containing cofactors [e.g., NAD(H), NADP(H)] [4–13].

Dye-ligands display several advantages compared to specific biological ligands due to their easy immobilization, high chemical and biological stability, high binding capacity, and low cost (Table 1). Triazine dyes can be considered to consist of two structurally distinct units joined together via an amino bridge (example structures are shown in Fig. 1). One unit, the chromophore (either azo, anthraquinone, or phathalocyanine), contributes the color, and the other, the reactive unit, provides the site for covalent attachment to the insoluble support. The first and most successful reactive unit that was explored in dye chemistry was cyanuric chloride (1,3,5-sym-trichlorotriazine) [5–7].

Many factors contribute to the successful operation of a dye-ligand chromatographic step. These include adsorbent properties, such as matrix type and ligand concentration, adsorption and elution conditions, flow rate, and column geometry [3, 8, 12]. Among them the adsorption and elution steps should be carefully optimized/designed for a successful separation [13–19]. The present chapter describes protocols for the synthesis of dye-ligand affinity adsorbents using as a model the triazine dye Cibacron Blue 3GA. In addition, protocols for screening, selection, and optimization of a dye-ligand purification step are also given and discussed.

2 Materials

2.1 Dye Purification and Characterization

1. Cibacron Blue 3GA (Sigma-Aldrich).
2. Diethyl ether.
3. Acetone.
4. Analytical TLC plates (e.g., 0.2 mm silica gel-60, Merck).
5. Sephadex LH-20 column (2.5 cm × 30 cm). Sephadex LH-20 is available from Sigma-Aldrich.

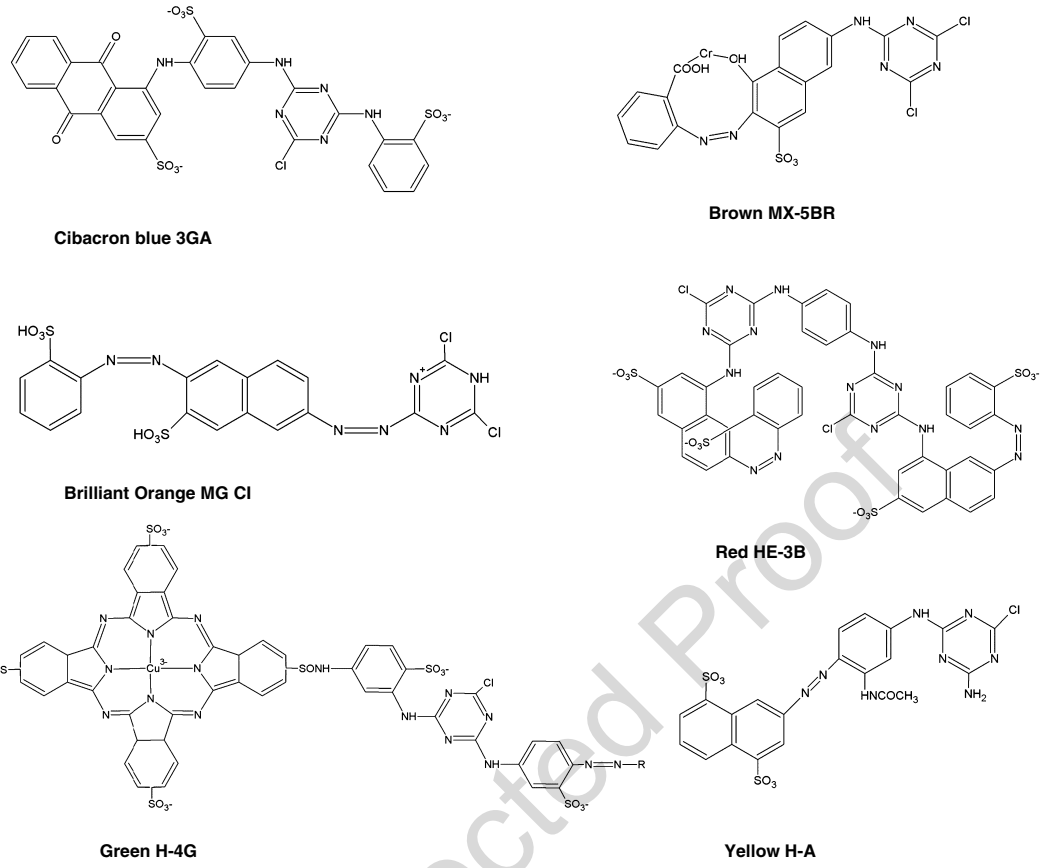

Fig. 1 Structure of some dye-ligands

6. Whatman filter paper, hardened ashless, Grade 542, diameter 70 mm.
7. Methanol/H_2O (50/50, v/v).
8. Solvent system for TLC: butan-1-ol/propan-2-ol/ethylacetate/H_2O (2/4/1/3, v/v/v/v).
9. Reverse phase HPLC column (e.g., C18 S5 ODS2 Spherisorb silica column, 250 mm × 4.6 mm, Gilson, USA).
10. *N*-cetyltrimethylammonium bromide (CTMB, HPLC grade, Sigma-Aldrich).
11. Solvent A: methanol/0.1 % (w/v) aqueous CTMB (80/20, v/v), solvent B: methanol/0.1 % (w/v) aqueous CTMB (95/5, v/v).
12. 0.45 μm cellulose membrane filter (e.g., Millipore).

2.2 Direct Dye Immobilization

1. Agarose-based support (e.g., Sepharose CL 6B, Sigma-Aldrich).
2. Solid Na_2CO_3.
3. 22 % (w/v) NaCl solution.

4. 1 M NaCl solution.
5. DMSO/H_2O 50 % (v/v) solution.

2.3 Synthesis of 6-Aminohexyl Derivative of Cibacron Blue 3GA

1. Cibacron Blue 3GA (Sigma-Aldrich).
2. 1,6-Diaminohexane.
3. Solid NaCl.
4. Concentrated HCl.
5. 1 M HCl solution.
6. Acetone.

2.4 Immobilization of 6-Aminohexyl-Cibacron Blue 3GA to Sepharose

1. Sepharose CL 6B (Sigma-Aldrich).
2. Water/acetone (2:1, v/v), water/acetone (1:2, v/v).
3. Dried acetone.
4. 1,1-Carbonyldiimidazole.
5. DMSO/water (50/50, v/v).
6. 2 M Na_2CO_3 solution.

2.5 Determination of Immobilized Dye Concentration

1. 5 M HCl.
2. 10 M NaOH.
3. 1 M Potassium phosphate buffer, pH 7.6.

2.6 Dye Screening: Selection of Dyes as Ligands for Affinity Chromatography

1. Dye-ligand affinity adsorbents: a selection of immobilized dye-adsorbents (0.5–1 mL) with different immobilized dye, packed in small chromatographic columns (0.5 cm × 5 cm). Adsorbent screening kits with prepacked columns are available commercially (e.g., Sigma-Aldrich).

2.7 Regeneration and Storage of Dye-Ligand Adsorbents

1. Sodium thiocyanate solution (3 M).
2. Aqueous ethanol solution, 20 % (v/v).

2.8 Purification of Phaseolus Vulgaris Glutathione Transferases on Cibacron Blue 3GA-Sepharose Affinity Adsorbent

1. *Phaseolus vulgaris* seeds (the common bean).
2. Mortar (diameter 10 cm) and pestle.
3. Potassium phosphate buffer, 20 mM, pH 6.0.
4. Cibacron Blue 3GA-Sepharose column (1 mL).
5. Cheesecloth.
6. Cellulose filter (0.45 μm pore size).
7. Glutathione solution (10 mM) in 20 mM potassium phosphate buffer, pH 6.0.
8. Sodium thiocyanate (3 M) solution.

3 Methods

Analytical grade chemicals and double-distilled water were used to prepare the buffers for ligand immobilization and affinity chromatography. All buffers were stored at 4 °C.

3.1 Dye Purification and Characterization

Commercial dye preparations are highly heterogeneous mixtures and are known to contain added buffers, stabilizers, and organic by-products [10, 11]. The following purification protocol, based on Sephadex LH-20 column chromatography, usually gives satisfactory purification (>95 %) (*see* **Note 1**):

1. Dissolve 500 mg of crude dye (e.g., Cibacron Blue 3GA, purity ~60 %) in 40 mL deionized water.
2. Extract the solution twice with diethyl ether (2×50 mL) and concentrate the aqueous phase approximately threefold using a rotary evaporator.
3. To the aqueous phase add 100 mL of cold acetone (−20 °C) to precipitate the dye.
4. Filter the precipitate through Whatman filter paper and dry it under reduced pressure.
5. Dissolve 100 mg dried dye in water/methanol (5 mL, 50/50, v/v) and filter the solution through a 0.45 μm cellulose membrane filter.
6. Load the dye solution on a Sephadex LH-20 column (2.5 cm×30 cm) which has been previously equilibrated in water/MeOH (50/50, v/v). Develop the column isocratically at a flow rate of 0.1 mL/min/cm.
7. Collect fractions (5 mL) and analyze by TLC using the solvent system: butan-1-ol/propan-2-ol/ethylacetate/H_2O (2/4/1/3, v/v/v/v). Pool the pure fractions containing the desired dye and concentrate the solution by 60 % using a rotary evaporator under reduced pressure (50 °C). Lyophilize and store the pure dye powder desiccated at 4 °C.

Analysis of dye preparations may be achieved by HPLC on a C18 reverse phase column (e.g., C18 ODS2 Spherisorb, Gilson, USA) using the ion-pair reagent *N*-cetyltrimethylammonium bromide (CTMB) [11].

1. Equilibrate the column using the solvent system methanol/0.1 % (w/v) aqueous CTMB (80/20, v/v) at a flow rate of 0.5 mL/min.
2. Prepare dye sample as 0.5 mM solution in the above system. Inject sample (10–20 μmol).

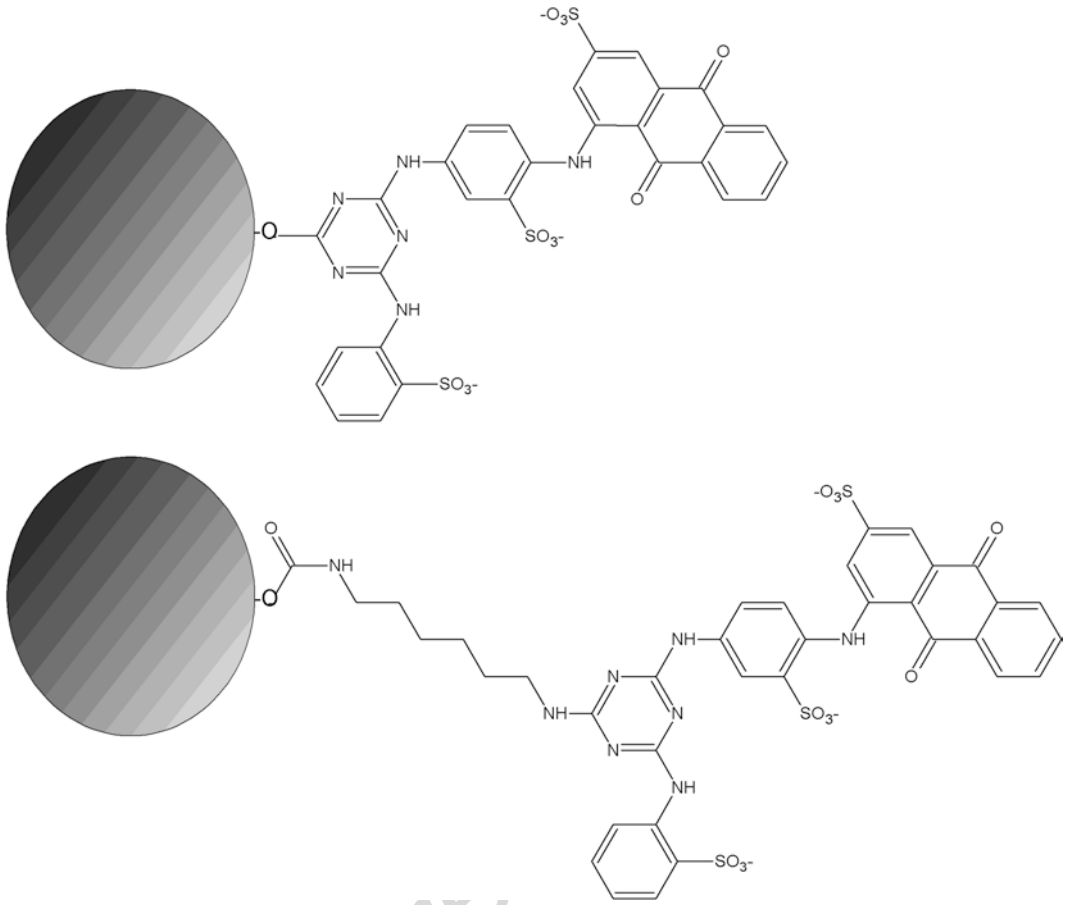

Fig. 2 Immobilization of chlorotriazine anthraquinone dyes. (**a**) Direct coupling via the chlorotriazine ring, (**b**) coupled to 1,1-carbonyldiimidazole-activated agarose by a triazine ring-coupled 6-aminohexyl spacer arm

3. Develop the column at a flow rate of 0.5 mL/min using the following gradients: 0–4 min 80 % B, 4–5 min 85 % B, 5–16 min 90 % B, 16–18 min 95 % B, and 18–30 min 95 % B. Elution may be monitored at both 220 nm and 620 nm.

3.2 Direct Dye Immobilization

Two different procedures have been used for dye immobilization to polyhydroxyl matrices: direct coupling of dyes via the chlorotriazine ring and coupling via a spacer molecule (Fig. 2) [4, 8, 9]. A hexamethyldiamine spacer molecule may be inserted between the ligand and the matrix. This leads to an increase in dye selectivity by reducing steric interference from the matrix backbone [4]. A hexyl spacer may be inserted by substitution of 1,6-diaminohexane at one of the chlorine atoms of the triazinyl group, and the dye-spacer conjugate may be immobilized to 1,1-carbonyldiimidazole-activated agarose (*see* **Note 2**).

1. To prewashed agarose gel (1 g) add a solution of purified dye (1 mL, 4–30 mg dye/g gel, *see* **Note 3**) and 0.2 mL of NaCl solution (22 % w/v).
2. Leave the suspension shaking for 30 min at room temperature (*see* **Note 4**).
3. Add solid sodium carbonate at a final concentration of 1 % (w/v) (*see* **Note 4**).
4. Leave the suspension shaking at 60 °C for 4–8 h for monochlorotriazine dyes and at room temperature for 5–20 min for dichlorotriazine dyes.
5. After completion of the reaction (*see* **Note 5**), wash the dyed gel to remove unreacted dye sequentially with water (100 mL), 1 M NaCl (50 mL), 50 % (v/v) DMSO (10 mL), 1 M NaCl (50 mL), and finally water (100 mL).

3.3 Synthesis of 6-Aminohexyl Derivative of Cibacron Blue 3GA

1. To a stirred solution of 1,6-diaminohexane in water (6 mmol, 10 mL), add a solution of purified dye (0.6 mmol, 25 mL) in water and increase temperature to 60 °C.
2. Leave the mixture stirring for 3 h at 60 °C.
3. Add solid sodium chloride to a final concentration of 3 % (w/v) and allow the solution to cool at 4 °C.
4. Add concentrated HCl to reduce the pH to 2.0. Filter off the precipitated product and wash it with hydrochloric acid solution (1 M, 50 mL), acetone (50 mL) and dry under vacuum.

3.4 Immobilization of 6-Aminohexyl-Cibacron Blue 3GA to Sepharose

Sepharose CL 6B first is activated with 1,1-carbonyldiimidazole to facilitate the immobilization of 6-aminohexyl dye analogue.

1. Wash agarose (1 g) sequentially with water/acetone (2:1, v/v; 10 mL), water/acetone (1:2, v/v; 10 mL), acetone (10 mL), and dried acetone (20 mL).
2. Resuspend the gel in dried acetone (5 mL) and add 0.1 g of 1,1-carbonyldiimidazole. Agitate the mixture for 15–20 min at 20–25 °C.
3. Wash the gel with dried acetone (50 mL). Add a solution of 6-aminohexyl-Cibacron Blue 3GA (0.1 mmol) in DMSO/water (50/50, v/v, 4 mL), the pH of which has been previously adjusted to 10.0 with 2 M Na_2CO_3.
4. Shake the mixture overnight at 4 °C. After completion of the reaction, wash the gel as in Subheading 3.2, **step 1**.

3.5 Determination of Immobilized Dye Concentration

Determination of immobilized dye concentration may be achieved by spectrophotometric measurement of the dye released after acid hydrolysis of the gel.

1. Suspend 30 mg of dyed gel in hydrochloric acid solution (5 M, 0.6 mL) and incubate at 70 °C for 3–5 min.

2. To the hydrolysate, add NaOH (10 M, 0.3 mL) and potassium phosphate buffer (1 M, pH 7.6, 2.1 mL).
3. Read the absorbance of the hydrolysate at 620 nm against an equal amount of hydrolyzed unsubstituted gel. Calculate the concentration of the immobilized dye as micromoles of dye per g wet gel.

3.6 Dye Screening: Selection of Dyes as Ligands for Affinity Chromatography

Dye-ligand affinity chromatography is an empirical approach to protein purification, and one cannot easily predict whether a specific protein will bind or not to a certain dye column. Thus, for efficient use of this technique, a large number of different dye-adsorbents need to be screened to evaluate their ability to bind and purify a particular protein [4, 5, 8, 9].

1. Degas the adsorbents, to prevent air bubble formation, and pack them into individual columns of 0.5–1 mL bed volume.
2. Dialyze the protein sample against 50 vol. of equilibration buffer. Alternatively this can be achieved using a desalting Sephadex G-25 gel-filtration column.
3. Filter the protein sample through 0.4 μm pore-sized filter or centrifuge to remove any insoluble material.
4. Wash the dye-adsorbents with 10 bed vol. of equilibration buffer. Load 0.5–5 mL of the protein sample (*see* **Note 6**) to the columns at a linear flow rate of 10–20 cm/mL.
5. Wash non-bound proteins from the columns with 10 bed vol. of equilibration buffer. Collect non-bound proteins in one fraction.
6. Elute the bound proteins with 5 bed vol. of elution buffer (*see* **Note 7**) and collect the eluted protein in a fresh new tube as one fraction.
7. Assay both fractions for enzyme activity and for total protein.
8. Determine the capacity, purification factor, and recovery achieved with each column. The best dye-adsorbent is the one that combines highest capacity, purification, and recovery (*see* **Note 8**).

3.7 Optimization of a Dye-Ligand Purification Step

After a dye-ligand adsorbent has been selected from a dye screening procedure (Subheading 3.6), optimization of the chromatographic step can be achieved by improving the loading and elution conditions using a small-scale column (1 mL).

The capacity of the dye-adsorbent (optimal column loading) for the target protein can be determined by frontal analysis [5, 8, 9]. This is achieved by continuous loading of the sample solution onto the column until the desired protein is detected in the eluate. The optimal loading is equivalent to 85–90 % of the sample volume required for frontal detection of the desired protein.

Attention should be paid to variables such as pH, buffer composition, and ionic strength of the equilibration buffer in order to maximize protein binding. In general, low pH (pH < 8.0) and ionic strength (10–50 mM), absence of phosphate ions, and the presence of divalent metal ions such as Mg^{+2}, Mn^{+2}, Ca^{+2} may increase binding (*see* **Note 9**) [12].

A simple test-tube method can be performed to determine the optimal starting pH and ionic strength of the equilibration buffer.

1. Set up five 1 mL columns. Equilibrate each adsorbent with a different pH buffer of the same ionic strength (e.g., 20 mM). Use a range from pH 6–8 in 0.5 pH unit intervals.
2. Load each column with sample and wash them with 5–10 bed vol. of equilibration buffer.
3. Elute the protein with 5 bed vol. of 1 M KCl and collect the eluted protein as one fraction.
4. Assay for protein and enzyme activity.
5. Determine the capacity of each column and the purification achieved.

When the optimum pH has been established, the same experimental approach may be followed to determine which ionic strength buffer can be used to achieve optimal purification and capacity. Use a range of ionic strength buffers with 10 mM intervals.

Special consideration should be given to the elution step in dye-ligand affinity chromatography. Selective or nonselective techniques may be exploited to elute the target protein [12]. Nonselective techniques (increase salt concentration and pH or reduce the polarity of the elution buffer by adding ethylene glycol or glycerol at concentrations of 10–50 %, v/v) normally give moderate purification (*see* **Note 10**). Selective elution is achieved by using a soluble ligand (e.g., substrate, product, cofactor, inhibitor, allosteric effector) which competes with the dye for the same binding site on the protein. This technique, although more expensive than nonselective methods, in general, provides a more powerful purification.

The selection of a suitable competing ligand is critical and often must be done empirically in small test columns using a number of substrates, cofactors, or inhibitors or in some instances a suitable combination of these [5]:

1. Load a 1 mL column with sample and wash with 5–10 bed vol. of equilibration buffer.
2. Wash the column with buffer of an ionic strength just below that required to elute the protein of interest to remove undesired proteins.

3. Elute the desired protein with 3 bed volumes of equilibration buffer containing appropriate concentration of a specific ligand (*see* **Note 11**).
4. Collect fractions and assay for protein and enzyme activity.
5. Evaluate the effectiveness of each specific ligand by determining the purification and recovery achieved.

3.8 Regeneration and Storage of Dye-Ligand Adsorbents

Dye-ligand adsorbents may be effectively regenerated by applying 3 column volumes of chaotropic solutions of urea or guanidine hydrochloride (6–8 M) or sodium thiocyanate (3 M). In some instances, where sterilizing and removing of pyrogens from the chromatographic columns are desired, regeneration with 1 M NaOH may be achieved. After regeneration, wash the column with 10 bed vol. water and finally with 20 % aqueous ethanol solution and store at 4 °C.

3.9 Purification of Phaseolus Vulgaris Glutathione Transferases on Cibacron Blue 3GA-Sepharose

Using this protocol the isoenzymes of glutathione transferase (GST, EC 2.5.1.18) from *Phaseolus vulgaris* can be purified. The optimum buffers for GSTs binding and elution on Cibacron Blue 3GA-Sepharose were established according to Protocol Subheading 3.7. All procedures were performed at 4 °C.

1. Soak *Phaseolus vulgaris* seeds (5 g) overnight in water.
2. Decant the water and transfer the seeds to the mortar with 15 mL of potassium phosphate buffer, pH 6.0 (20 mM). Crush the plant seeds in the mortar with the pestle. Squeeze the homogenate through cheesecloth, and collect the extract in a beaker.
3. Clarify the extract by centrifugation (14,000×*g*, 15 min). Collect the supernatant and clarify by filtration through a cellulose filter (0.45 μm pore size).
4. Equilibrate the adsorbent (Cibacron Blue 3GA-Sepharose, 1 mL) with 10 column volume of 20 mM potassium phosphate buffer, pH 6.0.
5. Apply the extract (~4 mL) to the affinity adsorbent (1 mL, 5 μmol immobilized dye per g wet gel).
6. Wash off non-adsorbed protein with equilibration buffer (~10 mL). This washing step removes unbound and weakly bound soluble contaminants from the chromatographic bed. Washing is performed by pumping starting buffer through the bed until the UV signal from the column effluent returns close to the baseline. This requires approximately 7 bed volumes of buffer.
7. Elute the bound GSTs with the equilibration buffer (20 mM potassium phosphate buffer, pH 6.0) containing 10 mM reduced glutathione (10 mL). Collect 1 mL fractions.

8. Assay for GST activity and protein. The protein content of each fraction may be estimated by the Bradford method [20]. Assay of enzyme activity may be achieved according to [21].
9. Regenerate the adsorbent by applying 3 column volumes of sodium thiocyanate (3 M).

4 Notes

1. Alternatively, purification may be accomplished by preparative TLC on Kieselgel 60 glass plates (Merck) using a solvent system comprising butan-1-ol/propan-1-ol/ethyl acetate/water 2/4/1/3 [13]. A typical protocol is as follows: Dissolve crude dye (approx. 50 mg) in water (0.5 mL). Apply the solution as a narrow strip onto the TLC plate and chromatograph at room temperature. Dry the plate and scrape off the band of interest. Elute the dye from the silica with distilled water, filter through 0.45 μm cellulose membrane filter, and lyophilize.
2. Immobilized ligand concentration plays an important role in dye-ligand affinity chromatography. This should be rigorously defined since it is this parameter which determines the strength of the interaction between the macromolecule and immobilized dye as well as the capacity of the adsorbent for the target protein [9, 14]. High ligand concentrations do not necessarily translate into equally high capacity for the target protein, since extreme levels of ligand substitution may lead to no binding due to the steric effect caused by the large number of dye molecules or even to nonspecific protein binding [9, 14]. On the other hand, low levels of ligand substitution reduce the capacity of the absorbent. An optimum ligand concentration which combines both specific protein binding and high capacity falls in the range of 2.0–3.0 μmol dye/g wet gel [4, 5, 8, 9, 15].
3. The amount of dye and the reaction time required to effect immobilized dye concentration in the range of 2.0–3.0 μmol dye/g gel depends on the chemical nature of the dye (e.g., dichlorotriazine dyes in general are more reactive than monochlorotriazines; thus less dye and shorter reaction times are required). In the case of biomimetic dyes, the nature of terminal biomimetic moiety (aliphatic or aromatic substituent) influences the electrophilicity of the triazine chloride and thus the reaction time [8].
4. This short incubation and the presence of electrolyte (e.g., NaCl) during the immobilization reaction are used in order to "salt out" the dye molecules onto the matrix and to reduce hydrolysis of the triazine chloride by the solvent. The presence of sodium carbonate provides the alkaline pH (pH 10–11)

necessary during the immobilization reaction in order to activate the hydroxyl group of the matrix to act as a nucleophile. The dye can be attached either by hydroxyl ions leading to dye hydrolysis or by carbohydrate-*O*- ions resulting in dye immobilization.

5. In the case of dichlorotriazine dye immobilization, residual unreacted chlorines in the coupled dye may be converted to hydroxyl groups by incubating the matrix at pH 8.5 at room temperature for 2–3 days or to amino groups by reaction with 2 M NH_4Cl at pH 8.5 for 8 h at room temperature [4, 8].
6. The total protein concentration of the applied sample may vary enormously. Ideally 20–30 mg total protein/mL of absorbent in a volume of 1–5 mL should be applied to each column assuming that the target protein constitutes 1–5 mg of the total protein. Column overloading should be avoided since it reduces the purifying ability of the absorbent, unless protein–protein displacement phenomena occur in the adsorption step. Such phenomena have been demonstrated, for example, during the purification of formate, lactate, and malate dehydrogenase on immobilized biomimetic dyes [8, 9, 15].
7. Elute bound protein either nonspecifically with high salt concentration (e.g., 1 M KCl) or specifically by inclusion in the buffer of a soluble ligand that competes with dye for the same binding site of the protein (e.g., 5 mM NAD^+, NADH, ATP, an inhibitor, a substrate). Salt elution leads to practically total protein desorption, therefore the technique reveals the adsorbent's affinity during the binding process. Specific elution of the protein provides information on the ability of the bound enzyme to elute biospecifically, leaving unwanted protein bound [8, 9, 15, 17].
8. Another procedure for screening dye-ligand adsorbents is dye-ligand centrifugal affinity chromatography [16]. This method is based on centrifugal column chromatography and uses centrifugal force rather than gravity to pass solutions through a column. Using this technique a large number of dye columns can be screened simultaneously and has been shown to be both satisfactory and faster compared with conventional gravity flow dye-ligand chromatography.
9. Normally raising the pH of the starting or eluting buffer will weaken the binding of proteins to dye-ligand adsorbents [12]. Below a pH of 6.0, many proteins will begin to bind nonspecifically due to ionic effects. Metal cations often promote binding of proteins to triazine dyes and may be added at concentrations in the range of 0.1–10 mM [12].
10. Elution by reducing the polarity of eluant often gives broad peak profiles compared to salt or pH elution.

11. The required concentration of competing ligand may vary from 1 μM to 25 mM, but most have been found to be in the range of 1–5 mM [3–5, 8, 9, 15]. Gradient elution is not usually as effective as stepwise elution because it broadens the elution peaks. However, such gradients can be used to determine the lowest required soluble ligand concentration for effective elution of the protein of interest.

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