Appl Biochem Biotechnol
https://doi.org/10.1007/s12010-018-2708-4

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# Immobilization of Carboxypeptidase A into Modified Chitosan Matrixes by Covalent Attachment

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Received: 20 November 2017 / Accepted: 19 January 2018
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**Abstract** Carboxypeptidase A (CPA) is a metalloexopeptidase that catalyzes the hydrolysis of the peptide bonds that are adjacent to the C-terminal end of a polypeptide chain. The enzyme preferentially cleaves over C-terminal L-amino acids with aromatic or branched side chains. This is of main importance for food industry because it can be employed for manufacturing functional foods from different protein sources with reduced hydrophobic amino acid content for patients with deficiencies in the absorption or digestion of the corresponding amino acids. In that way, strategies for effective multipoint covalent immobilization of CPA metalloenzyme on chitosan beads have been developed. The study of the ability to produce several chemical modifications on chitosan molecules before, during and after its coagulation to form carrier beads lead in a protective effect of the polymer matrix. The chemical modification of chitosan through the use of an N-alkylation strategy produced the best derivatives. N-alkyl chitosan derivative beads with D-fructose presented values of 0.86 for immobilization yield, 314.6 IU $g^{-1}$ bead for initial activity of biocatalyst and were 5675.64-fold more stable than the free enzyme at 55 °C. Results have shown that these derivatives would present a potential technological application in hydrolytic processes due to both their physical properties, such as low swelling capacity, reduced metal chelation ability and bulk mesoporosity, and increased operational stability when compared with soluble enzyme.

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Published online: 06 February 2018

**Keywords** Carboxypeptidase A · Immobilization · Multipoint covalent attachment · Chitosan · Swelling

## Introduction

Enzymes are proteins with proved industrial interests due to the innumerous catalytic reactions that can be performed with them. However, their stability and specificity in several bioprocesses is limited owe to the fact that they are active in a narrow range of physicochemical conditions causing enzyme degradation, altered selectivity, among other effects provoked when exposed to environments far from ideal [1]. So, enzyme immobilization inside a porous support can solve these problems. If the immobilization process is properly designed, the fixation of the enzyme to the support can improve the rigidity of the three-dimensional structure, enhancing the stability and resulting in a higher resistance to conformational changes induced by heat, organic solvents, and/or pH [2]. This effect can be achieved through multipoint covalent immobilization of each enzyme unit to several reactive groups present on the surface of an insoluble support [3], stablishing a concrete alternative to increase for the employment of biocatalysts in a larger number of industrial processes [4, 5].

One of the limitations for achieving derivatives with industrial feasibility is the high cost of some of the resins employed in enzyme immobilization. In that way, the searching of porous cheaper supports such as silica, alumina, glass, cellulose, chitin, and chitosan, among others, is a matter to be taken into consideration [6, 7].

In this direction, chitosan, a linear binary heteropolysaccharide which is composed of β-1,4-linked glucosamine (GlcN) with various degrees of N-acetylation of GlcN residues is an excellent alternative for achieving low cost supports being, in addition, non-toxic, biocompatible, biodegradable, and with antimicrobial effect against several pathogens [8]. It is also obtained from chitin, their natural precursor, through an alkaline deacetylation treatment responsible for the two key parameters to be always aware of in the existing types of chitosan: degree of deacetylation and molecular weight. In addition, it has a better solubility profile and loading (protein-loaded) properties, less crystallinity and viscosity, and improved particle properties such as tensile strength, elasticity, elongation, and moisture absorption in comparison with chitin [9–11].

On the other hand, its chemistry is of high interest because several reactions can be performed due to the presence of two hydroxyls, one acetamido, and one amine functional groups in the repeating hexosamide residue that chitosan has [12]. Moreover, due to the apparent pKa value of the free amino group of chitosan which is around 6.5, the polymer is only soluble in aqueous acidic solutions whereas it is insoluble in water and alkaline solutions. In that way, it can interact, in an acidic environment, with negatively charged surfaces and anionic systems, such as several polysaccharides, leading to the neutralization of chitosan amino groups which produce the modification of the physicochemical characteristics of these systems, ultimately giving rise to their unique functional properties [13–15].

The formation of physical hydrogels by chitosan without the need of any additive is based on electrostatic, hydrophobic, and/or hydrogen bonding reversible interactions (non-covalent nature) that occur between the polymer chains [16]. They are clearly dependent of physicochemical conditions such as pH, concentration, and temperature, among others, making these hydrogels unstable, and exhibiting a reversible gelation phenomenon.

Moreover, the swelling and the stiffness of those hydrogels can be tuned by adjusting the nature and the quantities of each component, in order to increase or decrease the number of interactions. In light of this, chitosan appears as an excellent biopolymer for the carrier preparation for enzyme immobilization due to these hydrogels that can be achieved by blending chitosan with other water-soluble non-ionic polymers through hydrophobic bonding [17–19].

Carboxypeptidase A (CPA) is a metalloenzyme containing $Zn^{2+}$ in the active site that hydrolyzes the peptide bonds of C-terminal residues with aromatic (tyrosine or phenylalanine) or large aliphatic side chains (isoleucine or leucine) amino acids. This exopeptidase rapidly removes, one at a time, hydrophobic amino acids from the C-terminal ends of a peptide chain. Its importance lies both on removing bitterness and reducing allergenicity from different foods, especially those from the Dairy Industry whose protein components are rich in hydrophobic amino acids, becoming a relevant enzyme for the production of hydrophobic amino acid-free hydrolyzates suitable for phenylketonuric patients [20, 21].

The fact that CPA needs a transition metal cation to be active, it represents a major issue in the immobilization process of this enzyme to chitosan matrixes, due to the ability of the latter to chelate the metal through the formation of dative covalent bonds with the amine groups present in the polysaccharide [22]. Hence, the metal ion would not be available for enzyme reaction and activity as well as stability would be compromised.

One way to solve this issue is to chemically modify chitosan polymer. In turn, it must be noted that any chemical modification performed on the chitosan molecule should not change the fundamental skeleton of the polymer, should keep the original physicochemical and biochemical properties, and finally, should bring new or improved properties to the carrier particles [23, 24]. Oligomerization, alkylation, acylation, thiolation, sulfation, phosphorylation, and graft copolymerization, among others, can be of potential applicability for this objective [25, 26]. Also, the formation of chitosan hybrids with sugars, cyclodextrins, and dendrimers have also used for new multifunctional macromolecules [27, 28].

In this paper, we analyzed some of the strategies to produce several chemical modifications on chitosan molecule before, during, and after its coagulation to form carrier beads. Additionally, we analyzed the behavior of biocatalysts obtained by multipoint covalent immobilization of the enzyme CPA on each prepared support as particle beads.

## Material and Methods

### Materials

Native chitosan (85.2% deacetylation degree) was acquired from Polymar (Fortaleza, Ce, Brazil). Carboxypeptidase A (EC 3.4.17.1) type II from bovine pancreas (44 IU $mg^{-1}$ of protein), N-benzoylglycyl-L-phenylalanine (hippuryl-L-phenylalanine) (HP), N-acetyl-L-tyrosine, 2,4,6-trinitrobenzenesulfonic acid (TNBS), polyvinyl alcohol (PVA, 99% hydrolyzed, with a molecular weight of 89,000–98,000 g $mol^{-1}$), N,N-dimethylformamide (DMF), sodium tri(poly)phosphate, polyaluminum chloride (PACl), calcium chloride, 25% (*v*/v) glutaraldehyde, 40% (*v*/v) glyoxal, sodium borohydride, D-lactose, D-fructose, and glycerol were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were of analytical grade.

## Methods

All experiments were performed at least three times. The data shown correspond to mean values of the three independent assays. Experimental error was never higher than 5%.

### *Preparation of Chitosan Beads*

Chitosan control particles were obtained following the protocol proposed by Manzo et al. [23].

**Chemical Modification of Chitosan Molecule Before Bead Preparation** For the preparation of N-alkyl supports, solid D-lactose or D-fructose was added up to a 4% (*w*/*v*) final concentration to freshly prepared 4% (*w*/*v*) chitosan solution in 5% (*v*/v) acetic acid and stirred until the sugars were dissolved. Then, the preparations were incubated at 50 °C during 4 h under constant stirring [23].

**Physical Modification of Chitosan Structure Before Bead Preparation** In order to produce thermogelation of chitosan, 1% (*w*/*v*) glycerol was added to chitosan solutions prepared in the same way as above [23].

**Bead Preparation with Chitosan-Polyelectrolyte Blends** Chitosan solution, as prepared above, were mixed with 1% (*w*/*v*) polyaluminum chloride, 10% (*w*/*v*) sodium tri(poly)phosphate or 1% (*w*/*v*) polyvinyl alcohol final concentration and each of them were stirred for 4 h at room temperature [23].

**Pre-Adsorption of an Agreeable Cation into Chitosan Beads** A solution of 4% (*w*/*v*) native chitosan in 5% (*v*/v) acetic acid was blown using a compressed air nozzle into a solution of 0.1 M NaOH (1:10) with the addition of 4% (*w*/*v*) $CaCl_2$ final concentration in order to achieve coacervate beads under slow stirring (50 rpm) for 24 h. The beads were filtered and rinsed thoroughly with distilled water, and then stored in refrigerator for further use [23].

**Superficial Chemical Modification of Chitosan Beads** Superficial primary amines react with TNBS or DMF modifying the hydrophobicity of the bead surface. Natural chitosan beads were prepared with the same procedure for control beads. Subsequently, they were re-suspended in 100 mM (1:10) bicarbonate buffer (pH 10.0) with a 1% (*v*/v) TNBS or 1% (*v*/v) DMF final concentration and agitated at 50 rpm and 55 °C for 6 h. The beads were filtered and rinsed thoroughly with an isopropanol-water mixture (1:1) followed by deionized water [19, 23].

**Preparation of Cross-Linked Chitosan Beads with Glyoxal** Glyoxal-treated chitosan beads were prepared with the same procedure as control beads. Then, particles were re-suspended in 100 mM (1:10) bicarbonate buffer (pH 10.05) with the addition of 3% (*v*/v) glyoxal and the suspension was stirred at 150 rpm and 25 °C for 16 h. The beads were filtered and rinsed thoroughly with distilled water [17, 23, 29].

In all cases, the modified chitosan beads were prepared and conserved using the same production system as that of the control beads [30].

### Physical Characterization of Beads

Chitosan beads' size distribution, density, and porosity were determined both by Matersizer 2000 particle size analyzer (Malvern Instrument, Malvern, UK), picnometry, and a gravimetric method, respectively. Furthermore, the swelling behavior of chitosan beads was measured at 25 °C using two buffer solutions of pH 7.0 and pH 10.0. The swollen weights, after removal of the surface liquid with a filter paper, were determined at certain time intervals until equilibrium swelling was attained. The swelling percentage was calculated by the following equation:

$$\%\text{Swelling} = \frac{W_t - W_0}{W_0} \times 100\% \quad (1)$$

where $W_0$ is the initial weight and $W_t$ is the final weight of the swollen hydrogel beads at a time $t$ when equilibrium swelling was achieved.

On the other hand, the effect of the different treatments performed over the surface and structure of the chitosan beads, were analyzed through both optical microscopy, with an Olympus BH-2 microscope (Olympus Corporation, Tokyo, Japan), and scanning electron microscopy, with a JEOL JSM 35C SEM (JEOL Ltd., Tokyo, Japan).

### Bead Activation with Glutaraldehyde

For activation with glutaraldehyde, 10 g of chitosan beads were suspended in 100 ml of 2.5% (*v*/v) glutaraldehyde solution in 100 mM buffer phosphate (pH 7.0). The suspension was stirred at 150 rpm and 25 °C for 6 h. The beads were filtered and rinsed thoroughly with distilled water to remove the excess of the activating agent.

### Immobilization Procedure

For immobilization, 10 mg of Carboxypeptidase A was dissolved in 10 ml of 100 mM (pH 10.0) bicarbonate buffer containing 10 mM N-acetyl-L-tyrosine. This latter compound is a competitive inhibitor included in the enzyme immobilization reaction to protect the enzyme's active site from coupling reagents [19]. Activated beads (1 g) were suspended in the enzyme solution under slow stirring (50 rpm) for 24 h at room temperature. Then, the derivative was filtered (Whatman filter paper 41), supernatant was recovered and immediately, the derivative was thoroughly rinsed with 25 mM Tris/HCl buffer (pH 7.5) containing 500 mM NaCl to remove unbound protein and finally, washed thoroughly with distilled water. All remaining solutions were recovered for protein and activity measurement.

CPA immobilization step was sealed through reduction of Schiff bases using a 120 mM sodium borohydride solution at 25 °C during 30 min under agitation. Beads were washed with distilled water to eliminate residual reducing agent and then rinsed with 50 mM phosphate buffer (pH 7.0) with 0.01% (*w*/*v*) sodium azide and stored at 4 °C until activity assays were done.

### Activity Assays for Soluble and Immobilized CPA Enzyme

Enzyme activity assay was performed by measuring the increase in absorbance at 254 nm produced by the hydrolysis of the synthetic substrate hippuryl-L-phenylalanine (HP). Soluble

enzyme or derivative suspension (100 μl) was added to the assay solution [2.9 ml of 1.0 mM HP prepared in 25 mM Tris/HCl buffer (pH 7.5) containing 500 mM NaCl] and the reaction was followed for 5 min. CPA activity test was carried out with a spectrophotometer Spectronic Genesys 5 (Thermo Fischer Scientific, Waltham, MA, USA), adapted with a thermostatic control and a mechanical stirring device, using 1 cm of light path quartz cuvette. In all assays, the interference of gel particles was taken into account using buffer without substrate for dispersion control. One international unit (1 IU) of CPA activity corresponds to the amount of enzyme that hydrolyzes 1 μmol of HP per minute under the assayed conditions.

*Enzyme Immobilization Parameters*

Immobilization performance was assessed through the calculation of enzyme loading capacity, recovered activity and thermal stability of each derivative.

The enzyme load on each biocatalyst was calculated by measuring the difference in concentration of protein in the supernatant (free enzyme) before ($P_0$) and after ($P_t$) of immobilization, taking into account the amount of protein recovered in the washing process ($P_w$) with 25 mM Tris/HCl, pH 7.5 (500 mM NaCl). Hence, the immobilization yield in terms of the incorporated protein to chitosan beads, $\eta_i$, was calculated according to:

$$\eta_i = \frac{P_0 - (P_t + P_w)}{P_0} \tag{2}$$

Protein content was determined through the methodology suggested by Bradford [31] using bovine serum albumin (BSA) as standard.

Recovered activity yield of the immobilization process was calculated by dividing the apparent specific activity of the immobilized enzyme derivatives, $A_{app}$, (IU $g_{beads}{}^{-1}$) and the theoretically immobilized (IU $g_{beads}{}^{-1}$) enzyme, because the offered enzyme load was known and the number of theoretically immobilized enzyme units per gram of gel beads can be calculated.

For evaluation of the thermal stability of each biocatalyst, both soluble enzyme and immobilized derivatives were incubated in a 25-mM Tris/HCl buffer (pH 7.5) with 500 mM NaCl at 55 °C. Periodically, aliquots of these suspensions were withdrawn and enzyme activities were assayed as described above. Thermal inactivation kinetics of the immobilized enzymes in chitosan are complex and cannot be described by a simple first-order model. Hence, pseudo half-lives ($\theta_{1/2}$) were defined as the operating time required for each biocatalyst to reduce its initial activity by 50% and it was obtained directly from the experimental tests. These values were representative of the stability of the derivatives and were used to compare it between all the synthesized CPA insoluble biocatalysts. Stabilization factor (SF) was calculated as the ratio between the half-life of the immobilized enzyme and that of the corresponding soluble enzyme.

## Results and Discussion

Chitosan content, coacervation medium composition, preparation process, and post-treatment of chitosan hydrogel beads are key factors for consideration of chitosan matrixes as supporting material for enzyme immobilization.

### 3.1. General properties of chitosan particles

Freeze-dried beads made from powder commercial chitosan, present a quasi-spherical shape and a compact structure (Fig. 1), having also a partially transparent appearance with a light-beige tone when appreciated in an optic microscope (Fig. 2). Moreover, there were no significant differences in shape and size of the particles obtained with the different materials and treatments, yielding an average diameter of about 750 ± 15 μm. The homogeneity of the beads regarding these physical properties may be explained due to the high shear force to which each droplet is subjected during the coacervation process where particles are formed. Also, carrier beads had a bulk density of 1.07 ± 0.02 g/ml, reinforcing the idea of the main influence of the technique adopted for particle synthesis on the characteristics of the supports obtained. The increase of chitosan concentration resulted in a higher hydrogel bead density which led to a more compact particle, while causing a decrease in the equilibrium water content, swelling, and porosity.

### Analysis of the Swelling Capacity of Chitosan Supports

First of all, the porosity and strength of the beads was influenced by the concentration of the chitosan acid solution, the degree of deacetylation of chitosan, and the type and concentration of the coacervation agents used. Higher differences between the different derivatives were found both in the porosity and in the swelling effect (Table 1) due to hydrogels that are highly swollen and hydrophilic polymer chains that can also absorb large amounts of water. The several treatments performed to chitosan conducted to different proportions of bulk, intermediate, and total bound water (primary and secondary bound water) for each support [32]. Furthermore, a direct relation was not seen between swelling ratios and the particle porous structure because the extent of hydration of the polymer structure; the presence of a cross-linking agent and the characteristics of the reaction media also had a profound influence on total trapped water. Moreover, these chemical and physical modifications done to the chitosan polymer also produced changes both in the functional groups present in the structure and in the interaction and disposition of the polymer chains, respectively, producing an alteration that affected the integrity and the elasticity of this polysaccharide. Hence, this led to different swelling ratios, porosities, and hydrophobic characteristics in the produced chitosan derivatives [33, 34].

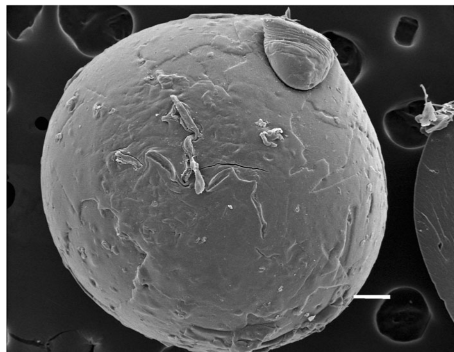


**Fig. 1** SEM micrographs of freeze-dried chitosan particles (110×)

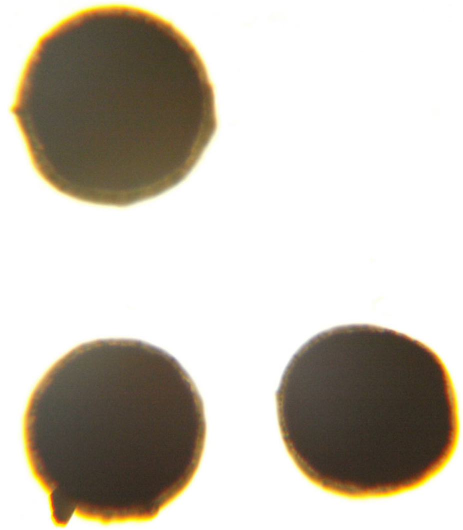


**Fig. 2** Optical photograph of obtained beads (20×)

In addition, and as a general tendency, the swelling capacity of ionic hydrogels in salt solutions was usually decreased in comparison to the values obtained in experiments with distilled water. Ions would capture bulk and bound water present in the interior of the particle due to a solvation effect and then migrate to the exterior of the bead due to an osmotic effect, causing a lesser enlargement of chitosan particle. The polarizability of ions would also have a main influence on polymer hydration and swelling factor because both polymer chemical groups and ions compete with strongly bound water in the first hydration layer finally causing a higher or lesser water retention depending on the type and concentration of polymer, charged groups and ions. In that way, a pH-dependent behavior due to the presence of ionizable groups was also seen when analyzing swelling capacity and experimenting a lower water retention in alkaline media in comparison with neutral pH. This was explained probably because as pH was increased, the net charge inside the hydrogel decreased so the osmotic effect due to electrostatic microenvironment between the interior and exterior of the particle was lower,

**Table 1** Porosity and swelling effect for the achieved supports

| Sample | Porosity | % swelling | |
|---|---|---|---|
| | | pH = 7.0 | pH = 10.0 |
| Native chitosan beads (control) | 0.62 ± 0.02 | 142 ± 9 | 115 ± 10 |
| N-alkyl chitosan derivative beads (lactose) | 0.80 ± 0.08 | 112 ± 8 | 102 ± 7 |
| N-alkyl chitosan derivative beads (fructose) | 0.81 ± 0.05 | 115 ± 7 | 98 ± 8 |
| Glycerol-chitosan beads | 0.51 ± 0.05 | 160 ± 8 | 134 ± 6 |
| PACl-chitosan beads | 0.69 ± 0.05 | 143 ± 9 | 123 ± 9 |
| Tri(poly)phosphate-chitosan beads | 0.57 ± 0.03 | 147 ± 8 | 96 ± 6 |
| Polyvinyl alcohol-chitosan beads | 0.56 ± 0.05 | 150 ± 10 | 118 ± 8 |
| Calcium-chitosan beads | 0.72 ± 0.08 | 134 ± 6 | 100 ± 8 |
| TNBS-chitosan beads | 0.49 ± 0.03 | 101 ± 8 | 72 ± 9 |
| DMF-chitosan beads | 0.47 ± 0.07 | 155 ± 10 | 136 ± 7 |
| Glyoxal-cross-linked chitosan beads | 0.59 ± 0.03 | 134 ± 9 | 125 ± 8 |

In all of cases, the data represents mean ± SD of three independent experiments

finally repelling a certain volume of intermediate and bulk water which was conducted to a particle shrinking [34–36].

On the other hand, considering that the swelling process is affected by specific relations between the molecules of the swelling medium and the polymer pendant groups (amines, amides, hydroxyls, and carboxyl), one can expect many kinds of polymer-solution interactions, and probably a complex enzyme kinetics with several diffusional constraints [36].

N-alkyl, calcium, TNBS, and glyoxal-cross-linked chitosan hydrogels are less sensitive to pH variation than control chitosan beads, showing better adaptability for their use in a subsequent alkaline hydrolysis process.

## Porosity of Chitosan Supports

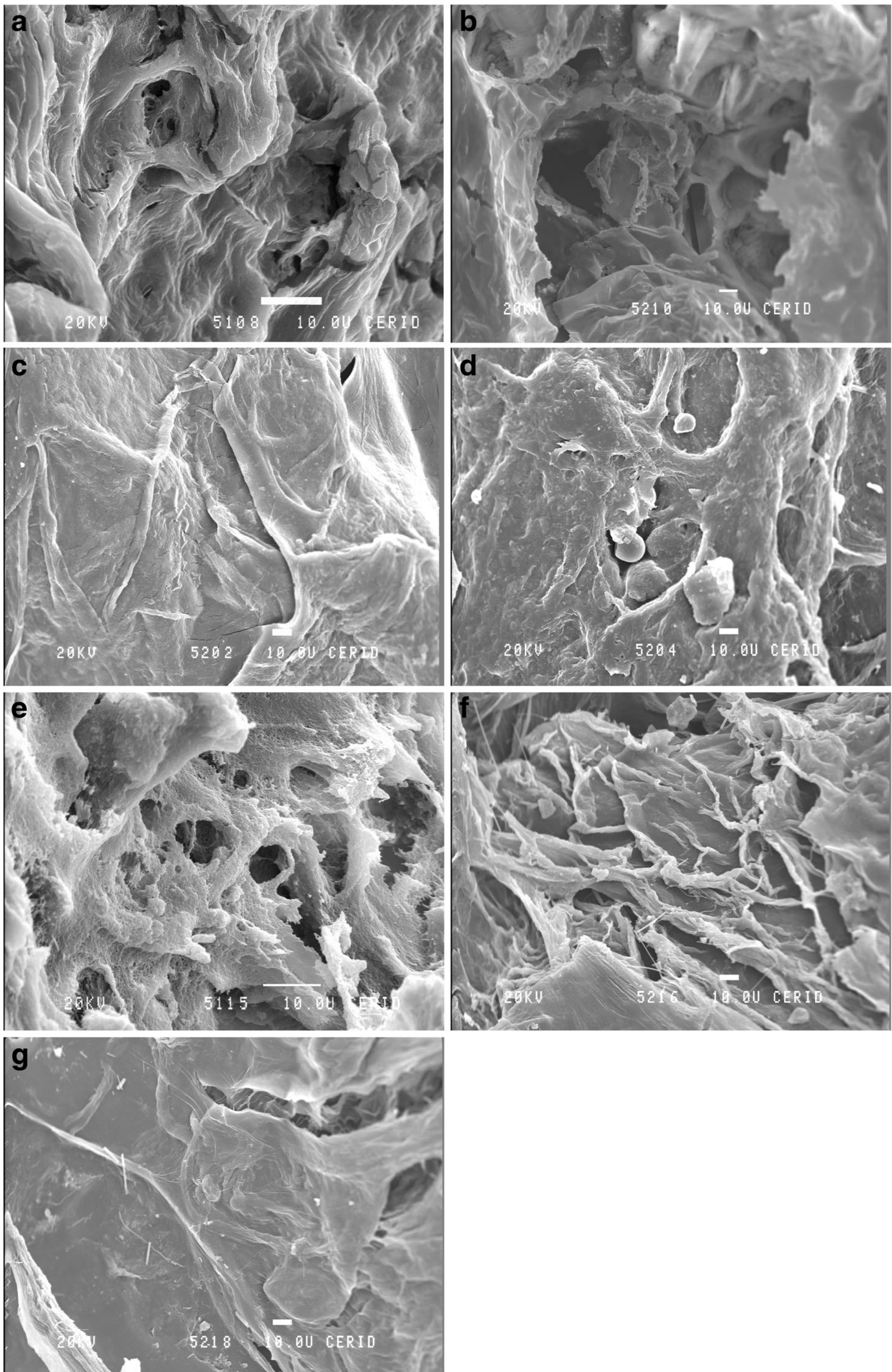
It can be observed that only N-alkyl chitosan derivatives (lactose and fructose), poly chloride-chitosan, and calcium-chitosan beads have higher porosity values than control (native chitosan beads), promoting a bigger protein loading capacity. Moreover, N-alkyl derivatives are those with the lowest swelling values, which ensure a higher mechanical stability of the particle.

Additionally, the dispersion between the porosity values obtained for each modified carrier was analyzed via microscopic observations. SEM micrographs showed that surface morphology of freeze-dried carrier particles revealed different porosities (Fig. 3a–g). Only control chitosan beads (Fig. 3a) and N-alkyl derivatives beads (Fig. 3b) exhibited surface roughness and bulk mesoporosity. Chitosan beads superficially treated either with TNBS (Fig. 3c) or DMF (Fig. 3d) showed a more condensed and flatter surface, but in their interior, the porosity was the same in comparison with control treatment. Hence, the difference in surface characteristics of the particles can subsequently lead to a steric effect on the network structure of these particles when comparing with control chitosan beads. Then again, the beads prepared with a blend of chitosan-polyaluminum chloride and chitosan with pre-adsorption of calcium ions (Fig. 3e), showed a more irregular and microporous surface.

On the other hand, the beads prepared with a blend of chitosan-sodium tri(poly)phosphate (Fig. 3f), glyoxal-cross-linked chitosan, chitosan-polyvinyl alcohol, and chitosan-glycerol (Fig. 3g) showed a smooth external area that showed interesting inner macropores when particle was sliced. This is because glyoxal, polyvinyl alcohol, and glycerol have been assumed that they may have a steric effect on the network structure of the beads in order to yield a right supramolecular structure with higher crystallinity [37–42]. Briefly, it can be observed that bead surface become smoother after treatment (addition) of chitosan with TNBS, DMF, sodium tri(poly)phosphate, polyvinyl alcohol, or glycerol.

## Achievement of CPA Chitosan Derivatives: Analysis of the Effects Produced by the Assayed Modifications to the Biopolymer

Table 2 presents the immobilization parameters for all obtained biocatalysts. Enzyme loading capacity of each chitosan derivative can be evaluated by analyzing the value of the immobilization yield. This value can be directly related to glutaraldehyde activation degree reached at the bead surface as well as the porosity of the derivative. Glutaraldehyde is one of the cross-linking agents most employed in protein immobilization due to its impressive ability to react with a wide number of functional groups through different reaction mechanisms in aqueous media. This versatility can be explained because glutaraldehyde can exist in multiple forms of different reactivity regarding pH, concentration, among other factors. As a counterpart, the


a
20KV 5108 10.0U CERID
b
20KV 5210 10.0U CERID
c
20KV 5202 10.0U CERID
d
20KV 5204 10.0U CERID
e
20KV 5115 10.0U CERID
f
20KV 5216 10.0U CERID
g
20KV 5218 10.0U CERID

◀ **Fig. 3** SEM micrographs of gel cross sections of the hydrogels. Control chitosan beads (**a**). N-alkyl derivative (D-fructose) (**b**). Chitosan beads superficially treated with TNBS (**c**) and DMF (**d**). Chitosan beads with pre-adsorption of calcium ions (**e**). Beads prepared with a blend of chitosan-sodium tri(poly)phosphate (**f**) and chitosan-glycerol (**g**)

generation of carrier-bound enzymes using this agent is complex due the difficulty of controlling the reactive species involved in enzyme-support interaction. Hence, the immobilization process using glutaraldehyde must be experimentally optimized for each enzyme and carrier used in the achievement of insoluble derivatives [43–47]. As can be observed, N-alkyl derivatives and chitosan beads with pre-adsorption of calcium ions showed the highest values for protein loading capacity, making evident a better surface characteristic for enzyme immobilization.

N-alkylation reaction is the reductive amination on the $-NH_2$ positions of the polymer matrix. Hence, the introduction of a non-charged entity (i.e., a neutral carbohydrate) gives to a random copolymer hydrophobic attractions that counterbalance the electrostatic repulsion produced by the $-NH_3^+$ groups, improving together the microenvironmental conditions that influences the immobilization process [48, 49]. Thus, D-lactose- and D-fructose-chitosan derivatives were 9 and 13 times more stable as the biocatalyst produced as control with the unmodified chitosan beads. In addition, as shown in L-arabinose isomerase (L-AI) immobilization process [23], chitosan treatment with sugars led to a decrease in chitosan-free amino groups causing a rapid reduction in the metal chelation power of the polymer. Hence, this chemical modification proved to be beneficial for immobilization of enzymes where a metal cofactor is essential for their activity, such as CPA and L-AI, among others.

On the other hand, physical modification of native chitosan with glycerol did not improve the immobilization parameters. The plasticizing effect of glycerol may have resulted in a smoother, less porous, and a more rigid polymer matrix that restricted the diffusion of the substrate to a greater extent [50]. The cross-link process promoted a steric impediment of the hybrid-chitosan beads pores, leading to a decrease on the pores size. As a consequence,

**Table 2** Immobilization parameters for the obtained biocatalysts

| Sample | Immobilization yield ($\eta_i$) | Initial activity of biocatalyst (IU $g^{-1}$ bead)$^+$ | Stabilization factor (SF)* |
|---|---|---|---|
| Native chitosan beads (control) | 0.75 ± 0.03 | 112.6 ± 0.1 | 455.38 |
| N-alkyl chitosan derivative beads (lactose) | 0.84 ± 0.04 | 260.9 ± 0.3 | 4152.45 |
| N-alkyl chitosan derivative beads (fructose) | 0.86 ± 0.02 | 314.6 ± 0.2 | 5675.64 |
| Glycerol-chitosan beads | 0.55 ± 0.02 | 92.4 ± 0.1 | 338.14 |
| PACl-chitosan beads | 0.79 ± 0.04 | 146.8 ± 0.3 | 874.66 |
| Tri(poly)phosphate-chitosan beads | 0.79 ± 0.05 | 135.8 ± 0.2 | 1093.33 |
| Polyvinyl alcohol-chitosan beads | 0.66 ± 0.03 | 82.5 ± 0.3 | 756.55 |
| Calcium-chitosan beads | 0.82 ± 0.05 | 212.4 ± 0.2 | 1664.97 |
| TNBS-chitosan beads | 0.47 ± 0.02 | 78.6 ± 0.1 | 1976.93 |
| DMF-chitosan beads | 0.34 ± 0.03 | 75.3 ± 0.1 | 1265.79 |
| Glyoxal-cross-linked chitosan beads | 0.69 ± 0.02 | 82.4 ± 0.3 | 970.58 |

In all of cases, the data represents mean ± SD of three independent experiments

These values were always in agreement with the reduced activity in the supernatant and in the washes performed to biocatalysts

$^+$ Initial soluble enzyme loading = 44 IU

*$t_{1/2}$ of soluble enzyme = 4.3 min (at 55 °C and pH 7.5)

molecule diffusion inside the beads was harmed and there is a possibility of having combined diffusive and steric effects. Thus, the real effective diffusion coefficient of substrate in derivative pores containing low quantity of immobilized enzyme will be lower than non-restrained particles.

The addition of poly aluminum chloride to chitosan improves its properties to absorb cations [51], which could finally produce an activation effect if desired metal ion was also present when blend was formed. However, experimentally, it has only been verified as a significant improvement in the stabilization factor.

Porous chitosan—tripolyphosphate beads, prepared through ionotropic cross-linking, did not substantially improve neither the protein load nor the biocatalyst initial activity, but the stabilization factor was significantly higher compared to control particles.

The intermolecular interaction between PVA and chitosan enhanced the mechanical properties (tensile strength and flexibility) and increased the number of hydrophilic groups (−OH) [52]. While it is true that bulk and surface hydrophilicity are very important parameters for application in enzyme immobilization, in this case, it was not improved neither the protein load nor the biocatalyst initial activity and only managed to increase the stabilization factor.

Pre-adsorption of calcium ions into chitosan beads did produce improvements in the immobilization yield, initial activity of biocatalyst and stabilization factor values. Although calcium ions act as protective agents of the enzyme while producing a considerable decrease in the affinity of chitosan towards zinc ion, this effect was less important than the achieved N-alkylation of the chitosan matrix, although this must be taken into account.

Chemical modification of native chitosan with TNBS or DMF did not promote the immobilization parameters. Moreover, they reacted with the amine groups of the support reducing the number of reactive sites for glutaraldehyde and increasing, simultaneously, the relative hydrophobicity of the support, which negatively impacts on multipoint covalent immobilization of the protein [23, 53]. However, the action of TNBS and DMF generally resulted in a higher half-live time and, consequently, in a higher stabilization factor compared to pure chitosan.

The glyoxal-cross-linked spheres were more compact with enhanced mechanical properties. Moreover, more hydrophobic beads were achieved in comparison with native chitosan. Therefore, better results in the stabilization factor related to pure chitosan were achieved. This is because glyoxal has been assumed that it may have a steric effect on the network structure of cross-linked particles compared to non-modified chitosan beads.

## Conclusions

Several strategies were conducted in order to produce several chemical modifications on chitosan molecule before, during, and after its coagulation to form carrier beads, resulted in a protective effect of the matrix. Thermal stability of synthesized derivatives resulted to be higher than the free enzyme allowing CPA to be more resistant to changes in the environmental parameters, such as temperature. In addition, many of these treatments performed to chitosan also reduced their abilities for metal chelation, successfully adapting the biopolymer for its use in the immobilization of metalloenzymes. Finally, the best chitosan-modified derivatives were obtained by means of the application of an alkylation strategy. In that way, N-alkyl chitosan derivative beads (fructose) presented values of 0.86 for the immobilization yield, 314.6 IU $g^{-1}$ beads for the initial activity of biocatalyst and were 5675.64-fold more stable than the free

enzyme at 55 °C. Actual studies are focused on employing the best CPA insoluble derivatives on producing hydrolysates of food proteins mainly from milk and by-products for achieving food preparations with low allergenicity and standardized for phenylketonurics consumers.

**Funding** This work was partially sponsored with funds of the projects CAI + D 2016 No. 50420150100051LI (Universidad Nacional del Litoral, Santa Fe, Argentina), PIP 2015-2017 No. 11220150100606CO (CONICET, Buenos Aires, Argentina), and PICT-2015-0365 (Agencia Nacional de Promoción Científica y Tecnológica, Buenos Aires, Argentina). The authors would also like to thank the financial support of the Brazilian Research Agencies CNPq, CAPES, FINEP, FUNCAP, and FAPESP.

**Compliance with Ethical Standards**

**Conflict of Interest** The authors declare that they have no conflict of interest.

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