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Original Article

Comparison of proteolytic, cytotoxic and anticoagulant properties of chromatographically fractionated bromelain to un-fractionated bromelain

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Abstract: Bromelain consisting of a number of proteolytic enzymes possess anticancer and thrombotic properties. Hence, four chromatically separated fractions were examined for their proteolytic, anticancer and antithrombotic activity. Bromelain fractions were separated using ion-exchange column chromatography. Proteolytic properties were assessed using standard azocasein assay. Anticancer properties were first assessed using four different cell lines PANC-1, HEP 2B, HEP 3G and OVCAR-3 on cells grown in 96 well plates. Subsequently, fraction 2 and fraction 3 combined with gemcitabine were tested in ASPC-1 cells. Then cytotoxicity of fraction 3 was compared to bromelain in combination with doxorubicin and N-acetylcysteine on HEP G2 and HEP 3B cells. Finally, the anticoagulation effect of fraction 3 or bromelain combined with N-acetylcysteine was evaluated using human blood. Fraction 3 showed the highest proteolytic activity (5% greater than standard bromelain) whilst others were less active. Cytotoxicity as assessed by IC50 indicated fraction 3 to be the most potent whilst the others did not follow their proteolytic potency order. OVCAR-3 was the most sensitive amongst the cell lines. Fraction 3 showed higher potency in combination with gemcitabine in ASPC-1 cells compared to fraction 2. Similarly, fraction 3 in combination with doxorubicin showed higher toxicity when compared to bromelain. Fraction 3 or bromelain only showed thrombolytic activity in combination with N-acetylcysteine. Fraction 3 may be developed for clinical use since it showed better cytotoxicity compared to bromelain.

Keywords: Bromelain, proteolysis, cytotoxic, coagulation, cancer

Introduction

Bromelain, an enzymic extract from the fruits and stem of pineapple plant (*Ananas comosus*) containing proteases, phosphatases, hydroxylases, peroxidases, glycoproteins etc, has shown anti-tumoural, anticoagulant, anticancer and a variety of therapeutic properties in a number of studies [1-4]. Further, it has mucolytic properties and in combination with N-acetylcysteine serves as an efficient mucolytic and anticancer agent [5-7]. Currently, it is undergoing clinical evaluation for the treatment of mucinous tumours secreted by a rare disease known as pseudomyxoma peritonei [8, 9]. More recently, we have shown that the addition of bromelain or N-acetylcysteine or their combinations with cytotoxics such as gemcitabine, doxorubicin, oxaliplatin etc, can potentiate the action of these chemotherapeutic agents in pancreatic cancer cell lines and in other cancers [10] with indication that the effective dosage of these chemotherapeutic agents may be dramatically reduced.

Since bromelain is made up of a number of enzymes and other proteinaceous component, researchers have separated them into fractions [11] in the hope of confining certain enzymic and therapeutic properties exclusively to these fractions. Hence, we have also separated bromelain into four fractions with varying proteo-

lytic activity. Although these fractions are components of bromelain, they do not necessarily share similar proteolytic potency, anti-tumoural and blood anti-coagulant properties. Since the anticancer effect of bromelain has been attributed to its proteolytic properties [12], we first examined this property using chromatographically separated bromelain fractions both, in their catalytically competent and irreversibly inactivated forms, with comparison to unfractionated bromelain after which, we examined the cytotoxic potency in four different cell lines such as pancreatic cancer (ASPC-1), hepatocellular cancer (HEP 3B, HEP G2) and ovarian cancer (OVCAR 3), *in vitro*. Their potency may enable us to select fractions that have good potential as chemotherapeutic adjuvant agents. Further, investigation of fractions with high anti-tumoural activity in combination with gemcitabine or doxorubicin, two common cytotoxics, along with the addition of N-acetylcysteine was carried out in the hope of developing a more effective anti-cancer agent.

Although the blood anticoagulant properties of bromelain are quite well known [13, 14], there are paradoxical reports suggest that bromelain may also cause minor coagulative disorder especially when delivered intraperitoneally in mice [15]. The intraperitoneal delivery of a combination of bromelain with N-acetylcysteine for mucinous tumours have resulted in elevation of inflammatory cytokines indicating that bromelain may act by degrading the surface layers of the peritoneum. The extrinsic pathway in blood coagulation may be at play in this instance since the intrinsic coagulative pathway is probably inhibited by bromelain [16]. The antithrombotic properties of bromelain have been quite well investigated in several studies and it has been shown to interfere with the coagulation cascade at several crucial points (intrinsic pathway and the common pathway), thus serving as an anticoagulant [17, 18]. Since the anticoagulative action of bromelain has been linked to its proteolytic properties, we investigated the antithrombotic properties of bromelain fraction with the highest proteolytic activity to give an indication of its safety in patients on anticoagulation therapy.

Materials and methods

Bromelain was purchased from Challenge Pty Ltd, Taiwan, China and Sigma-Aldrich. All other reagents used in this study are of analytical grade and purchased from Sigma-Aldrich. Iodoacetamide was purchased from Merck.

Fractionation and purification of Ananas comosus stem proteases

Stem bromelain proteases (Sigma-Aldrich, Ref. B4882; 3 units/mg protein) were fractionated as described previously [19]. Briefly, stem bromelain powder was suspended in 100 mM sodium acetate buffer, pH 5.0, in the presence of the reversible thiol-blocking reagent S-methyl methanethiosulfate (MMTS, Sigma-Aldrich, Ref. 64306) under constant moderate stirring for 2 hours at 4°C. MMTS is added to prevent autolysis and/or irreversible oxidation of the catalytic cysteine residues of stem bromelain proteases. The resulting suspension was ultracentrifuged (35000 × g, 4°C, 30 min) and the supernatant constituting the total soluble protein fraction (TE) was applied onto a home-made SP-Sepharose Fast Flow column (13 × 2.5 cm internal diameter; GE Healthcare) pre-equilibrated with 100 mM sodium acetate buffer pH 5.0 and eluted with a linear concentration gradient from 100 mM to 800 mM sodium acetate buffer, pH 5.0. The unbound material was washed away with ten column volumes of the pre-equilibrating buffer and elution of the bound proteins was performed with a linear concentration gradient of sodium acetate buffer pH 5.0. The chromatographic fractions were assayed for amidase activity using DL-BAPNA (Sigma-Aldrich, Ref. B4875) as a substrate as previously described [20]. The chromatographic fractions constituting the different proteases were pooled according to their amidase activity profile, concentrated by ultrafiltration and exhaustively dialyzed against water at 4°C. These fractions were lyophilized and stored at -20°C until use, in their reversibly inhibited forms, where the catalytic cysteine is S-thiomethylated. In this form, the fractions become fully active upon addition of an activator, such us L-cysteine.

Irreversible inhibition of stem bromelain fractions

Stem bromelain total extract (ET) and chromatographically obtained fractions (F1, F2, F3 and F4) were first activated with dithiothreitol (DTT, Sigma-Aldrich, Ref. 43815) at 5 mM final concentration for 10 minutes and subsequently irreversibly inhibited with a large excess of

iodoacetamide (Merck, Ref. 407710) at 10 to 20 mM final concentration, until the inhibition was completely achieved. After removing the iodoacetamide excess by exhaustive dialysis, residual proteolytic activity was checked fluorometrically using appropriate substrates [19]. The irreversibly inhibited samples were lyophilized and stored at -20°C until use.

Proteolytic properties of fractions (F1-4) in comparison to un-fractionated bromelain

Two hundred µg/ml of the different forms of bromelain (fractions 1-4, un-fractionated Sigma-Aldrich and Challenge bromelains) were prepared in 1X phosphate buffer saline at pH 7.0, containing 5.0 mM L-cysteine. To 250.0 µl of the different bromelain solutions was added 250.0 µl of azocasein (1.0% (w/v) solution in distilled water). The mixture was agitated in a shaker for 30 minutes at room temperature (23°C) and then 1.5 ml of trichloroacetic acid (5.0% (w/v) solution in distilled water) was added to the mixture and vortexed. The precipitated azocasein was centrifuged at 2500 rpm for 6 minutes and 150.0 µl of supernatant was pipetted out into a 400 µl microwell. To this, 150.0 µl of 1.0 N NaOH solution was added and the absorbance at 440 nm was measured [21]. The absorbance for the four fractions were then compared to assess their relative proteolytic activities.

Cytotoxic effect of the four bromelain fractions (F1-4)

The cancer cells were grown in cell culture medium (RPMI) containing 10.0% foetal bovine serum in humidifier at 37°C with 5.0% carbon dioxide, following a standard protocol. After three passages the cells were trypsinised and then seeded into a 96 well plate at 4000 cells/well and allowed to anchor overnight. The cells were then treated with varying concentrations of each fraction (F1-F4) and un-fractionated bromelain (control) in RPMI and incubated at 37°C, for 72 hours, at the end of which the media was decanted, and the plates were fixed using cold 10.0% trichloroacetic acid for 30 minutes at 4°C. The plates were then washed with tap water and dried overnight at 23°C, after which they were subjected to sulfhordamine B (SRB) assay following a standard protocol [22]. From the absorbance at 510 nm, the 50% inhibitory concentrations (IC50) in µg/ml were determined.

Cytotoxic effect of bromelain fractions 2 or 3 in combination with cytotoxic drugs

The tumour cell line ASPC-1 was seeded as before in a 96 well plate and treated with varying concentrations of bromelain fractions 2 or 3 and in combinations with the cytotoxic drug gemcitabine and then incubated over 72 hours at the end of which they were subjected to the SRB assay [22] as before with cells viability assessment at 510 nm. Similarly, the tumour cells HEP-G2 and HEP 3B were treated with varying concentrations of doxorubicin in combination with bromelain and NAC or with fraction 3 and NAC.

Interestingly, when irreversibly inactivated un-fractionated bromelain and chromatographically separated fractions 1, 2, 3 and 4 were assayed in the presence of L-cysteine on ASPC-1 cells in the range of 0-100.0 µg/ml, no cytotoxic activity was detected. These data clearly demonstrated that the cytotoxic effects of bromelain fractions are linked to their proteolytic activity (data not shown).

Determination of antithrombotic properties of fraction 3

Prothrombin time (PT), activated partial thromboplastin time (APTT), international normalised ratio: INR, F-10 (%): factor 10 as a percentage: The citrated blood samples prior to, and after addition of, bromelain fraction 3 were centrifuged first at 150 × g for 20 minutes and then at 1200 × g for 10 minutes. The resulting platelet poor plasma (PPP) was then obtained which was used for the determination of PT, INR, APTT and F10 using STA Neoptimal 10, STA TriniCLOT aPTT S, STA Deficient X kits (Diagnostica Stago Inc), respectively. All run on instrument Diagnostica Stago STA-R Evolution (Diagnostica Stago Inc).

Results

Fractionation and purification of Ananas comosus stem proteases

This first step of ion-exchange chromatography leads to four major protein populations according to amidase activity measurements (**Figure**

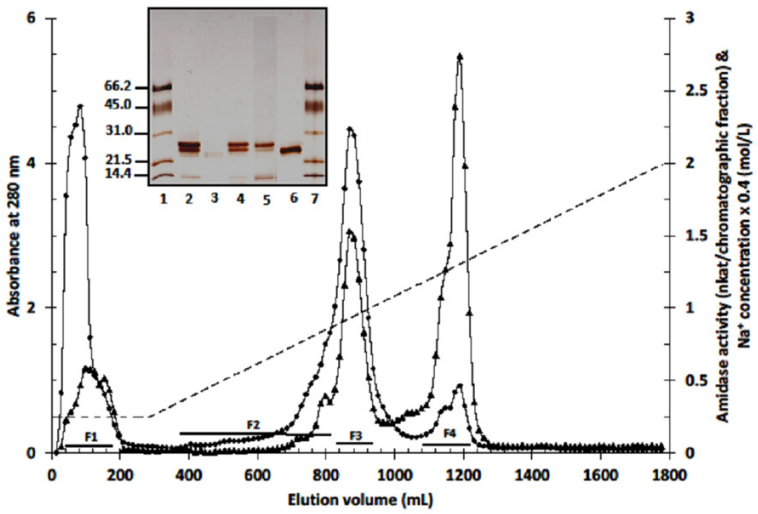

Figure 1. Fractionation on SP-Sepharose Fast Flow of the whole soluble protein fraction from Ananas comosus stem. Fractions of 14.0 mL were collected at flow rate of 60.0 mL/h and analyzed by absorbance measurements at 280 nm (●), Na^+ concentration (dotted line), and amidase activity (nkat/chromatographic fraction) against DL-BAPNA (▲). Inset, SDS-PAGE: lane 1 and 7: molecular weight standard, lane 2: whole soluble protein fraction, lane 3-6: fractions F1-F4, respectively.

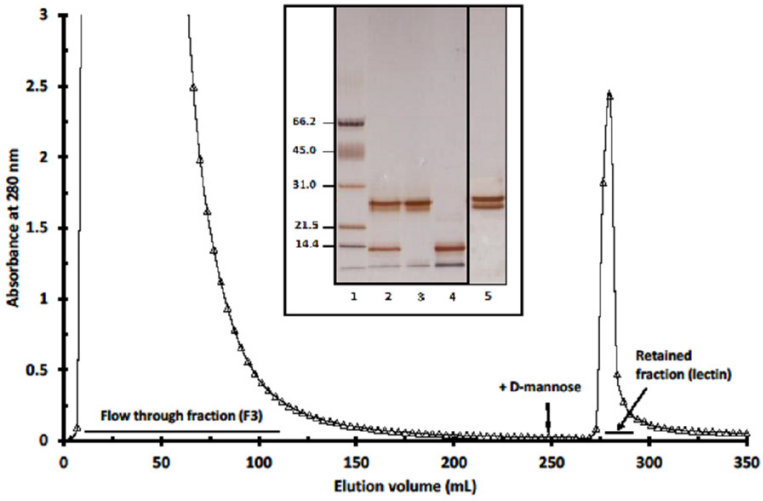

Figure 2. Affinity chromatography on D-mannose-Agarose of F3 fraction obtained after ion exchange chromatography on SP-Sepharose FF. The arrow indicates the starting elution with D-mannose. Inset: SDS-PAGE. Lane 1: molecular weight standard, lane 2: F3 fraction obtained after SP-Sepharose FF, lane 3: affinity chromatography flow through fraction (basic bromelain), lane 4: affinity chromatography retained fraction (lectin) and lane 5: affinity chromatography F2 flow through fraction.

1). According to SDS-PAGE analysis (Inset **Figure 1**), fractions 1 and 4 are nearly homogeneous and pure. For F2 and F3 fractions, we can see that in addition to the major protein band at around 24 kDa which corresponds to basic bromelain isoforms, another protein with an apparent molecular weight of 14 kDa is also visible. This later corresponds to the *Ananas comosus* lectin [23]. F2 and F3 fractions were thus further submitted to affinity chromatography on a mannose-agarose support [23] to obtain basic bromelain-enriched fractions. SDS-PAGE experiments (F3 taken as example in **Figure 2**) clearly indicated that the flow through fractions contained only the basic bromelain isoforms (lane 3 in Inset of **Figure 2**), lectin (lane 4 in Inset of **Figure 2**) being specifically retained on the affinity support.

For the anti-antitumor studies, fractions F1 and F4 were used without further purification, while for fractions F2 and F3, the mannose-agarose flow through fractions were assayed. Mass spectrometry and N-terminal sequencing analyses showed that F1 contained two acidic bromelain isoforms, F2 contained poorly active basic bromelain isoforms, F3 contained highly active basic bromelain isoforms and F4 contained ananain isoforms [19].

Comparison of proteolytic properties of fractions (F1-4) in comparison to un-fractionated bromelain

Proteolytic activities assessed with equivalent quantities of the various fractions indicates that F3 has the highest activity, about 5.0% higher than the control bromelain (un-fractionated). Fractions F1 (<21%), F4 (<37%) and F2 (<40%) had activities that were less compared to the bromelain (C) control. Fractions F2 and F4 had almost similar proteolytic activities. Hence, the order of proteolytic activities with highest are F3, F1, F4 and F2. (**Figure 3** and **Table 1**).

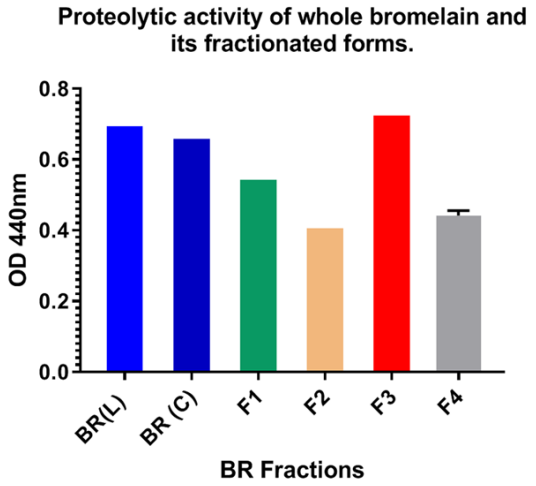

Figure 3. The picture indicates the relative proteolytic activities as compared to standard bromelain. BR (L) = bromelain from Sigma-Aldrich; BR (C) = bromelain from Challenge.

Table 1. The relative increase or decrease in proteolytic activity of different bromelain fractions (F1-F4) as compared to control bromelain (L)

	OD (440 nm)	*Z*
Bromelain (L)	0.705	
F1	0.555	<21
F2	0.417	<40
F3	0.736	>5
F4	0.441	<37

Fraction 3 shows a 5.0% increase whilst the others all show lesser proteolytic activity (<37-<21). Comparative proteolytic activity of fraction to control (Z) is calculated as follows: X = [OD (fraction)/OD (control) × 100]; 100-X = Y.

Cytotoxic effect of the four bromelain fractions (F1-4)

In ASPC-1 cells, F3 is comparably more potent than any of the other fractions and it is more than twice as potent when compared to bromelain (C) (4.22 vs 10.00 µg/ml). Noticeably, F2 is slightly more potent (about 20%) compared to bromelain (C) (8.10 vs 10.00 µg/ml). When compared to bromelain (C), F1 and F4 have reduced potency (**Figure 4A**).

In HEP 3B cells, a similar trend was seen with the different fractions, F3 being more potent, about twice as potent as control bromelain (C) (5.11 vs 11.98 µg/ml), whilst F2 is similar to control bromelain (C). For the remaining fractions, F1 is about 5 times weaker than control bromelain (C) whilst F4 is about four times as weak as control bromelain (C) (**Figure 4B**).

Fraction 3 shows superior cytotoxicity in HEP G2 cells and is twice as potent as control bromelain (C) (5.77 vs 11.67 µg/ml), whilst F2 shows similar potency to control bromelain (C). F1 shows almost about six-fold reduction in cytotoxicity when compared to control bromelain (C) (72.00 vs 11.67 µg/ml) (**Figure 4C**).

A similar trend is shown in OVCAR 3, F3 is again 2.5 times as potent as control bromelain (C) whilst F2 is only slightly more potent compared to control bromelain (C) (3.71 vs 4.73 µg/ml). The weakest fraction is again F1 followed by F4 (**Figure 4D**).

Hence, there seems to be trend in cytotoxicity in the different fractions, indicating that F3 is considerably more potent compared to the rest whilst F2 seems to be much more potent compared to F4 and F1 (**Table 2**).

Comparing the potency of F3 with that of F2 or un-fractionated bromelain in the different cell lines seems to indicate that F3 is twice as potent as F2. Further there is indication (based on IC50 values) that OVCAR 3 (ovarian tumour cells) is more sensitive to these two fractions, indicating that certain oncogenic cellular features are targeted by these fractions (**Figure 4E**).

Cytotoxic effect of bromelain fractions 2 or 3 in combination with gemcitabine on ASPC-1 cells and doxorubicin in combination with NAC and bromelain or fraction 3 on ASPC-1, HEP-G2 and HEP 3B cells

In ASPC-1 cells (pancreatic cancer cells), fraction 3 has slightly higher cytotoxicity in combination with gemcitabine (5.0 µM) at the concentrations investigated (**Table 3A**). At 5.00 and 10.0 µg/ml, fraction 3 shows a slightly greater difference in cytotoxicity. **Table 3B** indicates that at 2.5 and 5.0 µg/ml concentration, in the presence of 2.5 µM doxorubicin, un-fractionated bromelain showed slightly higher cytotoxicity compared to fraction 3. However, at 10.0 µg/ml, fraction 3 exhibited a much higher cytotoxicity (18% more) on hepatic cancer cells (HEP G2) when combined with doxorubicin (2.5 µM). **Table 3C** indicates that in the presence of

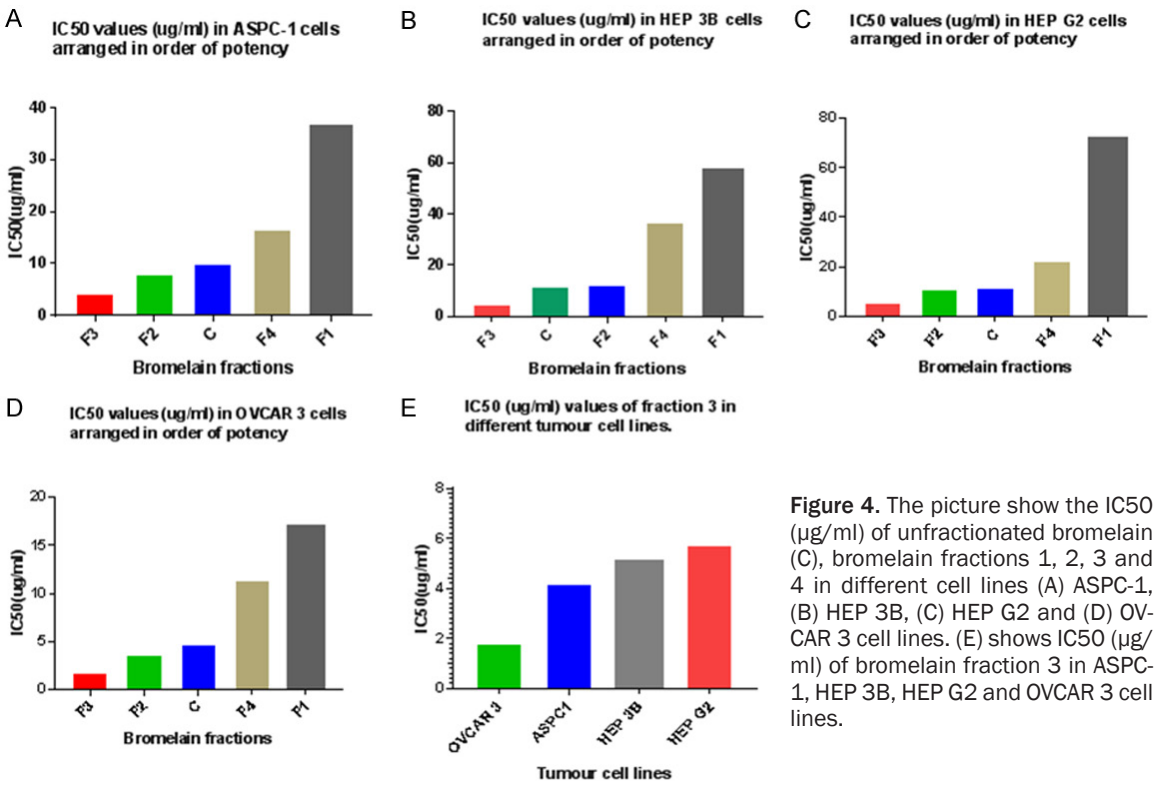

Figure 4. The picture show the IC50 (μg/ml) of unfractionated bromelain (C), bromelain fractions 1, 2, 3 and 4 in different cell lines (A) ASPC-1, (B) HEP 3B, (C) HEP G2 and (D) OVCAR 3 cell lines. (E) shows IC50 (μg/ml) of bromelain fraction 3 in ASPC-1, HEP 3B, HEP G2 and OVCAR 3 cell lines.

Table 2. Inhibitory concentration (IC50) μg/ml

FRACTION	*ASPC-1*	*HEP 3B*	*HEP G2*	*OVCAR 3*
F1	36.34	57.31	72.00	16.95
F2	8.10	12.24	11.15	3.71
F3	4.22	5.11	5.77	1.80
F4	16.53	37.14	22.52	11.38
Bromelain (C)	10.00	11.98	11.67	4.73

The IC50 values for the different fractions indicate that their cytotoxicity varies both, with the fractions and the cell lines.

Table 3A. ASPC-1cells after 72 hours treatment with gemcitabine (5.0 μM) in combination with bromelain (C) or bromelain fractions 3

Concentration of BR (C) or BR fraction 3 (mg/ml)	*% alive BR (C)*	*% alive (F3)*	*% reduction BR (C)*	*% reduction (F3)*
2.50	31.0	28.8	69.0	71.2
5.00	31.1	24.1	68.9	75.9
10.0	26.1	18.9	73.9	81.1

7.0 mM NAC in combination of 2.5 μM doxorubicin, there is no difference in cytotoxicity between un-fractionated bromelain and fraction 3 at concentrations of 2.50 and 5.00 μg/ml on HEP G2 cells (hepatic cancer cells). However, at 10.0 μg/ml bromelain fraction 3 showed greater activity (10% higher). The addition of 2.50-10.0 μg/ml bromelain (C) or fraction 3 with doxorubicin (2.5 μM) showed superior cytotoxic performance by fraction 3 on HEP 3B cells (hepatic cancer cells). A 20.0% increase in cytotoxic efficiency is obtained with fraction 3 compared to the un-fractionated bromelain (C) (**Table 3D**). In the presence of 7.0 mM NAC and 2.5 μM doxorubicin, at concentrations ranging from 2.5-10.0 μg/ml, there is only a marginal difference in cytotoxicity between the un-fractionated bromelain (C) and fraction 3 (**Table 3E**). **Figure 5** shows the cytotoxic effects of fractionated and un-fractionated bromelain combination therapies on pancreatic and hepatic cells *in vitro*.

Determination of antithrombotic properties of fraction 3

The prothrombin time (PT) indicates that the addition of bromelain (C) in the range of 5.0-10.0 μg/ml to blood had no effect; however the addition of 10.0 mM NAC to bromelain (5.0-

Table 3B. HEPG2 cells after 72 hours treatment with bromelain fraction 3 and doxorubicin (2.5 μM)

Concentration of BR (C) or BR fraction 3 (mg/ml)	% alive BR (C)	*% alive (F3)*	*% reduction BR (C)*	*% reduction (F3)*
2.50	25.4	28.6	74.6	71.4
5.00	24.4	29.5	75.6	70.5
10.0	23.9	7.60	76.1	92.4

Table 3C. HEPG2 cells after 72 hours treatment with BR Fraction 3, NAC (7.0 mM) and doxorubicin (2.5 μM)

Concentration of BR(C) or BR fraction 3 (mg/ml)	*% alive BR (C)*	*% alive (F3)*	*% reduction BR (C)*	*% reduction (F3)*
2.50	20.5	20.8	79.5	79.2
5.00	19.0	18.7	81.0	81.3
10.0	15.5	4.20	84.5	95.8

Table 3D. HEP3B cells after 72 hours treatment with BR (C) and fraction 3 in combination with doxorubicin (2.5 μM)

Concentration of BR (C) or BR fraction 3 (mg/mL)	*% alive BR (C)*	*% alive (F3)*	*% reduction BR (C)*	*% reduction (F3)*
2.50	27.1	20.1	72.9	79.9
5.00	26.1	20.9	73.9	79.1
10.0	21.9	2.10	78.1	97.9

Table 3E. HEP3B cells after 72 hours treatment with BR (C) or bromelain fraction 3 in combination with NAC (7.0 mM) and doxorubicin (2.5 μM)

Concentration of BR (C) or F 3 (mg/mL)	*% alive BR (C)*	*% alive (F3)*	*% reduction BR(C)*	*% reduction (F3)*
2.50	5.20	3.50	94.8	96.5
5.00	4.90	3.40	95.1	96.6
10.0	5.10	2.20	94.9	97.8

10.0 μg/ml) showed a significant increase in PT, indicating that the clotting mechanism will be delayed. The addition of fraction 3 also did not alter the PT whilst with the addition of NAC 10.0 mM to 10.0 μg/ml of fraction 3 increased the PT substantially (21.6 sec). The addition of 10.0 mM of NAC had a substantial increase in PT (25.4 sec). This indicates that both, bromelain (C) and fraction 3 should be combined to NAC to obtain an anticoagulant effect.

Compared to control APTT, the clotting time with the addition of bromelain (C) (5-10 μg/ml seems to have a very minor effect on the APTT (30.6-29.9 sec vs 31.5 sec for control). The addition of 10.0 mM NAC to 5.0 or 10.0 μg/ml bromelain (C) showed an increase in APTT (32.9-32.7 sec), indicating that there is an anticoagulant effect.

The addition of F3 showed a reduction of APTT value, 29.6 sec as compared to control value of 31.5 sec, similar to that obtained for bromelain (C). The addition of 10.0 mM NAC to 10.0 μg/ml of fraction 3 showed a small increase in APTT (33.4 sec vs 31.5 sec (control)), indicating a minor anticoagulant effect. However, the addition of 10.0 mM NAC by itself gave a value of APTT of 37.3 sec that shows a modest anticoagulant effect.

The INR values indicated that bromelain (C) (5.0-10.0 μg/ml) had no effect on coagulation time, whilst the addition of NAC (10.0 mM) to bromelain (C) (5.0-10.0 μg/ml) showed a substantial increase in INR that is indicative of delay in clotting (anticoagulant effect). Likewise, F3 on its own had no effect whilst in combination with 10.0 mM NAC it showed an anticoagulant effect (INR = 1.59). Finally, the addition of 10.0 mM NAC showed a high INR, indicating that it has a substantial anticoagulant effect (**Table 4**).

The addition of bromelain (C) (5.0-10.0 μg/ml) had no effect on F-10 (%), whilst the addition of NAC (10.0 mM) to bromelain (C) (5.0-10.0 μg/ml) showed a substantial drop in F-10 (%) (82-79.5), indicating an anticoagulant effect. Similarly, F3 on its own had no effect whereas in combination with 10.0 mM NAC it showed an anticoagulant effect (F-10% = 72). The addition of 10.0 mM NAC showed a high drop in F-10 (%), indicating that it has a considerable anticoagulant effect (**Table 4**).

Discussion

The anticancer properties of bromelain have been mainly attributed to its proteolytic component [12] although it contains a number of

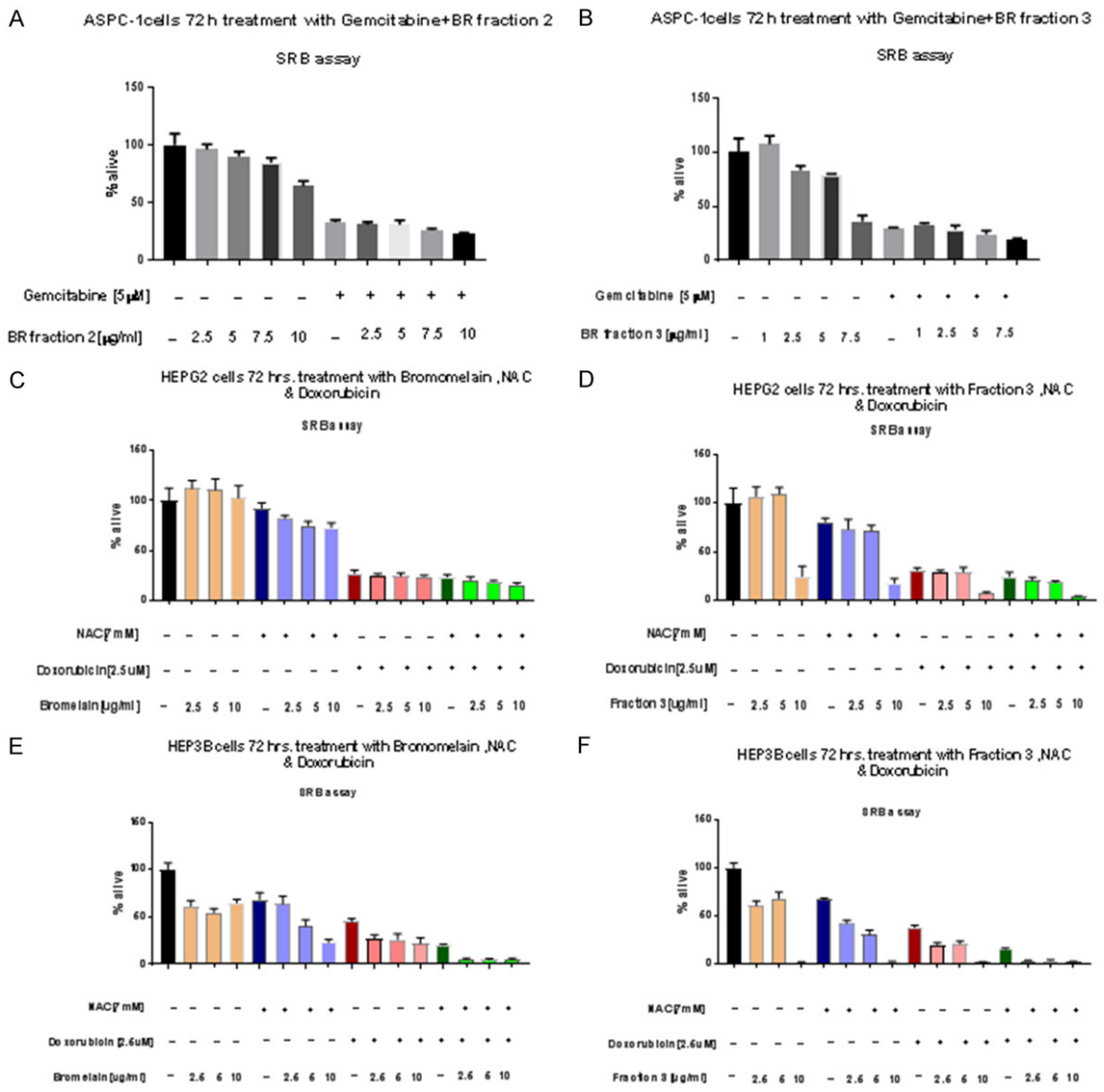

Figure 5. (A and B) show the cytotoxic effect of fractions 2 or 3 in combination with gemcitabine on ASPC-1 cell line. (C-F) show the cytotoxic effect of doxorubicin with NAC in combination with either fraction 3 or standard bromelain (C) on hepatic cancer cell lines HEP G2 and HEP 3B.

Table 4. It shows blood parameters that were determined in the presence of different concentrations of control bromelain (C) (BR) in µg/ml alone, control bromelain (C) (BR) + N-acetylcysteine mM (NAC), fraction 3 (F3) alone, fraction 3 + NAC, and NAC alone

Parameter	*Control*	*BR (5)*	*BR (10)*	*BR (5) + NAC (10)*	*BR (10) + NAC (10)*	*F3 (10)*	*F3 (10) + NAC (10)*	*NAC (10)*
PT (sec)	14.4	14.5	14.4	19.5	19.8	14.7	21.6	25.4
APTT (sec)	31.5	30.6	29.9	32.9	32.7	29.6	33.4	37.3
INR	1.04	1.05	1.04	1.43	1.45	1.06	1.59	1.88
F-10 (%)	115	115	115	82	79.5	108.5	72	55

PT: partial prothrombin time; APTT: activated partial prothromboplastin time. INR: International normalised ratio (patient PT/PT (control)), F-10: factor 10. Data highlighted in red indicate anticoagulant effect.

other enzymes such as carbohydrases, lectins, phosphatases, hydroxylases, peroxidases and glycosylases, etc [1, 4]. This is mainly due to the digestive nature of proteases that can disintegrate protein components of numerous receptors, signalling molecules and cancer related molecules that provide resistance, metastasis and accelerated replication in cancer cells [24]. Further, various mucins in cancer cells, both secretory and transmembrane, are glycoproteins providing a barrier to drug penetration in chemotherapy besides inducing accelerated replication and resistance [25]. These mucinous glycoproteins are disintegrated by a combination of bromelain and N-acetylcysteine with disruption of their cellular biochemical and physiological role [6, 7] and this mixture is currently used for treating mucinous tumours secreted by a rare cancer known as pseudomyxoma peritonei [9]. Besides, bromelain has also antithrombotic properties that affects various components in the coagulation cascade [17, 18]. Since bromelain is composed of several enzymes, fractions with proteolytic activity were isolated and their anticancer and antithrombotic properties were evaluated in the present study.

Proteolytic activity as assessed using the azocasein assay indicated that fraction 3 had the best activity compared to control (5% higher). However, the second potent fraction was F1 followed by F4 and then F2. F1 was 21% less active compared to control whilst F2 and F4 were almost of similar potency (40 and 37% less). The differential proteolytic activities may be related to the different enzyme composition such as highly active basic bromelain isoforms (F3), poorly active basic bromelain isoforms (F2), ananain (F4) and acidic bromelain isoforms (F1) [19]. This indicates that if cytotoxicity was mainly related to proteolysis, then the following investigation that relates to inhibitory concentration (IC50) of the four fractions should follow a similar order of activity, although other cellular features found within the cell types may influence cytotoxicity.

Cytotoxicity as assessed in pancreatic cancer cells (ASPC-1) indicated that fraction 3 was the most potent with the lowest IC50 value (twice as potent as control bromelain (C)) indicating that basic bromelain isoforms performs well in a cellular environment with pH 7.0, followed by poorly active basic bromelain isoforms (F2) that may be again indicative of pH effect on their proteolytic activity and hence their cytotoxicity. Fraction 4 that is composed of ananain isoforms is less active whilst fraction 1 (acidic bromelain isoforms) is least active. A similar trend existed in the other cell lines investigated such as hepatocellular cancer cells and the ovarian cell line. However, the trend in IC50 value as indicative of proteolytic potency only showed that F3 was the most active whilst fraction 4 also conformed to its order of proteolytic activity. Fractions 1 and 2 did not follow the order of their proteolytic activity in cellular cytotoxicity. Further, the proteolytic potency of un-fractionated bromelain and F3 were almost equal (F3 exceeding by 5%) but their IC50 values were very dissimilar and not proportional to their proteolytic activity. Hence, these findings suggest that other anti-tumour intrinsic factors other than their proteolytic properties contribute to their cytotoxic potency. Amongst the four cell lines investigated, OVCAR 3 was the most sensitive with the lowest IC50 values for all the fractions investigated. This suggests the presence of certain cellular features that are prone to the action of bromelain which needs further investigation. However, it is known that OVCAR 3 has high expression of mucins that are prone to the hydrolytic action of bromelain [26]. The second most sensitive cell line is the pancreatic tumour cells, ASPC-1. These cells are also highly mucinous expressing mainly of transmembrane types (MUC1, MUC16, etc.) [26, 27]. HEP 3B and HEP G2 that are of hepatic origin appear to be almost equally affected by the fractions but less sensitive (higher IC50 values). Further hepatic cell lines may have metabolic enzymes that are capable of deactivating bromelain and its chromatographically separated fractions since the liver is primarily involved in detoxifying xenobiotics [28].

Subsequent investigation of fractions 2 and 3 in combination with gemcitabine (5.0 μM) in pancreatic cancer cells indicated that at low concentration of fractions (2.5 μg/ml), the difference in % cellular inhibition was almost similar whilst at higher concentrations (5.0 and 10.0 μg/ml), fraction 3 was more potent. This may suggest the presence of certain other molecules that at higher concentrations may enable the increase in cytotoxic potency. In hepatocellular cell lines (HEP G2), the combina-

Table 5. It shows the order of proteolytic activities arranged from highest to lowest in relation to their cytotoxicity, along with their main components

Order of proteolytic activity	*Order of Cytotoxicity*	*Main components*
Fraction 3	Fraction 3	Highly active basic bromelain isoforms
Fraction 1	Fraction 2	Poorly active basic bromelain isoforms
Fraction 4	Fraction 4	Ananain isoforms
Fraction 2	Fraction 1	Acidic bromelain isoforms

The basic bromelain isoforms seem to have a higher cytotoxicity in all the cell lines inestigated.

tion of fraction 3 or un-fractionated bromelain (C) with doxorubicin (2.5 mM) indicated that un-fractionated bromelain (C) was slightly more cytotoxic at 2.0-5.0 μg/ml, however at 10.0 μg/ml, fraction 3 was significantly more potent, again indicating the presence of other molecules at trace amount that at certain concentrations provide this enhancement of cytotoxicity. A similar scenario existed when NAC (7.0 mM) was included in the treatment. NAC is known to enhance the cytotoxicity of bromelain in our previous studies in both hepatic and pancreatic cancer cell lines [10].

In the case of HEP 3B, the addition of doxorubicin (2.5 μM) indicated that fraction 3 was more potent at the three concentrations investigated when compared to un-fractionated bromelain (C). However, the addition of NAC (7.0 mM) to the mixture indicated that fraction 3 was only marginally more potent. This indicates that fraction 3 is relatively less enhanced by NAC as compared to un-fractionated bromelain that is made up of composite enzymes.

The variation of cytotoxic response of these hepatocellular cell lines to un-fractionated bromelain (C) and fraction 3 may be partially attributed to their individual cellular features [29]. On the other hand, the ratio of the agents that have been used either as dual or triple combinations may play a role in their efficiency. The combination of these agents is only efficient at certain molar ratio as it has been shown previously [10].

A comparison of proteolytic activity to cytotoxicity indicates that highly active basic isoforms of bromelain present in F3, with high proteolytic activity, shows the highest cytotoxicity as indicated by the IC50 values in all the cell lines investigated. The second most cytotoxic component is F2 composed of poorly active basic isoforms of bromelain but with low proteolytic activity (<40% compared to control), hence indicating that proteolytic activity does not really corresponds with degree of cytotoxicity. The third most cytotoxic component is F4 containing ananain and it corresponds with the comparative order of proteolytic activity of the assayed fractions. Finally, although F1 that is composed of acidic bromelains has relatively very high proteolytic activity (second most activity amongst the fractions), it seems to show low cytotoxicity. This again indicates that there is not usually a relationship between proteolytic activity and cytotoxicity (**Table 5**).

The basic bromelain isoforms seem to perform very well as cytotoxic agents perhaps owing to their basic optimum proteolytic activity at pH 7.0, whilst ananain and acidic isoforms may also be dependent on acidic environment for maximal proteolytic activity and hence their weaker cytotoxicity.

There are various reports indicating the antithrombotic properties of bromelain, as a total extract, and its potential as an anticoagulant for treatment of blood coagulative disorders [17, 18]. However, earlier evaluation on the proteolytic activity of fraction 3 showed that its activity is only slightly higher (by 5%) than un-fractionated bromelain, hence we did not expect to see a large variation in any of the coagulative parameters that we measured. The PT (prothrombin time) indicates that F3 was similar to controls and to un-fractionated bromelain (5.0-10.0 μg/ml). The APTT showed no change with addition of 10.0 μg/ml of bromelain. With the addition of 10.0 μg/ml of F3, the difference was being very small. Comparing the INR values indicated that the addition of bromelain (5.0-10.0 μg/ml) did not alter the values compared to a control which is similar to F3. Finally, the F-10 (%) also showed no difference as compared to un-fractionated bromelain with the addition of 5.0-10.0 μg/ml bromelain, however, fraction 3 showed a very small drop (108),

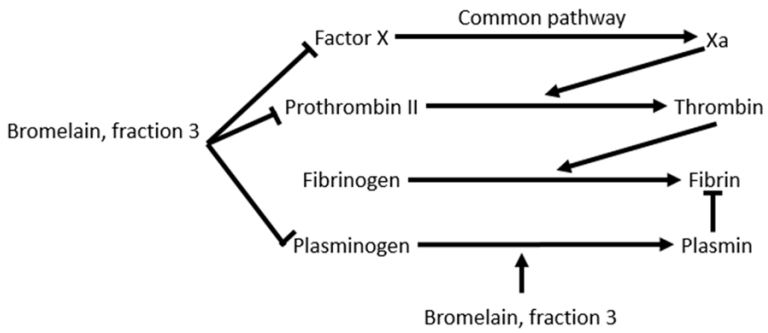

Figure 6. Coagulation cascade in the common pathway. Bromelain and fraction 3 may degrade factor X, prothrombin II and plasminogen and hence delay coagulation.

again indicating that there is no significant anti-coagulative action.

However, the addition of NAC showed significant difference, an increase in PT, APTT and INR values and a substantial drop in F-10 (%) values that is indicative of positive anticoagulative action. Likewise, the addition of NAC to un-fractionated bromelain in blood caused a significant increase in PT, APTT and INR values and a drop in F-10 (%) that is again indicative of anticoagulative action. Hence, NAC has substantial anticoagulative properties. This anticoagulative action is mainly due to its antioxidant properties that prevents the aggregation of platelets [30]. Further, it is able to disrupt the disulfide linkage found in fibrin fibrils in blood clot owing to its antioxidant properties [31]. Similarly, many other antioxidants increase the bleeding time [32]. Hence, from the present antithrombotic parameters measured, F3 does not have any significant anti-coagulant properties that may affect patients who are on warfarin or other anticoagulant therapy. However, one has to be careful since these measurements represent the intrinsic coagulation pathway. Studies with intraperitoneal delivery of bromelain in mice have indicated bleeding in the peritoneal cavity, owing to the abrasive action of bromelain on the peritoneal surface cells where the extrinsic pathway comes into action that unifies with the common pathway of coagulation (**Figure 6**). The common pathway where factor X, prothrombin II and plasminogen come into action to generate fibrin fibres may be affected by un-fractionated bromelain and fraction 3, and this needs further investigation. Although bromelain affects the kinin system (bradykinin, prekallikrin, etc) of the intrinsic coagulation pathway that connects with the common pathway, our investigation does not seem to suggest that the system is affected at the concentrations of bromelain and fraction 3 used in this study. Further it also does not affect the common pathway. However, the presence of NAC is crucial in affecting the coagulation parameters that we have monitored currently and hence when it is combined with bromelain, the coagulation parameters will be affected and hence increase bleeding time (**Figure 6**).

Therefore, the current study indicates that bromelain fractions (F1-F4) have different proteolytic activities that may affect their performance as an anti-cancer agent in the various cell lines investigated. However, their proteolytic potency does not appear to correlate well with their degree of anticancer action suggesting that these chromatically separated fractions do not only carry proteolytic enzymes but may have other components that affect the viability of tumour cells. Fraction 3 performs slightly better in combinations with gemcitabine when compared to fraction 2 in ASPC-1 cells (pancreatic cancer). In combination with doxorubicin, fraction 3 performs slightly better than standard (un-fractionated) bromelain. On the whole, as a single agent, bromelain (5.0-10.0 µg/ml) or fraction 3 (10.0 µg/ml), does not show any antithrombotic properties, however, in combination with NAC, they exhibit antithrombotic properties. Hence, for therapeutic purposes, a combination regime of NAC with either fraction 3 or bromelain may affect individuals who are on antithrombotic medication.

The reason why only fraction 3, composed of basic bromelain isoforms, seems to mimic the action of un-fractionated bromelain may be attributed to the fact that basic bromelain isoforms represent the major components of the bromelain total extract. The assessment of the dose of the bromelain complex mixture to be used for therapeutic purposes thus remains an important factor to be elucidated. Using a well characterized fraction, e.g. F3, will thus be a nice alternative and perspective. Actually, it

remains a challenge to understand at the molecular level the numerous effects of stem bromelain proteases, highlighting the need for more detailed studies combining both, cytotoxicity and proteomics investigations to identify potential specific targets affected by these promising proteases.

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Disclosure of conflict of interest

None.

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