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Cross-Linked Bromelain Inhibits Lipopolysaccharide-Induced Cytokine Production Involving Cellular Signaling Suppression in Rats

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Bromelain has been reported to have anti-inflammatory and immunomudulatory effects. It has been cross-linked with organic acids and polysaccharides by γ irradiation. The cross-linked (CL)-bromelain preparation resisted an acidic environment of pH 3 for 2 h and preserved 80% of its enzyme activity. Pretreatment of rats with CL-bromelain intragastrically for 7 days significantly reduced serum cytokine production induced by injected i.p. with 2.5 mg/kg of lipopolysaccharide (LPS). Bromelain significantly reduced serum glutamate−oxalacetate transaminase induced by LPS. The anti-inflammatory effect of CL-bromelain was correlated with reduced LPS-induced NF-κB activity and cyclooxygenase 2 (COX-2) mRNA expression in rat livers. In addition, CL-bromelain dose-dependently inhibited LPS-induced COX-2 mRNA and prostaglandin E2 (PGE2) in BV-2 microglial cells. CL-Bromelain also suppressed the LPS-activated extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinase (MAPK). In conclusion, the anti-inflammatory effects of the CL-bromelain preparation in vivo and in vitro suggest its therapeutic potentials.

KEYWORDS: Bromelain; cross-linking; cytokines; NF-κB; COX-2; MAPK

INTRODUCTION

Bromelain is a group of proteases extracted from the stem of the pineapple. Bromelain is known for its anti-inflammatory effect (*1*) and for dose-dependently providing relief for adults with mild knee pain (*2*). Other pharmacological activities of bromelain have been reported, such as the reduction of thrombogenesis, antihypertension, regulation of immune functions, antimicrobial infections, and inhibition of cancer cell growth (*3*−*7*). Bromelain can simultaneously enhance and inhibit immune cell responses in vitro and in vivo via a stimulatory action on accessory cells and a direct inhibitory action (*8*). In human macrophages/monocytes, bromelain induced a significant increase in interleukin (IL)-6, tumor necrosis factor α (TNF-α), and γ-interferon (IFN-γ) (*9*). Bromelain also enhanced IFN-γ-mediated nitric oxide (NO) and TNF-α production by murine macrophages. The effect of bromelain is independent of endotoxin receptor activation and is not caused by direct modulation of IFN-γ receptors. Instead, bromelain either enhances or acts synergistically with IFN-γ receptor-mediated signals (*10*). On the other hand, bromelain blocks activation of extracellular signal-regulated kinase (ERK)-2 in Th0 cells stimulated via the T cell receptor or stimulated with combined phorbol myristate acetate (PMA) and calcium ionophore. However, the inhibitory activity of bromelain is dependent upon its proteolytic activity (*11*).

Lipopolysaccharide (LPS) is an endotoxin from Gram-negative bacteria, which provokes inflammatory and immunological responses. LPS-stimulated murine microglia, macrophages, and Kupffer cells activated both phosphorylation and kinase activities of ERK1/2, c-Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinases (MAPKs) and, subsequently, TNF-α, IL-6, and IL-10 production (*12*−*14*). Pretreatment with the indolocarbazole Go6976, sesamin, or the p38 MAPK-specific inhibitors, SB202190 and SB203580, suppressed the LPS-induced NO, IL-6, and TNF-α production and phospho-p38 MAPK significantly in murine BV-2 cells (*14*, *15*). In human and rodent macrophages/monocytes, cytokine production and steady-state mRNA levels were increased in response to LPS. Expression of IL-1, IL-6, and TNF-α has been shown to be dependent upon the activation of the nuclear factor (NF)-κB (*16*, *17*). NF-κB can function upstream of cyclooxygenase (COX)-2 to control the transcription of this gene. Recent evidence indicates that the inducible COX may have both pro-

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and anti-inflammatory properties through the generation of different prostaglandins (*18*). Prostaglandin E_2 (PGE_2) strongly synergizes with the inflammatory cytokine, TNF-α, to promote NF-κB-dependent transcription and gene expression (*18*), and the COX-2 inhibitor also inhibits NF-κB activation (*19*). On the other hand, selective inhibition of either COX isoform resulted in an increased secretion of TNF-α; however, this effect is much greater with the COX-1 than with the COX-2 inhibitor (*20*). These results suggest that both COX-1 and COX-2 play significant roles in the regulation of the early response to endotoxemia.

Studies of the use of proteolytic enzymes in rheumatic disorders have mostly been conducted on enzyme preparations consisting of combinations of bromelain, papain, trypsin, and chymotrypsin (*21*−*23*). Thus, the mechanism of action of systemic enzyme therapy remains unclear. Becuase a cross-linking protein has been shown to be acid-resistant (*24*, *25*), we prepared bromelain by cross-linking it with a network of polymers to protect it from a gastric acid environment. Therefore, in this study, we were able to examine the effect of cross-linked (CL)-bromelain on rats with endotoxin administration. The signaling mechanism of CL-bromelain was also investigated.

MATERIALS AND METHODS

Materials. LPS from *Escherichia coli* serotype 0111:B4 was obtained from Sigma (St. Louis, MO). Bromelain was obtained from Come True Company (Taoyuan, Taiwan), and cell culture ingredients were obtained from Life Technologies (Grand Island, NY). Rabbit anti-mouse β-actin (Calbiochem, Temecula, CA) and anti-phospho-MAPKs (Promega, Madison, WI), streptavidin-horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson, West Grove, PA) were obtained separately. TNF-α, PGE−2, IL-1, and IL-6 ELISA kits were obtained from R&D (Minneapolis, MN).

Cross-Linked Bromelain Preparation. Konjac flour (Lu Ka Food company, Taipei, Taiwan; glucomannan, 92% pure) and lactic acid were mixed at 2:1 by weight; the mixture was heated at 80 °C for 5 min while stirring. The stirred mixture was irradiated with γ-ray (^{60}Co) of 15 kGy for 3 s to form an organic network polymer. The mixture of organic network polymer was neutralized with an appropriate amount of $NaHCO_3$ to about pH 7. The neutralized mixture was then added to the same weight of bromelain at 30 °C and then stirred for 3 min. The mixture was irradiated again with γ-ray (^{60}Co) of 15 kGy for 3 s to embed the bromelain into the network polymer (U.S. patent number 10/790,042).

Enzymatic Activity of Protein Hydrolysis. Casein was hydrolyzed with bromelain under the following conditions: substrate concentration, 2%; bromelain−substrate ratio, 5:100; pH, 3.0−7.0; and water-bath temperature, 37 °C. Each hydrolysate was rendered inactive by 10% trichloroacetic acid (TCA) and centrifugation at 300*g* for 5 min. Folin-Ciocalteau's (phosphomolybdate and phosphotungstate, Merck) phenol reagent reacts with tyrosine residues, and absorption values read at 578 nm. The protease activity was measured according to the method of Boyce and Walter (*26*, *27*) by spectrophotometrically monitoring the rate of tyrosine formation. One enzyme unit was defined as the amount of enzyme that releases 1 mmol of tyrosine from the substrate per minute under the conditions of enzyme assay and using crystalline tyrosine as the standard.

Animal Experiment. Male Sprague−Dawley (SD) rats, weighing about 250 g each, were maintained at 23 °C, with 12 h of day/night cycle, and provided ad libitum water and lab chow. Six sets of rats were treated differently, and each group included 8 rats. In the control groups, rats were maintained on a regular diet or supplemented with CL-bromelain (100 mg/kg) for 7 days. In the experiment groups, rats were maintained on a regular diet or supplemented with CL-bromelain (10, 50, or 100 mg/kg) for 7 days and then *E. coli* LPS (2.5 mg/kg) was injected into the abdominal cavity. After LPS injection, rats were maintained on a 24-h fast and sacrificed. Blood was collected and stored for immunological and biochemical studies.

ELISA Assay. TNF-α, IL-1β, IL-6, and PGE-2 were measured by ELISA kits (R&D, Minneapolis, MN). The absorbance at 450 nm was determined using a microplate reader (spectraMAX 340, Molecular Devices, Sunnyvale, CA).

Preparation of Nuclear Extract and NF-κB p65 Detection. The treated cells were washed and centrifuged and then resuspended in hypotonic buffer and incubated on ice for 15 min. After centrifugation at 14 000 rpm for 15 min, the supernatant cytoplasmic protein was collected. The remnants were resuspended in extraction buffer, incubated on ice for 30 min, and then centrifuged, after which the supernatant nuclear extract was collected. All proteins were stored at −80 °C. The protein concentration was determined by the Bradford assay (Bio-Rad, Hemel, Hempstead, U.K.). p65 subunit activity was detected by an ActivELISA kit, NF-κB p65 (IMGENEX, San Diego, CA). The individual wells of the plate were coated with capture antibody, followed by nuclear extract protein reaction, blocking, and finally an HRP-conjugated secondary antibody, which provided a sensitive colorimetric readout that was easily quantified by spectrophotometry. All of the procedures complied with the protocol.

Cell Culture. The murine BV-2 cell line was maintained in DMEM supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified incubator under 5% CO_2. Confluent cultures were passed by trypsinization. For experiments, cells were washed twice with warm DMEM and then treated in serum-free medium. In all experiments, cells were treated with CL-bromelain for the indicated times after LPS addition. CL-Bromelain was dissolved in phosphate-buffered saline (PBS).

MTT Reduction Assay. The cell viability was measured with blue formazan that was metabolized from colorless 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondria dehydrogenases, which are active only in live cells. BV-2 cells were preincubated in 24-well plates at a density of 5×10^5 cells per well for 24 h and then washed with PBS. Cells with various concentrations of CL-bromelain were treated with LPS for 24 h and grown in 0.5 mg/mL MTT at 37 °C. After 60 min, 200 μL of solubilization solution was added to each well and absorption values were read at 540 nm on an automated SpectraMAX 340.

Lactate Dehydrogenase (LDH) Release Assay. Cytotoxicity was determined by measuring the release of LDH. Cells with various concentrations of cross-linked bromelain were treated with LPS for 24 h, and the supernatant was used to assay LDH activity. The cell-free supernatant was mixed with potassium phosphate buffer containing NADH and sodium pyruvate in a final volume of 0.2 mL to a 96-well plate. The rate of absorbance values was read at 490/630 nm on an automated SpectraMAX 340 microtiter plate reader.

Isolation of RNA and RT-PCR. After treatment, total cellular RNA was extracted with a cold RNA extraction solution (Ultraspec RNA; Biotex Laboratory, Inc., Houston, TX). The RT-PCR assays were performed with a Titan One Tube RT-PCR system kit (Boehringer, Mannheim, Germany). Briefly, 1 μg of total RNA from each sample was added to 50 μL of a reaction mixture containing 0.2 mM dNTP, 0.4 μM each of sense- and antisense-specific primers, 5 mM dithiothreitol (DTT), 5 units of RNase inhibitor, 1 μL of AMV reverse transcriptase, and Expand High Fidelity enzyme mix. The primer sequences were as follows: 5'-TGCATGTGGCTGTGGATGTCATC, 3′-CACTAAGACAGACCCGTCATCTC for COX-1 (450-bp fragment), 5′-GAACATTGTGAACAACATCCC, 3′-TTGGTGGCATACATCATCAGA for COX2 (615-bp fragment), and 5-GTGGGCCGCTCTAGGCACCAA, 3′-CTCTTTGATGTCACGCACGATTTC for β-actin (540-bp fragment), as a control for the RNA isolation and reverse transcription. RT-PCR was conducted in a Perkin−Elmer Cetus thermocycler (Norwalk, CT). The preparations in the microtubes were incubated for 30 min at 50 °C and then amplified using a three-temperature PCR system usually consisting of denaturation at 94 °C for 45 s, primer annealing at 60 °C (for COX-1), 55 °C (for COX-2), or 60 °C (for β-actin) for 45 s, and extension at 72 °C for 2 min. The PCR product was visualized by electrophoresis in a 2.5% agarose gel, followed by staining with 0.5 μg/mL ethidium bromide. Verification of specific genes was established by their predicted size under UV light.

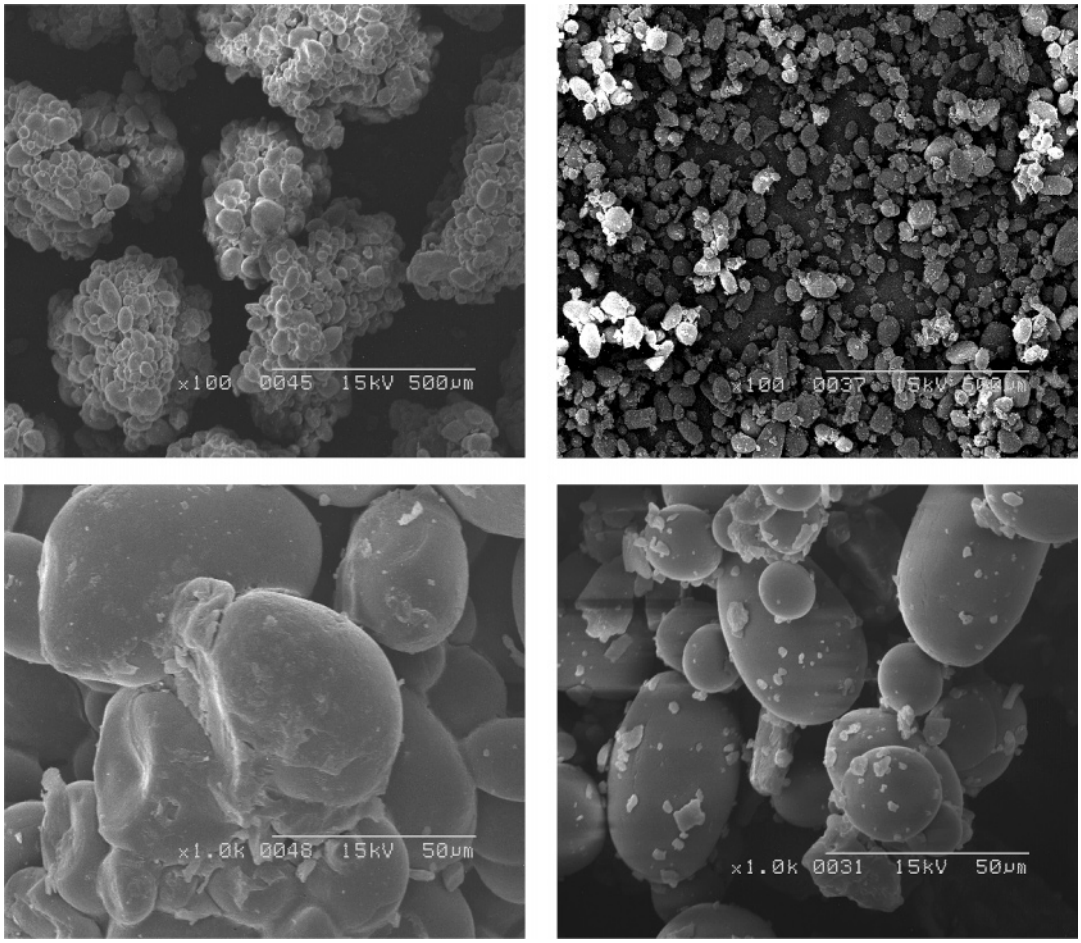

Figure 1. Bromelain, lactic acid, and konjac flour were cross-linked by irradiation with γ-ray (^{60}Co). The cross-linked bromelain (left) and bromelain powder (right) are shown in a scanning electron micrograph.

Quantification of the band density was performed by densitometric analysis (Digital Image Analysis System, PDI, Huntington Station, NY) and calculated as the optical density × area of band.

Preparation of Cell Extracts. Cells were collected and centrifuged at 200*g* for 10 min at 4 °C. The cell pellets were resuspended in an appropriate volume (approximately 4×10^7 cells/mL) of lysis buffer and sonicated. The protein concentration of samples was determined by the Bradford assay (Bio-Rad, Hemel, Hempstead, U.K.), and samples were equilibrated to 2 mg/mL with lysis buffer.

Western Blotting. Protein samples containing 50 μg of protein were separated on 12% sodium dodecyl sulfate−polyacrylamide gels and transferred to immobilon poly(vinylidene difluoride) membranes (Millipore, Bedford, MA). The membranes were incubated for 1 h with 5% dry skim milk in TBST buffer (0.1 M Tris-HCl at pH 7.4, 0.9% NaCl, and 0.1% Tween-20) to block nonspecific binding. Then, they were incubated with rabbit anti-mouse β-actin (1:5000) and anti-phospho-MAPKs (1:1000). Subsequently, the membranes were incubated with secondary-conjugated goat anti-rabbit IgG. β-Actin and phosphorylated MAPK proteins were detected by a chemiluminescence detection system according to the instructions of the manufacturer (ECL, Amersham, Berkshire, U.K.). The band intensity was quantified with a densitometric scanner (PDI, Huntington Station, NY).

Statistical Analysis. All data were expressed as the mean ± standard error of the mean (SEM). For single variable comparisons, Student's *t* test was used. For multiple variable comparisons, data were analyzed by one-way analysis of variance (ANOVA) followed by Scheffe's test. *p* values less than 0.05 were considered significant.

RESULTS

Proteolytic Activity of Bromelain. CL-Bromelain preparations (**Figure 1**) were found to release 60% bromelain activity at 20 min and 80% at 40 min. The stability of CL-bromelain in acid and alkali environments (pH 3−8) at 30 °C in the time range of 0−4 h was 74−96%. In contrast, the stability of original bromelain was less than 30% at pH 3 at 1 h and 50% at pH 8 at 4 h (data not shown).

Reduced Serum Cytokine Levels by CL-Bromelain. SD rats were pretreated intragastrically with different levels of CL-bromelain preparation (10, 50, or 100 mg/kg) and injected i.p. with LPS (2.5 mg/kg) and then maintained on a 24-h fasting. Serum concentrations of IL-1β, IL-6, and TNF-α were significantly elevated by LPS, and IL-1β and IL-6 were dose-dependently reduced by bromelain as compared with that of the LPS control. TNF-α was also reduced but to a lesser extent (**Table 1**).

Bromelain Suppressed LPS-Induced NF-κB Activation. Previous reports showed that agents decrease inflammatory cytokine through the inhibition of NF-κB activation (*14*, *28*). Indeed, pretreatment of CL-bromelain dose-dependently (10−100 mg/kg) inhibited the LPS-induced NF-κB p65 activities in rat livers by 27−70% (**Table 1**). Pretreatment of CL-bromelain did not alter the liver function but reduced LPS-induced serum levels of GOT ($p < 0.01$) and GPT.

Bromelain Suppressed LPS-Induced PGE_2 Release in BV-2 Cells. PGE_2 release from BV-2 cells was measured by an ELISA kit. Cells were treated with bromelain and/or 1 μg/mL LPS for 24 h. None of the LPS or bromelain treatments caused cell injury as judged by the MTT and LDH assays (data not shown). As shown in **Figure 2**, CL-bromelain dose-

Table 1. Effect of Bromelain on Cytokine Production in Endotoxemic Rats[a]

		bromelain (percentage of inhibition)		
concentration	control	10	50	100 (mg/kg)
serum				
IL-1β[b] (ng/dL)	800 ± 40	560 ± 45[c] (30)	300 ± 40[c] (64)	180 ± 42[c] (77)
IL-6 (ng/dL)	290 ± 35	210 ± 21[c] (27)	175 ± 20[c] (40)	145 ± 35[c] (50)
TNF-α (ng/dL)	105 ± 30	80 ± 20 (24)	75 ± 18 (29)	58 ± 22 (45)
GOT (IU/L)	140 ± 22	130 ± 16 (7)	106 ± 20 (24)	82 ± 16[c] (41)
GPT (IU/L)	62 ± 10	58 ± 10 (6)	55 ± 12 (11)	45 ± 11 (27)
liver				
NF-κB (pg/mL)	610 ± 57	450 ± 42[c] (26)	180 ± 40[c] (70)	160 ± 45[c] (74)

[a] Male SD rats were divided into groups (8 rats each) and supplemented with different levels of CL-bromelain for 7 days. After LPS (2.5 mg/kg) was injected into the abdominal cavity, rats were maintained on a 24-h fast. Blood was collected and stored at −70 °C before an ELISA assay or a liver-function test. The NF-κB p65 level was detected by an ActivELISA kit (IMGENEX). [b] Unstimulated control values for IL-1, IL-6, and TNF were 150, 130, and 40 (ng/dL), respectively; NF-κB levels were 100 pg/mL, and bromelain alone was 105 pg/mL. [c] $p < 0.01$ as compared with the LPS control group.

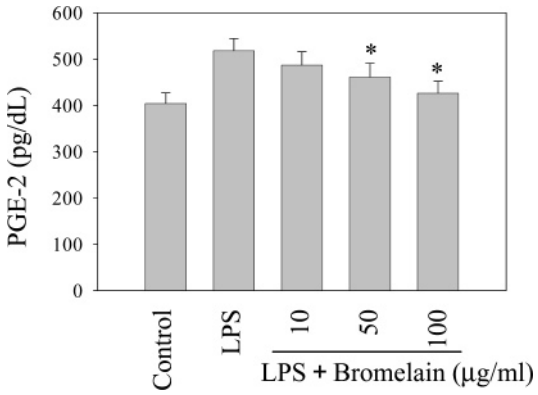

Figure 2. Bromelain-inhibited PGE_2 released by microglia BV-2 cells under LPS (1 μg/mL) stimulation. BV-2 cells were treated with/without LPS and CL-bromelain (10–100 μg/mL) for 24 h. Values represent mean ± SEM. (*) $p < 0.05$ indicates significant differences between LPS alone and LPS plus bromelain.

dependently (10−100 μg/mL) inhibited the LPS-induced PGE_2 in BV-2 cell culture ($p < 0.05$).

Bromelain Inhibited the Expression of COX-2 mRNA. RT-PCR analyses were performed to assess the effect of CL-bromelain on LPS-induced COX-1 and COX-2 mRNA steady-state levels. BV-2 cells were treated with bromelain and LPS for 4 h. The accumulation of COX-2 mRNA levels was inhibited dose-dependently (10−100 μg/mL) by CL-bromelain, whereas COX-1 mRNA showed no change after such treatment (**Figure 3a**). Similarly, LPS-induced COX-2 mRNA expressions in rat livers were reduced dose-dependently (from 10 to 100 mg/kg) by CL-bromelain pretreatment (**Figure 3b**). Therefore, the suppression of COX-2 mRNA expression by bromelain is due to transcription regulation through its inhibition of NF-κB activation.

Bromelain Suppressed LPS-Induced MAPK Activation. Studies indicate that LPS induces the activation of MAPKs and subsequently activates the transcription factors with the attendant induction of COX-2 (*29*, *30*). Therefore, we test whether the MAPK signaling pathways are involved in the anti-inflammatory effect of bromelain. As shown in **Figure 4**, CL-bromelain dose-dependently suppressed LPS-activated JNK, ERK1/2, and p38 MAPKs at 10 and 30 min.

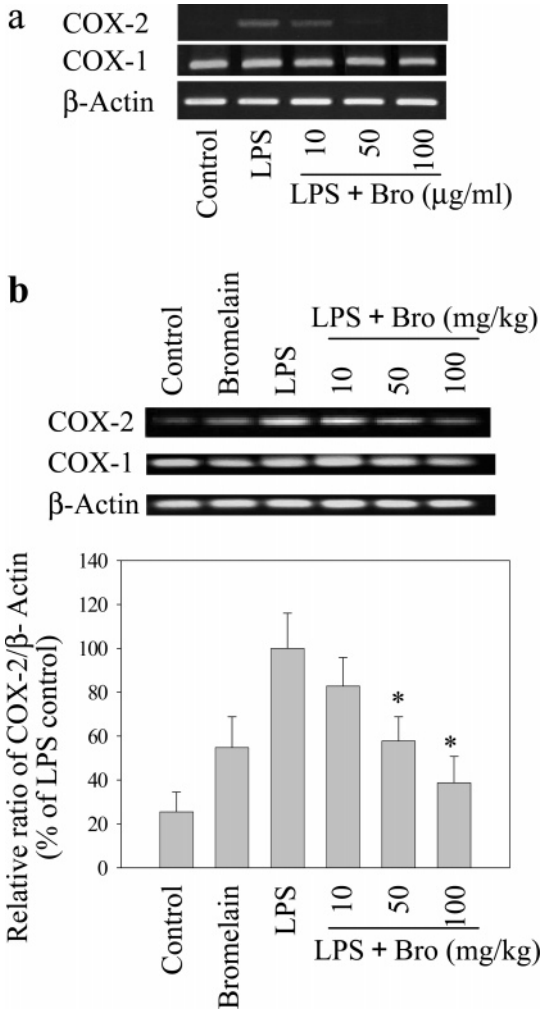

Figure 3. Effect of bromelain on LPS-induced COX-2 mRNA expression. (**a**) BV-2 cells were treated with 1 μg/mL LPS and various concentrations of CL-bromelain for 4 h, and (**b**) livers from rats were pretreated with bromelain for 7 days and injected with LPS for 24 h. Levels of COX-2, COX-1, and β-actin mRNA were assayed by RT-PCR. Values represent the mean from three independent experiments. Bromelain decreased COX-2 mRNA as compared with that of the LPS group, (*) $p < 0.05$.

DISCUSSION

Enteric preparation has been used for the therapeutic application of digestive enzymes (*31*, *32*). Previously, an enteric coating with Eudragit RS-100 was found to provide the acid stabilization to diastase or protein (*33*−*35*). Another method uses water-soluble polyphosphazene which cross-links when exposed to γ-rays to form hydrophilic, water-swellable membranes and hydrogels (*36*). We prepared the cross-linking of polysaccharide, lactic acid, and bromelain by γ irradiation that formed a polymer, readily released the bromelain moiety, and remained stable in an acidic (pH 3) environment. The present result demonstrated that rats supplemented with CL-bromelain had significantly reduced inflammatory responses.

We found that pretreatment with CL-bromelain reduced inflammatory cytokine in rats under endotoxemia. CL-Bromelain also reduced the LPS-induced COX-2 mRNA expression but not COX-1 in rat livers. COX-2 plays a prominent role in the inflammation associated with adjuvant arthritis, and COX-2-derived PGs upregulate COX-2 and IL-6 expression at inflammatory sites (*37*). Because COX-1 expression dominates normal tissues, whereas COX-2 mRNA is induced at the inflammatory site, COX-2-selective inhibitors are being used as anti-inflam-

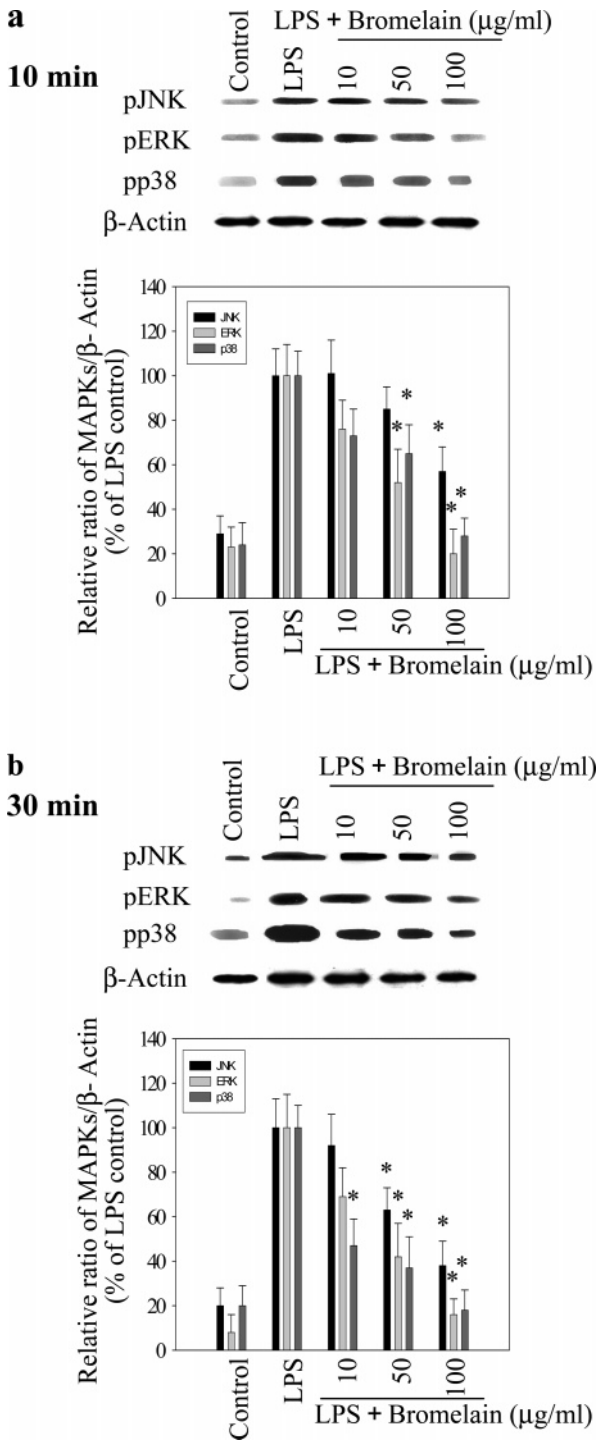

Figure 4. Bromelain-inhibited LPS-induced phospho-MAP kinases in BV-2 cells. Western blots showed that CL-bromelain (50–100 μg/mL) at 10 and 30 min significantly decreased the LPS-induced phospho-JNK, ERK, and p38 MAPKs. Values represent the mean from three independent experiments. (*) $p < 0.05$ as compared with the LPS group.

matory agents without gastric toxicity (*38*). Because IL-1, IL-6, and TNF-α productions are dependent upon NF-κB activation and NF-κB can promote COX-2 gene transcription, COX-2 inhibitors may exert an anti-inflammatory effect by inhibition of NF-κB (*16*−*19*). Our results of reduced cytokine, NF-κB activation, and COX-2 gene expression by CL-bromelain were consistent with these studies. PGE_2 production was increased in both blood and lung homogenates of mice in which LPS was administered i.p. and suppressed by the COX-2 inhibitor NS-398 (*39*). In line with this, bromelain dose-dependently inhibited the LPS-induced PGE_2 and COX-2 mRNA in BV-2 cells. Therefore, bromelain may be used as an anti-inflammatory agent.

LPS induced the activation of MAPKs and subsequently activated the transcription factors with the attendant induction of COX-2 (*27*−*30*). We found that bromelain dose-dependently suppressed LPS-activated phosphorylation of ERK1/2, JNK, and p38 MAPKs. Previously, pretreatment with agents such as the indolocarbazole Go6976, sesamin, or the p38 MAPK-specific inhibitors, SB202190 and SB203580, suppressed the LPS-induced NO, IL-6, and TNF-α production and phospho-p38 MAPK significantly in murine BV-2 cells (*14*, *15*, *40*). Bromelain-blocked activation of ERK-2 in stimulated T cells is dependent upon its proteolytic activity, because ERK-2 inhibition is abrogated by E-64, a selective cysteine protease inhibitor. However, inhibitory effects are not caused by nonspecific proteolysis, because the protease trypsin has no effect on ERK activation (*11*). Bromelain also significantly decreased the clinical and histological severity of colonic inflammation when administered to piroxicam-exposed IL-10−/− mice with established colitis. Proteolytic activity of bromelain is required for anti-inflammatory effects in vivo (*41*). When oral bromelain was formulated as an antacid, it retained substantial proteolytic activity throughout the gastrointestinal tract (*42*).

In conclusion, our results provide further support for the hypothesis that oral cross-linked bromelain may potentially modify inflammation in pretreated animals under LPS stimulation. The anti-inflammatory effect of CL-bromelain was correlated with reduced LPS-induced NF-κB activity and COX-2 mRNA expression and possibly through the suppression of LPS-activated ERK, JNK, and p38 MAPKs.

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