1 STRUCTURAL EFFECTS OF MICROALGAE ADDITIVES ON THE STARCH

2 GELATINISATION PROCESS

4 Marta Martínez-Sanz*, María José Fabra, Laura G. Gómez-Mascaraque and Amparo López-Rubio

7 Food Safety and Preservation Department, IATA-CSIC, Avda. Agustin Escardino 7, 46980
8 Paterna, Valencia (Spain).

10 *Corresponding author: Tel.: +34 963200022; fax: +34 963636301

11 E-mail address: mmartinez@iata.csic.es

12
Abstract

This work presents a detailed structural characterisation of the starch gelatinisation process and the effect of the addition of three microalgae species, *Nannochloropsis gaditana* sp., *Scenedesmus almeriensis* and *Spirulina*, by means of an advanced approach consisting of temperature-resolved simultaneous SAXS/WAXS experiments, combined with DSC. Furthermore, regular and high amylose corn starch were utilised to evaluate the impact of the amylose content.

The presence of microalgae has been seen to limit water accessibility towards the interior of starch granules, reducing granule swelling and, thus, hindering the arrangement of amylopectin helices into highly ordered crystalline structures. As a result, more heterogeneous lamellar structures, with reduced apparent crystallinity, are attained. Despite the existence of lipidic compounds in the three microalgae species, the tough cell walls in *Nannochloropsis* and *Scenedesmus* impede their release towards the aqueous medium. In contrast, the weak cell walls in *Spirulina* are disrupted by stirring, allowing cell components to be released. The diffused lipids form helical inclusion complexes with the amylose chains and promote the crystallisation of V-type structures. The presence of amylose-lipid complexes counteracts the limited water swelling effect and results in the formation of more crystalline and homogeneous lamellar structures. This result is relevant for the food industry due to the potential of *Spirulina* to affect the processability and nutritional characteristics of starch-based products.

Keywords: starch; microalgae; gelatinisation; SAXS; WAXS; DSC
1. Introduction

Starch is one of the most widely known dietary polysaccharides since it is the main component in raw and processed foods. Native starch is found in vegetal resources forming water-insoluble semi-crystalline granules which present a complex hierarchical architecture characterised by at least four structural levels: (i) granules, (ii) semi-crystalline amorphous growth rings, (iii) lamellae (i.e. stacks of alternating amorphous and crystalline structures) and (iv) linear amylose and branched amylopectin chains. The crystalline regions are thought to consist mainly of amylopectin side chains which are organized in double helices (Imberty, Buléon, Tran, & Pérez, 1991), whereas the amorphous regions are mainly composed of linear amylose chains and less ordered amylopectin branch points (Pérez & Bertoft, 2010). Depending on several factors such as the amylose/amylopectin ratio and the amylopectin chain length, these helices can be arranged differently into A-type (mainly found in cereal starches and related to short double helices) or B-type (long double helices found in tubers and high amylose starches) crystalline unit cells (Cheetham & Tao, 1998; Hoover, 2001; Paul J Jenkins, Cameron, & Donald, 1993; Salman, et al., 2009). Water has a great influence in the long range order of starches, constituting up to 4-7% and 25-27% of the crystalline unit cell in the A and B polymorphs, respectively (Imberty, Chanzy, Pérez, Buléon, & Tran, 1988; Imberty & Perez, 1988; Sarko & Wu, 1978).

Many food processing methods involve the use of combined heat and humidity, leading to starch gelatinisation which involves the break-up of the starch structure. Essentially, the gelatinisation process is initiated by the access of water towards the interior of starch granules, which swell and absorb water. Subsequently, structural changes occur at the different architectural levels, leading to crystallinity losses, disruption of the crystalline and lamellar structure of starch and amylose molecules leaking out of the starch granules forming...
a continuous gel. This complex gelatinisation phenomenon is governed by a number of factors such as starch botanical origin, plant growth conditions, extraction methods, water content, presence of additives, heating rate and thermal history (Imberty, et al., 1991; Liu, Yu, Xie, & Chen, 2006; Tester & Morrison, 1990; Varavinit, Shobsngob, Varanyanond, Chinachoti, & Naivikul, 2003; Waigh, Gidley, Komanshek, & Donald, 2000). A detailed understanding of the structural changes taking place during starch gelatinisation, at the different length-scales, as well as the effect of different parameters, is highly relevant to several scientific and industrial fields. For instance, in many food processing methods such as baking of bread, extrusion of cereal-based products, thickening, and gelling of sauces, starch gelatinisation is a key factor to produce a desirable texture or consistency of the end product (Biliaderis, Maurice, & Vose, 1980). From the human nutrition perspective, the degree of starch gelatinisation in food products is known to affect its digestion rate (Joergen Holm, Lundquist, Björck, Eliasson, & Asp, 1988). Furthermore, the gelatinisation process is the basis for the processing methods applied to produce starch-based biopolymeric materials (Li, et al., 2011; Liu, Xie, Yu, Chen, & Li, 2009).

Microalgae constitute one the most promising sustainable feedstocks for the manufacture of plant-derived products. Due to their ease of cultivation, high growing rates and productivity and possibility of adapting the harvesting conditions to modify their composition, microalgae have gained a great deal of interest for their use in a wide range of applications such as the production of biofuels (Mata, Martins, & Caetano, 2010) and food commodities (Draaisma, et al., 2013; Spolaore, Joannis-Cassan, Duran, & Isambert, 2006). Their interest from the human nutrition perspective lays in their interesting chemical composition. In particular, the high protein content of several species and their interesting lipidic profile, with relatively high amounts of ω3 and ω6 fatty acids (Spolaore, et al., 2006),
make them attractive for the production of nutritional supplements or food additives. The incorporation of microalgae into pasta and bakery products to enhance their nutritional profile is currently being investigated and has been reported in several recent works (De Marco, Steffolani, Martínez, & León, 2014; Monica Fradique, et al., 2010; Mónica Fradique, et al., 2013; Kadam & Prabhasankar, 2010). The components present in the microalgae may interfere with the gelatinisation process in starchy foods and, thus, the processing conditions may also be affected. Therefore, investigating the effect of different microalgae on the gelatinisation of starch may be of interest to determine their impact on the processability and digestibility of starch-microalgae blends. In this work, we have investigated the structural changes undergone by two starches, with different amylose contents, as well as the effect of three different microalgae species, during the gelatinisation process. To this end, an advanced approach of temperature-resolved simultaneous small angle and wide angle X-ray scattering (SAXS/WAXS) experiments, combined with DSC characterisation, has been utilised. The use of scattering techniques is particularly advantageous to study the starch gelatinisation process, since it enables the characterisation of different structural levels (crystalline and lamellar structures) in starch as the temperature is raised and at high relative humidity conditions.

2. Materials and methods

2.1 Materials

Corn starch (27-28% amylose) and high amylose starch (70% amylose) powders were supplied by Roquette (Roquette Laisa España, Benifaió, Spain). The three different microalgae species, i.e. *Nannocloropsis gaditana* sp., *Spirulina* and *Scenedesmus almeriensis*, in the form of dry powders, were kindly donated by Dr. Acién from the University of Almeria (Spain).
2.2 TEM characterisation of microalgae cell walls

The raw microalgae were dispersed in water by vortex stirring at a concentration of 4 g/L. One drop (8 μl) of the prepared suspensions was allowed to dry on a carbon-coated grid (200 mesh). The microalgae cell walls were stained with a 2 % (w/w) solution of uranyl acetate. TEM was performed using a JEOL 1010 equipped with a digital Bioscan (Gatan) image acquisition system at 80 kV.

2.3 Preparation of starch and starch/microalgae dispersions

Starch dispersions (samples designated as “corn” and “amylo” for the corn starch and high amylose starch, respectively) were prepared by adding 0.5 g of starch into 1 mL of water and subjecting the samples to vortex stirring for 2 min. For the starch/microalgae samples, 0.004 g of the microalgae powder were also added prior to stirring. The dispersions were immediately used for the SAXS/WAXS and the DSC experiments.

2.4 Temperature resolved SAXS/WAXS experiments

Combined small and wide angle X-ray scattering (SAXS and WAXS, respectively) experiments were carried out in the Non Crystalline Diffraction beamline, BL-11, at ALBA synchrotron light source (www.albasynchrotron.es). The starch and starch/microalgae dispersions were placed in sealed 2 mm quartz capillaries (Hilgenburg Gmbh, Germany). The energy of the incident photons was 12.4 KeV or equivalently a wavelength, λ, of 1 Å. The SAXS diffraction patterns were collected by means of a 9 CCD detector Quantum ADSC 315r with an active area of 315 x 315 mm², an effective pixel size of 102 x 102 μm² and a dynamic range of 16 bits. The sample-to-detector distance was set to 6488 mm, resulting in a q range with a maximum value of q = 0.25 Å⁻¹. Additionally, the WAXS
Diffraction patterns were collected by means of a 3 CCD detector Rayonix LX255-HS with an active area of 85 x 255 mm$^2$, an effective pixel size of 44 x 44 µm$^2$ and a dynamic range of 16 bits. In this case, the sample-to-detector distance was set to 144.9 mm, corresponding to a maximum q value of 7.87 Å$^{-1}$. This detector was tilted with a pitch of 26.4 degrees. Based on previous experiments, an exposure time of 2 seconds was selected for both detectors.

Samples were heated from 30 °C to 110 °C at a heating rate of 2 °C/min. Data were collected in frames of 30 seconds, followed by a period of 30 seconds in which the samples were protected from the beam by a local shutter. Each data frame thus corresponds to a temperature range of 1 °C, with one data frame every 2 °C. The data reduction was treated by pyFAI python code (ESRF) (Kieffer & Wright, 2013), modified by ALBA beamline staff, to do on-line azimuthal integrations from a previously calibrated file. The calibration files were created from well-known standards, i.e. Silver behenate (AgBh) and Cr$_2$O$_3$ for SAXS and WAXS respectively. The intensity profiles were then represented as a function of q (SAXS) and 2θ (WAXS) using the IRENA macro suite (Ilavsky & Jemian, 2009) within the Igor software package (Wavemetrics, Lake Oswego, Oregon).

2.5 SAXS/WAXS data fitting

SAXS data were fitted using the Igor NIST analysis macro suite (Kline, 2006) and applying a mathematical function consisting of a power-law term plus one Gaussian-Lorentzian peak, similar to that previously reported for several starch samples (A. Lopez-Rubio, et al., 2007; Salman, et al., 2009):
\[ I(q) = A \cdot q^{-m} + \left[ R \cdot \left( I_{\text{max}} \cdot \left( 1 + \frac{(2(q-q_{\text{max}}))^2}{\Delta q} \right)^{-1} \right) \right] + \left[ (1 - R) \cdot I_{\text{max}} \cdot \exp \left( -\frac{q^2}{2} \right) \right] + \text{bg} \]

The first term in equation (1) corresponds to the power-law function (where \( A \) is a prefactor and \( m \) is the power-law exponent) to account for the underlying diffuse scattering, the second and third terms correspond to the Lorentzian and Gaussian functions used to describe the starch lamellar peak (where \( q_{\text{max}} \) is the peak position, \( I_{\text{max}} \) is the intensity of the peak, \( \Delta q \) is the full width at half maximum and \( R \) is the Lorentzian to Gaussian ratio of the peak shape) and the fourth term accounts for the incoherent background.

WAXS peak fitting was performed in Igor, following the procedure described in a previous work (Amparo Lopez-Rubio, Flanagan, Gilbert, & Gidley, 2008). The obtained values from the fitting coefficients are those that minimize the value of Chi-squared, which is defined as:

\[ \chi^2 = \sum \left( \frac{y-y_i}{\sigma_i} \right)^2 \]  

(2)

where \( y \) is a fitted value for a given point, \( y_i \) is the measured data value for the point and \( \sigma_i \) is an estimate of the standard deviation for \( y_i \). The curve fitting operation is carried out iteratively and for each iteration, the fitting coefficients are refined to minimize \( \chi^2 \). The crystallinity index \( X_C \) was determined from the obtained fitting results by applying the following equation:

\[ X_C(\%) = \frac{\sum A_{\text{Crystal}}}{A_{\text{Total}}} \times 100 \]  

(3)

where \( A_{\text{Total}} \) is the sum of the areas under all the diffraction peaks and \( \sum A_{\text{Crystal}} \) is the sum of the areas corresponding to the crystalline peaks.
DSC measurements of starch and starch/microalgae dispersions were performed on a Perkin-Elmer DSC 8000 thermal analysis system using N₂ as the purging gas. Approximately 10 mg of samples were weighted and added into hermetically sealed aluminium sample pans. The sample treatment consisted of heating step from 0 ºC to 180 ºC at a heating rate of 2 ºC/min. Before evaluation, similar runs of an empty pan were subtracted from the thermograms. The DSC equipment was calibrated using indium as a standard. Measurements were done, at least, in triplicate.

3. Results and Discussion

3.1 Thermodynamic characterisation of the starch gelatinisation process

The gelatinisation process of starch and starch-microalgae suspensions in excess water was studied by means of DSC characterisation by heating the samples at a rate of 2ºC/min. Typical DSC profiles for the corn starch and high amylose based samples are shown in Figures 1A and 1B, respectively, and the extracted gelatinisation parameters are summarised in Table 1. All the samples show a broad melting peak that corresponds to the gelatinisation endotherm. In the case of corn starch the endotherm extends over a temperature range of ca. 60-70ºC with the maximum at 64 ºC, whereas the process takes place within the range of ca. 70-90ºC, with the maximum at 78ºC, for the high amylose starch. Similar gelatinisation temperatures have been previously reported for corn starch (Liu, et al., 2006; Yoshimura, Takaya, & Nishinari, 1996) and high amylose starch (Liu, et al., 2006) samples. The higher gelatinisation temperature for the high amylose starch is partly a result of the amylose fraction being less prone to swelling than the amyllopectin (Hermansson & Svegmark, 1996; Yuryev, Kalistratova, van Soest, & Niemann, 1998). Moreover, despite the higher packing...
density of the A-type polymorph, B-type starches have been seen to be more enzymatically resistant than A-type starches due to the greater crystallinity of the former ones in the outer granule regions (Sevenou, Hill, Farhat, & Mitchell, 2002). This could be an additional factor limiting water accessibility and therefore, hindering the gelatinisation process in high amylose starch.

It should be noted that the gelatinisation endotherm clearly presents a distinct shape of two overlapped peaks in the case of corn starch (see inset in Figure 1A). A similar behaviour has been previously reported for corn starch samples (46% w/w suspensions in water) with comparable amylose content (Biliaderis, Maurice & Vose, 1980). This is related to the existence of two different transitions that have been described to take place in the presence of moderate/high water contents: (i) the disruption of the inter-molecular hydrogen bonds leading to the dissociation of the amylopectin double helices from the sides of the crystallites, known as the smectic to nematic/isotropic phase transition and (ii) the unwinding of the amylopectin helices at higher temperatures, known as the helix-coil transition (Waigh, Gidley, Komanshek & Donald, 2000). In the case of the high amylose starch, the gelatinisation transition is characterised by a broad peak, similar to that previously detected for corn starch with 70% amylose content. This broad peak has been described to account for several processes: the disruption of amylopectin double helices, the dissociation of the amylose-lipid complexes and the melting of single-helical V-type crystallites (Matveev, et al., 2001).

Both types of starch present a similar gelatinisation enthalpy, although the peak height index (PHI), defined as the ratio between the enthalpy and the difference between the peak maximum and onset temperatures (Krueger, Knutson, Inglett, & Walker, 1987), which
provides a measure of the uniformity in the gelatinisation process, is greater for corn starch. This suggests that although a comparable amount of energy is needed to melt the amylopectin crystallites in both samples, a more heterogenous process takes place in the case of the high amylose starch, supporting the co-existence of different types of crystallite structures.

As deduced from Figure 1A and Table 1, the incorporation of microalgae into the corn starch suspensions does not lead to significant changes on the gelatinisation parameters estimated from the DSC thermograms. In contrast, the presence of microalgae seems to induce an increase in the maximum of the gelatinisation endotherm for the high amylose starch samples, although given the large associated standard deviation values the differences cannot be considered as significative. This effect is more evident for the *Spirulina* and *Scenedesmus* species and in the latter, it is accompanied by an increase in the gelatinisation enthalpy and the peak height index (i.e. a larger amount of energy is required to complete the phase transition and the gelatinisation process is more homogeneous). These results suggest that certain compounds in the microalgae are able to interact with the amylose fraction in the high amylose starch promoting the formation of more thermodynamically stable suspensions.
Figure 1. DSC thermograms from (A) corn starch samples and (B) high amylose starch samples. Curves have been offset for clarity.

Table 1. Onset (T₀), peak maximum (Tₚ) and conclusion (Tₖ) temperatures, entalphy (ΔH) and peak height index (PHI) values for the gelatinisation endotherm of pure starch and starch-microalgae suspensions. Entalphy values are expressed in J/g of dry starch. Standard deviation values on the last digit are shown in parantheses.
<table>
<thead>
<tr>
<th></th>
<th>62 (1)</th>
<th>64 (2)</th>
<th>67 (1)</th>
<th>10 (6)</th>
<th>5.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corn-Spirulina</td>
<td>62 (1)</td>
<td>64 (1)</td>
<td>69 (1)</td>
<td>9 (1)</td>
<td>4.5</td>
</tr>
<tr>
<td>Corn-Nannochloropsis</td>
<td>62.2 (8)</td>
<td>65.3 (5)</td>
<td>68.3 (1)</td>
<td>10.8 (8)</td>
<td>3.5</td>
</tr>
<tr>
<td>Corn-Scenedesmus</td>
<td>61.2 (2)</td>
<td>64.0 (1)</td>
<td>66 (2)</td>
<td>10.7 (6)</td>
<td>3.8</td>
</tr>
<tr>
<td>High Amylose</td>
<td>73 (1)</td>
<td>77.7 (8)</td>
<td>85.4 (5)</td>
<td>13 (6)</td>
<td>2.8</td>
</tr>
<tr>
<td>High Amylose-Spirulina</td>
<td>69 (6)</td>
<td>81 (3)</td>
<td>84 (6)</td>
<td>19 (5)</td>
<td>1.6</td>
</tr>
<tr>
<td>High Amylose-Nannochloropsis</td>
<td>73 (5)</td>
<td>79.7 (5)</td>
<td>82 (4)</td>
<td>8 (4)</td>
<td>1.2</td>
</tr>
<tr>
<td>High Amylose-Scenedesmus</td>
<td>77.4 (5)</td>
<td>80.5 (7)</td>
<td>88 (4)</td>
<td>22 (2)</td>
<td>7.1</td>
</tr>
</tbody>
</table>

259

3.2 Evolution of the starch crystalline and lamellar structure during the gelatinisation process

To investigate the structural changes undergone by starch during the gelatinisation process, the pure starch and starch-microalgae suspensions were characterised by means of temperature-resolved simultaneous SAXS/WAXS experiments. While SAXS is useful to characterise the lamellar structure of starch, WAXS provides information on the crystalline structure.

3.2.1. Crystalline structure

The crystalline structure of the starch and starch-microalgae samples was investigated by analyzing the WAXS patterns collected when heating the samples from 30ºC up to 110ºC. Figures 2 and 3 show the temperature-resolved WAXS curves (temperature steps of 10ºC) for the corn and high amylose starch samples, respectively. As shown in Figure 2, all the corn starch samples display typical A-type diffraction patterns, characterized by the main diffraction peaks located at 2θ = 15.0º, 17.3º, 18.0º and 23.0º. Interestingly, a weak peak located at ca. 20º, characteristic from the V-type crystalline structure (Amparo Lopez-Rubio, Flanagan, Gilbert, et al., 2008), appears in the spectra of the corn starch loaded with...
Spirulina (pointed out by an arrow in Figure 2B). Similarly, V-type crystallites have been reported to originate from the formation of complexes between the polar lipids found in native starches and the amylose fraction (Zobel, 1988). In addition, weak but sharp peaks, located at ca. 15.6°, 20.3°, 26.5°, 29.2° and 29.6°, are visible in the spectra of the sample loaded with Spirulina and two peaks at 22.9° and 29.6° appear in the sample containing Scenedesmus (indicated by discontinuous lines in Figures 2B and 2D). These peaks could arise from crystalline components, such as fatty acids, which are known to be found in the microalgae (Ahlgren, Gustafsson, & Boberg, 1992; James, Al-Hinty, & Salman, 1989; Ma, et al., 2017; Matos, et al., 2016). In fact, as shown in Figure S1, the X-ray diffraction patterns of the microalgae present several peaks, with the most intense ones located at 2θ values of ca. 16.6°, 20.3°, 29.0°, 30.8° and 31.6° for Spirulina, 27.3 ° and 31.5° for Nannochloropsis and 18.5°, 23.2° and 29.5° for Scenedesmus. The appearance of these peaks in the WAXS patterns from the aqueous suspensions (even at 30°C) is indicative of the presence of crystallised lipids in the Spirulina and Scenedesmus microalgae. However, only those lipids contained in the Spirulina microalgae seem to be able to establish strong interactions with amylose, leading to the formation of V-type crystallites. The formation of amylose-lipid complexes has been reported to depend on the chain size (with smaller lipid molecules being more prone to form complexes (Cui & Oates, 1999)) and the concentration of the lipids (Tang & Copeland, 2007). Wheat starch has been shown to form complexes with stearic acid up to a certain lipid concentration, above which the X-ray diffraction peaks characteristic of the V-type crystallites are accompanied by the appearance of additional peaks arising from the presence of lipid aggregates (Tang & Copeland, 2007). Thus, our results seem to indicate that the concentration of lipids in the Spirulina microalgae is above this critical point at which some of the lipids tend to self-associate.
The estimated crystallinity index values for the samples at the initial temperature of 30°C, listed in Table 2, evidence a structural effect of the microalgae in decreasing the apparent crystallinity of the starch granules, being this effect more obvious for the *Nannochloropsis* species. Hydration has been shown to be a relevant factor affecting starch crystalline structure, with a positive linear relationship between moisture content and crystallinity index (Cheetham, et al., 1998; Qiao, et al., 2017). This is due to the fact that water favors molecular motion and chain organization, thus promoting the formation of more ordered structures (Qiao, et al., 2017). Therefore, the decreased apparent crystallinity observed for the starch-microalgae suspensions is most likely a consequence of a limited moisture accessibility towards the interior of starch granules, i.e. a certain amount of the added water is taken up by the microalgae. The WAXS apparent crystallinity drop is in contrast with the DSC results, which show that the gelatinisation enthalpy of corn starch is unaffected by the presence of microalgae. The discrepancy between DSC and WAXS arises from (i) the lower sensitivity of DSC to determine crystalline transitions as compared to WAXS and (ii) the susceptibility of these techniques to identify crystalline or molecular order. With regards to (i), it has been shown that X-ray scattering is indeed able to detect residual crystallinity at temperatures higher than those at which the DSC gelatinisation endotherm is completed (Wang, Zhang, Wang, & Copeland, 2016). Furthermore, in relation to (ii), whereas the thermal transition determined by DSC accounts for the dissociation and unwinding of helical structures (amylopectin double helices, amylose-lipid complexes and V-type single helices), WAXS is only susceptible to those helical structures which are packed forming crystallites. Thus, the presence of microalgae does not perturb the molecular order (i.e. amount of helical structures) but restrains water access towards the interior of the granules, hindering the organisation of these helices into crystallites and hence, reducing the apparent crystallinity estimated by WAXS. The temperature range at which the crystallinity starts
decreasing for all the samples, i.e. 60-70°C (cf. Figure 4A), is well correlated to the gelatinisation temperature range determined from the DSC characterization (cf. Table 1).

As shown in Figure 3, the WAXS patterns from the high amylose corn starch samples differ significantly from the corn starch patterns, with peak positions characteristic of the B-type starch.
crystalline allomorph. A transition from A-type to B-type crystallinity has been previously reported for corn starches when increasing the amylose content across a range of 0-84% (Cheetham, et al., 1998). This is a consequence of longer amylopectin helical chains found in high amylose starches, which are preferentially crystallised into the B-type allomorph (Gernat, Radosta, Anger, & Damaschun, 1993). As observed for the corn starch, the peak indicative of V-type crystallinity is detected in the sample containing *Spirulina* and sharp peaks are visible in the scattering patterns of high amylose starch with *Spirulina* (peaks located at ca. 15.0°, 20.4°, 26.6° and 29.2°) and *Scenedesmus* (peaks located at ca. 23.0° and 29.7°). The formation of V-type crystallites as a result of amylose-lipid complexes may have important implications not only for the industrial processing of starch-based products, but also from the nutritional perspective. For instance, it has been reported that when amylose is complexed with lipids it is hydrolyzed and absorbed in the gastrointestinal tract to the same extent as free amylose but at a slower rate (J. Holm, et al., 1983). Additionally, the V-type amylose-lipid complexes have been shown to produce a decrease in the amylose solubility, hence increasing the gelatinisation temperature (Eliasson, Carlson, & Larsson, 1981) and postpone retrogradation, thus enabling longer storage times (Krog, 1971). It should be mentioned that the fact that a strong impact on the gelatinisation temperature is not clearly observed in this work is most likely related to the low microalgae loadings added into the starch aqueous suspensions (only 0.8 wt.-% with regards to the starch weight). The calculated crystallinity values at 30°C are 21.5%, 26.8%, 13.9% and 17.9% for the high amylose, high amylose-*Spirulina*, high amylose-*Nannochloropsis* and high amylose-*Scenedesmus* samples, respectively. It is also worth noting that, as seen in Figure 4B, the crystallinity of all the samples decreases significantly within the temperature range of ca. 70-90°C, which is in agreement with the temperature range determined from the DSC gelatinisation endotherms. However, it should be noted that, in agreement with previous
studies (Wang, et al., 2016), the samples still present residual structural order at temperatures higher than the DSC gelatinisation endotherm end temperature. It is also worth noting that even though at the final temperature of 110°C the starch crystalline structure has been almost completely destroyed, the peaks characteristic of the crystallised lipids present in the Spirulina and Scenedesmus microalgae are still detected, indicating the high thermal stability of these compounds.

The fact that the corn and high amylose starch present similar crystallinity values contrasts with previous works stating that the crystallinity index is negatively correlated with the amylose content (Gernat, et al., 1993). This may be true for low to intermediate amylose contents, where the A-type allomorph is preserved; however, it could be hypothesised that the longer helical structures present in starches with greater amylose contents and which are crystallised into the B-type conformation, do not significantly affect the overall crystallinity index. In fact, it has been shown that increasing the amylose content in B-type starch does not have a strong impact on the crystallinity as it does in A-type starch (Cheetham, et al., 1998). The complexation of amylose with the lipids from the added microalgae seems to have a positive impact on the starch crystallinity. Thus, while the general trend when incorporating microalgae into the suspensions is a reduction in the apparent crystallinity (especially in the case of the Nannochloropsis) due to a reduced swelling of the starch granules, this effect is reverted in the case of the high amylose starch loaded with Spirulina, as in this case a greater amount of lipid-amylose complexes are formed.
Figure 3. Temperature-resolved WAXS patterns of high amylose starch samples. (A) pure high amylose starch, (B) high amylose starch-Spirulina, (C) high amylose starch-Nannochloropsis and (D) high amylose starch-Scenedesmus.

Table 2. Lamellar peak parameters and crystallinity ($X_c$) for the native samples (T=30ºC).

The lamellar peak parameters were obtained by fitting the experimental SAXS data to the sum function of a power-law plus a Gaussian-Lorentzian peak and crystallinity was obtained from the peak fitting of the WAXS patterns. Standard deviation values on the last digit are shown in parentheses.
<table>
<thead>
<tr>
<th></th>
<th>Lamellar peak position (Å⁻¹)</th>
<th>Intensity of the lamellar peak (a.u.)</th>
<th>Width of the lamellar peak (Å⁻¹)</th>
<th>Lamellar repeat distance (Å)</th>
<th>Xc (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn</td>
<td>0.06358 (1)</td>
<td>110.4 (1)</td>
<td>0.0258 (1)</td>
<td>9.88</td>
<td>21.3 (3)</td>
</tr>
<tr>
<td>Corn-Spirulina</td>
<td>0.06335 (1)</td>
<td>72.6 (1)</td>
<td>0.0233 (1)</td>
<td>9.92</td>
<td>16.7 (4)</td>
</tr>
<tr>
<td>Corn-Nannochloropsis</td>
<td>0.06418 (2)</td>
<td>53.9 (1)</td>
<td>0.0269 (1)</td>
<td>9.79</td>
<td>13.9 (2)</td>
</tr>
<tr>
<td>Corn-Scenedesmus</td>
<td>0.06390 (1)</td>
<td>62.8 (1)</td>
<td>0.0257 (1)</td>
<td>9.83</td>
<td>18.0 (3)</td>
</tr>
<tr>
<td>High Amylose</td>
<td>0.06508 (1)</td>
<td>21.2 (1)</td>
<td>0.0239 (2)</td>
<td>9.65</td>
<td>21.5 (2)</td>
</tr>
<tr>
<td>High Amylose-Spirulina</td>
<td>0.06575 (2)</td>
<td>16.2 (1)</td>
<td>0.0197 (1)</td>
<td>9.56</td>
<td>26.8 (3)</td>
</tr>
<tr>
<td>High Amylose-Nannochloropsis</td>
<td>0.05239 (2)</td>
<td>24.6 (1)</td>
<td>0.0322 (3)</td>
<td>11.99</td>
<td>13.9 (2)</td>
</tr>
<tr>
<td>High Amylose-Scenedesmus</td>
<td>0.06456 (2)</td>
<td>14.4 (1)</td>
<td>0.0250 (2)</td>
<td>9.73</td>
<td>17.9 (2)</td>
</tr>
</tbody>
</table>

![Graph showing Xc (%) vs. T (°C) for different samples](image-url)
Figure 4. Evolution of the crystallinity index for the corn starch (A) and high amylose starch (B) samples.

3.2.2. Lamellar structure

The crystalline regions constituted by the tightly packed helical structures are alternated with amorphous regions, forming the next structural level known as lamellae. Due to their different physical density, the crystalline and amorphous domains present distinct X-ray scattering length density and, thus, the presence of these lamellar structures gives rise to the appearance of scattering features in the SAXS patterns of starch samples. To investigate the structural changes taking place in the lamellar arrangement during the gelatinisation process, as well as the effect of the microalgae addition, the SAXS patterns were collected simultaneously to the WAXS patterns when heating up the samples. As shown in Figure 5, at the initial temperature of 30°C all the samples present a well-defined peak located at ca. 0.06 Å⁻¹, which corresponds to the lamellar peak characteristic of hydrated native starch (Blazek & Gilbert, 2011; Chanvrier, et al., 2007; Salman, et al., 2009). The position of this peak is related to the thickness of crystalline and amorphous lamellae. Additionally, the peak
area depends on the scattering length density difference between the crystalline and amorphous domains. To extract detailed structural information from the obtained SAXS patterns, the experimental data within the q range of 0.045-0.2 Å⁻¹ were fitted using a mathematical function based on the sum of a power-law plus a Lorentzian-Gaussian peak (cf. section 2.5) and the main parameters extracted from the fits are gathered in Table 2. The first observation is that while the lamellar distance is not strongly affected by the starch amylose content, remaining within the range of 9-10Å typically reported for native starches (P. J. Jenkins & Donald, 1995), the intensity of the lamellar peak is greatly reduced for the high amylose starch. This is probably originated by a lower scattering length density contrast in this sample. In fact, it has been reported that while the lamellar distance remains constant, the size of the crystalline regions increases and the electron density difference between the crystalline and amorphous regions decreases with the amylose content (P. J. Jenkins, et al., 1995). This is a consequence of the larger amylopectin chain length observed for high amylose starches, which results in the formation of the less densely packed B-type crystallites. A-type structures are closely packed with water molecules between each double helical structure, whereas B-type are more open and water molecules are located in the central cavity formed by six double helices (Imberty, Buléon, Tran, & Pérez, 1991). Thus, in low amylose starches (with short amylopectin chains which are crystallised into the A-type structure) the crystalline lamellae are smaller but more densely packed, whereas in high amylose starches (with B-type crystallites formed by large amylopectin chains) crystalline lamellae become larger but more loosely packed.

As clearly observed in Figure 5 and confirmed by the fitting parameters, the incorporation of microalgae into the corn starch suspensions leads to a marked decrease in the relative intensity of the lamellar peak. Conversely, the peak position and width are not significantly
This indicates that the addition of microalgae reduces the scattering length density contrast but does not affect the lamellae structural parameters. Together with the DSC and WAXS characterisation, this suggests that the presence of microalgae does not affect the molecular order but impairs the organisation of these helical structures into densely packed and ordered crystalline domains by limiting to some extent the swelling of starch granules. The reduced apparent crystallinity would then contribute to the observed decrease in the scattering length density contrast. In the case of the high amylose starch, the presence of microalgae does not have a strong effect on the lamellar peak intensity, but the peak position and width are affected differently depending on the microalgae species. A significantly broader lamellar peak, with a greater associated lamellar repeat distance of ca. 12Å is observed upon incorporation of *Nannochloropsis*, being indicative of more heterogeneous lamellae in the presence of this microalgae species. This effect can also be explained by a decreased water accessibility towards the interior of starch granules, since it has been reported that in the dry state only a small fraction of starch helices can be aligned and packed to construct the semi-crystalline lamellar structure (Qiao, et al., 2017). Together with the reduced crystallinity estimated from the WAXS patterns, this result indicates that the presence of *Nannochloropsis* has a strong impact on the swelling mechanism of starch granules. In contrast, the addition of *Spirulina* has the opposite effect, i.e. a greater degree of crystalline order is attained (as indicated by the higher crystallinity index) and more homogeneous lamellae are formed (as suggested by the reduced lamellar peak width). The distinct effect of these microalgae on the starch structure and the gelatinisation process may be explained based on their different composition and, mostly, on their distinct cell wall structure. The total lipidic contents have been reported as ca. 6% for *Spirulina* (Matos, et al., 2016), 8-16% for *Nannochloropsis* (Matos, et al., 2016) and up to 30% for *Scenedesmus* microalgae (Jiang, et al., 2017; Ma, et al., 2017). However, the *Nannochloropsis* microalgae
have been shown to possess a tough cell wall, formed by a cellulosic inner layer and an outer layer rich in algaenans, which requires intense sonication treatments to be disrupted (Fabra, et al., 2017; Scholz, et al., 2014). The existence of such resistant cell walls would then impede the release of lipids from the cells towards the aqueous medium. The cell wall structure of the *Scenedesmus* species also contains an inner cellulosic layer, which is then covered by a thin middle layer and an outer pectic layer (Bisalputra & Weier, 1963). On the other hand, the cell walls in *Spirulina* seem to be richer in proteins and they present porous features which may facilitate the release of cell components (Van Eykelenburg, 1977). To corroborate their different cell wall integrity, raw microalgae were characterised by TEM and the crystalline components in their cell walls were stained with uranyl acetate. As observed in Figure S2, while the *Nannochloropsis* microalgae presented intact cell walls, intensely stained and with clearly defined edges, some of the cell walls in *Spirulina* microalgae seemed to be disrupted as a result of the sample preparation process. Therefore, despite the lower lipidic content of *Spirulina*, these components could more easily be released through the weak cell walls, being then able to interact with the amylose fraction in starch and forming complexes which induce a higher degree of crystallinity, as well as promoting the formation of more homogeneous lamellae.
The structural changes undergone by starch during the gelatinisation process were studied by heating the prepared suspensions up to 110°C and recording the SAXS patterns. The results for the pure corn starch and microalgae-loaded samples are shown in Figure 6. As observed from this figure, the relative intensity of the lamellar peak decreases gradually when the temperature increases, reaching a point at which the well-resolved peak is replaced by a very broad shoulder, which in some of the samples is eventually lost at the highest temperatures. A similar broad shoulder has been observed for retrograded starch and it has been correlated with the growth of crystals in an amorphous matrix (Amparo Lopez-Rubio, Flanagan, Shrestha, Gidley, & Gilbert, 2008). The distortion of the lamellar structure is evidenced by a strong increase in the width of the lamellar peak and in the associated lamellar repeating distance. As deduced from Figure S3, which shows the evolution of the calculated lamellar repeat distance as a function of the temperature, this transition takes place within the temperature range of 80-100°C.
Figure 6. Temperature-resolved SAXS patterns of corn starch samples and the corresponding fitting curves. (A) pure corn starch, (B) corn starch-\textit{Spirulina}, (C) corn starch-\textit{Nannochloropsis} and (D) corn starch-\textit{Scenedesmus}.

As shown in Figure 7, increasing the temperature has a similar effect on the lamellar peak in the high amylose starch samples. In that case, the transition from the lamellar peak to the broad shoulder also takes place within a temperature range of 80-100°C for the pure and
microalgae-loaded samples (cf. Figure S1). As opposed to the corn starch samples, the shoulder is still visible for all the high amylose samples at the final temperature of 110°C, indicating that starch still presents a residual long-range structure. This, together with the residual crystallinity detected in the WAXS patterns when raising the temperature above the gelatinisation endotherm end temperatures calculated from DSC, supports the hypothesis presented by Wang et al. that the gelatinization endotherm of starch does not represent complete starch gelatinization (Wang, et al., 2016).
Figure 7. Temperature-resolved SAXS patterns of high amylose starch samples and the corresponding fitting curves. (A) pure high amylose starch, (B) high amylose starch-\textit{Spirulina}, (C) high amylose starch-\textit{Nannochloropsis} and (D) high amylose starch-\textit{Scenedesmus}.

4. Conclusions
This study presents a detailed characterisation of the structural effects undergone by two types of starch with different amylose contents during the gelatinisation process as affected by the addition of different microalgae, carried out by means of temperature-resolved simultaneous SAXS/WAXS experiments, combined with DSC analyses.

The three different microalgae species incorporated into the starch aqueous suspensions, namely *Spirulina*, *Nannochloropsis* and *Scenedesmus*, have shown to induce structural changes during the gelatinisation process. Interestingly, the observed structural effects are strongly dependent on the microalgae cell wall structure and integrity. In general, the presence of microalgae limits water accessibility towards the interior of starch granules, hence impeding the organisation of amylopectin double helices into highly ordered crystalline regions, leading to the formation of more heterogeneous lamellar structures with decreased apparent crystallinity. This effect is more evident in the case of the *Nannochloropsis* species, whose resistant cell wall remains intact and does not allow the release of any component towards the aqueous medium. In contrast, the weak cell walls in the *Spirulina* microalgae are easily disrupted, enabling the release of the lipids contained within the cells. These free lipids form helical inclusion complexes with the starch amylose chains, promoting the formation of a certain fraction of V-type crystallites, increasing the overall crystalline index and resulting in the formation of more homogeneous lamellar structures. The formation of these lipid-amylose complexes is relevant from the processing and nutritional point of view. Starch granules become more thermodynamically stable, i.e. the gelatinisation temperature increases, and the complexed amylose is known to be more slowly digested in the gastrointestinal tract than free amylose.
These results support the potential of scattering techniques in providing a detailed structural information for starch samples, being able to simulate industrial-relevant processes such as the gelatinisation phenomenon by means of temperature-resolved experiments. The incorporation of microalgae with permeable and/or weak cell walls into starch may be interesting to generate more crystalline and thermodynamically stable products through the formation of lipid-amylose complexes.

Acknowledgements

Synchrotron experiments were performed at NCD beamline at ALBA Synchrotron with the collaboration of ALBA staff (2016021658 project). M.J. Fabra, Marta Martinez-Sanz and L.G. Gómez-Mascaraque are recipients of a Ramon y Cajal (RYC-2014-158), Juan de la Cierva (IJCI-2015-23389) and predoctoral (call 2013) contracts from the Spanish Ministry of Economy, Industry and Competitiveness, respectively.

References


thermodynamic and structural properties of low and high amylose maize starches. *Carbohydrate Polymers*, 44(2), 151-160.


Figure S1. X-ray diffraction patterns of the raw microalgae (freeze-dried).
**Figure S2.** TEM images of raw microalgae: (A) *Spirulina*, (B) *Nannochloropsis* and (C) *Scenedesmus*. 
Figure S3. Evolution of the lamellar distance extracted from the SAXS fitting parameters for the corn starch (A) and high amylose starch (B) samples.