

## Original Article

# Oral Bromelain Attenuates Inflammation in an Ovalbumin-induced Murine Model of Asthma

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Bromelain, a widely used pineapple extract with cysteine protease activity, has been shown to have immunomodulatory effects in a variety of immune system models. The purpose of the present study was to determine the effects of orally administered bromelain in an ovalbumin (OVA)-induced murine model of acute allergic airway disease (AAD). To establish AAD, female C57BL/6J mice were sensitized with intraperitoneal (i.p.) OVA/alum and then challenged with OVA aerosols for 3 days. Mice were gavaged with either (phosphate buffered saline)PBS or 200 mg/kg bromelain in PBS, twice daily for four consecutive days, beginning 1 day prior to OVA aerosol challenge. Airway reactivity and methacholine sensitivity, bronchoalveolar lavage (BAL) cellular differential, Th2 cytokines IL-5 and IL-13, and lung histology were compared between treatment groups. Oral bromelain-treatment of AAD mice demonstrated therapeutic efficacy as evidenced by decreased methacholine sensitivity ( $P \leq 0.01$ ), reduction in BAL eosinophils ( $P \leq 0.02$ ) and IL-13 concentrations ( $P \leq 0.04$ ) as compared with PBS controls. In addition, oral bromelain significantly reduced BAL CD19+ B cells ( $P \leq 0.0001$ ) and CD8+ T cells ( $P \leq 0.0001$ ) in AAD mice when compared with controls. These results suggest that oral treatment with bromelain had a beneficial therapeutic effect in this murine model of asthma and bromelain may also be effective in human conditions.

**Keywords:** airway inflammation – asthma – CD19+ B Cells – CD8+ T Cells – cysteine protease – eosinophils – IL-13 – immuno-modulation

## Introduction

The prevalence and severity of asthma continues to increase despite new pharmacological advances for both acute treatment and chronic disease management. The benefits of lifestyle changes and alternative asthma treatments are being investigated in the hope that safe and efficacious adjunctive therapies will be added to current treatment formulary (1–8). According to the National Institutes of Health, over 60% of Americans use complementary and alternative medicine with ~20%

using natural products or specific botanical formulations to supplement their health care needs (9). Botanicals such as *Boswellia serrata*, *Petasites hybridus*, *Astragalus membranaceus*, *Echinacea angustifolia* and *Ananas comosus* (common pineapple) and specific extracts such as bromelain from pineapple are being investigated as therapeutic agents in inflammatory conditions such as ulcerative colitis, multiple sclerosis and asthma (10–17).

Bromelain is a commonly used botanical extract, and is normally delivered as a powder either encapsulated in gelatin or prepared in an enteric coated tablet. Bromelain is available in combination with other natural products such as in Phlogenzym<sup>®</sup> (Bromelain, trypsin and rutosid trihydrate), Wobenzym<sup>®</sup> (Bromelain, trypsin, rutosid trihydrate, pancreatin, papain and phymotrypsin)

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and BCQ® (Bromelain, *Boswellia serrata*, *Curcuma longa* and quercetin) or as a single stand-alone product. Bromelain (enzyme classification 3.4.22.32) is a combination of sulfur-containing cysteine endopeptidases that have a broad specificity for the cleavage of proteins. One mechanism of action proposed to account for bromelain's therapeutic activity is the cleavage of lymphocyte cell surface receptors such as CD4, CD8, CD44 and CD62L. Receptor cleavage can result in altered cell communication, cell trafficking and cell signaling pathways leading to the modulation of pro- and anti-inflammatory cytokines such as IL-2, IL-4, IL-6 and TNF- $\alpha$  (18–22).

Experimental animal models, such as the ovalbumin (OVA)-induced model of allergic airway disease (AAD), are utilized to investigate the underlying mechanisms and potential therapies for asthma. Asthma is complex with characteristic airway hyperresponsiveness, airway inflammation, increased mucus and airway remodeling; measurable outcomes are produced similar to those found in humans. These outcomes include increases in inflammatory Th2 cytokines (IL-4, IL-5, IL-6 and IL-13), enhanced airway hyperresponsiveness, and increased total white blood cells, eosinophils and CD4+ T lymphocytes recovered from the bronchoalveolar lavage (BAL) (23–25).

The effect of intraperitoneally (i.p.) injected bromelain has been previously characterized in an OVA-induced murine model AAD (23). Bromelain treatment was found to be well-tolerated and non-toxic in both naive and AAD mice. Bromelain significantly reduced total BAL leukocytes, eosinophils and CD4+ T cells and lowered the concentration of IL-13 in the BAL. In order to better mimic the clinical environment and consider the effects of digestion and degradation on enzymatic activity, the oral administration of bromelain was adapted in this AAD model. The present study was designed to determine whether oral bromelain treatment had an anti-inflammatory or immunoregulatory effect in an OVA-induced murine model of acute AAD.

## Methods

### Mice

Female C57BL/6J mice, 3–6 months of age and weighing 17–24 g, were purchased from the Jackson Laboratory (Bar Harbor, ME, USA), and housed conventionally in plastic cages with corncob bedding. The mouse room was maintained at 22–24°C with a daily light/dark cycle (light from 0600 to 1800 h). Chow and water were supplied *ad libitum*. Seven to eight mice were used per group. The protocols for mice used were approved by the Animal Care Committee at the University of Connecticut Health Center.

### Ovalbumin Sensitization and Aerosol Exposure Protocol

Mice were immunized with three weekly i.p. injections of a suspension containing 25  $\mu$ g of OVA (grade V, Sigma Chemical Co., St. Louis, MO, USA) and 2 mg of aluminum hydroxide (alum) in 0.5 ml of 0.9% sodium chloride (pH 5.5, 308 mOsmol L<sup>-1</sup>; Baxter Healthcare Corporation, Deerfield, IL, USA). One week after the last injection the mice were exposed to 1% aerosolized OVA in sodium chloride, 1 h per day, for 3 days (acute AAD model) (23,24). The mice were placed in plastic restraint tubes (Research and Consulting Co., Basel, Switzerland) for nose-only aerosol exposure. The aerosols were generated by a BANG nebulizer (CH Technologies, Westwood, NJ, USA) into a 7.6 l inhalation exposure chamber to which restraint tubes were attached. Chamber airflow was 6 l min<sup>-1</sup>, and aerosol particle size of OVA was monitored by gravimetric analysis with a Mercer cascade impactor (In-Tox Products, Moriarty, NM, USA). The mass median aerodynamic diameter and geometric standard deviations were 1.4 and 1.6  $\mu$ m, respectively. The estimated daily inhaled OVA dose  $\sim$ 30–40  $\mu$ g per mouse. Twenty-four hours after the final aerosol exposure, the mice were sacrificed by overdose (0.15 ml i.p. injection per 20 g mouse of: 13 mg Ketamine HCL, Ketaset-III™ Fort Dodge Animal Health, Fort Dodge IA, USA, and 0.4 mg of xylazine, Tranquived™ Vedco, St Joseph, MO, USA) and exsanguination.

### Airway Hyperresponsiveness

Airway responses to methacholine were assessed by whole-body barometric plethysmography, using the Buxco system (Buxco Electronics, Troy, NY, USA), as previously described (23). Briefly, mice were evaluated for maximal enhanced pause (Penh), 24 h before treatment and 12 h after the third OVA aerosol was administered. Mice were placed in individual chambers and exposed for 2 min to aerosolized saline or increasing concentrations of methacholine from 3 to 300 mg ml<sup>-1</sup>. Respiratory system variables including tidal volume, respiratory frequency, inspiratory and expiratory times and changes in box pressure were recorded before and during aerosolization and for 4 min after each exposure. The Penh value response to methacholine was recorded at each dose. The entire dose–response relationship to methacholine was compared in individual animals before and after OVA aerosol exposures. In addition, individual animal sensitivity to methacholine was measured by the interpolated concentration of methacholine needed to increase the Penh value to two units (the 'Penh-2' value). The Penh-2 value was selected as the portion of the dose–response curve in which the greatest changes in sensitivity would be manifested (25). Increased sensitivity to methacholine during AAD was demonstrated by a decreased Penh-2 value (i.e. less methacholine needed to elicit the response).

### BAL Fluid Analysis

At time of sacrifice the lungs were lavaged *in situ* with five 1 ml aliquots of sodium chloride. The BAL fluid was centrifuged at 200 *g* for 10 min, the cellular pellet was resuspended in sodium chloride, and the total nucleated cells were counted with a hemocytometer using nigrosin exclusion as a measure of viability. Leukocyte differentials were determined in BAL fluid using cytocentrifuged (at 900 rpm for 5 min, Cytospin-4<sup>®</sup> Thermo Shandon, Astmoor, Runcorn, Cheshire, England, UK) preparations stained with May-Grünwaldstain and Giemsa (Accustain<sup>®</sup>, Sigma, St Louis, MO, USA). Briefly the slides were allowed to air dry for ~10 min, immersed in methanol, May-Grünwald and May Grünwald Buffer (pH 7.2, Sigma, St Louis, MO, USA) for 5 min each, and stained with Giemsa for 15 min. Stained BAL slide differentials were counted in a blind manner by 3 individuals. The remaining cells were analyzed phenotypically for T cell subpopulations using specific antibodies and fluorescence flow cytometry. BAL protein concentrations were measured in the supernatants by bicinchoninic acid (BCA) protein assay using bovine serum albumin as a standard (Pierce Biotechnology, Rockford, IL, USA).

### BAL-Cytokine Analysis

After collection of BAL, samples were centrifuged at 200 *g* for 10 min to remove cells. The BAL fluid component was concentrated using an Amicon Centriplus YM-10 filtration device (Millipore Corp., Bedford, MA, USA). Concentrated samples were then analyzed for Th2 cytokines IL-5 and IL-13 using enzyme-linked immunosorbent assay (ELISA) kits (Pierce Biotechnology Rockford, IL; R&D Systems Minneapolis, MN, USA) according to the manufacturer's directions. The limits of detection for IL-5 and IL-13 were 5 and 1.5 pg ml<sup>-1</sup>, respectively.

### BAL-Flow Cytometry and Immunofluorescence

BAL samples were prepared as previously described (23). Briefly the samples were washed in PBS (Dulbecco's Phosphate Buffered Saline, pH 7.4, Sigma St Louis, MO, USA) containing 0.2% bovine serum albumin and 0.1% NaN<sub>3</sub>. Aliquots containing 10<sup>4</sup> to 10<sup>5</sup> cells were incubated with 100 µl of appropriately diluted antibodies for 30 min at 4°C. After staining, the cells were washed twice with the above PBS solution, and relative fluorescence intensities were determined via flow cytometry using a FACSCalibur and analyzed with BD FACSDiva<sup>™</sup> Software v4.1 (Becton Dickinson, San Jose, CA, USA). Eosinophils were gated on forward and side scatter using a 4-decade log scale. The following fluorescence labeled monoclonal antibodies

was used: CD19-PE (1D3), CD4-PerCP (RM4-5) and CD8a-FITC (53-6.7) (Pharmingen, San Jose, CA, USA).

### Histology

After sacrifice, non-inflated lungs from separate animals were removed, fixed with 10% buffered formalin and processed in a standard manner. Tissue sections were stained with hematoxylin and eosin, Mallory's trichrome for collagen and periodic acid-schiff for mucus detection. All specimens were evaluated with a microscope-mounted Nikon Eclipse 400<sup>™</sup> camera (Tokyo, Japan). Digital images were created using Spot RT Slider<sup>™</sup> Software (Sterling Heights, MI, USA), and evaluated in Microsoft Photo Editor<sup>™</sup> (Redmond, WA, USA).

### Oral Bromelain administration

A stock solution of stem bromelain (EC 3.4.22.32), Lot # 1965 (Vital Nutrients, Middletown, CT, USA) was made using 60 mg of bromelain dissolved in 250 ml of PBS. Bromelain (200 mg kg<sup>-1</sup>) in 0.5 ml of PBS was prepared from the stock solution. Each animal received either bromelain or saline via gavage using a 22 gauge<sup>-1</sup> inch straight stainless steel feeding needle with a 1.25 mm ball tip (Braintree Scientific inc., Braintree, MA, USA), twice a day. Bromelain or saline treatments were administered 6–8 h apart, beginning 1 day prior to aerosolization of OVA. The dosages used were based on *in vivo* dose response studies performed in our laboratory (23). Bromelain was independently tested for authenticity, potency (2400–2660 GDU g<sup>-1</sup>), microbial contamination, residual solvents, heavy metals and aflatoxins (Vital Nutrients, Middletown, CT; ChromaDex, Clearwater, FL and Pharmline, Florida, NY, USA). Ananas comosus (common pineapple) was identified by the raw material provider (Indonesia) using organoleptic techniques and bromelain identity was confirmed with an Fourier transform infrared spectrometer (FTIR; Pharmline, Florida, NY, USA).

### Statistical Analysis

Statistical comparisons between groups were made with analysis of variance and unpaired *t*-tests using StatView 4.5 (Abacus Concepts, Inc., Berkeley, CA, USA) and JMP<sup>®</sup> Software (SAS Institute Inc., Cary, NC, USA). Dose-response data and cytokine levels were compared by repeated measures analysis of variance. Changes in Penh dose-response relationships were compared within and between groups by repeated measures analysis of variance. Changes in Penh-2 values before and after aerosol exposure within groups were made by paired *t*-tests and were compared between the control and bromelain-treated groups by repeated measures analysis of variance. All data were expressed as means ± standard

error of the mean, and differences were considered significant at  $P \leq 0.05$ .

## Results

### Treatment Protocol

Each animal received either bromelain ( $200 \text{ mg kg}^{-1}$ ) in  $0.5 \text{ ml}$  of PBS or  $0.5 \text{ ml}$  PBS alone via gavage (orally) twice daily beginning 6 days after the third OVA–Alum sensitization injection (Fig. 1). The treatments were administered 6–8 h apart, and all animals were sacrificed  $\sim 12 \text{ h}$  after the last treatment and the primary AAD outcomes collected and assessed.

### Analytical Testing and Quality Control

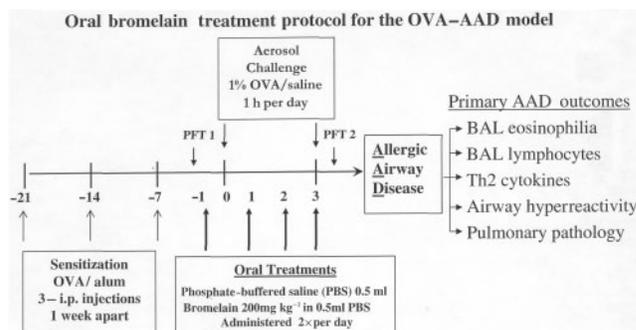
Bromelain was extracted from mature pineapple stems with methanol and bound with  $\leq 1\%$  rice starch. Upon importation, the certificate of analysis of bromelain was independently verified for its purity ( $\geq 99\%$ ), identity (via FTIR spectrometer) and potency (with a GDU assay) (Table 1). Further analyses for quality and contamination were performed for aflatoxins, heavy metals, chemical solvents pesticides, herbicides and microbial contaminants. All samples were stored at  $4^\circ\text{C}$  in opaque containers and source samples were stored for future analysis of stability and degradation.

### Decreased Pulmonary Eosinophilia Following Oral Bromelain Administration

Total BAL eosinophils were significantly elevated in AAD mice ( $167 \pm 33 \times 10^4$  cells;  $P \leq 0.0001$ ) as compared with naïve ( $1 \pm 2 \times 10^4$  cells) or naïve oral bromelain-treated ( $4 \pm 2 \times 10^4$  cells) mice (Fig. 2A). There was a significant reduction in total BAL eosinophils with bromelain treatment of AAD mice as compared with saline-treated AAD mice ( $P \leq 0.02$ ; Fig. 2A). Similar trends were noted on evaluation of BAL samples with flow cytometry (Fig. 2B). A representative FACS plot showing gated eosinophils on forward and side scatter from naïve saline-treated (upper left), naïve Bromelain-treated (upper right), AAD saline-treated (lower left), and AAD Bromelain-treated mice (lower right) are shown.

### Bromelain Modulates Lung Lymphocytes during Acute Asthma

As compared with naïve and naïve bromelain-treated mice, AAD mice displayed significant increases in the percentage of BAL CD4+, CD8+ and CD19+ lymphocytes (Fig. 3). Bromelain-treated AAD mice resulted in significant reductions in CD8+ ( $19 \pm 1$  versus  $9 \pm 2$ ;



**Figure 1.** Bromelain treatment protocol. Each animal was sensitized with 3 OVA–Alum i.p. injections 1 week apart ( $-21$  days,  $-14$  days,  $-7$  days). Six days after the third injection ( $-1$  day) each animal received either Bromelain ( $200 \text{ mg/kg}$ ) in  $0.5 \text{ ml}$  of phosphate buffered saline (PBS) or  $0.5 \text{ ml}$  PBS alone via gavage (orally). Treatment was administered twice daily (6–8 h apart) for 4 consecutive days. Animals were challenged with  $1\%$  OVA in saline for  $1 \text{ h}$  per day ( $0$ – $3$ ). All animals were sacrificed  $12 \text{ h}$  after the last treatment (day 3) and the primary AAD outcomes assessed.

$P \leq 0.002$ ), and CD19+ lymphocytes ( $2.4 \pm 0.3$  versus  $2.4 \pm 0.2$ ;  $P \leq 0.002$ ) when compared with AAD–saline-treated mice. No significant changes in CD4+ lymphocytes were noted in similar comparisons.

### Reduced IL-13 Levels in the BAL of Bromelain-treated Mice

A significant decrease in IL-13 concentration was observed in bromelain-treated AAD mice as compared with the saline treated AAD mice ( $41 \pm 12$  versus  $86 \pm 18 \text{ pg/ml}$ ; Fig. 4). There were no significant changes in BAL IL-5 concentrations with oral bromelain treatment of AAD mice as compared with saline treated AAD mice ( $20 \pm 4$  versus  $24 \pm 2 \text{ pg/ml}$ ).

### Shifts in Airway Responsiveness Following Bromelain Treatment

Airway responses to methacholine were assessed by whole-body barometric plethysmography using the Buxco system (Sharon, CT, USA). At baseline, before bromelain or saline treatment (open circles), both groups of animals had similar overall responsiveness (Fig. 5A;  $P = 0.50$ ) and sensitivity (Fig. 5B) to methacholine (Penh-2 values  $48 \pm 13 \text{ mg/ml}$  in the bromelain treatment versus  $64 \pm 15 \text{ mg/ml}$  in saline treatment group;  $P = 0.47$ ). At AAD (filled circles), saline-treated mice tended to be more generally responsive to methacholine ( $P = 0.069$ ; A) and were twice as sensitive to methacholine ( $P = 0.01$ ; B). No change occurred in bromelain-treated 3-day OVA mice terms of overall responsiveness ( $P = 0.52$ ; A) or methacholine sensitivity ( $P = 0.45$ ; B).

**Table 1.** Bromelain Identity and Quality Control Data

Item/test profile	Bromelain quality control and analytical testing data		
	Specification	Result	Method
<b>Botanical—Pineapple</b>	<i>Ananas cosmosus</i>	<i>Ananas cosmosus</i>	Visual
<b>Plant parts used</b>	Mature stem	Mature stem	Visual
<b>Botanical extract</b>	Bromelain (EC 3.4.22.31)	Bromelain (EC 3.4.22.31)	Methanol extraction
<b>Identification</b>	Standard match (Sigma)	Conforms to standard	FTIR, HPLC
<b>Activity, potency</b>	≥2400 GDU/g	2674 GDU/g	GDU assay
<b>Solvent residues</b>			
Methanol	≤3000 ppm	Negative	Gas chromatography
Ethanol	≤5000 ppm	Negative	Gas chromatography
Acetone	≤5000 ppm	Negative	Gas chromatography
Isopropanol	≤5000 ppm	Negative	Gas chromatography
Methylene chloride	≤600 ppm	Negative	Gas chromatography
Hexane	≤290 ppm	Negative	Gas chromatography
Ethyl acetate	≤5000 ppm	Negative	Gas chromatography
<b>Microbial profile</b>	Total plate count ≤3000/g	≤50/g	
Mold	≤300/g	≤50/g	STVCA
<i>E. Coli</i>	Negative	Negative	STVCA
<i>Salmonella</i>	Negative	Negative	STVCA
Coliforms	≤10/g	Negative	USP24p. 1814
<i>S. aureus</i>	Negative	Negative	USP24p. 1814
<i>P. aeruginosa</i>	Negative	Negative	USP24p. 1814
<b>Aflatoxin profile (G2, G1, B2, B1)</b>	Aflatoxins ≤20 ppb	≤1ppb	HPLC
<b>Heavy metal profile</b>			
Aluminum	≤1000 ppm	16 ± 0.5 ppm	ICP-MS
Arsenic	≤3ppm	≤0.50 ± 0.5 ppm	ICP-MS
Lead	≤10 ppm	0.07 ± 0.05 ppm	ICP-MS
Cadmium	≤3 ppm	≤0.25 + 0.25 ppm	ICP-MS
Mercury	≤2 ppm	≤0.10 + 0.1 ppm	ICP-MS
<b>Pesticide residues</b>			CDFA
Organochlorines	≤5 ppm	Negative	Gas and liquid
Organophosphates	≤7 ppm	Negative	chromatography
Organonitrates	≤5 ppm	Negative	coupled with mass
Carbamates	≤5 ppm	Negative	spectrometry

EC = enzyme classification; FTIR = Fourier transform infrared spectroscopy; HPLC = high-performance liquid chromatography; GDUs = gelatin dissolving units; STVCA = Scleris' total viable count assay; ICP-MS = inductively coupled plasma – mass spectrometry; CDFA = California Department of Food and Agriculture.

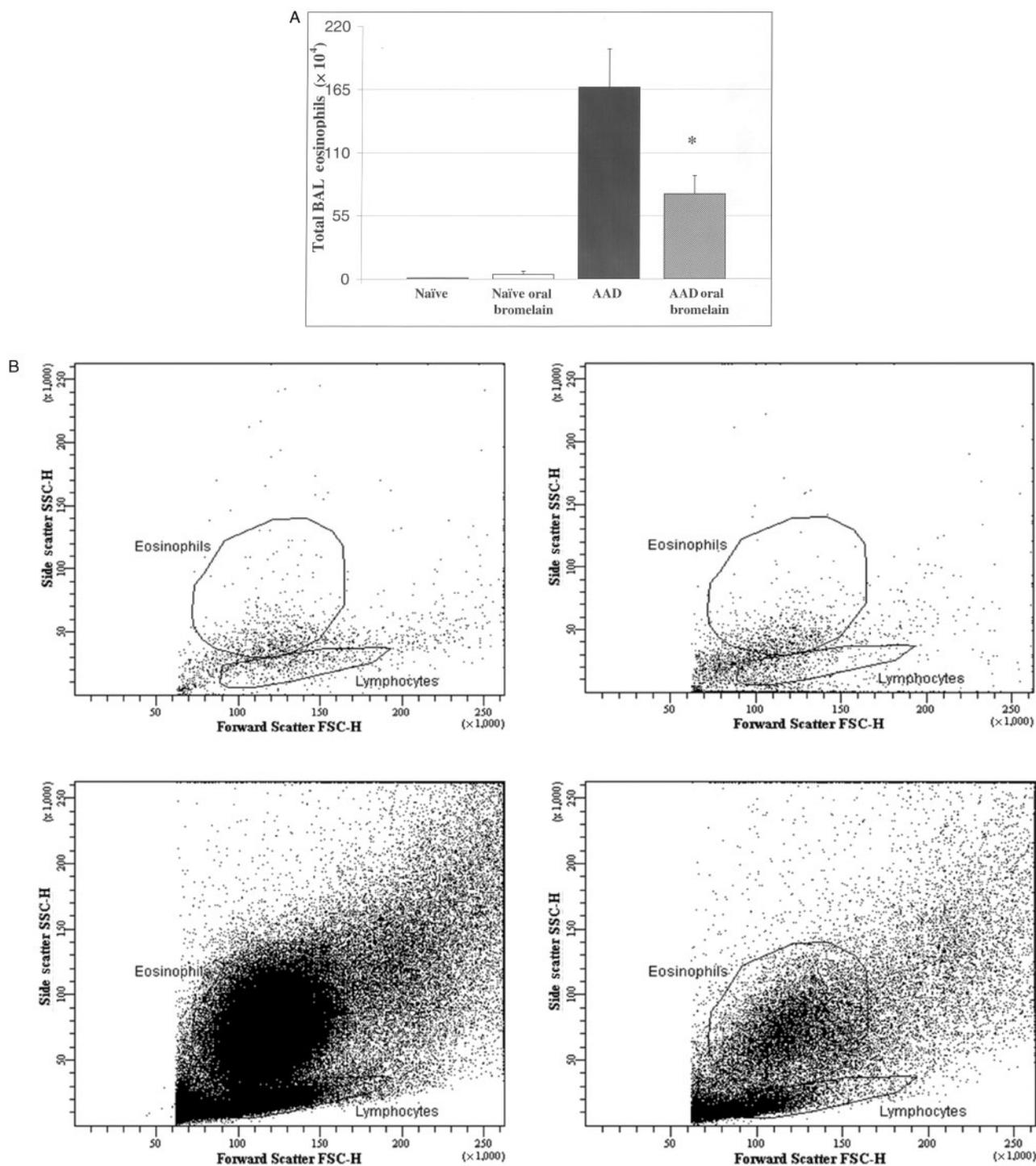
### The Effect of Oral Bromelain Treatment on Lung Pathology

Histological evaluations and Pathology Scores (PS), were performed on unmanipulated, uninflated formalin-fixed lungs from separate groups ( $n=4$ ) of naïve, naïve bromelain-treated, AAD saline- and AAD bromelain-treated mice stained with hematoxylin and eosin (H & E), Mallory's trichrome and periodic acid-schiff. The H & E stain has been included for comparison (Fig. 6). As demonstrated in the top panels, no evidence of histological injury was noted in naïve animals (A) or oral bromelain-treated naïve animals (B). Three day AAD mice develop mild pathologic changes, characterized by

perivascular and peribronchial (arrows) inflammation which includes cellular infiltrates (lymphocytes, plasma cells and eosinophils). These changes are present in the 3-day AAD saline-treated mice (C). Oral bromelain-treated AAD mice (D) appeared to have minimal histological injury. However, upon statistical comparisons of pathological scoring by four reviewers, there were no statistical changes in pathological scoring.

### Discussion

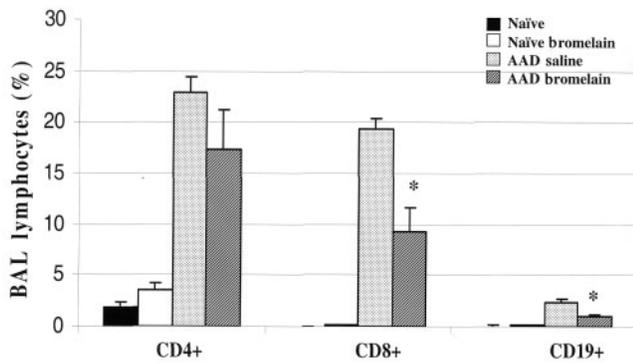
The current study is a continued characterization of the use of bromelain, a cysteine protease extracted from



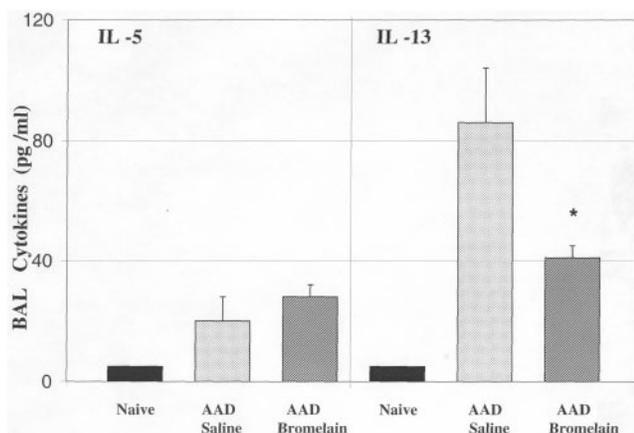
**Figure 2.** The effect of oral Bromelain treatment on BAL eosinophils. Total BAL eosinophils were significantly elevated in AAD mice as compared to naïve or naïve oral Bromelain-treated mice ( $P \leq 0.0001$ ; A). There was a significant reduction in total BAL eosinophils with Bromelain treatment of AAD mice as compared to saline treated AAD mice. Similar trends were noted on evaluation of BAL samples with Flow cytometry (B). A representative FACS plot showing gated eosinophils on forward and side scatter from; naïve-saline treated (upper left), naïve-Bromelain treated (upper right), AAD-saline treated mice (lower left), and AAD-Bromelain treated mice (lower right) are shown. Statistical comparisons were made by un-paired t-test,  $*P \leq 0.02$ ; Data represent means  $\pm$  SEM,  $n = 8$  animals per group.

pineapple, in asthma. We have previously shown that the i.p. administration of bromelain exerts anti-inflammatory effects in an OVA-induced murine model of AAD (23). The present study was designed to determine the efficacy

of oral bromelain treatment in a murine model of asthma. The data presented in this study demonstrate that oral bromelain therapy can attenuate inflammation in a well-characterized murine model of asthma (23–25)



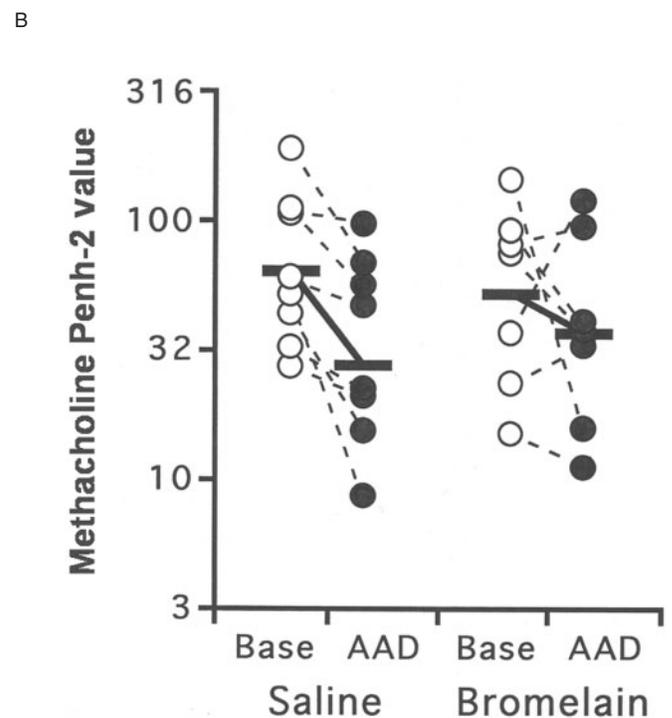
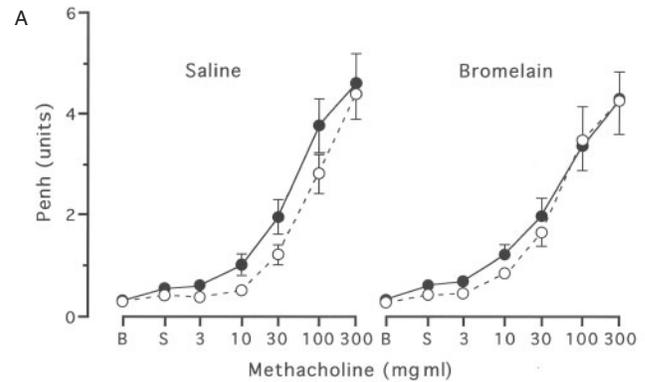
**Figure 3.** The effect of oral Bromelain treatment on BAL Lymphocytes. After 3 OVA aerosol exposures, there was a significant increase in the percentages of CD19+ B cells (A) CD8+ T cells (B) and CD4+ T cells (C) in the BAL of AAD mice, as compared to naïve controls. Oral Bromelain treatment significantly reduced CD19+ B cells ( $P \leq 0.00003$ ; A), and CD8+ T cells ( $P \leq 0.00002$ ; B). There was no change in the percentages of CD4+ T cells in the BAL between AAD and Bromelain treated AAD mice ( $P \leq 0.095$ ; C). Statistical comparisons were made by un-paired t-test, \* $P \leq 0.0001$ ; Data represent means + SEM,  $n = 8$  animals per group.



**Figure 4.** The effect of oral Bromelain treatment on IL-5 and IL-13 concentrations in BAL. There were no significant changes in BAL IL-5 concentrations with oral Bromelain treatment of AAD mice as compared to saline treated AAD mice. A significant decrease in IL-13 concentration was observed in oral Bromelain treated AAD mice as compared to the saline treated AAD mice. Statistical comparisons were made by un-paired t-test, \* $P \leq 0.04$ ; Data represent means  $\pm$  SEM,  $n = 8$  animals per group.

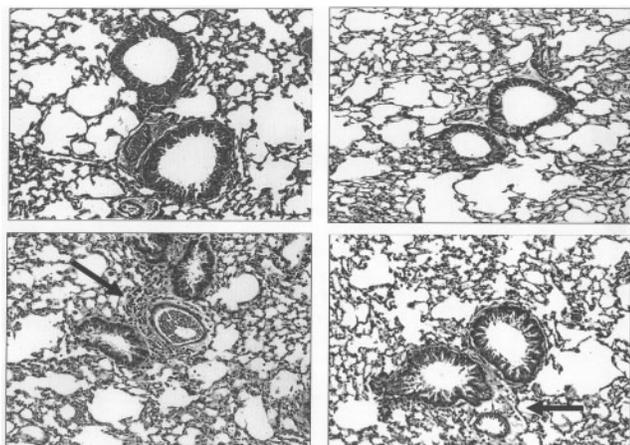
and support the existing literature that oral enzymes are bioavailable and efficacious for the treatment of inflammatory conditions (14,26–30).

The quality control and analytical verification of botanical products such as bromelain remain a concern as the use of these products continues to increase (6,31–33). Many botanicals are now undergoing intensive testing to ensure safety and quality for consumers and reproducibility for basic science and clinical research (34–36). The bromelain utilized for these experiments was obtained from a professional



**Figure 5.** The effect of oral Bromelain treatment on airway reactivity and methacholine sensitivity. At baseline (open circles), Bromelain-treated and saline-treated mice had similar reactivity ( $P = 0.50$ ) and sensitivity to methacholine (p2 values  $48 \pm 13$  mg/ml in Bromelain treated versus  $64 \pm 15$  mg/ml in saline treated;  $P = 0.47$ ). After 3 OVA aerosol exposures (filled circles), saline treated mice tended to be more reactive to methacholine ( $P = 0.069$ ; A) and were twice as sensitive to methacholine ( $P = 0.01$ ; B) whereas no change occurred in Bromelain treated mice in reactivity ( $P = 0.52$ ; A) and sensitivity ( $P = 0.45$ ; B). Comparisons were made by paired t-test and repeated measures ANOVA;  $n = 7-8$  animals per group.

natural product manufacturing company that produces products for clinical use. The bromelain extract was independently evaluated for purity, potency and contamination to ensure quality and reproducibility (Table 1). There were no solvent residues, microbial contamination, or greater than acceptable heavy metals or pesticide residues. There was no observed toxicity of oral bromelain treatment in either naïve or AAD animals



**Figure 6.** The effect of oral Bromelain treatment on lung pathology. **Top panels:** No evidence of histological injury was noted in naïve animals (A) or oral Bromelain treated naïve animals (B). **Lower panels:** As previously described, 3 day AAD mice develop pathological changes characterized by perivascular and peribronchiolar infiltration of lymphocytes, eosinophils and plasma cells (C), which was reduced in oral Bromelain treated AAD mice (D).

(as assessed by body weight, BAL protein concentrations and lung pathology).

The presence of increased BAL eosinophils is a hallmark of asthma. Compared with saline-treated AAD mice, bromelain-treated AAD mice had significantly reduced lung eosinophilia as observed by BAL differential and flow cytometry (Fig. 2). This observed effect of bromelain treatment on eosinophils may be due to a reduction in lymphocyte populations or a decrease in the Th2 cytokines that are responsible for the recruitment of eosinophils into the lung during asthma. Therefore, we examined the effect of oral bromelain treatment on Th2 cytokines (IL-5, IL-13) and lymphocyte subsets (CD4+, CD8+ and CD19+). Bromelain treatment reduced the percentages of CD8+ T cells and CD19+ B cells in the BAL (Fig. 3) and BAL IL-13 concentrations (Fig. 4). CD19+ B cells can act as primary antigen presenting cells in Th2 conditions such as asthma. A bromelain-mediated reduction in this subset may also diminish T cell driven inflammation thereby inhibiting the progression of allergic disease. Though, primarily a CD4+ T cell mediated condition, CD8+ T cells which secrete Th2 cytokines, have also been implicated in asthma (37). A reduction in these CD8+ T cells via bromelain may have additional protective effects by modulating lymphocyte-mediated cytotoxic asthma mechanisms such as granzymes or perforins. The effects of bromelain on the lymphocyte subpopulations were observed locally as represented by the BAL and not systemically (in peripheral blood or spleen, data not shown) suggesting that bromelain activity is focused at sites of inflammation.

Interestingly, the percentages of CD4+ T cells were not significantly reduced. This may be due to bromelain

altering the ratio of CD4+ T cell subsets, mainly the effector cells (CD4+CD25–Foxp3–) which cause airway inflammation, to regulatory cells (CD4+CD25+Foxp3+) which suppress it. Bromelain may enhance regulatory cell numbers and their function leading to the resolution of disease. The characterization of the effects of bromelain on these regulatory T cells is currently being investigated in our laboratory.

In addition, we examined the effects of bromelain treatment in asthma on both pulmonary function (PFT) and pathology. As a measure of pulmonary function, both saline and bromelain treatment groups were challenged to increasing doses (0–300 mg/ml) of methacholine, a common lung smooth muscle irritant, for assessment of airway reactivity and sensitivity (Fig. 5). During the baseline challenge, there was no difference between the groups. After OVA aerosols, the saline-treated group became more sensitive to methacholine whereas the bromelain group did not vary from baseline. This protective effect on lung function correlates well with the observed reductions in eosinophils, lymphocytes and cytokines that have been implicated in the pathogenesis of airway reactivity (38–41). In regards to lung pathology (Fig. 6), bromelain-treated mice appeared to qualitatively have reduced pulmonary injury as compared with saline-treated controls; however, independent blinded scoring did not reveal significant differences between the groups.

Future studies will evaluate the effects of bromelain in a 7–10 day AAD model with more advanced disease and bromelain administration will be varied after the onset of asthma (3 days of aerosol) to better simulate a clinical presentation and given during or before OVA sensitization to evaluate its effect on the priming response. Experiments are currently being designed to evaluate the effect of bromelain treatment on key immunoregulatory CD4+ T cell subsets such as Foxp3+ T regulatory cells which are known to modulate allergic responses (42,43) and to measure the retained enzymatic activity of bromelain in the serum of naïve and AAD mice after oral administration. In conclusion, the results obtained from this study suggest that the oral administration of bromelain attenuates inflammation in an OVA-induced murine model of asthma. These results may translate well in clinical trials.

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