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Enzymatic Acylation of Anthocyanin Isolated from Black Rice with Methyl Aromatic Acid Ester as Donor: Stability of the Acylated Derivatives

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1   **ABSTRACT**

2   The enzymatic acylation of anthocyanin from black rice with aromatic acid methyl

3   esters as acyl donors and *Candida antarctica* lipase B was carried out under a

4   reduced pressure. The highest conversion of 91% was obtained with benzoic acid

5   methyl ester as acyl donor, cyanidin 3-(6″-benzoyl)-glucoside, cyanidin

6   3-(6″-salicyloyl)-glucoside and cyanidin 3-(6″-cinnamoyl)-glucoside were

7   successfully synthesized. This is the first report on the enzymatic acylation of

8   anthocyanin form black rice with methyl aromatic esters as acyl donors and lipase as

9   biocatalyst. Furthermore, the acylation with aromatic carboxylic acids enhanced both

10   the thermostability and light-resistivity of anthocyanin. In particular, cyanidin

11   3-(6″-cinnamoyl)-glucoside was the most stable among the three acylated

12   anthocyanins synthesized.

13   **Keywords:** Black rice; Anthocyanin; Cyanidin-3-glucoside; *Candida antarctica*

14   lipase B; Enzymatic acylation

15 **INTRODUCTION**

16 Anthocyanins, the largest and most important group of water-soluble natural

17 pigments, represent one of the most widely distributed classes of flavonoids in

18 plants.[1] They are responsible for a variety of bright colors, e.g., blue, purple, red and

19 intermediate colorations of various plant tissues. As a group of flavonoids, they have

20 been reported to have pronounced beneficial health effects associated with

21 anti-oxidant, anti-viral, anti-microbial, anti-inflammatory, anti-tumor and

22 chemo-preventive activities.[2-12] Furthermore, epidemiological studies suggest that

23 the consumption of anthocyanins prevents the risk of cardiovascular disease,

24 oxidative stress and diabetes.[13]

25 Due to their attractive colors, broad spectrum safety and beneficial health effects,

26 there has been a growing industrial and academic interest in the use of anthocyanins

27 as natural food colorants for replacing synthetic dyes.[14-16] Unfortunately,

28 anthocyanins are highly unstable, and their low stability is therefore the primary

29 obstacle to the commercial application as colorants in food industry.[17,18] However, it

30 has been reported that rare forms of natural acylated anthocyanins exhibited high

31 color stability under different conditions.[15,19] In nature, the anthocyanin molecules

32 may be acylated through the esterification of sugar residues with a broad range of

33 organic acids such as *p*-coumaric, caffeic, ferulic, gallic, malonic, malic and succinic

34 acids. The acylation plays a significant role in the improvement of anthocyanin

35 stability through hydrophobic and "π-π"-interactions.[19-22] Therefore, acylated

36 anthocyanins may provide the desirable stability for food applications. However,

37   most commercial natural anthocyanin colorants are the mixtures of non-acylated and

38   acylated forms.

39   Enzymatic acylation of anthocyanins and other flavonoid glycosides *in vitro* can

40   increase their stability. The acylation of flavonoid glycosides can be achieved by

41   using lipases in organic solvents.[23] Two reactions can be catalyzed by lipases to

42   acylate flavonoid glycosides: direct esterification and transesterification. In the first

43   reaction, fatty acids or phenolic acids are used as acyl donors in organic solvents at

44   low water activity, and the by-product water is removed by molecular sieve.[24-27] In

45   the second reaction, fatty acids or aromatic carboxylic acid vinyl esters are used as

46   acyl donors,[28-31] but acyl donors need be synthesized for the reaction in advance.[32]

47   Methyl or ethyl esters can also be used as both acyl donor and reaction medium in

48   transesterification, in conjunction with a system for the online removal of the water

49   or alcohol under reduced pressure.[33-35] Compared with flavonoid glycosides such as

50   isoquercitrin, rutin and naringin, information on acylation of anthocyanin by lipases

51   is limited. In one study, the crude anthocyanin extract of jaboticaba (*Myrciaria*

52   *cauliflora*) fruits was acylated enzymatically by Novozym 435 with palmitic acid as

53   acyl donor,[36] and palmitic monoesters of delphinidin-3-glucoside and cyaniding

54   3-glucoside (**1**, **Figure 1**) were synthesized, but the conversion yields and acylation

55   position were not determined. In another study, *Candida antarctica* lipase B

56   (Novozym 435) was used to acylate directly the anthocyanins from blueberry with

57   phenolic acids, and several new acylated anthocyanins were synthesized,[25] but the

58   product of reaction was not separated and purified for further study.

59   Black rice (*Oryza sativa* L.) is widely cultivated and consumed in China and

60   other eastern Asia countries since ancient times. It has been regarded as a

61   health-promoting food since it contains high levels of anthocyanin pigments.[37-40] The

62   anthocyanin composition of black rice is simpler compared with those of other

63   materials such as black currant, purple corn and purple sweet potato. **1**, the best

64   known and most investigated non-acylated anthocyanin,[41] comprises more than 90%

65   of the total anthocyanins of black rice.[42-44] Thus, black rice is a more outstanding

66   source for the preparation of **1**. In the present study, therefore, the anthocyanin from

67   black rice was acylated by using aromatic carboxylic acid methyl ester as acyl donor

68   and lipase (Novozym 435) as biocatalyst, aiming to investigate the effects of acyl

69   donors on the regioselectivity and conversion yield of enzymatic acylation of

70   anthocyanin. Furthermore, the stability of the acylated anthocyanin was investigated.

71   **MATERIALS AND METHODS**

72   **Materials and Reagents**. Black rice (Longjin No.1, *Oryza sativa* L. subsp.

73   *Japonica*) was purchased from a local market in Nanjing, China. Novozym 435

74   (lipase B from *C. antarctica* immobilized on acrylic resin) was purchased from

75   Novozymes (Copenhagen, Denmark). All chemicals, esters and organic solvent used

76   were of the highest available purity and were purchased from Aldrich, Merck or

77   Sigma.

78   **Isolation of Anthocyanin form Black Rice.** The anthocyanin substrate of reaction

79   was prepared as follows. Black rice (1000 g) was extracted with 4000 mL aqueous

80   ethanol solution (ethanol/water/HCl, 80:20:0.5) at room temperature for 24 h. The

81   extraction filtrate was concentrated to 50 mL by using a RE-5250 rotary evaporator

82   and loaded onto an Amberlite XAD-7 column (800 × 40 mm i.d.). Then, the column

83   was washed with 1.25 L of water and 500 mL of ethanol/water solution (70:30, v/v),

84   respectively. The eluate of ethanol/water was concentrated to 50 mL under reduced

85   pressure at 55 ℃ and applied onto a polyamide resin column (800 × 40 mm i.d.).

86   The column was washed first with 1.25 L of water, then with 500 mL of a mixture of

87   ethanol/water (30:70, v/v). The resulting eluate was concentrated under reduced

88   pressure at 55 ℃ and lyophilized, affording the substrate for enzymatic reaction.

89   **Procedure of Enzymatic Acylation.** The substrate and lipase for reaction were

90   dried over silica gel under vacuum for at least 1 week before use, pyridine and acyl

91   donor (methyl benzoate, methyl salicylate and methyl cinnamate) were dried for at

92   least 5 d with 4 Å molecular sieves. The acylation reaction was performed in the 250

93   mL evaporation flask of a rotary evaporator. The pressure was reduced by using a

94   vacuum pump at the desired set point. For all reactions, 0.5 g substrate was dissolved

95   with 5 mL pyridine, and 10 mL acyl donor and 1 g lipase (Novozym 435) were then

96   added. The reaction was maintained at 40 ℃ and stirred at 30 rpm under vacuum of

97   900 mbar. After 48 h incubation, the reaction was stopped by filtration to remove the

98   enzyme.

99   **LC-MS Analysis.** An 1100 series HPLC system with diode array detector (DAD)

100   (Agilent Technologies) was used in this study. The chromatographic separation was

101   conducted by using a TSKgel ODS-100Z column (4.6 × 150 mm, 5 μm, Tosoh Corp.,

102   Tokyo, Japan). The mobile phase consisted of eluent A (6% acetic acid in water) and

103   eluent B (6% acetic acid in acetonitrile), and the linear gradient at a flow rate of 0.6

104   mL/min was programmed as follows: 0-25 min, 5% to 40% B; 25-35 min, 40% to

105   80% B; 35-45 min, 8% to 5% B; 45-50 min, 5% B. The temperature of the column

106   oven was set at 40 ℃, and the injection volume was 10 μL. Two wavelengths were

107   used, 520 nm for the detection of anthocyanins and 280 nm for the detection of all

108   other phenolic compounds. A T-split was used to reduce the flow before the sample

109   was introduced into the mass spectrometer (MS) (Agilent Technologies). The mass

110   analysis was performed with an electro-spray ionization ion-trap MS (ESI-MS). Full

111   mass spectra (m/z 100-2000) were recorded in a positive mode was using a capillary

112   voltage of 3.5 kV. The pressure of nebulizer gas (N_2) and flow rate of dry gas (N_2)

113   were set at 25 psi and 10 L/min, respectively. The capillary temperature was

114   controlled at 350 ℃.

115   **Purification of Acylated Anthocyanin.** The reaction mixture was dissolved and

116   loaded onto a Toyopearl HW-40S column (30 × 1.6 cm) pre-equilibrated with

117   solution of acetonitrile/water/acetic acid (15:79:6) at a flow rate of 2 mL/min. The

118   elution process was programmed as follows: acetonitrile/water/acetic acid (15:79:6)

119   for 30 min, acetonitrile/water/acetic acid (30:64:6) for 30 min, and 90% ethanol for

120   30 min. The elution was monitored by measuring the absorbance (Abs) at 280 nm

121   and auto-collected (3 mL/tube). The fractions were analyzed by HPLC, and the

122   desired fractions were collected, concentrated and dried, affording the desired

123   products.

124   **NMR Analysis.** The structures of acylated anthocyanins were characterized by [1]H

125 nuclear magnetic resonance (NMR) and ^{13}C NMR spectrometry with CD_3OD as

126 solvent, and the NMR spectra were recorded by using a Bruker high-resolution

127 AVANCE III 500NMR spectrometer. Data for **2**: ^{13}C NMR (CD_3OD) δ 67.0 (C″-6),

128 73.1 (C″-4), 75.5 (C″-2), 76.6 (C″-3), 78.7 (C″-5), 96.2 (C-8), 102.6 (C″-1), 102.9

129 (C-6), 116.4 (C-10), 116.9 (C′-5), 121.2 (C′-2), 121.7 (C′-1), 130.4 (C‴-5), 130.6

130 (C′-6), 131.9 (C‴-4), 132.2 (C‴-3), 134.0 (C‴-2), 135.3 (C-4), 145.8 (C-3), 146.4

131 (C′-3), 154.3 (C′-4), 155.9 (C-9), 159.5 (C-5), 169.3 (C‴-1), 169.4 (C-2, C-7). Data

132 for **3**: ^{13}C NMR (CD_3OD with 1% CF_3COOH) δ 63.7 (C″-6), 70.2 (C″-4), 73.3 (C″-2),

133 74.3 (C″-3), 76.4 (C″-5), 94.0 (C-8), 101.9 (C″-1), 102.0 (C-6), 111.2 (C‴-7), 111.7

134 (C-10), 114.0 (C′-5), 115.9 (C‴-6), 117.5 (C′-2), 119.6 (C′-1), 126.7 (C‴-4), 129.4

135 (C′-6), 135.4 (C-4), 143.2 (C-3), 146.0 (C′-3), 154.3 (C′-4), 155.9 (C-9), 159.5 (C-5),

136 169.7 (C‴-1), 164.0 (C-2), 172.5 (C-7). Data for **4**: ^{13}C NMR (CD_3OD) δ 63.4 (C″-6),

137 73.1 (C″-4), 75.5 (C″-2), 76.5 (C″-3), 78.8 (C″-5), 96.3 (C-8), 102.9 (C″-1), 102.9

138 (C-6), 115.0 (C-10), 116.4 (C′-5), 116.6 (C′-2), 116.8 (C‴-7), 121.1 (C′-1), 130.4

139 (C‴-5), 130.5 (C′-6), 130.6 (C‴-6), 131.0 (C‴-4), 132.4 (C‴-3), 132.4 (C-4), 137.0

140 (C′-4), 146.4 (C‴-2), 147.5 (C-3), 147.8 (C′-3), 154.3 (C′-4), 155.9 (C-9), 159.5

141 (C-5), , 168.5 (C‴-1), 169.3 (C-7), 170.0 (C-2).

142 **Determination of Anthocyanin Thermostability.** The stability of acylated

143 anthocyanin was evaluated with 0.01% (w/v) of pure anthocyanin dissolved in

144 0.01% aqueous HCl solution (pH 2.5). Effect of temperature on color stability of

145 anthocyanin solution was investigated in a water bath at 65, 80 and 90 ℃. After 2, 4,

146 6, 8, 10, 12, 16, 20 and 24 h of each treatment, change in color intensity was

147   determined with a DU730 Life Science UV/Vis spectrophotometer (Beckman

148   Coulter Inc.) by measuring the Abs at 520 nm.

149   **Determination of Anthocyanin Light-resistivity.** To evaluate the light stability,

150   the solutions of anthocyanin (5 mL) in glass open cuvettes were placed under the

151   fluorescent light (5000 lx) and ultraviolet illumination (253.7 nm, 2.1 mW/cm^2)

152   (Suzhou Purification Equipment Co., Ltd, Suzhou, China) at 20 ℃. After 3, 6, 12,

153   24, 48, 72, 120 and 168 h of each treatment, change in color intensity was

154   determined by using the DU730 UV/Vis spectrophotometer by measuring the Abs at

155   520 nm.

156   **Statistical Analysis.** All the analyses in the present study were repeated 3 times.

157   The data were analyzed by one way analysis of variance (ANOVA, version 8.15),

158   and the least significant difference (LSD) post hoc test was conducted. *P* value of

159   less than 0.05 was considered to be statistically significant.

160   **RESULTS AND DISCUSSION**

161   In this work, we firstly prepared an anthocyanin extract by use of black rice as

162   starting material. According to HPLC analysis, the content of **1** in the extract was

163   3.21%. Thus, the extract was purified by column chromatography of macroporous

164   resin (Amberlite XAD-7) and polyamide resin to afford **1** with a purity of 22.4% and

165   80.8%, respectively. Then, the partially purified anthocyanin was acylated by lipase

166   B from *C. antarctica* and aromatic esters as acyl donors. The reaction was monitored

167   by LC-MS, and the acylated derivatives of anthocyanin were purified from the

168   reaction mixture. Finally, the stability of acylated anthocyanin derivatives was

169   evaluated.

170   **Enzymatic Acylation of Anthocyanin.** The reaction mixture was submitted to

171   LC-MS analysis, and two wavelengths were used: 520 nm for the detection of

172   anthocyanins and 280 nm for the detection of all other phenolic compounds. **Figure**

173   **2A** shows the LC-MS profile of substrate for acylation reaction, and a main peak at

174   11.8 min, accounting for 95.4% (520 nm) of the total peaks area, was observed. The

175   molecular ion (*m/z*) for [M+H]$^+$ was 449.2, consistent with that of **1**. It has been

176   reported that **1** is the major anthocyanin present in black rice.[42-44] Thus, the major

177   substrate from black rice for present acylation reaction was confirmed as **1**.

178   **Figure 2B-D** shows the full scan spectra and MS data for the products of the

179   acylation of **1** with methyl benzoate, methyl salicylate or methyl cinnamate as donor.

180   Notably, one major product with similar retention time at 25-30 min was observed

181   for the reaction of each acyl donor. With methyl benzoate as donor, the [M+H]$^+$ ion

182   for the reaction product was *m/z* 553.4 (**Figure 2B**), which is consistent with that of

183   monoacylated cyanidin 3-glucoside with benzoyl (cyanidin 3-(6″-benzoyl)-glucoside,

184   **2**). Furthermore, the MS^2 data showed a characteristic base peak at *m/z* 287 for

185   cyanidin from the parent ion (*m/z* 553.4), indicating that the acylation reaction was

186   regioselective to the glycoside moiety and **2** was synthesized in the reaction system.

187   In addition, the structure of **2** was confirmed by NMR. In the ^{13}C NMR spectrum of

188   **2**, the signal for C″-6 of the glucose moiety shifted 4.6 ppm (from 62.4 to 67.0 ppm)

189   compared with that of **1**.[45] In a similar manner, the corresponding reaction products

190   (**3** and **4**) were obtained and characterized by LC-MS (**Figure 2C** and **D**) and NMR

191   when methyl salicylate and methyl cinnamate were used as the acyl donors.

192   **Effect of Acyl Donor on Acylation Reaction Efficiency.** The effect of the acyl

193   donor on the reaction efficiency was studied by using several available aromatic acid

194   methyl esters as acyl donors. As shown in **Figure 3**, all reactions reached their

195   reaction equilibrium between 30 and 40 h when methyl benzoate, methyl salicylate

196   and methyl cinnamate were used as acyl donors. The total conversion yield

197   decreased from 91% for methyl benzoate to 68% for methyl cinnamate. The

198   structure of acyl donor used slightly affected the reaction rate as well as the

199   conversion yield of **1**. The conversion yield decreased from 91% for methyl benzoate

200   as donor to 84% for methyl salicylate (methyl *ortho*-hydroxybenzoate) as donor. The

201   low conversion yield for methyl salicylate as donor might be caused by the steric

202   hindrance of the substrate. Conversion was also significantly affected by the

203   structure of carbon chain of aromatic acid methyl ester. When methyl cinnamate was

204   used as acyl donor under the same condition, the conversion yield of the reaction

205   was only 68%, decreasing significantly (23%) relative to methyl benzoate used as

206   acyl donor.

207   In this work, we obtained **1** from black rice by extraction and purification

208   through column chromatography and synthesized its acyl derivatives by use of *C.*

209   *antarctica* lipase B. The results demonstrated that Novozym 435 was a good catalyst

210   for the acylation of **1** when several available aromatic acid methyl esters were used

211   as acyl donors. The reaction was regioselective to the glycoside moiety and

212   monoacylated products of **1** were synthesized. With respect to the position of

213 acylation mediated by immobilized lipase B from *C. antarctica* for glycosylated

214 flavonoids, most studies show that the acylation always take place on the primary

215 hydroxyl group present on the glycoside moiety of the molecule, more precisely on

216 the C-6 carbon atom. As reported by Salem et al.,[46] the acylation of isoquercitrin

217 (quercetin 3-*O*-glucoside) with fatty acid esters of various carbon chain lengths and

218 Novozym 435 carried out in 2-methyl-2-butanol led to the synthesis of the sole

219 isoquercitrin 6"-ester. Nakajima et al.[28] and Stevenson et al.[25] used vinyl cinnamate

220 or 2-hydroxyphenylpropionic acid as acyl donors, leading to the same results.

221 Therefore, it is reasonable to believe that it is the 6″-OH group of **1** being acylated in

222 present study. In fact, the NMR data of the acylated anthocyanins confirmed the

223 hypothesis.

224 Compared with most enzymatic direct acylation of flavonoids with aromatic

225 acids as acyl donors,[46] the conversion yield of the present reaction was quite high.

226 Similar results were described in the literature for the synthesis of aromatic esters of

227 phloridzin under reduced pressure in the presence of a large excess of acyl donor

228 which also acted as a solvent for the acyl acceptor.[34] These results may be explained

229 by the nature of the reaction. The equilibrium of the reaction shifted towards

230 synthesis when the methanol was evaporated; thereby the conversion yield increased.

231 For acyl donor, the conversion yields were in the order of methyl benzoate > methyl

232 salicylate > methyl cinnamate. The differences in the conversion yields may be due

233 to the differences in structures of acyl donors, particularly the distribution of

234 hydroxyl groups on the aromatic ring. Such results are in agreement with the

235   conclusion of previous reports.[25,28] For example, naringin and isoquercetin are both

236   acylated by Novozym 435 with phenylpropionic acid (PPA) and its hydroxylated

237   derivatives as acyl donors, the conversion yields are in the following order:

238   2-hydroxy PPA > 3-hydroxy PPA > 3,4-dihydroxy PPA > PPA.[25] It has also been

239   reported that acylated isoquercitrins are synthesized by lipase-catalyzed

240   transesterification with vinyl cinnamate, vinyl cinnamate derivatives and other

241   carboxylic acid vinyl esters as acyl donors, but the conversion rates for vinyl

242   cinnamate derivatives are lower than that for vinyl cinnamate or vinyl

243   phenylpropionic acid ester.[28]

244   **Thermostability of Acylated Anthocyanins.** In order to investigate the effect of

245   acylation with aromatic acid methyl ester as donor on the thermostability of

246   anthocyanin, the monoacylated products (**2**, **3** and **4**) were synthesized and their

247   thermostablity at 65, 80 and 95 ℃ were examined. The logarithm of monomeric

248   anthocyanin content ($\ln(C/C_0)$) was plotted versus time (t) (**Figure 4**), and it was

249   found that the thermal degradation of **1** and its three monoacylated products (**2**, **3** and

250   **4**) followed first order reaction kinetics with respect to temperature. The results are

251   in agreement with those of previous studies, that is the degradation of monomeric

252   anthocyanins from various sources followed a first-order reaction model.[41,47,48] The

253   first order reaction rate constant (k) and half life time ($t_{1/2}$) were calculated by the

254   following equations:

255   $$\ln(C/C_0) = -k \times t \tag{1}$$

256   $$t_{1/2} = -\ln(1/2) \times k^{-1} \tag{2}$$

257 Dependence of the degradation rate constant on temperature is represented by the

258 Arrhenius equation:

259 $\quad Lnk = lnk_0 - Ea/RT$ $\hspace{6cm}$ (3)

260 where C_0 is the initial anthocyanin content and C_t is the anthocyanin content after t

261 minute heating at a given temperature, k_0 is the frequency factor (min^{-1}), Ea is the

262 activation energy (kJ/mol), R is the universal gas constant (8.314 J/mol K) and T is

263 the absolute temperature (Kelvin).

264 $\quad$ The kinetic parameters are summarized as shown in **Table 1**. The $t_{1/2}$ values

265 showed that the thermostability of the four anthocyanins (**1-4**) decreased with

266 increasing temperature. As expected, all $t_{1/2}$ values of the three monoacylated

267 products (**2**, **3** and **4**) were higher than that of **1** at the selected temperature. The

268 thermostability of the three synthesized products was in the following descending

269 order: **4** > **3** > **2**. The dependence of the degradation of **2**, **3** and **4** on temperature

270 was determined by calculating the value of Ea. High Ea reaction, normally, is more

271 susceptible to temperature change. Thus, as temperature increased, the increase in

272 the degradation rate of these anthocyanins was in the following order: **3** > **2** > **4**.

273 $\quad$ **Light-resistivity of Acylated Anthocyanins.** The $t_{1/2}$ values of **1-4** illuminated by

274 white fluorescent light and UV light at 20 ℃ were measured to examine the effects

275 of the acyl moieties on the light-resistivity. The results are summarized as shown in

276 **Table 2**. It is obvious that the decomposition rate of the anthocyanin under the UV

277 exposure was greater than the rate under the fluorescent light. **2**, **3** and **4** displayed

278 relatively higher stability than **1** in the response to both fluorescent and UV light.

14

279   The results implied that the acylation on **1** significantly improved its stability not

280   only under illumination but also under UV condition. Furthermore, **4** displayed the

281   greatest residual color intensity for fluorescent light treatment, while **3** exhibited

282   longer $t_{1/2}$ for UV treatment (**Table 2**). The results implied that the light-resistivity of

283   acylated anthocyanin was dependent on the structure of acyl moiety.

284   Natural acylated anthocyanins from plants such as purple sweet potato exhibit an

285   outstanding stability compared to non-acyl anthocyanins in different conditions.[19,49]

286   Furthermore, the stability of acylated anthocyanins synthesized with anthocyanin

287   extract of black rice and octenyl succinate anhydride has been evaluated, and it was

288   found that the acylated anthocyanins showed higher stability than non-acylated

289   anthocyanins.[50] Thus, for the first time, we explored the relative stability of **1** and its

290   acylated derivatives synthesized by lipase as catalyst. As for thermostability, all $t_{1/2}$

291   values of synthesized products were higher than that of **1** from black rice. These

292   results indicated that acylated substitution and the new ring formed through

293   transesterification between **1** and methyl aromatic acid esters were beneficial to

294   stabilize the anthocyanin molecules. The increasing color stability of anthocyanins is

295   associated with the higher steric-hindrance, which probably protects the anthocyanin

296   from hydration, and water is incapable of attacking the aglycone. In addition, it has

297   been reported that the acylation with aromatic carboxylic acids improved the

298   thermostability of isoquercitrin and isoquercitrin cinnamate, the stability of acylated

299   product being higher than that of isoquercitrin benzoate.[28] These results suggest that

300   thermostability of acylated molecules is dependent on the aromatic ring in the acyl

301   moiety.

302   In conclusion, the acylation offers an attractive strategy to improve the stability

303   of natural non-acyl anthocyanins. This is the first report on the enzymatic acylation

304   of anthocyanins form black rice with methyl aromatic acid esters as acyl donors and

305   lipase as biocatalyst. The acylation reaction, mediated by lipase under a pressure of

306   900 mbar, was demonstrated to be very efficient and regioselective, **2**, **3** and **4** were

307   successfully synthesized. The highest conversion of **1** was obtained when methyl

308   benzoic acid ester was used as acyl donor, indicating that the efficiency of the

309   synthesis depended on the acyl donor. The acylation with aromatic carboxylic acids

310   improved both the thermostability and light-resistivity of the anthocyanin. Among

311   the three acylated anthocyanins synthesized, **4** was the most stable under the

312   investigated temperature. Detailed studies including the bioactivity of acylated

313   anthocyanins are in progress.

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473 **Figure Captions**

474 **Figure 1.** Structures of cyanidin 3-glucoside (**1**) and its three acylated derivatives (**2**,

475 **3** and**4**) synthesized with lipase B from *C. antarctica* as biocatalyst.

476 **Figure 2.** LC-ESI-MS chormatograms showing the reaction substrate (**1**) from black

477 rice (A) and its reaction products (B, **2**; C, **3**; D, **4**).

478 **Figure 3.** Time-course acylation of **1** with methyl benzoate (△), methyl salicylate

479 (□) and methyl cinnamate (○) as acyl donors catalyzed by lipase B from *C.*

480 *antarctica* at 40 □ under a reduced pressure of 900 mbar.

481 **Figure 4.** Degradation of acylated anthocyanins during thermal treatment at different

482 temperatures.

Table 1

Thermal degradation parameters of anthocyanins

| Anthocyanin	Temperature (℃)	k (h^{-1})	$t_{1/2}$ (h)	E_a (kJ mol^{-1})
	65	0.0278 (0.9938)	24.93	
1	80	0.0864 (0.9982)	8.02	59.31 (0.9746)
	95	0.1543 (0.9947)	4.49	
	65	0.0107 (0.9980)	64.78	
2	80	0.0373 (0.9857)	18.58	61.37 (0.9574)
	95	0.0629 (0.9930)	11.02	
	65	0.0090 (0.9986)	77.01	
3	80	0.0325 (0.9946)	21.33	62.23 (0.9528)
	95	0.0542 (0.9854)	12.78	
	65	0.0082 (0.9913)	84.53	
4	80	0.0302 (0.9988)	22.95	59.46 (0.9299)
	95	0.0455 (0.9871)	15.23	

1, cyanidin 3-glucoside; **2**, cyanidin 3-(6″-benzoate)-glucoside; **3**, cyanidin

3-(6″-salicylate)-glucoside; **4**, cyanidin 3-(6″-cinnamate)-glucoside.

Table 2

Effects of light treatments on the color stability of anthocyanins

light	Half life time ($t_{1/2}$, h)			
	1	**2**	**3**	**4**
Dark	248.34 (0.9833)	744.23 (0.9534)	787.46 (0.9783)	803.21 (0.9873)
Fluorescent	113.32 (0.9750)	537.81 (0.9656)	563.22 (0.9897)	574.23 (0.9748)
UV	24.12 (0.9645)	132.32 (0.9879)	146.74 (0.9745)	142.34 (0.9856)

Numbers in parentheses are the determination coefficient. **1**, cyanidin 3-glucoside; **2**, cyanidin 3-(6″-benzoate)-glucoside; **3**, cyanidin 3-(6″-salicylate)-glucoside; **4**, cyanidin 3-(6″-cinnamate)-glucoside.
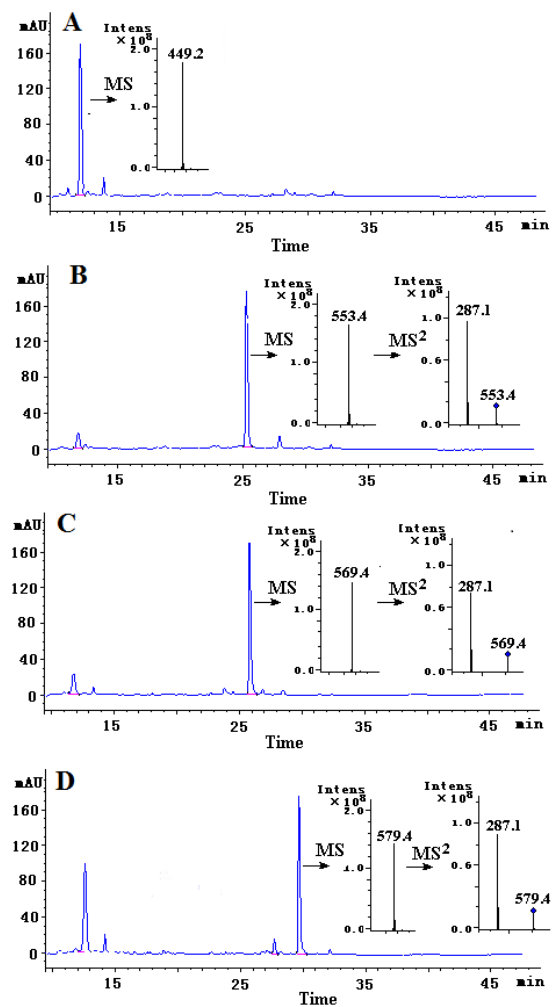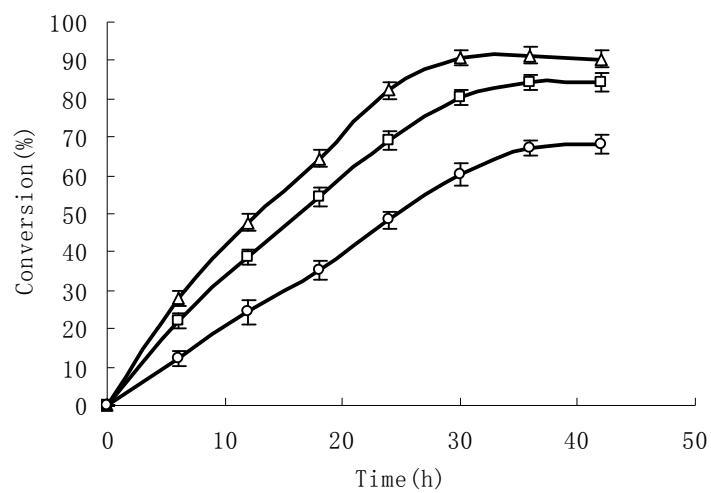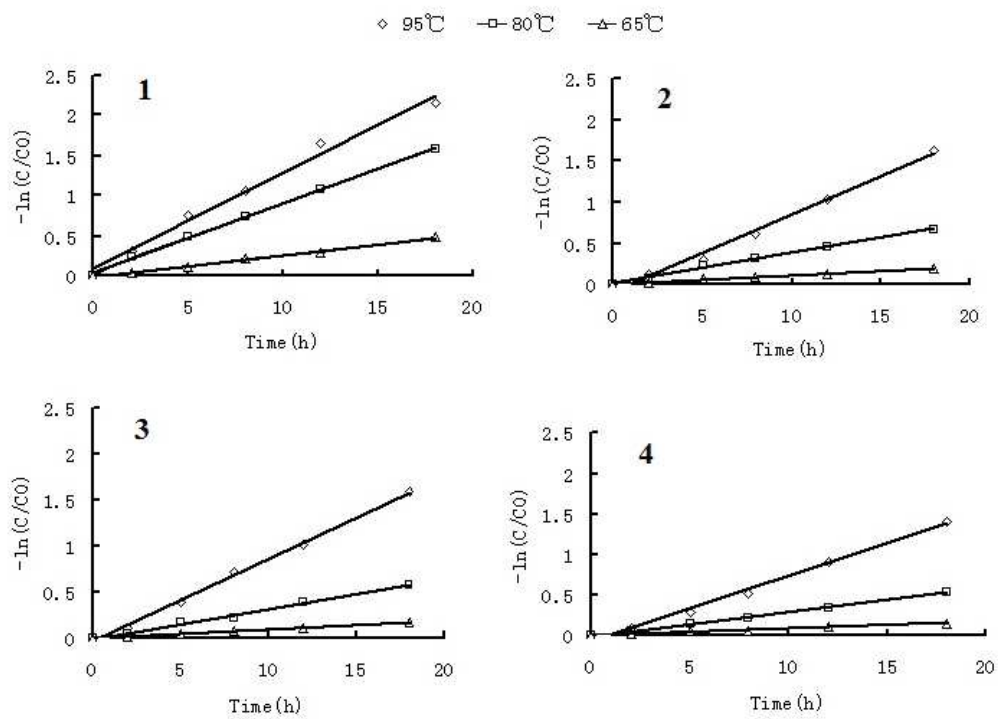
1   **2**   **3**   **4**
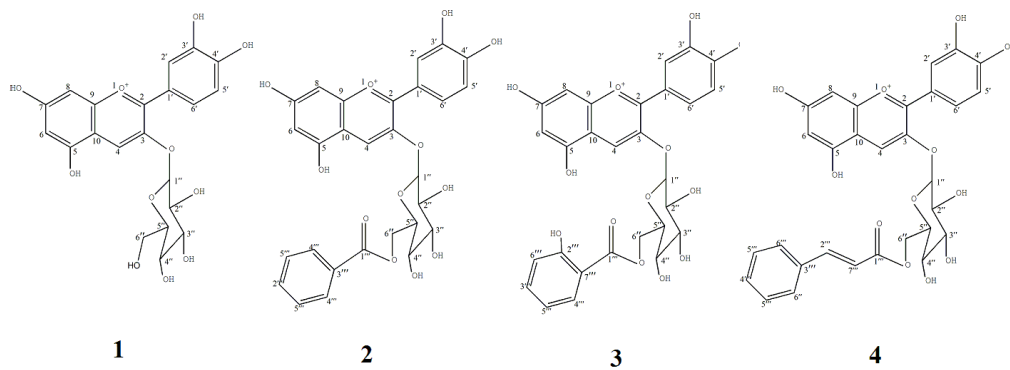
Figure 1

Figure 2

Figure 3

Figure 4

Graphic Abstract