CELLULOSIC MATERIALS FOR BIOTECHNOLOGICAL SEPARATIONS♦

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Abstract: New cellulosic materials functionalized with reactive dyes and metallic ions are of interest in affinity separation of enzymes. The purpose of this work is to present a review of our research activities, concerning the opportunities of cellulosic sorbents modified with reactive dyes and/or metallic ions to use in retention of biochemical compounds. We study the retention of lysozyme onto GE and AE microcrystalline ion exchange celluloses unmodified and functionalized with dye Reactive Red 120 and/or Cu^{2+} ions. The retention capacities of tested materials were evaluated by value of sorption capacity calculated using Langmuir

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parameters. The results of this study evidenced that celluloses materials tested proved to be an effective sorbent in retaining of proteic compounds traces from aqueous media. In the same time it was established the higher capacity of cellulose ion exchange for lysozyme uptake than that of the same cellulose modified with dye and Cu^{2+} ions.

Keywords: *affinity separation methods, sorption, cellulosic materials, lysozyme, reactive dyes - biomimetic ligands, metallic ions*

INTRODUCTION

During biotechnological processes there are generated a complex mixture of by-products and waste that contain several biocompounds: proteins, enzymes, peptides, nucleic acids or hormones. Among the techniques applied for isolation and purification of these mixtures are the chromatographic ones.

Highly selective affinity-based separations have greatly evolved over the past two decades to improve characteristics related to target specificity, dynamic adsorptive capacity, and chemical robustness of the affinity matrix. These separation matrices are used as screening devices for molecular interactions as well as for the purification of complex mixtures at the analytical, preparative, and large scale. Affinity adsorptive phases can be synthesized by the attachment of structures, called ligands, to immiscible macromolecular fluids, solvated gels, and porous solids. These ligands are selected for affinity properties for a soluble or colloidal target species. Advances in technology have been associated with the synthetic and biosynthetic design of affinity ligands and matrices. These advances have been made through a better understanding of phenomena associated with the transport of the target species to the ligand and the nature of molecular interactions between target and ligand.

Affinity chromatography is referred to as biospecific interaction chromatography. This is a technique that is based on specific interactions between molecular pairs from biological systems. Examples of ligands that can be used are antigens, antibodies, hormones, cofactors, receptors etc. Since some ligands employed in affinity chromatography originate from natural sources themselves they must also be purified, since they may contain host DNA, and viruses. One way to reduce these problems is to use synthetic ligands, such as the biomimetic dyes. Synthetic ligands, which are substances that are structurally similar to their *in vivo* parent molecule, are more stable or provide better selectivity for separation purposes. They are inexpensive, scalable, durable and reusable [1 - 4].

Use of cellulosic sorbents modified with dyes in biological systems study is based on both necessities for improvement of untreated materials affinity toward proteic substances and high dye stability of desorption from sorbents [5].

A number of ligand molecules (i.e., synthetic and natural) were immobilized for the modification of the surface of the chromatographic support materials. The change in the surface properties was found to affect the interaction of the surface with the surroundings. The amount of adsorbed target biological macromolecule on the support surface is primarily determined by the type of ligand molecule immobilized. The

surface hydrophobicity of the support materials can be changed depending on the polarity of immobilized ligand molecules used.
Membrane chromatography has been introduced as an alternative to the traditional column techniques. Affinity membranes operate in convective mode, which can significantly reduce diffusion limitations seen in column chromatography. As a result, higher throughput and faster processing times are possible in membrane systems [6].
Affinity extraction based on aqueous two-phase systems has many advantages for large-scale bioseparation processes. Introduction of affinity ligands into these systems has a profound and selective influence on the partitioning efficiency of the target proteins. Reversed micellar extraction systems by using dye ligands have been attracted as an alternative affinity separation technique due to its simplicity and scalability [2].
Affinity chromatography is a suitable technique for protein purification processes, with pseudo-biospecific ligands largely responsible for this success due to their low cost, availability, simple immobilization reaction, resistance to chemical and biological degradation as well as acceptable selectivity and capacity [1, 5]. Triazinyl dyes and immobilized metal ions are good examples of these ligands. Protein interaction with immobilized metal is a rather selective and versatile, high-affinity adsorption technique for which there is little quantitative information [1 - 4]. The affinity chromatography is characterized by advantages such as: good resolution for assurance of a high selectivity process uses of different materials like stationary phase, such as functionalized cellulosic materials with dyes and metallic ions. Sorbents based on cellulose (microcrystalline, beaded or active) modified with reactive dyes and/or metallic ions are new sorbents of high selectivity in separation of nucleic, proteins, enzymes or biosynthesis drugs [2].
The purpose of this work is to present a review of our research activities, concerning the opportunities of cellulosic sorbents modified with reactive dyes and/or metallic ions to use in retention of biomacromolecules. A comparative study regarding the lisozyme binding capacity of reactive dyes, Red Brilliant HE-3B, as well as the untreated or cellulosic sorbents functionalized with dye/metallic ion has also been made.

MATERIALS AND METHOD

Materials

Dye

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

Celluloses

Guanidoethyl (GE), and aminoethyl (AE) cellulose ion exchange unmodified and modified by sorption with reactive dyes Brilliant Red HE-3B and/or Cu^{2+} ions have been used.

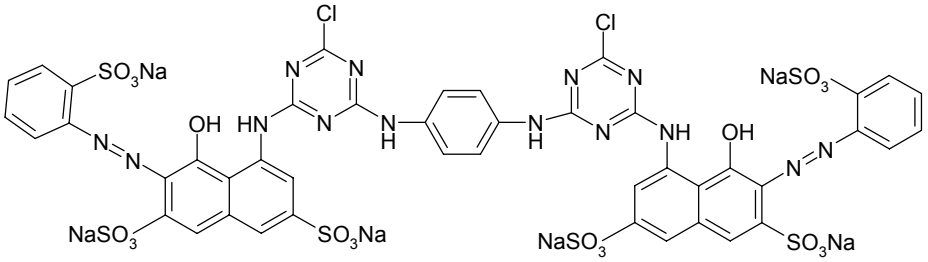

Figure 1. Structure of bis (monochloro-s-triazine) reactive dye (Brilliant Red HE-3B, C.I. Reactive Red 120)

Proteic compounds

The tested protein, the Lysozyme (I.U.B.3.2.1.17, muramidase), was prepared as a stock aqueous solution of 200 $\mu g.mL^{-1}$ concentration. The stock solution was diluted so that the concentration of the working solutions ranged from 1 $\mu g.mL^{-1}$ to 40 $\mu g.mL^{-1}$.

Working technique

The retention study was carried out by batch method, when samples of about 0.3 – 0.5 g sorbent (untreated or modified) were equilibrated with 50 mL protein solution containing variable amounts of active substance at 18 °C. After 1 hour, the phases were separated by filtration and the filtrate was analyzed using the Lowry method of water soluble proteins determination. The optical density of filtered solution was determined at the wavelength corresponding to the sorption maximum of amino copper (II) complexes, λ = 500 nm with respect to Lambert – Beer law, using a UV-Vis spectrophotometer of Jenway 6105 type.

The retention capacity of sorbents was evaluated by amount of adsorbed proteic substances:

$$q = \frac{C_0 - C}{G} \cdot V \cdot 10^{-3} \text{ (mg of lysozyme /g sorbent)}$$

where: C_0 and C are initial and equilibrium concentration of lysozyme ($mg.L^{-1}$),
G is amount of sorbent (g),
V is volume of aqueous system (mL).

RESULTS AND DISCUSSION

Lysozyme (E.C. 3.2.1.17) is a commercially important enzyme and is currently used in food technology as a potent antibacterial agent in milk products and in wine production processes, or in pharmaceutical field, as a drug for treatment of ulcers, infections, cancer, and HIV [6].

Affinity adsorption is a monolayer adsorption process, which means that adsorption equilibrium is reached when all the ligand molecules are combined with the complementary target molecules. This phenomenon may be described by simple adsorption equilibrium expressions, namely Langmuir and Freundlich equations. These equations can be used to predict the adsorption capacities of the affinity sorbents. The

binding site of a protein is a unique stereo chemical arrangement of ionic, polar, and hydrophobic groups in its three-dimensional structure, and where the polypeptide chains probably exhibit greatest flexibility. The dye-ligand molecules participate in non-covalent interaction with the protein to achieve tight and specific binding. Triazinyc dyes interact with an enzyme in a way involving the binding site the substrate or coenzyme binding site.

The unretained amount of protein after adsorption process was calculated on basis of absorbance values from calibration curve for proteins in aqueous solution determination.

In our previous work we established the condition to retain the lysozyme onto some cellulose materials functionalized with different reactive dyes and/ or metallic ions [7 – 10]. Results of our experimental studies were expressed as μg of protein taken up per 1 g sorbent, that, in fact, is represented the sorption isotherm and they are shown in figure 2.

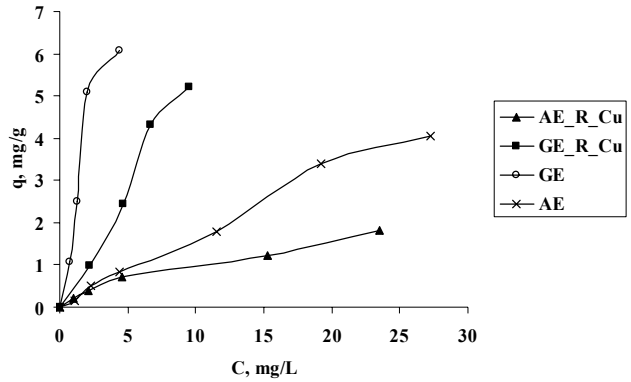

Figure 2. *Adsorption isotherms for lysozyme retention onto functionalized or not functionalized AE and GE cellulose*

The experimental equilibrium adsorption data for these four type of sorbents which shown Langmuir behaviors, were analyzed using two the most widely used adsorption isotherm models:

- the Freundlich [11], which is an empirical equation based on adsorption on heterogeneous surface. It is given as:

$$q = K_F \cdot C^{1/n} \tag{1}$$

The linearized form of Freundlich isotherm can be written as:

$$\log q = \log K_F + \frac{1}{n} \log C \tag{2}$$

where K_F parameter is related to the adsorption capacity;
n is a measure of adsorption energy.

A favorable adsorption correspond to a value of $1 < n < 10$. For $n = 1$, $K_F = K$ and isotherm is linear. K_F and n can be calculated by plotting ln q versus ln C (figure 3).

- the Langmuir [11] model, which is valid for monolayer adsorption onto a surface with a finite number identical site, and is expressed by following equations:

$$q = \frac{K_L \cdot C \cdot q_0}{1 + K_L \cdot C} \tag{3}$$

The linearized form of this equation can be written as:

$$\frac{1}{q} = \frac{1}{q_0} + \frac{1}{K_L \cdot q_0} \cdot \frac{1}{C} \tag{4}$$

where K_L is related to intensity of the adsorption process;
q_0 is the maximum value of adsorption capacity (corresponding to complete monolayer coverage).

The Langmuir constants can be calculated by plotting $1/q$ versus $1/C$ (figure 4).
The K, q_o, K_L, K_F, and n values derived from the linear Langmuir and Freundlich plots together with their correlation coefficients (R^2), are given in table 1.

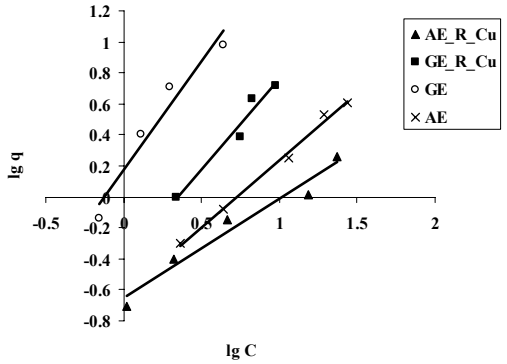

Figure 3. *Freundlich plots for the adsorption of the lysozyme on tested sorbent, at room temperature*

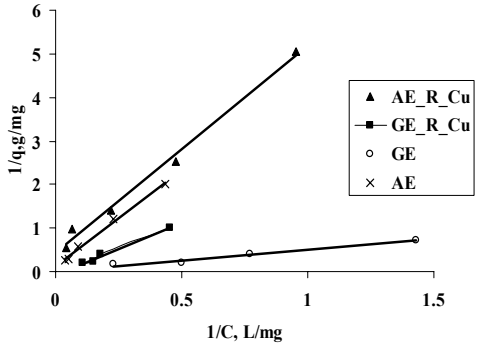

Figure 4. *Langmuir plots for the adsorption of the lysozyme on tested sorbent, at room temperature*

Using the values of binding Langmuir constant, K_L (L.mol^{-1}), and following equation [12] we can calculate the variation of apparent free energy (ΔG, kJ.mol^{-1}) of lysozyme sorption on different types of cellulosic sorbents (table 1).

$$\Delta G = -RT \ln K_L \tag{5}$$

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

The data presented in table 1 show the follows:

- regression values (R^2) indicate that the adsorption data onto cellulosic materials studied fitted well with the Langmuir isotherm;
- the cellulosic materials untreated presents the best values from the quantitative parameters of adsorption. This fact can be explained by blocked the active site favorable for lysozyme sorption by dye molecule and metallic ions. Both cellulosic materials prove to be an effective sorbent in removal of proteins traces from aqueous medium;
- the negative values of ΔG confirm that the lysozyme adsorption processes onto celluloses materials are a spontaneous.

Table 1. *The parameters of sorption process of lysozyme onto cellulosic materials modified with reactive dye and Cu^{2+} ions and unmodified, at room temperature*

Type of sorbent	AE	AE_R_Cu [8]	GE	GE_R_Cu [8]
Freundlich isotherm				
$\mathbf{K_F}$ (mg/g)(L/mg)$^{1/n}$	0.235	0.2219	1.489	0.3895
n	1.151	1.55	0.71	0.86
$\mathbf{R^2}$	**0.9932**	**0.9597**	**0.9521**	**0.9564**
Langmuir isotherm				
q_0 [μg.g^{-1}]	8.65	2.33	135.135	14.33
K_L [L.g^{-1}]	0.026	0.09024	0.0149	0.02932
$\mathbf{R^2}$	**0.9945**	**0.992**	**0.9719**	**0.985**
ΔG [kJ.mol^{-1}]	-14.423	-17.472	-13.076	-14.732

The lysozyme adsorptions strongly depend on the structure and distribution of functional groups on the surface of the different dye-ligand immobilized material. The lysozyme molecules should interact from different patches with each immobilized dye-ligand. After first lysozyme layer formation on each dye-ligand immobilized support surfaces, the adsorbed lysozyme molecules should reorganize and create another energetically favourable layer according to interaction side of the immobilized ligand molecules. It seems that metal ions play an important role in system, because the exposed electron-donating amino acid residues on the protein surface contribute to the binding of proteins to metal ions.

Affinity chromatography is a powerful protein separation method that is based on the specific interaction between immobilized ligands and target proteins. Affinity purification of protein complexes followed by identification of proteins by mass spectrometry serves as a tool for generating a map of protein–protein interactions and cellular locations of complexes for the study of protein posttranslational modification sites and quantitative proteomics [13]. Through applied chromatography techniques imposed for different separations and/or purifications of biocompunds, affinity techniques are good accepting grace of their high selectivity.

CONCLUSIONS

Affinity adsorption is a monolayer adsorption process, which means that adsorption equilibrium is reached when all the ligand molecules are combined with the complementary target molecules. This phenomenon may be described by adsorption equilibrium expressions, namely Langmuir and Freundlich equations.

The results of this study evidenced the higher capacity of cellulose ion exchange for lysozyme uptake than that of the same cellulose modified with dye and Cu^{2+} ions. The reactive dyes and the ions of transitional metals can be viewed as an example of a group - specific ligand that can bind more than one protein. These type sorbents are of interest in the proteins separation/concentration, and as well as in the active principles extraction in preparative or ecological processes. The system under study offers some new attractive possibilities of selective retention of lysozyme from aqueous effluents.

Starting from these preliminary results we can continuing the experimental research to establishes the operative parameters that influenced the sorption process of lysozyme and we can extend the working technique to dynamic system.

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REFERENCES

1. Clonis, D.Y.: Affinity chromatography as bioinformatic and combinatorial tools develop, *Journal of Chromatography A,* **2006**, **1101**, 1–24;
2. Gorduza, V.M., Tofan, L., Suteu, D., Gorduza, E.V.: *Biomaterials-Biotechnology-Biocontrol*, Ed. Cermi, Iasi, **2002**;
3. Rao, A.A.: Computational approach of protein analysis using affinity chromatography, *African J. Pure Applied Chem.*, **2007**, **1**(1), 001–007;
4. Powell, A., Siu, N., Inlow, J.K., Flurkey, W.H.: Immobilized metal affinity chromatography of Mushroom Tyrosinase, *Journal of Chemistry*, **2007***,* **1**(1), 1–13;
5. Ficarro, S. Salomon, B.A.R., Brill, L.M., Mason, D.E.: Automated immobilized metal affinity chromatography/nano-liquid chromatography/electrospray ionization mass spectrometry platform*, Rapid Commun. Mass Spectrom*., **2005**, **19**, 57–71;
6. Ghosh, R.: Protein separation using membrane chromatography: opportunities and challenge, *J. Chromatogr.,* **2002**, **952**,13–27;
7. Gorduza, V.M., Suteu, D., Tofan, L.: Cellulosic materials functionalized with reactive dyes and metallic ions, *Proceedings 30th Annual ARA Congress Chisinau,* Central Publishing House, **2005**, 605–608;
8. Suteu, D., Gorduza, V.M., Toma, O., Malutan, Th.: Cellulosic materials functionalized with reactive dyes and metallic ions for biotechnological separations, *Roum. Biotechnol. Lett*., **2008**, **13**(1), 3567–3574;
9. Gorduza V.M., Şuteu D., Tofan L: Multifunctionalized materials in performing separations, *Roum.J.Phys*., **2004**, **49**(9–10), 873–883;
10. Gorduza, V.M., Suteu, D., Tofan, L.: Biotechnological applications of sorbents functionalized with reactive dyes*, Annals of West University of Timişoara*, **2001**, **10**(3), 1049–1056;
11. Uddin, M.T., Islam, M.S., Abedin, M.Z.: Adsorption of phenol from aqueous solution by water hyacinth ash, *ARPN Journal of Engineering and Applied Sciences*, **2007**, **2**(2), 11–17;
12. Akkaya, G., Ozer, A.: Biosorption of Acid Red 274(AR 274) on Dicranella varia: Determination of equilibrium and kinetic parameters, *Process Biochem.*, **2005**, **40**(11), 3559–3568;
13. Wen-Chien Lee, Kelvin H. Lee: Applications of affinity chromatography in proteomics, *Analytical Biochemistry*, **2004**, **324**(1), 1–10.