A THESIS FOR THE DEGREE OF

MASTER OF SCIENCE IN FOOD AND NUTRITION

Bioconversion of Cyanidin-3-rutinoside to Cyanidin-3-glucoside in Black Raspberry by Crude α-L-Rhamnosidase from *Aspergillus* Species

Aspergillus 종에서 유래한 α-L-Rhamnosidase 를 이용한 Black Raspberry 의 Cyanidin-3-rutinoside 의 Cyanidin-3-glucoside 로의 생물전환

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ABSTRACT

Bioconversion of Cyanidin-3-rutinoside (C3R) to Cyanidin-3-glucoside (C3G) in Black Raspberry by Crude α-L-Rhamnosidase from *Aspergillus* Species

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The primary anthocyanins identified in black raspberry (*Rubus occidentalis*) were cyanidin-3-rutinoside (C3R), cyanidin-3-

xylosylrutinoside (C3XR), and cyanidin-3-glucoside (C3G). C3G has been known to be more bioavailable than C3R, the most abundant anthocyanin in black raspberry. In this study, in order to enhance bioavailability of anthocyanins in black raspberry, bioconversion of C3R to C3G in black raspberry was conducted by cleaving terminal L-rhamnose of C3R using crude enzyme extracts (CEE) from *A. usamii* KCTC 6956, *A. awamori* KCCM 60380, *A. niger* KCCM 11724, *A. oryzae* KCCM 12698, and *A. kawachii* KCCM 32819. Each *Aspergillus* species was grown in a medium containing L-rhamnose as an inducer, and the supernatant of medium was concentrated by filtration, centrifugation, and ultrafiltration in every 24 h. The concentrated filter residue was dissolved in 50 mM sodium acetate buffer (pH 3.8) to obtain CEE. The α-L-rhamnosidase and β-D-glucosidase activities of the CEE were determined by a spectrophotometric method using p-nitrophenyl-rhamnopyranoside and p-nitrophenyl-

glucopyranoside, respectively. Black raspberry juice (BRJ) (pH 3.8) was used as the substrate of bioconversion.

The CEE from *A. usamii* cultured for 8 days had the highest α-L-rhamnosidase activity with 2.73 U/mL, followed by those from *A. awamori, A. niger, A. oryzae,* and *A. kawachii*. The CEE from *A. usamii* cultured for 8 days also had the highest β-D-glucosidase activity with 2.75 U/mL, followed by those from *A. niger, A. awamori, A. kawachii,* and *A. oryzae*. All the CEE from *Aspergillus* species, except for *A. usamii*, showed the highest α-L-rhamnosidase activity on the 7^{th} day. α-L-Rhamnosidase had higher activity than β-D-glucosidase at all the times except for the CEE from *A. usamii* on the 8^{th} day. The *A. awamori* CEE showed the highest α-L-rhamnosidase activity at 30 and 40 ℃, while the enzyme activity of the *A. usamii* CEE was the highest at 50 and 60 ℃. *A. kawachii* and *A. oryzae* had no significant enzyme activities

under all tested conditions. When bioconversion of C3R to C3G in BRJ was analyzed by HPLC-DAD, the CEE from *A. usamii* and *A. awamori* hydrolyzed 95.7% and 95.6% of C3R to C3G, respectively, after 2 h incubation. The CEE from *A. kawachii* and *A. oryzae* did not convert C3R to C3G in BRJ. The result of the present study showed that *A. usamii* could be the most effective source for the bioconversion of C3R to C3G in BRJ.

Key words: *Rubus occidentalis*; cyanidin-3-rutinoside; cyanidin-3-glucoside; α-L-rhamnosidase; *Aspergillus*

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INTRODUCTION

Black raspberry (*Rubus occidentalis*) is one of the most abundant dietary sources of polyphenols and anthocyanins compared to other *Rubus* fruits (Wang et al., 2000; Moyer et al., 2002). Polyphenolic compounds such as anthocyanins have potential health benefits due to their strong antioxidant and anti-inflammatory activities (Scalbert et al., 2000). The primary anthocyanins identified in black raspberry are cyanidin-3-rutinoside (C3R), cyanidin-3-xylosylrutinoside (C3XR), and cyanidin-3-glucoside (C3G) (Tulio et al., 2008; Jung et al., 2014) (Fig. 1). C3R and C3XR in black raspberry have reported to exhibit high antioxidant activities (Tulio et al., 2008). C3G, which exists in a small amount in black raspberry, has potent antioxidant and anti-inflammatory effects (Tsuda et al., 2002; Choe et al., 2007; Jung et al., 2014). In the past

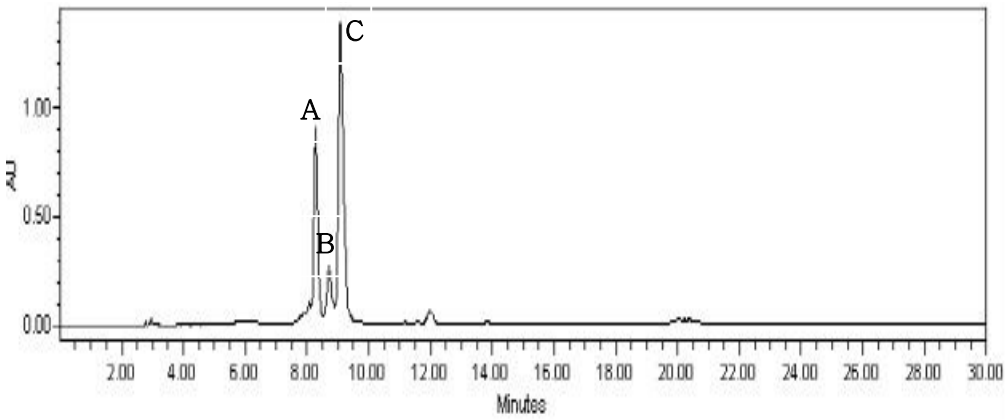


Figure 1. HPLC chromatogram of anothocyanin fractions in black raspberry juice: (A) cyanidin-3-xylosylrutinoside; (B) cyanidin-3-glucoside; (C) cyanidin-3-rutinoside

few decades, some studies focused on the enhancement of bioavailability of food by cleaving terminal L-rhamnose of flavonoid rutinoside. The absorption of flavonoid glucoside has been reported to be superior to that of flavonoid rutinoside (Hollman et al., 1999). It has been also reported that anthocyanins with an attached monoglycoside are more readily metabolized via methylation and/or glucuronide formation than those with di- or tri-glycoside (Wu et al., 2005). González-Barrio et al. (2004) reported that bioconversion using α-L-rhamnosidase from *A. aculeatus* could produce functional beverages based on their potentially increased flavonoid bioavailability. You et al. (2010) reported that transformation of rutin to quercetin-3-glucoside increased antiproliferative activity of the flavonol quercetin. In our lab, C3G was found to inhibit nitric oxide secretion specifically much more than C3R in the inflamed intestinal epithelial Caco-2 cells stimulated to conditioned medium from LPS-stimulated macrophage

RAW264.7 cells (unpublished). In an animal study, C3G was also found to appear rapidly in the plasma of rats when administered orally, while cyanidin was not detected in the plasma (Tsuda et al., 1999).

α-L-Rhamnosidase [EC.3.2.1.40] hydrolyzes the terminal α-L-rhamnose in naringin, hesperidin, rutin, terpenyl glycosides, and other glycosides containing α-L-rhamnose (Manzanares et al., 1997). The enzyme present in various fungi, bacteria, animal tissues, and plants (Kurosawa et al., 1973; Young et al., 1989) has been used for debittering citrus juice (Thomas et al., 1958) improving the flavor of grape juices or wine (Griffiths et al., 1959; Gallego et al., 2001) and preparing L-rhamnose (Fuse et al., 1988). Among the α-L-rhamnosidase-producing natural sources, *Aspergillus* species have been observed to be efficient enzyme producers for bioconversion of C3R to C3G in black raspberry due to their optimum pH ranging from 4 to 6 (Yadav et al., 2010). In

addition, various *Aspergillus* species are known to be non-toxic fungi (Takebe et al., 2003; Abe et al., 2006; Lee et al., 2007), and widely used in food industries. Many methods for assaying α-L-rhamnosidase have been reported by several researchers. Romero et al. (1985) reported that a convenient method using synthetic substrate p-nitrophenyl-α-L-rhamnopyranoside (pNPR) could assay the enzyme activity by monitoring the liberation of p-nitrophenolate ion at 400 nm (Fig. 2). This method could be easy to evaluate the enzyme activity; however, it cannot cover all substrates with terminal L-rhamnose due to the differences in structures of substrates and glycosidic linkages. In this study, α-L-Rhamnosidase and β-D-glucosidase activities of crude enzyme extracts (CEE) from five *Aspergillus* species (*A. usamii, A. awamori, A. niger, A. oryzae,* and *A. kawachii*) were determined by spectrophotometrical method using pNPR and p-nitrophenyl-β-D-glucopyranoside, respectively. The bioconversion of

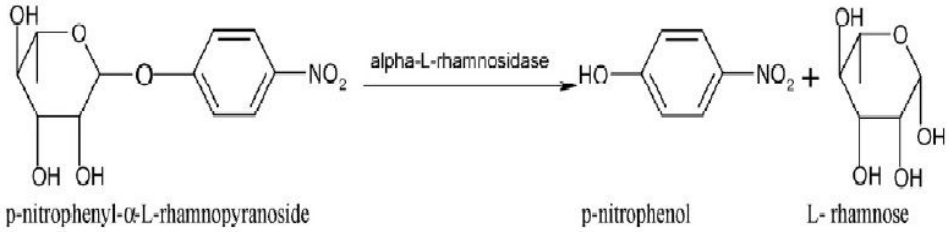


Figure 2. Determination of α −L−rhamnosidase activity with p−nitrophenyl− α −L−rhamnopyranoside

Cited from Yadav, 2010 (Yadav et al., 2010)

C3R to C3G in black raspberry juice (BRJ) by selected CEE was evaluated by HPLC analysis.

MATERIALS AND METHODS

1. Chemicals and reagents

C3G, C3R, p-nitrophenol, p-nitrophenyl-β-D-glucopyranoside (pNPG), and p-nitrophenyl-α-L-rhamnopyranoside (pNPR) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The protein assay reagents were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Sodium acetate and sodium carbonate were purchased from Samchun Chemical Co. (Korea).

2. Plant materials and sample preparation

Black raspberry (*Rubus occidentalis*) fruits, harvested in June 2008, were obtained from Gochang (Korea). The black raspberry was squeezed to get juice, which was then filtered through a Whatman No. 1 filter paper (Whatman International Ltd., Maidstone, England). The filtrate was centrifuged at 100 ×g for 10 min, and the supernatant, designated as black raspberry juice (BRJ), was stored at −20 ℃ before used as substrate of enzymatic bioconversion.

3. Fungal strains and culture conditions

A. usamii KCTC 6956 was purchased from Korean Collection for Type Cultures (Daejeon, Korea). *A. awamori* KCCM 60380, *A. niger* KCCM 11724, *A. oryzae* KCCM 12698, and *A. kawachii* KCCM 32819 were purchased from Korean Culture Center of Microorganisms (Seoul, Korea). *A. usamii*

and *A. awamori* were grown in maltose dextrose agar (Difco, Detroit, MI, USA), and the other species were grown in potato dextrose agar (Difco) under aerobic conditions at 30 ˚C. After incubation for 7 days, spores were suspended in 0.005% (w/v) Tween 80 solution with 0.9% (w/v) NaCl (Samchun Pure Chemical Co.) solution. At a concentration of 10^6/mL, the harvested spores were inoculated to 150 mL culture medium in a 500-mL Erlenmeyer flask, containing 0.1% (w/v) $NaNO_3$ (Junsei Chemical Co., Japan), 0.4% (w/v) yeast extract (Sigma Chemical Co.), 0.1% (w/v) tryptone (Sigma Chemical Co.), 0.05% (w/v) K_2HPO_4 (Sigma Chemical Co.), 0.05% (w/v) KCl (Samchun Pure Chemical Co.), 0.05% (w/v) $MgSO_4 \cdot 7H_2O$ (Samchun Pure Chemical Co.), 0.001% (w/v) $FeSO_4 \cdot 7H_2O$ (Samchun Pure Chemical Co.), and 0.5% (w/v) L-rhamnose (Sigma Chemical Co.) with pH adjusted to 6.0. The media with the spores were incubated in a shaking incubator (Changshin, Seoul, Korea) at 30 ˚C and 150 rpm for 8 days.

4. Preparation of crude enzyme extracts (CEE)

Five mL sample was taken from medium in every 24 hr, and the mycelia were removed by filtration using 0.45 μm syringe filter (Acrodisc, Pall Corporation, East Hills, NY, USA). Three mL of the filtrate was concentrated using Amicon Ultra - 4 centrifugal filters (Merck Millipore, Darmstadt, Germany) at 3000×g at 4 °C for 30 min, and the concentrated filter residue was dissolved in 500 μL 50 mM sodium acetate buffer (pH 3.8) to obtain CEE.

5. Spectrophotometrical enzyme assays

In order to find out the most effective *Aspergillus* species and conditions with respect to duration of incubating, reaction temperature for the bioconversion of anthocyanins in BRJ, α-L-Rhamnosidase and β-D-glucosidase activities of the CEE

were determined spectrophotometrically using pNPR and pNPG, respectively, as substrates (Romero et al., 1985). Ten μL of the CEE was added to 40 μL 5 mM pNPR or pNPG in 50 mM sodium acetate buffer (pH 3.8), followed by incubation at 30–60 °C for 10 min. The reaction was terminated by adding 150 μL 1 M sodium carbonate, and absorbance was measured at 405 nm. One unit of α-L-rhamnosidase or β-D-glucosidase activity was defined as the amount of enzyme that released 1 μmol of *p*-nitrophenol per min at each temperature and pH 3.8. The protein concentration was estimated by the Bradford method (Bradford, 1976) using bovine serum albumin as a standard.

6. Enzymatic bioconversion of C3R to C3G in BRJ

6.1. Determination of anthocyanins fraction in BRJ

The BRJ was dissolved in methanol (HPLC grade, J.T. Baker, Phillipsburg, NJ, USA) with 0.01% HCl (Samchun Pure Chemical Co.), and then fractionated using Sep-pak C18 cartridge (Waters Co., Milford, MA, USA), followed by filtration using 0.22 μm syringe filter (Pall Corporation). The anthocyanin composition was analyzed by HPLC (Waters 2996 Separation Module, Waters Co.) equipped with a photodiode array detector (Waters Co.) at 520 nm, and a XBridge C18 column (4.6 × 250 mm, 5 μm pore size, Waters Co.). The elution was performed using 5% (v/v) formic acid (Samchun Pure Chemical Co.)/water as solvent A and 100% acetonitrile (HPLC grade, J.T. Baker) as solvent B at a flow rate of 1 mL/min with the following gradient: 2% B (0-1 min); 2-10% B (1-2 min); 10-12.5% B (2-15.5 min); 12.5-60% B (15.5-21 min); 60-2% B (21-26 min); and 2% B (26-30 min). The injection of each sample was 20 μL.

6.2. Enzymatic bioconversion in BRJ with CEE

Twenty μL of the CEE was added to 200 μL of BRJ, followed by incubation at the temperature at which α-L-rhamnosidase activity showed the highest for every *Aspergillus* species. With a 30 min interval, the reaction was terminated by heating at 100 °C for 5 min. The reaction mixture was fractionated as mentioned in section 6.1.

7. Statistical analysis

All the experiments were performed in triplicate, and the data was expressed as means ± SEM. Unpaired t-test or one-way analysis of variance (ANOVA) was performed with SPSS program (SPSS Inc., Chicago, IL, USA). If significant by ANOVA, differences among the samples were determined using Duncan's multiple range test ($p<0.05$).

RESULTS AND DISCUSSION

1. Production and characterization of CEE

α-L-Rhamnosidase and β-D-glucosidase activities of the CEE from *Aspergillus* species were examined using pNPR and pNPG, respectively. The enzymatic characteristics of the CEE from the five *Aspergillus* species are shown in Table 1. The *A. usamii* CEE had the highest level of maximum α-L-rhamnosidase activity (*Rmax*) with 2.73 U/mL. The *A. awamori* CEE also had significantly higher level of *Rmax* than the *A. niger* CEE with 1.28 U/mL. The *A. kawachii* CEE had the lowest level of *Rmax* with 0.05 U/mL. Similar results were reported in previous papers for *A. niger* (1.64 U/mL) and *A. kawachii* (0.045 U/mL) (Puri et al., 2005; Koseki et al., 2008). In the present study, the *A. usamii* CEE also showed the highest level of β-D-glucosidase activity with 2.75 U/mL,

Table 1. Characteristics of crude enzyme extracts (CEE) from *Aspergillus* species

	Rmax[a] (U/mL)	*Gmax*[b] (U/mL)	*Tmax*[c] (°C)	t_{max}[d] (day)	*Pmax*[e] (μg)
A. usamii KCTC 6956	2.73±0.03[f]	2.75±0.00[f]	60	8	24.1±3.85
A. awamori KCCM 60380	2.21±0.13[g]	0.61±0.06[h]	40	7	25.5±4.09
A. niger KCCM 11724	1.28±0.09[h]	1.09±0.02[g]	50	7	78.3±2.26
A. oryzae KCCM 12698	0.12±0.01[i]	0.02±0.01[i]	50	7	3.74±0.85
A. kawachii KCCM 32819	0.05±0.01[i]	0.03±0.00[i]	50	7	9.30±3.07

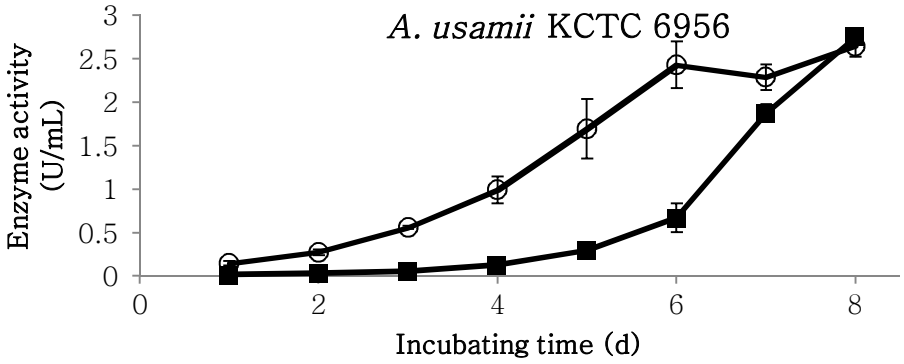
[a] The highest value of α-L-rhamnosidase activity at pH 3.8 (substrate: pNPR); [b] The highest value of β-D-glucosidase activity at pH 3.8 (substrate: pNPG); [c] Reaction temperature when the α-L-rhamnosidase activity of CEE reaches to *Rmax*; [d] Incubation time to reach *Rmax*; [e] Protein contents at *Tmax* on t_{max}; and [f],[g],[h],[i] Values with different superscripts within the same columns indicate significant differences.

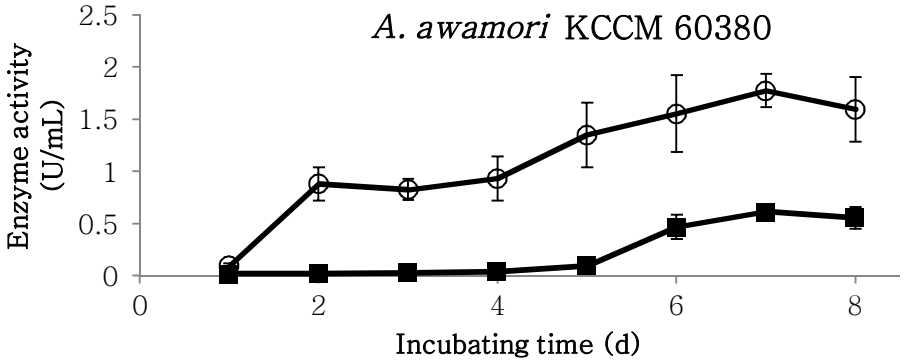
followed by the CEE from *A. niger, A. awamori, A. kawachii,* and *A. oryzae.* The CEE from *A. kawachii, A. niger,* and *A. oryzae* showed the highest *Rmax* at 50 ˚C, while the CEE from *A. awamori* and *A. usamii* showed the highest *Rmax* at 40 ˚C and 60 ˚C, respectively. All the CEE from *Aspergillus* species, except for *A. usamii*, showed the highest α-L-rhamnosidase activity on the 7^{th} day. Protein contents at the *Rmax* conditions ranged from 3.74 μg to 78.3 μg. The CEE from *A. niger* had the highest protein contents among the five, followed by those from *A. awamori* and *A. usamii. Rmax* of the CEE from *A. usamii* and *A. awamori* were significantly higher than that of the *A. niger* CEE, although the CEE from *A. niger* had 3-fold more proteins than those from *A. usamii* and *A. awamori*, these results imply that the CEE from *A. usamii* and *A. awamori* had more proteins related to α-L-rhamnosidase activity than that from *A. niger*. The CEE from *A. kawachii* and *A. oryzae* had less proteins compared to those from other

species.

2. Effects of incubating time of culture on enzyme activity

α-L-Rhamnosidase and β-D-glucosidase activities of the CEE from the five *Aspergillus* species measured at 50 °C for 8 days in every 24 h are shown in Fig. 3. Both of the enzymes had a tendency to increase their activities as incubating time passed, having the maximum activities on the 7th day. A previous study reported that naringinase extracted from *A. awamori* MTCC-2879 for debittering orange juice had the highest α-L-rhamnosidase activity on the 4th day (Puri et al., 2000). It has been reported that α-L-rhamnosidase purified from *A. kawachii* NBRC4308 which were grown in a medium containing 0.5% L-rhamnose as inducer had the highest activity on the 3rd day (Koseki et al., 2008). On the





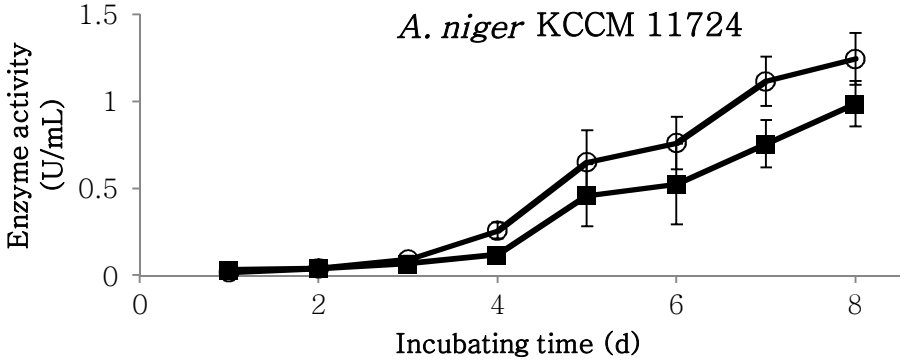


Figure 3. (*continued*)

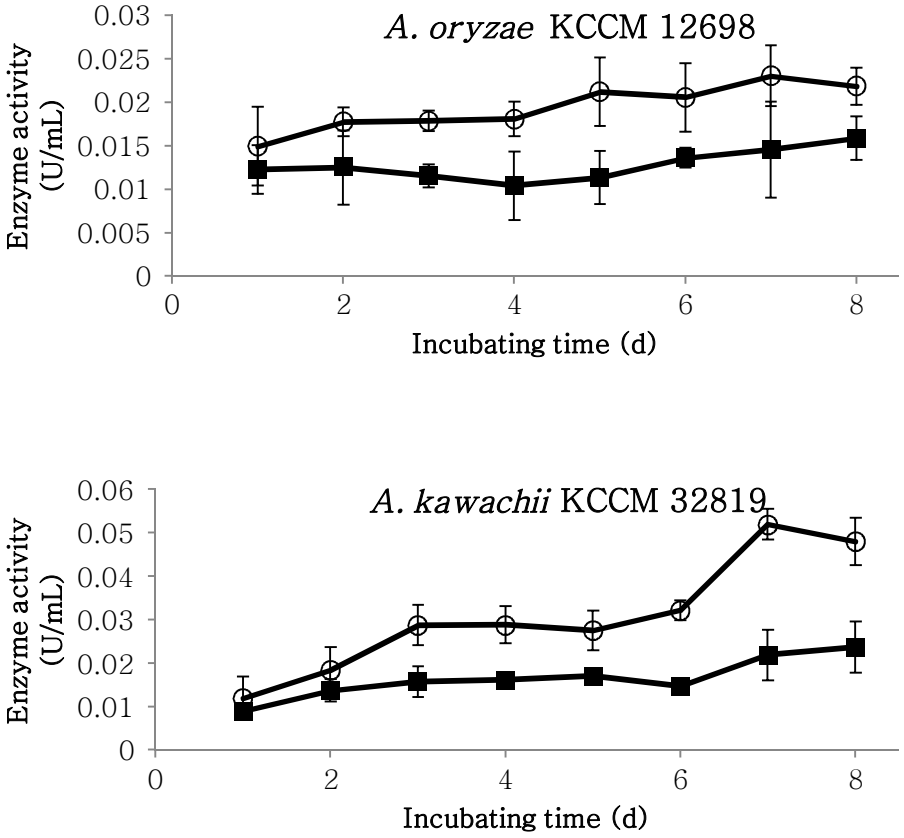


Figure 3. Time-course enzyme activities of crude enzyme extracts from *Aspergillus* species during incubation for 8 days at 50 ˚C: (◯) α-L-rhamnosidase activity; (■) β-D-glucosidase activity

other hand, in the present study, the CEE from *A. awamori* and *A. kawachii* were found to have the highest α-L-rhamnosidase activities on the 7th day with 1.77 U/mL and 0.05 U/mL, respectively. The reason why the incubating time to reach maximum enzyme activity showed the difference might be due to the difference in the basal medium composition. α-L-Rhamnosidase activity was higher than β-D-glucosidase activity at all the times except for the CEE from *A. usamii* on the 8th day. This result was expected because 0.5% (w/v) L-rhamnose was used as an inducer in this study. It has been reported that replacement of the carbon source could induce specific enzyme with no effects on growth of microorganisms (Puri et al., 2000). Concentrations of L-rhamnose have been also found to affect α-L-rhamnosidase activity (Koseki et al., 2008; You et al., 2010). The results of the previous studies imply that the quantity of carbon source remaining in medium during incubation might importantly affect enzyme secretion of

microorganisms. Generally, *Aspergillus* species have a strong activity of β-D-glucosidase, which converts a glucoside to an aglycone, as well as α-L-rhamnosidase activity (Bram et al., 1965). The high level of β-D-glucosidase activity is desirable for the role to hydrolyze naringin or terpenyl rutinoside (Manzanares et al., 1997). However, in this study, β-D-glucosidase is not desirable because an aglycone form may be less bioavailable than its glucoside form (Hollman et al., 1999). Both of the two enzymes had tendency to increase their activities as incubating time passed, having the maximum activity on the 7th day. A previous study reported that naringinase extracted from *A. awamori* MTCC-2879 for debittering orange juice had the highest α-L-rhamnosidase activity on the 4th day (Yadav et al., 2013). It has been reported that α-L-rhamnosidase purified from *A. kawachii* NBRC4308 which were grown in a medium containing 0.5% L-rhamnose as inducer had the highest activity on the 3rd day

(Koseki et al., 2008). On the other hand, the present study found that the CEE from *A. awamori* and *A. kawachii* had the highest α-L-rhamnosidase activity on the 7th day with 1.77 U/mL and 0.05 U/mL, respectively. The reason why the incubating time to reach maximum enzyme activity showed the difference might be due to the difference in culture conditions.

3. Effects of reaction temperature on enzyme activity

The effects of the temperature on α-L-rhamnosidase and β-D-glucosidase activities, the CEE obtained on the 7th day were measured at 30, 40, 50, and 60 °C (Fig. 4). As the temperature increased, α-L- rhamnosidase had a tendency to increase gradually. The *A. awamori* CEE showed the highest α-L-rhamnosidase activity at 30 and 40 °C, while the α-L-rhamnosidase activity of the *A. usamii* CEE was highest at 50 and 60 °C. These results imply that extracellular enzyme

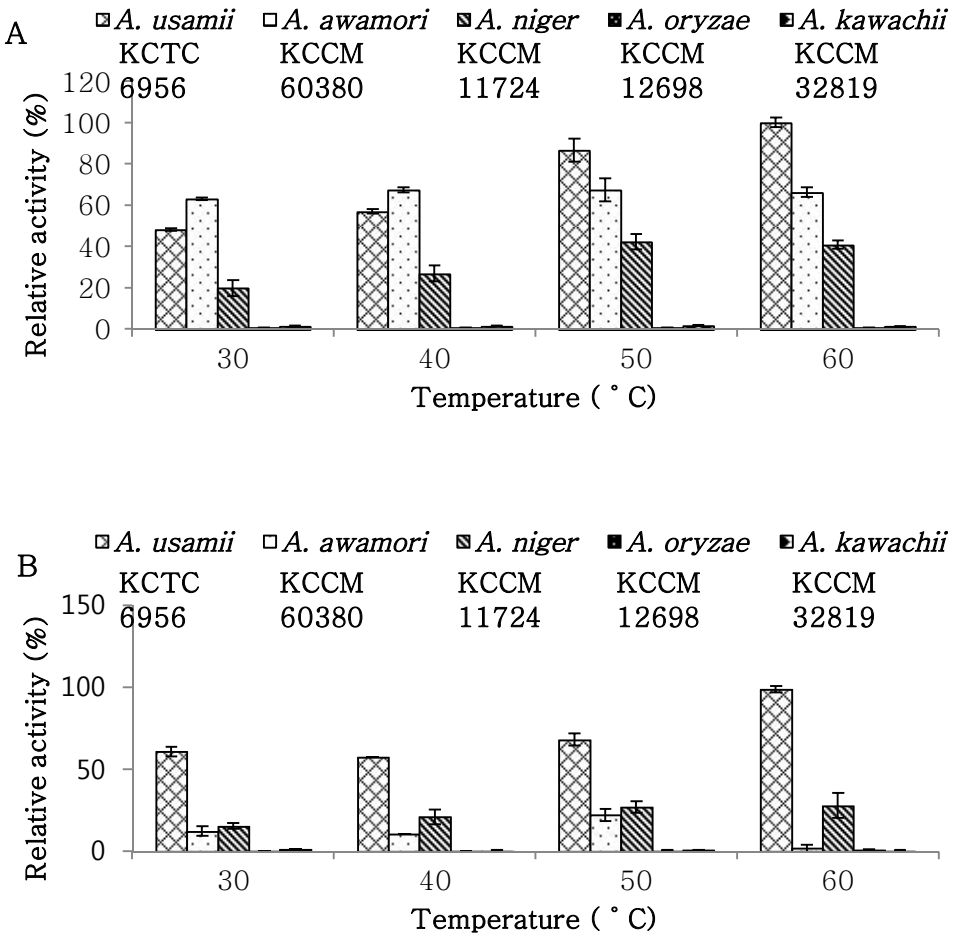


Figure 4. Effects of temperature on relative enzyme activities of crude enzymes extracted from *Aspergillus* species on the 7th day: (A) α−L−rhamnosidase; (B) β−D−glucosidase

from *A. usamii* might be more active at higher temperatures than that from *A. awamori*. *A. kawachii* and *A. oryzae* had no significant α-L-rhamnosidase activity under all the conditions. The optimum temperature for the α-L-rhamnosidase activity from at from *A. awamori*. *A. kawachii* and *A. oryzae* had no significant α-L-rhamnosidase activity under all the conditions. The optimum temperature for the α-L-rhamnosidase activity from *Aspergillus* species has been reported to range from 40 °C to 65 °C (Manzanares et al., 1997; Gallego et al., 2001; Koseki et al., 2008; Yadav et al., 2010; Yadav et al., 2013).

β-D-Glucosidase activity also had a tendency to increase gradually as the temperature increased, except for the *A. awamori* CEE (Fig. 4). β-D-glucosidase activity of the *A. awamori* CEE decreased rapidly at 60 ℃ unlike its α-L-rhamnosidase activity. At all the temperatures, the *A. usamii*

CEE was the highest β-D-glucosidase activity. *A. kawachii* and *A. oryzae* had also no significant β-D-glucosidase activity under all the conditions compared to other CEE from *Aspergillus* species.

4. HPLC analysis of the bioconversion of C3R to C3G in BRJ

Table 2 shows the most effective conditions of the CEE considering α- L-rhamnosidase and β-D-glucosidase activities based on the results described above. Anthocyanin fraction from BRJ consisted of C3XR, C3G, and C3R. The bioconversion of C3R to C3G in BRJ resulted in a decrease of C3R peak area and an increase of C3G peak area (Fig. 5). Table 3 shows that the changes of peak area for 2 h incubating under the conditions presented in Table 2. The CEE from *A. usamii,* which had the highest α-L- rhamnosidase activity for

Table 2. Profiles of selected crude enzyme extracts from *Aspergillus* species for bioconversion in black raspberry juice

	Incubating time[a] (day)	Temperature[b] (°C)	α-L-Rhamnosidase[c] (U/mL)	β-D-Glucosidase[d] (U/mL)
A. usamii KCTC 6956	5	60	2.36 ± 0.35	0.62 ± 0.13
A. awamori KCCM 60380	7	40	2.21 ± 0.13	0.21 ± 0.03
A. niger KCCM 11724	7	50	1.28 ± 0.09	0.75 ± 0.14
A. oryzae KCCM 12698	7	50	0.02 ± 0.00	0.01 ± 0.00
A. kawachii KCCM 32819	7	50	0.05 ± 0.00	0.02 ± 0.00

[a] Incubating time of culture; [b] Reaction temperature; [c] Enzyme activity at pH 3.8 (substrate: pNPR); [d] Enzyme activity at pH 3.8 (substrate: pNPG)

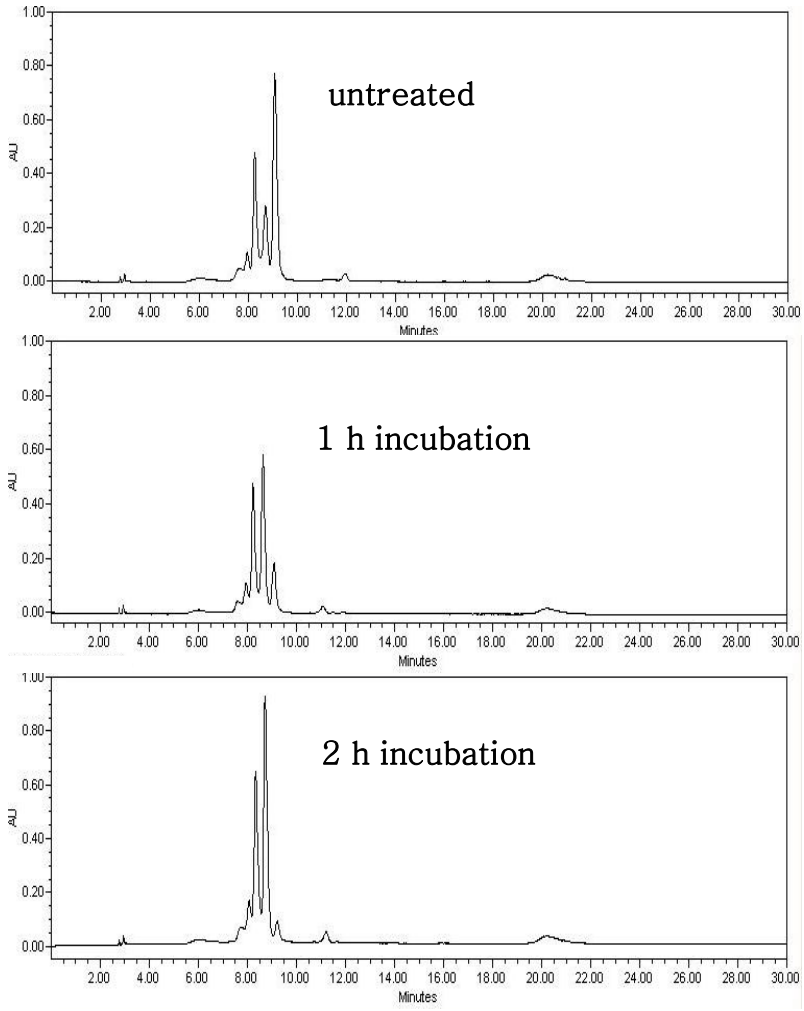


Figure 5. HPLC chromatogram of anthocyanin fractions from black raspberry juice treated with crude enzyme extract from *Aspergillus usamii* KCTC 6956

Table 3. Composition of cyanidin-3-rutinoside (C3R) and cyanidin-3-glucoside (C3G) in black raspberry juice after bioconversion for 2 h by crude enzyme extracts from *Aspergillus* species

(% by peak area)

	C3G	C3R	C3G/C3R
Black raspberry	13.5 ± 1.81	40.4 ± 4.16	0.33
A. usamii KCTC 6956	44.1 ± 0.18	1.60 ± 0.02	27.56
A. awamori KCCM 60380	41.5 ± 2.56	1.80 ± 0.03	23
A. niger KCCM 11724	27.4 ± 0.63	11.4 ± 0.35	2.4
A. oryzae KCCM 12698	13.6 ± 0.62	40.3 ± 0.25	0.34
A. kawachii KCCM 32819	13.5 ± 2.57	40.3 ± 0.25	0.33

pNPR, also had the most effective activity on the bioconversion in BRJ, hydrolyzing 95.7% of C3R to C3G. The CEE from *A. awamori* and *A. niger* hydrolyzed 95.6% and 64.7% of C3R to C3G, respectively. The CEE from *A. kawachii* and *A. oryzae* had no effects on the bioconversion in BRJ. α-L-Rhamnosidase has been reported that the enzyme activity varies depending on the substrate structures and the types of glycosidic linkages(Yadav et al., 2010). It is possible that α-L-rhamnosidase could only be active on pNPR, which has an α-1 glycosidic linkage, but not C3R, which has an α-1, 6 glycosidic linkage. In the present study, the bioconversion rate of C3R in black raspberry was found to be in the same order as the screened results using pNPR. The *A. niger* CEE, which had the highest β-D-glucosidase activity among the selected CEE with 0.75U/mL, reduced the sum of C3G and C3R peak areas from 53.9% to 38.8%. The CEE from *A. usamii* and *A. awamori*, which had less β-D- glucosidase activity than *A.*

niger, reduced the sum of C3G and C3R peak areas to 45.7% and 43.3%, respectively. These results imply that C3G was converted to cyanidin by β-D-glucosidase activity in the CEE. In conclusion, *A. usamii*, *A. awamori*, and *A. niger* were found to be effective α-L-rhamnosidase sources as determined by a spectrophotometric method using pNPR, and *A. usamii* could be the most effective source for the bioconversion of C3R to C3G in BRJ. The results of the present study could apply hydrolytic properties of the food-grade α-L-rhamnosidase from *A. usamii* to industrial bioconversion of other flavonoid rutinosides as well as C3R, which has an α-1,6 glycosidic linkage.

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국문 초록

Black raspberry (*Rubus occidentalis*)의 주요 anthocyanin은 cyanidin-3-rutinoside (C3R), cyanidin-3-xylosylrutinoside, cyanidin-3-glucoside (C3G) 등이다. Black raspberry 내에 가장 많이 존재하는 anthocyanin은 C3R이지만, C3G보다 생체이용률이 낮다고 알려져 있다. 본 연구의 목적은 5종류의 *Aspergillus* 곰팡이 (*A. usamii* KCTC 6956, *A. awamori* KCCM 60380, *A. niger* KCCM 11724, *A. oryzae* KCCM 12698, *A. kawachii* KCCM 32819) 에서 추출한 조효소액의 특성을 파악하고, α-L-rhamnosidase 활성이 높은 조효소액을 이용하여 black raspberry 과즙의 C3R을 C3G로 전환하는 것이다. α-L-Rhamnosidase 활성이 높은 조효소액을 얻기 위해 *Aspergillus* 포자를 rhamnose가 포함된 액체 배지에서 8일간 배양한 뒤, 매일 동시간에 배지 상층액을 얻고 여과 및 원심분리 등으로 농축시켜 조효소액으로 사용하였다. 조효소액의 효소 활성도는 p-nitrophenyl-

rhamnopyranoside와 p-nitrophenyl-glucopyranoside를 기질로 하여 측정하였다.

A. usamii 에서 추출한 조효소액은 배양 8일차에 2.73 U/mL로 최대 α-L-rhamnosidase 활성을 보였고, 이어서 *A. awamori, A. niger, A. oryzae, A. kawachii* 순이었다. *A. usamii*에서 배양 8일차에 추출한 조효소액은 2.75 U/mL로 최대 β-D-glucosidase 활성을 보였다. 50 °C에서 효소 활성도를 측정했을 때, *A. usamii*에서 추출한 조효소액을 제외한 다른 *Aspergillus* 종들의 조효소액은 배양 7일차에 최대 α-L-rhamnosidase 활성을 보였다. 대부분의 조건에서 α-L-rhamnosidase 활성은 β-D-glucosidase 활성보다 높았다. 배양 7일차 조효소액의 효소 활성도를 측정했을 때, 30 °C와 40 °C에서는 *A. awamori*에서 추출한 조효소액의 α-L-rhamnosidase 활성이 가장 높았고, 50 °C와 60 °C에서는 *A. usamii*에서 추출한 조효소액의 α-L-rhamnosidase 활성이 가장 높았다. *A. kawachii*와 *A. oryzae*의 배양 7일차 조효소액은 모든 반응 온도에서 α-L-rhamnosidase 활성이 거의 존재하

지 않았다. Black raspberry 과즙과 조효소액을 두 시간 동안 반응시켜 black raspberry 과즙의 anthocyanin 전환을 HPLC-DAD로 확인한 결과, *A. usamii* 배양 5일차 조효소액과 *A. awamori* 배양 7일차 조효소액은 각각 black raspberry의 C3R 95.7%와 95.6%를 C3G로 전환했다. *A. kawachii* 배양 7일차 조효소액과 *A. oryzae* 배양 7일차 조효소액은 black raspberry 과즙의 C3R을 C3G로 거의 전환하지 못했다.

주요어: black raspberry, cyanidin-3-rutinoside, cyanidin-3-glucoside, α-L-rhamnosidase, *Aspergillus*

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