Studying different dimensions of amylose–long chain fatty acid complexes: Molecular, nano and micro level characteristics

Shiran Zabar, Uri Lesmes, Itai Katz, Eyal Shimoni, Havazelet Bianco-Peled

Abstract

Recently amylose inclusion complexes, or V-amylose, have drawn much attention as a possible vehicle for the nanoencapsulation of unsaturated fatty acids. This study aimed to study three different structural strata of V-amylose, the molecular attributes using XRD, DSC and $^{13}$C CP/MAS NMR and the nanostructures using SAXS. Using these methods it was noted that decreased degree of fatty acid unsaturation induces the formation of more organized and well defined structures. Specifically, calculations based on SAXS data show that regardless of the crystallization temperature saturated SA yields the highest values for parameters like average crystalline lamellar thickness ($d_h$) and characteristic particle dimension ($R_g = 9.6$). SEM shows this trend extends even into the microscopic level. Overall, this study shows that in the case of long chain fatty acids, increased fatty acid unsaturation impairs the structure of amylose inclusion complexes.

1. Introduction

Since the middle of the past century, the inclusion behavior of starch, namely its linear glucose homo-polymer fraction named amylose, has been vastly studied both for scientific insights and for applicative objectives. Increasing in numbers as technology advances, these studies have shown that amylose complexes can molecularly include guest molecules within or in between hollow helices in a form termed V-amylose of nanometric dimensions (Biais, Le Bail, Robert, Pontoire, & Buleon, 2006; Conde-Petit, Escher, & Nuessli, 2006; Gelders, Goesaert, & Delcour, 2006; Kawada & Marchessault, 2004; Lalush, Bar, Zakaria, Eichler, & Shimoni, 2005; Lesmes, Cohen, Shener, & Shimoni, 2009).

Amylose–lipid complex formation is known to be affected by amylose degree of polymerization (DP), solution pH, crystallization temperature and the structure of the complexed lipid (mono-glyceride or fatty acid) (Akuzawa, Sawayama, & Kawabata, 1997; Biliaderis, 1998; Biliaderis & Galloway, 1989; Biliaderis, Page, & Maurice, 1986; Biliaderis, Page, Maurice, & Juliano, 1986; Biliaderis, 1998; Biliaderis & Galloway, 1989; Biliaderis, Page, & Maurice, 1986; Biliaderis, Page, Maurice, & Juliano, 1986; Biliaderis,

Page, Slade, & Sirett, 1985; Karkalas, Ma, Morrison, & Pethrick, 1995; Raphaëlides & Karkalas, 1988; Tufvesson, Skrabanja, Bjorck, Liljebärg Elmstahl, & Eliasson, 2001; Tufvesson, Wahlgren, & Eliasson, 2003a, 2003b). Other factors, such as concentration ratios, duration of complexation time, water content, concentration of amylose and that of the FA are also of importance, with 10:1 amylose:FA weight ratio deemed as optimal (Jovanovich & Maria, 1999; Jovanovich, Zamponi, Lupano, & Anon, 1992; Szejtli & Banky-Elod, 1975). In respect to the entrapment of fatty acids it has already been shown that increasing fatty acid (FA) chain length and decreasing unsaturation increase the thermal stability of V-amylose (Heinemann, Escher, & Conde-Petit, 2003; Karkalas et al., 1995; Tufvesson et al., 2003a, 2003b). The crystallinity of V-type amylose–lipid complexes are interspersed among amorphous areas, and may form various supramolecular structures, including spherulites and lamellae (Gelders, Duyck, Goesaert, & Delcour, 2005; Lalush et al., 2005). Spherulites of amylose complexed with conjugated linoleic acid have been reported to be about 50 nm in diameter (Lalush et al., 2005) and lamellae of amylose–alcohol complexes to have a lamella folding length of about 10 nm (Biliaderis & Galloway, 1989; Jovanovich & Maria, 1999). Transmission electron microscopy (TEM) micrographs of amylose–FA complexes (C16, C12, C8) also demonstrated uniaxial layout of amylose molecules, which were locally interrupted by amorphous segments. The crystal thickness increased with increasing chain length of the FA, and did not exceed...
4.6 nm, which corresponds to the total length of two palmitic acid molecules (Godet, Bouchet, Colonna, Gallant, & Buleon, 1996). Theoretical modeling also suggests V-amylose hosting fatty acids form an imperfect helix with the fatty acid partly inside, partly out, placing the carboxyl head outside the V-helix, leaving only the glycosidic C(4)–O–C(1) bonds as the greatest points of the helix flexibility (Lebail, Buleon, Shiftan, & Marchessault, 2000). Recently, even atomic force microscopy (AFM) was successfully used to study the nanostructures of V-amylose and other phenomena in starch systems (Dang, Braet, & Copeland, 2006; Lalush et al., 2005; Lesmes et al., 2009; Peng, Liu, & Kennedy, 2007; Tang & Copeland, 2007) illustrating that such novel technologies may provide new insights even to previously studied systems.

Long chain polyunsaturated fatty acids or LC-PUFA consumption is advocated due to their known beneficial effects on human health, however, their supplementation into functional foods is impaired by their chemical instability. In light of that, our overall aim was to deepen the understanding of the structure of V-amylose inclusion complexes in order to provide insights needed for its possible future use as a delivery system designed to deliver long chain fatty acids and possibly other nutraceuticals. The purpose of this study was to study the effects of fatty acid unsaturation on three different dimensions of V-amylose structure, i.e. the molecular level, nanometric level and microscopic level. This was achieved by characterizing different structural attributes of V-amylose complexes made with three fatty acids (FAs) having a constant chain length of 18 carbons but varying in chemistry, i.e. positioning and number of the double bonds along the FA chain. This study mainly focused on SAXS which was previously shown to provide insights and quantitative characterization of native and retrograded starch, however was not employed for the characterization of V-amylose before. Thus, small angle X-ray scattering (SAXS) was used to study the nanostructures of V-amylose in an attempt to link between the molecular attributes and the microscopic structures, using previously used techniques, such as DSC, solid state $^{13}$C CP/MAS NMR and SEM.

2. Experimental

2.1. Materials

2.1.1. Potato amylose

Potato amylose (Av. DP 900) 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.

2.1.3. Other reagents

Dimethylsulfoxide (DMSO) and all other reagents were analytical grade chemicals.

2.2. Methods

2.2.1. Formation of V-amylose molecular inclusion complexes

Production of V-amylose complexes via dilution of dimethylsulfoxide (DMSO) mixture was carried out based on a method previously described (Eliasson & Krog, 1985). Accordingly, DMSO preheated to 90 °C was used to gradually dissolve 500 mg of amylose. The resulting clear DMSO solution was then cooled to temperatures of 30 °C, 60 °C or 90 °C and 35 mg of the guest fatty acid (7% w/w relatively to amylose) was added at the right temperature. This DMSO mixture was rapidly added to 475 mL of distilled water at the corresponding temperature and incubated for 15 min under vigorous stirring at that temperature before chilling and separation.

2.2.1.1. Separation of the complexes. Separation of the V-amylose from the suspensions formed was done by centrifugation (2000g, 20 min) and the wet pellet was washed using 50% ethanol/water mixture (v/v) and centrifuged as before. As previous studies have demonstrated, such a treatment was found successful in the removal of excess or unbound ligands (Blais et al., 2006; Biliaderis & Galloway, 1989; Karkalas et al., 1995). Thus, 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. Overall, the separation process allowed material recovery yield similar of about 30–40% for all types of complexes and the powders produced were found to contain ~7% (w/w) fatty acid. These values were similar to those previously reported (Lalush et al., 2005; Lesmes et al., 2009).

2.2.2. XRD, DSC and $^{13}$C CP/MAS NMR – molecular level investigations

The formation of a V-type amylose–FA complex was verified by measuring the X-ray diffraction of powders produced from the suspensions. All freeze dried samples were kept in desiccators before their XRD characterization. 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 Cu K$_{\text{a}}$ radiation (0.154 nm), voltage 40 kV and current 40 mA. Approximately 200 mg of sample powders were loaded onto a plastic 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 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 $^{13}$C CP/MAS solid state NMR spectroscopy measured by a 300 MHz Chemagnetics-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 $^{13}$C spectra were obtained by direct excitation of the $^{13}$C nuclei and by cross polarization achieved via $^1$H nuclei adjacent to the resonating carbons.

2.2.3. SAXS based 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 the suspended state, i.e. before separation, and at the dry powder state after lyophilization.
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 diffraction 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 \( q = 2 \sin \theta / \lambda \). Normalization of the data was achieved using data obtained from the diffraction of water as a standard. The background was determined empirically using Equation (1) given by Balata-Calleja and Vonk (1989), in which \( k_1 \) and \( k_2 \) 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 (desmearing) 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.

2.2.4. Microscopic level investigation using SEM

The microscopic organization of the different amylose–FA complexes was done by scanning electron microscopy (SEM). SEM micrographs were obtained using a JEOL SEM (5400 model) from dry powders of amylose–FA complex samples plated with gold, in an acceleration voltage of 15 kV.

3. Results and discussion

3.1. 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 complex. The diffractograms (Fig. 1) confirm the formation of V-type structures as inferred from the peaks at \( 2\theta = 7.4°, 13.1° \) and 19.8°. The crystallization temperatures had no major effect on the XRD curves. All complexes hosting SA show an additional peak at \( 2\theta = 21.7° \), which some studies (Biliaderis & Galloway, 1989; Horii, Yamamoto, Hirai, & Kitamaru, 1987; Karkalas et al., 1995; Le Bail, Rondeau, & Buleon, 2005; Raphaelides & Karkalas, 1988) attribute to V-amylose polymorphism. It should be noted that the presence of uncomplexed FA can be excluded from the XRD data. This additional peak combines, to some extent, with that of pure SA probably physically trapped and not molecularly included, as recently suggested (Lesmes et al., 2009). LA and CLA are not crystalline at room temperature.

The thermal behavior of the complexes was studied using DSC measurements. Fig. 2 summarizes the melting temperatures and the enthalpies calculated from the thermograms obtained for complexes produced at different temperatures. As can be seen, the melting temperature increases slightly with increasing the production temperature, in agreement with previous reports (Eliasson & Krogius, 1985; Karkalas et al., 1995; Tufvesson et al., 2003a, 2003b). 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 meting enthalpy of the SA complexes is much higher than the other two FAs, 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.

3.1.1. 13C CP/MAS NMR

Analysis of the solid powders was also achieved by 1H–13C cross polarization (CP) 13C magic angle spinning (MAS) NMR according to assignments previously described (Snape, Morrison, Maroto-Valer, Karakals, & Pethrick, 1998) and indicated in Fig. 3 and cross referenced with data from the Spectral Database for Organic Compounds (http://riodb01.ibase.aist.go.jp/sdb), National Institute of Advanced Industrial Science and Technology, accessed January 2nd, 2007).

All powders were subjected to similar experiment conditions, e.g. same spinning speed, same contact time and similar data processing parameters. As can be seen in Fig. 4, besides DMSO traces
that were detected in all the powders, most of the FA methylene groups were poorly resolved with slight resolution differences between the fatty acids.

When specific regions of the spectra were analyzed more in depth (Fig. 5), the C2 and C3,5 of the amylose (Fig. 5A) and the methylene groups of the fatty acids (Fig. 5B) were the pivots of differences between samples. As to the fatty acid carboxylic head group, the NMR spectra suggest that all of the fatty acids were trapped to varying extents in both the acid form (COOH) and the salt form (COO−/C01) as can be inferred from the appearance of two signals at \( \pm 177 \text{ ppm and } \pm 182 \text{ ppm, which is in agreement with previous } ^{13}\text{C CP/MAS NMR studies of amylose complexes (Lebail et al., 2000; Snape et al., 1998). Since chemically CLA and LA differ only in the positioning of the double bonds, one would not expect significant differences in their pKa values which according to previous work should be over a pH of 11 (Kanicky & Shah, 2002).}

Interestingly, both CLA and LA complexed at acidic pH values were found to exist in both in the acid and salt form in all the powders produced.

NMR experiments also showed that differences in the complexation method are expressed at the molecular level of the amylose–FA complexes. These are evident in the significant differences in \(^{13}\text{C signal resolution between complexes made via DMSO or via acidification (Fig. 5). These differences are best viewed in the C1–O–C4 carbons of amylose and the fatty acid methylenes, which appear to be missing in the spectra of acidified complexes. From this one might conclude that acidification yields very ill-defined crystalline amylose powders other than V-amylose hosting SA. Contrary to this notion, both XRD patterns and enzymatic digestion tests (data not shown) show that the corresponding powders do contain SA and exhibit the typical V-type XRD pattern. Thus, it might be argued that the molecular level organization of acidified complexes differs from similar complexes made via DMSO.}

All the fore mentioned differences may indicate that the differences in the chemistry of the guests may affect the crystalline nature of the structures of the corresponding amylose complexes. Overall, it would appear that increasing the fatty acid unsaturation leads to the formation of ill-defined crystals compared to the crystalline structure of amylose complexes made with the fully saturated stearic acid.

3.2. 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. While dry powders produced at all three temperatures display a smooth pattern with no distinct peaks (Fig. 6a), suspending the powders in water resulted in the appearance of a shoulder at \( \pm 0.08 \text{ nm}^{-1} \) (Fig. 6a). This shoulder indicates that a slightly ordered structure with a typical Bragg distance of ca. 12.5 nm exists 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).

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

Fig. 3. Peak assignment to \(^{13}\text{C CP/MAS NMR spectrum of amylose complexes hosting stearic acid produced via DMSO dilution at 90 °C. \( \star \) Signal of }^{13}\text{C arising from DMSO.}\)
A better characterization of the nanostructure was gained by fitting the desmeared SAXS curves to appropriate theoretical models. As pointed out by Suzuki, Akio, and Yano (1997), SAXS patterns from dry starch samples are strongly influenced from the difference between the average density and the air, whereas the density fluctuation within the starch granules becomes less important. Therefore, we first fitted the curves from the suspended samples, for which the details of the internal inhomogeneities are more pronounced. Since the $-2$ slope in the double logarithmic plot (Fig. 6a) is indicative to a lamellar structure, we considered first simple models such as infinite large flat particle or a stack of oriented lamellar layers characterized by a single electron density. These models, however, failed to give a reasonable fit to the experimental data. A much better fit was obtained using the “modified lamellar model”. This model describes randomly oriented finite “domains”, made up of alternating layers of crystalline and amorphous 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, Shimoni, & Bianco-Peled, 2004; Zabar, Shimoni, & Bianco-Peled, 2008). The one-dimensional scattering intensity according to this model is given by:

$$i_1(s) = \frac{(\Delta \rho)^2}{2\pi^2 s^2 D} \left\{ \frac{1 - F_c}{1 - F_c F_a} (1 - F_a) \left[ F_a (1 - F_c) \right]^2 \right\} + \frac{(\Delta \rho_s)^2}{2\pi^2 s^2 N_D} \left\{ \frac{(\Delta \rho_a)^2}{(\Delta \rho)^2} \left[ 1 - (F_a F_c)^N \right] \right\}$$

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 difference between the crystalline lamellae $\rho_c$ and the amorphous lamellae $\rho_a$ and $\Delta \rho_s = (\rho_b - \rho_c)$ is the electron density differences between the amorphous lamellae and the background material $\rho_b$; $N$ 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. For an overall isotropic sample, where the stacks are randomly oriented with respect to the beam, one gets

$$i(s) = \frac{i_1(s)}{4\pi s^2}$$

The fitting procedure involved fixing the value of $\Delta \rho_s$ 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 (3), was calculated from the best-fit parameters listed in Table 1. The values of the average crystalline lamellar thickness $(x_c) = \varphi \times D$ and the average amorphous lamellar $(x_a) = (1 - \varphi) \times D$ 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 et al., 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 $I = \exp(-2\pi^2 R_g^2 s^2)/s^2$, 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 (Figs. 7 and 8). The “modified lamellar model” gave a good fit to the scattering from the suspended powders, 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. 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 $s \approx 0.25 \text{ nm}^{-1}$ (a Bragg distance of $\sim 4 \text{ nm}$). This observation indicates that the complexes hosting SA are more organized compared to the complexes prepared with the other fatty acids.

To summarize this part of the study, complexes produced with the three different fatty acids and at three different production temperatures seem to display a similar overall structure: crystalline regions embedded in an amorphous medium. Inside the crystalline regions, folded amylose chains (crystalline lamellas) are separated from one another by regions of imperfections, chain ends, and possibly residues of the fatty acid (amorphous lamellas). 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 amylopectin molecules leads to the formation of concentric regions of alternating amorphous and crystalline structures known as the growth rings. The lamellas of the $V_{II}$ 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.

### Table 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Crystallized at 30 °C</th>
<th>Crystallized at 60 °C</th>
<th>Crystallized at 90 °C</th>
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<tr>
<td>$\varphi$</td>
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<td>0.11</td>
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<td>$\rho$</td>
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<td>$D$ (nm)</td>
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<td>15.0</td>
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<tr>
<td>$\Delta\rho_0$</td>
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<td>9.8</td>
<td>4.9</td>
</tr>
<tr>
<td>$N$</td>
<td>55</td>
<td>22</td>
<td>34</td>
</tr>
<tr>
<td>$h_{\rho}$</td>
<td>5.8</td>
<td>3.9</td>
<td>6.0</td>
</tr>
<tr>
<td>$\varphi \times D$ (nm)</td>
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<td>2.5</td>
<td>3.0</td>
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<tr>
<td>$(1 - \varphi) \times D$ (nm)</td>
<td>15.8</td>
<td>20.5</td>
<td>12.0</td>
</tr>
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Fig. 6. SAXS curves of amylose-LA complexes produced at crystallization temperatures of (C) 30 °C, (A) 60 °C and (•) 90 °C. (a) Suspended powders (b) dry powders. Data and the corresponding fits were offset for clarity.

Fig. 7. SAXS curves of amylose-CLA complexes produced at crystallization temperatures of (C) 30 °C, (A) 60 °C and (•) 90 °C. (a) Suspended powders (b) dry powders. Data and the corresponding fits were offset for clarity.
When examining the values of the fitted parameters, summarized in Tables 1 and 2, it is hard to find a clear correlation between most fitted parameters and the crystallization temperature, the only exception being the average crystalline lamellar thickness which increases slightly at higher temperatures. On the contrary, the identity of the fatty acid seems to have a larger effect on the structural parameters. Decreasing the molecular flexibility (from LA to CLA to SA) resulted in increasing the order within the lamella: higher crystalline fraction $\varphi$, larger average crystalline lamellar thickness and larger characteristic particle dimension $R_g$. Moreover, the complexes formed from the stiffest fatty acid (SA) have a significantly more ordered structure which

Table 2

<table>
<thead>
<tr>
<th></th>
<th>CLA</th>
<th>SA</th>
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<tr>
<td>$\varphi$</td>
<td>0.19</td>
<td>0.24</td>
</tr>
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<td>$\beta$</td>
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<td>0.51</td>
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<td>$D$ (nm)</td>
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<tr>
<td>$\Delta\rho$</td>
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<td>1</td>
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<tr>
<td>$N$</td>
<td>38</td>
<td>40</td>
</tr>
<tr>
<td>$R_g$</td>
<td>5.2</td>
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<td>$\varphi \times D$ (nm)</td>
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<tr>
<td>$(1 - \varphi) \times D$ (nm)</td>
<td>10.8</td>
<td>12.4</td>
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Fig. 8. SAXS curves of amylose-SA complexes produced at crystallization temperatures of (○) 30 °C, (△) 60 °C and (■) 90 °C. (a) suspended powders (b) dry powders. Data and the corresponding fits were offset for clarity.

Fig. 9. SEM micrographs of (a) amylose-LA (b) amyloze-CLA and (c) amyloze-SA complexes produced at 60 °C. Bar=8 μm.
can explain the additional peak in the scattering pattern from the dry powders.

3.3. Microscopic level investigation using SEM

Scanning electron microscopy images of complexes hosting all three fatty acids aimed to study the microscopic attributes of the complexes. 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. 9. Complexes with LA and CLA appear to be smooth, with no distinct features on the microscopic level. Contrary, SEM micrographs of the amylase–SA complexes showed large crystals in complexes embedded in the matrix. The crystal thickness is estimated as 1 μm.

4. Conclusion

Molecular level investigations described hereby show that increased fatty acid unsaturation leads to the formation of ill-defined crystallites with decreased thermostability and spatial localizability. Fitting of the SAXS data to a modified lamellar model enabled the extraction of nano level characteristics of V-amylose. These characteristics and SEM micrographs indicate the trend observed in the molecular investigations extends even to the microscopic level of structure. Thus, the effect of fatty acid unsaturation on V-amylose structure has been shown to span throughout the different structural strata studied. Overall, these observations demonstrate that guest chemistry appears to universally affect some of the structural attributes of V-amylose. Further work should test and evaluate if such structural differences are also observed in food grade systems and whether these are related to varying functional performance of V-amylose as a delivery system.

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