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EFFECTS OF THERMAL TREATMENTS ON THE STABILITY OF *TRANS*-RESVERATROL AND YEAST INACTIVATION IN *TRANS*-RESVERATROL-AMPLIFIED GRAPE JUICE

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Abstract - For the production of *trans*-resveratrol-amplified grape juice, both *trans*-resveratrol quantity and microbial quality are important for the functionality and shelf life of the juice. Therefore, the thermal stability of *trans*-resveratrol and thermal inactivation of *Saccharomyces cerevisiae* within the grape juice was investigated at 60°C, 80°C and 100°C. Inactivation of *S. cerevisiae* was fitted to first-order kinetics, and the inactivation rate (k_1) and decimal reduction time (D-value) at each treatment temperature were estimated. The control grape juice had an inactivation rate of 0.0014 (s^{-1}) and a D-value of 11.90 min at 60°C, whereas the *trans*-resveratrol-amplified grape juice had an inactivation rate of 0.0016 (s^{-1}) and a D-value of 10.42 min at the same temperature. Similar inactivation kinetics of the control and *trans*-resveratrol-amplified grape juice were observed at 80°C and 100°C, which indicates that increased *trans*-resveratrol content does not affect the thermal inactivation of inoculated *S. cerevisiae*.

Key words: trans-Resveratrol-amplified grape juice, *Saccharomyces cerevisiae*, HPLC

INTRODUCTION

Grapes and red wines are rich sources of phenolic compounds, an important group of micronutrients present in plants, which include anthocyanins, catechins, proanthocyanidins, flavonols, stilbenes and other phenolics, all of which are potent antioxidants that show cardioprotective properties (Renaud and de Lorgeril, 1992; Zern and Fernández, 2005). Consumption of grape juice has been associated with a decrease in platelet aggregation (Keevil et al., 2000), inhibition of atherosclerosis and improvement in lipid and antioxidant parameters (Vinson et al., 2001). The "Kyoho" grape is an original Japanese table grape whose name means "great or big mountain", and was named after Mount Fuji because of its generous size. This grape is a cross between *Vitis vinifera* and *V. labrusca* grapes and has compact medium-to-large bunches with large irregular berries and a deep black-colored skin. The variety, famous for its large-sized berry and excellent taste, is an economically important grape cultivar in Korea (Deng et al., 2006). The taste, texture and juice are very similar to that of the Concord grape. Quite fragrant, the skin and seeds are not edible, as both are bitter, but the nearly one inch of meaty flesh is deliciously sweet and very juicy (Park et al., 2010).

Trans-resveratrol (3,5,4′-trihydroxystilbene) is a natural compound present in many vegetables and in related foods that is produced in response to fungal infections (particularly of *Botrytis cinerea*),

and to diverse abiotic factors, such as the presence of metallic ions, hydric stress or exposure to UV light. This action is similar to that of phytoalexins (Piñeiro et al., 2006). Trans-Resveratrol also assists in inhibiting mold growth on berries. There is increasing interest in trans-resveratrol research owing to its pharmacological activity (Dourtoglou et al., 1999). It has recently been discovered that trans-resveratrol has several biological effects, including anticancer, cardioprotective (Blanco et al., 1998; Adrian et al., 2000; Aznar et al., 2001; Careri et al., 2003; Jin et al., 2005; Padilla et al., 2005), antioxidant, platelet aggregation inhibitory and anti-inflammatory activities (Gambuti et al., 2004; Padilla et al., 2005).

The nutritional properties of grape juice are well known (USDA, 2012). Some kinetic data of nutrient destruction during the thermal processing of grape juice are also available (Villota and Hawkes, 2007). However, there is little information regarding the thermal stability of *trans*-resveratrol in conventional juice pasteurization conditions. For the production of *trans*-resveratrol-amplified grape juice, both *trans*-resveratrol quantity and microbial quality after thermal treatments are important to ensure the functionality and shelf life of the grape juice. Therefore, both the stability of *trans*-resveratrol and microbial inactivation during the thermal process were investigated in this study.

MATERIALS AND METHODS

Plant materials

Control and *trans*-resveratrol-amplified grapes (Kyoho) were obtained from Yeongcheon Agricultural Technology Extension Center, Yeongcheon, Korea.

Instruments and reagents

The HPLC system used in this study consisted of a Waters 1525 Binary HPLC (Tokyo, Japan), a TCM column oven and a Waters 2489 UV/visible detector, all controlled by a computer using Empower Pro 2.0 software. Separation was carried on a Discovery® C18 (25 cm × 4.6 mm, 5 μm) column (Sigma-Aldrich Co. Ltd., St. Louis, MO, USA). The column temperature was maintained at 33°C during the experiment. HPLC-grade methanol (MeOH) and distilled water (J. T. Baker®, Phillipsburg, NJ, USA) were used as the elution solution for HPLC. *Trans*-Resveratrol was purchased from Sigma-Aldrich Co. Ltd. (St. Louis, MO, USA).

Preparation of grape juice

Control and *trans*-resveratrol-amplified grapes were washed with tap water. Excess water was removed using tissue towels. The grape seeds were removed from the fruit flesh by hand using a clean spatula. The grape juice was extracted using a twin-screw juice extractor (NJE-2003, NUC Ltd., Daegu, Korea). The extracted juice was filtered through a sterilized cheesecloth and stored before use in an air-tight amber bottle at 4°C.

Preparation of test microorganism

Saccharomyces cerevisiae was selected as the test microorganism because yeast is frequently observed in thermally treated fruit juices and determines the shelf life of fruit juice. *S. cerevisiae* (KCCM 11693) was obtained from the Korean Culture Center of Microorganisms (Seoul, Korea). *S. cerevisiae* was transferred onto potato dextrose agar (BD, Franklin Lakes, NJ, USA) and incubated at 25°C for 3 days. A single colony of incubated *S. cerevisiae* was transferred to YM broth (BD, Franklin Lakes, NJ, USA) and grown in a shaking incubator at 25°C for 2 days.

Heat treatment of grape juice

Two different methods of thermal treatment were adopted to investigate the thermal inactivation of yeast and the thermal stability of *trans*-resveratrol within grape juice. For the thermal inactivation of yeast, 9 ml of previously sterilized grape juice was put into a glass test tube and placed into a water bath at 60°C, 80°C, or 100°C. When the previously sterilized grape juice reached the target temperature, 1 ml of yeast broth was injected into the pre-heated

grape juice and kept for the duration of the indicated treatment time. After heat treatment, the sample was removed from the water bath and cooled in an ice bath.

Determination of viable cell counts and kinetic analysis

One milliliter of each treated sample was serially diluted in 9 ml of sterile peptone water. Triplicate samples of 0.1 ml from an appropriate dilution were plated onto potato dextrose agar and incubated at 25°C for 3 days. After incubation, survivors (CFU/ml) were enumerated. The microbial inactivation data were analyzed by first order reaction kinetics. The general first-order kinetics equation is as follows:

$$-\frac{\mathrm{d}N}{\mathrm{dt}} = \mathrm{k} \cdot N \quad (1)$$

If the initial number of microorganism N_0 decreases to N_t after time t, then the ratio of N_t to N_0 can be obtained by integration

$$\log\left(\frac{N_t}{N_0}\right) = -k_1 \cdot t \quad (2)$$

or,

$$\log(N_t) = \log(N_0) - k_1 \cdot t \quad (3)$$

where, k_1 is the first-order rate constant.

Sample preparation for HPLC analysis

For the analysis of *trans*-resveratrol in the juice extract of control and *trans*-resveratrol-amplified grapes juice, a 3.0 g sample was extracted by reflux three times for 3 h with 200 ml EtOH. Each sample was then filtered with filter paper (Whatman No. 2, USA) and evaporated *in vacuo*. Each extract sample was prepared by dissolving 3.0 mg in 1.0 ml of absolute methanol. Injections were prepared by placing soluble samples in a Whatman 0.45 µm PVDF syringe filter (Cat No. 6779 1304, USA). The resulting samples were used for HPLC analysis.

Chromatographic conditions

For the quantification of *trans*-resveratrol by HPLC, the mobile phase was acetonitrile (A) and 0.1% acetic acid in distilled water (B). The program used increased solvent A in a linear gradient from 14 to 16% acetonitrile in solvent B over 0-8 min then increased solvent A to 27% acetonitrile over 15-40 min. The flow rate was 0.3 ml/min. Wavelength for the detector was 306 nm and the injection volume into the HPLC was 20 µl. All injections were performed in triplicate.

Calibration curve

Stock solutions of *trans*-resveratrol (0.6 mg/600 µL) in absolute MeOH were loaded into an HPLC for calibration. The calibration functions of *trans*-resveratrol were calculated with peak area (Y), concentration (X, mg/mL) and mean values (n = 12) ± standard deviation.

RESULTS AND DISCUSSION

Inactivation of inoculated S. cerevisiae in control and trans-resveratrol-amplified grape juices

To determine if temperature affects the inactivation of *S. cerevisiae* in control and *trans*-resveratrol-amplified grape juices, the number of surviving *S. cerevisiae* inoculated into the control grape juice was plotted on a semi-logarithmic scale. Fig. 1 shows the inactivation of *S. cerevisiae* inoculated into control grape juice. The initial count of inoculated *S. cerevisiae* was $1.3 \cdot 10^7$ CFU/ml. In this experiment, we found that the treatment temperature greatly affected the inactivation rate. At a treatment temperature of 60°C, yeast inactivation proceeded quite slowly compared to samples treated at temperatures of 80°C and 100°C. Therefore, an extra time axis was required to present the inactivation data of 60°C at the same time-temperature plan (top X-axis in Figs. 1A and 1B). We found that the rate of yeast inactivation increased rapidly as the treatment temperature increased. The inactivation data were fitted to first-order reaction kinetics (Eq. 3) which passes through

Table 1. Inactivation rate constant k_1 with correlation coefficient r^2, D value, and 5 decimal reduction times of *S. cerevisiae* in control (A) and *trans*-resveratrol-amplified grape juices (B).

Process Temp. (°C)	A				B			
	k_1 (s^{-1})	r^2	D-value (min)	5D reduction time (min)	k_1 (s^{-1})	r^2	D-value (min)	5D reduction time (min)
100	0.061	0.80	0.27	1.35	0.060	0.66	0.28	1.40
80	0.031	0.90	0.54	2.70	0.035	0.90	0.48	2.40
60	0.0014	0.83	11.90	59.50	0.0016	0.85	10.42	52.10

Table 2. The *trans*-resveratrol content in control (A) and *trans*-resveratrol-enriched grape juices (B)

Process Temp. (°C)	Process Time (sec)	**A** (mg/g)	**B** (mg/g)
100	Control	0.16 ± 0.01	0.36 ± 0.02
	15	0.18 ± 0.01	0.38 ± 0.01
	30	0.17 ± 0.01	0.35 ± 0.01
	45	0.17 ± 0.02	0.34 ± 0.02
	60	0.16 ± 0.02	0.35 ± 0.01
80	Control	0.19 ± 0.01	0.34 ± 0.03
	30	0.18 ± 0.01	0.34 ± 0.02
	60	0.16 ± 0.01	0.36 ± 0.02
	90	0.17 ± 0.01	0.38 ± 0.02
	120	0.18 ± 0.01	0.32 ± 0.01
60	Control	0.15 ± 0.01	0.29 ± 0.02
	600	0.13 ± 0.01	0.27 ± 0.02
	1,200	0.15 ± 0.01	0.23 ± 0.01
	1,800	0.18 ± 0.01	0.30 ± 0.02
	2,400	0.14 ± 0.01	0.25 ± 0.01

the initial microbial count (log N_0=7.1). The rate constants (k_1) at each temperature were estimated and the correlation coefficients, r^2, were calculated (Table 1).

Fig. 2 shows that the inactivation of *S. cerevisiae* inoculated into *trans*-resveratrol-amplified grape juice was similar to that seen in the control grape juice. The similarity in inactivation kinetics between control and *trans*-resveratrol-amplified grape juices can be verified by the similar rate constants (k_1) and decimal reduction times (D value) at each temperature. The D value is the time required at a certain temperature to inactivate 90% of the initial microorganisms. The D value is a negative reciprocal of the slope of a plot of log (N) against t, and can be easily obtained from the estimated rate constant k_1 (Toledo 1991). Table 1 shows the calculated D values at each processing temperature. The D values decrease rapidly as the treatment temperature increases.

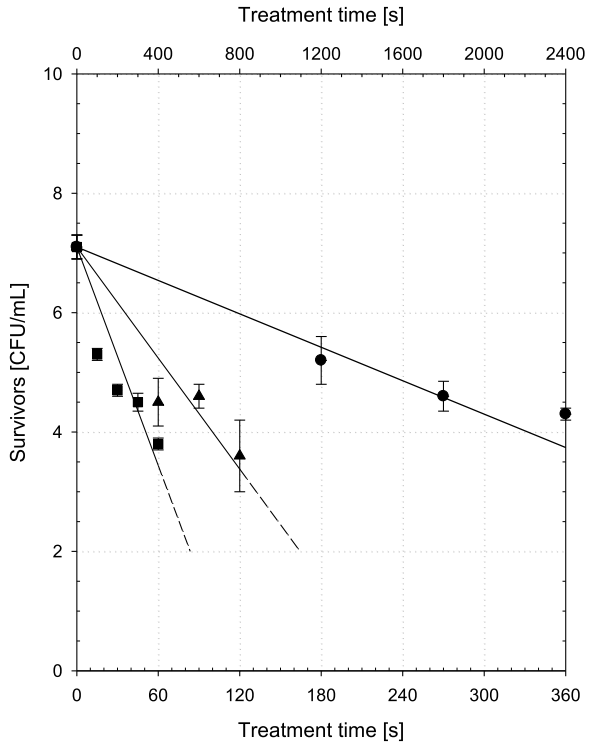

Fig. 1. Thermal inactivation of *S. cerevisiae* in control grape juice: 60°C (—●—), 80°C (—▲—), and 100°C (—■—). The top X-axis is the scale for the inactivation curve at 60°C, and the bottom axis is the scale for the 80°C and 100°C curves. The Y-axis is in logarithmic scale. The solid lines represent the regression line calculated using a first order inactivation model.

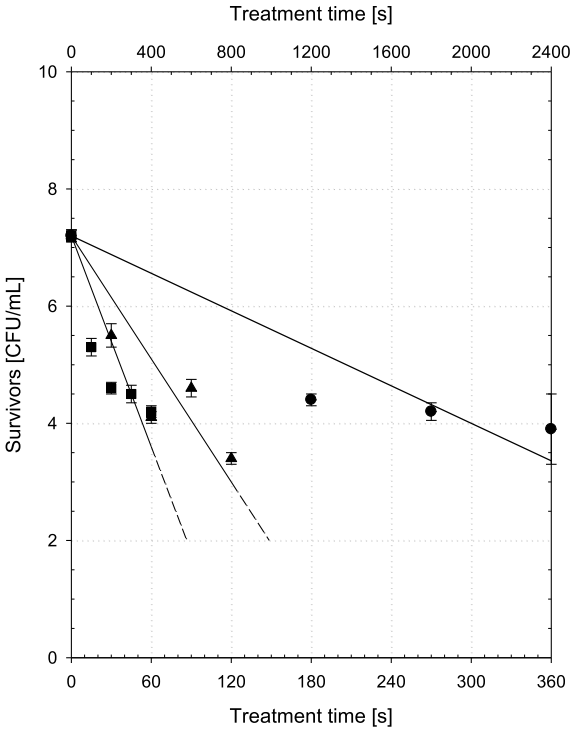

Fig. 2. Thermal inactivation of *S. cerevisiae* in *trans*-resveratrol-enriched grape juice: 60°C (—●—), 80°C (—▲—), and 100°C (—■—). The top X-axis is the scale for the inactivation curve at 60°C and the bottom axis is the scale for 80°C and 100°C curves. The Y-axis is in logarithmic scale. The solid lines represent the regression line calculated using a first order inactivation model.

The values of 5 decimal reductions of yeast are also shown in Table 1. The aim of the heat treatment of commercial fruit juice was to inactivate acid-resistant yeast (Buzrul et al., 2005). Other heat-resistant microorganisms may survive mild heat treatment conditions but cannot grow further due to the low pH of fruit juice (Silva et al., 1999). Therefore, processing conditions that include 5 decimal reductions will be adequate for the cold storage of grape juice. Furthermore, there were few differences in the yeast inactivation kinetics between the control and *trans*-resveratrol-amplified grape juices.

Content of trans-resveratrol in control and trans-resveratrol-amplified grape juices

The optimum mobile phase for the analysis of *trans*-resveratrol was determined using an HPLC flow with a solvent gradient (0 min, 14%; 8 min, 16%; 15 min, 27%; acetonitrile in 0.1% acetic acid in distilled water) for 40 min. At the wavelengths monitored, a peak was observed at 306 nm, and the retention time of *trans*-resveratrol was 9.38 min. In the HPLC profile of the sample solution, the retention time of the expected *trans*-resveratrol peak was the same as that of the standard compound. The calibration equation for *trans*-resveratrol was $Y = 8.27 \cdot 10^8 \cdot X - 3.58 \cdot 10^3$ ($r^2 = 0.999$) (Fig. 3).

The *trans*-resveratrol contents of both control and *trans*-resveratrol amplified-grape juices prepared using different processing conditions such as temperature and time and using non- and UV-processed grapes, were measured (Table 2). The *trans*-resveratrol contents in control- and *trans*-resveratrol-amplified grape juices ranged from 0.13 to 0.19 and 0.23 to

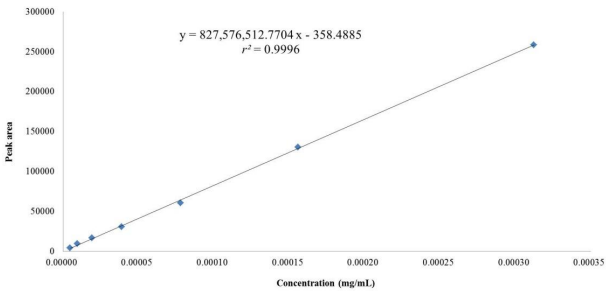

Fig. 3. Calibration curve of *trans*-resveratrol

0.38 mg/g, respectively. In previous papers, the *trans*-resveratrol content in grape juice was found to vary depending on the cultivation system, the producing region, the parts used and manufactured wines (Souto et al., 2001; Babikova et al., 2008; de Freitas et al., 2009; Zhang et al., 2011; Zhou et al., 2004). We found that the UV-processed samples had twice as much *trans*-resveratrol compared to district non-processed grapes. According to the results of both types of analysis, the processing time showed no correlation with the *trans*-resveratrol content. When we examined the effect of temperature, the *trans*-resveratrol content showed a slight decline in samples that were treated at 80°C and 100°C as compared to 60°C, with no differences between 80°C and 100°C. A similarity in the inactivation kinetics of control and *trans*-resveratrol-amplified grape juices was observed at 80°C and 100°C, which indicates that the increased *trans*-resveratrol content does not affect the thermal inactivation of inoculated *S. cerevisiae*.

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