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Scientific Essay

GRIN

Synthesis of chromatography adsorbent immobilized metal affinity (IMAC) from agarose and effect of the amount of $NaBH_4$ in the derivatization step

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ABSTRACT

A synthesis of IMAC adsorbent was performed. Agarose matrix was derivatized with epichlorohydrin using different amounts of $NaBH_4$. Furthermore, iminodiacetic acid was immobilized on the modified agarose. Finally, the derivatization efficiency was studied by measuring the epoxide groups and retention of Cu^{2+}. The results indicated that the matrices were modified efficiently and the use of $NaBH_4$ in small amounts is enough to provide a reducing environment to the reaction.

Keywords: Chromatography; Agarose; Iminodiacetic acid; copper; epichlorohydrin; $NaBH_4$

1. Introduction

Affinity chromatography is a type of adsorption chromatography in which a molecule to be purified is specifically and reversibly adsorbed by a ligand immobilized on a matrix, and the above is generally a natural polymer such as (agarose, cellulose, silica, alumina) or synthetic (derived from acrylamide, methacrylate, acrylate, polystyrene, etc.). The matrix needs to have certain properties as being solid, insoluble, and hydrophilic, with functional derivatizable groups and has a high chemical and mechanical stability.

To obtain an adsorbent for affinity chromatography are necessary two basic procedures: An activation of the matrix and then, a ligand immobilization. The mentioned steps are dependent each other. The first stage known as activation or derivatization is a chemical modification process on the functional groups of the matrix without change properties of the same, to subsequently adding a covalently bio-specific ligand, by reaction coupling or immobilization.

In this work, an adsorbent matrix for immobilized metal affinity chromatography (IMAC) was prepared [1]. To develop this matrix, the polysaccharide agarose (Aga) was used (the molecular formula of Aga is shown in Fig. 1), because was widely utilized to covalently immobilize biologically active substances (proteins), for the presence of hydroxyl groups, easily derivatizable. The hydroxyl groups were activated with epichlorohydrin [2,3] for subsequent ligand coupling step. Furthermore, the structure of the last also acts as a spacer arm to avoid steric hindrance problems. Once activated matrix, was carried out the binding of the metal chelating agent, iminodiacetic acid (IDA). The IDA was used to anchor copper ion (Cu^{2+})[4,5].

Because of the relevance of the matrix derivatization step [6], in this study were performed different synthesis conditions to react agarose with epichlorohydrin and two concentrations of $NaBH_4$. The use of $NaBH_4$ as a reducing agent medium is amply used in literature; however the recommended amounts are usually very different. Thus, the

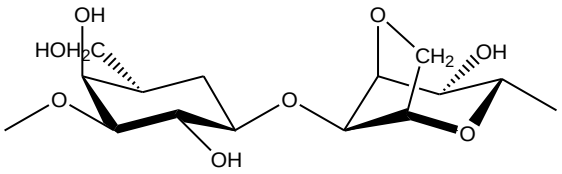

Fig. 1. Molecular structure of agarose

modification of the matrix under each of the recommended conditions was made, taking the ends as benchmarks with which to evaluate the amount of reagent needed to make this important step of modification.

2. Materials and methods

2.1. Reagents

Agarose (Aga), epichlorohydrin (Eph), $NaBH_4$, iminodiacetic acid (IDA) were purchased from Aldrich (Milwakee, WI, USA). Sodium hydroxide, ethanol, acetone, pyridine, phenolphthalein, $NaCO_3$, and $CuSO_4$ were purchased from Anhedra (Argentina). HCl was purchased from Cicarelli (Argentina). All reagents were used as received. All solutions were prepared in ultrapure water (18 MΩcm-1) from a Milli-Q system.

2.2. Activation of agarose

Duplicated assays using Erlenmeyer flasks with 3*g* of agarose each were added to 39*mL* of water and allowed to stand for 24 hours. After that, 3 and 40*mg* of $NaBH_4$ were added (called matrices *M1* and *M2*, respectively). Then, 30.6*mL* of epichlorohydrin (ECH) and 39.3*m*L of 1.0*M* sodium hydroxide solution were added. The reaction was reacted for 2 hours and half under reflux and stirred at 25°C. The reaction scheme is shown in Figure 2. After the reaction, the products were filtered and washed with 200*,* 150, and 50*mL* of water, acetone, and ethanol, respectively. Finally, the modified matrix was dried under vacuum to constant weight. The products obtained were called *M1-Ech* and *M2-Ech*.

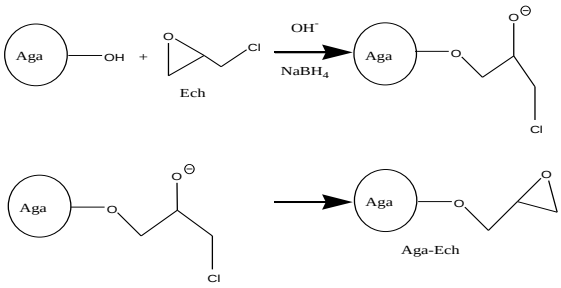

Fig. 2. Reaction of agarose with epichlorohydrin.

2.3. Quantification of epoxide groups

The epoxide groups of *M1-Ech* and *M2-Ech* were reacted with HCl in pyridine, as shown in Figure 3. A mass of 0.*2122g* and 0.1971*g* were weighed, respectively. Then, 1*mL* of HCl in pyridine (0.05M) was added to the reaction mixture and was reacted at 80°*C* for 45 minutes with magnetic stirring and reflux. After the reaction, 2*mL* of pyridine, 3 drops of phenolphthalein and HCl (excess) were added. The last was titrated with a solution of 0.05*M* sodium hydroxide.

The background was prepared by titrating 1*mL* of HCl, 2*mL* of pyridine, 3 drops of phenolphthalein with 0.05*M* sodium hydroxide

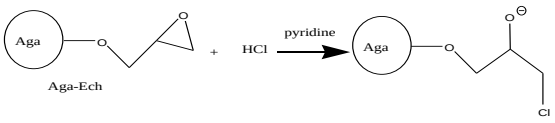

Fig. 3. Reaction of HCl with Aga-Ech

2.4. Coupling of iminodiacetic acid on activated matrix

Matrices *M1-Ech* and *M2-Ech* were allowed to hydrate for 24 hours. Once hydrated, the excess of water was removed and 1*g* of each matrix was transferred to Erlenmeyer flasks. Then, a 10*mL* of 2*M* Na_2CO_3 solution and 300*mg* of iminodiacetic acid (IDA) were added. The reaction mixture was homogenized for 5 minutes in an ultrasonic bath and was reacted under reflux and stirred at 60°*C* for 2.5 hours. After that, the reaction the mixture was filtrate in vacuum and washed with milli-Q water, 0.1*M* acetic

acid and milli-Q water again. Figure 4 shows a schematic of the reaction of IDA with Aga-Ech and the products obtained were called *M1-Ech-IDA* and M2-Ech-IDA.

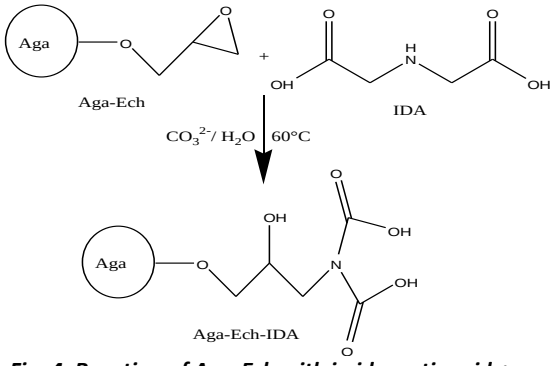

Fig. 4. Reaction of Aga-Ech with imidoacetic acid.+

2.5. Identification of carboxyl groups in the matrices Aga-Ech-IDA

The identification of carboxyl groups in the modified matrix, about of 0.2*g* of *M1-Ech-IDA* and *M2-Ech-IDA* were respectively weighed. Furthermore, 10*mL* water and 3 drops of phenolphthalein ethanol solution were added. Then, the matrix was reacted by adding drop wise a sodium hydroxide solution about 0.05*M*.

2.6. Complexation of cupric ions on the support of Aga-Ech-IDA

A small chromatographic column of 2*mL* was filled with 200*mg Aga-Ech-IDA*. Then, a 2*mL* $CuSO_4$ solution 0.1*M* was eluted through the same. The absorbance of the copper solution was quantified spectrophotometrically before and after of elution throughout the column. Measurements were realized at 800*nm* wavelength using a Shimadzu UV-pc spectrophotometer

3. Results

3.1 Quantification of epoxide groups of Aga-Ech

The agarose was modified with epichlorohydrin using low and high concentrations of sodium borohydride, as indicated in the experimental section. A quantification of epoxide groups present in the modified matrices was performed. For this reaction, the use of a non-protic solvent such as pyridine must be realized because, the epoxide are highly reactive and may react with protons from the medium.

For the determination of epoxide groups an excess of HCl was reacted with of Aga-Ech, and the protons were quantified using NaOH. The results of the back-titrations were:

- *Background (1 mL of HCl / 0.05M pyridine) consumed 0.91mL of 0.05M NaOH*
- *M1-Ech (0.2022g) were consumed 0.11mL of 0.05M NaOH*
- *M2-Ech (0.1971g) were consumed 0.16mL of 0.05M NaOH*

The concentration was determined for the matrices M1-Ech and M2-Ech and the results were shown in Figure 5.

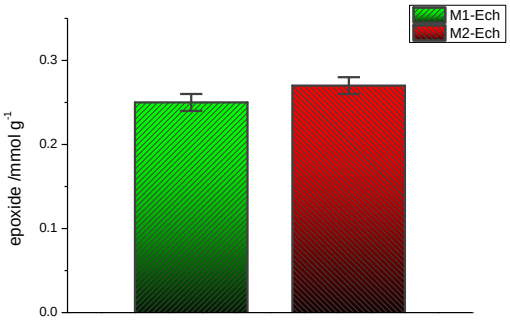

Fig. 5. Results of quantification of epoxide groups.

3.2. Identification of carboxyl groups in the matrices Aga-Ech-IDA

All matrices were tested with phenolphthalein and discoloration was observed, indicating the acid behavior of the modified matrix. The reaction of neutralization with phenolphthalein matrices required about 1*mL* of NaOH solution. The result indicates that the matrix was extensively functionalized IDA.

3.3. Immobilization of cupric ions on the support of Aga Ech-IDA

The ion Cu^{2+} was immobilized on the matrix through the formation of coordination complexes with IDA retained in the matrix. To demonstrate that the anchorage of the metal takes place only in the Aga-Ech-IDA matrix, the effect of the presence of this metal in contact with Aga matrices, Aga-Ech and Aga-Ech-IDA were qualitatively investigated (Figure 6). In the figure, the solutions of copper (a) in contact with the matrix Aga (b) and Aga-Ech (c) were found no visible change in color of the matrix or the solution. However, when the copper solution was in contact with Aga Ech-IDA (d), the matrix changes from white to deep blue, and the solution was significantly discolored. Therefore, the results indicated that only samples containing IDA effectively anchored the metal and removed the same from the solution.

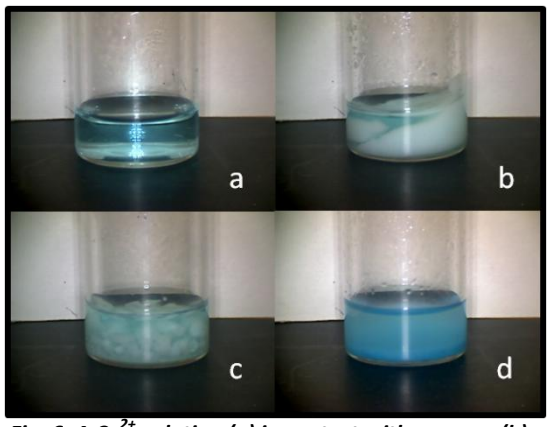

Fig. 6. A Cu^{2+} solution (a) in contact with agarose (b), aga-Ech (c), and aga-Ech-IDA (d).

To evaluate the efficiency of the immobilization of copper by *Aga-Ech-IDA* matrix, a solution of Cu^{2+} was passed through a chromatographic column containing 0.2*g* of matrix. To determine the amount of metal retained, the absorbance of copper solution was measured before and after passing through the column. The following results were obtained:

Abs H_2O (background): 0,041
Abs before elution of $CuSO_4$: 1.211
Abs after elution of $CuSO_4$: 0.058

With these values, the concentration of the solution once passed through the column was calculated:

[CuSO4] = 0,017 / 11.7 = 0.0015M

This means that the column retained 98.5% of the copper of the solution.

5. Conclusion

It was possible to synthesize an IMAC adsorbent. The agarose was modified using epichlorohydrin with different amounts of $NaBH_4$. The epoxide groups present in the matrices was quantified. The modification of the matrix with epichlorohydrin was independent of the amount of $NaBH_4$ used in these reactions. The Aga-Eph matrices were functionalized with IDA. The iminodiacetic acid was revealed for the presence of carboxylic groups and then for the efficient complexation of Cu^{2+} by the matrix *Aga-Eph-IDA*. For the modification reaction, low amounts of $NaBH_4$ can be used because it was only necessary to provide a reducing environment in the reaction of the matrix with epichlorohydrin. Furthermore, using the conditions employed in this work can be obtained an efficient modification of the agarose matrix.

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