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Effect of BTH on Anthocyanin Content and Activities of Related Enzymes in Strawberry after Harvest

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The effect of benzo-thiadiazole-7-carbothioic acid *S*-methyl ester (BTH) at 0.2 g L^{-1} on anthocyanin content and the enzymes involved in its metabolism such as glucose-6-phosphate dehydrogenase (G6PDH), shikimate dehydrogenase (SKDH), tyrosine ammonia lyase (TAL), phenylalanine ammonia lyase (PAL), cinnamate-4-hydroxylase (C4H), 4-coumarate/coenzyme A ligase (4-CL), and dihydroflavonol 4-reductase (DFR) in strawberry (*Fragaria* × *ananassa* Duch.) fruit was investigated in this study. The result showed that BTH treatment gave higher levels of anthocyanin in strawberries during 10 days of storage at 1 °C. Meanwhile, the treatment also increased the activities of G6PDH, SKDH, TAL, PAL, C4H, and DFR. These results indicated that the increase in anthocyanin content by BTH might result from the activation of its related enzymes. These data are the first evidence that BTH induces enzyme activities related to anthocyanin metabolism in strawberry fruit after harvest.

KEYWORDS: Anthocyanin; BTH; strawberry fruit; postharvest

INTRODUCTION

Anthocyanins belong to the widespread class of phenolic compounds collectively named flavonoids, which exhibit a wide range of biological effects including antioxidant and anticarcinogenic properties (*1*). In recent years, there has been convincing epidemilogical evidence showing that dietary anthocyanins may provide protection against coronary heart disease, stroke, and lung cancer (*2*). At least, part of these presumed health-promoting features can be attributed to the antioxidant properties of these compounds whose chemical structure appears ideal for free radical scavenging. Thus, high consumption of fruit rich in anthocyanins is beneficial to human health (*3*).

With the consciousness of the beneficial effects of anthocyanins on human health, one may create the idea to actively stimulate their biosynthesis and accumulation. Nowadays, anthocyanin's biosynthesis pathway has been almost completely elucidated (**Figure 1**), and most of the structural genes encoding the enzymes responsible for each step have been isolated from different sources (*4*, *5*).

Strawberry fruits are good sources of anthocyanins and have shown a remarkably high scavenging activity against chemically generated radicals (*6*). Recenty, it has been reported that anthocyanin composition and levels of anthocyanin in strawberries can be influenced by postharvest treatments such as ultraviolet light, altered gas composition, or application of signaling molecules (*7*−*11*). Benzo-thiadiazole-7-carbothioic acid *S*-methyl ester (BTH) is a functional analogue of the plant endogenous hormone-like compound salicylic acid. It has been shown to reduce the severity and development of gray mold disease caused by *Botrytis cinerea* on strawberry fruit (*12*). However, no information is available on the effect of BTH on anthocyanin content in strawberry fruit. The objective of this study was to determine whether postharvest application of BTH has an effect on strawberry anthocyanin content and, if so, whether these changes are associated with the modulation of enzymes important to anthocyanin synthesis.

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MATERIALS AND METHODS

Fruit and BTH Treatment. Strawberry (*Fragaria* × *ananassa* Duch. cv. Fengxiang) fruits were harvested by hand at the fully ripe stage from a farm at Nanjing, Jiangsu Province and transported, within 2 h, to the laboratory. All of the calyxes of strawberries were removed, and the fruits were sorted to eliminate damaged or unripe fruits, and selected for uniform size and color, and then divided randomly into two groups (n = 300 fruit per group).

The first group of fruits was immersed into a solution of 0.2 g L^{-1} BTH (Bion, Novartis Ltd., Basel, Switzerland) for 5 min, whereas the second group of fruits was considered as the control and was soaked in sterile deionized water for 5 min. All fruits were then air-dried for approximately 30 min and stored at 1 °C for 10 days (at 80%−90% relative humidity). There were 5 kg of fruit each per treatment, and the whole experiment was conducted twice. Twenty fruit samples were taken initially and at 2-day intervals during storage for measurements of anthocyanin content and activities of glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49), shikimate dehydrogenase (SKDH, EC 1.1.1.25), tyrosine ammonia lyase (TAL, EC 4.3.1), phenylalanine ammonia lyase (PAL, EC 4.3.1.5), cinnamate-4-hydroxylase (C4H, EC 1.14.13.11), 4-coumarate/coenzyme A ligase (4-CL, EC 6.2.1.12), and dihydroflavonol 4-reductase (DFR, EC 1.1.1.219).

Analysis of Total Anthocyanins. To prepare the fruit extracts, 2.5 g of flesh tissue from 10 fruits in each group was homogenized with 2.5 mL

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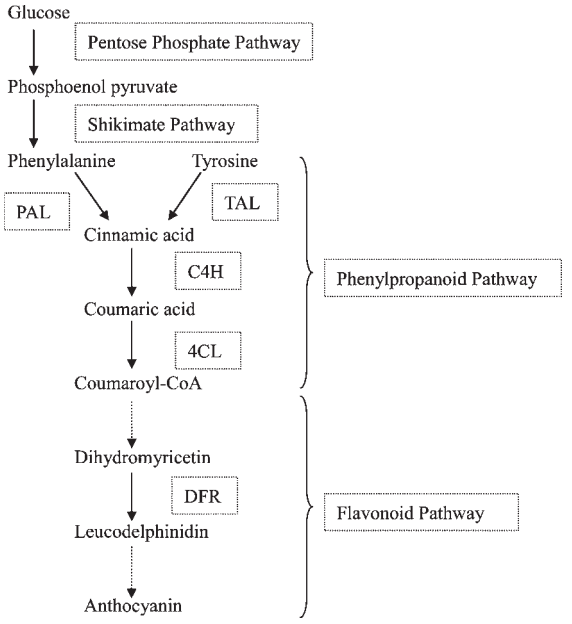

Figure 1. Anthocyanin biosynthetic pathway. Enzyme abbreviations: TAL, tyrosine ammonia lyase; PAL, phenylalanine ammonia lyase; C4H, cinnamate-4-hydroxylase; 4-CL, 4-coumarate: coenzyme A ligase; DFR, dihydroflavonol 4-reductase.

of 95% (v/v) cold ethanol containing 3% (v/v) formic acid and centrifuged at 10,000*g* for 15 min, and another 2.5 mL of 80% (v/v) cold ethanol containing 5% (v/v) formic acid extracted the residue again. The supernatants were combined and made the final volume of 10 mL. The ethanol extract was used for the analysis of total anthocyanins. Total anthocyanin content of strawberry fruit extract was measured using the pH differential method (*13*). Results were expressed as milligram of cyanidin 3-glucoside equivalents per 100 g of fresh weight.

Enzyme Assays. All enzyme extract procedures were conducted at 4 °C. G6PDH and SKDH were extracted with 0.1 M potassium phosphate buffer (pH 7.4) containing 0.5 mM dithiothreitol (DTT), 2 mM cysteine, and 2 mM EDTA. TAL and PAL were extracted using 0.2 M sodium borate buffer (pH 8.7) containing 20 mM β-mercaptoethanol. For C4H, 4-CL, and DFR, 5 g of fruit flesh was extracted with with 200 mM Tris-HCl buffer (pH 7.5) containing 25% (v/v) glycerol, and 0.1 M DTT The extracts were then homogenized and centrifuged at 10,000*g* for 20 min at 4 °C. The supernatants were used for the enzyme assays.

G6PDH activity was assayed according to Debham and Emes (*14*). The enzyme reaction mixture containing 5.88 μM NADP, 88.5 μM $MgCl_2$, and 53.7 μM glucose-6-phosphate was prepared. The increase in absorbance at 340 nm was monitored for over a period of 5 min. The rate of change in absorbance per minute was used to quantify the enzyme in the mixture using the extinction coefficient of NADPH (6.22 mM^{-1} cm^{-1}). One unit of enzyme activity was equivalent to the oxidation of 1 μmol of NADPH per min.

The spectrophotometric assay for SKDH activity was performed at 25 °C in a reaction medium containing 4 mM shikimic acid and 2.0 mM NADP in 0.1 M Tris-HCl buffer at pH 9.0 (*15*). One unit of enzyme activity was defined as the amount of protein that reduces 1 μmol of NADP per min. NADP reduction (NADH formation) was monitored at 340 nm using the extinction coefficient of NADP (6.22 mM^{-1} cm^{-1}).

TAL and PAL activities were assayed as described by Kozukue et al. (*16*) and Zucker (*17*), respectively. The assay medium contained 0.1 mL of enzyme extract and 1 mL of L-tyrosine for TAL or L-phenylalanine for PAL. After incubation at 40 °C for 1 h, the reaction was stopped by adding 0.2 mL of 6 M HCl. One unit of TAL was defined as the amount of enzyme causing an increase in A_{333} of 0.01 units h^{-1}. One unit of PAL activity was defined as the amount of enzyme causing an increase in A_{290} of 0.001 units h^{-1}.

C4H activity was assayed by using the method described by Bi et al. (*18*) with slight modifications. The extract (0.2 mL) was added to 2 mL of reaction buffer (50 mM phosphate buffer containing 2 mM of 2-mercaptoethanol, 2 mM *trans*-cinnamic acid, and 0.5 mM NADPH), which was incubated for 1 h at 37 °C. Absorbance values were measured at 290 nm after the reaction was stopped with 6 M HCl. One unit of C4H activity was defined as the amount of enzyme causing an increase in A_{290} of 0.001 units h^{-1}.

To assay 4-CL activity, the method of Voo et al. (*19*) was used, monitoring the increase in A_{333} using *p*-coumarate as the substrate. The 1 mL reaction mixture contained 50 μL of crude enzyme, 0.2 mM *p*-coumarate, 0.8 mM ATP, 7.5 mM $MgCl_2$, and 38 M CoA in 100 mM Tris-HCl buffer (pH 7.5). One unit of 4-CL was defined as the amount of enzyme that causing a decrease in A_{333} of 0.01 units min^{-1}.

DFR activity was measured according to Xie et al. (*20*) with some modifications. The reaction mixture contained 25 mM Tris-HCl (pH 7.0), 4 mM NADPH, 100 μM dihydroquercetin, and protein samples. The reaction was initiated by the addition of NADPH at 25 °C, followed by measuring the rate of NADPH oxidation at 340 nm. The enzyme activity was calculated by using the extinction coefficient of NADPH (6.22 mM^{-1} cm^{-1}). One unit of enzyme activity was equivalent to the oxidation of 1 μmol of NADPH per min.

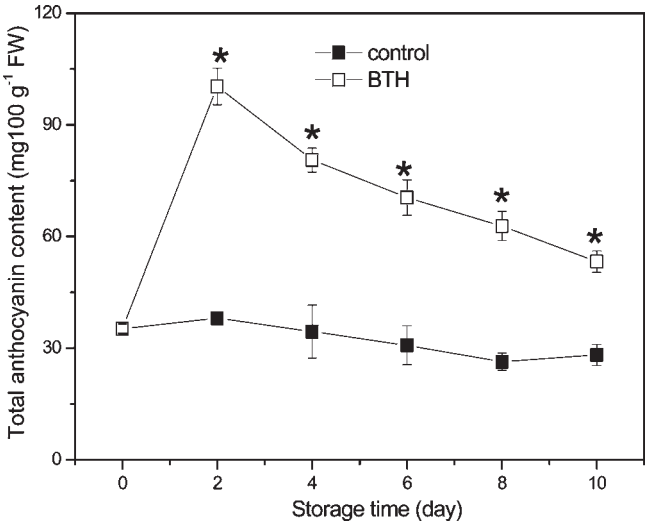

Figure 2. Effect of 0.2 g L^{-1} BTH treatment on anthocyanin content of strawberry fruit during storage at 1 °C. Values are the means ± SD of triplicate assays. Vertical bars represent the standard errors of the means. An asterisk indicates that the difference between the treatment and control is significant.

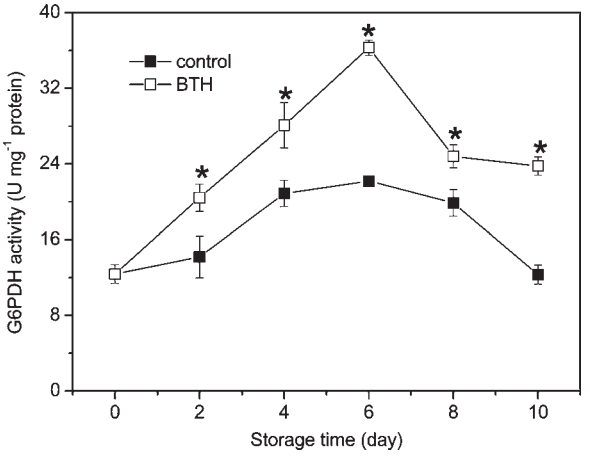

Figure 3. Effect of 0.2 g L^{-1} BTH treatment on glucose-6-phosphate dehydrogenase (G6PDH) activity of strawberry fruit during storage at 1 °C. Values are the means ± SD of triplicate assays. Vertical bars represent the standard errors of the means. An asterisk indicates that the difference between the treatment and control is significant.

Protein content in the enzyme extracts was estimated using the Bradford method, using bovine serum albumin as a standard (*21*). Specific activity of the enzymes was expressed as units per milligram protein.

Data Analysis. Experiments were performed using a completely randomized design. All statistical analyses were performed with SPSS (SPSS Inc., Chicago, IL, USA). The data were analyzed by one-way analysis of variance (ANOVA). Mean separations were performed by Duncan's multiple range tests. Differences at $p < 0.05$ were considered as significant.

RESULTS AND DISCUSSION

In our study, the total anthocyanin in strawberry fruit at harvest was around 35.2 mg 100 g^{-1} FW, and the content in control strawberries changed little during the 10 days of storage. However, the content in treated fruit increased dramatically and reached 100.2 mg 100 g^{-1} FW after 2 days of storage, and declined slightly during the following time (**Figure 2**). BTH treatment significantly ($p < 0.05$) enhanced anthocyanin content in strawberry fruits during the whole storage period. At the end of storage, anthocyanin content in control and treated strawberries was 28.2 and 53.2 mg 100 g^{-1} FW, respectively. Thus, BTH has a potential application in postharvest treatments for enhancing anthocyanin content and maintaining a high-quality product of strawberry fruit.

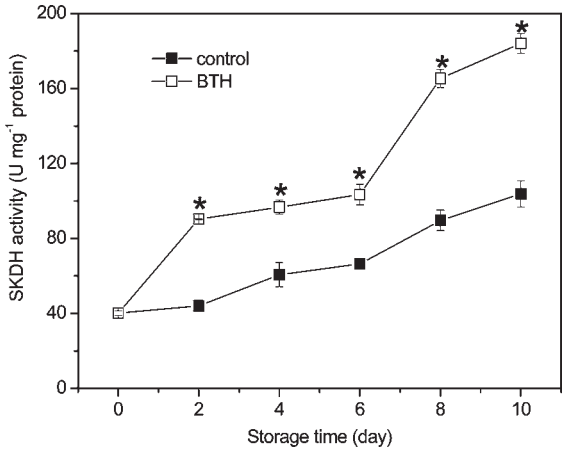

Figure 4. Effect of 0.2 g L^{-1} BTH treatment on shikimate dehydrogenase (SKDH) activity of strawberry fruit during storage at 1 °C. Values are the means ± SD of triplicate assays. Vertical bars represent the standard errors of the means. An asterisk indicates that the difference between the treatment and control is significant.

Anthocyanins are secondary metabolites, primarily synthesized through the pentose phosphate pathway and shikimate, phenylpropanoid, and flavonoid pathways (**Figure 1**). The oxidative pentose phosphate pathway provides precursor erythrose-4-phosphate for the shikimate pathway. The shikimate pathway converts these sugar phosphates to aromatic amino acids such as phenylalanine, which becomes the precursor for the phenylpropanoid pathway (*22*). The stimulatory effect of BTH on the total anthocyanin content leads to the investigation of the role of key regulatory enzymes in anthocyanin metabolism.

It is known that G6PDH activated by CO_2 stress results in phenolics accumulation in the root of *Panax ginseng* (*23*). In the present study, an increase in G6PDH activity was observed during early storage time (days 0−6), followed by a decline during the remaining time for the control and treated fruits (**Figure 3**). The activities in control and BTH-treated fruits were 22.2 and 36.3 U mg^{-1} protein, respectively, after 6 days of storage. The early increase is possibly due to the carbohydrate mobilization from the cotyledons directed toward the high nutrient requirements of the fruit (*22*). BTH-treated fruit had significantly ($p < 0.05$) higher activity of G6PDH compared to that of the control, which might be responsible for the higher anthocyanin content (**Figure 2**).

During storage at 1 °C, SKDH activity increased in all strawberries; however, BTH-treated fruits showed significantly ($p < 0.05$) higher enzyme activity than the control fruits (**Figure 4**), and the activity reached 184.0 U mg^{-1} protein. Since SKDH is one of the crucial enzymes in the biosynthesis of anthocyanin metabolites (*24*), the enhancement of anthocyanin content in treated strawberry fruit might result from the increased SKDH activity.

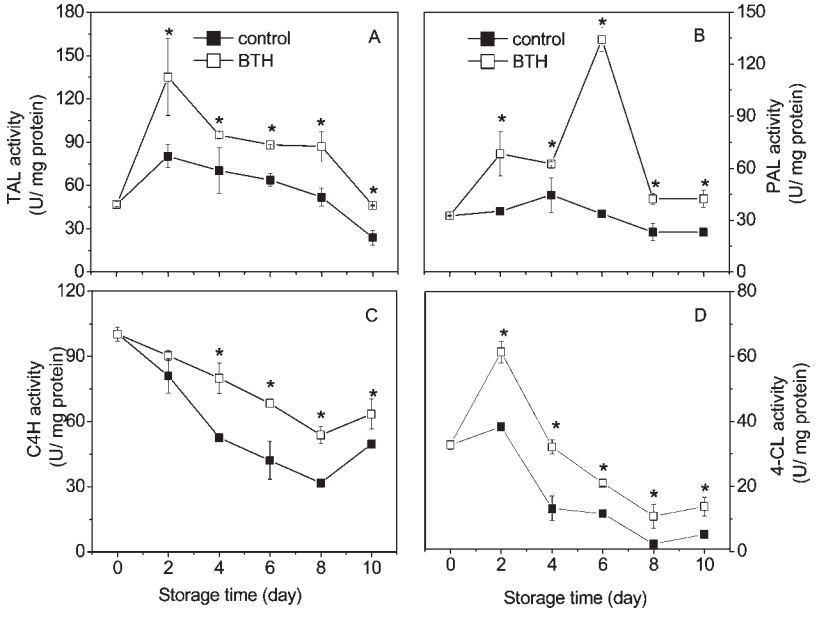

Figure 5. Effect of 0.2 g L^{-1} BTH treatment on activities of TAL (**A**), PAL (**B**), C4H (**C**), and 4-CL (**D**) of strawberry fruit during storage at 1 °C. Values are the means ± SD of triplicate assays. Vertical bars represent the standard errors of the means. An asterisk indicates that the difference between the treatment and control is significant.

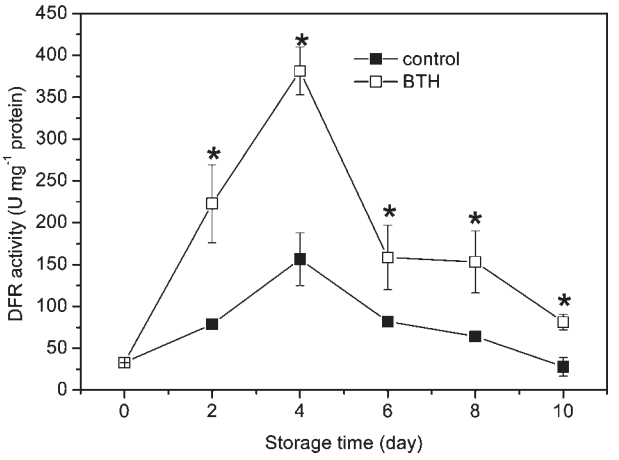

Figure 6. Effect of 0.2 g L^{-1} BTH treatment on DFR activity of strawberry fruit during storage at 1 °C. Values are the means ± SD of triplicate assays. Vertical bars represent the standard errors of the means. An asterisk indicates that the difference between the treatment and control is significant.

To study the BTH effect on anthocyanin change further, the enzymes involved in the phenylpropanoid pathway were also investigated. PAL and TAL are two key enzymes in the phenylpropanoid pathway. PAL catalyzes the conversion of L-phenylalanine to *trans*-cinnamic acid. As an activity analogous to PAL, TAL converts L-tyrosine to ammonia and *p*-coumaric (*25*). In this study, the activities of TAL and PAL in control fruit reached their peaks (80.3 and 44.5 U mg^{-1} protein, respectively) after 2 or 6 days of storage. BTH treatment caused increased activities of both enzymes, and the activities were 92.9% and 83.1%, respectively, higher than that in control fruits after 10 days of storage (**Figure 5A**,**B**). C4H and 4-CL are also pivotal regulatory enzymes in the phenylpropanoid pathway and play an important role in anthocyanin synthesis (*25*). C4H activity decreased gradually in all strawberries during the first 8 days of storage. Then, the activity increased with the prolonged storage time (**Figure 5C**). 4-CL activity increased and peaked to 38.3 U mg^{-1} protein on day 2 in control fruits (**Figure 5D**). During the whole storage period, the treated fruit gave significantly ($p < 0.05$) higher activities of these two enzymes than the control. A previous study has shown that treatment with gibberellic acid increased PAL and TAL activities, which was correlated to the anthocyanin accumulation in ripening strawberries (*26*). In this work, we demonstrated that an increase in anthocyanin content was accompanied not only by increases in the activities of PAL and TAL but also by increases in C4H and 4-CL activities.

The activity of DFR, a key regulatory enzyme of the flavonoid pathway (*5*), was examined with the aim of better understanding the process involved in strawberry's response to BTH treatment. The enzyme catalyzes the stereospecific reduction of dihydroflavonols to leucoanthocyanidins using NADPH as a cofactor. These leucoanthocyanidins are the immediate precursors for the synthesis of anthocyanins (*27*). The activity significantly increased during the first 4 days of storage and reached 156.4 U mg^{-1} protein in control strawberries. Then it declined gradually during the other times. BTH-treated fruit had significantly ($p < 0.05$) higher DFR activity. Four and 10 days after BTH treatment, the activities were 182 and 192%, respectively, higher than that of the control (**Figure 6**). Our result reveals that exogenous BTH treatment induces an increase in anthocyanin content in strawberry fruits.

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