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Chromatography of bovine lactoserum on the immobilized metallized dye Drimarene Rubine R/K 5BL Modulation of selectivity by metal exchange and application to purification of milk immunoglobulins

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Abstract

Chromatography on immobilized Drimarene Rubine R/K 5-BL, a copper-containing reactive dye, was evaluated as a method of purifying immunoglobulins from bovine lactoserum. Varying the eluent composition did not provide pure immunoglobulins. However, changing the metal from copper to zinc allowed the preparation of essentially pure immunoglobulins, in quantitative yield, using imidazole in 2 *M* NaCl as mobile phase. This procedure can probably be adapted to the very large-scale preparation of immunoglobulins from milk which are administered orally for human and animal therapy.

Keywords: Lactoserum; Milk; Selectivity; Stationary phases, LC; Immobilized metallized dyes; Dyes; Immunoglobulins

1. Introduction

Chromatography on immobilized dyes has been used to purify many proteins [1]. This type of chromatography has been successful because very selective supports are readily obtained by screening many dyes to find those which can be used for efficient purification [2–7]. Mobile phases (including these containing solutes that compete with dye–protein interactions) can also be screened for selectively eluting the protein of interest [8–12]. Some reactive dyes produced for tinctorial purposes that have been used for chromatography contain metals. Metallization of dyes is used in the dyes and pigments industry to improve light and wet fastness or to produce specific shades [13]. We have used a metallized dye (Sandoz Drimarene Rubine R/K 5BL) for chromatography of human immunoglobulins [12]. This copper-loaded dye was not satisfactory for preparing immunoglobulins from bovine whey, because the purification factor was poor. However, changing the metal linked to the dye resulted in very satisfactory performance in

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terms of yield and purity, as described in this paper.

The purification of bovine immunoglobulins from milk is not only a model system for evaluating the effect of metal nature on the performance of immobilized metallized dyes as affinity ligands. Oral administration of immunoglobulins is an efficient treatment for immunocompromised patients suffering from life-threatening *Cryptosporidium parvum* infections of the digestive tract [14–17], while most of the antibiotics tested to date have little effect [18–20]. However, the daily dose of immunoglobulins can be over 50 g. Up to now most studies have been performed using either plain or lyophilized bovine colostrum because the immunoglobulins content of colostrum is much higher (IgG 50 g/l, IgA 4g/l) than milk (IgG 0.6 g/l IgA 0.1 g/l). However, colostrum is produced by cows for only 2–4 days each year, whereas milk is produced for 11 months [21]. Only immunoglobulin preparations obtained from specially immunized cows seem to be suitable for therapeutic applications [18,22]. Because of the growing needs linked to the AIDS epidemic, oral administration of immunoglobulins for treatment of *Cryptosporidium parvum* infections might be based more on milk than on colostrum immunoglobulins. Hence the design of efficient milk immunoglobulin purification processes, amenable to large-scale operation at a resonable cost, is an attractive goal. Orally administered immunoglobulins are also used in humans to treat or prevent other intestinal infections [23–26] and to treat young domestic animals [27,28].

2. Experimental

2.1. Materials

Drimarene Rubine R/K-5BL was a gift from Sandoz Chimie (Rueil Malmaison, France). Sepharose CL 4B was obtained from Pharmacia (Uppsala, Sweden). Unpasteurized cow milk was bought from a local health store. All other chemicals were bought from Sigma (St. Louis, MO, USA), Merck (Darmstadt, Germany) or Bio-Rad (Richmond, CA, USA).

2.2. Dye immobilization [1]

Sepharose CL 4B was rinsed extensively with water and placed in 0.2 *M* NaOH solution containing 2% (w/v) NaCl. Gel was transferred in a vessel containing reactive dye (30 mg/ml gel; the dye was used as obtained from the manufacturer). A 0.2 *M* NaOH–2% NaCl solution was then added so that the final reaction volume was twice the gel volume. The gel suspension was tumbled at 60°C for 1 h. The gel was then rinsed with ten volumes of 0.2 *M* NaOH and then eventually re-treated as above with fresh dye up to six times. Finally, the immobilized dye was exhaustively washed with water, 8 *M* urea and 0.1 *M* NaCl.

Dye substitution was measured by hydrolysing the immobilized dye in 50% acetic acid at 110°C until the gel was completely dissolved [1]. The absorbance was then measured at 531 nm. Substitution was expressed as mg of dye per ml of support, using a solution of the reactive dye treated with 50% acetic acid as standard.

2.3. Preparation of a soluble metal-free dye derivative

Dye (900 mg) as received from the manufacturer was deactivated by incubating it for 1 h at 60°C in 60 ml of 0.2 *M* NaOH. Concentrated EDTA solution and sodium acetate buffer were added to give final concentrations of 0.1 and 0.05 *M*, respectively. The pH was adjusted to 5.4 with acetic acid. The solution was then loaded on to a 16×2.5 cm I.D. column, packed with Waters (Milford, MA, USA) preparative C_{18} support (mean particle diameter 55–105 μm, pore size 12.5 nm) equilibrated in 0.5% (v/v) triethylamine–1% (v/v) acetic acid in water [1].

After loading, the column was rinsed (flow-rate 5 ml/min) with the same mobile phase until no more blue EDTA–copper complex was eluted from the column. Finally, the demetallized

dye retained on the column was eluted with a 60-min linear gradient between the previous mobile phase and a mobile phase containing triethylamine and acetic acid as above, and 50% (v/v) acetonitrile. Red fractions were dried in a Speed Vac (Emeryville, CA, USA) apparatus. Deactivation and demetallization were monitored by analytical HPLC on a 25 cm × 0.46 cm I.D. C_{18} Nucleosil column (particle diameter 7 μm, pore diameter 8 nm) operated at a flow-rate of 1.5 ml/min with the same mobile phases as used for the preparative separations; a 20-min linear gradient between the initial and final mobile phases adequately separated the dye derivatives.

2.4. Difference spectral titration

Difference spectral titrations were performed in the buffers used for the chromatographic experiments using a Shimadzu (Kyoto, Japan) UV 160-A double-beam spectrophotometer. Cuvettes with a 10 cm optical path length were used in order to work with low dye concentrations. The sample and reference cuvettes contained initially identical dye solutions in buffer. Aliquots of metal ion solution in buffer and buffer without metal were thereafter added to the sample and reference cells, respectively. Difference absorption spectra were recorded after each pair of additions. Heights (A) of peaks (or troughs) of the difference spectra were plotted against metal ion concentration. Non-linear least-squares minimization (Kaleidagraph Software on an Apple Macintosh computer) was used to fit K_d and A_{max} as parameters in the following equation:

$$A = (A_{max}M_e)/(K_d + M_e)$$

where M_e is the equilibrium metal concentration (it was assumed, because of the low dye concentration used in the experiments, to be not very different from the total metal concentration), A_{max} is A at dye saturation with metal and K_d is the dissociation constant of the metal–dye equilibrium.

2.5. Formulations of buffers used for the chromatographic experiments

The following buffers were used: buffer A was 10 m*M* 2-(N-morpholino) ethanesulfonic acid (MES) containing 30 m*M* NaCl, adjusted to pH 6.0 with KOH; Buffer B was of the same composition as buffer A but contained 2 *M* NaCl; buffer C was of the same composition as buffer B but contained 50 m*M* imidazole; buffer D was prepared by mixing 1 volume of buffer B and 1 volume of ethylene glycol; buffer C′ was 10 m*M* N-2-(hydroxyeethyl) piperazine-N′-(2-ethanesulfonic acid) (HEPES) buffer containing 50 m*M* imidazole and 2 *M* NaCl adjusted to pH 6.8 with KOH; and buffer C″ was of same composition as buffer C but contained 150 m*M* imidazole.

2.6. Preparation of lactoserum

One volume of 10 m*M* sodium acetate buffer (pH 4.6) was added to one volume of skimmed milk. The pH of the mixture was adjusted to 4.6 with dilute acetic acid and allowed to stand in the cold for 1 h. It was centrifuged at 3000 *g* for 30 min and the supernatant (lactoserum) was recovered, its pH was adjusted to 6.0 with dilute sodium hydroxide and it was filtered through Whatman (Maidstone, Kent, UK) 3MM paper. The filtrate was usually loaded on to a Sephadex G-25 column equilibrated in buffer A, prior to chromatography on immobilized dye, or used as such without gel filtration (see Results and discussion).

2.7. Metal stripping and metal exchange of immobilized dye

Copper was removed from immobilized Rubine R/K-5BL by pumping 0.1 *M* ethylenediamine tetraacetic acid (EDTA) in buffer B through the column. The metal-stripped immobilized dye could be re-loaded with copper or loaded with the other tested metals by pumping ten column volumes of a 0.1 *M* solution of $CuSO_4$ in water or $NiCl_2$ or $ZnCl_2$ in buffer A. The column was then rinsed with ten column

volumes of water and buffer A (copper loaded columns) or with buffer A only (zinc- and nickel-loaded columns).

2.8. Chromatography of lactoserum on metal-loaded and metal-free Rubine R/K 5BL

Capacity was evaluated by frontal chromatography of lactoserum (containing 0.15 mg/ml of IgG) on 2.5 cm × 1.1 cm I.D. columns packed with three or six times coupled zinc-loaded gels (flow-rate 20 ml/h). Volumes of lactoserum which could be loaded on the columns before the immunoglobulin concentration in the collected fractions reached 10% of the value loaded were recorded. This allowed the derivation of a capacity value expressed as mg of IgG per ml of gel (the elution volume at the 10% value obtained with unsubstituted gel and the same experimental set-up was taken into account).

Analytical chromatographic experiments with immobilized metal-free or metallized dyes were performed using 2.5 cm × 1.1 cm I.D. columns packed with three times coupled gel. Columns were operated at a flow-rate of 20 ml/h. A Pharmacia UV-1 apparatus fitted with a 3-mm optical path cuvette was used to record the absorbance at the outlet of the column. Most of the studies used a protein load corresponding to 1.5 mg of IgG. Screening experiments to determine the capacities of various mobile phases to elute proteins from immobilized metallized and metal-free dyes were conducted using buffer A containing up to 2 *M* NaCl and/or up to 50 m*M* imidazole and/or up to 50% (v/v) ethylene glycol. The mobile phases were applied either as step or continuous gradients (results are not given in full). The results fully described below obtained with zinc-loaded, copper-loaded, nickel-loaded and demetallized Rubine R/K 5BL were obtained as follows: after sample loading the columns were rinsed in sequence with buffers A, B, C and D; after chromatography the columns were regenerated by pumping 0.1 *M* EDTA buffer (pH 8.0) containing 2 *M* NaCl and 6 *M* urea and subsequently re-metallized as needed; a parallel set of experiments were carried out with zinc-loaded immobilized dye using in place of buffer C, C′ or C″.

Larger scale experiments were performed using a 9 cm × 2.5 cm I.D. column filled with six times coupled gel. The flow-rate was 150 ml/h and the column was loaded with 350 ml of lactoserum without prior Sephadex G-25 gel filtration.

2.9. Identification and assays of proteins in eluted fractions

The proteins in the eluted fractions were identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting [29,30] using anbibodies against heavy and light chains of IgG (rabbit antiserum from Sigma, ref. B-8395), the secretory piece of IgA (affinity-purified antiserum prepared by Bethyl Laboratories, ref. A 10-108, purchased from Interchim, Montluçon France), bovine serum albumin (Cappel rabbit antiserum, ref. 55269, obtained from Organon Teknika, Durham, NC, USA) and lactoferrin (a rabbit antiserum provided by Professor Ribadeau Dumas, INRA, Versailles, France). Alkaline phosphatase or peroxidase tagged second antibodies were obtained from Sigma. Bovine IgG in starting material and in collected pools (for yield evaluation) was assayed by immunodiffusion using commercial bovine IgG (Sigma) as a standard and the antiserum indicated above. For the pilot experiments which were performed with a small amount of loaded IgG, IgGs were concentrated prior to immunodiffusion assay by precipitation with 2 *M* ammonium sulfate.

Measurements of IgG concentrations in individual chromatographic fractions, for capacity evaluation of the column, was performed by scanning SDS polyacrylamide gels after electrophoresis of aliquots of fractions. A SEBIA (Issy Les Moulineaux, France) Preference scanner was used.

Lactoperoxidase was assayed using an assay mixture containing 1 m*M* 2,2′-azinobis (2-ethylbenzthiazoline- 6-sulfonic acid) (ABTS) (Sigma), 25 m*M* sodium acetate buffer (pH 4.4) and 0.5 m*M* H_2O_2. The increase in absorbance at 412 nm was recorded in the Shimadzu UV-160-A spectrophotometer after addition of the sample to the assay cuvette [31].

3. Results and discussion

3.1. Characterization of the immobilized dye and the zinc–dye interaction

A single coupling step gave a dye substitution of 3.3 mg of dye per ml of gel, three coupling cycles gave 9.2 mg/ml and six coupling cycles gave 14 mg/ml.

The dissociation constants for the zinc–dye interactions were measured by differential spectroscopy (Fig. 1). The K_d in buffer A was 28.0 ± 0.4 μM, which is much lower than published values for the interaction of metals with other dyes not manufactured as metallized dyes [32]. The dissociation constant of the zinc Drimarene Rubine R/K 5BL equilibrium was significantly higher at 173.8 ± 2.2 μM in higher ionic strength buffer B. An increase in metal–dye dissociation constants with increasing ionic strength has also been reported by others [33]. The K_d of the zinc–dye equilibrium in imidazole-containing buffer C was 288.0 ± 9.1 μM and in ethylene glycol-containing buffer D 575.0 ± 24.5 μM.

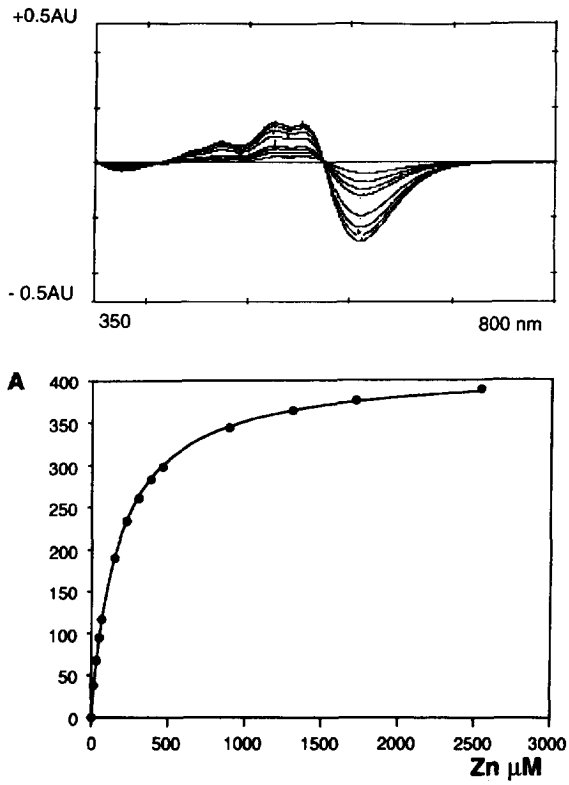

Fig. 1. Determination of the zinc ion–dye equilibrium dissociation constant in buffer B. Difference spectra were recorded in a double-beam spectrophotometer. Sample and reference cells (10 cm optical path length) contained 30 ml of demetallized dye solution dissolved in buffer B. Zinc ions in buffer B were added to the sample cell and buffer without metal to the reference cell. Difference absorption spectra were recorded after each pair of additions (top: overlay of several spectra). The depths (A) of troughs at 620 nm of the difference spectra were plotted against metal ion concentration (bottom). Filled circles are experimental points; the continuous line was derived by fitting experimental points to the equation $A = (A_{max}M_e)/(K_d + M_e)$, where M_e is the metal concentration added to the sample cuvette (probably not very different from the free metal concentration; see Experimental), A_{max} is the A value of metal-saturated dye and K_d is the dissociation constant of the metal–dye equilibrium.

3.2. Screening of mobile phases for eluting immunoglobulins from copper-loaded immobilized dye

Pilot experiments on copper-loaded immobilized dye were run using different buffer formulations. We tested the increase in ionic strength (with step or continuous gradients), with imidazole addition (as imidazole can elute proteins from immobilized metallized dyes [12]). Because of the limited success of these trials, metal exchange was tried and retained as the most powerful approach to modulate dye selectivity adequately as demonstrated below.

3.3. Evidence that the metal bound to the dye influences selectivity

Chromatograms obtained with zinc-loaded Drimarene Rubine R/K 5BL columns are shown in Fig. 2. SDS gels of fractions eluted from immobilized dye in four states are shown in Fig. 3. They demonstrate the marked effect of the metal load on selectivity.

Copper-loaded dye

β-Lactoglobulin was found mostly in fractions eluted from the column by buffer A. Buffer B

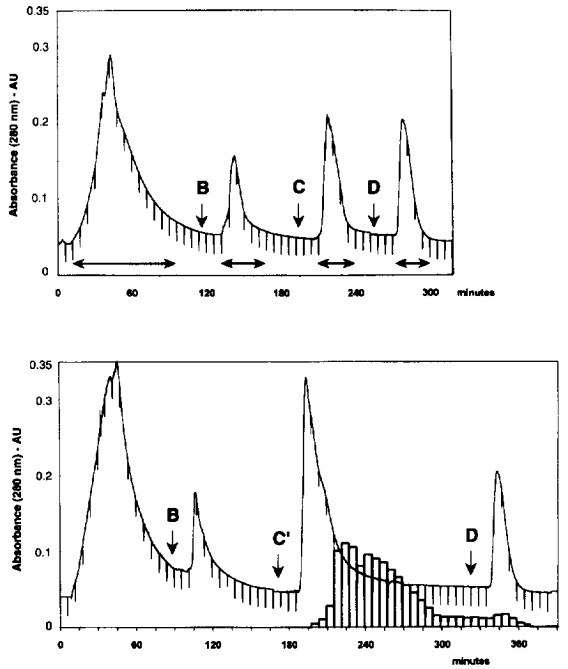

Fig. 2. Chromatography of acidic whey on a 2.5 cm × 1.1 cm I.D. immobilized Drimarene Rubine R/K 5-BL column. Flow-rate, 20 ml/h. Top: after loading with lactoserum column was rinsed with buffer A. Vertical arrows indicate changes to buffers B, C and D. Horizontal double-headed arrows show how fractions were pooled for electrophoresis. Bottom: development of the column with buffer C' was used instead of buffer C. The histogram superimposed on the absorbance record indicates the lactoperoxidase content of individual fractions.

eluted some β-lactoglobulin and albumin. Immunoglobulins were eluted by imidazole-containing buffer C, but were heavily contaminated with albumin, lactalbumin, lactoferrin and lactoperoxidase, and some β-lactoglobulin. Buffer D eluted little protein, as indicated by the faint bands on the electrophoresis gel.

Metal-free dye

All lactalbumin was found in A fraction together with most of the β-lactoglobulin. Buffer B eluted immunoglobulins contaminated with a small amount of β-lactoglobulin and all albumin and lactoperoxidase. No proteins were eluted by imidazole-containing buffer C, but ethylene glycol-containing buffer D eluted strongly retained lactoferrin.

Nickel-loaded dye

β-Lactoglobulin was recovered from nickel-loaded immobilized dye mostly in buffer A-eluted fractions. The buffer C-eluted fractions also contained some β-lactoglobulin, immunoglobulins, all the albumin, α-lactalbumin and lactoperoxidase. Lactoferrin was eluted with ethylene glycol-containing buffer D.

Zinc-loaded dye

β-Lactoglobulin and α-lactalbumin were mostly eluted by buffer A. Buffer B eluted small amounts of β-lactoglobulin and lactalbumin and most of the albumin. Buffer C eluted the immunoglobulins. The yield of IgG in buffer C-eluted fractions was quantitative. The immunoglobulins. The yield of IgG in buffer C-eluted fractions was quantitative. The immunoglobulins appeared as three bands on gels run under reducing conditions. Their molecular masses and reactivities with antibodies on Western blots identified them as the IgA secretory piece (70 000) and the heavy (53 000) and light (25 000) chains. Lactoperoxidase and lactoferrin were eluted by buffer D. Thus, adequate separation of immunoglobulins from most of the other protein constituents of milk was obtained only with the zinc-loaded immobilized dye. IgG and IgA were eluted together from the zinc-loaded immobilized dye. In this particular case this is an advantage, since it has been demonstrated in mice that oral administrations of both IgG and IgA are effective against *Cryptosporidium parvum* infections [18].

3.4. Insight into retention/elution mechanisms for the lactoserum proteins on the zinc-loaded column

Albumin was eluted from both zinc-loaded

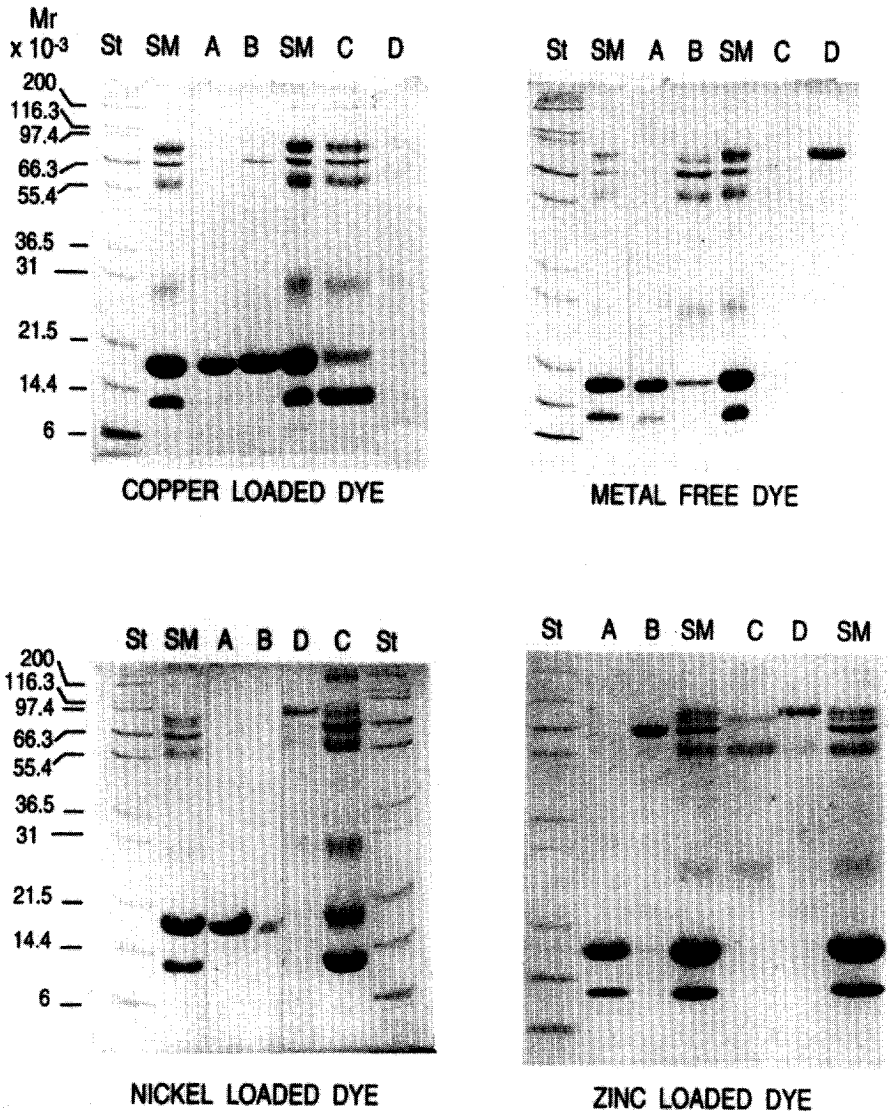

Fig. 3. SDS-PAGE under reducing conditions of fractions eluted from metal-loaded and metal-free immobilized Drimarene Rubine R/K 5BL. Lanes A, B, C and D are aliquots of pooled fractions eluted by the corresponding buffers. Lanes marked SM and St were -loaded with starting material (SM) and molecular mass standards (St).

immobilized dye and metal-free dye by an increase in ionic strength. This suggests that albumin was retained solely by ionic interactions, zinc playing no role in retention. An increased ionic strength did elute immunoglobulins from metal-free immobilized dye but not from a zinc-loaded column; this demonstrates that immobilized zinc interacts with bovine immunoglobulins. The eluting effect of imidazole is in agreement with this statement.

Lactoferrin was not eluted from zinc-loaded immobilized dye by buffer C′ (Fig. 2), although the higher pH of buffer C′ favours deprotonation of imidazole and thus increases its eluting power

(see Fig. 5 in Ref. [12]). Lactoferrin was also not eluted with a buffer containing 150 m*M* imidazole (buffer C″). Hence interactions with metal seem to be less important than hydrophobic interactions for retaining lactoferrin on immobilized dye; ethylene glycol necessary to elute lactoferrin from both zinc-loaded or metal-free immobilized dye probably worked by disrupting hydrophobic interactions.

Lactoperoxidase was eluted from metal-free immobilized dye by an increased ionic strength, suggesting that mostly ionic interactions were involved in retention of the protein on the unsubstituted dye. In contrast, when the zinc-loaded immobilized dye column was developed successively with buffers A, B, C and D, it was eluted only in D fractions. That lactoperoxidase was eluted from the zinc-loaded immobilized dye column with buffer C′ (Fig. 2, bottom) suggests that lactoperoxidase is retained more by interactions with the metal than by hydrophobic interactions. This shows that ethylene glycol, which breaks hydrophobic interactions, can also interfere with metal–protein interactions.

Ethylene glycol has seldom been used as a mobile phase modifier in standard immobilized metal affinity chromatography (IMAC), where the metal is retained on the chromatographic support by an immobilized chelator such as iminodiacetic acid. (IDA). Porath and Olin [34] nevertheless unambiguously showed that ethylene glycol interferes with the retention of proteins on nickel-loaded IMAC columns, and especially when dissolved in high ionic strength buffer. This effect was tentatively attributed to the fact that "ethylene glycol, being a vicinal diol, is able to form chelates" or to an effect on protein conformation [34]. The water structure-breaking properties of ethylene glycol might affect the ionization state of protein electron-donor groups responsible for retention on IMAC or metallized dye supports and/or deprotonation of the dye hydroxyl groups involved in metal bonding. Relevant to this latter point is the relatively high dissociation constant (575.0 ± 24.5 μM) for the zinc–dye equilibrium measured in buffer D.

3.5. Zinc-loaded Rubine R/K 5BL as a support for preparative immunoglobulin purification from bovine milk

The capacity was 2.7 mg of IgG per ml of gel treated three times with reactive dye. This increased to 7.2 mg/ml for the six times treated gel.

In larger scale experiments, 350 ml of acidic whey (containing 0.28 mg/ml of IgG) without prior gel filtration were loaded on a 45 ml column filled with six times coupled gel and 82% and 15% of loaded IgG were recovered in buffer C- and buffer D-eluted fractions, respectively. No attempt has been made to increase the IgG recovery in buffer C-eluted fractions by manipulating the mobile phase composition defined after small-scale experiments using less substituted gels (see above). The purity of IgG in buffer C-eluted fractions was identical with that observed in small-scale experiments. These results obtained with acidic whey without prior gel filtration prove that whey does not contain low-molecular mass substances which could impair the affinity interaction of immunoglobulins for the immobilized zinc-loaded dye.

4. General comments and conclusion

Dyes are very successful ligands for the purification of proteins, even though in all cases but one [35] no precise picture at the molecular level of the dye–protein interactions is available. In fact, the choice of useful dyes for a given purification procedure is largely empirical, although strategies have been devised to facilitate this choice [2–11]. When one dye is chosen and a purification process using this dye has to be implemented, attempts are often made to obtain a selective desorption procedure to separate the target protein from unwanted proteins which are also retained by the dye [8–12].

Other ways than manipulating mobile phase compositions can be used to ameliorate the performance of a selected immobilized dye.

Molecular modelling studies have shown that altering the structure of the terminal ring of Cibacron Blue F-3GA can change its affinity for liver alcohol dehydrogenase by three orders of magnitude, and that a dye analogue with a spacer between the terminal and triazine rings can separate two alcohol dehydrogenase isoenzymes whereas regular Cibacron Blue F-3GA cannot [36]. Other studies have demonstrated that it can be rewarding to introduce structural motives recognized by target enzymes (e.g., aminophenyl phosphonic acid for alkaline phosphatase) into a dye [37]. Chemical modifications such as reduction or splitting of azo bonds will also alter the affinity of dyes for proteins [38].

This paper has demonstrated that the properties of an immobilized metallized dye can be altered by simple metal exchange, and that, although essentially empirical, this approach can afford interesting selectivity gains. This is a new development in a long story, since the use of metals with dyes can be traced back to the late 1970s. Mg^{2+}, Al^{3+} and first-row transition metals may promote the binding of certain proteins to selected dyes [32,39–48]. This was shown by dye-induced enzyme inactivation [41,43,46] and also by chromatography [39–42,44,47,48]. Metals added to the mobile phase can promote adsorption of proteins to immobilized dyes, and the proteins can be eluted with a mobile phase devoid of metal [39–42] or containing chelating agents [42,45]. Dye–metal ion equilibrium dissociation constants have occasionally been measured and found to be in the millimolar range [32].

Other lines of research have used industrially produced metallized azo dyes for chromatography or phase partitioning experiments [12,49–52]. These dyes use as metal anchoring structures *o,o'*-dihydroxyazo, *o,o'*-carboxyhydroxyazo, *o,o'*-hydroxyaminoazo and *o,o'*-dihydroxyazomethine motives [13]. These dyes are liganded with metals so as to let free one or more metal coordination sites. These free sites may be involved in interactions with proteins. Hence proteins may be bound on these metallized dyes because of cooperation of both the "usual" electrostatic and hydrophobic interactions with the dye backbone and interactions with the immobilized metal. Proof of the interactions of proteins with metal is given by the eluting capacity of substances that coordinate metals, such as imidazole (this work and Ref. [12]). Imidazole also abolished the effect of adding metal-loaded dyed polyethylene glycol in partitioning experiments [52]. One study demonstrated that Cu^{2+} present in a metallized azo dye interacted with the essential histidine at the active site of alcohol dehydrogenase [50]. In short, interaction due to the metal of a metallized dye gives the opportunity to modulate the interaction between dye and protein, in particular, as demonstrated in this work, metal exchange can bring a favourable selectivity gain.

Zinc-loaded Drimarene Rubine R/K 5-BL is clearly suitable for purifying immunoglobulins from bovine milk. The techniques described to prepare immunoglobulins from bovine whey which might be suitable for large-scale operation at reasonable cost include chromatography on T gel [28] and IMAC [53]. Although so far applied to the preparation of IgG from cheese whey, these techniques could probably also be used with acidic whey. The capacities of two different T gel preparations, measured in a similar way as we did but using purified immunoglobulins (and not actual starting material), ranged from 12 to 18 mg of IgG per ml of gel. The described chromatographic procedure allowed the extraction of 0.3 mg of IgG per ml of whey (but the loaded amount of IgG was not given, so the fractional yield cannot be calculated). The purity of the eluted IgG was 74% [28]. As much as 33 mg of IgG per ml of gel were loaded on copper-loaded IDA gel but the column was deliberately overloaded so more strongly retained IgG displaced other proteins present in cheese whey. Elution of retained IgG ensured a recovery of 63% of input IgG with 85% purity [53].

In conclusion, metal exchange on immobilized Drimarene Rubine R/K 5-BL provides a support capable of one-step purification, with a quantitative yield of immunoglobulins (IgG + IgA) from

bovine whey. The capacity is satisfactory but might be increased by a greater dye substitution. This procedure seems amenable to large-scale production.

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