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# Proteolytic activity and immunogenicity of oral bromelain within the gastrointestinal tract of mice

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**Abstract**

Bromelain is a mixture of proteinases derived from pineapple stem that is marketed by health food stores as a "digestive aid". A number of studies suggest that bromelain may also have anti-inflammatory activity in vivo, including an anecdotal report describing potential efficacy in inflammatory bowel disease. We and others have previously shown that proteolytically active bromelain removes certain cell surface molecules and affects leukocyte migration, activation, and production of cytokines and inflammatory mediators in vitro. The purpose of this study was to determine whether ingested bromelain retains proteolytic activity within the murine gastrointestinal tract in vivo. The proteolytic activity of bromelain was determined in vitro using model substrates or immunofluorescence assays after administration of various doses and formulations orally to mice. Immune responses against bromelain were detected by enzyme immunoassays. When formulated in antacid, oral bromelain retained substantial proteolytic activity throughout the gastrointestinal tract. Bromelain concentrations within the colon were dependent on both dose and formulation and were sufficient to remove bromelain-sensitive molecules from both leukocytes and colon epithelial cells. Peak activity in the stool was observed 4 h after oral dosing. Although anti-bromelain IgG was detected in both serum and stool after long-term oral therapy, these antibodies did not prevent bromelain proteolytic activity within the gastrointestinal tract. These studies demonstrate that bromelain enzymes can remain intact and proteolytically active within the murine gastrointestinal tract. They provide further support for the hypothesis that oral bromelain may potentially modify inflammation within the gastrointestinal tract via local proteolytic activity within the colonic microenvironment.


*Keywords:* Proteinase therapy; Pharmacokinetics; Inflammatory bowel disease; Enteric protection

## 1. Introduction

Bromelain (EC 3.4.22.32) is a complex natural mixture of proteolytic enzymes that is derived from pineapple stems [1]. In the United States, bromelain is sold in health food stores as a nutritional supplement to "promote digestive health" and as an anti-inflammatory medication for horses. There is extensive data demonstrating the relative safety of oral bromelain, even in large doses. The $LD_{50}$ of bromelain is >10 g/kg in rodents [2]. Humans have been treated with up to 12 g/day orally without major side effects [3].

We and others have previously shown that in vitro treatment with bromelain removes certain cell surface molecules that affect lymphocyte migration and activation [4–7]. Bromelain treatment has also been

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shown to affect the production of cytokines and inflammatory mediators and to affect ERK-2-mediated signal transduction by leukocytes and colon epithelial cell lines in vitro [8,9]. These effects require that the bromelain be proteolytically active.

Based in part on these in vitro studies, trials of bromelain were conducted in a variety of inflammatory diseases and models of inflammation. These include carrageenan-induced pleurisy in the rat [10–12], immunologically mediated arteriosclerosis in rat aortic allografts [13], human rheumatologic diseases [14–16], the experimental allergic encephalomyelitis (EAE) model for the human autoimmune disease multiple sclerosis [17], and IgE-mediated perennial allergic rhinitis [18]. Beneficial effects were suggested or proven in each of these studies. In some studies, bromelain had efficacy similar to standard anti-inflammatory drugs. A dose of 10 mg/kg i.v. bromelain had activity similar to 0.3 mg/kg i.p. dexamethasone (equivalent to 20 mg prednisone in a 70-kg human) in rat pleurisy models [11,12]. Bromelain had efficacy similar to or better than classic non-steroidal anti-inflammatory agents (NSAIDs) in some rheumatologic studies in humans [14,16,19]. A recent anecdotal report described two patients with ulcerative colitis that was refractory to conventional treatments who rapidly entered and remained in clinical and endoscopic remission after self-treatment with oral bromelain obtained from a health food store [20]. The specific pathogenetic mechanisms vary in these different models and diseases, but each is characterized by excessive inflammatory activity. However, the mechanisms by which bromelain may decrease inflammation in these models are currently unknown.

It is not clear whether biologic effects of oral bromelain within the gastrointestinal tract occur due to local or systemic proteolytic activity. Although human adult intestinal epithelium has traditionally been thought to be impermeable to proteins, full-length proteolytically active bromelain has been documented in plasma from healthy male volunteers after oral administration of enteric-coated tablets. This was shown unequivocally by immunoprecipitation with anti-bromelain antibody, gel electrophoresis, and immunodetection with a second anti-bromelain antibody, as well as by immunoassay and proteolysis of model substrates [3]. However, bromelain was inefficiently absorbed in these subjects, with a plasma concentration of only 5–10 ng/ml after 48 h of oral treatment with divided doses totaling 4 g/day. No information is available regarding variability in bromelain absorption that may arise as the result of differences in age, gender, or disease states. Much of the bromelain present in the plasma is complexed with alpha-2 macroglobulin (α2M) and alpha-1-antichymotrypsin [3]. Although bromelain remains active proteolytically when complexed to α2M, its access to substrates is limited to small diffusible molecules [3,21]. In addition, bromelain–α2M complexes are also very rapidly taken up by scavenger receptors, primarily in the liver. This is important because α2M has been shown to be a potent adjuvant for induction of humoral immunity against the antigens which it complexes when administered subcutaneously or intradermally [22]. It is not known whether α2M complexes presented via other routes (e.g., systemically or mucosally) can result in antibody responses. Development of α2M-enhanced antibody responses to bromelain could limit patient responses to long-term bromelain therapy, particularly if mucosal immunity with concomitant secretion of IgA was generated. Alternatively, exposure to relatively large amounts of bromelain may induce oral tolerance similar to what is seen with most plant-based foods and no immune responses will be detected.

The purpose of this study was to determine whether the proteolytic activity of non-encapsulated bromelain is stable to passage though the murine gastrointestinal tract and whether oral administration of bromelain resulted in formation of anti-bromelain antibodies that could inhibit its proteolytic activity.

## 2. Materials and methods

### 2.1. Reagents

Bromelain (catalog no. B-4882) was obtained from Sigma (St. Louis, MO). Unless indicated, all other reagents were obtained either from Sigma or VWR (Atlanta, GA).

### 2.2. In vitro assays of bromelain proteolytic activity

Bromelain proteolytic activity was measured in a microplate assay format (modified from Ref. [23])

using the model peptide substrates, Z–Arg–Arg–pNA and Bz–Phe–Val–Arg–pNA, where Z = benzyloxycarbonyl, Bz = benzoyl, and pNA = *p*-nitroaniline (Bachem, King of Prussia, PA), at 250 μg/ml final concentration. Cleavage of the substrate results in free pNA that is detected colorimetrically at 405 nm. Assays were performed in buffer containing 10% dimethylformamide, 5 mM cysteine, 5 mM EDTA, and 0.1 M HEPES (pH 7.3). Soybean trypsin inhibitor (0.6–2 mg/ml) was added for assays of stool extracts to inhibit proteolytic activity from endogenous trypsin-like enzymes. Addition of soybean trypsin inhibitor had no effect on bromelain activity. Absorbance was measured at 30-s intervals for 10–60 min using a SpectraMax 250 plate reader and SoftMax Pro software (Molecular Devices, Sunnyvale, CA). Proteolytic activity was calculated from the change in absorbance vs. time, using the linear portion of the curve. The equivalent bromelain concentrations were calculated from standards of known concentration that were included in each assay.

### 2.3. In vivo studies of bromelain proteolytic activity

All animal studies were approved by the Institutional Animal Care and Use Committee. Wild-type female C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were fed escalating doses (2, 5, 10, and 20 mg) of bromelain formulated in 20–40 μl of water, 100 mg/ml $NaHCO_3$, or a 1:2 dilution of the commercial antacid Maalox (originally 400 mg $Al(OH)_3$ + 400 mg $Mg(OH)_2$ in 5 ml; Novartis Consumer Health, Parsippany, NJ) once daily for up to 5 days. Bromelain doses were placed directly into the mouth using a plastic pipet tip. Animals were allowed free access to food and water throughout the experiment. Stool pellets passed at various time points after dosing were extracted with PBS containing 1% bovine serum albumin (BSA) + 0.1% Kathon (a microbiocide; Supelco, Bellafonte, PA) and 0.6–2 mg/ml soybean trypsin inhibitor at a concentration of 100 mg stool/ml buffer. Solids were removed by centrifugation. In some mice, similar extracts were made at the time of sacrifice using luminal contents obtained at intervals throughout the stomach, small intestine (SI), and colon to determine how bromelain activity changes during transit through the gastrointestinal tract. For these studies, the SI was divided into three segments of approximately equal length (proximal SI, mid-SI, and distal SI). Colon was divided into two segments, one representing the cecum plus proximal colon and the other the distal colon. The molecular size of bromelain present in stool extracts was determined by electrophoresis through 15% SDS–PAGE gels, transfer to nitrocellulose, and reaction with polyclonal rabbit anti-bromelain antiserum. Bound antibodies were visualized using goat anti-rabbit IgG-horseradish peroxidase and enhanced chemiluminescence.

### 2.4. Analysis of cells and tissues

CT26 murine colon carcinoma cells were obtained from Dr. Lee Ellis (University of Texas M.D. Anderson Cancer Center, Houston, TX). The RAW264.7 murine macrophage cell line was obtained from the Tissue Culture Facility of the Duke University Comprehensive Cancer Center. Both of these cell lines express H-$2D^d$ MHC Class I molecules. Cells were incubated with 10–400 μg/ml bromelain in RPMI1640 or DMEM or with stool extracts additionally containing 2 mg/ml soybean trypsin inhibitor at 37 °C for 5–30 min. Cells were washed, then incubated with fluorescently labeled anti-CD44 (clone G44-2; Pharmingen/BD Biosciences, San Diego, CA) or anti-MHC Class I (H-$2D^d$, clone 34-5; Pharmingen) antibodies. Flow cytometric analysis was performed by the Flow Cytometry Facility of the Human Vaccine Institute at Duke University.

Immunohistochemistry was performed on frozen sections of murine colon tissue as described [24] using anti-CD44 (KM114; Pharmingen) or anti-CD3 (polyclonal; Dako, Carpenteria, CA) antibodies.

### 2.5. Determination of bromelain immunogenicity

Serum was obtained from mice who had been treated daily for 18 weeks with 2–20 mg oral bromelain formulated in water. Serum and stool were also obtained from mice given 2–20 mg bromelain orally in water, 1:2 Maalox, or 100 mg/ml $NaHCO_3$ five times weekly for 5 weeks. Serial dilutions of serum were analyzed by enzyme immunoassay, using 250 ng immobilized bromelain/well. Bound antibodies were detected using goat anti-mouse IgG coupled to horseradish peroxidase (HRP) (Jackson ImmunoResearch

Labs, West Grove, PA) and 3,3′ ,5,5′ -tetramethylbenzidine (TMB) substrate (KPL, Gaithersburg, MD). Stool extracts were analyzed for anti-bromelain antibodies by enzyme immunoassays that were detected with both goat anti-mouse IgG-HRP and goat anti-mouse IgA-HRP reagents (Southern Biotechnology Associates, Birmingham, AL). The titer of anti-bromelain antibodies was defined as the highest dilution at which color development in the immunoassay was $\geq 3\times$ the background signal obtained in the absence of immobilized bromelain. Antibody titers were expressed as the reciprocal and the geometric mean reciprocal titer was calculated for each dose group. Statistical comparison of groups was performed using Student's $t$ test after logarithmic transformation of reciprocal titers. A value of $p \leq 0.05$ was considered to be significant.

## 3. Results

### 3.1. Luminal bromelain activity following oral administration

To address how bromelain proteolytic activity changes with passage through the murine digestive tract, extracts were made from luminal contents obtained at measured intervals throughout the gastrointestinal tract of mice at several time points after oral administration of bromelain in water. The bromelain activity of these extracts was determined using the model substrates Z–Arg–Arg–pNA and Bz–Phe–Val–Arg–pNA. Under the assay conditions used, no substrate cleavage was observed in stool extracts from untreated mice. Thus, substrate cleavage corresponded directly to bromelain activity in the extracts. Bromelain rapidly moved through the murine upper gastrointestinal tract, with peak activity in the mid-small intestine 1 h following administration (Fig. 1). Colonic concentrations of bromelain rose thereafter, but the peak bromelain activity observed in the colon was less than that seen in the small intestine. Recovery of bromelain activity from the total gastrointestinal contents was low, with mean recoveries of $0.06 \pm 0.02\%$ of the administered dose ($n = 3$). The low bromelain activity observed, particularly in the upper portion of the murine gastrointestinal tract, suggested that that extensive gastric inactivation of bromelain may occur in vivo.

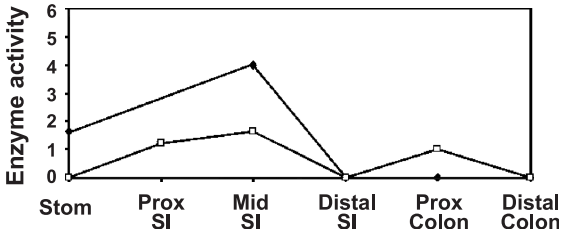


Fig. 1. Bromelain activity within the lumen of the gastrointestinal tract varies according to location and time since dosing. Mice were sacrificed 1 h (solid diamonds) or 2 h (open squares) after oral treatment with 20 mg bromelain in water. The bromelain activity of extracts made from the luminal contents of the stomach (Stom), small intestine (SI), and colon was determined using model substrates. Prox = proximal. Proteolytic activity against the Bz–Phe–Val–Arg–pNA substrate is shown.

### 3.2. Antacids enhance proteolytic activity of bromelain under simulated gastric conditions

Most proteins administered orally without enteric protection are hydrolysed by gastric acid and/or proteinases. Although enteric preparations have been shown to deliver proteolytically active bromelain at least to the small intestine in humans [3] and in pigs [25,26], the use of enteric coatings is problematic in mice due to their small size. Therefore, studies were performed to determine whether formulating bromelain in antacids could prevent its inactivation and/or degradation by low pH or by pH-dependent gastric enzymes. The proteolytic activity of 10 mg bromelain was determined after exposure to simulated murine gastric conditions in vitro when formulated in 20–40 μl of water, a 1:2 dilution of the commercial antacid Maalox (originally 400 mg $Al(OH)_3 + 400$ mg $Mg(OH)_2$ in 5 ml) with water, or 100 mg/ml sodium bicarbonate. Each formulation was incubated with 1 ml simulated murine gastric juice (PBS adjusted to pH 2 with HCl ± 1 mg/ml pepsin) for 2 h at 37 °C. The proteolytic activity of 100 μg/ml bromelain was then tested using the Z–Arg–Arg–pNA and Bz–Phe–Val–Arg–pNA model substrates. Essentially all bromelain activity against both substrates was destroyed by a 2-h exposure to pH 2 at 37 °C when bromelain was formulated in water (Fig. 2). No additional change in activity was seen when 1 mg/ml pepsin

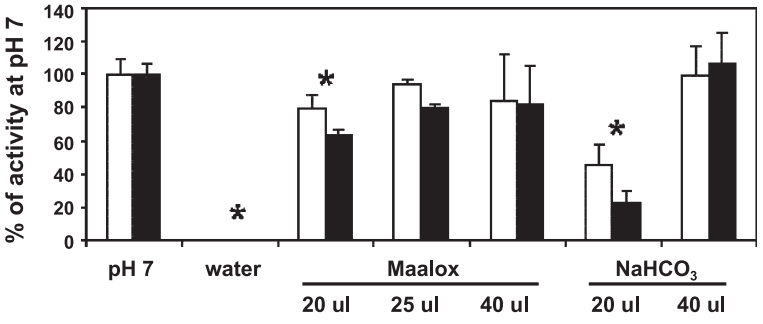


Fig. 2. Formulation in antacid preserves the proteolytic activity of bromelain during exposure to simulated gastric conditions. Proteolytic activity against the model substrates Z–Arg–Arg–pNA (open bars) and Bz–Phe–Val–Arg (solid bars) is compared for 10 mg bromelain exposed in vitro (37 °C, 2 h) to simulated gastric juice at pH 2 when formulated in water or with the indicated volumes of antacid. Data represents the mean rate of proteolysis of each model substrate ($\Delta A_{405\ nm}$/min), expressed as the % of the rate in PBS at pH 7 ± standard deviation for four independent assays. *Indicates conditions where bromelain activity was significantly decreased ($p < 0.05$) relative to activity at pH = 7 (Student's $t$ test).

was added to the solutions (not shown). However, formulation in either Maalox or sodium bicarbonate protected the proteolytic activity against both model substrates (Fig. 2). Full protection was achieved when 10 mg of bromelain was delivered in 25–40 μl of 1:2 Maalox or in 40 μl of 100 mg/ml $NaHCO_3$.

### 3.3. Antacids enhance proteolytic activity of stool extracts after bromelain treatment in vivo

If bromelain remains active throughout the gastrointestinal tract, then it should emerge in the feces in a proteolytically active form. Proteolytic activity was determined in stool extracts prepared at intervals after mice were treated orally with 2–20 mg bromelain formulated either in water, 25 μl 1:2 Maalox, or 40 μl $NaHCO_3$ (given as two "swallows" of 20 μl each). Minimal proteolytic activity was observed with stool extracts from mice treated with bromelain in water (Fig. 3A). Formulation in either Maalox or $NaHCO_3$ greatly increased the proteolytic activity of the stool extracts, with the highest stool proteolytic activity achieved using $NaHCO_3$. Bromelain activity was first detected in the stool at ~ 3 h, with peak bromelain activity observed at ~ 4 h following oral dosing (Fig. 3B). Essentially all proteolytic activity was gone by 8–24 h after dosing (Fig. 3B and not shown). Stool extracts that contained bromelain activity also contained full-length immunoreactive bromelain by Western blot (Fig. 4). These studies clearly show that, when administered orally in antacid, proteolytically active bromelain is present and potentially in contact with the intestinal mucosa for at least several hours following oral administration. However, due to the dehydration that occurs in the distal colon, it is not clear exactly how the activities measured in stool extracts correspond to luminal bromelain concentrations in other parts of the gastrointestinal tract.

### 3.4. Luminal bromelain concentrations are sufficient to proteolytically remove bromelain-sensitive molecules

We previously found that treatment with 1 mg/ml bromelain in vitro proteolytically altered 14 of the tested 59 markers that were constitutively expressed at >5 × background in human peripheral blood leukocytes [5]. To test the effects of lower bromelain concentrations similar to those measured in vivo on murine leukocyte and intestinal cell surface molecules, CT26 colonic epithelial and RAW264.7 murine macrophage cell lines were treated with 10–100 μg/ml bromelain for 5–30 min. The amount of the bromelain-sensitive marker CD44 remaining after treatment was compared to that of bromelain-resistant MHC Class I molecules using flow cytometry. As shown in Fig. 5A, CD44 removal from CT26 cells was rapid and dose-dependent, with only ~ 40% of the surface CD44 molecules remaining after a 5-min exposure to 10 μg/ml bromelain. Essentially all CD44 was removed when cells are exposed to 100 μg/ml bromelain for 30 min, with minimal effects on MHC

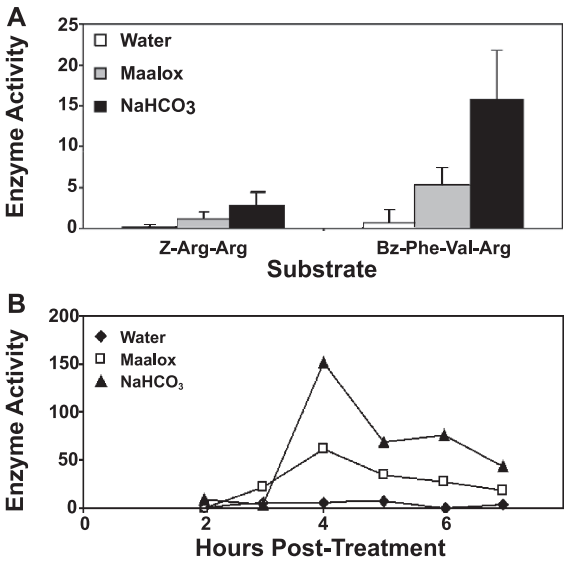


Fig. 3. Antacids enhance proteolytic activity of stool extracts after bromelain treatment in vivo. (A) Bromelain was formulated in water, a 1:2 dilution of the commercial antacid Maalox, or 100 mg/ml $NaHCO_3$ and given orally to mice 4 h prior to obtaining stool. Stool bromelain activity (mean ± standard deviation) is compared for groups of five mice that received 2 mg bromelain. A standard curve of known bromelain concentrations was used to express proteolytic activity as the equivalent of μg/ml bromelain present in 5 mg stool. *p* Values for differences in the activity of bromelain formulated in either Maalox or $NaHCO_3$ compared with water are $\leq 0.04$ using the Z–Arg–Arg–pNA substrate and $\leq 0.003$ using the Bz–Phe–Val–Arg–pNA substrate (Student's *t* test). (B) Proteolytic activity against the Bz–Phe–Val–Arg–pNA substrate is expressed as the equivalent of μg/ml bromelain present in 5 mg stool and shown as a function of time for stool extracts from mice given 5 mg oral bromelain formulated in either water, Maalox, or $NaHCO_3$ as described above. Maximal proteolytic activity is seen in the stool 4 h after treatment, with a markedly enhanced area under the curve (a measure of the time the mucosa is exposed to active enzyme) for bromelain formulated in $NaHCO_3$.

Class I molecules that are not sensitive to proteolysis by bromelain. Similar results were seen with the RAW264.7 macrophage cell line (Fig. 5B).

To further confirm the biologic activity of bromelain within the lumen of the gastrointestinal tract, CT26 cells were incubated with extracts prepared from stool obtained 4 h after mice were treated orally with 20 mg bromelain in 40 μl 100 mg/ml $NaHCO_3$. As shown in Fig. 6, stool extracts from mice treated with bromelain removed CD44 from the surface of CT26 cells. CD44 was not affected by extracts obtained from the same mice before bromelain treatment. These studies demonstrate that the activity of bromelain in stool extracts is not limited to diffusible model substrates. The luminal bromelain concentrations achieved by dosing oral bromelain in antacid are sufficient to modify expression of bromelain-sensitive molecules on cells under physiologic conditions. Colon tissues from control and bromelain treated mice were also examined to determine the effects of bromelain on CD44 in vivo. However, due to the very low to absent levels of immunohistochemically detectable CD44 on colonic surface epithelium of untreated mice, it was not possible to accurately measure changes in CD44 expression in the colon following bromelain treatment in vivo.

### 3.5. Immunogenicity of oral bromelain

Development of antibody responses to bromelain could limit patient responses to long-term bromelain therapy, particularly if mucosal immunity with concomitant secretion of IgA was generated. Immuno-

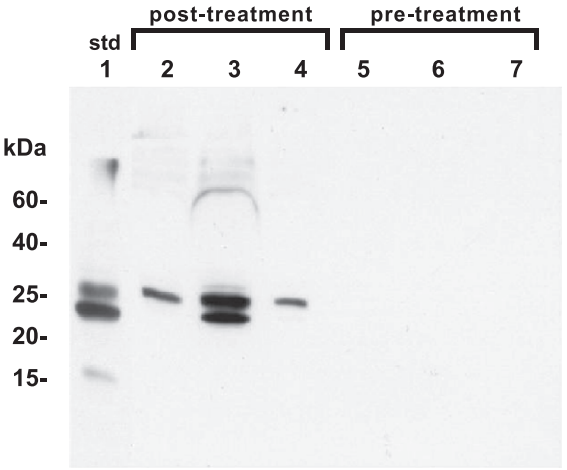


Fig. 4. Full-length bromelain is present in stool extracts from treated mice. Western blotting with polyclonal anti-bromelain antiserum shows that full-length (24–26 kDa) bromelain is present in the stool of bromelain-treated mice. Lane 1 = bromelain standard; lanes 2–4 = extract representing 3 mg stool from bromelain-treated mice; lanes 5–7 = stool extract from these mice prior to bromelain administration. The two bands seen in the bromelain standard are believed to represent different glycoforms of stem bromelain, the major proteinase component of natural bromelain. The proteolytic activities measured in the stool extracts using the Z–Arg–Arg–pNA model substrate were equivalent to 24, 53, and 8 μg/ml bromelain for lanes 2–4, respectively.

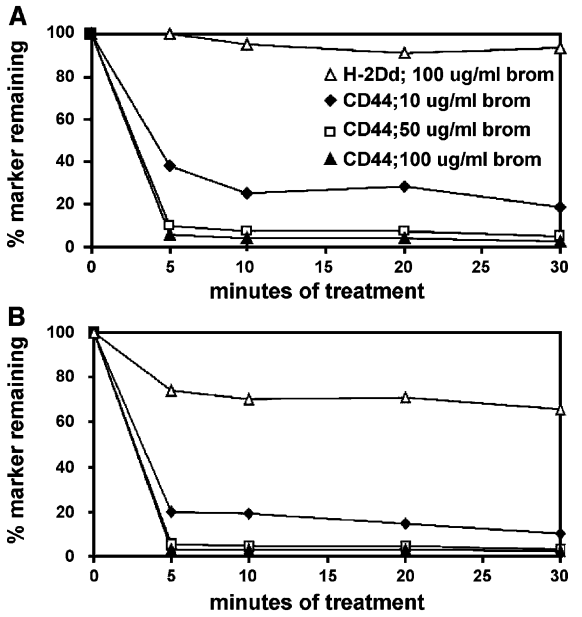


Fig. 5. Bromelain rapidly removes sensitive molecules from murine cells in a dose- and time-dependent fashion. CT26 intestinal epithelial cells (panel A) and RAW264.7 macrophages (panel B) were treated with the indicated concentrations of bromelain. The effects of treatment on cell surface levels of bromelain-sensitive CD44 and bromelain-resistant MHC Class I (H-$2^d$) molecules were determined by flow cytometry. Bromelain concentrations higher than 100 μg/ml removed essentially all cell surface CD44 molecules by the 5-min time point (not shown).

assays were therefore performed to determine whether anti-bromelain antibodies were present in serum from mice treated once daily for 18 weeks with oral bromelain formulated in water. Low titers of anti-bromelain antibodies were present in mice treated with 2 mg ($n=4$) or 5 mg bromelain/day ($n=5$), with higher titers in animals treated with 20 mg bromelain/day ($n=2$) (Fig. 7).

Next, serum and stool titers of anti-bromelain antibodies were compared in groups of three to five mice treated with escalating doses of bromelain formulated in either water, Maalox, or $NaHCO_3$ and given orally five times per week for 5 weeks. Mice given each of these formulations also developed anti-bromelain antibodies in their serum. The geometric mean reciprocal titer of anti-bromelain antibodies in the serum was 40,317 (range 2000–512,000) for mice given bromelain in water, 256,000 (range 128,000–512,000) for mice given bromelain in Maalox, and 80,635 (range 32,000–256,000) for mice given bromelain in $NaHCO_3$. These titers did not differ statistically. Positive IgG antibody titers in stool were not seen in five mice given bromelain formulated in water (average signal ± standard deviation for group, 1.43 ± 0.09-fold over background at a 1:8 dilution equivalent to 0.6 mg stool). Positive stool IgG titers were present in three of five mice given bromelain in Maalox (average for group, 3.62 ± 1.54-fold over background) and one of five mice given bromelain in $NaHCO_3$ (average for group, 2.23 ± 1.02-fold over background). None of the mice had positive titers of anti-bromelain IgA at a 1:8 dilution of stool extract equivalent to 0.6 mg stool. Bromelain proteolytic activity was readily detectable within the gastrointestinal tract of all mice, even those with the highest anti-bromelain titers in both serum and stool. The peak bromelain activity observed in stool or gastrointestinal contents did not appear to vary directly with serum anti-bromelain titer. In fact, the highest bromelain

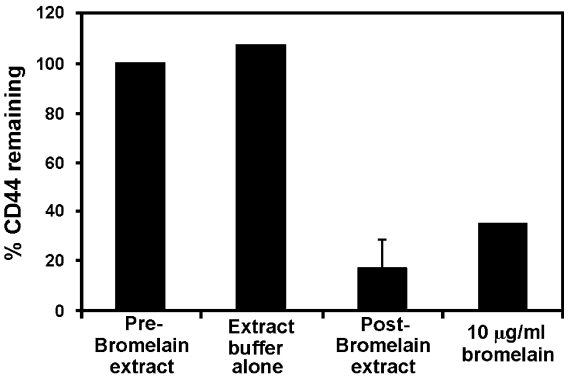


Fig. 6. Stool extracts from bromelain-treated mice remove bromelain-sensitive molecules in vitro. CT26 intestinal epithelial cells were treated for 30 min with buffer alone, stool extracts made prior to bromelain administration, or stool extracts prepared 4 h after dosing mice orally with 20 mg bromelain in 40 μl of 100 mg/ml $NaHCO_3$. The effects of treatment on cell surface levels of bromelain-sensitive CD44 and bromelain-resistant MHC Class I (H-$2^d$) molecules were determined by flow cytometry. Extracts of stool obtained prior to bromelain treatment did not remove CD44 from CT26 cells. However, extracts obtained post-bromelain treatment consistently removed bromelain-sensitive CD44 molecules from CT26 cells ($n=6$). The amount of CD44 removed was proportional to the bromelain proteolytic activity measured in the extracts using model substrate assays (not shown). MHC Class I reactivity following treatment with post-bromelain stool extracts was unchanged from pre-bromelain values (106 ± 9%; $n=6$).

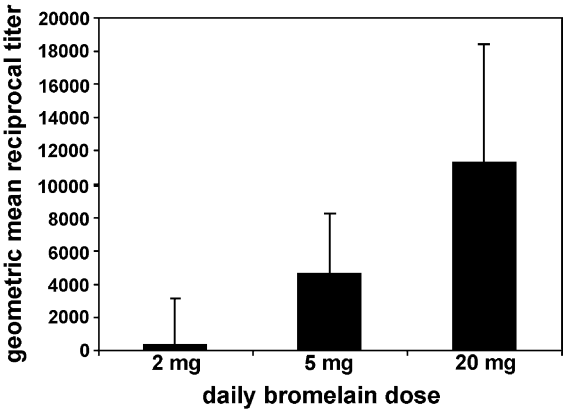


Fig. 7. Immunogenicity of oral bromelain. The geometric mean reciprocal titer of anti-bromelain antibodies in the serum is shown for mice given 2 mg ($n=4$), 5 mg ($n=5$), or 20 mg ($n=2$) bromelain formulated in water orally once daily for 18 weeks. Serum antibody titers were higher in mice receiving 20 mg as compared with 2 mg bromelain/day ($p=0.05$), but titers in mice receiving 2 or 5 mg bromelain/day were not statistically different ($p=0.10$).

activities were observed in the small intestine and colon of two mice with serum anti-bromelain titers of 1:64,000.

## 4. Discussion

These studies show that oral bromelain can exhibit substantial proteolytic activity within the gastrointestinal tract of mice. Passage through the murine gastrointestinal tract is rapid, with maximal activity observed in the stool 4 h after oral dosing. Although a high percentage of the administered bromelain is inactivated in the upper gastrointestinal tract, increased retention of proteolytic activity can be achieved by formulating bromelain in antacids such as Maalox or sodium bicarbonate. The residual bromelain activity present in stool is sufficient to remove bromelain-sensitive molecules from the cell surface of colon epithelial cells and macrophages. Repeated treatment with bromelain for 5–18 weeks results in development of anti-bromelain antibodies within the serum and in the stool, but these antibodies do not prevent bromelain proteolytic activity.

In vivo proteolytic activity of bromelain was previously documented only in the small intestine of pigs. Receptors for *Escherichia coli* adhesion were shown to be removed from small intestinal mucosa following administration of enterically protected oral preparations [25,26]. However, the current work is the first to document that oral bromelain can retain sufficient activity to proteolytically remove cell surface receptors throughout the entire gastrointestinal tract of mice, in the absence of encapsulation or other classic enteric protection techniques. Bromelain has been anecdotally reported to decrease intestinal inflammation in humans with ulcerative colitis [20]. The studies reported here provide support for the hypothesis that oral bromelain may potentially modify inflammation within the gastrointestinal tract via local proteolytic activity within the colonic microenvironment.

A moderate amount of inter-individual variability was observed in the amount and location of bromelain proteolytic activity recovered when the entire gastrointestinal tract was sampled. Similar variability was also observed when stool was obtained at given time points following oral bromelain dosing. Mice were allowed free access to food and water throughout all experiments. Food may have a buffering action that preserves bromelain activity, but it also induces acid and digestive enzyme secretion that can decrease bromelain activity. Variations in gastric acidity or gastrointestinal motility between animals likely contributes to variations in recovery of bromelain proteolytic activity, however, additional studies using larger numbers of animals and food restriction pre- and post-bromelain administration will be required to definitively address these issues.

CD44 was used as a model bromelain-sensitive marker in these studies, since it is typically highly expressed on the cell surface of a variety of cell types. Although most colon cell lines express surface CD44 in vitro, our studies showed that the immunoreactivity of colonic surface epithelium with CD44 mAbs is very low to absent in vivo, as previously reported [27]. Thus, it is unlikely the biologic effects of bromelain on cellular activation and cytokine secretion, especially within the colon, are solely due to proteolytic removal of CD44. Decreased extracellular regulated kinase (ERK-2) activation and/or decreased expression of mRNAs encoding pro-inflammatory cytokines are seen following bromelain treatment in both leukocyte and colon epithelial cell lines [8,9],

including in Jurkat T cells that lack CD44 expression [28]. Thus, the potential anti-inflammatory effects of bromelain most likely result from a removal of a bromelain-sensitive cell surface molecule other than CD44. The identity of this putative bromelain-sensitive molecule is currently unknown. None of the other cell surface molecules that are currently known to be bromelain-sensitive [5] are expressed on murine colonic epithelium.

The strong immunogenicity of bromelain following oral dosing was unexpected. Oral exposure to most proteins (e.g., those in food) results in tolerance rather than immunity. Co-administration with a mucosal adjuvant is generally necessary to develop immune responses against oral antigens [29]. Reciprocal serum antibody titers against cholera toxin, a xenogenic protein that also serves as a mucosal adjuvant, typically are in the range of $10^5$–$10^6$ by day 28 after three oral immunizations [30]. These titers are similar to what we observed in our bromelain-treated mice. Thus, even in the absence of a classical adjuvant, serum anti-bromelain responses are robust. It is known that bromelain is inefficiently absorbed into the systemic circulation, where it complexes with α2M [3]. α2M is a potent adjuvant for induction of humoral immunity against the antigens which it complexes when administered subcutaneously or intradermally [22]. However, the current data are insufficient to determine the site (mucosal vs. systemic) where anti-bromelain responses are generated and whether these responses are α2M-dependent. Further studies will be necessary to address these questions. However, the sustained in vivo proteolytic activity of bromelain despite the presence of relatively high anti-bromelain antibody titers suggests that the majority of antibody reactivity is directed at sites other than the active site. This has also been observed in rabbits, where subcutaneous immunization with bromelain in Freund's adjuvant results in high titers of anti-bromelain antibodies that can precipitate all bromelain enzymatic activity but do not affect proteolytic activity in the soluble state (L.P. Hale, unpublished data).

In summary, these studies show that biologically significant levels of bromelain enzymatic activity can be maintained throughout the gastrointestinal tract of mice for several hours after oral administration in vivo, particularly when using formulations containing antacid. This work is thus relevant for investigating potential mechanisms for the previously suggested anti-inflammatory effects of bromelain, especially in inflammatory bowel disease models. Furthermore, the information on formulations and gastrointestinal transit times reported here may also be relevant for oral administration of other protein-based therapies to mice.

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