Rhodamine B as Ligand for Affinity Chromatography: Chromatographic Studies on Derivatized Beaded Cellulose

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Abstract

Rhodamine B (RB) post-grafted onto beaded cellulose by a curing method is used as a biomimetic ligand in dye affinity chromatography. The grafted materials obtained are qualitatively characterized by scanning electron microscope and fourier transformed infrared spectroscopy. An amount of 76.79 mmol RB/g dyed cellulose is determined by elemental analysis. The RB affinity interaction with the trypsin, α-chymotrypsin, and BSA (bovine serum albumine) is analyzed using different mobile phase composition. The results show a selective separation of a mixture of BSA and trypsin into two single peaks by step elution with 1.75 M, 0.5 M, and 0 M ammonium sulphate in the eluent buffer. A good reproducibility of the retention time is obtained for these proteins in the mixture with typical values of 8.0 ± 0.2 min for BSA and 20.0 ± 0.2 min for trypsin, showing a possible application in the purification of samples with different composition.

Introduction

Affinity chromatography (AC) is the most specific chromatographic technique and one of the most powerful technologies for the purification of biomolecules. In general, affinity techniques focus on biomolecular recognition between biological macromolecules and complementary ligands, such as interactions between an antigen and its antibody or between an enzyme and its substrate (1–7). This biological recognition is known to involve a combination of specific types of intermolecular binding forces, namely electrostatic, hydrogen bonding, hydrophobic, and van der Waals interactions (2). Some of the advantages of techniques based on affinity interactions include the capacity to eliminate multi-purification steps, increase yields and thereby improve the economic of the process, although the stability and cost of the ligands are disadvantageous (5,8).

In general, ligands for protein AC are distinguished between two categories: synthetic and biological. The main advantage of biological ligands is their high selectivity and affinity. However, due to their biological origin, chemical nature, and production methods, these ligands tend to be expensive and unstable to the sterilization and cleaning conditions normally used in the manufacturing process of biologics, thus shortening the lifetime of the respective affinity adsorbents (4). Additional features include a high risk of contaminating the end-product with potentially dangerous leaches and a low protein-binding capacity. Synthetic ligands appear to tackle most of the problems discussed earlier in an effective manner with the exception of some lower selectivity/affinity in relation to their biologic counterparts. Indeed, they are easily coupled to a greater variety of macromolecular matrices, are resistant to biological degradation, and display the capacity to be complementary of the majority of biological macromolecules (4,9–16).

Nevertheless, Rhodamine B (RB) has been used in a large variety of applications, but the use of this cationic dye as a ligand in AC has never been investigated until now. Due to a combination of specific types of the previously mentioned interactions as spread out over the different moieties of the structure of the dye, a desirable selectivity between RB and the biomolecules to be purified can be expected.

Regarding the cationic nature of this dye, it is expected that it can be potentially used as a biomimetic ligand for the trypsin-like family of enzymes, which are known to show an uncommon binding preference for cationic substrates. These enzymes have similar catalytic mechanisms and form one of the largest groups of enzymes involved in digestion (trypsin), blood clotting (thrombin), and fibrinolysis (urokinase). The immobilized form of biomimetic cationic dyes binds trypsin, thrombin, carboxypeptidase B, and urokinase but neither the serine protease chymotrypsin, which prefers substrates with hydrophobic amino acids for its primary binding pocket, nor the unrelated albumin (17,18). One of the first studies examining the use of the cationic cyanine dyes immobilized onto agarose performed by our group showed a different chromatographic behavior between trypsin in relation to chymotrypsin and bovine serum albumin (BSA). It was also observed that dye-protein interaction was strongly dependent on the mobile phase composition (19).

A general consensus for an ideal matrix is that it should exhibit the following requirements: high specificity (implying a surface charge content approaching zero); absence of hydrophobic binding sites; good chemical stability; good mechanical rigidity;

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high binding capacity; good recoverability; high reproducibility and low cost (20–23). A variety of supports including agarose, cellulose, polyacrylamide, polyacrylate, and silica have been extensively used as matrices (20,23,24).

During the early days of chromatography, microcrystalline cellulose was used in powder form, even though its application was limited by poor flow characteristics and high pressure dropping during column operation. Since then, several techniques have been developed to replace the powder form by a more adequate particle shape, especially the spherical bead one. Beaded cellulose produced by solidification of cellulose xanthate in inert solvent and subsequently regenerated presents an excellent mechanical stability, even as a gel with large pores. Other important features and properties regarding its use in AC are its high hydrophilicity, narrow particle size distribution, high chemical resistance and compatibility with most commonly used solvents and buffers, and high temperature stability. Because of its rigid spherical particles, beaded cellulose has a high flow throughput of the gel even at low pressure, almost quintupling the flow rate in relation to agarose gel or cellulose not regenerated above 1.20 bar of pressure (25).

The carboxylic group present in RB structure makes this dye potentially reactive and, therefore, capable of binding to hydroxylic macromolecules like cellulose or agarose. In an earlier work, a new curing method to bind RB onto microcrystalline cellulose was developed by simple esterification, namely by heating a mixture of both using different temperatures of more than 180°C, eventually in the presence of catalytic amounts of zinc chloride or sodium hypophosphite (26).

Therefore, this curing method is herein used to derivatize beaded cellulose with RB, keeping in mind the promising capacities of RB as a ligand in AC, together with the good properties of beaded cellulose as a chromatographic matrix mentioned earlier. The grafted materials obtained were quantitatively and qualitatively characterized using scanning electron microscope (SEM), elemental analysis (EA), and fourier transformed infrared (FT-IR) spectroscopy. The affinity chromatographic behavior of three standard proteins (trypsin, BSA, and α-chymotrypsin) using chromatography columns with RB immobilized in beaded cellulose was studied using buffers of different composition.

Experimental

Reagents

Beaded cellulose Perloza ST (50–75 µm) was acquired from Iontosorb (Ústi nad Labern, Czech Republic) and was treated in vacuum at 100°C for 16 h in the presence of phosphorus pentoxide prior to use. RB and zinc chloride were purchased from Aldrich (St. Louis, MO) and used as received. Trypsin, α-chymotrypsin, and BSA were purchased from Sigma Aldrich. All other chemicals used were analytical-grade.

Apparatus

Infrared spectra (IR) were measured on a Mattson 5000-FTS FT-IR spectrometer (Thermo Scientific, Waltham, MA). All samples were prepared by mixing FT-IR-grade KBr (Aldrich Chemicals) with 1.0 wt.% of the dye or cellulose derivative and grinding to a fine powder. Spectra were recorded at 4 cm^{-1} (128 scans) over the 400–4000 cm^{-1} range without baseline corrections. Bands are given in cm^{-1}. The microanalyses were performed in triplicate using a Carlo-Erba CHNS-O AE-1108 Elemental Analyser (Thermo Scientific). SEM images were acquired in a Hitachi S-2700 with a UHV Dewar detector (Rontec EDX) (Tokyo, Japan). Beaded cellulose and derivatized beaded cellulose samples were magnified 100, 250, 500, and 5000x.

Procedures

Dye AC support

Beaded cellulose derivatization was carried out using a Büchi glass oven B-580 (Flawil, Switzerland) as a reaction vessel in the presence of phosphorus pentoxide inserted into the drying chamber. The curing method used was based on the cellulose microcrystalline derivatization already described (26) using beaded cellulose (2.0 g, 36.78 eq. OH), RB (1.0 g, 2.09 mmol), and zinc chloride (5 wt.%, 0.1 g) at a final temperature of 240ºC. The dyed cellulose obtained was sequentially washed-off with several portions of dimethylformamide (DMF), water, and ethanol and was continuously submitted to Soxhlet extraction process with DMF followed by ethanol until no bleeding was observed. The samples obtained were then well-dried at 50°C under vacuum in the presence of phosphorus pentoxide. Cellulose samples obtained were qualitatively and quantitatively characterized using SEM, EA, and FT-IR.

Chromatographic method and conditions

The chromatographic experiments were carried out at room temperature in a standard chromatographic system (Amersham Biosciences, Uppsala, Sweden). The beaded cellulose dye gel (9 mL) was packed in a column (4.5 × 1.6 cm i.d.) with deionized MilliQ water (Millipore, Billerica, MA). Prior to the sample application, the column was equilibrated with the desired mobile phase (10 mM Tris-HCl buffer, pH 8, with varying ammonium sulphate concentration) at a flow rate of approximately 0.25 mL/min. After the injection of a standard protein sample (150 µL of 10 mg/mL of trypsin, α-chymotrypsin, or BSA), the unbound protein was eluted with the same buffer. Desorption of the bound protein was carried out with 10 mM Tris-HCl buffer (pH 8). The elution profile was obtained by continuous measurement of the absorbance at 280 nm.

Results and Discussion

Succeeding our previous work where a curing method to derivatize microcrystalline cellulose with RB was developed (26), beaded cellulose was derivatized with the same dye envisioning its use in dye AC. The RB lactone formed during the process was assumed to be the principal responsible for the esterification of the dye onto cellulose (Figure 1).

The resulting dyed beaded cellulose was sequentially washed-off with DMF, water, and ethanol and was submitted to Soxhlet extraction with the latter solvent to ensure the removal of the unfixed dye. It was then well-dried. The resulting dyed beaded

cellulose was then characterized by SEM, EA, and FT-IR spectroscopy in order to confirm that a dye-cellulose covalent bond was present and to quantify the amount of dye bound onto cellulose.

The SEM with 100×, 250×, 500×, and 5000× magnification, performed on beaded cellulose samples before and after the dyeing process, did not present any significant differences between them (Figure 2). This observation showed the preservation of the original beaded cellulose morphology and, therefore, its mechanical properties after the dyeing process, which is crucial to its application in dye AC.

In addition to the IR spectra of cellulose (28), RB derivative herein obtained reveal the most intense RB C=C deformation band at 1596 cm^{-1} together with the expected C=O ester band at 1711 cm^{-1}. These bands are absent in both control samples spectra and match the cellulose one. The values found for the ester band are in agreement with several RB ester bands at 1707–1728 cm^{-1} (26,28).

The amount of ligand (*Q*, mmol dye/g dyed cellulose) was determined in the well-dried derivative beaded cellulose sample by EA as previously described (26). The amount of nitrogen was also determined in a sample of beaded cellulose prior to use (control sample 1) and in a sample of beaded cellulose submitted to the same curing method in the absence of dye (control sample 2) (Table I). The 76.79 mmol dye/g dyed cellulose thus obtained corresponds to a fixation yield of 38% and to a remarkable 380 mg of immobilized dye per gram of cellulose. Significantly, this value is 2.3 higher than those obtained under the same conditions with microcrystalline cellulose at a temperature of 250ºC. This difference can be explained by the increase of accessibility onto hydroxylic groups of the amorphous beaded cellulose in relation to the microcrystalline one.

It was not possible to determine *Q* by visible spectrophotometry akin to our previous work (26) because beaded cellulose is shown to be much more insoluble than the corresponding microcrystalline one, neither by FT-IR, because the obtained spectrum does not show a strong enough C=C band to be used in a reliable way.

The chromatographic experiments were performed in order to investigate the RB ability to separate and purify proteins as a dye AC ligand when immobilized onto beaded cellulose. Considering all intermolecular binding forces between immobilized RB dye and proteins previously referred, the retention profile of three model proteins (trypsin, α-chymotrypsin, and BSA) was studied as function of ionic strength and pH of the eluent. Both parameters could significantly affect the retention and adsorption mechanism of the proteins in AC. The structure and functions of the studied proteins are very well-known, and data is available so they can be used as suitable models. Trypsin and α-chymotrypsin are serine proteases from pancreas that belong to enzyme systems widely studied from a structural point of view and physical-chemical properties. Trypsin (MW 23 kDa, pI = 10.1–10.5) predominantly cleaves proteins at the carboxyl side of the amino acids lysine and arginine. On the other hand, α-chymotrypsin (MW 25 kDa, pI = 8.7) selectively catalyzes the hydrolysis of peptide bonds on the carboxyl side of tyrosine, tryptophan, and phenylalanine. BSA (MW 66 kDa, pI = 4.7) is the most abundant protein in blood plasma and presents important physiological functions, namely as a transporter. Due to its singular structure, BSA binds free fatty acids and other hydrophobic ligands like bilirubin, varfarin, and steroids (29).

The affinity interactions of the previous standard proteins with the RB derivatized support were evaluated using eluent buffers with varying pH. The retention was not strongly affected in the range of pH 7–9 (results not shown), thus all experiments were carried out at pH 8.

Table I. Percentage of Nitrogen Determined by EA and Amount of RB Bounded onto Beaded Cellulose*

Sample	% N†	Q × 10² (mmol dye/g dyed beaded cellulose)
Control 1 (beaded cellulose)	< 0.01	< 0.36
Control 2 (cured beaded cellulose)	0.033	1.18
Dye beaded cellulose	2.150	76.79

* Q = mmol dye/g dyed beaded cellulose.
† Standard deviation between 0.005 (control 1) to 0.061 (dye beaded cellulose) obtained in sets of three EA determinations.

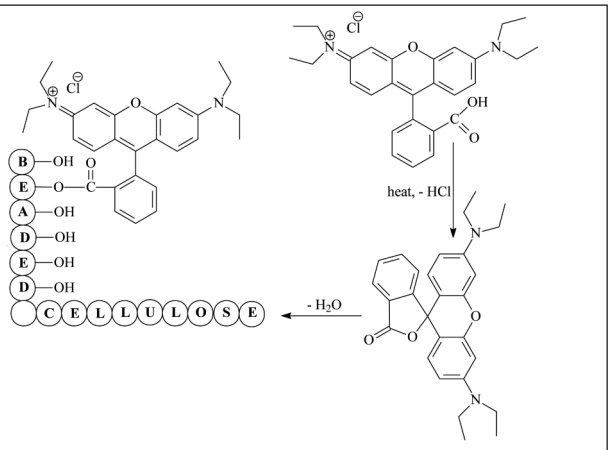

Figure 1. Beaded cellulose derivatization process with RB.

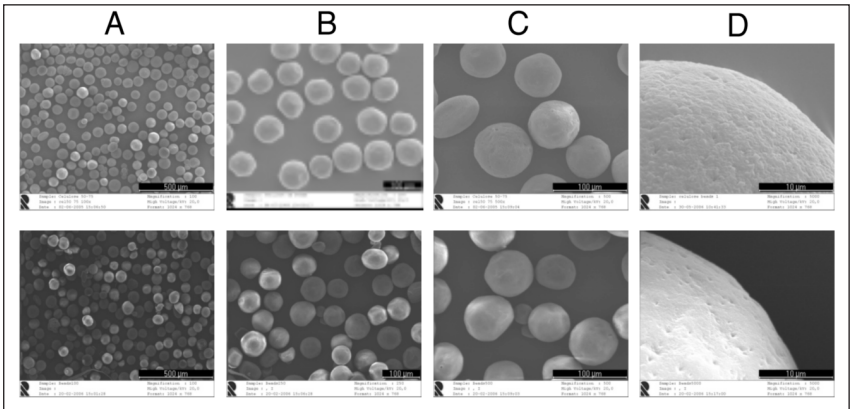

Figure 2. Representative SEM micrographs of beaded cellulose at 100× (A), 250× (B), 500× (C), and 5000× (D) magnification. Top row: prior to use and bottom row: dyed with RB.

The effect of the ionic strength on chromatographic profile of the three model proteins was evaluated using 10 mM Tris-HCl buffer (pH 8) with varying ammonium sulphate concentrations (0.5–1.75 M). The elution was performed using 10 mM Tris-HCl buffer (pH 8) without salt. The results showed that the presence of salt is important for the proteins to bind onto the derivatized column with RB because an increase in concentration of salt enhances the retention of all three studied proteins (Figure 3).

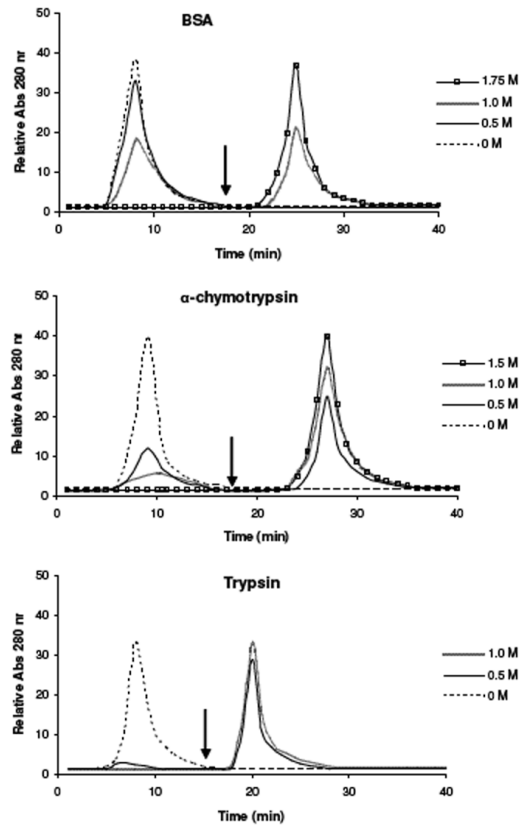

Figure 3. Affinity chromatography on beaded cellulose derivatized with Rodamine B. Sample: (A) BSA, (B) α-chymotrypsin, and (C) trypsin. Buffer: 10 mM Tris-HCl (pH 8) containing 0 M, 0.5 M, 1.0 M, 1.5 M, or 1.75 M ammonium sulphate. Elution (→): 10 mM Tris-HCl buffer (pH 8).

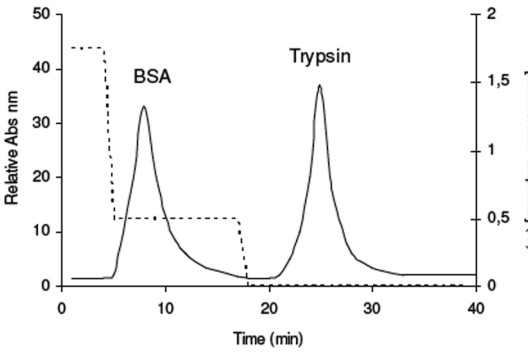

Figure 4. Fractionation of BSA and trypsin on beaded cellulose derivatized with Rodamine B. Initial buffer: 10 mM *tris*-HCl (pH 8) containing 1.75 M ammonium sulphate. Step elution (- - -) was performed by decreasing salt concentration from 1.75 to 0.5 M and 0 M.

These results suggest that, besides other specific intermolecular binding forces, hydrophobic interactions play an important role in protein retention on the dye-ligand of the stationary phase. In fact, no proteins were bound to the gel when using 10 mM Tris-HCl buffer (pH 8 without salt) as an eluent; however, on increasing the salt concentration, a selective retention of each protein in the RB derivatized gel was obtained. The increase of ammonium sulphate concentration in the mobile phase also enhances the amount of bound protein. The binding affinity of the chromatographic support decreases in this order: trypsin > α-chymotrypsin > BSA as evidenced by the elution profile of each model protein (Figure 3). Thus, for 1.0 M ammonium sulphate in eluent buffer, only trypsin was totally retained on the column (Figure 3C). On the other hand, the conditions for total binding of α-chymotrypsin and BSA were 1.5 M and 1.75 M, respectively (Figure 3A–3B). Desorption was then performed by lowering the ionic strength and differences in chromatographic behavior were also observed in conditions of total desorption being 0 M for trypsin and α-chymotrypsin and 0.5 M for BSA (Figure 3).

The ability of RB adsorbent to fractionate a mixture of proteins was confirmed using BSA and trypsin. These proteins were successfully separated into two single peaks (Figure 4) by step elution using different ammonium sulphate concentration in the eluent buffer. At 1.75 M, both proteins were retained on the dye support, but when the ionic strength was decreased to 0.5M, BSA was eluted in the first peak. Trypsin was totally unbound using eluent without salt.

The obtained retention times for the proteins in the mixture were 8.0 ± 0.2 min for BSA and 20.0 ± 0.2 min for trypsin. The good reproducibility showed by a typical standard deviation of ± 0.2 min, calculated for five chromatographic runs, indicate a possible application in purification of specific proteins from different crude extracts.

The stability of the column was demonstrated after one year of consecutive runs (approximately one hundred) with no changes in chromatographic performance.

Control experiments using cellulose beads submitted to the same dyeing process but in absence of RB did not result in any retention of proteins on the column, even when using a very effective salt (ammonium sulphate) in the eluent buffer in the promotion of hydrophobic interactions (30). Accordingly, the responsible for the selective retention of the model proteins is the dye RB immobilized on beaded cellulose, and therefore, there are different intermolecular forces involved in the pseudo-affinity process in addition to hydrophobic interactions. Depending on the specific conditions of the eluent buffer, RB displayed differential pseudo-affinity for BSA, α-chymotrypsin, and trypsin. These results suggest that the application of RB as ligand and the use of different eluting conditions can enable the resolution of mixtures of proteins because it was observed a selective retention of the studied model proteins.

A recent study regarding the use of dicarboxydecylthiacarbocyanines as ligands in dye AC showed a different retention profile for three model proteins (α-chymotrypsin, BSA, and lysozyme) using 10 mM Tris-HCl (pH 8) as buffer (31). In these conditions, α-chymotrypsin and lysozyme were totally retained, but BSA did not present any affinity for the dye support. However, α-chy-

motrypsin and lysozyme were totally unbound from the support when the ionic strength was increased by the use of 0.2 M and 1.0 M NaCl buffer solutions, respectively. These results showed that the selective interaction between dye ligands and the studied proteins depends not only on the ionic strength of eluent buffer but mainly on the characteristics of the immobilized dye because different cationic dyes exhibit specific and different molecular interactions in relation to the same proteins.

Conclusion

Beaded cellulose derivatized with RB was prepared and used as dye AC stationary phase. A recently developed curing method was used affording a ligand density of 76.79 mmol dye/g dyed cellulose determined by EA. Furthermore, whereas FT-IR pointed to the establishment of an ester bond between RB and beaded cellulose, SEM analysis showed the preservation of the original beaded cellulose morphology and therefore its mechanical properties after the dyeing process, which is crucial to its application in dye AC. This new dye AC stationary phase allowed the separation of model proteins showing a selective interaction depending not only on ionic strength of eluent buffer but mainly on the characteristics of the immobilized dye. Because the purification of proteins and enzymes from natural and recombinant sources is often a difficult task, especially when the target protein or enzyme is present at low concentration and/or with similar substrates specificity present in the starting material, this study is a contribution to the separation challenge of related biomacromolecules by the use of a mimic synthetic ligand immobilized into a natural macromolecule.

Acknowledgements

The authors are grateful to "Fundação para a Ciência e Tecnologia", Portugal, POCTI and FEDER, for their financial support (Project "Development of New Supports for Dye-Affinity Chromatography" POCTI/QUI/44776/2002) and for granting Andreia Silva an investigation fellowship. Finally, we also thanks to Centro de Óptica of Universidade da Beira Interior for the image acquisition from the Scanning Electron Microscope.

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Manuscript received November 15, 2007;
revision received October 1, 2008.