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Lipophilization and molecular encapsulation of *p*-coumaric acid by amylose inclusion complex

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ABSTRACT

p-Coumaric acid (CA) is a natural phenolic compound with a wide range of bioactivities, but its tendency to degrade during food processing and storage limits its application in functional foods. Forming amylose-guest inclusion complex is a technique to molecularly encapsulate guest molecules of interest and protect them from adverse environmental challenges. However, raw CA cannot be encapsulated into the helical cavity of amylose so this study used a strategy of lipophilization of CA to synthesize hexadecyl *p*-coumarate (HC), which might complex with amylose. The formation of the amylose inclusion complex was studied by complementary techniques: differential scanning calorimetry (DSC), X-ray diffraction (XRD), and Fourier transform infrared (FTIR) spectroscopy. To test the photo-stability of CA in the complex, a direct UV irradiation was performed. The *in vitro* release behavior of CA in the complex was also studied in both simulated gastric and intestinal fluids. The results showed that HC, but not CA, could form inclusion complex with amylose. Compared with raw CA, HC, and amylose-HC physical mixture, HC complexed with amylose showed a significant improvement in photo-stability ($P < 0.05$), with 90% retained, double the 43% retention of raw CA after 72 h of direct UV irradiation, and exhibited higher release rates of CA during *in vitro* digestion. These findings suggest that the amylose inclusion complex is a promising technique to protect CA in foods during processing and storage and to increase its bioaccessibility.

Keywords: p-Coumaric acid; Amylose; Lipophilization; Molecular encapsulation.

1. Introduction

p-Coumaric acid (CA), also known as trans-4-hydroxycinnamic acid, is a phytochemical present in cereals, fruits and vegetables such as wheat, apples and potatoes, which form a vital part of our daily diet (King & Young, 1999). Recent studies have reported that CA possesses a wide range of bioactivities, such as antioxidant (Cinzia, et al., 1995), anti-inflammatory (Pragasam, Venkatesan, & Rasool, 2013), anti-apoptotic (Peng, et al., 2018), antiplatelet (Luceri, et al., 2007), anti-nephrotoxic (Navaneethan & Rasool, 2014) and cardioprotective activities (Prasanna, Krishnan, & Rasool, 2013). Therefore, CA has the potential to be formulated into functional foods and nutritional supplements to improve health and prevent chronic diseases. However, CA is not stable and its properties can be compromised by environmental factors, such as light and oxygen, under certain processing and storage conditions.

To protect bioactive compounds from degradation and increase their stability and bioavailability, several encapsulation methods have been developed. For example, soluble starch has been used to formulate nanocomposite, which could increase the water solubility and stability of curcumin (Li, Shin, Lee, Chen, & Park, 2016) and a catechin-β-cyclodextrin inclusion complex has been fabricated to increase the storage stability of catechin (Ho, Thoo, Young, & Siow, 2017). The amylose inclusion complex is another effective encapsulation method, but has not yet been extensively studied, especially its ability to encapsulate and protect phenolic compounds.

Amylose is a linear polymer of glucose units connected by α-(1, 4)-glycosidic bonds. The polysaccharide can crystallize into A- and B-type allomorphs that both

consist of 6-fold left-handed double helices which are packed in parallel (Le, Choisnard, Wouessidjewe, & Putaux, 2018). Amylose also exists as single helix when crystallizing with some linear organic molecules, such as lauric acid, palmitic acid (C16), and stearic acid (Kong, Lee, Kim, & Ziegler, 2014; Lesmes, Cohen, Shener, & Shimoni, 2009; Zhang, Huang, Luo, & Fu, 2012). When forming inclusion complex, amylose adopts a left-handed single helical structure with a hydrophobic helical cavity, where the linear compound can be accommodated through the hydrophobic force between them (Putseys, Lamberts, & Delcour, 2010). It has been suggested that the amylose molecule can protect the entrapped guest molecules against adverse environmental challenges and regulate their release (Lesmes, Barchechath, & Shimoni, 2008; Lesmes, et al., 2009).

CA is relatively hydrophilic and adopts a bulk phenyl group, which cannot be entrapped by amylose directly. Therefore, to enable the amylose to encapsulate CA, a strategy of lipophilization of CA was conducted in the present study. CA was lipophilized by grafting on a C16 alkyl chain (provided by 1-hexadecanol) to obtain hexadecyl *p*-coumarate (HC). The ^{1}H-NMR spectrum and mass spectrum were used to confirm the synthesis of the target compound. The resultant samples were then examined by complementary techniques: differential scanning calorimetry (DSC), X-ray diffraction (XRD), and Fourier transform infrared (FTIR) spectroscopy, to verify the successful formation of the inclusion complex. The photo-stability and release behavior of CA in the inclusion complex were investigated to evaluate the effectiveness of this encapsulation technique.

2. Materials and methods

2.1. Materials

Potato amylose (free from amylopectin), *p*-coumaric acid, 1-hexadecanol, pancreatin from porcine pancreas (8 × United States Pharmacopeial Convention specifications), and pepsin (501 U/mg) were purchased from Sigma-Aldrich (St. Louis, MO, USA), triphenyl phosphine and diethylazodicarboxylate from TCI Co. (Shanghai, China), acetonitrile from J&K Scientific (Beijing, China), and tetrahydrofuran (THF), dimethyl sulfoxide (DMSO) and all other reagents of analytical grade from Sinopharm Chemical Reagent Co Ltd. (Beijing, China).

2.2. Lipophilization of CA

CA was lipophilized by the Mitsunobu reaction with some modifications (Katsumi, et al., 2009). 1-Hexadecanol (242 mg, 1 mmol), CA (328 mg, 1 mmol), and triphenylphosphine (524 mg, 2 mmol) were dissolved in THF (7 mL) by stirring. A solution of diethylazodicarboxylate (0.38 mL, 2 mmol) in THF (3 mL) was then added at 0 °C for 15 min then the mixture was stirred for 1 h. The reaction mixture was concentrated *in vacuo*, diluted with 25 mL ethyl acetate, washed with brine, dried, filtered then concentrated into solid form. The residue was then purified by silica gel column chromatography (hexane/ethyl acetate = 9/1) and concentrated to form a white solid powder. The mass and ^{1}H-NMR spectra were recorded to confirm that the synthesized product was HC (Fig. 1).

2.3. Preparation of amylose inclusion complex

The DMSO method was used to prepare the amylose inclusion complex with

slight modifications (Kong & Ziegler, 2014b; Ma, Floros, & Ziegler, 2011). Amylose (500 mg) was dissolved in 10 mL of 95% (v/v) aqueous DMSO by stirring at 90 °C for 30 min. Then 1 mL of pre-dissolved CA or HC (50 mg) solution in 95% (v/v) aqueous DMSO was mixed into the amylose dispersion. The mixture was vigorously stirred for 2 min using a vortex stirrer. The mixture was held at 90 °C for 30 min, diluted with 25 mL of deionized water at 90 °C then held for another 15 min before being allowed to cool to room temperature (20 °C) over at least 24 h. The precipitate was collected by centrifugation at 3000 × g for 10 min and washed 3 times with 50% (v/v) aqueous ethanol. The resulting pellet was transferred into an aluminum dish with a small amount of ethanol then allowed to dry at room temperature (20 °C) in a desiccator. The dried samples were pulverized into a fine powder for further analysis.

2.4. Differential scanning calorimetry

The thermal properties of the samples were studied using a Discovery DSC 250 differential scanning calorimeter equipped with an autosampler (TA Instruments, New Castle, DE, USA). Approximately 5 mg of each sample was weighed into a 60 µL stainless steel pan and deionized water added to obtain a 10% (w/v) suspension. For the raw CA and HC samples, deionized water was added to obtain a 5% (w/v) solution/dispersion. The pan was hermetically sealed then equilibrated at room temperature (20 °C) for at least 2 h. The samples were analyzed by heating from 20 °C to 150 °C at a rate of 5 °C/min. The data were analyzed using TA Trios software (TA Instruments).

2.5. X-ray diffraction analysis

Wide angle X-ray diffraction patterns were obtained using a Bruker D8 Discover X-ray diffractometer (Bruker Corporation, Billerica, MA, USA). Dried sample powders were exposed to Co Kα radiation (0.179 nm) then the data were converted to correspond to 2θ values based on Cu Kα radiation (0.154 nm).

2.6. Fourier transform infrared spectroscopy

Fourier transform infrared spectra of the samples were obtained using a PerkinElmer Spectrum 100 FTIR spectrometer (PerkinElmer Inc., Waltham, MA, USA) with an attenuated total reflection accessory. Spectra were recorded in the wavelength region between 650 and 4000 cm^{-1}. Each spectrum was obtained by averaging 32 scans at a resolution of 4 cm^{-1}.

2.7. Quantitative determination of p-coumaric acid and hexadecyl p-coumarate by High Performance Liquid Chromatography (HPLC)

All samples were analyzed as described in a previous study (Moron, Pozo-Morales, Benito Mora, Garvi, & Lebrato, 2018). The assay was carried out using a Shimadzu LC-20AT HPLC instrument (Kyoto, Japan) and an Agilent C-18 reverse phase column (250 mm × 4.6 mm × 5 µm) with UV detection at 280 nm for CA and 300 nm for HC. The injection volume was 10 µL, and an isocratic system using acetonitrile was used as the mobile phase at a flow rate of 1 mL/min. The calibration curve with CA and HC standard solutions ranging from 0 to 200 µM in concentration was constructed to quantify the amount of CA and HC.

2.8. Loading capacity determination

The total CA content encapsulated in the inclusion complex was determined using solvent extraction followed by HPLC measurement. About 10 mg of the inclusion complex powder was dissolved in 15 mL DMSO then diluted with 35 mL acetonitrile. The solution was treated in an ultrasonic cleaner for 15 min to extract the encapsulated HC. The sample was measured by the HPLC method described above (2.7). The loading capacity was calculated by the following formula:

$$\text{Loading capacity (\%)} = \frac{\text{Weight of HC}}{\text{Weight of amylose}} \times 100\%$$

2.9. Photo-stability analysis

The photochemical stability of the amylose-HC inclusion complex was estimated in comparison with CA, HC and amylose-HC physical mixture. The test was based on a previous study with some modifications (Li, et al., 2016). Samples (about 50 mg) were weighed into a small glass vial then subjected to UV irradiation (254 nm) from a UV lamp (16 W) at room temperature for 72 h in the dark. The distance between the samples and the UV lamp was 5 cm. After irradiation for 6, 12, 24, 48 and 72 h, the amylose-HC physical mixture and inclusion complex samples were dissolved in DMSO while the CA and HC samples were dissolved in acetonitrile to determine the retention of CA or HC using the HPLC method described above (2.7). The CA or HC content in each sample without UV treatment was regarded as 100% CA retention.

2.10. In vitro release of CA

The *in vitro* digestion was conducted in simulated gastric and intestinal fluids to compare the release profile of the inclusion complex with references, using methods in previous studies with some modifications (Jain, Jain, Gupta, & Ahirwar, 2007;

Tagliazucchi, Verzelloni, Bertolini, & Conte, 2010). Samples containing the equivalent amount of 10 mg of CA were first suspended in 50 mL of simulated gastric fluid (SGF, in 1 L distilled water: NaCl, 2.0 g; 36% HCl, 7.0 mL; Tween 80, 1.0 mL; pepsin, 0.6 g. pH = 1.2), then the suspensions were incubated at 37 °C in a shaker (150 rpm). The simulated intestinal digestion was conducted under the same conditions after 120 min of digestion in SGF: 10 mL SGF suspensions were collected then added to an equivalent amount of simulated intestinal fluid (SIF, in 1 L distilled water: KH_2PO_4, 6.8 g; NaOH, 0.616 g; Tween 80, 1.0 mL) The pH value was then adjusted to 7.4 with 1 M NaOH before adding 1.0 g/L pancreatin and 3 g/L porcine bile salts. During the *in vitro* release test, aliquots (1 ml) were obtained after 15, 30, 60, and 120 min of both the SGF and SIF digestions, then immediately centrifuged at 3000 × g for 10 min (Paramera, Konteles, & Karathanos, 2011). The supernatants were collected and their CA content was measured using the HPLC method described above (2.7).

2.11. Statistical analysis

All tests were done in triplicate. The data from the photo-stability analysis and *in vitro* release were expressed as mean and standard deviation (mean ± SD) then analyzed by two-way analysis of variance (ANOVA) with Tukey *post hoc* tests using IBM SPSS Statistics from Windows (Version 20.0; IBM Corp., Armonk, NY, USA). A difference between mean values with $P < 0.05$ was considered as statistically significant.

3. Results and discussion

3.1. Amylose inclusion complex with CA

3.1.1. Thermal properties

The DSC thermograms recorded from all the samples are shown in Fig. 2. Heating aqueous solutions of CA with and without amylose at over 100 °C probably resulted in its degradation, suggested by the endotherms ranging from 100-120 °C (Fig. 2a & 2c). The sample of amylose-HC inclusion complex exhibited an endotherm with a peak temperature at 96.72 ± 0.37 °C and an enthalpy of 19.91 ± 1.56 J/g (Fig. 2f). This endotherm of the amylose-HC inclusion complex was not caused by the melting of HC, which was around 80-82 °C as observed in the thermograms of both pure HC and the amylose-HC physical mixture (Fig. 2b & 2d). The dissociation temperature of the amylose-HC inclusion complex was very close to those found in amylose inclusion complexes with other C-16 lipid compounds, e.g. palmitic acid (Kong & Ziegler, 2014a) and ascorbyl palmitate (Kong & Ziegler, 2014b), indicating that amylose had complexed with the C-16 alkyl chain of hexadecyl *p*-coumarate. However, the dissociation enthalpy, proportional to the amount of guest compounds that have been complexed, was much higher than that found in inclusion complexes of amylose-palmitic acid and starch-ascorbyl palmitate (Kong & Ziegler, 2014a; Ma, et al., 2011) and was close to that of the starch-alkylresorcinol inclusion complex (Gunenc, Kong, Elias, & Ziegler, 2018). This result was actually in agreement with the loading capacity, 6.8%. In contrast, the tentative amylose-CA sample produced no noticeable endotherm (Fig. 2e), indicating that amylose had not formed inclusion complex with CA.

236 *3.1.2. X-ray diffraction analysis*

237 The XRD patterns of raw CA and HC, and their inclusion complex samples are
238 shown in Fig. 3. Both the raw CA and HC displayed sharp diffraction peaks, an
239 indication of their highly crystalline nature. The 23.7 ° peak seen in the XRD pattern
240 of HC has been observed as a major peak in many fatty acids and lipid molecules
241 (Lesmes, et al., 2009; Tang & Copeland, 2007), reconfirming the successful
242 lipophilization of CA. The absence of any characteristic peaks for CA and HC in the
243 inclusion complex samples, and the absence of their melting endotherms on DSC
244 thermograms, showed the effectiveness of the washing steps during the preparation
245 procedure: the uncomplexed CA and HC had been washed away, leaving only those
246 in the amylose single helices, if any. The tentative amylose-CA sample showed a very
247 diffuse pattern with hints of a B type, i.e. weak peaks at 17 °, 19.5 °, and 22 ° (Kong,
248 et al., 2014). The sample of amylose-HC inclusion complex, exhibiting peaks at 13 °
249 and 20 °, proved to be characteristic of the V_{6h} amylose XRD pattern seen in
250 amylose-lipid inclusion complexes (Kong, Bhosale, & Ziegler, 2018). This suggested
251 that HC had been entrapped intra-helically, instead of being precipitated into its only
252 phase.

253 *3.1.3. Fourier transform infrared investigation*

254 The FTIR spectra provide additional evidence for the presence of guest compounds
255 in the samples (Fig. 4). The FTIR spectrum of CA showed the characteristic phenolic
256 compound bands at around 1500, 1600, 1670, and 3375 cm^{-1}. The bands centered at
257 around 1500 and 1600 cm^{-1} were assigned to vibrations of the benzene ring; 3375

258 cm^{-1} and 1670 cm^{-1} were respectively attributed to O–H stretching and the vibrations
259 including C=C and C=O on CA (Ravikumar, Gaddamanugu, & Anand Solomon,
260 2013; Swisłocka, Kowczyk-Sadowy, Kalinowska, & Lewandowski, 2012). Compared
261 with CA, new bands appeared on the HC spectrum: bands at 2850 and 2920 cm^{-1},
262 both of which were attributed to the stretching vibrations on $–CH_2$ of the alkyl chains;
263 bands at 1710 and 1745 cm^{-1}, both of which were assigned to carbonyl stretching; and
264 a band at 720 cm^{-1} caused by the $–CH_2$ rocking of the alkyl chain (Ciesik, Koll, &
265 Grdadolnik, 2006), which jointly confirmed the structure of HC. On the spectrum of
266 the sample of amylose-HC inclusion complex, the broad O–H vibration ranging from
267 3000 to 3600 cm^{-1} was ascribed to the superposition of O–H bonds on the amylose
268 molecules (Kizil, Irudayaraj, & Eetharaman, 2002). Minor bands of benzene ring
269 vibration at around 1600 cm^{-1}, C=C and C=O on CA at 1670 cm^{-1}, and C–H vibration
270 on the alkyl chain at 2850 and 2920 cm^{-1} could be observed, indicating the presence
271 of HC in the inclusion complex. In contrast, the tentative amylose-CA sample showed
272 no band characteristics of CA, confirming that CA could not complex with amylose
273 and had been completely washed away.

274 *3.2. Photo-stability analysis*

275 UV irradiation is an effective and widely used method for evaluating
276 photo-stability. The retention of CA, HC, the amylose-HC physical mixture and the
277 amylose-HC inclusion complex are shown in Fig. 5. After 72 h of UV irradiation,
278 about 57% of the CA was degraded in the raw CA group, which corresponded to a
279 43% retention of CA. Lipophilization of CA improved the photo-stability of CA

slightly and resulted in a 57% retention after 72 h of UV irradiation. As expected, simply mixing amylose and HC into a physical mixture did not improve the photo-stability of HC. In contrast, the retention of CA (in the form of HC) was significantly higher when HC was encapsulated in the amylose inclusion complex ($P < 0.05$). As much as 90% of the loaded HC was still retained in the amylose-HC inclusion complex after 72 h of direct UV irradiation. Therefore, the results showed that amylose inclusion complexation can increase the photo-stability of CA. UV is electromagnetic radiation with a wavelength between that of visible light and that of X-rays. Although it is not an ionizing radiation, it can cause the photodegradation of many compounds, in combination with the actions of oxygen and moisture. By molecularly encapsulating HC in the crystalline V-type amylose inclusion complex, its exposure to oxygen and moisture could be largely blocked, thus limiting the extent of its photodegradation by oxidative and hydrolysis reactions.

3.3. In vitro release of CA

The release profiles of CA in SGF and SIF are shown in Fig. 6. A large amount of CA was detected in the raw CA group within the first 30 min in SGF. In contrast, HC, the amylose-HC physical mixture and the amylose-HC inclusion complex released a limited amount of CA in SGF, but burst immediately after being exposed to SIF. This phenomenon may be ascribed to the absence of lipase and amylase in SGF so that CA could not be released from HC or the inclusion complex. Similar results have reported that vitamin D_3 could not be released from starch nanoparticles until pancreatic amylase had been added to the SIF (Hasanvand, Fathi, Bassiri, Javanmard, &

Abbaszadeh, 2015). For raw CA group, it was notable that the CA concentration decreased after 30 min release in SGF, which may have been caused by the degradation of CA under acidic conditions (Yang, et al., 2018). Moreover, it appeared that significantly more CA was released from the inclusion complex sample than from the references in SIF ($P < 0.05$). These results implied that the amylose inclusion complex could protect CA from the acidic and enzymatic conditions of the stomach and thus increase the release of CA in the intestines, where the absorption of phenolic compounds occurs (Williamson & Clifford, 2017).

4. Conclusions

In this study, an approach for encapsulating CA based on amylose molecules through lipophilization was investigated. The DSC, XRD, and FTIR results indicated that the amylose-HC inclusion complex had been formed, but not the amylose complexation with CA. After 72 h of direct UV irradiation, the CA in the inclusion complex sample was significantly better retained than that in the other samples. The release profiles showed that the inclusion complex exhibited higher release rates of CA during *in vitro* digestion. These results have demonstrated that the molecular encapsulation of lipophilized bioactive compounds by amylose is a promising technique for enhancing the stability and bioaccessibility of bioactive compounds, which could be applied during the production of fortified foods.

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Conflict of interests

The authors have declared no conflicts of interest.

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448 **Figure Captions**

449 Fig. 1. Chemical structure of hexadecyl *p*-coumarate.

450

451 Fig. 2. DSC thermograms of (a) *p*-coumaric acid (CA, 5%, w/v), (b) hexadecyl
452 *p*-coumarate (HC, 5%, w/v), (c) amylose-CA physical mixture, (d) amylose-HC
453 physical mixture, (e) tentative amylose-CA inclusion complex, and (f) amylose-HC
454 inclusion complex.

455

456 Fig. 3. XRD patterns of (a) *p*-coumaric acid (CA), (b) hexadecyl *p*-coumarate (HC),
457 (c) tentative amylose-CA inclusion complex, and (d) amylose-HC inclusion complex.

458

459 Fig. 4. FTIR spectra of (a) *p*-coumaric acid (CA), (b) hexadecyl *p*-coumarate (HC), (c)
460 tentative amylose-CA inclusion complex, and (d) amylose-HC inclusion complex.

461

462 Fig. 5. Photo-stability of *p*-coumaric acid (CA), hexadecyl *p*-coumarate (HC),
463 amylose-HC physical mixture, and amylose-HC inclusion complex. The letters a, b,
464 and c indicate significant differences at each time point, $P < 0.05$ (a > b > c).

465

466 Fig. 6. The *in vitro* release of *p*-coumaric acid (CA) in simulated gastric and intestinal
467 fluids from raw CA, hexadecyl *p*-coumarate (HC), amylose-HC physical mixture, and
468 amylose-HC inclusion complex. The letters a, b, and c indicate significant differences
469 at each time point, $P < 0.05$ (a > b > c).