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Using Anthocyanin Extracts from Butterfly Pea as pH Indicator for Intelligent Gelatin Film and Methylcellulose Film

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Abstract

Among variety of intelligent food packaging, pH indicator packaging is becoming more popular, which can be made from synthetic and natural compounds. The search for natural pH indicator dyes that can be used in intelligent food packaging systems has recently focused on anthocyanins extracted from plants. Thus, this work aimed to develop and characterize an intelligent tag for pH indicator based on gelatin and methylcellulose-film with butterfly pea extract (BPE). The results showed that the colors of BPE solutions had a tendency to change from red to blue in a pH range of 4.0 to 8.0. The maximum absorption peak moved to a higher wavelength was observed at around 627 nm at pH 8.0 and shifted to 574 nm when the pH decreased to 5.0. After BPE was incorporated into the gelatin and methylcellulose-based films, the film's properties were characterized. The color of the incorporated films changed from purple to blue and blue to green in buffers with pH ranging from 2.0 to 6.0 and 7.0 to 10.0, respectively. The incorporated gelatin-based film containing BPE showed a clearer response to pH variation and showed a high pigment releasing rate when immerse in buffer of pH 10. The incorporated methylcellulose-based film containing BPE had higher water solubility than that of gelatin-based film ($p < 0.05$), as well as improved mechanical properties and water vapor permeability (WVP). Therefore, it is possible to use the BPE (anthocyanins) as a visual pH indicator for food package.

Keywords: anthocyanin; butterfly pea; intelligent packaging; pH indicator
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1. Introduction

Recently, packaging technologies have been developed for packaging and materials to be used as intelligent biodegradable packaging. Intelligent packaging is important in food and pharmaceutical industries to ensure food quality and safety. The main purposes of these new forms of packaging are to prolong shelf life, minimize environmental impact and communicate product changing over time to consumers. Among variety of intelligent food packaging, pH indicator packaging or real-time monitoring of food quality has become more favourite by consumer. Basically, visual pH indicators or pH sensitive dye can be made from synthetic and natural chemical compounds in which the former are avoided due to their side effects to human health. Many studies have reported prospective applications of natural pH indicator dyes in intelligent food packaging systems including anthocyanins extracted from sweet potato [1], red grapes [2], and purple cabbages [3].

The use of biopolymers in food packaging have gained increased interest in recent years because they represent an environmentally alternative to plastic-based polymers. Methylcellulose (MC) is natural biopolymer derived from cellulose, which has been widely applied in the food industry. MC presents some good properties such as water solubility, films forming ability, consistency, and hydrophilic property. Meanwhile, gelatin, fibrous protein, has been widely used as polymer base material in film formation due to its several advantages. Gelatin films present good film forming ability and oxygen barrier properties [4]. However, biopolymer-based materials can act differently when different types of ingredient are added. Protein can act as buffer systems due to their ionizable side groups resulting in uncertain responsiveness to pH changes. In addition, the mechanisms related to pH color responses of natural dye in MC films are still unknown. Formulation additives can provide a new functionality and physicochemical properties of pH indicator films.

Anthocyanins, natural plant compounds, are water soluble pigments that impart the red, purple and indigo color of many plants. Anthocyanins have the ability to change color with pH; changes that reflect structural transformations [5]. Their abilities can be suitable for use as parts of colorimetric pH indicators that can be utilized in intelligent food materials. Butterfly pea is known as a rich source of anthocyanin with blue color used for food and sweets. The butterfly pea's pigments can exhibit color at various pH, making them a natural alternative to synthetic indicators. Thus, the aims of this study are to develop pH indicator film incorporated with anthocyanin from butterfly pea and to characterize the physicochemical and mechanical properties of the indicator film.

2. Materials and Methods

2.1 Plant extract preparation

Butterfly pea (*Clitoria ternatea*) flowers were purchased from local market, Chonburi province, Thailand. The flowers were dried in hot air oven at 60°C for 24 h. The dried flowers were ground using blender. Then, sample powder (5 g) was immersed in 200 ml of 70% ethanol aqueous solution at 4°C for 24 h. After this period, the extracts were filtered through Whatman filter paper No.1. The filtrate obtained was referred to as "butterfly pea extract (BPE)." The extract was tested at different pH, ranging from pH 2 to 10 using 1 N HCl and 1 N NaOH. The pH was measured by pH meter and the sample was photographed. The absorption spectra of the BPE were measured using a UV-vis spectrophotometer. The BPE was adjusted for pH in the range of 2.0-10.0 using HCl and NaOH. The absorbances of the adjusted solutions were measured in the range of 400-700 nm.

2.2 Preparation of compostable film incorporated with anthocyanin extract

Compostable film incorporated with anthocyanins extract was prepared from commercial food grade bovine gelatin and methylcellulose. The gelatin film was prepared by mixing 3 g of gelatin and 0.75 g of glycerol with distilled water to obtain the film forming solution (FFS). The methylcellulose film was prepared by mixing 3 g of methylcellulose and 0.75 g of glycerol with 70% ethanol. The solution was incubated at 60ºC for 30 min in a water bath with occasionally stirring. The BPE with 10% final concentration was then incorporated into the FFS. De-aerated film-forming solution was cast onto a rimmed circular plastic plate (145 mm) and dried with a ventilated oven environmental chamber at 25 ± 0.5ºC and 50 ± 5% relative humidity for another 24 h. The obtained dried films were manually peeled and subjected to film characterization.

2.3 Determination of film thickness and color response to pH change of the indicator films

Film thickness was measured with a hand-held micrometer (Mitutoyo Absolute). Measurements were done at nine random points along the rectangular strips. The thickness values were used to calculate the mechanical properties of the films. Film color was determined using CIE-Lab colorimeter in the term of lightness (L^*), redness ($+a^*$) or greenness ($-a^*$), and yellowness ($+b^*$) or blueness ($-b^*$) of the films. The color response of the indicator films to pH changes in aqueous mediums (pH 2-10) were determined. The colorimeter was calibrated with a white standard plate. Before the measurement, the pH indicator films were immersed in 5 ml of each buffer solutions for 5 min. All measurements were taken in 4 replicates at random locations of each sample.

2.4 Determination of film solubility and water vapor permeability

The solubility and releasing rate of films were determined by cutting dried films into 10 × 10 mm pieces and weighted. The samples were immersed in sample cup with 5 ml pH buffer solutions at 25°C for 30 min. After that, the solutions were removed, and then dried. When the films had dried, the samples were weighed and calculated. Their solubility was calculated by the following equation:

$$\text{Film solubility (\%)} = (W_i\text{-}W_f)/W_i \times (100) \quad (1)$$

where W_i is initial weight and W_f is final weight. The releasing rate of the films was measured after solubility tests using the aqueous mediums (pH 2-10). The amounts of monomeric anthocyanins released under different pH conditions were determined by absorbance measurement at λmax using a UV/VIS spectrophotometer.

The WVP of the films was measured using a modified ASTM method [6]. The films were sealed onto an insertion cup containing water with grease and holder. The sample cups were placed in incubator with relative humidity at 30%. The cups were weighed every 6 h over a period of 3 days, and then the films' WVP were calculated as follows:

$$WVP = w \cdot x/A \cdot t \cdot (P_2\text{-}P_1) \quad (2)$$

where w is the weight loss of the cup (g), x is the film thickness (mm), A is the area of exposed films (m^2), t is the time of gain (s), and (P_2-P_1) is the vapor pressure differential across the film (Pa). The WVP was expressed as $gH_2O/s \cdot m \cdot Pa$. Each film was determined in triplication. Mechanical properties were measured using a Universal Testing Machine under conditioning for 48 h at 50 ± 5% RH at 25°C. Ten samples (2.5 x 8 cm) with an initial grip length of 3 cm were determined for

tensile strength (TS) and elongation at break (EAB). The cross-head speed was set at 30 mm/min with 1 kN load cell use. The wettability of the film was evaluated by contact angle measurement using a sessile-drop method. A 10 µl drop of distilled water was applied on the surface of each film, and then the angle between the drop and the surface of the film was measured.

2.5 Statistical analysis

The two-sample t-test was used for comparing two means of data whereas analysis of variance (ANOVA) was used for comparing three means of data. Tukey's range test was applied as Post Hoc test for multiple comparison of groups. Analysis was performed using MINITAB.

3. Results and Discussion

3.1 VIS-spectra of BPE solutions in various pH ranges

Color variations in the BPE solutions after adjusting pH with HCl or NaOH were tested and recorded. The results of BPE showed that the color change of BPE solution is caused by the dissociation of pH (Figure 1). The BPE's original color at pH 6 was purple and then turned violet when the pH of the solution was lower than 4.0. The color of BPE solutions turned blue, dark green, and green yellow when the pH of the solution was 7.0-8.0, 9.0-10, and 12.0, respectively. The BPE solution's colors had a remarkable change at pH 2.0 to pink and 12.0 to green-yellow. The maximum absorption peak movement to a higher wavelength was observed around 627 nm at pH 8.0 and shifted to 574 nm when the pH decreased to 5.0. When the pH decreased to 2.0, the maximum absorption peak shifted to 550 nm. The change of red to blue of anthocyanin might be caused by the loss of flavylium cation and the hydrolysis of anthocyanin molecules [7]. Bathochromic shift is usually found in anthocyanin compounds which shift their color when a change in environmental conditions, such as a change of charge occurs [8-10].

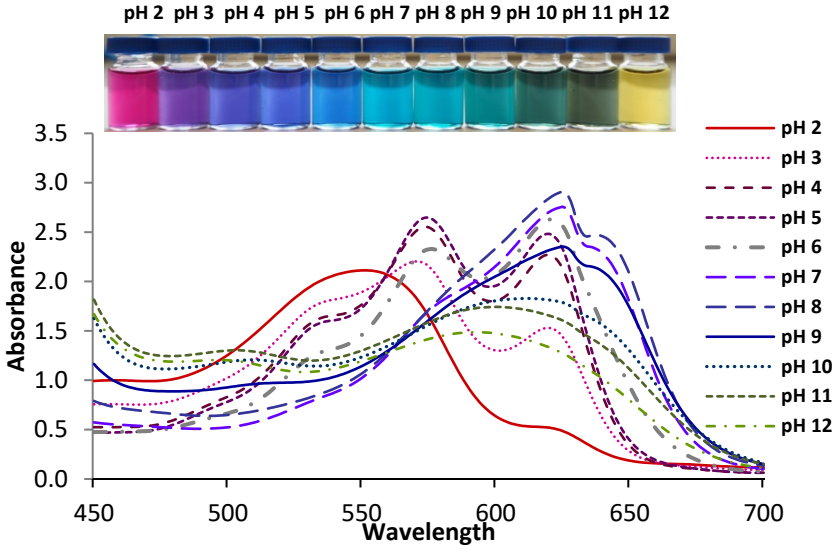

Figure 1. UV-Vis spectra of BPE solutions and color response variations of BPE at different pH (2-12)

3.2 Color response analysis of the indicator films exposed to different pH buffers

The color responses of the gelatin and methylcellulose films exposed to different pH buffers were examined by dripping the films in buffer solutions at pH range of 2.0 to 10.0. The result showing visible response color changes of each sample, inclining from pink to green in pH 2.0 to 10.0 buffers, are presented in Figure 2. The initial film color was blue, which turned purple when contacted with the pH 2.0 buffer solution and changed in color from blue to dark green with alkaline solution. The pH indicator film became purple in acid solution, and then showed a blue color when it was immersed in higher pH buffer solutions (pH 5.0-6.0). Thereafter, the film color changed to a light green color in pH 8.0 to 10.0 buffers. The color parameters of the pH indicator films after immersion in the pH buffers were measured using a CIE-Lab colorimeter. The colorimetric parameters of the gelatin and MC-based films containing BPE are shown in Table 1. Comparing color responses, $a*$ and $b*$ values showed significant differences in all pH buffers ($p < 0.05$). The indicator films immersed in alkaline solution showed lower $a*$ values, but increased in $b*$ values. The redness of the films increased with immersion in the acid buffers due to the flavylium cation forming in anthocyanins [10]. When it reaches pH 4.0, the anthocyanin solution were liberty blue due to the less amount of flavylium cation and quinonoidal anion. When pH is increased from 4.0 to 6.0, the hydration of the flavylium cation generates a colorless carbinol pseudo-base resulting from nucleophilic attack by water [11,12]. At alkaline pH (8.0-10.0), blue color solution were formed due to deprotonation of the quinonoid molecule [10]. In contrast, when pH is increased to alkaline condition, the films appear blue-green in color which is due to the anionic quinoidal base representing deep blue becoming more prominent [13]. Color properties in terms of $a*$ and $b*$ had significant color response variations influenced by acid-alkaline condition. It can be considered that the films incorporated with BPE had unequivocal colors in various pH environments.

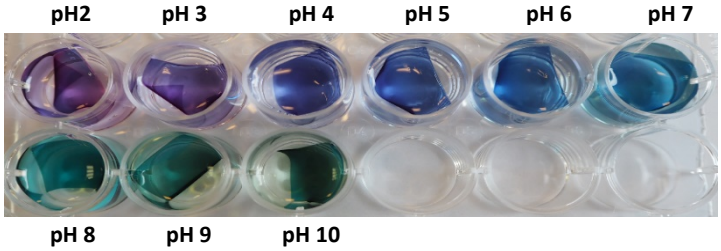

Figure 2. Color response of the gelatin film containing BPE in different pH buffers

Table 1. Colorimetric parameters of gelatin and MC-based films containing BPE

pH value	Gelatin-based film			MC-based film		
	L*	***a****	***b****	***L****	***a****	***b****
pH 2	58.47 ± 0.64	24.52 ± 0.97	-14.55 ± 0.59	53.16 ± 3.33	27.61 ± 1.70	-19.89 ± 4.41
pH 3	54.41 ± 0.83	17.71 ± 1.29	-17.76 ± 1.47	49.08 ± 1.16	24.75 ± 1.65	-22.00 ± 0.85
pH 4	50.33 ± 0.73	17.55 ± 1.81	-27.26 ± 1.22	49.28 ± 1.44	20.72 ± 0.19	-29.34 ± 0.90
pH 5	51.84 ± 1.21	15.91 ± 0.94	-28.63 ± 0.94	45.36 ± 1.27	19.41 ± 1.90	-28.44 ± 3.51
pH 6	54.67 ± 1.30	8.30 ± 0.51	-26.64 ± 1.16	47.39 ± 0.85	11.38 ± 0.58	-24.96 ± 1.38
pH 7	55.68 ± 2.25	-5.32 ± 1.73	-19.27 ± 1.51	47.39 ± 1.69	4.18 ± 1.85	-19.33 ± 0.72
pH 8	54.44 ± 0.74	-7.13 ± 0.30	-9.77 ± 0.31	50.27 ± 2.59	-6.12 ± 2.11	-11.49 ± 2.08
pH 9	55.04 ± 1.90	-3.71 ± 0.36	-4.99 ± 0.24	50.40 ± 0.66	-6.67 ± 0.49	-8.04 ± 0.73
pH 10	59.03 ± 1.71	-2.88 ± 0.83	-2.26 ± 0.67	44.32 ± 1.33	-0.47 ± 0.79	-3.08 ± 0.34

3.3 Film solubility and releasing rate of the pH indicator films

The solubility in different pH buffers is a significant property that determines the indicator film pertinence. The solubility of both gelatin and MC-based films was measured at pH 2.0-10.0. The gelatin films at neutral close pH showed a lower solubility (8.5% at pH 6), whereas the films in acid buffer presented the highest solubility (79.7% at pH 2) (Table 2). The difference in solubility of the films was affected by the pH of aqueous solution. Theoretically, proteins have a minimum solubility at the protein isoelectric point, at which the net charge of protein molecules is zero. Proteins basically present high solubility in acid or alkaline pH regions due to the excesses of charges on protein, which conduct repulsion among the molecules, thus favoring greater solubility. In contrast, MC-based films showed significantly higher solubility than that of gelatin films. The decreased film solubility of gelatin film compared with MC-based films was attributed to the cross linking effect of phenolic compounds presented in the butterfly pea extract. Similarly, reduced solubility of chitosan film incorporating anthocyanin in water was attributed to hydrogen bond development [14]. The higher solubility of methylcellulose films was due to a hydrophilic polymer involving the penetration of water to the polymer bulk, causing swelling, and then disruption of hydrogen and Van der Walls forces between MC polymer chains [15]. Anthocyanin molecules of PBE possibly caused a decrease in intermolecular interactions in the MC-based film. Therefore, the free side groups of the molecules might interact with water and increase water absorbency [16].

Table 2. Film solubility of BPE incorporated gelatin and MC-based films

pH	Film solubility (%)	
	Gelatin-based film	**MC-based film**
2	79.7 ± 4.8^{a}	89.8 ± 4.5^{a}
3	71.8 ± 4.5^{ab}	85.8 ± 3.5^{abc}
4	60.0 ± 10.2^{b}	77.0 ± 6.1^{abcd}
5	13.4 ± 8.5^{de}	73.5 ± 6.4^{bcd}
6	8.5 ± 3.1^{e}	69.2 ± 1.9^{cd}
7	24.7 ± 0.7^{cde}	70.7 ± 7.2^{cd}
8	31.8 ± 4.0^{cd}	62.8 ± 1.8^{d}
9	26.4 ± 3.4^{cde}	79.7 ± 2.3^{abc}
10	39.3 ± 11.8^{c}	80.5 ± 7.1^{abc}

Note: Different superscript letters indicate significant differences between means in the same column ($p < 0.05$).

The releasing properties of BPE in gelatin and MC-based film were determined under pH 2-10. The gelatin films showed that the releasing properties of the extract were in the range of 34.7% to 70.8%, whereas MC-based film had the releasing properties of 28.0% to 88.0% (Table 3). In acid solution, the releasing ability of the both films were lower than those in alkaline solution. Reduction in releasing properties of the extract from the gelatin and methylcellulose-based films was due to the interactions with the polymeric chains; between protein and phenol compounds and also methylcellulose and phenol compounds. These interactions, which lowered relaxation ability, led to lower swelling and thereby reduced the extract release rate. With increasing of pH to alkaline, the films significantly released anthocyanin into the solution. This indicates that the anthocyanin from the extract was dependently physico-chemically immobilized. At higher pH, negative charge unfolded the protein structure, and more anthocyanin residues were exposed to the solution and displayed increased anthocyanin release properties. Similar results were reported by Wu *et al*. [17],

Table 3. Releasing properties of BPE in gelatin and MC-based films

pH	λmax	Releasing properties of BPE in film (%)	
		Gelatin-based film	MC-based film
2	550	37.7 ± 1.8^{cdeA}	32.5 ± 5.4^{cdB}
3	570	35.4 ± 4.5^{deA}	33.4 ± 0.2^{cdA}
4	574	34.7 ± 3.2^{eA}	34.6 ± 4.2^{cdA}
5	574	41.4 ± 1.5^{bcdeA}	28.0 ± 2.3^{dB}
6	621	40.0 ± 1.0^{cdeA}	31.1 ± 1.8^{cdB}
7	625	48.5 ± 4.9^{bcA}	42.0 ± 2.5^{bcB}
8	625	46.7 ± 3.6^{bcdA}	49.1 ± 5.7^{bA}
9	625	51.9 ± 4.9^{bB}	79.2 ± 6.5^{aA}
10	600	70.8 ± 7.0^{aB}	88.0 ± 7.0^{aA}

Note: Two sample t-test was used for comparing 2 means in row. Different superscript letters indicate significant differences between means in the same column ($p < 0.05$) and different superscript capital letters indicate significant differences between means in the same row ($p < 0.05$).

who observed that the releasing properties of indicator dyes were affected by pH, and especially under alkaline solution.

3.4 Effect of BPE on the mechanical and physicochemical properties of films

The effect of BPE on the mechanical and physicochemical properties of the films were evaluated by testing the tensile strength (TS) and elongation at break (E%) of the films. The colored film thickness ranged between 50 and 55 μm (data not shown). The incorporation of BPE had no effect on the gelatin films' thickness. As the results shown in Table 4, colored films showed no significant difference in tensile strength and elongation at break when compared to control films, except in the case of tensile strength for gelatin film. Incorporation of BPE into formulations tended to improve the mechanical properties of MC-based films, but decreased those properties of gelatin-based films. Normally, the mechanical properties of compostable films rely on the interactions between the constituents of the film matrix. The enhanced TS and E% in MC-based film could be ascribed to intermolecular interactions between anthocyanins and methylcellulose chains. However, enormous incorporation of the phenolic compound sorely exhausted the molecular interaction between the methylcellulose polymers [18]. For gelatin-based film, the TS decreased after the addition of BPE, suggesting the anthocyanins-rich extract interrupted the formation of intra/interchain hydrogen bonds of gelatin. The decrease in TS is due to weakening of interactions between protein and phenol groups. Similar effects on mechanical properties were found in the case of anthocyanin used as color indicator in gelatin films [19].

The contact angle and WVP of pH indicator films are shown in Table 4. Incorporation of BPE into the films resulted in significantly higher WVP of films ($p < 0.05$). To develop the film as an indicator, a higher WVP should be examined. The films should have high volatile permeate ability for monitoring deterioration of food products. The pH indicator gelatin film showed significantly lower WVP than the methylcellulose-based film ($p < 0.05$). The increase in WVP for the pH indicator films might be due to the incorporation of the hydrophilic BPE. Polyphenolic molecules of BPE possibly caused low in intermolecular interactions in the film network. Thus, the hydroxyl groups of BPE molecules might cooperate with water and intervene in the network by

Table 4. Mechanical properties of gelatin and MC-based films incorporated with BPE

Film	Tensile (MPa)	Elongation at break (mm)	WVP $\times 10^8$ ($gH_2O/s \cdot m \cdot Pa$)	Contact angle (degree)
Gelatin-based film	51.1 ± 4.3^{abA}	58.9 ± 3.4^{aA}	10.54 ± 0.92^{bB}	70.56 ± 5.07^{bB}
Gelatin-based film with BPE	39.4 ± 1.2^{bB}	59.8 ± 2.3^{aA}	12.27 ± 0.82^{bA}	83.51 ± 9.09^{aA}
MC-based film	61.2 ± 22.2^{abA}	69.0 ± 7.0^{bA}	10.83 ± 0.44^{bB}	64.38 ± 3.23^{bcA}
MC-based film with BPE	65.5 ± 14.2^{aA}	71.0 ± 4.8^{bA}	20.20 ± 2.27^{aA}	54.62 ± 7.81^{cB}

Note: ANOVA and Tukey's test were used for comparison. Different superscript letters indicate significant differences between means in the same column ($p < 0.05$), and different superscript capital letters indicate significant differences between means in the same based film's type (with/without BPE) ($p < 0.05$).

hydrogen bonds and thus increase water vapor permeability [20]. The WVP increased when incorporated with bioactive compounds. Similar results were found in some reports of incorporating bioactive compounds containing the hydroxyl group into active films [21-23]. The contact angle technique is used to investigate the surface structures of the films for their hydrophobicity properties. Larger angle value represents more hydrophobicity of the pH indicator films. The contact angle for the gelatin-based film was higher after BPE incorporation, in contrast to the MC-based film, which showed a lower contact angle value after BPE incorporation (Table 4). This might be due to a greater number of hydrophilic sites for the water soluble components present on the MC-based film. In addition, the high amount of BPE in the MC-based films resulted in high polarity bonds groups of anthocyanin that can interact with water molecules and the contact angle consequently decreases by expanding water on the surface of the film.

4. Conclusions

The intelligent gelatin film incorporated with BPE presented a color variation as a function of pH ranges. Butterfly pea is suitable to be used as pH color response indicator in both gelatin and MC-based films due to its high sensitivity and variety of color shades. The pigment releasing properties showed that anthocyanin from butterfly pea in the extract was bonded loosely on the MC-based films. However, the mechanical properties and WVP need to be improved, when considering its application as a pH-responsive indicator for varieties of food products. Thus, development of color response film by incorporating anthocyanin from butterfly pea can be used as novel food smart packaging for pH sensitive product and spoilage detection for perishable food.

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