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Phenolic Compounds Responsible for the Superoxide Dismutase-like Activity in High-Brix Apple Vinegar

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High-Brix apple vinegar (HBAV) with palatable drinking qualities has been developed using a greater amount of apple ingredients. In HBAV and in regular apple vinegar (RAV), constituents of 4 kinds of organic acids, 20 kinds of amino acids, 3 kinds of sugars, 4 kinds of minerals, and phenols were determined. These constituents, except for acetic acid, in HBAV are of higher abundance than in RAV. HBAV had a 7.1 times greater superoxide dismutase (SOD)-like activity compared with RAV. Those constituents, except for phenols, had very low SOD-like activity, and total phenol levels in HBAV were comparable to 181 mg of gallic acid equivalents/100 mL, which was 6.0 times more abundant than in RAV. Nine kinds of phenols including two kinds of hydroxycinnamates, two kinds of hydroxybenzoates, and five kinds of hydroxycinnamoyl quinates, originating from raw material were determined, but there were no ascorbic acid and flavonoids in HBAV. Chlorogenic acid, 4-*p*-coumaroylquinic acid, and caffeic acid were the three major phenols, and their content levels were 19.6, 13.5, and 0.76 mg in 100 mL of HBAV, respectively. Sum of contents of chlorogenic acid and the isomers was 24.0 mg/100 mL, and the percentage was 56.9% in the total identified phenols in HBAV. In RAV, only chlorogenic acid was determined as phenols, and the content was 3.1 mg/100 mL. SOD-like activities of the constituents of HBAV were obtained through high-accuracy assays using vinegar reconstitutions, and each contribution to the total SOD-like activity was found. As a result, 77.2% for all SOD-like activity of HBAV was reconstituted using the determined nine phenols and other constituents. Chlorogenic acids were the most effective, and the contribution to the total activity was 41.7%. The most abundant phenols, chlorogenic acids, were the most important contributors to the SOD-like activity. These SOD-active phenols originated from raw material and remained through the acetic acid fermentation processes. In the fermentation process of HBAV, the active constituents were well maintained, providing an advantage in the production of a phenol-rich product.

KEYWORDS: Apple vinegar; high-Brix apple vinegar; phenolic compounds; SOD-like activity; high-accuracy assay

INTRODUCTION

Recently, beneficial health effects of vinegar, such as accelerating recovery from fatigue (*1*), enhancing calcium absorption (*2*), preventing hypertension (*3*), and reducing serum total cholesterol levels (*4*), have been reported. These health benefits suggest that we should consume vinegar more positively for our health promotion or disease prevention. In Japan, consumption of vinegar as a drink has increased considerably, presumably in anticipation of such health benefits (*5*). In 2006, a more easily drinkable apple vinegar product, "Kanjuku", which means sweet and ripe in Japanese, was developed. Kanjuku apple vinegar is characterized by an apple-sweet flavor with a high Brix value (the data described in this paper). This high-Brix apple vinegar (HBAV) is produced using a greater amount of ingredients.

The major health effects of vinegar are caused by acetic acid, the essential constituent in vinegar. The antihypertensive effect of acetic acid is demonstrated, leading to its utilization as a food for specified health use in Japan. Oral intake of a beverage containing 750 mg of acetic acid a day significantly decreased blood pressure of high-normal blood pressure and mild hypertensive patient (*6*). Acetic acid could reduce renin activity and subsequently decrease strong vasoconstrictive angiotensin II (*3*). In addition to the health benefit of acetic acid, we may expect those from the ingredient in vinegar. In regular apple vinegar (RAV), several functional food factors from apple existed through a two-stage production process; the first is ethanol fermentation of the raw

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material of apple juice, and the second is acetic acid fermentation of the apple cider wine. Health benefits of apple have been reported (*7*), and one of important category of apple active constituents is phenols. Phenols are secondary plant metabolites and act as antioxidant (*8*), antibacterial (*9*), and antiviral agents (*10*) in plants. In food materials, they affect flavor and color characteristics (*11*). Recently, health benefits and disease reduction properties of the phenols have attracted many researchers (*12*, *13*). It was expected that HBAV, which is produced using greater amounts of apple ingredients through a two-stage process in a similar manner to the production of RAV, exhibited improved health benefits with the presence of residual phenols in addition to acetic acid (*14*, *15*).

The excessive generation and accumulation of reactive oxygen species (ROS), including oxygen radicals, has been implicated in diseases of the blood vessels. ROS damage vascular endothelium directly through strong oxidizability and generate an opportunity for vascular diseases (*16*). The scavenging actions of ROS have been related to chemopreventive effects on vascular diseases by resisting inactivation of vasodilatory nitric oxide (NO), production of lipid peroxidation (*17*), and oxidation of low-density lipoproteins (*18*). Superoxide dismutase (SOD) is an important enzyme providing defense against the harmful effects of ROS in plants and animals. SOD converts two superoxide anions into an attenuated molecule of hydrogen peroxide and plays roles in vascular disease prevention and maintenance of normal blood vessel function (*19*). Specific nonenzymatic scavengers, such as tocopherols, ascorbic acid, flavonoids, and phenols, can prevent biological damages by trapping radical oxidants such as SOD (*20*). Therefore, SOD active constituents in vinegar would also have effects on maintaining normal vascular function. In this study, the constituents of organic acids, amino acids, sugars, minerals, and phenols in HBAV and RAV were determined and the SOD-like activities of the constituents were measured. On the basis of the analyses and assays, SOD-like activity of HBAV was reassembled to clarify the whole constituents possessing SOD-like activity. HBAV was expected to contain greater amounts of SOD active constituents from the apple ingredient. The changes of the major antioxidants following the ethanologenesis and acetification processes were also investigated.

MATERIALS AND METHODS

Vinegars and Intermediate Samples. HBAV and RAV were products of Mizkan Co., Ltd. (Aichi, Japan). Both products were made from the same quality of raw material apple juice. Fermentation intermediate samples from before and after ethanol fermentation as well as before and after acetic acid fermentation were prepared in the following laboratory-scale fermentations using the same lot of raw material. Alcoholic fermentation of the raw material with yeast was first performed. To 20 L of clear apple juice (Brix 25%) was added 25 g of *Saccharomyces cereviseae* (Oriental Yeast Co., Ltd., Tokyo, Japan) as starter, and the mixture was incubated at 30 °C for 96 h. After filtration, ethanol-fermented product with 15% (v/v) alcohol was obtained. The ingredient for acetic acid fermentation with 2.5% (v/v) alcohol was prepared by adding water to the ethanol-fermented product for RAV and also prepared by adding apple juice and water for HBAV. Bacteria culture including *Acetobacter aceti* with 7% acid degree was also prepared using the same ethanol-fermented product. The ingredient and the bacteria culture were mixed in the ratio of 7:3, and acetic acid fermentation was performed until the alcohol concentration reduced to 0.3% (v/v) in deep fermentation. During HBAV production process, the acetic acid fermentation process was performed in highly Brix condition. By adding water to the acetic acid fermentation product, the acid degree was adjusted to 5%. After filtration, RAV and HBAV samples were obtained. An equal amount of the raw material produced 3 times more RAV than HBAV.

Chemicals. For organic acids analyses, acetic acid, DL-malic acid, 50% gluconic acid, citric acid, *p*-toluenesulfonic acid (amino acid analysis grade), bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane (Bis-Tris), and ethylenediaminetetraacetic acid (EDTA) were purchased from Wako Pure Chemical Industries Co., Ltd. (Osaka, Japan). For analyses of sugars, D-fructose, D-glucose, and sucrose, and of amino acids, L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, and L-serine, from Wako Pure Chemical Industries Co., Ltd., and high-performance liquid chromatography (HPLC) grade acetonitrile from Sigma-Aldrich Japan K.K. (Tokyo, Japan) were used. For minerals analyses, calcium, sodium, potassium, and magnesium standard solutions (1000 mg/mL) were purchased from Wako Pure Chemical Industries Co., Ltd. For analysis of phenols, Folin−Ciocalteu phenol reagent, 5-hydroxymethylfurfural, chlorogenic acid, and caffeic acid from Sigma-Aldrich (St. Louis, MO), gallic acid monohydrate from Kanto Chemical Co., Inc. (Tokyo, Japan), sodium carbonate from Sigma-Aldrich Japan K.K., protocatechuic acid, *p*-hydroxybenzoic acid, and trifluoroacetic acid from Wako Pure Chemical Industries Co., Ltd., *p*-coumaric acid from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), and HPLC grade tetrahydrofuran (THF) from Nacalai Tesque, Inc. (Kyoto, Japan) were used. 4-*p*-Coumaroylquinic acid used as a standard was obtained by semipreparative HPLC purification of HBAV.

Measurement of Total Phenols. Total phenol concentrations were measured by using the Folin−Ciocalteu assay (*21*). To a 20 mL test tube were added 8.3 mL of water, 0.2 mL of vinegar sample, and 0.5 mL of double-diluted Folin−Ciocalteu reagent in water. The contents were mixed, and 1 mL of a 10% sodium carbonate aqueous solution was added to the mixture immediately. Solutions were mixed and allowed to stand at 30 °C for 30 min. Total phenol concentrations were determined using a Sefi IUV1240 spectrophotometer (AS ONE Co., Osaka, Japan) at 760 nm. Total phenol content was standardized against gallic acid and expressed as gallic acid equivalents (mg/100 mL). Correction of the results for sugars (fructose, glucose, and sucrose) and gluconic acid was performed on the basis of the results of the Folin−Ciocalteu assay for aqueous solutions using the same concentrations as in the vinegar samples. Ascorbic acid correction was not performed because ascorbic acid was not detected in all samples. Acetic and malic acids, which were Folin−Ciocalteu negative, and amino acids in low content did not affect the assays. Each measurement was performed in triplicate, and the results are expressed as mean ± standard deviation (SD).

HPLC Analysis for Acetic Acid, Malic Acid, Gluconic Acid, and Citric Acid. For organic acids analysis, the HPLC system (Shimadzu Corp., Kyoto, Japan) consisted of solvent delivery pump LC-10ADvp, buffer supply pump LC-10ADvp, autoinjector SIL-10ADvp, column oven CTO-10ACvp, electronic conductivity detector CDD-6A, data processor C-R7Aplus, and SCL-10Avp system controller. Separation was conducted with an Rspak KC-811 column (300 mm × 8 mm i.d., Showa Denko K.K., Tokyo, Japan). Elution was performed with a mobile phase of 4 mM *p*-toluenesulfonic acid aqueous solution. Detection was conducted with a postcolumn pH buffered method using 16 mM Bis-Tris aqueous solution containing 4 mM *p*-toluenesulfonic acid and 100 μM EDTA. Chromatography was performed at 50 °C with a flow rate of 0.6 mL/min and injection volumes of 50 μL for acetic acid and 20 μL for other organic acid analyses. Each measurement was performed in triplicate, and the results were expressed as mean ± SD.

HPLC Analysis for Fructose, Glucose, and Sucrose. The HPLC system for sugar analysis consisted of a PU-2080 solvent delivery unit and an RI-2031 refractive index detector (Jasco Co., Tokyo, Japan). Separations were conducted with a Shodex NH2P-50 column (250 mm × 4.6 mm i.d., Showa Denko K.K.). Elution was performed with the mobile phase of acetonitrile/water (75:25, v/v). Chromatography was performed at room temperature with a flow rate of 1.0 mL/min and an injection volume of 5 μL. Each measurement was performed in triplicate, and the results are expressed as mean ± SD.

HPLC Analysis for Amino Acids. Amino acids in HBAV and RAV were measured by ion exchange chromatography and spectrometric detection after ninhydrin reaction on a JEOL Aminotac JLC-500 V analyzer (JEOL, Tokyo, Japan). Samples of HBAV and RAV were directly analyzed without pretreatment. Each measurement was performed in triplicate, and the results are expressed as mean ± SD.

Atomic Absorption Analysis for Minerals. Atomic absorption spectrometer AA-6800 (Shimadzu) was used for analyses of Ca, Na, K, and Mg following the method described in the literature (*22*). Each

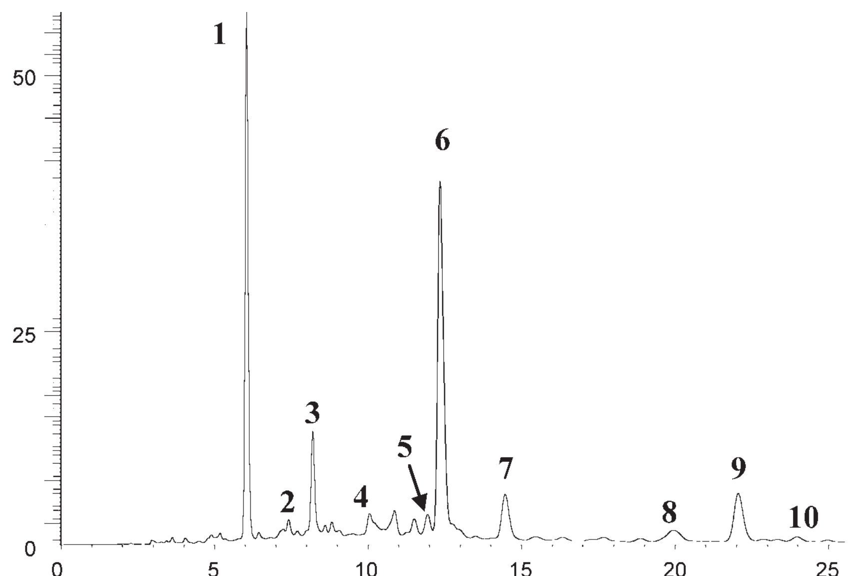

Figure 1. Typical HPLC chromatogram of HBAV. Peaks: 1, 5-hydroxymethylfurfural; 2, protocatechuic acid; 3, an isomer of chlorogenic acid; 4, *p*-hydroxybenzoic acid; 5, an isomer of *p*-coumaroylquinic acid; 6, chlorogenic acid; 7, caffeic acid; 8, an isomer of chlorogenic acid; 9, 4-*p*-coumaroylquinic acid; 10, *p*-coumaric acid. Detection wavelength was 280 nm.

measurement was performed in triplicate, and the results are expressed as mean ± SD.

HPLC Determination for Phenolic Compounds. Phenolic compounds were determined using an LC-2010 integrated HPLC system with a Class-VP workstation (Shimadzu). Separations were performed using a Cosmosil $5C_{18}$-MS-II reversed phase column (150 mm × 4.6 mm i.d., Nacalai Tesque, Inc.). Elution was performed with 0.025% (v/v) TFA in purified water (solvent A) and acetonitrile with 0.025% (v/v) TFA (solvent B) as mobile phase: 0 min, isocratic 5% solvent B; 0−5 min, gradient 5−9% solvent B; 5−15 min, isocratic 9% solvent B; 15−22 min, gradient 9−11% solvent B; 22−35 min, gradient 11−18% solvent B; and 35−40 min, isocratic 18% solvent B. A washing step of 8 min with 70% solvent B and a re-equilibration of the column during 10 min with 5% solvent B were carried out between individual runs. Chromatography was performed at 40 °C with a flow rate of 0.8 mL/min, injection volume of 10 μL, and detection at 280 nm. An external standard method was used for quantifications described in ref *23*. Each measurement was performed in triplicate, and the results are expressed as mean ± SD.

Liquid Chromatography−Mass Spectrometry (LC-MS) Analysis and Nuclear Magnetic Resonance (NMR) Analysis for Phenolic Compounds. LC-MS analysis was performed with a Waters 2695 (LC) and Quattro micro API (MS) system (Waters, Milford, MA). The HPLC conditions were the same as described for analysis for phenolic compounds. Mass spectra were acquired in electrospray ionization (ESI) mode using 3500 V capillary voltage, 20 V cone voltage, desolvation gas (N_2) flow of 350 L/h, cone gas (N_2) flow of 50 L/h, source temperature of 100 °C, and desolvation temperature of 350 °C. The mass spectrometer was operated in positive mode as 80 eV, target *m*/*z* 300, and scanning range *m*/*z* 50−1000.

NMR spectroscopic data were recorded on a Bruker DRX500 spectrometer (Bruker BioSpin Corp., Billerica, MA) at 500 MHz for ^{1}H and 125 MHz for ^{13}C NMR at 25 °C. Chemical shifts were referenced to the signal of trimethylsilane as an internal standard.

Identification of Phenols. 5-Hydroxymethylfurfural (**1**) and chlorogenic acid (**6**) were identified by comparing their relative retention times and NMR and ESI-MS spectra with those of standard compounds. Protocatechuic acid (**2**), *p*-hydroxybenzoic acid (**4**), caffeic acid (**7**), and *p*-coumaric acid (**10**) were identified by comparing relative retention times and ESI-MS spectra with those of standard compounds. ESI-MS and NMR spectra were used to identify 4-*p*-coumaroylquinic acid (**9**). Compounds **3**, **5**, and **8**, which were isomers of chlorogenic acid or 4-*p*-coumaroylquinic acid, were identified with ESI-MS spectra. After solid phase extraction with Sep-Pak Vac C18 cartridges (Waters), these compounds were purified by semipreparative HPLC through a CHEMCOBOND 5-ODS-W (20 × 250 mm, Chemco Scientific Co., Ltd., Osaka, Japan) in an isocratic condition as 0.025% TFA in acetonitrile/water (12:88, v/v) at a flow rate of 5 mL/min. Characteristics of compounds **1**−**10** in **Figure 1** are described below.

5-Hydroxymethylfurfural (***1***): amorphous powder; ESI-MS, *m*/*z* 127.0 $[M + H]^+$; ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.60 (s, 1H), 7.22 (d, J = 7.0 Hz, 1H), 6.52 (d, J = 7.0 Hz, 1H), 4.73 (s, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 177.6, 160.5, 152.5, 142.3, 122.5, 110.0, 57.7.

Protocatechuic acid (***2***): ESI-MS, *m*/*z* 155.1 $[M + H]^+$.

Isomer of chlorogenic acid (***3***): ESI-MS, *m*/*z* 355.3 $[M + H]^+$, *m*/*z* 163.1 $[M - \text{quinic acid}]^+$.

p-Hydroxybenzoic acid (***4***): ESI-MS, *m*/*z* 139.0 $[M + H]^+$.

Isomer of p-coumaroylquinic acid (***5***): ESI-MS, *m*/*z* 339.3 $[M + H]^+$, *m*/*z* 147.1 $[M - \text{quinic acid}]^+$.

Chlorogenic acid (***6***): white powder; ESI-MS, *m*/*z* 355.3 $[M + H]^+$, *m*/*z* 163.1 $[M - \text{quinic acid}]^+$; ^{1}H NMR (500 MHz, CD_3OD) δ 7.55 (d, J = 16 Hz,1H), 7.04 (d, J = 2 Hz, 1H), 6.95 (dd, 1H), 6.77 (d, J = 8.5 Hz, 1H), 6.25 (d, J = 16 Hz, 1H), 5.32 (m, 1H), 4.16 (m, 1H), 3.71 (dd, 1H), 2.02−2.24 (m, 2H); ^{13}C NMR (125 MHz, CD_3OD) δ 175.6, 167.3, 148.2, 145.7, 145.4, 126.4, 121.6, 115.1, 113.9, 113.8, 74.7, 72.1, 70.6, 69.9, 37.4, 36.8.

Caffeic acid (***7***): ESI-MS, *m*/*z* 181.2 $[M + H]^+$.

Isomer of chlorogenic acid (***8***): ESI-MS, *m*/*z* 355.3 $[M + H]^+$, *m*/*z* 163.1 $[M - \text{quinic acid}]^+$.

4-p-Coumaroylquinic acid (***9***): amorphous powder; ESI-MS, *m*/*z* 339.3 $[M + H]^+$, *m*/*z* 147.1 $[M - \text{quinic acid}]^+$; ^{1}H NMR (500 MHz, CD_3OD) δ 7.75 (d, J = 9 Hz, 1H), 7.50 (d, J = 8.5 Hz, 1H), 6.84 (d, J = 8.5, 1H), 6.77 (d, J = 9 Hz, 1H), 4.32 (dd, 1H), 4.26 (m, 1H), 4.24 (m, 1H), 2.01−2.25 (m, 2H); ^{13}C NMR (125 MHz, CD_3OD) δ 168.9, 161.2, 146.7, 131.2, 127.3, 116.8, 115.5, 79.1, 76.7, 69.5, 65.5, 42.3, 38.7.

p-Coumaric acid (***10***): ESI-MS, *m*/*z* 165.1 $[M + H]^+$.

Preparation of Reconstituted Apple Vinegar Samples. HBAV was first separated into three fractions using octadecylsilyl-silica gel (ODS) resin. HBAV (2.0 mL) was applied to a Sep-Pak Vac C18 cartridge (12 mL volume containing 2 g of resin). Fractions I, II, and III were eluted from the cartridge using 12 mL of 0.025% TFA in methanol/water (10:90, v/v), 12 mL of 0.025% TFA in methanol/water (70:30, v/v), and 24 mL of methanol, respectively. Each fraction was evaporated and then lyophilized to obtain 907.5 mg of residual fraction I, 24.2 mg of residual fraction II, and 3.1 mg of residual fraction III. Fraction I was dissolved with a solution of 3.08% of acetic acid in water (v/v), making up a final volume of 2.0 mL, to prepare fraction I reconstitution (reconstitution ***b***). A cocktail solution (reconstitution ***a***) containing 26.3 g of D-fructose, 10.5 g of D-glucose,

2.0 g of sucrose, 3.08 g of acetic acid, 1.97 g of malic acid, 407 mg of 50% gluconic acid, 95 mg of citric acid, 29 mg of L-alanine, 94 mg of L-asparagine, 66 g of L-aspartic acid, 11 mg of L-glutamic acid, 4.9 mg of L-serine, and 451 mg of potassium carbonate in 100 mL of purified water was prepared for reconstitutions containing fractions II and III (reconstitutions ***c*** and ***d***, respectively). Reconstitutions of these two fractions were prepared by adding the cocktail solution (reconstitution ***a***) to each fraction residue and making up a final volume of 2.0 mL. Eight different reconstitutions of apple vinegar samples, reconstitutions ***e−l***, were prepared by dissolving 4.1 mg of **1**, 0.41 mg of **2**, 0.77 mg of **4**, 24.0 mg of **5**, 0.76 mg of **7**, 16.0 mg of **9**, 0.21 mg of **10**, and a mixture of compounds **1−10** (each in the same amount as in reconstitutions ***e−k***) in 100 mL of cocktail solution ***a***, respectively. Reconstitution ***g*** contained the total amounts of **3**, **6**, and **8** as chlorogenic acids, and reconstitutions ***j*** contained **5** and **9** as 4-*p*-coumaroylquinic acid. These reconstitutions were subjected to a SOD-like activity assay to determine the active fractions or constituents contributing to the SOD-like activity of HBAV. The SOD-like activity of HBAV (33.3 times dilution) was set as 100%, and the activity of each tested sample was divided to find an activity ratio. The contribution value of the fraction or constituent to the whole SOD-like activity was determined by subtracting the activity ratio of reconstitution ***a*** from that of each sample.

SOD-like Activity Assay of Apple Vinegar. SOD-like activity of apple vinegar was evaluated on 96-well plates using a SOD Assay Kit-WST (Dojindo Laboratories, Kumamoto, Japan) according to a method reported previously by Ukeda et al. (*24*). Reduction of WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium monosodium salt) with a superoxide anion caused by the xanthine oxidase system produces a water-soluble formazan, which has a characteristic UV absorbance at 450−550 nm. A phosphate buffer solution (pH 7.0) was prepared by mixing 39 mL of 0.1 M NaH_2PO_3 solution and 61 mL of 0.1 M Na_2HPO_3. Vinegar samples were diluted in three dilution series with the phosphate buffer solution. Twenty microliters of sample solution and 200 μL of WST working solution were mixed in the well. The reaction was initiated by the addition of 20 μL of enzyme solution, and the plates were incubated at 37 °C for 20 min. The absorbance at 520 nm was measured with a model 680 microplate reader (Bio-Rad Laboratories Ltd., Hercules, CA). SOD-like activity (inhibition rate %) of each sample solution was calculated using following equation: SOD-like activity (inhibition rate %) = $\{[(A_{\text{blank}} - A_{\text{blank control}}) - (A_{\text{sample}} - A_{\text{sample control}})]/(A_{\text{blank}} - A_{\text{blank control}})\} \times 100$, where blank contained phosphate buffer in place of sample solution, sample control contained dilution buffer in place of enzyme solution, and blank control contained phosphate and dilution buffer in place of sample and enzyme solution. Assays of every dilution ratio were performed in triplicate, and inhibition rates were expressed as the mean value of the results. The dilution ratio (x) and the corresponding inhibition rate (y) were plotted, and the 50% inhibitory concentration (IC_{50}) dilution ratio for each sample was calculated from the regression curve. Coefficients of correlation (r^2) of all curves were >0.98.

Statistical Analysis. The values for contents of selected constituents in HBAV and RAV were all expressed as the mean ± SD. The quantified values for the constituents in HBAV were compared with those of RAV by a *t* test. The results were considered to be significant at $p < 0.05$ (*), highly significant at $p < 0.01$ (**), and very highly significant at $p < 0.001$ (***). Content changes of total phenols, compound **1**, and the three major phenols during HBAV production processes, ethanologenesis and acetification, were expressed as survival rates (%). The quantified values for the constituents after each process were compared with those before the process by a *t* test. The results were considered to be significant at $p < 0.05$.

RESULTS AND DISCUSSION

Chemical Quantification in Vinegars. Quantification of select chemical constituents of HBAV and RAV showed significant differences between the two vinegar samples (**Table 1**). Among the organic acids, acetic acid was the major ingredient in both, but the acetic acid content in HBAV was 64% of that in RAV. The other organic acids were more abundant in HBAV. The levels of malic, gluconic, and citric acids in HBAV were 4.9, 8.3, and 5.3 times higher than those in RAV, respectively. The total abundance of these organic acids in HBAV (5550 mg/100 mL) was greater than that in RAV (5277 mg/100 mL); however, the pH of RAV (3.1) was lower than that of HBAV (3.3). We suggest that the use of larger amounts of apple juice ingredients during production of HBAV resulted in the increase in those organic acids. Therefore, HBAV contained higher amounts of malic and citric acids from the raw apple juice and gluconic acid that had been converted from glucose during acetic acid fermentation (*25*). pK_a values of acetic acid, malic acid, gluconic acid, and citric acid are 4.76, 3.4 (pK_{a1}), 3.86, and 3.15 (pK_{a1}), respectively. HBAV, containing stronger acids in higher ratio, contained greater amounts of minerals as a countercation than RAV at the same level of pH. Each mineral in **Table 1** was much higher in abundance in HBAV than in RAV (**Table 1**). Potassium was the predominant element in both vinegars and was 28.6 times more abundant than sodium in HBAV and was 23.2 times greater in RAV. HBAV contained 3.6 times more potassium, 7.0 times more calcium, and 5.8 times more magnesium than RAV.

Table 1. Sugars, Organic Acids, Minerals, and Total Phenols Content in HBAV and RAV (Milligrams per 100 mL)[a]

constituent	HBAV	RAV
acetic acid	3075 ± 70***	4811 ± 82
malic acid	1973 ± 7***	399 ± 1
gluconic acid	407 ± 1***	49 ± 2
citric acid	95 ± 0***	18 ± 0
Na	15.8 ± 0***	5.4 ± 0.2
K	451.1 ± 8***	125.2 ± 4
Ca	31.4 ± 1***	4.5 ± 1
Mg	20.2 ± 3*	3.5 ± 0.4
alanine	29.3 ± 1	0.6 ± 0.2
asparagine	94.2 ± 6	2.0 ± 0.3
aspartic acid	65.6 ± 4	2.3 ± 0.1
glutamic acid	10.5 ± 0.4	0.4 ± 0.1
serine	4.9 ± 0.1	0.1 ± 0
total amino acids	217 ± 11	6.6 ± 1
D-fructose	$(26.3 \pm 0.3) \times 10^3$***	$(1.4 \pm 0) \times 10^3$
D-glucose	$(10.8 \pm 0.2) \times 10^3$***	$(0.6 \pm 0) \times 10^3$
sucrose	$(2.0 \pm 0.2) \times 10^3$**	ND[b]
total phenols[c]	181.1 ± 3***	30.0 ± 1

[a] Mean ± SD ($n = 3$). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, compared with RAV by *t* test. [b] Not detectable. [c] As gallic acid equivalents.

Amino acids were miner constituents in both HBAV and RAV. The contents of the 20 standard amino acids were 0.22 and 0.0066% in HBAV and RAV, respectively, of their composition. In HBAV asparagine was the most abundant amino acid, and the percentage of the five major amino acids, that is, alanine, serine, aspartic acid, asparagines, and glutamine, was 94.1% in total standard amino acids. Content of γ-aminobutyric acid (GABA), which has a blood pressure lowering effect, was only 3.3 ± 0.3 mg/100 mL in HBAV. In RAV, GABA was not detected.

During the manufacture of RAV, a minimal amount of apple juice is used for the fermentation processes. Therefore, most sugars are consumed during ethanol fermentation and remain as trace abundances in the final product. The fructose and glucose contents in RAV were only 1.4 and 0.6%, respectively, and no sucrose was detected (**Table 1**). In contrast, HBAV contained much greater amounts of sugars. The contents of fructose (26.3%) and glucose (10.8%) in HBAV were 17.5 times higher than the levels in RAV. Sucrose (2.0%) was detected only in HBAV. This high Brix level in HBAV is the result of using larger amounts of raw apple juice during production.

The total content of phenols in both vinegar samples were determined by using Folin−Ciocalteu assay. The accuracy of total phenol measurement must consider the influences of other reductive compounds. Therefore, the organic constituents listed in **Table 1** were tested by Folin−Ciocalteu assay (data not shown). Gluconic acid had very little effect, and the other coexisting organic acids did not have reductive property, whereas the sugars, which are the most abundant constituents in HBAV, had weak reductive properties. Contents of amino acids were too low to influence the results in both vinegar samples. Correction of the total phenol measurements for the interference effects of these organic constituents was performed. Ascorbic acid is a major reductive compound in apple; however, no ascorbic acid was detected in either apple vinegar. Apple phenolic compounds are great reductants. The corrected results of Folin−Ciocalteu assay are considered to be representative of apple phenolic composition. The total phenols in HBAV were 181 mg of gallic acid equivalents/100 mL and were 6.0 times higher than the total phenolic level in RAV (**Table 1**).

Apple Phenols Remaining through the Vinegar Production Processes. **Figure 1** shows a HPLC chromatogram at 280 nm of the phenolic fraction of HBAV. Six high-content compounds were identified and subsequently determined. Compound **1** contained in the peak at 6.1 min was identified as 5-hydroxymethylfurfural by ^{1}H and ^{13}C NMR and MS analyses. In the ESI-MS analysis of compound **6** appearing at 12.4 min, typical product ions at m/z 355.3 $[M + H]^{+}$ of caffeoylquinic acid and at m/z 163.1 of caffeoyl moiety in positive ion mode were observed. The NMR spectrum and retention time were identical with those of standard chlorogenic acid. Compound **9** was identified as 4-*p*-coumaroylquinic acid. The m/z 339.3 $[M + H]^{+}$ and m/z 147.1 of *p*-coumaroyl moiety were observed, and the ^{1}H and ^{13}C NMR spectra were identical with those reported previously (*26*). In the LC-MS analysis, the peak for compound **2** appeared at 7.4 min, that for **4** appeared at 10.1 min, that for **7** appeared at 14.5 min, and that ofr **10** appeared at 24.0 min, corresponding to retention times and typical product ions of standard samples and identified as protocatechuic acid, *p*-hydroxybenzoic acid, caffeic acid, and *p*-coumaric acid. Other compounds were identified as positional isomers of chlorogenic acid (**3** and **8**; m/z 355.3 $[M + H]^{+}$ and m/z 163.1 of caffeoyl moiety) or a positional isomer of 4-*p*-coumaroylquinic acid (**10**; m/z 339.3 $[M+H]^{+}$ and m/z 147.1 of *p*-coumaroyl moiety) by MS analyses. The apple polyphenols, procyanidin B-2, catechin, hyperoside, and quercetin, were not detected in either of the vinegar samples. Sample vinegars were pellucid and had no precipitate. The polyphenols conjugated to form cloudy materials and would be removable by filtration in the production processes to obtain a pellucid product. These results indicate that relatively stable water-soluble phenols can survive fermentation processes.

Table 2 shows the concentrations of compound **1** and the phenols **2**−**10** in HBAV and RAV. The content of compound **1**, which is derived from D-fructose by acid catalyzation (*27*), was 4.1 mg/100 mL in HBAV and 2.7 mg/100 mL in RAV. The differences were significant. HBAV contained 19.6 mg/100 mL of chlorogenic acid (**6**), which was 17.8 times greater than the level in RAV (1.1 mg/100 mL). This difference was very highly significant ($p = 6.5 \times 10^{-5}$). Compounds **2**, **4**, **7**, **9**, and **10** comprised 0.41, 0.77, 0.76, 16.0, and 0.21 mg/100 mL in HBAV, respectively, and were not detected in RAV. Isomers of chlorogenic acid, **3** and **8**, and an isomer of 4-*p*-coumaroylqunic acid, **5**, existed in HBAV, and their contents were 3.1, 1.3, and 2.5 mg/100 mL, respectively. The most abundant phenol was chlorogenic acid (**6**) in both vinegar samples. In RAV, chlorogenic acid was the only detectable phenol, and in HBAV the ratio in total of nine kinds phenols was 46.5%. The sum of chlorogenic acid and the isomers (**3** and **8**) was 24.0 mg/100 mL in HBAV, and the percentage was in 56.9% in the total phenols.

Table 2. Compound **1** and Phenol **2**−**10** Abundance Levels in HBAV and RAV (Milligrams per 100 mL)[a]

constituent	HBAV	RAV
5-hydroxymethylfurfural (**1**)	4.1 ± 0.4*	2.7 ± 0.04
protocatechuic acid (**2**)	0.41 ± 0.02**	ND[b]
isomer of chlorogenic acid (**3**)	3.1 ± 0.2*	ND
p-hydroxybenzoic acid (**4**)	0.77 ± 0.04**	ND
isomer of *p*-coumaroylquinic acid (**5**)	2.5 ± 0.1*	ND
chlorogenic acid (**6**)	19.6 ± 0.3***	3.1 ± 0.1
caffeic acid (**7**)	0.76 ± 0.1**	ND
isomer of chlorogenic acid (**8**)	1.3 ± 0.03*	ND
4-*p*-coumaroylquinic acid (**9**)	13.5 ± 0.4***	ND
p-coumaric acid (**10**)	0.21 ± 0.03**	ND

[a] Mean ± SD ($n = 3$). *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$, compared with RAV by a *t* test. [b] Not detectable.

The phenols **2**−**10** originated from the apple ingredients remaining in HBAV. Therefore, these content levels should represent the amount in the raw material less the amounts lost in the preparation process. Generally, the more raw material is used, the greater the quality of the apple vinegar. Thus, phenol content could be an important factor in improving the quality of apple vinegar.

Preparation of Apple Vinegar Reconstitutions for SOD-like Activity Assays. The SOD-like activities of HBAV and RAV are shown in **Figure 2**. IC_{50} dilution ratios, at which 50% of superoxide anion production was inhibited, in the assay system were used as an indicator of SOD-like activity for each sample. SOD-like activity of HBAV (33.3 times dilution) was 7.1 times greater than that of RAV (4.7 times dilution). Constituents possessing potent SOD-like activity should bring this superiority of HBAV. To investigate the constituents active in generating this higher SOD-like activity level, HBAV was separated by solid-phase extraction into three fractions: first, fraction I was eluted, then fraction II, and last fraction III. Volatile solvent was removed by rotary evaporator, then a phosphate buffer was added, and the original volume was made up to prepare the test solutions. The IC_{50} dilution ratios of fractions I and II were 4.9 and 36.3 times, respectively, and fraction III was inactive. Even the phosphate buffer solution of fraction II showed 9.0% higher activity than the SOD-like activity of HBAV (33.3 times dilution), and the total activity of fractions I and II infallible exceeded the original one. These data indicated that most of the active constituents were present in fraction II, but using phosphate buffer, it was difficult to determine their accurate relative contributions for the total SOD-like activity of HBAV. To determine the relative contribution of these fractions, it was necessary that a test sample contain the same amounts of not only the target constituent but also the other relevant components in HBAV, as accurately as possible. Thus, we produced a reconstitution of HBAV.

Nutrition factors in 100 g of HBAV were carbohydrates (including sugars), 43.0 g; protein (including amino acids), 0.3 g; fat, 0 g; sodium, 160 ppm; and acid proportion, 4.0%. Total amounts of the sugars and five kinds of major amino acids listed in **Table 1** accounted for 90.5% of the carbohydrates and 68.1% of the protein. Total mineral content was 518.5 mg/100 mL with 87.0% as potassium (451.1 mg/100 mL). The basic cocktail solution containing organic acids, sugars, amino acids, and potassium in the same level of those in HBAV was reconstitution ***a***. Effective acids in HBAV estimated 4.0 g/100 mL from the acid proportion, but the total amount of acids in **Table 1** was 5.6 g. By adding potassium carbonate, it was neutralized and proper acid

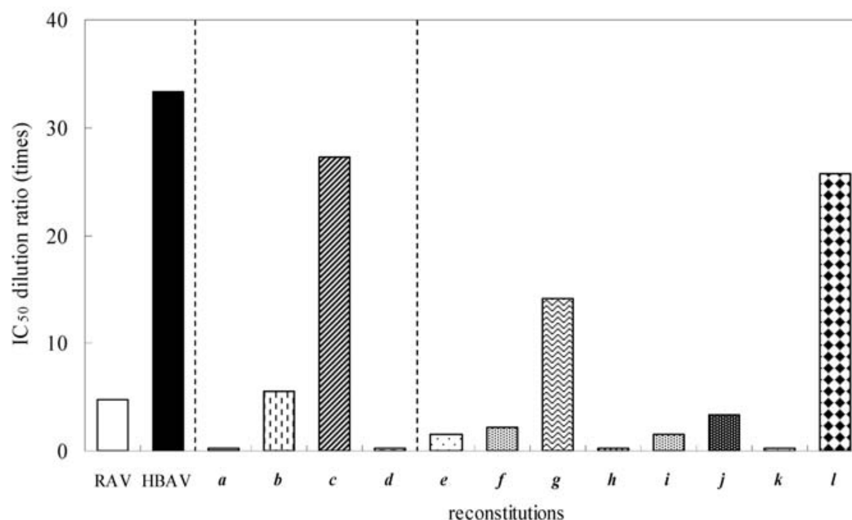

Figure 2. SOD-like activities of HBAV, RAV, and the vinegar reconstitutions. ***a***, solution containing HBAV organic acids, sugars, amino acids, and mineral; ***b***, fraction I dissolved in 3.08% of acetic acid; ***c***, ***a*** + fraction II; ***d***, ***a*** + fraction III; ***e***, ***a*** + **1**; ***f***, ***a*** + **2**; ***g***, ***a*** + **3**, **6**, and **8**; ***h***, ***a*** + **4**; ***i***, ***a*** + **5**, **9**; ***j***, ***a*** + **7**; ***k***, ***a*** + **10**; ***l***, ***a*** + mixture of compounds **1**−**10**.

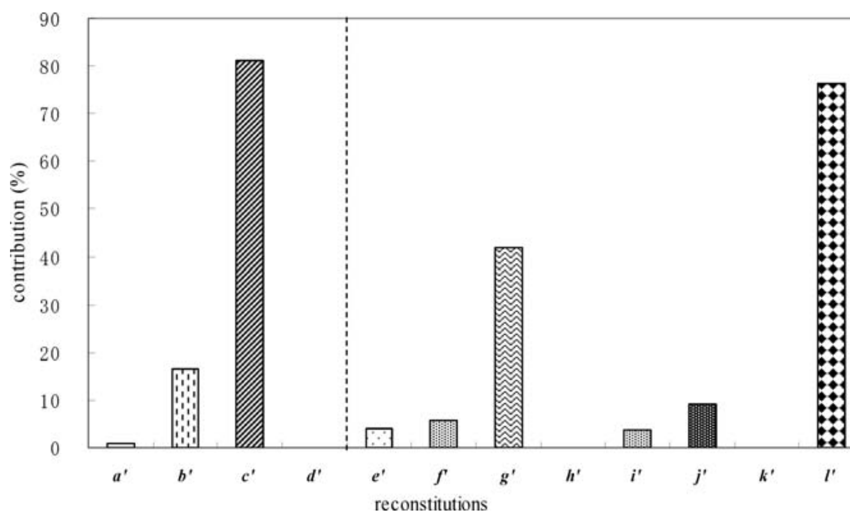

Figure 3. Relative contribution of fractions I, II, III, 5-hydroxymethylfurfural, and phenols to the SOD-like activity of HBAV. Descriptions of reconstitutions ***a'***−***l'*** correspond to ***a***−***l*** in the caption of **Figure 2**.

proportion was performed in the reconstitution. Using reconstitution ***a*** as a base, reconstitutions ***c*** and ***d*** containing dry residues of fractions II and III were prepared. Reconstitution ***b*** containing fraction I was prepared using aqueous acetate because volatile acetic acid was removed in the dry residue of fraction I. The results of the SOD-like activity assays indicated that the IC_{50} dilution ratio of reconstitution ***a*** (0.3 times dilution) was only 0.9% of that of HBAV, whereas the IC_{50} dilution ratios of reconstitutions ***b*** and ***c*** were 5.5 and 27.3 times, respectively. Reconstitution ***d*** containing fraction III had no SOD-like activity other than that of the basic cocktail solution. These results indicated that the source of the SOD-like activity was concentrated in fraction II. **Figure 3** shows the contribution of a series of apple vinegar reconstitutions to the full amount of SOD-like activity. Contributions of the cocktail solution (reconstitution ***a***), fraction I, and fraction II to the total SOD-like activity of HBAV were estimated to be 0.9, 16.5, and 81.1%, respectively. The sum comprised reasonably 98.5% of the full SOD-like activity level of HBAV. Although the residuals of fractions I and II were 45.4 and 1.2 g from 100 mL of HBAV, respectively, fraction II produced most of the SOD-like activity in HBAV. In addition, because fractions I and III did not have UV absorption at 254 nm on fluorescent TLC plates, we concluded that most of the phenols were included in fraction II.

Constituents Responsible for the SOD-like Activity of HBAV. An assay system using reconstitutions of apple vinegar helped determine more precisely the source of the SOD-like activity. Initially, we reconstituted apple vinegar to determine contributions of the constituents, including phenols, to the total SOD-like activity level according to the result of chemical determination of HBAV. To evaluate the contribution of 5-hydroxymethylfurfural (**1**), protocatechuic acid (**2**), chlorogenic acids (**3**, **6**, and **8**), *p*-hydroxybenzoic acid (**4**), caffeic acid (**7**), *p*-coumaroylquinic acids (**5** and **9**), and *p*-coumaric acid (**10**) from fraction II, test samples containing the same levels of these compounds were prepared using reconstitution ***a***. To evaluate the SOD-like activity of chlorogenic acids, the content of the isomers was qualified as chlorogenic acid equivalents, and the total content (24.0 mg of chlorogenic acid/100 mL) was used to prepare the reconstitution. The SOD-like activity of 4-*p*-coumaroylquinic acid and the

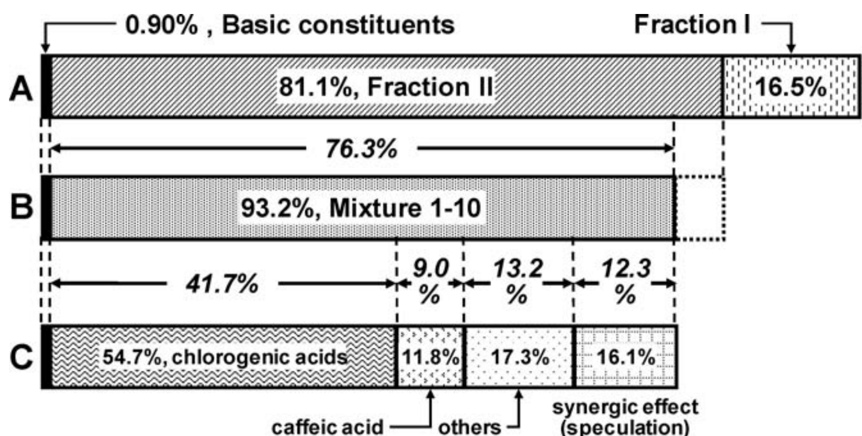

Figure 4. Reconstitution summary of SOD-like activity of HBAV: (**A**) reconstitution of SOD-like activity of HBAV with fractions I and II, and basic constituents; (**B**) reconstitution of SOD-like activity of fraction II with reconstitution ***l***; (**C**) composition of SOD-like activity with determined constituents in fraction II. Dotted frames indicate loss of activities.

isomer was evaluated in a similar manner. Reconstitutions ***e*** for compound **1**, ***f*** for **2**, ***g*** for **3**, **6**, and **8**, ***h*** for **4**, ***i*** for **5** and **9**, ***j*** for **7**, ***k*** for **10**, and ***l*** for a mixture of all **1**−**10** compounds were prepared, and their SOD-like activities were assayed. Resulting activity levels, represented as IC_{50} dilution ratios, are shown in **Figure 2**. The results for individual compounds suggest that adding chlorogenic acids was the most effective; that is, reconstitution ***g*** diluted in 14.2 times showed 50% inhibitory activity in the production of superoxide anion. Despite the low content, the SOD-like activity of caffeic acid was the second most effective, and the IC_{50} dilution rate was 3.3 times. The activity of *p*-coumaroylquinic acids was relatively weak considering the contents, and other activities were insignificant or none. The activity of mixture ***l*** indicated the sum of the effects of all 10 compounds and 25.7 times diluted solution expressed as the IC_{50}.

Figure 3 shows the relative contribution of this series of apple vinegar reconstitutions to the total SOD-like activity. The contribution of chlorogenic acids to the total activity (column ***g'*** in **Figure 3**) was 41.7%, among the best. The second best contribution rate of caffeic acid was 9.0%. The addition of **1**, **2**, and *p*-coumaroylquinic acids (columns ***e'***, ***f'***, and ***i'***) did not markedly improve the activity level, and their contributions were estimated to be only 3.9, 5.7, and 3.6%, respectively. Contributions of **4** and **10** for SOD-like activity of HBAV were not found. The contribution rate of **1**−**10** derived from reconstitution ***l*** was 76.3% for all SOD-like activity of HBAV and 93.2% for that of fraction II. This demonstrated that including caffeoyl components, a major phenol in HBAV plays the primary role in the SOD-like activity of HBAV, and the caffeoyl compounds accounting for approximately half of all the activity were also demonstrated to be the major SOD active source in HBAV. Chlorogenic acid (**6**) was the most effective single compound in the content (19.6 mg/100 mL) and the activity (IC_{50} = 40 μM, 14.2 μg/mL from our data). Although caffeic acid (**7**) has potent superoxide scavenging activity (IC_{50} = 19 μM, 3.4 μg/mL from our data), its relative content (0.76 mg/100 mL) was probably very small to produce a marked increase in the activity. There are other studies describing the health benefits of chlorogenic acid and caffeic acid, including its preventive effects on hypertension (*28*), cardiovascular disease (*29*), and diabetes and hyperlipemia (*30*), anti-inflammatory effects, and superoxide scavenging activities (*31*). We can also expect health benefits of caffeoyl compounds in HBAV, in addition to acetic acid.

Reconstitution of SOD-like Activity of HBAV. A reconstitution result of SOD-like activity of HBAV is summarized in **Figure 4**. SOD active fraction II was obtained through solid-phase extraction. Fraction I with 16.5% of the SOD-like activity should contain undetermined highly hydrophilic components. Dissolving these fractions to the basic cocktail containing the same levels of organic acids, sugars, amino acids, and potassium as HBAV, 98.5% of all SOD-like activity of HBAV was reconstituted (**Figure 4A**). Most of the SOD active components in fraction II were determined, and 94.1% of the SOD-like activity was reconstituted as reconstitution ***l***, but it is not clear which other constituents were responsible for the remaining 5.9% of the SOD-like activity. We suggest that it is due to other undetermined phenolic compounds (**Figure 4B**). Using determined constituents **1**−**10** from fraction II, 94.1% of the activity of fraction II was recovered and 77.2% of all SOD-like activity of HBAV was reconstituted. However, difference in activities between mixture of **1**−**10** and sum of individual components appeared as shown in **Figure 4C**. The sum of those **1**−**3** and **5**−**9** except for inactive **4** and **10** was found to be 64.0%. It was speculated that the difference of 12.3% could be caused by a synergic effect of coexistence of various type phenols.

In this study, a SOD-like activity assay system using reconstitutions of vinegar was employed. The results show that the major constituents of apple vinegar, including organic acids, amino acids, and sugars, were not effective antioxidants and that the phenols accounting for only 0.042% (w/v) played an important role in the SOD-like activity of the apple vinegar. In addition, the results suggest that increasing the amount of raw material used during processing will produce apple vinegar with greater potential health-related functions. We conclude that high-Brix apple vinegar is not only a palatable drinking product but also provides health benefits through its phenol and acetic acid constituents.

Changes of Phenol Levels in the Apple Vinegar Production Processes. We investigated the change of total phenols, 5-hydroxymethylfurfural (**1**), chlorogenic acids, caffeic acid, and *p*-coumaroylquinic acid during fermentation processes of HBAV in three lots. **Figure 5** shows survival rates of those compounds during the ethanol and acetic acid fermentation processes. In ethanol fermentation, total phenol abundance decreased to 88% of the original content, but increased to 105% significantly in the acetic acid fermentation. The measured value of total phenol for the fermentation product was corrected on the basis of the addition amount of water and ingredient in the process. The increase may be related to the production of reductive compound(s). Compound **1** decreased to 71.4% of the original level in the ethanol

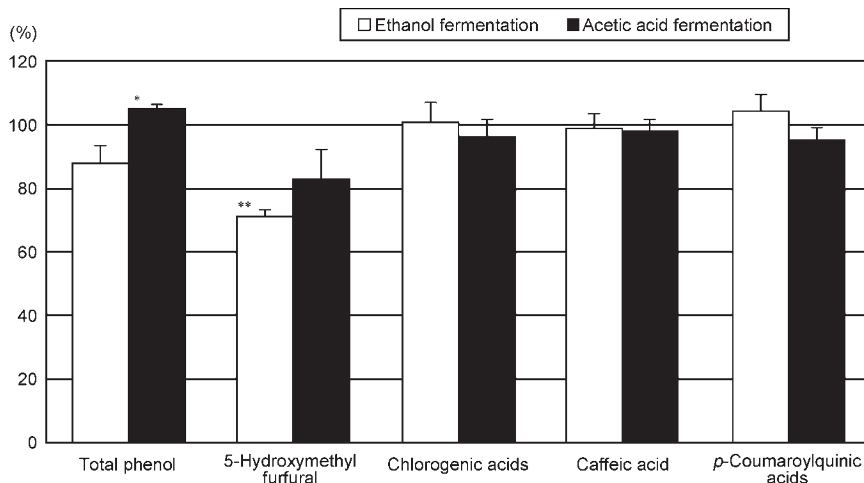

Figure 5. Survival rate of compound **1** and major phenols during the fermentation processes of HBAV (mean ± SD, $n = 3$). ∗, $p < 0.05$; and ∗∗, $p < 0.01$, compared quantitation results after process with those before process by a *t* test.

fermentation, significantly. In the acetic acid fermentation, it decreased to 82.6%. The abundance of the phenols remained at nearly original levels in the two fermentation processes. The contents of chlorogenic acids, caffeic acid, and *p*-coumaroylquinic acids were 101.2, 99.0, and 104.6% of the original in the ethanol fermentation, respectively, whereas in the acetic acid fermentation, 96.2% of the original level of chlorogenic acids, 98.3% of caffeic acid, and 94.7% of *p*-coumaroylquinic acids were maintained. These survival rates were obtained by comparing each of their total amounts in the raw apple juice used and in the ethanol fermentation product added apple juice with the corresponding value in the ethanol and in the acetic fermentation products. The total amount of each phenol remained unchanged during the fermentation process. A previous study suggested that 37.4% of total phenols, 20.0% of chlorogenic acid, and 15.8% of *p*-coumaroylquinic acid were lost during the acetification process of apple vinegar (*32*). Those results are distinctly different from our observations, in which there was no total phenol decrement in the acetic acid fermentation process. This suggests that the fermentation processes were advantageous for producing a phenol-rich apple vinegar.

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