Food Research 8 (2) : 248 - 256 (April 2024)
Journal homepage: https://www.myfoodresearch.com

FOOD RESEARCH

RESEARCH PAPER

Anthocyanins and phenolics content of Thai black soybean extracts and their prevention against myoglobin autoxidation

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Article history:
Received: 26 March 2022
Received in revised form: 2 May 2022
Accepted: 7 October 2022
Available Online: 3 April 2024

Keywords:
Black soybean,
Phenolics,
Anthocyanin,
Antioxidant activity,
Autoxidation kinetics,
Protein oxidation

DOI:
https://doi.org/10.26656/fr.2017.8(2).130

Abstract

Anthocyanin and phenolics found in black soybean are known as active antioxidants and have been linked to a variety of health benefits. The purpose of this study was to determine total monomeric anthocyanins (TMA), total phenolics (TP), and the retardation ability of protein autoxidation of an ethanolic extract of black soybeans planted in Thailand. We investigated the optimal conditions for extraction conditions. The ultrasonic-assisted approach successfully extracted both TMA and TP using 85% v/v ethanol as the solvent. The 15-min extraction time and solvent-to-sample ratio of 20:1 (v/w), at 30°C resulted in the highest concentration of TMA at 3.232±0.04 mg cyanidine-3-O-glucoside equivalent per gram dry weight whereas the longer time and higher temperature resulted in significantly decreased TMA content (P<0.05). The maximum TP concentration of 119.42±0.42 mg gallic acid equivalent per gram dry weight was obtained by using a method identical to that used to extract TMA, except that of the longer time, increased solvent consumption and temperature. The preventive effect of black soybean extracts on oxymyoglobin (oxyMb) autoxidation was investigated. The pseudo first-order rate constant of oxyMb oxidation is decreased in the presence of the extracts in comparison to oxyMb alone. Additionally, oxyMb's half -life was increased from 0.57 to 1.46 hrs in the presence of TMA-rich extracts and from 0.57 to 2.51 hrs in the presence of the highest concentration of TP extracts when compared to cyanidine-3-O-glucoside and gallic acid, respectively. The study revealed that the black soybean extract could stabilize the bound oxygen in the oxyMb lead to slow down autoxidation of the protein consumption of foods or products derived from black soybeans may help to reduce cellular oxidative stress and the risk of developing various diseases as the study's findings indicate.

1. Introduction

Along with the understanding of reactive oxygen species (ROS), It is important to understand how an imbalance between the quantity of free radicals and antioxidant defence enzymes in living cells can result in cell death, tissue injury and oxidative stress (Ebadi, 2001; Mohammed and Ibrahim, 2004). Other reactive species such as nitrogen and iron species may contribute to the production of ROS and oxidative stress (Silava and Coutinho, 2010; Weidinger and Kozlve, 2015). Free radicals are produced both endogenously and as a result of exposure to environmental oxidants (Kumar, 2011). The oxidative stress phenomena are related to a wide variety of human diseases such as vascular disease, skeletal muscle disorders, diabetes and cancer (Papaharalambus and Griendling, 2007; Kesarwala *et al*., 2016). The formation of oxygen free radicals and other ROS can be regulated only by the cellular antioxidant defence system which prevents endogenous free radical formation and by the consumption of synthetic antioxidants or food-derived antioxidants which increase ROS neutralization (Young and Woodside 2001; Husain and Kumar, 2012). As a result, increasing dietary consumption of antioxidant-rich foods may help reduce the risk of tissue injury or cancer (Cooper *et al*., 2005).

In numerous countries, black soybean (*Glycine max* (L.) merr) has been used as an ingredient in dietary foods, beverages and medicines (Galvano *et a*l., 2004; Lee *et al*., 2016). As a nutritional food, black soybeans contain important compounds with functional and antioxidant properties that promote human health and help prevent oxidative stress (Jhan *et al*., 2016). These include dietary fibers, essential amino acids, phenolic acids, flavones and anthocyanin in the seed coat (Xu and Chang 2008; Ciabotti *et al*., 2016; Sumardi *et al*., 2017; Genesan and Xu; 2017; Asan *et al*., 2019; Juliano *et al*., 2020). Anthocyanins are the distinctive and abundant compounds found in the seed coat of black soybeans which differentiate them from normal soybeans (Genesan and Xu, 2017; Koh *et al*., 2014). The principal anthocyanins are delphinidin-3-O-β-D-glucoside, cyanidin-3-O-β-D-glucoside and petunidin 3-O-β-D-glucoside (Koh *et al*., 2014). Previous research studies have demonstrated that anthocyanin derived from black soybeans has been shown to have anti-inflammatory (Miguel, 2011; Kim *et al*., 2015), anti-carcinogenic, chemoprotective properties (Okazaki *et al*., 2010), anti-fungal activity and also apoptosis induction activity (Kim *et al*., 2011). Black soybeans have a considerable inhibitory effect on lipoprotein oxidation (Takahashi *et al*., 2005) as well as DPPH radical scavenging

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eISSN: 2550-2166 /

activity, ferric reducing antioxidant power (FRAP) and oxygen radical absorbance capacity (ORAC) (Juan and Chou, 2010; Dajanta *et al*., 2013; Asan *et al*., 2019; Juliano *et al*., 2020). Consumption of black soybeans may help reduce the risk of developing a variety of diseases, including heart disease and breast cancer (Do *et al*., 2007). The physiological antioxidant activity of black soybean is due to anthocyanin pigments in the seed coat and other major phenolic compositions (Rice-Evans *et al*., 1996; Ha *et al*., 2009).

Myoglobin is required for oxygen transport and storage in the muscle cells, which contributes to oxidative stress reactions. The reaction is uncontrolled and takes place over time. It results in the production and release of reactive oxygen species, free radicals, lipid alkyl radradicals well as the formation of cytotoxic derivatives (Reeder and Wilson 2005). According to Chanthai *et al*. (2015), phenolic compounds in chilli extracts may retard myoglobin autooxidation. Thus, the phenolic and anthocyanin in black soybeans might be able to delay protein autooxidation, thereby contributing to disease prevention.

Nevertheless, no studies have been published on the antioxidant activity of black soybeans under physiological conditions using protein oxidation reactions. As a result of black soybeans' beneficial health effects, this study aimed to extract TMA and TP from whole black soybeans grown in Thailand. Additionally, the concentration of each compound in the black soybean extract was determined. Under physiological conditions, the synergistic antioxidant activity of black soybean extracts was evaluated in terms of its resistance to an autoxidation reaction of myoglobin protein.

2. Materials and methods

2.1 Preparation of black soybean sample

Black soybean seeds were purchased from the Royal Project Foundation (Thailand). The seeds were cleaned in water and dried for 24 hrs at a temperature of 50°C. The dried black soybean seeds were ground to a fine powder using a blender and then sealed in zip-lock bags and stored in a desiccator until further use.

2.2 Magnetic stirring extraction

Black soybean powder 2.50±0.01 g was mixed with 85% v/v ethanol as extracting solvent and stirred using a magnetic stirrer under various conditions, including a solvent-sample ratio of 20:1 and 40:1 w/v, the extraction temperature of 30 and 40°C and extraction times ranging from 15 mins to 2 hrs. The crude extract was filtered through filter paper and centrifuged at 2000×g for 10 mins. The supernatant was collected and then concentrated using a rotary evaporator at 50°C in a vacuum. Concentrated crude extracts were kept at a temperature of 4°C until further analysis.

2.3 Ultrasonic assisted extraction

Ultrasonic assisted extraction (UAE) was performed in a temperature-controlled ultrasonic cleaner (1825D; Crest-ultrasonics, USA). The black soybean powder was mixed with extracting solvent in Erlenmeyer flask and then immersed in an ultrasonic bath with water at 200W ultrasonic power. The extraction temperature, solvent-sample ratio and extraction time (0.25-2 hrs) were studied, as well as in a conventional magnetic stirring method. The crude extract obtained was kept at 4°C until further use. The crude extract obtained was quantified in triplicate for its TMA and TP contents. The antioxidant properties of the crude extract were determined by the retardation of the rate of the oxidation reaction.

2.4 Determination of total monomeric anthocyanins

The pH differential method was used to determine the total monomeric anthocyanins (TMA) content (Giusti and Wrolstad 2001). In brief, the methanolic crude extracts of black soybean were spectrophotometrically measured at 520 and 700 nm after being diluted with potassium chloride solution (pH 1.0) and sodium acetate solution (pH 4.5). The TMA concentration was determined using the following equation and expressed as mg of cyanidine-3-O-glucoside equivalent per gram dry weight (mg cy-3-gluE/ gDW).

$$\text{TMA content (mg/L)} = \frac{A \times MW \times DF \times 1000}{\varepsilon \times 1}$$

Where A is a difference in absorbance at various pH values = $(A_{510}\text{-}\ A_{700})_{pH1.0}$ - $(A_{510}\text{-}\ A_{700})_{pH4.5}$; MW (molecular weight) = 449.2 g.mol^{-1} for cy-3-glu; DF = dilution factor; l = pathlength in cm; e (molar extinction coefficient) = 26,900 L cm^{-1} mol^{-1} for cy-3-glu; 1000 = factor for conversion from g to mg.

2.5 Determination of total phenolics

The Folin-Ciocalteu technique was used to measure the total phenolics (TP) content in methanolic extracts of black soybean (Yawadio *et al*., 2007). After diluting the methanolic extracts with distilled water, Na_2CO_3 (7 %v/v) and the Folin-Ciocalteu re-agent were added. The mixture was incubated at room temperature for 1.5 hrs in the dark and then spectrophotometrically measured at 760 nm. The measurement of TP content in extracts was conducted in triplicate, and the TP concentration was determined using a gallic acid calibration curve and expressed as mg of gallic acid equivalent per gram of dry weight (mg GAE/gDW).

2.6 Determination of the prevention against myoglobin autoxidation

The antioxidation activity of methanolic crude extracts of black soybean was determined by their ability to inhibit protein oxidation. Myoglobin from equine skeletal muscle was used as a model for the autooxidation reaction in muscle (Sigma-Aldrich; Germany). The first-order kinetic analysis of myoglobin autoxidation in the presence of the extracts was performed under physiological conditions using the Chanthai *et al*. (2015) method. The antioxidation activity test was done as follows, the 300 mg/L of oxyMb and the 10 mg/L of crude extracts were mixed and measured for the absorbance at 581 nm at 37°C at the time interval of 5 min to 2 hrs. The absorbance data were analyzed by plotting $[MbO_2]_t/[MbO_2]_0$ versus time, where $[MbO_2]_t/[MbO_2]_0$ represents the ratio of the MbO_2 concentration after time *t* to that at time *t* = 0. The slope of each straight line was used to calculate the observed first-order rate constant, k_{obs}. Each experiment was also observed to have an average half-time ($t_{1/2}$).

2.7 Data analysis

The total monomeric anthocyanins, phenolics, and antioxidant activity of black soybean seeds were determined in triplicate. The information was presented as a mean standard deviation (n = 3). The data was analyzed using Duncan's multiple comparison test (P<0.05).

3. Results and discussion

Due to the fact that the analyte molecules contain one or more hydroxyl groups, which are easily soluble in a polar solvent like ethanol, the present study extracted TMA and TP from black soybean seeds with the help of the solvent ethanol (Yusnawan, 2018). The effects of extraction strategies, the solvent volume to mass sample ratio, the extraction temperature, and the extraction time on the extraction yield of both compounds in order to maximize the extraction yield were investigated. Table 1 shows the concentrations of TMA and TP in black soybean ethanolic extracts obtained using a variety of different extraction parameters.

3.1 Effect of various extraction techniques

All MSE and UAE experiments were performed at a temperature of 30°C in this investigation. The results indicate that using the UAE method resulted in a significantly (P<0.05) higher yield of TMA and TP (Figure 1). The significantly higher concentrations of TMA and TP in the UAE process compared to the MSE technique under the same conditions could be a result of the ultrasonic wave bursting the plant cells, allowing compounds to easily diffuse into the solvent (Ghafoor *et al*., 2009; Li *et al*., 2016). By and large, the longer the extraction time, the greater the amount of analyte recovered due to increased mass transfer and surface contact between the analyte and the extractant (Lapornik *et al*., 2005; Jing and Giusti 2007). Additionally, the effect of extraction time on the yields of TMA and TP across a range of time intervals (Figure 1 and Table 1) was investigated. The results indicate that there is no statistically significant difference in TMA and TP concentrations across a specified time range in both the MSE and UAE experimental runs (P<0.05). Increased extraction time has no effect on extraction yields because the analyte is distributed evenly between the solvent and solid materials and the extraction conditions are mild at a low temperature of 30°C (Zhang *et al*., 2018). As was the case with the 30°C extraction temperature and a solvent-sample ratio of 20:1 (v/w) (Table 1), these parameters affecting the extraction

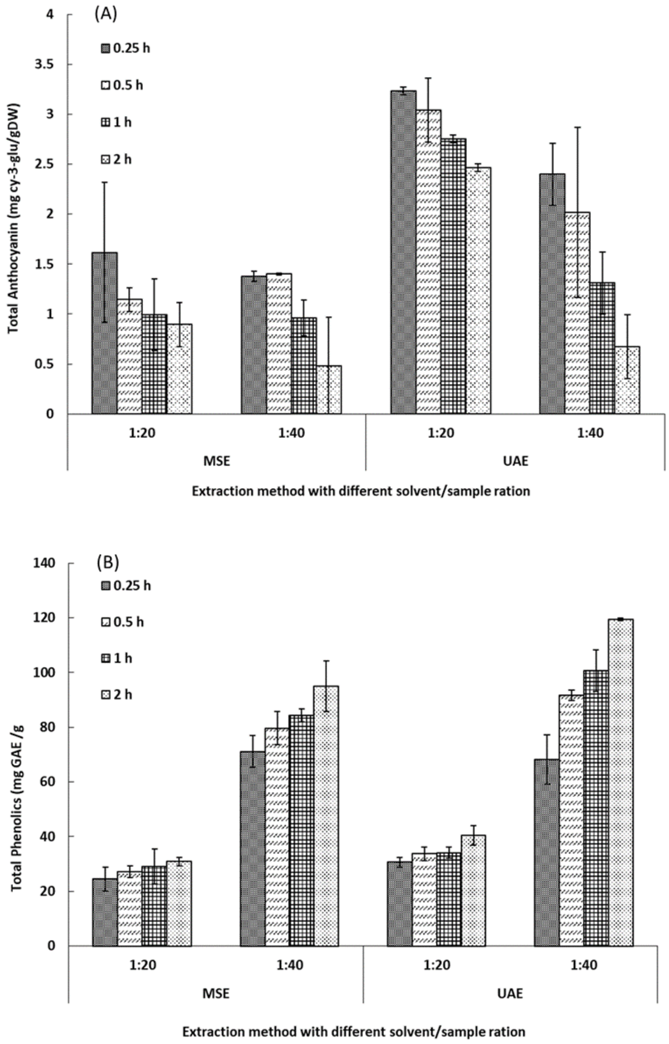

Figure 1. Effect of the extraction technique and solvent volume to the mass of the sample on TMA extraction yield (A) and TP extraction yield (B). Error bar indicates SD.

Table 1. The content of TMA (mg cy-3-gluE/gDW) and TP (mg GAE/gDW) obtained from an ethanolic extract of black soybean under MSE and UAE experiment at 30°C for a solvent-sample ratio of 20:1 (v/w) and 40:1 (v/w) respectively.

Solvent sample ratio	Extraction time (h)	The analyte contents in ethanolic extracts on black soybean			
		MSE		UAE	
		TMA	TP	TMA	TP
20:01	0.25	1.616±0.70[a]	24.44±4.32[a]	3.232±0.04[a]	30.69±1.76[a]
	0.5	1.144±0.12[abc]	27.12±2.12[a]	3.040±0.32[a]	33.72±2.51[ab]
	1	0.992±0.36[abc]	29.10±6.45[a]	2.752±0.04[ab]	34.07±2.02[ab]
	2	0.895±0.22[ab]	30.84±1.43[a]	2.464±0.04[a]	40.46±3.47[a]
40:01:00	0.25	1.376±0.05[ab]	71.08±5.79[b]	2.400±0.31[ab]	68.20±9.08[b]
	0.5	1.400±0.01[ab]	79.65±6.08[bc]	2.016±0.85[bc]	91.70±1.97[c]
	1	0.962±0.18[abc]	84.31±2.31[cd]	1.312±0.31[cd]	100.67±7.53[c]
	2	0.48±0.49[c]	94.96±9.16[d]	0.672±0.32[d]	119.42±0.42[d]

Values are presented as mean±SD. Values with different superscripts within the same column are statistically significantly different (p<0.05).

https://doi.org/10.26656/fr.2017.8(2).130

yield were further evaluated for both the MSE and UAE methods in order to obtain a higher concentration of target compounds.

3.2 The effect of the solvent volume on the mass of the sample

The volume of the solvent has a significant effect on the yields (Bąkowska *et al*., 2003). TMA and TP were extracted from black soybeans using a solvent volume to sample mass ratio of 20:1 and 40:1 (v/w) respectively. All experiments were carried out at 30°C over a four-point time period. The results in Figure 1 and Table 1 demonstrated that 40:1 (v/w) was significantly more efficient (P<0.05) at extracting a greater amount of TMA and TP than 20:1 (v/w) using both the MSE and UAE methods over a longer extraction time (1-2 hrs). The results are explained by the increased mass transfer from intracellular compounds into the solvent as a result of the ultrasound wave increasing the solvent penetration and diffusion ability and the contact area between the solvent and solid samples. However, no significant difference in TP content (P<0.05) was observed between the MSE and UAE methods using a solvent/sample ratio of 40:1 (v/w). However, no significant difference in TP content (P<0.05) was observed between the MSE and UAE methods using a solvent/sample ratio of 40:1 (v/w).

TMA is unstable and easily decomposed under light and temperature, the high volume of solvent had an impact on TMA extraction, particularly for the UAE method. TMA yield did not change significantly (P<0.05) from 0.25 to 0.5 hrs and decreased significantly (P<0.05) from 0.5 to 2 hrs in both methods of extraction. It is possible that a large volume of the solvent resulted in a short time for the solvent and TMA to reach equilibrium, ranging from 0.25 to 0.5 hrs. TMA content declines in both methods when a 40:1 v/w solvent sample ratio is combined with a long extraction time because the high volume of the solvent absorbs more heat energy, resulting in TMA (Pala *et al*., 2016). Furthermore, Le *et al*. (2019) found that using a higher liquid ratio for anthocyanin extraction resulted in lower anthocyanin content due to anthocyanin degradation caused by prolonged light exposure.

As previously reported, the UAE is a possible extraction method that can reduce both operating costs and lead time (Idris and Sulaiman 2017). This technique's increased extraction yield of bioactive compounds was also anticipated. Interestingly, TMA content determination using the UAE method with solvent/sample ratio of 20:1 and 40:1 (v/w) differed significantly over time (P<0.05). When compared to the 20:1 v/w ratio, using a high volume extractant to sample mass ratio of 40:1 v/w for the UAE technique results in a significantly decreased TMA yield of around 26% and up to 72% for 2 hrs. Whereas the TMA content determined using the MSE technique revealed a range of yields between 20:1 and 40:1 (v/w) at a concentration of 15 to 46%. Because ultrasonic irradiation may cause damage to heat-sensitive compounds and a larger volume of solvent absorbs a greater amount of heat energy, the TMA yield decreased significantly over time due to anthocyanin decomposition caused by ultrasonic irradiation (Idris and Sulaiman 2017; Vaga-Arrove *et al*., 2017).

3.3 Effect of extraction temperature

Due to the fact that temperature has a significant effect on the yield of TMA and TP in that it increases the solubility and mass transfer rate of compounds (Moldovan *et al*., 2016; Vaga -Arrove *et al*., 2017) the extraction temperature for the UAE process was increased to 40°C.

As illustrated in Figure 2B, increasing the extraction temperature resulted in an increase in TP extraction yields across all time intervals (Cacace and Mazza 2003). The TP content of crude extract increased significantly by approximately 40–70% when compared to the yield obtained with 30°C extractions, whereas the TMA content was significantly lower content than TP.

Comparing these results to previous reports, the TP contents were found to be lower than that found in whole black soybeans grown in China and Taiwan (Xu *et al*., 2007; Xu and Sam 2008). The differences in TMA and TP content between these and previous studies could be attributed to differences in the extraction method and solvent used, as well as the source of samples from different environments (Shih *et al*., 2002; Xu and Sam, 2008).

The highest TMA content of black soybean grown in Thailand was obtained using the UAE method which was used at 30°C with 40:1 solvent volume to sample mass ratio (Figure

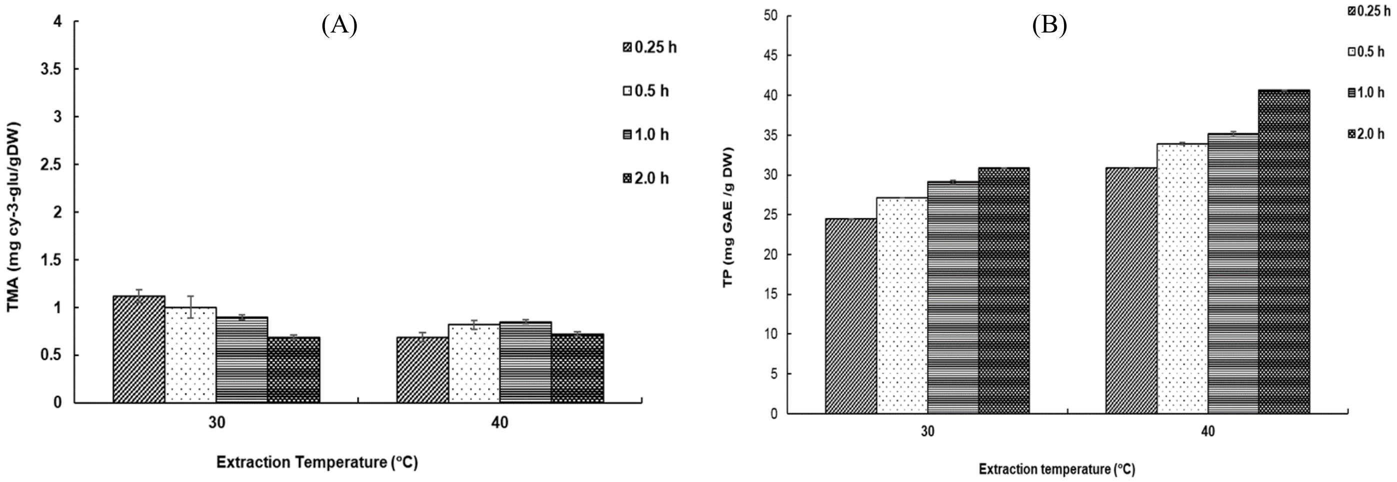

Figure 2. Effect of the extraction temperatures at different extraction time on TMA extraction yield (A) and TP extraction yield (B) obtained from UAE extraction of black soybean using methanol as the extractant. Error bar indicates SD.

https://doi.org/10.26656/fr.2017.8(2).130

2A). This TMA content value was significantly higher than that of several black soybean cultivars grown in China, Taiwan and Korea (Shih *et al*., 2002; Xu *et al*., 2007; Xu and Sam, 2008). The UAE method extracted most of TP with a volume to-sample mass ratio of 40:1 solvent and a longer extraction time (2 hrs).

Although some reports indicate that prolonged ultrasonic treatments, even when accompanied by a cooling system to maintain a constant temperature, alter the bioactive compounds which is most likely due to the competition between extraction and degradation (Saleh *et al*., 2016). As a result, short sonication times are preferable to minimize this effect while preserving the green extraction method (Achat *et al*., 2012; Jacotet-Navarro *et al*., 2015; Saleh *et al*., 2016; Ahmed *et al*., 2019). To minimize processing time and production costs, the shortest extraction time was suggested. The prolonged extraction could possibly increase the risk of TMA decomposition and TP oxidation.

3.4 The yields of total monomeric anthocyanins and total phenolics from black soybean seeds

The TMA and TP content of the ethanolic extract of black soybeans were compared (Table 1). For all extraction experiments, black soybean crude extract contains more TP than TMA. The maximum TMA concentration (3.232±0.04 mg cy-3-gluE/gDW) was obtained by utilizing the UAE method at a temperature of 30°C for 15 mins with a solvent-sample ratio of 20:1 (v/w).

The highest TP yield was 119.42±0.42 mg GAE/gDW when the UAE technique was used at 40°C for 2 hrs with a solvent-sample ratio of 20:1 (v/w). The results indicated that the TMA compounds in Thai black soybeans are less resistant to all the extraction conditions than those for TP, resulting in TMA degradation. It should be noted that the ethanolic extract of whole black soybean seeds grown in Thailand contains a greater concentration of TP than TMA. According to the extraction conditions used in this study, the extracts may contain a mixture of TMA and TP compounds. The solution of ethanolic extract of each of the highest yields of TMA and TP was chosen for antioxidation activity studies.

3.5 A kinetic study of the prevention of black soybean extracts against myoglobin oxidation

We determined the kinetics using oxymyoglobin (oxyMb) isolated from equine skeletal muscle and stored at 37°C in a pH 7.4 buffer. The synergistic inhibitory effect of black soybean extracts on oxyMb oxidation was determined by monitoring the change in absorbance at 580 nm, the isosbestic point, over a time interval of 5 to 25 mins (Chanthai *et al*., 2015; Pitchuanchom *et al*., 2021).

Since the antioxidants used are fixed concentration or constant, the rate equation would be Rate = k[MbO_2]. The slope of the linear plot of ln $[MbO_2]_t/[MbO_2]_0$ versus time (Figure 3) was used to calculate the pseudo-first-order rate constant (k_{obs}) for oxyMb in the presence of extracts and synthetic antioxidants at equivalent concentrations (Table 2).

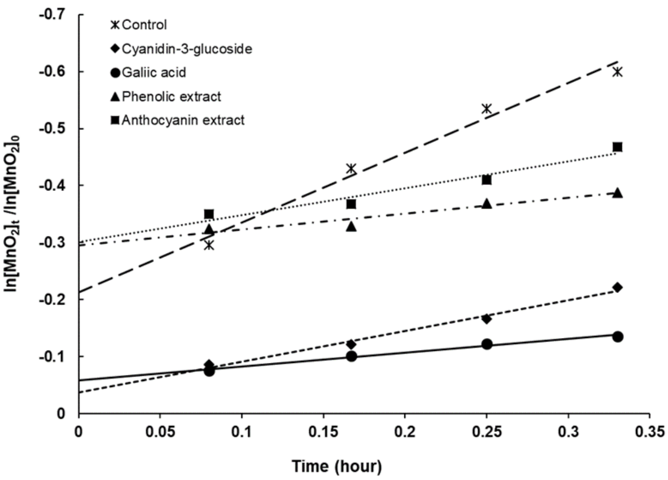

Figure 3. The first order plot for the autoxidation of oxymyoglobin in pH 7.4 buffer at 37°C with and without the extracts of black soybean and the antioxidant compound.

As shown in Table 2, the k_{obs} value for oxyMb with the presence of antioxidants was approximately 2 to 5 times lower than the value for oxyMb in the absence of antioxidants, indicating that the compounds slowed the autoxidation rate reaction. The rate constant for oxyMb oxidation with gallic acid was found to be lower, decreasing by 80.1% when compared to the rate constant for oxidizing oxyMb without the compound. Additionally, it was faster than oxyMb oxidation as well as the oxidation of oxyMb in the presence of cyanidine -3-glucoside.

This phenomenon is explained by the smaller structure of gallic acid being compared to cyanidine-3-glucoside. Mb, a heme-containing protein, is involved in the binding of oxygen to heme iron. However molecular oxygen is an oxidizing agent that can promote the oxidation of iron. A reactive oxygen species is the product of this autooxidation. Gallic acid was able to approach the heme pocket of Mb, neutralizing the oxygen atom on heme iron for delaying the formation of the molecular oxygen superoxide radical, resulting on gallic acid being more effective at slowing the initiation of the free radical chain reaction than cyanidine-3-glucoside (Tsuda *et al*., 1998; Chanthai *et al*., 2015; Pitchuanchom *et al*., 2021). With the addition of black soybean extracts, the decrease in k_{obs}

Table 2. First kinetic of oxyMb containing the black soybean extracts, anthocyanin and phenolics at 37°C.

Compounds	Rate constant (k_{obs})	$t_{1/2}$ (h)
Oxymyoglobin only (control)	1.224[a]	0.57[a]
Oxymyoglobin + Cyanidin-3-glucoside	0.541[b]	1.28[a]
Oxymyoglobin + Gallic acid	0.244[b]	2.84[a]
Oxymyoglobin + Anthocyanin extract	0.473[b]	2.51[a]
Oxymyoglobin + Phenolics extract	0.277[b]	1.46[a]

Values with different superscripts within the same column are statistically significantly different ($p<0.05$).

https://doi.org/10.26656/fr.2017.8(2).130

value was consistent with the oxyMb containing standard antioxidants, with a reduction of 77.4% for the TP-rich extract and 61.4% for the extract with the highest TMA content when compared to the pure oxyMb solution.

The mechanism of Mb's autoxidation is complex and is related to its physiological functions. The Mb oxidation reaction could occur via superoxide dissociation via an electron transfer process at the active site of oxyMb or via an oxidative bimolecular reaction with deoxyMb (Hegetschweiler *et al*., 1987; Wazawa *et al*., 1992). The following processes can be used to explain the autoxidation and can be used to explain the autoxidation of oxy Mb (Uri, 1961).

$$X\text{-}Fe^{II}O_2 \rightleftharpoons X\text{-}Fe^{II} + O_2$$

$$X\text{-}Fe^{II} + O_2 \rightleftharpoons X\text{-}Fe^{III} + O_2^{-}$$

The antioxidant compounds that stabilize the ferrous oxygen complex or donate the hydrogen atom or antioxidant compound may act as radical scavengers, resulting in a decrease in the rate of autoxidation. The kinetic analysis revealed that the spontaneous oxidation of oxyMb occurs in a pseudo-first-order reaction at constant oxygen concentration, pH and temperature (Chanthai *et al*., 2015; Pitchuanchom *et al*., 2021).

Additionally, the half-life ($t_{1/2}$) of the oxyMb containing the extracts was determined and compared to that of standard antioxidants. Because the half-life of a first-order-process is constant, the half-life is related to the rate constant of myoglobin autooxidation reaction with $t_{1/2}$ = ln2/k. The calculated $t_{1/2}$ values of oxyMb containing gallic acid and cyanidine-3-glucoside were increased from 0.57 to 2.84 and 1.28 hrs, respectively. Interestingly, black soybean extracts have the same half-life as antioxidant compounds, delaying myoglobin oxidation by 0.57 to 1.46 hrs and 2.51 hrs respectively. The greater improvement in $t_{1/2}$ suggests that the extracts of black soybeans could stabilize the bound dioxygen in oxyMb and scavenge superoxide anions or hydroxyl radicals due to their phenolic content (Chanthai *et al*., 2015; Pitchuanchom *et al*., 2021).

As a result, the black soybean extracts demonstrated a synergic effect of antioxidant activity when combined with TMA and TP, as the extracts were able to resist oxyMb oxidation by retaining the bound O_2 in the protein via hydrogen bonding, scavenging superoxide anion or free radical intermediates by contributing a hydrogen atom or an electron. Thus, black soybeans are a functional food that is used in healthy recipes, beverages and food ingredients for daily consumption to help prevent oxidative damage to the cells.

4. Conclusion

The study discusses the extraction of TMA and TP from whole black soybeans and their anti-autoxidation properties. The ultrasonic-assisted method yielded the highest amount of TMA and TP. The optimal conditions for obtaining the highest yield of TMA were a temperature and time of 30°C and 15 mins, respectively, with a solvent-to-sample ratio of 20:1. The highest yield of TP was obtained at 40°C for 2 hrs and a solvent-to-sample ratio of 40:1. The preventive effect of black soybean extract on oxyMb autooxidation demonstrated that both the TMA- and TP-rich extracts could slow the rate of oxyMb autooxidation by stabilizing the ferrous oxygen complex in heme pocket of Mb. As a result, black soybeans are a good source of antioxidants, which promote health and protect against diseases such as heart disease and cancer. However, the molecular mechanism of anthocyanin and phenolics in black soybean on the prevention of protein oxidation is necessary to study clearly and it will be further explored in future work.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

The authors wish to express their appreciation to Rajamangala University of Technology Isan, Nakhon Ratchasima for their kind assistance and support. Additionally, we would like to express our gratitude to our colleagues, whose vision and expertise aided the research.

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