



Yeast mannoproteins improve thermal stability of anthocyanins at pH 7.0



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ABSTRACT

Anthocyanins are food colourants with strong antioxidant activities, but poor thermal stability limits their application in neutral foods. In the present study, impacts of yeast mannoproteins on the thermal stability of anthocyanins were studied at pH 7.0. The degradation of anthocyanins at 80 and 126 °C followed first order kinetics, and the addition of mannoproteins reduced the degradation rate constant and increased the half-life by 4 to 5-fold. After heating at 80 and 126 °C for 30 min, mannoproteins improved the colour stability of anthocyanins by 4 to 5-fold and maintained the antioxidant capacity of anthocyanins. Visible light absorption, fluorescence spectroscopy, and zeta-potential results suggest that anthocyanins bound with the protein moiety of mannoproteins by hydrophobic interactions, and that the inclusion of anthocyanins in complexes effectively reduced the thermal degradation at pH 7.0. Therefore, mannoproteins may expand the application of anthocyanins as natural colours or functional ingredients.

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1. Introduction

Anthocyanins are the most abundant flavonoid glycosides and are widely used as water-soluble plant pigments in food products (Verbeyst, Van Crombruggen, Van der Plancken, Hendrickx, & Van Loey, 2011). The colour of anthocyanins varies with pH and advances through red, purple and blue to green as pH increases (Clifford, 2000). The significance of biological activities of anthocyanins to human health and disease prevention has also been frequently reported. Some of these activities include excellent antioxidant properties, neuroprotective, anticarcinogenic, and antidiabetic functions, visual acuity, and dermal health (Nizamutdinova et al., 2009; Rahman, Ichiyanagi, Komiyama, Sato, & Konishi, 2008; Zafra-Stone et al., 2007). However, anthocyanins have poor stability and can be easily hydrolysed to colourless compounds (Kopjar & Pilizota, 2011). Factors impacting their stability include temperature, pH, oxygen, enzymes, metallic ions, ascorbic acid, and sulphur dioxide (Idham, Muhamad, Setapar, & Sarmidi, 2012).

The structure of anthocyanins varies significantly with source, and the acetylated anthocyanins are generally more stable

(Mazza & Brouillard, 1987). Solution chemistry, particularly pH, is critical to the stability of anthocyanins (Mazza & Brouillard, 1987). The absorbance at a visible light wavelength corresponding to the maximum absorbance of petanin and cyanidin 3-glucoside was observed to be stable below pH 4.0 but became gradually diminished during storage at 10 °C and pH > pH 6.0 for 60 days (Fossen, Cabrita, & Andersen, 1998). The loss of absorbance and colour at pH 5–7 is caused by hydration at the 2-position of the anthocyanidin skeleton (Brouillard, 1982). Co-pigmentation is a well-known strategy to improve the stability of anthocyanins (Yan et al., 2013). β -Cyclodextrin was observed to improve the storage stability of chokeberry anthocyanins at pH 2.8–3.6 (Howard, Brownmiller, Prior, & Mauromoustakos, 2013). Pectin was observed to stabilise blackcurrant anthocyanins during storage at pH 3.0 (Buchweitz, Speth, Kammerer, & Carle, 2013; Lewis, Walker, & Lancaster, 1995). However, studies at neutral pH are scarce.

The thermal stability of anthocyanins is dependent on thermal treatment conditions (temperature and duration) and environmental factors such as pH, oxygen, light, and co-solutes (Patras, Brunton, O'Donnell, & Tiwari, 2010). Anthocyanins are known to be thermally stable below pH 3 and unstable at neutral pH (Tachibana, Kimura, & Ohno, 2014). Therefore, improving thermal stability of anthocyanins at neutral and weakly acidic conditions will expand their applications. The thermal stability of anthocyanins at pH 4.0 was improved after addition of β -cyclodextrin

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(Mourtzinou et al., 2008). Ferric ions were observed to stabilise anthocyanins after heating at neutral and weakly acidic conditions, and the aggregation after heating at 60 °C up to 80 min was prevented by further addition of alginate (Tachibana et al., 2014). The protection by the combination of ferric ions and alginate was proposed to have been enabled by complex formation (Tachibana et al., 2014). However, thermal sterilisation of low-acid (pH > 4.5) foods is regulated by the US Food and Drug Administration and requires more severe thermal treatments than 60 °C for 80 min, e.g., above 100 °C with the duration dependent on unit operations and products (Berk, 2013).

The potential for improving thermal stability and maintaining antioxidant properties of anthocyanins by yeast mannoproteins at pH 7.0 was studied in the present work. Yeast mannoproteins, with about 10% protein and 90% carbohydrate in composition, are highly glycosylated proteins that are available as commercial ingredients after being extracted from yeast cell wall (Goncalves, Heyraud, De Pinho, & Rinaudo, 2002). Mannoproteins are also present as one of the major polysaccharides in wine and are known for their ability to prevent crystallisation of tartrate and flocculation of proteins (Guadalupe & Ayestaran, 2008; Guadalupe, Martinez, & Ayestaran, 2010; Guadalupe, Palacios, & Ayestaran, 2007; Rodrigues, Ricardo-Da-Silva, Lucas, & Laureano, 2012). However, the addition of yeast mannoproteins in wine production was observed to reduce the dispersion and colour stabilities of the extracted polyphenols at pH 3.6 (Guadalupe et al., 2007). Another study reported no improvement in the storage stability of wine colour by commercial yeast mannoproteins at pH 3.8–4.0 (Rodrigues et al., 2012). Anthocyanins are cationic at highly acidic pH (Lee, Durst, & Wrolstad, 2005), and the polypeptide fraction of mannoproteins is also overall positively charged below the isoelectric point of pH 4.1 (Gomez et al., 1996). The repulsive electrostatic interactions between anthocyanins and mannoproteins may have resulted in the above observations. Formation of complexes between mannoproteins and anthocyanins may be more favourable at neutral pH when anthocyanins are not charged (Lee et al., 2005). The complexes may then improve the thermal stability of anthocyanins.

The first objective of the present study was to evaluate the effects of yeast mannoproteins on stability and antioxidant activity of anthocyanins at pH 7.0 after thermal treatment. The second objective was to study physicochemical properties of mannoprotein–anthocyanin mixtures before and after heating.

2. Materials and methods

2.1. Materials

Anthocyanins (ColorFruit® Violet 100WS, 10 colour units/kg liquid according to the manufacturer, with 54% w/w solids) was purchased from Chr. Hansen Inc. (Milwaukee, WI). The yeast mannoprotein product, with a brand name of Claristar™, was obtained from DSM Food Specialties (MA Delft, Netherlands). 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) was from Sigma-Aldrich Corp. (St. Louis, MO). Potassium persulphate, potassium ferricyanide, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and other chemicals were purchased from Fisher Scientific Inc. (Pittsburgh, PA).

2.2. Preparation of anthocyanin–mannoprotein solutions

Fresh mannoprotein and anthocyanin stock solutions (2% w/w) were prepared separately in distilled water, adjusted to pH 7.0, and kept at 4 °C before experiments. Five millilitre of a 2% w/w anthocyanin solution was mixed with 15 ml of a 0.67% w/w

mannoprotein working solution that was previously diluted from the stock solution in distilled water, corresponding to 0.5% w/w for both anthocyanins and mannoproteins. The mixture was stirred at room temperature for 30 min and the pH was adjusted to 7.0 using 1 M HCl or NaOH before subsequent experiments.

2.3. Thermal treatments

Two millilitre aliquots of the solutions were heated for 0, 5, 10, 20, 30, 60 and 90 min at 80 or 126 °C, used as representative pasteurisation and sterilisation temperatures (Berk, 2013) in a glycerol bath to establish thermal degradation kinetics. Samples heated for 30 min were used to characterise physicochemical properties. Control solutions with 0.5% w/w anthocyanins or mannoproteins alone at pH 7.0 were also prepared and treated similarly. Samples after heating were kept at 4 °C before further tests within 48 h.

2.4. Determination of anthocyanin concentration

The anthocyanin content in dispersions before and after heating was determined using absorbance at 570 nm (Abs_{570}) that corresponded to the maximum absorbance at pH 7.0 (Zheng, Bucheli, & Jing, 2009). Samples were diluted by 5-fold in deionised water before testing using an Evolution 201 UV–vis spectrophotometer (Thermo Scientific, Waltham, MA). Calibration curves of anthocyanins only, as well as of a mixture containing equal masses of anthocyanins and mannoproteins, were established separately to determine the concentration of anthocyanins in corresponding unknown samples.

2.5. Analysis of thermal degradation kinetics

The degradation data were fit to the first order kinetics shown in Eq. (1) (Kirca, Ozkan, & Cemeroglu, 2007; Wang & Xu, 2007). The half-life ($t_{1/2}$) at a heating temperature was determined to be the time when the anthocyanin concentration decreased to 50% of its initial concentration, as in Eq. (2) (Gris, Ferreira, Falcao, & Bordignon-Luiz, 2007).

$$c_t = c_0 e^{-kt} \quad (1)$$

$$t_{1/2} = -\ln(0.5)/k \quad (2)$$

where c_0 and c_t are the concentrations of anthocyanins before and after heating at a specific temperature for t min, and k (min^{-1}) is the degradation constant at the corresponding temperature.

2.6. Characterisation of dispersion colour changes after heating

The effects of heat treatment on the colour stability of anthocyanin dispersions were evaluated using Hunter L^* , a^* and b^* values. The instrument was a MiniScan XE Plus Hunter colorimeter (Hunter Associates Laboratory, Inc., Reston, VA). The hue angle [$H^\circ = \tan^{-1}(b^*/a^*)$] was additionally calculated (Larrauri, Sanchez-Moreno, & Saura-Calixto, 1998).

2.7. Visible light absorption and fluorescence spectroscopy

The visible light absorption spectra of anthocyanin dispersions were acquired between 350 and 700 nm at room temperature (21 °C) using an Evolution 201 UV–Vis spectrophotometer (Thermo Scientific, Waltham, MA). The fluorescence spectra of samples were recorded using an RF-1501 spectrofluorometer (Shimadzu Corp., Tokyo, Japan). The excitation wavelength was set at 370 nm, and

the emission spectra were recorded from 400 to 600 nm with the background fluorescence calibrated using distilled water.

2.8. Atomic force microscopy (AFM)

A Multimode VIII microscope (Bruker AXS, Billerica, MA) was used to study the morphology of particles in dispersions. Samples were diluted 100-fold using distilled water and pH was re-adjusted to 7.0. Two microlitre of the diluted sample was spread on a freshly-cleaved mica sheet and dried at room temperature for 120 min. The scanning was conducted at the tapping mode using a nano-probe cantilever tip (Bruker Nanoprobe, Camarillo, CA) at a frequency of 50–100 kHz. Particle dimensions were analysed using the AFM instrument software (Nanoscope Analysis, version 1.40, Bruker, Inc., Billerica, MA).

2.9. Zeta potential

After adjusting pH to 7.0, the above 100-fold diluted samples were characterised for zeta potential using a Delsa™ Nano-Zeta Potential and Submicron Particle Size Analyser (Beckman Coulter, Inc., Brea, CA) at room temperature.

2.10. Determination of in vitro antioxidant capacity

2.10.1. DPPH• scavenging capacity

The DPPH assay was carried out according to a literature protocol (Dudonne, Vitrac, Coutiere, Woillez, & Merillon, 2009) with slight modifications. The 0.125 mM DPPH was dissolved in ethanol daily as a fresh working solution. One millilitre of a sample solution was mixed with 1 ml of the DPPH-solution. Following incubation at room temperature (21 °C), in the dark, for 20 min, the absorbance of the mixture was measured at 517 nm using an Evolution 201 UV–Vis spectrophotometer (Thermo Scientific, Waltham, MA). The blank was prepared in the same manner by substituting the sample with distilled water. The DPPH• scavenging capacity was calculated by Eq. (3):

$$\text{Radical scavenging capacity (\%)} = [1 - (A_S/A_B)] \times 100 \quad (3)$$

where A_S and A_B are the absorbance value of the sample and blank, respectively.

2.10.2. ABTS•⁺ scavenging capacity

The ABTS assay followed a literature method (Dudonne et al., 2009) with some modification. The assay characterises the ability of an antioxidant to reduce the cationic ABTS radicals (ABTS•⁺). ABTS was dissolved in distilled water to a concentration of 7 mM and was reacted with 2.45 mM potassium persulphate at 21 °C in the dark for 16 h. The mixture was diluted 10-fold to prepare the final ABTS•⁺ working solution. Three millilitre of the ABTS•⁺ working solution was mixed with 0.1 ml of a sample and was reacted at 30 °C in the dark for 10 min before measuring the absorbance at 734 nm using the above spectrophotometer. Distilled water was used as a blank. The ABTS•⁺ scavenging capacity (%) was calculated similarly to Eq. (3).

2.11. Statistical analysis

All experiments were carried out in triplicate. One-way analysis of variance (ANOVA) was carried out for each treatment using SPSS 16.0 statistical analysis system (SPSS Inc., Chicago, IL). Tukey's honest significant difference (HSD) test was used to determine any significant differences ($P < 0.05$) of the mean.

3. Results and discussion

3.1. Effects of mannoproteins on the colour stability of anthocyanins after heating

Fig. 1A and Table 1 show the respective appearance and quantitative colour parameters of anthocyanin dispersions before and after heating at 80 and 126 °C for 30 min with and without an equal mass of mannoproteins. Anthocyanin dispersions showed a brilliant blue colour at pH 7.0 (Fig. 1A). After heating at 80 °C for 30 min, no visible difference was observed for the sample with mannoproteins, whilst the sample without mannoproteins became lighter. The visual difference between treatments with and without mannoproteins was more significant after heating at 126 °C for 30 min. The visual observations are further supported by the colour parameters of L^* , a^* , b^* , and hue angle that showed the significant improvement of colour stability after heating with mannoproteins (Table 1). As shown in Fig. 1A, anthocyanins are limited only to products that developed a blue colour at neutral pH, and the colour acceptance, especially after thermal processing, require verifications in specific food matrices using trained panels.

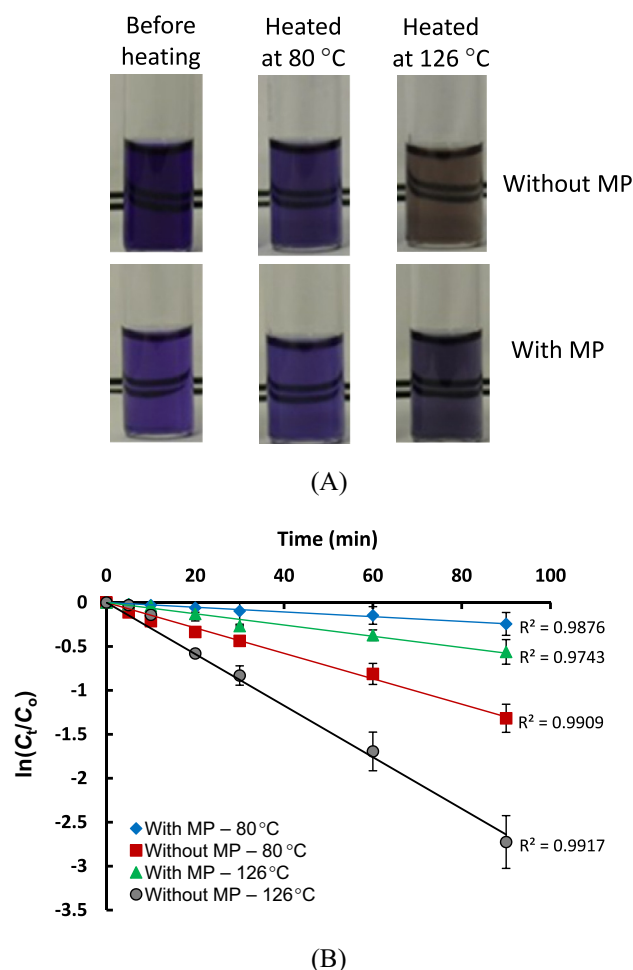


Fig. 1. (A) Appearance of 0.5% w/w anthocyanins with and without 0.5% w/w mannoproteins (MP) at pH 7.0 before and after heating at 80 or 126 °C for 30 min and (B) the degradation kinetics of anthocyanins after heating at 80 or 126 °C for 0–90 min.

Table 1
Colour parameters and absorbance at 570 nm (Abs_{570}) of 0.5% w/w anthocyanin solution at pH 7 before and after heating at 80 or 126 °C for 30 min without and with 0.5% w/w mannoproteins.^A

Solution	Parameter	Before heating	Heated at 80 °C for 30 min	Change ^B (%)	Heated at 126 °C for 30 min	Change ^B (%)
Anthocyanins only	L^*	-5.28 ± 0.05^g	3.11 ± 0.11^f	-158.9	15.1 ± 0.24^{cd}	-386.0
	a^*	-29.08 ± 0.36^f	-52.36 ± 0.11^a	80.1	-51.92 ± 0.27^b	78.5
	b^*	-47.67 ± 0.29^a	-29.06 ± 0.04^c	-39.0	-8.65 ± 0.13^f	-81.9
	Hue angle (°)	-0.07 ± 0.00^d	1.61 ± 0.01^b	2200.0	5.95 ± 0.06^a	8400.0
	a^*/b^*	0.61 ± 0.00^d	1.80 ± 0.01^b	195.1	6.01 ± 0.064^a	885.2
	Abs_{570}	0.61 ± 0.06^a	0.44 ± 0.05^b	-27.9	0.31 ± 0.03^c	-49.2
Anthocyanins and mannoproteins	L^*	13.93 ± 0.62^e	15.59 ± 0.03^c	11.9	14.39 ± 0.03^{de}	3.3
	a^*	-40.00 ± 0.32^e	-50.05 ± 0.08^c	7.6	-48.19 ± 0.13^d	20.5
	b^*	-36.41 ± 0.38^b	-28.58 ± 0.01^d	-10.5	-27.11 ± 0.07^e	-25.5
	Hue angle (°)	0.78 ± 0.03^c	1.56 ± 0.01^b	100.0	1.59 ± 0.01^b	103.8
	a^*/b^*	1.10 ± 0.02^c	1.75 ± 0.00^b	59.1	1.78 ± 0.01^b	61.8
	Abs_{570}	0.50 ± 0.01^a	0.47 ± 0.02^b	-6.0	0.38 ± 0.2^c	-24.0
Mannoprotein only	L^*	72.36 ± 0.05^b	73.18 ± 0.08^a	1.1	70.63 ± 0.72^a	-2.4
	a^*	-0.90 ± 0.01^g	-1.00 ± 0.05^g	11.2	-1.02 ± 0.03^g	13.0
	b^*	1.15 ± 0.05^g	0.90 ± 0.04^g	-21.7	-0.90 ± 0.14^g	-21.9
	Hue angle (°)	-0.30 ± 0.06^d	-0.79 ± 0.08^e	1.6	-0.84 ± 0.26^e	1.8
	a^*/b^*	-0.78 ± 0.04^e	-1.11 ± 0.06^f	0.4	-1.15 ± 0.19^f	0.5
	Abs_{570}	0.012 ± 0.002^d	0.004 ± 0.003^d	-0.7	0.007 ± 0.001^d	-0.4

^A Numbers are mean \pm standard deviation from triplicates. Different superscript letters in the same parameter indicate significant differences in the mean ($P < 0.05$).

^B Change% = $100 \times (\text{number after heating} - \text{number before heating}) / \text{number before heating}$.

3.2. Thermal degradation kinetics of anthocyanins as impacted by mannoproteins

The kinetics of anthocyanin degradation at 80 and 126 °C with and without mannoproteins is shown in Fig. 1B. The correlation coefficients (R^2) after linear regression of data according to Eq. (1) were all greater than 0.97. The first order kinetics, therefore, fit the data satisfactorily, as previously reported (Fischer, Carle, & Kammerer, 2013; Nayak, Berrios, Powers, & Tang, 2011; Verbeyst, Oey, Van der Plancken, Hendrickx, & Van Loey, 2010). The degradation rate constant and half-life of degradation were estimated according to Eqs. (1) and (2), listed in Table 2. The degradation rate at 80 °C without mannoproteins was about one order of magnitude higher than a study using black carrot anthocyanins, which may be due to protection of components in black carrot (Kırca, Özkan, & Cemeroglu, 2007). The rate of anthocyanin degradation was significantly ($P < 0.05$) higher at a higher temperature and was reduced 4.2 and 5.3-fold by mannoproteins at 80 and 126 °C, respectively. The half-life of degradation was significantly ($P < 0.05$) shorter at a higher temperature and significantly ($P < 0.05$) elongated by mannoproteins at each temperature. The half-life of degradation increased by 5.4-fold at both heating temperatures after addition of mannoproteins. As thermal degradation of anthocyanins is well-described by Arrhenius kinetics (Patras, Brunton, O'Donnell, & Tiwari, 2010), we did not attempt to study other temperatures to determine activation energy and thermodynamic parameters as impacted by mannoproteins.

In general, anthocyanins are more stable at a lower pH, and their stability is typically improved after self-association, co-pigmentation with compounds such as hydroxycinnamic acids,

flavonols, and flavone glycosides, and complexation with metallic ions such as magnesium and aluminium (Cavalcanti, Santos, & Meireles, 2011). In addition to the loss of absorbance due to hydration at the 2-position of the anthocyanidin skeleton at neutral pH (Brouillard, 1982), thermal degradation of anthocyanins has been discussed for the opening of the pyrylium ring to form a chalcone structure, deglycosylation, and cleavage of deglycosylated compounds to form products, such as protocatechuic acid, phloroglucinolaldehyde, and 4-hydroxybenzoic acid (Patras, Brunton, O'Donnell, & Tiwari, 2010). Most studies on thermal degradation of anthocyanins were conducted on sections of fruits, vegetables, and their juices that have other additional compounds present (some may act as co-pigments) and may have degradation kinetics that differ from studies using purified anthocyanins (Patras, Brunton, O'Donnell, & Tiwari, 2010). In a study, bog bilberry anthocyanin extract at pH 1–10 was heated at 80 °C for 60 min with and without casein, whey protein, and their mixture and conjugates with dextran (Zheng et al., 2009). The authors observed significant reductions of cyanidin-3-glucoside, malvidin-3-galactoside and malvidin-3-glycoside after heating at pH 8 based on high performance liquid chromatography. Although the constituents and their degradation products were not determined in the present work, it may be possible to compare our work with theirs based on the reduction of Abs_{570} . The anthocyanins in the present study (Fig. 1B) appeared to have a similar stability to their product that showed about 50% loss of Abs_{570} at pH 7 after heating for 60 min at 80 °C. The anthocyanin-mannoprotein mixture maintained 86.3% of Abs_{570} after heating at pH 7 for 60 min at 80 °C (Fig. 1B), which is higher than no more than 56.3% retention of Abs_{570} when anthocyanins were heated with caseins, whey proteins, and their conjugates.

3.3. Visible light absorption spectra of anthocyanins before and after heating

The visible light absorption spectra of anthocyanins before and after heating at 80 and 126 °C for 30 min are presented in Fig. 2A. Mannoproteins alone did not show significant Abs_{570} . The Abs_{570} of anthocyanin solution at pH 7 significantly reduced ($P < 0.05$, Table 1) and the centre of the absorption peak shifted to 550 nm (blue shift) after addition of mannoproteins (Fig. 2A). The blue shift is observed for anthocyanins in plants after aromatic acylation that also increases the stability (Tanaka, Sasaki, & Ohmiya, 2008). The

Table 2
Degradation reaction rate constant (k) and half-life ($t_{1/2}$) of anthocyanins during heating with and without yeast mannoproteins.^A

Temperature	Sample	k (min ⁻¹)	$t_{1/2}$ (min)
80 °C	Without mannoproteins	0.0114 ± 0.0010^b	50.4 ± 3.6^c
	With mannoproteins	0.0027 ± 0.0006^d	272.4 ± 22.2^a
126 °C	Without mannoproteins	0.0271 ± 0.0030^a	25.8 ± 3.0^d
	With mannoproteins	0.0051 ± 0.0009^c	143.4 ± 49.8^b

^A Numbers are means \pm standard deviations from triplicates. Different superscript letters in the same column indicate significant differences in the mean ($P < 0.05$).

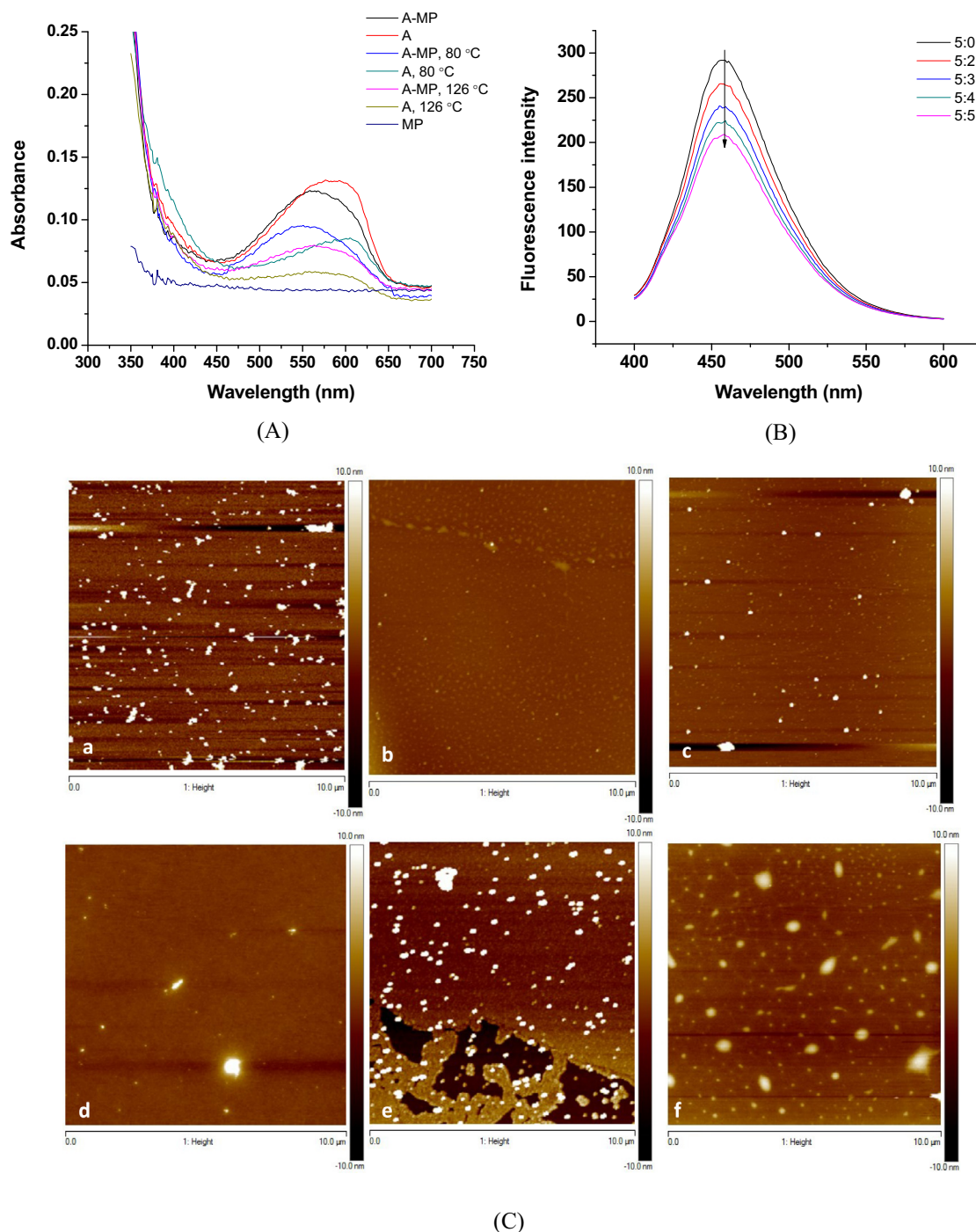


Fig. 2. (A) The visible light spectra of 0.5% w/w anthocyanins (A), 0.5% w/w mannoproteins (MP), and their equal mass mixtures (A-MP) at pH 7.0 before and after heating at 80 or 126 °C for 30 min, (B) fluorescence spectra of 0.5% w/w MP before and after mixing with A at A: MP mass ratios of 5:0, 5:2, 5:3, 5:4, and 5:5 at pH 7.0 and 21 °C, and (C) AFM topography images of MP (top) and their mixture with an equal mass of A (bottom) at pH 7.0 before (a and d) and after heating at 80 °C (b and e) or 126 °C (c and f) for 30 min.

UV-vis spectra suggest complex formation between anthocyanins and mannoproteins cause the lowered Abs_{570} and the blue shift.

After heating, a red shift (toward a longer wavelength, Fig. 2A) and a reduction in Abs_{570} (Table 1) were observed for the solution with anthocyanins only, indicating significant degradation. The red shift was insignificant after heating anthocyanins with mannoproteins at 80 °C for 30 min but was significant after heating at 126 °C for 30 min (Fig. 2A), with a significant reduction in Abs_{570} after heating at both temperatures (Table 1). The percentages of Abs_{570}

decrease after heating are listed in Table 1, showing significant improvement in heat stability of anthocyanins after mixing with mannoproteins, which agreed with visual appearance (Fig. 1A). Preliminary HPLC analysis possibly showed two types of anthocyanins in the commercial product (Supplementary Fig. S1 and Table S1). The areas of both chromatogram peaks decreased after heating, but the reduction percentage was different, which indicates the possibly different thermal stability of the two anthocyanins.

3.4. Molecular binding between mannoproteins and anthocyanins studied using fluorescence spectroscopy

As shown in Fig. 2B, the fluorescence intensity of anthocyanins decreased significantly after mixing with different concentrations of mannoproteins. The quenching of fluorescence intensity can be caused by dynamic and static mechanisms that result from intermolecular collision and complex formation, respectively (Vaughan & Weber, 1970). The fluorescence quenching data can be further analysed by the Stern–Volmer mode (Eq. (4)) (Vaughan & Weber, 1970).

$$F_0/F = 1 + k_q \tau_0 [Q] = 1 + K_{SV} [Q] \quad (4)$$

where F_0 and F are the fluorescence intensities without and with a quencher, $[Q]$ is the concentration of the quencher, K_{SV} is the Stern–Volmer quenching constant, k_q is the bio-molecular quenching constant, and constant τ_0 ($=10^{-8}$ s) is the life time of fluorescence in absence of a quencher (Vaughan & Weber, 1970).

For dynamic quenching, the maximum collisional quenching constant (k_q) of various quenchers is $2.0 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$ (Lange, Kothari, Patel, & Patel, 1998). The k_q from the anthocyanin–mannoprotein solutions estimated according to Eq. (4) was $7.67 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$. Therefore, the binding between anthocyanins and mannoproteins causes the static quenching of fluorescence, and complexes are formed between the two molecules at pH 7.0.

3.5. Physical structure changes of dispersions before and after heating

The topography characteristics of mannoproteins with and without an equal mass of anthocyanins were characterised using AFM before and after heating. The mannoprotein dispersion had irregular particles (Fig. 2C,a) with a mean dimension of 298.2 nm analysed using the AFM software. Since the average dimension of these structures is bigger than most single biopolymers, they may be aggregated mannoproteins themselves, other impurities, or both. After heating at 80 °C for 30 min (Fig. 2C,b), spherical particles were observed, and the mean dimension (90.2 nm) became smaller than that before heating, which may be caused by the improved hydration of mannoproteins or other compounds in the commercial product. After heating at 126 °C for 30 min (Fig. 2C,c), most particles were spherical and a few particles appeared to be aggregates of several particles, with the mean particle diameter (152.1 nm) bigger than that after heating at 80 °C. This suggests the aggregation of particles after heating at 126 °C but not 80 °C. Since this is a commercial mannoprotein product, we are not clear what causes the difference in the observed structural changes during heating at these two temperatures. When anthocyanins were mixed with mannoproteins (Fig. 2C,d), the structures became more heterogeneous than mannoproteins alone (Fig. 2C,a) and had a mean dimension of 279.4 nm, with the largest and smallest particles having a dimension of 360.8 and 224 nm, respectively. After heating at 80 and 126 °C for 30 min, the structures became bigger (Fig. 2C, e,f vs. a), increasing to a respective mean dimension of 320.9 and 386.4 nm. Therefore, the complexation by anthocyanins increased the thermal aggregation of mannoproteins.

To further understand structural changes of mannoproteins, caused by complex formation with anthocyanins and heating, zeta potentials of mannoprotein dispersions with and without anthocyanins were measured. As shown in Table 3, the zeta potential of mannoproteins was -31.13 mV before heating and became significantly more negative ($P < 0.05$), reducing to -42.35 and -49.53 mV after heating 80 and 126 °C for 30 min, respectively. Based on AFM (Fig. 2C), the structure of mannoproteins became smaller after heating, which enables the improved exposure of charged groups and therefore the bigger measured zeta-potential magnitude.

Table 3

Zeta potential of mannoproteins with and without anthocyanins in aqueous solutions at pH 7.0, before and after heating at 80 or 126 °C for 30 min.*

Heating conditions	Samples	Zeta potential (mV)
None	Without anthocyanins	-31.13 ± 2.08^d
	With anthocyanins	-36.83 ± 2.78^c
80 °C for 30 min	Without anthocyanins	-42.35 ± 4.37^b
	With anthocyanins	-52.08 ± 3.51^a
126 °C for 30 min	Without anthocyanins	-49.53 ± 3.47^a
	With anthocyanins	-50.70 ± 2.17^a

* Numbers are means \pm standard errors from triplicate measurements. Different superscript letters indicate significant differences in the mean ($P < 0.05$).

When mannoproteins were mixed with anthocyanins, the magnitude of negative zeta-potential (-36.83 mV) also significantly increased ($P < 0.05$, Table 3). Like mannoproteins alone, the zeta-potential of the mixture also became significantly more negative after heating, decreasing to -52.08 and -50.70 mV after heating at 80 and 126 °C for 30 min, respectively. Anthocyanins at neutral pH carry negligible charges (Lee et al., 2005) and their presence on the mannoprotein particle surface is not expected to increase the magnitude of negative zeta-potential, which is contrary to the results in Table 3. Since mannoproteins are glycosylated (Goncalves et al., 2002), anthocyanins likely bind with the protein moiety of mannoproteins by hydrophobic interactions, which changes the structure of mannoprotein particles that become more negatively charged. AFM and zeta-potential results suggest that anthocyanins are enclosed in the centre of mannoprotein structures and therefore have increased thermal stability. Specific molecular forces involving complex formation between anthocyanins and mannoproteins may require purified compounds and analytical techniques such as FTIR that has previously been used to study the affinity between proteins and phenolic phytochemicals (Li, Percival, Bonard, & Gu, 2011).

3.6. Impacts of heating on antioxidant properties of anthocyanins

The antioxidant capacity of anthocyanins before and after heating at 80 and 126 °C for 30 min with and without an equal mass of mannoproteins was evaluated for the ability to scavenge DPPH \cdot and ABTS \cdot^+ . As shown in Fig. 3, both DPPH \cdot and ABTS \cdot^+ scavenging activities of anthocyanin solutions decreased significantly after thermal treatment, more significant at a higher temperature ($P < 0.05$). This agrees with the literature that the degradation products of anthocyanins have much reduced antioxidant capacity (Zheng et al., 2009).

Mannoproteins had a weak antioxidant capacity that was not affected by heating (Fig. 3). Before heating, the mixture of mannoproteins and anthocyanins seemed to have additive effects from both components (Fig. 3). After heating, the radical scavenging capacity of the mixture did not show an apparent reduction, which may be partially contributed by the stronger antioxidant activity of the mixture than anthocyanins alone. Since the anthocyanin content decreased after heating with mannoproteins (Fig. 1B), Fig. 3 indicates the degradation products of anthocyanins are different after heating with the absence or presence of mannoproteins, which agrees with different visible light absorption spectra in Fig. 2A. The degradation products after heating with mannoproteins may have similar DPPH \cdot and ABTS \cdot^+ scavenging capacities as anthocyanins, which caused insignificant changes in the antioxidant capacity of the mixture. Identification of specific degradation products, however, requires future work using advanced analytical techniques such as nuclear magnetic resonance spectroscopy or liquid chromatography–mass spectroscopy.

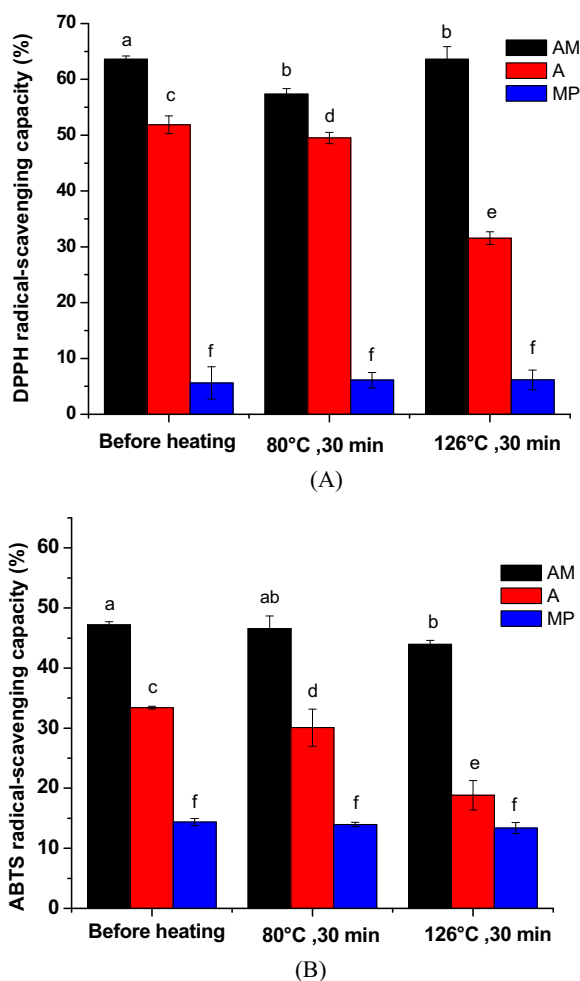


Fig. 3. DPPH[•] (A) and ABTS^{•+} (B) scavenging capacity of 0.5% w/w anthocyanins (A), 0.5% w/w mannoproteins (MP), and their mixture (AM) at pH 7.0 before and after heating at 80 or 126 °C for 30 min.

4. Conclusions

In conclusion, anthocyanins formed complexes with mannoproteins, probably by hydrophobic attraction. The complexes effectively protected anthocyanins from degradation at pH 7.0 during heating at 80 and 126 °C. The degradation of anthocyanins followed first order kinetics, and the addition of mannoproteins reduced the degradation rate constant and increased the half-life by 4 to 5-fold. The addition of mannoproteins improved the colour stability of anthocyanins by 4 to 5-fold and maintained the antioxidant capacity after heating at 80 and 126 °C for 30 min. Findings from the present work suggest that mannoproteins can be used to expand the application of anthocyanins as natural colours or functional ingredients.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2014.09.059>.

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