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Coupling lipophilization and amylose complexation to encapsulate chlorogenic acid

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ABSTRACT

Chlorogenic acid (5-caffeoylquinic acid) is a hydrophilic phenolic compound with antioxidant properties. Because of its high polarity, these properties may be altered when formulated in oil-based food. There is therefore an interest in trying to protect the natural antioxidant by molecular encapsulation. Amylose, the linear fraction of starch with essentially α(1–4) linkages, is well known for its ability to form semi-crystalline complexes with a variety of small ligands. Monoacyl lipids, as well as smaller ligands such as alcohols or flavor compounds, are able to induce the formation of left-handed amylose single helices. In contrast, chlorogenic acid is a bulky molecule whose topology requires the amylose helix to be distorted, which could prevent amylose complexation. An innovative strategy has been developed to overcome this problem by grafting an aliphatic chain onto chlorogenic acid then trapping this chain in the helical cavity. The lipophilization reaction was used to obtain a palmitoyl chlorogenic acid derivative and the amylose–palmitoyl chlorogenic acid assemblies were studied by X-ray diffraction, differential scanning calorimetry and NMR to elucidate the interaction. The results showed that such interactions between amylose and palmitoyl chlorogenic acid are effective.

1. Introduction

Antioxidants are frequently used in the formulation of foods, cosmetics, and pharmaceutical products with the objective of preventing the oxidation of lipids. They can slow down the oxidation of unsaturated fats and oils and the concomitant formation of off-flavors and unwanted volatiles.

There are many natural sources of antioxidants. Extracts rich in natural antioxidants, such as phenolic compounds, can be obtained from raw vegetable materials by extraction; one of the advantages of these compounds is that, because they are of natural origin, they are better accepted by consumers. Among phenolic compounds, polyphenols have stimulated a growing interest (Sun-Waterhouse, 2011) due to their wide range of biological activities such as antioxidant (Jiménez-Ecrig, Rinçon, Pulido, & Saura-Calixo, 2001; Kanazawa & Sakakibara, 2000; Moure et al., 2001; Nunez Sellés et al., 2002; Someya, Yoshiki, & Okubo, 2002), antimicrobial (Chun, Vattem, Lin, & Shetty, 2005; Oluwatuyi, Kaatz, & Gibbons, 2004), antiviral (Ikken et al., 1999), and anticarcinogenic (Chung et al., 1999; Sawa, Nakao, Akaike, Ono, & Maeda, 1999). They include chlorogenic acids, which are esters of caffeic acid and quinic acid. 5-Caffeoyl quinic acid (chlorogenic acid) is one of the major polyphenol compounds found in numerous plant species (Clifford, 1999) and possesses well documented biological (Morishita & Ohnishi, 2001), antioxidant (Kono et al., 1997; Zang, Cosma, Gardner, Castranova, & Vallyathan, 2003), and antimicrobial properties (Puupponen-Pimia et al., 2001; Zhu, Zhang, & Lo, 2004).

However, the enrichment of food products with chlorogenic acid is a challenging task due to its high hydrophilicity, its tendency to degrade under certain conditions during processing, storage, and transit in the digestive system, and its low bioavailability (Scheepens, Tan, & Paxton, 2010). Autoxidation of chlorogenic acid, which occurs during food processing for example, may lead to the formation of brown polymerized products (Ingraham & Corse, 1951). To overcome these drawbacks, several encapsulation and microencapsulation methods have been described to protect and improve chlorogenic acid stability. For example, Zhao, Wang, Yang, and Tao (2010) reported the use of β-cyclodextrin to form an inclusion complex with chlorogenic acid. Although Shi et al. (2007) described the yeast-cell-based microencapsulation of chlorogenic acid, molecular encapsulation with amylose, the linear component of starch, has never been reported. However, it is known from the literature that the use of amylose to form an inclusion complex can provide very interesting applications to protect

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bioactive molecules. Such a strategy may also allow the delivery of the molecules to be better controlled and targeted (Zuidam & Nedovic, 2010). Indeed, it is well established that amylose can form helical inclusion complexes with a variety of compounds such as iodine (Bluhm & Zugenmaier, 1981), dimethyl sulfoxide (Winter & Sarko, 1974), alcohols (Brisson, Chanzy, & Winter, 1991; Buléon, Delage, Brisson, & Chanzy, 1990; Rappenecker & Zugenmaier, 1981), and aroma compounds (Biais, Le Bail, Robert, Pontoire, & Buleon, 2006; Nuessli, Sigg, Conde-Petit, & Escher, 1997) in aqueous solution. The molecular organization of amylose complexes with various fatty acids has been extensively studied (Biliaderis & Galloway, 1989; Biliaderis, Page, Slade, & Sirett, 1985; Godet, Bizot, & Buléon, 1995; Godet, Buléon, Tran, & Colonna, 1993), and a model involving inclusion of the aliphatic part of the lipid in the cavity of amylose is commonly accepted (Godet, Tran, Colonna, Buléon, & Pezolet, 1995).

Amylose, the linear fraction of starch, forms crystalline complexes, known under the generic name of V amylose, with a variety of small ligands. Different types of V amylose, depending on the complexing molecule, have been reported in the literature. The best-known and best described complex is Vh amylose, which is obtained with linear alcohols (Brisson et al., 1991; Buléon, Duprat, Booy, & Chanzy, 1984; Le Bail, Bizot, Pontoire, & Buléon, 1995; Whittam et al., 1989) and monoacyl lipids (Godet, Bizot, et al., 1995; Godet, Buléon, et al., 1993). It consists of a six-fold left-handed helix repeating at 0.80 nm, in which the complexing agent is included.

Three other crystalline types of complex have also been highlighted with a branched alcohol. These complexes can initially be distinguished using the constructive amylose helix. Two families have been identified in the literature, namely V_6 and V_8, where 6 and 8 represent the number of D-glycosyl units per turn. For V_6 types, two trapping modes could be suggested: intra-helices inclusion V_{6I} (Vh) and intra–inter-helices inclusion V_{6II}, V_{6III}, where I, II and III represent the varying volume between helices in the crystalline stacking. For V_{6I} (Brisson et al., 1991), the small molecules could be entrapped only in the cavity of the helix (Godet, Tran, et al., 1995) and for V_{6II} and V_{6III}, the molecules could also be entrapped between helices. Another possibility is a larger cavity with eight D-glucose residues per turn, V_8, which allows the inclusion of bulky molecules (Le Bail, Rondeau, & Buléon, 2005; Winter, Chanzy, Putaux, & Helbert, 1998). Characteristic X-ray diffraction patterns (V_{6I}, V_{6II}, V_{6III}, V_8) are known (Le Bail et al., 2005; Yamashita & Monobe, 1971).

In this work, we propose to investigate the ability of amylose to form complexes with chlorogenic acid using the conventional hydrothermal method. An innovative process has been developed to allow this complexation. The strategy consists in first grafting a 16-carbon long aliphatic chain onto chlorogenic acid (Lorentz et al., 2010) then exploiting the ability of amylose to trap the carbon chain. The synthesis of a new chlorogenic acid derivative with a 16-carbon aliphatic chain has already been described under the name of the lipophilization reaction as a promising way to improve its antioxidant activity in emulsions (Chebil, Humeau, Falcimaigne, Engasser, & Ghoul, 2006; Figueroa-Espinoza & Villeneuve, 2005; Villeneuve, 2007). Differential scanning calorimetry, X-ray analysis and solid state nuclear magnetic resonance are used to investigate the physicochemical characteristics of inclusion complexes. To our knowledge, this is the first time that lipophilization has been used to improve encapsulation methods.

2. Materials and methods

2.1. Biological and chemical materials

The lipase preparation used was Novozym 435 purchased from Novozymes (Dk). It consists of lipase B from *Candida antarctica* immobilized on acrylic resin. All solvents and reagents were obtained from commercial sources and were either of HPLC or analytical grade. Palmitic acid and silica gel plates were purchased from Sigma–Aldrich (France). Chlorogenic acid was purchased from Acros (France) and was 99% pure. Potato amylose (type III), essentially free of amylopectin, was obtained from Sigma–Aldrich (France) and used as received.

2.2. Lipase-catalyzed synthesis of 4-O-palmitoyl chlorogenic acid

The chlorogenic acid derivative was enzymatically synthesized as previously described (Lorentz et al., 2010) with slight modifications. Briefly, chlorogenic acid, palmitic acid and Novozym 435 were dried for two days over P_2O_5 before use. In these conditions, the initial water activity (a_w) of the reaction medium, determined using an AqualabLite® (Decagon Devices Inc., USA), was below 0.2. The lipophilization reaction was carried out in 2-mL Eppendorf tubes in the dark. The reaction mixture consisted of 28 μmol of chlorogenic acid and 1.12 mmol of palmitic acid (substrate ratio of 40) in 1 mL of 2-methyl-2-butanol (2M2B). Palmitic acid and chlorogenic acid were first solubilized for 12 h in 2M2B under stirring at 1000 rpm and at 60 °C in an Eppendorf Thermomixer (Roucaire, France). Then 200 mg of molecular sieves (3 Å) previously dried overnight at 200 °C was added and the reaction was initiated by addition of 40 mg of enzyme.

After 7 days of reaction, the immobilized enzyme and molecular sieves were filtered off (0.22 μm), the solvent was evaporated under vacuum, and 4-*O*-palmitoyl chlorogenic acid was isolated by Sephadex LH-20 chromatography using chloroform/methanol (70/30, v/v) as the eluent. Purification was completed by means of preparative TLC. As determined by HPLC, the final product was at least 98% pure.

2.3. Preparation of amylose complexes

The complexation experiments were conducted using chlorogenic acid and 4-*O*-palmitoyl chlorogenic acid (Fig. 1). Before heating and mixing amylose and 4-*O*-palmitoyl chlorogenic acid, a nitrogen flow was first passed through the samples for 15 min to prevent their oxidation during heating.

Amylose was dispersed in pure water 1% (w/v) (200 mg/20 mL) at 145 °C for 45 min in a glass tube with a screw cap. Ten milligrams of 4-*O*-palmitoyl chlorogenic acid was preheated and solubilized in 5 mL water at 90 °C and added to the amylose solution after it had been cooled to 90 °C. The mixture was maintained at 90 °C for 10 min, cooled to room temperature and stored for 48 h. The precipitate was then collected by centrifugation (2000 × *g* for 10 min) and the water content adjusted by desorption at $a_w = 0.75$ over saturated NaCl solution before X-ray, DSC and solid state NMR analyses. Amylose solution without the addition of 4-*O*-palmitoyl acid was prepared under the same conditions and used as the reference.

All samples were prepared in triplicate and each measurement was made in triplicate.

2.4. Thermostability study

2.4.1. Chlorogenic acid

The thermostability of chlorogenic acid was determined by a UV spectrophotometric method using a Perkin-Elmer lambda 12 UV spectrophotometer with a bandwidth of 1 nm. A 10 μg/mL aqueous solution of chlorogenic acid was prepared and heated at 90 °C for 30 min. After cooling, the solution was diluted 40 times with water before measurement of UV spectra from 600 to 200 nm. UV spectra with and without the heating phase were compared.

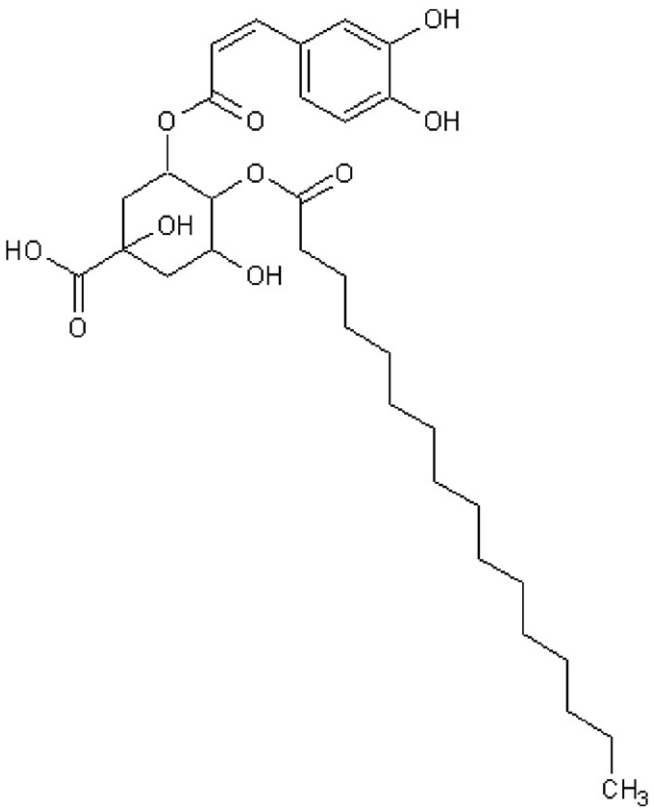

Fig. 1. Chemical structure of 4-*O*-palmitoyl chlorogenic acid.

2.4.2. 4-*O*-palmitoyl chlorogenic acid

As chlorogenic acid and 4-*O*-palmitoyl chlorogenic acid present the same UV spectra, a study of the thermostability of 4-*O*-palmitoyl chlorogenic acid was performed by HPLC. The protocol was adapted from Ishihara and Nakajima (2003) with major modifications described in Lorentz et al. (2010). An aqueous solution of 4-*O*-palmitoyl chlorogenic acid (1 g/L) was prepared with water previously passed through a nitrogen flow and samples were incubated at 90 °C for 30 min, 1 h or 2 h. HPLC analyses were carried out and compared before and after treatment.

2.5. Differential scanning calorimetry

DSC thermograms were recorded using an automated heat flux differential scanning calorimeter (T.A. Instruments, Q100). Stainless steel high pressure cells (T.A. Instruments, ref: 900825.902) were used. The system was calibrated with indium and a pan containing 12 μL of water was taken as the reference. Eight milligrams of sample was weighed into pans and 12 μL of water was added. Two successive scans were run in triplicate at 3 °C/min from 1 °C to 140 °C for the first scan and from 1 °C to 160 °C at 1 °C/min for the second, separated by a cooling stage at 3 °C/min.

2.6. X-ray diffraction analysis

Fifty milligrams of sample equilibrated at $a_w = 0.75$ was sealed in a copper ring between two sheets of adhesive tape to prevent any change in water content. The sample was examined by Wide Angle X-ray Scattering. Measurements were performed using a D8 Discover spectrometer with a GADDS detector and cross-coupled mirrors from Bruker-AXS, working at 40 kV and 40 mA, with a copper monochromator ($\lambda = 1.54059$ Å) and sample alignment by microscopic video and laser. Data were monitored by a 120° curve detector for 10 min and normalized between 3 and 30° (2θ).

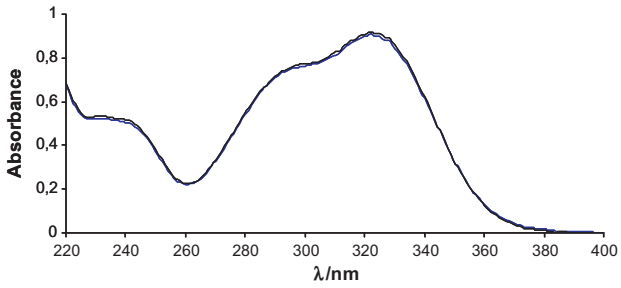

Fig. 2. UV spectra of chlorogenic acid before and after heating.

2.7. ^{13}C solid state NMR

NMR experiments were performed on a Bruker DMX-400 spectrometer operating at a ^{13}C frequency of 100.62 MHz and equipped with a double resonance H/X CP-MAS 4 mm probe. The MAS rate was fixed at 5000 Hz and each experiment was recorded at ambient temperature (294 ± 1 K). The Cross Polarization pulse sequence used a 3.75 μs 90° proton pulse, a 1 ms contact time at 66.7 kHz and a 10 s recycle time for an acquisition time of 17 ms during which dipolar decoupling was applied. A typical number of 5120 scans was acquired for each spectrum. Chemical shifts were calibrated with external glycine, assigning the carbonyl carbon at 176.03 ppm.

3. Results and discussion

3.1. Thermal stability of chlorogenic acid and its derivative

3.1.1. Chlorogenic acid

Heating to 90 °C was required to form complexes. At this temperature, the degradation of chlorogenic acid could be observed so its thermal stability was assessed by UV spectrophotometry.

The UV visible spectra before and after heating are shown in Fig. 2. No change was observed, proving that chlorogenic acid was stable at 90 °C.

3.1.2. 4-*O*-palmitoyl chlorogenic acid

Chromatograms of a solution of 4-*O*-palmitoyl chlorogenic acid obtained after 30 min, 1 h or 2 h exposure to 90 °C were compared with those of the same solution without treatment. The results (chromatograms not shown) indicated that heating at 90 °C for up to 2 h does not induce any modification. Therefore, 4-*O*-palmitoyl chlorogenic acid is stable at 90 °C and will not be altered or hydrolyzed during amylose complex formation.

3.2. Complexation analysis

3.2.1. Thermal analysis

In the present experiment, the complexing abilities of amylose with chlorogenic acid and 4-*O*-palmitoyl chlorogenic acid were determined by differential scanning calorimetry and compared to the reference (amylose without ligand).

Thermograms recorded on the two types of amylose complex studied and the reference are shown in Fig. 3. They have a variable shape according to the nature of the ligand. The second scan was used to prove reorganization during the cooling carried out between the two heating scans and, especially, the well known melting/recrystallization reversibility for amylose lipid complexes (Biliaderis & Galloway, 1989).

Thermograms obtained from amylose without ligand (reference) (Fig. 3A) and from the amylose–chlorogenic acid assembly (Fig. 3B) present a similar behavior with a broad endotherm at

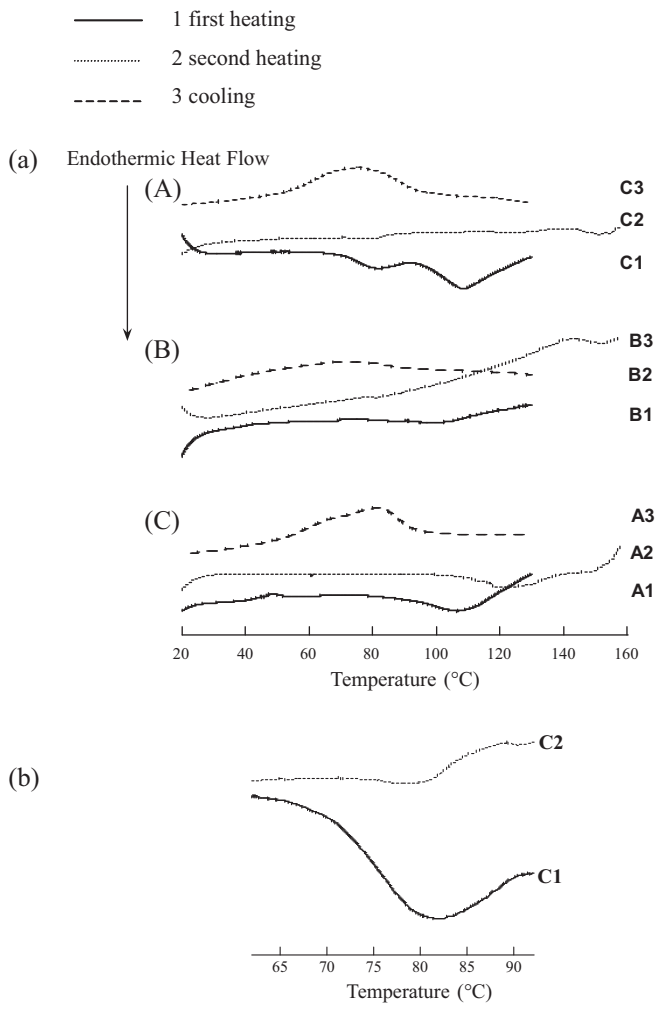

Fig. 3. (a) DSC thermograms of (A) amylose without ligand; (B) amylose complexed with chlorogenic acid; (C) amylose complexed with 4-*O*-palmitoyl chlorogenic acid. —, 1 first heating; · · · · ·, 2 second heating; - - - - -, 3 cooling. (b) Zoom of the preceding thermogram.

$107 \pm 2\,^{\circ}C$ during the first heating. This could be interpreted by a partial dispersion of amylose at 145 °C. Indeed, at this temperature, only one part of amylose was dispersed (less ordered amylose) and provoked a fast retrogradation during cooling and storage, which explains the low melting temperature of the retrograded amylose (107 °C). On the second scan, both endotherms are detected at around $120 \pm 2\,^{\circ}C$ and $150 \pm 2\,^{\circ}C$ which could be attributed, respectively, to retrograded amylose with poor crystallinity obtained during the cooling between the two heating scans and to the initial amylose possessing a good crystalline organization.

The thermogram of amylose–4-*O*-palmitoyl chlorogenic acid (Fig. 3C) presents one more endotherm at $80 \pm 2\,^{\circ}C$, obvious on the first and second scans (Fig. 3b) and highlighting the presence of complexes. However, the low melting temperature of the complexes reflects a very poor crystalline organization. Indeed, in the literature, the common melting temperature of amylose complexes is around 110 °C. For example, Godet, Buléon, et al. (1993) and Godet, Tran, Delage, and Buléon (1993) determined the melting temperature of amylose–palmitic acid complexes at 112 °C (Godet, Tran, et al., 1993). However, Biliaderis and Galloway (1989) identified two thermally distinct forms of the amylose–lipid complex, namely I (low Tm; amorphous complex) and II (high Tm; crystalline complex) (Biliaderis & Galloway, 1989).

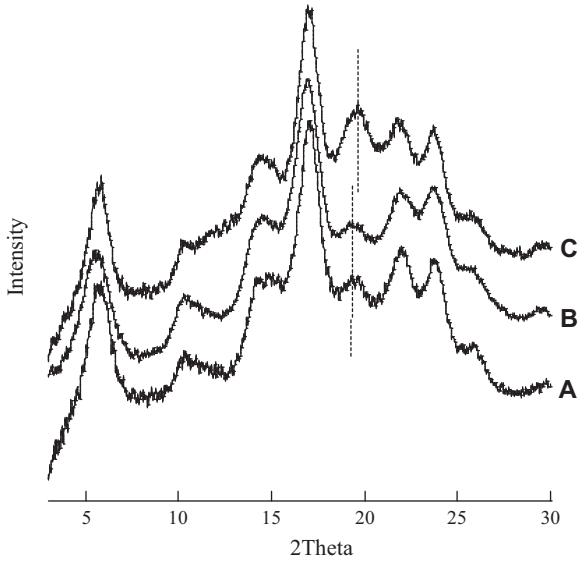

Fig. 4. X-ray diffraction diagrams of: (A) amylose without ligand; (B) amylose complexed with chlorogenic acid; (C) amylose complexed with 4-*O*-palmitoyl chlorogenic acid.

Based on our results, it can be concluded that chlorogenic acid does not induce interaction with amylose. However, the complexation of 4-*O*-palmitoyl chlorogenic acid was highlighted and the observed low melting temperature of the complexes could originate from a poorly ordered or amorphous complex.

3.2.2. *X-ray diffraction*

In amylose dispersions, the X-ray diagram obtained without antioxidant and used as a reference was characteristic of the B-type (Fig. 4A). In this case, the reflections showed that amylose had retrograded. In the presence of chlorogenic acid and 4-*O*-palmitoyl chlorogenic acid (Fig. 4B and C), reflections corresponding to the free antioxidants were not observed. These findings are in agreement with the DSC results, where no endotherm due to the free antioxidants appeared on the amylose-antioxidant assembly thermograms. The X-ray diffraction diagrams displayed only a B-type due to the presence of retrograded amylose. It is surprising that the amylose–4-*O*-palmitoyl chlorogenic assembly spectrum did not highlight a V-type structure. Nevertheless, on this spectrum, the peak at 19.5° (2Θ) corresponding to the B-type structure had shifted to a higher value: 19.8° (2Θ) characteristic of a V-type structure and with a greater intensity, but no conclusion can be drawn without additional work.

In conclusion, the reflections obtained were characteristic of the B-type for both assemblies, which shows that amylose, in this case, had retrograded, and indicates that chlorogenic acid did not form complexes with amylose. This result confirmed the inability of chlorogenic acid to form complexes with amylose during DSC experiments. Otherwise, it was difficult to conclude about the 4-*O*-palmitoyl chlorogenic acid complexation.

4-*O*-palmitoyl chlorogenic acid is a bulky molecule, which could: (i) induce the formation of the eight-fold helical conformation, (ii) cause a significant local distortion of the helix involving a spacing of the helices and therefore the emergence of a V_{6II} or V_{6III} crystalline structure, (iii) produce only complexation with the graft (aliphatic chain) with hindrance of the crystalline packing.

3.2.3. *^{13}C solid state NMR*

Solid state NMR has proved to be a powerful tool for characterizing some degrees of molecular order, such as helicity,

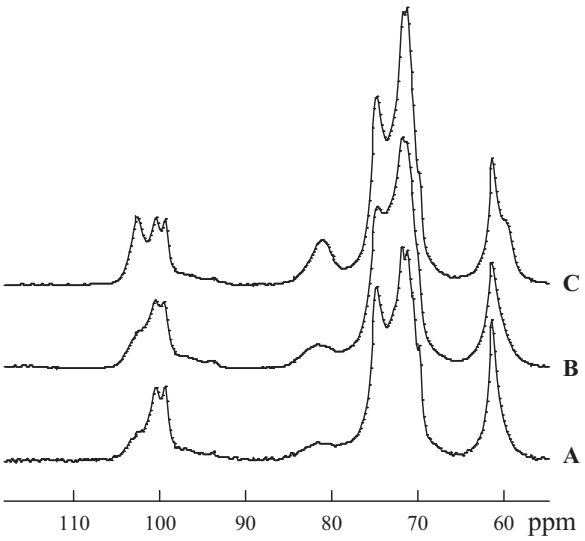

Fig. 5. ^{13}C CP/MAS NMR spectra of: (A) amylose without ligand; (B) amylose complexed with chlorogenic acid; (C) amylose complexed with 4-*O*-palmitoyl chlorogenic acid.

in the structure of starchy substrates (Gidley & Bociek, 1985; Horii, Yamamoto, Hirai, & Kitamaru, 1987; Paris, Bizot, Emery, Buzaré, & Buleon, 1999; Singh, Ali, & Divakar, 1993; Veregin, Fyfe, Marchessault, & Taylor, 1986).

The spectrum of the amylose–chlorogenic acid complex (Fig. 5B) is close to the reference spectrum (Fig. 5A), namely to the B-type spectrum; indeed the C1 region gives rise to a characteristic doublet at 100.5 and 99.3 ppm. According to Paris, Bizot, Emery, Buzaré, and Buléon (2001), the shoulder present on the doublet C1 detected in the spectrum of the amylose–chlorogenic acid complex may be attributed to the amorphous background.

The spectrum of the amylose–4-*O*-palmitoyl chlorogenic acid complex (Fig. 5C) displays the resonances the B-type amylose in addition of signals typical of V-type structure (Gidley & Bociek, 1985; Le Bail et al., 1999, 2005; Snape, Morrison, Maroto-Valer, Karkalas, & Pethrick, 1998). Peaks observed at 102.7, 81.4, 74.9, 71.6, 61.3 and 60 ppm were assigned to C1, C4, C3, C2–C5, and C6 carbons, respectively, and are characteristic of the V_{6I} form (Gidley & Bociek, 1985; Le Bail et al., 2005). For this crystalline form, only one signal is detected for each carbon site confirming their chemical environment in equivalent residues packed in a single-helix, typical conformation reported for the V-polymorphs of amylose (Gidley & Bociek, 1985; Le Bail et al., 1999; Snape et al., 1998). The doublet signal in the C1-region at 100.5 and 99.3 ppm was also observed and indicated the presence of double helices due to non-complexed B-type amylose (Gidley & Bociek, 1988; Veregin, Fyfe, Marchessault, & Taylor, 1987).

These results agreed with the X-ray diffraction and calorimetry studies, showing interactions between 4-*O*-palmitoyl chlorogenic acid and amylose, while chemical shifts observed for the carbon signals in the spectrum, especially C1, were clearly assigned to the spectrum of V_{6I} form (Le Bail et al., 2005). This confirms that only the graft was included inside the helical cavity of amylose.

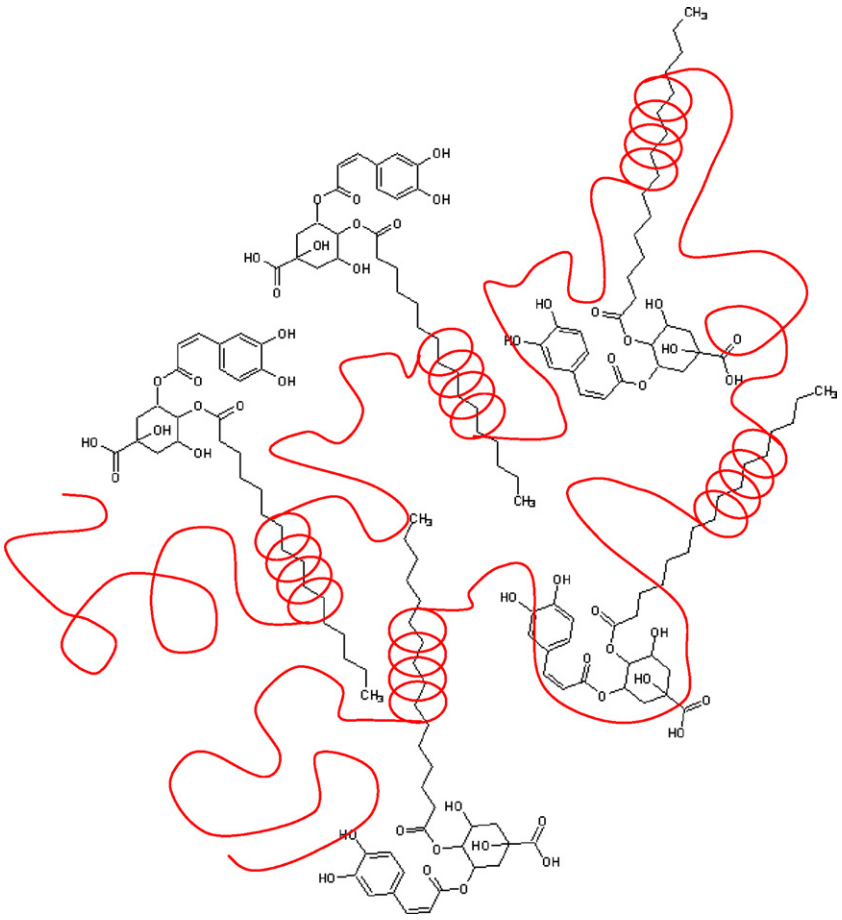

Fig. 6. Schematic model of the amylose–4-*O*-palmitoyl chlorogenic acid complex.

4. Conclusions

In this study, amylose–chlorogenic acid and amylose–4-*O*-palmitoyl chlorogenic acid assemblies and their corresponding calorimetry, X-ray and solid state NMR signatures were observed and compared to the reference (amylose without ligand). The techniques used show excellent complementarities in the determination of important structural features such as crystalline type, helical conformation and nature of the inclusion.

From differential scanning calorimetry data, it was possible to indicate the presence of an amylose complex for the amylose–4-*O*-palmitoyl chlorogenic acid assembly; indeed the thermogram obtained has a complex shape with a broad endotherm at 80 °C and, upon cooling, the complex forms again. The low melting temperature observed could be interpreted by the poor crystallinity of the complexes. In contrast to 4-*O*-palmitoyl chlorogenic acid, chlorogenic acid does not form a complex with amylose and leads to a similar behavior to that of the reference.

The X-ray data show only spectra of B-type structure. These results cannot determine the possible nature of the interactions, especially the presence or absence of inter-helical spaces in the unit cell of the structure. This reinforces the calorimetry results, namely that complexes formed with amylose and 4-*O*-palmitoyl chlorogenic acid are amorphous.

The ^{13}C CP/MAS NMR study of the amylose–chlorogenic acid assembly confirms the absence of complexes, whereas the resonances observed for carbon in the spectrum (especially C1 and C4) of the amylose–4-*O*-palmitoyl chlorogenic acid complex display the presence of a single helical conformation (V_6) while some shifts in the NMR C1 carbon signal represent more specifically a V_{6I} form.

Our results show that 4-*O*-palmitoyl chlorogenic acid forms amorphous complexes with amylose, with only the graft included in the helical cavity of the amylose. This study enables a schematic model of the complexes to be suggested as shown in Fig. 6.

Acknowledgments

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This work has been included in a patent.

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