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

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Cyanidin-3-glucoside (C3G) has been known to be more bioavailable than cyanidin-3-rutinoside (C3R), the most abundant anthocyanin in black raspberry (*Rubus occidentalis*). The aim of this study was to enhance the bioavailability of anthocyanins in black raspberry by cleaving L-rhamnose in C3R using crude enzyme extracts (CEEs) from *Aspergillus usamii* KCTC 6956, *A. awamori* KCTC 60380, *A. niger* KCCM 11724, *A. oryzae* KCCM 12698, and *A. kawachii* KCCM 32819. The enzyme activities of the CEEs were determined by a spectrophotometric method using ρ-nitrophenyl-rhamnopyranoside and ρ-nitrophenyl-glucopyranoside. The CEE from *A. usamii* had the highest α-L-rhamnosidase activity with 2.73 U/ml at 60°C, followed by those from *A. awamori* and *A. niger*. When bioconversion of C3R to C3G in black raspberry was analyzed by HPLC-DAD, the CEEs from *A. usamii* and *A. awamori* hydrolyzed 95.7% and 95.6% of C3R to C3G, respectively, after 2 h incubation. The CEEs from *A. kawachii* and *A. oryzae* did not convert C3R to C3G in black raspberry.

Keywords: *Aspergillus*, rhamnosidase, anthocyanin, black raspberry

Introduction

Black raspberry (*Rubus occidentalis*) is one of the most abundant dietary sources of polyphenols and anthocyanins compared with other *Rubus* fruits [15, 27]. Polyphenolic compounds such as anthocyanins have potential health benefits due to their strong antioxidant and anti-inflammatory activities [20]. The primary anthocyanins identified in black raspberry are cyanidin-3-rutinoside (C3R), cyanidin-3-xylosylrutinoside (C3XR), and cyanidin-3-glucoside (C3G) [10, 26]. C3R and C3XR in black raspberry exhibit high antioxidant activities [26]. C3G, which exists in a small amount in black raspberry, has potent antioxidant and anti-inflammatory effects [4, 10, 25].

In the past few decades, some studies focused on enhancement of the bioavailability of flavonoid rutinoside by cleaving the terminal L-rhamnose [7, 28]. The absorption of flavonoid glucoside has been reported to be superior to that of flavonoid rutinoside [9]. It has also been reported that anthocyanins with an attached monoglycoside are more readily metabolized *via* methylation and/or glucuronide formation than those with di- or tri-glycoside [29]. Talavéra *et al*. [22] demonstrated that the absorption of C3R was lower than that of C3G in rats. Tsuda *et al*. [25] reported that cyanidin, the aglycone form of C3G and C3R, showed low absorption rate in rats, whereas C3G appeared rapidly in the plasma when administered orally. However, the bioconversion of anthocyanins in the food matrix to enhance bioavailability has been rarely studied.

α-L-Rhamnosidase (E.C. 3.2.1.40) hydrolyzes the terminal α-L-rhamnose in naringin, hesperidin, rutin, terpenyl glycosides, and other glycosides containing α-L-rhamnose [14]. The enzyme present in various fungi, bacteria, animal tissues, and plants [12, 32] has been used for debittering citrus juice [23], enhancing the flavor of grape juice or wine [6, 8], and preparing L-rhamnose [5]. Among the α-L-rhamnosidase-producing natural sources, some *Aspergillus* species are known to be non-toxic fungi [1, 13, 21] and widely used in food industries. In addition, *Aspergillus* species have been observed to be efficient enzyme producers owing to their optimum pH ranging from 4 to 6 [31]. In most studies [11, 14], ρ-nitrophenyl-α-L-rhamnopyranoside (pNPR), naringin, hesperidin, and rutin have been used as substrates for the evaluation of α-L-rhamnosidase activity,

whereas C3R in the food matrix was rarely used.

In this study, therefore, the enzyme activities of crude enzyme extracts (CEEs) from five *Aspergillus* species (*A. usamii* KCTC 6956, *A. awamori* KCTC 60380, *A. niger* KCCM 11724, *A. oryzae* KCCM 12698, and *A. kawachii* KCCM 32819) were screened depending on the incubating time and temperature by spectrophotometric assays. The most effective CEE of each species was mixed with black raspberry juice (BRJ) to examine the bioconversion of C3R to C3G in the food matrix. To the best of our knowledge, this study is the first trial to compare the bioconversion of an anthocyanin in black raspberry using the five *Aspergillus* species.

Materials and Methods

Chemicals and Reagents

C3G, C3R, ρ-nitrophenol, ρ-nitrophenyl-β-D-glucopyranoside (pNPG), and pNPR were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Protein assay reagents were purchased from Bio-Rad Laboratories (Hercules, CA, USA).

Plant Materials and Sample Preparation

Black raspberry fruits, harvested in June 2013, 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, UK). The filtrate was centrifuged at 100 ×*g* for 10 min, and the supernatant, designated as BRJ, was stored at −20°C until used as substrate for enzymatic bioconversion.

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 solution. The harvested spores at a concentration of 10^6 spores/ml were inoculated to 150 ml of culture medium in a 500 ml Erlenmeyer flask, containing 0.1% (w/v) $NaNO_3$, 0.4% (w/v) yeast extract, 0.1% (w/v) tryptone, 0.05% (w/v) K_2HPO_4, 0.05% (w/v) KCl, 0.05% (w/v) $MgSO_4 \cdot 7H_2O$, 0.001% (w/v) $FeSO_4 \cdot 7H_2O$, and 0.5% (w/v) L-rhamnose, with the pH adjusted to 6.0. The media with the spores were incubated at 30°C and 150 rpm for 8 days.

Preparation of Crude Enzyme Extracts

A 5 ml sample was taken from the medium at every 24 h, and the mycelia were removed by filtration using a 0.45 μm syringe filter (Acrodisc, Pall Corporation, East Hills, NY, USA). Three milliliters of filtrate was concentrated using Amicon Ultra-4 centrifugal filters (10 kDa molecular weight cutoff; Merck Millipore, Darmstadt, Germany) at 3,000 ×*g* at 4°C for 30 min, and the concentrated filter residue was dissolved in 500 μl of 50 mM sodium acetate buffer (pH 3.8) to obtain CEEs for the determination of their enzyme activities.

Enzyme Assay

α-L-Rhamnosidase and β-D-glucosidase activities of the CEEs were determined spectrophotometrically using pNPR and pNPG, respectively, as substrates [19]. CEE (10 μl) was added to 40 μl of 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 of 1 M sodium carbonate, and the 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 ρ-nitrophenol per minute at each temperature and pH 3.8. The protein concentration was estimated by the Bradford method [2] using bovine serum albumin as the standard.

Enzymatic Bioconversion of C3R to C3G in BRJ by CEE

CEE (20 μl) 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 an interval of 60 min, the reaction was terminated by heating at 100°C for 5 min. The reaction mixture was fractionated using a Sep-Pak C18 cartridge (Waters Co., Milford, MA, USA), followed by filtration using a 0.22 μm syringe filter (Pall Corporation). The anthocyanin composition was analyzed by a HPLC (Waters 2996 Separation Module, Waters Co.) equipped with a XBridge C18 column (4.6 × 250 mm, 5 μm pore size; Waters Co.) and a photodiode array detector (Waters Co.) at 520 nm. The elution was performed using 5% (v/v) aqueous formic acid solution as solvent A and 100% acetonitrile 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).

Statistical Analysis

All the experiments were performed in triplicate, and the data were expressed as the mean ± standard error. One-way analysis of variance (ANOVA) was performed with the 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

Production and Characterization of CEEs

α-L-Rhamnosidase and β-D-glucosidase activities of the CEEs from *Aspergillus* species were examined using pNPR and pNPG, respectively. The enzymatic characteristics of

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

	R_{max} (U/ml)	G_{max} (U/ml)	T_{max} (°C)	t_{max} (d)	P_{max} (μg)
A. usamii	2.73 ± 0.03^a	2.75 ± 0.00^a	60	8	24.1 ± 3.85
A. awamori	2.21 ± 0.13^b	0.61 ± 0.06^c	40	7	25.5 ± 4.09
A. niger	1.28 ± 0.09^c	1.09 ± 0.02^b	50	7	78.3 ± 2.26
A. oryzae	0.12 ± 0.01^d	0.02 ± 0.01^d	50	7	3.74 ± 0.85
A. kawachii	0.05 ± 0.01^d	0.03 ± 0.00^d	50	7	9.30 ± 3.07

Values are the mean ± standard error (n = 3). Values with different superscripts within the same columns indicate significant differences. R_{max}: the highest value of α-L-rhamnosidase activity at pH 3.8 (substrate: pNPR); G_{max}: the highest value of β-D-glucosidase activity at pH 3.8 (substrate: pNPG); T_{max}: reaction temperature to reach R_{max}; t_{max}: incubation time to reach R_{max}; and P_{max}: protein contents at T_{max} on t_{max}.

the CEEs from the five *Aspergillus* species are shown in Table 1. The *A. usamii* CEE had the highest level of maximum α-L-rhamnosidase activity (R_{max}) with 2.73 U/ml. The *A. awamori* CEE also had a significantly ($p < 0.05$) higher level of R_{max} with 2.21 U/ml than the *A. niger* CEE with 1.28 U/ml. The *A. kawachii* CEE had the lowest level of R_{max} 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) [11, 18]. In the present study, the *A. usamii* CEE also showed the highest level of β-D-glucosidase activity with 2.75 U/ml, followed by the CEEs from *A. niger*, *A. awamori*, *A. kawachii*, and *A. oryzae*. The CEEs from *A. kawachii*, *A. niger*, and *A. oryzae* showed the highest R_{max} at 50°C, whereas the CEEs from *A. awamori* and *A. usamii* showed the highest R_{max} at 40°C and 60°C, respectively. All the CEEs from the *Aspergillus* species, except for *A. usamii*, showed the highest α-L-rhamnosidase activity on the 7th day. Protein contents at the R_{max} conditions ranged from 3.74 to 78.3 μg. The CEE from *A. niger* had the highest protein content among the five, followed by those from *A. awamori* and *A. usamii*. R_{maxs} of the CEEs from *A. usamii* and *A. awamori* were significantly ($p < 0.05$) 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 CEEs from *A. usamii* and *A. awamori* had more proteins related to α-L-rhamnosidase activity than that from *A. niger*. The CEEs from *A. kawachii* and *A. oryzae* had less proteins compared with those from the other species.

Effects of Incubation Time of Culture on Enzyme Activity

α-L-Rhamnosidase and β-D-glucosidase activities of the CEEs from the five *Aspergillus* species measured at 50°C for 8 days at every 24 h are shown in Fig. 1. Both of the enzymes had a tendency to increase their activities as the incubation time increased, 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 [30]. It has been reported that α-L-rhamnosidase purified from *A. kawachii* NBRC4308, which was grown in a medium containing 0.5% L-rhamnose as inducer, had the highest activity on the 3rd day [11]. On the other hand, in the present study, the CEEs from *A. awamori* and *A. kawachii* were found to have 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 incubation 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 a specific enzyme with no effects on the growth of microorganisms [17]. Concentrations of L-rhamnose have also been found to affect α-L-rhamnosidase activity [11, 28]. The results of the previous studies imply that the quantity of carbon source remaining in the medium during incubation might importantly affect the enzyme secretion of microorganisms. Generally, *Aspergillus* species have a strong activity of β-D-glucosidase, which converts a glucoside form to an aglycone form, as well as α-L-rhamnosidase activity [3]. A high level of β-D-glucosidase activity is desirable for the role to hydrolyze naringin or terpenyl rutinoside [14]. However, in this study, β-D-glucosidase is not desirable because an aglycone form may be less bioavailable than its glucoside form [9].

Effects of Reaction Temperature on Enzyme Activity

The effects of the temperature on α-L-rhamnosidase and β-D-glucosidase activities of the CEEs obtained on the 7th day were measured at 30°C, 40°C, 50°C, and 60°C (Fig. 2). As the temperature increased, the enzyme activities had a

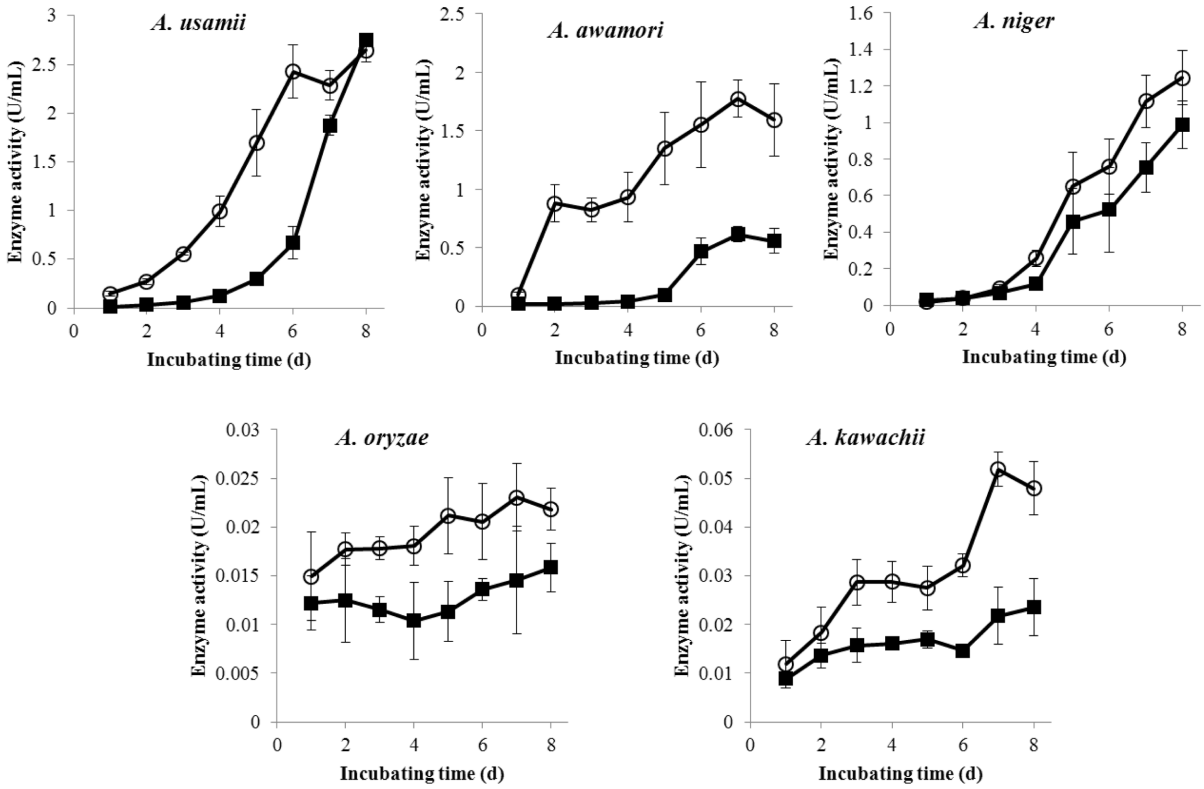

Fig. 1. Time-course of enzyme activities of crude enzyme extracts from *Aspergillus* species during 8 d of incubation at 50°C.
Values are the mean ± standard error (n = 3). (○) α-L-rhamnosidase activity; and (■) β-D-glucosidase activity.

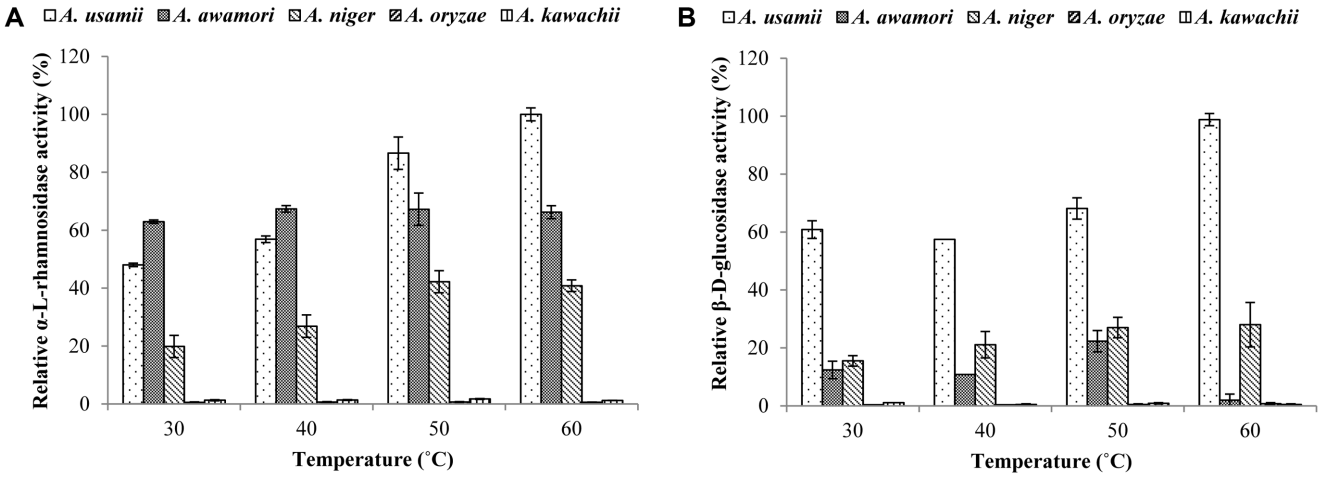

Fig. 2. Effect of temperature on the relative (**A**) α-L-rhamnosidase and (**B**) β-D-glucosidase activities of crude enzyme extracts from *Aspergillus* species after 7 d of incubation.
Values are the mean ± standard error (n = 3).

tendency to increase gradually. Among the CEEs produced by the five *Aspergillus* species, the *A. awamori* CEE showed the highest α-L-rhamnosidase activity at 30°C and 40°C, whereas the *A. usamii* CEE showed the highest at 50°C and 60°C. These results imply that the α-L-rhamnosidase from *A. usamii* might be more active at higher temperature than

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

	Time[a] (days)	Temperature[b] (°C)	α-L-Rhamnosidase[c] (U/ml)	β-D-Glucosidase[d] (U/ml)
A. usamii	5	60	2.36 ± 0.35	0.62 ± 0.13
A. awamori	7	40	2.21 ± 0.13	0.21 ± 0.03
A. niger	7	50	1.28 ± 0.09	0.75 ± 0.14
A. oryzae	7	50	0.02 ± 0.00	0.01 ± 0.00
A. kawachii	7	50	0.05 ± 0.00	0.02 ± 0.00

Values are the mean ± standard error (n = 3).

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

that from *A. awamori*. The optimum temperature for the α-L-rhamnosidase activity from *Aspergillus* species has been reported to range from 40°C to 65°C [6, 11, 14, 30].

Among the five *Aspergillus* species, the *A. usamii* CEE showed the highest β-D-glucosidase activity at all the tested temperatures. The β-D-glucosidase activity of the *A. awamori* CEE increased with temperature up to 50°C and dropped sharply at 60°C. *A. kawachii* and *A. oryzae* had little activities of both α-L-rhamnosidase and β-D-glucosidase at all the tested temperatures.

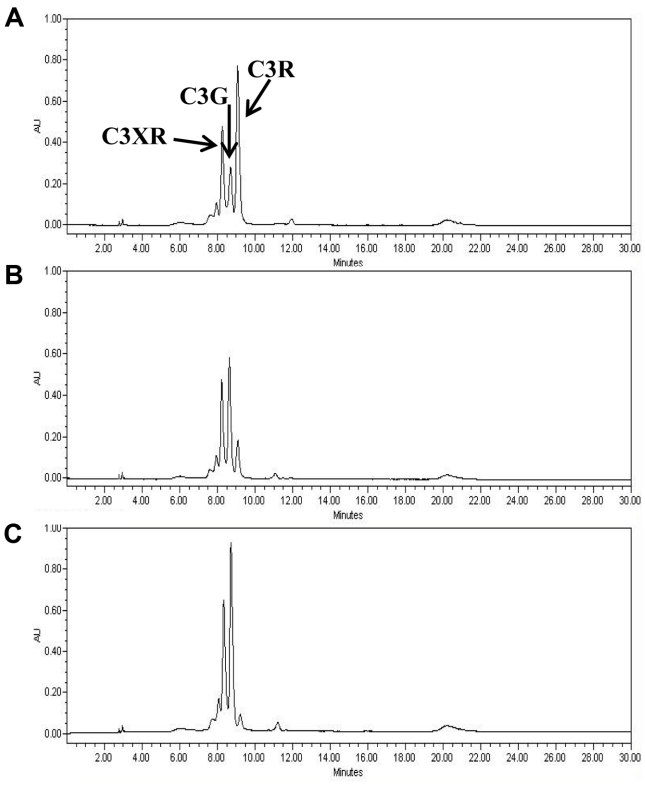

Fig. 3. HPLC chromatograms of anthocyanin fraction from black raspberry juice treated with crude enzyme extract from *Aspergillus usamii*.

(**A**) Black raspberry juice (initial); (**B**) after 1 h incubation; and (**C**) after 2 h incubation. C3XR: cyanidin-3-xylosylrutinoside; C3G: cyanidin-3-glucoside; and C3R: cyanidin-3-rutinoside.

Bioconversion of C3R to C3G in Black Raspberry

To determine the bioconversion of anthocyanins in black raspberry, the CEEs that had the highest α-L-rhamnosidase activity with lower β-D-glucosidase activity were selected on the basis of the above experiments (Figs. 1 and 2) and presented in Table 2. The anthocyanin fraction from black raspberry mainly consisted of C3XR, C3G, and C3R (Fig. 3). The bioconversion of C3R to C3G in black raspberry resulted in a decreased C3R peak area and an increased C3G peak area (Fig. 3). The contents of the anthocyanins in black raspberry when incubated for 2 h under the selected conditions are shown in Table 3. The CEE from *A. usamii* that had the highest α-L-rhamnosidase activity for pNPR was also the most effective on the bioconversion in black raspberry, hydrolyzing 95.7% of C3R to C3G. The CEEs from *A. awamori* and *A. niger* hydrolyzed 95.6% and 64.7% of C3R to C3G, respectively. The CEEs from *A. kawachii* and *A. oryzae* had no effects on the bioconversion of the

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

	C3G (μg/ml)	C3R (μg/ml)
BRJ	106.9 ± 11.9	283.7 ± 27.4
CEE[a]		
A. usamii	307.8 ± 1.2	28.6 ± 0.2
A. awamori	291.1 ± 16.8	29.7 ± 0.2
A. niger	198.2 ± 4.2	93.0 ± 2.3
A. oryzae	107.6 ± 4.1	282.8 ± 1.6
A. kawachii	106.6 ± 16.9	282.8 ± 1.6

Values are the mean ± standard error (n = 3).

[a]Prepared under the conditions presented in Table 2.

anthocyanin in black raspberry.

α-L-Rhamnosidase activity is known to be influenced by the anthocyanin aglycone and sugar moiety, and also by the types of glycosidic linkages [31], implying that a higher α-L-rhamnosidase activity when using either pNPR, naringin, or hesperidin as a substrate cannot ensure to hydrolyze C3R to C3G. However, in the present study, the bioconversion rate of C3R in black raspberry was found to be similar to the results screened by pNPR. The *A. niger* CEE, which had the highest β-D-glucosidase activity with 0.75 U/ml among the selected CEEs (Table 2), reduced the sum of C3G and C3R from 390.6 μg/ml in the juice to 291.2 μg/ml after the treatment. The CEEs from *A. usamii* and *A. awamori*, which had less β-D-glucosidase activity than *A. niger*, reduced the sum of C3G and C3R to 336.4 and 320.8 μg/ml, respectively. These results imply that C3G was also converted to cyanidin by β-D-glucosidase activity in the CEEs.

A. usamii, *A. awamori*, and *A. niger* were found to be effective α-L-rhamnosidase sources as screened by a spectrophotometric method using pNPR, and *A. usamii* could be the most effective source for the bioconversion of C3R to C3G in black raspberry. The results of the present study suggest that the hydrolytic properties of the food-grade α-L-rhamnosidase from *A. usamii* may be applied to industrial bioconversion of other flavonoid rutinosides as well as C3R, which have an α-1,6-glycosidic linkage.

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