RESEARCH ARTICLE

The chemopreventive action of bromelain, from pineapple stem (*Ananas comosus* L.), on colon carcinogenesis is related to antiproliferative and proapoptotic effects

Barbara Romano[1]*, Ines Fasolino[1]*, Ester Pagano[1], Raffaele Capasso[1], Simona Pace[1], Giuseppe De Rosa[1], Natasa Milic[2], Pierangelo Orlando[3], Angelo A. Izzo[1]** and Francesca Borrelli[1]

[1] Department of Pharmacy, University of Naples Federico II, Naples, Italy
[2] Department of Pharmacy, Faculty of Medicine, University of Novi Sad, Novi Sad, Serbia
[3] Institute of Protein Biochemistry, National Research Council, Naples, Italy

Scope: Colorectal cancer is an important health problem across the world. Here, we investigated the possible antiproliferative/proapoptotic effects of bromelain (from the pineapple stem *Ananas comosus* L., family Bromeliaceae) in a human colorectal carcinoma cell line and its potential chemopreventive effect in a murine model of colon cancer.

Methods and results: Proliferation and apoptosis were evaluated in human colon adenocarcinoma (Caco-2) cells by the ^{3}H-thymidine incorporation assay and caspase 3/7 activity measurement, respectively. Extracellular signal-related kinase (ERK) and Akt expression were evaluated by Western blot analysis, reactive oxygen species production by a fluorimetric method. In vivo, bromelain was evaluated using the azoxymethane murine model of colon carcinogenesis. Bromelain reduced cell proliferation and promoted apoptosis in Caco-2 cells. The effect of bromelain was associated to downregulation of $pERK_{1/2}$/total, ERK, and pAkt/Akt expression as well as to reduction of reactive oxygen species production. In vivo, bromelain reduced the development of aberrant crypt foci, polyps, and tumors induced by azoxymethane.

Conclusion: Bromelain exerts antiproliferative and proapoptotic effects in colorectal carcinoma cells and chemopreventive actions in colon carcinogenesis in vivo. Bromelain-containing foods and/or bromelain itself may represent good candidates for colorectal cancer chemoprevention.

1 Introduction

Colorectal cancer (CRC) is an important health problem across the world. In Europe, each year approximately 435 000 people are newly diagnosed with CRC [1]; about half of these patients die of the disease, making CRC the second leading cause of cancer deaths in Europe. Similarly, in 2013, an estimated 142 820 new cases of CRC will be diagnosed in the Unites States, with 50 830 estimated deaths [2]. In the majority of cases, CRC develops as a consequence of the progressive accumulation of genetic and epigenetic alterations that cause transformation and progression of normal colorectal mucosa to adenoma and, eventually, carcinoma [3]. Several factors can contribute to its development such as ethnicity, race, social status, family history of colon cancer, alcohol and smoking

Correspondence: Dr. Francesca Borrelli, Department of Pharmacy, University of Naples Federico II, Via D. Montesano 49, 80131 Naples, Italy
E-mail: franborr@unina.it
Fax: +39-081-678403

Abbreviations: ACF, aberrant crypt foci; **AOM**, azoxymethane; **Caco-2**, human colon adenocarcinoma cells; **CRC**, colorectal cancer; **DCFH-DA**, 2′,7′-dichlorofluorescin diacetate; **ERK**, extracellular signal-related kinase; **FBS**, fetal bovine serum; **MAP**, mitogen-activated protein kinase; **pERK**, phosphorylated extracellular signal-regulated kinase; **PI3K**, phosphoinositide 3-kinase; **ROS**, reactive oxygen species

*These authors equally contributed to this work.
**Additional corresponding author: Dr. Angelo A. Izzo,
E-mail: aaizzo@unina.it

abuse, diseases (diabetes, inflammatory bowel diseases, obesity), environment, and diet [4].

Although treatment options depend on the stage of the cancer, the patient's general health and whether the cancer has recurred, surgery is usually the primary or first treatment followed to adjuvant (additional) chemotherapy (5-fluorouracil, leucovorin, and oxaliplatin) or radiation therapy [5, 6].

In recent years, interest in primary prevention research has increased; in this regard, screening strategies and healthy lifestyle/dietary habits have been promoted to reduce the incidence of cancer. While screening strategies (blood in stool, endoscopic, and CT-colonoscopy) are associated with inconveniences (i.e. cost, associated morbidity) and, in any cases, they do not necessarily prevent the development of cancer or mortality; dietary factors are increasingly found to play important roles in chemoprevention [7, 8].

Recently, a great attention has been focused on a possible role of plant foods derived natural products in the prevention of CRC [9–12]. Bromelain is a mixture of proteolytic enzymes (or proteases) derived from pineapple stem (*Ananas comosus* L., family Bromeliaceae). In the United States and Europe, bromelain is sold in health stores as a nutritional supplement to promote digestive health, wounds healing, and as an anti-inflammatory agent [13, 14]. Preclinical studies indicate that bromelain exerts anti-inflammatory, antiedematous, antithrombotic/fibrinolytic, and immunomodulatory effects [15–17]. Relevant to the present investigation, bromelain has been shown to ameliorate experimental colitis in IL-10-deficient mouse and to decrease secretion of proinflammatory cytokines and chemokines in colon biopsies from patients with ulcerative colitis and Crohn's disease [18–20]. Considering the well-known association existing between intestinal inflammation and CRC, we investigated the possible antiproliferative/proapoptotic effects (and the mode of action) of this food component in a human colorectal carcinoma cell line and its potential chemopreventive effect in an animal model of colon cancer. In order to verify if the pharmacological effect of bromelain was due to its proteolytic activity, some in vitro experiments were performed by using proteolytically inactivated bromelain.

2   Materials and methods

2.1   Drugs

Bromelain, azoxymethane (AOM), celecoxib, and 2′,7′-dichlorofluorescin diacetate (DCFH-DA) were purchased from Sigma-Aldrich (Milan, Italy); ^{3}H-thymidine was purchased from PerkinElmer (Milan, Italy). All reagents for Western blot analysis and cell culture were obtained from Sigma-Aldrich, Bio-Rad Laboratories (Milan, Italy), and Microtech (Naples, Italy). Bromelain was dissolved in DMEM for in vitro experiments and in saline (2 mL/kg) for the in vivo experiments; celecoxib wase dissolved in 10% ethanol, 10%

Tween-20, 80% saline (0.2 mL/kg). The drug vehicles had no effect on the response under study.

2.2   Cell culture

Human colon adenocarcinoma Caco-2 and DLD-1 cells were purchased from the American Type Culture Collection (LGC Promochen, Italy) and used between passages 20 and 40. The cells were routinely maintained at 37°C in a 5% CO_2 atmosphere in 75 cm^2 polystyrene flasks in DMEM containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, 1 M Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] 2.5%, nonessential amino acid 1× and 2 mM L-glutamine. The medium was changed every 48 h. For ^{3}H-thymidine incorporation assay, Caco-2 and DLD-1 cells were incubated in FBS-deprived growth medium for 24 h before bromelain treatment. Visualization of the cells with optical microscopy revealed that cells were still adherent on the plates despite the presence of bromelain. For reactive oxygen species (ROS) assay, Caco-2 cells were led to differentiation (cells were used at postconfluence stage as a model of human enterocytes); preliminary experiments showed that a 5–7 day time of incubation was required for Caco-2 cells to undergo differentiation.

2.3   Animals

Experiments were performed on male imprinting control region (ICR) mice weighing 25–30 g (Harlan, S. Pietro al Natisone UD, Italy) after 1-wk acclimation period (temperature 23 ± 2°C; humidity 60%, free access to water). ICR mice were fed ad libitum with standard mouse food, except for the 12-h period immediately preceding their sacrifice. All animal procedures complied with the Italian D. L. no. 116 of January 27, 1992 and associated guidelines in the European Communities Council Directive of November 24, 1986 (86/609/ECC).

2.4   Bromelain inactivation and evaluation of its proteolytic activity

Inactivated bromelain (BR-WPA) was prepared as described by Borrelli and colleagues [21]. Briefly, 15 mg/mL bromelain in PBS (pH 8.0) was incubated with 15 mM DTT for 40 min at 37°C. Subsequently, the preparation was incubated for 80 min at 37°C with 130 mM iodoacetamide. Finally, to remove DTT and iodoacetamide, a dialysis (membranes MWCO = 3500) for 22 h against 0.9% saline was performed. This inactivation procedure reduced the activity to <5%. The bromelain integrity was investigated measuring its proteolytic activity against the proteic substrate p-Glu-Phe-Leu-p-nitroanilide (P-ANL; Sigma-Aldrich). Briefly, 100 μL of the bromelain solution were incubated at 37°C with 1.6 mL of PBS pH 6.5 and 0.2 mL P-ANL solution (1 mg/mL in DMSO). Subsequently, the mixture was incubated at 37°C for 15 min

Mol. Nutr. Food Res. 2013, *00*, 1–9

3

and then analyzed using a UV spectrofotometer (Shimadzu UV 1204) with the wavelength set at 410 nm. This assay also showed that bromelain was proteolitically active after 24 h incubation in the medium with serum. However, because this colorimetric assay does not allow to measure micromolar concentrations of bromelain, the assay was performed by using a concentration higher than those used in pharmacological studies (i.e. 10 mM instead of 1–10 μM).

2.5 Cell proliferation

Cell proliferation was evaluated in the colorectal carcinoma cell lines Caco-2 and DLD-1 using ^{3}H-thymidine incorporation assay as previously described [22]. Briefly, cells were seeded in 24-well plates at a density of 1×10^4 cells/well in DMEM supplemented with 10% FBS and grown for 48 h. Successively, cells were washed three times with 200 μL of PBS and then 1 mL of serum-free DMEM was added to each well. After 24 h of serum starvation, the cells were washed three times with PBS and incubated with DMEM supplemented with 10% FBS containing bromelain or inactivated bromelain (1–10 μg/mL) in presence of ^{3}H-thymidine (1 μCi/well) for 24 h. Cells were scraped in 1 M NaOH and collected in plastic miniature vials (PerkinElmer) filled up with liquid for scintillation counting (UltimaGold®, PerkinElmer). Cell proliferation was expressed as count per minute (cpm) on microgram of protein (cpm/μg protein) of incorporating ^{3}H-thymidine cells. The treatments were carried out in triplicate and three independent experiments were performed.

2.6 Measurement of caspase 3/7 activity

Apoptosis was evaluated by means of the Caspase-Glo(R) 3/7 Chemioluminescent Assay kit (Promega, USA) following the manufacturer's protocol. Caco-2 cells were seeded in 12-well plates at a density of 5×10^4 cells/well. After 48 h, the cells were incubated with bromelain or inactivated bromelain (1 and 10 μg/mL, 24 h). After incubation, cells were trypsinized, washed with PBS, and processed. The caspase assay was performed in 96-well white-walled plates, adding 100 μL of Caspase-Glo(R) 3/7 reagent to each well containing 1×10^4 and 2×10^4 cells in 100 μL of culture medium. After 1-h incubation in the dark at room temperature, luminescence (index of caspase 3/7 activity) was measured by a VersaDoc MP System equipped by the Quantity One(R) version 4.6 software (Bio-Rad). All samples were assayed at least in triplicate. Luminescence values from the blank reaction (vehicle-treated cells) were subtracted from experimental values.

2.7 Detection of ROS generation

Generation of intracellular ROS was estimated by the fluorescent probe, DCFH-DA [22]. DCFH-DA diffuses readily through the cell membrane and is enzymatically hydrolyzed by intracellular esterases to form nonfluorescent DCFH, which is then rapidly oxidized to form highly fluorescent dichlorofluorescein (DCF) in the presence of ROS. The DCF fluorescence intensity is paralleled to the amount of ROS formed intracellularly. For experiments, Caco-2 cells were plated in a 96-black well plates at the density of 1×10^4 cells/well and led to differentiation. Confluent Caco-2 cell monolayers were incubated for 24 h at 37°C with bromelain or inactivated bromelain (1–10 μg/mL). Then, the cells were rinsed and incubated for 1 hour with 100 μM DCFH-DA in Hanks' balanced salt solution containing 1% FBS. Finally, cells were rinsed and incubated with the Fenton's reagent (H_2O_2/Fe^{2+} 2 mM) for 3 h at 37°C. The DCF fluorescence intensity was detected using a fluorescent microplate reader (PerkinElmer), with the excitation wavelength of 485 nm and the emission wavelength of 538 nm.

2.8 Preparation of cytosolic lysates and Western blot analysis

Caco-2 cytosolic lysates were obtained as previously described [22]. Briefly, after bromelain (0.1–10 μg/mL) incubation for 24 h, the medium was removed and cells were washed with ice-cold PBS. The cells were collected by scraping for 10 min at 4°C with lysis buffer (50 mM Tris-HCl pH 7.4, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM NaF, 1% NP-40, 1 mM PMSF, 1 mM Na_3VO_4) containing complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). After centrifugation at $16\,200 \times g$ for 15 min at 4°C, the supernatants were collected and protein concentration was determined by Bio-Rad Protein Assay (Bio-Rad). For Western blot analysis, lysate aliquots containing 70 μg of proteins were separated on a 12% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Protran® Nitrocellulose Membranes, Schleicher & Schuell Bioscience, Dassel, Germany) using a Bio-Rad Transblot (350 mA, 3 h). Proteins were visualized on the filters by reversible staining with Ponceau-S solution (Sigma-Aldrich) and destained in PBS containing 0.1% Tween-20. Membranes were blocked at 4°C in milk buffer (5% nonfat dry milk in PBS/Tween 0.1%) and then incubated overnight at 4°C with mouse mAbs for $pERK_{1/2}$, ERK_2 (Santa Cruz, DBA S.r.l, Italy), phospho-Akt and rabbit polyclonal antibody for Akt (cell signaling from Euroclone, Milan, Italy). All antibodies were used at 1:1000 dilution in milk buffer (5% nonfat dry milk in PBS/Tween 0.1%). Subsequently, the membranes were incubated for 1 h at room temperature with 1:2000 dilution of antimouse or antirabbit IgG-horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences, UK). After washing in PBS/Tween 0.1%, the membranes were analyzed by enhanced chemiluminescence's (Amersham Biosciences). The optical density (OD) of the bands on autoradiographic films was determined by an image analysis system (GS 700 Imaging Densitometer, Bio-Rad) equipped with a software Molecular Analyst (IBM). The effect of bromelain on the mitogen-activated protein (MAP) kinase and

4   B. Romano et al.

Mol. Nutr. Food Res. 2013, *00*, 1–9

phosphoinositide 3-kinase (PI3K) activation was expressed as ratio of densitometric analysis of $pERK_{1/2}$/total extracellular signal-related kinase (ERK) bands and pAkt/Akt, respectively. Such assay was performed after 24-h bromelain exposure, based on previous work [22].

2.9 CRC AOM model

Mice were randomly divided into the following four groups (ten animals per group): group 1 (control) was treated with vehicles, group 2 was treated with AOM plus the vehicle used to dissolve bromelain, group 3 was treated with AOM plus bromelain (1 mg/kg), and group 4 was treated with AOM plus celecoxib (10 mg/kg). AOM (40 mg/kg in total, intraperitoneally) was administered, at the single dose of 10 mg/kg, at the beginning of the first, second, third, and fourth wk. Bromelain and celecoxib were given (intraperitoneally) three times a week for the whole duration of the experiment starting 1 wk before the first administration of AOM. The doses of bromelain and celecoxib were selected on the basis of previous published work dealing with the effects of these drugs in subchronic or chronic experiments [23–25]. All animals were euthanized by asphyxiation with CO_2 3 months after the first injection of AOM. Based on our laboratory experience, this time (at the dose of AOM used) was associated with the occurrence of a significant number of aberrant crypt foci (ACF, which are considered preneoplastic lesions), polyps, and tumors [26].

For ACF, polyps, and tumors determination, the colons were rapidly removed after sacrifice, washed with saline, opened longitudinally, laid flat on a polystyrene board, and fixed with 10% buffered formaldehyde solution before staining with 0.2% methylene blue in saline. Colons were examined using a light microscope at 20× magnification (Leica Microsystems, Milan Italy). The detection and quantitation of ACF, polyps, and tumors on the colon were performed as previously reported [27, 28]. Briefly, in comparison to normal crypts, aberrant crypts have greater size, larger and often elongated openings, thicker lining of epithelial cells, compression of adjacent crypts, and are more darkly stained with methylene blue. According to the number of constituent crypt, the total number of ACF and the number of foci containing four or more aberrant crypts (which are best correlated with the final tumor incidence) were evaluated. The criterion to dis-

tinguish polyps from tumors was established considering the main characteristic features of these two lesions (i.e. crypt distortion around a central focus and increased distance from luminal to basal surface of cells for polyps and high grade of dysplasia with complete loss of crypt morphology for tumors) [29]. For polyp and tumor evaluations, the colons of all mice were decolorized with 70% ethanol and embedded in paraffin; thereafter, 5-μm sections were deparaffinized with xylene, stained with hematoxylin-eosin and observed in a DM 4000 B Leica microscope (Leica Microsystems).

2.10 Statistical analysis

Statistical analysis has been carried out using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). Data are expressed as the mean ± SEM of *n* experiments. To determine statistical significance, Student's *t*-test was used for comparing a single treatment mean with a control mean, and a one-way analysis of variance followed by a Tukey–Kramer multiple comparisons test was used for analysis of multiple treatment means. *p*-Values <0.05 were considered significant.

3 Results

3.1 Cell proliferation and caspase 3/7 activity in CRC cells

Bromelain and proteolytically inactive bromelain (1–10 μg/mL), significantly and in concentration-dependent manner, reduced the ^{3}H-thymidine incorporation in proliferating Caco-2 cells (Fig. 1). The antiproliferative effect was significant starting from 3 and 1 μg/mL concentration for bromelain and inactivated bromelain, respectively (Fig. 1). Bromelain (1–10 μg/mL), significantly and in concentration-dependent manner, also reduced the ^{3}H-thymidine incorporation in DLD-1 cells (Fig. 2).

Bromelain, but not proteolytically inactive bromelain, at the concentration of 1 and 10 μg/mL, increased the activity of caspase 3/7, thus suggesting a proapoptotic effect in Caco-2 cells (Fig. 3).
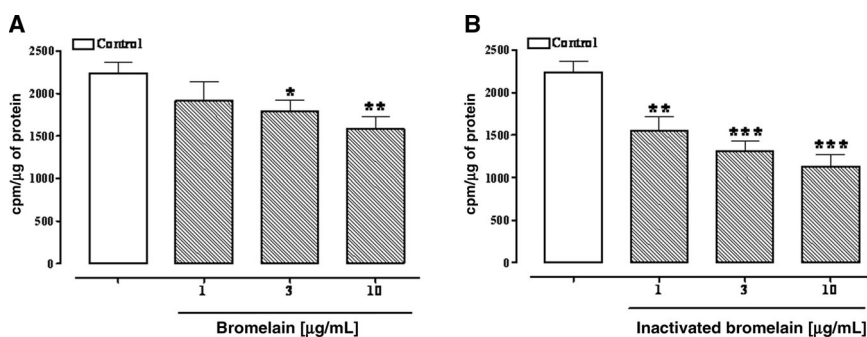
Figure 1. Antiproliferative effect of bromelain (1–10 μg/mL; A) and inactivated bromelain (1–10 μg/mL; B) in colorectal carcinoma (Caco-2) cells. Each bar represents the mean ± SEM of three independent experiments. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ versus control.
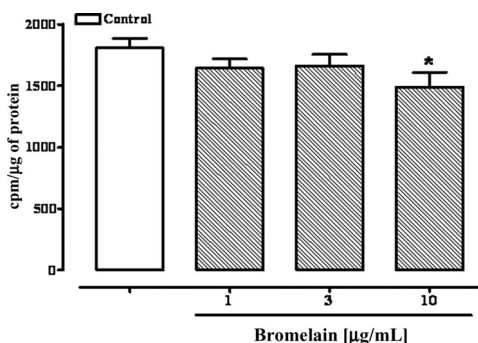
Figure 2. Antiproliferative effect of bromelain (1–10 μg/mL) in colorectal carcinoma (DLD-1) cell line. Each bar represents the mean ± SEM of three independent experiments. *$p < 0.05$ versus control.

3.2 MAP kinase and phospho-Akt expression in Caco-2 cells

The possible molecular mechanism of bromelain on cell proliferation and apoptosis was investigated by studying its effect on the MAP kinases and PI3K/Akt signaling pathways. The MAP kinases pathway involves two closely related kinases, known as ERK1 (p44) and ERK2 (p42) that come from dimerization of total cytosolic ERK. Bromelain (1–10 μg/mL), significantly and in concentration-dependent manner, reduced the expression of phosphorylated ERK_1 ($pERK_1$) and ERK_2 ($pERK_2$; Fig. 4). The effect was significant starting from the 3 μg/mL concentration. Similarly, the expression of PI3K was significantly reduced by bromelain (1–10 μg/mL; Fig. 5).

3.3 Intracellular ROS levels in Caco-2 cells

Hydrogen peroxide in the presence of iron (II) ions (Fenton's reagent, 2 mM), induced an oxidative stress in Caco-2 cells, resulting in an increased production of intracellular ROS (Fig. 6). Preincubation of Caco-2 cells for 24 h with bromelain (1–10 μg/mL) reduced the production of cytosolic ROS levels induced by 2 mM H_2O_2/Fe^{2+} (Fig. 6A). The effect was significant at the concentration of 10 μg/mL (Fig. 6A). By contrast, proteolytically inactive bromelain, did

not modify ROS levels increased by the Fenton's reagent (Fig. 6B).

3.4 AOM-induced CRC in mice

The carcinogenic agent AOM given alone induced the appearance of ACF, polyps, and tumors (Fig. 7) after 3 months of treatment. Bromelain (1 mg/kg) significantly reduced the total number of ACF/mouse, the number of ACF with four or more crypts, and the number of tumors/mouse, and completely prevented the formation of polyps (Fig. 7). Preliminary experiments showed that an optimal chemopreventive action was achieved for the 1 mg/kg bromelain dose, being a lower dose ineffective (0.3 mg/kg) and a higher dose (3 mg/kg) as active as 1 mg/kg. Bromelain did not induce appreciable toxic effects, as revealed by body weight measurements, absence of piloerection, and hypomotility. Moreover, visual observation of the peritoneal cavity did not reveal any adhesion or gross pathology following bromelain injection. Celecoxib (10 mg/kg), used as a reference drug, was also able to reduce the total number of ACF/mouse, the number of ACF with four or more crypts, the number of tumors, and the number of polyps (Fig. 7).

4 Discussion

Bromelain, a mixture of proteolytic enzymes derived from pineapple stem, has been reported to have beneficial effects in a variety of gastrointestinal diseases, including experimental inflammatory bowel disease [15–20]. In the present study, we have shown that bromelain exerts antiproliferative/proapoptotic actions in a colorectal carcinoma cell line and chemopreventive effects in a mouse model of experimental colon carcinogenesis in vivo.

Bromelain is known to exert both antiproliferative effects in different tumor cell lines, including gastric carcinoma cells, glioblastoma cells, ovarian cancer cells, and breast cancer cells [15–17] and proapoptotic effects in human epidermoid carcinoma cells and in breast cancer cells [30–32]. In the present study, we have shown that bromelain reduced cell proliferation and induced apoptosis (through activation of
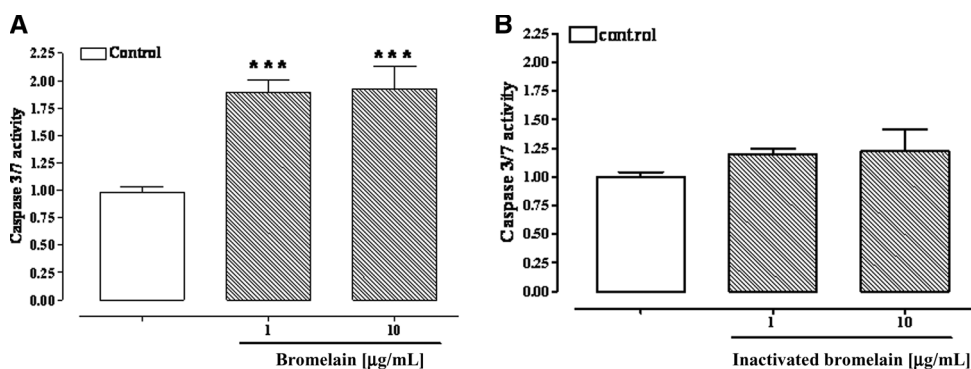
Figure 3. Effect of bromelain (1 and 10 μg/mL; A) and inactivated bromelain (1 and 10 μg/mL; B) on caspase 3/7 activity in colorectal carcinoma (Caco-2) cells. Each bar represents the mean ± SEM of three independent experiments. ***$p < 0.001$ versus control.
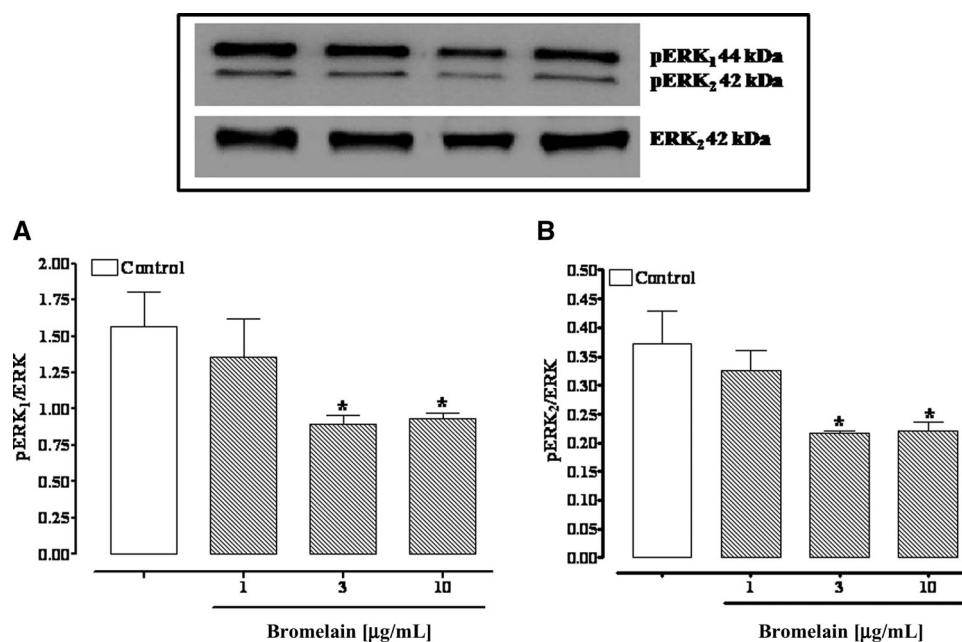
Figure 4. pERK$_1$ (A) and pERK$_2$ (B) expression (MAP kinase activation) in colorectal carcinoma (Caco-2) cells after 24 h of bromelain (1–10 μg/mL) incubation. The insert shows a representative e.g. of Western blot analysis. Each bar represents the mean ± SEM of three independent experiments. *$p < 0.05$ versus control.

caspase 3/7) in human colorectal carcinoma (Caco-2) cells. The proapoptotic effect of bromelain is not a consequence of its antiproliferative effect since bromelain, at the 1 μg/mL concentration, activated caspase 3/7 without inhibiting proliferation.

Because the proteolytic action has been supposed to be responsible for many of its pharmacological activities [15–17], we analyzed the effect of inactivated bromelain on Caco-2 cell proliferation and apoptosis. We found that the antiproliferative action of bromelain was not related to its proteolytic activity, since proteolytically inactive bromelain also exerted antiproliferative actions; the potential mechanisms able to explain the similarity in the effect of proteolytically active versus inactive bromelain on cell proliferation requires further studies. By contrast, the proteolytic activity is essential for bromelain to exert proapoptotic effects, since proteolytically inactive bromelain did not exert proapoptotic actions. It is worthy to note that other gastrointestinal actions of bromelain (i.e. antispasmodic, antidiarrhoeal, and anti-inflammatory) required proteolytic activity [18, 21].

To explore the possible mechanisms of bromelain effects (antiproliferative and proapoptotic), we analyzed two pathways that are essential in the regulation of tumor cell growth and apoptosis, i.e. PI3K/Akt and MAP kinase pathways [33, 34]. Our results showed that bromelain, concentration-dependently, downregulated Akt, ERK$_1$, and ERK$_2$ phosphorylation in Caco-2 cells suggesting a possible involvement of such pathways in the bromelain actions. Specifically, bromelain seems to promote apoptotic cell death in tumor cells and reduce cell proliferation of tumor cells by inhibiting Akt and ERK$_{1/2}$ phosphorylation, respectively. Our results are in agreement with previous observations that reported an in-
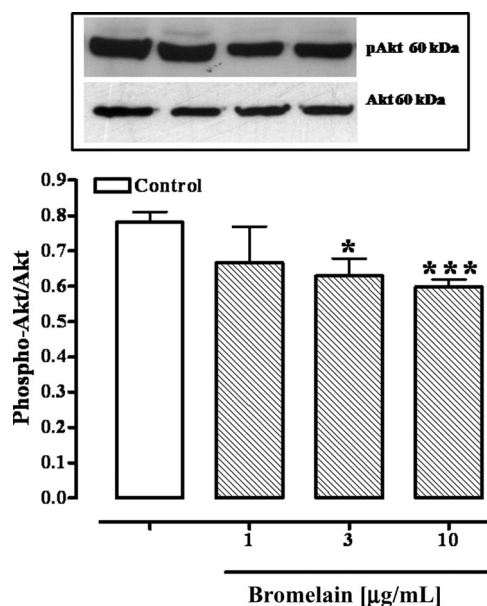
Figure 5. Phospho-Akt expression after 24 h exposure with bromelain (1–10 μg/mL) in colorectal carcinoma (Caco-2) cells. The insert shows a representative e.g. of Western blot analysis. Each bar represents the mean ± SEM of three independent experiments. *$p < 0.05$ and ***$p < 0.001$ versus control.

hibitory effect of bromelain on the activity of cell survival regulators such as Akt and ERK [35, 36].

A critical event in the development of gastrointestinal tumors is the impairment of antioxidant defense of intestinal epithelial cells leading to oxidative stress [37]. We thus evaluated the possible effects of bromelain on oxidative stress in
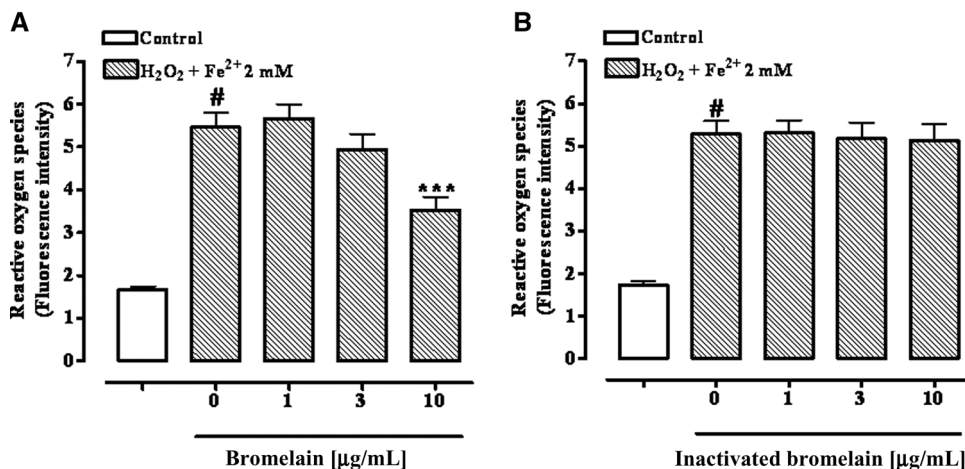
Figure 6. Effect of bromelain (1–10 μg/mL; A) and inactivated bromelain (1–10 μg/mL; B) on Fenton's reagent (H_2O_2/Fe^{2+} 2 mM)-induced ROS production in colorectal carcinoma (Caco-2) cells. Effect observed in differentiated Caco-2 cells after 24-h bromelain exposure. Data represent mean ± SEM of 6 experiments. $^{\#}p < 0.001$ versus control; $^{***}p < 0.001$ versus H_2O_2/Fe^{2+} alone.

Caco-2 cells. It has been previously shown that bromelain can stimulate ROS production in neutrophils in vitro as well as in neutrophils from healthy volunteers taking a polyenzyme preparation containing bromelain [38]. In the present study, we have found that bromelain, but not proteolytically inactive bromelain, reduced, in a concentration-dependent manner, ROS production induced by the Fenton's reagent in differentiated Caco-2 cells. Such data suggest that inhibition of

ROS production by bromelain requires enzymatic activity. It is possible to speculate that proteolytic activity is necessary for bromelain to remove cell surface molecules as reported in peripheral blood cells [39].

Economic analysis suggests that chemoprevention has the potential to represent a cost-effective intervention [40]. Moreover, proper nutrition and diet are important in helping to prevent many diseases including CRC. Several animal and
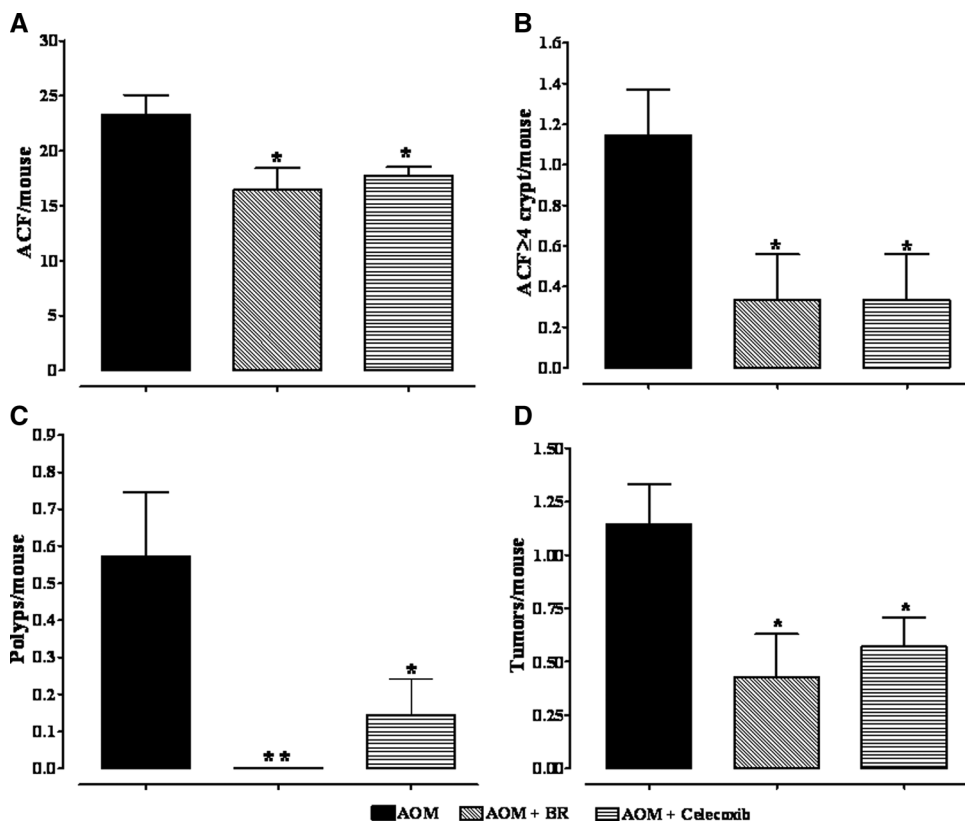
Figure 7. Effect of bromelain (1 mg/kg) on the formation of ACF (A), ACF with four or more crypts (B), polyps (C), and tumors (D) induced by the carcinogenic substance AOM in the mouse colon. AOM (40 mg/kg in total, intraperitoneally) was administered, at the single dose of 10 mg/kg, at the beginning of the first, second, third, and fourth wk. Bromelain (BR, 1 mg/kg) and celecoxib (10 mg/kg) were administered (intraperitoneally) three times a week for the whole duration of the experiment (starting from 1 wk before the first administration of AOM). Data represent mean ± SEM of ten animals $^{*}p < 0.05$ and $^{**}p < 0.01$ versus AOM alone.

human studies indicated that bromelain might have some anticancer activity [15–17], but its possible chemopreventive action against gastrointestinal tumors has been not explored to date. Because previous investigators have shown the efficacy of intraperitoneally-administered bromelain in experimental cancer [23, 41] and because bromelain administered orally without enteric protection is hydrolyzed by gastric acid, we have used the intraperitoneal route of administration. We have here shown that bromelain prevented the formation of preneoplastic lesions, polyps, and tumors induced in the mouse colon by the administration of the carcinogenic agent AOM. An optimal protective effect was achieved for the 1 mg/kg dose, being a lower dose ineffective (0.3 mg/kg) and a higher dose (3 mg/kg) as active as 1 mg/kg. This 1 mg/kg dose in mice is about 30–40 fold lower than the documented lethal dose of bromelain after intraperitoneal administration [15].

5   Conclusion

In conclusion, we have demonstrated that the food ingredient bromelain exerts antiproliferative/proapoptotic effects in colorectal carcinoma cells and chemopreventive actions against colon carcinogenesis in vivo. Studies in Caco-2 cells suggested that bromelain action could be related to inhibition of tumor cell proliferation as well as to stimulation of apoptotic processes by blocking the MAP kinase and (PI3K)/Akt signaling. However, whether or not such changes occur in ex vivo, colonic preparation has been not demonstrated in the present study. Bromelain-containing foods or bromelain itself—possibly as enteric-coated preparations, to prevent gastric acid inactivation—may represent good candidates for CRC chemoprevention.

The authors have declared no conflict of interest.

6   References

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