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Adsorption isotherms, kinetics and thermodynamic studies towards understanding the interaction between cross-linked alginate-guar gum matrix and chymotrypsin

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ABSTRACT

The adsorption kinetics of chymotrypsin, a pancreatic serine protease, onto an alginate-gum guar matrix cross-linked with epichlorohydrin has been performed using a batch-adsorption technique. The effect of various experimental parameters such as pH, salt presence, contact time and temperature were investigated. The pseudo-first-order and pseudo-second-order kinetic models were used to describe the kinetic data which shows that the adsorption of the enzyme followed the pseudo-second-order rate expression. The Langmuir, Freundlich and Hill adsorption isotherm models were applied to describe the equilibrium isotherms, and the isotherm constants were determined. It was found that Hill model was more suitable for our data because the isotherm data showed a sigmoidal behavior with the free enzyme concentration increasing in equilibrium. At 8 °C and at pH 5.0, 1 g hydrate matrix adsorbed about 7 mg of chymotrypsin. In the desorption process 80% of the biological activity of chymotrypsin was recovered under the condition of 50 mM phosphate buffer, pH 7.00–500 mM NaCl. When successive cycles of adsorption/washing/desorption were performed, it was observed that the matrix remained functional until the fourth cycle of repeated batch enzyme adsorption. These results are important in terms of diminishing of cost and waste generation.

1. Introduction

The adsorption of proteins in batch systems is an important method of protein concentration and purification especially when the target protein is in a feedstock. Adsorption has been widely used as one of the main steps of downstream operations in various fields, such as biology, medicine, biotechnology and food processing [1]. Within this context, the development of low-cost adsorbents with high adsorption capacity and selectivity has been a great challenge. Moreover, adsorption should be perfectly reversible to optimize recovery while preserving the activity of the desorbed (recovered) enzyme.

In the last years, the use of natural polymers to obtain non-soluble matrices has received much attention in the downstream process of industrial enzymes [2–5] as well as in the pharmaceutical field for controlled drug delivery [6,7]. The main reasons for this interest are that they are biocompatible, inexpensive and can be discarded into the environment without a negative impact. Alginate (Alg) and Chitosan are the most extensively studied polysaccharides used in the formation of non-soluble matrices for controlled drugs release and protein adsorption in a reversible manner [5,7–11]. This choice is based on the ability of both polymers to form, under certain conditions, matrices with defined and reproducible size and shape.

Alg is a water-soluble linear polysaccharide extracted from brown seaweed, composed of alternating blocks of 1–4 linked α-L-guluronic and β-D-mannuronic acid residues. The gelation of Alg can be carried out under an extremely mild environment using non-toxic reactants. The most important property of alginate is its ability to form gels by reaction with divalent cations. Alg beads can be prepared by extruding a solution of sodium alginate as droplets into a divalent cation solution such as Ca^{2+} or Ba^{2+} [12]. There are a great number of papers where alginate is transformated into a non-soluble matrix adding Ca^{++} to the medium. However, the working pH range of the matrix is limited and when calcium is

Abbreviations: GG, guar gum; Alg, alginate; Chy, chymotrypsin; BTEE, benzoil-L-tyrosine ethyl-ester.

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lost by the matrix, the Alg-calcium complex is destroyed, being the Alg soluble [2,8,10]. Others works report a chemical modification of the polylectrolyte by covalent bonding between the reactant and chemical groups of the polysaccharide, obtaining a non-soluble matrix at any pH which is independent from the presence of Ca^{2+} [13,14]. However, as the carboxylic groups of Alg participate in the chemical reaction, a significant number is lost, decreasing the charge of the matrix and its capacity to bind protein. Alg beads cross-linked with epichlorohydrin were used in a fluidized bed for the purification of proteins [14].

The incorporation of some substances could modify physical properties of calcium-Alg beads. This approach has been successfully used for the purification of α-amylase on cross-linked Alg-starch beads, based on the affinity of protein for the polymer [15]. Also, an Alg-guar gum bead has been used for the purification of jacalin [16].

Guar gum (GG) is comprised of a high molecular weight polysaccharides composed of galactomannans consisting of a (1 → 4)-linked β-D-mannopyranose backbone with branch points (i.e., 1 → 6-linked α-D-galactopyranose). There are between 1.5–2 mannose residues for every galactose residue [17]. This gum represents one of the most abundant industrial raw materials and has been subject of intensive research due to its sustainability and biosafety [18]. Alg-GG beads are not very stable, but the cross-linked Alg-GG matrix showed stability over a wide pH range and also in the presence of phosphate or Ca^{++} as chelator.

The development of new adsorbents is based on economic issues. Commercial beads for adsorption are formed by polysaccharides chemically cross-linked to make it non-soluble. Agarose generally is used with acid or basic chemical groups covalently attached to it. Besides, several commercial trademarks are available, such as Streamline™ and Sepharose™ but their cost is around 2000 USD per L which significantly affects the the final cost of production process in scaling-up. Alg-GG beads cross-linked with epiclorohydrin have properties similar to those of commercial adsorbents but are cheaper and easier to prepare. These advantages allows their use at scaling up level.

Because the downstream processing costs for enzyme production account for about 50–80% of the total process cost [19] the development of new materials with adsorption capacity for macromolecules is important. Our laboratory has been exploring the interaction between proteins and polysaccharides with net electrical charge, in order to use it as the first step in the concentration and purification of enzymes [20,21].

α-Chymotrypsin (Chy) is a serine endopeptidase produced by the pancreatic cells of mammals. It has a molecular weight of 25.7 kDa with an isoelectric point of 9.1 and optimum activity at pH 8.2. This enzyme has several applications in the industry and research. Chy is widely used in food and pharmaceutical industry, sequence analysis, peptide mapping, among other uses. A major limitation for the use of Chy is the high cost of the enzyme. Chy and related proteases are obtained by different protocols involving ammonium sulfate precipitation, adsorption and chromatographic techniques based on: affinity, ionic exchange, gel filtration and hydrophobic principles [22–25]. The need for a new protocol is based on economic and environmental issues. High amounts of salts are known to be contaminants and cannot be recycled. Chromatography is not a suitable technique to be used as an early unit operation in a downstream process from the economic point of view.

In the present work, beads made from a mixture of Alg and GG cross-linked with epichlorohydrin were used to study the adsorption of Chy. Although this adsorbent has relatively inexpensive production costs, few studies have reported its use as an adsorbent for this enzyme adsorption and purification studies.

2. Materials and methods

2.1. Chemical

Alginic acid, sodium salt (Alg), guar gum (GG), epichlorohydrin and α-chymotrypsin (Chy) were purchased from Sigma–Aldrich (St. Louis, MO, USA) and used without further purification. All other reagents were also of analytical grade. The solutions were prepared with distillated water.

2.2. Preparation of Alg-GG beads

250 mg of GG was dissolved in 50 mL of distilled water, after its complete dissolution 0.1 g of Alg was added and left stirring until complete dissolution. Beads were formed by dropping this solution through a syringe into a 100 mL of 0.1 M $CaCl_2$ solution according to the method previously reported [15]. The beads were maintained in stirring overnight and finally incubated in 6 mM $CaCl_2$ solution at 8 °C.

2.3. Preparation of non-soluble Alg-GG beads

Cross-linked Alg-GG particles were prepared by adapting the procedure of Roy et al. [16]. Alg-GG beads (10 g) were transferred to 50 mL of 50% P/V of ethanol containing 0.1 M $CaCl_2$ for 30 min at 45 °C. The beads were then kept in absolute ethanol containing 0.1 M $CaCl_2$ (50 mL) for 30 min. 2.65 mL of epichlorohydrin was added in small aliquots with stirring and the beads were kept for 10 min at 45°. 5.6 mL of 5 M NaOH was added to the above and kept overnight at 45 °C with stirring. Finally, acetic acid was added to the system till the pH became neutral. The matrix was washed with a 3:1 mixture (v/v) of absolute ethanol and water, followed by 95% ethanol and washed several times with distilled water. The matrix was finally re-suspended in distilled water and equilibrated with the work buffer before use.

2.4. Determination of Chy activity

Chy assay is based on the hydrolysis of benzoil-L-tyrosine ethyl-ester (BTEE) [22]. The reaction rate was determined by measuring the absorbance increase at 256 nm, at 25 °C, which results from the hydrolysis of the substrate at 0.6 mM concentration in 200 mM Tris-HCl buffer pH 8.2. One Chy unit (U) is defined as 1 μmol of substrate hydrolyzed per minute of reaction and was calculated as follow: UA (μmol/min) = ($\Delta Abs_{256\,nm}$/min) × 1000/964, where 964 is the benzoyl-tyrosine molar extinction coefficient [24].

2.5. Protein estimation

The concentration of protein in the medium was evaluated at 280 nm using a UV–vis spectrophotometer. A calibration curve was performed using dilutions of a standard Chy solution of 1 mg/mL.

2.6. Batch adsorption experiments

All adsorption experiments were conducted in batch mode with stirring of 30 rpm

2.6.1. Determination of the best adsorption conditions

For adsorption of commercial Chy, 3 mL of 0.15 mg/mL of Chy in 25 mM citrate buffer was incubated with 100 mg of hydrated cross-linked Alg-GG matrix. Two pH values –5.0 and 6.0- and the following ionic strengths –NaCl 50 mM, 100 mM and 200 mM- were assayed. The mixtures were stirred until the adsorption equilibrium was reached and the free protein in the supernatant was determined. The amount of Chy adsorbed was calculated from mass

balance. The data were processed as % protein adsorbed/g matrix or as mass of adsorbed Chy over mass unit of hydrated matrix (w). All measures were carried out at 25 °C.

2.6.2. Kinetic of adsorption

Experiments of batch kinetic adsorption of commercial Chy onto cross-linked Alg-GG beads were carried out measuring the concentration of free Chy in the supernatant over time. The mixtures were prepared with Chy 0.15 mg/mL in 25 mM citrate buffer at different pH and were stirred until the adsorption equilibrium was reached. All measures were carried out at 25 °C. To analyze the adsorption kinetic mechanism the adsorbed Chy amounts vs. time curves were fitted with two models, namely pseudo-first and pseudo-second order. Nonlinear regression analysis was applied to estimate the kinetic model parameters. Nonlinear regression was performed using trial and error method with the help of solver add-in functions using Sigma Plot v11 software.

2.6.3. Chy adsorption isotherm

Adsorption isotherm of Chy was determined by equilibrating different concentrations of Chy with 100 mg cross-linked Alg-GG beads at pH 5.00 and two temperatures: 25 °C and 8 °C. The mixtures were stirred until the adsorption equilibrium was reached and free protein in the supernatant was determined. The amount of Chy adsorbed at equilibrium time by unit of mass adsorbent (m) was determined by the following equation:

$$Q_{eq=}\frac{(C_{eq}-C_0)\times V}{m} \quad (1)$$

where Q_{eq} is the quantity of protein adsorbed per gram of adsorbent (mg/g), C_0 is the protein initial concentration in solution (mg/mL) and C_{eq} is the protein residual concentration in supernatant at equilibrium (mg/mL), V is the volume of solution (mL) and m is the mass of adsorbent (g). The results were expressed as Q_{eq} vs. C_{eq}.

In order to estimate the validity of isotherm models with experimental data the Langmuir, Freundlich and Hill models were tested.

2.7. Thermodynamic evaluation

The thermodynamic state functions are important indicators when estimating the mechanism of adsorption process. A distribution coefficient K_D reflects the binding ability of the surface for a molecule. The K_D is defined as follow:

$$K_D = \frac{a_s}{a_{eq}} \approx \frac{C_s}{C_{eq}} \quad (2)$$

where a_s and a_{eq} are respectively the activity of adsorbed Chy and Chy in liquid phase at equilibrium, C_s is the concentration Chy adsorbed in the solid matrix (mg/ g) and C_{eq} is the concentration of free Chy in solution in equilibrium with the Chy adsorbed (mg/mL). K_D can be obtained from extrapolating C_{eq} to zero in the plots of $\ln(C_s/C_{eq})$ vs. C_{eq} at both temperatures [26]. Extrapolating is performed since when the concentration approaches zero their value equals the activity.

The standard Gibbs energy (ΔG°) was estimated by applying the thermodynamic equation:

$$\Delta G^\circ = -RT\ln K_D \quad (3)$$

where R is the gas constant (1.9872 cal/mol K), T the absolute temperature (K) and K_D is the distribution coefficient. ΔH° (enthalpy change) was calculated from the van't Hoff equation:

$$\ln\frac{K_{D,298}}{K_{D,281}} = \frac{\Delta H^\circ}{R}\left(\frac{1}{281}-\frac{1}{298}\right). \quad (4)$$

and ΔS° (entropic change) from the well known equation:

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ \quad (5)$$

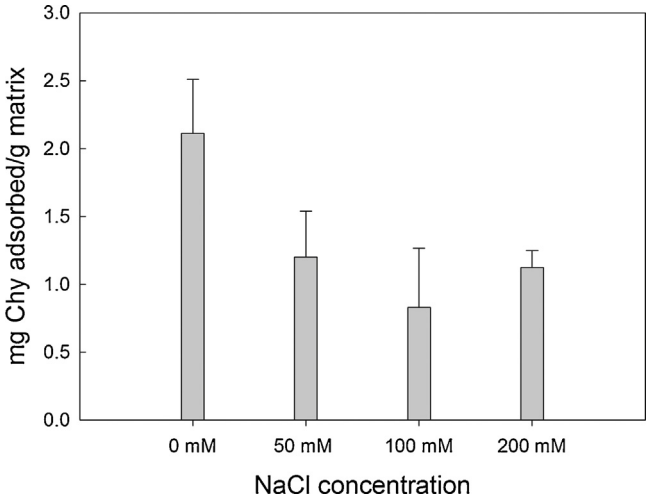

Fig. 1. Adsorption capacity of the matrix under different conditions. Medium: citrate buffer 25 mM pH 5.00 with different NaCl concentrations. Temperature: 25 °C. Initial Chy concentration 1 mg/mL. Total amount of matrix 100 mg.

2.8. Protein release from the matrix

Chy elution from the matrix was carried out at 25 °C. After adsorption, the matrix loaded with the enzyme was washed with the working buffer until a negligible 280 nm absorbance measurement. For desorption, the beads were suspended in different media and stirred for 24 h. Finally, the concentration of Chy in the supernatant was measured. The conditions assayed were: 25 mM phosphate buffer, pH 7.0 and NaCl 0 mM, 150 mM, 300 mM, 500 mM and 750 mM. First, time data were expressed as % released protein under the different conditions. Besides, desorption kinetics was measured under the best conditions determined above. All measures were carried out at 25 °C.

2.9. Statistic handle of the data

Non-linear regression analysis was applied to estimate the isotherm and kinetic model parameters. Nonlinear regression was performed using trial and error method with solver add-in functions of Sigma plot V11 software. In trial error procedure, isotherm and kinetic parameters were estimated by maximizing the coefficient of determination (R^2) (sum of squares) and minimizing the value of $\sum^2$ (residual sum of squares). Both coefficients have been widely employed to measure the fitting degree of model to adsorption data [27,28]. The coefficients were calculated according to the following equations:

2.10. Sum of squares of the error:

$$\Sigma_i^p \left(Q_{eq,\,cal} - Q_{eq,\,mean}\right)^2 \quad (6)$$

The R^2 coefficient

$$r^2 = \left(\frac{\left(q_{e,meas}-q_{e,calc}\right)^2}{\sum\left(q_{e,meas}-\bar{q}_{e,calc}\right)^2+\left(q_{e,meas}-q_{e,calc}\right)^2}\right) \quad (7)$$

3. Results

3.1. Determination of adsorption conditions

The extent of Chy adsorbed onto Alg-GG beads was highly dependent on pH and ionic strength of the medium. Fig. 1 shows the results obtained at pH 5.00. At pH 6.00 the extent of adsorp-

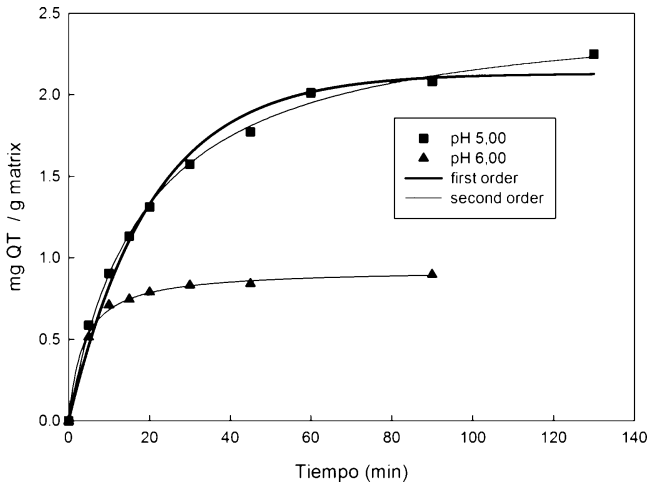

Fig. 2. Kinetics of Chy adsorption on the Alg-GG matrix. Medium: citrate buffer 25 mM. The initial Chy total concentration was 1 mg/mL. Total amount of Alg-GG matrix 100 mg. Temperature 25 °C.

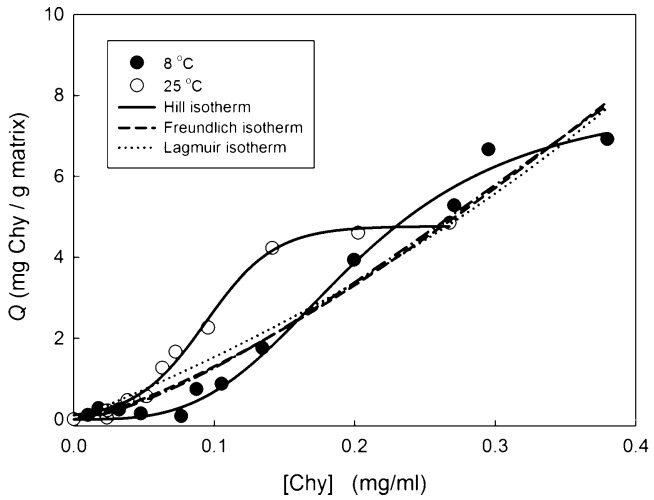

Fig. 3. Adsorption isotherm of Chy onto non-soluble Alg-GG beads. Medium: citrate buffer 25 mM pH 5.00. Temperature: 8 and 25 °C.

tion was only 0.5 mg Chy/g matrix. The best adsorption is achieved at pH 5.00 without added salt. This finding is consistent with an electrostatic mechanism of interaction between Chy and Alg-GG beads. So we selected the pH 5.0 to obtain the adsorption kinetics and isotherm curves.

3.2. Kinetic of Chy adsorption–desorption process on the matrix

The kinetics of the Chy adsorption was assayed as shown in Fig. 2. The experiments were performed at two pHs, in the range of negative electrical charge of the matrix. It can be seen that in both cases, the adsorbed protein increases with the contact time until it reaches a plateau. The point where the plateau begins is the equilibrium time required to achieve the maximum adsorption in these conditions. It can be seen that a pH 6.0 the adsorption is poor and needs only 14.14 ± 0.05 min to reach equilibrium. However, at pH 5.0 the percentage of adsorbed protein was 62% of the initial amount, requiring 45.67 ± 1.29 min to achieve equilibrium for the total process. These results are consistent with a Coulombic mechanism of interaction between Chy and Alg-GG beads which is strongly affected by pH and the presence of salts. We have used the following mathematical adsorption kinetics models to analyze the adsorption mechanism:

- *pseudo-first order model*

$$Q = Q_{\max}(1 - e^{-k_1 t}) \tag{8}$$

where Q and $Q_{\max}$ (mg/g) are the amounts of Chy adsorbed per unit weight of matrix in a time t and at the equilibrium respectively, and k_1 (min $^{-1}$) is the constant rate of pseudo-first order adsorption.

- *pseudo-second order model*

$$Q = \frac{k_{2t} Q_{\max 2}}{1 + k_{2t} Q_{\max 2}} \tag{9}$$

where Q and $Q_{\max}$ (mg/g) are the amounts of Chy adsorbed per unit weight of matrix in a time t and at the equilibrium respectively and k_2 (g/mg min) is the constant rate of pseudo-second order adsorption.

Table 1 shows a results comparison fitting the results with two kinetic models for the adsorption of Chy. It can be seen that $\sum^2$ value obtained from fitting to pseudo-second order kinetic is low, while R^2 value for this fitting is very near 1. Therefore, we conclude that the model that best explains the behavior of experimental data is the pseudo-second order kinetics. The adsorption mechanism of a solute onto polysaccharides matrix may be assumed to involve three steps: diffusion of the protein through the boundary layer, intra-particle diffusion, and adsorption of the protein on the matrix pores surface.

Finally, kinetics of the desorption process showed that the 80% of the total Chy adsorbed was released in a medium sodium phosphate pH 7.00. −NaCl 500 mM (data not shown) which suggests a reversibility in the adsorption-desorption process of Chy.

3.3. Adsorption isotherm of Chy on the Alg-GG beads

We performed the isotherm absorption at pH 5.0 because this pH value was found optimal to perform the adsorption process, so, the temperature effect on the adsorption was analyzed. We changed the temperature in a range where it did not produce any protein conformational change. Thus, any observed change on the isotherm should be due to a change in the matrix structure. By visual inspection of the adsorption isotherm experimental data (Fig. 3), a poor adsorption of enzyme at low protein concentration can be seen, but at increased Chy concentration the fraction of enzyme bound is significantly enhanced, in agreement with the presence of a cooperative effect in the adsorption process. Besides, an increase in the adsorption capacity of the matrix with the decreasing temperature is evident.

For adjustment of the experimental data the following isotherm models were chosen [29]:

(a) Freundlich isotherm is an empirical equation used for the description of multilayer adsorption with interaction between adsorbed molecules:

$$Q = K_F C_{eq}^{1/nF} \tag{10}$$

where C_{eq} indicates the equilibrium concentration of adsorbate and K_F and nF are the Freundlich constants characteristic of the system. K_F and nF are indicators of adsorption capacity and adsorption intensity, respectively.

(b) The Langmuir model is valid for monolayer sorption to a surface with a finite number of

identical sites. The well known expression of the Langmuir equation is:

$$Q = Q_{max} \frac{K_L C_{eq}}{1 + K_L C_{eq}} \tag{11}$$

Table 1
Kinetic parameters of adsorption of Chy onto Alg-GG beads. Medium sodium citrate buffer 25 mM pH 5.00. Temperature 25 °C.

R^2 (sum of squares)		$\sum^2$ (residual sum of squares)			
	Q_{max}	k_1 (min^{-1})	k_2 ($g\,mg^{-1}\,min^{-1}$)	R^2	$\sum^2$
Pseudo-first order	2.13 ± 0.05	(4.9 ± 0.3) ± 10^{-2}	NA	0.9877	0.0574
Pseudo-second order	2.54 ± 0.08	NA	(2.14 ± 0.09)10^{-2}	0.9992	0.00882

Table 2
Isotherm constants for Chy adsorption onto Alg-GG matrix.

Model	Temp (°C)	$Q_{máx}$ (mg/g hydrated matrix)	K_F	K_L	K_H	n_F n_H	R^2	$\sum^2$
Freundlich	8	NA	28.8 ± 5.5	NA	NA	0.8 ± 0.2	0.9553	3.75
	25	NA	19.4 ± 4.7	NA	NA	1.05 ± 0.1	0.9157	3.00
Langmuir	8	−18.1 ± 2.7	NA	−0.8 ± 0.4	NA	NA	0.9393	5.10
	25	25.8 ± 5.2	NA	1 ± 1	NA	NA	0.9237	2.71
Hill	8	8.0 ± 0.7	NA	NA	(6.4 ± 0.3) 10^{-3}	3.1 ± 0.4	0.9910	0.76
	25	5.1 ± 0.2	NA	NA	(8.6 ± 0.5) 10^{-3}	3.0 ± 0.3	0.9920	0.28

where Q and C_{eq} are the amount of protein adsorbed per unit weight of biomass and the unabsorbed protein concentration in solution at equilibrium, respectively. Q_{max} is the maximum amount of protein absorbed per unit weight of biomass to form a complete monolayer and K_L is the Langmuir adsorption constant related to the affinity of the binding sites.

(c) The Hill isotherm model has been applied to systems which present non-ideal competitive adsorption, to explain about the binding of a molecule onto homogeneous substrate. The model assumes that adsorption process as a cooperative phenomenon, with the ligand binding ability at one site on the matrix, may influence the binding capacity of other sites. The data fitted very well with a mathematical model that describes a sigmoidal function:

$$Q = Q_{max}\frac{C_{eq}^{H}}{\left(K_D + C_{eq}^{H}\right)} \quad (12)$$

where Q (mg/g matrix) is the amount of adsorbed protein at the equilibrium; Q_{max} (mg/g matrix) is the maximal capacity of adsorption of the matrix, C_{eq} is the free (unbound) Chy concentration, K_H is the Hill constant and H is the Hill cooperativity coefficient of the binding interaction.

Fig. 3 shows the fitting of the experimental data according to the three adsorption model proposed. Besides, Table 2 shows the parameters adsorption values obtained from the fitting of the data to the Freundlich, Langmuir and Hill model. The statistical analysis of the error function shows that the equation that best fisted the data is the Hill model (see Table 2), this occur at the two temperatures assayed.

The value of $Q_{máx}$ is higher at 8 °C, around 8 mg of enzyme per gram of matrix. If this value is expressed per gram of dried matrix, because the hydrated matrix has around 98% of water (data not shown) the adsorption capacity is 60 times higher, so, approximately 400 mg of Chy are adsorbed per dried gram of matrix. Moreover, it can be seen that even at room temperature the adsorption capacity of matrix is high, reaching a maximum of almost 5 mg of Chy per gram of hydrated matrix (300 mg of Chy per gram of dried matrix). This last finding shows the high capacity of adsorption of the matrix, making it suitable to be applied in the purification of Chy from natural sources, more complex in composition.

3.4. Thermodynamic parameters

Thermodynamic constants for Chy adsorption by Alg-GG beads were shown in Table 3. The negative adsorption standard free change (ΔG°) suggested that the adsorption was a spontaneous process. The positive ΔS° value indicates the increase disorder in the system due a loss of water from the matrix when the interaction is carried out.

Table 3
Thermodynamic constants for Chy adsorption onto Alg-GG beads.

Thermodynamic functions values	Temperature (K)	
	281	298
K_D	1.77 ± 0.2	2.48 ± 0.1
ΔG°(kcal/mol)	−0.318	−0.537
ΔS°(cal/mol K)	12.03	
ΔH°(kcal/mol)	3.27	

The positive standard enthalpy change (ΔH°) indicated that the adsorption process was endothermic in agree with the breaking of water-matrix interaction (water-hydroxyl bound by hydrogen bridge). Because the entropic change was positive, this function contributes to negatively the free energy of the adsorption, so it can be considered as entropically driven. At this point it should be noted that while the maximum adsorption capacity of the matrix is greater at 281 K, cooperativeness and adsorption kinetics are more favorable at 298 K. This can be due to the desolvation of the matrix and the decrease in the thickness of the boundary layer of water that surrounding the adsorbent with the increase in temperature. Besides, cooperative effect depends on different medium variables such as pH, temperature changes, etc. It can be seen that the cooperatively is higher at 25 °C, which might indicate a difference in the adsorption mechanism with temperature variations [28].

3.5. Desorption conditions

On the other hand, different desorption conditions were assayed as shown in Fig. 4. We selected a pH 7.0 to perform desorption process since under this condition the electrical charge of Chy is very low, so there is no interaction with the matrix. As shown in Fig. 4, the presence of increasing NaCl concentration between 0 to 500 mM induces an increase in the Chy release from the matrix. No addition effect on the desorption process was observed with respect to the NaCl 750 mM, because a fraction of Chy remained bound to the matrix, which suggests the presence of a hydrophobic effect in the Chy-Alg-GG interaction. The best conditions for desorption is the use of phosphate buffer with 500 mM NaCl. In this condition, the percentage of desorbed protein was 81%.

3.6. Regeneration study

Any adsorbent is economically viable if it can be regenerated and reused in many cycles of operation. For study the reutilization

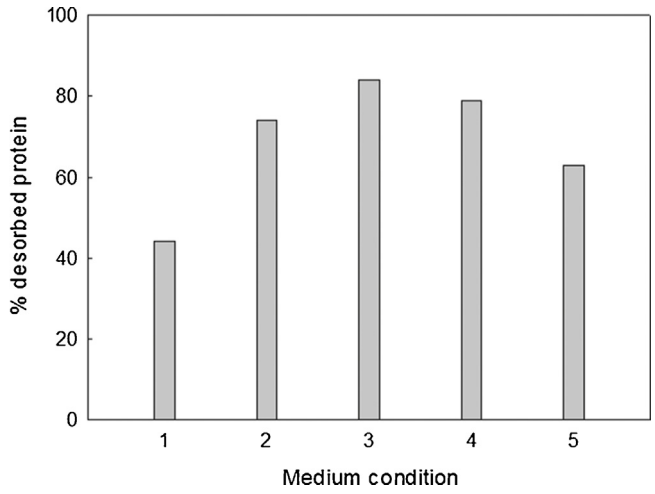

Fig. 4. Effect of salts on the desorption process of Chy bound to the Alg-GG matrix. Medium: 1 to 4: phosphate buffer 25 mM pH 7.00 100 mM, 500 mM and 750 mM NaCl respectively. 5: phosphate buffer 25 mM pH 7.00—ammonium sulphate 500 mM. Temperature: 25 °C.

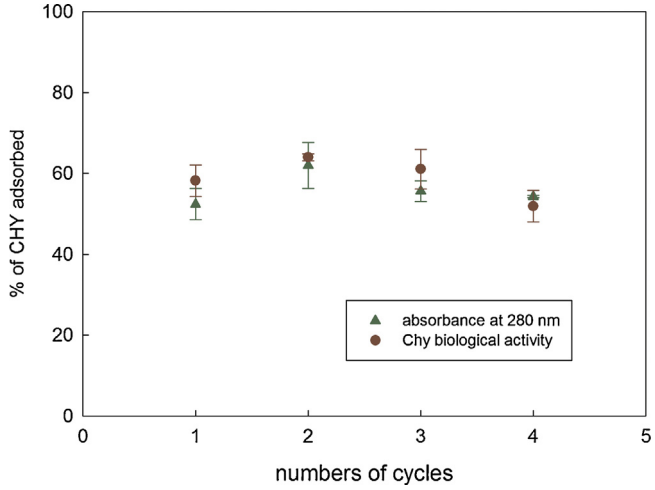

Fig. 5. Adsorption capacity of the matrix after successive cycles of use. The percentage of adsorbed protein was determined by measures of enzymatic activity and total protein concentration. Medium: citrate buffer 25 mM pH 5.00. Temperature: 25 °C.

of the matrix successive cycles of adsorption – elution – washing were carried out using the same matrix as adsorbent and an excess of protein. The concentration of recovered Chy was determined in each step. The process was repeated several times, for twenty days. Fig. 5 shows Chy recovered in each adsorption process. It can be seen that the adsorption capacity of the matrix was not modified in this period. However, in the fifth cycle a loss of functionality was observed, with only 40% of recovery of the enzyme (data not shown).

4. Discussion

In the last few years, our laboratory has been working on the study of the interaction between numerous enzymes and polysaccharides to be applied in affinity precipitation [20,21,30]. Such materials have the advantages of being non-expensive, easily available and non-toxic, so the bioseparation protocol is compatible with the application of the target enzyme in food processing. In the present work, we have evaluated the capacity of cross-linked alginate-guar gum to interact with Chy, a serine protease with various applications in different biotechnological processes. Cross-linked alginate-guar gum has been previously used as fluidized bed affinity media to isolate jacalin [16]. These studies were performed in a dynamic manner in column. However, there is no information about the interaction of the protein with this matrix at the equilibrium situation. In this work, we have worked in batch systems. The first step to apply an adsorption process must be the determination of the adsorption isotherms to evaluate the adsorption capacity of the system. The most widely-used adsorption models are the Langmuir or Freundlich isotherms. However, with the development of new adsorption material the experimental data show a behavior which cannot be described by the above two models.

Previous isotherms obtained for the adsorption of different proteins to a non-soluble polyelectrolyte matrix have been obtained starting at 1 mg/mL of free protein concentration at the equilibrium. The isotherms obtained in all these cases have been fitted using the Langmuir or Freundlich models, because the initial concentration of enzyme assayed was high and the first points of the isotherm data were lost [28,31].

With the development of new adsorption material the experimental data show a behavior which cannot be described by the above two models. We assayed the adsorption of Chy at low concentrations of enzyme (0 to 0.1 mg/mL) to obtain information about the adsorption process under this condition. Our results show that for both working temperatures the adsorption process has a sigmoid behavior in agreement with the presence of a cooperative effect. The data adjust very well to Hill model that assume that adsorption is a cooperative process. Alg-GG beads showed a maximal binding capacity of 8 mg Chy per gram of matrix at 8 °C commercial Capto S ion exchanger, for example, bind 103 mg Chy per mL using packing bed chromatography [32]. These differences can be explained taking into account the characteristics of the adsorbents. Commercial beads have a controlled particle diameter (around 20–100 um) and a major number of acids or basic groups bound to the polysaccharide chains, so the particles have a major exposed surface to the mobile phase. Alg-GG has a particle diameter of around 2 mm, the number of carboxylic groups is given for the alginate and is minor than the commercial beds. The highest binding capacity of commercial beds can be offset by some qualities that have Alg-GG matrix such as:

(1) Easy preparation, the matrix may be prepared in the laboratory in only 3 days with readily available materials without the need for specialized equipment or personnel.
(2) Shorter purification time due to faster adsorption and desorption process.
(3) Low cost of ~50 USD per L against 2000 USD per L of commercial matrices [32,33]. Considering also that the matrix can be reused up to 5 times, the cost reduction is even greater. The scaling up level is strong influenced by the cost of the unit operations, so it could to contribute to increase its application at this level.
(4) Minimal waste generation because of non-toxic reagents needed which can be discarded in the environment without negative effect on it.

The maximal enzyme amount absorbed by the matrix at pH 5.0 at 25 °C was around 61% of the total enzyme mass in solution. This value is the result of the equilibrium between free and bound Chy on the matrix, so the enzyme adsorption yield cannot be improved. The enzyme desorption from the matrix showed an 80% of the biological activity recovery of Chy under the best conditions assayed. Higher Chy recoveries could not be obtained due to the fact that NaCl does not displace the total of the enzyme bound to the matrix, demonstrating that the interaction is partly hydrophobic in character. When the adsorption – desorption process of Chy with the same matrix was tested repeatedly for 20 days, we observed that until the fourth reuse, the matrix maintained its functionality.

In conclusion, this work demonstrated the utility of the Alg-GG cross-linked matrix for adsorption of Chy in a dynamic manner, using a matrix of easy preparation, non-expensive and with potential of recycling. These results are important in terms of diminishing of cost and waste generation.

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