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Citrus unshiu Water Extract Inhibits Trypsin-induced TNF-α and Tryptase Productions by Blocking the ERK Phosphorylation and Trypsin Activity

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Abstract – *Citrus unshiu* (Rutaceae) has long been known as an anti-inflammatory and anti-allergic agent. In the present study, the inhibitory effect of CUWE (*Citrus unshiu* water extract) on the production of TNF-α and tryptase was examined. In addition, a possible mechanism for the inhibition of trypsin-stimulated human leukemic mast cell-1 (HMC-1) activation was determined. To do so, TNF-α production from the HMC-1 cells that were stimulated by trypsin (100 nM) in the presence or absence of CUWE (10, 100, and 100 μg/ml) was measured by enzyme-linked immunosorbent assay (ELISA) and reverse transcription-PCR. The tryptase production was evaluated by reverse transcription-PCR. Extracellular signal-regulated kinase (ERK) activation was analyzed by Western blot. Trypsin activity was measured by using Bz-DL-Arg-p-nitroanilide (BAPNA) as substrate. Results showed that the CUWE inhibited production of both TNF-α and tryptase from the trypsin-stimulated HMC-1 in a dose-dependent manner. The CUWE also inhibited the ERK phosphorylation and trypsin activity. These results indicate that the CUWE had an inhibitory effect on TNF-α and the tryptase productions by blocking the ERK phosphorylation and trypsin activity.
Keywords – *Citrus unshiu*, trypsin, tryptase, TNF-α, ERK

Introduction

Ulcerative colitis (UC) is an inflammatory bowel disease of undetermined etiology, characterized by chronic uncontrolled inflammation of the intestinal mucosa. In the colonic mucosa of patients with UC, the active inflammation is persistent and mucosal injury and repair is repeated. Trypsin is found at high levels in the colon lumen of patients with UC (Raithel *et al.*, 2001). Trypsin derives from digestive glands, inflammatory cells, and microbial pathogens. It has been shown that trypsin plays an important role in inflammation. Trypsin induces nitric oxide-dependent vasodilation, extravasation of plasma proteins, infiltration of neutrophils, and colonic inflam-mation (Kawabata *et al.*, 2001). Intracolonic injection of trypsin induced an inflammatory reaction characterized by granulocyte infiltration, increased wall thickness, tissue damage, and elevated T-helper cell type 1 cytokine (Cenac *et al.*, 2002). Trypsin also stimulates inflammatory mediator release from peritoneal macrophages (Lundberg *et al.*, 2000). Furthermore, mast cells play an important role in the pathogenesis of UC (Raithel *et al.*, 2001). The development of dextran sulphate sodium-induced experimental colitis was not shown in genetically mast cell-deficient *Ws/Ws* rats (Araki *et al.*, 2000). We previously showed that the levels of tryptase and tumor necrosis factor (TNF)-α expression in intestinal mast cells were significantly elevated in UC tissues compared to normal tissues (Kim *et al.*, 2003), indicating that mast cells is involved in the inflammatory features of UC. We previously showed that trypsin induced TNF-α secretion from human leukemic mast cell-1 (HMC-1) through extracellular signal-regulated kinase (ERK) cascade without any detectable phosphorylation of c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (p38 MAP kinase) (Kang *et al.*, 2003). Therefore, inhibitors of inflammatory mediator secretion or trypsin activity may be pharmacological strategies for the treatment of intestinal inflammation.

Citrus unshiu (Rutaceae) has long been used for treatment of allergy and inflammation in East Asian countries. Some investigators suggested that *Citrus* can be notable sources of anti-oxidative, anti-inflammatory, cancer preventive compounds (Murakami *et al.*, 2000). In the present study, we have investigated whether *Citrus unshiu* water extract (CUWE) inhibits trypsin-induced mast cell activation.

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Materials and Methods

Materials – Human trypsin, soybean trypsin inhibitor (SBTI), substrate Bz-DL-Arg-p-nitroanilide (BAPNA) and 3-(4,5-dimethylthiazol-2-yl)-2,5-dipheny lthiazolium br omide (MTT) were purchased from Sigma (St. Louis, MO). Anti-human ERK antibody and anti-human-phospho-ERK antibody were purchased from Santa Cruz Bio technology Inc. (CA, USA), and anti-IgG-horseradish peroxidase (HRP) was purchased from DAKO (High Wycomde, Bucks, UK). Anti-human tumor necrosis factor (TNF)-α antibody and recombinant human TNF-α were obtained from R & D Systems (Minneapolis, MN).

Plant material and fraction preparation – *Citrus unshiu* was obtained from an Oriental drug store (Iksan, Korea) and identified at the Department of Oriental Pharmacy, Wonkwang University. Voucher specimen has been deposited in the Herbarium at the Department of Oriental Pharmacy, Wonkwang University. The plant was cut into small pieces and boiled twice in water (100 g, 500 ml × 2) for 2 h. The residues were removed through a 0.45 μm filter, and the filtrate was lyophilized. The dried extract was weighed (yield, 8.5%) and reconstituted into aqueous solution of concentration, 50 mg/ml.

Cell culture – The human leukemic mast cell line HMC-1 (kindly provided by Dr. Y. Kitamura, Department of Pathology, Osaka University Medical School, Japan) was cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% heat inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 IU/ml penicillin, 50 μg/ml streptomycin, and 1.2 mM α-thioglycerol at 37°C under 5% CO_2 in air.

Cell viability – To determine the cell viability, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylthiazolium bromide (MTT, Sigma, St. Louis, MO) assay was performed. HMC-1 cells were seeded at 5×10^4/ml densities in 96-well plates (Nunc, Denmark) in 100 μl cell suspension per well. Each group had three wells with a non-treated group as control. CUWE (10, 100, and 1000 μg/ml) was added to each well and incubated at 37°C, 5% CO_2. After 48 h of incubation, MTT (5 mg/ml) 10 μl was added to each well and cultured for another 4 h. The supernatant was discarded and dimethyl sulphoxide (DMSO, Sigma, St. Louis) 100 μl added. When the formazan crystals were dissolved, optical density (O.D.) was measured using an ELISA reader at 540 nm. The optical density of formazan formed in control (untreated) cells was taken as 100% of viability.

Enzyme-linked immunosorbent assay for TNF-α – Cells were seed at 1×10^6 cells/ ml in 24 well tissue culture plates and pretreated with various concentration of CUWE (10, 100, and 1000 μg/ml) 30 min before trypsin (100 nM) stimulation. Eight hours after trypsin stimulation, TNF-α concentrations in the supernatant were measured as commercial instruction (Pharmingen assay, SanDiego, CA). Briefly, ELISA plates (Falcon, Becton Dickinson Labware, USA) were coated overnight at 4°C with anti-human TNF-α antibody diluted in coating buffer (0.1 M carbonate, pH 9.5) and then washed four times with PBS containing 0.05% tween 20 (PBS-T). Non-specific protein binding sites were blocked with assay diluent (PBS containing 10% FBS, pH 7.0) for at least 1 h, and 100 μl of each sample or TNF-α standards diluted in assay diluent were applied to wells. After incubation for 2 h, 100 μl of working detector (biotinylated anti-TNF-α monoclonal antibody and avidin-HRP reagent) was added and incubated for 1 h. Consequently, 100 μl of substrate solution (tetramethylbenzidine (TMB) and H_2O_2) was added to wells and incubated for 30 min in the dark before stopping the reaction by 50 μl stop solution (2 N H_2SO_4) and absorbance was read at 450 nm. All subsequent steps took place at room temperature, and all standards and samples were assayed in triplicate.

RNA extraction and reverse-transcription PCR (RT-PCR) for TNF-α and tryptase – CUWE-pretreated HMC-1 cells were stimulated with trypsin (100 nM) for 2 h. Total RNA was isolated from the harvested cells using the easy-BLUE™ RNA extraction kit (iNtRON Biotech, Korea). The concentration of total RNA in the final elute can be determined by spectrophotometry. Total RNA (5 μg) was converted to cDNA by reverse transcriptase at 37°C for 90 min using first-strand cDNA synthesis kit (Amersham Pharmacia Biotech). PCR amplication was performed as follows: tryptase (94°C for 45 sec, 50°C for 1 min, 72°C for 1 min: 30 cycles), TNF-α (94°C for 1 min, 60°C for 1 min, 74°C for 1 min: 30 cycles), GAPDH (94°C for 1 min; 60°C for 2 min; 72°C for 1 min: 30 cycles). Primers used in this study were as follows: tryptase (forward primer 5'-AGGATGCTG AATCTGCTGCTGCTG-3' and reverse primer 5'-TCACG GCTTTTTGGGGACATAGTG-3': 831 bp) (Vanderslice *et al.*, 1990), TNF-α (forward primer 5'-CAAAG TAG ACCTGCCCAGAC-3' and reverse primer 5'-GACCT CTCTCTAATCAGCCC-3': 490 bp), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward primer 5'-CCATGTTCGTCATGGGTGTGAACCA-3' and reverse primer 5'-GCCAGTAGAGGCAGGGA TGATGTTC-3': 251 bp) (Lee *et al.*, 2000). Final PCR products were separated on 1% agarose gels and photographed under UV light.

Western blot analysis for ERK – CUWE-pretreated

HMC-1 cells were stimulated with trypsin (100 nM) for 15 min. The cells were lysed with ice-cold lysis buffer (iNtRON Biotech, Korea). Western blot analysis was performed according to a standard procedure. Lysates (50 μg of protein) was separated by SDS-PAGE with 12% acrylamide gel and transferred on PVDF membrane (Millipore). After blocking with 3% BSA, membranes were blotted with anti-human-phospho-ERK antibody for 12 h at 4°C. HRP-conjugated antibody against rabbit IgG was used as a secondary antibody. Finally, epitopes on proteins recognized specifically by antibodies were visualized by using enhanced chemiluminescence (ECL) detection kit (Amersham, Milan). After stripping, the membranes were reprobed with anti-ERK antibody as respective loading controls.

Trypsin activity assay – Trypsin activity was assessed by the amidolytic ability of trypsin on a substrate BAPNA (Smith *et al.*, 1984). Samples were added to each tube containing 2.85 ml of 0.1 M Tris-HCl (pH 8.0) and 1 M glycerol, and the assay was started with the addition of 20 mM substrate in dimethyl sulfoxide (DMSO). After 30 min incubation at 37°C, the reactions were stopped by the addition of 50 μl of glacial acetic acid, and the absorbance was measured at 405 nm with spectrophotometer and compared to those given by standard curves of pnitroanilide to determine the concentration of product released. Trypsin concentration used in this study is 20 μg/ml and SBTI (2, 20, and 200 μg/ml) was used as control. Inhibition percentages of trypsin activity were calculated using the following equation:

$$\% \text{ of inhibition} = \frac{(A-B)}{A} \times 100$$

where A is a trypsin activity without CUWE and B is a trypsin activity with CUWE.

Statistical analysis – The results were expressed as mean±S.E. for the number of experiments. Statistical significance was compared between control and CUWE-pretreated group by the Student's *t*-test. Each experiment was repeated at least three times and yielded comparable results. Values with $p < 0.05$ were considered significant.

Results

Effect of CUWE on cell viability of HMC-1 cells – To examine the direct cytotoxic effect of CUWE, cell viability was examined after treatment of HMC-1 cells with three concentrations of CUWE (10, 100, and 1000 μg/ml) for 48 h. CUWE did not affect the viability of HMC-1 cells even 1000 μg/ml (Fig. 1).

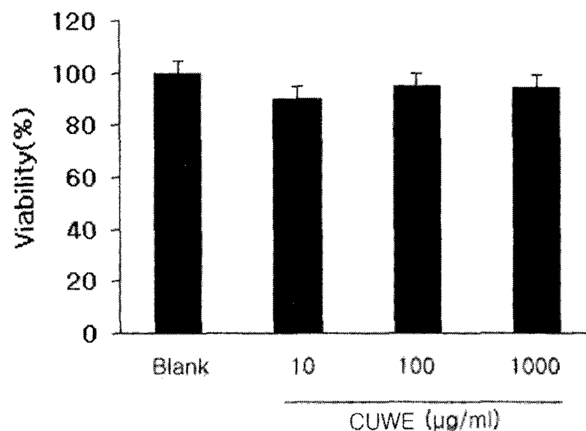

Fig. 1. Effect of CUWE on cell viability of HMC-1 cells. The cells were incubated with various concentration of CUWE (10, 100, and 1000 μg/ml). Cell viability was evaluated by MTT assay (expressed as percent of control). Values are mean±S.E. of three independent experiments.

Effect of CUWE on trypsin-induced TNF-α secretion – To study the effect of CUWE on trypsin-induced TNF-α secretion from HMC-1 cells, HMC-1 cells were pretreated with three concentrations of CUWE (10, 100, and 1000 μg/ml) for 30 min and then stimulated with trypsin (100 nM) for 8h. TNF-α secreted from HMC-1 cells were measured by ELISA. Trypsin (100 nM) induced a 10-fold increase in TNF-α secretion compare to trypsin-nontreated cells. CUWE (1000 μg/ml) significantly inhibited TNF-α secretion in a dose-dependent manner (Fig. 2). CUWE showed 48% inhibition for TNF-α in

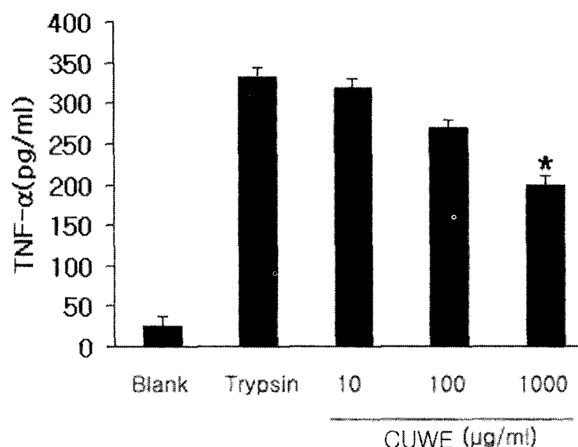

Fig. 2. Effect of CUWE on TNF-α secretion in trypsin-stimulated HMC-1 cells. The cells (1×10^6 cells/ml) were pre-incubated with three concentrations of CUWE (10, 100, and 1000 μg/ml) before stimulation with trypsin (100 nM) for 8 h. TNF-α levels in supernatant were measured by ELISA. Values are the mean±S.E. of duplicate determinations from three separate experiments (*$p < 0.05$).

concentration of 1000 μg/ml. This result demonstrates that CUWE modulate TNF-α secretion in trypsin-stimulated HMC-1 cells.

Effect of CUWE on trypsin-induced TNF-α and tryptase mRNA expression – To study the effect of CUWE on TNF-α synthesis in trypsin-stimulated HMC-1 cells, CUWE pretreated HMC-1 cells were stimulated with trypsin for 2 h. Tryptase and TNF-α mRNA expression levels in intracellular of HMC-1 cells activated by trypsin were determined by RT-PCR. Trypsin led to an increase of TNF-α and tryptase mRNA expression in HMC-1 cells. CUWE inhibited TNF-α mRNA expression in a dose-dependent manner and 1000 μg/ml CUWE significantly inhibited to near control level (Fig. 3). Especially, CUWE (1000 μg/ml) inhibited tryptase (Fig. 4). These results indicate that CUWE modulates TNF-α and tryptase synthesis in trypsin-stimulated HMC-1 cells.

Effect of CUWE on trypsin-induced ERK phosphorylation – To examine the effect of CUWE on trypsin-induced ERK phosphorylation, CUWE-pretreated HMC-1 cells were stimulated with trypsin for 15 min. Major immunoreactive band was identified with phosphorylated ERK by Western blot analysis and the level of

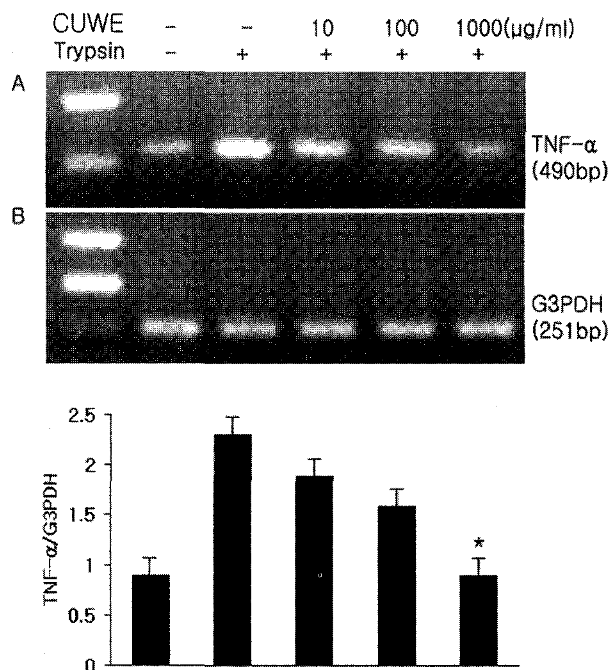

Fig. 3. Effect of CUWE on TNF-α mRNA expression in trypsin-stimulated HMC-1 cells. The cells were pre-incubated with three concentrations of CUWE (10, 100, and 1000 μg/ml) before stimulation with trypsin (100 nM) for 2 h. Total RNA (5 μg) was converted to cDNA by reverse transcriptase. GAPDH mRNA was carried out in parallel to confirm equivalency of cDNA preparation (B). Size marker is λDNA/HaeIII. *Significantly different ($P < 0.05$) from trypsin only-stimulated group.

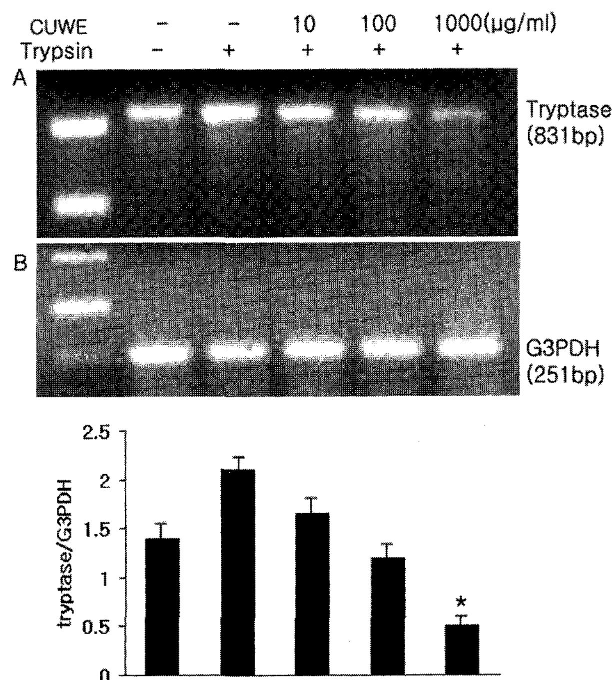

Fig. 4. Effect of CUWE on tryptase mRNA expression in trypsin-stimulated HMC-1 cells. The cells were pre-incubated with three concentrations of CUWE (10, 100, and 1000 μg/ml) before stimulation with trypsin (100 nM) for 2 h. Total RNA (5 μg) was converted to cDNA by reverse transcriptase. GAPDH mRNA was carried out in parallel to confirm equivalency of cDNA preparation (B). Size marker is λDNA/HaeIII. *Significantly different ($P < 0.05$) from trypsin only-stimulated group.

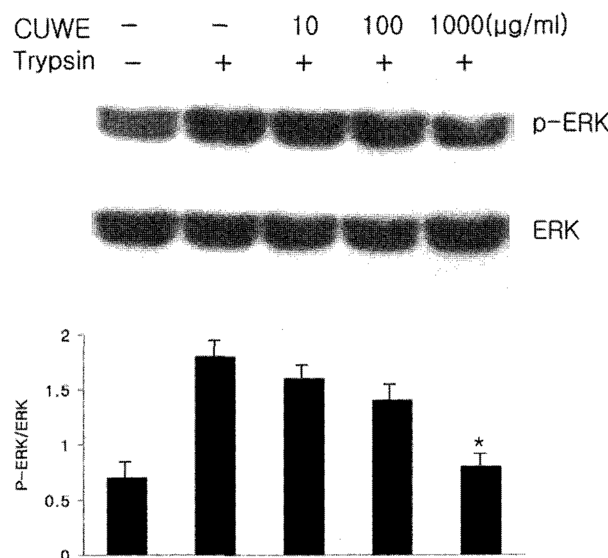

Fig. 5. Effect of CUWE on ERK1/2 phosphorylation in trypsin-stimulated HMC-1 cells. HMC-1 cells (1×10^6 cells/ml) pretreated with CUWE (10, 100, and 1000 μg/ml) were cultured for 15 min with trypsin (100 nM), and ERK1/2 (44/42 kDa) phosphorylation were analyzed by Western blot using specific antibodies against ERK1/2 and phospho-ERK1/2. *Significantly different ($P < 0.05$) from trypsin only-stimulated group.

Table 1. Inhibitory effect of CUWE on trypsin activity

Treatment		Inhibition(%)
SBTI	2	82.6 ± 1.1
(μg/ml)	20	88.0 ± 1.9
	200	94.0 ± 2.3
CUWE	10	0.5 ± 0.2
(μg/ml)	100	9.8 ± 0.5
	1000	30.0 ± 0.9*

SBTI : Soybean trypsin inhibitor, ND: not done, Trypsin concentration is 20 μg/ml. Values are the mean ± S.E. of duplicate determinations from three separate experiments (*$p < 0.05$).

phosphorylation was described as the relative ratio of band density against that of whole ERK (Fig. 5). Trypsin (100 nM) stimulation (Fig. 5, lane 2) induced 3-fold increase in the phosphorylation of ERK compared to that of trypsin-nontreated cells (Fig. 5, lane 1). CUWE pretreatment (1000 μg/ml) resulted in the blockade of trypsin-induced ERK phosphorylation, without affecting the levels of whole ERK (Fig. 5, lane 5). The result suggests that the inhibitory effect of CUWE on trypsin-stimulated mast cell activation could be caused through the suppression of ERK activation pathway.

Effect of CUWE on trypsin activity – To determine whether CUWE can modulate just trypsin activity, trypsin activity was measured by using BAPNA as substrate. Assay was performed with three concentrations of CUWE (10, 100, and 1000 μg/ml) and SBTI (2, 20, and 200 μg/ml) was used as control. CUWE inhibited trypsin activity in a dose-dependent manner although inhibition percentage of CUWE is much lower than that of SBTI. CUWE showed 30% inhibition in concentration of 1000 μg/ml (Table 1).

The result suggests that the inhibitory effect of CUWE on trypsin-stimulated mast cell activation could be caused through inhibition of trypsin activity.

Discussion

Trypsin is found at high levels in the colon lumen of patients with UC (Raithel *et al.*, 2001). We previously showed that the levels of tryptase and tumor necrosis factor (TNF)-α expression in intestinal mast cells were significantly elevated in UC tissues (Kim *et al.*, 2003) and trypsin induced TNF-α and tryptase production in human leukemic mast cell-1 (HMC-1) (Kang *et al.*, 2003). Therefore, agents that are nontoxic and can inhibit the production of TNF-α and tryptase in trypsin-stimulated intestinal mast cells can be useful for the inhibition of intestinal inflammation. Anti-TNF-α antibody (Infliximab) administration has a beneficial effect on inflammatory bowel disease (Gratz *et al.*, 2002; Kohn *et al.*, 2002). A tryptase inhibitor (APC 2059) treated mildly to moderately active ulcerative colitis (Tremaine *et al.*, 2002). We previously also showed that curcumin inhibited TNF-α secretion from trypsin-stimulated HMC-1 by block of ERK pathway (Baek *et al.*, 2003). In the present study, CUWE significantly reduced trypsin-induced TNF-α and tryptase production through the inhibition of ERK phosphorylation.

In order to examine another inhibitory mechanism of CUWE on inflammatory mediator production in trypsin-stimulated HMC-1, we tested the effect of CUWE on trypsin activity. Soybean trypsin inhibitor (SBTI) and Bowman-Birk inhibitor are trypsin inhibitors. SBTI inhibited collagenase-induced edema in the rat paw (Souza Pinto *et al.*, 1995). We previously showed that SBTI completely inhibited TNF-α secretion from trypsin-stimulated HMC-1 cells (Kang *et al.*, 2003). Moreover, Bowman-Birk inhibitor reduced colon inflammation in mice with dextran sulfate sodium-induced colitis (Ware *et al.*, 1999). Like this, trypsin inhibitors have anti-inflammatory effects. In this study, CUWE showed 30% inhibition on trypsin activity.

Taken together, CUWE might inhibit trypsin-induced TNF-α and tryptase production by inhibition of both ERK phosphorylation and trypsin activity. These results suggest that CUWE may be a therapeutic candidate for inflammatory disease due to trypsin.

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