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Research article

Controlled release of papain encapsulated in chitosan hydrogels and study of nematicidal action on *Panagrellus* sp.

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ABSTRACT

Alternatives to the use of chemical products traditionally used to combat agricultural pests and diseases have been a concern for society. Thus, based to sustainable agriculture, the use of enzymes has grown as an alternative to the control with traditional. However, some limitations prevent greater effectiveness of action of these important biological catalysts, such as variations in pH and temperature, the presence of inhibitory ions, among others. In this study, papain, a plant enzyme, was incorporated into chitosan hydrogels by cross-linking with glutaraldehyde at different concentrations (0; 0,25 %; 0,5 %; 0,75 %; 1 %). The results of scanning electron microscopy showed a difference in the porous morphology of the different treatments compared to chitosan without crosslinking and the FTIR analysis suggests crosslink formation in the hydrogels. The enzymatic activity of free papain was reduced by approximately 57 % of the initial value. On the outher hand, the results indicate that the activity of the enzyme released from some hydrogels increased and the diffusion of papain probably occurred in the solution from the surface of the formed chitosan hydrogels, represented by a parabolic diffusion model. The mortality of *Panagrellus* sp., a model nematode, was evaluated and the treatments with hydrogels demonstrated high mortality of *Panagrellus* sp. above 80 % after 5 days. The hydrogels containing papain were effective in the mortality of the nematodes evaluated and constituted a slow release system, sustainable environmentally, as it has the potential to reduce the number of applications needed to control pests and diseases in agriculture.

1. Introduction

Diseases and agricultural pests cause economic and production yield losses in the most diverse crops [1]. At the same time, sustainable measures, such as biopesticides, have emerged as alternative methods to the traditional chemical control used to mitigate the problem [2]. In this sense, the UN 2023 agenda highlights the importance of sustainable agriculture. However, chemical controllers are still widely used in the field and e.g. chemical nematicides are often used as one of the main methods in combating these agents [3].

In recent years, however, enzymes have emerged in biochemical control, especially those from microorganisms and plants, due to the diverse range of potential catalytic targets for proteases and chitinases [4]. Although the study of enzymes in the alternative control of agricultural diseases and pests has increased significantly, some limitations prevent greater effectiveness of action of these important biological catalysts. Enzymatic catalysis is influenced by environmental factors, such as variations in pH and temperature, the presence of inhibitory ions, among others [5]. Therefore, one way to maintain its action is to incorporate it into polymers, which form a suitable environment for storage and controlled release [6]

From this perspective, chitosan appears as an important enzyme incorporator, as it is a biodegradable, non-toxic, originating from the deacetylation of chitin from crustaceans and insects, which results in (1,4)-2-amino-2-deoxy-D-glucose, with antioxidant properties, in addition to being edible [7,8]. Therefore, it presents highly desirable properties for the conservation and release of enzymes in the most varied environments.

Zhang et al. [5] describe that the use of chitosans as encapsulants takes into account the protonation of amino groups present in the

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biopolymer in an acidic environment, which makes it an important cationic enzyme carrier. In this context, hydrogels are promising encapsulators for incorporating enzymes, such as proteases and laccases, for example [9].

The literature points out that, in the field of agriculture, biodegradable biopolymers, such as chitosan, are interesting innovations in the release of plant nutrients [10] and as potential for mitigating diseases occurring in these organisms [11].

On the other hand, in addition to the lack of studies on the use of papain in controlling pests and plant diseases, It is necessary to consider that its full potential must be guaranteed through the maintenance of enzyme activity, In this way, the encapsulation or immobilization of enzymes for the most diverse applications has the main objective the maintence of proteic stability [12].

Crosslinking consists of a way of chemically altering the structure of polymers in order to allow adequate control of retention or the release of incorporated molecules and improvement of structural characteristics of the material [13]. In this way, crosslinkers act as important regulators of the mechanical and biological properties of the hydrogel formed [14]. Thus, ultimately, they can even affect the release kinetics of molecules into the environment.

Currently, many studies focus on the search for coating and incorporation of natural biochemical compounds using plant extracts and active derivatives isolated from plant sources [15]. In this context, recently evaluated chitosan nano hydrogels and chitosan hybrids with polyethylene glycol as a model for protein delivery systems using papain [16] and PLGA (Poly (lactic-co-glycolic acid) nanoparticles loaded with papain were also used for enzyme release studies [17]. In addition to these materials, liposomes for topical application of papain, aiming at treating scars [18]. However, studies are still incipient regarding the use of incorporated enzymes in chitosan for the control of agricultural pests and diseases. It is worth highlighting that, regardless of the producing source (plants or microorganisms), the incorporation of enzymes into polymers provides improvements in the conditions of enzymatic catalysis with a view to controlling harmful agents of agricultural interest.

Controlled molecule delivery systems are advantageous because they tend to increase bioavailability and maintain a controlled and sustained release over time, there may be regulation of the release rate, increased efficiency and reduced environmental impacts [19,20]. Such characteristics are interesting for the application of a pesticide, for example, the papain, therefore, with the sustained release, it is possible to reduce the number of applications, much appreciated environmentally.

From an agricultural point of view, papain (EC 3.4.22.2) is a cysteine protease with great potential for use in controlling plant diseases and animal parasites and this important enzyme meets the growing need for sustainable control methods, but the protein is still little explored for this purpose [21]. However, In this context, proteases are promising enzymes as biocontrollers [22] and papain presents potential for use as a biopesticide [23].

Therefore, the present work evaluated the incorporation of papain into chitosan at different concentrations of glutaraldehyde as a crosslinker. Furthermore, a study was carried out on the enzyme's release kinetics in aqueous media and evaluation of its in vitro action on *Panagrellus* sp, a model organism used in studies analyzing nematicidal action [24] seeking to serve as a basis for using enzymes incorporated into biopolymers for biochemical control purposes. The porosity perfil of chitosan alone or with papain incorporated was carried out using scanning electron microscopy (SEM) images and the chemical characterization of the same materials occurred using Fourier transform infrared spectroscopy (FTIR).

2. Materials and methods

2.1. Materials

The chitosan used in the present work was purchased from Polymar (Fortaleza, Ceará, Brazil). Acetic acid (reagentplus, >99 % purity) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Pure papain was supplied by Dinâmica Química Contemporânea Ltda (Indaiatuba, São Paulo, Brazil) and glutaraldehyde (Grade II, 25 % in H_2O) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Preparation of hydrogels

Initially, a 1 % (w/v) chitosan solution was prepared by diluting the biopolymer in 1 % (v/v) acetic acid solution. The solution was then vacuum filtered with fabric filters to remove impurities. Subsequently, the pH of the filtered solution was corrected to 5.0 to avoid denaturation of papain [25]. Papain was inserted into this solution, so that the enzyme was at a concentration of 10 % (w/w) in relation to the mass of chitosan used, or 0,1 % (w/v) in relation to the solution volume. The Fig. 1 illustrates a calculation basis for preparing hydrogels.

Chitosan cross-linking occurred through the insertion of glutaraldehyde in different concentrations (v/v) in relation to the volume used to prepare the 1 % chitosan solution (w/v). The glutaraldehyde concentrations used were equal to 0; 0.25; 0.5; 0.75 and 1.0 % (v/v). After shaking for 10 minutes, at 26 °C, the solutions were lyophilized in a Labconco Freezone 2.5 apparatus for 48 h. Freeze-dried hydrogels containing papain were coded according to the concentration of glutaraldehyde used. Thus, G0, G025, G050, G075, and G1 correspond, respectively, to those materials whose glutaraldehyde concentrations were 0; 0.25; 0.5; 0.75 and 1.0 % (v/v) (Fig. 1 and Fig. 2). Additionally, a 1 % (w/v) chitosan solution without glutaraldehyde and papain was prepared and lyophilized (Quit1 %).

2.3. Scanning electron microscopy (SEM)

The porous morphology of the formed hydrogels was obtained using scanning electron microscopy. The analyses were carried out using a JEOL microscope, Model JSM6610LV, with adjustable acceleration voltage from 300 V to 30 kV. The freeze-dried samples were applied to double-sided carbon tape and metallized with gold for 120 s.

2.4. FTIR infrared analysis

For infrared analyses the IRAffinity-1 FTIR spectrophotometer (Shimadzu, Kyoto, Japan) was used. The method used a spectral range of 400–4000 cm-1, with 64 scans at a resolution of 2 cm^{-1}. The samples analyzed in the present work were inserted in KBr tablets [26].

2.5. Protein release study

The incorporated papain samples (G0, G025, G050, G075 and G1) were placed in water at a ratio of 1:3 (mass/volume) and incubated in an oven at 26 + /- 2 °C, in neutral pH.

The release of papain in solution was monitored by the quantification of total protein in solution using the method of Bradford [27]. This is a colorimetric method for quantifying proteins in solution very consolidated in the literature characterized by being fast, simple and cheap [28,29]. The tests were carried out in triplicate and the average values obtained were plotted as a function of time to evaluate the release kinetics.

Four mathematical models were studied (First order, Higuchi, Ritger-Peppas and parabolic diffusion) to evaluate the mechanism of papain release in chitosan hydrogels, according to the equations:

$$\ln(1- M_t/M_\infty) = - kt \quad (1)$$

$$M_t/M_\infty = kt^{1/2} \quad (2)$$

$$M_t/M_\infty = kt^n \quad (3)$$

$$(M_t/M_\infty)/t = k_p t^{-0,5} + b \quad (4)$$

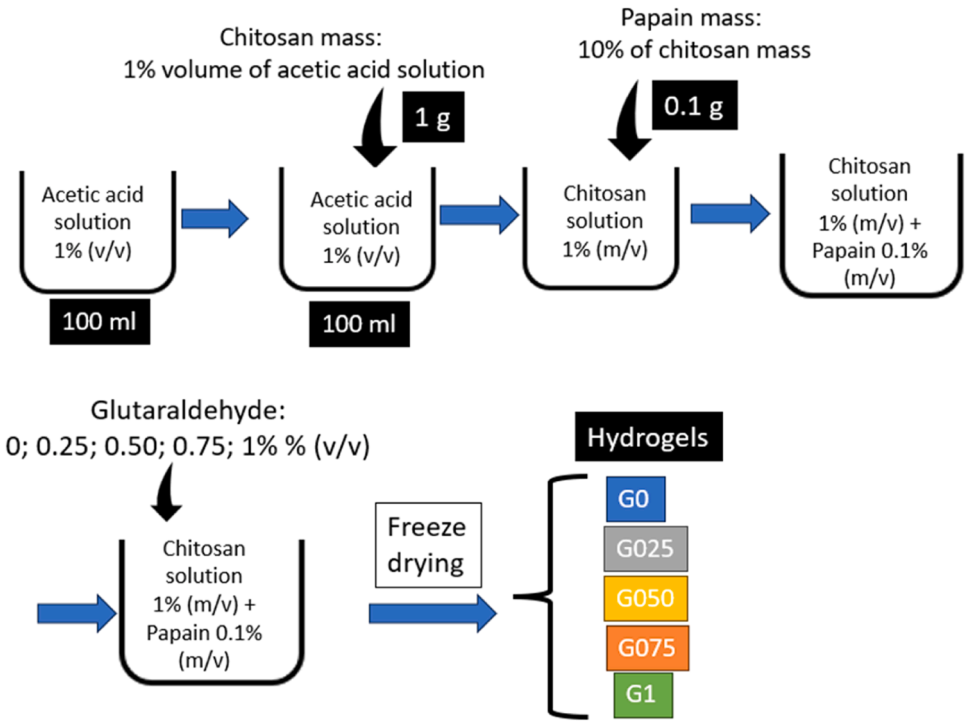

Fig. 1. Calculation basis to preparation of chitosan hydrogels.

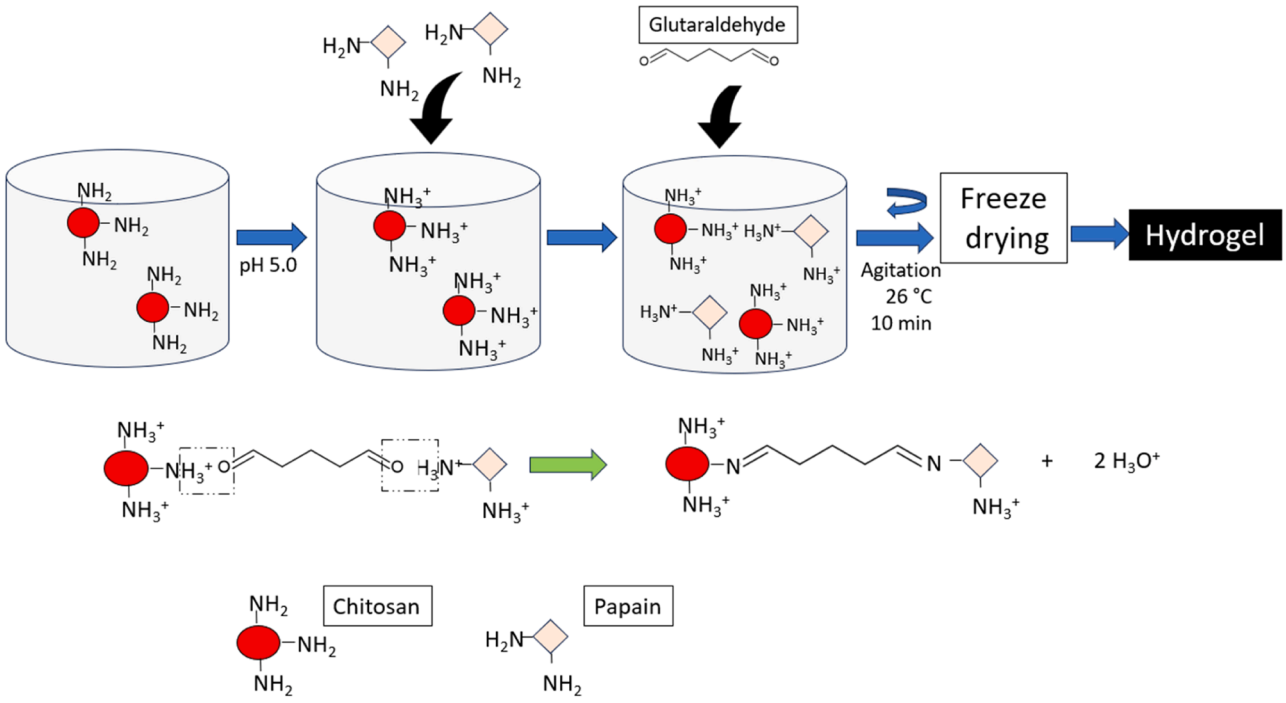

Fig. 2. Crosslinking of chitosan hydrogels.

In which Mt/M∞ is the fraction released of papain; n is the diffusion exponente; k, kp and b are kinetic constants. For $n \leq 0.43$, Fickian diffusion; If $0.43 < n < 1.0$, the transport is non-Fickian or anomalous [30–32].

2.6. Enzyme kinetics assay

In order to evaluate the enzymatic activity of papain in solution, samples of the incorporated enzyme (G0, G025, G050, G075 and G1) were placed in water at a ratio of 1:3 (mass/volume) and incubated in an oven at 26 + /- 2 °C.

The tests took place in triplicate, for 12 days, and the proteolytic enzymatic activity of papain was evaluated. Periodically, 50 µL aliquots were removed from the solution and protease assays were performed according to a methodology adapted from [33]. According to [33] the enzymatic unit was defined as the amount necessary for a 0.01 increase

in absorbance in 1 h under the assay conditions.

2.7. Nematicidal assay

The nematicidal assay was carried out according to the methodology adapted from [34]. Seven treated groups and a control group were assembled. Each group was formed by six replicates and around 100 juveniles from a mixed culture of *Panagrellus* sp. were added to each replicate.

Of the seven treated groups, five contained papain incorporated into chitosan (G0, G025, G050, G075 and G1). One of the groups was formed by free papain at a concentration of 0.30 % (w/v), which refers to the concentration if all papain were released of hidrogel in solution, and the other was treated only with 1 % (w/v) chitosan (Quit1 %). Masses of hydrogels were inserted into each replicate in a ratio of 1:3 in relation to the volume of water in the tubes. The control group contained *Panagrellus* sp. and water only.

2.8. Statistical analysis

Statistical analysis of the release kinetic data, as well as the results of the juveniles recovered in the treatments, were conducted using ANOVA at a significance level of 5 % for the Tukey test using the BioEstat Software 5.0 program. To calculate the average percentage reduction of larvae, the following equation was used [34]:

$$\text{Reduction}(\%) = \frac{(\textit{average of control larvae} - \textit{average treatment larvae})}{(\textit{average of control larvae})} \quad (5)$$

3. Results and discussion

3.1. Scanning electron microscopy

The results of scanning electron microscopy of hydrogel samples produced are shown in Fig. 3.

The SEM results show the surface morphology of the different treatments. The image from 1 % chitosan (w/v), without crosslinking and without the addition of papain (Quit1 %), appears as one with a presence of pores and the result is in accordance with [35]. The other images, referring to chitosan treatments with crosslinking and addition of enzyme, showed a reduced amount of porosity, as the concentration of glutaraldehyde increases, in comparison with QUI1 %. However, in G050, G075 and G1 similar porosity profiles are noted and these results are in accordance with the work of [36] in which the authors analyzed chitosan microparticles cross-linked with glutaraldehyde. In the results of [37], the cross-linked material presented a microporous surface without homogeneity and the cross-linking with glutaraldehyde provided the formation of porosity different from the chitosan particle structures without cross-linking. Those authors further observed that the surface of cross-linked chitosan was smoother compared to non-cross-linked chitosan microparticles.

Such observations are in line with the function of crosslinkers, as a crosslinking reaction alters the molecular properties of the polymer, such as increased chemical stability, rigidity, protein absorption and changes in the permeability of the material [38]. However, more studies are needed for better understanding, such as, for example, the analysis of encapsulation efficiency.

3.2. FTIR analysis

The chemical changes of chitosan hydrogels in the different treatments were analyzed by Fourier transform infrared spectroscopy (FTIR), according to Fig. 4.

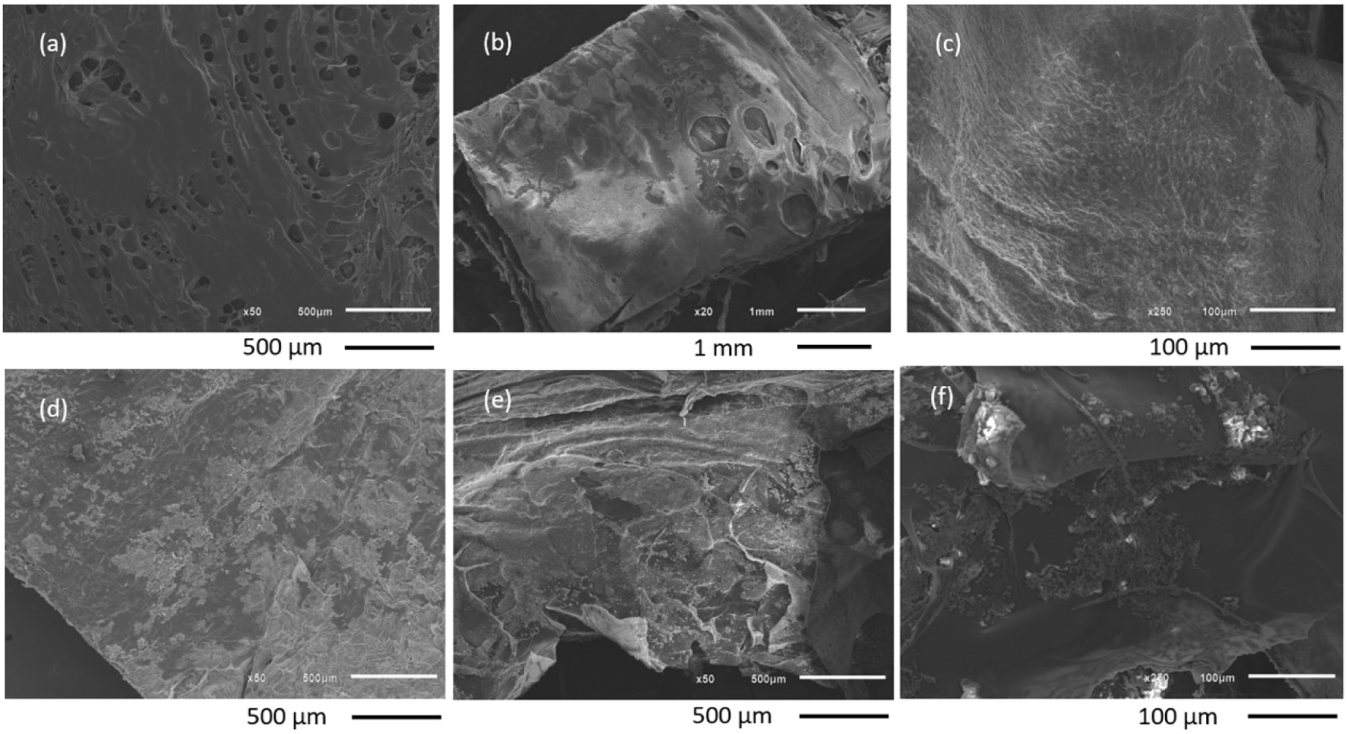

Fig. 3. SEM micrographs of cross-linked or non-cross-linked chitosan hydrogels. a- Quit1 %; b-G0; c-G025; d-G050; e-G075; f-G1. a- Quit1 % (Chitosan 1 % (w/v)). b) G0 (Chitosan 1 % (w/v) with papain without cross-linking). c) G025 (Chitosan 1 % (w/v) with papain and cross-linking to 0.25 % (v/v) glutaraldehyde). d) G050 (Chitosan 1 % (w/v) with papain and cross-linking to 0.50 % (v/v) glutaraldehyde). e) G075 (Chitosan 1 % (w/v) with papain and cross-linking to 0.75 % (v/v) glutaraldehyde).f) G1 (Chitosan 1 % (w/v) with papain and cross-linking with 1 % (v/v) glutaraldehyde).

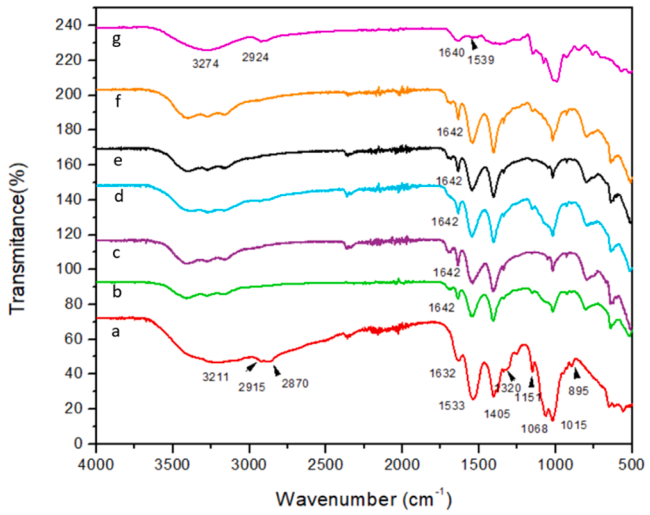

Fig. 4. Infrared spectra (FTIR). a- Quit1 % (Chitosan 1 % (w/v)). b) G0c) G025. d) G050. e) G075. f) G1. g) Free papain.

In the spectrum referring to Quit1 % (Fig. 4a), there is an accentuated band in the region around 3211 cm^{-1}. This region is representative of NH and OH stretching and intramolecular hydrogen bonds [44]. The FTIR spectrum in Fig. 4a also shows stretches around 2915 and 2870 cm^{-1}. They are characteristic of stretches υC-H, while the peak at approximately 1632 cm^{-1} is typical of amide I and, at around 1533 cm^{-1}, represents the deformation of NH present in the -NH_2 group. The spectrum also presents peaks around 1405 cm^{-1}, characteristic of υ-C-N axial deformation, and around 1320 cm^{-1}, characteristic of amide III [39].

Pavoni et al. [40] comment that the amino and hydroxyl groups of chitosan are the reactive sites of the polymer. Thus, as the authors present, the presence of the peak referring to the C═N bond, at 1650 cm^{-1}, is reasonable, derived from the reaction between chitosan (amine groups) and glutaraldehyde (carbonyl group), characteristic of crosslinking [41]. In this present work, the FTIR results, around this peak, more precisely 1642 cm^{-1}, increase as the concentration of glutaraldehyde increases (G0, G025, G050, G075 and G1), compared to chitosan without crosslinking (Quit1 %), which is a strong indication that crosslinking occurred.

As shown in the scheme in Fig. 2, the theoretical interaction of glutaraldehyde with papain occurs through the formation of the Schiff base structure (C═N), similarly in the reaction between glutaraldehyde and chitosan [42]. Therefore, the characteristic peak at 1642 cm^{-1} may have been formed by contributions from signals referring to the interactions of glutaraldehyde with the enzyme and with chitosan during the cross-linking process.

As for the FTIR spectrum of free papain, a long peak was observed between wavenumbers 3000 and 3630 cm^{-1}, approximately. According to [39] are characteristic of OH and secondary amine υ-N-H and, in addition, at 2924 cm^{-1} the peak represents υass-C-H (sp3). According to [43], at approximately 1650 cm^{-1} free papain has an amide-I band that is attributed to the stretching vibrational modes of the peptide carbonyl and provides information about the secondary structure of the protein. The authors report that the wavenumber range between 1540 and 1550 cm^{-1} is representative of the amide-II band, which characterizes the N-H bond of the enzyme. This information is in agreement with the present work, since, for free papain, Fig. 4g presents peaks close to 1640 and 1539 cm^{-1}.

3.3. Protein release study

The release profiles of papain in aqueous solution were plotted in Fig. 5.

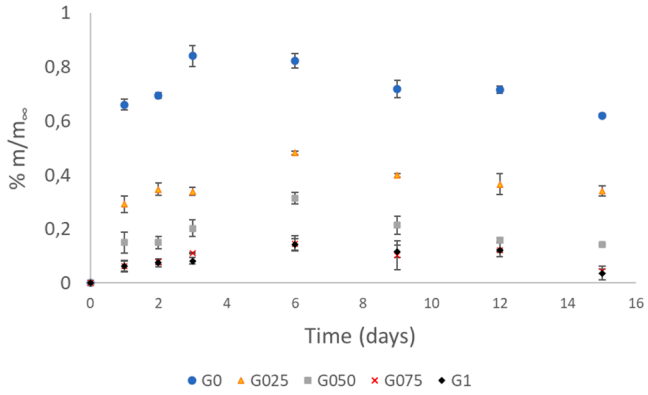

Fig. 5. Release behavior of the enzyme incorporated into chitosan.

From Fig. 5, an increase in the enzyme release rate is observed in the first six days in all treatments. However, after this period, the rate of release of the molecule decreases until the end of the analysis interval.

Of the four linearized mathematical models, the parabolic diffusion model was the one whose R^2 values were closest to 1 (Table 1). As described previously, the model is represented by the equation $(M_t/M_\infty)/t = k_p t^{-0,5} + b$, where Kp is the rate constant for the parabolic diffusion model and for the constant b there is no consensual chemical definition [31]. The Table 1 shows the kinetic parameters of the models.

The concentration of glutaraldehyde used in crosslinking influenced the release of papain, since in those treatments where the crosslinker concentration was higher, there was less amount of enzyme released. This was probably due to the decrease in the porosity of the hydrogel formed [44]. Furthermore, this phenomenon was corroborated by the diffusion coefficient values. K_p values decrease with increasing glutaraldehyde concentration, according to Table 1, indicating diffusion inversely proportional to the amount of crosslinker used in the study.

For the five experiments tested with incorporated enzyme, the parabolic diffusion model was well fitted ($R^2 > 0.95$) [31] and the diffusion of papain probably occurred into the solution from the surface of the chitosan hydrogels formed. The control step was the outer surface/edge diffusion process, since the parabolic diffusion model is widely used to elucidate this type of mechanism [30,31].

Papain is a protein that has amino acid residues in its structure. On the other hand, chitosan has amino groups (NH_2) in its constitution, therefore susceptible to protonation/deprotonation [45].

In the pH range between 5 and 6, specific protonation of papain amino acid residues occurs, in which the residues have positive charges [46]. According to [47], in situations with pH values lower than pKa values (pKa = 6.5), the chitosan molecule becomes protonated, since it has a specific amount of amino groups. These conditions give the polymer a positive charge and the molecule is polycationic at pH less than 6 [47].

Taking into account the system formed by chitosan and papain, at pH 5, chitosan amino acid groups are protonated and the amino acid residues in papain as well. Therefore, the tendency is for repulsion to occur between the papain and chitosan molecules.

Initially, papain is a cysteine protease of plant origin and, as such, is characterized by low stability in aqueous solutions, due to the autolysis of the molecule [48,49]. In addition to this issue, the papain release behavior in the present work is similar to the results obtained by Jafari et al. [16], in which the concentration of papain released from chitosan-based nanohydrogels increases until a certain time and then decreases considerably.

Jafari et al. [16] also comment that at acidic pH, the free amine and hydroxyl groups in chitosan polymer chains are converted into ammonium and hydronium ions, respectively. This protonation causes swelling in the hydrogel and, consequently, contribute to increases the

Table 1
kinetic parameters of the mathematical models.

Models										
	Higuchi		First-order		Ritger-Peppas			Parabolic		
	K	R^2	K	R^2	k	n	R^2	Kp	b	R^2
G0	0.1161	0.329	0.0207	0.0414	0.7308	−0.009	0.0067	0.819	−0.189	0.988
G025	0.0744	0.4827	0.0157	0.2139	0.3205	0.0782	0.2492	0.357	−0.074	0.989
G050	0.0335	0.2523	0.0047	0.0611	0.1718	0.0407	0.0205	0.178	−0.035	0.963
G075	0.0174	0.2382	0.0023	0.0647	0.0848	0.0345	0.0071	0.078	−0.013	0.970
G1	0.0183	0.2647	0.0028	0.0899	0.0784	0.0434	0.0089	0.075	−0.013	0.971

release of papain. At pH 5.0, those authors explain that there is a gradual increase in the rate of papain release.

This may occur due to an approximation with the pKa values of the ionizable groups of chitosan, and, in this way, the concentration of ammonium and hydronium ions in the medium is gradually reduced, decreasing swelling in nano hydrogels. This leads to a reduction in the rate of papain release. Furthermore, as shown in Fig. 5, from the sixth day onwards, the rate of enzyme released into the solution may have been lower, compared to the rate of enzyme hydrolysis due to autolysis.

The Bradford reagent is only form complex with protein molecules [29], not with free amino acids. This characteristic of the method corroborates the possible reduction in the accumulation of enzymes in solution.

However, more studies must be carried out to confirm these hypotheses. Along these lines, the lack of monitoring of pH values of the released papain-containing solution, over time, consists of a limitation to better understand the protonation profile of the molecules involved. Another limitation of the study was the lack of monitoring of the papain autolysis process, as it would be possible to confirm the profile related to the drop in the percentage of enzyme released in solution.

3.4. Released papain enzyme activity assay

According to the results, the enzymatic activity of free papain 0.30 % (w/v) decreased over time as shown in Fig. 6.

The profile of decreasing papain activity over time is in accordance with that found by [50] in which the authors evaluated free and immobilized papain with calcium alginate for twenty-eight days. The results of the analysis showed a decrease in proteolytic activity of around 94 % of the enzyme's initial activity at the end of the study and around 80 % in fifteen days. Although the activity of free papain in this study was not evaluated for a period similar to that mentioned, after twelvth days the enzymatic activity was reduced by approximately 57 % of the initial value, which demonstrates that the reduction trend is similar.

The enzymatic activities of papain released from hydrogels with higher degrees of crosslinking (G050, G075 and G1) showed similar profiles. Statistically, when comparing these three treatments, on each study day, there was no significant difference ($p > 0.05$) between them.

These results were supported by the FTIR results. The band in 1720 cm^{-1}, approximately, is characteristic of free aldehyde groups [42]. Thus, by Fig. 4 there are the similar signals in peaks close to 1720 cm^{-1} in G1 and G075 treatments and so, this hydrogels present the same numbers of free aldehyde groups. This would can explain the release result of G075 and G1 (Fig. 5) was not different, because the crosslink density maybe the same.

3.5. Nematicidal assay

Nematicidal assays with juveniles of *Panagrellus* sp. were carried out with free papain (0.30 % w/v) or incorporated, as shown in Fig. 7.

The mortality results (Fig. 7) indicate that the encapsulation of papain in chitosan hydrogel was statistically efficient ($p < 0.05$) in the mortality of juveniles of *Panagrellus* sp. in relation to the control composed of pure water. Another relevant point that was highlighted in this work refers to the nematicidal activity of pure chitosan (Quit1 %). Statistical analysis also demonstrated that treatments G0, G025, G050, G075, G1 and 0.30 % papain (w/v) do not differ in relation to mortality of *Panagrellus* sp.

Crosslinking with glutaraldehyde allowed the release of papain differently for G0 and G025 and very closely for the other treatments (G050, G075, G1) according to Fig. 5. On the other hand, the profiles of the enzymatic activities of papain released from G0 and G025 in solution also showed behavior analogous to that of the release study, with G0 and G025 differentiating from each other and the remainders being very similar (Fig. 6). The description of these profiles is corroborated by the porous morphology present in the SEM results presented in Fig. 3, in which the chitosan crosslinking treatments show little difference in the

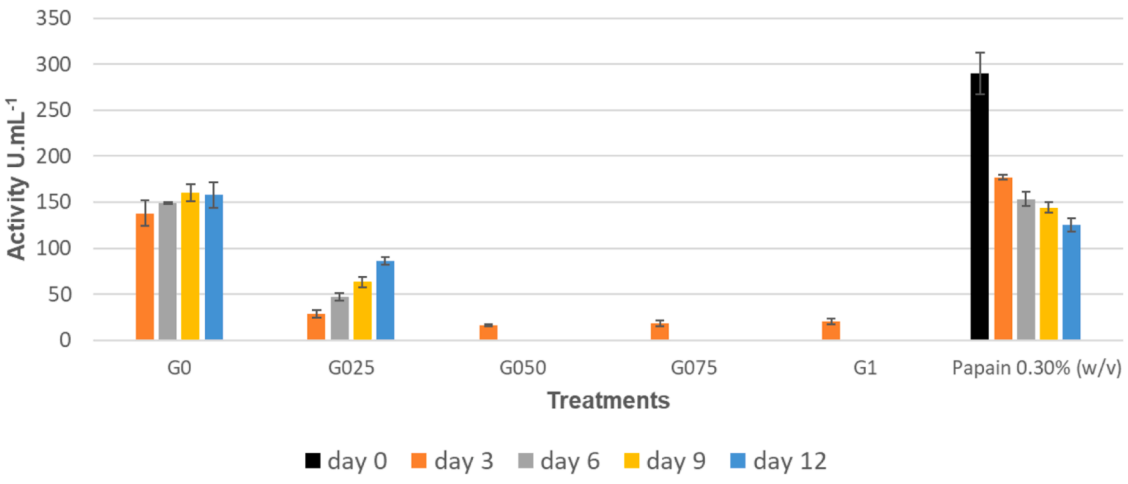

Fig. 6. Enzymatic activity profiles of papain released in aqueous solution.

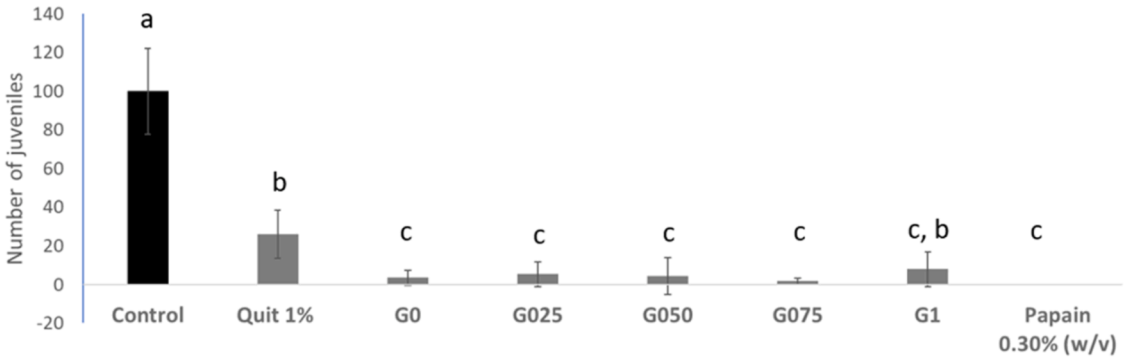

Fig. 7. Number of *Panagrellus* sp. juveniles recovered in the treatments (GO, G025, G050, G075, G1 and free papain 0.30 % (w/v) and in the control group after five days of incubation.

porosity of the hydrogels for G050, G075 and G1, also confirmed by the FTIR results.

The enzyme kinetics assay highlighted in Fig. 6 shows that, for G050, G075 and G1, the profiles demonstrates marked similarity, as previously discussed. For these three treatments, enzymatic activity was observed on the third day and it is worth noting that this event preceded the count of recovered nematodes, which occurred on the fifth day, highlighting the hypothesis that the enzyme has a considerable effect on juvenile mortality. Added to this is the fact that enzymatic action is easily observed by the digestion of cuticles and body contents of juveniles, according to Fig. 8. Thus, the proteolytic activities of papain in solution, resulting from the different treatments, were important in causing a high reduction in juveniles of *Panagrellus* sp., pointing to mortality rates above 80 % for those treatments with the enzyme encapsulated in hydrogels and above 70 % for chitosan without the enzyme.

In this context, other researchers obtained relevant results on nematode mortality using plant proteases. Gomes et al. [51] evaluated the action of extracts containing proteases from *Synadenium grantii*, a Euphorbiaceae plant, for 24 h and the mortality of juveniles of *Panagrellus redivivus* was 72 %. In another interesting work, Sufiate et al. [52] used latex containing *Euphorbia milii* proteases on *P. redivivus* larvae and, after 48 h, the reduction in juveniles was around 96 %. Castro et al. [23] studied the action of 0.5 % (w/v) papain (or 5 mg/mL) on *Panagrellus* sp. and, in 48 hours, the reduction was around 82 %.

It is worth mentioning that these results were obtained in incubation times of 24 and 48 h, unlike the present study, in which the incubation time was 5 days, with 100 % mortality. Consequently, the exposure time of *Panagrellus* juveniles. sp the action of papain was greater (Fig. 8). These results reaffirm the potential of papain in biochemical control.

Studies indicate that chitosan and its derivatives have known antimicrobial and antifungal properties [53,54]. This occurs due to the electrostatic interaction between the high positive charge density of chitosan and its derivatives, under certain conditions, such as pH, for example, and the anionic surface of microorganisms [55].

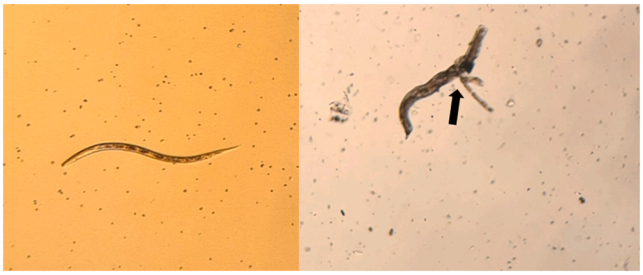

Fig. 8. (a) Juvenile of *Panagrellus* sp. intact (control group). (b) Juvenile of *Panagrellus* sp. with cuticle degradation after treatment with papain. The black arrow indicates the action of papain digestion on the nematode.

Chitosans also have multifunctional action, promoting increased growth and increased plant resistance [56]. Some studies demonstrate its activity in controlling various pathogens, such as the work of [57]. The researchers used chitosan alone or in the form of silver nanoparticles immobilized on pathogenic bacteria and on isolated fungi and the results were encouraging.

Within this perspective, Mouniga et al. [58] analyzed the effect of chitosan nanospheres on the development of nematodes *Meloidogyne incognita*, an important pathogen whose reproduction cycle occurs in part in the roots of various crops such as tomatoes. Along these lines, experiments in pots demonstrated a considerable reduction in the number of galls, egg mass and juveniles compared to the control after 45 days of inoculation. Khalil and Badawy [59] evaluated the in vitro action of chitosan on second-stage larvae of *M. incognita* at a concentration of 124.9 mg/L and there was a 50 % reduction in the number of nematodes after 48 h of treatment. These authors also studied the potential of chitosan in reducing *M. incognita* larvae in soil, however, at a concentration five times higher and, after two months of treatment, the reduction percentage was approximately 69 %.

In this present study, the action of encapsulated or free papain on *Panagrellus* sp. was evaluated unlike juveniles of the genus *Meloidogyne*. However, nematodes of the genus *Panagrellus* are models used to study mortality action due to the resistant constitution of its cuticle and, furthermore, they have peculiar characteristics, which allow the perception more efficient of sublethal toxic effects compared to phytonematodes [24,51,60]. Thus, the reduction of above 70 % (Fig. 7) of *Panagrellus* sp. juveniles, observed with treatment with chitosan alone, was very interesting, reinforcing the potential of the biopolymer as a nematicidal agent. The immobilization of enzymes provides the biocatalyst with greater stability by increasing rigidity and decreasing solubility [61]. From this perspective, in Fig. 7, a drop in the proteolytic activity of the free enzyme is observed, while, in some hydrogels (G0 and G025), It can be seen that activity grows and remains stable.

From the point of view of applicability in controlling pests of agricultural interest, this phenomenon is very appropriate. This is because, in the case of chemical pesticides, several applications are often required to ensure product efficiency in the field and the use of suitable carriers can guarantee the stability of the encapsulated molecule and the system tends to be more sustainable from an environmental point of view [62,63]. Similarly, as with traditional chemicals, repeated applications of the enzymes are necessary to control nematodes, as the stability of the molecule is often compromised due to the variation in temperature and pH of the environment [64,65].

Thus, the use of encapsulation is very appropriate to mitigate these problems and, therefore, hydrogels containing enzymes become promising as agents for controlling pests and diseases of agricultural interest.

4. Conclusion

Free papain as well as chitosan hydrogels containing the enzyme promoted the reduction of juveniles of *Panagrellus* sp., a nematode model for mortality studies. The different concentrations of glutaraldehyde used in the crosslinking impacted enzyme release kinetics in solution and enzyme activity profiles during the study period.

The literature reports the high potential of enzymes that exert relevant mitigating action on nematodes of agricultural interest and the results presented in this study reinforce this trend. Chitosan without cross-linking was also effective in the mortality of the nematodes evaluated and constitutes a slow release system, sustainable from an environmental point of view, as it has the potential to reduce the number of applications needed to control pests and diseases in agriculture.

Chitosan hydrogels with papain demonstrated a kinetic profile of slow release of papain, eficaz na redução de juvenis effective in reducing juveniles of *Panagrellus* sp., a model nematode for toxicity studies.

Free chitosan hydrogels, as well as free papain, showed nematicidal action. Therefore, they are a sustainable control alternative, due to the potential to reduce the consumption of traditional chemical nematicides.

The increase in glutaraldehyde concentration in chitosan hydrogels contributed to changing contributed to changing the surface morphology of hydrogels, compared to chitosan without cross-linking. There were also probably changes in the amount of amino groups in the chitosan polymer chain by reaction with glutaraldehyde in different concentrations. This also possibly changed the degree of swelling of the hydrogels, which may have contributed to the kinetic behavior of papain release into the medium.

Probably, the nematicidal action of chitosan hydrogels containing papain occurred through enzymatic digestion of the cuticle of juveniles.

5. Limitations of the study

The limitations of the present study are listed below:

Monitoring the pH values of the released papain-containing solution over time, to strengthen the discussion on chitosan protonation; perform analysis to prove the autolysis of the enzyme; previously test the efficiency of papain encapsulation; perform complementary analyzes to confirm the porosity profile of the hydrogels.

CRediT authorship contribution statement

Hugo Leonardo Andre Genier: Conceptualization, Methodology, Investigation, Data curation, Visualization, Writing-original draft. Juliano Elvis de Oliveira: Conceptualization, Funding acquisition, Investigation, and Writing - Review & Editing. Sthefania Ferreira dos Santos: Methodology and Investigation. Julia Carvalho Araújo: Methodology and Investigation. Filippe Elias de Freitas Soares: Supervision, Conceptualization, Resources, Project administration, Funding acquisition, Writing - Review & Editing.

All authors listed have contributed to the work, all authors have read, approved and agreed to this manuscript submission, no part of this work has been published yet and no portion of this work has been or is currently under consideration for publication elsewhere.

Declaration of Competing interest

The authors declare no interest conflicts.

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