Cellulosic materials functionalized with reactive dyes and metallic ions for biotechnologal separations

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Abstract

Affinity chromatography is a valuable method for the isolation of a specific biomolecule from complex mixtures in biotechnological processes. Affinity chromatography is a separation technique based on the use of specific and selective immobilized ligands able to associate reversibly to a desired biomolecule. New celluloses materials functionalized with reactive dyes and metallic ions are of interest in affinity separation of enzymes. This paper presents a sorption study regarding the testing the retained capacity of the microcrystalline aminoethyl (AE) and guanidoethyl (GE) celluloses modified with reactive dye Brilliant Red HE – 3B and Cu^{2+} ions, for the lysozyme molecules from aqueous solutions.

Keywords: affinity chromatography, functionalized sorbents, reactive dyes and metallic ions as biomimetic ligands, enzymes separations

Introduction

Affinity chromatography separates proteins based on a reversible interaction between a group of proteins and a specific ligand coupled to a matrix. A protein with a high affinity for a ligand can selectively bind to an affinity matrix on which the ligand is immobilized. After washing away the unbound proteins, the protein can be eluted and purified under conditions that disrupt the interaction between the protein and its ligand. Affinity chromatography is the only technique that enables the purification of a biomolecule based on its biological function or individual chemical structure. The high selectivity of affinity chromatography enables many separations to be achieved in one simple step, including common operations such as the purification of monoclonal antibodies or fusion proteins.

The major limitation of affinity chromatography, i.e. the limited number of natural ligands available, has been overcome by the introduction on of combinatorial chemistry approaches for the generation and screening of huge collections of chemical compounds able to recognize a desired biomolecule [1].

Affinity chromatography is a variant of liquid chromatography with the highest specificity for proteins. The intermolecular interactions (electrostatic, hydrophobic, van der Waals, hydrogen bonds) together with the biological ones, between a ligand and a target-protein, assure a high selectivity of separation using *affinity chromatography* for the proteins from a mixture [1-5].

Sorbents based on cellulose functionalized with reactive dyes are a type of materials of high selectivity in separation of biological compounds. The use of sorbents based on cellulose functionalized with dyes in biological systems study is due to the improvement of these untreated materials affinity toward proteic substances and the stability of the cellulose – dye [2]. In the same time, the low cost of the dyes, their ease of immobilization and stability to biological and chemical degradation, and the high protein – binding capacity of the corresponding adsorbents, has led to affinity chromatography materials that are much less expensive and more stable than those based on bioligands. This type of ligand mimics the structure and binding of natural biological ligands of the targeted protein. [5,6].

Considering as basic criteria the selectivity recently was imposed into the biomolecule separation and purification *the affinity chromatography with immobilized metal* [7-9]. This chromatography technique introduces a new mechanism into protein separation and complexation with ions of transitional metals (Cu^{2+}, Zn^{2+}, Ni^{2+}, Co^{2+}, Fe^{3+}) fixed on a support by a chelate ligand [10,11]. The biopolymers bonding depend on the structure of biochemical compound and the ion nature, being done with the help of imidazole, amino and thiol groups (from histidine, lysine and cysteine) by the Cu^{2+} and Ni^{2+} ions, phosphate groups presented into Fe(III) nucleic acids and the vicinal dihydric alcohols presented into sugars with cooper ions [11].

This paper presents a study regarding the testing the retained capacity of the microcrystalline aminoethyl (AE) and guanidoethyl (GE) celluloses modified with reactive dye Brilliant Red HE – 3B and Cu^{2+} ions, for the lysozyme molecules from aqueous solutions.

Materials and Method

A. Materials

Dye. The reactive dye bifunctional monochlortriazine Brilliant Red HE-3B from BEZEMA (Figure 1, MW =1463, adsorption maximum, λ_{max} = 530 nm, $\varepsilon = 38769.5\,L/mol\cdot cm$) was used as commercial salt. Working solution 0.1g/L were prepared by appropriate dilution with bidistilled water of the stock solution (5 g/L).

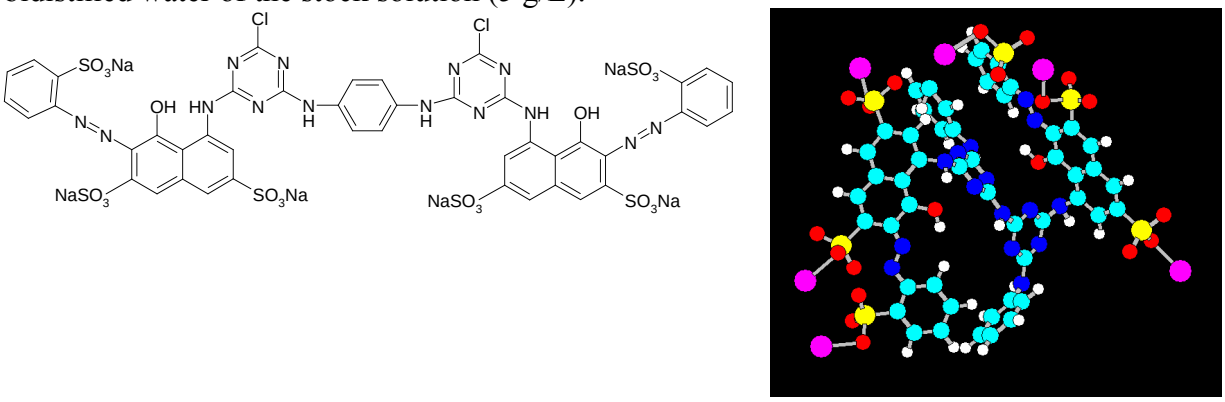

Figure1. *Bis* (monochloro-*s*-triazine) reactive dye (Brilliant Red HE-3B, C.I. Reactive Red 120)

Celluloses. Guanidoethyl (GE), and aminoethyl (AE) cellulose ion exchange (Table 1) modified by sorption of the reactive dyes Brilliant Red HE-3B have been used.

Table 1. The characteristics of selected ion exchange celluloses

Type of cellulos (Serva)	Functional group	Exchange capacity (mequiv /g)	pH for maximum exchange capacity
AE	- $OC_2H_4NH_2$	0.8 ±0.1	8.0
GE	- OC_2H_4NH-$C(NH_2)$=NI	0.4 ±0.1	7.5

Metallic ion. An aqueous stock solution of Cu (II) ions was prepared using $CuSO_4$ x $5H_2O$ salt. Working solutions, with 1200 mg Cu^{2+}/L concentration, were prepared from the stock solution 0.1M, by dilution with the double distilled water.

Proteic substance. The tested protein, the Lysozyme (I.U.B.: 3.2.1.17, muramidase), was prepared as a stock aqueous solution of 200 μg/mL concentration. The stock solution was diluted so that the concentration of the working solutions ranged from 1 μg/mL to 40 μg /mL.

B. Methods

Reactive dye attachment to microcrystalline cellulose. The sorbents based on celluloses modified with reactive dye Brilliant Red HE-3B were obtained by batch method, when samples of about 5 g of celluloses were equilibrated with 200 mL dye solution containing 0.1g/L at room temperature. After 24h, with intermittent stirring, the phases were separated by filtration. The filtrates containing residual concentrations of dye were spectrophotometrically analyzed at 530 nm with a DRELL DR 2000 Spectrophotometer (HACH Company). The amounts of dye sorbed on celluloses were calculated as the difference between the initial and final concentrations of the solutions and by amount of adsorbed dye: $q = \frac{C_0 - C}{G} \cdot V \cdot 10^{-3}$ (mg of dye/g cellulose), where: C_0 and C are initial and equilibrium concentration of dye in aqueous system (mg/L), G is amount of cellulose (g), and V is volume of aqueous system (L). [12,13]. After the dye attachments, the celluloses were washed several times with distilled water until all the physically adsorbed dye molecules were removed.

Incorporation of Cu (II) ions. Amounts of celluloses modified with reactive dye Brilliant Red HE-3B were contacted with aqueous solutions containing 1200 mg Cu^{2+}/ L, with respect the ratio: sorbent : solution of 1:50. The system was maintained at room temperature, with intermittent stirring and without pH adjustment. The concentration of Cu (II) ions in the filtrated solution was determined using a Perkin Elmer 3300 atomic absorption spectrophotometer. The amount of Cu^{2+} ions retained was expressed by removal degree: $R\% = \frac{C_0 - C}{C_0} \cdot 100$ where C_0 and C are initial and equilibrium concentration of Cu (II) in aqueous system (mg/L).

Lysozyme sorption procedure. The experimental studies of lysozyme retention onto the modified cellulose materials were carried out in batch condition, when samples about 0.05 and 0.09 g sorbent were equilibrated with 25mL protein solution containing variable amount of active substances (1-40 μg/mL). The experiments were carried out for 1hour at room temperature, at a magnetic stirring and at pH of 6-7. After 1 hour, the phases were separated by filtration, and the filtrates were analyses using the Lowry method of water soluble proteins determination. The optical density of filtered solutions were determined at the wavelength

corresponding to the adsorption maximum amine copper (II) complexes, λ= 500nm, with respect to Lambert – Beer law, using an UV –Vis Spectrophotometer of Jenway 6105 type.

Results and Discussion

A. Celluloses powders functionalization with dye and characterization

In our previous papers [12,13] concerning reactive dye Brilliant Red HE-3B retention on celluloses ion exchange, we establish that these materials have a good retention capacity. The strong binding of the dye to celluloses may have resulted from combination of different mechanism such as ion exchange, covalent bonding, electrostatic interactions, and hydrogen bodings.

B. Incorporating of Cu (II) ions

The retention of metallic ions on celluloses sorbents modified with dyes is possible if the dye containing functional groups able to forming complexes with transitional metallic cations. The azo dye presenting hydroxyl, carboxyl, amino groups in the o-o` positions towards the azo chromophore are useful [2]. The Cu is transition metals which can coordinate up to six electron donors. The metal ions can be immobilized onto celluloses materials by dye Brilliant Red HE-3B – chelating ligands, occurs especially through oxygen and nitrogen atoms and by the cellulose molecules. The amounts of Cu^{2+} ions retained onto celluloses are presented in table 2.

Table 2. The amounts of Cu (II) ions retained onto celluloses functionalized with reactive dye

Type of cellulose	Initial amount μg Cu^{2+}	Retained amount μg Cu^{2+}	R% Cu^{2+}
AE	14605	9167.5	62.77
GE	184150	178025	96.67

The values in Table 2 show that the amount of Cu (II) ions retained depends on type of cellulosic materials and amounts of dye immobilized onto the cellulosic sorbent.

C. Lysozyme sorption equilibrium

The unretained amount of protein after sorption process was calculated on basis of absorbance values from calibration curve for proteins in aqueous solution determination. The sorption capacity of celluloses materials was evaluated by amount of sorbed lysozyme $q = \frac{C_0 - C}{G} \cdot V \cdot 10^{-3}$ (mg of lysozyme/g of sorbent) where: C_0 and C are the initial ones and the equilibrium concentration of lysozyme in the solution (mg/L), G is the amount of sorbent (g) and *V* is the volume of the solution (mL). The dependence between the amount of protein retention and the initial lysozyme concentration is showed in figure 2.

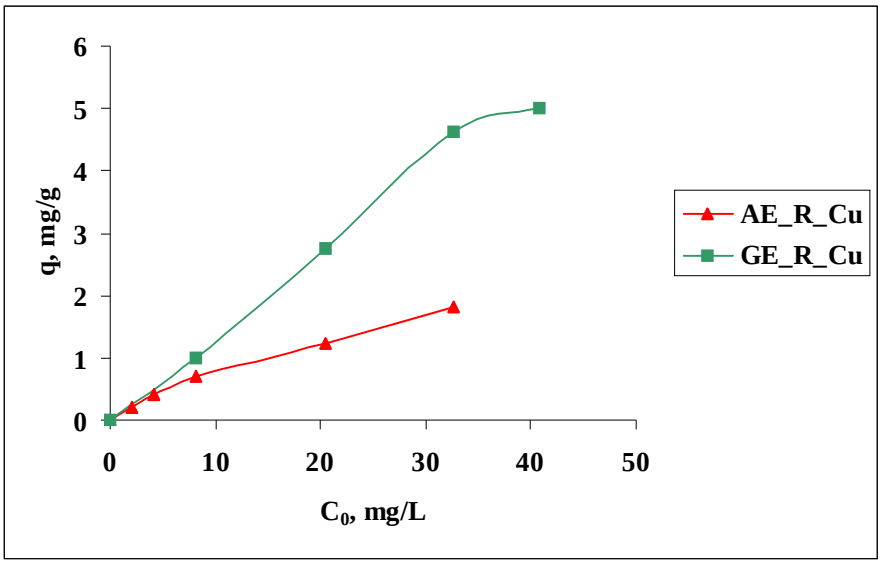

Figure 2. The influence of the initial lysozyme concentration on the sorption process

Isotherms - the equilibrium relation between the concentration of the sorbate on the solid phase and in the liquid phase- of the lysozyme on celluloses modified with reactive dye Brilliant red HE-3B and Cu (II) ions at room temperature of solutions are represented in Figure 3.

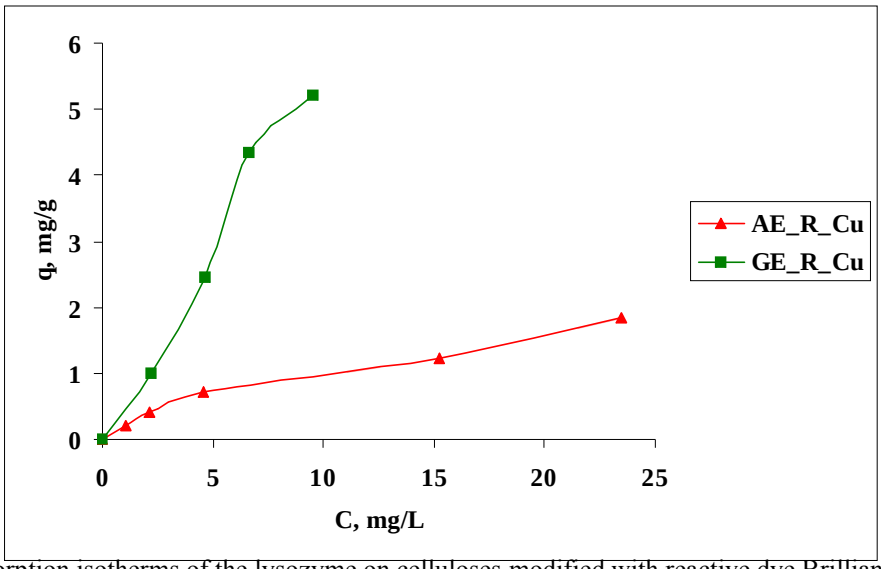

Figure 3. The sorption isotherms of the lysozyme on celluloses modified with reactive dye Brilliant Red HE-3B and Cu^{2+}ions (▲AE_R_Cu, ■GE_R_Cu)

The experimental equilibrium sorption data were analyzed by using three adsorption isotherm models: the linear [14], the Freundlich [15] and the Langmuir [16] expressed by following equations:

Linear isotherm: $q = K \cdot C$ (1)

Freundlich isotherm: $q = K_F \cdot C^{1/n}$ (2)

Langmuir isotherm: $q = \frac{K_L \cdot C \cdot q_0}{1 + K_L \cdot C}$ (3)

where the K_F parameter is related to the adsorption capacity and n is a measure of sorption energy; a favorable sorption correspond to a value of $1 < n < 10$. For $n = 1$, $K_F = K$ from Eq. (1), i.e. linear isotherm. The Langmuir constant, K_L is related to the intensity of the sorption process and q_0 is the maximum value of sorption capacity (corresponding to the complete monolayer coverage).

The Freundlich and Langmuir sorption parameters were determined by converting the corresponding equations in the linear forms as follows:

$$\log q = \log K_F + \frac{1}{n} \log C \tag{4}$$

$$\frac{1}{q} = \frac{1}{q_0} + \frac{1}{K_L \cdot q_0} \cdot \frac{1}{C} \tag{5}$$

The constants of the sorption isotherms, calculated from the intercepts and slopes of the corresponding linear plots (Figures 4 and 5) for reactive lysozyme sorption at room temperatures, together with their correlation coefficients (R^2), are presented in Table 3.

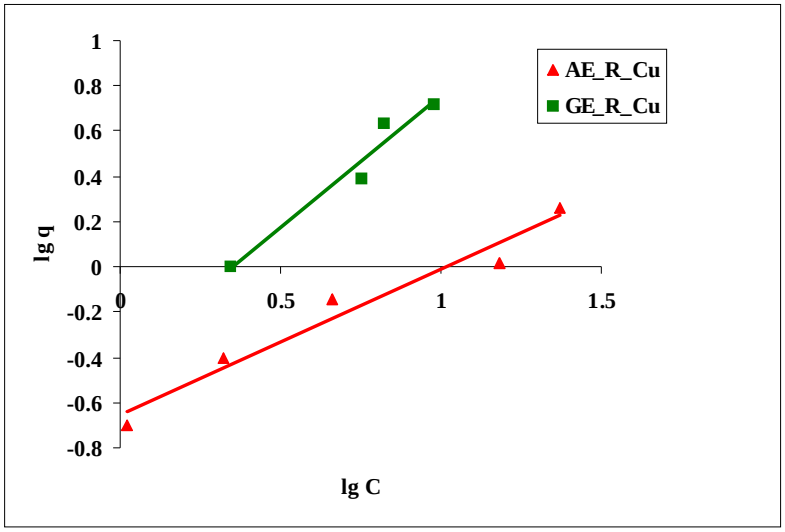

Figure 4. Freundlich plots for the sorption of the lysozyme on tested sorbent at 293.15 K

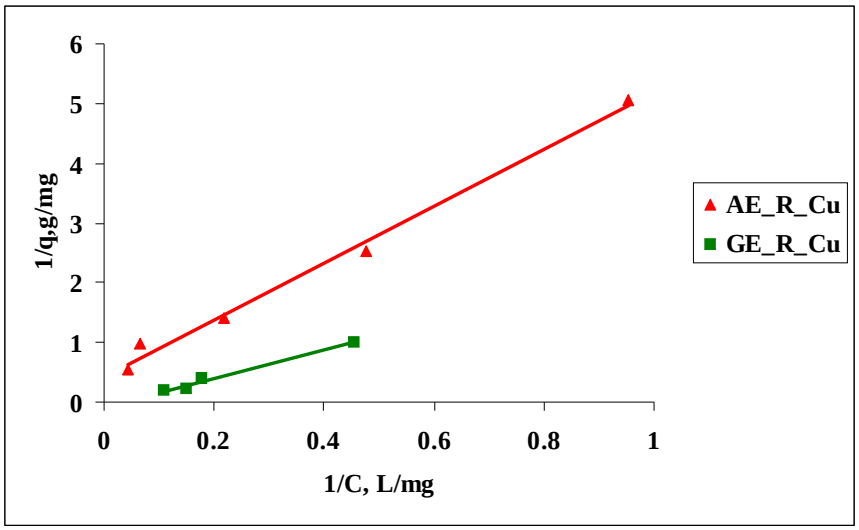

Figure 5. Langmuir plots for the sorption of the lysozyme on tested sorbent at 293.15 K

Table 3. The parameters of sorption process of lysozyme onto cellulosic materials modified with reactive dye and Cu^{2+} ions at room temperature

Type of cellulose	Freundlich isotherm			Langmuir isotherm			Linear isotherm	
	K_F $(mg/g)(L/mg)^{1/n}$	n	R^2	q_0 (μg/g)	K_L (L/g)	R^2	K (L/g)	R^2
AE	0.2219	1.55	0.9597	2.33	0.09024	**0.992**	0.0714	0.965
GE	0.3895	0.86	0.9564	14.33	0.02932	**0.985**	0.5834	0.978

The values in Table 3 showed that the experimental data were more suitable to the Langmuir model than to the Freundlich or linear models.
The small values of Langmuir constant K_L may suggest a weaker binding between lysozyme and the cellulose surface. As seen from Table 2 the values q_0 of maximum sorption capacity, corresponding to monolayer coverage of the binding sites available in the sorbent, was obtained in these conditions for GE cellulose functionalized that was in accordance with other our study [17].

D.Thermodynamic study

Using the values of binding Langmuir constant, K_L (L/mol), and following equation [16] we can calculate the variation of apparent free energy (ΔG, kJ/mol) of lysozyme sorption on different types of cellulosic sorbents (Table 4).

$$\Delta G = -RT \ln K_L \quad (5)$$

where R is the gas law constant and T is the absolute temperature (K)

Table 4. The apparent free energy of sorption process of lysozyme onto cellulosic materials modified with reactive dye and Cu^{2+} ions

Type of cellulose	T (K)	K_L L/mol	ΔG (kJ/mol)
AE	293	1298.37	-17.472
GE		421.86	-14.732

The negative values of ΔG showed in Table 4 confirm that the lysozyme sorption on modified cellulose materials is a spontaneous process.

E. IR study

Due to the small value of the sorbit concentration, in the IR spectrum of functionalized cellulose with reactive dye [13], Cu^{2+} ions and enzyme [13], the registered modifications are moved to higher wave numbers.

Conclusions

As new stationary phases in affinity chromatography of biomolecules, the properties of some celluloses materials namely AE - cellulose, GE – cellulose, modified with reactive dye Brilliant Red HE-3B and Cu^{2+} ions were tested. To establish the most suitable type of materials to be used as sorbent for the selected protein, the sorption isotherms were plotted. It was found that the celluloses modified with dye and metallic ions presents relative good values from the quantitative parameters of sorption.

The apparent thermodynamic parameters of sorption suggest an entropy-driven endothermic sorption process, most likely of physical nature.

The results of this study evidenced the capacity of cellulose ion exchange modified with dye and Cu^{2+} ions for lysozyme uptake.

The system under study offers some new attractive possibilities of selective retention of lysozyme from aqueous effluents.

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