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Poly(N-Isopropylacrylamide)-*co*-Acrylamide Hydrogels for the Controlled Release of Bromelain from Agroindustrial Residues of *Ananas comosus*

Authors Fernanda Croisfelt[1], Bianca C. Martins[1], Robson Rescolino[1], Diego F. Coelho, Beatriz Zanchetta[2], Priscila G. Mazzola[3], Luis Ricardo Goulart[1], Adalberto Pessoa Jr.[4], Elias B. Tambourgi[2], Edgar Silveira[1]

Affiliations
[1] Genetics and Biochemistry Institute, UFU, Uberlândia, MG, Brazil
[2] School of Chemical Engineering, Unicamp, Campinas, SP, Brazil
[3] Faculty of Pharmaceutical Sciences, Unicamp, Campinas, SP, Brazil
[4] School of Pharmaceutical Sciences, USP, São Paulo, SP, Brazil

Key words
- *Ananas comosus*
- Bromeliaceae
- N-isopropylpolyacrylamide
- hydrogels
- bromelain
- ethanol purification
- controlled release

Abstract

This works reports the purification of bromelain extracted from *Ananas comosus* industrial residues by ethanol purification, its partial characterization from the crude extract as well as the ethanol purified enzyme, and its application onto poly (N-isopropylacrylamide)-*co*-acrylamide hydrogels. Bromelain was recovered within the 30–70% ethanol fraction, which achieved a purification factor of 3.12-fold, and yielded more than 90% of its initial activity. The resulting purified bromelain contained more than 360 $U \cdot mg^{-1}$, with a maximum working temperature of 60 °C and pH of 8.0. Poly(N-isopropylacrylamide)-*co*-acrylamide hydrogels presented a swelling rate of 125%, which was capable of loading 56% of bromelain from the solution, and was able to release up to 91% of the retained bromelain. Ethanol precipitation is suitable for bromelain recovery and application onto poly(N-isopropylacrylamide)-*co*-acrylamide hydrogels based on its processing time and the applied ethanol prices.

Introduction

The largest pineapple *Ananas comosus* (L.) Merr. (Bromeliaceae) producers in the world are Thailand, Brazil, Philippines, Costa Rica, and India. Brazil alone harvested more than 2.4 million tons of pineapple in 2013 [1]. Pineapple industries seek maximum yields of raw and processed materials, but large percentages of waste are generated during processing. These waste residues, which make up nearly 60% of the weight of the raw product, contain significant amounts of bromelain [2].

Bromelain is the general name given to a group of endopeptidases found in pineapple as well as all proteases derived from members of the Bromeliaceae family. The downstream processing of enzymes such as bromelain corresponds to 70–90% of the total production cost [3]. As new and economically feasible biotechnological processes arise to maximise production, downstream strategies are the bottleneck within these processes. Current purification technologies are limited by design, since they were intended to function at a much smaller scale [4].

The industrial purification of biomolecules requires clarification steps, such as fractional precipitation with salts or organic solvents, which have low resolution with high processing capacity [5]. Although the use of such techniques reduces costs by concentrating and clarifying the target molecules, the degree of purification decreases significantly with increasing amounts of the precipitant agent [6]. In contrast, since the 1990s, bromelain has been prepared simply by ultrafiltration, centrifugation, and lyophilisation of the cooled pineapple juice, yielding a yellowish powder with a protein content of 40% [7].

Alternatively, bromelain has been purified by countercurrent chromatography [8], reverse micelles [9], aqueous two-phase systems [2, 10], expanded bed chromatography [11], and membrane separation processes [12]. However, even under the best operating conditions, these methods have not achieved a high enough purification factor to avoid polishing steps, such as exclusion molecular chromatography.

Precipitation with organic solvents is a technique with simple equipment requirements and low energy needs. It is also easy to scale up and provides the possibility of using a large number of precipitants, such as ethanol, which is widely produced in Brazil and worldwide. Furthermore, ethanol can be recycled in the final process by a simple distillation, reducing environmental impacts and processing costs.

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Correspondence
Prof. Dr. Edgar Silveira
Genetics and Biochemistry Institute
Federal University of Uberlândia
Av. Pará 1720, Sala 160, Bloco 2E
38400–902 Uberlândia, MG
Brazil
Phone: + 55 34 32 25 84 36
silveira@ingeb.ufu.br

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Table 1 Ethanol concentration effects on bromelain precipitation

	Protein concentration ($mg \cdot mL^{-1}$)	Bromelain activity ($U \cdot mL^{-1}$)	Specific activity ($U \cdot mg^{-1}$)	Protein yield (%)	Enzyme recovery (%)	Purification factor
Crude extract	0.208	24.05	115.62	n. a.	n. a.	n. a.
0–30%	0.052	0.00	0.00	25.00	0.00	0.00
30–70%	0.061	21.98	360.32	29.33	91.39	3.12

Bromelain can be classified as either fruit bromelain (E.C. 3.4.22.33) or stem bromelain (E.C. 3.4.22.32), each with unique characteristics. Stem bromelain is present in the stems, leaves, and bark of the pineapples, and is used as a drug for the oral systemic treatment of inflammatory, blood coagulation-related, and malignant diseases. It offers a wide spectrum of therapeutic applications with *in vitro* and *in vivo* antithrombotic and fibrinolytic activities [13]. Bromelain has been administered in the treatment of allergic airway disease [14], has been shown to possess anticancer activity [15], and has been used in cosmetic compositions [16].

The safe and efficient delivery of enzymes is the key to the commercial success of current and future biotechnology products [17]. Several approaches have been reported for the delivery of bioactive molecules [18–20], and over the past decade, hydrogels have been extensively used as matrices to control drug delivery [21,22]. Hydrogels are insoluble, cross-linked, hydrophilic polymers that are swollen in an aqueous medium, and their water holding capacity arises mainly from the presence of hydrophilic groups (amide, amine, carboxyl, hydroxyl groups, etc.) in the polymer chains. The water content of hydrogels may vary from 10% to thousands of times that of the dry weight of the polymer network [23].

The most studied polymer systems are thermosensitive hydrogels, and one of the most familiar polymers of this type is poly (N-isopropylacrylamide), or PNIPAAm. PNIPAAm is characterised by a lower critical solution temperature (LCST) of 32 °C in water, above which the hydrogels collapse.

The objectives and design principles of hydrogel formulation are: (a) to provide local and extended release of the loaded therapeutic agents to augment the therapeutic effect, and (b) to decrease the adverse reactions and preserve the bioactivity of the therapeutic agent [24]. To achieve the desired therapeutic efficacy *in vivo* or *in vitro*, the hydrogel should be capable of delivering therapeutics at the right dosage with preserved molecular bioactivity. The copolymerisation of PNIPAAm with acrylamide (AAm) permitted the synthesis of hydrogels with improved characteristics and considerably broadened their sphere of application.

This works reports the purification of bromelain extracted from the agro-industrial residues of *A. comosus*, the partial characterisation of crude extract bromelain and ethanol-purified bromelain, and the application of bromelain into poly(N-isopropylacrylamide)-*co*-acrylamide (PNIPAAm-*co*-AAm) hydrogels.

Results and Discussion

Prior works have effectively documented the purification of bromelain by several methods, such as aqueous two-phase systems [2], reverse micelles [9], membrane processes [12], and expanded bed [11]. Fileti et al. [9] described the extraction of bromelain by reversed micelles, whereby it was possible to achieve a purification factor of 4.96. Lopes et al. [12] showed that it was possible to recover nearly 100% of bromelain activity with a purification factor of 10, using an ultrafiltration process. Coelho et al. [2] reported bromelain purification using an unconventional aqueous two-phase system that recovered 66.38% of the biological activity and yielded an 11.8-fold purification factor. Silveira et al. [11] described an expanded bed process using an ion-exchange matrix, which was capable of recovering 32% of the original biological activity with a purification factor of 13. However, these methods have either generated a great deal of residues or have focused on cost-demanding techniques. On the other hand, little is known about the characteristics of bromelain after its purification.

The data of bromelain purification shown in **Table 1** reveals that it is possible to purify bromelain by a two-step ethanol precipitation with a purification factor of over 3.1. Evidently, no bromelain activity was observed with increasing concentrations of ethanol up to 30% (v/v), which indicates the substantially low hydrophobicity of bromelain. The two-step ethanol precipitation process of bromelain purification led to an almost 60% reduction in protein mass. Thus, it was possible to recover bromelain with no significant loss in activity and with a considerable purification factor.

These findings are in accordance with those described by Soares et al. [3], confirming that it is possible to achieve an enzymatic activity above 90%. However, with less than 8% loss at the recovery yield, the purification factor was increased to 3.12. Chaurasiya et al. [25] reported a maximum recovery with acetone precipitation, where a recovery yield of nearly 86% and a purification factor of 4.9 were achieved. Aqueous two-phase systems are another technique employed extensively by several researchers to purify biomolecules with low resolution and low cost [2, 10]. Ketnawa et al. [26] extracted bromelain with an aqueous two-phase system composed of PEG-$MgSO_4$, which yielded a recovery of 113.5% and a purification factor of 2.23.

As good as it may seem, all of these purification techniques generate a great deal of residue at high concentrations, such as polymers, organic solvents, synthetic surfactants, and salts. The use of ethanol as a precipitant agent is an environment-friendly technology since ethanol is produced from sucrose fermentation, which is a green process, and can be easily recovered by simple distillation. On the other hand, the pineapple processing industries generate nearly 60% of all vegetable biomass residues, including leaves, stems, and bark [2]. Forty-two percent of these residues can be extracted, generating nearly 250 mL of crude extract per kilogram of processed fruit. This extract yields more than 15.25 mg of protein and as much as 5500 bromelain units after precipitation.

Shortly after purification, samples of the crude extract and the purified bromelain were partially characterised for optimum pH and temperature simultaneously (**Fig. 1**). Before ethanol purification, bromelain exhibited optimum activity at a pH of 7.0 and suboptimum activity at a pH of 10.0. This evidence points to the likelihood that fruit bromelain was present in the initial pineap-

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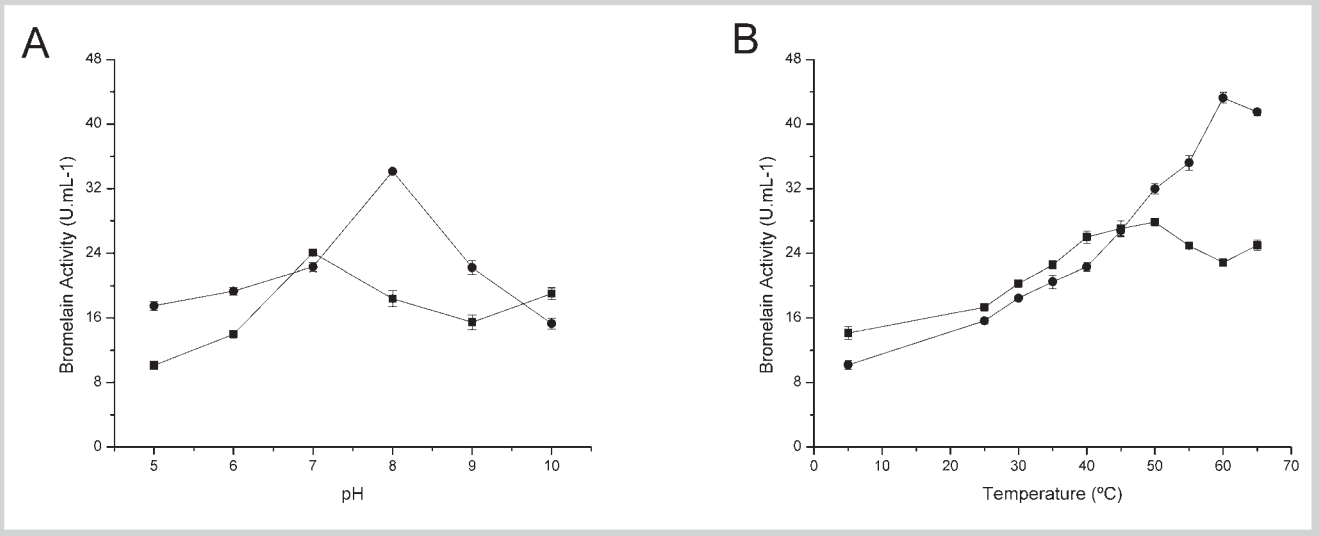

Fig. 1 Effect of (**A**) pH and temperature (**B**) on bromelain activity before (■) and after (•) ethanol purification. Bromelain activity was determined by the azocasein assay after incubation in various pH values and temperature. All data are presented as absolute activity values, and are represented as the mean ± standard deviation for at least three measurements.

ple residue and also in the crude extract. After purification, the pH at which optimum activity occurred shifted to 8.0, and no other peaks were observed.

Ketnawa et al. [27] reported an aqueous two-phase system to purify bromelain from pineapple bark and described its activity profile at pH values ranging from 3.0 to 10.0 in a solution containing poly(ethylene glycol). The maximum activity was observed at a pH of approximately 7.0, which was similar to that of bromelain from the crude extract. The ethanol-purified bromelain showed a similar behaviour, as its activity gradually decreased at acidic and more alkaline conditions.

It is apparent that the ethanol purification of bromelain is beneficial to its optimal temperature, as can be seen in **Fig. 1 A**. Prior to ethanol purification, the relative activity increased as the temperature increased up to a maximum of 50 °C, which was expected for the enzymes. However, after ethanol precipitation, the temperature of maximum bromelain activity rose to 60 °C, and at 65 °C, the decrease in activity was nearly imperceptible.

These results are similar to those reported by Ketnawa et al. [27], where the temperature of maximum activity was found to be around 50–60 °C. Enzymatic kinetics at low temperatures are ruled by the same principles as those in chemical kinetics, i.e., as the temperature increases, the reaction rate also increases since molecules have more kinetic energy to perform the reaction. Above a critical temperature of 50 °C for crude extract bromelain and 60 °C for ethanol-purified bromelain, the kinetic energy in the system is so high that the enzyme begins to unfold permanently, resulting in abrupt decreases in the reaction rate.

Comparing **Fig. 2 A** and **2 B**, it is clear that ethanol purification affects bromelain stability. Before purification, more than 80% of the initial activity of bromelain remained after 120 min for all pH values, except at 5.0 and 10.0. However, the purified bromelain retained nearly 50% of its initial activity after 180 min. Husain et al. [28] described that the active site of bromelain is close to the Cys-25 at the C-terminus and the His-159 at the C-terminus. Therefore, any structural or conformational changes near the active site might seriously affect the activity of bromelain.

According to Ahmad et al. [29], bromelain is in a fully denatured state when 80% of its native structure is lost, which is in accordance with the statement made by Husain et al. [28], who used casein as a substrate. This is true even if the active site of bromelain is fully inactivated. Thus, only ethanol-purified bromelain at a pH of 10.0 could be considered fully inactive.

Novaes et al. [30] found that bromelain at a pH of 8.0 at 35 °C lost 53.9% of its initial activity after 7 h, whereby a threshold of 40% was lost in the first 3 h of incubation. These results are similar to those of ethanol-purified bromelain, since crude extract bromelain was shown to be more stable.

As expected for plant enzymes, bromelain does not have good stability over time when exposed to high temperatures. It lost a significant amount of its relative activity at higher temperatures over time, as shown in **Fig. 3**. However, the enzyme could only be considered fully denaturated at temperatures higher than 60 °C for crude and higher than 50 °C for ethanol-purified bromelain. A better understanding of bromelain's denaturation process and stability over high temperature exposure could be obtained from a more detailed study of bromelain's thermodynamic profile through unfolding experiments. Moreover, the relative loss of activity by temperature exposure can be attributed to the unfolding and structural loosening of the initially fully active native state of bromelain at high temperatures.

During incubation and storage, bromelain could lose its proteolytic activity for two different reasons: autodigestion and inactivation due to harsh conditions. Autodigestion could be avoided by the use of conservatives such as glucose, sucrose, glycerol, and poly(ethylene glycol) [3]. Glycerol, sorbitol, sucrose, and trehalose [31] are also described as stabilisers of bromelain's native structure, which could prevent the loss of activity due to harsh processing conditions.

Temperature is the most widely used stimulus in environmentally responsive polymer systems. Changes in temperature are not only relatively easy to control, but are also easily applicable both *in vitro* and *in vivo*. PNIPAAm is a well-known polymer with temperature-sensitive characteristics, exhibiting constriction (lower critical solution temperature – LCST) at approximately

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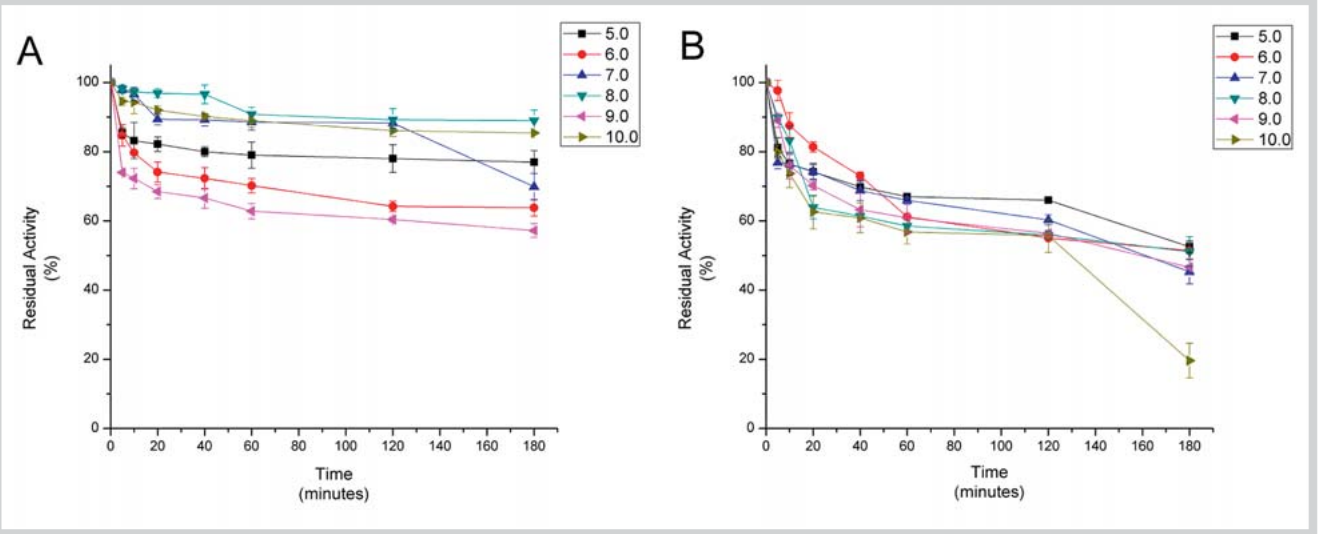

Fig. 2 Effect of environmental pH on bromelain activity before (**A**) and after (**B**) ethanol purification. Bromelain activity was determined by the azocasein assay after exposure to several pH values (5.0–10.0) for 180 min at 37 °C. All data are presented as absolute activity values, and are represented as the mean ± standard deviation for at least three measurements. (Color figure available online only.)

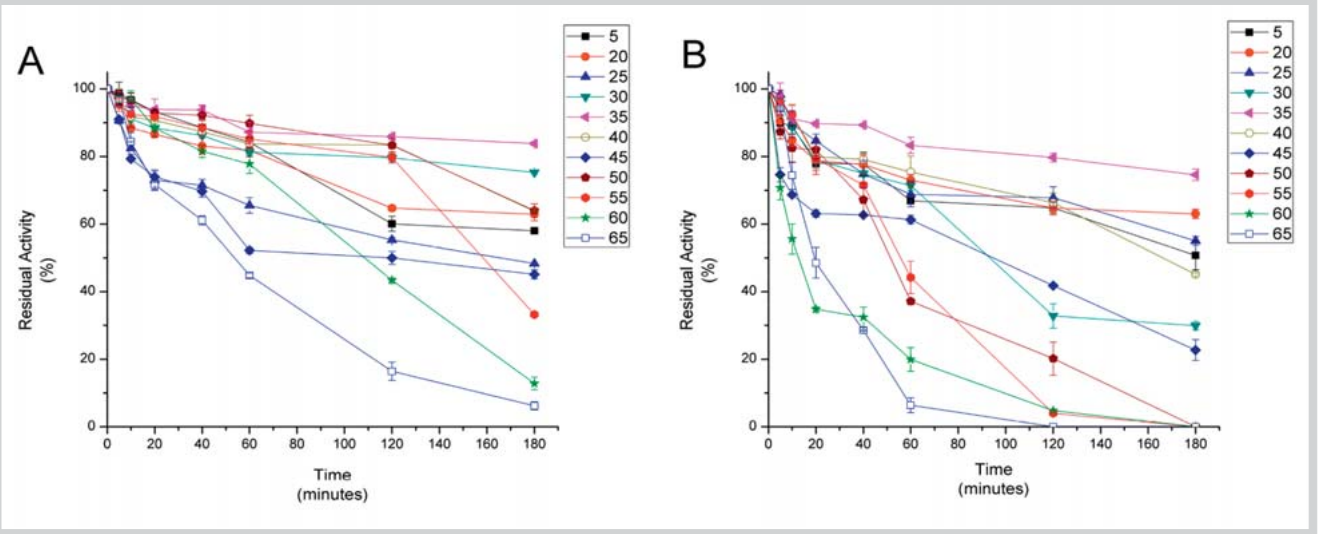

Fig. 3 Effect of environmental temperature on bromelain activity before (**A**) and after (**B**) ethanol purification. Bromelain activity was determined by the azocasein assay after exposure to several temperatures (4.0–65.0 °C) for 180 min at pH 7.0. All data are presented as absolute activity values, and are represented as the mean ± standard deviation for at least three measurements. (Color figure available online only.)

32 °C in aqueous solutions. Below this temperature, PNIPAAm is hydrophilic and exists in individual chains with a coiled conformation. Above 32 °C, it undergoes a sharp coil-to-globule transition, resulting in an abrupt shrinkage in the volume of the resulting hydrophobic hydrogel [32], thus having a capacity to release hydrophilic components such as bromelain.

The response surface methodology (RSM) is an optimisation technique based on factorial designs, as described by Box et al. [33], and it has been used with great success in industrial processes. RSM was applied to PNIPAAm-*co*-AAm hydrogel formulations to identify the optimum level and interaction of monomer concentration and reaction medium pH. The results are shown in ◉ **Table 2**. Run no. 10 and 12 exhibited maximum hydrogel swelling capacities, with a ratio of 125%, and the lowest swelling ratio was observed in run no. 13.

The competency of the RSM for the production of PNIPAAm-*co*-AAm hydrogels was verified using ANOVA and tested using Student's t-test statistical analysis. The results are described in ◉ **Table 3**. The p value represents the significance of the coefficients, and aids in understanding the patterns of the interactions between the variables. The p values suggest that the difference between all variables, including NIPAAm concentration, AAm concentration, and the pH of the reaction medium, showed statistical significance, aside from NIPAAm in the quadratic model and the cross-interaction between NIPAAm and AAm.

The interaction effects and optimal levels of the variables were determined by plotting the predicted profile of the response sur-

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Table 2 Design and results of response surface methodology.

Run	Levels			Swelling (%)
	X_1	X_2	X_3	
1	− 1	− 1	− 1	106
2	− 1	− 1	+ 1	105
3	− 1	+ 1	− 1	112
4	− 1	+ 1	+ 1	108
5	+ 1	− 1	− 1	105
6	+ 1	− 1	+ 1	104
7	+ 1	+ 1	− 1	122
8	+ 1	+ 1	+ 1	103
9	− 1.414	0	− 1	112
10	+ 1.414	0	+ 1	125
11	0	− 1.414	0	119
12	0	+ 1.414	0	125
13	0	0	− 1.414	102
14	0	0	+ 1.414	105
15	0	0	0	113
16	0	0	0	115
17	0	0	0	113
18	0	0	0	112

Table 3 Response surface methodology standardised effects and ANOVA.

Variables	Coefficient	t-test	P
X_1 (L)	1.820	5.344	0.0128
X_1 (Q)	0.418	1.181	0.3229
X_2 (L)	2.571	7.547	0.0048
X_2 (Q)	1.658	4.680	0.0184
X_3 (L)	− 1.463	− 4.294	0.0232
X_3 (Q)	− 4.896	− 13.819	0.0008
$X_1 X_2$	0.875	1.967	0.1439
$X_1 X_3$	− 1.875	− 4.215	0.0244
$X_2 X_3$	− 2.625	− 5.900	0.0097

Error = 1.583

face, as presented in **Fig. 4**. Lower and higher levels of pH did not result in better swelling behaviour in the PNIPAAm-*co*-AAm hydrogel. The profile of the swelling capacity indicates that the pH of the reaction medium for the production of PNIPAAm-*co*-AAm hydrogels should be around 8.0 for lower concentrations of NIPAAm and AAm. However, as the NIPPAm concentration rises, the pH should be more neutral.

According to Wang et al. [32], there is a hydrophilic/hydrophobic balance in the PNIPAAm hydrogels, resulting from the hydrophilic (amide) and hydrophobic (isopropyl) groups. The thermosensitive properties of these polymers are due to quick changes in the matrix hydrophilicity/hydrophobicity.

The incorporation of bromelain was performed by passive diffusion using dried PNIPAAm-*co*-AAm hydrogels. **Table 4** presents the concentration of bromelain loaded into the PNIPAAm-*co*-AAm hydrogels released at 35 °C. A study by de Moura et al. [34] investigated the loading of bovine serum albumin (BSA) into PNIPAAm-Alginate-Ca^{2+} hydrogels and the release of the protein, and the formulated hydrogels were capable of loading up to 35% BSA. However, increases in the NIPAAm concentration at the formulation stage did not affect the BSA loading capacity. All three formulations were able to load bromelain into the hydrogel matrix. However, formulation A was capable of loading up to 56% of the bromelain that was applied. Bromelain is 2.6 times smaller than BSA, which could explain the difference between the presented results and those of BSA. In addition, PNIPAAm-*co*-AAm hydrogels have a lower matrix compaction, presenting greater freedom of motion. Part of the energy is driven to sustain the motion, and part of it drives the hydrophilic-hydrophobic transition.

Formulation A of the PNIPAAm-*co*-AAm hydrogel was able to release up to 91% of the retained bromelain. The burst release of proteins from the formulations could be explained by the domain structure of the hydrogels. The partitioning of the protein between the hydrophilic and hydrophobic domains is a critical factor for protein release. At the sol-gel transition state, the system's volume contracts, leading to the expulsion of the aqueous phase in which proteins are dissolved [35].

However, the release rate of bromelain is greater than that of other proteins, such as BSA [34], bone morphogenetic protein [22], poly(γ-benzyl-L-glutamic acid) [36], and lysozyme [17]. This behaviour could be explained by the predominant role of polymer degradation. Bromelain was added to the PNIPAAm-*co*-AAm hydrogel after polymerisation. After initial diffusion from the gel, larger pore pathways were possible to form followed by a faster diffusion. Therefore, bromelain is released at a relatively faster rate than other proteins.

Bromelain has been used in the food industry for decades, where its crude stability and characterisation are quite well established. However, its nobler uses in industries such as cosmetics and pharmaceuticals industries are restricted because of the lack of information regarding its stability. Industrial processes often involve the use of a variety of solutions, various changes in pH, and, in most cases, the application of heat to achieve the final product. The present study demonstrated that greener processes, such as ethanol precipitation, could be used to purify bromelain by a factor up to 3.12 with a recover yield of 91.39%, and described the stability of the purified enzyme over varying pH and temperature. The concentration of the resulting purified bromelain was higher than 360 $U \cdot mg^{-1}$, with a maximum working temperature of 60 °C at pH 8.0. The ethanol-purified bromelain was less stable than that from the crude extract. However, it was fairly stable at mild temperatures and pH values. Ethanol precipitation seems to be quite suitable for bromelain recovery based on processing time and local ethanol prices, as Brazil is the largest ethanol producer in the world. Studies on the stability of purified bromelain are an important first step in the development of cost-effective industrial usage of pineapple residues.

Three formulations of PNIPAAm-*co*-AAm hydrogels were synthesised, and varying monomer concentrations and pH values were used in the delivery systems to control the release of bromelain. *In vitro* availability studies showed that all three formulations released almost all of the loaded bromelain. However, the major goals of our future studies are to characterise the formulated hydrogels, the *in vitro* biocompatibility of polymers in Franz's cell, and the *in vivo* absorption of bromelain. Further studies are also necessary to evaluate the effect of conservatives on the enzymatic activity of bromelain.

Materials and Methods

Chemicals and reagents

All chemicals and reagents were of analytical grade, unless otherwise stated. Ethanol (96% v/v) was purchased from local gas stations, and was stored in a cold chamber prior to use. The enzyme extract was obtained from a mixture of the bark, stems, and

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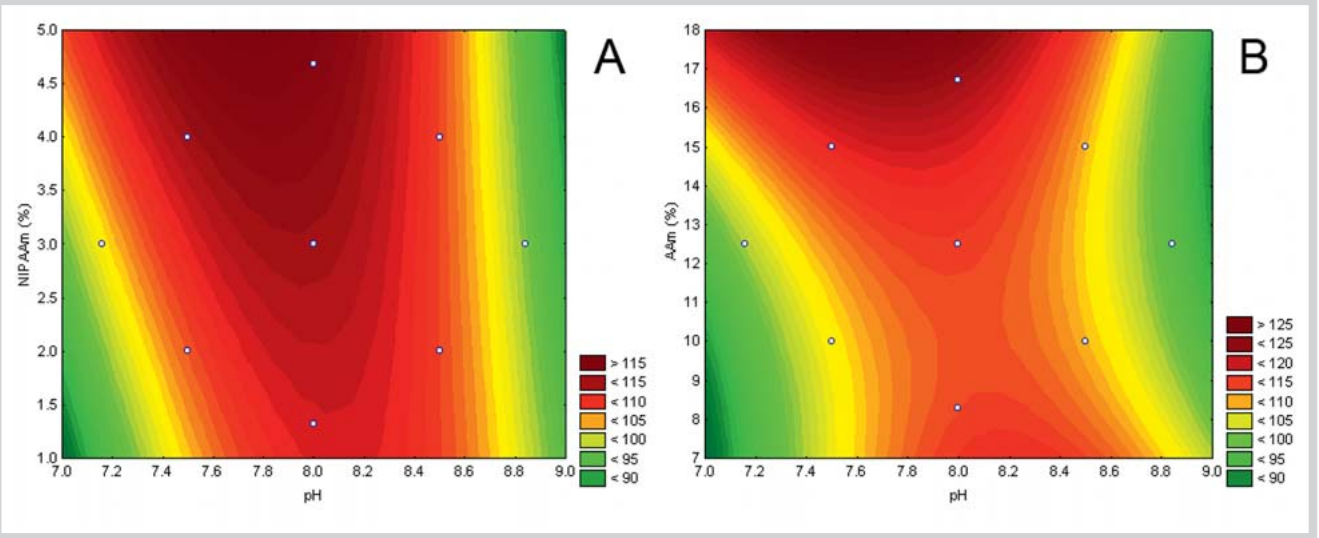

Fig. 4 Response surface of predicted PNIPAAm-*co*-AAm hydrogel swelling behaviour. **A** Swelling behaviour of pH and NIPAAm concentration interaction; **B** swelling behaiviour of pH and AAm concentration interaction. (Color figure available online only.)

Table 4 Protein and bromelain incorporated and released at PNIPAAm/AAm hydrogel's formulations.

Hydrogel formulation	Incorporated		Released	
	Protein ($\mu g \cdot mL^{-1}$)	Bromelain activity ($U \cdot mL^{-1}$)	Protein ($\mu g \cdot mL^{-1}$)	Bromelain activity ($U \cdot mL^{-1}$)
A	0.192	20.176	0.178	18.544
B	0.174	18.944	0.147	15.792
C	0.248	15.36	0.222	12.224

leaves of *A. comosus* (voucher No. 55877) from local pineapple processing industries. The plant material was identified by Dr. Luís Carlos Bernacci, Instituto Agronômico. A voucher specimen was deposited in the Instituto Agrônomico Herbarium, Campinas, SP, Brazil.

Enzyme extract

The pineapple residues were processed in a kitchen juicer and then centrifuged at 10000 × *g* for 20 min at 4 °C to remove insoluble particles. This aqueous extract is referenced as crude extract hereafter.

Protein quantification and enzymatic activity

Protein quantification was performed by the method described by Bradford [37]. The enzyme activity assay was performed by the azocasein method, in accordance with the method described by Coelho et al. [2]. Briefly, the reactor containing 125 µL of substrate and 125 µL of enzyme extract was incubated for 10 min at 37 °C. The enzymatic reaction was stopped with the addition of 750 µL of 5% trichloroacetic acid (w/v). The samples were centrifuged at 4000 × *g* for 10 min at 4 °C. The enzymatic activity was defined as the amount of enzyme needed to produce an optical density increase of 1.0 at 440 nm at 37 °C per hour.

Ethanol precipitation

The details of the methodology of bromelain precipitation can be found in a study by Englard et al. [38], and the procedure was conducted as follows: Ethanol was cooled at 0 °C and added in a dropwise manner to the crude enzyme extract until the desired concentration (10% to 90% v/v) was reached. The solution was centrifuged at 2000 × *g* for 20 min at 4 °C, and the resulting pellet was solubilised in 0.02 M phosphate buffer at a pH of 7.0.

Partial enzyme characterisation

Optimum pH and temperature: The optimum pH and temperature from bromelain activity were evaluated before and after each purification step. Optimum pH assays were performed by changing the reaction media with an appropriate buffer. Acetate buffer was used at a pH of 5.0, phosphate buffer was used at pH values ranging from 6.0 to 8.0, and Tris-HCl buffer was used at values of pH 9.0 and 10.0. Optimum temperature assays were performed by altering the incubation temperature of the original procedure from 5 °C to 65 °C.

pH and temperature stability: The pH and temperature stability of bromelain were evaluated before and after each purification step and assessed for 180 min during incubation at the respective pH or temperature. The assays were performed at values of pH and temperature ranging from 5.0 to 10.0 and from 5 to 65 °C, respectively.

Hydrogel synthesis

Hydrogels of PNIPAAm-*co*-AAm were synthesised by redox polymerisation using ammonium persulphate as an initiator and N,N,N′,N′-tetramethylethylenediamine (TEMED) as an accelerator, as described by Schild [39]. Briefly, an aqueous solution of 10% NIPAAm was mixed with a bis-acrylamide (AAm) solution (30% acrylamide and 0.8% N,N′-methylene-bis-acrylamide) in defined proportions and pH. Then, the accelerator (TEMED, 13 µL) and the aqueous solutions of the initiator (ammonium persulphate 15 $g \cdot L^{-1}$, 60 µL) were added to the former solution and

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Factors	Symbol	Levels				
		− 1.414	− 1	0	+ 1	1.414
NIPAAm (%)	X_1	1.32	2	3	4	4.68
AAm (%)	X_2	8.3	10	12.5	15	16.7
pH	X_3	7.16	7.5	8.0	8.5	8.84

Table 5 Monomers final concentration on hydrogel synthesis.

mixed gently at room temperature. Next, the hydrogels were synthesised in petri plates (diameter = 60 mm). After polymerisation, the gels were washed with distilled excess water and dried for 24 h in an oven at 60 °C.

Hydrogels were formulated using response surface methodology, as described by Barros Neto et al. [40]. To describe the nature of the response surface in the optimal region, a 23 central composite design with six star points ($\alpha = 1.414$) and six replications of the central points was used, leading to a total of 20 experiments. The levels of each factor are listed in **Table 5**. The swelling ratio was measured for each run. The model was fitted to the centred data on the response of biomass production consisting of a second-order polynomial function:

$$Y(x, \vartheta) = \vartheta_0 + \sum_{i=1}^{k} \vartheta_i x_i + \sum_{i=1}^{k-1} \sum_{j=2}^{k} \vartheta_{ij} x_i x_j + \sum_{i=1}^{k} \vartheta_{ii} x_i^2 \quad (1)$$

where Y is the predicted response, subscripts *i* and *j* take values from 1 to the number of variables (n), ϑ_0 is the intercept term, the ϑ_i values are linear coefficients, the ϑ_{ij} values are quadratic coefficients, and x_i and x_j are the levels of the independent variables. All experiments were analysed by Statistica 8.0 (Statsoft).

Hydrogel swelling capacity

Dried polyN-isopropylpolyacrylamide/acrylamide (PNIPAAm-*co*-AAm) hydrogels were immersed in an excess amount of water to attain swelling equilibrium at room temperature for 2 h prior to measurement. Swelling ratios are determined by the ratio of the weights of the swollen gel (W_s) to that of the corresponding dried gel (W_d) [41]. The three formulations with the best swelling ratios were selected for bromelain loading.

Bromelain hydrogel loading and release

After the response surface methodology, three PNIPAAm-*co*-AAm hydrogels were formulated. Formulation A contained 4.68% NIPAAm and 12.5% bis-AAm at pH 7.2, formulation B contained 4.68% NIPAAm and 15% bis-AAm at pH 8.0, and formulation C contained 3% NIPAAm and 15% bis-AAm at pH 8. A bromelain solution (0.1% w/v, in 0.1 M Tris-HCl and specific formulation pH, containing approximately 36 $U \cdot mL^{-1}$ bromelain) was prepared, and each dried hydrogel was soaked for 2 h. Thereafter, the swollen bromelain-loaded hydrogel was removed, and the amount of protein and bromelain activity were determined as described previously.

The amount of bromelain and its activity after being loaded into each hydrogel were determined by the following equation:

$$\text{Bromelain loaded} = [\text{Bromelain}]_{\text{before}} - [\text{Bromelain}]_{\text{after}} \quad (2)$$

$$\text{Activity loaded} = (U \cdot mL^{-1})_{\text{before}} - (U \cdot mL^{-1})_{\text{after}} \quad (3)$$

where the subscripts "before" and "after" represent the concentration and activity of bromelain in the solution before and after the loading process.

The *in vitro* experiments for bromelain release from the hydrogel was carried out at 35 °C using PBS. At the end of each experiment, the hydrogels were removed from the medium, and the bromelain concentration and activity were quantified. All measurements were performed at least in triplicate.

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Conflict of Interest

The authors have no conflicts of interest to declare.

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