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Changes of Angiotensin I-Converting Enzyme Inhibitory Activity, Fibrinolytic Activity and β-Secretase Inhibitory Activity of Red Wines During Fermentation and Post-Fermentation

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The cardiovascular angiotensin I-converting enzyme inhibitory activity, fibrinolytic activity and *bbb*-secretase inhibitory activity of four kinds of red wine were investigated during fermentation and post-fermentation. After 10 days of fermentation, the antihypertensive angiotensin I–converting enzyme (ACE) inhibitory activities of all the red wines ranged from 38.6% to 58.8%. However, the ACE inhibitory activities increased with the prolongation of the post-fermentation period; moreover, in the *Vitis hybrid* red wine, the ACE inhibitory activity reached its highest value, 76.9%, after 120 days of post-fermentation. During the fermentation and post-fermentation of all the red wines, fibrinolytic activity was weak or not detected. After 10 days of fermentation, *Vitis labrusca* B red wine exhibited the greatest antidementia β-secretase inhibitory activity of 54.8%, though β-secretase inhibitory activity decreased significantly to less than 10% during 120 days of post-fermentation. In conclusion, we obtained a highly valuable *Vitis hybrid* red wine that was fermented for 10 days at 25°C with *Vitis hybrid* and *S. cerevisiae* K-7 and then post-fermentation for 120 days at 4°C.

Key words: Angiotensin I- converting enzyme inhibitory activity, fibrinolytic activity, β-secretase inhibitory activity, red wines

Introduction

Grapes contain a large number of polyphenol compounds, including anthocyanin and proanthocyanidins [22], flavonoids and phenolic acids [7, 32]. Recently, it has come to light that these phenolic compounds have the following healthful biological effects: antioxidant activity [10] by scavenging of active harmful oxygen radicals [8, 33]; inhibition of oxidation on lipoprotein [31, 32] and low density lipoprotein [23]; anti-inflammatory action [24, 26]; lowering of blood cholesterol by resveratrol of grape and antimicrobial activity [30].

Many studies have reported the beneficial health effects of red wine [7, 27]. These effects of red wine may largely originate from the abundance of phenolics. Kallithraka *et al.* [9] reported that red wine may reduce the mortality rate from coronary heart disease. Amous *et al.* [1] reported that the DPPH radical scavenging activity and hydroxyl radical scavenging activity of red wine are closely correlated. Furthermore, there are some studies on the antioxidant activity of wines [4, 15], the angiotensin I-converting enzyme (ACE) inhibitory peptide from wine [33], and lactic acid bacteria in red wine [16]. Red wines, however, are limited with respect to offering unique characteristics, acceptability, and valuable physiological functionality [20, 21]. Therefore, there is a need to develop a new form of red wine with excellent acceptability, physiological functionality, and low alcohol toxicity.

In a previous paper [25], we investigated physicochemical properties and antioxidant activities of red wines during fermentation and post-fermentation. In this study, we vinified four kinds of red grapes and investigated the ACE inhibitory activity, fibrinolytic activity and antidementia β-secretase inhibitory activity during fermentation and post-fermentation for the purpose of developing a new functional red wine.

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Materials and Methods

Als and Methods

Grapes, yeast and chemicals. Four Korean varieties of grapes harvested in 2005: *Vitis labrusca* L (Gerbong), *Vitis labrusca* B (Campbell Early), *Vitis labrusca* (Muscat Bailey A), and *Vitis hybrid* (Sheridan) were purchased from a commercial grape market. *Saccharomyces cerevisiae* K-7 [11, 13], from the Laboratory of Food Biotechnology at Paichai University (Daejeon, Republic of Korea), was used in the preparation of the red wine.

The ACE was extracted from rabbit lung acetone powder (Sigma Chemical Co., St. Louis, MO, USA). Hip-His-Leu and fibrin were purchased from Sigma Chemical Co. Unless otherwise specified, all chemicals were of analytical grade.

Vinification of red grapes. After crushing four kinds of grapes, sugar was added to 24° Brix. We then added 150 ppm of $K_2S_2O_5$ and left the mixture for 5 hr. Next, we inoculated 1% of *S. cerevisiae* K-7 which was incubated for 24 hr in the must, and left the must to ferment for 10 days at 25°C. We then filtered the fermented broths and adjusted the SO_2 content to 150 ppm. The broths were then stored at 4°C for 120 days.

Preparation of red wine concentrates and assay of ACE inhibitory activity, fibrinolytic activity and β-secretase inhibitory activity. 50 mL of each red wine was concentrated to 5 mL by vacuum evaporator. Its solid content was 2.5 mg per ml and did not contain ethanol or any volatile acids.

ACE inhibitory activity, fibrinolytic activity and anti-dementia β-secretase inhibitory activity of the concentrates were determined as follows. First, ACE inhibitory activity was assayed using the method of Cushman and Cheung [5]. A mixture containing a 100 mM sodium borate buffer (pH 8.3), 300 mM NaCl, 3 units of ACE, and an appropriate amount of red wine was preincubated for 10 min at 37°C. The reactions were initiated by adding 50 μL of Hip-His-Leu at a final concentration of 5 mM, and the reaction was terminated after 30 min of incubation by adding 250 μL of 1.0 N HCl. After extracting the liberated hippuric acids with 1 mL of ethyl acetate, we dried 0.8 mL of the extracts using a Speed Vac concentrator (CVE-100; EYELA Co., Tokyo, Japan). The residue was then dissolved in 1 mL of sodium borate buffer. Absorbance at 228 nm was measured to estimate the ACE inhibitory activity.

Fibrinolytic activity was assayed by the method of Fayek *et al.* [6]. 0.5 mL of each sample was added to 3 mL of a substrate solution (0.6% fibrin in 0.1 M McIlvaine buffer, pH 7.0) and the mixture was incubated at 40°C for 10 min. The reaction was stopped by adding 3 mL of 0.4 M TCA for 30 min and then filtered with Whatman filter paper No. 2. 1 mL of the filtrates, 5 mL of 0.4 M Na_2CO_3, and 1 mL of 1 N Folin reagent were mixed and then left at room temperature for 30 min. To determine how much tyrosine was released from the fibrin, we used a tyrosine standard curve based on measurements of the absorbance at 660 nm. One unit of activity was defined as the production of 1 μg of tyrosine per minute for 1 mL of a sample.

The β-secretase (BACE1) inhibitory activity was determined according to the supplied manual with modifications. β-secretase inhibitory activity assay kit was the Wallac 1420 VICTOR^3 (PerkinElmer, Boston, MA, USA); β-secretase inhibitory activity assay kit was purchased from the PanVera Co. (Invitrogen, Madison, WI, USA). A mixture of 10 μL of an assay buffer (50 mM sodium acetate, pH 4.5), 10 μL of β-secretase (1.0 U/mL), 10 μL of the substrate (750 nM Rh-EVNLDAEFK-Quencher in 50 mM of ammonium bicarbonate), and 10 μL of a sample dissolved in the assay buffer was incubated for 60 min at 25°C under dark conditions. We excited the mixture at 530 nm and measured the emitted light at 590 nm. The inhibition ratio was obtained from the following equation: β-secretase inhibitory activity (%) = [1 - {(S-S_0)/(C - C_0)}] × 100, where C is the fluorescence of the control (enzyme, assay buffer, and substrate) after 60 min of incubation, C_0 is the fluorescence of the control at time zero, S is the fluorescence of the tested samples (enzyme, sample solution, and substrate) after 60 min of incubation, and S_0 is the fluorescence of the tested samples at time zero. All data are the mean of triplicate experiments. To check the quenching effect of the samples, we added the sample solution to reaction mixture C and investigated any reduction in fluorescence for each sample [3].

Ethanol determination and sensory evaluation. To determine the ethanol content, we used an alcohol meter (Ceti Optical Instruments, Antwerp, Belgium) after water distillation [11].

The sensory evaluation of the red wines was estimated with the aid of 50 sensory panels trained on the basis of a quantitative descriptive analysis [11]. The taste and odor of the red wines were evaluated on a scale of 1 to 5, in which

5 was the best score. We then plotted the means as a polygonal graph. The overall acceptability in terms of taste and odor was evaluated by using the mean value of a hedonic scale, where 1 means extremely disliked and 9 means extremely well liked. The Duncan Multiple Range test was used to investigate the 5% significance among the four kinds of wines.

Results and Discussion

Changes of physicochemical and functional properties of four red wines during fermentation

Physicochemical properties. Changes of physicochemical properties in four kinds of red wine during fermentation at 25°C for 10 days were investigated (Table 1). Ethanol contents did not change during 5 days and 10 days fermentation and there also was no difference among all the red wines, which showed ethanol content in a range of 11.4±0.6~12.8±0.5% (v/v) after fermentation for 10 days. Choi *et al.* [4] reported recently that alcohol content of sweet persimmon wine, which was produced from 24° Brix of sweet persimmon juice, was 12.8% (v/v) after 5 days of fermentation at 25°C.

Total acid content also showed no difference in all the red wines except *Vitis labrusca* L red wine. Total anthocyanin content and total phenol content, known as bioactive compounds, were the highest of 1.77±0.02 A_{520} and 0.93±0.05 mg/mL in *Vitis labrusca* B red wine, however it were the lowest of 0.25±0.02 A_{520} and 0.49±0.04 mg/mL in *Vitis labrusca* L red wine after fermentation for 10 days, respectiviely.

ACE inhibitory activity, fibrinolytic activity and β-secretase inhibitory activity. We investigated changes of ACE inhibitory activity, fibrinolytic activity and β-secretase inhibitory activity of four kinds of red wines during fermentation at 25°C for 10 days (Table 2). The antihypertensive ACE inhibitory activity was significantly increased from the 0.0~5.0% of the grapes themselves to 38.6~65.1% in the four kinds of red wines after 5 days fermentation; however, these figures did not significantly change after fermentation of 10 days. Furthermore, the highest level in the four kinds of red wine was reached in the *Vitis labrusca* L red wine (65.1±3.2%) after 5 days of fermentation, even though its total anthocyanin and phenol contents were very low as Table 1. This results suggest that its polyphenol compounds were not effected to ACE inhibitory activity. This results was also higher than those of traditional dandelion-wine (16.2%) [14], and chamomile (36.7%) [17], respectively, and had similar or lower activity relative to *Paecilomyces japonica*-wine (67.3%) [18], *Ganoderma lucidum*-wine (63.4%) [11], respectively. Tsutomu *et al.* [33] also reported six ACE inhibitory peptides from Muscat Bailey A red wine. The ACE (dipeptidyl carboxypeptidase I, E.C. 3.4.15.1) regulates blood pressure by converting the inactive decapeptide angiotensin I to the potent vasoconstrictor octapeptide angiotensin II; it also raises blood pressure by inactivating the vasodilating nonapeptide bradykinin [19]. Recently, various ACE inhibitors with antihypertensive effects have been isolated from the enzymatic digestion of food protein [2], sake and its by-products [29], cereals and legumes [28], and microbes such as yeast [13] and mushrooms [19].

Table 1. Physicochemical properties of various red wines from fermentation of 5 and 10 days at 25°C.

Ferment. period (days)	Red wines	Ethanol content (%)	pH	TA[1)] (%)	VA (%)	Ta (A_{520})	RS (mg/ml)	TP (mg/ml)	Color		
									L (light-ness)	a (red-ness)	b (yellow-ness)
5	VH.W[2)]	11.6±0.3	3.78±0.12	0.82±0.02	0.009±0.001	1.22±0.01	9.87±1.32	0.71±0.04	80.28±	22.72±	0.93±
	VIL.W	12.8±0.5	3.74±0.20	0.64±0.01	0.012±0.001	0.26±0.02	10.31±1.21	0.49±0.05	95.99±	4.99±	2.27±
	VI.W	12.0±0.7	3.67±0.15	0.80±0.01	0.011±0.001	1.77±0.01	8.57±2.45	0.62±0.01	75.85±	30.49±	0.89±
	VIB.W	12.4±0.6	3.68±0.04	0.84±0.03	0.008±0.001	2.14±0.01	8.34±0.01	0.98±0.10	66.44±	31.38±	5.96±
10	VH.W	11.6±0.2	3.82±0.02	0.78±0.02	0.011±0.001	1.37±0.02	7.40±0.52	0.70±0.02	77.51±	16.14±	1.17±
	VIL.W	12.0±0.4	3.83±0.06	0.61±0.04	0.011±0.001	0.25±0.02	7.00±0.45	0.49±0.04	96.39±	3.83±	1.64±
	VI.W	11.4±0.2	3.81±0.01	0.93±0.01	0.012±0.001	1.40±0.01	7.70±1.23	0.57±0.04	84.38±	22.25±	0.39±
	VIB.W	11.4±0.6	3.79±0.00	0.71±0.02	0.013±0.001	1.77±0.02	7.92±1.10	0.93±0.05	78.62±	23.96±	4.75±

TA, Total acid; VA, Volatile acid; Ta, Total antocyanin; RS, Reducing sugar and TP, Total phenol
Vh.W, *Vitis hybrid* (Sheridan) red wine; VIL.W, *Vitis labrusca* L (Gerbong) red wine; VI.W, *Vitis labrusa* (Muscat Bailey A) red wine and VIB.W, *Vitis labrusca* (Cambell Early) red wine.

Table 2. Angiotensin I- converting enzyme inhibitory activity, fibrinolytic activity and β-secretase inhibitory activity of various red wines from fermentation of 5 and 10 days at 25°C.

Fermentation periods (Days)	Red wines	ACE [1] inhibitory activity (%)	Fibrinolytic activivity (Clear zone : mm)	HMG-CoA reductase inhibitory activity (%)	β-Secretase inhibitory activity (%)
5	Vh. W [2]	52.2±0.1	2.2±0.1	ND [3]	13.2±0.1
	VlL. W	65.1±0.1	ND	ND	ND
	Vl .W	56.1±0.2	2.0±0.1	ND	34.1±0.2
	VlB. W	38.6±0.1	2.5±0.2	ND	64.8±0.1
10	Vh. W	58.8±0.1	3.0±0.1	ND	ND
	VlL. W	60.1±0.2	ND	ND	ND
	Vl .W	56.1±0.1	2.0±0.1	ND	15.2±0.1
	VlB. W	38.6±0.1	2.0±0.1	ND	54.8±0.2
Grapes	*Vitis hybrid*	ND	2.0	ND	ND
	Vitis labrusca L	5.0	0.1	ND	ND
	Vitis labrusca	ND	2.0	ND	ND
	Vitis labrusca B	4.5	ND	ND	ND

Table 3. Physicochemical properties of various red wines from post-fermentation for 120 days at 4°C.

Ferment. period (days)	Red wines	Ethanol content (%)	pH	TA[1] (%)	VA (%)	Ta (A_{520})	RS (mg/ml)	TP (mg/ml)	Color		
									L (light-ness)	a (red-ness)	b (yellow-ness)
30	BH.W[2]	11.8±0.4	3.72±	0.70±0.02	0.009±0.001	1.47±0.01	7.45±2.51	0.63±0.01	75.95	16.04	3.66
	VIL.W	12.6±0.2	3.63±	0.53±0.01	0.019±0.000	0.40±0.01	6.99±1.01	0.47±0.05	95.63	3.92	2.48
	VI.W	11.0±0.2	3.67±	0.88±0.02	0.007±0.001	1.43±0.00	7.59±0.52	0.52±0.03	85.20	20.17	1.89
	VIB.W	11.2±0.4	3.60±	0.70±0.01	0.010±0.001	2.09±0.01	8.10±1.11	0.97±0.00	80.26	23.89	7.83
60	BH.W	11.0±0.2	3.83±	0.86±0.01	0.007±0.000	1.43±0.00	7.83±2.20	0.79±0.02	77.20	16.2	3.35
	VIL.W	12.6±0.2	3.77±	0.53±0.01	0.019±0.000	0.36±0.00	7.42±0.03	0.58±0.02	94.16	4.03	3.88
	VI.W	11.6±0.2	3.76±	0.72±0.02	0.009±0.000	1.38±0.02	8.30±0.05	0.62±0.01	85.12	20.23	3.66
	VIB.W	11.8±0.2	3.72±	0.71±0.00	0.012±0.001	2.09±0.00	8.42±0.02	1.08±0.09	75.63	22.55	9.53
90	BH.W[2]	12.8±0.0	3.84±	0.68±0.00	0.007±0.000	1.21±0.01	7.80±1.05	0.63±0.02	77.13	14.80	3.86
	VIL.W	13.0±0.4	3.80±	0.54±0.01	0.010±0.001	0.31±0.02	7.65±2.01	0.48±0.06	91.03	4.88	5.97
	VI.W	12.4±0.2	3.85±	0.86±0.03	0.008±0.001	1.34±0.02	8.54±1.02	0.53±0.10	83.98	15.49	7.68
	VIB.W	11.6±0.0	3.79±	0.69±0.01	0.011±0.001	1.97±0.00	8.40±2.00	0.97±0.03	71.78	21.83	14.71
120	BH.W	12.5±0.0	3.90±	0.69±0.00	0.010±0.000	1.09±0.01	8.12±0.01	0.67±0.02	77.98	17.00	3.72
	VIL.W	12.8±0.2	3.78±	0.50±0.01	0.010±0.000	0.28±0.00	7.74±0.20	0.47±0.00	90.32	4.87	6.45
	VI.W	12.0±0.0	3.84±	0.89±0.02	0.007±0.001	1.33±0.00	8.60±0.32	0.58±0.00	81.02	15.48	8.69
	VIB.W	12.0±0.4	3.78±	0.70±0.02	0.009±0.001	1.72±0.02	8.70±0.01	1.23±0.02	70.99	21.53	14.77

*[1), 2)] and [3)] were same as Table 1.

Fibrinolytic activity were showed below 3.0 mm of the clear zone in grapes themselves as well as all the red wines after 10 days of fermentation without any change during fermentation and post-fermentation. No HMG-CoA reductase inhibitory activity was detected in grapes or in all red wines [12] (data not shown).

Antidementia β-secretase inhibitory activity reached its highest level (64.8%) in the *Vitis labrusca* B red wine after 5 days of fermentation from 0% of grapes themselves, though the level decreased to 54.8% after 10 days of fermentation. The β-secretase inhibitory activity of the *Vitis labrusca* red wine after 5 days of fermentation also decreased from 34.1% to 15.2% after 10 days of fermentation.

The difference of the three functionalities in the red wines may be caused by different chemical components between grapes varieties or wines components such as total

anthocyanin and phenol (Table 1) and organic acid, not by ethanol, because the red wine concentrates did not contain any alcohol.

Changes of physicochemical and functional properties of four red wines during post-fermentation

Physicochemical properties. Changes of physicochemical properties in the four kinds of red wine were investigated during post-fermentation periods at 4°C for 120 days (Table 3).

Ethanol contents of the four kinds of red wine were 12.0±0.4~12.8±0.2% (v/v) after post-fermentation for 120 days. *Vitis labrusca* B red wine showed the highest total anthocyanin and total phenol content after 120 days of post-fermentation.

ACE inhibitory activity, fibrinolytic activity and β-secretase inhibitory activity. Changes of ACE inhibitory activity, fibrinolytic activity and antidementia β-secretase inhibitory activity in the four kinds of red wines were investigated during a post-fermentation period of 120 days at 4°C. As shown in Fig. 1, the ACE inhibitory activity of the red wines increased with the prolongatio of the post-fermentation period and the highest level (76.9%) occurred in the *Vitis hybrid* red wine after a post-fermentation period of 120 days. The concentrates of *Vitis hybrid* red wine containing ACE inhibitors after post-fermentation were subjected to systematical solvent extraction using hexane, chloroform, ethylacetate, buthanol and D.W. Only the aqueous layer of final step showed a very high ACE inhibitory activity of 77.1%. This result suggests that the ACE inhibitor of *Vitis hybrid* red wine is a hydrophilic agent such as a protein, peptide, or sugars. We guess that increase of the ACE inhibitory activity is also caused by this hydrophilic agents, not by anthocyanin and phenol which were decreased during fermentation in Table 1. Further study is necessary to identify the ACE inhibitor.

Fibrinolytic activity, on the other hand, did not change within a clear zone of 0.5 mm to 2.5 mm. Moreover, HMG-CoA reductase inhibitory activity was not detected in any of the red wines during the post-fermentation. The β-secretase inhibitory activity decreased significantly to 5.2% in the *Vitis labrusca* B red wine and to 1.5% in the *Vitis labrusca* red wine (Fig. 2).

Sensory characteristics of the red wines. We investigated the acceptability of four kinds of red wines after 120 days of post-fermentation at 4°C and compared with those of commercial C red wine (Fig. 3). Generally, all the red wines had a strong sour taste and fruity flavor; sweetness and bitterness or alcoholic flavor were very weak and there was no difference in any of the red wines. From this sensory evaluation, we concluded that the *Vitis hybrid* red wine showed the best acceptability, which was similar that of commercial C red wine. Acceptability of the other red wines decreased in the following order: *Vitis*

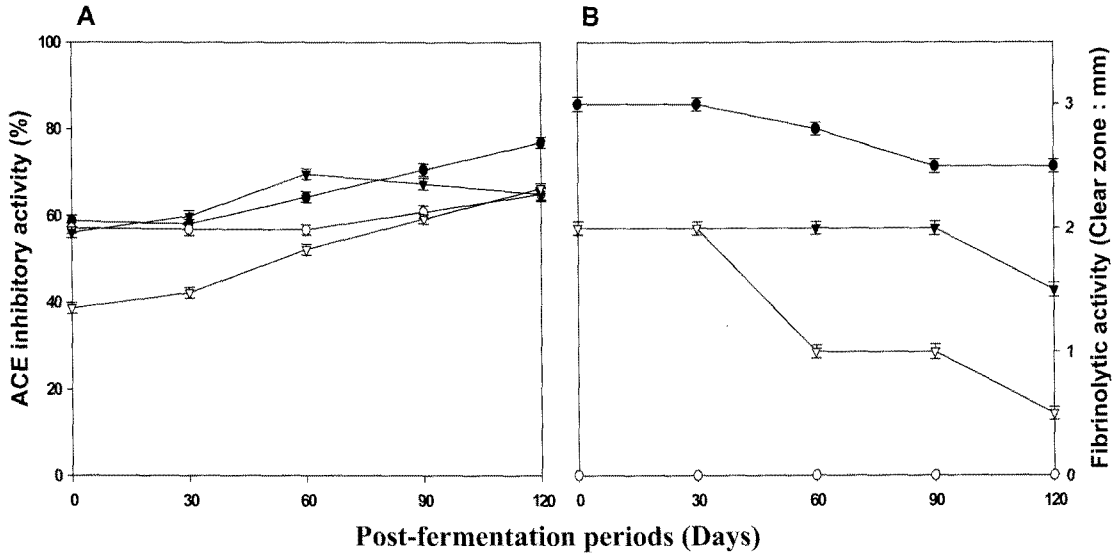

Fig. 1. Changes of angiotensin I-converting enzyme inhibitory activity (A) and fibrinolytic activity (B) in various red wines during post-fermentation at 4°C. ●, *Vitis hybrid* (Sheridan) red wine; ○, *Vitis labrusca* L (Gerbong) red wine; ▼, *Vitis labrusca* (Muscat Bailey A) red wine; ▽, *Vitis labrusca* B (Campbell Early) red wine. Values show means ±SE from three experiments performed in triplicate.

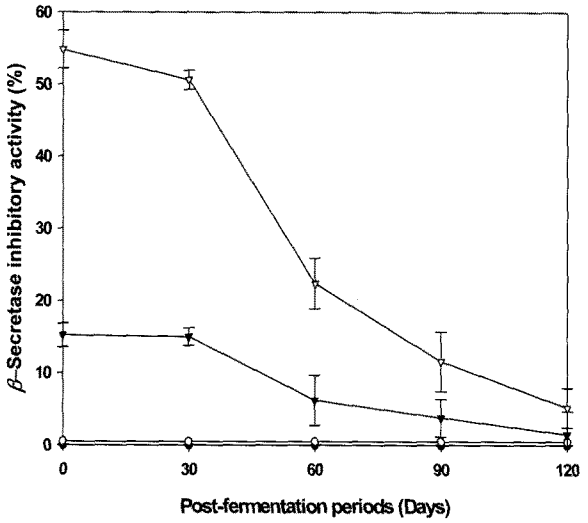

Fig. 2. Changes of β-secretase inhibitory activity in various red wines during post-fermentation at 4°C. ●, *Vitis hybrid* (Sheridan) red wine; ○, *Vitis labrusca* L (Gerbong) red wine; ▼, *Vitis labrusca* (Muscat Bailey A) red wine; ▽, *Vitis labrusca* B (Campbell Early) red wine. Values show means ±SE from three experiments performed in triplicate.

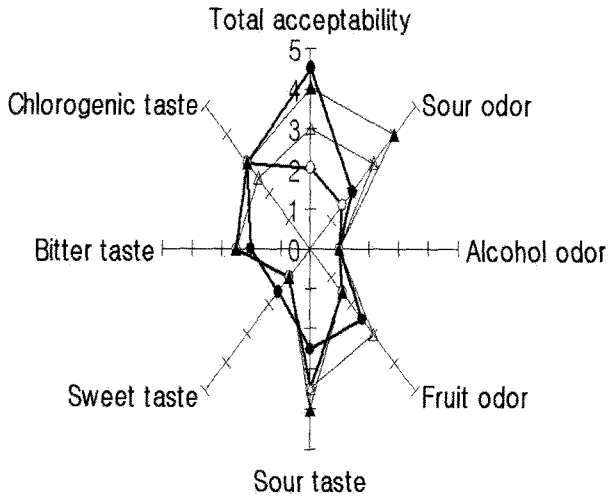

Fig. 3. The quantitative descriptive analysis profile for taste and odor of various red wines after post-fermentation for 120 days. ◆, *Vitis hybrid* (Sheridan) red wine; ■, *Vitis labrusca* L (Gerbong) red wine; ▲, *Vitis labrusca* (Muscat Bailey A) red wine; ●, *Vitis labrusca* B (Campbell Early) red wine; ×, commercial C red wine.

labrusca red wine, *Vitis labrusca* B red wine, and *Vitis labrusca* L red wine. The color of the *Vitis hybrid* red wine was also evaluated as good (bright reddish-pink). The results from the sensory evaluation were subjected to a dispersion analysis, and an F value of 2.58 was obtained. A significant difference of 5% was observed between the *Vitis hybrid* red wine and the other red wines. Lee *et al.* [20] reported that the *Vitis labrusca* B and *Vitis labrusca* varieties are suitable for red wine production.

In conclusion, we obtained a highly valuable *Vitis hybrid* red wine that was fermented for 10 days at 25°C with *Vitis hybrid* and *S. cerevisiae* K-7 and then post-fermentation for 120 days at 4°C. This wine has the potential to become a new functional red wine with high antihypertensive properties, a little antidementia and fibrinolytic activity as well as good acceptability. Therefore, we are confident that the *Vitis hybrid* red wine from this study will become excellent Korean functional red wine. Further studies should identify and characterize the ACE inhibitor of the *Vitis hybrid* red wine.

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적포도주들의 발효와 후 발효 중 심혈관 관련 Angiotensin I 전환효소 저해활성과 혈전용해활성 및 β-secretase 저해 활성의 변화

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본 연구는 4종류의 한국산 포도를 이용하여 포도주를 제조한 후 이들의 발효와 후 발효중의 심혈관 관련 angiotensin I 전환효소 저해 활성과 혈전 용해 활성 및 항치매성 β-secretase 저해활성을 조사하였다. 발효 10일 후 모든 시료 포도주들의 항고혈압성 엔지오텐신 전환효소(ACE) 저해활성은 38.6%~58.8% 이었다. 그러나 후발효가 진행됨에 따라 ACE 저해활성은 증가하여 세리단(*Vitis hybrid*) 포도주가 후발효 120일 후 최고인 76.9%에 도달하였다. 혈전용해활성은 모든 시료 포도주들에서 미약하거나 없었다. 발효 10일 후, 캠벨어리(*Vitis labrusca* B) 포도주가 54.8%의 가장 높은 항치매성 β-secretase 저해 활성을 보였으나 후발효 120일 후에는 10% 미만으로 현저하게 감소되었다. 결론적으로 본 연구에서는 세리단 포도를 *S. cerevisiae* K-7 효모로 25°C에서 10일간 발효 시킨 후 4°C에서 120일간 후발효 시켜서 고부가가치의 생리 기능성을 가진 세리단 적포도주를 제조하였다.