academicJournals

Vol. 12(3), pp. 35-39, March 2018
DOI: 10.5897/AJBR2017.0982
Article Number: 33EA90A56444
ISSN 1996-0778

http://www.academicjournals.org/AJBR

African Journal of Biochemistry Research

Full Length Research Paper

Effect of organic acids on the alteration of black rice anthocyanins by *Enterobacter aerogenes* NBRC 13534

Keiko YAMAMOTO, Noriaki SAIGUSA* and Yuji TERAMOTO

Department of Applied Microbial Technology, Faculty of Biotechnology and Life Science, Sojo University, Ikeda 4-22-1, Nishi Ward, Kumamoto 860-0082, Japan.

Received 19 December, 2017; Accepted February 27, 2018

In a previous study, it was reported that *Enterobacter aerogenes* and four organic acids are involved in the alteration of black rice anthocyanins. To investigate the factors involved in altering the coloration of black rice anthocyanins, organic acids (caffeic acid, ferulic acid, malonic acid, and *p*-hydroxybenzoic acid) were individually added to potato dextrose agar medium containing black rice anthocyanins and cultured with *E. aerogenes* NBRC 13534 at 37°C for five days. As a result, the color change of black rice anthocyanins was found in the medium supplemented with caffeic acid or ferulic acid among the four organic acids. HPLC analysis of anthocyanins in each medium revealed three new peaks not found in the original black rice anthocyanins in the sample supplemented with caffeic acid. Also, two new peaks were observed in the medium supplemented with ferulic acid.

Key words: Anthocyanin, anthocyanin modification, black rice, *Enterobacter aerogenes*, microorganism, color, pigment.

INTRODUCTION

Anthocyanins are a group of flavonoid-based pigments that impart various colors such as red, blue, and purple to flowers and fruits (Adachi and Yoshitama, 2004). The structure of anthocyanins is based on various combinations of anthocyanidin, sugar, and organic acid. Six types of anthocyanidins are predominantly found in nature: cyanidin, delphinidin, malvidin, pelargonidin, petunidin and peonidin (Zhao et al., 2014). As the sugar component, D-glucose, D-galactose, L-rhamnose, D-xylose, D-arabinose, and the like are often bound to anthocyanidins by a β-glycosidic bond. In addition, acylated anthocyanins are characterized by ester linkage of the organic acid to the sugar moiety and exhibit increased stability of color quality (Terahara, 1993). In contrast, anthocyanidins are characterized by low color stability, fading rapidly (Tsuda, 2012). Organic acids that bind to anthocyanins are largely divided into aromatic organic acids and aliphatic organic acids. Aromatics include hydroxycinnamic acids such as *p*-coumaric acid, caffeic acid, ferulic acid, and sinapinic acid, as well as hydroxybenzoic acids such as *p*-hydroxybenzoic acid and gallic acid. In addition to malonic acid and acetic acid, aliphatic organic acids include oxalic acid, succinic acid, and malic acid. Anthocyanins also undergo structural

*Corresponding author. E-mail: noriaki@bio.sojo-u.ac.jp Tel: +81-96-326-3111.

changes in response to pH, temperature, and the presence of enzymes. As a result of these properties, differences in components and environmental factors result in changes in the color quality of various plants (Ohba et al., 2000). Although anthocyanins are easily discolored and tend to fade, they are considered to be safer than artificial coloring compounds and are often used for coloring processed foods (Anna et al., 2014). Further, it has recently been reported that anthocyanins exhibit antioxidant activity (Tsuda, 2012). Thus, from the viewpoint of safety, anthocyanins are attractive compounds as colorants.

However, since anthocyanins are natural plant pigments, a multi-step process, including crop production, extraction, filtration, and purification, is necessary (Adachi and Yoshitama, 2004). Yields are also dependent on factors such as weather during crop cultivation (Aung et al., 2014). Therefore, in vitro approaches to the production of anthocyanins, where weather is not a factor, are being studied. Gregorio et al. (2017) reported that ethephon increased the content of anthocyanins in black carrot roots. However, despite advances in anthocyanin production in vitro, yields have not yet reached levels required commercially. To overcome this limitation, we speculated that the identification of microorganisms capable of modifying anthocyanins could lead to the rapid and inexpensive production of novel anthocyanins for use in foods. Anthocyanins are plant pigments. So, plant enzymes are usually used for structural modification of anthocyanins. However, this paper is an attempt to modify the structure of anthocyanin using microbial enzymes. Microbial growth and enzyme production is faster than that of plant. Therefore, microbial enzymes are very useful to convert from black rice anthocyanin to another anthocyanin as compared with that of plants. In addition, the main anthocyanin of black rice anthocyanin is Cyanidinn-3-glucoside, which is a very simple structure. Therefore, we believe it is easy to convert to various structures. We previously screened and identified microorganisms that could potentially modify the structure of anthocyanins (Saigusa et al., 2014).

The screened microorganisms were confirmed to have high homology with *E. aerogenes* NBRC 13534. *E. aerogenes* are known to exist in the soil or in the human intestinal tract. In addition, it has been reported that anthocyanin cleaves glucoside bonds and decomposes anthocyanidin heterocycle by intestinal microorganisms (Aura et al., 2005; Cheng et al., 2016; Mueller et al., 2017). From this, it was suggested that anthocyanin was degraded by *E. aerogenes*, and a change in anthocyanin occurred due to the binding of a new organic acid or sugar to the degraded anthocyanidin. In this study, *E. aerogenes* NBRC 13534 was used to investigate the effects of four organic acids (caffeic acid, ferulic acid, *p*-hydroxybenzoic acid and malonic acid) on alterations in black rice anthocyanins.

MATERIALS AND METHODS

Caffeic acid, ferulic acid, *p*-hydroxybenzoic acid, and malonic acid were purchased from Nacalai Tesque Co., Ltd. (Kyoto, Japan). Potato Dextrose Agar (PDA) medium was purchased from Nissui Co., Ltd. (Tokyo, Japan). All other reagents were of the highest grade available. Black rice (*Oryza sativa* var. *Indica* cv. *Shiun*) was purchased from Kajiwara Beikoku Co., Ltd. (Kyoto, Japan). *E. aerogenes* NBRC 13534 was purchased from NITE Bioresource Center (Chiba, Japan) as the microbial strain.

Incubation of *E. aerogenes* NBRC 13534

In this experiment, PDA medium containing anthocyanin extracted from black rice and organic acid was used to confirm the influence of organic acid on color quality change of black rice anthocyanin by *E. aerogenes* NBRC 13534. Black rice bran contains a lot of cyanidin - 3 - glucoside which is one kind of anthocyanin. (Nakagawa and Maeda, 2017). To separate the rice bran, unpolished black rice was subjected to flour mill "Hikiko" (Tokyo Unicom, Tokyo, Japan). It is necessary to always keep the pH acidic for extraction of pigment (Adachi and Yoshitama, 2004). So, to extract the anthocyanins, 25 g of bran was added to 250 mL of McIlvaine buffer (pH 4) and the resulting mixture was left in the dark at 15°C for 12 h. After filtering with gauze, the sample was centrifuged (10 min, 1309.23 ×g) and the supernatant was filtered using No. 1 filter paper (ADVANTEC Toyo Co. Ltd., Tokyo, Japan) to obtain the black rice anthocyanins. We prepared six different media. At first, PDA medium supplemented with 100 mL of black rice extract and 0.01 g of four organic acids (caffeic acid, ferulic acid, *p*-hydroxybenzoic acid, malonic acid) was prepared. Then, PDA medium containing 100 mL of black rice extract and 0.01 g of each organic acid was also prepared, respectively. As a control experiment, a medium supplemented with no organic acid was prepared. Each medium was sterilized in boiling water for 5 min. In order to maintain the anthocyanin structure, sterilization of the medium by autoclaving was avoided. After cooling, 20 mL of the medium was aseptically dispensed into a petri dish. After that, *E. aerogenes* NBRC 13534 was inoculated on the medium using a platinum loop and incubated at 37°C for 5 days. Changes in the color of the medium were assessed.

Absorption spectra

First, we extracted the anthocyanin from the medium. 15% acetic acid was used for anthocyanin extraction. The medium showing color changes was collected and added to a 15% acetic acid solution. After 12 h at 5 °C, anthocyanin was extracted from the medium. After that filtration of anthocyanin extract was performed using No. 1 filter paper (ADVANTEC Toyo Co. Ltd., Japan). Then, absorbance spectra of the anthocyanin extract were analyzed using a U-3010 spectrophotometer (Hitachi, Tokyo, Japan).

Anthocyanin analysis

Anthocyanins were analyzed using a high performance liquid chromatograph (HPLC) (Shimadzu, Kyoto, Japan) equipped with a CTO-10AC column oven and SPD-10AV UV-VIS detector. For analytical HPLC, an ODS-3 column (4.6 id×250 mm, GL Sciences, Inc. Tokyo, Japan) with solvents A (H_3PO_4:H_2O = 15:985) and B (H_3PO_4:AcOH:CH_3CN:H_2O = 15:200:250:535) under gradient conditions at a flow rate of 1 mL/min. Anthocyanin analysis was carried out using the following gradient conditions: 0-100 min, linear gradient from 35 to 55% of solvent B in solvent A; 100-140 min, linear gradient from 55 to 65% of solvent B in solvent A. Detection

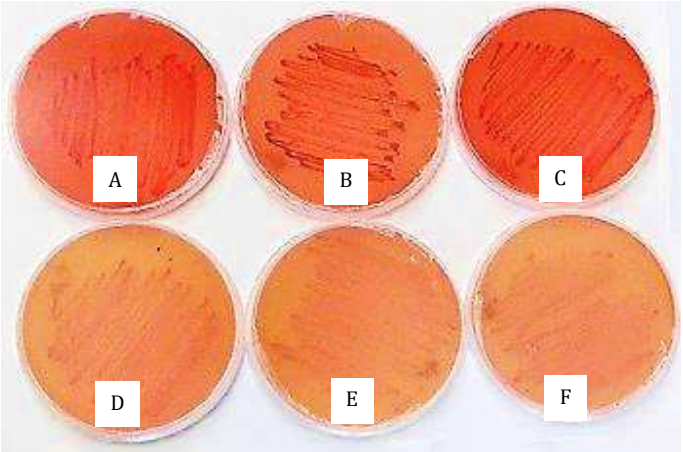

Figure 1. Effect of organic acids on alterations of black rice anthocyanins by *E. aerogenes*. (*E. aerogenes* NBRC 13534 was incubated at 37°C for 5 days in PDA medium supplemented with various organic acids) (A: All four added organic acids; B: ferulic acid; C: caffeic acid; D: without organic acids; E: *p*-hydroxybenzoic acid; F: malonic acid).

was performed at 520 nm. The column temperature was 35°C. The anthocyanin extract was diluted 2-fold with 15% acetic acid solution, then diluted 2-fold with solvent (solvent A: B = 65: 35) and filtered through disposable membrane filter DISMIC-13 HP (0.2 µm, ADVANTEC). The filtrate was measured by HPLC.

RESULTS AND DISCUSSION

Color changes were observed in PDA medium to which all four organic acids were added and in PDA to which caffeic acid or ferulic acid was added. These medium changed to deep red. It has been reported that the color quality of anthocyanins is increased by mixing caffeic acid with ferulic acid and gallic acid with anthocyanin (Qian et al., 2017). However, in a previous study report, color change was observed by culturing *E. aerogenes* in a medium containing phenolic compounds (caffeic acid and ferulic acid), but no color change was observed in the uncultured medium (Saigusa et al., 2014). From this, it was suggested that not only caffeic acid and ferulic acid but also *E. aerogenes* are important factors for changing the color quality of black rice anthocyanin. There was no color change in the PDA medium without organic acids or in that supplemented with malonic acid or *p*-hydroxybenzoic acid (Figure 1). The maximum absorption value of the PDA medium supplemented with all four organic acids was shifted from 520 nm, which is the maximum absorption value of black rice anthocyanins, to 500 nm. In addition, caffeic acid was shifted to 500 nm and ferulic acid to 510 nm. However, the PDA medium without organic acids and that containing malonic acid or *p*-hydroxybenzoic acid did not show changes in absorption (Figure 2). HPLC analysis revealed that the PDA medium to which all four organic acids were added showed four peaks not observed in black rice anthocyanins.

Three new peaks were observed in caffeic acid, while ferulic acid showed two new peaks. However, no new peaks were observed for PDA medium without organic acids or that containing malonic acid or *p*-hydroxybenzoic acid (Figure 3). From these results, it was confirmed that caffeic acid and ferulic acid are involved in the color changes of black rice anthocyanins. From our previous studies (Saigusa et al., 2014), among the four new peaks (peak 1-4) in a medium supplemented with four organic acids, it was confirmed that the two pigments corresponding to peaks 1 and 4 exhibit absorption maxima at 500 nm and peaks 2 and 3 have absorption maxima at 520 nm. From these results, it was suggested that a new peak may be involved in shifting to 500 nm in the medium supplemented with caffeic acid and in shifting to 510 nm in the medium supplemented with ferulic acid. Furthermore, since the structure of these two organic acids are similar, it was suggested that there is an important relationship between the change of color and the structure of the organic acid (Figure 4).

Since anthocyanin is a plant pigment, cultivation of plants is necessary to apply this pigment to foods. However, if anthocyanins can be synthesized *in vitro* freely, cultivation of plants for obtaining of anthocyanin becomes unnecessary, and it may be possible to obtain the necessary kinds of anthocyanins when necessary. Therefore, we tried modification and synthesis test of black rice anthocyanin using microbial enzyme. By incubation of microorganisms, microbial enzymes can be obtained much faster than plant enzymes. In the paper on the synthesis of anthocyanins, mainly plant enzymes are used (Yonekura et al., 2012). There is no paper on

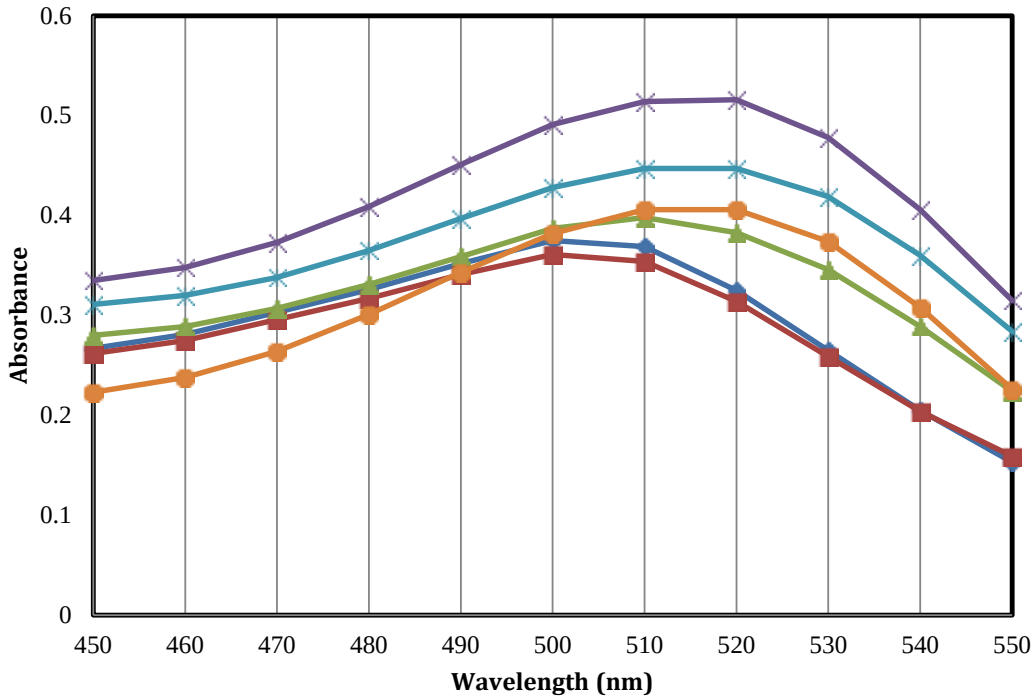

Figure 2. Absorbance spectrum of discolored black rice anthocyanin. Changes in maximal absorption value of black rice anthocyanins in due to *E. aerogenes* and organic acids. Incubate at 37°C for 5 days (◆- four organic acid, ■- caffeic acid, ▲- ferulic acid, ×- *p*-hydroxybenzoic acid, ✳- malonic acid, ●- no organic acids).

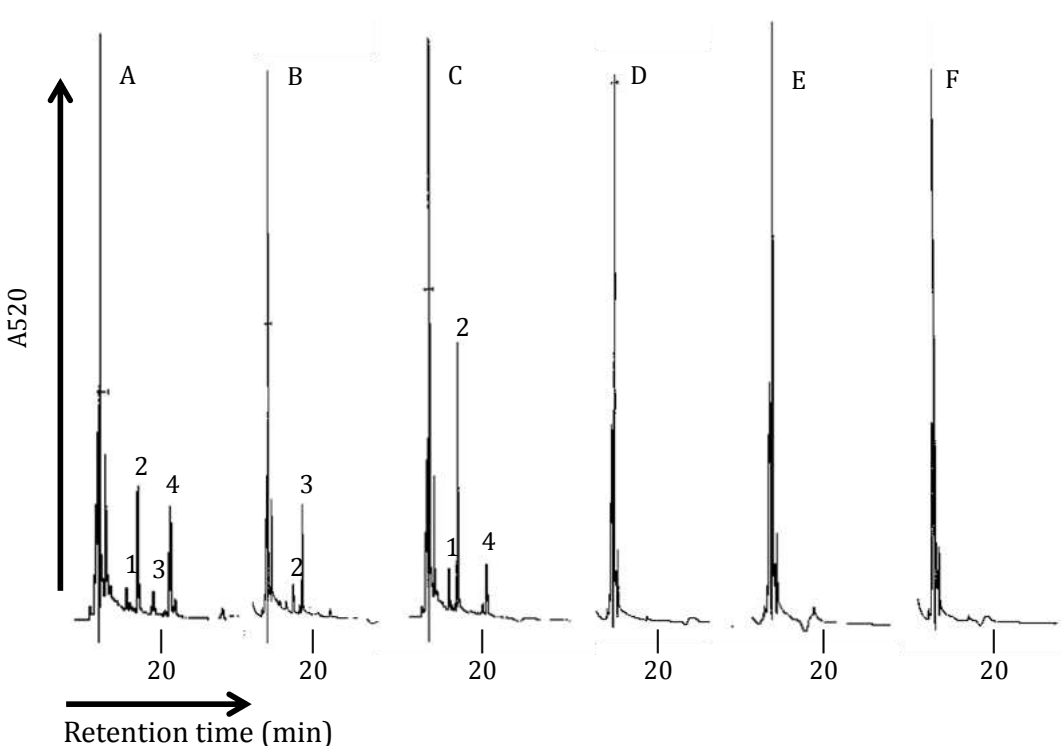

Figure 3. HPLC chromatograms of PDA medium with or without organic acids (A: four organic acids; B: ferulic acid; C: caffeic acid; D: *p*-hydroxybenzoic acid; E: malonic acid; F: no organic acids; Detection: 520 nm).

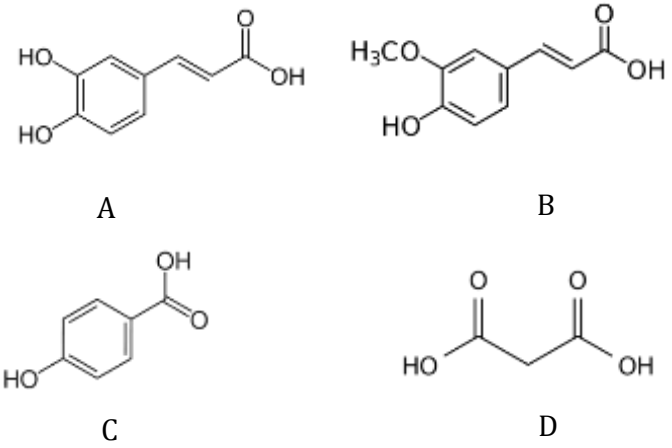

Figure 4. Structure of organic acid (A- caffeic acid; B- ferulic acid; C- *p*-hydroxybenzoic acid; D- malonic acid.

synthesis or modification by microbial enzymes. For that reason, we believe this paper is a very rare information. The main component of the black rice anthocyanin is cyanidin-3-glucoside (Terahara et al., 1994), which is a very simple structure. Also, as mentioned earlier, decomposition of anthocyanins by intestinal microorganisms has been reported (Aura et al., 2005; Cheng et al., 2016; Mueller et al., 2017). Also, improvement in color quality and stability of anthocyanins by organic acids has also been reported (Qian et al., 2017) which suggests that intestinal microorganisms other than *E. aerogenes* may also alter the structure of anthocyanins. For these reasons, depending on the combination of microbial enzyme and organic acid, various new anthocyanins may be born. However, we just got the possibility to produce anthocyanin *in vitro* from the result of this experiment. In the future, structural analysis of newly created peaks is necessary. The combination of various anthocyanins, organic acids and microbial enzymes must necessarily be further investigated. It will also be essential to establish an anthocyanin synthesis technology by microbial enzymes in the future.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Adachi T, Yoshitama K (2004). How to study on plant pigment. Research association of plant pigments. Osaka: Osaka Municipal Universities Press (OMUP).

Anna S, Alicja ZK, Katarzyna W, Antoni S, Agnieszka N, Paulina M, Dorota W (2014). Composition and antioxidant activity of red fruit liqueurs. Food Chem. 157:533-539.

Aung T, Muramatsu Y, Horiuchi N, Che J, Mochizuki Y, Ogiwara I(2014). Plant growth and fruit quality of blueberry in a controlled room under artificial light. J. Jpn. Soc. Hortic. Sci. 83:273-281.

Aura AM, Martin-Lopez P, O'Leary KA, Williamson G, Oksman-Caldentey KM, Poutanen K, Santos-Buelga C (2005). *In vitro* metabolism of anthocyanins by human gut microflora. Euro. J. Nutr. 44:133-142.

Cheng J, Liu X, Chen Z, Zhang Y, Zhang Y (2016). Mulberry anthocyanin biotransformation by intestinal probiotics. Food Chem. 213:721-727.

Gregorio B, Stephan G, Christoph C, Tsaneta D, Bjarne J, Finn O, Henrik L, Renate M (2017). Foliar-applied ethephon enhances the content of anthocyanin of black carrot roots (*Daucus carota ssp*. *sativus* var. *atrorubens* Alef.). BMC Plant Biol. 17(1):70.

Mueller D, Jung K, Winter M, Rogoll D, Melcher R, Richling E (2017). Human intervention study to investigate the intestinal accessibility and bioavailability of anthocyanins from bilberries. Food Chem. 231:275-286.

Nakagawa K, Maeda H (2017). Investigating pigment radicals in black rice using HPLC and multi-EPR. J. Oleo Sci. 66:543-547.

Ohba R, Igarashi Y, Tsukui A (2000). Anthocyanin -Food color and health, Japan, Tokyo: Kenpaku-sha Co. Ltd.

Qian BJ, Liu JH, Zhao SJ, Cai JX, Jing P (2017). The effects of gallic/ferulic/caffeic acids on colour intensification and anthocyanin stability. Food Chem. 228:526-532.

Saigusa N, Yamamoto K, Tsutsui M, Teramoto Y (2014). Microbial production of novel pigments from black rice anthocyanin. Food Sci. Technol. Res. 20:1013-1016.

Terahara N (1993). Studies on the structure determination for the acylated anthocyanin pigments and their stabilities. The Bulletin of the Faculty of Horticulture, Minamikyushu University 23:11-12 (Japanese).

Terahara N, Saigusa N, Ohba R, Ueda S (1994). Composition of anthocyanin pigments in aromatic red rice and its wine. J. Food Sci. Technol. 42:519-522.

Tsuda T (2012). Anthocyanins as functional food factors - chemistry, nutrition and health promotion. Food Sci. Technol. Res. 18:315-324.

Yonekura K, Fukushima A, Nakabayashi R, Hanada K, Matsuda F, Sugawara S, Inoue E, Kuromori T, Kazuo S, Wangwattana B, Yamazaki M, Saito K (2012). Two glycosyltransferases involved in anthocyanin modification delineated by transcriptome independent component analysis in Arabidopsis thaliana. Plant J. 69:154-167.

Zhao C, Chen Z, Bai X, Ding C, Long T, Wei F, Miao K (2014). Structure–activity relationships of anthocyanidin glycosylation. Mol. Divers. 18:687-700.