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Proteinaceous Constituents of Red Cabbage Juice Increase IL-10, but Decrease TNF-α Secretions Using LPS-Stimulated Mouse Splenocytes

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ABSTRACT

This study investigates the anti-inflammatory effects of proteinaceous constituents from red cabbage (*Brassica oleracea* L. var.) juice (RC). The RC was first absorbed using activated charcoal (RCA), then partially purified using a Sephadex LH-20 column and finally precipitated using saturated ammonium sulfate solution to obtain the protein-rich fractions. The crude and protein-rich samples from RC were administered to lipopolysaccharide (LPS) - stimulated BALB/c mouse splenocyte cultures for 48 hours. The results showed that the lyophilized RC powder was rich in protein (30.2 ± 0.2%), carbohydrate (33.8 ± 6.0%), phenolics (2.8 ± 0.3%), and flavonoids (1.1 ± 0.1%). After the activated charcoal absorption treatment, the levels of protein (5.2 ± 0.0%), phenolics (0.2 ± 0.0%), and flavonoids (0.0 ± 0.1%) in RCA, except carbohydrate (39.2 ± 2.5%), were significantly decreased. The ammonium sulfate precipitated proteins from red cabbage juice, especially the chromatographic fraction 4 (F4) through the Sephadex LH-20 column, significantly ($P < 0.01$) increased an anti-inflammatory interleukin (IL-10) from 1,547 ± 106 to 2,060 ± 83 pg/mL, but significantly ($P < 0.05$) decreased a pro-inflammatory tumor necrosis factor (TNF-α) secretions using LPS-stimulated mouse splenocytes from 1,137 ± 71 to 789 ± 167 pg/mL. This study suggests that the proteinaceous constituents of red cabbage juice show an anti-inflammatory potential via increasing IL-10, but decreasing TNF-α secretions using LPS-stimulated mouse splenocytes.

Key words: red cabbage, proteinaceous constituents, interleukin (IL-10), tumor necrosis factor (TNF-α), lipopolysaccharide (LPS) - stimulated mouse splenocytes

INTRODUCTION

Inflammation can be described as the local accumulation of fluid, plasma proteins and white blood cells resulting from physical injury, infection, or a local immune response. However, inhibiting the overproduction of inflammatory mediators such as pro-inflammatory cytokines interleukin (IL-1β, IL-6), and tumor necrosis factor (TNF-α)[(1)] or enhancing the secretion of anti-inflammatory cytokine, such as IL-10[(2)], by immune effector cells may prevent a variety of inflammation-derived diseases. In general, IL-10 is produced by Th2 cells, macrophages and some B cells during the late phase of inflammation. IL-10 is a cytokine synthesis inhibitory factor and is a potent suppressant of macrophage functions. Macrophages are called pro-inflammatory cells, therefore IL-10, in some cases, is called an anti-inflammatory cytokine for its inhibitory activities of cytokine synthesis and macrophage functions. Dietary fruits and vegetables have been reported to decrease the risks for chronic diseases, such as cardiovascular diseases, arthritis, chronic inflammation and cancers[(3-6)]. We have found that the bio-active components from certain fruits and vegetables help to stimulate cell proliferation[(7)] and modulate cytokine secretions[(8)] by primary murine splenocytes. Strawberry, loquat, mulberry, and bitter melon juices exhibit prophylactic effects on LPS-induced inflammation of murine peritoneal macrophages[(9)]. Reasonably, components from certain fruits and vegetables seem to have anti-inflammatory potential.

Cabbage is a popular dietary vegetable worldwide. Our previous study has indicated that red cabbage (*Brassica oleracea* L. var.) juice demonstrated *in vitro* anti-inflammatory effects against lipopolysaccharide (LPS) - induced inflammation of murine primary splenocytes via increasing IL-10 and decreasing IL-6 secretions[(10)]. The pigment compounds in red cabbage juice such as malvidin 3-glucoside (oenin), malvidin 5-glucoside and malvidin

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3,5-diglucoside are associated with inhibiting the secretion of IL-6 by LPS-stimulated splenocytes. However, the pigment compounds in red cabbage juice seem not singular attributes against the inflammation of LPS-stimulated splenocytes. Recently a novel 116 kDa glycoprotein isolated from *Ulmus davidiana* Nakai regulates the expression of iNOS and COX-2 *in vivo* and *in vitro*[11]. The isolated glycoprotein also regulates the activities of manganese-superoxide dismutase, activator protein-1, and nuclear factor-κB stimulated by reactive oxygen radicals in LPS-stimulated HCT-116 cells *in vitro*[12], and modulates inflammatory related factors in mouse colonic tissues *in vivo*[13]. Human recombinant lipocortin 1, which is a family of proteins that can inhibit phospholipase A2 activity in *in vitro* systems, exhibits acute local anti-inflammatory properties in the rat paw edema test[14]. A malleable matrix composed of fermented whey proteins and lactic acid bacteria shows an anti-inflammatory potential in an atopic dermatitis model[15]. Proteins seem to be another potentially anti-inflammatory food constituent, although anti-inflammatory proteins from fruits or vegetables are rarely discussed. This study attempted to determine whether proteins in the red cabbage juice have anti-inflammatory effects *in vitro*.

We hypothesize that some proteins are potentially valuable as anti-inflammatory agents. This study isolated proteins from red cabbage juice and evaluated their anti-inflammatory effects on LPS-stimulated mouse splenocytes.

MATERIALS AND METHODS

I. *Preparation of Red Cabbage Juice*

The *B. oleracea* L. var. red cabbage was purchased from a local supermarket in Taichung, Taiwan, in winter. The fresh sample was immediately, without storage, squeezed into juice using a manual stainless screw squeezer (Vegetable & Fruit Grinder, manual type, Mei-Er-Then Co., Ltd, Taipei, Taiwan, ROC). The juice was centrifuged at 9000 ×g (4°C) for 30 min, and the supernatant was collected using suction filtration through filter papers (Toyo No. 5B, Toyo Roshi Kaisha, Ltd.). The filtrate was measured, lyophilized, and stored at -30°C for future use[7]. The lyophilized crude red cabbage juice powder is referred to as RC. A part of the RC powder was dissolved in de-ionized water and then slowly mixed with activated charcoal (1:1, w/w) to absorb small molecules. After standing at 4°C for 30 min, the mixture was filtered with filter paper to obtain activated charcoal-absorbed juice. The supernatant was lyophilized to obtain an activated charcoal-treated sample and is referred to as RCA. The second part of the RC was re-dissolved and subjected to size-exclusion chromatography using Sephadex LH-20 column to further separate the potential bioactive components[16]. Chromatographic fractions through the LH-20 column were collected. Total protein, carbohydrate, phenolic or flavonoid contents in RC, RCA, and the selected chromatographic fractions were determined.

II. *Chromatographic Fractionation of Red Cabbage Juice*

An aliquot of 20 grams of dry Sephadex LH-20 (Amersham Biosciences, Uppsala, Sweden) was mixed with 80 mL of methanol (Mallinckrodt Chemicals, Phillipsburg, NJ, USA) for 3 h to activate the gel. The activated gel/methanol (3:1, v/v) was carefully poured into a glass chromatographic column (15 mm i.d. × 370 mm). The column wall was washed with 10 mL of methanol. The column stood at room temperature to allow the Sephadex gel to settle. To equilibrate the column, the column was drained and washed with methanol for 1 h before use[16]. About 2 grams of RC was dissolved in 10 mL de-ionized water and then separated (1 mL) on the Sephadex LH-20 gel filtration column at a flow of 0.5 mL/min controlled by peristaltic pump (Iwaki pst-110). Elution was carried out with 90 mL of 100% methanol, followed by 60 mL of 50% methanol/water (v/v) and finally by 60 mL of water. The eluent (3 mL/tube) was collected using a fraction collector (Isco Retriever 500, Teledyne Technologies, Inc., Lincoln, NE, USA) and detected by absorbance at 280 nm using spectrophotometer (Hitachi Model 100-20, Hitachi, Ltd., Tokyo, Japan). As Figure 1 shows, these collected fractions are referred to as fraction 1 (F1, tube number 1-10, 30 mL), F2 (tube No. 11-40, 90 mL), F3 (tube No. 41-48, 24 mL), F4 (tube No. 49-54, 15 mL), and F5 (tube No. 55-61, 21 mL) and were concentrated using a rotary vacuum evaporator (Eyela Model 81-1, Tokyo Rikakika Co. Ltd., Tokyo, Japan) and then lyophilized using a freeze dryer (Panchum CT-5000D, Panchum Scientific Corp., Kaohsiung, Taiwan, ROC). The yields of individual fractions (F1-F5) were 0.2, 39.0, 1.3, 2.1, and 0.4%, respectively. This fractionation process was repeated several times and each individual fraction was pooled together[10]. A portion of each fraction was heated at 100°C for 20 min and then lyophilized to procure the heat-treated fraction powder. Based on the yields and protein contents, fractions 2 and 4 were collected separately for anti-inflammatory activity evaluation and bioactive component identification.

III. *Ammonium Sulfate Precipitation*

To further extract protein from red cabbage juice and its chromatographic fractions, aliquots of 0.05 gram of the lyophilized powders including RC, RCA, F2, and F4 were respectively re-dissolved in 5 mL of de-ionized water. The solution was brought to saturation with solid ammonium sulfate under stirring conditions and maintained at 4°C for 30 min to precipitate the protein. After centrifugation (1500 × g, 10 min), the precipitate was collected and re-suspended in de-ionized water. The solution was dialyzed at 4°C using dialysis tube of 3500

MWCO (Pierce Biotechnology, Rockford, IL, USA) against phosphate-buffered solution (PBS, 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2-7.4) by replacing the buffer 5 times for 2 days. After dialysis, the protein in dialysis tube was lyophilized to obtain protein products (PRC, PRCA, PF2, and PF4) and stored at -30°C for future use[17]. Unfortunately, the recovery of obtained protein samples was not calculated and the obtained proteins were not subjected to the endotoxin contamination test. However these samples were prepared from fresh materials and partially purified through complicated purification processes at low temperature, the complicated purification process might eliminate or prevent possibly contaminated endotoxin from red cabbage juice. To moderately characterize the protein products from red cabbage juice, aliquots of the obtained proteins were respectively mixed with SDS-PAGE sample buffer containing 2-mercaptoethanol. Aliquots of 10 μL from each protein product were analyzed by SDS-PAGE electrophoresis in a 12.5% polyacrylamide gel containing 0.1% SDS using a Mighty small II cell (8 × 7 cm) (Pharmacia Biotech). The electrophoresis was carried out at 110 V, 30 mA for about 1.0 h. The gels were then stained with commassie brilliant blue R stain.

IV. *Analysis of Possible Bio-Components in Red Cabbage Juice Products*

The protein contents of lyophilized samples were analyzed using the BCA (bicinchoninic acid) protein assay kit (Pierce Biotechnology, Rockford, IL, USA), according to the accompanying instruction, using a 96-well microtitre plate. Briefly, aliquots of 25 μL of each standards (bovine serum albumin, BSA) or unknown samples were pipeted into the appropriate microwell plate wells (Nunc). Aliquots of 200 μL of the working reagent (containing sodium carbonate, sodium bicarbonate, bicinchoninic acid, sodium tartrate in 0.1 M sodium hydroxide and cupric sulfate) were added to each well and shaken thoroughly on a plate shaker for 0.5 min. The plate was covered and incubated at 37°C for 30 min. After incubation, the plate was cooled to room temperature and the absorbance was measured at or near 562 nm on a plate reader (enzyme-linked immunosorbent assay (ELISA) reader, ASYS Hitech GmbH, Austria). Using the standard curve, the protein concentration for each unknown sample was determined.

The carbohydrate content in the lyophilized samples was analyzed using the phenol-sulfuric acid method. The basic protocol of Dubois *et al*.[18] was followed, with the micro-plate format modifications[19,20]. Briefly, aliquots of 100 μL of each standard (1000, 250, 125, 62.5, 31.5, and 0 μg glucose/mL) (glucose, Sigma-Aldrich Co., St. Louis, MO, USA) or unknown samples were pipeted into the 96-well microplate wells (Nunc). Aliquots of 20 μL of 5% phenol (Sigma-Aldrich Co., St. Louis, MO, USA) in water were added to each well and mixed thorourghly on a plate shaker for about 0.5 min. Aliquots of 100 μL of concentrated sulfuric acid were then carefully added to each well. The plate was covered and incubated at room temperature for 30 min. After incubation, the plate was cooled to room temperature and the absorbance was measured at 490 nm on a plate reader (ELISA reader, ASYS Hitech GmbH, Austria). Using the standard curve, the carbohydrate concentration for each unknown sample was determined. The data were expressed as g glucose equivalents/100 g lyophilized powder.

V. *Determination of Total Phenolic and Flavonoid Contents*

Total phenolic contents in lyophilized samples were determined using the Folin-Ciocalteu method[21,22]. Briefly, aliquots of 0.1 g lyophilized powder samples were respectively dissolved in 1 mL de-ionized water. This solution (0.1 mL) was mixed with 2.8 mL of de-ionized water, 2 mL of 2% sodium carbonate (Na_2CO_3, Wako Pure Chemical Industries, Ltd., Osaka, Japan), and 0.1 mL of 50% Folin-Ciocalteau reagent (Wako Pure Chemical Industries, Ltd., Osaka, Japan). After incubation at room temperature for 30 min, the reaction mixture absorbance was measured at 750 nm against a de-ionized water blank on a spectrophotometer. Gallic acid (GA, Sigma-Aldrich Co., St. Louis, MO, USA) was chosen as a standard. Using a seven point standard curve (0-2000 mg/L), the levels of total phenolic contents in the samples were determined in triplicate, respectively. The data were expressed as grams of gallic acid equivalents (GAE)/100g lyophilized powder[7].

The total flavonoid content was determined according to the 2,4-dinitrophenylhydrazine (DNP) colorimetric method described by Chang *et al*.[23]. Briefly, aliquots of 0.02 g of lyophilized samples were respectively dissolved in 0.6 mL de-ionized water. This solution was mixed with 0.4 mL of DNP (Sigma-Aldrich Co., St. Louis, MO, USA) solution (0.5 g of DNP in 50 mL of 50% methanol). After incubation at 50°C for 50 min, aliquot of 1 mL of potassium hydroxide (KOH, Wako Pure Chemical Industries, Ltd., Osaka, Japan) solution (0.5 g of KOH in 50 mL of 50% methanol) was added to the reactant mixture. Two minutes later, aliquot of 8 mL of 50% methanol was added and the mixture was centrifuged at 1000 ×g for 15 min to obtain the supernatant. The reaction mixture absorbance was measured at 494 nm against a de-ionized water blank on a spectrophotometer. Naringenin (Sigma-Aldrich Co., St. Louis, MO, USA) was chosen as a standard. Using a seven point standard curve (0-2000 mg/L), the levels of total flavonoid contents in the samples were determined in triplicate, respectively. Data were expressed as grams of naringenin equivalents (NE)/100 g lyophilized powder.

VI. *Preparation of Splenocyte Cultures*

The animal use protocol listed below has been

reviewed and approved by the Institutional Animal care and Use Committee (IACUC), National Chung Hsing University, Taiwan, ROC. The female BALB/c mice (6 weeks old) were obtained from the National Laboratory Animal Center, National Applied Research Laboratories, National Science Council in Taipei, Taiwan, ROC and maintained in the Department of Food Science and Biotechnology at National Chung Hsing University, College of Agriculture and Natural Resources in Taichung, Taiwan, ROC. The mice were housed and kept on a chow diet (laboratory standard diet). After the mice were acclimatized for 2 weeks, the animals were weighed, anaesthetized with diethyl ether and immediately bled using retro-orbital venous plexus puncture to collect blood. Immediately after blood collection, the animals were sacrificed using CO_2 inhalation for primary splenocyte culture studies. The splenocytes were prepared by aseptically removing spleens from mice. Spleens were homogenized in TCM (Celox Laboratories Inc., Lake Zurich, IL, USA) medium. Single spleen cells were collected and treated by lysing the red blood cells with RBC lysis buffer [0.017 M Trizma base (Sigma-Aldrich Co., St. Louis, MO, USA), 0.144 M ammonium chloride (Sigma-Aldrich Co.), pH 7.2, 0.2 μm filtered]. Splenocytes were isolated from each animal and adjusted to 1×10^7 cells/mL in TCM medium with a hemocytometer using the trypan blue dye exclusion method. The splenocytes (0.50 mL/well) in the absence or presence of mitogens (0.50 mL/well), such as lipopolysaccharide (LPS, Sigma-Aldrich Co., L-2654, 10 μg/mL) only, or both LPS and red cabbage juice products. All samples were sterilized using a filter with 0.22 μm pore size before use. Inflammatory mediators were induced by the endotoxin LPS[24]. To evaluate the anti-inflammatory potential of red cabbage juice products, an inflammation-concurrent cell culture model was designed using LPS addition and test samples together[9]. The plates were incubated at 37°C in a humidified incubator with 5% CO_2 and 95% air for 48 h. The plates were centrifuged at 200 ×*g* for 10 min. The supernatants in cell cultures were collected and stored at -70°C for cytokine assays.

VII. *Measurement of Pro-Inflammatory and Anti-Inflammatory Cytokine Levels Secreted by Splenocyte Cultures using an Enzyme-Linked Immunosorbent Assay (ELISA)*

The splenocyte culture supernatants of three biological replicates (n = 3) in each individual treatment were collected to measure pro-inflammatory cytokine (IL-6, and TNF-α) and anti-inflammatory cytokine (IL-10) levels using sandwich ELISA kits, respectively. The IL-6, IL-10, and TNF-α concentrations were assayed according to the cytokine ELISA protocol from the manufacturer's instructions (mouse DuoSet ELISA Development system, R&D Systems, Minneapolis, MN, USA). The sensitivity of these cytokine assays was 15.6 pg/mL. One hundred microliters of 1:180 diluted (with PBS) anti-mouse captured antibodies were added to 96-microwell plate wells (Nunc, Thermo Fisher Scientific, Rockford, IL, USA) and incubated overnight at 4°C. After incubation, plates were washed four times with ELISA wash buffer (0.05% Tween 20 in PBS, pH 7.2-7.4). To block non-specific binding, 200 μL of block buffer [1% Bovine serum albumin (BSA, Sigma-Aldrich Co., St. Louis, MO, USA), 5% sucrose in PBS with 0.05% NaN_3] were added to each well. The plates were incubated at room temperature for 1 hour. After incubation, plates were washed three times with ELISA wash buffer. Volumes of 100 μL of sample or standard in reagent diluent [0.1% BSA, 0.05% Tween 20 in Tris-buffered Saline (20 mM Trizma base, 150 mM NaCl), pH 7.2-7.4, 0.2 μm filtered] were added to the 96-microwell plate wells and the plates were incubated at room temperature for 2 hours. A seven point (in duplicate) standard curve using 2-fold serial dilutions in reagent diluent, and a high standard of 1000 pg/mL were conducted. After incubation, plates were washed four times with ELISA washing solution. One hundred microliters of detection antibody (biotinylated goat anti-mouse monoclonal antibody at 1:180 dilution in reagent diluent) were then added to each well. Plates were incubated at room temperature for 2 hours. After incubation, plates were washed six times with ELISA wash buffer. One hundred microliters of working Streptavidin -HRP (horseradish peroxidase) dilution were added to each well and the plates were again incubated at room temperature for 20 min. After incubation, plates were washed six times with ELISA washing buffer. One hundred microliters of substrate solution (tetramethylbenzidine, TMB) were pipeted into the 96-microwell plate wells. Plates were incubated at room temperature for 20 min to develop color. Fifty microliters of stop solution (2N H_2SO_4) were added to each well to stop the reaction. The plates were measured for absorbance at 450 nm on a plate reader (ELISA reader, ASYS Hitech GmbH, Austria). Using the seven point standard curves, the levels of cytokines in splenocyte cultures were determined, respectively

VIII. *Statistical Analysis*

Data are expressed as mean ± S.D. of three determinations and analyzed statistically using one-way ANOVA followed using Dunnett's parametric type test or Duncan's New Multiple Range test. Differences between the control and other treatments were considered statistically significant if $P < 0.05$. Statistical tests were performed using SPSS version 12.0.

RESULTS AND DISCUSSION

I. *Total Protein, Carbohydrate, Phenolic or Flavonoid Contents in RC, RCA, F2, and F4*

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To confirm the anti-inflammatory components in red cabbage juice (RC), total protein, carbohydrate, phenolic or flavonoid contents in RC and its products were determined. The total protein, carbohydrate, phenolic and flavonoid contents in RC and RCA are given in Table 1. The results reveal that RC is rich in protein (30.2 ± 0.2%), carbohydrate (33.8 ± 6.0%), phenolics (2.8 ± 0.3%) and flavonoids (1.1 ± 0.1%). After RC was absorbed by activated charcoal, the recovery of RCA was 65 ± 3%. The levels of protein (5.2 ± 0.0%), phenolics (0.2 ± 0.0%), and flavonoids (0.0 ± 0.1%), except carbohydrate (39.2 ± 2.5%), in RCA were significantly ($P < 0.05$) decreased compared to those in RC. The results suggest that activated charcoal absorbed components with lower molecular weight including phenolics, flavonoids, and some proteins in RC. Since activated charcoal could not absorb the carbohydrate in RC (Table 1), the carbohydrate in RC or RCA is suggested to be polysaccharides for their higher molecular weight. The protein, phenolics, and flavonoid contents in RC indeed reflected to the UV-Vis spectra[10]. As our prediction, the activated charcoal absorbed most pigment and protein components, except the carbohydrate, in red cabbage juice and resulted in the disappearance of absorption peaks in RCA[10]. Polysaccharides from different origins have been reported to have immunomodulatory potential[25,26], however the immunomodulatory activity of polysaccharides from red cabbage juice remain to be further clarified.

To further purify the protein components in RC, the RC was subjected to the Sephadex LH-20 column. The chromatographic profiles and obtained fractions are shown in Figure 1. The major fractions, F2 and F4, were selected and analyzed. The results showed that both F2 and F4 have higher contents of protein (45.3 ± 0.8% and 62.0 ± 0.2%, respectively) (Table 2) than that of RC (Table 1). In contrast, the carbohydrate contents in F2 and F4 (25.3 ± 0.7% and 24.3 ± 1.5%, respectively) (Table 2) were decreased compared to that of RC or RCA. Heating slightly decreased protein and carbohydrate contents in F4 (Table 2). However, the thermo-stable properties of proteins and carbohydrates in red cabbage juice should be further studied. Red cabbage juice samples throughout the LH-20 column increased the protein contents compared to that of RC, suggesting that the Sephadex LH-20 column is suitable for protein purification of red cabbage juice.

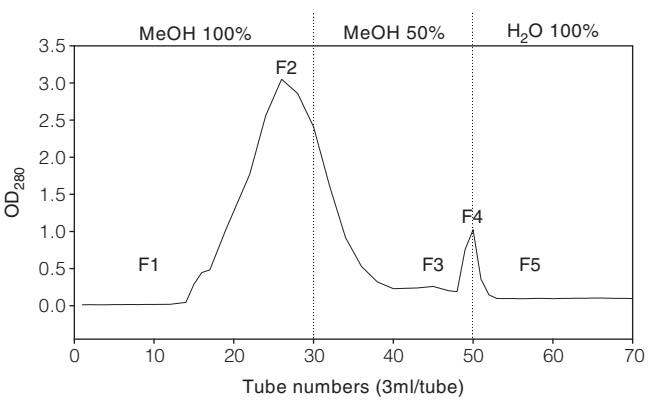

Figure 1. The chromatographic profile and obtained fraction from red cabbage juice through Sephadex LH-20 column detected by the absorbance at the wavelength of 280 nm.

II. *Effects of Protein from Red Cabbage Juice Product Administration to LPS-Stimulated Splenocytes*

To examine the anti-inflammatory potential of proteins from red cabbage juice (RC) products on LPS-stimulated murine splenocytes *in vitro*, isolated proteins from RC products were respectively administered to LPS-stimulated splenocyte cultures for 48 h. Secretions of pro-inflammatory cytokines including IL-6 as well

Table 1. Changes in compositions of red cabbage juice after absorbed by activated charcoal

Compositions (g/100 g)[a]	Treatments	
	Red cabbage juice (RC)[b,c]	Activated charcoal-absorbed red cabbage juice (RCA)[b,c]
Protein	30.2 ± 0.2 A	5.2 ± 0.0 B
Carbohydrate	33.8 ± 6.0 A	39.2 ± 2.5 A
Phenolics	2.8 ± 0.3A	0.2 ± 0.0 B
Flavonoids	1.1 ± 0.1 A	0.0 ± 0.1 B

[a]The contents of carbohydrate, phenolics, or flavonoids are expressed as grams of glucose, gallic acid, or naringenin equivalent/100g lyophilized powder, respectively.
[b]Values are mean ± SD of three determinations.
[c]Values within same row not sharing a common capital letter are significantly different ($P < 0.05$) analyzed using one-way ANOVA, followed by Duncan's New Multiple Range test.

Table 2. Protein and carbohydrate levels in selected chromatographic fractions from red cabbage juice throughout Sephadex LH-20 column

Compositions (g/100 g)[a]		Selected chromatographic fractions[b]	
		F2	F4
non-heated	Protein	45.3 ± 0.8 B	62.0 ± 0.2 A
	Carbohydrate	25.3 ± 0.7 A	24.3 ± 1.5 A
heated	Protein	44.9 ± 0.3 B	54.7 ± 0.4 A
	Carbohydrate	28.8 ± 4.5 A	22.5 ± 0.5 B

[a]The carbohydrate content is expressed as grams of glucose equivalent per 100 g lyophilized powder.
[b]Values are mean ± SD of three determinations. Values within same row not sharing a common capital letter are significantly different ($P < 0.05$) analyzed by one-way ANONA, followed by Duncan's New Multiple Range test.

as TNF-α, and an anti-inflammatory cytokine, IL-10, by LPS-stimulated splenocytes were determined. Tables 3-5 show the effects of isolated proteins from RC products on pro-inflammatory cytokines IL-6 as well as TNF-α, and an anti-inflammatory cytokine, IL-10, secretions by LPS-stimulated splenocytes. The results showed that PRC, PRCA, PF2 and PF4 (20-500 μg/mL), which respectively isolated proteins from RC, RCA, F2 and F4, administrations did not significantly ($P > 0.05$) affect IL-6 secretion by LPS-stimulated splenocytes (Table 3). However, PF2 and PF4 administrations significantly ($P < 0.05$) inhibited TNF-α secretions (Table 4). In the meantime, PRC and PF4 markedly ($P < 0.05$) increased IL-10 secretion (Table 5). The results from this study suggest that proteins especially PF2 and PF4 from red cabbage juice exhibit an anti-inflammatory potential. However, the isolated proteins from F4 (PF4) exhibit the better anti-inflammatory activity via decreasing a pro-inflammatory cytokine TNF-α, but increasing an anti-inflammatory cytokine IL-10 secretions (Tables 4 and 5).

In this study, proteins from red cabbage juice exhibited anti-inflammatory effects against LPS-induced inflammation of primary splenocytes via increasing an anti-inflammatory cytokine IL-10 and decreasing a pro-inflammatory cytokine TNF-α secretions. Lee *et al.* have reported that red cabbage extracts exhibited a neuroprotective action on brain *in vivo*[27]. Although IL-10 can serve as an anti-inflammatory cytokine, it is also a Th2-cytokine. A Th2-driven immune response might cause a change of TNF-α/IL-10, however it is a risk for allergy *in vivo*. Therefore, the use of novel plant proteins for therapeutic application should be prudent.

Table 3. Effects of ammonium sulfate precipitated-proteins from red cabbage juice products on the secretions of pro-inflammatory cytokine IL-6 by LPS-stimulated mouse splenocytes

Treatments	IL-6 secretion (pg/mL)[a,b,c,d]			
	PRC[f]	PRCA	PF2	PF4
LPS stimulation only[e]	566 ± 64	566 ± 64	566 ± 64	566 ± 64
LPS + sample (20 μg/mL)	622 ± 110	729 ± 17	468 ± 43	574 ± 72
LPS + sample (100 μg/mL)	664 ± 129	679 ± 99	508 ± 75	512 ± 78
LPS + sample (500 μg/mL)	763 ± 116	614 ± 148	612 ± 98	591 ± 70

[a]Values are mean ± SD of three biological replicates.
[b]There are no significant differences between sample treatments and the positive control of LPS stimulation in the same column analyzed using one-way ANOVA, followed by Dunnett's test of parametric type.
[c]The IL-6 level in the negative control cultures was 22 ± 16 pg/mL.
[d]The sensitivity of these ELISA kits was < 15.6 pg/mL.
[e]The lipopolysaccharides (LPS) concentration used in this study was 10 μg /mL.
[f]PRC: protein from crude red cabbage juice; PRCA: protein from crude red cabbage juices absorbed with activated charcoal; PF2: protein from F2 fraction; PF4: protein from F4 fraction.

Table 4. Effects of ammonium sulfate precipitated-proteins from red cabbage juice products on the secretions of pro-inflammatory cytokine TNF-α using LPS-stimulated mouse splenocytes

treatments	TNF-α secretion (pg/mL)[a,b,c,d]			
	PRC[f]	PRCA	PF2	PF4
LPS stimulation only[e]	1137 ± 71	1137 ± 71	1137 ± 71	1137 ± 71
LPS + sample (20 μg/mL)	1316 ± 208	1011 ± 252	636 ± 174**	904 ± 376
LPS + sample (100 μg/mL)	1266 ± 277	1028 ± 110	761 ± 296	840 ± 208
LPS + sample (500 μg/mL)	1066 ± 212	952 ± 176	778 ± 207*	789 ± 167*

[a]Values are mean ± SD of three biological replicates.
[b]Asterisks (*, **) mean significantly different from the positive control of LPS stimulation in the same column at the levels of $P < 0.05$, or $P < 0.01$, respectively, analyzed by one-way ANOVA, followed by Dunnett's test of parametric type.
[c]The TNF-α level in the negative control cultures was 75 ± 88 pg/mL.
[d]The sensitivity of these ELISA kits was < 15.6 pg/mL.
[e]The lipopolysaccharides (LPS) concentration used in this study was 10 μg /mL.
[f]PRC: protein from crude red cabbage juice; PRCA: protein from crude red cabbage juices absorbed with activated charcoal; PF2: protein from F2 fraction; PF4: protein from F4 fraction.

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To further unravel the immunomodulatory mechanism of proteins in red cabbage juice, the biochemical properties of all the fractions should be further clearly characterized and investigated. However, this study is just a preliminary study for investigating immune stimulating properties of proteins in red cabbage juice. Thus, the major purpose of this study focused on the immunomodulatory activity of proteinaceous constituents in red cabbage juice. However, the present study still tried to characterize the proteins in red cabbage juice. The proteins from red cabbage juice were subjected to 12.5% SDS-PAGE electrophoresis analysis (Figure 2.). The results showed that the protein subunits in crude red cabbage juice (PRC) are complicated with diverse molecular weights from 27 kDa to 84 kDa. As our expectation, activated charcoal could absorb most proteins in red cabbage juice, especially low molecular weight protein subunits (< 45 kDa). Reasonably, proteins in PF2 have higher molecular weight subunits than those in PF4 (< 35 kDa). Furthermore, PF4 demonstrated better anti-inflammatory activity than that of PF2 (Tables 4 and 5), suggesting that lower molecular weight proteins in red cabbage juice have better anti-inflammatory potential. This study has preliminarily characterized the proteins in red cabbage juice. Because antioxidant substances in plants often show an anti-inflammatory activity, we suggest that PF4 may also have an antioxidant activity, like superoxide dismutase[28]. Unfortunately, the obtained proteins were not completely purified and characterized.

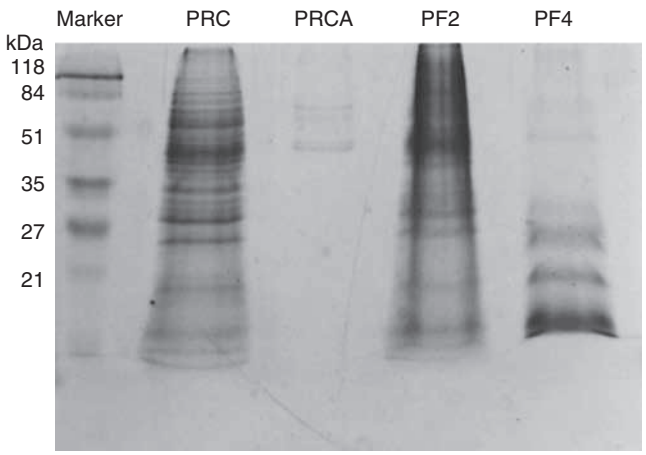

Figure 2. SDS-PAGE chromatograms of proteins precipitated with 100% saturation of ammonium sulfate from red cabbage juice.
Gel: 12.5% SDS-PAGE; PRC: protein from crude red cabbage juice; PRCA: protein from crude red cabbage juice absorbed with activated charcoal; PF2: protein from F2 fraction; PF4: protein from F4 fraction.

Our previous study has shown that pigment components including phenolics and flavonoids in the red cabbage juice increase an anti-inflammatory cytokine IL-10 but decrease a pro-inflammatory cytokine IL-6 secretions[10]. However, in this study the isolated proteins from red cabbage juice could not significantly affect IL-6 secretion of LPS-stimulated splenocytes (Table 3). It is, therefore, suggested that different bio-active components in red cabbage juice may exert differential immunomodulatory effects. However, the characteristic and bioavailability of bio-active proteins in red cabbage juice remain to be further clarified.

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Table 5. Effects of ammonium sulfate precipitated-proteins from red cabbage juice products on the secretions of anti-inflammatory cytokine IL-10 using LPS-stimulated mouse splenocytes

Treatments	IL-10 secretion (pg/mL)[a,b,c,d]			
	PRC[f]	PRCA	PF2	PF4
LPS stimulation only[e]	1547 ± 106	1547 ± 106	1547 ± 106	1547 ± 106
LPS + sample (20 μg/mL)	2055 ± 136**	1664 ± 59	1745 ± 102	1827 ± 106*
LPS + sample (100 μg/mL)	1891 ± 122*	1867 ± 264	1722 ± 70	1818 ± 102*
LPS + sample (500 μg/mL)	1825 ± 182	1848 ± 195	1771 ± 119	2060 ± 83**

[a]Value are mean ± SD of three biological replicates.
[b]Asterisks (*, **) mean significantly different from the positive control of LPS stimulation in the same column at the levels of $P < 0.05$, or $P < 0.01$, respectively, analyzed using one-way ANOVA, followed by Dunnett's test of parametric type.
[c]The IL-10 level in the negative control cultures was 24 ± 39 pg/mL.
[d]The sensitivity of these ELISA kits was < 15.6 pg/mL.
[e]The lipopolysaccharides (LPS) concentration used in this study was 10 μg /mL.
[f] PRC: protein from crude red cabbage juice; PRCA: protein from crude red cabbage juice absorbed with activated charcoal; PF2: protein from F2 fraction; PF4: protein from F4 fraction.

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