Inhibitory Effect of Fermented Grape Pomace on Degranulation in RBL-2H3 Cells and an Analysis of Its Active Ingredients

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We investigated the anti-allergic effect of fermented grape pomace from Vitis vinifera Koshu (FG), using rat basophilic leukemia (RBL-2H3) cells. FG showed greater inhibitory effect on degranulation than Lactobacillus plantarum NB strain (NB strain), which was used for fermentation, and grape pomace (G). Furthermore, we separated FG into three fractions with distilled water containing 0.1% trifluoroacetic acid (acidified water) (F1), ethyl acetate (F2) and methanol (F3) in order to investigate the active ingredients in FG, and analyzed their inhibitory mechanisms on degranulation. The F2 sample, which contained mainly low polymerized phenolic ingredients, showed a strong inhibitory effect, whereas the F3 sample, which contained mainly polymeric proanthocyandins, showed the highest inhibitory effect among all samples. It was confirmed that phenolic ingredients in FG act on various stages of degranulation. These results suggest that FG may exert anti-inflammatory and anti-allergic effects by acting on various stages of the degranulation response of mast cells.

Keywords: fermented grape pomace, degranulation, RBL-2H3 cells, polymeric proanthocyanidins, anti-allergy

Introduction

Recently, the number of people suffering from type-1 allergy (allergy) such as food allergy, pollen allergy and atopic dermatitis has been increasing throughout the world (Andersson and Lidholm, 2003; Madsen, 2005). Safe and effective functional foods for the prevention and treatment of such allergy are required because of its serious threat to not only patients but also their families. There are several steps to the onset of allergic symptoms. Mast cells and basophils play an important role in the development of allergic inflammatory reactions, which are caused by the release of various chemical mediators, such as histamine and serotonin, by degranulation responses in these cells (Metzger, 1992; Puxeddu et al., 2003). First, antigen specific IgE binds to high-affinity IgE receptors (FceRI) expressed on the membrane surface of mast cells and basophils. Next, antigens cross-link to IgE binding to FceRI, which leads to a series of signal transductions via phosphorylations and Ca²⁺ influx (Kopeć et al., 2006; Turner and Kinet, 1999). Several transcription factors, such as nuclear factor (NF)-κB, NF-AT and AP-1 are activated, followed by the production of metabolites of arachidonic acid and cytokines, and finally degranulation occurs (Kitaura et al., 2000; Krishnaswamy et al., 2001).

Several reports have been published demonstrating that various polyphenols in fruits and vegetables have anti-allergic effects. Fujimura et al. (2007) reported that a major green tea catechin, (-)-epigallocatechin gallate, inhibited FcεRI expression via interaction with the 67-kDa laminin receptor in human basophilic KU812 cells. Itoh et al. (2009) recently reported that flavonoids isolated from strawberry extract inhibited both Ca²⁺ influx and protein tyrosine kinase (Syk) activation in IgE-mediated degranulation in rat basophilic leukemia (RBL-2H3) cells. Tokura et al. (2005) reported that apple condensed tannin (ACT) inhibited binding between IgE and FceRI in bone marrow-derived cultured mast cells (BMMC).

Grape contains abundant polyphenols such as catechin,
epicatechin, gallic acid and polymeric proanthocyanidins mainly in the seeds and skin (Kammerer et al., 2004; Scalbert and Williamson, 2000). As about 80% of all grapes are used for wine making, a large quantity of grape pomace, including seeds and skin, is produced and discarded during processing (Schieber et al., 2001).

In this study, we utilized grape pomace from Vitis vinifera Koshu for the investigation of anti-allergic effect. We fermented grape pomace with lactic acid bacteria (LAB) and compared the inhibitory effect of LAB, grape pomace and fermented grape pomace (FG) on degranulation in RBL-2H3 cells. Furthermore, we explored the active ingredients in the FG sample and analyzed the mechanism of the inhibitory effect on degranulation.

Materials and Methods

Grape pomace  Pomace of Vitis vinifera Koshu, which includes the seeds, skin and pulp (grape pomace), was used. Grape pomace was collected from wine factories in Katsunuma City of Yamanashi prefecture in September 2009. Grape pomace was prepared by lyophilizing and crushing (G), and stored at 4°C.

Lactic acid bacteria  Lactobacillus plantarum NB strain (NB strain) isolated from grape was used. NB strain was incubated in lactic acid bacterial medium for food application as follows: distilled water, 900 ml; yeast extract (Nacalai Tesque, Kyoto, Japan), 10.0 g; KH₂PO₄, 1.0 g; KH₂PO₄, 2.0 g; CH₃COONa, 3.0 g; (NH₄)₂SO₄, 3.0 g; glucose, 30.0 g; protease treated skim milk (PSM, 20.0 g powdered skim milk (Yotsuba Co., Ltd., Sapporo, Japan) and 5 N orientase (HBI Enzyme Inc., Shiso, Japan)), 100 ml; MgSO₄·7H₂O, 0.58 g; FeSO₄·7H₂O, 0.034 g; MnSO₄·5H₂O, 0.12 g; a few drops of conc-H₂SO₄, pH 6.9), cultivated at 33°C for 48 h, harvested by centrifugation at 2000 rpm for 15 min, washed 3 times with distilled water, and lyophilized.

Standard phenolic substances  (+)-Catechin was purchased from Nacalai Tesque. (−)-Epicatechin was purchased from Sigma (St. Louis, MO). Procyanidin B1, B2 and B3 were kindly provided by Dr. Makabe (Graduate School of Agriculture, Shinshu University) (Mohri et al., 2009). Procyanidin C1 was purchased from Wako (Osaka, Japan).

Cells  RBL-2H3 cells were obtained from Cell Resource Center for Biomedical Research (Tohoku University). Cells were cultured in RPMI-1640 (RPMI, Sigma) containing 1% antibiotic antimycotic solution (Sigma) and 10% heat-inactivated fetal bovine serum (FBS, Biowest, Paris, France) in a 37°C, 5% CO₂ incubator.

Fermentation of grape pomace  The G sample, diluted with an equal amount of distilled water containing 0.5% glucose, was adjusted to pH 5.5 with NaOH and autoclaved at 121°C for 15 min. NB strain (5×10⁶ cells/ml) was added to this G fluid and fermented at 33°C for 48 h, then lyophilized and crushed (FG). The FG sample was stored at 4°C until experiments. NB strain accounted for about 10% of FG weight.

Fractionation of FG sample  Forty milligrams of the FG sample in 1 ml distilled water was held at 4°C overnight and then centrifuged at 1000 rpm for 5 min. The supernatant was collected and lyophilized (F0). F0 sample dissolved in distilled water was applied to a Sep-Pak (Vac 20 cc C18-5 g, Waters, Milford, MA) preconditioned with distilled water containing 0.1% trifluoroacetic acid (acidified water) and methanol. The column was eluted consecutively with acidified water, ethyl acetate and methanol. These fractionated samples were evaporated at 37°C under reduced pressure until all organic solvent was removed, and then lyophilized. Each fraction was termed F1, F2 and F3, respectively (Fig. 2).

β-Hexosaminidase assay  We measured the activity of β-hexosaminidase from RBL-2H3 cells as an indicator of degranulation using the method of Shinomiya et al. (2009) with some modifications. One hundred microliters of RBL-2H3 cells (2.5×10⁶ cells/ml) in HEPES-tyrode buffer (137 mM NaCl, 5.6 mM glucose, 2.7 mM KCl, 0.5 mM NaH₂PO₄, 1.0 mM CaCl₂, 10 mM HEPES and 0.1% BSA, pH 7.3) were cultured in 96-well microtitre plates (BD-Falcon, Franklin Lakes, NJ) with 10 μl of each sample dissolved in distilled water or distilled water only (control) at 37°C for 30 min. Fifty microliters of monoclonal anti-dinitrophenyl-IgE (DNP-IgE) (7.5 μg/ml, clone SEP-7, Sigma) was then added to cells and incubated for 2 h, followed by the additional incubation for 30 min with 50 μl of DNP-human serum albumin (DNP-HSA) (40 ng/ml, Sigma). Forty microliters of supernatant was transferred to 96-well NUNC-Immuno plates (Nunc, Roskilde, Denmark), and 40 μl of 1 mM p-nitrophenyl-N-acetyl-β-D-glucosaminide (PNAG, Sigma) was added to each well and incubated at 37°C for 1 h. The reaction was stopped with 160 μl of 0.1 M carbonate buffer (0.1 M NaHCO₃ and 0.1 M Na₂CO₃, pH 10.0) and absorbance at 405 nm was measured by a Model 680 microplate reader (Bio-Rad, Hercules, CA).

For the investigation of the inhibitory effect of each sample on various degranulation stages, each sample was added to cells at three different stages. These samples were added at 30 min before DNP-IgE addition (a) and 5 min before (b) and 5 min after DNP-HSA addition (c).

Total phenolic content measurement  Total phenolic contents of the F0, F1, F2 and F3 samples were determined by the Folin-Ciocalteu method (Singleton and Rossi, 1965). Five hundred microliters of 1 N Folin-Ciocalteu reagent (Nacalai Tesque) was added to 500 μl of each sample properly diluted with distilled water. After a reaction time of 3
ingredients in each sample were identified by retention time and spectral features of standard phenolic substances, or conformity to the studies carried out in our laboratories. In addition, oligomeric and polymeric proanthocyanidins in the F3 sample were judged by the result of the butanol-hydrochloric acid method and maximum absorbance among 270 – 290 nm.

Statistical analysis The results of the inhibitory effect of each sample on degranulation in RBL-2H3 cells are presented as mean ± standard deviation (SD). Statistical evaluation for the differences between control (cont) and each sample was performed by two-tailed Student’s t-test, and differences between each sample were assessed using the Bonferroni/Dunn post hoc test. In all cases, probability values of less than 0.05 were considered statistically significant.

Results Effects of NB strain, G and FG samples on degranulation in RBL-2H3 cells We first investigated the effects of NB strain, G and FG samples on degranulation in RBL-2H3 cells. The activity of β-hexosaminidase released from cells was measured as an indicator of degranulation. The significant inhibitory effect of the FG sample (300 μg/ml) was observed when each sample was added to cells at 30 min before the addition of DNP-IgE (P<0.05) (Fig. 1). On the other hand, NB strain (30 μg/ml) and the G sample (300 μg/ml) did not show a significant inhibitory effect. This result suggests that the FG sample may have a greater inhibitory effect on degranulation in RBL-2H3 cells than NB strain and the G

![Fig. 1. Effects of NB strain, G and FG samples on degranulation in RBL-2H3 cells.](image-url)
Inhibitory effect of each fraction from the FG sample on degranulation in RBL-2H3 cells

To identify active ingredients in the FG sample, we next separated the FG sample into three fractions and investigated the effect of each fraction on degranulation in RBL-2H3 cells. Figure 2 shows the method of fractionation and the recovery rate of each fraction from the FG sample. FG dissolved in distilled water (F0) was applied to a Sep-Pak and eluted with acidified water (F1), ethyl acetate (F2) and methanol (F3). The recovery rates of F1, F2 and F3 were 68.2%, 6.55% and 5.93%, respectively (% of F0).

Figure 3A shows the inhibitory effect of each sample at a concentration of 1 mg/ml. All samples other than the F1 sample showed strong inhibitory effects on degranulation in RBL-2H3 cells. Strangely, the F1 sample seemed to enhance degranulation rather than to inhibit it. The 50% inhibitory concentration (IC50) of each sample was evaluated as shown in Fig. 3B. The result was nearly the same as that shown in Fig. 3A. The F3 sample showed the highest inhibitory effect.

Fig. 2. Fractionation and the recovery rate of FG sample.

The FG sample, dissolved in distilled water, was centrifuged and the supernatant was collected and lyophilized (F0). The F0 sample, in distilled water, was applied to a Sep-Pak and eluted consecutively with acidified water (F1), ethyl acetate (F2) and methanol (F3). These fractionated samples were evaporated at 37°C under reduced pressure until all organic solvent was removed, and then lyophilized. After this, the yield was weighed.

Fig. 3. Inhibitory effect and the IC50 value of each fraction on degranulation in RBL-2H3.

Each sample (F0-F3) at a concentration of 1 mg/ml was added to RBL-2H3 cells, incubated and the activity of β-hexosaminidase was determined in the manner described in the Fig. 1 legend (A). The results are expressed as mean ± S.D. (n=3). The 50% inhibiting concentration (IC50) of each sample was calculated by regression analysis of the dose-response curves (B). Different letters indicate statistical differences (P<0.05) between each sample.
Fig. 4. Inhibitory effect of each fraction on various degranulation stages in RBL-2H3.
F0 (1 mg/ml, A), F1 (1 mg/ml, B), F2 (0.5 mg/ml, C) and F3 (0.5 mg/ml, D) were added to RBL-2H3 cells at three different stages of degranulation: addition at 30 min before DNP-IgE addition (a) and 5 min before (b) and 5 min after DNP-HSA addition (c). The cells were incubated and the activity of β-hexosaminidase was determined in the manner described in the Fig. 1 legend. The results are expressed as mean ± S.D. (n=3-6). *P<0.05, **P<0.001, ***P<0.0001 vs. the % of control.
among all samples (IC$_{50}$: 0.12 mg/ml). IC$_{50}$ values of the F0 and F2 samples were 0.92 and 0.37 mg/ml, respectively. The F1 sample did not inhibit degranulation, even at a concentration of 1 mg/ml. These results suggest that active ingredients are contained in the F2 and F3 samples.

**Variations in the inhibitory effects of each fraction on degranulation stages in RBL-2H3 cells**

To analyze the inhibitory mechanism of each fraction, we attempted to add samples at three different stages of degranulation in RBL-2H3 cells as follows: addition at 30 min before the addition of DNP-IgE (a), 5 min before (b) and 5 min after the addition of DNP-HSA (c). The F0 and F2 samples inhibited degranulation only when samples were added to cells at (a) stage ($P<0.05$ and $P<0.0001$, respectively) (Fig. 4A and C). The F0 sample showed the opposite enhanced effect by addition at (c) stage. On the other hand, the F3 sample inhibited degranulation at all stages ($P<0.001$) (Fig. 4D). Interestingly, the F3 sample showed an inhibitory effect even at (c) stage when degranulation might already have occurred and various chemical mediators might have been released from RBL-2H3 cells. The F1 sample did not show an inhibitory effect by addition at any stage. An enhanced effect was seen by addition at (a) and (c) stages (Fig. 4B).

**The properties of phenolic ingredients in each fraction from the FG sample**

We measured total phenolic and proanthocyanidin contents in each fraction by the Folin-Ciocalteu method and butanol-hydrochloric acid method, respectively. The F3 sample contained abundant total phenolic and proanthocyanidin contents, which were approximately 2 to 3-fold higher than those in the F2 sample (Fig. 5A and B).

To obtain more reliable confirmation, we analyzed the phenolic ingredients in the F1, F2 and F3 samples by HPLC. We identified the major ingredients in each fraction by the retention time and spectral features of standard phenolic substances, or conformity to studies carried out in our laboratories to date. Results suggested that the F2 sample contained monomeric (+)-catechin (3) and (-)-epicatechin (5), dimeric procyanidin B1 (1), B2 (4) and B3 (2) and trimeric procy-anidin C1 (7) (Fig. 6B). As all peaks (8) except 3 peaks (9-11) in the F3 sample had maximum absorbance among 270-

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![Fig. 5. Total phenolic and proanthocyanidin contents of each fraction.](image-url)

Total phenolic content of each sample (F0-F3) was determined by the Folin-Ciocalteu method (A). (-)-Gallic acid was used as the standard. Total phenol was expressed as mg of gallic acid equivalents (GAE eq)/g of sample. The butanol-hydrochloric acid method was used to compare proanthocyanidin content of each sample (F0-F3) (B). The result is expressed as optical density (OD).
Fig. 6. HPLC chromatogram for each fraction at 280nm.

F1 (A), F2 (B) and F3 (C) samples were analyzed by HPLC. Chromatographic separation was carried out on a Luna 5μ C18 column. Solvents were 0.1% trifluoroacetic acid (a) and 0.1% trifluoroacetic acid in acetonitrile (b). The gradient program was started with 5% (b) and changed to obtain 15% (b) at 30 min, 40% (b) at 45 min, and 75% (b) at 50 min. The 75% (b) was maintained until 65 min. Detection was performed at 280 nm. (mAU: milli absorbance units). 1, procyanidin B1; 2, procyanidin B3; 3, (+)-catechin; 4, procyanidin B2; 5, (-)-epicatechin; 6, unknown; 7, procyanidin C1; 8, oligomeric and polymeric proanthocyanidins; 9, unknown; 10, unknown; 11, unknown.
290 nm, the F3 sample contained mainly oligomeric and polymeric proanthocyanidins (Fig. 6C). On the other hand, the F1 sample seemed to contain no obvious major phenolic substances (Fig. 6A).

**Discussion**

Grape is one of the most cultivated fruits in the world, with global production exceeding 60 million tons per year (Schieber *et al.*, 2001). During the processing of grapes, a large quantity of pomace, including the seeds and skin, is produced and becomes food industrial waste. In this study, we utilized grape pomace for an investigation of anti-allergic effect due to its value as a source of various polyphenols, which are known as functional materials for human health (Fraga *et al.*, 2010). We fermented grape pomace with NB strain with the expectation that fermentation would improve the anti-allergic effect according to the conversion of phenolic ingredients and the probiotic effects of LAB.

To compare the inhibitory effects of NB strain, G and FG samples on degranulation, we added these samples to the culture of RBL-2H3 cells, which are frequently used as a mast cell model (Funaba *et al.*, 2003; Ortega *et al.*, 1988), and investigated the activity of β-hexosaminidase released from cells as an indicator of degranulation. The FG sample (300 µg/ml), but not NB strain (30 µg/ml) or the G sample (300 µg/ml), showed significant inhibitory effect on degranulation (Fig. 1). This result suggests that the FG sample has a greater inhibitory effect on degranulation in RBL-2H3 cells than NB strain and the G sample. Total phenolic content measured by the Folin-Ciocalteu method in the FG sample was approximately 1.5-fold higher than that in the G sample, and this augmentation was not due to the heating by autoclave, adjustment of pH or incubation of FG, because total phenolic content in the G sample was not changed by these treatments (data not shown). This result suggests that phenolic ingredients in FG have been changed in some way by fermentation. Thimothe *et al.* (2007) also reported that total phenolic content in grape pomace was increased by fermentation. In another study on fermented herbal extracts, it was reported that the glucosidic form of isoflavones changes to aglycones during fermentation with LAB (Seong *et al.*, 2009). Although the obvious change of phenolic ingredients during fermentation with NB strain remains unclear, fermentation seems to improve the inhibitory effect of grape pomace on degranulation in RBL-2H3 cells.

We attempted to identify active ingredients in the FG sample by fractionation using a Sep-Pak, and analyzed the inhibitory mechanisms of degranulation in RBL-2H3 cells. The F3 sample showed the highest inhibitory effect among all samples. The F2 sample also inhibited degranulation more strongly than the non-fractionated F0 sample (Fig. 3A and B). In this experiment, we observed that the F1 sample, which was eluted with acidified water, rather enhanced degranulation. This effect was observed again in the experiment shown in Fig. 4B. The yield of the F1 sample accounted for approximately 70% of the FG sample content (Fig. 2), and seemed to contain mainly saccharides such as pectin (Edashige *et al.*, 2008). As the FG sample is a mixture of various ingredients, certain ingredients in the F1 sample might hinder the inhibitory effect of the FG sample on degranulation in RBL-2H3 cells.

Measurement of the total phenolic and proanthocyanidin contents in each sample revealed that the F3 sample contained abundant proanthocyanidins (Fig. 5A and B). Furthermore, HPLC analysis demonstrated that the F2 sample contained monomeric and low polymerized phenolic ingredients such as catechin, epicatechin, procyanidin B1, B2, B3 and C1, while the F3 sample contained mainly oligomeric and polymeric proanthocyanidins. The F1 sample contained hardly any phenolic ingredients (Fig. 6).

Reflecting the variations of phenolic ingredients in each fraction, they showed various inhibitory effects on the three different degranulation stages (Fig. 4). Although the F3 sample inhibited degranulation at all stages, it is noteworthy that this sample inhibited the activity of β-hexosaminidase by addition at (c) stage when degranulation might already have occurred. Proanthocyanidins are known to bind to proteins and form soluble and insoluble complexes (Haslam, 1996; Okuda *et al.*, 1995). Therefore, it was speculated that one possible inhibitory mechanism of the F3 sample was direct interaction of proanthocyanidins with β-hexosaminidase released from RBL-2H3 cells to inactivate this protein. Since addition of the F3 sample at (a) stage showed slightly stronger inhibition than addition at (c) stage, the possibility remains that the F3 sample acts on any stage, from IgE binding to FceRI up to the release of chemical mediators. Study of these detailed mechanisms is now in progress. Although it has been reported that highly polymerized polyphenols are not usually absorbed in the intestine (Gonthier *et al.*, 2003), it is speculated that polymeric proanthocyanidins extracted from FG may be useful in diseases such as food allergy and inflammatory bowel disease (IBD) to suppress intestinal inflammation. Because the barrier function of the tight junction between intestinal epithelial cells is disturbed and intestinal permeability is increased in these diseases (Groschwitz and Hogan, 2009), inflammatory cells such as mast cells, basophils and eosinophils, could encounter these polyphenols in localized areas. On the other hand, the F2 sample inhibited degranulation only by addition at (a) stage. This result is similar to other studies reporting that monomeric or low polymerized
phenolic ingredients act on intracellular signaling cascades (Hämäläinen et al., 2007; Itoh et al., 2008; Kawai et al., 2007). The F0 sample also inhibited degranulation only by addition at (a) stage, and enhanced it by addition at (c) stage. This result suggests that certain ingredients in the FG sample may interfere with the inhibitory effect on degranulation as mentioned above.

In conclusion, we found that the FG sample inhibited degranulation in RBL-2H3 cells more effectively than NB strain and the G sample. FG contains various polyphenols, ranging from monomeric to polymeric, and proanthocyanidins in the F3 sample are specifically considered to be the most promising active ingredients, because this sample showed the smallest IC$_{50}$ value and inhibited degranulation by addition at all stages. It was speculated that phenolic ingredients in FG act on various degranulation stages. These results suggest that fermented grape pomace may be useful in anti-inflammatory and anti-allergic subjects. An evaluation of the anti-allergic effect of FG in in vivo experiments is now in progress.

References


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