Proteinase activity and stability of natural bromelain preparations

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Abstract

Bromelain is a complex mixture of proteinases typically derived from pineapple stem. Similar proteinases are also present in pineapple fruit. Beneficial therapeutic effects of bromelain have been suggested or proven in several human inflammatory diseases and animal models of inflammation, including arthritis and inflammatory bowel disease. However, it is not clear how each of the proteinases within bromelain contributes to its anti-inflammatory effects in vivo. Previous in vivo studies using bromelain have been limited by the lack of assays to control for potential differences in the composition and proteolytic activity of this naturally derived proteinase mixture. In this study, we present model substrate assays and assays for cleavage of bromelain-sensitive cell surface molecules can be used to assess the activity of constituent proteinases within bromelain without the need for biochemical separation of individual components. Commercially available chemical and nutraceutical preparations of bromelain contain predominately stem bromelain. In contrast, the proteinase activity of pineapple fruit reflects its composition of fruit bromelain>ananain~stem bromelain. Concentrated bromelain solutions (>50 mg/ml) are more resistant to spontaneous inactivation of their proteolytic activity than are dilute solutions, with the proteinase stability in the order of stem bromelain>fruit bromelain~ananain. The proteolytic activity of concentrated bromelain solutions remains relatively stable for at least 1 week at room temperature, with minimal inactivation by multiple freeze–thaw cycles or exposure to the digestive enzyme trypsin. The relative stability of concentrated versus dilute bromelain solutions to inactivation under physiologically relevant conditions suggests that delivery of bromelain as a concentrated bolus would be the preferred method to maximize its proteolytic activity in vivo.
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1. Introduction

Bromelain is a mixture of proteolytic enzymes that is derived from the stem of the pineapple plant, *Ananas comosus*. We and others have previously shown that bromelain proteolytically removes certain cell surface molecules that affect lymphocyte migration and activation [1–4]. In addition, bromelain treatment markedly affects the production of cytokines and inflammatory mediators by isolated leukocytes or colon epithelial cells in vitro [5,6]. These effects require that the bromelain be proteolytically active [1,5].

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Bromelain or proteinase mixtures containing bromelain have been suggested or proven to have anti-inflammatory effects in several different animal models of inflammation and human inflammatory diseases. These include carrageenan-induced pleurisy in the rat [7–9], immunologically mediated arteriosclerosis in rat aortic allografts [10], the experimental allergic encephalomyelitis (EAE) murine model for the human autimmune disease multiple sclerosis [11], collagen- or adjuvant-induced arthritis [12,13], IgE-mediated perennial allergic rhinitis [14], and some human rheumatologic diseases [15–17]. Oral bromelain was also anecdotally reported to induce clinical and endoscopic remission of ulcerative colitis in two patients whose disease was refractory to multi-agent conventional medical therapy [18]. Our studies thus far show that treatment with oral bromelain decreases the development of spontaneous colitis and also decreases the severity of established colitis in the IL-10 knockout model of murine inflammatory bowel disease (L.P. Hale et al., manuscript in preparation). Proteolytic activity is required for the anti-inflammatory effect of oral bromelain in this model.

Bromelain is only poorly absorbed when administered orally, generating plasma levels of less than 10 ng/ml in humans given 4 g/day [19]. We previously showed that, although 1 μg/ml is sufficient to remove at least 50% of bromelain-sensitive molecules from cells in the absence of plasma, concentrations of ≥500 μg/ml bromelain are required when cells are present in whole blood [2]. This is primarily due to inhibition of bromelain by the plasma proteinase inhibitor alpha-2-macroglobulin [2]. Taken together, these studies show that the concentration of bromelain achieved systemically following large oral doses is many orders of magnitude less that what is required to significantly affect cell surface bromelain-sensitive molecules. In contrast, we recently showed [20] that the concentration of proteolytically active bromelain in stool after oral administration was sufficient to remove bromelain-sensitive molecules from the surface of colon epithelial cells. Local proteolytic activity of bromelain has also been demonstrated in small intestine of pigs following oral administration [21,22]. Thus we feel that anti-inflammatory effects of oral bromelain in IBD are more likely due to local proteolytic activity within the intestinal lumen or lamina propria, rather than systemic activity.

Bromelain contains several distinct cysteine proteinases that have similar but distinct amino acid sequences, as well as differences in proteolytic specificity and sensitivity to inactivation. However, it is not clear how the different proteinases within bromelain contribute to its anti-inflammatory activity in vivo. Studies to test the potential efficacy of bromelain in clinical trials in animals or humans have been limited by the lack of assays to control for differences in the composition and proteolytic activity of naturally derived bromelain preparations. Stem bromelain (EC 3.4.22.32, formerly EC 3.4.22.4) is the most abundant proteinase within bromelain preparations derived from pineapple stem. Other proteinases that are present at lesser amounts include fruit bromelain (the major proteinase present in pineapple fruit; EC3.4.22.33, formerly EC 3.4.22.4 and 3.4.22.5) and ananain (EC 3.4.22.31). Several model peptide substrates of the form B–(P3)–P2–P1-indicator (where B=blocking group such as Z=benzyloxycarbonyl or Bz=benzoyl, and P1, P2, and P3 represent specific amino acids) have been used to characterize the proteolytic activity of purified bromelain enzymes. Amidolytic cleavage of the substrate results in release of free indicator that can be detected either fluorescently or colorimetrically. Stem bromelain preferentially cleaves the Z-Arg-Arg model substrate, whereas fruit bromelain and ananain show minimal activity against this substrate. In contrast, fruit bromelain and ananain but not stem bromelain efficiently cleave the Bz-Phe-Val-Arg substrate [23,24]. These enzymes also differ in their susceptibility to inactivation. Ananain is reported to be rapidly inactivated by the chicken egg white proteinase inhibitor cystatin and by the suicide substrate, E-64 (*trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane), but these inhibitors either very slowly or only minimally inactivate stem and fruit bromelain [23].

Development of assays that can assess its proteinase composition and enzyme activity are needed to facilitate the standardization of bromelain as a therapeutic product. The purpose of this study was to develop criteria for standardizing the content and activity of bromelain preparations and to determine the stability of their proteolytic activity under a variety of physiologically and therapeutically relevant conditions.

2. Methods

2.1. Reagents

Bromelain (catalog #B-4882) obtained from Sigma-Aldrich (St. Louis, MO) was used as the standard for these studies. Nutraceutical preparations of bromelain were obtained from GNC (Pittsburgh, PA), Natural Organics Laboratories (Amityville, NY), and Country Life (Hauppauge, NY). Unless specified, all other reagents were obtained from Sigma-Aldrich or Invitrogen (Carlsbad, CA).

2.2. Purification of bromelain proteinases

Bromelains were obtained from pineapple fruit by grinding fresh pineapple fruit with a mortar and pestle then centrifuging (4 °C, 10 min) at full speed in a microcentifuge. The supernatant was used without further fractionation. The bicinchoninic acid (BCA) assay (Pierce Chemical, Rockford, IL) was used to measure protein concentration.

The major component proteinases within bromelain were separated by cationic exchange chromatography, using a modification of previously published methods [23–27]. A solution of 30 mg/ml bromelain (Sigma-Aldrich) in 20 mM acetate buffer, pH 5.0, containing 0.1 mM EDTA (acetate buffer) was centrifuged at $13,000 \times g$ for 10 min to remove insoluble material, then loaded onto a 25 ml Fast flow SP-Sepharose column that had been pre-equilibrated with acetate buffer. Unbound protein was removed by extensive washing with acetate buffer. Bound proteins were eluted with a linear gradient of 0 to 1 M NaCl in acetate buffer. Fractions were collected and protein content and activity against model substrates (see below) were used to identify proteinase peaks. Fractions were pooled into 3 pools, dialyzed against PBS, concentrated, and further characterized as outlined below.

2.3. Assessment of proteolytic activity using model substrate assays

Bromelain proteolytic activity was measured in a microplate assay format (modified from 24) using the model peptide substrates, Z-Arg-Arg-*p*-nitroaniline (pNA), Bz-Phe-Val-Arg-pNA, and in some cases Z-Phe-Arg-pNA (Bachem, King of Prussia, PA), at 250 µg/ml final concentration. Cleavage of the substrate results in free pNA that was detected colorimetrically at 405–410 nm. Assays were performed in buffer containing 10% dimethylformamide, 5 mM cysteine, 5 mM EDTA, and 0.1 M HEPES (pH 7.3). Absorbance was measured at 30 s intervals for 20 min using a SpectraMax 250® plate reader and SoftMax Pro® software (both from 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. Where indicated, bromelain activity was converted to units of nmoles pNA produced/L min mg protein, using a molar absorptivity of 8480 M^{-1} cm^{-1} for pNA [24]. Protein concentration was measured using the BCA assay.

2.4. Assessment of proteolytic activity toward cell surface molecules

The CEM human T lymphocyte and the Raji B lymphocyte cell lines were obtained from the American Type Culture Collection (Manassas, VA) and grown at 37 °C in a humidified atmosphere containing 5% CO_2. CEM cells were grown in RPMI 1640 (4.5 g glucose/L+1 mM sodium pyruvate)+20% fetal bovine serum. Raji cells were similarly grown in RPMI 1640 with high glucose and pyruvate, however the fetal bovine serum was reduced to 10% and 10 mM HEPES was added. Cells were incubated with 100–1000 µg/ml bromelain or purified proteinase in RPMI 1640 at 37 °C for 30–60 min, washed, then incubated with fluorescently labeled antibodies (Pharmingen/BD Biosciences, San Diego, CA or Beckman Coulter, Brea, CA). Flow cytometric analysis was performed by the Flow Cytometry Facility of the Human Vaccine Institute at Duke University. The activity of bromelain was defined by the % marker removed (defined as [1 minus the ratio of the mean fluorescence intensity (MFI) of treated cells to the MFI of sham-treated cells]×100%). CD4, CD8, CD21, CD44, CD45RA, CD62L and CD80 are bromelain-sensitive, while CD3, CD20, and MHC Class I molecules are not affected by bromelain treatment and serve as controls [1–4,11].

2.5. Assays for bromelain stability

Solutions containing either 10 or 100 mg/ml bromelain in water, PBS, or 100 mg/ml NaHCO$_3$ were incubated with 1 mg/ml trypsin for 1 h at 37 °C, then residual bromelain activity was measured using model substrate assays. Soybean trypsin inhibitor was added at 1 mg/ml to inactivate trypsin. Controls showed that soybean trypsin inhibitor has no effect on bromelain activity. To test stability of bromelain to heat, aliquots of 10 to 125 mg/ml bromelain in water (~0.4 to 5 mM) were incubated at temperatures from 60 to 100 °C for 1 to 30 min. A small amount of precipitation that occurred when solutions were heated to >70 °C was removed by centrifugation. The BCA assay was used to determine the amount of protein remaining in solution and proteolytic activity against model substrates was corrected to reflect activity that remained per mg of recovered protein. For studies of chemical inactivation, 250 mg/ml bromelain in water (~10 mM) was incubated for 30 min at 37 °C with 100 mM dithrithreitol (DTT). Iodoacetamide was added to a concentration of 1 M and incubated further for 60–70 min at 37 °C. Precipitates were removed by centrifugation and the supernatant was dialyzed against water. The proteolytic activity of the recovered protein was determined using model substrates as described above.

3. Results

3.1. Purification and characterization of bromelain proteinases

Three major peaks (the largest one a doublet) were identified within bromelain using a combination of protein assays and enzymatic activity against model substrates (Fig. 1A). Fractions were then combined as shown to create 3 pools that were further analyzed for proteolytic activity using model peptide substrates and a panel of biologically relevant cell surface molecules. Western blotting with a rabbit polyclonal antiserum raised against the bromelain mixture showed that the 3 pools gave distinct and characteristic band patterns (Fig. 1B).

Pools 1 and 3 primarily cleaved the Bz-Phe-Val-Arg-pNA substrate, with minimal activity toward Z-
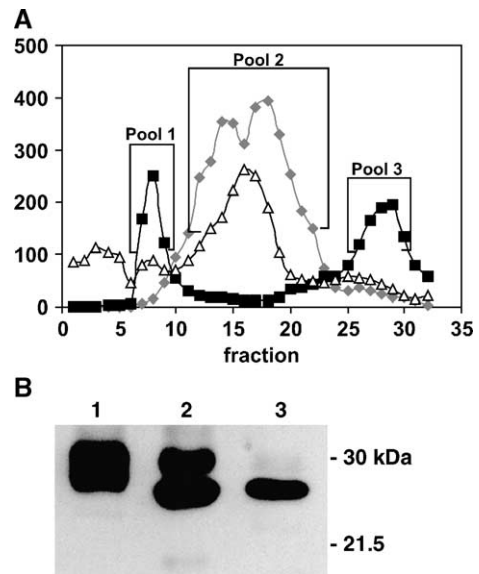

Fig. 1. Purification of bromelain component proteinases. A. Protein concentration and activity toward model peptide substrates are shown for FPLC fractions obtained as described in the text. Black squares show activity toward Bz-Phe-Val-Arg-pNA and gray diamonds show activity toward the Z-Arg-Arg-pNA substrate, in units of (ΔA_{410}/min/μg/ml protein)×100. The open triangles show the protein concentration of each fraction, in units of μg/ml. The brackets indicate the fractions used to make up Pools 1–3. B. Bromelain proteinase pools were separated on non-reducing 15% SDS-PAGE gels, transferred to nitrocellulose, and reacted with rabbit polyclonal antiserum raised against the bromelain mixture. Although the molecular weights of the bromelain component proteinases are in a similar range, the Western blot band pattern for each pool is unique.

Arg-Arg-pNA. Pool 2 had the opposite pattern, with strong activity against Z-Arg-Arg-pNA, and minimal activity against Bz-Phe-Val-Arg-pNA (Fig. 2A). A comparison of these results with published characteristics of these enzymes indicates that Pool 1 contains fruit bromelain, Pool 2 stem bromelain, and Pool 3 ananain [23].

Although Pools 1 and 3 had similar activities against the Bz-Phe-Val-Arg-pNA model substrate, these pools demonstrated differential activity towards cell surface molecules that are susceptible to cleavage by bromelain (Fig. 2B) [1,2]. Pool 1 proteolytically removed CD44, CD8, and CD45RA molecules, but had minimal effect on the other markers tested. Pool 3 additionally removed CD62L, CD21, and CD80 cell surface molecules. Despite their marked differences in cleavage of the model substrates tested, Pools 2 and 3
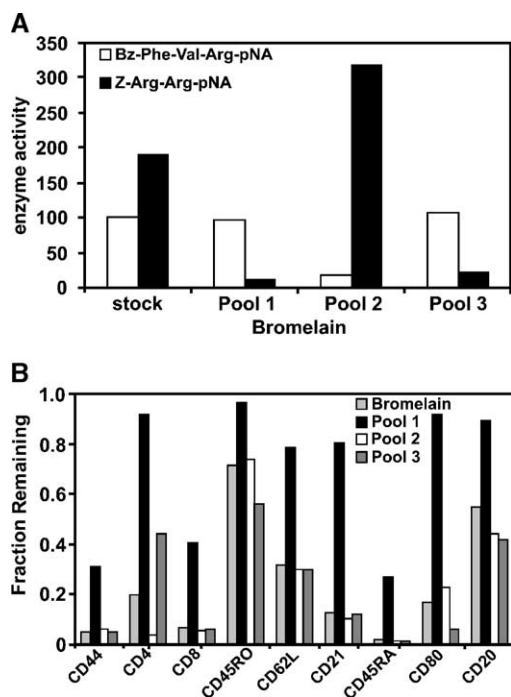
Fig. 2. Characterization of bromelain component proteinases. A. The proteinase activity of the pooled fractions (same units as Fig. 1) is shown against the indicated model substrates. Based on comparison with published data [24], Pool 1=fruit bromelain, Pool 2=stem bromelain, and Pool 3=ananain. Data is from a single experiment representative of >5 performed. B. CEM and Raji cells were treated with 200 μg/ml of natural bromelain or purified bromelain proteinase pools (1 h, 37 °C). The fraction of cell surface molecules remaining after treatment was determined using mAbs and flow cytometry and compared to that of media-treated cells. The three proteinase pools have differential activity toward these biologically relevant cell surface molecules and the activity of natural bromelain reflects its relative content of these proteinases.

had a similar spectrum of activity toward most of the cell surface molecules. Differences included the greater activity of Pool 2 toward CD4 and the greater activity of Pool 3 toward CD80. Clearly, the proteolytic specificity of bromelain proteinases can differ markedly toward biologically relevant molecules despite having similar specificity toward model substrates.

3.2. Characterization of proteinase activity within bromelain using model substrates

Model substrates have previously been used to determine the proteolytic specificity of individual proteinases purified from bromelain mixtures. Comparison of published K_m and k_{cat} values obtained using fluorescent peptide substrates showed that ananain and fruit bromelain had at least a 50-fold increased rate of cleavage of Bz-Phe-Val-Arg substrate and a >3000-fold decreased rate of cleavage for Z-Arg-Arg substrate compared to stem bromelain [23,24]. The activity of ananain could also be differentiated from that of fruit bromelain by its cleavage of the Z-Phe-Arg substrate. We determined whether these large differences in substrate specificity and rate of cleavage would allow the activity of individual proteinases within natural bromelain mixtures to be functionally characterized without the need for biochemical separation. The protein content of bromelain obtained from 4 different commercial sources varied as much as 10-fold (Fig. 3A). Analysis of these bromelain preparations using equivalent amounts of protein (Fig. 3B) also showed wide variations in proteolytic activity against model substrates. All 4 preparations had specific activity (activity/mg protein) against the Z-Arg-Arg-pNA substrate that was typically 1.5- to 2-fold higher than specific activity against the Bz-Phe-Val-Arg-pNA substrate. Activity against the Z-Phe-Arg-pNA substrate was limited by substrate solubility and by the low prevalence of ananain, the proteinase that specifically cleaves this substrate, in bromelain mixtures derived from pineapple stem. Although wide variations in absolute activity due to protein content were observed, the consistency of the relative activity of the different commercial bromelain preparations toward the 3 model substrates suggests that the ratios of the component proteinases that cleave these substrates are similar in the 4 preparations tested. Thus the choice of a specific source for use in clinical trials may be based on total content of active proteinases in the available preparations.

In contrast to the predominance of activity against the Z-Arg-Arg-pNA substrate that was seen with commercial bromelain preparations obtained from pineapple stem, fresh pineapple fruit predominantly contained activity against the Bz-Phe-Val-Arg-pNA substrate (Fig. 3C). Fruit bromelain was previously determined to be the major proteinase present in pineapple fruit [28] and our results are consistent with this. However, some activity was observed against the ananain-specific Z-Phe-Arg-pNA substrate, suggest-
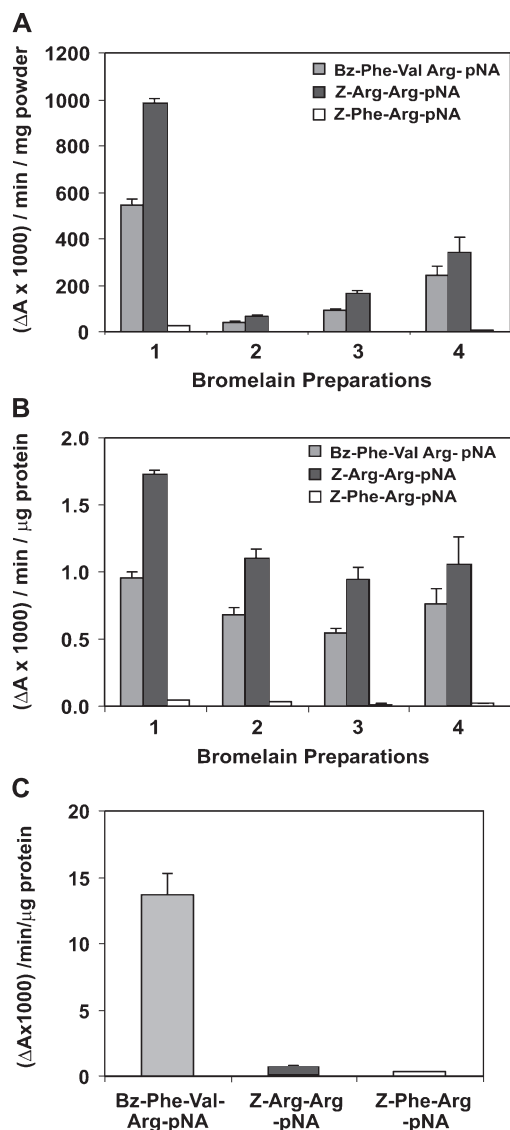
Fig. 3. Comparison of proteolytic activity of natural bromelain preparations. A. The activity of natural bromelain preparations obtained from 4 different manufacturers differed widely when considered per mg of powder, in part due to addition of silica and/or cellulose to aid in tablet formulation. B. The activity of these bromelain preparations was also determined toward the model substrates using a constant amount of protein per assay. Activity was assayed at 20 μg/ml protein for the Z-Arg-Arg-pNA and Bz-Phe-Val-Arg-pNA substrates and at 200 μg/ml for the Z-Phe-Arg-pNA substrate. Proteolysis of these substrates primarily reflects stem bromelain, fruit bromelain+ananain, and ananain activity, respectively. Data represents the mean±S.E.M. of 4 independent experiments. C. The proteinase activity of fresh pineapple extract is shown against three model substrates. Data represent the mean of 3–4 experiments performed.

ing that a minor component of the activity against the Bz-Phe-Val-Arg-pNA substrate is due to ananain. Minimal activity was seen against the Z-Arg-Arg-pNA substrate, indicating that stem bromelain is a minor component of proteinases present in pineapple fruit. To present the data from Fig. 3C in another way, extract from fresh pineapple containing 100 μg/ml protein has proteolytic activity equivalent to 120 ± 15 μg/ml standard bromelain against the Bz-Phe-Val-Arg-pNA substrate, 4.0 ± 0.8 μg/ml standard bromelain against the Z-Arg-Arg-pNA substrate, and 48 ± 8 mg/ml standard bromelain against the Z-Phe-Arg-pNA substrate. The amount of fruit extract protein needed to equal the Z-Arg-Arg-pNA activity of 1 mg of standard bromelain is thus ~25 mg. Extracts obtained from fresh pineapple fruit averaged 12 mg protein/g fruit. Thus, approximately 2 g of fresh pineapple fruit is needed to supply Z-Arg-Arg-pNA activity equivalent to 1 mg stem bromelain. Previous studies of bromelain in humans have used doses from 1 to 12 g/day [19]. Obtaining the stem bromelain activity of this dose solely from the fruit would therefore require consumption of 2000–24,000 g fresh pineapple fruit per day.

3.3. Spontaneous inactivation of bromelain solutions

The stability of the proteolytic activity of bromelain in water at room temperature was determined for solutions containing 0.6, 1.5, 6, 25, and 50 mg/ml bromelain. Although proteolytic activity toward cell surface CD44 decreased by ~50% over the first 24 h for the less concentrated (0.6 and 1.5 mg/ml) bromelain solutions (Fig. 4), 30–40% of the original activity was still retained at 96 h. The activity of more concentrated solutions (6, 25, or 50 mg/ml) toward CD44 remained stable at room temperature for at least 96 h (Fig. 4 and not shown). Thus the proteolytic activity of bromelain is more susceptible to spontaneous inactivation in water when the bromelain concentration (which in this case represented the total protein present in the solution) is dilute.

The proteolytic activity of bromelain toward cell surface molecules varies depends on the cell surface molecule chosen and may also reflect changes in the relative activities of stem bromelain, ananain, and/or fruit bromelain. Model substrate assays were used to better assess the resistance of these individual
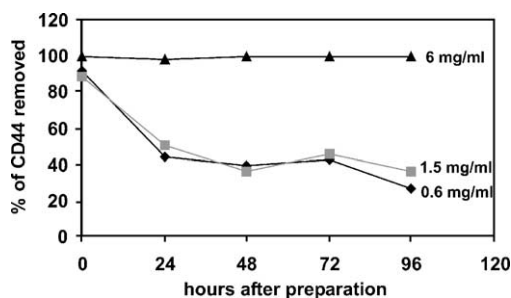
Fig. 4. Stability of bromelain solutions. Bromelain solutions of the indicated concentration were stored at room temperature for the indicated times after preparation. Their ability to remove CD44 from CEM T cells was then determined by flow cytometry. The 1.5 and 6 mg/ml solutions were diluted to 1 mg/ml immediately before incubation with the cells. Data is from a single experiment representative of two performed.

proteinase components within bromelain to spontaneous inactivation in solutions of different concentrations at room temperature. All solutions were diluted to 100 μg/ml for analysis of proteolytic activity. Activity toward the Z-Arg-Arg-pNA substrate was quite stable, with no detectable inactivation over 168 h if stored at 50 mg/ml (Fig. 5B). An approximately 20% decrease in activity toward Z-Arg-Arg-pNA was observed at 168 h if solutions were stored at 1 mg/ml (Fig. 5A). In contrast, activity toward both the Bz-Phe-Val-Arg-pNA and Z-Phe-Arg-pNA substrates decreased by ~30% over the first 24 h of storage, regardless of storage concentration (Fig. 5A,B). Activity toward these substrates remained relatively constant between 48 and 168 h for more concentrated solutions (50 and 125 mg/ml), but declined slightly for 1 mg/ml solutions. At least 40% of the original activity toward these substrates was still present at 168 h, regardless of storage concentration.

These data indicate that concentrated bromelain solutions are more resistant to spontaneous inactivation of their proteolytic activity than are dilute solutions, with stem bromelain more stable than fruit bromelain and ananain.

3.4. Stability of bromelain to pH and digestive enzymes

We previously showed that the proteolytic activity of bromelain toward both the Z-Arg-Arg-

pNA and Bz-Phe-Val-Arg-pNA substrates was completely abrogated when a 10 mg/ml bromelain solution was exposed to mock gastric conditions (PBS, pH $2.0\pm$pepsin) for 2 h at 37 °C [20]. However, we found that, when present at a concentration of 250 mg/ml, bromelain was resistant to inactivation by exposure to pH 2, with <5% loss of activity against the Z-Arg-Arg-pNA and Bz-Phe-Val-Arg-pNA substrates.

If oral bromelain survives exposure to low pH present in the stomach, it will next be exposed to pancreatic digestive enzymes within the small intestine. Incubation with trypsin (1 mg/ml, 1 h, 37 °C) in vitro slightly ($12\pm2\%$; $n=3$) decreased the activity of 10 mg/ml bromelain solutions against the Z-Arg-Arg-pNA substrate, but had no effect on the activity of bromelain at 100 mg/ml ($p=0.008$) (Fig. 6). Brome-
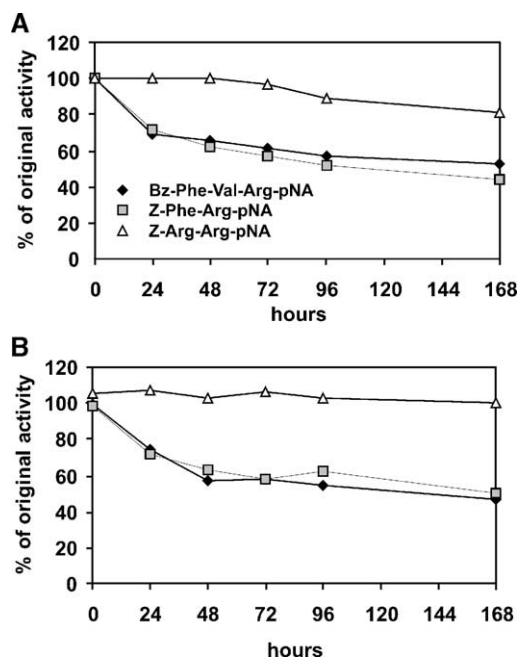

Fig. 5. Stability of bromelain solutions. Bromelain solutions of the indicated concentrations were stored at room temperature for the indicated times after preparation, then their ability to cleave model substrates was determined. All solutions were diluted to 100 μg/ml immediately before the assay. Solutions aged at 1 mg/ml (A) had decreased activity toward the Z-Arg-Arg-pNA substrate (open triangles) relative to solutions aged at 50 mg/ml (B). Data is from a single experiment representative of two performed and is expressed as a percentage of the activity of freshly prepared solutions assayed at the same concentration.
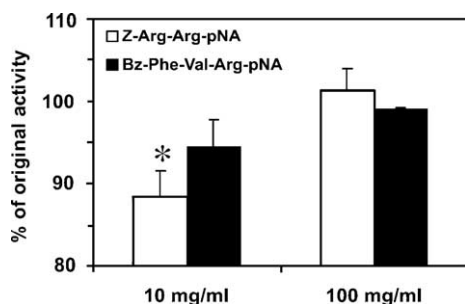
Fig. 6. Effect of trypsin on bromelain activity. Data represent the mean±S.E.M. of 3 independent experiments for bromelain solutions in PBS exposed to trypsin for 1 h at 37 °C (* indicates $p<0.05$ vs. freshly prepared solutions).

lain activity against the Bz-Phe-Val-Arg-pNA substrate was minimally affected by trypsin (<5% change in activity) and this did not differ according to the bromelain concentration during exposure ($p=0.28$). The use of water, PBS, or 100 mg/ml NaHCO$_3$ to make bromelain solutions did not influence their activity following exposure to trypsin.

Taken together, these studies suggest that providing large bolus concentrations of bromelain may prevent inactivation by low pH and by digestive enzymes such as trypsin, which could enhance the proteolytic activity of bromelain within the gastrointestinal tract in vivo.

3.5. Stability of bromelain toward heat and cycles of freeze/thaw

Slightly more than half of the original proteolytic activity remained after 30 min incubation at 60 °C (60% and 53% vs. the Z-Arg-Arg-pNA and Bz-Phe-Val-Arg-pNA substrates, respectively). Activity against these 2 substrates ranged from 9% to 22% of original after 15 min incubation at 70 °C. All proteolytic activity was consistently lost when bromelain solutions were heated at 100 °C for 1 to 10 min. Coomassie blue staining after SDS-gel electrophoresis showed that heating to 70 °C or above resulted in degradation of full-length bromelain (not shown). This was confirmed by Western blotting showing corresponding loss of immunoreactivity with polyclonal anti-bromelain antibody (Fig. 7). Thus the proteolytic activity of bromelain toward model substrates correlates with the presence of full-length, immunoreactive bromelain.

The proteolytic activity of solutions containing either 10 or 100 mg/ml bromelain in water or 100 mg/ml NaHCO$_3$ was not changed against the Z-Arg-Arg-pNA and Bz-Phe-Val-Arg-pNA substrates by 4 freeze/thaw cycles (data not shown).

3.6. Chemical inactivation of bromelain

Marked precipitation occurred following addition of iodoacetamide to solutions containing 250 mg/ml (DTT-treated) bromelain in water. Only ~20% of starting material remained in solution. Cleavage of the Z-Arg-Arg-pNA substrate by this reduced and alkylated bromelain was 21±1% ($n=5$) that of the bromelain starting material, per mg of soluble protein. Proteolytic activity of 250 mg/ml bromelain solutions against the Bz-Phe-Val-Arg-pNA substrate was more affected by reduction and alkylation, with only 6±2% of original activity detected. Solutions containing 10 mg/ml bromelain in PBS, pH 8.0 could be essentially totally inactivated by reduction with 10 mM DTT and alkylation with 50 mM iodoacetamide without precipitation, as described previously [2]. Thus, while more concentrated bromelain solutions are susceptible to denaturation and precipitation by exposure to DTT and iodoacetamide, their proteinase activity is resistant to chemical inactivation, similar to what was seen for spontaneous inactivation and inactivation by acid pH or trypsin of concentrated vs. dilute bromelain solutions.
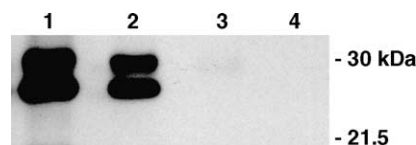

Fig. 7. Effect of heat on immunoreactivity and degradation of bromelain solutions. Aliquots of 10 mg/ml bromelain in water were incubated at room temperature (lane 1), 60 °C (lane 2) or 70 °C (lane 3) for 10 min, or boiled for 6 min (lane 4). Four μg from each sample was analyzed on a 15% SDS-PAGE gel, transferred to nitrocellulose, and reacted with anti-bromelain antibody. Under these conditions, bromelain-specific bands were visualized collectively as a broad doublet between 22 and 28 kDa. Although bromelain proteolytic activity (see text) and immunoreactivity were relatively stable up to 60 °C (compare lanes 1 and 2), marked loss of both proteolytic activity and immunoreactivity occurred upon heating to ≥70 °C (lanes 3 and 4). Loss of immunoreactivity correlated with loss of protein detectable by Coomassie blue staining (not shown), consistent with protein degradation.

4. Discussion

The existing data suggest that bromelain may have potential for therapy of inflammatory diseases, including arthritis and inflammatory bowel disease. The biological effects of bromelain depend on its proteolytic activity (1, 5; L.P. Hale, unpublished). Thus it is critical to accurately assess the proteinase content, activity, and specificity of the bromelain that is used pre-clinical studies in animals and in human clinical trials. The studies reported here provide methods to assess the proteolytic activity of natural bromelain preparations that can guide formulation and dosing of bromelain in future studies. Our data show that the ability of bromelain preparations to cleave various model substrates and specific cell surface molecules reflects the activity of its individual proteinase components.

Commercially available bromelain preparations contain predominately stem bromelain, with minor components of ananain and fruit bromelain. In contrast, the major proteinase present in pineapple fruit is fruit bromelain, with minor components of ananain and stem bromelain. Stem bromelain and fruit bromelain show differential activity toward cleavage of biologically relevant cell surface markers (Fig. 2B). Thus, it is reasonable to hypothesize that different biologic effects may occur following exposure to commercial bromelain tablets that contain primarily stem bromelain versus eating pineapple fruit. The sensitivity of bromelain proteinases to heat inactivation and their stability with respect to freezing suggests that fresh or frozen pineapple (not canned) would be required to obtain any potential health benefits from proteolytic enzymes present in pineapple fruit. However, fresh pineapple is not a concentrated source of bromelain enzymes, since kilogram quantities of fruit must be consumed to supply proteolytic activity similar to that of bromelain preparations used in previous studies [19].

The proteolytic activity of concentrated (≥50 mg/ml) bromelain solutions remains relatively stable for at least 1 week at room temperature, with minimal inactivation by freeze/thaw cycles or exposure to the digestive enzyme trypsin. This finding has direct relevance for formulation for use as an anti-inflammatory agent, as well as for the amount and frequency of dosing to optimize bromelain proteolytic activity in

vivo. We previously showed that the time required to re-express bromelain-sensitive cell surface molecules to 50% of sham treatment levels following bromelain treatment was 8–20 h for monocytes and 48 h for lymphocytes [1]. Administration of bromelain as a concentrated bolus once or several times daily would be expected to result in higher total proteolytic activity, particularly within the lumen of the gastrointestinal tract, compared with timed release dosing that generated lower peak concentrations.

Proteolytically active bromelain does access the circulation, but plasma levels following oral dosing are probably too low to significantly affect of expression of cell surface molecules [2,19]. However, bromelain concentrations within the gut lumen are clearly sufficient to remove at least a high percentage of bromelain-sensitive molecules present on cells exposed to the lumen [20]. This would include colon epithelial cells as well as lamina propria cells present in regions where surface integrity is compromised due to inflammation. About 10% of the bromelain applied to Caco-2 intestinal epithelial cell monolayers reaches the basolateral cell surface as intact biologically active molecules [29]. Thus, it is possible that bromelain may penetrate into intact lamina propria and thus also affect cell surface molecule expression on cells that are not directly exposed to the contents of the lumen.

We used cell-based assays to determine the activity of purified bromelain proteinases against a small panel of cell surface molecules known to be affected by commercially available bromelain [1,2]. We previously showed that proteolytic removal of a given bromelain-sensitive cell surface molecule is independent of the cell type on which it is expressed [2]. Instead, sensitivity to bromelain cleavage depends on the protein sequence and structure of that particular molecule. The spectrum of bromelain-sensitive molecules has been best worked out on leukocytes, for which many antibody reagents are available (e.g. CD1–247). Thus, leukocyte cell lines were used here as a convenient model in which to test bromelain activity. Additional studies will be required to determine the physiologic relevance of removing these and potentially additional molecules whose bromelain-sensitivity has not yet been recognized from cells present in the colonic microenvironment under conditions of inflammation.

For all of the molecules currently known to be bromelain-sensitive, the activity seen with the natural bromelain mixture is reflective of proteolytic activity seen in at least one of the fractions. The flow cytometric assay used in our studies cannot detect complementary or synergistic effects of component proteinases, since only a single cleavage may be sufficient to destroy reactivity with antibody, based on position of cleavage relative to the epitope recognized. Once a cleaved molecule has lost reactivity with specific antibody, additional cleavage by a different proteinase may make the "stump" left in the membrane shorter but would not affect scoring of the molecule as proteinase-sensitive. Mapping of proteinase cleavage sites of bromelain-sensitive molecules is of interest, but is beyond the scope of this manuscript.

The CD44 cell surface molecule is removed by each of the 3 main proteinases in bromelain and thus can serve as a useful indicator of total proteolytic activity. However, model substrate assays are more sensitive indicators of bromelain proteolytic activity and can detect changes that may be missed when analysis is limited to assays of effects on cell surface molecules such as CD44. Model substrate assays, with or without assays of cell surface molecules differentially targeted by fruit bromelain, stem bromelain, and/or ananain, can also assess differential activity of individual proteinases within bromelain and facilitate the standardization of natural bromelain derived from different sources as a therapeutic product.

Clearly, further studies need to be performed to rigorously establish the efficacy of bromelain as an anti-inflammatory drug and to determine how each of the component proteinases within bromelain contribute to its overall anti-inflammatory effects. By providing methods to standardize the proteinase activity of bromelain preparations, the studies described here will facilitate the rational design of future clinical trials of this promising complementary therapy and will strengthen the scientific knowledge base needed for informed use of bromelain in medical practice.

Acknowledgements

References

[1] Hale LP, Haynes BF. Bromelain treatment of human T cells removes CD44, CD45RA, E2/MIC2, CD6, CD7, CD8, and Leu 8/LAM1 surface molecules and markedly enhances CD2-mediated T cell activation. J Immunol 1992;149:3809–16.

[2] Hale LP, Greer PK, Sempowski GD. Bromelain treatment alters leukocyte expression of cell surface molecules involved in cellular adhesion and activation. Clin Immunol 2002;104:183–90.

[3] Kleef R, Delohery TM, Bovbjerg DH. Selective modulation of cell adhesion molecules on lymphocytes by bromelain protease 5. Pathobiology 1996;64:339–46.

[4] Munzig E, Eckert K, Harrach T, Graf H, Maurer HR. Bromelain protease F9 reduces the CD44 mediated adhesion of human peripheral blood lymphocytes to human umbilical vein endothelial cells. FEBS Lett 1994;351:215–8.

[5] Mynott TL, Ladhams A, Scarmato P, Engwerda CR. Bromelain, from pineapple stems, proteolytically blocks activation of extracellular regulated kinase-2 in T cells. J Immunol 1999;163:2568–75.

[6] Mynott TL, Crossett B, Prathalingam SR. Proteolytic inhibition of *Salmonella enterica* serovar typhimurium-induced activation of the mitogen-activated protein kinases ERK and JNK in cultured human intestinal cells. Infect Immun 2002;70:86–95.

[7] Majima M, Kawashima N, Hiroshi I, Katori M. Effects of an orally active non-peptide bradykinin B2 receptor antagonist, FR173657, on plasma exudation in rat carrageenin-induced pleurisy. Br J Pharmacol 1997;121:723–30.

[8] Majima M, Nishiyama K, Iguchi Y, Yao K, Ogino M, Ohno T, et al. Determination of bradykinin-(1–5) in inflammatory exudate by a new ELISA as a reliable indicator of bradykinin generation. Inflamm Res 1996;45:416–23.

[9] Ogino M, Majima M, Kawamura M, Hatanaka K, Saito M, Harada Y, et al. Increased migration of neutrophils to granulocyte-colony stimulating factor in rat carrageenin-induced pleurisy: roles of complement, bradykinin, and inducible cyclooxygenase-2. Inflamm Res 1996;45:335–46.

[10] Gaciong Z, Paczek L, Bojakowski K, Socha K, Wisniewski M, Heidland A. Beneficial effect of proteases on allograft arteriosclerosis in a rat aortic model. Nephrol Dial Transplant 1996;11:987–9.

[11] Targoni OS, Tary-Lehmann M, Lehmann PV. Prevention of murine EAE by oral hydrolytic enzyme treatment. J Autoimmun 1999;12:191–8.

[12] Rovenska E, Svik K, Stancikova M, Rovensky J. Enzyme and combination therapy with cyclosporin A in the rat developing adjuvant arthritis. Int J Tissue React 1999;21: 105–11.

[13] Rovenska E, Svik K, Stancikova M, Rovensky J. Inhibitory effect of enzyme therapy and combination therapy with cyclosporin A on collagen-induced arthritis. Clin Exp Rheumatol 2001;19:303–9.

[14] Thornhill SM, Kelly AM. Natural treatment of perennial allergic rhinitis. Altern Med Rev 2000;5:448–54.

[15] Wittenborg A, Bock PR, Hanisch J, Saller R, Schneider B. Comparative epidemiological study in patients with rheumatic diseases illustrated in a example of a treatment with non-steroidal anti-inflammatory drugs versus an oral enzyme combination preparation. Arzneimittel-Forschung 2000;50: 728–38.

[16] Brown AC. Lupus erythematosus and nutrition: a review of the literature. J Renal Nutr 2000;10:170–83.

[17] Masson M. Bromelain in blunt injuries of the locomotor system. A study of observed applications in general practice. Fortschritte der Medizin 1995;113:303–6.

[18] Kane S, Goldberg MJ. Use of bromelain for mild ulcerative colitis. Ann Intern Med 2000;132:680.

[19] Castell JV, Friedrich G, Kuhn CS, Poppe GE. Intestinal absorption of undegraded proteins in men: presence of bromelain in plasma after oral intake. Am J Physiol 1997;273:G139–46.

[20] Hale LP. Proteolytic activity and immunogenicity of oral bromelain within the gastrointestinal tract of mice. Int Immunopharmacol 2004;4:255–64.

[21] Mynott TL, Luke RK, Chandler DS. Oral administration of protease inhibits enterotoxigenic *Escherichia coli* receptor activity in piglet small intestine. Gut 1996;38:28–32.

[22] Chandler DS, Mynott TL. Bromelain protects piglets from diarrhoea caused by oral challenge with K88 positive enterotoxigenic *Escherichia coli*. Gut 1998;43:196–202.

[23] Rowan AD, Buttle DJ, Barrett AJ. The cysteine proteinases of the pineapple plant. Biochem J 1990;266:869–75.

[24] Napper AD, Bennett SP, Borowski M, Holdridge MB, Leonard MJC, Rogers EE, et al. Purification and characterization of multiple forms of the pineapple-stem-derived cysteine proteinases ananain and comosain. Biochem J 1994;301:727–35.

[25] Rowan AD, Buttle DJ, Barrett AJ. Ananain: a novel cysteine proteinase found in pineapple stem. Arch Biochem Biophys 1988;267:262–70.

[26] Harrach T, Eckert K, Schultze-Forster K, Nuck R, Grunow D, Maurer HR. Isolation and partial characterization of basic proteinases from stem bromelain. J Protein Chem 1995; 14:41–52.

[27] Harrach T, Eckert K, Maurer HR, Machleidt I, Machleidt W, Nuck R. Isolation and characterization of two forms of an acidic bromelain stem proteinase. J Protein Chem 1998;17: 351–61.

[28] Yamada F, Takahashi N, Murachi T. Purification and characterization of a proteinase from pineapple fruit, fruit bromelain FA2. J Biochem 1976;79:1223–34.

[29] Bock U, Kolac C, Borchard G, Koch K, Fuchs R, Streichhan P, et al. Transport of proteolytic enzymes across Caco-2 cell monolayers. Pharm Res 1998;15:1393–400.