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Original Article

Physical and chemical factors affecting the loading and release of bromelain from DC beads

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Abstract: Doxorubicin loaded DC beads (microspheres) has been used for treating un-resectable tumours by transarterial chemoembolization (TACE). We have shown that bromelain, an enzyme from the pineapple plant, enhances the cytotoxic effect of a number of chemotherapeutic drugs and in an earlier study we have demonstrated that it can be loaded into DC beads. Therefore, in the current study we have investigated how certain physical and chemical parameters affect its loading and release for future development of DC beads in cancer therapy. Aliquots of 40-60 µL of DC beads (100-300 µm) were treated to bromelain in distilled water and various parameters such as pH of solution, bromelain concentration, temperature, loading period, presence/absence of agitation and the cytotoxic effect of bromelain loaded beads were investigated. Further release kinetics was also studied with additional investigation of pH effect on the proteolytic activity of bromelain. Results indicate that higher loading of bromelin was achieved in the beads at lower pH, higher concentration of bromelain, with agitation, 24 hours loading and ambient room temperature. Proteolytic activity of bromelain was maximal at pH 4.5 whilst cytotoxicity was at par if not better in the bromelain loaded DC beads. Release kinetics indicated that bromelain can be delivered over several hours. Hence, we conclude that bromelain can be loaded more efficiently with manipulation of certain parameters with noticeable cytotoxicity in tumour cells.

Keywords: DC beads, micro-spheres, bromelain, temperature, agitation, proteolytic activity, cytotoxicity

Introduction

Drug eluting beads (DEB-TACE) were developed recently as a strategy for improving the performance of transarterial chemoembolization (TACE), a common treatment for un-resectable hepatic tumors. They consist of polymeric microspheres of specific sizes that can be loaded with various chemotherapeutic agents. The type of drug eluting bead that has been used in numerous studies is the DC bead manufactured by Biocompatibles UK. These are polyvinyl alcohol microspheres with sulphonate groups capable of actively sequestering cationic drugs through electrostatic attraction, hydrogen bonding and weak Van der Waals forces [1], and releasing them over a period of days to weeks. DC beads can provide sustained drug release over several days, as opposed to other commercial microsphere products which quickly elute within several minutes to hours [2]. Release kinetics were also found to follow the Higuchi equation, with the cumulative drug released being proportional to the square root of time elapsed [2, 3].

Bromelain is an extract from pineapples that contains proteases, phosphatases, glucosidases, peroxidases, cellulases, glycoproteins, carbohydrates, and proteinase inhibitors [4]. Its molecular weight is 28 KDa with 212 amino acid residues and amino groups that are well protonated at lower pH, hence, carrying net positive charge [5]. Its anticancer effect has been demonstrated in *in-vitro* and *in-vivo* models [6, 7]. Furthermore, bromelain in combination with N-Acetylcysteine has been evaluated as a treatment for peritoneal cancer with promising *in vivo* and clinical results [7, 8]. Bromelain can be tethered through hydrogen and vandervall forces to negatively charged sulphonate groups within the spheres. In previous studies,

we were able to load bromelain in distilled water or in phosphate buffer saline (PBS) at pH 2.2-4.2 by agitation at 37°C/24 hrs.

Hence, the present aim of our study is to investigate how certain physical and chemical parameters affect the loading and release of bromelain from DC beads (microspheres), to guide efficient loading and release of bromelain for therapeutic application. Therefore, we examined the effect of pH, temperature, bromelain concentration of loading solution as well as presence and absence of agitation, during loading with additional assessment of bromelain proteolytic activities at varying pH. Furthermore, we investigated the release kinetics of bromelain solution into phosphate buffer solution pH 6.5 at 37°C providing a basic model whereby drug diffusion out of spheres can be observed to mimic the tumor environment. Finally, we investigated the cytotoxic efficacy of bromelain loaded microspheres on ASPC-1 (pancreatic cancer) cells in comparison to free bromelain at equivalent concentration.

Materials and method

Materials

All chemicals used in the present study were purchased from Sigma-Aldrich, Australia. Hydrochloric acid (HCL) and sodium hydroxide (NaOH), PBS, azocasein, trichloroacetic acid (TCA) were of analytical grade. DC microspheres (100-300 μm) were purchased from BTG, Farnham, Surrey, UK. Bromelain was supplied by Challenge Bioproduce Co. Ltd, Taiwan, and China.

Methods

Measurement of bromelain concentration using azocasein assay: To measure concentrations of bromelain throughout our experiments, the azocasein assay was used similar to the procedure described by Coêlho et al., [9]. A serial dilution was performed with bromelain in phosphate buffer solution at pH 6.5 with concentration ranging from 0-200 μg/ml. An unknown concentration of bromelain solution is suitably diluted to fall within the range of the standard curve (0-200 μg/mL). A total of 250 μL of bromelain solution at each concentration was added to 250 μL of 1% azocasein solution and agitated on a shaker for 30-45 min/23°C. The reaction was stopped using 1500 μL of 5% TCA, vortex mixed, then centrifuged for 10 min at 2500 rpm. Then, 150 μL of supernatant from each concentration was placed onto a micro well to which 150 μL of 0.5 M sodium hydroxide was added and absorbance read at 400 nm using the PowerWave X (Bio-tek Instruments). A standard curve was then constructed using Microsoft Excel, with which the bromelain concentration of the unknown solution was determined (**Figure 1A**).

Measurement of bromelain concentration using sodium hydroxide Assay: Occasionally the sodium hydroxide assay was used to measure bromelain concentration. Again, a serial dilution of bromelain was performed in PBS with pH 6.5; however, higher concentrations were used (0-15 mg/mL). A volume of 200 μL of solution at each concentration of the serial dilution was added to each micro well. Likewise, 200 μL of solution of the sample in question is added to another micro well. Then 100 μL of PBS pH 13 (adjusted using 0.5 M sodium hydroxide) was added to each well already containing 200 μL of bromelain solution. Absorbance was read at 400 nm; a standard curve was constructed in Microsoft Excel and the unknown bromelain concentration was calculated (see **Figure 1B** for an example).

Effect of time and temperature on loading

To 40 μL of DC microspheres, 200 μL of bromelain solution was added (5 mg/mL with, pH 3.4) at 4°C and agitated either for 4 or 24 hours. A similar setup was used at ambient room temperature (23°C). Supernatant was collected to evaluate the amount of bromelain that has been loaded. Two separate standard curves were created - one for room temperature and the other for 4°C.

Effect of bromelain concentration on loading

Three aliquots of 40 μl microspheres in eppendorff tubes were incubated with a total quantity of 2000 μg of bromelain in varying solvent volumes: 200, 400, 800 μL, with agitation over 24 hours at 23°C. These varying solvent volumes correspond to varying concentrations - 10, 5, and 2.5 mg/mL respectively. The supernatant was retrieved for residual bromelain analysis through the azocasein assay.

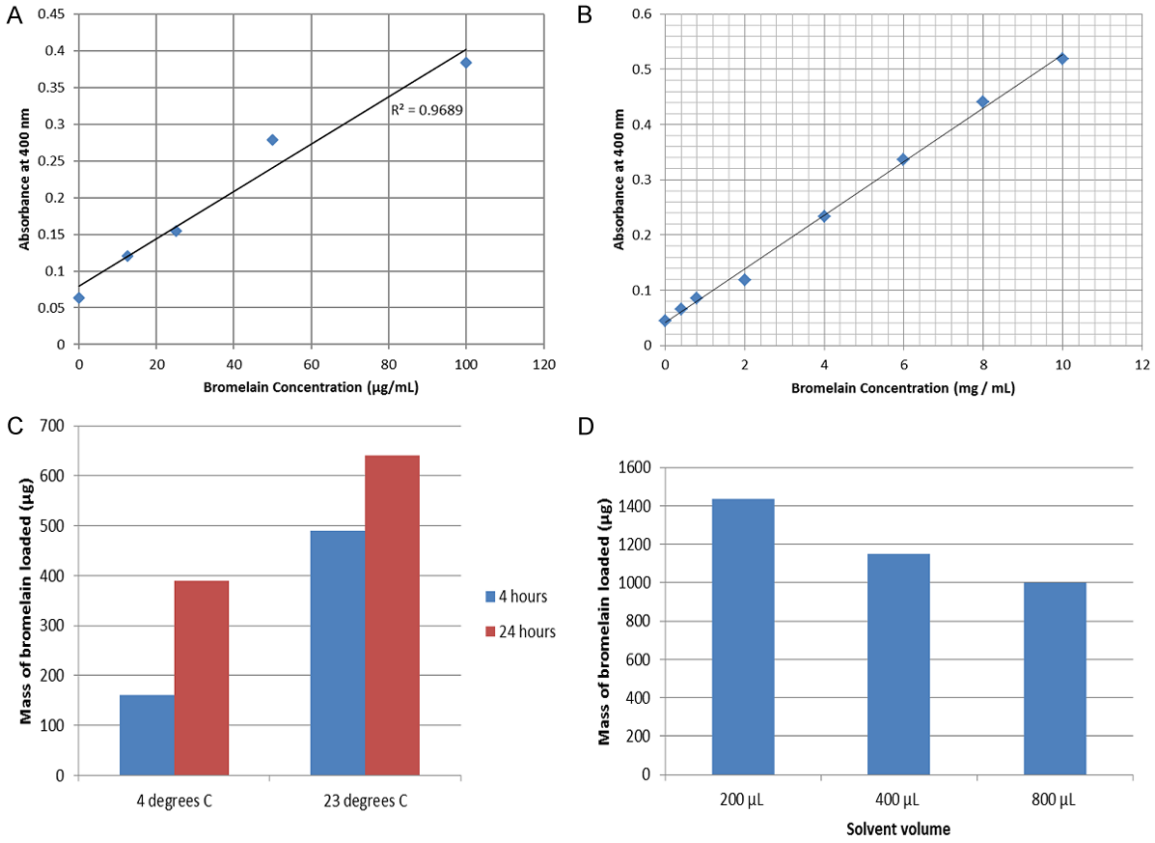

Figure 1. A. Calibration curve for bromelain using Azocasein assay for measuring bromelain in the µg range (0-200 µg/mL). It indicates good linearity between bromelain concentration and absorbance at 400 nm. B. Calibration curve for bromelain using sodium hydroxide assay for measuring higher concentration of bromelain in the mg range. The graph shows good linearity between bromelain concentration and absorbance at 400 nm. C. Impact of temperature and loading period (time) indicates that the total bromelain sequestered in the DC beads is time and temperature dependent, loading at 23°C for 24 hours achieves a much higher loading. D. Impact of solvent volume on amount of bromelain loaded shows that at high concentration (reduced solvent volume), the total amount of bromelain sequestered within the sphere is the highest.

Effect of pH on loading

Bromelain solution was prepared at 10 mg/mL in PBS, and pH was adjusted using 0.5 M sodium hydroxide and 0.1 N hydrochloric acid to 2.5, 3.5, 4.5, 5.5, 6.5 and 7.5 in different tubes. Next, 40 µL of DC microspheres were loaded with 200 µL of bromelain solution at different pH (2.5-7.5). The tubes were agitated overnight at ambient room temperature (23°C), and the supernatant was analysed for residual bromelain to calculate the amount that was loaded.

Bromelain loading cannot be accurately assessed with the azocasein assay because bromelain displays different activity at different pH, hence, bromelain solutions with different pH will give different absorbance readings. Hence, the sodium hydroxide assay was used from which bromelain concentrations could be determined, and loading amounts calculated.

Effect of pH on proteolytic activity of bromelain

An 8.0 mg/mL bromelain solution was prepared in distilled water and adjusted to different pH values (2.5, 3.5, 4.5, 5.5, 6.5, 7.5) using either 0.1 N sodium hydroxide or 0.1 N Hydrochloric acid. A volume of 1600 µg bromelain in 200 µl of PBS was added to 40 µL of spheres. Before analysis of the remnant bromelain in solution after loading is complete, the solution was diluted to 166 µg/mL and tested using azocasein assay to determine the impact of pH on enzymatic activity, compared to a standard curve constructed with bromelain solution at pH 3.5.

Effect of agitation during loading

Four aliquots of 40 µL of microspheres were placed into ependorff tubes. Then, 100 µL of bromelain solution at concentration 7.5 mg/mL was added to each tube. The 4 samples were separated into 2 groups - one without agitation, another with agitation at 300 rpm, and left for 4 hours before supernatant was aspirated. The azocasein assay was used to determine residual bromelain content, and hence infer the amount loaded into the microspheres.

Bromelain release from microspheres

We released microspheres pre-loaded with bromelain at pH of 3.5, 5.5, and 7.5, and also the microspheres from experiment 2 (200, 400 and 800 µL loading volume) into a centrifuge tube with 10 mL solution of PBS at pH of 6.4. The centrifuge tube was placed in a water bath at 37°C, and agitated, at half an hour, an hour, and every subsequent hour for 9 hours. Then 500 µL of PBS was removed from the centrifuge tube and replaced with fresh PBS. Afterwards, we analysed the bromelain concentration of each 500 µL sample using the azocasein assay. Using this method, we can calculate the amount of bromelain that has been eluted from the microspheres at each time point.

Cytotoxic efficacy of bromelain loaded DC microspheres (in vitro)

The human pancreatic cancer cell line ASPC1 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). ASPC1 cells were grown in RPMI (tissue culture media) in a flask over 3 passages in an incubator at 37°C following standard protocol [10]. The cells were then trypsinised, centrifuged, and suspended in fresh RPMI. Cell density was determined using standard protocols [11] after which they were plated in a 96 well plate at 6000 cells/well and allowed to attach overnight in an incubator at 37°C before treatment.

Bromelain solution was prepared at 20 µg/mL in RPMI and serially diluted through 10, 5, 2.5 and 1.25/mL for treatment of the free bromelain group. For the microsphere-loaded group, 40 µg of DC microspheres were loaded with 400 µg of bromelain (using 40 µL of a 10 mg/mL solution) at 23°C for 24 hours, and then likewise diluted down to 20, 10, 5, 2.5, and 1.25 µg/mL to form the treatment solutions. A control group of fresh RPMI with no bromelain was also used as a point of comparison.

Each treatment solution was used to treat 4 wells per plate and repeated in 3 plates. One plate was fixed at 24 hours, another at 48 hours, and the final one at 72 hours following treatment. Cells were fixed with 10% trichloroacetic acid at 4°C for 30 minutes, washed 5 times with tap water and air dried. Next, 100 µL of sulfhordamine was added to each well and allowed to stain the cells at 23°C/30 minutes after which it was emptied washed with 0.01% acetic acid. To the air-dried stained wells, 100 µL of 10 mM Tris base was added to each well with shaking [12], and the absorbance was read at 570 nm using Power Wave X (Bio-Tek Instruments).

Data analysis

Standard curves for the azocasein and sodium hydroxide assays were constructed using Microsoft Excel (Microsoft Corporation, Redmond, Washington, USA) and concentrations were read visually through the trend lines plotted. The sulforhodamine B assay data was analysed using Prism GraphPad 6 (GraphPad Software Inc, San Diego, California, USA). Treatment and control groups were compared using Dunnett's test, and statistical significance was determined at the cut-off of $P < 0.05$.

Results

Detection of bromelain using the azocasein assay or the sodium hydroxide assay

The azocaesin assay is used for measurement of small quantities of bromelain in the range of 1.0-200 µg/mL that depends on its proteolytic activity after which the linearity of the curve may be lost (**Figure 1A**). On the other hand, using the sodium hydroxide assay that does not depend on the proteolytic activity of bromelain but on the denaturation of protein, a higher quantity of bromelain may be detected (1-12 mg) (**Figure 1B**).

Effect of temperature on loading (4 vs 25°C)

The loading of bromelain into microspheres was increased at ambient room temperature (23°C) compared to 4°C. After 4 hours, the loading of bromelain was roughly 3 times high-

Table 1. Shows how temperature and duration of loading affects the amount of bromelain loaded into DC beads (microspheres). Room temperature (23°C) and 24 hours loading shows a much higher quantity of bromelain sequestered within the microspheres

Loading duration (hrs)	Mass of bromelain (μg)	
	Temperature (4°C)	Temperature (23°C)
4	160	490
24	390	640

Table 2. Shows that the quantity (mass) of bromelain loaded into the DC beads is dependent on the concentration of bromelain in solution, the higher the concentration, the greater the load when the process was conducted at ambient room temperature (23°C) over 24 hrs

Solvent Volume (μL)	200	400	800
Bromelain concentration of loading solution (mg/mL)	10	5	2.5
Mass of Bromelain loaded in beads (μg)	1435	1150	1000

er at 23°C compared to 4°C (490 μg vs 160 μg). After 24 hours of loading, the total loading was further increased at 23°C as compared to 4°C (640 μg vs 390 μg, 64% higher). Loading was much better at ambient room temperature compared to 4°C and loading over 24 hours achieved a much higher bromelain loading compared to 4 hours (**Table 1**; **Figure 1C**).

Effect of concentration of bromelain on loading

When the concentration of bromelain was increased in the loading solution, the total loading of bromelain within the microspheres also increased. Although the total mass of bromelain within the solution remained constant, the reduction of solution volume increased the concentration, and hence forth indicating that higher concentration bromelain led to a higher loading in the spheres. A bromelain concentration of 10.0 mg/mL showed a loading of 1435 μg, likewise 5 mg/ml had 1150, whilst 2.5 mg/ml had 1000 μg, showing a relationship between loading solution concentration and the total load in a fixed volume of microspheres (40 μL) (**Table 2**; **Figure 1D**).

Effect of pH on loading of bromelain into microspheres

A general trend towards an increase in the amount of bromelain loading was observed as the pH dropped. However, almost no loading was achieved when pH was greater than 6 in PBS. The maximum loading was achieved at a pH of 2.5, where about 44% of the bromelain was loaded that is equivalent to about 20 μg of bromelain per μL of microsphere (**Table 3**; **Figure 2A**). Although the loading solution carried a total load of 2000 μg, only 880 μg of bromelain was sequestered within the spheres at pH 2.5 which decreased as the pH increased, and above 6.5 no loading was observed.

Effect of pH on proteolytic activity of bromelain

The results showed that bromelain has maximal proteolytic activity at pH 4.5, with lower activity at either side of this maximum. At physiological pH (7.0-7.5) values, the activity of bromelain can be less than 20% of this maximum, which should be considered when determining bromelain dosages (**Table 4**; **Figure 2B**). However, when targeting tumours (pH 6.5), the proteolytic activity is again reduced by almost 50%, and hence adjustment in dosage may be required.

Effect of agitation during loading

It was found that without agitation, 250 μg of bromelain could be loaded into 40 μL of microspheres, while with agitation, 350 μg of bromelain was loaded. Using a 100 μl of solution containing 750 μg of bromelain, without agitation only 33% of the total load was sequestered whilst with agitation 47% of the bromelain present was sequestered indicating the advantage in increasing the motion of the bromelain molecules in solution (**Table 5**).

Bromelain release from microspheres

Our results from the release studies are shown in the following table and accompanying graphs (**Table 6**; **Figure 2C** and **2D**).

When comparing release kinetics of different pH loading solutions, the amounts released correspond to the amounts that have been

Table 3. Shows the difference in loading ratio of bromelain when the loading process was conducted at different pH of the solution at ambient room temperature (23°C) over 24 hrs with agitation. Loading ratio = Amount of bromelain sequestered within the microsphere/Total amount of bromelain in solution

pH of PBS	2.5	3.5	4.5	5.5	6.5	7.5
Amount of Bromelain loaded (mg)	0.88	0.74	0.58	0.08	-0.12	-0.02
Loading ratio (Amount loaded/total Bromelain)	0.44	0.37	0.29	0.04	-0.06	-0.01

loaded at those various pH levels. At lower pH values we observe greater loading, as well as increased release. Likewise, with greater loading volume and lower loading concentration, we observed reduced loading into microspheres. Release kinetics for greater loading volumes also corresponded to decreased rates of release, supporting the general principle that rates of release are proportional to the amount loaded. In all groups, we have observed that there is sustained release of bromelain for at least 9 hours.

Cytotoxic efficacy of bromelain loaded DC microspheres (in vitro)

Absorbance at 570 nm was used as a measure of the number of cells viable at specific time points as shown in the **Figure 3A-E**. The control group displayed steady growth through 24 to 72 hours, and we observe that bromelain solution only begins to have a noticeable effect on cell growth at a concentration of 5 μg/mL, gaining significance at $P < 0.05$ compared to the control group. At a concentration of 10 μg/mL, we observe that roughly half of the cells present in the control group at 72 hours are present in both the treatment groups. At 20 μg/mL of bromelain, cell growth is completely inhibited in both the treatment groups.

Discussion

The azocaesin assay is an established method for quantative analysis of bromelain in the micrograms range [10], usually below 200 μg/mL that utilizes the proteolytic properties of the enzyme. It shows good linearity between bromelain concentration and its proteolytic activity, however, for the detection of larger quantities, the linearity of the calibration curve is lost. The sodium hydroxide assay reported in this study may be used for detection of bromelain in the mg range (1-12 mg/mL). The assay depends on the yellow colour (detected at 400 nm) when a strong solution of sodium hydroxide is reacted with bromelain. Bromelain contains three cysteine residues that may form disulfide bonds with loss of some proteolytic activity. However, the addition of sodium hydroxide may disrupt these linkages (disulfide) leading to the development of yellow tinge that was monitored at 400 nm and this largely correlates with the quantity of bromelain present and does not indicate its proteolytic activity. The effect on disulfide bonds in similar molecules are said to be due to beta elimination as a result of hydrolysis by the negatively charged hydroxyl ions (OH^-) [13]. The loss of disulfide bonds can be monitored between 230-350 nm. However, this quantitative assay was developed in our laboratory, and it has not been reported before although it may be a convenient method for quantifying larger quantities of bromelain in a solution.

It is known that molecules in solution are subjected to random motion; the speed with which they move within the solution depends largely on the kinetic energy present in the solution that causes convection currents which are temperature dependent. Higher temperatures tend to provide greater motion within the solution [14]. Since, the sequestration of bromelain within the interior of the spheres is motion dependent, this may influence the number of molecules entering the sphere, a higher temperature such as room temperature (23°C) as compared to 4°C tends to have an advantage owing to an increase in thermal energy that is converted to kinetic motion which translates to increase in solvent and solute motion [15]. Henceforth, we observed a higher quantity of bromelain loading at 23°C. Temperature may also have a bearing on the strength of the hydrogen bond formation [16] between the NH^{3+} of bromelain and the negative sulphonate group within the spheres. This needs further investigation. Lower temperatures such as 4°C may affect these hydrogens bonds whilst tem-

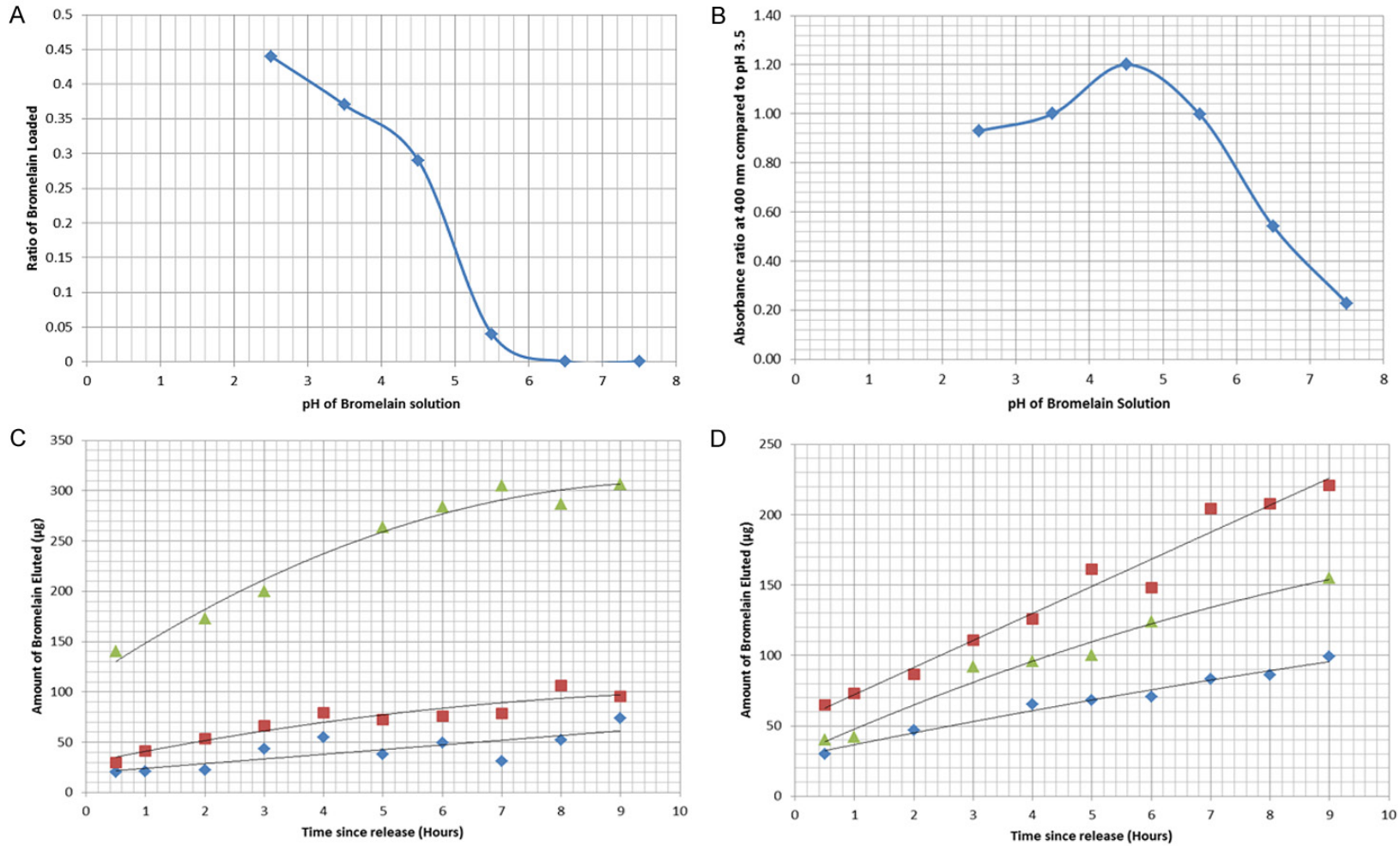

Figure 2. A. Impact of pH on bromelain loading into the DC microspheres. The graph shows that the best loading is achieved at pH 2.5 that gradually declines with increase in pH. B. Impact of pH on proteolytic activity of bromelain indicates that the activity is highest at 4.5 and that declines with increase in pH. C. Loading solution at pH 3.5 (green triangle), Loading solution at pH 5.5 (red square), Loading solution at pH 7.5 (blue diamond). Outliers have been omitted for clarity. Loading at pH 3.5 achieves a much higher loading of bromelain compared to pH 5.5 or 7.5 and the burst release and amount of bromelain release is correlated to the total load. D. Loading volume of 800 µL (red square), loading volume of 400 µL (blue diamond), loading volume of 200 µL (green triangle). Outliers have been omitted for clarity. A higher load of bromelain is achieved at higher bromelain concentration with corresponding burst release at 0.5 hr. and subsequent release quantities.

Table 4. Shows difference absorbance ratio of bromelain at different pH of bromelain solution. Absorbance ratio (X) = Absorbance at 400 nm/Absorbance at 400 nm of bromelain soln. at pH 3.5. AB = absorbance at 400 nm

pH (Bromelain solution)	2.5	3.5	4.5	5.5	6.5	7.5
AB 400 nm	0.414	0.431	0.46	0.43	0.355	0.288
AB Ratio (X)	0.93	1.0	1.20	1.0	0.54	0.23

Table 5. Shows the quantity (mass) of bromelain sequestered within the DC beads at ambient room temperature (23°C) when loading was carried out with a bromelain solution containing a total mass of 750 µg over 24 hours, with and without agitation

	Without agitation	With agitation
Mass of bromelain in supernatant (µg)	500	400
Mass of bromelain loaded (µg)	250	350

Table 6. Shows the quantity of bromelain (µg) that was eluted at various time intervals (0.5-9 hrs)

Sample	Elution sample collection Time (hrs)									
	0.5	1	2	3	4	5	6	7	8	9
a	140	317*	173	200	339*	263.5	284	305	287	307
b	30	42	53.5	66	79	72.5	75.5	78.5	106.5	96
c	20	21	22	43	55	37.5	49	31	52	74
d	65	73.25	87	111	125.8	161.3	148.3	204	208	221
e	30	112*	47	139	65.5	68	70.5	83*	86*	99
f	40	42	164*	92	96	100	124	89	92	155

(a) Loading solution at pH 3.5, (b) Loading solution at pH 5.5, (c) Loading solution at pH 7.5, (d) Loading volume of 800 µL, (e) Loading volume of 400 µL, (f) Loading volume of 200 µL. Outliers have been marked with an asterisk and removed from the following charts*.

peratures above a critical point may have similar effect owing to the disruption of these weak bonds. Temperature may also have effect on the size of the spheres since it is subjected to thermal contraction and expansion [17], and hence affect the pore dimensions, at different temperatures that may affect bromelain loading.

Further, molecules in motion when entering the pores of the spheres for sequestration within, depends on process duration time since only a certain quantity of molecules can penetrate the spheres within a certain time frame. The longer the period of exposure, the higher the sequestration until the reactive sites are saturated and this may follow saturation kinetics. Loading is a gradual process, and hence 24 hours loading showed a much greater quantity of sequestration as compared to 4 hours at ambient room temperature. Further studies to examine loading at different time points at ambient room temperature may indicate the optimum time required for maximal loading of certain quantity of bromelain.

When a microsphere is exposed to a solution containing solute (bromelain), the bromelain molecules impinge on the surface of the spheres at random and if it is in line with a micropore, then it enters the spheres and gets sequestered within. In such a scenario, the number of bromelain particles that impinges and enters the sphere will determine the amount of bromelain sequestered within, the larger the number present in solution (within limits to prevent stearic hindrance), the higher will be the impingement, and hence the sequestration within a time period until saturation sets in at the reactive site. Hence, the concentration of bromelain within the solution may dictate the number of bromelain molecules entering the pores of the sphere, the lower the concentration the lesser the number of molecules that are exposed to the pore. The concentration of a solution may be increased by increasing the

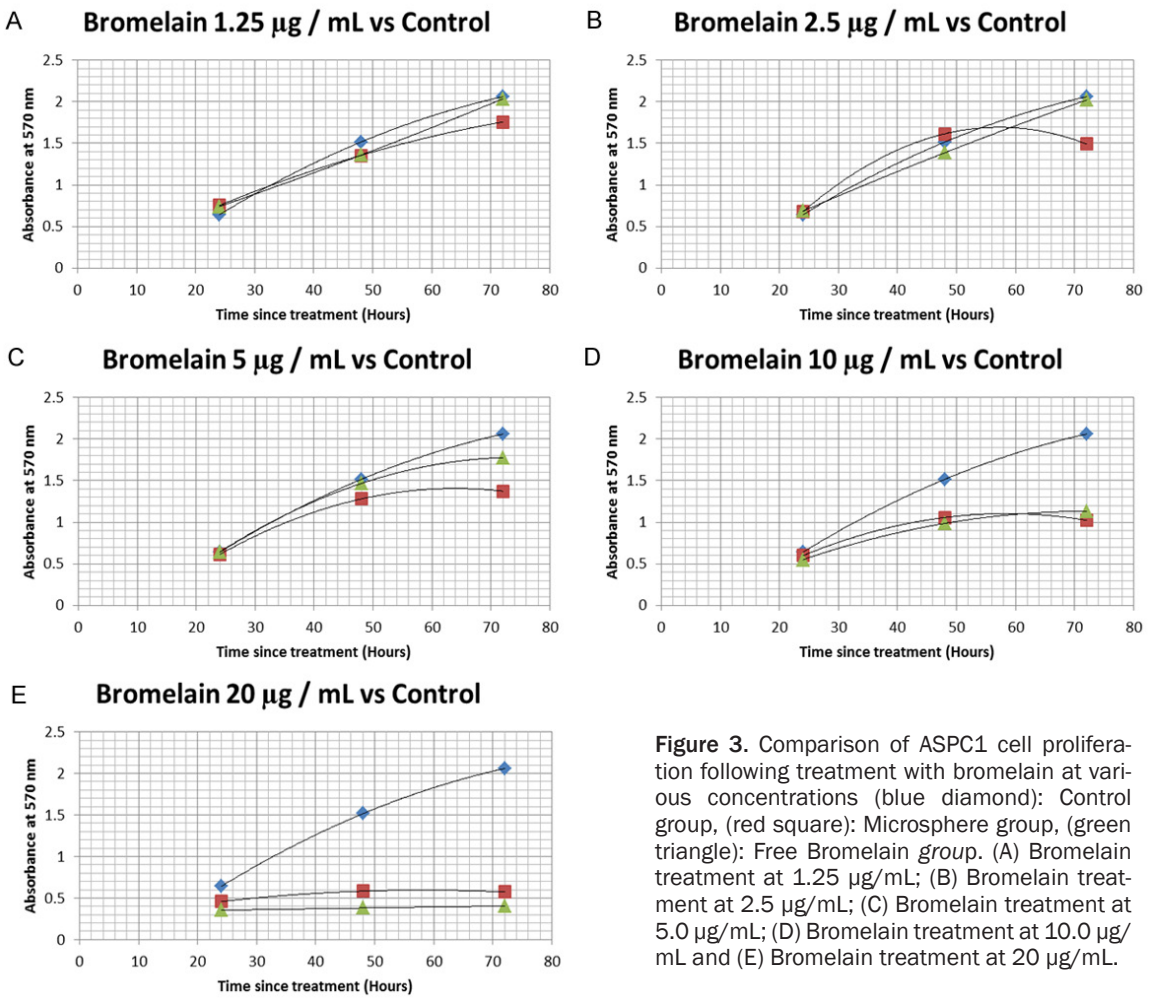

Figure 3. Comparison of ASPC1 cell proliferation following treatment with bromelain at various concentrations (blue diamond): Control group, (red square): Microsphere group, (green triangle): Free Bromelain *group*. (A) Bromelain treatment at 1.25 µg/mL; (B) Bromelain treatment at 2.5 µg/mL; (C) Bromelain treatment at 5.0 µg/mL; (D) Bromelain treatment at 10.0 µg/mL and (E) Bromelain treatment at 20 µg/mL.

solute quantity with a constant solvent volume or by maintaining the total solute quantity but reducing the solvent volume. In the present experiment, we adopted the second approach, and we were able to show that the higher the concentration of bromelain, the higher the percentage loading observed. Hence, by increasing the concentration of bromelain, a higher quantity may be loaded into the spheres. The bromelain concentration in the loading solution will reach maxima after which loading will be interfered and this paradigm needs further study. In addition, whether the increase in concentration affects the loading time needs investigation since a higher number of molecules in motion will be exposed to the pores in the spheres. Finally, maintaining the solvent volume constant and increasing the concentration of bromelain should be investigated since solvent volume may have a direct impact on loading.

At lower pH values, bromelain will be more significantly protonated, hence, being more positively charged [18, 19]. As the microspheres load through ionic exchange, the charge of bromelain is quite important in determining the rate of loading. More positively charged bromelain will correspond with faster loading through stronger electrostatic attraction to sulphonate groups inside microspheres, and we believe that this is the reason for the trend we observe. For future studies solutions of pH lower than 2.5 can be considered for investigation.

Bromelain is extracted from either the fruit or the stem, and maximum activity is pH dependent since the active sites carrying the -SH groups [20] often form disulfide bonds (-S-S-) owing to the folding of long chain of amino acid residues found in the molecule (212 residues). This may result in reduced proteolytic activity of bromelain depending on the number of disul-

fide bond formation. In a reducing medium (< pH 7.0) with substantial amount of H^+ present (acidic medium) will reduce these disulfide bonds, and hence restore its proteolytic activity. In the current investigation we found that the maximum activity was found at a pH of 4.5 that gradually declined towards neutral pH. At pH 6.5 (pH of tumour environment), the bromelain proteolytic activity is around 57% which is sufficient to disintegrate the matrix of the tumour with cytotoxic effect [21, 22]. On the other hand, tumours with even lower pH < 6.5 may experience a greater proteolytic effect from bromelain.

Our results also support the notion that agitation led to increased bromelain loading owing to an increase in kinetic energy in the loading mixture that involves both solute (bromelain) and the microspheres. Future studies are required to determine the agitation speed and time in relation to loading. This could potentially reduce loading time from 24 hours to probably to hours or even minutes, hence, reducing preparation time considerably for any bromelain loaded microsphere treatment.

From the cytotoxic plots for each concentration of bromelain, it appears that, both free bromelain and that encapsulated in the DC microspheres have noticeable effect on cell replication. However, comparing the free bromelain to that encapsulated, it appears that at 72 hrs, the latter has a greater effect compared to the former which indicates that bromelain is still released at 72 hours by the microspheres, and hence the slightly better effect (1.25-10.0 μg/mL). Free bromelain loses its activity in cell culture media with time at 37°C. This can be seen at concentrations ranging from 1.25-5.0 μg/mL. At 10.0 μg/mL bromelain, although the difference appears marginal, there is indication that bromelain from microspheres is released at a slower phase. However, at 20 μg/mL, free bromelain shows slightly greater effect (about 10% difference) compared to bromelain released by DC microspheres since at this high concentration, the loss of bromelain through degradation is probably accounted for and besides bromelain at high concentration is present from the beginning that displays no increase in cytotoxicity from the start. Further, free bromelain exists at high concentration immediately in solution that kills the cells whilst bromelain from microspheres is released slowly and still being released at 72 hours that may continue to do so for several more hours until exhaustion, since we found in our earlier work that it can release up to more than 120 hours depending on the bromelain load (paper under review). The prime disadvantage of delivery of free bromelain *in vivo* locally is that it would result in quick dissipation of bromelain through arteries into systemic circulation. On the contrary, delivery through microspheres allows slow release of bromelain since it depends on clearance at the site of delivery, as it is embolized at treatment site [23]. Further, the slow release prolongs the effect of cytotoxics over extended period of time enabling a more successful treatment outcome [24].

Remarkably, these results provide preliminary evidence that microspheres offer a mechanism for slow, sustained release of bromelain over at least 9 hours. Future researchers may want to continue these experiments past 9 hours until the completion of bromelain release. Several limitations exist with this methodology. We have simulated tumor clearance through removal of 500 μL of PBS every hour; however, in the body, drug clearance is a continual process rather than a discrete one occurring every hour. Furthermore, our model cannot account for drug metabolism which occurs within the body. Another limitation is that the tumor environment contains membrane barriers between fluid spaces which prevent drug diffusion, and hence will alter drug elution. Our experiment therefore only approximates what happens in the body, so *in vivo* measurements of serum drug concentrations, as well as tumor drug concentrations are more desirable and should be considered as potential future avenues of research.

The investigation is limited in its application currently because it is placed in a broader context where usefulness of drug-eluting bead TACE is not completely established, nor is the clinical utility of bromelain for the treatment of HCC. Superiority of microspheres even over more conventional forms of TACE still has not been strongly established, and the question of whether TACE is superior to simple arterial infusions is still unanswered. Crucially, the purported advantages of microspheres for TACE, namely their embolization effect, as well as their slow-release mechanism, have not been shown to provide any benefit to survival [25,

26]. More research is needed to compare between pure intra-arterial injections of drug, and drug delivery through microspheres. Furthermore, while current research on bromelain as an anti-cancer agent is promising, considerably more research is necessary in order to lead towards clinical trials which can prove or disprove its utility in specific patient groups.

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Disclosure of conflict of interest

Mucpharm Pty Ltd is commercially developing BromAc and is the owner of the intellectual property surrounding this area of research. D.L.M is the Chief Executive Officer. K.K., K.P., A.H.M., J.A., and S.J.V. are employees of Mucpharm Pty Ltd.

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