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The synergic retardation effects of both total capsaicinoids and phenolic extracts from some hot chilli peppers on the autooxidation kinetics of oxymyoglobin

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ABSTRACT

Both of total capsaicinoids and phenolic compounds were extracted from hot chilli pepper by magnetic stirring method using 90% methanol (at 60℃ for 2 h) and 90% acidified (0.05% HCl) methanol (at ambient temperature) as the extraction solvents, respectively. The capsaicinoids contents in the extract of eight chilli samples were found in the range of 63.76-106.6 mg/g DW with some residual phenolics containing of 16.56-21.91 mg/g DW. For total phenolics extraction, the phenolic contents were almost found in the range of 27.99-39.03 mg/g DW, but the ranges of capsaisinoids still resembled (59.02-113.8 mg/gDW). Thus, these extracts gave rather fixed ratios of both total phenolic antioxidants. For application, the autooxidation kinetics of oxymyoglobin (oxyMb) in the presence of both capsaicinoids and phenolic compounds in the extracts was monitored at 581 nm for 2 h using freshly prepared oxyMb solution. The results showed that the crude extracts could retard effectively the autooxidation reaction of oxyMb. Their observed first-order rate constant (k_{obs}) ratios in the presence of the extracts for both capsaicinoids and phenolic compounds were found in the same range between 0.38-0.50 h^{-1} and 0.29-0.48 h^{-1}, respectively, with respect to those of control. It is, therefore, implied that the phenolic antioxidants in hot chilli can keep serving this stabilized function on the oxygen storage of the protein in muscle.*

Key words: myoglobin, autooxidation, capsaicinoids, phenolic compounds, chilli pepper

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INTRODUCTION

Hot chilli peppers are known to be good sources of phytochemical compounds including vitamin C, E and A, alkaloids, flavonoids, and carotenoids [1]. Capsaicinoids, the pungent alkaloids, are the main active component of chilli peppers. The naturally occurring capsaicinoids present in hot chilli pepper are capsaicin and other structurally related compounds, namely dihydrocapsaicin, nordihydrocapsaicin, homocapsaicin and homodihydrocapsaicin [2]. Capsaicin and dihydrocapsaicin are responsible for approximately 80-90% of the total capsaicinoids present and contribute greatly to overall pungency [3]. The capsaicinoids accumulate in pericarp and placenta of chilli pepper and their concentrations vary, depending on hotness of species varieties, growing conditions and the harvested time. Capsaicinoids have many useful properties, especially antioxidant properties. Many reports reveal substantial antioxidant activity of chilli pepper extracts of capsaicinoids [4-7]. Phenolic compounds found in hot chilli pepper include both simple phenols and flavonoids [8]. Phenolic compounds arethe common antioxidants which can retard or inhibit lipid oxidationby acting scavengers and can protect against propagation of the oxidative chain like capsaicinoids [9,10]. Based on the antioxidant properties of capsaicinoids and phenolics, these compounds were extracted from various kinds of hot chilli pepper samples and their concentrations were evaluated. In addition, this

study was aimed to investigate the influence of both extracts from the hot chilli pepper samples on the autooxidation rate of oxymyoglobin.

MATERIALS AND METHODS

2.1 Chemicals

All the reagents usedwere analytical reagent (AR) grade including dihydrocapsaicin, gallic acid monohydrate, sodium dithionite, Tris-HCl, sodium carbonate and horse heart myoglobin (Sigma-Aldrich, Germany), Folin-Ciocarteu phenol reagent, methanol, hydrochloric acid (Carlo Erba, Italy).

2.2 Apparatus

Capsaicinoids and phenolics contents in hot chilli pepper extracts and their antioxidant activities were determined using Diode Array UV-Visible spectrophotometer (Agilent 8453,USA). Centrifuge (Hitachi EBA 20, Germany), rotary evaporator (Model B-490 Buchi, Switzerland) and magnetic stirrer (Iowa 52001, Germany) were used for sample preparation.

2.3 Chilli pepper fruits

Eight samples (green, orange and red pepper fruits as labeled sample A-H as shown in Figure 1) used in this study were randomly purchased from a fresh market in Khon Kaen, Thailand.

Figure (1): Pictures of different kinds of hot chilli pepper samples used in this study

2.4 Analysis of total capsaicinoids in the chilli extract

Extraction of capsaicinoids in hot chilli pepper samples were carried out following by Juagsamut et al. (2012) [3]. Briefly, all chilli pepper fruits were dried at 60°C for 4 days and ground to a fine powder using a blender (Philips, Netherlands). The powder was shifted through a 500 µm mesh laboratory sieve (Endecotts Limited, England). Two grams of the ground samples were weighed into a 15 mL centrifuge tube, and 20 mL of 90% methanol was added. The extraction was done by magnetic stirring method at 60°C for 2 h. The extract was centrifuged at 5000 rpm for 10 min and then filtered through a Whatman No.42 filter paper. The solvent in the extract was evaporated to dryness using a rotary evaporator and the residue was dissolved with methanol to make a final volume of 5 mL. Stock standard solution of dihydrocapsaicin (1000 mg/L) was prepared by dissolving dihydrocapsaicin in methanol. Working solutions were prepared by dilution of the stock solution with Tris-HCl buffer solution pH 7.2. The calibration curve of capsaicinoids was obtained in the range of 10-60 mg/L. The absorbance of the solutions was measured at 279 nm [3,4] and total capsaicinoids was determined directly from the calibration curve.

2.5 Analysis of total phenolic compounds in the chilli extract

The total phenolics extract was obtained using the same extraction method as mentioned above [10] but the extraction was carried out at room temperature by using methanol: 0.05% HCl (90:10) as an extraction solvent. The extract was centrifuged, filtered, evaporated and the residue was dissolved with the extraction solvent to make a final volume of 20 mL. The Folin-ciocalteau method as previously described using gallic acid as a standard compound was used to estimate total phenolics content [12]. Stock standard solution (1000 mg/L) of gallic acid was prepared by accurately dissolving with methanol and kept in a dark vial and stored at 4°C prior to use. Working standard solution was prepared by diluting the stock solution. The calibration curve in the range of 1.0-4.5 mg/L was generated by using gallic acid solution with deionized water, Na_2CO_3 and Folin-Ciocalteau reagent. After incubated at room temperature for 90 min, the absorbance of the blue color solution was measured at 745 nm. The total phenolics content in the extract was, therefore, determined from the calibration curve.

2.6 Autooxidation kinetics of oxymyoglobin

Myoglobin, an oxygen-binding protein, was used as an autooxidation reaction model for studying the antioxidant properties of hot chilli extract. The aim of this study was to investigate the effect of dihydrocapsaicin, gallic acid and hot chilli extracts (total capsaicinoids and phenolic compounds) on retardation of the autooxidation reaction of horse heart oxymyoglobin. Myoglobin in ferrous form can bind oxygen molecule reversibly as shown in Figure 2 [13].

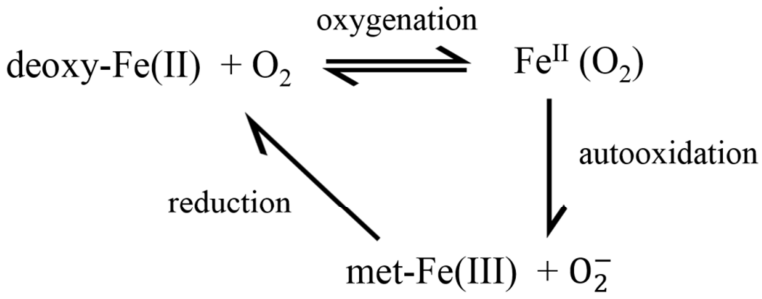

Figure (2): some functional complexes of myoglobin with oxygen molecule [13]

The mechanism for the autooxidation reaction of oxymyoglobin (oxy-Fe^{2+}) to metmyoglobin (met-Fe^{3+}) can be explained by the Fenton reaction as follows [14,15]:

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{-\cdot}$$
$$2O_2^{-\cdot} + 2H^+ \rightarrow H_2O_2 + O_2$$
$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\cdot}$$

Since temperature is one of important factors affecting the autooxidation kinetics of oxymyoglobin (oxyMb), this reaction rate becomesfaster as the temperature increases. To obtain the optimum temperature for studying the effect of an antioxidant on retardation of the autooxidation of oxyMb, various incubation temperatures were also determined bymonitoring the changes inan oxidation state of iron from (oxy-Fe^{2+}) to (met-Fe^{3+}). The stock solution of horse heart Mb (about 6.2 g/L) was prepared by dissolving the protein with Tris-HCl buffer pH 7.2. An aliquot of the protein solution was converted to oxyform by addition of 10 µL of fresh sodium dithionite solution (100 mg/L) and makes a volume to 4 mL to obtain the final concentration of oxyMb of 300 mg/L. The solution was gently agitated with urging of oxygen gas for a few min until the bright red colored appeared. The oxyMb solution was

quickly transferred to a quartz cell and placed in thermostat connecting with UV-Visible spectrophotometer. The changes in the absorbance in the region of 450-650 nm were recorded at an interval of 5 min for 2 h by varying the incubation temperatures (25, 30, 33, 35, 37, 40 and 50°C). The solution was subject to the absorbance measurement at 581 nm. The absorbance data were analyzed by plotting in terms of $[MbO_2]_t/[MbO_2]_0$ versus time, where the ratio of MbO_2 concentration after time t to that at an initial time $t = 0$). From the slope of each straight line, the observed first-order rate constant, k_{obs} in h^{-1}, was determined. According to the autooxidation reaction of oxyMb, the superoxide radical ($O_2^{\cdot-}$) and hydroxyl radical ($OH^{\cdot}$) which produced by the reaction of ferrous iron in the heme and hydrogen peroxide (H_2O_2) are concerned. The present work was aimed to study the effect of antioxidants including capsaicinoids and phenolic compounds on the autooxidation rate of oxyMb because these compounds could retard the oxidation by scavenging free radical intermediate ($O_2^{\cdot-}$, $OH^{\cdot}$) by donating a hydrogen atom as follows:

$$ROO^{\cdot} + AH \rightarrow ROOH + A^{\cdot}$$
$$RO^{\cdot} + AH \rightarrow ROH + A^{\cdot}$$

Where $ROO^{\cdot}$, $RO^{\cdot}$and AH represents $O_2^{\cdot-}$, $OH^{\cdot}$and antioxidant, respectively. The oxyMb was prepared as described above and followed by the addition of each of various amounts of dihydrocapsaicin or gallic acid. The final concentrations of both dihydrocapsaicin and gallic acid were 5, 10, 20, 30, 40 and 50 mg/L. The oxyMb and the antioxidant solutions were then mixed and measured for k_{obs} values at 37°C at the time interval of 5 min to 2 h. The hot chilli extract was added to the oxyMb solution to get the final concentration of the extract of 10 mg/L. The observed autooxidation rate constant, k_{obs} was determined under the same optimum conditions as described above.

RESULTS AND DISCUSSION

3.1 Total capsaicinoids in hot chilli samples

Total capsaicinoids in eight varieties of hot chilli samples were determined based on dry weight basis as shown in Table 1. The capsaicinoids contents found in these samples were ranged of 63.76-106.62 mg/g DW. Sample A gave the highest content of capsaicinoids, while sample F had the lowest one. According to the extraction solvent for both groups of the compounds, they could be extracted using methanol or the acidified methanol. It is noted that some phenolic compounds can certainly be released into the methanol extract due to very similar organic structure. Therefore, the total phenolics contents in the methanol extract were also found using Folin-Ciocalteau method. It was found that the total phenolics always appeared in the extract of capsaicinoids in the range of 16.56-21.91 mg/g DW.

Table (1): Total capsaicinoids and phenolics contents in hot chilli samples based on dry basis (mg/g DW; *n* = 3)

	Capsaicinoids extract		Phenolics extract	
Sample	Capsaicinoids content	Phenolics content	Phenolics content	Capsaicinoids content
A	106.6	20.22	32.06	113.8
B	83.45	17.98	34.27	90.16
C	64.35	20.13	32.62	60.77
D	74.99	21.40	35.33	80.97
E	75.89	20.28	30.88	92.23
F	63.76	21.91	39.03	83.99
G	64.68	17.98	27.99	59.02
H	72.76	16.56	28.47	64.33

3.2 Total phenolics in hot chilli samples

Total phenolics contents in these eight varieties of chilli samples were also found in the same range (27.99-39.03 mg/g DW) as mentioned above (Table 1) when using methanol: 0.05%HCl (90:10) as the extraction solvent. Sample F gave the highest total phenolics content and sample G showed the lowest one. Based on the phenolic structure, the capsaicinoids contents in the acidified methanol extract were also determined. The results showed that the capsaicinoids also presented in the same range of 59.02-113.81 mg/g DW, slightly higher than those of the methanol extract. It is suggested that an acidified methanol can be effectively used to extract capsaicinoids from the chilli samples. Therefore, it is evident that the each of the hot chilli extracts obtained from both extraction methods contains both of total capsaicinoids and phenolic compounds.

3.3 Effect of temperature on the autooxidation of oxymyoglobin

The horse heart Mb solution was oxygenated to be oxyMb and recorded the Soret peak at 410 nm and visible band at 543 nm and 581 nm (Figure 3). When autooxidation reaction of the protein occurred, the Soret peak tend to slightly blue shifted and the charge transfer bands (543 nm and 581 nm) disappeared. Because of the oxidation of oxyMb is

favored by higher temperature, the observed first-order rate constant (k_{obs}) for the autooxidation reaction at various temperatures was determined.

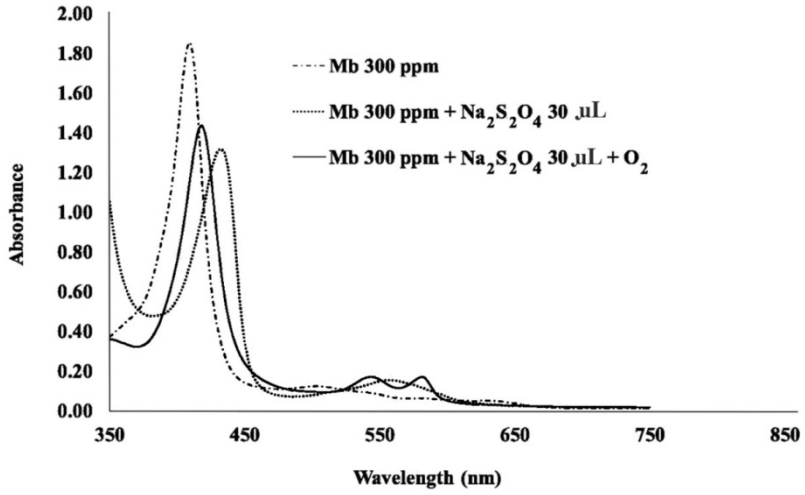

Figure (3): Absorption spectra of metMb, reduced Mb and oxyMb (each 300 mg/L)

The first-order plots for the autooxidation of oxyMb at various temperatures are shown in Figure 4. The results showed that when increase in an incubation temperature, the slope of straight line (k_{obs}) also increased. The k_{obs} values of the plots are 0.179, 0.329, 0.443, 0.626, 0.779, 1.21 and 1.64 attributing from the incubation temperature of 25, 30, 33, 35, 37, 40 and 50°C, respectively. However, 37°C was chosen for studying the effects of antioxidants and hot chilli extracts on retardation of autooxidation reaction of oxyMb because the k_{obs} could be obviously observed. By contrast, the autooxidation rate at higher incubation times was rather difficult to observe because the oxyMb solution was rapidly oxidized.

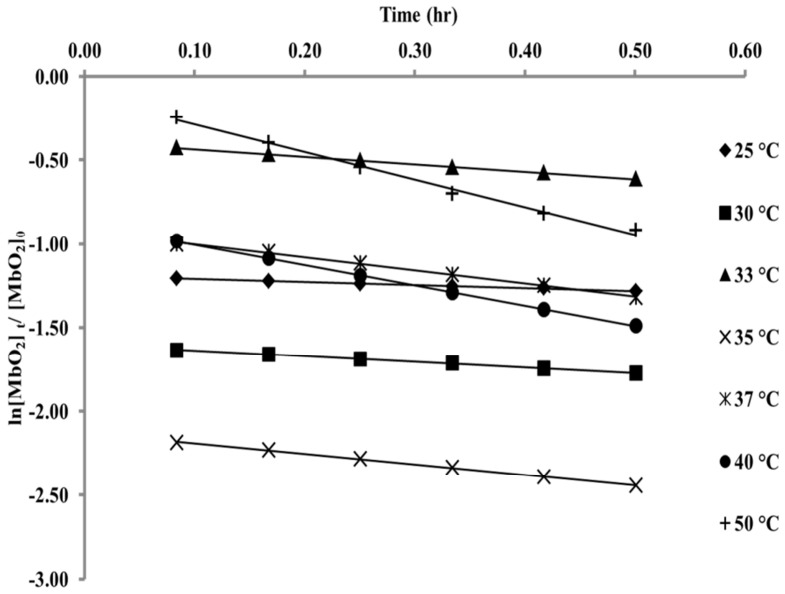

Figure (4): First-order plots for the autooxidation rate of oxyMb at various incubation temperatures

3.4 Effects of dihydrocapsaicin and gallic acid on the autooxidation of oxymyoglobin Dihydrocapsaicin and gallic acid solutions were chosen for capsaicinoids and phonolic compounds as standard antioxidants, respectively. The effect of dihydrocapsaicin and gallic acid concentrations on the autooxidation of oxyMb was preliminary studied. It was found that an increasing of concentrations of both compounds decreased the k_{obs} values compared with oxyMb solution without antioxidants. It is suggested that both dihydrocapsaicin (about 10x less) and gallic acid could stabilize the autooxidation reaction of oxyMb (Table 2).

Table (2): The $*k_{obs}$ ratios of the autooxidation rate of oxyMb in the presence of dihydrocapsaicin and gallic acid at various concentrations

Dihydrocapsaicin (ppm)	$*k_{obs}$ (h^{-1})	Gallic acid (ppm)	$*k_{obs}$(h^{-1})
Control	-	Control	-
5	0.067	5	0.667
10	0.067	10	0.552
20	0.057	20	0.468
30	0.049	30	0.370
40	0.043	40	0.257
50	0.038	50	0.222

$*k_{obs} = k_{obs\ (sample)} / k_{obs\ (control)}$; *where* $k_{obs\ (control)}$ *for dihydrocapsaicin* $= 83.2 \times 10^{-2}$ *and* $k_{obs\ (control)}$ *for gallic acid* $= 78.4 \times 10^{-2}$

According to both capsaicinoids and phenolic extract, no difference in the k_{obs} values of all samples was found (Figure 5 and Table 3). From the results, it is shown that although various chilli samples have different amounts of both capsaicinoids and phenolic compounds, they still exhibit the same retardation of the autooxidation rate of oxyMb, since their antioxidants used are fixed at the same level. In addition, both of the extracts composed of mixed ratios of both total capsaicinoids and total phenolic compounds. Therefore, the effects of the antioxidant capacities of the chilli extracts on retardation of the autoxidation rate of oxyMb were demonstrated under the optimum conditions of their kinetic study.

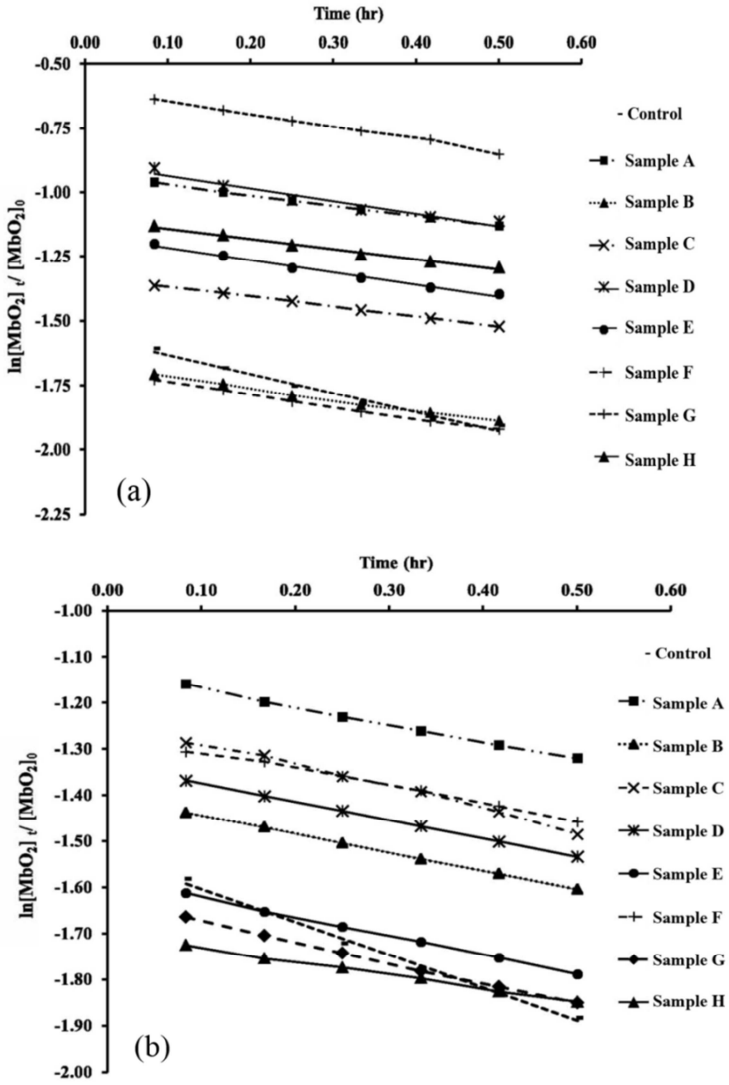

Figure (5): The effect of crude extracts of hot chilli on retardation of the autooxidation rate of oxyMb: (a) capsaicinoids extract and (b) phenolics extract

Table (3): The $*k_{obs}$ ratio of autooxidation rate of oxyMb in the presence of total capsaicinoids and phenolics extracts

Sample	Capsaicinoids extract $*k_{obs}(h^{-1})$	Phenolics extract $*k_{obs}$ (h^{-1})
Control	-	-
A	0.545	0.546
B	0.579	0.565
C	0.522	0.673
D	0.668	0.554
E	0.646	0.583
F	0.644	0.521
G	0.679	0.628
H	0.539	0.411

$*k_{obs} = k_{obs\ (sample)} / k_{obs\ (control)}$; where $k_{obs\ (control)}$ for capsaicinoids = 74.1×10^{-2} and $k_{obs\ (control)}$ for phenolics = 71.1×10^{-2}

Total capsaicinoids and total phenolics were extracted using 90% (v/v) methanol and methanol: 0.05% HCl (90:10, v/v), respectively. The contents of both phenolic compounds in various chilli samples were comparable. Interestingly, these extracts showed their effective antioxidant activities on the retardation of the autooxidation reaction of the oxyMb solution. Therefore, daily consumption of hot chilli by either direct or indirect way of food cooking would be fruitfully benefited for health, in particular to serve stabilization for oxygen storage in such the protein structure.

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