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Improving the water solubility of *Monascus* pigments in acidic conditions with gum arabic

Wenjie Jian [a,b,c], Yuanming Sun [c], Jian-Yong Wu [a,*]

[a] *Department of Applied Biology & Chemical Technology, State Key Lab of Chinese Medicine and Molecular Pharmacology in Shenzhen, The Hong Kong Polytechnic University, Kowloon, Hong Kong*

[b] *Department of Medical Technology, Xiamen Medical College, Xiamen, 361000, China*

[c] *College of Food Science, South China Agricultural University, Guangzhou, 510642, China*

*Corresponding author:

Telephone: (852)3400 8671; fax: (852) 2364 9932; email: jian-yong.wu@polyu.edu.hk

Abstract

BACKGROUND: *Monascus* pigments (Mps) are natural food colorants and their stability of *Monascus* pigments (Mps) in acidic solutions is important for their applications in the food industry. This study was to evaluate the use of gum arabic (GA) as a stabilizer for maintaining the solubility of Mps in an acidic aqueous solution exposed to a high temperature and to analyze the molecular interactions between GA and Mps.

RESULTS: Mps dispersed (0.2 g kg^{-1}) in deionized water at pH 3.0-4.0 without GA formed precipitates, but remained in a stable solution in the presence of GA (1 g kg^{-1}). The significant improvement of Mps water solubility in acidic conditions was attributed to the formation of pigment and GA complexes, as indicated by a sharp increase in the fluorescence intensity. The results on particle size, zeta-potential and transmission electron microscopy further suggested that molecular binding of Mps to GA, electrostatic repulsion and steric hindrance of GA prevented the aggregation of Mps in acidic solutions.

CONCLUSION: GA was proven an effective stabilizer of natural food colorants in acidic solutions.

Keywords: Natural pigments, gum arabic, solution stability, acidic pH, high temperature

Introduction

Monascus pigments (Mps), the secondary metabolites of *Monascus purpureus* or *M. anka*, have been widely used as natural food colorants in the food industry in East Asia and other regions[1-3]. Mps are composed of various azaphilone structures exhibiting yellow, orange, and red color [4, 5]. The molecular composition of Mps varies with *Monascus* strains, fermentation processes, and culture conditions [6]. So far a total of 54 Mps structures have been documented. Two red pigments, rubropunctatin and monascorubrin, represent the major constituents of Mps, which have very similar structures but only difference in the length of side chains (as shown in Supplemental data) [7]. Both rubropunctatin and monascorubrin are insoluble in water and several methods have been developed to convert them into water soluble pigments [5]. An effective method is the addition of alkaline to the alcohol used for extracting Mps from fermentation broth or fermented rice, with which the lactone bond is hydrolyzed to water-soluble pigments [8]. Most of the Mps used in the food industry have been produced by this method. The Mps remain soluble up to 150 g kg^{-1} in water at pH5.0-10.0 but become insoluble to form precipitates in an acidic condition with pH $\leq$ 4.0. In soluble form, the Mps are very sensitive to heat and light, turning pale upon exposure to sunlight and higher temperature over 90°C [9]. It is of interest to develop safe and effective methods for improving the solution stability of Mps in a wider range of pH and thermal conditions.

Biopolymers such as polysaccharides, proteins and their complexes are widely applied as stabilizers of food additives such as flavors and colorants in aqueous dispersions[10-12]. Gum arabic (GA), a polysaccharide-protein complex composed of arabinogalactan (800-900 g kg^{-1}), glycoprotein (20-40 g kg^{-1}), and arabinogalactan-protein (100-200 g kg^{-1}), is a popular emulsifier and carrier of food ingredients in the food industry[13-15] In an aqueous solution, the glycoprotein constituents of GA form a wattle blossom-like structure with the protein portion aggregating internally and the polysaccharide chain extending outward into the aqueous phase. Such an aggregate structure confers the GA macromolecule an amphiphilic characteristic, which contributes to its emulsification and encapsulation function [16]. Moreover, GA is negatively charged at pH over 2.0 and can remain in a stable solution over a wide range of acidic and ionic conditions [17]. Very recently, GA and Fe^{2+} through a synergistic action have shown effective for the improvement of norbixin thermal stability in acidic condition [18].

The present study was performed to evaluate the thermal stability of Mps in an acidic aqueous solution at pH 3.0-5.0 with the addition of GA in terms of the color and solubility. In addition, the physicochemical properties including zeta potential, particle size and morphology of the GA-Mps complexes were determined to gain further understanding of the molecular interactions and stability mechanism of the GA-Mps complex in the acidic aqueous

solution.

Materials and methods

Chemicals

Gum arabic (GA) was purchased from Sigma-Aldrich (Catalog number G9752, weight-average molecular weight 250 kDa) with insoluble residues <0.2 g kg^{-1}. Red Mps were obtained from Jiangmen Kelong Biological Technology Corporation (Jiangmen, Guangdong, China) and purified by ion exchange resin as described in our previous study[19]. All other chemicals were attained from certified suppliers with sufficient quality and purity.

Preparation of GA-Mps solution

Mps (0.2 g) and GA (1.0 g) powder were added together to 150 mL deionized (DI) water and stirred at 21 ºC for 30 min (Mps/GA mass ratio 0.2; GA concentration 6.7 g kg^{-1}). The liquid was then filtered through a Millex-HN nylon clarification kit with a pore size of 0.2 μm (Fisher Scientific). The filtrate solution was diluted 5 times with DI water, and the solution pH was adjusted to 3.0, 4.0 and 5.0, respectively, using 1.0 M HCl instead of buffer solution for convenience and reducing the interference induced by salts in buffer solution . Control without GA was prepared in parallel with the GA-Mps mixture solutions. All solutions were heated at 90°C or 121°C in glycerol bath (VLEP heater, Italy) for 30 min and then cooled in a

21ºC water bath.

Colorimetric analysis of Mps solution

Colorimetric characteristics of the Mps and GA-Mps solution were determined by spectrophotometric measurement as reported previously [9]. The absorption spectrum in the range of 390-600 nm and the peak absorbance at 490 nm was measured with an Agilent Cary 60 UV-Vis spectrophotometer.

Fluorescence spectroscopy

Fluorescence spectroscopy was performed on a spectrofluorometer (Fluoromax-4, Horiba Scientific, France). The Mps and GA samples were dissolved in DI water, and DI water was used as the blank. The emission spectra were recorded in the range of 330-550 nm with the excitation wave length set at 300 nm and the excitation and emission slit widths set at 5 nm.

Measurement of particle size and zeta-potential

Particle size of Mps and GA solutions was determined by dynamic laser light scattering (DLS) analysis on a Malvern Zetasizer Nano-ZS90 instrument at room temperature. The DLS analysis was performed with a digital auto correlator at a scattering angle of 90° and a wavelength of 533 nm to attain the hydrodynamic diameter of samples. Zeta-potential of the

sample solutions was measured in the same instrument using a folded capillary cuvette DTS1060, with five repeating measurements taken for each sample.

Transmission Electron Microscope

The sample solution was deposited on a carbon-coated copper mesh grid and then dried naturally in the open air at room temperature. The mesh grid was loaded onto a Jeol EM-2011 microscope at an accelerating voltage of 120 kV. The TEM images were recorded with a Gatan MultiScan 794 camera and processed with Gatan Digital Micrograph 3.1 software. It is well known that TEM imaging of particle morphology is based on the transmission of an electron beam through the particle specimen to reflect its three dimensional structure.

Statistical Analysis

All experiments were carried out in triplicate and the results were expressed as the mean ± standard deviation (SD). Analysis of variance (ANOVA) was performed with the SPSS 16.0 (SPSS Inc., Chicago, IL) software.

Results and discussions

Color stability of Mps solution

In our preliminary experiments, GA and several other biopolymers were tested as stabilizers

in the Mps solution in water, including xanthan, *Konjac* glucomannan, maltodextrin, sodium caseinate, bovine serum albumin with the same method as described in part 2.2. With all biopolymers except GA, Mps formed precipitates when pH was below 4.0 at 21ºC (data not shown). To our interest, no precipitation was observed in the GA-Mps solution even at a low pH of 1.0 and a Mps/GA mass ratio of 5.0. Considering the practical conditions for Mps in the food industry above pH 2.0 and the concentration 0.2 g kg^{-1}, the experiments were performed in the range of pH 3.0-5.0 at 0.26 mg/ml Mps concentration. As the suitable GA concentration was chosen at 1.4 g kg^{-1}, the Mps/GA mass ratio was set at 0.2 in the following experiments. The temperatures (90°C and 121°C) and thermal treatment period (30 min) applied in the experiments were corresponding to those for pasteurization (60 - 90°C) and pressurized steam sterilization (121°C) which are most commonly applied in the food process industry. In the present study, only the colorimetric parameters of Mps were evaluated after thermal treatment but not the discoloration constants because the later have been measured in several previous studies [9, 20, 21].

Photographs of samples before and after heating are presented respectively in Fig 1. As stated previously[20], the carboxylic radical in the backbone of Mps confers its solubility in neutral and alkaline solution. When pH is below 4.0, the Mps is sparingly soluble, and forms precipitate by aggregation. However, the Mps mixed with GA remained in clear solution at

pH 3.0-5.0. The results indicate that the acid stability of Mps in solution was improved by GA. As the pH value of GA mother solution was about pH 6.5 and Mps was highly stable in neutral condition, the Mps-GA should be in a solution state instead of microemulsion. The stability of Mps in GA solution may be attributed to the formation of a complex between GA and Mps. The steric hindrance of GA can limit the aggregation of Mps and prevent the formation of precipitates in acidic conditions.

The addition of GA into the Mps solution did not affect the solution color (Fig. 1) or the UV-Vis absorbance (Table 1). All Mps solutions with or without GA faded significantly without precipitation after heating, especially at temperature over 90°C. Heating caused a blue shift of the maximum absorbance peak (to a lower wave length) and a significant decrease in the absorbance intensity, corresponding to the loss of solution color (Fig. 1). At pH 3.0, the peak absorbance of Mps solution (at 490 nm wavelength) after the thermal treatment was decreased by 10.7% at 90°C and 66.5% at 121°C, respectively (Table 1). The color loss of Mps solution was attributed to thermal degradation of Mps molecules, such as the disruption of the ester linkage in the Mps azaphilone structure [20]. While thermal treatment caused significant color loss of the Mps solution, the acid pH condition at a constant temperature did not have a significant effect on the color ($P<0.05$). This is in agreement with the report by [22] and attributed to the stability of azaphilone chromophore of Mps in acidic

conditions.

Overall the results showed that the GA improved the aqueous solubility of Mps in acidic conditions but not the thermal stability. In a recent study by Guan and Zhang (2014), the GA and Fe^{2+} in combination synergistically improved the thermal and acidic stability of a natural pigment, norbixin. However, no significant improvement of the color stability was observed in our preliminary experiments when we added several metal ions such as Fe^{2+}, Mg^{2+}, Ca^{2+} and Cu^{2+} and vitamin C at 0.15 mM individually to the Mps-GA solutions at pH 3.0-5.0, (data not shown). Our observation was consistent with that from the similar tests on Mps at pH 4.0-7.0 reported previously [20]. The different effects of GA and metal ions on the acidic solubility and thermal stability of Mps and norbixin may be due to the different structures of the two pigments and their interactions with GA in the solution.

Fluorescence spectra of the Mps-GA solutions

Fig. 2 presents the fluorescence spectra of GA with or without Mps before and after heating at 90 °C and 121°C for 30 min. The fluorescence spectrum of GA peaked at 426 nm and the intensity showed a linear correlation to the concentration. No fluorescence was observed with Mps when exited at 300 nm (data not shown). At room temperature and with thermal treatment at 90°C for 30 min, fluorescence enhancement occurred only when the mass ratio

of Mps/GA was 0.1 or below. When the mass ratio Mps/GA was over 0.1, the fluorescence intensity was decreased sharply, and was completed quenched when the ratio reached 0.5 (Fig. 2a and Fig. 2b). The similar phenomenon was observed with thermal treatment at 121°C for 30 min at a higher mass ratio of 0.1 (Fig. 2c).

The alteration between enhancing and quenching mode of fluorescence spectrum observed with the variation of GA/Mps mass ratios suggests the existence of two major binding sites between GA and Mps. The molecular interactions between GA and Mps may occur through binding sites in both the protein moiety and polysaccharide chain of GA. In contrast, only fluorescence quenching but no enhancement effect was found with the norbixin-GA complex reported previously [18].

Fluorescence spectrometry provides useful information for the non-bonded interactions between molecules, which cause two major fluorescence effects, enhancement and quenching, due to different interaction mechanisms [23]. The fluorescence quenching may be attributed to either intermolecular collision or complex formation, which can be identified via Stern-Volmer equation [24],

$$F_o/F = 1 + k_q \tau_o [Q] \quad (1)$$

where F and F_o are the fluorescence intensity units with and without a quencher, respectively,

[Q] the concentration of the quencher, k_q the quenching constant, and τ_o (=10^{-8} s) the life time of fluorescence in the absence of any quencher. Quenching is attributed to complex formation at $k_q > 2.0\times10^{10}\ M^{-1}s^{-1}$, and to intermolecular collision at lower k_q. The k_q values of GA-Mps solutions derived from equation 1 (Table 2) were in the range of 3.77×10^{12}–$3.10\times10^{12}\ M^{-1}s^{-1}$ at pH 3.0-5.0 before heating, but were decreased significantly to 2.21×10^{12}–$1.85\times10^{12}\ M^{-1}s^{-1}$ after heating for 30 min at 90ºC and more significantly to 0.93×10^{12}–$0.62\times\times10^{12}\ M^{-1}s^{-1}$ after heating at 121ºC for 30 min.

Nevertheless, all the k_q values of the GA-Mps solutions were over $2.0\times10^{10}\ M^{-1}s^{-1}$, indicating that the fluorescence quenching can be attributable to complex formation between Mps and GA via binding interactions. Furthermore, the significant decrease of k_q values after thermal treatment suggests the decrease in the number of binding sites between GA and Mps. Similarly, heating also resulted in the decrease of k_q value in the norbixin-GA complex solution [18]. According to Stern-Volmer equation, higher k_q value suggests more binding sites and stability between two components. Thus, the observed decrease of k_q values after thermal treatment indicated the lower binding stability of Mps with GA at high temperature, partly induced by thermal degradation of Mps molecules.

Changes in GA particle size

Particle size is an important characteristic of biopolymers that is closely related to aggregation or dispersion and molecular interactions in solution. Therefore, the GA particle size may also be affected by the addition of Mps to the solution due molecular interactions between GA and Mps. Fig. 3 shows the number-average mean diameters of GA in aqueous solution with and without Mps at various pH and temperature conditions. In solution without Mps (Fig. 3a), the particle size of GA was always less than 50 nm before and after heating at pH 3.0–5.0 and was also significantly influenced by both temperature and pH (P <0.05). The pH value seemed no effect on the particle size of GA before and after heating at 90°C, but a significant increase was observed at pH 3.0 after heating at 121°C, rather higher than those at pH 4.0- 5.0 (P <0.05). These results indicated that GA could be stably dispersed in the above conditions, despite of the partly aggregation promoted by heating and low pH value.

However, the particle size of GA showed a notable increase in the presence of Mps in comparison with the GA solution without Mps (P <0.05) (Fig. 3b). Temperature had no significant effect on the particle size of GA-Mps solutions except at pH 4.0. At pH 4.0, a marked increase in the particle size of GA-Mps was found after heating at 121°C comparing to heating at 90°C and before heating (P <0.05). Furthermore, a maximum particle size was always revealed at pH 3.0 regardless of the temperature of heating. By comprehensive

analysis, it was found that the maximum particle size of GA-Mps solution occurred at pH 3.0 after heating at 121°C for 30 min. Nevertheless, the number-average mean diameter of GA-Mps solutions at all pH levels was smaller than 90 nm both before and after thermal treatment. Such a small averaged particle size is desirable for the formation of a stable solution or dispersion. These findings fully demonstrated the stable solubility of GA-Mps observed at Fig. 1.

Overall the experimental results (Fig. 3a and Fig. 3b) indicate that Mps had a much more significant influence than heating and pH change on the particle size of GA. Significant increase in the particle size of GA with the addition of Mps may be attributed to the interaction and complex formation between GA and Mps which promoted the aggregation of GA particles. On the other hand, the formation of GA-Mps complexes was effective to prevent the aggregation of Mps molecules and to maintain a stable solution at acidic pH. An even more significant increase in the GA particle size was found in the norbixin-GA solution, from < 80 nm to 300 nm hydrodynamic diameter [18]. However, the large particle size of norbixin-GA complexes resulted in the instability of norbixin-GA complexes and the precipitation of norbixin. In comparison, the average particle size of GA-Mps complexes at all conditions was smaller than 90 nm, conferring its higher stability in the solution.

The significant effect of pH on the hydrodynamic diameter of GA was similar to that reported by Guan[18], but much less significant effects have been reported by others [25-27]. The discrepancies in the acidic pH effects with previous studies are most probably attributed to differences in the experimental and measurement methods and in the actual properties of GA material. The composition and properties vary with the source plants including the species, age and the plantation environment [28].

Polydispersity index is another important characteristic of biopolymer size distribution. As shown in Fig. 3c, the polydispersity index of GA in all conditions was in the range of 0.40- 0.60. This range is common for biopolymers, indicating the multimodal size distribution of GA. The polydispersity index was not significantly affected by the temperature change but decreased with the decrease of pH from pH 3.0 to pH 5.0. The effect of pH change was probably attributed to the GA aggregation as shown in Fig. 3a. Similarly to the change in particle size, the polydispersity index of GA showed a significant increase with the addition of Mps (Fig. 3d), due probably to the aggregation through the formation of GA-Mps complexes. Thermal treatment reduced the polydispersity index of GA-Mps with the maximum of 0.70 occurring at pH 3.0 and 21°C, which is the threshold value of suitable polydispersibility index. Overall, a suitable polydispersibility index (<0.70) and number-average diameter (< 90 nm) in GA and GA-Mps prevented the formation of large GA aggregates to maintain a stable solution.

Changes in zeta potential of GA

Electrostatic charge is another important property of biopolymers affecting molecular interation, aggregation and particle size in solution [29]. Therefore, the zeta potential of GA-Mps solution was measured to determine the nature and magnitude of electrastatic charges in various conditions. As shown in Fig. 3e, GA exhibited negative zeta potentials (being negatively charged) at pH 3.0-5.0 and the negative charge increased with the increase in pH (or increase in the concentration of OH^-). The negative zeta potential of GA was decreased slightly by heating from -15.8 ~ -33.0 mV to -14.2 ~ -31.3 mV at 90 °C and to -10.4 ~ -30.7 at 121°C. Similarly heating also decreased the negative zeta potential of GA-Mps solution (Fig. 3f). The addition of Mps significantly increased the negative zeta potential of GA ($P<0.05$) because of the negative charges in Mps molecules and the formation of GA-Mps complexes. This implied Mps favored the stable dispersion of GA by electrostatic charge despite of the increased particle size. The COO- groups on the surface of polysaccharides should be accountable for the negative charge of GA[17] and their protonation at lower pH value led to the decrease in negative charge [30]. Despite the decrease in negative charge of GA, the steric hindrance of the polysaccharide chain and the suitable particle size (<90 nm) and polydispersibility index (<0.70) maintained the stability of GA solution. Therefore, zeta potential measurement together with the above molecular binding and particle

size analyses have shown that Mps in acidic solutions can be stabilized by the molecular binding and steric repulsion of GA.

Changes in particle morphology

Transmission electron microscopy (TEM) was performed to detect the morphological characteristics of GA and GA-Mps particles in water. Fig. 4a shows the TEM images of GA and GA-Mps particles at pH 3.0 before and after thermal treatment. Free GA particles were in spherical shape with different diameters and the shape was not significantly affected by heating at 90°C for 30 min. After heating at 121°C for 30 min the GA particles formed larger aggregates with diameter increased from 30 nm to 50 nm (Fig. 4a). The similar TEM images of GA on carbon background have been reported previously [31]. Furthermore, clear TEM images of GA and sodium caseinate mixtures have also been documented in the literature [32], with a lower concentration of GA sample solution (GA: 1 g kg^{-1}; sodium caseinate: 1 g kg^{-1}) than in this study (GA: 1.3 g kg^{-1}).

As for the GA-Mps solutions (Fig. 4a), GA-Mps particles also presented spherical morphology with larger diameters at room temperature and were not significantly affected by thermal treatment. Similar morphology was also observed for the GA and GA-Mps solution at pH 4.0 (Fig. 4b) and pH 5.0 (Fig. 4c). Uniform and spherical fine particles were observed

for GA at pH 4.0 and pH 5.0 before and after thermal treatments, whereas the addition of Mps promoted the aggregation of GA leading to the formation of relatively large and irregular particles. These further confirmed the observed result that polydispersity index of GA increased with addition of Mps (Fig. 3d). Therefore, the results of TEM images were consistent with the results of particle size distribution as shown in Fig. 3.

Besides the above TEM images, AFM (atomic force image) images of GA with Mps were also determined (Supplement data). In addition to TEM image, AFM image is also an important tool in observation of biopolymer's morphology. As revealed in AFM image of GA with Mps in pH3.0 after heating at 121°C for 30 min, spherical particles with varying heights occurred. About 50% of particles' heights were 40nm, as found in height distribution of GA particles with Mps, and all of the heights were within 100nm. The similar phenomenon was also observed for GA with or without Mps in other conditions, and the topography and height of particles varied according to the treatment conditions (Data not shown). These observations were in accordance with literature [18], which judged the changes of GA particles upon treatment using height of particles in AFM images.

The particle morphology was attributed to the state of aggregation of the GA polysaccharide chains in solution. The relatively uniform and spherical GA particles were formed by

aggregation of several GA molecules. Mps molecules were attached to the GA particles by molecular binding to form GA-Mps complexes, which further promoted the aggregation of free particles to form even larger particles. With the electrostatic repulsion and steric hindrance provided by GA molecules, further aggregation of larger GA-Mps particles was inhibited when there were changes in pH or temperature. In other words, molecular binding between GA and Mps and the intrinsic property of GA were the chief factors for maintaining the Mps solution stability.

Indeed, a suitable LC-MS system would be an effective quantification of Mps. We attempted to do LC-MS analysis for Mps with addition of GA, but it failed and none successful tests were done. Generally, the condition for analysis of Mps using LC-MS included ODS C18 column, and an elution gradient of distilled water/methanol from 100:0 to 30:70 [33, 34]. However, the condition is not suitable for GA-Mps, as GA would precipitate and form larger particle in the presence of organic alcohol such as methanol and ethanol [35], which blocks the column. Alternatively, gel permeation chromatography is not suitable either, which use salt solution as mobile phase for analysis of polysaccharides [36], whereas, non-volatile salt is prohibited for mass spectrometry [5]. Furthermore, the weighted average molecular weight of GA is about 250 kDa, far higher than the maximum mass limit of MS spectrometry (6000 Da). Hence, it is unsuitable to quantitatively analyze Mps in the presence of GA based on the

current technology.

According to above results and discussion, we propose a schematic model to illustrate the interactions between GA and Mps and the structures of GA-Mps complexes formed in the aqueous solution (Fig.5). The amphiphilic nature of GA macromolecules allows for their aggregation through hydrophobic interaction of the protein moieties and the extension of the hydrophilic polysaccharide chains into the water phase, forming a wattle blossom-like structure. The quenching constant values (k_q>2.0×10^{10} M^{-1}s^{-1}) were indicative of molecular binding between GA and Mps responsible for the formation of stable GA-Mps complexes. Such binding interaction is favorable for aggregation of GA molecules to form larger aggregate particles, which may be contributable to the particle size increase (from about 47 nm to 80 nm). The change in fluorescence (from enhancement to quenching) suggests the existence of two major binding sites of Mps to GA, one on the polysaccharide chain (major portion) and the other on the protein moiety (small portion). The molecular binding keeps Mps well distributed on the various parts of GA and the steric hindrance inhibits the aggregation of Mps in acidic conditions. As a result, the precipitation of Mps is prevented and solution is maintained. Based on all above results and discussion, suitable particle size (<90 nm) and electrostatic repulsive potential (−15 mv to −40 mv) were all favorable and contributing factors for maintaining stable dispersion of GA-Mps in various conditions.

Conclusions

The results have shown that the acid stability of Mps in an aqueous solution could be significantly improved by the addition of gum arabic (GA) at a suitable concentration (1 g kg^{-1}). The addition of GA had no significant influence on the color of Mps solutions or the thermal stability of Mps solution at 90°C or a higher temperature. Addition of metal ions was not effective to prevent the color loss and to maintain the color stability. The mechanisms for the improved acid stability of Mps with GA were most probably attributed to the formation of GA-Mps complexes through molecular binding interactions and also to the prevention of aggregation of Mps by electrostatic repulsion and steric hindrance offered by the GA polymer chains. Overall the present study has demonstrated the potential of gum arabic as an effective additive for improving the acid stability of natural food colorants such as Mps.

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<Figure captions>

Fig. 1. Photographs of Mps solutions with or without GA at pH 3.0-5.0 before and after heating at 90 or 121 °C for 30 min.

Fig. 2. Fluorescence spectra of GA-Mps at various mass ratios in acidic aqueous solution: (a) at room temperature (21°C); (b) after heating at 90 °C for 30 min; (c) after heating at 121°C for 30 min. (Fluorescence spectroscopy performed at 300 nm excitation and 5 nm excitation, and emission slit width)

Fig. 3. Properties of GA and GA-Mps particles in acidic aqueous solutions before and after heating at 90°C and 121°C for 30 min: particle sizes (number-average diameters) of GA (a) and GA-Mps (b); polydispersibility index of GA (c) and GA-Mps (d); zeta potential of GA (e) and GA-Mps (f).

Fig. 4. TEM images of GA (upper) and GA-Mps (down) in aqueous solutions at pH 3.0 (a), 4.0 (b) and 5.0 (c) before (left) and after heating for 30 min at 90 °C (center) or 121 °C (right).

Fig. 5. A hypothesized schematic model for the interactions between GA and Mps and the structures of GA-Mps complexes formed in an aqueous solution.

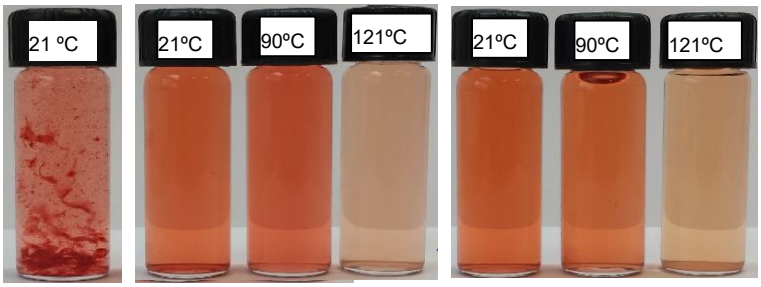
Table 1 Spectrophotometric (calorimetry) properties of Mps solution with or without GA at pH 3.0-5.0 before and after heating at 90 or 121°C for 30 min.

Heating temperature	pH	Maximum absorbance wavelength, nm		Absorbance at 490 nm, AU_{490}	
		Mps	GA-Mps	Mps	GA-Mps
None	3	N/D	498.7±1.2	N/D	1.03±0.21
	4	498.0±1.3	497.3±1.2	1.14±0.20	1.08±0.32
	5	495.3±1.6	496.0±1.7	1.17±0.15	1.11±0.14
90°C	3	N/D	498.0±1.4	N/D	0.92±0.13
	4	496.3±1.4	495.0±.1.5	1.10±0.15	0.95±0.11
	5	494.3±1.1	492.0±1.2	1.06±0.17	1.01±0.23
121°C	3	N/D	495.3±1.9	N/D	0.35±0.10
	4	494.3±1.0	492.0±1.4	0.34±0.10	0.37±0.13
	5	488±1.2	488.0±1.2	0.46±0.20	0.48±0.14

*N/D: not determined due to precipitation.

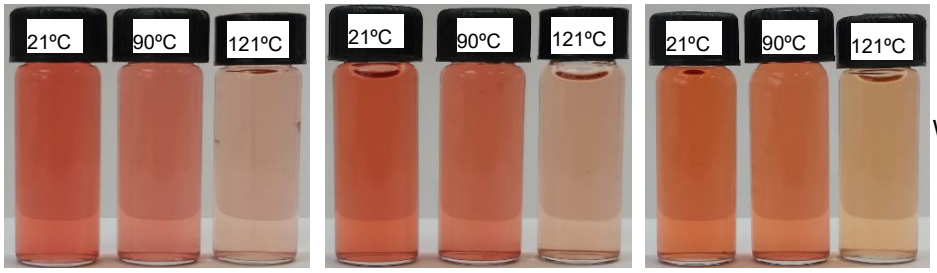
Table 2 Quenching constants k_q of GA-Mps solutions before and after heating at 90 and 121ºC for 30 min

Heating temperature	pH	k_q (10^{12} $M^{-1}s^{-1}$)
None	3.0	3.10±0.07
	4.0	3.77±0.04
	5.0	3.76±0.06
90ºC	3.0	1.80±0.08
	4.0	2.21±0.08
	5.0	1.85±0.07
121ºC	3.0	0.93±0.05
	4.0	0.79±0.04
	5.0	0.62±0.05



Without GA

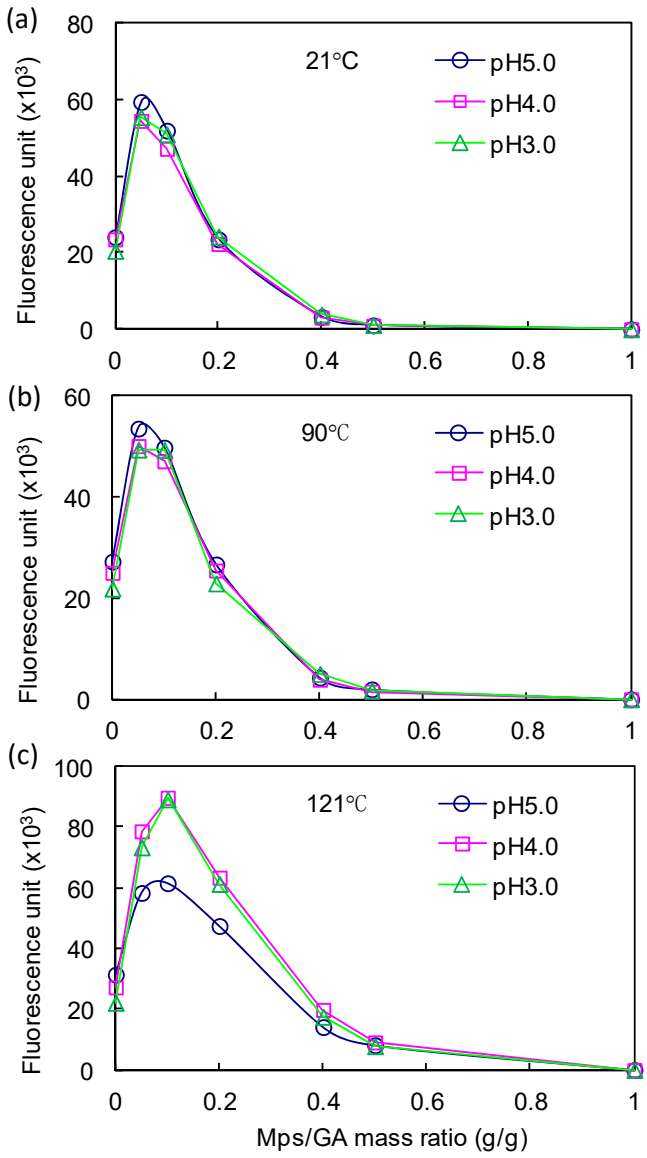
pH3.0 pH4.0 pH5.0



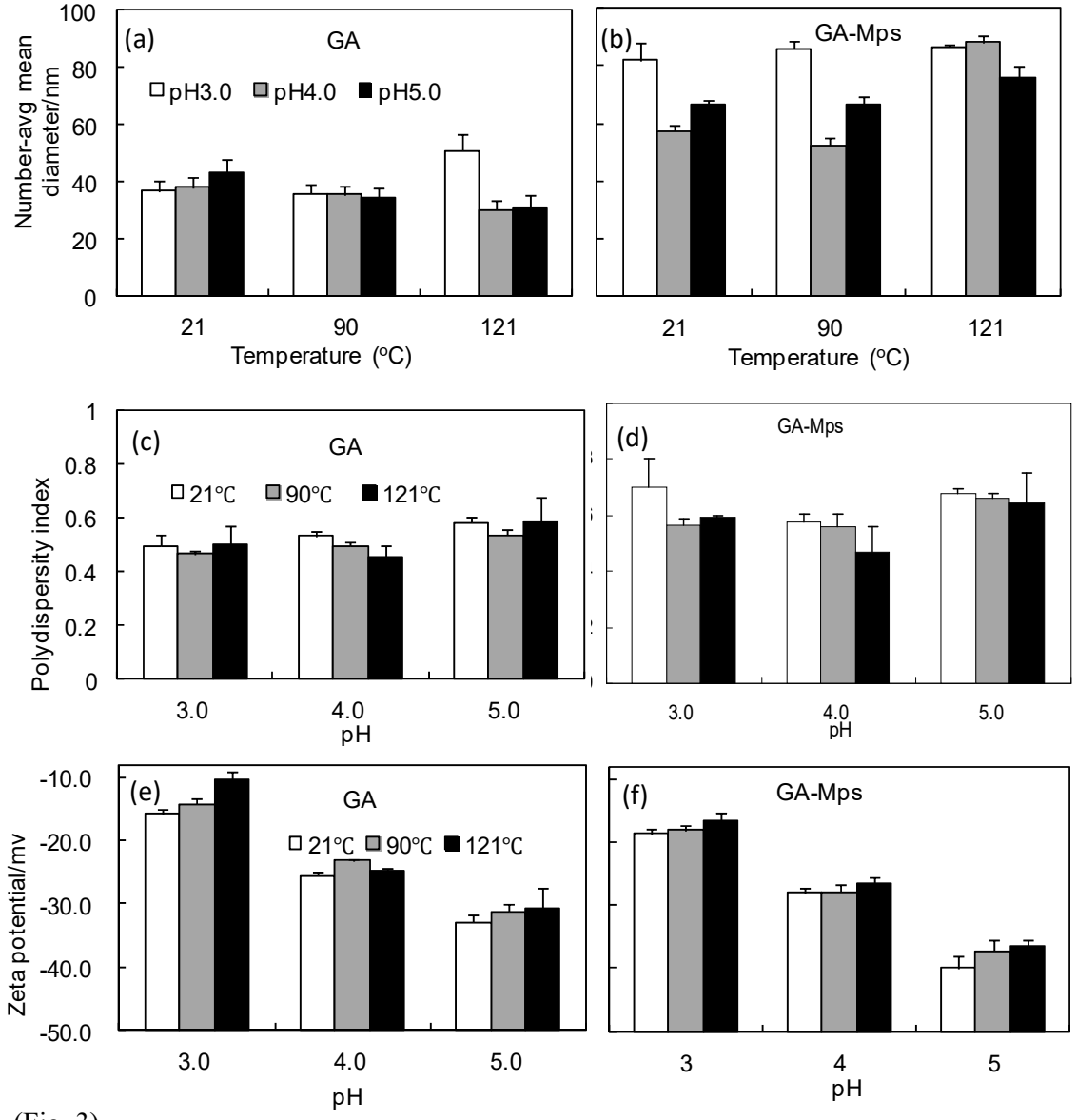
With GA

pH3.0 pH4.0 pH5.0

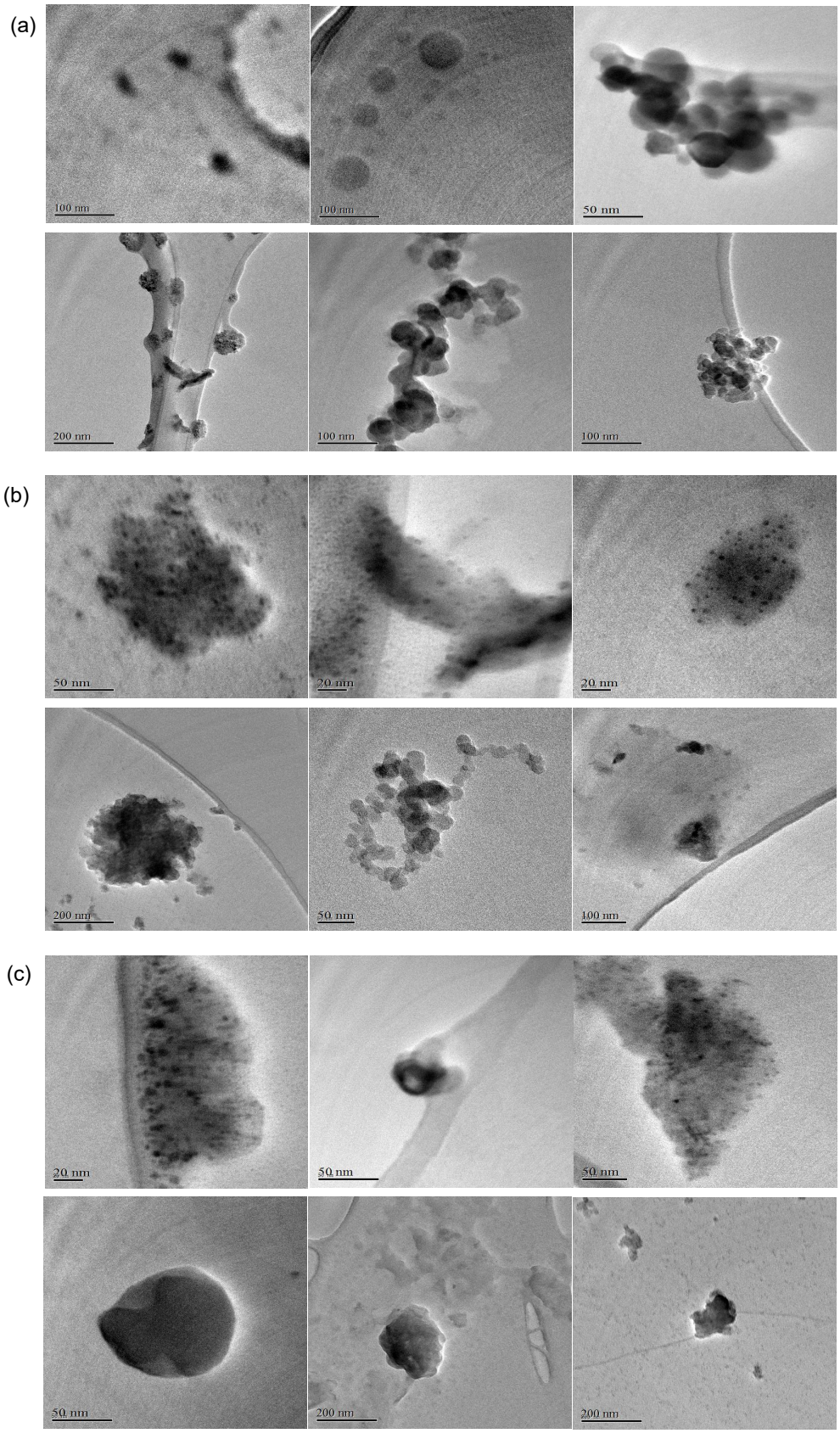
(Fig.1)


(a)
21°C
pH5.0
pH4.0
pH3.0
Fluorescence unit (x10³)
80
60
40
20
0
0
0.2
0.4
0.6
0.8
1
(b)
90℃
pH5.0
pH4.0
pH3.0
Fluorescence unit (x10³)
60
40
20
0
0
0.2
0.4
0.6
0.8
1
(c)
121℃
pH5.0
pH4.0
pH3.0
Fluorescence unit (x10³)
100
80
60
40
20
0
0
0.2
0.4
0.6
0.8
1
Mps/GA mass ratio (g/g)

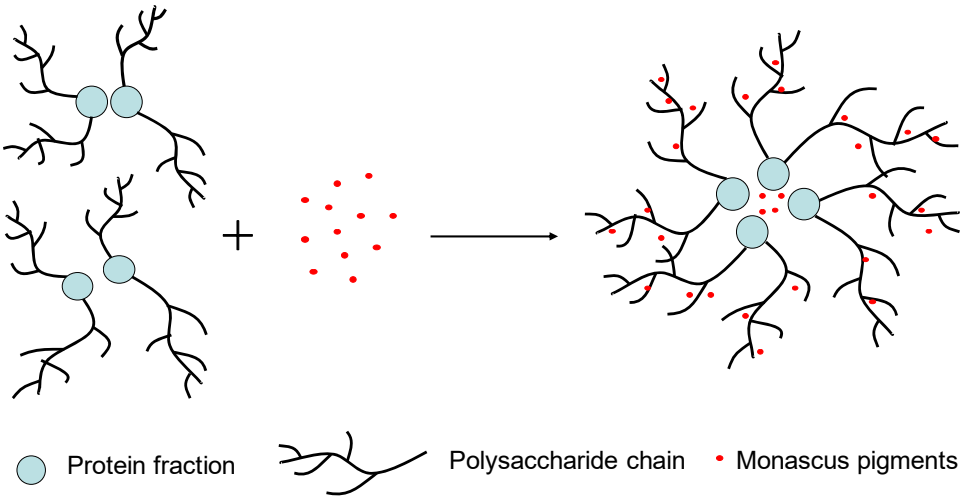
(Fig. 2)


(a)
GA
pH3.0
pH4.0
pH5.0
Number-avg mean diameter/nm
100
80
60
40
20
0
21
90
121
Temperature (°C)
(b)
GA-Mps
21
90
121
Temperature (°C)
(c)
GA
21℃
90℃
121℃
Polydispersity index
1
0.8
0.6
0.4
0.2
0
3.0
4.0
5.0
pH
(d)
GA-Mps
3.0
4.0
5.0
pH
(e)
GA
21℃
90℃
121℃
Zeta potential/mv
-10.0
-20.0
-30.0
-40.0
-50.0
3.0
4.0
5.0
pH
(f)
GA-Mps
3
4
5
pH

(Fig. 3)


(a)
100 nm
100 nm
50 nm
200 nm
100 nm
100 nm
(b)
50 nm
20 nm
20 nm
200 nm
50 nm
100 nm
(c)
20 nm
50 nm
50 nm
50 nm
200 nm
200 nm

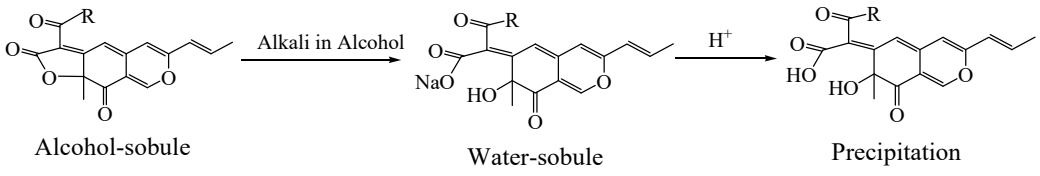
1 (Fig. 4)


Protein fraction
Polysaccharide chain
Monascus pigments

(Fig. 5)

Supplemental data:

1. Chemical structure of major Mps



R=C_7H_{15} Monascorubin ; R=C_5H_{11} Rubropunctation

2. AFM image of GA with Mps in pH3.0 after heating at 121°C for 30 min, (a) 2D image; (b) 3D image; (c) Distribution of particles' heights