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Purification of recombinant ricin A chain with immobilised triazine dyes

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Abstract

Immunotoxins, such as those based on ricin A chain, must be rigorously purified before they can be administered in vivo. The work described in this paper investigates the interaction between recombinant ricin A chain and several triazine dyes and other ligands that may be of value in its purification. All ligands displayed a high affinity (dissociation constants 3–20 μM) and are displaced from their binding sites on the protein by polynucleotides, heparin and synthetic polyphosphates, but not by mono- or dinucleotides. Affinity chromatography on the immobilised dyes, Procion Red H-3B, Procion Red HE-3B, Procion Red HE-7B and Procion Yellow HE-4R, resulted in a one-step purification of recombinant ricin A chain from an *Escherichia coli* fermentation extract to 94–98% purity and with a >95% yield. These materials are far superior to purification on the conventional dye, Cibacron Blue F-3GA, and show promise for the isolation of immunotoxins from immunoconjugation mixtures.

1. Introduction

Ricin, a potent cytotoxin from the seeds of the castor bean (*Ricinus communis*), is a heterodimer consisting of a ribosome-inactivating A chain, linked by a disulphide bond to a cell-surface receptor binding (B) chain [1]. The B chain binds ricin to cell surfaces through galactose-containing receptors, triggering endocytosis of the toxin [2]. The disulphide bond between the two chains is broken, and once within the cytosol, ricin A chain enzymatically inactivates the 60S ribosomal subunit, inhibiting protein synthesis and causing the death of the cell [3]. Ricin A chain is an N-glycosidase, which hydrolyses a specific adenine base from a highly conserved loop region of 28S rRNA [4]. A comparison of the amino acid sequence of ricin A with other ribosome-inactivating proteins revealed thirteen conserved residues [5]. The three-dimensional structure of ricin has been solved by X-ray crystallography [6] revealing that many of these invariant residues are clustered in a putative active site cleft. Site-directed muta-

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genesis of some of these residues [7–9] has led to a proposed mechanism of ricin A chain action [10]. Around the putative active site cleft of ricin A chain are several patches of arginine residues; such arginine-rich clusters are commonly found in RNA-binding proteins and may also play an important role in specific RNA recognition [11].

The toxicity of ricin A chain within the cytosol has led to its use in immunotoxins for the treatment of certain cancers [12]. However, glycosylation of native ricin A chain can result in the rapid clearance of immunotoxins from the bloodstream by the liver [13]. The cDNA encoding ricin has been cloned and expressed in *Escherichia coli* [14] and the non-glycosylated recombinant ricin A chain has been used in the construction of second-generation immunotoxins [15].

Immunotoxins must be rigorously purified before they can be administered in vivo and affinity chromatography on immobilised dyes has proved to be a convenient method for this [16–18]. Immobilised triazine dye adsorbents are increasingly being used for protein purifications [19], the low cost of reactive dyes combined with their resistance to chemical and biological degradation and ease of immobilisation, has led to affinity adsorbents that are more stable and less expensive than those based on natural biological ligands. The most commonly used dye, Cibacron Blue F-3GA, has been shown to interact with native ricin A chain but not with ricin B chain or intact ricin, both in solution [20] and with the dye immobilised onto agarose [21]. Cibacron Blue F-3GA was found to reduce the ability of native ricin A chain to inactivate ribosomes in an in vitro translation assay suggesting that the dye interacted at, or near, the active site [22].

This work describes an investigation into the interaction of a recombinant ricin A chain (r-ricin A) with a range of reactive dyes in order to improve the specificity and efficiency of their use in the purification of ricin A. The use of these dyes immobilised onto agarose in the purification of r-ricin A from an *E. coli* fermentation extract is compared with the more conventional immobilised Cibacron Blue F-3GA.

2. Experimental

2.1. Chemicals

C.I. Reactive Blue 2 and Procion dyes Red H-3B, Red H-E3B, Red H-E7B and Yellow H-E4R were supplied by ICI Organics Division (Blackley, Manchester, UK). r-Ricin A samples were supplied by Zeneca Pharmaceuticals (Macclesfield, UK). Sephadex LH-20 and Sepharose CL-4B, deoxy-CTP and polynucleotides poly(A), poly(C), poly(G) and poly(U) were obtained from Pharmacia (Uppsala, Sweden). Heparin and polyphosphates were obtained from Sigma (Poole, UK). ATP, $NADP^+$ and NAD^+ were obtained from Boehringer Mannheim (Lewes, UK). Herring testes DNA and yeast RNA were supplied by Dr. J.A.H. Murray, Institute of Biotechnology, Cambridge, UK. Heparin agarose was obtained from Affinity Chromatography Ltd. (Ballasalla, Isle of Man, UK). All other reagents and solvents were of analytical grade and were obtained from UK suppliers.

2.2. Spectral difference titrations

The dissociation constants (K_d) of triazine dyes with r-ricin A were determined by difference spectral titrations at 25°C. A stock solution (approximately 0.5 mg ml^{-1}) of r-ricin A (filtered through a 0.45-μm pore size filter) in 2-(N-morpholino)ethanesulphonic acid (MES)–3-(N-morpholino)propanesulphonic acid (MOPS)–N-[tris(hydroxymethyl)methyl]glycine (Tricine)·NaOH buffer, pH 7.5 (33.3 or 3.33 m*M* in each buffering species, to give a total anion concentration of 0.1 or 0.01 *M*) was prepared and the protein concentration was determined from the absorbance at 280 nm using an extinction coefficient of 1.1 ml mg^{-1} cm^{-1} and a subunit M_r of 29 900 [23]. A stock solution of r-ricin A (10–20 nmol; 1 ml) was added to black-walled silica cuvettes (10 mm pathlength) and placed in the sample beam of a Perkin-Elmer Lambda 7 spectrophotometer. A buffer blank was placed in a similar cuvette in the reference beam and a background correction was performed between

700 and 450 nm. Identical aliquots (2–10 μl) of purified dye solution (1 mM) were added to both cells and the difference spectrum recorded after each pair of additions. The difference absorption, $\Delta\lambda_{max}$, was estimated. Data were corrected for the effect of dilution and the K_d value was calculated by fitting the data to the following equation on an ENZFITTER program [24] using a non-linear regression analysis in an identical manner to that described by Cleland [25]:

$$K_d = \frac{[P_T] - [PD]([D_T] - [PD])}{[PD]}$$

where $[P_T]$ is the total protein concentration, $[D_T]$ is the total dye concentration and [PD] is the concentration of the protein–dye complex.

2.3. Competitive binding experiments

A complex of r-ricin A and dye was formed in solution and the ability of mono-, di- and polynucleotides or other ligands to displace the dye was measured as follows. A 10 mm pathlength black-walled silica cuvette containing r-ricin A (0.3 mg, 10 nmol) and Procion Red H-3B (11.6 ng, 15 nmol) in 1 ml MES–MOPS–Tricine · NaOH buffer, pH 7.5 (3.33 mM in each anion, to give a total anion concentration of 10 mM) was placed in the sample beam of a UV–Vis spectrophotometer. A buffer blank contained the same concentration of dye. The absorption maximum was recorded after the addition of 2–10 μl increments of mono-, di- or polynucleotide, or synthetic ligand as a 5 or 10 mg ml^{-1} solution in deionised water, to both the sample and reference cuvette. Subsequently, identical volumes of dye solution were added to both sample and reference cuvettes. The ligands used were: mononucleotides (ATP, deoxy-CTP), dinucleotides (NAD^+, $NADP^+$), polynucleotides [herring testes DNA, yeast RNA, poly(A), poly(C), poly(G), poly(U)] and other ligands (heparin, polyphosphate glasses of the general formula P_nO_{3n+1}/Na_{n+2} where n is the average number of phosphorous atoms in the chain for $n = 3, 4, 5, 15, 65$).

Data were presented as the absorbance at $\Delta\lambda_{max}$ against amount of ligand added (μg).

2.4. Immobilisation of triazine dyes to agarose by direct coupling

Monochlorotriazine dyes were directly coupled to a cross-linked agarose (Sepharose CL-4B, Pharmacia) according to the method of Lowe et al. [26]: to exhaustively washed agarose (5 g moist mass) was added a solution of purified dye (50 mg) in water (5 ml), followed by NaCl solution (22%, w/v; 1 ml). The mixture was agitated for 30 min at room temperature before adding solid sodium carbonate (50 mg), followed by agitation overnight at 60°C. The dyed gels were washed sequentially with water (200 ml), 1 M NaCl solution (100 ml), water (100 ml), 50% (v/v) dimethyl sulphoxide solution (20 ml) and water (200 ml) to ensure complete removal of any uncoupled dye, and stored in sodium azide solution (0.02%, w/v) at 4°C until required.

2.5. Determination of immobilised dye concentration

Dyed agarose (30 mg moist mass) was hydrolysed at 60°C for 5 min with HCl solution (5 M; 0.6 ml). The hydrolysate was neutralised by the addition of NaOH solution (10 M; 0.3 ml) and potassium phosphate buffer, pH 7.6 (1 M; 2.1 ml). The absorbance at the λ_{max} of the hydrolysate was read against an agarose blank treated in an equivalent manner. Molar extinction coefficients were determined from hydrolysed dye solution (1–25 μM) prepared in a manner identical to that of the hydrolysed gel. Immobilised dye concentrations were calculated as μmol dye per gram moist mass gel.

2.6. Determination of molar capacity of immobilised ligands for r-ricin A chain

Adsorbent (approximately 0.15 ml) was fully equilibrated with Tris · HCl buffer (0.1 M, pH 7.0) before r-ricin A (0.45–0.75 mg, 15–25 nmol) was added in equilibration buffer (1 ml)

and gently agitated for 5 min. The adsorbent was allowed to settle and the supernatant removed for protein determination. Protein was determined by the method of Bradford [27], and a standard curve for r-ricin A prepared for the range 0–0.75 mg ml^{-1} from dilutions of a solution of known concentration. This procedure was repeated until significant amounts of r-ricin A were found in the supernatant. The molar capacity of adsorbents was calculated as mol r-ricin A bound per mol of immobilised ligand.

2.7. Purification of r-ricin A from an E. coli fermentation extract by chromatography on immobilised triazine dyes

Column chromatography experiments were performed using a Pharmacia fast protein liquid chromatography (FPLC) system at 20–25°C. Dyes immobilised onto agarose were packed into Pharmacia HR 5/10 columns to a volume of approximately 1 ml, and equilibrated with Tris · HCl buffer, pH 7.0 (100 m*M*). A solution of r-ricin A *E. coli* fermentation extract was filtered (0.45-μm pore size filter) and loaded onto the column via a 0.2-ml injection loop at a flow-rate of 1 ml min^{-1}. After all unbound protein had washed through, r-ricin A chain adsorbed was eluted by a stepwise gradient of 0.25 *M* NaCl (for 8 min) to 0.55 *M* NaCl (for 9 min) in 100 m*M* Tris · HCl, pH 7.0. Eluted protein was monitored by absorbance at 280 nm. The collected fractions were analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE).

2.8. Analysis of purified r-ricin A by SDS-PAGE

The general procedure employed was SDS-discontinuous PAGE previously described by Laemmli [28]. Stacking gel (4%, w/v; 0.125 *M* Tris · HCl, pH 6.8) and separating gel (12%, w/v; 0.375 *M* Tris · HCl, pH 8.8) were used to electrophorese the loaded samples (1–50 μg). The gels were run at 200 V, 40 mA for approximately 1 h before staining with either silver stain according to the method described by Merril et al. [29] or with Coomassie Blue R-250. Molecular mass markers (low range) were used and consisted of rabbit muscle phosphorylase *b* (M_r 97 400), bovine serum albumin (M_r 66 200), hen egg white ovalbumin (M_r 45 000), bovine carbonic anhydrase (M_r 31 000), soybean trypsin inhibitor (M_r 21 500) and hen egg white lysozyme (M_r 14 400). The stained gels were scanned and bands were quantified using whole band analysis on a Bio Image system (Millipore, MI, USA)

3. Results

Commercial dye preparations frequently contain impurities such as reaction intermediates, hydrolysis products, buffer salts and de-dusting agents [30], and it is essential to remove these before studying dye–protein interactions [31]. The reactive dyes used in this study were purified by Sephadex LH-20 column chromatography and the degree of purification assessed by high-performance liquid chromatography (HPLC) as previously described [32]. In all cases, the purified dyes were estimated by peak integrations to be >96% pure.

Difference spectroscopy is a useful technique for studying the interactions between dyes and proteins [33]. The dye Cibacron Blue F-3GA has been most commonly used for such studies and found to be a sensitive spectroscopic probe for the binding sites of enzymes [34]. The dyes chosen for this study were polyaromatic, polysulphonated molecules that might prove useful mimics of the repeating patterns of heterocyclic bases and ribose-polyphosphate backbones of polynucleotides such as RNA. Fig. 1 shows an example of a spectral difference titration for r-ricin A with Procion Red H-3B. The difference spectra show maxima at 558 nm (a "red shift" of 24 nm from the absorbance maximum of the unbound dye), minima at 490 and 528 nm and an isosbestic point at 542 nm. The maxima at 558 nm were used to calculate the K_d value of r-ricin A for the dye using a non-linear regression analysis. Table 1 shows the structure, λ_{max} and molar extinction coefficient of this and several

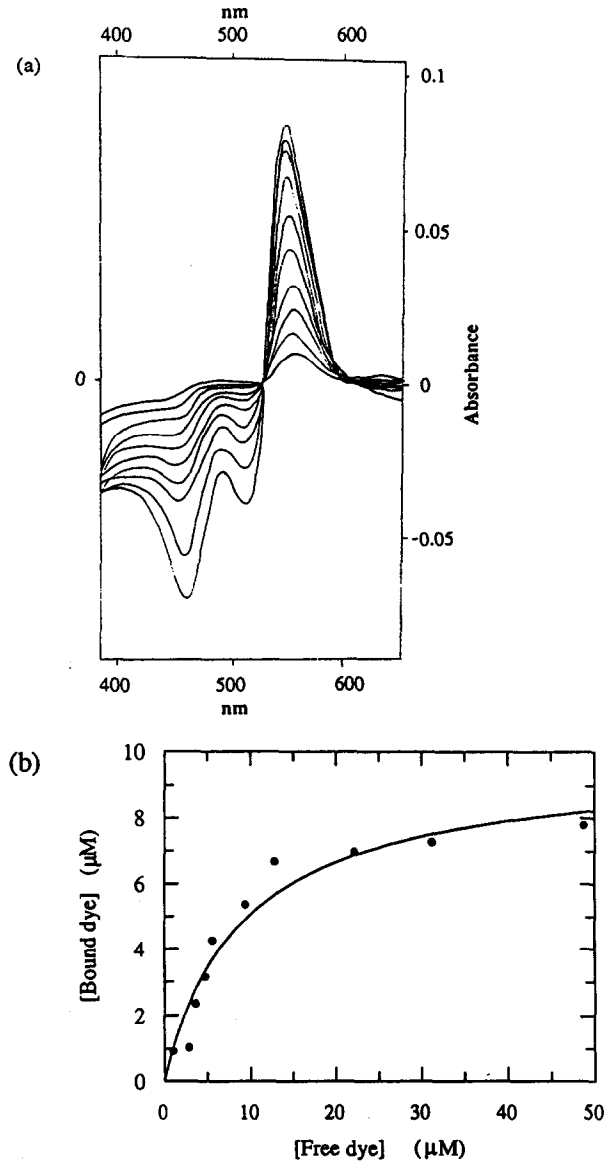
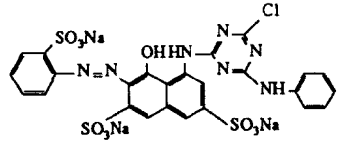
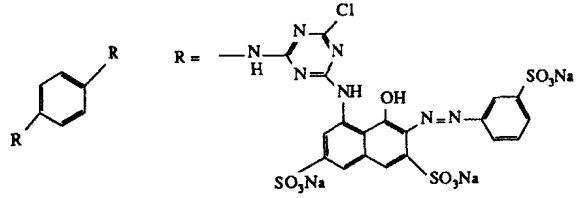
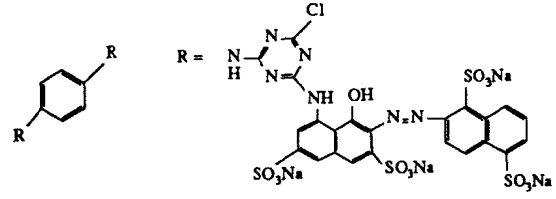
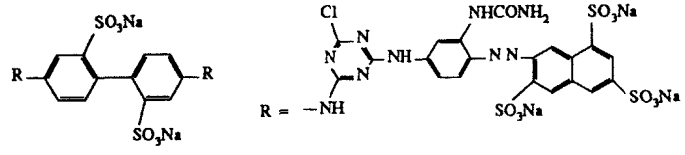
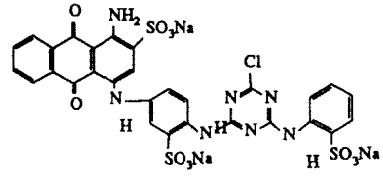


Fig. 1. Determination of the dissociation constant for r-ricin A and Procion Red H-3B in solution. r-Ricin A (0.33 mg, 10.4 nmol subunits) in 1 ml MES–MOPS–Tricine · NaOH buffer, pH 7.5 (0.01 *M*) and a buffer reference were titrated with aliquots (2–10 μl) of Procion Red H-3B solution (1 m*M*); the difference spectra were recorded at 25°C following each pair of additions. (a) The spectral difference titration of r-ricin A (10.4 μ*M*) with Procion Red H-3B (2–9.9 μ*M*). (b) The difference absorbance spectrum plotted as a function of the bound dye versus the free dye concentrations. The K_d value for the complex was determined from these data according to the method of Thompson and Stellwagen [34] and calculated using a non-linear regression analysis.

other dyes and their dissociation constants with r-ricin A determined at pH 7.5 in 10 m*M* buffer. The K_d values were all in the μ*M* range and varied little from dye to dye despite widely differing structures. In general, the values of K_d for the dye–r-ricin A complexes were two to sixfold greater in higher-ionic-strength buffer (results not shown), indicating that electrostatic interactions are important in dye–r-ricin A interactions. These observations suggest that the dyes may bind electrostatically at the arginine-rich clusters on the surface of r-ricin A. The value of the dissociation constant for Cibacron Blue F-3GA with r-ricin A determined in this work (5.6 μ*M*) is considerably higher than that calculated by Watanabe and Funatsu [22] (0.77 μ*M*) and may be due to differences between native and recombinant ricin A chain.

The effect of a variety of mono-, di- and polynucleotides and other ligands on r-ricin A–dye complexes was studied by competitive binding experiments. The results for Procion Red H-3B are shown in Fig. 2 and were typical for all the dyes. Mononucleotides (ATP and deoxy-CTP) and dinucleotides (NAD^+ and $NADP^+$) did not displace the dye from the dye–r-ricin A complex (Fig. 2a) while polynucleotides [RNA, DNA and poly(U)] did displace the dye (Fig. 2b). Readdition of the dye after displacement with polynucleotides restored the difference peak at 558 nm, suggesting that the dye and polynucleotide were competing for the same site on r-ricin A. These results agree with those of a similar experiment by Watanabe and Funatsu [22] for Cibacron Blue F-3GA. Control experiments confirmed that the spectrum of the dye alone was not altered by the addition of any of the ligands used. In addition, it was found that the polyanionic ligands heparin and synthetic polyphosphates also displaced Procion Red H-3B from a dye–r-ricin A complex. A range of polyphosphates of the general formula P_nO_{3n+1}/Na_{n+2} where n is the number of phosphate units in the chain varying between 3 and 65 were used in competitive binding experiments with Procion Red H-3B for r-ricin A (Fig. 2c). The data are presented as absorbance at 558 nm against concentration of phosphate units (PO_4^{2-}) to offset

Table 1
Spectral difference titrations of r-ricin A chain with Cibacron Blue F-3GA and Procion dyes

Ligand	Structure	λ_{max} (nm)	Molar extinction coefficient[a] ($l\ mol^{-1}\ cm^{-1}$)	$\Delta\lambda_{max}$ (nm)	K_d (μM)
Procion Red H-3B		534	23 315	558	8.9 ± 2.1
Procion Red HE-3B		535	40 796	552	3.7 ± 0.7
Procion Red HE-7B		543	53 250	575	18.0 ± 2.5
Procion Yellow HE-4R		402	34 205	457	19.1 ± 2.9
Cibacron Blue F-3GA		617	12 600	683	5.6 ± 1.4

[a]In deionised water.

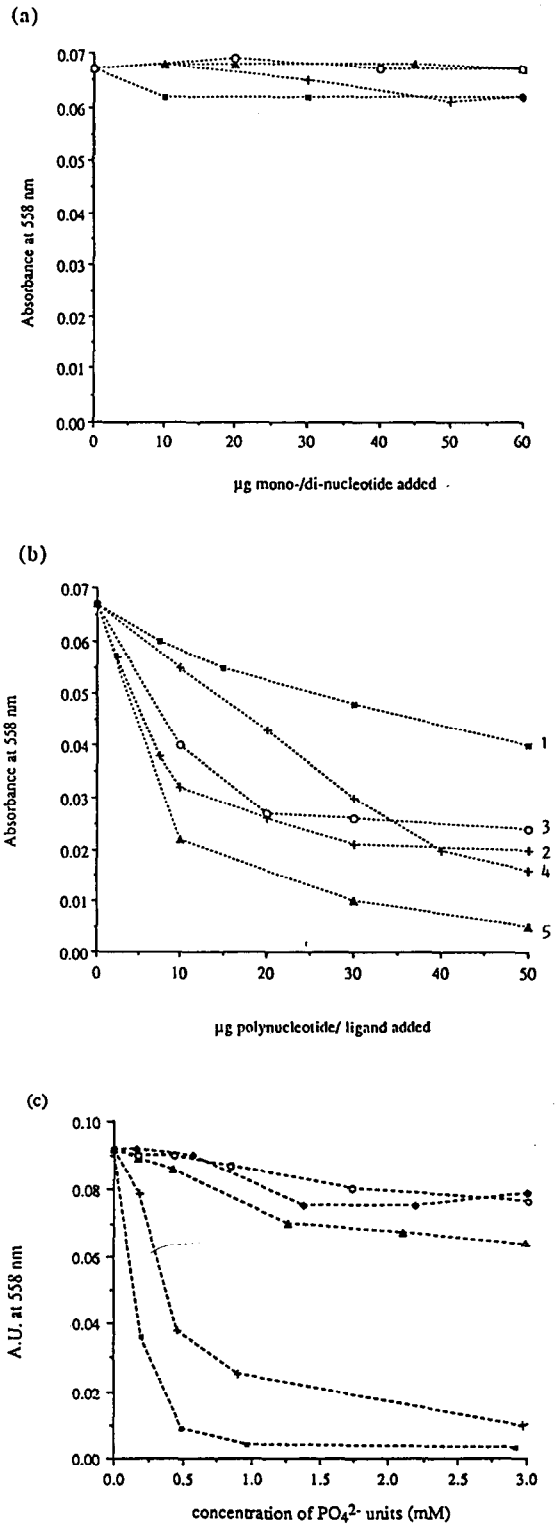


Fig. 2. The effect of various (a) mono- and dinucleotides, (b) polynucleotides and other ligands, and (c) polyphosphate glasses of general formula $P_nO_{3n+1}Na_{n+2}$ with between $n = 3$ and $n = 65$ phosphate units in a linear chain, on the maximum difference absorbance of Procion Red H-3B in the presence of r-ricin A. The sample cuvette initially contained r-ricin A (10 nmol) and 15 nmol dye in 1 ml of MES–MOPS–Tricine · NaOH buffer, pH 7.5 (0.01 *M*), and the reference cuvette contained the same concentration of dye in 1 ml of buffer. The maximum difference absorptions were recorded after the addition of 2–10-µl increments of ligand. (a) ○ = $NADP^+$; ▲ = NAD^+; + = ATP^+; ■ = deoxy-CTP. (b) 1 = DNA; 2 = RNA; 3 = poly(U); 4 = heparin; 5 = polyphosphate ($n = 65$). (c) (◆) $n = 3$; (△) $n = 4$; (○) $n = 5$; (+) $n = 15$; (■) $n = 65$.

the effects of displacement by ionic strength increase alone. Polyphosphates of 3 to 5 units were unable to displace the dye from a complex with Procion Red H-3B, but polyphosphates of 15 and 65 units did displace the dye. The ability of polyphosphates to displace effectively dyes suggests the importance of the interactions between negatively charged dye sulphonates and positively charged protein groups in the interaction with r-ricin A. It is not clear why phosphate ligands of different lengths have different effects on the r-ricin A–dye complex. It may be that polyphosphates of 15 to 65 units are better analogues of the RNA substrate, or they may induce a conformational change in r-ricin A.

The affinity of proteins for dyes immobilised onto a chromatographic support can sometimes vary considerably from the K_d values determined in solution studies [31]. Therefore, the interaction between r-ricin A and immobilised dyes was also studied. The immobilised dye concentration is an important determinant of the affinity of a protein for a dye adsorbent and the use of lightly substituted gels (approximately 2 µmol dye per g moist mass gel) lead to more satisfactory purifications [35]. The Procion dyes Red H-3B, Red HE-3B, Red HE-7B and Yellow HE-4R and Cibacron Blue F-3GA, were immobilised onto Sepharose CL-6B, a cross-linked agarose, by direct coupling and the immobilised dye concentrations were determined to be between 2.3 and 2.8 µmol dye per g moist mass gel (Table 2). The molar capacities of immobilised

Table 2
Comparison of molar capacity for r-ricin A chain of triazine dyes and heparin immobilised on agarose

Immobilised ligand	Ligand concentration (μmol ligand per g moist mass gel)	Molar capacity (μmol r-ricin A per μmol immobilised ligand)
Procion Red H-3B	2.8	0.06
Procion Red HE-3B	2.7	0.07
Procion Red HE-7B	2.7	0.07
Procion Yellow HE-4R	2.3	0.13
Cibacron Blue F-3GA	2.7	0.03
Heparin	1	0.09

dyes and of heparin agarose for r-ricin A were determined batchwise (Table 2). Procion Red H-3B, Procion Red HE-3B and Procion Red HE-7B exhibited a twofold higher molar capacity for r-ricin A than immobilised Cibacron Blue F-3GA, while immobilised Procion Yellow HE-4R exhibited a fourfold increase in molar capacity over Cibacron Blue F-3GA and a 1.5-fold higher capacity than heparin agarose. Recombinant ricin A was not retained on Sepharose CL-6B alone. Molar capacities of approximately 10% of the total ligand are typical for immobilised dyes [36].

The selectivity of dyes immobilised onto agarose for r-ricin A was examined by the purification of r-ricin A from an *E. coli* fermentation extract by FPLC. A non-selective desorption technique was adopted (NaCl, 0.25–1 *M*) so that an indication of adsorbent specificity could be obtained. An example of the FPLC purification of r-ricin A from an *E. coli* fermentation extract on immobilised Procion Red HE-7B is shown in Fig. 3. Crude extract (lanes 3 and 8) was applied to the column and protein that did not bind to the column appeared at the void volume (2.5 ml, fraction I; lane 4), adsorbed proteins were eluted with a stepwise gradient of 0.25 *M* and 0.55 *M* NaCl in Tris · HCl buffer, pH 7.0 (0.1 *M*). r-Ricin A was eluted with 0.55 *M* NaCl (fraction III) and showed few contaminating proteins on the silver-stained SDS-PAGE gel (lane 6). It was estimated by band quantification that r-ricin A was purified to approximately 95% purity with a yield of >95% from a crude extract containing 10% r-ricin A. A comparison by Coomassie-stained SDS-PAGE of the purification of r-ricin A from an *E. coli* fermentation extract by dyes immobilised on agarose and by heparin agarose is shown in Fig. 4. Both Procion Yellow HE-4R and Procion HE-7B show good purifications of r-ricin A (lanes 4 and 5, respectively, both 98% purity) with only a few minor contaminants remaining from the crude extract (lanes 3 and 10). Heparin agarose and immobilised Cibacron Blue F-3GA gave less satisfactory purifications [lane 6 (85% purity) and lane 7 (40% purity), respectively], with both ligands retaining *E. coli* proteins. Analysis by silver-stained SDS-PAGE of the purification of r-ricin A from an *E. coli* fermentation extract by Procion Red H-3B and Procion Red HE-3B immobilised on agarose [Fig. 5, lane 5 (98% purity) and lane 7 (94% purity), respectively] showed that these dyes also gave good, one-step purifications comparable to that obtained on Procion Red HE-7B (Fig. 3). Heparin agarose (lane 10, 75% purity) performed poorly in comparison.

4. Discussion

A recombinant ricin A (expressed in *E. coli)* is increasingly being used in the construction of immunotoxins. Immobilised dyes could be exploited in two procedures in the production of ricin-based immunotoxins —for the purification of ricin A chain from the recombinant *E. coli* extract and for the purification of ricin A—

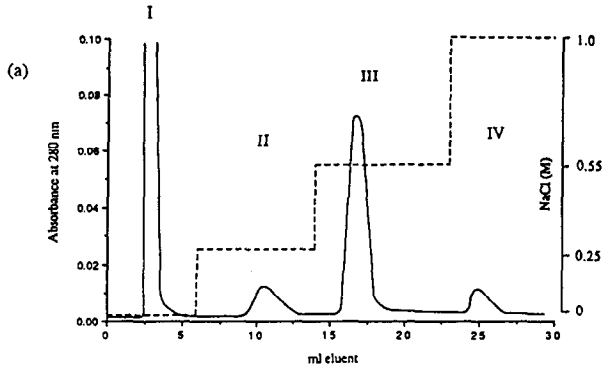


Fig. 3. The purification of r-ricin A from an *E. coli* fermentation extract on Procion Red HE-7B immobilised on agarose. (a) A sample (200 μl) of *E. coli* fermentation extract was loaded onto a column (1 ml) of Procion Red HE-7B immobilised onto agarose, equilibrated in Tris·HCl buffer (0.1 *M*), pH 7.0, at a flow-rate of 1 ml ml^{-1}. After unbound protein had washed through, a stepwise gradient was employed at 25, 55 and 100% (v/v) of NaCl (1 *M*) in Tris·HCl buffer, pH 7.0 (0.1 *M*). Eluted protein was measured at 280 nm and fractions (0.5 ml) were collected and analysed by SDS-PAGE. (b) Silver-stained SDS-PAGE gel of the purification of r-ricin A from an *E. coli* fermentation extract on Procion Red HE-7B-agarose. Lanes: 1, 10 = Molecular mass markers (kDa = kilodalton); 2 = r-ricin A standard; 3, 8 = *E. coli* extract; 4 = fraction I, not retained on Procion Red HE-7B column; 5 = fraction II, eluted with 0.25 *M* NaCl; 6 = fraction III, r-ricin A eluted with 0.55 *M* NaCl; 7 = fraction IV, eluted with 1 *M* NaCl; 9 = blank; 10 = r-ricin A purified on heparin agarose.

containing immunotoxins from immunoconjugation mixtures. This work describes the first example of the purification of r-ricin A from crude extracts using immobilised dyes.

Purification of r-ricin A from an *E. coli* fermentation extract on immobilised ligands Procion Red H-E3B, Procion Red H-E7B and Procion Yellow H-E4R resulted in a one-step purification of r-ricin A with high yield (94–98% purity with >95% yield, as only minor contaminants were visualised by silver staining of an SDS-PAGE gel). In comparison, heparin agarose

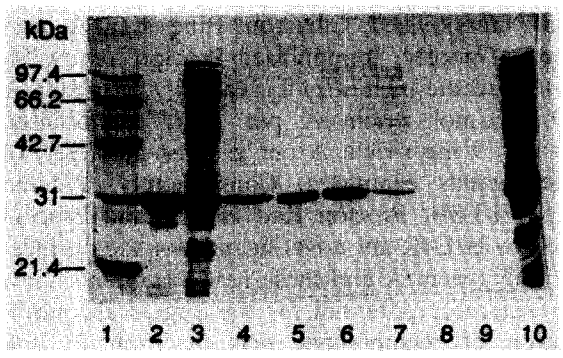


Fig. 4. Coomassie-stained SDS-PAGE gel of the purification of r-ricin A from an *E. coli* fermentation extract on dyes immobilised onto agarose. Lanes: 1 = Molecular mass markers (kDa = kilodalton); 2 = r-ricin A standard; 3, 10 = *E. coli* fermentation extract; 4 = r-ricin A purified on immobilised Procion Yellow HE-4R; 5 = r-ricin A purified on immobilised Procion Red HE-7B; 6 = r-ricin A purified on heparin agarose; 7 = r-ricin A purified on immobilised Cibacron Blue F-3GA; 8, 9 = blank.

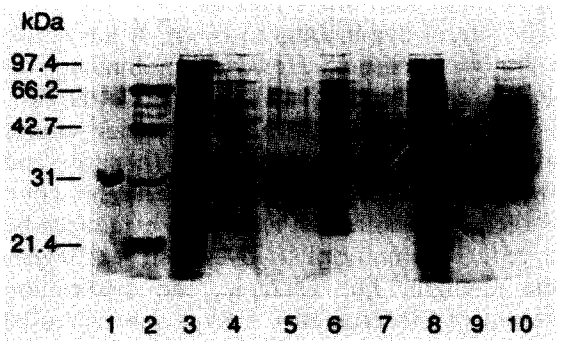


Fig. 5. Silver-stained SDS-PAGE gel of the purification of r-ricin A from an *E. coli* fermentation extract on dyes immobilised on agarose. Lanes: 1, 9 = r-ricin A standard; 2 = Molecular mass markers (kDa = kilodalton); 3, 8 = *E. coli* fermentation extract; 4 = protein not retained on immobilised Procion Red HE-3B column; 5 = r-ricin A purified on immobilised Procion Red HE-3B; 6 = protein not retained on immobilised Procion Red H-3B column; 7 = r-ricin A purified on immobilised Procion Red H-3B; 10 = r-ricin A purified on heparin agarose.

and immobilised Cibacron Blue F-3GA performed poorly. Immobilised Procion Yellow H-E4R had the highest molar capacity for r-ricin A (0.13 μmol r-ricin A per μmol immobilised ligand; 9 mg r-ricin A per g moist mass gel) of the ligands examined. Consequently, Procion Red H-E3B, Procion Red H-E7B and Procion Yellow H-E4R are novel ligands for the purification of r-ricin A and these new adsorbents show considerable potential for the large-scale purification of recombinant ricin A from *E. coli* fermentation extracts and for the purification of immunotoxins from immunoconjugation mixtures. In a recent publication, Li et al. [37] report the high level expression and simplified purification of a recombinant ricin A chain from *E. coli*. However, this procedure required further S-200 gel filtration to achieve equivalent purity. The overall yields of recovered protein also appeared to be considerably lower than those reported here. The similarities between ricin A and other ribosome-inactivating proteins such as abrin and modeccin [38] may also enable effective purifications of these other proteins to be achieved. Furthermore, the purification of the recently developed fusion toxins (wholly recombinant immunotoxins consisting of toxins and antibody fragments produced by genetic fusion [39,40]) may also prove possible.

The site of dye-binding to r-ricin A has yet to be firmly established. However, preliminary results presented in this paper suggest that the dyes bind to a site competitive with polynucleotides and their polyanionic analogues (heparin and polyphosphates). It appears that this interaction is largely electrostatic in nature, as evidenced by the reduced affinity of all dyes tested at higher ionic strength. For example, the dissociation constant of Cibacron Blue F3G-A was increased from 5.6 ± 1.4 μM in 10 mM buffer pH 7.5 to 33.1 ± 14.7 μM in 100 mM buffer pH 7.5. A plausible explanation for this is that the dyes bind r-ricin A at the arginine-rich clusters involved in co-ordinating RNA. Further work is in progress to identify the detailed nature of the interaction and the binding site topography in order to design a novel ligand based on these dyes for the purification of r-ricin A and its immunoconjugates [41]. Furthermore, these studies should rationalize the selective elution strategy based on the competitive interactions between the dyes and known competitive ligands, such as heparin and polyphosphates. It is anticipated that these studies will lead to a comprehensive purification strategy for ricin A and its immunoconjugates.

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