SULFANILIC ACID-MODIFIED CHITOSAN MINI-SPHERES AND THEIR APPLICATION FOR LYSOZYME PURIFICATION FROM EGG WHITE

*Daniela B. Hirsch[1, 2], María F. Baieli[1, 2], Nicolás Urtasun[1, 2], Juan M. Lázaro- Martínez[3, 4], Romina J. Glisoni[5, 2], María V. Miranda[1, 2], Osvaldo Cascone[1, 2] and Federico J. Wolman[1, 2], * *

[1] *Universidad de Buenos Aires, Facultad de Farmacia y Bioquímica, Cátedra de Biotecnología. Junín 956, 1113 Buenos Aires, Argentina*

[2] *CONICET- Universidad de Buenos Aires, Instituto de Nanobiotecnología (NANOBIOTEC), Junín 956, 1113 Buenos Aires, Argentina*

[3] *Universidad de Buenos Aires, Facultad de Farmacia y Bioquímica, Departamento de Química Orgánica, Junín 956 (C1113AAD), CABA, Argentina.*

[4] *CONICET- Universidad de Buenos Aires, Instituto de Química y Fisicoquímica Biológicas (IQUIFIB), Junín 956 (C1113AAD), CABA, Argentina.*

[5] *Universidad de Buenos Aires, Facultad de Farmacia y Bioquímica, Cátedra de Tecnología Farmacéutica II. Junín 956, 1113 Buenos Aires, Argentina.*

* Corresponding author: Federico Javier Wolman

Junín 956, (1113) Buenos Aires, Argentina.

Tel.: +54 11 5287 4679

Fax: +54 11 4508 3645.

E-mail address: fwolman@ffyb.uba.ar

Abstract

A cation exchange matrix with zwitterionic and multi-modal properties was synthesized by a simple reaction sequence coupling sulfanilic acid to a chitosan based support. The novel chromatographic matrix was physico-chemically characterized by ss-NMR and ζ potential, and its chromatographic performance was evaluated for lysozyme purification from diluted egg white. The maximum adsorption capacity, calculated according to Langmuir adsorption isotherm, was 50.07 ± 1.47 mg g^{-1} while the dissociation constant was 0.074 ± 0.012 mg mL^{-1}. The process for lysozyme purification from egg white was optimized, with 81.9% yield and a purity degree of 86.5%, according to RP-HPLC analysis. This work shows novel possible applications of chitosan based materials. The simple synthesis reactions combined with the simple mode of use of the chitosan matrix represents a novel method to purify proteins from raw starting materials.

Keywords: Multi-modal ion exchange chromatography, chitosan, chromatographic matrix, sulfanilic acid, egg white lysozyme.

1. Introduction

Chitin, a linear high-molecular weight polymer of *N*-acetylglucosamine (GlcNAc), is an abundant, available and renewable source found mainly in the exoskeleton of arthropods [1-3]. Whereas chitin is insoluble in common solvents at any pH value, chitosan, the total or partial *N*-deacetylated form of chitin, is soluble in aqueous acid solutions. Due to its free amino and hydroxyl groups in its structure, chitosan is susceptible to undergo chemical modifications [4]. Both chitin and chitosan present the advantage of being natural, biodegradable and environmentally safe biopolymers [1,5]. Both polymers have many potential uses and applications in different fields that are gaining interest. Such applications include areas as different as drug delivery systems and novel biomaterials for 3D modeling [6,7]. Chitosan can also be applied in protein downstream processing, where chromatographic matrices made with this support material are valuable products [2,8]. When purifying proteins from natural complex sources, such as egg white, whey or recombinant sources like larval extracts, due to their high viscosity, high solute concentration and the presence of particulate material, the purification method must be carefully studied because conditioning and clarification steps are usually needed [1,9]. Noteworthy, these additional steps imply more time and a greater processing cost. Lysozyme (Lz) is a well characterized enzyme present in animal and human tissues, body fluids and secretions, whose principal natural source industrially used is the egg white (Lz represents between 3-4% of the total egg white proteins) [10]. This enzyme is commercially interesting mainly due to its antimicrobial activity, because it damages bacterial cell walls by catalyzing the hydrolysis of 1,4-β-bonds between GlcNAc and muramic acid [11,12]. This enzyme has several applications in pharmaceutical, food and veterinary industries as fungistatic, anti-inflammatory, antitumor, antiviral, a natural food preservative, etc. [13-15].

4

Therefore, the search for efficient and scalable strategies for its purification involves both an academic and an industrial challenge. Many purification methods such as precipitation, ultrafiltration and liquid chromatography, have been developed [16]. However, these methods present disadvantages such as low selectivity in the case of precipitation and the need of special equipment in the case of ultrafiltration and liquid chromatography. Cation exchange chromatography is one of the most effective methods for the purification of Lz from egg white because of the high isoelectric point of this protein (10.7) in comparison with the other proteins present in this source [12,17-20]. Bayramoglu et al [20] have published an interesting approach involving the development of chitosan based magnetic beads provided with sulphonic groups, and their utilization for Lz purification from egg white. The sulphonic groups were incorporated after the beads were grafted with poly (glycidyl methacrylate) by the epoxy groups treatment with sodium sulfite.

In this study, we explored the development of a new cation exchange matrix in the form of mini-spheres with multi-modal and zwitterionic properties by chitosan chemical modification using sulfanilic acid as an immobilized ligand. In order to bind the sulfanilic acid to the mini-spheres, a simple reaction synthesis scheme was performed based on two reactions with epichlorohydrin using two different concentrations. The first reaction was performed to crosslink the chitosan mini-spheres and the second to activate them for the ligand immobilization.

We characterized this matrix by evaluating the chemical structure, process parameters and conditions for the purification of Lz from egg white.

2. Materials and methods

2.1 Materials

5

Chitosan (medium molecular weight), Lz standard, sulfanilic acid (4-aminobenzenesulfonic acid) and *Micrococcus lysodeikticus* were from Sigma- Aldrich (St. Louis, MO, USA). Epichlorohydrin was from Fluka Analytical (Buchs SG, Switzerland). The Quick Start Bradford reactive for total protein determination was from BioRad (Hercules, CA, USA). Hen eggs were purchased in a local market. All other reagents were analytical reagent grade.

2.2 Matrix synthesis

Chitosan mini-spheres were obtained according to the procedure described in a previous work [4]. Briefly, a chitosan solution was prepared by dissolving 2% of medium molecular weight chitosan powder in a 2% acetic acid solution [21]. This solution was dripped through a 15 G needle on a 2 M NaOH solution with soft continuous stirring, resulting in 1.60 – 1.80 mm size chitosan mini-spheres [4]. Sixteen hours later, the matrix was rinsed with distilled water until a neutral pH value. The mini-spheres were then crosslinked with a 250 mM epichlorohydrin solution, pH 10.0 at 60°C for 4 h under continuous gentle stirring. For the coupling of sulfanilic acid, the matrix was first activated by a second treatment with epichlorohydrin, using a 2.55 M solution (60°C, 16 hours, pH 10.0) followed by the coupling reaction with a 0.46 M sulfanilic acid solution, pH 10.0 under stirring for 40 h at 60°C.

2.3 Physico-chemical characterization of the matrix

2.3.1 Solid-state Nuclear Magnetic Resonance (ss-NMR)

Solid-state Nuclear Magnetic Resonance (ss-NMR) experiments were performed at room temperature in a Bruker Avance II-300 spectrometer equipped with a 4-mm MAS probe.

6

The operating frequency for protons and carbons was 300.13 and 75.46 MHz respectively. High-resolution ^{13}C solid-state spectra for the different chitosan mini-sphere samples were recorded using the ramp 1H→^{13}C CP-MAS (cross-polarization and magic angle spinning) sequence with proton decoupling during acquisition (SPINAL64) with a contact time of 1.5 ms and a recycle delay time of 5 s. The spinning rate for all the samples was 10 kHz. Elemental analysis was performed with a LECO CHNS 932 elemental analyzer device.

2.3.2 Zeta potential (ζ potential)

To perform a comprehensive study of the Zeta potential (ζ potential) versus the pH values of the micro-sized matrices, mini-spheres were previously subjected to mechanical disruption to generate a homogeneous micro-particle suspension. The suspension was obtained by breaking 100 mg of each matrix using a spatula and then maintaining it under vigorous magnetic stirring for 48 h in 10 mL of distilled water. Finally, the micro-sized suspensions obtained were filtered (1.2 μm nitrocellulose membrane, Osmonics, Inc.) to homogenize the particle size. Then, 2 mL aliquots from the suspension were diluted 1:1 with distilled water and NaCl was added up to a final 10 mM solution concentration. The pH values were adjusted to a range between 2.0 and 9.0 by adding an adequate volume of 50 mM HCl or NaOH solutions.

Size (D_h, hydrodynamic diameter), size distribution (polydispersity index, PDI) and ζ potential were measured by Dynamic Light Scattering using a Zetasizer Nano-ZS (Malvern Instruments, Malvern, UK) with a measurement range of 0.6 nm to 6 μm, at a scattering angle of 173° and a fixed measurement position of 4.65 mm. The Nano-ZS is provided of a Scatter Technology (NIBS®). Samples were contained in disposable polystyrene and disposable folded capillary cells for size and ζ potential measurements, respectively

[11,16]. The viscosities of the samples were 0.8872 cP (± 0.0001) at 25ºC. Results were expressed as the mean ± standard deviation (SD) of three independent samples prepared under identical conditions. Data for each single specimen was the result of at least six runs.

2.4 Lysozyme adsorption isotherms

Adsorption isotherms were performed by incubating 50 mg of hydrated matrix with 1 mL of Lz solutions (0.063-10 mg mL^{-1}) at different pH values using acetate buffer (20 mM, pH 4.0 and 5.0), phosphate buffer (20 mM, pH 6.0, 7.0 and 8.0) and carbonate buffer (20 mM, pH 9.0) with gentle agitation (16 h, 20°C). The absorbance of the supernatant was measured at 280 nm and the difference between the final and the initial Lz concentration was used to calculate the equilibrium protein concentration adsorbed to the matrix. Maximum adsorption capacity (q_m) and dissociation constant (K_d) parameters were determined according to the Langmuir model [22,23]. This model is described by the following equation:

$$q = q_m C/(K_d + C) \tag{1}$$

Where q is the amount of protein adsorbed on the matrix at equilibrium, q_m is the maximum adsorption capacity, C is the equilibrium concentration of the protein in the solution and K_d is the dissociation constant.

All determinations were performed in triplicate and the results are expressed as the average ± SD.

2.5 Effect of ionic strength on lysozyme adsorption

The impact of ionic strength on Lz adsorption was determined by incubating 50 mg of matrix with 1 mL of Lz 1 mg mL^{-1} solution in phosphate buffer (20 mM, pH 7.0) at five

different NaCl concentrations (0-200 mM). Incubation was performed at 20ºC for 4 h with

gentle agitation. The absorbance of the supernatant was measured at 280 nm and the

difference between the final and the initial Lz concentration was used to calculate the

protein concentration adsorbed to the matrix.

All determinations were performed in triplicate and the results are expressed as the average

± SD.

2.6 Elution studies

Elution studies were carried out using 50 mg of matrix saturated by a previous treatment

with 10 mg mL^{-1} Lz solution. The effect of pH was studied using elution buffers with

different pH values (3.0 - 9.0), an increase in ionic strength (0.0 – 1.5 M NaCl) and the

addition of an organic component to the buffer (25% propylene glycol). The buffers used

were 50 mM acetate-acetic buffer pH 3.0 and pH 5.0, 20 mM phosphate buffer pH 7.0 and

20 mM carbonate buffer pH 9.0.

All determinations were performed in triplicate and the results are expressed as the average

± SD.

2.7 Lysozyme purification from egg white

2.7.1 Adsorption kinetics and optimization of the sample/matrix ratio

Increasing quantities of matrix (25, 50 and 100 mg/mL) were incubated with egg white

diluted 1:4 in adsorption buffer. Samples of the supernatant were taken at 0, 0.5, 1, 2, 4, 6

and 16 h and analyzed for Lz activity.

All determinations were performed in triplicate and results are expressed as the average ±

SD.

2.7.2 Purification process

Batch adsorption was carried out with 100 mg of matrix in contact with 1 mL of egg white (diluted 1:4 with 20 mM potassium phosphate buffer, pH 7.0), under gentle agitation for 4 h. The adsorption was analyzed by measuring Lz activity. After four washing steps with the adsorption buffer, the protein was eluted with 1 mL of 25% propylene glycol + 20 mM potassium phosphate buffer pH 7.0, 1.5 M NaCl.

All samples (diluted egg white, supernatant and eluted fractions) were analyzed by RP-HPLC and the total protein content by the Bradford method.

All determinations were performed in triplicate and results are expressed as the average ± SD.

2.8 Analytical assays

2.8.1 Measurement of lysozyme activity

The enzymatic activity of Lz was measured by its lytic action on *Micrococcus lysodeikticus* [1]. To this end, 25 µL of the sample was added to 975 µL of a *M. lysodeikticus* suspension (0.5 mg mL^{-1}) in 20 mM potassium phosphate buffer pH 7.0. Absorbance at 450 nm was measured every 10 s for 2 min, defining one unit of Lz activity as a decrease in 0.001 absorbance units per minute.

2.8.2 RP-HPLC

The RP-HPLC analysis was performed on a Shimadzu LC-20AT System. A C18 ACE HPLC column (4.6 mm x 25 cm, 300 Å, 5 µm, Advanced Chromatography Technologies Ltd, Aberdeen, Scotland) was used to analyze all samples from the purification process.

The flow rate was 1 mL min^{-1}, the injection volume was 20 µL, and the effluent was monitored by its absorbance at 215 and 280 nm. The solvents were 0.065% trifluoroacetic acid (TFA) in water (A) and 0.050% TFA in acetonitrile (B). The elution gradient was: 0-5 min, 0-25% B; 5-25 min, 25-80% B; 25-26 min, 80-100% B; 26-30 min, 100% B. The peak areas were determined for the calculation of purity degree (%).

2.9 Statistical analysis

Results of the elution studies and adsorption kinetics were subjected to analysis of variance (ANOVA) ($P < 0.05$) using the statistical program Statgraphics Centurion XV (Statgraphics, Warrenton, USA).

3. Results and discussion

3.1 Physico-chemical characterization of the matrix

The objective of the present work was to contribute to expanding the uses of chitosan in chromatographic applications by synthesizing a novel multi-modal cationic exchange matrix. In this sense, chitosan mini-spheres were subjected to a first reaction with a bifunctional agent (epichlorohydrin) to crosslink the formed mini-spheres, followed by a second reaction using an excess of epichlorohydrin to functionalize the chitosan hydroxyl and amino groups for immobilization of sulfanilic acid through its amine group (Figure 1). Sulfanilic acid was chosen as ligand since this molecule is a precursor for the triazine dyes synthesis and our group has previously reported interesting results using them as ligands for affinity purification processes [4,24]. The sulfanilic acid presents the advantage, over the triazine dyes, of not having regulatory restrictions if the matrix or product obtained from the process is used in the food industry. As mentioned in the introduction section, due to its

high isoelectric point, Lz purification from egg white by cation exchange chromatography represents an effective strategy. Considering the moderate/high total solute concentration present in egg white, the multi-modal characteristic of the cation exchange matrix would allow the adsorption of the Lz directly from this source with no other treatment than a dilution.

To characterize the chemical modifications in the modified crosslinked chitosan mini-spheres, ^{13}C CP-MAS spectra were performed on each synthetic step and the results are shown in Figure 2. The characteristic resonance signals for the chitosan structures (Figure 2 A) remained in the mini-spheres crosslinked with 250 mM of epichlorohydrin solution (Figure 2 B) [25]. In addition, a significant broadening of the NMR signals was observed due to the random confinement of the chitosan structure during the formation of the spheres. This behavior can be particularly observed in the C_1 signal at around 97-107 ppm since it is a well resolved signal without the superposition with another NMR signal. Particularly, a new signal at 72.6 ppm was assigned to the epichlorohydrin residues that remained chemically bound to the chitosan mini-spheres ($C_{9,10}$) after the nucleophilic attack of the hydroxyl groups in C_6 and the opening of the epoxy group by another hydroxyl group. Also, a small resonance signal at 47.4 ppm appeared associated with the substitution of the chlorine atom in the epichlorohydrin molecule by the amine group (C_{11}) leaving a pendant epoxy group that can react with another hydroxyl or amino group. Although the *ss-*NMR experiments performed here were not quantitative, some semi quantitative information can be obtained with the consecutive treatments with different epichlorohydrin solutions taking into account that all the ^{13}C CP-MAS spectra were acquired with the same contact time and recycle delay time.

The consecutive reaction of the crosslinked chitosan mini-spheres with 2.55 M epichlorohydrin solution led to a significant increase in the signals at 72.6 and 64.1 ppm (Figure 2 C). This reaction allowed the binding of the sulfanilic residues to the chitosan mini-spheres according to the aromatic signals observed in the range of 110-160 ppm in the ^{13}C CP-MAS spectrum in Figure 2 D. Also, the C_1 resonance signal in the sulfanilic-modified mini-spheres was broader than in the rest of the materials due to the complex chemical structures, where a proportion of native chitosan coexists together with different degrees of crosslinking and chemical functionalization with sulfanilic acid.

Elemental analysis was done to quantify the degree of functionalization with sulfanilic acid. According with the amount of sulfur content (1.28 g% = 0.04 mole%) the sulfanilic acid content was estimated as 70 mg (0.4 mmol) per gram of modified chitosan mini-spheres. The amount of amine groups was determined as 0.48 mole% (%N: 6.67 and %C: 38.80) in the unmodified chitosan. Then, the amount of secondary amine groups in the functionalized chitosan mini-spheres, corresponding to the sulfanilic residues, was estimated as 0.04 mole% according to Eq. 2 where the %NCS and %NSCH are the %N of the chitosan and sulfanilic acid bounded to the chitosan structures, respectively. The %N Total (4.95% = 0.35 mole%) was obtained from the elemental analysis and the %NSCH (0.04 mole%) was estimated since it is equivalent to the sulfur content (%S = 1.28 = 0.04 mole%). The nitrogen content corresponding to the polysaccharide chitosan structure was calculated as 0.31 mole%. The reduction in the amount of the nitrogen associate to the chitosan clearly indicated that the crosslink with epichlorohydrin took place increasing the carbon content (%C: 41.19).

$$\%NTotal = \%NCS + \%NSCH \tag{2}$$

For the preparation of the samples to analyze the ζ potential, the particle disruption process displayed an average particle size of 1216.0 ± 254.7 nm and PDI values of 0.265 ± 0.124. The curves obtained from the ζ potential versus pH allowed concluding that the incorporation of the sulfonic groups onto the chitosan mini-spheres was successful since a charge inversion can be observed at pH 5.70 (Figure 3). The curve obtained for the sulfanilic acid-modified mini-spheres reveals the zwitterionic nature of the synthesized matrix in which positive amino groups coexist with negatively charged sulfonic groups [26,27]. This suggests better adsorptive properties for the capture of proteins from raw materials and a better and more selective elution of the adsorbed protein from the chromatographic support, by changing the net charge of the matrix through the modification of the pH value and/or the ionic strength of the elution buffer. Moreover, given the intrinsic nature of the sulfanilic acid used as ligand and the chitosan itself as chromatographic support, the resulting matrix may interact with the target protein in a multi-modal approach, involving principally both ion-exchange and hydrophobic interactions together with Van der Waals interactions [28]. These types of interactions may be useful to adsorb the protein from a complex raw medium directly to the matrix without any pretreatment or conditioning.

3.2 Equilibrium adsorption isotherms

To characterize the interaction between Lz and the synthesized matrix, maximum adsorption capacities were compared at different pH values (4.0-9.0) as shown in Figure 4A. The highest maximum adsorption capacity was obtained at pH 7.0. The adsorption isotherm performed at pH 7.0 is shown in Figure 4B; the q_m calculated for Lz was 50.07 ± 1.47 mg g^{-1}. The K_d value calculated was 0.074 ± 0.012 mg mL^{-1}, which is lower than the

14

Lz concentration in egg white (1-3 mg mL^{-1}), thus making this matrix able to ensure an efficient adsorption from egg white.

The information obtained from the adsorption maximum capacities vs pH analysis was consistent with the ζ potential curves since the negative charge of the matrix seems to reach its maximum at the pH value of 7.0. Other cation exchange matrices with sulfonate groups as ligands and high adsorption capacity [20] showed their maximum adsorption capacity at pH 5.0, where the beads are charged negatively while the protein has a higher positive charge.

In comparison with other chromatographic materials with immobilized triazine dyes as ligands our mini-spheres showed higher maximum adsorption capacities. The maximum adsorption capacities obtained by Arica et al. [18] using Procion Brown MX-5BR and Procion Green H-4G immobilized on poly (2-hydroxyethylmethacrylate) resulted in 32.6 mg mL^{-1} and 45.0 mg mL^{-1} respectively. In other work published by the group [19] Procion Green H-4G was immobilized on poly (2-hydroxyethylmethacrylate)/chitosan membranes and the maximum adsorption capacity obtained for Lz was 20.28 mg mL^{-1}. Noteworthy, in these mentioned cases the maximum adsorption capacities were found at pH values of 7.0 and 6.0, which are similar to our synthesized mini-spheres and consistent with the adsorptive type of interaction.

3.3 Effect of ionic strength on lysozyme adsorption

As seen on Figure 5, the increase of the ionic strength (in the studied range) had almost no effect on the binding of Lz to the matrix. This behavior reveals the multi-modal nature of the presented matrix; therefore this matrix could be potentially applied to purification of proteins from a raw material containing moderate ionic strength. The importance of this relays on the lack of need of including a conditioning step in the purification process, like

the desalinization requirement of the sample before the chromatographic step, resulting in a lower cost of the purification process. In comparison with other cation exchange matrices, as the reported by Bayramoglu et al.[20] where the adsorption capacity decreased almost to half of the full capacity in the presence of 100 mM KCl, the results shown in this work account for the benefit of including chemical groups involving both hydrophobic and electrostatic interactions when designing a ligand.

3.4 Elution studies

The elution percentages obtained from the mini-spheres, previously saturated with Lz, when varying the pH and ionic strength of the elution buffer are shown in Figure 6. None of the eluents tested achieved a complete desorption of the protein bound to the matrix. The results obtained were lower than 60% elution degree. However, the different results obtained when varying the pH and ionic strength of the elution buffer revealed various features related to the interactions between the mini-spheres and Lz. At neutral and alkaline pH, we observed a correspondence between the increase in ionic strength and the amount of protein eluted, until a plateau was reached at 1 M NaCl for pH 7.0 and at 1.5 M NaCl for pH 9.0. A slight improvement in desorption was also observed between pH 9.0 and pH 7.0 (37.9% $\pm$ 1.12 for pH 9.0 and 31.0% $\pm$ 1.43 for pH 7.0).

Noteworthy, when acid elution buffers were used an opposite effect was observed since the elution capacity decreased as the ionic strength increased. This effect was intensified at lower pH values. At these acidic pH values, as shown in the ζ potential studies, both the matrix and the protein were positively charged. Under this condition, the electrostatic component of the interaction may be minimized –or even a repulsive effect may occur between the positively charged matrix and the protein- and the elution could be a

consequence of the disruption of hydrophobic forces. This disruptive effect increased with a decrease in the salt concentration. By studying the effect of the pH value and other variables in protein purification processes mediated by hydrophobic interactions, Hjertén et al. [29] and Fang Xia et al. [30] found that Lz adsorption is maximized when the pH value of the adsorption buffer is close to the isoelectric point of the protein. In this case, while studying the effect of pH from the desorption perspective, the opposite effect can be appreciated, which is coherent with that published by these authors.

The addition of 25% propylene glycol to each condition previously studied led to a positive outcome in the elution, especially for pH 7.0 and 3.0, with higher elution percentages at pH 7.0. As shown in Figure 7, there was a synergistic effect between the addition of propylene glycol and the increase in ionic strength, mainly at pH 7.0 with 1.5 M NaCl, since the elution results were 4.72 ± 0.95 % for the addition of 25% propylene glycol to the pH 7.0 buffer without NaCl and 5.39 ± 0.42 % for the addition of 25% propylene glycol to the pH 9.0 buffer. This effect was also observed by Wolman et al. [31] when working with membranes functionalized with triazine dyes for bovine lactoferrin purification, as the elution was maximized with the use of ethylene glycol in addition to the ionic strength. Under these conditions, the inclusion of a component that impairs the hydrophobic interaction and of another that affects the electrostatic forces causes a synergistic effect on the elution of the adsorbed protein in chromatographic matrices such as the one presented in the present work and the ones with triazine dyes.

Buffers at pH 3.0 and 5.0 were also studied adding propylene glycol without NaCl, and an improvement in the elution results was obtained, especially for the eluent at pH 3.0. This is coherent since the hydrophobic interactions were disrupted even more than the buffer without this component, probably because propylene glycol has an effect on the decrease of

the dielectric constant of the elution solution. This effect was also observed by Låås [32] in an early work on the development of hydrophobic chromatography matrices. This author found that the use of ethylene glycol combined with a decrease in the salt concentration resulted in a better elution performance of the adsorbed protein to the hydrophobic matrix. As already mentioned, the importance of hydrophobic interactions over electrostatic ones under this condition might be explained by the change in the net charge of the matrix at these pH values (as shown in Figure 3).

Since the eluent 20 mM phosphate buffer, pH 7.0, with 1.5 M NaCl and 25% propylene glycol was significantly better than 50 mM acetate, pH 3.0, with 25% propylene glycol according to the ANOVA, the former was chosen for the purification process of Lz from egg white.

3.5 Lysozyme purification from egg white

Due to the complexity of the egg white as a starting material and the premise of preserving the process economy by avoiding the conditioning of the sample, batch was selected as the most simple and effective operative mode for the present process. However, a dilution 1:4 was needed to facilitate the recovery of the matrix after the adsorptive step and improve the diffusiveness of the protein to reduce the time required for the adsorption step.

In order to optimize the purification process some parameters must be established to guarantee cost effectiveness. The optimum relation between the adsorption time and the amount of matrix to be used was determined to ensure maximum Lz adsorption at the minimum time with the minimum amount of matrix. The results shown in Figure 8 indicate that, for all the samples taken at different times, the adsorption was always higher with the greatest amount of matrix tested. For this reason, 100 mg matrix per 1 mL egg white was

the quantity of matrix chosen for further studies. In a previous work, Fee and Chand [33] established that there was a given value for the relation between the sample volume and the matrix amount, in which by increasing the matrix quantity there was no increase in the target protein bound to it, but there was a reduction of the time required to complete the adsorptive step. This effect was also verified in a previous work published by Baieli et al. [4] on lactoferrin purification from sweet whey. In reference to the time required for Lz adsorption, 4 h was chosen as the time for the adsorptive step, based on productivity terms (amount of purified protein per time and matrix amount) and as there was no significant difference (p<0.05), between 4 to 24 h using 100 mg matrix per 1 mL egg white (90.18% ± 1.54 and 96.36% ± 0.62, respectively).

Taking into account all the results from previous studies, the whole process was evaluated for the purification of Lz using 20 mM phosphate buffer, pH 7.0, with 1.5 M NaCl and 25% propylene glycol as eluent. As it can be appreciated from the results shown in Table 1, the elution percentage was high enough to ensure high yields. In comparison with other cation exchange matrices such as alcohol-insoluble solids from pea pod as chromatographic media [15], which had a yield of 71.7% using a pretreatment for albumin precipitation with ethanol, the results obtained in this work were better taking also into account that the only pretreatment was a dilution. The pretreatment of egg white solution with ethanol was also used in other cation-exchange-based method published by Chiu, Lin and Suen [17], working with glass fiber-based membranes, whose yield was 72.6 / 68.8% depending on the flow rate used.

Noteworthy, some commercial matrices such as Spec 70 SLS from Pall Corporation (Port Washington, New York, USA) have been tested in the same conditions as the mini-spheres, but the purification process was not possible because the matrix was not able to be

recovered after the adsorption step using egg white diluted 1:4 as the starting material.
Although these commercial matrices are larger than the typical chromatography matrices
(260 – 600 µm) for a successful purification the starting material must be more diluted than
1:4.

Purity was determined from the RP-HPLC chromatogram shown in Figure 9. As it can be
seen, the process is selective since the pattern of peaks of most proteins is maintained after
the adsorptive step with the exception of the Lz peak (minute 15.3). In addition, the Lz
eluted had a 86.5% purity degree, achieved in only one chromatographic step. The first
peak observed at 3.37-3.75 minutes in the chromatogram of the eluted fraction corresponds
to the eluent used. Another interesting aspect of the developed purification process is that
Lz activity was not affected during the adsorption and elution steps.

In order to test the matrix reusability, three consecutive purification processes were
performed without applying regeneration steps in between cycles. For each process both
yield and purity degree parameters remained almost constant, with a minor decay in the
obtained yield for the third one (77.4 %). After applying a regeneration step using 0.05 M
NaOH, the next purification cycle performed restored the initial parameters values.

There are many published works on the purification of Lz using chitosan based matrices in
different forms such as membranes or beads. Using magnetic chitosan beads dotted with
poly (cation-exchange) groups [20] the results obtained in the purification process were a
purity degree of 93% and a yield of 86.29%, which are in the same order as the results
obtained in the present work. There are several differences between the mentioned
magnetic beads and the mini-spheres here developed; one of these is that the sulfanilic
matrix has a simple reaction synthesis and no use of organic solvents. Another difference is
the lower costs associate with the equipment needed in the purification process, only a

20

sieve is needed for the separation of the mini-spheres after each chromatographic step while a magnet is required for the magnetic beads. In addition, the multi-modal property of the mini-spheres allows an efficient protein adsorption even in the presence of moderate ionic strength. In a previously mentioned publication, the purity degree and yield percentages achieved in the purification process of Lz were similar to those reported in the present work [18]. In the cited paper, authors used chitosan affinity membranes with triazine dyes as immobilized ligands; however these approaches may represent a bigger challenge to scale up.

4. Conclusions

In this work, we presented and fully characterized a novel multi-modal cation exchange chromatographic matrix based on chitosan mini-spheres with sulfanilic acid immobilized as ligand through ^{13}C CP-MAS experiments. We evaluated its potential application by exploring Lz purification from egg white. The matrix developed allowed the recovery and purification of Lz with a low-cost matrix that presented adequate mechanic resistance and was easy to recover after the process steps because of its size and density. In addition, this characteristic allowed egg white diluted 1:4 to be the starting material without any further pretreatment, which contributed to achieving a short-time and low-cost process. For these reasons, this matrix is a promising potential alternative for industrial use. Moreover, this work emphasizes the usefulness of chitin/chitosan-based materials for chromatographic purposes, thus increasing the commercial value of these natural polymers.

Acknowledgements

21

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Figure legends
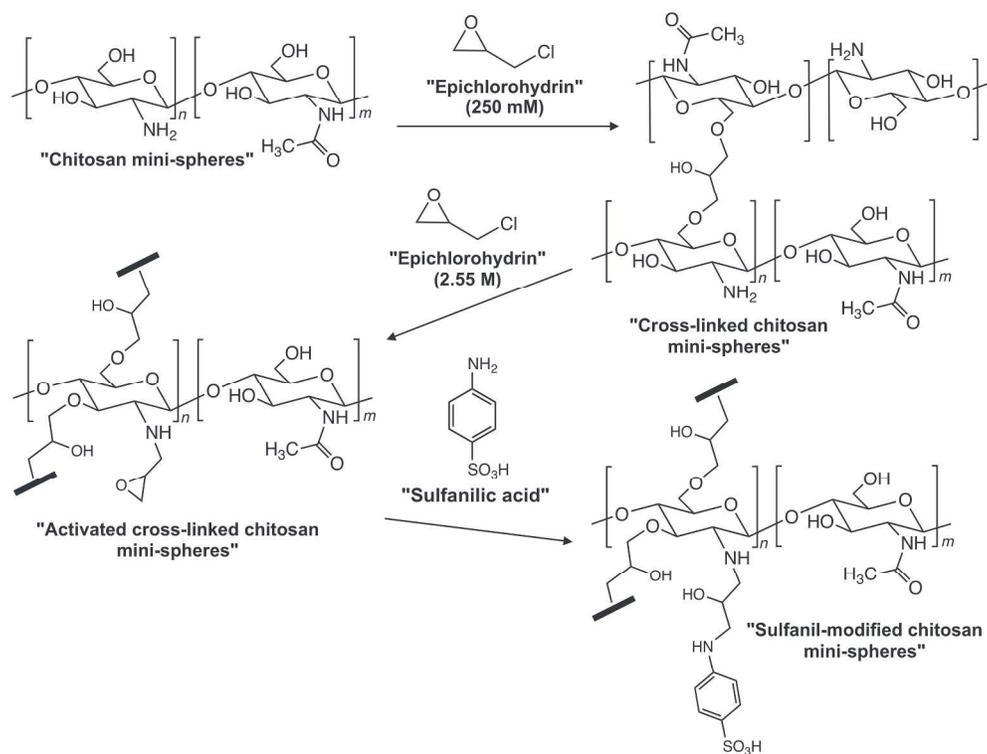
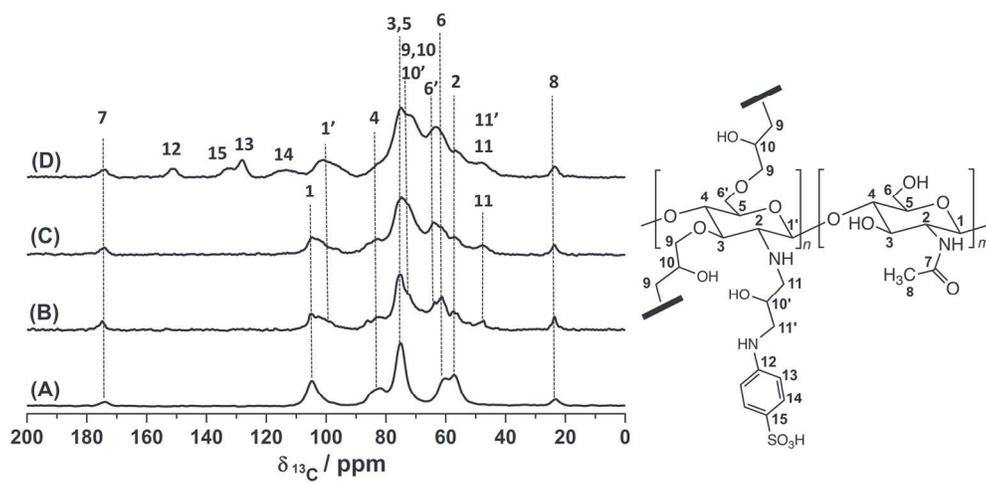Figure 1. Scheme of the synthetic reactions used to obtain the mini-spheres.

Figure 2. *ss*-NMR analysis. A, chitosan mini-spheres; B, epichlorohydrin 250 mM crosslinked mini-spheres; C, epichlorohydrin 2.55 M activated mini-spheres; D, sulfanilic acid-modified mini-spheres.
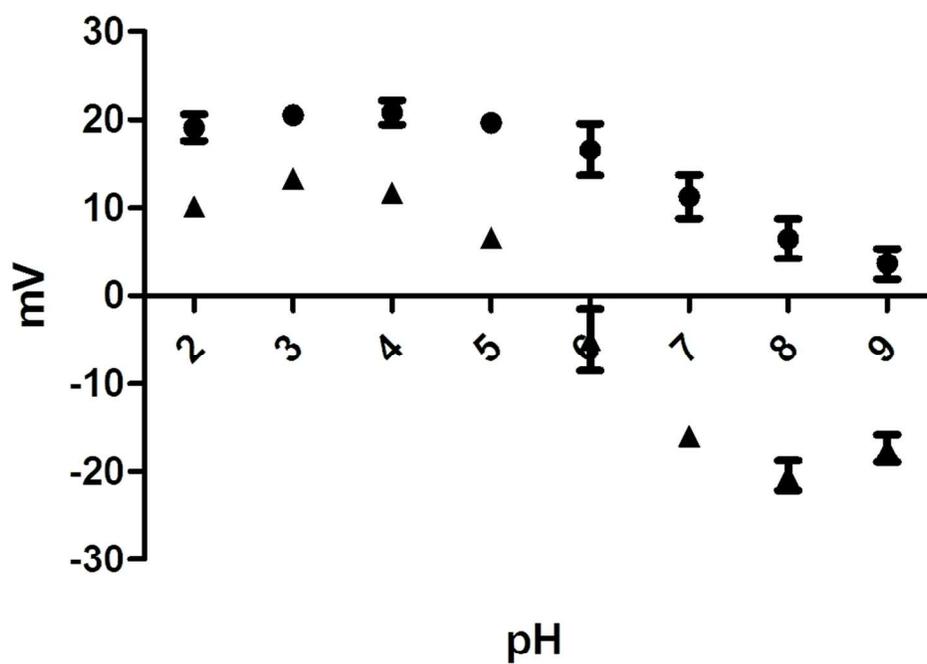
Figure 3. ζ potentials at different pH values of disrupted (•) chitosan mini-spheres and (▲) chitosan mini-spheres with sulfonic group.
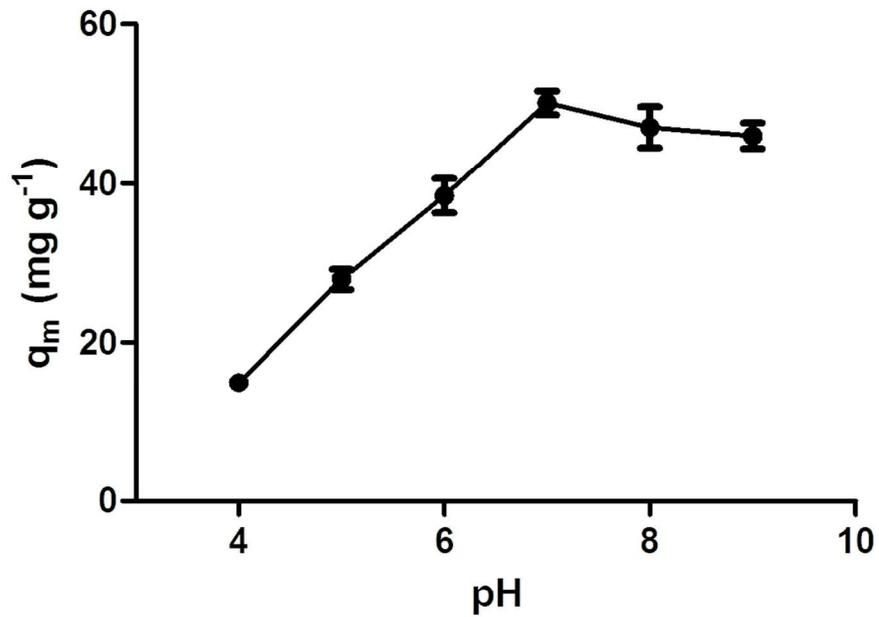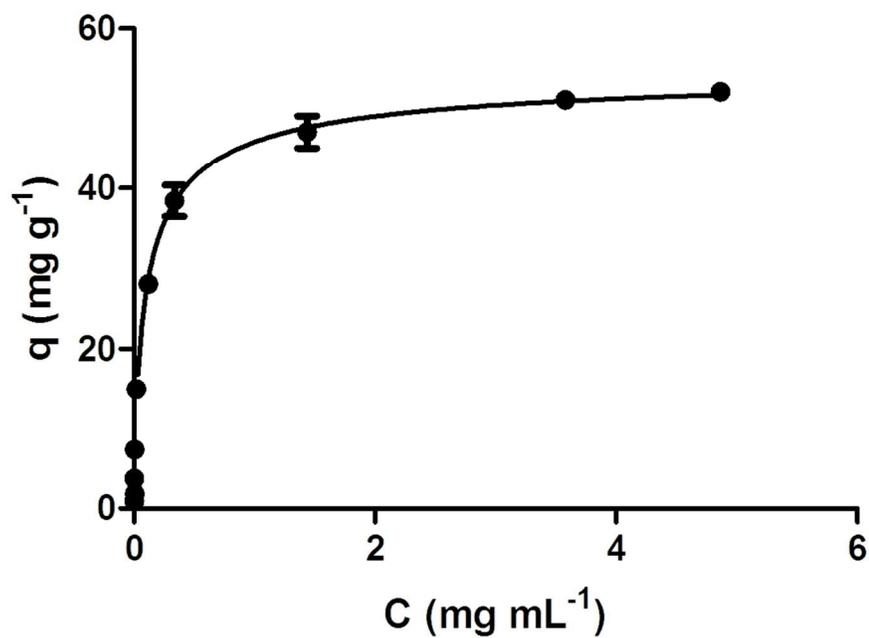
Figure 4. Pure protein adsorption studies. (A) Maximum adsorption capacity at different pH values, (B) Pure Lz adsorption isotherm at pH 7.0. C, equilibrium concentration of Lz in the supernatant; q, equilibrium concentration of Lz bound to the hydrated matrix (mg/g)
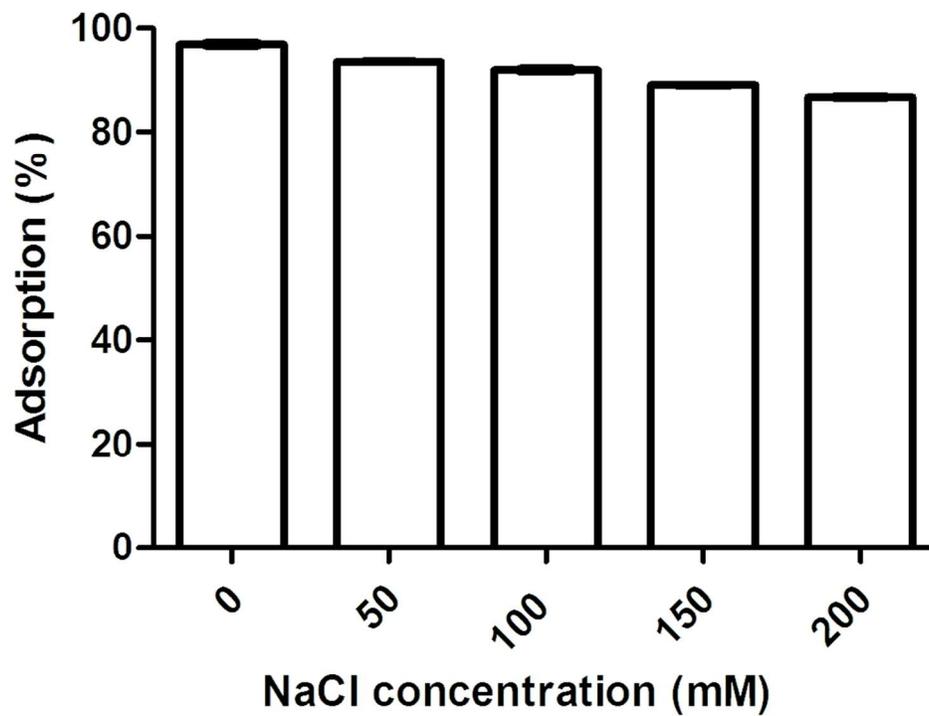
Figure 5. Effect of increasing ionic strength on Lz adsorption

Figure 6. Performance of different eluents for Lz elution. Different concentrations of NaCl were tested for elution buffers at different pH values: 3.0 (•), 5.0 (○), 7.0 (■) and 9.0 (□).

Figure 7. Optimization of the elution. Effect on the addition of propylene glycol to the best eluent for each pH; (■) without NaCl; (■) 25% propylene glycol; (■) 1.5 M NaCl; (□) 1.5 M NaCl + 25% propylene glycol.

Figure 8. Adsorption kinetics of Lz from egg white. For adsorption of Lz from egg white, 25, 50 or 100 mg/mL of matrix was incubated with diluted egg white 1:4 for 24 h at room temperature with gentle agitation. Samples were taken at 0.5 – 24 h. 25 mg/mL (•), 50 mg/mL (■) and 100 mg/mL (▲).

Figure 9. RP-HPLC chromatograms (215 nm) from the Lz purification process: egg white (A), egg white after adsorption (B), eluted sample (C), Lz standard 0.5 mg mL^{-1} (D).

Table 1. Lz purification process parameters

Adsorption (%)	Elution (%)	Yield (%)	Purity (%)
92.76 ±1.08	88.27 ± 4.78	81.90 ± 5.39	86.47

Figure 1. Scheme of the synthetic reactions used to obtain the mini-spheres.

129x99mm (600 x 600 DPI)

Figure 2. ss-NMR analysis. A, chitosan mini-spheres; B, epichlorohydrin 250 mM crosslinked mini-spheres; C, epichlorohydrin 2.55 M activated mini-spheres; D, sulfanilic acid-modified mini-spheres.

86x43mm (600 x 600 DPI)

Figure 3. ζ potentials at different pH values of disrupted (●) chitosan mini-spheres and (▲) chitosan mini-spheres with sulfonic group.

95x76mm (300 x 300 DPI)

Figure 4. Pure protein adsorption studies. (A) Maximum adsorption capacity at different pH values, (B) Pure Lz adsorption isotherm at pH 7.0. C, equilibrium concentration of Lz in the supernatant; q, equilibrium concentration of Lz bound to the hydrated matrix (mg/g)

105x78mm (300 x 300 DPI)

Figure 4. Pure protein adsorption studies. (A) Maximum adsorption capacity at different pH values, (B) Pure Lz adsorption isotherm at pH 7.0. C, equilibrium concentration of Lz in the supernatant; q, equilibrium concentration of Lz bound to the hydrated matrix (mg/g)

105x75mm (300 x 300 DPI)

Figure 5. Effect of increasing ionic strength on Lz adsorption

96x77mm (300 x 300 DPI)
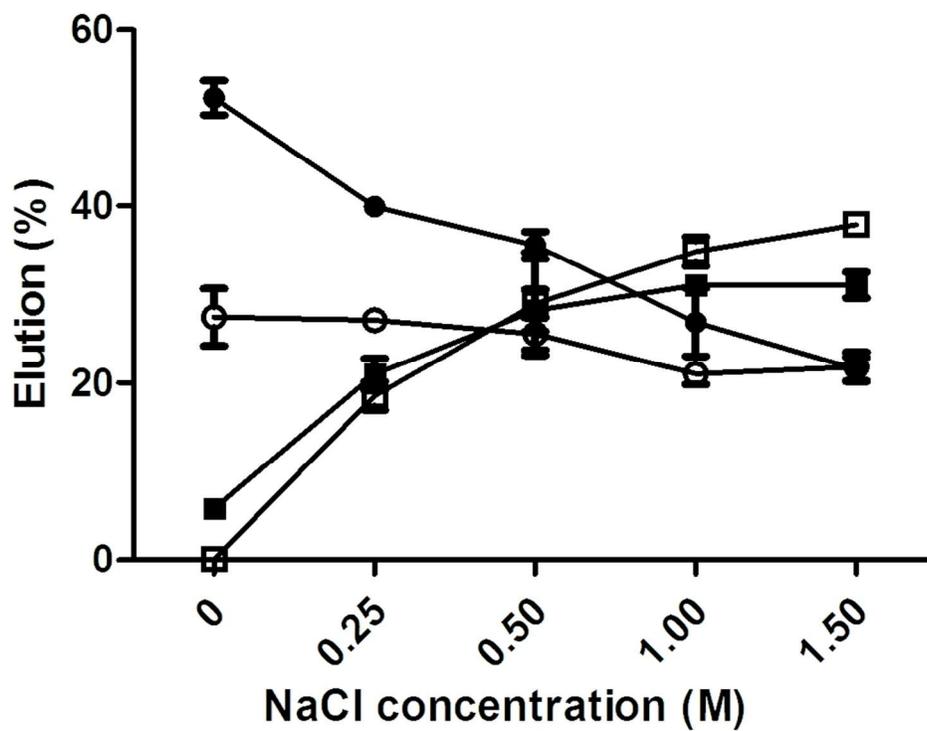
Figure 6. Performance of different eluents for Lz elution. Different concentrations of NaCl were tested for elution buffers at different pH values: 3.0 (●), 5.0 (○), 7.0 (■) and 9.0 (□).

94x77mm (300 x 300 DPI)
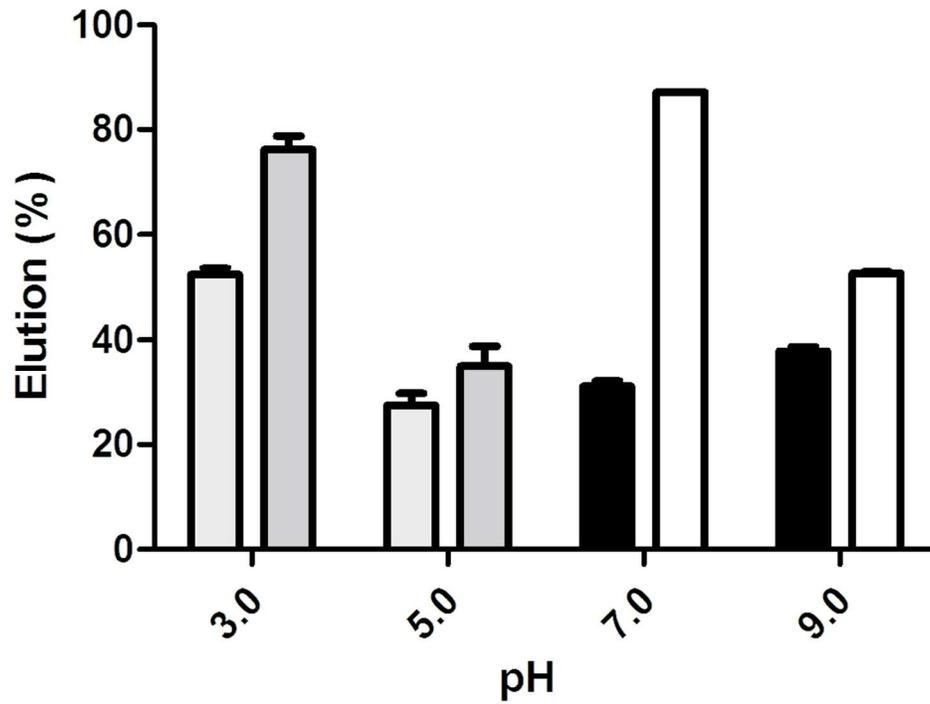
Figure 7. Optimization of the elution. Effect on the addition of propylene glycol to the best eluent for each pH; (■) without NaCl; (■) 25% propylene glycol; (■) 1.5 M NaCl; (□) 1.5 M NaCl + 25% propylene glycol.

96x81mm (300 x 300 DPI)
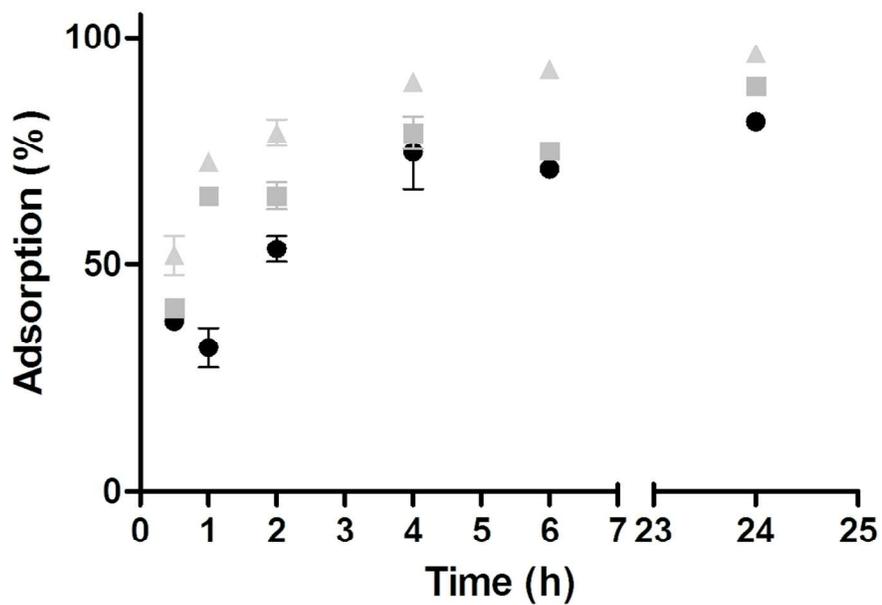
Figure 8. Adsorption kinetics of Lz from egg white. For adsorption of Lz from egg white, 25, 50 or 100 mg/mL of matrix was incubated with diluted egg white 1:4 for 24 h at room temperature with gentle agitation. Samples were taken at 0.5 – 24 h. 25 mg/mL (●), 50 mg/mL (■) and 100 mg/mL (▲).
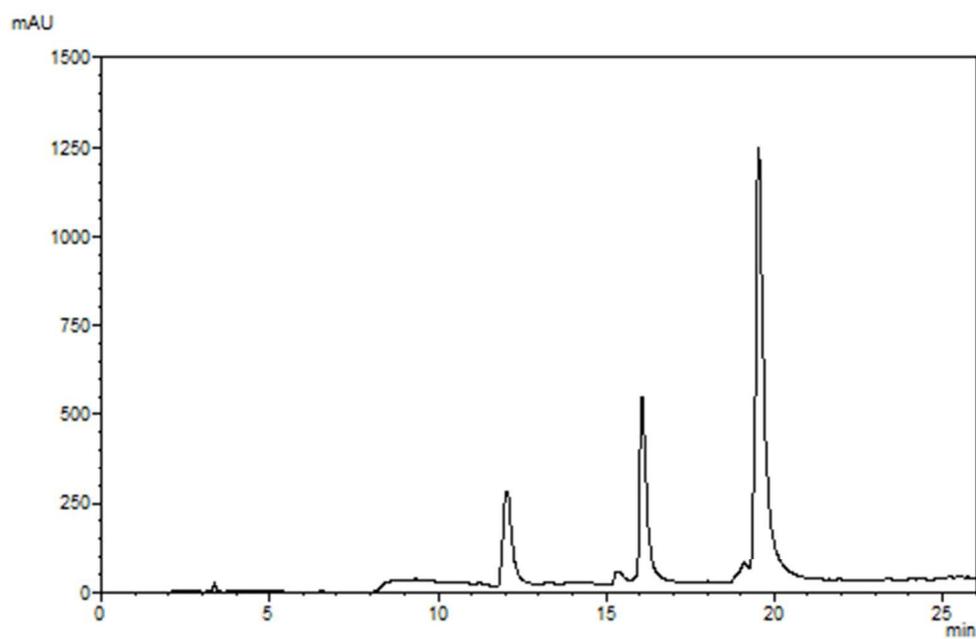
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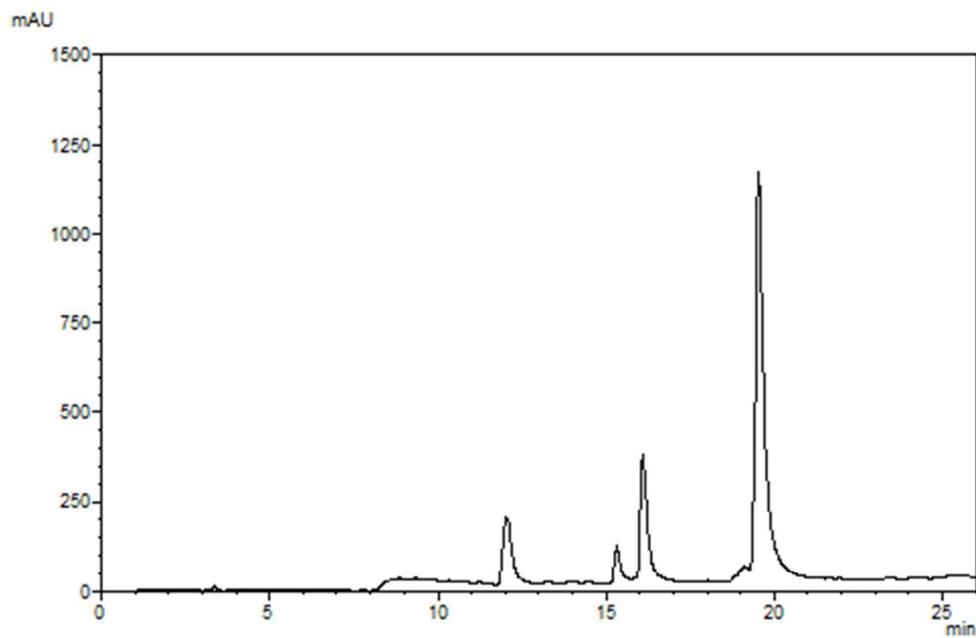
Figure 9. RP-HPLC chromatograms (215 nm) from the Lz purification process: egg white (A), egg white after adsorption (B), eluted sample (C), Lz standard 0.5 mg mL-1 (D).

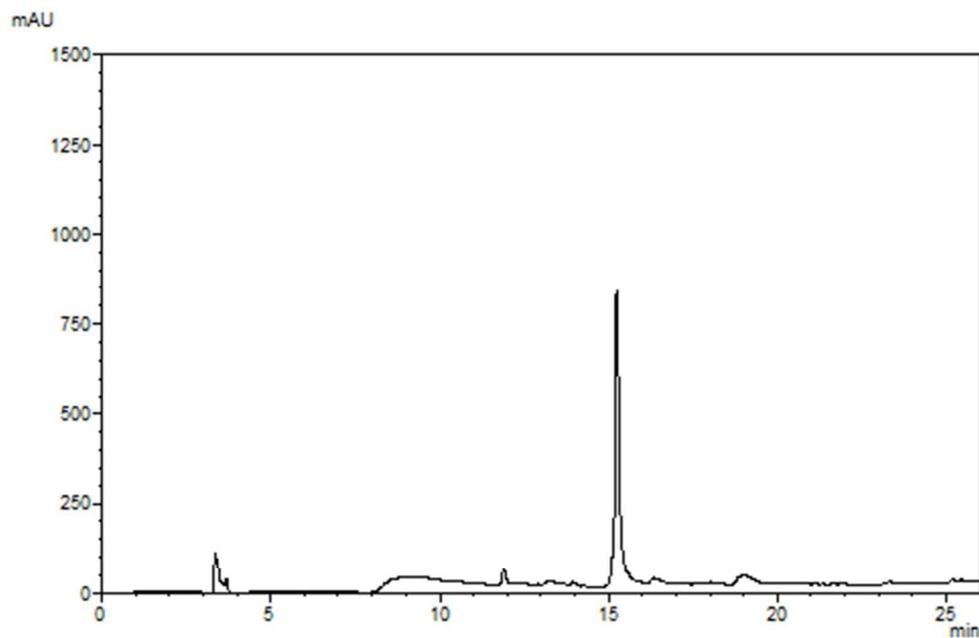130x86mm (300 x 300 DPI)

Figure 9. RP-HPLC chromatograms (215 nm) from the Lz purification process: egg white (A), egg white after adsorption (B), eluted sample (C), Lz standard 0.5 mg mL-1 (D).
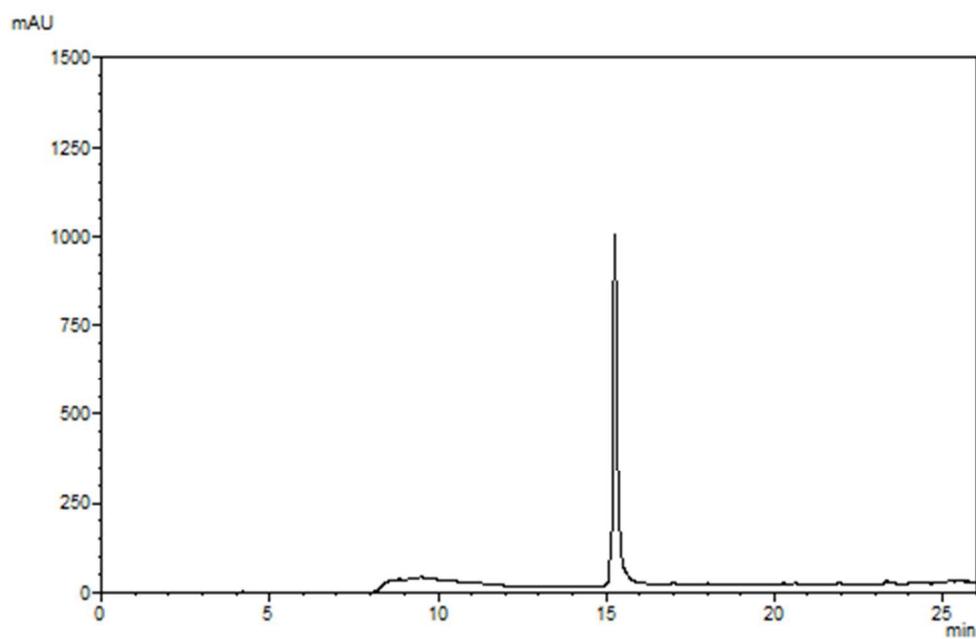
130x84mm (300 x 300 DPI)

Figure 9. RP-HPLC chromatograms (215 nm) from the Lz purification process: egg white (A), egg white after adsorption (B), eluted sample (C), Lz standard 0.5 mg mL-1 (D).

129x85mm (300 x 300 DPI)

Figure 9. RP-HPLC chromatograms (215 nm) from the Lz purification process: egg white (A), egg white after adsorption (B), eluted sample (C), Lz standard 0.5 mg mL-1 (D).

130x84mm (300 x 300 DPI)