Structural characterization of amylose-long chain fatty acid complexes produced via the acidification method

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Abstract

Amylose molecular inclusion complexes, or V-amylose, have been studied as a possible nano-sized delivery system for unsaturated fatty acids. This study aimed to study three different structural levels of V-amylose produced via an acidification method. Molecular attributes were studied using XRD, DSC and 13C CP/MAS NMR, nanostructures using SAXS and AFM, and the microscopic level by SEM and AFM. 13C labeled fatty acids revealed head groups were entrapped in both COO- and COOH forms. SAXS data, showed that conjugated linoleic acid yield particles with the highest values for parameters like average crystalline lamellar thickness (\(d = 0.46\)) and characteristic particle dimension (\(R_g = 1011\)). AFM revealed surface roughness increases from 7.72 \(\pm\) 4.34 nm to 11.54 \(\pm\) 6.05 nm during the formation of V-amylose. The insights described contribute to the understanding of V-amylose structure and help establish a model for V-amylose structure which may prospectively be used in the fabrication of a novel delivery system.

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1. Introduction

Consumption of poly-unsaturated fatty acids (PUFA) has been enthusiastically advocated due to their essential role in human health. Studies have shown PUFA support improved performance of the immune system, reduce blood pressure, decrease the chance for heart attacks and the recurrence rate of certain cancers (Shahidi & Miraliakbari, 2004, 2005). Familiar to the public as omega 3 and 6, PUFA include 2 or 3 methylenes interrupted by double bonds which render them susceptible to heat, light and oxidation. PUFA cannot be synthesized by the human body and should be provided with the diet however the daily uptake is, on average, lower than the recommended amount. Enrichment of food products with PUFA is a challenging technological task due to their tendency to degrade and autoxidize at high rates during production, storage, and passage in the digestive system. Various encapsulation platforms have been suggested to be suitable for the controlled delivery of lipophilic nutraceuticals such as PUFA and omega 3 rich oils (Barrow, Nolan, & Jin, 2007; Lalush, Bar, Zakaria, Eichler, & Shimoni, 2005; McClements, Decker, & Weiss, 2007; Semo, Kesselman, Danino, & Livney, 2007). This study focused on amylose-based molecular inclusion complexes or V-amylose as a prospective controlled delivery system for PUFA, as suggested in previous work (Lalush et al., 2005; Lesmes, Barzechath, & Shimoni, 2008; Lesmes, Cohen, Shener, & Shimoni, 2009; Zabar, Lesmes, Katz, Shimoni, & Bianco-Peled, 2009).

The molecular organization of amylose complexes with various fatty acids has been studied extensively and complex formation has been shown to be affected by parameters such as complexation temperature and lipid structure (monoglyceride or free fatty acid), with increased fatty acid (FA) chain length and decreased unsaturation increasing V-amylose thermal stability (Biliaderis & Galloway, 1989; Godet, Buleon, Tran, & Colonna, 1993; Godet, Bizot, & Buleon, 1995; Tufvesson, Wahlgren, & Eliasson, 2003b). Also, two main crystalline polymorphic forms have been identified. Type I is considered to be amorphous, while the semi-crystalline type II displays three peaks at Bragg angles of 7.4°, 13.1° and 19.8° in its X-ray diffraction pattern (Biliaderis & Galloway 1989; Lesmes et al., 2009). However, the nanostructure of V-amylose has been explored to a lesser extent compared to its molecular structure. Transmission electron microscopy (TEM) micrographs of amylose fatty acid complexes revealed uniaxial layout of amylose molecules, which were locally interrupted by amorphous segments with a thickness of no more than 4.6 nm (Godet, Bouchet, Colonna, Gallant, & Buleon, 1996). Other studies suggest amylose–alcohol complexes have...
a lamellae folding length of about 10 nm (Biliaderis & Galloway 1989; Jovanovich & Maria, 1999). Recently AFM work has shown that V-amylose also exhibits an aggregative nature, with aggregates being comprised of small spherulites of ~50–100 nm, lamellae of a few microns in length and ~10 nm of thickness and some other ill-defined structures (Lalush et al., 2005; Lesmes et al., 2009).

Understanding the structure and physicochemical properties of V-amylose is believed to be important for the future design and fabrication of such systems (Lesmes & McClements, 2009; McClements, Decker, & Park, 2009; McClements, Decker, Park, & Weiss, 2008). Accordingly, we have already investigated the effect of guest chemistry on the structure of V-amylose inclusion complexes produced via dilution of dimethylsulfoxide (Zabar et al., 2009). To the best of our knowledge, this was the first study to combine molecular level investigations with nanostructure and microscopic characterization. The effect of fatty acid unsaturation on V-amylose structure has been shown to span throughout the different structural strata studied. In the current study, we broaden our previous research toward a different production method more suitable for food applications. Thus, amylose complexes with fully saturated 18:0 stearic acid (SA), 18:2 linoleic acid (LA) and naturally occurring 18:2 conjugated linoleic acid isomer mixture (CLA) were produced using a previously described acidification method. X-ray diffraction (XRD), $^{13}\text{C}$ solid state CP/MAS NMR and differential scanning calorimetry (DSC) were used to study the molecular organization and thermal properties of the amylose complexes produced while Small angle X-ray scattering (SAXS), scanning electron microscopy (SEM) and atomic force microscopy (AFM) probed the nano and micro structures of the complexes. The effect of the production method as well as the effects of the various guest molecules on the structural characteristics is discussed.

2. Experimental

2.1. Materials

2.1.1. Potato amylose (Av. DP 900)

Potato Amylose (Av. DP 900) is essentially free of amylopectin was purchased from Sigma Co., Israel (A0512) and used as received.

2.1.2. Long chain fatty acids

The complexation experiments were conducted using three different fatty acids of various degrees of unsaturation. Fully saturated 18:0 (18 carbons and 0 double bonds) stearic acid (SA) (Sigma S-4751), 18:2 cis-9,cis-12-octadecadienoic acid or linoleic acid (LA) (Sigma L-1376); and 18:2 mixture of cis-and trans-9,11 and −10,12-octadecadienoic acids or conjugated linoleic acid (CLA) (a mixture of cis-and trans-9,11 and −10,12-octadecadienoic acids. Linoleic acid <1%) – (Sigma 0-5507); all of at least 99% purity. Additionally, molecular level studies aimed at determining the positioning of fatty acid in V-amylose used uniformly labeled $^{13}\text{C}_{16}$-stearic acid (605 581) and uniformly labeled $^{13}\text{C}_{18}$-linoleic acid (605 735), both with at least 99% isotope enrichment were used. Complexes Produced with these fatty acids were used in solid state $^{13}\text{C}$ CP/MAS NMR experiments.

2.1.3. Other reagents

Potassium Hydroxide (KOH), Hydrochloric Acid (HCl), and all other reagents were analytical grade chemicals.

3. Methods

3.1. Formation of V-amylose molecular inclusion complexes

Production of V-amylose complexes via acidification of an alkali solution mixture of amylose and guest fatty acid dilution was carried out based on a method previously described (Eliasson & Krog, 1985; Karkalas, Ma, Morrison, & Pethrick, 1995; Lalush et al., 2005). 600 mg of amylose were dissolved in 40 mL of preheated (90 °C) 0.1 M KOH then cooled to crystallization temperature of 30 °C, 60 °C or 90 °C. Similarly, an alkali fatty acid (FA) solution (60 mL, 1 mg/ml, 0.1 M KOH) preheated to 90 °C, was cooled to the same crystallization temperature of 30 °C, 60 °C or 90 °C and then mixed together with amylose solution. The solution mixture was titrated under gentle stirring to a final pH of 4.7 using 2 M HCl solution. The resulting suspension was incubated at a constant temperature for 24 h under gentle stirring. At the end of incubation phase, the suspension was cooled to 25 °C.

3.1.1. Separation of the complexes

Separation of the V-amylose from the suspensions was done by centrifugation (2000 g, 20 min). The wet pellet was washed using 50% ethanol/water mixture (v/v) and centrifuged as before. This step was repeated three times to remove residues of uncomplexed FA, and to obtain salt-free complexes, before the resulting pellet was transferred to petri dishes, freeze dried and pulverized into a fine powder.

3.2. Molecular level investigations

Investigation of the molecular level characteristics of the amylose-FA complexes powders produced was studied through X-ray diffraction (XRD), $^{13}\text{C}$ solid state CP/MAS NMR (ssNMR) and differential scanning calorimetry (DSC). These methods have already been successfully and extensively used by others to verify formation and study V-amylose inclusion complexes (Biais, Le Bail, Robert, Pontoire, & Buleon, 2006; Bulpin, Welsh, & Morris, 1982; Godet, Bizot, et al., 1995; Godet et al., 1993; Godet, Tran, Colonna, Buleon, & Pezolet, 1995; Jouquand, Ducruet, & Le Bail, 2006; Kawada & Marchessault, 2004; Lalush et al., 2005; Le Bail, Rondeau, & Buleon, 2005; Tozuka et al., 2006).

The formation of a V type amylose-FA complex was verified by measuring the X-ray diffraction of powders produced from the suspensions. These XRD measurements were carried out on a Philips PW 3020 powder diffractometer equipped with a graphite crystal monochromator (Philips, The Netherlands). The operating conditions were CuKα1 radiation (λ = 0.154 nm), voltage 40 kV and current 40 mA. Approximately 200 mg of sample powders were loaded onto a poly(methylmethacrylate) plate and scanned over the angular range of 2θ from 5° to 35° with step size 0.02°. Counting time was 4 s per step.

The thermal properties of the amylose-FA complexes were studied by DSC. These were obtained from the heating curves of obtained for 7 mg of powdered sample suspended using 21 mg of double distilled water placed in a sealed stainless steel DSC pan (Perkin–Elmer stainless steel pressure-tolerant pans). These curves were determined using a Perkin Elmer DSC-7 system (The Perkin Elmer corp., Norwalk Conn, USA). The system was first calibrated using Indium and then samples were measured against a 20 mg pure water reference pan. Samples were scanned from 25 °C to 150 °C with a 5 °C/min ramping. The transition temperatures and enthalpies were calculated using the Pyris thermal analysis system version 3.72 of Perkin Elmer LLC.

Further molecular level characterization of the amylose-FA complexes was achieved through $^{13}\text{C}$ CP/MAS solid state NMR spectroscopy measured by a 300 MHz chemagments-Infinity spectrometer operating at 75.45 MHz. Lyophilized powder samples were packed into 7.5 mm zirconia rotors and spun at 5000 Hz at the Magic Angle. The $^{13}\text{C}$ spectra were obtained by direct excitation of the $^{13}\text{C}$ nuclei and by cross polarization achieved via $^1\text{H}$ nuclei adjacent to the resonating carbons.
3.3. Nanometric level investigations

In order to study the structural properties of the amylose-FA complexes at the nanometric level small angle X-ray scattering (SAXS) was used. These analyses probed the nanostructure of amylose-FA complexes at two states: lyophilization dry powder and the powder suspended in distilled water.

SAXS experiments were performed with Cu-Kα radiation at an acceleration voltage of 20 kV and current of 10 mA radiating through a 20 μm entrance slit leading to the collimation block, with slit length delimiters set at 15 mm. The X-ray scattering was collected using compact Kratky camera having a linear position sensitive detector system (Raytech) with pulse-height discrimination and a multichannel analyzer (Nucleos). Prior to analysis amylose-FA complex samples were suspended in miliQ water overnight and then placed in sealed quartz capillaries with 2 mm diameter (Muller). During measurements sample temperature was kept at 25 ‘C by a temperature controller (A. peer Co.).

Scattering data was used to generate scattering curves, expressed in term of the scattering intensity I as a function of the scattering vector q = 2sinθ/λ. Normalization of the data was achieved using data obtained from the scattering of water as a standard. The background was determined empirically using Equation (1) given by Balata-Calleja and Vonk (Balata-Calleja & Vonk, 1989), in which k1 and k2 are empirical constants.

\[ I = k_1 + k_2 (s)^n \]  

(1)

The background intensity was subtracted from the raw data. The correction of the effect of the beam dimension (desmeasuring) was performed according to the Indirect Transformation Method (Glatter, 1977; Porod, 1982) using the program ITP. Data analysis was based on fitting of the desmeared curve to an appropriate model using a least-square procedure.

4. Results and discussion

4.1. XRD, DSC and 13C CP/MAS NMR – molecular level investigations

X-ray diffraction was used in order to verify the formation of the amylose-FA molecular inclusion complexes. The diffractograms (Fig. 1) confirm the formation of V type structures, as inferred from the peaks at Bragg angles of 2θ = 7.4°, 13.1° and 19.8°. The diffractograms of amylose complexes with SA did not seem to be affected by crystallization temperatures. Contrary, diffractograms arising from complexes hosting LA or CLA and crystallized at 90 °C showed additional low-intensity peaks at Bragg angles of 2θ = 14.9°, 17.1° and 22.6°. This observation suggests that a high crystallization temperature induces the formation of A-type amylose crystals along side with the V type structures. All complexes hosting SA show an additional peak at a Bragg angle of 2θ = 21.7°, which some studies
The thermal behavior of the complexes was studied by DSC. Fig. 2 summarizes the melting temperatures and the enthalpies calculated from the thermograms obtained. As can be seen, the melting temperature increases slightly with increasing the production temperature, in agreement with previous reports (Eliasson, 1994; Karkalas et al., 1995; Tufvesson, Wahlgren, & Eliasson, 2003a). The melting temperatures of the complexes seem to be correlated with the melting temperature of the fatty acids (LA < CLA < SA), however this dependence is not statistically significant. Notably, the melting enthalpy of the SA complexes crystallized at 60 °C and 90 °C is much higher than the other two FA's, which may suggest higher degree of crystallinity. The differences between the thermal behavior of the amylose-CLA and amylose-LA complexes are much less pronounced, as can be expected from their chemical similarity.

In the past years there has been a growing number of studies utilizing solid state 13C CP/MAS NMR to probe the molecular level attributes of the V-amylose complexes with various ligands, mainly revealing insights regarding the host amylose (Biais et al., 2006; Biliaderis & Galloway 1989; Gidley & Bociek, 1988; Kawada & Marchessault, 2004; Snape, Morrison, Maroto-Valer, Karakals, & Pethrick, 1998). In this study, analysis of the solid powders was achieved by direct excitation of the 13C nuclei and by H–13C cross polarization (CP) 13C magic angle spinning (MAS) NMR according to assignments previously described (Snape et al., 1998) and cross referenced with data from the Spectral Database for Organic Compounds (http://riodb01.ibase.aist.go.jp/sdbs/, National Institute of Advanced Industrial Science and Technology, accessed January 2nd, 2007). These experiments helped produce 13C NMR spectra for amylose complexes with stearic acid or linoleic acid (Fig. 3), which concur with previous work (Kawada & Marchessault, 2004; Snape et al., 1998; Zabar et al., 2009).

As can be seen in Fig. 3, the differences in the guest, namely of the fatty acid, were expressed in the 13C NMR spectra of the samples. The first observation made was that the resolution of the C1 and C4 carbons of amylose was highly sensitive to the guest type. This was noted both for 13C direct excitation and CP NMR spectra. In this respect, amylose complexes with linoleic acid yielded more resolved spectra than complexes produced with stearic acid. A similar and parallel trend, expressed in signal resolution, was also noted for the peaks assigned to the carbons of the fatty acids in the chemical shift range of 15–35 ppm. A possible explanation to these differences might be the mobility of the molecules in the samples which is generally considered to be inversely related to solid state NMR peak resolution. If so, one can argue that the data implies that both the amylose and the entrapped fatty acid have increased molecular mobility in the case of saturated stearic acid compared to the unsaturated linoleic acid. However, a reservation has to be made since solid state NMR peak resolution is directly related to the spatial localizability of the samples and vs a vi to molecular mobility depending on the molecules in question. Thus, it would be better to state that the findings directly suggest that amylose complexes produced with linoleic acid produce more spatially defined structures than complexes produced with stearic acid.

Fig. 2. The dependence of the average melting temperatures (bottom panel) and the melting enthalpies (top panel) on the crystallization temperature of amylose complexed LA, CLA and SA.

Fig. 3. 13C solid state NMR spectra of amylose complexes hosting Stearic acid (SA) or linoleic acid (LA). [A] Direct excitation 13C MAS NMR spectra. [B] 13C CP/MAS NMR spectra.
Further molecular investigation of the structure of amylose complexes was achieved through the use of stearic acid and linoleic acid uniformly labeled with $^{13}$C. The corresponding complexes formed with these $^{13}$C labeled FA's were subjected to XRD analysis which is given in Fig. 4. These XRD patterns fully correspond to various previously described studies (Conde-Petit, Escher, & Nuesl, 2006; Lalush et al., 2005; Lesmes et al., 2009; Zabar et al., 2009), thus, verifying the formation of a V type structure. Following these results and those previously described (Zabar et al., 2009), these samples were also subjected to solid state $^{13}$C NMR experiments. The spectra obtained were used not only as a basis of comparison with previous studies but also to help provide some insights regarding the molecular state of the included fatty acid. Thus, $^{13}$C CP/MAS NMR spectra of amylose-$^{13}$C-U-LA, given in Fig. 5, did not show any marked changes in the chemical shift of the carbons assigned to the fatty acid tail. A slight difference was noted in the carboxyl carbon, which was expected to appear as a single peak in the range of 175–178 ppm since the fatty acids in question had pKa values of over eight (Kanicky & To appear as a single peak in the range of 175–178 ppm since the difference was noted in the carboxyl carbon, which was expected 

4.2. SAXS based nanometric level investigations

Characterization of the nanostructure of various complexes was performed using SAXS. Fig. 6 compares the scattering patterns from dry powders of V-amylose LA complexes to the scattering patterns of the same complexes suspended in water. The scattering patterns of both the dry and the suspended complexes display a $\sim$2 slope, indicative to lamellar structures. While dry powders display a smooth pattern with no distinct peaks (Fig. 6b), suspending the powders in water resulted in the appearance of a shoulder at $s = 0.08$ nm$^{-1}$ (Fig. 6a). This shoulder indicates that a slightly ordered structure with a typical Bragg distance of ca. 12.5 nm exist in the sample. Since the complex is not soluble in water, suspending in water is not expected to alter its nanostructure thus the changes in the shape of the scattering pattern can be attributes to changes in the contrast – the electron density difference between the complex and the medium (air or water). The fact that changing the contrast modifies not only the scattered intensity but also its $s$-dependence suggests that the complexes have heterogeneous internal structure with at least two electron densities (Glatter, 1980). We note that the scattering pattern of complexes produced of at high crystallization temperature of 90 °C could not be acquired due to high adsorption.

A better characterization of the nanostructure was gained by fitting the desmeared SAXS curves to appropriate theoretical models. We have followed the same analysis procedure detailed in our previous publication (Zabar et al., 2009). Briefly, curves from the suspended samples were fitted using the “modified lamellar model”, describing randomly oriented finite “domains” made up of alternating layers of crystalline and amorphous material, embedded in an amorphous background material (Wenig & Bramer, 1978). This model was previously used to analyze scattering patterns from native starches (Cameron & Donald, 1992; Wenig & Bramer, 1978) and resistant starch (Shamai, Bianco-Peled, & Shimoni, 2003; Shamai, Shimon, & Bianco-Peled, 2004; Zabar, Shimon, & Bianco-Peled, 2008). For an overall isotropic sample, where the domains are randomly oriented with respect to the beam, the scattering intensity $I(s)$ is given by:

$$I(s) = \frac{1}{4\pi} \frac{1}{s^2} \int_0^\infty \int_0^\infty \int_0^\infty \phi(\mathbf{r}) \phi(\mathbf{r} + \mathbf{s}) \sin(s \cdot \mathbf{r}) \, d^2 \mathbf{r} \, ds,$$

where $\phi(\mathbf{r})$ is the electron density distribution, $s = \frac{4\pi}{\lambda} \sin(\theta/2)$ is the momentum transfer vector, $\theta$ is the scattering angle, and $\lambda$ is the wavelength of the scattered X-rays. The scattering intensity is proportional to the square of the electron density contrast between the complex and the medium, and the total scattering amplitude is given by the integral of the product of the electron density distribution of the complex and its Fourier transform over all possible spatial frequencies.


where \( F_c \) and \( F_a \) are Fourier transformation of the Gaussian thickness distribution functions \( f(x_c) \) and \( f(x_a) \), respectively; the average lamellar repeat distance, \( D \), is given by \( D = x_c + x_a \) where \( x_c \) and \( x_a \) are the average thickness of the crystalline and amorphous regions, respectively; \( \Delta \rho = (\rho_c - \rho_a) \) is the electron density differences between the crystalline lamellae \( \rho_c \) and the amorphous lamellae \( \rho_a \) and \( \Delta \rho_{am} = (\rho_m - \rho_a) \) is the electron density differences between the amorphous lamellae and the background material \( \rho_m \); \( N \) is the number of repeats in a stack; \( \varphi \) is the crystalline fraction of the total lamellar thickness; and \( \beta \) is a factor related to the width of the distributions of lamellar thickness.

The fitting procedure involved fixing the value of \( \Delta \rho_{am} \) to 1 and fitting the values of the other 5 parameters \( \Delta \rho, N, D, \varphi \) and \( \beta \) using a least-square procedure. The solid line in Fig. 6a, which represents the best fit to Equation (2), was calculated from the best-fit parameters listed in Table 1. The values of the average crystalline lamellar thickness \( \langle x_c \rangle = \varphi D \) and the average amorphous lamellar \( \langle x_a \rangle = (1 - \varphi) D \) are also listed.

Next, an attempt to fit the same “modified lamellar model” to the SAXS curve of the dry complexes was made. As the only structural difference between the dry and suspended samples is the contrast, we used \( \Delta \rho \) as the only fitted parameter while keeping the values of all other parameters at their previously determined values. This fit, shown as a dashed line in Fig. 6b, clearly underestimates the scattering intensity at low angles. A probable cause for this excess scattering is surface scattering, arising from the shape of the particles, which is masked in the suspended samples due to the lower contrast (Suzuki, Akio, & Yano, 1997). The –2 slope suggests that the overall shape is two dimensional. Although several structural models could be considered here, we decided to avoid complicated models that would add several additional parameters to the five already fitted. Alternatively, we fitted the appropriate Guinier approximation \( I = \exp(-2\pi^2 R_g^2/s^2) \), where \( R_g \) is the radius of gyration. This fit is shown as a solid line in Fig. 6b. The fitted values of \( R_g \) a measure to the particle’s size, are listed in Table 1.

The fitting procedure described above was also found to be suitable for the complexes hosting CLA and SA at all three temperatures (Figs. 7 and 8). The “modified lamellar model” gave a good fit to the scattering from the suspended powders (solid lines in Figs. 7a and 8a), however underestimated the scattering from the dry powders. As for the LA containing complex, an initial –2 slope was observed in the patterns from the dry powders, and an appropriate Guinier approximation was fitted to this data (solid lines in Figs. 7b and 8b). The best–fit parameters, and the values of the average crystalline lamellar thickness and the average amorphous lamellar, are summarized in Table 2. Notably, the scattering curves of dry samples hosting SA produced at all crystallization temperatures a significant peak at \( s \sim 0.25 \text{ nm}^{-1} \) (a Bragg distance of ~4 nm). This observation indicates that the complexes hosting SA are more organized compared to the complexes prepared with the other fatty acids, in accordance with the DSC and the SEM results.

To summarize this part of the study, crystallization of LA-amylose complexes at high temperature of 90 °C leads to formation of isotropic nanostructure with large dimensions. Other complexes produced via the acidification method are characterized by crystalline regions embedded in an amorphous medium. Inside the crystalline regions, folded amylose chains (crystalline lamellae) are separated from one another be regions of imperfections, chain ends, and possibly residues of the fatty acid (amorphous lamellae). An important point to be made is that although the structural model used in this work was adopted from SAXS studies of native starch, the overall structure of the V type complex is expected to be fundamentally different than that of native starch. In native starch, radial orientation of the amylpectin molecules leads to the formation of
concentric regions of alternating amorphous and crystalline structures known as the growth rings. The lamellae of the VII complexes are formed by crystallization from solution and thus a typical crystallization pattern of linear polymers, i.e. laminated structures arranged in spherulites, could be expected. Nevertheless, from SAXS point of view, the measured dimensions are very small compared to the spherulite or the growth ring dimension. Therefore, the analytic calculations of both systems can be based on models of ideal lamella.

When examining the values of the fitted parameters, summarized in Tables 1 and 2, it seems that increasing the crystallization temperature results in increasing the order within the lamella: higher crystalline fraction \( q \), larger average crystalline lamellar thickness and larger characteristic particle dimension \( R_g \), the only exception being LA-amylose complexes produced at 90°C. Similarly, decreasing the molecular flexibility (from LA to CLA to SA) leads to formation of more ordered structures. The complexes formed from the stiffest fatty acid (i.e. stearic acid) have a significantly more ordered structure which can explain the additional peak in the scattering pattern from the dry powders. Furthermore, comparing the values in Tables 1 and 2 to those described in our previous work (Zabar et al., 2009) reveals that producing V-amylose through the acidification method produces larger particles than when produced via DMSO method. This trend is also supported by the particle size distribution curves described in other work (Eliasson, 1994; Karkalas et al., 1995; Lesmes et al., 2009; Tufvesson et al., 2003a).

4.3. Microscopic level characterization using AFM and SEM

Atomic force microscopy was applied as a novel nanotechnology tool to help elucidate the micro and nano scale attributes of V-amylose complexes. Since the link between fatty acid unsaturation...
and particle size distribution and morphology was recently established based on light scattering particle sizing and AFM (Lesmes et al., 2009), this study aimed to probe the structural changes occurring during complexation. Thus, sampling amylose-SA complexes after titration to acidic conditions ($t = 0$) and at the end of the crystallization process ($t = 24$ h) illustrated the structural changes occurring during complexation (Fig. 9).

These AFM images not only demonstrated the aggregative nature of amylose complexes but also reveal a marked change occurs in the morphology of the amylose particles during complexation. The structures viewed after titration (Fig. 9A) appear to be in agreement with a structural model in which amylose chains closely interact with each other to form unique structures, as put forth by others (Cui, 2005). After 24 h of complexation such structures were not detected, but rather aggregates of spheroids (Fig. 9B), coinciding with previous AFM work (Lalush et al., 2005; Lesmes et al., 2009). This visual structural change was also noted in surface roughness measurements. Cross sectioning of corresponding AFM images revealed the average surface roughness of the amylose particles increases from $7.72 \pm 4.34$ nm at $t = 0$ to $11.54 \pm 6.05$ nm after 24 h.

Scanning electron microscopy images of complexes hosting all three fatty acids aimed to study the microscopic attributes of the complexes. Complexes with LA produced at 30 °C and 60 °C appear to be smooth, with no distinct features on the micronic level (Fig. 10a and b). Contrary, production at 90 °C lead to the formation of a porous structure (Fig. 10c and d), presumably due to the presence of bubbles at this temperature with is close to the boiling point of the aqueous solution from which the complex was precipitated. The pores are few microns is size, however the SAXS results suggest that smaller pores exist as well.

![Fig. 9.](image_url) Atomic Force Microscopy (AFM) topography images (3 μm × 3 μm) of V-amyllose complexes hosting stearic acid. [A] Sample immediately after titration to acidic condition (crystallization time $t = 0$). [B] Sample at the end of the crystallization process (crystallization time $t = 24$ h). Samples deposited on mica and freely dried.

![Fig. 10.](image_url) SEM micrographs of amylose-LA complexes produced at (a) 30 °C; (b) 60 °C; (c) and (d) 90 °C.

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For complexes with CLA and SA, the production temperature did not have a significant effect on the results, therefore only representative micrographs taken from samples produced at 60 °C are shown in Fig. 11. Complexes with CLA appear to be smooth. Contrary, SEM micrographs of the amylose-SA complexes showed large crystals in complexes embedded in the matrix. The crystals thickness is estimated as 1 μm.

In summary, data presented in this paper shows amylose interactions with fatty acids lead to the formation of molecular inclusion complexes organized in lamellae packed in spheroids which tend to aggregate, as illustrated in Fig. 12. Basically, the presence of fatty acids induces segments in the amylose chain to form helices entrapping the fatty acids. These molecular segments tend to form lamellae of distinct nanometric dimensions as described here and elsewhere (Biais et al., 2006; Godet et al., 1996; Zabar et al., 2009). Furthermore, this study shows that the lamellae are interspersed by amorphous segments of the amylose chains into spheroids of submicron size which tend to form aggregates well into the microscopic range. Thus, this study not only provides quantitative insights into the lamellar structures of V-amylose but also links molecular insights with microscopic ones, allowing the extension of a previous model suggested for V-amylose (Biais et al., 2006) well into the microscopic range.

5. Conclusion

This research aimed to study three different strata of V-amylose structure harboring long chain fatty acids. To the best of knowledge this is the first study to use SAXS to probe the nanostructures of V-amylose formed by acidification and combine the results with
molecular and microscopic level investigations. This study shows that all FA's induce V-amylose formation however to a varying extent. As in other studies, molecular level investigations show that increased fatty acid saturation leads to the formation of ill-defined crystallites with decreased thermostability and spatial localization, as observed in previous studies (Smith, 2007; McClements, 2009; Park, 2009). Significant structural changes occur at the molecular level, resulting in the formation of variable-sized crystallites with decreased thermostability and spatial localization as compared to the particles formed through the use of dimesylsulfoxide (DMSO) (Lesmes et al., 2009; Zabar et al., 2009).

These insights demonstrate that guest chemistry universally affects some of the structural attributes of V-amylose probably manifesting in other aspects of V-amylose such as susceptibility to hydrolysis by amylases. This in turn may affect the release of the guest molecules and/or other aspects of its functionality as a controlled release system targeted to the lower gastrointestinal tract, as recently suggested (Gelder, Goevaert, & Delcour, 2006; Lalush et al., 2006). The structural and stoichiometric studies of complexes between aroma compounds and amylose. Polymorphic transitions and quantification in amorphous and crystalline areas. Carbohydrate Polymers, 68(3), 306–315.


References


