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Bromelain Loading and Release from a Hydrogel Formulated Using Alginate and Arabic Gum

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ABSTRACT

An ideal wound dressing ensures a moist environment around the wound area and absorbs exudates from the wound surface. Topical application of bromelain to incised wounds has been shown to reprogram the wound microenvironment to promote effective tissue repair. Combining the characteristics of hydrogels and bromelain is therefore of great interest. Herein, we describe the development of a hydrogel, formulated using alginate and Arabic gum, for bromelain loading and release. The hydrogel formulation was evaluated using response surface methodology, considering the pH value and the concentration of alginate and Arabic gum. Bromelain loading and release were evaluated based on passive diffusion. Differential scanning calorimetry and Fourier transform infrared spectroscopy were performed to confirm bromelain immobilization in the hydrogel. The final hydrogel formulation had a swelling ratio of 227% and incorporated 19% of bromelain from a bromelain solution. Bromelain immobilization in the hydrogel was the result of hydrogen bond formation and was optimal at 4 °C after 4 h of contact. This evidence suggests that bromelain entrapment into a hydrogel is a promising strategy for the development of wound dressings that support the debridement of burns and wounds.

Introduction

Wound healing is a dynamic and complex process that requires the restoration of anatomic continuity and function and involves a sequence of interdependent and overlapping physiological events. The important phases of wound healing can be summarized as follows: inflammation, proliferation, and maturation [1].

Current treatment options involve repeated doses, bandage replacement, supervision by nursing personnel, and hospitalization, making treatment expensive and limited [2]. A major drawback is the need for frequent dressing changes, which increases the risk of infection. In the case of chronic wounds with insufficient blood flow and local edema, wound healing is very difficult to achieve without active treatment [3]. Various devices, dressings, drugs, and delivery systems have been extensively investigated in order to accelerate wound healing and expand their application to multiple types of wounds.

Topical application of bromelain to incised wounds has been shown to reprogram the wound microenvironment to promote effective tissue repair [4]. The term "bromelain" originally described any member of the Bromeliaceae family [5]; however, it is now used as a collective term for the proteolytic enzymes found in the stem, fruit, or leaf tissues of the plants of the Bromeliaceae family, which includes the well-known pineapple species, *Ananas comosus* L. [6–8].

A cream containing 35% bromelain in a lipid base is beneficial for the debridement of necrotic tissue and the acceleration of wound healing [9]. Singer et al. [10] showed that an enzymatic bromelain-based debriding agent rapidly removed the necrotic layer of the dermis and preserved unburned tissue after a single 4 h topical application in a porcine comb burn model. The topical bromelain used on the incised wound tracks accelerated the recovery of blood perfusion, increased pO_2 in the wound tissue, controlled the expression of tumor necrosis factor alpha (TNF-α), and upregulated the expression of transforming growth factor beta (TGF-β) [11]. Enzymatic debridement using bromelain is preferred over surgical debridement because a surgical incision is painful and nonselective, and exposes the patient to the risks of repeated anesthesia and significant bleeding [12, 13].

An ideal wound dressing ensures a moist environment around the wound and absorbs exudates from the wound surface [14]. Hydrogel dressings are designed to hold moisture on the surface of the wound, which prevents bacteria and oxygen from reaching the wound, thereby providing a barrier against infection [15]. This type of dressing also minimizes the loss of body fluids and accelerates the healing process [16].

Further, using hydrogels in wound dressings allows for the inclusion of additives that add desirable functionalities to the dressing, such as the encapsulation of cells [17] or large molecules like insulin [18]. Moreover, hydrogel-based drug-loaded networks have been developed for medical and pharmaceutical applications, especially for use as controlled drug delivery systems [18–20].

Momin et al. [15] reported the use of a chitosan-alginate hydrogel loaded with curcumin and honey for wound healing. This hydrogel provided a dry wound bed, and the curcumin and honey effectively contributed to faster wound healing. Nayak et al. [21] developed and characterized alginate-Arabic gum beads containing glibenclamide to reduce the need for multiple doses and improve the sustained release of the medication. These optimized beads exhibited a high drug encapsulation efficiency and a suitable sustained drug release pattern over a prolonged period of time [21]. Poly(N-isopropylacrylamide)-co-acrylamide hydrogels with a swelling rate of 125% have been developed; these hydrogels are capable of loading 56% of bromelain from a bromelain solution and releasing up to 91% of the retained bromelain [22].

Recently, the exploration of natural polymers for use in the development of various pharmaceutical dosage forms has increased to include polymers with more diversity and different properties. Natural polymers offer specific advantages over synthetic polymers, including ease of availability, biocompatibility, biodegradability, non-toxicity, and pollution-free processing [23]. These properties make natural polymers attractive for use in hydrogel formulations in order to provide acceptable biodegradability and biocompatibility. However, in order to use them for this purpose, the development of new synthesis and cross-linking methods to achieve these desirable characteristics is necessary [24].

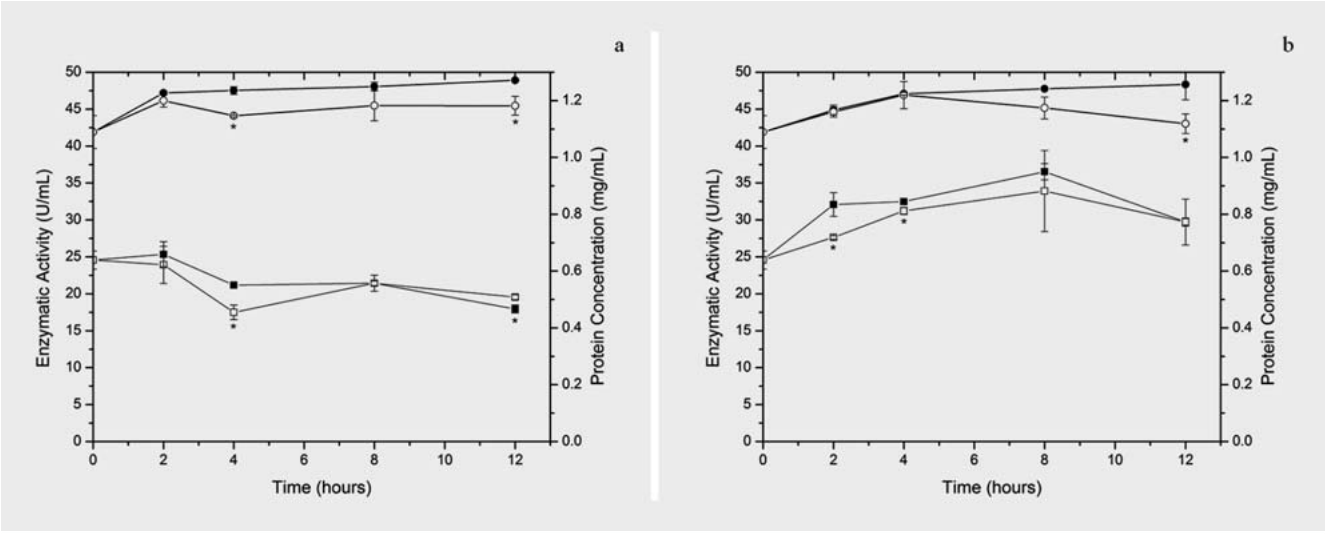
Polysaccharides, such as alginates, have been used extensively in the food, cosmetic, pharmaceutical, and biomedical industries for their gel-forming properties in the presence of multivalent cations [25]. Additives may modify the physical and chemical properties of hydrogels by altering the network structure, rheological properties [26], or hydrophobicity. Mixing alginate with other polymers also influences pore size and network complexity. For example, the presence of gum helps overcome the fast dissolution of a drug from a sodium alginate hydrogel at a higher pH, which was found to be the major limitation for alginate use [27].

Arabic gum is used extensively in the pharmaceutical and food industries as an emulsifier in the encapsulation process and is considered a natural amphiphilic surfactant [28]. It can be used in hydrogel production because of its biodegradability, biocompatibility, and non-toxicity [29]. However, Arabic gum cannot polymerize on its own and must be mixed with other polymers such as alginate.

Here, we describe the development of a hydrogel formulation using alginate and Arabic gum. Bromelain loading and release from the formulated hydrogel has been described, aiming at the development of a wound dressing. During hydrogel formulation, swelling capacity and weight loss were determinate as well as hydrogel characterization by differential scanning calorimetry (DSC) and Fourier transform infrared (FTIR) spectroscopy. During bromelain loading and release, enzyme stability was evaluated using quantification of total protein and enzymatic activity.

Results and Discussion

Divalent calcium cations have been reported to induce cross-linking between alginate chains, leading to the formation of strong, water-resistant gels [30–32]. When calcium ions are added to a sodium alginate solution, alignment of the G blocks (from the alginate structure) occurs and the calcium ions bind between the two chains like eggs in an egg carton [33, 34]. Therefore, the reac-



▶ **Fig. 1** Analysis of bromelain incorporation into hydrogel disks at (**a**) 4 °C and (**b**) 25 °C. Both figures show the protein concentration in control bromelain solution (●) and in the remaining solution after hydrogel immersion (○), and enzymatic activity in control (■) and remaining (□) solutions. *Statistically significance ($p < 0.05$) between control and remaining solutions. Error bars indicated the standard deviation of three replicates.

tion of alginate with calcium is the result of calcium-induced dimerization of the G-block regions.

Alginate (at concentrations of 1, 2, and 3%, w/w) and Arabic gum (at concentrations of 1, 2, and 3%, w/w) were tested at three different pH conditions (5, 6, and 7) according to the factorial design 2^3. In this first study, surface response methodology indicated that the relationship between the concentrations of alginate and Arabic gum was the most significant variable affecting the hydrogel polymerization process, followed by the concentration of alginate. Once the buffer pH was found to be the least significant variable affecting the polymerization process, a pH of 6 was used in all subsequent formulation studies.

Further studies were conducted to optimize the concentrations of alginate and Arabic gum required for the hydrogel formulation. Hydrogel rehydration ability (water absorption) was the criterion used to determine the optimal alginate and Arabic gum concentrations. According to the factorial design 2^3, hydrogel rehydration ability was greater when using the highest alginate concentration and the lowest Arabic gum concentration, 3 and 1% respectively. A new central composite design was used to complete the analysis of the concentrations of alginate (0.59 to 3.41%, w/w) and Arabic gum (0.79 to 2.21%, w/w).

The second factorial design indicated that rehydration ability was greater when alginate and Arabic gum concentrations were high [2.00 and 2.21% (w/w), respectively]. With this formulation, the hydrogel absorbed 2.27 times its original weight in water.

A hydrogel dehydration test was performed with the final hydrogel formulation. For the duration of the study, the hydrogel presented a significant weight loss of approximately 4%, which was attributed to a constant loss of water.

After 4 h of bromelain loading at 4 °C (▶ **Fig. 1 a**), the protein concentration of bromelain solution after hydrogel immersion significantly decreased to 93% of that of the control bromelain solution, indicating that the hydrogel disks absorbed the protein. After 4 h, a significant decrease (17%) in bromelain enzymatic activity in the immersion solution was also recorded, which suggested that bromelain was absorbed by the hydrogel disks.

After 2 and 4 h of bromelain loading at 25 °C (▶ **Fig. 1 b**), there were significant decreases in the enzymatic activity of the bromelain solutions before and after contact with the hydrogel disks (16 and 4%, respectively), which indicated that bromelain was incorporated into the hydrogel. However, at those incorporation times, no significant difference in protein content was observed. After 12 h, there was a significant decrease in protein concentration (12%), but there was not a significant difference in enzymatic activity. The optimal bromelain loading time was determined to be 4 h at 4 °C and these conditions were used to incorporate bromelain into the hydrogel disks for all subsequent studies.

Hydrogels with bromelain were prepared as described above for the bromelain release study. ▶ **Table 1** indicates the bromelain content of the hydrogels prior to the release study. The results indicated a decrease in protein content and enzymatic activity (7 and 19%, respectively) in the bromelain solution after hydrogel immersion compared with the initial bromelain solution. Bromelain release from hydrogel disks was also studied using passive diffusion by using alginate-Arabic gum hydrogel disks swollen in bromelain solution. The protein concentration and enzymatic activity of the buffer solution before and after contact with hydrogel loaded with bromelain are presented in ▶ **Fig. 2**.

After 15 min of release, a significant increase in enzymatic activity and protein concentration in the buffer solution was recorded. The amount of released bromelain after the first 15 min was higher than the amount of loaded bromelain. According to the supplier, commercial stem bromelain (Sigma-Aldrich) is 35% pure, containing other compounds with a molecular weight higher than 30 kDa [35], which may not have been incorporated into the hydrogel. Therefore, this fact may indicate that the hydrogel selective absorbed bromelain, enhancing its enzymatic activity.

▶ **Table 1** Total protein concentration and enzymatic activity of bromelain solution (3 mg/mL) after 4 h of incorporation at 4 °C into hydrogels for the desorption study (n = 18).

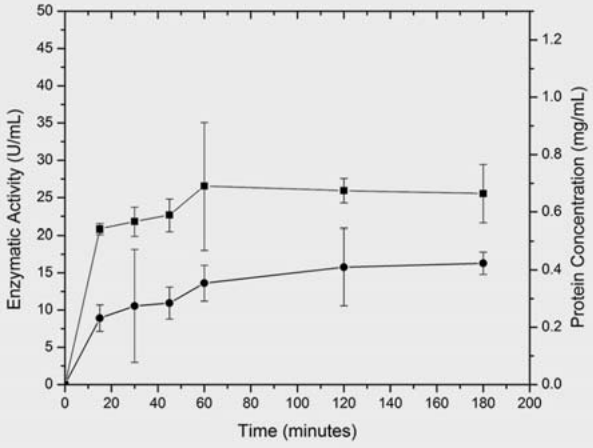
	Initial bromelain solution	Bromelain solution after hydrogel immersion	Hydrogel disks
Protein concentration (mg/mL)	1.71 ± 0.02	1.59 ± 0.02	0.12 ± 0.01
Enzymatic activity (U/mL)	33.14 ± 1.91	26.98 ± 0.22	6.16 ± 1.92
Specific activity (U/mg)	19.39 ± 1.36	16.92 ± 0.17	53.99 ± 2.39

Bromelain release increased during the first hour and then plateaued, as shown in ▶ **Fig. 2**. This happened because of the passive diffusion of bromelain, which required the substance to be distributed non-uniformly between the media. Once uniform distribution was achieved, there was no further observable macroscopic change [36].

The thermic properties of free bromelain, hydrogel, and hydrogel with bromelain were studied using DSC, presented in ▶ **Fig. 3**. Glass transition temperature (T_g) is defined as the temperature at which an amorphous system changes from a glass to the rubbery state, and it is associated with an endothermic relaxation peak. The high T_g value indicates greater enzyme stability at a high temperature, while the low T_g value indicates the vulnerability of the product during handling and mixing with others compounds. The DSC thermogram for free bromelain (▶ **Fig. 3a**) presented enthalpy relaxation with onset at 50 °C and ends at 130 °C, indicating a greater product stability. The other thermic events at 151.05 °C and 215.35 °C (▶ **Fig. 3a**) indicate the presence of crystalline material in the free bromelain.

A DSC hydrogel thermogram (▶ **Fig. 3b**) showed an exothermic peak, due to the degradation of the polymer, at 287 °C. The second endothermic event (175 °C) is associated with T_g of both cross-linked polymers. The more important DSC event is the lower T_g of hydrogel with bromelain showing low thermal stability in the formulation with Arabic gum and sodium alginate (212.86 and 198.55 °C).

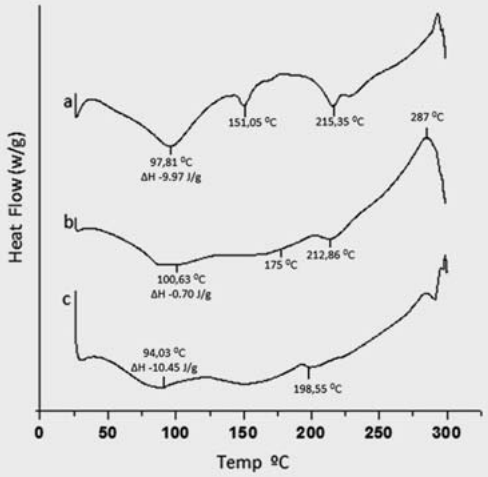
In endothermic processes, heat energy is absorbed by the surrounding environment, a phenomenon known as enthalpy (ΔH) [37]. Free bromelain (▶ **Fig. 3a**), hydrogel (▶ **Fig. 3b**), and hydrogel with bromelain (▶ **Fig. 3c**) presented ΔH of fusion of 97.81 °C and − 9.97 J/g, 100.63 °C and − 0.70 J/g, and 94.03 °C and − 10.45 J/g, respectively. The ΔH values obtained in this study differed from those obtained in the study by Amid et al. [38]; however, the T_g values obtained in this study were similar to the results presented for recombinant bromelain analyses. The amount of heat required to break the bonds between amino acid chains for free bromelain and hydrogel with bromelain was low. In contrast, the high T_g was an indicator of protein thermic stability, and denaturation occurred at low temperatures [39]. When bromelain was incorporated into the hydrogel, only a slight change in the hydrogel degradation temperature (from 214.06 to 212.86 °C) was observed. This suggested that bromelain absorption did not interfere with the intermolecular polymeric chains of alginate and Arabic gum.



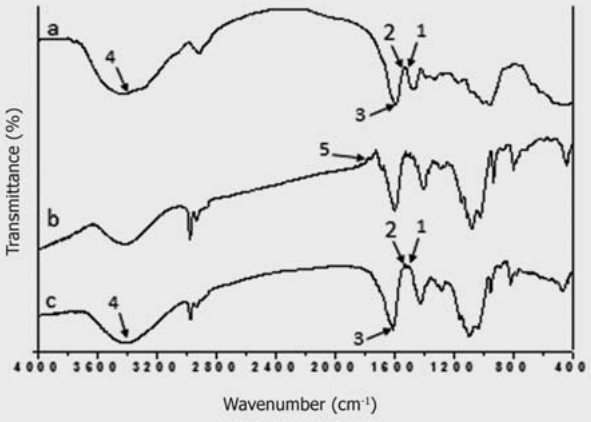
▶ **Fig. 2** Analysis of protein concentration (●) and enzymatic activity (■) released from hydrogels loaded with bromelain at different times at 36 °C. Error bars indicate the standard deviation of three replicates.

The characteristic chemical groups of bromelain that were present in the hydrogel with bromelain were determined using FTIR spectroscopy, as shown in ▶ **Fig. 4**. Bromelain immobilization into hydrogel occurred by the formation of hydrogen bonds between the –OH and $–COO^-$ groups of sodium alginate and the NH_2 groups of bromelain [40]. Hydrogel with bromelain presented stretches at 1635 cm^{-1} (▶ **Fig. 3c**, stretch 3), which were indicative of C=O groups. This confirmed the presence of amino acids containing amide I in their side chains, such as asparagine and glutamine. This sample also presented stretches at 1541 cm^{-1} (▶ **Fig. 3c**, stretch 2), which corresponded to the C–N group (amide II) of monoalkyl and guanidium [41]. The analysis indicated a vibration displacement at 1519 cm^{-1} (▶ **Fig. 3a, b**, stretch 1) relative to bromelain. The bromelain enzymatic peptide bond (3398 cm^{-1}, stretch 4) was observed at 3404 cm^{-1} in the hydrogel with bromelain [40]. The hydrogel with bromelain does not show stretches at 1764 cm^{-1} (▶ **Fig. 3b, c**, stretch 5) corresponding to the carboxylic group ($-COO^-$) presented in the hydrogel, which suggests the possible interconnection with bromelain.

The present study showed that alginate and Arabic gum were successfully combined to formulate a hydrogel; however, their concentration ratio was crucial for hydrogel polymerization and



▶ **Fig. 3** DSC thermogram curves of (**a**) free bromelain, (**b**) hydrogel, and (**c**) hydrogel bromelain.



▶ **Fig. 4** FTIR spectra of free bromelain (**a**), hydrogel (**b**), and hydrogel with bromelain (**c**). The numbers 1, 2, 3, and 5 correspond to stretches at 1519 cm^{-1}, 1541 cm^{-1}, 1635 cm^{-1}, and 1764 cm^{-1}, respectively. The stretch at 3398 cm^{-1} (5) in unpurified bromelain was observed at 3404 cm^{-1} in the hydrogel with bromelain.

rehydration ability. The highest bromelain loading was at 4 °C after 4 h, with an increase in bromelain-specific activity. Bromelain release increased during the first hour and then plateaued, owing to technique limitations. DSC and FTIR analyses indicated that bromelain incorporation into hydrogels occurred by hydrogen bond formation and did not interfere with alginate or Arabic gum intermolecular polymeric chains. These results provide evidence that bromelain entrapment into hydrogel provides a promising strategy for skin applications, such as burn and wound debridement.

Materials and Methods

Materials

Bromelain (35 % protein), azocasein, and bicinchoninic acid assay kits were purchased from Sigma-Aldrich. Alginate (purity > 90 %) and Arabic gum (purity > 90 %) were purchased from Dinâmica Química Contemporânea Ltda. Other reagents were of reagent or food grade.

Hydrogel formulation

Blank hydrogel matrix was prepared using an ionotropic external gelation technique, and calcium chloride was used as a cross-linking medium. Calcium chloride was chosen because divalent calcium cations have been reported to induce cross-linking between alginate chains, leading to the formation of a strong, water-resistant gel [30–32]. In brief, alginate and Arabic gum were first dissolved in phosphate buffer, in defined proportions and pH, and the solution was placed in a 12-well plate (diameter = 21.4 mm). The alginate and Arabic gum mixture was covered with an aliquot of a 0.5 M calcium chloride-isopropanol solution to initiate the polymerization process required to form the hydrogel. After 30 min, the calcium chloride-isopropanol solution was replaced with an aqueous 0.5 M calcium chloride solution for 1 h. Upon completion of polymerization, the hydrogel matrix was rinsed under running water to remove residual calcium ions and other non-polymerized components, and finally dried for 24 h in an oven at 45 °C.

Hydrogels were formulated using response surface methodology, as described by Neto et al. [42]. To describe the nature of the response surface in the optimal region, a 2^3 full experimental design with three variables, two levels, and four replications of the central point was used, leading to twelve experiments. The variables considered were alginate concentration (1 and 3 %, w/w), Arabic gum concentration (1 and 3 %, w/w), and buffer pH (5 and 7). The swelling ratio was measured for each run. Based on the results of the full experimental design, a 2^2 central composite design was chosen. All experiments were analyzed using Statistica 7.0 software (Statsoft).

Hydrogel swelling capacity and weight loss

Dried hydrogel was immersed (at 4 °C) in phosphate buffer at the same pH as that of the alginate and Arabic gum mixture for 12 h. Hydrogel samples from the immersion bath were weighed, dried for 12 h at 45 °C, and weighed again. The weight variation was used to estimate product swelling ratios according to Eq. 1.

$$P = \frac{W_s}{W_d} \times 100 \quad (1)$$

Eq. 1. Percentage of water absorption calculation used as a measurement of hydrogel hydration ability, where W_s is the weight of swollen hydrogel and W_d is the weight of dried hydrogel.

For weight loss, samples were removed from the immersion bath, weighed, and dried for 12 h at 45 °C. Thereafter, the samples were weighed again and incubated at 45 °C. For 35 days, the samples were weighed daily and the weight was compared with the

initial weight of the dried samples. Changes in weight were used to estimate hydrogel weight loss according to Eq. 2.

$$WL = \frac{W_t - W_i}{W_i} \times 100 \quad (2)$$

Eq. 2. Percentage of weight loss (*WL*) calculation, where W_t is the hydrogel weight at day t and W_i is hydrogel initial weight.

Bromelain loading and release

A swelling method was utilized to incorporate bromelain into the hydrogel matrix. Hydrogel disks (diameter = 15 mm) were immersed in 1 mL bromelain (3 mg/mL)-McIlvaine buffer (pH 5) using 24-well plates. The plates were agitated at 4 or 25 °C for 2, 4, 8, or 12 h. Thereafter, swollen bromelain-loaded hydrogel was removed from the solution and the protein concentration and bromelain activity were determined in the remaining solution. To test bromelain stability during incorporation, a separate bromelain solution was maintained under the same experimental conditions, but without the hydrogel matrix, as a control. Samples were collected at the same time points for comparative evaluation. The decreases in protein amount and enzymatic activity between the control and the solution after hydrogel immersion were used to determine bromelain loading according to Eq. 3.

$$HD = S_c - S_a \quad (3)$$

Eq. 3. Bromelain loading in hydrogel disks (*HD*), where S_c is the control solution and S_a is the solution after hydrogel immersion.

Bromelain release from the hydrogel was carried out at 36 °C in PBS (pH 7). Hydrogel disks, loaded with bromelain, were immersed in 1 mL of PBS buffer in 24-well plates and stored at 36 °C. At 15, 30, 45, 60, 120, and 180 min, the hydrogel was collected from the medium and the bromelain concentration and enzymatic activity of the medium were quantified. Increases in protein concentration and enzymatic activity of the medium were used to determine bromelain release.

Enzymatic activity and protein quantification

Enzymatic activity was determined using the azocasein method [43, 44]. Bromelain was allowed to cleave the substrate, azocasein, at 37 °C for 10 min. Trichloroacetic acid was then added to precipitate non-hydrolyzed azocasein and stop the reaction. Cleavage of azocasein caused the release of tyrosine residues, which were detected at 440 nm by spectroscopy. Enzymatic activity was calculated in activity units (U/mL) and was defined as the amount of bromelain needed to produce 1 mmol of tyrosine per min at 37 °C. The protein concentration was determined using a bicinchoninic acid assay [45].

Differential scanning calorimetry and Fourier transform infrared spectroscopy

For thermoanalytical characterization, thermal profiles of pure bromelain, hydrogel, and hydrogel with bromelain were analyzed using DSC analyses (Thermal Analyzer TA 60 W-Shimadzu DSC-60) under an oven atmosphere with nitrogen gas and a dynamic flow rate between 20–50 mL/min, a heating ratio of 10 °C/min, and a temperature between 25 and 350 °C. Hydrogels were incubated in the oven at 30 °C and were mortar crushed after drying.

FTIR (Shimadzu, FTIR IRAffinity-1 S), using transmittance modes, was used to characterize the specific chemical groups of the pure bromelain, hydrogel, and hydrogel with bromelain. Hydrogels were incubated in the oven at 30 °C and were mortar crushed after drying. Approximately 2 mg of hydrogel sample were mixed with potassium bromide for tablet formation. Spectra were drawn in the wavelength band between 4000 and 400 cm^{-1} after 64 readings, with 4 cm^{-1} of resolution. All spectra were normalized and the vibration bands were associated with the principal chemical groups.

Data analysis

All measurements were performed in triplicate. All results are expressed as the mean ± standard deviation values, and were calculated using one-way analysis of variance or Student's t-test. Statistical significance was established at $p < 0.05$.

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Conflict of Interest

The authors declare no conflict of interest.

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