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# Kinetic study on the effects of sugar addition on the thermal degradation of phycocyanin from *Spirulina* sp.

Hadiyanto[a,b,c,*], Marcelinus Christwardana[c,d,**], Heri Sutanto[e], Meiny Suzery[f], Devita Amelia[a], Ruth Febrina Aritonang[a]

[a] Department of Chemical Engineering, Diponegoro University, Jl Prof. Soedharto, SH, Tembalang, Semarang 50275, Indonesia
[b] Master Program of Environmental Science, Diponegoro University, Jl Imam Bardjo, SH, Semarang 50275, Indonesia
[c] Center of Biomass and Renewable Energy (C-BIORE), Diponegoro University, Jl Prof. Soedharto, SH, Tembalang, Semarang 50275, Indonesia
[d] Graduate School of Energy and Environment, Seoul National University of Science and Technology, 232 Gongneung-ro, Nowon-gu, Seoul 01811, Republic of Korea
[e] Physic Department, Faculty of Science and Mathematic, Diponegoro University, Indonesia
[f] Chemistry Department, Faculty of Science and Mathematic, Diponegoro University, Indonesia

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ABSTRACT

Phycocyanin is a source of antioxidants and natural colorants extracted from microalgae. It undergoes color, concentration, and antioxidant activity degradations during heating processes, such as in food processing. The effects of different kinds of sugars as stabilizer and elevation of temperature were studied. Glucose, sucrose, or fructose as stabilizer were mixed in phycocyanin solution, which was heated to 40 °C, 60 °C, and 80 °C for 60 min. Results showed that phycocyanin degraded significantly without any sugar addition. The addition of glucose could increase the activation energy by up to fourfold due to the polymerization of protein phycocyanin by sugar, and glucose could prevent damage of the phycocyanin structure. Phycocyanin underwent discoloration from bright blue to faint blue after heating at 80 °C. Fructose addition in phycocyanin could minimize color degradation at 80 °C, whereas glucose addition could increase the antioxidant activity of phycocyanin by decreasing $IC_{50}$ up to 18.47%.

## 1. Introduction

Food colorants are widely used as an additive material in both manufacturing and consumer food industries due to their compatibility with food processes (Yamjala, Nainar, & Ramisetti, 2016). Natural food colorants from natural sources, such as plants or fruits, have been used since 1500 BCE, but they were replaced by synthetic colorants in the early 19th century because of the industrial revolution (Downham & Collins, 2000). Natural colorants currently dominate 31% of the dye market, whereas 40% are synthetic colorants (Mapari, Thrane, & Meyer, 2010). The rising market in natural colorants is caused by awareness of diet and health, which starts with the reduced consumption of synthetic material.

Several plants and fruits are currently used as natural colorants, such as *Rosella* (red), grape (purple), carrots (orange), blueberry (blue), or *Pandanus* leaves (green) (Duangmal, Saicheua, & Sueeprasan, 2008; Clydesdale, Francis, & Damon, 1978; Kirca, Özkan & Cemeroğlu, 2007; Fracassetti et al., 2013; Ningrum, Minh, & Schreiner, 2015). However, the utilization of microalgae extract as a natural colorant is also increasing, particularly phycocyanin (blue) from *Spirulina* sp., β-carotene (orange) from *Dunaliella* sp., or astaxanthin (red) from *Haematococcus* sp. (Dufossé et al., 2005). Most food colorants are also used as antioxidants to reduce free radicals.

Phycocyanin is a natural colorant extracted from microalgae. This pigment–protein complex compound can be extracted from blue-green algae (cyanobacteria) *Spirulina* sp., and it is classified as a phycobiliprotein (Markou & Nerantzis, 2013). The molecular weight, position, and maximum absorption intensity of phycocyanin depend on the state of aggregation, which is affected by pH solution, temperature, protein concentration, and origin of the algae itself (Mishra, Shrivastav, & Mishra, 2008). Phycoyanin captures oxygen radicals because it contains an open tetraphyrrole chain that can bind a peroxy radical by donating hydrogen atoms, which are bonded in the 10th C atom from a tetraphyrrole molecule (Estrada, Bescos, & Fresno, 2001; Romay, González, Ledón, Remirez, & Rimbau, 2003).

In its application as a natural food colorant, degradation of color, concentration, and antioxidant activity often occurs due to high temperatures during food processing. Phycocyanin gradually changes from

* Corresponding author at: Department of Chemical Engineering, Diponegoro University, Jl Prof. Soedharto, SH, Tembalang, Semarang 50275, Indonesia.
** Corresponding author at: Center of Biomass and Renewable Energy (C-BIORE), Diponegoro University, Jl Prof. Soedharto, SH, Tembalang, Semarang 50275, Indonesia.
*E-mail addresses:* hadiyanto@live.undip.ac.id (Hadiyanto), marcelinus@seoultech.ac.kr (M. Christwardana).

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light blue to faint blue, and this transformation is a disadvantage in the food industry because of the resulting unattractive hue. Several techniques have been used to prevent the thermal degradation of phycocyanin, such as the addition of a stabilizer (Antelo, Costa, & Kalil, 2008; Sun, Wang, & Qiao, 2006), pH adjustment (Wu, Wang, Xiang, Li, & He, 2016), and encapsulation (Chen et al., 1996).

Stabilizer addition is commonly used to minimize the thermal degradation of phycocyanin due to its simplicity and economy. Sugar is a stabilizer that is typically used to stabilize proteins that are easily degraded, and it has a simple structure such as monosaccharide (glucose, fructose, and galactose) or disaccharide (maltose, lactose, and sucrose). Sugar can bind with protein via an N-linked glycosidic bond, which can minimize thermal degradation during food processing (Allison, Chang, Randolph, & Carpenter, 1999; Imamura, Ogawa, Sakiyama, & Nakanishi, 2003). The utilization of sugar as a stabilizer in the food industry has been studied by Martelli, Folli, Visai, Daglia, and Ferrari (2014), who used sugar to reduce the anthocyanin degradation of raspberry. Meanwhile, Vikram, Ramesh, and Prapulla (2005) used sugar to reduce the nutrient degradation of orange juice. In another experiment, Sadilova, Stintzing, Kammerer, and Carle (2009) attempted to add sugar to fruit juices to stabilize the anthocyanin content. Therefore, sugar is a promising stabilizer that can be used to prevent the thermal degradation of phycocyanin.

In the present study, commercial sugar with simple structures (i.e., glucose, fructose, and sucrose) was used as a promising phycocyanin stabilizer to minimize thermal degradation. The reaction kinetics of the thermal degradation of phycocyanin and the activation energy were studied. Phycocyanin color changes were also observed before and after heating using a colorimeter. Enhanced antioxidant activity after heating will be helpful to establish a baseline protocol for antioxidant stability.

## 2. Experimental

### 2.1. Materials and method

Phycocyanin extract from *Spirulina platensis* (in powder form) was purchased from CV Neoalgae (Sukoharjo, Indonesia). Glucose, sucrose, and fructose (industrial grade) were purchased from a local supermarket in Semarang in June 2016. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), citric acid, and sodium citrate dihydrate were obtained from Sigma–Aldrich (St. Louis, MO, USA)

### 2.2. Sample preparation and heat processing

Phycocyanin samples were prepared for heating. Approximately 7.2 ml of 0.1 M citric acid and 42.8 ml of 0.1 M sodium citrate dihydrate were mixed together, and deionized (DI) water was added to dissolve the mixture until the volume was 100 ml to form citrate buffer (pH 6). Subsequently, 50 mg of phycocyanin extract and sugar as stabilizer agent (10 or 15 wt%) was added and mixed into 50 ml of citrate buffer (pH 6). An Erlenmeyer flask containing the sample solution covered with aluminium foil to protect from further photo-degradation (Bahnemann, Lawton, & Robertson, 2013) then was placed into a water bath and heated for 60 min via a water bath thermostat (Clifton Water Bath Ne1 28 L). DI water was filled into the water bath, and the thermostat was set to 45 °C, 65 °C, or 85 °C to obtain actual temperatures of 40 °C, 60 °C, or 80 °C in Erlenmeyer flasks containing the samples. The samples were collected before heating, and UV–Vis measurements were obtained for all the heat levels studied every 10–60 min while heating. The processed samples were stored in a vial, and their color and antioxidant activity were measured on the same day. The processed samples were stored in vials at 4 °C to avoid protein degradation until further analysis.

### 2.3. Phycocyanin content analysis

Total phycocyanin content (TPC), C-phycocyanin content (C-PC), and total A-phycocyanin content (A-PC) of the samples were determined using UV–Vis spectrophometry (SP-3000, Optima, Japan). About 5 ml of samples was added into cuvettes and allowed to stand for 3 min at room temperature. The absorbance was then measured at 620 and 652 nm. The absorbance values were converted to C-PC and A-PC values following Eqs. (1) and (2) (Bennet & Bogorad in 1973):

$$C - PC = \frac{OD_{620} - 0.475 * OD_{652}}{5.34} \quad (1)$$

$$A - PC = \frac{OD_{652} - 0.280 * OD_{620}}{5.09} \quad (2)$$

The values of C-PC and A-PC were summed to obtain the TPC values. TPC at *t* time was divided by the initial TPC values and multiplied by 100 to determine the relative concentration of phycocyanin (% C).

### 2.4. Kinetic model analysis

The degradation kinetics of the phycocyanin content were determined by fitting a zero-order, first-order, or second-order kinetic model to experimental data as shown in Eqs. (3), (4), and (5):

$$C_t - C_0 = -kt \quad (3)$$

$$\ln\frac{C_t}{C_0} = -kt \quad (4)$$

$$\frac{1}{C_t} - \frac{1}{C_0} = -kt \quad (5)$$

where $C_t$ is the phycocyanin concentration at *t* time, $C_0$ is the initial phycocyanin concentration, t is the heat processing time, and *k* is the kinetic rate constant. All the data were fitted to models, and the coefficient of determination ($R^2$) was used as a criterion for adequacy of fit.

### 2.5. Activation energy measurement

The activation energy of phycocyanin degradation was measured by relating the kinetic model as shown in Eqs. (3)–(5) and the Arrhenius equation as shown in Eqs. (6) and (7)

$$k = C^{-E_a/(RT)} \quad (6)$$

$$\ln k = -\frac{E_A}{R}\left(\frac{1}{T}\right) - \ln C \quad (7)$$

where C is the phycocyanin concentration, *k* is the kinetic rate constant, $E_a$ is the activation energy, R is the ideal gas constant, and T is temperature. The slope that was determined by fitting was $E_a/R$, so $E_a$ was determined by multiplying the slope with R (8.314 J $mo^{l-1}$ $K^{-1}$).

### 2.6. Color degradation measurement

The color of phycocyanin before and after heating was analyzed using a colorimeter (Konica Minolta CR-400 Chroma Meter; Tokyo, Japan). The colorimeter was calibrated using a white reference tile before measuring the samples. About 50 µl of samples was filled into a transparent glass surface (10 cm × 15 cm) and then analyzed. The lab coordinates (L* for lightness, a* for red-green, and b* for yellow-blue) were determined using a CIE scale, and color change (E*) was measured with Eq. (8):

$$\Delta E^* = \sqrt{\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2}} \quad (8)$$

where ΔE* is the color change, ΔL* is the difference in L before and after heating, Δa* is the difference in *a* before and after heating, and Δb* is the difference in *b* before and after heating.

### 2.7. Antioxidant activity measurement

In this experiment, DPPH radical scavenging capacity was used to determine the antioxidant activity of phycocyanin before and after heating. This method was selected because it is a simple, fast, easy, accurate, reliable, and practical screening technique. The DPPH radical scavenging capacity assay was carried out by mixing 1 ml of DPPH with 1 ml of sample. The mixture was diluted using methanol until 5 ml (final DPPH concentrations were 1, 2, 4, and 8 ppm) and left to stand for 24 h. DI water was used as blank solution. The samples were measured using a UV–Vis spectrophotometer at 515 nm (Gomez-Alonso, Fregapane, Salvador, & Gordon, 2003), and the antioxidant activity or % inhibition was determined by Eq. (9):

$$\text{Antioxidant activity} = \left(1-\frac{A_{sample}}{A_{blank}}\right)\times 100\% \quad (9)$$

where $A_{sample}$ is the absorbance of the sample, and $A_{blank}$ is the absorbance of the blank solution at 515 nm. The values of % inhibition in several concentrations were plotted, and the linear regression equation was measured. The inhibitor concentration at 50% inhibition ($IC_{50}$) was determined by entering the y-value of the linear regression equation by 50 which means 50% inhibition process. The result of x-value from linear regression equation represents the inhibitor concentration at 50% inhibition ($IC_{50}$).

## 3. Results and discussion

### 3.1. Effect of different kinds of sugar in heat processing on the phycocyanin concentration

The effect of different kinds of sugar added to phycocyanin subjected to heat treatment on the phycocyanin concentration must be determined. Fig. 1a shows the decrease in the phycocyanin concentration during heating at 40 °C. At this temperature, phycocyanin mixed with glucose could stabilize the concentration up to 95–97%, whereas phycocyanin mixed with sucrose, fructose, and without sugar demonstrated phycocyanin concentration stability of up to 70%, 71%, and 67%, respectively. At this condition, glucose and fructose could easily bond with protein in phycocyanin by a glycosidic bond. The low concentration stability of phycocyanin with fructose compared with that of phycocyanin with glucose was due to fructose having a more stable structure than glucose (Assary & Curtiss, 2012). Thus, fructose had difficulty in making a sugar–protein bond compared with glucose. Sucrose, which has a complex structure, had difficulty in bonding with protein due to the O-linked glycosidic bond between its glucose and fructose structure, thereby explaining why it is known as a non-reducing sugar. Fig. 1b shows the decrease in the phycocyanin concentration under heating at 60 °C. At 60 °C, phycocyanin mixed with glucose was dominant and could stabilize the phycocyanin concentration up to 54%, which was higher than other samples (concentrations of phycocyanin mixed with sucrose, fructose, and without sugar addition were 37%, 47%, and 33%, respectively). This finding indicates the presence of an N-linked glycosidic bond between glucose and protein at this temperature, whereas fructose started to break down and sucrose showed difficulty in bonding with protein. Fig. 1c shows the decrease in the phycocyanin concentration during heating at 80 °C. The O-linked glycosidic bond on the sucrose structure started to break down into glucose and fructose at relatively high temperatures. The resulting glucose and fructose from sucrose formed an N-linked glycosidic bond with protein and could stabilize the phycocyanin concentration up to 28%, whereas the phycocyanin concentration was maintained at 35% with glucose addition. Phycocyanin with fructose addition could stabilize the phycocyanin concentration up to 25%, whereas that without sugar addition could maintain the phycocyanin concentration up to 22%.

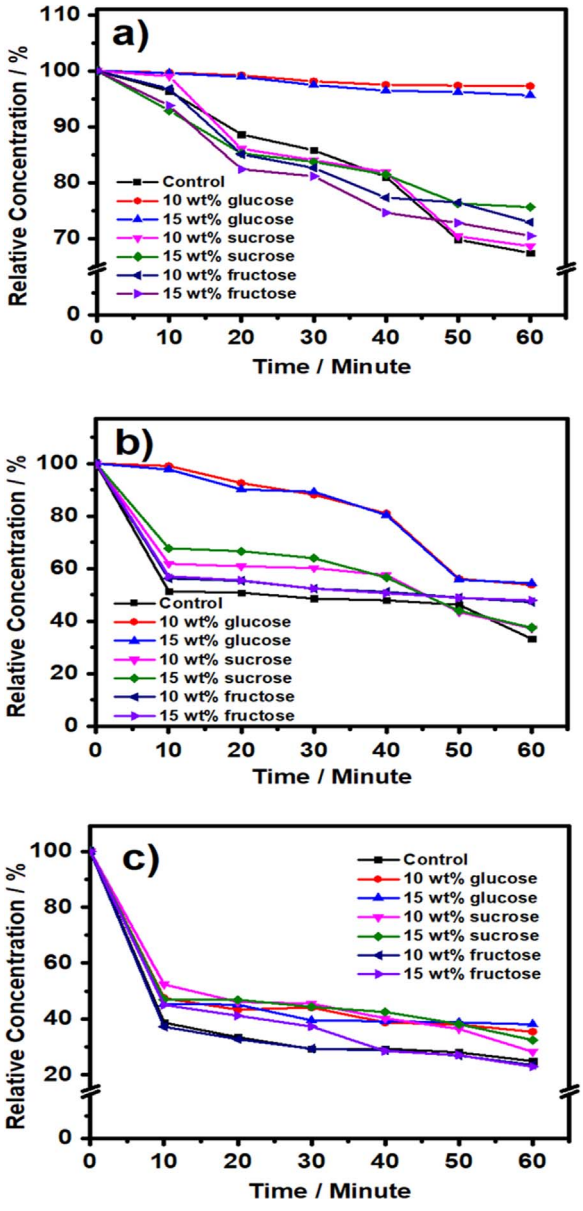


**Fig. 1.** Phycocyanin concentration degradation during heating process at temperature (a) 40 °C, (b) 60 °C, and (c) 80 °C.

### 3.2. Kinetic analysis of the thermal degradation of phycocyanin

The thermal degradation of phycocyanin was modeled using zero-order (Eq. (3)), first-order (Eq. (4)), and second-order kinetics (Eq. (5)), and the determination coefficient ($R^2$) of all samples was measured using Excel-software. Fig. 2a–c show that the thermal degradation of phycocyanin without any sugar addition was modeled by first-order kinetics, which had a higher $R^2$ value than zero-order or second-order kinetics ranging from 0.962 to 0.983 ($R^2$ range for zero-order and second-order kinetics were 0.703–0.929 and 0.675–0.946, respectively) (Gonçalves, Pinheiro, Abreu, Brandão, & Silva, 2010; Jaiswal & Abu-Ghannam, 2013; Martins, Jongen, & van Boekel, 200026–28). A similar trend in first-order kinetics was also observed in phycocyanin added with glucose, sucrose, or fructose. Table 1 illustrates that $R^2$ exceeded 0.95. The value of $k$ increased with increasing temperature. Thus, temperature affected the degradation rate of phycocyanin.

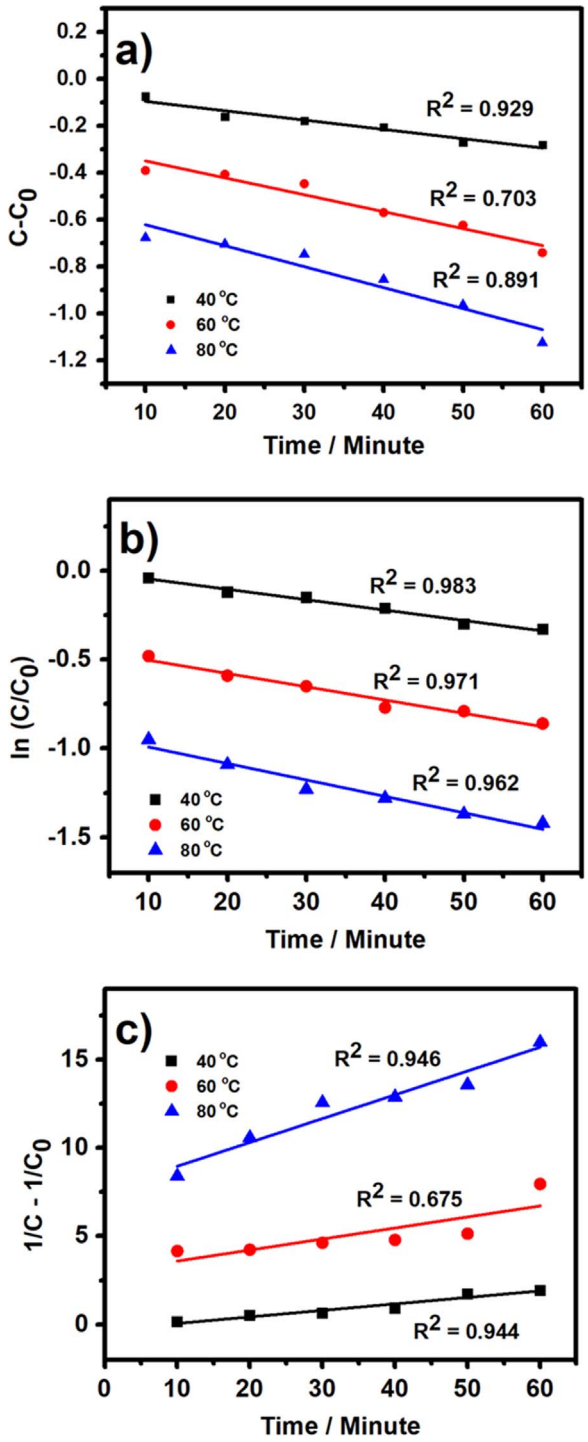


**Fig. 2.** (a) zero order, (b) first-order, and (c) second-order of phycocyanin thermal degradation kinetic model.

### 3.3. *Effect of different kinds of sugar on the activation energy of the thermal degradation of phycocyanin*

The activation energy of the thermal degradation of phycocyanin must be studied to determine the minimum energy required to initiate the degradation reaction of phycocyanin. The values of ln *k* were used to determine $E_a$ via Eq. (7) (Table 1), and the values were plotted in a graph shown in Fig. 3. Fig. 3 and Table 2 clearly indicate that phycocyanin mixed with glucose had a higher slope than the other stabilizers. The average $E_a$ value of phycocyanin mixed with glucose was 52.41 kJ $mol^{-1}$ $K^{-1}$, whereas that of phycocyanin mixed with sucrose, fructose, and without sugar addition were 15.5, 10.9, and 10.51 kJ $mol^{-1}$ $K^{-1}$, respectively. The addition of sugar in phycocyanin under heat treatment could increase $E_a$ by up to fourfold. However, at different concentrations (10 and 15 wt%), the addition of sugar with 15 wt% resulted in a higher $E_a$ than the addition of 10 wt%, which indicated that a high sugar concentration can produce more bonds with the protein in phycocyanin. According to Chaiklahan, Chirasuwana, and Bunnag (2012), a substance with a low $E_a$ can degrade easily. The results in Figs. 2 and 3 suggest that glucose was a

**Table 1**
Kinetic reaction rate and its determination coefficient on phycocyanin thermal degradation with and without sugar addition.

| Stabilizer | Temperature (°C) | *k* (× $10^{-4}$ $s^{-1}$) | $R^2$ | ln *k* (ln $s^{-1}$) |
|---|---|---|---|---|
| Control (no stabilizer) | 40 | 0.98 | 0.983 | −9.227 |
| | 60 | 1.3 | 0.971 | −8.987 |
| | 80 | 1.6 | 0.962 | −8.772 |
| 10 wt% Glucose | 40 | 0.08 | 0.957 | −11.359 |
| | 60 | 0.6 | 0.987 | −9.721 |
| | 80 | 0.95 | 0.974 | −9.262 |
| 15 wt% Glucose | 40 | 0.13 | 0.963 | −11.695 |
| | 60 | 0.62 | 0.983 | −9.778 |
| | 80 | 0.97 | 0.967 | −9.297 |
| 10 wt% Sucrose | 40 | 1.2 | 0.956 | −9.056 |
| | 60 | 1.8 | 0.956 | −8.623 |
| | 80 | 2 | 0.986 | −8.526 |
| 15 wt% Sucrose | 40 | 0.67 | 0.959 | −9.616 |
| | 60 | 1.2 | 0.963 | −9.028 |
| | 80 | 1.5 | 0.958 | −8.816 |
| 10 wt% Fructose | 40 | 0.87 | 0.956 | −9.297 |
| | 60 | 1.4 | 0.964 | −8.886 |
| | 80 | 1.5 | 0.961 | −8.839 |
| 15 wt% Fructose | 40 | 0.9 | 0.964 | −9.334 |
| | 60 | 1.5 | 0.955 | −8.898 |
| | 80 | 1.6 | 0.977 | −8.862 |

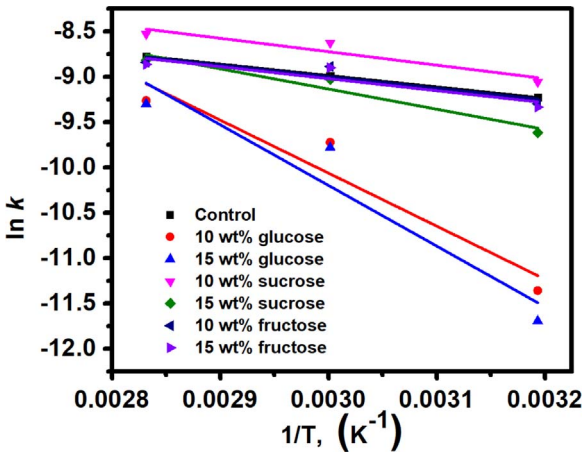


**Fig. 3.** Arrhenius plot of phycocyanin thermal degradation to determine activation energy.

**Table 2**
Activation energy and its determination coefficient on phycocyanin thermal degradation with and without sugar addition.

| Stabilizer | $E_a$/R | $E_a$ (kJ $mol^{-1}$ $K^{-1}$) | $R^2$ |
|---|---|---|---|
| Control (no stabilizer) | 1264 | 10.51 | 0.956 |
| 10 wt% Glucose | 5880 | 48.89 | 0.927 |
| 15 wt% Glucose | 6832 | 56.8 | 0.912 |
| 10 wt% Sucrose | 1490 | 12.39 | 0.902 |
| 15 wt% Sucrose | 2239 | 18.62 | 0.947 |
| 10 wt% Fructose | 1401 | 11.65 | 0.905 |
| 15 wt% Fructose | 1450 | 12.06 | 0.931 |

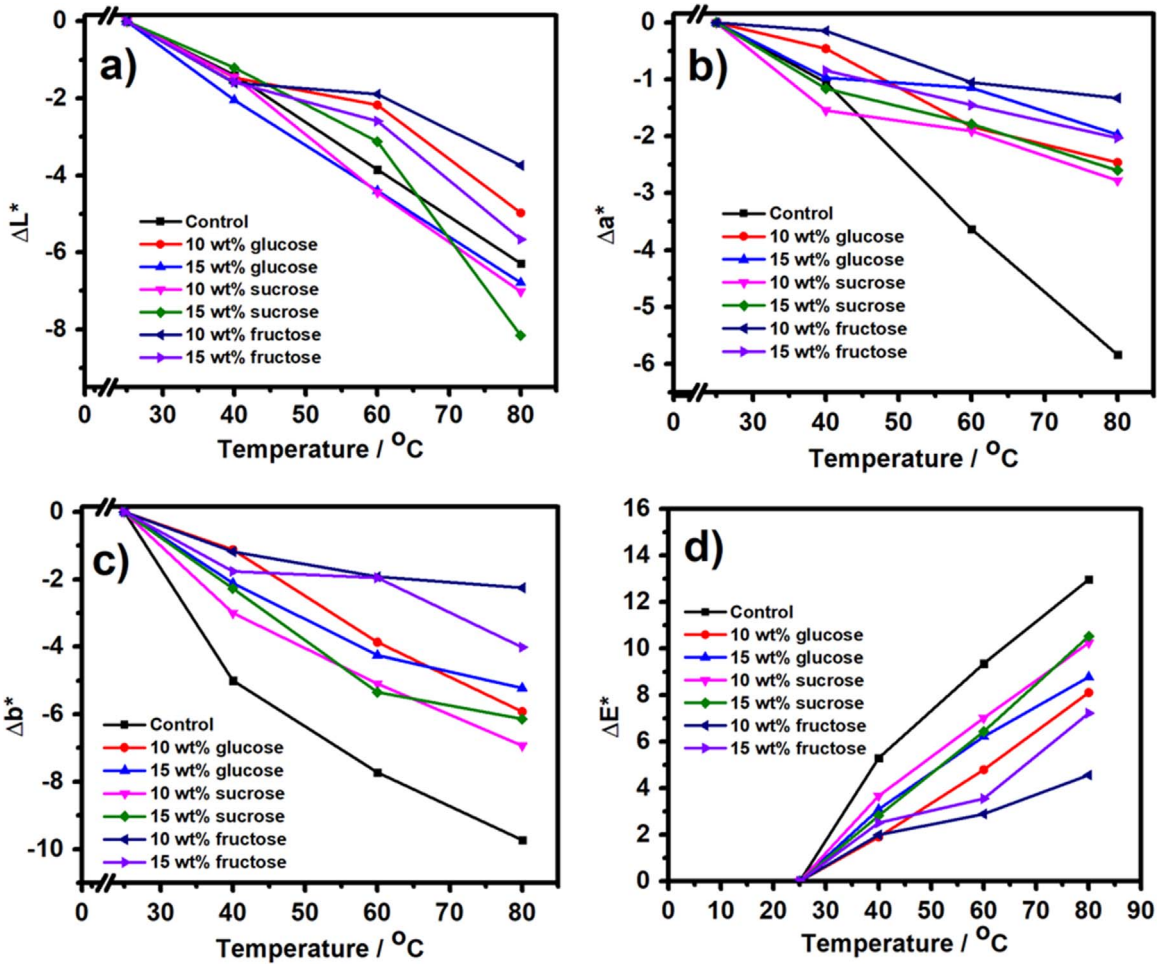


**Fig. 4.** Correlation of (a) ΔL*, (b) Δa*, (c) Δb*, and (d) ΔE* in heating process temperature 40 °C, 60 °C, and 80 °C.

promising stabilizer that could maintain the phycocyanin concentration from thermal degradation.

### 3.4. Effect of different kinds of sugar and temperature on the color degradation of phycocyanin

The color of fresh phycocyanin solution was light blue, as indicated by CIE scale with coordinates L* = 66.69; a* = −14.42; and b* = −15.35. Fig. 4a-c show that ΔL*, Δa*, and Δb* decreased as the temperature and time increased, and this result was compatible with the literature (Jaiswal & Abu-Ghannam, 2013). By contrast, ΔE* increased (Fig. 4d). For the effect of sugar addition, phycocyanin mixed with fructose exhibited a bright light compared with the other samples because of its low ΔE* value (Fig. 4d). As ΔE* increased, the discoloration became increasingly apparent. In general, the color of phycocyanin with sugar still looks good (bright blue) during heating at 40 °C and 60 °C. However, the color become faint under heating at 80 °C. The discoloration of phycocyanin from bright blue to faint blue may be due to (i) the polymerization of protein phycocyanin by sugar (Krifi, Bondurant, & Metche, 2000) and (ii) damage of the phycocyanin protein. The polymerization of protein phycocyanin by sugar resulted in enzymatic reaction resistance and further protection from thermal degradation (Huang, 1956). Sucrose needed more time and energy to break down into sucrose and glucose and bond with protein. During this time, phycocyanin structures were broken prior to bonding with glucose and fructose (from the breakdown of sucrose). Meanwhile, phycocyanin mixed with fructose exhibited a bright blue color, which indicated that fructose only prevented the hydration reaction on phycocyanin pigment active sites (Huang, 1956) but failed to polymerize the phycocyanin structure. The addition of excess sugar also weakened the color of phycocyanin due to the growth of microorganisms (Garzón & Wrolsta, 2001).

### 3.5. Effect of different kinds of sugar and heating process on antioxidant activity

The antioxidant activity after heating must be determined to assess the quality of phycocyanin. All phycocyanin samples without and with sugar addition were mixed with DPPH as inhibitor at 1, 2, 4, and 8 ppm. In this experiment, DPPH functioned as a free radical agent. Fig. 5 and Table 3 show that phycocyanin mixed with 15 wt% of glucose had a low $IC_{50}$ value of 100.98 ppm, and antioxidant activity was categorized as mid-level. By contrast, phycocyanin without any sugar addition had a high $IC_{50}$ value of 123.86 ppm, and antioxidant activity was categorized as weak level. Thus, $IC_{50}$ could decrease up to 18.47% with the addition of 15 wt% of sugar. A low $IC_{50}$ indicated high antioxidant activity. Most of the phycocyanin samples were categorized with weak antioxidant activity due to the utilization of crude phycocyanin extract, not pure extract. The purity of the phycocyanin extract could decrease the value of $IC_{50}$. The sugar–protein bond in phycocyanin mixed with glucose also could maintain the antioxidant level during heating. As the sugar amount increased, the $IC_{50}$ value decreased due to the presence of more sugar–protein bonds. However, under saturated sugar conditions, sugar could form a bond with flavonoid, thereby decreasing antioxidant activity.

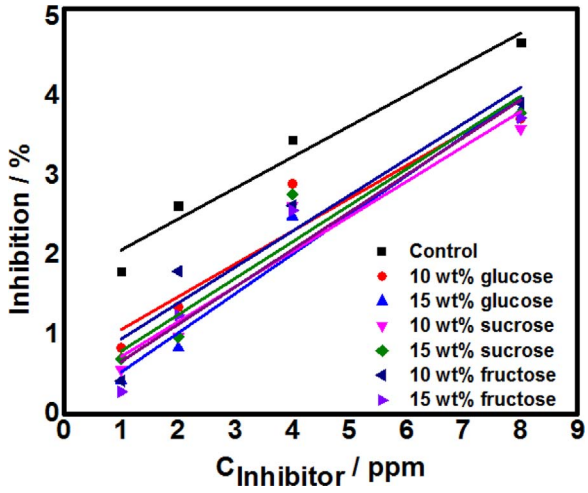


**Fig. 5.** Correlation of % inhibition in several DPPH concentrations.

**Table 3**
$IC_{50}$ and its determination coefficient on phycocyanin thermal degradation with and without sugar addition.

| Stabilizer | $IC_{50}$ | $R^2$ |
|---|---|---|
| Control (no stabilizer) | 123.856 | 0.965 |
| 10 wt% Glucose | 111.873 | 0.95 |
| 15 wt% Glucose | 100.975 | 0.959 |
| 10 wt% Sucrose | 112.65 | 0.926 |
| 15 wt% Sucrose | 108.216 | 0.926 |
| 10 wt% Fructose | 109.395 | 0.91 |
| 15 wt% Fructose | 105.811 | 0.934 |

## 4. Conclusion

This study showed that the addition of sugar affected the stability, activation energy, color, and antioxidant activity of phycocyanin. The stability of phycocyanin was affected by the sugar–protein interaction via glycosidic bond, which could polymerize phycocyanin and prevent degradation reactions. In the thermal degradation kinetic model, the first-order kinetic model demonstrated an average $R^2$ value of 0.967. The addition of glucose to phycocyanin could increase the activation energy by about fourfold and decrease the degradation rate. Phycocyanin mixed with fructose presented a slight discoloration due to fructose's ability to prevent the hydration reaction of phycocyanin's color active site without polymerizing the phycocyanin structure. The color change on phycocyanin became faint because of two possibilities: (i) polymerizing proteins by sugars or (ii) the phycocyanin protein structure broke. Phycocyanin with glucose addition exhibited mid-level antioxidant activity with $IC_{50}$ value of 100.98, which was 18.47% lower than phycocyanin without any sugar addition. This difference was due to the polymerization of protein phycocyanin by glucose and inhibition of antioxidant degradation during heating. Therefore, glucose is a promising stabilizer for phycoyanin as a source of antioxidants and natural colorant in the food industry.

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