



HEALTH SCIENCES

β -galactosidase Encapsulated in Carrageenan, Pectin and Carrageenan/Pectin: Comparative Study, Stability and Controlled Release

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Abstract: The present study investigated the encapsulation of β -galactosidase in carrageenan, pectin and its hybrid hydrogels by using the ionotropic gelation method. The material obtained was characterized by Fourier transform infrared spectroscopy (FTIR), thermogravimetric analysis (TG/DTG) and scanning electron microscopy (SEM). The effects of pH, temperature and storage time were evaluated in terms of the catalytic activity of the free and encapsulated enzyme. Addition studies were conducted evaluating the performance of catalytic activity in vitro conditions. Carrageenan, pectin and hybrid hydrogels presented encapsulation efficiency of $58 \pm 1\%$, $72 \pm 1\%$ and $77 \pm 2\%$, respectively. The pectin hydrogel showed the higher β -galactosidase activity in pH and temperature tests. However, the carrageenan hydrogel exhibited best stability after been stored for three months. Carrageenan and pectin hydrogels were 2.0 and 2.4 times more efficiently than commercial tablet in the releasing β -galactosidase under in vitro conditions, respectively. The results suggest that pectin and carrageenan hydrogels may be useful for the development of new formulation of β -galactosidase.

Key words: biopolymer, encapsulation, enzymatic activity, ionotropic gelation, lactase.

INTRODUCTION

The enzyme β -galactosidase is responsible by catalyzing the hydrolysis of milk lactose into its monosaccharides glucose and galactose. In the absence or insufficiency of β -galactosidase, gastrointestinal problems occur, such as the intolerance to lactose that affects about 75% of the world population causing symptoms like diarrhea, flatulence, severe abdominal pain and distention and swelling (Nichele et al. 2011, Facin et al. 2015, Zhang et al. 2016).

The use of β -galactosidase represents an alternative for the treatment and relief of symptoms caused by such intolerance, which is orally administered as tablets or capsules (Montalto et al. 2006). However, the clinical use

of enzymes presents some limitations like the elevated cost, instability and short life span after ingestion. Moreover, the β -galactosidase passes through the acid pH of stomach after oral ingestion, what may compromise the structural integrity of the enzyme and consequently its activity on hydrolyzing the lactose (Nichele et al. 2011). The oral administration of drugs containing enzyme as active components is not suitable due to the high susceptibility to digestive enzymes along the gastrointestinal tract. An alternative to protect the enzymes from degradation and allow its release in specific organs is their immobilization (Muheem et al. 2016).

The immobilization of enzymes has been studied during several years, what indicates

a continuous interest by this area, probably in function of the already known benefits provided by the enzymatic immobilization and the desire to improve matrices and methods. The immobilization of β -galactosidase may be obtained through diverse methods like adsorption, covalent bonding, chemical aggregation, imprisonment and encapsulation (Ansari & Husain 2010). The β -galactosidase has been immobilized using a great variety of solid supports with the purpose of being used in different applications (Panesar et al. 2010, Wang et al. 2018, McClements 2018).

The immobilization of β -galactosidase may preclude the chemical and biological degradation, making it more stable, therefore tables containing the encapsulated enzyme may represent a promising alternative to complement the absence of this enzyme in humans intolerant to lactose (Facin et al. 2015, Montalto et al. 2006). The enzymes remain entrapped in a porous matrix that isolate them from the direct contact with the surrounding medium, thus making them more stable (Zhang et al. 2016, Eş et al. 2015).

The ionotropic gelation was chosen as immobilization method in the present work, which consists in the complexation between one polymer and opposed charge ions that suffer ionic reticulation when enter in contact with the polymer, then forming intra and intermolecular crossed bonding (Patil et al. 2012). In this method, a polymeric solution containing the enzyme is injected in other hardening solution under conditions that promote the polymer gelation. This procedure results in the formation of hydrogels, which have important properties for biological, pharmaceutical and medical applications: they are biodegradable, biocompatible and do not present toxicity (Van Vlierberghe et al. 2011).

The ionotropic gelation has been used to encapsulate the β -galactosidase. Zhang et al. (2016) investigated the potential of carrageenan-based hydrogel to encapsulate β -galactosidase and results indicated that it may be useful in encapsulating systems. However, the authors did not conduct β -galactosidase delivery studies in simulated gastrointestinal conditions. In Wahba (2016) research, the β -galactosidase was covalently immobilized onto calcium pectinate gel beads that were cross-linked with the polyethyleneimine and glutaraldehyde for the applications of industrial enzymes. Despite of good results found, mainly related to efficient encapsulation and mechanical stability allowing its reusability, this hydrogel could not be employed for pharmaceutical and medicinal applications due to presence of toxic reticulant agent.

The present work aimed to encapsulate β -galactosidase, using ionotropic gelation, in hydrogels of carrageenan, pectin (without employed reticulate agents) and a hybrid containing the two biopolymers. The hybrid hydrogel was performed based on the fact that such biopolymers used provide advantages from possible synergistic effects on the mechanical properties. The objective is obtained hydrogels biocompatible that shows better thermal and β -galactosidase delayed release *in vitro* conditions (simulated gastric and intestinal fluids) than commercial tablets containing this enzyme. Posteriorly, the material obtained was characterized by Fourier transform infrared spectroscopy (FTIR), thermogravimetric analysis (TG/DTG) and scanning electron microscopy (SEM). The effect of pH and temperature was also assessed to verify the capacity of hydrogels to protect the encapsulated β -galactosidase under such specific conditions. Moreover, the influence of storage time on the stability of hydrogels was evaluated. Finally, the hydrogels

were submitted to the study of β -galactosidase release *in vitro* to evaluate the behavior of simulated gastrointestinal condition. The result of this test was compared with the commercial tablet containing β -galactosidase in the same conditions. In the current article, special emphasis has been placed on a systematic and comparative study involved three hydrogel prepared to encapsulate β -galactosidase with minimal chemical modification.

MATERIALS AND METHODS

Materials

β -galactosidase *Aspergillus Oryzae*, Sigma Aldrich (specific activity: 13.4 U/mg); kappa-type Carrageenan, Sigma Aldrich; pectin with low esterification (35%) degree type LM -101 AS, CP Kelco; O-nitrophenol- β -D-galactoside (ONPG), Sigma Aldrich, commercial tablet containing β -galactosidase (9,000 U) and gastro-resistant capsule of hypromellose, Fagron were employed. The other reagents used presented degree of analytical purity of the trademark Synth. Ultra-pure water type I (Genaka) was also use.

β -galactosidase encapsulation

The process of β -galactosidase encapsulation in carrageenan, pectin and hybrid hydrogels (containing these two polymers) was conducted using a method similar to those described by Zhang et al. (2016). All the hydrogels were prepared in triplicates and followed the same procedure. Firstly, 20 mL of solution of β -galactosidase diluted in buffer Tris/HCl, 50 mM pH 7.0 were prepared containing 2,000 units (U) of enzyme activity. In sequence, 20 mL of polymeric solution were prepared by dissolution of the polymer in distilled water under agitation at 60 °C during 1 h and after the temperature was reduced to 37 °C. The solutions of enzyme and polymer were then mixed (1:1

v/v) under continuous agitation. The hydrogel spheres charged with enzymes were prepared using a syringe with needle of 25 mm to drip the β -galactosidase/polymer solution in 40 mL of saline solution (KCl for the carrageenan hydrogel, CaCl₂ for the pectin hydrogel and both salts in the hybrid hydrogel) under continuous agitation. The hydrogel spheres remained for more than one hour at room temperature to complete reticulation and then were collected by filtration and washed with distilled water and buffer Tris/HCl 50 mM, pH 7.0 to remove any excess of ions from the surface. After this step, hydrogels were stored in refrigerator (4 °C) until reaching constant mass to obtain dehydrated hydrogel employed in others essays. The same procedure was conducted for the control hydrogel (without enzymes). In this case the enzymatic solution was substituted by 20 mL of buffer Tris/HCl, 50 mM, pH 7.0.

The carrageenan hydrogel was obtained using carrageenan solution 1.75% (m/v) and KCl 6.0% (m/v). For the pectin hydrogel, a pectin solution 3.0% (m/v) and CaCl₂ 0.50% (m/v) was used. Finally, for the hybrid hydrogel, 3.0 and 0.75% (m/v) of pectin and carrageenan and 1.0 and 2.0% (m/v) of the salts CaCl₂ and KCl were used, respectively. The concentration of polymers and salt were obtained after a univariate study considering some physical aspects of the hydrogels, like the spherical form, the resistance to handling, the smooth surface and homogeneity among spheres.

Catalytic activity of β -galactosidase

The method described in USP Pharmacopoeia (2005) was applied to evaluate the enzymatic activity during the preparation of hydrogels (efficiency of encapsulation) as well as in the tests of pH and temperature (stability of hydrogels) and dissolution (study of controlled release *in vitro*). This method consists in a

spectrophotometric reaction using O-NPG as substrate. O-NPG is a colorless compound that forms galactose (colorless) and O-NP (yellow) after hydrolysis. Initially, 2,000 μ L of solution of the substrate ONP-G 3.7 mg/mL prepared in buffer acetate pH 4.5 were incubated during 10 min in water bath maintained at 37.0 ± 0.1 °C. After this time, 500 μ L of sample were added, the system was agitated in vortex for 10 s and incubated during 15 min in water bath maintained at 37.0 ± 0.1 °C. Posteriorly, 2,500 μ L of solution of sodium carbonate 10% (m/v) was added to stop the enzymatic reaction. The solution was transferred to a beaker where 20 mL of distilled water was added. The diluted content was analyzed in spectrophotometer at 420 nm using a glass bucket of optical path of 1 cm by means of the spectrophotometer model Evolution Thermo Scientific® 60 (Waltham, EUA). The concentration of β -galactosidase was calculated using an analytic curve previously determined with different concentrations (range from 50 to 500 U/L) from a stock solution 500 U/L prepared in buffer Tris/HCl, 50 mM, pH 7.0. All analyses were conducted in triplicate. In this case, one unit of enzymatic activity (U) was defined as value of enzyme catalyzing the hydrolysis of 1 μ mol of ONPG per minute.

Efficiency of encapsulation

During preparation of hydrogels, two aliquots were collected to monitor the encapsulation efficiency of the enzymatic solution (considered 100% of β -galactosidase used in the system) and of the supernatant after the hydrogel was collected by filtration. The activity of β -galactosidase in the aliquots was determined by the method described in the section previous. The efficiency of β -galactosidase encapsulation in hydrogels was defined as the difference between the activity of β -galactosidase present in the enzymatic solution and the supernatant.

The yield of encapsulation was calculated as follows:

$$E \text{ (\%)} = \left(\frac{A_i - A_f}{A_i} \right) \times 100 \quad (1)$$

EE = efficiency of encapsulation (%), A_i = activity of β -galactosidase (U) available in the system and A_f = activity of β -galactosidase (U) recovered from the supernatant.

Methods for the characterization of hydrogels

Fourier transform infrared spectroscopy (FTIR) analysis

The FTIR spectra of hydrogels after β -galactosidase immobilization were obtained by means of spectroscope Thermo Scientific, USA, model iS50 FTIR. The samples of hydrogels were evaluated in the region from 400 to 4000 cm^{-1} with resolution of 4 cm^{-1} and 64 scanings using the accessory of de ATR GladiATR diamond (Pike Technologies, USA).

Thermogravimetric analysis

Thermal analyses of pure hydrogels and after β -galactosidase immobilization were conducted using the thermos-balance Hitachi (Japan), model TG / DTA7300. The temperature ranged from 30 to 500 °C with heating rate of 10°C / min under nitrogen atmosphere. Analyses were conducted using aluminum crucibles with approximately 3 mg of sample. The equipment was previously calibrated with indium pattern.

Scanning electron microscopy (SEM)

Images of SEM were obtained using microscope TM3000 Hitachi Analytical Table Top (Tarrytown, NY, EUA) with voltage acceleration at 15 kV. Samples were placed in scotch tape of double carbon without any pre-treatment to obtain the micrographies. Quantitative analyses of image

data was performed using free software *ImageJ* version 1.52a developed by Schneider et al. (2012).

Effects of pH and temperature

The effect of pH on the enzymatic activity was evaluated incubating 20 mg of each hydrogel in 10 mL of solution with pH ranging from 1.0 to 7.0 during 30 min at 37°C, in rotating shaker (model NT 232, Novatecnica). In this test, solutions of HCl 0.1 M at pH 1.0 and 2.0 and buffer solution Tris/HCl, 50 mM were used, which were corrected with NaOH 0.1 M for pH between 3.0 and 7.0.

The effect of temperature on the enzymatic activity was determined by the incubation of 20 mg of each hydrogel in 10 mL of buffer solution Tris/HCl, 50 mM, pH 7.0 at 25 – 60 °C during 30 min, also in rotating shaker.

Both for the essays of pH and temperature, analyses were conducted using dehydrated hydrogels recently prepared and with dehydrated hydrogels hermetically packaged after three months of storage in refrigerator at 4 °C. For comparative purposes, analyses were also done using 8.7 U of β -galactosidase pattern (free enzyme). The activity of β -galactosidase was evaluated using the methodology described previously. The data were expressed as relative activity (%), considering the absolute value of 8.7 U of β -galactosidase as 100% obtained by pectin hydrogel at pH 7.0. The results were compared using Student t test with confidence interval of 95%. All the analyses were conducted in triplicates.

Study of β -galactosidase release *in vitro*

The dehydrated hydrogels containing β -galactosidase (60 mg) were placed in gastro-resistant capsules of hypromellose Fagron. The effect of simulated conditions of gastrointestinal tract was made using 30 mL of simulated gastric fluid (SGF, HCl 0.1 M, pH 1.2) during two hours.

After this period, the medium was changed by simulated intestinal fluid (SIF, buffer Na_2HPO_4 , 100 mM, pH 6.8) in which capsules remained for one more hour. The system remained under agitation at 100 rpm at 37 °C by means of a rotative shaker.

At specific intervals (30, 60, 90, 120, 135, 150, 165 and 180 min) one aliquot of 500 μL was collected and the corresponding medium was replenished to maintain the volume constant. The β -galactosidase activity in the aliquots was determined by the method described previously. The same procedure was made using 3.17 mg of the commercial tablet containing β -galactosidase for comparative purposes. This mass contained approximately 26.1 U of enzyme. The data were expressed as relative activity (%), considering the absolute value of 29 U of β -galactosidase as 100% obtained by pectin hydrogel at pH 7.0. The results were compared using Student t test with confidence interval of 95%. All analyses were conducted in triplicates.

RESULTS AND DISCUSSION

Encapsulation of β -galactosidase in hydrogels

Preliminary experiments were conducted to establish the adequate conditions to form the hydrogel with proper physical stability and consequently higher percentage of the enzyme encapsulation. In this study, the efficiency of encapsulation was defined as the percentage of enzyme confined within the hydrogen spheres in relation to the total amount of active enzyme initially present in the solution used to form the hydrogel. Thereunto, as described in section of catalytic activity of β -galactosidase, the activity of the remaining enzyme was analyzed by spectrophotometry after removing the hydrogel spheres formed in the medium. Carrageenan, pectin and hybrid hydrogels presented efficiency of encapsulation of $58 \pm 1\%$, $72 \pm 1\%$ and $77 \pm$

2%, respectively. This fact could be related to different mechanism of ionotropic gelification performed by carrageenan and pectin. Besides, it could explain due to concentration of polymer solution used in the process.

The carrageenan hydrogel provided efficiency of encapsulation similar to those presented by Zhang et al. (2016), who also produced carrageenan hydrogel by ionotropic gelation and attained efficiency of 63%. In Facin et al. (2015) work, the β -galactosidase was covalently immobilized in carrageenan hydrogel coated by chitosan providing an immobilization efficiency of 50%, what is inferior to the result found in the present work. Moreover, Facin et al. (2015) immobilized β -galactosidase in hydrogels grafted with chitosan copolymerized with methylene-bisacrylamide and polyacrylic acid and obtained an efficiency of encapsulation approximately 3 times lower (only 19% of the enzyme was immobilized).

Characterization of hydrogels

Fourier Transform Infrared Spectroscopy (FTIR) analysis

FTIR is an important technique to investigate the miscibility of polymer mixture. When chemical groups interact molecularly, changes are observed in FTIR spectra, such as the displacement of absorption bands. Such changes may be an indicative of a good miscibility of polymers (Martins et al. 2012).

FTIR spectra of carrageenan, pectin and hybrid hydrogels are shown on Fig. 1, in which common and specific absorption bands can be observed. Common bands are: a wide band in the region from 3000 to 3600 cm^{-1} attributed to the hydrogen bonding of the groups OH and band in 2994 – 2900 cm^{-1} of C-H stretching (Martins et al. 2012, Pascalau et al. 2012). Specific absorption

bands may be observed in the carrageenan spectrum: band 845 cm^{-1} relative to the bonding C-O-SO₃ in the position C₄ of the galactose ring; band of 1234 cm^{-1} attributed to the bonding S=O of sulfate esters; band of 924 cm^{-1} relative to the bonding C-O of 3,6-anhydrogalactose and band of 1070 cm^{-1} attributed to the glycosidic bonding (Sharifzadeh et al. 2016). The pectin spectrum presented typical bands at 1600 cm^{-1} characteristics of the group C=O (COO⁻) and other at 1740 cm^{-1} attributed to the vibration C=O (Mahmood et al. 2014, Wang et al. 2018). The spectrum of hybrid hydrogel presented bands similar to those detected in the carrageenan and pectin hydrogels, however less intense or slightly displaced, indicating a good miscibility of polymers.

Thermogravimetric analysis

The thermogravimetric analysis was conducted to evaluate the stability of hydrogels containing β -galactosidase as well as to determine how the interaction of both polysaccharides occurs on the hybrid hydrogel. The TG curves (Fig. 2a) present the temperature of events for all the hydrogels.

The derived thermogravimetry (DTG) indicated at least three significant thermal events at similar ranges (Fig. 2b). The first step (until 120 °C) may be attributed to the water evaporation in which there was an increase of temperature in all analyzed hydrogels (until 165 °C for the carrageenan hydrogel, until 150 °C for the pectin hydrogel and until 160 °C for the hybrid). In this first event, there was a mass loss of 6, 13 and 9% for carrageenan, pectin and hybrid hydrogels, respectively. The destruction of the glycosidic chain occurred between 120 and 200 °C, which was observed mainly in carrageenan and hybrid hydrogels. The third stage occurred between 230 and 330 °C and is

related to the decomposition of polysaccharides (Wahba 2016, Martins et al. 2012, Mahmood et al. 2014, Ma et al. 2011). The hybrid hydrogel present similarities both with the carrageenan and pectin hydrogels, thus indicating the occurrence of interaction between the polymers. The second event occurred between 160 and 250 °C, what confers a mass loss of 34% with peak at 180 °C, a behavior similar to those presented in the second event of the carrageenan hydrogel (peak

at 185 °C). Finally, other event occurred from 250 to 500 °C with mass loss of 12% with peak at 271°C, temperature intermediate between the third event of carrageenan hydrogel with peak at 305 °C and second event of pectin hydrogel with peak at 236 °C.

Scanning Electron Microscopy (SEM)

The morphology of dehydrated hydrogels of carrageenan, pectin and hybrid may be observed

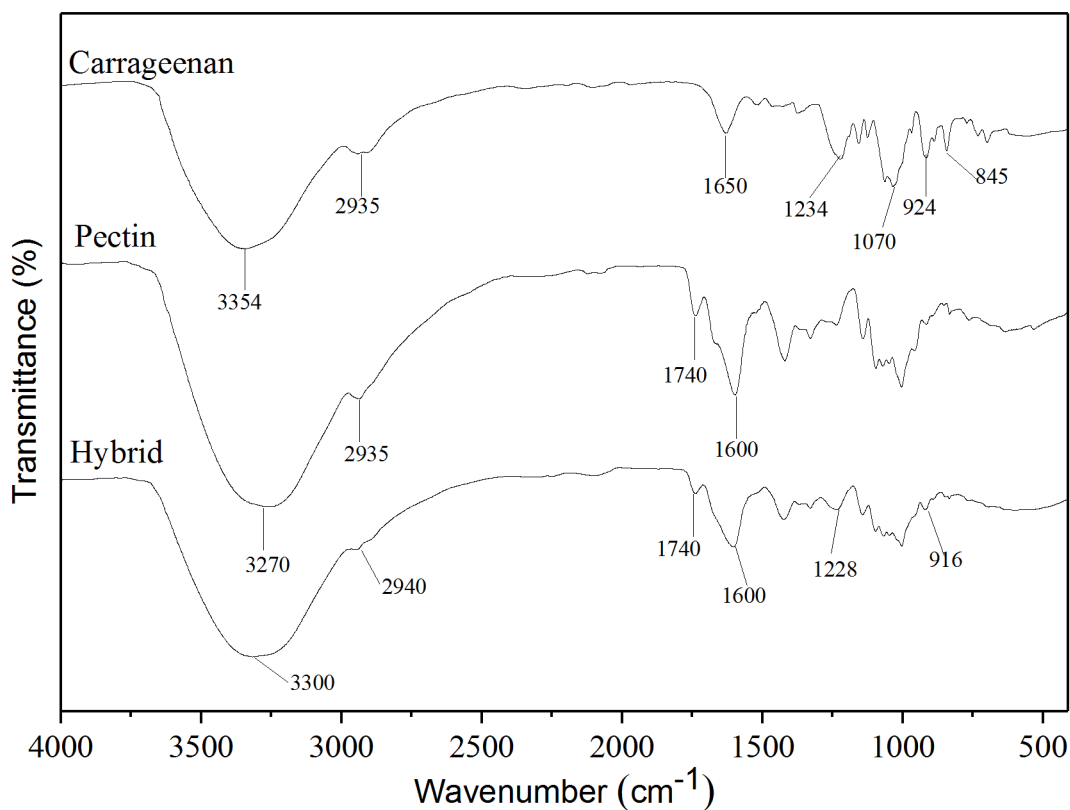


Figure 1. FTIR spectra of carrageenan, pectin and hybrid hydrogels (n=3). The samples of hydrogels were evaluated in the region from 400 to 4000 cm^{-1} with resolution of 4 cm^{-1} and 64 scanning.

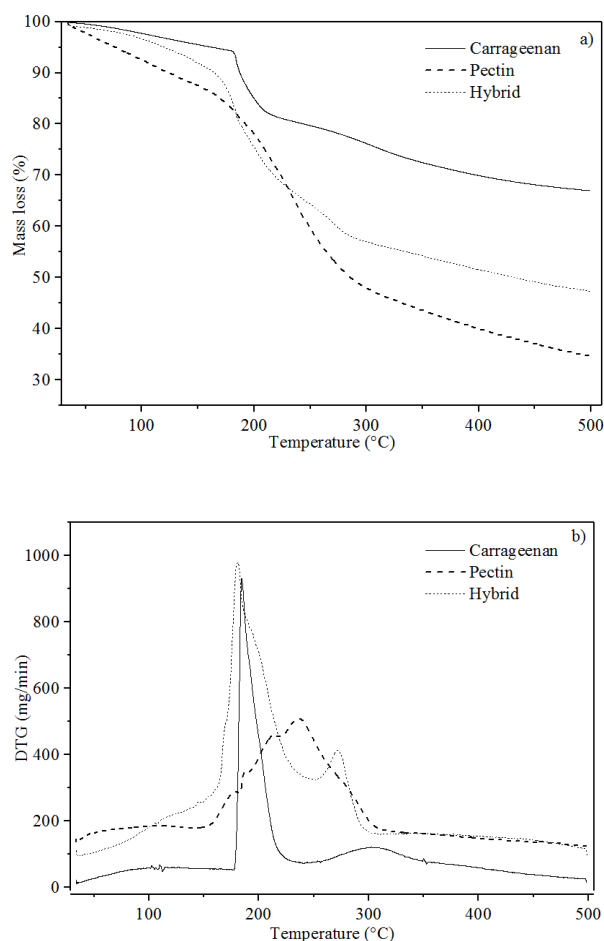


Figure 2. Thermal analysis of carrageenan, pectin and hybrid hydrogels (n=3): a) TG and b) DTG curves. The temperature ranged from 30 to 500 °C with heating rate of 10°C /min under nitrogen atmosphere using aluminum crucibles with approximately 3 mg of sample.

on Fig. 3. According image-analysis *ImageJ*® software, hydrogels spheres of carrageenan, pectin and hybrid presented different diameter of approximately 0.82, 0.58 and 1.08 mm, respectively and volume of 0.29, 0.10 and 0.66 mm³, respectively. The beads of all hydrogels presented spherical form distorted with some wrinkles in the surface presumably due to the drying process.

Since the hydrogels were formed in the presence of a great amount of water, in function of the low concentration of the polymeric solution

they suffer a strong deformation during the drying process. The spherical form of hydrogel in the wet state is generally altered after drying, thus generating a very irregular surface, and in the case of pectin hydrogels, the spheres tend to agglomerate. The evaporation of water during the drying process probably is responsible by determining the obtained morphologies (Jung et al. 2013).

The carrageenan hydrogel presents grains with irregular geometric forms grouped into conglomerates, as shown by Fig. 3b. The same characteristic is present on carrageenan hydrogels prepared in the work of Pascalau et al. (2012), however in the present work the grains were not homogeneously distributed along the sphere surface (Fig. 3a), while the pectin hydrogel presented rough surface with large cracks. The hybrid hydrogel also presented grains like the carrageenan one, however in the former they are smaller and more disperse along the sphere surface, as shown by Fig. 3f.

Influence of pH and temperature on the enzymatic activity

In this study, pH values between 1.0 and 7.0 were selected to evaluate the conditions found in the gastrointestinal tract. Furthermore, the activity released (8.7 U) by the pectin hydrogel at pH 7.0 (material that presented the best results in this condition) was used as parameter to determine the amount of free enzyme employed in the essay.

According to results presented on Fig. 4a, at pH 1.0 and 2.0 the enzymatic activity was close to the quantification limit (\leq LOQ = 0.01 U) in all hydrogels, as well as in the free enzyme. This effect may be attributed to changes in the tridimensional structure of the enzyme when the pH is reduced, what leads to alterations on the morphology and in the surface of the respective active sites, thus indicating that the hydrogel

spheres do not inhibit the loss of enzymatic activity in acid medium. This probably occurred due to the fact that H^+ ions from the medium are small enough to migrate through the pores of spheres, then entering in the hydrogel matrix, consequently altering the properties of the enzyme active site (Zhang et al. 2016).

The carrageenan hydrogel also did not present significant activity at pH 3.0, thus indicating that the β -galactosidase was not released in the medium under such condition since the free enzyme was active in this pH. This hydrogel presented the best result at pH 4.0, value close to the optimum of the enzyme (4.5). On the other hand, the pectin hydrogel at pH 3.0 presented 5.0 ± 0.3 U, it means, 57.4% of the expected activity at pH 7.0 (8.7 ± 0.3 U). When comparing to the free enzyme, the pectin hydrogel activity was 5 times superior. All these

data demonstrated the character of preservation of the tridimensional structure of the enzyme encapsulated in this material, at least partially.

The pectin hydrogel presented the greatest activity of β -galactosidase between pH 4.0-7.0. At pH 5.0 and 7.0 the observed activity was statistically equal (Student t test with confidence interval of 95%) to the free enzyme. The pectin hydrogel presented activity statistically equal in the pH range from 4.0 to 7.0. Specifically, at pH 7.0 this hydrogel generated activity 1.7 and 5.8 times greater than the carrageenan and hybrid hydrogels, respectively.

A similar behavior was observed by Zhang et al. (2016). The study of stability of carrageenan hydrogel in different pHs demonstrated that the enzymatic activity was null between 2.0 and 4.0, despite it was incapable to protect the enzyme from the acid, thus leading to the loss

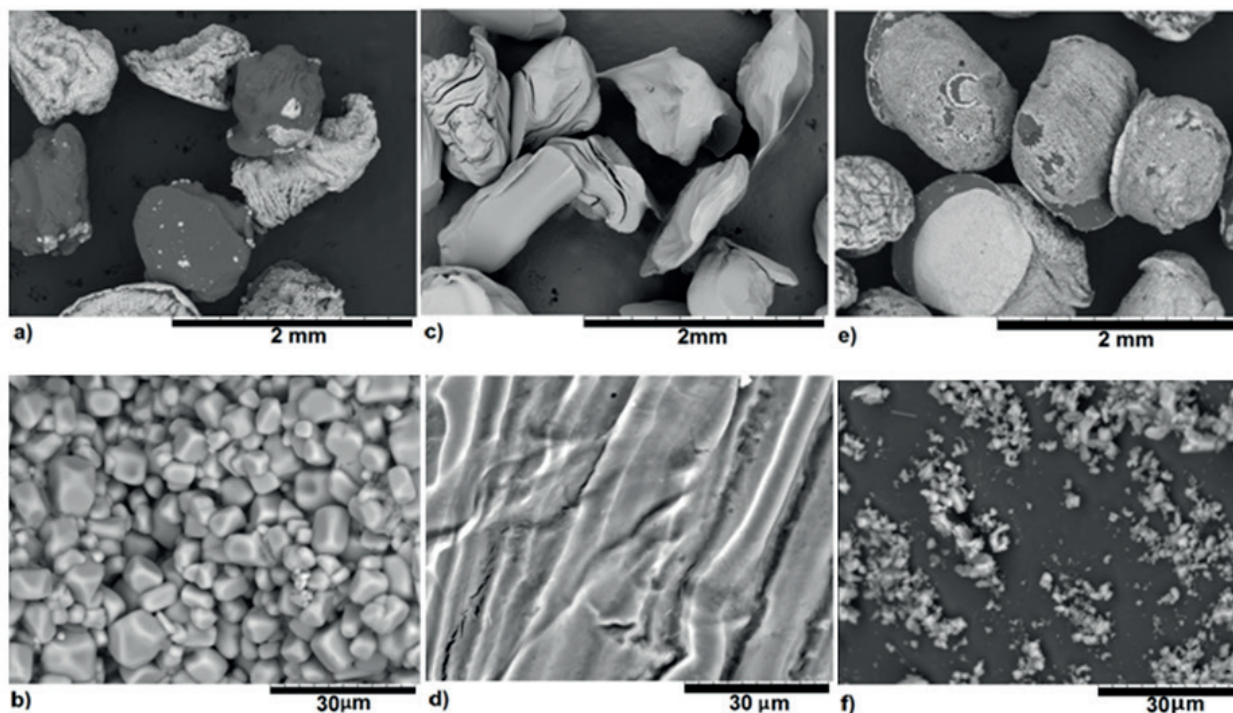


Figure 3. SEM images of dry hydrogels: a) Carrageenan at 50x magnification, b) Carrageenan at 2000x magnification, c) Pectin at 50x magnification, d) Pectin at 2000x magnification, e) Hybrid at 50x magnification and f) Hybrid at 2000x magnification.

of activity. The encapsulation of β -galactosidase in spheres of hydrogel led to an increase of 76% on activity when compared to the free enzyme (β -galactosidase produced by fermentation of a selected strain of the yeast *Kluyveromyces lactis*) at pH 7.0.

The hybrid hydrogel presented the lowest amount of released enzyme, what was a negative surprise since this hydrogel presented the highest efficiency of encapsulation. One possible explanation may be attributed to the fact that a higher amount of enzyme may have been encapsulated by the hybrid hydrogel, however without preserving its tridimensional structure, thus affecting the result of activity in different pHs.

All hydrogels presented greater activity in higher pH (pH 7.0) than in relation to the acid condition (pH 3.0). This may be explained by the fact that anionic hydrogels (as hydrogels formed by carrageenan and pectin) have groups (for instance, $-\text{COO}^-$ or $-\text{SO}_4^-$) for which the deprotonation occurs when the pH of medium is above their ionization capacity, which improve the hydrogel swelling, thus expanding the polymeric network and allowing the enzyme release (Ullah et al. 2015).

The enzymatic activity depends on the medium temperature and the optimal temperature of β -galactosidase is 50 °C. Above this temperature the enzymatic activity decreases due to the denaturation of the biomolecule. In general, the β -galactosidase encapsulated in pectin hydrogel presented behavior similar to the free enzyme, as presented on Fig. 4b, remaining stable, it means, with the same activity between 35 and 55 °C (data statistically equal according to the Student test, confidence interval of 95%). On the other hand, the carrageenan hydrogel was not influenced by the temperature between 40 and 55 °C, while the hybrid hydrogel presented the same activity

of enzyme along all the evaluated range of temperature, except at 60 °C.

At lower temperatures such as 25 and 30 °C, all hydrogels presented a strong decrease of enzymatic activity. The pectin hydrogel, for instance, presented a loss of 51 and 42% at the temperatures of 25 and 30 °C, respectively, when compared to its activity at optimal temperature (50 °C), indicating that at lower temperatures the polymeric network expands less and consequently releases less enzyme in the medium. It is important to highlight that any hydrogel remained stable at 60 °C. According to the obtained results, there was a reduction of 59, 68 and 47% for the hydrogels of carrageenan, pectin and hybrid, respectively, considering the highest value of enzymatic activity found for each material in the respective test. The profile of β -galactosidase release found in the present work differs from those related by Zhang et al. (2016). These authors found a lower activity of β -galactosidase encapsulated in carrageenan from 40 °C. In this sense, hydrogels prepared with the here presented methodology may be considered more stable at higher temperatures.

Influence of storage time on the stability of hydrogels

Fig. 5 represents the behaviour of hydrogels after three months of storage hermetically packaged in refrigerator at 4°C. This test also verified whether the pH of medium influence the profile of release/activity of β -galactosidase after storage.

The hybrid hydrogel demonstrated the greatest relative loss of activity since after storage any activity was observed (\leq LOQ = 0.01 U). The pectin hydrogel presented an activity loss of 79% at pH 4.0 and 69% at pH 7.0, despite it presented the greatest β -galactosidase activity/release in all the pH range evaluated. On the other hand, the carrageenan hydrogel presented

the greatest stability. A decrease of activity of approximately 8 and 29% were observed at pH 4.0 and 7.0, respectively, what provided better results of activity/release to the carrageenan hydrogel stored for three months when compared to the pectin hydrogel incubated in the same conditions.

It is also important to mention that the profiles of β -galactosidase release in hydrogels stored for three months in refrigerator at 4 °C

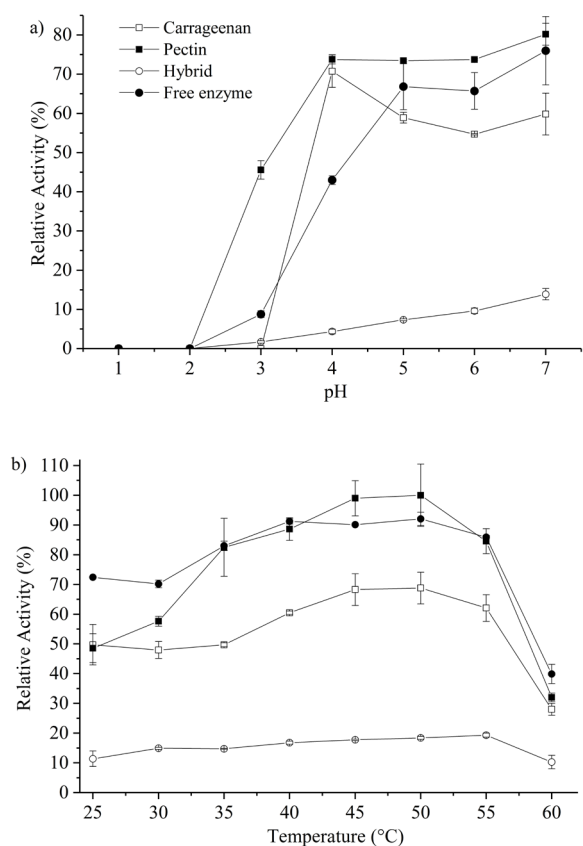


Figure 4. pH and temperature influence in catalytic activity, (n=3): a) pH activity profiles for activity of free and immobilized β -galactosidase. The activity of free and immobilized β -galactosidase was measured in the buffers of various pH (1.0–7.0) at 37 °C, for 30 min. The buffers used were HCl 0.1 M in pH 1.0 and 2.0 and buffer solution Tris/HCl, 50 mM. b) Temperature activity profiles for activity of free and immobilized β -galactosidase. The activity of β -galactosidase was measured in buffer solution Tris/HCl, 50 mM, pH 7.0 at various temperatures (25–60 °C), for 30 min.

are similar to the results of the pH influence conducted with recently prepared hydrogels (data not shown).

Study of β -galactosidase release *in vitro*

The present study was conducted to evaluate the behaviour of hydrogels simulating conditions in the gastrointestinal tract. For that, hydrogels were placed in gastrointestinal capsules of hypromellose, since the test of pH influence revealed that at values close to the stomach pH (1.2) the enzyme did not present activity, even when encapsulated. Moreover, a similar procedure involving the β -galactosidase for evaluation of delivery system was adopted by He et al. (2014) and Perissinato et al. (2017).

Recent studies conducted by our team have revealed that commercial tablets containing β -galactosidase presented disintegration time of approximately 2 minutes when exposed to the simulated conditions of gastrointestinal tract, even in acid medium, what indicates that the tablets present an immediate release of enzyme. In this sense, we proved that the commercial tablet did not provide protection in the simulated gastric fluid, since $86.0 \pm 0.8\%$ of β -galactosidase was released in 5 minutes of the dissolution essay, remaining only 14.0% of the activity indicated in the label in the end of the test (210 min). However, when the commercial tablets were included in gastro-resistant capsules the enzymatic activity was 6.7 times higher (Perissinato et al. 2017).

According to the profile presented on Fig. 6, it is possible to note that gastro-resistant capsules presented protection during all the period of permanence in the gastric medium (until 120 min) and there was no enzyme release (\leq LOQ = 0.01 U). In this way, all samples are considering statistically equals in gastric medium. After this medium was changed by the intestinal one, capsules disintegrated in

approximately 8 minutes. After disintegration, the carrageenan and pectin hydrogels released β -galactosidase as the time advanced, attaining a greater activity than the commercial tablet in the end of test (180 min).

At 150 min of release *in vitro* test (30 min in the simulated intestinal medium), the carrageenan, pectin and hybrid hydrogels obtained release of 14 ± 2 , 22 ± 2 and 3.0 ± 0.3 U, respectively. These data corroborate the activity found in the test of pH influence, in which the release of 5.0 ± 0.5 , 8.5 ± 0.5 and 1.5 ± 0.2 U were observed for the carrageenan, pectin and hybrid hydrogels at pH 7.0 (the mass of hydrogel used was threefold lower). At 135

min of release *in vitro* test, carrageenan, pectin hydrogels and commercial product showed data released statistical equal. At 150 min, only carrageenan hydrogel and commercial product are considering statistical equal. After this time, all materials can be considered statistical different using Student t test with confidence interval of 95%.

It was also possible to note that the release of β -galactosidase from the carrageenan hydrogel occurs more slowly than the pectin hydrogel. The same activity presented by the carrageenan hydrogel at 150 min is noticed at 165 min in the pectin hydrogel. The same behavior occurs when comparing the activity released by the

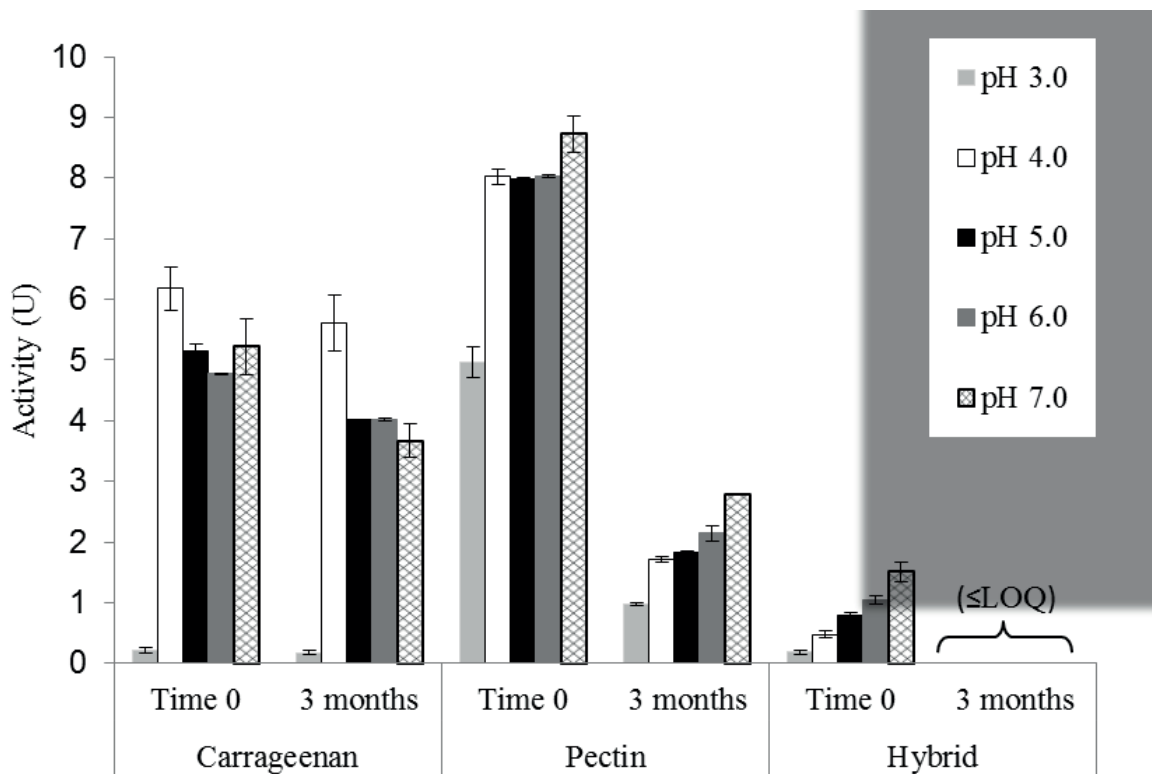


Figure 5. Comparison of enzyme amount released ($n=3$) at different pH (1.0–7.0) for 30 min in hydrogels using freshly prepared samples and after stored at 4°C for 3 months. The buffers used were HCl 0.1 M in pH 1.0 and 2.0 and buffer solution Tris/HCl, 50 mM. The catalytic activity was determined by spectrophotometric (LOQ = 0.01 U).

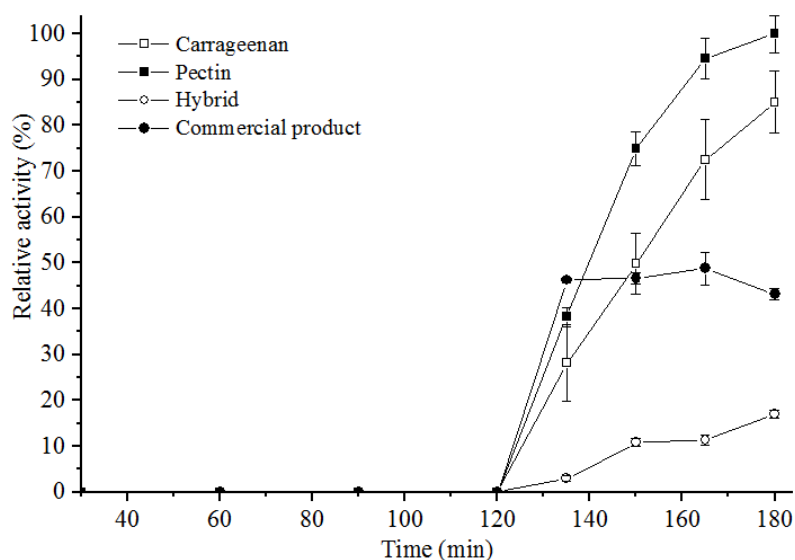


Figure 6. Release profile of hydrogels (60 mg) and the commercial product (3.17 mg) placed in the gastro-resistant capsule of hypromellose (n=3). The effect of simulated conditions of gastrointestinal tract was made using 30 mL of simulated gastric fluid (SGF, HCl 0.1 M, pH 1.2) during two hours. After this period, the medium was changed by simulated intestinal fluid (SIF, buffer Na₂HPO₄, 100 mM, pH 6.8) during one hour.

pectin hydrogel at 180 min. This result may be related to the fact that the carrageenan hydrogel generates larger spheres. According to the SEM results, the spheres diameter of carrageenan hydrogel is 1.4 times larger than the pectin ones. The dimensions of hydrogel have an important role related to its capacity to release immobilized bioactive agents. The rate of release decreases as the hydrogel size increases since the molecules have an additional distance to migrate until the surrounding medium. A careful control of dimensions of the hydrogel is therefore required to guarantee an adequate profile of retention and release of an encapsulated bioactive agent (McClements 2017).

From 135 min on, the activity released by the commercial tablet remained constant, thus indicating that all encapsulated enzyme was available when it disintegrated. Furthermore, there was a decrease of 50% in relation to the activity expected for this gastrointestinal material (26 U). The carrageenan and pectin hydrogels were 2.4 and 2.0 times, respectively,

more efficient on releasing than the commercial tablet at 180 min.

A similar result was presented by He et al. (2014), who prepared polymeric nano-capsules compounded by poly-lactic acid containing β -galactosidase. The nano-capsules were freeze-dried and placed in gelatin capsules that posteriorly were covered with hydroxypropyl methyl cellulose phthalate (HP55). The covering with HP55 was made immersing the gelatin capsules containing the nano-capsules in solution of HP55 (15% m/v) of dichloromethane (DCM) and acetone (4:1, v/v) followed by drying at room temperature. *In vitro* results revealed that the capsule covered with HP55 remained intact in the simulated gastric fluid and protected the β -galactosidase from the acid denaturation. The enteric covering dissolved rapidly and released the nano-capsules containing β -galactosidase under the simulated intestinal condition, which presented more stability than the free β -galactosidase.

Additionally, it is important to report that it would be conduct the evaluation of *in vitro* release in the presence of some digestive enzyme (i.e. pepsin and trypsin). In this sense, the capacity of the hydrogel to preserve the catalytic activity of β -galactosidase could be tested in conditions more similar to those of the digestive tract.

CONCLUSIONS

β -galactosidase release *in vitro* revealed that carrageenan and pectin hydrogels were more efficient on releasing than the commercial tablet. However, the β -galactosidase release by the carrageenan hydrogel occurs more slowly when compared to the pectin one, what may be related to the fact that the carrageenan hydrogel generates volume sphere around 3 times larger than the pectin one, and therefore the molecules have a longer distance to migrate until the surrounding medium.

The study of pH influence on the enzymatic activity indicated that the enzyme immobilization in spheres of hydrogel was not sufficient to avoid the loss of activity induced by extremely acid pHs. The study of temperature influence revealed that all hydrogels presented thermal stability between 40 and 55 °C and the pectin hydrogel presented activity similar to the free enzyme. After three months stored the carrageenan hydrogel presented the best stability, and it presented activity/release superior to the pectin hydrogel.

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