# Stability and antioxidant activity of acylated jambolan *(Syzygium cumini)* anthocyanins synthesized by lipase-catalyzed transesterification

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## Abstract

The acylated antocyanins of jambolan fruit were synthesized by lipase-catalyzed transesterification reaction with cinnamic acid (intramolecular interaction). The native anthocyanins of jambolan (non-acylated form) were successfully converted to anthocyanin cinnamate (acylated form) by the enzymatic system. The products of synthesis were determined by reverse phase/RP-HPLC and their spectral pattern by spectrophotometer. Acylation of jambolan anthocyanins with cinnamic acid gave change of color from red to purplish-red in beverage model system, pH 3. The acylated anthocyanins of jambolan showed higher thermal and light stability than non-acylated anthocyanins. Acylation of jambolan anthocyanins with cinnamic acid partially decreased their antioxidant activity.

## Introduction

Jambolan *(Syzygium cumini)* fruit, a tropical fruit found in Indonesia, is edible fruit, ovoid form with a deep purple peel when ripe, containing a fleshy pink or almost white pulp with sour-sweet taste, and rather astringent (Figure 1). In Indonesia, the ripe fruits are usually eaten fresh and the seeds used to treat diabetes. Jambolan fruit has attractive color, which the color of this fruit is due to the presence of anthocyanins especially in its peel part. The content of total monomeric anthocyanin in peel of the ripe fruit is 731 mg/100 g fresh weight. Jambolan fruit anthocyanins had been also identified as 3,5-diglucosides derivatives of delphinidin, petunidin, malvidin, cyanidin, and peonidin (Sari *et al.*, 2009).



Figure 1. Ripe jambolan fruit

Anthocyanins obtained from jambolan fruit peel are attractive and potential as novel source of natural colorants for food system. However, they showed low color intensity and stability. Our previous research showed that intermolecular interaction of jambolan fruit anthocyanins with aromatic acid (sinapic acid, caffeic acid, ferulic acid) and rosemary polyphenol extract could enhance color intensity, but reduce color stability on thermal treatment (Sari *et al.*, 2012), because intermolecular interaction has weak hydrophobic forces. Therefore, improving stability of jambolan fruit anthocyanins through intramolecular interaction (acylation reaction) is still needed to obtain more stable of color. In intermolecular interaction, an organic acid or aromatic acyl group is covalently linked to an anthocyanin resulting stable acylated anthocyanin. Previous study of some researchers showed that acylated anthocyanins through intramolecular interaction showed high stability (light-resistance and thermostability) (Dyrby *et al.*, 2001; Nakajima *et al.*, 2003; Cevallos-Casals and Cisneros-Zevallos, 2004).

Acylated anthocyanin could be synthesized by lipase-catalyzed transesterification reaction using aromatic acyl group as acyl donor (intramolecular interaction). Enzymatic acylation of anthocyanins

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(pelargonidin 3-O-ß-D-glucopyranoside and cyanidin 3-O-ß-D-glucopyranoside) with cinnamic acids by lipase-catalyzed transesterification had been successfully conducted by Nakajima *et al.* (1997); Nakajima *et al.* (2003). By the regioselective transesterification with lipase from *Candida antartica*, these anthocyanins were converted to their corresponding cinnamate esters via esterification of the primary alcohol residues of D-glucose of anthocyanin with carboxyl residue of cinnamic acid. Hence, in this study cinnamic acid as acyl donor and lipase-catalyzed transesterification method were selected for jambolan anthocyanins acylation. Lipase from *Candida antartica* is used because it is specific to catalyze the esterification for primary alcohol of D-glucose of anthocyanin, stable and commercially available.

The purposes of this study are 1) to synthesize acylated anthocyanins of jambolan fruit with cinnamic acid by lipase-catalyzed transesterification, 2) to characterize stability of acylated anthocyanins at color, temperatures and light compared to non-acylated anthocyanins with a view to elucidate their potential applications as natural food colorants, and 3) to evaluate antioxidative capability.

## Materials and Methods

### Chemicals and reagents

Organic solvents, acids, and salts used were pro analysis purchased from Merck (Darmstadt, Germany). Vinyl cinnamate, lipase acrylic resin from *Candida antartica*, Trolox (6-hydroxy-2,5,7,8-tetramethyl-chromane-2 carboxylic acid), and DPPH (2,2-diphenyl-1-picrylhydrazyl) were obtained from Sigma-Aldrich (St. Louis, MO).

### Sample preparation

Fully ripened jambolan fruits were obtained from a traditional market in Jember district, Indonesia. Jambolan fruits were selected for their highly colored peel (deep purple), washed, manually peeled, and steam-blanched at approximately 80°C for 4 min. Jambolan peels were packed in polyethylene plastic and stored at -20°C.

### Anthocyanin extraction and purification

Anthocyanin extraction was done following the modified procedure described by Sari *et al.* (2012). Anthocyanins were extracted using methanol at room temperature under vigorous stirring for 1 hour and separated by centrifugation (6000 rpm). Filtrates were collected and the residu was re-extracted with the same solvent (three times). Filtrates were

combined and filtered through Whatman No. 1 filter paper then concentrated in a rotavapor at 40°C under reduced pressure to obtain aqueous extract (Figure 2). The extract was shaken in a separatory funnel with 2 volumes of chloroform (four times) and ethyl acetate (seven times), respectively. The aqueous portion was collected and evaporated in a rotavapor at 40°C, until all residual chloroform and ethyl acetate was evaporated.



Figure 2. Anthocyanin extract of jambolan fruit

Anthocyanins were partially purified by Amberlite XAD-7 resin on open column (30 x 1.5 cm i.d). The extract was carefully put into open column, then washed with water. Anthocyanins that absorbed on Amberlite XAD-7 resin were subsequently eluted with ethanol. The eluate was concentrated using a rotavapor at 40°C to dryness and stored at -20°C until used.

### Acylation of anthocyanins

Acylated anthocyanins were enzymatically synthesized from their non-acylated forms with cinnamic acid vinyl ester by lipase-catalyzed transesterification according to methods of Nakajima *et al.* (1997); Nakajima *et al.* (2003). Cinnamic acid was used as the acyl donor. The reaction mixture contained purified anthocyanins (non-acylated forms) from jambolan fruit, vinyl cinnamate, and lipase (lipase acrylic resin from *Candida antartica*) in acetone containing 10% DMSO. After incubation of the reaction mixture by shaking for 2 days in a water bath at 40°C, the ester products (acylated anthocyanins) were isolated from the reaction mixture by sentrifugation, evaporation of the solvent, and purification of the acylated anthocyanins using solid phase extraction on C18 Sep-Pak cartridge. Before purification, the excess of vinyl cinnamates were removed by hexane. Non-acylated and acylated anthocyanins in the mixture were carefully put into cartridge for purification, and subsequently eluted with 10%, 20%, and 30% acetonitrile in 4% phosphoric acid solution, respectively. The eluates were concentrated using a rotavapor at 40°C and analyzed by HPLC. The first and second eluates contained non-acylated anthocyanins and the third eluate was contained acylated anthocyanins.

## HPLC analysis of anthocyanins

Acylated and non-acylated anthocyanins of jambolan fruit were analyzed by high performance liquid chromatography (HPLC). The HPLC analysis was performed in a Shimadzu liquid chromatography LC-2040 (Kyoto, Japan). The HPLC conditions were done following procedure described by Durst and Wrolstad (2001). The solvents used were: (A) HPLC-grade acetonitrile and (B) 4% phosphoric acid in water. The elution profile consisted of a linear gradient from 6% to 25% A for 55 min, from 25% to 25% A for 10 min, and from 25% to 6% A for 25 min at a flow rate of 1 mL/min. The UV-vis detector was set at 520 nm. A Shimadzu-ODS LC column (250 x 4 mm i.d, 5 μm particle size), thermostated at 30°C, was used to separate acylated or non-acylated anthocyanins.

## Spectral analysis of anthocyanins

UV-visible absorption spectra of acylated and non-acylated anthocyanins were determined on spectrophotometer (Genesys 10, USA). Spectral measurements were made on the range of 200-650 nm.

## Pigment stability studies

Beverage model systems were prepared using anthocyanins of jambolan fruit (acylated or non-acylated forms) and citrate buffer (0.1 M, citric acid-sodium citrate) at pH 3, adjusted to give an absorbance reading of ~0.6 units at $\lambda_{vis-max}$. The color stability of anthocyanins, both acylated and non-acylated forms, was analyzed in which the effects of heating temperature and fluorescent light were taken into account. The influence of heating temperature on color stability was done with test samples inside test tubes immersed in a water bath at 80 and 99°C for 0, 15, 30, 45, 60, and 75 min. Light effect on color stability of pigment was performed with test samples inside transparent glass bottles exposed to white fluorescent light of 23 watt (Philips lamp) in the box dimension 58 x 72 x60 cm (4000 lux) for 0, 1, 2, 3, 4, 5, and 6 days at 32°C.

The absorbance of the sample solutions was measured periodically, depending on the treatments. The color retention of anthocyanin for each time period was calculated as percentage of zero-time absorbance readings, taken as 100% retention. The percent color retention (R%) values were calculated according to equation 1, as described by Gris *et al.* (2007).

$$\% \text{ retention } (R\%) = C_t/C_o \text{ x } 100 \qquad (1)$$

where $C_o$ is the initial absorbance value (zero-time)

and $C_t$ is the absorbance value after a certain treatment time *t*. Degradation kinetic study of anthocyanins followed a first-order reaction (Kirca and Cemeroglu, 2003). The first-order reaction rate constants *(k)* and half-life time *(t_{1/2})* values, i.e. the times needed for 50% degradation of anthocyanins, were calculated according to equations 2 and 3, respectively.

$$\ln (C_t/C_o) = -k \text{ x } t \qquad (2) \qquad t_{1/2} = -\ln 0.5 \text{ x } k^{-1} \qquad (3)$$

where $C_o$ is the initial absorbance value (zero-time), $C_t$ is the absorbance value after a certain treatment time *t*, *k* is the first-order kinetic constant, and $t_{1/2}$ is the half-life time.

## Antioxidant activity

Antioxidant activity of acylated or non-acylated anthocyanins was determined using DPPH radical according to the method of Yamaguchi *et al.* (1998) with some modifications. The sample solution was added to test tube containing mixture of ethanol and DPPH˙ solution (100 μM) in ethanol (freshly prepared). After incubation for 30 min at room temperature, the decrease in absorbance of DPPH˙ at 517 nm was immediately measured on an UV-visible spectrophotometer (Genesys 10, USA). Antioxidant activity was expressed in term of trolox equivalent antioxidant capacity, TEAC.

## Data analysis

Analyses of data were obtained from 3 replications. Values were expressed as means and standard deviations.

## Results and Discussion

### Acylation of anthocyanins by lipase-catalyzed transesterification (intramolecular interaction)

The acylated anthocyanins of jambolan fruit were enzymatically synthesized from their non-acylated forms with vinyl cinnamate in acetone by the lipase enzyme. Lipase CAL (lipase from *Candida antarctica*) was used to synthesize. The ester products (acylated anthocyanins) were synthesized most efficiently in organic solvent acetone, but not in methanol (data not shown). Figure 3 shows HPLC chromatogram of non-acylated and acylated anthocyanins of jambolan fruit. HPLC chromatogram shows that the acylated anthocyanins by lipase-catalyzed acylation reaction (Figure 3b) have longer retention time than non-acylated anthocyanins (Figure 3a). According to Durst and Wrolstad (2001), elution order of anthocyanins on reversed-phase chromatography is based on polarity; non-acylated anthocyanins typically are
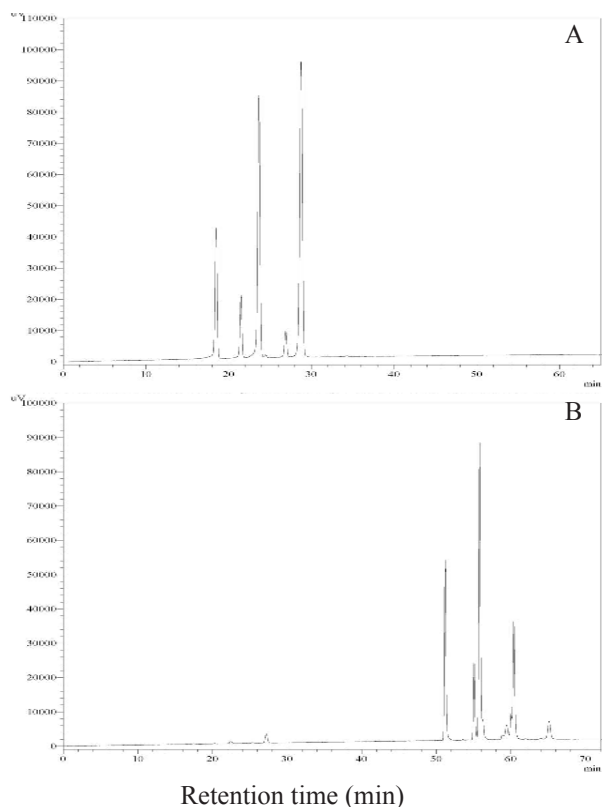
Figure 3. HPLC chromatogram of non-acylated anthocyanins (A) and acylated anthocyanins (B) of jambolan fruit

eluted before acylated anthocyanins.

The spectra of anthocyanin can provide information about the presence of acylating groups. UV-visible spectral of non-acylated and acylated anthocyanins of jambolan is shown in Figure 4, which the patern of both spectral is different. Acylating group is found at 275 nm. Spectra of the acylated anthocyanins show higher peak (at 275 nm) than non-acylated anthocyanins. Cinnamic acid that used as the acyl donors also determined and shown the same maximum wavelength at 275 nm.
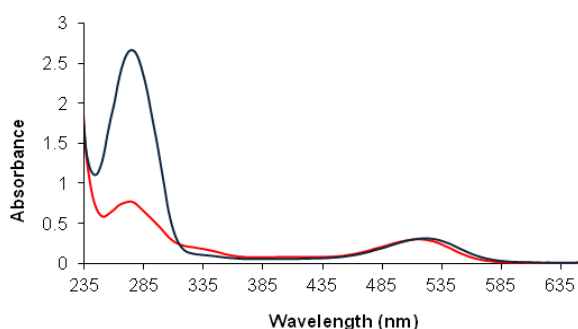


Figure 4. UV-visible spectral of non-acylated (red line) and acylated anthocyanins (black line), dissolved in buffer acetate pH 3

*Color stability of acylated anthocyanin*

The color property of non-acylated and acylated anthocyanins of jambolan fruit is presented in Table 1. The color of non-acylated jambolan anthocyanins

Table 1. Color property of non-acylated and acylated jambolan anthocyanins

| Anthocyanin* | Visual Color | Wavelength Maximum ($\lambda_{vis-max}$) |
|---|---|---|
| Non-acylated anthocyanin | Red | 515 |
| Acylated anthocyanin | Purplish-red | 521 |

*anthocyanin dissolved in buffer citrate, pH 3

in beverage model system (pH 3) was changed to purplish-red color due to acylation of cinnamic acid. Acylation of jambolan anthocyanins also promoted an increase in the maximum absorption wavelengtah (bathochromic shift) from 515 to 521 nm. Gonet (1998); Francis (1989) explained that the bluing effect was consistently reported on copigmentation/acylation reaction as the consequence of the bathochromic shift affecting the pigment visible absorption band.

The influence of heating process 80 and 99°C, and white fluorescent light on color stability of non-acylated and acylated jambolan anthocyanin were studied in beverage model system pH 3, determining as % color retention ($R\%$) at $\lambda_{vis-max}$ as function at time (Figure 5) and kinetic parameters such rate constant ($k$) and half-life time ($t_{1/2}$) (Table 2).

Table 2. Kinetic parameters of non-acylated and acylated jambolan anthocyanins during heating and exposure to fluorescent light

| Anthocyanin | Kinetic Parameters | |
|---|---|---|
| | Rate Constant ($k$) | Half-life Time ($t_{1/2}$) |
| | Heating at 80°C[a] | |
| Non-acylated anthocyanin | 0.0083 | 1.40 h |
| Acylated anthocyanin | 0.0048 | 2.41 h |
| | Heating at 99°C[a] | |
| Non-acylated anthocyanin | 0.0193 | 0.60 h |
| Acylated anthocyanin | 0.0108 | 1.07 h |
| | Exposure to white fluorescent light[b] | |
| Non-acylated anthocyanin | 0.1174 | 5.91 d |
| Acylated anthocyanin | 0.0750 | 9.30 d |

[a]Exposed to heat for 1.25 h. [b]Exposed to white fluorescent light for 6 d. Time unit: h = hours, d = days

At the two heating temperature 80 and 99°C, the acylated anthocyanin revealed higher thermostability than the non-acylated anthocyanin, as confirmed by % color retention, $k$, and $t_{1/2}$ values. The acylated anthocyanins had the values of $k$ smaller and the values of $R\%$, $t_{1/2}$ higher than non-acylated anthocyanins. Similar results were also apparent in light treatment. During light exposure, the acylated anthocyanins were degraded slower than non-acylated anthocyanins.

Acylated anthocyanin contributed to an increase in the color stability, suggesting a protective effect of intramolecular copigmentation. The structure-stability relationship due to hydrophobic interaction between the anthocyanin skeleton and aromatic acid
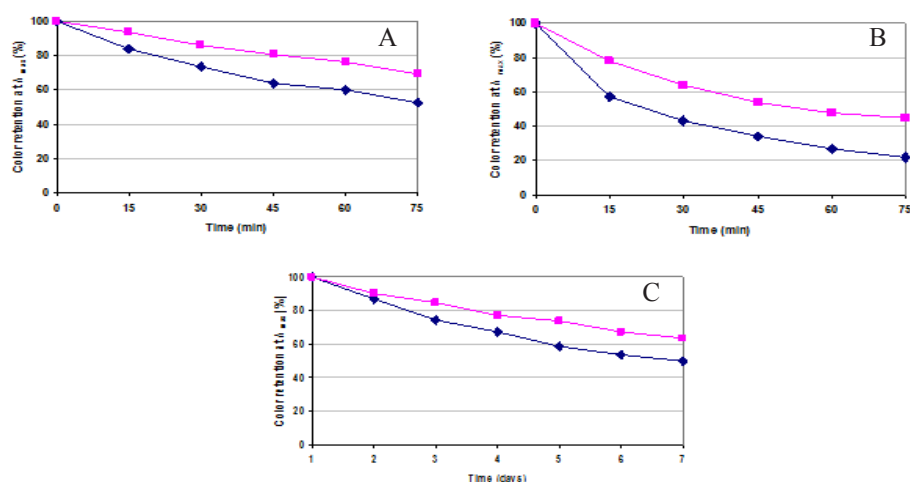
Figure 5. Color retention of non-acylated (◆) and acylated jambolan anthocyanins (■) in citrate buffer pH 3 during heating of 80°C (A) and 99°C (B); and exposure to white fluorescent light (C)

moiety (cinnamic acid) in the acylated anthocyanin structure that synthesized by lipase-catalyzed transesterification. Further, mechanism in protection of copigmentation effect was explained by Williams and Hrazdina (1979); Malien-Aubert *et al.* (2001), that copigmentation consists of the stacking of the copigment molecule on the planar polarizable nuclei of the anthocyanin-colored forms (flavylium ion, quinonoidal forms), with efficiently delocalized $\pi$-electrons, resulting in an overlapping arrangement of the two molecules. Thereby, the nucleophilic attack of water on the anthocyanin molecule, which leads to colorless carbinol (hemiketal) and chalcone forms, is prevented.

*Antioxidant activity of acylated anthocyanin*

Antioxidant activity of anthocyanin was measured in the DPPH$^\bullet$ system, expressed as Trolox equivalents (Figure 6).
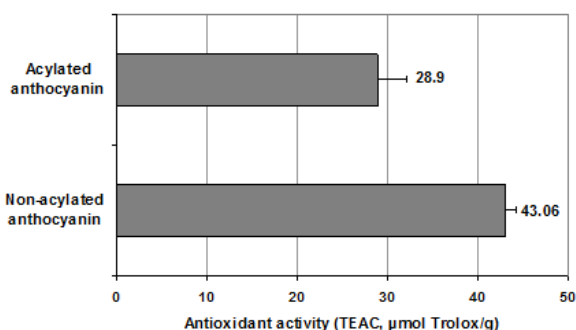


Figure 6. Antioxidant activity of non-acylated and acylated jambolan anthocyanins. Data are expressed as micromoles of Trolox equivalent per g sample

The TEAC values of non-acylated and acylated jambolan anthocyanins were 43.06 and 28.90 µmol Trolox/g, respectively. Antioxidant activity was partially decreased by the acylation of jambolan anthocyanins with cinnamic acid. Substitution acyl moiety of non-acylated jambolan anthocyanin by lipase-catalyzed transesterification resulted in a decrease in antioxidant activity. Nakajima *et al.* (2003) also explained that the antioxidant potentials of the anthocyanins was decreased by the introduction of the acyl moiety in their glucoside molecules. Acylation with *p*-coumarate revealed higher antioxidant activity than acylation with cinnamic acid of anthocyanins.

**Conclusion**

Acylated jambolan anthocyanins were successfully synthesized by lipase-catalyzed transesterification (acylation) with cinnamic acid as acyl donor in acetone. Acylation with cinnamic acid gave change of color from red to purplish-red in beverage model system, pH 3. Thermal and light stability of acylated anthocyanis could be enhanced due to the introduction of cinnamic acid into glucoside molecule of jambolan anthocyanins by the enzymatic system. In addition, acylation of the jambolan anthocyanins with cinnamic acid partially decreased their antioxidant activity. However, the decreasing antioxidant activity was not essentially changed by acylation with cinnamic acid. The lipase-catalyzed transesterification should be applicable to synthesize stable acylated jambolan anthocyanins with slightly decreasing of antioxidant activity.

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